The Effect of Temperature on the Performance of Anaerobic Membrane Bioreactors for Treatment of Domestic Wastewater

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THE EFFECT OF TEMPERATURE ON THE PERFORMANCE OF ANAEROBIC MEMBRANE BIOREACTORS FOR TREATMENT OF DOMESTIC WASTEWATER

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Engineering and Earth Science

by
Emily Blair Evans
August 2019

Accepted by:
Dr. Sudeep Popat, Committee Chair
Dr. David Ladner
Dr. David Freedman
ABSTRACT

Anaerobic membrane bioreactors (AnMBRs) offer a potentially energy efficient means of treating domestic wastewater, but their performance with seasonal temperature variations must be understood to determine their feasibility in replacing conventional activated sludge processes. A bench-scale AnMBR treating primary clarifier effluent from a WWTP in Greenville, SC was found to achieve a similar chemical oxygen demand (COD) removal during operation at 35°C, 25°C, and 15°C, with average permeate COD concentrations of 70.5 mg/L, 60.7, and 77 mg/L respectively. Methane yields averaged 109 L CH₄/kg COD_{removed} at 35°C, 114 L CH₄/kg COD_{removed} at 25°C, and 64 L CH₄/kg COD_{removed} at 15°C. Reduced methane yield is attributed to a decrease in methanogen abundance, seen by an almost 3-fold decrease in mcrA gene abundance during operation at 15°C. The conclusion that the AnMBR achieves similar methane yields at 35°C and 25°C is significant because substantial energy savings could be obtained from not heating the reactor to 35°C as is common in conventional anaerobic processes. Energy savings could especially be significant if the wastewater is already near 25°C for a portion of the year. By understanding the microbial components of AnMBR treatment through molecular microbial analysis and relating them with the performance of the AnMBR at different temperatures, we can better understand the functionality of specific microbial communities and therefore better inform, operate, and design anaerobic resource recovery processes for maximum effectiveness.
ACKNOWLEDGEMENTS

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<thead>
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<tbody>
<tr>
<td>AnMBR</td>
<td>Anaerobic Membrane Bioreactor</td>
</tr>
<tr>
<td>BOD</td>
<td>Biochemical Oxygen Demand</td>
</tr>
<tr>
<td>BOD$_5$</td>
<td>5-day Biochemical Oxygen Demand</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical Oxygen Demand</td>
</tr>
<tr>
<td>CSTR</td>
<td>Constantly Stirred Tank Reactor</td>
</tr>
<tr>
<td>DWW</td>
<td>Domestic Wastewater</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HRT</td>
<td>Hydraulic Retention Time</td>
</tr>
<tr>
<td>mcrA</td>
<td>Methyl Coenzyme M reductase</td>
</tr>
<tr>
<td>MG-RAST</td>
<td>Metagenomic Rapid Annotations using Subsystems Technology</td>
</tr>
<tr>
<td>NPDES</td>
<td>National Pollutant Discharge Elimination System</td>
</tr>
<tr>
<td>NTC</td>
<td>No Template Control</td>
</tr>
<tr>
<td>OLR</td>
<td>Organic Loading Rate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcription</td>
</tr>
<tr>
<td>sCOD</td>
<td>Soluble Chemical Oxygen Demand</td>
</tr>
<tr>
<td>SOP</td>
<td>Standards of Procedure</td>
</tr>
<tr>
<td>SRT</td>
<td>Solids Retention Time</td>
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<td><strong>Full Form</strong></td>
</tr>
<tr>
<td>------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>TMP</td>
<td>Transmembrane Pressure</td>
</tr>
<tr>
<td>TSS</td>
<td>Total Suspended Solids</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile Fatty Acids</td>
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1.0 INTRODUCTION

As society continues to develop, commonplace processes are being analyzed and reinvented to optimize their effectiveness. Wastewater treatment plants have become a recent focus of this reinvention as their goal has transformed into producing a superior quality effluent to protect natural environments while additionally recovering resources otherwise wasted, which are considered higher value end products.\(^1\) One development that has gained attraction is the incorporation of anaerobic treatment into the water treatment train.\(^2\) Anaerobic treatment allows for the conversion of waste into biogas that is high in methane content and can be captured and utilized as an energy source.\(^3\) While this is commonly used for sludge stabilization in digesters,\(^4\) it has potential for achieving similar effluent qualities while offsetting the aeration costs in traditional activated sludge systems, which accounts for approximately 0.75% of the total U.S. energy consumption.\(^5,6\) Anaerobic processes additionally produce a smaller amount of waste solids, which would decrease the total energy needed to process these solids.\(^4\) By decreasing the energy demands of wastewater treatment, we will decrease its environmental footprint and design a more sustainable water management strategy.

Due to the slower growth rate of anaerobic microbial populations compared to aerobic microbes, a much longer solids retention time (SRT) must be achieved to obtain similar treatment levels.\(^4,7\) Since anaerobic bacteria are poor settling, traditional gravity separation is not sufficient to maintain the desired SRT.\(^8\) However, the incorporation of membranes allow solids to stay in the system long enough to achieve effective
This kind of system is known as an anaerobic membrane bioreactor (AnMBR). AnMBRs allow for short hydraulic retention times (HRTs), which is desired to treat high flow rates of wastewater.

One of the chief goals in wastewater treatment is the removal of constituents contributing to the chemical oxygen demand (COD) of the water. If these constituents were not removed, they would deplete the water they are being discharged into of oxygen that is critical for natural ecosystems existing in that water body. Consequently, the oxygen demand of waste streams are regulated with typical National Pollutant Discharge Elimination System (NPDES) limits consisting of 30 mg/L BOD₅. BOD₅ is a measurement of biological oxygen demand, or the extent to which a biological system, simulating what would be present in a natural body of water, would oxidize the discharged waste stream. The BOD₅ test is a 5-day long test. Due to the tedious nature of the test, COD is often used as a replacement because it can be completed in a matter of hours. Instead of using a biological system to oxidize the discharged waste stream, a COD test uses a strong chemical oxidizer, potassium dichromate, that will oxidize all organic compounds in the sample. In domestic wastewater COD values can be approximated to be 2.1 times the BOD₅.

In the United States, anaerobic systems are commonly used for high strength wastewater with COD concentrations larger than 4-5 g/L due to their ability convert this COD into the commodity of biogas at a high enough rate to heat a bioreactor to mesophilic temperatures of 35°C. Since the temperature of untreated domestic wastewater (DWW) in the United States ranges from 3 to 27°C with an average value of
16°C, it is necessary to characterize the performance of AnMBRs for DWW treatment within these ranges of seasonal variation. This temperature range has an additional significance when considering the growth rates of methanogens. Figure 1 shows the relative growth rates of methanogens at different temperatures as they relate to the optimum growth rate of thermophiles, showing that mesophilic methanogens have the greatest growth during operation between 35°C and 40°C. It also shows that psychrophilic methanogens have greatest growth at 15°C with an apparent decline in dominant growth between 20-25°C. Since influent COD concentrations needed to heat a bioreactor to mesophilic temperatures are an order of magnitude higher than typical domestic wastewater influents, AnMBRs must be proven to operate in ambient temperatures in order to be economically viable. Furthermore, wastewater in the upstate of South Carolina ranges in seasonal temperature from 13-27°C, giving this temperature range...
additional importance and contributing towards the rational behind selecting 15-35°C as the focus of this study. The performance of the reactor is likely to change as a result of the changing microbial community that is responsible for converting complex organics in the waste (like carbohydrates, proteins, and lipids) to their end product of methane through a complex series of biochemical reactions. Since microbial growth and activity is affected by temperature, there exists a need to have a more comprehensive understanding of the changing microbial community that exists within AnMBRs in changing climates. By further understanding the microbial components and relating them with the performance of the AnMBR, we can better understand the functionality of specific microbial communities and therefore better inform, operate, and design anaerobic resource recovery processes for maximum effectiveness.

Better understanding of the microbial components will come through DNA and RNA analysis of the biomass in the AnMBR reactor. Results from DNA analysis will be able to show us the community structure and potential function of the system, as not all DNA present is actively being used for the ultimate synthesis of proteins. Changes in the community structure and potential function as a result of temperature will show how closely operational temperature affects AnMBR performance and could give a hint as to whether or not temperature control would be needed in a full-scale AnMBR. RNA analysis will be able to show a glimpse into the functional aspects of the microbial community, as RNA is only translated if it is actively being used to transcribe proteins. These results offer a more quantitative relationship between the microbial community and their function.
While AnMBRs theoretically offer an energy efficient means of treating wastewater, it should be noted that the goal of wastewater treatment exceeds simply the removal of COD and solids from the water stream. The aim of treatment also includes nutrient control, pathogen control, and meeting whole effluent toxicity standards. When understanding the significance of the microbial analysis, it should also be noted that there are some natural variations in microbial community and function. Communities can adapt, and multiple communities can offer the same function. This study contributes only a piece in the overall effort to determine the effectiveness of AnMBR application for domestic wastewater treatment as a function of the operating temperature.
2.0 PREVIOUS RESEARCH

The desire to decrease energy requirements for domestic wastewater treatment along with the development in membrane technology has sparked an academic and commercial interest in the performance and application of AnMBRs. Studies have covered a variety of topics including reactor configuration, types of membrane, and parameters that influence AnMBR operation. Reactors have varied in configuration by having the membrane unit submerged internally as well as located in an external reactor. A variety of membranes of have been tested for domestic wastewater treatment, i.e., flat sheet, tubular, and hollow fiber. Other studies look at different aspects of the AnMBR system, including operational strategies to prevent membrane fouling, effects of HRT and SRT on system performance, impact of the biofilm layer, impact of the inoculum source on the ability for AnMBRs to adapt to temperature changes, and the effects of changing reactor temperature.

Understanding the microbial community responsible for wastewater treatment and methane production in AnMBR systems has become more popular as this technology is proving its feasibility for treatment of various kinds of wastewater. Understanding the microbial community responsible for treatment is not simply an academic interest, but could also lead to a more informed operation and design of AnMBRs. Some studies have identified and classified bacterial and archaeal communities in the suspended biomass and biofilm layer. However, the development of polymerase chain reaction (PCR) techniques offers a more precise method for contributing data similar to those found in studies using outdated techniques. One study using PCR techniques and DNA sequencing
compares 16S rRNA relative abundance and activity in suspended biomass and a biofilm layer on the membrane surface at a range of psychrophilic temperature conditions (15, 12, 9, 6, and 3°C). Another study compares 16S rRNA relative abundance and activity in suspended biomass over a larger range of temperatures (35, 25, 20, and 15°C), to treat recycled food wastewater rather than treating domestic wastewater. Smith et. al published a study reporting the 16S rRNA relative abundance and activity alongside the relative methyl coenzyme-M reductase (mcrA) gene expression in suspended biomass and varying biofilm layers at an operating temperature of 15°C. The mcrA gene is a functional gene that has been related to the production of methane by methanogenic bacterial communities. It promotes the reduction of the methyl group attached to coenzyme-M and release of methane which allows for the characterization of the methanogenic community function when targeted in PCR. The mcrA gene is demonstrated to be a useful biomarker of methanogens by the significant positive correlation found between methane production and mcrA gene copy numbers and transcripts. The expression of this mcrA gene in AnMBRs has been focused on psychrophilic temperatures (<15°C) and has yet to be analyzed as a consequence of complete seasonal temperature variation. Pilot-scale AnMBRs have reported operation between 17-35°C, yet have not completed advanced microbial analysis within this temperature range. Additionally, many of the studies preforming advanced microbial analysis have been completed using synthetic wastewater. These techniques have yet to be applied for an AnMBR treating real domestic wastewater.
Metagenomic analysis has been helpful in the study of anaerobic communities, particularly for the optimization of anaerobic digestion, as it has led to a deeper understanding of the microbial community and potential methane-producing pathways. This technique has been useful towards understanding anaerobic digestion, but has not yet been applied to AnMBRs used for treatment of domestic wastewater and offers a potential means to better optimize AnMBR design and operation. Since this treatment technology relies on the anaerobic microbial community to achieve proper performance, understanding that community and how it relates to performance is crucial to better correlating how the performance is affected by the design and operation of the system.

These previous studies serve as a basis of comparison as this study will seek to compare mcrA gene abundance and expression in suspended biomass samples taken from a wide range of temperatures (35, 25, and 15ºC). How the entire metagenome changes as a function of temperature will also be compared in an effort to understand the effect of operational temperature on both community structure and potential function. Additionally, a bench scale AnMBR has not analyzed the changing microbial community in a reactor that has operated long term (e.g., ≥ 2 months) with domestic wastewater as the feed. Most previous studies having only used synthetic feeds made to mimic domestic wastewater. Operating with real DWW for a long-term study will aid in understanding the true performance of AnMBRs’ treatment capabilities as well as promote a microbial community that more accurately reflects what might exist if implemented in the field.
3.0 RESEARCH OBJECTIVES

The main objective of this research is to investigate the relationship between the microbial community and AnMBR performance as a function of temperature and develop an understanding of possible inhibitions to pathways involved in the conversion of primary effluent to methane and carbon dioxide. Specific objectives are as follows:

1. **Evaluate change in reactor performance as a function of varying bioreactor temperature**

   **Hypothesis:** As the operational temperature of the AnMBR decreases from 35°C to 25°C and 15°C, the system will be able to achieve similar COD removal percentages due to the membranes ability to maintain a high solids concentration in the system; however, there will be a decrease in methane production at 15°C due to temperature inhibition of the microbial community.

2. **Quantify mcrA gene abundance and expression in RNA as a function of varying bioreactor temperature**

   **Hypothesis:** As the operational temperature of the AnMBR decreases from 35°C to 25°C and 15°C, the mcrA gene will show similar abundance and expression for 35°C and 25°C and decrease at the lowest operational temperature due to temperature inhibition of the methanogen community.
3. Establish a relationship between the microbial community structure and function at different temperatures, and the membrane performance.

**Hypothesis:** As the operational temperature of the AnMBR decreases from 35°C to 25°C and 15°C, the microbial community structure will shift to favor organisms that thrive in colder climates due to nature’s inclination towards adaptation.
4.0 MATERIALS AND METHODS

4.1 AnMBR Operation

A bench-scale AnMBR system with a 2-L continuously stirred tank reactor (CSTR) and an external membrane module with continuous operation was used in this study. Primary clarifier effluent was collected weekly from ReWa’s Mauldin Road Water Resource Recovery Facility in Greenville, SC and used as the feed. This plant was chosen due to its proximity and continuously operating primary clarifier. Due to the nature of this feed source, slight variations in the AnMBR feed over time were expected although samples were collected at approximately the same time each collection day to prevent sampling inconsistencies. The collected wastewater was sparged with Argon gas, stored in a refrigerator kept at 4°C, and then pumped (Cole-Parmer Masterflex peristaltic pump, drive model number: 07551-20, pump head model number: 07516-00) into the 2-L bioreactor, previously seeded with 10% mesophillic sludge from ReWa’s anaerobic digester at the Mauldin Road Water Resource Recovery Facility. The bioreactor was mixed by a magnetic mixer and a high recirculation rate through the external membrane module.

The AnMBR was operated with a hydraulic retention time (HRT) ranging from 0.6 to 1.5 days, or 14 to 36 hours. Solids were only wasted for sampling; consequently, the AnMBR operated with a high solids retention time (SRT), averaging 550 days over the 275-day operational period. A list of wasting events and calculation of the average SRT are seen in Appendix A. The bioreactor was fit with a pH and temperature sensor (Sensorex pH/ORP Transmitter TX 300). Over the 275-day operation, the pH in the
bioreactor stayed between 7.0 and 7.4 with no pH control. With the help of an image processing technique, a webcam constantly monitored the bioreactor and was programmed to keep the water level stable by altering the feed pump speed. This technique is described in further detail in Appendix B. Biogas produced in the reactor was collected in a 1-L Tedlar bag. The anaerobic sludge then passes through an external cross-flow hollow fiber membrane module. This module was fitted with Koch Puron hollow fibers with a pore size of 0.03 µm. A permeate pump (Cole-Parmer Masterflex peristaltic pump) was operated at a constant flow rate (2.8 mL/min), while a pressure sensor (Omega PX309-015CGI) recorded the transmembrane pressure. A weighing scale was used to monitor the effluent mass. During operation, the membrane was cleaned by backwashing for 200 seconds at 32 mL/min every 30-60 minutes. If increasing fouling occurred, such that it compromises the achievable HRT, the membrane was taken offline and chemically cleaned using 1% NaOCl, followed by 1% NaOH, and lastly followed by 1% citric acid. On Day 232, the membrane unit was additionally attempted to be cleaned through sonication, where the module was placed in a sonicator for 4 hours. The recirculating concentrate passed through a water bath with controlled temperature using a heater/chiller (LabTech RH23-6A). AnMBR operation and data acquisition was automated and computer controlled using LabView. The AnMBR temperature was initially maintained at 35ºC until stable conditions were achieved. Stable conditions are defined as a period of two weeks with relatively stable methane production and COD removal. The temperature of the bioreactor was then decreased to 25ºC over the course of one week and maintained at 25ºC until stable conditions were achieved. This process was
repeated for operation at 15°C. This temperature range was chosen due to its relevance in average wastewater temperatures in South Carolina as well as the locations of many pilot scale plants. Operation at 35°C was conducted to have a point of comparison with the other two operating temperatures, since its performance was assumed to be best. Operational parameters discussed in this section are summarized in Table 1. A schematic of the bench-scale AnMBR system can be seen in Figure 2. A picture of the system can be seen in Figure 3. Additional pictures of the AnMBR set up can be seen in Appendix C.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<td>Volume of Reactor</td>
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</tr>
<tr>
<td>Reactor Type</td>
<td>CSTR</td>
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<tr>
<td>HRT</td>
<td>0.6 - 1.5 d</td>
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<tr>
<td>SRT</td>
<td>550 d</td>
</tr>
<tr>
<td>Temperatures</td>
<td>35°C, 25°C, and 15°C</td>
</tr>
<tr>
<td>Feed Source</td>
<td>Primary Clarifier Effluent</td>
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<td>Inoculum</td>
<td>10% mesophilic anaerobic digester sludge</td>
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<tr>
<td>Membrane Unit</td>
<td>External Koch Puron®</td>
</tr>
<tr>
<td>Fouling Control</td>
<td>Backwashing with permeate for 200 s every 30-60 min</td>
</tr>
<tr>
<td>Chemical Cleaning</td>
<td>As needed with 1% NaOCl, 1% NaOH, followed by 1% citric acid</td>
</tr>
</tbody>
</table>
Figure 2. Schematic of bench-scale AnMBR

Figure 3. Image of the bench-scale AnMBR in operation
4.2 Changes in Operation During Course of Study

4.2.1 Water Level Control

As previously stated, during operation at 35°C and 25°C, the water level of the system was controlled through image processing. A webcam captured an image of the reactor, which was then processed using MatLab, determining the percentage of headspace in the reactor. If the percentage of headspace was above a set value, then the feed pump would stop. If the percentage of headspace was below the set value, the feed pump would turn on and add feed to the bioreactor at 2.9 mL/min. During operation at 15°C, condensation inside the reactor prevented the effectiveness of image processing for water level control. To allow for accurate water level control, the configuration of the system was altered by placing the scale previously used for permeate collection under the bioreactor and using the weight as a water level sensor. If the weight went above a set value, the feed pump would stop. If the weight was below the set value, the feed pump would turn on to 2.9 mL/min. This alteration proved successful and is further described in Appendix B.

4.2.1 Membrane Module

Since the start of AnMBR operation with domestic wastewater, the membrane module consisted of an external submerged hollow fiber membrane module. During the first operational period of 148 days, the module consisted of Koch Puron® fibers with a pore size of 0.03 µm and a total surface area of 151 cm² (Figure 4a). Due to irreversible fouling, the module was replaced on day 148 with another Koch Puron hollow fiber membrane module with a higher surface area of 290 cm² (Figure 4b). This change was
made to maintain the HRT and hydraulic flux in a desired range. HRT and flux are further discussed in Section 5.1.1.

Figure 4. Hollow fiber membrane module with a) 151 cm² and b) 290 cm² surface area. Red arrows depict the direction of recirculation flow. The permeate is pulled out of the top of the membrane module.

4.3 Objective I: AnMBR Performance Analysis

4.3.1 Reactor Performance

Reactor performance was analyzed by monitoring solids concentrations, COD removal and effluent nutrient concentrations, and methane production. Duplicate samples of the feed and permeate were taken every 2-3 days and analyzed for the following parameters: TSS, VSS, COD, and sCOD. TSS and VSS were measured using Standard Methods. COD concentrations were quantified using COD kits from Hach. Soluble COD was determined by passing the samples through a 0.20 µm filter prior to the Hach kit analysis. Duplicate samples were taken of the feed and permeate and analyzed for
ammonia, sulfate, and total phosphorus every week. Ammonia, sulfate, and total phosphorus were quantified using their respective Hach kits. Percent removal of each of these constituents were calculated from the results and compared between the different operating temperatures.

The volume of biogas produced in the Tedlar bag was measured every 2 days and analyzed for percentage of methane and carbon dioxide through gas chromatography. A 0.25 mL sample was collected using a Hamilton Gastight® GC Syringe and injected into the gas chromatography unit (Shimadzu GC 2014). The carrier gas was ultra-high purity Argon at 415 kPa and a flow rate of 10 mL/min. Each sample run was 5 minutes with the injector and thermal conductivity detector set to 150°C and the column set to 120°C. The dissolved methane content in the bioreactor was calculated from these results using published Henry’s Law constants and are listed below.\textsuperscript{36,37} Calculations are shown in Appendix E.

\begin{equation}
HLC = \text{Henry's Law Constant} = \frac{c_{\text{aqueous}}}{c_{\text{gaseous}}}
\end{equation}

\begin{align*}
HLC_{35°C} &= 0.030 \\
HLC_{25°C} &= 0.034 \\
HLC_{15°C} &= 0.040
\end{align*}

From day 150 to 275, the dissolved methane inside the permeate was measured experimentally by filling a 160 mL serum bottle with argon gas then sealing it with a septum. Half of the volume (80 mL) was filled with permeate from the AnMBR and then placed into an incushaker (VWR Incubating Mini Shaker) set at 55°C for 1 hour in order for the methane to equilibrate between the gas and liquid phase. A gas sample was then taken out of the serum bottle and analyzed through gas chromatography to find the
concentration of methane in the gas phase in the serum bottle. This was used to calculate the concentration still remaining in the liquid phase using Henry’s Law constant at 55°C of 0.023. The permeate methane that was gaseous was added to that which was still dissolved and taken to be the experimental value of dissolved methane in the permeate. The volume of gaseous and dissolved methane achieved was compared between the different operating temperatures. This same process was used prior to day 150, but with different permeate volumes and collection vials in an effort to find the most effective measurement. From day 1-48, a 5 mL syringe was used to collect 2 mL of permeate and 2 mL of argon gas. The syringe was incubated in a water bath kept at 55°C for 1 hour. From day 49-149, a 27 mL serum bottle was used with 10 mL permeate and 17 mL of argon gas, but the results were still very variable, so a larger volume was taken with the 160 mL serum bottle as described earlier.

The calibration curve for the gas chromatography was preformed by completing a GC analysis on triplicate samples of the following known methane volumes: 0%, 10%, 30%, and 50%. These methane concentrations were created using a known volume of methane gas (Airgas 50% methane, 50% air) and diluting it inside the gastight GC syringe with air. These samples were processed through the GC under the operating conditions listed above. The averages of their peak areas were peak areas were plotted to determine the slope, or reference factor, which was later used to associate a certain peak area with a given percentage volume of methane.

Membrane performance was assessed through the continual recording of transmembrane pressure (TMP) and flux through the membrane.
Every 1-2 weeks a suspended biomass sample was taken from the bioreactor through a sampling valve located on the recirculation line and analyzed for TSS and VSS by the methods mentioned above.

4.3.2 Volatile Fatty Acids Analysis

In anaerobic environments, complex biodegradable particulates undergo hydrolysis, where they are converted to volatile fatty acids (VFAs) such as butyric and propionic acid. These VFAs then undergo acidogenesis where they are converted into acetic acid and/or hydrogen. Acetic acid and hydrogen are then converted to methane and carbon dioxide through a biochemical process referred to as methanogenesis. To understand the pathways to methane production occurring at varying temperatures, samples were taken from the bioreactor at each temperature and analyzed for VFA composition using high performance liquid chromatography (HPLC). By better understanding the important microbial pathways in the reactor, one can better link of microbial activity to changes in these intermediary products, and thus better understand bioreactor performance.

A HPLC unit fitted with an Aminex® HPX-87H Ion Exclusion column was used for the VFA analysis. The unit operates with 5 mM H₂SO₄ operating at a flow rate of 0.6 mL/min and a 210 nm wavelength. Each sample was processed in 45 minute runs with the temperature of the column set to 30°C. Each sample (5 mL) was taken from the bioreactor, centrifuged at 10000 × g for 5 minutes and then filtered through a 0.20 µm filter to separate the solids. A portion (1.5 mL) of the filtered sample was injected into the
HPLC unit. If the HPLC could not be run at the same time as sample collection, the samples were stored in vials at 0°C.

A calibration curve for VFA analysis using HPLC was created for each of the following VFAs: formate, acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate. Duplicates of the following concentrations were created of each VFA and processed using the operating conditions listed above: 0.1 mM, 0.5 mM, 1 mM, 5 mM, 10 mM, 25 mM. The peak areas and peak retention times were recorded for each concentration of each VFA and averaged. The values were plotted to determine the slope, or the reference factor, which was later used to associate a certain peak area with a given concentration of that VFA. These calibration curves can be seen in Appendix F.

4.4 Objective II: mcrA Gene Quantification

4.4.1 Quantitative Polymerase Chain Reaction on Bioreactor DNA Samples

DNA samples were processed using quantitative polymerase chain reaction in order to see how the abundance of the mcrA gene changed with respect to operational temperature of the bioreactor. While RNA processed through RT-qPCR is able to give us quantitative data on the number of mcrA transcript copies in functional use in the AnMBR, processing DNA through qPCR allows the analysis of relative abundance of mcrA genes at different operation temperatures, although not all genes present might be functionally in use.

Two times at steady operation at each temperature, triplicate homogeneous suspended biomass samples were taken for DNA analysis from the bioreactor and
processed onsite. Sampling occurred on days 42, 77, 162, 173, 261, and 270. If the samples could not be immediately processed, they were stored at -20°C. After thawing on ice, the biomass samples were centrifuged at 4000 \( \times g \) for 7 minutes. The supernatant was decanted, resulting in close to 0.2 g of pelletized biomass in the centrifuge tubes. DNA was extracted following the guidelines DNeasy® PowerLyzer® PowerSoil® Kit (Qiagen, 2016). Once extracted, the DNA was quantified using Qubit DNA kit and then stored at -20°C.

The samples were thawed on ice before processing for qPCR. Two of the technical replicates for each collection date were plated in triplicates in the 96-well plate. These samples were processed using SYBR Green MasterMix along with 5 \( \mu \)M mixed solution of forward primer mlas\(^{24} \) (5’-GGYGGTGTMGGNTTCACHCARTA-3’) and reverse primer \( mcrA \)-rev\(^{39} \) (5’-CGTTACATBGCGTAGTTVGGRTAGT-3’), sequence specific primers used to quantify methyl coenzyme M reductase (\( mcrA \)) gene expression. In preparing the 96-well plate, 19 \( \mu \)L of SYBR-green master mix was used and 1 \( \mu \)L of sample was added since the concentration of DNA was between 35-85 ng dsDNA/\( \mu \)L.

Thermocycling conditions consisted of an initial 2 min denaturation at 95°C, followed by 5 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 45 s, followed by a slow ramp of 0.1 °C/s to an extension at 72°C for 30 s. This was followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 45 s, followed by a faster ramp of 1.6 °C/s to an extension at 72°C for 30 s. This was then followed by a final extension at 72°C for 5 min. A melt curve analysis was completed after the last cycle by denaturing at 95°C for 15 s, annealing at 50°C for 60 s and then slowing ramping at 0.15
°C/s until all samples are denatured again at 95°C, which was held for 15 s. This melt curve contributes to evaluating the specificity of the amplifications that occur.

There were three controls that were established in every 96-well plate. The first was an extraction blank in which a DNA extraction was completed without the addition of a sample. This assesses the quality of the extraction to see if there was any outside genomic contamination in the extraction process. The second control was a no template control (NTC) in which nuclease free water was used instead of the DNA template. This control monitored contamination and primer-dimer formation that could lead to false positive results. The last control was a positive control of DNA extracted from an anaerobic digester that the \textit{mcrA} gene was known to exist and amplify. This sample was taken from another study completed in the same lab at Clemson University.

From these qPCR results, we were able to achieve a quantification of the number of \textit{mcrA} gene copies present in the sample using a standard curve analysis. The standard curve was created by making a tenfold series dilution containing $10^7$, $10^6$, $10^5$, $10^4$, and $10^3$ copies of a pooled culture of \textit{mcrA} sequences. This pooled culture of DNA clones contained six different \textit{mcrA} sequences that were isolated and sequenced from various methanogens common in anaerobic digestion.\textsuperscript{26} Their nucleotide sequences can be found in GenBank® under accession numbers: HM800542, HM800549, HM800560, HM800574, HM800581, and HM800611. Having a variety of \textit{mcrA} genes allows the standard curve to more accurately reflect the diverse community that exists within the AnMBR. A standard curve was generated with 72.5% efficiency and an R\textsuperscript{2} value of
0.985. The slope was -4.222 and y-intercept 42.062. The threshold was set at 0.2 and baseline calculated between 3 and 5 cycles.

4.4.2 Reverse Transcription-Quantitative Polymerase Chain Reaction on Bioreactor RNA Samples

Two times at steady operation at each temperature, triplicate homogeneous suspended biomass samples were taken from the bioreactor and processed onsite. Sampling occurred on days 42, 77, 162, 173, 261, and 270. The biomass samples were centrifuged at 4000 × g for 7 minutes. The supernatant was decanted, resulting in close to 0.2 g of pelletized biomass in the centrifuge tubes. RNALater (Qiagen 2016) was added to the pelletized biomass and used for RNA preservation. The samples were incubated in RNALater at 4ºC for 24 hours and then stored at -20ºC until they could be further processed. RNA was extracted from these biomass samples following the guidelines from RNeasy® PowerSoil® Total RNA Kit (Qiagen 2017). Once extracted, the RNA was quantified using Qubit RNA kit then frozen at -80ºC.

The RNA concentrations of samples taken from the bioreactor were below detection limits and could not be amplified in the PCR, so the technical replicates at each sampling date were pooled together through an ethanol precipitation procedure. The RNA samples were thawed on ice and then the technical replicates of each sampling date were combined in a 2 mL centrifuge tube. Molecular grade 3M sodium acetate at a pH of 5.2 (ThermoScientific) was added at 1/10 the sample volume. Molecular grade 20 µg/µL glycogen (ThermoScientific) was then added so that the final glycogen concentration was 1 µg/µL. Ice-cold pure ethanol (200 proof molecular grade) was added at 2.5 volumes of
the sample. The sample was then incubated at -20ºC overnight. After incubation, the samples were centrifuged at 13,000 × g for 30 minutes. The supernatant was decanted as to not remove the pellet and then 2.5 volumes of ice-cold 70% molecular grade ethanol was added and allowed to contact the pellet for 2 minutes. The tubes were then centrifuged at 13,000 × g for 15 minutes. The supernatant was decanted so as to not remove the pellet, which was then air-dried for 5 minutes. This pooled RNA sample was then resuspended in TE buffer.

The pooled RNA sample was then transcribed to complementary DNA (cDNA) and later amplified using reverse transcription quantitative polymerase chain reaction (RT-qPCR). A two-step assay was performed. The first step of the assay was reverse transcription (RT) where the RNA was transcribed into cDNA using SuperScript™ II Reverse Transcriptase and a gene specific primer. The primer used was *mcrA*-rev. The resulting cDNA was quantified using a Qubit cDNA kit. The cDNA then served as the template for the second step of the assay: qPCR.

The cDNA was plated on a 96-well plate and PowerUp SYBR Green MasterMix was added to the samples along with 5 µM mixed solution of forward primer mlas and reverse primer *mcrA*-rev, sequence specific primers used to quantify *mcrA* gene expression. Thermocycling conditions were consistent with those listed in Section 4.4.1.

Four controls were established in every 96-well plate to accurately monitor the RT-qPCR process. The first was no reverse transcriptase control (-RT) where reverse transcription was completed with nuclease free water as the template rather than RNA. This control monitored if there was any genomic DNA contamination in the RT reagents
used. The second was an RNA only control where RNA was used as the template rather than the cDNA generated from RT. This control showed how pure the RNA sample is and if the cDNA could potentially be contaminated with genomic DNA. The last two controls are the same as those included in Section 4.4.1: a no template control (NTC) and a positive control. Again, the NTC monitored contamination of the qPCR reagents and primer-dimer formation. The same positive control sample as described in Section 4.4.1 was used to complete RT-qPCR.

From these RT-qPCR results, quantification of the \textit{mcrA} transcripts present in the sample was achieved using a standard curve analysis prepared in the same manner described in Section 4.4.1. The number of transcripts was normalized per mass of cDNA in that sample. The effect of temperature on the number of \textit{mcrA} gene transcripts was further analyzed as discussed later in this thesis.

\textbf{4.4.3 Reverse Transcription-Quantitative Polymerase Chain Reaction on Batch Samples at Varying Temperatures}

Because RNA samples taken from the bioreactor and stored in RNALater were not at high enough concentration to amplify using RT-qPCR, a mini batch scale experiment was designed to assess the effect of bioreactor temperature on the functional expression of the \textit{mcrA} gene in biomass from the AnMBR. Six 12 mL samples were taken from the AnMBR bioreactor and placed in a sterile 50 mL centrifuge tube. Each sample was spiked with 2 mM acetate (made from sodium acetate) and thoroughly mixed. The tubes were sparged with Argon gas to remove oxygen and then sealed to keep the system anaerobic. Duplicate samples were placed in an incubator operating at 35°C.
and another two were placed in an incusaker operating at 25°C. The final two samples were kept at 15°C by submerging the vials in the temperature controlled water bath of the AnMBR. The samples were kept at these temperatures for 72 hours. At the end of 72 hours, RNA was extracted in the same manner described in Section 4.4.2. After extraction, the total RNA was then cleaned using Qiagen DNase Max Kit to remove any remaining genomic DNA, leaving behind cleaned RNA for further processing. This cleaned RNA was then processed through RT-qPCR in the same manner described in Section 4.4.2. In preparing the 96-well plate, 18 µL of SYBR-green master mix was used and 2 µL of sample was added with concentrations of cDNA ranging between 3.7-5.0 ng cDNA/µL. A standard curve was generated with 65.7% efficiency and an R² value of 0.998. The slope was -4.559 and y-intercept 43.852. The threshold was set at 0.2 and baseline calculated between 3 and 5 cycles.

4.5 Objective III: Metagenomic Community Analysis

The DNA samples that were used for qPCR analysis, as mentioned in Section 4.4.1, were prepared for metagenomic sequencing. First, 5 µL of the extracted DNA was portioned off to analyze with a bioanalyzer. The bioanalyzer was used to evaluate the quality of DNA by determining the DNA strand lengths in the sample. This was completed to ensure good quality DNA samples were sent to be sequenced. In this case, good quality DNA samples were determined by having long strand fragments, or a large number of base pairs (>500 bp fragments). Methods for analysis follow the Agilent DNA 12000 kit (Agilent Technologies 2018).
Once the quality of DNA was assured, the samples were aliquoted and sent to Novogene Corporation in Sacramento, CA for metagenomic sequencing using Illumina high-throughput sequencer with paired-end sequencing strategy. The remaining DNA samples were frozen at -80 °C for possible future analysis. Data shown in this thesis was processed and analyzed using MG-RAST in order to see how the community structure changes in response to changes in temperature. Due to sampling dates and the time it took for Novogene to sequence the samples, at the time of this thesis, the sequenced data was only received for operation at 35°C and 25°C. Once the 15°C sequences are received, they will be included in the comparison among the operating temperatures. It should also be noted that the analysis included in this thesis shows simple community structure shifts; however, with entire metagenomic sequencing, there is potential for extended analysis into functional microbial changes in the AnMBR as a consequence of temperature variation.
5.0 RESULTS AND DISCUSSION

5.1 Objective I: AnMBR Performance

Table 2 shows the characteristics of the primary clarifier effluent wastewater used as the feed to the AnMBR. All values reported are averages from at least 43 days, and so standard errors are listed instead of standard deviation due to the large sample size. VFAs are the exception to this, whose value reported is averaged over 6 samples. The table shows a significant concentration of COD due to VFAs in the feed. This concentration could be due to the fact that Mauldin Road WWTP allows the solids to ferment in the primary clarifier to produce VFAs necessary for biological phosphorous removal occurring downstream.

Table 2. Average influent wastewater characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Mean</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSS</td>
<td>mg TSS/L</td>
<td>111.2</td>
<td>7.9</td>
</tr>
<tr>
<td>VSS</td>
<td>mg VSS/L</td>
<td>108.9</td>
<td>7.8</td>
</tr>
<tr>
<td>Total COD</td>
<td>mg COD/L</td>
<td>424.7</td>
<td>13.0</td>
</tr>
<tr>
<td>Soluble COD</td>
<td>mg COD/L</td>
<td>205.7</td>
<td>5.3</td>
</tr>
<tr>
<td>VFA</td>
<td>mg COD/L</td>
<td>50.1</td>
<td>6.0</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>mg SO$_4^{2-}$/L</td>
<td>59.3</td>
<td>1.7</td>
</tr>
<tr>
<td>NH$_3$-N</td>
<td>mg NH$_3$-N/L</td>
<td>36.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Total P</td>
<td>mg PO$_4^{3-}$/L</td>
<td>25.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>

5.1.1. Hydraulic Residence Time, Hydraulic Flux, and Transmembrane Pressure

As mentioned in Section 4.2.1, during the first operational period of 148 days, the module consisted of Koch Puron fibers with a pore size of 0.05 μm and a total surface area of 151 cm$^2$ (Figure 4a). The initial HRT during 35°C operation was 14 hours, with a membrane flux of 10 L/(m$^2$·h). Towards the end of 35°C operation, the HRT increased
and membrane flux declined due to irreversible membrane fouling. Specifically, HRT and membrane flux varied between 14 to 24 hours, and 10 to 6 L/ (m$^2$·h) respectively as shown in Figure 5 and 6. At the start of operation at 25°C, the membrane module was taken offline and chemically cleaned using 1% NaOCl followed by a 1% solution of NaOH and lastly followed by a 1% solution of citric acid. This accounts for the slight drop in HRT and increase in hydraulic flux seen at the beginning of operation at 25°C. However, during AnMBR operation at 25°C the HRT kept increasing while membrane flux was declining, and backwashing the membrane could not control fouling any further. Consequently, another hollow fiber membrane module with a higher surface area of 290 cm$^2$ was potted (Figure 4b) and replaced the old membrane module on day 148. From day 148 till 232 the HRT and the membrane flux varied between 12 to 19 hours and 4 to 6 L/ (m$^2$·h) respectively. On Day 232, the membrane unit with higher surface area was taken offline for cleaning via sonication. Upon reinstallation into the system, the membrane was found to be torn. The original membrane module was then placed back in-line and continued operation from Day 232 to Day 275. During this period the HRT increased from 18.5 to 36 hours and flux decreased from 8.4 to 3.9 L/ (m$^2$·h). The overall variations in HRT between 14 and 36 hours falls within range of published literature on AnMBRs, for both bench and pilot scale. As previously mentioned, one advantage to AnMBRs is that they effectively separate the HRT from the SRT, so with decreasing HRT they can treat a larger flow of wastewater with a smaller footprint. Many studies operate at the lower end of the range achieved in this study, obtaining HRTs between 2.2-19 hours. The changing HRT should be considered when comparing the
performance of the AnMBR at different operational temperatures because as the HRT decreases, the organic loading increases, causing an increase in biogas production.\(^7\) The organic loading rate (OLR) inside the reactor stayed between 300 and 800 mg COD/(L·d) during 275 days of AnMBR operation. Apart from the affecting HRT, the unstable OLR was also due to the varied COD concentration of the domestic wastewater due to rain events. COD concentrations and the OLR are further discussed in Section 5.1.3.

![Figure 5](image)

**Figure 5.** Hydraulic retention time of the AnMBR over the 275-day operation of the AnMBR.

The changes in flux seen in **Figure 6**, with variances between 10 L/ (m\(^2\)·h) and 3 L/ (m\(^2\)·h), are consistent with published literature; however, pilot-scale systems typically operate with higher fluxes, ranging from 6-17 L/ (m\(^2\)·h).\(^{20,28,42,43}\) The changing flux achieved by the system is due to changing membrane performance, most likely induced by fouling.
The reduced membrane performance can additionally be seen by analyzing the TMP. **Figure 7** shows the average TMP taken every 10th day of operation of the AnMBR. Its performance shows the same trend as the changes in HRT seen in **Figure 5**. On day 1, the TMP was 6 psi. This somewhat high starting TMP is due to the previous use of the membrane unit in another study with synthetic wastewater. After 20 days of operation with domestic wastewater the membrane reached the maximum transmembrane pressure for the Koch Puron® hollow fiber membranes of 9 psi. This quick deterioration in membrane performance is attributed to the initial long intervals between backwashing. Operational parameters were then changed to backwash every 30-60 minutes and membrane performance was recovered. For the rest of operation at 35°C, membrane performance was controlled through interval backwashing. As previously mentioned, on day 97 the membrane was chemically cleaned, restoring the TMP to 6.5 psi. However,
the TMP continuously increased until day 148 when it was decided to install a new membrane unit with increased surface area. The TMP increased gradually from 1.6 to 4.4 psi over the course of 84 days. The first membrane module was then put back in place and the TMP increased from 3.5 to 5.7 psi. Although the TMP was smaller at the end of operation at 15°C than it was at the beginning of operation at 35°C, the flux was still lower due to an increase in viscosity of the water. All TMPs reported in Figure 7 are normalized to the viscosity of water at 35°C. Viscosities of water at each temperature were taken from Kestin et al. 44

![Figure 7](image_url)  
**Figure 7.** Transmembrane pressure experienced by hollow fiber membrane unit over the 275-day operation of the AnMBR. All TMPs are normalized to the viscosity of water at 35°C.
5.1.2. Solids Removal

The total suspended solids of the feed, permeate, and bioreactor are shown in Figures 8 and 9. The TSS of the feed averaged around 120 mg/L. The TSS of the permeate remained consistent during operation at 35°C, 25°C, and 15°C averaging 3 mg/L. The standard error of this measurement is 0.8 mg/L. It was expected that the AnMBR would produce an effluent with a small TSS concentration due to the small pore size of the membranes in use. The concentration of solids in the permeate is most likely due to a small level of microbial growth in the permeate collection system. The TSS of the bioreactor averaged around 3000 mg/L. As previously mentioned, at the start of operation the bioreactor was seeded with sludge from an anaerobic digester to have a solids concentration of 2500 mg/L.

![Graph showing TSS](image)

**Figure 8.** Total suspended solids of the feed and permeate of the 275-day operation of the AnMBR.
As seen in Figure 9, the solids concentration increased during operation at 35°C, eventually averaging at 2960 mg/L during operation at 25°C and 15°C. The concentration of solids decreased during operation at 25°C due to operational mishaps and unplanned loss of solids from the reactor. There was an additional loss of solids when the membrane unit was changed. Higher variation of TSS in the bioreactor during operation at 15°C is most likely due to nonuniform mixing prior to sampling related to the moving of the electric mixer described in Section 4.2.1.

As mentioned in Section 4.1, the average SRT over the 275-day operation was 550 days. Long SRTs in AnMBRs promote the growth of methanogenesis, helping the reactor become more efficient in terms of methane production and have little impact on COD removal. However, long SRTs are also coordinated with increased biomass concentrations, which lead to membrane fouling. Due to random sampling times and loss of biomass due to operational mishaps, although the SRT averaged 550 days, it is likely to have fluctuated over the 275-day operation. Even considering variations in SRTs, the
SRT achieved is an order of magnitude higher than common in anaerobic digestion.\textsuperscript{4} As regulated by U.S. 503, anaerobic digesters operating at 35°C must have a minimum SRT of 15 days to ensure proper pathogen reduction in solids treatment.\textsuperscript{4} AnMBRs ability to keep high SRTs allow the system to support enough biomass for effective treatment during low temperature operation, when biomass growth rates are reduced.\textsuperscript{13,45}

These results show the capability of the AnMBR to keep a high solid concentration in the reactor while still meeting regulated low permeate solids concentrations below 30 mg TSS/L.

5.1.3. Chemical Oxygen Demand Removal

The AnMBR feed had an average total COD concentration of 425 mg/L and soluble COD concentration of 206 mg/L over the 275 day operational period included in this study. Due to the day-to-day variations that occur at a wastewater treatment plant, the feed COD varied in concentration as shown in Figure 10. This variation gives a more practical understanding of how an AnMBR would operate receiving domestic wastewater. The COD concentration in the permeate averaged 70.5 mg/L during operation at 35°C, 60.7 mg/L during operation at 25°C, and 77.0 mg/L during operation at 15°C. Increases in the permeate COD concentration occurred after chemically cleaning the membrane on day 97, and changing the membrane module on days 148 and 232 and can be distinguished in Figure 10. These average effluent concentrations are consistent with some pilot-scale plants;\textsuperscript{30} however, bench-scale AnMBRs using synthetic feed have achieved lower COD concentrations around 30 mg/L in the permeate.\textsuperscript{15,20} Given the assumption that in domestic wastewater COD values can be approximated to be 2.1 times
the BOD₅,⁴ the permeate produced in this study has a BOD₅ very close to the typical NPDES permit of 30 mg/L. However, considering the fact that many VFAs are non-detect in the permeate, there is reason to assume that this COD: BOD₅ ratio would be much higher than 2.1. Studies treating real domestic wastewater that have reported BOD₅ measurements in the permeate measure COD: BOD₅ ratios between 3.2 and 4.8.²¹,³⁰ Using these ratios signify that the permeate BOD₅ concentrations achieved in this study would be safely below the typical NPDES permit of 30 mg/L, proving that AnMBRs are a feasible alternative to conventional activated sludge when considering biochemical oxygen demand removal. If slightly excess BOD₅ concentrations occurred, they could be removed in downstream operations such as a trickling filter.

The percentage of COD removal remained relatively constant with variations due to membrane cleaning, changing the membrane module, and large variations in the

![Figure 10. Chemical oxygen demand concentrations in the feed and permeate over the 275-day operation of the AnMBR.](image)
organic loading rate (OLR). The OLR organic loading rate ranged between 400 and 1300 mg COD/(L·d) during 275 days of AnMBR operation. The varying OLR can be attributed to changing HRT caused by membrane fouling and varied COD concentration of the domestic wastewater due to rain events. The relationship between the OLR and the percentage of COD removal achieved by the AnMBR is seen in Figure 11. Operational results were averaged from days 39-79 for 35°C, days 155-184 for 25°C, and days 210-275 for 15°C to produce the bar graphs in Figure 12, which displays the average percentage removal at each of the three operating temperatures, all averaging between 82.2 and 83.2 % COD removal with no obvious difference in-between the operating temperatures. This performance is close to, but slightly below the 88% COD removal achieved by most studies using actual domestic wastewater.\textsuperscript{34,43,46} Again, studies using synthetic wastewater obtained a higher COD removal.\textsuperscript{18,20,47}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure11.png}
\caption{COD removal and loading rate over the 275-day operation of the AnMBR. Moving averages shown are calculated with a 10 period intervals.}
\end{figure}
If undesired electron acceptors are in the feed or bioreactor, they are expected to consume COD in the otherwise electron-acceptor-less reactor. Each feed tank was sparged with Argon gas to remove dissolved oxygen, the thermodynamically most favorable electron acceptor. However, trace amounts of oxygen were continuously measured in the biogas. This oxygen could be responsible for some COD removal as well as decreased methane production. The feed was also tested for sulfate, a common electron acceptor. The feed had consistently high sulfate concentrations. Reducing sulfate to sulfide is more energetically favorable than reducing CO\textsubscript{2} to methane; therefore, sulfate reduction is often difficult to suppress in methanogenic systems.\textsuperscript{15} Consequently, the sulfate was measured in the feed and permeate in order that its reduction might be accounted for in later calculations. On average, 65% (37 mg/L) of sulfate was reduced in the bioreactor. During operation at 35°C, 58.7% of sulfate was reduced. At 25°C and

![Figure 12. Average COD removal of the AnMBR at 35°C, 25°C, and 15°C. Average values were taken for each temperature from days 39-79, 155-184, and 210-275 respectively. Standard errors are included.](image-url)
15°C, 70.8% and 66.8% of sulfate was reduced, respectively. The average feed and permeate concentrations are displayed in Figure 13. While the sulfate reduction percentages are slightly different between each temperature, these differences are most likely due to sulfate loading differences that are similar to the OLR differences shown in Figure 11. By calculating the COD equivalence of the sulfate that was reduced, the percentage of the total COD removed that was due to sulfate reduction could be calculated. Over the three temperature operations, an average of 6.5% of the total COD removal was due to the sulfate reduction. The average fraction of COD that is associated with the removal of sulfate at each temperature is displayed in Figure 12. Although the reduction of sulfate to sulfide does not technically represent COD removed due to sulfide contributing to COD itself, it represents a loss in COD that can eventually be converted to methane. If sulfate were not present, this portion of COD could be converted to methane;

![Figure 13](image-url)  
**Figure 13.** Average sulfate concentrations in the feed and permeate at 35°C, 25°C, and 15°C. Average values were taken for each temperature from days 39-79, 155-184, and 210-275 respectively. Standard errors are included.
therefore, it should be accounted for when comparing methane production rates to the theoretical values where sulfate is not included. A review on pilot scale AnMBRs noted that the methane yield is highly impacted by the presence of sulfate in the feed, with the average yield almost doubling for systems with low sulfate concentrations. Additional reviews have stated that high sulfate concentrations can be a significant microbial barrier to the application of AnMBRs especially as low temperatures become favorable to hydrogentrophic sulfate reducers. Sulfate reduction dominates over methanogenesis, but it does not completely inhibit methane production if enough electron donor is present. Methane production and yield will be discussed more thoroughly in the following sections.

5.1.4 Methane Production

The gaseous methane production increased gradually, stabilizing at approximately 58 mL CH₄/d, (0.029 m³ CH₄/ [m³ bioreactor d]) for 35°C, 56 mL CH₄/d (0.028 m³ CH₄/ [m³ bioreactor-d]) at 25°C, and 27 mL CH₄/d (0.014 m³ CH₄/ [m³ bioreactor-d]) at 15°C operational temperatures. The methane production is shown over time in Figure 14. Variations in methane production could be related to changes in HRT, OLR, and microbial community shifts and adaptation periods. Instrument errors occurred with LabView on days 55, 195, and 250 causing the water level to drop in the AnMBR bioreactor. The water level dropping caused a vacuum effect inside the reactor, which allowed air to enter into the reactor. When this occurred, the bioreactor was sparged with Argon gas, effectively removing the oxygen from the system, but also bringing the methane concentration down. After these events, the AnMBR would have to recover as it
slowly picked up methane production once again. The difference in gaseous methane production at 15°C was expected as more methane is partitioned into the dissolved phase, as understood by its higher solubility at colder temperatures along with lower Henry’s Law constants (gas-liquid). The dissolved methane concentration over the AnMBR operational period is shown in Figure 15. Dissolved methane concentration was calculated using Equations 2-4. The percentage of methane in the dissolved phase is shown in Figure 16, showing that a slightly higher percentage of methane is in the aqueous phase at colder temperatures.
Figure 15. Dissolved methane concentration in the permeate over the course of 275 day operation of the AnMBR. The moving average shown was calculated with 10 period intervals.

Figure 16. Percentage of methane in the dissolved phase in the permeate over the course of 275 day operation of the AnMBR. The moving average shown was calculated with 10 period intervals.
As described in Section 4.3.1, there were three different methods of measuring dissolved methane that were used over the course of the study. The results from Day 1-150 were very variable and far from the calculated value, so their validity could be questioned. This variability was probably due to a lack of a sealed system when using a syringe, or lack of sufficient volume to accurately measure methane using the GC. The method using the 160 mL serum bottles gave results similar to the calculated value. Figure 15 shows that the dissolved methane concentration stays relatively constant between all three operating temperatures, with oversaturation of methane in the permeate not observed as seen in Smith et al.20 Again, variations in dissolved methane production could be related to the same changes in HRT, OLR, sparging events, and/or microbial community shifts and adaptation periods. The average dissolved methane concentration stabilizes at approximately 8.6 µg CH₄/L for 35°C, 8.4 µg CH₄/L at 25°C, and 7.6 mL µg
CH4/L at 15°C operational temperature. The standard errors for these averages are 0.3, 0.1, and 0.4 µg CH4/L respectively. Knowing that 3.99 µg COD/µg CH4, it can be seen that the methane in the permeate contributes to a very small portion of the remaining COD in the permeate: averaging 34.4 µg COD/L, 33.5 µg COD/L and 30.3 µg COD/L during operation at 35°C, 25°C, and 15°C, respectively. While there was not a vast difference in the concentration of dissolved methane concentration between the three operational temperatures, it can be seen that a larger percentage of methane is found in the aqueous phase at colder temperatures. This ratio is related to Henry’s Law mentioned in Equations 1, with methane having a higher propensity for the aqueous phase in colder temperatures. This trend can be seen in Figure 16. Calculations for the dimensionless Henry’s Law constant at each temperature are shown in Appendix D. Again, these were calculated from published Henry’s Law constants and are listed in Section 4.3.1.36,37 The dissolved methane exits the AnMBR system in the permeate, which could escape as a gas later causing undesired greenhouse gas emissions. Managing this dissolved methane must be a consideration in AnMBR design. Additional figures relating to methane production can be found in Appendix F.

As mentioned, there are a lot of factors that potentially skew the methane production rates as they are displayed in Figures 14. Another way to approach and compare methane production at different temperatures is analyzing the differences in methane yield, or the volume of methane produced over the mass of COD consumed. This gives an idea of how much of the influent COD (found mainly in biodegradable particulates and VFAs) is being converted all the way into methane. Methane yield in this
thesis includes the methane produced in both the gaseous and aqueous phases. **Figure 17** shows how this ratio changed over the 275-day operation of the AnMBR. While some variations occur, the value appears similar during operation at 35°C and 25°C while decreasing at 15°C. The average values at each temperature range are 109 L CH₄/kg COD at 35°C, 114 L CH₄/kg COD at 25°C, and 64 L CH₄/kg COD at 15°C operational temperature. The standard errors for these averages are 7.1, 7.6, and 7.6 respectively. A pilot AnMBR receiving similar influent feed with a sulfate concentration 1.6 times higher than the sulfate in this thesis reports average methane yields of 82.1, 60.1, and 15.4 L CH₄/kg COD for operation at 30°C, 25°C, and 17°C.²⁸ These values show a similar trend to the values reported in this study of having a significant decrease in methane yield between operation at 25°C and the lowest temperature. However, the study shows lower total methane yields than what was found in this study, which could be attributed to various differences in operation including a much higher SRT.⁷ The pilot-scale reactor

![Figure 17: Methane yield over the 275-day operation of the AnMBR. The moving average shown was calculated with 10 period intervals.](image-url)
operated with an SRT of nearly 30 days. A study comparing AnMBR performance as a function of temperature for food waste recycling wastewater reports a similar methane yields during operation at 35°C and 25°C, but undetectable methane production during operation at 15°C. Another study comparing AnMBR performance as a function of temperature for domestic wastewater again reports similar methane yields of 190 L CH₄/kg COD during operation at 35°C and 25°C, but a decrease to 140 L CH₄/kg COD during operation at 15°C. The differences in the methane yields achieved in this study could be due to differences in feed content, including feed source and the presence of sulfate, and AnMBR design and operation.

These average methane yields are broken up into their gaseous and dissolved phases in Figure 18. This shows that 35% of the total methane yield exists in the aqueous phase during operation at 35°C, 44% exists in the aqueous phase during operation at 25°C, and 45% exists in the aqueous phase during operation at 15°C. Figure 17 and 18

![Figure 18. Average methane yield in gaseous and dissolved phase at 35°C, 25°C, and 15°C. Average values were taken for each temperature from days 39-79, 155-184, and 210-275 respectively. Standard errors are included.](image)
again show that the AnMBR was able to convert a similar mass of COD into methane at 35°C and 25°C, but is able to convert less COD into methane at 15°C. This does raise a question: if the AnMBR was able to achieve a similar COD removal at 15°C but did not produce the same volume of methane, where is the COD going? This question will be addressed further with the aid of microbial analysis to understand how microbial community shifts or functional changes could explain the differences in methane production. The results of this analysis can be found in Sections 5.2 and 5.3.

Theoretically, 1 kg of COD should be able to produce 350 L of methane. This is derived from Equations 5 and 6. As Figure 17 shows, the AnMBR treating primary clarifier effluent averaged around 100 L CH₄/kg COD, 28.5% of the theoretical value. This value is similar to pilot scale reactors run under similar conditions²⁸,³¹. The system did however peak at 164 L CH₄/kg COD during operation at 35°C, 221 L CH₄/kg COD during operation at 25°C and 109 L CH₄/kg COD during operation at 15°C.

\[
\text{CH}_4 + 2\text{O}_2 \rightarrow \text{CO}_2 + 2\text{H}_2\text{O} \tag{5}
\]

\[
\frac{1 \text{ mol CH}_4}{2 \text{ mol } \text{O}_2} \times \frac{0.224 \text{ m}^3}{\text{ mol CH}_4} \times \frac{\text{ mol } \text{O}_2}{32 \text{ g}} \times \frac{10^3 \text{ g } \text{O}_2}{\text{ kg } \text{O}_2} = \frac{0.35 \text{ m}^3}{\text{ kg COD}} \tag{6}
\]

Most studies reporting methane production account for the differences between the theoretical value and the observed value by suggesting sulfate reduction contributes significantly to the reduced efficiency.²⁸,³¹ In this study, it was calculated that on average 6.5% of the available COD went to sulfate reduction and thus was not able to be used to produce methane. This thesis affirms that measuring the influent sulfate concentration and determining the sulfate to COD ratio is critical when determining if AnMBR application is appropriate for a particular wastewater. Knowledge of the sulfate
concentration can help estimate the potential methane production to determine if implementing an AnMBR would be energy-effective. The sub-theoretical methane yield can also be attributed to decreased rates of hydrolysis of particulate organic matter under cold temperatures, which lead to an increase in particulates in the system that decreases the methane production. It is assumed that 20% of the COD went to microbial growth, cell lysis, and decay. It is additionally assumed that there is a 20-25% energy loss during conversion of biodegradable organics to methane. All of these factors together contribute to measurable methane yields being below the theoretical.

The conclusion that the AnMBR operates similarly at 35°C and 25°C is significant based upon our understanding of anaerobic processes. Typically anaerobic digesters operate at 35°C because they function at the highest efficiency at this temperature. However, AnMBRs vary from anaerobic digesters in a number of ways including the type of feed they receive. AnMBRs receive a feed that is much easier to break down, while digesters receive sludge that contains constituents that are much harder to degrade. The higher temperature in digesters supports hydrolysis of these constituents. Since these constituents do not make up as much of the reactor volume, such extravagant hydrolysis is not needed. The solids wasted from an AnMBR could be further treated to remove these slowly biodegradable and inert constituents. If AnMBRs are only required to operate at 25°C rather than the assumed 35°C for traditional anaerobic processes, significant energy savings could be obtained, especially if the wastewater is already near that temperature for a portion of the year.
5.1.5. Volatile Fatty Acids Analysis

Samples of the feed, bioreactor, and permeate from days 30, 79, 85, 111, 194, 210, 226, 240, and 245 were analyzed utilizing HPLC for VFAs including formate, acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate. The feed had consistent concentrations of propionate, isobutyrate, butyrate, and isovalerate, totaling an average concentration of 50.1 mg COD/L as seen in Table 1. Again, this concentration of VFAs in the feed could be due to the WWTPs operation of the primary clarifier as a fermentation basin to provide VFAs that are needed in biological phosphorous removal.

Propionate was the only VFA consistently detected in the bioreactor and permeate. The propionate concentration in the bioreactor averaged 9.2 mg COD/L, 8.4 mg COD/L, and 6.5 mg COD/L during operation at 35°C, 25°C, and 15°C respectively. The propionate concentration in the permeate averaged 12.2 mg COD/L, 11.4 mg COD/L, and 7.9 mg COD/L during operation at 35°C, 25°C, and 15°C respectively. These results are listed in Table 3.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Mean Concentration in Feed [mg COD/L]</th>
<th>Standard Deviation</th>
<th>Mean Concentration in Bioreactor [mg COD/L]</th>
<th>Standard Deviation</th>
<th>Mean Concentration in Permeate [mg COD/L]</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>35°C</td>
<td>23.2</td>
<td>1.1</td>
<td>9.2</td>
<td>2.0</td>
<td>12.2</td>
<td>0.8</td>
</tr>
<tr>
<td>25°C</td>
<td>21.5</td>
<td>1.6</td>
<td>8.4</td>
<td>2.5</td>
<td>11.4</td>
<td>0.4</td>
</tr>
<tr>
<td>15°C</td>
<td>13.7</td>
<td>7.2</td>
<td>6.5</td>
<td>0.9</td>
<td>7.9</td>
<td>0.6</td>
</tr>
</tbody>
</table>
These results show that while most of the VFAs in the feed and other VFAs produced as an intermediary product were consumed through either fermentation or anaerobic oxidation, propionate could not be completely removed at any of the operational temperatures. The concentrations in the bioreactor are not significantly different from each other. Propionate reaches a slightly smaller concentration when operating at 15°C compared to the two higher temperatures suggesting slightly better propionate reduction. However, the difference between the propionate concentrations in the permeate could be due to the varying propionate concentrations in the feed which averaged 23.2 mg COD/L, 21.5 mg COD/L, and 13.7 mg COD/L during operation at 35°C, 25°C, and 15°C respectively, as shown in Table 3. Nevertheless, with any complex biological system with multiple intermediary products like propionate present in the AnMBR, percent removal cannot be merely judged based on the measured input and output since there cannot be an accurate measurement of propionate production rates inside the bioreactor. Inhibition of complete propionate metabolism shows evidence of slight inhibition of anaerobic fermentation. Additionally, it has been related to low methane production rates and typically has the slowest degradation rate of all VFAs in anaerobic systems so should be specially monitored. Analyzing the microbial community structure as it relates to propionate metabolism might shed some light on this propionate buildup. The results discussed here differ from other studies that show an accumulation of VFAs, increasing at lower temperatures. Differences in results could be attributed to different reactor design and operation, including higher HRTs in this study.
5.1.6. Nitrogen and Phosphorous Analysis

Anaerobic processes lack the ability in and of themselves to remove nutrients from the waste stream and therefore produce a stream high in nutrients that could be used for agriculture irrigation or further nutrient recovery.\(^3\) \(\text{Figures 19 and 20}\) are consistent with this knowledge of anaerobic systems as neither nitrogen (measured in the form of ammonia) nor phosphorous (measured in the form of phosphate) were significantly reduced between the feed and permeate. Nitrate values were initially measured at the beginning of this study, but measurements were stopped after getting consistently undetectable levels in both the feed and the permeate, further confirming that nitrification was not occurring as it would if significant levels of oxygen were present.

The slight decrease in phosphate levels between the influent and the effluent seen in \(\text{Figure 19}\) is most likely due to the utilization of phosphorous for biomass growth. The

\[\text{Figure 19. Average phosphate concentrations in the feed and permeate of the AnMBR at 35°C, 25°C, and 15°C. Average values were taken for each temperature from days 39-79, 155-184, and 210-275 respectively. Standard errors are included.}\]
slight increase in ammonia seen in Figure 20 is most likely due to protein hydrolysis releasing ammonia under anaerobic conditions.\textsuperscript{54}

Wastewater treatment plants that have permit limitations on these nutrients would be required to implement a post-treatment unit operation in order to remove or recover these nutrients. However, if permit limitations were not in place, the effluent of the AnMBR that is high in nutrient content could also be used as a resource. For example, it could be used as irrigation water, providing necessary nutrients for plants to grow.

5.2 Objective II: \textit{mcrA} Gene Quantification

5.2.1 Relative Abundance of \textit{mcrA} Gene Copies through qPCR

Processing DNA through qPCR allows the analysis of relative abundance of \textit{mcrA} genes at different operation temperatures, although not all genes present might be functionally in use. The results shown in Figure 21 display the relative abundance of
mcrA gene copies normalized to the mass of DNA in the sample. Each bar on the graph is the averaged value of six replicates from that sample date (three replicates in the qPCR of two technical replicates). The first two sample dates were taken at the end of operation at 35°C, the middle two sample dates were taken at the end of operation at 25°C, and the last two samples dates were taken at the end of operation at 15°C. From Figure 21 it can be seen that the mcrA abundance is very similar between AnMBR operation at 35°C and 25°C with the first four sample dates having relative abundances of $1.2 \times 10^5$ copies/ng DNA, $9.6 \times 10^4$ copies/ng DNA, $8.6 \times 10^4$ copies/ng DNA, and $1.3 \times 10^5$ copies/ng DNA respectively. The gene abundance during operation at 15°C shows a slight decrease with the last two sample dates having relative abundances of $3.2 \times 10^4$ copies/ng DNA and

![Graph showing relative mcrA gene abundance](image)

**Figure 21.** mcrA gene abundance during each sampling date. Each date shows the average of six data points. Standard deviations are included.
Although there is not a significant order of magnitude change, the mcrA gene abundance still decreases nearly 3 fold when operating at 15°C. This lower abundance of the mcrA gene shows evidence of slightly reduced methanogen abundance in the microbial community, which could be responsible for lower methane yield at this lower temperature. Studies have shown that there is a positive and significant correlations between mcrA abundance and methane production rate in anaerobic digesters, which is affirmed by these results. Studies that have analyzed the mcrA copies in 16S rRNA in AnMBR suspended and biofilm biomass again correlate higher mcrA abundance with higher methanogenic activity, although this has not yet been related to changing bioreactor temperature and has only been completed with synthetic wastewater as the feed. The similarity between the abundance at 35°C and 25°C shows additional evidence that AnMBRs can operate with similar performance at these temperatures, providing cost savings to operators who wouldn’t need to heat the reactor to 35°C.

5.2.2 Relative Abundance of mcrA Gene Transcripts Through RT-qPCR in Bioreactor RNA Samples

Due to an error in the RNA extraction process, the RNA samples taken from the bioreactor did not produce concentrations of RNA that could amplify and be read using qPCR, even after the technical replicates from each sampling date were pooled through the ethanol precipitation described in Section 4.4.2. The error was most likely due to RNase contamination of the samples, reagent contamination in the RNA extraction kit, or contamination in the RNALater solution.
5.2.3 Relative Abundance of \textit{mcrA} Gene Transcripts Through RT-qPCR in Batch Samples

RNA from the batch test was successfully extracted, transcribed in RT, and amplified in qPCR. By sampling the RNA, the functional impact of temperature on the microbial community’s ability to produce methane is seen. Figure 22 shows the average transcript number normalized to the cDNA concentration. Each bar on the graph is the averaged value of six replicates from that sample date (three replicates in the qPCR of two technical replicates). The batch samples held at 35°C, 25°C, and 15°C for 72 hours had average relative \textit{mcrA} gene expressions of $7.1 \times 10^4$ transcripts/ng cDNA, $7.7 \times 10^4$ transcripts/ng cDNA, and $1.4 \times 10^5$ transcripts/ng cDNA respectively. These values have standard deviations of $3.2 \times 10^4$ transcripts/ng cDNA, $2.9 \times 10^4$ transcripts/ng cDNA, and $5.3 \times 10^4$ transcripts/ng cDNA respectively. These results show that there is just under a 2
fold increase in \textit{mcrA} gene expression in the sample taken at 15°C compared to the samples taken at 35°C and 25°C.

These results are surprising, since they show a nearly opposite trend to those of the relative \textit{mcrA} gene abundance discussed in \textbf{Section 5.2.1}. Since other studies report decreasing \textit{mcrA} expression with decreasing methane production, that trend was expected.\textsuperscript{20,56} The slight difference in \textit{mcrA} expression could be a consequence of temperature shock as the biomass samples were taken from the AnMBR reactor, which was held at 15°C, and immediately incubated on incushakers at 35°C and 25°C. A change in temperature by 10-20°C in a matter of minutes could stunt methanogen activity.\textsuperscript{4,23} Since the batch samples were only incubated at each temperature for 72 hours, they may not have had enough time to recover. In one study, a temperature shock of 10°C to a bioreactor operating at 45°C took 16 days to recover its methane production.\textsuperscript{23}

\textbf{5.4 Objective III: Metagenomic Community Analysis}

Novogene preformed metagenomic sequencing for two technical replicates from each sampling date. This thesis includes the results from the sequencing preformed on the DNA of the AnMBR biomass during operation at 35°C and 25°C. At the time of this thesis, results from operation at 15°C were still being sequenced and will be included in the comparison between the operating temperatures when they arrive.

Taxonomic analysis was preformed using the MG-RAST server. Again, it should be noted that the analysis included in this thesis shows simple community structure shifts with the changing operational temperature; however, with entire metagenomic sequencing, there is potential for extended analysis into functional microbial changes in
the AnMBR as a consequence of seasonal variation. **Figures 23 and 24** show the percent abundance of archaea and bacteria at a genus-level, respectively. **Figure 23** shows archaea with a percent abundance greater than 1%, totaling a maximum of 88% of the archaea present in the sample. *Methanoseta*, an aceticlastic methanogen, had the highest abundances, averaging 19.6% and 15.0% at 35°C and 25°C, respectively. The abundance decreased with a decrease in operational temperature. *Methanospirillum*, a hydrogenotrophic methanogen, had high abundances averaging 9.3% and 13.9% at 35°C and 25°C, respectively. The abundance slightly increased with decreasing temperature. Another hydrogenotrophic methanogen, *Methanotermobacter*, increased in abundance with decreasing temperature, averaging 4.1% at 35°C and 6.0% at 25°C. From these three genera, we can see a shift in the microbial community from aceticlastic methanogenesis to hydrogenotrophic methanogenesis. *Methanosarcina*, had relatively large abundances within the samples, averaging 12.0% and 11.6% at 35°C and 25°C, respectively. Here the abundance slightly decreased with a decrease in operational temperature. *Methanosarcina* is known to produce methane using all three metabolic pathways, thus its presence at a large abundance suggests an adaptable methanogenic community in the bioreactor.
Figure 23. Taxonomic profiling at the Genus level for AnMBR archaea operating at 35°C and 25°C.

Figure 24 shows bacteria with a percent abundance greater than 0.6%, totaling a maximum of 45% of the bacteria present in the sample. This shows that there is a diverse bacterial culture, with 55% of the population made up of a variety of genus’ with abundances <0.6%. Candidatus Cloacamonas, a propionate oxidizing and hydrogen producing syntroph, had the highest abundance of 12.8% during operation at 35°C and decreased to 4.2% at 25°C. Syntrophus, another fatty acid-degrading syntrophic bacterium, also decreased from 4.1% at 35°C to 3.5% at 25°C. However, many other
genus’ with relatively high abundances increased in abundance with decreasing temperature including *Bacteroides* (formate producers), *Chlorobium* (sulfide oxidizers), and *Desulfovibrio* (sulfate reducers), which increased by 1.7%, 2.6%, and 0.4%, respectively. Notable abundances of *Geobacter* (CO₂ reducers) and *Clostridium* (formate producers) were additionally detected in the biomass samples; however, their abundances were relatively stable between the two operating temperatures.

**Figure 24.** Taxonomic profiling at the Genus level for AnMBR archaea operating at 35°C and 25°C.
6.0 RELEVANCE

AnMBRs could be a means of decreasing the energy consumption associated with conventional wastewater treatment by eliminating aeration costs and reducing the cost of processing solids since fewer solids are produced in anaerobic systems than aerobic systems.\textsuperscript{4,13} After long-term operation, this study has shown that an AnMBR treating real domestic wastewater is able to produce an effluent that meets the total suspended solids regulations as well as achieving effective COD removal at all operational temperatures spanning typical seasonal variation in South Carolina and other temperate, subtropical, and tropical climates.

The conclusion that the AnMBR operates similarly in its methane yield at 35°C and 25°C is significant because substantial energy savings could be obtained from not heating the reactor to 35°C as is common in conventional anaerobic digesters. Energy savings could especially be significant if the wastewater is already near 25°C for a portion of the year as it is in tropical climates.\textsuperscript{51} This study also reveals that methanogenesis is slightly reduced during operation at 15°C as lower amounts of methane are produced; however, the ability of the system to still meet regulated standards, namely COD and solids removal, signifies that AnMBRs are still feasible within this temperature range. A higher methane yield is expected for wastewater low in sulfate.\textsuperscript{9} An energy analysis would need to be completed to discern if the lower methane production during the winter months would make AnMBRs a truly energy-saving wastewater treatment technology.
Exact roles of many organisms in anaerobic environments have not even been defined, much less quantified. This thesis reveals how the microbial community structure in an AnMBR treating DWW changed between 35°C and 25°C using advanced techniques and metagenomic sequencing. Comparing these results to the coming data from operation at 15°C will offer a complete picture of how the functional potential of the microbes responsible for treatment respond to seasonal temperature variation.

Furthermore, if we are able to consider community structure in a quantitative way, it can result in better system modeling, design, and performance as AnMBRs can be designed for optimum microbial performance. By quantitative PCR of AnMBR DNA, this study has shown that methanogen abundance is slightly reduced when operating at 15°C due to the suppressed abundance of \textit{mcrA} genes. This knowledge, if affirmed in other studies under different operating conditions, could be incorporated into future models created to predict methane production of AnMBR systems, which is a crucial part to the design process and feasibility studies.

In this study we have better understood the relationship between temperature, microbial communities, and AnMBR performance, and have thus provided evidence for the implementation of AnMBRs for treatment of domestic wastewater.
7.0 FUTURE WORK

As mentioned at the beginning of this study, the work described above contributes only a piece in the overall effort to determine the effectiveness of AnMBR application for domestic wastewater treatment. Further work must be completed to understand the feasibility of AnMBRs considering other wastewater treatment goals including: nutrient control, pathogen control, and meeting whole effluent toxicity standards. Further processes should be assessed for nutrient treatment or recovery from the AnMBR effluent, which is high in both ammonia and phosphate. Additionally, processes should be considered to properly treat the dissolved methane in the permeate before it is released and contributes to greenhouse gas emissions.

This study revealed the importance of membrane fouling control to maintain constant flux through the system. Alterations to the membrane unit and fouling control operations should be considered in future systems. Membrane units with easy access to biofilm development should be considered to evaluate the significance of the biofilm in AnMBR treatment.24

When understanding the significance of the microbial analysis, it should also be noted that there are natural variations in microbial communities and function. Communities can adapt, and multiple communities, although different, can offer the same function. The microbial analysis in this study should be replicated in an AnMBR treating domestic wastewater under different operating conditions to affirm the significance of the microbial community analysis. Furthermore, future studies can continue to evaluate the relationship between temperature, microbial communities, and AnMBR performance at
smaller temperature intervals between 25°C and 15°C to have a better understanding of AnMBR performance during spring and fall months. These results could then be further quantified and utilized in AnMBR modeling.

These studies combined will work together to provide evidence for the feasibility of anaerobic membrane bioreactors for treatment of domestic wastewater and promote more effective modeling, design, and operation.
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https://doi.org/10.1002/bit.10628.


(45) O’Flaherty, V.; Collins, G.; Mahony, T. The Microbiology and Biochemistry of


**APPENDIX A: SRT CALCULATION**

**Table 4. List of wasting events from the bioreactor**

<table>
<thead>
<tr>
<th>Day</th>
<th>Volume wasted [mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>32</td>
<td>10</td>
</tr>
<tr>
<td>43</td>
<td>39</td>
</tr>
<tr>
<td>56</td>
<td>10</td>
</tr>
<tr>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>77</td>
<td>40</td>
</tr>
<tr>
<td>79</td>
<td>20</td>
</tr>
<tr>
<td>85</td>
<td>50</td>
</tr>
<tr>
<td>96</td>
<td>10</td>
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<tr>
<td>104</td>
<td>10</td>
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<tr>
<td>111</td>
<td>10</td>
</tr>
<tr>
<td>118</td>
<td>10</td>
</tr>
<tr>
<td>133</td>
<td>10</td>
</tr>
<tr>
<td>145</td>
<td>10</td>
</tr>
<tr>
<td>148</td>
<td>55</td>
</tr>
<tr>
<td>162</td>
<td>56.5</td>
</tr>
<tr>
<td>163</td>
<td>15</td>
</tr>
<tr>
<td>173</td>
<td>151.5</td>
</tr>
<tr>
<td>182</td>
<td>10</td>
</tr>
<tr>
<td>194</td>
<td>10</td>
</tr>
<tr>
<td>209</td>
<td>10</td>
</tr>
<tr>
<td>212</td>
<td>10</td>
</tr>
<tr>
<td>226</td>
<td>10</td>
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<tr>
<td>230</td>
<td>120</td>
</tr>
<tr>
<td>236</td>
<td>10</td>
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<tr>
<td>232</td>
<td>55</td>
</tr>
<tr>
<td>240</td>
<td>10</td>
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<tr>
<td>252</td>
<td>10</td>
</tr>
<tr>
<td>260</td>
<td>10</td>
</tr>
<tr>
<td>261</td>
<td>70</td>
</tr>
<tr>
<td>262</td>
<td>10</td>
</tr>
<tr>
<td>270</td>
<td>72</td>
</tr>
</tbody>
</table>
Average SRT calculation:

\[
\text{Average SRT} = \frac{\text{Inventory}}{\text{Wastage}} = \frac{\forall_{\text{reactor}} \times \text{MLSS}}{Q_{\text{waste}} \times X_{\text{waste}}}
\]

where MLSS = average mixed liquor suspended solids of the bioreactor = 2960 mg/L

\[
Q_{\text{waste}} = \text{average wasting rate from the bioreactor} = 3.6 \frac{mL}{d}
\]

and \(X_{\text{waste}} = \text{average suspended solids of the wated biomass} = \text{MLSS} = 2960 \frac{mg}{L}\)

assuming a completely mixed system

\[
Q_{\text{waste}} = \frac{\text{sum of wasting events listed in Table 4}}{\text{days of operation}} = \frac{1000 mL}{275 d} = 3.6 \frac{mL}{d}
\]

\[
\text{Average SRT} = \frac{\forall_{\text{reactor}} \times \text{MLSS}}{Q_{\text{waste}} \times X_{\text{waste}}} = \frac{2000 mL \times 2960 \frac{mg}{L}}{3.6 \frac{mL}{d} \times 2960 \frac{mg}{L}} = 550 d
\]
APPENDIX B: IMAGE PROCESSING WATER LEVEL CONTROL

To maintain a constant water level in the bioreactor, an image processing technique was developed. A webcam (Logitech HD Webcam C615) constantly monitored the bioreactor, taking an image every minute and processed it through a Matlab code embedded into the Labview program. The side of the bioreactor opposite of the camera was covered in red tape and a green strip of tape was placed on the bioreactor facing the camera. The green tape was placed at the target level in the reactor so that the total volume in the system equaled 2 L.

The Matlab script, enclosed below, crops the image and applies a filter so that any red color is turned to black. The percentage of black pixels is then quantified, and thus provides a measurement of the level of headspace in the reactor. If the percentage of headspace is below a pre-determined value, then the water level surpassed the desired level and the feed pump turns off. If the headspace is above the pre-determined value, then the water level fell too low and the feed pump turns on. This process is depicted below.
Matlab script embedded in LabView:

clear
clc
close all
folder='C:\Users\Popatlab\Desktop\Negin\AnMBR data from labview\AnMBR-DWW\3-16-19';
format shortg

n=0;
percentageofblackpixel=0;
camList = webcamList
.cam = webcam(2)

format shortg Realtime = clock;
img = snapshot(cam);
h=figure;
image(img);
baseFileName = sprintf('Image.png');
fullFileName = fullfile(folder, baseFileName);
saveas(h, fullFileName);

f=imread(fullFileName);
path(path, folder)
[BW,RGB] = createMask5(f);

IRGB=imshow(RGB);
baseFileName2 = sprintf('RGBMImage.png');
fullFileName2 = fullfile(folder, baseFileName2);
saveas(IRGB, fullFileName2);

RIRGB=imread(fullFileName2);
RIRGB=imcrop(RIRGB ,[1080 298 50 648]);
baseFileName3 = sprintf('RGBMRImage.png');
fullFileName3 = fullfile(folder, baseFileName3);
CRGB=imshow(RIRGB);

saveas(CRGB, fullFileName3);
A=imread(fullFileName3);
close (h)
dimension=size(A);
S=double(A);
SR=S(:,:,1);
SG=S(:,:,2);
SB=S(:,:,3);
compareG=SG./255;
for i=50:1055
    for j=(dimension(2)-50)/2:(dimension(2)-50)/2+50
        if SR(i,j)==0 && SG(i,j)==0 && SB(i,j)==0
            n=n+1;
            percentageofblackpixel=n/(1005*50)*100;
        end
    end
end
APPENDIX C: ANMBR SET UP

Figure 25. A) Hollow fiber membrane module B) Temperature control system with heater/chiller connected to water bath that passes recirculation C) Tedlar bag used to collect biogas on top of recirculation and permeate pumps and D) Permeate collection and backwash system
APPENDIX D: HENRYS LAW CONSTANT CALCULATIONS

Calculating the dimensionless Henry’s Law Constant for methane (CH₄) at 35°C:

\[ H_{35}^{CP} = 0.0014 \exp \left( 1600 \times \left( \frac{1}{273 + 35} - \frac{1}{298.15} \right) \right) = 0.001179 \frac{L \cdot \text{atm}}{K \cdot \text{mol}} \]

\[ H_{35}^{CC} = H_{35}^{CP} \times RT = 0.001179 \times (0.082057 \times (273 + 35)) = 0.0298 \]

Calculating the dimensionless Henry’s Law Constant for methane (CH₄) at 25°C:

\[ H_{25}^{CP} = 0.0014 \exp \left( 1600 \times \left( \frac{1}{273 + 25} - \frac{1}{298.15} \right) \right) = 0.001404 \frac{L \cdot \text{atm}}{K \cdot \text{mol}} \]

\[ H_{25}^{CC} = H_{25}^{CP} \times RT = 0.001404 \times (0.082057 \times (273 + 25)) = 0.0343 \]

Calculating the dimensionless Henry’s Law Constant for methane (CH₄) at 15°C:

\[ H_{15}^{CP} = 0.0014 \exp \left( 1600 \times \left( \frac{1}{273 + 15} - \frac{1}{298.15} \right) \right) = 0.001691 \frac{L \cdot \text{atm}}{K \cdot \text{mol}} \]

\[ H_{15}^{CC} = H_{15}^{CP} \times RT = 0.001691 \times (0.082057 \times (273 + 15)) = 0.0399 \]
**APPENDIX E: HPLC CALIBRATION CURVES**

![Formate Calibration Curve]

- **Equation:** \( y = 0.0047x \)
- **R²:** 0.99996

![Acetate Calibration Curve]

- **Equation:** \( y = 0.0058x \)
- **R²:** 0.99994

![Isobutyrate Calibration Curve]

- **Equation:** \( y = 0.0037x \)
- **R²:** 0.99997
Butyrate Concentration [mM] vs. Peak area

- Equation: $y = 0.0051x$
- $R^2 = 0.99983$
APPENDIX F: ADDITIONAL FIGURES

F. 1 Soluble COD

In addition to total COD analysis, soluble COD was also analyzed in the feed and used to calculated percent soluble COD removal achieved using the AnMBR. The average sCOD removal achieved at each operational temperature is shown in Figure 26. Similar to total COD removal, the sCOD removal did not vary between the operational temperatures in a large degree. Similar sCOD removal was achieved during operation at 25°C and 15°C, while operation at 35°C showed slightly higher removal. This is most likely because hydrolysis is more effective at higher temperatures, so it is able to break down more of the complex biodegradable components in the water. Even though operation at 35°C shows slightly higher sCOD removal capabilities, the total COD removal was not significantly different between the three temperatures. Since total COD is a better indicator of what is actually regulated, BOD₅, operating at 35°C does not prove

![Figure 26](image_url)

**Figure 26.** Average soluble COD removal at 35°C, 25°C, and 15°C. Average values were taken for each temperature from days 39-79, 155-184, and 210-275 respectively. Standard errors are included.
to be sufficiently advantageous for the AnMBR. The trend of sCOD removal over time and sCOD loading can be seen in Figure 27. Decreases in the sCOD removal occurred after chemically cleaning the membrane on day 97, and changing the membrane module on days 148 and 232 and can be distinguished in Figure 27. The soluble COD loading had a smaller range that the total COD loading, ranging between 32 mg COD/(L·d) and 489 mg mg COD/(L·d). Variations in loadings could be due to changes in feed concentrations due to rain events, or changes in the HRT of the AnMBR.

![Figure 27](image)

**Figure 27.** Soluble COD removal and soluble COD loading rate over the 275-day operation of the AnMBR. The moving averages shown were calculated with 10 period intervals

**F.2 Methane Production**

In the same manner that the gaseous methane production rate was shown in Section 5.1.4, the aqueous methane production rate can be seen in Figure 28. It was calculated using Equation 7, shown below. Again, variations in dissolved methane production could be related to the same changes in HRT, OLR, and/or microbial
community shifts and adaptation periods. The spike in dissolved methane production at the end of operation at 25°C is most likely due to the decrease in HRT and consequential increase in organic loading rate and increase in methane production in both aqueous and gaseous phases. The average dissolved methane production stabilized at approximately 29 mL CH₄/d for 35°C, 44 mL CH₄/d at 25°C, and 21 mL CH₄/d at 15°C operational temperature. The standard errors for these averages are 1.3, 1.75, and 1.3 mL CH₄/d respectively. While there was not a vast difference in the rate of dissolved methane production between the three operational temperatures, it can be seen that the bioreactor produced less methane, in both the gaseous and dissolved phase, when operating at 15°C.

\[
Dissolved \ CH₄ \ Production \ in \ Permeate = \frac{Fraction \ CH₄ \ in \ biogas \times V_{reactor} \times HLC}{HRT} \quad (7)
\]

where \( V_{reactor} = \) volume of the bioreactor = 2000 mL

Figure 28. Dissolved methane production rate in the permeate over the 275-day operation of the AnMBR. The moving averages shown were calculated with 10 period intervals
Methane concentrations in the gas and aqueous phase are seen in Figures 29 and 30, showing that the gas phase concentration is much higher than the permeate concentration.

**Figure 29.** Gaseous methane concentration over the 275-day operation of the AnMBR. The moving averages shown were calculated with 10 period intervals

**Figure 30.** Dissolved methane concentration in the permeate over the 275-day operation of the AnMBR. The moving averages shown were calculated with 10 period intervals
F. 3 Nutrient Concentrations Over AnMBR Operational Period

In the main body of the thesis, nutrient concentrations were reported as their averaged concentrations during pseudo-steady state of each operational temperature. Sulfate, ammonia, and phosphate concentrations in the feed and permeate are displayed over the 275 day operational period of the AnMBR in Figures 31, 32, and 33 respectively. These figures show that nutrient concentrations were relatively stable throughout the study, with some variation due to rain events.

![Sulfate Concentrations](image)

**Figure 31.** Sulfate concentrations in the feed and permeate over the 275-day operation of the AnMBR displaying continuous sulfate removal.
Figure 32. Ammonia concentrations in the feed and permeate over the 275-day operation of the AnMBR

Figure 33. Phosphate concentrations in the feed and permeate over the 275-day operation of the AnMBR