Biotransformation of Atenolol and Ibuprofen under Methanogenic Conditions and their Influence on Gas Production and the Microbial Community

By

Julia K. Campbell

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ABSTRACT OF THE THESIS

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By JULIA K. CAMPBELL

Thesis Director:
Professor Lily Y. Young

Pharmaceuticals and personal care products (PPCPs) are used on a daily basis by people worldwide and are released into the environment by human excretion as well as improper disposal. These emerging contaminants make their way to a wastewater treatment plant where they are incompletely removed and are released into the environment via wastewater effluent. Atenolol, a beta-blocker, is commonly found in wastewater, treated effluent, and in the open environment. This research investigated the microbial transformation of atenolol under methanogenic conditions. Two sets of enrichment cultures were established with sediment from Iona marsh and from anaerobic digester sludge from a wastewater treatment plant as inoculum; both were fed 0.5 mM of atenolol. The Iona marsh primary enrichment cultures completely removed atenolol by day 45 with the formation of the metabolite atenolol acid. The anaerobic sludge enrichment cultures completely removed atenolol by day 98 with the formation of atenolol acid. Atenolol amended cultures produced more total gas and methane than the unamended backgrounds in both enrichment culture set-ups. Results from the Anaerobic Toxicity Assay (ATA) and Biochemical Methane Potential (BMP) showed that atenolol was not chronically toxic and has the potential to be biodegraded. However, atenolol was

only transformed. Using 16S rDNA, the microbial community was characterized via Illumina sequencing and displayed a difference between the atenolol transforming communities compared to unamended background controls; addition of atenolol selected for a fermentative and methanogenic community. This is the first demonstration of the transformation of atenolol to atenolol acid under anaerobic conditions to our knowledge. Atenolol acid was not further degraded or transformed in either culture set-up throughout the course of the experiment. These findings indicate atenolol can be transformed to atenolol acid in methanogenic environments such as anaerobic sediments, digesters, and the rumen, but the metabolite atenolol acid is recalcitrant in these environments. This finding provides further support that pharmaceutical metabolites remain in the environment and that these metabolites should also be tested like pharmaceuticals to see if they elicit adverse effects on the organisms living in the environment. Another set of enrichment cultures were set up in the same manner but with the pharmaceutical ibuprofen, a Nonsteroidal anti-inflammatory drug (NSAID), that is also found throughout the environment and in wastewater treatment plants. Ibuprofen was not degraded in either set of cultures, but the ibuprofen amended cultures had higher total gas and methane production than the unamended backgrounds. Results from the Anaerobic Toxicity Assay (ATA) and Biochemical Methane Potential (BMP) showed that ibuprofen was not chronically toxic and has the potential to be biodegraded; however, it was not biodegraded. Community analysis of the ibuprofen amended Iona cultures revealed that ibuprofen selected for a methanogenic community.

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DEDICATION

To my mother, Katrina: you have made so many sacrifices and worked so hard in order to provide me with the best life with the most opportunities. I can never say enough how grateful I am for this. The most important lesson you've taught me through example is to be kind to all and to have an open heart even in the most difficult of times; I truly believe it is this that has allowed me to be where I am today.

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Chapter 1

Literature Review

1.1 Introduction

Since the late 19th century, the world has enjoyed the advances of modern pharmacology. Beginning with the discovery and synthesis of such compounds as aspirin, chloroform, and barbiturates, society saw an increase in the quality of life with advent of modern pharmaceuticals (Jones, 2011). Today, Pharmaceuticals and Personal Care Products (PPCPs) are used on a daily basis by people worldwide, and are integral to public health. North America is the largest market for both over-the-counter and prescription pharmaceutical sales, with a total of 406.2 billion dollars in sales in 2014 (IMS health). Daily use of prescription drugs alone by adults 20 years or older is estimated to be 59% (Kantor, Rehm, Haas, Chan, & Giovannucci, 2015). However, the pharmaceuticals that have given humanity greater quality of life and extended it are now found throughout the environment and even in drinking water (Loraine & Pettigrove, 2006). Aside from their intended use, the additional risks of being exposed to these pharmaceuticals are not well understood for humans. Many pharmaceuticals are endocrine disruptors and have been known to cause feminization in fish as well as alter their behavior (Fent, Weston, & Caminada, 2006). The potential for creating antibiotic resistant bacteria has also become a concern due to the detection of antibiotics throughout the environment and wastewater treatment plants (Kolpin et al., 2002). Due to these potential risks, researchers have begun to study where pharmaceuticals are found in the environment, their effects, and how to remove them.

1.2 Fate and Transport

Concentrations of pharmaceuticals in the environment range from ng/L to µg/L and have been found in all parts of the environment including surface waters, sediment, and soil (Hernando, Mezcua, Fernández-Alba, & Barceló, 2006). In the environment these pharmaceuticals can cause unintended effects to the ecosystem. The intended purpose of pharmaceuticals is to elicit a physiological response inside the user's body. When released into the environment, pharmaceuticals can cause unwanted physiological responses to the non-target organisms living there (Kümmerer, 2009). Examples of effects of exposure to pharmaceuticals in the environment include feminization of fish, altered development of aquatic organisms including fish and frogs, and antimicrobial resistance in environmental microorganisms (Fent, Weston, & Caminada, 2006; Kostich, Batt, Glassmeyer, & Lazorchak, 2010; Khetan & Collins, 2015). Bioaccumulation in aquatic organisms is also a concern. Studies have shown the accumulation of many types of pharmaceuticals in fish intended for human consumption (Ramirez et al., 2009; Nakamura et al., 2008). These findings are not only a potential public health concern, but raise concerns about the health of the ecosystem and overall quality of the water.

When a pharmaceutical is ingested and the body uptakes it, the pharmaceutical elicits a response from the body, and then excretes it via waste elimination (Xia, Bhandari, Das, & Pillar, 2005). After excretion to sewage, waste enters a wastewater treatment plant. The main design of a wastewater treatment plant is to remove biochemical oxygen demand (BOD) of human waste and perhaps excess nutrients such as nitrogen and phosphate, not to remove complex organic contaminants such as pharmaceuticals. Once this wastewater has been treated, it is released as effluent into

streams, rivers, or other bodies of water. PPCPs are incompletely removed during wastewater treatment and then released into the environment in two ways: direct release into open waters, or into soil when biosolids are used as fertilizer (Kolpin et al., 2002; Fent, Weston, & Caminada, 2006; Xia, Bhandari, Das, & Pillar, 2005).

Another way pharmaceuticals are released into the environment is improper disposal of pharmaceuticals, in which a person directly dumps unwanted or expired pharmaceuticals in a sink or toilet (Glassmeyer et al., 2009). This then has the same path into the environment as the excreted PPCPs. Pharmaceutical manufacturing plants also serve as a source of PPCPs (Ternes, Joss, & Siegrist, 2004). Antibiotics for livestock production are produced and used in larger volumes than those for humans; this also serves as a source of pharmaceutical contamination in the environment (Halling-Sorensen et al., 1998).

1.3 Anaerobic Digestion Process

After wastewater has gone through preliminary and primary treatment to remove large debris, large solids, and grit, it is then processed by aerobic microbes in what is known as the aeration tank (Fig. 1) (Bitton, 2005). Here, aerobic microbes break down organic matter and proliferate. After the organic matter has been broken down by the aerobic microbes, the wastewater is moved to a sedimentation tank where solid material settles out and is separated from the aqueous portion (Bitton, 2005). These solids, called sludge, contain new cell biomass, which makes up the majority of sludge; this is then either recycled back into the aeration tank or is may be movied to an anaerobic digester (Hammer & Hammer, 2004). In the anaerobic digester, the sludge is reduced by a community of anaerobic microbes breaking it down and producing gas, which consists of

methane, carbon dioxide, and trace amounts of hydrogen sulfide (Hammer & Hammer, 2004). The remaining sludge or biomass is dewatered or air dried to remove excess water; this water is then returned to the beginning of the treatment plant (Fig. 1) (Hammer & Hammer, 2014). Then, one of several things happens to the dried sludge: it is either transported to a landfill, incinerated, or further processed to become biosolids (Hammer & Hammer, 2004). In the lattermost case, biosolids are then utilized as agricultural fertilizer (Kinney, Burkhardt, Werner, & Jorgensen, 2006).

Anaerobic digestion is a favorable process because it can save the plant money by reducing the volume of solids that would have to be transported to a landfill or incinerated; also, plants can recover the methane in order to help power the plant (Carballa, Omil, Ternes, & Lema, 2007). However, there are two ways by which this process can transport pharmaceuticals into the environment. The excess water that is returned to the beginning of the plant during the dewatering process will eventually make it out into the open environment in the outgoing treated water (effluent), in which PCPPs have been found (Kostich, Batt, & Lazorchak, 2014). Also, biosolids are land applied, pharmaceuticals that remain in these biosolids can be taken up by plants that may be consumed by people or animals; run-off from these biosolids can also transport pharmaceuticals to receiving waters (Czajkowski, 2010; Topp, Sumarah, & Sabourin, 2012).

1.4 Methanogens and Anaerobic Degradation

Methanogens cannot utilize complex compounds and rely on anaerobic bacteria to break down matter into C-1 and C-2 compounds such as acetate and carbon dioxide (and hydrogen) that methanogens can then utilize to produce energy (Schink, 1997). A major

by-product of this reaction is methane gas (Schink, 1997;Whitman, Bowen, & Boone, 2006). This process is called methanogenesis. Methanogenesis is an important process in the degradation of organic matter in anaerobic environments as it converts solid organic matter to gas (Schink, 1997). This process helps industries such as wastewater anaerobic digestion and industrial waste processing in two ways: firstly, the conversion of solid waste to gas by methanogenesis saves time and money by avoiding transportation of the solid wastes to a landfill or incineration facility; secondly, the methane gas produced by methanogens can be captured and used to generate energy (Chen, Cheng, & Creamer, 2008). However, methanogens can be inhibited by certain compounds such as phenols, surfactants, and detergents, which causes low methane yield (Chen et al., 2008; Owen, Stuckey, Healy, Young, & McCarty, 1979). Some pharmaceuticals such as propranolol and diclofenac sodium have also been shown to have an inhibitory effect on the methanogenic community (Fountoulakis, Stamatelatou, & Lyberatos, 2008). Methane production a good indicator of how well the methanogens are functioning. Assays such as the Biochemical Methane Potential and Anaerobic Toxicity Assay (to be discussed in further detail in chapter 3) have been developed in order to see if different substrates could possibly be inhibitory to a methanogenic community (Owen et al., 1979).

1.5 Compounds of Interest

1.5.1 Atenolol

Atenolol is a synthetic cardio-selective beta-adrenergic blocker also known as a‘beta blocker’ (Fig. 2) (AstraZeneca, 2008). Beta blockers have been in use since the mid-1960s to treat and manage a variety of medical conditions such as hypertension, angina (insufficient blood flow to the heart), atrial arrhythmia, migraines, heart failure,

and post-mycocardial infarction (post-heart attack) (Helfand, Peterson, Christensen, Dana, & Thakurta, 2009). Beta blockers work by fully or partially blocking hormones called catecholamines from activating adrenergic receptors (Frishman, 2003). When catecholamines activate these receptors they cause an increase in heart rate, heart muscle contraction, and blood pressure. Beta blockers help slow this response and therefore alleviate the symptoms of the aforementioned conditions (Frishman, 2003). Atenolol, being cardio-selective, is approved to treat conditions specific to the heart which include hypertension, angina, and post-mycocardial infarction (Helfand et al., 2009). When taken orally, only 50% is bioavailable (AstraZeneca, 2008). This means only 50% is absorbed into the body and functions for its intended purpose, while the other 50% is immediately excreted unchanged in the feces (AstraZeneca, 2008). Once utilized by the body, the absorbed 50% is excreted via urine with 90% being the unchanged compound and the remaining being either the metabolite atenolol-glucuronide or hydroxyatenolol (J Radjenović, Pérez, Petrović, & Barceló, 2008). In total, of the bioavailable dose, 90% is excreted unchanged while 10% is the other two metabolites (J Radjenović et al., 2008).

Atenolol is a popular beta blocker with prescriptions for it in the United States reaching 44.2 million; that means that almost 14% of the population is taking atenolol (Palmer et al., 2008). With such a high percentage of the population taking the pharmaceutical, combined with the fact that when one dose of atenolol is taken, a total of 95% of the parent compound is excreted, it is not surprising that atenolol has been found throughout the wastewater treatment system and in the open environment.

A study looked at the concentrations of pharmaceuticals in a total of 50 wastewater treatment plant effluents throughout the United States (Kostich et al., 2014);

atenolol was detected in 48 of the 50 at ng/L magnitude. In one study that looked into the partitioning of pharmaceuticals within a wastewater treatment plant, it was found that atenolol concentrations in the incoming water (influent) were between 0.4-1.4 µg/L (Jelic et al., 2011). The effluent concentration was 0.4 µg/L while the concentration in the digested sludge was in the magnitude of µg/kg (Jelic et al., 2011). Similarly, in another study, concentrations of a New York wastewater treatment plant's effluent had a median concentration of 1415 ng/L, with the highest reading in this study being 14,200 ng/L (Palmer et al., 2008). Atenolol has also been detected in the open water at a concentration of 145ng/L (Hernando, Mezcua, Fern, & Barcel, 2006).

Since it is detected in the open environment, atenolol has been investigated for adverse effects on fish. For example, studies have shown that atenolol can affect the function of vital cellular activities in fish. Environmentally relevant concentrations of atenolol have been shown to cause the reduction of hemoglobin in trout (Steinbach et al., 2014). The production of glucose in trout has also been shown to be greatly decreased when in an environment with concentrations as low as 0.01 nM (Ings, George, Peter, Servos, & Vijayan, 2012). These effects from atenolol have the possibility of decreasing the overall fitness of fish exposed to even small concentrations.

1.5.2 Ibuprofen

Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) (Fig. 3) (Bushra & Aslam, 2010). Since its introduction in 1969, ibuprofen has become a popular pain reliever (Bushra & Aslam, 2010). NSAIDs such as ibuprofen work by inhibiting cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2); this inhibition leads to anti-inflammatory, pain relieving, and fever reducing effects (Bushra & Aslam, 2010). Unlike

atenolol, ibuprofen is available without a prescription. However, there are higher doses of ibuprofen available with a prescription. Eight percent of the United States' population has a prescription for this stronger version of the over-the-counter ibuprofen (Palmer et al., 2008).

Once it enters the body, up to 15% of the ibuprofen is excreted as the parent compound, while the remainder is excreted as the metabolites hydroxy-ibuprofen and carboxy-ibuprofen (Weigel et al., 2004). However, even though humans are only excreting a maximum of 15% of the unchanged parent compound, ibuprofen is still detected throughout the environment. In a study that looked at 84 streams throughout the United States, ibuprofen was detected at a frequency of 9.5% and at a median concentration of 0.2 µg/L with its maximum detection being 1 µg/L (Kolpin et al., 2002). In treated effluent ibuprofen has been detected at concentrations of up to 14.6 µg/L (Palmer et al., 2008). Average concentrations of ibuprofen in treated effluent have been reported to be 2.1 µg/L (Hernando, Mezcua, Fern, et al., 2006).

Aquatic organisms such as fish have been shown to experience adverse health effects when exposed to ibuprofen. In one study on Japanese killifish, exposure to the environmentally relevant concentration of 0.1 µg/L has shown delayed egg hatching even when they were transferred to clean water (Han et al., 2010). The study also found that exposure caused decreased survival of adult fish (Han et al., 2010). In another study with killifish, it was shown that ibuprofen increases the number of eggs laid, but decreases the number of spawning events (Flippin, Huggett, & Foran, 2007). These changes may alter the food chain and overall health of the ecosystem.

1.6 Microbial Interactions with Pharmaceuticals

Pharmaceuticals are being released into wastewater treatment plants and the open environment where microbial communities are exposed to these contaminants. Microorganisms have been shown to transform or degrade a variety of pharmaceuticals and even utilize them for growth, while other microorganisms can be inhibited or killed by the same pharmaceutical (Helbling, Hollender, Kohler, Singer, & Fenner, 2010; Kagle, Porter, Murdoch, Rivera-Cancel, & Hay, 2009; Murdoch & Hay, 2005). For example, *Sphingomonas* Sp. Strain Ibu-2 has the ability to aerobically metabolize the pharmaceutical ibuprofen (Murdoch & Hay, 2005). Ibuprofen has also been shown to inhibit growth for a variety of microorganisms (Obad, 2015). In some cases, metabolites produced during microbial degradation of pharmaceuticals cannot be further broken down (Helbling et al., 2010). These metabolites can still be pharmacologically active and can sometimes exhibit toxicity to eukaryotic organisms (Celiz, Tso, & Aga, 2009;López-serna, Petrovi, & Barceló, 2012). While the effects of pharmaceutical metabolites have been studied on eukaryotic organisms, these effects have not yet been documented on prokaryotic organisms such as bacteria and archaea. As prokaryotes are vital to ecosystems in all environments, it is important to further investigate the effects of pharmaceutical metabolites on prokaryotes.

1.7 Microbial Degradation of Pharmaceuticals

Solutions have been proposed to remove pharmaceuticals from wastewater before they can reach the environment, such as implementing new technology like advanced oxidation processes and membrane bioreactors (Radjenović, Petrović, & Barceló, 2009; Stasinakis, 2008). These new technologies have shown promising results at pilot

treatment plants with higher removal rates than conventional treatment technologies (Radjenović et al., 2009). However, due to high costs from installing new equipment, cleaning, spare parts, and maintenance, this technology is not feasible for most municipal wastewater treatment plants (Judd, 2008; Stasinakis, 2008).

Researchers have started to investigate whether microorganisms that already inhabit the wastewater treatment plant and the environment are able to degrade pharmaceuticals as a potential solution to reduce the amounts of these emerging contaminants. The degradation rate varies greatly depending on the pharmaceutical (Onesios et al., 2009). During biological treatment, studies have shown that a variety of pharmaceuticals can be removed from water with removal efficiencies from 0% up to 99% due to biodegradation (Onesios et al., 2009; Radjenović et al., 2009).

Removal in the environment has been studied to a lesser extent. one study looked at the degradation of pharmaceuticals in terms of degradation half-lives in estuarine and seawater microcosms (Benotti & Brownawell, 2009). Again, the degradation half-lives varied greatly depending on the pharmaceutical. For example, degradation half-lives ranged from 0.68-11 days for nicotine, acetaminophen, and fluoxetine and from 35 over 100 days for antipyrine, carbamazepine, and sulfamethoxazole (Benotti & Brownawell, 2009). Due to the thousands of compounds that fall into the category of pharmaceuticals, it is important that researchers look at frequently used pharmaceuticals and their metabolites in order to determine whether or not they can be removed by microbial means.

1.8 Conclusions

Exceedingly large amounts of pharmaceuticals are used throughout the world on a daily basis and their excretion into the environment is becoming both a public and environmental health concern. It is therefore important to find a way to remove these pharmaceuticals from the environment as well as to remove them before they reach the environment. The following experiments aim to enrich for a microbial community that is capable of metabolizing the selected compounds, atenolol or ibuprofen, as a nutrient or carbon source under methanogenic conditions, identify the members of this community, and assess the biodegradability and toxicity of the selected compounds to the microbial community.

1.9 Figures:

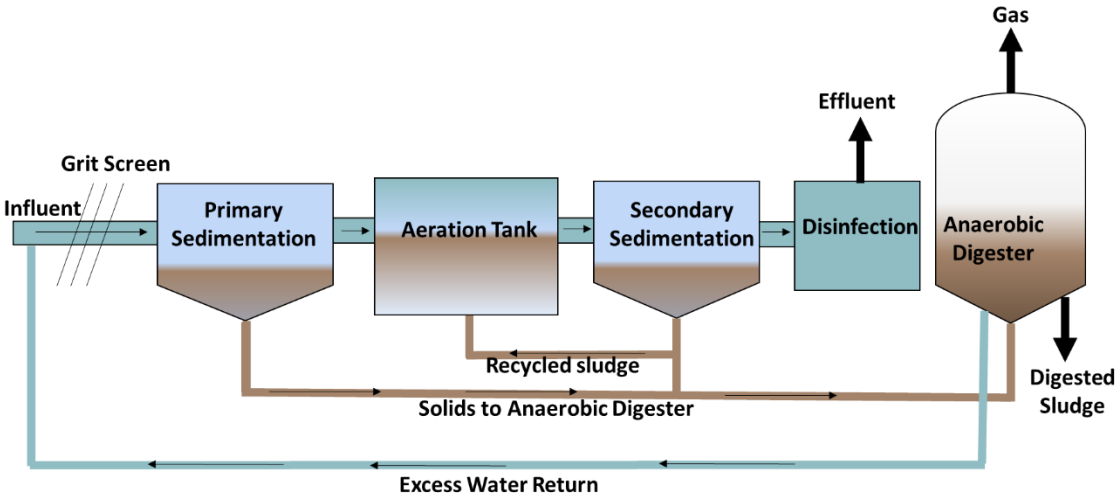

Figure 1: Diagram of simplified wastewater treatment process

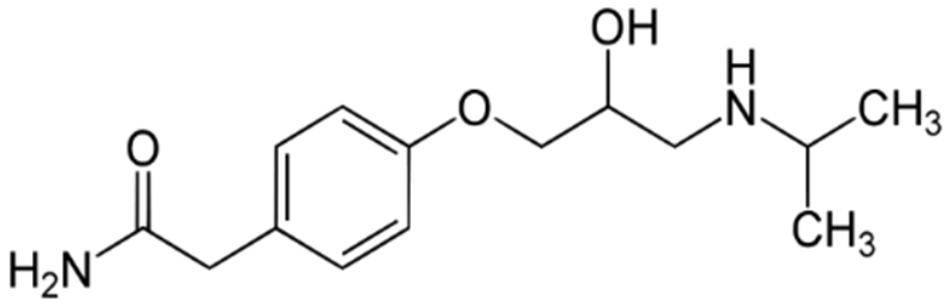

Figure 2: Structure of atenolol

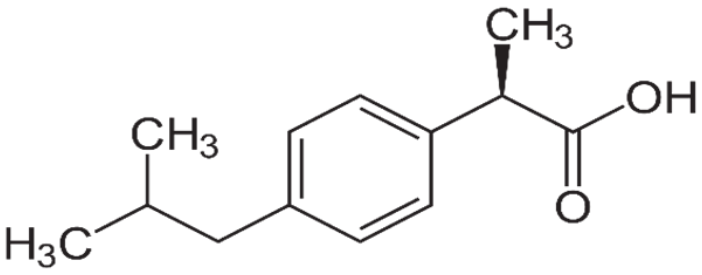

Figure 3: Structure of ibuprofen

Chapter 2

Transformation of Atenolol and Gas Production

2.1 Introduction

Atenolol has been detected throughout the environment and enters through incomplete removal during wastewater treatment (Monteiro, 2014; Kostich, Batt, & Lazorchak, 2014). Its presence in the environment indicates that atenolol is not completely removed by conventional wastewater treatment. The microbial transformation of atenolol to the metabolite atenolol acid has been documented under aerobic conditions in both activated sludge and soil (Radjenović, Pérez, Petrović, & Barceló, 2008; Koba, Golovko, Kode, & Grabic, 2016). Barbieri et al. (Barbieri et al., 2011) demonstrated microbial degradation of atenolol under sulfate, manganese, and iron reducing conditions as well as denitrifying conditions.

The microbial degradation of atenolol under methanogenic conditions to our knowledge has not been reported. Under methanogenic conditions it is possible to measure microbial activity by measuring the production of methane, an end product of methanogenesis (Balch, Fox, Magrum, Woese, & Wolfe, 1979). The aim of this study was to examine the activity of the microbial community via gas and methane production and to monitor the transformation of atenolol via High Performance Liquid Chromatography (HPLC) under methanogenic conditions in two sets of enrichment cultures. One set up used anaerobic digester sludge as the inoculum, a semi controlled environment where the microbial community is exposed to pharmaceuticals on a daily

basis. The second set up used anaerobic sediment from a marsh, an open environment where the microbial community is seldom exposed to pharmaceuticals.

2. 2 Materials and Methods:

2.2.1 Enrichment Culture Establishment

Two sets of enrichment cultures were established. Inocula were obtained from an anaerobic digester in Rockland County Sewer District One in Rockland County, New York (anaerobic sludge cultures) and sediment from the Iona Island Marsh (Iona cultures), a marsh located on the Hudson River in Rockland County, New York. Both sets of enrichment cultures were set up in the same manner, with only the source of the inoculum differing. In 160 mL serum bottles, 100 mL slurries, comprised of 20 mL anaerobic sludge or Iona Marsh sediment and 80 mL of defined medium was anaerobically added under a 70% N_2/ 30% CO_2 atmosphere (Owen et al., 1979) (see appendix for media components). A headspace of 60 mL allowed measurement of gas production. The serum bottles were sealed with blue rubber butyl stoppers and crimped. Atenolol was added at 0.5 mM as the sole carbon source. Since atenolol has low solubility in water, an atenolol stock was created in which atenolol was dissolved in methanol and added to silica at 0.5 mmol per gram of silica. The methanol was then evaporated off leaving atenolol adsorbed to the silica, and the atenolol-sorbed silica was then suspended in sterile methanogenic media and 1.0 mL was injected via syringe into the sealed cultures. Cultures were established in triplicate. Duplicate sterile controls were prepared in the same manner but were autoclaved once each day for three consecutive days to ensure sterilization. Duplicate background controls were prepared in the same manner as the actives but with no addition of atenolol. The Iona cultures were incubated

at 25°C in the dark while the anaerobic sludge cultures were incubated at 37°C in the dark, as that is the average temperature of the digester (Linda Hoffman, Personal Communication, Rockland Co. Sewer District One, 2015). After the Iona cultures completely transformed the first amendment, they were reamended in the manner described above. Once the reamendment was transformed, the cultures then underwent a 50% transfer into fresh medium and were reamended with atenolol. Anaerobic sludge cultures only underwent one amendment.

2.2.2 HPLC Analysis

Loss of the substrate atenolol was monitored using a Beckman (Brea, Ca.) System Gold HPLC with an ultrasphere ODS (250 mm by 4.6 mm; particle size 5 µm) column and UV detector. After, 0.6 mL samples were taken via syringe from the cultures by insertion through the rubber stopper seal, 0.6mL of N_2/CO_2 was injected back into the cultures in order to prevent negative pressure. A 1:1 methanol extraction was then performed, gently shaking the sample after the addition of methanol. The extraction then sat for ten minutes. 0.8 mL of the extraction is then filtered through SpinX 0.22 µm filter tubes and transferred to a glass vial for HPLC analysis. The following HPLC procedure is adapted from (Sasaki et al., 1995). The mobile phase was 20% methanol and 80% 50 mM NaH_2PO_4 at a flow rate of 1 mL/min. Ultraviolet detection was set to an absorbance of 226 nm, with atenolol retention time of five minutes; the total run time was extended to eight minutes to ensure the detection of any metabolites.

2.2.3 Metabolite Identification

Identification of the metabolite was achieved using an Agilent 1100 series (Santa Clara, Ca.) HPLC-MS equipped with a quaternary pump, DAD, and a G2445A MSD trap with electrospray ion source set to positive mode. HPLC was performed using a Phenomenex (Torrance, Ca.) Prodigy column, 250 mm x 4.6 mm x 5 µm. The flowrate was set to 1.0 mL/min with a 1:3 splitter leading to reduce flow into MS. The mobile phase consisted of solvent A (0.1% formic acid in water (v/v)) and solvent B (0.1% formic acid in acetonitrile) (v/v)). The gradient starts at 1% B to 20% B from 0 to 19 minutes and 20% to 1% B from 19 to 20 minutes. The DAD was set at 210, 254, 280, 370, and 450nm. For the MSD trap, capillary voltage was set to 3.5kV. Nitrogen flowrate was 9 L/min and the capillary temperature was set at 350°C. Nitrogen was set as a nebulizer at 40 psi and helium as a collision gas using 80% collision energy. To quantify the concentration of the metabolite atenolol acid, standards were made at varying concentration using the pure chemical atenolol acid (Toronto Research Chemicals, Toronto, Canada) and the same HPLC method in section 2.2.2 was utilized.

2.2.4 Total Gas and Methane Production

Total gas production was monitored and analyzed for methane production every three to five days for a thirty-five day period. Anaerobic sludge cultures were allowed to come to room temperature prior to gas monitoring. To monitor total gas production, a wetted glass syringe equipped with a needle was flushed with N_2/CO_2 several times to displace air and atmospheric oxygen. Inserting the needle into the septa of the serum bottle and holding the syringe horizontal to allow free movement allows the excess gas in the serum bottles to freely displace the plunger. The gas volume was then recorded as

described in Healy and Young (1979). One milliliter of gas was then returned back into the culture to be sampled and used separately for methane analysis while excess was released. If gas produced was less than or equal to one milliliter, the entire volume was returned back to the culture.

The Fisher Gas Partitioner Model 1200 (Waltham, Ma.), a GC-TCD, was used to monitor methane production. Immediately after recording the excess gas produced by a culture, 0.5 mL was injected into the GC-TCD, then the percentage of methane measured was then multiplied by the excess gas production found earlier to find the total volume (mL) of methane produced.

2.3 Results

2.3.1 Transformation of Atenolol in Iona Cultures

As seen in Figure 4, atenolol concentration in active cultures decreased 98% by day 15. By day 45 the atenolol had been completely transformed with no detectable atenolol remaining. As the atenolol was transformed a metabolite begins to appear. At day 0 no metabolite is present; by day 15 the concentration has increased to 0.35 mM. At day 45, with atenolol depleted, the metabolite concentration increased to 0.4mM. It is important to note that the initial concentration of atenolol was quantified at 0.4mM and once atenolol was depleted the metabolite concentration was 0.4mM. This indicates that atenolol is being completely transformed to this metabolite.

Upon reamendment with 0.5 mM of atenolol (day 64 in Figure 4), the cultures continued to metabolize 61% of the atenolol after day 7 (represented as day 71 in Figure 4). By day 34 (represented as day 98 in Figure 4), no atenolol was detected in the

amended cultures. The concentration of the metabolite continued to increase. In its first refeeding, the same pattern of rapid transformation provides further evidence that this community is robust and does not seem to be affected by the accumulating metabolite. No loss of atenolol was seen in sterile controls. The unamended backgrounds (not shown) did not have any background atenolol or atenolol metabolite. The ability of these cultures to transform the majority of the compound within 15 days indicates that a community was already established in this environment with the ability to transform atenolol to the metabolite.

After two amendments and complete loss of atenolol, the 100 mL cultures were split; 50 mL of culture from each bottle was transferred into 50 mL fresh medium making the total volume 100 mL. The remaining 50 mL of culture in the original bottles was given 50 mL of fresh media. The split cultures were monitored for transformation of atenolol (Fig. 5). Due to the split, there was carryover of the metabolite, making the initial concentration 0.49 mM. On day 7, 28% of the atenolol had been transformed. At day 45, over 99% of atenolol had been transformed to the metabolite. The ending concentration of the metabolite was 1.0 mM, again, atenolol was completely transformed to the metabolite. No loss of parent compound was seen in sterile controls. The split cultures were slower to transform atenolol to the metabolite. While 98% of atenolol was transformed to the metabolite by day 15 in the first two amendments (Fig. 4), it was only by day 28 that the split cultures transformed 98% of atenolol to the metabolite (Fig. 5). During the split, biomass and organic matter is reduced to half of its original volume. This likely explains why the split cultures took longer to transform the atenolol than the first two amendments.

2.3.2 Total Gas and Methane Production in Iona Cultures

Methane production in the amended cultures illustrated in Figure 6, was not observed until day 23. By day 31, a dramatic increase in methane production was seen, producing 48% of the total methane produced during the initial feeding. Gas production rate started to decrease on day 45. Day 63, the day prior to refeeding, had a cumulative methane production of 35 mL. Cultures were refed on day 64. By day 7 after the refeeding (represented as day 71 in Figure 6) 29 mL of methane had been produced, bringing the cumulative methane production to 64 mL. The methane production continued to slowly climb until day 98 with total cumulative methane production reaching 73 mL. The unamended backgrounds produced little methane totaling 9 mL by day 98. The sterile controls produced no methane. Total gas of the active cultures was similar to methane production until day 31, with methane accounting for over 90% of the total gas produced during that time. Total gas production started to increase by day 38 and continues to further separate from methane production. By day 98 total gas production reached 105 mL. After day 38, methane accounted for 66% to 75% of total gas. Background cultures produced almost the same ratio of total gas to methane. Sterile controls produced no gas. Amended cultures produced more than eight times the amount of methane and total gas as the background controls. Increase in both total gas and methane in active cultures indicate that both fermentative and methanogenic communities benefit from the addition of atenolol. In comparison, background cultures seemed to select for a community that produced mostly methane.

The bottles were opened during the culture split, and reamended with 0.5 mM of atenolol. This began a new timeline of measuring methane production. Similar to the

primary enrichments, a spike in methane production is seen on day 7 with total cumulative methane increasing from 2 mL on day 2 to 29 mL on day 7 (Fig. 7). After day 7, methane continues to be slowly produced. Total gas production in the amended cultures saw the same spike in production at day 7 that was seen for methane production but at a higher magnitude. Methane accounted for 59% to 69% of total gas after day two. The split seemed to decrease the percent of methane in the total gas. This indicates that a methanogenic community is still abundant, but less methane is being produced which could indicate that other anaerobic gas producers such as fermenters are becoming more robust.

2.3.3 Transformation of Atenolol in Anaerobic Sludge Cultures

The amended cultures transformed more than 30% of the atenolol by day 15 (Fig. 8). A formation of metabolite began in the same manner as the Iona cultures. By day 71, 87% of the atenolol had been transformed and the metabolite concentration continued to increase. By day 98 atenolol was no longer detected. The metabolite concentration was 0.36 mM. Similar to the Iona cultures, atenolol was completely transformed to the metabolite. The initial concentration of atenolol was quantified at 0.39 mM; the ending concentration of the metabolite was 0.36 mM. The sterile controls had no loss throughout the experiment. The background cultures (not shown) had no atenolol or atenolol metabolite detected. The transformation of atenolol to its metabolite took almost 100 days to complete. This indicates that while transformation of atenolol to its metabolite is possible in anaerobic sludge, it is a slow process and the microbial community with the ability to transform atenolol may not exist in high numbers in this environment.

2.3.4 Total Gas and Methane Production in Anaerobic sludge Cultures

A spike of methane production in the amended cultures was seen by day 15, with 44% of the total cumulative methane produced by this time point (Fig. 9). A steady production of methane continued until day 63. After this point methane production was slower. Slow production continued until day 98, bringing the total cumulative methane production to 68 mL. A spike in total gas production was seen by day 15, producing 37% of the cumulative total gas. Total gas production kept steadily increasing until day 63. Total gas production after day 63 did start to slow down but not to the same extent that methane production did. Cumulative total gas production reached 109 mL by day 98. Background cultures produced a total of 31 mL of methane and did not experience the same spike in methane production as the amended cultures. Background cultures steadily produced total gas until day 52; afterwards, they slowly produced total gas reaching a cumulative total of 70 mL by day 98. Methane production in active cultures accounted for an average of 61% of the total gas, while methane production in backgrounds accounted for an average of 46% of the total gas. Amended cultures had both higher total gas production and methane production than the backgrounds indicating that atenolol enhances both the methanogenic and the anaerobic community as a whole. However, methane accounted for the majority of total gas in the amended cultures as opposed to the backgrounds, in which methane accounted for little under half the total gas. While atenolol seems to enhance the methanogenic community and the anaerobic community as a whole in comparison to the backgrounds, when compared solely to gas production within the active cultures methanogens, are favored.

2.3.5 Identification of the Metabolite

In both culture set ups the metabolite was identified as atenolol acid (Fig. 10). The transformation to atenolol acid involves a hydrolysis of the amide group and its replacement with a hydroxyl group, resulting in a carboxyl in the 6 position. Its appearance coincided with the degradation of atenolol. This was the only metabolite identified and since it molar concentration coincided quantitatively with the loss of atenolol, it appears that atenolol was completely transformed to atenolol acid.

2.4 Discussion

2.4.1 Identification of the Metabolite

Atenolol acid has been identified as the microbial metabolite of atenolol degradation in multiple studies and in a variety of environments. The transformation of atenolol to atenolol acid has been documented in soil, wetland, and activated sludge microcosms (Koba et al., 201; J Radjenović et al., 2008; Svan et al., 2016). These microcosms were all under aerobic conditions. The experiments carried out for this thesis were done under anaerobic conditions in both freshwater marsh sediment and anaerobic sludge from a wastewater treatment digester. To our knowledge, this is the first documentation of the transformation of atenolol to atenolol acid under anaerobic conditions. The variety of environments and conditions in and under which atenolol is microbially transformed to atenolol acid indicates this ability is spread throughout a variety of bacteria. Atenolol has structural similarity to aromatic amino acids. Amino acids, whether synthesized or taken in, are needed by all life forms. This may explain

why the similarly structured atenolol is transformed in a variety of environments as bacteria throughout all types of environments interact with amino acids.

2.4.2 The Transformation of Atenolol and Methane and Total Gas Production

Both the Iona cultures and anaerobic sludge cultures were able to transform atenolol to atenolol acid. Atenolol amended cultures in both set-ups had an increase of both total gas production and methane production compared to their backgrounds, indicating that atenolol enhanced the activity of both the fermentative and methanogenic communities. However, the amount of time that it took to transform atenolol to atenolol acid and the amount of gas produced varied between the two set-ups. The anaerobic sludge cultures took a total of 98 days to transform 0.5 mM of atenolol to atenolol acid (Fig. 8). Meanwhile, the Iona primary cultures were able to transform 0.5 mM of atenolol to atenolol acid, were refed, and transform another 0.5 mM of atenolol to atenolol acid within the same time period of 98 days (Fig 4). Interestingly, the anaerobic sludge cultures quickly produce both methane and total gas; by day 22, total gas production was 71 mL and methane production was 47 mL (Fig. 9). The Iona cultures had under 4 mL of both total gas and methane production by day 22 (Fig. 6). It took two amendments of atenolol in the Iona cultures to produce the same amount of total gas and methane that the anaerobic sludge cultures produced with one amendment of atenolol. The Iona cultures were faster at transforming atenolol while the anaerobic sludge cultures were better at producing both total gas and methane.

These inocula are from extremely different environments and it is probable that this is the reason why these cultures behaved so differently. The Iona Island marsh is an intertidal marsh with sediments comprised of peat and silt (Yozzo et al., 2005). The

marsh is home to a variety of microbial life, but also wildlife such as marsh nesting birds, frogs, salamanders, and fish; the marsh also provides a spawning and nursery habitat for many fish such as blueback herring, white perch, striped bass, and mummichog (Yozzo et al., 2005). The marsh is lush with plants such as pickerel-weed, cattail, and the invasive common reed (Yozzo et al., 2005). The Iona Island marsh is in the open environment and constantly changing; wildlife will be born in this marsh and then die and decay, and plants will grow and shed their foliage into the marsh with the changing seasons. These factors contribute to the amount of carbon and solids found in the marsh; Iona Island marsh sediment has an average of 55% total dry solids (EPA STORET data). These solids contain carbon which is vital for the growth of microorganisms. No carbon is being removed in the transformation of atenolol to atenolol acid, only nitrogen. There is not carbon within the defined medium. It is probable that the high amount of solids in the inoculum is the carbon source for the microorganisms. While nitrogen was added to the media in the form of ammonia, the Iona Island marsh area has a low nitrogen concentration in the water phase. Total nitrogen ranges from 0.5-0.6mg/L with less than half being ammonia (*Draft Environmental Impact Statement for State Pollutant Discharge Elimination System Permits for Bowline Point, Indian Point 2& 3, and Roseton Steam Electric Generating Stations*, 1998). Atenolol is similar to large complex nitrogen sources such as aromatic amino acids like phenylalanine. The microorganisms in this environment may prefer or need complex nitrogen sources. This explains why these microorganisms need complex nitrogen sources such as atenolol, even though nitrogen was already provided in the form of ammonia. Nitrogen is a key macronutrient that provides building blocks for cells to make proteins and nucleic acids and therefore helps

synthesize new cells (Bitton, 2005). The removal of nitrogen from atenolol can provide the nitrogen that helps cells proliferate. The increase in cells then increases the amount of cells that can metabolize the carbon to C-1 and C-2 compounds. These C-1 and C-2 compounds can be utilized by the methanogens. Therefore, atenolol indirectly increases methane production.

In contrast, the anaerobic digester in which the sludge is contained is a closed environment. Factors such as temperature, the amount of sludge, and how long the sludge is kept in the digester, along with the entire wastewater treatment process are all controlled. Anaerobic sludge is only home to microbial life that have been enriched to perform a specific function, which is to reduce and stabilize solids and to produce methane and carbon dioxide. This inoculum has already been highly enriched with microorganisms that produce methane and other gases. Unlike the Iona cultures, which were incubated at 25°C, the anaerobic sludge cultures were incubated at 37°C. This is the same temperature of the digester from which the inoculum of anaerobic sludge cultures was obtained. Methanogens have been shown to be more metabolically active (i.e., methane producing) at higher temperatures (Whitman et al., 2006).This explains why the anaerobic sludge cultures produced the same amount of total gas and methane as the Iona cultures. However, unlike the Iona cultures, which received two amendments before being split, anaerobic sludge cultures only received one amendment of atenolol. Anaerobic sludge has a much higher nitrogen level than the Iona sediment. The average total nitrogen concentration in domestic wastewater is 35mg/L; 25% of this is removed through sedimentation (Bitton, 2005). The sludge collected from sedimentation is sent to the digester; this makes the theoretical concentration of total nitrogen in the digester

about 9mg/L. This is much higher than the amount of total nitrogen found in Iona Island marsh. In the anaerobic sludge cultures, it took twice the amount of time to transform one amendment of atenolol to atenolol acid compared to the Iona cultures. The high nitrogen concentration in the anaerobic sludge cultures may have slowed the degradation rate of atenolol as the microorganisms did not immediately need to utilize atenolol for nitrogen.

2.5 Conclusions

Atenolol was transformed to atenolol acid under methanogenic conditions by both the Iona and anaerobic sludge cultures. However, the time and amount of gas produced varied between the two cultures. The Iona cultures were able to transform atenolol to atenolol acid at a faster rate than the anaerobic sludge cultures. The transformation of atenolol to atenolol acid also seems to increase methane and total gas production, implying it increases the microbial activity in both culture set-ups. However, the one-time amended anaerobic sludge cultures could produce the same amount of methane and total gas as the Iona cultures that had been amended twice. Key differences that could be attributing to these variations are the amount and type of nitrogen as well as the microbial community that exists within each inocula. What is novel about this study is that this transformation has yet to be reported under anaerobic conditions in known literature.

2.6 Figures

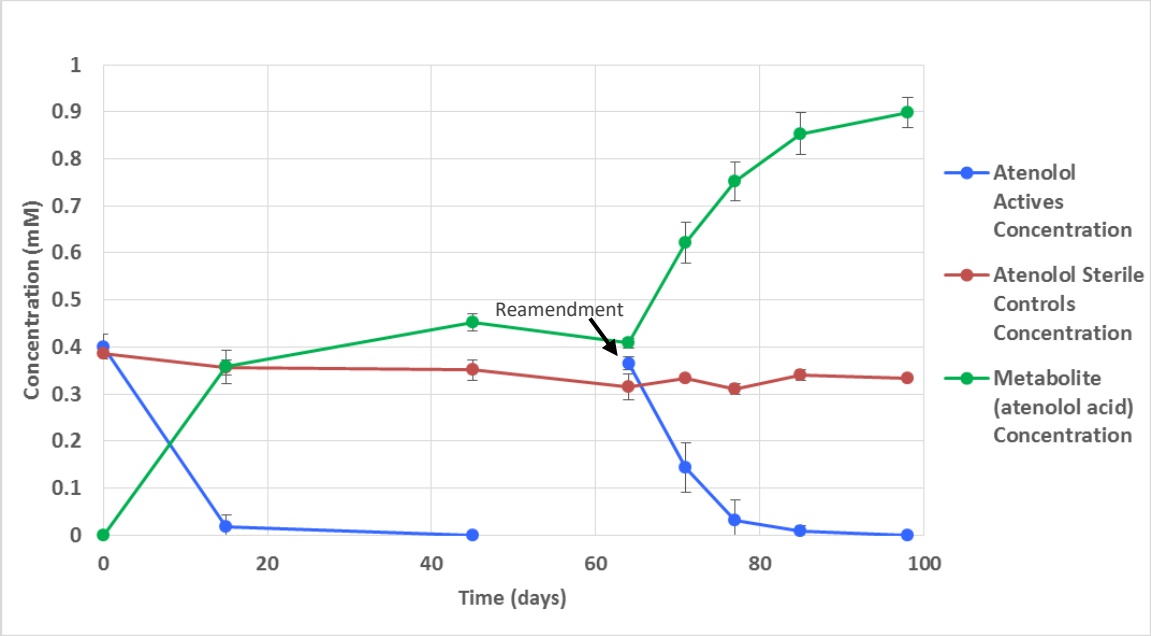

Figure 4: Transformation of atenolol in Iona primary cultures (note: some error bars cannot be seen due to the size of the marker)

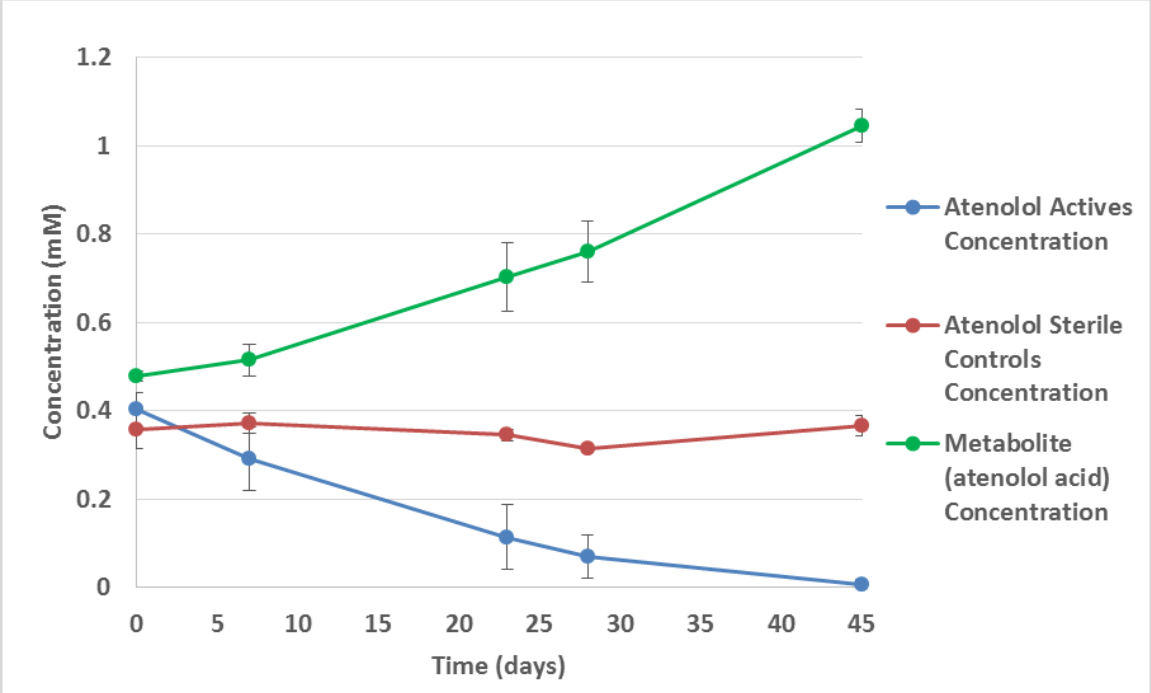

Figure 5: Transformation of atenolol in Iona split cultures (note: some error bars cannot be seen due to the size of the marker)

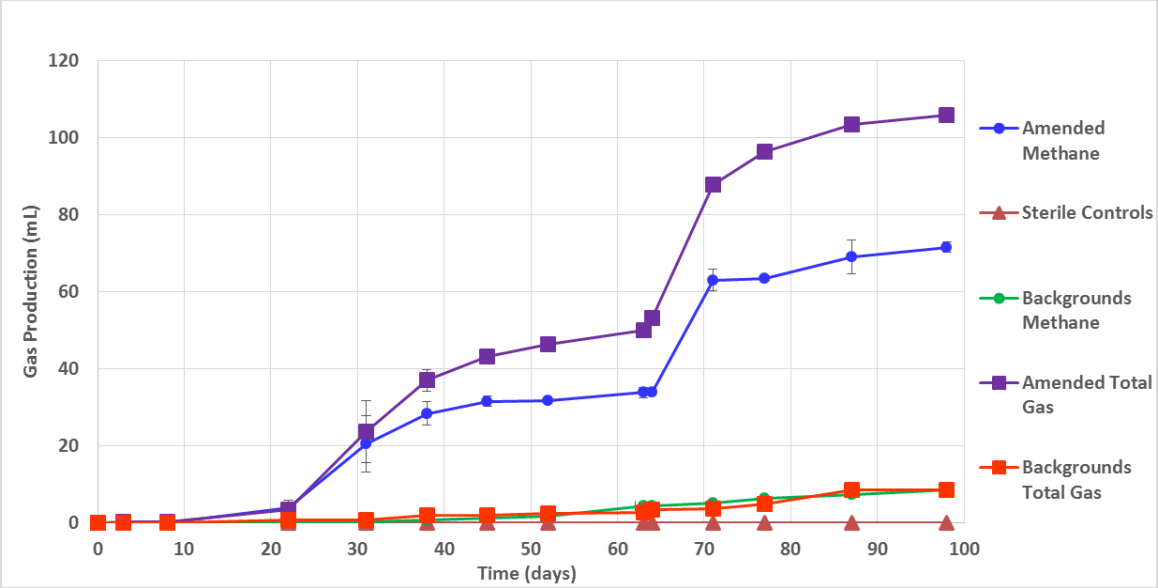

Figure 6: Cumulative methane and total gas production in atenolol amended Iona primary cultures (note: some error bars cannot be seen due to the size of the marker)

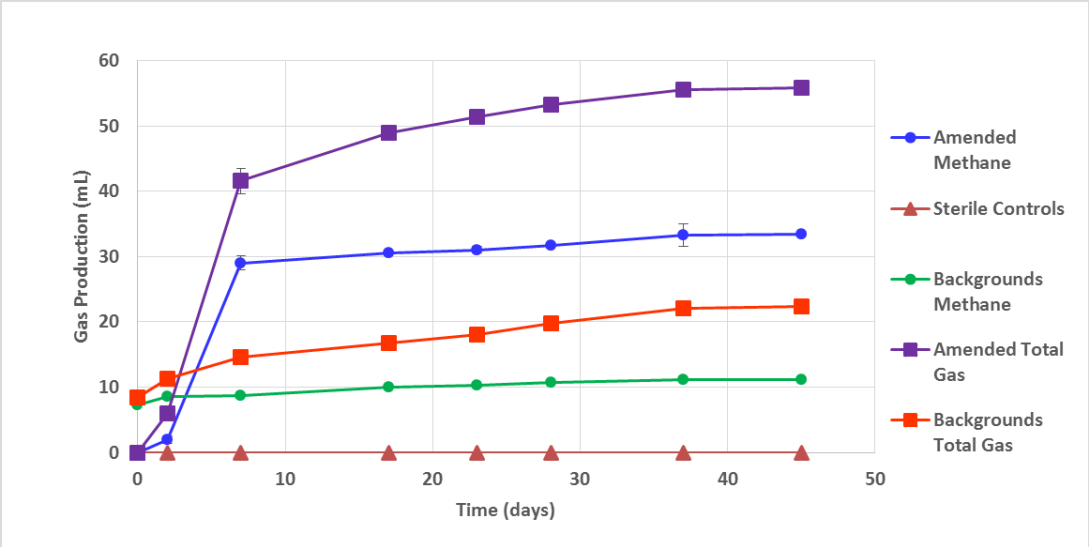

Figure 7: Cumulative methane and total gas production in atenolol amended Iona split cultures (note: some error bars cannot be seen due to the size of the marker)

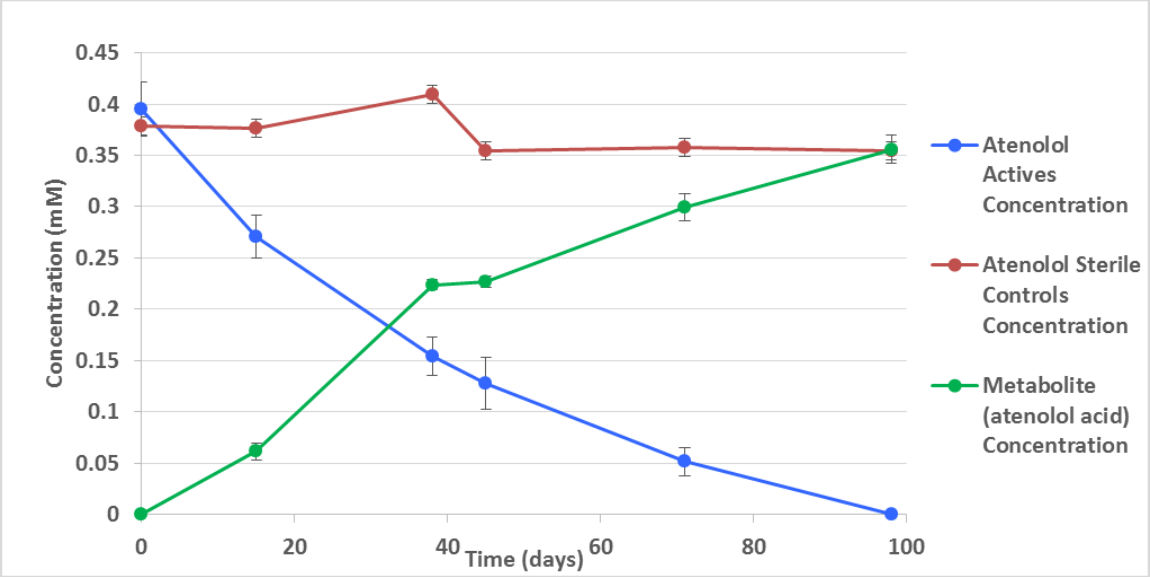

Figure 8: Transformation of atenolol in anaerobic sludge cultures (note: some error bars cannot be seen due to the size of the marker)

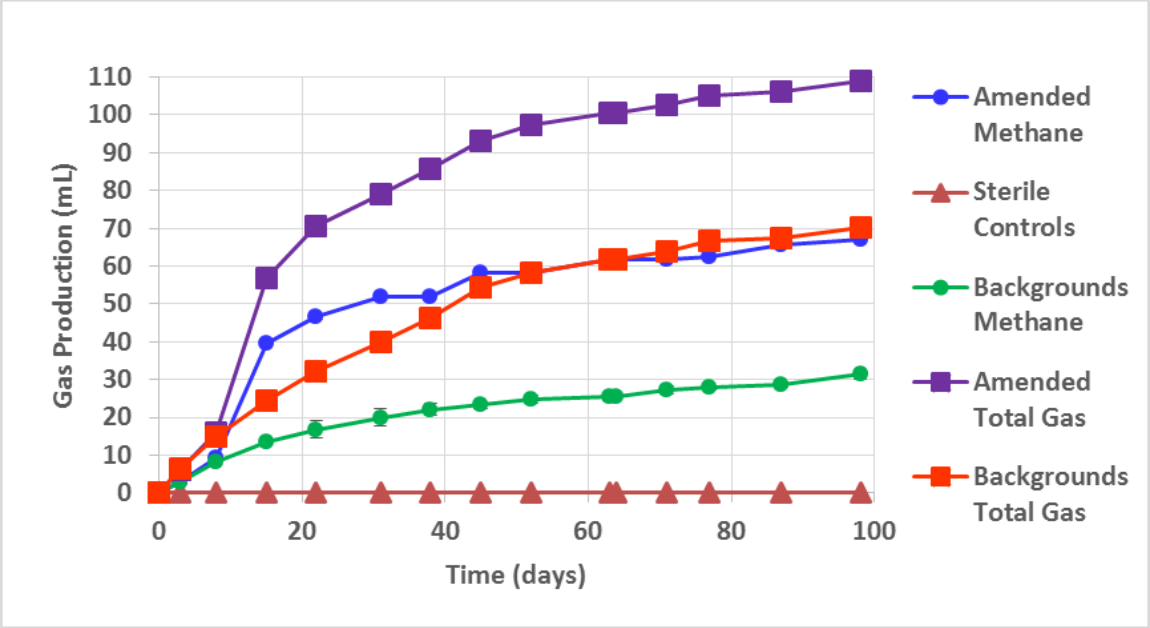

Figure 9: Cumulative methane and total gas production in atenolol amended anaerobic sludge cultures (note: some error bars cannot be seen due to the size of the marker)

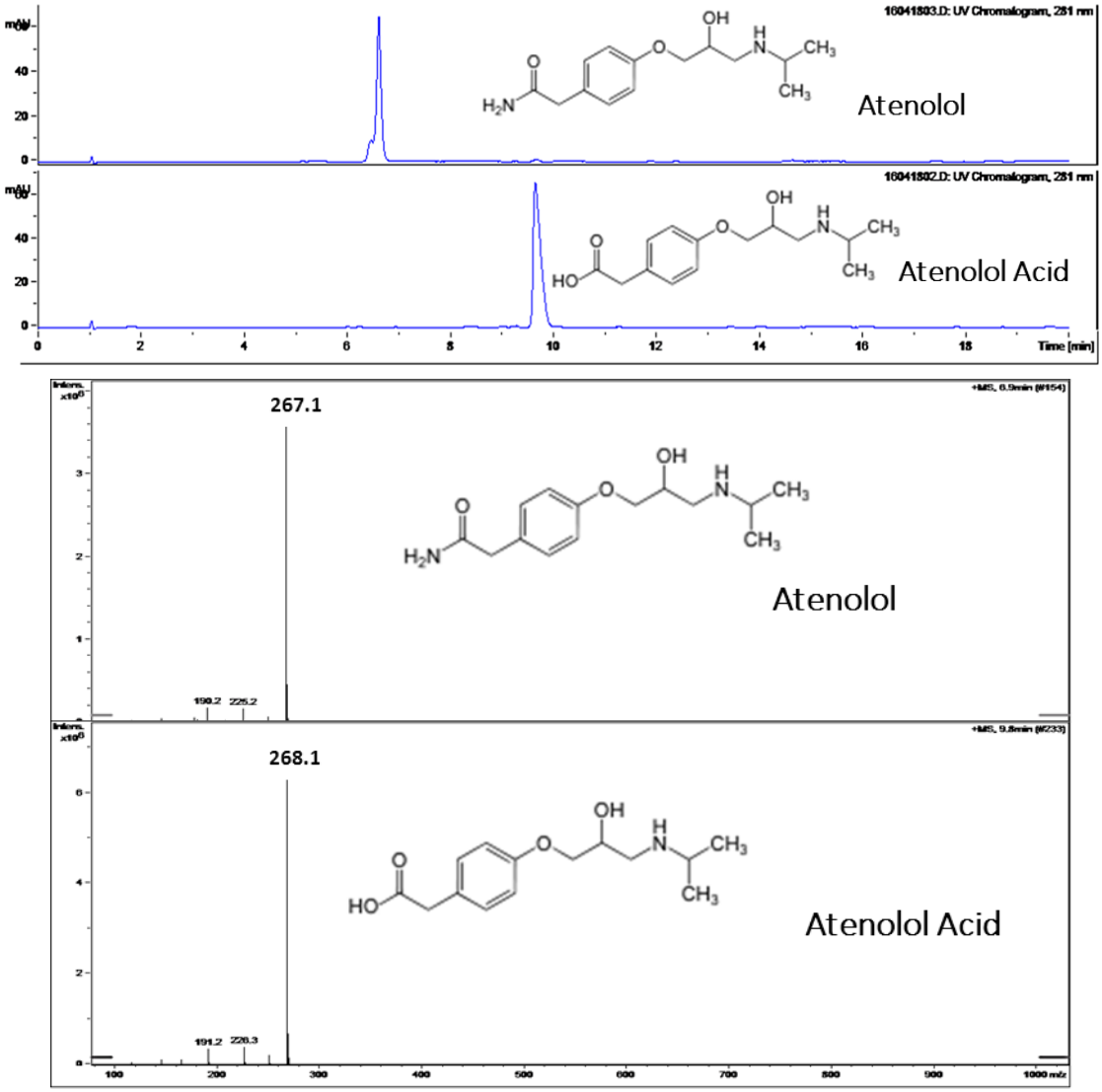

Figure 10: Peak and mass spectrum of atenolol (in sterile controls) and atenolol acid (in active cultures)

Chapter 3

Anaerobic Toxicity Assay and Biochemical Methane Potential of Atenolol Amended Cultures

3.1 Introduction

Anaerobic digestion utilizes microbial metabolism to break down and stabilize organic matter during which carbon dioxide and methane gas are produced (Hammer and Hammer, 2004). This is a vital process to many municipal, agricultural, and industrial waste/wastewater treatment facilities. Because of the reliance of this treatment process on active microorganisms, it is important to identify any toxic or inhibitory compounds and non-biodegradable compounds that could potentially cause the system to fail (Chen et al., 2008). If a compound is not biodegradable, it cannot be converted to gas. Toxic or inhibitory compounds can inhibit or kill the methanogens and fermentative bacteria responsible for converting the solids to methane and carbon dioxide. Examples of such compounds include ammonia, sulfide, heavy metals, organics, and xenobiotics such as surfactants and detergents (Chen et al., 2008). In order to test the biodegradability, inhibition, and toxicity of organics, two techniques were developed by Owen, et al (1979): The Anaerobic Toxicity Assay (ATA) and the Biochemical Methane Potential (BMP) (Owen et al., 1979).

The BMP measures a compound's biodegradability by measuring the amount of methane produced by cultures amended with the compound of interest and comparing it to the background that contains only the inoculum (Owen et al., 1979). If

the amended cultures produce more methane than the background cultures the compound is biodegradable; if it produces the same or less it is not biodegradable.

The ATA measures whether or not a compound is toxic or inhibitory. Amended cultures are fed the compound of interest and acetate and propionate; methanogens can utilize acetate and anaerobic bacteria can utilize both acetate and propionate (Owen et al., 1979). The amended cultures are then compared to active cultures that have only been fed acetate and propionate. If amended cultures produce the same or more total gas and methane than that of the unamended active cultures, the compound of interest is not toxic to the anaerobic community; if the amended cultures produce less total gas and methane than the unamended active cultures, the compound of interest is toxic to the anaerobic community (Owen et al., 1979).

In this study, atenolol is investigated for any potential inhibitory or toxic effects to the microbial community by performing an anaerobic toxicity assay. Its biodegradability potential is investigated by performing the biochemical methane potential.

3.2 Materials and Methods

3.2.1 Biochemical Methane Potential and Anaerobic Toxicity Assay

The BMP inocula was obtained from Rockland County Sewer District One in Rockland County, New York, from their anaerobic digester. The inoculum was incubated at 37°C for one week to further reduce any readily degradable organic material in the collected sample. Cultures were established in the same manner as described in section 2.2.1. Triplicate amended active cultures were prepared at three different concentrations of atenolol: 1.0 mM, 0.5 and 0.25. Duplicate sterile controls with 1.0 mM atenolol were

prepared in the same manner as the amended active cultures but were autoclaved once each day for three consecutive days to ensure sterilization. Duplicate background controls were prepared in the same manner as the actives but with no addition of the atenolol in order to assess the amount of gas that is produced due to the addition of atenolol in the amended cultures.

The ATA inoculum was obtained from the same source and was allowed to incubate for one day to reduce any readily degradable organic material. In 60 mL serum bottles, 20% volume of inoculum was anaerobically added under an N_2/CO_2 atmosphere to a defined methanogenic media as described in (Owen et al., 1979). This gave a total liquid volume of 35 mL. A headspace of 25 mL was left in the enrichment cultures order to measure future gas production. The serum bottles were then sealed with a rubber butyl stopper and crimped with aluminum seals. Active amended cultures were prepared in triplicate at: 0.5 mM and 0.25 mM. Each culture was then given a solution of acetate and propionate of 18.3 mM and 5.5 mM, respectively. Duplicate sterile controls were prepared in the same manner but were autoclaved once each day for three consecutive days to ensure complete sterilization. Triplicate active controls were only given the acetate and propionate, not atenolol. Duplicate sterile controls were then made for the active controls. Two unfed controls that only received the 20% inoculum in methanogenic medium were made. Table 1 provides further clarification of the ATA experimental set up.

3.2.2 Monitoring Total Gas and Methane Production

The methods were carried out in the same manner as found in section 2.2.4.

3.2.3 HPLC Analysis

The methods were carried out in the same manner as found in section 2.2.2.

3.3 Results

3.3.1 The Biochemical Methane Potential of Atenolol

Methane production at all atenolol concentrations and in unamended background cultures showed a similar amount of methane production until day 8 (Fig. 11). At day 8, amended cultures had started to produce slightly more methane than the unamended backgrounds. Amended cultures at all concentrations saw a spike in methane production by day 14. 60% of total methane production was seen on day 14 for 1.0 mM amended cultures, 49% for 0.5mM amended cultures, and 45% for 0.25 mM amended cultures. No spike in methane production of this magnitude was seen again through the duration of this experiment. Methane production then continued at a lower rate in all live cultures. At the end of the experiment, day 35, the total cumulative methane productions for the amended cultures were as followed: 1.0 mM: 83 mL; 0.5 mM: 55 mL; and 0.25 mM: 40 mL. The unamended backgrounds never saw any dramatic increase or spikes in methane production as the amended cultures experienced with a total cumulative methane production of 22 mL at end of the experiment.

All amended cultures showed an increase in methane production compared to the unamended backgrounds, indicating that atenolol is biodegradable. However, as stated in the previous chapter atenolol is only transformed to atenolol acid and no carbon was removed. However, the presence of atenolol increased methane production. As the concentration of atenolol increased, so did the amount of methane produced. The 0.25

mM atenolol amended cultures produced 181% more methane compared to the background cultures. The 0.5 mM amended cultures produced 250% more methane and 1.0 mM atenolol amended cultures produced 377% more compared to the backgrounds. Methane production is an end product of active methanogens; the increase in methane production with the increase in atenolol concentration indicates that the transformation of atenolol to atenolol acid increases the activity of the methanogenic community.

3.3.2 Anaerobic Toxicity Assay of Atenolol

Total gas production of the atenolol amended cultures and active controls saw an immediate spike in total gas production; the amount of total gas produced by atenolol amended cultures and active controls remained similar until day 5 (Fig. 12). By day 9, the amended cultures start to produce more total gas than the active controls. Production in total gas started to differentiate among the two amended cultures after day 9 and continued until the end of the experiment. Day 30 concluded the experiment; at this point 1.0 mM atenolol amended cultures had a total cumulative total gas production of 75 mL; 0.5 mM atenolol amended cultures produced 62 mL. The active controls produced a total of 49 mL of total gas by day 30. Seed backgrounds never had the same spike in total gas production as the atenolol amended cultures and active controls had; by day 30 the seed backgrounds produced at total of 18 mL of total gas. The sterile cultures (not shown) produced no gas. Overall total gas production in atenolol amended cultures was greater than active controls, indicating that atenolol is not toxic or chronically inhibitory to the anaerobic community.

Methane production of the atenolol amended cultures and active controls had an immediate increase in methane production (Fig. 13). Unlike total gas production, which

remained similar between amended cultures and active controls until day 5, methane production was similar between amended cultures and active controls until day 2. Afterwards atenolol amended cultures produced more methane than active controls. The atenolol amended cultures had almost identical methane production at both concentrations until day 9, after which the 1.0 mM atenolol amended cultures started to produce more methane than 0.5 mM atenolol amended cultures. A small spike in methane production was seen at day 12 for the atenolol amended cultures at both concentrations. No further spikes in methane production was seen for the rest of the experiment. By the end of the experiment on day 30, 1.0 mM atenolol amended cultures had a cumulative methane production of 53 mL; 0.5 mM atenolol amended cultures had a cumulative methane production of 45 mL. Actives controls ended the experiment with a total cumulative methane production of 37 mL. The seed backgrounds did not see the same early spike of methane production. At the end of the experiment, the seed background cultures produced at total of 15 mL of methane. Overall, total methane production was greater in the atenolol amended cultures at both concentrations when compared to the active controls. This indicates that atenolol is not toxic or chronically inhibitory to the methanogenic community.

Samples for HPLC analysis were taken at the beginning and end of the experiment to determine whether atenolol was transformed during the ATA. Figure 14A shows the transformation of atenolol in the 0.5 mM amended cultures. Day 0 began with a concentration of 0.41 mM in the active cultures. On day 34, the amended cultures had a concentration of 0.16 mM, for a total loss of 0.26mM. Atenolol acid at day 34 had a total concentration of 0.25 mM. The 1.0 mM amended cultures also saw loss in the active

cultures. In Figure 14B, the amended cultures started with a concentration of 0.88 mM and by day 34 they were down to a concentration of 0.56 mM for a total loss of 0.32 mM. As with the 0.5 mM amended cultures, atenolol acid appears on day 34 with a total concentration of 0.34 mM. Sterile controls in both set-ups saw no measured loss in atenolol. The 1.0 mM amended cultures lost only 0.07 mM more atenolol than 0.5 mM amended cultures. This shows that even when given twice the concentration the culture can still only transform so much in a certain amount of time.

3.4 Discussion

3.4.1 Biochemical Methane Potential of Atenolol

Methane production in atenolol cultures was higher than the background at every concentration than the backgrounds (Fig. 11). Theoretically, this indicates that atenolol is biodegradable at these concentrations. However, it is not biodegradable; it is only transformed. In chapter two, the metabolite was identified as atenolol acid, in which only the nitrogen group was removed from atenolol. This shows that the biochemical methane potential assay must be used with caution. It is important to look for metabolites at the end of the BMP to ensure complete mineralization.

Since no carbon is being utilized from atenolol and no additional carbon was added in the medium, the carbon must be from any residual carbon left in the inoculum. Although this inoculum was incubated for one day prior to the experimental set-up, degradable residual carbon still could be available. Even with no carbon being removed from atenolol, the amended cultures are producing higher amounts of methane than the backgrounds. It is probable that the removal of the nitrogen is contributing to this

increase in methane production. Nitrogen is an important macronutrient and its role in methanogenesis is starting to be more understood. One study looking at variety of carbon/nitrogen sources and the C/N ratio (Wagner, Hohlbrugger, Lins, & Illmer, 2012) found that amount of nitrogen and the type of nitrogen source may have a more important role in methane production than once thought. For example, casamino acids which have a C/N ratio of 3.74 when fed to the cultures produced approximately 600 mL methane per mole of nitrogen; L-arginine has a C/N ratio of 1.29 when fed to the cultures, this produced approximately 1400 mL methane per mole of nitrogen (Wagner et al., 2012). Atenolol is increasing the pool of nitrogen in size and in type to utilize and decreasing the C/N ratio in these cultures and this could be the reason for the enhancement of methane production. Another explanation could be that atenolol is selecting for bacteria that can not only utilize complex nitrogen sources such as atenolol as a nitrogen source, but also have the ability to ferment carbon into C-1 and C-2 compounds that methanogens can use (discussed in further detail in chapter 4). Many fermenters that produce acetate and carbon dioxide also can and sometimes prefer to utilizing complex nitrogen sources (Baena et al., 2016;Smith & Macfarlane, 1997). Atenolol could provide the nitrogen to these fermenters that then utilize the residual carbon to produce the needed C-1 and C-2 for methanogens in order to produce methane.

3.4.2 Anaerobic Toxicity Assay of Atenolol

Comparing total gas and methane production throughout the ATA can give a better understanding as to how the community is being affected. Anaerobic digestion is a complex microbial ecosystem; while methane is only produced by the methanogenic community, the other major component of total gas, carbon dioxide is produced by a

variety of anaerobic bacteria and methanogens (Bitton, 2005). Therefore, looking at total gas production can give insight into the effect of atenolol on the anaerobic community as a whole, while looking at methane production specifically looks at the effects of atenolol on the methanogenic community and the bacteria that provide the C-1 and C-2 compounds to the methanogens.

For total gas production, the maximum rate ratio (MRR), the equation given in Table 2, can help determine if the selected substrate is inhibitory (Owen et al., 1979). MMR of less than 0.95 suggests possible inhibition, while a MRR of less than 0.9 suggests significant inhibition. Table 2 shows the MRR of three time periods throughout the experiment. There was inhibition at the beginning of the assay in both 1.0 mM and 0.5 mM amended cultures. The anaerobic community quickly acclimated to the presence of atenolol, and by day 12 showed no inhibition and continued to be unaffected until the end of the experiment at day 30.

Similar to MRR, the maximum methane ratio (MMR), the equation pictured in Table 3, is used to see if the selected substance is inhibitory to methanogenesis (O'Connor, Rivera, & Young, 1988). A value of less than 1 suggests inhibition while a ratio value over 1 suggests substrate metabolism (O'Connor et al., 1988). Similar to that of the maximum rate ratio, atenolol shows initial inhibition on day 2 under both amendment concentrations (Table 3). Day 12 and day 30 show a value of over one, suggesting substrate metabolism. Results from HPLC analysis (Fig. 14A and 14B) confirmed this. These ratios suggest that methanogens and the anaerobic community as a whole, while initially inhibited, can quickly acclimate to the presence of atenolol and utilize it. However, atenolol was not completely degraded, only transformed to atenolol

acid. Similar to the BMP, it is important to proceed with caution when utilizing the ATA.

Comparing total gas production to methane production can give insight to how the anaerobic community has shifted. In seed backgrounds, methane accounted for 83% of total gas production. This inoculum is from an anaerobic digester where methanogens thrive which explains why the majority of total gas is methane. When amended cultures were normalized by subtracting out the total gas or methane of active controls, methane was still found to be the majority of total gas. However, the percent of methane decreased compared to the seed backgrounds; 65% of the total gas was methane in 1.0 mM amended cultures and 62% of the total gas was methane in 0.5 mM amended cultures. This indicates that while atenolol increases both total gas and methane production, the percent of methane that makes up the total gas decreases.

3.5 Conclusions

The BMP and ATA were utilized in order to better understand the effects and potential biodegradability of atenolol. However, the results of these assays did not coincide with the HPLC data. The BMP data shows that atenolol is biodegradable, but it is only transformed to atenolol acid. The data from the ATA revealed that atenolol had an initial inhibitory effect on the total gas and methane production, but the community was able to quickly acclimate and utilize atenolol and ultimately produce excess gas. It also suggested that atenolol is metabolized, but again atenolol is only transformed to atenolol acid. These set of experiments show that the BMP and ATA should be used with caution as they did not yield the expected results.

Another beta-blocker, propranolol was investigated to see if it was inhibitory or biodegradable using the BMP and a modified ATA; it was found to be inhibitory and non-biodegradable (Fountoulakis et al., 2008). This shows that although pharmaceuticals fall into the same class of drugs and act upon the human body in a similar manner; their impact on the microbial community can differ from compound to compound. Since over 13% of the United States population is on this pharmaceutical (Palmer et al., 2008) and with 76%-90% of atenolol excreted into the treatment system (AstraZeneca, 2008) it is important to know that atenolol is not overtly affecting the anaerobic community found in wastewater sludge digesters in a negative manner. However, atenolol did exert an effect on the cultures that is different than the seed background, showing that atenolol has the ability to change the community and the long term effects of how that could impact a digester is unknown.

3.6 Table and Figures

	20% inoculum	Acetate/Propionate	Atenolol	Autoclaved
Amended Cultures	+	+	+	-
Unamended Active Controls	+	+	-	-
Seed Backgrounds	+	-	-	-
Amended Culture Sterile Controls	+	+	+	+
Unamended Active Sterie Controls	+	+	-	+

Table 1: ATA Set up

$$\text{Maximum Rate Ratio (MRR)} = \frac{\text{gas production of substrate fed} - \text{gas production of seed background}}{\text{gas production of active background} - \text{gas production of seed background}}$$

Concentration	Day 2	Day 12	Day 30
1.0mM	0.79	1.78	2.03
0.5mM	0.86	1.45	1.58

Table 2: Maximum rate ratio of atenolol

$$\text{Maximum Methane Ratio (MMR)} = \frac{\text{methane production of substrate fed} - \text{methane production of seed background}}{\text{methane production of active background} - \text{methane production of seed background}}$$

Concentration	Day 2	Day 12	Day 30
1.0mM	0.93	1.82	2.03
0.5mM	0.95	1.45	1.56

Table 3: Maximum methane ratio of atenolol

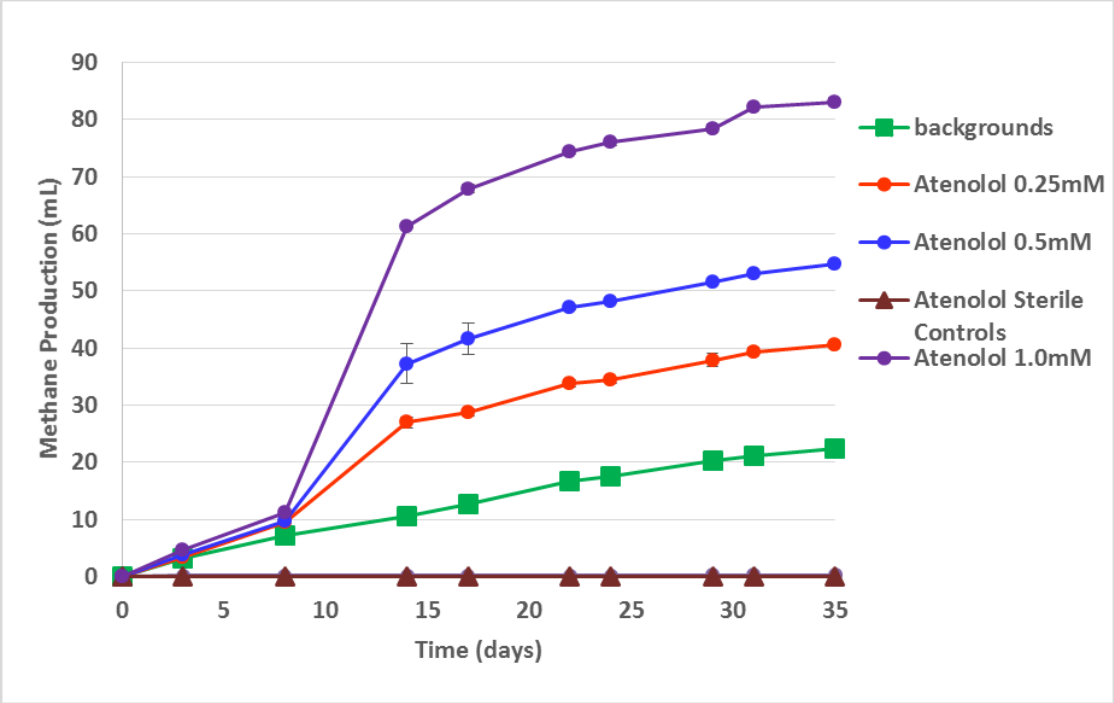

Figure 11: Biochemical methane potential of atenolol (note: some error bars cannot be seen due to the size of the marker)

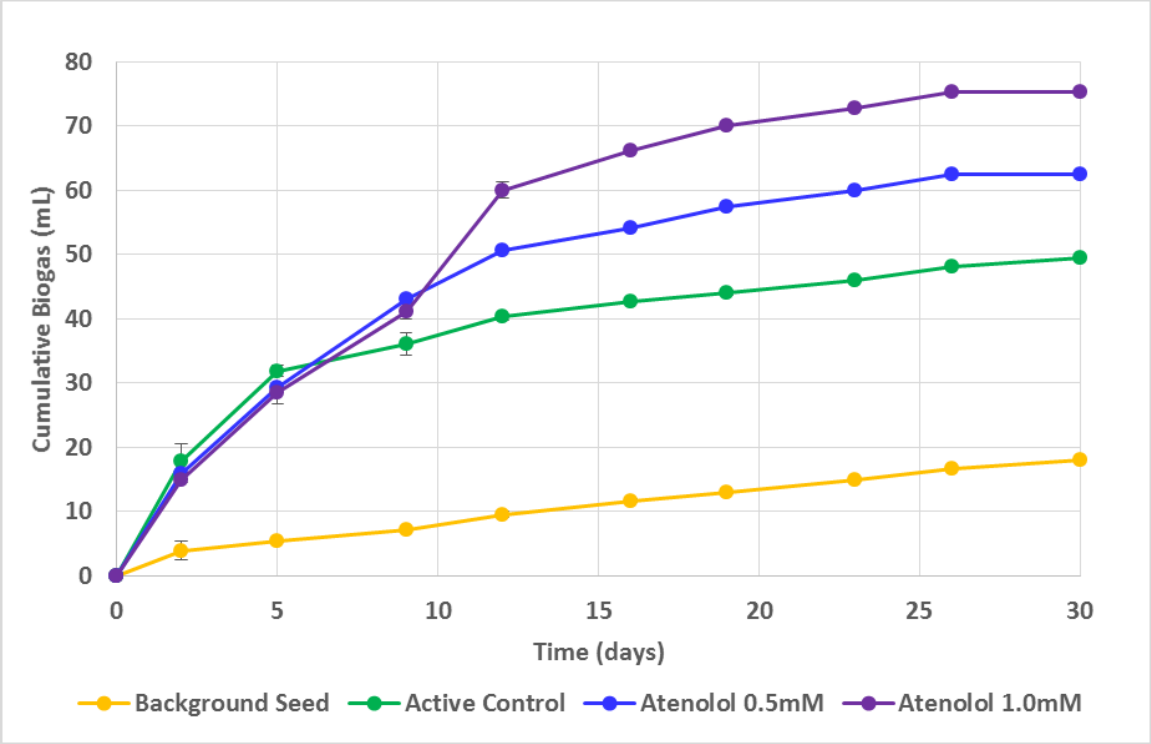

Figure 12: Atenolol ATA total gas production (note: some error bars cannot be seen due to the size of the marker) Note: no gas production in sterile controls (not shown).

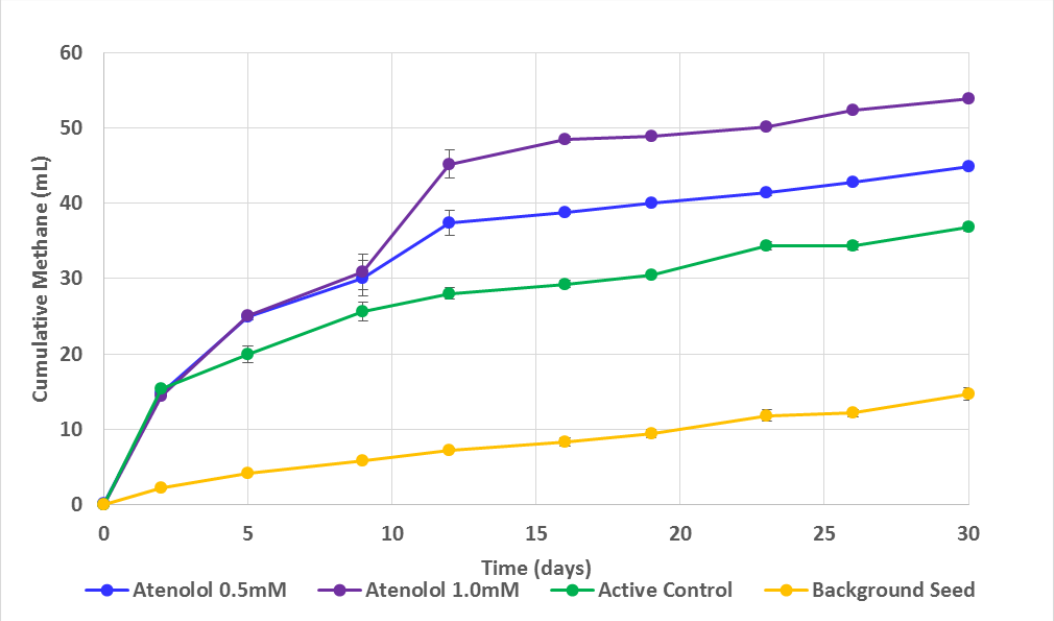

Figure 13: Atenolol ATA methane production (note: some error bars cannot be seen due to the size of the marker) Note: no gas production in sterile controls (not shown).

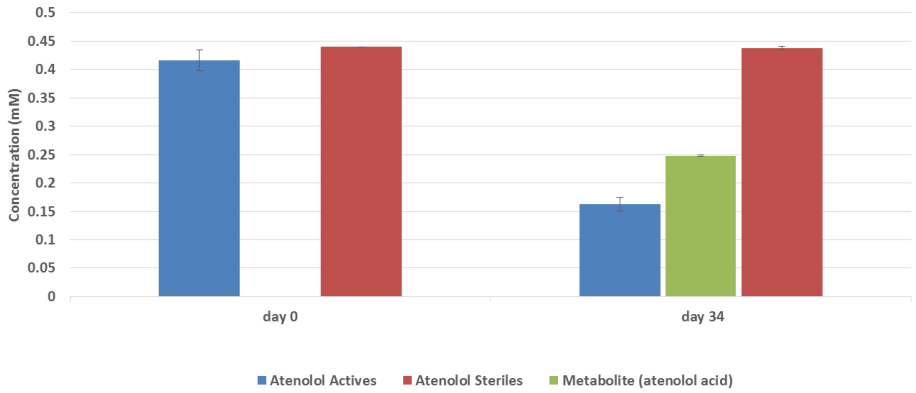

Figure 14A: ATA 0.5 mM atenolol transformation

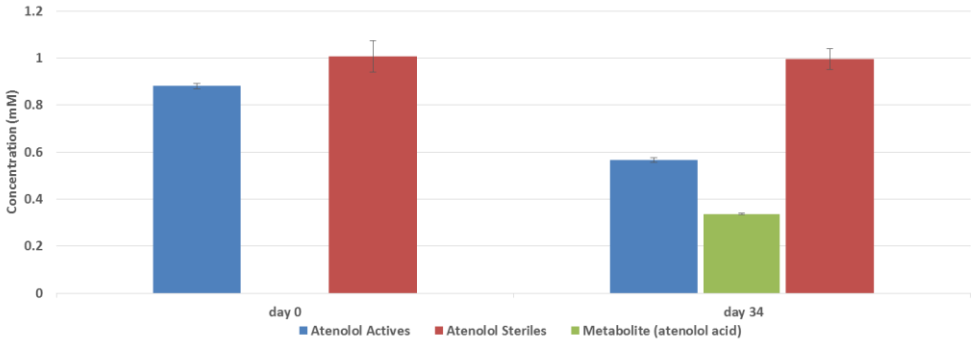

Figure 14B: ATA 1.0 mM atenolol transformation

Chapter 4

Community Analysis of Atenolol Amended Cultures

4.1 Introduction

Bacteria and archaea are the most metabolically diverse groups of organisms on the planet. These organisms have been documented to grow on and metabolize complex compounds normally considered hazardous or toxic such as dyes, polycyclic aromatic hydrocarbons from fossil fuels, and pesticides, along with more traditional substrates (Seo, Keum, & Li, 2009; Soleim & Scheline, 1972). Bacterial degradation of pharmaceuticals such as NSAIDs, beta-blockers, and antibiotics has also been documented (Barbieri et al., 2011; Lahti & Oikari, 2011; Onesios et al., 2009b).

However, not all bacteria and archaea are created equal when it comes to metabolic capabilities. As with any ecosystem, there is competition and niches in the microbial world. When xenobiotics are introduced into a microbial system, this can create stress and cause changes in the community make-up. Community analysis can help better understand which microorganisms can tolerate and perhaps utilize the compound of interest.

Outside of showing that antibiotics can select for antibiotic resistant bacteria within the wastewater treatment system, little research has been done on how pharmaceuticals change the community structure (Rizzo et al., 2013). However, one study showed that when activated sludge bioreactors were given a variety of pharmaceuticals there was reduced diversity compared to the control, and also a reduction in species key to nitrification, an important microbial process in wastewater

treatment (Kraigher, Kosjek, Heath, Kompare, & Mandic-mulec, 2008). In this study, the influence of atenolol on the anaerobic bacterial and archaeal community is investigated.

4.2 Materials and Methods

DNA was extracted from the two culture set ups described in chapter two using the commercial kit FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA). The protocol given with the kit was followed. DNA was extracted in duplicate in order to sequence for both bacterial and archaeal DNA. Both active Iona and anaerobic sludge atenolol cultures were analyzed as well as their backgrounds. For the anaerobic sludge cultures, DNA was extracted on day 22 of its first refeeding. For Iona cultures, DNA was extracted on day 23 of its first refeeding.

These extractions were then sent to MR.DNA for PCR purification and Illumina MiSeq sequencing (MR.DNA, Shallowater, TX.). Mr.DNA used 16S rRNDA gene PCR primers 27F for bacteria and 344F for archaea with barcode on the forward primer, they were then used in a 28 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA). The following method was utilized: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed, 5 cycle was used on the PCR products. Success of amplification was ensured by running a 2% agarose gel and checking the relative intensity of the bands. Samples were pooled and purified using Ampure XP beads. The purified PCR product was then used to prepare illumina DNA library. The sequencing followed the Miseq manufacturing protocol. The data was then processed using the MR.DNA analysis pipeline (MR DNA, Shallowater, TX,). Operational taxonomic units (OTUs) generated by the MR.DNA analysis pipeline were

defined by clustering at 3% divergence (Glassing et al., 2015). The final OTUs were classified using BLASTn against a database derived from RDPIII and NCBI. Data was then graphed to show sequences that accounted for 1% or more of the total sequences.

4.3 Results

4.3.1 Atenolol Iona Cultures -Bacterial

Figure 15 displays the diversity of the bacterial community in the atenolol amended cultures and the unamended background cultures. In Iona cultures the unamended backgrounds showed an extremely diverse community with a variety of metabolic abilities. The five highest families in the unfed background were *Prolixibacteraceae* (12%), *Bacteroidaceae* (12%), *Acidobacteriaceae* (6%), *Geobacteraceae* (6%), and *Flavobacteriaceae* (5%). These families are extremely diverse in their metabolic capabilities. *Prolixibacteraceae* has many species that reduce nitrate and fix nitrogen; *Geobacteraceae* are anaerobes that can use a variety of metals as electron acceptors (Iino, Sakamoto, & Ohkuma, 2015; Huang et al., 2016; Lovley, Krushkal, Yan, & Methe, 2004). The anaerobic family *Bacteroidaceae* can ferment a variety of substrates, while acidophilic *Acidobacteriaceae* has members that range from aerobic to anaerobic. *Flavobacteriaceae* also range from aerobic to anaerobic with many members thriving in areas with high carbon dioxide (Agronomique, Nakagawa, Holmes, & Segers, 2002). This community snapshot illustrates the microbial heterogeneity that exists within Iona Island marsh. All other families found in the unamended backgrounds represented under 5% each of the total sequences. The amended cultures had a dramatic increase in two families in particular: *Eubacteriaceae* going from 4% in the unfed backgrounds to 26% and *Erysipelotrichaceae* with an increase from 2% to 16%. These

two families contain an overwhelming amount of fermenters and are known for their utilization of complex nitrogen sources (Wolfgang, Schleifer, & Whitman, 2009; Verbarg et al., 2009). There were also increases in the following families: *Spirochaetaceae* from 1% to 3%, *Acholeplasmataceae* from 3% to 4%, *Bacillaceae* from 0.7% to 6%, *Clostridiaceae* from 5% to 6%, *Sphingobacteriaceae* from 0.3% to 11%, and *Marinifilaceae* from 0.1% to 2%. *Spirochaetaceae, Acholeplasmataceae, Bacillaceae*, and *Clostridiaceae* are also known to contain fermenters (Brown, Bradbury, & Johansson, 2010;Paster, 2010;Paster, 2010). The 5 families that dominated in the unfed background all showed a significant decrease in the atenolol amended cultures. The addition of atenolol decreased the microbial heterogeneity in the Iona Island marsh sediment and favored bacteria with the ability to ferment.

4.3.2 Atenolol Anaerobic Sludge Cultures- Bacterial

Figure 16 displays the diversity of the bacterial community of the anaerobic sludge cultures in both atenolol amended and the unamended backgrounds. Unlike the Iona cultures, the anaerobic sludge cultures showed little change between atenolol amended and unamended cultures. *Clostridiaceae*, *Eubacteriaceae*, *Spirochaetaceae*, *Cytophagaceae*, *Anaerolineaceae*, and *Sphingobacteriaceae* were the dominant families in both the atenolol amended cultures and backgrounds. The rest of the families only contributed 5% or less each to the total sequences. Within this group of families there were some differences; the family *Clostridiaceae* went from 18% in the backgrounds to 15% in the amended cultures. *Sphingobacteriaceae* increased in abundance in the amended cultures, going from 10% in the backgrounds to 13% in the amended cultures. *Eubacteriaceae* and *Spirochaetaceae* only had a 0.5% increase in the amended cultures,

pushing their abundance in the amended cultures to 13% each. *Cytophagaceae* decreased in amended cultures going from 7% to 6% in unamended backgrounds. *Anaerolineaceae* had a 1% decrease, going from 5% in unamended backgrounds to 4% in the amended cultures. One other family established themselves in the amended cultures: *Bacteroidaceae* went from 0.5% in the backgrounds to 2% in the amended cultures. Anaerobic digesters are exposed to a variety of contaminants, including pharmaceuticals, on a daily basis (Stasinakis, 2012). The community is already acclimated to exposure to such compounds as atenolol, which may explain why there is little difference between atenolol amended and unamended cultures.

4.3.3 Atenolol Iona Cultures- Archaeal

Shown in Figure 17 is the diversity of the archaeal community from Iona cultures, both atenolol amended and the unamended background cultures. The unfed background cultures had three orders that comprised over half the community: *Thermoproteales* (31%), *Thermoplasmatales* (15%), and *Methanosarcinales* (13%). Amended cultures had one order that overwhelmingly dominated: *Methanosarcinales* (73%). All other orders decreased in the amended cultures. The largest order in the unfed backgrounds, *Thermoproteales*, had decreased to 2% in the amended cultures. The second largest order inunamended background cultures, *Thermoplasmatales*, dropped to 11% in the amended cultures. However, even with this decrease, *Thermoplasmatales* is still the second largest order in amended cultures. Atenolol amended cultures saw a dramatic increase in the acetoclastic methanogenic order *Methanosarcinales*, and a decrease in not only non-methanogenic orders but the other methanogenic orders *Methanobacteriales*, *Methanocellales*, *Methanomicrobiales*, and *Methanomassiliicoccales*. This illustrates that

the addition of atenolol not only selects for methanogens, but specifically for acetoclastic methanogens.

4.3.4 Atenolol Anaerobic sludge Cultures- Archaeal

Illustrated in Figure 18 is the diversity of the archaeal community from anaerobic sludge cultures, both atenolol amended and the unamended cultures. Anaerobic sludge cultures had about half the amount of orders represented in their community compared to Iona cultures. This highlights not only the microbial heterogeneity of Iona cultures, but also highlights how anaerobic sludge has already been enriched due to the specific environment it comes from; methanogens are the overwhelming majority of archaea in anaerobic digesters (Wilkins, Lu, Shen, Chen, & Lee, 2015). The most dominant orders in the backgrounds were *Methanobacteriales* (35%) followed by *Methanomicrobiales* (27%) and then *Methanosarcinales* (19%). In amended cultures there was a decrease in *Methanobacteriales* going from 34% in the backgrounds to 22% in the amended cultures. A dramatic increase was seen in the order *Thermoplasmatales*, going from 15% in the backgrounds to 26% in the amended cultures. A slight increase in *Methanomicrobiales* and *Methanosarcinales* was seen in the amended cultures when compared to the background cultures. Even with the increase in the non-methanogenic order Thermoplasmatales, methanogens were still the majority of the archaeal community in amended cultures making up 73% of the total sequences.

4.4 Discussion

4.4.1 Atenolol Iona Cultures-Bacterial

As illustrated in Figure 15, the two most predominant families, *Eubacteriaceae* and *Erysipelotrichaceae* make up more than 40% of the community sequences in the atenolol amended cultures; in unamended background cultures both families combined made up 6% of the total sequences. The family *Eubacteriaceae* is known for its members being obligate anaerobes, with the majority of its members having a fermentative lifestyle (Wolfgang et al., 2009). *Erysipelotrichaceae* has members ranging from aerobic to facultative anaerobic; members who are facultative anaerobes, utilize fermentation (Verbarg et al., 2009). Some of the other families that increased in atenolol amended cultures, *Clostridiaceae, Spirochaetaceae, Acholeplasmataceae* and *Bacteroidaceae,* also contain a large number of fermenters and facultative anaerobes (Dai, Wu, & Zhu, 2011; Krieg et al., 2010; Paster, 2010; Martini, Marcone, Lee, & Firrao, 2014). Besides their lifestyles, what all these families have in common are their metabolic by-products. Major fermentation products that are formed by *Eubacteriaceae* members are butyrate, formate, and acetate, while *Erysipelotrichaceae* members form lactate and acetate (Flint et al., 2014;Wolfgang et al., 2009;Verbarg et al., 2009). *Clostridiaceae*, *Spirochaetaceae, Acholeplasmataceae* and *Bacteroidaceae* produce C-1 and C-2 compounds such as carbon dioxide as well as acetate and hydrogen (Dai, Wu, & Zhu, 2011; Krieg et al., 2010; Paster, 2010; Martini, Marcone, Lee, & Firrao, 2014). The products formate, acetate and carbon dioxide/hydrogen can be utilized by methanogens to produce methane (Whitman et al., 2006). In order to be producing these compounds, the bacteria mentioned must be getting carbon from the inoculum as atenolol only undergoes

deamination. As mentioned in chapter 2, Iona Island marsh sediment has a high solid content and variety of wildlife and plants which die and decay, inputting a wide array of carbon into the system. This carbon can then be utilized by these families. Then the C-1 and C-2 compounds produced can be utilized by methanogens.

Atenolol is only undergoing a deamination, which raises the question as to what family or families are responsible for this transformation. What is unique about *Eubacteriaceae* and *Erysipelotrichaceae* compared to the other families is that they have members known for their association with and utilization of complex nitrogen sources (Kaakoush, 2015;Wolfgang et al., 2009). For example, many members of *Eubacteriaceae* are known for their utilization and preference of proteinaceous nitrogen sources; these are much more complex and larger than non-proteinaceous nitrogen sources such as ammonia (Wolfgang et al., 2009). Interestingly, atenolol has a similar structure to aromatic amino acids such as phenylalanine and could be consider a complex nitrogen source.

The family *Erysipelotrichaceae* has been shown to be associated with high protein diets and is also been shown to ferment amino acids (Hugenholtz, n.d.; Wolfgang et al., 2009). People with the metabolic disorder phenylketonuria, a disorder in which the body builds up the aromatic amino acid phenylalanine, have been shown to have an increased abundance of *Erysipelotrichaceae* in comparison to people who do not have this disorder (Oliveira, Mendes, & Dobbler, 2016). While this study did not look into if they utilize phenylalanine, it further emphasizes their association with complex nitrogen sources. While cultures were supplemented with a nitrogen source, it was ammonia, which is not a complex nitrogen source. *Eubacteriaceae* and *Erysipelotrichaceae* are

known for their utilization and association with complex nitrogen sources, and are the members likely responsible for the deamination of atenolol.

4.4.2 Atenolol Anaerobic Sludge Cultures-Bacterial

Background and atenolol amended anaerobic sludge cultures had little variation when compared to one another (Fig. 16). Unlike the inocula from the Iona cultures, which was obtained from an open, uncontrolled environment, anaerobic sludge is from a semi-controlled environment. The flow of sludge, how long it remains in the system, temperature, the atmosphere of the system, and to a certain extent, the composition of the sludge are controlled (Hammer & Hammer, 2004). This is done to ensure that the microbial anaerobic food chain will perform properly and accomplish waste stabilization (Bitton, 2005). The community in the anaerobic sludge cultures had already been enriched prior to inoculating which may explain the little variation seen among the background and atenolol amended cultures. Microbial communities in anaerobic digesters, such as the microbes in these inocula are exposed to pharmaceuticals during the treatment process (Jelic et al., 2011; Stasinakis, 2012). Therefore, the community may have already been acclimated to the presence of pharmaceuticals such as atenolol, and as such its introduction would not greatly affect the community. Domestic wastewater makes up a large portion of wastewater that a municipal treatment plant receives; potential carbon sources are coming from urine and fecal material as well as any personal care products or food scraps that may have found their way down the drain (Bitton, 2005). This carbon is broken down in the aeration process by aerobic bacteria, while remaining carbon from this as well as dead and living cells make it to the anaerobic

digester; this carbon can then be utilized by the bacteria in the digester (Bitton, 2005). This carbon was in the inoculum and served as the carbon source for microorganisms.

Eubacteriaceae was a dominant family at 13% in atenolol amended anaerobic sludge cultures (Fig. 16). As explained in section 4.4.1, *Eubacteriaceae* prefer complex nitrogen sources and are one of the two members (the other being *Erysipelotrichaceae*) likely responsible for the transformation of atenolol acid in Iona cultures (Wolfgang et al., 2009). Atenolol amended anaerobic sludge cultures had half the amount of *Eubacteriaceae* that the atenolol amended Iona cultures. The lower abundance of *Eubacteriaceae* and complete lack of *Erysipelotrichaceae* in the atenolol amended anaerobic sludge cultures compared to the Iona cultures could also explain why it took twice the amount of time to see complete transformation of atenolol.

4.4.3 Atenolol Iona Cultures-Archaeal

While background cultures did have some methanogenic orders of archaea, two out of the three most abundant orders, *Thermoplasmatales*, and *Thermoproteales*, are not methanogens (Fig. 17) (Huber & Stetter, 2006;Huber, Huber, & Stetter, 2006). The orders mentioned above include a variety of archaea that range from anaerobic to aerobic and can utilize complex organic substrates (Huber & Stetter, 2006;Huber et al., 2006). The third most abundant order in the unamended backgrounds, *Methanosarcinales*, increased even further in atenolol amended cultures. Members of this order can utilize carbon dioxide, methanol, acetate, methylamines, and methyl sulfides as substrates (Kendall & Boone, 2006). The bacterial community in the atenolol amended culture described in the previous section has the ability to produce metabolites such as carbon dioxide, acetate, and hydrogen that can then be utilized by members of

Methanosarcinales as well as other methanogens (Dai et al., 2011; Krieg et al., 2010; Paster, 2010; Whitman et al., 2006). This increase in fermentative bacteria and therefore in C-1 and C-2 metabolites for methanogens to use in the amended culture helps explain why the amended culture experienced higher methane production.

4.4.4 Atenolol Anaerobic Sludge Cultures- Archaeal

Unlike the archaeal community in the Iona cultures, methanogens were the dominant archaea in the unamended background in the anaerobic sludge cultures (fig. 18). Digesters are enriched for methanogens in order to produce methane, which explains why the unamended backgrounds had a rich methanogenic community. Toxicity to specific members of the methanogenic community may have contributed to specific shifts within the community. One study found that a similarly structured beta blocker, propranolol, can inhibit methanogenesis (Fountoulakis et al., 2008). Beta blockers function by acting on receptors on the cell surface to produce the intended effect (Frishman, 2003). The order *Methanobacteriales,* which was lower in the atenolol amended cultures, has a pseudomurein cell wall (Bonin & Boone, 2006). In contrast, orders *Methanosarcinales* and *Methanomicrobiales*, which saw a slight increase in the atenolol-fed cultures, have a glycoprotein cell wall (Bonin & Boone, 2006). This suggests that *Methanobacteriales*' differing cell wall could account for its sensitivity to atenolol.

Interestingly, a non-methanogenic order, *Thermoplasmatales,* had the greatest increase in the amended culture. Members of this family are facultative anaerobes and are known to survive on decomposing cellular matter (Huber & Stetter, 2006). Iona cultures have a higher amount of solids than anaerobic sludge cultures; Iona Island Marsh sediment has an average of 55% total dry solids while the sludge entering system can

have anywhere from 5%-9% (EPA storet data; Tchobanoglous, Burton, & Stensel, 2003). This increases the competition for the carbon within in the solids found in the anaerobic sludge cultures. Therefore some members of the community will die; *Thermoplasmatales* can then utilize the dead cellular matter for their carbon source. During their metabolism, they produce C-1 and C-2 compounds such a carbon dioxide and acetate (Huber & Stetter, 2006). These C-1 and C-2 metabolites provide methanogens with substrates to produce methane. The large increase in *Thermoplasmatales* in the anaerobic sludge amended cultures and therefore its production in these metabolites could contribute to the increase in methane production.

4.5 Conclusions

The bacterial and archaeal community in Iona cultures showed a large shift in their community structure when compared to the backgrounds, indicating that addition of atenolol reduces microbial heterogeneity and selected for a specialized community that can not only tolerate the presence of atenolol, but also selected for members that can possibly utilize it as a nitrogen source. This community ended up being one that is fermentative that can utilize the carbon available in the inocula and produces C-1 and C-2 compounds. The production of these C-1 and C-2 compounds selected for methanogens in the archaeal community, specifically the acetoclastic order *Methanosarcinales*. Even though transformation of atenolol to atenolol acid happened in the anaerobic sludge cultures as well, the community greatly differed. This is from the vast environmental difference that each inocula was taken from. Anaerobic sludge cultures had smaller changes in the bacterial community revealing that this community has the ability to remain fairly stable when atenolol is introduced. Interestingly, the most drastic increase

in the anaerobic sludge archaeal community was the order *Thermoplasmatales*, a non-methanogenic order. This order seemed to take the place of the fermenters found in atenolol amended Iona cultures, as *Thermoplasmatales* produces C-1 and C-2 compounds for methanogens to utilize. Both set-ups had a large community of *Eubacteriaceae*, a community member that is likely responsible for the deamination of atenolol. The implications of this experiment are that atenolol has the ability to induce change in the microbial community in two very different environments. These analyses also gave insight into who is transforming atenolol. The impact that this may have on the microbial ecosystem in the open environment is unknown. Further investigation into the activity of functional genes involved in nitrogen and carbon acquisition and production can help us to better understand which microbes are functional in these cultures.

4.6 Figures

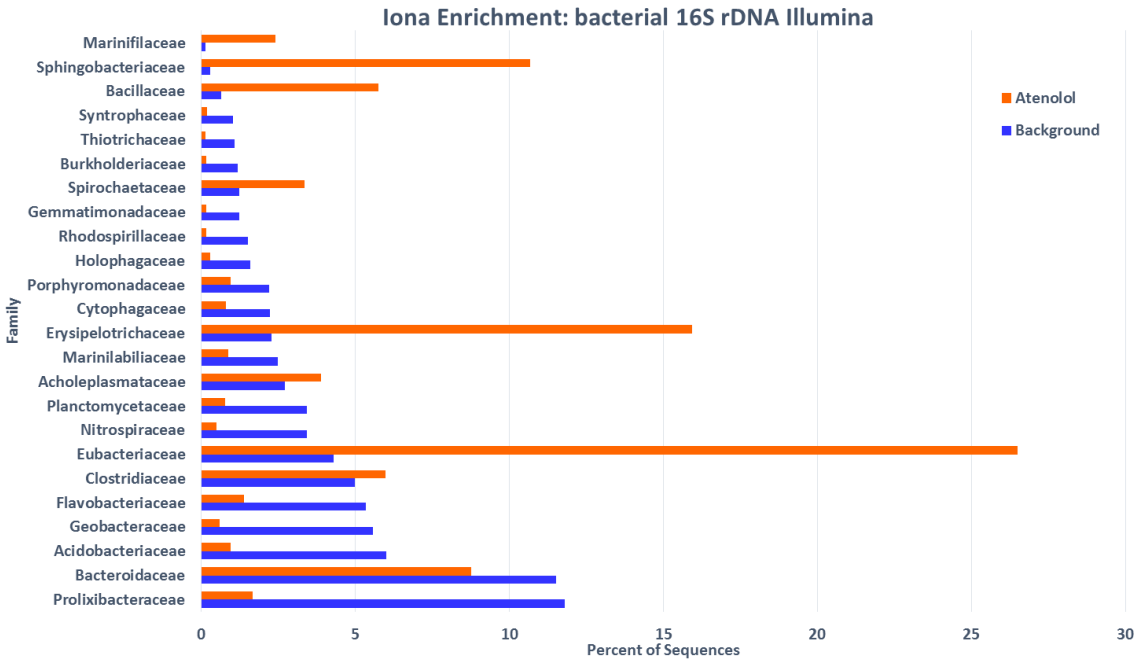

Figure 15:16S rDNA family level bacterial composition of atenolol amended and background Iona cultures

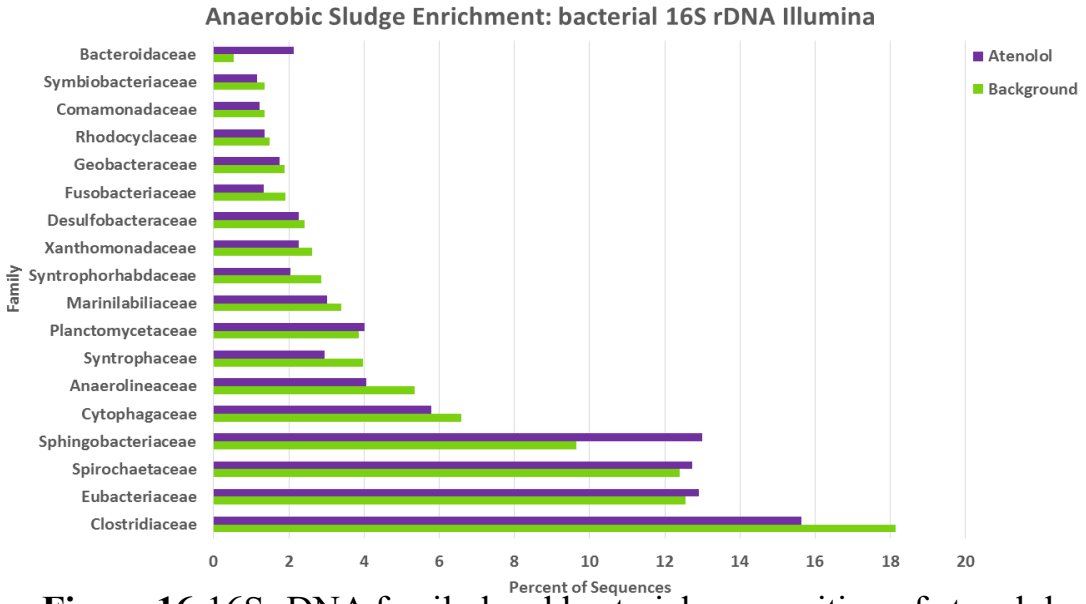

Figure 16:16S rDNA family level bacterial composition of atenolol amended and background anaerobic sludge cultures

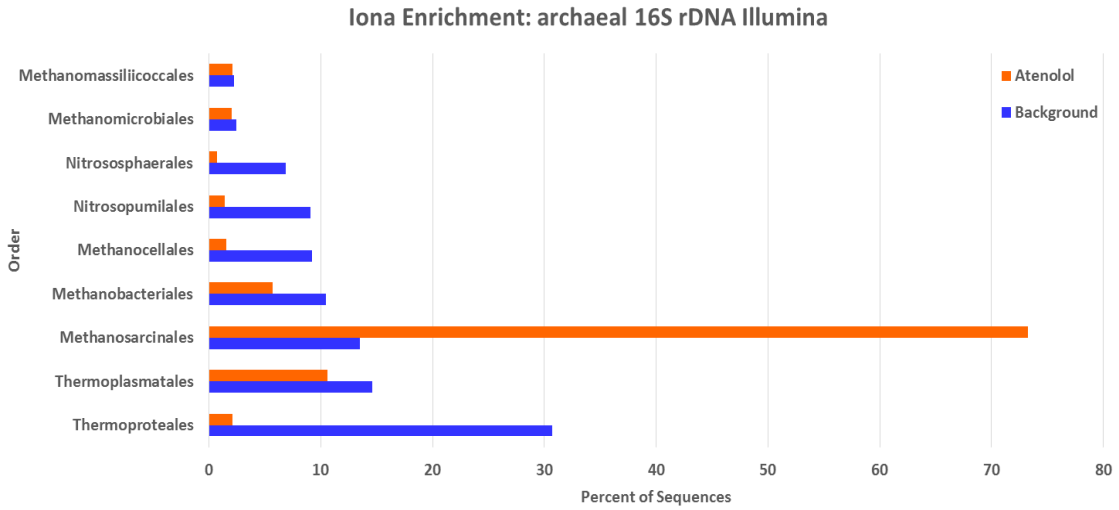

Figure 17: 16S rDNA order level archaeal community composition of atenolol amended and background Iona cultures

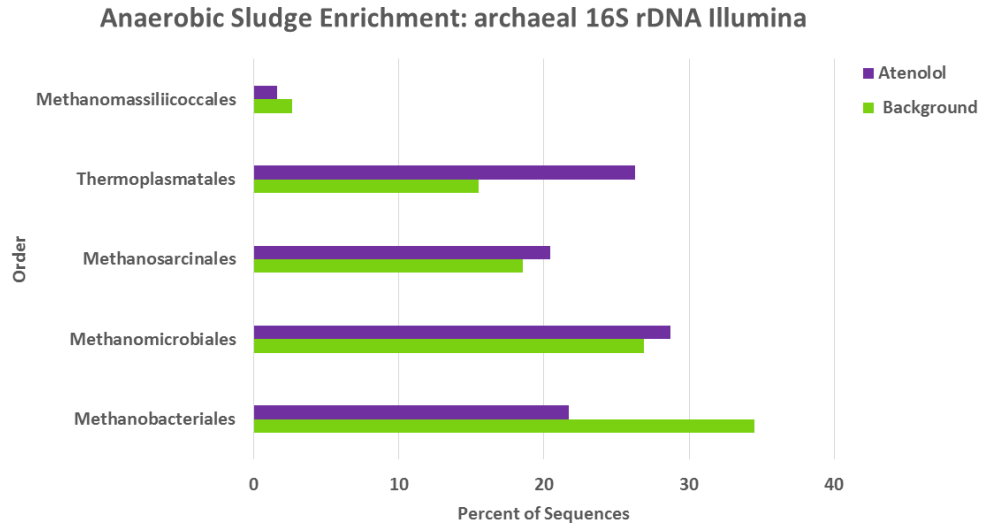

Figure 18: 16S rDNA order level archaeal community composition of atenolol amended and background anaerobic sludge cultures

Chapter 5

The Effect of Ibuprofen on Gas Production and the Microbial Community

The following is experimental data and results from experiments set up in the same manner as in the atenolol experiments, but using ibuprofen as a substrate instead.

5.1. HPLC Analysis and Gas Production of Ibuprofen amended cultures

5.1.2 Materials and Methods

5.1.2.1 Enrichment Culture Establishment

Enrichment cultures were set up in the same manner as described in section 2.2.1 with the following changes: ibuprofen was added at 0.5 mM as the sole carbon source.

5.1.2.2 HPLC Analysis

Loss of the substrate ibuprofen was monitored using a Shimadzu Class VP HPLC system with a Kinetex C18 100 (250 mm by 4.6 mm; particle size 5 µm) column and UV detector. Samples (0.6 mL) were taken via syringe from the cultures; 0.6 mL of N_2/CO_2 was injected back into the cultures to maintain headspace volume. A 1:1 methanol extraction was then performed, gently shaking the sample after the addition of methanol, after which the extraction sat for ten minutes. After ten minutes, 0.8 mL of the extraction was filtered through a SpinX 0.22 µm then transferred to a glass vial for HPLC analysis. The following HPLC protocol was adapted from (Murdoch & Hay, 2005): The mobile phase was 70% methanol and 30% 40 mM acetic acid at a flow rate of 1 mL/min with a retention time set at 12 minutes. Ultraviolet detection was set to an absorbance of 285 nm.

5.1.2.3 Gas and Methane Production

The monitoring of gas and methane production was done in the same manner as described in section 2.2.4.

5.1.3 Results and Discussion

No degradation or transformation of ibuprofen was seen throughout the duration of the experiment in either enrichment set up (Fig. 19 and Fig. 20). However, enhanced total gas and methane production with the addition of ibuprofen was seen in both set ups. As seen in Figure 21, Iona amended cultures had a total of 28 mL of methane produced and 44 mL of total gas was produced by the end of the experiment. Iona backgrounds had a total production of 13 mL of methane and 16 mL of total gas (Fig. 21). Interestingly, in the amended cultures, 63% of the total gas production was contributed from methane while in the unamended backgrounds 81% of the total gas was methane through the duration of the experiment. This implies that ibuprofen selected for both an increase in the methanogenic and fermentative community, while the backgrounds selected for a small methanogenic community in the Iona cultures.

The anaerobic sludge cultures produced more total gas and methane than the Iona cultures. Seen in Figure 22, amended cultures had a total of 56 mL of methane and 99 mL of total gas produced by the end of the experiment. The background cultures only produced 39 mL of methane and 80 mL of total gas produced (Fig. 22). Unlike the Iona cultures, the ratio of methane to total gas when comparing amended to unamended was not as drastically affected in the anaerobic sludge cultures. Methane comprised 56% of the total gas produced by the amended cultures. Methane comprised 48% of the total gas

produced by the unamended backgrounds. The main difference between the amended cultures and the backgrounds is the magnitude in which gas was produced. Amended cultures produced a higher magnitude of both methane and total gas compared to the backgrounds. This indicates that a fermentative and methanogenic community were already well established in the inoculum and that the amendment of ibuprofen further enhanced these communities.

Higher amounts of methane and total gas production in amended compared to background cultures generally implies some sort of transformation or degradation of the selected substrate. However, there is no transformation or degradation in either experimental set up. Studies have shown that ibuprofen has anti-microbial properties (Al-janabi, 2010; Elvers & Wright, 1995; Obad, 2015). One possible explanation is that ibuprofen eliminated competition between microorganisms by killing some of the community members and therefore reducing competition for resources that remained in the original inoculum. There could then be more resources available for microorganisms involved indirectly or directly with total gas and methane production. The differences in methane and total gas production between the two experimental set ups can be attributed to the extremely different environments the inocula come from as explained in section 2.4.2.

5.1.4 Conclusions

Ibuprofen was not transformed or degraded in either experimental set up. However, methane and total gas production increased in the amended cultures in both set-ups. This shows that just the presence of ibuprofen can dramatically shift the microbial community and its activities. Ibuprofen has been shown to have anti-microbial properties

and could be killing microorganisms not responsible for gas and methane production. This then reduces the competition for resources and can increase gas and methane production. This shift in activity in ibuprofen amended cultures in both experimental set-ups is an important finding, as the pharmaceutical ibuprofen is found throughout the open environment and wastewater treatment plant (Hernando, Mezcua, Fern, et al., 2006; Kolpin et al., 2002). Therefore, ibuprofen could be eliciting these changes in microbial activity in these environments. The microbial ecosystem is no different than any other, in that each member plays an important role in contributing to the overall welfare of the ecosystem. For example, one study that fed an activated sludge bioreactor a 50 µg/L mix of the pharmaceuticals naproxen, ketoprofen, diclofenac, colfibric acid, and ibuprofen showed loss in genera key to activated sludge processes when compared to the unamended bioreactors (Kraigher et al., 2008). Further research should be done not only with ibuprofen, but with other commonly detected pharmaceuticals to see if their presence changes the microbial activity within a community.

5.2. Biochemical Methane Potential and Anaerobic Toxicity Assay

5.2.1 Materials and Methods

5.2.1.1 Biochemical Methane Potential and Anaerobic Toxicity Assay

Methods for this experiment were done in the same manner as described in section 3.2.1 but with ibuprofen used as the substrate of interest.

5.2.1.2 Gas and Methane Production

The monitoring of gas and methane production was done in the same manner as described in section 2.2.4.

5.2.2 Results

5.2.2.1 The Biochemical Methane Potential of Ibuprofen

Methane production remained similar between the amended cultures and backgrounds up until day 8 (Fig. 23). After day 8, there was a large increase in methane production in the amended cultures while the backgrounds continued a steady production in methane. Day 14 showed the largest spike in methane production among the amended cultures. Ibuprofen amended at 1.0 mM produced 61% of the total cumulative methane that was produced during the course of the experiment between day 8 and day 14 alone. By day 14, 0.5 mM and 0.25 mM amended cultures produced 49% and 33% of the total cumulative methane, respectively. No spike in methane production of this magnitude was seen again through the duration of this experiment. Steady methane production among the amended cultures continued until day 31 and had little to no methane production by day 35. The total cumulative methane production for the amended cultures was as follows: 1.0 mM: 57 mL; 0.5 mM: 41 mL, and 0.25 mM: 35 mL. Backgrounds produced a total of 22 mL of methane by the end of the experiment. No methane production was seen in the sterile controls. As the concentration of ibuprofen increased, so did the amount of methane produced. 0.5 mM ibuprofen amended cultures had a 15% increase in methane production compared to the 0.25 mM ibuprofen amended cultures. 1.0 mM amended cultures had a 28% increase in methane production compared to the 0.5 mM

ibuprofen amended cultures. This shows that the increase in concentration of ibuprofen increases the activity of the methanogenic community. It is important to note that the BMP is utilized to see with a compound can be mineralized to methane. Enhanced methane production compared to the background cultures generally indicates that the compound of interest is being mineralized. Ibuprofen increased methane production but was not mineralized or transformed in anyway. This shows that compounds with little degradation information such as ibuprofen, should be used cautiously when testing them using the BMP.

5.2.2.2 Anaerobic Toxicity Assay of Ibuprofen

The amended cultures and active backgrounds saw an immediate spike in total gas production, while the seed backgrounds produced little total gas (fig. 24). Active controls produced more total gas up until day 5. Total gas production among the amended cultures remained similar until day 9. On day 12, the amended cultures started to differ in their total gas production. The amended cultures continued to produce more total gas than the active backgrounds throughout the duration of the experiment. By the end of the experiment the 1.0 mM amended cultures produced a total of 87 mL of total gas; the 0.5 mM produced 81 mL. Active controls produced a total of 71 mL of total gas. Seed backgrounds only produced 43 mL of total gas. No gas production was seen in the sterile controls. Overall total gas production in ibuprofen amended cultures was greater than active controls, indicating that ibuprofen is not toxic or chronically inhibitory to the anaerobic community.

Similar to the total gas production results, amended cultures and active controls saw an immediate spike in methane production, while the seed backgrounds produced

little methane (fig. 25). Amended cultures started to produce more methane than the active controls on day 5. Production of methane among amended cultures remained similar until day 9. On day 12 1.0 mM amended cultures started to produce more methane than 0.5mM amended cultures. The 1.0 mM amended cultures continued to produce the most methane until the end of the experiment. On day 30 1.0 mM amended cultures had a total cumulative methane production of 44 mL; 0.5 mM amended cultures had 40 mL and active backgrounds closely followed with 37 mL. Seed backgrounds produced 15 mL of cumulative methane by the end of the experiment. No methane production was seen in the sterile controls. Total methane production was greater in the ibuprofen amended cultures at both concentrations when compared to the active controls. This indicates that ibuprofen is not toxic or chronically inhibitory to the methanogenic community. Similar to the BMP, the ATA should be used with caution as enhanced gas production generally indicates that some sort of degradation of the compound of interest is happening. However, ibuprofen is not being degraded and seems to be having some other effect that enhances gas production.

5.2.3 Discussion

5.2.3.1 The Biochemical Methane Potential of Ibuprofen

While results of the BMP indicate that ibuprofen is biodegradable, from previous HPLC analysis of the enrichment cultures, it's known that this is not the case. Similar to the explanation in the appendix section 1.2, it is possible that ibuprofen may have killed off members of the community and therefore lessened the competition for resources in the inoculum for other members of the bacterial and methanogenic communities. If more

resources for the methanogenic community are available then there is the potential for more methane production.

5.2.3.2 Anaerobic Toxicity Assay of Ibuprofen

Total gas production can give further insight into the effect of ibuprofen on the anaerobic community as a whole while methane production can look at the effect of ibuprofen on the methanogenic community. For total gas production, the maximum rate ratio (MRR), the equation pictured in table 4, is utilized to determine whether or not the selected substrate is inhibitory (Owen et al., 1979). A MRR of less than 0.95 suggests possible inhibition, while a MRR of less than 0.9 suggests significant inhibition. Day 2 has significant inhibition with a MRR of 0.81 for both 1.0 mM and 0.5 mM amended cultures (Table 4). However, by day 12 both 1.0 mM and 0.5 mM amendments had MRRs of 1.46 and 1.21 and continued to have MRRs above 0.95. This indicates that there may have been acute inhibition to the anaerobic community but not chronic inhibition.

Similar to the MRR, the maximum methane ratio (MMR), the equation pictured in table 5, is used to see if the selected substance is inhibitory to methanogenesis (O'Connor et al., 1988). A value of less than 1 suggests inhibition while a ratio value of over 1 suggests substrate metabolism (O'Connor et al., 1988). Day 2 MMR showed inhibition to the methanogenic community (Table 5), but the community quickly acclimated and had MMRs above 1, indicating that ibuprofen is not chronically inhibitory.

5.2.4 Conclusions

The BMP and ATA were utilized in order to better understand the effects and potential biodegradability of ibuprofen. The BMP data shows that ibuprofen is biodegradable; however, this is not the case. Ibuprofen could be inhibiting or killing off members of the community which could then free up carbon and other resources for the anaerobic and methanogenic community to utilize. This would then increase the methane and total gas production when compared to the backgrounds. The ATA revealed that ibuprofen had an initial inhibitory effect to the entire anaerobic community but was able to quickly acclimate and ultimately to produce excess total gas and methane. This shows that the presence of ibuprofen has the ability to change how the community functions. Although it is not overtly negative, ibuprofen is still changing the dynamic of the anaerobic community found in the anaerobic digester. Further research needs to be done to better determine the possible long term effects of ibuprofen. These experiments also show that the BMP and ATA should be used with caution as the data does not coincide with the expected results.

5.3. Community Analysis of Iona Ibuprofen-Amended Cultures

5.3.1 Materials and Methods

The DNA extractions were taken on day 87 of the experiment. Community Analysis was done in the same manner as described in section 4.2.

5.3.2 Results

5.3.2.1 Ibuprofen-Bacterial

Figure 26 illustrates the bacterial diversity in both ibuprofen amended and unamended backgrounds. Only three families saw an increase in the ibuprofen amended cultures compared to the background cultures: *Spirochaetaceae* (1% to 3%), *Planctomyceataceae* (3% to 5%), and *Acholeplasmataceae* (3% to 28%) (Fig. 26). The rest of the families in the background culture had an overall decrease when compared to the ibuprofen amended culture. *Acholeplasmataceae* had the largest increase. These three families are known for containing many fermenting members and their ability to thrive in low carbon environments (Ward, 2010;Brown et al., 2010; Paster, 2010). Ibuprofen is not being degraded and therefore increasing competition for the carbon remaining in the inoculum. Therefore, these families being able to thrive in low carbon environments gives them a competitive advantage.

5.3.2.2 Ibuprofen-Archaeal

Illustrated in Figure 27 is the diversity of the archaeal community in both the ibuprofen amended cultures and the unamended backgrounds. *Thermoproteales* was the dominating order in the unamended backgrounds, representing 31% of the community (Fig. 27). In the ibuprofen amended cultures, *Thermoproteales* dramatically decreased to 6%. The addition of ibuprofen selected for a methanogenic archaeal community. The methanogenic order, *Methanosarcinales*, saw a dramatic increase in the ibuprofen amended cultures, going from 13% in the background to 64% in the ibuprofen amended cultures. Another methanogenic order, *Methanobacteriales*, increased from 10% in the

unamended background to 16% in the ibuprofen amended cultures. *Methanomicrobiales* increased from 2% in the unamended to 4% in the ibuprofen amended cultures.

5.3.3 Discussion

5.3.3.1 Ibuprofen-Bacterial

Acholeplasmataceae, a facultative anaerobe, is the dominant family in the ibuprofen amended cultures. Members of this family have been isolated in a variety of including sewage, soil, compost, plants, rumens, and fish (Windsor, Windsor, & Noordergraaf, 2010). Many members of this family have a saprophytic lifestyle, meaning that they utilize dead and decaying organic matter for carbon and nutrient requirements (Edward & Freundt, 1970;Siewert et al., 2014). One study added dead C^{13} labeled *E. coli* cells a pig manure slurry and found that the *Acholeplasma* species were the predominant community members that assimilated C^{13} and proved to have a competitive advantage for utilizing dead bacterial cells (Hanajima, Aoyagi, & Hori, 2015). There were large reductions in every family except for three in the ibuprofen amended cultures. This means that there was dead cellular material available for *Acholeplasmataceae* to utilize for growth. *Acholeplasmataceae* also contains many members known to ferment (Brown, Bradbury, & Johansson, 2010;Martini, Marcone, Lee, & Firrao, 2014). The other two families that had an increase in the ibuprofen amended cultures were *Planctomycetaceae* and *Spirochaetaceae*. Planctomycetaceae can be found in a variety of aquatic environments including marine, freshwater, and estuarine (Ward, 2010). Little research has been done on the metabolism of *Planctomycetaceae*; however, *Planctomycetaceae* has members capable of fermentation (Ward, 2010). Members *Planctomycetaceae* are also known for their slow growth and ability to easily survive in low carbon

environments (Lage & Bondoso, 2012). Since ibuprofen is not being degraded the only carbon that is available is what is left in the inoculum; this may have been advantageous to *Plantcomycetaceae* due to their ability to survive with lower amounts of carbon. The anaerobic *Spirochaetaceae* is free living or can live in a variety of hosts; members of this family can produce metabolites such as acetate, ethanol, and carbon dioxide/hydrogen during their anaerobic carbohydrate metabolism (Paster, 2010). One study has shown that *Spirochataetaceae* plays in important role in providing methanogens with C-1 and C-2 compounds in low carbon environments (Dollhopf et al., 2001). It is probable that this is what is happening in the ibuprofen amended cultures.

All three dominant families not only produce C-1 and C-2 during fermentation, put also have the ability to thrive in low carbon environments. Saprophytic *Acholeplasmataceae* could be utilizing not only the organic matter in the inoculum for fermentation, but also the organic matter of the decaying cells that have been killed by the presence of ibuprofen. *Planctomycetaceae* and *Spirochaetaceae* could also be utilizing the carbon in the inoculum to produce C-1 and C-2 compounds. These products of fermentation could serve as a carbon source for the methanogenic community and could explain the increase in methane production in the ibuprofen amended cultures compared to the background cultures.

5.3.3.2 Ibuprofen-Archaeal

While methanogens were present in the background cultures, they were dominated by the non-methanogenic orders *Thermoplasmatales* and *Thermoproteales* (Huber & Stetter, 2006;Huber, Huber, & Stetter, 2006).The ibuprofen amended cultures shifted to a methanogenic community with the order *Methanosarcinales* making up more

than half of the ibuprofen amended community. The methanogenic orders *Methanobacteriales* and *Methanomicrobiales* also increased in the ibuprofen amended cultures. This increase in methanogens explains the increased methane production seen in the amended cultures compared to the background cultures (fig. 21).

Methanosarcinales can utilize a variety of C-1 and C-2 compounds such as carbon dioxide, acetate, methanol, methylamines, and methyl sulfides as substrates (Kendall & Boone, 2006). *Methanobacteriales* are generally hydrogenotrophic using hydrogen to reduce carbon dioxide to methane; *Methanomicrobiales* contains hydrogenotrophic members as well, but also contains members that can utilize formate, propanol, and ethanol (Bonin & Boone, 2006;Garcia, Ollivier, & Whitman, 2006) Many of these C-1 and C-2 compounds are fermentation products that could be produced by the family *Acholeplasmataceae*, as that family contains many strong fermenters (Brown et al., 2010). *Spirochaetaceae* can produce a variety of the C-1 and C-2 compounds mentioned above (Paster, 2010). This would then give *Methanosarcinales*, *Methanobacteriales*, and *Methanomicrobiales* substrates to utilize to produce methane.

5.3.4 Conclusions

The bacterial and archaeal community in the ibuprofen amended Iona cultures showed a large shift in comparison to the unamended backgrounds. This indicates that the amendment of ibuprofen has selected for a community that can withstand the presence of ibuprofen and utilize the carbon within the inoculum. The dominant bacterial families can either ferment or anaerobically metabolize carbohydrates to C-1 and C-2 compounds. These C-1 and C-2 compounds then enhance and help the methanogenic community grow. Ibuprofen has selected for a fermentative and methanogenic community. These

findings follow the same theme found in the other ibuprofen experiments: ibuprofen not only changes microbial activity but the overall community. Ibuprofen reduces microbial diversity and selects for a specialized community.

5.4. Tables and Figures

$$\text{Maximum Rate Ratio (MRR)} = \frac{\text{gas production of substrate fed} - \text{gas production of seed background}}{\text{gas production of active background} - \text{gas production of seed background}}$$

Concentration	Day 2	Day 12	Day 30
1.0mM	0.81	1.46	1.57
0.5mM	0.81	1.21	1.35

Table 4: Maximum rate ratio of ibuprofen

$$\text{Maximum Methane Ratio (MMR)} = \frac{\text{methane production of substrate fed} - \text{methane production of seed background}}{\text{methane production of active background} - \text{methane production of seed background}}$$

Concentration	Day 2	Day 12	Day 30
1.0mM	0.93	1.64	1.53
0.5mM	0.95	1.41	1.30

Table 5: Maximum methane ratio of ibuprofen

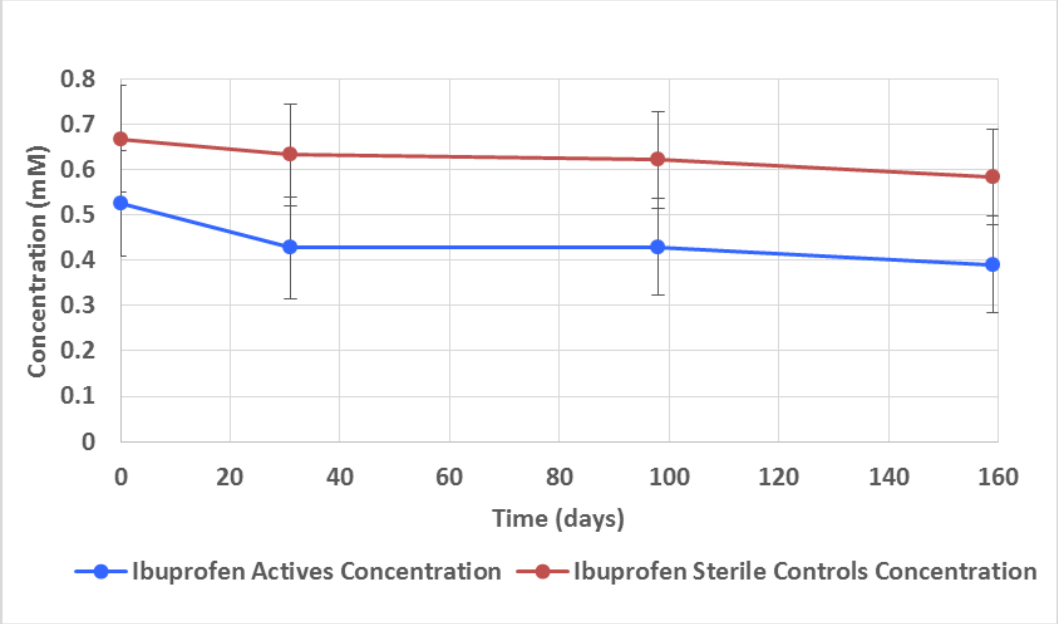

Figure 19: HPLC Analysis of ibuprofen amended Iona cultures

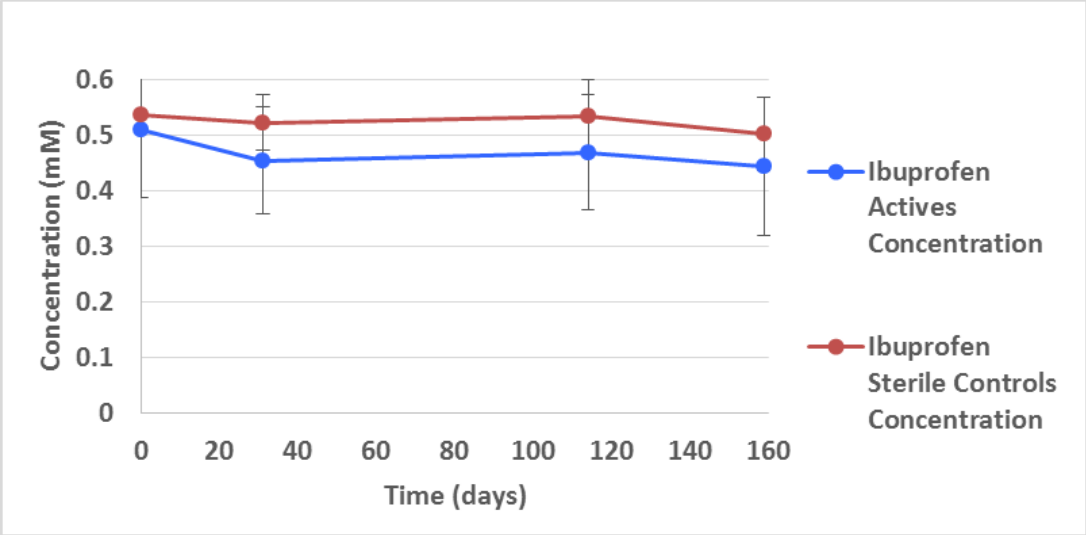

Figure 20: HPLC Analysis of ibuprofen amended anaerobic sludge cultures

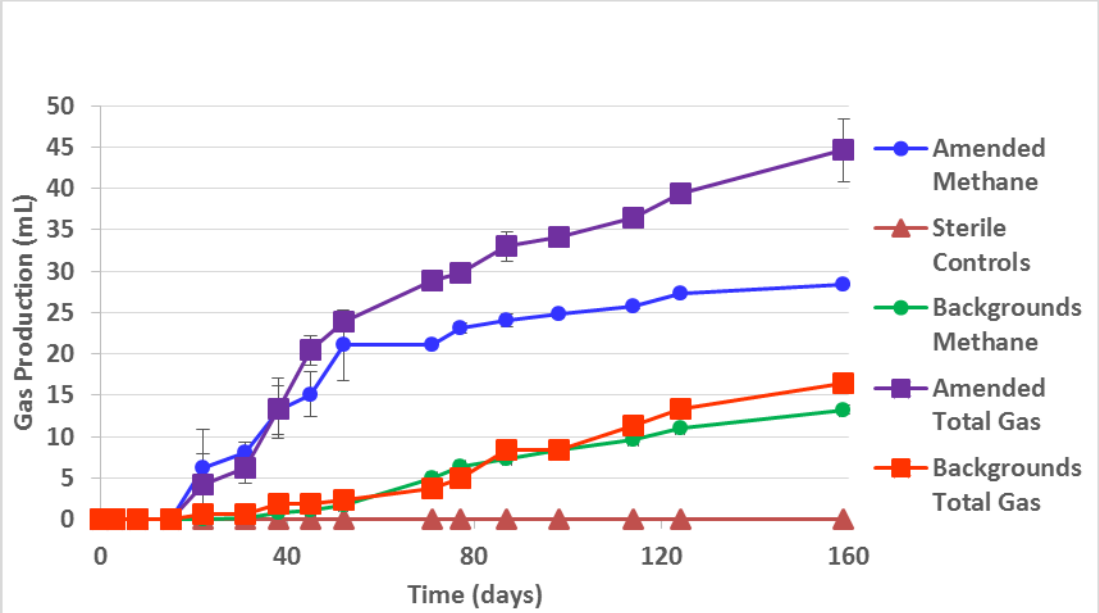

Figure 21: Cumulative methane and total gas production in ibuprofen amended Iona cultures (note: some error bars cannot be seen due to the size of the marker)

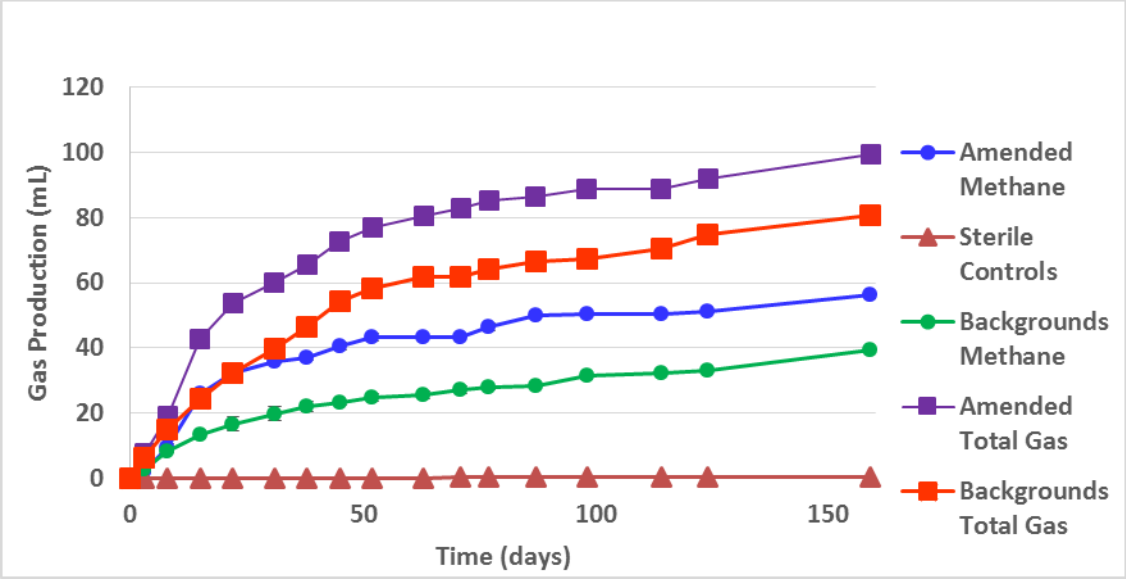

Figure 22: Cumulative methane and total gas production in atenolol amended anaerobic sludge cultures (note: some error bars cannot be seen due to the size of the marker)

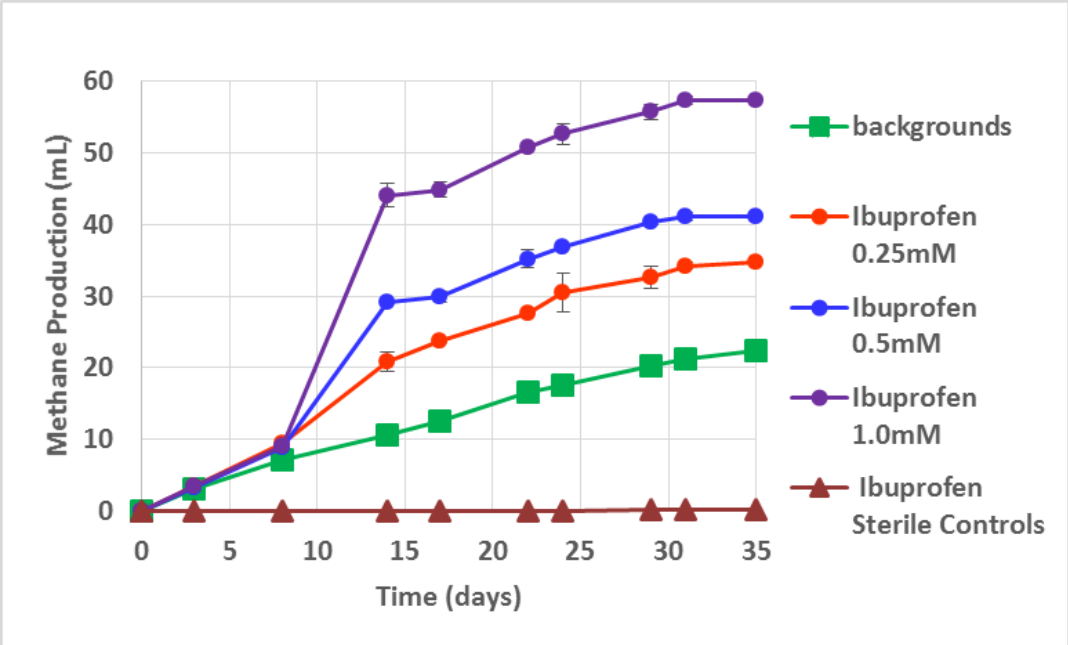

Figure 23: Biochemical methane potential of ibuprofen (note: some error bars cannot be seen due to the size of the marker)

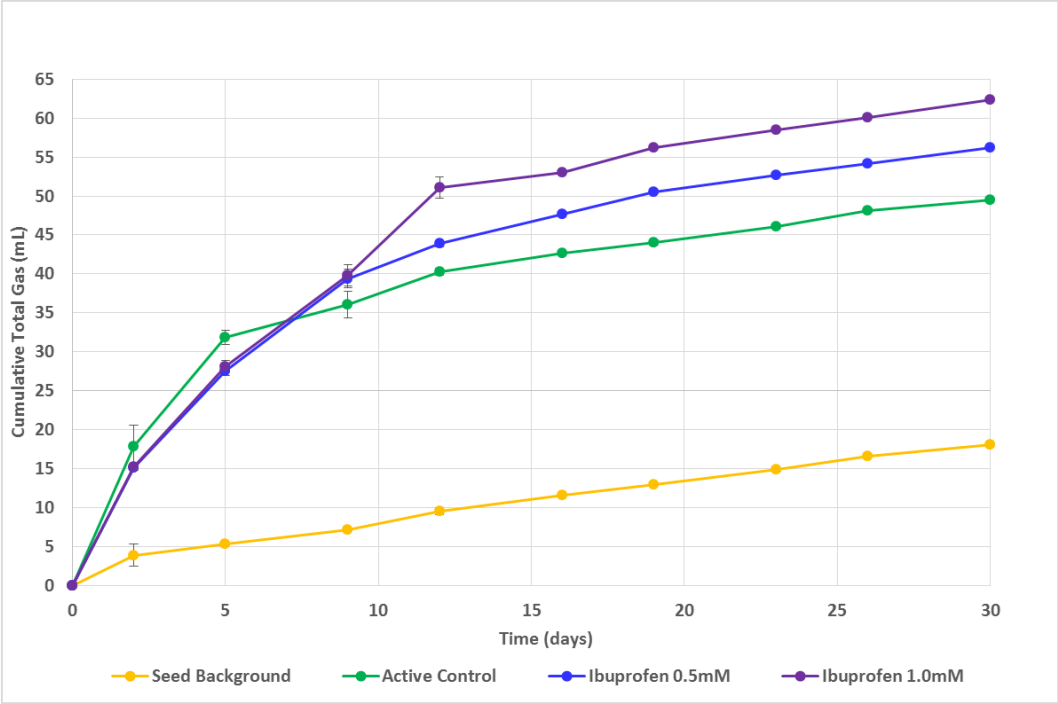

Figure 24: Ibuprofen ATA total gas production (note: some error bars cannot be seen due to the size of the marker) Note: no gas production in sterile controls (not shown).

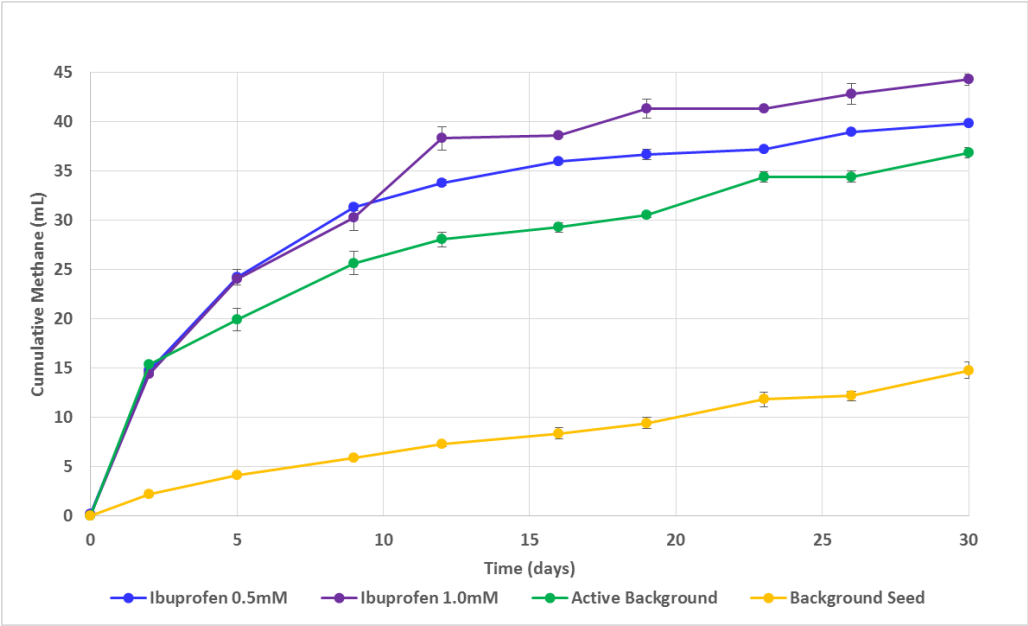

Figure 25: Ibuprofen ATA total methane production (note: some error bars cannot be seen due to the size of the marker) Note: no gas production in sterile controls (not shown).

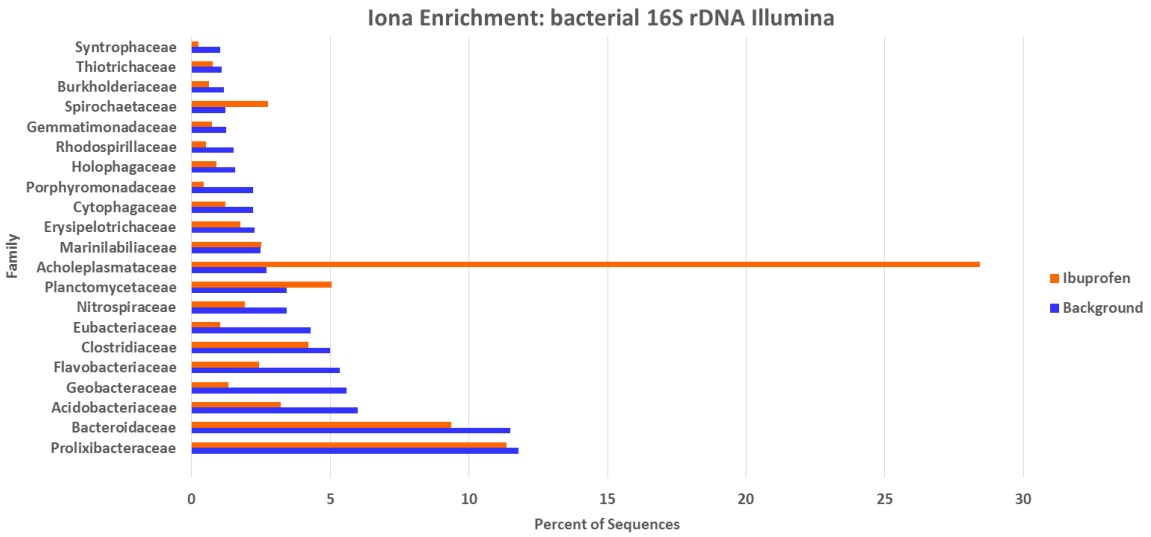

Figure 26: 16S rDNA family level bacterial community composition of ibuprofen amended and background Iona cultures

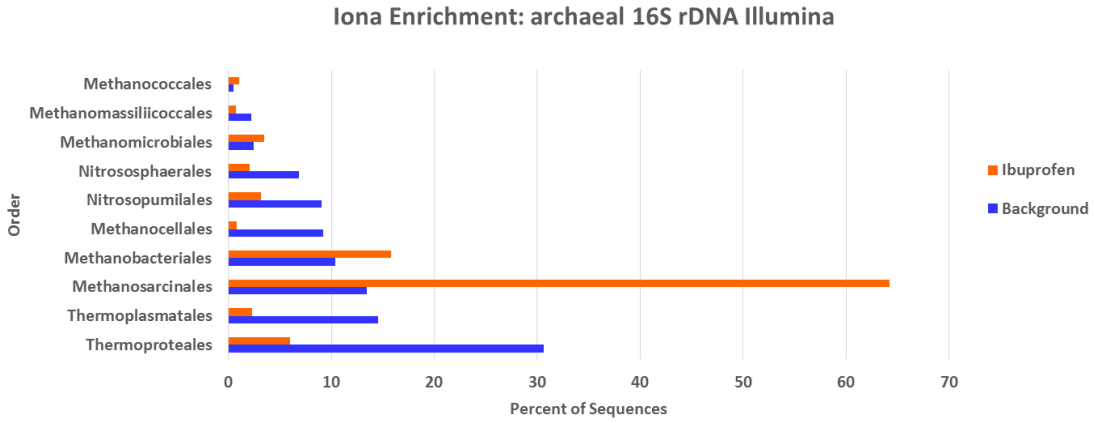

Figure 27: 16S rDNA order level archaeal community composition of ibuprofen amended and background Iona cultures

Chapter 6

Concluding Remarks and Future Directions

Atenolol was found to be transformed to atenolol acid in both culture set-ups. The transformation of atenolol to atenolol acid has been previously described under aerobic conditions (Koba et al., 2016; J Radjenović et al., 2008). To our knowledge, this is the first report of transformation of atenolol to atenolol acid under anaerobic conditions. It is interesting that the metabolite atenolol acid has been identified not only in aerobic cultures, but in a variety of environments including activated sludge and soil. This raises questions about the mechanism(s) used during this transformation and if they differ between aerobic and anaerobic conditions.

The monitoring of methane production during transformation as well as during the BMP and ATA assays showed that atenolol was biodegradable and not chronically inhibitory to the anaerobic community. However, atenolol was not biodegraded, it was only transformed. Even though no carbon was removed from atenolol, methane production was enhanced with the addition of atenolol in both assays. The presence of atenolol favored a community that could utilize the residual carbon in the inoculum. The Iona cultures had more solids in its inoculum than the anaerobic sludge cultures and therefore most likely had more carbon available. This explains why the Iona cultures were able transform atenolol faster than the anaerobic sludge cultures.

Some bacterial families dominate in atenolol amended were known to favor complex nitrogen sources; atenolol could possibly be used as a complex nitrogen source. One study revealed that some nitrogen sources enhance methane production more than

others (Wagner et al., 2012). Casamino acids when fed to cultures produced approximately 600 mL methane per mole of nitrogen; L-arginine fed to the cultures produced approximately 1400 mL methane per mole of nitrogen (Wagner et al., 2012). This could be tested in our assay by using a variety of nitrogen sources ranging from simple structured ammonia and urea to more complex sources such as amino acids and peptones, but keeping the amount of carbon the same throughout all amendments. This can confirm if changing the nitrogen source can enhance methane production.

The inoculum from the Iona cultures comes from an uncontrolled open environment; the constantly changing environment and variety of niches increases the diversity of the community. The inoculum from the anaerobic sludge cultures are from a closed environment that is semi-controlled and physically mixed; this reduces the heterogeneity of the environment and therefore the community by selecting for organisms that can compete in such a specific environment. Community analysis of unamended backgrounds showed the vast differences between the Iona cultures and anaerobic sludge cultures. The Iona unamended cultures had a greater diversity in its community, while the unamended anaerobic sludge cultures had a community with a smaller range of metabolic capabilities. Further community analysis revealed that the presence of atenolol did affect the composition. In particular, the Iona cultures' bacterial community had shifted its composition to a community abundant in members with the ability to ferment and an affinity toward complex nitrogen sources. Anaerobic sludge cultures remained fairly stable even with the introduction of atenolol. This result not only spoke to the community's stability but also indicated that there is more than one type of bacterial community responsible for the transformation of atenolol, as the anaerobic sludge

community greatly differed from the Iona community. This study could be built upon by looking into the functional genes that are active during the transformation of atenolol.

Ibuprofen was not degraded or transformed in either culture set up. However, the BMP and ATA results showed that it was biodegradable and not chronically toxic. This shows that the BMP and ATA should be used with caution as the data did not coincide with what actually occurred. While ibuprofen was not degraded or transformed it still had an effect on the community. Ibuprofen enhanced gas and methane production and also changed the community structure in both cultures set ups. As previously discussed, ibuprofen has antimicrobial effects and this could have caused the community shift (Obad, 2015). This could be tested by plating the cultures under anaerobic conditions at a variety of ibuprofen concentrations and looking for zones of clearing.

Much of the research regarding the microbial degradation of pharmaceuticals has been black box research, where studies only look for the presence of a selected pharmaceutical and then the absence of said pharmaceutical. It was only recently that studies have begun to look at any metabolites produced. A recent monitoring study of the Hudson River Estuary found that not only atenolol acid was present in each sample but also the metabolite atenolol acid at the magnitude of ng/L (Pochodylo & Helbling, 2015). Few studies have been done on how these pharmaceutical metabolites affect organisms living the environment. Some studies have shown that metabolites can still be pharmacologically active and can sometimes exhibit toxicity on eukaryotic organisms, but have not looked at effects such as the ability to be an endocrine disruptor (Celiz, Tso, & Aga, 2009;López-serna, Petrovi, & Barceló, 2012). Therefore, pharmaceutical

metabolites could also be contributing to the feminization of fish and frogs that some pharmaceuticals are known for, but it is not being monitored.

This research helps build the case that pharmaceuticals can indeed be transformed, but that the metabolites remain. While atenolol was transformed to atenolol acid, it remained during the course of the experiment indicating that it may be a dead end metabolite and may be being released into the environment. Therefore, this metabolite should also be analyzed when looking for the presence of pharmaceutical residues in the environment. It is unknown if atenolol acid is still pharmacologically active, but metabolites of other pharmaceuticals such as carbamazepine, diazepam (Valium®), and atorvastatin (Lipitor®) have been documented to still be pharmacologically active (López-serna et al., 2012). Presently, standard wastewater treatment does completely remove pharmaceuticals and their metabolites. Further research is needed to improve current wastewater treatment to remove micropollutants such as pharmaceuticals. New treatment processes such as the Membrane Bioreactor (MBR), have shown higher removal rates of pharmaceuticals than conventional treatment (Jelena Radjenović et al., 2009). This work further emphasizes the importance of looking for pharmaceutical metabolites in the environment as well as the need to determine if a pharmaceutical is truly being mineralized or only transformed. If pharmaceuticals such as atenolol are not being mineralized, it is important to investigate other options beyond conventional treatment.

Appendix

Components to Methanogenic Media

Component	Concentration (g/L)
Resazurin	0.001
$(NH_4)_2PO_4$	0.04
NH_4Cl	0.2
$MgCl \circ 6H_2O$	1.8
KCl	1.3
$MnCl_2 \circ 4H_2O$	0.02
$CoCl_2 \circ 6H_2O$	0.03
H_3BO_3	0.0057
$CaCl_2 \circ 2H_2O$	0.0027
$Na_2MoO_4 \circ 2H_2O$	0.0025
$ZnCl_2$	0.0021
$FeCl_2 \circ 4H_2O$	0.368
$NaHCO_3$	2.64
$Na_2S \circ 9H_2O$	0.5
Vitamin Solution	1% (vol/vol)

Table 6A: Components to methanogenic media

Component	Concentration (g/L)
Biotin	0.002
Folic acid	0.002
Pyridoxine hydrochloride	0.01
Riboflavin	0.005
Thiamin	0.005
Nicotinic acid	0.005
Pantothenic acid	0.005
B_{12}	0.0001
p- aminobenzoic	0.005
Thioctic acid	0.005

Table 6B: Components to vitamin stock

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