THESIS

ANAEROBIC DIGESTION OF ORGANIC WASTES:

THE IMPACT OF OPERATING CONDITIONS ON HYDROLYSIS EFFICIENCY

AND MICROBIAL COMMUNITY COMPOSITION

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ABSTRACT

ANAEROBIC DIGESTION OF ORGANIC WASTES:

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MICROBIAL COMMUNITY COMPOSITION

Anaerobic digestion (AD) is an environmentally sustainable technology to manage organic waste (e.g., food, yard, agricultural, industrial wastes). Economic profitability, however, remains a key barrier to widespread implementation of AD for the conversion of specific feedstocks (e.g., manure, the organic fraction of municipal solid waste (OFMSW), and agricultural residue) to energy. Specifically, high capital and operating costs and reactor instability have continually deterred the use of AD. In order to develop AD systems that are highly efficient and more cost-effective, it is necessary to optimize the microbial activity that mediates the digestion process. Multi-stage AD systems are promising technologies because they allow for separate process optimization of each stage and can enable processing of high-solids content waste. Leachate is recycled through the system, which reduces heating and pumping costs, as well as conserving water. The leachate recycle, however, leads to an increase in ammonia and salinity concentrations. At this time, the impact of reactor conditions (ammonia and salinity concentrations) on hydrolysis is not well understood. As hydrolysis is one rate-limiting step of the process in the conversion of refractory wastes (e.g., lignocellulosic materials), optimization of hydrolysis has the potential to radically improve the economic profitability of AD. The specific objectives of this research were to: 1) determine the effects of operating conditions on hydrolysis efficiency for a variety of solid wastes

(manure, food waste, and agricultural residue); 2) determine hydrolysis kinetic parameters as a function of the operating conditions; and 3) identify characteristics of microbial communities that perform well under elevated ammonia and salinity concentrations.

To this end, small-scale batch reactors were used to determine hydrolysis efficiency and kinetic rates. Initially, the AD sludge inoculum was exposed directly to the high ammonia and salinity concentrations (1, 2.5, 5 g Total Ammonia Nitrogen (TAN)/L and 3.9, 7.9, 11.8 g sodium/L) as would occur in a reactor treating organic waste with leachate recycle. Results demonstrated a need to acclimate, or adapt, the microorganisms to high concentrations, as methane generation was significantly inhibited with high concentrations. Thus, the organisms were acclimated for two to four months to these testing conditions. The batch studies were repeated, and results demonstrated substantial improvement in hydrolysis efficiency and methane generation. However, although differences in kinetic rates were not statistically significant, general trends in hydrolysis rates suggested that hydrolysis efficiency decreases with increased ammonia and salinity concentrations for a variety of feedstocks (i.e., manure, food waste, agricultural residue). Additionally, results demonstrated that acclimation was necessary to achieve optimal hydrolysis rates. Furthermore, microbial community composition changes in the inocula post-acclimation indicated that reactor inoculation could help improve tolerance to elevated levels of ammonia and salinity to minimize reactor start-up times and improve economic viability.

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1.0 Introduction

1.1 Research Motivation

Recent increases in environmental regulations for pollutant minimization have demonstrated the need for safe and effective methods of organic waste disposal. Anaerobic digestion (AD) is an environmentally sustainable technology for converting a variety of feedstocks (waste sources) including manure, the organic fraction of municipal solid waste (OFMSW), and agricultural residue to energy in the form of methane (Demirer et al., 2005a). Furthermore, the digestion of multiple feedstocks (co-digestion) has the potential to increase overall energy generation through synergistic interactions. Anaerobic digestion involves a series of processes that first break down organic matter into sugars and amino acids via hydrolysis. Acidogenic and acetogenic bacteria convert these products into acetic acid, which is then converted to methane via methanogenesis.

Current AD technologies for the treatment of manure and OFMSW are often nonviable in the U.S. due to lack of economic profitability. In arid regions, manure is often collected via means that result in a high-solids waste, and since water is a scarce resource, reactors that are able to effectively digest this high-solids feedstock are needed. Furthermore, since the cost of landfilling (the most common method of MSW management) in the U.S. is low, it is difficult for existing sustainable alternative waste treatments, such as AD, to compete as viable treatment methods. Additionally, reactor instabilities specific to the treatment of high-solids manure and OFMSW (e.g., build-up of toxic products such as rapid volatile fatty acid (VFA) accumulation) have contributed to decreased digestion efficiency and loss of profits. Therefore, digesters capable of

stable performance and economic profitability are needed to allow for the widespread AD implementation treating high-solids manure and OFMSW.

Multi-stage AD systems are a promising technology for the treatment of manure and OFMSW, but key operating parameters that determine reactor stability and efficiency are still not fully understood. AD is a microbially-mediated process, and previous research has demonstrated that the organisms which mediate each process perform optimally under different conditions (Ward et al., 2008). Multi-stage AD systems separate each process of the system, allowing for individual process optimization. This type of system may also recycle leachate through the system to conserve water and reduce energy costs; however, this recycle may result in a build-up of ammonia and salinity, which can inhibit microbial waste conversion at high concentrations. Fresh water can be used to decrease the concentrations of these inhibitors, but this additional consumption leads to an increase in heating and pumping costs. Thus, the optimal balance of energy and water consumption versus process efficiency must be determined to maximize the economic benefit of the system.

1.2 Research Objective

Since hydrolysis is a rate-limiting step in the breakdown of lignocellulosic material, optimization of this step has the potential to greatly increase economic profitability. However, hydrolysis remains one of the least understood steps in the process (Miron et al., 2000; Gavala et al., 2003), and studies examining the impact of operating conditions (e.g., ammonia and salinity concentrations) on hydrolysis are needed to guide reactor design and operation. Thus, the objective of this research was to investigate the effects of operating conditions (e.g., ammonia and salinity concentrations)

on hydrolysis efficiency of manure, OFMSW, and agricultural residue and determine hydrolysis kinetic parameters as a function of ammonia and salinity concentrations. Additionally, this study sought to identify microbial community characteristics that lead to optimal process performance.

1.3 Thesis Overview

The following chapter (Chapter 2) presents an introduction to AD, designed to familiarize the reader with the current state of technology for manure and OFMSW and associated challenges that are barriers to economically profitable implementation. Research was conducted to determine hydrolysis kinetic rates and microbial community composition for the AD of manure and food waste (a component of OFMSW) and is presented in Chapters 3 and 4, respectively. The last chapter (Chapter 5) is dedicated to summarizing the findings and implications of this study and recommends future work. The appendices include additional research conducted on hydrolysis for agricultural residues (Appendix A) and supplemental methods and data involved in the determination of hydrolysis rates.

2.0 Background and Literature Review

AD has the potential to address two large issues facing today's society: manure management and OFMSW treatment. First, the issue of manure management has become increasingly important as current practices of manure disposal (e.g., composting for land application, anaerobic lagoons) by concentrated animal feeding operations (CAFOs) contribute to surface and groundwater pollution and release significant volumes of methane to the atmosphere (US EPA, 2011; Gloy, 2011). Because methane has 21 times the Global Warming Potential (GWP) of carbon dioxide (Gloy, 2011), avoiding methane emissions is critical. Secondly, heightened interest in diverting MSW from landfills has been demonstrated by recently issued programs (e.g., recycling programs) and policies (Levis et al., 2010). Currently, approximately 54% of MSW is disposed of in landfills (US EPA, 2008), with approximately 50-70% of MSW being organic material (Verma, 2002). Although the OFMSW is a potential energy source, difficulties associated with the diversion of the organic fraction (e.g., expensive and complex equipment), alternative treatment costs, and process reliability have slowed landfill diversion. AD provides a means for utilizing valuable organic waste (e.g., waste that would ultimately end up in landfills), but future work is needed to improve process reliability and economic benefits.

This chapter provides a discussion of the AD microbiological process and available AD designs, followed by a description of the current state of AD technology for manure and OFMSW. It also presents an in-depth discussion on benefits, challenges, and operating parameters associated with multi-stage AD systems. Finally, because on-going efforts to develop and optimize AD technologies can be guided by molecular biology

tools to track the required microorganisms, a description of appropriate molecular biology tools is included.

2.1 Microbiology of AD

To engineer economically viable AD systems for the treatment of manure and OFMSW, it is first necessary to understand the microbiology that mediates the digestion process. The AD system is divided into three processes: hydrolysis, acidogenesis/ acetogenesis, and methanogenesis. As shown in Figure 2.1, first hydrolytic bacteria convert complex organic matter (proteins, fats, and carbohydrates) present in the waste feedstock into soluble fatty acids and other organic monomers. This process is mediated by hydrolytic enzymes including proteases, which degrade proteins, lipases, which degrade fats, and cellulases, which degrade cellulose. Next, acidogenesis converts the hydrolysis products (amino acids, fatty acids, and sugars) into VFAs, alcohols, carbon dioxide, and hydrogen. Byproducts, including ammonia and hydrogen sulfide, are also produced (Strik et al., 2005). Acetogenesis further converts the acidogenesis products into acetic acid, carbon dioxide, and hydrogen. In the final process, methanogenic Archaea produce methane from the products of acido/acetogenesis. There are two main types of microorganisms responsible for methane formation: acetoclastic methanogens and hydrogen-utilizing methanogens. Acetoclastic methanogens split acetate (an electron donor) into carbon dioxide and methane (Lachavanne et. al., 1997). Hydrogen-utilizing methanogens use hydrogen and reduce carbon dioxide to form methane (Mara et al., 2003). The former pathway is the dominant mechanism and typically accounts for approximately 70% of methane production in AD reactors because hydrogen is limited in AD (Mara et al., 2003; Monnet, 2003; Verma, 2002).
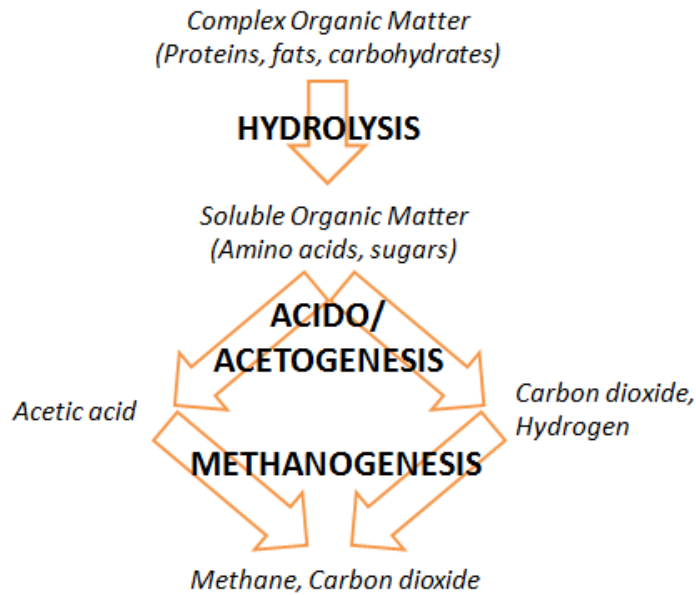
Figure 2.1 AD process flow chart

2.2 Currently Available AD Reactor Designs

A variety of different AD reactor designs have been developed to date. These AD systems may be classified according to the following characteristics:

- Reactor configuration (batch or continuous)

- Solids content (wet or dry)

- Number of stages (single-stage, two-stage, or multi-stage)

2.2.1 Reactor Configurations

In batch systems, a reactor is loaded with feedstock, run to completion, emptied and reloaded. Batch reactors benefit from technical simplicity and low operating costs. However, they require a larger footprint due to lower organic loading rates than continuously fed reactors (Verma, 2002). Additionally, batch reactors often suffer from instability in microbial populations, and since AD is a microbially-mediated process, the

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efficiency of the digestion process depends upon the stability of the microorganisms present.

In continuous systems, reactors are continuously fed feedstock, allowing a steady-state to be reached in the reactor with a constant gas yield. Two conventional continuous feed reactor configurations are completely-stirred reactors (contents of reactor are mixed by mechanical agitation or effluent or biogas recirculation) and plug-flow reactors (contents of reactor are pushed along a horizontal reactor). Although continuous reactors have higher operating costs due to pumping requirements, these reactors are able to maintain microorganisms within the system, thereby avoiding lag times associated with microorganism growth in batch reactors (Chaudhary, 2008).

2.2.2 Solids Content

Digesters may be further classified according to the total solids (TS) content of the waste to be digested. Batch reactors may be operated as either wet reactors (less than 15% TS) (Tchobanoglous et al., 1993) or dry reactors (22-40% TS) (Verma, 2002). Conventional wet technologies include complete mix reactors and plug-flow digesters and operate at maximum solids contents of 3-10% and 10-14%, respectively (Wilkie, 2005; Demirer and Chen, 2005b). For high-solids wastes including manure and OFMSW, to maintain a solids content less than 14%, high volumes of water may be required to dilute wastes, which increases capital and operating costs because large reactor volumes are required and heating and pumping requirements are increased (Verma, 2002). Therefore, dry reactors are often used to digest high-solids waste. Dry reactors are characterized by smaller AD reactor footprints than wet reactors (Verma, 2002) and often operate as vertical, high-solids plug-flow reactors (Rapport et al., 2008). However,

conventional dry reactors also require an increased energy input to move and/or pump the waste through the reactor in comparison to a diluted, low-solids content reactor (e.g., complete mix or low-solids plug-flow systems) (Verma, 2002).

2.2.3 Number of Stages

Digesters also can be operated as single-stage, two-stage, or multi-stage reactors. Single-stage reactors are the simplest of reactor configurations. In single-stage systems, all the digestion processes take place in one reactor, and these systems benefit from lower capital and operating costs (Vandevivere, 2002; Kelleher, 2007). In contrast, two-stage reactors separate the hydrolysis and acido/acetogenesis processes from methanogenesis. In the first stage, digestion is limited by the rate of hydrolysis of cellulosic materials; the second stage is typically limited by the rate of microbial growth (Chaudhary, 2008). Two-stage designs allow for separate optimization of each process (e.g., longer biomass retention times in the methanogenesis reactor), which can increase biogas yield because hydrolytic and methanogenic bacteria are known to have different optimal conditions (Verma, 2002), Additionally, this type of reactor is more stable than single-stage reactors (Chaudhary, 2008; Vandevivere, 2002), as the latter are more subject to process failures due to pH changes or ammonia build-up (Chaudhary, 2008; Rapport et al., 2008). For example, in a two-stage system, pH may be adjusted prior to flow through the methanogenesis reactor. As with continuously fed reactors, microorganisms may be retained in this system. Furthermore, multi-stage reactors may be used to separate each process (e.g., hydrolysis, acido/acetogenesis, and methanogenesis) into three reactors, providing further process control and optimization over each process and increased methane yields.

2.3 Current State of Technology

2.3.1 AD of Manure

Historically, traditional methods of manure management have not been environmentally sustainable. Anaerobic lagoons are a common method of treating livestock waste (Wang et al., 2004) that have been successfully implemented since the early 1960s (Safley and Westerman, 1992), but they accounted for over 60% of North American methane emissions to the atmosphere from animal waste in 1990 (Sharpe and Harper, 1999; Adler, 1994). In 2010, methane emissions from livestock manure management (primarily lagoons) continued to rise relative to the 1990s and accounted for approximately 8% of total U.S. anthropogenic methane emissions (US EPA, 2010). Thus, alternative manure management technologies are needed.

AD is an attractive alternative for manure management because it allows methane to be captured and used as an energy source. However, farm-based AD reactors traditionally have experienced high failure rates largely due to issues such as poor installation or equipment failure (e.g., generators) (Frame et al., 2001). For example, up until 1998, more than 60% of on-farm digesters failed (Lusk, 1998). Improved system design and installation practices, as well as heightened understanding of operational parameters (e.g., optimal pH or VFA concentrations), has enhanced the performance of farm-based AD systems; furthermore, recent increased use of co-digestion on farms has helped increase process stability through feedstock synergisms and improved methane yields to become more economically feasible (Paul, 2008). Currently, the majority of farm-based AD systems treat mostly manure (AgSTAR EPA, 2011), and it is estimated that there are 176 anaerobic digesters in operation at commercial livestock farms in the

U.S. (AgSTAR EPA, 2011). As seen in Table 2.1, the majority of digesters are located in the Eastern U.S., with a few digesters operating along the western coast.

Table 2.1 Excerpt of operational digesters treating manure in the U.S. (AgSTAR EPA, 2011)

State	Number of digesters treating manure
New York	22
Vermont	13
Pennsylvania	22
North Carolina	5
Washington	5
California	11
Oregon	4
Colorado	**1**
Wyoming	**1**
Kansas	**0**
Nebraska	**1**

Clearly, regional factors play a large role in the feasibility of AD systems treating manure. Of the digesters in operation, approximately 30% of the AD systems are operated as complete-mix systems and 47% are plug-flow reactors (AgSTAR EPA, 2011). As manure is often collected by wet scraping (using large volumes of water to scour manure from concrete gutters) in the Eastern U.S., which results in solids contents of approximately 12% (Demirer and Chen, 2005b), these conventional technologies are suitable and economically viable. However, this type of manure collection results in a high water usage that is simply not an option in arid regions. As a result, dry scraping of manure is employed, utilizing tractor-mounted blades to scrape manure from the lots. Dry scraping is also used in cold regions, where freezing conditions prevent the use of flushing or necessitate infrequent collection. The solids content of manure collected via dry scraping is greater than 25% (Hall et al., 1985), and further dilution of the waste with

water is often either impractical or unfeasible because of increased costs and resource consumption. Consequently, pumping and mixing of the manure are unachievable, thereby rendering continuously-stirred and plug-flow reactors nonviable or non-profitable for application in arid regions. As a result, alternative designs capable of maintaining reactor stability and that require minimal water usage are needed to effectively digest high-solids waste including dry scraped manure.

2.3.2 AD of OFMSW

Municipal solid waste (MSW), encompassing refuse generated by a community with the exception of industrial, construction, hazardous, or agricultural waste (Tchobanoglous et. al, 1993; US EPA, 2012), is a prevalent waste source that has historically caused environmental concern. In 2009, Americans alone generated 243 million tons of MSW, over half of which ended up in landfills (US EPA, 2009). Because landfills are estimated to be the second highest source of anthropogenic methane emissions in the U.S. (Levis and Barlaz, 2011; US EPA, 2010) and require a large land footprint, waste diversion is needed. Since OFMSW (e.g., food waste, wood, paper) accounts for approximately 50-70% of the total waste material (Braber, 1995; Kayhanian, 1994), this waste represents a valuable, underutilized energy source.

The implementation of AD systems treating OFMSW varies greatly by world regions due to differences in economic profitability. Currently, AD of OFMSW is widespread in Europe; approximately 200 plants spanning over 17 countries that treat mixed MSW and biowaste (source separated, biodegradable MSW) are currently in operation (De Baere and Mattheeuws, 2010). This successful application is due largely to economic initiatives (high energy prices, high tipping fees, and tax incentives) in Europe

and to European Union requirements that by 2016 Member States must reduce the amount of landfilled organic waste by 65% relative to 1995 levels (Levis et al., 2010; Rapport et al., 2008). Despite such an abundance of digesters in Europe, there is only full-scale digester treating OFMSW in North America (Canada). The city of San Jose, CA recently received permission to implement a digester treating commercial organic waste and construction is underway (Zero Waste Energy, 2012). The main factors contributing to lack of use of AD for OFMSW treatment in the U.S. are cost and process reliability. Overall cost could be improved by energy-supported regulations (e.g., tipping fees, tax credits, carbon credits) (Rapport et al., 2008) or more efficient, economically viable AD technologies through reduced capital and operating costs and increased profits from energy generation.

Reactor instabilities and operational challenges in conventional technologies treating OFMSW decrease process performance and lead to a loss of economic profits. OFMSW can form scum layers that cause clogging in pipes due to its heterogeneous nature and tendency to separate (Rapport et al., 2008), and pretreatment of OFMSW is often required for homogenization prior to digestion which requires complex equipment and leads to a loss in organic solids (Rapport et al., 2008). As OFMSW typically contains 30-60% solids, large volumes of water also are required to dilute the waste to appropriate levels for application in wet digesters (Rapport et al., 2008). Additionally, toxic compounds present in OFMSW (e.g., heavy metals) can diffuse through reactors in wet systems and inhibit microbial activity (Vandevivere, 2002). Most notably, since portions of OFMSW (e.g., non-cellulosic food waste components) degrade rapidly during hydrolysis, acid build-up in reactors is a common occurrence, and pH drops have been

shown to greatly inhibit methanogenic activity (Cho et al., 1995). <u>Thus, AD systems that</u>
<u>are economically profitable and stable (e.g., prevention against pH shock) are necessary</u>
<u>to allow for implementation in the U.S.</u>

2.4 Benefits and Challenges of Multi-Stage AD Technology

Multi-stage AD systems that utilize a "dry" hydrolysis process are promising technologies that can be designed to overcome the main barriers associated with digestion of dry-scraped manure and OFMSW: high-solids content and rapid VFA accumulation, respectively. As shown in Figure 2.2, fresh organic matter can be packed in leachate bed reactors (LBRs), which can be operated as batch reactors with leachate recirculation. Water is largely conserved through leachate recirculation (Shahriari et al., 2012), thus making this system a suitable applicable in arid regions, and heating and pumping requirements are also reduced. Leachate from the LBRs can be pumped and held in a compositing tank, where acido/acetogenesis occurs. Leachate is then fed to the high-rate methanogenic reactor continuously. The high-rate methanogenic reactor may be a fixed-film reactor (reactor that utilizes support materials to maintain biofilm and prevent washout of microorganisms) or an upflow anaerobic sludge blanket (USB) reactor (reactor containing a thick sludge layer that degrades waste flowing upward). The acido/acetogenic and methanogenic stages act as continuous reactors, which results in constant gas production (Chaudhary, 2008).
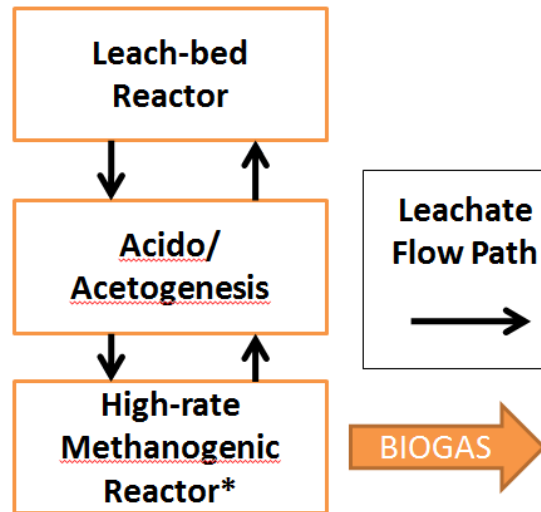
Figure 2.2 Multi-stage AD process
*High-rate methanogenic reactor may be fixed-film reactor or upflow anaerobic sludge
blanket reactor.

Although the leachate recirculation is highly desirable to conserve water, it also
creates challenges including build-up of ammonia (by-product of AD) and salinity in the
aqueous phase (Shahriari et al., 2012). While there is a great wealth of knowledge
concerning optimum operating conditions for methanogenesis (Chen et al., 2008),
currently there is little understanding of the impact of operating conditions on hydrolysis
rates. This knowledge will help guide reactor design by determining leachate recycling
ratios, and, consequently, the amount of additional fresh water and energy (e.g., heating
and pumping) required to dilute elevated ammonia and salinity concentrations. As a
result, reactor performance can be maximized in spite of the challenging conditions
created by elevated ammonia and salinity concentrations. Hydrolysis is a rate-limiting
step in the digestion of recalcitrant wastes such as lignocellulosic matter (Colberg, 1988;
Nielsen et al., 2004; Pavlostathis and Giraldo-Gomez, 1991), and thus, optimization of
this step has the potential to radically increase economic profitability of AD by lowering

required reactor volumes, decreasing operating costs, and increasing methane generation. Therefore, to render these systems more economically viable, additional research is needed to determine optimum operating conditions for hydrolysis.

2.5 Impact of Operating Conditions on Hydrolysis

2.5.1 Ammonia Inhibition

Inhibition of the overall AD process by ammonia is a common occurrence during the digestion of feedstocks with naturally high ammonia concentrations such as manure (Angelidaki and Ahring, 1993). Numerous studies have been conducted to determine the inhibitory levels of ammonia concentrations on methane production (Chen et al., 2003). Hulshoff Pol et al. (1982) reported a significant lag phase during the start-up of a USB reactor treating wastewater operating at 1 g NH_4-N/L as compared to USB reactors begun with 0.4 g NH_4-N/L or less. Van Velsen (1979) reported gradually increasing methanogenic inhibition to occur at concentrations from 0.72 to 4.95 g NH_4-N/L during the treatment of sewage sludge; however, methane production still occurred at concentrations of 4.96 g NH_4-N/L. Several studies have reported half maximal inhibitory concentration (IC_{50}) for methanogenesis to occur at ammonium nitrogen concentrations ranging from 4.0 to 12.8 g NH_4-N/L (Gallert and Winter, 1997; Sung and Liu, 2003). For example, Lay et al. (1998) determined the IC_{50} for methane production to occur from 4.09 − 5.55 g NH_4-N/L, and toxicity levels (point at which methanogenesis levels were not detectable) were reached at 5.88 to 6 g NH_4-N/L. Hendriksen and Ahring (1991) determined IC_{50} levels for methanogenesis to occur at 6 g NH_4-N/L, and complete inhibition was observed at 9.0 g NH_4-N/L.

Past research has shown that the free ammonia (FA) concentration (NH_3) in a system is responsible for the ammonia inhibition observed (Braun et al., 1981; De Baere et al., 1984; Angelidaki and Ahring, 1993). The concentration of FA is dependent on three main parameters: total ammonia concentration, pH, and temperature (Hansen et al., 1998). For a given total ammonia concentration, FA concentration increases with increasing pH values (Figure 2.3). As the temperature of a system increases, the fraction of total ammonia that is in the form of FA also increases (Hansen et al., 1998). Thus, thermophilic digesters have been shown in several studies to be much more sensitive to ammonia toxicity (Braun et al., 1981; Van Velsen, 1981; Parkin and Miller, 1983; Angelidaki and Ahring, 1994).
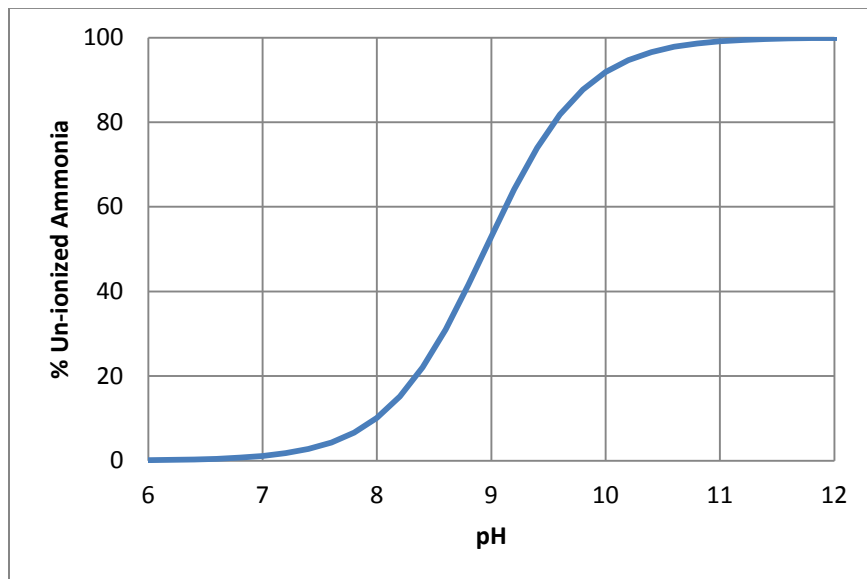
Figure 2.3 pKa table for ammonia

As a result, reactors operating at high pH values (e.g., 8) and thermophilic temperatures may experience up to ten times the FA concentrations regarded as inhibitory (Hansen et al., 1998; Angelidaki and Ahring, 1993). Since not all papers report specific operating

conditions such as temperature or pH (or do not maintain constant pH values), it is often difficult to directly compare literature values; this lack of knowledge is one explanation for the wide range of inhibitory values reported because the values of total ammonia vary greatly with slight operational changes. Furthermore, differences in microbial communities could cause reactor inhibition at different ammonia concentrations. However, it is clear from the literature that ammonia inhibition studies have focused on the effects to methanogenesis, and research is needed to investigate the inhibition levels specifically on hydrolysis.

2.5.2 Salinity Inhibition

The concentration of dissolved solids (e.g., potassium, magnesium, calcium, and sodium) in an AD system at high levels can inhibit microbial activity (Chen et al., 2003). Sodium is one of the most prevalent cations found in organic wastes (e.g., manure, certain foods) that can interfere with microbial metabolism at high concentrations (Kugelman and McCarty, 1964; Rinzema et al., 1988; Gourdon et al., 1989). Several studies have been conducted to determine optimal and maximum sodium levels for methanogens. Sodium concentrations ranging from 0.1-0.2 g/L were found to be favorable for the growth of mesophilic anaerobes (McCarty, 1964). Similarly, Kugelman and Chin (1971) determined 0.23 g sodium/L to be the optimum concentration for mesophilic aceticlastic methanogens. Fang et al. (2011) found that methanogenic activity was reduced by 50% at 11 g sodium/L in the anaerobic digestion of manure. Numerous additional studies have determined a 50% inhibition level to occur from 5.6-53 g sodium/L for methanogenesis (Chen et al., 2003; Liu and Boone, 1991; Soto et al., 1993; Vallero et al., 2003). Despite the extensive research done on methanogenesis, however,

little research has been conducted to determine the impact of salinity on hydrolysis rates. Additionally, causes of the wide range of inhibitory values are not fully understood but could be attributed to several variables, such as differences in microbial communities present in the inocula or length of acclimation periods. Thus, research is also needed to determine the effects of acclimation on hydrolysis.

2.6 Acclimation of Microbial Inocula

Acclimation, the process of adapting microorganisms and microbial communities to a given set of environmental conditions, has been reported to increase the activity (e.g., methane generation rates) of microorganisms in the presence of inhibitors (Chen et al., 2008). Koster and Lettinga (1984) determined that methanogenic sludge could adapt to gradually increasing concentrations of ammonium-nitrogen up to 1.7 g NH_4-N/L without causing a significant lag in methane production; sludge exposed to concentrations of 2.1 g NH_4-N/L immediately produced methane, but at lower rates. Hashimoto (1986) reported ammonia nitrogen inhibition on methanogenesis at 4 g/L for acclimated cultures as compared to 2.5 g/L for unacclimated cultures. Koster and Lettinga (1988) studied the anaerobic digestion of potato waste and observed methanogenic activity at total ammonia nitrogen (TAN) concentrations (NH_3-N + NH_4^+-N) as high as 11.8 g/L after adaptation, in contrast to observed methanogenic toxicity at 1.9 g/L before adaptation. Similarly, studies also have demonstrated improved reactor performance as a result of acclimation to high sodium concentrations (Chen et al., 2008). Mendez et al. (1995) reported an increase from 12.0 to 17.0 g sodium/L in the 90% inhibition level of methanogens from anaerobic sludge after an acclimation period of 719 days. Chen et al. (2003) reported

methanogenesis toxicity increased from 12.7 to 22.8 g sodium/L after acclimation to 4.1 and 12.0 g sodium/L, respectively.

However, this phenomenon of acclimation is not fully understood (Chenowyth et al., 1987). There are two possible mechanisms of adaptation: (1) changes within the predominant existing microorganisms (i.e., changes in activity due to enzyme regulation), and (2) microbial community population shifts due to selective growth of specific microorganisms (Fry and Day, 1992). Interestingly, acclimation of microorganisms (specifically, methanogens) to higher salinity concentrations has produced mixed results. Rinzema et al. (1988) was unable to adapt a pure culture of *Methanothrix* sp. to higher salinity concentrations of 14 g sodium/L after a 12 week period. This suggests that the *Methanothrix* sp. is not capable of adaptation via mechanism #1, and the *Methanothrix* sp. would not be selected for during the adaptation of a mixed community. Additionally, Vallero et al. (2002, 2003) reported that sodium tolerance levels of sulfate-reducing microorganisms cultured under thermophilic conditions could not be increased through gradual additions of sodium chloride to 9.8 g sodium/L. Thus, since the results of acclimation studies have demonstrated varying effects on performance, the process of acclimation as a technique for improving performance cannot be considered fully reliable at this time. Furthermore, since the acclimation strategies that maximize performance are not well documented, and methods of acclimation can vary widely (e.g., acclimation time, sudden or gradual chemical additions), indicators of acclimation performance (e.g., methane generation, microbial community response) need to be monitored to determine the most efficient means of acclimation.

2.7 Determining Hydrolysis Rates

The study of kinetics can provide a quantitative measure of hydrolysis behavior (Luo et al., 2012) and help guide the design and operation of reactors by determining the effects of operating conditions on hydrolysis. A wide range of different hydrolysis kinetic models have been successfully applied to AD systems, including first order models and modified first order models. Hydrolysis in AD systems has been traditionally modeled using a first-order kinetic rate via Equation 1 (Eastman and Ferguson, 1981):

$$\frac{dS}{dt} = -kS \qquad \text{Equation 1}$$

S is the volatile solids concentration, t is the time (days) and k is the first-order rate coefficient (day^{-1}). Thus, the cumulative processes taking place in hydrolysis, such as the different degradation rates of various particle sizes or compositions, are simplified into one first-order model (Eastman and Ferguson, 1981).

Hydrolysis, however, is often affected by the degradability of the substrate, especially with complex matter comprised of lignocellulosic materials (e.g., manure, components of food waste) (Vavilin et al., 2008). For example, research has reported the following organic fractions for various feedstocks: 82% (office paper, food waste), 72% (yard waste), and 22% (newsprint) (Kayhanian et al., 1991; Kayhanian and Tchobanoglous, 1992). When the biodegradability is less than 100%, it is important to modify Equation 1 to include the substrate biodegradability, which can be determined by batch studies. The first-order kinetic rate model including a biodegradability factor is as follows:

$$\frac{dS}{dt} = -k(S - \beta S_0) \hspace{3cm} \text{Equation 2}$$

β is the non-degradable fraction of the substrate, and S_0 is the initial substrate concentration.

Additionally, recent studies have suggested that hydrolysis rates also are affected by high inoculum-to-feedstock ratios (Fernandez et al., 2001). As a result, Equation 2 may be modified to account for biomass concentration or activity:

$$\frac{dS}{dt} = -kX^n(S - \beta S_0) \hspace{3cm} \text{Equation 3}$$

X is the hydrolytic biomass concentration and n is a power index. For example, Sanders (2001) demonstrated different hydrolysis rates for the degradation of gelatin with different initial sludge concentrations using Equation 3. Thus, it is necessary to use Equation 3 to normalize hydrolysis rates when AD systems are limited by microorganism concentrations. However, if the system is saturated with biomass and additional inoculum does not improve rates, Equations 1 or 2 may be used to calculate hydrolysis rates. Additional variables, such as particle diameter (e.g., surface area limits rate) or shape, may also be included in kinetic models to allow for individual experiment optimization.

2.8 Molecular Biology Tools Useful to Engineers

Traditionally, engineers have relied upon macroscopic measurements (e.g., chemical oxygen demand (COD), VFA concentration) of the reactor influent and effluent as indicators of reactor performance, but this provides limited understanding of the microbial communities that mediate the process. Now, advanced molecular biology tools are able to provide detailed knowledge of microbial composition *inside* the reactors (Talbot et al., 2008). Anaerobic digesters contain complex microbial consortia (Riviere et

al., 2009), and it has been documented that microbial community composition can have notable impacts on reactor performance (McHugh et al., 2004; Carballa and Smits, 2011). For instance, several studies theorize that shifts in composition or quantity of microbial archaeal and bacterial communities can signal reactor instabilities (e.g., overload of organic input waste) and allow for preventative measures to be taken (McHugh et al., 2004; Lee et al., 2008).

Molecular biology tools can be used to establish correlations between the quantity of specific types of microorganisms present in AD systems and reactor stability and performance (Carballa and Smits, 2011). Although the majority of studies have focused on targeting the 16S rRNA gene to determine phylogenetic profiles of the microbial communities present in the reactors (Bouallagui et al., 2004; Sasaki et al., 2007; Chachkhiani et al., 2004), these tools provide limited information because phylogenetically related organisms displaying different functional capabilities cannot be readily distinguished. Tools targeting functional genes (e.g., genes involved in hydrolysis, acido/acetogenesis, or methanogenesis) provide more detailed information with respect to the quantities of microorganisms with specific functional capabilities (e.g., cellulose-hydrolyzing bacteria and methanogens). Quantitative polymerase chain reaction (qPCR) is a molecular technique that amplifies and quantifies a targeted gene (Hurst et al., 2007). qPCR targeting the 16S rRNA gene and *mcrA* gene (which encodes the alpha subunit of methyl-coenzyme M reductase) has been used successfully to characterize and quantify microorganisms in digesters. Conklin et al. (2006) utilized qPCR to determine if microbial community dominance of *Methanosarcina* leads to more stable digestion in response to engineered feeding frequencies. Traversi et al. (2011) used

qPCR to demonstrate the importance of *Methanosarcina* as bioindicators of reactor performance. Song et al. (2010) quantified methanogenic groups via the 16S rRNA gene to more accurately estimate methane production and biokinetic parameters to guide AD design and operation. Westerholm et al. (2011) used qPCR to conclude that the quantity of acetogens in an anaerobic digester was not affected by high ammonia levels (6.9 g NH_4-N/L). This suggests that the acido/acetogenesis process in a multi-stage system could be successfully operated at high ammonia levels, thus rendering the process more economically viable. However, very few studies have used qPCR to quantify cellulose-degrading bacteria in anaerobic systems to further optimize the hydrolysis process. Pereyra et al. (2010) recently developed a qPCR assay targeting the *cel5* and *cel48* families of cellulose-degrading bacteria for use in characterizing the microbial communities in sulfate-reducing bioreactors; however, these assays have not been applied to anaerobic digesters.

In addition to quantification, the identification of specific microorganisms present in reactors can help predict or improve reactor performance (e.g., bioaugment reactors with bacteria known to perform well under specific reactor conditions). Terminal restriction fragment length polymorphism (T-RFLP) is a rapid, low-cost molecular tool for microbial community profiling that can be used to track changes in microbial communities in response to engineered variables (e.g., feedstock, nutrient concentrations). For example, Collins et al. (2003) utilized T-RFLP targeting Archaea and Bacteria as a biomonitoring tool to demonstrate the stability of the archaeal communities and relative diversity of bacterial communities during the start-up of a psychrophilic anaerobic digester. Wang et al. (2009) reported community structure

differences in co-digesters depending upon the feedstock or organic loading rate; this knowledge suggests that reactors receiving different seasonal waste input could require an acclimation time to process the new waste.

However, the identification of hydrolytic organisms that are tolerant to high ammonia and salinity concentrations remains to be elucidated. Furthermore, assays that target functional genes (e.g., genes involved in cellulose degradation) are still very limited and only target specific families (e.g., *cel48*) of hydrolyzing genes.

2.9 Summary

Although AD is an ideal technology for the treatment of manure and OFMSW, feedstock-specific challenges to digestion (e.g., high solids) have prevented widespread implementation of AD systems. An appropriate multi-stage technology was proposed in this thesis that incorporates a leachate recycle to conserve water and reduce heating and pumping requirements. However, this recycle leads to an increase of ammonia and salinity concentrations, and currently there is a lack of knowledge concerning the effects of these specific operating conditions (e.g., ammonia and salinity concentrations) on hydrolysis efficiency. Increased understanding of reactor response and efficiency from these conditions will help guide reactor design and operation, ultimately maximizing economic benefit.

3.0 Anaerobic Digestion of Manure: The Impact of Process Conditions on

Hydrolysis Efficiency and Microbial Community Composition

3.1 Introduction

Livestock manure generates over one billion tons of waste annually in the United States (Labatut et al., 2011). In 2010, methane emissions from livestock manure management (e.g., lagoons) accounted for approximately 8% of total U.S. anthropogenic methane emissions (US EPA, 2010). Because methane has a Global Warming Potential of 21 times carbon dioxide (Gloy, 2011), the need to reduce methane emissions is paramount. Furthermore, conventional methods of manure management (e.g., land application and lagoons) contribute to groundwater and surface water contamination (Wen et al., 2005; US EPA, 2011; Burkholder et al., 2007). As environmental regulations are becoming increasingly strict (such as requiring discharge permits for concentrated animal feeding operations (US EPA, 2008)), the development of efficient, environmentally-conscious methods of manure disposal is needed (Wen et al., 2004).

The AD of manure represents an environmentally sustainable technology for manure management that offers several advantages over conventional methods, including solids reduction, odor control, potential for nutrient recovery through post-AD composting, and biogas production for energy generation (Demirer and Chen, 2005a; Holm-Nielsen et al., 2009). While conventional wet AD reactor designs are able to effectively treat manure collected as a slurry (e.g., via wet-scraping), these systems operate at a maximum solids content of 10-16% (Ward et al., 2008; Demirer and Chen, 2005b), and regional climatic factors (i.e., arid conditions and freezing temperatures) often necessitate dry-scraping of manure or infrequent manure collection, which typically

leads to greater than 25% solids content (Hall et al., 1985). Dilution of these wastes is either impractical or unfeasible. Thus, conventional AD reactor designs are not suitable for application to manure in arid regions, and alternative designs are needed.

Multi-stage AD systems that utilize leachate-bed hydrolysis reactors are a promising technology that can convert high-solids wastes to biogas (Yang et al., 2003; Shin et al., 2010; Bouallagui et al., 2004). In these systems, leachate is trickled over the high-solids waste to promote hydrolysis. The leachate, which contains hydrolyzed waste in the form of VFAs, can be transferred to an acido/acetogenesis storage tank and subsequently passed to a high-rate methanogenesis reactor (e.g., fixed-film reactor) where biogas is produced. Physical separation of the processes (i.e., hydrolysis, acido/acteogenesis, and methanogenesis) results in a more stable and robust system that is resistant to reactor perturbations such as leachate acid spikes. Furthermore, each process can be optimized independently to maximize the activity of the specific microbes that mediate each process (Song et al., 2004; Ince, 1998; Demirer and Chen, 2005b; RIS International, 2005). Additionally, leachate can be recycled through the system to conserve water and reduce heating and pumping costs. However, one drawback of this approach is that leachate recycle leads to an increase in the aqueous phase concentrations of ammonia (by-product of AD) and salinity, which are originally present in the manure.

High ammonia and salinity concentrations have been shown to negatively impact methanogenesis and overall process performance (Kayhanian, 1994; Kugelman and McCarty, 1964). Although nitrogen is an essential nutrient for microorganisms and low concentrations of ammonia (less than 0.2 g/L) are beneficial to the digestion process (Liu and Sung, 2002; Chen et al., 2003), TAN concentrations ranging from 1.7 to 14 g TAN/L

have been shown to inhibit methane production by 50% (Sung and Liu, 2003; Bujoczek et al., 2000; Chen et al., 2008). Sodium is also a required nutrient for growth of microorganisms, and optimal sodium concentrations ranging from 0.1 to 0.23 g/L for mesophilic anaerobes have been reported (McCarty, 1964; Kugelman and Chin, 1971). However, higher sodium concentrations have been shown to inhibit methanogenesis; half maximal inhibitory concentration (IC_{50}) values of 5.6 to 53 g/L of sodium have been documented (Feijoo et al., 1995; Chen et al., 2003; Omil et al., 1995; Vallero et al., 2002; Liu and Boone, 1991; Soto et al., 1993; Vallero et al., 2003). The reasons for the discrepancies in reported IC_{50} values are unknown, but, for the studies reported in the literature, reactor configurations, operating conditions, and microbial community adaptation periods varied. As past research has focused on optimization of methanogenesis or the overall performance of single-stage systems, the impact of ammonia and salinity concentrations on hydrolysis rates is not well understood.

The knowledge of the impact of ammonia and salinity concentrations on digester performance is needed to guide reactor design to determine optimal leachate recycling ratios, and, consequently, the amount of additional fresh water and energy (e.g., heating and pumping) required. Furthermore, strategies are needed to improve process performance in the presence of elevated ammonia and salinity concentrations (e.g., develop improved microbial seeds) to improve the economic viability of AD in arid regions. Hydrolysis is a rate-limiting step in the digestion of recalcitrant wastes such as lignocellulosic matter (Colberg, 1988; Nielsen et al., 2004; Pavlostathis and Giraldo-Gomez, 1991), and thus, optimization of this step has the potential to radically increase

economic profitability of AD by lowering required reactor volumes, decreasing operating costs, and increasing methane generation.

Therefore, the objective of this study was to determine the effects of operating conditions (e.g., ammonia and salinity concentrations) on hydrolysis rates, as well as identify characteristics of microbial communities that can effectively hydrolyze manure under conditions of high ammonia and salinity concentrations.

3.2 Methods

3.2.1 Manure and Inoculum Collection

Manure was collected from Five Rivers Cattle Feeding LLC in Greeley, CO. The manure was ground and homogenized using a food blender (Hamilton Beach, Southern Pines, NC) and then sifted through a 0.5 mm mesh sieve. The ground manure was stored at 4°C for subsequent use. Microbial inoculum for batch reactor tests was collected from the Drake Municipal Wastewater Treatment Plant (DWWTP) mesophilic digester (Fort Collins, CO). The inoculum was purged with nitrogen gas and maintained at 35°C after collection. Manure and inoculum were analyzed for total and volatile solids (TS and VS, respectively) content, and total and dissolved chemical oxygen demand (TCOD and DCOD, respectively) prior to each experimental set-up as described in Section 3.2.4. The same initial manure and inoculum were used for every batch test to minimize compositional differences due to different collection periods. Initial characteristics of the inocula and substrate are shown in Table 3.1.

Table 3.1 Characteristics of inocula and substrate

	Parameter			
	TCOD	DCOD	% TS	% VS
DWWTP Inocula	25.6 g COD/L	0.36 g COD/L	1.8	1.3
Manure	0.82 g COD/g substrate	--	71.2	41.3

3.2.2 Hydrolysis Batch Reactor Set-up

140-ml luer lock plastic syringes (Sherwood Medical, Northern Ireland) fitted with three-way luer lock valves (Cole Parmer, Vernon Hills, IL) were used as small-scale batch reactors to allow for biogas collection at constant pressure and anaerobic sampling (Figure 3.1). Digestion tests were conducted with 60 ml nutrient solution (Owen et al., 1978) (Appendix B) and 60 ml acclimated inoculum (Section 3.2.3). Manure was supplied at an initial concentration of 5 g COD/L. Ammonia (supplied as NH_4Cl) and salinity (supplied as NaCl) were added to the nutrient solution to produce nutrient solutions with high levels of ammonia (1, 2.5, and 5 g TAN/L) or salinity (3.9, 7.9, and 11.8 g sodium/L). Digestion tests were also conducted for "baseline" ammonia and salinity concentrations (0.14 g TAN/L and 1.2 g sodium/L, respectively) that were originally present in the nutrient solution. Syringes without manure were run as controls to measure gas production from the inoculum alone, and syringes with inoculum fed glucose as the sole carbon source were run to ensure that methanogenesis was not rate-limiting (Appendix C). Syringes were incubated in an incubator shaker (100 rpm) at 35°C. All tests were performed in triplicate. Hydrolysis reaction periods lasted approximately 10 to 15 days.

Figure 3.1 Batch reactor for determining hydrolysis kinetic rates

3.2.3 Acclimation of Microbial Inocula

Initial tests demonstrated the need for acclimation of the microbial inocula to the elevated ammonia and salinity concentrations tested (Appendix D). Thus, 1-L glass flask batch reactors were set up to acclimate microbial seeds to each of the ammonia and salinity concentrations listed above. Inoculum (200 ml) from DWWTP and nutrient solution (800 ml) were mixed and adjusted to the appropriate amount of ammonia (1, 2.5, and 5 g TAN/L) or sodium (3.9, 7.9, and 11.8 g sodium/L), and the pH was adjusted to approximately 7.1. All batch reactors were fed 5 g manure every two weeks, and 500 ml of the reactor contents were removed and replaced with 500 ml of fresh nutrient solution once a month to prevent buildup of inhibitory compounds, such as ammonia or hydrogen sulfide. Hydrolysis rates were determined for each culture after four months of acclimation. Since the baseline ammonia and salinity concentrations present in the

nutrient solution were not considered to be elevated, inocula subjected to 0.14 g TAN/L or 1.2 g sodium/L were not acclimated to the testing conditions prior to kinetic rate testing.

3.2.4 Analytical Methods

The volume of biogas produced during the reaction period was measured by determining the distance the syringe plunger moved, and gas volumes measured at 35ºC were converted to gas volumes at standard temperature and pressure using the Ideal Gas Law (Bettelheim et al., 2009). Gas samples were analyzed at room temperature via gas chromatography for methane composition using a Hewlett Packard Series 2180 gas chromatograph equipped with an Alltech column packed with HayeSep Q 80/100 mesh operating at injection and detector temperatures of 100°C. TS and VS of the inocula and substrate were measured according to the procedures documented in *Standard Methods for the Examination of Water and Wastewater* (APHA, 1995). Liquid samples were collected and analyzed for TCOD and DCOD using Hach's COD High Range Vials and digestion colorimetric method according to the manufacturer's instructions (Hach, Loveland, CO). Samples were filtered through a 0.2 μm syringe filter to remove particulate COD prior for DCOD analysis. To monitor the concentration of ammonia throughout the acclimation period and ensure that there was not a notable increase in the targeted concentrations (1-5 g TAN/L) resulting from a small volume of added manure in the flasks, Hach's Nitrogen-Ammonia High Range Reagent Set was utilized according to the manufacturer's instructions. Ammonia concentrations did not vary largely due to amounts contributed from the manure; thus, it was concluded that additional salt concentrations inherent in the manure were also negligible and were not factored into the

amount of sodium added to the nutrient solution. Free ammonia concentrations were calculated as a function of pH, temperature, and total ammonia nitrogen concentrations.

3.2.5 Calculation of Hydrolysis Rates

Hydrolysis rates were estimated in each batch reactor by isolating the hydrolysis step from methanogenesis. To determine hydrolysis kinetic rates, each immediate hydrolysis product (e.g., soluble products) and downstream products that already underwent acido/acetogenesis (e.g., methane) were included to account for all solubilized material in the system. Thus, hydrolysis kinetic rates were calculated for each batch test as follows. All of the hydrolysis products (immediate and downstream) were converted to COD equivalents, summed for each time point and divided by the initial COD input to the system to yield the extent of substrate solubilization using Equation 1 as described previously (O'Sullivan et al., 2008):

$$\text{Extent of Solubilization} = \frac{COD_{soluble} + COD_{CH_4} + COD_{biomass}}{COD_{initial}} \quad \text{(Equation 1)}$$

Each reaction period lasted approximately 10-14 days, and it was concluded that any new biomass formed in the system was negligible in comparison to the COD from soluble compounds and methane formation; thus, this term was excluded from Equation 1. To determine the rate of hydrolysis, a first-order kinetic model was applied to the data according to the following equation:

$$\frac{dS}{dt} = -k(S - \beta S_0) \quad \text{(Equation 2)}$$

S is the substrate concentration (1-Extent of Solubilization), t is the time (days), k is the first-order hydrolysis rate constant (day^{-1}), β is the non-degradable fraction of the substrate, and S_0 is the initial substrate concentration (Vavilin et al., 2008). β was

determined through long-term batch digestion tests. Kinetic rates were calculated for each individual reactor and then averaged over the triplicate reactors to estimate hydrolysis rates for each operating condition. Attempts to modify Equation 2 by including a biomass factor are described in Appendix E, but it was concluded that the methods of biomass measurement utilized were non-representative of actual biomass in the system.

3.2.6 Microbial Community Composition Analysis

To track changes in the microbial community composition as a function of each operating condition, functional gene-based terminal restriction length polymorphism (T-RFLP) analyses were conducted on DNA isolated from each acclimated and un-acclimated culture. At the end of each inocula acclimation period (4 months), DNA was extracted from culture samples using the MoBio PowerSoil DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the protocol and stored at -20°C. In an attempt to track changes to the hydrolyzing microbial communities, extracted DNA was subjected to PCR amplification of the *cel5* and *cel48* genes (genes encoding glycoside hydrolases of families 5 and 48) as described previously (Pereya et al., 2010; Lefevre, 2011). Despite repeated efforts utilizing DNA samples from a variety of reactor conditions (e.g., varying ammonia, salinity, and feedstock compositions), it was not possible to amplify *cel* genes present in the microbial communities examined in the present study using existing primers; thus, T-RFLP analysis was not possible. To track changes to the methane-producing microbial communities, T-RFLP analysis was conducted targeting *mcrA* (gene encoding the alpha subunit of methyl-coenzyme M reductase). Briefly, DNA was PCR-amplified using *mcrA* primers developed by Luton et al. (2002). The forward primer was labeled on the 5' end with 6-carboxylfluorescein

dipivalate-6-aminohexyl amidite (6-FAM) dye. The amplifications were performed using a Bio-Rad S1000 Thermocycler (Bio-Rad Laboratories, Hercules, CA). Reactions consisted of 1X Reaction Buffer (5 Prime, Gaithersburg, MD), 1X PCR Enhancer (5 Prime), 1 mM Mg(OAc)$_2$, 0.05 mM each dNTP (New England Biolabs, Ipswich, MA), 0.5 µl of formamide, 0.2 µM of each primer, 7 U Taq polymerase (5 Prime), 2 µl DNA template, and nuclease-free water to a final volume of 50 µl. The thermocycling program was as follows: 3 min at 95ºC, followed by 40 cycles of 40 s at 95ºC, 30 s at 56ºC, and 30 s at 68ºC, and a final extension of 7 min at 68ºC. Amplicons were treated with Klenow fragment as follows: 50 µl of PCR product was incubated with 2.5 U of Klenow fragment (New England Biolabs), 5.7 µl of 10X Buffer N2 (New England Biolabs), and 0.3 µl of 10mM each dNTP for 1 hr at 20ºC. The products were then purified using NucleoSpin Extract II (Macherey-Nagel, Bethlehem, PA) according to the manufacturer's instructions and re-suspended in 35 µl of elution buffer. To gel purify correctly sized amplicons, 35 µl of the products were run on a 1% agarose gel; the bands were extracted using the NucleoSpin Extract II kit (Macherey-Nagel) according to the manufacturer's instructions and suspended in 50 µl nuclease-free water. The purified amplicons were digested with 10 U each of restriction enzymes MspI and HaeIII (New England Biolabs) and 1X Buffer N4 (New England Biolabs) for 12 hr at 37ºC. The digestion products were cleaned-up using the QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany), re-suspended in 50 µl elution buffer, and stored at -20 ºC. Products were submitted to the Colorado State University Proteomics and Metabolomics Facility for T-RFLP analysis.

3.2.7 Microbial Community Composition Statistical Analysis

T-RFLP data was analyzed via non-metric multi-dimensional scaling (NMDS) to identify and characterize microbial community changes. Estimated fractional abundances for each operational taxonomic unit (OTU) were calculated by dividing each fragment peak area by the total peak for a given electropherogram. Terminal restriction fragments (T-RFs) representing less than 5% of the total area were excluded to focus on the dominant members of the community and eliminate possible "background" T-RFs caused by the amount of labeled DNA loaded onto the separation gels (Sait et al., 2003; Rees et al., 2004). Then, T-RFLP data was analyzed using the statistical software Primer v6 (Primer-E Ltd, Plymouth, United Kingdom). The similarity matrices were calculated according to the Bray-Curtis coefficient:

$$S_{jk} = 100 \left\{ 1 - \frac{\sum_{i=1}^{p} |y_{ij} - y_{ik}|}{\sum_{i=1}^{p} (y_{ij} + y_{ik})} \right\}$$

S_{jk} represents the similarity between the jth and kth samples and y_{ij} represents the data in the ith row and jth column (Clarke, 1993). NMDS plots were generated using 100 restarts and plots with stress values greater than 0.2 were omitted (Clark, 1993).

3.3 Results and Discussion

3.3.1 Hydrolysis Rate Determination

Regression analyses of the hydrolysis data according to Equation 2 are presented in Figures 3.2 and 3.3 as a function of the ammonia and salinity concentrations (1 - 5 g TAN/L and 3.9 - 11.8 g sodium/L), respectively.
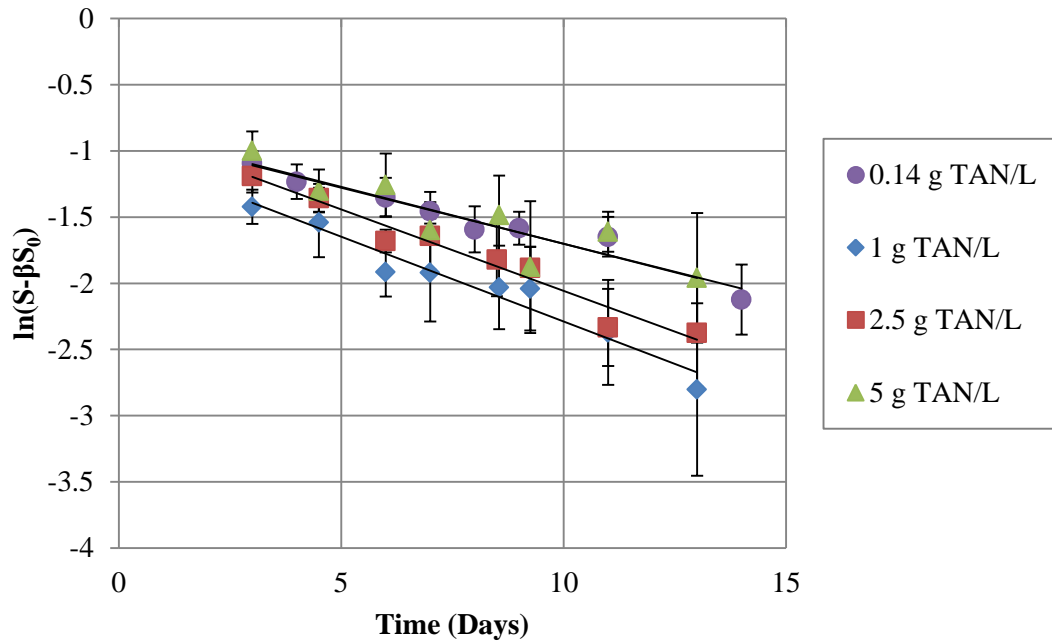
Figure 3.2 Regression analyses for a range of ammonia concentrations (β = 0.49)
Error bars represent standard deviations for triplicate reactors.
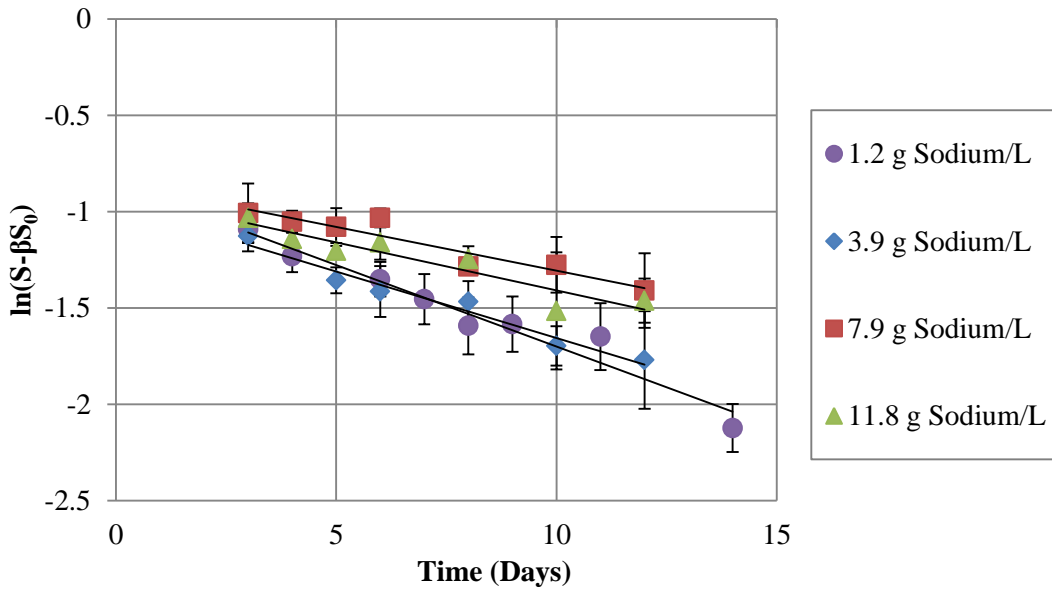
Figure 3.3 Regression analyses for a range of salinity concentrations (β = 0.49)
Error bars represent standard deviations for triplicate reactors.

The hydrolysis rate constants determined from the slopes calculated via regression analysis are shown in Table 3.2. Calculated coefficients of determination (R^2) indicate that the data fit the first-order hydrolysis kinetic model used (Table 3.2).

Table 3.2 Kinetic rate values according to regression analyses

Reactor*	Kinetic rate, k (day^{-1})*	R^2
0.14 g TAN/L (0.0021 g NH_3-N)	0.089 (0.009)	0.95
1 g TAN/L (0.015 g NH_3-N)	0.128 (0.061)	0.95
2.5 g TAN/L (0.038 g NH_3-N)	0.123 (0.029)	0.96
5 g TAN/L (0.075 g NH_3-N)	0.086 (0.017)	0.80
1.2 g Sodium/L	0.089 (0.009)	0.95
3.9 g Sodium/L	0.069 (0.013)	0.96
7.9 g Sodium/L	0.045 (0.010)	0.90
11.8 g Sodium/L	0.050 (0.018)	0.87

*In the Reactor column, numbers in parentheses indicate free ammonia concentrations. In the Kinetic rate column, numbers in parentheses indicate standard deviations for triplicate reactors.

The hydrolysis rates calculated for cattle manure in this study (0.045-0.128 day^{-1}) are similar to rates documented in literature (Vavilin et al., 1997; Vavilin et al., 2008). Observed hydrolysis rates can vary widely depending on substrate composition, experimental conditions (e.g., temperature), and biomass to substrate ratios (Vavilin et al., 2008) making direct comparison difficult; however, previously reported first-order rates for cellulose, a major component of manure, range from 0.04-0.13 day^{-1} (Gujer and Zender, 1983; Liebetrau et al., 2004; O'Sullivan et al., 2008). Other studies have documented hydrolysis rates for livestock waste in the same range; first-order kinetic rates of 0.1 day^{-1} and 0.13 day^{-1} have been reported for pig manure and cattle manure, respectively (Vavilin et al., 1997; Vavilin et al., 2008). Crops and crop residue, which

also contain cellulose, have demonstrated hydrolysis rates ranging from 0.009-0.094 day^{-1} (Lehtomaki et al., 2005; Tong et al., 1990).

Despite an extensive amount of research conducted on the impact of ammonia and salinity concentrations on methanogenesis (Vallero et al., 2002; Chen et al., 2008), the effect of these concentrations on hydrolysis has been largely understudied (Lim et al., 2008). In one of the only studies to look specifically at the effects of sodium on hydrolysis, Lim et al. (2008) reported that increasing amounts of salinity up to 35 g/L had no significant effect on the overall degree of hydrolysis of blue mussels. Hansen et al. (1998) determined the effects of ammonia up to 6 g TAN/L on methanogenesis, but also drew the conclusion that the activity of hydrolytic bacteria in the system remained constant and thus hydrolysis was not impacted. However, the seed used in this study came from an established lab reactor digesting cattle manure at a concentration of 3 g TAN/L, thus, the seed was partially acclimated to elevated ammonia concentrations. Additionally, these studies do not attempt to quantify the *rates* of hydrolysis for a range of ammonia and sodium concentrations in order to provide reactor operation guidance to maximize economic profit, such as leachate recycling ratios. Thus, the research in this study is novel because quantitative measures of the impact of ammonia and salinity concentrations were determined, and the effect of acclimation to elevated concentration was shown to be necessary to prevent reactor failure.

As a result of limited research in this area, it is not possible to directly compare rates in this study to other rates reported in the literature determined under similar operating conditions. In this study, an analysis of variance (ANOVA) test indicated that there were no statistically significant differences (p-value = 0.56) between the kinetic rate

constants for the range of ammonia concentrations likely because the acclimation period allowed the microbial inocula to adapt to the conditions tested. However, calculated hydrolysis rates were faster at 1 g TAN/L (0.015 g NH_3-N) and 2.5 g TAN/L (0.0375 g NH_3-N) than at 5 TAN g/L (0.075 g NH_3-N) suggesting that even after an acclimation period of 4 months, elevated ammonia concentrations might have a moderate inhibitory affect. For example, estimated methane generation from a day's production of manure at a 3,000 cattle feedlot in Colorado ranges from 57,940 ft^3 methane to 52,050 ft^3 methane for 1 – 5 g TAN/L, respectively, over a 20 day digestion period. Interestingly, the lowest TAN concentration (0.14 g TAN/L) demonstrated a slightly slower kinetic rate than was observed for 1 g TAN/L or 2.5 g TAN/L. A possible explanation for this slower rate could be that the inoculum used in this test was anaerobic digester sludge that was not acclimated to the testing conditions because this ammonia concentration (0.14 g TAN/L) was not considered to be elevated. However, lack of acclimation to the nutrient solution and feedstock used (e.g., manure) may have led to the reduced hydrolysis rate observed. Further experiments would be required to validate this hypothesis, but the results of this study suggest that acclimation to reactor conditions is critical for achieving optimal performance.

Interestingly, in contrast to the results obtained for hydrolysis rates for a range ammonia concentrations, an ANOVA test revealed statistical significance in the differences for hydrolysis rates obtained for a range of salinity concentrations (1.2 - 11.8 g sodium/L) (p-value = 0.013). Thus, it is suggested that slight hydrolysis inhibition occurs at even low sodium levels (3.9 g sodium/L). However, an ANOVA test conducted on the hydrolysis rates for the three acclimated inocula to elevated salinity concentrations

(3.9, 7.9, and 11.8 g sodium/L) revealed that differences in the kinetic rates were not statistically significant (p-value = 0.17), but gradual decreases in hydrolysis rates with increasing salinity concentrations were observed. Therefore, it is suggested that moderate inhibition on hydrolysis occurs with increasing salinity concentrations. As demonstrated for ammonia, the slight effects of hydrolysis inhibition are depicted as estimated methane production for a 3,000 cattle feedlot ranges from 49,850 to 43,270 ft^3 of methane for 3.9-11.8 g sodium/L, respectively. As was observed for elevated ammonia conditions, acclimation appears to be critical for achieving optimal hydrolysis rates; however, a trade-off still exists between maximizing performance and expending resources to maintain reduced salinity levels

3.3.2 Microbial Community Composition Analyses

Functional-gene based T-RFLP analyses were conducted to determine if changes to the microbial community composition of the inocula occurred as a result of acclimation. It was found that available assays targeting genes encoding enzymes involved in hydrolysis (e.g., *cel* genes) were not suitable for the microbial communities investigated in this study, and thus, the analysis of microbial communities reported is based on *mcrA*-targeted T-RFLP. Future work is required to develop broadly applicable functional-gene based assays for hydrolyzing microbial communities and to characterize changes to hydrolyzing microbial communities as a function of acclimation. Via *mcrA*-targeted T-RFLP, the acclimated and un-acclimated microbial inocula investigated in the present study were found to contain between 1 and 5 major T-RFs (Figure 3.4).
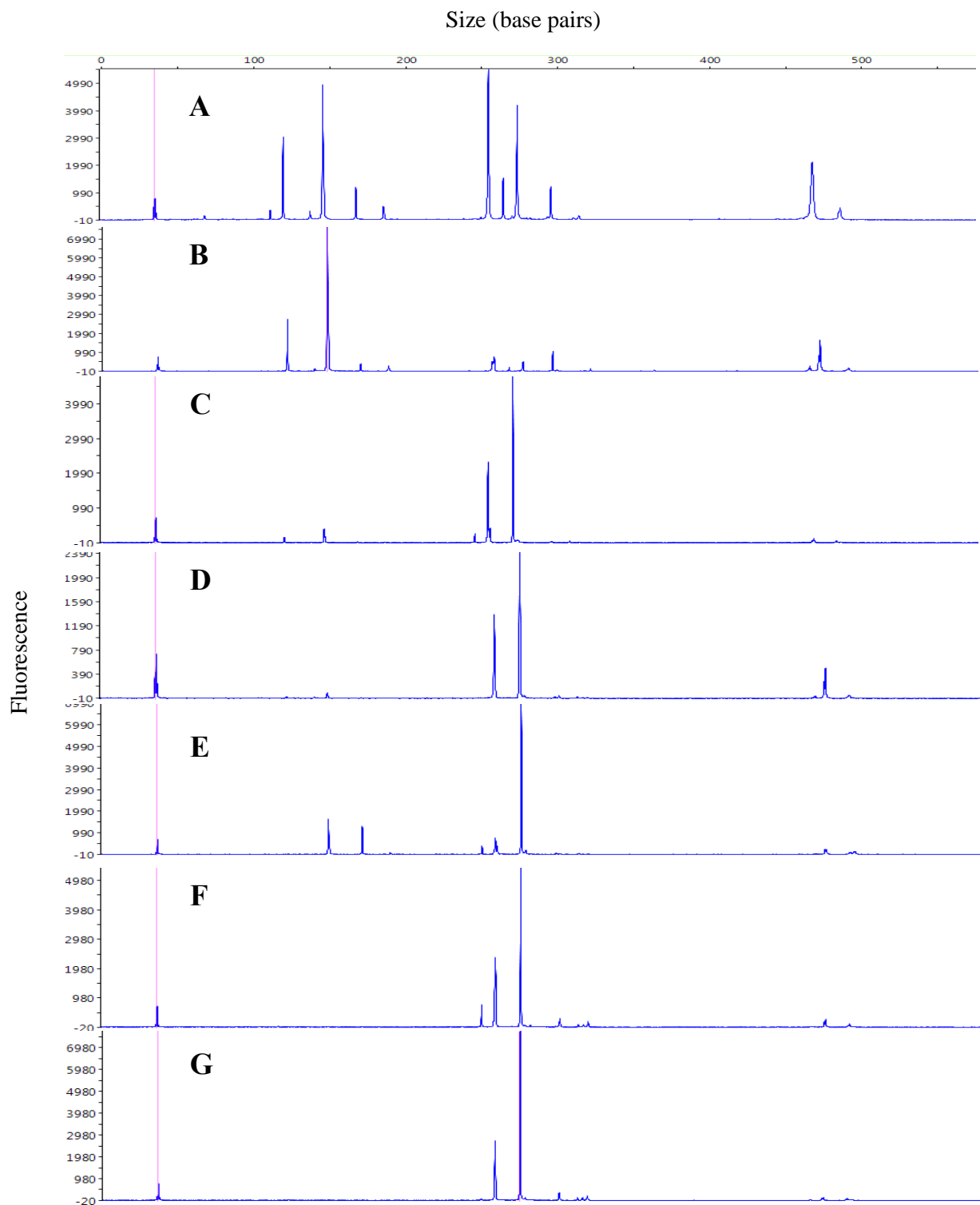
Figure 3.4 Electropherograms illustrating *mcrA*-targeted peaks
Original inocula (A), Inocula acclimated to 1 g TAN/L (B), 2.5 g TAN/L (C), 5 g TAN/L (D), 3.9 g sodium/L (E), 7.9 g sodium/L (F), 11.8 g sodium/L (G).

NMDS plots provide a means of visualizing T-RFLP data where each point represents a distinct microbial community. Distance between points reflects relative dissimilarity of microbial community composition (Clarke, 1993), and the orientation of axes is arbitrary. A NMDS plot of the data shows that post-acclimation communities were distinct from the original inocula indicating that microbial community shifts occurred, as opposed to changes solely to the *activity* of the microbial community present in the original inoculum source (DWWTP sludge), due to acclimation for inocula subjected to both elevated ammonia and elevated salinity concentrations (Figure 3.5).
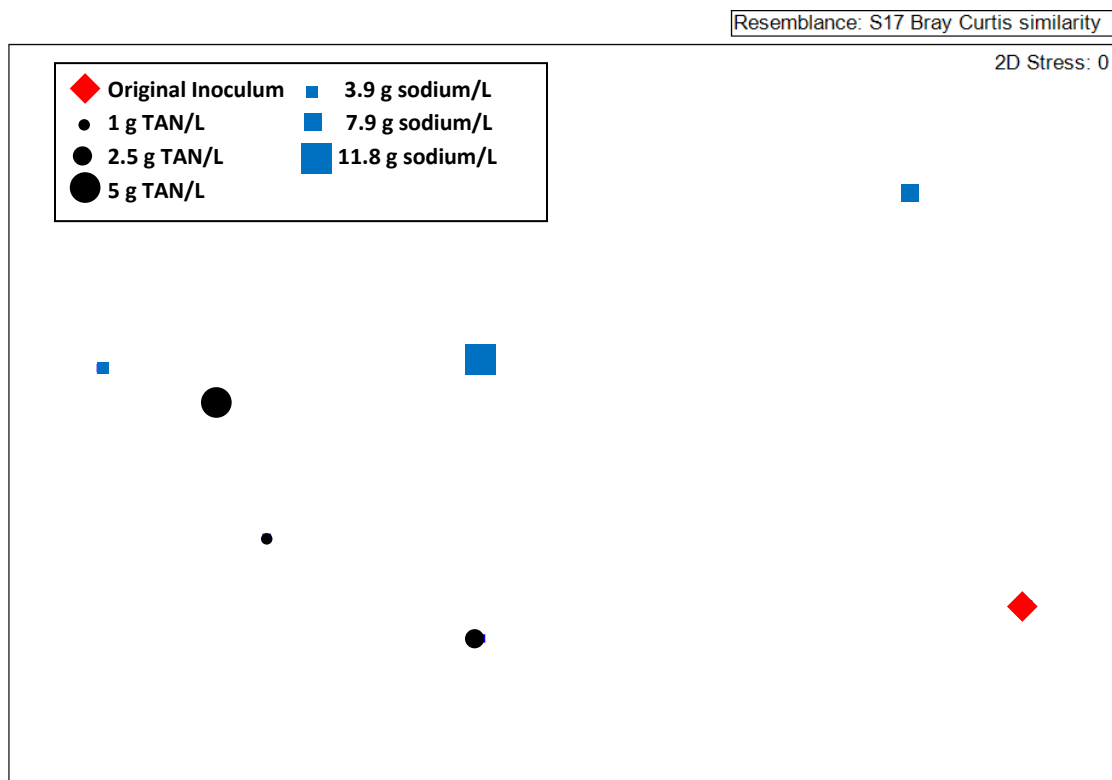
Figure 3.5 Multidimensional scaling plot based on *mcrA*-targeted T-RFLP

Interestingly, the majority of post-acclimation inocula (1 - 5 g TAN/L and 3.9, 11.8 g sodium/L) were located in the same proximity, suggesting that similar organisms were able to function under the range of conditions investigated (with the exception of

the inoculum acclimated to 7.9 g sodium/L). The reason for the divergence in the community structure of the inoculum acclimated to 7.9 g sodium/L is unknown. Given that inocula subjected to both elevated ammonia and salinity concentrations were located fairly close together, acclimation could have selected for general tolerance to extreme conditions or tolerance to ammonia and salinity could be present in similar microorganisms. Alternatively, although the observed microbial community shifts are consistent with the improved hydrolysis rates observed after acclimation, the reason for microbial community changes could be attributed to variables aside from the ammonia and salinity concentrations including nutrient solution components and/or the feedstock provided (manure). Thus, current work is underway to identify the underlying causes of the microbial community composition changes and to extend findings to the hydrolyzing and aceto/acidogenic microbial communities (e.g., via *cel*-targeted, *hydA*-targeted and 16S rRNA-gene targeted assays).

3.4 Conclusions

The results of this study suggest that hydrolysis efficiency decreases with increased ammonia and salinity concentrations (although decreased rates were not statistically significant). Additionally, it was determined that acclimation of microbial inocula to elevated concentrations of ammonia and salinity was necessary to prevent reactor failure and achieve optimal hydrolysis rates. Thus, determination of the microbial community changes that lead to tolerance to elevated levels of ammonia and salinity can help guide reactor inoculation and operation for systems with leachate recycle (or feedstocks that have naturally high concentrations of ammonia and salinity). Detailed case-specific economic analyses are needed to characterize the tradeoff between water

and energy requirements required to maintain low ammonia and salinity concentrations and increased process performance. Preliminary estimations demonstrate slight energy savings (approximately $170 and $190 for reactors operating at 1 g TAN/L and 3.9 g sodium/L compared to 5 g TAN/L and 11.8 g sodium/L, respectively, for the feedlot scenario previously described in Section 3.3.1) from increased process performance at decreased ammonia and salinity concentrations, although current estimations do not factor the increased water and energy consumption required to dilute elevated concentrations into cost comparison. However, the results of this study have demonstrated that the impact of this resource consumption/ process performance tradeoff can be minimized via acclimating microbial inocula to specific reactor operating conditions.

4.0 Anaerobic Digestion of Food Waste: The Impact of Process Conditions on

Hydrolysis Efficiency and Microbial Community Composition

4.1 Introduction

The need for landfill alternatives in the United States has been demonstrated by recently issued programs (e.g., recycling programs) and policies (Levis et al., 2010). AD of OFMSW is a favorable technology because it diverts waste from landfills, recovers nutrients in the form of composting, and generates energy in the form of methane (Demirer et al., 2005a; Holm-Nielsen et al., 2009). However, lack of economic feasibility has slowed the implementation of AD technologies in the U.S. (Rapport et al., 2008).

The digestion of OFMSW presents several challenges that affect reactor stability and can lead to a loss in profits. The waste can cause clogging in pipes in single-stage, wet digesters, necessitating the removal of inert solids prior to digestion which requires complex equipment (Rapport et al., 2008). Since OFMSW typically contains 30-60% solids, large volumes of water are required to dilute the waste to levels appropriate for wet digestion; furthermore, toxic compounds present in OFMSW (e.g., heavy metals) are able to diffuse through wet systems more easily and cause microbial inhibition (Vandevivere, 2002). Finally, portions of OFMSW (e.g., non-cellulosic food waste components) are able to rapidly degrade during hydrolysis and cause inhibitory levels of acid build-up (Cho et al., 1995). Thus, the development of AD systems capable of maintaining reactor stability and proving economically profitable are necessary to allow for increased implementation in the U.S.

Multi-stage AD systems that incorporate leachate-bed hydrolysis reactors are a favorable technology for the conversion of waste to methane (Yang et al., 2003; Shin et

al., 2010; Bouallagui et al., 2004). Leachate flows through the high-solids waste to promote hydrolysis and is then transferred to an acido/acetogenesis storage tank in the form of VFAs. The leachate then passes through a high-rate methanogenesis reactor and is converted to methane. Separation of each process (i.e., hydrolysis, acido/acteogenesis, and methanogenesis) creates a stable system that is more resilient to reactor perturbations (e.g., pH changes) and allows for individual process optimization to maximize microbial activity (Song et al., 2004; Ince, 1998; Demirer and Chen, 2005b; RIS International, 2005). Additionally, leachate recycle through the system can be incorporated to conserve water and reduce operational costs. However, leachate recycle causes an increase in ammonia (by-product of AD) and salinity concentrations.

Elevated ammonia and salinity concentrations have been demonstrated to inhibit methanogenesis and overall digester performance (Kayhanian, 1994; Kugelman and McCarty, 1964). Although low ammonia concentrations (less than 0.2 g/L) are favorable to AD performance (Liu and Sung, 2002; Chen et al. 2003), TAN concentrations in the range of 1.7 to 14 g/L are reported to reduce methane production by 50% (Sung and Liu, 2003; Bujoczek et al., 2000; Chen et al., 2008). Furthermore, concentrations of sodium ranging from 0.1 to 0.23 g/L have been shown to be beneficial to the process (McCarty, 1964; Kugelman and Chin, 1971). Half maximal inhibitory concentration (IC_{50}) values ranging from 5.6 to 53 g sodium/L have been reported (Feijoo et al. 1995; Chen et al. 2003; Omil et al. 1995; Vallero et al. 2002; Liu and Boone, 1991; Soto et al., 1993; Vallero et al., 2003). For the studies documented in the literature, reactor designs, operating parameters, and acclimation periods have varied, thus likely accounting for the wide range of reported IC_{50} values.

The impact of ammonia and salinity concentrations must be determined to guide reactor design to optimize leachate recycling ratios. Additionally, strategies that maximize digester performance under elevated ammonia and salinity concentrations (e.g., develop improved microbial seeds) are needed to render AD processes economically viable. Since hydrolysis is a rate-limiting step in the digestion of recalcitrant wastes (e.g., lignocellulosic matter such as fruit rinds) (Colberg, 1988; Nielsen et al., 2004; Pavlostathis and Giraldo-Gomez, 1991), optimization of this process could radically improve economically profitability of AD by lowering reactor volumes, decreasing operation costs, and improving methane generation.

Therefore, the objectives of this study were to investigate the impact of operating conditions (e.g., ammonia and salinity concentrations) on hydrolysis rates and identify characteristics of microbial communities that successfully hydrolyze food waste under elevated salinity and ammonia concentrations.

4.2 Methods

4.2.1 Food Waste and Inoculum Collection

Food waste was collected from Colorado State University Academic Village Ram's Horn dining facility, which has a separate collection for biodegradable food waste. In this system, the waste is separated, pulped to less than 2 cubic centimeters, centrifuged to remove water, and collected in bins. After collection for testing, the ground food waste was then stored at 4°C for subsequent use.

Microbial inoculum for the batch reactor tests was collected from the Drake Municipal Wastewater Treatment Plant (DWWTP) mesophilic digester (Fort Collins, CO), purged with nitrogen gas and maintained at 35°C. Food waste and inoculum were

analyzed as described in Section 3.2.1. Initial characteristics of the inocula and substrate are shown in Table 4.1.

Table 4.1 Characteristics of inocula and substrate

	Parameter			
	TCOD	DCOD	% TS	% VS
DWWTP Inocula	25.6 g COD/L	0.36 g COD/L	1.8	1.3
Food Waste	0.48 g COD/g substrate	--	29.3	28.1

4.2.2 Hydrolysis Batch Reactor Set-up

Hydrolysis batch reactors were assembled and conducted as described in Section 3.2.2 using food waste as feedstock.

4.2.3 Acclimation of Microbial Inocula

After initial tests revealed the need for organism acclimation to the different ammonia and salinity testing concentrations (Appendix D), batch reactors were set up as described previously in Section 3.2.3 to acclimate the microbial inocula to the testing conditions (1-5 g TAN/L and 3.9 – 11.8 g sodium/L). Each culture was tested after two months of initial setup.

4.2.4 Analytical Methods

To collect data necessary for hydrolysis rate determination, COD and methane analyses were measured as described in Section 3.2.4.

4.2.5 Calculation of Hydrolysis Rates

Hydrolysis kinetic rates were calculated for each batch test as described in Section 3.2.5.

4.2.6 Microbial Community Composition Analysis

At the end of each inoculum acclimation period (two months), DNA was extracted from the flasks and subjected to T-RFLP analysis as described in Section 3.2.6.

4.2.7 Microbial Community Composition Statistical Analysis

Non-metric multi-dimensional scaling (NMDS) was used to provide a visual interpretation of the T-RFLP data. Data was analyzed according to the methods described in Section 3.2.7. Relative distance indicates the level of similarity between points, and the stress value provides an indication of plot accuracy.

4.3 Results and Discussion

4.3.1 Hydrolysis Rate Determination

Regression analyses of the hydrolysis data according to Equation 2 for a range of ammonia and salinity concentrations are displayed in Figures 4.1 and 4.2, respectively.
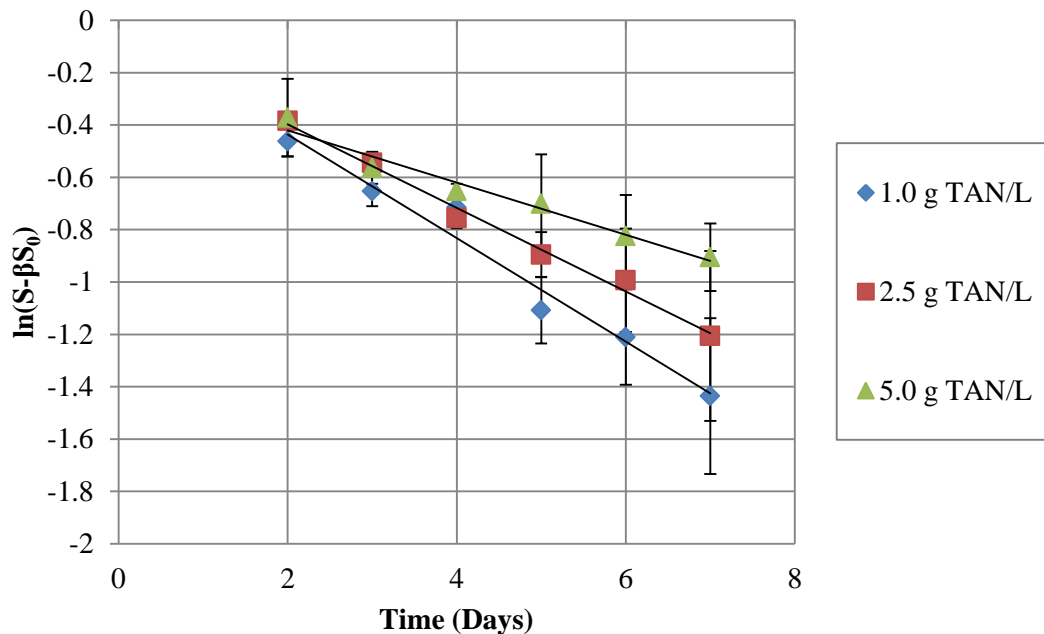
Figure 4.1 Regression analyses for a range of ammonia concentrations ($\beta = 0.11$)
Error bars represent standard deviations for triplicate reactors.
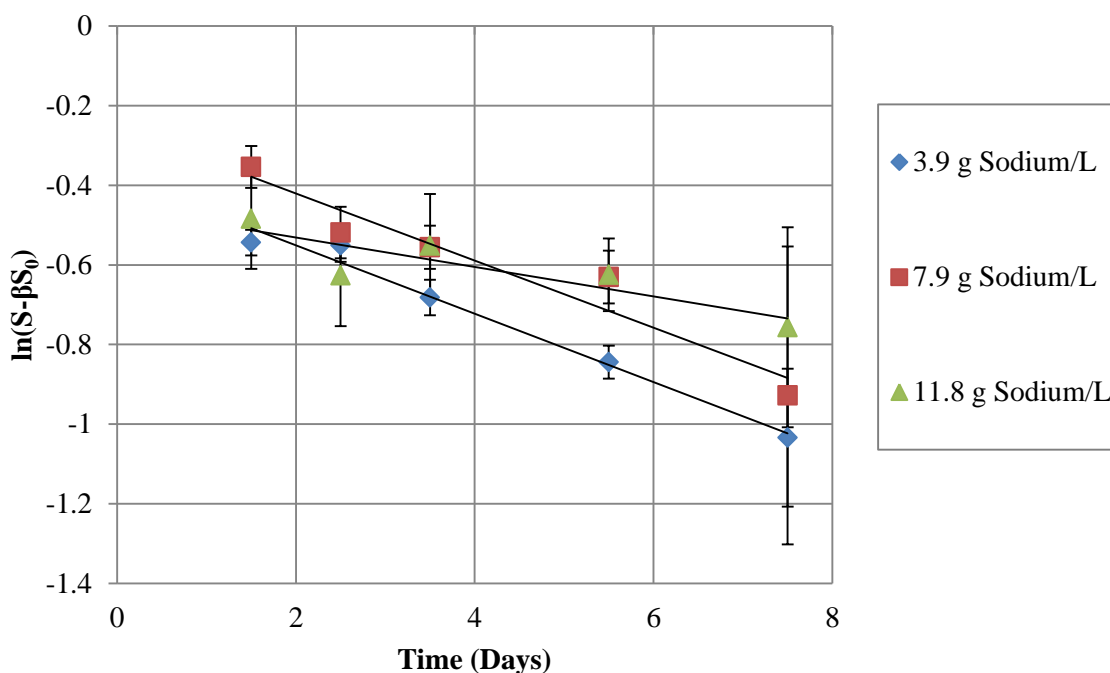
Figure 4.2 Regression analyses for a range of salinity concentrations (β = 0.11)
Error bars represent standard deviations for triplicate reactors.

The hydrolysis rate constants determined from the slopes of the regression analyses in

Figures 4.1 and 4.2 are shown in Table 4.2. Calculated coefficients of determination (R^2)

demonstrate that the data was appropriately modeled using a first-order hydrolysis kinetic

rate (Table 4.2).

Table 4.2 Kinetic rate values according to regression analyses

Reactor	Kinetic rate, k (day^{-1})*	R^2
1 g TAN/L (0.015 g NH_3-N)	0.198 (0.061)	0.97
2.5 g TAN/L (0.038 g NH_3-N)	0.160 (0.066)	0.99
5 g TAN/L (0.075 g NH_3-N)	0.100 (0.042)	0.97
3.9 g Sodium/L	0.086 (0.020)	0.98
7.9 g Sodium/L	0.084 (0.061)	0.93
11.8 g Sodium/L	0.037 (0.026)	0.77

*Numbers in parentheses indicate standard deviations
for triplicate reactors.

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The hydrolysis rates determined in this study for food waste (0.037-0.198 day^{-1}) are similar to rates reported in the literature (Vavilin et al., 2008), although rates can vary drastically depending on the composition of the waste and reactor conditions. Previously reported rates for MSW and biowaste are 0.1 day^{-1} and 0.12 day^{-1}, respectively (Liebetrau et al., 2004; Bolzonella et al., 2005). Additional studies have documented hydrolysis rates for a variety of household biowaste, such as kitchen waste (0.34 day^{-1}), food waste (0.55 day^{-1}), orange peels (0.145 - 0.474 day^{-1}), and wholewheat bread (0.195 day^{-1}) (Liebetrau et al., 2004; Vavilin et al., 2004; Veeken and Hamelers, 1999). Other MSW (e.g., office paper, cardboard, and newsprint) hydrolysis rates range from 0.036 – 0.057 day^{-1} (Vavilin et al., 2004).

As discussed in Section 3.3.1, previous research on the impact of elevated ammonia and salinity concentrations on hydrolysis is not well understood and thus makes direct comparison to rates observed in this study difficult. An ANOVA analysis on the hydrolysis rates in this study indicated that there were no statistical differences in rates for the ranges of ammonia and salinity concentrations (p-values = 0.055 and 0.29, respectively), thus suggesting the same conclusions as discussed in Section 3.3.1. For example, estimated methane generation from a day's production of manure at a 3,000 cattle feedlot in Colorado ranges from 59,125 ft^3 to 56,770 ft^3 and 46,560 ft^3 to 25,012 ft^3 of methane for 1 – 5 g TAN/L and 3.9 – 11.8 g sodium/L, respectively, over a 20 day digestion period. Furthermore, the similar results obtained for the hydrolysis of manure and food waste suggest that these results may be extended to a variety of feedstocks.

To determine if hydrolysis rates would improve with additional microbial acclimation, the cultures were acclimated for an additional 2 months (4 months total).

However, the relationships among the rates remained extremely similar (Appendix F). Therefore, it is concluded that these rates are indicative of reactor performance over time and suggested that increases in rates due to additional acclimation are unlikely for higher ammonia and salinity concentrations. Additionally, these results suggest that it takes less than 2 months to acclimate the inoculum used in this study. Since the rate of acclimation of organisms determines reactor performance, these findings are particularly key for estimating the time required to achieve stable reactor performance.

4.3.2 Microbial Community Composition Analyses

To determine if microbial community composition changes occurred due to acclimation, functional-gene based T-RFLP was conducted as discussed in Section 3.3.2. The acclimated and un-acclimated microbial inocula examined in this study contained between 3 and 6 major T-RFs (Figure 4.3).
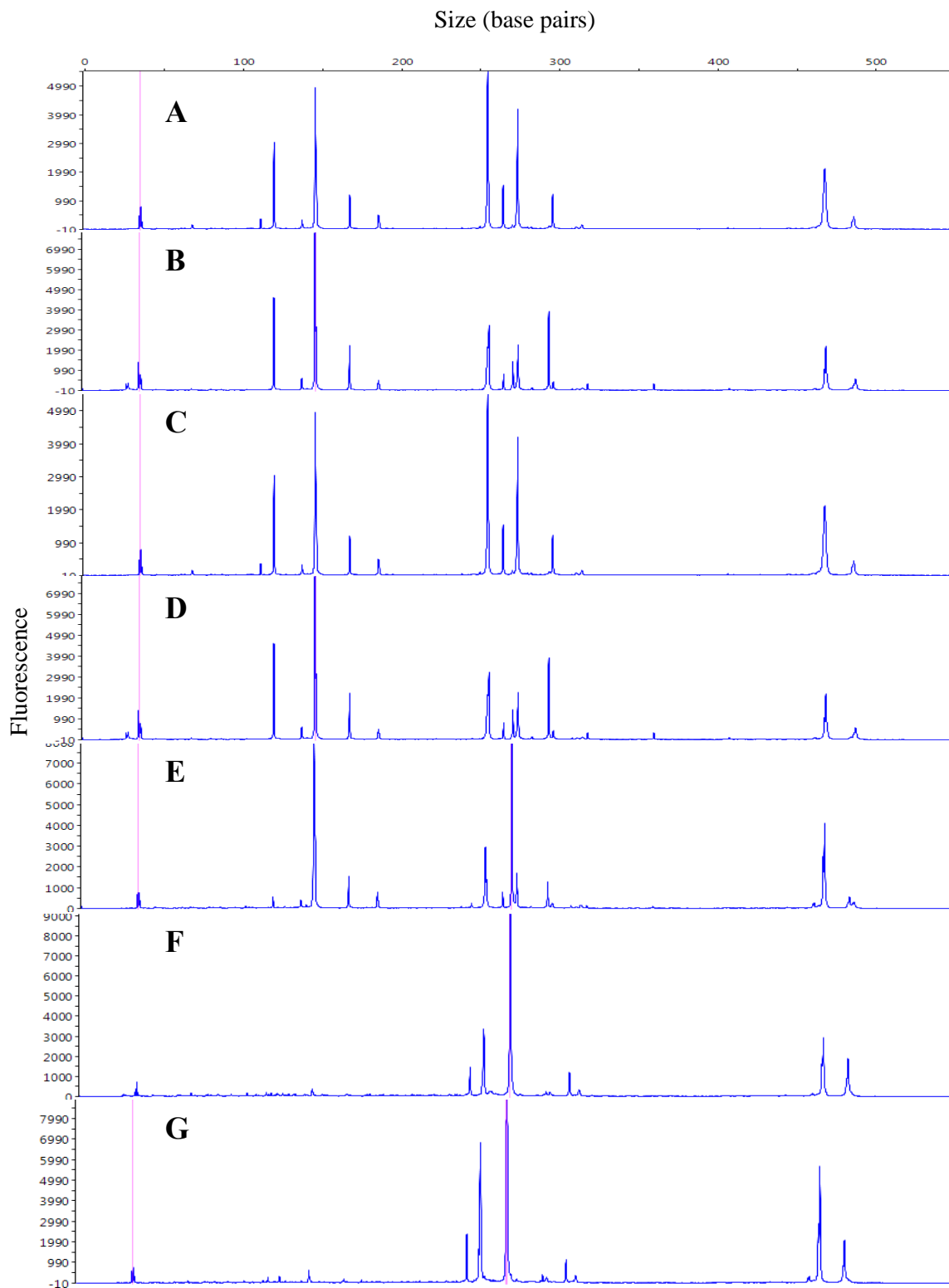
Figure 4.3 Electropherograms illustrating *mcrA*-targeted peaks
Original inoculum (A), Inocula acclimated to 1 g TAN/L (B), 2.5 g TAN/L (C), 5 g TAN/L (D), 3.9 g sodium/L (E), 7.9 sodium/L (F), 11.8 g sodium/L (G).

A NMDS plot of the data shows that post-acclimation communities were distinct from the original inocula. Therefore, microbial community shifts occurred due to acclimation for inocula subjected to both elevated ammonia and elevated salinity concentrations (Figure 4.4).
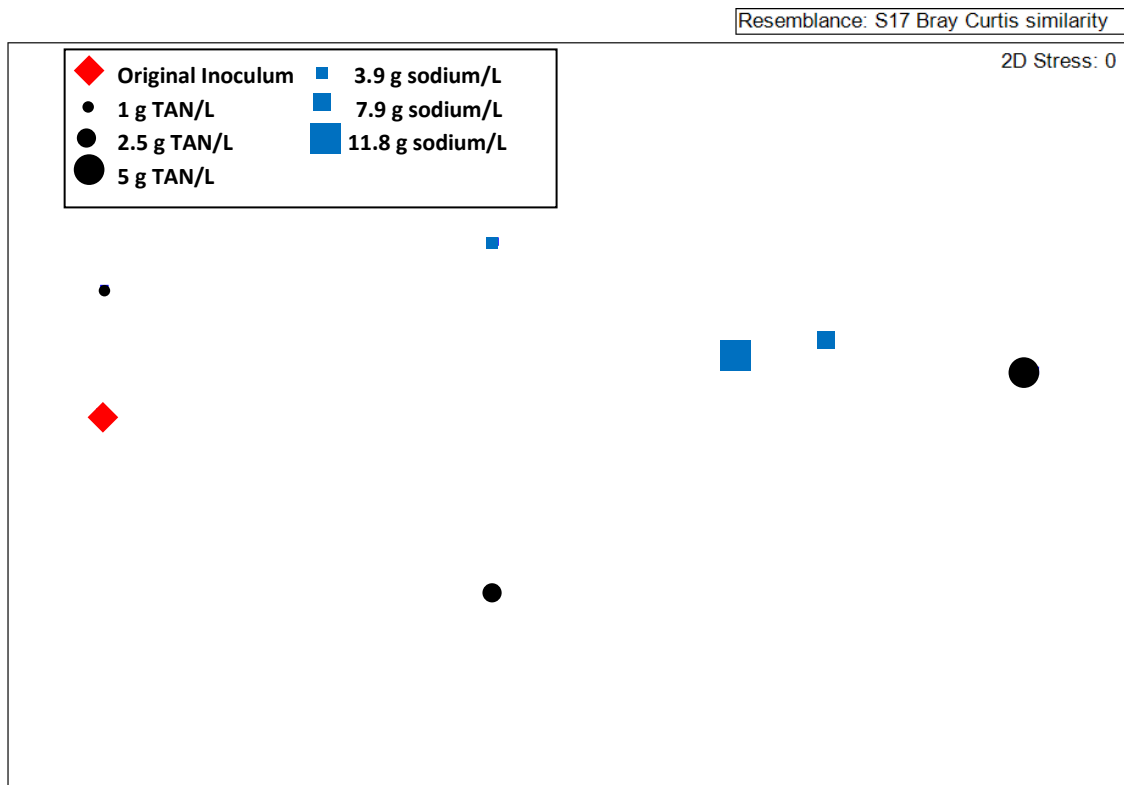
Figure 4.4 Multidimensional scaling plot based on *mcrA*-targeted T-RFLP

The inocula acclimated to the elevated concentrations of ammonia showed increasingly further distance from the original inoculum, indicating that a more significant microbial community shift occurred during acclimation to extremely high ammonia concentrations. In comparison, the salinity-acclimated inocula communities were more clustered, indicating that changes occurred in the microbial communities from the original inoculum, but there was less variance between the saline inocula post-acclimation. As discussed in Section 3.3.2, the shifts in microbial communities were

consistent with improved hydrolysis rates post-acclimation. The reason for microbial community changes could be attributed to a variety of variables. First, the high ammonia and salinity concentrations could have selected for organisms tolerant to the extreme conditions. It is also possible that the nutrient solution and/or food source also could have caused distinct shifts in the microbial communities; however, NMDS plots containing data for inocula acclimated to ammonia and salinity concentrations for manure and food waste do not appear to cluster according to feedstock. Thus, current work is underway to identify the underlying cause for microbial community composition changes in these experiments.

Since this study is targeted toward hydrolysis, microbial community analysis would ideally focus on cellulose-degrading genes. However, to our knowledge, there are no molecular tools available targeting *cel* genes for T-RFLP analysis. Since shifts in the methanogenic communities have been shown, it is likely that similar shifts occurred in the hydrolytic communities. As a result, future work will focus on developing assays that target cellulose-degrading genes to provide additional insight into the hydrolytic process.

4.4 Conclusions

The results of this experiment suggest that hydrolysis efficiency decreases with elevated ammonia and salinity concentrations regardless of feedstock. Furthermore, it was determined that acclimation of microbial organisms to higher concentrations of ammonia and salinity was required to achieve optimal hydrolysis rates. Therefore, determination of microbial community characteristics that lead to increasing levels of ammonia and salinity can help guide reactor design (e.g., microorganism inoculation) for systems with leachate recycle. A detailed economic analysis is required to recommend

specific operating parameters to achieve a balance between process efficiency and resource consumption. Preliminary estimations, however, demonstrate slight energy savings (approximately \$315 and \$630 for reactors operating at 1 g TAN/L and 3.9 g sodium/L compared to 5 g TAN/L and 11.8 g sodium/L, respectively, for feedlot scenario previously described in Section 4.3.1) from increased process performance at decreased ammonia and salinity concentrations, although current estimations do not factor increased water and energy consumption required to dilute elevated concentrations into the cost comparison. Results of this study, however, have demonstrated the importance of acclimation as a means of minimizing the economic tradeoff.

5.0 Conclusions

Acclimation periods of the microbial inocula for 2-4 months resulted in substantial improvement in hydrolysis rates such that the vast majority of differences in hydrolysis rates due to increasing ammonia or salinity concentrations were statistically insignificant. General trends indicated that hydrolysis is slightly inhibited by elevated ammonia and salinity concentrations, but again, steps may be taken to minimize inhibitory effects through the process of acclimation. Furthermore, results demonstrated shifts in microbial communities of the inocula pre- and post-acclimation, indicating that specific microbes were selected for and required for the reactor to perform well under the stressed operating conditions. Thus, with the application of microbial inocula acclimation or reactor bioaugmentation with organisms that are tolerant to extreme conditions, it is suggested that hydrolysis reactors could be operated at increased concentrations of ammonia and salinity to help minimize the tradeoff between energy consumption and process efficiency.

Since similar results on hydrolysis rates were observed for a variety of feedstocks (e.g., manure, food waste, agricultural residue), it is suggested that the findings in this study are applicable to a broad range of feedstocks. This result is particularly important because interest in co-digestion is growing due to wide varieties of available waste and improved overall reactor performance from feedstock synergisms. For example, seasonal variations in waste sources could also necessitate changing reactor feedstock, but results from this study suggest that optimal hydrolysis rates are able to be maintained.

Although molecular assays were successfully utilized targeting the *mcrA* gene to demonstrate microbial composition changes in inocula post-acclimation, further research

is required to develop assays suitable for genes targeting cellulose-degradation. This is desirable because tracking genes involved in cellulose-degradation could provide key information used to understand and enhance reactor performance during hydrolysis (e.g., bioaugmentation with microbes tolerant to elevated ammonia and salinity concentrations). Currently, molecular tools for *cel*-targeted genes are limited and have not been applied previously to AD systems. In this study, it was found that current assays were not suitable for the microbial communities investigated. Therefore, future work will focus on developing functional-gene based assays that target hydrolyzing bacteria and are broadly applicable to track microbial community changes as a function of acclimation.

Although it has been demonstrated that acclimation improves performance, little research has been conducted to determine the best acclimation procedures (e.g., sudden or gradual chemical additions, acclimation time). Furthermore, since the rate of acclimation of the inocula determines startup reactor times, the response of different microbial seeds (e.g., fresh manure, landfill leachate, wastewater treatment plant AD sludge) should be monitored in batch laboratory-scale reactors (e.g., glass flasks) to determine which source leads to the fastest hydrolysis rates post-acclimation. Microbial communities should be tracked during acclimation via the aforementioned developed functional gene-based tools to determine the rate at which communities adapt to reactor conditions.

Finally, additional research is required to verify that the results obtained in this study can be applied to a variety of AD systems that encounter elevated ammonia and salinity concentrations. Thus, performance should be monitored via larger laboratory-scale reactors to ensure that the microorganisms respond in the same manner as observed

in the batch systems and long-term stable performance can be achieved after an acclimation period. To this end, laboratory-scale reactors (e.g., 8-in diameter polycarbonate pipes) should be operated under elevated ammonia and salinity concentrations using the microbial seed that demonstrates the fastest rate of acclimation and monitored to determine if hydrolysis rates remain comparable to hydrolysis rates occurring in reactors operating under baseline conditions. This will help verify the broad extent of AD systems to which the findings of this study may be applied.

Appendix A: Hydrolysis Kinetic Rates for Agricultural Residue

Co-digestion is a viable option in Colorado because of the abundant agricultural residues (e.g., potato waste, corn stover, onion waste), synergistic effects of the substrates (e.g., pH buffer) during digestion, and subsequent increased methane yields. In this study, hydrolysis rates were also determined for corn stover (stalk, leaves, husk, and cob leftover after harvest) for a range of ammonia concentrations. Figure A.1 depicts the regression analyses for corn stover. Table A.1 provides a summary of the kinetic rates and coefficients of determination.
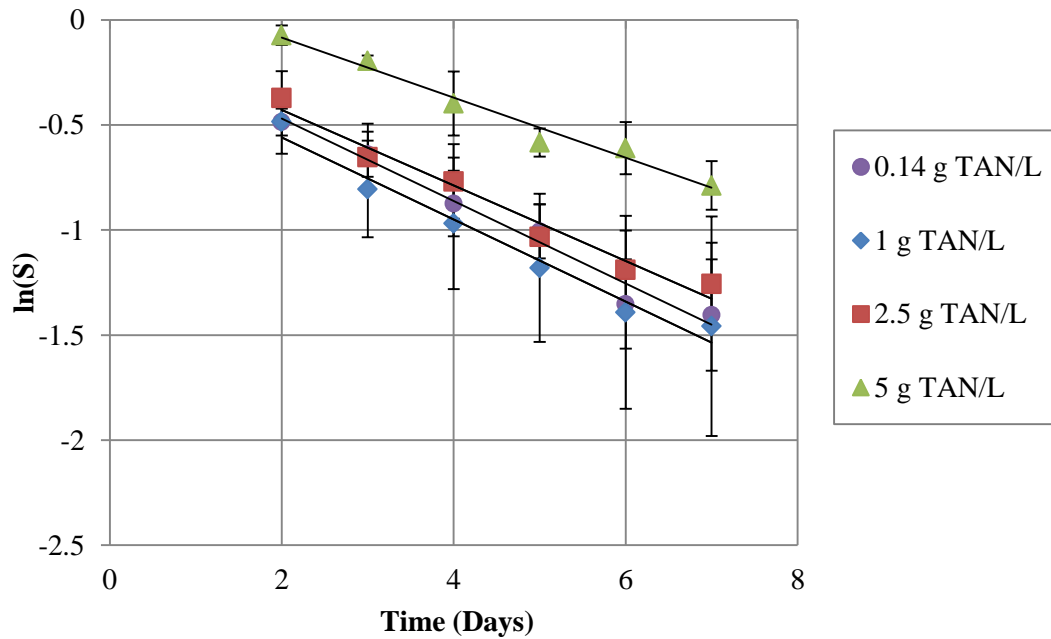
Figure A.1 Regression analyses for a range of ammonia concentrations
Error bars represent standard deviations in triplicate reactors.

Table A.1 Kinetic rate values according to regression analyses

Reactor	Kinetic rate, k (day^{-1})*	R^2
0.14 g TAN/L	0.196 (0.045)	0.98
1 g TAN/L	0.195 (0.062)	0.97
2.5 g TAN/L	0.179 (0.023)	0.97
5 g TAN/L	0.143 (0.013)	0.98

*Numbers in parentheses indicate standard deviations for triplicate reactors.

As seen for inocula fed with manure and food waste, kinetic rates decrease with increasing ammonia concentrations. Once again, differences in rates are statistically insignificant, but the general trends can be noted. Thus, it is again suggested that mild inhibition on hydrolysis occurs with elevated ammonia concentrations (>2.5 g TAN/L).

Appendix B: Nutrient Solution Preparation

Concentrated Stock Solutions:

Solution	Compound	Concentration (g/L)
S1	Sample	<2g/L degradable COD
S2	Resazurin	1
S3	$(NH_4)_2HPO_4$	26.7
S4	$CaCl_2 - 2H_2O$	16.7
	NH_4Cl	26.6
	$MgCl_2 - 6H_2O$	120
	KCl	86.7
	$MnCl_2 - 4H_2O$	1.33
	$CoCl_2 - 6H_2O$	2
	H_3BO_3	0.38
	$CuCl_2 - 2H_2O$	0.18
	$Na_2MoO_4 - 2H_2O$	0.17
	$ZnCl_2$	0.14
S5	$FeCl_2 - 4H_2O$	370
S6	$Na_2S - 9H_2O$	500
S7	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 acid	0.005
	Thioctic acid	0.005

Defined Media Preparation (Owen et al., 1978):

1. Add one liter of deionized water to a two liter volumetric flask.
2. Add the following:
   a. 1.8 ml S2
   b. 5.4 ml S3
   c. 27 ml S4
3. Add deionized water up to the 1800 ml mark.
4. Boil for 15 minutes while flushing with nitrogen gas at approximately 1L/min.

5. Cool to room temperature while continuing to flush with nitrogen gas.
6. Add the following:
   a. 18 ml S7
   b. 1.8 ml S5
   c. 1.8 ml S6
7. Change gas to 30% CO_2: 70% N_2 mixture and continue flushing.
8. Add 8.40g $NaHCO_3$ as powder.
9. Bubble the CO_2:N_2 gas mixture until media pH stabilizes at approximately 7.1.
10. Carefully seal volumetric flask while minimizing the introduction of air into the container.

Appendix C: Glucose Controls

Syringes with inocula fed glucose as the sole carbon source were run to ensure that methanogenesis was not rate-limiting in the batch tests. The following figures C.1 and C.2 depict methane production for the inocula fed glucose used in the manure batch tests.

Figure C.1 Methane production over time for a range of ammonia concentrations for inocula used in manure batch tests.

Figure C.2 Methane production over time for a range of salinity concentrations for inocula used in manure batch tests.

The following figures depict methane production for the inocula fed glucose used in the food waste batch tests.
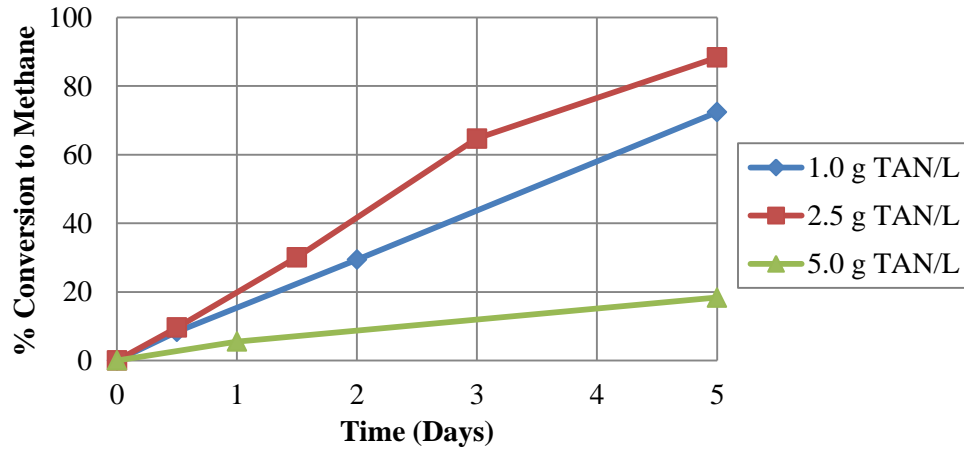

Figure C.3 Methane production over time for a range of ammonia concentrations for inocula used in food waste batch tests

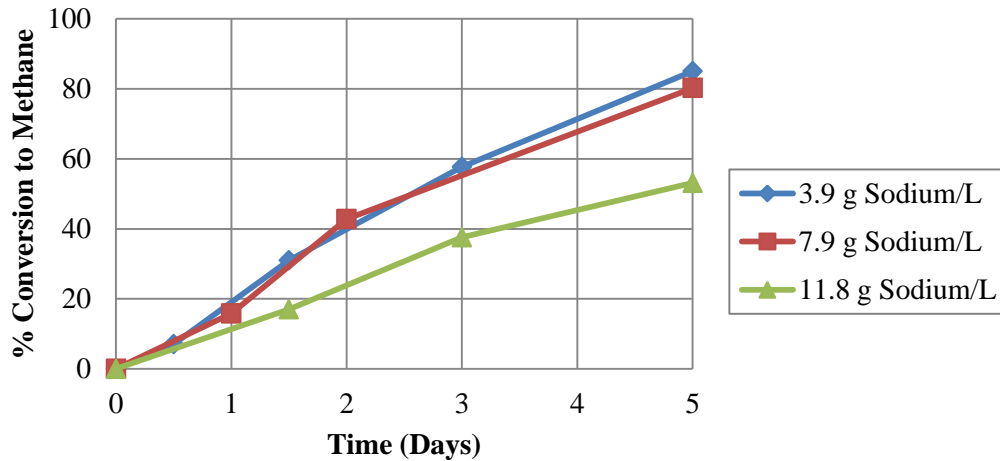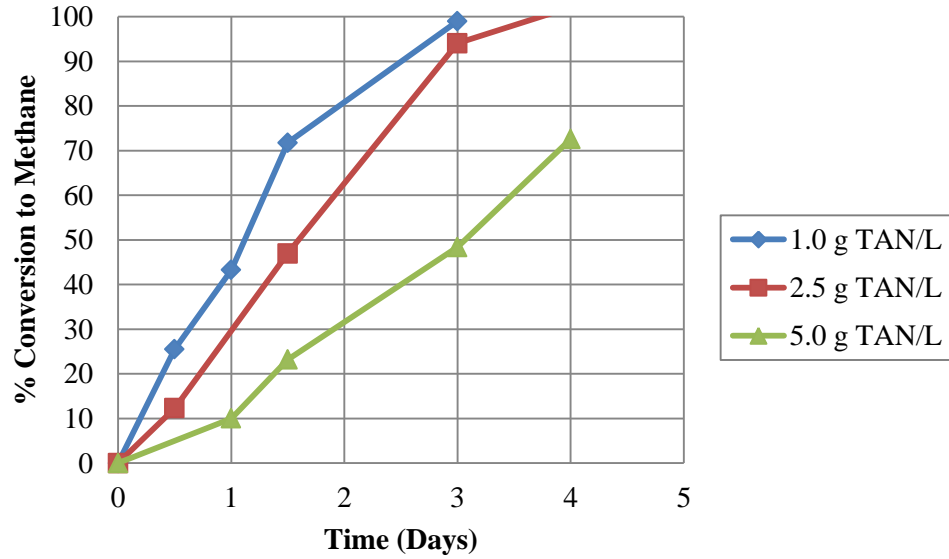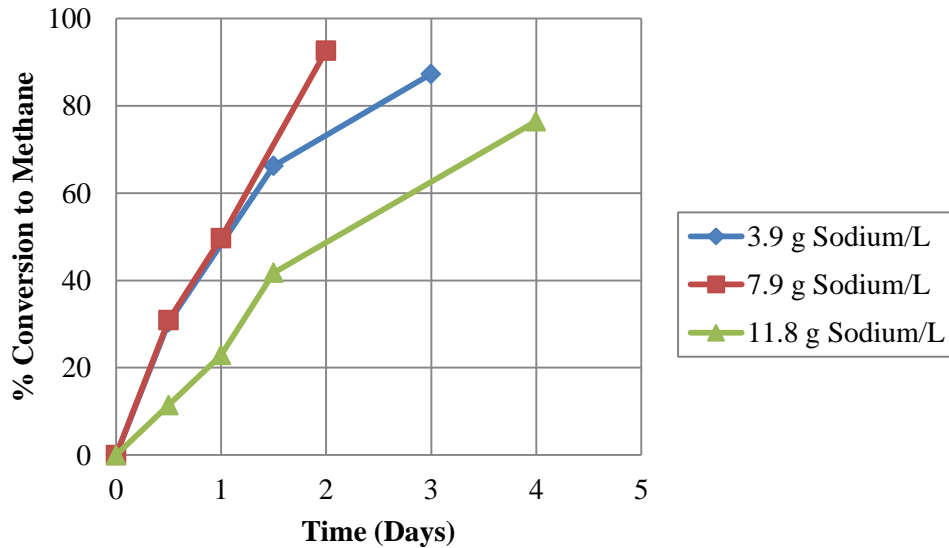**Figure C.4 Methane production over time for a range of salinity concentrations for inocula used in food waste batch tests.**

In every case, methane production rates were inhibited by increasing ammonia or salinity concentrations; however, in all but one case, the rates of methane production far exceeded the hydrolysis rates. Thus, the results indicated that methanogenesis was not rate-limiting in the batch systems. One exception was the inocula used for the manure test operating under 5 g TAN/L, which demonstrated a slower methanogenesis rate than the hydrolysis rate. However, hydrolysis rates would only be inhibited if the rate-limiting methanogenesis caused a build-up of inhibitory intermediates (e.g., VFAs) that affected hydrolysis. Since the amount of manure added to the system was extremely small (~1g), a build-up of intermediates to inhibitory levels is unlikely. Additional calculations verified that any possible VFA accumulation in the system was well below concentrations regarded as inhibitory. Furthermore, pH was monitored throughout the reaction period, and pH drops that could indicate acid build-up were not observed. Therefore, observed trends in the measured hydrolysis rates as a function of ammonia and salinity concentration can be assumed to represent the activity of the hydrolyzing microbial communities.

Appendix D: Acclimation of Inoculum

Initial tests conducted in an attempt to measure hydrolysis rates for unacclimated inoculum (DWWTP digester sludge) with elevated ammonia and salinity concentrations (1 to 5 g TAN/L or 3.9 to 11.8 g sodium/L) showed that pre-acclimation of the microbial communities was necessary to yield measurable hydrolysis rates. High ammonia and salinity concentrations substantially inhibited methane production (Fig D.1 and D.2, respectively) and caused a significant drop in the ATP concentration (Fig. D.3 and D.4, respectively).
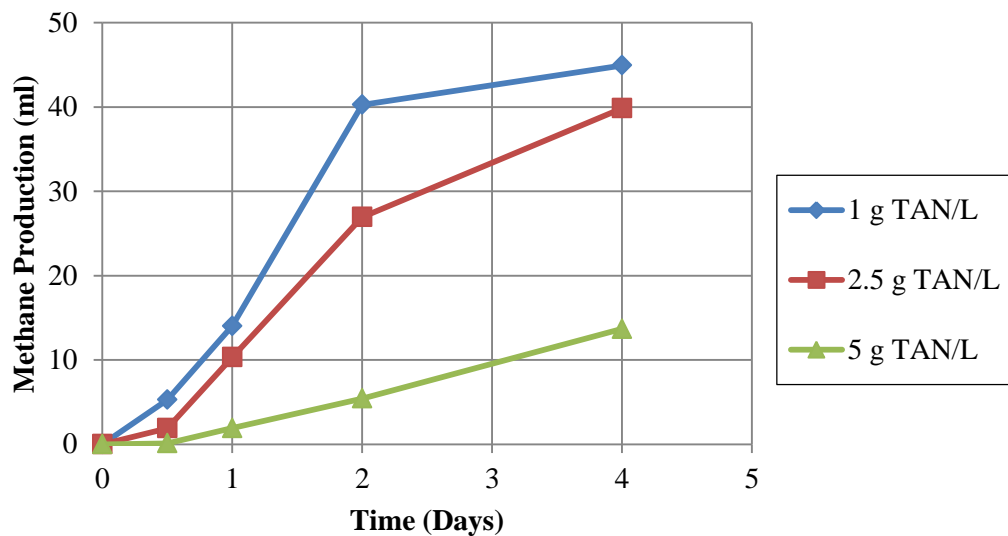
Figure D.1 Methane production for directly exposed inoculum to high ammonia concentrations (glucose as substrate)
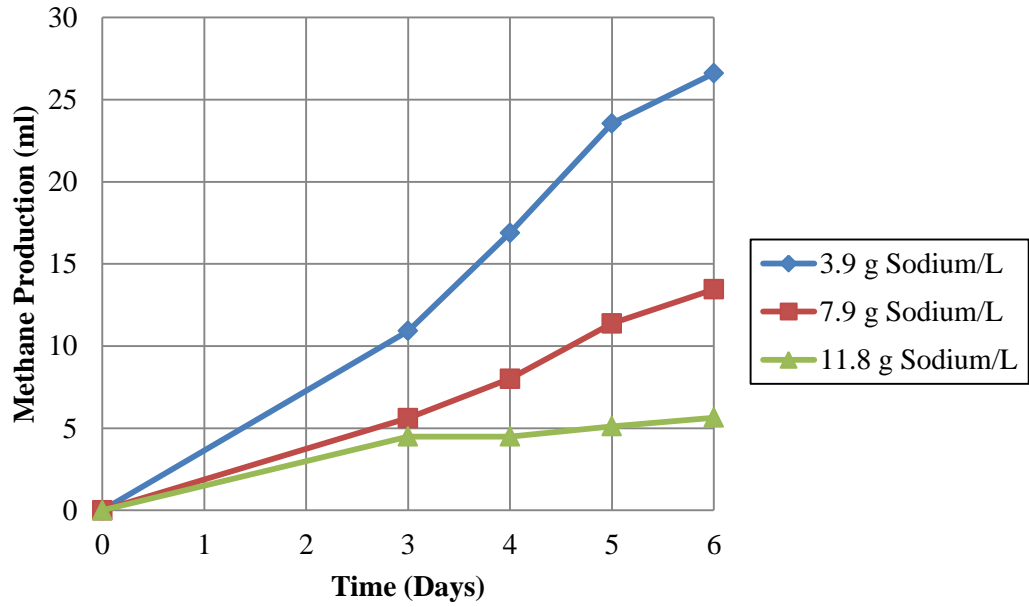
Figure D.2 Methane production for directly exposed inoculum to high salinity concentrations (glucose as substrate)
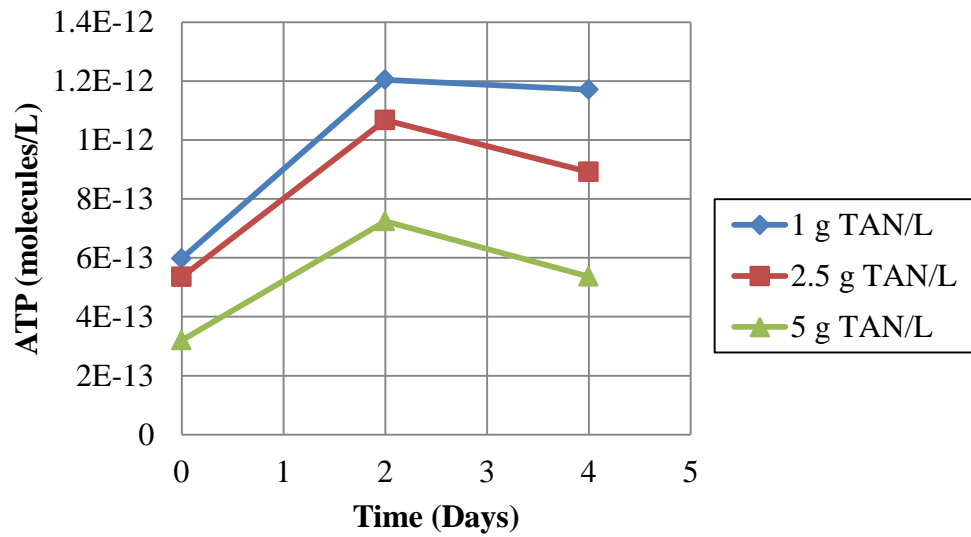
Figure D.3 ATP concentration for directly exposed inoculum to high ammonia concentrations (glucose as substrate)
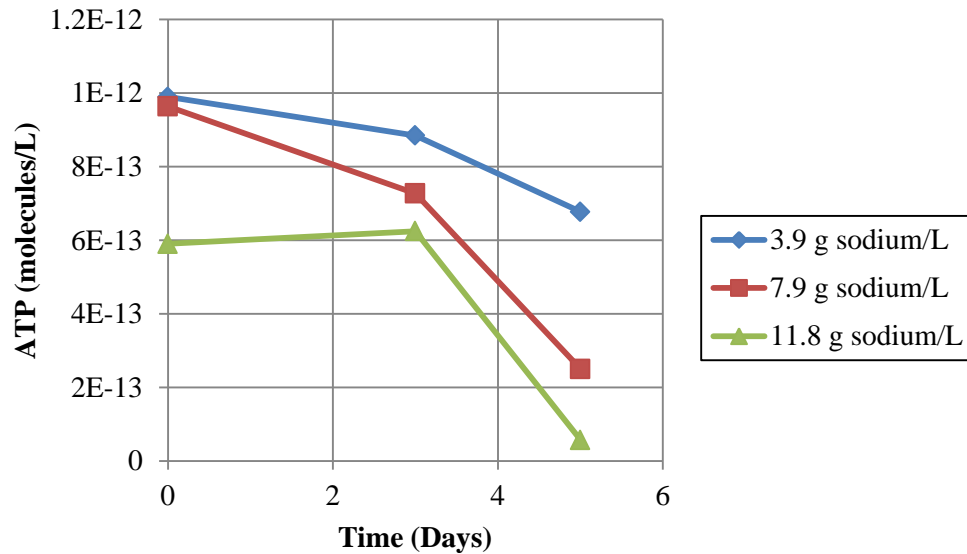
Figure D.4 ATP concentration for directly exposed inoculum to high salinity concentrations (glucose as substrate)

After acclimation for 2-4 months, microbial activity increased as evidenced by measureable hydrolysis rates, elevated ATP values, and increased methane production (Fig. D.5). After a four month acclimation period, results demonstrated substantial improvement in biogas composition (>50 % methane as determined by gas chromotagraphy), and methane production was similar for the lowest sodium concentration (3.9 g sodium/L) and the highest sodium concentration (11.8 g sodium/ L). The hydrolysis rates reported in Chapter 3 and 4 were determined for these acclimated microbial inocula.
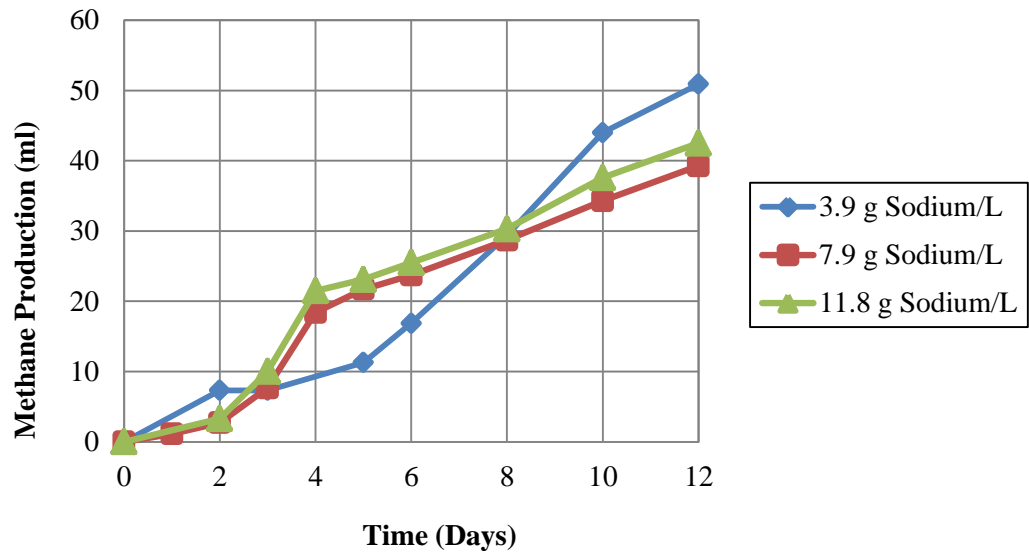
Figure D.5 Methane production for acclimated inoculum to high salinity concentrations (glucose as substrate)

Thus, it may be concluded that during full-scale operation, it may be necessary to seed reactors with pre-acclimated inoculum or it might be possible to develop acclimated microbial communities during startup via operating with reduced feeding rates (to accommodate reduced hydrolysis rates) and by gradually allowing ammonia and salinity to increase. Future research is required to determine optimal approaches to achieving maximum hydrolysis rates in full-scale systems.

Appendix E: Biomass Factor

The amount of biomass in an anaerobic digester has been shown to affect hydrolysis kinetic rates (O'Sullivan et al., 2008; Vavilin et al., 2008). For example, Mourino et al. (2001) reported an increase in hydrolysis rates for rumen-inoculated systems as the inoculum volumes increased from 5 to 20% on a volume per volume basis. However, common biomass measurement methods (e.g., VSS, cell culturing, or organic nitrogen) are often inaccurate or are not appropriate for AD of solid substrates (Chung and Neethling, 1988). For example, VSS is inappropriate for AD reactor systems that contain particulate substrates because in this case both biomass and substrates will be detected as VSS (Chung and Neethling, 1989). Thus, most studies forego measuring biomass concentrations and assume that biomass density is constant across the reactors being compared (O'Sullivan et al., 2008). Therefore, the objective of this research was to investigate the need for a biomass factor in the current studies and determine an accurate method of biomass quantification.

Methods

Adenosine triphosphate (ATP) measurement is a simple, rapid, and increasingly accepted method of biomass quantification that measures only metabolically active cells, thus excluding inactive biomass such as dead cells (Chung and Neethling, 1989). Thus, ATP levels were used as an indicator of the quantity of active cells and were measured using the Promega BacTiter-Glo Microbial Cell Viability Assay (Madison, WI) according to the manufacturer's protocol.

qPCR also was used to estimate the quantity of Bacteria and methanogenic Archaea present in the batch reactors using assays targeting bacterial 16S rRNA genes

and *mcrA*, respectively. Bacterial 16S rRNA genes were quantified according to Li et al. (2010) except where differences are noted. *mcrA* genes were quantified according to Pereya et al. (2010) except where differences are noted. Primer sequences are shown in Table E.1. Briefly, all amplifications were performed using an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA). Reactions consisted of 1X *Power* SYBR green PCR master mix (Applied Biosystems), 1 mM $Mg(OAc)_2$, 0.15 µM (Bacteria) or 0.2 µM (*mcrA*) of each primer, 2 µl DNA template, and nuclease-free water to a final volume of 25 µl. The thermocycling program was as follows: 10 min at 95ºC, followed by 40 cycles of 30 s (Bacteria) or 40 s (*mcrA)* at 95ºC, 30 s at 56ºC, and 30 s at 60ºC.

Table E.1 qPCR primer sequences*

Target and Primer	Sequence (5' $\rightarrow$ 3')
Bact_1369F	CGGTGAATACGTTCYCGG
Bact_1492R	GGWTACCTTGTTACGACTT
*mcrA*_1035F	GGTGGTGTMGGATTCACACARTAYGCWACAGC
*mcrA*_1530R	TTCATTGCRTAGTTWGGRTAGTT

*Primers Bacter_1369F and Bact_1492R were designed by Li et al. (2010). Primers *mcrA*_1035F and *mcrA*_1530R were designed by Pereya et al. (2010).

To model the impact of biomass concentration on measured hydrolysis rates, a modified version of the first-order kinetic model (Equation 2) including a biomass factor was utilized (Vavilin et al., 2008):

$$\frac{dS}{dt} = -\hat{k}X^n(S - \beta S_0) \qquad \text{(Equation 3)}$$

X is the biomass concentration (measured via ATP analysis or qPCR), and n is an empirical constant that must be determined for a given inoculum and substrate.

Results

     To determine if the amount of biomass was limiting for the small-scale AD reactors used herein, a batch study was conducted using varying amounts of biomass under baseline conditions (1.2 g sodium/L and 0.14 g TAN/L). Manure was used as the carbon source. As shown in Figure E.1 and Table E.2, hydrolysis rates increased as the volume percentage of inoculum added increased, suggesting that biomass limited measured hydrolysis rates when less than 15% inoculum was used.
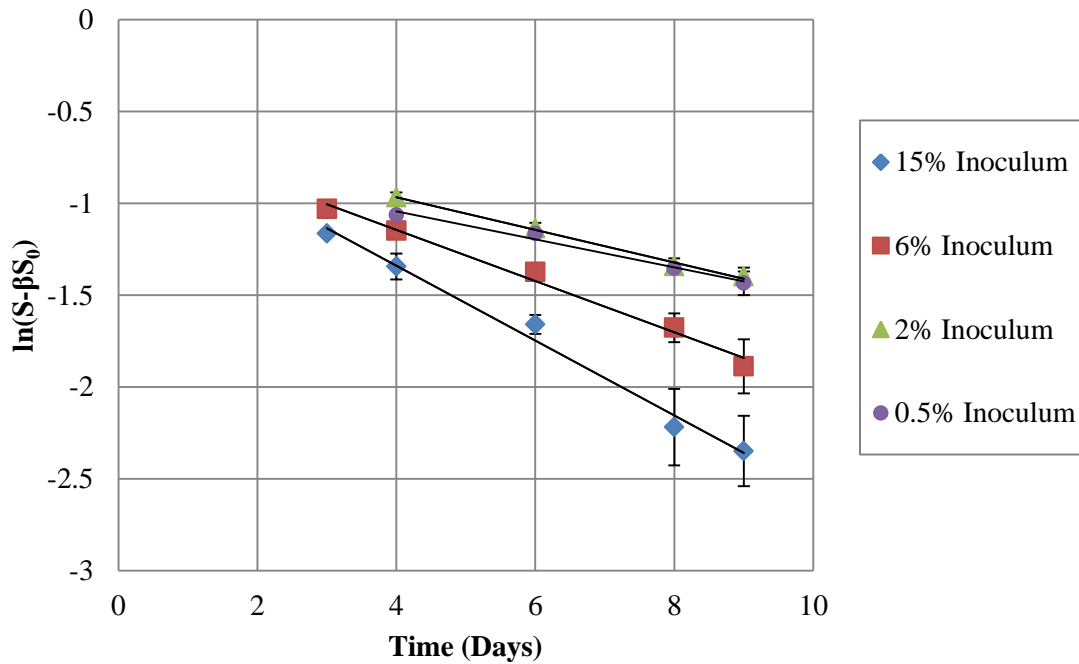

Figure E.1 Regression analyses demonstrating the impact of biomass (by volume)

Table E.2 Kinetic rate values determined via regression analyses

Reactor (% Inoculum, by Volume)	Kinetic rate, k (day^{-1})*	R^2
15%	0.204 (0.031)	0.99
6%	0.139 (0.021)	0.99
2%	0.089 (0.009)	0.99
0.5%	0.076 (0.014)	0.98

* Numbers in parentheses indicate standard deviations in triplicate reactors.

Given that the biomass concentration did affect measured hydrolysis conversion rates, it was considered desirable to include biomass concentration in the hydrolysis rate model used. Thus, the experimental data (as shown in Table E.2) was analyzed and *n* was determined via three different methods of biomass measurement (ATP, 16S rRNA, and *mcrA* quantification) using Equation 3. Next, the value determined for *n* for each method was applied to the experimental data for a range of ammonia and salinity concentrations as determined in Chapters 3 and 4, and subsequent hydrolysis rates were determined that included a biomass factor. Tables E.3 and E.4 summarizes biomass measurements for manure-fed inocula according to each method and the resulting measured hydrolysis rates determined from Equation 3, respectively.

Table E.3 Biomass measurements for manure-fed inocula

Reactor	Biomass Measurement		
	ATP Concentration (μM)	Copy # of 16S rRNA gene*	Copy # of *mcrA* gene*
1 g TAN/L	0.090	6.2×10^5 (35.6×10^3)	7.6×10^4 (8.3×10^3)
2.5 g TAN/L	0.075	4.4×10^5 (46.1×10^3)	16.3×10^4 (33.8×10^3)
5 g TAN/L	0.050	5.0×10^5 (32.4×10^3)	17.3×10^4 (23.0×10^3)
3.9 g Sodium/L	0.148	4.4×10^5 (35.5×10^3)	10.9×10^4 (37.5×10^3)
7.9 g Sodium/L	0.147	5.1×10^5 (84.8×10^3)	6.3×10^4 (33.6×10^3)
11.8 g Sodium/L	0.092	5.2×10^5 (12.3×10^3)	18.1×10^4 (23.1×10^3)

*Numbers in parentheses represent standard deviations for triplicate reactors.

Table E.4 Effect of biomass concentration on measured hydrolysis rates

	Hydrolysis Rate (day^{-1})*		
Reactor	Based on ATP Concentration (μM^{-1}-day^{-1})	Based on 16S rRNA gene (copy # x 10^{-7}-day^{-1})	Based on *mcrA* gene (copy # x 10^{-7}-day^{-1})
1 g TAN/L	0.706 (0.339)	0.412 (0.198)	0.581 (0.279)
2.5 g TAN/L	0.774 (0.183)	0.396 (0.094)	0.558 (0.133)
5 g TAN/L	0.727 (0.139)	0.288 (0.034)	0.303 (0.058)
3.9 g Sodium/L	0.269 (0.052)	0.121 (0.023)	0.281 (0.030)
7.9 g Sodium/L	0.190 (0.047)	0.078 (0.018)	0.219 (0.026)
11.8 g Sodium/L	0.271 (0.097)	0.085 (0.030)	0.173 (0.036)

*The calculated n (power index) for ATP concentration, 16S rRNA gene, and *mcrA* genes was 0.71, 0.18, and 0.31, respectively. Numbers in parentheses represent standard deviations for triplicate reactors.

The effects of biomass concentration on measured hydrolysis rates varied depending on the method of biomass quantification. Interestingly, utilizing ATP levels for X in Equation 3 resulted in calculated kinetic rates for the highest concentrations of ammonia and salinity (5 g TAN/L and 11.8 g sodium/L, respectively) that were faster than the calculated rates for the lowest concentrations (Table E.4). This occurrence was due to the fact that as concentrations of ammonia and salinity increased in the reactors, ATP concentrations generally decreased likely because the microorganisms present were under increasingly stressed reactor conditions. As was observed for kinetic rates calculated from Equation 2, differences in the rates for the range of ammonia and salinity concentrations were found to be statistically insignificant, but as noted previously, the general trends changed. Thus, it was concluded that ATP levels are not a viable method for measuring biomass in a small-scale batch hydrolysis rate tests. Furthermore, ATP measurements were variable across the testing period since cells are responsive to

environmental conditions (e.g., substrate level, pH or temperature) further complicating analysis of these findings.

Because DNA-based assays are not sensitive to short-term changes in environmental conditions, qPCR assays were investigated to determine if they represented a viable alternative for biomass measurement. Interestingly, utilizing 16S rRNA gene quantities as a biomass indicator did not yield expected results. When the biomass was analyzed via qPCR targeting 16S rRNA for varying levels of input inoculum, the quantities of 16S rRNA genes did not change as the percentage of inoculum added changed. It was first verified that inhibition during amplification was not occurring due to inhibitors such as humic acids. Thus, it is possible that the majority of the biomass present in the inocula was methanogens, and therefore the differences in the percentage of hydrolyzing bacteria were below the detection limit of the assay. It was hypothesized that the quantity of 16S rRNA genes would decrease with increasing ammonia and salinity concentrations. Consequently, no significant trends were observed correlating 16S rRNA gene copy number to ammonia or salinity concentrations. The highest sodium concentration actually yielded the highest 16S rRNA copy number. As a result, despite the modified kinetic rates demonstrating a gradually decreasing rate as ammonia and salinity concentrations increased (although statistically insignificant), the validity of this biomass measurement is questioned. The 16S rRNA gene primers used only target Bacteria in the system and not Archaea (e.g.,, methanogens); however, given that hydrolysis is mediated by Bacteria and not Archaea this does not explain the observed result. However, to verify that the observed results could not be explained by differences in the quantity of methanogens, *mcrA* gene quantities were calculated as a

function of inoculum percentage and ammonia and salinity concentrations. However, again, there was no logical correlation between the *mcrA* gene quantity and inoculum volume, and the resultant general trend for the modified rates as a function of ammonia and salinity concentrations remained approximately the same as rates calculated from Equation 2. Since the kinetic rates measured were *hydrolysis* rates, it is not surprising that *mcrA* quantification data did not yield rational correlations.

Since none of the three methods of biomass quantification proved to be viable approaches, Equation 2, which does not include a biomass factor, was used. To overcome this barrier, all tests were set up with the same volume of inoculum so rates could be compared across ammonia and salinity concentrations.

Appendix F. Hydrolysis Kinetic Rates for Food Waste

After hydrolysis rates suggested a moderate degree of inhibition with increasing ammonia and salinity acclimation after a two month inoculum acclimation period, the inoculum was acclimated for an additional two months to see if rates improved. Figures F.1 and F.2 depict regression analyses after a four month total acclimation period for a range of ammonia and salinity concentrations, respectively. Table F.1 provides a summary of the kinetic rates and coefficients of determination.
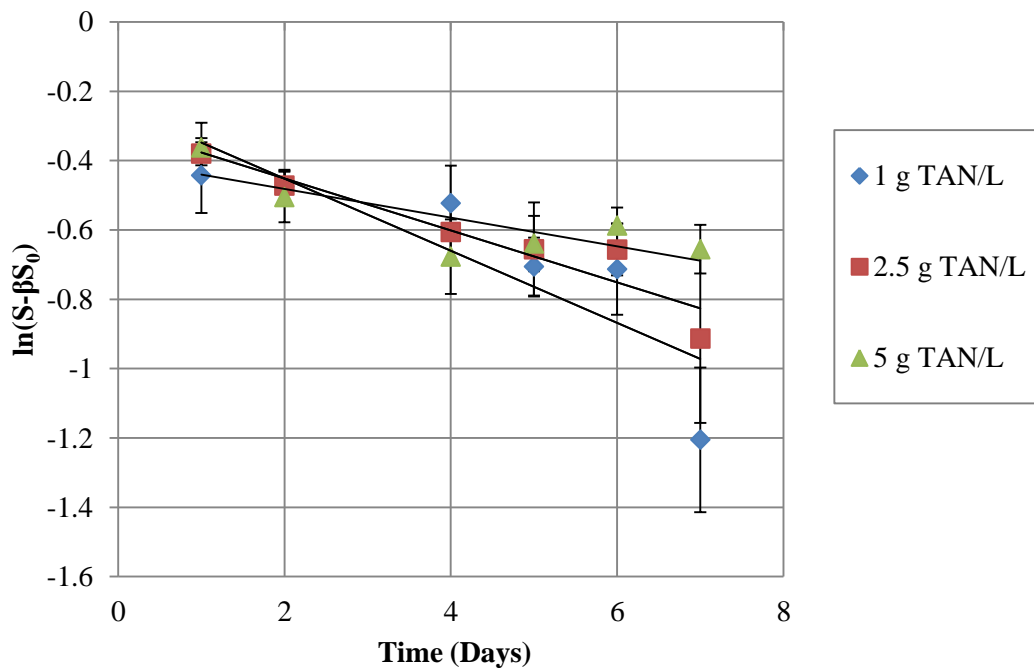
Figure F.1 Regression analyses for a range of ammonia concentrations (β = 0.11)
Error bars represent standard deviations in triplicate reactors.
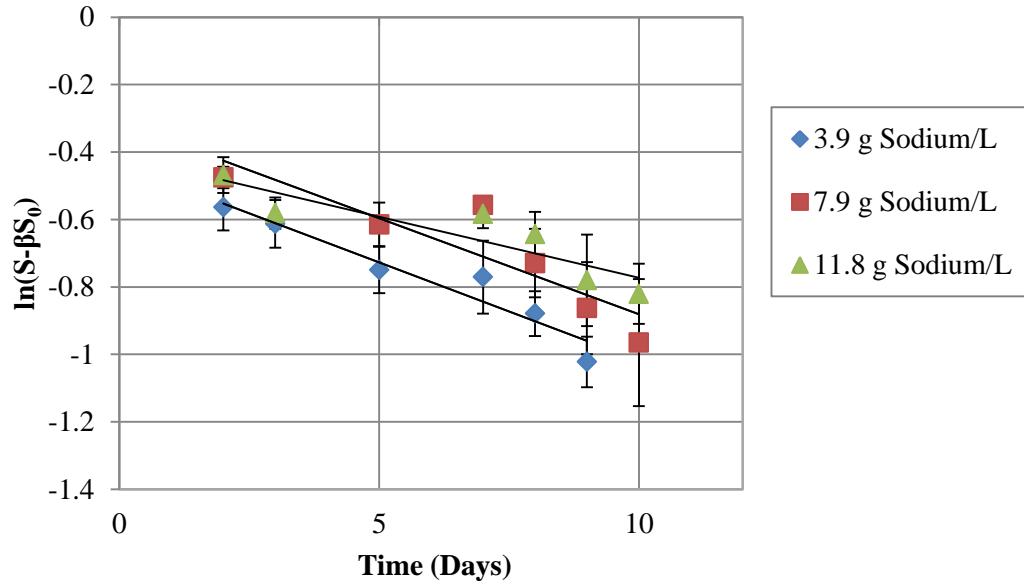
Figure F.2 Regression analyses for a range of salinity concentrations (β = 0.11)
Error bars represent standard deviations measured in triplicate reactors.

Table F.1 Kinetic rate values according to regression analyses

Reactor	Kinetic rate, k (day^{-1})*	R^2
1 g TAN/L	0.097 (0.005)	0.72
2.5 g TAN/L	0.069 (0.017)	0.90
5 g TAN/L	0.043 (0.020)	0.65
3.9 g sodium/L	0.053 (0.011)	0.93
7.9 g sodium/L	0.054 (0.017)	0.79
11.8 g sodium/L	0.036 (0.013)	0.80

*Parentheses indicate standard deviations in triplicate reactors.

The trends of the kinetic rates (Table F.1) are extremely similar to the rates obtained after only two months of acclimation. Interestingly, the actual rates are slower than the rates obtained at two months, but this could be explained potentially by a food source with a different composition at four months (i.e., more cellulosic material). As the ammonia concentration increased, the kinetic rate gradually decreased. The trends resulting from increasing salinity concentrations were almost identical to the results

obtained two months prior; the two lowest sodium concentrations (3.9 and 7.9 g sodium/L) demonstrated the same kinetic rates, whereas the highest concentration (11.8 g sodium/L) yielded a slower kinetic rate. These results suggest that additional acclimation of inocula beyond two months will not further improve kinetic rates, and two months is sufficient to accommodate the rate of change of microorganisms to elevated ammonia and salinity concentrations.

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