DISSERTATION

DEVELOPMENT OF ADVANCED MICROBIAL COMMUNITIES FOR ENHANCING WASTE HYDROLYSIS PROCESSES: INSIGHTS FROM THE APPLICATION OF MOLECULAR BIOLOGY TOOLS

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ABSTRACT

DEVELOPMENT OF ADVANCED MICROBIAL COMMUNITIES FOR ENHANCING WASTE HYDROLYSIS PROCESSES: INSIGHTS FROM THE APPLICATION OF MOLECULAR BIOLOGY TOOLS

Anaerobic digestion (AD) is an environmentally attractive technology for conversion of various solid wastes to energy. However, despite numerous benefits, AD applications to OFMSW remain limited in North America due to economic barriers with existing technologies. Suboptimal conditions in anaerobic digesters (e.g., presence of common inhibitors ammonia and salinity) limit waste hydrolysis in AD and lead to unstable performance and process failures compromising economic viability. To guide development of microbial management strategies to avoid process upsets and failures due to inhibitors, hydrolysis rates were determined in batch, single-stage digesters seeded with unacclimated or acclimated inocula under a range of ammonia and salinity concentrations for two model feedstocks (food waste and manure). Using unacclimated inocula, hydrolysis was found to be severely inhibited for elevated ammonia (decrease of nearly 4-fold relative to baseline) and salinity (decrease of up to 10-fold relative to baseline). However, for inocula acclimated over 2 to 4 months, statistically significant inhibition was not detectable except in the case of food waste subjected to elevated ammonia concentrations (p-value = 0.01). Inhibitors and feedstock were found to have a major influence on bacterial community structure. Next, a more detailed analysis of the acclimation process revealed that microbial communities under stressed conditions (elevated ammonia) adapt more slowly (weeks) to feedstock changes (from wastewater sludge to manure or filter paper) than

under non-stressed conditions (days). Molecular tools were utilized to separate temporal effects on hydrolyzers from temporal effects on methanogens. Bacterial and archaeal sequencing results identified multiple organisms (e.g., *Clostridiales vadinBB60, Ruminococcaceae, Marinilabiaceae, Methanobacterium,* and *Thermoplasmatales Incertae Sedis*) that were selected for in microbial communities in stressed reactors under perturbed conditions (feedstock changes). Collectively, results from these studies suggested that weeks of acclimation are required to build up sufficient quantities of desired hydrolyzing microbes; thus, hydrolysis processes operated in batch mode should be inoculated with each new batch, and desired microorganisms should be maintained in the system via properly developed inoculation strategies. To identify improved methods of maintaining such communities in multi-stage reactor systems, reactor performance under elevated ammonia and salinity was compared for leach bed reactors (LBRs) seeded with unacclimated inoculum and different ratios of acclimated inoculum (0-60% by mass) at start-up. Additionally, the effect of seeding methods was examined by identifying the optimal ratio of fresh waste to previously digested waste in multi-stage systems incorporating leachate recycle during long-term operation. Results demonstrated that high quantities of inoculum (~60%) increase waste hydrolysis and are beneficial at start-up or when inhibitors are increasing. After start-up (~112 days) with high inoculum quantities, leachate recirculation leads to accumulation of inhibitor-tolerant hydrolyzing bacteria in leachate. During long-term operation, low inoculum quantities (~10%) effectively increase waste hydrolysis relative to without solids-derived inoculum. Additionally, molecular analyses indicated that combining digested solids with leachate-based inoculum doubles quantities of Bacteria contacting waste over a batch and supplies additional desirable phylotypes *Bacteriodes* and *Clostridia*. To provide detailed insight into microbial community activity during degradation,

metatranscriptomic analyses were conducted on reactors fed food waste and manure under low ammonia, and several common active (e.g., *Methanomicrobia*, *Methanosaeta concilii*, and *Clostridia)* and unique active (e.g., *Enterobacteriaceae*, *Clostridium thermocellum,* and *Clostridium celluloyticum*) phylotypes between the reactors were identified. Functional classification of the active microbial communities generally revealed several similarities between the reactors despite the differences in feedstock. However, similarities or differences in transcript abundance for specific gene categories (e.g. one-carbon metabolism or fermentation) might indicate some potentially useful biomarkers for monitoring process health. Additionally, data from this experiment expanded the gene sequence database for assay development, which is particularly key for improving current functional gene-targeted assays to more accurately characterize microbial communities. Overall, results from this study have provided operational guidance for establishing and maintaining desired microbial communities as inocula to enhance waste hydrolysis for a variety of feedstocks.

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1.0 Introduction

Increased regulations for waste management have augmented the need for cost-effective and sustainable methods of organic waste disposal. For example, the state of California recently implemented a statewide goal to divert 75% of total waste from landfills (NRDC, 2014), and several states have passed laws or are proposing regulations to prevent commercial food scraps from being landfilled (BioCycle, 2013). Additionally, recent regulations and public interest have heightened interest in diverting the organic fraction of municipal solid waste (OFMSW) from landfills (Levis et al., 2010). Anaerobic digestion (AD) is a valuable waste-to-energy technology that provides a means of utilizing organic waste (e.g., waste that would otherwise ultimately end up in landfills) to produce useful products including methane and soil amendments. Furthermore, AD is capable of treating two prevalent waste streams that are difficult to sustainably manage: manure and OFMSW. For example, traditional methods of manure disposal (e.g., anaerobic lagoons or land application) may contribute to surface water and groundwater contamination and result in discharge of significant quantities of methane to the atmosphere (Gloy, 2011; US EPA, 2011). Despite numerous benefits to AD, however, applications of AD for management of dry-scraped manure and OFMSW remain limited in the U.S. because currently available technologies are not economically viable; thus, future work is needed to improve process reliability and performance and to provide for widespread adoption in the U.S.

1.1 Problem overview and specific research needs

One challenge to optimizing bioenergy generation via AD is the presence of microbial inhibitors that can lead to suboptimal performance or even process failures. For example, elevated levels of inhibitors, including ammonia and salinity, are often found in AD systems,

particularly those digesting high-salinity (food waste) or high-ammonia (manure) feedstocks. Inhibition of AD performance due to elevated ammonia and salinity has been demonstrated to occur at concentrations >1.7 g TAN/L and 3.5 g Na^+/L, respectively (Chen et al., 2008). Additionally, inhibitors may accumulate in systems that recycle leachate (Chen et al., 2008; Kayhanian, 1994; Shahriari et al., 2012), which is a common practice for high-solids AD technologies. Leachate recycle may be employed to retain methanogens in single-stage AD configurations and to reduce water usage in both single- and multi-stage systems.

Multi-stage AD technologies are promising options to treat a variety of wastes because these systems use separate reactors for each stage, allowing for individual optimization of hydrolysis and methanogenesis processes. Hydrolysis may be conducted in leach bed reactors (LBRs) operated in dry batch mode because LBRs are designed to accept high solids waste (>15% solids) (Mata-Alvarez et al., 2000). For example, AD technologies treating food waste recently implemented in the U.S. operate as LBR-based systems often due to the high solids content of food waste (30-60% solids); similarly, dry-scraped manure may contain greater than 26% solids content (Hall et al., 1985; Rapport et al., 2008). Often, reactors are fed a constant feedstock composition to prevent reactor upset or failure, although the ability to accept additional substrates is often desirable for increasing bioenergy production but has associated risks with respect to process stability. Next, methanogenesis is conducted in high-rate reactors including fixed film (FF) or upflow anaerobic sludge blanket (UASB) reactors (Najafpour et al., 2006). Numerous studies have focused on improving methanogenesis under inhibitory conditions (Chen et al., 2008), but studies focused on enhancing hydrolysis in the presence of inhibitors are lacking. Hydrolysis is the first and often rate-limiting step in AD (Nielsen et al., 2004). Thus,

advancements in hydrolysis processes have the potential to improve AD performance and economic viability.

In particular, operational procedures need to be developed to guide the establishment and maintenance of optimal microbial communities required for digestion in the presence of elevated ammonia and salinity. In practice, little attention is given to microbial community management at a highly technical level, but the types of microbes that are favored and maintained in a system (e.g., microbial residence times) have important implications for process performance. Acclimation, the process of adapting microbial populations 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). For example, Hashimoto (1986) demonstrated that inhibition of methanogenesis occurs at a lower ammonia level for un-acclimated methanogenic communities (2.5 g TAN L^{-1}) than for acclimated methanogenic communities (4 g TAN L^{-1}). However, the impact of acclimation on hydrolysis rates and the microbial ecology of hydrolyzing bacterial communities under conditions of elevated ammonia and salinity have not been well studied. Furthermore, since reactor performance also is controlled by the quantity of required microorganisms present in a system, inoculation guidelines (e.g., mass of inoculum required) for reactors that operate in batch mode (LBRs) are needed to ensure that sufficient desirable microorganisms are provided at the start of each new batch. Similarly, knowledge of the time required for bacterial and archaeal communities to acclimate to perturbation under stress (e.g., allowing ammonia and salinity concentrations to increase through leachate recycle or changing feedstock composition) is critically needed to inform digester start-up, operation (e.g., solids residence times and amount of inoculum required), and approaches for

microbial communities management (via inoculation) during changes in reactor conditions or operation.

Since microbial community composition is linked with reactor performance (Liu et al., 2002), tracking microbial communities over time via molecular biology assays could elucidate key changes in composition or quantities required for maintaining optimal performance. Specifically, direct information about the functional capabilities of community members is desirable (Pereyra et al., 2012) since the phylogenetic composition of a system does not necessarily correlate to functionality of a system (Langille et al., 2013). Unfortunately, currently available functional gene-targeted assays (e.g., assays targeting genes involved in cellulose degradation) were developed for other anaerobic applications (e.g., reactors treating acid mine drainage) and are not always successful for microbial communities found in AD. For example, in some cases target genes in our study could not be detected even though reactor performance data indicated that genes encoding enzymes with the target function were present (data not shown). Others have found assays targeting cellulose genes unsuccessful as well (*personal communications*). These results suggest that some published primers targeting relevant functional genes are not as broadly applicable as desired. Thus, improved assays are needed to provide more accurate and robust methods for monitoring microbial community composition in anaerobic digesters. Molecular polymerase chain reaction-based assays may be improved by modifying primers to eliminate mismatches with gene targets to ensure that assays accurately quantify functional genes in the target microbial communities. Primer modification requires knowledge of the target gene sequences, which are often unknown for uncultured mixed microbial communities in reactor systems. However, target gene sequences can now be obtained directly from environmentally relevant communities via next generation sequencing and may be

utilized to modify existing primers or create relevant new primers that will specifically target bacteria associated with AD.

1.2 Research objectives

The overarching goal of this work was to enhance AD performance under stresses or during perturbations via optimizing microbial seeding methods and microbial management strategies. To this end, the following specific project objectives were to:

1a) determine the effects of ammonia and salinity concentrations on hydrolysis rates, and to

1b) determine the impact of microbial community acclimation on hydrolysis rates under these conditions for two model substrates (manure and food waste),

2a) investigate bacterial and archaeal community dynamics under a model stress (elevated ammonia) during a digester perturbation (changing the feedstock from wastewater sludge to one of two model feedstocks), and

2b) identify Bacteria and Archaea that are selected for in digesters operating under elevated ammonia with the two model feedstocks (manure and filter paper),

3a) evaluate initial and long-term inoculation methods to establish enriched inhibitor-tolerant hydrolyzing communities and enhance performance of LBRs, and to

3b) advance understanding of hydrolyzing microbial communities in multi-stage AD systems, and

4a) elucidate the impact of different feedstocks on the composition of the active microbial community and the functional microbial processes, and

4b) obtain sequences of genes expressed during the degradation of two model substrates to identify putative functional gene biomarkers of hydrolysis for monitoring bacterial community health in AD systems.

1.3 Research tasks

The tasks below were designed to comprehensively address the overarching research goal and specific objectives. The specific tasks were as follows:

1) **Operate laboratory-scale batch reactors to determine hydrolysis kinetics as a function of inhibitor concentrations with and without inoculum acclimation**.

   Hypothesis 1: Elevated levels of ammonia and salinity negatively impact hydrolysis rates, and acclimation of microbial inocula will mitigate these negative impacts leading to higher hydrolysis rates.

   The approach involved monitoring reactor performance (e.g., dissolved chemical oxygen demand [DCOD]) for inoculum exposed directly or acclimated to elevated ammonia and salinity. Additionally, molecular tools (e.g., terminal restriction fragment length polymorphism [T-RFLP] assays) were utilized to track changes in the bacterial and methanogenic communities as a function of ammonia and salinity concentrations as well as feedstock.

2) **Operate batch-scale reactors to track dynamic bacterial and archaeal community responses during acclimation to perturbation under stress.**

   Hypothesis 2: Bacterial and archaeal community acclimation times will differ with archaeal acclimation times being longer.

   Hypothesis 2a: Acclimation periods will be on the order of weeks.

This time period is similar to or longer than the length of a batch digestion period, suggesting the need to actively maintaining inhibitor-tolerant strains in leach bed reactors operated in batch mode under high ammonia and salinity conditions.

The approach involved tracking reactor performance and microbial community composition via molecular biology assays (e.g., T-RFLP) to determine bacterial and archaeal dynamics during acclimation. Ammonia was used as a model stress, and digester perturbation was simulated via changing feedstocks from wastewater sludge to manure or filter paper. Next-generation sequencing was utilized to identify microorganisms present in communities post-acclimation.

3) **Conduct laboratory-scale, multi-stage AD reactor microbial seeding tests.**

Hypothesis 3: Reactors seeded initially with acclimated inocula will operate at higher rates of hydrolysis and methanogenesis than reactors operating with unacclimated inocula.

Hypothesis 4: Waste reduction will be limited by the quantity of organisms provided as inoculum in the LBRs under elevated ammonia and salinity.

The approach was to operate laboratory-scale, multi-stage AD systems fed post-consumer OFMSW under elevated ammonia and salinity conditions. The impact of inoculum type on hydrolysis performance was investigated by comparing performance for LBRs seeded with unacclimated inoculum or acclimated inoculum. After tests revealed that acclimated inoculum performed better than unacclimated inoculum, ratios of fresh waste to previously digested waste (used as the inoculum source of microorganisms for each successive batch of waste) were tested via seeding reactors with decreasing percentages (60 - 0%) of inoculum over time and tracking reactor performance. Molecular tools were utilized to

quantify the bacteria present in the leachate compared to inocula and fresh waste, and key bacteria linked with improved performance were identified via next-generation sequencing.

4) **Sequence and identify functional genes expressed during hydrolysis of manure and food waste.**

This last task was not hypothesis-driven, but rather was an effort to expand the databases for functional genes that could serve as potential biomarkers for molecular assay development. The approach was to operate semi-continuous, single-stage reactors fed manure or food waste. Metatranscriptomic analyses were utilized to identify active Bacteria and Archaea and profile active functional genes for each reactor as a function of feedstock.

1.4 Dissertation organization

Relevant background information is provided in this first chapter as well as at the beginning of each of the following chapters. Chapters 2-4 describe and discuss experimental results related to objectives 1-3. Each chapter is based on a published paper (chapter 2) or a manuscript prepared for submission as a journal article (chapters 3-4). Thus, each chapter is formatted to meet the requirements of the appropriate journal. The second chapter, "Microbial community acclimation enhances waste hydrolysis rates under elevated ammonia and salinity," compares the performance of small-scale batch reactors inoculated with acclimated or unacclimated inoculum exposed to elevated ammonia and salinity. Molecular analyses tracking microbial community changes during acclimation demonstrated that feedstock and stress impact microbial communities. This work has been published in *Bioresource Technology*. The third chapter, "Stress impacts microbial community responses to feedstock changes during bioenergy generation," presents performance data and detailed microbial community dynamics in reactors that are operating under stress (elevated ammonia) and impacted by perturbation (feedstock

changes). This manuscript was prepared for submission to *Bioresource Technology*. Chapter four, "Enhanced anaerobic digestion performance via combined solids- and leachate-based hydrolysis reactor inoculation," discussions the construction, operation, and performance of laboratory-scale reactors operating under stress with unacclimated or varying ratios of acclimated inoculum. This manuscript was prepared for submission to *Bioresource Technology.* Chapter five, "Comparative metatranscriptomic analysis of microbial communities digesting manure or food waste," presents an analysis of RNA sequencing data for reactors operating with two different feedstocks. This work is part of a larger effort and will become part of a longer collaborative paper. My contribution to that manuscript is presented herein. The final chapter presents overall conclusions gained from these studies and suggests future research that could be undertaken as an extension of this work. Appendices are included that detail specific methods and protocols utilized during this study.

2.0 Microbial Community Acclimation Enhances Waste Hydrolysis Rates under Elevated Ammonia and Salinity Conditions[1]

2.1 Introduction

The need for rapid start-up, cost-effective, renewable energy generation technologies in conjunction with the need for sustainable waste management has led to increased interest in development of waste-to-energy technologies such as AD. Over one billion tons of manure and 155 million tons of biodegradable municipal solid waste (i.e., OFMSW) are generated annually in the United States at present (Cuellar & Webber, 2008; US EPA, 2011). Currently employed waste management strategies, including anaerobic lagoons and landfilling, represent major contributors to greenhouse gas emissions. Alternatively, organic wastes can serve as bioenergy feedstocks. Livestock manure has the potential to meet approximately 1% of the U.S. energy demand (Cuellar & Webber, 2008), and it has been estimated that OFMSW generated in California could supply approximately 8% of the state's energy demands (Rapport et al., 2008). Furthermore, AD can be followed by composting to recover nutrients and generate valuable soil amendments. Thus, AD can be integral to the development of environmentally sustainable closed-loop resource management systems.

Despite clear benefits, applications of AD for management of manure and OFMSW remain limited in the U.S. because currently available technologies are not economically viable. Furthermore, conventional AD technologies are intended for treatment of slurry wastes (solids content < 10%) (Demirer & Chen, 2005) and require large volumes of water when applied to

[1] Wilson, L.P., Loetscher, L.H., Sharvelle, S.E., De Long, S.K. 2013. Microbial community acclimation enhances waste hydrolysis rates under elevated ammonia and salinity conditions. *Bioresource Technology*, **146**, 15-22.

high solids feedstocks (e.g., manure and food waste), making these systems technically infeasible in water-constrained regions. Multi-stage, leachate-bed systems are a promising technology with the potential to overcome technical and economic challenges (Demirer & Chen, 2005; Shahriari et al., 2012). These systems can be designed with high-solids dry fermentation LBRs for hydrolysis and operated with leachate recirculation to minimize water usage. Thus, multi-stage systems are suitable for application to feedstocks including manure collected in arid regions via dry scraping, which results in greater than 26% solids content (Hall et al., 1985), as well as to OFMSW, which contains 30-60% solids (Rapport et al., 2008). Furthermore, multi-stage AD systems also benefit from higher methane yields because each process stage (e.g., hydrolysis, acido/acetogenesis, and methanogenesis) can be independently optimized (Shahriari et al., 2012; Ward et al., 2008). However, design and operational challenges, such as the accumulation of inhibitors due to leachate recirculation, remain (Cysneiros et al., 2011).

Although energy inputs and fresh water consumption are reduced by leachate recirculation, the resulting elevated levels of inhibitors, including ammonia and salinity, may decrease process performance (Shahriari et al., 2012). Total ammonia nitrogen (TAN) and sodium concentrations ranging from 1.7 to 14 g TAN L^{-1} and 5.6 to 53 g sodium L^{-1} have been shown to decrease methane production by 50% (Chen et al., 2008). However, hydrolysis kinetic rates as a function of ammonia and salinity concentrations have not been reported. Knowledge of the impact of elevated ammonia and salinity concentrations on hydrolysis rates is needed to guide reactor design to determine the minimum mass input ratio of fresh water to recirculated leachate. Since hydrolysis is rate-limiting in the digestion of lignocellulosic wastes (Nielsen et al., 2004), optimization has the potential to greatly increase AD economic profitability by lowering required reactor volumes, decreasing operating costs, and increasing methane

generation. Higher capital costs are associated with multi-stage systems (Ward et al., 2008), and in order to remain economically viable, these systems must be fully optimized.

Moreover, the development of strategies to achieve and maintain optimal microbial communities in AD systems would likely enhance the economic viability of AD (Steinberg & Regan, 2008), and could potentially lead to optimal hydrolysis rates even in the presence of elevated ammonia and salinity. Acclimation, the process of adapting microbial populations 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., 2003b; Chen et al., 2008; Hashimoto, 1986). However, the impact of acclimation on hydrolysis rates and the microbial ecology of hydrolyzing bacterial communities under conditions of elevated ammonia and salinity have not been well studied.

The study described herein was based on the hypothesis that elevated levels of ammonia and salinity would negatively impact hydrolysis rates, and further, that acclimation of microbial inocula would mitigate these negative impacts leading to higher hydrolysis rates. Thus, the objectives of this study were: 1) to determine the effects of ammonia and salinity concentrations on hydrolysis rates, and 2) to determine the impact of microbial community acclimation on hydrolysis rates under these conditions for two model substrates (manure and food waste). To this end, hydrolysis kinetics were determined via laboratory-scale batch tests as a function of inhibitor concentrations with and without inoculum acclimation. Additionally, molecular tools (T-RFLP assays) were utilized to track changes in the bacterial and methanogenic communities as a function of ammonia and salinity concentrations as well as feedstock.

2.2 Methods

2.2.1 Waste and inocula collection

Manure samples were collected in 5-gallon buckets from Five Rivers Cattle Feeding LLC in Greeley, CO. Representative samples from 3 buckets were combined, ground and homogenized using a food blender (Hamilton Beach, Southern Pines, NC), and then sieved (0.5 mm mesh). Food waste was collected from Colorado State University Ram's Horn dining facility where food waste is separated, passed to a pulper, and mixed with water to produce a pulpable slurry. Next, the slurry is transported to an attached Hydra-Extractor (Somat Company, Lancaster, PA), which removes the majority of water, and the remaining pulp is deposited in bins. Samples from 3 bins were collected, and the pulp was ground and homogenized further in a food blender at high speed for 3 minutes. After collection, the manure and food waste were stored at 4°C for subsequent use for approximately 1 – 4 months. Microbial inocula were collected from the Drake Municipal Wastewater Treatment Plant (DWWTP) mesophilic anaerobic digester (Fort Collins, CO). The inocula were purged with nitrogen gas, maintained at 35°C, and used within 2 days of collection. Waste and inocula 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 (Table 1). Manure and inocula were collected twice during this study, and samples 1 and 2 were used to determine hydrolysis rates pre- and post-acclimation, respectively. Within each set of pre- or post-acclimation experiments, the same manure and inoculum samples (1 or 2) were used for every test to avoid compositional differences due to different collection periods (Table 2.1).

Table 2.1. Characteristics of inocula and feedstock

	Parameter			
Sample	TCOD	DCOD*	TS (%)	VS (% of TS)
Manure 1	0.82 g COD/g waste	ND	71.2	41.3
Manure 2	0.53 g COD/g waste	ND	77.3	48.4
Food	0.48 g COD/g waste	ND	29.3	95.9
Inoculum 1	25.6 g COD/L	0.36 g COD/L	1.8	72.1
Inoculum 2	22.3 g COD/L	0.28 g COD/L	1.6	74.6

*ND indicates not determined

Since different waste and inocula characteristics were observed, calculated hydrolysis rates may be compared only for reactors operating with the same manure and inoculum samples.

2.2.2 Hydrolysis batch reactor set-up

140-ml plastic syringes (Sherwood Medical, Northern Ireland) fitted with three-way luer lock valves were used as small-scale batch reactors with a 120-ml working volume to allow for biogas collection at constant pressure and anaerobic sampling. Digestion tests were conducted in triplicate or quintuplicate for manure and food waste, respectively, with 50% nutrient solution (Owen et al., 1979) and 50% unacclimated or acclimated inocula (see *Section 2.2.3*). Five replicates were used for food waste to ensure accurate results due to the greater heterogeneity of the feedstock. Feedstock was supplied at an initial concentration of 5 g COD L^{-1}. Ammonia (supplied as NH_4Cl) and salinity (supplied as NaCl) were added to the nutrient solution to

produce elevated levels of ammonia (1.0, 2.5, 5.0, and 10.0 g TAN L^{-1}) or salinity (3.9, 7.9, 11.8, and 20.0 g sodium L^{-1}); these concentrations were selected because they could occur in reactors that incorporate leachate recirculation (Shahriari et al., 2012) and are in the range of salinity and ammonia levels that have been reported previously to decrease methane production (Angelidaki & Ahring, 1993; Chen et al., 2008). Digestion tests also were conducted for the "baseline" ammonia and salinity concentrations (0.14 g TAN L^{-1} and 1.2 g sodium L^{-1}) present in the nutrient solution using manure as a substrate to ensure that rates under non-stressed conditions were comparable to literature values. Syringes were incubated in an incubator shaker (100 rpm) at 35°C for approximately 10 to 15 days. Syringes without waste were run as controls to measure gas production from the inocula alone. Additionally, syringes were run with glucose (substrate that does not require hydrolysis) as the sole carbon source to measure rates of methanogenesis in the batch reactors. Methanogenesis rates were found to be faster than hydrolysis rates (data not shown); and thus, it was concluded that sufficient levels of methanogens were present in all inocula to ensure that hydrolysis was the rate-limiting step.

2.2.3 Acclimation of microbial inocula

Initial tests demonstrated the need for acclimation of the microbial inocula to the elevated ammonia and salinity concentrations tested. Thus, inoculum (sample 2) (200 ml) from DWWTP and nutrient solution (800 ml) were mixed in 1-L glass flasks and adjusted to the concentrations of ammonia (1, 2.5, and 5 g TAN L^{-1}) or sodium (3.9, 7.9, and 11.8 g sodium L^{-1}) that were utilized in the experiments to determine hydrolysis rates, and the pH was adjusted to approximately 7.1. All reactors were fed 5 g of manure sample 1 or food waste 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. Hydrolysis rates for inocula

fed manure were determined for each culture after four months of acclimation. Because tests indicated there were no statistically significant differences in the hydrolysis rates calculated for a range of ammonia and salinity concentrations for inocula fed manure (p-values = 0.43 and 0.18, respectively), inocula fed food waste were tested after two months to see if similar results could be obtained with less acclimation time. Inocula used for determination of hydrolysis rates under baseline conditions were not acclimated to the testing conditions prior to kinetic rate testing since these ammonia and salinity concentrations were not considered to be inhibitory (Chen et al., 2008).

2.2.4 Analytical methods

The volume of biogas produced was determined by measuring the distance the syringe plunger moved with digital calipers to ensure a high degree of accuracy, and gas volumes measured at 35 ºC were converted to volumes at standard temperature and pressure using the Ideal Gas Law. Methane content was determined using a Hewlett Packard Series 2180 gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with an Alltech column (Alltech, Deerfield, IL) packed with HayeSep Q 80/100 mesh (HayeSeparation, Inc., Bandera, TX) operating at injection and detector temperatures of 100°C. TS and VS were measured according to standard methods (APHA, 1995). Liquid samples were analyzed for TCOD and DCOD using Hach's COD High Range Vials and digestion colorimetric method (Hach, Loveland, CO). Samples were filtered through a 0.2 μm syringe filter prior for DCOD analysis. Hach's Nitrogen-Ammonia High Range Reagent Set was utilized to verify that there was not a notable increase in ammonia concentrations due to ammonia contributed from the waste feedstocks. Free ammonia concentrations were calculated as a function of pH, temperature, and total ammonia nitrogen concentrations. Adenosine-triphosphate (ATP) concentrations of the inocula were quantified

using the BacTiter-Glo Microbial Cell Viability Assay (Promega, Madison, WI) according to the manufacturer's protocol.

2.2.5 Calculation of hydrolysis rates

Hydrolysis rates were calculated in each reactor by isolating the hydrolysis step from methanogenesis. All of the hydrolysis products (i.e., soluble products and methane) were converted to COD equivalents, summed for each time point, and divided by the initial COD input to calculate the extent of substrate solubilization with Equation 1 as described previously (O'Sullivan et al., 2008):

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

Each reaction period lasted approximately 10-14 days, and it was determined that new biomass formed during this time was negligible (< 1%) compared 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 as described previously (Vavilin et al., 2008):

$$dS/dt = -k(S - \beta S_0) \quad (Eq.2)$$

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-biodegradable fraction of the substrate, and S_0 is the initial substrate concentration. β was determined by measuring the amount of methane and DCOD produced over an 8-week period, and substrate remaining in the reactors after 8 weeks was assumed to be non-biodegradable (β = 0.49 and 0.11 for manure and food waste, respectively). Kinetic rates were calculated for each reactor by plotting $\ln(S-\beta S_0)$ as a function of time, and then kinetic rates were averaged for the replicate reactors.

2.2.6 Microbial community composition analyses

T-RFLP analyses were conducted on DNA isolated from the unacclimated and acclimated inocula. DNA was extracted using the MoBio PowerSoil® DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) and stored at -20°C. To track changes to the bacterial and methane-producing microbial communities, T-RFLP analyses were conducted targeting the 16S rRNA gene and the *mcrA* gene (which encodes the alpha subunit of methyl-coenzyme M reductase). T-RFLP analysis was conducted targeting the 16S rRNA gene using primers 8F and 1392R as described by Dollhopf et al. (2001), except where differences are noted; T-RFLP analysis targeting the *mcrA* gene was conducted using primers 1035F and 1530R developed by Luton et al. (2002) as described by Lefèvre et al. (2013), except where differences are noted. Forward primers were labeled on the 5' end with 6-carboxylfluorescein (6-FAM) dye. Amplifications were performed using a Bio-Rad S1000 Thermocycler (Bio-Rad Laboratories, Hercules, CA). For analyses targeting the 16S rRNA gene, reactions consisted of 1X Reaction Buffer (5 Prime, Gaithersburg, MD), 1.5mM of $MgCl_2$ solution (5 Prime), 0.2 mM of each dNTP (New England Biolabs [NEB], Ipswich, MA), 0.2 mg/ml of BSA (NEB), 0.5 µM of each primer, 2 U of Taq polymerase (5 Prime), 2 µl of 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 25 cycles of 30 s at 94 ºC, 30 s at 55 ºC, and 30 s at 72 ºC, and a final extension of 7 min at 72 ºC. For analyses targeting the *mcrA* gene, reactions consisted of 1X Reaction Buffer (5 Prime), 1X PCR Enhancer (5 Prime), 1 mM of $MgCl_2$ solution (5 Prime), 0.05 mM of each dNTP (NEB), 0.5 µl of formamide, 0.2 µM of each primer, 7 U of Taq polymerase (5 Prime), 2 µl of 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. All amplicons were treated with 2.5 U of Klenow fragment. The products were then purified using NucleoSpin Extract II (Macherey-Nagel, Bethlehem, PA) and re-suspended in 35 µl of elution buffer. 35 µl of the products were run on a gel, and the bands were extracted using the NucleoSpin Extract II kit (Macherey-Nagel). The purified amplicons were digested with 10 U each of restriction enzymes MspI and HaeIII (*mcrA*) or HhaI and MspI (16S rRNA) (NEB) and 1X Buffer N4 (NEB) for 12 hr at 37ºC. The digestion products were cleaned-up using the QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany) and stored at -20 ºC. T-RFLP analyses were conducted at the Colorado State University Proteomics and Metabolomics Facility.

2.2.7 Statistical analyses

For the batch reactor tests, analysis of variance (ANOVA) tests were conducted using R Statistical Software (R Core Team, 2012) to determine if the slope of fitted regression lines for kinetic rates as a function of ammonia and salinity concentrations were statistically different from 0 as well as to determine statistical differences in average hydrolysis kinetic rates for a range of ammonia and salinity concentrations. Results yielding a p-value less than 0.05 were considered to be significant. To analyze the T-RFLP data, estimated fractional abundances for each operational taxonomic unit (OTU) were calculated by dividing each fragment peak area by the total peak area for a given electropherogram. Terminal restriction fragments (T-RFs) representing less than 5% of the total area were excluded as described previously (Rees et al., 2004). T-RFLP peaks were manually aligned among samples, and T-RFs were considered to be the same if their respective sizes did not differ by more than 1.5 base pairs (bp). Matrices of T-RF relative abundance were assembled, and similarity matrices were calculated according to the Bray-Curtis coefficient using the statistical software Primer V6 (Primer-E Ltd, Plymouth, United

Kingdom), and non-metric multidimensional scaling (MDS) plots were produced to analyze microbial community differences. Stress values less than 0.1 represent 2-dimensional ordinations with little risk of misinterpretation, values less than 0.2 correspond to useful ordinations, and values over 0.2 represent nearly random plots (Clarke, 1993; Rees et al., 2004). Plots were generated using 100 restarts.

2.3 Results and discussion

2.3.1 Determination of hydrolysis rates with unacclimated inocula

The batch system hydrolysis data were found to fit a first-order hydrolysis kinetic model (Eq. 2) (data not shown), and rates determined for manure under baseline conditions (0.03 and 0.09 day^{-1} for samples 1 and 2, respectively) were comparable with rates previously reported in the literature. For example, Vavilin et al. (2008; 1997) reported hydrolysis rates for pig and cattle manure to be 0.1 day^{-1} and 0.13 day^{-1}, respectively. Previously reported first-order rates for cellulose, a major component of manure, range from 0.04-0.13 day^{-1} (Gujer & Zehnder, 1983; Liebetrau et al., 2004; O'Sullivan et al., 2008). The hydrolysis rates for manure reported herein are likely slightly lower than previously reported values because the manure used in this study had an extremely high solids content because it was not collected fresh or via wet-scraping as is commonly done in regions with abundant water supplies. The observed variability in measured hydrolysis rates between manure samples 1 and 2 was not unexpected because both the manure composition and microbial inocula varied between collection events (Table 1). Thus, for tests conducted to determine hydrolysis rates as a function of inhibitor concentrations, both feedstock sources and inoculum sources originated from the same collection event.

Initial attempts to determine hydrolysis rates with inocula subjected directly to elevated ammonia and salinity concentrations revealed that these unacclimated inocula performed poorly (Fig. 2.1a, b).

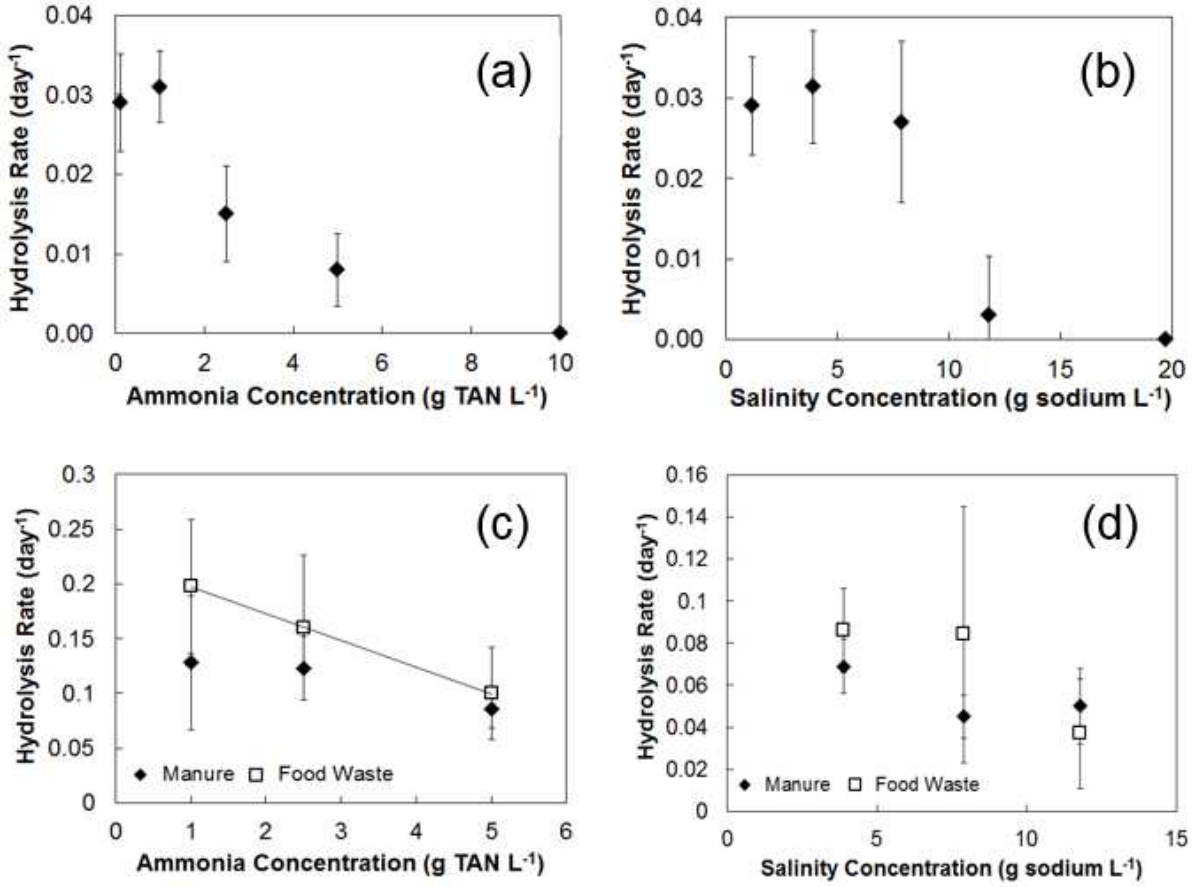

Figure 2.1. Hydrolysis kinetic rates for the unacclimated and acclimated inocula for a range of ammonia and salinity concentrations.Tests with unacclimated inocula as a function of ammonia (a) or salinity (b) concentration were conducted with manure and inoculum samples 1. Tests conducted with acclimated inocula as a function of ammonia (c) or salinity (d) concentration were conducted with manure (sample 2) or food waste. All acclimated inocula were derived from inoculum sample 2. Since feedstocks and inocula originated from different collection events, hydrolysis rates from unacclimated and acclimated inocula (Figs. 1a and c, Figs. b and d) should not be compared directly. Error bars represent standard deviations for triplicate reactors. The regression line shown for hydrolysis rates as a function of elevated ammonia with food waste has a slope of -0.024 L g^{-1} TAN day^{-1} (p-value less than 0.05); no other regression lines were found to be statistically significant, and thus, are not shown.

Although hydrolysis rates were comparable to rates under baseline conditions for up to 1 g TAN L^{-1}, hydrolysis rates were substantially reduced (~ 4-fold) for 5 g TAN L^{-1} (Fig. 2.1a), and hydrolysis was not detectable at 10 g TAN L^{-1} (i.e., measureable levels of DCOD and methane were not produced). Likewise, hydrolysis rates were similar to rates under baseline conditions for up to 7.9 g sodium L^{-1}, but rates decreased sharply as salinity concentrations increased up to 11.8 g sodium L^{-1} (Fig. 2.1b). The average rate measured at 11.8 g sodium L^{-1} was 10-fold lower than the average rate at baseline conditions. Hydrolysis was not detectable for 20 g sodium L^{-1}. Furthermore, ATP concentration (the energy currency of living cells) was monitored throughout the batch tests and demonstrated dramatic reductions (up to 60%) in response to elevated ammonia and salinity concentrations (data not shown), indicating that the inocula responded poorly to the stressed conditions. Because maximizing hydrolysis rates is critical to achieving economic viability of AD systems, optimizing the hydrolyzing microbial community via acclimation was explored to determine if this strategy could improve performance for systems with leachate recirculation. 5 g TAN L^{-1} and 11.8 g sodium L^{-1} were selected as the upper limits for the post-acclimation tests because these were the maximum concentrations for which measurable hydrolysis was observed.

2.3.2 Determination of hydrolysis rates as a function of ammonia concentration with acclimated inocula

Acclimated cultures demonstrated hydrolysis kinetic rates in the range of previously reported rates for both manure and food waste (Fig. 2.1c, d; Table 2.2). For manure, the measured hydrolysis rate at the minimum ammonia concentration tested (1 g TAN L^{-1}) was 0.128 day^{-1}, and for the food waste, the rate at this ammonia level was 0.198 day^{-1}. Similarly, Bolzonella et al. (2005) reported a rate of 0.1 day^{-1} for OFMSW, and Liebetrau et al. (2004)

reported a rate of 0.12 day^{-1} for biowaste. However, since feedstocks and inocula originated from different collection events, hydrolysis rates from unacclimated and acclimated inocula (Figs. 2.1a and 2.c, Figs. 2.b and 2.d) should not be compared directly. As expected, food waste hydrolysis rates were higher than manure hydrolysis rates because manure is a more recalcitrant waste due to its higher lignocellulosic content.

Table 2.2. Summary of hydrolysis kinetic rates as a function of ammonia, salinity, and feedstock

Ammonia concentration (g TAN L^{-1})	Substrate	Hydrolysis rate (day^{-1})*	Salinity concentration (g sodium L^{-1})	Substrate	Hydrolysis rate (day^{-1})*
1 (0.015 g NH_3-N)	Manure	0.128 (0.06)	3.9	Manure	0.069 (0.01)
2.5 (0.038 g NH_3-N)	Manure	0.123 (0.03)	7.9	Manure	0.045 (0.01)
5 (0.075 g NH_3-N)	Manure	0.086 (0.02)	11.8	Manure	0.050 (0.02)
1 (0.015 g NH_3-N)	Food Waste	0.198 (0.06)	3.9	Food Waste	0.086 (0.02)
2.5 (0.038 g NH_3-N)	Food Waste	0.160 (0.07)	7.9	Food Waste	0.084 (0.06)
5 (0.075 g NH_3-N)	Food Waste	0.100 (0.04)	11.8	Food Waste	0.047 (0.03)

*Numbers in parentheses represent standard deviations for triplicate reactors. R^2 values ranged from 0.77 to 0.98.

Interestingly, acclimated cultures demonstrated hydrolysis kinetic rates in the same range even in the presence of elevated ammonia concentrations (Fig. 2.1c, d; Table 2.2). For acclimated inocula fed manure, statistical analyses revealed there were no significant differences among the average kinetic rates for a range of ammonia concentrations (p-value = 0.43) (Fig. 2.1c). Analyses also revealed that the slope of the hydrolysis regression line for inocula fed manure for a range of ammonia concentrations was not statistically different from 0 (p-value = 0.20). Thus, these findings suggest that acclimation substantially improved hydrolysis kinetic

rates. Hashimoto et al. (1986) reported similar findings concerning the effects of ammonia on methanogenesis of cattle manure; methane inhibition began at 2.5 g TAN L^{-1} for unacclimated methanogens but not until 4 g TAN L^{-1} for acclimated methanogens. In this study, hydrolysis rates were substantially reduced at 2.5 g TAN L^{-1} prior to acclimation, but hydrolysis rates comparable to rates demonstrated at baseline values were observed at 5 g TAN L^{-1} for acclimated inocula.

For food waste, microbial community acclimation substantially mediated ammonia inhibition effects; however, hydrolysis rates were found to decrease by 2-fold at 5 g TAN L^{-1} relative to 1 g TAN L^{-1} (Fig. 2.1c), suggesting that even after acclimation, elevated ammonia concentrations likely still have a moderate inhibitory effect. Although an ANOVA test did not detect significant differences among the average kinetic rates for a range of ammonia concentrations (p-value = 0.06), the slope of the food waste hydrolysis rate regression line (slope = -0.024 L g^{-1} TAN day^{-1}; Fig. 2.1c) was found to be statistically different from 0 (p-value = 0.01). This finding is not unexpected given that inhibition of microbial activity (e.g., methanogenesis) has been documented under conditions of high ammonia (Angelidaki & Ahring, 1993). Two possible explanations may describe the greater ammonia inhibition observed for reactors fed food waste. First, organisms present in the manure that was added to the reactors could have been more resilient to higher ammonia concentrations since manure typically contains elevated total nitrogen levels (approximately 3 – 4 g N L^{-1}) (Angelidaki & Ahring, 1993; Demirer & Chen, 2005) due to uric acid and undigested proteins, which produce elevated ammonia levels when anaerobically digested (Abouelenien et al., 2010). Second, the type of organisms required for the hydrolysis of food waste may not have been as resilient to elevated ammonia concentrations as the organisms selected for in reactors fed manure. Thus, even if

optimal inhibitor-tolerant microbial communities can be maintained in hydrolysis reactors, a tradeoff exists between the fresh water required to decrease inhibitory ammonia levels and increased process performance. The regression function developed herein can be used to predict relative hydrolysis rates at full-scale as a function of ammonia concentration, and therefore, provide guidance for determining the optimal fraction of recirculated leachate.

2.3.3 Determination of hydrolysis rates as a function of salinity concentration for acclimated inocula

Similar to the results obtained for elevated ammonia conditions, results showed that acclimation substantially enhanced hydrolysis rates in the presence of elevated salinity (Fig. 2.1d). Despite apparent trends of decreasing hydrolysis rates with increasing salinity, there were no statistically significant differences in hydrolysis rates detectable via ANOVA analysis for the range of salinity concentrations tested for reactors fed manure or food waste (p-values = 0.18, 0.29, respectively). Additionally, analyses indicated that the slopes of the manure and food waste hydrolysis rate regression lines (Fig. 2.1d) were not statistically different from 0 (p-values = 0.16, 0.16, respectively). Similarly, Chen et al. (2003b) reported an increase from 13 to 23 g sodium L^{-1} in the 100% methane inhibition level for anaerobic sludge after a longer acclimation period of approximately nine months, although hydrolysis was not investigated separately from methanogenesis. Thus, since acclimation of the inocula substantially improved reactor performance, results suggest that AD systems could perform well operating at or below the maximum inhibitor concentrations (5 g TAN L^{-1} and 11.8 g sodium L^{-1}) used in this study with acclimated inocula. Collectively, these findings suggest that during the acclimation period, the microbial communities adapted such that they displayed increased ammonia and salinity tolerance. Therefore, molecular biology analyses were conducted to examine this hypothesis.

2.3.4 Analysis of microbial community composition changes during acclimation

T-RFLP analysis of both the bacterial (via 16S rRNA gene-targeted T-RFLP) and archaeal (via *mcrA* gene-targeted T-RFLP) microbial communities revealed distinct differences in T-RFs between unacclimated and acclimated inocula (Fig. 2.2). Analysis of the 16S rRNA gene-targeted data revealed that all inocula contained between 3 and 8 major T-RFs (T-RFs that comprised greater than 5% of the total peak area for a given electropherogram) (Fig. 2.2a through e). Interestingly, one T-RF (83 bp) was unique to all acclimated inocula fed manure (with the exception of inocula fed manure acclimated to 3.9 g sodium L^{-1}) (Fig. 2.2b, d) but was not detectable in the original inocula and was not dominant in the inocula fed food waste (Fig. 2.2a, c, e). Furthermore, an additional T-RF (323 bp) was present in all acclimated inocula fed manure under elevated ammonia concentrations (Fig. 2.2b) but was not present in the original inocula or acclimated inocula fed food waste. Similarly, acclimated inocula fed food waste under elevated salinity concentrations each contained 2 T-RFs (159 and 256 bp) (Fig. 2.2e) that were distinct from the original inocula. *mcrA*-targeted T-RFLP analysis revealed that all inocula contained between 1 and 6 major T-RFs (Fig. 2.2f through j). Interestingly, 11 out of 12 acclimated inocula (with the exception of inocula fed food waste acclimated to 1 g TAN L^{-1}) contained the same T-RF (269 bp) (Fig. 2.2g through j) that was not detectable in the original inocula. These findings could indicate that certain bacterial or methanogenic phylotypes profited from the stressed conditions and became active during acclimation (Pesaro et al., 2004).

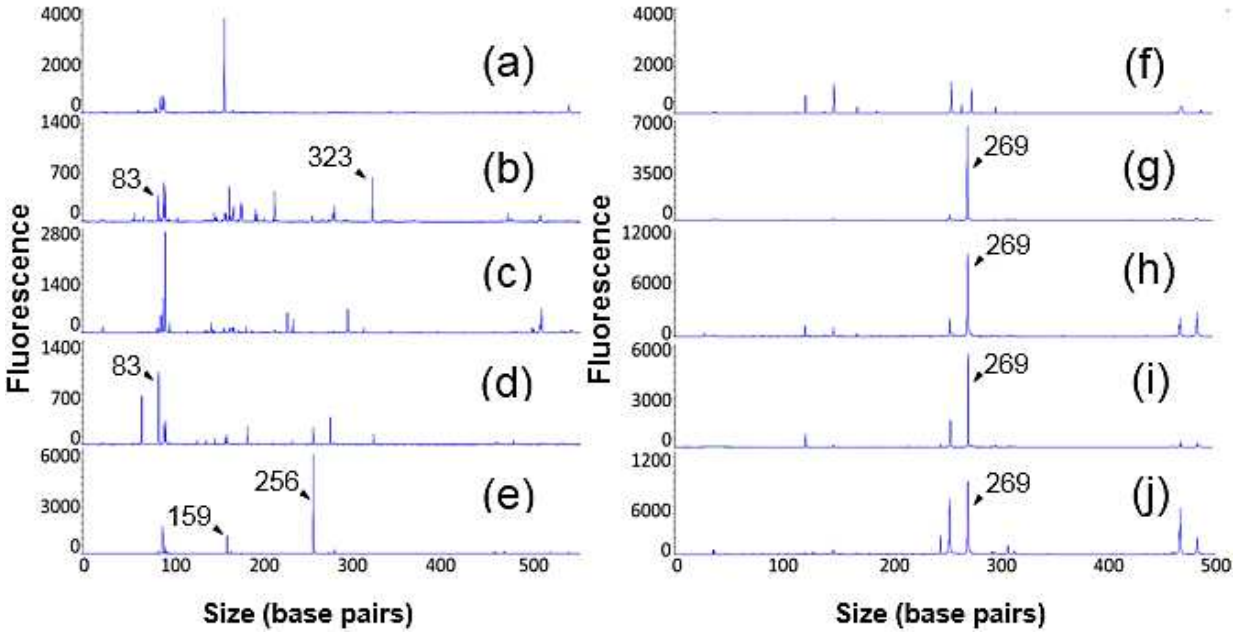

Figure 2.2. Representative electropherograms for T-RFLP analysis of the original inoculum and acclimated microbial inocula.16S rRNA gene-targeted T-RFLP electropherograms are shown for the original inoculum (a), inocula acclimated to 5 g TAN L^{-1} fed manure (b) and food waste (c), and inocula acclimated to 11.8 g sodium L^{-1} fed manure (d) and food waste (e). Electropherograms based on *mcrA*-targeted T-RFLP analysis are shown for the original inoculum (f), inocula acclimated to 5 g TAN L^{-1} fed manure (g) and food waste (h), inocula acclimated to 11.8 g sodium L^{-1} fed manure (i) and food waste (j).

MDS analysis also showed that the post-acclimation microbial community compositions were distinct from the original inoculum (Fig. 2.3). The stress value for the MDS plot based on T-RFLP analysis targeting the bacterial 16S rRNA gene was 0.12 (Fig. 2.3a). Although this value is higher than the ideal value of 0.1, this plot still represents a useful ordination for visualizing differences in bacterial communities for unacclimated and acclimated inocula. The MDS plot depicting differences in archaeal microbial communities has a stress value of 0.07 (Fig. 2.3b), which represents an ideal ordination with little chance of misinterpretation. The community shifts do not appear to be due to components in the nutrient solution alone because microbial communities acclimated to elevated ammonia that were fed food waste clustered with

the original inoculum source. Results indicating that substantial microbial community shifts occurred suggest that observed improvements in hydrolysis rates were not solely due to changes in the activity of the microbial community present in the original inoculum source.

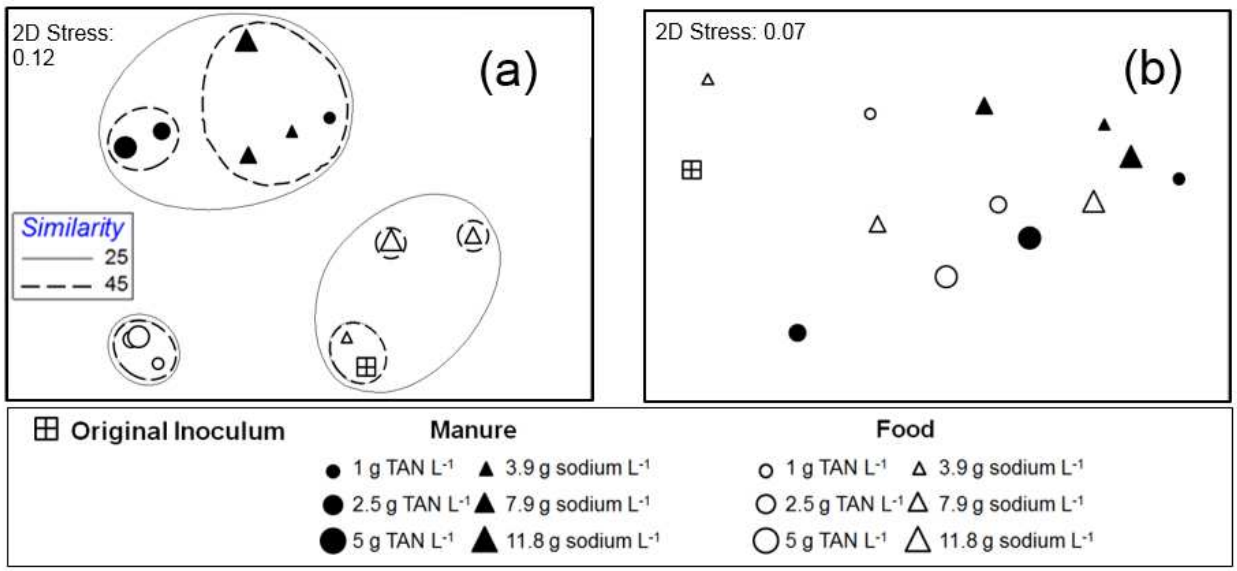

Figure 2.3. MDS plots based on 16S rRNA gene-targeted (a) and *mcrA*-targeted (b) T-RFLP analysis.Inoculum sample 2 was used for the unacclimated inoculum.

Furthermore, results suggest that the presence of the specific inhibitors, ammonia or salinity, as well as the feedstock source, played critical roles in shaping the bacterial community structures. T-RFLP patterns for the bacterial 16S rRNA gene-targeted assay indicated that the bacterial communities generally formed four clusters: 1) communities fed manure under elevated ammonia, 2) communities fed manure under elevated salinity, 3) communities fed food waste under elevated ammonia, and 3) communities fed food waste under elevated salinity (Fig. 2.3a). For manure, ammonia-acclimated communities were found to cluster separately at the 45% similarity level, and salinity-acclimated communities were found to cluster separately at the 45% similarity level, with the exception that the community acclimated to the lowest salinity

concentration clustered with the communities subjected to elevated ammonia. However, this finding is not unreasonable given that the microbial communities acclimated to 3.9 g sodium L^{-1} did not have to adapt to extremely high salinity concentrations (as compared to communities acclimated to 11.8 g sodium L^{-1}); and therefore, feedstock may have played a larger role in microbial community selection as compared to salinity concentration. For food, salinity-acclimated bacterial communities clustered at the 45% similarity level, and ammonia-acclimated communities clustered at the 25% similarity level. By contrast, although *mcrA*-targeted T-RFLP analyses revealed that substantial changes in the archaeal communities occurred, no clear trends were revealed according to ammonia, salinity, or feedstock (Fig. 2.3b). This result suggests that additional variables play a role in the selection of methanogens, and further research is required to identify the driving factors for archaeal community structure.

The microbial community composition shifts reported herein are consistent with previous studies, which have shown that operating conditions (e.g., solids content and temperature) play a substantial role in microbial community selection (Cho et al., 2013; Shi et al., 2013). For example, recently methanogenic communities were shown to decrease in genus-level diversity and shift from hydrogenotrophic to acetoclastic methanogens in response to acclimation to dry conditions (Cho et al., 2013). Cirne et al. (2007) also reported that very few similarities in microbial community composition for bacterial and archaeal groups (determined via fluorescence *in situ* hybridization) were observed for digesters operating with different feedstocks (e.g., beets and grass) despite otherwise similar operating conditions when hydrolysis was rate-limiting. Interestingly, microbial communities acclimated to food waste and a range of ammonia concentrations appeared to require less overall bacterial community shifts from the original inocula than inocula acclimated to manure as evidenced by the greater distance between

the manure-acclimated communities and the original inoculum (Fig. 2.3a). Since wastewater treatment plant digesters (the source of the original inoculum) typically digest waste that is derived from food and are not exposed to manure, this result is not unexpected. Alternatively, bacteria originally present in the manure supplied as feedstock during acclimation may have outcompeted the inoculum-derived bacteria, and thus, played a major role in shaping the structure of the manure-acclimated communities. Additionally, it appears likely that ammonia-tolerant strains are distinct from salinity-tolerant strains given the general lack of overlap between salinity-acclimated and ammonia-acclimated communities. Thus, results suggest that the presence of multiple inhibitor-tolerant phylotypes would be required in reactor systems operating under conditions of both elevated ammonia and salinity (e.g., systems with leachate recirculation). However, for the range of elevated ammonia and salinity concentrations tested, the specific inhibitor concentration likely was not a major driver for MDS plotting position; and thus, salinity- and ammonia-tolerant communities of a given composition could likely perform well under a range of salinity and ammonia concentrations.

Collectively, the microbial community composition analyses suggest that ammonia- and salinity-tolerant microorganisms were present in the original inoculum, but those particular strains were not dominant initially. After acclimation, increased hydrolysis rates were likely due to an increase in the quantity of inhibitor-tolerant strains. Acclimation has been shown to select for tolerant microorganisms in response to stressed conditions for a variety of natural and engineered systems. For example, according to a recent study focused on methanogenesis, digesters treating manure and food waste demonstrated lower Archaea diversity (1 dominant species as compared to 2 to 3) after acclimation to elevated ammonia concentrations as compared to before acclimation, indicating that conditions selected for ammonia-resistant methanogens

(*Methanosarcinaceae* spp.) (Fotidis et al., 2013). In another recent study, enhanced tolerance to salinity was observed in sequencing batch reactors treating wastewater after an acclimation period as demonstrated by stable process performance, and results demonstrated that microbial communities changed in response to the salt concentrations, suggesting that acclimation resulted in the selection of specific microorganisms (Bassin et al., 2012). In this study, the observed maximum inhibitor concentrations at which measurable hydrolysis was observed (5 g TAN L^{-1} and 11.8 g sodium L^{-1}) might have been due to a lack of strains capable of withstanding higher levels of ammonia and salinity in the wastewater treatment plant digester-derived inoculum. The possibility remains that these maximum inhibitor concentrations could be increased if the original inocula were supplemented with microbes derived from saline (e.g., estuaries) or ammonia-rich environments. Further research would be required to test this possibility.

2.4 Conclusions

Results from this study demonstrate the importance of the inoculum for achieving optimal hydrolysis rates and show that resource consumption (e.g., water use) may be minimized while achieving high AD performance via acclimating microbial communities to elevated concentrations of inhibitors (ammonia and salinity). Furthermore, results suggest that changing feedstocks (e.g., due to changing availability) may require an adaptation period before optimal hydrolysis rates are achieved. Alternatively, bioaugmentation with feedstock-specific or inhibitor-tolerant inocula might be a viable strategy for avoiding periods of sub-optimal performance. Sustaining high hydrolysis rates will require that optimal microorganisms are maintained over the course of reactor operation.

3.0 Stress Impacts Microbial Community Responses to Feedstock Changes during Bioenergy Generation

3.1 Introduction

Maintaining optimal, stable energy generation is critical for the economic viability of bioenergy technologies including anaerobic digestion (AD), which is currently the best available technology for producing energy from organic waste materials. Widespread adoption of AD for application to high solids waste materials (the organic fraction of municipal waste and dry-scraped manure) hinges on shifts in energy prices and regulatory incentives but also critically on advances that lead to more optimal performance. However, stress (e.g., inhibitory levels of salinity and ammonia) may occur in digesters and upset reactor performance (Chen et al., 2008). For example, high salinity (>3.5 g Na^+/L) and ammonia (>1.7 g TAN/L) may originate from feedstocks high in these inhibitors (e.g., food waste [salinity] and manure [ammonia]) or due to leachate recycle, which is typically employed in high-solids digesters (Chen et al., 2008). Additionally, digesters may experience perturbations throughout the course of operation including feedstock changes (e.g., switching to co-digestion). Accepting additional substrates is often desirable for increasing bioenergy production but has associated risks with respect to process stability. Digester microbial communities must possess the ability to efficiently adapt or acclimate to changing conditions to maintain stable performance. Stress and perturbations can even lead to system failure if not properly managed (Franke-Whittle et al., 2014).

Although many studies have examined the impact of stress or perturbation individually (Fernandez et al., 2000; LaPara et al., 2002), in field-scale systems, both can occur simultaneously. Microbial community dynamics in response to perturbations under stressed

conditions are not well understood for bioenergy systems. In practice, little attention is given to microbial community management at a highly technical level, but the types of microbes that are favored and maintained in a system have important implications for process performance and operation (e.g., microbial residence times). Although the impact of residence times on microbial community structures is well known in the wastewater field (Ahmed et al., 2007), fewer studies have looked at timescales for microbial change in the context of AD under stress and perturbation. This knowledge is needed to inform microbial community management approaches to maintain optimal, stable performance in anaerobic digesters for bioenergy operation.

The general types of microbial community responses to disturbances have been well-characterized, and the particular response mode ultimately may influence process performance. Dynamic community responses to disturbances can be categorized as resistance (microbial community composition stays the same), resilience (returns to original composition after disturbance), or functional redundancy (changes composition but performs like original community) (Allison & Martiny, 2008). Process functional stability typically does not correlate with stability in community structure (Collins et al., 2003; Fernandez et al., 1999; Fernandez et al., 2000; Lefevre et al., 2013; Pereyra et al., 2012). For example, Fernandez et al. (2000) demonstrated that methanogenic reactors with dynamic populations demonstrated greater functional stability in response to substrate shock compared to those with populations that varied little over time. Community adaptability is critical for maintaining functional stability and preventing failure, and functional redundancy protects against reactor failure since the various microorganisms tolerate a range of environmental conditions (Jurburg & Salles, 2015). Thus, reactor populations must be developed and maintained that have functional redundancies to support long-term process stability.

Acclimation is one method of developing microbial communities with resilience to stress and can potentially promote functional redundancies. Acclimation has been shown to increase hydrolysis and methanogenesis rates under stress. Increased hydrolysis rates occurred in wet digesters operating under elevated ammonia when inoculum were acclimated for 2-4 months (Wilson et al., 2013). Similarly, Chen et al. (2003b) reported increased tolerance by methanogens to elevated sodium after a 9 month acclimation period.

Knowledge of the time required for bacterial and archaeal communities to acclimate to perturbation under stress is critically needed to inform digester start-up, operation (e.g., solids residence times and amount of inoculum required), and approaches for microbial community management (via inoculation) during changes in reactor conditions or operation. For example, leach bed hydrolysis reactors in AD systems may benefit from re-inoculation with hydrolyzers with each new batch if substantial time is required for stress-tolerant bacterial and archaeal communities to develop for the feedstock used; however, re-inoculation of low-solids digesters is not performed in the field. Further, development of optimal and practical microbial management strategies for digesters impacted by stress would benefit from molecular tools designed to track desired microbes. However, acclimation timescales are not well understood, and key stress-tolerant AD microorganisms have not been identified.

The study described herein was based on the hypothesis that bacterial and archaeal community acclimation times will differ with archaeal acclimation times being longer, and acclimation periods will be on the order of weeks. Thus, the objective of this study was to investigate bacterial and archaeal community dynamics under a model stress (elevated ammonia) during a digester perturbation (changing the feedstock from wastewater sludge to one of two model feedstocks). Additionally, we sought to identify Bacteria and Archaea that are selected

for in digesters operating under elevated ammonia with the two model feedstocks (manure and filter paper). The approach involved tracking reactor performance and microbial community composition via molecular biology assays (e.g., T-RFLP) to determine microbial community dynamics during acclimation. Next-generation sequencing was utilized to identify microorganisms present in communities post-acclimation.

3.2 Methods

3.2.1 Feedstock and inoculum collection

Manure samples were collected from Five Rivers Cattle Feeding LLC (Greeley, CO) and stored at 4 °C for approximately 1 week. Microbial inoculum was collected from the DWWTP mesophilic anaerobic digester (Fort Collins, CO). The inoculum was purged with nitrogen gas, maintained at 35 °C, and used within 1 day of collection. Feedstock and inoculum were analyzed for TS and VS and TCOD prior to experiment set-up (Table 3.1).

Table 3.1. Characteristics of feedstock and inocula

	Parameter		
Sample	TS (%)	VS (%)	TCOD (g/g manure, g/L)
Manure	82.1 (±4.1)	47.6 (±1.3)	0.45 (±0.1)
DWWTP Inoculum	1.6 (±0.2)	77.1 (±0.3)	22.3 (±1.2)

3.2.2 Reactor configuration and operation

1-L semi-continuous glass digesters were fitted with liquid and gas sampling ports, and gas was collected via water displacement (Fig. 3.1).

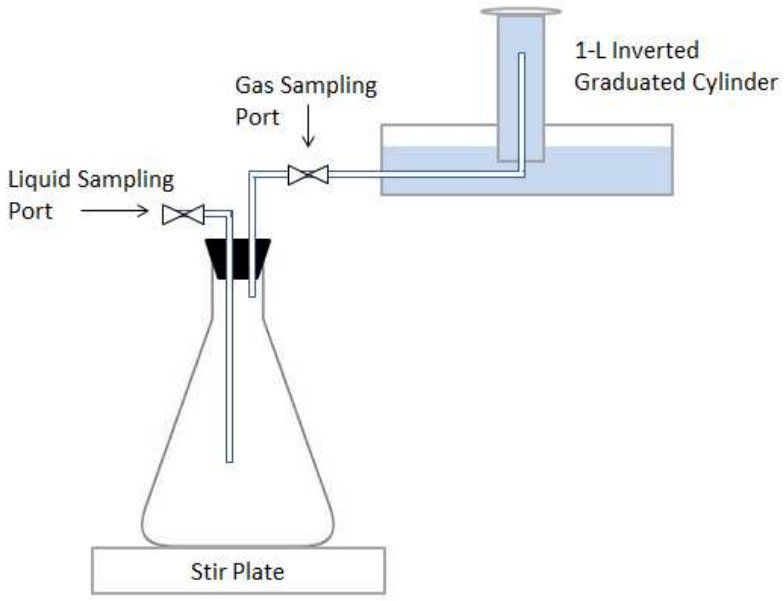

Figure 3.1. Laboratory-scale, semi-continuous system experimental set-up

Triplicate digesters were seeded with microbial inoculum (30% by volume) and nutrient solution (70% by volume) (Owen et al., 1979) to ensure that nutrients were not limiting in the system. Digesters were fed 1 g COD/L of filter paper (Whatman No. 1) (Soundar & Chandra, 1990) or manure weekly under anaerobic conditions, and equal volumes of sludge were removed for downstream chemical and biological analyses. Prior to feeding, the feedstocks were blended (Hamilton Beach blender) separately with nutrient solution to create a slurry. At each feeding, the pH of each flask was adjusted to approximately 7.2 using NaOH. During the experimental period, the average pH of the reactors ranged from 6.90 to 7.38 (with the exception of reactors fed manure averaging a pH of 6.68 at week 2) (Fig. 3.2).

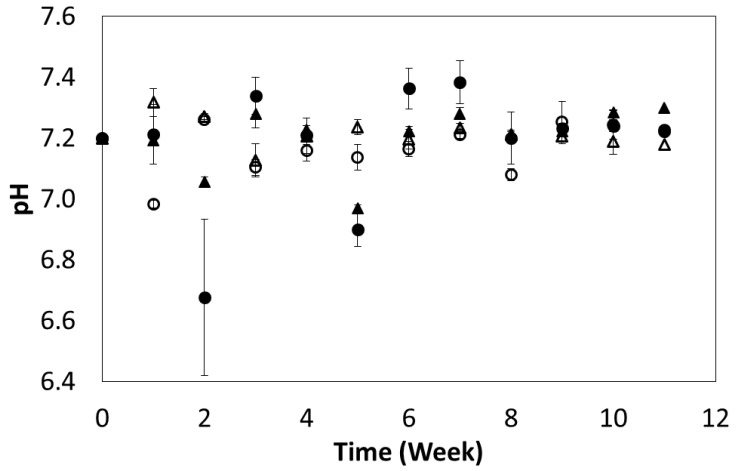

Figure 3.2. pH concentration for reactors fed paper under low (○) and high (●) ammonia, and reactors fed manure under low (△) and high (▲) ammonia.Error bars represent averages for triplicate reactors.

Digesters were operated under elevated ammonia concentrations (5 g TAN/L, supplied as NH_4Cl) previously shown to be inhibitory in reactors operating with unacclimated microbial inoculum (Wilson et al., 2013). Digesters were also operated for "baseline" ammonia concentrations (0.1 g TAN/L) present in the nutrient solution to demonstrate performance of digesters operating under non-stressed conditions. The flasks were incubated at 35 °C and continually mixed via stir plates for 11 weeks.

3.2.3 Chemical analyses

The volume of biogas produced each week was measured by determining the amount of gas captured in the inverted graduated cylinders. Biogas samples were collected weekly, and methane content was determined via gas chromatography using a thermal conductivity detector as described previously (Wilson et al., 2013). TS and VS of the feedstock and inoculum were measured according to standard methods (APHA, 1995), and liquid samples were analyzed for pH and electrical conductivity (EC) using probes (VWR #89231-604 and #11388-382,

respectively). DCOD concentrations were determined using Hach's COD High Range Vials and digestion colorimetric method (Hach, Loveland, CO). Volatile fatty acids (VFAs) were measured using Hach's Volatile Acids TNTplus Reagent Set and esterification method. Samples were filtered through 0.2 μm syringe filter prior for DCOD and VFA analyses. Ammonia concentrations were measured weekly using Hach's Nitrogen-Ammonia High Range Reagent Set and found to remain constant over time.

3.2.4 Microbial community analyses

3.2.4.1 T-RFLP analyses

To track microbial community composition responses to acclimation, DNA was extracted weekly from all digesters and analyzed via T-RFLP. Prior to DNA extraction, 50-ml samples were centrifuged at 5,000 g for 10 min at 4 °C. The supernatant was discarded, and the remaining pellet was used for DNA extraction. DNA was extracted using MoBio's PowerSoil kit according to the manufacturer's instructions. T-RFLP analyses targeted the 16S rRNA gene and the *mcrA* gene as previously described (Dollhopf et al., 2001; Wilson et al., 2013).

3.2.4.2 Next-generation sequencing analyses

DNA samples extracted from the original inoculum and reactors operating after 11 weeks were sent to Research and Testing Laboratories LLC (Lubbock, Texas) for bacterial and archaeal sequencing using Illumina's MiSeq platform. Since all digesters operating under low ammonia performed similarly, DNA from 1 of the triplicate digesters fed filter paper and manure was chosen as representative microbial communities. However, since digesters operating under high ammonia demonstrated greater variability via T-RFLP, DNA from 2 digesters for each feedstock were sent for next-generation sequencing in efforts to capture representative compositions. For next-generation sequencing via Illumina MiSeq, quality control of the raw sequences was

performed by Research and Testing Laboratory according to their standard protocol (Table 3.2). Briefly, the running average quality score was determined along each sequence and the sequence was trimmed such that the total average quality score was above 25. The average sequence read length was 534.86 bp, and sequences shorter than 100 bp were discarded. Sequences were clustered at 4% divergence using USEARCH, and clusters with only one member (singletons) were discarded (Edgar, 2010). Clusters were checked for chimeras based on the consensus sequence of the centroid of each cluster using the *de novo* method in UCHIME (Edgar et al., 2011). The sequences in clusters in which the consensus sequence was determined to be chimeric were discarded. Each sequence was compared to the consensus sequence in its cluster to correct for differences. If a given base in the sequence was different than the corresponding base in the consensus sequence and the quality score for that base was less than 30, the base was changed, inserted, or deleted to match the consensus sequence. Quality controlled sequences were classified using mother v.1.37.0 (Schloss et al., 2009). Unique sequences were aligned to the SILVA reference database v 123 (Quast et al., 2013) and classified using a naïve Bayesian classifier with a cutoff of 90 (Wang et al., 2007). The final community structure was based on all sequences that passed quality control. Shannon diversity was calculated for the aligned sequences using mothur with a cutoff of 0.03.

Table 3.2. Characteristics of raw reads

	Inoculum	Substrate: Paper			Substrate: Manure		
		Low Ammonia	High Ammonia		Low Ammonia	High Ammonia	
Number of reads: Bacteria	19,501	13,103	16,142	18,965	20,693	20,752	10,327
Number of reads: Archaea	50,147	34,700	65,976	88,039	68,974	56,392	26,992

3.2.5 Statistical analyses

ANOVA and Tukey's Honest Significant Difference (HSD) tests were conducted using R Statistical Software (R Core Team, 2012) to determine if the slopes of the fitted regression lines for VFA concentrations and methane production were statistically different from 0 as well as to determine statistical differences in weekly average VFA concentrations and methane production. Results yielding a p-value less than 0.05 were considered to be significant. T-RFLP data was analyzed and similarity matrices were calculated as previously described (Wilson et al., 2013). Stress values less than 0.1 represent 2 dimensional pictures with little risk of misinterpretation, values less than 0.2 correspond to useful ordinations, and values over 0.2 illustrate nearly random plots (Clarke, 1993).

3.3 Results and discussion

3.3.1 Digester performance

Digesters operating under low ammonia demonstrated consistent, and high performance throughout the experimental period (Fig. 3.3). VFA and DCOD concentrations remained low, indicating that hydrolysis products did not build-up (Fig. 3.3a, b).

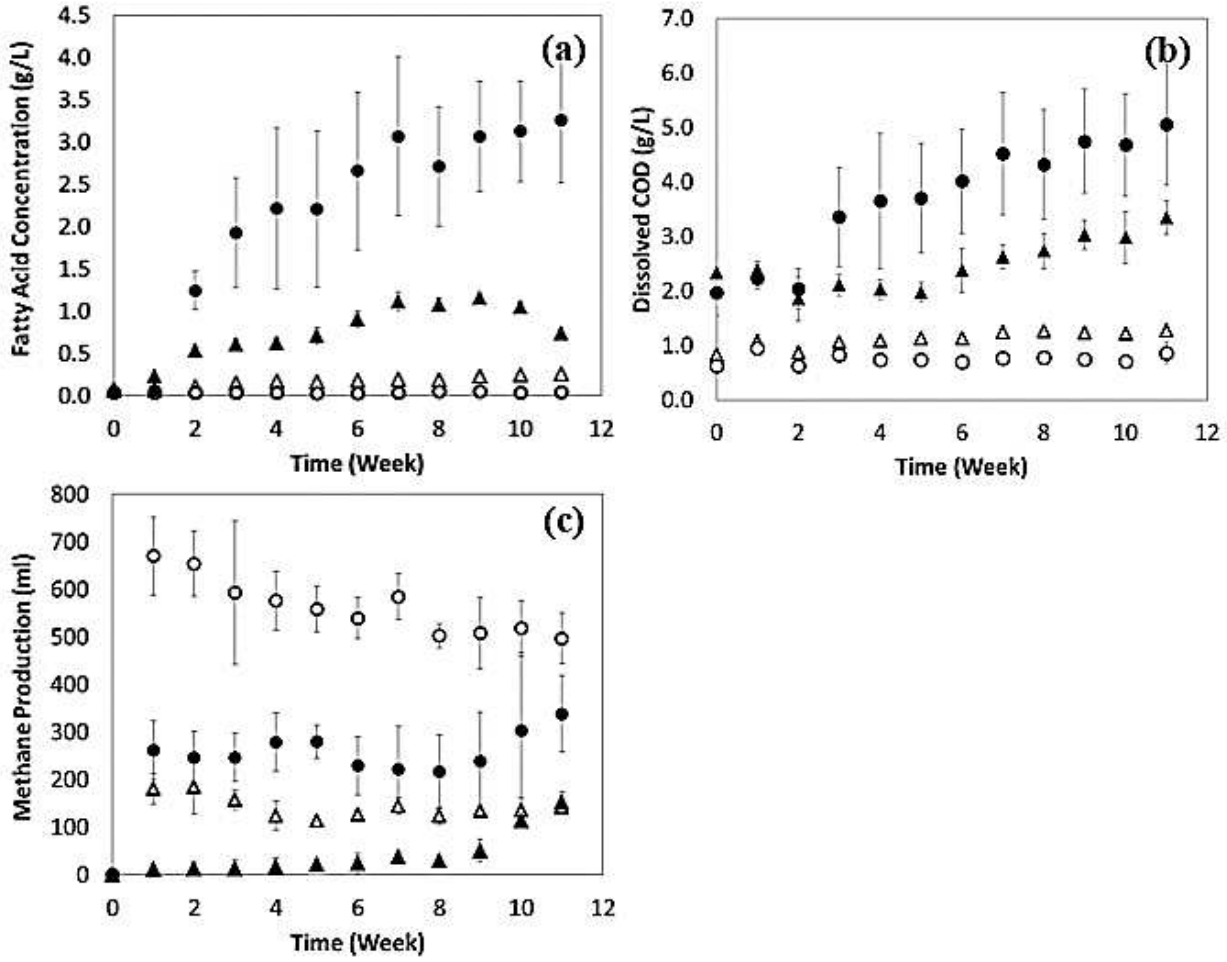

Figure 3.3. Fatty acid (a) and DCOD (b) concentrations and methane production (c) for reactors fed paper under low (○) and high (●) ammonia, and reactors fed manure under low (△) and high (▲) ammonia.Error bars represent averages for triplicate reactors.

Methane production was high and comparable to literature values (Fig. 3.3c) (Wilson et al., 2013). Thus, non-stressed digesters acclimated to the feedstock changes (paper or manure after wastewater sludge) and digester conditions rapidly (~days).

Digesters operating under high ammonia fed paper initially demonstrated low, but consistent methane generation, followed by improved performance during the final weeks of the experimental period. VFA concentrations began building up at week 2 and reached a peak of 3.3 g/L at week 11 (Fig. 3.3a). pH decreased substantially from 7.2 to 6.7 by week 2, coinciding with

the increase in VFA concentration. However, the pH was manually adjusted each week and did not decrease below 6.9 at any other time point. Similarly, DCOD concentrations (Fig. 3.3b) increased by week 3 and reached a peak of 5 g/L at week 11. Methane generation occurred by the end of week 1, but remained low and relatively stable for the first 9 weeks (Fig. 3.3c). During week 10, methane production began increasing, although this trend was not statistically significant.

Collectively, VFA concentration and methane generation data from digesters fed paper operating under high ammonia suggest that hydrolysis was occurring to some extent during the first week. These results indicate that microorganisms capable of degrading paper under ammonia stress were present initially. However, the quantity of stress-tolerant methanogens was not sufficient since digesters experienced VFA accumulation and methane production was limited. The increase in methane production observed at the end suggests that ammonia-tolerant methanogens increased in quantity after 10 weeks, although methane generation under stressed conditions was still 1.5-fold lower than methane generation observed at low ammonia.

For digesters operating under high ammonia fed manure, overall bioenergy generation performance was initially extremely low, but increased over time and ultimately was comparable to manure-fed digesters operating under low ammonia. Initially, VFA concentrations increased over first two weeks (p-value = 3.7×10^{-6} for regression line different than 0), and then remained high and stable for another 2 weeks (Fig. 3.3a). Concentrations then increased for an additional 3 weeks to a peak of 1.1 g/L (p-value = 4.3×10^{-5}) and remained stable for 2 more. Then, VFA concentrations decreased for the final two weeks (p-value = 0.002). DCOD concentrations increased beginning in week 6 (Fig. 3.3b). Methane production was low for the first 9 weeks of operation, but increased dramatically in the final 2 weeks and reached production levels

comparable to digesters fed manure operating under low ammonia (Fig. 3.3c). An ANOVA test indicated differences among weekly-averaged methane generation rates (p-value = 5.12×10^{-11}), and further analyses revealed that average methane production at weeks 10 and 11 was statistically greater than average production during weeks prior (highest Tukey's HSD p-value = 0.002).

Performance data for all digesters under high ammonia shows that stressed digesters required a longer acclimation period than digesters operating under non-stressed conditions. In one of our previous studies (Wilson et al., 2013), results demonstrated that hydrolysis occurs after direct exposure to elevated ammonia in digesters fed manure, but rates were ~4-fold slower than rates observed in digesters under low ammonia. Thus, we expected hydrolysis to occur slowly at the start and for rates to improve with time. The initial accumulation of VFAs for reactors operating with both paper and manure under high ammonia suggests that, after a couple of weeks, hydrolyzing communities were more acclimated than methanogen communities. VFA concentrations not only are controlled by the rate of production, but also the rate of consumption by methanogens. Thus, VFA concentrations are dependent upon both the quantity and type of Bacteria and Archaea present. The decrease in VFAs in the final weeks and corresponding increase in methane generation suggests that methanogens required a longer acclimation time, which is not surprising given their slow growth rates (Lens, 2005). Interestingly, methane production was immediately better in reactors fed paper as opposed to manure. This result suggests that a microbial community more dissimilar from the inoculum was required to digest manure. Additionally, longer time may have been required because manure is a more recalcitrant feedstock, particularly since reactors were fed equal amounts of manure and paper on a COD basis. However, the interdependent nature of hydrolysis and methanogenesis makes

distinguishing impacts on each process separately difficult based on operational data alone; thus, molecular analyses were conducted to provide an in-depth understanding of the effect of acclimation on hydrolyzers and methanogens separately.

3.3.2 Microbial community dynamics

Microbial community diversity analyses did not reveal robust trends over time or in response to stress. Weekly Shannon indices calculated using T-RFLP data indicated that stress increased diversity (Fig. 3.4).

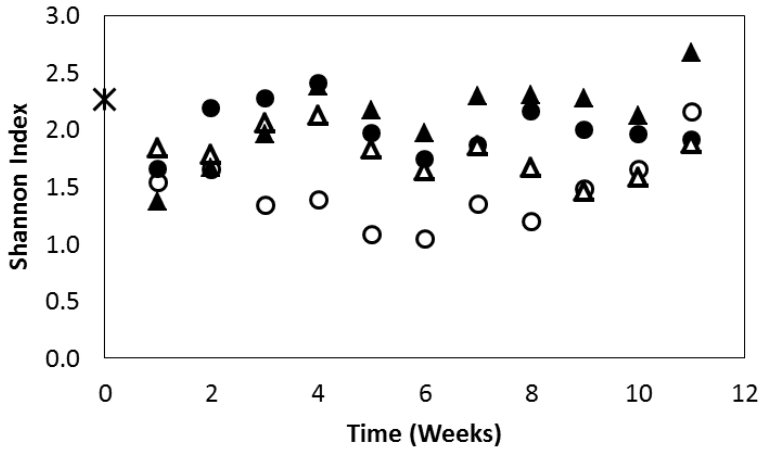

Figure 3.4. Shannon indices based on 16S rRNA T-RFLP datafor the original inoculum, reactors fed paper under low (○) and high (●) ammonia, and reactors fed manure under low (▵) and high (▲) ammonia.

However, for next-generation sequencing data, Shannon indices indicated decreased diversity under stress (Table 3.3).

Table 3.3. Shannon indices and species richness analyses based on 16S rRNA bacterial and archaeal pyrosequencing data

	Inoculum	Substrate: Paper			Substrate: Manure		
		Low Ammonia	High Ammonia		Low Ammonia	High Ammonia	
Shannon Index: Bacteria	3.52	2.85	1.89	2.42	3.63	2.81	2.78
Shannon Index: Archaea	2.19	1.69	1.62	1.52	1.94	1.79	1.73
Species Richness: Bacteria	144	97	99	108	147	135	116
Species Richness: Archaea	18	17	16	17	22	20	18

Bacterial 16S rRNA gene-targeted analyses via MDS plots revealed differences among the microbial communities present in the reactors. Bacterial communities generally formed four main groups: reactors fed paper under low ammonia, reactors fed paper under high ammonia, reactors fed manure under low ammonia, and reactors fed manure under high ammonia (Fig. 3.5).

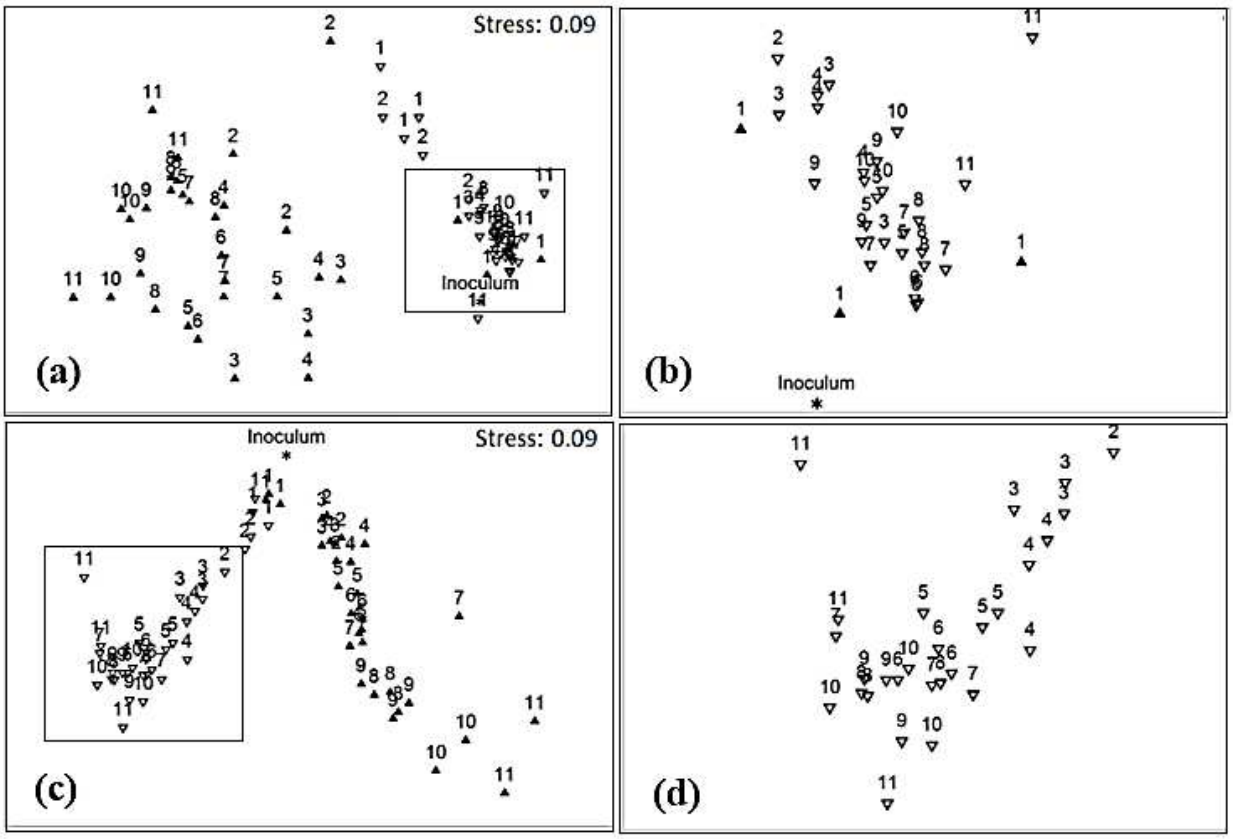

Figure 3.5. MDS plot of bacterial community compositionfor reactors fed paper (a) and manure (c). Boxes within plots indicate areas of the full MDS plots that were enlarged for detail for reactors fed paper (b) and manure (d).Open and closed symbols indicate reactors operating under low and high ammonia, respectively. Numbers above symbols indicate week of acclimation. Multiple points at each week represent triplicate reactors.

Moreover, community structures changed substantially as a function of time for all groups, but the dynamic behavior differed between non-stressed and stressed communities. Bacterial communities under low ammonia fed paper became relatively stable after approximately 2 weeks and grouped closely to original seed (Fig. 3.5a, b). Similarly, communities fed manure stabilized after approximately 5 weeks (Fig. 3.5c, d). Conversely, under high ammonia, all communities were highly dynamic over the entire period. For reactors digesting manure, communities experienced the greatest changes at weeks 10 and 11, corresponding to the time at which a dramatic increase in methane production was observed.

These results are consistent with previous studies that demonstrate feedstock and inhibitors affect microbial community development (Chae et al., 2009; Wilson et al., 2013), and perturbations are known to drive changes in community composition because dominant microorganisms under steady-state conditions are often not the best adapted to the perturbed conditions (Lefevre et al., 2013; Pereyra et al., 2012). Further, these results corroborate the conclusion that a microbial community more dissimilar from the inoculum was required to digest manure, as suggested by the performance data. Moreover, existing organisms in the inoculum may have gained additional AD functional capabilities effective under elevated ammonia via horizontal gene transfer, although more research is required to test this suggestion. Rapid acclimation to new the feedstock under low ammonia conditions can be explained by similarities in substrate composition; wastewater sludge and manure have been shown to contain similar cellulose (13-29% and 14-27%, respectively) and protein (18-32% and 17-25%, respectively) abundances (Champagne & Li, 2009; Chen et al., 2003a). However, the more dynamic community behavior observed under stressed conditions has not been previously reported for bioenergy systems.

Similarly, *mcrA*-targeted MDS plots demonstrated unique responses for methanogenic communities under low and high ammonia. However, the timescales for community structure change differed between Bacteria and Archaea. All methanogenic communities under low ammonia remained relatively consistent, with exception of communities at week 11 (Fig. 3.6).

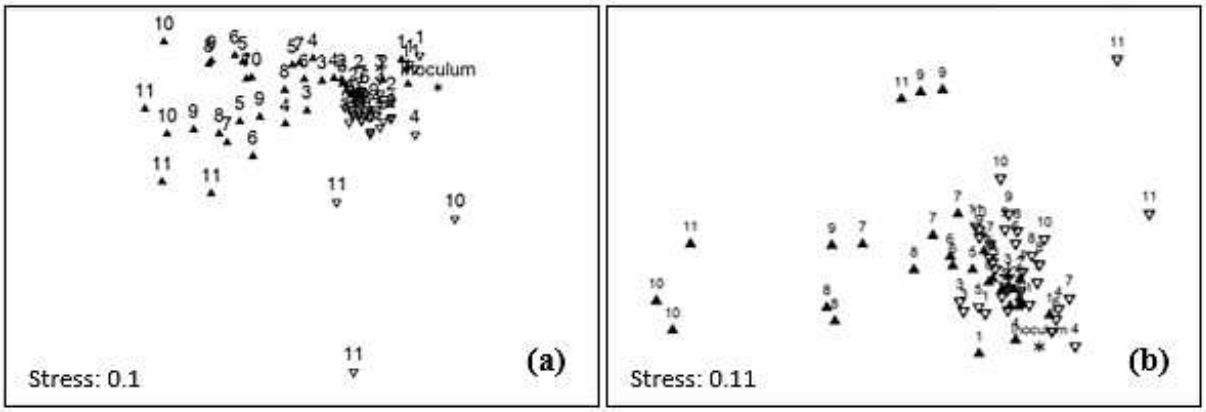

Figure 3.6. MDS plot of *mcrA*-targeted community compositionfor reactors fed paper (a) and manure (b).Open and closed symbols indicate reactors operating under low and high ammonia, respectively. Numbers above symbols indicate week of acclimation. Multiple points at each week represent triplicate reactors.

Reasons for changes during the final week of the experimental period are unknown, as conditions remained stable. Conversely, digesters operating under high ammonia demonstrated highly dynamic communities throughout the experimental period. For digesters fed paper, communities clustered with the original inoculum and the low ammonia communities for approximately the first 3 weeks but became increasingly dissimilar from original inoculum as a function of time (Fig. 3.6a). Interestingly, for reactors fed manure, communities generally grouped close to the original inoculum for the first 7 weeks, but demonstrated more substantial changes beginning at approximately week 8, which corresponds to the onset of methane production (Fig. 3.6b).

The increased performance observed after week 8 for stressed reactors combined with microbial community analyses suggest that functional redundancy occurred in all of our reactors. Functional redundancy is key for stable performance. Further, the ability of microbial communities to remain flexible under stress suggests a positive long-term prognosis. Stress may even increase the ability of reactors to respond to additional stresses by selecting for tolerant

microbes (Lefevre et al., 2013). However, further work is required to fully understand how initial ammonia stress may impact how well AD reactors respond to subsequent increased ammonia stress or other stresses (e.g., salinity).

Collectively, results demonstrate that stress differentially impacted the ability of bacterial and archaeal communities to acclimate to new feedstocks and reactor conditions. Under non-stressed conditions, microbial communities readily adjusted to feedstock changes. Bacterial microbial communities under low ammonia fed paper or manure demonstrated shifts over time, but ultimately stabilized after 2-5 weeks. Similarly, LaPara et al. (2002) observed rapid shifts over a 15-day period in microbial community composition in response to changes in influent wastewater composition in biological reactors treating pharmaceutical wastewater, and functional stability was maintained as determined by high effluent quality. In our study, under stress and perturbation, the acclimation process took substantially longer as the communities had to shift to tolerate the stress while responding to feedstock changes. Performance and microbial community composition data suggest that acclimation to a feedstock change during stress occurs on the order of weeks for hydrolyzers to months for methanogens. Several studies have demonstrated acclimation under stress (without other changes) occurs on the order of months. For example, Borja et al. (1996) demonstrated that stable operation of upflow anaerobic sludge blankets at elevated ammonia concentrations was achieved after an acclimation period of 6 months, although the methane yield was lower compared to reactors operating at low ammonia levels. In a more recent study, we postulated that acclimation to elevated ammonia required 2-4 months based on methane generation (Wilson et al., 2013), although that study did not include detailed microbial analysis as a function of time nor distinguish temporal impacts on Bacteria and Archaea separately. Thus, our data and literature findings clearly indicate that acclimation to

stress requires longer time periods than acclimation to new feedstocks. In light of this knowledge, management of microbial communities in AD systems should involve monitoring stresses carefully and adjusting inoculation practices when stresses are observed.

3.3.3 Identification of beneficial phylotypes

To identify beneficial microbial phylotypes, detailed analysis of weekly 16S rRNA gene-targeted T-RFLP data was conducted. For reactors fed paper under low ammonia (Fig. 3.7a), a single T-RF (157 bp) dominated the community over the entire study; interestingly, this T-RF was non-detect in reactors fed paper under high ammonia by week 3. In contrast, for reactors fed manure under low ammonia, T-RFs of 81, 89, and 91 bp increased in abundance for approximately the first 3-5 weeks, and then collectively dominated (>75% total abundance) microbial communities after the communities stabilized at week 5 (Fig. 3.7c).

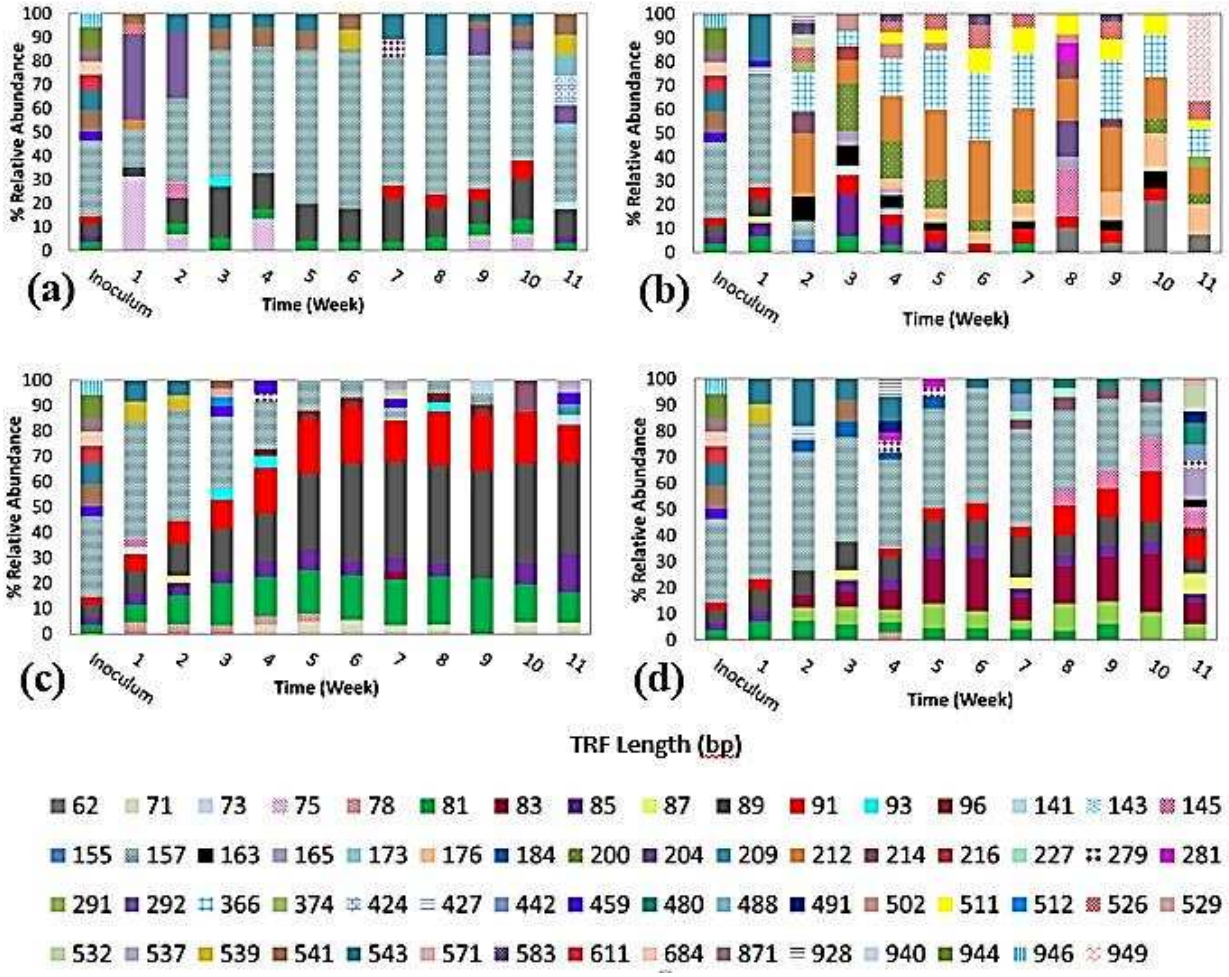

Figure 3.7. Representative histograms of 16S rRNA gene-targeted TRFLP databy week for reactors fed paper under low (a) and high (b) ammonia and reactors fed manure operating under low (c) and high ammonia (d).

Interestingly, microbial communities in reactors operating under high ammonia showed little similarity to other reactors. For reactors fed paper, T-RFs of 212 and 366 bp appeared in high abundance beginning in week 2 and were present only under these reactor conditions (Fig. 3.7b). Very few overlapping T-RFs between reactors fed paper under low and high ammonia were present. For reactors fed manure under high ammonia, microbial communities were not dominated by a few specific T-RFs as seen in reactors under low ammonia (Fig. 3.7d). Throughout the study, the T-RF of 83 bp generally increased in abundance, but this T-RF was

not detectable in the inoculum and did not appear in high abundance in any other reactors. Likewise, the T-RF of 145 bp was first detected in week 8, and remained present until the end of the experimental period; this T-RF was never present for more than one week in microbial communities in the other reactors.

Similarly, *mcrA*-targeted T-RFLP data revealed that microbial communities under stress were dominated by few abundant phylotypes that were distinct from those dominating under non-stress conditions. Reactors fed paper under low ammonia generally were dominated by the same T-RFs throughout the entire study period with only slight variations in T-RF abundance (Fig. 3.8a).

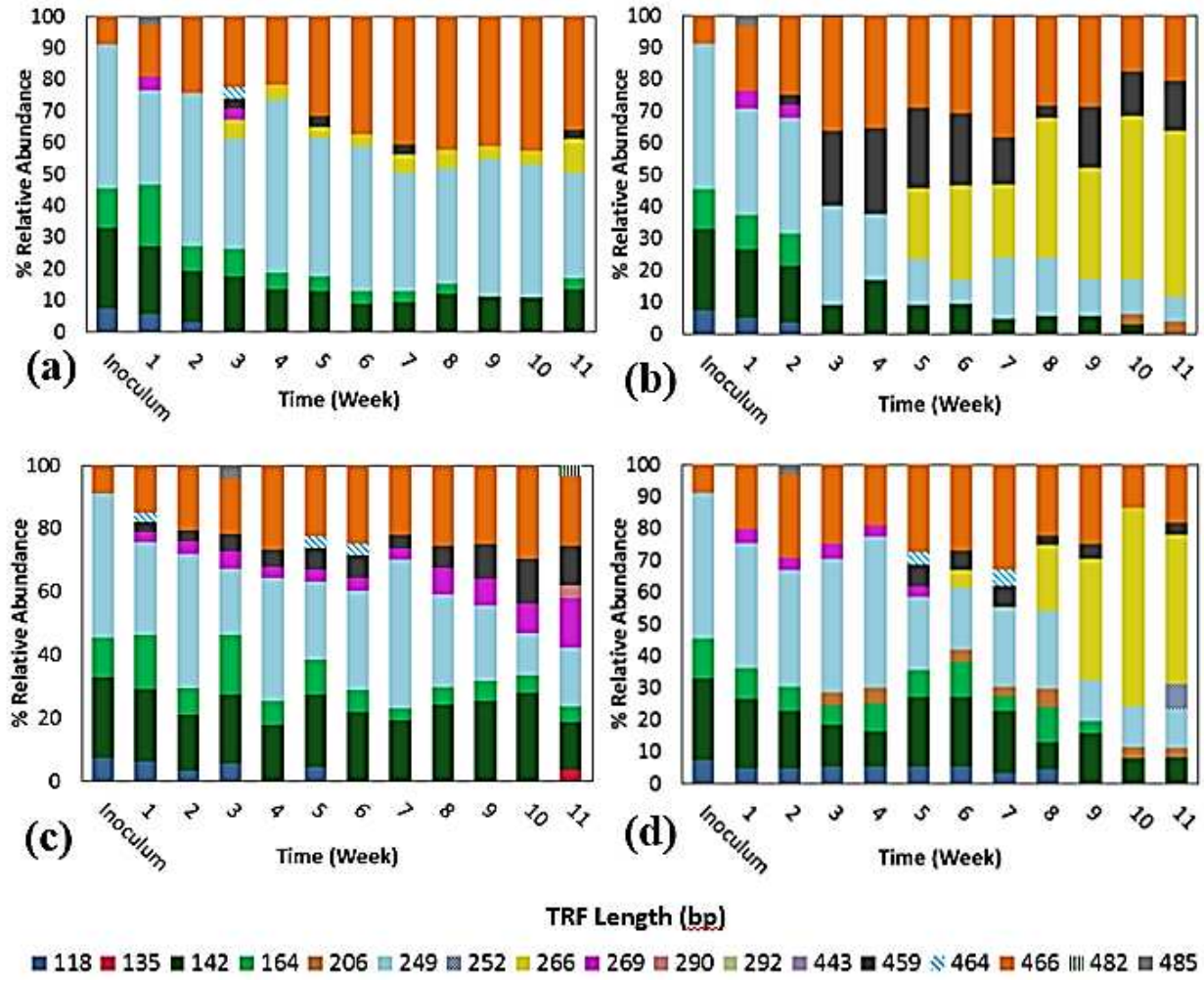

Figure 3.8. Representative histograms of *mcrA* gene-targeted TRFLP databy week for reactors fed paper under low (a) and high (b) ammonia and reactors fed manure operating under low (c) and high ammonia (d).

By week two, T-RFs of 249 and 466 bp dominated and remained in high abundance (≥30% and 25%, respectively). For reactors fed manure under low ammonia, the T-RF of 269 bp increased in abundance (from 3% to 16% at week 11) but was present at relatively low levels during all weeks (Fig. 3.8c). All reactors under high ammonia demonstrated a decrease in the T-RF of 249 bp. Additionally, for reactors fed paper under high ammonia, the T-RF of 459 bp was noticeably present (3- 25% abundance) in weeks 2-11, but was not present in the original inoculum (Fig. 3.8b). Similarly, the T-RF of 266 bp was first detected at week 5 and was present at high

abundance (>20%) each week thereafter. These T-RFs were detected only at low levels in reactors fed paper at low ammonia. For reactors fed manure under high ammonia, the T-RF of 266 bp was detected at week 6, and was present in higher abundance (>15%) at weeks 8 and beyond (Fig. 3.8d). This is a similar trend to reactors fed paper under high ammonia, and since this T-RF was present for both feedstocks, these results suggest that T-RF of 266 bp is an ammonia tolerant methanogen.

16S rRNA-gene amplicon sequencing identified the beneficial microbes that were selected for during acclimation (Fig. 3.9, 3.10a). At the phylum level, *Bacteroidetes* and *Proteobacteria* were present in high abundance (>19%) in the original inocula, although *Proteobacteria* decreased to less than 10% abundance in all reactors post-acclimation, with the exception of reactors fed manure under low ammonia (Fig. 3.9).

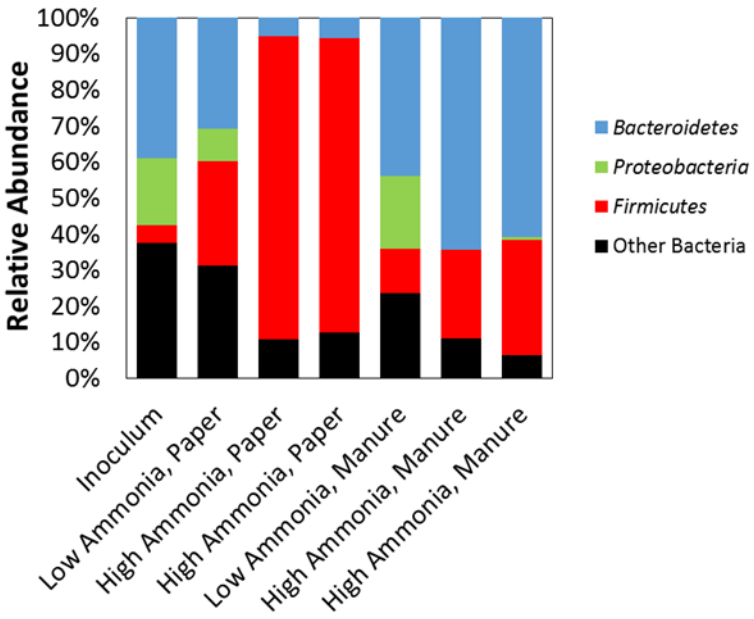

Figure 3.9. Bacterial 16S rRNA gene-targeted next-generation sequencing data for inoculum and selected reactors.Phylogenetic identities are given at the phylum level. "Other Bacteria" indicates grouped phylotypes that individually comprised less than 5% abundance. Two samples were analyzed for reactors operating under elevated ammonia because microbial communities among triplicate reactors appeared to be more variable based on MDS plots compared to microbial communities in reactors operating under low ammonia.

Interestingly, *Bacteriodetes* was present at >60% abundance in reactors fed manure under high ammonia, but comprised less than 6% of the microbial communities in reactors fed paper under high ammonia. In contrast, microbial communities in reactors fed paper were dominated by the phylum *Firmicutes* (>82% abundance).

To identify more detailed differences between the microbial communities in reactors as a function of feedstock and stress, lower taxonomic levels were analyzed (Fig. 3.10a). For reactors operating under low ammonia, genus *Smithella* (phylum *Proteobacteria*), family *Draconibacteriaceae* (phylum *Bacteroidetes*), and class *Bacteroidetes vadinHA17* (phylum *Bacteroidetes*) were present in higher abundance compared to reactors operating under high ammonia indicating their sensitivity to elevated ammonia levels. The type species *Smithella*

propionica of the genus *Smithella* is an anaerobe that syntrophically degrades propionate with butyrate as a significant product (Liu et al., 1999). *Ruminococcaceae*, members of the *Firmicute* phylum, were present at high abundance in reactors fed paper regardless of ammonia level, which was logical considering species within this family are known to digest cellulosic waste (Chassard et al., 2012). Interestingly, the genus *Ruminococcaceae UCG-012* specifically was selected for in reactors operating under high ammonia which indicates tolerance to ammonia, while unclassified members of *Ruminococcaceae* were selected for in reactors under low ammonia. For reactors operating under high ammonia, order *OPB54* within phylum *Firmicutes* and genus *Halocella*, a halophilic cellulolytic bacterium, were present (between 1% - 23% abundance), but were not detectable under low ammonia. Similarly, all reactors operated under high ammonia demonstrated a higher abundance of family *Clostridiales vadinBB60* group within the class *Clostridiales*, which are obligate anaerobes previously identified in the digestate of reactors treating manure (Sun et al., 2015). Interestingly, the family *Marinilabiaceae* of the phylum *Bacteroidetes* was noticeably present only in reactors fed manure under high ammonia. Although this family was comprised of unclassified members in our microbial communities, a notable species in this family is *Alkaliflexus imshenetskii*. This species has been isolated from alkaline lakes (pH of 7.5) and digested municipal solid waste and also has been shown to utilize soluble products formed by uncultured hydrolytic cellulose degraders (Cardinali-Rezende et al., 2012; Zhilina et al., 2004).

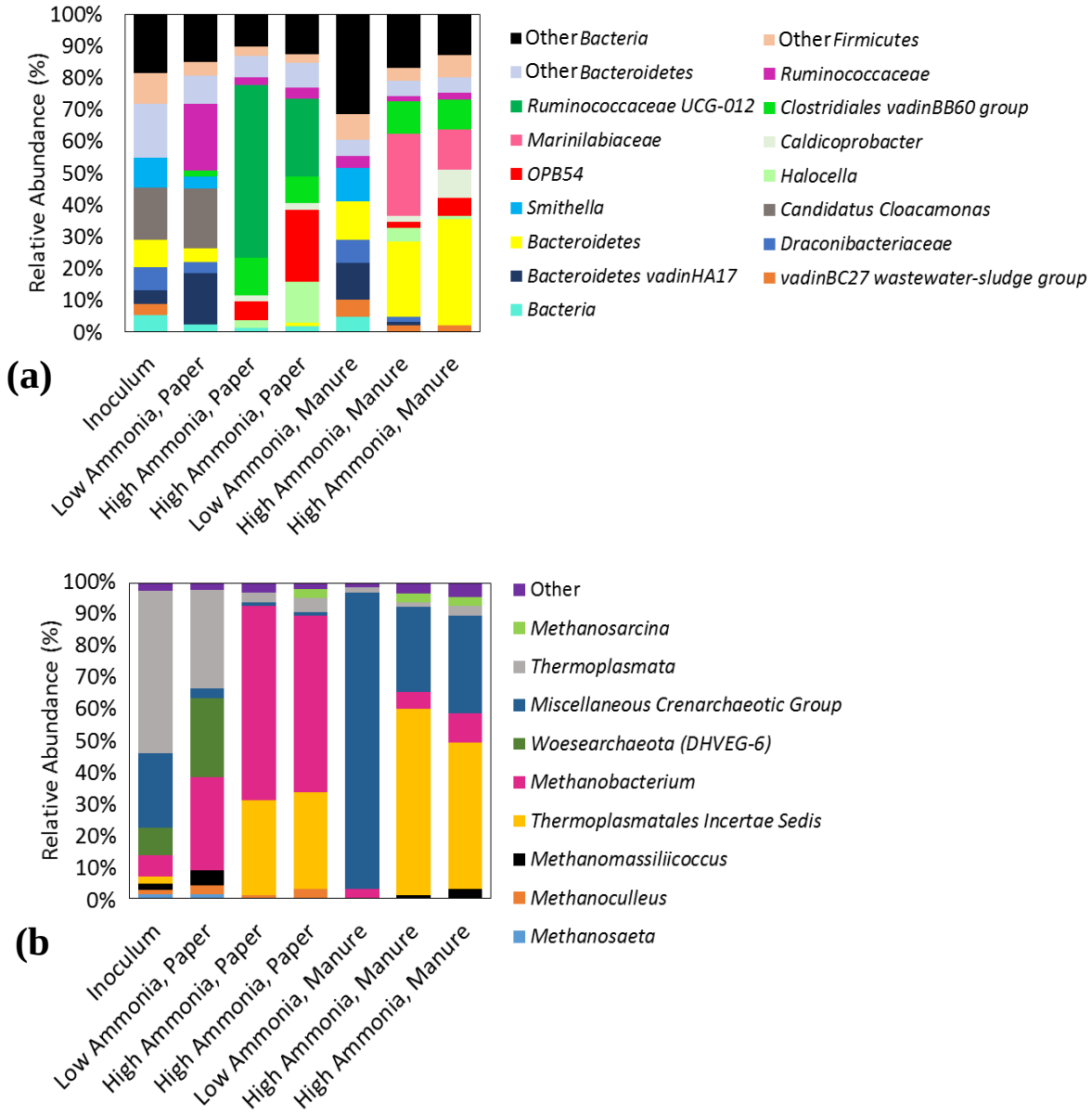

Figure 3.10. Bacterial (a) and Archaeal (b) 16S rRNA gene-targeted next-generation sequencing data for inoculum and selected reactors.Phylogenetic identities are given at the lowest classified taxonomic level. Labels identified as "Other" indicate grouped phylotypes that individually comprised less than 5% abundance. Two samples were analyzed for reactors operating under elevated ammonia because microbial communities among triplicate reactors appeared to be more variable based on MDS plots compared to microbial communities in reactors operating under low ammonia.

Archaeal-targeted sequencing data yielded high quantities of the highly diverse

Miscellaneous Crenarchaeotal Group in the inoculum and reactors fed manure (Fig. 3.10b).

Although these microorganisms have been identified in widespread environments (e.g., terrestrial hot springs, deep oceanic subsurface sediments, termite guts, landfill leachate, and anaerobic wastewater reactors), little is known about the functional roles these microbes (Kubo et al., 2012). A recent study suggested that this community is not active in methanogenesis or sulfur cycling, but these organisms potentially may access fermentative substrates or could be linked to breakdown of refractory organic matter (Kubo et al., 2012). Thus, the recalcitrant nature of manure may have selected for these particular organisms. Additionally, the single sample used for DNA extraction from reactors fed manure under low ammonia may not have been representative of the microbial communities since the Miscellaneous Crenarchaeotal Group was unexpectedly present in >90% abundance. In contrast, this phylum was present in less than 4% abundance in reactors fed paper.

All reactors operating under high ammonia contained a high abundance (>30%) of the order *Thermoplasmatales Incertae Sedis*. This order was present at only 2% abundance in the inoculum and was not detected in reactors operating under low ammonia. However, the class *Thermoplasmata* containing this genus was abundant in the inoculum and reactors fed paper under low ammonia. *Thermoplasmata* are facultative anaerobes capable of sulfur respiration (Chojnacka et al., 2015) and may be non-methanogenic. Interestingly, a recent study has suggested that methanogens, *Crenarchaeota*, and *Thermoplasmata* commonly co-exist in anaerobic digesters, although additional research is needed to determine the impact of interactions among these microorganisms (Chouari et al., 2015). However, the dominant presence of the order *Thermoplasmatales Incertae Sedis* in reactors under high ammonia suggests that this phylotype is an ammonia-tolerant organism, which competes with methanogens for resources when ammonia levels are high.

Hydrogenotrophic methanogens were selected for in response to perturbation and stress in reactors fed paper (Fig. 3.10b). All reactors fed paper had a high abundance of *Methanobacterium*, a hydrogenotrophic methanogen, whereas reactors fed manure contained less than 9% of this genus. Interestingly, for reactors fed paper, a greater abundance (>55%) of *Methanobacterium* was observed in reactors operating under high ammonia compared to reactors operating under low ammonia (30%). Thus, hydrogentrophic methanogens appeared to be more dominant under stressed conditions. Higher VFA concentrations present in reactors under high ammonia compared to concentrations in reactors under low ammonia may have contributed to the selection for hydrogenotrophic methanogens. Elevated acid levels have been shown to select for hydrogenotrophic methanogens and lead to a decrease in acetoclastic methanogens (Angenent et al., 2002). Further, acetate-utilizing methanogens have been shown to be more sensitive to ammonia than hydrogentrophic methanogens (Hori et al., 2006; Karakashev et al., 2005). Although VFA concentrations remained low in reactors fed paper under low ammonia, higher rates of VFA production and utilization may have selected for the presence of hydrogen-utuilzing methanogens compared to lower rates of production in reactors fed manure. Collectively, bacterial and archaeal sequencing identified multiple microorganisms (e.g., *Clostridiales vadinBB60, Marinilabiaceae, Methanobacterium,* and *Thermoplasmatales Incertae Sedis*) that were selected for under stressed conditions. To our knowledge, these organisms have not previously been associated with high performance under elevated ammonia, and thus additional research should investigate the ability of these organisms to improve system performance large-scale systems. Additionally, identified beneficial microbes can be targeted via molecular assays to monitor the health of microbial communities in AD reactors that experience common stresses at full-scale.

3.4 Conclusions

This study has demonstrated that microbial communities under stressed conditions adapt more slowly to feedstock changes than under non-stressed conditions. Thus, particular attention must be given to microbial community management when inhibitors are present. For example, because acclimation periods of weeks are required to build up sufficient quantities of desired microbes, hydrolysis processes operated in batch mode, such as dry AD processes, should be inoculated. Properly developed inoculation strategies may be applied to avoid system upsets and failures. Future research is needed to develop microbial community management strategies for full-scale implementation to avoid the negative impacts of inhibitors on bioenergy generation.

4.0 Enhanced Anaerobic Digestion Performance via Combined Solids- and Leachate-based Hydrolysis Reactor Inoculation

4.1 Introduction

AD is an environmentally attractive technology for conversion of various wastes to energy. However, despite numerous benefits, AD applications to OFMSW remain limited in North America due to economic barriers with existing technologies. To achieve economic viability, improved reactor performance is needed to increase bioenergy generation. One challenge to optimizing bioenergy generation is the presence of microbial inhibitors that can lead to suboptimal performance or even process failures. For example, elevated levels of inhibitors, including ammonia and salinity, are often found in AD systems, particularly those digesting high-salinity (food waste) or high-ammonia (manure) feedstocks or that recycle leachate (Chen et al., 2008; Kayhanian, 1994; Shahriari et al., 2012). Inhibition of AD performance due to elevated ammonia and salinity has been demonstrated to occur at concentrations >1.7 g TAN/L and 3.5 g Na^+/L, respectively (Chen et al., 2008). Leachate recycle may be employed to retain methanogens in single-stage AD configurations and to reduce water usage in both single- and multi-stage systems. Thus, strategies are needed to maintain high energy generation even when inhibitors are present.

Multi-stage AD technologies are advantageous because these systems use separate reactors for each stage, allowing for individual optimization of hydrolysis and methanogenesis processes. For OFMSW, hydrolysis is preferably conducted in LBRs operated in dry batch mode because LBRs can accept high solids waste (>15% solids) (Mata-Alvarez et al., 2000). Methanogenesis is conducted in high-rate reactors including FF or UASB reactors (Najafpour et

al., 2006). Numerous studies have focused on improving methanogenesis under inhibitory conditions (Chen et al., 2008), but studies focused on enhancing hydrolysis in the presence of inhibitors are lacking. Hydrolysis is the first and often rate-limiting step in anaerobic digestion (Xu et al., 2011). Thus, advancements in hydrolysis processes have the potential to improve AD performance and economic viability.

Previous research has demonstrated that hydrolysis rates can be increased in the presence of elevated ammonia and salinity via inoculation (Wilson et al., 2013). Further, inoculum acclimation has been shown to enhance hydrolysis rates in wet reactors treating food waste and manure by selecting for optimal microbial communities capable of performing under inhibitory conditions (Wilson et al., 2013). However, inoculation with hydrolyzers is not used in practice in high-solids systems. Since the quantity of microorganisms in LBRs can limit reactor performance, establishing and maintaining desirable microbial communities in reactors is key to optimal operation. To promote establishment of desirable microorganisms in LBRs, acclimated inoculum may be used as initial seed. After start-up (~2 weeks to 3 months [Angenent et al., 2002; Griffin et al., 1998]), LBRs may benefit from re-inoculation with each new batch because substantial time (several days to weeks) is required to develop inhibitor-tolerant hydrolyzing bacterial communities (Wilson et al., 2016). Thus, development of successful inoculation approaches for high-solids systems is needed.

To maintain desired microorganisms in AD systems, inocula derived from previously digested waste are promising because surface-attached bacteria are known to mediate hydrolysis of the recalcitrant feedstock cellulose (Wang et al., 2010). Digested waste inoculum has been shown to increase hydrolysis rates in LBRs under low ammonia conditions. Xu et al. (2012) recommended 20% inoculum (by mass) for LBRs, although that study used artificial food waste

and LBRs were run in isolation (i.e., not connected to methanogenesis reactors). Another study suggested leachate recycle (as opposed to single pass) may provide inoculum for improving process performance in LBRs treating manure (El-Mashad et al., 2006). By contrast, Degueurce et al. (2016) suggested that the beneficial role of leachate recirculation in LBR-based AD is abiotic and mainly due to leachate nutrient content, pH, and buffering capacity. However, none of these studies investigated the impact of inhibitors, and studies only examined one batch period. Thus, further research was needed to investigate optimal inoculation approaches and amounts for post-consumer waste in multi-stage systems during start-up and long-term operation with elevated ammonia and salinity.

Optimal inoculation approaches, including use of digested waste inoculum or leachate recycle, will most readily be developed with a fundamental understanding of the composition and distribution of microbial communities present in digested waste, leachate and fresh waste. Different microbial communities are selected for as a function of different reactor conditions including inhibitor concentrations and feedstock (Wilson et al., 2013). Thus, by extension, microbial communities are expected to differ substantially between digested waste, leachate and fresh waste, but the distribution and composition of microbial communities in multi-stage AD systems over long-term operation has yet to be investigated. This knowledge will support optimization of operational methods, potentially leading to improved economic viability via increased methane production, or reduced reactor volumes and batch times.

Thus, the objectives of this study were to (1) evaluate initial and long-term inoculation methods to establish enriched inhibitor-tolerant hydrolyzing communities and enhance performance of LBRs, and (2) to advance understanding of hydrolyzing microbial communities in multi-stage AD systems. The approach was to operate laboratory-scale, multi-stage AD

systems fed post-consumer OFMSW under elevated ammonia and salinity conditions. The impact of operational approaches on hydrolysis performance was investigated by comparing performance for LBRs seeded with unacclimated inoculum and different ratios of acclimated inoculum at start-up. Additionally, the effect of seeding methods was examined during long-term operation by identifying the optimal ratio of fresh waste to previously digested waste in multi-stage systems incorporating leachate recycle. Molecular tools (e.g., T-RFLP and 16S rRNA gene amplicon sequencing) were utilized to track microbial community development in the inoculum, feedstock, and leachate.

4.2 Materials and methods

4.2.1 Feedstock and inoculum collection

The OFMSW feedstock utilized in this study was a combination of food and yard waste. Food waste was collected from the Colorado State University Ram's Horn dining facility as previously described (Wilson et al., 2013). Food waste was stored in bins for less than 2 days prior to experimental use. Yard waste (leaves and grass clippings) and wood chips (used as a bulking material) were collected from a local landscaping company (Hageman Earth Cycle, Fort Collins, CO). After collection, OFMSW was stored at 4°C until subsequent use the following day. Immediately prior to experimental use, food waste (82% by wet mass), grass clippings (7.25%), leaves (3.5%), and wood chips (7.25%) were mixed thoroughly by hand to create the OFMSW feedstock loaded into LBRs. This food waste to yard waste ratio is consistent with ratios that may be used in the field (Beanie, 2013). Original microbial inoculum was collected from the DWWTP mesophilic anaerobic digester (Fort Collins, CO) and used immediately after collection. Waste and inoculum were analyzed for TS and VS content prior to digestion (Table 4.1).

Table 4.1. Characteristics of waste and inocula

	Parameter	
Sample	TS (%)	VS (%)
Food waste	20.2 (±3.6)	95.7 (±1.4)
Grass clippings	30.2 (±2.1)	88.5 (±1.4)
Leaves	88.1 (±3.1)	90.3 (±0.9)
DWWTP Inoculum	1.8 (±0.1)	76.9 (±0.4)

*Numbers in parentheses indicate standard deviations of 2-3 replicate batches (waste) or samples (inoculum).

4.2.2 Reactor configurations

4.2.2.1 Start-up test configuration

Two different configurations were used in this study to investigate the effects of inoculum percentage at start-up and over time. For experiments focused on reactor start-up, 4 LBRs were connected to individual leachate storage tanks (LSTs) without connections to methanogenic reactors (Fig. 4.1a, d).

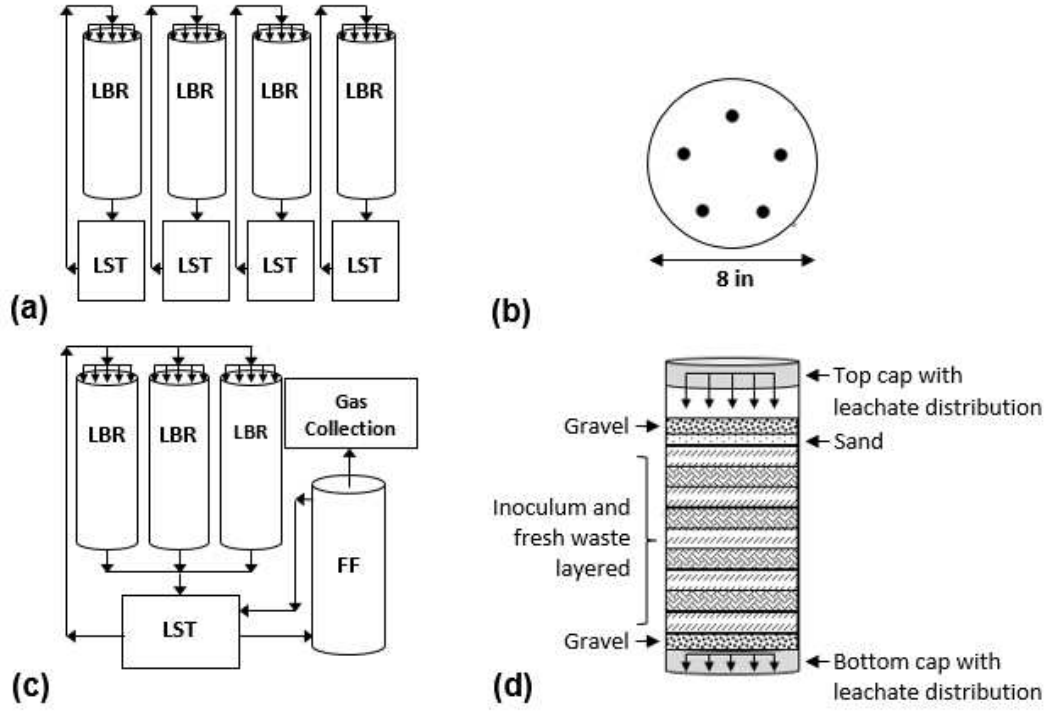

Figure 4.1. Reactor configurationfor determining performance at start-up (a). Plan view of LBR depicting spacing of leachate distribution ports (b). Reactor configuration for determining performance over time (c). Configurations include leach bed reactors (LBRs), leachate storage tank (LST), and a fixed film (FF) reactor. Arrows depict leachate flow path. LBR schematic depicting layered inoculum and fresh waste (d).

Utilizing separate LSTs allowed for isolation of the effects of inoculum percentages without the influence of microbial community development from the other LBRs or methanogenic reactors. LBRs were clear polycarbonate pipe with a diameter and height of 20 cm and 91 cm, respectively. The LBRs were sealed with Cherne adjustable pipe caps that were fitted with 5 evenly-spaced ports for leachate distribution (Fig. 4.1b). The volume of waste in each LBR varied depending on the percentage of inoculum used and was ~10 - 25L. 5-gal plastic carboys (~4 gal leachate) were utilized as LSTs. Leachate from each LBR was gravity-fed into an individual LST, and then pumped (via Cole Parmer Masterflex L/S Digital Drive peristaltic pumps) back to the top of the LBR at a rate of 20 ml/min. Flow rates were digitally controlled,

and the accuracy of the flow rate was manually confirmed three times during the experimental period. 1.27 cm and 0.32 cm vinyl tubing were used for leachate delivery to the LSTs and LBRs, respectively.

4.2.2.2 Long-term operation configuration

For long-term operation, a multi-stage system was utilized that contained a triplicate set of LBRs (previously described in *Section 4.2.2.1)*, 1 LST (54.6 cm x 38.1 cm x 66 cm), and 1 FF reactor (cylindrical drum with a radius and height of 23 cm and 79 cm, respectively) (Fig. 4.1c). The LST and FF had working volumes of ~10 and 30 gal, respectively. Leachate from the 3 LBRs flowed into 1 LST and then was pumped to the FF at a rate of 9 ml/min. Effluent from the FF was pumped at the same rate back to the LST. Leachate also was pumped from the LST to the top of the LBRs as described in *Section 4.2.2.1.* Vinyl tubing (1.27 cm inside diameter) was used for leachate delivery to and from the FF. The FF was filled with plastic packing material (Bioportz moving media, Entex Technologies). Leachate samples for liquid analysis were collected from the LST and FF reactor effluent (post-treatment). Samples were collected from the LST, rather than LBR effluent directly, as a means of monitoring pH and VFA concentrations in the composited leachate delivered to the LBRs and FF to ensure conditions were not inhibitory (e.g., >6.5 pH in the LST) (Ahn et al., 2010).

4.2.3 Microbial acclimation for LBRs and FF

To develop a large volume of acclimated inoculum to seed LBRs for tests under elevated ammonia and salinity concentrations, 2 LBRs were loaded with 3600 g (wet weight) of OFMSW and inoculated with DWWTP AD sludge (20% of total material by mass) by layering (*Section 4.2.1*). During digestion, leachate generated was collected in one LST (initially filled with nitrogen-purged deionized [DI] water) and recirculated back to the top of each LBR using the

configuration in Fig. 4.1a. Leachate was analyzed at least twice a week for DCOD, VFAs, ammonia, pH, and EC. After the first batch (16 days), the LBRs were emptied and refilled by layering fresh waste with previously digested waste (60% wet weight). Fresh waste and inoculum were alternately layered (4 and 5 layers of fresh waste and inoculum, respectively) to provide contact between waste and inoculum; layers were separated by mesh (5 mm) so that they could be distinguished post-digestion. Fresh waste layers were 900 g (wet weight) each, and the mass of inoculum layers varied depending on the inoculum percentage utilized. Columns were purged with nitrogen gas after each refilling to avoid prolonged exposure of the inoculum to oxygen. LBRs were operated over a 16-week period, and ammonia and salinity concentrations in leachate gradually were elevated artificially to 3.5 g TAN/L and a conductivity of 45 µS/cm (surrogate measure of salinity, ~6 g Na^+/L), respectively, by adding ammonium chloride and sodium chloride. Acclimation was considered complete when final solids destruction did not vary by more than 10% for consecutive batches.

To acclimate a microbial inoculum for the FF reactor (Fig. 4.1c), AD sludge was incubated at 35 ºC for 2 weeks and fed glucose while the ammonia and salinity concentrations were increased gradually to 1.5 g TAN/L and 3 g Na^+/L using ammonia chloride and sodium chloride. Next, the inoculum was mixed with a nutrient solution (50% v/v basis) (Owen et al., 1979) to ensure that nutrients were not limiting in the system, and the reactor was fed glucose or leachate generated from the LBRs at an organic loading rate (OLR) of 1 g COD/L·day while ammonia and conductivity concentrations were slowly increased further to 3.5 g TAN/L and 45 µS/cm. The effluent was recycled continuously at a rate of 50 ml/min, and the pH was adjusted to 7 using NaOH as needed. Throughout the 16-week period, the methanogenic reactor was operated as a “wet” (low-solids) digester (i.e., recycled liquid flowed through a sludge of

suspended biomass) rather than as a FF reactor to avoid sub-optimal microorganisms (non-inhibitor-tolerant microorganisms) attaching to the plastic packing. After 16 weeks, packing material was added to the reactor, and the FF reactor was operated for 3 weeks prior to subsequent experiments.

4.2.4 Reactor operation

4.2.4.1 Determining optimal seeding methods at start-up

To determine optimal seeding methods at start-up or when inhibitors become elevated, LBRs operating under elevated ammonia and salinity (3.5 g TAN/L and 45 µS/cm, respectively) were seeded by layering initially with unacclimated (60% by wet mass) or different percentages of acclimated inoculum (60%, 10%, and 0% by mass) and operated for a total of 3 batches with the set-up described in *Section 4.2.2.1* (Fig. 4.1a). The unacclimated inoculum utilized was previously digested waste from an identical multi-stage system operating under low ammonia and salinity. The source of acclimated inoculum was previously digested waste from the acclimated LBRs (*Section 4.2.3).* For all 3 batches, the mass of fresh waste (3600 g) loaded into each LBR remained constant. Since a FF reactor was not utilized in this set-up, leachate was manually diluted 50% by volume daily (Xu et al., 2011) with nitrogen-purged DI water to simulate the FF function (i.e., to reduce the concentration of soluble organics typically consumed in the FF). The DI water was spiked with appropriate concentrations of ammonium chloride and sodium chloride to maintain elevated ammonia and sodium in the system.

4.2.4.2 Determining optimal seeding methods over time

To determine the optimal ratio of previously digested waste to fresh waste over time, LBRs operating under elevated ammonia and salinity were seeded initially with a high percentage (60% by mass) of acclimated inoculum with the set-up described in *Section 4.2.2.2*

(Fig. 4.1c). After each batch (16 days), the LBRs were emptied and refilled using previously digested waste as described in *Section 4.2.3*. Then, LBRs were reseeded with decreasing percentages of inoculum (40%, 20%, 10%, and 0%) to determine the minimum amount of inoculum needed to maintain optimal microorganisms in the system. For each inoculum percentage, LBRs were operated for 2 - 3 batches. Post-digestion, separate inoculum and digested waste layers were mixed thoroughly by hand for 5 minutes, and 5 random samples were analyzed for TS and VS. Thus, for each inoculum percentage, hydrolysis performance results were based on a minimum of 30 samples (3 columns x 2 batches x 5 samples).

4.2.5 Chemical analyses

TS and VS of the inoculum and waste were measured pre- and post-digestion according to Standard Method 2540 (APHA, 1995). TS and VS removal efficiencies were calculated according to Xu et al. (2011). TS removal efficiencies were calculated according to the following equation:

$$TS_{removed}\% = \frac{TS_{initial} - TS_{final}}{TS_{initial}} * 100\% \quad (Eq. 3)$$

The same equation was applied to calculate VS removal efficiencies (Xu et al., 2011). Liquid samples extracted from the LST and FF effluent were analyzed for pH and EC using probes (VWR #89231-604 and #11388-382, respectively). DCOD concentrations were determined using Hach's COD High Range Vials and digestion colorimetric method (Hach, Loveland, CO). VFAs were determined using Hach's Volatile Acids TNTplus Reagent Set and esterification method. Samples were filtered through a 0.2 μm syringe filter prior to DCOD and VFA analyses. Hach's Nitrogen-Ammonia High Range Reagent Set was utilized weekly to verify that ammonia concentrations remained constant over time. Biogas volume was measured utilizing a wet tip gas

meter (Speece Co., Nashville, TN), and biogas composition was determined via gas chromatography using a thermal conductivity detector as described previously (Wilson et al., 2013).

4.2.6 Microbial community analyses

Motivated by the long-term study results, experiments were conducted to compare quantities and compositions of the microbial communities among the leachate, inoculum, and fresh waste. Samples used for quantification of Bacteria in leachate and previously digested inoculum analyses were collected on day 192 of the long-term experiment. To prepare samples for DNA extraction, 50-ml leachate samples were centrifuged at 5,000 g for 10 minutes at 4°C. The supernatant was discarded, and the remaining pellet was used for DNA extraction. For solid samples, 25 g of fresh waste or inoculum were blended with 125 ml of sterile DI water for 1 minute to ensure representative samples. The blended mixture was then centrifuged at 5000 g for 5 minutes at 4°C. The supernatant was discarded, and the pellet was used for DNA extraction. For all samples, DNA was extracted using MoBio's PowerSoil® DNA isolation kit according to the manufacturer's instructions.

SYBR green™ assays were utilized to quantify the number of bacterial 16S rRNA gene copies in each sample. Genomic DNA extracted from *Thauera aromatica* (ATCC #7002265D) was used to generate calibration curves. The primer set 1369F and 1492R was used for amplification (Li et al., 2010), and all assays were conducted using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Each 25-µl SYBR green™ qPCR reaction contained: 1X Power SYBR green™ (Life technologies, Grand Island, NY), forward and reverse primers (0.15 µM), PCR grade water, and 4 ng DNA template. Thermocycling conditions were as follows: 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s, 56ºC for 20 s, and 68ºC for

30 s (Li et al., 2010). Dissociation curve analysis was conducted to confirm amplicon specificity. For all inoculum percentages, bacterial 16S rRNA gene quantities were estimated based on a single sample (192-day sample) to eliminate the impact of changes over time. The number of 16S rRNA genes per mass of solid inoculum was determined, and the total quantity of Bacteria added to the LBRs was projected for each inoculum percentage. All numbers are expressed as a function of the amount of fresh waste added, which was constant over time. For leachate, the quantity of 16S rRNA gene copies was determined per volume of leachate, and multiplied by the amount of leachate distributed to the LBRs over the experimental period.

Community composition was determined via T-RFLP analyses of DNA isolated from leachate, fresh waste, and previously digested inoculum and waste. Previously digested waste was collected from three LBRs operated with fresh waste and inoculum (40% by mass) layered (LI), fresh waste and inoculum (40% by mass) thoroughly mixed by hand (MI), and fresh waste only with no inoculum (NI) utilizing the set-up described in *Section 4.2.2.2*. To determine microbes present early in the batch period, digested samples were extracted from each layer of the LBRs after 5 days of operation; leachate and previously digested inoculum samples were collected as described in this section. To track changes to bacterial, fermenting, and hydrolyzing communities, T-RFLP analyses were conducted targeting the 16S rRNA gene, *hydA* gene (encodes the alpha subunit of iron hydrogenase), and *cel48* gene (encodes glycoside hydrolases of family 48). T-RFLP analysis targeting the 16S rRNA gene was conducted as described by Wilson et al. (2013). T-RFLP analyses targeting the *hydA* gene and *cel48* gene were conducted as previously described by Lefèvre et al. (2013).

DNA from leachate (collected on day 192), fresh waste, and inoculum (collected on day 192) were submitted to Research and Testing Laboratories LLC (Lubbock, Texas) for bacterial

16S rRNA gene amplicon sequencing using Illumina MiSeq. Primers 28f and 388R were used, targeting the V1-V2 hypervariable regions of 16S rRNA genes (Sundquist et al., 2007; Turnbaugh et al., 2009). Bioinformatic analysis was conducted as described previously (*Section 3.2.4.2)*.

4.2.7 Statistical analyses

ANOVA and Tukey's HSD tests were conducted using R Statistical Software (R Core Team, 2012) to determine if the slope of the fitted regression line for VS reduction as a function of inoculum percentage was statistically different from 0 as well as to determine statistical differences in average VS reduction for a range of inoculum percentages. Results yielding a p-value less than 0.05 were considered to be significant. T-RFLP data were analyzed and similarity matrices were calculated according to Wilson et al. (2013), except terminal restriction fragments (T-RFs) representing less than 1% of the total area were excluded (Rees et al., 2004). Pairwise distances were calculated aligned sequences, followed by clustering and estimation of Shannon diversity.

4.3 Results and discussion

4.3.1 Impact of inoculum percentage on waste hydrolysis at start-up

We hypothesized that waste reduction would be limited by the number of organisms provided as inoculum in the LBRs under elevated ammonia and salinity conditions. As expected, results demonstrated that higher percentages of inoculum are beneficial and improve VS reduction. Initially, with acclimated inoculum at 60% and a digestion period of 16 days, reactors demonstrated an average VS reduction of 69.5% (±3.5) (Fig. 4.2a).

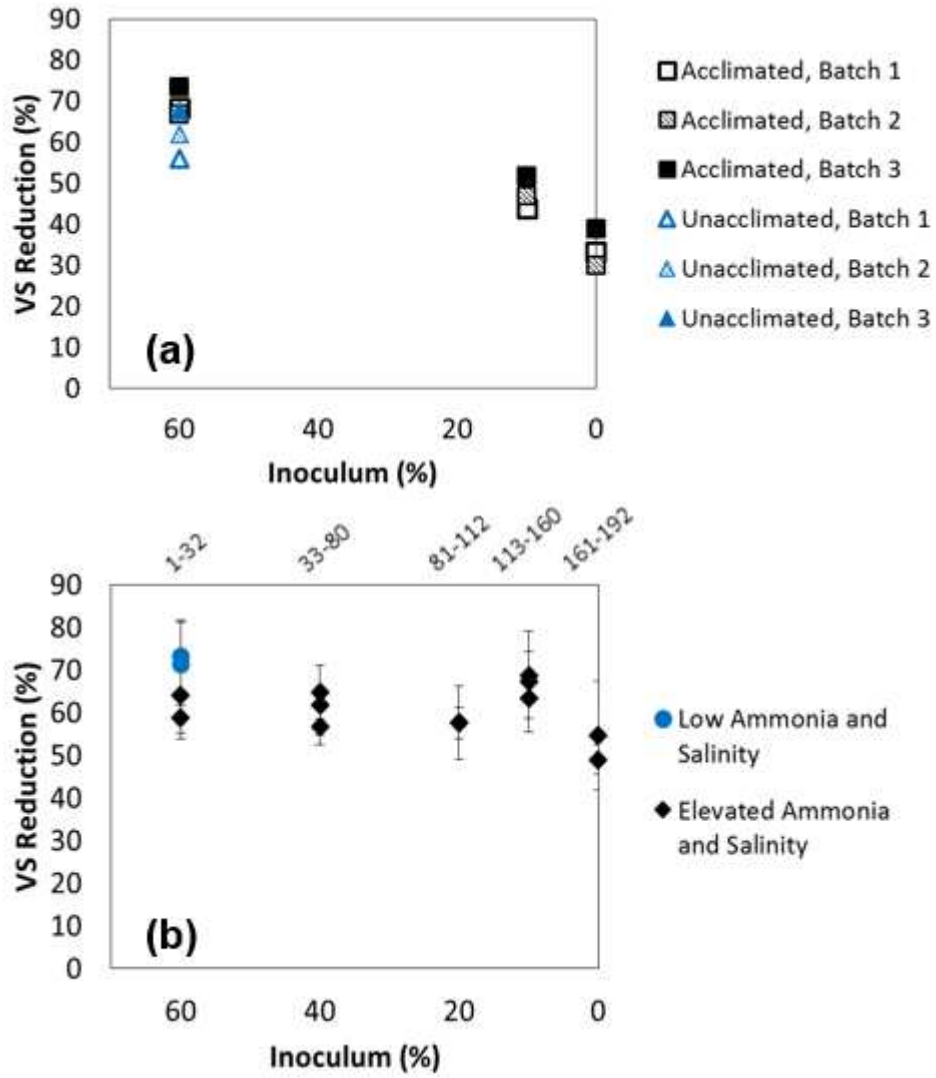

Figure 4.2. VS reduction as a function of percentage of inoculumat start-up (a) and over time (b). At start-up (a), reactors were seeded with acclimated (▲) and unacclimated (■) inoculum operating under elevated ammonia and salinity. Three batches (starting on days 0, 16, and 32) were run for each percentage. Over time (b), reactors were seeded with unacclimated inoculum under low ammonia (●) to demonstrate optimal reactor performance without inhibition or acclimated inoculum under elevated ammonia and salinity (◆). Multiple data points at each percentage demonstrate results for successive batches (a and b). Data points and error bars represent average performance and standard deviation for the set of triplicate reactors for a single batch (b only). Days of operation are indicated at the top of the figure.

Results from our study demonstrated that hydrolysis performance was better than or comparable to previously reported performances for dry digesters operating under lower ammonia and salinity, despite the elevated levels present. For example, Xu et al. (2014) achieved 45.4 - 69.4%

VS reduction in LBRs treating food waste inoculated with WWTP AD sludge with a 16-day residence time. Similarly, Yan et al. (2014) demonstrated 58.7 – 68.1% VS reduction in LBRs fed simulated food waste and inoculated with AD sludge and cow manure. Lu et al. (2008) demonstrated 63% VS reduction in LBRs connected to a UASB with leachate recirculation treating vegetable and flower waste over a 10 day period. Thus, results indicated that high solids conversion can be achieved even in the presence of inhibitors, if required organisms are provided in sufficient quantities via inoculation.

For lower acclimated inoculum percentages, average VS reduction was reduced ~1.5- and 2-fold (10% and 0%, respectively) (Fig. 4.2a). An ANOVA test on triplicate batches at each inoculum percentage indicated that significant differences exist among means for VS reduction (p-value = 0.0001). The significant reduction in solids removal with reduced inoculum percentages demonstrates that the quantity of hydrolyzing bacteria present in LBRs limits performance. VS reduction improved by the end of batch 3 for all inoculum percentages (with VS reduction increasing to 73% for the 60% acclimated inoculum). Performance increases for successive batches likely indicate that the concentration of inhibitor-tolerant hydrolyzing bacteria increased in the systems (e.g., in the digestate). However, VS reduction with 0% inoculum was still 1.9-fold lower than VS reduction with 60% inoculum for the third batch. In the only other study to investigate the impact of inoculum percentage at start-up in LBRs, Xu et al. (2012) also observed a decrease in waste hydrolysis with reduced inoculum percentages (80%, 20%, 5%, and 0%), albeit a less dramatic trend than observed in our study. The less dramatic decrease was likely because Xu et al. (2012) investigated low ammonia and salinity conditions. Specifically, their study demonstrated 1.13- and 1.14-fold lower VS removals for inoculum percentages of 20% and 5%, respectively, compared to an inoculum percentage of 80%. Xu et al. (2012)

recommended a 20% inoculum; however, the study did not test multiple batches over time or 10% inoculum. Ultimately, the substantial reduction in VS removal observed in our study at lower inoculum percentages suggests that higher volumes of acclimated inoculum are needed at start-up, or when salinity and ammonia are observed to increase in leachate, for optimal performance.

To determine if acclimated inoculum enhanced performance compared to unacclimated inoculum, both inocula were tested in parallel at 60%. Interestingly, acclimated inoculum initially demonstrated 68% VS reduction, compared to 56% VS reduction for unacclimated inoculum (Fig. 4.2a). However, both reactors performed similarly by the end of batch 3. Thus, results suggest that inhibitor-tolerant organisms accumulated in as little as 3 batches when a high quantity of inoculum was used (60%). Several studies have demonstrated that acclimated inoculum performs better than unacclimated inoculum in wet systems (Calli et al., 2005; Chen et al., 2008; Omil et al., 1995; Sung & Liu, 2003). For example, we previously demonstrated that an AD sludge-derived inoculum acclimated to elevated ammonia (5 g TAN/L) fed manure and food waste performed significantly better than unacclimated sludge directly exposed to elevated ammonia concentrations in wet batch reactors (Wilson et al., 2013). Thus, results herein extend this finding to LBRs and provide guidance on the quantity of inoculum needed to maximize solids reduction at start-up (~40-60%) or when inhibitors build up.

4.3.2 Impact of inoculum percentage on system performance long-term operation

To determine how inoculum percentages and connection to a FF affect maintenance of desired organisms and performance over long-term operation, experiments were conducted with multi-stage reactors that incorporated leachate recycle for over 190 days. Hydrolysis performance and methane generation were monitored across a range of inoculum percentages

(60% - 0%). Average VS reduction for LBRs with low ammonia and salinity at 60% inoculum was slightly better (1.17-fold) than for LBRs with high ammonia (~3.5 g TAN/L) and salinity (45 µS/cm) (Fig. 4.2b). This slight inhibition is consistent with the observations of others (Chen et al., 2008); however, in our study inhibition likely was minimized via the usage of acclimated inoculum. In one of the first studies to determine the effects of elevated ammonia, Kayhanian (1994) demonstrated that high solids digesters performed best at TAN concentrations in the range of 0.6-1 g/L. More recently, Duan et al. (2012) reported significant inhibition at 3 - 4 g TAN/L in high solids AD of sewage sludge with the highest VS reduction reaching only 40% (compared to ~60% for uninhibited reactors) and VFA concentrations exceeding 10 g/L (Song et al., 2004). In contrast, our study demonstrated VS reduction levels up to an average of 62 (±7)% at 60% inoculum under elevated ammonia and salinity, indicating a benefit for use of inoculums acclimated to elevated ammonia and salinity.

Interestingly, in contrast to start-up, over time VS reduction remained relatively constant for decreasing inoculum percentages (Fig. 4.2b). At 60% initial inoculum, VS reduction averaged 62 (±7)% over the first two batches. When inoculum percentages were then decreased to 40% and 20%, VS reduction appeared to decrease slightly. Unexpectedly, VS reduction increased to an average of 67 (±7)% when the inoculum percentage was lowered further to 10%, which was the highest VS reduction demonstrated for any of the percentages. Moreover, this performance at 10% inoculum is comparable to performance demonstrated in reactors operating under low ammonia and salinity. However, at 0% inoculum, VS reduction once again decreased and demonstrated the lowest reductions of any inoculum percentages. Despite a decreasing trend for VS reduction as a function of inoculum percentage (with the exception of 10% inoculum), the slope of the linear regression for all percentages with acclimated inoculum was not significantly

different from zero, suggesting that inoculum percentage did not substantially affect VS reduction over time. However, an ANOVA test revealed significant differences in mean VS reductions among the various inoculum percentages (p-value = 0.0074), and Tukey's HSD test identified significant differences between the mean VS reduction at 10% and 0% inoculum (p-value = 0.003). No other significant differences between mean VS reductions were observed. Thus, regardless of inoculum percentage, over time reactors demonstrated VS reduction comparable to systems running under optimal conditions despite the elevated ammonia and salinity levels (Lu et al., 2008; Xu et al., 2014; Yan et al., 2014).

Consistent with solids reduction data, VFA concentrations demonstrated steady performance for decreasing inoculum percentages over time (Fig. 4.3a). At the start of a new batch, leachate VFA concentrations spiked in the LST due to the influx of fresh waste but gradually reduced as readily hydrolysable substrates were converted to soluble products and methane. Steady, low VFA concentrations in FF effluent demonstrated the FF was effective despite elevated inhibitor levels. pH values ranged from 6.6 – 8.3 and 7.8 - 8.5 in the LST and FF effluent, respectively, over the course of the experiment (data not shown).

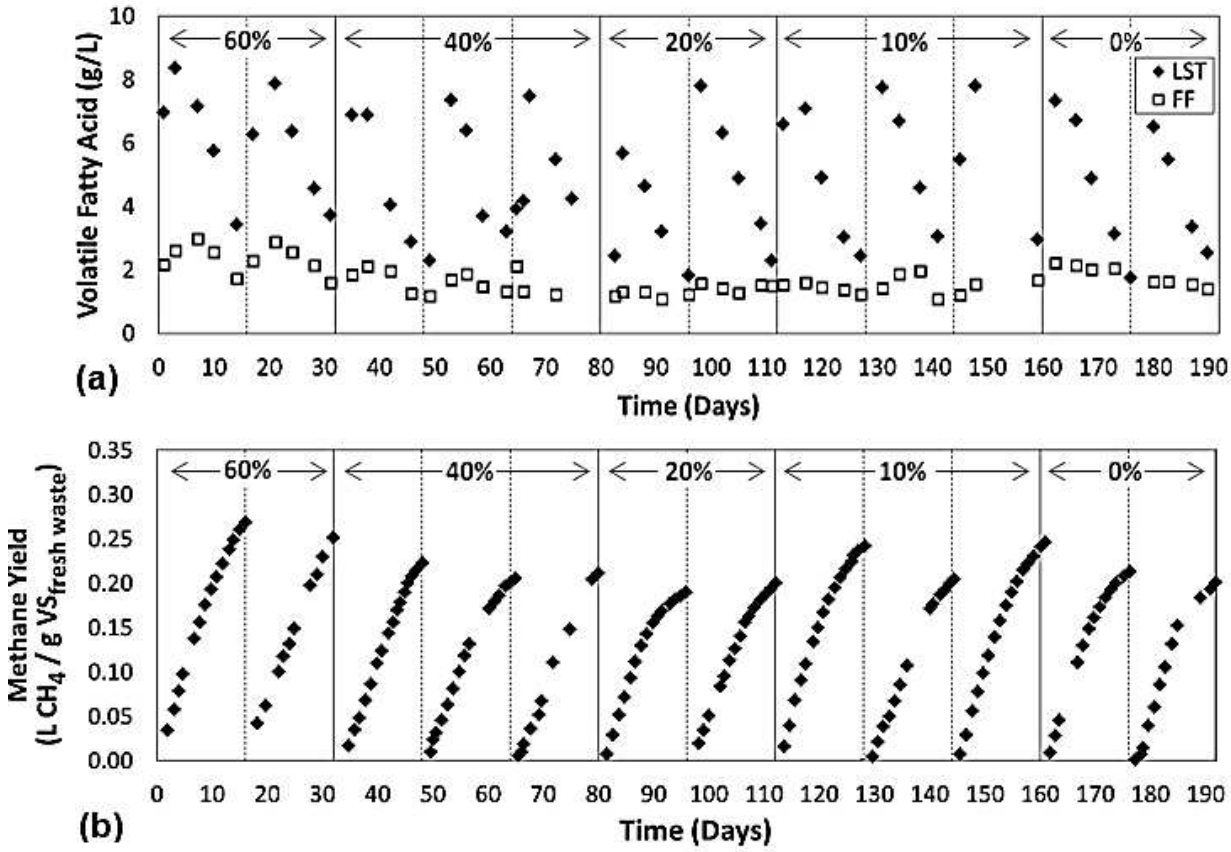

Figure 4.3. Long-term performance data for multi-stage reactorsoperating under elevated ammonia and salinity (~3.5 g TAN/L and 45 µS/cm). (a) VFA concentrations and (b) methane yield are shown for a range of acclimated inoculum percentages over time. Inoculum percentages are noted at the top of each graph section.

Overall, our reactors demonstrated comparable or higher methane generation to previous literature values for reactors operating under low ammonia and salinity conditions (Fig. 4.3b). The highest average methane yield, observed with the greatest inoculum percentage, was 0.260 [± 0.01] L CH_4/ g $VS_{fresh\ waste}$) (Figure 4.3b). By contrast, lower methane production (0.182 L CH_4/ g VS) has been demonstrated in LBRs coupled with a UASB fed simulated food waste for a 17-day digestion period at start-up (Xu et al., 2011). Additionally, Han et al. (2002) observed 0.27 L CH_4/ g VS for sequential batch, two-phase anaerobic composting systems fed food waste (with impurities removed) and inoculated with rumen microorganisms with a 10 day solids

retention time. Dearman et al. (2007) demonstrated similar or slightly lower methane yields (0.214, 0.229 L CH_4/ g VS) for dry food waste digesters using 10% (w/w) inoculum despite an extremely long digestion period (73 days). Solids reduction is strongly impacted by feedstock composition, and thus lower rates reported by others may also be due, in part, to use of more recalcitrant feedstocks. However, superior microbial populations likely improved methane production herein.

Interestingly, despite remaining within the range of reported methane yields, average yields in our study decreased with decreasing inoculum at 40% and 20% (0.212 [±0.01] and 0.195 [±0.01] L CH_4/ g $VS_{fresh\ waste}$, respectively), but then increased at 10% inoculum and decreased at 0% inoculum, consistent with solids reduction data (Fig. 4.3b). At 10% inoculum, methane yield was 0.230 (±0.02) L CH_4/ g $VS_{fresh\ waste,}$ and at 0% inoculum yield was 0.207 [±0.01] L CH_4/ g $VS_{fresh\ waste}$). Thus, collectively our results suggest that after an initial start-up period, low quantities of solid inoculum (~10%) are sufficient under elevated ammonia and salinity, and importantly, that even after start-up, solid-phase inoculum addition improves performance relative to no inoculum. The increase in performance observed at 10% was unexpected considering the declining trend for higher percentages and the idea that hydrolyzers are solids-associated (Wang et al., 2010; Zhang et al., 2007). These results led us to hypothesize that desired microorganisms built up in the leachate over time and thus leachate recirculation could also provide a source of hydrolyzing bacteria. Similarly, others have speculated that leachate recirculation enhances performance based on results from a high-solids, single-stage AD system fed cattle waste operated at 40ºC and 50ºC (El-Mashad et al., 2006). To test this hypothesis and develop a mechanistic understanding of microbial community dynamics in multi-stage AD systems, bacterial communities were characterized over long term operation.

4.3.3 Quantification of leachate-derived and solids inoculum bacteria

We sought to determine if leachate recirculation could provide a source of hydrolyzing bacteria and first quantified Bacteria in the leachate and solid waste inoculum to determine how the quantities of Bacteria present compared. qPCR data revealed 60% inoculum resulted in the addition of $3.1x10^{11}$ bacterial 16S rRNA genes/ g fresh waste to the LBR (without including bacteria contributed by leachate), while 10% inoculum provided $2.3x10^{10}$ bacterial genes/ g fresh waste (Fig 4.4).

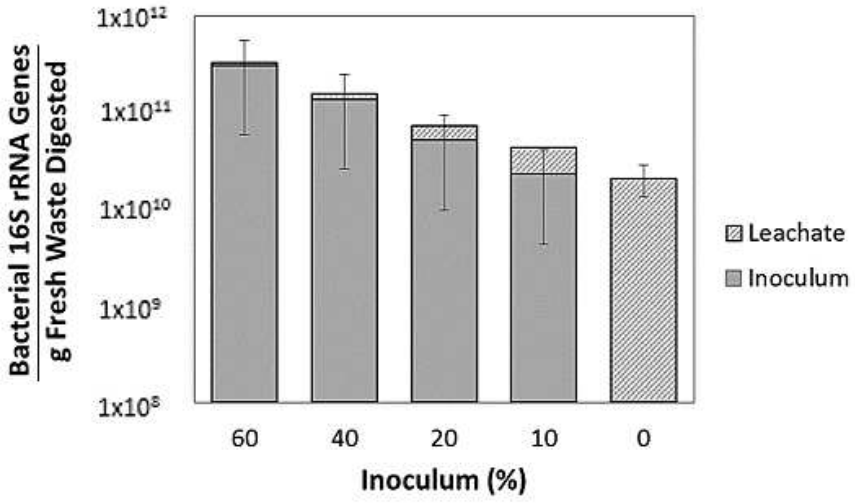

Figure 4.4. Comparison of quantities of bacterial 16S rRNA genes added to leach beds via acclimated inoculum and leachate recirculationthroughout a 16-day batch period. The assay utilized in our study only targets bacteria and thus avoids methanogen detection. Leachate and inoculum analyzed were collected on day 192. Thus, for all of the inoculum percentages (10-60%), bacterial 16S rRNA gene quantities were estimated based on a single sample to eliminate the impact of changes over time. The amount of fresh waste added to each reactor remained constant over time, and the amount of inoculum was adjusted accordingly. Error bars correspond to standard deviations for qPCR reactions run on DNA extracted from quintuplicate samples.

Interestingly, data indicate that the quantity of Bacteria in 10% inoculum ($2.3x10^{10}$ genes/ g fresh waste) and in the leachate (0% inoculum) after 192 days of operation ($2.1x10^{10}$ genes/ g fresh waste) are very similar. These data are 16S rRNA gene-based and thus do not indicate the type or activity of bacteria, but these results support the hypothesis that leachate is a good source of

inoculum for hydrolysis after sufficient time has allowed microorganisms to build up in the leachate. Notable improvements in performance were not seen until day 112, so high percentage of inoculum may be beneficial initially for at least 2-4 batches. The initial percentage of inoculum used likely will affect the number of batches required to establish sufficient hydrolyzing populations because high initial volumes of inoculum will add greater quantities of microorganisms to the system. The inoculum percentage could also be decreased slowly after 2 batches as was done here to continue building up desired organisms, and simultaneously avoid system overload by slowly introducing higher quantities of OFMSW. Further work is necessary to clarify the most advantageous method of decreasing inoculum over time. However, within ~3-4 months, results suggest that low volumes of inoculum may be utilized consistently. Importantly, molecular data also indicate that addition of 10% inoculum doubles the quantity bacteria that contact fresh waste over the course of a batch; this finding is consistent with the superior performance observed at 10% inoculum compared to leachate alone.

4.3.4 Microbial community composition dynamics

Because the type, as well as the quantity, of Bacteria control performance, we sought to determine if microbial community compositions between the solid inoculum and leachate after 192 days of operation were similar. Thus, we examined the total bacterial (via 16S rRNA gene-targeted T-RFLP), fermenting (via *hydA* gene-targeted T-RFLP), and hydrolyzing (via *cel48* gene-targeted T-RFLP) microbial communities among fresh waste, leachate, and inoculum. Additionally, digested samples were analyzed after a 5 day digestion period to determine microbes present at the early stages of a batch.

Ribosomal and functional gene-targeted MDS analysis demonstrated that over time the leachate microbial communities became similar to digestate-based communities (Fig. 4.5), while the

microbial communities in the feedstock remained distinct. Similarly, Staley et al. (2012) demonstrated distinct shifts in microbial community composition in decomposed refuse compared to fresh refuse in landfills.

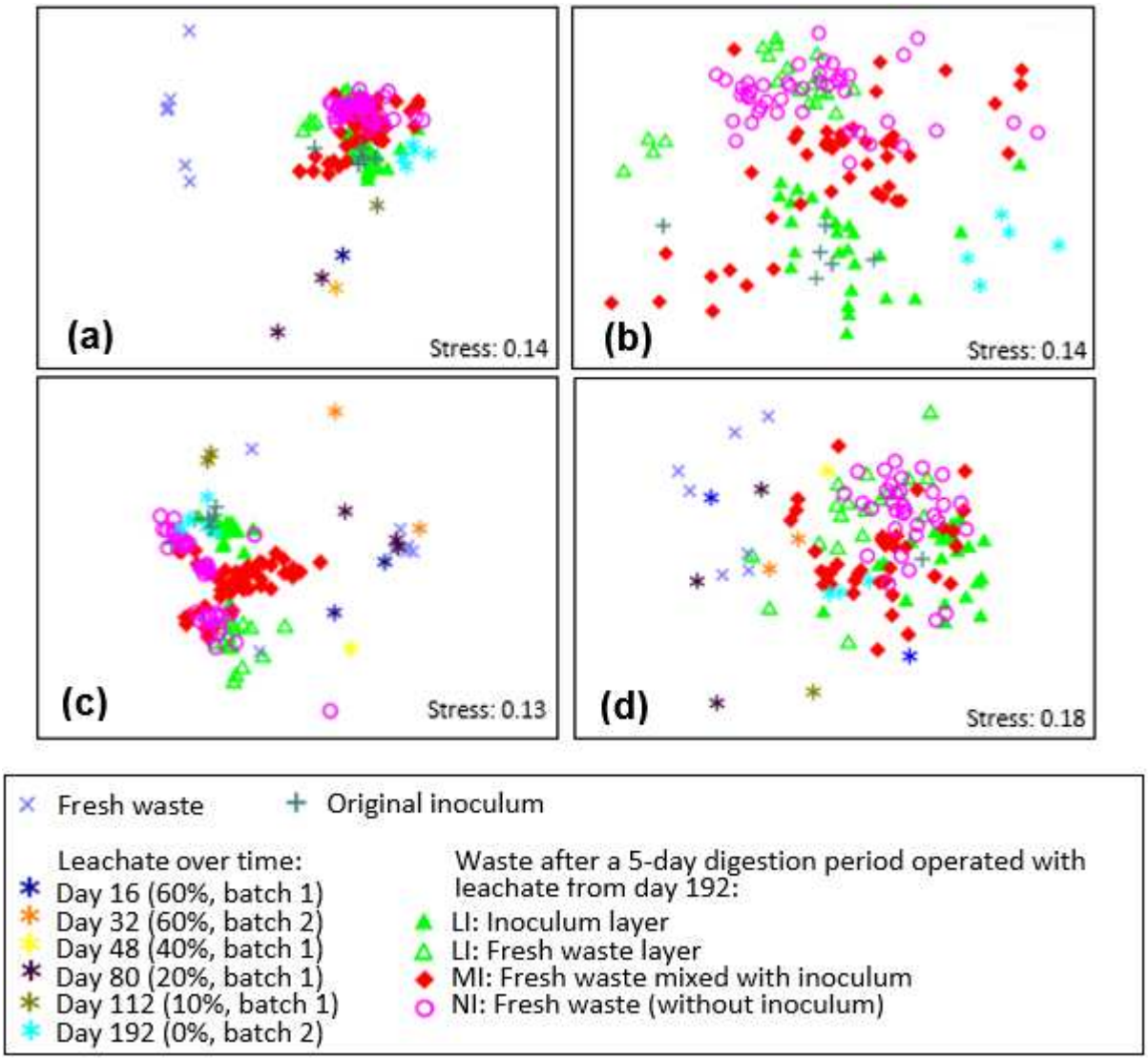

Figure 4.5. MDS plots based on (a) 16S rRNA gene-targeted, (b) enlarged section of plot (a), (c) hydA gene-targeted, and (d) cel48 gene-targeted T-RFLP analysis.

Initially, through day 80, leachate bacterial communities were distinct from other communities. However, by day 112, the communities had undergone a large shift, and by day 192, the leachate

communities clearly clustered with inoculum and digested waste communities (Fig. 4.5a, b). Similarly, by day 192, hydrolyzing communities in leachate converged with digested waste communities, even though hydrolyzers have been considered solids-associated (Fig. 5d). Interestingly, *cel48* microbial communities did not cluster as tightly as the bacterial or fermenting communities (Fig. 4.5c), which indicates that cellulose hydrolyzers are less different among fresh waste, inoculum, and leachate. Thus, molecular data suggests that recirculated leachate can indeed be a useful inoculum for hydrolysis after a lag period if the leachate microbial communities are properly developed. Interestingly, in one of the few studies to investigate microbial community dynamics in AD leachate, Degueurce et al. (2016) concluded there was little transfer of microorganisms between leachate and solid waste in LBRs digesting manure, and, instead, microorganisms inherently present in the manure strongly impacted reactor performance. The contrasting results observed in our study may be explained by the difference in feedstocks; manure often is used as an inoculum due to its high quantity of microorganisms, which likely dominated reactor communities, whereas OFMSW microorganisms were readily outcompeted by optimized inoculum microbes. The OFMSW feedstocks likely did not contain high levels of bacteria tolerant of elevated salinity and ammonia. Additionally, lower redox in the AD system likely contributed to selection of different microbes despite storage of the food waste in large bins that may have become anaerobic.

Results also indicated that the inoculum delivery approach impacted development of desired microbial communities within fresh waste during the digestion process. For LI, original inoculum and 5-day digested waste formed a tight cluster when compared to fresh waste and early leachate, indicating relatively successful transfer of inoculum microbes to the fresh waste (Fig. 4.5a). However, inoculum and fresh waste layers were distinguishable from each other (Fig.

4.5b). For MI, the microbial community composition grouped generally between the LI waste and LI inoculum layers; these results suggest that thorough mixing is likely beneficial. Interestingly, the NI microbial communities generally clustered with the LI waste layer and MI, indicating that after 192 days, leachate delivered generally the same types of bacteria to the fresh waste as the solid waste inoculum. However, NI communities grouped closer to the LI fresh waste layer than the inoculum layer, consistent with the observed benefits of inoculation with digested waste. These results are similar to previous research indicating that surface-attached bacteria are important for hydrolysis, particularly for cellulose (Lu et al., 2008; Wang et al., 2010). In one of the few studies to look at inoculation methods, El-Mashad et al. (2006) demonstrated that adding inoculum throughout the reactor height compared to leaving previously digested waste at the bottom of reactors increased methane generation. Thus, enhanced delivery methods (e.g., mixing) may increase hydrolysis performance further. Future research is needed to develop enhanced inoculation approaches suitable for full-scale implementation in dry digestion technologies.

4.3.5 Identification of inhibitor-tolerant microbial phylotypes

Bacterial-targeted 16S rRNA gene amplicon sequencing results revealed distinct phylotypes present in inoculum, leachate, and fresh waste. The highest species diversity was observed in the inoculum and one leachate sample (Shannon values of 4.7 and 3.37, respectively), whereas the lowest species diversity was observed in the other leachate sample (1.0) (Table 4.2). The majority (>95%) of microorganisms in all samples were identified beyond the domain level (Fig. 4.6).

Table 4.2. Shannon indices

Sample	Shannon Index
Fresh Waste	2.92
Fresh Waste	2.67
Inoculum	4.69
Inoculum	4.12
Leachate	1.00
Leachate	3.37

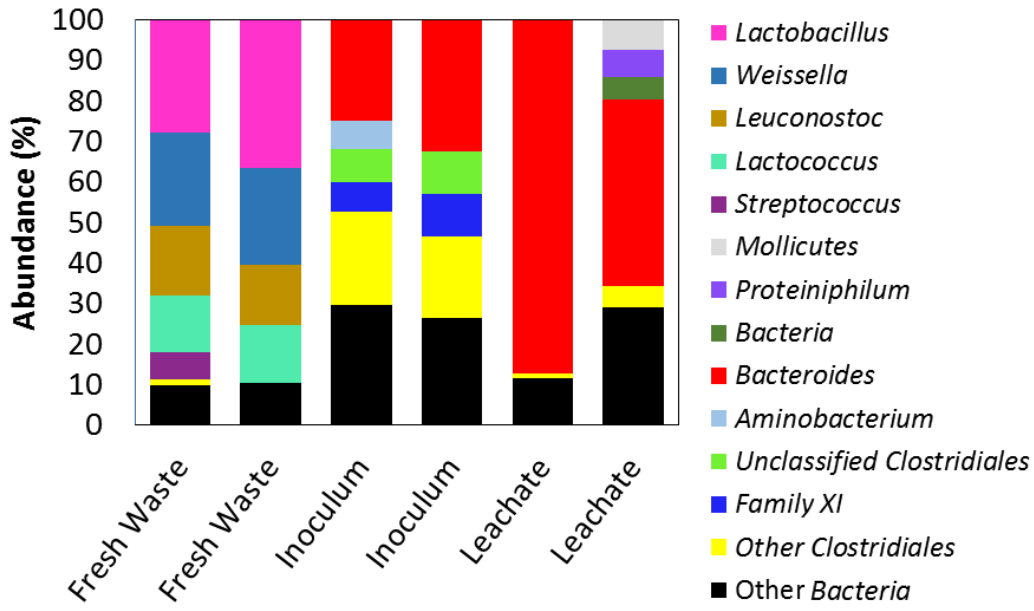

Figure 4.6. Relative genus-level abundance of 16S rRNA bacterial-targeted OTUs based on Illumina MiSeq sequencing.Higher taxonomic levels are given when genus-level identification was not possible. The label identified as "Other" indicates grouped phylotypes that individually comprised less than 5% abundance. Two representative samples each of inoculum, leachate, and fresh waste were chosen for analysis. Leachate and inoculum analyzed were collected on day 192.

Interestingly, the microbial community in fresh waste was dominated by anaerobes, as well as microorganisms associated with food waste. At the order level, *Lactobacillales* accounted for over 89% of the microorganisms in the fresh waste, although its presence was less than 1 and 7% abundance in the leachate and inoculum, respectively. Within this order, 4 main genera (*Leuconostoc, Lactococcus, Weissella,* and *Lactobacillus*) were identified (Fig. 4.6), all of which are acid-tolerant anaerobes largely used in the fermentation of dairy products and vegetables. The dominance of these anaerobes was somewhat unexpected and could reflect the particular waste utilized in this study or storage method of food waste prior to collection.

Although microbial communities in the leachate and inoculum were similar, differences observed suggest complimentary capabilities. *Bacteroidales* accounted for the majority of the organisms in the leachate (94 and 66%) and comprised 37 and 42% abundance in the two inoculum samples (data not shown). *Bacteroides* was the dominant genus in leachate and comprised over 25% of the solid waste inoculum. *Bacteroides* are known to be cellulolytic and carbohydrate-utilizing (Wang et al., 2010). The particular species (*Bacteroides coprosuis*) dominating the genus *Bacteroides* observed in our reactors was first isolated in 2005 from a swine-manure storage pit (Whitehead et al., 2005) and has been documented since then in digesters treating municipal sludge and waste from cattle farms. Similar to the *cel48* gene-targeted T-RFLP data, results indicated that hydrolyzing bacteria in the form of *Bacteroides* (which contain the *cel48* gene) are present in the leachate. Based on these observations, *Bacteroides* are likely ammonia and salinity tolerant and contribute to improved performance under these conditions. Class *Clostridiales*, bacteria well known for their cellulolytic capabilities (Wang et al., 2010), comprised 40% of the inoculum communities, but were present at very low levels in the leachate (1 and 5%) and fresh waste (<2%). This observation is consistent with

previous studies indicating *Clostridia* are solids-associated (Lu et al., 2008; Wang et al., 2010) and may help explain observed performance improvements when solid inoculum was provided. Yard waste accounted for just over 10% of the total mass added to reactors and is a main source of cellulose in the system, and this feedstock may have been hydrolyzed more completely when *Clostridia* were provided.

4.4 Conclusions

The challenges associated with inhibitory ammonia and salinity concentrations encountered in AD systems can be overcome via hydrolysis seeding methods. Inoculum should be established by maintaining high levels of digested waste (~40-60% by mass) in hydrolysis reactors to seed successive batches at start-up or when salinity and ammonia are observed to be elevating. After a lag period, desired hydrolyzers accumulate in recirculated leachate. Thus, leachate recirculation combined with digested-waste inoculum (~10% by mass) is recommended for improved performance. Optimized hydrolysis may reduce capital and operating costs and ultimately increase the economic viability of dry AD systems that incorporate leachate recycle.

5.0 Comparative Metatransciptomic Analysis of Microbial Communities Digesting Manure or Food Waste

5.1 Introduction

Molecular biology tools can be used to link microbial community composition with operational parameters and overall reactor performance. DNA-based molecular approaches are widely utilized to identify the presence or abundance of microorganisms in a reactors. Although DNA-based approaches are relatively simple to execute, knowledge of the activity (rather than presence) of microorganisms via more difficult RNA-based approaches generally is more valuable for determining key microbes in a system. Further, the majority of AD studies have determined phylogenetic profiles of the microbial communities present in reactors via targeting the 16S rRNA gene since this gene is highly conserved between different species (Isenbarger et al., 2008; Yang et al., 2014a). However, since the phylogenetic diversity of a system does not necessarily correlate to functionality of the system, a better understanding of metabolic capabilities, functional redundancies, and microbial community interactions in digesters would be valuable for optimizing digester performance (Langille et al., 2013; Pereyra et al., 2012; Vanwonterghem et al., 2014). Thus, functional gene-based approaches often are desirable and may yield more relevant and informative results.

Unfortunately, functional gene-based approaches applied with commonly used molecular tools suffer from limitations. Quantitative polymerase chain reaction (qPCR) and real-time PCR (RT-qPCR) are molecular techniques used to quantity genes (DNA) or gene expression (RNA), respectively. T-RFLP is a molecular fingerprinting tool utilized for profiling microbial communitie structure (Schutte et al., 2008). Each of these tools amplifies specific genes in the

DNA or RNA using primers, which are short fragments of complementary DNA designed to target specific regions (e.g., genes) in a sample. Numerous studies have been conducted to develop primers with appropriate sequences targeting the 16S rRNA gene. Unfortunately, functional gene-based approaches often suffer from a general lack of characterization and unavailability of target sequences for microorganisms in anaerobic mixed cultures; thus, challenges encountered with functional gene-based assays typically are the result of inadequate primer sets that often are more appropriate for pure cultures (Pereyra et al., 2010). Currently available functional gene-targeted assays (e.g., assays targeting genes involved in cellulose degradation) were developed for other anaerobic applications (e.g., reactors treating acid mine drainage) and are not always successful for microbial communities found in AD. For example, in some cases target genes in our previous studies could not be detected even though reactor performance data indicated that genes encoding enzymes with the target function were present. Others have found assays targeting functional genes unsuccessful as well (e.g., those encoding cellulases; *personal communications*). These results suggest that some published primers targeting relevant functional genes are not as broadly applicable as desired.

The emerging field of metatranscriptomics is a means for obtaining target gene sequences from environmentally relevant communities via sequencing messenger RNA (mRNA) (RNA copies of expressed genes) extracted from AD samples. Metatranscriptomic approaches are by nature non-targeted and do not require complete *a priori* knowledge of target genes sequences; thus, these approaches overcome the barriers associated with qPCR and RT-qPCR. Metatranscriptomic methods utilize a sequencing platform (e.g., Ion Torrent or Illumina) to generate millions of reads, which may be mapped against annotated reference genomes to predict gene function (Moran, 2009; Vanwonterghem et al., 2014). Alternatively, genes may be

identified after *de novo* assembly (transcriptome assembly without the aid of a reference genome). Gene expression may be measured *in situ*, which allows for the determination of abundance and function of microorganisms that are metabolically active. For example, metatranscriptomics may be utilized to determine immediate microbial metabolic responses in digesters as a result of reactor operational changes or perturbations (e.g., feedstock changes). To date, very few studies have used metatranscriptomics to characterize microbial communities in AD applications. In one of the first studies to target the metatranscriptome of anaerobic digesters, taxonomic profiling revealed dominant phyla of *Euryarchaeota* and *Firmicutes* in microbial communities extracted from a production-scale digester treating agricultural waste, and results also indicated a high transcriptional activity of archaeal species (Zakrzewski et al., 2012). However, the experimental design did not lead to operational recommendations. Interestingly, conflicting results have been documented regarding the impact of operating conditions on microbial communities at the metagenomics level (DNA-based characterization). For example, similar community profiles have been observed at high taxonomic ranks for reactors operating under different process conditions, substrates, and technologies (Stolze et al., 2015). In contrast, St.-Pierre and Wright (2014) demonstrated limited overlap in bacterial community structure among multiple digesters fed different feedstocks and suggested that microbial community populations may require manipulation for optimal performance depending on the feedstock used. However, these studies were DNA-based and thus only considered presence, rather than activity, of microorganisms. Additional research is needed to investigate the differences in upregulated genes (genes showing increased activity) in response to different feedstocks and reactor conditions.

An additional benefit of metatrascriptomics is the potential opportunity to leverage acquired sequences for improved primer design. This opportunity is particularly key since minor changes in primer sequences have been shown to yield significantly different quantification results because of differences in gene sequences between organisms (De Long et al., 2010; Guy et al., 2004; Ledeker & De Long, 2013). For genes identified that are of particular interest for AD processes, the newly available sequence data may be compared to existing primer sets and utilized to modify these primers or create relevant new primers that will accurately target bacteria associated with AD. The resultant improved assays could yield more accurate microbial community composition and activity results, leading to enhanced operational methods.

In this study, my objective was to 1) elucidate the impact of different feedstocks on the composition of the active microbial community and the functional microbial process, and 2) obtain sequences of genes expressed during the degradation of two model substrates to identify putative functional gene biomarkers for hydrolysis for monitoring health of bacterial communities in AD systems. To this end, I sought to compare active microbial pathways as a function of feedstock. I also sought to sequence and identify functional genes expressed during hydrolysis of manure and food waste in an effort to expand the databases for functional genes that could serve as potential biomarkers for molecular assay development. The approach was to operate semi-continuous, single-stage reactors fed manure or food waste under low ammonia. Metatranscriptomic analyses were utilized to identify active Bacteria and Archaea and profile expressed functional genes for each reactor as a function of feedstock.

5.2 Materials and methods

5.2.1 Feedstock and inoculum collection

Manure samples were collected from Five Rivers Cattle Feeding LLC (Greeley, CO) and stored at 4ºC for 1 day prior to initial use. Food waste was prepared manually according to Chen et al. (2006) immediately prior to use. Microbial inoculum was collected from the DWWTP mesophilic anaerobic digester (Fort Collins, CO). The inoculum was purged with nitrogen gas, maintained at 35 °C, and used within 5 hours of collection. Feedstock and inoculum were analyzed for TS and VS prior to experiment set-up (Table 5.1).

Table 5.1. Characteristics of feedstock and inoculum

	Parameter	
Sample	TS (%)	VS (%)
Food waste	29.2 (±4.1)	94.1 (±1.9)
Manure	71.3 (±3.4)	51.5 (±2.1)
DWWTP Inoculum	1.6 (±0.1)	78.2 (±0.6)

5.2.2 Reactor configuration and operation

Two 1-L semi-continuous reactors were operated under low ammonia (<0.5 g TAN/L) for a period of 28 days. Reactors were initially seeded with inoculum (20% by volume), supplemented with a nutrient solution (Owen et al., 1979) to ensure that nutrients were not limiting in the system, and fed manure or food waste. Digesters were fed 1 g COD/L of manure or food waste every working day (5 days per week), and equal volumes (50 mL) of sludge were removed from both

reactors via a sampling port for downstream chemical and biological analyses. Prior to feeding, the feedstocks were blended (Hamilton Beach blender) separately with nutrient solution to create a slurry. Digesters were operated at a hydraulic retention time (HRT) of 20 days, and produced biogas was collected via water displacement.

5.2.3 Chemical analyses

The following parameters were monitored every other sampling day: DCOD, pH, VFA and ammonia concentrations, and methane generation. The volume of biogas was measured by determining the amount of gas captured in the inverted graduated cylinders, and methane content was determined via gas chromatography using a thermal conductivity detector as described previously (Wilson et al., 2013). TS and VS of the feedstock and inoculum were measured according to standard methods (APHA, 1995), and liquid samples were analyzed for pH and EC using probes (VWR #89231-604 and #11388-382, respectively). DCOD concentrations were determined using Hach's COD High Range Vials and digestion colorimetric method (Hach, Loveland, CO). VFAs were measured using Hach's Volatile Acids TNTplus Reagent Set and esterification method. Samples were filtered through 0.2-μm syringe filter prior for DCOD and VFA analyses. Ammonia concentrations were measured using Hach's Nitrogen-Ammonia High Range Reagent Set.

5.2.4 Metatranscriptomics analyses

Total RNA was extracted from the AD reactors using the Power Microbiome RNA Isolation kit (MoBio). To ensure that representative active microbial communities were sequenced, total RNA was extracted once steady-state operation was achieved on days 26, 27, and 28 of operation. RNA quantity and quality were analyzed via the Experion Bioanalyzer. mRNA was enriched in the total RNA using the MicrobExpress kit (Ambion).

Metatranscriptomic libraries were prepared using the Ion Total RNA-Seq Kit V2 Library (Life Technologies) and sequenced on a Life Technologies Ion Proton System using the Ion Seq 200 kit (Life Technologies) by the Colorado State University Next Generation Sequencing Core. One lane per metatranscriptome was sequenced. Subsequent metatranscriptomic and bioinformatics analyses were conducted by Karen Rossmassler, a post-doctoral researcher at Colorado State University in the Civil and Environmental Engineering department. First, sequences were filtered and trimmed for quality (Qscore per basepair ≥17, minimum length 35 basepairs) by the program FASTQC. After quality control, millions of high-quality sequences representing hundreds of millions of basepairs were selected for analysis. The raw high-quality sequences were submitted to an online portal for high-throughput sequence analysis, MG-RAST (Meyer et al., 2008), for rRNA and protein-encoding transcript identification, taxonomic assignment, and annotation.

5.2.5 Bioinformatic analyses

62 and 60% of the total reads were identified as rRNA in microbial communities from reactors fed manure and food waste, respectively. These reads were removed from the dataset, and subsequent analyses were based on mRNA only (Table 5.1). The taxonomic affiliation of mRNA sequences was assigned based on the non-redundant M5NR database in MG-RAST using the lowest common ancestor method from MEGAN (Huson & Weber, 2013). Annotated mRNA transcripts were assigned to a SEED subsystem in MG-RAST with the default parameters (maximum e-value of 10^{-5}, minimum identity cutoff of 60%, and minimum alignment length cutoff of 15). The number of mRNA transcripts assigned to a given SEED subsystem was normalized by dividing by the total number of annotated mRNA reads for each reactor, to allow comparison between datasets.

Table 5.2. Characteristics of raw reads

Inoculum (%)	Manure	Food Waste
Number of total reads	5,759,844	14,539,807
Number of rRNA reads	3,550,635	8,708,149
Number of non-rRNA high quality reads	2,209,209	5,831,658
Range of read lengths (bp)	35-225	35-231
Average read length (bp)	53	64

The relative abundances as a percentage of mRNA transcripts of genes involved in important ecosystem functions, methanogenesis and lysis of cellulose, were determined. Relative abundances of methanagenesis transcripts were determined by searching for that subcategory of SEED subsystems on MG-RAST. Furthermore, transcripts of the methyl-coenzyme A reductase gene subunit (*mcrA*) were quantified by searching for its KEGG EC number, 2.8.4.1 (Kanehisa et al., 2016). Relative abundances of genes encoding enzymes with cellulolytic activity were determined by searching for transcripts encoding cellulases and endoglucanases (3.2.1.4), beta-glucosidases (3.2.1.74), and cellobiohydrolases (3.2.1.91 and 3.2.1.76) (Kanehisa et al., 2016).

To determine if *mcrA* or cellulase reads were present that were not annotated as such in MG-RAST, a less stringent strategy was used to select reads that were similar to *mcrA* or cellulose-encoding genes. A reference set of *mcrA* and cellulolytic genes were downloaded from GenBank based on their assigned EC number (Benson et al., 2015). The reference set of *mcrA* genes contained 2877 sequences totaling 1,319,471 bp. The reference set of cellulase genes

contained 1133 sequences totaling 2,056,264 bp. These reference sets were used to recruit metatranscriptomic reads using FR-HIT (Niu et al., 2011). Transcripts were recruited with FR-HIT with an e-value cutoff of 10, a kmer of 11, a kmer overlap of 8, and a sequence identity cutoff of 75%. The transcript recruitment rates were normalized for comparison between metatranscriptomes and reference sets by dividing the number of transcripts recruited by the total number of basepairs in the recruiting reference set.

5.3 Results and discussion

5.3.1 Reactor performance

Stable process performance in each reactor was achieved over the 4-week operational period (i.e., the volume of methane generated per week did not vary by more than 20%). DCOD and VFA values did not build up over time, demonstrating that hydrolysis and fermentation products were not accumulating (Fig. 5.1a, b).

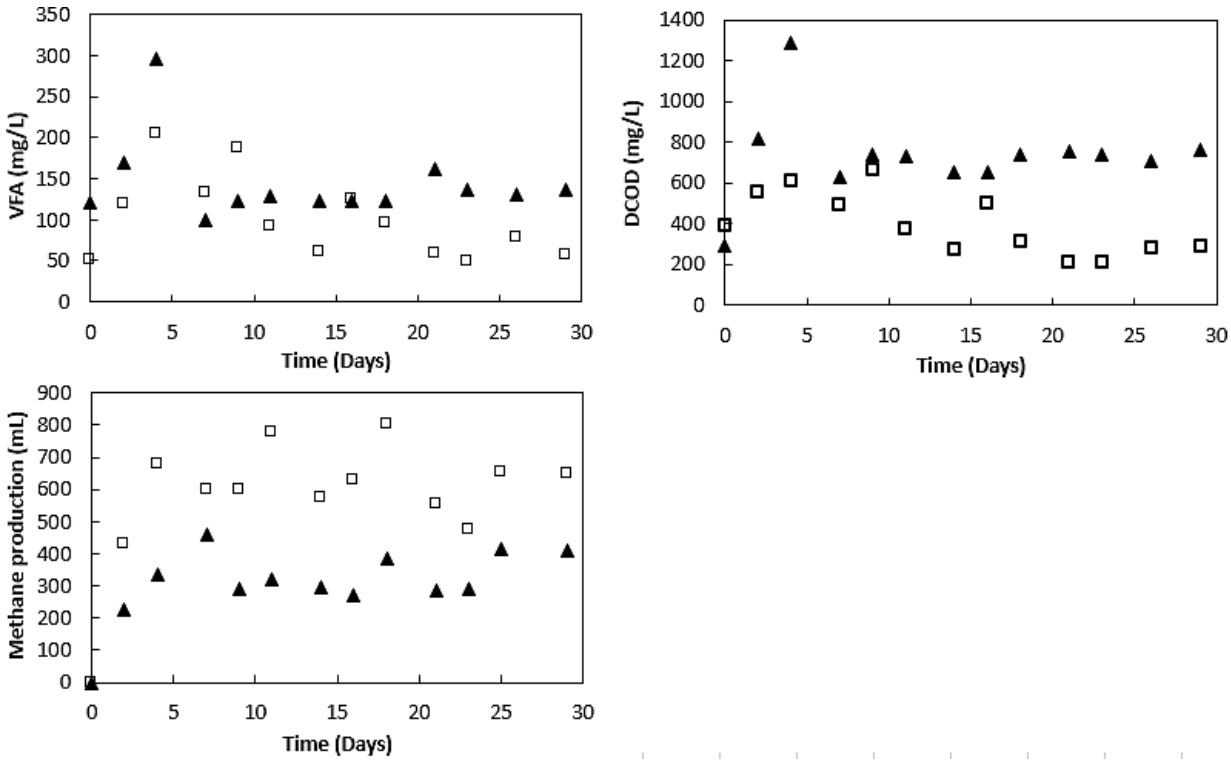

Figure 5.1. VFA (a) and DCOD (b) concentrations and methane production (c) over time for reactors fed manure (▲) and food waste (□).RNA was extracted on days 26, 27, and 28 for metatranscriptomic analyses.

After the first week of operation, pH and ammonia concentrations remained within the range of 7.08-7.46 and 0.30-0.37 g TAN/L, respectively (data not shown). Methane production was comparable to literature values (Gungor-Demirci & Demirer, 2004; Lee et al., 2010; Wilson et al., 2013). Reactors fed food waste produced approximately 1.5 – 2 times the amount of methane generated by reactors fed manure per week, which was expected given the difficulties associated with manure hydrolysis due to its high cellulose and lignin content) (Fig. 5.1c).

5.3.2 Taxonomic classification of microbial communities

Metatranscriptomic analyses revealed similarities in active microbial communities in reactors as a function of feedstock. Microbial communities in both reactors demonstrated activity by *Clostridia*, although communities in reactors fed food waste contained approximately twice

the abundance (26%) of *Clostridia* transcripts (RNA that has been transcribed from a gene and encodes a protein) compared to abundances found in communities fed manure (Fig. 5.2a, b).

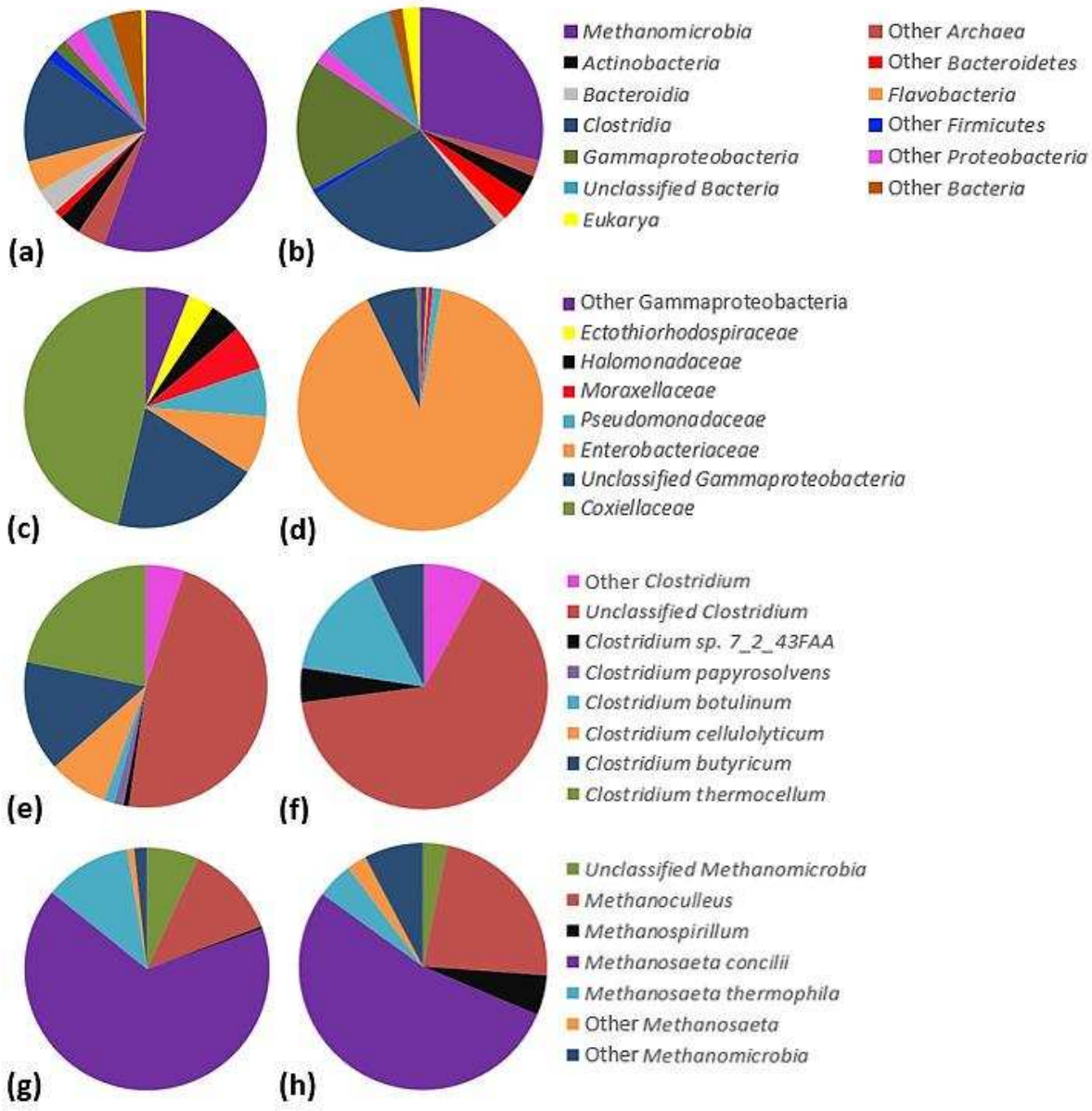

Figure 5.2. Taxonomic profiling of microbial communities in reactors fed manure and food wastefor overall composition (a and b, respectively), families within Gammaproteobacteria (c and d), species within Clostridia (e and f), and communities within Methanomicrobia (g and h). Labels identified as "Other" indicate grouped phylotypes that individually comprise less than 1% of transcripts.

Clostridia often are highly active in digesters fed a variety of feedstocks due to their ability to degrade numerous complex substrates, such as cellulose. For example, *Clostridia* produce cellulosomes and participate in hydrolysis of plant fibers that are often found in manure (St-Pierre & Wright, 2014). Additionally, *Clostridia* are capable of degrading amino acids and fermenting carbohydrates (Minton & Clarke, 1989). Thus, the high activity of *Clostridia* in both reactors was not surprising, although *Clostridia* were not necessarily performing the same functional role in both reactors. Microbial communities fed food waste also demonstrated uniquely high activity of *Gammaproteobacteria* (17.5% of transcripts).

Interestingly, microbial communities in reactors fed manure demonstrated a substantially greater abundance of archaeal transcripts compared to communities in reactors fed food waste (Fig. 5.2a, b). Specifically, greater than 50% of all transcripts were associated with the class *Methanomicrobia* in reactors fed manure, compared to 29% of transcripts in reactors fed food waste (Fig. 5.2a, b). Similarly, metagenomic analyses of a full-scale anaerobic reactor digesting activated sludge revealed a high abundance of *Methanomicrobia* (85.4% of all Archaea) (Guo et al., 2015). However, methanogen-associated transcripts accounted for an unexpectedly large proportion of mRNA sequences in our studies compared to previous results which documented only 5-17% archaeal abundance in metagenomic AD studies (Guo et al., 2015; Yang et al., 2014b). Thus, results from our study suggest that methanogens are highly active although they do not produce high quantities of biomass or cells (Grady et al., 2011). Additionally, manure may have contributed additional methanogens to the system, resulting in higher methanogenic activity compared to the activity in reactors fed food waste.

To gain a more detailed understanding of active microorganisms as a function of feedstock, additional analyses were conducted at lower taxonomic levels within key classes

found to differ in the overall analysis. Within the class *Gammaproteobacteria*, microbial communities demonstrated dramatic differences in activity (Fig. 5.2c, d). In reactors fed manure, activity was dominated by *Coxiellaceae* (46% of transcripts) and unclassified *Gammaproteobacteria* (20% of transcripts), and multiple families comprised the remainder of the activity. *Coxiella burnetii*, the only species of *Coxiellaceae* present in this study, is a pathogen found in cattle manure (Guatteo et al., 2006). In contrast, microbial community activity in reactors fed food waste was dominated solely by *Enterobacteriaceae* (>90% of transcripts), a facultative anaerobe responsible for fermentative hydrogen production that contains many members responsible for spoilage of food (Baylis et al., 2011).

Interestingly, within the class *Clostridia,* both microbial communities showed high activity of unclassified *Clostridium* (>47% of transcripts) (Fig. 5.2e, f). This finding may indicate that microbial data for hydrolyzers associated with AD reactors is lacking, and additional research is required to improve databases. Alternatively, unique families may have the same sequences for a target gene and therefore cannot be distinguished from each other. Further, both communities displayed activity by *Clostridium butyricum*, although communities fed manure demonstrated twice the abundance of this species compared to communities fed food waste. This species has been shown to be effective in producing hydrogen from organic substrates, particularly carbohydrates (Chen et al., 2005). Since manure often contains a high carbohydrate content resulting from high energy cattle diets (He & Zhang, 2014), the greater activity observed in reactors fed manure is not surprising. However, despite similarities in microbial community activity, results also indicated differences in species selected for as a function of feedstock. Specifically, *Clostridium thermocellum* and *Clostridium celluloyticum* (cellulose-degrading microbes) were active only in microbial communities fed manure, whereas

Clostridium botulinum (an anaerobic species that may cause botulism) was active only in reactors fed food waste.

Although microbial communities demonstrated differences in relative activity levels of *Methanomicrobia* as a function of feedstock, within this class microbial activities were similar between the reactors. Activity levels in both reactors were dominated by *Methanosaeta concilii* (>50% of transcripts of *Methanomicrobia*) and *Methanoculleus* (≥12% of transcripts) (Fig. 5.2g, h). Additionally, communities fed manure demonstrated activity by *Methanosaeta thermophila* at higher abundance than communities fed food waste. In previous metagenomic studies, *Methanosaeta* was shown to be present in high abundance for multiple anaerobic digesters fed a variety of substrates (Nelson et al., 2011; Yang et al., 2014b). Overall, reactors demonstrated greater activity by acetate-utilizing methanogens (*Methanosaeta concilii*, *Methanosaeta thermophila*) than hydrogenotrophic methanogens (*Methanoculleus*). These results are in agreement with several metagenomic studies that suggest the acetate-utilizing pathway generally is dominant in digesters, responsible for more than 70% methane in most digesters (Yang et al., 2014b).

Collectively, results suggest that co-digestion or the addition of food waste to reactors fed manure could synergistically increase hydrolysis performance. The higher abundance of overall *Clostridia* transcripts in microbial communities in reactors fed food waste indicates that cellulose may be more efficiently hydrolyzed in these reactors compared to reactors fed manure. Thus, manure hydrolysis might benefit further from the additional activity of species within *Clostridia* uniquely selected for by food waste. Future work is required to test the proposed idea and extent of synergistic effects of manure and food co-digestion.

5.3.3 Functional classification for microbial communities

Overall, the reactors demonstrated similar functional profiles. The major functional categories for both reactors were those involved in respiration, protein metabolism, amino acids and derivatives, carbohydrates, and clustering-based systems (Fig. 5.3a, b).

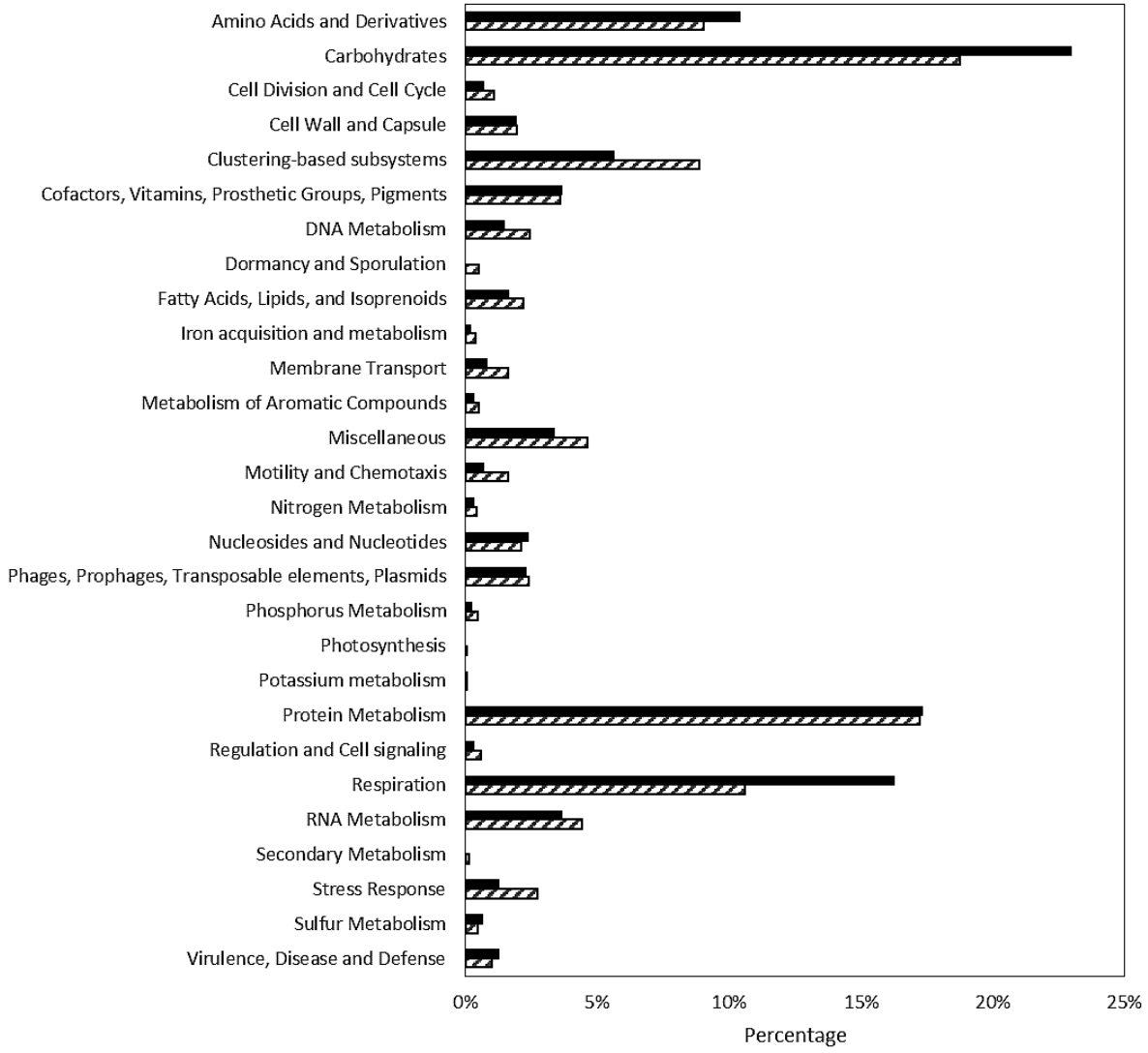

Figure 5.3. Functional categories of microbial communities in reactors fed manure (■) and food waste (▨).

Previous studies have demonstrated similar results at the metagenomic level (Guo et al., 2015; Yang et al., 2014b); this study is one of the few to demonstrate these results at the metatranscriptomic level. Microbial communities in both reactors contained similar abundances of transcripts involved in protein metabolism. The comparable activity observed is likely due to the similar protein composition documented in manure and food waste (17% and 11%, respectively) (Chen et al., 2003a; Matsakas et al., 2014). Although statistically significant conclusions may not be drawn from the data due to the lack of replication in this study, microbial communities demonstrated differences in transcript abundance in several of the main functional categories. Microbial communities fed manure demonstrated a higher abundance of transcripts involved in both respiration and carbohydrates (Fig. 5.3). Conversely, microbial communities fed food waste demonstrated higher abundance of transcripts involved in clustering-based systems and stress response. Clustering-based systems contain groups of genes whose functional attributes are not well understood, and the relatively high abundance of these subsystems suggests that additional research is needed to identify key roles these groups may have in the AD process (Delmont et al., 2012). Interestingly, the higher abundance of transcripts involved in stress response in reactors fed food waste suggests that these communities were experiencing more stress. Reasons for this occurrence are unknown, although rapid VFA production commonly associated with the AD of food waste may have invoked a stress response. Additional research would be required to test this hypothesis. The fatty acids and lipids category demonstrated higher activity in microbial communities in reactors fed food waste, which was expected considering the food waste likely had a higher lipid content. However, overall activity in this functional classification was low (≤2% abundance of transcripts) in both reactors.

Relevant active subsystems were investigated further at Level 2 and 3 subsystems to provide more detailed functional analyses. Lower (e.g., Levels 2 and 3) subsystems provide greater functional resolution for the categories defined in Level 1. The classifications analyzed at lower subsystems were chosen due to the key role (e.g., methanogenesis, fermentation) that each function contributes in the overall AD process. Within the carbohydrate classification, microbial communities fed manure demonstrated higher activity (more transcripts) involved in one-carbon metabolism (Fig. 5.4a).

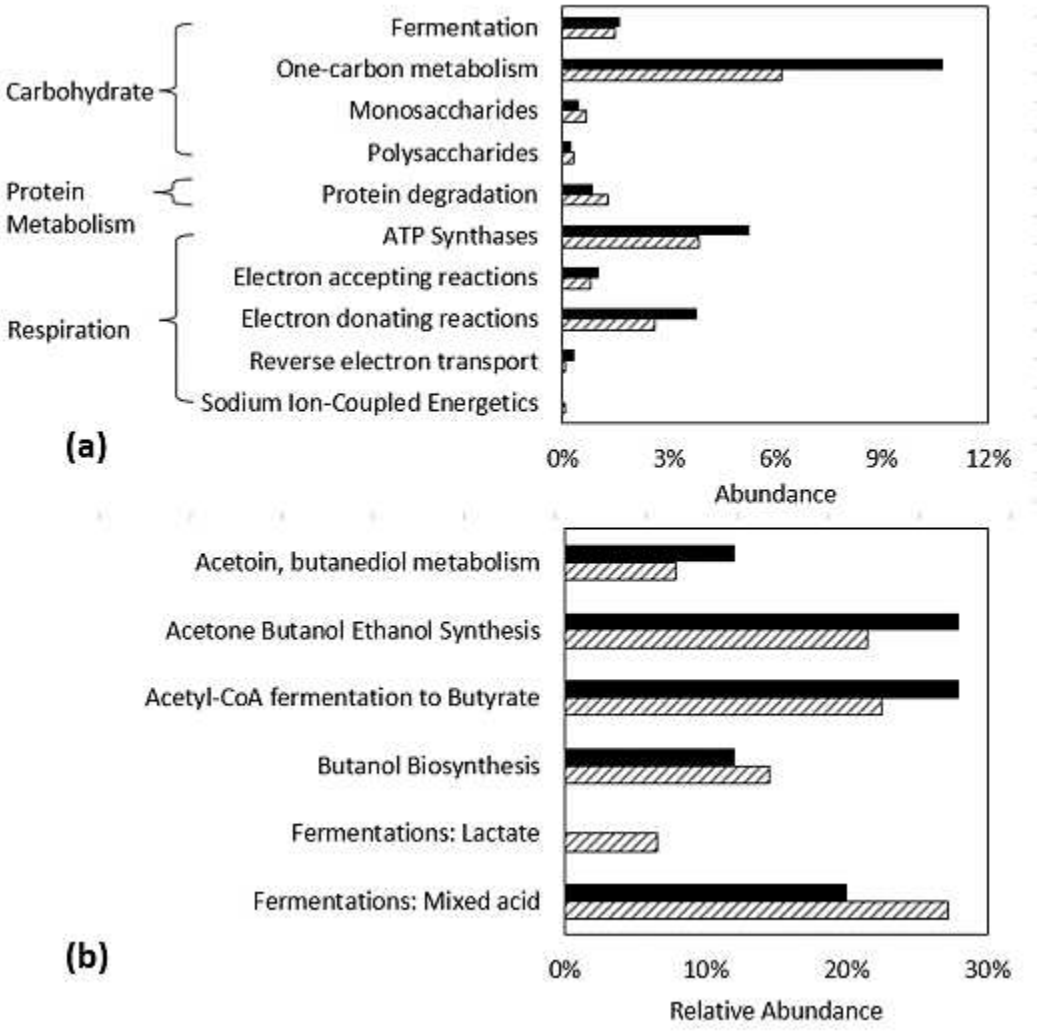

Figure 5.4. Abundance of selected Level 2 (a) and Level 3 (b) subsystems in microbial communities for reactors fed manure (■) and food waste (▨).

One-carbon metabolism involves converting complex organic matter to simple compounds, which plays an important role in methanogenesis (Guo et al., 2015). This result is consistent with the abundance of active methanogens in reactors fed manure demonstrated previously in Fig. 5.2a. As suggested earlier, manure may have contributed additional methanogens as compared to food waste. Although both microbial communities demonstrated similar transcript abundance involved in active Level 2 fermentation, results demonstrated some functional differences between feedstocks in the microbial communities at the Level 3 subsystem (Fig. 5.4b). Reactors fed food waste demonstrated more transcripts involved in fermentation of lactate and mixed acid. VFA concentrations were similar between the two reactors, but this result is consistent with VFAs being produced and utilized at a higher rate in the reactor fed food waste.

Collectively, results observed from this study show that feedstock influences microbial community composition and function. These results have important implications for reactor operation. In practice, changing feedstocks or shifting to co-digestion could occur due to changes in available waste or the desire to increase bioenergy generation by incorporating additional feedstocks. Active microbial community management, including targeted inoculation strategies, may be beneficial for optimal performance when feedstocks are changed. For example, data reported herein suggests particular phylotypes that should be included in inocula developed for application during a feedstock switch involving manure or food waste. Further, observed differences in transcript abundance in several gene categories (e.g. one-carbon metabolism or fermentation) might indicate some new and potentially useful biomarkers for monitoring process health. Future work might involve selecting a suite of apparently relevant genes and using these genes to monitor microbial communities in reactors fed different feedstocks over time to determine which genes emerge as useful biomarkers.

5.3.4 Putative biomarkers

In efforts to identify putative biomarkers in our system, analyses were conducted targeting cellulose-encoding and *mcrA* genes. These genes were chosen due to the importance of hydrolysis and methanogenesis processes in AD. Unfortunately, straightforward approaches (general keyword searches and specific queries using KEGG ID number and EC number in MG-RAST) did not indicate that many cellulase genes were present in our system. These results were unexpected considering that cellulose likely was present in our systems, and I hypothesize that these conventional approaches yielded inaccurate results. Alternatively, cellulose degradation pathways truly may have been inactive in our system at the time of sampling due to the recalcitrant nature of cellulose. In efforts to verify the results obtained, less stringent approaches (e.g., FR-HIT) were applied to the data, and several cellulase genes were putatively identified. However, the rate of gene recruitment was still lower than originally predicted (data not shown), particularly considering the wide range of cellulolytic genes targeted. Further, results were impacted substantially by the parameters used during analysis. Thus, efforts are ongoing to apply different cutoff parameters to determine if more putative cellulase genes are identified.

In contrast to the results obtained for cellulase genes, FR-HIT analyses indicated a high abundance of transcripts involved in methanogenesis. The rate of recruitment was approximately 10-fold greater for the *mcrA* gene compared to cellulolytic genes. These results provide a new opportunity to explore the diversity and variance of methanogenic genes without the use of specific PCR primers. In particular, although the *mcrA* gene has been widely investigated using targeted primers (Luton et al., 2002; Pereyra et al., 2010; Steinberg & Regan, 2009), it is unclear if these primers capture all the types of *mcrA* genes present. Thus, additional work is needed to investigate if the reads annotated as *mcrA* in this study would be identified using existing

primers. If some phylotypes would be under-quantified or even missed, new primers could be designed and optimized.

5.4 Conclusions

This study elucidated microbial community activity responses to two model feedstocks at the level of gene expression. Thus, the data expands on previous DNA-based work by identifying phylotypes whose activity can be linked with the degradation of these feedstocks. Specifically, taxonomic analyses identified several common active (e.g., *Methanomicrobia*, *Methanosaeta concilii*, and *Clostridia)* and unique active (e.g., *Enterobacteriaceae*, *Clostridium thermocellum*, and *Clostridium celluloyticum*) phylotypes between the reactors. The application of carefully developed inocula including these shared phylotypes may enhance waste hydrolysis for a variety of feedstocks, and the inclusion of feedstock-specific phylotypes likely will enhance AD performance when feedstocks are changed or during co-digestion. Functional classification for the active microbial communities revealed several similarities despite the differences in feedstock. Differences and similarities in transcript abundance in specific gene categories (e.g. one-carbon metabolism or fermentation) might indicate some potentially useful biomarkers for monitoring processes that are healthy or progressing toward failure. Additionally, data from this study has expanded the gene sequence database for assay development, which is particularly key for improving current functional gene-targeted assays. The development of additional primer sets targeting the hydrolysis of proteins, cellulose, or fats (shown to occur in our system) could yield more informative microbial community composition results. However, additional work is required to identify cellulases present in our system since preliminary results indicated a surprising lack of cellulase transcripts. Thus, future work may investigate genes identified by FR-HIT to determine if these genes may be useful biomarkers or exploring factors influencing

cellulase expression to identify opportunities for engineering interventions (e.g., bioaugmentation of specific phylotypes or strategies to enhance expression of desired pathways) to improve performance further.

6.0 Conclusions

Collectively, results from these studies demonstrate the importance of the inoculum for achieving optimal hydrolysis rates and improved overall performance in reactors operating under stress. Firstly, results demonstrated that significantly improved hydrolysis performance may be achieved via acclimating microbial communities to elevated concentrations of the inhibitors ammonia and salinity. Further, inhibitors and feedstock were found to have a major influence on bacterial community structure indicating that acclimation was due to changes in the types of hydrolyzing bacteria present. A more detailed analysis of the acclimation process revealed that microbial communities under stressed conditions (elevated ammonia) adapt more slowly (weeks) to feedstock changes than under non-stressed conditions (days). Bacterial and archaeal sequencing results identified multiple organisms (e.g., *Clostridiales vadinBB60, Marinilabiaceae, Methanobacterium,* and *Thermoplasmatales Incertae Sedis*) that were selected for in microbial communities in stressed reactors subjected to a feedstock change. Jointly, results from these studies suggested that weeks of acclimation are required to build up sufficient quantities of desired hydrolyzing bacteria; thus, hydrolysis processes operated in batch mode should be inoculated, and desired microorganisms should be maintained in the system via properly developed inoculation strategies applied to each new batch. Additional studies to identify improved methods of maintaining such microorganisms in laboratory-scale, multi-stage reactors demonstrated that high quantities of inoculum (~60%) increase waste hydrolysis and are beneficial at start-up or when inhibitor concentrations are increasing. After start-up (~112 days) with high inoculum quantities, leachate recirculation leads to accumulation of inhibitor-tolerant hydrolyzing bacteria in leachate and low inoculum quantities (~10%) effectively increase waste

hydrolysis relative to without solids-derived inoculum. Further, additional phylotypes (e.g., *Clostridiales*) were provided by the solid waste inoculum but were present at very low levels in the leachate. Metatranscriptomic analyses revealed several common active (e.g., *Methanomicrobia*, *Methanosaeta concilii*, and *Clostridia)* and unique active (e.g., *Enterobacteriaceae*, *Clostridium thermocellum*, and *Clostridium celluloyticum*) phylotypes between reactors fed manure and food waste. Results also indicated that certain microbial community functions (e.g., fermentation and methanogenesis) were affected by feedstock differences.

Overall, results demonstrate that the challenge of inhibitory ammonia and salinity concentrations encountered in AD systems may be overcome via hydrolysis seeding methods. Thus, particular attention must be given to microbial community management when inhibitors are present, and properly developed inoculation strategies may be applied to avoid system upsets and failures. In practice, changing feedstocks or operating under co-digestion applications could occur due to changes in currently available waste, and active microbial community management, including targeted inoculation strategies, may be beneficial for optimal performance when feedstocks are changed. For example, data reported herein suggests particular microbial phylotypes that should be included in inocula developed and quantities for application during the accumulation of inhibitors or a feedstock switch involving manure or food waste. Ultimately, the improved hydrolysis operational guidelines demonstrated in these studies may reduce capital and operating costs and minimize resource consumption while achieving high AD process performance, thereby increasing the economic viability of AD systems and allowing for more widespread implementation in North America.

The unique results demonstrated have highlighted additional knowledge gaps in the AD field and opportunities to build upon the conclusions drawn herein. Small-scale or laboratory-scale reactors were operated in this study to allow for replicate reactors operated under a variety of operating conditions. However, demonstration-scale operation of multi-stage reactors operating under elevated ammonia and salinity with various inoculum percentages is needed to determine if similar results are obtained regardless of reactor size. Difficulties associated with demonstration-scale operation (e.g., prolonged oxygen exposure to the inocula while refilling LBRs) potentially could impact reactor performance and provide additional insights not observed via laboratory-scale experiments. This knowledge is particularly valuable since supplementary future work could be conducted at laboratory-scale if results are transferrable across reactors sizes, thus allowing for more cost-effective and efficient studies.

Additionally, since microbial community activity and function were shown to be impacted by feedstocks (e.g., manure and food waste), future work is needed to determine if similar results are observed with other feedstocks. Additional research also might involve selecting a suite of apparently relevant genes and using these genes to monitor microbial communities in reactors fed different feedstocks over time and during optimal and sub-optimal performance to determine which genes emerge as useful biomarkers. Thus, for reactors operating under co-digestion or seasonal feedstock changes, additional active phylotypes that should be included in the engineered inocula to achieve enhanced performance may be identified via targeted functional gene-based tools.

Data generated from the comparative metatranscriptomic experiment has expanded the current gene sequence database. However, additional work is required to improve existing functional gene-based assays utilizing sequences obtained from this study. Although initial

results demonstrated that cellulase genes were present in low abundance, work is ongoing to determine if additional cellulase genes may be detected via alternate, less-stringent methods of gene recruitment. Identified transcripts of both putative cellulase and methanogenic genes may be compared against existing primer sets and queried *in silico* to determine if mismatches would result in a biased amplification. If such mismatches are present, new primers may be designed and optimized to amplify the previously unamplified cellulase and *mcrA* genotypes.

7.0 References

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8.0 Appendix A: Nutrient Solution Preparation

Table 8.1. 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., 1979):

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.

9.0 Appendix B: Gas Chromatography Protocol

GC Preparation

1. Press the power button on the GC.
2. Open the gas values connected to nitrogen and air.
3. Open ChemStation (Online) program on the desktop computer.
4. Select program CH4_pw from available list.
5. Wait for program to load and GC to warm up (approximately 10 minutes). When the light turns green on the computer program, the GC is ready to begin.

Running samples

1. To run standards, fill a 50-ml plastic syringe with 80% methane gas and 2 additional syringes with 50% and 25% (by volume) nitrogen gas. Pull samples for analysis directly from plastic syringes and inject 20-µl of biogas directly into the TCD port. Press the start button on the GC.
    a. Results from each run automatically appear after completion. Approximate run times were 90 seconds.
    b. Inspect the electropherograms to ensure the peaks are clean and do not tail off substantially in either direction. If abnormalities are present, re-run the sample.
    c. Record the area of the peaks of interest.
    d. Generate a calibration curve plotting area of the peak versus methane %.

2. Collect gas samples in plastic syringes capped with a rubber septum, and pull samples directly from plastic syringes and inject 20-µl of biogas directly into the TCD port. Press the start button on the GC.
    a. Record the area of the methane peak for each sample, and determine the % methane from the calibration curve.
3. When finished, close GC computer program.
4. Turn off power to GC and close gas valves.

Notes:

1. Replace the septa on the TCD port every 50-100 injections.
2. Clean GC syringes often according to manufacturer's instructions.

10.0 Appendix C: Bioinformatics Analyses

Bioinformatics analyses described in Chapter 3 originally were conducted by Research and Testing Laboratory using their data analysis pipeline. Briefly, sequences were denoised, chimeras were removed using UCHIME, and then reads were quality scanned to remove poor reads. Taxonomic analysis was performed using USEARCH. Relative abundance for phyla, order, family, genus, and species were generated according to the following criteria. Sequences with >97% identity to well-characterized 16S rRNA gene sequences were considered to be from the same species, sequences with 95% to 97% identities were considered to be from the same genus, sequences with 90% to 95% identity were considered to be from the same family, sequences with 85% to 90% identity were considered to be from the same order, sequences with 80% to 85% identity were considered to be from the same class, and sequences with 77% to 80% identity were considered to be from the same phylum. Any match below 77% identity was discarded. The resulting bacterial and archaeal microbial community data were assembled at the genus level (Fig. 10.1).

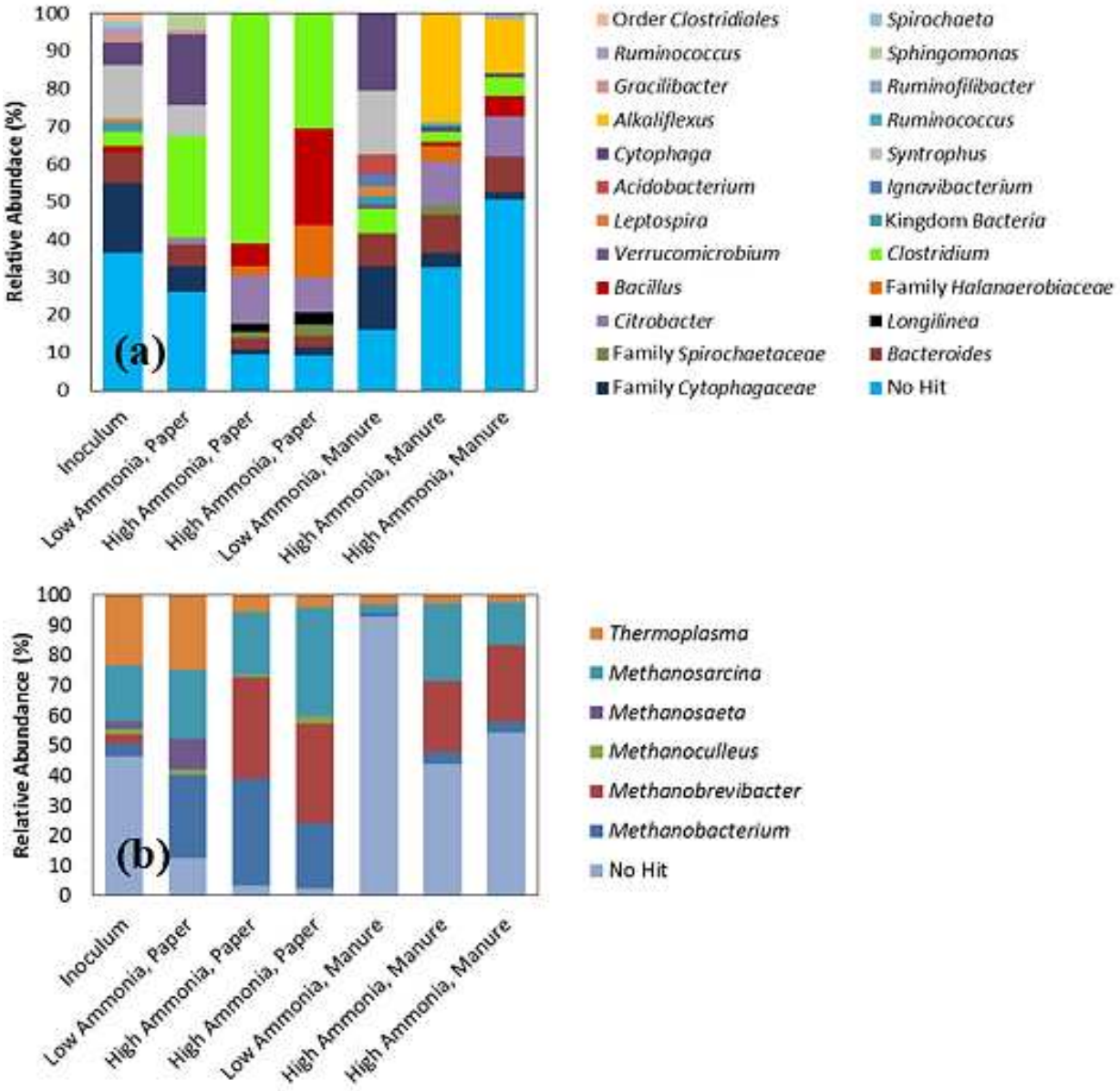

Figure 10.1. Bacterial (a) and Archaeal (b) 16S rRNA gene-targeted pyrosequencing data for inoculum and selected reactors utilizing Greengenes database.

Sequence data also was analyzed by Karen Rossmassler according to the methods described in Chapter 3 using the SILVA database as opposed to the Greengenes database utilized by Research and Testing Laboratory. Results obtained with the SILVA database were depicted in Fig. 3.9. Taxonomic classifications clearly varied depending upon the method of analyses

utilized, particularly with regards to the relative abundance of identified microorganisms. Results herein are included to highlight the important of the query database.