ABSTRACT

WANG, LING. Influencing the Anaerobic Microbiome through Substrate Selection and Overloading Stresses to Achieve High-Performance Co-Digestion of Grease Interceptor Waste (Under the direction of Dr. Francis Lajara de los Reyes III).

Anaerobic co-digestion of grease interceptor waste (GIW) integrates waste management and resource recovery to improve economics and sustainability. There have been many attempts to improve the performance and stability of digester microbiome, but a universal approach regardless of the GIW origins that can enhance community resistance and resilience and avoid GIW overload and inhibition of methanogenesis is still lacking. In this dissertation, I conducted, for the first time for GIW co-digestion, a series of common garden experiments to directly investigate the causality between microbial community composition and functional dynamics in response to different GIW loading rates. The impacts of substrate history (feed selection and intensity of historical pre-conditioning) and overloading GIW stresses on methanogenic microbiomes were evaluated. Subsequently I demonstrated that microbial community dissimilarity due to substrate history and the presence of key microbial populations determined digester survivability to overloading GIW stresses. Based on the results, I developed a feed-specific community adaptation strategy to achieve resilient and resistant GIW co-digestion. This strategy was successfully validated in two independent bioreactor experiments. Step feeds of GIW resulted in better microbial adaptation compared to the control and reduced the inhibitory effect of GIW. Gradual addition of GIW, up to 75% (w/w) of volatile solids (VS) added, increased methane yield by 336% from 0.180 to 0.785 L-methane/g-VS added, the highest value reported to date for co-digestion of GIW. Pulse feeds of GIW increased methane yield from 0.134 to 0.748 L-methane/g-VS added at 70% (w/w as VS) GIW and increased digester resistance against GIW inhibition. Lastly, I...
presented the first comprehensive time-series investigation of long-term (>200 days) microbial community and functional dynamics of GIW co-digesters with different loading stress histories. I evaluated the relationships between community composition, function, the environment under different levels of stress and stress history, and identities and ecological potentials of core populations within different metabolic networks. This dissertation represents the first complete demonstration on exact approaches using substrate selection and overloading stresses to pre-select for more stress-tolerant members across anaerobic functional guilds and achieve high-performance GIW co-digestion.
Influencing the Anaerobic Microbiome through Substrate Selection and Overloading Stresses to Achieve High-Performance Co-Digestion of Grease Interceptor Waste

by
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DEDICATION

This dissertation is dedicated to God my heavenly Father, for His mercy and grace that have given me new birth through the resurrection of His son, Jesus Christ; for His blessing and deliverance that restore my soul and spirit; for His comfort and guidance that lead me in paths of righteousness; for He is my rock, my shield, my strength, my hope, and the source of all my joys; for He listens my prayers and sees my affliction and distress; for He is my shepherd and all I ever need.
BIOGRAPHY

Ling Wang grew up in Tainan, Taiwan. From 2007 to 2010, Ms. Wang worked as an undergraduate research assistant in the air pollution control laboratory of Dr. Hsin Chu at National Cheng Kung University, from where she received a Bachelor of Science in Environmental Engineering. In Fall 2010 she began graduate studies in the Department of Civil, Construction, and Environmental Engineering at North Carolina State University, and worked as a graduate research and teaching assistant beginning in 2011. Her graduate course work has focused on anaerobic co-digestion of grease interceptor waste to enhance biogas production, under the direction of Dr. Francis de los Reyes III. She received a Master of Science in Environmental Engineering in June 2012 and continued her work on this topic during her Ph.D. Her dissertation integrates biosystem engineering, microbial community ecology, next-generation molecular techniques and bioinformatics. After graduate studies, Ms. Wang aspires to pursue a career as a research scientist.
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Chapter 1 Introduction

Anaerobic digestion, including hydrolysis, acidogenesis (fermentation), acetogenisis, and methanogenesis, is carried out by different consortia of microbes occupying distinct ecological preferences. To improve economics and sustainability, anaerobic co-digestion of grease interceptor waste (GIW) has shown great potential to substantially increase biogas production and methane content. Despite its potential, studies have shown inhibition of microbial community during degradation of lipid-rich materials and a threshold of GIW addition that led to inhibited and unstable methanogenesis and process failure. Indeed, a long-standing question in bioreactor engineering is what ecological patterns and processes determine the performance and functional stability of methanogenic biosystems and how this complex microbial network can be improved. The major questions that remain unanswered and continue to limit broader and more cost-effective applications of this biotechnology include: (1) identification of the maximum allowable GIW loading rate and the corresponding maximum methane yield achieved before inhibition, (2) core microbial populations across metabolic networks that play important roles in reducing substrate toxicity and enhancing functional resilience and resistance, and (3) a universal approach regardless of the GIW origins that can “push the limit” of GIW co-digestion and achieve high-yield yet stable operation.

In this dissertation, I addressed these questions by integrating (1) biosystem engineering using the common garden concept from the discipline of microbial community ecology, (2) a conceptual and hypothesis-driven community enrichment approach to selectively establish
digester microbiomes that had different niche preferences, (3) 16S metagenomic surveys using high-throughput DNA sequencing, and (4) novel microbial co-occurrence network analysis to statistically assess key microbiome members to high-performance GIW co-digestion. In chapter 2, I presented a literature review on anaerobic co-digestion of GIW-related substrates, major biological and operational challenges, microbial adaptation and community ecology of digester microbiomes in GIW-disturbed environments. In chapter 3, I presented the experimental objectives of this dissertation.

Chapters 4 to 7 are composed of four manuscripts for future journal paper submission. In chapter 4, I focused on the common garden experiments which allowed direct identification of the impacts of substrate history (feed selection and intensity of historical pre-conditioning) and overloading GIW stresses on digester performance and stability. In chapter 5, I evaluated the causality between microbial community composition and functional dynamics in response to different GIW loading rates and community adaptation strategy to achieve resilient and resistant GIW co-digestion. In chapters 6, I validated this strategy in two independent long-term (>200 days) bioreactor experiments using GIW of different origins. In chapter 7, I assessed the microbial community and functional dynamics of GIW co-digesters with different loading stress histories and validated how substrate history can be used to actively direct digester microbiomes to improve a specific function.
Chapter 2 Anaerobic co-digestion of grease interceptor waste

2.1 Overview

The major metabolic processes in anaerobic digestion include hydrolysis, acidogenesis (fermentation), acetogenesis, and methanogenesis (Figure 2-1). These processes are carried out by different groups of microbes occupying distinct ecological niches. During hydrolysis and acidogenesis, larger polymers (carbohydrates, proteins, and lipids) are broken down into smaller organic compounds (amino acids, sugars, long chain fatty acids (LCFAs), and alcohols) that are further degraded into short chain fatty acids (e.g. propionate and butyrate), acetate, hydrogen, and carbon dioxide. During acetogenesis and methanogenesis, syntrophic bacteria degrade short chain fatty acids into acetate, hydrogen, and carbon dioxide and subsequently methanogenic archaea convert acetate, hydrogen, and carbon dioxide into methane (McCarty, 1964; Parkin and Owen, 1986; Speece, 1996; Stuckey et al., 2011).

The microbiota in anaerobic digesters are engineered under mesophilic or thermophilic environments to harbor complex bacterial and archaeal metabolic networks for biosolids degradation and energy recovery. In recent years the operation of engineered biosystems, such as anaerobic digesters, has been challenged by the demand for better performance, efficiency, and stability in the face of sustainable energy and waste management needs. One attractive application is anaerobic co-digestion of biosolids with grease interceptor waste (GIW) to enhance biogas production that can be used to offset energy requirements at
wastewater treatment facilities. GIW is one of the most abundant waste-based organic substrates in the U.S. with high energy potential but limited disposal options (Austic, 2010; Dayton, 2010; Long et al., 2012; Skaggs et al., 2018). GIW typically comprises three major components: fat, oil, and grease (FOG), food particles, and associated wastewater generated from food service establishments (Chapin, 2008; Aziz et al., 2011; Gallimore et al., 2011; Long et al., 2012). Similar FOG-based wastes include: animal fats and waste oils from food or processing plants, the edible oil industry, the dairy products industry, and slaughterhouses; mixed FOG wastes from receiving or dewatering facilities, and trapped grease wastes from wastewater treatment facilities (EPA, 2004; Appels et al., 2011; Long et al., 2012).

**Figure 2-1** Metabolic pathways and corresponding microbial communities of anaerobic digestion
Co-processing FOG-rich materials with sewage sludge increased methane production by 9% to 317% (compared to the base case with no co-digestion), depending on the source of startup substrate and co-substrate, total organic loading rate (OLR), OLR of co-substrate, solid retention time (SRT), mixing intensity, and feeding strategy and frequency (Davidsson et al., 2008; Kabouris et al., 2009; Luostarinen et al., 2009; Wan et al., 2011; Wang et al., 2013). Successful applications of anaerobic co-digestion with FOG wastes have been reported worldwide in lab scale (Kabouris et al., 2009; Luostarinen et al., 2009; Wan et al., 2011; Wang et al., 2013; Ziels et al., 2016), pilot scale (Davidsson et al., 2008), and full scale (Bailey, 2007; Gabel et al., 2009; York and Magner, 2009; Cesca et al., 2010; Downey, 2010; Muller et al., 2010; Johnson et al., 2011). Lipid-rich wastes from different origins have been extensively tested, including raw (un-dewatered) FOG from a receiving facility (Wan et al., 2011), dewatered FOG from grease traps (Kabouris et al., 2009), GIW from a meat processing plant (Luostarinen et al., 2009), GIW from a restaurant (un-dewatered) (Wang et al., 2013), grease trap sludge (Davidsson et al., 2008), and trapped FOG wastes from wastewater treatment facilities (Martín-González et al., 2011; Silvestre et al., 2011; Noutsopoulos et al., 2013), waste cooking oil (Ziels et al., 2016), and food waste and FOG (Amha et al., 2017).

2.2 Challenges of GIW co-digestion

When co-digesting FOG-rich wastes with sewage sludge, the main challenge is to avoid overloading and inhibition of methanogenesis. Efficient degradation of major intermediates, such as LCFAs, acetate, propionate, and butyrate, is crucial to prevent drops in pH,
imbalance of the major metabolic steps, and consequently, process inhibition. The inhibition and toxicity of LCFAs to digester microbiomes, including limitations in substrate and product transport, damage to cell membrane, increased lag phase of methane production, loss of methanogenic activity, and sludge flotation and washout, have been documented in many studies conducted with synthetic LCFAs (Hanaki et al., 1981; Koster and Cramer, 1987; Angelidaki and Ahring, 1995; Hwu et al., 1998; Palatsi et al., 2010; Rinzema et al., 2013), and reviewed (Chen et al., 2008; Alves et al., 2009; Long et al., 2012).

Although co-processing FOG-rich materials with sewage sludge has been reported to increase methane production, the performance profiles can differ markedly in terms of (1) the maximum methane yield that can be achieved and (2) the corresponding maximum allowable substrate loading rate before process failure which can be observed by drops in pH, alkalinity, and methane yield. For microbial communities, this threshold may be considered as the maximum degree of tolerance a digester microbiome has against GIW inhibitory effects based on their ecological roles and interaction networks (or “combined efforts”). Indeed, different consortia of microbes may be affected by and respond to the same abiotic manipulation differently, both in terms of their community composition and functional/metabolic networks (Nemergut et al., 2013). Despite the potential of GIW co-digestion to substantially improve biogas production, its application and bioenergy potential are still limited due to a lack of understanding of microbial community dynamics associated with GIW disturbance and approaches to improve digester performance and community stability (resilience and resistance) and avoid substrate overload and inhibition of methanogenesis.
2.3 Microbial adaptation in GIW bioreactors

Challenging the microbial community with GIW to enhance methane production increases the risk of substrate overdose and LCFA inhibition, but also creates an opportunity for microbial adaptation of methanogenic archaeal and LCFA-degrading bacterial communities. Exposures to inhibitors such as ammonia, sulfide, sodium, insoluble organic compounds, and LCFAs at various concentrations have been reported to lead to adaptation of microorganisms to various degrees (Chen et al., 2008). Biomass adaptation and digester recovery from LCFA inhibition were observed in microbial communities treated with synthetic LCFAs (Alves et al., 2001; Cavaleiro et al., 2001, 2008, 2009, Palatsi et al., 2009, 2010), and real FOG-based wastes (Nadais et al., 2006; Silvestre et al., 2011; Wang et al., 2013; Ziels et al., 2016). The adaptation may be the result of population adaptation when the microbial population shifts towards the better-adapted microbiome members (Zeeman et al., 1985; Chen et al., 2008; Palatsi et al., 2010; Kougias et al., 2016). Although the exact microbiome assembly mechanisms and related functional dynamics are still not clear, it is believed that adaptation of microorganisms may decrease the inhibitory effect of toxicity shock and increase the biodegradability of undesired substrates (Wu et al., 1993; Chen et al., 2008; Silvestre et al., 2011; Stuckey et al., 2011).

In our recent studies (Wang et al., 2013), we used step feeding (slow increases of substrate loading rates) to induce microbial adaptation and showed the highest performance reported to date for co-digestion of GIW. Gradual addition of GIW, up to 66% (w/w) of volatile solids (VS) added, enhanced methane yield by 318% from 0.180 to 0.752 L-methane/g-VS added. This improvement can be attributed to the increase of biodegradable
substrates available when co-processing sewage sludge with GIW and to the step feeding of GIW with intermittent feeding patterns (every-other-day) that showed positive effects on the development of better microbial adaptation and reduction in inhibition of methanogenesis. Indeed, some studies have documented that during FOG co-digestion microbial adaptation correlated to reduced inhibition and improved digester performance (Silvestre et al., 2011; Ziels et al., 2016; Amha et al., 2017).

Positive effects of integrating intermittent and step feedings on digester performance were observed in previous studies. Compared to daily-fed communities, De Vrieze et al. (2013) observed higher degrees of bacterial dynamics and tolerance to ammonium loading shock in anaerobic communities treated with every-two-day feeding (without co-processing FOG-rich materials), although no difference in methane production and methanogenic community composition was found. Cavaleiro et al. (2009) showed that five pulse-feed cycles followed by step feeding of synthetic dairy wastewater with sodium oleate promoted sludge acclimation and more efficient degradation of LCFA-rich wastewater in an up-flow anaerobic column reactor. This feeding pattern also contributed to higher methane production. Biomass adaptation and increases in methane yield have been reported in a lab-scale continuous stirred tank reactor treated with step feeding of trapped grease wastes from a WWTF in a twice-a-day feeding pattern (Silvestre et al., 2011) and in an upflow anaerobic sludge blanket reactor treated with step feeding of dairy wastewater in an intermittent 48hr-feed-48hr-feedless mode (Nadais et al., 2006). Stabilization or “feedless” period was demonstrated to aid in retaining and entrapping organic matter (Nadais et al., 2006). It was also shown that a slow increase in FOG-rich co-substrate dose was found to induce biomass
adaptation, allowing more efficient degradation of LCFAs and reduction of LCFA inhibition (Silvestre et al., 2011).

Additionally, several researchers have used pulse feeds of synthetic LCFAs and other waste-based materials along with batch methanogenic activity and toxicity tests to evaluate LCFA inhibition (Cavaleiro et al., 2001, 2008, 2009; Neves et al., 2009; Palatsi et al., 2009, 2010). These studies demonstrate the possibility of using a pulse feeding strategy to induce tolerance and adaptation in methanogenic archaeal and syntrophic acidogenic populations.

2.4 Microbial ecology in GIW digesters

Recent advances in microbial community ecology have sought to identify and explain patterns and processes of community assembly that govern natural ecological systems such as soil (Zhou et al., 2010; Barberán et al., 2012), ocean (Steele et al., 2011; Gilbert et al., 2012), as well as the human microbiomes of individuals (Fierer et al., 2008; Nasidze et al., 2009; Turnbaugh et al., 2009) or across body habitats (Costello et al., 2012; Human Microbiome Project Consortium, 2012). Microbial community assembly has been considered historically as a deterministic process in which interspecies interactions (e.g. mutualism/syntrophy, commensalism, parasitism/predation, amensalism, and competition) (Lidicker, 1979; Faust and Raes, 2012) and niche differentiation (ecologically meaningful differences between species) are dominant drivers in determining the relationship between taxon traits and the environment (Weiher and Keddy, 1999; Chase and Leibold, 2003). In contrast, neutral theory suggests that community assembly is mainly shaped by stochastic factors such as birth, death, immigration, and speciation, and assumes ecological equivalence
of community members (Bell, 2001; Harpole, 2010). Others recognize the attributes of combined deterministic and stochastic processes in shaping the microbial community (Ofiteru et al., 2010). A conceptual synthesis of these theories has been proposed to include evolutionary forces, and concentrate these community assembly processes into four categories: diversification (generation of new genetic variation), dispersal (movement of species across space), selection (deterministic structuring via abiotic factors) and drift (stochastic changes in species abundance) (Vellend, 2010; Nemergut et al., 2013).

Along with the recognized practical significance of microbial resource management (Read et al., 2011; Koch et al., 2014; Carballa et al., 2015; Sales and Lee, 2015), it is believed that theories and patterns of community ecology can be applied to study and better manage the complex community assembly and interaction webs of reactor microbiota in engineered environments. Specifically, there is a need to understand the degree to which reactor microbiome is structured by ecological processes, and the relationships and interactions between individual taxa and the environmental factors (Curtis et al., 2003; Rittmann et al., 2006; Faust and Raes, 2012; Pholchan et al., 2013; Gude, 2015; Rodríguez et al., 2015).

With advances in high-throughput sequencing and bioinformatics analysis techniques, several researchers have profiled microbiomes of anaerobic digesters performing single-substrate digestion of sewage sludge or co-digestion of multiple waste streams in lab scale (Wirth et al., 2012; Ziganshin et al., 2013) or full scale (Werner et al., 2011; Lee et al., 2012; Zhang et al., 2012; Sundberg et al., 2013). These efforts were made to link community dynamics such as composition shifts, species richness, and evenness, to environmental
factors and community function. More recent studies actively manipulated community assembly in lab scale reactors to examine potential patterns and processes guiding the population and functional dynamics, such as deterministic niche and stochastic neutral theories and divergence and convergence patterns (Pholchan et al., 2013; Zhou et al., 2013; Vanwonterghem et al., 2014). Additionally, to identify ecological roles of core populations in microbiome assembly and functional dynamics, co-occurrence or microbial network inference analysis has been applied to soil (Barberán et al., 2012), wastewater treatment plants (Ju et al., 2014; Ju and Zhang, 2015), and household biogas digesters (Rui et al., 2015).

Reactor microbiomes are optimally engineered and structured for the metabolic cooperation of specialized groups of microbes. However, these complex microbial communities and interaction networks in reactors have been traditionally treated as black boxes and their community ecology across space and time remains largely unknown. This has posed a substantial challenge to maintaining community function and stability when microbiomes are subject to dynamic changes following environmental disturbances. We believe that fundamentally understanding how the reactor microbiome is shaped by and interacts with the environmental traits, both compositionally and functionally, can provide deeper insights into approaches towards better community structuring. Additionally, understanding the important ecological mechanisms directing the abiotic and biotic characteristics of reactor microbiomes can facilitate prediction and improvement of community function in response to different stress conditions in the environment.
Chapter 3 Research goals

The overall objective of this dissertation is to understand the ecological patterns and processes that determine the performance and functional stability of methanogenic biosystems and how the digester microbiome can be improved to achieve high-performance GIW co-digestion.

Through a series of common garden experiments, in chapter 4, I evaluated (1) whether pre-disturbance experience matters (do they really need to be step-fed to achieve higher methane yield); (2) if substrate selection matters, which substrate leads to better performance; (4) comparing mixed FOG and food solids with GIW, does dewatering matter; and ultimately (5) how can we used this knowledge to build a “map” to high-performance, resilient and resistant co-digestion of GIW. In chapter 5, I assessed (1) the causal relationship between microbial community, function, and environment, (2) impacts of substrate history (feed selection and the intensity of preshocks) on community survivability to GIW stresses, (3) the microbial co-occurrence/interaction network of key digester populations that play important role in reducing GIW inhibition, and (4) approaches to improve community performance and stability.

By shaping the digester microbiomes using step and pulse feeding strategies, in chapter 6, I evaluated (1) if step feeding can create more robust microbial communities against GIW inhibition by inducing microbial adaptation; (2) if both GIW-adapted and non-GIW-adapted communities can recover from a onetime loading shock and if so (3) if they can achieve the same level of methane production or even tolerate a higher GIW loading rate; and (4) if
challenging the anaerobic co-digester with periodic pulse feeds can result in more robust microbial communities. In chapter 7, I analyzed (1) how different levels of GIW disturbance direct community assembly and related functional dynamics; (2) how stress history links to community survivability and function; and (3) how ecological roles and niche characteristics of key digester microbiome members relate to their responses to different adaptation approaches and disturbance intensities.
Chapter 4 Common garden experiment identifies approaches to achieve resilient and resistant anaerobic co-digestion of grease interceptor waste

4.1 Abstract

Anaerobic co-digestion of grease interceptor waste (GIW) has shown great potential to substantially improve biogas production. However, its application and bioenergy potential are still limited due to a lack of understanding of approaches to improve digester performance and stability and to avoid substrate overload and inhibition of methanogenesis. In this study, we conducted, for the first time for GIW co-digestion, a series of common garden experiments to directly identify substrate history and operating strategies that can lead to resilient and resistant digester function. Eight sets of digesters in triplicates were seeded with the inoculum from a full-scale digester and further enriched at the same organic loading rate (OLR) using different substrates, including: (1) thickened waste activated sludge (control), (2) carbohydrate, (3) protein, (4) lipid, (5) acetic acid, (6) propionic and butyric acids, (7) mixed fats, oils and greases (FOG) and food solids, (8) GIW composed of FOG, food solids and associated wastewater. After the selective enrichment, the digester garden was exposed to a common environmental disturbance at low-GIW loading rate and repeated for mid- and high-GIW loading rates. Throughout the experiments, digester function
(methane production and other parameters associated with effluent quality) and stability (ecological resilience and resistance) were monitored to directly attribute performance to community difference. Despite identical inoculum, operating conditions (e.g. substrate loading rate, digester system setup, solid retention time) and performance (methane production and effluent quality) during the pre-disturbance stage, we observed feed-driven heterogeneity in response to GIW perturbations of increased intensity. Specifically, functional resistance and resilience were enhanced, resulting in a substantial enhancement of methane yield only when the substrate history (feed selection and intensity of preshocks) was favorable. The highest GIW loading rate achieved without inhibition was 42 % (v/v), 63% COD, or 58% (w/w) of volatile solids (VS) added, with a total OLR of 1.74, or OLR of substrate of 1.01 g-VS/L/day. Methane content was increased from 62.3% to 74.1%, and the highest methane yield achieved was 453 mL-methane/g-VS added, or 218.6 mL-methane/g-COD (450% increase) compared to the control digester. Combined, this study advances our understandings of the impacts of substrate selection and overloading stresses on methanogenesis and demonstrates specific operating approaches to enhance digester performance and susceptibility to different GIW loading rates.

4.2 Importance

This study is the first direct demonstration based on ecological concepts of a common garden experiment to directly identify substrate history and operating strategies that can lead to enhanced functional resistance and resilience in anaerobic co-digesters treating grease interceptor waste.
4.3 Introduction

Anaerobic digesters are engineered under specific environmental conditions to allow the metabolic cooperation of specialized consortia of microbes for biosolids degradation and energy recovery from wastewater streams. To improve energy production and waste-to-energy management, co-digestion of sewage sludge with lipid-rich wastes that are high in biological methane potential has been widely studied using wastes from different origins such as raw (un-dewatered) fats, oils, and greases (FOG) from a receiving facility (Wan et al., 2011) (Wan et al., 2011), dewatered FOG from grease traps (Kabouris et al., 2009), grease interceptor waste (GIW) from a meat processing plant (Luostarinen et al., 2009), GIW from a restaurant (un-dewatered) (Wang et al., 2013), grease trap sludge (Davidsson et al., 2008), and trapped FOG wastes from wastewater treatment facilities (Martin-González et al., 2011; Silvestre et al., 2011; Noutsopoulos et al., 2013), waste cooking oil (Ziels et al., 2016), and food waste and FOG (Amha et al., 2017).

The major metabolic processes in anaerobic digestion, including hydrolysis, acidogenesis (fermentation), acetogenesis, and methanogenesis, are carried out by different consortia of microbes occupying distinct ecological preferences. One major challenge at elevated FOG concentrations is maintaining digester stability to allow efficient degradation of major intermediates such as long chain fatty acids (LCFAs), acetate, propionate, and butyrate. LCFA toxicity to digester microbiomes, including limitations in substrate and product transport, damage to cell membrane, increased lag phase of methane production, loss of methanogenic activity, and sludge flotation and washout, have been demonstrated in previous studies conducted with synthetic LCFAs (Hanaki et al., 1981; Koster and Cramer,
1987; Angelidaki and Ahring, 1995; Hwu et al., 1998; Palatsi et al., 2010; Rinzema et al., 2013), and reviewed (Chen et al., 2008; Alves et al., 2009; Long et al., 2012). (Martín-González et al., 2011; Silvestre et al., 2011; Noutsopoulos et al., 2013)

To avoid FOG overload and inhibition of methanogenesis, it is crucial to determine the maximum allowable FOG loading rate and the corresponding maximum methane yield achieved before inhibition. Different organic loading rates (OLRs) have been widely tested, ranging from 0.05 (4% (w/w) VS of FOG added) to 3.40 g-VS/L/day (75%(w/w) VS of FOG added), but yielded inconsistent results (Davidsson et al., 2008; Kabouris et al., 2009; Luostarinen et al., 2009; Martín-González et al., 2011; Silvestre et al., 2011; Wan et al., 2011; Noutsopoulos et al., 2013; Wang et al., 2013; Ziels et al., 2016). Many encountered inhibitions at low doses and yielded low biogas production. Others achieved higher methane yields, but the performance profiles differed markedly even at the same OLR or % FOG added. Co-digestion of FOG wastes appears to be dependent on many factors, including the source of startup substrate and co-substrate, total OLR, OLR of GIW, operating conditions (e.g. temperature, solid retention time, mixing intensity, and feeding strategy and frequency). Additionally, the characteristics of FOG materials are source-specific and can have VS ranging from 17% to 93% (w/w), chemical oxygen demand (COD) up to 1211 kg/m3 and other chemical compounds such as detergents or polymers used for dewatering (Davidsson et al., 2008; Kabouris et al., 2009; Luostarinen et al., 2009; Wan et al., 2011; Long et al., 2012; Wang et al., 2013).

In an earlier study, we used step feeding (slow increases of substrate loading rates) from 0%, 44%, 66%, to 84% (w/w) of VS added to determine the limit of GIW co-digestion
(Wang et al., 2013). The threshold input of GIW without digester failure was determined to be in the range of 66% to 84%. A narrower range was identified from 66% to 71% (w/w) of VS added if combined with results from other studies using various GIW sources (Davidsson et al., 2008; Kabouris et al., 2009; Luostarinen et al., 2009; Wan et al., 2011; Wang et al., 2013). Under the same GIW addition, non-step-fed digester experience inhibition and process failure, while the step-fed digester showed improved performance. Step feeding from 44% to 66% achieved the highest methane yield reported to date for co-digestion of GIW, increasing methane yield by 318% from 0.180 to 0.752 L-methane/g-VS added, biogas production from 2.2 to 21.6 L/day, and methane content from 60.2% to 68.6%. Compared to other single-level studies, step feeding of GIW achieved higher methane yield at the same GIW OLR and higher GIW OLR without digester inhibition. Indeed, previous studies have documented that microbial adaptation through step feeding or other acclimation strategies correlated to reduced inhibition of methanogenesis and improved performance in bioreactors treated with LCFAs (Cavaleiro et al., 2009; Kougias et al., 2016; Ziels et al., 2017), FOG-based wastes (Silvestre et al., 2011; Ziels et al., 2016), or other waste streams (Nadais et al., 2006; Goux et al., 2015). However, specifically how the adaptation mechanism can be initiated and to what degree this mechanism needs to be developed according to fluctuating stresses remain unclear.

There have been many attempts to improve the performance and stability of FOG co-digestion, but a universal approach regardless of the FOG origins that can “push the limit” of GIW co-digestion and achieve high-yield yet stable operation is still lacking. In this study, we conducted a series of common garden experiments using different substrates, including:
(1) thickened waste activated sludge (control), (2) carbohydrate, (3) protein, (4) lipid, (5) acetic acid, (6) propionic and butyric acids, (7) mixed FOG and food solids, (8) GIW composed of FOG, food solids and associated wastewater, to selectively cultivate eight sets of digesters in triplicates at the same OLR. When exposed to a common disturbance at low-, mid- and high-GIW OLRs, we monitored digester function (methane production and effluent quality) and stability (ecological resilience and resistance) to directly identify the impacts of substrate history and operating approaches to achieve digester resilience and resistance.

Specifically, through this unique approach we ask: (1) whether pre-disturbance experience matters (do they really need to be step-fed to achieve higher methane yield), (2) if substrate selection matters, which substrate leads to better performance, (4) comparing mixed FOG and food solids with GIW, does dewatering matter, and ultimately (5) how can we use this knowledge to build a “map” to high-performance, resilient and resistant co-digestion of GIW.

4.4 Materials and methods

**Substrate selection, collection and preparation.** Eight different substrates were used for selective enrichment, including (1) thickened waste activated sludge (TWAS) (control), (2) carbohydrate, (3) protein, (4) lipid, (5) acetic acid, (6) mixed propionic and butyric acids, (7) mixed FOG and food solids, (8) mixture of FOG, food solids and wastewater (i.e. the entire content of GIW). Anaerobic sludge (inoculum) and TWAS (base substrate) was collected from South Durham Water Reclamation Facility in North Carolina. To compare digester performance between synthetic and real wastes, we included major GIW components, food solids and FOG, as individual feed sources. GIW from a food service
establishment (FSE) in Cary, North Carolina was used as co-substrate. The three primary components of GIW, i.e. FOG, food particles, and wastewater, were collected separately at the FSE and stored at 4°C immediately after collection. GIW was sterilized prior to use using ionizing radiation (Cobalt-60 gamma irradiation) at 22 Gray/hr for a 24-hours exposure at the research facility of the Department of Nuclear Engineering, North Carolina State University, NC. The carbohydrate substrate was composed of glucose (50% COD) and starch (50% COD). The protein substrate was whey-based protein powder containing 20g protein, 4g carbohydrate and <0.5g fat per 28.2g of powder. Lipid substrate was 100% pure rice bran oil containing 3.5g saturated, 6g polyunsaturated and 4.5g monounsaturated fats, and 0% protein and carbohydrate. Substrate characterizations are shown in Table 4-1. The control reactor was fed with 100% TWAS and the treated reactors were fed with selective substrate (28-30% COD added from substrate) mixed with TWAS at similar OLR of substrate (Table 4-2).

**Digester operation.** The digester garden was established through the operation of mother and daughter digesters. For the low-GIW dose experiment, during the cultivation period (Days 1-66), different functional communities were developed by selective addition of substrates at similar OLR at 2.03-2.41 g-COD/L/day (Table 4-2). From Days 1 to 38, eight 1.2L mother digesters were selectively enriched using TWAS (control), carbohydrate, protein, lipid, acetic acid, mixed propionic and butyric acids, mixed food solids and FOG, and GIW. The total OLR was 1.69 g-COD/L/day for the control reactor (TWAS) and 2.03-2.41 g-COD/L/day for the treated reactors. If considering the OLR of each substrate, approximately 0.58-0.73 g-COD/L/day of substrate were loaded in each treated reactor during the cultivation period. On day 38, the content of each mother digester was equally distributed
into three 0.4L daughter digesters and continued to be cultivated at the same OLR with the same substrate type from Days 38 to 66. During disturbance period (Days 66-86), each triplicated daughter digester system was exposed to a low GIW disturbance (63% COD added from GIW) for a total of five feeding cycles (Table 4-2). To evaluate digester function and stability, we continuously monitored methane yield, methane content (Figure 4-1), effluent concentration and reduction rate of volatile solids, pH, and alkalinity after the disturbance (Figures 4-S1 to 4-S4).

For mid- and high-GIW dose experiments, four digester systems were set up to perform mid and high GIW dose experiments simultaneously using two types of substrate at a time. For example, during the cultivation period, TWAS (control, 100% TWAS) and carbohydrate (28% COD added from carbohydrate mixture) digester systems were maintained in two 0.8L mother digesters in triplicate. At least 2 feeding cycles (8 days) before the disturbance experiment was performed, mother digesters were combined and equally distributed into three 0.4L daughter digesters for mid-GIW dose and three 0.4L daughter digesters for high-GIW dose. The protein, lipid, acetic acid, mixed propionic and butyric acids, mixed food solids and FOG, and GIW digester systems were performed accordingly at similar OLRs (Table 4-2). During the disturbance period, one triplicated daughter digester system was exposed to a mid-GIW dose disturbance (77.9% COD added from GIW) and another system to a high-GIW dose disturbance (95.5% COD added from GIW) for a total of five feeding cycles (Table 4-1 and Figure 4-2). The amount of substrate added was chosen as a good starting loading rate ensuring enough stress intensity to develop community assembly.
without causing a process failure, and preparing microbial communities for higher level disturbances, according to our previous studies (Wang et al., 2013).

**Digester design and environment.** Special columns were constructed to allow easier sampling and subsequent dividing of the biomass (Figures 4-S5 and 4-S6). Each digester consisted of a polypropylene screw lid container with a unique grip lug and a leak proof seal ring. The lid was inserted with a nylon straight barbed connector with a silicon washer to keep the reactor airtight. A high-performance precision tygon tubing was connected the barbed connector to a PVDF 3-way stopcock for biogas sampling and subsequently to a flex-foil gas bag fitted with a hose valve for biogas collection. Another PVDF 3-way stopcock was fitted on the bottom side of the digester chamber serving as a feed and decant port.

All digesters were placed on a platform shaking table operated for mixing in a temperature-controlled room to maintain mesophilic conditions (37°C). All digesters were fed every four days in a draw-and-fill semi-continuous mode with a solids retention time (SRT) of 20 (during cultivation period) and 14.7 days (during disturbance period). Effluent was collected, and an equal amount of feedstock was introduced into the digesters.

**Digester function.** Chemical and physical characteristics of the sludge and biogas were analyzed to evaluate digester microbiome function. Every four days before decanting and feeding the digesters, biogas production was recorded and normalized to standard temperature and pressure (STP) conditions. Methane content in biogas was assessed using a gas chromatograph (GC, SRI 8610C) equipped with a thermal conductivity detector. Every four days effluent sludge was collected and analyzed for total solids (TS), volatile solids (VS), alkalinity, and pH according to Standard Methods (American Public Health
Association (APHA), 2005). Concentrations of individual volatile fatty acids (VFA; e.g., acetic, propionic, butyric, and valeric acids) were determined using a GC (GC-2014 Shimadzu) equipped with a flame ionization detector according to Method 5560 D in Standard Methods.

4.5 Results

Eight sets of digesters in triplicate were inoculated with the inoculum from a full-scale digester and cultivated using different feed sources including: (1) TWAS (control), (2) carbohydrate, (3) protein, (4) lipid, (5) acetic acid, (6) mixed propionic and butyric acids, (7) mixed FOG and food solids, (8) GIW composed of FOG, food solids and associated wastewater at the same OLR (Tables 4-1 and 4-2). After cultivation, we exposed them to a common environmental disturbance using low, mid and high GIW loading rates described below in more detail.
**Table 4-1** Substrate characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Anaerobic sludge (inoculum)</th>
<th>TWAS (base substrate)</th>
<th>Lipid (rice bran oil)</th>
<th>GIW Wastewater FP and FOG layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS (g/kg-wet sample)</td>
<td>25.9 ± 0.05</td>
<td>24.4 ± 1.45</td>
<td>1005 ± 5.97</td>
<td>2.70 ± 0.10 352 ± 7.14</td>
</tr>
<tr>
<td>VS (g/kg-wet sample)</td>
<td>16.0 ± 0.05</td>
<td>17.0 ± 1.22</td>
<td>1004 ± 6.03</td>
<td>2.39 ± 0.05 349 ± 6.33</td>
</tr>
<tr>
<td>VS/TS (%)</td>
<td>61.8</td>
<td>69.8</td>
<td>100</td>
<td>88.6 99.3</td>
</tr>
<tr>
<td>COD (g/L)</td>
<td>28.7 ± 3.20</td>
<td>33.8 ± 5.81</td>
<td>3032 ± 565</td>
<td>5.65 ± 0.67 744 ± 229</td>
</tr>
</tbody>
</table>

*a* Total solid concentration  
*b* Volatile solid concentration  
*c* Chemical oxygen demand concentration

**Table 4-2** Substrate composition, total organic loading rate (OLR) and OLR of substrate during cultivation (A) and disturbance periods (B).

<table>
<thead>
<tr>
<th>Reactor microbiome</th>
<th>Substrate concentration</th>
<th>Total OLR</th>
<th>OLR of substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%vol</td>
<td>%VS&lt;sup&gt;c&lt;/sup&gt;</td>
<td>%COD&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cultivation period</td>
<td>TWAS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>-</td>
<td>45.2</td>
<td>28.0</td>
</tr>
<tr>
<td>Protein</td>
<td>-</td>
<td>43.2</td>
<td>28.1</td>
</tr>
<tr>
<td>Lipid</td>
<td>0.5</td>
<td>19.3</td>
<td>30.2</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>1.2</td>
<td>-</td>
<td>28.1</td>
</tr>
<tr>
<td>Propionic and butyric acids</td>
<td>4.1</td>
<td>-</td>
<td>28.2</td>
</tr>
<tr>
<td>Food particles and FOG</td>
<td>1.8</td>
<td>24.1</td>
<td>28.2</td>
</tr>
<tr>
<td>GIW</td>
<td>15</td>
<td>24.9</td>
<td>29.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disturbance period</th>
<th>Low GIW dose</th>
<th>Mid GIW dose</th>
<th>High GIW dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>42</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>57.7</td>
<td>73.8</td>
<td>94.4</td>
</tr>
<tr>
<td></td>
<td>63.0</td>
<td>77.9</td>
<td>95.5</td>
</tr>
<tr>
<td></td>
<td>1.74</td>
<td>2.03</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td>3.61</td>
<td>4.17</td>
<td>5.11</td>
</tr>
<tr>
<td></td>
<td>1.01</td>
<td>1.50</td>
<td>2.42</td>
</tr>
<tr>
<td></td>
<td>2.28</td>
<td>3.25</td>
<td>4.88</td>
</tr>
</tbody>
</table>
**Low-GIW dose experiment.** During the cultivation period (Days 1-66), different functional communities were developed by selective addition of substrates at similar organic loading rates (OLR) (Table 4-2). From Days 1 to 38, eight 1.2L mother digesters were selectively enriched using the different substrate types (Figure 4-1), and on day 38, each mother digester was equally distributed into three 0.4L daughter digesters (Figure 4-1). To maintain anaerobic conditions, nitrogen was supplied during distribution, which led to a quick drop in methane content and methane yield on Day 38 (Figure 4-1). Methane content recovered after one to two feeding cycles as the biogas filled up the reactor chamber. After this transition, each daughter digester continued to be cultivated at the same OLR with the same substrate type from Days 38 to 66. The average methane content during cultivation was 62.2% for TWAS reactor, 59.3% for carbohydrate reactor, and approximately 66-69% for protein, lipid, acetic acid, mixed propionic and butyric acids, mixed food solids and FOG, and GIW reactors (Table 4-3). The average methane yield during cultivation was 48.9 mL/g-COD for the control reactor (TWAS) and approximately 100-130 mL/g-COD for the treated reactors.

During the disturbance period (Days 66-86), each triplicated daughter digester system was exposed to a low GIW disturbance (63% COD added from GIW) for a total of five feeding cycles (Table 4-1 and Figure 4-1). Overloading was observed immediately after the end of the first feeding cycle (from Days 66-70) in five types of digester cultivated by TWAS, carbohydrate, protein, acetic acid, and mixed propionic and butyric acids, according to the digester performance profiles (methane production and effluent quality) (Figures 4-1, 4-S1, 4-S2). The average methane yield of TWAS reactor decreased from 48.9 mL/g-COD to 14.8
mL/g-COD (Day 86), and average methane content from 62.2% to 55.2% (Day 86). The average methane yield of carbohydrate reactor decreased from 112 to 11.0 mL/g-COD, and average methane content from 59.3% to 53.8% (Day 86). The average methane yield of protein reactor decreased from 111 to 17.9 mL/g-COD, and average methane content from 69.7% to 54.7% (Day 86). The average methane yield of acetic acid reactor decreased from 129 to 14.1 mL/g-COD, and average methane content from 66.3% to 52.1% (Day 86). The average methane yield of propionic/butyric acids reactor decreased from 128 to 13.1 mL/g-COD, and average methane content from 69.4% to 56.3% (Day 86).

On the other hand, lipid, food solids/FOG, and GIW digesters showed improved performance since Day 66 (Figure 4-1 and Table 4-3). Comparing the average methane yield before and after the disturbance, the methane yield of lipid reactor increased from 102 to 206 mL/g-COD added, and methane content from 69.1% to 73.2%. The methane yield of food solids/FOG reactor increased from 126 to 218 mL/g-COD added, and methane content from 69.9% to 73.8%. The methane yield of GIW reactor increased from 118 to 216 mL/g-COD added, and methane content from 69.5% to 73.4%.
Figure 4-1 Low stress experiment: methane yield and methane content during cultivation and disturbance periods.
Table 4-3 Low stress experiment: average methane yield and methane content

<table>
<thead>
<tr>
<th>Reactor microbiome</th>
<th>Methane yield (mL/g-COD)</th>
<th>Methane content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TWAS</td>
<td>48.9</td>
<td>62.2</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>112</td>
<td>59.3</td>
</tr>
<tr>
<td>Protein</td>
<td>111</td>
<td>69.7</td>
</tr>
<tr>
<td>Lipid</td>
<td>102</td>
<td>69.1</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>129</td>
<td>66.3</td>
</tr>
<tr>
<td>Propionic and butyric acids</td>
<td>128</td>
<td>69.4</td>
</tr>
<tr>
<td>Food particles and FOG</td>
<td>126</td>
<td>69.9</td>
</tr>
<tr>
<td>GIW</td>
<td>118</td>
<td>69.5</td>
</tr>
<tr>
<td>TWAS</td>
<td>11.7</td>
<td>52.0</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>17.1</td>
<td>54.0</td>
</tr>
<tr>
<td>Protein</td>
<td>17.4</td>
<td>51.9</td>
</tr>
<tr>
<td>Lipid</td>
<td>206</td>
<td>73.2</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>13.8</td>
<td>53.5</td>
</tr>
<tr>
<td>Propionic and butyric acids</td>
<td>16.0</td>
<td>57.0</td>
</tr>
<tr>
<td>Food particles and FOG</td>
<td>218</td>
<td>73.8</td>
</tr>
<tr>
<td>GIW</td>
<td>216</td>
<td>73.4</td>
</tr>
</tbody>
</table>
**Mid- and high-GIW dose experiments.** Four digester systems were set up to perform mid and high GIW dose experiments simultaneously using two types of substrate at a time (Figure 4-2). Similarly, a quick decrease and recovery pattern of methane content and methane yield were observed during the transition of mother and daughter digesters, after which all digester systems continued to be cultivated at the same OLR with the same substrate type until challenged in the disturbance experiment. The average methane content during cultivation was 64.9% for TWAS reactor, 60.4% for carbohydrate reactor, and approximately 67-70% for protein, lipid, acetic acid, mixed propionic and butyric acids, mixed food solids and FOG, and GIW reactors (Table 4-4). The average methane yield during cultivation was 76.8 mL/g-COD for the control reactor (TWAS) and approximately 120-140 mL/g-COD for the treated reactors. The small difference in methane yield during cultivation period between low-GIW dose and mid- and high-GIW dose experiments was likely due to the differences in system setup, working volume, and duration of operation before disturbance experiments were performed.

During the disturbance period, a daughter digester system (in triplicate) was exposed to a mid-GIW dose disturbance (77.9% COD added from GIW) and another system (in triplicate) to a high-GIW dose disturbance (95.5% COD added from GIW) for a total of five feeding cycles (Table 4-1 and Figure 4-2). After the first disturbance, all eight digester systems showed decreases in methane content and methane yield (Figure 4-2). Other effluent parameters also indicated digester overloading consistently across all digester types (Figures 4-S3 and 4-S4).
Figure 4-2 Mid- and high-GIW dose experiment: methane yield and methane content during cultivation and disturbance periods.
Table 4.4 Mid- and high-GIW dose experiment: average methane yield and methane content

<table>
<thead>
<tr>
<th>Reactor microbiome</th>
<th>Methane yield (mL/g-COD)</th>
<th>Methane content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cultivation period</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TWAS</td>
<td>76.8</td>
<td>64.9</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>130</td>
<td>60.4</td>
</tr>
<tr>
<td>Protein</td>
<td>141</td>
<td>70.9</td>
</tr>
<tr>
<td>Lipid</td>
<td>139</td>
<td>70.5</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>129</td>
<td>67.3</td>
</tr>
<tr>
<td>Propionic and butyric acids</td>
<td>120</td>
<td>69.8</td>
</tr>
<tr>
<td>Food particles and FOG</td>
<td>134</td>
<td>68.2</td>
</tr>
<tr>
<td>GIW</td>
<td>127</td>
<td>67.3</td>
</tr>
<tr>
<td><strong>Mid-GIW dose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TWAS</td>
<td>11.7</td>
<td>52.5</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>11.4</td>
<td>53.1</td>
</tr>
<tr>
<td>Protein</td>
<td>4.41</td>
<td>41.6</td>
</tr>
<tr>
<td>Lipid</td>
<td>17.2</td>
<td>52.9</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>3.31</td>
<td>42.5</td>
</tr>
<tr>
<td>Propionic and butyric acids</td>
<td>3.81</td>
<td>41.4</td>
</tr>
<tr>
<td>Food particles and FOG</td>
<td>24.1</td>
<td>56.4</td>
</tr>
<tr>
<td>GIW</td>
<td>14.6</td>
<td>49.6</td>
</tr>
<tr>
<td><strong>Disturbance period</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TWAS</td>
<td>9.15</td>
<td>52.8</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>10.5</td>
<td>50.6</td>
</tr>
<tr>
<td>Protein</td>
<td>6.29</td>
<td>31.4</td>
</tr>
<tr>
<td>Lipid</td>
<td>10.0</td>
<td>50.5</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>2.79</td>
<td>37.0</td>
</tr>
<tr>
<td>Propionic and butyric acids</td>
<td>3.48</td>
<td>37.0</td>
</tr>
<tr>
<td>Food particles and FOG</td>
<td>16.9</td>
<td>56.4</td>
</tr>
<tr>
<td>GIW</td>
<td>7.34</td>
<td>38.5</td>
</tr>
</tbody>
</table>
Comparison of digester performance. Before the low-GIW dose disturbance, all 7 treated digester systems were cultivated under the same environmental conditions (digester system setup, total OLR, OLR of substrate, SRT) and generated the same level of methane yield at approximately 125 mL/g-COD. After the low-GIW dose disturbance, 5 digesters including the control encountered inhibition that significantly reduced their methane production and effluent quality. Only digesters cultivated by lipid, food solids/FOG, and GIW survived and achieved approximately 100% increase in methane yield (Figures 4-1 and 4-3). Specifically, comparing the methane yield before (Day 66) and after five feeding cycles of disturbance (Day 86), the methane yield of lipid reactor increased from 106 mL/g-COD to 187 mL/g-COD (76% increase), and methane content from 69.8% to 73.9%. The methane yield of food solids/FOG reactor increased from 105 mL/g-COD to 205 mL/g-COD (95% increase), and methane content from 69.6% to 74.5%. The methane yield of GIW reactor increased from 104 mL/g-COD to 219 mL/g-COD (110% increase), and methane content from 70.4% to 74.1%. Compared to the controlled digester system on Day 66, the addition of lipid, food solids/FOG, and GIW at low-GIW dose increased the methane yield by approximately 370 to 450%.
Figure 4-3 Average methane yield and methane content during low-, mid- and high-GIW dose experiments.
**Digester stability assessment.** The influence of a disturbance on digester function was assessed using ecological parameters of functional stability, including resistance (the maximum accumulation of key intermediate product), resilience (the time it takes for the accumulated product to return to its original state) (Botton *et al.*, 2006; Werner *et al.*, 2011). We monitored individual VFA concentrations under regular and disturbed environments for one feeding cycle with one feeding/disturbance event. An increase in concentrations of acetic, propionic, butyric and valeric acids was observed in all triplicated digester systems that were previously cultivated by lipid, food solids/FOG and GIW on Day 67, 12 hours after the low-GIW dose disturbance (Figure 4-4). The concentration of acetic acid increased from less than 30 mg/L to 200 mg/L and continued to increase on Day 69. However, no occurrence of methanogenic inhibition was identified by the measured biogas production, methane content, effluent VS concentration, pH, and alkalinity. The concentrations of propionic, butyric and valeric acids also increased on Day 67 but subsequently decreased.

![Figure 4-4 Effluent volatile fatty acids (VFAs) during low stress experiment](image.png)
4.6 Discussion

To compare performance of step feeding with previous studies, we adopted the idea of Noutsopoulou et al. (2013) to plot biogas yield versus OLR of FOG wastes (calculated by FOG waste content (% w/w of VS added) times total OLR) and replaced biogas yield with methane yield to obtain a more accurate result that includes the contribution of methane content in biogas (Figure 4-5).

Methane yields at various OLRs of FOG-based wastes were categorized into four zones. Zone 1 ranges from 0 to ~1.03 g-VS/L/day where both non-step-fed (white symbols) and step-fed digesters (black symbols) were able to function without process failure, with average methane yields ranging from 0.1 to 0.5 L-methane/g-VS added. Within this range, methane yield is proportional to the OLR of FOG wastes; i.e. the more biodegradable VS added, the more methane produced within zone 1.

The threshold for FOG waste addition resulting in an inhibited digestion process is identified to be at approximately 1.03 g-VS/L/day where Zone 2 starts, ranging from 1.3 to ~2.0 g-VS/L/day. Within Zone 2, the degree of digester tolerance against LCFA inhibition varied. Process failures indicated by zero methane production were observed in both step-fed (Martín-González et al., 2011) and non-step-fed (Wang et al., 2013) digesters at ~1.4 g-VS/L/day. On the other hand, stable results were found in both step-fed (Luostarinen et al., 2009; Wang et al., 2012; Wang et al., 2013) and non-step-fed (Kabouris et al., 2009; Noutsopoulou et al., 2013; Wan et al., 2011) digesters, producing methane yields of 0.45 to 0.75 L-methane/g-VS added.
Figure 4-5 Methane yields at various OLRs of FOG-based wastes categorized into four zones. OLR of FOG-based wastes is calculated by multiplying FOG waste content (% w/w of VS added) and OLR of total substrate. White symbols represent methane yields without step feeding treatment. Black symbols represent methane yields with step feeding treatment. Methane yields at zero represent the occurrence of process failure when data of stable methane yield was unavailable.
Favorable substrate history (feed selection and intensity of pre-conditioning) enhances resilience and resistance of GIW co-digestion. From our previous study (Wang 2013), step feeding at 46% (w/w) of VS added (0.73 g-VS/L/day OLR of GIW) first increased methane yield from 0.18 to 0.50 L-methane/g-VS added, and at 66% (w/w) of VS added (1.43 g-VS/L/day OLR of GIW) methane yield was increased to 0.75 L-methane/g-VS added (318% increase). As shown in Figure 4-5, this production enhancement is the highest reported to date for co-digestion of GIW.

In this study, we used a common garden approach and further demonstrated that substrate history (in terms of selection and dosage) during pre-disturbance cultivation phase played an importance role in reducing GIW toxicity and the risk of overloading. In Figures 4-1, 4-3 and 4-4, despite identical inoculum, environmental conditions (operation and OLR of substrate), and digester function (methane production and effluent quality) during the pre-disturbance cultivation period, only digester systems cultivated using lipid, FOG/food solids and GIW at 0.20 to 0.27 g-VS/L/day survived a more intense GIW perturbation at 1.01 g-VS/L/day. The process failure observed in digesters cultivated by TWAS (control), carbohydrate, protein, acetic acid, and propionic/butyric acids indicated the importance of substrate selection. Our results showed that the stress response of anaerobic digesters was feed-specific, and only step feeding using similar substrates (i.e. lipid, FOG/food solids and GIW) improved digester resistance and resilience to an elevated stress condition. The pre-conditioning of lipid was conducted using rice bran oil (Table 4-1), which showed a similar cultivation effect in reducing GIW toxicity and increasing digester performance, compared to using real food solids/FOG and GIW.
To demonstrate the importance of a “preshock” cultivation period (the step feeding approach), we mapped the methane yields from this study during low- (1.01 g-VS/L/day), mid- (1.50 g-VS/L/day) and high-GIW disturbances (2.42 g-VS/L/day) in Figure 4-5. When using lipid, FOG/food solids and GIW at 0.20 to 0.27 g-VS/L/day to step-feed during cultivation, methane content was increased from 62.3% to 74.1%, and the highest methane yield achieved was 453 mL-methane/g-VS added, or 218.6 mL-methane/g-COD (450% increase), compared to the control digester (TWAS). Without a pre-conditioning treatment, the control digester (TWAS) encountered process failure.

The process failure of all digester systems (including lipid, FOG/food solids and GIW digesters) at mid- (1.50 g-VS/L/day) and high-GIW disturbances (2.42 g-VS/L/day) demonstrated that step feeding at 0.20 to 0.27 g-VS/L/day was not sufficient to reduce the inhibition at mid- and high-GIW doses. To “properly enter Zone 2” and overcome GIW toxicity at 1.0 g-VS/L/day, pre-conditioning the digester at around 0.20 to 0.27 g-VS/L/day achieved resilient and resistant operation at 1.01 g-VS/L/day. To overcome higher level of disturbance, such as at 1.43 g-VS/L/day OLR of GIW from Wang et al. (2013), a pre-conditioning treatment at 0.73 g-VS/L/day OLR of GIW was required. These two observations from two independent studies using different GIW origins consistently revealed that in order to allow better adapted digesters, it is recommended not to “jump too far” after the cultivation period. A cultivation at 0.20 to 0.27 g-VS/L/day only allowed a “jump” of 0.7-0.8 g-VS/L/day OLR to achieve resilient and resistant GIW co-digestion at 1.01 g-VS/L/day. Similarly, a cultivation at 0.73 g-VS/L/day also allowed a “jump” of 0.7-0.8 g-VS/L/day OLR to achieve stable operation at 1.43 g-VS/L/day. Indeed, gradual addition of
substrate allowed development of microbial adaptation when the community shifts towards the better adapted microorganisms (Zeeman et al., 1985; Chen et al., 2008; Palatsi et al., 2010; Kougias et al., 2016) and reduction of substrate inhibition (Cavaleiro et al., 2009; Kougias et al., 2016; Ziels et al., 2017). Our results indicated that a cultivation period for at least one SRT cycle of 20 days and a “jump” of 0.7-0.8 g-VS/L/day OLR for each step feeding is beneficial to increase community tolerance to GIW inhibitory effects and reduce risks of overloading. Combined, our results showed that the differences in digester response to an elevated GIW loading rates were determined by substrate selection (type of feed) and pre-disturbance experience (intensity of preshocks).

**Approaches to achieve resilient and resistant GIW co-digestion.** Here we propose, for the first time for GIW co-digestion, a “map” to achieve better performance and robustness to GIW toxicity (Figure 4-5). First of all, the most convenient and widely practiced measure to characterize GIW is biodegradable VS, compared to COD that is relatively difficult to measure due to the nature of FOG. This map is created based on performance data of VS basis. Second, comparing methane production and OLR of GIW from different FOG sources, our results demonstrated the feasibility of this map as a universal guidance suitable for different types of GIW origins. Third, our common garden approach directly identified the importance of feed selection and the beneficial effect of pre-disturbance experience using step feeding at the right dosing window to ensure resilient and resistant GIW co-digestion. Fourth, we examined the difference of cultivation using FOG/food solids (dewatered GIW) and (non-dewatered) GIW and concluded that both approaches resulted in substantial enhancement of methane yield. For engineering
applications, this result provides deeper insights for future economical evaluations on dewatering, as dewatering GIW reduces cost of transportation.

4.7 Conclusions

Using eight different types of substrate, we conducted the first common garden experiment for GIW co-digestion and directly identified substrate history (feed selection and intensity of preshocks) and operating strategies that enhanced digester performance (methane production) and stability (resistance and resilience) in response to different GIW disturbance levels.
4.8 Supporting information

Figure 4-S1 Total solids (TS) and volatile solids (VS) concentrations at low GIW dose.
Figure 4-S2 Total solids (TS) and volatile solids (VS) concentrations at mid and high GIW doses.
Figure 4-S3 pH and alkalinity at mid and high GIW doses.
**Figure 4-S4** Platform shaking tables with wooden grid frame.

**Figure 4-S5** Digester column design.
Figure 4-S6 Experimental design of one set of mother-daughter system that consisted of three mother (Task 1A) and six daughter (Task 1B) replicate digesters. There are a total of eight mother-daughter systems cultivated with (1) TWAS (control), (2) carbohydrate-, (3) protein-, (4) lipid-, (5) acetic acid-, (6) propionic and butyric acids, (7) Food particles and FOG, and (8) GIW-based substrates.
Chapter 5 Common garden experiment identifies causality between key microbial populations and resilient and resistant anaerobic co-digestion of grease interceptor waste

5.1 Abstract

Anaerobic co-digestion of grease interceptor waste (GIW) integrates waste management and resource recovery to improve economics and sustainability. Despite its potential, studies have shown inhibition of microbial community during degradation of lipid-rich materials and a threshold of GIW addition that led to inhibited methanogenesis and process failure. The major metabolic processes in anaerobic digestion, including hydrolysis, acidogenesis (fermentation), acetogenesis, and methanogenesis, are carried out by different consortia of microbes occupying distinct ecological preferences. Identifying microbial populations across these metabolic networks that play important roles in reducing substrate toxicity and enhancing functional resilience and resistance can expand our knowledge on approaches to achieve high-performance GIW co-digestion. In this study, we conducted a series of common garden experiments to directly investigate the causality between microbial community composition and functional dynamics in response to different overloading stresses. During the cultivation period, we used the methanogenic microbiome originated from a full-scale
digester and developed eight sets of microbial communities in triplicates using different feed sources, including: (1) thickened waste activated sludge (control), (2) carbohydrate, (3) protein, (4) lipid, (5) acetic acid, (6) propionic and butyric acids, (7) mixed fats, oils and greases (FOG) and food solids, (8) GIW composed of FOG, food solids and associated wastewater. During the disturbance period, these substrate-enriched microbial communities were further exposed to three independent disturbance events at low-, mid- and high-GIW loading rates. This approach allowed us to directly attribute differences in community function to differences in community composition. A comprehensive analysis on community dynamics and digester performance was performed using high-throughput sequencing of 16S rRNA gene. Despite identical seed, environment (digester operation, substrate loading rate, feeding patterns, feed microorganisms) and general whole-community function (methane production and effluent quality) observed during cultivation period, microbial community dissimilarity due to substrate history (feed selection and intensity of preshocks) determined digester survivability to overloading GIW stresses. Based on ecological concepts of a common garden experiment, our results directly identified the presence of key microbial populations that determined whether methanogenesis was inhibited or enhanced during GIW co-digestion. Combined, this study represents the first demonstration on exact approaches using substrate selection and overloading stresses to develop feed-specific community adaptation mechanisms and achieve resilient and resistant GIW co-digestion.
5.2 Importance

This study demonstrates the potential to achieve better bioenergy resource management when biological engineering questions are tested using ecological frameworks. For the first time for GIW co-digestion, a common garden approach was applied to directly identify the causal relationships between microbial community, function, and environment in triplicated anaerobic microbiomes. We evaluated the impacts of substrate history (feed selection and intensity of historical pre-conditioning) and overloading stresses of GIW on methanogenic microbiomes and demonstrated how reactor microbiomes can be manipulated to improve its resilience and resistance to GIW inhibition.

5.3 Introduction

Major metabolic processes such as hydrolysis, acidogenesis, acetogenesis, and methanogenesis in anaerobic digestion are carried out by different groups of microbes with unique ecological preferences. In anaerobic co-digesters treating grease interceptor waste (GIW), one major challenge is to ensure efficient degradation of major intermediates such as long chain fatty acids (LCFAs), acetate, propionate, and butyrate. LCFA toxicity to digester microbiomes, including limitations in substrate and product transport, damage to cell membrane, increased lag phase of methane production, loss of methanogenic activity, and sludge flotation and washout, have been demonstrated in previous studies conducted with synthetic LCFAs (Hanaki et al., 1981; Koster and Cramer, 1987; Angelidaki and Ahring, 1995; Hwu et al., 1998; Palatsi et al., 2010; Rinzema et al., 2013).
To avoid GIW overload and inhibition of methanogenesis, it is crucial to better understand microbial community dynamics associated with disturbance and identify approaches to improve community resilience and resistance. Studies have documented that the microbial community dynamics in methanogenic bioreactors are largely driven by deterministic factors (selective pressure via abiotic variables) (Vellend, 2010; Nemergut et al., 2013), for example, operating conditions such as pretreatment, temperature and salinity (Mei et al., 2017), sludge retention time and substrate loading rate (Ju et al., 2017), feed selection (Wagner et al., 2013; Vanwonterghem et al., 2014; De Francisci et al., 2015), and feeding patterns (Goux et al., 2015; Ziels et al., 2017). These results suggest that microbial community may be actively shaped by changing the environmental factors to improve a specific whole-community function. Indeed, microbial adaptation (community shifts towards the better adapted microorganisms) (Zeeman et al., 1985; Chen et al., 2008; Palatsi et al., 2010) through step feeding of substrate or other acclimation strategies correlated to reduced inhibition of methanogenesis and improved performance in bioreactors treated with LCFAs (Cavaleiro et al., 2009; Kougas et al., 2016; Ziels et al., 2017), FOG-based wastes (Silvestre et al., 2011; Ziels et al., 2016), or other waste streams (Nadais et al., 2006; Goux et al., 2015). The influence of historical pre-conditioning on the response of microbial community to re-exposure of past disturbance or other stressors has also been demonstrated in soil microbiomes (Cregger et al., 2012; Evans and Wallenstein, 2012; Pagaling et al., 2014; Azarbad et al., 2016). However, the specific causality of microbial community, whole-community function under different environmental stresses has not been established in GIW co-digesters.
In this study we conducted a series of common garden experiments to investigate the causal relationship between adaptation mechanisms of microbial community and whole-community resilience and resistance to GIW overloading. We shaped methanogeneic microbiome originated from a full-scale digester into eight microbial communities in triplicate using different feed sources including: (1) TWAS (control), (2) carbohydrate, (3) protein, (4) lipid, (5) acetic acid, (6) mixed propionic and butyric acids, (7) mixed fats, oils, and greases (FOG) and food solids, (8) entire content of GIW composed of FOG, food solids and wastewater. Each functional guild of anaerobic digestion is fulfilled by a reservoir of phylogenetically diverse populations that share common resources (niche overlap). By limiting the feed source to a specific type of substrate (e.g., lipid), we selectively enriched microbial members that are key to the target metabolic pathway (e.g., lipid degradation). When exposed to overloading GIW stresses, we monitored microbial community dynamics and whole-community function (methane production, effluent quality, ecological resilience and resistance) to directly identify the impacts of substrate history (feed selection and the intensity of preshocks) on community survivability to GIW stresses and key microbial populations in reducing substrate toxicity and enhancing functional resilience and resistance.

5.4 Materials and methods

Substrate selection, collection and preparation. Eight different substrates were used for selective enrichment, including (1) TWAS (control), (2) carbohydrate, (3) protein, (4) lipid, (5) acetic acid, (6) mixed propionic and butyric acids, (7) mixed FOG and food solids, (8) mixture of FOG, food solids and wastewater (i.e. the entire content of GIW). These feeds
were selected because they are key metabolic materials during anaerobic digestion. Additionally, we used mixture of more than one substrate such as mixed propionic and butyric acids to enrich multiple microbial communities across different functional networks.

Anaerobic sludge (inoculum) and thickened waste activated sludge (TWAS) (base substrate) was collected from South Durham Water Reclamation Facility in North Carolina. To compare digester performance between synthetic and real wastes, we included major GIW components, food solids and FOG, as individual feed sources. GIW from a food service establishment (FSE) in Cary, North Carolina was used as co-substrate. The three primary components of GIW, i.e. FOG, food particles, and wastewater, were collected separately at the FSE and stored at 4°C immediately after collection. GIW was sterilized prior to use using ionizing radiation (Cobalt-60 gamma irradiation) at the research facility of the Department of Nuclear Engineering, North Carolina State University, NC. Carbohydrate was supplied by half glucose half starch, protein was supplied by protein powder, and lipid was supplied by rice bran oil. Substrate characterizations are shown in Table 5-S1.

**Digester design and environment.** Special columns were constructed to allow easier sampling and subsequent dividing of the biomass. Each digester consists of a polypropylene screw lid container with a unique grip lug and a leak proof seal ring. The lid was inserted with a nylon straight barbed connector with a silicon washer to keep the reactor airtight. A high-performance precision tygon tubing was connected the barbed connector to a PVDF 3-way stopcock for biogas sampling and subsequently to a flex-foil gas bag fitted with a hose valve for biogas collection. Another PVDF 3-way stopcock was fitted on the bottom side of the digester chamber serving as a feed and decant port.
All digesters were placed on a platform shaking table operated for mixing in a temperature-controlled room to maintain mesophilic conditions (37°C). All digesters were fed every four days in a draw-and-fill semi-continuous mode with a solids retention time (SRT) of 20 (during cultivation period) and 14.7 days (during disturbance period). Effluent was collected, and an equal amount of feedstock was introduced into the digesters.

**Digester operation.** The digester garden was established through the operation of mother and daughter digesters. For the low-GIW dose experiment, during the cultivation period (Days 1-66), different functional communities were developed by selective addition of substrates at similar OLRs (Table 5-S2). From Days 1 to 38, eight 1.2L mother digesters were selectively enriched using TWAS (control), carbohydrate, protein, lipid, acetic acid, mixed propionic and butyric acids, mixed food solids and FOG, and GIW. The control reactor was fed with 100% TWAS. The treated reactors were fed with selective substrate (28-30% COD added from substrate) mixed with TWAS (Table 5-S2). The total OLR of each reactor was 1.69 g-COD/L/day for the control reactor (TWAS) and approximately 2.03-2.41 g-COD/L/day for the treated reactors. If considering the OLR of each substrate, approximately 0.58-0.73 g-COD/L/day of substrate were loaded in each treated reactor during the cultivation period. On day 38, each mother digester was equally distributed into three 0.4L daughter digesters and continued to be cultivated at the same OLR with the same substrate type from Days 38 to 66. During the disturbance period (Days 66-86), each triplicated daughter digester system was exposed to a low GIW disturbance (63% COD added from GIW) for a total of five feeding cycles (Table 5-S2). To evaluate digester
function and stability, we continuously monitored methane yield, methane content (Figure 5-S1) throughout the experiment.

For mid- and high-GIW dose experiments, four digester systems were set up to perform mid and high GIW dose experiments simultaneously using two types of substrate at a time. For example, during cultivation period, TWAS (control, 100% TWAS) and carbohydrate (28% COD added from carbohydrate mixture) digester systems were maintained in two 0.8L mother digesters in triplicates. At least 2 feeding cycles (8 days) before the disturbance experiment was performed, mother digesters were combined and equally distributed into three 0.4L daughter digesters for mid-GIW dose and three 0.4L daughter digesters for high-GIW dose. The protein, lipid, acetic acid, mixed propionic and butyric acids, mixed food solids and FOG, and GIW digester systems were performed accordingly at similar OLRs (Table 5-S2). During disturbance period, one triplicated daughter digester system was exposed to a mid-GIW dose disturbance (77.9% COD added from GIW) and another system to a high-GIW dose disturbance (95.5% COD added from GIW) for a total of five feeding cycles (Table 5-S1 and Figure 5-S2). The amount of substrate added was chosen as a good starting loading rate ensuring enough stress intensity to develop community assembly without causing a process failure, and preparing microbial communities for higher level disturbances, according to our previous studies (Wang et al., 2013).

**Digester function.** Chemical and physical characteristics of the sludge and biogas were analyzed to evaluate digester microbiome function. Every four days before decanting and feeding the digesters, biogas production was recorded and normalized to standard temperature and pressure (STP) conditions. Methane content in biogas was assessed using a
gas chromatograph (GC, SRI 8610C) equipped with a thermal conductivity detector. Every four days effluent sludge was collected and analyzed for total solids (TS), volatile solids (VS), alkalinity, and pH according to Standard Methods (American Public Health Association (APHA), 2005). Concentrations of individual volatile fatty acids (VFA; e.g., acetic, propionic, butyric, and valeric acids) were determined using a GC (GC-2014 Shimadzu) equipped with a flame ionization detector according to Method 5560 D in Standard Methods.

**DNA extraction, 16S rRNA gene amplicon sequencing, and bioinformatics.** A total of 276 sludge samples were extracted for genomic DNA (gDNA) components using a modified aluminum sulfate DNA extraction method (Staley et al., 2011). Forward and reverse primer pair sequences, modified 341F and modified 806R, respectively, were used to amplify a DNA fragment of ∼460 bp length flanking the V3 and V4 regions of the 16S rRNA gene of bacteria and archaea in the gDNA samples (Yu et al., 2005; Sundberg et al., 2013). Library preparation, quantification, normalization, and pooling were performed according to the Illumina 16S metagenomics protocol. High Sensitivity DNA Analysis on a Bioanalyzer was performed to ensure library quantity and quality. Pooled libraries were run on an Illumina MiSeq platform for 300 bp paired-end read sequencing at the Genomic Sciences Laboratory, North Carolina State University, NC. Sequences were deposited to the National Centre for Biotechnology Information (NCBI) Sequence Read Archive with the accession number SRP077521.

Amplicon sequence pairs were merged, trimmed to remove primer sequences, and quality filtered using the QIIME pipeline (Caporaso et al., 2010) and Trimmomatic 0.33
(Bolger et al., 2014), if not otherwise specified, with default settings. Operational taxonomic units (OTU) clustering was performed on a total of 276 samples (96 and 180 samples including triplicates from low- and mid- and high-GIW dose experiments, respectively) at the ≥97% sequence similarity level using QIIME open-reference OTU picking workflows (Edgar, 2010; Rideout et al., 2014). RDP Classifier 2.2 (Wang et al., 2007) was used to assign taxonomy to each cluster representative based on the Greengenes taxonomy and reference database (McDonald et al., 2012; Werner et al., 2012). Sequences were aligned based on the Greengenes core reference alignment (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010). Chimeric sequences were identified using ChimeraSlayer (Haas et al., 2011) and removed from the alignment to build a phylogenetic tree using FastTree 2.1.3 (Price et al., 2010).

Quality filtered and chimera-free sequences of triplicate microbiomes were normalized to correct for differences in sampling efforts. Taxon abundance profiles were visualized using R ggplot package (Wickham, 2009). The compositional differences between microbiomes were analyzed by nonmetric multidimensional scaling (NMDS) statistics using R vegan package (Oksanen et al., 2015) applying the Bray-Curtis similarity index and visualized using R ggplot2 package (Wickham, 2009). Co-occurrence network analysis was conducted using the quality filtered and chimera-free sequences from the low-GIW dose experiment. Only samples from Day 62 during the cultivation period were used, including Tw-23 to 25, Cb-23 to 25, Pr-23 to 25, Li-23 to 25, Ac-23 to 25, Bp-23 to 25, FF-23 to 25, and Gw-23 to 25. Sequences from each of the eight triplicates were merged and analyzed in
QIIME using script make_otu_network.py and the co-occurrence network was constructed using Cytoscape 3.4.0 (Shannon et al., 2003).

5.5 Results

Different functional consortia in the digester common garden were developed by selective addition of substrates. We shaped a methanogenic microbiome originated from a full-scale digester into eight microbial communities using different feed sources including: (1) TWAS (control), (2) carbohydrate, (3) protein, (4) lipid, (5) acetic acid, (6) mixed propionic and butyric acids, (7) mixed FOG and food solids, and (8) entire content of GIW composed of FOG, food solids and wastewater. The dynamic community shifts and function of the anaerobic microbiome system were studied in three common garden experiments using low-, mid- and high-GIW doses. For all experiments, we used anaerobic sludge freshly collected before the initiation of each experiment from the same full scale anaerobic digester as the inoculum. During cultivation period, all digester systems were operated under the same environmental conditions but using different feed sources mixed with the same base substrate (thickened waste activated sludge, TWAS). We sequenced samples and triplicates from each experiment during different performance phases, as well as the feedstock and inocula, for a total of 276 microbiome samples. A total of 37,631,583 quality filtered and chimera-free sequences were generated and mapped to bacterial and archaeal 16S ribosomal RNA (rRNA) genes in the Greengenes database with an average of 136,346 sequences per sample. Sampling points are indicated in Figures 5-S1 and 5-S6.
**Low-GIW dose experiment.** During the cultivation period, the total OLR of each reactor was 1.69 g-COD/L/day for the control (TWAS) and approximately 2.03-2.41 g-COD/L/day for the treated digester systems (Figure 5-S1). All 7 treated digester systems were cultivated using different feed sources under the same environmental conditions and generated the same level of methane yield at approximately 100-130 mL/g-COD. During the low-GIW dose disturbance at 3.61 g-COD/L/day, 5 digesters including the controlled encountered inhibition that significantly reduced their methane production and effluent quality. Only digesters cultivated by substrates enriched with lipid, food solids/FOG, and GIW survived and achieved approximately 100% increase in methane yield (Figure 5-S1).

We analyzed microbial community dynamics during the cultivation (Days 0-66) and disturbance periods (Days 66-86) using NMDS statistics (Figure 5-1). During the cultivation period, one microbiome sample from Day 34 (e.g. microbiome Tw-22) and triplicate samples from Day 62 (e.g. Tw-23 to 25) were analyzed. During the disturbance period, triplicate samples from Day 74 (e.g. Tw-26 to 28) and Day 86 (e.g. Tw-29 to 31) were analyzed. During the cultivation period, digester systems showed dissimilarity where microbiomes enriched with lipid, food solids/FOG and GIW became more closely clustered over time from Day 33 to Day 62. Over time, protein- and TWAS-treated microbiomes gradually shifted away from acid- and carbohydrate-treated microbiomes.

During the low-GIW dose disturbance, the productive (Li, FF and Gw) and inhibited microbiomes (Tw, Cb, Pr, Ac, and Bp) diverged even more apart from each other over time. The productive (Li, FF and Gw) microbiomes consistently showed similar microbial structure and achieved higher methane yield with improved functional resilience and
resistance (Figure 5-S1). The inhibited microbiomes (Tw, Cb, Pr, Ac, and Bp) shifted further apart over time consistently as their performance profile continued to indicate the occurrence of process failure.

Figure 5-1 NMDS statistics applying Bray-Curtis similarity index on taxa abundance profiles of bacterial and archaeal 16S rRNA amplicon sequences obtained from the low-GIW dose experiment, including samples of the inoculum (ANA), digester systems enriched with TWAS (Tw), carbohydrate (Cb), protein (Pr), lipid (Li), acetic acid (Ac), butyric/propionic acids (Bp), FOG/Food solids (dewatered GIW) (FF), and grease interceptor waste (Gw). Arrows indicate the order of sampling. One microbiome sample from Day 34 (e.g. microbiome Tw-22) and triplicate samples from Day 62 (e.g. Tw-23 to 25) were analyzed during cultivation period (Days 0-66). Triplicate samples from Day 74 (e.g. Tw-26 to 28) and Day 86 (e.g. Tw-29 to 31) were analyzed during disturbance period (Days 66-86). Sampling days are listed in Figure 5-S1 in more details.
Taxon abundance profiles on Day 62 during the cultivation period (Days 0-66) are shown in Figure 5-2. Complete taxon profiles are shown in Figures 5-S2 to 5-S5. Microbiomes during cultivation were dominated by archaeal members of *Methanosaeta*, and bacterial classes Actinobacteria, Bacteroidia, Clostridia, Gammaproteobacteria, Synergistia and Cloacamonea. Specifically, dominant bacterial populations included orders Actinomycetales (Actinobacteria), Bacteroidales (Bacteroidia) and SHA-98 (Clostridia), families SB-1 (Bacteroidia) and *Thermovirgaceae* (Synergistia), genera vadinCA02 (Synergistia) and W22 (Cloacamonea). Sub-dominant bacterial populations included families *Intrasporangiaceae* (Actinobacteria) and *Porphyromonadaceae* (Bacteroidia), genera *Sporanaerobacter*, *Syntrophomonas* (Clostridia) and *Acinetobacter* (Gammaproteobacteria).

We observed feed-specific taxon distribution for acids-, protein, lipid, food solids/FOG and GIW digesters. Specifically, *Methanosaeta* were found more abundant in butyric/propionic acids-treated microbiomes, and much more abundant in acetic acid-, lipid-food solids/FOG- and GIW-treated microbiomes, compared to TWAS-, protein- and carbohydrate-treated microbiomes. Archaea from order Methanosarcinales (including genera *Methanosaeta* and *Methanosarcina*) are known aceticlastic methanogens, while some members are capable of performing other methanogenic pathways (Demirel and Scherer, 2008; Mori *et al*., 2012; Zhu *et al*., 2012; Borrel *et al*., 2013). A direct substrate enrichment of acetic acid increased the relative abundance of acetate-degrading methanogens from 4.3-4.6% to up to 10.8%, compared to the control (TWAS). A less direct substrate enrichment of butyric/propionic acids increased the relative abundance of acetate-degrading methanogens from 4.3-4.6% to up to 7.6%, compared to the control (TWAS). A much less-direct substrate
enrichment of lipid-based materials (lipid, food solids/FOG and GIW) also increased the relative abundance of acetate-degrading methanogens from 4.3-4.6% to 7.8%-12.4%, compared to the control (TWAS). Genus *Syntrophomonas* (Clostridia) showed low abundance (0.01 to 0.04%) in microbiomes enriched with TWAS-, carbohydrate-, protein-, and acetic acid-treated microbiomes. On the contrary, the relative abundance of *Syntrophomonas* was enriched in butyric/propionic acids microbiomes (1.2 to 1.6%), and much more abundantly (up to 7.3%) consistently in microbiomes treated with lipid-based substrates. In protein-treated microbiomes, members of family *Porphyromonadaceae* (Bacteroidia) were significantly more abundant (4.5 to 5.2%) across all triplicate samples, suggesting that these populations may be responsible for metabolizing protein.
Figure 5-2 Community relative abundance of taxa from triplicate microbiomes cultivated by TWAS (Tw), carbohydrate (Cb), protein (Pr), lipid (Li), acetic acid (Ac), butyric/propionic acids (Bp), FOG/Food solids (FF), and grease interceptor waste (Gw) on Day 62 during cultivation period (Days 0-66). Bacterial and archaean taxa with more than 8% relative abundance in at least one sample are listed. Taxa with less than 8% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 3% relative abundance. p: phylum, c: class, o: order, f: family, g: genus.
Figure 5-3 Community relative abundance of taxa from triplicate microbiomes cultivated by TWAS (Tw), carbohydrate (Cb), protein (Pr), lipid (Li), acetic acid (Ac), butyric/propionic acids (Bp), FOG/Food solids (FF), and grease interceptor waste (Gw) on Day 86 during cultivation period (Days 66-86). Bacterial and archaenal taxa with more than 8% relative abundance in at least one sample are listed. Taxa with less than 8% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 3% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Taxon abundance profiles on Day 86 during the disturbance period (Days 66-86) are shown in Figure 5-3. At low-GIW dose disturbance, microbiomes cultivated by lipid-based substrates (lipid, food solids/FOG, and GIW) showed enhanced community function with increased methane production and stable operation (Figure 5-S1). Compared to pre-disturbance cultivation period on Day 62, pre-dominant archaeal *Methanosaeta* increased in relative abundance from 7.8%-12.4% to up to 19.5% on Day 86 in survivor communities. Relative abundance of pre-dominant bacterial genus *Syntrophomonas* (Clostridia) also increased from 4.1%-7.3% to up to 18.9%. Significant increases of family *Porphyromonadaceae* (Bacteroidia) from <1% to up to 13% and genus *Sporanaerobacter* (Clostridia) from <0.5% to up to 6.3% were observed consistently in all survivor communities. In the inhibited communities, family *Porphyromonadaceae* increased to up to 5.9% and genus *Sporanaerobacter* to up to 33.7%.

After the disturbance, pre-dominant order Bacteroidales (Bacteroidia), family *Thermovirgaceae* (Synergistia) and genus W22 (Cloacamonae) were replaced by other consortia and became sub-dominant. A shift within order Bacteroidales (Bacteroidia) was observed where members of family *Porphyromonadaceae* displaced the pre-dominant populations that belong to the same order. Additionally, during cultivation 48.8%-63.7% of the microbial community was composed of sub-dominant populations less than 8%, however, after the disturbance the sub-dominant community was reduced to 27.3%-49.4% and members from different consortia were enriched in response to the disturbance.
**Mid- and high-GIW dose experiment.** During cultivation period, the total OLR of each reactor was 1.69 g-COD/L/day for the control (TWAS) and approximately 2.03-2.41 g-COD/L/day for the treated digester systems (Figure 5-S6). All 7 treated digester systems were cultivated using different feed sources under the same environmental conditions and generated the same level of methane yield at approximately 120-140 mL/g-COD. During the mid-GIW dose disturbance at 4.17 g-COD/L/day and high-GIW dose disturbance at 5.11 g-COD/L/day, all eight digester systems encountered process failure and showed decreases in methane content and methane yield (Figure 5-S6).

Community analysis showed similar feed-driven dissimilarity patterns as the low-GIW dose experiment during cultivation period (Figure 5-S7). During the disturbance period, all microbiomes became functionally inhibited and regardless of the GIW loading rate, communities treated with acid- and lipid-based materials shifted toward the same direction over time (i.e. A_07, B_07, L_07, F_09, and G_09).

Taxon abundance profiles during the cultivation and disturbance periods at mid- and high-GIW doses are shown in Figures 5-S8 to 5-S15. Microbiomes during cultivation were dominated by archaeal members of *Methanosaeta*, and bacterial classes Actinobacteria, Bacteroidia, Clostridia, Betaproteobacteria, and Synergistia. Genus *Syntrophomonas* (Clostridia) showed <1% relative abundance in microbiomes enriched with TWAS-, carbohydrate-, protein-, and acetic acid-treated microbiomes, 1.3%-1.9% in butyric/propionic acids microbiomes, and 2.8%-5.5% in microbiomes treated with lipid-based substrates. Similar to low-GIW disturbance, both the mid- and high-GIW overloading stresses let to a dynamic shift of dominant populations from classes Bacteroidia to Clostridia consistently
across all inhibited microbiomes regardless of the intensity of overloading stress. Specifically, genus *Sporanaerobacter* and family *Ruminococcaceae* increased in relative abundance from <1% to up to 30% and 6%, respectively. Genus *Caloramator* also showed increased relative abundance from <15 to up to 12% in microbiomes treated with carbohydrate, butyric/propionic acids, food solids/FOG and GIW after the overloading disturbance.

**Co-occurrence network analysis.** To better understand the inter-species network of core digester populations, we statistically analyzed and constructed a co-occurrence network based on the taxonomic community profiles of microbiomes enriched using different substrates during the cultivation period (Figure 5-4). Distribution of sample-nodes revealed feed-specific community dissimilarity between microbiomes cultivated by protein, carbohydrate and lipid- and acids-based materials. Microbiomes cultivated by lipid-based substrates (i.e. lipid, food solids/FOG and GIW) clustered closely and shared multiple taxa that were exclusive to this community type. Similar patterns were also observed for other substrates. We identified four major clusters of consortia that were specific to each substrate source. Archaeal family *Methanospirillaceae*, bacterial orders Bacteroidales (Bacteroidia) and Rhizobiales (Alphaproteobacteria), families *Saprospiraceae* (Bacteroidia) and *Methylocystaceae* (Alphaproteobacteria), and genera *Paludibacter* (Bacteroidia), *Sporanaerobacter* and *Syntrophomonas* (Clostridia), *Comamonas* (Betaproteobacteria), *Syntrophus* (Deltaproteobacteria), and *Methylocaldum* (Gammaproteobacteria) were found to be the core taxa that were uniquely in microbiomes cultivated by lipid-based substrates. Acetic acid and butyric/propionic acids-treated microbiomes shared more similar
membership with the control (TWAS) compared to others. Protein consortia showed the most distinct network and mainly harboured families *Porphyromonadaceae* (Bacteroidia), genera *Enterococcus* (Bacilli), *Sedimentibacter* (Clostridia) and *Tissierella* (Clostridia). Our results demonstrated that feed variability directed the community dynamics and similar feeds led to similar community niche profiles.

Two highly inter-connected clusters were also identified, revealing clusters of co-occurring microbes shared between different communities, despite difference in substrate used during cultivation. Complete lists of populations identified through co-occurrence analyses are shown in Table 5-S3 to 5-S5.
Figure 5-4 Co-occurrence network of digester microbiomes on Day 62 during cultivation period visualized using clusters of “nodes” (OTU-nodes and sample-nodes) connected by “edges” (lines connecting shared OTUs between nodes). Closer clustering implies more shared OTUs (i.e. more shared taxa) are found between two samples. The relationship between nodes and edges is weighted according to the number of sequences within an OTU based on the microbial abundance profile. Larger nodes represent more connections; vice versa.
5.6 Discussion

In this study we performed a series of common garden experiments to investigate the causality between microbial population dynamics and whole-community function (methane production, effluent quality, ecological resilience and resistance) to GIW overloading stresses. During the cultivation period, all digester microbiomes were given identical inoculum, operated under the same environment (digester operation, substrate loading rate, feeding patterns) and showed similar community function (methane production, effluent quality). However, under low-GIW stress, all microbiomes previously cultivated by non-lipid-based materials encountered process failure and inhibition of methanogenesis. On the contrary, microbiomes pre-disturbed by lipid-based materials, including lipid (new rice bran oil), food solids/FOG, and GIW, showed significant enhancement of methane production and community robustness to the same loading stress (Figure 5-S1).

Community analysis showed that different substrates let to different community structure and after the disturbance, survivor and inhibited communities further diverged and showed greater community dissimilarity (Figure 5-1). Taxon profiling also showed a clear difference between survivor and inhibited communities before the disturbance. Specifically, aceticlastic *Methanoseta* and syntrophic fatty acid-degrading *Syntrophomonas* were found much more abundant in the survivor microbiomes. However, compared to other inhibited communities in which *Methanoseta* showed only 3.6% to 6.2% relative abundance, microbiomes cultivated by acetic acid and butyric/propionic acids also possessed *Methanoseta* at similar abundance level (6.5% to 10.8) as the lipid-cultivated microbiomes (7.8% to 12.4%), but could not survive the same GIW loading rate (Figure 5-2). This observation suggested that
Syntrophomonas, rather than Methanosaeta, possibly played more important roles in reducing GIW toxicity and enhancing community resilience and resistance. Both Methanosaeta and Syntrophomonas were demonstrated to be “necessary”, but “sufficient” members of Syntrophomonas at >4% relative abundance was proved to be the key to reduce GIW inhibitory effects, particularly from the accumulation of LCFAs and other intermediates such as propionate and butyrate, at the low-GIW dose.

The results of co-occurrence analysis indicated that other microbial populations may also play important roles in increasing community resilience and resistance (Figure 5-4). Microbiomes cultivated by different substrates shared different taxa and four major clusters of consortia that were specific to different substrate sources were identified. Microbiomes cultivated by lipid-based substrates shared many taxa that were exclusive to this community type, including family Methanospirillaceae, bacterial orders Bacteroidales (Bacteroidia) and Rhizobiales (Alphaproteobacteria), families Saprospiraceae (Bacteroidia) and Methylocystaceae (Alphaproteobacteria), and genera Paludibacter (Bacteroidia), Sporanaerobacter and Syntrophomonas (Clostridia), Comamonas (Betaproteobacteria), Syntrophus (Deltaproteobacteria), and Methylocaldum (Gammaproteobacteria). While Methanosaeta and Syntrophomonas were the major contributors to community survivability, these taxa were found to be the “supportive” taxa that were uniquely possessed by the survivor microbiomes.
Under both mid- and high-GIW dose stresses, all eight digester systems were inhibited and showed significant decreases in methane content and methane yield (Figure 5-S6). Our community analysis showed that the specialized microbial populations developed by cultivation at 0.20 to 0.27 g-VS/L/day ensured stable operation at low-GIW loading rate (1.01 g-VS/L/day) but was not sufficient to reduce the GIW toxicity at mid- (1.50 g-VS/L/day) and high-GIW (2.42 g-VS/L/day) loading rates. The cultivation at 0.20 to 0.27 g-VS/L/day only allowed a “jump” of 0.7-0.8 g-VS/L/day OLR to 1.01 g-VS/L/day. Similarly, a cultivation at 0.73 g-VS/L/day allowed a “jump” of 0.7-0.8 g-VS/L/day OLR to 1.43 g-VS/L/day (Wang et al., 2013). Gradual addition of substrate allowed development of microbial adaptation when the community shifts towards the better adapted microorganisms (Zeeman et al., 1985; Chen et al., 2008; Palatsi et al., 2010; Kougiás et al., 2016) and reduction of substrate inhibition (Cavaleiro et al., 2009; Silvestre et al., 2011; Musat et al., 2012; Kougiás et al., 2016; Ziels et al., 2017). However, our results indicated that even though the beneficial community shifts occurred after the cultivation period, these community shifts only allowed a “jump” of 0.7-0.8 g-VS/L/day OLR for one step feeding stage. To achieve GIW co-digestion at higher OLRs, more step feeds are necessary to induce sufficient level of microbial adaptation, or more “necessary” taxa such as *Methanosaeta* and *Syntrophomonas* and more specialized “supportive” populations such as those identified by the network analysis across anaerobic metabolic pathways.

After the low-GIW dose disturbance, in the survivor communities some pre-dominant populations, including members of the order Bacteroidales (Bacteroidia), family *Thermovirgaceae* (Synergistia) and genus W22 (Cloacamonae), were replaced by other
consortia and became sub-dominant, whereas other pre-dominant communities, including *Methanosaeta* and *Syntrophomonas*, continued to thrive over time (Figures 5-3, and 5-S2 to 5-S5). We also observed significant increases in previously-sub-dominant family *Porphyromonadaceae* (Bacteroidia) and genus *Sporanaerobacter* (Clostridia) consistently in both survivor and inhibited communities. For the mid- and high-GIW dose experiments, significant increases in genus *Sporanaerobacter* and family *Ruminococcaceae* were observed in all inhibited communities regardless of the intensity of overloading stress (Figures 5-S7 to 5-S15). Our results identified a substantial shift of dominant microbial communities across many consortia after the disturbance. Niche differentiation clearly played an important role in selecting the key populations to reduce GIW overloading stress and allow other better-adapted communities to thrive.

Comparing the community susceptibility of (1) non-cultivated TWAS-community (control), (2) carbohydrate, protein, and acids-cultivated communities, and (3) lipids-cultivated communities, our results demonstrated that substrate history in terms of feed selection (right feed) and the intensity of preshocks (right dose) directed the community niche profiles and in turn, determined community survivability to GIW stresses. Based on ecological concepts of a common garden experiment and the results of community profiling and co-occurrence network analyses, our results directly identified key microbial populations whose contribution reduced GIW inhibition. This study represents the first demonstration that substrate selection and overloading stresses can lead to feed-specific community adaptation mechanisms and influence the anaerobic microbiome to improve community resilience and resistance to GIW inhibition.
5.7 Conclusions

A long-standing question in bioreactor engineering is what ecological patterns and processes determine the performance and functional stability of methanogenic biosystems and how this complex microbial network can be improved. This study represents the first investigation of the causal relationships between microbial community, function, and environment for GIW co-digestion in triplicate anaerobic microbiomes. We established a common garden composed of eight types of digester microbiomes that had specific metabolic preferences and directly tested the effect of microbial community structure on community function in controlled environments. Microbial community dynamics due to substrate history (feed selection and intensity of preshocks) played significant roles in achieving better resilience and resistance under different GIW overloading stresses. Our results demonstrated that these microbial dynamics reflected the force of environmental selection and can be actively directed by engineering approaches to improve a specific function.
### 5.8 Supporting information

**Table 5-S1** Substrate characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Anaerobic sludge (inoculum)</th>
<th>TWAS (base substrate)</th>
<th>Lipid (rice bran oil)</th>
<th>GIW Wastewater FP and FOG layer</th>
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</thead>
<tbody>
<tr>
<td>TS (g/kg-wet sample)</td>
<td>25.9 ± 0.05</td>
<td>24.4 ± 1.45</td>
<td>1005 ± 5.97</td>
<td>2.70 ± 0.10 352 ± 7.14</td>
</tr>
<tr>
<td>VS (g/kg-wet sample)</td>
<td>16.0 ± 0.05</td>
<td>17.0 ± 1.22</td>
<td>1004 ± 6.03</td>
<td>2.39 ± 0.05 349 ± 6.33</td>
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<tr>
<td>VS/TS (%)</td>
<td>61.8</td>
<td>69.8</td>
<td>100</td>
<td>88.6 99.3</td>
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<tr>
<td>COD (g/L)</td>
<td>28.7 ± 3.20</td>
<td>33.8 ± 5.81</td>
<td>3032 ± 565</td>
<td>5.65 ± 0.67 744 ± 229</td>
</tr>
</tbody>
</table>

*a* Total solid concentration  
*b* Volatile solid concentration  
*c* Chemical oxygen demand concentration

**Table 5-S2** Substrate composition, total organic loading rate (OLR) and OLR of substrate during cultivation (A) and disturbance periods (B).

<table>
<thead>
<tr>
<th>Reactor microbiome</th>
<th>Substrate concentration</th>
<th>Total OLR</th>
<th>OLR of substrate</th>
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<tr>
<td></td>
<td>%vol</td>
<td>%VS</td>
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<td>100</td>
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<tr>
<td>Protein</td>
<td>-</td>
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<tr>
<td>Lipid</td>
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<td>19.3</td>
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<td>Acetic acid</td>
<td>1.2</td>
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<tr>
<td>Propionic and butyric acids</td>
<td>4.1</td>
<td>-</td>
<td>28.2</td>
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<tr>
<td>Food particles and FOG</td>
<td>1.8</td>
<td>24.1</td>
<td>28.2</td>
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<tr>
<td>GIW</td>
<td>15</td>
<td>24.9</td>
<td>29.3</td>
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</tbody>
</table>

(A) Cultivation period

- Low GIW dose  
- Mid GIW dose  
- High GIW dose

(B) Disturbance period

- Low GIW dose  
- Mid GIW dose  
- High GIW dose
Table 5-S3 Co-occurring taxa within each consortium. Highlight indicates taxa that were more abundantly found in each group.

<table>
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<tr>
<th>Li FF Gw consortia</th>
<th>Archaea</th>
<th>Euryarchaeota</th>
<th>Methanomicrobia</th>
<th>Methanomicrobiales</th>
<th>Methanospirillaceae</th>
<th>Euryarchaeota</th>
<th>Methanomicrobia</th>
<th>Methanomicrobiales</th>
<th>Methanospirillaceae</th>
<th>Gastrospira</th>
<th>Closterium</th>
<th>Bacteroidia</th>
<th>Bacteroidales</th>
<th>Porphyromonadaceae</th>
<th>Paludibacter</th>
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<td>Bacteroidia</td>
<td>Bacteroidales</td>
<td>Porphyromonadaceae</td>
<td>Paludibacter</td>
</tr>
<tr>
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<td>Euryarchaeota</td>
<td>Methanomicrobia</td>
<td>Methanomicrobiales</td>
<td>Methanospiroplasma</td>
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Table 5-S4 Shared taxa between all consortia. Highlight indicates taxa that were more abundantly shared.

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</table>
Table 5-S5 Shared taxa between all consortia excluding protein consortia. Highlight indicates taxa that were more abundantly shared.

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<th>Phylum</th>
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**Figure 5-S1** Total organic loading rate (OLR), DNA sampling points, methane content and methane yield during cultivation (Day 0-66) and disturbance periods (Day 66-86) in low-GIW dose experiment. During cultivation period, one microbiome sample from Day 34 (e.g. microbiome Tw-22) and triplicate samples from Day 62 (e.g. Tw-23 to 25) were analyzed. During disturbance period, triplicate samples from Day 74 (e.g. Tw-26 to 28) and Day 86 (e.g. Tw-29 to 31) were analyzed.
Figure 5-S2 Community relative abundance of taxa from microbiomes cultivated by TWAS (Tw) and carbohydrate (Cb) during cultivation (Days 34 and 62) and disturbance periods (Days 74 and 86). During cultivation period, one microbiome sample from Day 34 (e.g. microbiome Tw-22) and triplicate samples from Day 62 (e.g. Tw-23 to 25) were analyzed. During disturbance period, triplicate samples from Day 74 (e.g. Tw-26 to 28) and Day 86 (e.g. Tw-29 to 31) were analyzed. Bacterial and archaeal taxa with more than 8% relative abundance in at least one sample are listed. Taxa with less than 8% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 10% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Figure 5-S3 Community relative abundance of taxa from microbiomes cultivated by protein (Pr) and lipid (Li) during cultivation (Days 34 and 62) and disturbance periods (Days 74 and 86). During cultivation period, one microbiome sample from Day 34 (e.g. microbiome Tw-22) and triplicate samples from Day 62 (e.g. Tw-23 to 25) were analyzed. During disturbance period, triplicate samples from Day 74 (e.g. Tw-26 to 28) and Day 86 (e.g. Tw-29 to 31) were analyzed. Bacterial and archaeal taxa with more than 8% relative abundance in at least one sample are listed. Taxa with less than 8% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 10% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Figure 5-S4 Community relative abundance of taxa from microbiomes cultivated by acetic acid (Ac) and butyric/propionic acids (Bp) during cultivation (Days 34 and 62) and disturbance periods (Days 74 and 86). During cultivation period, one microbiome sample from Day 34 (e.g. microbiome Tw-22) and triplicate samples from Day 62 (e.g. Tw-23 to 25) were analyzed. During disturbance period, triplicate samples from Day 74 (e.g. Tw-26 to 28) and Day 86 (e.g. Tw-29 to 31) were analyzed. Bacterial and archaeal taxa with more than 8% relative abundance in at least one sample are listed. Taxa with less than 8% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 10% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Figure 5-S5 Community relative abundance of taxa from microbiomes cultivated by food solids/FOG (FF) and GIW (Gw) during cultivation (Days 34 and 62) and disturbance periods (Days 74 and 86). During cultivation period, one microbiome sample from Day 34 (e.g. microbiome Tw-22) and triplicate samples from Day 62 (e.g. Tw-23 to 25) were analyzed. During disturbance period, triplicate samples from Day 74 (e.g. Tw-26 to 28) and Day 86 (e.g. Tw-29 to 31) were analyzed. Bacterial and archaeal taxa with more than 8% relative abundance in at least one sample are listed. Taxa with less than 8% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 10% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Figure 5-S6 Total organic loading rate (OLR), DNA sampling points, methane content and methane yield during cultivation and disturbance periods in mid- and high-GIW dose experiments. During cultivation period, we analyzed triplicate microbiome samples from the first sampling point and triplicate samples from digesters treated with mid- and high-GIW doses from the second sampling point. During disturbance period, triplicate samples from digesters treated with mid- and high-GIW doses were analyzed for each sampling point. Non-highlighted samples are from mid-GIW dose experiment. Highlighted samples are from high-GIW dose experiment.
Figure 5-S7 NMDS statistics applying Bray-Curtis similarity index on taxa abundance profiles of bacterial and archaeal 16S rRNA amplicon sequences obtained from the mid- and high-GIW dose experiments, including samples of the digester systems enriched with TWAS (T), carbohydrate (C), protein (P), lipid (L), acetic acid (A), butyric/propionic acids (B), FOG/Food solids (F), and grease interceptor waste (G). Arrows indicate the order of sampling. Triplicate samples from each sampling day were merged and normalized. Sampling days are listed in Figure 5-S6 in more details.
Figure 5-S8 Community relative abundance of taxa from microbiomes cultivated by TWAS (Tw) during cultivation and disturbance periods. Bacterial and archaeal taxa with more than 10% relative abundance in at least one sample are listed. Taxa with less than 10% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 3% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
**Figure 5-S9** Community relative abundance of taxa from microbiomes cultivated by carbohydrate (Cb) during cultivation and disturbance periods. Bacterial and archaeal taxa with more than 10% relative abundance in at least one sample are listed. Taxa with less than 10% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 3% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Figure S10 Community relative abundance of taxa from microbiomes cultivated by protein (Pr) during cultivation and disturbance periods. Bacterial and archaeal taxa with more than 10% relative abundance in at least one sample are listed. Taxa with less than 10% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 3% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Figure 5-S11 Community relative abundance of taxa from microbiomes cultivated by lipid (Li) during cultivation and disturbance periods. Bacterial and archaeal taxa with more than 10% relative abundance in at least one sample are listed. Taxa with less than 10% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 3% relative abundance. p_ : phylum, c_ : class, o_ : order, f_ : family, g_ : genus.
Figure 5-S12 Community relative abundance of taxa from microbiomes cultivated by acetic acid (Ac) during cultivation and disturbance periods. Bacterial and archaeal taxa with more than 10% relative abundance in at least one sample are listed. Taxa with less than 10% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 3% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Figure 5-S13 Community relative abundance of taxa from microbiomes cultivated by butyric/propionic acids (Bp) during cultivation and disturbance periods. Bacterial and archaeal taxa with more than 10% relative abundance in at least one sample are listed. Taxa with less than 10% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 3% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Figure 5-S14 Community relative abundance of taxa from microbiomes cultivated by food solids/FOG (FF) during cultivation and disturbance periods. Bacterial and archaeal taxa with more than 10% relative abundance in at least one sample are listed. Taxa with less than 10% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 3% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Figure S15 Community relative abundance of taxa from microbiomes cultivated by GIW (Gw) during cultivation and disturbance periods. Bacterial and archaeal taxa with more than 10% relative abundance in at least one sample are listed. Taxa with less than 10% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 3% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Chapter 6 Step and pulse feedings increase community resistance in anaerobic digesters treating grease interceptor waste

6.1 Abstract

Improving anaerobic co-digestion of thickened waste activated sludge with grease interceptor waste (GIW) was studied by operating lab scale semi-continuous digesters with a solids retention time of 20 days at mesophilic conditions (37 °C). The first experiment extended previous research on the use of step feeding to develop microbial adaptation and enhance recovery from overload of GIW. The second experiment applied the ecological concepts of resistance and resilience to determine the possibility of creating more robust digesters against GIW inhibition using periodic pulse feeds of GIW. Step feeds of GIW resulted in better microbial adaptation compared to the control, and reduced the inhibitory effect of GIW. Gradual addition of GIW, up to 75% (w/w) of volatile solids (VS) added, increased methane yield by 336% from 0.180 to 0.785 L-methane/g-VS added, the highest value reported to date for co-digestion of GIW. Upon overloading, both GIW-adapted and non-GIW-adapted digesters were able to recover from process failure, although GIW-adapted digesters did not achieve the same level of methane production and encountered more fluctuations compared to the results prior to overloading at the same GIW loading rate. Pulse feeds of GIW increased methane yield from 0.134 to 0.748 L-methane/g-VS added at 70% (w/w as VS) GIW and increased digester resistance against GIW inhibition.
6.2 Introduction

Anaerobic co-digestion of biosolids with grease interceptor waste (GIW) enhances biogas production that can be used to achieve energy independence and sustainability at wastewater treatment facilities (WWTFs). GIW is one of the most abundant waste-based organic substrates in the U.S. with high methane potential (Austic, 2010; Dayton, 2010; Long et al., 2012; Skaggs et al., 2018), and typically comprises three major components: fat, oil, and grease (FOG), food particles, and associated wastewater generated from food service establishments (FSEs) (Chapin, 2008; Aziz et al., 2011; Gallimore et al., 2011; Long et al., 2012). Similar FOG-based wastes include: animal fats and waste oils from food or processing plants, the edible oil industry, the dairy products industry, and slaughterhouses; mixed FOG wastes from receiving or dewatering facilities, and trapped grease wastes from WWTFs (EPA, 2004; Appels et al., 2011; Long et al., 2012). Co-processing FOG-rich materials with sewage sludge increased methane production by 9% to 317%, depending on the source of startup substrate and co-substrate, total organic loading rate (OLR), OLR of co-substrate, solid retention time (SRT), mixing intensity, and feeding strategy and frequency (Davidsson et al., 2008; Kabouris et al., 2009; Luostarinen et al., 2009; Wan et al., 2011; Wang et al., 2013). Successful applications of anaerobic co-digestion with FOG wastes have been reported worldwide in lab scale (Kabouris et al., 2009; Luostarinen et al., 2009; Wan et al., 2011; Wang et al., 2013; Ziels et al., 2016), pilot scale (Davidsson et al., 2008), and full scale (Bailey, 2007; Gabel et al., 2009; York and Magner, 2009; Cesca et al., 2010; Downey, 2010; Muller et al., 2010; Johnson et al., 2011).
Anaerobic digestion includes hydrolysis, acidogenesis, acetogenesis, and methanogenesis in either a mesophilic or a thermophilic environment. This complex metabolism involves many microbial groups and close syntrophic cooperation between acetogenic bacteria and methanogenic archaea (McCarty, 1964; Parkin and Owen, 1986; Speece, 1996; Stuckey et al., 2011). When introducing FOG-rich wastes that are high in biodegradable volatile solid (VS), chemical oxygen demand (COD), and lipid concentrations, the challenge is to avoid overloading and inhibition of methanogenesis. Efficient degradation of major intermediates such as long chain fatty acids (LCFAs), acetate, propionate, and butyrate is crucial to prevent drops in pH, imbalance of the major metabolic steps, and consequently, process failure. The inhibition and toxicity of LCFAs to microorganisms have been documented in many studies conducted with synthetic LCFAs (Hanaki et al., 1981; Koster and Cramer, 1987; Angelidaki and Ahring, 1995; Hwu et al., 1998; Palatsi et al., 2010; Rinzema et al., 2013). Inhibitory effects of LCFAs including substrate and product transport limitation, damage to cell membrane, increased lag phase of methane production, methanogenic activity loss, sludge flotation and washout (Hanaki et al., 1981; Koster and Cramer, 1987; Angelidaki and Ahring, 1995; Hwu et al., 1998; Palatsi et al., 2010; Rinzema et al., 2013), and reviewed (Chen et al., 2008; Alves et al., 2009; Long et al., 2012).

Challenging the microbial community with GIW to enhance methane production increases the risk of co-substrate overdose and LCFA inhibition, but also creates an opportunity for microbial adaptation of methanogenic archaeal and LCFA-degrading bacterial communities. Exposures to inhibitors such as ammonia, sulfide, sodium, insoluble organic compounds, and LCFAs at various concentrations have been reported to lead to
adaptation of microorganisms to various degrees, as summarized in Chen et al. (2008). Biomass adaptation and digester recovery from LCFA inhibition were observed in microbial communities treated with synthetic LCFAs (Alves et al., 2001; Cavaleiro et al., 2001, 2008, 2009, Palatsi et al., 2009, 2010), and real FOG-based wastes (Nadais et al., 2006; Silvestre et al., 2011; Wang et al., 2013; Ziels et al., 2016). The adaptation may be the result of population adaptation when the microbial population shifts towards the better adapted microorganisms or due to phenotypic adaptation (physiological acclimation) when internal changes of existing microorganisms occur (Zeeman et al., 1985; Chen et al., 2008; Palatsi et al., 2010; Kougias et al., 2016). Although the exact mechanism is still not clear, it is believed that adaptation of microorganisms may decrease the inhibitory effect of toxicity shock and increase the biodegradability of undesired substrates (Wu et al., 1993; Chen et al., 2008; Silvestre et al., 2011; Stuckey et al., 2011).

In our recent study we challenged an anaerobic digester with step feedings of GIW from 46%, 66%, to 84% (w/w as VS) in an attempt to induce microbial adaptation and evaluate the limit of anaerobic co-digestion of GIW (Wang et al., 2013). However, unlike lab-scale experiments where GIW loading rate can be customized, full-scale WWTFs can encounter fluctuations in GIW loading because of availability, seasonal changes in characteristics and collection areas, and introduction of new sources. These factors may make a step feeding approach, which relies on a stable GIW loading that increases in discrete steps, difficult. Several researchers have used pulse feeds (short periods of overloading) of synthetic LCFAs and other waste-based materials to evaluate LCFA inhibition (Cavaleiro et al., 2001, 2008, 2009; Neves et al., 2009; Palatsi et al., 2009, 2010). These studies demonstrate the possibility
of using a pulse feeding strategy to induce tolerance and adaptation in methanogenic archaeal and syntrophic acidogenic populations. Pulse feeds of GIW may be a feasible feeding strategy instead of step feeding to better simulate the fluctuating strengths in feedstock and to evaluate the ability of digesters to accept GIW at different concentrations.

Two sets of experiments were performed in this study. In experiment I, we continued our previous study (Wang et al., 2013) to further evaluate (i) if step feeding can create more robust microbial communities against GIW inhibition by inducing microbial adaptation; (ii) if both GIW-adapted and non-GIW-adapted communities can recover from a onetime loading shock and if so (iii) if they can achieve the same level of methane production or even tolerate a higher GIW loading rate. Additionally, in experiment II we sought to evaluate (i) if challenging the anaerobic co-digester with periodic pulse feeds can result in more robust microbial communities, (ii) if pulse feeding can enhance methane production, and (iii) the use of ecological parameters such as resistance (magnitude of change in accumulated concentration of major intermediates) and resilience (time taken by the accumulated intermediates to return to its referential state) (Botton et al., 2006; Werner et al., 2011) as measures of digester robustness.
6.3 Materials and methods

**Substrates and inoculum.** Anaerobic sludge (12 L) from South Durham Water Reclamation Facility in North Carolina was used as inoculum; thickened waste activated sludge (TWAS) from North Cary Water Reclamation Facility in North Carolina was used as base substrate, and GIW from two separate restaurants (food service establishments, FSEs) in Cary, North Carolina was used as co-substrate. The three primary components of GIW: FOG, food particles, and wastewater, were collected separately at each FSE and stored at 4°C immediately after collection.

**Experimental setup and design.** Two (8 L) lab-scale anaerobic reactor systems (one control and one treated) were operated at mesophilic conditions (37°C) and fed every other day with a SRT of 20 days. For a detailed reactor description, see Wang et al., 2013.

In experiment I, we continued our previous experiment by challenging both the control and treated digesters at 66 and 75% (w/w as VS) (Figure 1-2a). In experiment II, six research phases were designed to evaluate the feasibility of applying repeated pulses of GIW to anaerobic digesters to develop more robust communities and produce higher methane production (Figure 6-3a and Table 6-3). During the start-up periods, both reactors were fed only TWAS at an OLR of 1.33 g-VS/L/day in phase 1, and a mixture of TWAS (70% (w/w) as VS) and GIW (30% (w/w) as VS) at an OLR of 1.64 g-VS/L/day in phase 2. In phase 3, four pulse-fed cycles were applied to the treated digester at an OLR of 2.24 g-VS/L/day which corresponds to a GIW input of 60% (w/w as VS) in addition to regular feeding at 1.64 g-VS/L/day (30% (w/w) as VS) (Figure 6-3a). During phases 4 to 6, to evaluate whether more robust microbial populations can be enhanced by pulse treatment, both digesters were
challenged at higher GIW loads of 70 and 90% (w/w) as VS at 2.95 and 4.48 g-VS/L/day, respectively (Figure 6-3a).

**Analytical methods.** Biogas production was measured with a wet tip gas meter (Wet Tip Gas Meter.com, Nashville, TN) and normalized to STP based on the daily local climatological data (NCDC, 2012; NCDC, 2013). Methane content in biogas was analyzed by a gas chromatograph (GC, SRI 8610C) equipped with a thermal conductivity detector. Total solids (TS), VS, alkalinity, and pH were measured according to Standard Methods (APHA, 2005). Concentrations of individual volatile fatty acids (VFA; e.g., acetic, propionic, butyric, and valeric acids) were determined by acidification, centrifugation, filtration, and direct injection into a GC (GC-2014 Shimadzu) equipped with a flame ionization detector according to the VFA gas chromatographic method (5560 D) in Standard Methods (APHA, 2005). Total VFA (TVA; VFAs up to six carbon atoms) concentration was measured by centrifugation, acidification, distillation, and titration according to the TVA distillation method (5560 C) in Standard Methods (APHA, 2005).
6.4 Results

Substrate characterization. TWAS and co-substrate (GIW that contains FOG, food particles, and wastewater mixed in ratios of 10%, 40%, and 50% by volume, respectively) were mixed thoroughly into feedstock and subdivided into desired portions before use (see Wang et al., 2013 for characterization of raw substrates and prepared feedstock used in experiment I). The characterization of raw TWAS, GIW, and individual GIW components used in experiment II is presented in Table 6-1; that of the prepared mixtures of TWAS and GIW is shown in Table 6-2.

Table 6-1 Characterization of raw substrates and co-substrates used in experiment II.

<table>
<thead>
<tr>
<th></th>
<th>TWAS</th>
<th>Wastewater</th>
<th>Food particles</th>
<th>FOG</th>
<th>GIW&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS (g kg&lt;sub&gt;wet sample&lt;/sub&gt;&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>34.02</td>
<td>0.99</td>
<td>78.07</td>
<td>625.5</td>
<td>96.14</td>
</tr>
<tr>
<td>VS (g kg&lt;sub&gt;wet sample&lt;/sub&gt;&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<td>0.75</td>
<td>75.94</td>
<td>623.3</td>
<td>95.07</td>
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<tr>
<td>VS/TS(%)</td>
<td>78.0</td>
<td>75.8</td>
<td>97.3</td>
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<td>5.2</td>
<td>4.2</td>
<td>7.1</td>
<td>4.5</td>
</tr>
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</table>

<sup>a</sup> GIW composition: FOG (10 vol%), food particles (40 vol%), and wastewater (50 vol%).
Table 6-2 Characterization of prepared feedstock used in experiment II.

<table>
<thead>
<tr>
<th>Composition (% w/w as VS)</th>
<th>TWAS</th>
<th>GIW</th>
<th>Start up</th>
<th>Pulse feed training</th>
<th>Perturbation test</th>
<th>Recovery test</th>
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</thead>
<tbody>
<tr>
<td>Phase 1</td>
<td>Phase 2</td>
<td>Normal feeds</td>
<td>Pulse feeds</td>
<td>Phase 4</td>
<td>Phase 5</td>
<td>Phase 6</td>
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<tr>
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<td>70</td>
<td>70</td>
<td>40</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>GIW</td>
<td>0</td>
<td>30</td>
<td>30</td>
<td>60</td>
<td>70</td>
<td>90</td>
</tr>
<tr>
<td>TS (g kg\text{wet sample}^{-1})</td>
<td>34.02</td>
<td>39.57</td>
<td>39.57</td>
<td>50.30</td>
<td>64.23</td>
<td>92.59</td>
</tr>
<tr>
<td>VS (g kg\text{wet sample}^{-1})</td>
<td>26.53</td>
<td>32.76</td>
<td>32.76</td>
<td>44.87</td>
<td>58.93</td>
<td>89.54</td>
</tr>
<tr>
<td>VS/TS(%)</td>
<td>78.0</td>
<td>82.8</td>
<td>82.8</td>
<td>89.2</td>
<td>91.7</td>
<td>96.7</td>
</tr>
</tbody>
</table>
Experiment I: step feeding. Experiment I was initiated following our previous study (Wang et al., 2013) to confirm the possibility of inducing microbial adaptation and enhancing recovery of inhibited digesters by step feeding of GIW.

The OLR, biogas production, methane yield, and methane content over time in previous (Figure 6-1, left column) and current (Figure 6-2, right column) experiments are shown in Figure 1. Previously the treated digester was challenged by step feeds of 0%, 46%, 66%, to 84% GIW (w/w of VS added) (Figure 6-1) to identify the maximum methane production and determine the limit of anaerobic co-digestion of TWAS with GIW (Wang et al., 2013). The highest GIW loading rate achieved without digester failure was 66% (w/w of VS added) at an OLR of 2.16 g-VS/L/day, increasing methane production from 0.180 to 0.752 L-methane/g-VS added during phase 4 (Figure 6-1) (Table 6-3). The treated digester was overloaded during phase 5 as the GIW loading rate was increased to 84% (w/w of VS added). Inhibition of fatty acid oxidation and methanogenesis was identified by the decreases in effluent pH, alkalinity, and COD and VS reduction rates and increases in TVA concentration (Wang et al., 2013). Following the digester failure, the treated digester was subjected to 100% TWAS to allow recovery in phase 5. Stable biogas production and methane content and decreases in effluent pH, alkalinity, and VFA concentrations back to the desired ranges were viewed as indicators of stable anaerobic processes and digester recovery, although the microbial composition might have changed in response to the GIW overloading.

During experiment I (Figure 6-1), we spiked both the control and treated digesters with 66% (w/w as VS) GIW at an OLR of 2.16 g-VS/L/day to evaluate if microbial communities previously overdosed at 84% (w/w) GIW in the treated digester can still produce the same
amount of methane upon recovery. The spike also tested if microbial communities that have never experienced GIW feeds in the control digester since phase 1 would respond differently from those in the treated digester when facing a higher GIW loading rate. We observed a severe digester upset shortly after we introduced 66% (w/w as VS) GIW into the control digester. Immediate decreases in methane yield, methane content, biogas production (black symbols in Figure 6-1 during perturbation test), VS and COD reduction rates, pH, and alkalinity (black symbols in Figure 6-2 during perturbation test) and increases in effluent VS, COD, and TVA concentrations, and TVA/alkalinity ratio (black symbols in Figure 2 during perturbation test) indicate buildup of undigested substrates and inhibition of methanogenesis in the control digester. On day 258 the 66% (w/w) GIW feed was discontinued and replaced by 100% TWAS to allow the inhibited control digester to recover. As the control digester recovered, the methane yield gradually reached 0.599 L-methane/g-VS added on day 279 (Figure 6-1). This increase in methane yield was due to the consumption of accumulated substrates that possibly were undigested FOG in GIW. It required approximately three SRT (60 days) for the inhibited control digester to achieve stable biogas production and methane content. The step-treated digester (digester B) was overloaded at 84% (w/w) GIW during phase 5 and recovered at 100% TWAS (white symbols in Figure 6-1). When challenged by 66% (w/w) GIW during the perturbation test, the step-treated digester stabilized within 10 days, producing biogas at 12.6 L day-1, methane yield at 0.665 L-methane/g-VS added, and methane content at 69.6% (Figure 6-1 and Table 6-4).

During the recovery test (Figure 6-1), we examined if microbial populations in the treated digester can tolerate higher GIW addition upon recovery from a process failure at
84% (w/w) GIW followed by a step treatment at 66% (w/w) GIW and if the control digester overloaded at 66% (w/w) GIW can survive the same GIW feed this time upon recovery. The treated digester was challenged with 75% (w/w) on day 319, increasing methane yield from 0.665 to 0.785 L-methane/g-VS added and biogas production from 12.6 to 21.6 L day-1 (Figure 6-1 and Table 6-4). The control digester was again spiked with 66% (w/w) GIW which increased methane yield from 0.197 to 0.740 L-methane/g-VS added and biogas production from 2.3 to 16.5 L day-1. The control digester was further pushed to 75% (w/w) GIW, producing methane yield at 0.783 L-methane/g-VS added and biogas at 21.4 L day-1 (Figure 6-1 and Table 6-4).
Figure 6-1 Organic loading rate (OLR) and biogas characteristics over time in previous (Wang et al., 2013) and current experiments (experiment I). (a) OLR: black line is for control digester and grey line is for treated digester; (b) methane yield; (c) biogas production; (d) methane content.
Table 6-3 GIW content, total organic loading rate (OLR), OLR of GIW, and average biogas production, methane yield, and methane content of control and treated digesters in step and pulse feeding experiments.

<table>
<thead>
<tr>
<th>GIW content in feedstock (v/v %)</th>
<th>GIW content in feedstock (w/w %)</th>
<th>Total OLR (g- VS/L/day)</th>
<th>OLR of GIW (g- VS/L/day)</th>
<th>Methane yield (L g VS added(^{-1}))</th>
<th>% increase (L/day)</th>
<th>% increase (%)</th>
<th>Biogas (L/day)</th>
<th>Methane content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control digester (A)</td>
<td>0</td>
<td>0</td>
<td>1.24</td>
<td>0.197</td>
<td>n.d.</td>
<td>2.3</td>
<td>n.d.</td>
<td>64.4</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>66</td>
<td>2.16</td>
<td>1.43</td>
<td>n.d. b</td>
<td>n.d. b</td>
<td>n.d. b</td>
<td>624</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>66</td>
<td>2.16</td>
<td>1.43</td>
<td>0.740</td>
<td>276</td>
<td>16.5</td>
<td>69.9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>75</td>
<td>3.14</td>
<td>2.36</td>
<td>0.783</td>
<td>298</td>
<td>21.4</td>
<td>837</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step feed(^{a}) (experiment I)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control digester (B)</td>
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<td>0</td>
<td>1.24</td>
<td>0.180</td>
<td>n.d.</td>
<td>2.2</td>
<td>n.d.</td>
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<td></td>
<td>10</td>
<td>46</td>
<td>1.58</td>
<td>0.73</td>
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<td>209</td>
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<td></td>
<td>20</td>
<td>66</td>
<td>2.16</td>
<td>1.43</td>
<td>0.752</td>
<td>318</td>
<td>13.7</td>
<td>523</td>
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<tr>
<td></td>
<td>40</td>
<td>84</td>
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<td>0.630 b</td>
<td>250 b</td>
<td>19.6 b</td>
<td>791 b</td>
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<td>2.16</td>
<td>1.43</td>
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<tr>
<td></td>
<td>30</td>
<td>75</td>
<td>3.14</td>
<td>2.36</td>
<td>0.785</td>
<td>336</td>
<td>21.6</td>
<td>883</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated digester (A)</td>
<td>n.d.</td>
<td>0</td>
<td>1.33</td>
<td>0.141</td>
<td>n.d.</td>
<td>2.0</td>
<td>n.d.</td>
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<tr>
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<td>30</td>
<td>1.64</td>
<td>0.49</td>
<td>0.419</td>
<td>n.d.</td>
<td>6.4</td>
<td>n.d.</td>
<td>64.3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.64</td>
<td>0.49</td>
<td>0.424</td>
<td>n.d.</td>
<td>6.5</td>
<td>n.d.</td>
<td>64.4</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>2.95</td>
<td>2.06</td>
<td>0.704</td>
<td>68</td>
<td>18.6</td>
<td>189</td>
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<tr>
<td></td>
<td>90</td>
<td>4.48</td>
<td>4.03</td>
<td></td>
<td>n.d. b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulse feed (experiment II)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control digester (B)</td>
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<td>1.33</td>
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<td>n.d.</td>
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<td>0.49</td>
<td>0.422</td>
<td>n.d.</td>
<td>6.5</td>
<td>n.d.</td>
<td>63.6</td>
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<tr>
<td></td>
<td>60</td>
<td>2.24</td>
<td>1.35</td>
<td>0.515</td>
<td>n.d.</td>
<td>8.7</td>
<td>n.d.</td>
<td>64.2</td>
</tr>
<tr>
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<td>0.748</td>
<td>77</td>
<td>19.8</td>
<td>203</td>
<td>66.9</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>4.48</td>
<td>4.03</td>
<td></td>
<td>n.d. b</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\(^{a}\) Data of step feeds of 0%, 46%, 66%, and 84% (w/w) GIW in the treated digester are obtained from Wang et al., 2013.

\(^{b}\) Digester failures observed.

Figure 6-2 Effluent characteristics over time in control (●) and treated (○) digesters during phase 5 (Wang et al., 2013) and perturbation test (current study, experiment I). (a) VS concentration; (b) VS reduction rate; (c) COD concentration; (d) COD reduction rate; (e) pH; (f) alkalinity; (g) total volatile acid; (h) ratio of total volatile acid and alkalinity.

Figure 6-2 Effluent characteristics over time in control (●) and treated (○) digesters during phase 5 (Wang et al., 2013) and perturbation test (current study, experiment I). (a) VS concentration; (b) VS reduction rate; (c) COD concentration; (d) COD reduction rate; (e) pH; (f) alkalinity; (g) total volatile acid; (h) ratio of total volatile acid and alkalinity.
Table 6-4 Previous studies performing anaerobic co-digestion by single-level, step, or pulse feeding.

<table>
<thead>
<tr>
<th>Continuous feeding at a frequency of once or more a day</th>
<th>Single-level feeding</th>
<th>Step feeding</th>
<th>Pulse feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Davidsson et al., 2008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Luostarinen et al., 2009&lt;sup&gt;a&lt;/sup&gt;; Silvestre et al., 2011&lt;sup&gt;a,e&lt;/sup&gt;; Martin-González et al., 2010&lt;sup&gt;d&lt;/sup&gt;; Wan et al., 2011&lt;sup&gt;a&lt;/sup&gt;; Noutsopoulos et al., 2013&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Current study&lt;sup&gt;a,e&lt;/sup&gt;; Cavaleiro et al., 2001&lt;sup&gt;b&lt;/sup&gt; (synthetic dairy wastes ← oleate&lt;sup&gt;b,e&lt;/sup&gt;); Cavaleiro et al., 2008&lt;sup&gt;b&lt;/sup&gt; (oleate and palmitate ← dairy wastewater&lt;sup&gt;b,e&lt;/sup&gt;); Cavaleiro et al., 2009&lt;sup&gt;b&lt;/sup&gt; (synthetic dairy wastewater with sodium oleate; five pulse feed cycles followed by continuous step feeds)&lt;sup&gt;b,e&lt;/sup&gt;; Nielsen and Ahring, 2006&lt;sup&gt;b&lt;/sup&gt; (cattle and pig manure ← oleate&lt;sup&gt;b,e&lt;/sup&gt;); Neves et al., 2009&lt;sup&gt;b&lt;/sup&gt; (cow manure and food waste ← oily waste&lt;sup&gt;b,e&lt;/sup&gt;); Palatsi et al., 2009&lt;sup&gt;b&lt;/sup&gt; (cow manure ← synthetic LCFAs)&lt;sup&gt;b,e&lt;/sup&gt;; Palatsi et al., 2010&lt;sup&gt;b&lt;/sup&gt; (manure ← synthetic LCFAs)&lt;sup&gt;b,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Intermittent feeding at a frequency of once every other day or less</td>
<td>De Vrieze et al., 2013&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Nadais et al., 2006&lt;sup&gt;b&lt;/sup&gt; (dairy wastewater)&lt;sup&gt;b,e&lt;/sup&gt;; Wang et al., 2013&lt;sup&gt;a,e&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Co-digestion of sewage sludge (containing primary and/or waste activated sludge) with FOG-based wastes.

<sup>b</sup> Digestion of synthetic LCFA-based materials. The feeding materials and pattern are indicated with an arrow in the table: base substrate ← pulse feeds of substrate.

<sup>c</sup> Digestion of sewage sludge only.

<sup>d</sup> Co-digestion of municipal solid waste with FOG-based wastes.

<sup>e</sup> Microbial/biomass adaptations observed.
**Experiment II: pulse feeding.** In experiment II the treated digester was spiked with periodic pulse feeding of GIW to analyze its influence on methane production and community robustness measured by resistance and resilience. The unique feature of pulse feeding is its ability to simulate situations when full-scale facilities accept high-strength wastes with fluctuating concentrations.

Experiment II contains two start-up periods in phases 1 and 2, repeated pulses of GIW in phase 3, followed by perturbation and recovery tests (Figure 6-3). The GIW loading rates in phases 1 and 2 were 0% and 30% (w/w as VS), respectively, for both digesters (Figure 6-3a). The average biogas productions, methane yields, and methane content during phases 1 and 2 (Table 6-3) and the effluent VS concentration and reduction rate, pH, and alkalinity over time (Figure 6-4) show the reactor performance. During phase 3, four repeated pulses of GIW at 60% (w/w as VS) were conducted in the treated digester, while the control digester was still treated with 30% (w/w as VS) GIW (Figure 6-3a). GIW pulse feeding at 60% (w/w as VS) was selected based on our previous results in Wang *et al.* (2013) where step feeding of GIW equal to or less than 66% (w/w as VS) was able to produce stable biogas without process failure. The pulses of GIW increased methane yield from 0.422 to 0.515 L-methane/g-VS added and biogas production from 6.5 to 8.7 L day-1 in the treated digester (Figure 6-3b and Table 6-3).

The first perturbation test for both digesters involved addition of 70% (w/w as VS) GIW. The increases in methane yield of pulse-treated and control digesters were 0.748 and 0.704 L-methane/g-VS added respectively (Table 6-3). The enhancement in methane yield due to pulse feeding was not significant. We proceeded to evaluate the ecological parameters by
measuring changes in individual VFAs concentrations during the first perturbation test (Figure 6-5). The ecological parameters are resistance (changes in concentrations of major intermediates) and resilience (time taken to return to the baseline) defined by Botton et al. (2006) and Werner et al. (2011). Due to the every-other-day feeding frequency, concentrations of individual VFAs fluctuate over time (Figure 6-5).

During the second perturbation test, both digesters were further challenged with 90% (w/w as VS) GIW (Figure 6-3a). In our previous study (Wang et al., 2013), the step-treated community (treated from 46% to 66% GIW) failed to survive addition of 84% (w/w as VS) GIW. This GIW shock at 90% (w/w as VS) is expected to exert different influences on the treated (pulse-treated at 60% GIW and step-treated at 70% GIW) and control (step-treated from 30% to 70% GIW) digesters. Both digesters were intentionally overloaded in an attempt to observe responses of two different microbial communities towards a high GIW shock. We observed severe process failure shortly after 90% (w/w) GIW shock in both digesters indicated by immediate drops in methane yield, biogas production, and methane content (Figure 6-3). Following the digester upset, 90% (w/w) GIW feeding was replaced by 30% (w/w) GIW during the recovery test (Figure 6-3a) where the changes in individual VFAs concentrations were measured as shown in Figure 6-6. Upon application of the 90% GIW shock, concentrations of all VFAs significantly increased in both control and treated digesters (Figure 6-6). Propionate and valerate continued to accumulate over the test period while acetate and butyrate recovered within one SRT cycle in both digesters.
Figure 6-3 Organic loading rate (OLR) and biogas characteristics over time in experiment II. (a) OLR; (b) methane yield; (c) biogas production; (d) methane content.
Figure 6-4 Effluent characteristics over time in experiment II. (a) OLR; (b) VS concentration; (c) VS reduction rate; (d) pH; (e) alkalinity.
**Figure 6-5** Organic loading rate (OLR) and effluent characteristics over time in control (●) and treated (○) digesters during perturbation test at 70% (w/w) GIW loading rate in experiment II. Gray arrows indicate that both digesters were fed every other day. (a) OLR: black line is for control digester and grey line is for treated digester; (b) acetate; (c) propionate; (d) butyrate; (e) valerate.
Figure 6-6 Organic loading rate (OLR) and effluent characteristics over time in control (●) and treated (○) digesters during perturbation and recovery tests in experiment II. (a) OLR: black line is for control digester and grey line is for treated digester; (b) acetate; (c) propionate; (d) butyrate; (e) valerate.
6.5 Discussion

Previously in Wang et al. (2013) we explored the limit of anaerobic co-digestion by step feeds of GIW from 0%, 46%, 66%, to 84% (w/w) (Figure 6-1). The highest GIW addition rate achieved without significant process inhibition was 66% (w/w as VS). In this study, we designed two sets of experiments to further evaluate the microbial adaptation and recovery capacity by manipulating the feeding patterns at various GIW loading rates. The goal is to compare digester stability, performance, and ecological robustness of GIW-adapted (step-treated and pulse-treated) and non-GIW-adapted microbial communities by measuring changes in methane production and individual VFA concentrations. The following sections describe our results.

**Step feeding of GIW from 46% to 66% (w/w) at an every-other-day frequency created a more robust digester against overloading by inducing microbial adaptation.** In experiment 1, following the recovery from GIW inhibition at 84% (w/w) GIW (phase 5, Figure 6-1), we conducted perturbation and recovery tests at 66% and 75% (w/w) GIW to further push the digester limit (Figure 6-2). The step-treated digester was able to tolerate 66% (w/w) GIW (gray box in phase 4, Figure 6-1), while the non-step-treated digester encountered major process failure at 66% (w/w) GIW loading rate (black box pictured top left in perturbation test, Figure 6-1). The GIW-adapted and non-GIW-adapted digesters showed different levels of tolerance at the same GIW loading rate, suggesting that the previous step feed at 46% (w/w) GIW (phase 3, Figure 6-1) very likely induced a certain level of microbial adaptation, creating more robust microbial communities against GIW inhibition at a higher loading rate.
Positive effects of integrating intermittent and step feedings on digester performance were also observed in previous studies. Compared to daily-fed communities, De Vrieze et al. (2013) observed higher degrees of bacterial dynamics and tolerance to ammonium loading shock in anaerobic communities treated with every-two-day feeding (without co-processing FOG-rich materials), although no difference in methane production and methanogenic community composition was found. Cavaleiro et al. (2009) showed that five pulse-feed cycles followed by step feeding of synthetic dairy wastewater with sodium oleate promoted sludge acclimation and more efficient degradation of LCFA-rich wastewater in an up-flow anaerobic column reactor. This feeding pattern also contributed to higher methane production. Biomass adaptation and increases in methane yield have been reported in a lab-scale continuous stirred tank reactor (CSTR) employing step feeding of trapped grease wastes from a WWTF in a twice-a-day feeding pattern (Silvestre et al., 2010) and in an upflow anaerobic sludge blanket (UASB) reactor treated with step feeds of dairy wastewater in an intermittent 48hr-feed-48hr-feedless mode (Nadais et al., 2006). Stabilization or “feedless” period was demonstrated to aid in retaining and entrapping organic matter (Nadais et al., 2006), and a slow increase in FOG-rich co-substrate dose was found to induce biomass adaptation, allowing more efficient degradation of LCFAs and reduction of LCFA inhibition (Silvestre et al., 2010).

In this study, the use of step feed at an every-other-day feeding frequency was proven feasible and beneficial to the development of a more robust digester where microorganisms were trained to adapt to higher GIW loads.
Both the GIW-adapted and non-GIW-adapted digesters recovered from GIW inhibition with the termination of GIW addition, careful maintenance, and adequate rest period. We intentionally overloaded both the GIW-adapted (treated) and non-GIW-adapted (control) digesters at 84% (phase 5, Figure 6-1) and 66% (days 244 to 258 during perturbation test, Figure 6-1), respectively, to evaluate their recovery capacity. Upon overloading at 84% (w/w) GIW followed by a recovery period, the step-treated digester was able to function once again at 66% (w/w) GIW, producing methane at 0.665 L-methane/g- VS added (gray box pictured top middle in perturbation test, Figure 6-1). This methane production is slightly lower compared to that of 0.752 L-methane/g- VS added during phase 4 before the digester was inhibited at 84% (w/w). Also, during the second 66% (w/w) GIW feeding, the methane production and methane content fluctuated more compared to those in phase 4. These observations suggest that the microbial communities in the step-treated digester might have not completely recovered from the 84% (w/w) GIW shock. However, the rest period in phase 5 restored a portion of its capacity against GIW inhibition to maintain its function without significant process upset. A longer recovery phase may be needed for complete recovery. We further pushed the recovered step-treated digester to 75% (w/w) GIW in the recovery test, which increased methane production to 0.785 L-methane/g- VS added (gray box pictured top middle in perturbation test, Figure 6-1 and Table 6-3). After a recovery period, the non-step-treated (control) digester overloaded at 66% (w/w) GIW was able to tolerate 66% (w/w) GIW this time and even higher at 75% (w/w) GIW, producing methane at 0.740 and 0.783 L-methane/g- VS added, respectively (black box pictured top right in perturbation test, Figure 6-1 and Table 6-3).
When a long period of recovery was provided, both the GIW-adapted and non-GIW-adapted digesters were able to recover from GIW shocks (at 84% and 66% (w/w), respectively) that previously caused severe process inhibition. Based on these results, the highest GIW loading rate achieved without digester upset increased from 66% (Wang et al., 2013) to 75% (w/w), and the range of GIW addition leading to inhibition increased from 66% - 84% (Wang et al., 2013) to 75% - 84% (w/w) of VS added.

**Four pulse feeds of GIW at 60% (w/w) increased methane production and community resistance against inhibition at 70% (w/w) GIW loading rate.** Since pulse feeding can be a better option to simulate changes in concentrations of FOG-rich wastes in real-world digester operation, we explored the potential of challenging the digester with periodic pulse feeds in developing a similar adaptation effect and enhancement in methane yield that we observed in step feeding experiments. After four repeated pulses of GIW at 60% (w/w as VS) during phase 3 (Figure 6-3), the pulse-treated digester was subjected to a 70% (w/w) GIW shock, along with the non-pulse-treated control digester.

The treated digester was step-treated at 30% (w/w) GIW and pulse-treated at 60% GIW, while the control digester was only step-treated at 30% GIW (Figure 6-3). When spiked with 70% (w/w) GIW, the enhancement in methane production was slightly greater in the treated digester (Table 6-3). To measure digester robustness, the ecological concepts of resistance and resilience were applied in this study. As adopted from Botton et al. (2006) and Werner et al. (2011), community resistance can be determined by the maximum concentration of major intermediates accumulating due to GIW inhibition against normal anaerobic metabolism; community resilience can be measured by the time required for the accumulated
intermediates to be transformed and return to the baseline. When subjected to 70% (w/w) GIW, the amounts of acetate, propionate, butyrate, and valerate accumulated in the pulse-treated digester were all less than those in the non-pulse-treated digester throughout the first perturbation test (Figure 6-5), indicating that the pulse-treated digester was more resistant than the non-pulse treated digester. Similarly, when treated with 90% (w/w) GIW during the second perturbation test, the pulse-treated digester accumulated a lower amount of acetate, compared to the non-pulse-treated digester (Figure 6-6b). As the concentrations of propionate and valerate kept accumulating, acetate was slowly processed, suggesting that aceticlastic methanogens may be the more resistant populations against GIW inhibition.

On the other hand, no significant difference in the time taken to digest accumulated acetate, propionate, butyrate, and valerate were observed either at 70% (w/w) GIW (Figure 6-5) or in the recovery test (Figure 6-6). Therefore, whether the digester resilience was increased due to pulse feeding could not be determined at this GIW loading rate. We initially suspected that GIW addition at 70% (w/w) probably did not provide sufficient perturbation to show difference in time required for recovery between pulse-treated and non-pulse-treated digesters. However, when overloaded with 90% (w/w) GIW, the degrees of resilience in both digesters were similar as the time taken to consume accumulated acetate and butyrate was consistent in both digesters (Figures 6-6b and 6-6d). More in-depth investigation on developing and testing a resilient and resistant digester system is needed. For example, it is possible that the five pulse-feeds of 70% (w/w) GIW in the perturbation test may have allowed the control digester to achieve a level of adaptation, thus making the succeeding perturbation at 90% (w/w) GIW addition ineffective in showing differences between the two
digesters. In essence the perturbations at 70% GIW “trained” the control reactor. The increases in methane production and digester resistance observed in this study suggest the potential of using pulse feeding to develop more resistant microbial populations against GIW inhibition and enhance methane production.

Changes in feeding strategy, microbial ecology, and interactions between biomass inhibition and adaptation play an important role in maximizing methane production in anaerobic co-digesters. When exploring maximizing methane production by developing microbial adaptation, we discovered that step or pulse feeding produced the highest methane yield reported compared to researchers that performed single-level and daily feedings at similar FOG waste loading rates (Davidsson et al., 2008; Luostarinen et al., 2009; Kabouris et al., 2009; Wan et al., 2011). Table 4 lists previous studies performing single digestion of sewage sludge, co-digestion of sewage sludge with FOG-based wastes, and digestion of synthetic LCFA-based materials. These studies are categorized by feeding strategy and frequency. Many studies conducted pulse feeds of synthetic LCFAs along with batch methanogenic activity and toxicity tests to investigate LCFA inhibition (Alves et al., 2001; Cavaleiro et al., 2001, 2008, 2009, Palatsi et al., 2009, 2010). Others evaluated the potential of enhancing methane production by co-processing sewage sludge or municipal solid waste with FOG-based wastes such as waste industrial FOG and animal fats from food processing plants, GIW from grease interceptors at FSEs and FOG receiving facilities, and trapped grease wastes from dissolved air flotation units at WWTFs (Davidsson et al., 2008; Kabouris et al., 2009; Luostarinen et al., 2009; Martín-González et al., 2011; Silvestre et al., 2011; Wan et al., 2011; Noutsopoulos et al., 2013; Wang et al., 2013; Ziels et al., 2016).
We adopted the idea of Noutsopoulos et al. (2013) to plot biogas yield versus OLR of FOG wastes (calculated by FOG waste content (% w/w of VS added) times OLR of total substrate) based on results of the studies listed in Table 4 and replaced biogas yield with methane yield to obtain a more accurate result that includes the contribution of methane content in biogas (Figure 6-7). Methane yields at various OLRs of FOG-based wastes were categorized into four zones in Figure 7. Zone 1 ranges from 0 to ~1.3 g-VS/L/day where both non-FOG-adapted (white and gray symbols) and FOG-adapted digesters (black symbols) were able to function without process failure, with average methane yields ranging from 0.1 to 0.5 L-methane/g-VS added. Within this range, methane yield is proportional to the OLR of FOG wastes; i.e. the more biodegradable VS added, the more methane produced in zone 1. The threshold for FOG waste addition resulting in an inhibited digestion process is identified to be at approximately 1.3 g-VS/L/day. Zone 2 ranges from ~1.3 to ~2.1 g-VS/L/day where the degree of digester tolerance against LCFA inhibition varied. Process failures indicated by zero methane yield were observed in both FOG-adapted (Martín-González et al., 2011) and non-FOG-adapted (current study) digesters at ~1.4 g-VS/L/day. On the other hand, stable results were found in both FOG-adapted (current study; Luostarinen et al., 2009; Wang et al., 2013) and non-FOG-adapted (Kabouris et al., 2009; Noutsopoulos et al., 2013; Wan et al., 2011) digesters, producing methane yields of 0.45 to 0.75 L-methane/g-VS added. In general, step or pulse feeding showed potential in increasing methane yield up to ~0.75 L-methane/g-VS added in zone 2 (current study; Wang et al., 2013). In Zone 3 ranging from ~2.1 to ~4.0 g-VS/L/day, single-level digesters without step or pulse feed treatment to develop biomass adaptation encountered process upset (Davidsson et al., 2008; Noutsopoulos et al., 2013; Wan et al., 2011). Step-treated digesters seemed to be inhibited but were still able to function within this range (Luostarinen et al., 2009; Wang et al., 2013 and current study). Zone 4 ranges from ~4.0 to ~7.5 g-VS/L/day where neither FOG-adapted (current study) nor non-FOG-adapted (Noutsopoulos et al., 2013) digesters could survive LCFA inhibition.

By comparing referenced methane yields and different feeding strategies, we were able to identify four zones of OLRs of FOG-based wastes in Figure 6-7 to provide guidance for
future applications of FOG co-digestion. If prevention of severe process upset is a top priority, we recommend operating digesters in zone 1 where the implementation of step or pulse feeding is not necessary to maintain active methanogenesis. If significant enhancement in methane yield is the goal, operating in zone 2 with proper step or pulse treatment to promote biomass adaptation can very likely result in high methane yield based on the experimental results. Operating digesters in zone 3 or 4 is not recommended without more investigations on its feasibility. Adapting digesters with step or pulse feeds of FOG waste increased digester resistance and subsequently methane production. By comparing available data from FOG co-digestion studies, we were able to suggest the first guideline proposed for operating anaerobic co-digestion of FOG-based wastes at various OLRs (as represented by Figure 6-7).
Figure 6-7 Methane yields at various OLRs of FOG-based wastes categorized into four zones. OLR of FOG-based wastes is calculated by multiplying FOG waste content (% w/w of VS added) and OLR of total substrate. Black symbols and the white symbol with thicker black line represent methane yields of step or pulse feeding. Gray symbols represent methane yields of single-level feeding. White symbols represent methane yields of non-step feeding (i.e. those methane yields of startup periods prior to step feeding of GIW). Methane yields at zero represent the occurrence of process failure when data of stable methane yield was unavailable.
6.6 Conclusions

Step feeding of GIW showed positive effects on the development of better microbial adaptation and reduction in inhibition of methanogenesis. Step feeding of GIW at 75% (w/w) enhanced methane yield from 0.180 to 0.785 L-methane/g-VS added, biogas production from 2.2 to 21.6 L/day, and methane content from 60.2% to 68.6%. GIW overloadings at 66% and 84% (w/w) confirmed the recovery capacity of both GIW-adapted and non-GIW-adapted digesters when proper maintenance and recovery period from GIW addition were provided. Pulse feeding of GIW at 60% (w/w) increased methane yield and community resistance against GIW inhibition. Step feeds of GIW at 30% and 70% (w/w) integrated with four pulse feeds of GIW at 60% (w/w) increased methane yield from 0.134 to 0.748 L-methane/g-VS added, biogas production from 2.0 to 19.8 L/day, and methane content from 54.2% to 66.9%.

Both step and pulse feeding strategies at an every-other-day frequency produced the highest methane yield reported, compared to previous studies conducted with daily single-level feeding at similar loading rates. GIW overloading at 90% (w/w) revealed that aceticlastic methanogens may be better adapted communities against GIW inhibition compared to syntrophic bacteria. More comprehensive research on the development of biomass adaptation due to changes in the microbial composition in response to GIW overloading is needed to create more robust populations and maximize methane production. Based on different feeding patterns, four zones identified by comparing various OLRs of FOG-based wastes and referenced methane yields are presented to provide guidelines on future implementations of anaerobic co-digestion of FOG-based wastes.
Chapter 7 Microbial community assembly and dynamics in anaerobic bioreactors with different loading stress histories

7.1 Abstract

By analyzing the shifts in microbial communities across operational environments, we showed that microbiomes in anaerobic digesters could be selectively assembled using specific operational conditions to improve general community function (bioenergy production from grease interceptor waste). We evaluated the relationships between community composition, function, the environment under different levels of stress and stress history, and identities and ecological potentials of core populations. We used substrate loading to increase or reduce stress, which in turn resulted in functionally and compositionally representative assemblies. Microbiomes with different stress histories became compositionally dissimilar but remained functionally similar under similar disturbance intensity. When re-stressed at a new or similar level, microbiomes that have been previously disturbed converged to a common structure, regardless of the difference in disturbance histories. We identified functional redundancy and selection-driven shifts of dominant community members within different metabolic networks before and after overloading. Our results demonstrate how stress history can be used to pre-select for more stress-tolerant members across functional guilds. We also demonstrated that at higher substrate loadings, communities became less diverse and less even, but more specialized with
better function. These ecological insights may lead to enhancement of microbiota in engineered reactors via active control of stress histories.

7.2 Importance

The experiments reported in this paper represent the first comprehensive time-series investigation of long-term (>200 days) community assembly and functional dynamics of anaerobic co-digesters treating grease interceptor waste with different loading stress histories. This approach allowed us to show the following: microbial community assembly appears to be primarily determined by selection processes that can be directed using changes in substrate loading; the disturbance history (whether a community has experienced disturbance) is a factor in subsequent community composition; putative identification of organisms involved in specific anaerobic metabolic processes; the mapping of specific taxa to GIW overloading and recovery is a novel approach that led to identification of potentially key taxa (e.g., Christensenella) for lipid degradation. Our results also show that substrate loading patterns, such as step loading, can be used to “actively guide” microbial communities to achieve higher methane production or better response to overloading stress.

7.3 Introduction

Microbial community assembly has been considered historically as a deterministic process in which interspecies interactions (e.g. mutualism/syntrophy, commensalism, parasitism/predation, amensalism, and competition) (Lidicker, 1979; Faust and Raes, 2012) and niche differentiation (ecologically meaningful differences between species) are dominant
drivers in determining the relationship between taxon traits and the environment (Weiher and Keddy, 1999; Chase and Leibold, 2003). In contrast, neutral theory suggests that community assembly is mainly shaped by stochastic factors such as birth, death, immigration, and speciation, and assumes ecological equivalence of community members (Bell, 2001; Harpole, 2010). Others recognize the attributes of combined deterministic and stochastic processes in shaping the microbial community (Ofiteru et al., 2010). A conceptual synthesis of these theories has been proposed to include evolutionary forces, and concentrate these community assembly processes into four categories: diversification (generation of new genetic variation), dispersal (movement of species across space), selection (deterministic structuring via abiotic factors) and drift (stochastic changes in species abundance) (Vellend, 2010; Nemergut et al., 2013).

We posit that the framework of microbial community assembly can be used to examine and better manage the complex community ecology and interaction webs of microbiota in engineered reactors. While reactor microbiomes are structured for the metabolic cooperation of specialized groups of microbes, the complex communities and interaction networks in reactors have been traditionally treated as “black boxes” (Koch et al., 2014; Sales and Lee, 2015). This has posed a challenge to maintaining function and stability when microbiomes are subject to dynamic environmental disturbances, and to evaluating recovery after stress or process failure. We believe that understanding how the reactor microbiome is shaped by, and interacts with, inhibitory environments, can provide deeper insights in predicting and improving community function in response to different stress conditions and stress histories.
Several researchers have profiled microbiomes of anaerobic digesters (ADs) performing single-substrate digestion of sewage sludge or co-digestion of multiple waste streams in lab or full scale (Werner et al., 2011; Zhang et al., 2012; Sundberg et al., 2013; Ziganshin et al., 2013). These efforts were made to link community dynamics to environmental factors and community function. Other studies actively manipulated community assembly in lab scale ADs to examine potential processes guiding the population and functional dynamics, such as deterministic niche and stochastic neutral theories (Vanwonterghem et al., 2014; Lucas et al., 2015). AD microbiomes can be used for waste management and resource recovery from lipid-rich materials that are high in biological methane potential, such as grease interceptor waste (GIW) from food service establishments. The challenges are to maximize production of bioenergy while maintaining stability and function, and to avoid (or recover from) overloading of inhibitory substrates. The potential of GIW co-digestion in enhancing waste-to-energy production has been extensively demonstrated at different loading rates (Davidsson et al., 2008; Alves et al., 2009; Kabouris et al., 2009; Luostarinen et al., 2009; Noutsopoulos et al., 2013), although the performance profiles and inhibitory concentrations differed markedly due to the “black box” approach. To better understand the microbial community assembly associated with substrate inhibition in ADs, more recent studies identified disturbance-driven community and functional dynamics using GIW-related substrates (Kougias et al., 2016; Amha et al., 2017; Ziels et al., 2017) and other substrates (Goux et al., 2015; Mosbæk et al., 2016; Vanwonterghem et al., 2016; De Vrieze et al., 2017). Others demonstrated that microbial adaptation in ADs, particularly the shifts in abundance of syntrophic fatty acid-degrading populations, correlated to reduced inhibition and improved
community performance in GIW-disturbed environments (Silvestre et al., 2011; Ziels et al., 2016; Amha et al., 2017). However, the ecological potentials, stress tolerance, and recovery (Botton et al., 2006; Shade et al., 2012; Mosbæk et al., 2016) of AD populations in response to different levels of disturbance remain largely unknown in GIW co-digesters. Moreover, there is very limited research on microbial community dynamics and responses to overloading, subsequent recovery, and further overloading.

By shaping the methanogenic reactor microbiome from a full-scale anaerobic digester into microbial communities with distinct disturbance histories and niche preferences during two independent experiments, we were able to gain insights on the following: (1) how different levels of disturbance direct community assembly and related functional dynamics; (2) how history of microbiome assembly links to community survivability and function; and (3) how ecological roles and niche characteristics of key digester microbiome members relate to their responses to different assembly approaches and disturbance intensities.

7.4 Materials and methods

Digester microbiome seeding, environment, operation, and function. This study integrates two independent experiments and applies GIW as the source of selective stress. Experiment I used gradual increases of GIW loading rates, while Experiment II used sudden spikes of GIW at higher concentrations. For Experiment I, two 8 L lab-scale anaerobic digesters (Community 1 and Community 2) were set up in parallel and inoculated with anaerobic digester biosolids from the South Durham Water Reclamation Facility in North Carolina (NC). TWAS from the North Cary Water Reclamation Facility in NC was used as
base substrate, and GIW from a restaurant in Cary, NC was used as co-substrate. The three primary components of GIW: fat, oil, and grease (FOG), food particles, and wastewater, were collected separately and stored at 4°C immediately after collection. Digesters were supplied with a mixture of TWAS and GIW every other day and operated with a solids retention time of 20 days at 37°C (for mesophilic organisms). For a detailed reactor description, see Wang et al. (Wang et al., 2013) The same setup was used for Experiment II under the same environmental conditions, while using a different feeding strategy and a source of GIW from a different restaurant.

Digester microbiome function was assessed by measuring chemical and physical characteristics of the sludge and biogas. Biogas production was recorded daily and normalized to standard temperature and pressure (STP) conditions. Methane content in biogas was analyzed daily using a gas chromatograph (GC, SRI 8610C) equipped with a thermal conductivity detector. Every other day during each feed time, effluent was removed and an equal amount of feedstock was introduced into the digesters. Effluent sludge was analyzed for total solids (TS), volatile solids (VS), alkalinity, and pH according to Standard Methods (American Public Health Association (APHA), 2005). Concentrations of individual volatile fatty acids (VFA; e.g., acetic, propionic, butyric, and valeric acids) were determined by acidification, centrifugation, filtration, and direct injection into a GC (GC-2014 Shimadzu) equipped with a flame ionization detector according to Method 5560 D in Standard Methods. Total VFA (TVA; VFAs up to six carbon atoms) concentration was measured by centrifugation, acidification, distillation, and titration according to Method 5560 C in Standard Methods (American Public Health Association (APHA), 2012).
DNA extraction, 16S rRNA gene amplicon sequencing, and bioinformatics. A total of 120 sludge samples were extracted for genomic DNA (gDNA) components using a modified aluminum sulfate DNA extraction method (Staley et al., 2011). Forward and reverse primer pair sequences, modified 341F and modified 806R, respectively, were used to amplify a DNA fragment of ~460 bp length flanking the V3 and V4 regions of the 16S rRNA gene of bacteria and archaea in the gDNA samples (Yu et al., 2005; Sundberg et al., 2013). Library preparation, quantification, normalization, and pooling were performed according to the Illumina 16S metagenomics protocol. High Sensitivity DNA Analysis on a Bioanalyzer was performed to ensure library quantity and quality. Pooled libraries were run on an Illumina MiSeq platform for 300 bp paired-end read sequencing at the Genomic Sciences Laboratory, North Carolina State University, NC. Sequences were deposited to the National Centre for Biotechnology Information (NCBI) Sequence Read Archive with the accession number SRP077521.

Amplicon sequence pairs were merged, trimmed to remove primer sequences, and quality filtered using the QIIME pipeline (J Gregory Caporaso et al., 2010) and Trimmomatic 0.33 (Bolger et al., 2014). Operational taxonomic units (OTU) clustering was performed on a total of 120 samples (20 samples in triplicate from Experiments I and II) at the ≥97% sequence similarity level using QIIME open-reference OTU picking workflows (Edgar, 2010; Rideout et al., 2014). RDP Classifier 2.2 (Wang et al., 2007) was used to assign taxonomy to each cluster representative based on the Greengenes taxonomy and reference database (McDonald et al., 2012; Werner et al., 2012). Sequences were aligned based on the Greengenes core reference alignment (DeSantis et al., 2006) using PyNAST.
(Caporaso et al., 2010). Chimeric sequences were identified using ChimeraSlayer (Haas et al., 2011) and removed from the alignment to build a phylogenetic tree using FastTree 2.1.3 (Price et al., 2010).

Quality filtered and chimera-free sequences of triplicate microbiomes were merged and normalized to correct for differences in sampling efforts. Taxon abundance profiles were visualized using R ggplot package (Wickham, 2009). The compositional differences between microbiomes were analyzed by nonmetric multidimensional scaling (NMDS) statistics using R vegan package (Oksanen et al., 2015) applying the Bray-Curtis similarity index and visualized using R ggplot2 package (Wickham, 2009). Diversity analysis was performed in QIIME using a series of subsamplings to calculate the number of distinct OTUs (observed OTU counts), and Shannon, Simpson’s evenness, and Gini indices at the same rarefaction depth (24,000 randomly selected reads per sample).

7.5 Results

The dynamic community assembly and function of AD systems were studied in two independent experiments (I and II) using GIW as the source of selective stress. For both experiments, we used microbial communities from the same full scale anaerobic digester as the inoculum. Both experiments were run under the same environmental conditions, but using different feeding strategies and sources of co-substrate (GIW from two different restaurants) mixed with the same base substrate (thickened waste activated sludge, TWAS). We sequenced triplicate samples from each experiment during different performance phases, for a total of 120 microbiome samples. A total of 8,925,849 quality filtered and chimera-free
sequences were generated and mapped to bacterial and archaeal 16S ribosomal RNA (rRNA) genes in the Greengenes database with an average of 74,382 sequences per sample.

**Microbiome assembly approaches and related functional dynamics.** In Experiment I (Figure 7-1), Community 1 was subjected to discrete loading steps from 0% GIW (w/w of VS added) to 46% then 66%. At 84% GIW loading, the reactor community appeared to be overloaded, and allowed to recover at 0% GIW, after which GIW loading was re-increased to 66% then 75% GIW. Community 2 (Figure 7-1) that served as a control system from Days 1 to 243 at 0% GIW was challenged and overloaded at a higher loading rate of 66%, allowed to recover at 0% and subsequently fed at 66% and 75% GIW. This approach led to the development of several representative microbiomes: startup (S1, S14, S15), developing (S2-S6), overloaded (S7, S16), recovering (S17), recovered (S8, S18), and developed (S9-S13, S19-S20), categorized based on the performance profiles and operating conditions (Figures 7-1, 7-S1 and 7-S2). These six types of reactor microbiomes were identified for the first time in this work, and defined as: (1) startup - representing community assembly prior to exposure to a selective stress; (2) developing - assembly during a lower level of stress; (3) overloaded - assembly during a process failure at a higher level of stress; (4) recovering - assembly during recovery from a process failure; (5) recovered - assembly after recovery from a process failure; and (6) developed - assembly after re-exposure to the same or higher level of stress after recovery from a process failure.

In Experiment II (Figure 7-2), Community 3 treating a stable 30% GIW was challenged with four periodic spikes at 60%, then 70%, experienced process failure at 90% GIW feed, and allowed a recovery phase with 30% GIW feed. Community 4 (Figure 7-2) was step fed
from 30% to 70%, showed overloading at 90% GIW, and allowed to recover at 30% GIW. Representative microbiomes created through the combined step and pulse feeding strategy are startup (P1, P2), developing (P3-P12), overloaded (P13, P14), and recovering (P15-P20) microbiomes, categorized based on the performance profiles and operating conditions (Figures 7-2, 7-S3 and 7-S4). These experiments represent the first comprehensive time-series investigation of long-term community assembly and functional dynamics of GIW co-digesters with different loading stress histories.
**Figure 7-1** Experiment I: Community relative abundance (A) and functional (B) dynamics of startup (S1, S14, S15), developing (S2-S6), overloaded (S7, S16), recovering (S17), recovered (S8, S18), and developed (S9-S13, S19-S20) microbiomes. Each microbiome was sampled and sequenced in triplicate. Sampling points are labeled with pink circles. Bacterial and archaeal taxa with more than 8% relative abundance in at least one sample are listed. Taxa with less than 8% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 10% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
**Figure 7-2** Experiment II: Community relative abundance (A) and functional (B) dynamics of startup (P1, P2), developing (P3-P12), overloaded (P13, P14), and recovering (P15-P20) microbiomes. Each microbiome was sampled and sequenced in triplicate. Sampling points are labeled with yellow circles. Bacterial and archaeal taxa with more than 8% relative abundance in at least one sample are listed. Taxa with less than 8% relative abundance across all samples are grouped into one bar. Bars without labels represent less than 10% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Linking community dynamics and function to environmental stress level in AD microbialomes. The functional dynamics and compositional differences across microbiomes S1-20 were analyzed using NMDS statistics to link environmental condition (stress level), microbial community dissimilarity and overall function (methane yield) (Figure 7-3). In Experiment I (Figure 7-3A), Community 1 first showed selection-driven compositional shifts in microbiomes S1-S6 as the stress level was increased from 0%, to 46% then 66% GIW. Overloading was observed according to the functional profiles (Figure 7-1B, Community 1, Days 190-210) of S7 when the stress level was elevated to 84% GIW. When overloaded, S7 remained compositionally similar to previous assemblies, but functionally inhibited. After recovery, the pre-disturbance assembly was displaced by a new community (S8) which rebounded when re-stressed at 66% (S9-11) then 75% GIW (S12, S13). Community 2 was first overloaded at 66% GIW (S16) and became compositionally dissimilar to the previous startup communities (S14, S15). According to the reactor performance profiles (Figure 1B, Community 2, Days 263-280), the community gradually recovered as the methane yield and methane content increased, which led to assembly S17 that replaced the pre-disturbance populations. After recovery, community function was restored (Figure 7-1B, Community 2, Days 300-320), and became functionally similar to the startup microbiomes S14 and S15, while the compositional dissimilarity persisted (S18). Finally, Community 2 converged with Community 1 when re-stressed at 66% (S19) then 75% GIW (S20) (Figure 7-3A).
**Figure 7-3** NMDS statistics linking environmental conditions (arrow size representing stress level), microbial community dissimilarity (circles representing microbiome composition) and whole community function (environmental contours representing methane yield). NMDS statistics were constructed applying Bray-Curtis similarity index on taxa abundance profiles of bacterial and archaeal 16S rRNA amplicon sequences obtained from experiments I (A) and II (B). Environmental contours were calculated using methane yield data shown in Figures 1 and 2. Arrows indicate the order of sampling. The size of the arrow increases with stress level (% GIW loading).
Increasing levels of stress led to functionally and compositionally representative assemblies. We assessed how increasing levels of stress directed community assembly, and how changes in the community structure related to whole community performance in microbiomes P1-20 (Figure 7-3B). Initially, both startup microbiomes P1 and P2 clustered together. As the stress condition was increased, microbiomes gradually shifted to more functionally productive (in terms of methane yield) and compositionally dissimilar assemblages (P3-P12). This trend was observed regardless of the additional pulse-fed stress on Community 3, suggesting that the effect of pulse feeding on community assembly was minimal at the selected disturbance level (microbiomes P6-P9, Figures 7-2B and 7-3B). The pulse-fed pattern of stress at 60% GIW slightly shifted the community structure of P6-P9, compared to P5 and P10 communities that have been maintained at 30% GIW stress, but did not significantly influence the effect of higher levels of disturbance at 70% and 90% GIW on the subsequent community assembly in P11-P12 and P13-P20, respectively (Figure 7-3B).

During process failure on Day 138 (Figure 7-2B), overloaded microbiomes P13 and P14 became functionally and compositionally dissimilar from both the startup P1 and P2 and the productive assemblies P3-P12 (Figure 7-3B). These dissimilarities remained significant in the recovering microbiomes P15-P20 as the performance and effluent characteristics profiles (Figures 7-2B, 7-S3 and 7-S4) continued to indicate process inhibition. Taken together, the data suggested that increasing levels of stress guided the development of representative startup, productive, and recovering assemblies that were functionally and compositionally dissimilar from each other.
7.6 Discussion

Different stress histories led to different communities, which determined their susceptibility to the same or a new disturbance. To evaluate the effect of environmental stress history on community structure and performance, we compared assemblies of four reactor microbiomes S3, S8, S15, and S18 (Figures 7-1 and 7-3A). Microbiome S3 was influenced at 46% GIW and had not experienced any overloading; microbiome S8 resulted from gradual stresses of 46% and 66%, overloaded at 84% and recovered at 0% GIW; microbiome S15 was maintained at 0% GIW, representing the startup microbiome without any prior experience with GIW; microbiome S18 was overloaded at 66% and recovered at 0% GIW.

The startup microbiome S15 clustered more closely with other startup communities S1 and S14 (Figure 7-3A). Compositionally, S3, S8, S15 and S18 showed dissimilar taxonomic structures after exposure to different assembly patterns. However, except for S3 that was maintained at 46% GIW, the environmental contours show that the 0% GIW environment provided for S8, S15 and S18 resulted in similar methane yield of about 0.2-0.3 L/g-VS. The distribution of these communities suggested that microbiomes S8, S15, and S18 were functionally similar but remained compositionally dissimilar after disturbance. The assembly history affected the community structure, and these selection-driven changes in the community remained altered even when a common condition (the 0% GIW feed) was introduced. This indicates that either the new assemblies (S8 and S18) possessed taxa that were functionally redundant with the taxa in the original startup assemblies (S1, S14 and S15), or the new assemblies possessed taxa that were functionally dissimilar but resulted in
the same combined function (Allison and Martiny, 2008). Similar observations were also reported in other types of bioreactors (Vuono et al., 2015; De Vrieze et al., 2017) where a new assemblage that replaced the pre-dominant community after a disturbance persisted under similar conditions and performed similar function. Our results further demonstrate the presence of functionally redundant populations in two communities with different assembly histories (Figure 7-3A, Community 1 and Community 2) after a strong selection manipulation.

We applied a common environmental stress (66% GIW loading rate) to S3, S8, S15, and S18, in which populations were distinctly shaped by different disturbance histories as described previously. For microbial communities in S3 that were still adapting to the stress condition, this loading rate was a step up from the 46% GIW experienced earlier. For members in S8 that have recovered from a process inhibition, this selection pressure was less inhibitory compared to the 84% GIW feed experienced previously. Similarly, this disturbance was not foreign to populations in S18 that have just recovered from a process failure at 66% GIW. The resulting communities S6, S11, and S19 were more functionally productive (in terms of methane yield) and compositionally dissimilar compared to S16, the resulting assembly from S15 (Figure 7-3A). The performance data (Figure 7-1B) indicated inhibition of methanogenesis in S16, as the methane production dropped from 0.2 L-CH₄/g-VS added to close to zero, and methane content dropped from 60% to 40%. This was consistent with other signature parameters indicating process inhibition such as drops in pH, alkalinity, and reduction of COD and VS removal rates (Figures 7-S1 and 7-S2). Additionally, taxonomic structural distribution and dynamics showed convergence of
communities S6, S11, and S19, clustering with other functionally productive assemblies (Figure 7-3A). Conversely, inhibited community S16 and the following community S17 became compositionally and functionally more dissimilar from the productive populations.

Several studies on soil microbiomes have identified the influence of historical conditions on the response of microbial community to re-exposure of past disturbance or other stressors (Cregger et al., 2012; Evans and Wallenstein, 2012; Pagaling et al., 2014; Azarbad et al., 2016). Similarly, studies on AD microbiomes in disturbed environments demonstrated the link between improved community function and microbial adaptation due to specialized inoculum or strategic operating methods that led to favorable community shifts (Silvestre et al., 2011; Kougias et al., 2016; Ziels et al., 2016; Amha et al., 2017). Our results provide clear evidence that stress history is a key factor to community susceptibility. Compared to the relatively “inexperienced” microbiome S15, the presence of (or sufficient numbers of) specialized taxa obtained from different disturbance histories in microbiomes S3, S8, and S18 served as a “good starting point”, allowing them to be functionally less inhibited and compositionally more prepared for the same or higher level of stress. For these “experienced” microbiomes, the same or a new disturbance level resulted in an ecological convergence toward a common structure, irrespective of the difference in their stress histories (Figure 7-3A).

**Ecological shifts of dominant communities in response to different stress conditions reflected the niche preferences of individual taxa.** The taxon abundance profiles of Community 1 (Figure 7-1) showed that the abundance of individual taxa changed as the
stress intensity increased over time. Specifically, taxa of microbiomes S1 to S6 that had not experienced any overloading changed in abundance when the GIW feeding was increased.

The same stress condition incited different responses from different taxa consistently across time-series investigations in controlled environments, suggesting that these changes reflected their stress tolerance, or ecological niche preferences to the selective stresses. For example, microbiome S1 represents a startup assembly prior to any exposure to stress (at 0% GIW), while S6 represents a developing assembly in response to a lower level of stress (at 66% GIW) (Figure 1B). As the GIW loading was increased from 0%, to 46%, then 66%, taxa that decreased in abundance across microbiomes S2-S6 likely had a lower tolerance, or less favorable substrate preference to GIW disturbance. These populations included members of families SB-1 (Bacteroidetes) and Thermovirgaceae (Synergistetes), and genera SC103 (Thermotogae) and Candidatus Cloacamonas (WWE1) (Figure 7-1A).

Taxa that remained abundant or increased in abundance had a higher stress tolerance, or more favorable ecological strategies in response to the selective filtering. These consortia included bacterial members of order Bacteroidales, families Christensenellaceae (Firmicutes), Ruminococcaceae (Firmicutes), and Aeromonadaceae (Proteobacteria), and genera Sedimentibacter (Firmicutes), Syntrophomonas (Firmicutes), and vadinCA02 (Synergistetes), and W22 (WWE1), and archaeal members of Methanosaeta. The syntrophic relationship between methanogens and members of Firmicutes family Syntrophomonadaceae capable of using fatty acids with carbon chain lengths ranging from C4 to C18 has been well established (McInerney et al., 2008, 2009; Sieber et al., 2012). Our temporal investigations consistently reveal the increasing dominance of Syntrophomonas and Methanosaeta,
followed by *Methanospirillaceae* members, with increasing stress level before overloading (Figures 7-1A and 7-S5). *Syntrophomonas* increased in abundance from less than 1% (in S1, startup microbiome) to 21.7% (in S13, developed microbiome). *Methanosaeta* increased in abundance from less than 2.3% (in S1) to 10.6% (in S13). *Methanospirillum* increased in abundance from less than 1% (in S1) to 4.6% (in S13). Other closely related members of family *Methanospirillaceae* also increased in abundance from less than 1% (in S1) to 3.7% (in S13). These observations are consistent with Ziels et al. (Ziels et al., 2016) where *Syntrophomonas, Methanosaeta* and *Methanospirillum* were dominant populations in the AD treating waste cooking oil.

On the other hand, microbiome S17 of Community 2 (Figure 7-1A) represents a recovering assembly after a higher level of stress that caused a process failure (Figure 7-1B). Taxa that remained abundant or increased in abundance upon overloading as those identified in S17 likely were the “survivor members” that had specialized niche preferences and were more competitive under a higher stress condition. These members included genera *Prevotella* (Bacteroidetes), *Sporanaerobacter* (Firmicutes), and vadinCA02 (Synergistetes), and family *Ruminococcaceae* (Firmicutes).

*Phylogenetically diverse and functionally redundant populations filled specialized ecological roles and shifted at different stress levels.* To evaluate the ecological shifts of dominant communities across functional guilds, we mapped community members of microbiomes S1, S6, S15 and S17 (Figure 7-1) onto their corresponding metabolic functions in the anaerobic network (Figure 7-4). The major metabolic processes in anaerobic digestion include hydrolysis, acidogenesis, acetogenesis, and methanogenesis, which are carried out by
different groups of microbes occupying distinct ecological niches. We divided this process into five major groupings including three bacteria-driven and two archaea-driven metabolic pathways: (1) degradation of carbohydrates/proteins into amino acids/sugars and subsequently short chain fatty acids (SCFAs)/acetate; (2) degradation of lipids into long chain fatty acids (LCFAs)/alcohols and subsequently SCFAs/H₂/CO₂; (3) degradation of SCFAs into acetate/H₂/CO₂ and the conversion between acetate and H₂/CO₂; (4) degradation of acetate into methane; and (5) degradation of H₂/CO₂ into methane. Changes in the individual taxa abundance are displayed as percentage increase or decrease in relative abundance by comparing microbiomes S6 (developing assembly from a lower level of stress) with S1 (startup assembly prior to any exposure to stress) and S17 (recovering assembly from a process failure at a higher level of stress) with S15 (startup assembly prior to any exposure to stress). We identified several bacterial and archaeal clades that were dominant (>8% relative abundance) in assemblies S1, S6, S15 and S17. We acknowledge the limitations of 16S rRNA gene-based analysis in definitive correlations to function (Vanwonterghem et al., 2014; Prosser, 2015). However, the metabolic pathways in anaerobic digestion are well known, and mapping changes in relative abundance to their metabolic pathways based on inferred uses of substrates from previous literature (as detailed in the Supplemental Information) can be justified as a first step in community-function analysis.

The results of metabolic network analyses (Figure 7-4) show that depending on the stress levels, microbiomes developed diverse groups of core populations that filled specialized ecological roles, and these deterministic processes occurred across different functional guilds. At zero disturbance level (startup microbiome S1), each of the major
anaerobic metabolism niches, such as hydrolysis, acidogenesis, acetogenesis, and methanogenesis, was fulfilled by a reservoir of phylogenetically diverse populations that share common resources. At a lower level of disturbance (developing microbiome S6), community assembly appeared to shift to a more selective population where each metabolic network was occupied by a smaller pool of phylogenetically diverse communities. At a higher level of disturbance that caused a process overloading (recovering microbiome S17), each guild was selectively enriched into an even smaller subset of potentially functionally redundant populations that showed higher ecological tolerance.

Within the metabolic pathway of carbohydrate/protein/amino acid/sugar degradation, SB-1 (Bacteroidetes), *Thermovirgaceae* (Synergistetes), and *Candidatus Cloacamonas* (WWE1) showed a lower tolerance to GIW stress. Some of the other Bacteroidetes members, *Sedimentibacter* (Firmicutes), *Aeromonadaceae* (Proteobacteria), and W22 (WWE1) had a more favorable niche at a lower level of GIW. *Prevotella* (Bacteroidetes), *Sporanaerobacter* (Firmicutes), and *Ruminococcaceae* (Firmicutes) appeared to be the survivor populations for community recovery from a higher level of stress that caused a process failure. In the lipid/LCFA degradation pathway, *Christensenellaceae* (Firmicutes) had a higher tolerance to a lower level of GIW, while vadinCA02 (Synergistetes) was identified as a survivor member under a higher level of GIW stress. In SCFA degradation and conversion between acetate and H₂/CO₂, *Thermotogaceae* (Thermotogae) showed less preference to the selective pressure, while *Syntrophomonas* (Firmicutes) had more tolerance to a lower level of stress. In the conversion of acetate to methane, *Methanoseta* members survived and dominated at a lower level of stress; while *Methanosarcina* community increased significantly during recovery.
after exposure to a higher level of stress. In the conversion of H₂/CO₂ to methane, WSA2 (Methanobacteriales), *Methanomassiliicoccus* (E2), and *Methanolinea* (Methanomicrobiales) showed less tolerance to GIW. On the other hand, Methanomicrobiales members *Methanoculleus* and *Methanospirillaceae* increased in abundance at a lower level of stress, while *Methanoregulaceae* (Methanomicrobiales) appeared to be the dominant survivor group during recovery.

Previous studies on co-digestion of GIW or related substrates have predominantly focused on the abundance dynamics of syntrophic bacteria and methanogenic archaea (Kougias et al., 2016; Ziels et al., 2016, 2017; Amha et al., 2017). Our results indicate dynamic shifts of dominant members within each functional guild that had more tolerance to the selective stress. The population shifts not only reflected the niche preferences of individual taxa, but also indicated functional redundancy of different populations across metabolic pathways. This mechanism ensured the maintenance of an ecological function when a group of taxa was removed or inhibited under stress (Briones and Raskin, 2003; Botton et al., 2006; Allison and Martiny, 2008). This niche overlap has also been observed in bioreactor microbiomes driven by selective pressure of cellulose treatment (Vanwonterghem et al., 2014), change of solids retention time (Vuono et al., 2015) and salinity (De Vrieze et al., 2017).
Figure 7-4 Metabolic network of anaerobic digestion mapped with associated functional profiles of selected bacterial (>8% relative abundance) and all archaeal taxa identified in the step feeding experiment. Changes in the individual taxa abundance are presented as percent increase or decrease in relative abundance by comparing microbiomes S6 with S1 (small arrow) or S17 with S15 (large arrow). S1 and S15 communities represent a startup assembly prior to exposure to stress. S6 community represents a developing assembly (at 66% GIW stress) before overloading. S17 community represents a recovering assembly after overloading (at 66% GIW stress). This reconstruction is based on inferred uses of substrates from previous literature and not intended to provide a complete description of the functional potentials and ecological roles of these taxa.
Community dynamics of low-abundance populations. The abundance and function profiles (Figures 7-1, 7-2, 7-4 and 7-S5) demonstrate that the initially low-abundance populations were capable of becoming dominant after a major disturbance event. Bacterial populations such as Prevotella, Sporanaerobacter, Ruminococcaceae, Christensenellaceae and Aeromonadaceae were initially low-abundance (<0.01% relative abundance) but became prevalent (>8% relative abundance) at one or multiple time points when exposed to different levels of stress. The archaeal community remained low-abundance (<0.01% relative abundance) at all time points (except Methanosaeta) (Figures 7-1, 7-2 and 7-S5) but the metabolic network analyses (Figure 4) reveal significant increases in both aceticlastic (Methanosarcina) and hydrogenotrophic populations (Methanoculleus, Methanospirillaceae, and Methanoregulaceae) at higher levels of stress.

Exploration of the low-abundance microbial populations (the rare biosphere) in bioreactors is currently limited, unlike other ecosystems (Galand et al., 2009; Sjöstedt et al., 2012; Shade et al., 2014; Lynch and Neufeld, 2015). Our results demonstrate that in bioreactors low-abundance members across metabolic networks responded to selective pressure and some low-abundance taxa switched from rare to abundant after a disturbance event as key “survivors”. However, more deeply sequenced data and time-series validation are needed to determine their contribution to community assembly and function after GIW disturbance.

Are Christensenellaceae populations key to lipid metabolism in GIW co-digesters? The abundance and network profiling (Figures 7-1, 7-2 and 7-4) revealed Christensenellaceae as potential lipid/LCFA metabolizers with higher tolerance to GIW
stress. Interestingly, Everard et al. (Everard et al., 2014) observed significant increases in abundance of vadinCA02 (Synergistetes) under a high-fat diet in gut microbiomes of prebiotic-treated mice, while Goodrich et al. (Goodrich et al., 2014) identified potential keystone characteristics of Christensenellaceae (Firmicutes) associated with a lean body mass index in human gut microbiomes. Their results also indicated reduced weight gain associated with Christensenellaceae when inoculating germ-free mice with lean and obese human fecal samples and when amending an obese-associated microbiome of a donor stool with Christensenella minuta. Although the exact roles of these populations remain to be established, these findings, as well as our results revealing their increases in abundance under high levels of GIW stress (Figures 7-1, 7-2 and 7-4), raise questions about (1) whether the ecological role of these populations was associated with enhanced degradation of lipid-rich substrates in gut environments, and (2) whether an increased methane yield in a GIW-challenged digester would also be related to the enrichment of these populations. Although lipid-degrading consortia have been identified in pure and mixed cultures (Serikovna et al., 2013; Phong et al., 2014), the identities of core populations, elucidation of their ecological roles, and functional networks associated with GIW degradation and methane production have not been identified in digester microbiomes at high GIW loadings.

**Productive microbiomes became less diverse and less even under niche selection.**

The startup microbiomes were more diverse and even (Figure 7-5), while microbiomes with a stress history (i.e., developing and developed microbiomes) became less diverse and less even. This linear relationship only applies to the functioning communities and not the overloaded and recovering microbiomes. Studies have suggested that higher community
diversity or evenness resulted in more robust function (Wittebolle et al., 2009; Werner et al., 2011, 2014) because it ensures more suites of populations with a wide range of metabolic possibilities. On the other hand, decreases in diversity and evenness associated with enhanced function were also observed (Pholchan et al., 2013). Here we show that in anaerobic bioreactors, higher diversity or evenness did not correlate with increased community function under high levels of GIW stress. Rather, upon exposure to stress, communities were selectively re-assembled into a less diverse and less even, but more specialized community associated with better function.
Figure 7-5 The relationship between community function and diversity of microbiomes in Experiments I and II at different stress levels. Diversity was calculated from 10 rarefactions of 24,000 sequences each. Higher rarefaction depths showed similar results. Methane yield of each sample (shown in Figures 1 and 2) is plotted against (A) community richness (observed OTU counts), (B) Shannon index, (C) Simpson’s evenness index, and (D) Gini coefficient.
7.7 Supporting information

EXPERIMENTAL METHODS

Functional implications of key taxa identified in the metabolic network analyses (Figure 7-4)

We identified the following bacterial lineages as the core members involved in the metabolism of carbohydrate/protein/amino acid/sugar: (1) order Bacteroidales (Bacteroidetes); (2) families Ruminococcaceae (Firmicutes), Aeromonadaceae (Proteobacteria), Thermovirgaceae (Synergistetes), and Cloacamonaceae (WWE1); and (3) genera Prevotella (Bacteroidetes), Sedimentibacter (Firmicutes), and Sporanaerobacter (Firmicutes).

- Bacteroidetes populations have been identified as the main potential metabolizers of carbohydrate-based substrates among those dominant taxa in various types of microbiomes, such as in maize-fermenting digesters (Hanreich et al., 2013), digester treating alpha-cellulose (Vanwonterghem et al., 2014), and combined mesophilic anaerobic and thermophilic aerobic digesters treating food wastewater (Jang et al., 2015).

- Members of Firmicutes family Ruminococcaceae have been studied in diverse gut communities and identified as polysaccharide degraders. For example, cellulosolytic rumen specialist Ruminococcus flavefaciens possesses a cellulosome complex that consists of a scaffoldin(s) and cellulosomal enzymes for efficient degradation of plant cell walls (Ding et al., 2001; Flint et al., 2008). Amylolytic bacterium Ruminococcus bromii has been shown to play an important role in the human colon as its degradative capability and activities with other gut microbiome members were
found key in the utilization of dietary resistant starches (Abell et al., 2008; Ze et al., 2012). In replicate anaerobic reactors, Vanwonterghem et al. (2014) observed convergence in population dynamics during hydrolysis and digestion under the selective pressure of alpha-cellulose where members of order Bacteroidales and genus *Ruminococcus* had a significant correlation to higher VFA concentrations, and were likely the main cellulose degraders during the initial hydrolysis. Based on these results, we placed *Ruminococcaceae* in the carbohydrate/protein/amino acid/sugar degradation pathway.

- Members of *Aeromonas* can utilize a wide range of sugars such as D-glucose, D-galactose, maltose, D-mannitol, D-mannose, and sucrose, among others (Hickman-Brenner et al., 1987; Binet et al., 1998). Thus sequences affiliated with Proteobacteria family *Aeromonadaceae* were categorized into the carbohydrate/protein/amino acid/sugar metabolism, although other genera such as *Oceanimonas* and *Tolumonas* in this family may occupy different niches.

- Sequences affiliated with family *Thermovirgaceae* belong to phylum Synergistetes, which has been found to degrade amino acids (Vartoukian et al., 2007; Göker et al., 2012). Members of Synergistetes, though normally found subdominant in bacterial ecosystems (Vartoukian et al., 2007), have been identified in a lab-scale anaerobic digester during an acetate crisis (Delbès et al., 2001). Ito et al. (2011) confirmed the metabolic function of Synergistetes members to utilize acetate, as well as their competitive significance to aceticlastic methanogen *Methanosaeta* at high acetate concentrations. Members in the phylum Synergistetes include *Thermovirga lienii*, a
strain originally isolated from hot oil-well production water from a reservoir in the North Sea (Dahle, 2006). Members of this community have a fermentative metabolism and utilize amino acids, proteinaceous substrates, and selective organic acids (but no sugars, fatty acids, or alcohols), producing ethanol, acetate, propionate, isovalerate/2-methylbutyrate, H₂, and CO₂ (Dahle, 2006; Göker et al., 2012). Based on the above studies, we tentatively classified Thermovirgaceae as involved in carbohydrate/protein/amino acid/sugar degradation, while recognizing other inferred niche potentials of these populations (e.g. acetate degradation).

- Member of the WWE1 family Cloacamonaceae have been implicated in the syntrophic metabolism of amino acid and oxidative degradation of propionate (Pelletier et al., 2008; Sieber et al., 2012). Thus we placed this group in the pathway of carbohydrate/protein/amino acid/sugar metabolism.

- Rumen Prevotella populations, members of the Bacteroidetes group Prevotellaceae, are capable of utilizing various carbohydrates, producing formate, acetate, and succinate that can be subsequently decarboxylated into propionate by rumen bacteria (Bryant et al., 1957; Strobel, 1992; Purushe et al., 2010). Mitsumori et al. (2012) linked the shifted dominance of Prevotella to their possible use of hydrogen for fermentation of sugars and lactate, resulting in production of propionate. In a study of human microbiome, analyses of fecal samples from 98 individuals revealed that gut enterotype clustering was strongly correlated with long-term diets of the host, and differed primarily between levels of two microbial taxa: Prevotella associated with carbohydrate-based diet, and Bacteroides associated with protein
and animal fat-based diet (Wu et al., 2012). Based on these results, *Prevotella* was identified as metabolizers of carbohydrate/protein/amino acid/sugar.

- Members of Firmicutes genus *Sedimentibacter* are capable of utilizing amino acid and pyruvate, producing acetate and butyrate as the main fermentation products, and propionate, lactate and traces of isobutyrate and isovalerate as the minor products (Breitenstein et al., 2002). *Sedimentibacter* is therefore classified into the carbohydrate/protein/amino acid/sugar metabolic pathway.

- *Sporanaerobacter acetigenes*, a member of Firmicutes family *Tissierellaceae*, was identified as an acetogenic, sulfur-reducing bacterium capable of utilizing some sugars, peptides, and various single amino acids with acetate, isobutyrate, and isovalerate as end products (Hernandez-eugenio et al., 2002; Alauzet et al., 2014). Thus we classified this group in the degradation pathway of carbohydrate/protein/amino acid/sugar.

We categorized members of the family *Christensenellaceae* (Firmicutes) and genus vadinCA02 (Synergistetes) into the lipid/LCFA degradation pathway. More discussions on these populations are in Results and Discussion (main text).

- Host genetics and gut microbiome association with heritability and obesity were assessed by comparing microbiota across fecal samples obtained from populations of twin pairs (Goodrich et al., 2014). *Christensenellaceae* was identified as both the most highly heritable taxon and the hub in a microbial network of co-occurring heritable microorganisms associated with a lean body mass index. Fecal microbiota
transplant studies show that *Christensenellaceae* was associated with reduced weight gain in germ-free mice inoculated with lean and obese human fecal samples. Moreover, addition of *Christensenella minuta* to an obese-associated microbiome of a donor stool lacking detectable *Christensenella* resulted in reduced weight gain and altered microbiomes of the recipient mice. Based on the above, we classified Firmicutes family *Christensenellaceae* into the metabolic pathway of lipid/LCFA degradation to emphasize their potential role in the degradation of lipid-rich materials.

- Sequences affiliated with Synergistetes member vadinCA02 (within family *Synergistaceae*) were identified in gut microbiomes of prebiotic-treated mice under a high-fat diet (Everard *et al.*, 2014). The study showed significant increases in abundance of genera vadinCA02 (affiliated with phylum Synergistetes) and decreases of *Prevotella* (affiliated with phylum Bacteroidetes) to be associated with a high-fat diet. Their exact function in anaerobic environments remains to be identified, but based on the above studies, we tentatively classified vadinCA02 as members involved in lipid/LCFA degradation.

We classified *Thermotogaceae* (Thermotogae) and *Syntrophomonas* (Firmicutes) as the core members involved in SCFA degradation and conversion between acetate and H$_2$/CO$_2$.

- Thermotogae populations have been implicated in the fermentation of carbohydrate-based materials (Conners *et al.*, 2006; DiPippo *et al.*, 2009; Schut and Adams, 2009; Kazanov *et al.*, 2013). The genetic potentials of members of Thermotogae and
candidate phylum WWE1 were revealed to syntrophically oxidize butyrate to CO₂, H₂, and acetate, in addition to the syntrophic metabolism by *Syntrophus* (Lykidis *et al.*, 2011; Sieber *et al.*, 2012). In a subsequent ecogenomics study, Nobu *et al.* (2015) suggested that Thermotogae-related *Mesotoga* and *Pseudothermotoga* populations perform syntrophic oxidation of acetate. Thermotogae members were proposed as unconventional syntrophic acetate degraders due to the lack of a complete butyrate degradation pathway detected. Here we identified this group to be involved in the degradation of SCFA and conversion between acetate and H₂/CO₂. The syntrophic potential of Thermotogae revealed by Lykidis *et al.* (2011) and Nobu *et al.* (2015) represents an addition to the known syntrophic metabolism fulfilled by *Syntrophomonas* or *Syntrophus*. In this study, *Syntrophomonas* and *Syntrophus* were identified dominant (over 8% relative abundance) and subdominant (less than 8% relative abundance), respectively, across samples from both step and pulse feeding experiments.

- Members of Firmicutes family *Syntrophomonadaceae* are specialists capable of using fatty acids with carbon chain lengths ranging from C4 to C18 in syntrophic association with methanogens (McInerney *et al.*, 2008, 2009; Sieber *et al.*, 2012), and thus categorized in SCFA degradation and conversion between acetate and H₂/CO₂.

Archaea from order Methanosarcinales (including genera *Methanosaeta* and *Methanosarcina*) are known aceticlastic methanogens, while some members are capable of performing other methanogenic pathways (Demirel and Scherer, 2008; Mori *et al.*, 2012; Zhu
Members from orders Methanobacterales and Methanomicrobiales (including genera *Methanolinea*, *Methanospirillum*, and *Methanoculleus*) are hydrogenotrophic methanogens that can grow on H$_2$/CO$_2$ and produce methane (Demirel and Scherer, 2008; Thauer *et al.*, 2008; Maus *et al.*, 2012; Sakai *et al.*, 2012; Parshina *et al.*, 2014). Archaeal members of *Methanomassiliicoccus*, representing a previously unknown putative new lineage of methanogens belonging to the candidate order E2, have been proposed to be obligate H$_2$-dependent methylotrophic methanogens that can reduce methylated compounds to methane with H$_2$ as the electron donor (Dridi *et al.*, 2012; Borrel *et al.*, 2013; Iino *et al.*, 2013; Poulsen *et al.*, 2013).
Figure 7-S1 Step fed microbiome: organic loading rate (OLR) and effluent characteristics (volatile solids (VS) and COD concentrations, and VS and COD reduction rates).
Figure 7-S2 Step fed microbiome: organic loading rate (OLR) and effluent characteristics (pH, alkalinity, total volatile acids, and total volatile acids/alkalinity ratio).
Figure 7-S3 Pulse fed microbiome: organic loading rate (OLR) and effluent characteristics (volatile solids (VS) concentration, VS reduction rate, pH, and alkalinity).
Figure 7-S4 Pulse fed microbiome: organic loading rate (OLR) and effluent characteristics (acetate, propionate, butyrate, and valerate).
Figure 7-S5 Abundance dynamics of archaeal community in Experiments I (S1-20) and II (P1-20). Each microbiome was sampled and sequenced in triplicate. Bars without labels represent less than 2% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.
Chapter 8 Conclusions

A long-standing question in bioreactor engineering is what ecological patterns and processes determine the performance and functional stability of methanogenic biosystems and how this complex microbial network can be improved.

In chapter 4, using eight different types of substrate, I conducted the first common garden experiment for GIW co-digestion, and showed that:

- Despite identical inoculum, operating conditions (e.g., substrate loading rate, digester system setup, solid retention time), and performance (methane production and effluent quality) during the pre-disturbance stage, feed-driven community structure in response to GIW perturbations of increased intensity was observed.

- Substrate history (feed selection and intensity of preshocks) influenced functional resistance and resilience and overall digester survivability when stressed at high GIW concentrations.

- The highest GIW loading rate achieved without inhibition was 42 % (v/v), 63% COD, or 58% (w/w) of VS added, with a total OLR of 1.74, or OLR of substrate of 1.01 g-VS/L/day. Methane content was increased from 62.3% to 74.1%, and the highest methane yield achieved was 453 mL-methane/g-VS added, or 218.6 mL-methane/g-COD (450% increase) compared to the control digester.
In chapter 5, I investigated the causality between microbial community composition and functional dynamics in response to different overloading stresses, and demonstrated that:

- Microbial community dissimilarity due to substrate history (feed selection and intensity of preshocks) and the presence of key microbial populations determined the digesters’ ability to survive GIW overloading stresses.

- While *Methanosaeta* and *Syntrophomonas* were the major contributors to community survivability, other sub-dominant co-occurring taxa were exclusively harbored in resilient and resistant digester microbiomes, indicating their potential roles in reducing GIW toxicity and enhancing functional resilience and resistance.

- These sub-dominant taxa included family *Methanospirillaceae*, bacterial orders Bacteroidales (Bacteroidia) and Rhizobiales (Alphaproteobacteria), families *Saprospiraceae* (Bacteroidia) and *Methylcystaceae* (Alphaproteobacteria), and genera *Paludibacter* (Bacteroidia), *Sporanaerobacter* (Clostridia), *Comamonas* (Betaproteobacteria), *Syntrophus* (Deltaproteobacteria), and *Methylocaldum* (Gammaproteobacteria).

- Based on the results, a feed-specific community adaptation strategy using substrate selection and overloading stresses was developed to achieve resilient and resistant GIW co-digestion.
In chapter 6, the community adaptation strategy was successfully validated in two independent bioreactor experiments using step and pulse feedings of GIW. Specifically, the results showed that:

- The community adaptation strategy resulted in better microbial adaptation compared to the control, and reduced the inhibitory effect of GIW.

- Gradual addition of GIW, up to 75% (w/w) of VS added, increased methane yield by 336% from 0.180 to 0.785 L-methane/g-VS added, the highest value reported to date for co-digestion of GIW.

- Pulse feeds of GIW increased methane yield from 0.134 to 0.748 L-methane/g-VS added at 70% (w/w as VS) GIW and increased digester resistance against GIW inhibition.

In chapter 7, I conducted the first comprehensive time-series investigation of long-term (>200 days) microbial community and functional dynamics of GIW co-digesters with different loading stress histories. The results indicated that:

- Microbiomes with different stress histories became compositionally dissimilar but remained functionally similar under similar disturbance intensity.

- When re-stressed at a new or similar level, microbiomes that have been previously disturbed converged to a common structure, regardless of the difference in disturbance histories.

- Dominant microbial populations showed functional redundancy and selection-driven shifts within different metabolic networks before and after overloading.
At higher substrate loadings, communities became less diverse and less even, but more specialized with better function.

Microbiomes in anaerobic digesters could be selectively assembled using specific operational conditions to improve general community function.

In this dissertation, I demonstrated the potential to achieve better bioenergy resource management when biological engineering questions are tested using ecological frameworks and showed how reactor microbiomes can be influenced to improve their resilience and resistance through selection of specialized (necessary and supportive) microbial populations. Based on the results, I developed a feed-specific community adaptation strategy to reduce GIW inhibition and successfully validated this strategy in two independent bioreactor experiments. The community adaptation strategy through substrate selection and proper intensity of preshocks resulted in significant enhancement of methane production and development of resistant GIW co-digestion microbiomes. This dissertation represents the first complete demonstration on exact approaches using substrate selection and overloading stresses to pre-select for more stress-tolerant members across anaerobic functional guilds and achieve high-performance GIW co-digestion.
Chapter 9 Future work

**Full scale applications.** One key finding of this research is that we were able to develop a “map” to high-performance GIW co-digestion. This map is constructed based on hypothesis-driven experiments and comprehensive microbial community analyses, and was successfully validated in two independent experiments using GIW from different sources. For full-scale applications, operators can follow the directions to use “the right feed” at the “right dose” for each step feeding phase and actively pre-select more GIW-tolerant microbial populations to achieve high-yield yet resilient and resistant digester operation.

Specifically, if starting from 0% GIW addition, operators can step feed at an OLR of 0.4-0.6 g-VS of GIW/L/day (within Zone 1) to increase methane production by at least 100%, operate for at least one cycle of SRT, and further step feed at an OLR of 1.0-1.2 g-VS of GIW/L/day (entering Zone 2) to achieve at least another 100% increase in methane yield. After two step feeding phases, methane content in the biogas can be increased from 60% to at least 70%, and up to 74%, most likely in three SRT cycles. Based on bioreactor experiments and microbial community analyses, “no step feeding”, “jumping too far” and “using the wrong feeds” were the main contributors that led to inhibited microbial community and digester failure at OLR of GIW higher than 1.0 g-VS of GIW/L/day. The results also demonstrated that after overloading at high GIW stresses, digester community was robust enough to bounce back to the original community structure when a proper “resting” period was allowed and achieved the same level of performance when re-stressed.
This map has been validated using four different lipid-rich materials, including pure rice bran oil (cooking oil) and GIW from three types of restaurants (serving steaks, burgers, or buffet) and uses the most common and more easily measured parameter (volatile solids) to quantify GIW concentration in the feedstock. Future work to further improve the adaptation map include: (1) in addition to GIW from the restaurants, validating this approach using a much more diverse GIW category (e.g., meat processing plants, trapped FOG wastes from wastewater treatment plants), (2) evaluating the microbial recovery mechanisms that can lead to faster recovery, and (3) comparing microbial data from the common garden and step and pulse feeding experiments within Zone 1 to evaluate the exact mechanisms behind the “0.6-0.7 OLR per jump” limitation.

**Microbial community assembly.** Understanding the microbial assembly mechanisms directing the function and stability of engineered microbiomes will enable further development and optimization of GIW co-digestion engineering. Specifically, knowledge gained from the identities of core populations that coexist across metabolic networks and their ecological potentials can lead to more fundamental design and guide future full-scale applications. For example, if there is an “optimal” assembly of core populations whose combined ecological roles exert an optimal network of functions, this assembly will reduce inhibition at high GIW levels and enhance biogas production with stable inter-species metabolic interactions. I expect this dissertation to bring more insights on such assembly and the “rules” for more fundamental engineering of digester startup. Future work for GIW co-digestion includes: (1) a comprehensive investigation of the metabolic pathways enhanced through the developed community adaptation strategy using metatranscriptomics; (2) key
pathways that lead to high-performance GIW co-digestion; (3) possible approaches for faster assembly; and finally (4) the construction of a “map” that can direct the assembly of target microbial communities and metabolic networks based on site-specific (different plant configurations) conditions at full-scale facilities and predict the “health” of digester microbiomes facing dynamic environmental disturbances. These discoveries will enable broader and more cost-effective applications of this biotechnology.
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APPENDICES
Appendix A

GIW sampling at a FSE.
Steps: At the manhole, collect FOG at the top layer using a scoop, service provider pumped out remaining FOG, collect wastewater at the middle layer, service provider pumped out remaining wastewater, collect food solids at the bottom layer, service provider pumped out remaining entire content of the GI, finishing their routine pumping service.
Appendix B

Reactor design

Design 1 for substrate containing smaller particles (e.g. TWAS, carbohydrate)

Design 2 for substrate containing larger particles (e.g. food solids)
## Appendix C

### List of supplies

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<thead>
<tr>
<th>Vender</th>
<th>Description</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reactor body</strong></td>
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<tr>
<td>US Plastic</td>
<td>1 gallon PP Jar; 4.91&quot;Dia. x 8.38&quot;H</td>
<td>71168</td>
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<tr>
<td></td>
<td>1/2 gallon PP Jar; 4.91&quot;Dia. x 8.38&quot;H</td>
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<tr>
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<td>1 Quart PP Jar; 4.91&quot;Dia. x 8.38&quot;H</td>
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<tr>
<td><strong>Sampling/Feeding port</strong></td>
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<tr>
<td>Cole-Parmer</td>
<td>Bulkhead Connectors, 1/4&quot; NPT(F) to 27/32&quot; OD, PVC</td>
<td>EW-06445-10</td>
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<tr>
<td></td>
<td>Female luer x female luer adapter, PP, 25/pk</td>
<td>45508-22</td>
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<td></td>
<td>Male luer lock x 1/4&quot; NPT, Black Polypropylene, 10 Pack</td>
<td>EW-30505-52</td>
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<tr>
<td>Mcmaster</td>
<td>Miniature PVC Ball Valve, Low-Flow, NPT Male x Male, 1/4&quot; Pipe Size</td>
<td>4757K16</td>
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<td>Miniature PVC Ball Valve Low-Flow, NPT Female x Male, 1/4&quot; Pipe Size</td>
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<td><strong>Reactor lid</strong></td>
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<tr>
<td>Mcmaster</td>
<td>Lightweight Quick-Turn Tube Coupling, Chemical Resistant Barbed Socket, for 1/4&quot; Tube ID, packs of 10</td>
<td>51525K29</td>
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<td>Lightweight Quick-Turn Tube Coupling, Chemical Resistant Barbed Plug, for 1/4&quot; Tube ID, packs of 10</td>
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<td>High-Strength Quick-Turn Tube Coupling, Brass Barbed Plug, for 1/4&quot; to 5/16&quot; Tube ID</td>
<td>51465K117</td>
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<td>High-Strength Quick-Turn Tube Coupling, Brass Barbed Socket, for 1/4&quot; to 5/16&quot; Tube ID</td>
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<tr>
<td>Cole-Parmer</td>
<td>Tygon Lab Tubing, Non-DEHP, 1/4&quot;ID X 3/8&quot;OD, 50 FT/PACK</td>
<td>WU-06407-80</td>
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<td>Stopcocks with Luer Connections; 3-way; male lock; 5 flow pattern; Non-sterile, pk pf 100</td>
<td>EW-30600-07</td>
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<td>Thermo Scientific Nalgene® barbed bulkhead fitting kit, 1/4&quot; tubing ID, pk of 2</td>
<td>EW-06259-10</td>
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<td><strong>Clamps</strong></td>
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<tr>
<td>Speedy</td>
<td>Size &quot;BB&quot; Double Grip Hose Clamp (Min .36&quot; - Max .41&quot;) 100 pieces</td>
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<td><strong>Feedstock</strong></td>
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<tr>
<td>Fisher</td>
<td>Starch, Soluble (Powder/Certified ACS), Fisher Chemical; 500g</td>
<td>S516-500</td>
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<td>Dextrose (D-Glucose) Anhydrous (Granular Powder/Certified ACS), Fisher Chemical; 3kg</td>
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<td></td>
<td>GNC Pro Performance® AMP Amplified Wheyolic Extreme 60™ - Vanilla</td>
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<td>XX Rice bran oil</td>
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<td><strong>COD analysis</strong></td>
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<tr>
<td>HACH</td>
<td>COD Digestion Vials, High Range Plus, 200 to 15,000 mg/L COD, pk/150</td>
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<td><strong>VFA analysis</strong></td>
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<tr>
<td>VWR</td>
<td>Acrodisc® 0.8/0.2 µm Syringe Filters 25 mm with Supor® Membrane, Pall Laboratory, pack of 50</td>
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<td>MicroLiter 0.2 µm Nylon Syringe Filters 17 mm with 1 µm glass fiber Prefilter Pack of 100</td>
<td>97048-610</td>
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<td>MicroLiter 0.2 µm Nylon Syringe Filters 13 mm with 1 µm glass fiber Prefilter Pack of 100</td>
<td>97048-598</td>
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</tbody>
</table>
Appendix D

Reactor setup
Run 1: two types of feeds, two mother digesters and then three daughters, repeat run 4 times (for 8 types of feeds) at mid and high GIW dose.

Run 2: eight types of feeds with triplicates at low GIW dose.