INVESTIGATION OF THE DEGRADATION OF LIGNOCELLULOSIC MATERIALS IN ANAEROBIC DIGESTION

By

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ABSTRACT

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Biogas produced through anaerobic digestion of lignocellulosic materials, is largely recognized as one of the only carbon negative fuel sources. The study consisted of two parts; 1) codigestion of AFEX^{TM}-pretreated corn stover and cow manure and 2) degradation of plant cell wall components (different compounds) in agricultural biogas plants. It was concluded from the first part of the study that AFEX^{TM}-pretreated corn stover promotes conversion of methane production in anaerobic digestion but has a smaller impact on consumption of the plant cell wall components. The corresponding biogas production (213 L/kg VS loading) of the AFEX treated co-digestion was 22% higher than that (175 L/kg VS loading) of the untreated co-digestion. The second part of the study led to the conclusion that biogas is produced mainly from nonstructural carbohydrates in the influent, and the plant cell wall makes a smaller contribution to biogas generation. Was observed greater correlations between the biogas productivity and the reduction of two organic components (TOC=17.8% & Protein=18.1%). On the other hand, lower correlations were detected between the consumptions of the plant cell was components (Lignin=12.5%, Cellulose=3.7% & Xylan=0%) and the biogas productivity.

I want to dedicate this work to my friend Dr. Shenpan Lin, for being such an important part of my journey, we miss you! You’re the man of a thousand hearts!!

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1. INTRODUCTION

1.1. Statement of the Problem

The world is currently transitioning from fossil fuel–dominated energy sources to more renewable-energy–based approaches, like bioenergy technologies. Bioenergy generation has been promoted in many countries (e.g., Germany, Sweden, Italy, and USA) by tax incentives and public policies. However, the extensive application of bioenergy technologies is not yet a reality around the world. Many technologies such as pyrolysis, gasification, transesterification, and saccharification are still at the bench and pilot scale. Anaerobic digestion (AD), however, the most widely applied bioenergy technology, has been deployed globally at residential and commercial scales. The advantages of AD over other bioenergy technologies are the production of renewable energy in addition to organic-waste disposal, environmental protection, and greenhouse emission reduction (Mao, Feng, Wang, & Ren, 2015).

Application of AD to convert recalcitrant carbon into usable carbon—methane—attracted increasing attention in recent years. Recalcitrant carbon is most commonly found in nonfood biomass, or lignocellulosic biomass (e.g., corn stover, wood biomass), specifically in the plant cell wall (Keegstra, 2010). This advancement may significantly increase the diversity of feedstocks for methane production and contribute to non–food-based bioenergy generation. Because of the recalcitrant nature of lignocellulosic biomass, pretreatment needs to be utilized to treat biomass before digestion. Numerous efforts have been made to develop pretreatment processes. One of the most developed pretreatments is Ammonia Fiber Expansion (AFEX^{TM}). The goal of the AFEX^{TM} process is to break important linkages between plant cell wall components, thus paving an accessible way to usable carbon sources. The AFEX^{TM} -technology has reached the pilot scale operation and could be a valid pretreatment process for AD of lignocellulosic biomass.

Many countries worldwide have established public policies to promote the AD technology and its implementation. For example, countries like the United States (US) have the Renewable Fuel Standard (RFS). The RFS promotes the production of biogas from lignocellulosic sources and manufacture of "renewable natural gas" in the D3/D7 category known as cellulosic fuel (from a lignocellulosic source) (EPA, 2014). This framework requires obligated parties involved in the vehicle fuel supply chain to source a portion of the fuel from renewable lignocellulosic feedstocks. For biogas producers utilizing animal manures, biosolids, or landfill gas this provides new opportunities to market renewable natural gas (methane sourced from biogas). However, the degradation of cellulosic biomass and its contribution to biogas production via AD are not fully understood.

To fulfill the needs mentioned above, this thesis covers two important topics: codigestion of pretreated lignocellulosic biomass and degradation of lignocellulosic biomass during the digestion process.

1.2. Goals & Objectives

To achieve the project goals, the following specific objectives were pursued:

"*Dynamic microbiome assembly and the effect on the performance of AD of AFEX-pretreated corn stover and conventional corn stover.*"

a) To study the impact of codigestion of conventional corn stover and AFEX-pretreated corn stover on digestion performance.
b) To elucidate dynamic changes of microbial communities during the codigestion.
c) To identify correlations in order to explain the relation between digestion performance and microbial communities.

"*Degradation of lignocellulosic feedstocks at agricultural biogas plants*"

d) To perform broad characterization of the influents and effluents of different commercial agricultural biogas plants (ABPs) by different methods from the literature.

e) To carry out the biomethane potential testing of from influent samples.

f) To determine the consumption of different components in the influent samples and their contribution to biogas production.

g) Conduct a sensitivity analysis to rank the importance of the components in terms of biogas productivity.

2. LITERATURE REVIEW

In 2017, renewable power accounted for 70% of net additions to global power-generating capacity (REN21, 2018). The uses of this energy are diverse in such sectors as heating and cooling, power, and transportation. Contributing to this progress are advancements in enabling technologies and political efforts to develop the institutional conditions that encourage the use of more renewable sources in the worldwide energy matrix. This review focuses specifically on the status of this bioenergy transition in the US and the application of AD of lignocellulosic biomass to produce biogas and eventually renewable natural gas (RNG).

2.1. Transition to a renewable-based portfolio in the United States

To produce more renewable energy, the US government has established the RFS, created under the Energy Policy Act of 2005, which amended the Clean Air Act (EPA, 2017). The Energy Policy Act establishes requirements for the minimum volume of renewable fuel production to replace fossil fuel production. In 2007, the program was renewed, and changes were made in the long-term goals to redefine various parameters such as greenhouse gas (GHG) emissions and the definitions of renewable biomass. The new RFS approved and updated the "fuel pathways," categorizing renewable fuel into different types depending on the sources. Fuel pathways include four critical parameters serving to categorize different fuels. The parameters are (1) the feedstock, (2) production process (type of technology), (3) fuel type (form of fuel), and (4) capacity for reduction of GHG emissions as compared to 2005 petroleum baseline.

By statute, the RFS includes four categories of renewable fuel each with a specific fuel pathway and a renewable identification number (RIN D-Codes). The categories are advanced biofuel (D5), biomass-based diesel (D4), cellulosic biofuel (D3 & D7), and renewable fuel (D6). Figure 1 shows how the Environmental Protection Agency (EPA) categorizes each fuel according

to the GHG emissions reduction capacity. Categories D4 and D5 (Advanced & Biodiesel fuel) must meet 50% of lifecycle GHG reduction and can be produced from renewable biomass (excluding cornstarch). The renewable fuel (D6) typically refers to ethanol derived from corn starch and must meet 20% of the GHG reduction.

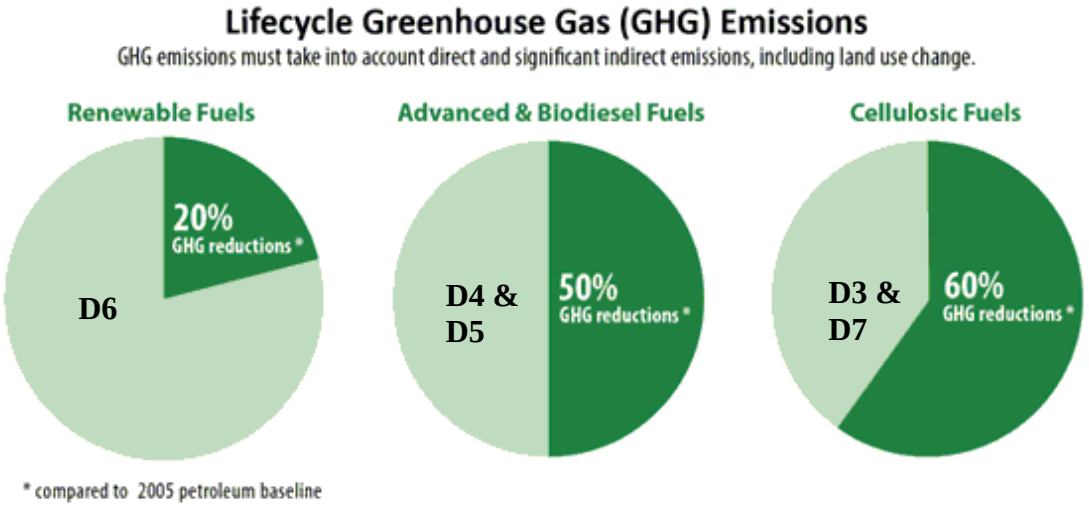


Figure 1. Categorization of renewable fuels by the RFS Act. Adapted from ref. (EPA, 2017).

The third category is D3 and D7, called Cellulosic Fuels. The sources of these types of fuel include cellulose, hemicellulose, and lignin (plant cell wall components also called lignocellulosic biomass). Cellulosic fuel can reach 60% of the GHG reduction as compared to 2005 petroleum baseline. With this categorization, the EPA has approved several pathways such as ethanol made from sugarcane, cellulosic ethanol made from corn stover, biogas from landfills, municipal wastewater facilities, agricultural digesters, and any other biogas from the cellulosic components.

In the case of biogas, there are two kinds of possible pathways: Q and T. The Q pathway is designated for biogas produced from cellulosic biomass transformed by AD and is assigned the

D3 code (cellulosic fuel). In pathway T, biogas[1] is produced from no lignocellulosic feedstocks (carbohydrates, proteins, and lipids among others) and will be assigned code D5. Nowadays, many US biogas plants are registered as energy producers at the EPA, splitting between D3 and D5, using both categorizations to produce and sell their energy on the market. Within this framework, biogas is required to be purified to pipeline quality natural gas standard, resulting in a fuel commonly called RNG. Since 2014, there has been a linear positive increase in RNG production in the US, and a stable increase is predicted until 2022 (Hanson, 2017). Hanson (2017) showed that in 2016, the RNG produced was used to reach 82% of the federal targets set for cellulosic fuel. Producers are utilizing feedstocks such as dairy manure (DM), biosolids, and landfill gas with codigestion of food waste or starch, to produce D5 RNG. Biogas producers have been able to increase their revenue by 78%, whereas the ones being compared register only as D5 producers.

Although AD and biogas production look promising, the capacity for energy production from plant cell wall components and the rate at which this production is possible continue to be explored. At this moment, it is important to more deeply understand the ability of AD to process these materials to link them with the actual market of RNG.

2.2. AD

AD is a microbiome-based bioprocess that consumes organic matter and produces mainly a mixture of methane (CH_4) and carbon dioxide (CO_2) called biogas, under warm conditions (typically >35°C) and in reactors with minimal oxygen (<1%). This mixed culture forms a microbial community where ecological principles drive metabolic fluxes and affect the operation

[1] Also called in the Act and US Policies as “Renewable Natural Gas” (RNG).

and performance of the process. The biogas can be transformed into usable energy in several ways, including engine combustion or purification to a natural gas standard to produce RNG.

AD can turn organic residues to high-value resources such as renewable energy and fertilizer in comparison with conventional disposal as wastewater (activated sludge) or solid waste (landfill) (McCarty, Bae, & Kim, 2011). Currently, over 2,000 AD plants produce biogas in the United States (USDA, USEPA, & USDOE, 2015), whereas Europe has over 17,000 biogas plants (Brijde, Dumont, & Blume, 2014). China is the world leader in biogas production, with 17 million digesters (Changda, Xiang, Wu, & Yifeng, 1994). Countries in Africa and South and Central America have been developing (and investing in) biogas technologies in the last decade owing to their high potential in those regions (Flavin et al., 2014; Roopnarain & Adeleke, 2017). These regions have high viability of residual organic biomass and high solar radiation: both conditions are favorable for integration of these technologies with AD exploitation (Aguilar Alvarez et al., 2016).

2.3. AD principles

AD involves a complex syntrophic association of producers/consumers that interact to attain effective transport of electrons. The digestion process is based upon three stages: (1) hydrolysis of complex compounds, (2) volatile fatty acid (VFA) formation, and (3) methane production (Fang & Liu, 2002). AD proceeds in the absence of oxygen; therefore, organic acids assume the role of an electron acceptor in the fermentation process and turn into methane at the methanogenic stage (Rittmann & McCarty, 2001). There exists a direct correlation between carbon mineralization and microbial community structure.

The first metabolic pathway involved here is hydrolysis. The hydrolyzed compounds are broken into monomers and oligomers by different enzymes such as amylase, cellulose, and protease, among others (Nayono, 2010; Singh nee' Nigam & Pandey, 2009). The hydrolysis flux

is proportional to the organic loading rate (OLR) applied to a reactor. Penaud et al. (1997) showed that the best conditions for high consumption of organic matter and high VFA concentrations occur at pH 8.5 and at high OLRs (~5 kg of chemical oxygen demand [COD] per m^3 per day) with solid retention times (Θ_x) higher than 2 days; this condition is normally not considered in the design of AD reactors, owing to the high variability in other factors (like feedstock composition) with time. With respect to modeling of AD, the hydrolysis step normally is not considered because of the complex waste pool. The combination of lysis, nonenzymatic decay, phase separation, and physical breakdown is defined as hydrolysis thus creating a lot of complexities when modeling is attempted (Batstone et al., 2002). After this initial step, the products of hydrolysis are fermented.

Acidogenesis (VFA formation) follows hydrolysis. During acidogenesis, fermentative bacteria consume monosugars and fatty acids formed during the hydrolysis to produce organic acids, hydrogen, and CO_2 (Batstone et al., 2002). During acidogenesis, most of the energy and mass flow from the organic polymers thereby going to organic acids (76%), such as acetic, propionic, isobutyric, butyric, valeric, isovaleric, isocaproic, and caproic, among others. There is less molar production of H_2, acetate, and CO_2 (Speece, 1983). The acidogenesis rate grows with the OLR. Goux et al., (2015) increased the OLR of mesophilic reactors until reaching acidosis and studied the microbial community of the reactors before, during, and after, thus demonstrating that there is a shift of the fermenters under these unbalanced conditions. Acidogenesis is a crucial upstream process for methane production. It should to be controlled, monitored, and maintained at low concentrations. Nevertheless, it is most important to monitor the consumption of VFA by the downstream processes. Moreover, maintaining low VFA concentrations keeps alkalinity and pH in the appropriate range for the downstream processes of acetogenesis and methanogenesis.

Acetogenesis proceeds simultaneously with acidogenesis. Acetogenesis uses products from acidogenic fermentation (e.g., propionate, butyrate, and valerate) and generates more acetic acid. From a thermodynamic standpoint, this process does not happen spontaneously because the standard Gibbs free energy of the reaction is positive ($\Delta G^0 > 0$). This condition changes its window from pH ~4 to pH ~6. Elevated temperatures will also influence the process of switching the reaction into a spontaneous state. Digestion of VFAs will be more effective with supplemental heat. This whole process may happen at 35 °C for mesophilic conditions or at 47 °C under thermophilic conditions (Oh & Martin, 2010).

Finally, methanogenesis is the last metabolic process necessary to create methane. This process derives from two main metabolic pathways: the hydrogenotrophic pathway and acetotrophic pathway (Demirel & Scherer, 2008). They are classified as based on two different groups of methanogens: acetate fermenters and hydrogen oxidizers (Rittmann & McCarty, 2001). The acetate fermenters employ acetate as an electron donor and as a carbon source and are slow growers. Hydrogen oxidizers grow faster but require a high concentration of H_2 to favor the process (Demirel & Scherer, 2008). Because methanogens must be present to produce methane, the normal pH requirement is in the range of 6.5 to 8.2 (Safferman, Kirk, Faivor, & Wu-haan, 2012). Furthermore, due to the energy available from the electron donor–acceptor setup, AD has a slow growth rate ($f_s^0 = 0.05$) and requires long solids retention time (θ_x) because of the methanogens' growth requirements (Rittmann & McCarty, 2001). During AD startup, the concentration of VFAs increases, and the conditions become less favorable for the growth of methanogens. In this stage, it is also important to maintain pH in the right range for methanogens. If pH is not maintained, the methanogenic population could be hurt and gas production may decrease (Goux et al., 2015). Normally, during the establishment process, it is possible to observe low gas production. During

this establishment, VFA concentrations are normally high (12,000 mg kg^{-1}). Then, the concentration of VFAs decreases, and the abundance of methanogens increases, leading to increased alkalinity and CO_2 concentration (Rittmann & McCarty, 2001). The increase in alkalinity directly affects pH; the initial alkalinity depends directly on the feedstock but can increase during the process owing to the formation of cation salts. Regardless of this stable state, the systems always require normal evaluation of the parameters. AD systems are stable when the culture strikes a balance between VFA formation and methane production (Y. Chen, Cheng, & Creamer, 2008; Demirel & Scherer, 2008). This condition is the most important factor to ensure good operation and effective energy production. By following this recommendation, it is possible to maintain a healthy process and a productive anaerobic digester.

On the other hand, the process has also been studied from the energetic or stoichiometric point of view. After assuming that carbon dioxide is the electron acceptor, one can write the stoichiometric equation for generalized organic waste (Rittmann & McCarty, 2001):

$$C_nH_aO_bN_c + \left(2n + c - b - \frac{9df_s}{20} - \frac{df_e}{4}\right)H_2O \rightarrow \frac{df_e}{8}CH_4 + \left(n - c - \frac{df_s}{5} - \frac{df_e}{8}\right)CO_2 + \frac{df_s}{20}C_5H_7O_2N + \left(c - \frac{df_s}{20}\right)NH_4^+ + \left(c - \frac{df_s}{20}\right)HCO_3^- \quad (1)$$

where $d = 4n + a - 2b - 3c$, and f_s represents the fraction of waste organic matter synthesized or converted to cells, and f_e denotes the portion converted to energy, such that $f_s + f_e = 1$. The value of f_s depends on the energy generation and synthesis reactions as well as the decay rate b and θ_x. For a reactor in operation steady state, f_s can be estimated using equation 2:

$$f_s = f_s^0 \left[\frac{1 + (1 - f_d) b\theta_x}{1 + b\theta_x}\right] \quad (2)$$

Table 1 summarizes the values of f_s^0 and b for methane-producing fermentation of common organic compounds.

Table 1. Coefficients for stoichiometric equations for anaerobic treatment of various organic materials (Rittmann & McCarty, 2001).

Waste component	**Typical Chemical Formula**	f_s^0	**Y ($gVSS_a/g\ BOD_L$ removed)**	b **(d^{-1})**
Carbohydrates	$C_6H_{10}O_5$	0.28	0.20	0.05
Proteins	$C_{16}H_{24}O_5N_4$	0.08	0.056	0.02
Fatty acids	$C_{16}H_{32}O_2$	0.06	0.042	0.03
Municipal sludge	$C_{10}H_{19}O_3N$	0.11	0.077	0.05
Ethanol	CH_3CH_2OH	0.11	0.077	0.05
Methanol	CH_3OH	0.15	0.110	0.05
Benzoic acid	C_6H_5COOH	0.11	0.077	0.05

2.4. AD: Microbial communities

As explained before, biogas is produced by a biological process called AD. The process is performed by a specialized and sophisticated microbial community, where different actors have different roles in the structure of the joint organization (Campanaro et al., 2016). The different members of the organization have different functions, and they are linked by interactions forming networks. If these interactions are established, then the consortium can turn diverse kinds of macromolecules into methane and carbon dioxide mainly. Figure 2 shows the gene diversity present in an AD microbial community, and how the organization looks like a “funnel.”

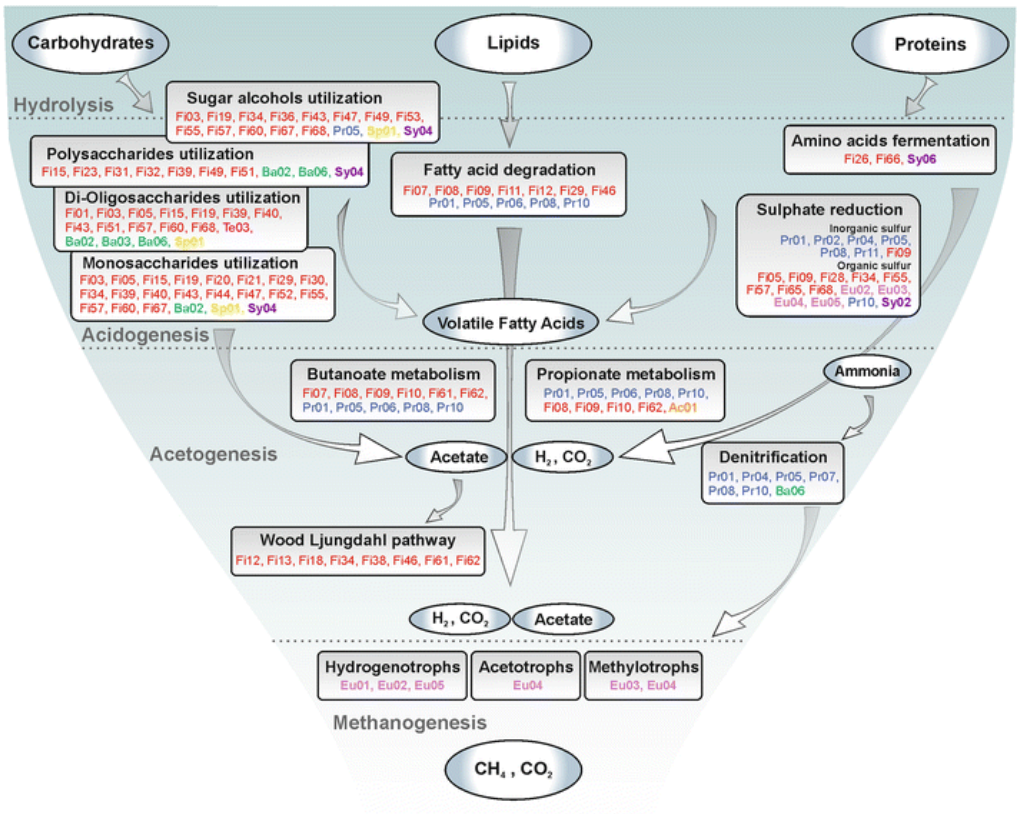


Figure 2. Functional roles in the biogas production. Each box shows a list of genes (and their abundance) identified for each metabolic pathway of all the processes (Campanaro et al., 2016).

This functional structure manifests an increase of specialization as it reaches the final metabolic pathway of the process. This means that there are more diverse kinds of genes able to act in the first main steps of AD, and later steps require more unique kinds of microbes to finally produce biogas. That is why it is possible to see high species diversity in the fermentation steps and more unique species of microbes that are involved in methane formation. That is why it is mandatory to study the guild (metabolic diversity) and the clade (phylogenetic diversity) of an AD microbial community.

The phylogeny in an AD community is based on two main domains: bacteria and archaea. The archaea population mainly belongs to the phylum *Euryarchaeota* and is mainly dominated by acetoclastic methanogenic genera such as *Methanosaeta*, followed by hydrogenoclastic genera

such as *Methanospirillum* and *Methanobrevibacter* (Demirel & Scherer, 2008). Thermophilic reactors are dominated by the hydrogenoclastic genus *Methanothermobacter* followed by versatile *Methanosarcina* (Kirkegaard et al., 2017). On the other hand, the phylogeny of the bacterial community is more diverse (≈46 phyla) and more abundant (≈90% of total abundance) (Kirkegaard et al., 2017; Rojas-Sossa et al., 2017). Phyla such as Firmicutes, Proteobacteria, Chloroflexi, Actinobacteria, Bacteroidetes, Synergistetes, and Acidobacteria, are the most abundant in AD reactors. What is still not clear is whether the microbes are enriched in the reactors or if the microbes immigrate to the reactors via the feedstocks. Kirkegaard et al., (2017) tested whether the immigrating microorganisms tend to die, survive, or grow in the reactors. It was found that there was a peak of microorganisms who were highly enriched in the reactor. Those authors found that the microorganisms that were enriched represented 60% of the total abundance in the reactors. Improving the understanding of these relationships will allow scientists and engineers to focus on controlling and changing those relations with the objective of improving the feedstock-to-methane conversion or attenuating the inhibitory effects in the process. Some of the substrates whose conversion to methane needs to be improved are plant cell wall components, lignocellulosic biomass. These compounds are the most abundant carbon source on Earth (Tye, Lee, Wan Abdullah, & Leh, 2016).

2.5. Plant cell wall components

The term “lignocellulosic biomass” or nonfood plant biomass refers to a portion of plant matter composed mainly of structural carbohydrates that form the plant cell wall (Chundawat, Donohoe, et al., 2011). These structures have been mostly categorized into three groups—two kinds of polysaccharides (cellulose and hemicellulose) and one type of polymer (lignin)—that form the complex and strong structures providing support to a plant as well as protection against microbial

invasion (Keegstra, 2010). These materials have been driven by evolution to become highly recalcitrant toward bacterial enzymes. The main components of the plant cell wall are cellulose, hemicelluloses, lignin, and pectin (V Balan, Sousa, Chundawat, Humpula, & Dale, 2012). In Figure 3, readers can see the reported chemical structures of cellulose, hemicelluloses, and lignin.

2.5.1. Cellulose

In Figure 3a, there is a diagram of the crystalline cellulose nanofibril structure, with the formula $(C_6H_{10}O_5)_n$. These compounds form straight cylinders strongly bound and giving rigidity to the plant cell wall. Glucose is a homogeneous polysaccharide held together via covalently (1,4)-linked β-D-glucans. These carbon chains interact with one another via hydrogen bonds to form a crystalline structure (Keegstra, 2010). This material is the main component of the plant cell wall (V Balan et al., 2012).

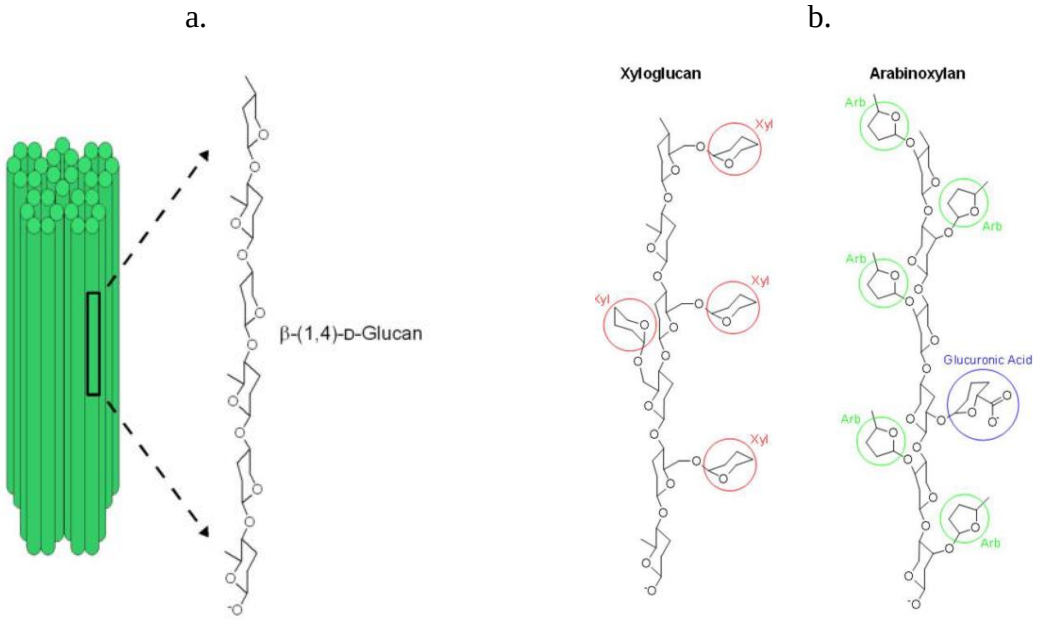
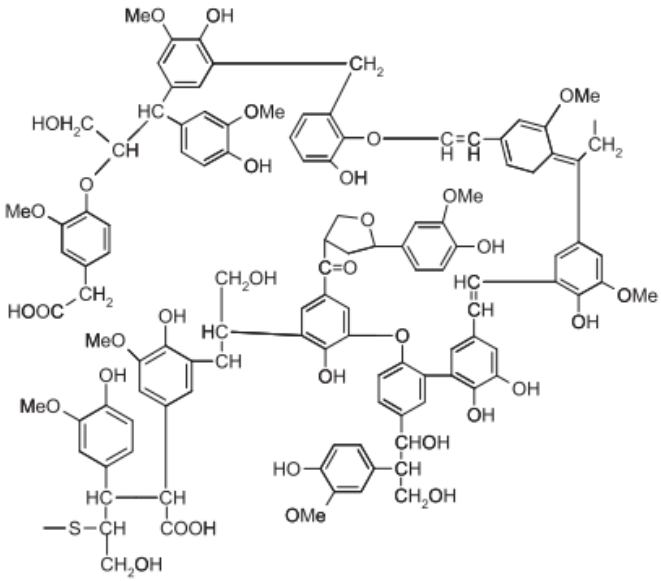


Figure 3. Chemical structure of polysaccharides and polymers that constitute the plant cell wall; these materials are also called lignocellulosic biomass. a. Cellulose (V Balan et al., 2012). b. Hemicelluloses (V Balan et al., 2012). c. Lignin (Sarkanen & Ludwig, 1971).

Figure 3 (cont'd)

c.



2.5.2. Hemicelluloses

Hemicellulose has two common structures: xyloglucan and arabinoxylan (Figure 3b). Scheller & Ulvskov, (2010) described hemicelluloses as a group of polysaccharides that are neither cellulose nor pectin and contain linked backbones of glucose, mannose, or xylose. These polysaccharides are more heterogeneous in their structure and in their physicochemical properties than cellulose is (Scheller & Ulvskov, 2010). The presence of the different kinds of hemicelluloses varies among plant families or species, but it is known that hemicelluloses bind tightly to cellulose microfibrils via hydrogen bonds (Keegstra, 2010). Hemicellulose engages in complex binding to lignin, called the lignin–carbohydrate complex (LCC).

2.5.3. Lignin

Lignin is the second most abundant constituent after cellulose and is reported to be more abundant (by mass) in plant cell walls (Norgren & Edlund, 2014). Figure 3c provides an overview of lignin structure. It is a heterogeneous and amorphous macromolecule with variable composition

dependent on the plant source (Sjöström, 1993). However, lignin can be classified by three monomers: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, which all differ in the positions of the aromatic rings (V Balan et al., 2012; Norgren & Edlund, 2014).

2.5.4. Pectin

Voragen, Coenen, Verhoef, & Schols, (2009) described pectin as one of the most complex macromolecules in nature, because it can be composed of as many as 17 different monosaccharides and with 20 different linkages. Due their anionic nature, these polysaccharides are involved in the transport of ions and in the porosity of the plant cell wall. On the other hand, several studies indicate the importance of the characterization of these compounds in the plant cell wall and of their complex interactions with the other components of the cell wall (V Balan et al., 2012; Keegstra, 2010; Scheller & Ulvskov, 2010).

2.6. Lignocellulosic-biomass degradation in AD

AD of lignocellulosic materials is inefficient because most of the carbon is recalcitrant. These materials show poor solubilization, thus resulting in low biogas production and poor digestion performance (Y. Chen et al., 2008; Mao et al., 2015; Raposo, De La Rubia, Fernández-Cegrí, & Borja, 2012). Important findings on this topic started with advances in the quantification of fiber content in forage samples (Goering & Van Soest, 1975). The publication of this method allowed researchers to address the degradation of different components of the plant cell wall. Hills, (1979) showed how pure microcrystalline cellulose is consumed in AD and how cellulose combined with other organic compounds in complex waste may not be totally bioavailable for bacteria. Later, Hills & Roberts, (1981) published one of the first optimization efforts to maximize gas production during the degradation of dairy manure and field crop residues. They found optimum performance was achieved when the nonlignin carbon-to-nitrogen ratio was ~25 and ~32. On the other hand,

Yue et al., (2013) demonstrated how the codigestion of dairy manure and corn stover promotes biogas productivity. However, the conversion of nonrecalcitrant carbon from corn stover directly correlates with the microbial community structure. Besides, some plants generate resin extracts, and these extracts could be inhibitory for AD (Speece, 1987). Finally, there is evidence of inhibition of AD by lignin derivates obtained with aldehyde; they are highly toxic to methanogens (Y. Chen et al., 2008). To optimize this process, it is necessary to pretreat these materials to improve the bacterial enzyme accessibility or detoxify the feedstock to make it bioavailable as an energy source to microbes.

2.7. Pretreatment technologies for lignocellulosic materials

The nature of lignocellulosic materials makes them very resistant to an enzymatic attack. The main objective of the pretreatment is to change or weaken these properties to prepare the material for the downstream process (AD and ethanol production, among others). Different kinds of pretreatment methods exist, including physical, chemical, physicochemical, and biological (Taherzadeh & Karimi, 2008). Different challenges make the pretreatment of these materials effective, this due the high disparity presented in the lignocellulose structure between specific plants, specific plant tissues, and plant cells. Actually this materials shows a complexity paradigm across scale of systems who are tried to be studied (Chundawat, Donohoe, et al., 2011). Factors such as crystallinity of cellulose, surface area protection by lignin and hemicellulose, the degree of cellulose polymerization, and acetylation of hemicelluloses affect the pretreatment of lignocellulosic biomass (Karimi & Taherzadeh, 2016). Analytical methods, imaging, and crystallinity analyses are normally used to evaluate pretreatment methods. Existing pretreatments include milling, irradiation, microwaving, steam explosion, supercritical CO_2, alkaline and acid hydrolysis, AFEX^{TM}, and others (Taherzadeh & Karimi, 2008).

2.8. AFEX™

AFEX™ is an important pretreatment technology. It is an ammonia-based pretreatment that has proven to be a cost-effective way to reduce the recalcitrance of plant cell wall components (e.g., lignocellulosic materials) and improve microbial fermentation (Chundawat et al., 2013). This technology is currently being scaled up for potential commercialization. One of the important features of AFEX™ is the reversible nature of the interaction of ammonia (NH_3) with biomass; this feature allows most of the ammonia to be recovered (Chundawat et al., 2013). The conventional method is to use liquid ammonia (0.3–2.0 [g NH_3]/[g dry biomass]) in moist biomass (0.1–2.0 g in H_2O per gram of dry biomass) with supplemental heat at high pressure (~2.25 MPa). The process takes place in a batch reactor, but could occur in a plug flow reactor or in a packed bed reactor (Chundawat et al., 2013). First, ammonia reacts with water to cause a rapid increase in temperature. Later, more heating is supplied to maintain a constant reaction temperature (~100 °C); this process lasts for 30 min, then NH_3 is exhausted, thereby releasing the pressure from the reactor (Perez-Pimienta et al., 2016).

Three physicochemical changes exist in AFEX; (1) the LCC split and product formation, (2) lignin/hemicellulose redistribution, and (3) cellulose decrystallization (Chundawat, Beckham, Himmel, & Dale, 2011; da Costa Sousa, Chundawat, Balan, & Dale, 2009). The LCC linkages are some of the most important impediments to cellulase. By ammonolysis (an NH_3 reaction with esters) and hydrolysis (acid formation), AFEX™ breaks these bonds and produces amides and organic acids (Balan et al., 2012). The decrystallization effect could be observed scanning sample surfaces at the microscale by atomic force microscopy (AFM). In Figure 4, AFM pictures of conventional corn stover are presented (Figure 4a), as is AFEX™-pretreated corn stover (Figure 4b). In the AFM images, is possible to observe visually how AFEX™ changed the geometry on

the architecture of the cell wall. With the AFEX^{TM} technology is not possible to observes the normal array of the typical microfibril. Here is possible to observe changes in the normal crevices and cracks that formed part of the natural cell wall surface landscape, changing the AFM surface roughness factor from 16 to 30. The action mechanism of AFEX^{TM} consists in several chemical processes. Ammonia first reacts and evaporates; this event results in the formation of nanoporous (10 to 500 nm width) tunnel-like networks due to the rapid decompression and volatilization of ammonia; then, the extractives redeposit out of the cell wall surface. Chundawat et al., (2011) found on AFEX^{TM}-reaction products a strong enrichment of lignin-derived phenolics. This, due a strong stain by Safranin die between samples of untreated corn stover (Figure 4c) and AFEX^{TM} corn stover (Figure 4d).

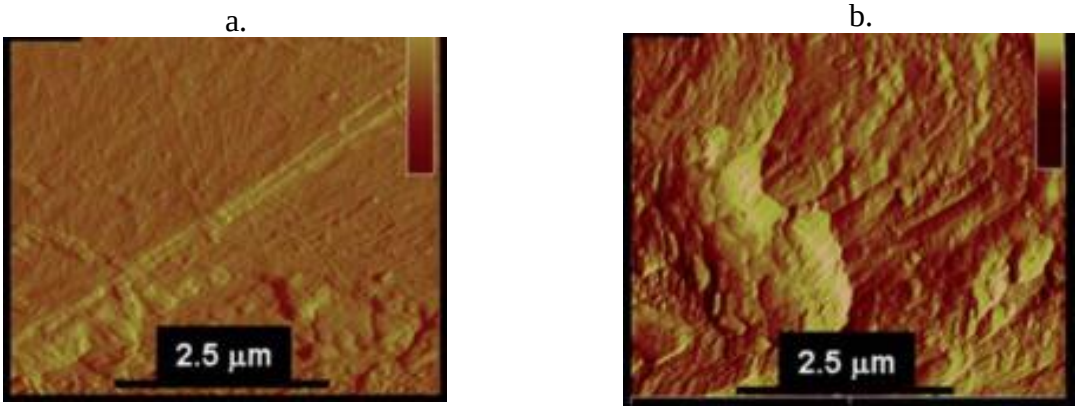
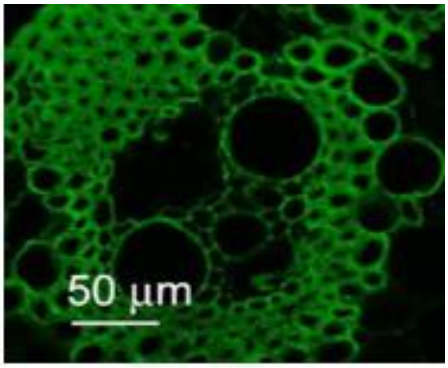


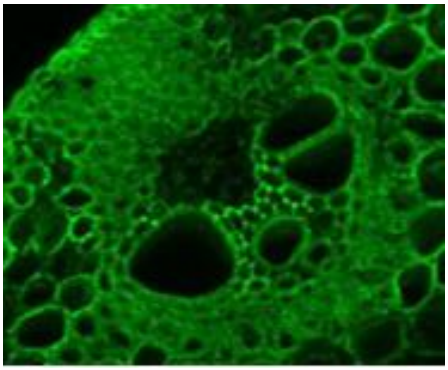
Figure 4. AFM images of untreated a. and AFEX^{TM}-pretreated b. corn stover. Confocal fluorescence imaging analysis of untreated c. and AFEX^{TM}-pretreated d. corn stover. Adapted from ref. (Chundawat, Donohoe, et al., 2011) with permission.

Figure 4 (cont'd)

c.



d.



The nanoporous networks enhance a microbial attack (Lau & Dale, 2009) without removing any of lignin and hemicelluloses into separate liquid streams. Interest in the testing of AFEX^{TM} materials as an AD substrate comes from the ability of the AFEX^{TM} process to improve activity of the enzymes toward the cellulose/hemicellulose/lignin system at the same time releasing acetic and lactic acids (V Balan et al., 2012; Venkatesh Balan, Bals, Chundawat, Marshall, & Dale, 2009).

In conclusion, it is necessary to optimize AD. This optimization requires the use of different social and scientific disciplines. For example, it is crucial to develop better policies regarding biogas production where the framework is aligned to the abilities of the technology. Additionally, it is important to understand the ecological principles of the microbial communities that control the process. Research should focus on understanding the nature of these cooperating species and the relations among them. Finally, it is necessary to improve analytical characterization of the AD feedstocks, specifically lignocellulosic compounds, in order to better determine what they are and improve their exploitation in AD.

3. MATERIALS & METHODS

To obtain the results mentioned in the Objectives section, two experiments were performed and are described in detail in this section. The experimental design was different between the two projects. Nonetheless, the experiments are structured in the same way: sample collection and description, experimental setup, analytical methods, biological analysis (if necessary), performance evaluation, and statistical analysis.

The first experiment is a novel evaluation of two kinds of semicontinuous codigestion of manure and corn stover,conventional corn stover (CS) and AFEX^{TM}-pretreated corn stover (AFEX) . The continuous digestion was monitored for performance and microbial community dynamics for 75 days. All the statistical analyses were conducted using nonparametric statistics and ecological statistics.

The second experiment was evaluation of the digestion of plant cell wall components at ABPs in the state of Michigan. Here samples were collected from the influent and effluent and characterized; then, the samples were evaluated in terms of the biogas potential, and finally, statistical analysis was performed to try to explain the correlation of these characteristics with the observed biogas productivity. The main objective was to examine the contribution of the plant cell wall components to the biogas production from the plants.

Below is a description of both experiments.

3.1. Dynamic microbiome assembly and the effect on the performance of AD of AFEX-pretreated corn stover and CS

3.1.1. Feedstock sample collection

Corn Stover, corn stover pretreated[2] with AFEX™, and DM samples served as feedstocks for this study. CS samples were collected from the Michigan State University (MSU) Beef Cattle Teaching & Research Center in November 2015; AFEX™ samples were obtained from the Michigan Biotechnology Institute in October 2015, and DM samples were collected from the MSU Dairy Cattle Teaching & Research Center in January 2016. After collection the samples, CS and AFEX were stored at room temperature (~20 °C) in airtight bags, after which they were air dried and milled using a Willey Mill (Standard Model No. 3; Arthur H. Thomas, Philadelphia, PA). Finally, CS and AFEX samples were sieved through a 2 mm coarse mesh (No. 8, W.S. Tyler, Cleveland, Ohio) prior to use. Meanwhile, the DM samples were stored at −18 °C and thawed 2 days before the experiment.

The AFEX samples were pretreated in a high-pressure Parr® stainless-steel reactor. For this purpose, anhydrous liquid ammonia was added into the reactor at a 2:1 ratio (dry mater basis); the reaction was allowed to proceed for 30 min at a temperature of 102 °C and pressure 2.25 MPa.

3.1.2. Feedstock mixture preparation

Two feedstock mixtures containing DM, one with CS and the other with AFEX, were prepared for the laboratory scale AD experiment. Both feedstock mixtures were prepared 2 to 5 days prior to use and stored at −4 °C. The feedstock mixtures were a 4:1 dry matter ratio of DM to CS or AFEX. Distilled water was added to the mixtures to prepare a group with a TS content of 5%. The mass of manure needed was calculated using equation 3:

[2] For the benefit of usage, AFEX™ is going to continue being called AFEX.

$$m_{DM} = \frac{4}{5}\left(\frac{5\% \cdot 100\ g}{TS_{DM}}\right) \quad (3)$$

where m_{DM} is the mass of raw manure needed to obtain a feedstock mixture containing 5% of TS (g), and TS_{DM} is the TS content of raw manure (%). On the other hand, the necessary mass of CS or AFEX was calculated via equation 4:

$$m_{CS\ or\ AFEX} = \frac{1}{5}\left(\frac{5\% \cdot 100}{TS_{CS\ or\ AFEX}}\right) \quad (4)$$

where $m_{CS\ or\ AFEX}$ is the mass of CS or AFEX needed to obtain a feedstock mixture containing 5% of TS (g), and $TS_{CS\ or\ AFEX}$ is the TS content of CS or AFEX (%).

3.1.3. Semicontinuous AD experiment

Nine semi—continuous—feed completely stirred tank reactors (CSTRs) as anaerobic digesters with a liquid volume of 0.75 L were setup in triplicate for each feedstock mixture. Wheaton® bottles with rubber septa screw caps served as the CSTR vessels. The working volume of the digesters was 0.75 L. Needles were used to puncture the septa to release the biogas. The biogas production was measured by the water displacement method. Figure 5 shows the configuration of the digestion unit.

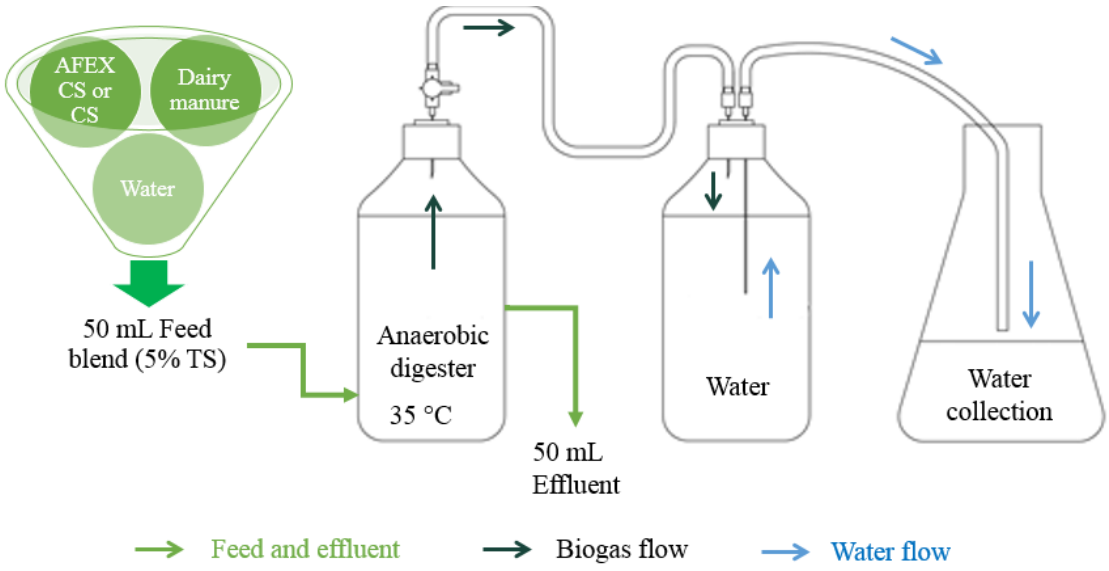


Figure 5. Flow direction of biogas and water in the water displacement method.

Experiments were carried out on MaxQ 4000 incubator shakers (Thermo Scientific, Odessa, TX) at a temperature of 35 ± 0.5 °C and a shaking speed of 150 rpm. The hydraulic retention time (HRT) of the digesters was 20 days, and the duration of the digestion experiment was 75 days. Fifty milliliters of the AD effluent was discharged, and 50 mL of the feedstock mixture was fed every other day. pH was controlled in a range from 6.9 to 7.1 using a 30% (v/v) sodium hydroxide solution. All these operations were carried out in an anaerobic chamber (PLAS Lab, Lansing, MI), and the chamber was purged with a medical grade specialty gas (85% N_2, 10% H_2, 5% CO_2). A palladium catalyst heater was employed to ensure that the chamber was completely anaerobic. Two milliliters of the AD effluent were stored at –80 °C for microbial-community analysis. Ten milliliters of this effluent was used to quantitate TS and volatile solids (VS); the rest of the AD effluent was employed for quantitation of VFAs and structural carbohydrates.

3.1.4. Analytical methods

3.1.4.1. Gas Chromatography (GC)

Biogas concentrations of CH_4 and CO_2 were measured on an SRI 8610C GC system equipped with a HayeSep® column and a thermal conductivity detector. Biogas samples were collected at standard temperature from the bottle headspace using a 5 mL Hamilton® syringe after gas production was recorded. Hydrogen (H_2) and helium served as carrier gases with pressure set to 145 kPa. The thermal conductivity detector was kept at a constant temperature of 150 °C. The injection volume was 3 mL with 100 µL transferred to the GC column.

3.1.4.2. TS/VS

Ten-milliliter samples were used to measure the TS/VS ratio following the standard method (APHA, 1989). The samples were dried for 24 h at 105 °C in a convection oven to quantify TS; the dried sample was then volatilized at 550 °C to obtain the VS.

3.1.4.3. National Renewable Energy Laboratory (NREL) Structural Carbohydrates

Fiber content was measured by the NREL method "Determination of Structural Carbohydrates and Lignin in Biomass" developed by the NREL (A. Sluiter et al., 2012). Raw samples were dried at 45 °C in a food dehydrator (Tribest Sedona® SD-P9000). Then, low-concentration hydrolysis was performed in an autoclave, Getinge® 533LS. A Shimadzu® UV-1800 spectrophotometer was employed to measure absorbance for lignin quantitation. The monosugars from the hydrolysis of cellulose and hemicellulose were quantified using a Shimadzu® HPLC system equipped with a Bio-Rad Aminex HPX-87H analytical column and a refractive index detector. The mobile phase was 0.005 mol/L sulfuric acid at a flow rate of 0.6 mL/min. The column temperature was set to 65 °C.

3.1.4.4. Concentrations of VFAs

Ten milliliters of the AD effluent was centrifuged at 7,025 × g for 10 min in a Beckman® centrifuge (Allegra X-12R, Beckman Coulter, Inc., Brea, CA) to collect the supernatant for measurement of VFA concentration. The supernatant was washed with 25% (w/w) metaphosphoric acid at a ratio of 1 to 5 (acid to sample) to remove remaining solids. The VFAs were quantified on a Shimadzu GC system (GC-2010, Shimadzu Corp., Kyoto, Japan) equipped with a capillary column (122-3232 DB-FFAP, Agilent Technologies, Santa Clara, CA) and a flame ionization detector (Shimadzu Corp., Kyoto, Japan). Helium served as a carrier gas with the pressure set to 79 kPa. The injection volume was 10 µL with 1 µL transferred to the GC column. The column temperature was set to 150 °C for 2 min and raised to 220 °C at a rate of 15 °C/min, then maintained at 220 °C for 1 min. The temperatures of the injector and detector were set to 250 and 270 °C, respectively. The volatile free acid mixture (CRM46975, Sigma–Aldrich, St. Louis, MO) served as the VFA standard. The acids quantified were acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid, and heptanoic acid. A final VFA concentration was determined by summing all the concentrations of the VFA profile described above. Due to the importance of the behavior of acetic (Hac) and propionic acid (Hpa), these were separately studied and plotted.

3.1.4.5. Carbon and Nitrogen

Raw materials dried at 45 °C were used to measure carbon and nitrogen content. Total organic carbon (TOC) (TMECC 04.01-A) and total nitrogen (TMECC 04.02-D) were measured to calculate the carbon-to-nitrogen ratio (TMECC 05.02-A) by the Test Methods for the Examination of Composting and Compost (USDA & CCREF, 2001).

3.1.5. Microbial Community Analysis

3.1.5.1. Amplicon preparation and sequencing procedures

The PowerLyzer® PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad) was used to extract DNA from the DM sample and DNA from the AD effluent of the CS and AFEX reactors at various time points. The DNA concentrations were measured on a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The DNA samples with the DNA concentration less than 25 ng/μL were concentrated using 5 M NaCl and cold ethanol (200 proof). The extracted DNA was stored at –80 °C before use as a template for amplicon preparation with universal primers: forward primer Pro 341 F (5′-CCTACGGGNBGCASCAG-3′) and reverse primer Pro 805 R (3′-GACTACNVGGGTATCTAATCC-5′), to amplify both archaeal and bacterial DNAs (Takahashi, Tomita, Nishioka, Hisada, & Nishijima, 2014). Twenty-five microliters of the master mix solution containing 12.5 μL of the GoTaq® Green Master Mix (Promega, Madison, WI), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 0.5 μL BSA, and 1 μL of extracted DNA (~40 ng/ μL), were mixed with 9 μL of DNase- and RNase-free water for PCR. The PCR program started with a denaturing step at 95 °C for 5 min, followed by 30 cycles of the touchdown steps (denaturing at 95 °C for 2 min, annealing at 58 °C for 5 s, and elongation at 48 °C for 5 s), and ended with a final extension at 72 °C for 5 min. PCR products were loaded onto a Bio-Rad® 1% TAE Mini ReadyAgarose™ precast gel with 1% ethidium bromide and were visualized using an electrophoresis unit (Bio-Rad, Hercules, CA).

After the PCR products showed correct bands on the electrophoresis gel (1% agarose, dyed with ethidium bromide) for all the samples, samples containing the original DNA template were analyzed at the Research Technology Support Facility at MSU. Then, at this facility, the V3-V4 region (positions 341–806) of the 16S rRNA gene was amplified by nested PCR with a set of

primers designed to detect bacteria and archaea (Takahashi et al., 2014). First, primary PCR was carried out with chimeric primers containing target-specific portions (as described in Takahashi's report) and Fluidigm CS oligos at their 5′ ends. Secondary PCR targeting the CS oligos was then carried out to add sequences necessary for Illumina sequencing and unique barcodes. The PCR products were normalized via Invitrogen SequalPrep DNA normalization plates, and normalized eluates from the plates were pooled. After validation and quantification, a pool was sequenced in an Illumina MiSeq flow cell (v2) with a 500-cycle reagent kit (2×250 bp paired-end reads). Custom sequencing primers matching the Fluidigm CS1 and CS2 oligos were used. Base calling was done by Illumina Real Time Analysis (RTA) v.1.18.54 software, and the output of RTA was demultiplexed and converted to FASTQ format in Illumina Bcl2fastq v.1.8.4.

3.1.5.2. Bioinformatics

The FASTQ files from Illumina sequencing were analyzed with BION, a semi-commercial open-source package for microbial-community analysis from the Danish Genomic Institute, Aarhus, Denmark. Primer sequences were utilized to extract the paired sequences from the raw reads, and minimum quality of 99% was set as a requirement for at least 14 of 15 bases for forward reads and 28 of 30 for reverse reads. A minimum length of 50 was imposed. Paired reads were joined where there was at least a 25-base overlap and 85% similarity. Sequences were then filtered for length (250 minimum) and quality (99.6%), dereplicated, preclustered at 99%, and checked for chimeras by an algorithm unique to BION. Nonchimeric sequences were clustered at 99% stringency and a minimum length of 300. The sequences were then matched to reference sequences using a K-mer length of 8 with a step size of 4 and were compared with the region 340–807 in RDP 11.04. The sequence similarities of each sample were converted to a taxonomic profile, using

the RDP taxonomy, and the profiles were combined into abundance tables and counted as operational taxonomic units (OTU).

3.1.6. Evaluation of Digestion Performance

To evaluate the performance of the digesters, it was assumed that the reactors are under steady-state conditions, where the mass is being conserved and there is no change in stored mass with time. Under these assumptions, four performance parameters were determined to evaluate the digestion: biogas productivity, VS concentration reduction, and xylan and cellulose content reductions. Biogas productivity was determined using equation 5:

$$BP = \frac{V_{sample} \cdot 1E-6}{20 \cdot m_{feed} \cdot VS_{Feed}} \quad (5)$$

where BP is biogas productivity (m^3 of biogas/[kg VS]), V_{sample} is the accumulated produced biogas for each HRT (mL), m_{feed} is the total mass fed into the reactors around each HRT (g), and VS_{Feed} is the VS percentage in feed mixtures (%). Meanwhile, the VS content reduction was determined via equation 6:

$$VS_{Reduction} = \frac{m_{feed}VS_{feed} - m_{effluent}VS_{effluent}}{m_{feed}VS_{feed}} \cdot 100 \quad (6)$$

where $VS_{Reduction}$ is the VS reduction in the reactor (%), VS_{feed} is the VS percentage in feed mixtures (%), $m_{effluent}$ is the total mass of the AD effluent (g), and $VS_{effluent}$ is the VS percentage in the AD effluent (%). The cellulose reduction was determined by means of equation 7:

$$Cellulose_{Reduction} = \frac{m_{feed}Cellulose_{feed} - m_{effluent}Cellulose_{effluent}}{m_{feed}Cellulose_{feed}} \cdot 100 \quad (7)$$

where $Cellulose_{Reduction}$ is the cellulose reduction in the reactor (%), $Cellulose_{feed}$ is the cellulose content (%) of feed mixtures (%), and $Cellulose_{effluent}$ is the cellulose content (%) in the effluent (%).

The xylan reduction was determined using equation 8:

$$Xylan_{Reduction} = \frac{m_{feed}Xylan_{feed} - m_{effluent}Xylan_{effluent}}{m_{feed}Xylan_{feed}} \cdot 100 \quad (8)$$

where $Xylan_{Reduction}$ is the xylan reduction in the reactor (%), $Xylan_{feed}$ is the xylan content (%) of feed mixtures, and $Xylan_{effluent}$ is the xylan content (%) of the effluent. Because we assumed steady-state conditions (i.e., m_{feed} = $m_{effluent}$), the mass terms are cancelled out in equations 6 to 8, making the calculation of the performance parameters simpler.

3.1.7. Statistical analysis of microbial-community data & of digestion performance

To understand the microbial ecological conditions of the reactors and their effect on the operation of the reactors, a series a statistical analysis was performed. First ANOVA was conducted, with the objective to find statistically significant differences among the operational parameters. Then alpha (α) and beta (β) diversity were calculated and analyzed. α-Diversity describes the structure of the community by itself, in the context of the study; this is the structure of each sample sequenced (Bolker, 2008). Meantime, β-diversity measures the turnover of species between two places. In the context of this analysis, β-diversity is the comparison between different reactor communities (Bolker, 2008).

The OTU table from the bioinformatics pipelines was employed to interpret the microbial data and correlate the microbial communities with digestion performance. The R statistical software (version 3.5.0) was used to carry out the analysis. Software code is presented in the Appendix.

Three data files—the OTU table, taxonomy table, and metadata table—were generated for R to run the analysis. All these tables were saved as tab limited TXT files.

3.1.7.1. ANOVA & Normality

The Shapiro–Wilk normality test was performed on the digestion performance data transformed to a logarithmic scale (R function `log`). The digestion performance data included biogas productivity, VS content reduction, VFA concentration, cellulose content in the AD effluent, xylan content in the AD effluent, and lignin content of the AD effluent. The R function `shapiro.test` was executed. A one-way ANOVA was then performed separately for each HRT (1, 2, & 3) using the R function `aov`. On the ANOVA results, the Tukey pairwise comparison was performed to find statistically significant differences between the various operational parameters via the R function `TukeyHSD`.

3.1.7.2. α-Diversity

By means of R libraries `vegan` (Oksanen et al., 2016), `phyloseq` (McMurdie & Holmes, 2013), `MASS` (Venables & Ripley, 2002), and `tidyverse` (Wickham, 2009), a detailed α-diversity analysis was performed, to study the diversity in each sample. Several diversity indices (i.e., Shannon's, Simpson, Inverse Simpson, and Fisher) were calculated using the `diversity` function. The sampling curve or richness curve was calculated using the `rarecurve` formula; this approach allows us to observe the change in diversity. Both formulas are a part of the `vegan` package. The sampling curve is a plot of the species accumulation versus the sample size; the rate at which new species are added reflects useful richness diversity (Bolker, 2008).

3.1.7.3. β-Diversity

A class object was constructed to unify all data at the experiment level using `phyloseq()` from library `phyloseq`. Before the samples were analyzed, the dataset was rarefied. Rarefication allows a researcher to normalize a dataset thereby enabling a comparison of the relative species richness in a normalized base. Because the rarefication procedure requires random subsampling, a constant random number was set using `set.seed()`. This technique allowed to reproduce the results every time the same code was running. The dataset was rarefied by means of the function `rarefy_even_depth` from `phyloseq`. Four hundred thirty OTUs were removed from the datasets thereby allowing me to normalize the abundance of all the datasets. The function `merge_samples()` was executed to merge the duplicates of each run. The data were transformed to relative values using the function `transform_sample_counts` in `phyloseq`. The relative values served to compare relative abundance levels of individual taxa between different samples. Based on the normalized data, abundance bar plots were built. In addition, some ordination analysis was performed as well. Finally, to obtain a multivariate understanding of this effect in the microbial communities, nonmetric multidimensional scaling analysis was performed on the OTU data at the family level using the `metaMDS()` function from `vegan`. Bray distance was chosen as a dissimilarity index. Functions `subset_taxa()` and `taxa_glom()` were employed to cluster similar taxa at different levels from a domain to genus.

3.2. Degradation of lignocellulosic feedstocks at ABPs

3.2.1. Influent and effluent sampling at ABPs

Fresh influent and effluent samples were collected at three ABPs in Michigan. The samples were obtained on two farms (Farm A and Farm B) and from the MSU South Campus Anaerobic

Digester (SCAD) on a weekly basis. Farms A and B were sampled on May 22 and 30 and June 6, whereas the SCAD samples were collected on June 5, 11, and 19, 2018. The main characteristics of the sampled ABPs are listed in Table 2. The samples were transported and stored at a temperature of 3 °C prior to the analysis at the Anaerobic Digestion Research and Education Center (ADREC) of MSU.

Table 2. Description of the ABPs sampled

Name	Reactor Units	Effluent streams per reactor	Feedstocks	Type
Farm A	3	1	Dairy manure/FOG	CSTR
Farm B	2	1	Dairy manure	Plug-Flow
SCAD	1	1	Dairy manure/FOG	CSTR

The ABPs have different reactor designs, effluents streams and configurations. For instance, Farm A and SCAD are CSTR reactors. For this type, the feedstock is introduced in tanks which are generally stirred by impellers or pumps (Doran, 1995). The performance of these reactors is dependent of reaction kinetics and the HRT in which the reactor operates (Fogler, 1999). The digester at Farm A consists of three reactor units each with a mass flow of feedstock of 604,74 m^3 per day. Therefore, one sample from each the influent and the effluent were sampled for each unit. Then, the samples were mixed using a volume ratio 1:1:1 in the laboratory. Regarding SCAD, this ABP consists of only one reactor tank and a single input–output stream; therefore, the samples obtained from the streamlines were used as is. On the other hand, Farm B is a plug-flow digester, in which the fluid is pumped through a pipe or tunnel where the feedstock reacts. Here, the chemical reactions proceed as the feedstock travels through the reactor volume like a piston. Additionally, Farm B has two effluent lines (Table 2). Thus, in the case of Farm B, the composites were prepared at a volume ratio 1:1. In addition, jugs of 5 L with the composites were prepared and stored at 4 °C.

3.2.2. Analytical Methods

Influent and effluent samples were characterized in terms of a variety of parameters. The samples were dried at 45 °C for 3 days. Then, the dry sample material was characterized following the method presented by Templeton, et al., (2010). Such parameters as cellulose, xylan, lignin, ash, protein content, water extractives (H_2O.Ext), and ethanol extractives (C_2H_6O.Ext) were measured. Quantification of the extractives in the samples was based on the method "Determination of Extractives in Biomass" NREL/TP-510-42619 (A Sluiter, Ruiz, Scarlata, Sluiter, & Templeton, 2008). The extraction procedures were performed in 600 mL Tall Form Kimble® Berzelius Beakers using a reflux system called Labconco® Crude Fiber Apparatus (Kansas City, MO); the process lasted for 2 h. For the aqueous extraction, 2 g of a raw dry sample was placed in 100 mL of deionized (DI) water mixed with 4 mL of 30% v/v thermostable Novozymes Thermamyl® 120 L (Franklinton, North Carolina). For the ethanol extraction, 1 g of a sample already water extracted was placed with 100 mL of 71.25% v/v ethanol and digested for 2 h again. After the samples were digested, they were transferred to 50 mL vials. Next, these samples were centrifuged at 4,427 × *g* on a HERMLE Z206A (Wehingen, Germany). After that, the supernatant was discarded, and the tubes were centrifuged again with 50 mL of DI water. This process was repeated twice. Finally, the precipitate and some of the supernatant were transferred to a 20 mL tube for drying and were dehydrated at 45 °C. In both extraction procedures, a small sample was dried at 105 °C to correct the final extractive content for moisture. Protein content was calculated using a nitrogen protein conversion multiplier of 6.25 (J. B. Sluiter, Ruiz, Scarlata, Sluiter, & Templeton, 2010).

Parameters such as COD, soluble chemical oxygen demand (sCOD), total Kjeldahl nitrogen (TKN), and ammonia (NH_3) were analyzed in the wet samples (Patel & Nakhla, 2006). COD was

measured by an EPA-approved Hach method 8000. For measuring sCOD, the samples were centrifuged at 4427 x *g* and then passed through a 0.45 µm filter with the help of a vacuum pump. Next, the filtered samples served as input for COD Hach method 8000. TKN was quantified by the EPA-approved Hach method 10242. Ammonia analysis involved EPA-approved Hach method 10205. The samples were analyzed at room temperature. All the described Hach methods involved a Hatch DRB200 reactor and Hatch DR5000 spectrophotometer (Loveland, Colorado). In addition, TS/VS, TOC, total organic nitrogen, structural carbohydrates, methane content, and biogas were quantitated by the method described in subsection 1.1.4.

3.2.3. Biochemical Methane Potential (BMP) in ABPs influents

BMP assays were performed on the samples from the ABPs influents. Considering the need for a relatively large amount of a dry sample for fiber content analysis, a modified BMP method was adopted (Faivor & Kirk, 2011). The samples were blended using a Nutri-Ninja Professional BL450 900 Watt without the addition of water (Hansen et al., 2004). The blended samples were mixed with the digestion filtrate at a sample-filtrate VS ratio of 2:1. The mixtures contained 200 g of the filtrate and blended sample. DI water was used to bring the reactor volume to 1 L. Each sample was analyzed in triplicates. The control contained 200 mL of filtrate and 800 mL of DI water without the blended samples. The samples of the mixtures were mixed on a stir plate for 10 min and then poured into a 0.75 L graduated Wheaton® bottle. The bottles were sealed with Wheaton® screw caps that have a septum for poking needles to measure the gas production. The bottles were flushed with nitrogen at a constant flow rate of 750 mL min^{-1} for 15 min and incubated under mesophilic conditions (35 °C) on a Thermolyne Bigger Bill Oscillator shaker. After 2 h of incubation, the gas was released from the bottles. Then, the experiment was started, lasting for 50

days. Finally, once the experimental data were obtained, biogas productivity was calculated using equation 9, usually called the raw method productivity:

$$BP_{raw\ method} = \frac{(V_{sample} - \bar{V}_{seed})}{(VS_{raw} \cdot m_{sample})} \cdot 1000 \quad (9)$$

where $BP_{raw\ method}$ is the biogas productivity of the raw sample (L/[kg initial VS]), V_{sample} is the accumulated volume of the gas produced in the BMP (mL), $\bar{V}_{seed}$ denotes the average accumulated volume of gas produced by the control BMP (mL), VS_{raw} is the VS of feedstock mixtures (mg/kg), and m_{sample} represents the sample mass added into the BMP (kg).

3.2.4. Statistical analysis of ABP data

3.2.4.1. ANOVA & Normality

An R script was written in-house to analyze the dry matter, characterize the raw samples, and to model the possible effect of this characterization on biogas productivity. First, all the data were transformed to dry matter basis by dividing each obtained data point by the TS determined in the sample. The parameters studied were sCOD, COD, NH_3, TKN, protein, TOC, H_2O.Ext, C_2H_6O.Ext, cellulose, xylan, acid lignin, and biogas productivity. Then, the Shapiro–Wilk test was performed with transformation to the logarithmic scale for each parameter measured, with clustering of the samples for each biogas plant, and by taking a difference between the influent and effluent. Function `shapiro.test` was utilized to perform this analysis. Later, one-way ANOVA was performed separately for each parameter measured by means of the factors: “plant” (Farm A, Farm B, or SCAD) and “flow” (influent or effluent). The ANOVA was performed via function `aov`. Due to the high variation observed in the data, for significance, a p value of 0.1 was selected. On the ANOVA results, the Tukey pairwise comparison was performed to find

statistically significant differences in various operational parameters, using the `TukeyHSD` function .

3.2.4.2. Average percentage of a parameter decrease on dry matter

In addition, an average percentage of reduction in each parameter was calculated via the average of the influent and effluent data, by means of equation 10:

$$\bar{R} = \frac{\bar{I} - \bar{E}}{\bar{E}} \tag{10}$$

where $\bar{R}$ is the average content reduction percentage (%), $\bar{I}$ is the average concentration of each substance (% dry matter), and $\bar{E}$ is the average effluent concentration of the same substance (% dry matter basis). These values were used as inputs of the variance-based sensitivity analysis.

3.2.4.3. Variance-based sensitivity analysis

To gain a deeper insight into the impact of the characteristics of the influents and their decrease on the BMP biogas productivity, a sensitivity analysis was performed assuming a linear model and with calculation of the covariance matrix of the reduction samples. The model assumed noninteraction among the parameters. The description of the model is given in equation 11:

$$BP = f(R_{sCOD}, R_{COD}, R_{NH_3}, R_{TKN}, R_{Protein}, R_{TOC}, R_{(H_2O)_{Ext}}, R_{(C_2H_6O)_{Ext}}, R_{Cellulose}, R_{Xylan}, R_{Lignin}) \tag{11}$$

where BP is the biogas productivity obtained in the BMP experiments (L/[kg initial VS]), R_{sCOD} is the average decrease in sCOD (%), R_{COD} is the average decrease in COD (%), R_{NH_3} is the average decrease in NH_3 content (%), R_{TKN} is the average decrease in TKN content (%), $R_{Protein}$ is the average decrease in protein content (%), R_{TOC} is the average reduction in TOC content (%), $R_{(H_2O)_{Ext}}$ is the average reduction in $(H_2O)_{Ext}$ (%), $R_{(C_2H_6O)_{Ext}}$ is the average reduction in

$(C_2H_6O)_{Ext}$ content (%), $R_{Cellulose}$ is the average decrease in cellulose content (%), R_{Xylan} is the average decrease in xylan content (%), and R_{Lignin} is the average decrease in acid lignin content (%).

The programming code for the analysis is presented in Appendix 7.4 in part 9 of the Rscript developed. The code was written via Oracle Crystal Ball specifically, and this analysis was performed using the newly developed function `contribution_to_variance()`[3]. Here, the variance contribution is calculated by squaring the rank correlation coefficients and is normalized to obtain 100%; these results are only an approximation and are not precisely variance decomposition. The rank correlation shows that positive coefficients indicate an increase in the assumption, whereas negative coefficients imply the opposite situation. The larger the absolute value of the correlation coefficient, the stronger is the relation. Finally, the constructed plot showed a contribution and rank correlation.

[3] http://mattgrogan.info/stats/contribution-to-variance/

4. RESULTS

The results section will be described in two subsections (4.1 and 4.2). The first subsection describes the evaluation of the codigestion of manure with the two kinds of corn stover, CS and AFEX. Here, readers can see a description of feedstock mixtures and their characterization, the digestion performance observed, and the details about the microbial community established during both kinds of digestion.

On the other hand, in subsection 4.2, readers will see dry-matter and raw characterization of the ABPs studied, the results of the BMP experiments performed with the ABP influents, and the sensitivity analysis of biogas productivity on the basis of the reductions observed in the parameters being measured.

4.1. Dynamic microbiome assembly and the effect of the performance of AD of AFEX-pretreated corn stover and CS

4.1.1. Feedstock characterization and codigestion mixture mass ratios

Table 3 summarizes the characteristics of DM, CS, and AFEX used in the study. It is apparent that the TS concentrations of the CS and AFEX samples are much higher in comparison with DM samples. This phenomenon is mainly caused by rumen digestion. DM is the digested residue of plant biomass and other nutrients in the animal feed. These three feedstocks all contain significant amounts of cellulose, xylan, and lignin and are considered lignocellulosic biomass. Among them, CS has the highest cellulose content; at 37.30%, AFEX has the highest hemicellulose content (21.73%), and DM has the highest lignin concentration (21.86%). Meanwhile, DM has a much lower C/N ratio (18.2) than CS and AFEX do. This result makes it possible to mix DM with CS and AFEX to obtain feedstock mixtures with desired C/N ratios (between 15 and 30) for healthy and efficient AD. In addition, DM has a much higher moisture content of 85.55% than do CS

(7.40%) and AFEX (2.64%). Mixing DM, CS, and AFEX can significantly reduce the demand for water needed to carry out the digestion.

Table 3. Characteristics of assay feedstocks.

Characteristic	DM	CS	AFEX
Raw Total solids (%)	14.45 ± 0.59	92.60 ± 0.19	92.68 ± 0.14
Volatile solids (% raw TS)	88.40 ± 0.64	95.66 ± 0.23	97.36 ± 0.07
TOC (% raw TS)	37.6	46.61 ± 0.16	46.40 ± 0.04
Nitrogen (% raw TS)	2.07	0.51 ± 0.08	1.55 ± 0.08
C/N	18.2	91.39	29.94
Cellulose (% TS)	22.63 ± 0.27	37.30 ± 0.24	28.96 ± 1.60
Xylan (% raw TS)	9.29 ± 0.32	19.61 ± 0.55	21.73 ± 0.21
Lignin (% raw TS)	21.86 ± 1.14	17.62 ± 1.06	18.44 ± 0.68

4.1.2. Digestion Performance

Figure 6 summarizes different patterns of biogas production, methane content, VS content reduction, cellulose/xylan content reduction, and VFA content as parameters of the anaerobic codigestion of two feedstock mixtures (CS and AFEX). During the 75-day semi-continuous digestion, three stages: 1 (days 0–20), 2 (days 21–40), and 3 (days 41–75), were chosen based on the HRT of 20 days to investigate dynamic changes during the anaerobic codigestion.

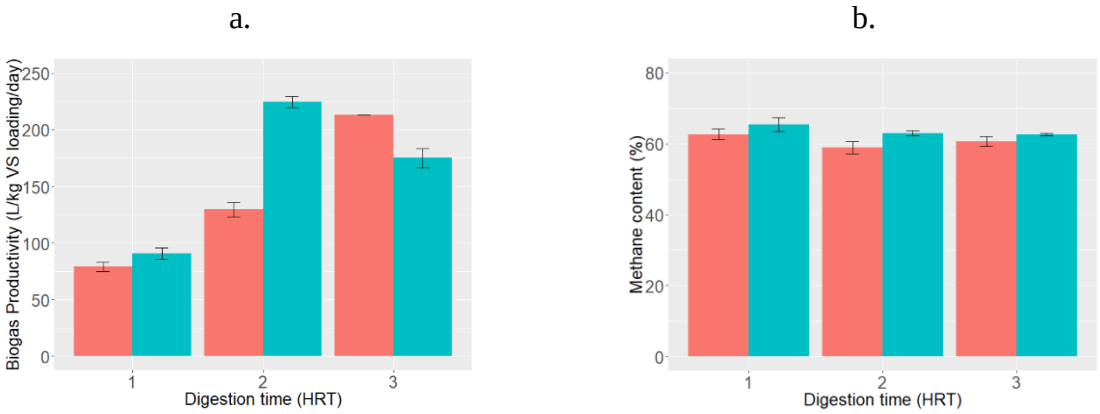
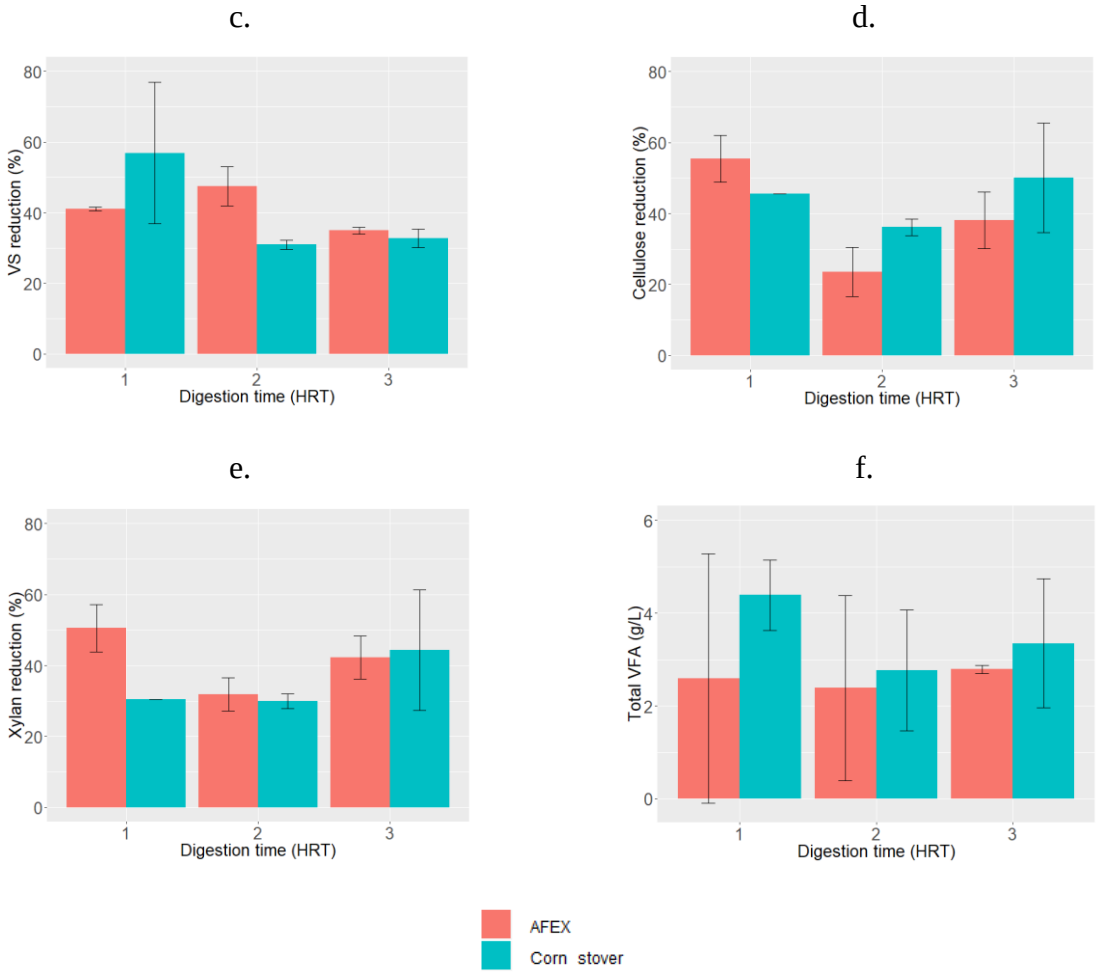


Figure 6. Digestion performance of AFEX and CS during the digestion (three HRTs). a. Biogas productivity, b. methane content, c. VS content reduction, d. cellulose content reduction, e. xylan content reduction, f. total VFA concentration.

Figure 6 (cont'd)



The experimental data revealed that the factors of feedstock and digestion time had a significant ($p < 0.05$) influence on biogas production (Table 3), and both digestion procedures had a lag phase in biogas production (Figure 6a). The lag phases for the CS and AFEX-pretreated corn stover digestion were approximately one and two HRTs, respectively. Biogas production by the CS codigestion rapidly increased to 225 ± 5 L/[kg VS loading] per day in the 2nd HRT relative to the 1st HRT (91 ± 5 L/[kg VS loading] per day), and then leveled off in the 2nd HRT to reach stable biogas production of 175 ± 8 L/[kg VS loading] per day (Figure 6a). As for the AFEX-pretreated corn stover codigestion, biogas production kept increasing from 79 ± 4 L/[kg VS loading] per day in the 1st HRT gradually to 130 ± 6 L/[kg VS loading] per day, and then stabilized

at 213 ± 0 L/[kg VS loading] per day in the 3rd HRT. In the stabilized 3rd HRT, the AFEX-pretreated corn stover codigestion indicated significantly ($p < 0.05$) higher biogas production in comparison with the CS codigestion.

In terms of CH_4 content, feedstock and HRT had a significant ($p < 0.05$) impact on methane content in the biogas during the digestion (Table A1). Both codigestion procedures started at slightly but significantly ($p < 0.05$) higher methane contents (62% ± 1% and 65% ± 2% for the AFEX-pretreated corn stover and CS digestion reactions, respectively) in the 1st HRT than the subsequent HRTs (Figure 6b). Methane contents stabilized at 61% ± 1% and 62% ± 0% for the corresponding digestion reactions in the 3rd HRT without a significant ($p > 0.05$) difference between the two digestion groups (Figure 6b). The variation observed in the content of the biogas samples is normal during the establishment of methanogenic communities.

As for the VS content reduction, it was similar to VFA in that feedstock and digestion time generally had no significant ($p > 0.05$) influences on the VS content reduction in both digestion reactions (Table A1). However, the VS content reductions largely fluctuated in the first two HRTs (Figure 6c). After the 1st HRT, the AFEX-pretreated corn stover codigestion yielded a greater VS content reduction than did the CS codigestion, particularly in the 2nd HRT where the VS content reduction of the AFEX-pretreated corn stover codigestion (47% ± 5%) was significantly higher than that (31% ± 1%) in the CS codigestion. This observation along with the higher biogas productivity of the AFEX-pretreated corn stover codigestion could be explained by the chemical and structural changes of the corn stover during the AFEX treatment. Anhydrous ammonia in the AFEX pretreatment reacts with feruloyl or coumaryl ester bonds in biomass to form amides, especially acetamide (Chundawat et al., 2013; Chundawat, Donohoe, et al., 2011). Guyot et al. demonstrated that amide compounds can be easily degraded by anaerobes and methanogens

(Guyot, Ferrer, & Florina, 1995). Accordingly, the AFEX-pretreated corn stover codigestion manifested more efficient biogas formation from VS than did the control CS codigestion.

Both factors—digestion time and feedstock—generally had no significant ($p > 0.05$) impact on cellulose and xylan content reduction, except that the digestion time was a significant factor for cellulose content reduction. However, high variations of cellulose and xylan degradation were observed during AD (Figure 6d & 6e). The AD of the AFEX-pretreated corn stover yielded significantly ($p < 0.05$) higher cellulose and xylan content reductions in the 1st HRT than that of the CS digestion. After the 2nd HRT, the differences in cellulose and xylan content reductions between the two codigestion experiments were not significant ($p > 0.05$). The original mixtures of both codigestion reactions had higher concentrations of fresh AFEX-pretreated corn stover and CS in the reactor at the beginning of the digestion (the 1st HRT). Accordingly, more cellulose and xylan from AFEX-pretreated corn stover and CS were released into the reactors. Because the cellulose and xylan in the AFEX-pretreated corn stover were relatively easy to digest by microbes owing to the loose carbohydrate–lignin bonds (V Balan et al., 2012), the larger amount of the AFEX-pretreated corn stover in the 1st HRT led to significantly greater cellulose and xylan content reductions than did the CS digestion. After the 1st HRT, 20% of AFEX-pretreated corn stover and CS in the feed for codigestion might be too little to reveal significant impacts on the overall cellulose and xylan content reductions (Figure 6d & 6e).

The VFA data further verified the performance patterns of these two digestion reactions (Figure 2f). A large variation (2.6 ± 2.7 g/L) between replicates of the AFEX corn stover digestion and a high VFA concentration (4.3 ± 0.8 g/L) of the CS digestion indicated unstable digestion during the 1st HRT. With progression of the digestion, the VFA concentrations stabilized at 2.8 ±

0.1 and 3.4 ± 1.4 g/L for the AFEX-pretreated corn stover and CS digestion reactions, respectively (Figure 2f).

In Figure 7, the concentration of two VFAs being quantified is plotted: Hac and Hpa. On average, the sum of the concentrations of these two VFAs represents 80% of the total acid-producing fermentation measured in the effluents. The ANOVA results (Table A1) suggested that in the 2nd HRT, the concentration of Hac was significantly lower ($p = 0.01$) in group AFEX (0.41 g/L) in comparison with group CS (1.82 g/L).

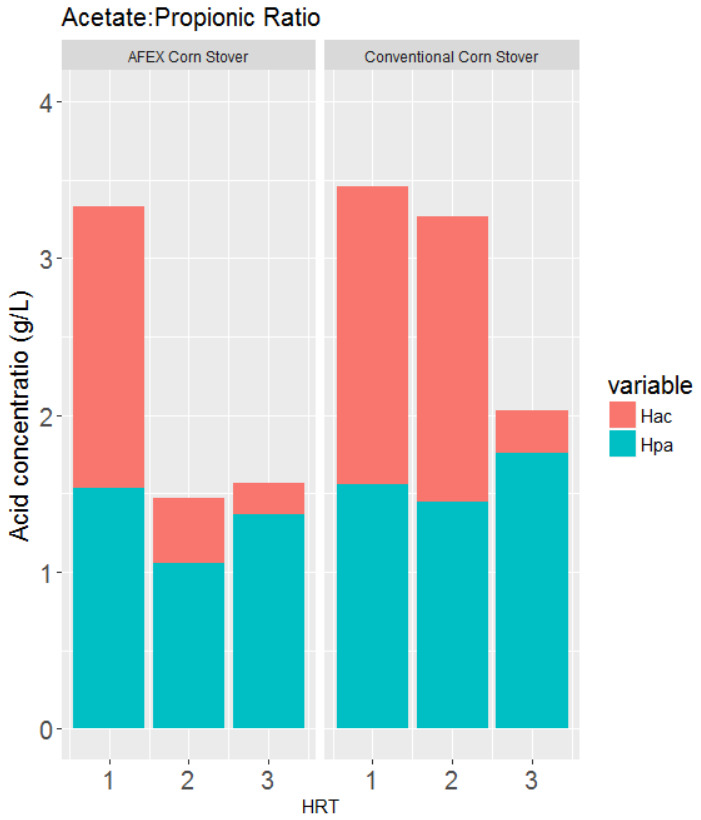


Figure 7. Average acetate (Hac) and propionic acid (Hpa) concentrations in the reactors.

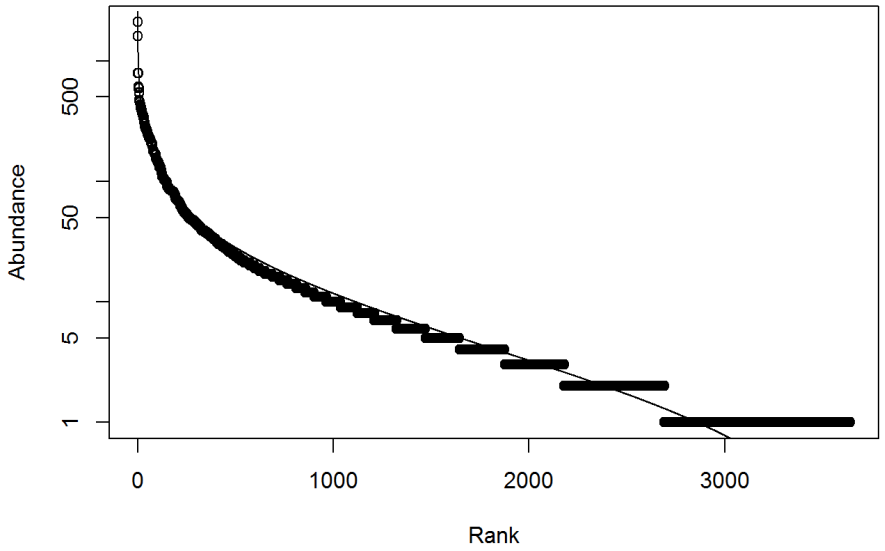
On the other hand, Hpa concentration did not show significant differences between the different digestion reactions and at the same time did not undergo any reduction throughout the whole experiment. The concentrations of these two acids could be having a strong effect on biogas

productivity and on microbial ecology. Wang, Zhang, Wang, & Meng, (2009) reported that 0.9 g/L Hpa has a significant inhibitory effect on a methanogen's growth ($p < 0.01$). Those authors also found that this inhibition resulted in the accumulation of acetate and affected the total methane yield. By contrast, such accumulation was not seen in the AFEX reactors according to Figure 7, and the concentration of acetate was significantly lower in the AFEX reactors in comparison with CS reactors. Moreover, Chundawat, Beckham, et al., (2011) described how the AFEX pretreatment produces a series of ammonolytic and hydrolytic reactions that cleave various ester linkages, thereby resulting in the formation of amides and acids such as acetate. This evidence may confirm a possible influence of AFEX pretreatment on the acetate formation and consumption by codigestion in the AFEX reactors. It is important to remember that the group of methanogens are acetate reducers, and the concentration of acetate will have a major impact on the growth of this group and on methane formation (Rittmann & McCarty, 2001).

4.1.3. Microbial Community Analysis

Figure 8 illustrates the evenness and richness diversity of the microbial species in each reactor (α-diversity). The average relative abundance of microbial species indicates a good fit to the lognormal distribution of the microbial communities and reveals high rarity of the species (Figure 8a). Similar evenness was observed during codigestion of different feedstock mixtures elsewhere (R. Chen et al., 2016; Rojas-Sossa et al., 2017). The sampling richness curves for each digester are presented in Figure 8b. The diversity of the microbial communities in the codigestion experiment was significantly different from that of the inoculum. The shifts could be caused by changes of nutrients in the feedstock mixture as well as introduction of new microbial species in groups CS and AFEX (Kirkegaard et al., 2017).

a.



b.

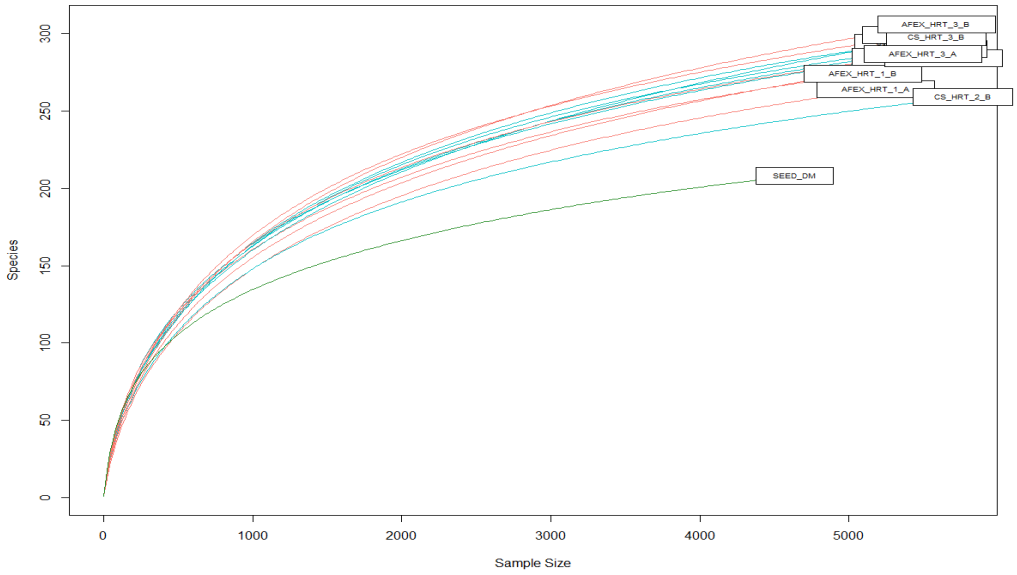


Figure 8. Diversity of microbial communities in both digestion reactions. a. The rank abundance (Whittaker) plots of relative abundance of OTUs in both digesters. The dots represent the logarithmic percentage of the relative abundance of each species, and then the lognormal curve was plotted on the data. b. Examples or the diversity curves seen in the digesters.

Figure 8b also indicates that during the stable digestion performance (the 3rd HRT), microbial communities of the AFEX codigestion reaction were slightly more diverse than those of

the CS codigestion reaction. This is because the sampling curves showed higher steepness in the 3rd HRT. This finding indicates that AFEX-pretreated corn stover allowed for digestion to maintain a higher number of microbial species and a large number metabolic fluxes could be happening inside the reactors (Colwell & Rangel, 2009). The diversity indices of the digestion reaction for three HRTs are presented in Figure 9.

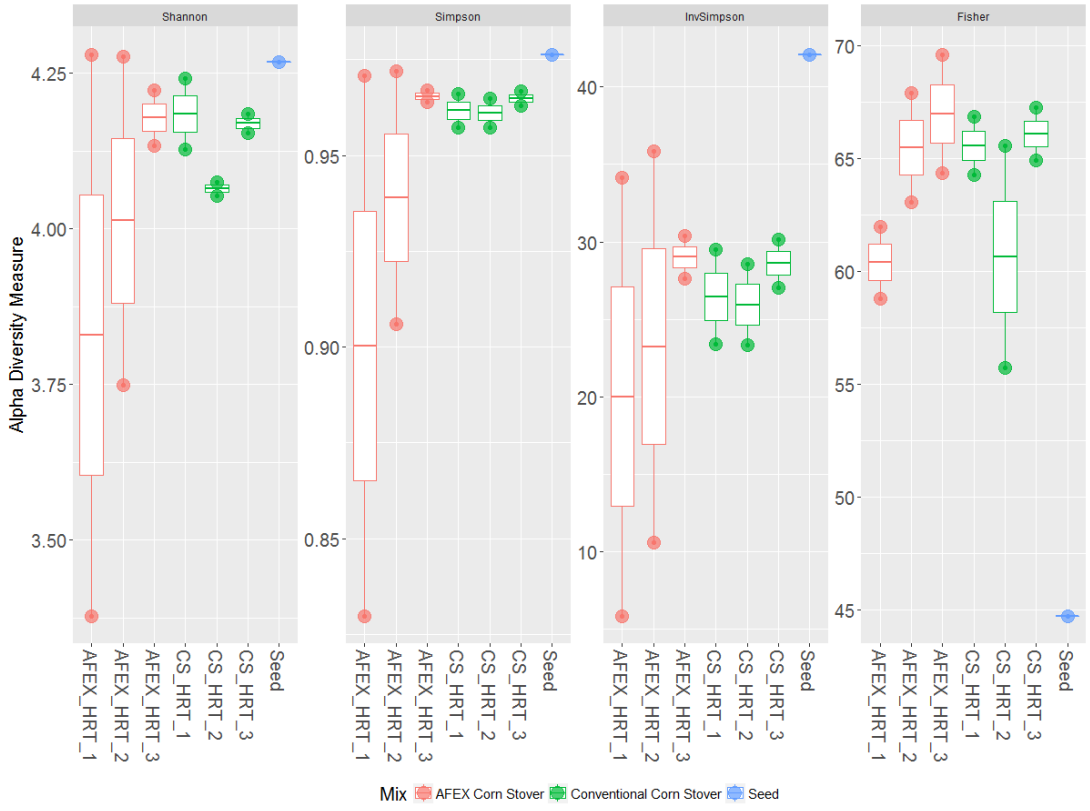


Figure 9. Ecological diversity indices (Shannon, Simpson, Inverse Simpson, and Fisher) for each AD reactor.

Much larger variation in Shannon, Simpson, and Inverse Simpson indices for the AFEX replicates was observed in the 1st and 2nd HRTs as compared to the CS replicates. The variation significantly diminished in the 3rd HRT. The results once again show that AFEX certainly has a bigger impact on the microbial communities of AD. Meanwhile, the Fisher diversity index indicates a large difference between the seed and digestion samples, consistently with the

rarefaction curve (Figure 8b), where the seed diversity was much lower than that of the digestion samples.

Figure 10 shows the relative abundance of AFEX and CS samples at four taxonomic levels. At the domain level, as presented in Figure 10a, the total relative abundance of archaea was higher in the AFEX digesters than CS reactors in the 2nd and 3rd HRTs. The data suggest that AFEX codigestion has a potential to enrich the archaea population. The distribution of dominant phyla (Bacteroidetes, Proteobacteria, Spirochaetes, and Verrucomicrobia) is depicted in Figure 10b. These phyla have been detected in other digestion studies. The phylum Bacteroidetes of AFEX and CS digesters showed significant increases in its relative abundance as compared to the seed sample. Considering the high carbohydrate content in groups AFEX and CS, Bacteroidetes as carbohydrate-degrading microbes correspondingly increased in number at the beginning of the digestion to satisfy the need for nutrient utilization to support healthy digestion. With progression of the digestion, stable digestion was achieved, and balanced communities formed correspondingly. The abundance of the phylum Bacteroidetes decreased. In contrast to Bacteroidetes, phyla Verrucomicrobia and Proteobacteria did not undergo enrichment relative to the seed. Both decreased in abundance with the digestion duration. This phenomenon may be caused by the increased carbohydrate content and reduced protein amount of AFEX and CS feedstocks. Of note, the phylum Spirochaetes got enriched during the digestion of AFEX and CS (Figure 10b). In the 3rd HRT, the AFEX digesters showed much higher relative abundance of these genera compared to other genera. The rich nonrecalcitrant carbohydrates of the AFEX reaction could have played a key role in this shift. Two main genera from the phylum Spirochaetes were found: *Treponema* and *Sphaerochaeta* (Figure 10c). Turroni et al., (2016) detected a significant

increase in the abundance of *Treponema* in human gut microbiomes of hunter-gatherers who lived on high-plant-fiber diets. The relative abundance of archaeal genera is illustrated in Figure 10d.

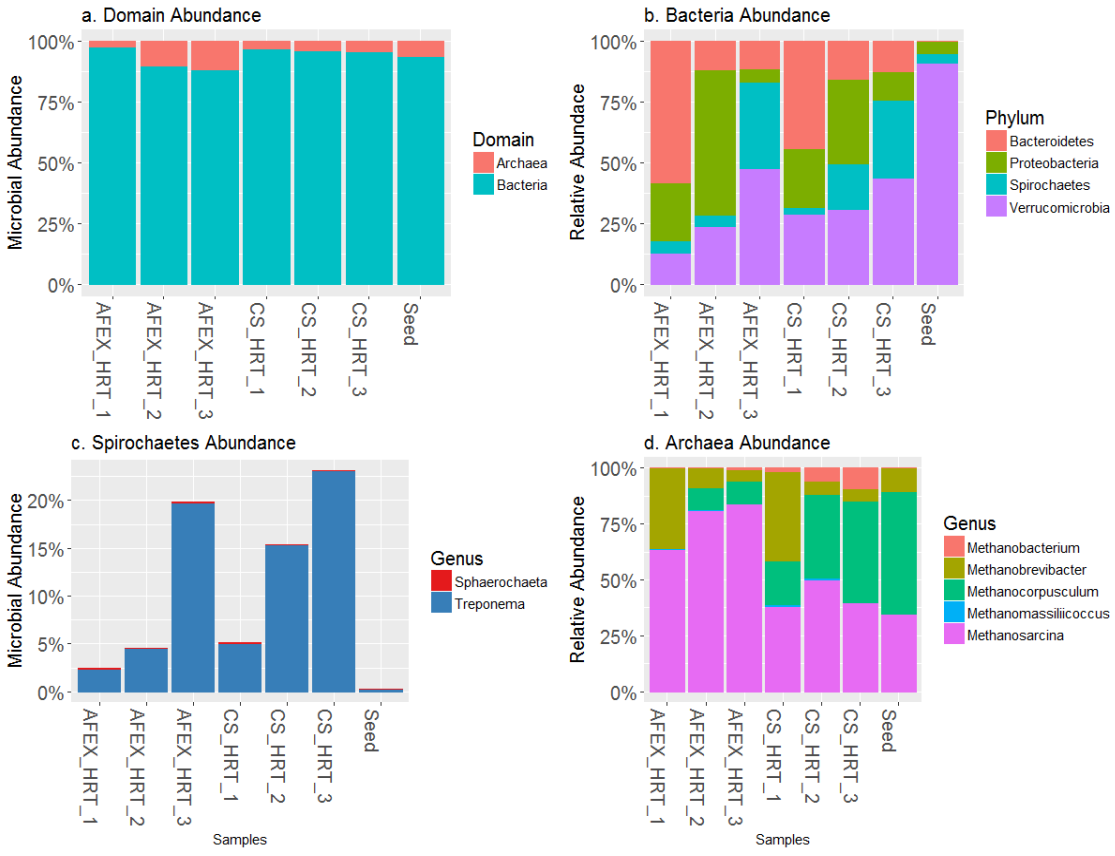


Figure 10. Relative abundance of different taxa found in the reactors. a. Relative abundance of the microbial domains. b. Relative bacterial phylum abundance. c. Relative Spirochaetes genera abundance. d. Relative Archaea genera abundance.

Methanosarcina, *Methanocorpusculum*, and *Methanobrevibacter* were three dominant archaea in both AFEX and CS digestion reactions. *Methanocorpusculum* and *Methanosarcina* got enriched with the digestion duration. Greater enrichment of *Methanosarcina* was present in the AFEX digestion reaction than in the CS digestion, whereas more *Methanocorpusculum* was present in the CS digestion reaction than in the AFEX digestion reaction, however total archaea enrichment in group CS was lower than that in group AFEX (Figure 10a). On the other hand, the genus *Methanobrevibacter* showed a decrease in the relative abundance with an increase in HRTs.

This genus is known as a hydrogenotroph that oxidizes hydrogen to produce methane. This could be happening because of propionate accumulation, which could be affecting the growth of this group. Propionate is an important intermediate during AD, and this degradation produces Hac, H_2, and CO_2 (Li, Ban, Zhang, & Jha, 2012). In addition, the genus *Methanobacterium* manifested good enrichment in the AFEX digestion reaction as the digestion progressed. The changes in relative microbial abundance of this Archaea genus are consistent with the observed performance and the structural characteristics of lignocellulosic materials as the feed. The microbial community analysis led to the conclusion that the AFEX reaction significantly enriched Archaea communities during the digestion, and accordingly, biogas productivity significantly increased.

Finally, nonmetric multidimensional scaling visualization of the microbial abundance was conducted to elucidate the relations between microbial communities and digestion performance; the results are presented in Figure 11. The visualization uncovered important correlations among microbial communities, biogas productivity, feedstocks, and xylan/VS content reduction. A similar trend has been observed in other similar studies. There was a possible inverse linear correlation between the AFEX biogas productivity and Hac metabolic fluxes in the reactor.

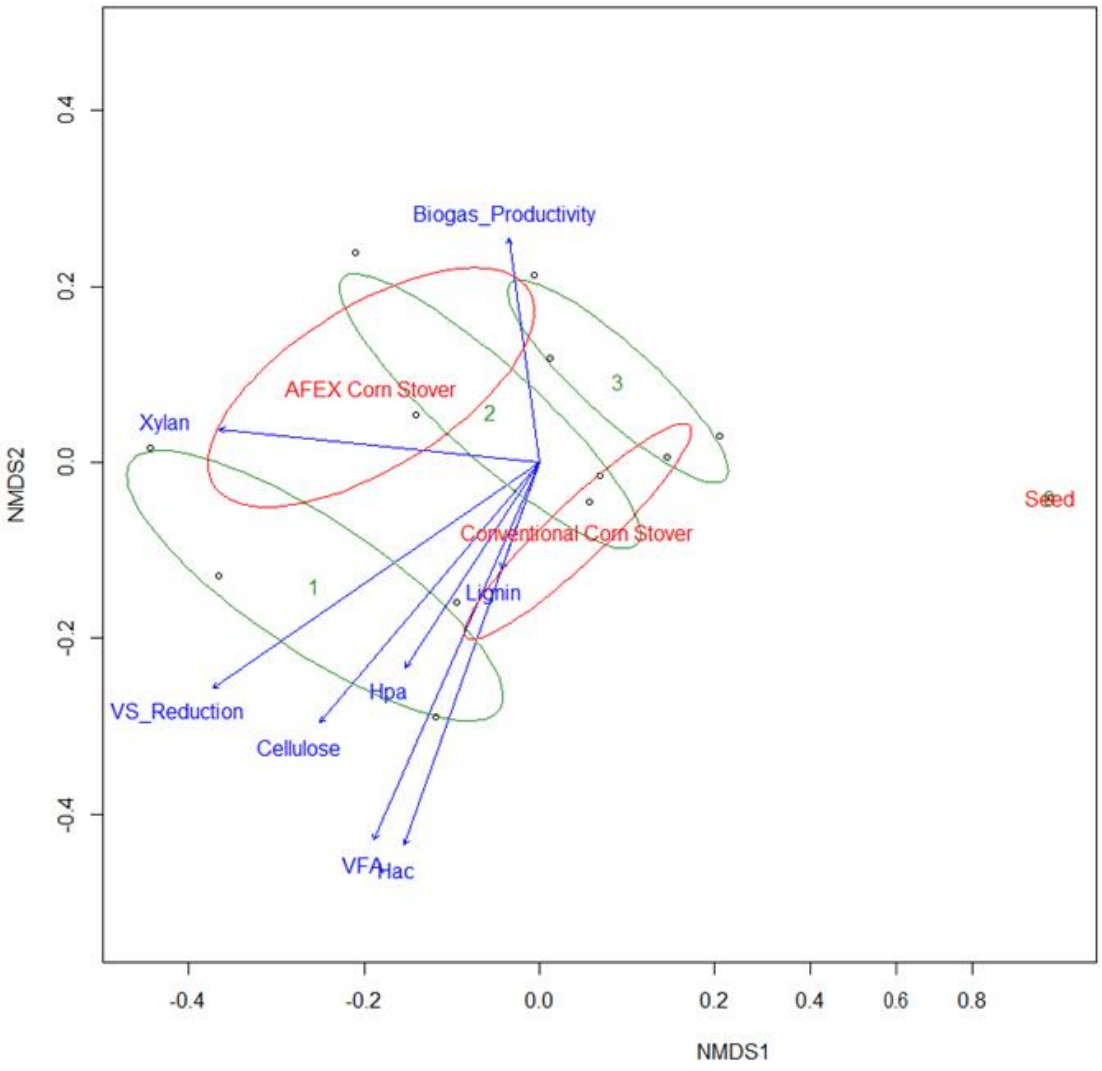


Figure 11. Nonmetric multidimensional scaling of the relative abundance of microbial communities in the digesters.

4.2. Degradation of Lignocellulosic Feedstocks at ABPs

4.2.1. Influent and effluent dry matter and characterization of raw samples

Figure 12 is a plot of the average influent and effluent composition observed at different ABPs.

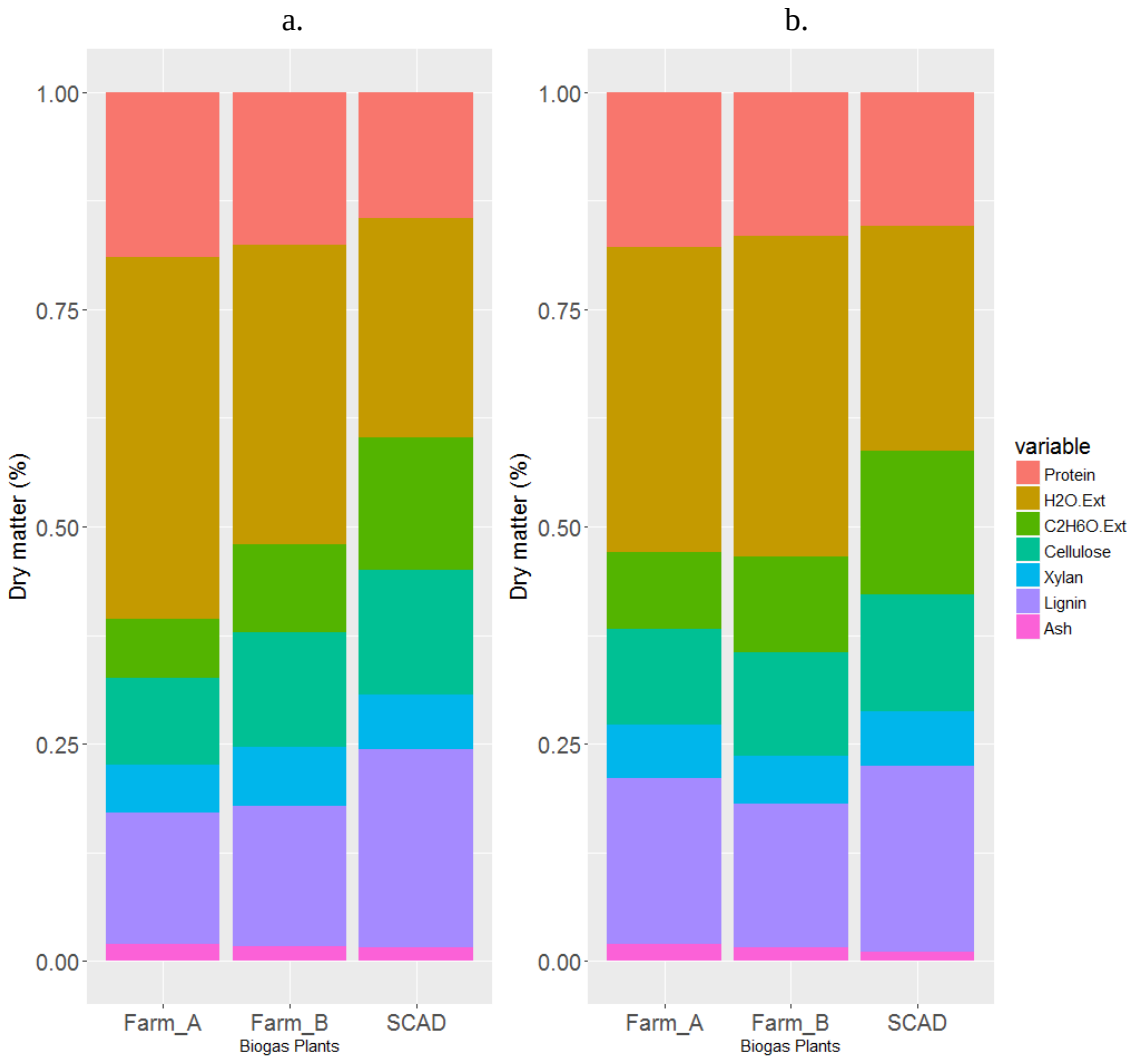


Figure 12. a. Influent and b. effluent composition analysis on a dry-matter basis.

In Figure 12a, the relative composition of the influents of biogas plants. Table A2 shows a pairwise comparison for this analysis. According to the data, there were significant differences in the protein content and ethanol extractives. The SCAD protein content was significantly lower in

comparison with Farm A ($p = 0.002$) and Farm B ($p = 0.014$). In contrast, the amount of SCAD ethanol extractives was significantly higher than that on Farm A ($p = 0.09$); there were no significant differences in the pairwise comparison SCAD–Farm B. Meanwhile, there was some variation of this composition, and we found very similar data on lignocellulosic content: 12% cellulose, 5% xylan, and 18% lignin. These values are lower than the ones EPA recommends as AD feedstocks: 22% cellulose, 36% hemicellulose, and 21% lignin (EPA, 2014; Sun & Cheng, 2003). Most of the ABPs studied practice recycling of the effluent. The digestate is normally filtered in a liquid–solid separator and is mixed with a fresh influent; then, it is fed into the reactor. This procedure increases alkalinity of the influent and maintains healthy digestion. This procedure may dilute the influents and could be responsible for low content of plant cell wall components at the ABPs studied. On the other hand, in the case of effluents, more significant differences were found (Table A2).

Significant differences were found in protein, water extractives, ethanol extractives, and lignin content of the effluent samples. As for protein, we found again a lower concentration in the SCAD reactor in comparison with Farm A ($p = 0.02$), suggesting possible major consumption of protein by Farm B in comparison with the other two biogas plants. Moreover, H_2O.Ext content was significantly lower at the SCAD ABP in comparison with the Farm B effluents ($p = 0.07$). The C_2H_6O.Ext concentration of the effluents showed significant differences between SCAD and Farm A ($p = 0.04$); if this parameter is compared between influents and effluents, any reactor showed substantial consumption of this substance.

Significant differences were found in lignin content between SCAD and Farm B ($p = 0.05$); this pattern is suggestive of possible storage of lignin in the Farm A and B reactors, and this storage seems to be higher in the Farm A reactor. Yue et al. (2013) reported that AD codigestion of DM

normally can homogenize the AD effluents, producing very similar concentrations of plant cell components relative to one another in the AD effluent. Finally, ash content was evaluated here: all the reactors had similar contents of ash in the effluent, which contained ~2% of dry matter.

Figure 13 presents plots of the proportions (%) of sCOD, COD, NH_3 (ammonia), and TKN in the influent samples and effluent samples from the three ABPs sampled. In Table A2, pairwise comparisons of these raw parameters are detailed.

There was higher COD relative content in the SCAD reactor in comparison with Farm A ($p = 0.06$) and Farm B ($p = 0.06$), and this concentration was similar between Farms A and B. As Figure 13A shows, effluent sCOD concentration was lower on Farm A than at SCAD ($p = 0.09$).

On the other hand, Figure 13B shows the averages of nitrogen forms quantified in the streams: NH_3 and TKN. NH_3 content of influents was higher on Farm B than at SCAD ($p = 0.08$). NH_3 content of effluents was higher at SCAD than on Farm B ($p = 0.02$), indicating a possibly higher ammonification rate in the SCAD reactor in comparison with Farm B. As for Farm A, almost nonexistence of ammonification was found. The TKN content of effluents was higher on Farm A than on Farm B ($p = 0.02$). TKN concentration (Figure 13B) increased in all the reactors, and on Farms A and B, this could be happening because these ABPs had a higher protein concentration in the diet.

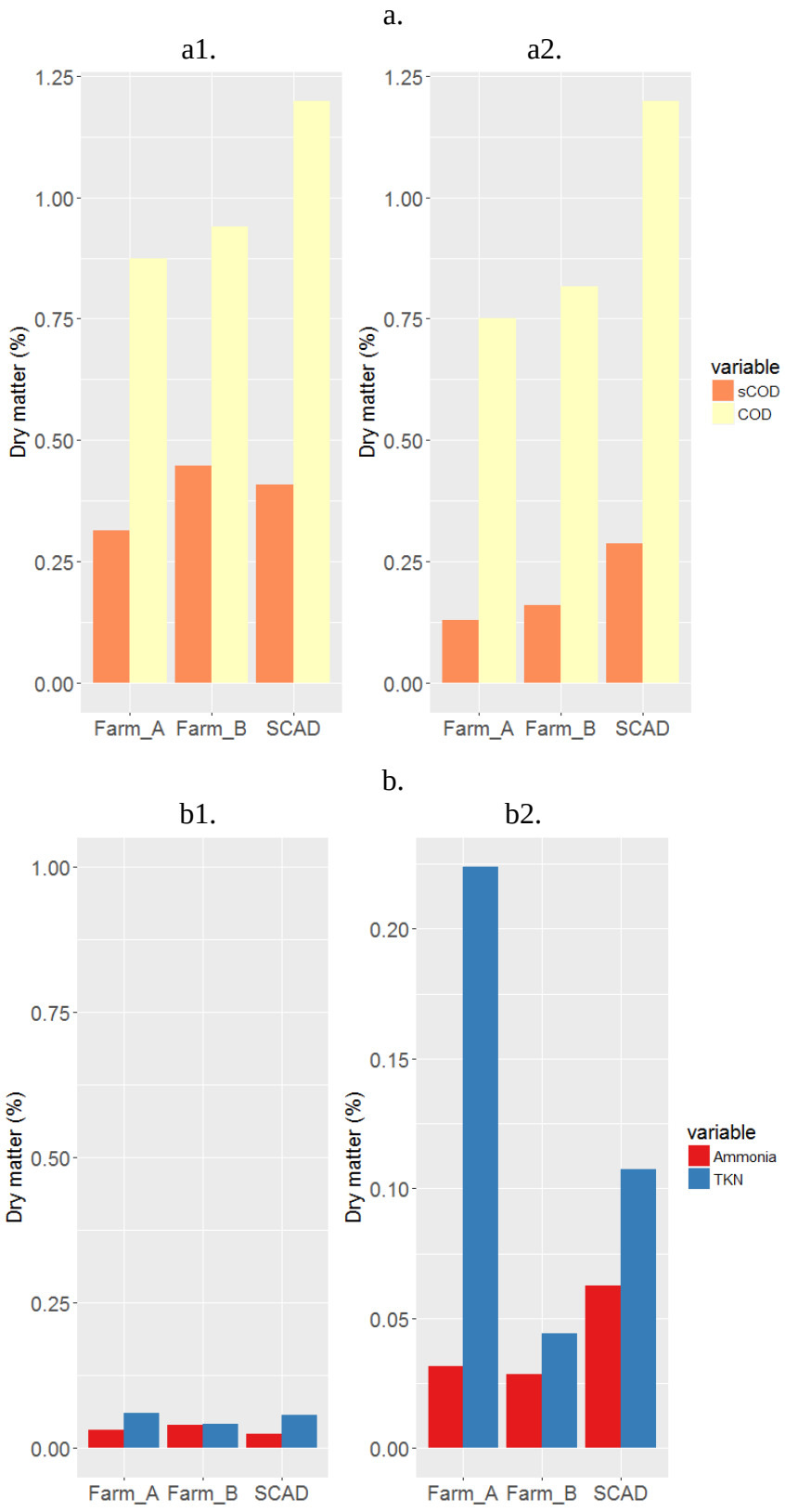


Figure 13. Raw sample characterization. a. Chemical oxygen composition of a1. influents, a2. influents. b. Concentrations of nitrogen forms: b1. influents, b2. effluents.

Finally, the last parameter studied was TOC; ANOVA analysis uncovered significantly higher TOC contents in Farm B ($p = 0.08$) and SCAD ($p = 0.005$) influents in comparison with Farm A influents; significant differences were not found in the effluent samples. Figure 14 depicts the variation of this parameter in the scatterplot on the different dates of sampling. These data indicate how the only reactor that showed clearly substantial consumption of TOC throughout the dates of sampling was the SCAD reactor. Farm A and Farm B influents fluctuated a lot throughout the experiment; actually, very similar TOC influent and effluent concentrations were observed.

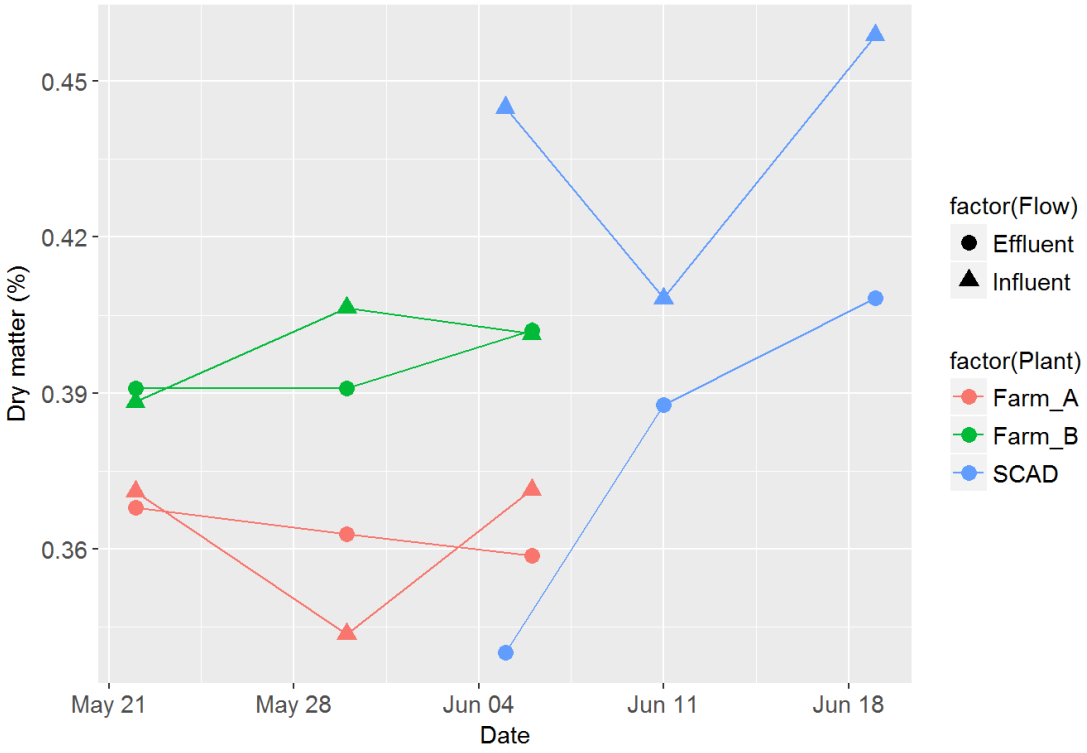


Figure 14. TOC variation in the dry matter of the influents and effluents throughout the sampling period.

Table 4 presents an average reduction in each parameter across the sampling time points for the different ABPs. All the data showed degradation of sCOD, COD, protein, and TOC. By contrast, almost all the ABPs showed an increase in NH_3, TKN, H_2O.Ext, and C_2H_6O.Ext concentrations. Finally, in terms of decreases in cellulose, xylan, and lignin concentrations, the

reactors showed differences among the different ABPs. Farm A did not show a reduction in the amount of plant cell wall components; in contrast, Farm B manifested a stronger decrease in cellulose (11% ± 6%) and xylan (18% ± 3%) and lignin.

Table 4. Decreases in the averages dry matter content of each parameter in the reactors.

	Percentage of decrease		
Variables	**Farm A**	**Farm B**	**SCAD**
R_{sCOD}	53% ± 11%	44% ± 31%	42% ± 16%
R_{COD}	14% ± 9%	7% ± 21%	11% ± 62%
R_{NH_3}	-14% ± 1%	34% ± 2%	-208% ± 3%
R_{TKN}	-206% ± 9%	-33% ± 2%	-102% ± 7%
$R_{Protein}$	8% ± 2%	6% ± 1%	-10% ± 3%
R_{TOC}	0% ± 3%	1% ± 2%	13% ± 8%
$R_{(H_2O)_{Ext}}$	17% ± 30%	-6% ± 28%	-6% ± 10%
$R_{(C_2H_6O)_{Ext}}$	-26% ± 7%	-10% ± 5%	-13% ± 16%
$R_{Cellulose}$	-9% ± 9%	11% ± 6%	4% ± 11%
R_{Xylan}	-9% ± 5%	18% ± 3%	-3% ± 5%
R_{Lignin}	-23% ± 9%	-1% ± 7%	3% ± 6%

Finally, SCAD showed a reduction in cellulose (4% ± 11%) and lignin (3% ± 6%) contents but yielded an increase in xylan concentration (negative reduction: –3% ± 5%). It should be mentioned that this plant's amounts of cell components are still lower in comparison with the amounts seen in R_{sCOD} and R_{COD}.

4.2.2. BMP Experiments on ABP influents

The BMP experiments revealed a significant difference in biogas productivity from the influents between SCAD and Farm A, with SCAD being significantly better than Farm A ($p = 0.06$): almost threefold higher biogas productivity in comparison with Farm A. Nonsignificant

differences were noted between Farm B or SCAD and Farm A BMP. Figure 15 is a plot of the accumulation of gas production in the reactors; it is not possible to detect any inhibition at the beginning of the experiment.

The biogas productivity obtained was 278 ± 68, 390 ± 117, and 659 ± 71 L/[kg initial VS] for Farm A, Farm B, and SCAD, respectively.

a.

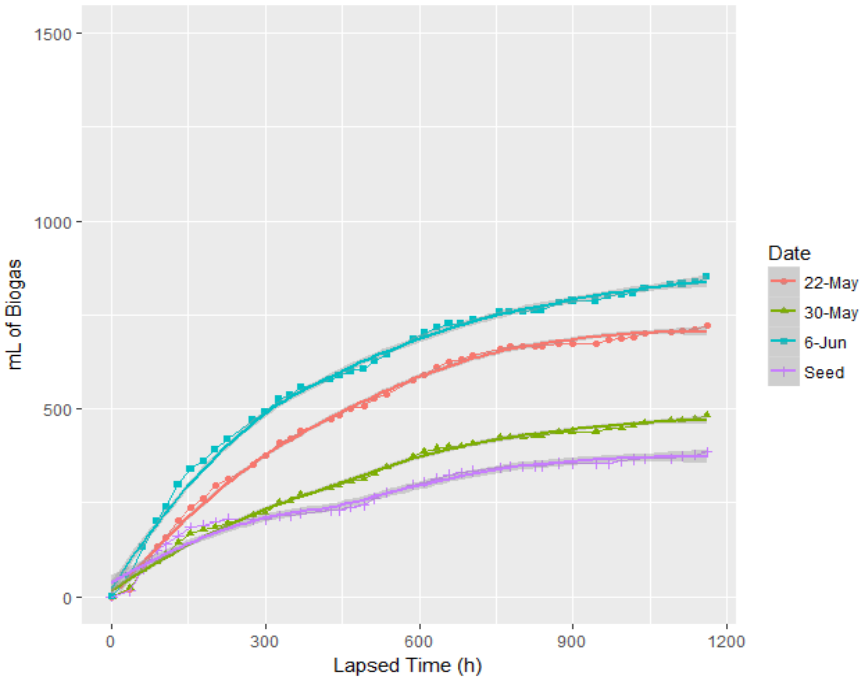
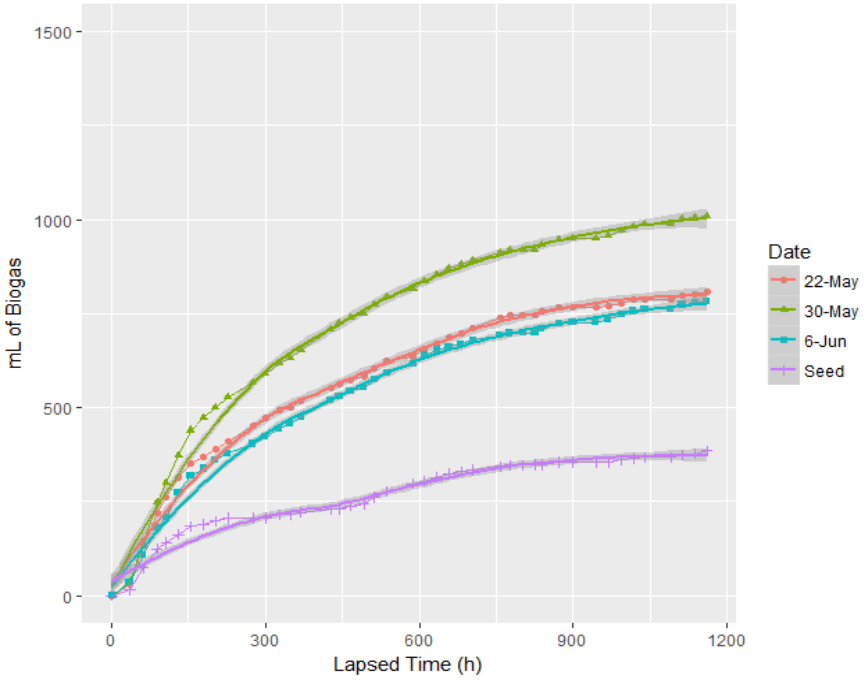


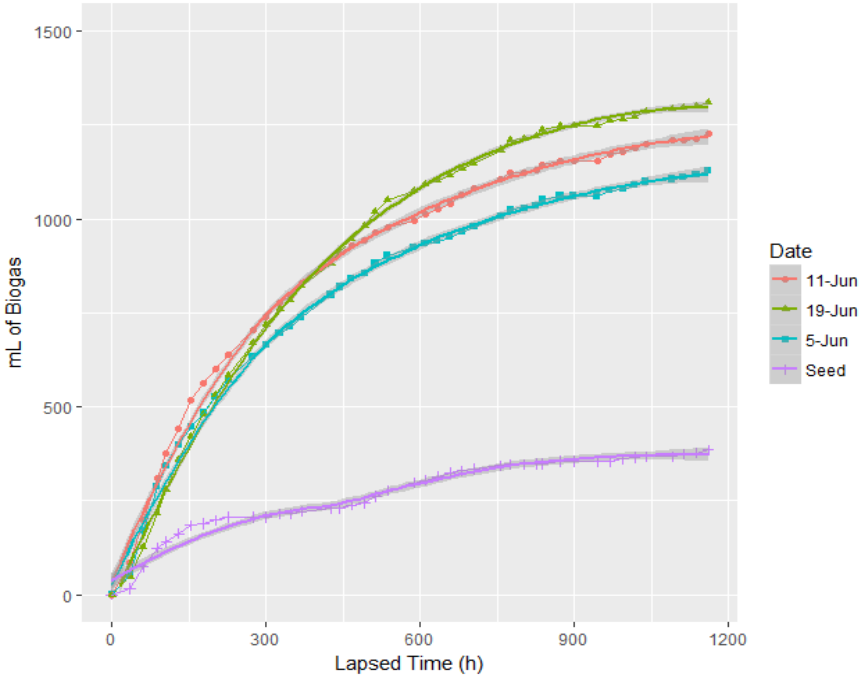
Figure 15. BMPs: accumulated gas production on different dates of sampling of influents. a. Accumulated gas production of Farm A, b. accumulated gas production of Farm B, c. accumulated gas production of the MSU south campus digester.

Figure 15 (cont'd)

b.



c.



4.2.3. Variance-based sensitivity analysis of the downregulation of each compound and the effect on biogas productivity

The objective of this analysis was to explain the biogas productivity observed in the BMP with the data on downregulation of substances obtained above. The input of the model (equation 11) is the data on concentration reductions (Table 4). Figure 16 illustrates the output of the analysis. The contribution to the variance was ranked for each parameter. This approach allows us to infer possible sensitivity of biogas productivity to each parameter. There were possible positive correlations with R_{TOC}, R_{Lignin}, $R_{(C_2H_6O)_{Ext}}$, $R_{Cellulose}$, and R_{TKN}. On the other hand, there was a possible negative correlation of biogas productivity with R_{COD}, $R_{(H_2O)_{Ext}}$, R_{sCOD}, R_{NH_3}, and $R_{Protein}$. The magnitude of a correlation is dependent on the absolute value of the contribution to the variance. It can be noted that there were greater correlations of biogas productivity with R_{TOC} (17.8%) and $R_{Protein}$ (−18.1%), relative to the other parameters, with R_{TOC} showing a positive correlation and $R_{Protein}$ a negative one. These two parameters are direct measurements of carbon (TOC) and nitrogen (protein). The ABPs have different diets namely Farm A and Farm B diets contain a low concentration of carbon and high content of protein. On the other hand, SCAD nutrition contains more TOC in comparison with the other two ABPs studied. Then, the other parameters (with lesser correlation) were ranked: R_{Lignin} (12.5%), $R_{(C_2H_6O)_{Ext}}$ (6.6%), $R_{Cellulose}$ (3.7%), and R_{TKN} (2.9%). The negative ranking was $R_{(H_2O)_{Ext}}$ (−9.9%), R_{sCOD}(−13.0%), and R_{NH_3} (−14.7%). These results can be explained by the high productivity observed at SCAD in comparison with the other ABPs. SCAD had a higher content of lignin and C_2H_6O.Ext in the influents and effluents and manifested substantial production of NH_3 (Table 4).

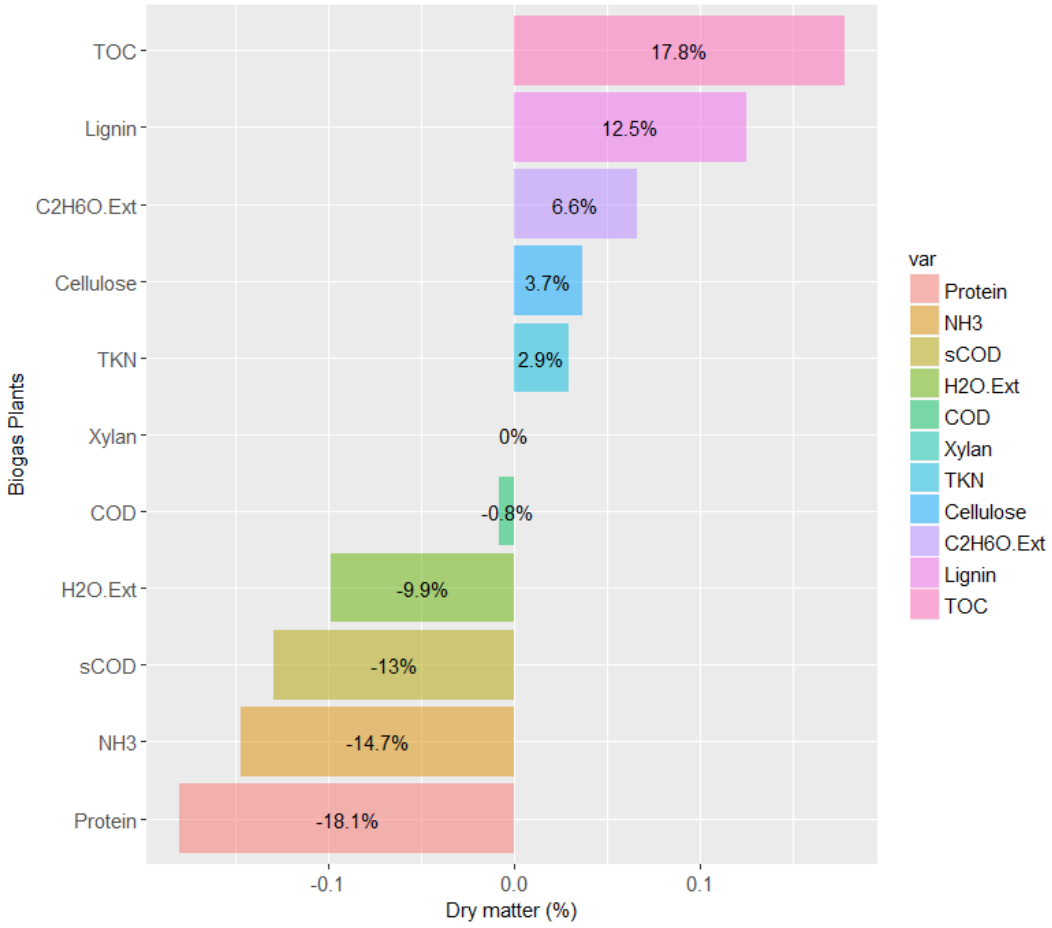


Figure 16. The ranking of contributions to the variance of biogas productivity by each of the parameters measured.

Moreover, the negative correlation could be explained by the different reductions in concentrations of substances among the different ABPs. For example, there is a substantial correlation with $R_{(H_2O)_{Ext}}$ and R_{NH_3}; these phenomena could be due to the activities on Farm A, where production of NH_3 was important and consumption of H_2O.Ext was important too. Nonetheless, this ABPs also has lower biogas productivity. Finally, regarding R_{sCOD}, most of the biogas plants studied had very similar R_{sCOD} values but different biogas productivity; therefore, this parameter is not important for the explanation of differences in biogas productivity.

Finally, the importance of R_{Xylan} for biogas productivity was equal to 0%, indicating insignificance change in the amount of this plant cell wall component for biogas productivity observed at the ABPs under study.

5. CONCLUSIONS

The results show a big picture of actual stages in the use of plant cell wall components as an energy source in AD. Presented below are specific conclusions from both research projects described above.

5.1. Dynamic microbiome assembly and the effect on the performance of AD of AFEX-pretreated corn stover and CS

The digestion performance and the microbial community analysis indicate that AFEX-pretreated corn stover promotes a positive linear correlation between a reduction in cellulose content and biogas productivity.

AFEX does not promote degradation of lignocellulosic materials in the reactors studied; rather, the impact of AFEX manifested itself in chemical reduction of acetic acid and its production rate in the reactors.

AFEX codigestion promotes the enrichment with *Methanosarcina* and possibly increases the role of acetic acid as an electron acceptor because *Methanosarcina* is an acetic acid reducer.

The tested CS mixtures promote the enrichment with acid fermenter *Treponema*; this genus has been proven to get enriched with high lignocellulosic inputs.

There is possible migration of various bacteria found in the reactors, which is important. However, there is no migration for Archaea genera. The enrichment could be a consequence of AFEX codigestion.

5.2. Degradation of lignocellulosic feedstocks at the ABPs

There is big variability of the inputs of the digesters sampled.

The results revealed positive and negative effects on biogas productivity. Positive influences were seen where carbon concentration diminished, and negative influences were detected where nitrogen was abundant in the diet.

The ABPs studied have different diets, where Farms A and B show low content of carbon and high concentration of protein in the diet. On the other hand, SCAD has high TOC concentration in comparison with the other two ABPs and is the most productive plant among those studied.

Ranking the AD influents constituents that correlate with biogas productivity. Furthermore, it was revealed that the recalcitrant carbon in ADP influents does not contribute to biogas productivity.

Finally, at the biogas plants sampled, there is no evidence of a possible contribution to biogas productivity from degradation of lignocellulosic materials: most of the contribution results from destruction of carbon-based materials that are not structural carbohydrates.

6. RECOMMENDATIONS

To improve the results of operation of the AD reactors, it is recommended to operate semicontinuous AD for five or six HRTs and with a DM control to measure against a true baseline. In this case, it will be possible to attain a bigger change in the dominate microbial taxa and at the same time to observe uniformity in the characteristics of inputs and outputs. It will also be important to examine these results with a control setup that involves manure digestion. Feedback from the control will enable better monitoring and observation of the metabolic fluxes that take place inside the reactors. Furthermore, a manure control will allow investigators to examine the seed dynamics and the positive effect of corn stover on the diversity of possible enrichment with microbial immigrants fed in with the feedstock from outside. It is strongly recommended to expand research into possible lignocellulosic bacterial fermenter spirilla like *Treponema*. These studies may allow researchers to use this genus as a possible indicator of healthy lignocellulosic AD. Besides, it is important to investigate more thoroughly the relation between *Treponema* enrichment and AFEX^{TM} digestion and their acid formation effects. To decrease the high variation observed at the ABPs sampled, it will be advisable to expand the study to more biogas plants and more sampling dates. Moreover, it will be useful to study this degradation in a more controlled model, for example, in a BMP reactor, where it is possible to change the number of experimental conditions and create triplicates. These two recommendations will decrease the observed statistical insignificance of the results.

APPENDIX

APPENDIX

RSCRIPT for microbial community analysis of digestion from an AD reactor

```
## Dynamic microbiome assembly in performance
## of co-anaerobic digestion of AFEX corn stover
## MICHIGAN STATE UNIVERSITY
## ADREC
##Version 2.0
## Using as inputs 16S OTU tables and Performance reactors data
## Made by Juan Pablo Rojas, 2018
cat("\014") #Erase console

# 1. LOADING LIBRARY AND TABLES ------
library(vegan)
library(phyloseq)
library(MASS)
library(ggplot2)
library(grid)
library(gridExtra)
library(readr)
library(VennDiagram)
library(tidyverse)
library(Rmisc)
library(gtable)
library(devtools)
library(proto)
library(reshape2)

##Choose the OTU.Table should be a .txt
con <- file.choose(new = FALSE)
##Now choose the Taxanomy table should be .txt to
con1 <-file.choose(new = FALSE)

OTU_Table <- read.table(con, header = T, row.names = 1)

OTU_Table_taxonomy <- read.delim(con1, header = T, row.names = 1)

metadata <- read.delim("~/Thesis/ADonAFEXfiber(2016)/DNA-
data/No_manure/Metadata_v2_no_seed.txt", row.names=1)

Methane <- read_delim("Methane.txt", "\t", escape_double = FALSE, trim_ws = TRUE)

# 2 NORMALITY TEST FOR EACH DATA SET----

metadata$Lignin <- NULL

#CONVENTIONAL CORN STOVER

for (i in 4:10) {
  print("#CONVENTIONAL CORN STOVER")
  fit <- metadata %>% filter(Mix == "Conventional Corn Stover")
  print(colnames(fit[i]))
  print(shapiro.test(log(unlist(fit[,i]))))
}
```

```r
#AFEX CORN STOVER

for (i in 4:10) {
  print("#AFEX CORN STOVER")
  fit <- metadata %>% filter(Mix == "AFEX Corn Stover")
  print(colnames(fit[i]))
  print(shapiro.test(log(unlist(fit[,i]))))
}

# 3 ANOVA TEST OF THE PARAMETERS----

metadata$Mix <- factor(metadata$Mix) ##Factor Statement
#Biogas_Productivity
for (i in 1:3) {
  fit <- metadata %>% filter(HRT == i)
  print("HRT")
  print(i)
  ANOVA<- aov(log(unlist(Biogas_Productivity))~Mix, fit) #ONE WAY ANOVA for Productivity
  print(TukeyHSD(ANOVA))  #Plot results
}

#VS_Reduction
for (i in 1:3) {
  fit <- metadata %>% filter(HRT == i)
  print("HRT")
  print(i)
  ANOVA<- aov(log(unlist(VS_Reduction))~Mix, fit) #ONE WAY ANOVA for Productivity
  print(TukeyHSD(ANOVA))  #Plot results
}

#VFA
for (i in 1:3) {
  fit <- metadata %>% filter(HRT == i)
  print("HRT")
  print(i)
  ANOVA<- aov(log(unlist(VFA))~Mix, fit) #ONE WAY ANOVA for Productivity
  print(TukeyHSD(ANOVA))  #Plot results
}

#Hac
for (i in 1:3) {
  fit <- metadata %>% filter(HRT == i)
  print("HRT")
  print(i)
  ANOVA<- aov(log(unlist(Hac))~Mix, fit) #ONE WAY ANOVA for Productivity
  print(TukeyHSD(ANOVA))  #Plot results
}

#Hpa
for (i in 1:3) {
  fit <- metadata %>% filter(HRT == i)
  print("HRT")
  print(i)
  ANOVA<- aov(log(unlist(Hpa))~Mix, fit) #ONE WAY ANOVA for Productivity
  print(TukeyHSD(ANOVA))  #Plot results
}

#Cellulose
for (i in 1:3) {
  fit <- metadata %>% filter(HRT == i)
  print("HRT")
```

```
  print(i)
  ANOVA<- aov(log(unlist(Cellulose))~Mix, fit) #ONE WAY ANOVA for Productivity
  print(TukeyHSD(ANOVA))  #Plot results
}

#Xylan
for (i in 1:3) {
  fit <- metadata %>% filter(HRT == i)
  print("HRT")
  print(i)
  ANOVA<- aov(log(unlist(Xylan))~Mix, fit) #ONE WAY ANOVA for Productivity
  print(TukeyHSD(ANOVA))  #Plot results
}

#Methane
for (i in 1:3) {
  fit <- Methane %>% filter(Mix == "AFEX Corn Stover")
  print(shapiro.test(log(unlist(fit$CH4))))
  fit <- Methane %>% filter(Mix == "Conventional Corn Stover")
  print(shapiro.test(log(unlist(fit$CH4))))
  fit <- Methane %>% filter(HRT == i)
  print("HRT")
  print(i)
  ANOVA<- aov(log(unlist(CH4))~Mix, fit) #ONE WAY ANOVA for Productivity
  print(TukeyHSD(ANOVA))  #Plot results
}

# 4. PLOTTING PERFORMANCE PARAMETERS VS HRT -----
metadata <- read.delim("~/Thesis/ADonAFEXfiber(2016)/DNA-
data/No_manure/Metadata_v2_no_seed.txt", row.names=1)

#Gas Production
Gas_prod <- read_delim("Gas_prod.txt", "\t", escape_double = FALSE, col_types = cols(Date =
col_date(format = "%m/%d/%Y")), trim_ws = TRUE)
gasprod<- ggplot(Gas_prod, aes(Date, Gas, color=Mix)) + geom_point(aes(shape=Mix, color=Mix))
+
  labs(x = "Date", y="mL", title="a. Biogas Production")+
  theme(plot.title = element_text(hjust = 0.5))+
  theme(legend.position="none", axis.text.x = element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))
gasprod

metadata<-metadata[-13, ]
##Calculation of summary of the data
tgc_Biogas <- summarySE(metadata, measurevar="Biogas_Productivity", groupvars=c("Mix","HRT"))
tgc_VS <- summarySE(metadata, measurevar="VS_Reduction", groupvars=c("Mix","HRT"))
tgc_VFA <- summarySE(metadata, measurevar="VFA", groupvars=c("Mix","HRT"))
tgc_Hac <-summarySE(metadata,measurevar="Hac", groupvars=c("Mix","HRT"))
tgc_Hpa <-summarySE(metadata,measurevar="Hpa", groupvars=c("Mix","HRT"))
tgc_Cellulose <- summarySE(metadata, measurevar="Cellulose", groupvars=c("Mix","HRT"))
tgc_Xylan <- summarySE(metadata, measurevar="Xylan", groupvars=c("Mix","HRT"))
tgc_Lignin <- summarySE(metadata, measurevar="Lignin", groupvars=c("Mix","HRT"))
tgc_CH4 <- summarySE(Methane, measurevar="CH4", groupvars=c("Mix","HRT"))

##Make a table
tgc_head <-tgc_Biogas[,1:4]
sum_table<- data.frame(tgc_head, tgc_VS$VS_Reduction, tgc_VFA$VFA,tgc_Hac$Hac,tgc_Hpa$Hpa,
                       tgc_Cellulose$Cellulose,tgc_Xylan$Xylan,tgc_Lignin$Lignin)
sum_table$N<- NULL
```

```
names(sum_table)[4]<-paste("VS_Reduction")
names(sum_table)[5]<-paste("VFA")
names(sum_table)[6]<-paste("Cellulose")
names(sum_table)[7]<-paste("Xylan")
names(sum_table)[8]<-paste("Lignin")
names(sum_table)[9]<-paste("Hac")
names(sum_table)[10]<-paste("Hpa")
head(sum_table)

#CH4 Productivity vs HRT
Biogas<- ggplot(tgc_Biogas, aes(x=HRT,y=Biogas_Productivity, fill=Mix)) +
  geom_bar(stat="identity",position=position_dodge(0.9))+
  geom_errorbar(aes(ymin=Biogas_Productivity-se, ymax=Biogas_Productivity+se), show.legend=F,
width=.1,position=position_dodge(0.9))+
  labs(x = " ", y=expression(m^3~kg~VS^-1), title="b. Biogas Productivity")+
  theme(plot.title = element_text(hjust = 0.5))+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))

# Methane
tgc_CH4$CH4<- tgc_CH4$CH4 * 100
tgc_CH4$se<- tgc_CH4$se * 100
CH4<- ggplot(tgc_CH4, aes(x=HRT,y=CH4, fill=Mix)) +
  geom_bar(stat="identity",position=position_dodge(0.9))+
  guides(fill=FALSE)+labs(x = " ", y="%", title="c. Methane content")+
  geom_errorbar(aes(ymin=CH4-se, ymax=CH4+se), show.legend=F,
width=.1,position=position_dodge(0.9))+
  theme(plot.title = element_text(hjust = 0.5))+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))
CH4

  #VS Reduction vs HRT
tgc_VS$VS_Reduction<- tgc_VS$VS_Reduction * 100
tgc_VS$se<- tgc_VS$se * 100

VS<- ggplot(tgc_VS, aes(HRT, VS_Reduction, fill=Mix)) +
  geom_bar(stat="identity",position=position_dodge(0.9))+
  geom_errorbar(aes(ymin=VS_Reduction-se, ymax=VS_Reduction+se), show.legend=F,
width=.1,position=position_dodge(0.9))+
  guides(fill=FALSE)+labs(x = " ", y="%", title="d. VS Reduction")+
  theme(plot.title = element_text(hjust = 0.5))+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))

#VFA Concentration vs HRT
VFA<- ggplot(tgc_VFA, aes(HRT, VFA, fill=Mix)) +
  geom_bar(stat="identity",position=position_dodge(0.9))+
  geom_errorbar(aes(ymin=VFA-se, ymax=VFA+se), width=.1,show.legend=F,
position=position_dodge(0.9))+
  guides(fill=FALSE)+ labs(x = " ", y="g/L", title="e. Total VFA Concentration")+
  theme(plot.title = element_text(hjust = 0.5))+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
```

```
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))

#Cellulose vs HRT
tgc_Cellulose$Cellulose<- tgc_Cellulose$Cellulose * 100
tgc_Cellulose$se<- tgc_Cellulose$se * 100
Cellulose<- ggplot(tgc_Cellulose, aes(HRT, Cellulose, fill=Mix)) +
  geom_bar(stat="identity",position=position_dodge(0.9))+
  geom_errorbar(aes(ymin=Cellulose-se, ymax=Cellulose+se), width=.1,show.legend=F,
position=position_dodge(0.9))+
  guides(fill=FALSE)+ labs(x = " ", y="%", title="f. Cellulose Reduction")+
  theme(plot.title = element_text(hjust = 0.5))+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))

#Xylan vs HRT
tgc_Xylan$Xylan<- tgc_Xylan$Xylan * 100
tgc_Xylan$se<- tgc_Xylan$se * 100
Xylan<- ggplot(tgc_Xylan, aes(HRT, Xylan, fill=Mix)) +
  geom_bar(stat="identity",position=position_dodge(0.9))+
  geom_errorbar(aes(ymin=Xylan-se, ymax=Xylan+se), width=.1,show.legend=F,
position=position_dodge(0.9))+
  guides(fill=FALSE) + labs(x = "Reactors HRT", y="%", title="g. Xylan Reduction")+
  theme(plot.title = element_text(hjust = 0.5))+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))

#Lignin vs HRT
tgc_Lignin$Lignin<- tgc_Lignin$Lignin * 100
tgc_Lignin$se<- tgc_Lignin$se * 100
Lignin<- ggplot(tgc_Lignin, aes(HRT, Lignin, fill=Mix)) +
  geom_bar(stat="identity",position=position_dodge(0.9))+
  geom_errorbar(aes(ymin=Lignin-se, ymax=Lignin+se), width=.1,show.legend=F,
position=position_dodge(0.9))+
  guides(fill=FALSE) + labs(x = "Reactors HRT", y="%", title="g. Lignin Reduction")+
  theme(plot.title = element_text(hjust = 0.5))+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))

get_legend<-function(myggplot){
  tmp <- ggplot_gtable(ggplot_build(myggplot))
  leg <- which(sapply(tmp$grobs, function(x) x$name) == "guide-box")
  legend <- tmp$grobs[[leg]]
  return(legend)
}

legend <- get_legend(Biogas)

#Save the plot and addition to all plots in one page
ga <- grid.arrange(gasprod, Biogas + guides(fill=FALSE) , CH4,
                   VS, VFA, Cellulose,
                   Xylan, legend, ncol=2)
```

```
tgc_Hac$HRT <- NULL
tgc_Hac$N <- NULL
tgc_Hac$sd<- NULL
tgc_Hac$se<- NULL
tgc_Hac$ci<- NULL
tgc_Hac<-melt(tgc_Hac, na.rm = FALSE, value.name = "value")

## Using Mix as id variables

tgc_Hpa$HRT <- NULL
tgc_Hpa$N <- NULL
tgc_Hpa$sd<- NULL
tgc_Hpa$se<- NULL
tgc_Hpa$ci<- NULL
tgc_Hpa<-melt(tgc_Hpa, na.rm = FALSE, value.name = "value")

## Using Mix as id variables

tgc_Ac_Pa<-rbind(tgc_Hac,tgc_Hpa)
HRT<- c(1,2,3,1,2,3,1,2,3,1,2,3)
tgc_Ac_Pa<-data.frame(tgc_Ac_Pa,HRT)

#Acetate & Propionate vs HRT
Ac_Pa<- ggplot(tgc_Ac_Pa, aes(fill=variable, y=value, x=HRT)) +
  geom_bar(stat="identity")+facet_grid(.~Mix)+ ylim(0,4)+ labs(x = "HRT", y="Acid concentratio
(g/L)", title="Acetate:Propionic Ratio")+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))
Ac_Pa

# 5. ALPHA DIVERSITY----
#UPLOAD OTU.Table with seeded
##Choose the OTU.Table should be a .txt
OTU_Table_seeded <- read.delim("~/Thesis/ADonAFEXfiber(2016)/DNA-data/No_manure/OTU_v1.txt",
row.names=1)
set.seed(711)
## Now we create the data.frame used for OTU Table, let's watch it!
## Now we create a matrix object with the data frame

t.OTU.table.seeded <- t(OTU_Table_seeded)  # Conversion a matriz y transposición de tabla
# Let's make some Alpha diversity analysis indexes
#First Shannon
H <- diversity(t.OTU.table.seeded, index = "shannon", MARGIN = 1, base = exp(1))
#Then Simpson
D <- diversity(t.OTU.table.seeded, "simpson", MARGIN = 1, base = exp(1))
#Third inverse Simpson
iD <- diversity(t.OTU.table.seeded, "inv")
# The last is Pielou's evenness
J<-H/log(specnumber(t.OTU.table.seeded)) #Pielou's evenness
print("ALPHA DIVERISTY WITH SEED SAMPLE")

#Sampling Curve
col <- c("#00BFC4", "#00BFC4", "#F8766D", "#F8766D", "#00BFC4", "#00BFC4", "#F8766D",
"#F8766D","#00BFC4", "#00BFC4", "#F8766D", "#F8766D","forestgreen")
lty <- c("solid")
pars <- expand.grid(col = col, lty = lty, stringsAsFactors = FALSE)
ra <- rarecurve(t.OTU.table.seeded, step = 20, col =col,lty = lty, cex = 0.6) # curvas de
rarefracción
```

```
rad <- rad.lognormal(t.OTU.table.seeded) # Rank of Abundance
rad1 <- plot(rad, xlab = "Rank", ylab = "Abundance") # Plotting the rank

S <- specnumber(t.OTU.table.seeded) # observed number of species
(raremax <- min(rowSums(t.OTU.table.seeded)))

Srare <- rarefy(t.OTU.table.seeded, raremax)
rarecurve(t.OTU.table.seeded, step = 20,col =col,lty = lty, sample = raremax, label = FALSE)

# 6. BETA DIVERSITY----
## 6.1 ABUNDANCE PLOTS AND RICHNESS

metadata_seeded <- read.delim("~/Thesis/ADonAFEXfiber(2016)/DNA-
data/No_manure/Metadata_v2_no_seed.txt", row.names=1)

#Phyloseq
OTU <- otu_table(OTU_Table_seeded,taxa_are_rows = TRUE) # OTU Table production for phyloseq
TAX <- tax_table(as.matrix(OTU_Table_taxonomy)) ## Taxanomy production for phyloseq
SAM <-sample_data(metadata_seeded)
physeq <- phyloseq(OTU, TAX, SAM) ##physeq document production

#Rarefication and normalization of abundance data
physeq.r = rarefy_even_depth(physeq, rngseed = TRUE) #Function for normalize physeq object

#Richness
r=plot_richness(physeq, x = "Duplicate", measures = c("Shannon", "Simpson",
"InvSimpson","Fisher"), color = "Mix") + geom_boxplot()
r+geom_point(size = 5, alpha = 0.7)+xlab("")+
  theme(legend.position="bottom", axis.title.x = element_blank(), axis.text.x =
element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))

#Normalize Abundace Plotbar Bacteria
physeq1 <-tax_glom(physeq.r, taxrank=rank_names(physeq.r)[4], NArm=TRUE, bad_empty=c(NA, "", "
", "\t"))
mergedGP = merge_samples(physeq1, "Duplicate")
physeq_1 = transform_sample_counts(mergedGP, function(x) x/sum(x))
p = plot_bar(physeq_1, fill = "Phylum")
p + geom_bar(aes(color=Phylum, fill=Phylum), stat = "identity",position = "stack")+
  scale_y_continuous(labels=scales::percent)+
  ylab("Relative Abundace") + labs(title = "Bacteria Abundance") +
  theme(legend.position="right", axis.title.x = element_blank(), axis.text.x =
element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))

#Abundace Plotbar Spirochaetes
physeq6 <-subset_taxa(physeq.r, Phylum== "Spirochaetes")
physeq6_1 <-tax_glom(physeq6, taxrank=rank_names(physeq6)[6], NArm=TRUE, bad_empty=c(NA, "", "
", "\t"))
l = plot_bar(physeq6_1,x="Duplicate", fill = "Genus")+ geom_bar(aes(color=Genus, fill=Genus),
stat = "identity",position = "stack") +
  ylab("Microbial Abundance") + xlab("Samples") + scale_fill_brewer(palette="Set1")+
  scale_colour_brewer(palette="Set1")+
  labs(title = "c. Spirochaetes Abundance") +scale_y_continuous(labels=scales::percent)+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.x = element_text(15), axis.title.y = element_text(size = 15),
        axis.text.y = element_text(size = 15),
```

```
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15),
        legend.direction="vertical")
l

# Top 20 Abundace Plotbar Bacteria
topN = 20
most_abundant_taxa = sort(taxa_sums(physeq.r), TRUE)[1:topN]
physeq.r20 = prune_taxa(names(most_abundant_taxa), physeq.r)
ntaxa(physeq.r20)
length(get_taxa_unique(physeq.r20, "Phylum"))
topp(0.3)
f1 = filterfun_sample(topp(0.3))
print(f1)
## function (x)
## {
##     fun = flist[[1]]
##     fval = fun(x)
##     for (fun in flist[-1]) {
##         fval = fval & fun(x)
##     }
##     return(fval)
## }
## <bytecode: 0x0000000024956680>
## <environment: 0x00000000249572c0>
## attr(,"class")
## [1] "filterfun"

wh1 = genefilter_sample(physeq.r20, f1, A = round(0.5 * nsamples(physeq.r20)))
sum(wh1)

ex2 = prune_taxa(wh1, physeq.r20)
mergedGP = merge_samples(ex2, "Duplicate")
ex2_r = transform_sample_counts(mergedGP, function(x) x/sum(x))
physeq8_1 <-tax_glom(ex2_r, taxrank=rank_names(ex2_r)[2], NArm=TRUE, bad_empty=c(NA, "", " ",
"\t"))
b = plot_bar(physeq8_1, fill = "Phylum") + geom_bar(aes(color=Phylum, fill=Phylum), stat =
"identity",position = "stack") +
  ylab("Relative Abundace") + labs(title = "b. Bacteria Abundance") +
scale_y_continuous(labels=scales::percent)+
  theme(legend.position="right", axis.title.x = element_blank(), axis.text.x =
element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))
b

#Abundance Plotbar Domain

physeq1 <-tax_glom(physeq.r, taxrank=rank_names(physeq.r)[1], NArm=TRUE, bad_empty=c(NA, "", "
", "\t"))
mergedGP = merge_samples(physeq1, "Duplicate")
physeq_dom = transform_sample_counts(mergedGP, function(x) x/sum(x))
a = plot_bar(physeq_dom,fill = "Domain") +
  geom_bar(aes(color=Domain, fill=Domain), stat = "identity",position = "stack") +
  ylab("Microbial Abundance") + labs(title= "a. Domain Abundance")
+scale_y_continuous(labels=scales::percent)+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.x = element_blank(), axis.title.y = element_text(size = 15),
        axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
```

```
15),plot.title= element_text(size = 15))
a

#Abundace Plotbar Archaea
physeq5 =subset_taxa(physeq.r, Domain== "Archaea")
physeq5_1 <-tax_glom(physeq5, taxrank=rank_names(physeq5)[6], NArm=TRUE, bad_empty=c(NA, "", "
", "\t"))
mergedGP = merge_samples(physeq5_1, "Duplicate")
physeq5_2 = transform_sample_counts(mergedGP, function(x) x/sum(x))
d = plot_bar(physeq5_2, fill = "Genus")+ geom_bar(aes(color=Genus, fill=Genus), stat =
"identity",position = "stack") +
  ylab("Relative Abundance") + xlab("Samples") + labs(title = "d. Archaea Abundance")
+scale_y_continuous(labels=scales::percent)+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.x = element_text(15), axis.title.y = element_text(size = 15),
        axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15),
        legend.direction="vertical")
d

ec <- grid.arrange(a,b, l,d, ncol=2)

## 6.2 PCOA WITH_SEED_FILE
GP.ord <- ordinate(physeq.r, "NMDS", "bray")

p1 = plot_ordination(physeq.r, GP.ord, type="taxa", color="Phylum", title="taxa")
print(p1)

p2 = plot_ordination(physeq.r, GP.ord, type="samples", color = "HRT" ,shape="Mix")
p2+ stat_ellipse(geom = "polygon", alpha = 0.45, aes(fill = Mix))+  geom_point(size=5)

## Too few points to calculate an ellipse

HRT <- list()
HRT[[1]] <- c("CS_HRT_1_A","CS_HRT_1_B",
              "AFEX_HRT_1_A","AFEX_HRT_1_B","SEED_DM")
HRT[[2]] <- c("CS_HRT_2_A","CS_HRT_2_B",
              "AFEX_HRT_2_A","AFEX_HRT_2_B","SEED_DM")
HRT[[3]]<- c("CS_HRT_3_A","CS_HRT_3_B",
             "AFEX_HRT_3_A","AFEX_HRT_3_B","SEED_DM")

# Principal components analysis for each HRT

  beta <- vegdist(t.OTU.table.seeded, binary = TRUE)
  pcoa.obj <- capscale(t.OTU.table.seeded ~ 1, distance = "bray")
  plot(pcoa.obj) #plot the PcoA plot
  text(scores(pcoa.obj)$sites[,1], scores(pcoa.obj)$sites[,2]) # change of the labes

  #labels=row.names(t.OTU.table.seeded)

  #SECOND trial with metaMDS
  vare.mds <- metaMDS(t.OTU.table.seeded, trace = FALSE)
  vare.mds

  stressplot(vare.mds)

  metadata_fil <- read.delim("~/Thesis/ADonAFEXfiber(2016)/DNA-
data/No_manure/Metadata_v2_no_seed.txt", row.names=1)

  ef <- envfit(vare.mds,env = metadata_seeded[4:11], permutations = 999, p.max =0.95, na.rm =
```

```
TRUE)
  plt <-plot(vare.mds, display = "sites", type = "p")
  identify(plt, what = "sites")

  pl <- plot(ef)
  with(metadata_fil,ordiellipse(vare.mds, HRT,col="forestgreen",kind = "se",conf = 0.95, label
= TRUE))
  with(metadata_fil,ordiellipse(vare.mds, Mix,col="red",kind = "se",conf = 0.95, label =
TRUE))
```

Shapiro-Wilk & ANOVA results of performance data of AD reactor

Table A1. Statistical results from the AD performance around the three HRT.

Parameter	Degree of freedom	Feedstock	HRT	Feedstock & HRT	Residuals
Biogas productivity	Sum square	1,564.0	27,646.0	9,037.0	180.0
	F value	52.30	462.10	151.00	
	P (>F)	*0.0004	*0.00003	*0.000007	
Methane content	Sum square	25.5	22.2	2.3	11.3
	F value	13.6	5.9	0.6	
	P (>F)	*0.01	*0.04	0.57	
VS reduction	Sum square	2.7	470.8	528.1	440.2
	F value	0.04	3.20	3.60	
	P (>F)	0.85	0.11	0.09	
Cellulose reduction	Sum square	18.8	1,070.7	464.2	399.2
	F value	0.28	8.05	3.50	
	P (>F)	0.61	*0.02	0.10	
Xylan reduction	Sum square	142.6	405.8	261.8	427.7
	F value	2.00	2.85	1.84	
	P (>F)	0.21	0.14	0.24	
VFA concentration	Sum square	2.5	1.6	1.2	15.4
	F value	0.97	0.32	0.23	
	P (>F)	0.36	0.74	0.80	

*Significance is selected with a p-value of 0.01

Calculation and assumptions for increasing BMP volume for the ABPs experiments

The objective of this calculation is to determine a dry-matter mass balance of anaerobic degradation of structural carbohydrates. The requirements of dry mass necessitate changing the volume of the normal BMP. The calculation requirements are based on the material for one sample and on dry-matter material. With this sample size, it is possible to obtain two NREL content points per sample. In addition, it is assumed that predigestion TS is 7,000 mg/L and postdigestion TS is 4,000 mg/L for each BMP, and the TS concentration of our control/seed is ~4,000 mg/L. Similarly, it is assumed that the samples will be dried at 45 °C, and therefore, the sample will have ~10% moisture content. Finally, the BMP VS:VS Inoculum:Feed ratio will be 2. The experiments are based on the protocols developed by the NREL for quantitation of structural carbohydrates and determination of extractives in the biomass that could affect the results on the carbohydrates (A Sluiter et al., 2008; Amie Sluiter et al., 2004). The sample size calculation is described below:

$$TS_{PRE} \approx 7\frac{g}{L}$$

$$\rho_{AD\ culture} \approx \rho_{H2O}$$

$$\%\ moisture \approx 10\%$$

$$\%\ extractives \approx 82\%$$

Mass Required for Predigestion Analysis

$$(m_T)_{PRE} = (m_{TSVS} + m_{NREL} + m_{BMP})(1 + \%\ moisture)$$

$$m_{TSVS} = (V_{TSVS})_{sample} \cdot TS$$

$$m_{TSVS} = 0.04\ L \cdot 7\frac{g}{L} = 0.28 \approx 0.5\ \text{g}$$

$$m_{NREL} = (m_{dry\ matter} + (m_{NREL})_{sample})(1 + \%\ extractives)$$

$$(m_{NREL})_{PRE} = (0.3 + 0.7)(1 + 0.82) = 1.82\ \text{g}$$

$$V_{NREL} = \frac{m_{NREL}}{TS}$$

$$V_{NREL} = \frac{1.82}{7} = 0.26 \text{ L}$$

$$m_{BMP} = (V_{BMP})_{sample} \cdot TS$$

$$m_{BMP} = 0.150\ L \cdot 7\frac{g}{L} = 1.05$$

$$(m_T)_{PRE} = (0.5 + 1.82 + 1.05)(1 + 0.1) = 3.7 \approx 4\ g$$

$$(V_T)_{PRE} = 0.04 + 0.26 + 0.15 = 0.45\ \approx 0.5\ L$$

The above calculation means that it is not possible to obtain enough dry material required to run NREL with the actual volume size of a BMP test (0.150 L). The standard volume has to be increased for the preparation for the experiment (Faivor & Kirk, 2011), to obtain the dry mass required to run the BMP analysis and obtain the mass required for characterization of the lignocellulosic content in the BMP experiment. This observation implies that it is necessary to increase the sample volume of the BMP from 0.150 to 0.5 L. Accordingly, the final mass for the BMP pre- and postdigestion will be as follows: Blend the preparation for 1 L predigestion. The final volume of the BMP test for postdigestion characterization is 0.5 L. One should use 7 g of a dry sample for one sample as explained in the following calculation:

Mass Required for Postdigestion Analysis

$$m_{BMP} = (m_{NREL})_{POST} + m_{TSVS} = 1.82 + 0.5 = 2.32 \text{ g}$$

$$(V_T)_{POST} = \frac{(m_{NREL})_{POST}}{TS_{POST}} = \frac{2.32}{4} = 0.58 \text{ L}$$

RSCRIPT for ABP variance-based sensitivity analysis

```
## Fiber Analysis in ABP
## MICHIGAN STATE UNIVERSITY
## ADREC
## Version 2.0
## Made by Juan Pablo Rojas, 2018
cat("\014") #Erase console

# 1. Loading Library and Tables ------
library(vegan)

library(MASS)
library(ggplot2)
library(grid)
library(gridExtra)
library(readr)
library(VennDiagram)

library(tidyverse)

library(Rmisc)

library(reshape2)

Biogas_Plants <- read.delim("~/Thesis/Fiber Study/Biogas_Plants_071618.txt", row.names=1)

# 2. Evaluate the normal test of the independen variables----------

# Influent_Farm_A
Influent_Farm_A <- Biogas_Plants %>%  filter(Flow == "Influent" & Plant== "Farm_A")
for (i in 4:14) {
  print(colnames(Influent_Farm_A[i]))
  print(shapiro.test(log(unlist(Influent_Farm_A[,i]))))
}

# Influent_Farm_B
Influent_Farm_B <- Biogas_Plants %>%  filter(Flow == "Influent" & Plant== "Farm_B")
for (i in 4:14) {
  print(colnames(Influent_Farm_B[i]))
  print(shapiro.test(log(unlist(Influent_Farm_B[,i]))))
}

# Influent_SCAD
Influent_SCAD <- Biogas_Plants %>%  filter(Flow == "Influent" & Plant== "SCAD")
for (i in 4:14) {
  print(colnames(Influent_SCAD[i]))
  print(shapiro.test(log(unlist(Influent_SCAD[,i]))))
}

# Effluent_Farm_A
Effluent_Farm_A <- Biogas_Plants %>%  filter(Flow == "Effluent" & Plant== "Farm_A")
for (i in 4:14) {
  print(colnames(Effluent_Farm_A[i]))
  print(shapiro.test(log(unlist(Effluent_Farm_A[,i]))))
}

# Effluent_Farm_B
Effluent_Farm_B <- Biogas_Plants %>%  filter(Flow == "Effluent" & Plant== "Farm_B")
for (i in 4:14) {
  print(colnames(Effluent_Farm_B[i]))
```

```
  print(shapiro.test(log(unlist(Effluent_Farm_B[,i]))))
}

# Effluent_SCAD
Effluent_SCAD <- Biogas_Plants %>%  filter(Flow == "Effluent" & Plant== "SCAD")
for (i in 4:14) {
  print(colnames(Effluent_SCAD[i]))
  print(shapiro.test(log(unlist(Effluent_SCAD[,i]))))
}

# 3. Plotting BMP Results----

Farm_A_BMP <- read.delim("~/Thesis/Fiber Study/Farm_A_BMP_080318.txt")

Farm_B_BMP <- read.delim("~/Thesis/Fiber Study/Farm_B_BMP_080318.txt")

SCAD_BMP <- read.delim("~/Thesis/Fiber Study/SCAD_BMP_080318.txt")

#Farm_A Gas Production
Farm_A_gasprod<- ggplot(Farm_A_BMP, aes(Time, Gas, color=Date)) + geom_point(aes(shape=Date,
color=Date)) + ylab("mL of Biogas") +
  geom_line(data=Farm_A_BMP[Farm_A_BMP$Date!="Gas", ])+geom_smooth()+ labs(title = "a.
Acumulated Gas Production Farm A")+
  xlab("Lapsed Time (h)")+
  ylim(0,1500)

#Farm_B Gas Production
Farm_B_gasprod<- ggplot(Farm_B_BMP, aes(Time, Gas, color=Date)) + geom_point(aes(shape=Date,
color=Date)) + ylab("mL of Biogas") +
  geom_line(data=Farm_B_BMP[Farm_B_BMP$Date!="Gas", ])+geom_smooth()+ labs(title = "b.
Acumulated Gas Production Farm B")+
  xlab("Lapsed Time (h)")+
  ylim(0,1500)

#SCAD Gas Production
SCAD_gasprod<- ggplot(SCAD_BMP, aes(Time, Gas, color=Date)) + geom_point(aes(shape=Date,
color=Date)) + ylab("mL of Biogas") +
  geom_line(data=SCAD_BMP[SCAD_BMP$Date!="Gas", ])+geom_smooth()+ labs(title = "c. Acumulated
Gas Production SCAD")+
  xlab("Lapsed Time (h)")+ylim(0,1500)

BMPS <- grid.arrange(Farm_A_gasprod, Farm_B_gasprod,SCAD_gasprod ,ncol=3)

# 4. ANOVA test of the independen variables----------

Biogas_Plants$Plant <- factor(Biogas_Plants$Plant) ##Factor Statement
Biogas_Plants$Flow <- factor(Biogas_Plants$Flow) ##Factor Statement

#ANOVA Test for parameters
#Characterization Data
for (i in 4:14) {
  #Influent
  fit <- Biogas_Plants %>% filter(Flow == "Influent")
  print(fit[1,3])
  print(colnames(fit[i]))
  ANOVA<- aov(log(unlist(fit[,i]))~Plant, fit) #ONE WAY ANOVA for Productivity
  print(TukeyHSD(ANOVA))  #Plot results
#Effluent
  fit <- Biogas_Plants %>% filter(Flow == "Effluent")
```

```
  print(fit[1,3])
  print(colnames(fit[i]))
  ANOVA<- aov(log(unlist(fit[,i]))~Plant, fit) #ONE WAY ANOVA for Productivity
  print(TukeyHSD(ANOVA))  #Plot results
  print("------------------------------------------------------------------------")
}

#Plant Operation
for (i in 15:16) {
  #Influent
  fit <- Biogas_Plants %>% filter(Flow == "Influent")
  print(fit[1,3])
  print(colnames(fit[i]))
  ANOVA<- aov(log(unlist(fit[,i]))~Plant, fit) #ONE WAY ANOVA for Productivity
  print(TukeyHSD(ANOVA))  #Plot results
    print("------------------------------------------------------------------------")
}

# 5. Plotting the the dry matter constituents. ----

#Dry Matter
Biogas_Plants$Date<- NULL
Biogas_Plants$sCOD<- NULL
Biogas_Plants$COD<- NULL
Biogas_Plants$Ammonia<- NULL
Biogas_Plants$TKN<- NULL
Biogas_Plants$TOC<- NULL
Biogas_Plants$CH4_Prod<- NULL
Biogas_Plants$Mass_Flow<- NULL

dat<-melt(Biogas_Plants, na.rm = FALSE, value.name = "value")

## Using Plant, Flow, Reactor as id variables

Influent<- dat %>% filter(Flow == "Influent")
Effluent<- dat %>% filter(Flow == "Effluent")


Dry_matter_influent<- ggplot(Influent, aes(fill=variable, y=value, x=Plant)) +
  geom_bar( stat="identity", position="fill")+ labs(x = "Biogas Plants", y="Dry matter (%)",
title="a. Influents")+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))


Dry_matter_effluent<- ggplot(Effluent, aes(fill=variable, y=value, x=Plant)) +
  geom_bar( stat="identity", position="fill")+
  guides(fill=FALSE)+ labs(x = "Biogas Plants", y="Dry matter (%)", title="b. Effluents")+
  theme(legend.position="right", axis.text.x = element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))

get_legend<-function(myggplot){
  tmp <- ggplot_gtable(ggplot_build(myggplot))
  leg <- which(sapply(tmp$grobs, function(x) x$name) == "guide-box")
  legend <- tmp$grobs[[leg]]
  return(legend)
```

```
}

legend <- get_legend(Dry_matter_influent)

#Save the plot and addition to all plots in one page
dry_1 <- grid.arrange(Dry_matter_influent + guides(fill=FALSE), Dry_matter_effluent, ncol=2,
right=legend)

# 6. Plotting the the raw constituents. ----

Biogas_Plants <- read.delim("~/Thesis/Fiber Study/Biogas_Plants_071618.txt", row.names=1)

Biogas_Plants$Ash<- NULL
Biogas_Plants$TKN<- NULL
Biogas_Plants$Ammonia<- NULL
Biogas_Plants$TOC<- NULL
Biogas_Plants$Protein<- NULL
Biogas_Plants$H2O.Ext<- NULL
Biogas_Plants$C2H6O.Ext<- NULL
Biogas_Plants$Cellulose<- NULL
Biogas_Plants$Xylan<- NULL
Biogas_Plants$Lignin<- NULL
Biogas_Plants$CH4_Prod<- NULL
Biogas_Plants$Mass_Flow<- NULL



dat<-melt(Biogas_Plants, na.rm = FALSE, value.name = "value")

## Using Date, Plant, Flow, Reactor as id variables

Influent<- dat %>% filter(Flow == "Influent")
Effluent<- dat %>% filter(Flow == "Effluent")


C_influent<- ggplot(Influent, aes(fill=variable, y=value, x=Plant)) +
  geom_bar( stat="identity",position=position_dodge(0.9))+ labs(x = "Biogas Plants", y="Dry
matter (%)", title="a. Influent ")+
  theme(plot.title = element_text(hjust = 0.5))+
  scale_fill_brewer(palette="Spectral")+
  theme(legend.position="right", axis.title.x = element_blank(), axis.text.x =
element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))


C_effluent<- ggplot(Effluent, aes(fill=variable, y=value, x=Plant)) +
  geom_bar( stat="identity",position=position_dodge(0.9))+
  guides(fill=FALSE)+ labs(x = "Biogas Plants ", y="Dry matter (%)", title="b. Effluent")+
  theme(plot.title = element_text(hjust = 0.5))+
  scale_fill_brewer(palette="Spectral")+
  theme(legend.position="right", axis.title.x = element_blank(), axis.text.x =
element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))
```

```
get_legend<-function(myggplot){
  tmp <- ggplot_gtable(ggplot_build(myggplot))
  leg <- which(sapply(tmp$grobs, function(x) x$name) == "guide-box")
  legend <- tmp$grobs[[leg]]
  return(legend)
}

legend <- get_legend(C_influent)



#Save the plot and addition to all plots in one page
liq_1 <- grid.arrange(C_influent + guides(fill=FALSE) , C_effluent, ncol=2, right=legend)

#Nitrogen Balance
Biogas_Plants <- read.delim("~/Thesis/Fiber Study/Biogas_Plants_071618.txt", row.names=1)

Biogas_Plants$Ash<- NULL
Biogas_Plants$sCOD<- NULL
Biogas_Plants$COD<- NULL
Biogas_Plants$TOC<- NULL
Biogas_Plants$Protein<- NULL
Biogas_Plants$H2O.Ext<- NULL
Biogas_Plants$C2H6O.Ext<- NULL
Biogas_Plants$Cellulose<- NULL
Biogas_Plants$Xylan<- NULL
Biogas_Plants$Lignin<- NULL
Biogas_Plants$CH4_Prod<- NULL
Biogas_Plants$Mass_Flow<- NULL



dat<-melt(Biogas_Plants, na.rm = FALSE, value.name = "value")

## Using Date, Plant, Flow, Reactor as id variables

Influent<- dat %>% filter(Flow == "Influent")
Effluent<- dat %>% filter(Flow == "Effluent")



N_influent<- ggplot(Influent, aes(fill=variable, y=value, x=Plant)) +
  geom_bar( stat="identity",position=position_dodge(0.9))+ labs(x = "Biogas Plants", y="Dry
matter (%)", title="a. Influent ")+
  theme(plot.title = element_text(hjust = 0.5))+ylim(0,1)+
  scale_fill_brewer(palette="Set1")+
  theme(legend.position="right", axis.title.x = element_blank(), axis.text.x =
element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
15),plot.title= element_text(size = 15))



N_effluent<- ggplot(Effluent, aes(fill=variable, y=value, x=Plant)) +
  geom_bar( stat="identity",position=position_dodge(0.9))+
  guides(fill=FALSE)+ labs(x = "Biogas Plants ", y="Dry matter (%)", title="b. Effluent")+
  theme(plot.title = element_text(hjust = 0.5))+
  scale_fill_brewer(palette="Set1")+
  theme(legend.position="right", axis.title.x = element_blank(), axis.text.x =
element_text(size = 15),
        axis.title.y = element_text(size = 15), axis.text.y = element_text(size = 15),
        legend.text = element_text(size = 11),legend.title= element_text(size =
```

```
15),plot.title= element_text(size = 15))

legend <- get_legend(N_influent)


#Save the plot and addition to all plots in one page
liq_1 <- grid.arrange(N_influent + guides(fill=FALSE) , N_effluent, ncol=2, right=legend)

# 7. Plotting TOC influent and Effluent-----

Biogas_Plants <- read_delim("Biogas_Plants_071618.txt","\t",
                            escape_double = FALSE, col_types = cols(Date = col_date(format =
"%m/%d/%Y")),trim_ws = TRUE)



Biogas_Plants$sCOD<- NULL
Biogas_Plants$COD<- NULL
Biogas_Plants$TKN<- NULL
Biogas_Plants$Ammonia<- NULL
Biogas_Plants$Reactor<- NULL
Biogas_Plants$Protein<- NULL
Biogas_Plants$H2O.Ext<- NULL
Biogas_Plants$C2H6O.Ext<- NULL
Biogas_Plants$Cellulose<- NULL
Biogas_Plants$Xylan<- NULL
Biogas_Plants$Lignin<- NULL
Biogas_Plants$CH4_Prod<- NULL
Biogas_Plants$Mass_Flow<- NULL



TOC<- ggplot(Biogas_Plants, aes(x=Date,y= TOC, shape=factor(Flow),colour = factor(Plant))) +
  geom_point(size = 3)+geom_line()+ theme(plot.title = element_text(hjust = 0.5))
TOC

# 8. Reduction of average influet and Effluent----
Biogas_Plants <- read.delim("~/Thesis/Fiber Study/Biogas_Plants_071618.txt", row.names=1)
Biogas_Plants$CH4_Prod<- NULL
Biogas_Plants$Mass_Flow<- NULL
Biogas_Plants$Date<- NULL
Biogas_Plants$Reactor<- NULL

#Calculating averages

tgc_Reduction <- data.frame()

for (i in 3:13) {
  fit<- summarySE(Biogas_Plants,measurevar=colnames(Biogas_Plants[i]),
groupvars=c("Flow","Plant"))
  #Nulling non requiring columns
  fit$N<- NULL
  fit$se<- NULL
  fit$ci<- NULL

  dat<-melt(fit, na.rm = FALSE, value.name = "value")

  #Average reduction
  Influent<- dat %>% filter(Flow == "Influent" & variable != "sd")
  Effluent<- dat %>% filter(Flow == "Effluent"& variable != "sd")
```

```
  Substract <- (Influent$value-Effluent$value)
  Total<- Influent$value
  Reduction<- Substract/Total
  tgc_head <-Influent[,2:3]
  tgc_Reduction<- data.frame(tgc_head, Reduction)
  print(colnames(Biogas_Plants[i]))
  print(head(tgc_Reduction))

    #Standard deviation
  Influentsd<- dat %>% filter(Flow == "Influent" & variable == "sd")
  Effluentsd<- dat %>% filter(Flow == "Effluent"& variable == "sd")
  Substractsd <- (Influentsd$value)+(Effluentsd$value)
  rel_Substractsd <- Substractsd
  rel_Total <-  Influentsd$value
  SD <-rel_Substractsd+rel_Total
  tgc_Reduction<- data.frame(tgc_head, SD)
  print(colnames(Biogas_Plants[i]))
  print(head(tgc_Reduction))


  print("------------------------------------------------------------------------")
}

for (i in 3:13) {
  fit<- summarySE(Biogas_Plants,measurevar=colnames(Biogas_Plants[i]),
groupvars=c("Flow","Plant"))
  #Nulling non requiring columns
  fit$N<- NULL
  fit$value<- NULL
  fit$se<- NULL
  fit$ci<- NULL

  dat<-melt(fit, na.rm = FALSE, value.name = "sd")

  Influent<- dat %>% filter(Flow == "Influent")
  Effluent<- dat %>% filter(Flow == "Effluent")
  Reduction <- (Influent$sd - Effluent$sd)/Influent$sd
  tgc_head <-Influent[,2:3]
  tgc_Reduction<- data.frame(tgc_head, Reduction)
  print(colnames(Biogas_Plants[i]))
  print(head(tgc_Reduction))

  # tgc_Reduction<- rbind(output, mtcars[i, ])

  print("------------------------------------------------------------------------")
}

#9. Contribution of the variance----
rm(list=ls())
setwd('/Users/rojasju2/Documents/Thesis/Fiber Study/R_Scripts/Code/Code')

source('/Users/rojasju2/Documents/Thesis/Fiber
Study/R_Scripts/Code/Code/contribution_variance.R') # Load function

filename <- 'Reduction_Biogas_Plants_082918.csv'

input.data <- read.csv(filename)
var_data<-
contribution_to_variance(CH4~sCOD+COD+NH3+TKN+Protein+TOC+H2O.Ext+C2H6O.Ext+Cellulose+Xylan+Li
```

```
gnin, data=input.data)



h <- ggplot(var_data, aes(x = var, y = pct, fill = var)) +
  geom_bar(alpha = 0.50, stat = "identity") +
  geom_text(aes(y = pct / 2, label = paste0(round(pct * 100, 1), "%"))) +
  coord_flip() + labs(x = "Biogas Plants", y="Dry matter (%)", title="Contribution to
Variance")+
  theme(plot.title = element_text(hjust = 0.5))+
  theme(legend.position="right", axis.text.x = element_text(size = 11),
        axis.title.y = element_text(size = 11), axis.text.y = element_text(size = 11),
        legend.text = element_text(size = 11),legend.title= element_text(size =
11),plot.title= element_text(size = 11))


print(h)
```

Shapiro–Wilk & ANOVA results on ABP characterization

Table A2. Statistical results from characterization of the influents and effluents of three different reactors

Parameter	Flow	Farm A			Farm B			SCAD		
		W	$\bar{X}$	T	W	$\bar{X}$	T	W	$\bar{X}$	T
sCOD [% dry matter]	In	0.9	0.26±0.05	A	0.9	0.27±0.15	A	0.7	0.35±0.04	A
	Eff	0.9	0.12±0.01	B	0.8	0.15±0.01	AB	0.9	0.21+0.07	A
COD [% dry matter]	In	0.9	0.84±0.03	B	0.7	0.84±0.08	B	0.9	1.04±0.13	A
	Eff	0.9	0.72±0.02	A	0.8	0.78±0.04	A	0.8	0.78±0.04	A
Ammonia [% dry matter]	In	0.9	0.02±0.01	AB	0.9	0.03±0.01	A	0.9	0.01±0.01	B
	Eff	0.9	0.02±0.00	AB	0.9	0.02±0.01	B	0.7	0.05±0.02	A
TKN [% dry matter]	In	0.9	0.04±0.01	A	0.9	0.03±0.01	A	0.9	0.03±0.02	A
	Eff	0.7	0.14±0.06	A	0.9	0.04±0.00	B	0.9	0.06±0.03	AB
Protein [% dry matter]	In	0.9	0.18±0.00	A	0.8	0.17±0.01	A	0.9	0.14±.01	B
	Eff	0.9	0.17±0.01	A	0.8	0.16±0.00	AB	0.9	0.15±0.01	B
TOC [% dry matter]	In	0.7	0.36±0.01	B	0.9	0.39±0.01	A	0.9	0.43±0.02	A
	Eff	0.9	0.36±0.00	A	0.7	0.39±0.01	A	0.9	0.37±0.03	A
H_2O.Ext [% dry matter]	In	0.9	0.40±0.14	A	0.8	0.32±0.10	A	0.9	0.23±0.04	A
	Eff	0.8	0.33±0.02	AB	0.9	0.35±0.08	A	0.9	0.24±0.02	B
C_2H_6O.Ext [% dry matter]	In	0.8	0.06±0.03	B	0.9	0.09±0.02	AB	0.8	0.14±0.05	A
	Eff	0.9	0.08±0.01	B	0.9	0.10±0.01	AB	0.9	0.16±0.05	A
Cellulose [% dry matter]	In	0.7	0.10±0.04	A	0.8	0.13±0.02	A	0.9	0.13±0.05	A
	Eff	0.9	0.10±0.00	A	0.9	0.11±0.02	A	0.8	0.13 ±0.02	A
Xylan [% dry matter]	In	0.7	0.05±0.02	A	0.8	0.06±0.01	A	0.9	0.06±0.02	A
	Eff	0.9	0.06±0.00	A	0.9	0.05±0.01	A	0.8	0.06±0.01	A
Lignin [% dry matter]	In	0.9	0.15±0.04	A	0.8	0.15±0.02	A	0.9	0.21±0.02	A
	Eff	0.7	0.18±0.01	AB	0.9	0.16±0.03	B	0.9	0.21±0.02	A
Productivity [m^3/kg VS]	In	0.8	53.4±72	B	0.9	86.9±99	AB	0.9	154.13±170	A

*T = Tukey's test, significance is assumed at a p value of 0.01.

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