ANAEROBIC BIODEGRADABILITY OF COMPLEX SUBSTRATES:
PERFORMANCE AND STABILITY AT MESOPHILIC AND THERMOPHILIC
CONDITIONS

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

by
Rodrigo A. Labatut
January 2012
Performance and stability of the anaerobic digestion of complex substrates was investigated in three separate studies. First, an array of materials was subjected to the biochemical methane potential (BMP) batch assay to evaluate the influence of the substrate’s characteristics on the biomethane potential and overall biodegradability. The applicability of stoichiometry-based methods for predicting biomethane yields of complex substrates was also assessed. Results showed that, readily-degradable carbohydrates and less-degradable, but high energy-density lipid-rich substrates produce higher biomethane yields than recalcitrant, low energy-density lignocellulosic materials. Also, when substrate biodegradability was accounted for, predicted biomethane yields were within 10% of those obtained via the BMP assay.

In the second study, biodegradability and biomethane potential of the main biomolecules comprising composite substrates were determined at mesophilic and
thermophilic temperatures using the BMP assay. Also, stabilization of individual constituents of co-digested substrates was investigated using continuously-stirred anaerobic digesters (CSADs) operated at either temperature. Lastly, the feasibility of using either BMP data or stoichiometry-based methods to predict substrate stabilization and biomethane yields of semi-continuously-fed CSADs, was evaluated. Results revealed that, with the exception of starch, lignin, and lipids at inhibitory concentrations, most biopolymers are biodegradable under both thermal ranges. As previously reported, biodegradability (and biomethane potential) of lignocellulosic substrates is correlated with their lignin content. Finally, a methodology to estimate the biodegradable fraction based on the chemical composition of substrates was developed. Results were between 5% and 18% of those observed at steady-state conditions; differences using the BMP assay were within the same range.

Lastly, the influence of the operating temperature and substrate chemical composition on performance and stability of anaerobic digestion was evaluated. At shorter hydraulic retention times (HRTs) and high manure-to-dog food ratios, the thermophilic CSAD outperformed the biomethane production rates and substrate stabilization of the mesophilic CSAD. Mesophilic digestion appeared more stable regardless of the HRT and substrate composition. The thermophilic CSAD was less stable, particularly at low lipid-to-manure ratios, and more sensitive to changes in environmental and operating parameters, i.e., temperature and mixing intensity.
BIOGRAPHICAL SKETCH

Rodrigo received an Aquacultural Engineering degree from Universidad Católica del Norte, Chile, where his work was primarily focused on wastewater treatment technologies and recirculating aquaculture systems (RAS) using biological filtration and advance oxidation processes (AOP). Rodrigo moved to the U.S. to begin graduate studies in the Department of Biological and Environmental Engineering at Cornell University. Under the guidance of Mike Timmons, he researched the hydrodynamics of aquaculture tanks using experimental and computational fluid dynamics (CFD) methods, and received his M.S. degree in 2005. Later that year, motivated by his interest in bioenergy, he helped to write a proposal, which provided funding to pursue a Ph.D. in the same department under the direction of Norm Scott. Rodrigo’s work was mainly focused on the co-digestion of dairy manure with complex substrates, to develop an understanding of how different chemical components biodegraded under mesophilic and thermophilic conditions.

In 2007, Rodrigo met his future wife, Jenny. In 2010, he joined Dairy Environmental Systems, where he currently works as a Research Associate to promote anaerobic digestion in N.Y.S. In May 2011, Jenny and Rodrigo had a beautiful boy, named Mateo. In August 2011, Rodrigo finally took his B-Exam, graduated from Cornell, and is now ready to enjoy life together with his family.
Dedicada a mi hijo Mateo, que me dió la fuerza necesaria para poder terminar este proyecto y comenzar el más importante de mi vida…
ACKNOWLEDGEMENTS

I would like to thank my wife, Jenny, for her continuous support throughout this physical and emotional rollercoaster called PhD. To my family, who always sent me their words of encouragement from the far lands of Chile.

I would also like to thank my entire committee, and especially, Norm Scott, for his guidance to try to keep this research down to earth, and as applicable to the real world as possible. To all the members Angenent Lab, who enlightened my research during the weekly group discussions.

I would like to recognize Kristen Vitro, an undergraduate student at Cornell University, for her continued assistance and thorough laboratory analyses in conjunction with this study. In addition, I would like to thank Curt Gooch, P.E., from the Cornell PRO-DAIRY Program, for his insightful contributions to this research.

Lastly, I would like to thank the New York State Energy Research and Development Authority (NYSERDA) and Conycit, Chile, for the financial support provided to this study.
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1.1. Introduction

Anaerobic digestion has been used to stabilize animal manure, reduce greenhouse gas (GHG) emissions, control odors, promote environmental management of nutrients, and produce clean and local renewable energy. Waste stabilization is the primary objective of anaerobic digestion. While animal manure is probably the main waste subjected to anaerobic digestion, the organic fraction of municipal solid waste and waste activated sludge have been also treated via this process. In addition, a growing number of anaerobic digesters using bioenergy crops have been installed, particularly in Europe, with the sole objective of producing biogas.

Most anaerobic treatment facilities, however, perform co-digestion. Anaerobic co-digestion of animal manure with other organic substrates, such as food residues, has been primarily used to improve the economic viability of farm treatment processes as well as to provide additional high-strength constituents which can potentially increase biomethane yields. Furthermore, ideal digestion conditions for waste stabilization are rarely provided by the co-substrates alone – if mono-digestion is intended, some degree of chemical pre-conditioning will be certainly needed. Although fairly undegradable, animal manure can provide the necessary environmental conditions (e.g., alkalinity, pH, nutrients) to maximize waste stabilization, and thereby biomethane production.
Despite the benefits of co-digestion, the highly complex nature of the potential co-substrates makes it difficult to predict the overall impact that additional materials could have on the digestion processes. The anaerobic stabilization of particulate matter is a highly dynamic, multi-step process, where physicochemical and biochemical reactions take place in a sequential and parallel way as a result of continuous interactions between the main components that make up the system, namely, substrates, microorganisms/enzymes, and environment (Figure 1-1).

Figure 1-1. Main factors determining performance and stability of anaerobic digestion

The delicate balance between these components is what determines the performance and stability of the digester, and ultimately, the overall energy output and waste treatment efficiency of the system. Therefore, there is a need for information with
regards to the impact that the influent substrate characteristics and system operating temperature have on the process, in particular in complex substrate mixtures, such as those observed in co-digestion operations. 

Today, performance and stability of the anaerobic digestion of composite substrate mixtures, such as those found in co-digestion operations, are usually evaluated via analytical methods. Long-term digestion batch tests, such as the biochemical methane potential (BMP) assay, can be used to assess the extent of biodegradability of substrates and ultimate biomethane yields. In addition, semi- or continuous-flow studies can be used to evaluate performance and stability of anaerobic digesters, where microbial populations are acclimated for specific substrates and product inhibition can be properly assessed over long-term periods.

In this study, three different studies were conducted under batch and semi-continuous conditions to help in answering the following research questions:

i) What makes a substrate more or less biodegradable?

ii) How do the thermal regime and substrate chemical composition affect biodegradability, biomethane yields, and the stability of the process?

iii) Is it possible to predict steady-state anaerobic biodegradability and biomethane yields of continuously-fed anaerobic digesters using analytical or theoretical methods?

iv) How feasible would it be to use such methods for typical, large-scale situations?
The first and second questions were addressed in the first study, i.e., Chapter 2. The biochemical methane potential (BMP) assay was conducted on a diverse array of complex materials, including co-digestion samples, to evaluate how substrate’s physical and chemical characteristics affected biomethane potential and overall biodegradability. The influence of the operating temperature was evaluated in the second study, i.e., Chapter 3. The biomethane potential and biodegradability of the main biomolecules comprising composite substrates were evaluated at mesophilic and thermophilic conditions using the BMP assay. In addition, fate and stabilization efficiency (biodegradability) of selected individual constituents of co-digested substrates were evaluated at mesophilic and thermophilic conditions using continuously-stirred anaerobic digesters (CSADs) operated at semi-continuous conditions. Furthermore, the feasibility of using either BMP data or stoichiometry-based methods to estimate substrate stabilization and biomethane yields at semi-continuous, steady-state conditions was also evaluated in Chapter 3. Lastly, performance and long-term stability of the CSADs were evaluated in the third study, i.e., Chapter 4.
CHAPTER 2

BIOCHEMICAL METHANE POTENTIAL AND BIODEGRADABILITY OF COMPLEX ORGANIC SUBSTRATES

Abstract

The biomethane potential and biodegradability of an array of substrates with highly heterogeneous characteristics, including mono- and co-digestion samples with dairy manure, was determined using the biochemical methane potential (BMP) assay. In addition, the ability of two theoretical methods to estimate the biomethane potential of substrates and the influence of biodegradability was evaluated. The results of about 175 individual BMP assays indicate that substrates rich in lipids and easily-degradable carbohydrates yield the highest biomethane potential, while more recalcitrant substrates with a high lignocellulosic fraction have the lowest. Co-digestion of dairy manure with easily-degradable substrates increases the specific biomethane yields when compared to manure-only digestion. Additionally, biomethane potential of some co-digestion mixtures suggested synergistic activity. Evaluated theoretical methods consistently over-estimated experimentally-obtained biomethane yields when substrate biodegradability was not accounted. Upon correcting the results of theoretical methods with observed biodegradability data, an agreement greater than 90% was achieved.

**Nomenclature**

- **BMP** Biochemical methane potential, mL CH\(_4\)/g VS added (equivalent to \(B_o\))
- **\(B_o\)** Observed SMY, mL CH\(_4\)/g VS added (measured, e.g., via BMP)
- **\(B_u\)** Ultimate SMY, mL CH\(_4\)/g VS added (theoretical, e.g., calculated using stoichiometry)
- **\(COD_D\)** Degradable chemical oxygen demand, mg/L
- **\(COD_T\)** Total chemical oxygen demand, mg/L
- **\(f_D\)** Substrate biodegradable fraction or extent of biodegradability, decimal
- **\(n\)** Number of moles of gas
- **\(P\)** Absolute pressure of gas, kPa
- **\(R\)** Universal gas constant, 8.3145 L kPa/K · mol
- **SMY** Specific biomethane yield, mL CH\(_4\)/g VS added
- **\(T\)** Absolute temperature of gas, K
- **\(V\)** Volume of gas, L
- **WSMY** Weighted SMY, mL CH\(_4\)/g VS added
2.1. Introduction

Today, a diverse range of organic substrates is subjected to the process of anaerobic digestion – waste stabilization is the primary objective, and livestock manure, a substrate which amounts to over a billion tons produced per year in the United States, is probably the main waste treated via this process in the world (Kellogg et al., 2000). Given that the primary sources of biomethane in livestock operations come from animal enteric fermentation and uncovered raw manure-stabilization lagoons (Amon et al., 2001), the benefits of anaerobic digestion of animal manure are evident. Manure-associated greenhouse gas (GHG) emissions comprise a significant contribution to total GHGs released by the U.S. agricultural sector, with biomethane, from conventional livestock practices, estimated at 8% of the total anthropogenic biomethane emissions (USEPA, 2010). Along with mitigating biomethane gas emissions, anaerobic digestion of animal manure has the potential to reduce farm-generated odors, improve crop-based nutrient management, and produce local, renewable energy. Food residues and waste activated sludge (WAS) are additional examples of organic wastes stabilized through anaerobic digestion. In Germany there are over 500 anaerobic digestion facilities for the treatment of the organic fraction of municipal solid waste (Kübler et al., 2000). A growing number of on-farm digester operations throughout New York State are currently co-digesting livestock manure with a range of easily-degradable food residues, such as cheese whey and wastes from
ice cream and onion operations (Gooch & Pronto, 2009). Waste activated sludge, generated by municipal wastewater treatment plants (WWTP) in amounts that reach over 10 million dry tons per year in the European Union (Appels et al., 2008), is usually digested on-site with concomitant production of electricity and heat. In Germany, the use of short rotation crops for bioenergy generation has been increasing, and in 2007 had an agricultural area of 500,000–550,000 ha dedicated exclusively to produce energy crops to sustain its 3750 biogas plants (Rosch et al., 2009).

To anticipate the overall impact and biomethane yields of such a diverse range of substrates on large-scale, semi- or continuous-flow anaerobic digesters, long- and short-term, laboratory-scale experimental methods have been developed. Long-term studies (i.e. 1-2 years) conducted in bench-scale, semi- or continuous-flow reactors, are designed to emulate the conditions of commercial-scale digesters and study their overall performance over time. Short-term (i.e. 1-2 months), batch-mode anaerobic digestion tests, such as the biochemical methane potential (BMP) assay, are primarily intended to determine biomethane yields and biodegradability of substrates.

In addition, a considerable number of theoretical approaches has also been developed. In the early stages of anaerobic digestion, stoichiometrical-based methods that predicted the major final products of fermentation were developed, (Symons & Buswell, 1933; McCarty, 1972). Most recent approaches are more complex models
that simulate the biochemical and physicochemical reactions of anaerobic digestion to predict the major transient and final products of the fermentation process (Angelidaki et al., 1999; Batstone et al., 2000). Regardless of the theoretical method used, its accuracy will largely depend on the knowledge of the substrate composition, and particularly, on its biodegradable fraction. Thus, the need for a simple, quick, and accurate method to estimate biomethane yields and biodegradability of organic substrates is apparent.

2.2. Objectives

In this study, the biomethane potential of more than 30 substrates, including mono- and co-digestion samples, was determined using the BMP assay. Based on the substrate characteristics and observed biomethane yields, their biodegradable fraction was determined. Similarly, the co-digestion of dairy manure with several organic substrates was evaluated for its potential to increase biomethane production over conventional manure-only digestion methods. In addition, the feasibility of using two common theoretical methods to estimate the biomethane yields of complex substrates was evaluated. Selection of substrates was based on their frequency of inclusion in anaerobic digesters in New York State and to cover a wide range of material biodegradabilities and chemical compositions.
2.3. Materials and Methods

2.3.1. Analytical methods

The anaerobic digestion of substrates was performed in batch mode using the biochemical methane potential (BMP) assay. The biomethane potential of substrates was evaluated based on their specific biomethane yield (SMY) – defined here as the total volume of biomethane produced during the digestion period per amount of substrate initially added (i.e. mL CH₄/g VS added).

2.3.1.1. Biochemical methane potential assay

The BMP protocol followed in this study was based on the principles described by Owen et al. (1979) and revised by others (Chynoweth et al., 1993; Hansen et al., 2004). Briefly, known amounts of substrate and an active anaerobic inoculum were added to 250-mL serum bottles. pH was measured, and bottles were gassed with N₂ and sealed immediately using rubber septa and aluminum crimp caps. Once sealed, the bottles were placed in an incubator and maintained at a constant mesophilic temperature (35±1°C). In each BMP trial, two additional bottles containing only inoculum were included to account for background (i.e. endogenous) biomethane production. Mixing was performed manually to each bottle every two days during the entire incubation period. The duration of the BMP assay was specifically determined for each substrate, and the test was ended when the cumulative biogas curve reached the plateau phase, usually after 30 days.
2.3.1.2. Biological inoculum and nutrient requirements

The inoculum was obtained from a farm-based completely-mixed anaerobic digester operated at a 25-day hydraulic retention time (HRT), which co-digested dairy manure with an array of food residues (i.e. cheese whey, milk slop, and raw onions). The inoculum was harvested from the supernatant of the digester’s effluent after 24 hours of quiescent settling. No additional external nutrients/trace elements were added to the BMP bottles – it was assumed that basic nutrient requirements for anaerobic microorganisms were provided by the manure-based inoculum, as Gustafson (2000) found in significant amounts in dairy manure.

2.3.1.3. Biogas production measurement

Biogas production was determined indirectly, by measuring the cumulative pressure inside the bottles via gage pressure transducers (Model PX26, Omega Engineering, Inc.). Pressure was continuously measured using a data acquisition (DAQ) system interfaced with a computer, and controlled via LabVIEW® (National Instruments Co., Austin, TX). In addition, a pressure-control bottle containing the equivalent volume of sample replaced by tap water was included to account for abiotic, external pressure variations due to temperature and atmospheric pressure changes. Similarly, temperature was monitored through thermocouples measuring gas-phase temperature changes in tap water-containing bottles. Finally, pressure data were converted to
volume of biogas at standard temperature and pressure (STP), according to the ideal law of gases (Eq. 2-1).

\[ PV = nRT \]

where, \( P, V, n, \) and \( T \) are respectively: absolute pressure (kPa), volume (m\(^3\)), moles, and absolute temperature (K) of the gas; and \( R \) is the universal gas constant (8.3145 L kPa/K · mol).

2.3.2. Analytical methods

All substrates were mixed and blended thoroughly to reduce particle size and create uniform and representative specimens. Total solids (TS), volatile solids (VS), and chemical oxygen demand (COD) (colorimetric dichromate closed reflux method) were determined according to Standard Methods (APHA, 1995). 10-day biochemical oxygen demand (BOD) tests were performed using a HACH BODTrak™ (HACH Co., Loveland, CO). Methane and carbon dioxide content in the biogas was determined with an SRI 8610C (SRI Instruments, Torrance, CA) gas chromatograph equipped with a thermal conductivity detector (TCD), using Helium as a carrier gas in a 0.3-m HaySep-D packed Teflon® column under isothermal conditions at 105°C. Additional analyses to determine the precise chemical composition of dairy manure were conducted. Hemicellulose, cellulose, and lignin content were determined according to the neutral detergent fiber (NDF) and acid detergent fiber and lignin (ADF/ADL) analyses described by Mertens (2002) and Möller (2009), respectively.
Total Kjeldahl nitrogen (TKN) concentration was determined according to the Standard Methods (APHA, 1995). Total ammonia-N (TAN) concentration was measured using an ion selective electrode (Thermo Fisher Scientific, Inc.). Total organic nitrogen was calculated by subtracting TAN from TKN. Total protein content was calculated based on the assumption that an average protein contains 16% organic N. Neutral lipids were determined according to method of Loehr and Rohlich (1962). Non-lignocellulosic carbohydrates (e.g. sugars, starch) were obtained by difference.

2.3.3. Biomethane yield estimation methods

The ability of theoretical methods to accurately estimate specific biomethane yields of complex substrates was evaluated by comparing the observed SMY ($B_o$) of selected substrates to the ultimate SMY ($B_u$). Only a brief description of the theoretical methods is presented here – the reader is referred to the original cited literature for further details.

2.3.3.1. Bioenergetics and Stoichiometry of Biological Reactions (McCarty, 1972)

Heterotrophs consume organic matter both for energy and for synthesis. The free energies of microbiologically-mediated reactions can be used to estimate cell yields and the overall stoichiometry associated with growth, and determine the fraction of a
particular organic substrate (i.e. electron donor) that is used for energy \( (f_e) \) and the fraction used for synthesis of cellular material \( (f_s) \).

2.3.3.2. Buswell Formula (Symons and Buswell, 1933)

This equation simply represents a balanced redox reaction where the only products of anaerobic digestion are methane, carbon dioxide, and ammonia. In contrast to the method of McCarty, the Buswell Formula assumes that all the electrons donated are exclusively used for metabolic energy, i.e. cellular synthesis is neglected.

The two methods described above do not account for substrate biodegradability, or in other words, it is assumed that all the electrons from the donor are available for the electron acceptors. The ability of either method to accurately estimate biomethane yields primarily depends on two fundamental substrate characteristics, namely chemical composition and biodegradability. With the exception of the chemical composition of dairy manure, which was analytically determined in this study, and switchgrass, which was as reported by Lemus et al. (2002), the composition of all the substrates covered in this study was obtained from the Nutrient Data Laboratory (NDL) database (USDA, 2009). For both theoretical methods, calculations were based on the molecular formulae of the substrates’ constituents, as stated previously.

A more difficult parameter to estimate is substrate biodegradability. The rate at which substrates are degraded will be mainly determined by its physical and chemical properties as well as its susceptibility to produce inhibitory intermediate products.
throughout the bioconversion processes. Physicochemical characteristics, such as particle size, lignin content or degree of crystallinity of the lignocellulosic matrix, will mainly affect the kinetics of the hydrolysis step, while pH, un-ionized ammonia, or fatty acid (long- and short-chain) concentrations, could affect one or multiple step(s) of the anaerobic digestion process (i.e. hydrolysis, acidogenesis/β-oxidation, acetogenesis, and methanogenesis). Therefore, depending on the residence time, the rate of degradation of the substrate will determine its extent of biodegradability, and thereby its biomethane yield. Conventional methods described in the literature to estimate the biodegradability of organic substrates are experimental. In fact, the BMP assay is one of the most widely used analytical methods to determine the portion of substrate that can be biologically degraded under anaerobic conditions. This fraction can be estimated by the ratio between the degradable and total chemical oxygen demand (Eq. 2-2):

Eq. 2-2

\[ f_D = \frac{COD_D}{COD_T} \]

where, \( f_D \) is the substrate biodegradable fraction (decimal), \( COD_D \) is the degradable chemical oxygen demand (mg/L), and \( COD_T \) is the total chemical oxygen demand (mg/L). \( COD_D \) can be calculated from the observed specific biomethane yields (\( B_s \)) and the theoretical 350 mL of CH\(_4\) (at STP) per g of COD stabilized (McCarty, 1964). Also, \( COD_T \) was determined analytically for each substrate (see Table 2-1 and Table 2-2).
2.4. Results and discussion

2.4.1. Characterization of substrates

The substrates investigated in this study cover a wide range of material biodegradabilities and chemical compositions, and include mono-digestion (digestion of a single substrate) and co-digestion (digestion of more than one substrate) samples. All co-digestion samples consisted of substrates co-digested with dairy manure. Main physical and chemical characteristics of the mono- and co-digestion samples are presented in Table 2-1 and Table 2-2, respectively. Additionally, the chemical composition of selected substrates as obtained from the NDL database is shown in Table 2-3. As described in the Materials and Methods section, the chemical composition of dairy manure was determined analytically in this study.
Table 2-1. Physical and biochemical characteristics of the mono-digestion samples

<table>
<thead>
<tr>
<th>Mono-digestion samples</th>
<th>BOD (g/kg)</th>
<th>COD (g/kg)</th>
<th>TS (g/kg)</th>
<th>VS (g/kg)</th>
<th>BOD/COD</th>
<th>VS/TS</th>
<th>VS/COD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw manures (M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw dairy manure</td>
<td>45.8</td>
<td>128.9</td>
<td>124.0</td>
<td>102.1</td>
<td>0.36</td>
<td>0.82</td>
<td>0.79</td>
</tr>
<tr>
<td>Manure separated liquid</td>
<td>33.2</td>
<td>71.0</td>
<td>57.5</td>
<td>40.5</td>
<td>0.47</td>
<td>0.71</td>
<td>0.57</td>
</tr>
<tr>
<td>Food residues (FR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheese whey</td>
<td>64.9</td>
<td>128.3</td>
<td>71.4</td>
<td>59.8</td>
<td>0.53</td>
<td>0.84</td>
<td>0.53</td>
</tr>
<tr>
<td>Plain pasta</td>
<td>188.7</td>
<td>934.3</td>
<td>422.6</td>
<td>407.7</td>
<td>0.20</td>
<td>0.97</td>
<td>0.44</td>
</tr>
<tr>
<td>Meat pasta</td>
<td>205.8</td>
<td>562.8</td>
<td>381.8</td>
<td>340.6</td>
<td>0.37</td>
<td>0.89</td>
<td>0.61</td>
</tr>
<tr>
<td>Used vegetable oil</td>
<td>ND</td>
<td>2880.0</td>
<td>991.0</td>
<td>988.8</td>
<td>ND</td>
<td>1.00</td>
<td>0.34</td>
</tr>
<tr>
<td>Ice cream</td>
<td>ND</td>
<td>266.8</td>
<td>113.8</td>
<td>109.1</td>
<td>ND</td>
<td>0.96</td>
<td>0.41</td>
</tr>
<tr>
<td>Fresh dog food</td>
<td>ND</td>
<td>530.4</td>
<td>132.2</td>
<td>125.6</td>
<td>ND</td>
<td>0.95</td>
<td>0.24</td>
</tr>
<tr>
<td>Cola beverage</td>
<td>ND</td>
<td>121.5</td>
<td>93.6</td>
<td>88.7</td>
<td>ND</td>
<td>0.95</td>
<td>0.73</td>
</tr>
<tr>
<td>Cabbage, raw</td>
<td>ND</td>
<td>90.9</td>
<td>78.6</td>
<td>72.0</td>
<td>ND</td>
<td>0.92</td>
<td>0.79</td>
</tr>
<tr>
<td>Potatoes, raw</td>
<td>53.5</td>
<td>261.8</td>
<td>177.4</td>
<td>163.5</td>
<td>0.20</td>
<td>0.92</td>
<td>0.63</td>
</tr>
<tr>
<td>Invasive aquatic plants (AP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frogbit (Oneida lake)</td>
<td>32.9</td>
<td>49.5</td>
<td>51.8</td>
<td>38.7</td>
<td>0.67</td>
<td>0.75</td>
<td>0.78</td>
</tr>
<tr>
<td>Water Chestnut (Oneida river)</td>
<td>40.4</td>
<td>46.2</td>
<td>89.0</td>
<td>74.2</td>
<td>0.87</td>
<td>0.83</td>
<td>1.61</td>
</tr>
<tr>
<td>Eurasian Milfoil (Oneida lake)</td>
<td>26.4</td>
<td>27.8</td>
<td>106.1</td>
<td>66.7</td>
<td>0.95</td>
<td>0.63</td>
<td>2.40</td>
</tr>
<tr>
<td>Water Celery (Oneida lake)</td>
<td>27.9</td>
<td>33.6</td>
<td>92.9</td>
<td>47.0</td>
<td>0.83</td>
<td>0.51</td>
<td>1.40</td>
</tr>
<tr>
<td>Chara (Tully lake)</td>
<td>27.9</td>
<td>31.5</td>
<td>148.8</td>
<td>37.7</td>
<td>0.89</td>
<td>0.25</td>
<td>1.20</td>
</tr>
<tr>
<td>Others (O)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Switchgrass</td>
<td>88.6</td>
<td>706.7</td>
<td>930.1</td>
<td>904.9</td>
<td>0.13</td>
<td>0.97</td>
<td>0.68</td>
</tr>
<tr>
<td>Corn silage</td>
<td>ND</td>
<td>ND</td>
<td>217.3</td>
<td>200.7</td>
<td>ND</td>
<td>0.98</td>
<td>0.91</td>
</tr>
<tr>
<td>Corn leachate</td>
<td>50.7</td>
<td>122.3</td>
<td>49.2</td>
<td>35.4</td>
<td>0.42</td>
<td>0.72</td>
<td>0.29</td>
</tr>
<tr>
<td>Mouthwash</td>
<td>ND</td>
<td>160.5</td>
<td>130.2</td>
<td>118.4</td>
<td>ND</td>
<td>0.91</td>
<td>0.74</td>
</tr>
<tr>
<td>Suspended fat, oil and grease (FOG)</td>
<td>155.5</td>
<td>600.1</td>
<td>267.2</td>
<td>229.7</td>
<td>25.9</td>
<td>0.86</td>
<td>0.38</td>
</tr>
<tr>
<td>Settled fat, oil and grease (FOG)</td>
<td>97.0</td>
<td>290.0</td>
<td>128.4</td>
<td>112.6</td>
<td>33.4</td>
<td>0.88</td>
<td>0.39</td>
</tr>
</tbody>
</table>

ND: not determined
Table 2-2. Physical and biochemical characteristics of the co-digestion samples

<table>
<thead>
<tr>
<th>Co-digestion samples (M = dairy manure)</th>
<th>Mix ratio (VS basis)</th>
<th>BOD (g/kg)</th>
<th>COD (g/kg)</th>
<th>TS (g/kg)</th>
<th>VS (g/kg)</th>
<th>BOD/COD</th>
<th>VS/TS</th>
<th>VS/COD</th>
</tr>
</thead>
<tbody>
<tr>
<td>M:Cheese whey</td>
<td>90:10</td>
<td>45.5</td>
<td>103.2</td>
<td>83.2</td>
<td>68.4</td>
<td>0.44</td>
<td>0.82</td>
<td>0.66</td>
</tr>
<tr>
<td>M:Cheese whey</td>
<td>75:25</td>
<td>46.4</td>
<td>100.3</td>
<td>68.5</td>
<td>57.7</td>
<td>0.46</td>
<td>0.84</td>
<td>0.58</td>
</tr>
<tr>
<td>M:Plain pasta</td>
<td>90:10</td>
<td>91.8</td>
<td>158.5</td>
<td>132.0</td>
<td>116.7</td>
<td>0.58</td>
<td>0.88</td>
<td>0.75</td>
</tr>
<tr>
<td>M:Plain pasta</td>
<td>75:25</td>
<td>97.2</td>
<td>293.6</td>
<td>222.5</td>
<td>211.4</td>
<td>0.33</td>
<td>0.95</td>
<td>0.67</td>
</tr>
<tr>
<td>M:Meat pasta</td>
<td>90:10</td>
<td>70.4</td>
<td>151.2</td>
<td>101.9</td>
<td>89.8</td>
<td>0.47</td>
<td>0.88</td>
<td>0.59</td>
</tr>
<tr>
<td>M:Meat pasta</td>
<td>75:25</td>
<td>98.8</td>
<td>233.0</td>
<td>148.5</td>
<td>136.9</td>
<td>0.42</td>
<td>0.92</td>
<td>0.59</td>
</tr>
<tr>
<td>M:Used vegetable oil</td>
<td>75:25</td>
<td>ND</td>
<td>922.0</td>
<td>263.6</td>
<td>235.4</td>
<td>ND</td>
<td>0.89</td>
<td>0.26</td>
</tr>
<tr>
<td>M:Dog food:ice cream</td>
<td>50:25:25</td>
<td>ND</td>
<td>317.0</td>
<td>106.9</td>
<td>96.9</td>
<td>ND</td>
<td>0.91</td>
<td>0.31</td>
</tr>
<tr>
<td>M:Cola beverage</td>
<td>75:25</td>
<td>38.6</td>
<td>122.4</td>
<td>102.7</td>
<td>83.8</td>
<td>0.32</td>
<td>0.82</td>
<td>0.68</td>
</tr>
<tr>
<td>M:Potatoes</td>
<td>75:25</td>
<td>58.0</td>
<td>122.0</td>
<td>134.4</td>
<td>114.3</td>
<td>0.48</td>
<td>0.85</td>
<td>0.94</td>
</tr>
<tr>
<td>M:Switchgrass</td>
<td>75:25</td>
<td>17.1</td>
<td>413.6</td>
<td>308.0</td>
<td>284.4</td>
<td>0.04</td>
<td>0.92</td>
<td>0.69</td>
</tr>
<tr>
<td>M:Mouthwash</td>
<td>75:25</td>
<td>51.1</td>
<td>168.6</td>
<td>110.5</td>
<td>86.0</td>
<td>0.30</td>
<td>0.78</td>
<td>0.51</td>
</tr>
<tr>
<td>M:Cola:mouthwash</td>
<td>75:12.5:12.5</td>
<td>53.5</td>
<td>140.7</td>
<td>108.8</td>
<td>86.8</td>
<td>0.38</td>
<td>0.80</td>
<td>0.62</td>
</tr>
</tbody>
</table>

ND: not determined

2.4.2. Experimental parameters

Rate (and extent) of biomethane production are maximized when the right pool of enzymes and microorganisms for degrading a particular substrate are present in the medium in sufficient concentrations. The inoculum used in this study was obtained from a well-established on-farm anaerobic digester acclimated to degrade lignocellulosic materials as well as easily degradable carbohydrates, and a fraction of proteins and lipids. Therefore, it was assumed to be microbiologically adequate for degrading the diverse range of substrates proposed for the BMP assays. Similarly, the amount of inoculum used in the test bottles was determined on the basis of the amount of organic substrate available for degradation, i.e. an inoculum-to-substrate (I/S) ratio (VS basis), which is equivalent to the inverse value of the food-to-
microorganism (F/M) ratio. In this study, preliminary trials (data not shown) were conducted with dairy manure to determine appropriate substrate concentrations and I/S ratios for the assay. It was concluded that for manure concentrations ≥3 g VS/L, a minimum I/S ratio of 0.5 was required to ensure process start-up during the first three days of the assay. These results are supported by Hashimoto (1989), who also found a minimum ratio of 0.5 when digesting wheat straw at concentrations of 10 – 40 g VS/L. Furthermore, Hashimoto showed that maximum biomethane production rates were achieved at I/S ratios ≥2. Similarly, studies conducted by Owen et al. (1979) and Chynoweth et al. (1993) suggested I/S ratios of 1 and 2, respectively. In contrast, Fernández et al. (2001) concluded that I/S ratios as low as 0.03 were sufficient to achieve maximum degradation of brewery spent grains at a concentration of 70 g/L (56 g VS/L). In this study, an I/S = 1 was used to maximize degradation rates and ensure that the biomethane potential was achieved.

2.4.3. Experimental biomethane yields

A summary of the average observed specific biomethane yields ($B_o$) of all substrates analyzed in this study is presented in Figure 2-1. Despite biodegradability limitations discussed in the next section, it is apparent that substrates high in lipids and easily degradable carbohydrates (e.g., used oil, ice cream) have the highest $B_o$. On the other hand, lignocellulosic substrates, such as switchgrass and most of the substrates co-digested with manure, show the lowest $B_o$. Noticeably, most invasive freshwater
aquatic plants exhibit a high $B_r$. From the co-digestion samples, the mixture of manure with fresh dog food and ice cream waste presented the highest biomethane yield, which is expected due to the high biomethane yield found in both mono-digestion experiments with these two substrates.

Table 2-3. Chemical composition of selected substrates (% VS basis)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mix ratio (VS basis)</th>
<th>VFA</th>
<th>Protein</th>
<th>Lipids</th>
<th>Hemicelluloses</th>
<th>Cellulose</th>
<th>Lignin</th>
<th>Sugars, starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw dairy manure</td>
<td>-</td>
<td>3.5</td>
<td>5.7</td>
<td>16.1</td>
<td>9.6</td>
<td>32.6</td>
<td>13.8</td>
<td>16.5</td>
</tr>
<tr>
<td>Cheese whey</td>
<td>-</td>
<td>0</td>
<td>13.4</td>
<td>5.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>80.9</td>
</tr>
<tr>
<td>Plain pasta</td>
<td>-</td>
<td>0</td>
<td>16.5</td>
<td>3.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>80.1</td>
</tr>
<tr>
<td>Meat pasta</td>
<td>-</td>
<td>0</td>
<td>19.3</td>
<td>14.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>66.7</td>
</tr>
<tr>
<td>Used vegetable oil</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ice cream</td>
<td>-</td>
<td>0</td>
<td>8.3</td>
<td>38.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>53.1</td>
</tr>
<tr>
<td>Fresh dog food</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cola beverage</td>
<td>-</td>
<td>0</td>
<td>0.7</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>99.1</td>
</tr>
<tr>
<td>Cabbage, raw</td>
<td>-</td>
<td>0</td>
<td>17.8</td>
<td>1.4</td>
<td>0</td>
<td>36.2</td>
<td>0</td>
<td>44.6</td>
</tr>
<tr>
<td>Potatoes, raw</td>
<td>-</td>
<td>0</td>
<td>10.5</td>
<td>0.8</td>
<td>0</td>
<td>9.5</td>
<td>0</td>
<td>79.2</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>-</td>
<td>0</td>
<td>0.7</td>
<td>0</td>
<td>42.2</td>
<td>48.8</td>
<td>8.3</td>
<td>0</td>
</tr>
<tr>
<td>Corn silage</td>
<td>-</td>
<td>0</td>
<td>13.8</td>
<td>5.0</td>
<td>0</td>
<td>11.5</td>
<td>0</td>
<td>69.7</td>
</tr>
<tr>
<td>Suspended FOG</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Settled FOG</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M:Cheese whey</td>
<td>90:10</td>
<td>3.2</td>
<td>6.5</td>
<td>15.1</td>
<td>10.5</td>
<td>29.4</td>
<td>12.5</td>
<td>23.0</td>
</tr>
<tr>
<td>M:Cheese whey</td>
<td>75:25</td>
<td>2.6</td>
<td>7.6</td>
<td>13.5</td>
<td>8.8</td>
<td>24.5</td>
<td>10.4</td>
<td>32.6</td>
</tr>
<tr>
<td>M:Plain pasta</td>
<td>90:10</td>
<td>3.2</td>
<td>6.8</td>
<td>14.9</td>
<td>10.5</td>
<td>29.4</td>
<td>12.5</td>
<td>22.9</td>
</tr>
<tr>
<td>M:Plain pasta</td>
<td>75:25</td>
<td>2.6</td>
<td>8.4</td>
<td>13.0</td>
<td>8.8</td>
<td>24.5</td>
<td>10.4</td>
<td>32.4</td>
</tr>
<tr>
<td>M:Meat pasta</td>
<td>90:10</td>
<td>3.2</td>
<td>7.0</td>
<td>15.9</td>
<td>10.5</td>
<td>29.4</td>
<td>12.5</td>
<td>21.5</td>
</tr>
<tr>
<td>M:Meat pasta</td>
<td>75:25</td>
<td>2.6</td>
<td>9.1</td>
<td>15.6</td>
<td>8.8</td>
<td>24.5</td>
<td>10.4</td>
<td>29.1</td>
</tr>
<tr>
<td>M:Used vegetable oil</td>
<td>75:25</td>
<td>2.6</td>
<td>4.3</td>
<td>37.1</td>
<td>8.8</td>
<td>24.5</td>
<td>10.4</td>
<td>12.4</td>
</tr>
<tr>
<td>M:Cola beverage</td>
<td>75:25</td>
<td>2.6</td>
<td>4.4</td>
<td>12.2</td>
<td>8.8</td>
<td>24.5</td>
<td>10.4</td>
<td>37.2</td>
</tr>
<tr>
<td>M:Potatoes</td>
<td>75:25</td>
<td>2.6</td>
<td>6.9</td>
<td>12.3</td>
<td>8.8</td>
<td>26.8</td>
<td>10.4</td>
<td>32.2</td>
</tr>
<tr>
<td>M:Switchgrass</td>
<td>75:25</td>
<td>2.6</td>
<td>4.4</td>
<td>12.1</td>
<td>19.3</td>
<td>36.7</td>
<td>12.4</td>
<td>12.4</td>
</tr>
</tbody>
</table>

2.4.3.1. Previous studies

Dairy manure is probably one of the most thoroughly and frequently studied substrates in anaerobic digestion. As such, it constitutes an ideal substrate for biomethane potential comparisons. However, dairy manure is highly variable in
nature, as it originates from a wide range of dairy operations, involving different animal breeds, ages, diets, as well as management practices. Interestingly, there seem to be a considerable good agreement among the $B_o$ reported in the literature and those presented in this study, as we discussed below.

The $B_o$ of manure found in this study was based on a total of 47 individual BMP assays performed on manure samples collected from six different dairy farms at various times of the year. The average and range of distribution of $B_o$ was respectively, 243±60 and 127 to 329 mL of CH$_4$ per g VS added. The average $B_o$ found in this study compares well to the overall average value reported by the IPCC (1997) of 240 mL CH$_4$/g VS added. Also, it is within the range of distribution reported by Vedrenne et al. (2008) of 204 – 296 mL CH$_4$/g VS added, and compares quite well with the value reported by El-Mashad and Zhang (2010) of 241 mL CH$_4$/g VS added. Particularly remarkable is the fact that the average $B_o$ obtained in this study also compares well with the $B_o$ of 241 mL CH$_4$/g VS added, determined by Hoffmann et al. (2008) from a study with four CSADs and throughout three different HRTs. A rather lower $B_o$ was reported by Moller et al. (2004) in BMP studies, i.e. 148±41 mL CH$_4$/g VS added; however, it is still within the range of distribution of the observed SMYs found in this study. All preceding $B_o$ values were obtained at mesophilic conditions ($\sim$35°C). At thermophilic conditions (55°C), this study’s average $B_o$ also agrees quite well with the values reported in the literature for both batch and
continuous studies. Nielsen et al. (2004), for example, reported a $B_v$ of 227 mL CH$_4$/g VS added in batch operation, and 236 to 241 mL CH$_4$/g VS added in continuously-stirred tank reactors (CSTRs) operated at 15-d HRT. Likewise, Mladenovska et al. (2006) reported 233 and 238 mL /g VS added at thermophilic conditions in batch and CSTRs, respectively.

In spite of the intrinsic variability of dairy manure among the studies discussed above, their average biomethane potential are comparable, and this study constitutes no exception. Particularly interesting, is the fact that the aforementioned studies have been conducted under a wide range of experimental conditions, from batch to continuous mode, and from mesophilic to thermophilic range temperatures. The latter is especially important if BMP results are to be used for estimating approximate biomethane potential and biodegradability of specific substrates in large-scale, continuous-flow anaerobic digesters (see below discussion on scopes and limitations of the BMP assay).
Figure 2-1. Summary of the observed specific biomethane yields ($B_o$) at STP, as obtained from the BMP assay of ca. 35 substrates. The value outside the bars is the average $B_o$ with the sample number in parenthesis. Error bars represent the standard deviation of $B_o$ for each substrate.

2.4.3.2. BMP production curves

During a BMP assay, biogas production curves can follow a diverse array of patterns. These patterns are not by any measure trivial, but have meaningful implications.
Biodegradability characteristics of substrates and production of inhibitory intermediate products will mainly control the kinetics of the different steps of anaerobic digestion and define the shape of the biogas production curve. This, can aid to identify important characteristics of substrates and anticipate digestion issues. Figure 2-2 depicts four distinctive biogas production patterns from four different substrates during the course of a 40-d BMP assay. Figure 2-2A depicts the cumulative biogas production of dairy manure, a slowly-degradable substrate due to its composition, which consists of approximately 60% lignocelluloses (Table 2-3). It is apparent that the biogas production rate approaches zero near 25 days sludge residence time, which would indicate its biochemical biogas potential (equivalent to $B_0$). Conversely, Figure 2-2B shows the steep biogas production pattern of cheese whey – a substrate mostly composed of easily-degradable sugars (Table 2-3), which appears to achieve its maximum biogas potential in less than 15 days. Figure 2-2C shows the BMP curve of used vegetable oil where biogas production appears to be highly inhibited during the first 12 days of digestion. Lipid-rich substrates are easily degradable (mostly short-chain fatty acids), but are prone to produce biochemical inhibition due to long-chain fatty acid (LCFA) accumulation coming from the hydrolysis of neutral lipids. Therefore, the limiting factor of biodegradability in this case is mainly attributed to LCFA accumulation and inhibition, rather than substrate recalcitrance as in the case of dairy manure, where the lignocellulosic matrix is
primarily responsible for its low biodegradability. Figure 2-2D illustrates corn silage, which as discussed next, it is one of the most biodegradable substrates, but which paradoxically exhibits a rather slow biodegradability rate, maybe due to the acidic characteristics of this substrate and insufficient buffering capacity.

2.4.3.3. Scopes and limitations of the BMP assay

Interpretation of the BMP assay results is of paramount importance. A valid concern exists regarding the suitability of using the results of the BMP assay, a laboratory-scale, batch test, to predict the performance of semi- or continuous-flow, commercial-size anaerobic digesters. In addition to the physical differences in the fluid- and thermo-dynamic characteristics given by the reactor’s scale and geometry, batch reactors and semi- or continuous-flow digesters essentially differ in their mode of operation. The way the reactor is fed has a fundamental impact on the thermodynamic equilibrium of the anaerobic process – and thus, on the food-web interactions. Semi- and continuous-flow digesters are characterized by dynamic changes due to periodic substrate feeding and product removal – thus, unless the digester undergoes shock loads or sudden environmental changes, process unbalance (and product accumulation) rarely occurs under steady-state conditions. In contrast, in a batch reactor, unless removed via biologically-mediated processes, substrates, microorganisms, enzymes, intermediate products, and (sometimes) final products are accumulated within the system. When the concentration of an intermediate product
(particularly, volatile fatty acids and hydrogen) reaches the homeostatic threshold of a certain organism, or group of organisms, the thermodynamic balance is altered, and one or several metabolic reactions may be inhibited, causing further product accumulation and delay of substrate degradation. In most cases, product inhibition is reversible, and as soon as thermodynamic conditions become favorable, reactions resume.

Figure 2.2. BMP assay curves for a 40-day run showing four distinctive biogas production patterns (mL @ STP) from four different substrates; A: dairy manure, B: cheese whey, C: used vegetable oil, D: corn silage; error bars represent the standard deviation for the replicates.
The BMP assay is designed to provide ideal anaerobic conditions and prevent any form of biochemical inhibition. To ensure this, three important conditions should be met throughout the BMP assay: 1) appropriate microbial community, enzyme pool, and nutrients are present; 2) environmental conditions are optimal; and 3) substrate and intermediate product concentrations are well below inhibitory/toxic levels. Nevertheless, product inhibition is difficult to prevent, and indeed occurs in some BMP assays. Fortunately, product inhibition primarily affects reaction kinetics, and thus, provided that adequate digestion time is allowed, stabilization of the substrate’s biodegradable fraction and maximum biomethane yields should be achieved. However, a more difficult problem to foresee, which directly affects the biomethane potential, is trace element deficiency. This can occur during long-term anaerobic digestion of certain substrates lacking an essential element, such as cobalt in thin stillage (Agler et al., 2008). Accordingly, the BMP assay may not be a suitable test to predict biomethane yields, stabilization performance, or possible process failure due to shock loads and product inhibition, over long-term semi- and continuous-flow anaerobic digestion operations. BMP results should be limited to a relative interpretation of the substrate’s biomethane potential, and not for an absolute estimation of daily biomethane yields or the overall performance and stability of large-scale digesters. The BMP assay is best suited when used to elucidate what types of substrates, from an array of potential substrates, have the highest biomethane
potential. In addition, the assay can be used to estimate the potential ideal ratios between co-substrates when co-digestion is intended. Lastly, BMP assay results can be used to determine the extent of anaerobic biodegradability of substrates, and thus, relative residence times required for complete digestion.

2.4.4. Theoretical specific biomethane yields

A comparison between observed ($B_o$) and theoretical ($B_u$) specific biomethane yields (SMYs) for 17 selected substrates is depicted in Figure 2-3A. $B_u$ using McCarty’s method were in average 11.6% lower than the values obtained using the Buswell Formula, and therefore, closer to the $B_o$. This is expected, since McCarty’s method accounts for the fraction of electron donor which is lost in cell protoplasm synthesis, while the Buswell Formula does not. In fact, Symons & Buswell (1933) reported that during the digestion of pure carbohydrates, an average of 12% of the total carbon fed was lost in the cell protoplasm which was not accounted for by their formula. Regardless of the method used, however, $B_u$ is consistently higher than $B_o$. A potential source of difference may come from the use of theoretical substrate compositions, rather than actual, experimentally-determined substrate constituent’s concentrations. However, data suggest that the largest contribution of difference is due to the fact that the two methods do not account for substrate biodegradability. The biodegradable fraction ($f_D$) was calculated using Eq. 2 and the substrates characteristics reported Table 2-1 in and Table 2-2. The results are presented in Table
2-4. With the exception of the co-digestion of manure and plain pasta, data suggest that most degradable substrates are sugar- and starch-rich carbohydrates. The high biodegradability for co-digestion of manure and plain pasta could be due to experimental error, or more likely, due to a synergistic mixture as discussed in the next section. Furthermore, it is noticeable that the lower the substrate biodegradability is, the poorer the estimation is, i.e. recalcitrant lignocellulosic substrates and oil-rich substrates where product inhibition is likely to occur.

The importance of using substrate biodegradability information to estimate biomethane yields is demonstrated in Figure 2-3B, which corrects the theoretical calculations depicted in Figure 2-3A using the substrate biodegradability fractions reported in Table 2-4. In comparison, Figure 2-3B exhibits a considerable better agreement between the theoretical and observed data for both theoretical methods. As shown in Figure 2-4, after biodegradability data are included in the calculations both methods exhibit an agreement higher than 90% with the observed data, as determined by their coefficient of determination, i.e. $R^2 = 0.91$ and $R^2 = 0.93$ for McCarty and Buswell methods, respectively. The difference that prevails can be attributed to variation of the observed SMYs, as suggested by the standard deviation (SD) of $B_o$ (Figure 2-1), which in most cases is greater than 10%. It is also apparent that of both methods, the Buswell Formula produces results closer to observed biomethane values after correcting for substrate biodegradableability – this is explained by
the fact that McCarty’s method accounts for cell synthesis, which is already factored in the observed biodegradability fraction. This leads to the conclusion that when biodegradability data is available, the Buswell Formula is the method of choice for estimating biomethane yields.

Table 2-4. The anaerobic biodegradability fraction ($f_D$) of selected substrates sorted by decreasing biodegradability. Data calculated using Eq. 2-2 and the experimental parameters presented in Table 2-1 and Table 2-2

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mix ratio (VS basis)</th>
<th>$B_0$ (mL CH$_4$/g VS added @ STP)</th>
<th>COD$_{CH4}$ (g COD/g VS added)</th>
<th>$f_D$ (g COD/g COD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cola beverage</td>
<td>-</td>
<td>373.1</td>
<td>1.066</td>
<td>0.78</td>
</tr>
<tr>
<td>Corn silage</td>
<td>-</td>
<td>296.1</td>
<td>0.846</td>
<td>0.77</td>
</tr>
<tr>
<td>M:Plain pasta</td>
<td>75:25</td>
<td>353.5</td>
<td>1.010</td>
<td>0.68</td>
</tr>
<tr>
<td>Used vegetable oil</td>
<td>-</td>
<td>648.5</td>
<td>1.853</td>
<td>0.64</td>
</tr>
<tr>
<td>Cheese whey</td>
<td>-</td>
<td>423.6</td>
<td>1.210</td>
<td>0.64</td>
</tr>
<tr>
<td>M:Potatoes</td>
<td>75:25</td>
<td>227.7</td>
<td>0.651</td>
<td>0.61</td>
</tr>
<tr>
<td>Potatoes</td>
<td>-</td>
<td>334.5</td>
<td>0.956</td>
<td>0.60</td>
</tr>
<tr>
<td>Ice cream</td>
<td>-</td>
<td>502.3</td>
<td>1.435</td>
<td>0.59</td>
</tr>
<tr>
<td>Cabbage</td>
<td>-</td>
<td>256.5</td>
<td>0.733</td>
<td>0.58</td>
</tr>
<tr>
<td>Manure</td>
<td>-</td>
<td>242.7</td>
<td>0.693</td>
<td>0.55</td>
</tr>
<tr>
<td>M:Meat pasta</td>
<td>75:25</td>
<td>285.6</td>
<td>0.816</td>
<td>0.48</td>
</tr>
<tr>
<td>M:Plain pasta</td>
<td>90:10</td>
<td>224.0</td>
<td>0.640</td>
<td>0.48</td>
</tr>
<tr>
<td>Settled FOG</td>
<td>-</td>
<td>413.4</td>
<td>1.181</td>
<td>0.46</td>
</tr>
<tr>
<td>M:Cola</td>
<td>75:25</td>
<td>235.0</td>
<td>0.671</td>
<td>0.46</td>
</tr>
<tr>
<td>M:Whey</td>
<td>90:10</td>
<td>237.6</td>
<td>0.679</td>
<td>0.45</td>
</tr>
<tr>
<td>Suspended FOG</td>
<td>-</td>
<td>402.3</td>
<td>1.149</td>
<td>0.44</td>
</tr>
<tr>
<td>Plain pasta</td>
<td>-</td>
<td>326.1</td>
<td>0.932</td>
<td>0.41</td>
</tr>
<tr>
<td>M:Whey</td>
<td>75:25</td>
<td>252.4</td>
<td>0.721</td>
<td>0.41</td>
</tr>
<tr>
<td>M:Switchgrass</td>
<td>75:25</td>
<td>207.8</td>
<td>0.594</td>
<td>0.41</td>
</tr>
<tr>
<td>M:Meat pasta</td>
<td>90:10</td>
<td>232.1</td>
<td>0.663</td>
<td>0.39</td>
</tr>
<tr>
<td>Meat pasta</td>
<td>-</td>
<td>216.2</td>
<td>0.618</td>
<td>0.37</td>
</tr>
<tr>
<td>Fresh dog food</td>
<td>-</td>
<td>426.6</td>
<td>1.219</td>
<td>0.29</td>
</tr>
<tr>
<td>M:Oil</td>
<td>75:25</td>
<td>360.6</td>
<td>1.030</td>
<td>0.26</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>-</td>
<td>122.2</td>
<td>0.349</td>
<td>0.24</td>
</tr>
<tr>
<td>Corn leachate</td>
<td>-</td>
<td>106.5</td>
<td>0.304</td>
<td>0.09</td>
</tr>
</tbody>
</table>
In summary, it is apparent that the use of stochiometric methods together with biodegradability information is able to produce reasonable estimations of specific biomethane yields. The use of empirical methods, employing single and multiple regression models to estimate SMYs, has also been described (Gunaseelan, 2007). However, the use of purely empirical (as opposed to descriptive) methods to estimate anaerobic digestion products will most likely compound the effects of substrate chemical composition, biodegradability and bioenergetics; disregarding the stoichiometry therein.
Figure 2-3. Observed and estimated biomethane yields of 17 selected substrates using McCarty’s bioenergetics and Buswell’s formula; A: theoretical estimations as is, i.e. not accounting for substrate biodegradability, B: theoretical estimations corrected using the substrate biodegradable fraction ($f_D$) reported in Table 2-4.
2.4.5. Effects of co-digestion on biomethane yields: synergistic substrate mixtures?

Co-digestion of certain substrates can produce synergistic or antagonistic effects. The synergistic effect of co-digesting swine manure with oil mill waste was reported by Angelidaki & Ahring (1997). Synergism would be seen as an additional biomethane yield for co-digestion samples over the weighted average of the individual substrates’ SMY, namely the weighted specific biomethane yield (WSMY). Similarly, evidence of antagonism would be translated into a lower biomethane yield in the co-digestion samples as compared to the WSMY. Synergistic effects may arise from the contribution of additional alkalinity, trace elements, nutrients, enzymes, or any other amendment which a substrate by itself may lack, and could result in an increase in substrate biodegradability, and therefore, biomethane potential. Antagonistic effects can come from several factors, such as pH inhibition, ammonia toxicity, high volatile
acid concentration, among others. Table 2-5 summarizes this analysis for co-digestion mixtures of dairy manure with food residue substrates, depicting the differences between the biomethane yields from co-digestion samples and the WSMYs calculated from mono-digestion biomethane yields. For example, the WSMY of manure co-digested with cheese whey is 288 mL/g VS; however, the observed SMY of this co-digestion sample was 252 mL CH₄/g VS. Since the negative differential in biomethane yield is within its SD (109 mL/g VS), it is not clear if this difference is indeed the result of an antagonistic effect (Table 2-5). The similarity of the $B_o$ and WSMY values for the co-digestion of manure with switchgrass and its SD suggest that the co-digestion of these two substrates does not produce either synergistic or antagonistic effects. Furthermore, it is evident that a mixture of lignocellulosic substrates will not produce high biomethane yields unless some kind of pretreatment is applied. Data suggest, however, that the co-digestion of manure with both plain pasta and meat pasta is synergistic, since biomethane yields are 30% higher than the digestion of manure and pasta separately. Similarly, the positive differential suggests that the co-digestion of manure with oil is synergistic; however data in this case are not conclusive, since this differential is within the SD of $B_o$.

Due to the variability of the data it is not possible to draw definitive conclusions on the synergism and absence of antagonism observed in our co-digestion trials. Further co-digestion studies with dairy manure, a lignocellulosic substrate, and a co-substrate
with a well-balanced composition of proteins, lipids, and easily-degradable carbohydrates should be conducted to elucidate which components within the co-digestion mixture show further degradation as compared to their mono-digestion condition.

Table 2-5. Observed SMYs ($B_o$) from the co-digestion samples as compared to weighted SMYs (WSMY), calculated as the sum of the individual contributions of the mono-digestion samples

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$B_o$ (mL/g VS @ STP)</th>
<th>SD</th>
<th>WSMY</th>
<th>Differential ($B_o$ - WSMY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manure</td>
<td>242.7</td>
<td>60.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Co-substrates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25% Cola</td>
<td>235.0</td>
<td>118.5</td>
<td>275.3</td>
<td>-40.3</td>
</tr>
<tr>
<td>25% Potatoes</td>
<td>227.7</td>
<td>81.1</td>
<td>265.7</td>
<td>-38.0</td>
</tr>
<tr>
<td>25% Cheese whey</td>
<td>252.4</td>
<td>109.0</td>
<td>287.9</td>
<td>-35.5</td>
</tr>
<tr>
<td>25% Mouthwash</td>
<td>220.1</td>
<td>96.5</td>
<td>250.6</td>
<td>-30.5</td>
</tr>
<tr>
<td>10% Plain pasta</td>
<td>224.0</td>
<td>34.7</td>
<td>251.0</td>
<td>-27.0</td>
</tr>
<tr>
<td>10% Cheese whey</td>
<td>237.6</td>
<td>69.3</td>
<td>260.8</td>
<td>-23.2</td>
</tr>
<tr>
<td>10% Meat pasta</td>
<td>232.1</td>
<td>31.0</td>
<td>240.0</td>
<td>-7.9</td>
</tr>
<tr>
<td>12.5% Cola and 12.5% Mouthwash</td>
<td>258.0</td>
<td>42.8</td>
<td>262.9</td>
<td>-4.9</td>
</tr>
<tr>
<td>25% Switchgrass</td>
<td>207.8</td>
<td>4.5</td>
<td>212.6</td>
<td>-4.8</td>
</tr>
<tr>
<td>25% Used oil</td>
<td>360.6</td>
<td>168.1</td>
<td>344.1</td>
<td>16.4</td>
</tr>
<tr>
<td>25% Meat pasta</td>
<td>285.6</td>
<td>29.0</td>
<td>236.1</td>
<td>49.5</td>
</tr>
<tr>
<td>25% Plain pasta</td>
<td>353.5</td>
<td>36.6</td>
<td>263.6</td>
<td>89.9</td>
</tr>
<tr>
<td>25% Dog food and 25% Ice cream</td>
<td>467.3</td>
<td>39.9</td>
<td>353.6</td>
<td>113.7</td>
</tr>
</tbody>
</table>

**2.5. Conclusions**

Substrates highly rich in lipids and easily-degradable carbohydrates exhibited higher biomethane potential – more recalcitrant, lignocellulosic-materials presented lower biomethane yields. Experimental biomethane yields were consistently overestimated by the theoretical methods evaluated; by including the experimentally-obtained biodegradability fraction in the calculations, an agreement of over 90% was achieved.
Co-digestion of dairy manure with easily-degradable substrates increases the biomethane yields when compared to manure-only digestion; Synergistic biomethane yields were observed in a number of substrates co-digested with dairy manure; however, further testing is necessary to validate this conclusion.

References


CHAPTER 3
TOWARDS A GENERAL UNDERSTANDING OF ANAEROBIC
BIODEGRADABILITY – FATE AND CONVERSION OF BIOPOLYMERS
DURING THE DIGESTION OF COMPOSITE SUBSTRATES

Abstract

Biodegradability information is fundamental to make reliable product yield estimations from biologically-mediated reactions. It influences the stoichiometry of the anaerobic digestion food web, and thus it affects biomethane production rates, concentration of the effluent products, and ultimately, the overall energy output of the system and its waste treatment efficiency. The purpose of this study was to develop a method to predict the extent of biodegradability and biomethane potential of complex, composite substrates based on their carbohydrate, protein and lipid composition. Anaerobic digestion studies were conducted under both batch and continuous operating conditions, and mesophilic (37°C) and thermophilic (55°C) temperature ranges. Batch reactors were intended to determine the extent of biodegradability and biomethane potential of a variety of substrates, including, pure biomolecules from the major chemical groups, lignocellulosic materials, and complex substrates represented by co-digestion mixtures. Lignocellulosic substrates included an array of fibrous materials of different chemical composition. Semi-continuously-fed reactors were used to investigate both biomethane yields and fate and
biodegradability of individual substrate constituents over long-term anaerobic digestion of complex wastes. Lignocellulosic dairy manure and a high-strength, multi-component waste, emulated by dry dog food, were co-digested at two different ratios and three hydraulic retention times (HRT). The degradable fraction and biomethane yields of an array of proteins, carbohydrates and lipids were obtained under batch conditions. Co-digestion samples were assessed using cow manure and dog food, and a linear decrease in the biodegradability and biomethane yields was observed with the proportional increase of the dairy manure. Two models were developed to predict extent of degradation and biomethane yields of lignocellulosic materials using lignin as a predictor. Batch tests are able to produce reasonable predictions of the biodegradability and biomethane yields of semi-continuous studies at steady-state conditions and a specific retention time. Finally, a methodology that allows to estimate the degradable fraction and biomethane yields of lignocellulosic composite substrates was developed on the basis of substrate chemical composition and exiting stoichiometry methods.

Nomenclature

BMP Biochemical methane potential, mL CH₄/g VS added (equivalent to \( B_o \))

\( B_o \) Observed SMY, mL CH₄/g VS added (measured, e.g., via BMP)
Introduction

Anaerobic biodegradability information is fundamental to make reliable product yield estimations from biologically-mediated reactions. The fraction of influent substrate available for biological degradation influences the stoichiometry of all the reactions involved in anaerobic digestion. From an engineering point of view, substrate biodegradability influences the rate of biomethane production (and thus, the retention time of the system), ultimate biomethane yields, stability of the system, degree of
waste stabilization, and concentration of the effluent products, all of which ultimately impact the overall energy output and waste treatment efficiency of the system.

A number of theoretical methods to predict the stoichiometry of the biochemical and physicochemical reactions of anaerobic digestion have been developed in the past (Angelidaki et al., 1999; Batstone et al., 1997; Batstone et al., 2002; Costello et al., 1991; McCarty, 1972; McCarty, 1964b; Sotemann et al., 2005; Symons & Buswell, 1933). Regardless of the level of complexity, a common characteristic of these methods is that they are all based on the premise that influent substrates are completely biodegradable. Thus, for readily-degradable, soluble substrates, such as volatile fatty acids or sugars, predictions are, in general, in agreement with observations; however, for less degradable, complex, composite-substrate mixtures, biodegradability information is not only limited, but also inaccurate, and so are theoretical predictions.

Today, most anaerobic treatment facilities co-digest mixtures of two or more substrates concomitantly. In Europe, animal waste (i.e. manure) is co-digested with non-agricultural (usually high-strength) organic wastes, harvesting residues, and energy crops (Weiland, 2006); while in the U.S., a growing number of anaerobic co-digestion projects is in place, particularly on dairy farms (Scott et al., 2010). The extent of biodegradability (and biomethane potential) of composite substrate mixtures, such as those found in co-digestion operations, are usually assessed via analytical methods.
For many years, the standard method used has been the biochemical methane potential (BMP) assay (Owen et al., 1979) – a long-term digestion batch test, which despite its limitations (see Chapter 2), is probably the most economical and practical alternative to analyze an array of prospective substrates for anaerobic digestion. However, a recurrent question that appears to be always left unanswered in the literature is, how representative are the results obtained through batch methods of those observed under semi-continuous, steady-state conditions? Given the inherently different mode of operation of these systems (Pavlostathis & Giraldo-Gomez, 1991), and its impact on the substrate degradation kinetics, can ultimate parameters (rather than rates), such as extent of substrate biodegradability and biomethane potential, be predicted using the BMP assay?

In an attempt to circumvent lengthy fermentation tests, such as the BMP, few studies have evaluated the feasibility of predicting the biodegradability fraction of organic substrates exclusively from their chemical characteristics. The studies of Chandler et al. (1980) and Hashimoto et al. (1981) assessed the biodegradability of lignocellulosic materials, and developed a model to predict the biodegradability fraction of these materials based on the lignin content. Gossett & Belser (1982), and later Pavlostathis & Gossett (1986), created models to predict the extent of biodegradability of biological sludge, which were primarily based on the biochemical characteristics of the biomass and the retention time of the activated sludge process. The validity of
applying such models to semi- or continuous systems will still depend on the source of the data (i.e., batch, continuous) and conditions (e.g. thermal regime) used to develop it, as well as the availability (and accuracy) of the substrates’ chemical composition.

Either analytical or theoretical, a methodology to estimate the degradable fraction of composite streams, which could be applicable to semi- or continuous systems, would serve as a decision support tool for co-digestion operations in selecting co-substrates that would potentially produce increased energy outputs.

3.2. Objectives

The purpose of this study was to investigate the compositional factors that determine the extent of biodegradability of complex, particulate substrates under ideal, semi-continuously-fed, steady-state anaerobic conditions. This was accomplished by addressing the following objectives:

1. To assess the extent of biodegradability and biomethane potential of the main biomolecules comprising composite substrates
2. To evaluate the feasibility of using both analytical and theoretical methods to estimate waste stabilization and biomethane potential of composite substrates under ideal, semi-continuous, steady-state conditions
3. To evaluate the impact of influent substrate composition and hydraulic retention time on biodegradability and reactor effluent characteristics
3.3. Background and discussion of concepts

3.3.1. Definition of biodegradability

Substrate biodegradability is primarily characterized in terms of rate and extent of degradation. Rate is the speed of substrate utilization (degradation), which under ideal, steady-state conditions (absence of inhibition) is directly related to the rate of (intermediate) product(s) formation. Extent (or ultimate biodegradability) represents the hypothetical maximum biological degradation achieved at a solids retention time equal to infinity. Under batch conditions, ultimate biodegradability is usually assumed to be reached when the rate of degradation approaches to zero, i.e. stabilization is deemed to be completed.

Ultimate biodegradability of organic substrates is determined by physicochemical and biochemical factors. The structural and conformational characteristics of the biomolecules composing the influent material, and the interaction between them, determine the degree of complexity of the substrate and its surface area available for enzymatic hydrolysis, thus constituting a physicochemical limitation for biodegradability. In addition, biochemical inhibition constitutes a significant factor determining substrate biodegradability by influencing the rate, and eventually the extent, of any biologically-mediated reaction occurring in the anaerobic digestion processes. Contrarily, biochemical inhibition (and toxicity) usually arises in secondary
digestion stages, e.g. fermentation, oxidation, methanogenesis, primarily as a result of reactant-product imbalance and consequential intermediate product accumulation (or vice versa) to levels that may disrupt the metabolic processes of the microorganisms and/or create thermodynamic barriers for subsequent biochemical conversions. Consequently, in absence of biochemical inhibition, the biodegradability of complex organic substrates should be primarily dependent on the physicochemical characteristics of the substrate and their effect on the extent of hydrolysis. It is therefore apparent that, under steady and optimal biological and environmental conditions – that is, a system with (1) a well established/acclimated and selected microbiological community and extracellular enzymatic pool for degrading a particular waste, (2) absence of substance(s) at inhibitory/toxic concentrations, and (3) constant operating parameters, particularly, temperature and sludge retention time – the rate and extent of biodegradability is solely dependent on the physicochemical characteristics of the influent substrate, and thus, on its hydrolysis of the composite substrates.

3.3.2. Substrate composition

Although a small portion of inorganic/inert material is normally present, substrates are almost entirely organic, made up of a mixture of carbohydrates, proteins and lipids. Depending on the degree of complexity of the substrate, these three major
substrate constituents can be found in a variety of structural forms, from simple monomers or dimers to complex associations of branched, long chain-polymers.

3.3.2.1. Carbohydrates

This group encompasses a diverse array of biomolecules, from readily-degradable sugars, to lignin-bound biofibers. Among the factors that determine the biodegradability characteristics of carbohydrates are: the nature of the glycosidic linkage, inter-molecular forces and linkage between chains, molecular size, and the affinity for the medium (Van Soest, 1982).

Structural carbohydrates – lignocellulose

Lignocellulosic materials are probably one of the most common substrates subjected to anaerobic digestion processes, with applications ranging from treatment of animal waste (e.g. cattle, swine, chicken) to bioenergy production, using crop residues (e.g. corn stover) or energy crops (e.g. switchgrass, short rotation woody crops). Furthermore, cellulose constitutes the major organic component of municipal solid waste (Stinson & Ham, 1995).

The lignocellulose matrix is primarily made up of three biopolymers, i.e. cellulose, hemicellulose, and lignin, which are strongly intermeshed and chemically bonded together by non-covalent forces and by covalent cross-linkages (Perez et al., 2002). Cellulose and hemicellulose together make up of 63 – 78% of the fiber lignocellulose structure, while lignin makes up of 15 – 38% (Angelidaki & Ahring, 2000).
addition, the lignocellulosic matrix includes other biopolymers, primarily pectin and proteins, which add additional complexity and thus resistance to its degradation (Leschine, 1995).

Cellulose is an unbranched polymer, insoluble in water, which consists of several thousands of D-glucose units joined by β-1,4-glycosidic linkages. Pure cellulose is rare in nature; all structural cellulosics are combined to some degree with lignin, hemicellulose, cutin and minerals in the plant cell wall structure (Van Soest, 1982). The biodegradability of cellulose varies from total indigestibility to complete digestibility, depending largely upon the degree lignification (Van Soest, 1982); in fact, there seems to be a consensus that extent of biodegradability of lignocellulosic materials is related to the lignin content, as previous studies suggest (Chandler et al., 1980; Van Soest, 1982; Zeikus, 1980). Hydrolysis of cellulose produces D-cellobiose (β-1,4-bond), which upon complete hydrolysis D-glucose is released (Colberg, 1988). Cellulose molecules are strongly associated through inter- and intra-molecular hydrogen-bonding and van der Waals forces that result in the formation of microfibrils, which in turn form biofibers (Leschine, 1995). Microfibrils form highly ordered crystalline domains interspersed by more disordered, amorphous regions; the amorphous component is digested more easily by enzymes than the crystalline component (Dale et al., 2005).
Hemicellulose is a complex carbohydrate structure that consists of different polymers, such as pentoses (e.g. xylose and arabinose), hexoses (e.g. mannose, glucose and galactose), and sugar acids. The dominant component of hemicelluloses is xylan, which primarily comes from agricultural plants, such as grasses and straw (Hendriks & Zeeman, 2009). Xylans have a \(\beta\)-1,4-linked xylopyranose backbone with attached side groups of acetate, arabinofuranose, and O-methyl glucuronic acid (Leschine, 1995). Hemicellulose has a lower molecular weight than cellulose, and branches with short lateral chains that consist of different sugars, which are easily hydrolysable polymers (Hendriks & Zeeman, 2009). Considering that hemicelluloses surround the cellulose microfibrils and occupy spaces between fibrils, this biopolymer must be degraded, at least partially, before cellulose can be effectively degraded by cellulolytic bacteria (Leschine, 1995).

Lignin is a highly branched, hydrophobic polyphenolic aromatic compound, primarily located in the cell walls of vascular plants (Colberg, 1988; Hendriks & Zeeman, 2009). Lignin provides rigidity to plant cell walls and resistance to biodegradation, and constitutes the most significant factor limiting biodegradability of lignocellulose in anaerobic digestion systems (Van Soest, 1982). Lignin is tightly associated to hemicellulose, which covers cellulose and creates a physical barrier for hydrolytic enzymes (Angelidaki & Ahring, 2000). In fact, biodegradability of hemicellulose is directly related to that of cellulose and inversely related to lignification (Van Soest,
To date, only few studies have assessed the influence of lignin on the extent of biodegradability of lignocellulosic materials (Chandler et al., 1980; Hashimoto et al., 1981; Jung et al., 1997). Lignin in itself is believed to be recalcitrant in anaerobic environments (Zeikus, 1980); however, previous studies have shown that degradation of lignin is possible under anaerobic conditions (Colberg, 1988; Symons & Buswell, 1933), and particularly by rumen microorganisms (Hu et al., 2008).

**Non-structural carbohydrates – starch**

In addition to sugars, starch is one of the most frequently found non-structural carbohydrates in anaerobic digesters, usually composing primary municipal wastes and food processing wastes. Starch ubiquitous in foods, coming primarily from grains, such as corn and wheat, and tubers, such as potato and tapioca (Shogren, 1998). Starch consists of two primary biopolymers, i.e. amylase, which is a linear chain of α-1,4-linked D-glucose units, and amylopectin, which is chain of α-1,4-linked D-glucose with branches of α-1,6-linked D-glucose (Kaplan, 1998). Starch, which is partially soluble in water, is the primary polysaccharide for storing energy in higher plants (Shogren, 1998; Van Soest, 1982). Some forms of starches are insoluble and resistant to degradation (e.g. wheat breads), while others are partially bioavailable (e.g. raw potatoes, banana) (Van Soest, 1982).
3.3.2.2. Proteins

In complex substrates subjected to anaerobic digestion, proteins are mainly found as a constituent of either foods (e.g. food processing wastes) or active/dead biomass (e.g. waste activated sludge and animal wastes). Proteins are natural polymers composed of amino acid units linked covalently one to another through peptide (or amide) bonds, which are formed after dehydration of the $\alpha$-carboxyl group of one amino acid and the $\alpha$-amino group of another (Nelson et al., 2008). The protein chemical structure is one of the main factors determining the extent of hydrolysis, and thereby its biodegradability.

There are two major groups of proteins based on their chemical structure: fibrous proteins, with polypeptide chains arranged in long strands or sheets, and globular proteins, with polypeptide chains folded into a spherical or globular shape. Fibrous proteins, such as keratin and collagen, are structural, insoluble-proteins, which are more difficult to hydrolyze (Batstone, 2000). Globular proteins, such as casein and albumin, are functional proteins, which are usually readily hydrolysable (McInerney, 1988).

Proteins are hydrolyzed by extracellular enzymes into their constituent polypeptides and amino acids. Protein hydrolysis in anaerobic environments has not been well studied except for regions in the gut of animals, such as in the rumen (McInerney, 1988). There seems to be major differences between protein degradation in the
rumen and in anaerobic digesters. For instance, in the rumen, carbohydrate-fermenting bacteria degrade proteins and the fermentation of amino acids alone does not provide sufficient energy for growth. In anaerobic reactors, however, proteolytic bacteria predominantly mediate protein degradation and the processes involved are energy yielding (McInerney, 1988).

3.3.2.3. Lipids

Lipids are commonly found in wastewater treatment plants as a mixture of fats, oil and grease (FOG). FOG normally account for about 30% of the volatile solids in raw wastewater sludge; however, over 50% of the COD reduction and biomethane production during anaerobic digestion comes from FOG degradation (Novak & Carlson, 1970). The simplest lipids are triglycerides, or neutral lipids, which are composed of three fatty acids each in ester linkage with a single glycerol (Nelson et al., 2008). Triglycerides contribute to waste solids from food processing industries and can comprise up to 65% (w/w) of meat industry wastes (Broughton et al., 1998). Lipids are differentiated by its fatty acid chain length, degree of chemical saturation (number of double bonds), and thereby their physical state, i.e. liquid, solid. Hydrolysis rates of fats vary depending on these chemical characteristics as well as their specific surface area (Martinelle & Hult, 1994).

The longer the fatty acyl chain and the fewer the double bonds, the slower the rate of degradation (Novak & Carlson, 1970); probably because of a lower solubility in water
Biodegradability of fat-rich wastes in anaerobic digestion can also be affected, because the floating properties of lipids can result in cell biomass washout. In addition, the low surface area to volume ratios of these floating aggregates slows their biological degradation (Gujer & Zehnder, 1983). In addition, inhibition of the anaerobic process can occur due to long-chain fatty acid (LCFA) accumulation (Ahring & Nielsen, 2006; Angelidaki & Ahring, 1992; Lalman & Bagley, 2000; Lalman & Bagley, 2001), which can negatively impact the extent of degradation.

Triglycerides are hydrolysed to glycerol and LCFA by a group of esterases, called lipases (Pavlostathis & Giraldo-Gomez, 1991). Glycerol is fermented to a variety of VFA, alcohols and formic acid (Broughton et al., 1998). LCFA is primarily degraded via β-oxidation (Jeris & McCarty, 1965) by H₂-producing acetogenic bacteria (Bryant, 1979) to acetate and hydrogen (Hanaki et al., 1981), while methanogens consume acetate, formate and hydrogen to produce biomethane.

3.4. Materials and Methods

3.4.1. Experimental design

Anaerobic digestion studies were conducted under both batch and semi-continuous operating conditions. Batch and semi-continuous studies were conducted at both mesophilic (37°C) and thermophilic (55°C) thermal conditions.
Batch reactors were intended to determine the extent of biodegradability and biomethane potential of a variety of substrates, including, pure biomolecules, lignocellulosic materials, and complex substrate mixtures. Pure biomolecules included monomers, dimers and polymers, from the major chemical groups, i.e. carbohydrates, proteins, and lipids. Lignocellulosic substrates included an array of fibrous materials of different chemical composition. Finally, several co-digestion samples were also assessed as discussed below.

Semi-continuous reactors were used to investigate both biomethane yields and fate and biodegradability of individual substrate constituents over long-term anaerobic digestion of complex wastes. Lignocellulosic dairy manure and a high-strength, multi-component waste, emulated by dry dog food, were co-digested at two different ratios and three hydraulic retention times (HRT). Additionally, batch tests were conducted to evaluate the extent of biodegradability of the same co-digestion mixture at an array of different co-digestion ratios. A steady source of inoculum, microbiologically acclimated for degrading the various substrates evaluated in the batch tests, was provided by the semi-continuous reactors throughout the study.

**3.4.2. Batch studies**

Anaerobic digestion studies were performed in accordance with the biochemical methane potential (BMP) assay (Owen et al., 1979).
3.4.2.1. Substrate specimens

The pure biomolecules subjected to the assay were selected from each major chemical group, to assess the effect of different physicochemical characteristics on substrate biodegradability. These substrates are presented in Table 3-1, organized by their main chemical group.

The two main non-lignin components of structural carbohydrates, i.e. cellulose, hemicelluloses (represented here by Xylan), were studied in a pure (lignin-free) form. Starch, a non-structural polysaccharide, commonly found in food wastes, was also assessed in this study, together with the main products of carbohydrate hydrolysis, i.e. glucose and cellobiose.

The proteins selected are the most commonly found in food and animal wastes, and exhibit distinctive physicochemical characteristics, such as solubility, which is usually regarded as a main determinant for biodegradability (McInerney, 1988). Casein is globular and insoluble, albumin is globular and soluble, and collagen is a fibrous and insoluble protein.

The lipids included in the BMP assay are the most abundant fatty acids found in many types of wastewaters (Hatamoto et al., 2007; Miron et al., 2000; Novak & Carlson, 1970). We evaluated these fatty acids in their most prevalent, naturally-occurring form, i.e. as neutral lipids, or triglycerides. Three triglycerides were evaluated: saturated 16-carbon glycerol tripalmitate (GTP), saturated 18-carbon glycerol
tristearate (GTS), and monounsaturated 18-carbon glycerol trioleate (GTO). The fatty acids account for variation in fatty acid chain length and degree of chemical saturation (thereby, physical state), which ultimately affects their biodegradability. Although long-chain fatty acids (LCFA) are regarded to be degradable, they are potentially inhibitory to anaerobic systems at extremely low concentrations (Angelidaki & Ahring, 1992; Lalman & Bagley, 2000). Therefore, an equally-balanced mixture of GTP, GTS and GTO was evaluated at three different concentrations, with the purpose of assessing LCFA inhibition.

Table 3-1. Pure substrates evaluated in the BMP assays

<table>
<thead>
<tr>
<th>Biomolecule</th>
<th>Available details</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>From cotton linters, Microcrystalline, 50 µm</td>
<td>Sigma Aldrich Co.</td>
</tr>
<tr>
<td>Hemicellulose, Xylan</td>
<td>From Beechwood, ≥90% xylose residues</td>
<td>Sigma aldrich Co.</td>
</tr>
<tr>
<td>Starch</td>
<td>Powder</td>
<td>B&amp;A, Allied Chemical</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>D(+), &gt;98% purity</td>
<td>Acros Organics</td>
</tr>
<tr>
<td>Glucose</td>
<td>D(+), ACS</td>
<td>Sigma-Aldrich Co.</td>
</tr>
<tr>
<td>Proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>Pure</td>
<td>Acros Organics</td>
</tr>
<tr>
<td>Albumin</td>
<td>From eggs, powder</td>
<td>Acros Organics</td>
</tr>
<tr>
<td>Collagen</td>
<td>Insoluble</td>
<td>MP Biomedicals, LLC</td>
</tr>
<tr>
<td>Lipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol tripalmitate</td>
<td>Pure</td>
<td>TCI</td>
</tr>
<tr>
<td>Glycerol tristearate</td>
<td>Pure</td>
<td>TCI</td>
</tr>
<tr>
<td>Glycerol trioleate</td>
<td>Pure</td>
<td>TCI</td>
</tr>
</tbody>
</table>

The lignocellulosic substrates subjected to the BMP assay were primarily selected with the purpose of covering a wide spectrum of lignin content to assess its influence on the substrate’s biodegradability. A secondary objective was to evaluate the
biomethane potential of these materials due to their occurrence in anaerobic digestion, as wastes, refuse, or bioenergy crop materials. Six lignocellulosic materials were considered: (1) dairy manure, (2) red onions, (3) corn silage, (4) wheat straw, (5) miscanthus, and (6) switchgrass. Lignocellulosic substrates were first air-dried at 55°C and ground to a size of 1 mm using a Wiley mill in preparation for both the fiber analysis (see section 3.2) and the BMP assay.

The co-digestion mixtures evaluated through the BMP assay combined various ratios of dairy manure-to-dog food pellets, as follows (VS basis): (1) 100% Manure – 0% Dog food, (2) 75% Manure – 25% Dog food, (3) 50% Manure – 50% Dog food, (4) 25% Manure – 75% Dog food, (5) 0% Manure – 100% Dog food. Dairy manure and the dog food pellets were prepared in the same fashion as they were for the semi-continuous studies, as detailed in Section 3.5.1.2. Also, no external nutrients were added in an attempt to replicate the conditions existent in the semi-continuous studies.

3.4.2.2. Biomethane potential and biodegradability fraction

The biomethane potential was evaluated in terms of the specific biomethane yield (SMY), i.e. the total volume of biomethane produced during the digestion period divided by the amount of substrate initially added to the reactor (i.e. mL CH₄/g VS added). The extent of substrate biodegradability is directly related to the SMY, and in this study it was determined in one of two ways. For substrates where chemical
composition was either known (i.e. pure biomolecules) or determined analytically during this study, the biodegradability fraction was determined by the ratio of observed to ultimate (theoretical) SMY (Eq. 3-1):

\[ f_D = \frac{B_o}{B_u} \]  

where, \( f_D \) is the substrate biodegradable fraction (decimal) and \( B_o \) and \( B_u \) correspond to the observed and ultimate SMY (mL CH\(_4\) per g VS added), respectively. \( B_u \) was determined directly from the BMP assay whereas \( B_o \) was calculated using the Buswell Formula, because as compared to other methods, such as the one developed by McCarty using bioenergetics (McCarty, 1972), the Buswell Formula produces results closer to observed biomethane values after correcting for substrate biodegradability (see Chapter 2). For all lignocellulosic substrates (except for dairy manure), which only fiber composition was known, as determined in this study, \( f_D \) was obtained by the ratio of degradable to total chemical oxygen demand (COD) (Eq. 3-2):

\[ f_D = \frac{COD_D}{COD_T} \]  

where, \( COD_D \) is the degradable chemical oxygen demand (g/L), and \( COD_T \) is the total chemical oxygen demand (g/L). \( COD_D \) was calculated from the theoretical 350 mL of biomethane (at STP) per g of COD stabilized (McCarty, 1964a) and \( B_o \), which was obtained from the BMP assay. \( COD_T \) was determined analytically for each lignocellulosic substrate.
3.4.2.3. Biochemical methane potential assay

The biochemical methane potential assay protocol used in this study was based on the principles described by Owen et al. (1979) and revised by others (Chynoweth et al., 1993; Owens & Chynoweth, 1993; Hansen et al., 2004; Angelidaki et al., 2009). A step-by-step protocol for setting-up the BMP assay used in this study is included in the appendix; a brief description of this procedure is as follows. Known amounts of substrate and a previously prepared mixture of active anaerobic inoculum and nutrient/trace element solution, as described below, were added to either 160-mL or 250-mL serum bottles. pH was measured, and bottles were gassed with pure nitrogen and sealed immediately using rubber septa and aluminum crimp caps. Two identical sets of bottles were prepared; each set was placed in a separate incubator and maintained at either mesophilic (37°C) or thermophilic (55°C) temperature conditions. Throughout the digestion period, incubators were maintained within 1°C of the corresponding operating temperature. In all BMP assays, each sample bottle was evaluated in duplicates or triplicates, and two additional bottles containing only the inoculum/nutrient mixture were included to correct for endogenous biomethane production (subtracted form the sample bottles). Mixing was performed continuously via an orbital shaker throughout the incubation period. As mentioned above, the duration of the BMP assay was specific for each substrate, and determined as the biogas production rate approached zero.
Assay parameters

As recommended in Chapter 2, an inoculum-to-substrate ratio of 0.5 (VS basis) was used in this study to maximize degradation rates and ensure that the full extent of biomethane potential was achieved. A total volume of 50 mL was used in all BMP bottles, and included, the sample, nutrient medium, and inoculum. To minimize the possibility of process inhibition, in all the BMP assays, except for lipids, the sample concentration was set to 1.5 g VS/L, which was half of the concentration used in previous BMP studies conducted by the authors (Chapter 2). Concentrations as low as 0.2 g/L of oleic acid have been reported to be inhibitory of methanogenesis in batch tests at thermophilic conditions. Therefore, a concentration of 0.1 g VS/L was used for all samples of pure triglycerides, and for the equally-balanced mixture of triglycerides described above, three concentrations were evaluated, i.e. 0.3, 0.9, and 1.8 g VS/L.

Inoculum source and nutrients supplement

The inoculum for all BMP assays was obtained from two lab-scale continuously-stirred anaerobic digesters (CSADs) operated at either operating temperature. The CSADs were acclimated to degrading a complex substrate consisting of the co-digestion of manure and a balanced mixture of carbohydrates, proteins and lipids (see semi-continuous studies, below). The inoculum was harvested from the supernatant of each digester’s effluent after 24 hours of quiescent settling. In addition, a medium
solution, containing essential nutrients, trace elements, and vitamins, was included in all BMP assays, but in the co-digestion experiments for the reasons stated previously.

The medium formulation used in this study is presented in Table 3-2.

Table 3-2. Nutrient and trace element concentrations used in the BMP assays

<table>
<thead>
<tr>
<th>Basal medium</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>200</td>
</tr>
<tr>
<td>KCl</td>
<td>100</td>
</tr>
<tr>
<td>MgCl₂ 6H₂O</td>
<td>600</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>138</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>176</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td>100</td>
</tr>
<tr>
<td><strong>Trace elements</strong></td>
<td></td>
</tr>
<tr>
<td>FeCl₃ 6H₂O</td>
<td>200</td>
</tr>
<tr>
<td>MnCl₂ 4H₂O</td>
<td>4</td>
</tr>
<tr>
<td>CoCl₂ 6H₂O</td>
<td>10</td>
</tr>
<tr>
<td>NiCl₂ 6H₂O</td>
<td>10</td>
</tr>
<tr>
<td>ZnCl₂ 2H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>Na₂SeO₃</td>
<td>0.1</td>
</tr>
<tr>
<td>Na₂MoO₄ 2H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>CaCl₂ 2H₂O</td>
<td>100</td>
</tr>
<tr>
<td>CuCl₂ 2H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>KI</td>
<td>10</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.5</td>
</tr>
<tr>
<td>Na₂S 9H₂O</td>
<td>100</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
</tr>
<tr>
<td>Resazurin</td>
<td>1</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>4200</td>
</tr>
</tbody>
</table>

Biogas production measurement
Biogas production was measured via gage pressure transducers (Model PX26, Omega Engineering, Inc.), which were attached to hypodermic needles and inserted through the bottle’s rubber septa in a procedure comparable to that described in Chapter 2. Cumulative pressure inside the bottles was measured continuously during the entire BMP assay using a data acquisition (DAQ) system interfaced with a computer, and controlled via LabVIEW® (National Instruments Co., Austin, TX). In addition, a pressure-control bottle containing the equivalent volume of sample replaced by tap water was continuously measured to account for abiotic, external pressure variations due to temperature and atmospheric pressure changes. Similarly, temperature was monitored through copper-constantan thermocouples measuring gas-phase temperature changes in tap water-containing bottles. Pressure data gathered by the DAQ system were converted to volume of biogas at standard temperature and pressure (STP), according to the ideal law of gases. Biogas production obtained using this method was validated repeated times by measuring the biogas volume directly from the bottles using the syringe technique as described by (Owen et al., 1979).

3.4.3. Semi-continuous studies

Two identical CSADs with a working volume of 4.5 L were operated at either mesophilic (37°C) or thermophilic (55°C) thermal conditions, and fed semi-continuously (see Section 3.5.1.1). Two influent substrate compositions, representing both upper and lower manure-to-dog food ratios, and three different hydraulic
Retention times (HRTs) were evaluated in both CSADs throughout the course of the study. Our experimental design combined these variables to produce a total of four different operating periods for the study. Each period (P), indicating its duration, influent substrate composition, and HRT, for each reactor, is presented in Table 3-3. The temperature of the CSADs was maintained within 1°C through built-in water jackets and heated water recirculators (PolyScience, Model 210). Also, each CSAD was sealed with an air-tight head-plate where the influent, effluent and biogas lines were located, and a biogas sample port and temperature probe were also in place.

Table 3-3. Summary of the operating conditions for each CSAD during the four periods (P) evaluated in this study

<table>
<thead>
<tr>
<th>Period (P)</th>
<th>Days</th>
<th>HRT (d)</th>
<th>Composition (% VS basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mesophilic CSAD</td>
</tr>
<tr>
<td>Start-up</td>
<td>0 – 62</td>
<td>0 – 62</td>
<td>30</td>
</tr>
<tr>
<td>P-1</td>
<td>63 – 330</td>
<td>63 – 330</td>
<td>20</td>
</tr>
<tr>
<td>P-2</td>
<td>331 – 430</td>
<td>331 – 360</td>
<td>15</td>
</tr>
<tr>
<td>P-3</td>
<td>431 – 498</td>
<td>361 – 498</td>
<td>15</td>
</tr>
<tr>
<td>P-4</td>
<td>499 – 544</td>
<td>499 – 544</td>
<td>10</td>
</tr>
</tbody>
</table>

3.4.3.1. CSAD’s operation and start up

Reactors were fed manually every 48±1 h by first withdrawing a known amount of effluent (calculated according to the reactors’ volume and current HRT) and then adding the same amount of the prepared feed. Biogas production was continuously measured in each reactor via gas meters (Actaris Meterfabriek bv, Delft, The
Netherlands) and recorded every 48-h cycle, right after feeding. Methane and carbon dioxide content in biogas was measured periodically using a gas chromatograph as described below. pH was measured with the same frequency from the withdrawn effluent using a single-reference electrode (Thermo Fisher Scientific, Inc.). Gas data were corrected for temperature and pressure variations and reported at standard conditions (0°C and 1 atm). Each reactor was continuously stirred with a mechanical mixer (Model 5vb, EMI, Inc., Clinton, CT) using a 62-mm diameter axial flow impeller (Lightnin A-310, Rochester, NY). A more complete description of the reactors experimental setup and operation is presented in Chapter 4.

The inoculum used to start the anaerobic digesters was obtained from a farm-based mesophilic completely-mixed anaerobic digester (Ridgeline Dairy Farm, Clymer, NY) operated at a 20-day hydraulic retention time (HRT), which co-digested raw dairy manure with an array of high strength food residues (i.e. milk, ice cream, grapes, and salad dressing) at the time of sampling (Pronto & Gooch, 2008). The inoculum was harvested from the supernatant of the CSAD’s effluent after 24 hours of quiescent settling.

3.4.3.2. Substrate feed

The anaerobic digestion of complex, particulate substrates was represented by the co-digestion of dairy manure with dog food dry pellets. Rumensin® free dairy manure mixed with urine was collected at the beginning of the study from the influent pit of a
dairy farm anaerobic digester treating the daily waste of 600 cows (AA Dairy, Candor, NY). The raw manure was homogenized, blended and initial analyses were performed before storing it in individual 1-L containers and maintained at -20°C throughout the study. Only at the beginning of the operating condition P-3, was a new batch of raw manure from the same dairy farm used – a fact with no implications to the results of the study, as discussed in Chapter 4.

Dog food was used in this study because its characteristics approach the multi-component chemical composition of a generic food residue and it is also reproducible and stable over time. The pelleted dry dog food from Science Diet (Hill Pet Nutrition, Inc.) was specifically selected because it contains a certified, well balanced mixture of carbohydrates, lipids, and proteins. The pellets were ground in an industrial blender and sieved afterwards to produce a feed particle size in the range of 1 – 2 mm.

Dairy manure and dog food were mixed at two different ratios to produce an influent of either 25% manure and 75% dog food or 75% manure and 25% dog food (VS basis) depending on the period (see Table 3-3). The overall influent volatile solids concentration of the combination of these two substrates was set to be constant at 30 g VS/L during the study, mainly to prevent solids settling problems and avoid ammonia inhibition throughout the three increasing organic loading rates (shorter HRTs). Raw dairy manure (ca. 100 g VS/L) was diluted with tap water prior to
feeding to achieve the required VS concentration and influent volume when combined with dry dog food.

3.4.3.3. Steady-state conditions

A reactor was considered to be at steady state only after two criteria were met: (1) it had been operated for a period of time equal to three times the hydraulic retention time (HRT) being evaluated, and (2) the volumetric biogas production rate (L/L-d) was stable and had not varied more than 10% for the last HRT. Once at steady-state conditions, a comprehensive set of samples and measurements was obtained from each digester to determine final, steady-state parameters

3.4.4. Analytical methods

Methane and carbon dioxide content in biogas was measured using a SRI 8610C (SRI Instruments, Torrance, CA) Gas Chromatograph (GC) equipped with a thermal conductivity detector (TCD) and a flame photometric detector (FPD), using Helium as a carrier gas in a 1-m Rtx-XLSulfur® packed column and a ramped temperature program. Calibration of the GC was conducted periodically with standard mixtures of methane and carbon dioxide (Airgas, Inc.).

pH was measured using a single-reference electrode (Thermo Fisher Scientific, Inc.). Total solids (TS) and volatile solids (VS) were determined according to Standard Methods, sections 2540B and 2540E, respectively (APHA, 1995). Chemical oxygen demand (COD) was determined according to the closed reflux titrimetric method, as
described in section 5220C of Standard Methods (APHA, 1995). Total volatile fatty acids were measured by the distillation method as described by the Standard Methods (APHA, 1995). Similarly, individual VFAs were determined with a HP Agilent GC model 5890 equipped with a flame ionization detector (FID), using helium as a carrier gas in a NUKOL® capillary column, and following the program described in the appendix. A commercially prepared 10 mM volatile fatty acids (VFA) standard mixture, containing, acetic (C2), propionic (C3), isobutyric (C4), butyric (C4), isovaleric (C5), valeric (C5), isocaproic (C6), caproic (C6), and enanthic (C7) acids, was obtained from Sigma Aldrich Co. Long chain fatty acids (LCFA) were determined using the same GC setup but following a different temperature program and sampling preparation adapted from Neves et al. (2009), as described in the appendix. The LCFA stock solution was prepared in our lab using dichloromethane as a solvent, and a mixture of capric (C10), lauric (C12), myristic (C14), palmitic (C16), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids as standards, and petadecanoic acid (C15) as an internal standard (IS) – all reagents were HPLC grade and obtained from Sigma-Aldrich Co.

Total Kjeldahl nitrogen (TKN) concentration was determined according to the Standard Methods (APHA, 1995). Total ammonia-N (TAN) concentration was measured using an ion selective electrode (Thermo Fisher Scientific, Inc.). Total organic nitrogen (TON) was calculated by subtracting TAN from TKN.
protein content for solid or semi-solid samples (TS > 10%) was determined from the TON data and based on the assumption that an average protein contains 16% organic N, i.e. TON/0.16. For liquid samples (TS < 10%), which included all digester effluent samples, total protein content was determined according to the method of Lowry (Lowry et al., 1951), using bovine serum albumin (BSA) as a protein standard, which was obtained from Sigma-Aldrich Co. Neutral lipids were determined according to method of Loehr and Rohlich (1962) for wastewaters.

Hemicellulose, cellulose, and lignin content were determined according to the neutral detergent fiber (NDF) and acid detergent fiber and lignin (ADF/ADL) analyses methods adapted from Mertens et al. (2002) and Moller (2009), respectively. Total carbohydrates were determined using the method of Gaudy (1962). Non-lignocellulosic carbohydrates (e.g. sugars, starch, pectin) were determined by subtracting NDF from the total carbohydrates measurement. Detailed protocols of the analytical procedures used in this study, including additional information about the equipment employed, are found in the appendix.

3.5. Results and discussion

3.5.1. Batch Studies

3.5.1.1. Degradation of carbohydrates

The observed specific biomethane yields \( (B_o) \) of the six pure carbohydrates evaluated in this study are shown in Figure 3-1. The theoretical specific biomethane yields \( (B_t) \)
were calculated using the Buswell Formula, and the following molecular formulae: \((C_6H_{10}O_5)_n\) for cellulose and starch, \((C_5H_{8}O_4)_n\) for xylan, \(C_{12}H_{22}O_{11}\) for cellobiose, and \(C_6H_{12}O_6\) for glucose. Details of the theoretical specific biomethane yield calculation using the Buswell Formula are found in the appendix.

![Graph showing specific biomethane yield of carbohydrate components at each thermal regime](image)

**Figure 3-1.** Observed specific biomethane yield of carbohydrate components at each thermal regime. Black bars represent the theoretical specific biomethane yields, calculated using the Buswell Formula. Error bars represent the standard deviation of the replicates. Values are reported at STP conditions.

Results indicate that pure microcrystalline cellulose was fairly degradable under both thermal conditions. While 80% (i.e. \(f_D = 0.80\)) of the initial amount of cellulose was degraded under mesophilic conditions, 87% was degraded at thermophilic. These results are within the range of the values reported in the literature. Tong et al. (1990)
reported 89% and 90% biodegradability under mesophilic conditions for Whatman no. 5 filter paper and Solka floc BW200, respectively. On the other hand, Wang et al. (1994) observed 84% biodegradability when added cellulose spikes in mesophilic BMP studies, whereas Stinson and Ham (1995) reported 88% cellulose destruction under the same thermal conditions.

In long-term batch digestion tests, Symons and Buswell (1933) observed xylose degradation to be lower at mesophilic temperatures compared to thermophilic (i.e. 86% vs. 96%, respectively). Although, the extent of degradation of xylan found in this study was higher at thermophilic temperatures, $f_D$, were considerable lower at both thermal regimes compared to those reported in the former study, i.e. 53% and 66% under mesophilic and thermophilic temperatures, respectively. This could be partially explained by the fact that in our study we used xylan as the substrate, whereas the former authors used xylose, i.e. xylan had to be hydrolyzed to sugars ($\geq90\%$ xylose, see Table 3-1) prior to being fermented.

Biodegradability of starch was comparable to that of xylan in this study. A 46% biodegradability was observed at mesophilic conditions, whereas a 65% was attained at thermophilic. In this case, however, Symons and Buswell (1933) observed a significantly higher biodegradability at both thermal regimes, i.e. 95% at mesophilic and 80% at thermophilic. The reasons for these differences are not obvious, especially considering the high repeatability found in these samples for both thermal
regimes (n = 3, SD = 0.5% at mesophilic conditions; n = 3, SD = 2.9% at thermophilic conditions).

As expected, cellobiose was highly degradable at both thermal regimes (i.e. 92% at mesophilic; 86% at thermophilic). Similarly, in our study, 87% of the added glucose was degraded at mesophilic conditions and 84% at thermophilic. This agrees well with the 90% degradation reported in other studies (Tong, 1990; Chen and Hashimoto, 1996).

3.5.1.2. The effect of lignin on the extent of biodegradability of structural carbohydrates

The results of the fiber analyses conducted on all six lignocellulosic substrates, following the neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) procedures, are presented in Table 3-4. Results confirmed varied amounts of lignin as well as great diversity in the lignocellulosic fiber composition. Dairy manure exhibited not only the highest content of lignin but also the greatest proportion of lignin to other fiber components, as evidenced by the ratios shown in Table 3-4. Over 70% of the VS of grasses, i.e. switchgrass, wheat straw, and miscanthus, consisted of fiber, whereas 46% in corn silage and only 11% in red onions. More than 85% VS in onions are carbohydrates, from which 45% consists of sugars (USDA, 2009). On the other hand, corn silage VS is composed of 87% of carbohydrates, where 40% consist of starch (Mertens et al., 2002).
A good linear correlation was observed between the lignin content of the various lignocellulosic materials and the anaerobic biodegradability fraction and specific biomethane yield at mesophilic thermal conditions (Figure 3-2). The $f_D$ and $B_o$ prediction models based on the lignin content of the lignocellulosic material at mesophilic conditions are, respectively:

Eq. 3-3 \[ f_D = 0.822 - 2.743 \cdot \text{Lignin} \quad (SE = 0.018; \ R^2 = 0.99) \]

Eq. 3-4 \[ B_o = 325.9 - 784.3 \cdot \text{Lignin} \quad (SE = 9.2; \ R^2 = 0.95) \]

Based on Eq. 3-3, the maximum degradable fraction of lignocellulosic substrates under mesophilic conditions, which is obtained when the lignin content is set to zero, is 0.82. This value compares well to that obtained for pure cellulose in this study, i.e. 0.80±0.09. Likewise, the intercept of Eq. 3-4 reveals that the specific biomethane yield would be 326 mL per g VS added, which is also in good agreement with the $B_o$ obtained for pure cellulose, i.e. 331±38 mL CH$_4$ per g VS added. The narrow confidence intervals at a 95% level, high coefficient of determination ($R^2$), and small standard error (SE), suggest that both models produce results with relatively good precision. This is especially true considering that the standard deviations (SD) of the lignocellulosic materials are, in general, greater than the SE produced by the models, as seen in the summary table (Table 3-5). In other words, the variability of $f_D$ and $B_o$ when determined using the BMP assay may be higher than the error introduced by the model predictions.
At thermophilic conditions, a considerably lower linear correlation between the lignin content of the lignocellulosic materials and their anaerobic biodegradability extent and specific biomethane yield, was found (Figure 3-3). The $f_D$ and $B_o$ prediction models as a function of the lignin content of the lignocellulosic material at thermophilic conditions are, respectively:

Eq. 3-5  \[ f_D = 0.853 - 3.221 \cdot \text{Lignin} \quad (SE = 0.075; R^2 = 0.84) \]

Eq. 3-6  \[ B_o = 338.6 - 998.8 \cdot \text{Lignin} \quad (SE = 28.3; R^2 = 0.78) \]

The relatively higher dispersion of the thermophilic-obtained data is apparent when compared to those obtained at mesophilic temperatures. This is evidenced by the wider confidence intervals exhibited by the thermophilic data (Figure 3-3), which implies that there is a greater degree of variability associated with the predictions obtained using above models. However, a close observation of the data suggests that the substrate, which deviates more notably from the set, is miscanthus. In fact, if this substrate is excluded from the data set, a coefficient of determination ($R^2$) of 0.97 and 0.90 is achieved for $f_D$ and $B_o$, respectively. However, no apparent reasons for the deviation of this substrate were found, and therefore miscanthus data were not excluded for the calculation of Eq. 3-5 and Eq. 3-6. Despite this, the biodegradability fraction, as indicated by the intercept of Eq. 3-5 (i.e. for a lignin-free substrate), is 0.85, which compares well with the $f_D$ of pure cellulose found in this study at thermophilic temperatures, i.e. 0.87±0.06. However, due to the large confidence
limits observed for these data, there is no way to know how accurate the slope of the model is in describing the data. Furthermore, the specific biomethane yield prediction model (Eq. 3-6) shows an even greater difference between the intercept, i.e. 339 mL CH$_4$ per g VS added, and the $B_0$ of pure cellulose, i.e. 359±26 mL CH$_4$ per g VS added. This indicates not only that the intercept of the model, but also the slope, are considerably deviated, which could lead to significant errors in the calculations. This is confirmed by the large SE reported for this equation, i.e. 28.3 mL CH$_4$ per g VS added.

A close look at the slope of the $f_D$ prediction models of both thermal regimes suggests that biodegradability of lignocellulosic materials is more adversely affected by the lignin content at thermophilic temperatures than at mesophilic. Furthermore, it appears that lignocellulosic substrates with a lignin content below about 8% (VS basis) would be more degradable at thermophilic conditions, whereas at higher levels of lignin, lignocellulosic substrates would be more degradable at mesophilic. Although this hypothesis might be hampered by the confidence limits of the thermophilic model, the actual data support this hypothesis. When we compare the extent of biodegradability of lignin-free cellulose and hemicellulose (Figure 3-1), and the two substrates with the lowest lignin content (<8% VS basis), i.e. red onions and corn silage (Table 3-4), at both thermal conditions, it is apparent that higher degradabilities are produced at thermophilic conditions. However, the two lignocellulosic substrates
with a >8% VS lignin content, i.e. switchgrass and dairy manure (Table 3-4), are more degradable at mesophilic conditions. Because the thermophilic prediction model also has a steeper slope, it is possible that the same occurs for $B_o$; however, it is more difficult to draw conclusions in this case, because of the dispersion of the thermophilic data.

3.5.1.3. Lignin as a predictor of $f_D$ and $B_o$

Additional predictors of biodegradability fraction and specific biomethane yield were assessed using the mesophilic data set (data not shown). A linear correlation with a $R^2 \geq 0.90$ was observed between $f_D$ (or $B_o$) and the ratio of lignin to other fiber components (e.g. lignin/NDF, lignin/cellulose, lignin/hemicelluloses) only when dairy manure was excluded from the data set. When correlated with above ratios, dairy manure was a clear outlier, apparently due to its higher lignin content relative to the other fiber components (see Table 3-4). The most plausible explanation comes from the determination of lignin in itself (i.e. ADL method), which relies on a cationic detergent solution and strong acidic conditions ($72\% \text{ H}_2\text{SO}_4$) to remove acid-labile carbohydrates, lipids, and proteins. However, it is impossible to remove proteins completely, as it has been reported that a variable small amount of insoluble/indigestible nitrogen ($\geq 1.5\%$), which is assumed to be mostly in the form of protein, can be found bound to ADL lignin (Van Soest, 1982).
Table 3-4. Results of the fiber analysis conducted in the lignocellulosic substrates evaluated in this study; results reported in g/g VS

<table>
<thead>
<tr>
<th></th>
<th>Cow manure</th>
<th>Red onions*</th>
<th>Switchgrass</th>
<th>Wheat straw</th>
<th>Miscanthus</th>
<th>Corn silage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase-treated neutral detergent fiber (aNDF)</td>
<td>0.560</td>
<td>0.105</td>
<td>0.830</td>
<td>0.822</td>
<td>0.726</td>
<td>0.460</td>
</tr>
<tr>
<td>Acid detergent fiber (ADF)</td>
<td>0.464</td>
<td>0.125</td>
<td>0.579</td>
<td>0.579</td>
<td>0.503</td>
<td>0.301</td>
</tr>
<tr>
<td>Acid detergent lignin (ADL)</td>
<td>0.138</td>
<td>0.004</td>
<td>0.098</td>
<td>0.056</td>
<td>0.078</td>
<td>0.029</td>
</tr>
<tr>
<td>Hemicellulose (aNDF-ADF)</td>
<td>0.096</td>
<td>0.000</td>
<td>0.251</td>
<td>0.243</td>
<td>0.223</td>
<td>0.159</td>
</tr>
<tr>
<td>Cellulose (ADF-ADL)</td>
<td>0.326</td>
<td>0.121</td>
<td>0.481</td>
<td>0.523</td>
<td>0.425</td>
<td>0.272</td>
</tr>
<tr>
<td>Lignin (ADL)</td>
<td>0.138</td>
<td>0.004</td>
<td>0.098</td>
<td>0.056</td>
<td>0.078</td>
<td>0.029</td>
</tr>
<tr>
<td>Lignin/hemicellulose</td>
<td>1.44</td>
<td>-</td>
<td>0.39</td>
<td>0.23</td>
<td>0.35</td>
<td>0.18</td>
</tr>
<tr>
<td>Lignin/cellulose</td>
<td>0.42</td>
<td>0.03</td>
<td>0.20</td>
<td>0.11</td>
<td>0.18</td>
<td>0.11</td>
</tr>
<tr>
<td>Lignin/(cellulose+hemicellulose)</td>
<td>0.33</td>
<td>0.03</td>
<td>0.13</td>
<td>0.07</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>Lignin/(cellulose+hemicellulose+lignin)</td>
<td>0.25</td>
<td>0.03</td>
<td>0.12</td>
<td>0.07</td>
<td>0.11</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*Based on aNDF-ADF, hemicellulose is negative; however, it was assumed to be zero, see text
### Table 3-5. Summary of the observed specific biomethane yields ($B_o$) and biodegradable fractions ($f_D$) of pure biomolecules obtained in the BMP assays

<table>
<thead>
<tr>
<th>Carbohydrates</th>
<th>Theoretical ($B_o$)</th>
<th>Mesophilic</th>
<th>SD</th>
<th>Thermophilic</th>
<th>SD</th>
<th>Mesophilic</th>
<th>SD</th>
<th>Thermophilic</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>414.5</td>
<td>331.3</td>
<td>37.9</td>
<td>359.3</td>
<td>26.2</td>
<td>0.80</td>
<td>0.09</td>
<td>0.87</td>
<td>0.06</td>
</tr>
<tr>
<td>Hemicellulose, Xylan</td>
<td>423.9</td>
<td>222.6</td>
<td>49.9</td>
<td>279.1</td>
<td>31.5</td>
<td>0.53</td>
<td>0.12</td>
<td>0.66</td>
<td>0.07</td>
</tr>
<tr>
<td>Starch</td>
<td>414.5</td>
<td>191.8</td>
<td>2.1</td>
<td>268.8</td>
<td>12.1</td>
<td>0.46</td>
<td>0.01</td>
<td>0.65</td>
<td>0.03</td>
</tr>
<tr>
<td>D(+) - Cellobiose</td>
<td>392.6</td>
<td>359.3</td>
<td>63.2</td>
<td>338.9</td>
<td>40.4</td>
<td>0.92</td>
<td>0.16</td>
<td>0.86</td>
<td>0.10</td>
</tr>
<tr>
<td>D(+) - Glucose</td>
<td>373.0</td>
<td>323.9</td>
<td>22.6</td>
<td>313.9</td>
<td>3.3</td>
<td>0.87</td>
<td>0.06</td>
<td>0.84</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proteins</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>460.4</td>
<td>407.3</td>
<td>31.1</td>
<td>375.5</td>
<td>9.0</td>
<td>0.88</td>
<td>0.07</td>
<td>0.82</td>
<td>0.02</td>
</tr>
<tr>
<td>Albumin</td>
<td>462.7</td>
<td>390.0</td>
<td>36.9</td>
<td>354.6</td>
<td>15.7</td>
<td>0.84</td>
<td>0.08</td>
<td>0.77</td>
<td>0.03</td>
</tr>
<tr>
<td>Collagen</td>
<td>396.9</td>
<td>302.5</td>
<td>32.0</td>
<td>353.9</td>
<td>4.1</td>
<td>0.76</td>
<td>0.08</td>
<td>0.89</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lipids*</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol tripalmitate</td>
<td>1005.8</td>
<td>915.0</td>
<td>-</td>
<td>655.7</td>
<td>37.7</td>
<td>0.91</td>
<td>-</td>
<td>0.65</td>
<td>0.04</td>
</tr>
<tr>
<td>Glycerol tristearate</td>
<td>1023.9</td>
<td>903.9</td>
<td>-</td>
<td>1056.6</td>
<td>-</td>
<td>0.88</td>
<td>-</td>
<td>1.03</td>
<td>-</td>
</tr>
<tr>
<td>Glycerol trioleate</td>
<td>1011.9</td>
<td>1101.2</td>
<td>-</td>
<td>919.6</td>
<td>72.5</td>
<td>1.09</td>
<td>-</td>
<td>0.91</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*Blank spaces in SD due to replicates of some lipid samples that did not produce biogas
Figure 3-2. Correlation between acid detergent lignin (ADL) content of six different lignocellulosic materials and their (A) anaerobic biodegradability fraction and (B) observed specific biomethane yield (SMY), at mesophilic thermal conditions. The solid line represents the line of perfect fit and segmented lines set the limits of the confidence intervals at a 95% level.
Figure 3-3. Correlation between acid detergent lignin (ADL) content of six different lignocellulosic materials and their (A) anaerobic biodegradability fraction and (B) observed specific biomethane yield (SMY), at thermophilic thermal conditions. The solid line represents the line of perfect fit and segmented lines set the limits of the confidence intervals at a 95% level.
The content of lignin-bound indigestible protein can be higher in dairy manure, because of the presence of Maillard nitrogen, tannin-bound nitrogen and keratins of animal origin (Van Soest, 1982). This can be especially true in the case of the dairy manure used in this study, which exhibited a total protein content of 14.9% VS basis (see Table 3-6). As a result, the ADL content of the dairy manure determined in this study (i.e. 13.8% VS basis) could have been overestimated by at least 1.5% as a result of indigestible protein cross-linked with lignin. The true ADL content of dairy manure could have been determined by measuring the protein content of the ADL residue; however, this was not done in the present study. For all practical purposes, using the fraction of lignin of the entire substrate VS (i.e. not only the fiber fraction) can circumvent this issue, because the substrate VS not only includes the lignocellulosic matrix, but also the lignin-bound proteins, whose biodegradability is also dependent on the lignin content. That is probably the cause of why the fraction of lignin with respect to the entire substrate VS appears to be a better predictor of biodegradability and biomethane yields than the ratio of lignin to lignocellulosic components. Finally, the robustness of lignin as a single predictor of biodegradability is further validated when considering the diverse composition of the lignocellulosic substrates tested in this study.
3.5.1.4. Degradation of proteins

The observed specific biomethane yields \((B_s)\) of the three proteins evaluated in this study are shown in Figure 3-4. The theoretical specific biomethane yields \((B_t)\) for these proteins were also calculated using the Buswell Formula. In this case, the molecular formula of each protein was derived from its specific amino acid composition. The amino acid composition of casein was obtained from the study of Ramsay and Pullammanappallil (2001), from which the resultant molecular formula was \(C_{5.21}H_{0.92}O_{2.67}N_{1.21}S_{0.03}\). For egg albumin, the amino acid composition reported by Lewis et al. (1950) was used, and the derived molecular formula was \(C_{5.24}H_{10.13}O_{2.62}N_{1.27}S_{0.05}\). Finally, the amino acid composition of collagen was obtained from Eastoe (1955) which resulted in the formula \(C_{3.67}H_{7.49}O_{2.13}N_{1.16}S_{0.01}\).

As expected, both globular proteins were highly degradable at both thermal regimes. The mesophilic digester, however, showed a slight edge on extent of degradation. Although the specific biomethane yield of collagen is lower than that of globular proteins, it was more degradable despite being a structural, fibrous protein. In this case, the thermophilic digester showed a higher specific biomethane yield (and thus biodegradability).
Figure 3-4. Observed specific biomethane yield (SMY) of three types of proteins at mesophilic and thermophilic conditions. Black bars represent the theoretical specific biomethane yields, calculated using the Buswell Formula. Error bars represent the standard deviation of the replicates. Values are reported at STP conditions.

3.5.1.5. Degradation of lipids

The observed specific biomethane yields of the pure triglycerides evaluated in this study are shown in Figure 3-5. In the particular case of lipids, significant variability in the data was observed among the replicates. Some replicates produced a significantly lower amount of biogas, while others did not observe any biogas production during the entire digestion time. This could, in part, be attributed to poor homogenization of the samples, which would result in a decrease of substrate availability for enzymatic
hydrolysis. Indeed, to prevent this issue, previous studies have homogenized/emulsified the lipid-containing samples using ultrasonification prior to digestion (Angelidaki & Ahring, 1992). On the other hand, the small amount of fatty acid required for the sample concentrations used in this study (i.e. 5 – 30 μg), could have also contributed to some differences, as suggested by some replicates where $B_w$ was greater than $B_u$. Finally, free long-chain fatty acid inhibition may also have resulted in lower (or no) biogas yields in some samples as widely reported in the literature (Angelidaki & Ahring, 1992; Angelidaki et al., 1990; Lalman & Bagley, 2002; Lalman & Bagley, 2000; Neves et al., 2009).

Regardless of the data variability, overall results agree with the literature. With the exception of glycerol tripalmitate (GTP) at thermophilic conditions, all pure triglycerides were almost fully degraded at both thermal regimes (Figure 3-5). In batch degradation experiments using C$^{14}$ tracers, Jeris and McCarty (1965) found that 89% of palmitic acid was recovered as biogas at mesophilic conditions. Similarly, in our experiments, 91% of glycerol tripalmitate was degraded at mesophilic conditions (Figure 3-5, Table 3-5). Furthermore, in batch experiments at thermophilic conditions, Angelidaki et al. (1990) found that ca. 90% of GTO at a concentration of 1.5 g/L, or lower, was degraded. Novak and Carlson (1970) conducted comprehensive mesophilic degradation experiments under completely-mixed, continuous conditions with an array of several fatty acids. They found that free long-
Chain fatty acids were completely biodegradable as long as enough retention time was provided. Similarly, O'Rourke (1968) in continuous studies, showed that neutral lipids were also fully degraded at mesophilic temperatures and a minimum hydraulic retention time of 35 – 40 days. It is not apparent why GTP at thermophilic temperatures produced only 66% of its theoretical biomethane potential. Since both replicates were degraded fairly equally, it is possible that in this case it was indeed due to process inhibition, rather than a difference in substrate availability.

Figure 3-5. Observed specific biomethane yield of three triacylglycerols at mesophilic and thermophilic conditions. GTP: glycerol tripalmitate; GTS: glycerol tristearate; GTO: glycerol trioleate. Concentration of each triglyceride is 0.1 g/L. Black bars represent the theoretical specific biomethane yields, calculated using the Buswell Formula. Error bars represent the standard deviation of the replicates. Values are reported at STP conditions.
The results of the digestion of the triglyceride mixtures are presented in Figure 3-6. Results suggest that fatty acids are between 80 and 100% degradable at both thermal conditions, provided that concentrations are below 0.9 g/L. The large SD of the 0.3-g/L mesophilic sample is the result of one replicate producing only 65% of the theoretical biomethane yield, and a second producing 97%. Thus, it is likely that the replicate showing less biodegradability was the result of decreased fatty acids availability due to poor sample homogenization, rather than inhibition, as discussed above. However, at a concentration of 1.8 g/L, the degradation of the fatty acid mixture was apparently hindered by inhibition at both thermal regimes. In the case of the mesophilic sample, both replicates observed a similar biomethane yield after 90 days of incubation, which corresponded to a 57% average degradation. At thermophilic temperatures, one replicate observed a biomethane yield equivalent to 30% substrate degradation, while the other 88%, which resulted in an average of 59% biodegradability. To elucidate whether the long-chain fatty acid (LCFA) mixture was indeed inhibitory at 1.8 g/L we can look at the BMP curves shown in Figure 3-7. The plot shows the digestion of the LCFA mixture of one of the two replicates at the three concentrations\(^1\) (Figure 3-6 presents the average). It can be observed that the lag phase and degradation rate of the 0.3-and 0.9-g/L LCFA mixtures are appreciable

\(^1\) Only one replicate was connected to the continuous data acquisition system
lower at mesophilic conditions compared to thermophilic, which is expected due to the inherent faster kinetics at thermophilic temperatures. On the other hand, at both thermal regimes, the 1.8-g/L LCFA mixture (inverted triangles) presents a longer lag phase and apparent slower degradation rate compared to those observed at lower concentrations; thus, it is possible that this could be the result of LCFA inhibition. Although the effect seems to be more dramatic at thermophilic conditions, it is only apparent, since the second replicate of this sample produced 88% of its biomethane potential (see Figure 3-6). Thus, even though it is apparent that some degree of inhibition takes place at 1.8 g/L, it is not clear whether it affects both thermal regimes equally and to what extent. In the case of the CSAD studies, a significant drop in the biogas production was observed in the thermophilic digester when the LCFA concentration reached 2.1 g/L; an issue that was directly attributed to LCFA inhibition (see Chapter 4).
Figure 3-6. Observed specific biomethane yields at mesophilic and thermophilic conditions for the balanced mixture of GTP, GTS, and GTO at three different initial sample concentrations. Segmented line represents the theoretical specific biomethane yield calculated using the Buswell Formula. Error bars represent the standard deviation of the replicates. Values are reported at STP conditions. Only one replicate was available for the thermophilic sample of 0.3 g/L LCFA.
Figure 3-7. BMP curves showing the specific biomethane yields (at STP) for one of the replicates of the balanced mixture of GTP, GTS, and GTO at three initial sample concentrations; (A) mesophilic and (B) thermophilic. The average theoretical specific biomethane yield for the long-chain fatty acid (LCFA) mixture is 1,015 mL CH₄ per g VS added, as calculated using the Buswell Formula. Only 10 days of data are available for the thermophilic sample of 0.3 g/L LCFA due to leaking problems.
3.5.1.6. Degradation of complex substrate mixtures – the case of co-digestion

Results of the co-digestion trials are shown in Figure 3-8. Co-digestion ratios are in volatile solids (VS) basis. The theoretical specific biomethane yields for each co-digestion ratio were calculated using the Buswell Formula and the chemical composition of dairy manure and dog food, as determined analytically in this study, and which results are presented in the next section (Table 3-6).

A clear linear trend of increasing specific biomethane yields with the decrease of the manure-to-dog food ratio is observed. This is expected, not only because dog food has a higher $B_u$, but also because its chemical components are significantly more degradable than those of dairy manure, as seen in Table 3-6. The influence of the biodegradability fraction on the biomethane potential becomes apparent when we compare the $B_u$ of 100% manure and 100% dog food, which differ a bit more than 100 mL CH$_4$ per g VS added, but nearly 400 between $B_o$.

Based on the data, the calculated $f_D$ for dairy manure was 0.46 and 0.42 at mesophilic and thermophilic temperatures, respectively. For dog food, the $f_D$ was calculated to be 0.94 and 0.99 for the mesophilic and thermophilic regimes, respectively. Thus, dog food is virtually twice as degradable as manure. In fact, data suggest that for 50:50 or higher manure-to-dog food co-digestion ratios, higher biomethane yields are obtained at mesophilic relative to thermophilic conditions (Figure 3-8). Although the differences fall within the SD range obtained from the replicates, it is somewhat
expected that manure or any high-lignin content material would be more degradable, and produce higher $B_o$, at mesophilic conditions with respect to thermophilic; as discussed in the lignocellulosic analysis section, above.

In addition, the linear trend observed with the proportional change of manure to dog food suggests that no synergistic or antagonistic effects were produced for this co-digestion mixture. Specifically, a synergistic co-digestion mixture would observe a higher biomethane yield than the sum of the biomethane yields of the weighted individual contributions produced by the independent digestion of manure and dog food. Similarly, antagonism would reflect a lower biomethane yield in the co-digestion mixture as compared to the weighted biomethane yield of that mixture. Evidence of synergistic mixtures was observed in previous studies conducted by the author (see Chapter 2). Synergistic effects should specially be observed in manure-based co-digestion, because dairy manure contributes with alkalinity, trace elements, nutrients, and/or enzymes to the fermentation medium, which the co-substrate may lack. In fact, to evaluate possible synergistic effects, the co-digestion BMP trials purposely did not include nutrient solution in the media. However, the co-substrate used in this study, i.e. dog food, included vitamins and some nutrients in its composition, which may have helped pure dog food to reach its theoretical maximum biomethane yield.
3.5.2. Semi-continuous studies

3.5.2.1. Influent characterization

Results of the analyses to determine the chemical composition of dairy manure and dog food are shown in Table 3-6. In addition, the resulting influent compositions of the two co-digestion ratios evaluated in this study are also presented in Table 3-6 and graphically in Figure 3-9.
Table 3-6. Chemical composition of dairy manure, dog food and resulting influent for the two co-digestion ratios; values in g/100 g (VS basis)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Dairy manure (Mn)</th>
<th>Dog food (DF)</th>
<th>MnDF2575</th>
<th>MnDF7525</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>14.9</td>
<td>34.5</td>
<td>29.6</td>
<td>19.8</td>
</tr>
<tr>
<td>Lipids</td>
<td>4.7</td>
<td>30.8</td>
<td>24.3</td>
<td>11.2</td>
</tr>
<tr>
<td>Sugars, starch</td>
<td>24.4</td>
<td>29.9</td>
<td>28.5</td>
<td>25.8</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>9.6</td>
<td>-</td>
<td>2.4</td>
<td>7.2</td>
</tr>
<tr>
<td>Cellulose</td>
<td>32.6</td>
<td>4.8</td>
<td>11.7</td>
<td>25.6</td>
</tr>
<tr>
<td>Lignin</td>
<td>13.8</td>
<td>-</td>
<td>3.5</td>
<td>10.4</td>
</tr>
</tbody>
</table>

(A) MnDF2575

(B) MnDF7525

Figure 3-9. Substrate constituent contribution in the reactors influent feed at (A) 25:75 and (B) 75:25 manure-to-dog food co-digestion ratios
The differences between the two influent chemical characteristics are fairly apparent. At a 25:75 manure-to-dog food ratio, there is a well-balanced contribution between proteins, lipids, non-structural carbohydrates, and lignocellulosic carbohydrates. At a 75:25 manure-to-dog food ratio, the amount of lignocellulosic material is almost half of the influent substrate.

3.5.2.2. Stabilization of substrate constituents

The stabilization of the substrate constituents for the four operating periods (P) of the semi-continuous studies is shown in Figure 3-12 and Figure 3-13, and is discussed below.

In P-1, both reactors were maintained at 20 days HRT, corresponding to an organic loading rate (OLR) of 1.5 g VS/L-d, and a manure-to-dog food ratio of 25:75. While over 71% of the influent volatile solids were stabilized at mesophilic conditions, ca. 65% was under thermophilic. It is worth mentioning that the thermophilic reactor experienced a major upset during this period as a result of a short- and long-fatty acid imbalance (see Chapter 4). Although the reactor was considered to be at steady state and recovered for more than three HRTs, volatile fatty acid levels had started to accumulate by the end of this P, and were in the 1 g/L range when constituent measurements were taken. At mesophilic conditions, a high extent of degradation was observed for proteins, lipids and cellulose, and interestingly, more than 40% of the influent lignin was degraded at this thermal regime. However, only 52% of the
non-structural carbohydrates were degraded at mesophilic conditions. At thermophilic conditions, lipids, non-structural carbohydrates, and hemicelluloses appeared to be more degradable; however, proteins appeared to be fairly undegradable as compared to the mesophilic reactor, and almost no lignin was degraded at this thermal regime in P-1. Literature regarding lignin degradation is scarce; however, it has been demonstrated that lignin degradation is indeed possible under anaerobic conditions (Colberg, 1988). As early as 1933, Boruff and Buswell described the anaerobic fermentation of lignin, where apparently, ca. 50% of lignin was destroyed from cornstalks at a temperature between 25-30°C.

Only the mesophilic reactor reached steady state conditions of P-2; the thermophilic reactor crashed for a second time, again due to fatty acid imbalance, as detailed in Chapter 4. During this period, the mesophilic reactor OLR was increased to 2 g VS/L-d, which reduced the HRT to 15 days, while keeping the same co-digestion ratio as in P-1. By shortening the HRT in 5 days, the overall VS destruction was decreased by 10%. This overall decrease was mostly impacted by the destruction of proteins, which constituted 30% of the influent substrate and went from 80% degradation in P-1 to 66% in P-2. As expected, lignocellulosic components were highly impacted by the 15-d HRT. Data show that lignin degradation apparently stopped and accumulated in the digester, which consequently impacted hemicelluloses, cellulose, and probably, even protein degradation, which can be
associated with lignin (Van Soest, 1982). Although a decrease of lignin degradation is expected, accumulation is more difficult to explain. A possible explanation, however, may arise from the protein interference of the fiber analysis, which can be accounted as lignin (ADL) when lignified, especially in substrates containing indigested forage protein, such as those present in animal manure (see previous discussion).

In P-3, the manure content of the influent feed was increased to 75% (VS basis), which set the co-digestion ratio to 75:25. The HRT in both reactors was maintained at 15-d HRT. As expected, the increase of the manure content significantly impacted the overall degradation of both reactors, which VS destruction decreased to ca. 40%. With the change in influent composition, the fiber loading rate increased nearly 3-fold relative to P-2, i.e. from 0.35 to 0.86 g/L-d. Here, not only the degradation of the proteinaceous and lignocellulosic substrate components was affected, but also the lipid degradation, which stabilization relative to P-1 and P-2 decreased almost by half under both thermal regimes. This is certainly not expected, as the HRT was kept the same as in P-2; however, because the lipid concentration in the effluent at P-2 and P-3 was virtually the same for both reactors, as expected for no change in HRT (see Table 3-10), and the lipid content in the influent feed was decreased more than half, i.e. from 24% to 11% (Table 3-6), an apparent decrease in lipid degradation was expected to be seen. With respect to lignin, a small increase of degradation was observed in the mesophilic digester, which probably translated into the increase of hemicelluloses
destruction. On the other hand, at thermophilic conditions, 14% of the lignin was degraded and an increased amount of hemicellulose could be stabilized.

In the last period, P-4, the HRT was decreased from 15 to 10 days, while the influent co-digestion ratio was maintained in 75:25 manure-to-dog food. Interestingly, in this period the overall VS stabilization was improved under both thermal regimes, regardless of the shorter hydraulic retention time. As seen in Figure 3-12, in the mesophilic reactor, the greater substrate stabilization comes from the increase in the degradation of lignin, hemicelluloses, and ultimately, from the degradation of non-structural carbohydrates. A similar analysis can be done for the thermophilic reactor, which also increased its overall VS destruction by significantly increasing carbohydrate destruction, particularly lignin and non-structural carbohydrates. Given these results, it is possible that the increase in VS destruction in this period was the result of a steady acclimation of the microbial community to the increasing proportion of lignocellulosic material of the influent feed – or simply, the addition of a greater amount of lignocellulosic hydrolytic enzymes, coming from the increased volumes of manure, which loading rate (in terms of fiber) increased from 0.86 to 1.30 g VS/L-d. Either alternative would improve the kinetics of substrate degradation, which would result in a more efficient treatment.

Another unexpected result which occurred in this period, was the accumulation of protein in both reactors. The trend of increasing protein concentration in the effluent
was more noticeable after the change in composition conducted in P-3 (Table 3-10). This is not related to the influent composition, which protein content in P-3 and P-4 was actually 10% lower than that in P-1 and P-2. Also, possible inaccuracies in the Lowry protein analysis (usually due to interferences) were discarded using an alternative method (TKN) for protein determination, which confirmed the Lowry results within a 5%. Instead, two possible factors that could have separately contributed to the accumulation of protein were identified. One factor, that could explain the decrease in protein degradation, is the change from a more degradable to a more recalcitrant type of proteins resulting from the change in the influent composition in P-3. Indeed, proteins that compose dairy manure are, for the most part, hardly degradable, as they primarily consist of undigested proteins from cow’s forage, fibrous proteins (e.g. keratin), and microbial biomass. The proteins contained in the dog food are primarily degradable, food-based proteins derived from corn grains and chicken meal (Hill Pet Nutrition, Inc.). A second, and a probably more determinant factor in creating protein accumulation, comes from the fact that carbohydrates are more energy yielding than other biomolecules, that is, a larger fraction of the electron donor’s energy is used for cell synthesis. Using the concepts developed by McCarty based on stoichiometry and bioenergetics (McCarty, 1972), it is possible to estimate the microbial yields ($Y_{\mu}$) and the fractions of electron donor used for energy ($f_e$) and microbial synthesis ($f_s$) from any particular substrate (electron
donor). Based on these principles, and assuming a cell decay rate, $b$, of 0.03 day$^{-1}$ and a HRT of 30 days, the following overall stoichiometric reactions were developed for carbohydrates, proteins and lipids.

**Carbohydrates**

\[ C_6H_{12}O_6 + 0.214HCO_3^- + 0.214NH_4^+ \]
\[ \rightarrow 2.667CO_2 + 0.810H_2O + 0.214C_5H_7O_2N + 2.476CH_4 \]

**Proteins**

\[ C_{16}H_{24}O_5N_4 + 0.589H_2O + 3.974H^+ + 0.237HCO_3^- \]
\[ \rightarrow 7.423CO_2 + 3.775NH_4^+ 0.024C_5H_7O_2N + 7.682CH_4 \]

**Lipids**

\[ C_8H_{16}O + 3.182H_2O + 0.045HCO_3^- + 0.045NH_4^+ \]
\[ \rightarrow 2.091CO_2 + 0.064C_5H_7O_2N + 5.545CH_4 \]

Where the microbial biomass is represented by $C_5H_7O_2N$, and where the molecular weight is 113 g/mol. Thus, there are 113 g of biomass (i.e. volatile suspended solids, VSS) per mol. Using this information, and from above reactions, $Y_{obs}$, $f_s$, and $f_e$ were estimated (Table 3-7).

Table 3-7. Biomass yield, and fractions of electron donor going to energy and synthesis, as calculated from stoichiometry and bioenergetics

<table>
<thead>
<tr>
<th>Biomolecule</th>
<th>$Y_{obs}$ (g VSS/g COD)</th>
<th>$f_s$ (fraction)</th>
<th>$f_e$ (fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>0.122</td>
<td>0.172</td>
<td>0.828</td>
</tr>
<tr>
<td>Proteins</td>
<td>0.051</td>
<td>0.072</td>
<td>0.928</td>
</tr>
<tr>
<td>Lipids</td>
<td>0.020</td>
<td>0.028</td>
<td>0.972</td>
</tr>
</tbody>
</table>
From the results, it is apparent that the microbial yields obtained from carbohydrates are significantly higher than those obtained from proteins (2x) and lipids (6x). With the change of the co-digestion ratio in P-3, the amount of carbohydrates in the influent increased from 46 to 69%. This confirms that protein concentration could have increased steadily since P-3 as a result of a microbial synthesis increase, due to favorable bioenergetic conditions.

3.5.2.3. Effluent characterization

The change in the contribution of the substrate constituents in the reactors’ effluent throughout the four periods of the study is shown in Figure 3-14. In addition, a thorough profile of the short- and long-chain fatty acids sampled in this study for each period (P) is presented in Figure 3-15 due their relevant role in determining reactor stability, particularly at thermophilic conditions (see Chapter 4).

In P-1, over 45% of the mesophilic reactors’ effluent consisted of non-structural carbohydrates, while a similar percentage of VS was found as proteins in the effluent of the thermophilic reactor. Cellulose was more degradable at mesophilic conditions while hemicelluloses under thermophilic conditions. Both CSADs observed a similar concentration of lipids in the effluent, but the mesophilic CSAD observed higher concentrations of total LCFA, i.e. 0.91 vs. 0.72 g/L (2.57 and 2.00 g COD/L, respectively) and almost zero of VFAs, as compared to 1.39 g COD/L of the thermophilic CSAD. This supports the discussion presented in Chapter 4 – that the
faster rates of β-oxidization under thermophilic conditions can break the kinetic balance between VFA consumers and hydrogenotrophic methanogens.

In the mesophilic reactor, the concentration of lipids in the effluent did not change regardless of the decrease of HRT at P-2; however, the high concentration of some VFAs relative to P-1 suggests that the OLR increase could have created an imbalance in the kinetics of fatty acid degradation.

With the change in influent composition in P-3, the lignocellulosic constituents appear to contribute nearly 50% to the effluent VS in both reactors. Also, with a shorter HRT, the concentration of LCFAs decreased considerably in P-3 compared to P-1. Indeed, the extent of lipid hydrolysis decreased from 60% to 6% and from 48% to 8% in the mesophilic and thermophilic CSADs, respectively. Furthermore, with the increase in the manure-to-dog food ratio, it is expected that some LCFAs could have been adsorbed onto the manure biofibers (see Chapter 4).

In P-4, the increase of lignocellulosic material degradation decreases its concentration in the effluent, whereas protein, mostly from biomass, increases its concentration. Although the lipid concentration of both reactors at P-3 and P-4 is virtually the same, there is again an increase in the concentration of some VFAs relative to P-3, which is probably the result of insufficient retention time for their degradation. As seen in Table 3-10, the effluent lipid concentration of both reactors increased only slightly as the HRT was shortened from 20 through 10 days; however, the effluent total LCFA
concentration decreased considerably. It was calculated that the extent of lipid hydrolysis through the HRTs was reduced from 60% to 9% in the mesophilic digester, and from 48% to 7% in the thermophilic digester. This indicates that at shorter HRTs, hydrolysis of lipids is the rate-limiting step at both thermal regimes; however, at 20 days or longer HRT’s, it is probable that LCFA degradation becomes the rate-limiting factor for lipid degradation.

**3.5.3. Batch vs. semi-continuous studies**

It is intrinsically difficult to compare results of batch and semi-continuous studies, and to draw conclusions from one system that could be applicable to the other. In general, kinetics of semi- or continuously-fed systems cannot be emulated under batch conditions; these two systems differ in their mode of operation, and the usefulness of comparisons and extrapolations between each other has several limitations (see Chapter 2). The application of biodegradation rates should be limited to relative comparisons between different substrates, rather than different systems. However, the biodegradability fraction \( f_D \) and observed specific biomethane yield \( B_o \) obtained via the BMP assay may be comparable to those obtained in CSADs at long HRTs, provided no inhibition processes are in place (see Section 3.6.5, below).

**3.5.4. Destruction of individual substrate constituents**

Based on the results of the biodegradability studies of individual biomolecules presented in Table 3-5 and the results of the stabilization of the individual
constituents of the semi-continuous study at P-1 (Figure 3-12), it was found that; at mesophilic conditions, hemicellulose, non-structural carbohydrates\textsuperscript{2} and protein degradation compare fairly well; however, cellulose was under predicted by the BMP and lipids were somewhat over predicted. At thermophilic conditions, only lipids degradation is within a 5% difference compared to the semi-continuous studies. Although differences are expected given the variability of $f_D$ found for some constituents using the BMP (see Table 3-5), there is also a possibility that the upset and constant instability of the thermophilic reactor during P-1 had affected the degradation of the individual components. In fact, during P-3 and P-4, when the thermophilic reactor was very stable, VS stabilization and SMY are comparable in both CSADs (see Chapter 4). On the other hand, the degradation of the separated, individual lignocellulosic components may not have the same degradation kinetics of the individual components as an integral part of the lignocellulosic matrix (due to their association with lignin). Indeed, it seems that in the case of lignocellulosic materials, a more realistic (and practical) approach would be to lump together the biodegradation of the lignocellulosic components (e.g., Eq. 3-3 – Eq. 3-6).

\textsuperscript{2} Non-structural carbohydrates consist of mostly starch in the dog food pellets used (Hill Pet Nutrition, Inc.)
3.5.5. Using BMP data to predict specific biomethane yields (SMY) obtained at steady-state in semi-continuous CSADs

The observed SMY ($B_o$) determined via the BMP assay is obtained when the biomethane production rate approaches to zero and the degradation of the substrate material has essentially stopped. Depending on the type of substrate, this usually occurs after 25 – 30 days under BMP conditions. Although, as discussed previously, degradation rates of batch (i.e., BMPs) and semi-continuously-fed (i.e., CSADs) systems are not necessarily analogous, we can expect that at sufficiently long HRTs, the extent of substrate degradation could be comparable in both systems. Thus, SMYs obtained in CSADs should approach observed SMYs ($B_o$) determined via the BMP assay. The BMP curves for the 25:75 and 75:25 manure-to-dog food co-digestion ratios are presented in Figure 3-10 and Figure 3-11, respectively. These plots were built using the only two replicates being monitored continuously via pressure transducers, although two more replicates were observed for each sample. As observed, depending on the co-digestion ratio, the biomethane potential was obtained after about 25 days of incubation. Results of the CSADs studies presented in Chapter 4 are shown in Table 3-8, for convenience.
Table 3-8. Volatile solids stabilization (decimal) and specific biomethane yields, SMY (mL CH₄ per g VS added) obtained at steady-state conditions in the CSADs at each operating period (P)

<table>
<thead>
<tr>
<th>Period (P)</th>
<th>Stabilization</th>
<th>SMY</th>
<th>Stabilization</th>
<th>SMY</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1: 20d HRT – MnDF2575</td>
<td>0.715</td>
<td>443.6</td>
<td>0.647</td>
<td>373.9</td>
</tr>
<tr>
<td>P-2: 15d HRT – MnDF2575</td>
<td>0.609</td>
<td>424.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P-3: 15d HRT – MnDF7525</td>
<td>0.394</td>
<td>257.2</td>
<td>0.409</td>
<td>270.6</td>
</tr>
<tr>
<td>P-4: 10d HRT – MnDF7525</td>
<td>0.421</td>
<td>252.6</td>
<td>0.451</td>
<td>269.1</td>
</tr>
</tbody>
</table>

As expected, the $B_o$ obtained at 30 days using the BMP, over-predicts SMY obtained in the CSADs at both co-digestion ratios, and particularly during P-1 for the thermophilic CSAD, most probably as a result of the lower performance exhibited by this reactor due to VFA and LCFA inhibition (see Chapter 4). However, at P-3 and P-4, the SMYs of the CSADs are within the standard deviation of the BMP assay. As suggested by the lower concentrations of LCFAs and VFAs observed in P-3 and P-4 relative to P-1 (see Figure 3-15), it is possible that the increase in the manure-to-dog food co-digestion ratio in P-3 decreased the inhibition of both CSADs due to adsorption of LCFAs on manure biofibers (see Chapter 4). For this co-digestion ratio, the difference between specific biomethane yield of the BMP and the semi-continuously-fed CSADs was within 5 – 15%.
Figure 3-10. BMP curves of the co-digestion samples of 25:75 manure-to-dog food ratio (MnDF2575) at mesophilic (above) and thermophilic (below) temperatures; error bars represent the standard deviation for two replicates, although a total of four replicates were observed for this sample (Figure 3-8); solid line represents the non-linear fit to the curve $B_0 = (1-e^{-kt})$; to build these curves, the methane content measured at the end of the assay, was assumed to be constant throughout the incubation time.
Figure 3-11. BMP curves of the co-digestion samples of 75:25 manure-to-dog food ratio (MnDF7525) at mesophilic (above) and thermophilic (below) temperatures; error bars represent the standard deviation for two replicates for this sample, although a total of four replicates were observed for this sample (Figure 3-8); solid line represents the non-linear fit to the curve $B_0 = (1-e^{-kt})$. 
3.5.6. Predicting steady-state SMY and substrate stabilization of semi-continuous CSADs by integrating biodegradability data and stoichiometry

Ideally, we would want to make (reliable) estimations of SMYs and extent of substrate stabilization of complex substrate mixtures based on a set of parameters and equations. Based on the results of this study and the stoichiometric relationships developed by Symons & Buswell (1933), which were discussed previously, we attempted to estimate the biodegradable fractions ($f_D$) and observed SMYs ($B_o$) of several co-digestion ratios. As discussed above, $f_D$ and $B_o$ are respectively homologous to substrate stabilization and SMYs obtained at long HRTs in semi-continuously-fed CSADs.

In order to estimate SMY and substrate (VS) stabilization of CSADs, the following methodology was followed. First, $f_D$ and $B_o$ of dairy manure were calculated using the prediction models developed in this study for lignocellulosic substrates (i.e., Eq. 3-3 – Eq. 3-6). Second, the $B_o$ for dog food was calculated using the Buswell formula and corrected using the $f_D$ values obtained in the BMP studies for the individual constituents (Table 3-5). Lastly, for a specific co-digestion ratio, a weighted average SMY was calculated based on the individual SMY contributions of manure and dog food. A flowchart of this methodology is included in the Appendix. Predictions of $f_D$ and $B_o$ at mesophilic and thermophilic temperatures for several co-digestion ratios are presented in Table 3-9.
Table 3-9. Prediction of biodegradability fraction (decimal) and specific biomethane yields (mL CH₄ per g VS added) for several manure (Mn)-to-dog food (DF) co-digestion ratios at mesophilic and thermophilic conditions

<table>
<thead>
<tr>
<th>Co-digestion ratio</th>
<th>Mesophilic</th>
<th>Thermophilic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( f_D ) (Eq. 3-3)</td>
<td>( B_o ) (Eq. 3-4)</td>
</tr>
<tr>
<td>Mn100</td>
<td>0.443</td>
<td>217.6</td>
</tr>
<tr>
<td>MnDF7525</td>
<td>0.541</td>
<td>298.0</td>
</tr>
<tr>
<td>MnDF5050</td>
<td>0.639</td>
<td>378.5</td>
</tr>
<tr>
<td>MnDF2575</td>
<td>0.736</td>
<td>458.9</td>
</tr>
<tr>
<td>DF100</td>
<td>0.834</td>
<td>539.4</td>
</tr>
</tbody>
</table>

From results reported in Chapter 4, at P-1, where the manure-to-dog food co-digestion ratio was 25:75, the VS stabilization (equivalent to \( f_D \)) of the mesophilic CSAD was 0.715 and the SMY was 444 mL CH₄ per g VS added. The predicted \( f_D \) and \( B_o \) for this co-digestion ratio at mesophilic conditions are 0.736 and 459 mL CH₄ per g VS added (Table 3-9). Thus, the predicted \( f_D \) and \( B_o \) are within 3% of the observed values reported for the semi-continuous study at mesophilic conditions.

In the case of the thermophilic reactor, 0.647 of the substrate was stabilized at P-1, and 374 mL CH₄ per g VS added were produced. Here, data do not agree as well as for the mesophilic reactor, probably because of the instability of the thermophilic reactor during P-1, as mentioned above. Regardless, predicted values are still within a reasonable 10-15% of the observed ones.

Prediction of \( f_D \) and \( B_o \) the 75:25 manure-to-dog food co-digestion ratio (P-3 and P-4) pose more challenges, particularly for two reasons: first, lignocellulosic prediction...
equations and biodegradability data are based on ultimate data – that is, long-term digestion, which is usually not shorter than 30 days. P-3 and P-4 were conducted at 15 and 10-d HRTs, a significantly shorter degradation time as to that provided for complete digestion in BMPs; secondly, with nearly 50% lignocellulosic material in the influent in P-3, this substrate requires even longer detention times to complete digestion than that at P-1 and P-2. At P-3 and P-4, the stabilized VS fractions of the mesophilic reactor were 0.394 and 0.421, respectively, those of the thermophilic digester were 0.409 and 0.451, respectively. The prediction values in Table 3-9 for $f_D$ at this co-digestion ratio are between 11 and 27% of the observed values, which it is still reasonable, considering that the error of some analytical techniques, such as the BMP, are well within this range. Contrarily, the observed specific biomethane yields at P-3 and P-4 were unexpectedly in close agreement with their predictions, especially at thermophilic conditions. At P-3 and P-4, the observed specific biomethane yields of the mesophilic reactor were 257 and 253 mL CH$_4$ per g VS added, respectively; compared to those of the thermophilic digester of 271 and 279 mL CH$_4$ per g VS added, respectively. Thus, in this case, the predictions of $B_a$ for this co-digestion ratio in Table 3-9 are within 15% for the mesophilic digester, and 4%, for the thermophilic digester.

This prediction method, which uses a combination of biodegradability data, including the lignocellulosic-based models (Table 3-5), and stoichiometric-based equations
(Buswell Formula), should be applicable whenever the composition of the substrate to be digested is available. This is especially feasible in co-digestion operations, where usually well-characterized industrial wastes, such as food wastes, are digested with manure. A comprehensive online database maintained by the Nutrient Data Laboratory (NDL) contains detailed information about the chemical composition of foods, and it is available at http://www.ars.usda.gov/ba/bhnrc/ndl (U.S. Department of Agriculture, 2009). Dairy manure composition can vary somewhat from farm to farm and throughout the year; however, its specific biomethane yield and biodegradability fraction have been well characterized by this and other studies. In this case, manure from several farms throughout New York State was measured by the authors for numerous years with no significant variation (see Chapter 2).
Figure 3-12. Stabilization of substrate constituents under mesophilic (left) and thermophilic (right) conditions at steady state; hatched filled-area in lignin represents accumulation; P-1: 20-d HRT, MnDF2575; P-2: 15-d HRT, MnDF2575
Figure 3-13. Stabilization of substrate constituents under mesophilic (left) and thermophilic (right) conditions at steady state; hatched fill in protein represents accumulation; P-3: 15-d HRT, MnDF7525; P-4: 10-d HRT, MnDF7525
Figure 3-14. Effluent characterization of reactors at the different periods representing the percent contribution of the substrate constituents to the total VS
Table 3-10. Concentration of constituents in the CSADs’ effluent at each period (g VS/L)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>P-1</th>
<th>P-2</th>
<th>P-3</th>
<th>P-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>T</td>
<td>M</td>
<td>T</td>
</tr>
<tr>
<td>Proteins</td>
<td>1.77</td>
<td>4.73</td>
<td>3.00</td>
<td>4.25</td>
</tr>
<tr>
<td>Lipids</td>
<td>1.53</td>
<td>1.48</td>
<td>1.80</td>
<td>1.89</td>
</tr>
<tr>
<td>Sugars, starch</td>
<td>4.09</td>
<td>2.18</td>
<td>3.09</td>
<td>3.85</td>
</tr>
<tr>
<td>Hemicelluloses</td>
<td>0.33</td>
<td>0.06</td>
<td>0.58</td>
<td>1.56</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.25</td>
<td>1.15</td>
<td>1.51</td>
<td>3.68</td>
</tr>
<tr>
<td>Lignin</td>
<td>0.59</td>
<td>1.00</td>
<td>1.74</td>
<td>2.94</td>
</tr>
</tbody>
</table>

M: Mesophilic; T: Thermophilic
Figure 3-15. Concentrations of volatile fatty acids – VFA (left axis) and long-chain fatty acids – LCFA (right axis), in the effluent of the mesophilic (above) and thermophilic (below) reactors at steady-state conditions for each period (P); VFA and LCFA on the horizontal axis correspond to the sum of individual fatty acids.
**Main findings**

With the exception of starch and lignin, sugars, and lignin-free cellulose and hemicellulose are fairly degradable at both thermal regimes, but structural carbohydrates are, in general, more degradable at thermophilic conditions. Globular and fibrous proteins are degraded well and fairly equally in both thermal regimes. With the exception of tripalmitin at thermophilic conditions, lipids are highly degraded at both regimes, provided that the concentrations are maintained low enough to not produce inhibition.

Lignin content is negatively correlated with the extent of degradation and specific biomethane yield of lignocellulosic substrates. One model to predict extent of degradation and another to predict biomethane yields of lignocellulosic materials were developed using lignin as a single predictor for the mesophilic and thermophilic thermal ranges.

A linear decrease in the biodegradability and biomethane yields is observed with an increase of the dairy manure-to-dog food ratio in the batch co-digestion studies. At low dairy manure-to-dog food ratios, influent stabilization is maximized at long HRTs, at lower HRTs the stabilization of lignocellulosic materials decreases considerably. Protein accumulates in the effluent at shorter HRTs due to an increase in the proportion and loading of carbohydrates in the influent, which are more synthesis yielding than proteins and lipids.
BMP results somewhat over predicted biomethane yields of semi-continuous reactors, particularly during inhibition processes taking place in the thermophilic digester. However, in absence of inhibition, it is possible to use BMP curves to obtain estimations of the specific biomethane yields of complex substrates within 15% of those observed during a determined HRT at steady-state conditions.

Finally, a methodology to predict the degradable fraction and specific biomethane yields of lignocellulosic composite substrates was developed on the basis of substrate chemical composition and stoichiometry, which produces comparable results to those observed at steady-state conditions.

3.6. Conclusions

Based on the batch and semi-continuous studies, the following conclusions can be drawn.

- Two models to predict extent of degradation and biomethane yields of lignocellulosic materials were developed using lignin as a single predictor
- In absence of inhibition, BMP assays can be used to estimate specific biomethane yields within 15% of those observed in continuous studies
- A theoretical methodology to predict the degradable fraction and biomethane yields of lignocellulosic composite substrates at steady-state conditions was developed based on substrate chemical composition and stoichiometry
3.7. References


CHAPTER 4

INFLUENCE OF HYDRAULIC RETENTION TIME AND SUBSTRATE CHEMICAL COMPOSITION ON PERFORMANCE AND STABILITY OF ANAEROBIC DIGESTION AT MESOPHILIC AND THERMOPHILIC CONDITIONS

Abstract

The anaerobic digestion of complex particulate substrates is a continuum of physicochemical and biochemical reactions resulting from constant interactions between microorganisms, enzymes, and chemical substances, as constrained by the environmental conditions of the system. It is the delicate balance between these components that ultimately determines how stable and efficient the anaerobic digestion process is. The system temperature and the physicochemical characteristics of the substrates being degraded are probably the most important parameters influencing this balance, as they determine the consortia of microorganisms involved, the pathways of biochemical conversions, and the overall kinetics of the process. In this work, anaerobic digestion of complex particulate substrates was studied under both, mesophilic and thermophilic temperature ranges, by co-digesting dairy manure and dry dog food in semi-continuously-fed continuously-stirred anaerobic digesters
(CSADs). The effect of three distinct hydraulic residence times (HRT) and two co-digestion ratios (i.e., manure-to-dog food) on the performance and stability of the process was evaluated over a period of 18 months. It was found that the temperature range, influent substrate composition, and HRT, all played an important role in influencing performance and stability of the process. Anaerobic digestion at mesophilic temperatures was stable throughout all HRTs and outperformed thermophilic digestion in both biogas production and volatile solids stabilization efficiencies at 20 and 15-d HRTs with low manure-to-dog food ratios. At high manure-to-dog food ratios and at 15 and 10-d HRTs, higher biogas production and volatile solids stabilization was observed at thermophilic temperatures; however, at low manure-to-dog food ratios, thermophilic digestion proved to be highly sensitive to organic loading rates (OLR), particularly lipids, and reactor operating characteristics.

Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCFA</td>
<td>Long chain fatty acids</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile fatty acids</td>
</tr>
<tr>
<td>VS</td>
<td>Volatile solids</td>
</tr>
<tr>
<td>TKN</td>
<td>Total Kjeldahl nitrogen</td>
</tr>
<tr>
<td>CSAD</td>
<td>Continuously-stirred anaerobic digester</td>
</tr>
</tbody>
</table>
HRT  Hydraulic retention time (d)
OLR  Organic loading rate (g/L-d)
COD  Chemical oxygen demand (g/L)
TAN  Total ammonia nitrogen (g/L)
NDF  Neutral detergent fiber
ADF  Acid detergent fiber
ADL  Acid detergent lignin

4.1. Introduction

The anaerobic stabilization of complex particulate matter is a highly dynamic, multi-step process, where physicochemical and biochemical reactions take place in sequential and parallel ways. Such reactions occur as a result of continuous interactions between the main components that make up the system (i.e., microorganisms, environment, and substrates). The continuous balance between these components is what basically determines the performance and stability of anaerobic digestion. Both temperature and substrate characteristics dictate the microbial groups involved in the degradation process, and may be the most important external parameters for the process design and control of anaerobic digesters. As temperature increases, the rate of biochemical and enzymatic reactions within cells, and thus growth and decay rates, also increase. Furthermore, with a rise above a
specific temperature, which is characteristic of each species, inhibition and then
mortality occur, as proteins and structural components of the cell become irreversibly
denatured (Anderson et al., 2003). Although there is a continuum of microorganisms
that grow from temperatures as low as –12°C (Brock & Madigan, 1997) to greater
than 200°C (Amend & Shock, 2001), there is no single species capable of growing
over this entire temperature spectrum. Anaerobic microorganisms are generally
divided into three thermal groups: psychrophiles, mesophiles, and thermophiles
(VanLier et al., 1997); however, most anaerobic digesters are operated at either
mesophilic or thermophilic temperature regimes (Ward et al., 2008). Operated at a
temperature range between 35 and 40°C, mesophilic digesters are less energy intensive
and usually more commonly employed in full-scale facilities than thermophilic
digesters, which are usually operated between 55°C and 60°C. In addition,
thermophilic digesters have been reported to be less stable than mesophilic digesters,
being more susceptible to inhibition (Angelidaki & Ahring, 1994; Hansen et al., 1999;
Hwu & Lettinga, 1997) and sudden environmental changes (Biey et al., 2003; Khanal
et al., 2010; Nguyen et al., 2007; VanLier et al., 1996; Zinder, 1986). Nevertheless,
thermophilic anaerobic digestion presents several important advantages over
mesophilic digestion. The capability of thermophilic digestion to inactivate
pathogenic organisms has been recognized to be a proven method for producing
Class A biosolids (Kim et al., 2002), which are essentially pathogen free streams, with
no restrictions on crop type, harvesting, or site access for land application (USEPA, 2000). Additionally, increased growth rates of microorganisms and accelerated interspecies hydrogen transfer at thermophilic temperatures (Gavala et al., 2003) can lead to faster degradation rates, higher solids destruction, and ultimately to increased biomethane yields at shorter retention times.

Equally important to the performance and stability of anaerobic digestion process, is the substrate being digested. The physical properties and chemical composition of the substrate have a major impact on the dynamics of the food network of anaerobic digestion, by influencing the microbial groups involved in the processes, the biochemical conversion pathways, the thermodynamic balance of the reactions, the rate of the biochemical reactions, and the stoichiometry of the products formed. The array of organic substrates subjected to anaerobic digestion today varies widely in both diversity and complexity – from soluble, readily-degradable substrates, such as milk processing wash water, to complex, multi-component mixtures of lignocellulosic and high-strength particulate substrates, such as animal manure and primary solid waste. Under optimal steady state conditions, formation and consumption of intermediate products proceed until all the bioavailable electrons reach the terminal electron acceptors. However, the array of substances formed is degraded at different rates, and transient accumulation of intermediate products is common. The ability of the digester to withstand transient accumulation of intermediates without causing
process perturbation depends on the type of substance and the concentration reached. Accordingly, the type of constituents composing the substrate mixture, their proportion in the influent, and the loading rate of the digester, are key factors in maintaining process stability and digestion performance. Furthermore, the operating thermal regime, digester configuration, and stability of operating parameters also play an important role.

Parallel studies comparing mesophilic and thermophilic anaerobic digestion are few in the literature. In addition, the effects of substrate chemical composition and thermal regime are usually analyzed separately, and the interrelation of both factors is not considered.

4.2. Objective

The objective of this study was to evaluate long-term performance and stability of the anaerobic digestion of complex, particulate substrates under both mesophilic (37°C) and thermophilic (55°C) operating temperatures – specifically, the semi-continuously-fed co-digestion of dairy manure and a generic high-strength, multi-component substrate.

4.3. Background and discussion of concepts

4.3.1. Anaerobic bioconversion of complex substrates

The main biochemical conversion pathways believed to occur during anaerobic digestion are depicted in Figure 4-1. In the first step of this process, the
organic/degradable fraction of the composite materials, which is made up of an array of carbohydrates, proteins, and lipids, is hydrolyzed to soluble products via extracellular enzymes that allow their passage across the cell membrane. Once in the cell, sugars, amino acids, and long-chain fatty acids are fermented or oxidized to volatile fatty acids, alcohols, carbon dioxide, hydrogen, as well as nitrogenous and sulfurous compounds. With the exception of acetate, volatile fatty acids are converted to additional acetate, hydrogen, and carbon dioxide. In the final step, methane is produced via either cleavage of acetate or reduction of carbon dioxide with hydrogen. The major groups of organisms mediating the biochemical reactions depicted in Figure 4-1 are (1) fermentative bacteria, (2) hydrogen-producing acetogenic bacteria, (3) hydrogen-consuming acetogenic bacteria, (4) carbon dioxide-reducing methanogens, and (4) acetoclastic methanogens (Pavlostathis & Giraldo-Gomez, 1991). The most important biochemical reactions involved directly or indirectly in the anaerobic digestion processes are presented in Table 4-1.
Figure 4-1. Major pathways in the anaerobic digestion of complex substrates; numbers represent the microbial groups described in the text; adapted from McCarty & Smith (1986), Pavlostathis & Giraldo-Gomez (1991), and Batstone et al. (2002)
Table 4-1. Principal reactions involved in the bioconversion process of complex organic substrates and their associated thermodynamic Gibbs free-energy changes ($\Delta G^\circ_r$) at standard 25°C, and mesophilic (37°C) and thermophilic (55°C) temperatures.a

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Equation</th>
<th>(\Delta G^\circ_r) (kJ/reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>25°C</td>
</tr>
<tr>
<td>Eq. 4-1</td>
<td>Acetate cleavage (\text{CH}_3\text{COO}^-(aq) + \text{H}_2\text{O}(l) \rightarrow \text{CH}_4(g) + \text{HCO}_3^-(aq))</td>
<td>-31.0</td>
</tr>
<tr>
<td>Eq. 4-2</td>
<td>Propionate oxidation (\text{CH}_3\text{CH}_2\text{COO}^-(aq) + 3 \text{H}_2\text{O}(l) \rightarrow \text{CH}_3\text{COO}^-(aq) + \text{HCO}_3^-(aq) + \text{H}^+ + 3\text{H}_2(g))</td>
<td>76.5</td>
</tr>
<tr>
<td>Eq. 4-3</td>
<td>Palmitate oxidation (\text{CH}_3(\text{CH}<em>2)</em>{14}\text{COO}^-(aq) + 14 \text{H}_2\text{O}(l) \rightarrow 8 \text{CH}_3\text{COO}^-(aq) + 7\text{H}^+ + 14\text{H}_2(g))</td>
<td>401.2</td>
</tr>
<tr>
<td>Eq. 4-4</td>
<td>Hydrogenotrophic methanogenesis (\text{HCO}_3^-(aq) + 4 \text{H}_2(g) \rightarrow \text{CH}_4(g) + 3 \text{H}_2\text{O}(l))</td>
<td>-135.5</td>
</tr>
<tr>
<td>Eq. 4-5</td>
<td>Syntrophic oxidation of propionate (4 \text{CH}_3\text{CH}_2\text{COO}^-(aq) + 3 \text{H}_2\text{O}(l) \rightarrow 4 \text{CH}_3\text{COO}^-(aq) + \text{HCO}_3^-(aq) + \text{H}^+ + 3\text{CH}_4(g))</td>
<td>-100.7</td>
</tr>
</tbody>
</table>

a Temperature corrections other than standard were conducted using the Gibbs-Helmholtz Equation.
4.3.2. Influence of temperature and substrate chemical composition on inhibition

There are several substrates capable of producing intermediate products with the potential to cause inhibition and process instability. The accumulation of these substances in the system can slow down or interrupt biologically-mediated processes, either by disrupting the homeostatic equilibrium of organisms or by imposing thermodynamics constraints to biochemical reactions. Urea- and protein-rich substrates, such as animal manures and industrial wastes, are an excellent source of nitrogen and alkalinity, but can also create high levels of ammonia in anaerobic digesters. Total ammonia ($\text{NH}_3 + \text{NH}_4^+$) and particularly its unionized form, are inhibitory to methanogens, by diffusing into the cell and causing proton imbalance (Angelidaki & Ahring, 1993; Kayhanian, 1994; Koster & Lettinga, 1988). Furthermore, most studies conclude that the inhibitory effect of ammonia increases with temperature, with thermophilic organisms being more susceptible than mesophiles (Angelidaki & Ahring, 1994; Chen et al., 2008; Hansen et al., 1998). Anaerobic digestion of fats and oil-containing wastes is often hampered because of the inhibitory effect of long-chain fatty acids (Chen et al., 2008), which are the major intermediate products of lipid degradation. Although the inhibitory effect of long-chain fatty acids has not been well-established, it has been theorized that is of a physicochemical nature, where the surface-active fatty acids adheres to the organisms’
cell wall and impedes the passage of nutrients (Henderson, 1973; Rinzema et al., 1994). Similarly, long-chain fatty acids inhibition has been shown to be more pronounced under thermophilic temperatures as compared to mesophilic (Hwu & Lettinga, 1997). Long-chain fatty acids usually accumulate in anaerobic digesters when molecular hydrogen, a major product of their oxidation, reaches thermodynamically limiting levels for the hydrogen-producing organisms to be able to function. Furthermore, the degradation of propionate, a major intermediate of anaerobic digestion of complex substrates depends on even lower partial pressures of hydrogen, accumulates as well (McCarty & Smith, 1986; Schmidt & Ahring, 1993). As with LCFA, higher accumulation of propionate and resulting inhibition has been related to increasing temperatures (Kim et al., 2002; Speece et al., 2006; Wilson et al., 2008). Simple carbohydrates, such as sugar- or starch-rich substrates, degrade fast and nearly to completion, but usually require supplementation of external nutrients and/or alkalinity. Although biochemical conversion of simple carbohydrates does not produce highly inhibitory substances, shock loads of volatile fatty acids and resulting low pH, could be produced due to the high biodegradability of these substrates and the conversion rate can be further increased with temperature.

4.3.3. Microbial syntrophic associations and bioenergetics

Successful bioconversion of complex substrates to methane and carbon dioxide depends on delicate associations among different groups of microorganisms. A
primary example of these associations is the syntrophic relationship between hydrogen producers and hydrogen consumers, which is a mechanism known as interspecies hydrogen transfer (Zehnder & Stumm, 1988). The balance between these two groups of organisms is of foremost importance to prevent reactor instability (Demirel & Yenigün, 2002). By consuming hydrogen, hydrogenotrophs can create conditions for obligate hydrogenogens to perform catabolic oxidations, which in the absence of hydrogenotrophs would not have been energy-yielding (Dolfing, 1988). Some of these reactions can occur within narrow thermodynamic limits, and in absence of syntrophic associations, these reactions cannot proceed. This point can be illustrated with the oxidation of propionate. The energy available, as determined by the standard Gibbs free energy ($\Delta G_r^\circ$), to convert propionate to acetate and hydrogen is highly positive (Eq. 4-2), which means that the reaction does not proceed spontaneously; however, the use of hydrogen by hydrogen-utilizing methanogens is highly favorable (Eq. 4-4), and when the two species act in syntrophic association (Eq. 4-2 + Eq. 4-4), the combined reaction become energetically favorable (Eq. 4-5). The concentration of either acetate or hydrogen, or both together, can be reduced sufficiently to produce a favorable free-energy change for propionate oxidation; however, it is the partial pressure of hydrogen that tends to control the process (McCarty & Smith, 1986).
The standard Gibbs free energy ($\Delta G_r^\circ$) represents the energy available for a reaction to proceed when reactants and products are at unit activity and pH is 7, and absolute temperature and pressure are 25°C and 1 atm, respectively (McCarty & Smith, 1986; Thauer et al., 1977):  

$$\Delta G_r^{\circ'} = \sum [\Delta G_r^\circ (products) - \Delta G_r^\circ (reactants)]$$

For temperatures other than 25 °C, Gibbs free energy values can be corrected using the Gibbs-Helmholtz Equation (Eq. 7).  

$$\frac{\Delta G_r^{\circ'}(T_2)}{T_2} - \frac{\Delta G_r^{\circ'}(T_1)}{T_1} = \Delta H^\circ (P) \left[ \frac{1}{T_2} - \frac{1}{T_1} \right]$$

The $\Delta G_r^{\circ'}$ values for the reactions presented in Table 4-1 were corrected for mesophilic (37°C) and thermophilic (55°C) temperatures, using this equation and enthalpy values for compounds. The overall, actual free-energy of a reaction ($\Delta G_r$) to determine the direction it will proceed can be calculated from the expression below, which takes into account the actual concentrations compounds other than unity.  

$$\Delta G_r = \Delta G_r^\circ + RT \ln Q_r$$

where $\Delta G_r^\circ$ is the Gibbs free-energy at a pH other than 7, $R$ is the universal gas constant, $T$ is the absolute temperature, and $Q_r$ is the activity product. The actual Gibbs free-energy limits for the key reactions involved in the interspecies hydrogen

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3 A more detailed explanation of the thermodynamic concepts and the equations used for the calculations are presented in the appendix section of this manuscript.
transfer were calculated using this expression at mesophilic and thermophilic temperatures as a function of the hydrogen partial pressure (Figure 4-2). The colored boxes in the figure show the limits where all the reactions are thermodynamically possible. It is apparent that higher hydrogen partial pressures ($P_{H_2}$) are possible at thermophilic temperatures as compared to mesophilic. It is also interesting that palmitate oxidation can occur at much higher $P_{H_2}$ (i.e., 1.5 orders of magnitude), which suggests that, in general, further degradation of long chain fatty acids (LCFA) could be expected in thermophilic digesters under high loads of molecular hydrogen ($P_{H_2} = 10^{-2}$ atm). This could lead to free LCFA inhibition and accumulation of propionate because $P_{H_2}$ could be over its threshold for oxidation (i.e., $10^{-3.2}$ atm).
Figure 4-2. Thermodynamic thresholds for propionate and palmitate oxidation, and hydrogenotrophic methanogenesis at mesophilic, 37°C (blue line), and thermophilic, 55°C (red line), temperatures; the limits where all the reactions are possible at mesophilic and thermophilic temperatures are shown in the blue and red colored boxes, respectively. The plot was built assuming 1mM for all fatty acids, 100 mM for bicarbonate, 0.7 atm for methane, and 0.3 atm for carbon dioxide.

4.3.4. Propionate and molecular hydrogen: major nodes involved in the bioconversion processes of complex substrates

In the anaerobic degradation of complex substrates, approximately 30% of the electron flow associated with methane production goes through propionate (Jeris & McCarty, 1965; McCarty & Smith, 1986). The energy available for the degradation of propionate is very small, and requires partial pressures of hydrogen below $10^{-4}$ atm at
25 °C (McCarty & Smith, 1986; Schmidt & Ahring, 1993). As mentioned above, such low hydrogen partial pressures in methanogenic systems are only possible by interspecies transfer of molecular hydrogen from hydrogen-producing bacteria to hydrogen-oxidizing methanogens (Bryant, 1979). *Syntrophomonas* and *Syntrophobacter* genera live in symbiotic association with the methanogens to ferment complex mixtures of volatile fatty acids. *Syntrophomonas wolfei* is a versatile anaerobic bacterium capable of fermenting caprylic (C8), enanthic (C7), caproic (C6), valeric (C5), and butyric (C4) acids into a mixture of acetic (C2) and propionic acids (C3) (McCarty & Mosey, 1991). This generates the hydrogen gas that *Methanobacterium bryantii* and other methanogens will use to reduce bicarbonate and produce methane gas (Eq. 4-4). Propionic acid produced by *Syntrophomonas wolfei*, or directly by acid forming bacteria, is fermented to acetic acid by *Syntrophobacter wolinii*, a slow-growing bacterium, which also relies in the presence and activity of hydrogen-utilizing methanogens to act as its hydrogen-scavenger (McCarty & Mosey, 1991). Aside of trans-2-butenolic acid, which is unlikely to occur in anaerobic digesters, *Syntrophobacter wolinii* has no alternative substrates (Boone & Bryant, 1980). This limited metabolic repertoire is what McCarty & Mosey (1991) suggested as a key point to explain the persistence of propionate that sometimes occurs in anaerobic digesters. Furthermore, some studies have reported lower diversity of both bacterial and archaea communities in thermophilic compared to mesophilic digesters (Leven et al., 2007; Raskin et al., 1994), which can also explain
the higher propionate concentrations as well as the overall poorer stability of thermophilic digesters. Thus, a higher microbial diversity could be important for process stability, by providing a wider repertoire of microorganisms and enzymes which could allow the development of additional syntrophic relationships and alternatives pathways for a more efficient degradation of certain chemical products before they accumulate and produce inhibition.

4.4. Materials and methods

4.4.1. Experimental design and operating conditions

Two identical lab-scale continuously-stirred anaerobic digesters (CSADs) with a working volume of 4.5 L were operated at either mesophilic (37°C) or thermophilic (55°C) temperature conditions. Two influent substrate compositions, representing both high and low manure-to-dog food ratios, and three different hydraulic retention times (HRTs) were evaluated in both CSADs through the course of the study. Our experimental design combined these variables to produce a total of four operating periods for the study. Each period (P), indicating the duration, influent substrate composition, and HRT, for each reactor, is presented in Table 4-2.
Table 4-2. Summary of the operating conditions for each CSAD during the four periods (P) evaluated in this study

<table>
<thead>
<tr>
<th>Period (P)</th>
<th>Days</th>
<th>HRT (d)</th>
<th>Composition (% VS basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mesophilic CSAD</td>
<td>Thermophilic CSAD</td>
<td></td>
</tr>
<tr>
<td>Start-up</td>
<td>0 – 62</td>
<td>0 – 62</td>
<td>30</td>
</tr>
<tr>
<td>P-1</td>
<td>63 – 330</td>
<td>63 – 330</td>
<td>20</td>
</tr>
<tr>
<td>P-2</td>
<td>331 – 430</td>
<td>331 – 360</td>
<td>15</td>
</tr>
<tr>
<td>P-3</td>
<td>431 – 498</td>
<td>361 – 498</td>
<td>15</td>
</tr>
<tr>
<td>P-4</td>
<td>499 – 544</td>
<td>499 – 544</td>
<td>10</td>
</tr>
</tbody>
</table>

**4.4.1.1. CSAD description**

The reactor experimental setup is shown in Figure 4-3. The temperature of the reactors was maintained within 1°C through built-in water jackets and heated water recirculators (PolyScience, Model 210). Each reactor was sealed with an air-tight head-plate where the influent, effluent and biogas lines were located, and a biogas sample port and temperature probe were also in place. The gas collection system of each reactor consisted of the following elements: (1) a foam trap flask; (2) a flexible rubber balloon, used as a biogas reservoir/pressure buffer during effluent withdrawal; (3) a steel wool scrubber, used to reduce hydrogen sulfide and minimize system corrosion; (4) a glass bubbler, for visual determination of gas production; (5) and a gas meter (Actaris Meterfabriek bv, Delft, The Netherlands). Each reactor was continuously stirred with a mechanical mixer (Model 5vb, EMI, Inc., Clinton, CT) using a 62-mm diameter axial flow impeller (Lightnin A-310, Rochester, NY). Both
reactors were mixed at 100 RPM during P-1 and P-2, but increased to 125 RPM for
the remaining part of the study (P-3 and P-4) to keep the solids in suspension when
the content of manure was increased to 75% (VS basis) (see Table 4-2).

Figure 4-3. The two 4.5-L CSADs used in the study, showing the mechanical mixers, biogas flow meters, and
heated-water recirculators

4.4.1.2. Anaerobic digesters startup

The inoculum used to start the anaerobic digesters was obtained from a farm-based
mesophilic completely-mixed anaerobic digester (Ridgeline Dairy Farm, Clymer, NY)
operated at a 20-day hydraulic retention time (HRT), which co-digested raw dairy
manure with an array of high strength food residues (i.e., milk, ice cream, grapes, and
salad dressing) at the time of sampling (Pronto & Gooch, 2008). The inoculum was
harvested from the supernatant of the digester’s effluent after 24 hours of quiescent settling.

4.4.1.3. Substrate feed

The anaerobic digestion of complex, particulate substrates was represented by the co-digestion of dairy manure with dog food dry pellets. Rumensin® free dairy manure mixed with urine was collected at the beginning of the study from the influent pit of a dairy farm anaerobic digester treating the daily waste of about 600 cows (AA Dairy, Candor, NY). The raw manure was homogenized, blended and initial analyses were performed before putting it in individual 1-L containers and maintained at -20°C throughout the study. Only at the beginning of P-3, a new batch of raw manure from the same dairy farm was collected – a fact with no implications to the results of the study, but nonetheless discussed later in the manuscript.

Dog food was used in this study because its characteristics approach the multi-component chemical composition of a generic food residue and it is also reproducible and stable over time. The pelletized dry dog food from Science Diet (Hill Pet Nutrition, Inc.) was specifically selected because it contains a certified, well balanced mixture of carbohydrates, lipids, and proteins. The pellets were ground in an industrial blender and sieved afterwards to produce a feed particle size in the range of 1 – 2 mm.
Dairy manure and dog food were mixed at two different ratios to produce an influent of either 25% manure and 75% dog food or 75% manure and 25% dog food (VS basis) depending on the period (see Table 4-2). The overall influent volatile solids concentration of the combination of these two substrates were set to be constant at 30 g VS/L during the study, mainly to prevent solids settling problems and avoid ammonia inhibition throughout the three increasing organic loading rates due to shorter HRTs. Raw dairy manure (ca. 100 g VS/L) was diluted with tap water prior to feeding to achieve the total VS concentration when combined with dry dog food. During the entire duration of this study, reactors were fed semi-continuously and manually every 48±1 h, by first withdrawing a known amount of effluent (calculated according to the reactors’ volume and current HRT) and adding the same amount of the prepared feed subsequently.

4.4.2. Analytical methods

Biogas production was measured using the previously described gas meter every 48-h cycle right after feeding. pH was measured with the same frequency from the withdrawn effluent using a single-reference electrode (Thermo Fisher Scientific, Inc.). Methane, carbon dioxide, and hydrogen sulfide content in the biogas were measured periodically using a SRI 8610C (SRI Instruments, Torrance, CA) gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and a flame photometric detector (FPD), using Helium as a carrier gas in a 1-m Rt-XLSulfur® packed column
and a ramped temperature program. Gas data were corrected for temperature and pressure variations and reported at standard conditions (0 °C and 1 atm). Total solids (TS) and volatile solids (VS) were determined according to Standard Methods, sections 2540B and 2540E, respectively (APHA, 1995). Chemical oxygen demand (COD) was determined according to the closed reflux titrimetric method, as described in section 5220C of Standard Methods (APHA, 1995).

Total volatile fatty acids (VFA) were measured by the distillation method as described by the Standard Methods (APHA, 1995). Similarly, individual VFAs were determined with a HP Agilent GC model 5890 equipped with a flame ionization detector (FID), using helium as a carrier gas in a NUKOL® capillary column. A commercially prepared 10 mM volatile fatty acids (VFA) standard mixture, containing, acetic (C2), propionic (C3), isobutyric (C4), butyric (C4), isovaleric (C5), valeric (C5), isocaproic (C6), caproic (C6), and enanthic (C7) acids, was obtained from Sigma Aldrich Co. Long chain fatty acids (LCFA) were determined using the same GC setup but following a different temperature program and sampling preparation adapted from Neves et al. (2009), as described in the appendix. The LCFA stock solution was prepared in our lab using dichloromethane as a solvent, and a mixture of capric (C10), lauric (C12), myristic (C14), palmitic (C16), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids as standards, and petadecanoic acid (C15) as an internal standard (IS) – all reagents were HPLC grade and obtained from Sigma-Aldrich Co.
Total Kjeldahl nitrogen (TKN) concentration was determined according to the Standard Methods (APHA, 1995). Total ammonia-N (TAN) concentration was measured using an ion selective electrode (Thermo Fisher Scientific, Inc.). Total organic nitrogen was calculated by subtracting TAN from TKN. Total protein content was calculated based on the assumption that an average protein contains 16% organic N. Neutral lipids were determined according to method of Loehr & Rohlich (1962) for wastewaters. Hemicellulose, cellulose, and lignin content were determined according to the neutral detergent fiber (NDF) and acid detergent fiber and lignin (ADF/ADL) analyses described by Mertens et al. (2002) and Möller (2009), respectively. Non-lignocellulosic carbohydrates (e.g. sugars, starch) were obtained using the method of Gaudy (1962). Detailed protocols of the analytical procedures and information about the equipment used in this study can be found in the Appendix.

4.4.3. Steady-state conditions and feeding cycle monitoring

A reactor was considered to be at steady state only after two criteria were met: (1) it had been operated for a period of time equal to three times the hydraulic retention time (HRT) being evaluated, and (2) the volumetric biogas production rate (L/L-d) was stable and had not varied more than 10% for the last HRT. Once at steady-state conditions, a comprehensive set of samples and measurements was obtained from each digester to determine final, steady-state performance parameters. In addition, a
continuous monitoring of biomethane production, pH, and selected performance parameters was performed in both reactors throughout an entire 48-h feeding cycle to investigate degradation kinetics.

4.5. Results and Discussion

Both the mesophilic and thermophilic anaerobic digesters were operated continuously for a period of over 540 days. Five of the parameters periodically monitored during the study are shown in Figure 4-4. HRT and feed composition changes are labeled in the graph according to the operating conditions and timeline shown in Table 4-2. Similarly, all the unplanned/unexpected incidents occurred during the course of the study, which may have affected the operating parameters, and potentially the performance and stability of the reactors, are labeled in the graph with event numbers that are briefly described in Table 4-3. Influent composition and loading rates of the individual substrate components for each period are shown in Table 4-4.
Table 4-3. Description of unanticipated events occurred during the 544 days of digester operation, which are flagged with a circular callout in Figure 4-4.

<table>
<thead>
<tr>
<th>Callout #</th>
<th>Day(s)</th>
<th>Reactor</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>84</td>
<td>Thermophilic</td>
<td>Problem with flow meter, wrong reading</td>
</tr>
<tr>
<td>(2)</td>
<td>146</td>
<td>Both</td>
<td>Perturbation during feeding cycle monitoring</td>
</tr>
<tr>
<td>(3)</td>
<td>200-204</td>
<td>Both</td>
<td>No feeding due to high VFA concentration in thermophilic reactor</td>
</tr>
<tr>
<td>(4)</td>
<td>232</td>
<td>Mesophilic</td>
<td>Heated water pump not recirculating; temperature drop to ~ 20 °C for approximately 15 hours</td>
</tr>
<tr>
<td>(5)</td>
<td>260-264</td>
<td>Mesophilic</td>
<td>Heated water pump not recirculating; temperature drop to ~ 20 °C for at least 4 days</td>
</tr>
<tr>
<td>(6)</td>
<td>348</td>
<td>Mesophilic</td>
<td>Heated water pump not recirculating; temperature drop to ~ 20 °C for undetermined number of hours</td>
</tr>
<tr>
<td>(7)</td>
<td>360-370</td>
<td>Mesophilic</td>
<td>Intermittent not proper mixing after increasing manure loading rate; solids settled on the bottom of reactor; impeller shaft lifted 3 cm to suspend solids</td>
</tr>
<tr>
<td>(8)</td>
<td>454</td>
<td>Both</td>
<td>Change to second batch of raw manure</td>
</tr>
</tbody>
</table>
Figure 4-4. Operating parameters measured during the operating period of 544 days; from top to bottom: volumetric biogas production rate (@ STP), pH, total volatile fatty acids (VFA), volatile solids (VS), and total COD (TCOD); square callout labels indicate dates where changes in feed composition were made as percent of manure added balanced with dog food, e.g. 25 = 25% manure, 75% dog food (see Table 4-2); circular callouts indicate unanticipated events occurred during the operating period as described in Table 4-3.
Table 4-4. Influent composition data vs. stability of digesters and their ability to reach steady state at each operating condition

<table>
<thead>
<tr>
<th>Period (P)</th>
<th>Organic loading rate (g/L-d)</th>
<th>Ratios (VS basis)</th>
<th>Stable/Steady state?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proteins</td>
<td>Lipids</td>
<td>Carbohydrates</td>
</tr>
<tr>
<td>P-1</td>
<td>0.41</td>
<td>0.41</td>
<td>0.40</td>
</tr>
<tr>
<td>P-2</td>
<td>0.55</td>
<td>0.54</td>
<td>0.53</td>
</tr>
<tr>
<td>P-3</td>
<td>0.26</td>
<td>0.40</td>
<td>0.39</td>
</tr>
<tr>
<td>P-4</td>
<td>0.39</td>
<td>0.59</td>
<td>0.59</td>
</tr>
</tbody>
</table>

\(^1\)Neutral detergent fiber (NDF): cellulose, hemicelluloses, and lignin; M: mesophilic CSAD, T: thermophilic CSAD

4.5.1. Startup and acclimation of anaerobic digesters

Acclimation of anaerobic digesters was conducted at a 30-d HRT by feeding only raw manure first and then introducing dog food in a step-wise manner until the target co-digestion proportion was reached (i.e., 75% VS basis). During this period, reactors experienced a slow and variable increase of biogas production, but greater accumulation of total VFA and resultant low pH were observed in the mesophilic digester (Figure 4-3). When biogas production rate and total VFAs were steady and comparable in both reactors, on day 62, feeding rate was increased to reach the target HRT of 20 days, the equivalent of an organic loading rate (OLR) of 1.5 g VS/L-d (Table 4-4).

4.5.2. Period 1 (20-d HRT, 25% manure – 75% dog food)

The first of two major upsets occurred in the thermophilic digester were observed during P-1. A few days after performing a feeding cycle monitoring (callout #2 in Figure 4-4), the concentration of VFAs of the thermophilic digester experienced a
steep increase from an average of 0.2 g/L to a value of approximately 6.4 g/L. The pH dropped below 6.3, and concurrently methanogenesis stopped. Recovery of the thermophilic digester was first attempted by suspending feeding for two feeding cycles (which was done in both CSADs), and secondly by adding granulated activated carbon (GAC). As discussed in more detail later, neither of these approaches produced any apparent changes; however, when feeding was restarted with a 100% manure influent, VFAs concentration started decreasing and methanogenesis resumed. Dog food was reintroduced in a step-wise manner during the course of several feeding cycles to reach the target co-digestion ratio of 25:75 manure-to-dog food. Steady-state conditions in the thermophilic digester regained after 100 days of the initial perturbation, which we assumed was caused by the 48-h monitoring. By the end of this period, VFAs had accumulated to an average of 1.0 g/L (15.9 mM) in the thermophilic digester, but a steady-state biogas production rate was observed. Conversely, the mesophilic digester showed no evidence of perturbation throughout the thermophilic digester upset, and steady-state conditions for this CSAD were observed after one HRT cycle at this period.

4.5.3. Period 2 (15-d HRT, 25% manure – 75% dog food)

An increase of the biogas production rate was observed in both reactors immediately after shortening the HRT from 20 to 15 days and increasing the OLR to 2 g VS/L-d (Table 4-4). However, further accumulation of VFAs with a concomitant decrease in
the biogas production was observed in the thermophilic digester (Figure 4-4). Biogas production virtually stopped between 25 and 30 days after P-2 started. Based on the reactor’s previous upset, we decided to increase the ratio of manure-to-dog food from 25:75 to 75:25 in order to keep the thermophilic digester operating at 15-d HRT. Upon making this change, biogas production resumed quickly, and complete recovery of the thermophilic digester was observed in ca. 20 days. Once again, the biogas production rate of the mesophilic digester was steady during the entire P-2, and the observed total VFA concentration was low (i.e., <0.05 g/L).

4.5.4. Period 3 (15-d HRT, 75% manure – 25% dog food)

In this period, the co-digestion ratio of manure-to-dog food was increased from 25:75 to 75:25. The thermophilic CSAD started this period ca. 70 days earlier than the mesophilic (see Table 4-2) due to the aforementioned premature failure experienced in P-2. As a result, the thermophilic digester spent double the time in this operating condition as compared to the mesophilic digester. Throughout this period, biogas production was steady and comparable in both digesters. A slight decrease in the rate of biogas production was observed in both reactors during the first third of the period, following a change in the source of raw manure (callout #6 in). This depression was stabilized and both reactors reached steady-state conditions soon after.
4.5.5. Period 4 (10-d HRT, 75% manure – 25% dog food)

With an HRT of 10 days, the OLR was increased to 3 g VS/L-d (Table 4-4). A comparable increase of over 30% in the biogas production rate was observed in both reactors, which were at steady-state after one HRT cycle. In P-4, the thermophilic digester exhibited a more marked (higher) difference in the biogas production rate, which was already visible in P-3. Furthermore, both reactors were stable during this entire period.

4.5.6. First upset of the thermophilic digester

4.5.6.1. Accumulation of LCFAs, VFAs, and hydrogen can cause digester failure at thermophilic temperatures

A clear relationship between the 48-h feeding cycle monitoring and the reactor failure is observed in Figure 4-4, because after being at steady-state for more than four HRT cycles, the thermophilic CSAD suddenly crashed only a few days after the monitoring was conducted. What are the causes that led to the thermophilic reactor failure? Our hypothesis is that accumulation of LCFAs and VFAs, and possibly high partial pressure of molecular hydrogen, ultimately caused the failure of the thermophilic reactor.

As shown in Figure 4-5, approximately one month before the perturbation triggered by monitoring (day 120), the total concentration of LCFAs in the thermophilic digester was nearly 40% higher than that measured in the mesophilic digester (4.48 vs. 
2.77 g COD/L, respectively). By the time methanogenesis had stopped in the thermophilic digester (ca. day 210), total LCFAs had been reduced to less than a half in this reactor (i.e. 2.14 g COD/L), while there was only ca. 16% reduction in the mesophilic digester (i.e. 2.21 g COD/L). Acetate and propionate concentrations in the thermophilic digester increased from less than 0.05 g COD/L each, on the day of the monitoring (day 148), to 2.31 g COD/L (36.15 mM) and 1.28 g COD/L (11.42 mM), respectively, on the day of the reactor failure. It is apparent that LCFA degradation rates proceeded faster in the thermophilic digester soon after the cycle monitoring, as evidenced by the sustained increase of total VFAs observed in Figure 4-4. By contrast, total VFA concentrations in the mesophilic digester decreased from ca. 0.7 g COD/L (ca. 98% acetate) to less than 0.1 g COD/L, showing no evidence of an upset. Even-carbon LCFAs are primarily degraded by H₂-producing bacteria to acetate and molecular hydrogen via β-oxidation (Jeris & McCarty, 1965; McInerney, 1988), while odd-carbon LCFAs are mainly degraded to acetate and propionate (Gottschalk, 1986; Nelson et al., 2008). Therefore, considering that all the LCFAs measured were even-carbon (Figure 4-5), it is reasonable to assume that most of the acetate observed on day 210 came from LCFA degradation. In fact, 77.4% of the total LCFA-COD could be accounted for by acetate-COD, which compares well with the 68.4% of palmitate oxidized to acetate reported by Jeris & McCarty (1965) from a study with radio-labeled carbon. The remaining LCFA-COD not reflected in acetate-
COD could be accounted for by molecular hydrogen; however, our laboratory did not have a reduction gas detector at the time of the study, and it was not possible to measure hydrogen in the gas phase. The source of propionate increase is more difficult to account for, because it comes from an array of different sources (e.g., amino acids, sugars, odd LCFAs); however, most of its accumulation was probably due to free-energy limitations imposed by the products of its oxidation, i.e., acetate and molecular hydrogen (Figure 4-2). Accumulation of propionate due to high partial pressures of hydrogen in thermophilic digesters has been reported many times in the literature, and in particular at thermophilic temperatures (Kim et al., 2002; McCarty & Smith, 1986; Speece et al., 2006).

The bioenergetic limitations for the oxidation of propionate and palmitate at the actual conditions observed in the mesophilic and thermophilic digesters are shown in Figure 4-6. The plot shows that at the observed acetate concentration when LCFA degradation had essentially stopped (day 210), palmitate degradation could only have proceeded if the partial pressure of hydrogen ($P_{H_2}$) was lower than $10^{-3.2}$ atm (Figure 4-6). Because LCFAs were not degrading, it is apparent that hydrogen levels were higher than this value. This is further supported by the fact that propionate, which exhibited a comparable hydrogen threshold at the observed acetate concentration, was already accumulating in the thermophilic CSAD (Figure 4-5).
Not surprisingly, propionate was not accumulating in the mesophilic digester on day 210, as its degradation was ca. 1.4 orders of magnitude less limited by the partial pressure of hydrogen (Figure 4-5). Interestingly, the comparable free energy limitations for the degradation of palmitate at both operating temperatures (Figure 4-6) could partially explain why both CSADs exhibited similar accumulation of LCFAs after the thermophilic crash (day 214).
Figure 4-5. Long- and short-chain (volatile) fatty acids and biogas production rate observed in the mesophilic (above) and thermophilic (below) CSADs before and after the mixing perturbation occurred on day 148, during the 48-h monitoring period; no LCFA measurements could be performed on days 148 (either reactor) and 210 (mesophilic CSAD).
Figure 4-6. Lines of constant, zero $\Delta G_r$ for propionate and palmitate oxidation, and hydrogenotrophic methanogenesis at mesophilic 37°C (left) and thermophilic 55°C (right) temperatures, as a function of $P_{H_2}$ and acetate; the plots were constructed using the actual concentrations of methane, carbon dioxide, LCFAs (as palmitate), and propionate at the days shown in the graphs; bicarbonate was assumed to be 100 mM.
4.5.6.2. Accelerated reaction rates at thermophilic temperatures can increase product inhibition

Why did the thermophilic digester exhibit higher concentrations of LCFAs than the mesophilic before the perturbation? Our hypothesis is that hydrolysis rates of neutral lipids are faster at thermophilic temperatures, which produced shock loads of LCFAs and a higher degree of inhibition in the thermophilic relative to the mesophilic CSAD. Although hydrolysis of particulate substrates is usually the rate-limiting step of anaerobic digestion, in lipid-containing substrates, degradation of LCFAs via β-oxidation can be the slowest conversion step and control the overall kinetics of the digestion process (Novak & Carlson, 1970; O'Rourke, 1968; Pavlostathis & Giraldo-Gomez, 1991; Rinzema et al., 1994). Differences between the rates of neutral lipids hydrolysis and β-oxidation of fatty acids can result in a reactant-product imbalance and a significant accumulation of LCFAs overtime. Certainly, in this case, accumulation of LCFAs in both digesters contrasted with the (lower) levels of acetate observed on day 120, especially in the thermophilic CSAD, suggesting that β-oxidation was limiting the extent of methanogenesis (Figure 4-5). This explanation also agrees with the results of Hanaki et al. (1981), who in batch digestion tests conducted at 37°C, observed that LCFAs hydrolyzed from whole milk (39% palmitic acid) accumulated within a day, albeit their degradation occurred over the course of several days. The same authors reported that LCFA accumulation could also be
accentuated at concentrations greater than 0.5 g/L because at this level, LCFAs were inhibitory for the \( \beta \)-oxidizing organisms themselves. Furthermore, at a concentration of 1.0 g/L the authors observed a lag phase of nearly 10 days for LCFA degradation, which consequently extended that of methanogenesis to over 20 days. On the other hand, Angelidaki & Ahring (1992), found that concentrations as low as 0.2 g/L of unsaturated oleic acid (C18:1) and 0.5 g/L of saturated stearic acid (C:18:0) increased the lag phase of methanogenesis in batch tests conducted at 55°C. Koster & Kramer (1987) observed a sharp decrease in the methanogenic activity at concentrations over 3 mM for capric (C10:0), myristic, (C14:0) and oleic acids, and of over 1 mM for lauric acid (C12:0). Although no apparent differences in biomethane production rates were observed between the mesophilic and thermophilic CSADs before the perturbation (see Figure 4-4 and Figure 4-5), above studies suggest that both digesters were within the inhibitory range during that period. This offers an explanation for the greater accumulation of LCFAs observed in the thermophilic digester (1.0 and 1.6 g LCFA /L, respectively), which could be attributed to a higher degree of inhibition, particularly of \( \beta \)-oxidizers, relative to the mesophilic digester. It is apparent, however, that methanogens in the thermophilic CSAD were not affected to a greater extent by the higher LCFA concentrations observed in this digester. The average biogas production rate of the thermophilic digester throughout the 20-d HRT cycle, and prior to the perturbation, was only slightly lower (ca. 4.8%) than that of the
mesophilic digester (see Figure 4-4 and Figure 4-5). Thus, it is plausible that, if the thermophilic (and mesophilic) reactor was in fact inhibited, the most affected process was $\beta$-oxidation, rather than methanogenesis. This is supported by the data, as practically no acetate accumulation was observed before the perturbation, indicating that, at least, acetoclastic methanogens were not affected. In addition, inhibition concentrations reported in above studies were obtained under batch conditions, and thus, results should be interpreted with caution. In semi- or continuous reactors, shock loads and inhibition are minimized. For example, Nielsen & Ahring (2006) found no inhibitory effects on the biogas production when adding pulses of 0.5 and 1.0 g/L of oleic acid to CSTRs, while Angelidaki & Ahring (1992) reported that a concentration of 0.2 g/L of the same fatty acid was inhibitory under batch conditions. In addition, most inhibition studies use LCFAs in a free form – a condition that does not necessarily represents real digester conditions, as it does not consider the gradual release of free, non-glycerol bound LCFAs from the hydrolysis of neutral lipids, and thus, their addition is likely to produce shock loads and inhibition.

In summary, accumulation of LCFAs in both reactors could have occurred as a result of differences between the rates of hydrolysis and those of $\beta$-oxidation. It is not clear, however, if these differences were caused by inhibition, or they can be attributed to purely dissimilar reaction rates of the two bioconversion processes. The answer is probably a combination of both. The greater accumulation of LCFAs in the
thermophilic digester operated in this study can probably be explained by faster hydrolysis rates relative to the mesophilic digester, which brought about greater accumulation of LCFAs in a shorter period of time and produced a relatively higher degree inhibition and additional accumulation.

4.5.6.3. Mixing intensity of CSADs is increased at thermophilic temperatures and can accelerate degradation of lipids

What triggered accelerated rates of LCFA degradation during the monitoring? As the angular velocity of the reactor’s impeller was the only operating parameter modified during the monitoring, it is believed that the accelerated LCFA degradation rates were related to the increased in the mixing intensity of the CSADs.

During the monitoring period, the angular velocity of both CSADs’ impeller was repeatedly increased from its regular operation of 100 RPM, to approximately 1,500 RPM. This operation was performed ca. 10 times during the 48-h monitoring. Although it has been shown that high shear rate (high mixing intensity) can reduce particle size and decrease diffusion limitations (Lanting, 2003), several other studies have shown that high mixing intensity and duration had a detrimental effect on reactor performance, as it can disrupt the syntrophic relationships between the hydrogen-producing bacteria and the hydrogen-utilizing methanogens (Hansen et al., 1999; Hoffmann et al., 2008; McMahon et al., 2001; Speece et al., 2006; Stroot et al., 2001; Vavilin & Angelidaki, 2005). However, if indeed hydrogen syntrophic
associations were affected by the vigorous mixing of the digestate, what caused accelerated LCFA degradation rates? High shear rates are used in to homogenize (disperse) immiscible substances, such as oil-in-water emulsions in the dairy processing industry. It is possible that in a similar fashion, naturally-occurred fatty acid emulsions in the digestate could have been homogenized by the vigorous mixing of the monitoring. With dispersed, smaller-diameter lipid emulsions in the digestate, the LCFA-to-water interface area could have been increased, thereby favoring the contact between substrate and fatty-acid oxidizing bacteria, and thus increasing β-oxidation rates. Even though, an increase of LCFA degradation was observed in both reactors after the mixing perturbation, a greater extent was achieved in the thermophilic digester (52.1% vs. 21.6% in the mesophilic). This difference could be explained by the effect of temperature on three physical properties, in particular: system entropy, viscosity of water, and physical state of fatty acids. As temperature rises, the entropy of the system increases as does the number of molecular collisions within the system. This produces an increase in the rate of reactions as described by the Arrhenius Equation. On the other hand, as temperature increases, water viscosity decreases, which in turn lowers the resistance for the mechanical torque of the impellers and results in an increase of the mixing intensity, i.e. shear rate, as described by the Reynolds number:
Eq. 4-9

\[ N_{Re} = \frac{\omega D_a^2 \rho}{2\pi \mu} \]

where, \( N_{Re} \) is the Reynolds number (dimensionless), \( \omega \) is the angular velocity of the impeller (rad/s), \( D_a \) is the impeller diameter (m), and \( \rho \) and \( \mu \) are the density (kg/m\(^3\)) and dynamic viscosity (kg/m-s) of the fluid, respectively. However, since \( \omega = 2\pi f \), Eq. 4-9 can be also expressed as:

Eq. 4-10

\[ N_{Re} = \frac{v D_a^2 \rho}{\mu} \]

where, \( v \) is the frequency of rotation (cycles/s), which allows an easier conversion between RPM and \( v \) (\( RPM = v/60 \)).

Figure 4-7. Mixing intensity as represented by the Reynolds number (\( N_{Re} \)) plotted against temperature. The blue and red lines marks the values at mesophilic and thermophilic temperature conditions. Calculated based on \( v = 1.67 \text{ s}^{-1} = 100 \text{ RPM} \), \( D_a = 0.062 \text{ m} \), and with \( \rho \) and \( \mu \) obtained from tabulated values as a function of temperature.
A plot of $N_{Re}$ as a function of temperature reveals that the 18°C difference between both anaerobic digesters creates a mixing intensity that is 36% higher in the thermophilic as compared to the mesophilic digester (Figure 4-7). This suggests that during the monitoring mixing events, the mixing intensity in the thermophilic digester corresponded to a value closer to 2,044 RPM rather than 1,500 RPM. With higher shear rate, a greater degree of homogenization, and therefore, an increased rate of LCFA degradation could have been observed in the thermophilic digester.

4.5.6.4. Physical state of fatty acids change with temperature and can impact their degradation

Can the rate of LCFA degradation be affected by their physical state? We postulate that fatty acids in a more liquid state can be more easily dispersed and therefore bioavailable for microbial/enzymatic degradation.

Depending on its degree of saturation and chain length, fatty acids have different melting points, which essentially change their physical state as a function of temperature. At room temperature, saturated LCFAs are usually found in a crystalline/solid state, whereas unsaturated LCFAs are observed in a liquid state. A plot of the melting points of saturated LCFAs reveals an apparent linear trend with their degree of saturation ($R^2 = 0.98$), which could be proven to be a useful relationship to be used in predicting LCFA degradation. As observed in Figure 4-8, for example, palmitic acid (C16), which constituted 42% of the total LCFA pool of
the thermophilic digester before the mixing perturbation, has a melting point of 62.9°C. This suggests that palmitic acid should have been in a practically liquid state in the thermophilic digester, whereas closer to a solid state in the mesophilic digester. With palmitic acid in a more liquid form, a higher extent of emulsification should have been achieved through mixing (and especially at 30% higher shear rate), which could have further increased its availability for β-oxidizers and therefore the rate of LCFA degradation.

Figure 4-8. Melting point (°C) of long chain fatty acids as a function of the number of carbon in the polymer chain (data from Boere Rogers et al., 2001); segmented blue and red lines mark mesophilic and thermophilic temperatures, respectively.

Another scenario that could have influenced the LCFA degradation rates as a result of increased mixing intensity is an increase in the mass transfer rate of hydrogen from the liquid to the gas phase, which could have lowered the partial pressure of H₂ (P_{H₂})
in the digestate and decreased the free energy required for LCFA oxidation. This scenario seems to be only plausible if $P_{H_2}$ were high enough to produce thermodynamic limitations for LCFA degradation at the time of the monitoring, i.e. $>10^{-2}$ atm (Figure 4-2). However, and although hydrogen was not measured, it is apparent that $P_{H_2}$ was lower than $10^{-4}$ atm as propionate was not accumulating at the time of the monitoring.

4.5.7. Second upset of thermophilic digester

4.5.7.1. Stability of lipid-rich co-digestion operations is increased at mesophilic temperatures and high manure-to-lipid ratios

The second upset of the thermophilic digester occurred after the hydraulic retention time (HRT) was shortened from 20 to 15 days, which effectively increased the overall organic loading rate (OLR) of the reactors from 1.5 to 2 g VS/L-d, and in particular, lipid loading rate from 0.41 to 0.54 g/L-d (Table 4-4). Is stability dependent on the lipid loading rate or the proportion between manure and lipid substrates? Results suggest that rather than lipid loading (i.e., concentration), it is the ratio of manure-to-lipid-containing substrate what determines stability.

In this upset, an increase of VFA concentrations and possibly $H_2$ led to LCFA accumulation and further accumulation of VFAs. As observed in Figure 4-9, on day 352, the concentration of acetate was comparable to that on day 210. This created similar free energy limitations for the degradation of propionate and LCFAs (as
palmitate) due to hydrogen, which suggests that the partial pressure of hydrogen was again over $10^{-3.2}$ atm. In fact, to decrease the lipid loading, the same recovery strategy used for the first crash was used this time, and the thermophilic digester was fed with only raw manure on day 352 and for the next three feeding cycles. Unexpectedly, by the next feeding cycle, on day 354, the LCFA concentration decreased by 47% (8.28 to 4.35 mM); however, the concentration of acetate or biomethane production did not increase, suggesting that the decrease of LCFAs was not the result of degradation. Although part of the LCFA reduction could be attributed to digestate dilution, only 13% of the digester volume was replaced with manure for that feeding cycle. If these compounds were not removed physically from the reactor and apparently no biological processes took place, could LCFAs have been removed via other means? It has been reported that the addition of adsorbents, such as bentonite, granular activated carbon, or biofibers, can effectively reduce LCFAs from solution, which in turn can reduce its bioavailability and therefore inhibitory effects (Nielsen & Ahring, 2006; Palatsi et al., 2009). Furthermore, Neves et al. (2009) conducted a study where a 26-L CSTR digester fed with cow manure was subjected to pulses of an oily waste with 99.8% lipid content, and found that LCFAs were adsorbed onto the biomass. This suggests that in this study, manure, and particularly its fiber component, could have acted as an adsorbent, where LCFAs accumulated. The question is, if these compounds were adsorbed onto the manure biomass, could they still be
extracted/detected through the analysis used in this study? The analysis conducted by Neves et al. (2009) involved a separate process for extracting LCFAs from the solid phase (i.e. attached to the solid matrix), where samples were dried to improve the extraction with the solvent. In the LCFA analysis performed in this study samples were treated as a whole, and no previous drying was performed before solvent extraction, which could have significantly lowered the recovery of LCFAs from the solid phase. This could be especially true in samples containing large portions of solid materials with adsorptive and/or absorptive properties. Indeed, Neves et al. (2009) found no traces of LCFAs in the liquid phase of their 100% raw manure digester following any of the oily-waste pulses conducted in their study. Therefore, it is possible that in our study, part (if not all) of the LCFAs adsorbed onto the biomass could not be recovered by our analysis, especially after only manure was added to the digester, as in the analysis of day 354. In this study, palmitic acid, which represented 44% (3.61 mM) of the total LCFA pool before adding manure, was reduced by 65% during the 48-h of the feeding cycle (days 352 – 354). Additionally, stearic and oleic acids, which combined constituted 23% of the total LCFAs before adding manure, were also reduced each in exactly 65% during the same time period, while capric, lauric, myristic and linoleic acids did not experience any significant change. This is in agreement with the study of Neves et al. (2009), who reported that the main LCFAs adsorbed/accumulated onto the sludge were palmitic and oleic acids (stearic acid was
negligible, as it constituted less than 2% of the LCFAs present in the oily-waste pulses). Furthermore, the rapid accumulation of LCFA onto the manure fibers observed in our study is in accordance with the observations of the previous authors and those of Hanaki et al. (1981), who suggested that LCFA could be accumulated on the digester biomass within 24 h. Above discussion suggests that, if not enough adsorbent material (i.e. manure fibers) is available for the adsorbate (i.e. LCFAs), or in other words, if the proportion between manure and LCFAs is not adequate, the adsorbent will eventually saturate, which will result in accumulation of LCFAs in the liquid phase. It is apparent that for P-1 and P-2 of this study, the ratio of manure-to-lipid-containing substrate (i.e. dog food) was too low for the amount of hydrolyzed lipids, i.e. free LCFAs, in the thermophilic digester. This was evidenced by the accumulation of these compounds in the liquid phase, which produced inhibition, instability, and ultimately failure of the digester. Thus, an excess of adsorbent material (manure) should decrease the bioavailability of free LCFAs, which in turn would reduce possible inhibition and increase the stability of the process. Indeed, even though the lipid loading rate was equally high at P-3, or even higher at P-4, no accumulation of LCFAs was observed in both periods, which maintained the thermophilic reactor exceptionally stable (Table 4-4). Therefore, it is evident that the amount of LCFAs that can be absorbed onto the manure fibers will depend on the proportion between manure and the lipid material, rather than the digester’s lipid
loading. In fact, this proportion can be more accurately assessed by the amount of adsorbent material of manure over the lipid substrate, i.e. fiber-to-lipid ratio. The fiber consists of the sum of cellulose, hemicelluloses, and lignin, as determined by the neutral detergent fiber (NDF). As observed in Table 4-4, the fiber-to-lipid ratio was 0.65 for P-1 and P-2, while it was nearly four times higher (i.e. 2.18) for P-3 and P-4, which helps to explain why no accumulation of LCFAs was observed in either reactor. Interestingly, lower accumulation of LCFAs was observed in the liquid phase of the mesophilic digester for Stages 1 and 2, despite the fact that both reactors had the same fiber-to-lipid ratio. As discussed previously, this was attributed to slower lipid hydrolysis rates, and it is the most likely reason why the mesophilic digester was stable in P-1 and P-2 whereas the thermophilic digester was not. Therefore, it can be concluded that the stability of the co-digestion processes is influenced by two main operating parameters: the ratio of manure-to-lipid and the anaerobic digestion thermal regime (as it affects the rate of LCFA production, i.e. lipid hydrolysis). Finally, it is apparent that relatively higher manure-to-lipid ratios should be required at thermophilic temperatures as compared to mesophilic temperatures.
Figure 4-9. Long- and short-chain (volatile) fatty acids and biogas production rate observed in the mesophilic (above) and thermophilic (below) CSADs before and after the HRT was decreased from 20 to 15 days on day 330.
4.5.8. Performance of anaerobic digesters at steady-state conditions

Main effluent parameters measured at steady state conditions during the four periods of this study are shown in Table 4-5. As expected, TS, VS and COD concentrations increased in the effluent as the HRT decreased due to hydrolysis rate limitations. The same was observed when the content of slowly degradable lignocellulosic manure in the mesophilic digester was increased from 25 to 75% while maintaining a constant HRT of 15 days. With the exception of the thermophilic digester at P-1, total measured VFAs at steady-state conditions were below 0.7 g/L for all digester periods. As discussed previously, total VFA concentrations were over 1.0 g/L by the end of P-1 in the thermophilic digester due to additional accumulation of LCFAs, which increased mostly acetate and propionate (Figure 4-5). Also, the pH, which appeared to be fairly constant throughout all the periods in both digesters, was lower in the thermophilic digester during steady-state conditions at P-1, probably due to the higher levels of VFAs. Also, it is apparent that the thermophilic digester exhibited higher pH relative to the mesophilic digester, a fact that has been reported in several studies (Gavala et al., 2003; Song et al., 2004; Watanabe et al., 1997), and, which it is attributed to the temperature difference. Higher ammonia-N concentrations were observed under thermophilic temperatures at P-3 and especially at P-4. This is expected, since the concentration of ammonia-N is positively related to the amount of
proteinaceous material degraded during digestion, which is usually higher under thermophilic temperatures (Parkin & Owen, 1986).

Biogas and biomethane production rates, specific biomethane yields, and volatile solids destruction at steady-state conditions during the four periods of this study are shown in Figure 4-10A – C, respectively. As previously discussed, no steady-state conditions could be attained for the thermophilic digester at P-2, as it failed at the beginning of this stage.

As observed in Figure 4-10A, biogas/biomethane production rates were higher in the mesophilic digester at P-1 (p < 0.01). This is expected given the difference in accumulation of LCFAs, acetate, and propionate between the thermophilic and mesophilic digesters (Figure 4-5). On the other hand, biogas/biomethane production rates were lower than those observed in the thermophilic digester at P-3 (p < 0.01) and P-4 (p < 0.01). Also, the methane content of biogas in both thermal regimes was between ca. 60% and 64% during the entire study; however, large variation was observed in both reactors during the feeding cycle. This is reported later in the manuscript. Also, a considerable decrease in the biogas/biomethane production rate of the mesophilic digester was observed when the co-digestion ratio was changed from 25% to 75% manure VS at a constant HRT of 15 days (i.e. P-2 to P-3). Additionally, independent of the digester operating temperature, biogas/biomethane production rates increased as the HRT decreased. This is expected, as the feeding rate
(i.e. OLR) was increased by 25% from P-1 to P-2 and 33% from P-3 to P-4. In the case of the mesophilic digester, rates increased with decreasing HRTs regardless of the co-digestion ratio. However, in the thermophilic digester, stability was strongly dependent on the co-digestion ratio, and reactor failure occurred soon after the HRT was decreased from 20 to 15 days and the manure content was 25%.

The observed specific biomethane yields (SMY), or $B_o$, of the mesophilic and thermophilic digesters at the four periods are shown in Figure 4-10B. The theoretical SMY ($B_u$), which is represented by a segmented line in Figure 4-10B, was calculated for the two co-digestion ratios evaluated in this study using the Buswell Formula, as described in Chapter 2, and detailed substrate chemical composition, as reported in Chapter 3.

At P-1, the observed SMYs ($B_o$) represented a 76% and 64% of the theoretical SMY ($B_u$) in the mesophilic and thermophilic digesters, respectively. Interestingly, despite of the shorter HRT, $B_o$ of the mesophilic digester at P-2 was only slightly lower than that at P-1, and represented 72% of $B_u$ ($p < 0.05$); in fact, the difference was within the SD of the observed SMY at P-1 and P-2. This suggests that the substrate utilization efficiency was only slightly affected by shortening the HRT from 20 to 15 days when the manure content was 25%. Even more interesting, the differences between $B_o$ at P-3 and P-4 in both digesters were not significant ($p > 0.05$), and represented 49% and 52% of $B_u$ for the mesophilic and thermophilic digesters,
respectively. Likewise, this suggests that substrate biodegradability at both thermal regimes was not affected by decreasing the HRT from 15 to 10 days when the content of manure was 75%. This is also supported by the degree of volatile solids stabilization achieved at P-3 and P-4, as discussed below. A more in depth discussion about substrate biodegradability is presented in Chapter 3.

As expected, the stabilization (i.e. destruction) of volatile solids (VS) during the four stages of the study followed a similar trend as the $B_0$ (Figure 4-10C). In P-1, the thermophilic digester exhibited a significantly lower VS destruction relative to the mesophilic (p < 0.05), probably attributed to its higher levels of LCFAs and depressed kinetic rates due to acetate and/or propionate inhibition (Figure 4-5). However, although not statistically significant (p > 0.05), slightly higher VS destruction was achieved by the thermophilic digester in P-3. Not surprisingly, in P-4, the thermophilic digester presented a significantly higher VS destruction relative to the mesophilic digester (p < 0.05), as the effects of faster reaction rates (particularly hydrolysis) in the thermophilic digester at P-3 and P-4 should become more noticeable at shorter hydraulic retention times. Also, the change in co-digestion composition at P-3 produced a decrease from 60.9% to 39.4% in VS destruction, a statistically significant difference (p< 0.05), which was due to an increase in manure content that decreased the biodegradability of the substrate mixture.
Figure 4-10. (A) Biogas and biomethane production rates (@STP), (B) observed specific biomethane yields (@STP), and (C) volatile solids destruction at steady-state conditions for P-1 and P-2 (25% manure at 20d and 15d HRT, respectively) and P-3 and P-4 (75% manure at 15d and 10d HRT, respectively); percent value in lower bars of (A) indicates the methane concentration in biogas; the theoretical specific biomethane yield (@STP) in (B) is represented by a segmented line; error bars in (A), (B), and (C) represent the standard deviation of at least three different measurements at steady-state.
Table 4-5. Main effluent parameters measured during steady-state conditions at each period

<table>
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<tr>
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<th>20d HRT MnDF2575 P-1</th>
<th>15d HRT MnDF2575 P-2</th>
<th>15d HRT MnDF7525 P-3</th>
<th>10d HRT MnDF7525 P-4</th>
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<td>VS (g/L)</td>
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<td>10.60</td>
<td>11.72 F</td>
<td>18.17</td>
</tr>
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<td>COD (g/L)</td>
<td>10.87</td>
<td>17.21</td>
<td>15.75 F</td>
<td>22.33</td>
</tr>
<tr>
<td>Total VFA (mg/L)</td>
<td>60.4</td>
<td>1240.5</td>
<td>622.9 F</td>
<td>19.9</td>
</tr>
<tr>
<td>pH (pH units)</td>
<td>7.24</td>
<td>7.37</td>
<td>7.27 F</td>
<td>7.28</td>
</tr>
<tr>
<td>Ammonia (mg/L as N)</td>
<td>1038.2</td>
<td>884.3</td>
<td>1318.8 F</td>
<td>763.6</td>
</tr>
</tbody>
</table>

Mn: manure; DF: dog food; M: mesophilic; T: thermophilic; F: reactor failure
Similarly, with the decrease in HRT from 20 to 15 days at constant 25% manure content (i.e. P-1 to P-2), the percent of VS destruction of the mesophilic digester decreased significantly (p < 0.05), from 71.5% to 60.9%. Interestingly, shortening the HRT from 15 to 10 days increased the VS destruction from 39.4% to 42.1% and from 40.9% to 45.1% in the mesophilic and thermophilic digesters, respectively. However, this change was statistically significant in the thermophilic digester (p < 0.05), but not in the mesophilic digester (p > 0.05).

An overall analysis of Figure 4-10 suggests that biomethane production rates can be significantly increased by shortening the HRT (increasing OLR) up to the limiting value determined by the washout of microorganisms; however, destruction of volatile solids and efficiency of biomethane produced per mass of substrate fed do not necessarily decrease as HRT is decreased.

4.5.9. Feeding cycle monitoring

In a semi-continuous reactor, feeding is done in pulses. During the four periods conducted in this study, both mesophilic and thermophilic digesters were fed in 48-h pulses. During the feed-to-feed cycle period parameter fluctuations can be observed. Once at steady-state conditions, both reactors were subjected to a feeding cycle monitoring, where biogas production and methane and carbon dioxide content were frequently measured. The change in these parameters over the 48-h cycle is shown in Figure 4-11 through 11, for each period, respectively.
Figure 4-11 confirms that biogas and biomethane production rates were significantly higher in the mesophilic digester at P-1, as shown in Figure 4-10A; however, Figure 4-11 also shows that the rate differences were not apparent until approximately 8 hours after feeding. With already elevated concentrations of VFAs in the thermophilic digester, methanogens could have been inhibited due to the shock load of VFAs following feeding. On the other hand, the mesophilic digester exhibited faster but variable biomethane production rates during the first 24 hours and lower but more stable rates towards the end of the cycle. Although both reactors experienced a significant fluctuation in methane content during the feeding cycle at P-1, a considerable larger variation as represented by the standard deviation (SD) was observed in the thermophilic digester (SD = 6.54%) as compared to the mesophilic (2.40%). Indeed, a more significant and faster drop of methane concentration was observed in the thermophilic digester during the first 9 hours after feeding, probably due to faster hydrolysis and consequently acidogenesis and acetogenesis, which also decreased the reactor’s pH during the same time period (data not shown).

Relative to P-1, a more significant initial drop in methane content was observed in the mesophilic digester at P-2, when the HRT was decreased to 15 days (SD = 5.97%); however, a more sustained biomethane production rate was observed throughout the feeding cycle (Figure 4-12).
At P-3, biogas production was evidently faster in the thermophilic digester; however, when looking at the biomethane production rates, the difference between both reactors is only obvious after 32 hours of feeding (Figure 4-13). This is explained by the initial lower methane content observed in the thermophilic digester, which tends to increase faster than that of the mesophilic towards the last 16 hours of the feeding cycle. As in P-1, the methane content variation was more significant in the thermophilic (SD = 5.36%) than in the mesophilic (SD = 2.61%) digester. In addition, probably due to the substrate composition change, the biomethane production rate of the mesophilic digester during the 48-h cycle decreased considerably faster at P-3 than at P-2. At P-2, the larger portion of the influent substrate was easily-degradable dog food (i.e. 75% VS basis), which explains the rather sustained biomethane production rate throughout the feeding cycle.

Faster biogas and biomethane production rates of the thermophilic digester relative to the mesophilic become more evident at P-4 (Figure 4-14). In addition, even though the initial drop of methane concentration appears to be earlier in the cycle, the recovery also occurs faster. Again, the variation of methane content is greater in the thermophilic digester than in the mesophilic (SD of 5.04% and 3.93%, respectively). Also, the biomethane production rates over the 48-h cycle appear to decrease at a comparable pace as those at P-3.
Figure 4-11. Change in the biogas parameters following reactor feeding at $t = 0$ for P-1 at steady-state conditions (feeding was conducted every 48 hours); (A) cumulative biogas volume, (B) cumulative biomethane volume, (C) methane content in biogas, and (D) biogas production rate; all volumes reported at STP.
Figure 4-12. Change in the biogas parameters following reactor feeding at $t = 0$ for P-2 at steady-state conditions (feeding was conducted every 48 hours); (A) cumulative biogas volume, (B) cumulative biomethane volume, (C) methane content in biogas, and (D) biogas production rate; all volumes reported at STP.
Figure 4-13. Change in the biogas parameters following reactor feeding at \( t = 0 \) for P-3 at steady-state conditions (feeding was conducted every 48 hours): (A) cumulative biogas volume, (B) cumulative biomethane volume, (C) methane content in biogas, and (D) biogas production rate; all volumes reported at STP.
Figure 4-14. Change in the biogas parameters following reactor feeding at $t = 0$ for P-4 at steady-state conditions (feeding was conducted every 48 hours); (A) cumulative biogas volume, (B) cumulative biomethane volume, (C) methane content in biogas, and (D) biogas production rate; all volumes reported at STP.
4.5.10. Rate-limiting steps at steady-state conditions

The slowest step in a sequence of reactions is usually called the rate-limiting step (Hill, 1977). In anaerobic digestion of complex substrates, such as primary or secondary sludge, the hydrolysis of particulate matter to soluble products has been reported to be the rate-limiting step (Eastman & Ferguson, 1981; Ghosh, 1981; Pavlostathis & Giraldo-Gomez, 1991). This study was not the exception. Figure 4-15 shows the contribution of particulate and soluble COD to the total COD pool measured in the effluent of the digesters at steady-state conditions during the four stages of the study. The graph suggests that hydrolysis of particulate material to soluble products is the rate-limiting step under both mesophilic and thermophilic temperatures for the three HRTs and two feed compositions.

At P-1, the particulate material contributes over 60% of the total effluent COD at either thermal regime, while from P-2 – P-4, it is over 80%. Also, at P-3 and P-4, the thermophilic digester achieved a relatively greater extent of particulate material solubilization than the mesophilic due to faster hydrolysis rates. It is puzzling, however, that the effluent total COD was slightly higher in the thermophilic digester compared to the mesophilic – but, it is possible that this difference is due to the large variability of the COD measurements evidenced by the large SD.

Effluent COD data confirms the VS destruction pattern observed in both reactors depicted in Figure 4-10C, where greater stabilization is observed at 20-d HRT and
25% manure content (P-1), and comparable VS destruction is observed at both 15 and 10-d HRTs and 75% manure content (i.e., P-3 and P-4).

It has been reported that in anaerobic digestion, the rate-limiting step is related to the nature of the substrate, process configuration, temperature, and loading rate (Pavlostathis & Giraldo-Gomez, 1991). Likewise, hydrolysis of particulate material was also influenced by the reactor thermal regime (i.e. operating temperature), loading rate (i.e., HRT), and substrate composition.

![Figure 4-15](image-url)

Figure 4-15. Particulate (PCOD) and soluble (SCOD) COD contribution in the effluent of the mesophilic and thermophilic anaerobic digesters at the four periods; values inside the bars represent the percent share to total COD; error bars represent the standard deviation of at least three different measurements at steady-state.
4.5.11. Final Remarks

Results, from this study, indicate that at longer HRTs, the mesophilic digester yields greater biogas production and volatile solids destruction than the thermophilic digester. As discussed previously, the reason is attributed to product inhibition, which is a direct consequence of the feed composition. During the 20-d HRT stage of this study, 75% dog food (VS basis) was added in the influent, and with that, 27% of lipid material. Long chain fatty acids (LCFA), which arise from the hydrolysis of neutral lipids, accumulated in the thermophilic digester probably as a result of the differences between the reaction kinetics of lipid hydrolysis and LCFA oxidation. The thermodynamic implications of substrate-to-product imbalance have been previously discussed, because it causes the accumulation of intermediate products, such as LCFAs, VFAs and H₂, which can create inhibition, toxicity and further thermodynamic limitations for the reactions.

Thus, what appears to be a comparative disadvantage of the mesophilic digester is actually an advantage at long HRTs and high lipid influents, because under mesophilic conditions lipids may be flushed out before they get hydrolyzed, while in a thermophilic digester, LCFAs may be produced faster than they can break down. In contrast, at shorter HRTs and high manure content, thermophilic digestion offers comparative advantages over mesophilic digestion by increasing the rates of
biomethane production, efficiency of substrate utilization, and organic matter stabilization.

The HRT and process thermal regime should be chosen depending on whether the objective of anaerobic digestion is stabilization or biogas production – If it is both, as it normally is, it will be a trade-off between the two goals, where higher HRTs will favor better stabilization, and lower HRTs will favor biogas yields. Likewise, thermophilic digestion will offer comparable process stability and better organic matter stabilization than mesophilic digestion at lower HRTs, which has the potential of decreasing reactor volumes, thereby reducing capital costs; however, the mesophilic conditions will ensure a more stable process over a wider variety of HRTs. Overall, the optimal HRT for process performance and stability will depend on the thermal regime and the chemical composition of the substrate being digested.
4.6. Conclusions

- From this study, it can be concluded that the stability of the co-digestion processes is influenced by two main operating parameters: the ratio of manure-to-lipid and the anaerobic digestion thermal regime (as it affects the rate of LCFA production, i.e. lipid hydrolysis).

- Based on the results of this study and the literature, stability of AD under thermophilic temperatures appears to be quite sensitive to the hydrodynamics of the reactor/vessel, specifically to turbulence and shear rate created by mixing intensity.

- Hydrolysis of particulate components appear to be the rate limiting step at all HRTs and feed compositions evaluated under mesophilic and thermophilic temperatures.

- At shorter HRTs and high manure content, thermophilic digestion offers advantages over mesophilic digestion by increasing the rates of biomethane production, efficiency of substrate utilization, and organic matter stabilization.

- Mesophilic digestion appears to be a more stable process regardless of the HRT and substrate chemical composition.
4.7. References


CHAPTER 5

5.1. Conclusions and future work

The results from this study yielded the following conclusions according to the research questions set forth in Chapter 1:

*What makes a substrate more or less biodegradable?*

Results showed that substrates rich in easily-degradable carbohydrates and a high volatile solid content have high biomethane yields; lipid-rich substrates were less degradable, but exhibited the highest biomethane yields as a result of their significantly higher energy density, i.e., chemical oxygen demand. More recalcitrant lignocellulosic-materials led to lower biomethane yields as a result of both low biodegradability and chemical oxygen demand.

A more detailed analysis of the biodegradation of individual substrate constituents revealed that, with the exception of starch, lignin, and lipids at inhibitory concentrations, sugars, lignin-free cellulose and hemicellulose, and proteins are highly biodegradable under both mesophilic and thermophilic conditions. This study also confirms previous reports that the biodegradability fraction and biomethane potential of lignocellulosic substrates are strongly correlated with the lignin content.
How do the operating temperature and substrate chemical composition affect biodegradability, biomethane yields and the stability of the process?

Results suggest that in absence of inhibition, overall biodegradation rates are faster at thermophilic conditions, but biodegradability and biomethane yields at steady-state are comparable under both operating temperatures.

Under semi-continuous conditions, stability of process is strongly influenced by the manure-to-lipid ratio and the anaerobic digestion operating temperature. Product inhibition is more likely to occur at thermophilic temperatures due to increased lipid hydrolysis rates and resulting long-chain fatty acid accumulation. Additionally, process stability at thermophilic temperatures appears to be more sensitive to temperature fluctuations and the hydrodynamics of the reactor/vessel, specifically to mixing intensity.

At shorter retention times and higher manure content, the thermophilic digester outperformed the mesophilic digester with increased rates of biomethane production, efficiency of substrate utilization, and organic matter stabilization. Mesophilic digestion, however, appears to be a more stable process regardless of the operating parameters, e.g., retention time, substrate chemical composition, mixing intensity.
Is it possible to predict steady-state anaerobic biodegradability and biomethane yields of semi-continuously-fed anaerobic digesters using analytical or theoretical methods?

The accuracy of stoichiometry-based methods for predicting biomethane yields of complex substrates strongly depended on the inclusion of the biodegradability fraction. Based on these results, a methodology to estimate the biodegradability fraction based on the chemical composition of the substrate, was developed. Combining this methodology and existing stoichiometric methods (i.e., Buswell Formula) to predict biomethane yields it was possible to produce results that were mostly within 15% of those observed at steady-state conditions in the semi-continuous CSADs; acceptable results, considering that the differences of the analytical techniques, such the BMP assay used in this study, are well within this range.

How feasible would it be to use such methods in practical situations?

The methodology developed for estimating the biodegradable fraction of complex substrates uses biodegradability data obtained in this study to correct the stoichiometry-based equations to predict biomethane yields. Thus, the method can produce satisfactory predictions provided that an approximate composition of the substrate to be digested is available. This is especially feasible for on-farm co-digestion operations, where substrates of known composition, particularly food wastes, are digested with a well-characterized substrate, such as manure.
Future work

From the findings of this study, several research proposals have been identified:

- Investigate adsorption (bio-sorption) characteristics of manure fibers, particularly on reducing the inhibitory effects of long-chain fatty acids

- Evaluate long-term process stability under semi-continuous conditions and thermophilic temperatures using different manure-to-dog food co-digestion ratios

- Investigate possible synergistic effects on biodegradability and biomethane yields when co-digesting manure with high-strength substrates

- Validate biomethane and biodegradability predicting methods against large-scale semi- or continuous anaerobic digesters

- Survey the microbial community structure of co-digestion operations under mesophilic and thermophilic temperatures to relate specific microbial groups and/or diversity to performance and stability of the process
6.1. Analytical Methods

6.1.1. Amylase-treated neutral detergent fiber (aNDF) analysis

*Adapted from Mertens et al. (2002)*

**Description**

Plant cells can be divided into less digestible cell walls comprised of hemicelluloses, cellulose, and lignin, and mostly digestible cell contents, comprised of starch and sugars. These two components can be separated by the use of two distinct types of detergents, i.e., a neutral detergent (ND) and an acid detergent (AD). Neutral detergent solution and heat-stable α-amylase are used to dissolve easily digested proteins, lipids, sugars, starches, and pectins in organic materials, leaving a fibrous residue that is primarily cell wall components in plant materials (cellulose, hemicelluloses, and lignin) and indigestible nitrogenous matter in animal products.

**Reagents**

- Sodium sulfite ACS – Na$_2$SO$_3$ anhydrous (Sigma-Aldrich, Co.)
- Heat-stable alpha amylase solution – ANKOM FAA (Ankom Technology, Co.)

Neutral-detergent (ND) solution – to 500 mL distilled water, add 18.6 g disodium EDTA, 4.56 g dibasic sodium phosphate (Na$_2$HPO$_4$), 6.81 g sodium borate decahydrate (NaB$_4$O$_7$·10H$_2$O), and mix until dissolved (heat if necessary). Under a hood, add 30 g sodium lauryl sulfate and additional 200 mL of DDI water. Mix until detergent is dissolved, and add 10 mL triethylene glycol to suppress foam.

Add 290
mL of DDI water to reach a total volume of 990 mL. Mix thoroughly. Verify that pH is between 6.95 and 7.05, and adjust with concentrated HCl or NAOH, as required (if pH is off by > 0.5 units, discard). Store ND solution at room temperature or, if cool storage causes precipitation, warm to 25°C, and mix before use. RECORD DATE SOLUTION WAS PREPARED, pH MEASUREMENTS, AND ADJUSTMENTS IN REAGENT LOG BOOK.

**Determination**

1. Dry empty crucibles for >4 h at 105°C and weigh (hot directly from the oven or room temperature after desiccation). Record empty crucible weight for test portions ($W_{crucible}$) to nearest 0.0001 g. Mix thoroughly and weigh 0.5 ($\pm 0.0500$) g of air-dry material, or equivalent amount of wet material into refluxing beaker ($W_{sample}$). To report results in a dry matter basis, weigh a second test portion at the same time for dry matter determination (DM).

2. Preheat calibrated reflux units. Add 0.5 ($\pm 0.1$) g sodium sulfite and 50 ($\pm 5$) mL ND to each refluxing beaker and swirl (critical for starchy feeds that stick to bottom during refluxing). Note: do not add ND and sodium sulfite to test portions more than 60 min before refluxing. Heat to boiling within 4 – 5 min, add 250 uL working amylase solution, resuspend particles stuck to bottom or sides, and swirl.

3. Reflux for 60 min at boiling temperature that creates vigorous particle movement, but not excessive foaming that carries particles up the side of the beaker. Mixtures may foam vigorously for 1 – 2 min. Note: do not reduce temperature of heating unit. 5 – 10 min after amylase is added, rinse sides of beaker using a bottle with fine nozzle to resuspend particles attached to the sides with minimum amount of ND.
4. Remove extracted mixture from heating unit and let particles settle for 30 – 60 s. Before transfer, observe mixture to determine if lipid globules are present on surface, or if solution is milky, which indicates that test sample should be rerun after acetone pre-extraction. Place Teflon stirring rod in crucible and preheat by adding 40 mL boiling water for 40 – 60 s.

5. Remove water with vacuum, and immediately pour top 30 – 40 mL of solution, keeping beaker inverted over crucible. Use minimum vacuum to evacuate excess liquid. Note: make sure to close vacuum before residue becomes dry, as excessive vacuum and evacuating to dryness cause some residues to clog crucible and not wash properly. Rinse all unattended particles into crucible using a fine stream of boiling water. Fill crucible half full with hot water. Add 250 µL of amylase and stir.

6. React with amylase for a minimum of 45 – 60 s while scrapping remaining particles from bottom and sides of reflux beaker with rubber policeman. Evacuate amylase solution and transfer any remaining residue from reflux beaker into crucible with 20 – 30 mL boiling water. Two rinses are usually sufficient. After transferring residues from beaker, fill crucible ¾ full with boiling water and soak for 1 – 3 min.

7. Evacuate water, add 40 – 50 mL boiling water, soak 3 – 5 min, and repeat. If residues are difficult to filter after first soak, add additional 250 µL amylase solution.

8. Evacuate water, but do not evacuate fiber residues to dryness, just remove enough water to leave a damp or moist residue before adding acetone. Refill crucible with 40 – 50 mL acetone, stir to disperse particles, soak 3 – 5 min, and repeat, rinsing stir rod to remove attached fiber particles.

9. Vacuum residue dry, remove crucible from manifold, and air dry for 10 – 60 min to remove acetone. Dry crucibles at 105°C for a minimum of 8 h and weigh
(W\textsubscript{dry}). Ignite crucible and fiber in 500°C furnace for 5 h. Temper in 105°C oven for at least 1 h and weigh (W\textsubscript{ash}). Weigh crucibles containing fiber or ash residues (hot or desiccated to room temperature) in same order as empty crucibles.

**Calculations**

Eq. 6-1
\[
aNDF = \frac{(W\text{dry} - W\text{crucible})}{(W\text{sample} \cdot \text{DM})}
\]

Eq. 6-2
\[
aNDF\text{om} = \frac{(W\text{dry} - W\text{ash})}{(W\text{sample} \cdot \text{DM})}
\]

where, aNDF is amylase-treated neutral detergent fiber (decimal), aNDF\text{om} is the organic matter portion of aNDF (decimal), W\text{X} corresponds to the weight of the x component (g), and DM is the dry matter content of the air-dried sample (decimal).

**6.1.2. Acid detergent fiber (ADF) and lignin (ADL) determination**

*Adapted from Moller (2009)*

**Description**

Acid detergent fiber (ADF) is determined in the first stage of the method. Cationin detergent solution is used to remove acid-labile carbohydrates, protein that is not complexed into Maillard products (heat-damaged), and fats. The remaining fibrous residue is primarily cellulose and lignin. The residue is weighted to determine ADF. In the second stage, the remaining residue is solubilized by 72% H\textsubscript{2}SO\textsubscript{4}, leaving the acid detergent lignin (ADL), or simply, lignin, which is determined gravimetrically (Moller, 2009).
Reagents

- Acid detergent solution – add 20 g cetyl trimethyl-ammonium bromide, technical grade (Sigma-Aldrich, Co.) to 1 L 0.5 M H₂SO₄, previously standardized. Agitate to aid solution.
- Sulfuric acid 72% by weight – standardize >95% sulfuric acid to specific gravity 1.634 at 20°C or 12 M. Calculate grams acid and water needed to prepare 1000 mL of solution using the following formula:

\[
\text{Grams acid needed} = \frac{100 \times 98.09 \times 12}{\text{H}_2\text{SO}_4 \text{ assay} \%}
\]

where, 1.634 is the density of 72% H₂SO₄. Weigh water into 1000 mL volumetric flask and add the calculated amount of H₂SO₄ slowly with occasional swirling. The flask must be cooled in water in order to add the required weight of acid. Cool to 20°C and check to make sure the volume is correct. Meniscus should be within 0.5 cm of the calibration mark at 20°C.
- Acetone – technical grade

Determination ADF

1. Dry empty crucibles and record tare weight \(W_{\text{crucible}}\)
2. Weigh ca. 1 g of dried material to an accuracy of ±0.002 g \(W_{\text{sample}}\)
3. Weigh a portion of sample for dry matter determination (DM)
4. Immediately before refluxing, add 100 mL of acid detergent solution at room temperature (do not add acid detergent >10 min before refluxing)
5. Heat to boiling in 5-10 min, reflux for 60±5 min from the time of onset boiling
6. After 5-10 min of refluxing, rinse down sides of beaker using a fine stream of acid detergent solution (<5 mL)
7. After refluxing, remove each beaker from the heating unit, swirl and filter into crucible – use a fine stream of boiling water to rinse all particles into the crucible.

8. Remove all acid detergent and rinse water using minimum vacuum.

9. Close vacuum and fill crucible with ca 40 mL of hot water (90-100°C), stir to break up the residue filter mat, and let soak 3-5 min – vacuum dry.

10. Repeat step 9 twice.

11. Rinse sides and bottom of crucible to remove acid completely.

12. Add 30-40 mL acetone, stir to break up all lumps and expose all particles to acetone, and let soak 3-5 min – vacuum dry.

13. Repeat step 12 twice.

14. Dry crucibles >3 hrs, preferably overnight, at 105°C.

15. Cool crucibles at least 15 min in desiccator and weigh \( W_{\text{dry}} \).

**Determination ADL**

1. Place crucibles in a shallow metallic tray and cover contents with cooled (15°C) 72% \( \text{H}_2\text{SO}_4 \) and stir with glass rod to smooth paste, breaking all lumps.

2. Fill crucible about halfway with acid and stir, leave glass rod in crucible, refill with 72% \( \text{H}_2\text{SO}_4 \) and stir hourly as it drains, keeping crucible at 20-23°C (cool of necessary).

3. After 3 h, filter as completely as possible with vacuum, and wash with hot water, rinsing sides of the crucible and glass rod well until acid free – remove glass rod.

4. Place crucibles in hot oven and dry >3 h at 105°C, cool in desiccator and weigh \( W_{\text{acid dry}} \).

5. Ignite crucibles at 525°C in furnace for 3 h.
6. While still hot, place crucibles in oven at 105°C for 1 h, cool in desiccator and weigh ($W_{\text{acid ash}}$).

**Calculations**

Eq. 6-5

$$ADF = \frac{(W_{\text{dry}} - W_{\text{crucible}})}{(W_{\text{sample}} \cdot DM)}$$

Eq. 6-6

$$ADL = \frac{(W_{\text{acid dry}} - W_{\text{crucible}})}{(W_{\text{sample}} \cdot DM)}$$

Eq. 6-7

$$ADL_{om} = \frac{(W_{\text{acid dry}} - W_{\text{acid ash}})}{(W_{\text{sample}} \cdot DM)}$$

where, ADF is acid detergent fiber (decimal), ADL is acid detergent lignin (decimal), ADL_{om} is the organic matter portion of ADL (decimal), $W_x$ corresponds to the weight of the x component (g), and DM is the dry matter content of the air-dried sample (decimal).
6.1.3. Total carbohydrates

Adapted from Gaudy (1962)

Reagents

- Anthrone, 100 mL portion – dissolve 0.2 grams of Anthrone in 100 mL of 95% H₂SO₄. Store until ice cold - highly reproducible results are achieved when the Anthrone reagent is prepared fresh and refrigerated 2-6 hours prior performing the analysis.

Determination

Total and soluble/dissolved carbohydrates are determined through the same method, with exception that for soluble carbohydrates the sample is first passed through a 0.22-µm membrane filter.

- A 2.5 mL sample (or a smaller amount diluted to 2.5 mL with distilled-de-ionized (DDI) water), containing less than 75 mg/L of sugars, a reagent blank, and at least 4 dilutions of a standard glucose solution are prepared in screw-cap culture tubes.
- All tubes are placed in a cool-water bath (3-5 ºC) to equilibrate.
- 5 mL of ice cold freshly prepared Anthrone reagent are added to the tubes while still in the water bath. The tubes are closed with the screw caps, shaken thoroughly, and then placed in a boiling water bath for exactly 15 minutes.
- After the boiling time has elapsed, the tubes are placed in a cool-water bath. After reaching ambient temperature, absorbance is measured at 620 nm.
6.1.4. Lipids by wet extraction

*Adapted from Loehr and Robich (1962)*

**Apparatus and reagents**
- Waring blender
- Buchner funnel (12.5 cm)
- Whatman #40 filter paper or equivalent (12.5 cm)
- 500-mL separatory funnel
- Hydrochloric acid (1N)
- Methanol (analytical reagent)
- Chloroform (analytical reagent)

**Determination**
1. Acidify a 25-mL sample to pH ~ 3 with approximately 1N HCl.
2. Transfer acidified sample to a Waring blender and add 20 mL chloroform and 50 mL methanol in this order, using the same graduate cylinder for the sample, chloroform, and methanol.
3. Blend sample and solvents for 1 min.
4. Add 30 mL chloroform and blend for 30 secs.
5. Add 25 mL DI water and blend for 30 secs.
6. With suction, filter the blender contents through the filter paper in a Buchner funnel into a 500-mL suction flask.
7. Rinse the blender with 10 mL of methanol and add washings to the filter.
8. Transfer flask contents to a 500-mL separatory funnel, wash flask with 12 mL chloroform and 12 methanol, and pour washings into the separatory funnel.
9. Allow funnel contents to stand until separation is complete.

10. Withdraw the chloroform bottom layer into a previously-tared 125 or 250-mL Erlenmeyer flask containing a few glass beads, leaving a small amount of chloroform behind. The phases should break calmly after 2-5 mins. If an interfacial emulsion remains, it should not be withdrawn into the tared flask, but should be left behind with the methanolic layer.

11. Add 12 mL of chloroform to the separatory funnel, agitate, and after the phases separate, take off the bottom layer into the same tared flask.

12. Evaporate almost all of the solvent from the tared flask in a water bath at 80°C and remove the remaining solvent from the flask with a jet of dry air.

13. Dry the tared flask in a 103°C oven for five mins, or until any water clinging to the sides of the flask has evaporated. Cool the dried flask in a desiccator for 30 mins and weigh.

Calculation

Eq. 6-8

\[ \text{Total lipids (mg/L) = } \frac{(A-B) \times 1000}{\text{ml sample}} \]

where, A is the increase in weight of the tared flask plus sample (mg) and B is the increase in weight of the tared flask plus blank, i.e. DI water + solvents (mg)
6.1.5. Total proteins (Lowry method)

Adapted from Lowry (1951) and Olson & Markwell (2001)

Apparatus and reagents

- 1 mg/ml protein standard (e.g., BSA, albumin, or γ-globulin)
- Sample
- Lowry assay mix
- Freshly prepared diluted Folin-Ciocalteu reagent
- Test tubes (e.g., 16 × 125–mm)
- Spectrophotometer warmed up and set to 660 nm and cuvette

Procedure

1. Prepare standards containing a range of 1 to 100 μg protein (e.g., albumin or γ-globulin) to a volume of 1 ml
2. Add sample to water in a 16 × 125–mm test tube to yield a final volume of 1 ml. Include two separate tubes containing water for water blanks
3. To each tube, add 5 ml of freshly prepared Lowry assay mix and thoroughly vortex.
4. Incubate tubes 10 min at room temperature
5. Add 0.5 ml of diluted Folin-Ciocalteu reagent to each tube and vortex immediately.
6. Incubate 30 min at room temperature
7. Vortex the tubes, zero the spectrophotometer with the blank, and measure absorbance at 660 nm
6.1.6. Ammonia (ion-selective electrode method)

Reagents
Distilled-Deionized water
Stock ammonium chloride solution (1M)
Diluted standards of ammonium chloride (.001M, .01M, and .1M)

Procedure
1. Place 25mL of standard solution or sample into 100 mL beaker
2. Immerse electrode while mixing with magnetic stirrer on a relatively low rate to minimize evaporation
3. Add 1 mL of ammonia adjusting ISA solution to beaker
4. Allow time for electrode to equilibrate, and record mV reading

Calculations
Prepare a standard curve of concentration of ammonium chloride vs. mV readings on a semi-logarithmic graph. Use graphing tools to fit a logarithmic curve to the points, with $R^2 > 0.98$. Last, fit sample points to the standard curve to find concentrations

6.1.7. Total volatile fatty acids (VFA) – Titrimetric method

Reagents
1:1 Sulfuric acid : DDI water
Standard sodium hydroxide titrant, 0.1N
Acetic acid stock solution, 2000 mg/L
Procedure

1. If the sample contains high solids, centrifuge to obtain 100mL supernatant
2. Add 100 mL sample and 100 mL DI water (you may use 50 mL sample and 150 mL DI water if VFA levels are high) to 500 mL flask
3. Add 5mL 1:1 H$_2$SO$_4$:DI water, and mix
4. Add several clay chips and connect to distillation apparatus
5. In a 250 mL graduated cylinder, collect 150 mL, pouring off the first 15 mL
6. Titrate the distillate with 0.1N NaOH to pH of 8.3

Calculations.

Eq. 6-9 \[ \text{VFA (mg/L)} = \frac{(\text{mL NaOH} \times 0.1 \text{ mol/L} \times 60,000)}{\text{mL sample} \times F} \]

To determine ‘F’ for your distillation column – dilute an appropriate volume of acetic acid stock solution to approximate the expected sample concentration and distill as for a sample. Use the following calculation:

Eq. 6-10 \[ F = \frac{\text{VFA recovered}}{\text{VFA standard}} \]

where VFA recovered is the volatile acid concentration recovered in distillate (mg/L) and VFA standard is the volatile acid concentration of the standard solution used (mg/L)
6.2. Background information

6.2.1. Bioenergetics and thermodynamics

The thermodynamic equilibrium of microbiologically-mediated reactions is given by the value of the Gibbs free energy ($\Delta G^\circ$), as mentioned above. $\Delta G^\circ$, defined below, represents the energy available for a reaction to proceed when reactants and products are at unit activity and standard conditions, i.e. pH = 7, T = 25°C, and P = 1 atm (Thauer et al., 1977; McCarty and Smith, 1986).

$$\Delta G' = \Delta G^\circ' + RT \sum_{i=1}^{mk} v_{ik} \ln a_i$$

Where $v_{ik}$ is the stoichiometric coefficient for component $i$ in reaction $k$ with $mk$ components, $a_i$ is the activity of component $i$, $R$ is the universal gas constant, and $T$ is the absolute temperature. However, Gibbs free energy of reactions can be also calculated for other than standard conditions. For temperatures other than 25°C, $\Delta G$ values can be corrected using the Gibbs-Helmholtz Equation. Enthalpy values can be assumed to be constant within a 50°C range, and therefore this equation is fairly accurate only within this range.

In addition, other authors, such as Amend and Shock (2001), have compiled comprehensive, internally-consistent data sets with the $\Delta G_r^\circ$ of hundreds of reactions at temperatures from 2 to 200°C.

6.2.2. Stoichiometric methods for the calculation of biomethane yields

*Method A. Buswell Formula*

Substrate stabilization during anaerobic digestion is directly related to the biomethane production. An equation to describe substrate stabilization based on knowledge of its chemical composition was developed by Symons and Buswell (1933) to predict
biomethane yields and other fermentation end products. The equation, which was later modified by Buswell and Mueller (1952), is presented below:

\[
\begin{align*}
C_nH_\alpha O_\beta N_\gamma + \left[ n - \frac{a}{4} - \frac{b}{2} + \frac{3c}{4} \right] H_2O \rightarrow \\
\left[ \frac{n}{2} - \frac{a}{8} + \frac{b}{4} + \frac{3c}{8} \right] CO_2 + \left[ \frac{n}{2} + \frac{a}{8} - \frac{b}{4} - \frac{3c}{8} \right] CH_4 + cNH_3
\end{align*}
\]

The method described by Symons and Buswell (1933) assumes that 100% of the substrate is biodegradable and that all the electrons donated are used to produce energy, i.e. cellular synthesis is neglected.

**Method B. Bioenergetics and stoichiometry of biological reactions**

The thermodynamic equilibrium of microbiologically-mediated reactions can be used to predict cell yields and the overall stoichiometry associated with growth. Bioenergetics, along with some observations of the efficiency with which bacteria capture free energy, allows determining which either fractions of a particular organic substrate (electron donor) will be used for energy and for synthesis of cellular material. The general reaction \((R)\) can then be obtained as follows (McCarty, 1972; McCarty, 1975):

\[
R = R_d - f_eR_a - f_sR_c
\]

The overall stoichiometric equation requires three half-reactions, one for the oxidation of the electron donor \((R_d)\), one for the formation of the electron acceptor \((R_a)\), and one for the oxidation of cells \((R_c)\). The fraction of electron donor which is coupled with the electron acceptor to produce energy is represented by \(f_e\). The fraction of electron donor which is coupled with cell synthesis is represented by \(f_s\). For the equation to balance, the sum of \(f_e\) and \(f_s\) must equal to 1. The method of bioenergetics also assumes complete biodegradability of the substrate, but in this case, as opposed to the Symons and Buswell (1933) method, not only energy is taken into account, but synthesis of cellular material as well.
6.3. Characteristics of chemical substances and conversion factors

<table>
<thead>
<tr>
<th>Molecule</th>
<th>COD (g /g molecule)</th>
<th>Melting point (°C)</th>
<th>Formula</th>
<th>Molecular weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methane</td>
<td>2.857(^1)</td>
<td>-</td>
<td>CH(_4)</td>
<td>16.04</td>
</tr>
<tr>
<td>Carbohydrates (as glucose)</td>
<td>1.066</td>
<td>-</td>
<td>C(_6)H(_12)O(_6)</td>
<td>180.16</td>
</tr>
<tr>
<td>Proteins (average)</td>
<td>1.498</td>
<td>-</td>
<td>C(<em>{16})H(</em>{24})O(_3)N(_4)</td>
<td>352.39</td>
</tr>
<tr>
<td>Lipids (average)</td>
<td>2.895</td>
<td>-</td>
<td>C(<em>{12})H(</em>{34})O(_2)</td>
<td>274.45</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>1.066</td>
<td>16.5</td>
<td>C(_2)H(_6)O(_2)</td>
<td>60.05</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>1.514</td>
<td>-21</td>
<td>C(_3)H(_6)O(_2)</td>
<td>74.08</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>1.818</td>
<td>-7.9</td>
<td>C(_4)H(_8)O(_2)</td>
<td>88.11</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>2.039</td>
<td>-34.5</td>
<td>C(_5)H(_10)O(_2)</td>
<td>102.13</td>
</tr>
<tr>
<td>Caproic acid</td>
<td>2.207</td>
<td>-3.4</td>
<td>C(_6)H(_12)O(_2)</td>
<td>116.16</td>
</tr>
<tr>
<td>Enanthic acid</td>
<td>2.338</td>
<td>-7.5</td>
<td>C(_7)H(_12)O(_2)</td>
<td>130.18</td>
</tr>
<tr>
<td>Caprylic acid</td>
<td>2.444</td>
<td>16.7</td>
<td>C(_8)H(_16)O(_2)</td>
<td>144.21</td>
</tr>
<tr>
<td>Capric acid</td>
<td>2.605</td>
<td>31.6</td>
<td>C(_10)H(_20)O(_2)</td>
<td>172.26</td>
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<tr>
<td>Lauric acid</td>
<td>2.720</td>
<td>44.2</td>
<td>C(_12)H(_24)O(_2)</td>
<td>200.32</td>
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<tr>
<td>Myristic acid</td>
<td>2.807</td>
<td>54.4</td>
<td>C(_14)H(_28)O(_2)</td>
<td>228.37</td>
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<tr>
<td>Palmitic acid</td>
<td>2.875</td>
<td>62.9</td>
<td>C(_16)H(_32)O(_2)</td>
<td>256.42</td>
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<tr>
<td>Stearic acid</td>
<td>2.930</td>
<td>69.9</td>
<td>C(_18)H(_36)O(_2)</td>
<td>284.48</td>
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<tr>
<td>Oleic acid</td>
<td>2.894</td>
<td>13.5</td>
<td>C(_18)H(_34)O(_2)</td>
<td>282.46</td>
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<td>Linoleic acid</td>
<td>2.857</td>
<td>-5</td>
<td>C(_18)H(_32)O(_2)</td>
<td>280.45</td>
</tr>
</tbody>
</table>

\(^1\)g/L @ STP
\(^2\)From Beare-Rogers (2001)
6.4. Example of the developed methodology to predict \( B_o \) and \( f_D \) of semi-continuous digesters at steady-state using biodegradability data and stoichiometric methods

A flowchart example of the calculation for the efficiency of substrate stabilization (equivalent to \( f_D \)) and SMY (equivalent to \( B_o \)) in the semi-continuously-fed CSADs used in this study is shown below:

Chemical composition data
- Carbohydrates
- Proteins
- Lipids

Stoichiometry
Buswell or Bioenergetics

Biodegradability data biomolecules (non-lignocellulosic)

Lignocellulosic substrate models
\[ f_D (\text{mesophilic}) = 0.822 \cdot 2.743 \cdot \text{Lignin} \]
\[ f_D (\text{thermophilic}) = 0.853 \cdot 3.221 \cdot \text{Lignin} \]

Differences (%) between predictions and continuous studies (CSADs) at steady-state during period 1

<table>
<thead>
<tr>
<th></th>
<th>Mesophilic</th>
<th>Thermophilic</th>
</tr>
</thead>
<tbody>
<tr>
<td>( f_D )</td>
<td>-2.9%</td>
<td>-3.4%</td>
</tr>
<tr>
<td>( B_o )</td>
<td>-11.3%</td>
<td>-17.8%</td>
</tr>
</tbody>
</table>

Chemical composition of food wastes can be found in the Nutrient Data Laboratory (NDL) website, available at [http://www.ars.usda.gov/ba/bhnrc/ndl](http://www.ars.usda.gov/ba/bhnrc/ndl) (U.S. Department of Agriculture, 2009)

Chemical composition of manure does not vary greatly, data for manure SMY can be found in Chapter 2, or samples of manure can be sent to forage analysis laboratories.
perform fiber analysis to obtain its lignin content, from which equation developed in Chapter 3 can be used to predict $f_D$ and $B_0$.

6.5. References


