Mechanisms of Methanogenic Inhibition in Advanced Anaerobic Digestion

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ABSTRACT

A series of lab-scaled digestion studies including conventional mesophilic anaerobic digestion (MAD), thermophilic anaerobic digestion (TAD) at a range of treatment temperatures, and mesophilic high solids digestion of thermally pretreated wastewater sludge (THD) were carried out. Enhanced digestion performance in terms of solids destruction and methane generation by THD relative to MAD was achieved, and was largely attributable to the solubilization and subsequent biodegradation of energy-rich substrates within blended primary and secondary sludge. TAD was observed to underperform MAD, especially at elevated temperatures as methanogenic inhibition resulted in the accumulation of headspace hydrogen, thus resulting in poor removal of volatile fatty acids. The thermodynamics of fatty acid metabolism was favorable at each digestion temperature, thus it was concluded that microbial inhibition was the controlling factor in poor thermophilic performance.

Inhibition by free unionized ammonia (NH_3) was characterized for THD and MAD biomass. Acetic acid degradation was equally affected over a range of NH_3 concentrations; however, methane generation by THD was less sensitive to ammonia inhibition, thus suggesting that methanogenesis by THD was less dependent on the NH_3-sensitive process of aceticlastic methanogenesis. Total ammonia nitrogen (TAN) and bicarbonate alkalinity were stoichiometrically produced from proteinaceous material during thermal hydrolytic pretreatment and subsequent high solids anaerobic digestion. Combined effects of TAN and high pH resulted in NH_3-inhibition during THD. Kinetic evaluations suggested that a growth rate reduction of approximately 65% was associated with in-situ NH_3 concentrations of the THD reactor.

NH_3-inhibition was apparently responsible for a shift in dominant methanogenic community of the aceticlastic *Methanosarcina barkeri* in MAD to the hydrogenotrophic *Methanoculleus bourgensis* in THD. A similar shift in methanogenic community was observed between low-temperature thermophilic digestion at 47°C, where the dominant order was *Methanosarcinales*,

to high temperature thermophilic digestion at 59°C where the dominant order was *Methanobacteriales*. These findings support a process-driven pathway shift from aceticlastic to non-aceticlastic methanogenesis between 180 and 290 mg/L NH_3-N. Such a threshold is supported by previous literature related to ammonia tolerance of pure cultures of methanogens and has significant implications for the kinetic design of advanced anaerobic digestion processes.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Biosolids management is an increasingly large contributor to the overall operational cost of a wastewater treatment plant. In very large applications, the money available for capital improvement and the land available for new construction also represent further managerial constraints on biosolids systems. Biosolids treatment processes also have a direct impact on material that leaves the plant, and as such, significant risk is associated with the implementation of a management plan. When each of these factors are considered together, we are often left with the following: *The search for a compact, reliable, and efficient solids treatment process that often needs to be tailored to the unique demands of a particular plant.*

The most widely used method for biosolids stabilization is anaerobic digestion since is satisfies both the reliability and efficiency requirements mentioned above. The operational and managerial constraints (i.e. the minimization of capital, land, and risk) placed on biosolids management systems have fueled interest in advanced anaerobic digestion technologies (AAD). There is considerable interest in AAD technologies since they pose the potential for increased degradation of rates, better pathogen inactivation, and increased energy recovery. However, as has been well established and documented for thermophilic anaerobic digestion, advanced anaerobic digestion processes are often more susceptible to upset due to potentially high concentrations of toxic substances (e.g. ammonia) combined with relatively high substrate pressures (i.e. high fatty acid concentrations). Previous studies have shown that aceticlastic methanogens tend to be the most sensitive community to environmental and toxic stress imposed by AAD processes (Ahring *et al.*, 2001; Hajarnis and Ranade, 1993; Hajarnis and Ranade, 1994a; Schnurer *et al.*, 1999).

The relatively few, simple compounds that serve as methanogenic substrates have been widely summarized (Madigan *et al.*, 2003; McCarty and Smith, 1986; Parkin and Owen, 1986; Speece,

2002), and they fall into two general categories: hydrogen (H_2) plus carbon dioxide (CO_2) and/or formic acid (HCOOH), and methylated two-carbon compounds such as methanethiol (CH_3SH) and acetate (CH_3COOH). The formation of methanogenic substrates from complex organic material is dependent on effective shuttling of carbon from complex organic material to single carbon compounds, the rate limiting stage of this process being hydrolysis. Enhanced hydrolysis processes can reasonably be expected to greatly affect the ratio of readily fermentable organic compounds (e.g. simple sugars, short-chain fatty acids) to slowly biodegradable organics. As such, enhanced hydrolysis generally imposes high substrate pressure not only on the organisms that rely on readily fermentable substrates (Tanaka and Karniyama, 2002), but also those organisms that benefit from the byproducts of fermentation, i.e. the methanogens. Other researchers have reported that K_S values for aceticlastic methanogenesis of 0.3-0.5 µmol/mL acetate (Zinder *et al.*, 1984) Recent studies have discussed from a modeling viewpoint, the impact of solids hydrolysis on aceticlastic methanogenesis (Straub *et al.*, 2006), and also the competitive kinetic advantage of certain methanogenic species over others at varied substrate pressures (Conklin *et al.*, 2006). While these studies have focused largely on aceticlastic methanogens, it is not hard to imagine that altering overall carbon flow of anaerobic digestion by the enhanced production of readily fermentable substrate within a reactor would have a similar effect on the growth and population dynamics of the CO_2-reducing methanogens (based on fermentative hydrogen production) and possibly cause the selective dominance of one group of methanogens over another. The information gleaned from these studies can be applied with actual digestion processes in mind by observing the effect of enhanced hydrolysis (and thus, readily fermentable substrate availability) on methanogenic population dynamics during mesophilic anaerobic digestion (MAD).

Ammonia and short chain fatty acids are produced from the hydrolysis of proteins and lipids in wastewater sludge, respectively; therefore AAD processes that focus on the enhancing the hydrolysis of complex organic material have the potential to impose a strong inhibition on aceticlastic methanogenesis. Aceticlastic methanogens are generally thought to be responsible for two-thirds of the methane production from an anaerobic digester, and as such, are directly linked to the recoverable energy yield of a digestion system. Furthermore, since aceticlastic methanogens are the terminal community involved in fatty acid degradation, reduction in their

activity could result in poor organic removal leading to high strength recycle streams. On the other hand there appears to be some evidence that high fatty acid processes may reduce the occurrence of volatile organic sulfur odors within biosolids (Örlygsson *et al.*, 1994; Örlygsson *et al.*, 1995). In any event, care must be taken during process selection to determine the overall effect of an advanced anaerobic digestion system within the broader scope of a wastewater treatment plant. In order to assess the potential for inhibition of acetic acid utilization, we investigated factors that impact the production of inhibitory compounds during enhanced hydrolytic pretreatment, the effect of these compounds on the kinetics and ecology of methanogenesis, and the occurrence of similarities between what has been observed with better understood advanced digestion systems, such as thermophilic anaerobic digestion (TAD), and a novel enhanced hydrolysis processes, thermal hydrolysis (THD).

1.2 RESEARCH QUESTIONS

Question 1: How are fatty acid and free ammonia concentrations within the reactor linked to the type and operation of the advanced anaerobic digestion process? The production of inhibitory compounds by enhanced hydrolytic processes is linked to the hydrolysis of protein and lipid at either high temperature, high pressure, or under acidic (fermentative) conditions. It has been previously observed that the primary inhibitory agents tied the hydrolysis of relatively large biomolecules (ammonia and short chain fatty acids) exist at relatively high concentrations shortly after thermal hydrolysis (Bougrier *et al.*, 2008) and in high-temperature digestion processes (Angelidaki and Ahring, 1994; vanLier, 1996; vanLier *et al.*, 1996). In order to establish the link between pretreatment process and any potential downstream methanogenic inhibition, it is important to determine the operational conditions that significantly affect the conversion of proteins and lipids to their corresponding substrates.

Question 2: What is the kinetic nature of the inhibition that is imparted on acetate-utilizing methanogens by the conditions within the reactor (i.e. what are the growth and half-saturation parameters of the Monod expression for acetate utilization compared with conventional digestion)? It has been widely reported that under the environmental conditions of certain

anaerobic digestion technologies aceticlastic methanogens may become inhibited. Among the conditions that have reportedly elicited aceticlastic inhibition are high temperature (Angelidaki and Ahring, 1994; Schnurer *et al.*, 1999; Wilson *et al.*, 2008), high ammonia concentrations (Angelidaki and Ahring, 1994; Sung and Liu, 2003), and high fatty acid concentrations (Hajarnis and Ranade, 1994b; Yang and Okos, 1987). Since it is hypothesized that the advanced anaerobic technologies that are of interest to this study may result in the large-scale production of inhibitory agents within the digester feed, it is important to determine the nature of any inhibition of aceticlastic methanogenesis caused by these processes in order effectively predict the effect of operational and design considerations (e.g. hydraulic retention time, temperature) on digester performance.

Question 3: Does the kinetic inhibition of acetate-utilizing methanogens result in a meaningful shift in methanogenic community? Kinetic design requires understanding of the microbial populations present, as well as the kinetic parameters (whether inhibited or uninhibited) that govern the populations' rates of growth and substrate utilization. For example, anaerobic digesters exhibiting non-aceticlastic methanogenesis by syntrophic acetate oxidizing cultures have been observed possess lower substrate utilization (and by association, growth) rates (0.5-5.3 mM acetate/day) than conventional aceticlastic processes (4.8-10.0 mM acetate/day) (Karakashev *et al.*, 2006). In addition, the apparent half saturation coefficient, K_S, for acetate has been measured as 0.65 mM for non-aceticlastic methanogenesis (Petersen and Ahring, 1991), lower than reported values for aceticlastic methanogens *Methanosarcina sp.* (5.33 mM acetate) and *Methanosaeta sp.* (1.5 mM acetate). Understanding which populations are significant within the operational ranges of a particular treatment process is crucial for accurate design of novel processes.

Question 4: Is the resultant mechanism of methanogenic inhibition unique to thermal hydrolysis processes or general across several "extreme" anaerobic digester conditions (e.g. high temperature and/or ammonia processes)? By addressing this question, we elucidate fundamental mechanisms by which AAD processes are found to differ ecologically and kinetically from conventional anaerobic digestion. The goal in answering this question is to better understand novel AAD processes within the context of classical AAD processes such as

thermophilic anaerobic digestion, and to assist in predictive modeling of mechanistic digester operation based on the intrinsic physical-chemical conditions of each process.

1.3 ANNOTATED DISSERTATION OUTLINE

Chapter 1: ***Introduction*** The introduction chapter provides background, defines the focus by stating the four major research questions, and briefly summarizes the material presented in this dissertation by including an annotated dissertation outline.

Chapter 2: ***The effect of temperature on the performance and stability of thermophilic anaerobic digestion*** This manuscript provides a mechanistic description of lab-scaled thermophilic anaerobic digester operation over a range of reactor temperatures from 49°C to 57.5°C. Thermophilic process data serve as a background for addressing *Research Question 4* in later chapters. This work was presented at the 2007 IWA Biosolids Specialty Conference in Moncton, New Brunswick Canada, and has since been accepted for publication:

Wilson, C.A., Fang, Y., Novak, J.T., Murthy, S.N., (2008) The effect of temperature on the performance and stability of thermophilic anaerobic digestion. *Water science and technology* **(57)** 2: 297-304.

Chapter 3: ***Anaerobic digestion of raw and thermally hydrolyzed sludge under various operational conditions*** Comparative digester performance data are presented for seven lab-scaled THD systems and two MAD systems, including biogas production, solids destruction, sludge dewatering, and odor generation. Probable mechanisms relating to the outperformance of THD relative to MAD are discussed. *Research Question 1* is directly addressed in this chapter. Process data serve as background information for addressing *Research Questions 2 and 4* in later chapters.

Chapter 4: ***Hydrolysis of macromolecular components of primary and secondary wastewater sludge by thermal hydrolytic pretreatment*** A laboratory simulation of thermal hydrolytic pretreatment (THP) process was performed on wastewater sludge, as well as key

macromolecular components: proteins, lipids, and polysaccharides. Production of specific low molecular weight hydrolysis products as well common aggregate wastewater constituent measurements were used to assess the breakdown of different wastewater fractions, as affected by hydrolysis temperature. *Research Question 1* is directly addressed in this chapter. This work has been accepted for publication:

Wilson C.A. and Novak, J.T., (2009) Hydrolysis of macromolecular components of primary and secondary wastewater sludge by thermal hydrolytic pretreatment. *Water Research* **(43)** 18: 4489-4498.

Chapter 5: ***The kinetics of process dependent ammonia inhibition of methanogenesis from acetic acid*** The results of batch ammonia toxicity assays using THD and MAD biomass are presented. Monod kinetic parameters are evaluated over a range of NH_3 concentrations from approximately 20-320 mg/L NH_3-N in order to observe differences in response between biomass samples cultivated at low and high in-situ NH_3 concentrations. *Research Question 2* is directly addressed in this chapter.

Chapter 6: ***Methanogenic community structure in mesophilic, thermophilic, and thermal hydrolytic pretreatment anaerobic digester systems*** Methanogenic communities from MAD, THD, low-temperature TAD (47°C), and high temperature TAD (59°C) were analyzed by order-specific real-time quantitative PCR (qPCR) and archaeal clone library analysis. Distinct shifts in methanogenic community and evidence of an alternate acetate degradation pathway for high NH_3 digestion processes are discussed. *Research Questions 3 and 4* are directly addressed in this chapter.

Chapter 7: ***Concluding Remarks and Engineering Significance*** The concluding chapter discusses specific significant contributions of this research to the wastewater engineering field, and proposes several valuable extensions to the work described throughout.

Appendix A: ***Comprehensive Enhanced Digestion Evaluations at Blue Plains Advanced Wastewater Treatment Plant*** This paper was presented at the 2009 Water Environment

Federation Residuals and Biosolids Specialty Conference in Portland, Oregon. The manuscript presents performance data obtained over seven years of lab-scaled anaerobic digestion studies performed at Virginia Tech, funded by the District of Columbia Water and Sewer Authority, including performance data from MAD (8), TAD (14), THD (2), acid-gas anaerobic digestion (1), temperature-phased anaerobic digestion (5), and sequential anaerobic-aerobic digestion (9). As this thesis discusses the effect of process-driven physical-chemical changes in a digester environment of methanogenic community and performance, the broad scope of process performance data contained in this paper is a pertinent addition to the discussion contained herein.

Appendix B: ***Supplemental information for Chapter 5*** Raw data for batch ammonia toxicity assays are presented, including acetic acid progress curves and associated model fits.

Appendix C: ***Supplemental information for Chapter 6*** Raw qPCR data for methanogenic orders *Methanobacteriales, Methanococcales, Methanomicrobiales,* and *Methanosarcinales* including information on order-specific PCR primer and TaqMan® probe sets are presented.

Appendix D: ***Biosolids odor compound characterizations for THD and MAD biosolids*** Raw biosolids odor compound data are presented. A significant amount of work within freshwater sediments has revealed the direct link between methylotrophic methanogenesis and the degradation of volatile organic sulfur compounds (VOSC), (Lomans *et al.*, 2002; van Leerdam *et al.*, 2006) which have been directly correlated to the human perception of biosolids odor (Witherspoon *et al.*, 2004). The relative presence of methanogens within an anaerobic digester has been positively correlated to the suppression of VOSC after dewatering (Chen *et al.*, 2005). Recent studies (Muller *et al.*, 2003; Muller *et al.*, 2004) have shown that VOSC production from anaerobically digested biosolids is irrevocably linked to the shear imparted during biosolids conditioning and dewatering (most notably through the application of high solids centrifugation). It has been hypothesized that high shear energy is responsible for the release of iron bound protein complexes that are (a) available for microbial degradation, and (b) rich in the sulfur containing amino acids, CYS and MET, which have been shown to be precursors of VOSC via Stickland transformations (Higgins *et al.*, 2006; Higgins *et al.*, 2008; Ramsay and

Pullammanappallil, 2001). These proteins persist though conventional anaerobic digestion since they are relatively tightly bound within insoluble complexes. Since biosolids odor production (as VOSC) is linked to methanogenic activity, the effect of AAD on methanogenic kinetics and population dynamics could reasonably be expected to impact odors. These data will be prepared for publication in 2010.

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CHAPTER 2

THE EFFECT OF TEMPERATURE ON THE PERFORMANCE AND STABILITY OF THERMOPHILIC ANAEROBIC DIGESTION

2.1 AUTHORS

Christopher A. Wilson, Yuan Fang, John T. Novak, Sudhir N. Murthy

2.2 ABSTRACT

Sustainable operation of an anaerobic sewage sludge digester requires the effective shuttling of carbon from complex organic material to methane gas. The accumulation of intermediates and metabolic products such as volatile fatty acids and hydrogen gas not only reveal inefficiency within the digestion process, but can be detrimental to reactor operation at sufficiently high levels. Eight anaerobic digesters (1 mesophilic and 7 thermophilic) were operated in order to determine the effect of steady-state digestion temperature on the operational stability and performance of the digestion process. Replicate reactors operated at 57.5°C, the highest temperature studied, were prone to accumulation of volatile fatty acids (4052 and 3411 mg/L as acetate) and gaseous hydrogen. Reactors operated at or below 55°C showed no such accumulation of intermediate metabolites. Overall methanogenesis was also greatly reduced at 57.5°C (0.09 L CH_4/g VS fed) versus optimal methane formation at 53°C (0.40 L CH_4/ g VS fed). Microbial community assessment (PCR-DGGE) and free energy calculations suggest that the accumulation of fatty acids and hydrogen, and relatively poor methanogenic performance at 57.5°C are likely due to temperature limitations of thermophilic aceticlastic methanogens.

2.3 KEYWORDS

Acetate
Free energy calculations
Hydrogen
Methane
PCR-DGGE
Propionate
Temperature
Thermophilic anaerobic digestion

2.4 NOMENCLATURE

ADP	Average daily production
CH_4	Methane
CO_2	Carbon dioxide
DGGE	Denaturing gradient gel electrophoresis
$\Delta G^{o'}$	Standard change in Gibbs free energy
H_2	Hydrogen
PCR	Polymerase chain reaction
VFA	Volatile fatty acids
VS	Volatile solids
VSR	Volatile solids reduction

2.5 INTRODUCTION

Stringent pathogen regulations required for the land application of domestic wastewater biosolids have revitalized interest in thermophilic anaerobic digestion. Since biological activity at these temperatures requires specialized organisms equipped with thermostable cell components and enzymes, thermophilic anaerobic digestion has recently been recognized as holistically unique biological process, rather than a close variation of mesophilic anaerobic digestion. As such, much of the fundamental discussion regarding anaerobic digestion technology, keeping mesophilic anaerobic digestion in mind, is inadequate for the development of a thorough

understanding of the design parameters and operational variables that greatly affect thermophilic performance.

Much of the recent research regarding thermophilic anaerobic digestion deals with previous reports of digester instability and the accumulation of organic intermediates as a result of reactor perturbation. Several previous studies have looked at the effect of intentional shifts in digester temperature and intermittent temperature fluctuations on the stability of a thermophilic anaerobic digester. While these studies are important in assessing the response and adaptation of a pre-existing microbial community to an alternate condition, few studies have focused on the effect of steady-state temperature on the development of a unique digester community. The objectives of this study were to determine the effect of different temperatures within a chosen thermophilic range (49°-57.5°C) and to assess the role of digestion temperature in determining the steady-state performance of an anaerobic digester.

2.6 METHODOLOGY

2.6.1 Anaerobic Digester Setup and Operation

High-density polyethylene batch fermentation reactors supplied by Hobby Beverage Equipment Company (Temecula, California) were chosen for this study. The conical bottom of these vessels was thought to be advantageous in terms of mixing and suspension of grit, similar to the full-scale application of egg-shaped anaerobic digesters. The nominal volume of each vessel was 25 liters (L) and was operated with an active volume of 22.5 L. The reactor vessels were modified to accept a threaded stainless steel thermometer, also supplied by Hobby Beverage. Digesters were operated at 35°C, 49°C, 51°C, 53°C, 55°C, and 57.5°C. Replicate digesters at 53°C and 57.5°C were operated in order to investigate the reproducibility of the results of this study. For the purpose of this paper, the reactors are named according to their operating digestion temperature. Replicate reactors of the same temperature are designated by appropriate subscripts. The digesters were heated using an external circulating water bath (Haake DC10-W19) attached to 13 mm i.d. vinyl tube water jacket around each digester. This heating method controlled the internal temperature of the digesters within ± 0.2°C of the nominal temperature.

A conical stainless steel batch fermentation reactor produced by Blichmann Engineering, LLC was used in the operation of reactor 49°C. The reactor had similar dimensions and the same nominal volume as the HDPE reactors produced by Hobby Beverage and was therefore determined to be an equivalent reaction vessel for anaerobic digestion. Unfortunately, the reactor lid consisted of a stainless steel to stainless steel joint and leaking of digester gas from the reactor was unpreventable. Compositional analysis of the digester headspace gas did not yield significant quantities of ambient air (N_2 plus O_2), so the digester was thought to be functioning in a manner similar to the other units. Seeding, start-up, daily feed preparation, and mixing were performed as described previously (Wilson et al. 2006). Stabilization of reactor operation typically occurred within 30 days of commencement of regular feeding and wasting. In the case of the 49°C digester from which quantification of biogas production was not possible, monitoring of pH was used as the primary indicator of steady-state performance.

2.6.2 Gibbs Free Energy Change

The Gibbs free energy for the reactors under actual reactor conditions ($\Delta G'$) was calculated on the basis of previously published standard Gibbs free energies for the individual reactions involved in syntrophic propionate oxidation (McCarty and Smith 1986; Rossini et al. 1952). The calculation of $\Delta G'$ included temperature corrections to standard Gibbs free energy ($\Delta G^{o\prime}$) for the various reactor conditions by applying the constant enthalpy from of the Van't Hoff equation as well as measured concentrations of appropriate reactants and reaction products.

2.6.3 Headspace Analyses

Headspace methane (CH_4) and carbon dioxide (CO_2) were analyzed on a Shimadzu Gas Chromatograph (Model GC-14A) with a thermal conductivity detector (TCD). The column was constructed from a 4 meter length of copper tubing with a 6.35 mm inner diameter. The column was coiled to fit in the GC-14A oven and packed with Haysep Q media (Supelco, Bellefonte, PA). Helium was used as the carrier gas at a flow rate of approximately 17 ml/min.

Headspace hydrogen (H_2) concentrations were analyzed using a reduction gas detector (Trace Analytical RGA5). Nitrogen was used as the valve actuator at 414 kPa and carrier at a flow rate

of 50 ml/min. The RGA5 was operated isothermally with a bed temperature of 265°C and a column temperature of 80°C.

2.6.4 Volatile Fatty Acid Analysis

Volatile fatty acids (VFA) were measured weekly on the solution phase of each digester. Samples for VFA analysis were passed through a 0.45 µm nitrocellulose membrane filter and frozen prior to analysis. VFA were measured using a Shimadzu gas chromatograph (Model GC-14A) with flame ionization detector (FID). Species separation was performed using a Nukol™ fused silica 15 m x 0.53 mm capillary column with 0.5 µm film thickness. A Shimadzu computer integrator (Model CR501 Chromatopak) was used for data analysis. Helium was used as the carrier gas at a flow rate of 17 ml/min. Additional gasses are as follows: Hydrogen, 45 ml/min; Air, 450 ml/min; Nitrogen, 13 ml/min. Volatile fatty acids are expressed as mg/L of individual species (C2-C7 fatty acids). Individual acid concentrations are converted to acetate on a theoretical oxygen demand basis and summed to report Total VFA as mg/L as acetate.

2.6.5 PCR-DGGE Analysis

Samples were collected aseptically from the digesters and feed source, and shipped on dry ice to Bucknell University (Lewisburg, PA) for community analysis. Prior to analysis samples were stored at -50°C to prevent changes in community structure. DNA extraction and amplification (Ovreas et al., 1997) and denaturing gradient gel electrophoresis (DGGE) (LaPara et al., 2000) were performed as previously described. DGGE was carried out for bacterial and archaeal analyses at run times of 5.5 hours and 3.5 hours, respectively, and with an applied voltage of 200V as previously described (Nakatsu et al., 2000). Primers PRBA338f and PRUN518r were used for community analysis of organisms in the bacterial domain, and primers PARCH340f and PARCH519r were used for analysis of the archaeal community (Chen et al., 2005).

2.6.6 Statistical Analysis

Statistical analyses of reactor pH and methane production rate were performed to determine repeatability of replicate reactors and to determine significant difference in reactor operation at

various temperatures. These parameters were chosen since they were used to define steady-state reactor operation and have been classically been viewed as indicators of reactor upset and performance. Statistical analyses were performed using NCSS statistical software package (Kaysville, Utah). All statistical analyses were carried out at the 95% confidence level (α = 0.05). Reactor pH data ($n \geq 25$) were found to be normally distributed over the period of steady-state data collection based; random variation in methane production rates caused these data ($n \geq 21$) to be non-normal. Since it was desired that the same statistical analysis be used for pH and methane data, a nonparametric test was required for this condition. The Kruskal-Wallis Z-test was used in this case since it did not require the assumption of data normality.

2.6.7 Additional Analyses

Total and volatile solids concentrations (Method 2540-G) as well as total alkalinity (Method 2320-G) were analyzed as specified in Standard Methods for the Examination of Water and Wastewater (APHA, 1995).

2.7 RESULTS AND DISCUSSION

An observed optimal thermophilic temperature range for methanogenesis in terms of both methane partial pressure (P_{CH4}, Figure 2.1.a.) and average daily methane production (ADP_{CH4}, Table 2.1) is approximately 53-55°C. This temperature range corresponds with the classic definition of thermophilic anaerobic digestion (generally 55°C). There are insufficient data to determine whether suboptimal temperatures (49°C, 51°C) resulted in significantly reduced methane production; however, operation of replicate digesters at 57.5°C showed that ADP_{CH4} was reduced by up to 77% at digestion temperatures only slightly above optimal. While random variation in ADP_{CH4} by digesters operated at 51-55°C did not allow us to make any statement as to the significance of varied ADP_{CH4} in that range, both digesters operated at 57.5°C showed ADP_{CH4} that was significantly ($p < .05$) less than each of lower temperature digesters. It is interesting to note that corresponding trends in P_{CO2} and ADP_{CO2} were also observed at 57.5°C. P_{CO2} increased from an average 32.9 ± 1.4 kPa at temperatures ranging from 49-55°C to 39.1 at 57.5°C. Across the same temperature change, ADP_{CO2} was observed to decrease by 36-67%.

The accumulation of volatile fatty acids (VFA) has long been associated with anaerobic digester upset conditions (Harper and Pohland 1986). Such accumulation has been observed to be more pronounced at thermophilic temperatures (Gray et al. 2006; Speece et al. 2006), likely due to temperature sensitivity of aceticlastic methanogens under elevated thermophilic temperatures (Ahring et al. 2001; Chen 1983; Leven et al. 2007). In this study, operation of digesters at 57.5°C resulted in the accumulation of C2-C7 fatty acids. Specifically, acetate and propionate accumulated to maximum concentrations of 624 and 1485 ppm as acetate in digester 57.5°C_2, respectively, accounting for approximately 62% of total VFA in that reactor. In digester 57.5°C_1, acetate and propionate showed substantial accumulation relative to lower temperature reactors, however these individual acids only comprised 41% of total VFA. The reason for the preference for longer chain fatty acids (C4-C7, individual VFA species data not shown) in digester 57.5°C_1 has not been determined, but the overall accumulation of relatively simple organic acids in both reactors operated at 57.5°C suggests that some form of digester upset is being induced at this temperature.

While the presence of VFA within an anaerobic digester is representative of inefficient conversion of complex organic substrates to CH_4, fatty acids can also be particularly damaging to the digester environment in themselves. The accumulation of VFA serves as a pool of weak acid, of which, dissociation would cause the release of free hydrogen ions into solution, thus lowering the reactor pH. Since the pKa values of acetate, propionate, and butyrate are all less than 5.0, these important VFA species tend to exist in a predominantly deprotonated form at near neutral pH (Rittmann and McCarty 2001). The effect of VFA in this context is that the steady-state operating pH of digesters operated at 57.5°C ($\bar{x}$ = 7.13) was significantly lower ($p < .05$) than that of digesters operated at 55°C or below ($\bar{x}$ = 7.43 ± 0.04), contributing to the relative instability of the higher temperature thermophilic digesters (Table 2.1). Reactor pH again follows the previous discussion of relatively good reactor performance at temperatures ranging from 49-55°C, and relatively poor reactor performance at 57.5°C. Over the course of the study, the steady-state pH of each reactor tended to fluctuate very little, which suggests that these reactors were quite well buffered in light of relatively large variations in VFA concentration. Stability of an anaerobic digester is often defined as the reactor's α-value, the ratio of the concentration of total organic acids (VFA) to that of total alkalinity within a digester. Digesters

57.5°C_1 and 57.5°C_2 have α-values of 1.07 and 0.87, respectively, showing a decreased capacity for resisting additional perturbation (e.g. toxicants, organic slug loading, temperature and mixing fluctuations). Digesters operating at 49-55°C proved to have much lower α-values (0.13-0.36) and are considered to be more stable systems.

Volatile solids reduction (VSR) proved to be an unreliable metric for the assessment of digestion performance due to differences in effluent VFA concentrations with respect to digestion temperature. The average steady-state VSR for each thermophilic reactor in this study is listed in Table 2.2. It can be reasonably expected that a direct correlation between biological methane production and VSR would be observed during stable anaerobic digestion; however, a plot of ADP_{CH4} versus VSR for each reactor (Figure 2.2) shows a relatively poor correlation ($R^2 = 0.66$).

In order to determine whether the volatilization of VFA had a significant impact on VSR measurements, a calculated correction factor for effluent VFA concentration was applied to each reactor's measured volatile solids concentration. The characterization of biological volatile solids fed to the laboratory digesters is assumed to be approximated by the previously published empirical formula for heterotrophic biomass, $C_{10}H_{19}O_3N$. Alternate empirical formulas are available to characterize various sludges (e.g. nitrifying activated sludge, BioP sludge); however, no data were available during this study that supported their use. Measurements of organic nitrogen in the digester feed throughout this study reveal an average concentration of 2130 mg/L ± 240 mg/L as nitrogen (measured on 4% TS blended sludge, 78% volatile), or 6.8% of volatile solids by mass. These data support the use of the empirical formula for wastewater sludge, $C_{10}H_{19}O_3N$, which exhibits reasonable agreement with our organic nitrogen measurements at approximately 7.0% nitrogen, by mass.

Using the concentration of VFA in each digester, converted to an equivalent concentration of biological volatile solids, the corrected VSR for each digester as shown in Table 2.2 could then be calculated. When the corrected values for VSR are compared to daily CH_4 production in Table 2.1, the predicted linear correlation ($R^2 = 0.91$) between the two analyses can be seen (Figure 2.2). The remaining variation between corrected VSR and CH_4 is likely the result of various solubilized digestion products including protein, polysaccharides, and reduced alcohols.

The difference between raw and corrected VSR data suggests that a significant portion of organic material that is solubilized at 57.5°C does not undergo complete fermentation and persists as unstabilized COD within the digester in the form of VFA. It is significant to note that, once effluent VFA are taken into account, the digesters operated at 57.5°C both exhibit a VSR less than the 38% metric cited by the CFR 40 Part 503 regulations for vector attraction reduction.

Hydrogen has been found to play a key role in the conversion of VFA to acetate, and eventually CH_4. A relatively narrow window of hydrogen concentrations exists (0.1-10 Pa) within which propionate can be successfully metabolized (McCarty and Smith 1986). This can be partially attributed to the potentially large molar quantities of hydrogen produced during propionate metabolism, causing feedback inhibition on the oxidation of propionate and other organic acids (de Bok et al. 2001; Seeliger et al. 2002). Hydrogen in digester 57.5°C_2 accumulated to a concentration of approximately 11.4 Pa, only slightly higher than the previously published upper limit for efficient metabolism of propionate. Such concentrations of hydrogen in themselves are unlikely to result in a substantial buildup of propionate, however past studies focused on the development of online early warning systems for digester upset used hydrogen partial pressures as low a 6.5 Pa to indicate potential onset of overload conditions (CordRuwisch et al. 1997).

Bioenergetics and thermodynamics can be used in order to assess the implication of reactor conditions as observed in this study, and in conjunction with previous literature describing the microbiology of thermophilic anaerobic digestion, can help to establish a mechanism to explain previous reports of temperature induced instability. The concentrations of H_2, CH_4, CO_2, H^+, acetate, and propionate were used to calculate actual Gibbs free energies for reactions directly involved in propionate and acetate metabolism (listed in Table 2.3) under methanogenic conditions at a selection of temperatures (Figures 2.1.c.-2.1.d.). The mesophilic reactor was not considered in this analysis since propionate was below detectable levels.

Previous studies have shown that the minimal energy quantum required to sustain microbial life corresponds to ⅓ of the quantum required for the synthesis of 1 mol ATP (~70 kJ/mol ATP) (Ahring and Westermann 1988; Scholten and Conrad 2000). This means that for a

microorganism to survive by carrying out any of the metabolisms listed in Table 2.3, the reaction must provide a minimum free energy release of greater than 23 kJ/reaction (ΔG = -23 kJ/mol).

The results of this analysis show that, under actual reactor conditions, propionate oxidation, hydrogenotrophic methanogenesis, and aceticlastic methanogenesis are all near the theoretical threshold of viable energy gain from their respective metabolism. It has been well documented that hydrogenotrophic methanogens and acid-oxidizing bacteria occur in syntrophic clusters that enhance the intercellular transport of hydrogen (de Bok et al. 2004). As such, the energy produced through these two reactions (syntrophic propionate oxidation) is observed to be much greater than the energy available to either community in itself (Figure 2.1.b.). Additionally, past research has shown that in complex competitive environments, the actual energy requirements for microbial life may indeed be slightly lower (Hoehler et al. 2001). In their study, Hoehler et al. found that hydrogenotrophic methanogenesis was able to proceed with a free energy release as low as $\Delta G = -10.6$ kJ/mol.

While data from previous studies regarding syntrophic metabolism and energetic limits for microbial life may provide an explanation as to how effective methanogenesis can occur in the thermophilic reactors operated in this study at or below 55°C, strictly looking at microbial energetics alone does not explain the poor methanogenic performance of the digesters operated at 57.5°C. Microbial community assessment via PCR-DGGE analysis was used to observe changes in overall microbial community structure at the various digester temperatures. The archaeal community analysis (Figure 2.3.b.) provided useful insight into methanogenic inhibition occurring at high digestion temperatures. In an anaerobic digester, analysis of total archaeal population is considered to be representative of the methanogenic population (Chen et al. 2005). Analysis of reactors 35°C, 49°C, and 53°C_2 yielded the presence of distinct bands, representing individual populations of methanogenic organisms. Interestingly, fewer archaeal bands were identified in the mesophilic reactor, signifying less microbial diversity among methanogens. Similar results in previous research have been interpreted as a community lacking the biological diversity to resist significant environmental stressors; however, data from this study suggest that the limited archaeal community observed at 35°C is indeed effective in regulating fatty acids and H_2 within the digester. Methanogenic communities observed in reactors 49°C and 53°C_2 were

robust, having multiple well defined bands. Reactor 57.5°C_2, however, failed to produce any well defined bands representing methanogenic DNA. The minimal production of CH_4 by this digester suggests that the poorly defined shadows seen in Figure 2.3.b. at 57.5°C represent some quantity of methanogenic biomass.

Past research has shown aceticlastic methanogens are typically more temperature sensitive than hydrogenotrophic methanogens or acetatrophic bacteria. Ahring et al (2001) found that an increase in reactor temperature from 55°C to 65°C actually resulted in a marginally increase in the activity of hydrogenotrophic methanogens, while aceticlastic activity and overall methane yield decreased.

The results of this study with regard to methane production, as well as the accumulation of acetate and propionate are consistent with the findings of previous research. The greatly simplified methanogenic community observed at 57.5°C may be the result of temperature inhibition of aceticlastic methanogens, resulting in the reliance on hydrogenotrophic methanogenesis as the dominant source of methane gas.

The implication of this hypothesis on overall carbon flow within the 57.5°C reactors is that acetate produced during the fermentation of organic acids and other complex substrates must be channeled around aceticlastic methanogenesis in order to produce methane gas. Thermophilic species of bacteria capable of carrying out acetate oxidation as shown in Table 2.3 have been recognized (Hattori et al. 2000; Lee and Zinder 1988); however, acetate oxidation is primarily thought to dominate at lower acetate concentrations than were observed during this study (Shigematsu et al. 2004). The highly exergonic condition for acetate oxidation shown in Figure 2.1.d. clearly exhibits the competitive advantage that acetate-oxidizing bacteria would have under the conditions within our thermophilic digesters. Perhaps the elimination of a functional aceticlastic methanogenic population would naturally result in the channeling of acetate through acetate oxidation. Community analysis of the bacterial population (Figure 2.3.a.) present at 49°C, 53°C, and 57.5°C shows that the overall bacterial community in not greatly affected by the digestion temperature, and supports the hypothesis that bacterial oxidation of acetate is an important metabolism in our digesters.

2.8 CONCLUSION

The observed mechanism of temperature induced instability observed in this study agrees with previous discussions of high-temperature methanogenic environments. The accumulation of fatty acids corresponded with low observed methane output, and suggests that the temperature limitation of aceticlastic methanogenesis may be responsible for reactor instability and a significantly depressed pH at 57.5°C. Hydrogen accumulation was observed to gradually increase throughout the range of thermophilic temperatures, revealing that the onset of temperature induced instability was not merely a binary phenomenon at 57.5°C, but the result of a gradual change in energy flow that became apparent only once an inhibitory reactor condition was reached. This study supports past efforts to establish on-line hydrogen monitoring as an early warning detector of digester upset in instability. Following the protocol established by CordRuwisch et al (1997), the reactor operating a 55°C would have signaled digester upset conditions, and the type of failure observed at 57.5°C would potentially be avoided.

A better understanding of the role that digestion temperature plays on the accumulation of anaerobic digestion intermediates is critical to the design of high-temperature digestion systems to achieve sludge hygienisation. Additional microbial community and kinetic studies are required in order to test the proposed mechanism of digester instability, and to determine the importance of acetate oxidizing bacteria during high-temperature anaerobic digestion.

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2.10 TABLES

Table 2.1

Observed reactor conditions at various temperatures.

Reactor	pH	Alkalinity (ppm as $CaCO_3$)	VSR (%)	Daily Gas Production (litres per day)		VFA Concentrations (ppm as Acetate)		
				CH_4	CO_2	Acetate	Propionate	VFA_{Total}
37°C	NA[1]	NA	NA	7.0 ± 0.3	3.8 ± 0.1	24	BDL[3]	39 ± 4
49°C	7.45 ± 0.04	3391 ± 115	57.9 ± 3.9	NA	NA	259	500	1226 ± 564
51°C	7.38 ± 0.04	4180 ± 143	48.8 ± 1.6	10.8 ± 3.0	6.1 ± 1.7	219	517	1039 ± 151
$53°C_1$	7.40 ± 0.06	4269 ± 112	57.8 ± 4.9	12.9 ± 1.2	6.8 ± 0.8	75	417	537 ± 88
$53°C_2$	7.46 ± 0.05	4819 ± 96	56.8 ± 1.8	14.1 ± 1.1	7.6 ± 0.7	254	466	814 ± 362
55°C	7.46 ± 0.03	4695 ± 73	48.4 ± 3.7	13.0 ±1.3	6.3 ± 2.4	70	471	590 ± 235
$57.5°C_1$	7.13 ± 0.07	3722 ± 123	45.4 ± 5.1	3.2 ± 0.5	2.5 ± 0.4	334	1318	4052 ± 255
$57.5°C_2$	7.13 ± 0.04	3965 ± 89	45.5 ± 1.9	4.8± 0.5	3.9 ± 0.5	624	1485	3411 ± 523

[1] Data not available for reactor [2] Average ± Standard Deviation [3] Below detection limit (<10 ppm)

Table 2.2

Standard and VFA-corrected VSR of laboratory digesters at various temperatures.

	Volatile Solids Reduction (%)	
	Standard	**VFA Correction**
49°C	57.9	55.0
51°C[b]	48.8	46.4
$53°C_1$	53.6	52.4
$53°C_2$	56.8	55.0
55°C	48.4	47.0
$57.5°C_1$	45.4	36.1
$57.5°C_2$	45.5	37.7

Table 2.3

Energetic reactions involved in acetate and propionate metabolism during anaerobic digestion.

Reaction	Equation	$\Delta G^{0'}$ (kJ/reaction)	
Hydrogenotrophic Methanogenesis	$H_2 + {}^{1}\!/_{4}\, CO_2 \rightarrow {}^{1}\!/_{4}\, CH_4 + {}^{1}\!/_{2}\, H_2O$	-130.7	(Scholten and Conrad 2000)
Aceticlastic Methanogenesis	$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-31.0	(Rothfuss and Conrad 1993)
Syntrophic Propionate Oxidation	$CH_3CH_2COO^- + {}^{1}\!/_{2}\, H_2O \rightarrow CH_2COO^- + {}^{3}\!/_{4}\, CH_4 + {}^{1}\!/_{4}\, CO_2$	-26.0	(Scholten and Conrad 2000)
Propionate Oxidation	$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + 3H_2 + CO_2$	68.4	(Scholten and Conrad 2000)
Acetate Oxidation	$CH_3COO^- + 4H_2O \leftrightarrow 2HCO_3 + 4H_2 + H^+$	104.6	(Rothfuss and Conrad 1993)

2.11 FIGURES

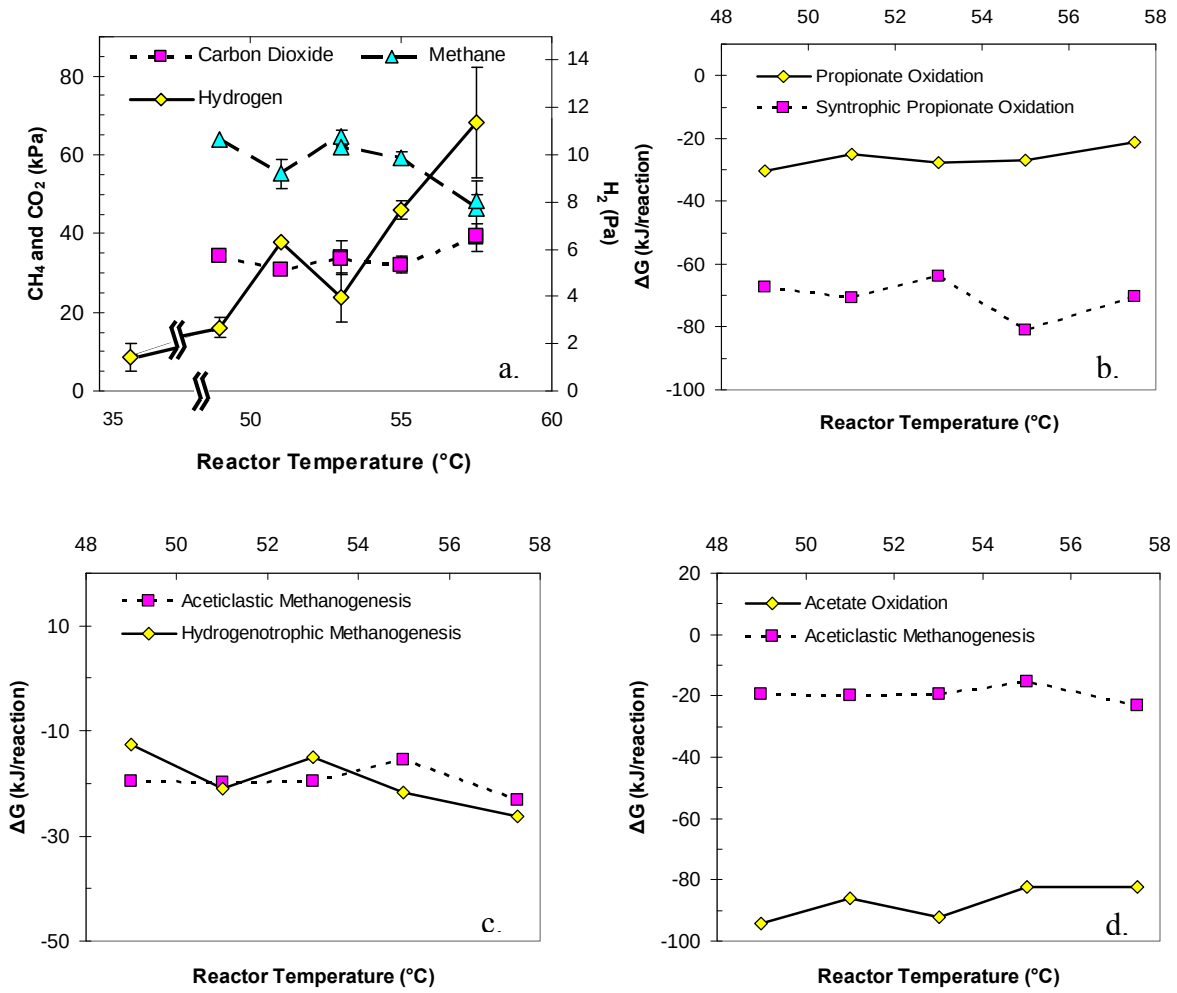

Figure 2.1

Effect of digestion temperature on (a.) headspace gas composition and free energy release associated with (b.) propionate metabolism, (c.) methane formation, and (d.) acetate metabolism.

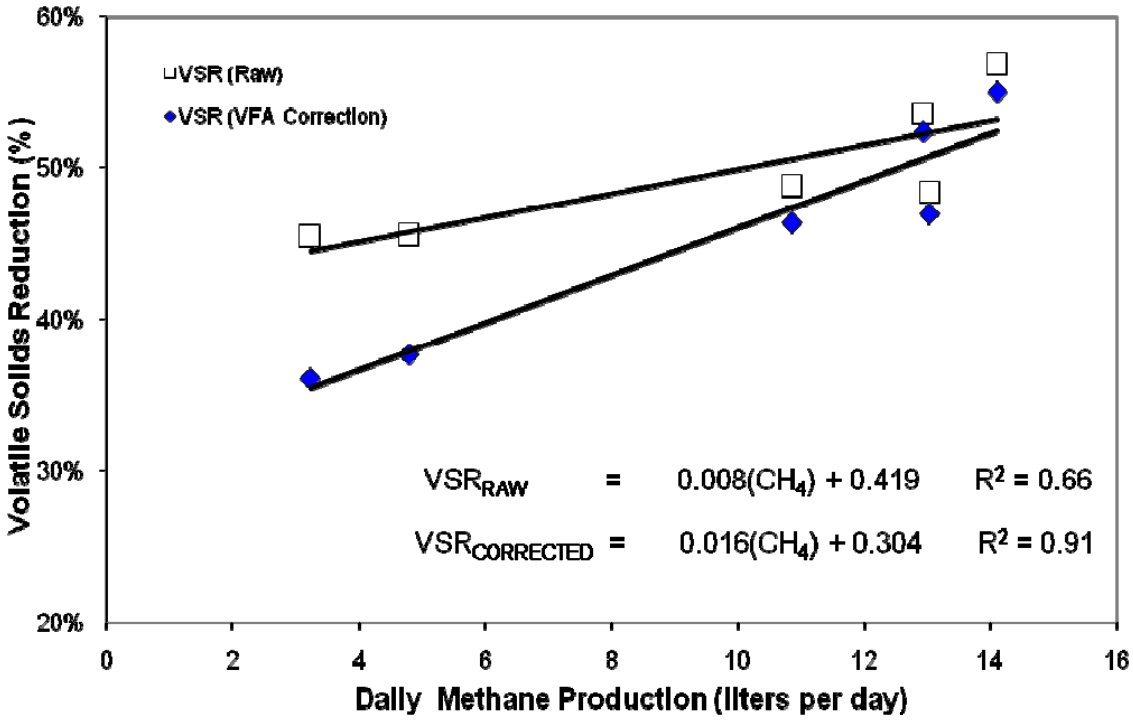

Figure 2.2

Correlation of average steady-state CH4 production with raw and VFA-corrected VSR.

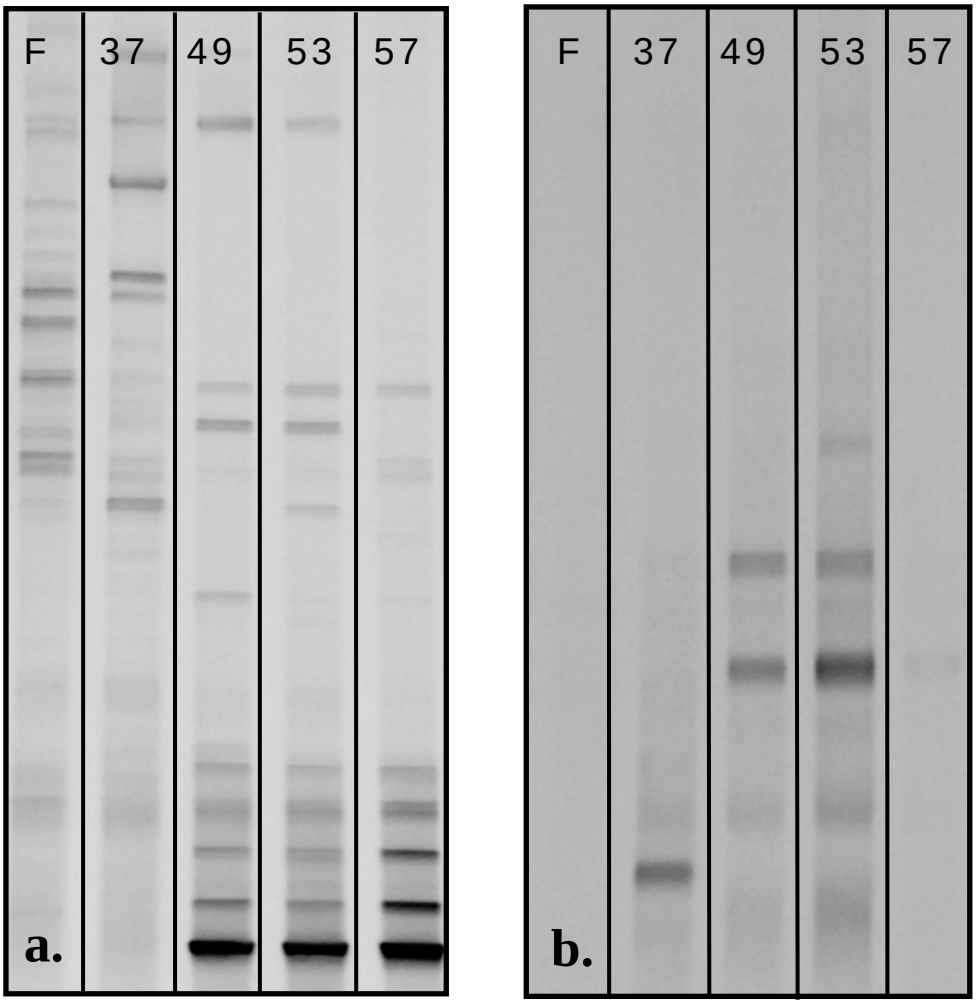

Figure 2.3

Bacterial (a.) and archaeal (b.) community profiles of reactor feed (F), mesophilic digester (37°C) and selected thermophilic digesters (49°C, 53°C2, and 57.5°C2) by PCR-DGGE.

CHAPTER 3

ANAEROBIC DIGESTION OF RAW AND THERMALLY HYDROLYZED SLUDGE UNDER VARIOUS OPERATIONAL CONDITIONS

3.1 AUTHORS

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3.2 ABSTRACT

Interest in energy recovery and sludge minimization from anaerobic digestion has resulted in the development and application of thermal hydrolytic pretreatment processes upstream of mesophilic anaerobic digestion (THD). Typically, waste activated sludge (WAS) has been targeted for thermal pretreatment due to its relatively poor biodegradability compared to primary solids. However, increased demand for low-pathogen biosolids requires thermal pretreatment of primary solids in addition to WAS. There are comparatively few performance data available to evaluate the operation of a THD process treating combined primary sludge and WAS under a range of operational conditions.

Volatile solids reduction (VSR) by THD ranged from 56-62%, compared to approximately 50% for conventional mesophilic anaerobic digestion (MAD). Higher VSR contributed to 24-59% increased specific biogas production from THD relative to MAD. The high solids conditions of the THD feed resulted in high total ammonia nitrogen (proportional to solids loading) and total alkalinity concentrations in excess of 14 g/L as $CaCO_3$. Increased pH in THD reactors caused 5-8 time more unionized ammonia to be present than in MAD, and this likely led to inhibition of aceticlastic methanogens resulting accumulation of residual volatile fatty acids between 2-6 g/L as acetic acid. THD produced high quality biosolids cake that possessed low organic sulfur-based biosolids odor and dewatered to between 33-39% total solids.

3.3 KEYWORDS

Ammonia release
Anaerobic digestion
Biosolids dewatering
Biosolids odor
Methane energy recovery
Sludge minimization
Thermal hydrolysis

3.4 NOMENCLATURE

CST	Capillary suction time
MAD	Conventional mesophilic anaerobic digestion
NH_3	Free unionized ammonia
SRT	Solids retention time
TAD	Thermophilic anaerobic digestion
TAN	Total ammonia nitrogen
THD	High-solids anaerobic digestion of thermally pretreated wastewater sludge
THP	Thermal hydrolytic pretreatment
TS	Total solids
VFA	Volatile fatty acids
(t)VOSC	(total) Volatile organic sulfur compounds
VS	Volatile solids
VSR	Volatile solids reduction
WAS	Waste activated sludge

3.5 INTRODUCTION

With the exception of final effluent, biosolids represent the most significant end product of modern wastewater treatment systems. In this sense, wastewater treatment cannot be considered "complete" until the end-use of biosolids is considered and implemented. Beneficial disposal

routes for biosolids are becoming increasingly limited as biosolids generation increases. Simultaneously, the encroachment of land development into once rural areas applies more stringent requirements on the quality of land-applied biosolids. These factors contribute to drive the need for in-plant sludge minimization, and reduced overall biosolids generation. These requirements are underscored by defined research efforts such as the Water Environment Research Foundation's (WERF) Optimization of Wastewater Solids Operations (OWSO) project. This effort includes the development and identification of innovative solids management processes that reduce biosolids volume and encourage energy recovery. In many cases, anaerobic digestion of wastewater sludge is the major process component of biosolids generation. Energy recovery and sludge minimization goals are better achieved by improving the methanization of waste sludges.

The rate limiting step in anaerobic digestion is considered to be particulate hydrolysis and solubilization of organic material (Eastman and Ferguson, 1981). Physical hydrolysis has been applied using high-temperature thermal pretreatment in both laboratory studies (Bougrier *et al.*, 2008; Eskicioglu *et al.*, 2006; Li and Noike, 1992) and full-scale applications (Barr *et al.*, 2008; Kepp *et al.*, 2000; Potts and Jolly, 2004). Researchers have shown that the degree of sludge hydrolysis prior to anaerobic digestion resulted in corresponding improvements in overall solids destruction during anaerobic digestion (Bougrier *et al.*, 2006) and as a result, biogas production from waste-activated sludge (WAS) is improved by 20-100% with THD relative to MAD (Barjenbruch and Kopplow, 2003; Bougrier *et al.*, 2006; Li and Noike, 1992; Stuckey and McCarty, 1978; Tanaka *et al.*, 1997; Valo *et al.*, 2004). Common temperature ranges for thermal hydrolysis are from 150-190°C, and corresponding pressures of 4.8 to 12.6 bar. It appears that the hydrolysis temperature, rather than digestion parameters such as solids retention time (SRT) and digester temperature, impacts the degree to which biogas generation is improved via THD (Li and Noike, 1992; Pinnekamp, 1989; Stuckey and McCarty, 1978). Drawbacks to THD process have been identified as the production of inert organic material (Phothilangka *et al.*, 2008), highly colored liquor (Dwyer *et al.*, 2008), and ammonia release (Kepp *et al.*, 2001). These drawbacks can largely be mitigated by reducing pretreatment temperature or solids loading (Dwyer *et al.*, 2008; Wilson and Novak, 2009).

Generally, positive results regarding methanization of sludge and sludge minimization have been attained through the use of THD; however, these studies focused primarily on THD of sludges consisting of WAS only. Long-term beneficial reuse of biosolids requires consideration of sludge sanitization by THD (Potts and Jolly, 2004). To achieve Class A biosolids as defined by CFR 40 Part 503.32, primary solids must undergo thermal treatment as well, and little information regarding THD of combined sludges exists. In addition, case studies of full scale installations are inherently limited in their ability to include side-by-side data from concurrently operated conventional MAD and laboratory batch digestion studies are inadequate to investigate the operability of the process under various operational conditions (e.g. SRT, digester temperature, pretreatment temperature) for a consistent sludge source.

The objective of this study was to operate a series of semi-continuous THD systems treating combined primary sludge and WAS from a single source in order to determine the potential for sludge minimization and methanization relative to concurrently operated MAD. Operational conditions for the reactors in this study were selected based on those being considered for a full-scale THD process installation at the District of Columbia Water and Sewer Authority (DCWASA) Blue Plains Advanced Wastewater Treatment Plant (Blue Plains).

3.6 METHODOLOGY

3.6.1 Reactor Setup and Operation

Conical high-density polyethylene reactors supplied by Hobby Beverage Equipment Company (Temecula, California) were used as pilot scale anaerobic digesters. The conical bottom of these vessels was thought to be advantageous in terms of mixing of dense high solids sludge feed to THD. The nominal volume of the reactor vessels was 25 liters. Reactors were operated with active volumes of either 15 liters (15-day SRT) or 20 liters (20-day SRT) in order to maintain a constant volumetric feeding and wasting rate across each system. Daily batch feeding was performed at a rate of 1 L/day. The reactors were modified to accept a threaded stainless steel thermometer. Digestions were carried out at either 37°C (maintained within a constant temperature room) or at 42°C with the addition of Thermolyne heating tape (Fisher Scientific). Reactors were continuously mixed by digester gas recirculation from the headspace through the conical reactor bottom.

Conventional mesophilic anaerobic digesters (MAD) were initially seeded with sludge from Pepper's Ferry Regional Wastewater Treatment Plant (Radford, VA). Thermally hydrolyzed sludge digesters (THD) were initially seeded with reconstituted dewatered biosolids from Ringsend Wastewater Treatment Works (Dublin, Ireland) which was commissioned in 2003 and operates THD.

Digester feed was obtained from DCWASA Blue Plains and consisted of a 1:1 ratio of primary and secondary solids. Sludge to be subjected to MAD was shipped on ice to Virginia Tech (Blacksburg, VA). These solids had a concentration of approximately 6% total solids (TS). Sludge that was thermally pretreated was dewatered to approximately 15% TS by DCWASA and shipped on ice to RDP Technologies, Inc. (Norristown, PA) for thermal treatment. The dewatered cake was pretreated for 30 minutes at either 150°C (4.80 bar) or 170°C (7.90 bar), followed by an immediate flash to ambient temperature and pressure. Sludge hydrolysate was then shipped on ice to Virginia Tech. These solids had concentration ranging from 10-12% TS, depending on the hydrolysis temperature. Since the temperatures required for thermal hydrolytic pretreatment (THP) were achieved via high pressure steam injection, the quantity of steam (and thus dilution of the 15% TS cake) was slightly higher for higher temperature thermal treatments. Additional ammonia nitrogen in the form of NH_4HCO_3 was added during one phase of this research in order to achieve an artificial increase in ammonia nitrogen concentration from 2380 to 2880 mg/L as N. Reactors were each operated for a period of at least five months in order to ensure that acclimation of the biomass occurred, and representative steady-state data could be collected. The general operating conditions (reactor SRT, digester temperature, and pretreatment temperature) of the anaerobic digesters are listed in Table 3.1.

3.6.2 Analyses

Total (TS) and volatile solids (VS) concentrations (Method 2540-G) as well as ammonia nitrogen (Method 4500-C) and total alkalinity (Method 2320-B) were determined as specified in Standard Methods for the Examination of Water and Wastewater. Wet tip gas meters (Nashville, TN) were used to determine total biogas production from the anaerobic digesters. Reported

values and associated error bars are indicative of average ± standard deviation of sample replicates over a period of steady-state reactor operation.

Volatile fatty acids (VFA) were measured weekly on the solution phase of each digester during steady-state operation. Samples for VFA analysis were passed through a 0.45 μm nitrocellulose membrane filter and frozen prior to analysis. Thawed samples were acidified by adding 85% phosphoric acid at a rate of 1% v/v and analyzed via HP5890 GC-FID and Supelco Nukol column with the following gas flow rates: (Nitrogen) 14 mL/min, (Air) 450 mL/min, (Hydrogen) 44 mL/min, (Helium) 16 mL/min. The initial oven temperature was set to 80°C and ran isothermally for 3 minutes, then increased at 6°C/min for 10 minutes.

A biosolids dewatering simulation method was used that has been shown to adequately mimic full-scale dewatering through the use of a high solids centrifuge. The laboratory simulation of high solids centrifugation involved polymer conditioning, shear application, lab-scaled centrifugation, and mechanical compression. Details of this process can be found elsewhere (Glindemann *et al.*, 2006; Muller *et al.*, 2004).

Incubation of dewatered biosolids were conducted in order to analyze the accumulation of specific volatile organic sulfur compounds (VOSC) as indicators of biosolids odor production (Higgins *et al.*, 2006; Novak *et al.*, 2006). Specific VOSC species associated with dewatered biosolids were measured using a cryotrapping gas chromatograph (GC, HP 5890) – mass selective detector (MS, HP 5970). The GC column was a Supelco Equity-5, 30 m x 0.25 mm capillary column with a 1.0 μm film thickness. A liquid nitrogen cryotrap was used to achieve species separation and was controlled by AMA Systems (Pforzheim, Germany) cryotrap heaters and control equipment. Compounds of interest, including methanethiol, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide were individually assessed using HP Chemstation integration software and then summed on the basis of sulfur mass to report total headspace VOSC (tVOSC) concentrations in ppmv. The reported concentrations were normalized with respect to the mass of volatile solids loaded to each headspace bottle in order remove the effect of heterogeneity in sample dryness and mass.

3.7 RESULTS AND DISCUSSION

3.7.1 Overall Solids and Biogas Performance

Regarding the comparison of the operational conditions of THD, seven distinct operational conditions were used to compare digester performance including an SRT range of 15 and 20 days, digester temperatures of 37 and 42°C, and hydrolysis temperatures of 150 and 170°C. Generally, 3 or 4 digesters (including MAD and THD) were operated concurrently throughout the approximately 2-year study (Figure 3.1). Temporal variations in feed sludge make it difficult to compare average steady-state data across the entire study; however, time course data from concurrently operated digesters reveal the nature of this variability (Figure 3.2). Periods of low VSR (e.g. days 280-290), high VSR (e.g. days 307-323), and periods of relative stability (e.g. days 378-443) are well correlated with respect to time, suggesting that valuable comparisons may be made between side-by-side digesters.

The highest VSR and relatively high biogas production was achieved from the digester receiving additional ammonia in the form of ammonium bicarbonate (THD IV). This process also exhibited a relatively high residual VFA concentration of approximately 6 g/L as acetic acid. A preference for non-aceticlastic methanogenesis via acetate oxidation has been observed in other high ammonia anaerobic processes (Karakashev *et al.*, 2006; Petersen and Ahring, 1991; Schnurer *et al.*, 1997; Schnurer *et al.*, 1999). Interestingly, lower solids reduction and biogas production were achieved for a side-by-side replicate reactor (THD III) that was not subjected to external ammonia addition. It is hypothesized that a more complete shift to a non-aceticlastic methanogenic pathway is an important characteristic of high-solids/ammonia processes such as THD for enhanced digester performance. Further microbial and molecular investigations of THD and MAD archaeal communities are under way in order to test this hypothesis.

For concurrently operated THD processes THD V, VI, and VII, a comparison of reactor performance relative to hydrolysis temperature can be made. While VSR for the THD pretreated at 150°C (THD V and VII) was just slightly higher than for that at 170°C (THD VI), a notable increase in biogas generation was attained with the reduction in hydrolysis temperature. It is unclear by which mechanism biogas generation (independent of VSR) is improved via hydrolytic

pretreatment at 150°C relative to 170°C. In the case of both MAD and THD, the selection of digester SRT of 15 or 20 days did not cause a significant increase in solids destruction or biogas generation that was consistent across all side-by-side processes.

While such comparisons can be dawn among THD systems operated in this study, it is clear that the most important distinction in reactor performance is that between THD and MAD in general. Stable operation of THD was obtained at very high volatile solids loading rates of approximately 5.05 kg VS/m^3-day (compared to 2.97 and 2.23 kg VS/m^3-day for the 15 and 20-day MAD, respectively). VFA/Alkalinity ratios routinely varied between 0.2 and 0.4 for THD throughout the study and pH remained consistent between 7.8 and 7.9 (data not shown). Given the increased solids loading, the THD outperformed the MAD in terms of both volatile solids destruction (Figure 3.3) and biogas generation (Figure 3.4). Additional volatile solids reduction (VSR) of 6-12% was achieved by THD and specific biogas production was improved by 24-59%.

3.7.2 Specific Biogas Production and VSR

Specific volumetric biogas production is most often normalized on the basis of VS destroyed. Such normalization provides a valuable measure of the methanization potential of the feed sludge, rather than the degree to which that feed sludge is consumed within the digester. Based on a macromolecular COD fractionation of wastewater sludge of roughly 52% protein, 31% lipid, and 17% carbohydrate (Tanaka *et al.*, 1997) and typical gas production rates for these fractions (Buswell and Neave, 1939), one would expect 0.938 cubic meters of biogas per kg of VSR (m^3/kg VSR). Considering conventional MAD, the specific biogas production measured in this study is in relatively good agreement with the literature-derived value (MAD I @ 1.017 m^3/kg VSR; MAD II @ 1.023 m^3/kg VSR). However, it is clear that THD results in a higher specific biogas production rate relative to VSR (Figure 3.4).

The incremental improvement in specific biogas production relative to VSR (noted as dimension A in Figure 3.4) suggests that more energetic volatile solids are degraded during THD. Since both protein and carbohydrates have typical gas production rates less than 1 m^3/kg VSR, the increased biogas production relative to VSR by THD directly suggests better degradation of the

lipid fraction of the feed sludge. The hydrophobicity of fats and grease (primarily long-chain fatty acids and associated triglycerides) greatly limits the rate of their degradation at mesophilic temperatures (Moen *et al.*, 2003; O'Rourke, 1968). Previous research has shown that lipids are readily fragmented and solubilized to form short-chain fatty acids and other low molecular weight aliphatic compounds via thermal hydrolysis (Li and Noike, 1992; Tanaka *et al.*, 1997; Wilson and Novak, 2009). As evidenced by published octanol-water partition coefficients (K_{OW}), carboxylic acid length has a distinct affect on partitioning between the organic and aqueous phases (Scherrer and Howard, 1979). It is reasonable to conclude that the increased bioavailability of lipid derived material resulted in an increased production of biogas per mass of volatile solids degraded.

As mentioned previously, improvements in solids destruction were obtained with THD relative to MAD (Figure 3.3). It is unclear as to whether this improvement is attributable to increased rates (e.g. a shift from slowly to readily biodegradable organic fractions) or an increase in the overall extent (e.g. a shift from hardly to slowly biodegradable organic fractions) of solids degradation caused to thermal hydrolytic pretreatment. The question lies in whether relatively complete stabilization occurred during MAD. If so, one could conclude that THD altered the extent of potential anaerobic digestion. If not, one could conclude that a larger fraction of biodegradable material was indeed degraded during 15-20 days via THD relative to MAD. In either case, the extent to which volatile solids were stabilized by THD further contributes to the advantage of hydrolytic pretreatment in terms of normalized biogas production.

In disparate processes such as THD and MAD where insight into the overall methanization of sewage is of interest, normalizing biogas generation to VS *fed* is perhaps more descriptive than to VS *destroyed*. As such, the observed 24-59% increase in biogas production relative to VS fed via THD relative to MAD (noted as dimension B in Figure 3.4) is dependent both on the degree of solids destruction and the yield of biogas per VS destroyed. The range of improvement is within, but on the lower end of previously reported increases in specific methane production (Barjenbruch and Kopplow, 2003; Bougrier *et al.*, 2006; Li and Noike, 1992; Stuckey and McCarty, 1978; Tanaka *et al.*, 1997; Valo *et al.*, 2004); however, as these other studies largely

focused on thermal pretreatment of sludges consisting of waste-activated sludge only, the somewhat reduced benefit for a combined sludge is expected.

3.7.3 Ammonia Release

The release of ammonia arising from the anaerobic degradation of wastewater sludge is predictable based on influent solids loading and subsequent hydrolysis/solubilization during anaerobic digestion (Wett *et al.*, 2006). Thus, high solids digestion facilitated by thermal hydrolytic pretreatment produces higher reactor ammonia concentrations than are achieved during conventional MAD (Table 3.1). Adjusting for VSR and solids loading, the average ammonia release for MAD was 36.7 mg NH_3-N/gram VSR. The combined ammonia release for the THD process was 35.8 (± 1.9) mg NH_3-N/gram VSR, including 6.2 (± 0.5) and 29.5 (± 1.8) mg NH_3-N/gram VSR for the pretreatment and digestion steps, respectively. These similar values for nitrogen release by solids destruction provide additional support to the hypothesis that additional solids destruction during THD was attributable to solids low in organic nitrogen (i.e. lipid rather than protein).

The higher organic loading to the THD, as well as higher VSR combined to produce elevated ammonia concentrations within these digesters. A secondary effect of the high solids loading to the THD was a corresponding increase in digester alkalinity, resulting in a higher characteristic pH for THD (7.82 ± 0.04) relative to MAD (7.40 ± 0.07). The combination of elevated pH and increased total ammonia nitrogen concentrations resulted in 5-8 times the concentration of free unionized ammonia as in MAD (Table 3.1). Unionized ammonia is a well-established inhibitor of anaerobic digestion, specifically reducing the activity of aceticlastic methanogenic populations (Angelidaki and Ahring, 1994; Eldem *et al.*, 2004; Lay *et al.*, 1998; Liu and Sung, 2002; Sung and Liu, 2003). The occurrence of high residual VFA concentrations in the anaerobic digestion process has been associated with NH_3 inhibition in both modeling (Angelidaki and Ahring, 1993) and full-scale investigations (Karakashev *et al.*, 2006). In this study, between 2-6 g/L of residual VFA (mostly as acetic acid) was measured for THD and was dependent on the SRT. Residual VFA concentrations for the MAD were 0.23 and 0.28 g/L for the 15 and 20 day SRT reactors, respectively. Unless this residual organic material is recovered,

such as for in-plant use as a carbon source for denitirification processes, the high levels of residual VFA after THD represent a significant loss of energy potential as methane gas. Additionally, this undegraded acetate must be further treated, either as a sludge dewatering recycle stream or as a return flow to the liquid side treatment plant.

3.7.4 Organic Sulfur-Based Odor Compounds and Sludge Dewaterability

Biosolids odor research has typically been limited to full-scale processes and field-produced sludge cakes. Although embryonic or innovative technologies could be successfully operated in the lab, no method existed to replicate the effects of full-scale dewatering processes for the small sludge volumes produced at lab-scale. This limitation led to the development of a laboratory simulation method for biosolids produced from high solids centrifugation, as well as the quantification of sulfur based biosolids odor compound accumulation during storage (Glindemann *et al.*, 2006). Since these methylated sulfur compounds undergo fairly rapid interconversion between one another, biosolids headspace sulfur odor is often quantified as total volatile organic sulfur compounds (tVOSC) (Higgins *et al.*, 2006). Included in this measurement are methanethiol, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide. Hydrogen sulfide is not typically included in this measurement since its production may be strongly dependent on sulfate reduction in addition to VOSC cycling, and is largely sequestered as iron-sulfide in moderate to high iron sludges such as those from DCWASA.

Biosolids from the THD process produced low headspace tVOSC relative to MAD (Figure 3.5). Several interpretations may be drawn with regard to these data. First, the additional solids destruction of TAD relative to MAD may have specifically targeted material that serves as precursors to tVOSC generation. This material is material is largely derived from protein degradation (Higgins *et al.*, 2006; Lomans *et al.*, 1999; Lomans *et al.*, 2002). As has been demonstrated throughout this paper, it is unlikely that additional VSR observed for THP was attributable to protein degradation. In addition, it has previously been shown for a wide variety of disparate processes treating DCWASA sludge, that VSR is a poor indicator of tVOSC generation from biosolids (Wilson *et al.*, 2009).

Second, the degradation of tVOSC via demethylation is viewed as the dominant mechanism for the removal of sulfur-based odor compounds associated with anaerobically digested and dewatered biosolids (Higgins *et al.*, 2006). The enhancement of methanogenic activity among THD digester communities relative to MAD may serve to limit the accumulation of tVOSC. However, the production of NH_3 concentrations within the THD leads to the presence of 2-6 g/L residual VFA after digestion. Due to this inhibition, it is unlikely that demethylation of tVOSC by the aceticlastic methanogenic population of THD is enhanced within the biosolids, and as such, it is unlikely that methanogenic activity is responsible for reduced odor production from THD relative to MAD.

Finally, processes with higher concentrations of residual VFA (including thermal hydrolytic pretreatment and TAD) have been observed to produce relatively low tVOSC-generating biosolids (Wilson *et al.*, 2006; Wilson *et al.*, 2009). One could speculate that the presence of residual bioavailable substrate for methanogens aids in their rapid recovery following oxidative and mechanical stresses during dewatering activities. Additionally, tVOSC production may be limited by the thermodynamics of sulfur-amino acid degradation (Orlygsson *et al.*, 1995) due to the accumulation of the high concentrations of acetate, propionate, ammonium, and hydrogen gas (Equations 3.1 and 3.2).

$$\text{Cysteine} + 3H_2O \rightarrow \text{acetate} + NH_4^+ + HCO_3^- + H_2S + H_2$$

$$\Delta G_o' = -57.9 \text{ kJ/rxn} \qquad (3.1)$$

$$\text{Methionine} + 3H_2O \rightarrow \text{propionate} + NH_4^+ + HCO_3^- + CH_3SH + H_2$$

$$\Delta G_o' = +137.4 \text{ kJ/rxn} \qquad (3.2)$$

It is clear that, especially based on Equation 3.2 regarding the production of methanethiol, the degradation of sulfur-containing amino-acids requires the efficient management of product inhibition. It is reasonable to assume that given the 60-80% moisture content of dewatered biosolids, the chemical environment with respect to the concentrations of low-molecular weight

compounds such as VFA and ammonia is similar to what occurs in the bulk liquid of the upstream digester. The high concentrations of these constituents typical of THD could impose a thermodynamic limitation on the conversion of proteinaceous material to tVOSC, and rather, force degradation of biosolids residuals through alternate pathways. Further exploration of the production and degradation of particular organic sulfur and amino acid species is needed in order to more fully support this hypothesis.

Traditional methods of assaying sludge dewaterability (e.g. capillary suction time, specific resistance to filtration, etc.) suggested relatively poor dewaterability of THD biosolids relative to MAD biosolids. For example, optimally polymer conditioned THD sludge samples had CST values raging from 54-59 seconds, compared with 24-27 seconds for polymer conditioned MAD sludge. However, cake solids after dewatering exhibited greatly improved sludge dewaterability that was not explained by CST measurements (Figure 3.6). While the mechanism for increased cake dryness following THD has not been well established, improved dewaterability of thermally hydrolyzed digested sludges is widely reported in previous studies (Neyens and Baeyens, 2003). A full-scale application of the THD process (Oxley Creek Water Reclamation Plant, Brisbane, Australia; 17.2 MGD) resulted in savings of approximately $71K (USD) per month, attributable to reduced transportation and disposal costs (Barr et al., 2008).

3.8 CONCLUSION

While this work is consistent with the growing body of literature regarding the THD process, it is unique among these studies because combined primary and secondary sludge is used, and a long-term laboratory evaluation of THD with respect to digester performance and biosolids quality under varied operational conditions is presented. Additionally, analysis of dewatered cake biosolids odor potential was incorporated into the study. Based on analysis of the nine reactor systems operated in this study and described herein, we can draw the following conclusions:

- Thermal hydrolytic pretreatment caused an increase in specific biogas production among all THD systems, relative to conventional MAD. This increase in specific biogas production was attributable to increased VSR by THD and a preference for degrading high-energy solids (e.g. lipids). THD resulted in improved overall specific biogas production by 24-59% relative to MAD.

- Total ammonia release per mass of volatile solids destroyed was equivalent for conventional MAD (digestion) and THD (pretreatment *and* digestion). However, higher solids loading to THD resulted in TAN concentrations between 2050 and 2510 mg/L NH_3-N. Furthermore, alkalinity release from solids destruction caused higher reactor pH in THD. The result was 5-8 times more unionized ammonia in THD than MAD. Inhibition of the THD aceticlastic methanogenic community was evidenced by residual VFA concentrations of approximately 2-6 g/L as HAc.

- The accumulation of tVOSC, an indicator of biosolids odor, from THD digested and dewatered biosolids was reduced by 52-92% relative to MAD. Additionally, THD biosolids were much easier to dewater than MAD biosolids. Low-odor, high-solids biosolids cake represents a significant benefit of thermal hydrolytic pretreatment.

- Based on broad measures of digester performance (e.g. VSR and biosolids production), there was little effect of varying operational parameters of THD (i.e. digester SRT, and digester and pretreatment temperatures). The conclusion of this research then, is that enhanced energy recovery, reduced sludge disposal, and stable operation can be achieved relative to MAD under the range of varied operational conditions described in this study.

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3.11 TABLES

Table 3.1

General operating conditions of lab-scaled semi-continuous flow digester systems.

Digester ID	SRT (days)	Digester Temperature (°C)	Pretreatment Temperature (°C)	TAN (NH_3) mg/L N
MAD I	20	37	NA†	1430 ± 80 (40)
MAD II	15	37	NA	1330 ± 60 (37)
THD I	15	37	170	2050 ± 80 (190)
THD II	15	37	170	2220 ± 300 (200)
THD III	15	42	170	2280 ± 170 (250)
THD IV	15	42	170	2880 ± 180 (320)‡
THD V	15	42	150	2510 ± 80 (280)
THD VI	15	42	170	2470 ± 130 (270)
THD VII	20	42	150	2130 ± 430 (230)

† No pretreatment was applied prior to MAD.

‡ Includes additional 500 mg/L NH_3HCO_3-N artificially added with digester feed. TAN concentration of 2380 mg/L TAN-N was considered for ammonia release calculations for THD IV.

3.12 FIGURES

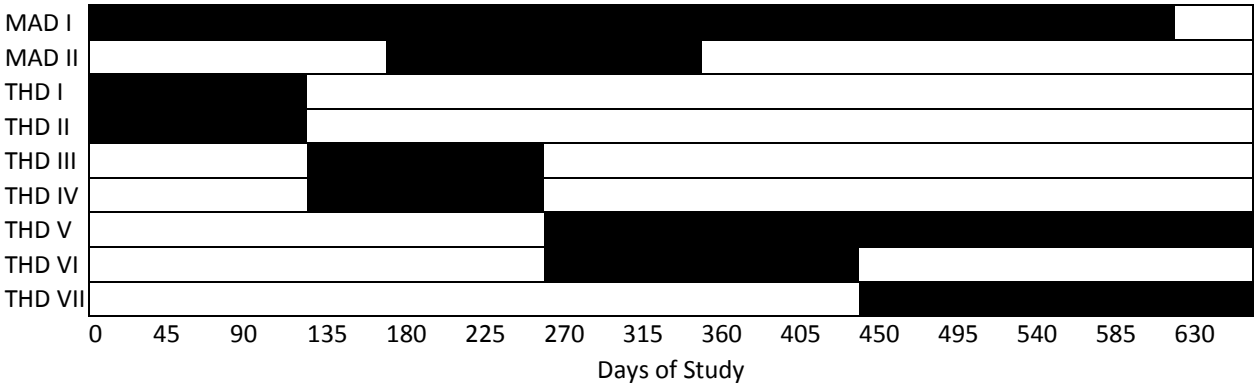

Figure 3.1

Schedule of operation of nine anaerobic digestion systems throughout this 2-year study. Each digester was operated for a minimum of 4 months to ensure steady-state conditions, and 3-4 digestion systems were operated at any given time.

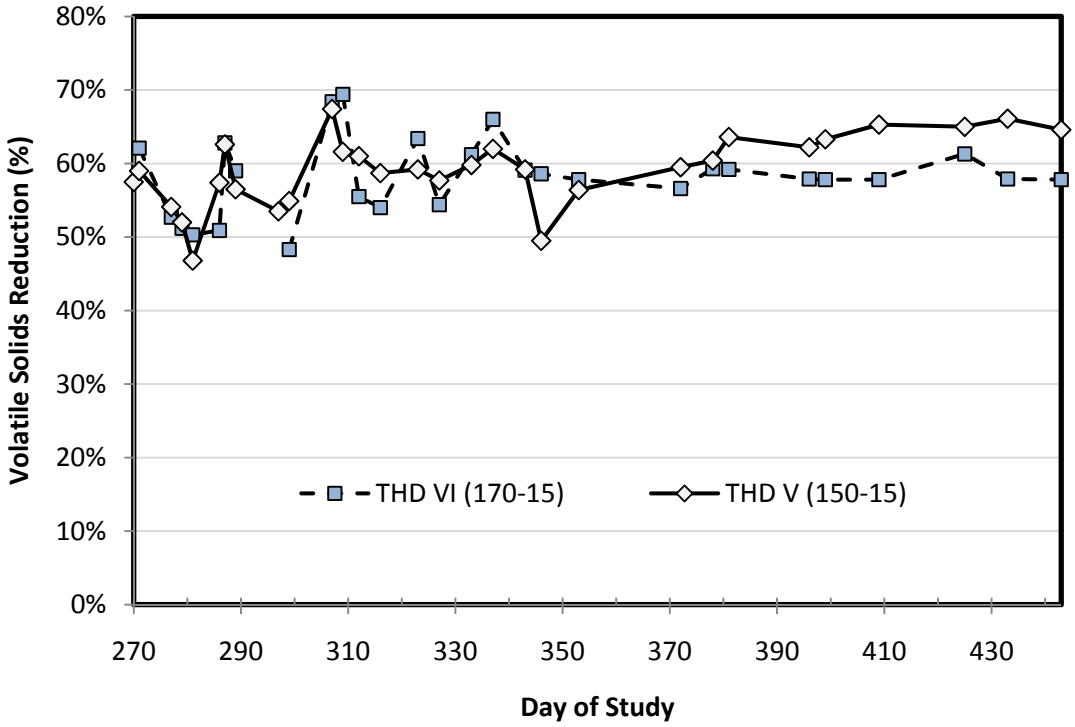

Figure 3.2

Time-course VSR data for THD digesters operated concurrently. Variability in sludge feed causes similar trends in VSR with respect to time for side-by-side digestion systems

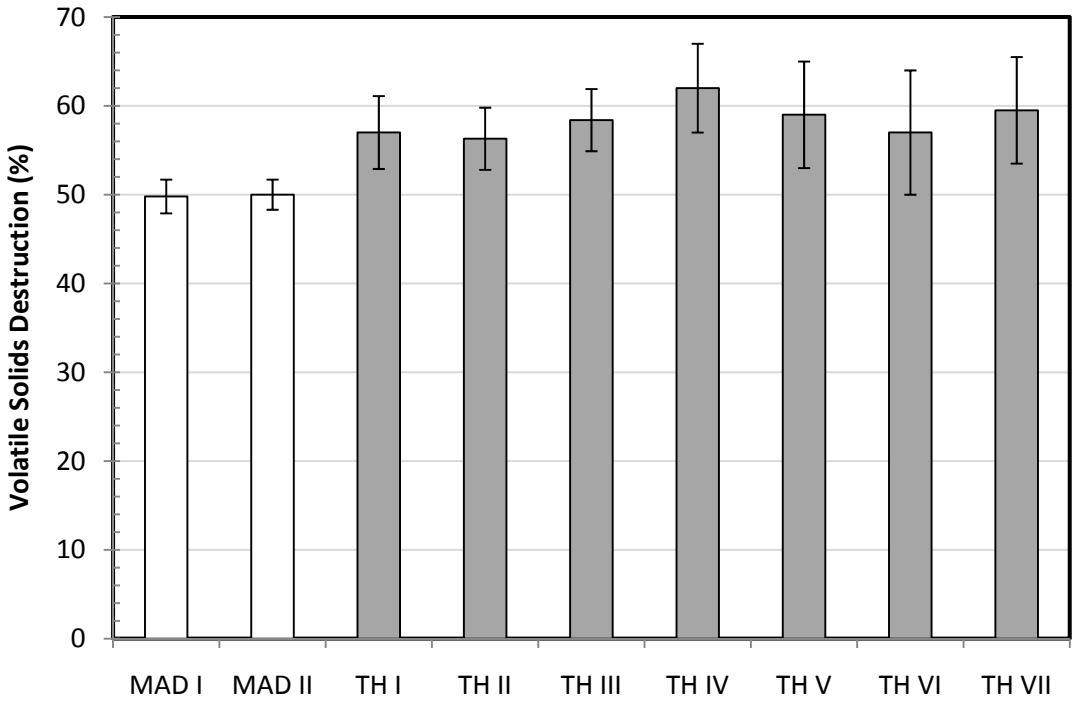

Figure 3.3

Average steady-state VSR in MAD (white) and THD (shaded) systems

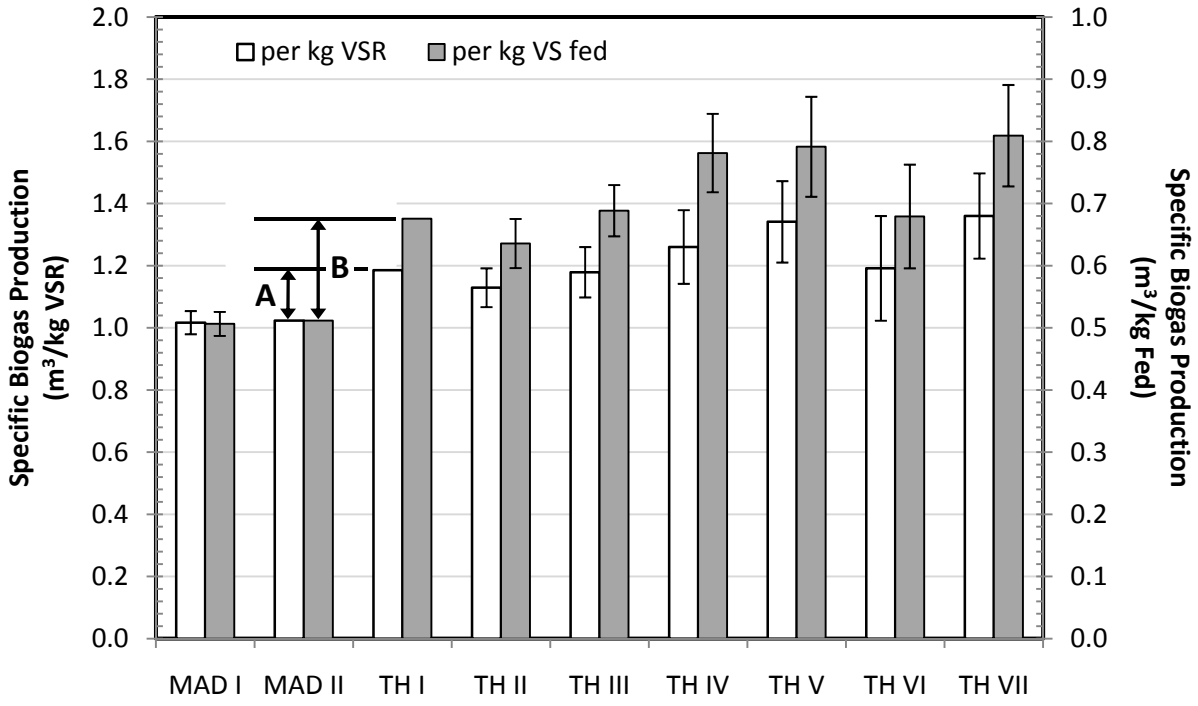

Figure 3. 4

Specific biogas production rates normalized to kg *VSR* (white) and kg *VS fed* (shaded). See in-text discussion for explanation of dimensions A and B.

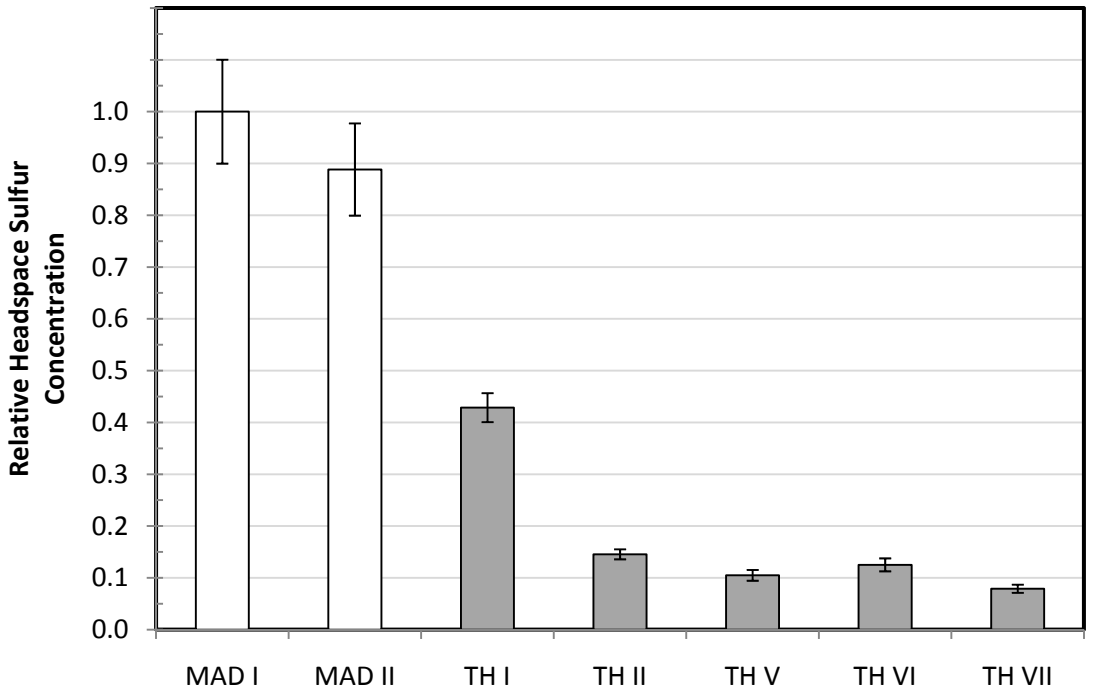

Figure 3.5

Peak headspace tVOSC from incubated dewatered MAD (white) and THD (shaded) biosolids samples. Peak headspace tVOSC concentration associated with MAD I biosolids (890 ppmv/g VS) was normalized to 1.0.

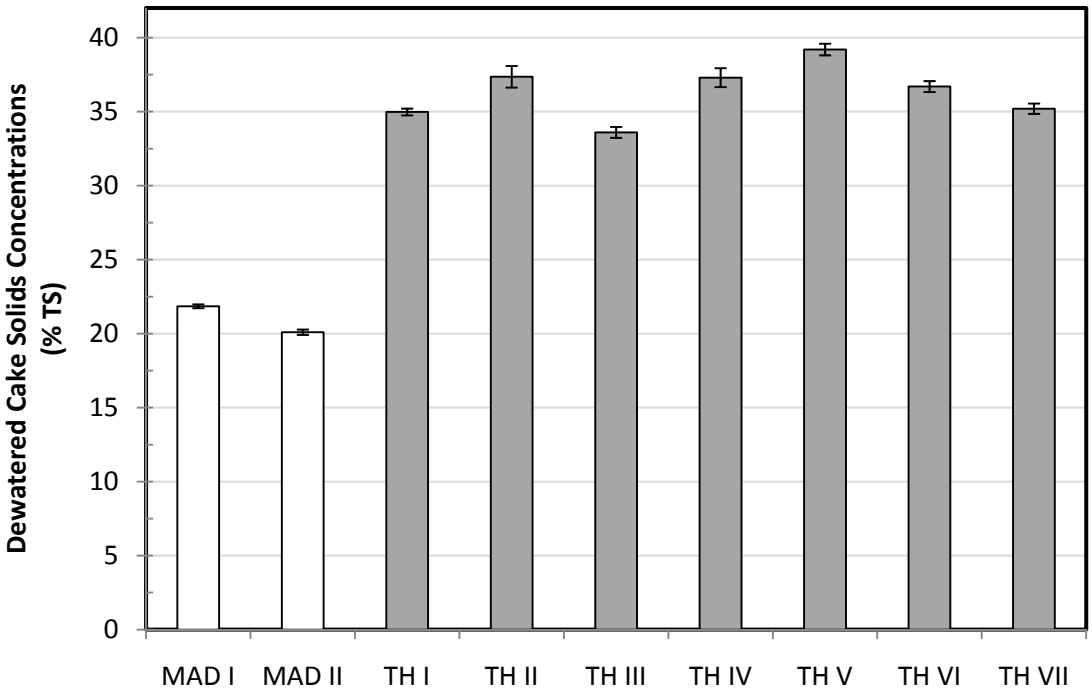

Figure 3.6

Cake solids of dewatered MAD (white) and THD (shaded) biosolids using lab-scaled high solids centrifuge simulation.

CHAPTER 4

HYDROLYSIS OF MACROMOLECULAR COMPONENTS OF PRIMARY AND SECONDARY WASTEWATER SLUDGE BY THERMAL HYDROLYTIC PRETREATMENT

4.1 AUTHORS

Christopher A. Wilson and John T. Novak

4.2 ABSTRACT

A laboratory simulation of the thermal hydrolytic pretreatment (THP) process was performed on wastewater sludge, as well as key macromolecular components: proteins, lipids, and polysaccharides. Hydrolysis temperatures from 130-220°C were investigated. The objectives of this study were to determine how and over which temperature range THP specifically affects sludge components, and whether hydrolysis temperature can be used to minimize the previously reported drawbacks of THP such as high total ammonia nitrogen (TAN) loads and the production of highly-colored recalcitrant organics. In addition, the applicability of THP to primary sludge (PS) was investigated.

The breakdown of proteins, lipids, and polysaccharides was determined to be temperature dependent, and both waste activated sludge (WAS) and PS responded similarly to THP apart from intrinsic differences in lipid and protein content. Pure carbohydrate solutions were not largely converted to mono- or dimeric reducing sugar units at temperatures below 220°C, however significant caramelization of starch and production of dextrose and maltose was observed to occur at 220°C. Volatile fatty acid production during thermal hydrolysis was largely attributed to the breakdown of unsaturated lipids, and long chain fatty acid production was not significant in terms of previous reports of methanogenic inhibition. Ammonia was produced from protein during thermal hydrolysis, however solids loading rather than thermal hydrolysis

temperature appeared to be a more meaningful control for ammonia levels in downstream anaerobic digestion.

4.3 KEYWORDS

Ammonia
Lipid
Methanogenic inhibition
Polysaccharide
Protein
Sludge minimization
Thermal hydrolysis

4.4 NOMENCLATURE

(s)COD	(soluble) Chemical oxygen demand
LCFA	Long chain fatty acid
PS	Primary sludge
TAN	Total ammonia nitrogen
THP	Thermal hydrolytic pretreatment
TS	Total solids
UV_{254}	Ultraviolet (254 nanometers)
VFA	Volatile fatty acid
VS	Volatile solids
WAS	Waste activated sludge

4.5 INTRODUCTION

The treatment and disposal of wastewater sludge is a major contributor to the operational costs of modern wastewater treatment. While treated wastewater sludge (or *biosolids*) are most often beneficially reused for agricultural fertilization, hauling biosolids over long distances to appropriate land applications sites is expensive and poorly justified in terms of fuel usage. In

terms of a life-cycle assessment of various costs and activities involved in wastewater treatment, biosolids hauling may account for 4-5 times the consumer paid economic cost and nearly double the net greenhouse gas emissions of treatment plant electricity usage (Rebitzer *et al.*, 2003). Still, the common practice of anaerobic digestion followed by land application seems to be the most economical and environmentally sensible widely employed practice for biosolids management (Murray *et al.*, 2008). Enhanced anaerobic digestion processes of particular interest are those that have the potential to reduce the overall amount of biosolids to be disposed, while maximizing the acceptability of the biosolids by increased pathogen inactivation and reduced biosolids odors.

Reduction of solids and methanization of sewage sludge can be improved by enhancing its rate limiting step, organic matter hydrolysis (Li and Noike, 1992). Several processes have been developed to enhance the solubilization of complex particulate substrate so that they can be more rapidly and completely consumed within the anaerobic process. Ultrasonic cavitation (Lehne *et al.*, 2001), ozonation (Battimelli *et al.*, 2003, Chiavola *et al.*, 2007), high pressure homogenization (Stephenson *et al.*, 2007) and thermal hydrolytic pretreatment (THP) (Bougrier *et al.*, 2008, Climent *et al.*, 2007, Li and Noike, 1992, Tanaka *et al.*, 1997) have been shown to have a positive effect on either the solubilization of organic material, or the reduction of wastewater sludge. Of these, ultrasonic cavitation, ozone, and high-pressure homogenization are largely targeted at the disruption of waste activated sludge (WAS) floc, while THP can be targeted at both primary sludge (PS) and WAS, and thus has potential to produce Class A biosolids as defined by United States CFR 40 Part 503.32 (USEPA, 1999). Because THP holds the combined benefits of decreasing biosolids quantity and increasing biosolids acceptability, it is of interest to the wastewater industry.

Most studies have shown an optimal range for thermal hydrolysis of wastewater sludge of 160 to 180°C, with increases in methane yield during subsequent anaerobic digestion up to that range. Higher temperatures lead to a sharp reduction in biodegradability of sludge hydrolysate (Bougrier *et al.*, 2008, Stuckey and McCarty, 1978). Possible explanations for this behavior include the production of recalcitrant soluble organics (Bougrier *et al.*, 2007, Pinnekamp, 1989) or toxic/inhibitory intermediates during the THP process (Barlindhaug and Odegaard, 1996,

Haug *et al.*, 1983). Hydrolysis temperature, more importantly than contact time, seems to govern the extent to which sludge disintegration occurs (Li and Noike, 1992). Several studies have reported the solubilization of aggregate chemical properties of sludge samples (e.g. lipids, polysaccharides, proteins, COD, etc.) and its effect on biological methane potential at specific temperatures (Haug *et al.*, 1978, Li and Noike, 1992, Stuckey and McCarty, 1978, Tanaka *et al.*, 1997). However, few have investigated thermal hydrolysis over a broad temperature range with for a single sludge source (Bougrier *et al.*, 2008), and no references to the effect of thermal hydrolysis on primary sludge or individual macromolecular components of wastewater sludge have been made.

Successful application of an advanced anaerobic digestion process requires the efficient channeling of carbon from complex particulate substrates to methane gas. The hydrolysis of lipids to long chain fatty acids (LCFA) and volatile fatty acids (VFA) as well as the hydrolysis of protein to ammonia and VFA has been discussed in detail (O'Rourke, 1968, Stuckey and McCarty, 1978, Wett *et al.*, 2006). Since wastewater sludge contains significant fractions of both lipid and protein (Tanaka *et al.*, 1997), it is reasonable to surmise that compounds which are inhibitory to methanogenesis such as ammonia (Lay *et al.*, 1998, Liu and Sung, 2002) and hydrophobic fatty acids (Chowdhury *et al.*, 2007, Lalman and Bagley, 2002) could arise through the thermal hydrolysis of proteins and lipids, respectively; however, it is unclear which intermediates may be produced in sufficient quantities to impose a meaningful disruption or inhibition on the methanization process. The purpose of this study is to investigate the effect of THP temperature on specific macromolecular components of wastewater sludge, and to discuss the potential for inhibition by hydrolysis products on methanogenic activity.

4.6 METHODOLOGY

4.6.1 Hydrolysis Experiments

Hydrolysis experiments were carried out in a Parr No. 4745 acid digestion reactor. Heat was applied by a Fisher Scientific 220V muffle furnace. Samples were hydrolyzed for 2 hours (including initial heating time) and were then chilled at -20°C for 30 minutes until the reactor could be easily handled before storing overnight at 4°C to minimize volatilization of hydrolysate.

The experiments were performed on a variety of macromolecular compounds considered to be representative of the key fractions of wastewater sludge; carbohydrates, lipids, and protein. PS and WAS obtained from the District of Columbia Water and Sewer Authority Blue Plains Advanced Wastewater Treatment Plant were also subjected to hydrolysis experiments. These sludges were obtained thickened by gravity (PS) or by dissolved air floatation (WAS), and normalized to approximately 6% TS prior to experimentation. Hydrolysis temperatures ranging from 130°C to 220°C were investigated in most cases, though 110°C was warranted in the case of glyceryl trilinolenate (C18:3) for additional clarity. The corresponding pressures to the selected hydrolysis temperatures were as follows: 110°C (140kPa), 130°C (280kPa), 150°C (510kPa), 170°C (890kPa), 190°C (1460kPa), 220°C (2870kPa). The absolute maximum pressure rating for the Parr No. 4745 acid digestion reactor is 8270kPa. Individual substrates within each class of chemical are listed in Table 4.1. Chemical characteristics of the sludge samples are listed in Table 4.2.

4.6.2 Sample Analysis

Soluble fractions of wastewater sludge were defined as passing 0.45µm filtration (Corning® cellulose acetate polystyrene filter apparatus). Total solids (TS), volatile solids (VS), total ammonia nitrogen (TAN) and chemical oxygen demand (COD) were determined as per *Standard Methods, 21st Ed.* (APHA, 1998). Ultraviolet absorbance at 254 nm (UV_{254}) of the soluble fraction of sludge hydrolysate was measured with a Beckman DU640 spectrophotometer.

Soluble proteins in sludge and protein hydrolysate were each measured using the Hartree (1972) modification of the Lowry *et al.* (1951) protein assay using bovine serum albumin (BSA) as a standard. Protein hydrolysate was size-separated by ultrafiltration at 1kDa, 10kDa, and 30kDa with Millipore regenerated cellulose membranes prior to analysis. Ammonia yield from protein was normalized based on the organic nitrogen (org-N) content of BSA based on typical amino acid composition (BSA = 15.95% org-N, 66.43 g/mol) (Swiss-Prot Database).

Soluble polysaccharides were measured as described by Dubois *et al.* (1956) using D-Glucose as a standard. Cellulose and starch hydrolysates were filtered through 0.45µm and dextrose,

cellobiose, and maltose were measured via HP1090 HPLC-RID (refractive index detector) with a Biorad Aminex HPX-87P column and nanopure water as the eluent.

Lipid hydrolysate was fractionated between water- and organic-soluble fractions by acidification then agitation in contact with equal volumes of hexane and sample. Volatile fatty acid (VFA) concentrations in filtered and acidified aqueous samples were measured via HP5890 GC-FID and Supelco Nukol column with the following gas flow rates: (Nitrogen) 14 mL/min, (Air) 450 mL/min, (Hydrogen) 44 mL/min, (Helium) 16 mL/min. The initial oven temperature was set to 80°C and ran isothermally for three minutes, then increased at 6°C/min for ten minutes. Analysis of hexane-soluble hydrocarbons was performed via Agilent 6870/5973 GC-MS and Agilent DB-5MS Column. The initial oven temperature was 100°C and ran isothermally for three minutes, increased by 10°C/min for 20 minutes, then held at 300°C for 4 minutes.

In all cases, error bars are used to indicate standard deviation from mean values of triplicate samples.

4.7 RESULTS AND DISCUSSION

The susceptibility of the macromolecular components of wastewater sludge to thermal treatment was largely evaluated on two bases, (1) the destruction of the parent molecule to form lower molecular weight derivatives, and (2) the release of small soluble *monomeric* substances. The production rates of hydrolysis products were defined on a mass yield basis (i.e mg/g parent compound) in the case of the pure chemical hydrolysis experiments, and on a concentration basis (i.e. mg/L sludge) in the case of sludge hydrolysis experiments. In order to correlate the yields derived from pure chemicals in this study to relevant sludge concentrations, an assumption is made that THP precedes a high TS anaerobic digestion process and that the pre-hydrolysis sludge cake has a COD of approximately 150,000 mg/L (see sample calculation in supplemental material). The fractionation of COD in WAS (40% protein, 25% lipid, 15% polysaccharide) as determined by Tanaka *et al.* (1997) was considered as “typical” and was used to arrive at estimated concentrations of various chemical species in wastewater sludge based on the measured yields derived from the hydrolysis of pure chemicals.

4.7.1 Protein Hydrolysis

Protein comprises the largest fraction of wastewater organic material, and as such, its destruction is intimately linked to desirable increases in volatile solids destruction (VS). Additionally, ammonia is produced through the hydrolysis of proteinaceous material (Kayhanian, 1994), and is well established as a significant inhibitor of methanogenesis in high TS (Lay *et al.*, 1998) and thermophilic (Sung and Liu, 2003) digestion systems.

Truly soluble protein is a subjective measurement that is operationally defined by filter pore size. BSA (66.4 kDa) would likely meet the often used definition for soluble substrates of passing a 0.45µm nominal pore size filter, however this says nothing of the requirement for proteolysis before the BSA can be used for either catabolic or anabolic purposes by a cell. Eskicioglu *et al.* (2006) discuss that anaerobic digesters being fed thermally pretreated WAS with smaller molecular weight fractions of both proteins and polysaccharides resulted in higher methane yields and better degradation kinetics than digesters receiving conventional WAS. In this case, a rather coarse definition of solubility is insufficient to discern between the degradability of various substrates.

Proteins are hydrolyzed during anaerobic digestion to peptides and individual amino acids which are in turn oxidatively degraded to short-chain fatty acids (McInerney, 1988). TH of the protein BSA was observed to have similar effects of this biological hydrolysis process in that protein was converted both to smaller molecular weight peptides and VFA. Hydrolysis temperature was observed to have an impact on the size fractionation of BSA (Figure 4.1). While very small polypeptides (< 1 kDa) were not produced at any temperature used, the dominant protein size fraction progressively decreased with increased temperature. From a fractionation standpoint 130°C and 150°C behaved very similarly, with 66% and 60% of protein being > 30 kDa, and 30% and 31% being 10-30 kDa, respectively. Higher temperatures beyond 150°C caused a further reduction in the dominant size fraction, down to 1-10 kDa at 220°C. Destruction of amino acids was apparent by the production of VFA from BSA (Figure 4.2) at temperatures greater than 150°C which could partially explain the absence of a pronounced < 1 kDa fraction at high hydrolysis temperatures. At temperatures below 220°C, the amount of VFA yield from protein was 15-30% of the yield measured from lipids.

Ammonia was also produced during the thermal hydrolysis of BSA. The evolution of ammonia reached a maximum at 170°C, whereas further molecular weight reduction proceeded at temperatures of 190°C and 220°C (Figure 4.3). It is noteworthy that at 220°C nearly 30% of the initial protein was apparently lost. Protein was measured by the Lowry assay which relies on the reduction of Cu^{2+} to Cu^{+} as it binds to peptide bonds in order to produce a color-change reaction with Folin reagent. It is possible that proteins were hydrolyzed to individual amino acids that did not react positively in the Lowry assay. At 130°C, approximately 4% of the total protein was not measured. Such relatively small protein destruction rates could stoichiometrically account for the observed TAN yield across the investigated temperature range, assuming that ammonia was produced not only through the cleavage of amine functional groups of amino acids (e.g. Histidine, Arginine), but also from the destruction of the peptide bonds.

Based on the assumed wastewater sludge composition, thermal hydrolysis of protein results in the following TAN concentrations: 770 mg/L (130°C), 1400 mg/L (150°C), 1690 mg/L (170°C), 1720 mg/L (190°C), and 1760 mg/L (220°C). This value is likely an overestimate of the potential for ammonia production by THP of a combined sludge since (a) the calculation involves the COD fractionation of WAS, which can be expected to be higher in protein than PS, (b) the nature of the clean-water experiment does not provide the complex multi-ligand chemical environment that wastewater sludge would provide, thus complexation and reaction of the ammonia produced did not occur, and (c) loss of TAN through volatilization was not allowed to occur in the experimental setup.

It is also important to note that the TAN concentration calculated here does not include that which would be produced through additional protein hydrolysis and VS destruction during anaerobic digestion. Though it is difficult to accurately predict the TAN concentration of an anaerobic digester without extensive characterization of a particular feed sludge, using the value 0.022 g NH_3-N/g TS to estimate ammonia release during conventional anaerobic digestion (Wett *et al.*, 2006) we obtain 2640 mg/L NH_3-N for 12% TS sludge. Considering a conventional digestion process without the addition of THP (solely biological hydrolysis), the TAN concentrations due to solids loading approach the previously reported threshold for ammonia inhibition of aceticlastic methanogens (Angelidaki and Ahring, 1994, Lay *et al.*, 1998, Sung and

Liu, 2003). Operation of THP at lower than 170°C may have some benefit in reducing the potentially inhibitory accumulation of ammonia within an anaerobic digester.

4.7.2 Lipid Hydrolysis

VFA production due to lipid hydrolysis was observed to be dependent on both the hydrolysis temperature and the degree of saturation of the fatty acid chain (Figure 4.2). The increased susceptibility of highly unsaturated fatty acids to the oxidative stress imposed by thermal hydrolysis is likely analogous to the susceptibility of the same compounds to rancidity during food spoilage and rancidification (Bernardier *et al.*, 2007). Further evidence of the role of hydrogenation in fatty acid susceptibility to thermal hydrolysis is the comparative contribution of C2-C7 short-chain fatty acids to the total quantified VFA after thermal hydrolysis of triglycerides of stearic and linolenic acid (systematically *all-cis-9,12,15-octadecatrienoic acid*) (Figure 4.4). The production of VFA was relatively low from glyceryl tristearate, contributing approximately 60 mg/L as HAc at 170°C based on the assumed lipid fraction of WAS. These VFA were predominantly distributed among C5 and longer fatty acids (89% of total C2-C7 fatty acids). Similarly, the production of VFA from glyceryl trilinolenate at 170°C contributed an approximate concentration in WAS of 460 mg/L as HAc consisting of 87% acetate and propionate. Subtracting this mass of acetic and propionic acid, most likely produced as a result of breaking of the ω-3, -6, and -9 double bonds, results in remarkably similar VFA yield to that which was observed from glyceryl tristearate. These data suggest that a significant fraction of VFA produced during thermal hydrolysis is from unsaturated fatty acids.

Perhaps of greater concern than VFA accumulation though, is the potential accumulation long-chain fatty acids (LCFA) in an anaerobic digester. Lalman and Bagley (2000) reported that unsaturated LCFA which are produced through the hydrolysis of fats have been found to be moderately inhibitory to methanogenesis at concentrations as low as 10-30 mg/L, whereas little or no inhibition was observed from saturated LCFA at 100 mg/L. thermal hydrolysis of glyceryl trioleate (1.5% w/v) in clean water at temperatures ≥ 170°C yielded free oleic acid near the reported threshold for inhibition of aceticlastic methanogenesis by unsaturated fatty acids (Table 4.3). Nevertheless, evidence exists that processes receiving threshold inhibitory loads of LCFA

outperform conventional processes in terms of methane yield since the addition of a rapidly biodegradable substrate enhances the extant enzymatic rates for biological hydrolysis and acidogenesis (Kabouris *et al.*, 2008).

The observed production of diverse chemical functionalities, rather than simple fragmentation of the lipid molecule, is not entirely unexpected considering the highly oxidizing environment within the reaction vessel and the gradual heating to each nominal hydrolysis temperature. Since it has been shown that THP is more strongly dependent on temperature than time of treatment, it can be considered that sludge thermally hydrolyzed at any temperature is actually subjected to lower temperature thermal hydrolysis conditions as the temperature gradually rises to each nominal condition. This may describe why certain functionalities requiring fewer redox reactions for their formation (e.g. aldehydes) from LCFA are produced in significant qualities at 130-150°C, while functionalities more distant to LCFA (e.g. alkanes and alcohols) require higher treatment temperatures. Comparatively little data is available to quantify the anaerobic toxicity of non-LCFA hydrocarbons produced via lipid hydrolysis; however the relatively random fractionation of the triglyceride to these compounds is of note since it may suggest a significantly different temperature-dependent chemical pathway for the degradation of lipid-derived material in thermally hydrolyzed sludge than through the well studied β-oxidation pathway of a conventional wholly biological process (Novak and Carlson, 1970).

A major implication of this work for wastewater treatment is that THP alters the chemical makeup of the hydrocarbon chains associated with lipid compounds. Lipids were fragmented to form VFA, as well as reacted to form various other functionalities. Potentially, an ancillary effect of the physical transformation of organic molecules by thermal hydrolysis is either the breakdown or conversion of xenobiotic compounds such as synthetic estrogens and other endocrine disrupting compounds (EDC) to less active chemical species. Adsorption to biosolids is an effective removal mechanism for certain hydrophobic EDC such as the various alkylphenols from a liquid wastewater stream, however conventional sludge stabilization processes are not effective in degrading these compounds (La Guardia *et al.*, 2001). There is reasonable question as to whether EDC sequestration in this manner is appropriate considering the broad and beneficial process of land application. The seemingly random fragmentation and

transformation of hydrocarbons observed in this study seems to suggest the benefit of further exploring THP for EDC degradation.

4.7.3 Polysaccharide Hydrolysis

Polysaccharides such as cellulose and starch are not expected to be converted to inhibitory derivatives via hydrolysis. However, evidence exists that exceptionally high temperature and short reaction time could cause non-enzymatic browning of sugar, either through caramelization (pyrolysis) of carbohydrates or reaction with N-terminal amines of proteins via the Maillard reaction (Labuza and Baisier, 1992). The production of Maillard products (i.e melanoidin) is a well studied area of the food processing and medical industry that has particular implications for the thermal treatment of sewage sludge. It is possible that the condensation of reducing sugars with amino acids to produce the characteristic "tea-colored" liquor of THP processes could be enhanced by the fragmentation of protein to expose additional free amines. The products of these reactions have been implicated in the poor degradability of sludge hydrolysate produced at temperatures above 180°C (Bougrier *et al.*, 2008, Pinnekamp, 1989), thus interrupting the channeling of carbon from complex particulate substrates to methane gas. In addition, the occurrence of centrate color has been positively correlated to increases in the operating temperature of THP (Dwyer *et al.*, 2008).

Diluted (100x) cellulose and starch hydrolysate did not absorb in the near UV range when produced at temperatures at or below 170°C. At 190°C, starch hydrolysate showed a UV_{254} absorbance of 0.02. Hydrolysis at 220°C resulted in a UV_{254} absorbance of cellulose hydrolysate of 0.03, and UV_{254} absorbance of starch hydrolysate of 0.42. The strong near-UV absorbance of starch, and less so of cellulose, at very high hydrolysis temperatures is apparently a direct result of caramelization rather than Maillard reaction since free protein/free amines were absent.

Few mono- and disaccharides were produced from starch hydrolysis at 130-190°C or from cellulose at any temperature. The onset of the apparent caramelization of starch at 220°C corresponded with approximately 89 ± 4.1% of the total mass of cellulose being recovered as either dextrose (37 ± 2.3%) or maltose (52 ± 3.4%) (Table 4.4). These small sugars should be

readily degradable under anaerobic conditions (Higuchi *et al.*, 2005), so the non-enzymatic browning of a pure starch solution seems not to correlate with the production of recalcitrant organic carbon. While the analysis of hydrolysate of pure polysaccharide solutions says little about the potential for recalcitrant compound production resulting from interaction with hydrolyzed protein at high temperatures, it appears that only a small mass of non-reducing long-chain polysaccharides (specifically starch and cellulose) is actually converted to reducing sugars (i.e. dextrose, cellobiose, and maltose) which can partake in this type of condensation reaction via thermal hydrolysis. Further investigation of the potential for reducing sugar formation from alternate sugars and long chain polysaccharides (e.g. hemicelluloses) is important to assess the potential effect of Maillard products on the biodegradability of wastewater sludge following THP.

Although the thermal hydrolysis of polysaccharides does not seem to be of particular importance in terms of the degradability of wastewater sludge, it is noteworthy that production tea-colored compounds that absorb in the near UV range are of importance when considering the potential for UV disinfection of the final plant effluent. While little UV_{254} absorbance was observed from polysaccharides alone, the small masses of dextrose, cellobiose, and maltose that were produced at moderate thermal hydrolysis temperatures may impart significant UV_{254} absorbance and interfere with UV disinfection when present in a complex organic mixture of wastewater sludge.

4.7.4 Wastewater Sludge Hydrolysis

In general, it is thought that the effectiveness of THP is inversely related to the initial biodegradability of the raw sludge (Bougrier *et al.*, 2007, Pinnekamp, 1989). For example, THP has historically been applied to WAS rather than primary sludge. Thus, thermal pretreatment of WAS is comparatively well studied (Bougrier *et al.*, 2008). As this study has shown, the macromolecular makeup of a particular sludge has an inherent impact on the effectiveness of thermal pretreatment, the potential for inhibition by the hydrolysate, and the applicable range over which THP is amenable. In light of the advantages of primary sludge hydrolysis in terms of pathogen inactivation and biosolids odor control as well as the difference in chemical

composition of primary sludge from WAS, it makes sense to revisit the differential effect of specific THP temperatures for these separate waste streams.

Each measured chemical or physical property of PS and WAS hydrolysate was dependent on the hydrolysis temperature (Figure 4.5). As a primary indicator of hydrolytic efficiency, the release of soluble COD (sCOD) shows that at least initially (Raw-130°C), THP is indeed more effective on WAS than PS. However, these data suggest that the apparent advantage for hydrolyzing WAS is based on the release of relatively easily hydrolysable material, and that there was little difference between WAS and PS in terms of the incremental increase in sCOD per increase in hydrolysis temperature after the initial release of sCOD at 130°C.

Soluble biopolymers (i.e proteins and polysaccharides) were strongly affected by thermal hydrolysis 150°C and above. The rapid rise in soluble biopolymer concentrations between 130°C and 150°C was more pronounced than the corresponding fragmentation of pure proteins and polysaccharides at higher temperatures over the same temperature range where few < 1kDa peptide fragments (Figure 4.1) or monomeric sugars (Table 4.4) were produced. This comparison between the increase in *soluble* material concurrently with the sparse production of small molecular weight compounds underscores the inadequacy of defining the degradability of a hydrolysate based on its filterable fraction. There appeared to be little difference in the overall trend of biopolymer release between PS and WAS, although concentrations of soluble protein and polysaccharide in WAS samples was consistently, if only slightly, higher than for PS. This can be reasonably related to the amount of biopolymer within biological floc (e.g. WAS total protein content of 31.2g/L compared to PS total protein content of 13.4g/L, Table 4.2). Thermal hydrolysis of WAS at 130°C had a larger impact on soluble polysaccharide release (Figure 4.5.D, 420 mg/L increase) than soluble protein release (Figure 4.5.C, 35 mg/L increase) at the same temperature. This phenomenon has been previously observed between 130 and 150°C (Bougrier *et al*., 2008) and it was then concluded that the relative susceptibility of polysaccharides to solubilization was related to their loose association to biological cell membranes and distal location within sludge floc.

While it was determined that the hydrolysis of complex carbohydrates was not likely of importance to the overall production of readily degradable solids from sludge, non-enzymatic browning of the hydrolysate was far more pronounced at lower temperatures within the milieu of wastewater sludge than was observed for pure polysaccharide solutions (Figure 4.5.E). However, for plants interested in thermal hydrolytic pretreatment of sludge and UV disinfection of final effluent, there appears to be marginal benefit of lower hydrolysis temperatures in terms of the production of UV quenching compounds. These data suggest that the UV_{254} of sludge thermal hydrolysate is not solely dependent on the caramelization of sugars, but also on the presence of other hydrolyzed organics (e.g. protein). Though it must be noted that other complex polysaccharides not tested in this study may yield additional reducing sugars, thermal hydrolysis did not seem to be effective in producing a relatively large mass of reducing sugars from either glucose or starch. It is reasonable to conclude that thermal hydrolysis does not seem to be particularly effective in increasing the apparent degradability of the total COD fraction attributed to cellulose or starch. Additionally, the contribution of Maillard products to previous reports of poor degradability of high temperature hydrolysates is likely dependent on the nature and composition of polysaccharides that serve as precursors for non-enzymatic browning.

The findings of this research generally corroborate previous investigations of thermal hydrolysis temperature and centrate (Dwyer *et al.*, 2008). This study determined that measurements of centrate color, UV_{254}, dissolved organic nitrogen, and sCOD each increased as hydrolysis temperature was increased from 145-165°C, and largely attributed these values to the production of melanoidin with increased temperature. The study described in this paper builds on the work of Dwyer et al. by investigating the mechanism of UVA_{254} increase at temperatures of 130-220°C. The primary findings of this study with respect to the temperature dependence of UVA_{254} are that, (1) carmelization was determined to be a significant contributor to UVA_{254} only at a very high temperature (220°C), and that two important polysaccharide sludge components, starch and cellulose, were not degraded to mono- or dimeric reducing sugars at common THP temperatures.

Although the solubilization of protein was similar between PS and WAS samples, ammonia release due to thermal hydrolysis was drastically more pronounced for WAS than for PS at

190°C and 220°C (Figure 4.5.A). These were the same temperatures at which actual protein degradation was observed for the pure BSA experiment, thereby linking protein destruction during thermal hydrolysis to ammonia production. It is unclear as to why high temperatures were not as effective in ammonia production from PS, but one can speculate that exocellular protein in WAS may be more susceptible/accessible to hydrolysis than the particulate organic nitrogen in PS.

The amount of ammonia release relative to raw samples at moderate hydrolysis temperatures was low. Assuming that sludges would be blended at a 50/50 mass ratio before treatment, the average TAN concentrations of the raw sludges and hydrolysates at any temperature (Figure 4.5.A) can be used to represent a composite sludge mixture. Based on these calculations, the incremental effect of thermal hydrolysis on the feed sludge TAN concentration is as follows: 130°C (+48 mg/L NH_3-N); 150°C (+79 mg/L NH_3-N); 170°C (+114 mg/L NH_3-N); 190°C (+364 mg/L NH_3-N); 220°C (+526 mg/L NH_3-N).

While the ammonia release from 130-170°C may seem insignificant in terms of the potential for ammonia inhibition in the reactor, one must keep in mind that (1) thermal hydrolytic pretreatment is most often applied to sludges having concentrations greater than 6% TS, and (2) these TAN concentrations are in addition to the ammonia produced through the biological hydrolysis of organic nitrogen containing compounds during subsequent anaerobic digestion. Although hydrolysis experiments with protein (Figure 4.3) and wastewater sludge (Figure 4.5.A) suggest that operating a THP process at 150°C versus 170°C may reduce TAN loading to a digester by 20-30%, it is unclear what the effect on this reduced loading would have on steady-state TAN concentrations within a continuous-flow anaerobic digester. Though it is difficult to accurately predict the TAN concentration of an anaerobic digester without extensive characterization of a particular feed sludge, using the value 0.022 g NH_3-N/g TS to estimate ammonia release during anaerobic digestion (Wett *et al.*, 2006) we obtain 2640 mg/L NH_3-N for 12% TS sludge. Since this value, depending on reactor temperature and pH, approaches the previously reported threshold for ammonia inhibition of aceticlastic methanogens (Angelidaki and Ahring, 1994, Lay *et al.*, 1998, Sung and Liu, 2003), the incremental increases in TAN due

to THP are important considerations when specifying an appropriate thermal hydrolysis temperature.

By comparing the TAN concentrations produced from the hydrolysis of PS and WAS against VFA production (Figure 4.5.F), the macromolecular distinction between these sludges as substrates for THP and anaerobic digestion becomes apparent. VFA concentrations of the PS hydrolysate were found to be 4-7 times greater than for WAS, likely due to the high lipid fraction of PS. As described previously, the protein content of WAS made it particularly prone to ammonification at high hydrolysis temperatures. Hence, the macromolecular composition of these sludges resulted in the differences that were observed in VFA and TAN release during THP. On the contrary, the aggregate chemical components (COD, soluble biopolymers, and UV_{254}) of PS hydrolysate suggest a similar benefit of thermal hydrolytic pretreatment for PS compared with WAS.

4.8 CONCLUSION

The effect of temperature on the hydrolysis of proteins, lipids, and polysaccharides, both in pure form and as part of the macromolecular makeup of primary and secondary wastewater sludge, has been studied. The main conclusions that can be drawn from this study are as follows:

- Both PS and WAS respond similarly to THP in terms of solubilization of COD, proteins, and polysaccharides. Significantly more VFA were produced from PS, which were linked to the hydrolysis of unsaturated (e.g. glyceryl trilonolenate) rather than saturated (e.g. gylceryl tristearate) lipids. Additional ammonia was produced from WAS, which correlates well with its higher total protein content relative to PS.

- At common THP temperatures (less than 220°C), caramelization did not appear to contribute to the UV_{254} of sludge hydrolysate at temperatures (less than 220°C), and starch and cellulose were note largely converted to mono- or dimeric reducing sugars.

- Ammonification of protein by thermal hydrolysis may be an important consideration in the event that methanogenic inhibition becomes apparent. The production of ammonia could be marginally reduced by operating a thermal hydrolytic pretreatment process at 150°C. Solids loading likely dominates the steady-state TAN concentration within an anaerobic digester, and may provide a more meaningful control against ammonia induced methanogenic inhibition.

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4.10 TABLES

Table 4.1

Substrates for hydrolysis experiments

Carbohydrate [1]	Lipid [2]	Protein [3]
Potato Starch	**Glyceryl Tristearate (C18:0)**	**Bovine Serum Albumin (66.4kDa)**
CAS No. 9005-25-8	*CAS No. 555-43-1*	*CAS No. 9048-46-8*
Cellulose	**Glyceryl Trioleate (C18:1)**	
CAS No. 9004-34-6	*CAS No. 122-32-7*	
	Glyceryl Trilinoleate (C18:2)	
	CAS No. 537-40-6	
	Glyceryl Trilinolenate (C18:3)	
	CAS No. 14465-68-0	

[1] Prepared as 1% solution (w/v) in distilled water
[2] 100µL added to 5mL distilled water (2% v/v)
[3] 2000 mg/L solution in 1x phosphate buffered salt solution, pH 7.8

Table 4.2

General chemical properties of primary and secondary wastewater sludges used in this study

General Property	Primary Sludge	Secondary Sludge
tCOD	73200 ± 3200 mg/L	65700 ± 4300 mg/L
sCOD	6300 ± 100 mg/L	820 ± 10 mg/L
Total Protein	13400 ± 1700 mg/L	31200 ± 1300 mg/L
Soluble Protein	32 ± 16 mg/L	400 ± 29 mg/L
Soluble Polysaccharide	110 ± 23 mg/L	58 ± 12 mg/L
TS (TS/VS)	6.0% (0.81)	6.0% (0.76)
UVA_{254}	0.053 ± 0.009	0.025 ± 0.010
Total Organic Nitrogen	3140 mg/L N	6160 mg/L N
VFA	4720 mg/L as HAc	680 mg/L as HAc

Table 4.3

Yield of selected hydrocarbon types from the hydrolysis of 2% solution of glyceryl trioleate

Hydrolysis Temperature	Yield (mg hydrocarbon/g lipid hydrolyzed)					
	LCFA	Aldehyde	Alkane, -ene	Alcohol	Ketone	Acetylene
130°C	0.123	0.021	BDL[3]	BDL	BDL	BDL
150°C	0.139	0.206	0.014	BDL	BDL	BDL
170°C	0.269[1,2]	1.722[1]	0.019	0.222	BDL	0.049
190°C	0.378[1,2]	1.976	0.024	0.367[1]	0.468	0.062

[1] Significant increase from immediately lower temperature ($\alpha = 0.05$).
[2] Assuming 150,000 mg/L COD WAS with 25% COD fraction lipid, hydrolysis at 170°C and 190°C would produced LCFA concentrations of 29 mg/L and 40 mg/L, respectively. See sample calculation in supplemental material for explanation.
[3] BDL, below detectable level

Table 4.4

Yield of mono- and disaccharides from 1% solution of cellulose and potato starch

Hydrolysis Temperature	Yield (mg *mono-, disaccharide*/g polysaccharide hydrolyzed)			
	Cellulose		Potato Starch	
	Dextrose	Cellobiose	Dextrose	Maltose
130°C	< 0.1 ± < 0.1	0.6 ± 0.1	0.1 ± < 0.1	7.2 ± 0.2
150°C	< 0.1 ± < 0.1	1.0 ± 0.1	0.2 ± 0.1	7.3 ± 0.6
170°C	0.1 ± < 0.1	3.8 ± 0.4	0.2 ± 0.1	9.0 ± 0.4
190°C	0.1 ± 0.1	5.0 ± 0.3	1.0 ± 0.3	13 ± 1.1
220°C	3.4 ± 0.3	4.9 ± 0.3	370 ± 23	520 ± 34

4.11 FIGURES

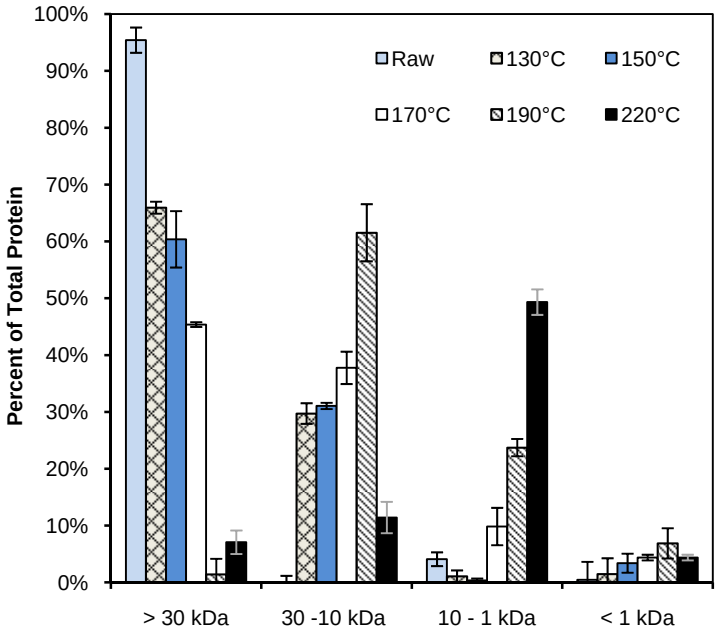

Figure 4.1

Size fractionation of BSA through thermal hydrolysis at various temperatures. Initial protein concentration of 2000 mg/L in 1x PBS.

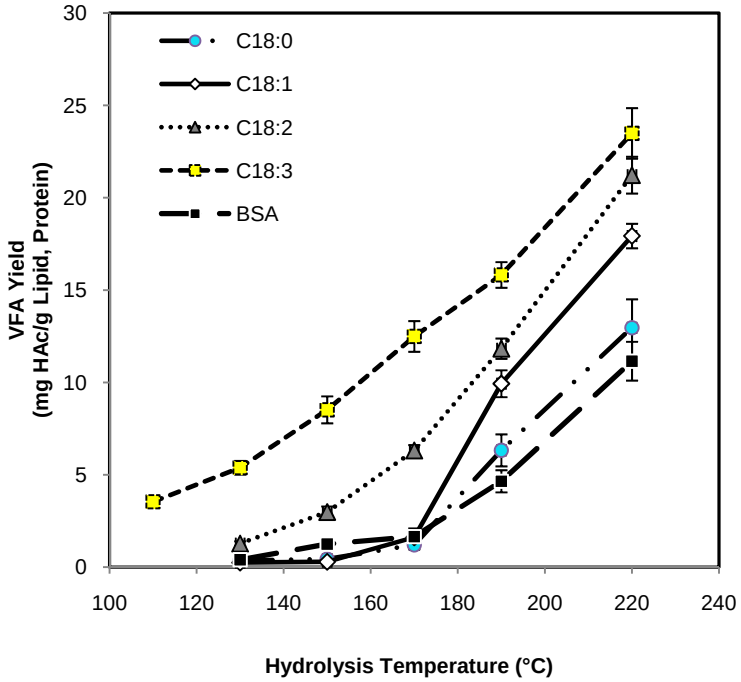

Figure 4.2

VFA yields from lipids and BSA (protein). Neither protein nor saturated fatty acids seem to be a significant source of VFA during thermal hydrolysis.

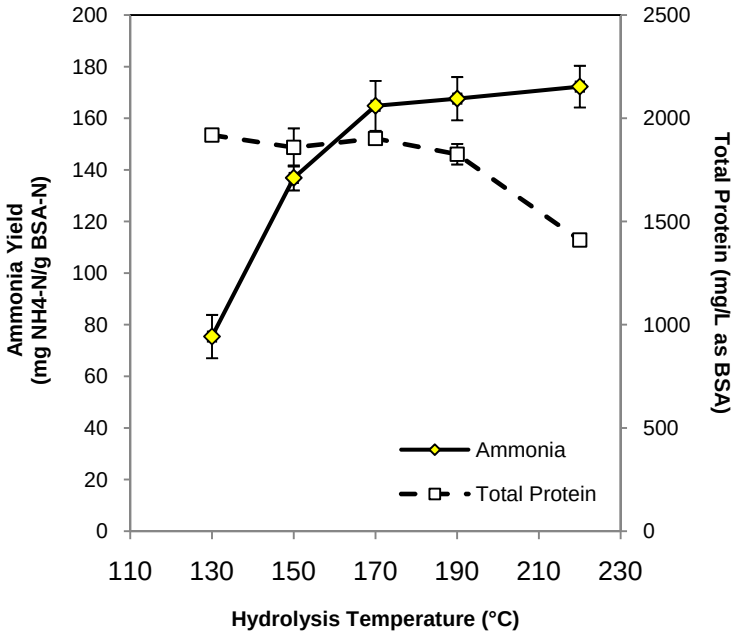

Figure 4.3

Temperature effect on ammonia production from BSA via thermal hydrolysis, and associated degradation of total protein. Initial protein concentration of 2000 mg/L in 1x PBS.

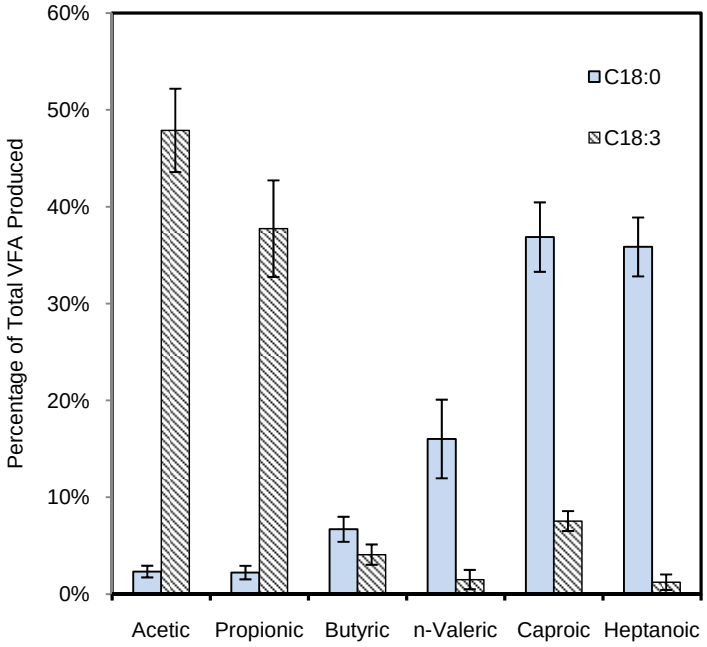

Figure 4.4

Distribution of VFA from the thermal hydrolysis of glyceryl trilinolenate (C18:3) and glyceryl tristearate (C18:0) at 170°C.

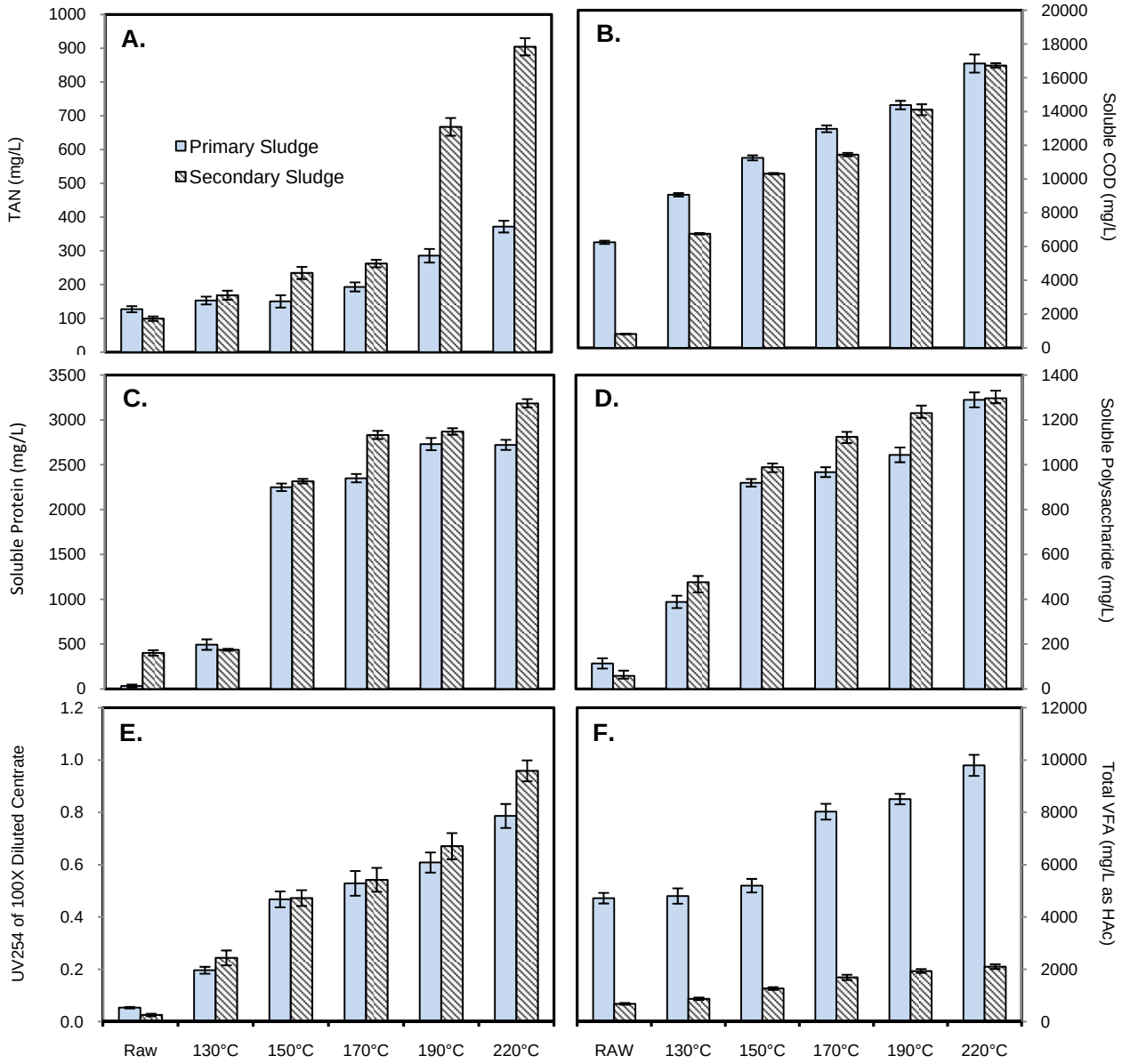

Figure 4.5

Effect of hydrolysis temperature on key chemical characteristics of PS and WAS hydrolysate: (A) TAN, (B) soluble COD, (C) soluble protein, (D) soluble polysaccharide, (E) UV_{254} absorbance, (F) VFA.

4.12 SUPPLEMENTAL MATERIAL

Sample calculation relating ammonia yields in Figure 4.3 to theoretical sludge ammonia concentrations. Similar calculations are used to arrive at sludge concentrations of ammonia, hydrocarbons, and volatile fatty acids based on measured yields from pure chemicals:

Empirical formula for bovine serum albumin (BSA): $C_{2880}H_{5778}O_{1474}N_{757}S_{40}$

$\downarrow$

$C_{3.8}H_{7.6}O_{1.9}NS_{0.05}$
(Swiss-Prot Database)

$$C_{3.8}H_{7.6}O_{1.9}NS_{0.05} + \boldsymbol{3.75}\ O_2 \rightarrow \boldsymbol{3.80}\ CO_2 + \boldsymbol{1.80}\ H_2O + NH_4^+$$

128 g BSA = ***120*** g O_2

1.07 g BSA/g COD and ***0.160*** g BSA-N/ g BSA

$$\frac{165\ \text{mg NH}_4\text{-N}\ \dagger}{\text{g BSA-N}} \left| \frac{0.40\ \text{g BSA}\ \ddagger}{\text{g sludge COD}} \right| \frac{0.160\ \text{g BSA-N}}{\text{g BSA}} \left| \frac{1.07\ \text{g BSA}}{\text{g BSA COD}} \right.$$

$$= \frac{11.3\ \text{mg NH}_4\text{-N}}{\text{g sludge COD}}$$

Assume raw solids having 150,000 mg/L COD:

$$\frac{11.3\ \text{mg NH}_4\text{-N}}{\text{g sludge COD}} \left| \frac{150\ \text{g sludge COD}}{\text{L}} \right. \boxed{= \frac{1690\ \text{mg NH}_4\text{-N}}{\text{L}}}$$

† From Figure 4.2 @ 170°C
‡ From measured fractionation of total sludge COD in Tanaka et al. (1997)

CHAPTER 5

THE KINETICS OF PROCESS DEPENDENT AMMONIA INHIBITION OF METHANOGENESIS FROM ACETIC ACID

5.1 AUTHORS

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5.2 ABSTRACT

Advanced anaerobic digestion processes aimed at improving the methanization of sewage sludge may be potentially impaired by the production of inhibitory compounds (e.g. free ammonia). The result of methanogenic inhibition is relatively high effluent concentrations of acetic acid and other soluble organics, as well as reduced methane yields. An extreme example of such an advanced process is the thermal hydrolytic pretreatment of sludge prior to high solids digestion (THD). Compared to a conventional mesophilic anaerobic digestion process (MAD), THD operates in a state of constant inhibition driven by high free ammonia concentrations, and elevated pH values. As such, previous investigations of the kinetics of methanogenesis from acetic acid under uninhibited conditions do not necessarily apply well to the modeling of *extreme* processes such as THD. By conducting batch ammonia toxicity assays using biomass from THD and MAD reactors, we compared the response of these communities over a broad range of ammonia inhibition. For both processes, increased inhibitor concentrations resulted in a reduction of biomass growth rate ($r_{max} = \mu_{max} \cdot X_{AC}$) and a resulting decrease in the substrate half saturation coefficient (K_S). These two parameters exhibited a high degree of correlation, suggesting that for a constant transport limited system, the K_S was mostly a linear function of the growth rate. After correcting for reactor pH and temperature, we found that the THD and MAD biomass were both able to perform methanogenesis from acetate at high free ammonia concentrations (equivalent to 3-5 g/L total ammonia nitrogen), albeit at less than 30% of their respective maximum rates. The reduction in methane production was less pronounced for the THD biomass than for MAD, suggesting that the long term exposure to ammonia had selected

for a methanogenic pathway less dependent on those organisms most sensitive to ammonia inhibition (i.e. aceticlastic methanogens).

5.3 KEYWORDS

Acetic acid
Ammonia inhibition
Kinetic modeling
Methanogenesis
Thermal hydrolysis

5.4 NOMENCLATURE

α_A	Conjugate acid fraction
ATA	Ammonia toxicity assay
C_L	Concentration of the limiting substrate
$\Delta G^{o'}$	Standard change in Gibbs free energy
HAc	Unionized acetic acid
HRT	Hydraulic retention time
I_{NH3}	Ammonia inhibition factor
K_S	Half saturation coefficient
LI_{NH3}	Logistic model estimation of I_{NH3}
MAD	Conventional mesophilic anaerobic digestion
NH_3	Free unionized ammonia
r_{max}	Maximum growth rate
SRT	Solids retention time
TAA	Total acetic acid
TAN	Total ammonia nitrogen
THD	High-solids anaerobic digestion of thermally pretreated wastewater sludge
TPA	Total propionic acid
TS	Total solids
VFA	Volatile fatty acids

5.5 INTRODUCTION

The anaerobic digestion of sludges is, and has been for many years, a broadly applied operation in biological wastewater treatment. Anaerobic digestion has many distinct benefits, including a low biomass yield, a potentially desirable energy balance, and its ability to treat very high concentrations of organic waste. These advantages have long been considered to outweigh the disadvantages of very large high hydraulic retention time (HRT) systems, and relatively poor effluent quality. These drawbacks in conjunction with more stringent discharge requirements have fueled the development of processes that can be more heavily loaded (and thus potentially smaller) while outperforming the conventional digestion systems of the past in terms of sludge minimization and energy recovery.

One issue that arises in the operation of advanced anaerobic digestion systems is that of methanogenic sensitivity to chemical and environmental stressors. Thermophilic and high solids anaerobic digestion processes have been studied from the standpoint of *ammonia inhibition* (Angelidaki and Ahring, 1994; Karakashev *et al.*, 2006; Liu and Sung, 2002) and these processes are understood to be wholly different in their biology and biochemistry than conventional anaerobic digestion (Iranpour and Cox, 2006). In order to make use of these new processes, designers must understand the distinctions between advanced and conventional digestion processes in order to apply predictive modeling tools.

Compelling evidence exists that the consideration of the unionized forms of both methanogenic substrates and inhibitors (Angelidaki and Ahring, 1994; Eldem *et al.*, 2004; Sung and Liu, 2003) as the determinative species for the kinetics of aceticlastic methanogenesis. Specifically, methane production from VFA by acetate and propionate acclimatized anaerobic digester biomass has been observed to be dependent on both total organic acid concentration and pH, or rather, solely dependent on the concentration of undissociated acid species (Fukuzaki *et al.*, 1990a; Fukuzaki *et al.*, 1990b). The dependence of methane production rates on nondissociated free acetic (HAc) and propionic (HPr) acid concentrations followed the second order substrate inhibition model originally proposed by Andrews (1978), suggesting that the effects of pH on methanogenic efficiency typically observed for anaerobic systems may be a result of the dependence on either substrate availability or inhibition. Similarly, ammonia inhibition has been

related to the presence of free unionized ammonia (NH_3) driven by changes in pH (Eldem *et al.*, 2004), thermophilic temperature (Angelidaki and Ahring, 1994), or both (Sung and Liu, 2003).

It is interesting that kinetic data derived from a simple non-pH-dependent model for methanogenic degradation of TAA and TPA is still widely employed (Kugelman and Chin, 1971; Lawrence and McCarty, 1969; VanLier *et al.*, 1996). Perhaps separate processes with very similar characteristic pH could be adequately modeled and evaluated without consideration of the unionized substrates and inhibitors. However, the development of advanced anaerobic digestion processes which operate at the margins of the well understood physiologic ranges of anaerobic digestion (e.g. high temperature, high/low pH, high concentrations of chemical inhibitors) make the inclusion of substrate/inhibitor speciation critical.

The objective of this study is to investigate the kinetics of methanogenesis from HAc under a condition of process-dependent inhibitory conditions. Thermal hydrolytic pretreatment followed by mesophilic anaerobic digestion (THD) was studied, as well as a side by side conventional mesophilic digestion process (MAD). THD was chosen since abnormally high solids loading to the anaerobic digester is facilitated by reduced viscosity during thermal pretreatment (Kepp *et al.*, 2000; Potts and Jolly, 2004). Destruction of protein and other complex organics results in high characteristic reactor pH and stoichiometric production of ammonia during THD (Kepp *et al.*, 2001). Thus, this process is particularly amenable to this study. By presenting kinetic data from these disparate processes, the importance of ammonia and acetic acid speciation for the performance of diverse anaerobic digestion processes is recognized.

5.6 METHODOLOGY

5.6.1 Anaerobic Digester Operation

Conical high-density polyethylene reactors supplied by Hobby Beverage Equipment Company (Temecula, California) were used as pilot scale anaerobic digesters. The conical bottom of these vessels was thought to be advantageous in terms of mixing of dense high solids sludge feed to THD. The nominal volume of the reactor vessels was 25 liters. Reactors were operated with active volumes of 15 liters, and daily batch feeding was performed at a rate of 1 L/day. The

reactors were modified to accept a threaded stainless steel thermometer. Reactor temperatures were 37°C and 42°C for MAD and THD, respectively. Reactors were continuously mixed by digester gas recirculation from the headspace through the conical reactor bottom.

Conventional mesophilic anaerobic digesters (MAD) were initially seeded with sludge from Pepper's Ferry Regional Wastewater Treatment Plant (Radford, VA). Thermally hydrolyzed sludge digesters (THD) were initially seeded with reconstituted dewatered biosolids from Ringsend Wastewater Treatment Works (Dublin, Ireland) which was commissioned in 2003 and operates THD.

Digester feed was obtained from DCWASA Blue Plains and consisted of a 1:1 ratio of primary and secondary solids. Sludge to be subjected to MAD was shipped on ice directly to Virginia Tech (Blacksburg, VA). These solids had a concentration of approximately 6% total solids (TS). Sludge that was thermally pretreated was dewatered to approximately 15% TS by DCWASA and shipped on ice to RDP Technologies, Inc. (Norristown, PA) for thermal treatment. Thermal treatment took place at 150°C and 4.8 bar for approximately 30 minutes. Sludge hydrolysate was then shipped on ice to Virginia Tech. These solids had concentration of approximately 12% TS. Both reactors had been operating for greater than a year at the time of this study. Other operating data for the digesters is shown in Table 5.1.

5.6.2 Batch Ammonia Toxicity Assays

Ammonia toxicity assays were conducted on biomass from semi-continuous flow lab-scaled digesters. Incubations with total ammonia nitrogen (TAN) concentrations of 16 to 230 mM (220 to 3220 mg/L) as N for THD and 36 to 357 mM (500 to 5000 mg/L) as N for MAD were performed. Ammonia was added as a 7.15 M stock solution of NH_4HCO_3 in D.I. water. Initial acetic acid concentrations for MAD incubations were 80-100 mM, and were 220 mM for THD. Acetate was added as a 10% w/w glacial acetic acid stock. Such disparate ranges of ammonia and acetic acid for MAD and THD were necessary in order to achieve similar ranges in free unionized species in reactors exhibiting differing pH levels. Each 125 mL serum bottle received 10 mL of digester biomass, 10 mL 1.0 M phosphate buffered saline solution (prepared according

to the natural pH of the parent digesters), appropriate volumes of ammonia and acetic acid stock solutions, and filled to 100 mL with D.I. water. Biomass samples were effectively diluted 10x in order to reduce the carryover of TAN and volatile fatty acids (VFA) from the parent digesters so that low ammonia batch incubations could be performed. Bottle headspaces were purged with nitrogen gas before sealing with a butyl rubber stopper. Serum bottles were submerged in a shaking water bath at the either 37°C (MAD) or 42°C (THD) according to the operating temperature of the parent digester.

Biogas production was periodically monitored via a liquid column manometer filled with pH indicating acid/salt solution as described in *Standard Methods* (APHA, 1989). Bottles were sampled periodically for acetic acid concentration and pH. Samples for VFA analysis were passed through a 0.45 µm nitrocellulose membrane filter and frozen prior to analysis. Thawed samples were acidified by adding 85% phosphoric acid at a rate of 1% v/v and analyzed via HP5890 GC-FID and Supelco Nukol column with the following gas flow rates: (Nitrogen) 14 mL/min, (Air) 450 mL/min, (Hydrogen) 44 mL/min, (Helium) 16 mL/min. The initial oven temperature was set to 80°C and ran isothermally for 3 minutes, then increased at 6°C/min for 10 minutes.

5.6.3 Kinetic Parameter Estimation

The Monod expression relating substrate utilization to substrate concentration can be written as:

$$-\frac{dC_L}{dt} = r_g = -(\mu_{max} \,/\, Y) \cdot S/[(K_S + S) \cdot X_{AC}] \tag{5.1}$$

where C_L is the concentration of the limiting substrate in its bioavailable form, t is time in days, μ_{max}, Y, and X_{AC} are the maximum specific growth rate, yield coefficient, and concentration of the active population degrading C_L, and K_S is the half-saturation constant for C_L. This expression can be integrated as:

$$t = -\frac{Y}{r_{max}\left[K_S \cdot ln\left(\frac{C_L}{C_{LO}}\right) + C_L - C_L\right]} \qquad where\ r_{max} = \mu_{max} \cdot X_{AC} \tag{5.2}$$

Non-linear least squares regression was used to fit acetic acid progress curve data to the integrated Monod expression via Microsoft Excel's *solver* add-in. The inclusion of C_{LO} as a fitting parameter provides a measure of quality assurance, as the fitted parameter can be directly compared against analytical data. An estimate of the yield coefficient for anaerobic growth on acetic acid of 0.04 $^{\text{g biomass}}/_{\text{g acetic acid}}$ (Rittmann and McCarty, 2001) was applied as a constant for both MAD and THD assays.

Estimates of uncertainties of fitted parameters from progress curve data were performed using a method described in detail elsewhere (Smith *et al.*, 1997; Smith *et al.*, 1998). In general, the uncertainty estimation calculates an approximate 95% confidence interval for each fitting parameter based on the effect on the model fit of a small change in that fitting parameter's value (i.e. specific parameter sensitivity), and also the sum of squared errors (i.e. overall model fit quality) across the entire progress curve. A joint (3-parameter) 95% percent confidence interval was calculated for each ammonia concentration based on a one-way ANOVA (F-test). The resulting joint confidence interval represents all points within a 3-dimensional (3D) ellipsoid the lack statistical difference from the model-fitted parameters (e.g. $C_{LO,FIT}$). Because we were primarily interested in the values of r_{max}, and K_S, the joint confidence intervals presented in this study are shown as 2D ellipses that represent a slice through the 3-parameter joint confidence interval at $C_{LO} = C_{LO,FIT}$.

Initial slopes of cumulative biogas production curves were used to determine the degree of inhibition imposed by unionized ammonia. These data are plotted as the fraction of biogas production activity remaining versus the concentration of unionized ammonia. For example, a reduction in the biogas generation rate by 20% would be plotted as an inhibition factor (I_{NH3}) of 0.80, thus a high I_{NH3} value (i.e. close to 1.0) would be indicative of no methanogenic inhibition. Previous research shows that ammonia inhibition of aceticlastic methane generation is relatively mild at low unionized ammonia concentrations and follows a logistic reduction in

methanogenesis as ammonia is increased, theoretically leading to full inhibition of methanogenesis at sufficiently high concentrations (Angelidaki and Ahring, 1994). As such, the following logistic model has been applied to calculated LI_{NH3} values via least-squares regression (Wett *et al.*, 2009):

$$LI_{NH3} = \frac{1}{1+e^{-I_{NH3,SLOPE}\cdot(K_{I,NH3}-\{NH_3\}}} \tag{5.3}$$

where $I_{NH3,SLOPE}$ is a curve fitting parameter, and $K_{I,NH3}$ is the molar unionized ammonia concentration at which $I_{NH3} = 0.50$.

Acid-base partitioning was calculated based on published pKa values for ammonia and acetic acid, and corrected for temperature using the constant enthalpy form of the van't Hoff equation (CRC, 2009).

5.7 RESULTS AND DISCUSSION

5.7.1 Values and Trends of Kinetic Fitting Parameters

Kinetic parameters obtained from non-linear least squares regression, relative to HAc, are presented in Table 5.2. The low magnitude of K_S values is striking in comparison with previously published TAA-based data. Furthermore, the reported HAc half-saturation values between 0.45 and 6.8 mg/L for anaerobic digester communities in this study are of the same order of magnitude as literature reported values for various microbial communities pertinent to aerobic wastewater treatment (Button, 1985). Single parameter confidence intervals shown in Table 5.2 reveal a higher degree of certainty for MAD data, which were largely driven by the higher frequency of data collection for MAD ATA experiments. However, characteristic model fits to HAc progress curve data suggest for both processes suggest high-quality fitting parameter estimates (Figure 5.1). In addition, model-fitted C_{LO} values varied minimally from intended spiked HAc concentrations.

A direct linear correlation exists between corresponding model-estimated values for K_S and r_{max} (Figure 5.2). Because r_{max} is dependent both on the maximum specific growth rate, μ_{max}, and the active HAc-degrading population concentration, X_{AC}, it is concluded that K_S is not a constant, but rather, dependent on the microbial growth rate. Such phenomena in which K_S is observed to vary linearly with biomass growth rate have been described in the past in both aerobic and anaerobic processes (Lawrence and McCarty, 1969; Novak, 1974). As NH_3 concentration increases, r_{max} is observed to decrease as a result of kinetic inhibition. Considering a family of Monod curves in which r_{max} is observed to vary, the different shapes of the Monod curves (and as such, the different values of r_{max}) can be obtained either by shifts in K_S or by shifts in the initial *slopes* of the Monod curves. This slope, a parameter identified elsewhere as a particular community's *specific affinity* for a substrate of interest (Button, 1985; Laanbroek and Gerards, 1993), is denoted as α_A^o and mathematically defined as the ratio of the parameters r_{max}/K_S, (Equation 5.4).

$$\text{Specific Affinity } (\alpha_A^o) = \frac{\cancel{\text{mg substrate}}}{\cancel{\text{mg biomass}}\text{–day}} \text{x} \frac{\cancel{\text{mg biomass}}}{\cancel{\text{L volume}}} \text{x} \frac{\cancel{\text{L volume}}}{\cancel{\text{mg substrate}}} \tag{5.4}$$

This parameter is particularly well suited for describing the affinity of a biomass as it is indicative of the effect of increased substrate concentrations on growth at very low substrate concentrations ($C_{LO} \approx 0$). The linear correlation observed between K_S and r_{max} for THD and MAD assays suggests a constant affinity over various levels of NH_3, meaning that the inhibition of r_{max} rather than variations in *specific affinity* dictate the shapes of the family of Monod curves obtained from these kinetic values (Figure 5.3).

While the implication of the covariance of r_{max} and K_S on the constant substrate affinity of THD and MAD communities is clear, the cause of this covariance is not well described in the literature. For sludges comprised of floc-forming particles that exhibit somewhat viscous bulk fluid properties, it is expected that diffusion limited transport of substrate to actively degrading cells dictates the concentration of substrate at the cellular interface that drives biological reactions. For a diffusion limited system, this substrate concentration is inherently lower than that measured from the bulk fluid and used for kinetic determinations. Previous observations have suggested that unstirred boundary layers adjacent to a biological membrane tend to place artificial upward bias on measured half-saturation coefficients (Winne, 1973). It is possible that

the reduction in r_{max} at increasing inhibitor concentration reduces the effect of diffusion limitation across unstirred boundary layers near the sludge floc surface, and this the artificial upward bias on K_S at high r_{max} is alleviated. This finding suggests that under for diffusion limited constant transport of bulk-phase substrate to the surface of a biological floc where uptake occurs, the magnitude of K_S is dependent as its name implies on saturation kinetics. Hence, the values of kinetic parameters obtained through modeling of biological systems are specific to the controlled experimental conditions, especially those that dictate substrate transport and utilization to and by the biomass (e.g. inhibitor and substrate concentration, incubation temperature, biomass and particulate matter densities, etc.). As these experimentally controlled variables were inherently different between THD and MAD toxicity assays, care is taken to consider only the relative effects of NH_3-inhibtion on methane production and HAc degradation when drawing comparisons between these different processes.

5.7.2 NH_3 Inhibition of Maximum Growth Rates, r_{max}

A plot of r_{max} values for both THD and MAD against either TAN or NH_3 concentrations shows that the effect of increased nitrogen concentration has a clear negative effect on substrate utilization. Growth rate reductions of 63% and 76% were achieved at the highest ammonia concentrations tested in this study for MAD and THD, respectively. Based on data in Figure 5.4a, one would conclude that the impact of increased ammonia loads HAc utilization is more dramatic for THD compared with MAD. For example at a TAN concentration of 1220 mg/L as N, the THD process is approximately 50% inhibited, whereas the MAD process shows 17% inhibition at a TAN concentration of 1250 mg/L as N. However, high solids loading to THD resulted in a stochiometric production of bicarbonate alkalinity based on volatile solids destroyed (Kepp *et al.*, 2001; Wilson *et al.*, 2009) and resulted in a characteristic pH of 7.85, compared with 7.35 for MAD. Such disparate pH conditions within these separate anaerobic digestion processes support the necessity for considering the substrate and inhibitor speciation. Taking pH and temperature effects into account, the conjugate acid fractions of ammonia (α_A) for the THD and MAD processes are 0.890 and 0.972, respectively. This means that at characteristic process pH and any given TAN concentration, the functional inhibitor concentration is approximately four times higher in THD.

A plot of growth rate reduction due to NH_3-inhibition reveals that biological growth on acetate is very similarly affected by increased NH_3 concentration for both digestion processes (Figure 5.4.b). However, an important distinction for the operation of these processes is made by considering not only the partitioning of total ammonia to the conjugate base form, but also the typical TAN concentration of the two digesters. Applying the calculated α_A values to the typical TAN concentrations for both THD and MAD, in situ NH_3 concentrations are approximately 260 and 40 mg/L N, respectively. At these NH_3 concentrations, we would expect that biological growth on acetate would be inhibited by 15% for MAD, and by 65% for THD. While these numbers are exacerbated by the high solids loading to THD, it is clear that comparative evaluations of MAD and THD could not be accurately conducted without consideration for free ammonia inhibition.

5.7.3 Implications of K_S Values for THD Operation

Optimization of reactor operation requires knowledge of factors limiting biological growth, and by association, substrate utilization. High VFA concentrations after THD represent a significant loss of recoverable energy as methane gas. Additionally, undegraded soluble organics must receive further treatment, either as a dewatered centrate side stream process or as a return flow to the liquid side treatment plant. Unlike with NH_3, the lower pH of MAD tends to increase the useable fraction of HAc compared with higher pH THD; however, the magnitude of VFA accumulation results in higher reactor substrate concentrations (C_L) for THD (4.8 mg/L HAc) than MAD (0.60 mg/L HAc). Compared with applicable values for K_S given the in-situ NH_3 concentration typical of these processes, respective digester HAc concentrations are much higher than K_S for THD at 260 mg/L NH_3-N (0.95 mg/L HAc) and much less than K_S for MAD at 40 mg/L NH_3-N (5.8 mg/L HAc).

Practically, THD is operated in the portion of its Monod growth curve where the substrate utilization rate (dC_L/dT) is governed by r_{max}, whereas the substrate utilization rate for MAD is more strongly dependent on K_S. Achieving lower effluent concentrations of VFA from THD requires either (a) providing a longer SRT to achieve more complete stabilization of HAc

(O'Rourke, 1968), or (b) implementing measures to alleviate NH_3 inhibition of r_{max}. Reduction in NH_3 concentrations by reduced pH could be achieved by introduction of a rapidly acidifiable substrate, such as in the codigestion of fats, oils, and grease (FOG), or by the metered addition of a mineral acid to the digester. At a concentration of 2380 mg/L TAN, reducing the pH from 7.85 to 7.50 would cause a reduction of NH_3-N concentration from 260 to 125 mg/L, and result in an approximate 25% increase in r_{max}.

Additionally, loading of proteinaceous sludges can be reduced in order to achieve the combined effects of lower TAN concentrations and lower pH. The yield and concentration of soluble products such as TAN and bicarbonate alkalinity (HCO_3^-) are predictable based on sludge composition and organic loading rates, respectively (Wett *et al.*, 2006). Since a widely cited advantage of THD is reactor volume reduction due to increased biologically and mechanically attainable solid loading rates, major reductions in feed solids as a method for lowering alkalinity and TAN concentration are undesirable. However, the nature of feed solids, whether proteinaceous or not, significantly affects yields of HCO_3^- and TAN during anaerobic digestion. For example, methanogenic degradation of glycine theoretically yields 181 mg TAN and 650 mg alkalinity as $CaCO_3$ per gram glycine degraded. Comparatively, methanogenic degradation of palmitic acid (HPa) theoretically yields 125 mg alkalinity as $CaCO_3$ and *consumes* approximately 19 mg TAN per gram HPa (Rittmann and McCarty, 2001); see supplemental material for calculations.

This previous analysis has broad implications for the operation of THD. It is clear that the THD process is not necessarily solids loading limited, but more specifically *organic-nitrogen* loading limited. This suggests that THD is amenable to codigestion of low-nitrogen wastes (e.g. FOG). Previous studies have reported volatile solids total Kjeldahl nitrogen fraction for FOG of 1.3% compared to 6-9% for combined primary and secondary sludge (Kabouris *et al.*, 2008). While mixing and incorporation of FOG within an anaerobic digester poses a significant operational concern for MAD, the fragmentation of lipids and conversion to VFA during thermal hydrolytic pretreatment (Wilson and Novak, 2009) is likely to mitigate lipid aggregation during digestion. Additionally, FOG exists as a high-solids liquid at mesophilic temperatures. By taking advantage of this distinction between a *solids* loading limited and an *organic nitrogen* loading

limited process as revealed by the in-situ HAc saturation during THD, we propose that solids loading to and energy recovery from the THD process can be maximized.

5.7.4 Evidence of an Alternate Methanogenic Pathway in THD

Methanogenic inhibition factors (I_{NH3}) related to the initial slope of cumulative biogas production curves from batch ammonia toxicity assays are presented in Figure 5.5.a. It is clear that the degree of methanogenic inhibition attributable to NH_3 is less for THD than MAD at equivalent NH_3 concentrations. The logistic model that was applied to these data exhibited good statistical fit. Coefficients of determination (R^2) of LI_{NH3} for THD and MAD were 0.947 and 0.951, respectively. While ammonia toxicity assays were not conducted for MAD at NH_3 concentrations greater than 150 mg/L, plotted ratios of LI_{NH3} THD to LI_{NH3} MAD over the concentration range of THD experiments show that at overall methanogenesis during MAD is much more sensitive to ammonia inhibition than THD (Figure 5.5.b). This is especially true as NH_3 levels are increased above the characteristic in-situ ammonia concentrations of MAD. These trends are distinct from previously discussed data depicting similar responses of the kinetics of HAc degradation by THD and MAD biomass over a range of NH_3 concentrations. Acclimation of the methanogenic community may partially explain the relatively low sensitivity methane production by THD biomass to high NH_3 concentrations. However, the contrasting responses of acetate utilization kinetics and methane production rate to increased NH_3 levels suggests that the cause-effect relationship between acetate degradation and methane production differs among THD and MAD reactors. It is thus hypothesized that methanogenesis during THD is less dependent on ammonia-sensitive aceticlastic methanogenesis, and rather, non-aceticlastic methanogenesis from HAc is important for THD.

High NH_3 concentrations as a result of thermophilic temperatures or high solids loading have been associated with a methanogenic pathway shift from aceticlastic to non-aceticlastic methanogenesis. Non-aceticlastic methanogenesis refers to HAc metabolism by a syntrophic co-culture containing a hydrogenotrophic methanogenic species and an organism capable of oxidizing acetate to CO_2 plus H_2 (Petersen and Ahring, 1991; Zinder and Koch, 1984). Examples of such previously identified organisms include the thermophilic *Thermoacetegenium*

phaeum (Hattori *et al.*, 2000) and mesophilic *Clostridum ultunense* (Schnurer *et al.*, 1999), suggesting that non-aceticlastic methanogenesis is dependent on chemical rather than physical environmental conditions. Previous research has shown that the dominance of non-aceticlastic methanogenesis is related to the inhibition of aceticlastic methanogens resulting in an available ecological niche, rather than competitive advantage of syntrophic HAc oxidation. Aceticlastic methanogenesis is exergonic ($\Delta G^{0'}$= -31.0 kJ/mol), thus proceeds in the absence of syntrophic associations. Acetate oxidation however, is highly endergonic ($\Delta G^{0'}$ = 104.6 kJ/mol) and requires consumption of reaction products, primarily gaseous hydrogen (similar to fatty acid oxidation), in order to proceed (Rothfuss and Conrad, 1993). It has been calculated elsewhere that given the following in-situ conditions for a methanogenic coculture with *C. ultunense* : 25 mM TAA, 100 mM HCO_3^-, 31 kPa methane; the total energy release available for syntrophic acetate oxidation and hydrogenotrophic methanogenesis is approximately -17 kJ/mol (Ahring and Westermann, 1988; Hattori, 2008; Schink, 1997; Scholten and Conrad, 2000). Several studies have suggested that the minimum quantum of energy required to sustain microbial life corresponds approximately 1/3 the quantum of energy required for the production of 1 mol ATP (i.e. 70 kJ/mol ATP/ 3 protons/mol ATP = 23 kJ / proton; equal to the energy obtained from the translocation of one proton across a charged cellular membrane) (Ahring and Westermann, 1988; Hattori, 2008; Schink, 1997; Scholten and Conrad, 2000). Lower minimum free energy requirements for growth of hydrogenotrophic methanogens of approximately 10 kJ/mol have been observed in either stressed physiologic states (Jackson and McInerney, 2002) or in highly competitive environments (Hoehler *et al.*, 2001), suggesting that the growth of a syntrophic acetate oxidizing coculture can be supported these low energy yields. In addition, hydrogenotrophic methanogens having high hydrogen affinity such as *Methanoculleus sp.* are most commonly associated with syntrophic acetate oxidation (Schnurer *et al.*, 1999; Shigematsu *et al.*, 2004), a characteristic that supports the low hydrogen concentrations that are required for the maintenance of acetate oxidation near its thermodynamic limit.

Because acetate oxidation is not thermodynamically advantageous relative to aceticlastic methanogenesis at characteristic HAc levels of anaerobic digestion, it is generally believed that inhibition of aceticlastic methanogens is necessary to promote acetate oxidation as a dominant methanogenic pathway. In the context of THD and the kinetic data presented herein, it is clear

that elevated NH_3 concentrations impose meaningful inhibition of aceticlastic methanogenesis such that the alternate, less thermodynamically preferred metabolism may dominate. Past research has shown that hydrogenotrophic methanogenic species (including *Methanobacterium bryantii*, *Methanobacterium formicicum, Methanobrevibacter smithii,* and *Methanobrevibacter arboriphilus*) were less sensitive to ammonia toxicity than aceticlastic species (including *Methanosarcina barkeri,* and *Methanosaeta concilii*). The hydrogenotrophic species were found to be insensitive to TAN concentrations of 5600 mg/L (Sprott and Patel, 1986) and recovered more quickly from long-term incubation at TAN concentrations as high as 7700 mg/L (Hajarnis and Ranade, 1993; 1994). Other researchers have found a direct correlation between anaerobic digester TAN concentration, the absence of *Methanosaeta sp.* from the digester community, and the corresponding dominance of non-aceticlastic methanogenesis as the preferred degradation pathway for HAc (Karakashev *et al.*, 2006). Such findings directly implicate inhibition of aceticlastic methanogenesis in an observed pathway shift to non-aceticlastic methanogenesis.

5.8 CONCLUSION

Both THD and MAD communities were able to produce methane from acetate at NH_3 concentrations corresponding with 3-5 g/L TAN, albeit at less than 30% of their respective uninhibited rates. Degradation of high organic loads led to generation of excess alkalinity during THD, resulting in elevated pH levels. The preference for NH_3 relative to NH_4^+ at elevated pH was the primary compounding factor that led to NH_3- inhibition during THD. Higher organic loading rate could be maintained with limited methanogenic inhibition by either artificially lowering reactor pH, removal of free ammonia by recuperative thickening, or by preferential feed sourcing of low organic nitrogen waste sludges such as FOG.

Consideration of unionized species of inhibitors and substrate was crucial to properly understand the kinetic implications of process variations between THD and MAD, as well as operational considerations (such as solids loading and pH) within individual processes. In addition, physical sludge characteristics were observed to play a role of the evaluation of substrate affinity and saturation kinetics that are not well explained by classical models of anaerobic digestion kinetics. Further understanding of the process dependent variations in digester chemistry, ecology, and

thermodynamics will allow predictive evaluations of advanced digestion processes to be better incorporated within the engineering design process.

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5.10 TABLES

Table 5.1

General operating parameter of parent (lab-scaled) anaerobic digesters

Parameter	Conventional	Cambi
SRT	15 days	15 days
Temp	37°C	42°C
pH	7.35	7.85
TAN (α_A), mg/L as N	1400 ± 80 (*0.972*)	2380 ± 170 (*0.890*)
VFA_{TOTAL} (α_A), mg/L as HAc	230 ± 50 ($2.617x10^{-3}$)	5700 ± 160 ($8.461x10^{-4}$)

Table 5.2

Summary of modeled kinetic parameters for conventional and Cambi processes as effected by TAN concentration

Reactor	Ammonia Concentration mg/L as N		K_S (mg/L as HAc)	r_{MAX} ($mg/L \cdot day^{-1}$)	C_{LO} (mg/L as HAc)
	NH_3	TAN			
MAD	15	500	6.810 ± 0.191	0.593 ± 0.009	13.79 ± 0.20
	35	1250	5.738 ± 0.394	0.492 ± 0.018	13.50 ± 0.48
	56	2000	5.221 ± 0.054	0.455 ± 0.003	13.32 ± 0.07
	77	2750	4.013 ± 0.119	0.377 ± 0.006	13.53 ± 0.21
	77	2750	4.139 ± 0.055	0.384 ± 0.003	16.23 ± 0.11
	98	3500	3.210 ± 0.026	0.309 ± 0.001	16.11 ± 0.07
	119	4250	2.450 ± 0.026	0.265 ± 0.002	16.16 ± 0.09
	140	5000	1.588 ± 0.110	0.217 ± 0.008	16.78 ± 0.37
THD	24	220	5.322 ± 2.019	0.125 ± 0.039	11.63 ± 1.23
	79	720	3.820 ± 1.801	0.093 ± 0.033	11.37 ± 1.47
	134	1220	2.276 ± 1.103	0.063 ± 0.019	10.91 ± 1.31
	189	1720	1.565 ± 0.721	0.052 ± 0.013	11.24 ± 1.10
	299	2720	0.933 ± 0.442	0.040 ± 0.007	11.16 ± 0.85
	354	3220	0.453 ± 0.236	0.030 ± 0.004	10.59 ± 0.54

5.11 FIGURES

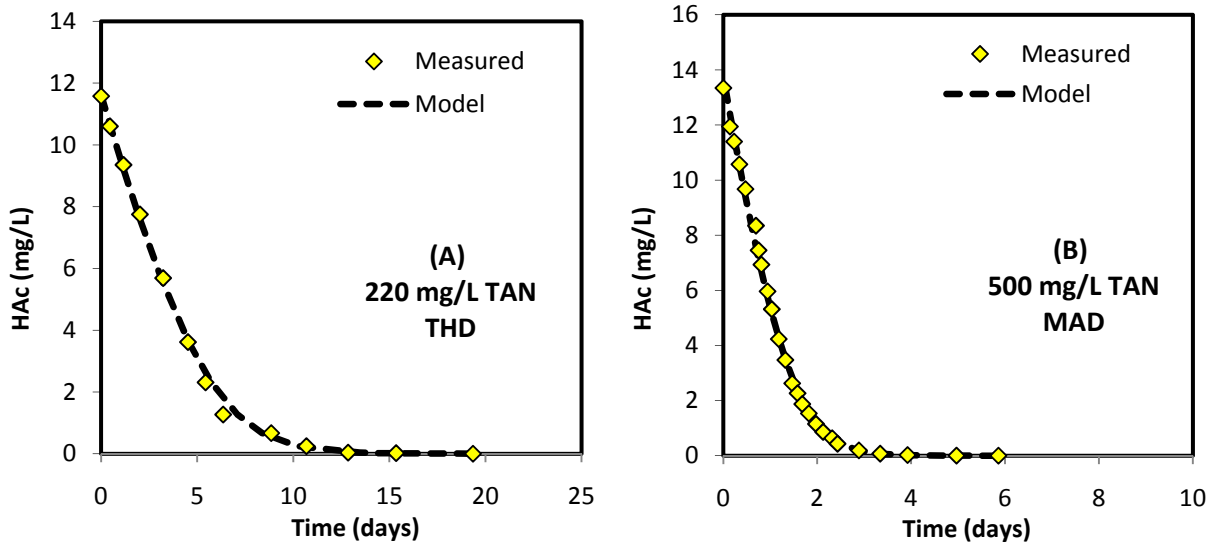

Figure 5.1

Representative acetic acid progress curves and associated modeled curve fit for the (A) THD, and (B) MAD processes at low ammonia concentrations.

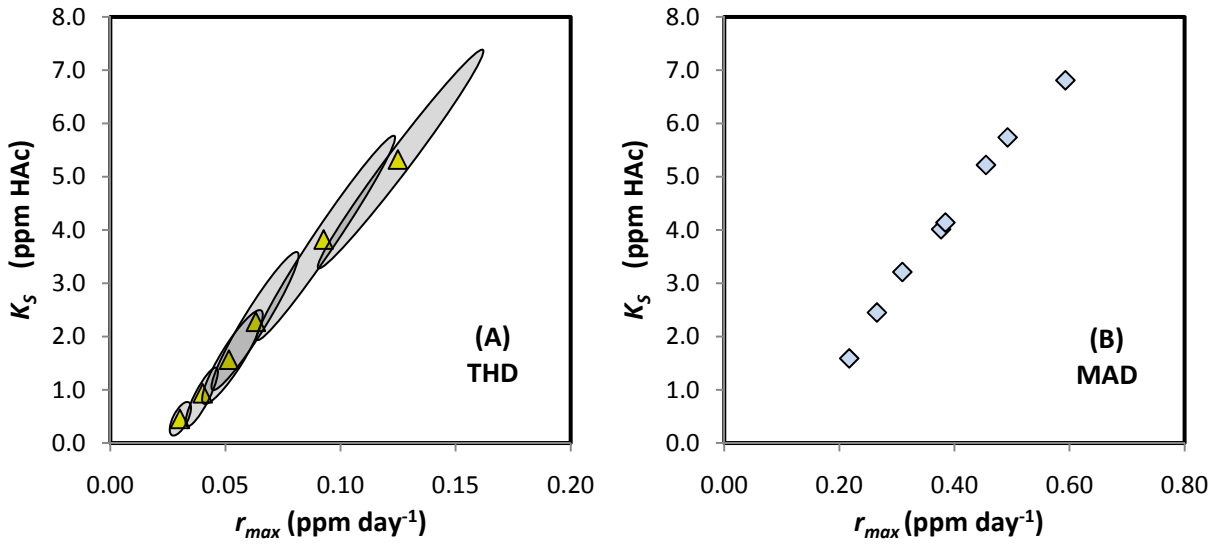

Figure 5.2

Correlation between modeled K_S and r_{MAX} (μ_{MAX}·X) for the (A) THD and (B) MAD process at various ammonia concentrations. Grey ellipses represent thin slices of 95% joint confidence intervals for the three curve fitting parameters: K_S, r_{MAX}, C_{LO}. Displayed slice of each 3-dimensional ellipsoid taken at $C_{LO} = C_{LO\ (Modeled)}$. Both the overall correlation between K_S and r_{MAX}, as well as the inclination of each ellipsoid in the x-y plane suggest as high degree of dependence between the values obtained for K_S and r_{MAX}. Due to the tightness of fit to MAD data, graphical display of joint confidence intervals is not practical and is thus omitted.

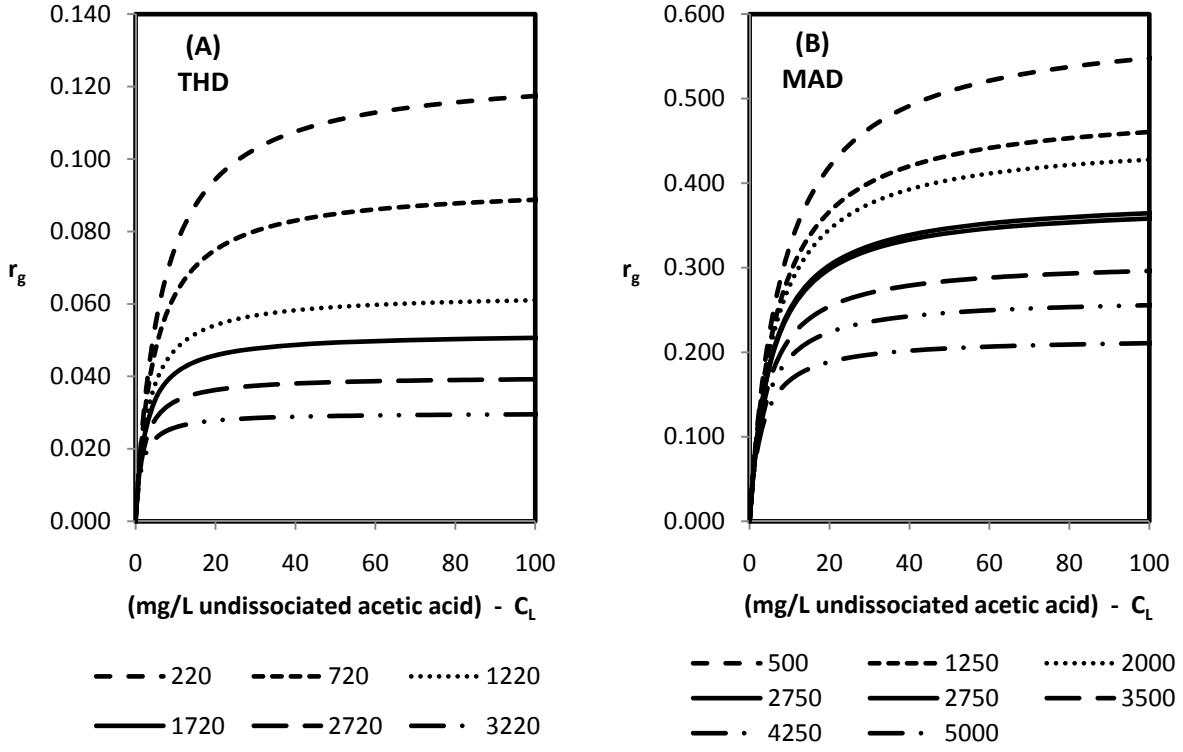

Figure 5.3

Derived Monod curves using model-estimated kinetic parameters for THD (A) and MAD (B). Data labels refer to TAN concentrations. The initial slope of each curve, α_A^o, is equivalent to r_{max}/K_S.

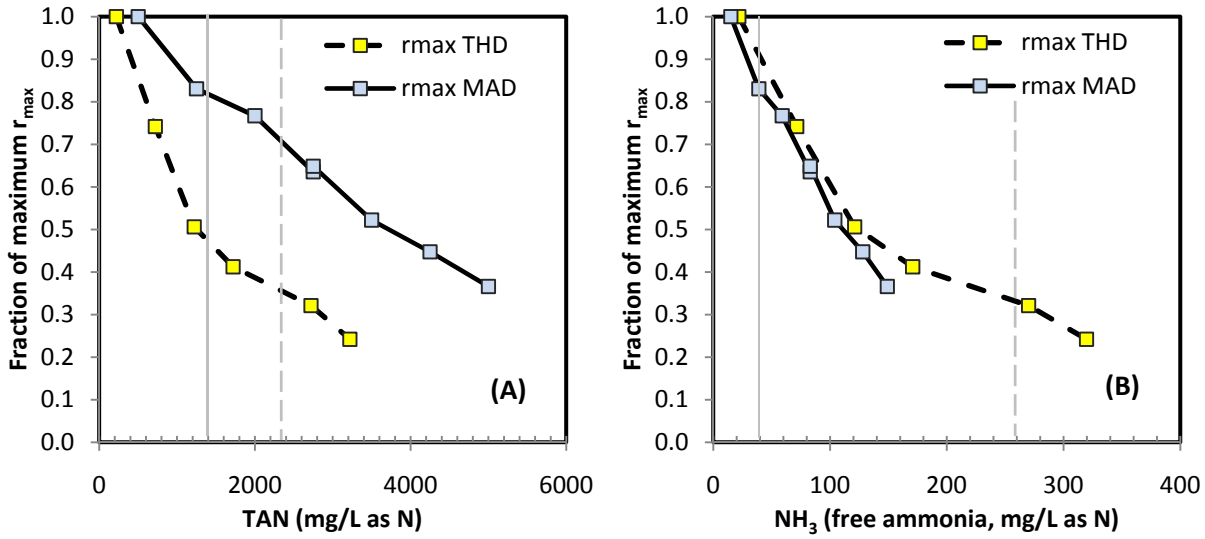

Figure 5.4

Maximum growth rates (r_{max}) for acetic acid degradation expressed as a fraction of r_{max} at the lowest ammonia level. The apparent effect of TAN concentration on growth rate inhibition is greater for THD than MAD (A); however, considering only the unionized form of ammonia (NH_3), it is clear that THD and MAD respond similarly to increasing inhibitor concentrations (B). Vertical lines denote in-situ nitrogen species concentrations for THD (dashed line) and MAD (solid line).

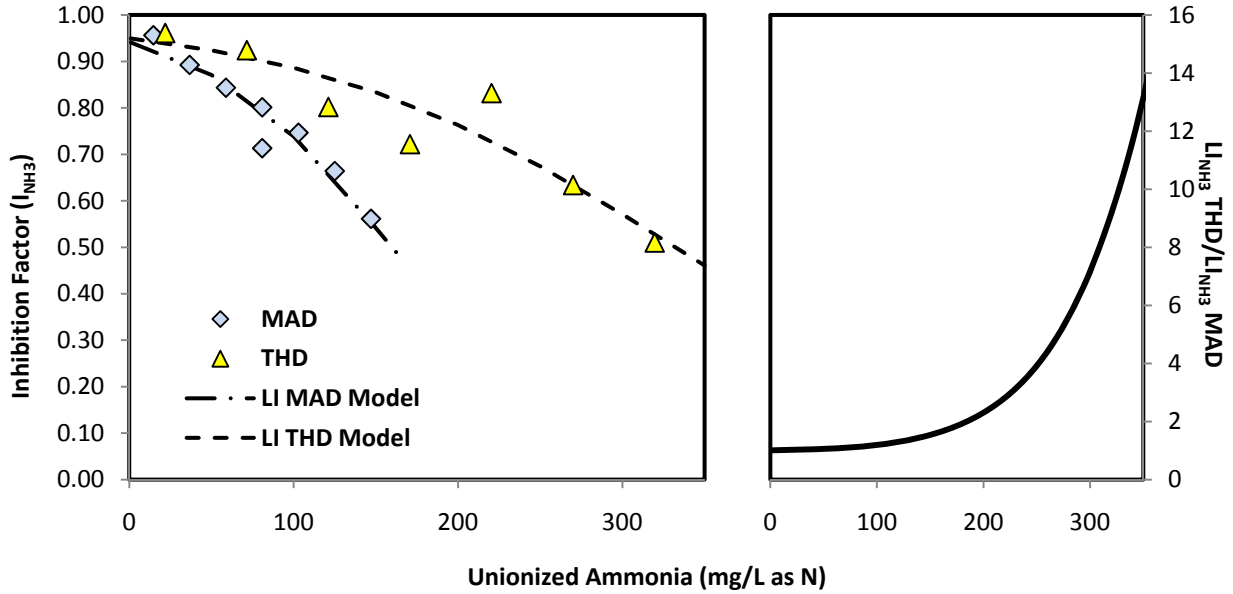

Figure 5.5

(A) Inhibition factors (I_{NH3}) related to the initial slope of cumulative methane production curves from batch ammonia toxicity assays. The rate of methane generation from the Cambi process is less affected by increased ammonia concentration, especially at high NH_3 concentrations (B). This suggests that methanogenesis (from acetate) is less dependent on the activity of aceticlastic methanogens during THD (i.e. dominance of non-aceticlastic methanogenesis is apparent).

5.12 SUPPLEMENTAL MATERIAL

Assume: fraction of substrate to energy, $f_e = 0.92$

fraction of substrate to cell synthesis, $f_S = 0.08$

Methanogenic degradation of glycine:

Overall reaction:

$$0.167\ CH_2NH_2COOH + 0.234\ H_2O = 0.004\ C_5H_7O_2N + 0.163\ HCO_3^- + 0.036\ CO_2 + 0.163\ NH_4^+ + 0.115\ CH_4$$

(Glycine, Amino Acid) (Biomass) (Alkalinity) (TAN)

75.07 g/mol glycine x 0.167 mol = 12.54 g glycine

18.05 g/mol NH_4^+ x 0.163 mol = 2.93 g NH_4^+ (2.28 g NH_4^+-N)

2.28 g NH_4^+-N/12.54 g glycine = 181 mg NH_4^+-N/g glycine (RELEASE)

0.163 mol HCO_3^- x 50,000 mg/mol = 8150 mg Alkalinity as $CaCO_3$

8150 mg Alkalinity as $CaCO_3$/12.54 g glycine = 650 mg Alkalinity as $CaCO_3$/g glycine (RELEASE)

Methanogenic degradation of palmitate:

Overall reaction:

$$0.011\ CH_3(CH_2)_{14}COO^- + 0.071\ H_2O + 0.004\ NH_4^+ = 0.004\ C_5H_7O_2N + 0.007\ HCO_3^- + 0.032\ CO_2 + 0.115\ CH_4$$

(Palmitate, LCFA) (TAN) (Biomass) (Alkalinity)

255.42 g/mol palmitate x 0.011 mol = 2.81 g palmitate

18.05 g/mol NH_4^+ x - 0.004 mol = - 0.072 g NH_4^+ (-0.054 g NH_4^+-N)

-0.054 g NH_4^+-N/2.81 g palmitate = -19.2 mg NH_4^+-N/g palmitate (UPTAKE)

0.007 mol HCO_3^- x 50,000 mg/mol = 350 mg Alkalinity as $CaCO_3$

350 mg Alkalinity as $CaCO_3$/2.81 g palmitate = 125 mg Alkalinity as $CaCO_3$/g palmitate (RELEASE)

Source: Rittman, B.E., and P.L. McCarty. 2001. Environmental biotechnology: Principles and applications. McGraw-Hill, Boston.

CHAPTER 6

METHANOGENIC COMMUNITY STRUCTURE IN MESOPHILIC, THERMOPHILIC, AND THERMAL HYDROLYTIC PRETREATMENT ANAEROBIC DIGESTER SYSTEMS

6.1 AUTHORS

Christopher A. Wilson, John T. Novak, Chad McKinney, Tim LaPara, Sudhir Murthy

6.2 ABSTRACT

Methanogenic communities from four laboratory-scaled sludge anaerobic digestion processes were investigated by quantitative real-time PCR (qPCR) and clone library analysis, including mesophilic (MAD), high temperature (HT) and low temperature (LT) thermophilic, and thermally pretreated sludge anaerobic digestion (THD). There was a correlation between reactors (THD and HT) exhibiting accumulation of volatile fatty acids (VFA), free unionized ammonia (NH_3) concentrations equal or greater than 290 mg/L NH_3-N, and the dominance of hydrogenotrophic methanogens. Alternatively, lower NH_3 processes (LT and MAD) showed low residual VFA concentrations and methanogenic communities predominantly consisted of aceticlastic methanogens. *Methanosaeta sp*. was missing from the THD digester, suggesting that the probable occurrence of non-aceticlastic methanogenesis was related to the high NH_3 concentration (310 mg/L NH_3-N) of that process. Although the fraction of clones attributable to aceticlastic methanogens was less for THD (13%) than MAD (67%), the overall methanogenic population was approximately ten times more densely concentrated for THD. It is speculated that methanogenic density of THD is related to the bioavailability of methanogenic substrates for both aceticlastic and hydrogenotrophic populations. While the concentration of NH_3 tolerant hydrogenotrophic methanogens did not vary between LT and HT reactors, aceticlastic methanogens were 44 times more concentrated in the low temperature reactor, resulting in a shift in dominant methanogenic population from *Methanobacteriales* (88%) in LT to

Methanosarcinales (85%) in HT. A threshold for a pathway shift between aceticlastic and non-aceticlastic methanogenesis appears to exist between 180 and 290 mg/L NH_3-N.

6.3 KEYWORDS

Archaeal clone library
Methanogenic community
Non-aceticlastic methanogenesis
Real-time quantitative PCR (qPCR)
Thermal hydrolysis
Thermophilic anaerobic digestion

6.4 NOMENCLATURE

AAD	Advanced anaerobic digestion
CH_4	Methane
CO_2	Carbon Dioxide
$\Delta G^{o'}$	Standard change in Gibbs free energy
H_2	Hydrogen
HT	High temperature (59°C) thermophilic
K_S	Monod half-saturation coefficient
LT	Low temperature (47°C) thermophilic
MAD	Mesophilic anaerobic digestion
NH_3	Free (unionized) ammonia
OTU	Operational taxanomic unit
qPCR	Quantitative real-time PCR
THD	High-solids anaerobic digestion of thermally pretreated wastewater sludge
TAN	Total ammonia nitrogen
TS	Total solids
VFA	Volatile fatty acids (C2-C7)
VS	Volatile solids

6.5 INTRODUCTION

Advanced anaerobic digestion processes (AAD) including high-temperature, high-solids, and pretreated sludge digestion present potential improvements in sludge stabilization, pathogen reduction, and energy recovery relative to conventional digestion processes. However, application of AAD requires an understanding of the fundamentals (e.g. microbiological and biochemical) that dictate reactor stability and performance. Stable anaerobic digester operation requires the cultivation and maintenance of a stable methanogenic population, including acetate and hydrogen (H_2) utilizing species. While hydrogenotrophic methanogens are responsible for maintaining low gaseous H_2 concentrations and supporting the thermodynamic viability of fatty acid fermentation (McCarty and Smith, 1986), acetic acid is thought to be the dominant intermediate involved in methane (CH_4) production (Gujer and Zehnder, 1983; Mackie and Bryant, 1981; Smith and Mah, 1966).

Aceticlastic methanogens are believed to be more sensitive to inhibition by free unionized ammonia (NH_3) than hydrogenotrophic species (Hajarnis and Ranade, 1993; Hajarnis and Ranade, 1994). This is a particularly critical distinction for AAD processes. High-temperature, high-solids, and pretreated sludge digestion each impose upward pressure NH_3 concentrations. Total ammonia nitrogen (TAN) is produced at a stoichiometric rate as organic solids are destroyed, either during biological sludge digestion (Wett *et al.*, 2006) or under hydrolytic pretreatment (Stuckey and McCarty, 1978; Wilson and Novak, 2009). In addition, hydrolytic pretreatment increases the applicable solids loading rates to an anaerobic digester by reducing the viscosity of raw sludge, thus further increasing associated nitrogen loading and release during anaerobic digestion (Bougrier *et al.*, 2008). Thermophilic conditions further exacerbates high total nitrogen concentrations, as the ratio of NH_3/NH_4^+ increases with increasing temperature resulting in sensitivity of aceticlastic methanogenesis and VFA accumulation at high temperatures (Angelidaki and Ahring, 1994; Speece *et al.*, 2006; vanLier, 1996; vanLier *et al.*, 1996).

Previous research has shown that anaerobic digesters subjected to long-term aceticlastic inhibition select for an alternate methanogenic pathway from acetate, independent of aceticlastic methanogens (Blomgren *et al.*, 1990; Schnurer *et al.*, 1994). Specifically, this alternate pathway,

termed non-aceticlastic methanogenesis refers to HAc metabolism by a syntrophic co-culture containing a hydrogenotrophic methanogenic species and an organism capable of oxidizing acetate to CO_2 plus H_2 (Petersen and Ahring, 1991; Zinder and Koch, 1984). Examples of such previously identified organisms include the thermophilic *Thermoacetegenium phaeum* (Hattori et al., 2000) and mesophilic *Clostridum ultunense* (Schnurer et al., 1996). Anaerobic digesters exhibiting non-aceticlastic methanogenesis by syntrophic acetate oxidizing cultures have been observed possess lower substrate utilization (and by association, growth) rates (0.5-5.3 mM acetate/day) than conventional aceticlastic processes (4.8-10.0 mM acetate/day) (Karakashev *et al.*, 2006). In addition, the apparent half saturation coefficient, K_S, for acetate has been measured as 0.65 mM for non-aceticlastic methanogenesis (Petersen and Ahring, 1991), lower than reported values for aceticlastic methanogens *Methanosarcina sp.* (5.33 mM acetate) and *Methanosaeta sp.* (1.5 mM acetate). In practice, the design of anaerobic digestion processes relies on accurate assessments of microbial growth and substrate utilization. Currently, design considerations are made for the maintenance of a stable population of aceticlastic methanogens due to their inherent sensitivity to perturbation; however, long term inhibition of aceticlastic methanogens giving way to the dominance of a non-aceticlastic pathway is not well described by the kinetics of *Methanosarcina* and *Methanosaeta sp*. It is useful to clarify the nature of the anaerobic digester acetate utilizing population, especially when considering the application of AAD processes where aceticlastic inhibition is common. In the present study, the steady-state aceticlastic and hydrogenotrophic methanogenic populations were investigated for a conventional mesophilic anaerobic digester (MAD), as well as two AAD processes: thermophilic anaerobic digestion at 47°C (LT) and 59°C (HT) and high-solids mesophilic anaerobic digestion of thermally hydrolyzed sludge (THD). The aim of this study is to determine the extent to which methanogenic pathway shifts are prevalent for classic (HT and LT) and novel (THD) AAD processes.

6.6 METHODOLOGY

6.6.1 Anaerobic Digester Operation and Monitoring

Conical high-density polyethylene reactors supplied by Hobby Beverage Equipment Company (Temecula, California) were used as pilot scale anaerobic digesters. The conical bottom of these vessels was thought to be advantageous in terms of mixing of dense high solids sludge feed to THD. The nominal volume of the reactor vessels was 25 liters. Reactors were operated with active volumes of 15 liters, and daily batch feeding was performed at a rate of 1 L/day. The reactors were modified to accept a threaded stainless steel thermometer. Reactor temperatures were 37°C, 42°C, 47°C and 59°C for MAD, THD, LT, and HT reactors, respectively. Reactors were continuously mixed by digester gas recirculation from the headspace through the conical reactor bottom.

MAD, LT, and HT reactors were initially seeded with sludge from Pepper's Ferry Regional Wastewater Treatment Plant (Radford, VA). Thermally hydrolyzed sludge digesters (THD) were initially seeded with reconstituted dewatered biosolids from Ringsend Wastewater Treatment Works (Dublin, Ireland) which was commissioned in 2003 and operates THD.

Digester feed was obtained from DCWASA Blue Plains and consisted of a 1:1 ratio of primary and secondary solids. Sludge to be subjected to MAD, LT, and HT was shipped on ice directly to Virginia Tech (Blacksburg, VA). These solids had a concentration of approximately 6% total solids (TS). Sludge that was thermally pretreated was dewatered to approximately 15% TS by DCWASA and shipped on ice to RDP Technologies, Inc. (Norristown, PA) for thermal treatment. Thermal treatment took place at 150°C and 4.8 bar for approximately 30 minutes. Sludge hydrolysate was then shipped on ice to Virginia Tech. These solids had concentration of approximately 12% TS. Reactors had been operating for a minimum of 8 months at the time of community analyses.

Samples for VFA analysis were passed through a 0.45 μm nitrocellulose membrane filter and frozen prior to analysis. Thawed samples were acidified by adding 85% phosphoric acid at a rate of 1% v/v and analyzed via HP5890 GC-FID and Supelco Nukol column with the following gas

flow rates: (Nitrogen) 14 mL/min, (Air) 450 mL/min, (Hydrogen) 44 mL/min, (Helium) 16 mL/min. The initial oven temperature was set to 80°C and ran isothermally for 3 minutes, then increased at 6°C/min for 10 minutes.

Total and volatile solids (VS) concentrations (Method 2540-G) as well as TAN (Method 4500-C) were determined as specified in Standard Methods for the Examination of Water and Wastewater. Reported values and associated error bars are indicative of averages of sample replicates over a period of steady-state reactor operation. Pertinent operating data for the digesters is shown in Table 6.1.

6.6.2 Extraction of Genomic DNA from Biomass Samples

Digested sludge was collected aseptically and pelleted at 14,000xG for 30 minutes. Four samples were taken over a period of 20 days during steady-state reactor operation. Pure cultures of methanogenic archaea were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Each pure culture was concentrated to a final volume of 200 µl in its own growth medium. Total DNA extraction of approximately 10 mg of homogenized pellet or 100 µL of concentrated pure culture suspension was performed using the FastDNA® Spin Kit for soil from MP Biomedicals (Carlsbad, CA) following the protocol specified by the manufacturer. Final volume of elutriation solution was 100 µl. Genomic DNA extracts were frozen at -80°C prior to use or analysis.

6.6.3 Standard Curves for Enumeration of Methanogenic 16s rRNA Gene Copies

Standard curves were generated for the 16S rRNA gene of five methanogenic orders. Genomic DNA extracts from pure cultures of *Methanobacterium bryantii* (DSMZ 863), *Methanococcus voltae* (DSMZ 1537), *Methanospirillum hungatei* (DSMZ 864), and *Methanosarcina barkeri* (DSMZ 800) were amplified using order-specific primers (Yu *et al.*, 2005). Genomic DNA from a member of the methanogenic order *Methanocellales* was amplified from a purified genomic DNA extract of THD biomass using the order-specific primer set specified by Franke-Whittle (2009) (Table 6.2). PCR products were prepared for quantitative real-time PCR (qPCR) by cloning into T1 phage chemically competent *Escherichia coli* using the Invitrogen™ (Carlsbad,

CA) TOPO TA Cloning® Kit following the protocol specified by the manufacturer. Recovered inserts were quantified on 3% agarose using a Bio-Rad (Hercules, CA) ChemiDoc XRS imaging system and Quantity One software. Stock standards of 10^{10} copies per ul were produced by dilution in sterilized nanopure water, and serial dilution to 10^{1} copies per ul was performed immediately preceding analysis. All standard curves were highly linear ($R^2 > 0.98$) within a concentration range of 10^{1}-10^{9} copies per ul (Table 6.3).

6.6.4 Order-Level Quantification of Methanogenic Communities by qPCR

Real-time qPCR was performed using an ABI (Foster City, CA) 7300 thermal cycler system and TaqMan® Environmental Master Mix 2.0 kit for *Methanococcales, Methanobacteriales, Methanomicrobiales,* and *Methanosarcinales,* and SYBR® Green PCR Master Mix for *Methanocellales*. The reaction mixture was prepared according to manufacturers recommendations. PCR was performed according to the following cycles: for *Methanococcales, Methanobacteriales,* and *Methanosarcinales,* 95°C initial denaturation for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, and simultaneous annealing an extension at 60°C for 60 seconds; for *Methanomicrobiales,* 95°C initial denaturation for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, and simultaneous annealing an extension at 63°C for 60 seconds; and for *Methanocellales*, 95°C initial denaturation for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for 40 seconds.

6.6.5 Clone Libraries Construction and Analyses

A single biomass sample taken from each reactor during steady-state reactor operation was used for clone library construction. Archaeal 16S rRNA gene segments were amplified from total genomic DNA extracts of THD and MAD biomass using the primers pARCH340F (5'-CCC TAC GGG GYG CAG CAG G-3') (Ovreas *et al.*, 1997) and ARC915R (5'-GTG CTC CCC CGC CAA TTC CT-3') (Muyzer *et al.*, 1993). Amplicons were cloned using the Promega (Madison, WI) pGEM®-T Easy Vector system following the protocol specified by the manufacturer. In total, 161 genomic DNA inserts (88 from THD, 73 from MAD) were recovered from successful clones at *EcoR1* restriction sites. All products were sequenced using a BigDye Terminator cycle sequencing kit (version 3.1) with an Applied Biosystems (Foster City, CA)

3730XL capillary electrophoretic DNA analyzer at the University of Minnesota DNA Sequencing and Analysis Facility. Data from highly similar sequences (greater than 99% sequence similarity) were defined as operational taxonomic units. Each sequence was searched for phylogenetic relatives using the nucleotide BLAST (BLASTn) program (Altschul *et al.*, 1990).

6.7 RESULTS AND DISCUSSION

Clone library analysis was used to determine the relative distribution of aceticlastic (heterotrophic) and hydrogenotrophic (autotrophic) methanogens within THD and MAD reactors. These processes, among those described in this paper, represent the highest (310 mg/L N) and lowest (50 mg/L N) measured steady-state free NH_3 concentrations, respectively. There was an overall preference for the growth of hydrogenotrophic methanogens during THD as aceticlastic methanogens were represented by only 13% of THD clones (Figure 6.1). Conversely, the MAD methanogenic community was dominated (67%) by aceticlastic methanogens. Generally, it is observed that for the anaerobic digestion of domestic sewage sludge under conventional conditions (i.e. near neutral pH, mesophilic temperature, low inhibitor concentrations etc.) acetic acid serves as an intermediate substrate for 70-90% of total CH_4 production (Gujer and Zehnder, 1983; Mackie and Bryant, 1981; Smith and Mah, 1966). Assuming typical stoichiometries for CH_4 generation from acetic acid and hydrogen, and respective yield coefficients of 0.04 g VS/gram acetic acid and 0.45 g VSS/g H_2 (Rittmann and McCarty, 2001), we expect that the ratios of methanogenic populations that utilize either acetic acid or H_2 are representative of source of CH_4 generation from a particular biomass sample. It is noted that the growth of methanogens during MAD in this study corresponds well with previous literature, while the relative growth of aceticlastic and hydrogenotrophic methanogens during THD is distinct.

Aceticlastic methanogenesis, describing the fermentative sequential oxidation of the carbonyl carbon atom of acetic acid producing carbon dioxide (CO_2) and reduction of the methyl carbon atom of acetic acid producing CH_4, has been observed as the primary pathway for methanogenic degradation of acetic acid in conventional mesophilic anaerobic digesters (Mackie and Bryant,

1981; Zinder *et al.*, 1984; Zinder and Koch, 1984). Non-aceticlastic methanogenesis from acetic acid, which describes a two-step process of acetic acid oxidation to CO_2 and hydrogen (H_2) and subsequent reduction of CO_2 to CH_4 by hydrogenotrophic methanogens (Barker, 1936) has been previously described. As this metabolism ($\Delta G^{\circ\prime}$ = 104.6 kJ/mol) is not as energetically favorable as aceticlastic methanogenesis ($\Delta G^{\circ\prime}$ = -31.0 kJ/mol) (Rothfuss and Conrad, 1993), dominance of non-aceticlastic methanogenesis generally limited to environments having high concentrations of aceticlastic inhibitors (e.g. NH_3) or alternatively, to relatively dilute environments such as freshwater sediments where reaction products (e.g. H_2) are nearly immediately dispersed and reaction thermodynamics are enhanced (Karakashev *et al.*, 2006; Schnurer *et al.*, 1999). In a survey of thirteen methanogenic reactors conducted by Schnurer *et al*. (1999), it was noted that in the four of thirteen reactors that operated with steady-state VFA concentrations in excess of 1.2 g/L (an indication of aceticlastic inhibition), 38-82% of methyl ^{14}C-labelled carbon from acetic acid was recovered as labeled $^{14}CO_2$. Two of these four digesters had NH_3 concentrations of 440, and 880 mg/L as N, directly implicating NH_3 inhibition in the occurrence of non aceticlastic methanogenesis. The remaining two digesters possessed reportedly inhibitory concentrations of either Na^+ or K^+. Lower VFA digesters all channeled between 10-25% of acetic acid through non-aceticlastic methanogenesis. Based on previous work, it appears that the high steady-state NH_3 concentration for the THD reactor described in this study is the likely cause of the shift away from aceticlastic growth. substantially

Methanogenic diversity was also affected by THD relative to MAD. The THD methanogenic community consisted of three operational taxonomic units (OTU), two hydrogenotrophic and one aceticlastic; whereas the MAD methanogenic community consisted of five OTUs, three hydrogenotrophic and two aceticlastic (Table 6.4). Previous work has shown that there is a high degree of correlation between the absence *Methanosaeta sp*. from an anaerobic digester community and the occurrence of non-aceticlastic methanogenesis (Karakashev *et al.*, 2006). In addition, it was determined by the authors that a single dominant methanogenic species comprised greater than 90% of the archaeal community in each reactor sample. These findings led the authors to the proposal of a bimodal system for methanogenesis where the aceticlastic pathway dominated in the presence of *Methanosaeta sp.* and syntrophic acetate oxidation was preferred in their absence. Pure cultures of *Methanosaeta concilii* have been found to be

particularly sensitive to NH_3 inhibition, showing a reduction of growth and CH_4 production of approximately 80% with an NH_3 concentration of 29 mg/L NH_3-N (Sprott and Patel, 1986). It is clear that NH_3 concentrations in the MAD reactor were to a large extent inhibitory to *Methanosaeta sp*. at 50 mg/L NH_3-N; however, full inhibition of this population did not occur, although they are greatly suppressed relative to *Methanosarina sp*. At 310 mg/L NH_3-N, no THD clones were attributable to *Methanosaeta sp*. In contrast with Karakashev *et al.* (2005), diversity was observed among non-dominant methanogenic groups, and these groups were responsible for 22.7% of clones for THD and 34.2% of clones for MAD. While these data generally support the bimodal system for methanogenesis proposed by Karakashev *et al*. (2006), it appears that the disappearance of *Methanosaeta sp.* may serve an overly conservative indicator of a shift in methanogenic acetic acid degradation pathway as aceticlastic methanogens (i.e. *Methanosarcina sp.*) remained dominant within the MAD reactor at reported strongly inhibitory NH_3 levels for *Methanosaeta sp.*, and accumulation of VFA observed in other instances of non-aceticlastic methanogenesis was not observed.

Methanogen 16S rRNA gene copy concentrations are presented for THD and MAD (Figure 6.2), and HT and LT (Figure 6.3). As THD received higher solids loading than the other reactors, 16S gene copies for all reactors are normalized to mass of dry volatile solids within each biomass sample. Hydrogenotrophic orders *Methanobacteriales, Methanococcales,* and *Methanomicrobiales* (and later to include *Methanocellales*) are summed in order to represent the population of hydrogen-utilizing methanogens relative to aceticlastic members, all of the order *Methanosarcinales*. While data for *Methanocellales* are not yet available, there is strong evidence that members of this order are not likely to be present in sufficient concentrations to affect inferences drawn related to methanogenic ecology from the MAD reactor. From the clone library analysis, members of the order *Methanocellales* were represented by 4.1% (3/73) of MAD clones, whereas organisms of the order *Methanomicrobiales* showing 96.7% similarity to *Methanospirillum hungatei* and those of the order *Methanobacteriales* showing 99.8% similarity to *Methanobacterium congolense* were together responsible for 27.4% (20/73) of archaeal clones. Based on these ratios, it appears highly likely that quantification of members of the order *Methanocellales* from the MAD digester by qPCR would marginally increase the enumeration of total hydrogenotrophic methanogenic biomass from this reactor.

Previously identified *Methanoculleus sp.* were generally observed to be halotolerant organisms having slightly acidic pH optima (Harris *et al.*, 1984; Maestrojuan *et al.*, 1990; Ollivier *et al.*, 1986), suggesting that these organisms are unlikely to find an advantageous competitive niche within the domestic sewage sludge digesters of this study. However, members of the order *Methanocellales* have been associated with environments having high inorganic salt concentration (e.g. TAN and sodium salts) (Schnurer *et al.*, 1999), as well as thermophilic environments (Harris *et al.*, 1984; Spring *et al.*, 2005). It has been previously noted that these organisms' presence in conjunction with inhibition of aceticlastic methanogens, especially by NH_3, may be driven by their tolerance to inorganic salt concentrations in excess of several grams per liter. In addition, several reports have been made of members of the methanogenic family *Methanomicrobiaceae* (including genera *Methanogenium* and *Methanoculleus*) requiring acetate as a necessary non-electron donor growth factor (Ollivier *et al.*, 1986; Spring *et al.*, 2005). It is likely that an elevated acetate level due to the occurrence of aceticlastic inhibition creates a preferential environment for the autotrophic growth of this specialized group of methanogens. In any event, conclusions drawn regarding the population of hydrogenotrophic methanogens in the absence of quantitative data pertaining to the order *Methanocellales* are tentative, and will be reassessed as these data become available.

LT and HT reactors possess essentially equivalent population densities of hydrogenotrophic methanogens of the order *Methanobacteriales*, each at 1.66×10^7 copies/g VS. These populations comprise 99.2% and 99.4% of the sum of *Methanobacteriales, Methanococcales,* and, *Methanomicrobiales* for LT and HT reactors respectively, suggesting even in the absence of quantitative *Methanocellales* data that *Methanobacteriales* may be the dominant hydrogenotrophic order in both thermophilic reactors. While the concentration of NH_3 tolerant hydrogenotrophic methanogens did not vary between LT and HT reactors, aceticlastic methanogens were approximately 44 times more concentrated in the low temperature reactor.

Assuming that total hydrogenotrophic methanogenic quantities are represented in Figure 6.3, the variation in aceticlastic methanogens between thermophilic digesters represents in a shift in dominant methanogenic population from *Methanobacteriales* (88%) in LT to *Methanosarcinales* (85%) in HT. NH_3 concentrations in LT (180 mg/L NH_3-N) and HT (290 mg/L NH_3-N) systems

are bounded by concentrations in MAD (50 mg/L NH_3-N) and THD (310 mg/L NH_3-N), regarding which a shift towards the preferential growth of hydrogenotrophic methanogens at high NH_3 concentrations has been previously discussed. Based on the assumptions made regarding the order *Methanocellales* throughout this discussion (i.e. that the percentage of *Methanoculleus sp*. obtained from clone library analysis of MAD and THD reactors is valid, and contribution of *Methanocellales* to the overall population of hydrogenotrophic methanogens under thermophilic conditions is small), the contribution of *Methanosarcinales* to the overall methanogenic population for the four digestion systems can be calculated (Figure 6.4). It appears that a threshold exists between approximately 180 mg/L NH_3-N and 290 mg/L NH_3-N above which growth of non-aceticlastic methanogens is preferred. This concentration range seems appropriate in the context of previously published NH_3 toxicity data for pure culture *Methanosarcina barkeri*, showing that 20%, 50%, and complete inhibition of growth occurred at NH_3 concentrations of 90, 160, and 260 mg/L NH_3-N (Hajarnis and Ranade, 1993). Other research has shown that small concentrations of sodium ion (e.g. 50 mg/L Na^+) are beneficial in counteracting NH_3 inhibition of *Methanosarcina barkeri* such that the survival of *Methanosarcinales* observed for THD could be adequately explained (Sprott and Patel, 1986).

6.8 CONCLUSION

The implications of a process dependent shift in methanogenic community as presented in this study is broad in terms of our understanding of methanogenic kinetics in AAD systems and our ability to apply predictive models to process design. While evidence of non-aceticlastic methanogenesis has been previously discussed with respect to thermophilic, high-solids, and high NH_3 processes, data regarding a methanogenic community shift away from aceticlastic methanogens within a THD process do not exist elsewhere in the literature. An apparent threshold for the for preferred growth of hydrogenotrophic methanogens relative to aceticlastic methanogens appears to exist between 180 and 290 mg/L NH_3-N for a variety of anaerobic digestion processes, suggesting that a common mechanism exists (i.e. NH_3 inhibition of aceticlastic methanogenesis) that selects for aceticlastic or non-aceticlastic methanogenesis during anaerobic digestion. This conclusion is well supported by previous literature related to NH_3 tolerance and toxicity of the aceticlastic methanogen, *Methanosarcina barkeri*.

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6.10 TABLES

Table 6.1

Operating parameter of anaerobic digestion systems

Digester	pH	Temperature	$\alpha_A = f(T,pH)$	TAN	NH_3	tVFA
Thermo-60°C	7.50	59°C	0.8641	2170 ± 80	290	9270 ± 2020
Thermo-47°C	7.60	47°C	0.9134	2100 ± 90	180	1100 +230
THD	7.85	42°C	0.8900	2800 ± 110	310	5700 ± 60
MAD	7.35	37°C	0.9724	1400 ± 80	50	230 ± 50

Table 6.2

Primer and probe combinations for order-specific quantification of methanogens by qPCR

Primer/Probe	Function	Sequence (5'→3')	Target Group	Reference
MCC495F	*forward*	TAAGGGCTGGGCAAGT		
MCC686F	*TaqMan*	TAGCGGTGRAATGYGTTGATCC	*Methanococcales*	Yu *et al*., 2005
MCC832R	*reverse*	CACCTAGTYCGCARAGTTTA		
MBT857F	*forward*	CGWAGGGAAGCTGTTAAGT		
MBT929F	*TaqMan*	AGCACCACAACGCGTGGA	*Methanobacteriales*	Yu *et al*., 2005
MBT1196R	*reverse*	TACCGTCGTCCACTCCTT		
MMB282F	*forward*	ATCGRTACGGGTTGTGGG		
MMB749F	*TaqMan*	TYCGACAGTGAGGRACGAAAGCTG	*Methanomicrobiales*	Yu *et al*., 2005
MMB832R	*reverse*	CACCTAACGCRCATHGTTTAC		
MSL812F	*forward*	GTAAACGATRYTCGCTAGGT		
MSL860F	*TaqMan*	AGGGAAGCCGTGAAGCGARCC	*Methanosarcinales*	Yu *et al*., 2005
MSL1159R	*reverse*	GGTCCCCACAGWGTACC		
MCL298F†	*forward*	GGAGCAAGAGCCCGGAGT	*Methanocellales*	Franke-Whittle *et al*., 2009
MCL586R	*reverse*	CCAAGAGACTTAACAACCCA		

† Primer set MCL (*Methanoculleus)* was previously optimized for SYBR Green real-time PCR, thus no TaqMan probe is provided (Franke-Whittle *et al*., 2009).

Table 6.3

16S rRNA gene source cultures and standard curve QA/QC data for qPCR analysis

Primer Set	Source DNA		Slope	Intercept	R^2
MCC	DMSZ 1537	*Methanococcus voltae*	-4.171	51.513	0.984
MBT	DMSZ 863	*Methanobacterium bryantii*	-3.608	45.565	0.997
MMB	DMSZ 864	*Methanospirillum hungatei*	-4.191	53.561	0.997
MSL	DMSZ 800	*Methanosarcina barkerii*	-4.367	51.466	0.987
MCL	This study	*Uncultured Methanoculleus clone*	*Not yet available*		

Table 6.4

Distributions of 16S rRNA gene clones obtained from clone library analysis of THD and MAD biomass

Digester	OTU No.	Total Clones (%)	Closest Relative	Accession No.	Match (%)
THD	**THD-Arc1**	68 (77.3)	*Methanoculleus bourgensis*	AY196674	515/515 (100)
THD	**THD-Arc2**	9 (10.2)	Uncultured *Methanomicrobiales* archaeon	CU917280	513/515 (99.6)
THD	**THD-Arc3**	11 (12.5)	Uncultured *Methanosarcina sp.*	EU888016	519/519 (100)
		∑ = 88			
MAD	**MAD-Arc1**	16 (21.9)	Uncultured *Methanomicrobiales archaeon*	CU917418	514/515 (99.8)
MAD	**MAD-Arc2**	3 (4.1)	*Methanoculleus bourgensis*	AB065298	515/515 (100)
MAD	**MAD-Arc3**	4 (5.5)	*Methanobacterium congolense*	NR028175	518/519 (99.8)
MAD	**MAD-Arc4**	2 (2.7)	Uncultured *Methanosaeta sp.*	EU888815	502/515 (97.5)
MAD	**MAD-Arc5**	48 (65.8)	*Methanosarcina barkeri*	AY196682	514/519 (99.0)
		∑ = 73			

6.11 FIGURES

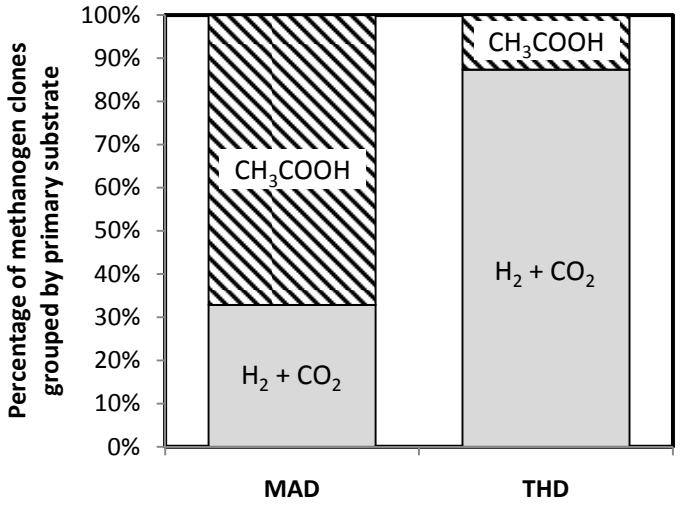

Figure 6.1

Fractions of aceticlastic and hydrogenotrophic methanogens obtained from clone library analysis of MAD and THD biomass

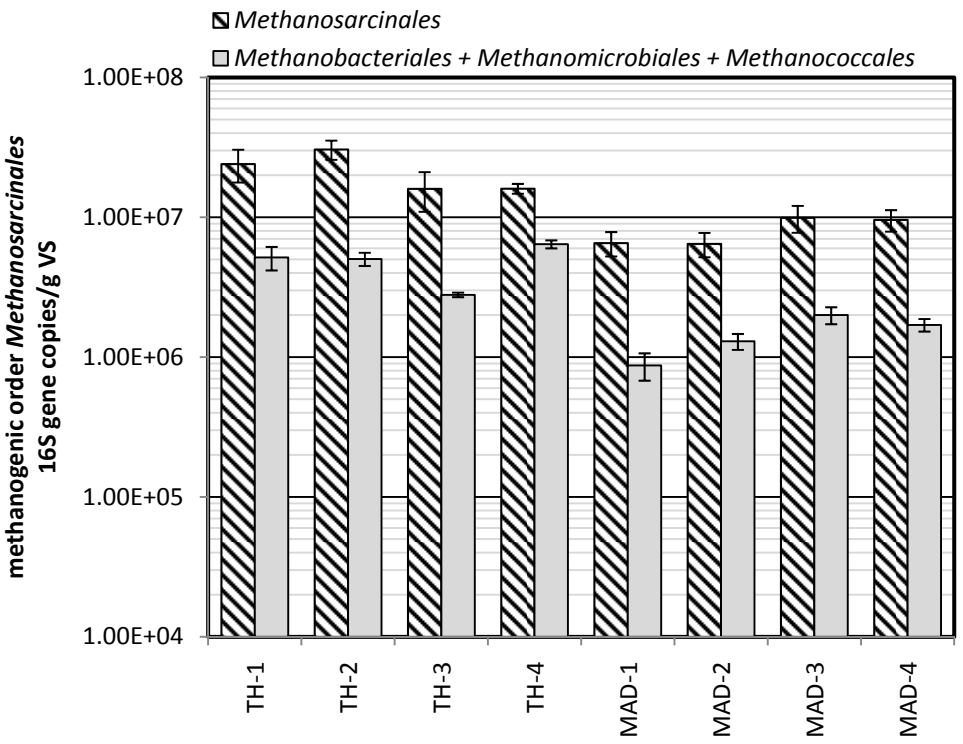

Figure 6.2

16S rRNA gene copies associated with aceticlastic (*Methanosarcinales*) and hydrogenotrophic (*Methanobacteriales* + *Methanomicrobiales* + *Methanococcales*) methanogenic populations for replicate samples of THD (TH-1-4) and MAD (MAD-1-4) biomass.

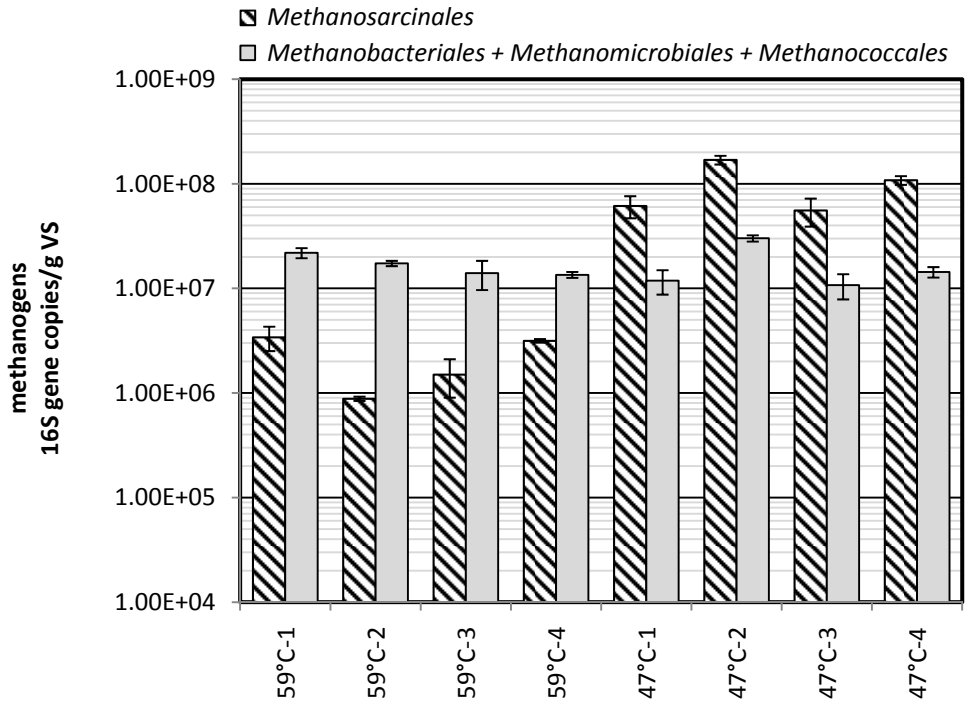

Figure 6.3

16S rRNA gene copies associated with aceticlastic (Methanosarcinales) and hydrogenotrophic (Methanobacteriales + Methanomicrobiales + Methanococcales) methanogenic populations for replicate samples of HT (59°C-1-4) and LT (47°C-1-4) biomass.

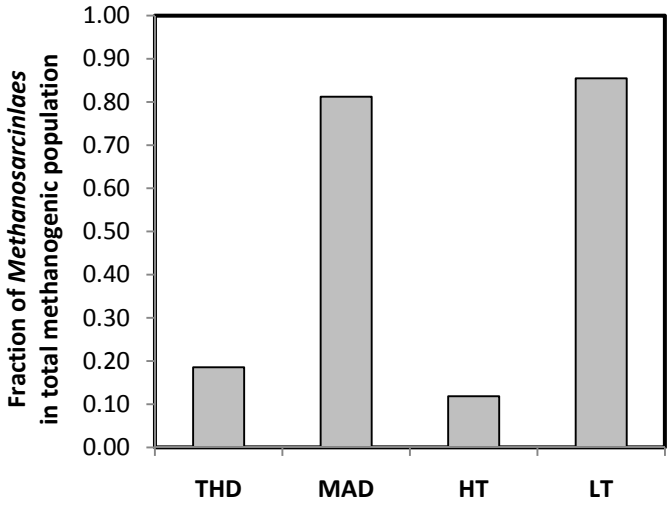

Figure 6.4

Proposed fractional contribution of methanogens of the order *Methanosarcinales* to overall methanogenic populations. Calculation of these fractions is based on assumptions made regarding the relative importance/dominance of methanogens of the order *Methanocellales* within each digester community as described in-text.

CHAPTER 7

CONCLUDING REMARKS AND ENGINEERING SIGNIFICANCE

Engineered ecosystems are at the core of domestic wastewater treatment. Success of biological treatment schemes requires the maintenance of key microbial populations necessary to achieve various ends: *scavenging of low-level organic carbon, nitrification and denitirification, phosphorous uptake, solids-liquid separation, and methanization of dense organic material*. This thesis has aimed at improving the understanding of the fundamental mechanisms that control methanogenic populations and performance under extreme environmental and chemical conditions. Such an understanding is crucial to our capacity to design and operate digestion processes at the classical limits of operability. This research examined two advanced digestion processes, one classic and one novel, that push these classical limits of operability. Thermophilic anaerobic digestion is an old and, in many ways, well understood process. However, lack of diversity at high temperatures and the presence of chronic inhibitors (e.g. unionized ammonia) have allowed us to view digester ecology and syntrophy under a state of sustained perturbation that is only otherwise observable during dynamic failure or upset of conventional (mesophilic) digestion systems. The distinction between process stability and sensitivity which are respectively characteristic of mesophilic and thermophilic digestion help to develop a continuum over which changes in methanogenic pathway, accumulation of digestion intermediates and byproducts, and wide shifts in ecology may be observed. Specific contributions to our understanding of thermophilic anaerobic digestion by this research include the following:

- ***Low level accumulation of anaerobic digestion intermediates serves as an early warning of digester failure.*** A distinction exists between the gradual onset of physical-chemical conditions which lead to digester failure, and the dramatically sharp occurrence of digester failure itself.

- ***Volatile solids analysis of advanced anaerobic digestion systems is prone to large errors due to analytical artifacts.*** Routine evaluation of digestion performance by analysis of volatile solids reduction is interfered upon by the presence of soluble digestion intermediates. Significant errors were detected in the estimation of sludge stability when influent and effluent VFA or ammonia concentrations were widely dissimilar. A broad study focused on describing such analytical artifacts that affect the evaluation of digester performance would serve as a significant additional benefit to digester operations.

While much work was performed with respect to thermophilic digestion throughout this research, it was largely engaged in to build upon and to develop understanding of the broad base of existing knowledge in mechanistic thermophilic digester operation. Substantial and unique contributions have been made in the field of thermal hydrolytic sludge pretreatment. Among the most important of these contributions, are:

- ***The identification of high solids anaerobic digestion of thermally hydrolyzed sludge as an organic nitrogen loading limited process***. Ammonia and bicarbonate release from proteinaceous material are the combined causes of inhibition of acetate utilization by free unionized ammonia. Higher solids loading (and thus additional energy recovery) could be achieved by amending the digester would high-carbon, low nitrogen feeds. For domestic wastewater treatment where cellular material comprises a significant fraction of process feed, organic nitrogen represents a significant and fundamental limitation of this process at high organic loading rates.

  Investigation of process-based modifications to counter this limitation is a valuable future extension of this work. Such investigations may include recuperative thickening/elutriation of ammonia from an internal recycle stream, chemical amendments (such as mineral acid) to lower digester pH and thus alter the ratio of NH_3/NH_4^+, and the exploration of the suitability of thermal hydrolytic pretreatment applied to codigestion of fats, oils, and greases with wastewater sludge.

- ***Unionized species of acetic acid and ammonia are the important active form of those molecules for describing substrate utilization by, and ammonia inhibition of acetate degrading methanogenic communities.*** This represents a paradigm shift from previously reported kinetic parameters for methanogenesis from acetic acid in terms of total acetic acid, and thresholds for ammonia inhibition in terms of total ammonia nitrogen. To be broadly applicable, future research pertaining to anaerobic digestion kinetics should take care to ascertain the effect of the physical-chemical environment on the bioavailable forms of ionizable species.

- ***This is the first reported combined kinetic and ecological evaluation of a high solids anaerobic digestion process treating thermally hydrolyzed wastewater sludge.*** As described throughout, kinetic based design requires knowledge of methanogenic ecology as well as the impact of relevant inhibitors in order to appropriately size and obtain predictable performance from new anaerobic digestion systems. This work greatly increases the practical applicability of a novel advanced anaerobic digestion process with potentially large environmental and economical benefits. Similarly, combined kinetic and ecological evaluations of digestion processes are somewhat rare in the literature, making our evaluation of the control process (conventional mesophilic anaerobic digestion) a valuable contribution to anaerobic digestion literature as well. As we gain understanding of the role that process design and operation plays in selecting for specific methanogenic communities, and in turn, dictating the kinetics of methane production, combined kinetic and ecological evaluations of a wide variety of digester processes and process-dependent physical/chemical environments will help to further elucidate the continuum between conventional and extreme digestion processes.

APPENDIX A

APPENDIX A

COMPREHENSIVE ENHANCED DIGESTION EVALUATIONS AT BLUE PLAINS ADVANCED WASTEWATER TREATMENT PLANT

A.1 AUTHORS

Christopher A. Wilson, John T. Novak, Sudhir N. Murthy, Walter F. Bailey

A.2 ABSTRACT

The District of Columbia Water and Sewer Authority (DCWASA) has undertaken a long-term study to evaluate the performance and operational aspects of various sludge digestion alternatives. While the initial goal of this study was to determine an appropriate digestion technology for Blue Plains Advanced Wastewater Treatment Plant, the outcome has been an increased awareness of the fundamental digestion parameters that impact solids degradation. These studies have been performed since 2003 at Virginia Polytechnic Institute and State University (VT) with a consistent reactor design and operational protocol so as to allow direct comparison between digestion technologies. In addition, conventional mesophilic anaerobic digestion has been maintained nearly continuously at VT over the course of the project in order to observe any potential shifts in the biodegradability of the source sludge from Blue Plains.

To date, detailed investigations have been performed on the following technologies (each with significant operational variation, e.g. solids retention time, temperature, etc.):

Single Stage Digestion:

- Mesophilic anaerobic digestion (MAD)*
- Thermophilic anaerobic digestion (49-58°C)*

Multiple Stage Digestion:

- Acid Gas Anaerobic Digestion (AGAD)
- Temperature Phased Anaerobic Digestion (TPAD)
- Cambi Thermal Hydrolysis followed by MAD *

*Post aerobic digestion following these technologies has also been investigated.

This paper summarizes these digestion process evaluations by investigating the effect of global system variables (e.g. digester temperature, anaerobic staging, anaerobic/aerobic staging) on solids removal efficiency and when applicable, nutrient and odor reduction potential.

A.3 KEYWORDS

Aerobic digestion,
Anaerobic digestion
Biosolids odor
Nitrogen
Reactor staging
Retention time
Sludge minimization

A.4 INTRODUCTION

For the past six years, the District of Columbia Water and Sewer Authority (DCWASA) has been pursuing an appropriate process train that satisfies the following requirements:

1. Biosolids minimization (enhanced solids destruction, increased cake solids)
2. Energy production and reduction in direct and indirect greenhouse gas emissions
3. Enhanced biosolids acceptability (reduce biosolids odor/ fecal coliforms/ microconstituents)
4. Compatibility within plant (reducing nitrogen loading in return centrate streams)

As part of the Water Environment Research Federation's project 05-CTS-3: *Evaluation of Processes to Reduce Activated Sludge Solids Generation and Disposal*, Sandino *et al*. (2008) presented a literature review of recent advances in sludge minimization technologies. From this review, one can see that there is a broad approach from a process standpoint of achieving goals such as those set forth by DCWASA. The process evaluations in this study have largely focused on biological processes (rather than chemical or mechanical), although one thermal alternative was investigated.

The goal of this paper is to summarize the work performed by DCWASA and VT over the past six years in order to provide guidance as to which processes and operational parameters seem to have a positive effect of sludge stabilization, odor control, and nutrient reduction.

A.5 DESCRIPTION OF PROCESSES

A.5.1 Single-Stage Digestion

Single-stage digestion processes included conventional mesophilic anaerobic digestion (MAD) and thermophilic anaerobic digestion (TAD). Both of these processes are among the oldest and best understood of all anaerobic digestion technologies. The application of TAD has been somewhat limited in the past due to concerns with process stability and in-plant odors; however interest has recently increased due to the need for readily implementable Class A digestion technologies (Iranpour, 2006, Iranpour and Cox, 2006). The investigation of TAD allowed us to determine whether digestion temperature was an important factor in determining solids destruction. The thermophilic temperatures investigated were 49°C, 51°C, 53°C, 55°C, and 58°C.

A.5.2 Multi-Stage Anaerobic Digestion

The prevalent multi-stage digestion technologies are temperature-phased anaerobic digestion (TPAD) and acid-gas anaerobic digestion (AGAD). In either case, the intent is to create distinct sequential biological environments that are particularly suited to organisms grown in the stages. In the case of TPAD, the temperature phasing results in separate niches for thermophilic and

mesophilic methanogens. For AGAD, the niches are occupied by fermenting (hydrolytic) and methanogenic organisms, respectively (Fox and Pohland, 1994, Han and Dague, 1997). The operational variable of the TPAD systems was digester solids retention time (SRT), and the initial thermophilic stages was maintained at 55°C. For AGAD systems, the initial acid stage was mesophilic (37°C).

A.5.3 Thermal Hydrolytic Pretreatment

Thermal hydrolysis (TH) is applied to raw sludge in order to decrease the viscosity and thus double the digester loading and capacity, and increase the biodegradability and dewaterability of raw sludges. Since the thermal hydrolysis pretreatment process achieves enhanced sludge hydrolysis and pathogen deactivation, it is most often followed by conventional MAD (TH-MAD), however other enhanced anaerobic digestion processes such as TPAD may benefit from hydrolysis pretreatment (Jolis, 2008). In this study, hydrolysis temperatures of 150°C and 170°C, digestion temperatures of 37°C and 42°C, and SRT of 15 days and 20 days were investigated. Data from these operational variations did not differ significantly, so data from TH-MAD with a hydrolysis temperature of 150°C, a digester temperature of 37°C, and retention times of 15 and 20 days are reported.

A.5.4 Anaerobic/Aerobic Staged Digestion

Novak et al (2003) have suggested that different sludge fractions degrade via anaerobic and aerobic metabolism. In addition, the careful application of aerobic digestion of anaerobically digested sludge within a sludge stabilization train allows for the removal of nitrogen through nitrification/denitrification processes. In this study, post aerobic digestion was applied following: MAD (MAD-AER), TAD (TAD-AER), and TH-MAD (TH-MAD-AER). The SRT of these post aerobic digestion processes ranged from 3-9 days.

A.6 METHODOLOGY

A.6.1 Reactor Configuration

The anaerobic digesters chosen for this work were constructed as previously described (Wilson *et al.*, 2008). In general, the digesters were 25-L high-density polyethylene conical-bottom tanks (Figure A.1). Mixing of the digester contents was achieved by recirculating of headspace gasses. The tanks were obtained from Hobby Beverage Equipment Company (Temecula, CA).

Construction of aerobic digesters was less standard than anaerobic digesters. One of two configurations was most commonly used:

1. A vented laboratory fermenter with a paddle mixer. Air was supplied from a breathing-quality air cylinder or an industrial oxygen blend (50% oxygen, 50% nitrogen). Anoxic/Aerobic cycling was attained either by a timer controlled solenoid valve or by a dissolved oxygen (DO) controller. A representative DO profile for this type of reactor is shown in Figure A.2.A.

2. An open-top Eckenfelder-type acrylic reactor where aeration served as the only mixing. Air was supplied via air stones from either a peristaltic or aquarium pump. Anoxic cycling was attained by slug feeding anaerobically digested biosolids under continued aeration. A representative oxidation-reduction potential (ORP) profile for this type of reactor is shown in Figure A.2.B.

Solids and nitrogen analyses were performed in accordance with *Standard Methods, 21st Ed.* (APHA, 1998), and biosolids headspace total volatile organic sulfur compound (tVOSC) measurements were performed as described previously (Wilson *et al.*, 2006).

Since the data from this study is, by nature, specific to sludge produced at DCWASA Blue Plains, it is important to note that the correlations and conclusions that were drawn from these data are sludge specific as well. For example, the addition of iron for phosphorous control at Blue Plains has an effect on both biosolids odor (due to the formation of FeS) and sludge

degradability (Park *et al.*, 2006). The fact that these digestion studies were performed on sludge from only one source, although a critically important fact for drawing comparisons between different processes, should be considered along with the data included in this paper.

A.7 RESULTS AND DISCUSSION

A.7.1 Volatile Solids Reduction

Two general assumptions generally guide process selection when attempting to enhance VSR within a sludge digestion process train: (1) the kinetics of solids destruction at high temperatures are faster than those at lower temperature, and (2) staged treatment enhances solids destruction by providing distinct ecological niches for microorganisms, as well as better representing plug-flow hydraulic characteristics. The selection of digestion technologies that were operated as part of this overall process evaluation were often driven by these two assumptions, although they did not prove to be universally true.

Average steady-state VSR data obtained from the operation of 39 distinct reactor studies are shown in Figure A.2. Several processes (e.g. MAD 15-day SRT, TAD 55°C 15-day SRT) were operated several times over the six years of this research program. Data from these replicates systems show the varied nature of laboratory sludge digestion experiments and the importance of such a large data set in order to draw meaningful process comparisons.

Single stage TAD appeared to be the overall poorest performing process, suggesting that the Arrhenius-based assumption of better performance with increased temperature while true, may not completely explain all the process mechanisms. Higher temperature thermophilic systems (TAD 58°C 15-day SRT) generally performed more poorly than lower temperature (TAD 49°C 15-day SRT) systems. This type of temperature dependence on VSR likely points to free ammonia inhibition (Sung and Liu, 2003) or temperature sensitivity of methanogenic communities as a cause for the poor performance of TAD (Inman *et al.*, 2005). An increase in temperature increases the free ammonia concentration in a process, thus resulting in greater inhibition of the acetoclastic methanogens in the thermophilic digesters. This inhibition results in an accumulation of volatile fatty acids in the digesters.

Anaerobic staged digestion systems, particularly TPAD, were among the best performers in terms of VSR. It appears that the free ammonia inhibition that caused poor performance of TAD in this study was relieved within the subsequent mesophilic stage.

Other enhanced anaerobic digestion systems (i.e. TH-MAD and AGAD) performed moderately well compared with other reactor trains. TH-MAD in particular performed better than 19 of 22 single-stage anaerobic digestion systems in this study, noting that two single stage MAD reactors that outperformed TH-MAD (15 or 20-day) operated at an SRT of 20 and 25 days. It was also noted that TH-MAD 20-day achieved a slightly higher VSR than TH-MAD 15-day (59.6% vs. 57.5%). AGAD was the median performer in terms of VSR. It was noted that the acid phase was successful in achieving sludge hydrolysis, producing total volatile fatty acid (VFA) concentrations of greater than 7 g/L. However, release of inorganic carbon during acid phase digestion maintained the pH of the digester at approximately 7.0. Because of this phenomenon, AGAD was not effective in creating separate ecological niches for fermentative/hydrolytic bacteria and methanogens, and may have limited its performance relative to TPAD.

Post aerobic digestion of anaerobically digested sludge nearly always resulted in a significant additional VSR. The application of an ANA-AER process train was equally effective for low VSR anaerobic systems (see TAD 55°C 7.5-day SRT vs. TAD-AER 55°C 7.5/3-day SRT) and higher VSR anaerobic systems (see TH-MAD 15-day SRT vs. TH-MAD-AER 15/6-day SRT) where post aerobic digestion provided an additional 12% and 11% VSR, respectively. Additional VSR data during aerobic digestion obtained from other process trains is listed in Table A.1. The consistent additional VSR provided by ANA-AER would likely result from a distinct "aerobically degradable fraction" of sludge solids that is not effectively degraded during anaerobic treatment (Novak *et al.*, 2003) or a lowering of substrate Ks that allows for better digestion of solids. Some research suggests that this aerobically degradable fraction may be related to exocellular polymeric substances produced by flocculent bacteria (Park *et al.*, 2006). For the Blue Plains AWTP solids, the maximum VS destruction appears to be approximately 70% regardless of treatment combinations employed. The remaining 30% likely is composed of non-degradable or recalcitrant organic debris that resist further digestion.

A.7.2 Biosolids Sulfur Odor Production

Biosolids odor research was originally limited to full-scale processes, and field-produced sludge cakes. Although embryonic or innovative technologies could be successfully operated in the lab, no method existed to replicate the effects of full-scale dewatering processes for the small sludge volumes produced at lab-scale. This limitation led to the development of a laboratory simulation method for biosolids produced from high solids centrifugation, as well as the quantification of sulfur based biosolids odor compound accumulation during storage (Glindemann *et al.*, 2006). Since these methylated sulfur compounds undergo fairly rapid interconversion between one another, biosolids headspace sulfur odor is often quantified as total volatile organic sulfur compounds (tVOSC) (Higgins *et al.*, 2006). Included in this measurement are methanethiol, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide. Hydrogen sulfide is not typically included in this measurement since its production may be strongly dependent on sulfate reduction in addition to VOSC cycling, and is largely sequestered as FeS in moderate to high iron sludges such as DCWASA's.

To our knowledge, a process based investigation of biosolids odor from a single sludge source has not been presented to date. While all 39 reactor studies included in Figure A.3 did not have associated biosolids headspace tVOSC data, sufficient data exists to make comparisons between single stage MAD, single stage TAD, ANA-AER processes, and TH-MAD (Figure A.4).

An often cited drawback of thermophilic processes is the resultant biosolids odor. This assumption is likely drawn from in-plant observations of nondewatered sludge, rather than biosolids. Kumar *et al*. (2006) found that the headspace tVOSC concentration within a thermophilic reactor was 11.5 ppmv as sulfur while a concurrently operated mesophilic reactor had a tVOSC concentration of 1.5 ppmv as sulfur. Such differences are largely driven by liquid/gas partitioning and the temperature dependence of Henry's law. The magnitude of either digester headspace concentration was significantly less than was observed for any biosolids sample however (Figure A.4). Digester headspace tVOSC is largely controlled by the active methanogenic biomass through the mechanisms described elsewhere (Chen *et al.*, 2004, Higgins *et al.*, 2006).

Thermophilic biosolids, rather than mesophilic, resulted in less accumulation of tVOSC, and TAD-AER and MAD-AER processes were interspersed between the single stage anaerobic systems. Biosolids odor reduction can be attributed to a combination of several mechanisms: (1) preferential degradation of VOSC precursors during anaerobic digestion, (2) preferential degradation of VOSC in dewatered biosolids, or (3) thermodynamic limitations on VOSC production from protein as a result of by product (ammonia, VFA, hydrogen) accumulation.

Preferential degradation of VOSC precursors is apparent by comparing MAD with MAD-AER. As discussed before, the aerobically degradable fraction of sludge seems to consist of exocellular polymers (e.g. proteins) which are the precursors for VOSC generation (Chen *et al.*, 2004, Higgins *et al.*, 2006). The loss of this material during aerobic digestion resulted in a lower odor biosolids cake. On the other hand, the degradation of VOSC in biosolids is interrupted by aerobic conditions, an effect observed in the increase in biosolids tVOSC between TAD and TAD-AER systems. Finally, processes with higher concentrations of residual VFA (including thermal hydrolytic pretreatment and TAD) produced relatively low odor biosolids. One could speculate that the presence of residual substrate for methanogenesis limits the production of VOSC in these biosolids. Additionally, the VOSC production may be limited by the thermodynamics of sulfur-amino acid degradation (Örlygsson *et al.*, 1995) due to the accumulation of the high concentrations of acetate, propionate, ammonium, and hydrogen gas (Equations A.1 and A.2).

$$\text{Cysteine} + 3H_2O \rightarrow \text{acetate} + NH_4^+ + HCO_3^- + H_2S + H_2$$

$$\Delta G^{o`} = -57.9 \text{ kJ/rxn} \qquad \text{(A.1)}$$

$$\text{Methionine} + 3H_2O \rightarrow \text{propionate} + NH_4^+ + HCO_3^- + CH_3SH + H_2$$

$$\Delta G^{o`} = +137.4 \text{ kJ/rxn} \qquad \text{(A.2)}$$

An additional finding during this study dealt with the correlation between solids destruction and biosolids odor. Evidence of the assumption that VSR and biosolids odor can be found in United States CFR 40 Part 503.32. In order to meet vector attraction (presumably due to biosolids odors) reduction criteria for biosolids land application, a benchmark of 38% VSR must be

achieved. It is clear from this study that, at any level of sludge stabilization, VSR is not a good indicator of biosolids odor reduction following high-solids centrifuge or like equipment. Rather, specific processes that tend to produce unfavorable environments for VOSC production (due to protein degradation) or favorable conditions for VOSC degradation (due to methanogenic activity) are effective in biosolids odor reduction.

A.7.3 Nitrogen (Ammonia) Removal

Anaerobically digested sludges, due in part to protein hydrolysis, are typically high in ammonia nitrogen. While the return of dewatering liquors from these processes may increase a treatment plant's nutrient load, the concentration of ammonia in a relatively small flow stream provides the opportunity for efficient nitrogen removal via sidestream processes. Sidestream removal of ammonia is often applied to dewatered centrate streams, however the potential for additional VSR during an ANA-AER process as described in this study was attractive in terms of meeting the *biosolids minimization* requirement set forth by DCWASA.

Ammonia removal rates by several ANA-AER processes are shown in Table A.1. Although removal of total nitrogen generally did not exceed 50-60% for any reactor configuration, much of the organic nitrogen was tied up in the particulate biomass, and would be sequestered within the biosolids upon dewatering. Ammonia removal rates (noting that oxidized nitrogen species were relatively low) were considered to be a good indicator of the performance of the ANA-AER process train in terms of DCWASA's requirement for plant-wide process compatibility.

In general, TAD-AER and TH-MAD-AER processes performed better in terms of nitrogen removal than MAD-AER processes. Although nitrate and nitrite concentrations were generally below our detection threshold of 5 mg/L as N, it is possible that the high VFA load from TH-MAD and TAD processes may have benefitted the aerobic reactors by providing a preferred carbon source for denitrification, thus improving the reactant/product balance for nitrification. In any event, the removal of greater than 95% of ammonia from the return centrate stream in combination with an additional 10% VSR is a significant benefit for biosolids management.

Other benefits of post-aerobic digestion include the degradation of estrogens (Fang *et al.*, 2007) and limiting the fugitive methane (a greenhouse gas) produced from biosolids cake storage.

A.8 CONCLUSION

The evaluation of numerous biosolids stabilization process trains by DCWASA has allowed us to reliably evaluate several processes against each other, as well as to test several long-held assumptions relating VSR and biosolids odor production to process configuration. In the end, one particular process was able to best meet the four biosolids management program goals set by DCWASA, *TH-MAD-AER 15&20/6-day SRT*. This process train achieved 69% VSR, 95% ammonia removal from the centrate stream, and low biosolids VOSC levels. In addition, considering the recent questions that have been posed regarding the reactivation and regrowth of indicator organisms following thermophilic sludge digestion and high-shear dewatering (Higgins *et al.*, 2007), the extreme conditions of thermal hydrolytic pretreatment suggest that TH-MAD may best be able to reliably meet Class A requirements. The application of this process train at DCWASA Blue Plains is under continuing review.

A.9 ACKNOWLEDGMENTS

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A.11 TABLES

Table A.1

Ammonia removal characteristics of selected ANA-AER processes

Process Train	Influent VFA mg/L as Acetate	Influent NH_4^+ mg/L as N	NH_4^+-N Removal [1] %	Additional AER VSR %
TH-MAD-AER 15/6-day [2]	2300	1900	95	11
TAD-AER 55°C 15/3-day [3]			86	14
TAD-AER 55°C 15/6-day	6000	500	84	13
TAD-AER 55°C 15/9-day			96	NA
MAD-AER 15/3-day [3]			66	10
MAD-AER 15/6-day	360	700	61	12
MAD-AER 15/9-day			76	16

[1] Nitrate and nitrite were able to be maintained below 10 mg/L as N for each reactor at steady-state, with the exception of MAD-AER 15/3-day which had a nitrite concentration of 38 mg/L as N.

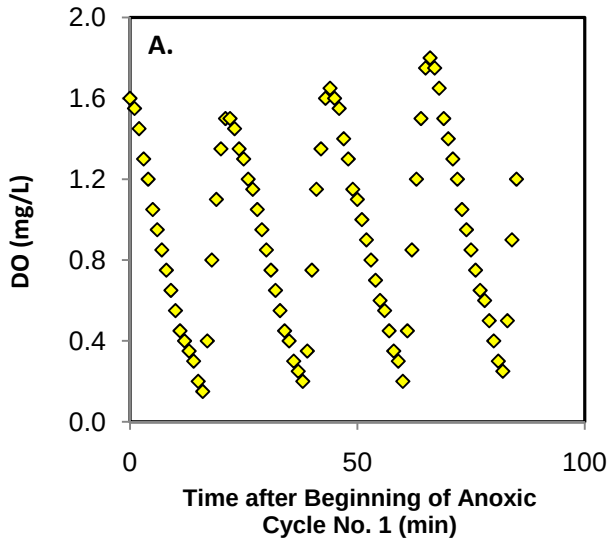
[2] Operated with short aerobic/anoxic cycles as shown in Figure A.2.A.

[3] Operated with diurnal aerobic anoxic cycle with anaerobic slug load as shown in Figure A.2.B.

A.12 FIGURES

Figure A.1

Typical anaerobic digester construction



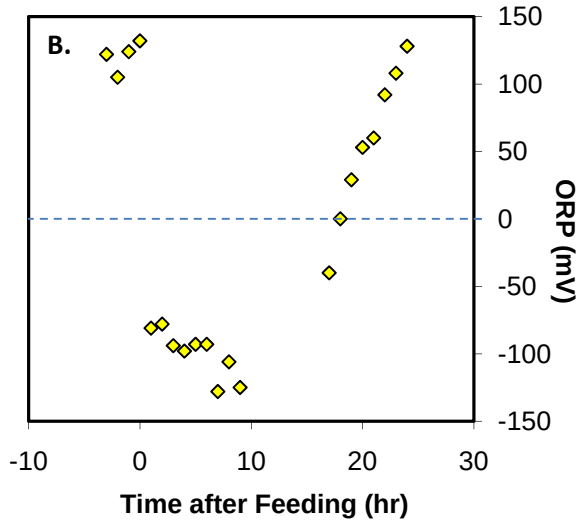


Figure A.2

Methods for aerobic/anoxic cycling during post-aerobic digestion: (a) Low and high DO setpoints provided relatively short AER/ANX cycles, and (b) constant aeration and slug-feeding of anaerobically digester sludge resulted in a diurnal AER/ANX cycle. Both methods were able to be optimized for nitrogen removal by nitrification/denitrification process.

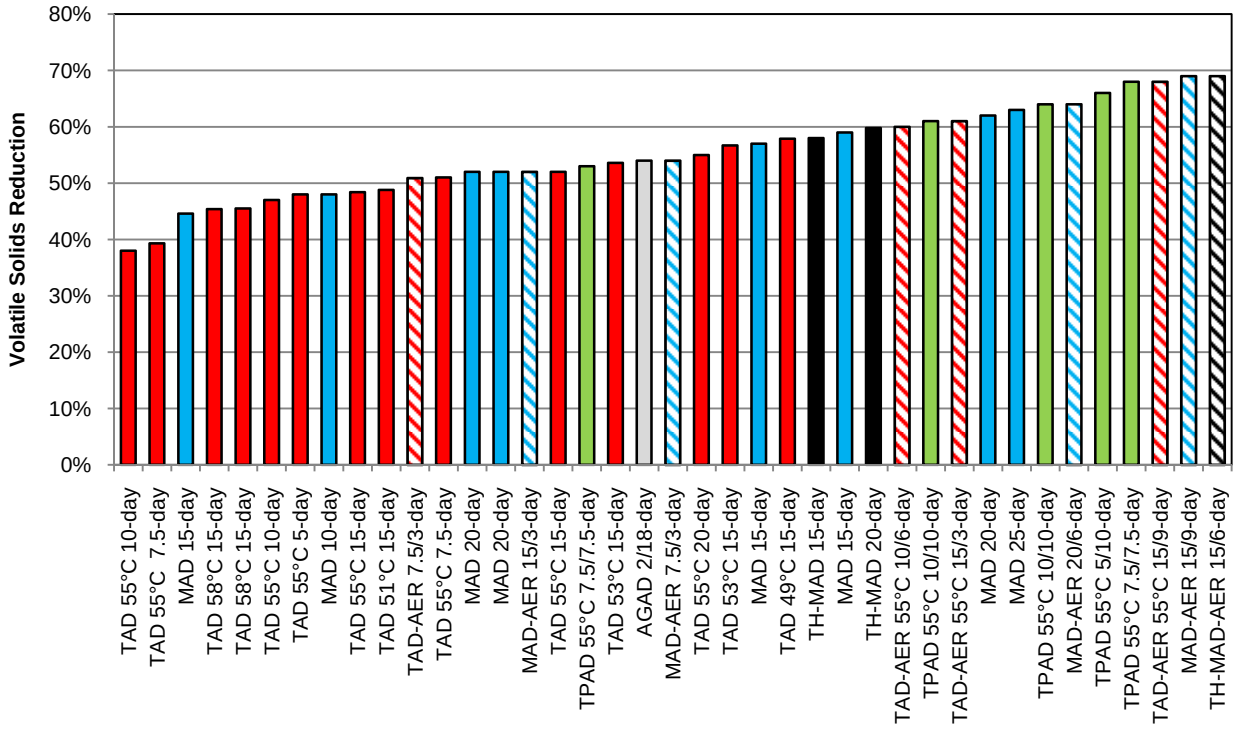


Figure A.3

Steady-state VSR of laboratory-scaled sludge treatment trains. Bar colors are as follows: Red, TAD; Blue, MAD; Green, TPAD; Grey, AGAD; Black, TH-MAD. White striped bars represent post-aerobic digestion of TAD, MAD, and TH-MAD.

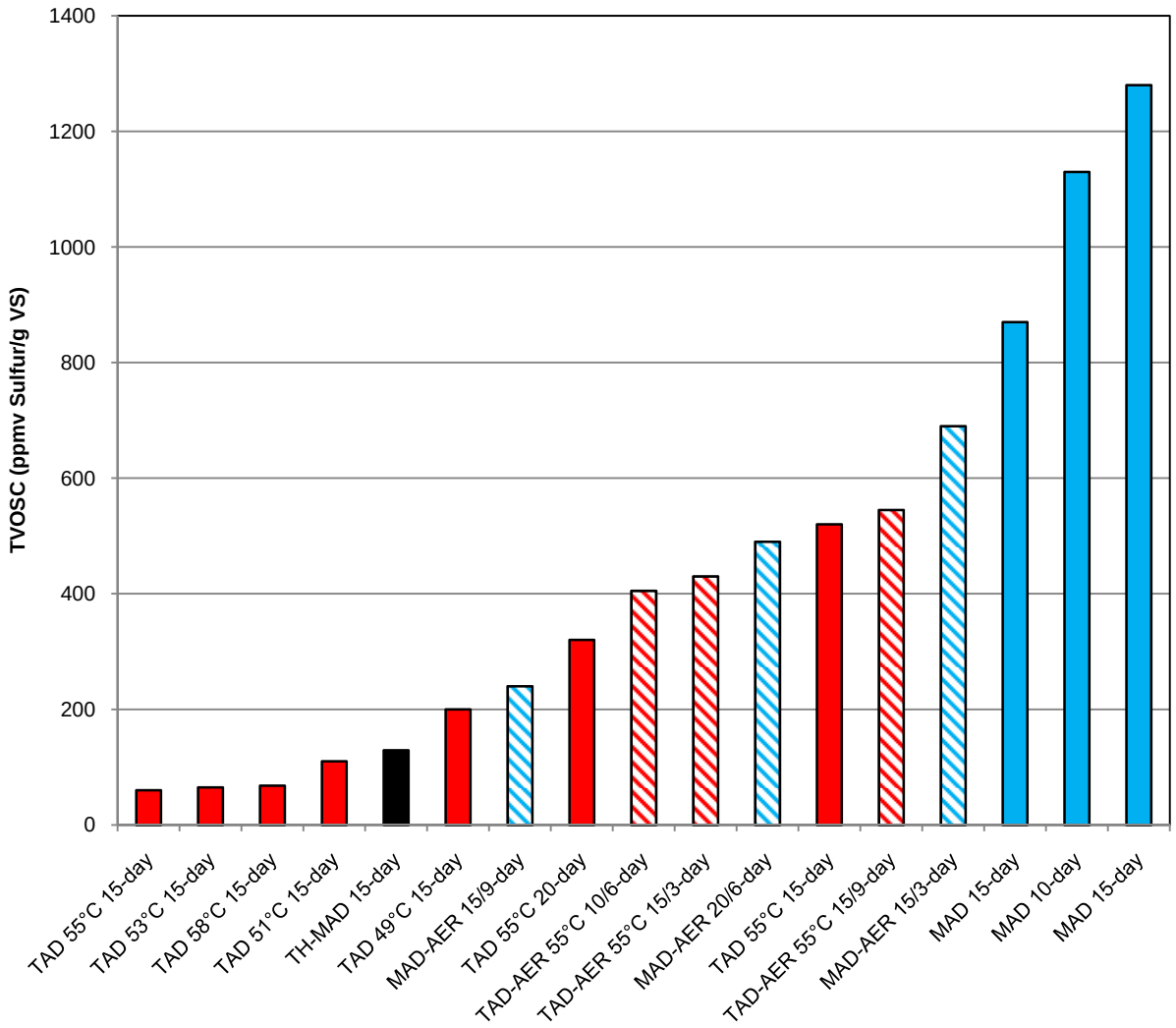


Figure A.4

Peak tVOSC values from biosolids incubation (summation of methanethiol, dimethyl sulfide, and dimethyl disulfide). Bar colors as described for Figure A.3.

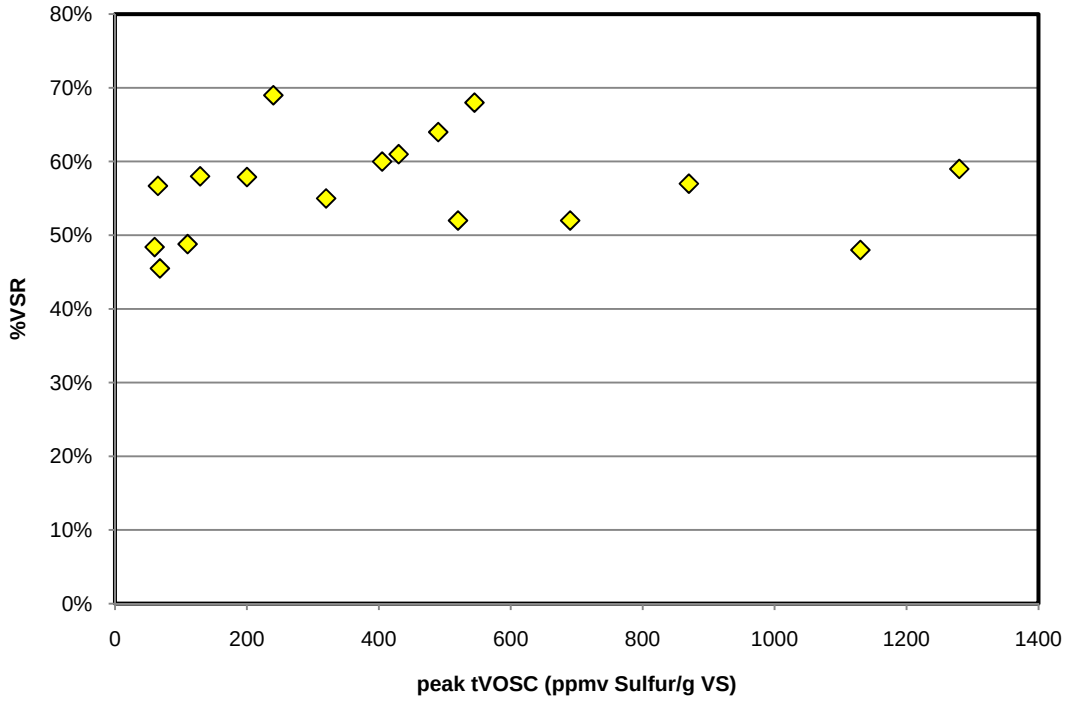


Figure A.5

Correlation between tVOSC and VSR.

APPENDIX B

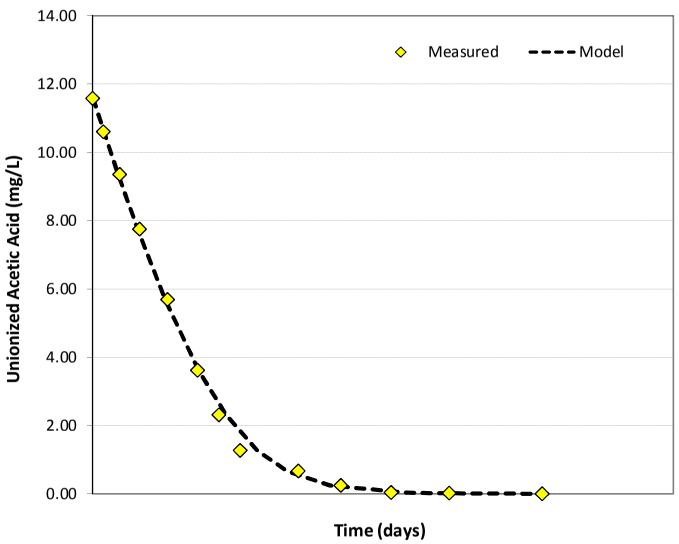
Y (g/g) =	0.040	(constant)
r (μ_{MAX}·X) =	0.125	fitting parameter (varies)
K_S (ppm) =	5.322	fitting parameter (varies)
C_{min} (ppm) =	2.830	(constant)
C_{LO} (ppm) =	11.626	fitting parameter (varies)

(ppm·day^{-1})

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00								-0.82
1.13	0.08								-0.74
4.98	0.36								-0.47
11.54	0.82	14.41	11.58	0.02	1	42.86	0	0.00	0.00
17.97	1.28	13.43	10.60	0.48	1	57.80	0	0.00	0.46
27.83	1.99	12.18	9.35	1.10	1	355.51	0	0.00	1.16
39.78	2.84	10.58	7.75	1.93	1	633.06	0	0.00	2.02
56.70	4.05	8.52	5.69	3.12	1	984.91	0	0.00	3.23
74.78	5.34	6.45	3.62	4.55	1	119.83	0	0.00	4.52
87.62	6.26	5.14	2.31	5.74	0	0.00	1	7830.04	5.43
100.45	7.18	4.10	1.27	7.09	0	0.00	1	47105.57	6.35
135.45	9.68	3.50	0.67	8.37	0	0.00	1	19732.42	8.85
161.12	11.51	3.08	0.25	10.21	0	0.00	1	19296.84	10.68
191.45	13.68	2.87	0.04	13.45	0	0.00	1	31364.58	12.85
226.45	16.18	2.85	0.02	14.50	0	0.00	1	61988.87	15.35
282.45	20.18	2.83	0.00	19.68	0	0.00	1	9230.91	19.35

Σ = 2194 + 196549

= 198743

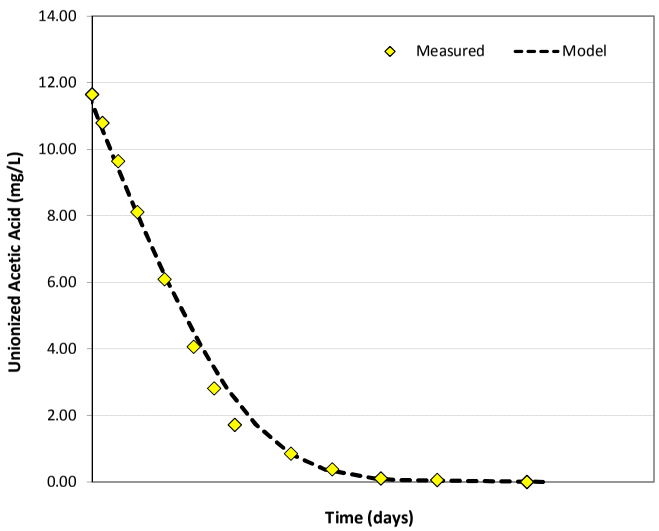


THD 220 ppm TAN

Y (g/g) =	0.040	(constant)
r (μ_{MAX}·X) =	0.093	fitting parameter (varies)
K_S (ppm) =	3.820	fitting parameter (varies)
C_{min} (ppm) =	2.807	(constant)
C_{LO} (ppm) =	11.375	fitting parameter (varies)

(ppm·day^{-1})

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00								-0.82
1.13	0.08								-0.74
4.98	0.36								-0.47
11.54	0.82	14.45	11.64	-0.15	1	2038.86	0	0.00	0.00
17.97	1.28	13.59	10.79	0.34	1	1193.06	0	0.00	0.46
27.83	1.99	12.44	9.64	1.02	1	1654.84	0	0.00	1.16
39.78	2.84	10.92	8.11	1.97	1	220.10	0	0.00	2.02
56.70	4.05	8.90	6.09	3.31	1	610.41	0	0.00	3.23
74.78	5.34	6.87	4.06	4.85	1	9751.41	0	0.00	4.52
87.62	6.26	5.62	2.81	6.00	1	27591.83	0	0.00	5.43
100.45	7.18	4.53	1.72	7.29	0	0.00	1	75688.86	6.35
135.45	9.68	3.65	0.85	8.83	0	0.00	1	44.06	8.85
161.12	11.51	3.19	0.38	10.35	0	0.00	1	9580.94	10.68
191.45	13.68	2.91	0.11	12.57	0	0.00	1	6679.02	12.85
226.45	16.18	2.87	0.06	13.56	0	0.00	1	276975.28	15.35
282.45	20.18	2.81	0.00	20.31	0	0.00	1	80105.05	19.35
					Σ =	**43061**	**+**	**449073**	
							=	**492134**	

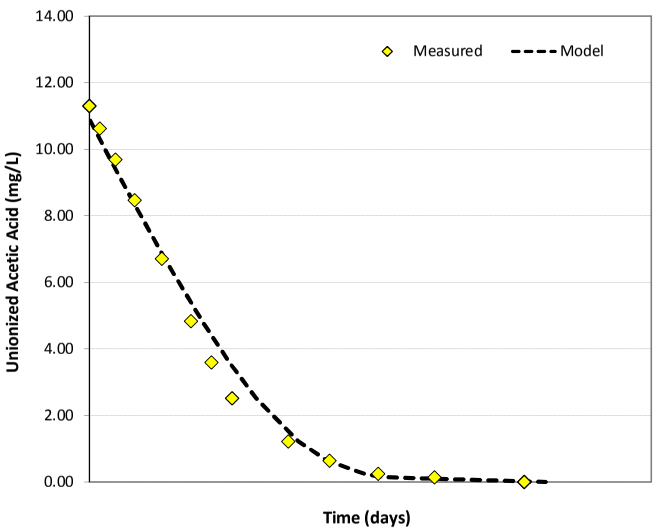


THD 720 ppm TAN

Y (g/g) =	0.040	(constant)
r (μ_{MAX}·X) =	0.063	fitting parameter (varies)
K_S (ppm) =	2.276	fitting parameter (varies)
C_{min} (ppm) =	3.409	(constant)
C_{LO} (ppm) =	10.911	fitting parameter (varies)

(ppm·day^{-1})

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00				0		0		-0.82
1.13	0.08				0		0		-0.74
4.98	0.36				0		0		-0.47
11.54	0.82	14.71	11.30	-0.30	1	7625.92	0	0.00	0.00
17.97	1.28	14.03	10.62	0.22	1	4832.46	0	0.00	0.46
27.83	1.99	13.10	9.69	0.94	1	4125.13	0	0.00	1.16
39.78	2.84	11.88	8.47	1.91	1	1026.77	0	0.00	2.02
56.70	4.05	10.12	6.71	3.36	1	1610.66	0	0.00	3.23
74.78	5.34	8.24	4.83	5.02	1	21941.64	0	0.00	4.52
87.62	6.26	7.00	3.59	6.23	0	0.00	1	55067.47	5.43
100.45	7.18	5.93	2.52	7.42	0	0.00	1	99627.96	6.35
135.45	9.68	4.63	1.22	9.29	0	0.00	1	16876.23	8.85
161.12	11.51	4.05	0.64	10.58	0	0.00	1	903.11	10.68
191.45	13.68	3.66	0.25	12.21	0	0.00	1	35633.37	12.85
226.45	16.18	3.55	0.14	13.05	0	0.00	1	455993.43	15.35
282.45	20.18	3.41	0.00	20.30	0	0.00	1	77655.68	19.35
					Σ =	**41163**	**+**	**741757**	
							=	**782920**	

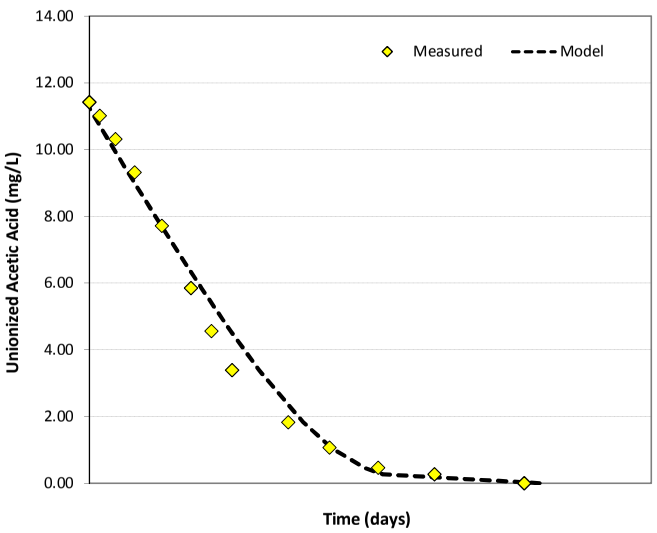


THD 1220 ppm TAN

Y (g/g) =	0.040	(constant)
r (μ_{MAX}·X) =	0.052	fitting parameter (varies)
K_S (ppm) =	1.565	fitting parameter (varies)
C_{min} (ppm) =	3.451	(constant)
C_{LO} (ppm) =	11.241	fitting parameter (varies)

(ppm·day^{-1})

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00				0		0		-0.82
1.13	0.08				0		0		-0.74
4.98	0.36				0		0		-0.47
11.54	0.82	14.87	11.42	-0.16	1	2164.08	0	0.00	0.00
17.97	1.28	14.47	11.01	0.20	1	5761.81	0	0.00	0.46
27.83	1.99	13.76	10.31	0.83	1	9834.16	0	0.00	1.16
39.78	2.84	12.77	9.31	1.72	1	7412.64	0	0.00	2.02
56.70	4.05	11.16	7.71	3.20	1	71.19	0	0.00	3.23
74.78	5.34	9.30	5.85	4.98	1	18566.34	0	0.00	4.52
87.62	6.26	8.01	4.56	6.29	0	0.00	1	63255.00	5.43
100.45	7.18	6.84	3.39	7.55	0	0.00	1	124352.28	6.35
135.45	9.68	5.28	1.83	9.52	0	0.00	1	38188.90	8.85
161.12	11.51	4.52	1.07	10.76	0	0.00	1	527.41	10.68
191.45	13.68	3.92	0.46	12.24	0	0.00	1	32037.88	12.85
226.45	16.18	3.72	0.27	13.06	0	0.00	1	455203.49	15.35
282.45	20.18	3.45	0.00	20.07	0	0.00	1	44529.46	19.35
					Σ =	**43810**	**+**	**758094**	
							=	**801905**	

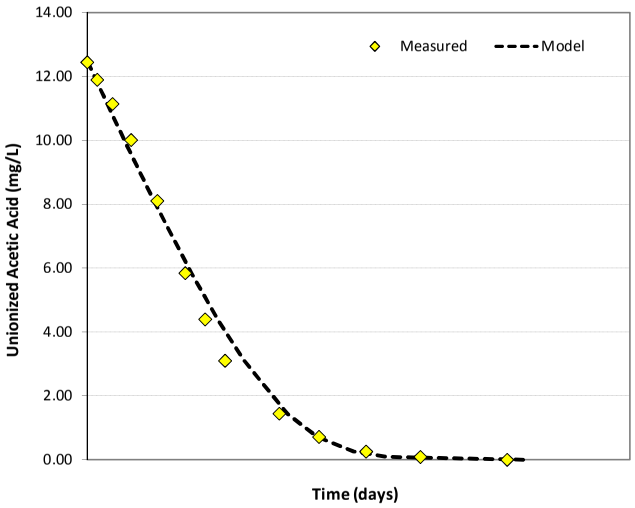


THD 1720 ppm TAN

Y (g/g) =	0.040	(constant)
r (μ_{MAX}·X) =	0.071	fitting parameter (varies)
K_S (ppm) =	2.460	fitting parameter (varies)
C_{min} (ppm) =	2.483	(constant)
C_{LO} (ppm) =	12.498	fitting parameter (varies)

(ppm·day^{-1})

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00				0		0		-0.82
1.13	0.08				0		0		-0.74
4.98	0.36				0		0		-0.47
11.54	0.82	14.93	12.44	0.04	1	110.92	0	0.00	0.00
17.97	1.28	14.38	11.90	0.41	1	232.15	0	0.00	0.46
27.83	1.99	13.62	11.14	0.92	1	4919.68	0	0.00	1.16
39.78	2.84	12.49	10.01	1.71	1	8209.88	0	0.00	2.02
56.70	4.05	10.58	8.10	3.08	1	1904.55	0	0.00	3.23
74.78	5.34	8.33	5.84	4.80	1	7047.53	0	0.00	4.52
87.62	6.26	6.88	4.40	6.01	0	0.00	1	28963.89	5.43
100.45	7.18	5.58	3.10	7.23	0	0.00	1	66089.99	6.35
135.45	9.68	3.93	1.45	9.22	0	0.00	1	11633.69	8.85
161.12	11.51	3.20	0.72	10.60	0	0.00	1	660.17	10.68
191.45	13.68	2.74	0.26	12.28	0	0.00	1	28402.24	12.85
226.45	16.18	2.57	0.09	13.85	0	0.00	1	194590.48	15.35
282.45	20.18	2.48	0.00	20.12	0	0.00	1	50758.95	19.35
					Σ =	**22425**	**+**	**381099**	
							=	**403524**	

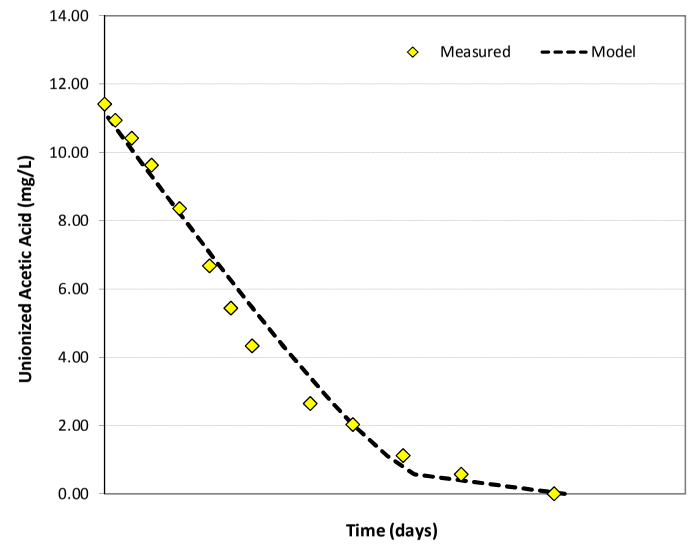


THD 2220 ppm TAN

Y (g/g) =	0.040	(constant)
r (μ_{MAX}·X) =	0.040	fitting parameter (varies)
K_S (ppm) =	0.933	fitting parameter (varies)
C_{min} (ppm) =	3.519	(constant)
C_{LO} (ppm) =	11.156	fitting parameter (varies)

(ppm·day^{-1})

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00				0		0		-0.82
1.13	0.08				0		0		-0.74
4.98	0.36				0		0		-0.47
11.54	0.82	14.94	11.42	-0.28	1	6843.46	0	0.00	0.00
17.97	1.28	14.46	10.95	0.23	1	4607.71	0	0.00	0.46
27.83	1.99	13.94	10.42	0.80	1	11464.71	0	0.00	1.16
39.78	2.84	13.15	9.63	1.66	1	11052.13	0	0.00	2.02
56.70	4.05	11.88	8.36	3.06	1	2366.00	0	0.00	3.23
74.78	5.34	10.20	6.68	4.94	1	15584.40	0	0.00	4.52
87.62	6.26	8.96	5.44	6.37	0	0.00	1	75507.20	5.43
100.45	7.18	7.85	4.33	7.69	0	0.00	1	153892.18	6.35
135.45	9.68	6.16	2.64	9.83	0	0.00	1	82710.47	8.85
161.12	11.51	5.55	2.03	10.69	0	0.00	1	2.18	10.68
191.45	13.68	4.64	1.12	12.15	0	0.00	1	42353.19	12.85
226.45	16.18	4.09	0.57	13.32	0	0.00	1	355016.94	15.35
282.45	20.18	3.52	0.00	19.80	0	0.00	1	17456.39	19.35
					Σ =	**51918**	**+**	**726939**	
							=	**778857**	

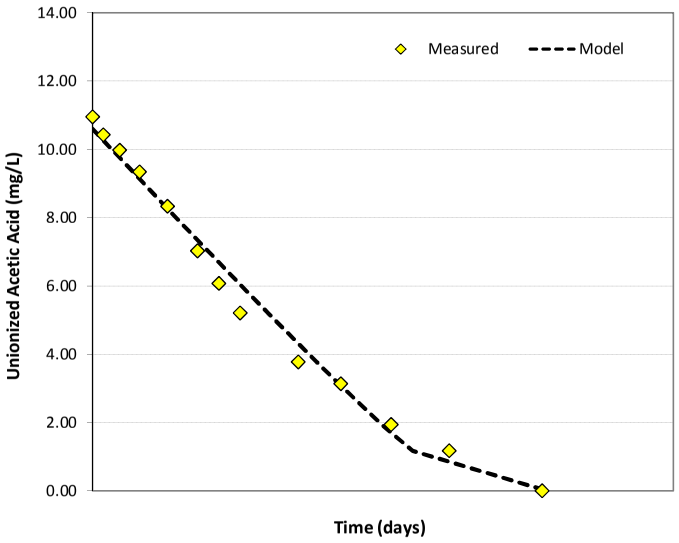


THD 2720 ppm TAN

Y (g/g) =	0.040	(constant)
r (μ_{MAX}·X) =	0.030	fitting parameter (varies)
K_S (ppm) =	0.453	fitting parameter (varies)
C_{min} (ppm) =	4.062	(constant)
C_{LO} (ppm) =	10.591	fitting parameter (varies)

(ppm·day^{-1})

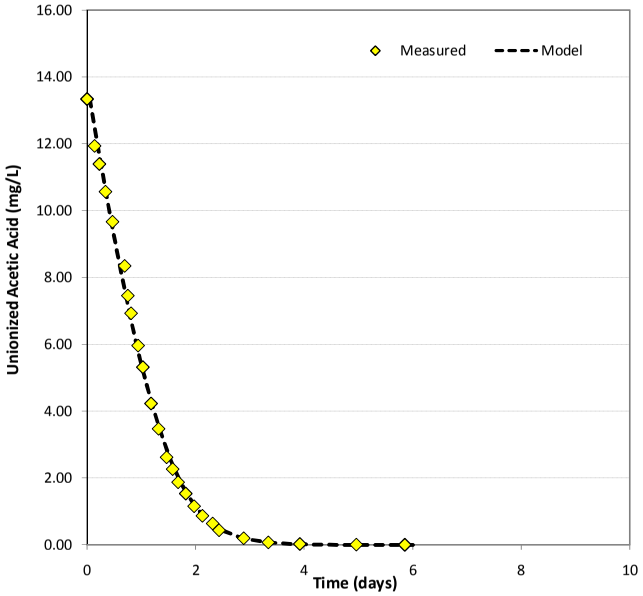
Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00				0		0		-0.82
1.13	0.08				0		0		-0.74
4.98	0.36				0		0		-0.47
11.54	0.82	15.02	10.96	-0.50	1	21799.10	0	0.00	0.00
17.97	1.28	14.49	10.43	0.22	1	4791.81	0	0.00	0.46
27.83	1.99	14.04	9.98	0.84	1	8763.91	0	0.00	1.16
39.78	2.84	13.41	9.34	1.73	1	7356.92	0	0.00	2.02
56.70	4.05	12.40	8.33	3.13	1	813.81	0	0.00	3.23
74.78	5.34	11.09	7.03	4.96	1	16697.87	0	0.00	4.52
87.62	6.26	10.14	6.08	6.31	0	0.00	1	65762.74	5.43
100.45	7.18	9.27	5.21	7.54	0	0.00	1	123171.78	6.35
135.45	9.68	7.84	3.78	9.64	0	0.00	1	53167.89	8.85
161.12	11.51	7.20	3.14	10.59	0	0.00	1	757.03	10.68
191.45	13.68	6.00	1.94	12.46	0	0.00	1	13247.15	12.85
226.45	16.18	5.24	1.17	13.78	0	0.00	1	214224.17	15.35
282.45	20.18	4.06	0.00	19.56	0	0.00	1	3967.12	19.35
					Σ =	**60223**	**+**	**474298**	
							=	**534521**	



THD 3220 ppm TAN

Y (g/g) =	0.040	(constant)	
r (μ_{MAX}·X) =	0.593	fitting parameter (varies)	(ppm·day^{-1})
K_S (ppm) =	6.810	fitting parameter (varies)	
C_{min} (ppm) =	0.057	(constant)	
C_{LO} (ppm) =	13.791	fitting parameter (varies)	

Experimental Data				Predicted Data:		zero order		first order	
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00	13.40	13.34	0.05	1	178.04	0	0.00	-1.03
1.96	0.14	12.00	11.94	0.19	1	223.08	0	0.00	-0.89
3.21	0.23	11.46	11.40	0.25	1	34.76	0	0.00	-0.80
4.76	0.34	10.63	10.57	0.34	1	0.05	0	0.00	-0.69
6.58	0.47	9.73	9.67	0.44	1	73.27	0	0.00	-0.56
9.72	0.69	8.40	8.35	0.60	1	792.27	0	0.00	-0.34
10.50	0.75	7.51	7.45	0.71	1	134.74	0	0.00	-0.28
11.34	0.81	6.99	6.93	0.78	1	83.90	0	0.00	-0.22
13.16	0.94	6.02	5.96	0.91	1	60.40	0	0.00	-0.09
14.43	1.03	5.37	5.32	1.01	1	38.09	0	0.00	0.00
16.52	1.18	4.29	4.23	1.19	1	5.23	0	0.00	0.15
18.48	1.32	3.53	3.47	1.33	1	8.65	0	0.00	0.29
20.51	1.47	2.68	2.62	1.52	1	227.87	0	0.00	0.43
22.12	1.58	2.32	2.26	1.61	1	70.12	0	0.00	0.55
23.52	1.68	1.93	1.87	1.72	1	150.41	0	0.00	0.65
25.48	1.82	1.59	1.53	1.84	1	24.14	0	0.00	0.79
27.61	1.97	1.21	1.15	1.99	1	39.00	0	0.00	0.94
29.75	2.13	0.92	0.86	2.15	0	0.00	1	37.84	1.09
32.44	2.32	0.69	0.63	2.30	0	0.00	1	14.95	1.29
34.03	2.43	0.49	0.43	2.49	0	0.00	1	319.17	1.40
40.42	2.89	0.25	0.19	2.88	0	0.00	1	5.48	1.86
46.76	3.34	0.13	0.07	3.33	0	0.00	1	4.15	2.31
54.92	3.92	0.08	0.02	3.86	0	0.00	1	298.54	2.89
69.51	4.97	0.06	0.00	4.77	0	0.00	1	3172.65	3.93
82.01	5.86	0.06	0.00	6.00	0	0.00	1	1863.92	4.83

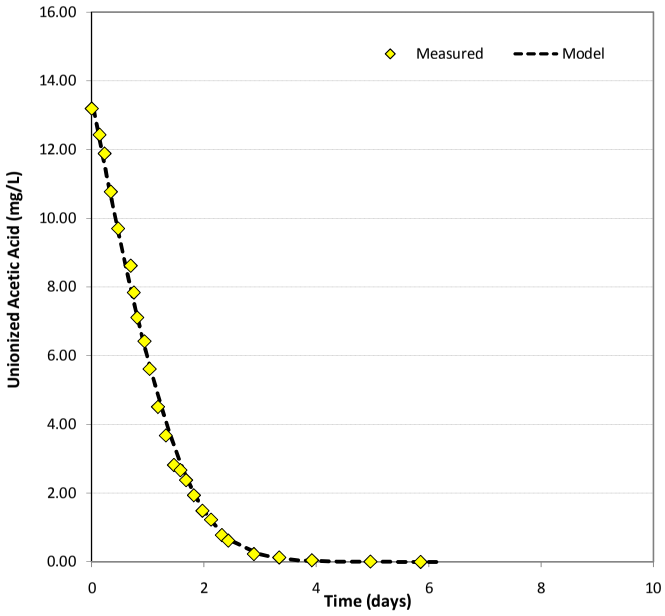


Σ =	**2144**	**+**	**5717**
		=	**7861**

MAD 500 ppm TAN

Y (g/g) =	0.040	(constant)	
r (μ_{MAX}·X) =	0.492	fitting parameter (varies)	(ppm·day^{-1})
K_S (ppm) =	5.738	fitting parameter (varies)	
C_{min} (ppm) =	0.210	(constant)	
C_{LO} (ppm) =	13.504	fitting parameter (varies)	

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00	13.40	13.19	0.04	1	114.51	0	0.00	-1.03
1.96	0.14	12.64	12.43	0.13	1	17.36	0	0.00	-0.89
3.21	0.23	12.09	11.88	0.19	1	123.11	0	0.00	-0.80
4.76	0.34	10.98	10.77	0.33	1	13.42	0	0.00	-0.69
6.58	0.47	9.91	9.70	0.46	1	3.92	0	0.00	-0.56
9.72	0.69	8.83	8.62	0.61	1	668.37	0	0.00	-0.34
10.50	0.75	8.05	7.84	0.71	1	114.21	0	0.00	-0.28
11.34	0.81	7.32	7.11	0.82	1	6.28	0	0.00	-0.22
13.16	0.94	6.63	6.42	0.92	1	27.43	0	0.00	-0.09
14.43	1.03	5.83	5.62	1.05	1	30.82	0	0.00	0.00
16.52	1.18	4.72	4.51	1.24	1	332.14	0	0.00	0.15
18.48	1.32	3.89	3.68	1.40	1	613.42	0	0.00	0.29
20.51	1.47	3.03	2.82	1.60	1	1533.57	0	0.00	0.43
22.12	1.58	2.88	2.67	1.64	1	269.96	0	0.00	0.55
23.52	1.68	2.59	2.38	1.71	1	94.44	0	0.00	0.65
25.48	1.82	2.15	1.94	1.84	1	50.21	0	0.00	0.79
27.61	1.97	1.70	1.49	2.00	0	0.00	1	86.81	0.94
29.75	2.13	1.44	1.23	2.11	0	0.00	1	10.05	1.09
32.44	2.32	0.99	0.78	2.36	0	0.00	1	183.51	1.29
34.03	2.43	0.83	0.62	2.48	0	0.00	1	234.42	1.40
40.42	2.89	0.44	0.23	2.98	0	0.00	1	697.17	1.86
46.76	3.34	0.34	0.13	3.25	0	0.00	1	690.30	2.31
54.92	3.92	0.26	0.05	3.70	0	0.00	1	4245.08	2.89
69.51	4.97	0.22	0.01	4.45	0	0.00	1	23198.79	3.93
82.01	5.86	0.21	0.00	6.24	0	0.00	1	12292.10	4.83

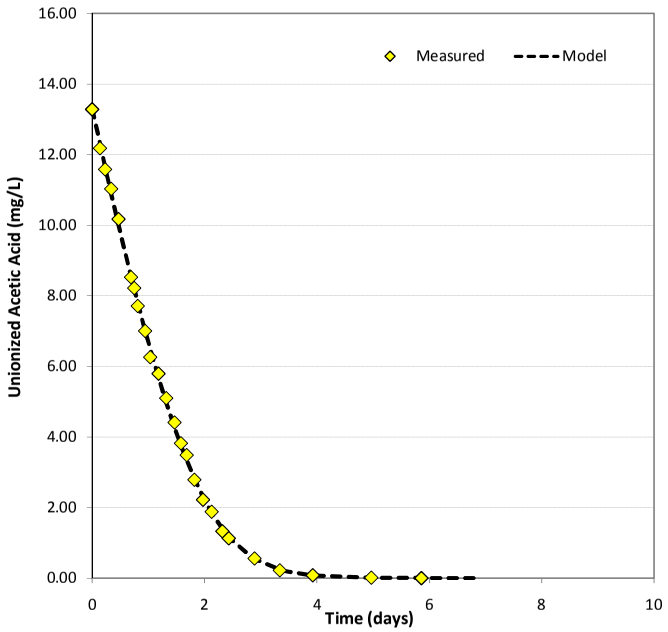


Σ =	**4013**	**+**	**41638**
		=	**45651**

MAD 1250 ppm TAN

Y (g/g) =	0.040	(constant)	
r (μ_{MAX}·X) =	0.455	fitting parameter (varies)	(ppm·day^{-1})
K_S (ppm) =	5.221	fitting parameter (varies)	
C_{min} (ppm) =	0.119	(constant)	
C_{LO} (ppm) =	13.323	fitting parameter (varies)	

Experimental Data				Predicted Data:		zero order		first order	
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00	13.40	13.28	0.01	1	2.77	0	0.00	-1.03
1.96	0.14	12.30	12.18	0.16	1	25.96	0	0.00	-0.89
3.21	0.23	11.70	11.58	0.24	1	14.11	0	0.00	-0.80
4.76	0.34	11.15	11.03	0.32	1	33.24	0	0.00	-0.69
6.58	0.47	10.29	10.17	0.45	1	50.19	0	0.00	-0.56
9.72	0.69	8.64	8.53	0.70	1	0.74	0	0.00	-0.34
10.50	0.75	8.34	8.22	0.75	1	1.62	0	0.00	-0.28
11.34	0.81	7.83	7.71	0.83	1	27.19	0	0.00	-0.22
13.16	0.94	7.12	7.00	0.95	1	3.60	0	0.00	-0.09
14.43	1.03	6.38	6.26	1.08	1	170.85	0	0.00	0.00
16.52	1.18	5.91	5.79	1.16	1	30.42	0	0.00	0.15
18.48	1.32	5.22	5.10	1.29	1	58.31	0	0.00	0.29
20.51	1.47	4.53	4.41	1.44	1	76.46	0	0.00	0.43
22.12	1.58	3.94	3.82	1.57	1	14.31	0	0.00	0.55
23.52	1.68	3.61	3.49	1.65	1	102.63	0	0.00	0.65
25.48	1.82	2.91	2.79	1.83	1	4.81	0	0.00	0.79
27.61	1.97	2.34	2.22	2.00	0	0.00	1	66.69	0.94
29.75	2.13	2.00	1.88	2.12	0	0.00	1	4.00	1.09
32.44	2.32	1.45	1.33	2.35	0	0.00	1	83.67	1.29
34.03	2.43	1.25	1.13	2.45	0	0.00	1	33.77	1.40
40.42	2.89	0.68	0.56	2.86	0	0.00	1	44.59	1.86
46.76	3.34	0.34	0.22	3.37	0	0.00	1	92.46	2.31
54.92	3.92	0.20	0.08	3.90	0	0.00	1	54.66	2.89
69.51	4.97	0.13	0.01	4.96	0	0.00	1	0.43	3.93
82.01	5.86	0.12	0.00	6.92	0	0.00	1	97589.15	4.83



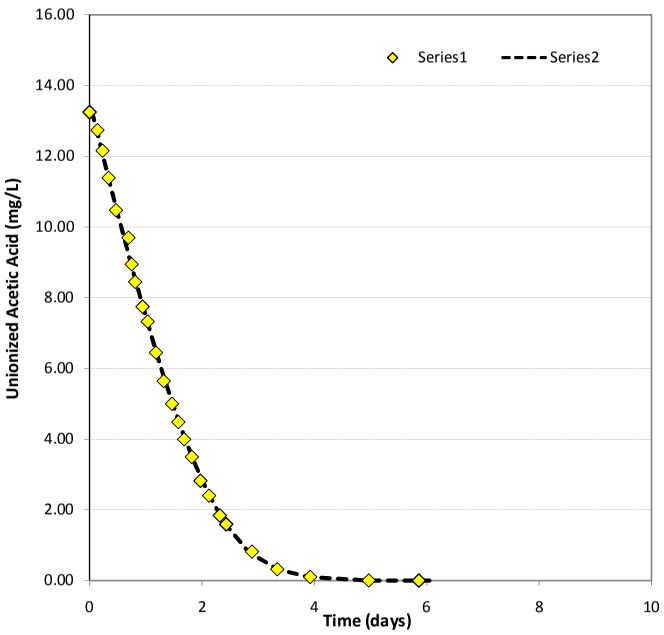
Σ =	617	+	380
		=	997

MAD 2000 ppm TAN

Y (g/g) =	0.040	(constant)
r (μ_{MAX}·X) =	0.377	fitting parameter (varies)
K_S (ppm) =	4.013	fitting parameter (varies)
C_{min} (ppm) =	0.158	(constant)
C_{LO} (ppm) =	13.534	fitting parameter (varies)

(ppm·day^{-1})

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00	13.40	13.24	0.04	1	172.73	0	0.00	-1.03
1.96	0.14	12.89	12.73	0.12	1	23.54	0	0.00	-0.89
3.21	0.23	12.31	12.16	0.21	1	20.64	0	0.00	-0.80
4.76	0.34	11.54	11.38	0.34	1	1.47	0	0.00	-0.69
6.58	0.47	10.63	10.47	0.48	1	13.99	0	0.00	-0.56
9.72	0.69	9.85	9.69	0.61	1	586.77	0	0.00	-0.34
10.50	0.75	9.10	8.94	0.74	1	12.30	0	0.00	-0.28
11.34	0.81	8.60	8.44	0.82	1	17.71	0	0.00	-0.22
13.16	0.94	7.90	7.74	0.95	1	5.41	0	0.00	-0.09
14.43	1.03	7.48	7.32	1.02	1	4.60	0	0.00	0.00
16.52	1.18	6.60	6.44	1.19	1	6.05	0	0.00	0.15
18.48	1.32	5.80	5.64	1.35	1	56.46	0	0.00	0.29
20.51	1.47	5.16	5.00	1.48	1	16.31	0	0.00	0.43
22.12	1.58	4.64	4.48	1.59	1	11.32	0	0.00	0.55
23.52	1.68	4.15	3.99	1.70	1	50.15	0	0.00	0.65
25.48	1.82	3.65	3.49	1.83	1	3.61	0	0.00	0.79
27.61	1.97	2.98	2.82	2.01	0	0.00	1	102.25	0.94
29.75	2.13	2.56	2.40	2.13	0	0.00	1	4.57	1.09
32.44	2.32	2.00	1.84	2.32	0	0.00	1	4.29	1.29
34.03	2.43	1.75	1.59	2.42	0	0.00	1	6.08	1.40
40.42	2.89	0.98	0.82	2.83	0	0.00	1	316.82	1.86
46.76	3.34	0.48	0.32	3.33	0	0.00	1	10.32	2.31
54.92	3.92	0.26	0.10	3.90	0	0.00	1	50.89	2.89
69.51	4.97	0.16	0.00	4.99	0	0.00	1	60.24	3.93
82.01	5.86	0.16	0.00	6.13	0	0.00	1	6548.21	4.83



Σ =	1003	+	555
		=	1559

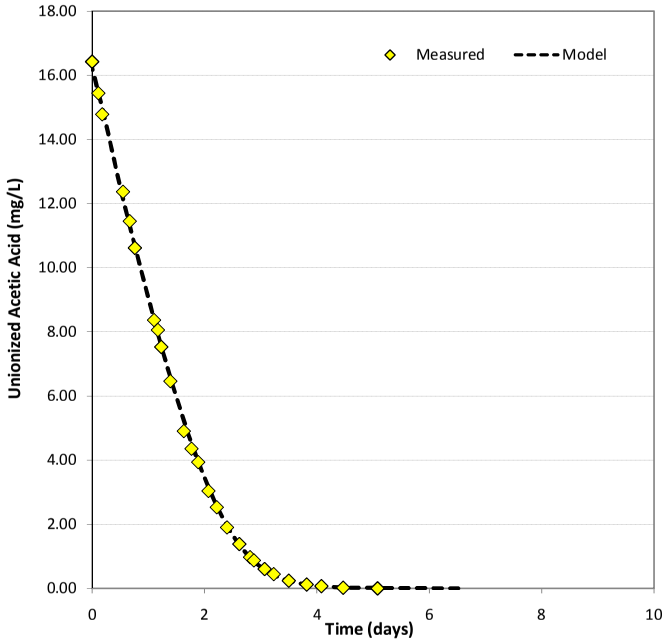
MAD 2750 ppm TAN
Replicate 1

Y (g/g) =	0.040	(constant)
r (μ_{MAX}·X) =	0.384	fitting parameter (varies)
K_S (ppm) =	4.139	fitting parameter (varies)
C_{min} (ppm) =	0.240	(constant)
C_{LO} (ppm) =	16.229	fitting parameter (varies)

(ppm·day^{-1})

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00	16.67	16.43	-0.03	1	73.55	0	0.00	-0.55
1.54	0.11	15.69	15.45	0.11	1	1.17	0	0.00	-0.44
2.52	0.18	15.03	14.79	0.21	1	84.01	0	0.00	-0.37
7.70	0.55	12.61	12.37	0.52	1	85.96	0	0.00	0.00
9.38	0.67	11.69	11.45	0.65	1	46.01	0	0.00	0.12
10.64	0.76	10.86	10.62	0.77	1	3.30	0	0.00	0.21
15.40	1.10	8.61	8.37	1.10	1	1.15	0	0.00	0.55
16.38	1.17	8.30	8.06	1.15	1	29.62	0	0.00	0.62
17.22	1.23	7.77	7.53	1.24	1	3.04	0	0.00	0.68
19.46	1.39	6.70	6.46	1.41	1	46.91	0	0.00	0.84
22.82	1.63	5.15	4.91	1.69	1	341.15	0	0.00	1.08
24.78	1.77	4.60	4.36	1.80	1	83.98	0	0.00	1.22
26.46	1.89	4.18	3.94	1.89	0	0.00	1	0.19	1.34
28.98	2.07	3.28	3.04	2.09	0	0.00	1	49.20	1.52
31.08	2.22	2.77	2.53	2.23	0	0.00	1	2.56	1.67
33.60	2.40	2.14	1.90	2.41	0	0.00	1	19.25	1.85
36.68	2.62	1.62	1.38	2.61	0	0.00	1	15.15	2.07
39.34	2.81	1.21	0.97	2.80	0	0.00	1	6.62	2.26
40.32	2.88	1.11	0.87	2.86	0	0.00	1	39.90	2.33
42.98	3.07	0.84	0.60	3.05	0	0.00	1	47.29	2.52
45.22	3.23	0.68	0.44	3.20	0	0.00	1	95.28	2.68
49.00	3.50	0.48	0.24	3.48	0	0.00	1	39.90	2.95
53.48	3.82	0.36	0.12	3.79	0	0.00	1	82.15	3.27
57.12	4.08	0.31	0.07	4.03	0	0.00	1	252.23	3.53
62.58	4.47	0.26	0.02	4.57	0	0.00	1	819.78	3.92
71.12	5.08	0.24	0.00	6.51	0	0.00	1	177875.21	4.53

Σ =	800	+	1470
		=	2269



MAD 2750 ppm TAN

Replicate 2

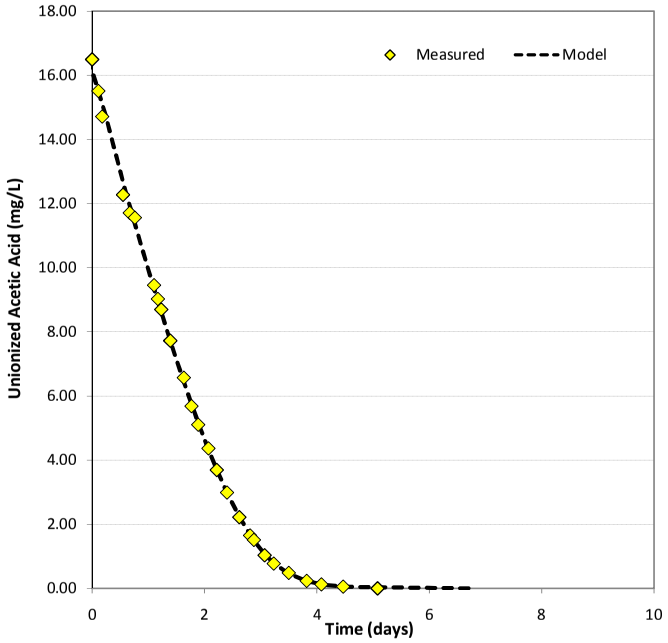
Y (g/g) =	0.040	(constant)
r (μ_{MAX}·X) =	0.309	fitting parameter (varies)
K_S (ppm) =	3.210	fitting parameter (varies)
C_{min} (ppm) =	0.179	(constant)
C_{LO} (ppm) =	16.115	fitting parameter (varies)

(ppm·day^{-1})

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00	16.67	16.49	-0.06	1	361.40	0	0.00	-0.55
1.54	0.11	15.69	15.51	0.10	1	2.65	0	0.00	-0.44
2.52	0.18	14.89	14.71	0.24	1	350.36	0	0.00	-0.37
7.70	0.55	12.45	12.27	0.61	1	310.14	0	0.00	0.00
9.38	0.67	11.89	11.71	0.70	1	86.75	0	0.00	0.12
10.64	0.76	11.74	11.56	0.73	1	97.39	0	0.00	0.21
15.40	1.10	9.63	9.45	1.08	1	25.55	0	0.00	0.55
16.38	1.17	9.20	9.02	1.16	1	13.05	0	0.00	0.62
17.22	1.23	8.87	8.69	1.22	1	17.34	0	0.00	0.68
19.46	1.39	7.90	7.72	1.39	1	0.01	0	0.00	0.84
22.82	1.63	6.75	6.57	1.61	1	50.13	0	0.00	1.08
24.78	1.77	5.86	5.68	1.78	1	11.14	0	0.00	1.22
26.46	1.89	5.28	5.10	1.90	0	0.00	1	10.48	1.34
28.98	2.07	4.54	4.36	2.06	0	0.00	1	5.94	1.52
31.08	2.22	3.87	3.69	2.22	0	0.00	1	0.52	1.67
33.60	2.40	3.17	2.99	2.40	0	0.00	1	1.92	1.85
36.68	2.62	2.40	2.22	2.62	0	0.00	1	0.24	2.07
39.34	2.81	1.83	1.65	2.82	0	0.00	1	2.21	2.26
40.32	2.88	1.69	1.51	2.87	0	0.00	1	8.78	2.33
42.98	3.07	1.21	1.03	3.09	0	0.00	1	36.49	2.52
45.22	3.23	0.95	0.77	3.24	0	0.00	1	18.70	2.68
49.00	3.50	0.66	0.48	3.48	0	0.00	1	42.21	2.95
53.48	3.82	0.41	0.23	3.81	0	0.00	1	2.80	3.27
57.12	4.08	0.30	0.12	4.10	0	0.00	1	23.18	3.53
62.58	4.47	0.23	0.05	4.46	0	0.00	1	4.77	3.92
71.12	5.08	0.18	0.00	6.73	0	0.00	1	235118.73	4.53

Σ = 1326 + 158

= 1484



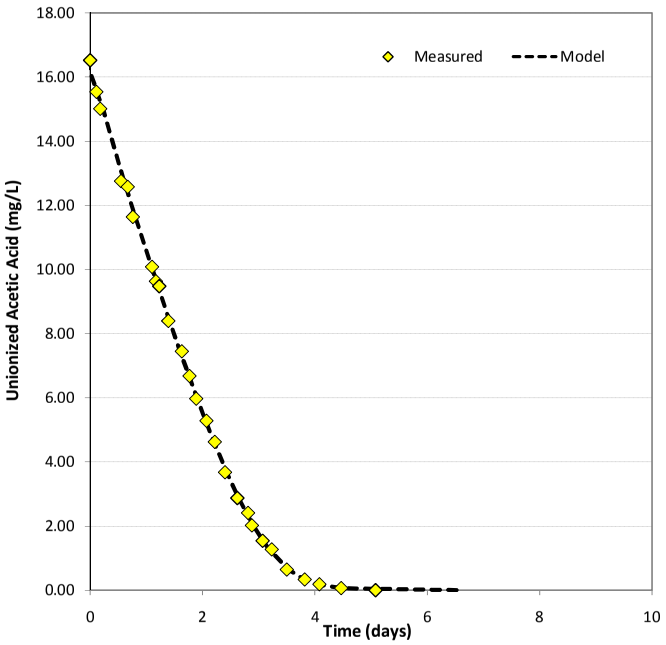
MAD 3500 ppm TAN

Y (g/g) =	0.040	(constant)	
r (μ_{MAX}·X) =	0.265	fitting parameter (varies)	(ppm·day^{-1})
K_S (ppm) =	2.450	fitting parameter (varies)	
C_{min} (ppm) =	0.147	(constant)	
C_{LO} (ppm) =	16.163	fitting parameter (varies)	

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00	16.67	16.52	-0.07	1	414.91	0	0.00	-0.55
1.54	0.11	15.69	15.54	0.12	1	8.93	0	0.00	-0.44
2.52	0.18	15.15	15.01	0.22	1	168.04	0	0.00	-0.37
7.70	0.55	12.90	12.75	0.60	1	232.28	0	0.00	0.00
9.38	0.67	12.72	12.57	0.63	1	110.43	0	0.00	0.12
10.64	0.76	11.79	11.64	0.80	1	159.02	0	0.00	0.21
15.40	1.10	10.23	10.08	1.09	1	6.50	0	0.00	0.55
16.38	1.17	9.78	9.63	1.18	1	3.17	0	0.00	0.62
17.22	1.23	9.63	9.48	1.21	1	52.38	0	0.00	0.68
19.46	1.39	8.55	8.40	1.41	1	44.35	0	0.00	0.84
22.82	1.63	7.59	7.44	1.60	1	70.38	0	0.00	1.08
24.78	1.77	6.83	6.68	1.76	1	17.34	0	0.00	1.22
26.46	1.89	6.12	5.97	1.90	0	0.00	1	18.49	1.34
28.98	2.07	5.43	5.28	2.05	0	0.00	1	21.99	1.52
31.08	2.22	4.77	4.62	2.20	0	0.00	1	25.30	1.67
33.60	2.40	3.82	3.67	2.43	0	0.00	1	83.84	1.85
36.68	2.62	3.02	2.87	2.64	0	0.00	1	43.97	2.07
39.34	2.81	2.56	2.41	2.78	0	0.00	1	97.57	2.26
40.32	2.88	2.17	2.02	2.90	0	0.00	1	35.75	2.33
42.98	3.07	1.69	1.54	3.07	0	0.00	1	0.67	2.52
45.22	3.23	1.42	1.27	3.18	0	0.00	1	178.31	2.68
49.00	3.50	0.79	0.64	3.53	0	0.00	1	87.78	2.95
53.48	3.82	0.48	0.33	3.82	0	0.00	1	0.23	3.27
57.12	4.08	0.33	0.18	4.07	0	0.00	1	18.67	3.53
62.58	4.47	0.21	0.06	4.48	0	0.00	1	3.95	3.92
71.12	5.08	0.15	0.00	6.58	0	0.00	1	193264.46	4.53

Σ = 1288 + 617

= 1904

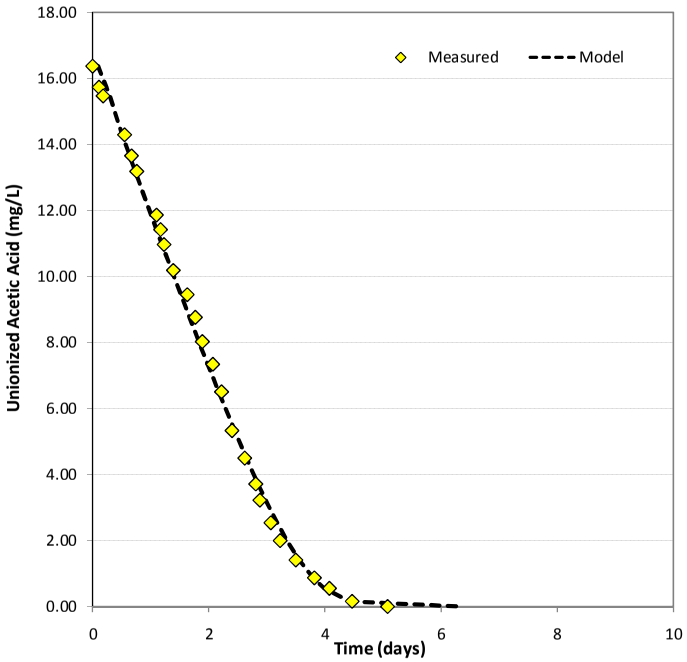


MAD 4250 ppm TAN

Y (g/g) =	0.040	(constant)
r (μ_{MAX}·X) =	0.217	fitting parameter (varies)
K_S (ppm) =	1.588	fitting parameter (varies)
C_{min} (ppm) =	0.300	(constant)
C_{LO} (ppm) =	16.783	fitting parameter (varies)

(ppm·day^{-1})

Experimental Data				Predicted Data:	zero order		first order		
		CH_3COOH (Acid Form)							
Time,hours	Time, d	HAc	HAc_{ADJ}	Time, d	On/Off	T error (s^2)	On/Off	T error (s^2)	Corrected Time, d
0.00	0.00	16.67	16.37	0.09	1	744.30	0	0.00	-0.55
1.54	0.11	16.03	15.73	0.24	1	1377.91	0	0.00	-0.44
2.52	0.18	15.77	15.47	0.30	1	1163.31	0	0.00	-0.37
7.70	0.55	14.59	14.29	0.51	1	164.59	0	0.00	0.00
9.38	0.67	13.95	13.65	0.64	1	93.14	0	0.00	0.12
10.64	0.76	13.48	13.18	0.73	1	57.63	0	0.00	0.21
15.40	1.10	12.16	11.86	1.01	1	713.94	0	0.00	0.55
16.38	1.17	11.72	11.42	1.10	1	405.26	0	0.00	0.62
17.22	1.23	11.27	10.97	1.20	1	97.35	0	0.00	0.68
19.46	1.39	10.48	10.18	1.36	1	64.31	0	0.00	0.84
22.82	1.63	9.75	9.45	1.52	1	1041.91	0	0.00	1.08
24.78	1.77	9.06	8.76	1.67	1	885.65	0	0.00	1.22
26.46	1.89	8.33	8.03	1.83	0	0.00	1	311.64	1.34
28.98	2.07	7.64	7.34	1.98	0	0.00	1	660.00	1.52
31.08	2.22	6.81	6.51	2.17	0	0.00	1	203.45	1.67
33.60	2.40	5.63	5.33	2.45	0	0.00	1	188.42	1.85
36.68	2.62	4.80	4.50	2.65	0	0.00	1	78.08	2.07
39.34	2.81	4.01	3.71	2.85	0	0.00	1	143.30	2.26
40.32	2.88	3.52	3.22	2.98	0	0.00	1	908.20	2.33
42.98	3.07	2.84	2.54	3.18	0	0.00	1	1028.77	2.52
45.22	3.23	2.30	2.00	3.35	0	0.00	1	1212.72	2.68
49.00	3.50	1.71	1.41	3.56	0	0.00	1	301.14	2.95
53.48	3.82	1.17	0.87	3.80	0	0.00	1	35.71	3.27
57.12	4.08	0.85	0.55	3.99	0	0.00	1	668.88	3.53
62.58	4.47	0.46	0.16	4.43	0	0.00	1	147.34	3.92
71.12	5.08	0.30	0.00	6.38	0	0.00	1	146572.92	4.53
					Σ =	**6809**	**+**	**5888**	
							=	**12697**	



MAD 5000 ppm TAN

DERIVATION OF EQUATION 5.2:

$$\frac{-ds}{dt} = \frac{-\left({}^{\mu_{max}}/_{Y}\right) \cdot S}{K_S + S} \cdot X$$

$$\frac{-ds}{dt} = \frac{-\mu_{max} \cdot X}{Y} \cdot \frac{S}{K_S + S}$$

$$\frac{-ds}{dt} = \frac{1}{k_1} \cdot \frac{S}{K_S + S} \qquad where\ k_1 = \frac{-Y}{\mu_{max} \cdot X}$$

$$dt = k_1 \cdot \frac{K_S + S}{S}\ ds$$

$$dt = k_1 \left[\frac{K_S}{S} + 1\right]\ ds$$

Assume constant biomass, X

i.e. Low F/M or low yield

$$\int_0^t dt = k_1 \int_{S_0}^{S} \left[\frac{K_S}{S} + 1\right]\ ds$$

$$t = k_1 [K_S \cdot \ln(\mathrm{S}) + S]_{S_0}^{S}$$

$$t = k_1 [K_S \cdot \ln(S) + S - K_S \cdot \ln(S_0) - S_0]$$

$$t = [K_S \cdot \ln(S) - \ln(S_0) + S - S_0]$$

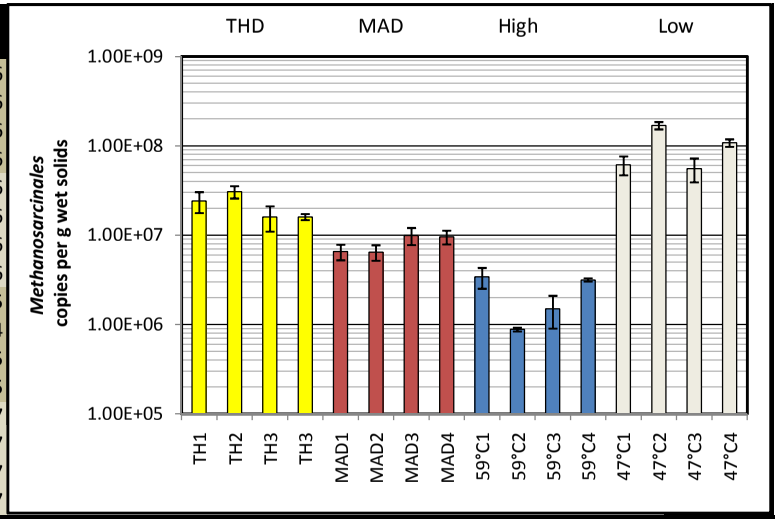
$$t = k_1 \left[K_S \cdot \ln\left(\frac{S}{S_0}\right) + S - S_0\right]$$

$$t = -\frac{Y}{\mu_{max} \cdot X} \left[K_S \cdot \ln\left(\frac{S}{S_0}\right) + S - S_0\right]$$

$$t = -\frac{Y}{r_{max}} \left[K_S \cdot \ln\left(\frac{S}{S_0}\right) + S - S_0\right] \qquad where\ r_{max} = \mu_{max} \cdot X$$

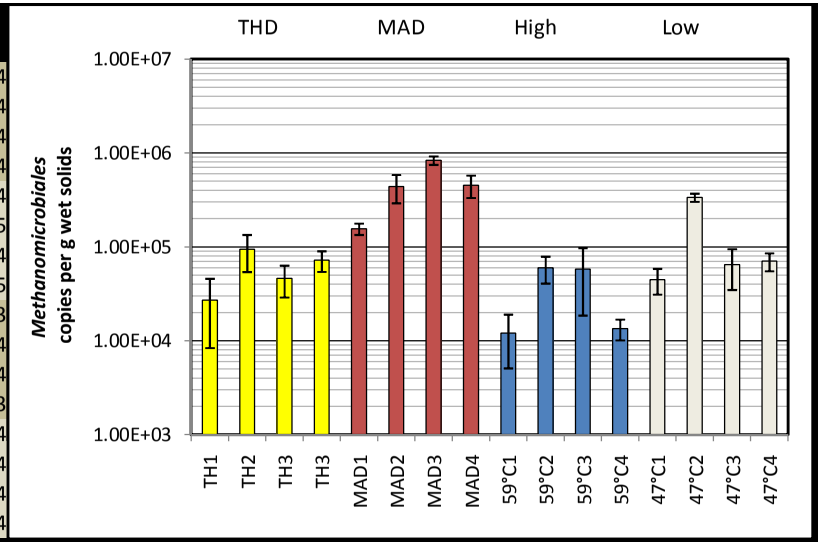
APPENDIX C

Sample	copies/ul		copies/ml	g/100 ul	copies/g wet		%VS	copies/g VS	
	Average	Stdev			Average	Stdev		Average	Stdev
TH1	5.02E+05	1.32E+05	5.02E+08	0.1121	4.48E+06	1.18E+06	18.60%	2.41E+07	6.35E+06
TH2	5.58E+05	8.74E+04	5.58E+08	0.1079	5.17E+06	8.10E+05	16.90%	3.06E+07	4.79E+06
TH3	3.20E+05	1.01E+05	3.20E+08	0.1148	2.78E+06	8.77E+05	17.40%	1.60E+07	5.04E+06
TH3	2.87E+05	2.32E+04	2.87E+08	0.0942	3.05E+06	2.46E+05	19.00%	1.60E+07	1.29E+06
MAD1	1.13E+05	2.24E+04	1.13E+08	0.1259	8.97E+05	1.78E+05	13.70%	6.55E+06	1.30E+06
MAD2	1.16E+05	2.30E+04	1.16E+08	0.1270	9.17E+05	1.81E+05	14.20%	6.46E+06	1.27E+06
MAD3	1.78E+05	3.87E+04	1.78E+08	0.1229	1.45E+06	3.15E+05	14.60%	9.91E+06	2.15E+06
MAD4	1.45E+05	2.54E+04	1.45E+08	0.1090	1.33E+06	2.33E+05	13.90%	9.58E+06	1.68E+06
59°C1	5.30E+04	1.39E+04	5.30E+07	0.1064	4.98E+05	1.31E+05	14.60%	3.41E+06	8.95E+05
59°C2	1.37E+04	6.36E+02	1.37E+07	0.1027	1.33E+05	6.19E+03	15.10%	8.81E+05	4.10E+04
59°C3	2.61E+04	1.04E+04	2.61E+07	0.1087	2.40E+05	9.60E+04	16.00%	1.50E+06	6.00E+05
59°C4	4.89E+04	1.96E+03	4.89E+07	0.1040	4.71E+05	1.88E+04	14.90%	3.16E+06	1.26E+05
47°C1	1.03E+06	2.45E+05	1.03E+09	0.1102	9.35E+06	2.22E+06	15.20%	6.15E+07	1.46E+07
47°C2	2.97E+06	2.83E+05	2.97E+09	0.1178	2.52E+07	2.41E+06	14.90%	1.69E+08	1.61E+07
47°C3	9.01E+05	2.69E+05	9.01E+08	0.1006	8.96E+06	2.68E+06	16.10%	5.56E+07	1.66E+07
47°C4	2.03E+06	1.95E+05	2.03E+09	0.1234	1.64E+07	1.58E+06	15.20%	1.08E+08	1.04E+07



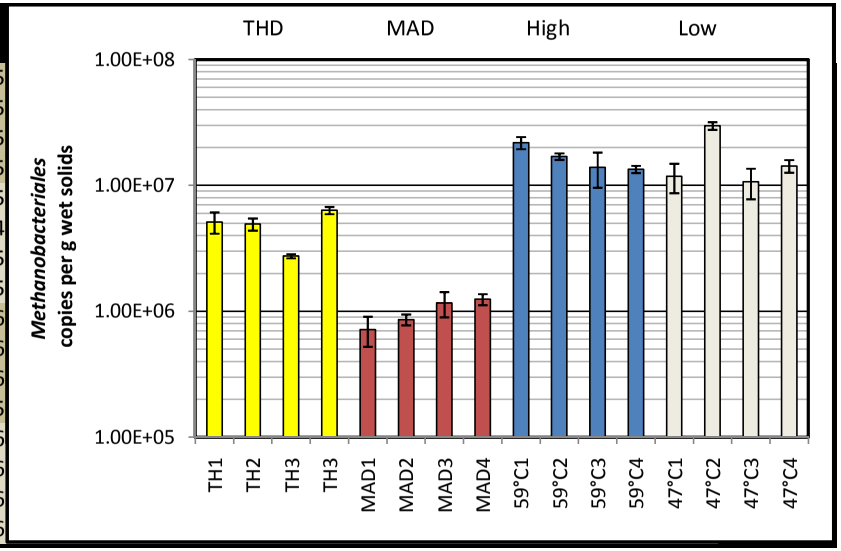
Slope:	-4.365726
Intercept:	51.465683
R^2	0.987318
Methanosarcinales	

Sample	copies/ul		copies/ml	g/100 ul	copies/g wet		%VS	copies/g VS	
	Average	Stdev			Average	Stdev		Average	Stdev
TH1	5.66E+02	3.91E+02	5.66E+05	0.1121	5.05E+03	3.49E+03	18.60%	2.71E+04	1.88E+04
TH2	1.72E+03	7.34E+02	1.72E+06	0.1079	1.59E+04	6.80E+03	16.90%	9.43E+04	4.03E+04
TH3	9.22E+02	3.44E+02	9.22E+05	0.1148	8.03E+03	3.00E+03	17.40%	4.62E+04	1.72E+04
TH3	1.29E+03	3.19E+02	1.29E+06	0.0942	1.37E+04	3.39E+03	19.00%	7.21E+04	1.78E+04
MAD1	2.69E+03	3.68E+02	2.69E+06	0.1259	2.14E+04	2.92E+03	13.70%	1.56E+05	2.13E+04
MAD2	7.90E+03	2.64E+03	7.90E+06	0.1270	6.22E+04	2.08E+04	14.20%	4.38E+05	1.46E+05
MAD3	1.50E+04	1.55E+03	1.50E+07	0.1229	1.22E+05	1.26E+04	14.60%	8.36E+05	8.64E+04
MAD4	6.89E+03	1.85E+03	6.89E+06	0.1090	6.32E+04	1.70E+04	13.90%	4.55E+05	1.22E+05
59°C1	1.87E+02	1.08E+02	1.87E+05	0.1064	1.76E+03	1.02E+03	14.60%	1.20E+04	6.95E+03
59°C2	9.27E+02	2.96E+02	9.27E+05	0.1027	9.03E+03	2.88E+03	15.10%	5.98E+04	1.91E+04
59°C3	1.01E+03	6.86E+02	1.01E+06	0.1087	9.29E+03	6.31E+03	16.00%	5.81E+04	3.94E+04
59°C4	2.09E+02	5.20E+01	2.09E+05	0.1040	2.01E+03	5.00E+02	14.90%	1.35E+04	3.36E+03
47°C1	7.51E+02	2.30E+02	7.51E+05	0.1102	6.81E+03	2.09E+03	15.20%	4.48E+04	1.37E+04
47°C2	5.90E+03	5.79E+02	5.90E+06	0.1178	5.01E+04	4.92E+03	14.90%	3.36E+05	3.30E+04
47°C3	1.05E+03	4.85E+02	1.05E+06	0.1006	1.04E+04	4.82E+03	16.10%	6.48E+04	2.99E+04
47°C4	1.32E+03	2.85E+02	1.32E+06	0.1234	1.07E+04	2.31E+03	15.20%	7.04E+04	1.52E+04



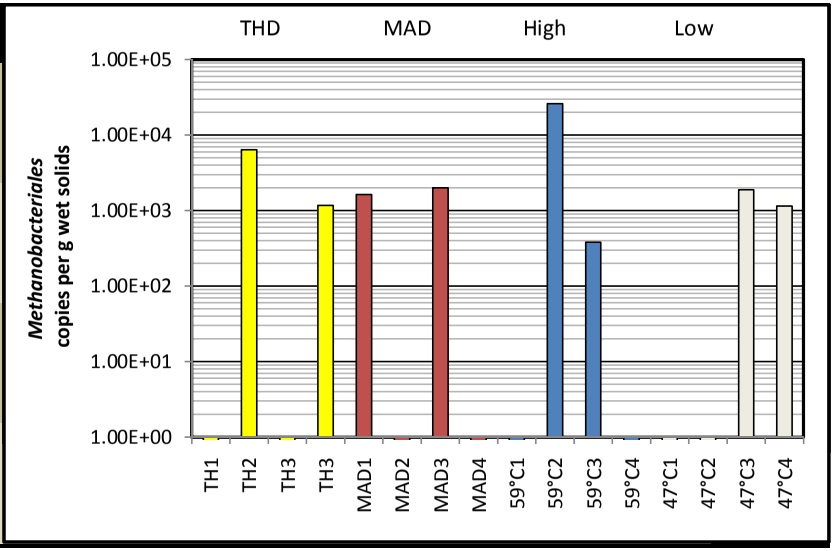
Slope:	-4.191324
Intercept:	53.560913
R^2	0.996674
Methanomicrobiales	

Sample	copies/ul		copies/ml	g/100 ul	copies/g wet		%VS	copies/g VS	
	Average	Stdev			Average	Stdev		Average	Stdev
TH1	1.07E+05	2.06E+04	1.07E+08	0.1121	9.54E+05	1.83E+05	18.60%	5.13E+06	9.86E+05
TH2	8.99E+04	9.91E+03	8.99E+07	0.1079	8.34E+05	9.18E+04	16.90%	4.93E+06	5.43E+05
TH3	5.48E+04	2.06E+03	5.48E+07	0.1148	4.77E+05	1.80E+04	17.40%	2.74E+06	1.03E+05
TH3	1.14E+05	7.56E+03	1.14E+08	0.0942	1.21E+06	8.03E+04	19.00%	6.35E+06	4.23E+05
MAD1	1.23E+04	3.32E+03	1.23E+07	0.1259	9.79E+04	2.64E+04	13.70%	7.15E+05	1.92E+05
MAD2	1.55E+04	1.54E+03	1.55E+07	0.1270	1.22E+05	1.21E+04	14.20%	8.59E+05	8.54E+04
MAD3	2.08E+04	4.72E+03	2.08E+07	0.1229	1.69E+05	3.84E+04	14.60%	1.16E+06	2.63E+05
MAD4	1.89E+04	1.91E+03	1.89E+07	0.1090	1.73E+05	1.75E+04	13.90%	1.25E+06	1.26E+05
59°C1	3.40E+05	3.76E+04	3.40E+08	0.1064	3.19E+06	3.54E+05	14.60%	2.19E+07	2.42E+06
59°C2	2.64E+05	1.56E+04	2.64E+08	0.1027	2.57E+06	1.52E+05	15.10%	1.70E+07	1.00E+06
59°C3	2.42E+05	7.56E+04	2.42E+08	0.1087	2.23E+06	6.95E+05	16.00%	1.39E+07	4.35E+06
59°C4	2.09E+05	1.35E+04	2.09E+08	0.1040	2.01E+06	1.30E+05	14.90%	1.35E+07	8.73E+05
47°C1	1.98E+05	5.21E+04	1.98E+08	0.1102	1.79E+06	4.73E+05	15.20%	1.18E+07	3.11E+06
47°C2	5.23E+05	3.63E+04	5.23E+08	0.1178	4.44E+06	3.08E+05	14.90%	2.98E+07	2.07E+06
47°C3	1.73E+05	4.71E+04	1.73E+08	0.1006	1.72E+06	4.69E+05	16.10%	1.07E+07	2.91E+06
47°C4	2.68E+05	3.09E+04	2.68E+08	0.1234	2.17E+06	2.50E+05	15.20%	1.43E+07	1.65E+06



Slope:	-3.607636
Intercept:	45.563583
R^2	0.996674
Methanobacteriales	

Sample	copies/ul		copies/ml	g/100 ul	copies/g wet		%VS	copies/g VS	
	Average	Stdev			Average	Stdev		Average	Stdev
TH1			1.00E-01	0.0921	1.09E-03	0.00E+00	18.60%		
TH2	1.16E+02		1.16E+05	0.1079	1.08E+03	0.00E+00	16.90%	6.38E+03	
TH3			1.00E-01	0.1079	9.27E-04	0.00E+00	17.40%		
TH4	2.10E+01		2.10E+04	0.0942	2.23E+02	0.00E+00	19.00%	1.17E+03	
MAD1	2.80E+01		2.80E+04	0.1259	2.22E+02	0.00E+00	13.70%	1.62E+03	
MAD2			1.00E-01	0.1270	7.87E-04	0.00E+00	14.20%		
MAD3	3.58E+01		3.58E+04	0.1229	2.91E+02	0.00E+00	14.60%	1.99E+03	
MAD4			1.00E-01	0.1090	9.17E-04	0.00E+00	13.90%		
59°C1			1.00E-01	0.1064	9.40E-04	0.00E+00	14.60%		
59°C2	4.04E+02		4.04E+05	0.1027	3.93E+03	0.00E+00	15.10%	2.61E+04	
59°C3	6.65E+00		6.65E+03	0.1087	6.12E+01	0.00E+00	16.00%	3.82E+02	
59°C4			1.00E-01	0.1040	9.62E-04	0.00E+00	14.90%		
47°C1			1.00E-01	0.1102	9.07E-04	0.00E+00	15.20%		
47°C2			1.00E-01	0.1178	8.49E-04	0.00E+00	14.90%		
47°C3	3.05E+01		3.05E+04	0.1006	3.04E+02	0.00E+00	16.10%	1.89E+03	
47°C4	2.16E+01		2.16E+04	0.1234	1.75E+02	0.00E+00	15.20%	1.15E+03	



Slope:	-4.171569
Intercept:	51.513435
R^2	0.984452
Methanococcales	

Primer and probe binding locations and specificity for pure methanogenic cultures used for qPCR standard curve generation

Methanococcus voltae (DSM 1537)
Order *Methanococcales*

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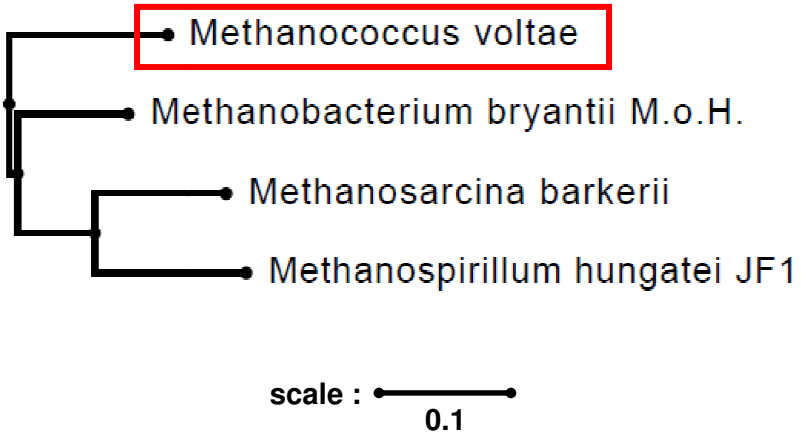

Forward Primer		TaqMan Probe		Reverse Primer

Methanosarcina barkeri fusaro (DSM 800)
Order *Methanosarcinales*

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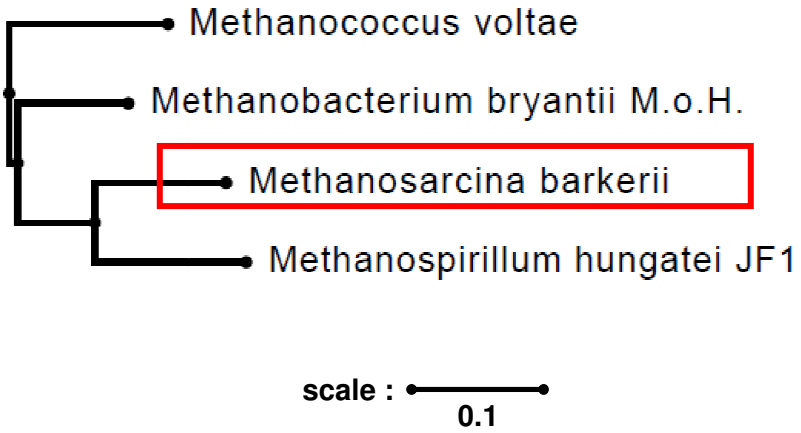

Forward Primer		TaqMan Probe		Reverse Primer

Methanobacterium bryantii M.o.H (DSM 863)
Order *Methanobacteriales*

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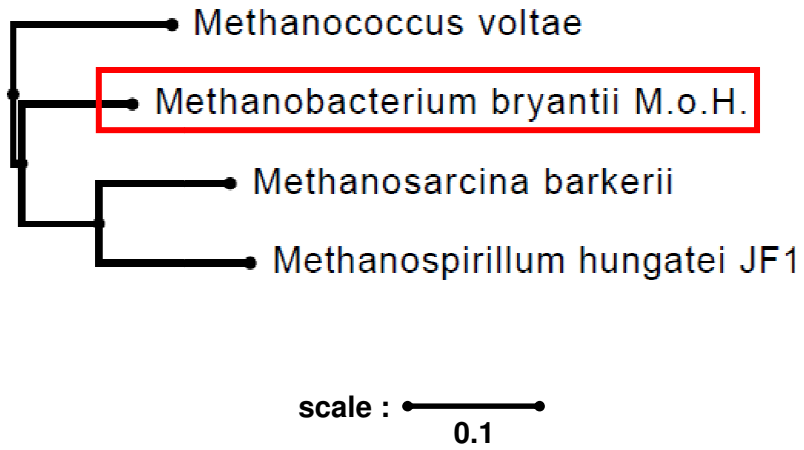

Forward Primer		TaqMan Probe		Reverse Primer

Methanospirillum hungatei JF1 (DSM 864)
Order *Methanomicrobiales*

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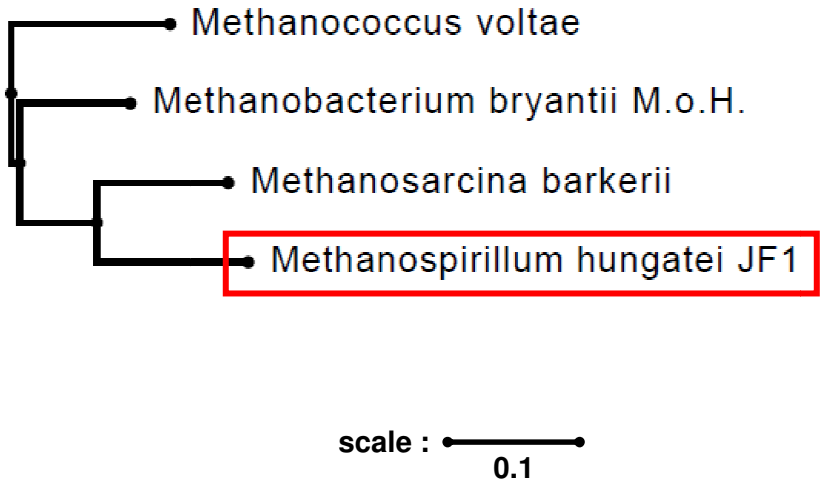

Forward Primer		TaqMan Probe		Reverse Primer

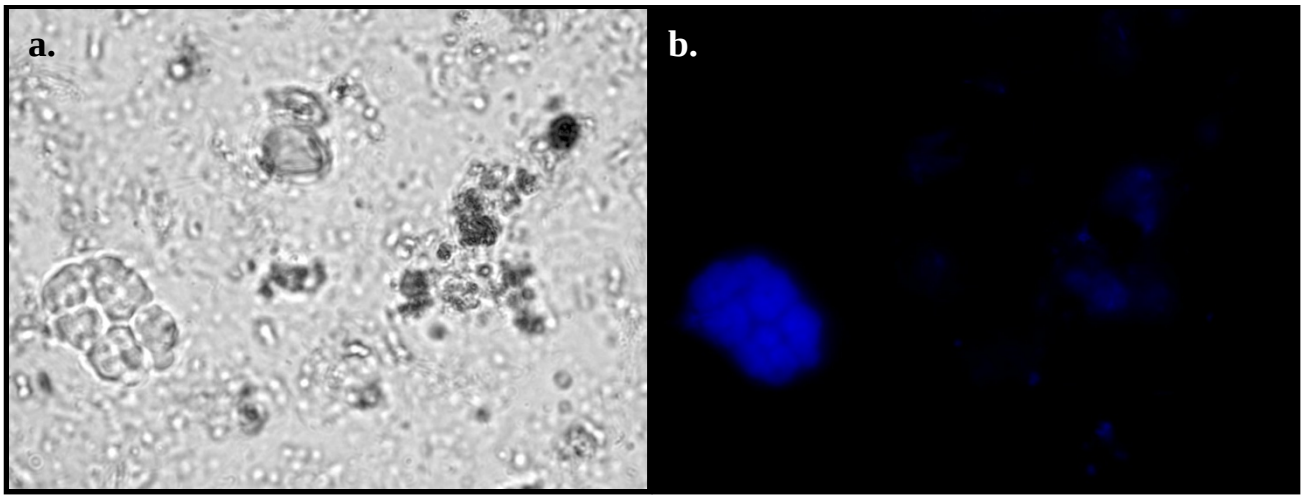

Figure C.1

(a) Phase contrast and (b) epifluorescence micrographs of THD biomass. Few small clusters of *Methanosarcina*-like cells were observed and there was a broad presence of small cocci and rod-shaped cells that exhibited autofluorescence.

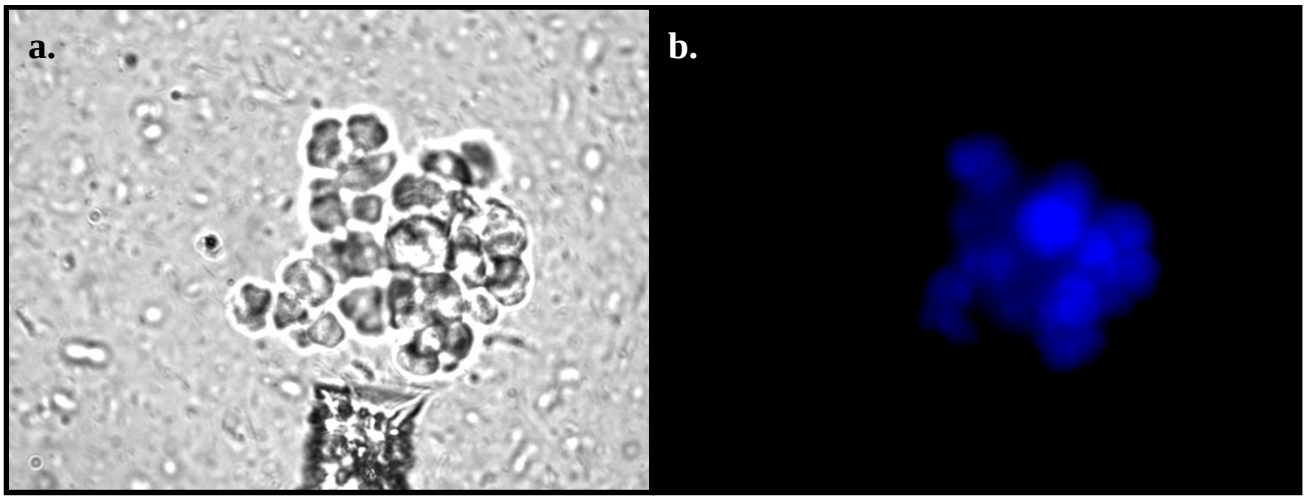

Figure C.2

(a) Phase contrast and (b) epifluorescence micrographs of MAD biomass. Autofluorescent *Methanosarcina*-like clusters were comparatively larger and more numerous than was observed within the THD sample.

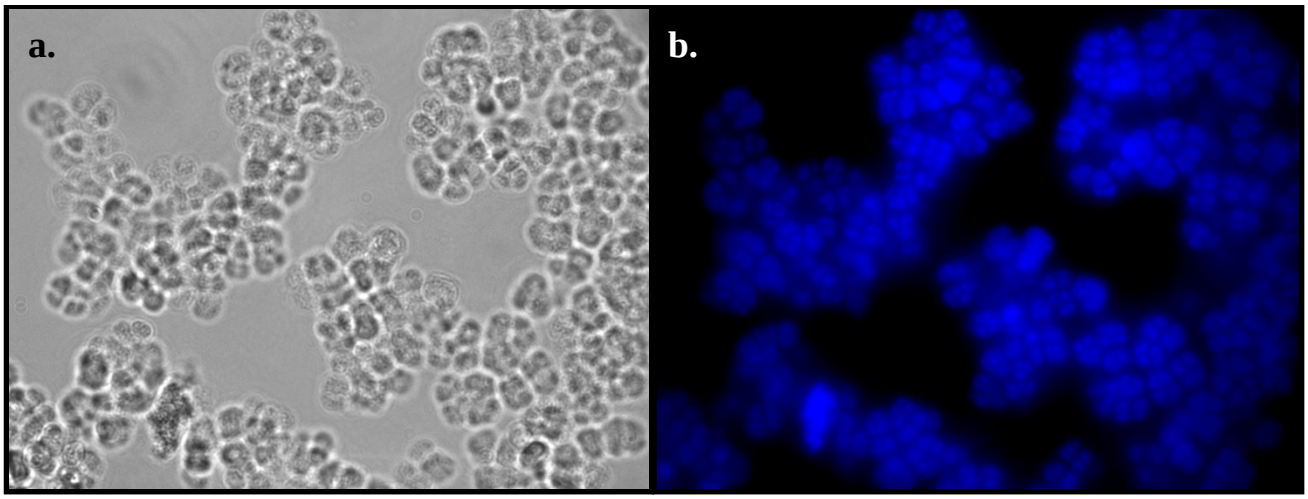

Figure C.3

(a) Phase contrast and (b) epifluorescence micrographs of *Methanosarcina barkeri* (DSM 800).

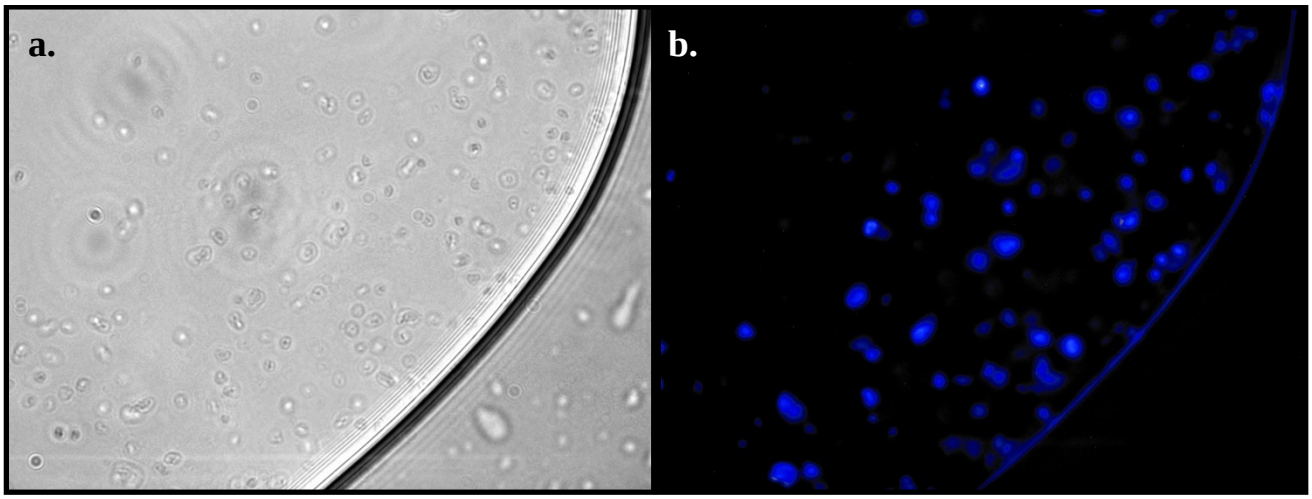

Figure C.4

(a) Phase contrast and (b) epifluorescence micrographs of *Methanoculleus bourgensis* (DSM 3045).

APPENDIX D

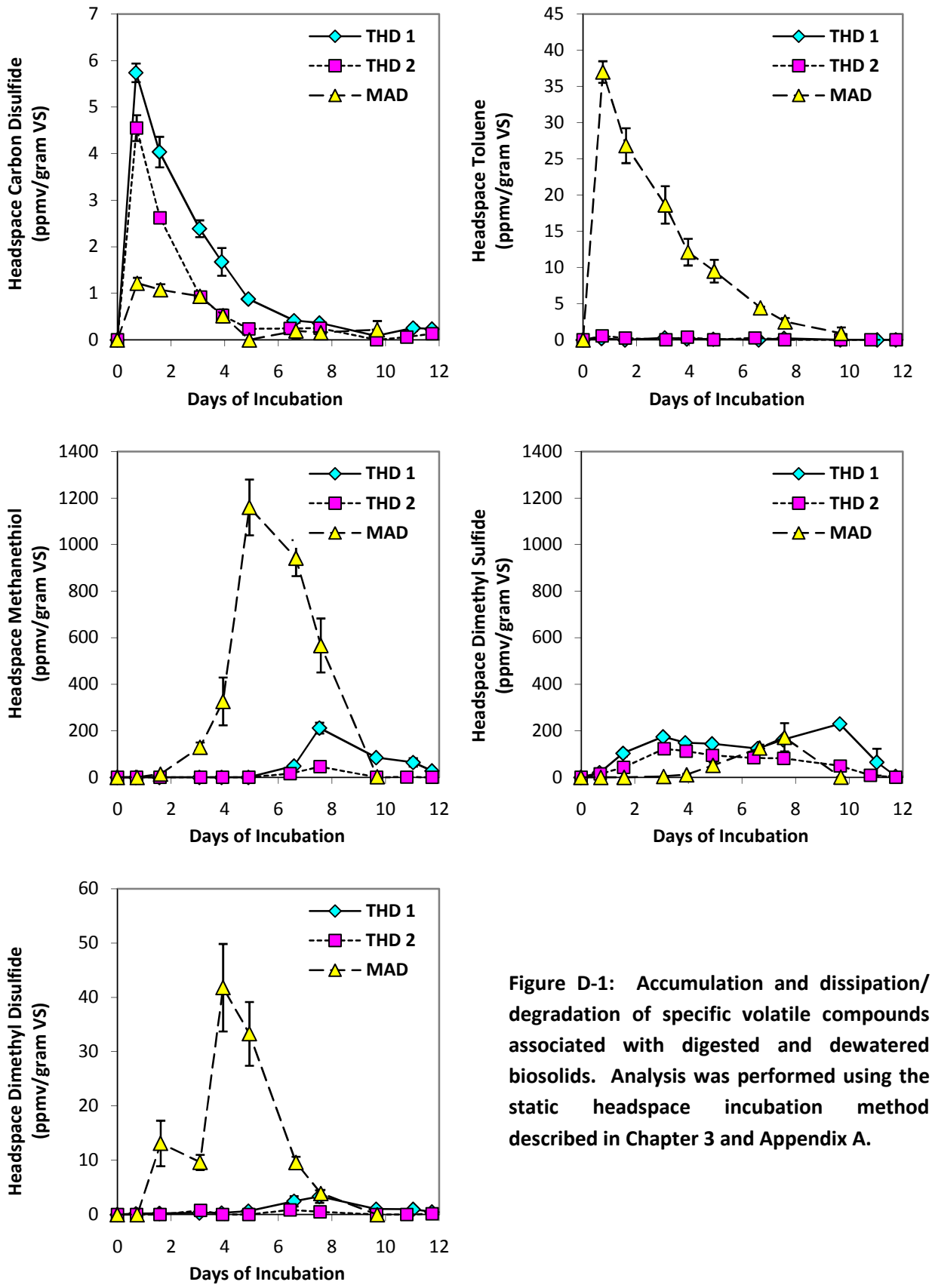

Figure D-1: Accumulation and dissipation/ degradation of specific volatile compounds associated with digested and dewatered biosolids. Analysis was performed using the static headspace incubation method described in Chapter 3 and Appendix A.

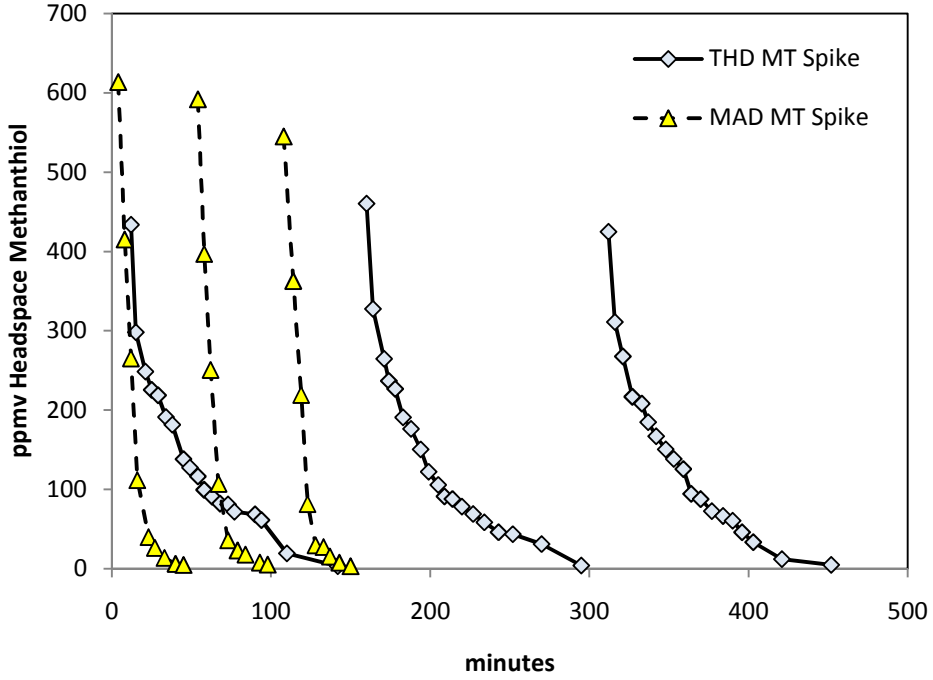

Figure D-2: Results of methanethiol (MT) degradation experiment. Solids-equalized digested THD and MAD biosolids were spiked with MT and continuously agitated. Headspace was periodically analyzed for MT. Closed vials were spiked a total of three times in order to achieve experimental replicates.