ABSTRACT

CREAMER, KURT SERENO. Impact of Ammonia and Long Chain Fatty Acids on Thermophilic Anaerobic Digestion of Swine Wastes. (Under the direction of Dr. Jay Cheng).

Environmentally sound treatment of by-products in a value-adding process is an ongoing challenge in animal agriculture. Thermophilic anaerobic digestion of wastes originating from agricultural production and animal processing represents a potential waste treatment technology to address environmental concerns such as odor emissions and removal of pathogenic microorganisms, while at the same time producing renewable energy (biogas) as a by-product. However, thermophilic digestion is subject to inhibition by ammonia and long chain fatty acids (LCFA), both of which are prevalent in manure and animal processing wastewater. Several swine manure collection methods under development separate the urine from the feces, which creates the opportunity to operate a digester on feces only, greatly reducing the ammonia load to the digester. One objective of this study was to determine whether operation on feces only would yield significant performance improvements for a thermophilic anaerobic digester operating on swine waste. Effluent from a continuously stirred tank reactor (CSTR) was used as the inoculum for batch tests in which the substrate contained three different concentrations of urine (urine-free, as-excreted urine:feces ratio and double the as-excreted urine:feces ratio). Inocula were acclimated to these same urine:feces ratios to determine methane production. Results show that both urine-free and as-excreted substrates were not inhibitory to anaerobic inocula. Anaerobic microorganisms can be readily acclimated to substrate with double the as-excreted urine concentration, which contained

TKN concentrations up to 7.20 g-N liter^{-1}. The sludge collected from the dissolved air flotation (DAF) wastewater treatment process in swine processing facilities is an example of a high-lipid substrate containing potentially inhibitory levels of LCFA. A second objective of this study was to determine the fundamental performance parameters for thermophilic anaerobic digestion of DAF sludge. Testing in a semi-continuous stirred tank reactor and in batch reactors was conducted to determine substrate degradation rates and biogas yield. Stable operation could not be achieved using pure DAF sludge as a substrate, possibly due to inhibition by long chain fatty acids or to nutrient deficiencies. However, a 1:1 ratio (w/w, dry basis) of DAF sludge and swine manure (feces only), resulted in stable and productive digester operation. In the semi-continuous stirred reactor at 54.5°C, a hydraulic residence time of 10 days, and an organic loading rate of 4.68 gVS/day/L, the methane production rate was 2.19 L/L/day and the specific methane production rate was 0.47 L/gVS (fed). Maximum specific methanogenic activity (SMA) in batch testing was 0.15 mmolesCH_4 hr^{-1} gVS^{-1} at a manure/DAF substrate concentration of 6.9 gVS liter^{-1}. Higher substrate concentrations cause an initial lag in methane production, possibly due to long chain fatty acid or nitrogen inhibition.

Impact of Ammonia and Long Chain Fatty Acids on Thermophilic Anaerobic Digestion of Swine Wastes

by
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DEDICATION

To my lovely wife, Melissa, without whose steadfast love and support this Dissertation would simply not have been possible.

BIOGRAPHY

Kurt Creamer is currently the Connectivity Manager for Novozymes North America. In this role, he facilitates collaborations that can bolster Novozymes' internal R&D efforts, such as partnerships with government agencies and university researchers. He started his career at Battelle Memorial Institute as a Research Engineer, leading both product and process development tasks in the Fuels and Combustion Department. Prior to joining Novozymes, Kurt held several positions with NC State University (NCSU), beginning with Assistant Director of the Office of Technology Transfer, where he and his colleagues managed university intellectual property and worked to commercialize university inventions. He later held a joint appointment with NCSU's North Carolina Solar Center and Animal & Poultry Waste Management Center, managing projects in the areas of biomass energy, biofuel production, and swine waste treatment. On behalf of the NC State Energy Office, Kurt organized the North Carolina Biomass Council to promote economic development, a reduction in greenhouse gas and other emissions, and energy security through the utilization of biomass (including biofuels, bioproducts and biopower), in the State of North Carolina. Kurt holds four patents and is a licensed Professional Engineer (Mechanical). He graduated Phi Beta Kappa from Brown University and received an M.S. in Engineering from the University of California at Davis.

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CHAPTER 1. LITERATURE REVIEW

1.1. Overview

In anaerobic digestion, microbes degrade organic matter in the absence of oxygen, leading to the formation of biogas (a mixture of carbon dioxide and methane). Biopolymers, including lipids, proteins and complex carbohydrates, are degraded into monomers by hydrolytic bacteria. Lipids in the form of triglycerides are hydrolyzed into glycerol and long chain fatty acids (LCFA). Proteins are hydrolyzed into their component amino acids and complex carbohydrates are hydrolyzed into sugar monomers. From these substrates, acidogens produce acetic acid and other volatile fatty acids (VFA) that can be converted into methane by aceticlastic methanogens.

As a wastewater treatment process, anaerobic digestion has an inherent appeal in that renewable energy is produced as a by-product. In comparison with aerobic wastewater treatment processes, anaerobic processes have two distinct advantages: (1) they do not require aeration equipment and the capital and operating expenses associated therewith, and (2) they produce a much smaller amount of sludge per unit of volatile solids (or chemical oxygen demand (COD)) metabolized.

Anaerobic digesters typically operate in one of three temperature regimes:

- Psychrophilic - in this type of digester, no external heat is applied to the digester. The temperature in the digester is allowed to equilibrate with ambient temperature. At ambient temperatures, degradation rates are slow and residence

times correspondingly long. Because of the seasonal variations in digester temperature, substantial variation in biogas production occurs.

- Mesophilic – a typical temperature for a mesophilic digester is 37 °C. At mesophilic temperatures, the degradation rate is substantially greater than that at ambient temperature. Typical HRT for mesophilic digesters are in the 15-30 day range.
- Thermophilic – thermophilic digesters operate at temperatures of around 55 °C and as a result have a microbial community distinct from either a mesophilic or ambient temperature digester. At these temperatures, degradation is rapid, leading to residence times in the 5-15 day range.

Thermophilic digestion has several advantages over its lower temperature counterparts. The lower retention times translate directly into greater methane productivity per unit of reactor working volume. In a study on cattle manure, Mackie and Bryant (Mackie and Bryant 1995) found that a thermophilic digester produced four times the amount of methane per unit volume of reactor as compared to a mesophilic digester. The thermophilic digester also achieved better volatile solids reduction and specific methane yield. For treating a given wastewater flow rate, the thermophilic reactor will be smaller and therefore the capital cost will be reduced.

All digesters are subject to ammonia and long chain fatty acid (LCFA) inhibition. However, thermophilic digesters are especially sensitive to ammonia inhibition because of the impact of temperature on the equilibrium between NH_3 and NH_4^+. Free ammonia is

believed to be the form of ammonia that is toxic to anaerobic microbes and higher temperatures increase the equilibrium ratio of free ammonia to total ammonia nitrogen (TAN).

Co-digestion can be an effective technique for dampening the impacts of high-ammonia or high-lipid wastes. The effects of ammonia inhibition, for example, can be mitigated through simple dilution with a low-ammonia co-substrate. As another example, high-lipid feedstocks are often deficient in certain nutrients needed for anaerobic fermentation; a co-substrate could be selected to provide the missing nutrients (Ahring et al. 1992). Manure, in particular, can be an excellent co-substrate for a variety of industrial wastes (Angelidaki and Ellegaard 2003). For one, due to its high alkalinity, manure has a strong buffering capacity that can protect the digester against catastrophic drops in pH that can cause digester failure resulting from the temporary accumulation of VFA. Secondly, manure is rich in nutrients necessary for optimal microbial growth. Because of its relatively high nitrogen content, manure is best paired with substrates deficient in nitrogen.

1.2. Research Topic 1: High-Ammonia Substrates and Ammonia Inhibition

1.2.1. Nitrogen Mineralization in Anaerobic Digesters

Total ammonia nitrogen (TAN) present in an anaerobic digester comes from the mineralization of organic nitrogen in the substrate as well as any TAN already present in the substrate. Animal wastes contain compounds, such as urea and proteins, which are readily

degraded into ammonia. As it is for all living organisms, nitrogen is an important nutrient for anaerobes, but at high concentrations ammonia nitrogen is inhibitory.

Few studies, however, have specifically focused on the mineralization of organic nitrogen in anaerobic digesters, so the impact of, for example, hydraulic residence time on mineralization level is not well known. In the range of operating conditions normally found in anaerobic digesters, digester effluent typically contains more than 50% of the nitrogen in the form of NH_3-N. Operating on swine manure at a 25-day HRT, Hashimoto (1983) measured total ammonia nitrogen (TAN) concentrations in the effluent that represented 69% and 61% of total Kjeldahl nitrogen (TKN) at thermophilic and mesophilic temperatures, respectively. The TAN/TKN fraction in the substrate was only 38.2%, indicating substantial mineralization during digestion. Lo et al. (1985) presented data from which the overall mineralization in the digester could be calculated. At an HRT of 10 days under thermophilic conditions, the TAN/TKN fraction rose from 19.2% in the influent to 57% in the effluent for a substrate having a volatile solids concentration of 3-4%. Sayed et al. (1988) concluded that the degradation of proteins proceeded rapidly in the batch fed and recirculating batch fed digesters used in their study as evidenced by the NH_3-N concentrations measured at the termination of the experiment. NH_3-N represented 85% and 76% of the total Kjeldahl nitrogen at digester temperatures of 30 °C and 20 °C respectively. Krylova et al. (1997) studied the anaerobic digestion of poultry manure over a range of temperatures and concentrations of total solids in batch tests. As a percentage of TKN, TAN represented from 77.1% to 92.3% after 15 days of incubation and from 80.0% to 96.0% after 30 days of

incubation. In these tests, the raw manure had high TAN/TKN fractions of 63.6-73.7%. Salminen et al. (2000) found that 50-60% of the nitrogen in the feed was mineralized to ammonia within 3-6 days of incubation in batch tests. The TAN/TKN fraction in the substrate was only 1.6%. The mineralization rate was independent of other test parameters (inoculum to substrate ratio and substrate concentration). In semi-continuous testing of the same substrate, Saliminen et al. (2002) found that NH_3-N represented 56-57% of the TKN in the digesters at HRT of 13-50 days. At HRT of 50-100 days, NH_3-N represented 52-67% of the TKN.

Hill et al. (1985) used swine waste collected from a production barn with a sloped floor such that urine was subject to runoff and the remaining material was assumed to be feces-only. This feedstock was digested in a semi-continuous 378 liter, intermittently-stirred reactor under thermophilic conditions (55 °C), an organic loading rate of 3.28 g-VS d^{-1}, and several HRT. Compared to whole waste (manure and urine), mineralization of organic nitrogen in the feces was lower. As a percentage of TKN, the TAN nitrogen concentration in the effluent was 48%, 47% and 51% at HRT of 25, 15, and 10 days, respectively. The TAN/TKN fractions in the feed were 33%, 36%, and 35% for HRT of 25, 15, and 10 days, respectively. The lower mineralization rate for feces-only substrate is not surprising, since the mineralization of whole waste includes the mineralization of readily degradable urea in the urine.

Once mineralized, the equilibrium of ammonia/ammonium can be represented as follows:

$$K_a = \frac{[H^+][NH_3]}{[NH_4^+]}$$

Hafner and Bisogni (2009) point out that this simple equilibrium model is based on an assumption of a dilute solution; non-ideal behavior due to interaction between ions is ignored. The magnitude of the error increases with total solids content, but even at 3% total solids, the free ammonia concentration in a swine waste digester would be overestimated by more than 40% by using the simple equilibrium model. Inclusion of an activity coefficient for NH_4^+ can reduce the error to below 10%.

1.2.2. Studies Using High-Ammonia Feedstocks

High ammonia concentrations are inhibitory to the microbial community in anaerobic digesters. Animal manures tend to be ammonia-rich feedstocks. As an extreme example, as part of a study of full-scale biogas plants in Denmark, Angelidaki et al. (2005) identified a biogas plant treating mink manure with a feedstock TAN concentration exceeding 10 g-N $liter^{-1}$, leading to unstable operation as evidenced by a VFA concentration that on occasion rose above 14 g $liter^{-1}$ (as acetate). As part of this same study, the authors found a correlation between nitrogen content and VFA concentration in full-scale anaerobic digesters in Denmark. VFA concentration is often used as an indicator of anaerobic digester stability; the authors suggest that a healthy digester is one with a VFA concentration below 1.5 g $liter^{-1}$.

While not in the same league as mink manure, swine manure has a relatively high ammonia nitrogen content when compared to dairy or beef cattle manure. As far back as 1973, Hobson and Shaw (1973) recognized that the ammonia concentration in swine waste

may be the reason for difficulty in achieving stable digester operation when the organic loading rate exceeded 3.2 g-VS liter^{-1} d^{-1} or the solids content of the digester exceeded 4% TS. Hashimoto (1983) conducted both semi-continuous (daily-fed) and batch tests to determine the performance of swine manure at both mesophilic (35 °C) and thermophilic temperatures (55 °C). The batch tests were used to determine the ultimate methane yield, which was 0.49 liters gVS^{-1} fed. Under both mesophilic and thermophilic conditions, stable operation was achieved at 15, 10, and 5 HRT. Interestingly, at a 25-day HRT, thermophilic operation was not stable at an influent substrate concentration of 62.5 g-VS liter^{-1}; methane production decreased and volatile acids increased up until the experiment was terminated. Mesophilic operation was achievable at the 25-day HRT. Hashimoto notes that while the TKN was very similar for the mesophilic and thermophilic digesters, the free ammonia concentration was estimated to be 410 mg liter^{-1} in the thermophilic digester but only 170 mg liter^{-1} in the mesophilic digester because of the dependence of the equilibrium concentrations of NH_3 and NH_4^+ on temperature. Higher temperatures increase the equilibrium ratio of free ammonia to total ammonia nitrogen (TAN).

Hansen et al. (1998) used mixtures of swine waste, with an already high background concentration of ammonia nitrogen, and cattle manure to evaluate the inhibition by ammonia on digester performance. In batch tests using 58 mL serum vials, various ratios of fresh swine:cattle manure were used to achieve a range of ammonia nitrogen concentrations. Inoculum for the batch tests came from a laboratory CSTR digester operating on cattle manure at a HRT of 15 days and a temperature of 55 °C. The background TAN

concentration in the inoculum source digester was 3 g-N liter^{-1}. The uninhibited specific growth rate (measured when methane production was increasing exponentially in the batch tests) was 0.150 days^{-1} when the calculated free ammonia concentration was 1.1 g-N liter^{-1}. This concentration signaled the onset of inhibition. At a calculated free ammonia concentration of 1.3 g-N liter^{-1}, the growth rate decreased by 33%.

Poultry manure from laying hens is another potential digester feedstock with a high ammonia content. Pechan et al. (1987) were able to successfully digest poultry manure at mesophilic temperatures using an influent total solids content of 11.3-14.1% and HRT of 27-58 days. TAN concentrations in the effluent were typically in the range of 4.07 to 5.85 g-N liter^{-1} with excursions as high as 7.5 g-N liter^{-1}. Specific methane production was 0.239-0.370 liters gVS^{-1} fed, methane concentration in the biogas was 59-67% and volatile solids reduction was 58-64%. VFA concentrations were quite high, 4.6-9.3 g liter^{-1} (as acetic acid), but did not to appear to have an overt impact on digester performance.

In a study on cow manure, Zeeman et al. (1985) encountered significant difficulty in trying to digest the substrate at thermophilic temperatures (50 °C) with a TAN concentration above 3 g-N liter^{-1}. The operating parameters of the 120 liter digester used in these tests were as follows: 40 day HRT, intermittent stirring, and semi-continuous feeding. Performance was poor, characterized by low specific methane production, high VFA concentrations, and unstable operation. The authors hypothesized that the reason for poor performance was ammonia inhibition but for a given substrate, changing the concentration of ammonia through dilution, for example, would change the concentration of volatile solids or

other components in the substrate that are potentially inhibitory. To separate the impact of reduced ammonia concentration vs. reduced concentration of other components, a test was conducted wherein exogenous ammonia was added after the substrate was diluted to bring the total ammonia concentration back to the undiluted value. In this case, performance was not improved. However, when the substrate was diluted without addition of exogenous ammonia, performance was substantially improved. Hence, volatile solids (and other component) concentration, in the range tested, did not impact digester performance. Additional work with the introduction of exogenous ammonia is described below.

1.2.3. Inhibition Studies Using Exogenous Ammonia

Free ammonia is believed to be the form of ammonia that causes ammonia inhibition in anaerobic digesters (McCarty and McKinney 1961). The threshold levels found to be inhibitory are in the range of 80-150 mg-N liter^{-1} (Koster and Lettinga 1984; Debaere et al. 1984; Braun et al. 1981; McCarty and McKinney 1961).

In one of the landmark studies on ammonia inhibition, Angelidaki and Ahring (1993) conducted tests in a continuously fed digester operating on cattle manure and found that at TAN concentrations of 4 and 6 g-N liter^{-1}, methane production was reduced by 75% in comparison to the control reactor operating at 1.5 g-N liter^{-1}, from 0.2 liters g-VS^{-1} to 0.05 liters g-VS^{-1}. Methane production did not decrease immediately – it occurred gradually over the course of more than 3 weeks. As the methane production fell, there was a corresponding rise in the VFA concentration to 4 g liter^{-1} (as acetate). After an additional 30 days, methane production recovered somewhat to 0.15 liters g-VS^{-1} and the VFA decreased to 3 g liter^{-1} (as

acetate). To determine the impact of a TAN concentration of 6 g-N liter^{-1} on methanogenesis, specific methanogenic activity (SMA) testing was conducted. In these batch tests, substrate was fed to inoculum from the digester maintained at 6 g-N liter^{-1} and to inoculum from the control reactor. The initial methane production is then measured. The SMA is defined as the methane production rate divided by the VSS in the inoculum, which serves as a proxy for the active biomass in the inoculum. Using acetate as the substrate, methane production from the high-ammonia reactor sludge was 73% lower than the control. Using H_2/CO_2 as the substrate, methane production from the high-ammonia reactor sludge was only 52% lower than the control. From these results, the authors conclude that the high ammonia concentrations are more inhibitory to the aceticlastic methanogens than to the hydrogenotrophic methanogens. The authors note that to some extent, ammonia inhibition is self-correcting. As the ammonia level is increased and methanogenesis is inhibited, VFA tend to build up, thereby reducing the pH and increasing the equilibrium ratio of NH_4^+ to NH_3, resulting in a lower but stable methane production rate.

The same team (Angelidaki and Ahring 1994) continued work on ammonia inhibition by studying the impact of temperature at a given total ammonia load in semi-continuous (fed 4 times daily), stirred 3-liter (working volume) reactors operating on cattle manure at a 15 day HRT. Two ammonia levels were used in testing: the background TAN content of the manure (2.5 g-N liter^{-1}), and a high level (6.0 g-N liter^{-1}) achieved through the addition of NH_4Cl. The temperature of the low-ammonia reactor was increased in two steps of 3 °C each from 55 °C to 61 °C. After each step, biogas production decreased but eventually

recovered to the original level, although an increase in VFA was noted with increasing temperature. For the high-ammonia reactor, the biogas production at 55 °C was already lower than the low-ammonia reactor at the same temperature. After the first step increase to 58 °C, biogas production dropped but recovered to the baseline value. However, after the next step (to 61 °C), biogas production dropped sharply and did not recover. As with the low-ammonia reactor, VFA concentration rose with temperature and correlated well with the calculated concentration of free ammonia. A calculated free ammonia concentration of 0.7 g-N liter^{-1} was a threshold value beyond which VFA rose sharply. The authors concluded from the study that sensitivity to ammonia is greater at higher temperatures and that free ammonia is the inhibitory form of ammonia.

Borja et al. (1996) also conducted experiments on the effect of ammonia inhibition during digestion of cattle manure in six 3-liter (working volume) continuously-fed UASB reactors at a 15 day HRT under thermophilic (55 °C) conditions. The inoculum used in the UASB reactors was sludge from a lab-scale thermophilic CSTR digester fed cattle manure with a TAN concentration of 0.5 g-N liter^{-1}. NH_4Cl was added to give the desired TAN concentration in the reactors. The results showed that TAN concentrations above 5 g-N liter^{-1} were inhibitory. After six months of adaptation, the digester operated stably up to 7 g-N liter^{-1}, but methane yield was reduced as compared to the control at 3 g-N liter^{-1}. VFA concentrations were also higher: 3 g liter^{-1} (as acetic acid) as compared to 1 g liter^{-1} in the control. The cycle often seen with a sudden increase in ammonia concentration – a large fall in methane production and corresponding rise in VFA concentrations followed by partial

recovery – could be avoided by gradually increasing the TAN concentration to the highest level tested, 7 g-N liter^{-1}. However, regardless of whether the change in ammonia concentration is gradual or sudden, the final specific methane production was approximately the same: 0.15 liters-CH_4 g-VS at 7 g-N liter^{-1} (corresponding VFA concentration was 3 g liter^{-1}). Specific methanogenic activity (SMA) tests conducted on inoculum acclimated to 7 g-N liter^{-1} indicated that both the aceticlastic and hydrogenotrophic methanogens are affected by ammonia inhibition. Using acetate as a substrate, the SMA was 72% lower for the high-ammonia sludge as compared to the control sludge. Using hydrogen as substrate, the SMA was 56% lower. Toxicity testing suggests that the aceticlastic methanogens are more sensitive to ammonia than the hydrogenotrophic methanogens. The ammonia concentration at which the microbial growth rate (as measured indirectly by methane production rate) was reduced by 50% was 4.0 g-N liter^{-1} and 7.5 g-N liter^{-1} for aceticlastic and hydrogenotrophic methanogens, respectively.

Hansen et al. (1998) conducted batch tests at 55 °C on swine manure with elevated concentrations of ammonia achieved by adding NH_4Cl. Inoculum for the batch tests came from a laboratory CSTR digester operating on cattle manure at an HRT of 15 days and a temperature of 55 °C. The specific growth rate of the microbial consortium, as estimated from the portion of the batch test where methane production increased exponentially, was not affected by the TAN concentration until the level reached 5.1 g-N liter^{-1}, corresponding to a free ammonia concentration of 1.4 g-N liter^{-1}. At this level, the growth rate decreased by 36% compared to the growth rate at 3.1 and 4.1 g-N liter^{-1}. At a TAN concentration of 8.1 g-

N liter^{-1} (the highest level tested), corresponding to a free ammonia concentration of 1.9 g-N liter^{-1}, the growth rate decreased by 79% compared to the growth rate at 3.1 and 4.1 g-N liter^{-1}. The authors conclude that the threshold for ammonia inhibition is a free ammonia concentration of 1.1 g-N liter^{-1}. The increase in VFA concentration with increasing ammonia concentration indicates that methanogenesis is the rate-limiting process under these conditions; acidogenesis and acetogenesis are independent of ammonia concentration in the range tested.

In batch testing, ammonia inhibition is characterized by a lag phase, in which the onset of methane production is delayed in comparison to the control. Braun et al. (1981) found that the lag phase increased rapidly at TAN concentrations of 2.2 g-N liter^{-1} and above when the nitrogen was added as ammonia gas. At 3.4 g-N liter^{-1} of TAN, despite a lag phase of 80 days, the volume of biogas produced was the same as at 2.2 g-N liter^{-1}. Interestingly, when ammonia nitrogen was added as NH_4Cl, inhibition was not observed even at concentrations as high as 4.8 g-N liter^{-1}. Lay et al. (1998) hypothesized that the effect on lag phase may be mechanistically distinct from the effect on growth rate. They further hypothesized that the lag phase may be related to adaptation of the microbes. Batch testing was conducted under mesophilic conditions (37 °C) in 120-mL glass bottles using sludge cake from a municipal wastewater treatment plant as the substrate and seed sludge from a lab digester that had been operating on the sludge cake for 1.5 years. The independent variables were ammonium concentration and pH. For each bottle, cumulative methane production was measured over time and the resulting data was fit using the modified Gompertz equation

(Cho et al. 1996; Zwietering et al. 1990; Zwietering et al. 1992). The model contains a parameter that represents the lag time and another parameter that represents the maximum specific methane production rate (mL-CH_4 gVS^{-1} d^{-1}) during incubation. The authors concluded that the maximum specific methane production rate correlates better to the ammonium nitrogen concentration than to the free ammonia concentration. As the ammonium concentration (NH_4^+-N) increased to 4.09-5.55 g-N $liter^{-1}$, methane production fell 50% and ceased altogether at 5.88-6.00 g-N $liter^{-1}$. On the other hand, the lag phase seemed to depend more on the free ammonia concentration; concentrations of free ammonia nitrogen above 0.5 g-N $liter^{-1}$ represented a shock load to unacclimated bacteria and caused a lag phase.

Much of the work on ammonia inhibition has been conducted as CSTR or batch tests. Recently, Garcia and Angenent (2009) studied ammonia inhibition in 5-liter anaerobic sequencing batch reactors operating on swine waste at 25 °C using inoculum from an expanded granular sludge bed (EGSB) reactor treating brewery wastewater. These digesters used biogas recirculation for intermittent mixing. During test period 1, baseline digester performance was established. Methane yield was 0.31 liters-CH_4 g-VS^{-1} at the background ammonium concentration in the manure of 1.2 g-N $liter^{-1}$. When the ammonium concentration was artificially raised to >4.0 g-N $liter^{-1}$ (corresponding to a free ammonia concentration >80 mg-N $liter^{-1}$), methane yield decreased by 45% as compared to the low-ammonia control reactor. By increasing the reactor temperature to 35 °C, the methane yield rebounded such that it was only 13% less than the low-ammonia control reactor, despite an

increase in the calculated free ammonia concentration to ~250 mg-N liter^{-1}. The authors assert that the 10 °C increase in temperature doubled the rate constants of methanogenesis, which at 25 °C is rate limiting, thereby offsetting the tendency of the increasing free ammonia concentration to inhibit methanogenesis.

Hendriksen and Ahring (1991) examined the effect of ammonia on individual species of thermophilic hydrogenotrophic methanogens. The four species tested were: (1) *Methanobacterium thermoautotrophicum* strain ΔH, (2) *Methanobacterium thermoformicicum*, (3) a *Methanogenium* species and (4) a putative *Methanobacterium thermoautotrophicum* strain BA. For all strains tested the threshold inhibitory level of TAN was 3.0-4.0 g-N liter^{-1}. *M. thermoautotrophicum* strain ΔH was the most sensitive of the species tested, with a sharp reduction in growth rate at TAN concentrations above 4.0 g-N liter^{-1}. At a TAN concentration of 6.0-6.5 g-N liter^{-1}, a 50% reduction in growth rate was observed among all species. *Methanobacterium thermoformicicum* and the *Methanogenium* sp. were able to grow, albeit slowly (25% of initial growth rate), even at 9.0 g-N liter^{-1}.

Jarrell (1987) also examined ammonia inhibition on individual methanogens: *Methanospirillum hungatei*, *Methanosarcina barkeri*, *Methanobacterium thermoautotrophicum* and *Methanobacterium formicicum*, all hydrogenotrophic methanogens. *M. hungatei* was the most sensitive; a 50% reduction in methanogenesis was observed at an NH_4Cl concentration of 4.2 g-N liter^{-1}. The NH_4Cl concentrations causing a 50% reduction in methanogenesis was 10.5, 11.9, and 19.8 g-N liter^{-1} for *M. formicicum*, *M. barkeri*, and *M. thermoautotrophicum* respectively. These tests were only intended to

evaluate the initial methane production (2.5 hours) from the samples. As Hashimoto (1986) found, the effects of ammonia inhibition can take days to become apparent.

Selected results from ammonia inhibition studies are summarized in Table 1. A simple regression analysis was conducted to help identify any correlation between the independent variables of temperature, total ammonia nitrogen and free ammonia versus the dependent variable of methane yield reduction (relative to the control). The experimental results shown in this Table do not bear out the assertion that free ammonia is the form of nitrogen responsible for inhibition. Methane yield reduction correlated poorly ($R^2 = .006$) with free ammonia concentration; methane yield reduction correlated much better ($R^2 = .76$) with total ammonia nitrogen (TAN). This observation is consistent with the data presented by Sung and Liu (2003), who showed a dramatic decrease in methane production as the TAN concentration increased from 4.92 to 5.77 g liters^{-1}. As the TAN concentration increased, the pH decreased due to an increase in VFA concentration. The calculated free ammonia concentration was actually lower at a TAN concentration of 5.77 g liters^{-1} than at a TAN concentration of 4.92 g liters^{-1}, because of the pH reduction. Despite the lower free ammonia concentration at 5.77 g liter^{-1} TAN, methane production nonetheless decreased.

When the methane yield reduction was regressed against both TAN and temperature, the R^2 value was 0.80, but the temperature coefficient was negative, resulting in a predicted modest decrease in the methane yield reduction with increasing temperature, in contrast to predictions based on considerations of free ammonia, which increases with temperature at a given TAN concentration. The wide variation in temperature effects on ammonia inhibition

is exemplified by Hashimoto (1986) and Gallert & Winter (1997), who saw very little difference in ammonia inhibition at mesophilic and thermophilic temperatures, versus Hansen et al. (1998), who saw a dramatic reduction in methane yield with temperature at a fixed TAN concentration. In general, ammonia inhibition results are highly variable, and may reflect differences in reactor type/configuration, degree of adaptation of the microbial consortia, substrate type, etc.

Table 1. Selected Results from Ammonia Inhibition Studies

TAN Conc. g liter^{-1}	Free Ammonia Conc. g liter^{-1}	Temp °C	Methane Reduction (relative to control)	Reactor Type/ Analysis Type	Feed	Reference
2.500	0.200	55	0.0%	CSTR	cattle manure	(Hashimoto 1986)
3.075	0.084	30	19.8%	Batch/SMA	VFA	(Vanvelsen 1979)
4.000	0.020	30	50.0%	Batch/SMA	acetate	(Soubes et al. 1994)
4.000	0.080	25	45.0%	ASBR	swine manure	(Garcia and Angenent 2009)
4.000	0.390	55	0.0%	CSTR	cattle manure	(Hashimoto 1986)
4.920	0.096	55	39.0%	CSTR	non-fat dry milk	(Sung and Liu 2003)
4.990	0.137	30	55.0%	Batch/SMA	VFA	(Vanvelsen 1979)
5.000	1.174	55	25.0%	CSTR	cattle manure	(Angelidaki and Ahring 1993)
5.020	0.257	35	71.4%	CSTR	swine manure	(Kroeker et al. 1979)
5.100	1.400	55	36.0%	Batch/growth rate	swine manure	(Hansen et al. 1998)
5.734	0.164	30	56.5%	Batch/SMA	potato juice	(Koster and Lettinga 1988)
5.770	0.055	55	64.0%	CSTR	non-fat dry milk	(Sung and Liu 2003)
7.000	1.644	55	72.0%	Batch/SMA	acetate	(Borja, Sanchez, and Weiland 1996)

1.2.4. Adaptation and Mitigation

van Velsen (1979) found that digested sewage sludge acclimated to a TAN concentration of 815 mg-N liter^{-1} can produce methane in the presence of TAN concentrations up to 5 g-N liter^{-1}, although the lag phase increases dramatically above a TAN concentration of 1.2 g-N liter^{-1}. At a TAN concentration of 5 g-N liter^{-1} (the highest concentration tested), the lag phase lasted 50 days. As methane production begins, VFA are simultaneously degraded, except in the case of propionic acid, whose degradation was delayed in comparison with other VFA. In fact, at 5 g-N liter^{-1} of TAN, propionic acid remained undegraded even after 90 days of incubation. van Velsen conducted similar experiments with swine manure and found that digested manure is well acclimated to TAN concentrations in the range of 0.6-3.1 g-N liter^{-1}. For both sewage sludge and digested swine manure, the maximum gas production rate decreased slowly with increasing TAN concentrations in the range of 0.6-3.0 g-N liter^{-1}.

In tests on cattle manure at thermophilic temperatures (55 °C) with a 5 day HRT, Hashimoto (1986) found that volatile solids reduction was higher in the reactor with inoculum acclimated to higher levels of ammonia nitrogen. At the highest NH_4Cl loading rate, which resulted in a TAN concentration of approximately 5.4 g-N liter^{-1}, reactor productivity (liters-CH_4 liter^{-1} reactor working volume d^{-1}) was over four times higher in the acclimated reactor (1.77 liters-CH_4 liter^{-1} d^{-1}) as compared to the unacclimated reactor receiving the same NH_4Cl dose (0.39 liters-CH_4 liter^{-1} d^{-1}). In addition, VFA were much lower in the acclimated reactor (2.40 g liter^{-1}) compared to the unacclimated reactor (5.47 g

liter^{-1}). According to Hashimoto, for unacclimated mesophilic and thermophilic reactors, the threshold inhibitory level of TAN is approximately 2.5 g-N liter^{-1}. For acclimated reactors, in this case to a TAN level of 1.4 to 3.3 g-N liter^{-1}, the corresponding threshold inhibitory level is approximately 4 g-N liter^{-1}.

Koster and Lettinga (1988) examined the adaptation of granular sludge from an industrial UASB reactor treating wastewater from a sugar beet processing plant. Potato juice was used as substrate in batch reactors operated at 30 °C, with various amounts of NH_4Cl added to achieve the desired TAN concentration. Prior to adaptation, a TAN level of 1.9-2.0 g-N liter^{-1} was enough to cause a complete cessation of methane production. The highest TAN concentration at which methane was produced was 11.8 g-N liter^{-1}, which is 6.2 times the toxic TAN concentration for unadapted sludge; the authors denoted this ratio as the "adaptation potential". Although growth was observed at a TAN concentration of 11.8 g-N liter^{-1}, the specific methanogenic activity (SMA), measured in units of g-COD (methane) g-VS^{-1} (inoculum biomass) d^{-1} was only 1/10 of the activity at 2.3 g-N liter^{-1}. Buildup of VFA concentrations was concomitant with the decrease in SMA. From an economic viability standpoint, the authors suggest that the lowest feasible SMA in a UASB reactor is 0.1-0.15 g-COD (methane) gVS^{-1} d^{-1}, which occurs in a TAN range of 5-7.5 g-N liter^{-1}. Angelidaki and Ahring (1993) also found that the microbial consortia could be acclimated to higher levels of nitrogen if the concentration was increased gradually. With a gradual increase in TAN, 4 g-N liter^{-1} had only a slight effect on methane yield, whereas with a sudden increase in TAN to the same level, methane production decreased dramatically.

Sung and Liu (2003) examined both the chronic and acute affects of ammonia toxicity in 14-liter CSTR anaerobic reactors operated under thermophilic conditions (55 °C) with a 7-day SRT and an organic loading rate of 4 g-COD liter^{-1} d^{-1} using soluble non-fat dry milk as the substrate. TAN concentrations of 0.40, 1.20, 3.05, 4.92, and 5.77 g-N liter^{-1} were tested in a stepwise fashion; at each step, the microbial consortia was given time to adapt to the TAN concentration, achieved by adding NH_4Cl to the incoming feed. At TAN concentrations of 4.92 and 5.77 g-N liter^{-1}, methane production decreased by as much as 39% and 64%, respectively, as compared to the control. Batch testing of specific methanogenic activity (SMA) revealed an interesting pattern as the inoculum adapted to higher and higher levels of TAN. While acclimated inoculum could tolerate higher levels of ammonia, it produced less methane than unacclimated inoculum at non-inhibitory ammonia concentrations, as if the physiological changes required allowing it to tolerate high ammonia levels come at a cost; maximum methane production is reduced. Acclimated inoculum could also tolerate a wider pH range than its unacclimated counterpart. Methanogenesis ceased completely in the 8-13 g-N liter^{-1} range, depending on degree of acclimation and pH. Using a Monod-based model to fit the data, the authors were able to show that ammonia exhibits characteristics of uncompetitive inhibition.

Several researchers have evaluated ways to mitigate the effect of ammonia on anaerobic digestion. Borja et al. (1996) added zeolite (2% w/v) to both continuous and batch reactors, under the hypothesis that zeolite's ion exchange capacity can reduce the activity of ammonium ions. In the continuous reactors, at TAN concentrations of 4 g-N liter^{-1}, the

methane yield decreased in the control reactors but not in the zeolite reactors. Methane yield in the control reactor did recover over a period of 29 days, suggesting acclimation of the microbial community. At 5 g-N liter^{-1}, specific methane yield in both reactors dropped precipitously, but the zeolite reactor recovered to its original level (0.28 liters-CH_4 gVS^{-1}). Methane yield in the control reactor improved to 0.19 liters gVS^{-1}, but did not reach the original level (0.25 liters gVS^{-1}). VFA concentrations mirrored methane production. At 4 g-N liter^{-1}, the control reactor exhibited an increase in VFA concentration but the zeolite reactor did not. At 5 g-N liter^{-1}, both reactors were affected but VFA in the zeolite reactor eventually returned to the original levels. Similar behavior was found in the batch tests. With the addition of zeolite, the lag phase prior to the onset of gas production was shorter for zeolite reactors, with the difference more pronounced at higher TAN concentrations. At 7 g-N liter^{-1}, the control reactor produced only inconsequential amounts of methane even aver 50 days of incubation, whereas the zeolite reactor began producing methane normally after 20 days.

Hansen et al. (1999) tested five different techniques for improving specific methane yield in a CSTR digester operating on swine waste in which the TAN concentration was 6.0 g-N liter^{-1}. Addition of glauconite improved specific methane yield at thermophilic temperatures (55 °C) from 67 to 90 mL-CH_4 g-VS^{-1}. The proposed mechanism was the same as that for zeolite – the glauconite's ion exchange characteristics reduced the activity of ammonium. Activated carbon also gave the specific methane yield a boost; the authors suggest that the benefit of activated carbon may be in the removal of sulphide from solution,

a known inhibitor of anaerobic digestion (Chen et al. 2008). Because the sulphide concentration was lower than reported in the literature as inhibitory, the authors theorized that the sensitivity to sulphide may be greater when the ammonia concentration is high. Another technique used to improve methane yield was to allow the digester contents to settle prior to removal of effluent and addition of substrate. The principle, like that used in anaerobic sequencing batch reactors, is to more effectively retain the active biomass in the reactor, thereby improving the solids retention time for a given HRT. Increasing HRT also had a positive effect on methane yield, as might be expected, since this will also lead to a higher biomass concentration in the reactor. Adding granular sludge from an operational UASB reactor also improved methane yield. As with settling and increasing HRT, adding granular sludge effectively increases the amount of active biomass in the digester.

Krylova et al. (1997) added 10% (w/v) powdered phosporite ore to batch anaerobic digesters using poultry manure as a substrate in an effort to mitigate the effects of high ammonia concentrations. Substantial improvements in methane production were observed at TAN concentrations as high as 7.8 g-N liter^{-1}. At higher TAN concentrations (13.2 g-N liter^{-1}), an irreversible inhibition of methanogenesis occurred; at this level, addition of phosphorite was unable to reverse the effects.

Co-digestion with a lower ammonia feedstock is an obvious way to mitigate ammonia inhibition. Balancing the carbon:nitrogen ratio in the feedstock is one method for ensuring that ammonia nitrogen will not exceed inhibitory levels. Sievers and Brune (1987) studied the impact of carbon:nitrogen ratio on digester performance. Testing was conducted in 2-

liter reactors using organic loading rates of 1.12, 2.24, and 4.00 g-VS liter^{-1} d^{-1} with a HRT of 15 days. Inoculum was sourced from a 189-liter digester operating on swine waste. At each loading rate, five C/N ratios were tested: 2/1, 6/1, 16/1, 20/1, and 25/1. The baseline feedstock was swine waste; urea or glucose was added as necessary to achieve the desired C/N ratio as measured by total organic carbon (TOC) and total Kjeldahl nitrogen (TKN). Over the range of C/N ratios tested, the authors found that at the higher ratios (19/1 and above), digesters were unstable and were sensitive to any increases in organic loading rate. At lower ratios, inhibition by free ammonia was a suspect in decreased specific methane production, as measured by mL-CH_4 g-TOC^{-1}. The maximum specific methane production occurred at a C/N ratio of 19/1 at an organic loading rate of 2.24 g-VS liter^{-1} d^{-1}. However, because of unstable operation, the authors recommend a C/N ratio of 16/1, which resulted in more stable operation with minimum loss in specific methane production.

The technique of co-digestion of a high-ammonia feedstock with a low-ammonia feedstock was also utilized effectively by Gelegenis et al. (2007). In this study, diluted poultry manure with a TAN concentration of 4.9 g-N liter^{-1} was co-digested with olive mill wastewater (OMW) with a very low TAN concentration (<1 mg-N liter^{-1}). This strategy reduced the concentration of ammonia in the digester to below the threshold level above which digester performance is impaired. A second benefit of combining poultry manure with OMW is that the alkalinity of OMW is near zero; a digester operating on OMW alone would be very sensitive to imbalances between acid production and consumption in the digester. Poultry manure, like most manure, is high in alkalinity; the alkalinity of the manure used in

this study was 20.2 g liter^{-1} (as $CaCO_3$), thereby providing excellent buffering capacity. For a mixture of 25% OMW and 75% manure (v/v), the reactor productivity was 0.37 liters-CH_4 liter^{-1} d^{-1}.

In the Southeastern United States, the primary method for removing manure from swine production facilities is to flush the waste out with copious amounts of water. The resulting wastewater is very dilute and utilizing this wastewater in mesophilic or thermophilic digesters would be cost-prohibitive because of the amount of energy required to heat up the excess water. Hill et al. (1986) re-concentrated this wastewater using a Sweco vibrating separator. A portion of the methane potential ends up in the filtrate and is therefore lost during this process, estimated to be approximately 40% of the total (Hill et al. 1985). As compared to the as-excreted waste, however, both TKN and TAN levels are substantially reduced from roughly 5 to 2 g-N liter^{-1} for TKN and from 2-2.5 to 0.4-0.5 g-N liter^{-1} for TAN. Excellent performance was achieved on the re-concentrated waste; at a 10 day HRT under thermophilic conditions with an organic loading rate of 5.8 g-VS liter^{-1} d^{-1}, reactor productivity was 2.71 liters-CH_4 liter^{-1} (working volume) d^{-1}, specific methane productivity was 0.72 liters gVS^{-1} destroyed, and VS reduction was 65.6%.

Recovery of digesters that are in an ammonia-inhibited state is an important practical consideration because of inevitable variations in feedstock composition. Nielsen and Angelidaki (2008) evaluated several strategies for digester recovery following ammonia inhibition. Both batch and continuous tests were conducted on cattle manure at 55 °C. Inoculum for the batch tests came from a lab-scale CSTR digester treating cattle waste and

for the CSTR tests from a pilot-scale digester treating cattle waste. The CSTR operated at a 15 day HRT. At time zero, both the batch and continuous reactors were injected with a pulse load of NH_4Cl designed to raise the ammonia nitrogen concentration to an inhibitory level. Based on earlier work by the same lab, the target was to exceed a calculated free ammonia concentration of 1.1 g-N liter^{-1}. Recovery strategy 1 (RS1) was non-intervention; for batch tests this meant continued incubation; for CSTR tests, this meant continuation of daily feeding with fresh manure such that the TAN concentration would gradually decrease through washout. Recovery strategy (RS2) was to dilute the digesters with distilled water down to a pre-chosen ammonia concentration. Recovery strategy (RS3) was to dilute the digesters with effluent from an operational digester treating cattle manure. Recovery strategy 4 (RS4) was to dilute the digesters with fresh manure to cause an immediate lowering of the ammonia concentration. In the batch tests, in the 3 days following the ammonia pulse but prior to recovery strategy implementation, the methane production decreased by 53%. The results of the batch testing indicated that RS4 (dilution with fresh manure) might be the best strategy because it led to the quickest recovery (full recovery in just 3 days) and greatest overall methane production. RS2 (dilution with water) also led to full recovery but it took much longer. Comparison in the batch test is challenging, however, because each recovery strategy led to a different volatile solids content in the batch vials. For the CSTR reactors, the ammonia pulse caused an immediate and precipitous drop in methane production. The corresponding rise in VFA concentration was modest, prompting the researchers to conclude that the overall process was inhibited, not just methanogenesis. Because of the slow dilution

of the RS1 strategy, methane production did not resume until 13 days after the ammonia pulse. Interestingly, a sharp rise in VFA occurred just prior to the resumption of methane production, suggesting that acidogenic fermentation recovered just prior to methanogenesis. As in many other digesters that have experienced upsets, propionate is slow to return to pre-upset concentrations, possibly making it the best indicator of the return of the digester to normal operation. Despite the immediate dilution with water, the RS2 CSTR recovered only one day faster than the RS1 CSTR and because of the lost substrate due to dilution the RS2 CSTR had an overall lower methane output by the end of the experiment. In the RS3 CSTR, addition of active non-inhibited biomass that also served to lower the ammonia concentration resulted in a rapid recovery of the digester, as defined by resumption of methane production and lowering of VFA, in only 5 days after the ammonia pulse. In the RS4 CSTR (dilution with fresh manure), recovery was also rapid (5-6 days); this digester produced a surplus of methane during recovery because of the additional organic loading associated with this recovery strategy in comparison to the other recovery strategies. But the higher organic loading caused instability in the RS4 CSTR, as evidenced by higher VFA concentrations and lower pH. The fastest and most stable recovery process was RS3, but economics and the practicality of retaining effluent at a commercial digester site may dictate some combination between RS2, RS3 and RS4.

1.3. Research Topic 2: High-Lipid Substrates and LCFA Inhibition

1.3.1. Lipid Metabolism in Anaerobic Digesters

Feedstocks with high lipid content are an attractive feedstock for anaerobic digestion because they can greatly boost the amount of methane produced per unit of volatile solids fed. In theory, 1.01 liters-CH_4 (at STP[1]) can be produced from one gram of oleate (a common fatty acid in animal and food processing wastes) as compared to 0.37 liters-CH_4 from one gram of glucose (Kim et al. 2004)

Lipids contained in waste streams from the animal or food processing industry are primarily in the form of triglycerides (which consist of three long chain fatty acids (LCFA) esterified to a glycerol molecule) and free fatty acids. In anaerobic digesters, triglycerides are readily hydrolyzed into their component fatty acids and glycerol with the aid of extracellular lipase enzymes produced by the microbial consortia (Weng and Jeris 1976). Hydrolysis of sheep tallow triglycerides under mesophilic conditions proceeds rapidly (Broughton et al. 1998). Non-esterified LCFA accounted for 90% of total extractable lipids within 48 hours of substrate addition.

LCFA are characterized by the number of carbon atoms and the number and location of C=C double bonds in the carbon backbone. Saturated fatty acids are those with no double bonds, mono-unsaturated fatty acids have one double bond, and poly-unsaturated fatty acids

[1] STP is an acronym for standard temperature and pressure, in this case a temperature of 0 °C and a pressure of 1 atmosphere.

have two or more double bonds. Common LCFA in food and animal processing wastewaters include linoleic (C18:2), oleic (C18:1), stearic (C18:0) and palmitic (C16:0) acids.

Syntrophic LCFA-degrading bacteria metabolize fatty acids by the mechanism of β-oxidation (Weng and Jeris 1976). Compared to the degradation of other biopolymers such as proteins or starch, β-oxidation is relatively slow. The bacteria that are capable of degrading LCFA have low growth rates (Mackie et al. 1991). In β-oxidation, one mole of a fatty acid with an even number of carbon atoms with n carbons in the backbone is oxidized into n/2 moles of acetate. H^+ serves as the electron acceptor for this oxidative degradation; thus hydrogen is produced. The thermodynamics of β-oxidation are such that if the hydrogen partial pressure builds up, further LCFA degradation will be unfavorable (Mackie et al. 1991). Thus the actions of hydrogenotrophic methanogens that utilize H_2 as an electron donor to reduce CO_2 are important in the overall synergy of the process.

The initial steps in LCFA degradation have been studied. In treating effluents from olive oil mills, Beccari et al. (1998) deduced that the saturation of oleic acid to form stearic acid was the rate limiting step in the conversion of oleic acid to palmitic acid via stearic acid. Since stearic acid could not be detected, it was assumed that a rate-limiting saturation of oleic to stearic was followed by a relatively rapid conversion of stearic to palmitic. Pereira et al. (2002) found that palmitic acid was the primary component of the LCFA that accumulate on biomass sludge upon feeding a digester with oleic acid-rich substrate, representing over 80% of the total LCFA. In further studies, Pereira et al. (2003) concluded that conversion of oleic to palmitic acid is not rate-limiting in the overall oxidation of LCFA. The accumulation of

palmitic acid on digester biomass, on the other hand, suggests that the rate of palmitic acid degradation is relatively slow. On a molar basis, palmitic acid was also the predominant LCFA identified during the anaerobic degradation of sheep tallow (Broughton et al. 1998). In this same study, the ratio of oleic acid to stearic acid under mesophilic conditions remained below 0.7 during days 3 to 30 of the digestion process. This ratio is lower than in the saponified tallow, suggesting that oleic acid was being hydrogenated to form the corresponding saturated acid (stearic). During degradation of solid poultry slaughterhouse wastes in batch tests, Salminen et al. (2000) also found a preponderance of palmitic acid, with lesser amounts of oleic, stearic and myristic acids.

1.3.2. Studies Using High-lipid Substrates

By-products from animal processing facilities typically contain high lipid contents and have been evaluated frequently as a substrate for anaerobic digestion. Despite their high methane potential, lipid-rich substrates can cause operational problems in anaerobic digesters. Depending on the source, lipid-rich substrates are often characterized by low nutrient content and low alkalinity (Angelidaki and Ahring 1997). Low nutrient content could prevent vigorous microbial growth. Low alkalinity will have a major impact on the stability of digester operation. Inevitably, small perturbations in digester operation can cause an imbalance between acid-producing acidogens and acid-consuming methanogens. With sufficient buffering, the pH of the digester remains stable and the digester can recover. Without sufficient buffering, however, such an imbalance can cause a significant drop in pH, which will further impair methanogenesis. This feed-forward inhibition of methanogenesis

can lead to a total cessation of methane production and a catastrophic drop in pH from which the digester cannot recover.

Another challenge for utilizing high-lipid substrates in digesters is flotation and scum formation. Adsorption of lipids onto active biomass can decrease the density of the biomass, causing it to float on the surface and, depending on the digester configuration, making it subject to washout (Hwu and Lettinga 1997). Similarly, foam formation can also complicate digester operation and impact the bioavailability of the substrate (Hejnfelt and Angelidaki 2009).

Using milk fat as a substrate, Perle et al. (1995) saw a 75-90% reduction in methanogenic efficiency[2] for sludges exposed to milk fat concentrations of 500 mg liter^{-1}. One objective of this study was to determine whether the inhibitory component of the milk fat triglycerides was the LCFA or the glycerol. In batch tests, the methane production from the glycerol-fed digester was equivalent to the casein-fed control, indicating that glycerol is non-inhibitory. In contrast, the oleic acid-fed (representative of LCFA in milk fat) batch reactor produced 37.5% less methane than the glycerol-fed batch reactor at the same COD loading. The ATP concentration of the oleic acid-fed reactor was approximately 5 times lower than the glycerol-acid fed reactor, another indication of reduced physiological activity.

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[2] Defined as the ratio of the specific methanogenic activity (in units of mlCH_4 gVSS^{-1} h^{-1}) of the tested sludge to the specific methanogenic activity of the reference sludge

Sheep tallow is another high-lipid substrate from the animal processing industry. Under mesophilic conditions at sheep tallow concentrations of 0 and 5 g liter^{-1}, Broughton et al. (1998) found no lag period prior to the onset of methanogenesis after substrate addition to the 20 liter batch anaerobic digesters used for the tests. The concentration of LCFA corresponded to 10 mM at 5 g liter^{-1} tallow. At higher tallow doses, lag periods of 13 and 48 days were observed for tallow concentrations of 10 g liter^{-1} and 20 g liter^{-1} respectively. Under these conditions, high levels of LCFA and VFA were observed. These results infer that syntrophic LCFA oxidation and aceticlastic methanogenesis are rate limiting at these tallow levels. Under thermophilic conditions, addition of 5 and 10 g liter^{-1} of tallow delayed the onset of methanogenesis by 43 and 48 days respectively. Even after 90 days, no methane was produced under thermophilic conditions at 20 g liter^{-1} of tallow, inferring that thermophilic reactors are more sensitive to LCFA inhibition than mesophilic reactors. Other than at this one condition, methanogens were not permanently inhibited by the tallow, despite long lag times prior to the onset of methanogenesis. LCFA originating from the tallow were completely converted to biogas. LCFA and VFA concentrations as a function of time suggest that acetogens are inhibited prior to the complete cessation of methane production, eventually causing a buildup of LCFA large enough to completely inhibit methanogenesis.

Based on studies of slaughterhouse waste in batch digesters, Sayed et al. (1988) concluded that the reduction in methanogenic activity seen after exposure to different wastewater fractions from a slaughterhouse was due to the physical adsorption of colloidal particles to the inoculant (sludge from a UASB). Adsorption of the soluble fraction of

wastewater was low, whereas high-lipid content colloidal material adsorbed quickly to the sludge upon exposure. The authors believe that the film formed around the biomass interferes with physical transport of substrates and nutrients into the active biomass. To prevent inhibition of the active biomass with lipid-rich animal processing wastes, an organic loading rate of approximately 0.34 g-COD g-VSS^{-1} d^{-1} should not be exceeded (Sayed et al. 1987). Five days per week feeding is also recommended to allow liquefaction of complex substrates to take place on the two non-feeding days.

The degradation pattern for solid poultry slaughterhouse wastes (lipids represent 10% of TS) matches that for other lipid-rich substrates (Salminen et al. 2000). Measurement of LCFA, VFA and methane production over time for a batch assay revealed rapid hydrolysis and acidogenesis with a concomitant rise in LCFA and VFA. After a lag period of 6 to 9 days, methane production rose rapidly with a corresponding decline in LCFA and VFA. After 27 days of incubation, LCFA were below the detection limits. The ratio of substrate to inoculum affected both the extent of the lag period and the maximum methane production rates. Higher inoculum to substrate ratios had shorter lag times and higher methane production rates.

In a follow-up study, Salminen and Rintala (2002) studied the impact of hydraulic retention time and loading on the anaerobic digestion of solid poultry slaughterhouse waste in a semi-continuous 2-liter mesophilic (31 °C) digester. Stable operation was achieved at organic loading rates up to 0.8 g-VS $liter^{-1}$ d^{-1} and hydraulic retention times of 50-100 days. Methane yield per gram of volatile solids added was 0.52-0.55 liters g-VS^{-1}. Compared to

operation on manure substrates, the maximum achievable organic loading rate is quite low and the hydraulic residence times remarkably long. The authors suggest that co-digestion with complementary wastes could enable a stable process at higher organic loadings and/or lower HRT. Attempts to increase the organic loading rate or reduce the hydraulic residence time caused accumulation of VFA and LCFA and a reduction in methane yield. This inhibition was reversible, however, by a suspension in feeding or a reduction in the organic loading rate.

Edstrom et al. (2003) studied several residues from animal processing (animal by-products, blood, stomach contents, and sludge from wastewater treatment) in various combinations with food waste and manure. The animal by-products had a fat content of 35-40% (of total solids). Tests were conducted in a 3-liter (working volume) fed-batch reactor along with a lab-scale CSTR. At an organic loading rate of 2 g-VS liter^{-1} d^{-1}, the fed-batch reactor produced 0.76 liters-CH_4 g-VS^{-1} fed for substrate that had been pasteurized prior to addition to the reactor. Specific methane production for unpasteurized substrate was much lower at 0.31 liters-CH_4 g-VS^{-1} fed. Establishment of stable operation in the lab CSTR was challenging. The highest organic load achieved was 5 g-VS liter^{-1} d^{-1} at an HRT of 22 days. At these conditions, specific methane production was 0.56 liters-CH_4 g-VS^{-1} fed. Unstable operation for the substrates used in these tests could be due to inhibition by either ammonia or LCFA or both. While LCFA levels were not measured, the high fat content of the substrates make LCFA an inhibition suspect. Total ammonia nitrogen levels were measured;

calculated free ammonia concentrations were in the range of inhibitory concentrations reported in the literature.

Jeganathan et al. (2006) evaluated anaerobic digestion of an oily wastewater from a rendering plant in three lab-scale UASB reactors operating at 35 °C. Operational problems caused by the high-lipid substrate were not caused by inhibition. Instead, the loading of the sludge granules with fatty material decreased the density of the sludge, eventually leading to flotation and washout. Even in the absence of washout, floating sludge is not available for the conversion of substrate, so the effective biomass in the reactor is reduced accordingly. The threshold fats, oil, and grease (FOG) loading above which floating and washout occurred was found to be 1.04 g-FOG g-VSS^{-1}. The primary constituent of the FOG was palmitic acid.

Swine processing by-products were the subject of a recent study by Hejnfelt and Angelidaki (2009). They looked at five different by-products: fat, blood, raw waste (meat, fat and bones), intermediate product (pressed raw waste) and bone flour. In addition, they also evaluated homogenized mixed pork waste representing all non-commercial waste from one processed pig. Tests were conducted under thermophilic (55 °C) conditions in 0.5 or 2.0 liter batch reactors. Inoculum came from a full-scale commercial digester in Denmark. Specific methane yields were measured for each substrate and compared to the maximum theoretical methane potential based on composition. In addition to the batch tests, semi-continuous tests were conducted in 3.2 liter (working volume) CSTRs using different ratios of manure and the homogenized mixed pork waste and the same inoculum as for the batch

tests. HRT was 21 days. Two different organic loading rates were tested, 8.3 g-VS d^{-1} and 12.5 g-VS d^{-1}. In general, the wastes studied were characterized by high protein levels and high lipid levels. In the batch tests, specific methane yields were in the range of 0.225-0.619 liters g-VS^{-1}. Dilution of the by-products was found to improve methane yield because LCFA and/or ammonia concentrations were inhibiting digestion. LCFA concentrations higher than 5 g-lipids $liter^{-1}$ and TAN concentrations higher than 7 g-N $liter^{-1}$ were found to be inhibitory. Lag times prior to the onset of methanogenesis were 3-5 days, with the exception of fat, which had a lag time of 20 days, indicative of LCFA inhibition. For the continuous tests, stable operation could not be achieved at thermophilic temperatures at an organic loading rate of 12.5 g-VS d^{-1} on a mixture of manure and homogenized mixed pork waste. At 8.3 g-VS d^{-1}, a very low specific methane yield of 0.061 liters g-VS^{-1} was achieved. Under mesophilic conditions at the same organic loading rate and HRT, the specific methane yield of the manure and homogenized mixed pork waste (0.489 liters g-VS^{-1}) was 40% higher than the yield of manure alone.

In a 5-liter anaerobic sequencing batch reactor (ASBR) operating at 35 °C, Martinez-Sosa et al. (2009) evaluated the digestion of the skimmings from a dissolved air flotation system treating wastewater from a cooked pork processing plant. Fats represented 74% of total solids and volatile solids represented over 99% of total solids. The fat fraction was primarily in a non-hydrolyzed state; triglycerides represented 80% of the fat fraction and diglycerides represented another 15.5%. Of the LCFA present, the most predominant were myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and

linoleic acid (C18:2). Inoculum used for testing was sludge from an anaerobic reactor treating distillery vinasse. The specific methane achieved was a remarkable 0.88 liters gVS^{-1} fed, which is approaching the theoretical potential for a typical fatty acid (1.01 liters for oleate). Consistent with the high methane yield, the total solids conversion was also very high (97%), indicating a substrate with extraordinary biodegradability.

1.3.3. Inhibition Studies Using Exogenous LCFA

It has long been known that LCFA inhibit bacterial growth and that inhibition depends on both the number of carbons and the degree of unsaturation (Galbraith et al. 1971). In particular, inhibition increases with the number of double bonds (Nieman 1954). For saturated fatty acids, maximum inhibition occurs for fatty acids having around 12 carbons (Nieman 1954).

Koster and Cramer (1987) were among the first to look at the inhibition of anaerobic digestion by LCFA. The impact of four saturated LCFA (caprylic, capric, lauric and myristic) along with one mono-unsaturated LCFA (oleic) on methanogenesis was evaluated in batch tests at 30 °C using acetate as the feedstock. The inoculum used in the tests was granular sludge from a commercial UASB reactor treating potato processing wastewater. The measure of performance was the maximum specific methanogenic activity, in units of μliters-CH4 $g\text{-}VS^{-1}$ h^{-1}. Two key concentrations were identified for each LCFA, the threshold concentration above which specific methanogenic activity starts to decline and the concentration at which specific methanogenic activity has declined by 50%. The inhibition threshold concentration was 6.75mM, 2.6mM, 1.6mM, 2.6mM, and 2.4mM for caprylic,

capric, lauric, myristic, and oleic acids respectively. The concentrations causing a 50% reduction in specific methanogenic activity were >10mM, 5.9 mM, 4.3mM, 4.8mM and 4.35 mM for caprylic, capric, lauric, myristic, and oleic acids respectively. Additional tests were conducted on lauric acid to separate the effects of concentration and LCFA:biomass ratio. In this study, the concentration of the LCFA was a better predictor of inhibition than the LCFA:biomass ratio. The buildup of VFA under inhibitory conditions is an indicator that methanogenesis is more readily inhibited than acidogenesis. Combinations of LCFA were also studied, the results of which showed that the inhibitory level of a particular LCFA was lower in the presence of a second LCFA. These synergistic effects are important since fatty substrates will almost always contain a mixture of LCFA.

Angelidaki and Ahring (1992) examined the degradation of acetate, propionate, and butyrate in the presence of variable amounts of oleate and stearate. Oleate was slightly more inhibitory than stearate; initial inhibitory levels for oleate were 0.1-0.2 g liter^{-1} as compared to approximately 0.5 g liter^{-1} for stearate. The effect of the LCFA on batch anaerobic digestion was to increase the lag time prior to the onset of substrate degradation. Both oleate and its corresponding triglyceride (glycerol trioleate) were tested. Addition of oleate caused immediate cessation of methane production from which the authors concluded that the free fatty acid was the inhibitory compound, not the other product of hydrolysis (glycerol) or the triglyceride itself. The effect of LCFA appeared to be concentration dependent and not dependent on the ratio of LCFA to active biomass, since the inhibitory concentration was the same regardless of the amount of active biomass in the test vial. This is not consistent with

the view of Henderson (1973) that surface active fatty acids adhere to the cell wall and interfere with transport processes through the cell membrane. This mechanism would suggest that it is the fatty acid to biomass ratio that is the relevant indicator of inhibition, not concentration.

Rinzema et al. (1994) evaluated the impact of shock loads of LCFA (using capric acid as a model LCFA) on the activity of granular sludge from a UASB reactor. Testing was conducted in stirred batch reactors. Like Angelidaki and Ahring (1992), these authors also concluded that inhibition is related to LCFA concentration, not LCFA-to-biomass ratio. Concentrations exceeding 6.7-9.0 mM "virtually completely" kill the acteogenic and methanogenic microbial populations. The exponential growth of small numbers of survivors accounts for the eventual resumption of microbial growth, starting with acetogenic bacteria and hydrogenotrophic methanogens followed by aceticlastic methanogens. This hypothesis differs from other researchers who conclude that the bulk population recovers, not just a few survivors. This point may be somewhat academic; a long lag phase is industrially impractical regardless of the mechanism involved. Even at sub-toxic concentrations, the lag period between substrate addition and methane production increases with increasing LCFA concentration. The lag phase is interpreted by the authors as an imbalance between β-oxidation and methanogenesis due to the inhibition of acetotrophic methanogens.

Hwu and Lettinga (1997) conducted similar tests on oleate in batch tests at mesophilic and thermophilic temperatures using acetate as a substrate. The concentration at which the specific methanogenic activity decreased by 50% varied widely (factor of 12)

depending on the source of the sludge and the temperature. In general, however, they concluded that the sensitivity to oleate was higher at thermophilic temperatures (55 °C) as compared to mesophilic temperatures (both 30 °C and 40 °C were tested). At 55 °C the IC_{50} for oleate was 0.79mM and 0.35mM for the two thermophilic sludges tested. At 30 °C, the IC_{50} for oleate for the most tolerant sludge, 4.30mM, was quite comparable to the value (4.35mM) found by Koster and Cramer (1987). The difference in sensitivity exhibited by the two thermophilic sludges is attributed by the authors to morphological differences in the sludges. The more resistant thermophilic sludge was a dense granular sludge and the more susceptible sludge was a flocculent, high surface area sludge. Earlier work by Hwu et al. (1996) had concluded that sludge specific surface area plays a role in acute oleate toxicity.

In later work, Hwu et al. (1998) tested a mixture of LCFA consisting of 82% (w/w) oleate (C18:1) and palmitate (C16:0) in an expanded granular sludge bed (EGSB) reactor operating at 55 °C. EGSB, like UASB, are characterized by short HRT. After startup on glucose, acetate, and caprylate, the EGSB were fed the LCFA mixture as their sole carbon and energy source. Volatile solids reductions could not be accounted for by the methane production in the reactor. The researchers discovered that fats were accumulating in the digester in the form of a white film on the sludge granules. A floating layer of fat and sludge granules also formed that caused clogging of the biogas collector. Not surprisingly, this floating matter consisted largely of oleate and palmitate. Miranda et al. (2006) also evaluated the degradation of LCFA in high throughput, short HRT reactors, both conventional UASB reactors and downflow anaerobic expanded bed (DAEB) reactors. In all

reactors tested, biogas production, biogas methane content, and specific methanogenic activity of the sludge decreased with an increase in LCFA. Correspondingly, VFA concentrations increased. As with other studies in high throughput reactors, LCFA accumulate on the surface of the sludge granules, leading to flotation and washout. The threshold LCFA concentration for impaired performance in the UASB was 500-1000 mg-LCFA liter^{-1}. In the DAEB, the biomass is in the form of a fixed film, preventing washout. However, adsorption of LCFA on the biomass impedes contact between the biomass and the substrate.

Lalman and Bagley (2000) focused their efforts on linoleic acid (C18:2) and the metabolism thereof. Batch testing was conducted in 160 mL serum bottles at 21 °C using an inoculum mixture from digesters at a wastewater treatment plant and a food processing plant. At all concentrations tested, linoleic acid could not be detected after 25 days of incubation. No 16-carbon fatty acid with two double bonds (C16:2) was observed, indicating that hydrogenation of one double bond was at least one path for degradation, since oleic acid (C18:1) was observed transiently, as was palmitoleic acid (C16:1). Palmitic acid (C16:0) and myristic acid (C14:0) were produced stoichiometrically and may have inhibited their own further degradation; degradation times were very long (~60 days). All measured fatty acids were eventually degraded with the exception of acetic acid which accumulated. Aceticlastic methane production ceased completely in fermenters receiving 30 mg liter^{-1} or more of linoleic acid and acetate substrate (the authors note that inhibition may have been enhanced by diethyl ether, the co-solvent used to prevent linoleic acid from precipitating in the bottles).

Hydrogenotrophic methanogenesis, on the other hand, was only slightly inhibited at this same linoleic acid concentration.

Following their work with linoleic, Lalman and Bagley (2001) next turned their attention to oleic and stearic acids. The observable anaerobic degradation products at 21 °C for oleic acid (C18:1) were palmitic (C16:0) and myristic (C14:0) acids, which in turn were eventually degraded to acetate and finally methane. Interestingly, no LCFAs were observed for stearic (C18:0) acid degradation although the stearic acid was very slowly metabolized. Stearic acid was less inhibitory to aceticlastic methanogenesis than oleic acid. Whereas concentrations of oleic acid >30 mg liter^{-1} inhibited acetate degradation, concentrations of stearic acid up to 100 mg liter^{-1} did not inhibit aceticlastic methanogenesis. As with earlier work on linoleic acid, hydrogenotrophic methanogenesis was only slightly inhibited by oleic and stearic acids.

Alosta et al. (2004) looked at the inhibitory effects of oleic acid (C18:1), stearic acid (C18:0), and linoleic acid (C18:2) on glucose degradation. Batch tests were conducted in 160 mL serum bottles using inoculum originally sourced from a wastewater treatment plant and maintained in a 4 liter semi-continuous digester utilizing glucose (1.0 g liter^{-1}) as a substrate. Results showed that glucose degradation matched the control for linoleic, oleic, and stearic acid concentration of 50 and 100 mg liter^{-1}, wherein all glucose was degraded within four hours of the initiation of incubation. At concentrations at or greater than 300 mg liter^{-1} of linoleic or oleic acid, however, glucose (5-25% of the original concentration) could be detected after 8 hours. The pattern of VFA production and degradation changed

dramatically with increasing LCFA concentration. For example, butyrate was only found in cultures receiving ≥300 mg liter^{-1} linoleic, indicating that the presence of linoleic inhibits the degradation of butyrate. Also, the peak acetate concentration and the timing of that peak were significantly altered by addition of LCFA. At concentrations of linoleic acid ≥500 mg liter^{-1}, for example, acetate concentration was above 150 mg liter^{-1} and still rising after 20 days of incubation. By contrast, at concentrations of stearic acid ≥500 mg liter^{-1}, acetate concentration peaked on or before day 6 and was below 150 mg liter^{-1} after 20 days of incubation. Patterns for propionate were similar. For a given LCFA concentration, glucose degradation rates were lower for linoleic acid than for the other two LCFA, indicating that polyunsaturated LCFA may be more inhibitory than monounsaturated or saturated LCFA.

Kim et al. (2004) also looked at LCFA inhibition in batch testing. Tests were conducted in 125 mL serum bottles at 35 °C using granular sludge from a lab-scale UASB reactor treating dairy wastewater as the inoculum and acetate as the carbon and energy source. A distinct pattern emerged with regard to the inhibition by LCFA; the inhibition increased with the number of double bonds in the LCFA. The concentration at which the methane production rate dropped by 50% was 3.10mM, 0.72mM, 5.71mM, and 5.37mM, for oleate (C18:1), linoleate (C18:2), palmitate (C16:0), and stearate (C18:0) respectively. Results using propionate as the carbon and energy source were similar although the inhibitory concentrations were higher. Modeling of aceticlastic methanogenesis as a function of LCFA concentration showed that inhibition followed a non-competitive pattern. The lag phase preceding the onset of methanogenesis in batch testing was also dependent on the

LCFA concentration. By fitting the data with a modified Gompertz model, the lag phase could be mathematically modeled. The concentrations of each LCFA that induce a 5 day lag time are 5.93, 2.24, 4.02, and 2.81 mM for oleate, linoleate, palmitate, and stearate, respectively.

Ortega et al. (2008) conducted batch tests for biochemical methane production using oleic acid as substrates. In these tests, sludge from a mesophilic UASB digester treating wastewater from a cheese plant was used as an inoculant for thermophilic (after adaptation) batch tests. A specific amount of substrate (4.5 g-COD liter^{-1}) was added to 120ml test bottle and the mmoles of methane measured until no more methane was produced. The oleic acid was degraded into acetic acid only after 49 days. They concluded that this remarkable lag period was due to inhibition of aceticlastic methanogens by long chain fatty acids.

In LCFA toxicity assays, Palatsi et al. (2009) found that LCFA concentrations exceeding 1 g liter^{-1} caused inhibition. The LCFAs used were a mixture of sodium oleate, sodium stearate and sodium palmitate in a ratio of 40:10:50 (w/w/w) respectively. At LCFA concentrations of 2.5, 4.0 and 6.0 g liter^{-1}, methane production ceased temporarily but eventually recovered and bypassed the control methane production after lag periods of 12 to 20 days.

In a recent study of LCFA toxicity, Palatsi et al. (2009) concluded that the mechanism of LCFA inhibition is binding of the LCFA to the cell surface and physical inhibition of nutrient transport through the cell membrane. Pererira et al. (2002) showed that this binding is reversible and that eventually the LCFA on the biomass surface were degraded. However,

a lag phase of 500 hours preceded resumption of methanogenic activity for a sludge containing 4,570 mg-COD g-VSS^{-1}. In further studies, Pereira et al. (2003) found that once the loading of an oleic acid-based synthetic substrate reached a level of 2,861 mg-COD g-VSS^{-1}, no methanogenic activity could be detected in specific methanogenic activity testing using acetate, propionate and butyrate as substrates. Only hydrogenotrophic methanogenesis could be detected, consistent with their theory that hydrogen could diffuse through the accumulated LCFA whereas the VFA substrates could not. The accumulated LCFA were converted to methane. The methane production was quantified, giving an indirect measurement of the mass of the biomass-associated substrate. Once the accumulated LCFAs were metabolized, the specific methanogenic activity of the remaining biomass was actually enhanced as compared to the initial activity (prior to LCFA accumulation). While these tests show that inhibition is not permanent, the authors acknowledge that the inhibition mechanism could either be caused by mass transfer limitations resulting from the LCFA barrier, or a temporary inhibition of metabolic processes. The authors suggest that the ratio of absorbed LCFA to active biomass (represented as VSS) may be a better way to represent toxicity, because of the clear relationship between absorbed LCFA and methanogenic activity. At steady-state, the degree of saturation of the active biomass with LCFA appears to be a function of the LCFA concentration, not the LCFA loading rate. Pereira et al. (2004) found that the most efficient methane production rate occurs at an LCFA loading of about 1,000 mg-COD g-VSS^{-1}.

Selected results from LCFA inhibition studies are summarized in Table 2. A simple regression analysis of LCFA inhibitory level as a function of the number of carbons in the LCFA, the number of double bonds in the LCFA, and temperature gave interesting results (for this simple analysis, the threshold values were lumped in with the 50% inhibition values). The equation took the following form:

$$Inhibitory\ Level, mM\ = 12.9 - 0.123 * (T, ℃) - 0.228 * (\#\ of\ C) - 1.51 * (\#\ of\ C = C)$$

The R^2 value for the regression was 0.80. The equation is consistent with conclusions found in the literature referenced earlier which suggest that the concentration of LCFA causing inhibition decreases with temperature, the number of carbons, and the number of double bonds.

Table 2. Selected Results from LCFA Inhibition Studies

LCFA	LCFA Type	Temp °C	Inhibitory Level, mM	Methane Reduction	Reference
Caprylic	C8:0	30	10.00	50%	(Koster and Cramer 1987)
Capric	C10:0	30	5.90	50%	(Koster and Cramer 1987)
Lauric	C12:0	30	4.30	50%	(Koster and Cramer 1987)
Myristic	C14:0	30	4.80	50%	(Koster and Cramer 1987)
Palmitic	C16:0	35	5.71	50%	(Kim et al. 2004)
Stearic	C18:0	55	1.76	threshold	(Angelidaki and Ahring 1992)
Stearic	C18:0	35	5.37	50%	(Kim et al. 2004)
Oleic	C18:1	30	4.35	50%	(Koster and Cramer 1987)
Oleic	C18:1	55	0.53	threshold	(Angelidaki and Ahring 1992)
Oleic	C18:1	55	0.79	50%	(Hwu and Lettinga 1997)
Oleic	C18:1	30	4.30	50%	(Hwu et al. 1998)
Oleic	C18:1	35	3.10	50%	(Kim et al. 2004)
Linoleic	C18:2	35	0.72	50%	(Kim et al. 2004)

1.3.4. Adaptation and Mitigation

As with ammonia inhibition, some research suggests that the microbial community can be adapted to levels of LCFA that would have previously been inhibitory. Palatsi (2009) provides evidence for such adaptation in experiments wherein LCFA were fed to both batch and semi-continuous digesters as a series of LCFA pulses. From the first pulse to the fourth pulse, the recovery time was reduced from 40 days to 10 days and the maximum accumulation of VFA after each pulse reduced from 92.8 mM to 60.4 mM.

Neves (2009) also used pulses of LCFA to examine the response of a 26-liter CSTR mesophilic digester fed with cow manure at an HRT of 26 days and an organic loading rate of 1.2 g-COD liter^{-1} d^{-1}. After 123 days of operation, weekly pulses of an oily effluent (99.8% fat content) from a fish cannery were injected into the digester. The combined organic loading rate for days where the manure and oil effluent were added together was 5.0 g-COD liter^{-1} d^{-1}. The primary LCFA components of the oily effluent were oleic acid (C18:1, 43.2%), linoleic acid (C18:2, 38.3%), and palmitic acid (C16:0, 12.5%). Apparently this digester was not a true CSTR because the authors refer to a solid phase in the bottom of this reactor. In fact, the LCFA added to the reactor were quickly adsorbed to this solid phase; following a pulse, the LCFA could not be detected in the liquid phase. The primary components of the adsorbed LCFA on the solid phase were oleic (C18:1) and palmitic (C16:0), although C14:0, C18:0 and C18:2 were also detected. After the last two pulses, a decrease in oleic was observed in the solid phase, an indication that the biomass was adapting to the presence of LCFA and becoming more effective in degrading oleic.

In a similar study, Nielsen and Ahring (2006) have also provided evidence of adaptation to LCFA following increasing pulses of oleate. Two thermophilic CSTR digesters fed with mixtures of pig and cattle manure at different TS and VS concentrations were used for the studies. In both digesters, performance was severely impaired following an injection of 2 g-oleate liter^{-1}. However, 20 days after exposure, both reactors exhibited lower VFA concentrations than before the pulse, indicating that the oleate had stimulated biomass activity. In toxicity testing, the specific methanogenic activity of the active biomass in the CSTR became more tolerant to the presence of oleate after repeated exposure during the course of the experiment. The inhibitory level on day 160 of the experiment was 0.6-0.8 g-oleate liter^{-1} as compared to 0.2-0.4 g-oleate liter^{-1} on day 100. This increase in tolerance suggests an adaptation of aceticlastic methanogens to oleate over the course of the experiment. Another indicator of adaptation was an improved recovery of the CSTR after a second pulse of 2.0 g-oleate liter^{-1} was injected, as measured by a shorter lag prior to the resumption of methane production and a lower peak in the concentrations of VFA following the second pulse.

Enrichment techniques are often used to help isolate microbes within the community that are adapted to metabolism of a given substrate. The simplest form of enrichment is where the microbial community is grown on the substrate of interest, then at some point a portion of the growing culture is removed and placed on fresh substrate. Enrichment is more challenging when the desired microbe is known to only grow in syntrophic associations with other microbes. Such is the case in the β-oxidation of LCFA, where hydrogenotrophic

methanogens are needed to keep the partial pressure of hydrogen low in order to keep the thermodynamic conditions favorable for the LCFA degraders. Roy et al. (1986) used enrichment on a substrate containing oleate as the carbon and energy source to isolate a fatty acid degrading acetogenic bacterium. This Gram-negative, obligately syntrophic bacterium can only use protons as electron acceptors and was grown in association with the hydrogenotrophic methanogen, *Methanospirillum hungatei*. This strain can ferment all linear unsaturated fatty acids from 4 to 18 carbons, as well as several unsaturated LCFA (oleate and linoleate). Despite its morphological and substrate-utilizing similarities to *Syntrophomonas wolfei*, the authors have proposed this strain as a new species, *Syntrophomonas sapovorans* sp. nov., because of its broader substrate range.

Menes et al. (2001) also used classical enrichment techniques to look for LCFA degraders in a culture known to degrade the effluent from the wool scouring process, an effluent known to be lipid-rich. A portion of the growing culture was repeatedly transferred to fresh mineral medium containing oleate as a carbon and energy source. To ensure proper hydrogen scavenging, *Methanobacterium thermoautotrophicum* was added to the medium as well. After successive transfers, an oleate-degrading enrichment culture emerged that could successfully metabolize linear, saturated fatty acids with an even number of carbons from 2 to 18, with methane as the final product. The culture could also metabolize the tested seven and nine carbon linear, saturated fatty acids, with methane and propionate as the final products. Amplified ribosomal DNA restriction analysis (ARDRA) was used to identify Domain Bacteria microbes in the enrichment culture. Sequencing of one of the dominant

restriction patterns showed homology with *Syntrophothermus lipocalidus* and *Thermosyntropha lipolytica*, which are known to degrade 4-carbon LCFA and higher. However, the divergence from these known organisms is such that the authors believe that the LCFA-degrading isolate represents a new species.

Hatamoto et al. (2007) also used molecular methods to determine the microbes present in enrichment cultures grown on each of four substrates (palmitate, stearate, oleate, or linoleate). 16S rRNA gene clone libraries were constructed from the LCFA-degrading enrichment cultures. Sequencing of representative rRNA gene clones revealed that the palmitate and stearate degrading cultures were dominated by bacteria affiliated with the family Syntrophomonadaceae. Isolated bacteria from the oleate degrading culture, on the other hand, belonged to the phylum *Firmicutes*, and were not closely related to other known organisms except for a recent isolate from the same laboratory. Following isolation, however, this strain was unable to utilize palmitate as the sole carbon source. Thermophilic enrichment cultures grown on linoleate were not stable and did not survive successive transfers, which the authors attribute to the relative toxicity of linoleate compared to other LCFA and also to the difficulty in enriching syntrophic associations of microorganisms that depend, for example, on interspecies hydrogen transfer.

How the microbial community adapts to repeated exposure to LCFA in real-world conditions is not well understood. One possible scenario is enrichment of lipid-degrading organisms for digesters fed high-lipid substrates. Angelidaki and Ahring (1995) used non-traditional enrichment techniques to establish a thermophilic, stearate-degrading culture. A

fed-batch technique was used for culture enrichment wherein the culture was intermittently fed additional substrate (manure supplemented with 2% rapeseed oil) without any removal of cultural from the vessel. In this way, stearic acid levels could be carefully controlled and the culture was not shocked by transfer to fresh medium. The enriched thermophilic culture, which had a temperature optimum of 55 °C, completely converted stearate to CH_4 and CO_2. Acetate and trace amounts of butyrate were observed as intermediate products. Other LCFA with an even number of carbon atoms could also be completely degraded. Interestingly, LCFA with an odd number of carbon atoms produced, along with CH_4 and CO_2, an equivalent concentration of propionate, which could not be further degraded by the culture. The degradation patterns provide strong support for the β-oxidation degradative pathway.

Pereira et al. (2002) used denaturing gradient gel electrophoresis (DGGE) to characterize the microbial community in a digester fed increasing amounts of oleic acid. Two parallel reactors, one inoculated with granular sludge and the second inoculated with suspended sludge were used for the experiment. The test was divided into four sequential periods, corresponding to four levels of oleate in the feed. DNA was extracted, cloned, amplified and subjected to DGGE for each test period. The operating conditions resulted in the formation of a floating biomass layer and a settled biomass layer in the digester. For the Domain Bacteria, the similarity between the top and bottom communities diverged significantly from periods 1 to 4. The similarity was 86.8% in period 1 and dropped to 56.7% in period 4. Moreover, both sludges diverged from the original inoculum, although the top sludge diverged more; it was only 17.3% similar to the inoculum (the bottom sludge

was 42.8% similar to the inoculum). The archaea present in the sludges were associated with the aceticlastic *Methanosaeta* and the hydrogenotrophic *Methanobacterium*. In the reactor inoculated with suspended sludge, the DGGE bands corresponding to the *Methanosaeta*-like clones became progressively weaker as LCFA concentration increased.

Shigematsu et al. (2006) made the observation that classic enrichment techniques may lead to isolation of only those LCFA-degrading bacteria that can tolerate high concentrations of LCFA. To avoid this bias, they conducted tests in a 1.85 liter (working volume) CSTR reactor operating at 37 °C with an HRT of 2.5 days fed a wastewater enriched with emulsified oleic and palmitic acids. The inoculum used in the CSTR was mesophilic digested sludge from a domestic wastewater treatment plant. While known LCFA-degrading bacteria from the family Syntrophomonadaceae were identified in the reactor, the predominant bacteria in the reactor were from the phyla *Bacteroidetes* and *Spirochaetes*, which are not at all closely related to known LCFA-degrading bacteria. The authors suggest that perhaps these bacteria are important in LCFA degradation in environments with lower concentrations of LCFA.

Kuang et al. (2006) used fluorescence in situ hybridization (FISH) to examine changes in bacterial and archaeal populations in four 2-liter (working volume) reactors after the reactors had been fed with oleate at levels that caused complete cessation in methane production. The control continued to be fed oleate whereas the others were fed glucose, cysteine and a glucose/cysteine mix. Prior to oleate feeding, archaeal cell counts were greater than bacterial cell counts. As the methane production decreased during oleate

feeding, both bacterial and archaeal populations decreased, but the bacteria were less impacted by oleate than the archaea, consistent with research showing that LCFA are more inhibitory towards methanogenesis than acidogenesis/acetogenesis (Beccari et al. 1996). During recovery, counts of methanogens correlated negatively with the amount of residual oleate on the biomass. In the reactor fed both glucose and cysteine, the microbial populations recovered to their pre-inhibition state within forty days.

Sousa et al. (2008; 2007) examined which microbes were dominant after feeding a continuous digester with oleate and palmitate followed by batch degradation of the LCFA that accumulated in the digester during continuous operation. Analysis of 16S rRNA showed that the LCFA-degrading bacteria in the enrichment cultures were predominantly in the families *Clostridiaceae* and *Syntrophomonadaceae*. Hydrogenotrophic methanogens were represented by *Methanobacterium* and aceticlastic methanogens were represented by the genera *Methanosaeta* and *Methanosarcina*, all in the Domain Archaea. The cultures grown on oleate and palmitate were taxonomically distinct from each other as well as from the starting inoculum. In the oleate-degrading culture, a new obligately syntrophic bacterium, given the name *Syntrophomonas zehnderi*, was isolated as a co-culture with *Methanobacterium formicicum*. Denaturing gradient gel electrophoresis (DGGE) was used to monitor changes in the microbial community during the feeding of LCFA in continuous testing. At the end of the continuous testing period, the similarity between the oleate-fed sludge and the inoculum sludge was only 23.5% and the similarity between the palmitate-fed sludge and the inoculum sludge was only 34.9%. Known LCFA-degrading members of the

family Syntrophomonadaceae were not detected in DGGE profiles of the inoculum but were predominant in the LCFA-fed reactors at the end of the continuous testing period. Together, these two findings provide strong evidence of adaptation of the microbial community to the prescience of LCFA by enhancement of species that prior to LCFA exposure are minor members of the community. Also at the end of the continuous testing period, similarity between bacterial communities in the oleate-fed and palmitate-fed reactors was low (54% Pearson similarity) whereas the archaeal communities were nearly identical (99% Pearson similarity). The dissimilarity is possibly due to differences in the substrates: oleic acid is mono-unsaturated whereas palmitic is a saturated fatty acid. Only a small portion of LCFA-degraders are able to metabolize unsaturated fatty acids. Bacterial communities also changed in composition after batch degradation for both the oleate-fed and palmitate-fed reactors such that the similarity before and after batch degradation was only 61% and 75% respectively. Archaeal populations shifted even more dramatically than the bacterial populations during batch degradation. This shift was determined to be a wholesale replacement of *Methanosaeta* species with *Methanosarcina* species during batch degradation.

If LCFA inhibition is indeed a toxic effect, several researchers have shown that recovery from the inhibited state is possible. Palatsi et al. (2009) evaluated several methods for augmenting the recovery of a digester deliberately inhibited with 4.0 g liter^{-1} of an LCFA mixture consisting of sodium oleate, sodium stearate and sodium palmitate in a ratio of 40:10:50 (w/w/w). The fastest recovery period was achieved by dilutive replacement of 40% of the digester contents with inoculum stored prior to addition of the LCFA. Within 3 days

the digester recovered, as indicated by a resumption of methane production and a decrease in VFA. When fresh manure was used as the diluent, recovery was comparable. However, when water was used as the diluent, the recovery time was much longer. The researchers attributed the longer recovery to the fact that diluting with water results in a higher LCFA to biomass ratio than diluting with either inoculant or fresh manure. This finding is consistent with work that suggests that a given concentration of LCFA is more inhibitory in reactors with a lower solids content; adsorption of the LCFA to biomass fibers in the reactor biomass provides a buffer for LCFA exposure, especially in situations where LCFA input is non-steady (Nielsen and Ahring 2006). The worst recovery strategy (based on time to recovery) was to simply stop feeding. Diluting the LCFA with inoculum or fresh manure led to much faster recovery. Because inoculum storage for upset recovery would add significant capital cost to a digester facility, dilution with fresh substrate was deemed the most practicable recovery strategy. These same researchers identified the addition of adsorbents as another industrially relevant method for digester recovery. Adsorbents used in testing included bentonite and fibers separated from the effluent of an operational digester. Adsorbents compete with the active biomass for LCFA adsorption and therefore reduce the effective ratio of LCFA to actively growing biomass.

Dilution of high-lipid substrates with low-lipid co-substrates is a straightforward method for lowering the concentration of LCFA and therefore the inhibitory threat. Manure is an excellent co-substrate for anaerobic digestion because of its strong buffering capacity and its broad array of nutrients that are critical for robust microbial growth (Angelidaki and

Ellegaard 2003). The lipid content of manure is modest; the crude lipid in cattle manure (using an ether extraction) was 5.9% of the total solids (Mackie and Bryant 1995). High-lipid substrates such as slaughterhouse wastes and olive oil mill efflulents, by contrast, have a high methane potential but are often characterized by low nutrient content and low alkalinity (Angelidaki and Ahring 1997). These two substrates are clearly complementary. In a mixture of manure and lipid-rich substrate, the manure will provide needed nutrients and buffering capacity, whereas the lipid-rich substrate will greatly boost the methane production capacity of the digester. This strategy has been used extensively to increase the economic performance of anaerobic digesters in Denmark (Tafdrup 1994), where manure is often mixed with various food wastes of higher methane potential. Alvarez and Liden (2008) found that co-digestion of a mixture of manure, slaughterhouse waste and fruit/vegetable waste performed better than any single substrate and also better than mixtures of any two substrates. A mixture of 17% slaughterhouse waste, 17% manure, and 67% fruit/vegetable waste (w/w/w based on volatile solids) gave a methane yield of 0.35 liters g-VS^{-1} fed. Reactor productivity and volatile solids reduction were also highest for this mixture. In the absence of the buffering capacity and broad array of nutrients provided by manure, mixtures of slaughterhouse waste and fruit/vegetable waste were inhibited. Mladenovska et al. (2003) compared a mixture of manure and lipids to manure alone at 37 °C and found that the manure/lipid mixture showed a higher specific methane yield (0.38 liters g-VS^{-1} as compared to 0.22 liters g-VS^{-1}) and a higher VS reduction (51% as compared to 37%) than manure alone.

Olive mill effluent (OME), which has a high lipid content, is another industrially relevant substrate for anaerobic digestion that can be co-digested with manure (Angelidaki and Ahring 1997). These two co-substrates are particularly complementary: (1) the manure provided much needed alkalinity since the OME had relatively low buffering capacity and therefore low resistance to upsets that drive down the pH, (2) the manure had a relatively high content of TAN (2.5 g-N liter^{-1}), nicely balancing the nitrogen-poor OME (0.1 g-N liter^{-1}), (3) the improved methane potential of the mixture over manure alone, as noted above. The high lipid content translates to a high methane potential; the methane production from a 50:50 combination of OME:manure was more than double that of manure alone at an equivalent organic loading rate.

1.4. Other Current Research Topics in Anaerobic Digestion

1.4.1. Pathogen Destruction

Pathogen control has become an increasingly important aspect of wastewater treatment and utilization of the effluent therefrom. Thermophilic anaerobic digesters offer a particularly effective means for pathogen destruction. Lee and Shih (1988) studied the effect of anaerobic digestion on oocysts from the Protozoan *Eimeria tenella*, an enteric parasite in poultry that causes coccidiosis. After a five day HRT in a thermophilic digester, the infectivity of both sporulated and unsporulated oocysts was greatly reduced, as measured by body weight gain and lesion score in comparison to uninfected control chicks. By contrast, mesophilic digestion only resulted in a modest decrease in infectivity, particularly for

sporulated oocysts. Olsen and Nansen (1987) examined the fate of bovine parasites in anaerobic digesters: *Cooperia oncophora*, *Dictyocaulus viviparus*, and *Eimeria* spp. Three-liter batch fermentations of manure slurries were conducted at mesophilic (35 °C) and thermophilic (53 °C) temperatures. *C. oncophora* was inactivated within two days under mesophilic conditions and 15 minutes under thermophilic conditions. Larvae of *D. viviparus* were also rapidly inactivated. Oocysts of *Eimeria* spp., on the other hand, were not significantly reduced in morphology or number by anaerobic digestion, but their infective viability was not tested.

The fate of viruses in anaerobic digesters has also been studied. McKain and Hobson (1987) conducted testing on porcine enteroviruses, a virus that causes reproductive disease in swine. The enteroviruses were suspended in dialysis tubing (to prevent washout and thereby simplify destruction calculations) and placed in both a mesophilic and thermophilic continuously stirred tank reactor (CSTR). In the mesophilic digester seeded with the enteroviruses, viable viruses could be detected for up to 9 days, whereas for the thermophilic digester, the virus was totally inactivated in 1 hour. The authors conclude that any practical thermophilic digester design would lead to the destruction of this enterovirus. Monteith et al. (1986) also studied inactivation of viruses (bovine enterovirus and bovine parvovirus) by anaerobic digestion (both mesophilic and thermophilic), heat treatment, gamma irradiation, ensilage and composting. These viruses were selected as being representative of the most resistant bovine enteric viruses. Under thermophilic conditions (55 °C), deactivation was rapid (infectivity could not be detected after 30 minutes for the enterovirus and after two

days for the parvovirus), whereas bovine enterovirus remained viable for up to 13 days and bovine parvovirus remained viable for up to 8 days under mesophilic conditions (35 °C). For the use of the manure as a substrate for producing single cell protein for re-feeding, only thermophilic anaerobic digestion was deemed by the authors to be reliable enough to ensure total viral deactivation.

Other studies have focused on bacterial destruction in anaerobic digesters. Olsen et al. (1985) examined the fate of *Mycobacterium paratuberculosis*, a non- sporing bacteria, in the anaerobic digestion of manure at both mesophilic and thermophilic temperatures in batch reactors. *M. paratuberculosis* causes paratuberculosis in ruminants, is spread through contaminated water or feed, and is extremely resistant to degradation under a variety of environmental conditions. Under mesophilic conditions (35 °C), viable bacteria could be detected on days 7, 14, and 21 but not on day 28. Under thermophilic conditions (53-55 °C), viable bacteria could not be detected after just 3 hours. The authors conclude that where hygiene is concerned, thermophilic digesters must be given priority. Olsen and Larsen (1987) continued work on bacterial fate in anaerobic digesters with a study on the reduction of pathogenic and indicator bacteria at laboratory scale (2-3 liters) in cattle and pig manure slurries, again at both mesophilic and thermophilic temperatures. At mesophilic temperatures, the time required for 90% inactivation (T_{90}) of pathogenic bacteria in a vegetative state was 0.9-2.4 days. For indicator organisms, T_{90} values were longer: 3.1, 3.2, and 7.1 days for total coliforms, fecal coliforms and group D streptococci respectively. At thermophilic temperatures, T_{90} values for vegetative pathogenic bacteria were substantially

shorter, ranging from 0.3 to 1.2 hours. In contrast, spores of *Clostridium perfringens* type C and *Bacillus cereus* were not substantially reduced in number at either temperature. In fact, viable spores were identified after 180 days at 53 °C. The authors conclude that 12-24 hours residence time in a thermophilic anaerobic digester gives a "hygienically acceptable" reduction of vegetative pathogenic bacteria.

Dugba and Zhang (1999) also evaluated the destruction of coliform bacteria but in another type of reactor -- a two-stage anaerobic sequencing batch reactor. Dairy wastewater was treated in three different systems, two thermophilic-mesophilic configurations and one mesophilic-mesophilic configuration. The thermophilic-mesophilic configurations effectively eliminate total coliforms – none were detected in the effluent until the organic loading rate reached 6 g-VS liter^{-1} d^{-1}, at which point the overall digester performance also began to deteriorate. Only a 1-2 log reduction in total coliforms was achieved in the mesophilic-mesophilic configuration, regardless of organic loading rate. The thermophilic-mesophilic configurations also achieved better VS conversion and higher biogas productions rates (per unit working volume) at a given organic loading rate.

Martens and Bohm (2009) have provided a thorough overview of techniques available for pathogen destruction in manure. After reviewing the methods available, the authors conclude that for cost reasons, the only viable options at the farm level are

composting and anaerobic treatment. However, a practical hydraulic residence time[3] (HRT) in a mesophilic anaerobic digester is not adequate to ensure reliable inactivation of pathogens. Additional physical, chemical or thermal treatment would be required. Thermophilic anaerobic treatment at 53-55 °C or above would be effective for the destruction of vegetative bacteria, moderately-resistant bacteria, and parasites in an infections stage. Operation would need to include a minimum of 20 hours without the addition of feed, which would be consistent with semi-continuous once-daily feeding.

The superior pathogen reduction capability of thermophilic anaerobic digestion enables the effluent to meet Class A Biosolids specifications, as codified in U.S. Code of Federal Regulations, Title 40 (40 CFR), Part 503. Land application of effluents from waste treatment processes are based on the presumed pathogen content of the treated waste. Class A biosolids are considered pathogen-free and enjoy much less restricted usage for land application than their Class B counterpart, which have undergone a process to "significantly" reduce pathogens but are not considered pathogen-free. In addition to pathogen destruction requirements, Class A biosolids must also meet a vector attraction reduction target. Using a temperature-phased anaerobic digestion process, which features a thermophilic primary stage and a mesophilic secondary stage, Han et al. (1997) found that total and fecal coliforms were

[3] Hydraulic residence time (HRT) is the average amount of time that the liquid influent entering a digester remains in the digester. It can be calculated by dividing the working volume of the reactor by the influent volumetric flow rate.

reduced to levels that never exceeded the 1000 MPN[4] g-TS^{-1} limit for meeting Class A Biosolids specifications.

1.4.2. Pretreatment and Bioaugmentation to Enhance Performance

Many potential feedstocks for anaerobic digestion contain lignocellulosic fibers that are recalcitrant to degradation in the time frame available in a typical digester. Increasing the degradability of these fibers is a logical target for efforts to improve the performance of digesters; success would open up a much larger array of feedstocks that could be used as feedstocks for anaerobic digestion. For farm-based digesters, Kaparaju and Rintala (2003) estimate that as much as 30% of the methane potential is lost in the effluent.

Kaparaju and Rintala (2005) attempted to improve the anaerobic digestion of the particulate matter in digester effluent that exceeded 2mm using several methods including thermal and chemical (incubation with NaOH) treatments, maceration, and freezing and thawing. The substrate was prepared by sieving the effluent from an operational farm-scale digester. The liquid fraction from sieving was used as the inoculant. Following treatment, methane potential of the solid fraction was measured in batch tests in 120 mL glass bottles over a range of temperatures (5-55 °C). In the mesophilic-thermophilic temperature range (35-55 °C), only the chemical plus thermal treatment resulted in a specific methane

[4] MPN is an acronym for "most probable number", a technique for determining the concentration of a microorganism through serial dilution to the point where the subsample is likely to include only one microorganism. The subsample is then cultured in (or on) an appropriate media and the presence or absence of the microorganism in a large number of replicates can be mathematically related to the original concentration.

production that exceeded the untreated controls. Other treatments actually caused a decrease in specific methane yield.

Nielsen et al. (2004) tested a 3-day thermal pretreatment at 68 °C prior to thermophilic digestion at 55 °C. Both batch and CSTR testing were performed. In CSTR testing, the 3-day pretreatment was followed by digestion in a reactor with a 12-day HRT and an organic loading rate of 3 g-VS liter^{-1} d^{-1}. The control reactor was a one-stage reactor with a 15-day HRT and comparable organic loading rate. In the batch tests, an increase in the specific methane yield of 24-56% was observed when cattle manure and its component fractions (fiber and liquid) were pretreated for various times (36, 108 and 168 hours). Methane yield from fibers recovered from the effluent of a full-scale reactor was also improved by the 68 °C pretreatment, with the improvement increasing with pretreatment time. In the CSTR tests, the thermal pretreatment boosted overall methane yield by 6-8% and volatile solids reduction by 9% in comparison with the control reactor.

Mladenovska (2006) focused on thermal pretreatment of swine/cattle manure mixtures at 100-140 °C (using an autoclave) as a means for improving digester performance. Tests were conducted in both batch and CSTR reactors operating at 55 °C using inoculum from a full-scale thermophilic digester. For the CSTR reactor, the HRT was 18 days and the organic loading rate was 2.5 g-VS liter^{-1} d^{-1}. Only the solid fraction of the manure, obtained by screening the substrate through a sieve with a 600mm mesh size, was pretreated. Results of batch testing showed that both methane yield and production rate were enhanced by the thermal pretreatment, ranging from 9-24% for the 20 minute pretreatment and 10-17% for the

40 minute pretreatment. For the CSTR tests, reactor productivity, specific methane yield and VS removal were all higher in the test reactor (utilizing thermally pretreated substrate) than in the control. The authors conclude that thermal pretreatment at the conditions utilized in their testing improves the degradability of the solids fraction of the substrate, possibly by removal of hemicellulose from lignocellulosic fibers, thereby opening of the structure and allowing better access by hydrolytic enzymes, in accordance with research on biomass pretreatment for enzymatic saccharification (Mosier et al. 2005).

Hejnfelt and Angelidaki (2009), in testing homogenized mixed swine processing waste (non-commercial by-products from one slaughtered animal), found that thermal pretreatments (pasteurization at 70 °C or sterilization at 133 °C) and alkali hydrolysis (NaOH) had no effect on methane yields in subsequent anaerobic digestion of the pretreated feedstocks. However, in this case, the researchers believed that the reason pretreatment was not effective was because the mixed waste was readily degradable in the absence of pretreatment, as indicated by specific methane yield relative to the maximum theoretical yield (based on volatile solids content).

Luste et al. (2009) evaluated several pretreatment methods for trying to improve the anaerobic digestion of four by-products from the animal processing industry (digestive tract contents, drumsieve waste, dissolved air flotation sludge from the slaughterhouse, and grease trap sludge from the meat-processing plant). Physicochemical pretreatments included thermal pretreatment (70 °C for 60 minutes), ultrasound (5600 kJ/kg-TS), base (2 M NaOH, pH 12-12.2, 4 hours) and acid (6 M HCl, pH 2-2.5, 4 hours). The effectiveness of the

pretreatments was, in part, measured by the increase in the amount or rate of methane production in batch tests in 2 liter glass bottles at 35 °C. The source of inoculum was digester effluent from a municipal wastewater treatment plant. For drumsieve waste, thermal pretreatment substantially improved the specific methane yield, from 0.23 liters g-VS^{-1} fed to 0.34 liters g-VS^{-1} fed. For dissolved air flotation sludge, all pretreatments improved the specific methane yield. Base was the most effective; the yield increased from 0.340 liters g-VS^{-1} fed for the control to 0.390 liters g-VS^{-1} fed for the base-pretreated. For the grease trap sludge, acid pretreatment increased the specific methane yield from 0.90 liters g-VS^{-1} fed for the control to 1.01 liters g-VS^{-1} fed. The specific methanogenic activity (SMA) of dissolved air flotation sludge was improved by ultrasound pretreatment by 19%. All pretreatments improved the SMA of drumsieve waste and grease trap sludge, with average improvements of 15% and 14% respectively, excluding thermally pretreated grease trap sludge, which caused an increase in SMA by 22%.

For anaerobic digesters utilizing lipid-rich substrates, lipases (triacylglycerol ester hydrolases), which hydrolyze triglycerides into glycerol and fatty acids, are at first glance obvious candidates for enhancing digester performance. Research has shown, however, that hydrolysis of triglycerides is not the rate-limiting step in the degradation of lipid-rich substrates (Broughton et al. 1998). Nonetheless, these enzymes have been shown to improve degradation of lipid-rich substrates such as dairy wastewater. Mendes et al. (2006) used a porcine pancreas enzyme preparation for evaluating enzymatic pretreatment as a means to improve digestion of dairy wastewater. In addition to lipase activity, the enzyme preparation

also included protease and amylase activities. Following pretreatment, the substrate was digested in 500 mL batch reactors incubated at 35 °C using inoculum from a UASB digester treating dairy wastewater. Biogas production from the pretreated wastewater ranged from 0.354 to 0.445 liters, depending on the length of pretreatment, with the longest pretreatment time tested, 12 hours, giving the highest biogas yield. Biogas production from the control was 0.209 liters, less than half of the maximum from enzymatic pretreatment. In this study, the enzymatic pretreatment appears to improve the liquefaction and bioavailability of lipids, eliminating inhibition and sludge flotation problems observed in the control.

Rosa et al. (2009) also used an enzyme preparation with lipase activity to improve the anaerobic digestion of dairy wastewater in an anaerobic sequencing batch reactor operating at 30 °C. Fat removed from a dissolved air flotation unit was used to artificially raise the lipid content of the wastewater to 1200 mg liter^{-1}. The enzyme preparation, obtained from the solid state fermentation of a *Penicillium* sp. fungus, was added in a 24-hour hydrolytic pretreatment process at 30 °C that preceded digestion. When the digester was fed pre-hydrolyzed wastewater, COD removal was 90%. When the prehydrolysis step was eliminated, however, the digester operated unstably and the COD removal dropped to 32%. The lipid content of the digester effluent rose and lipids accumulated on the digester biomass.

A strategy to introduce a lipolytic microbe into an anaerobic digester treating high-lipid substrates to improve hydrolysis of the triglycerides could backfire. Improved hydrolysis could lead to an imbalance between the release of LCFA (from the triglycerides) and their subsequent degradation. Cirne et al. (2006) hypothesized that introduction of an

anaerobic lipolytic microorganism could perhaps improve hydrolysis and β-oxidation by a more steady release of LCFA from triglycerides. Batch tests were conducted in 100 mL serum bottles at 37 °C with continuous stirring (150 RPM) using triolein as a model substrate. As fermentation proceeded, the bioaugmented treatment had higher concentrations of stearate and palmitate than the control, an indication that bioaugmentation was improving hydrolysis. The methane production was also increased for the bioaugmented treatment, which would translate to shorter HRT and associated reductions in digester capital cost. However, survival of the bioaugmenting organism, *Clostridium lundense* (DSM 17049T), could not verified.

Nielsen et al. (2007) evaluated a biological approach to improving the degradability of lignocellulosic fibers for subsequent anaerobic digestion. Two hyperthermophilic bacteria isolated from hot springs in Iceland were used in a 3-day, 68 °C pretreatment followed by digestion at 55 °C: (1) *Caldicellulosiruptor lactoaceticus* (strain 6A), which can degrade cellulose along with xylan, starch, pectin, cellobiose, xylose, and maltose (Mladenovska et al. 1995), and (2) *Dictoyoglomus* (strain B4a), which can utilize xylan and xylose as sole substrates. In batch experiments, the pretreatment was applied to the fiber and liquid fractions of the raw feedstock (cattle manure), which were obtained by centrifugation, as well as the whole manure feedstock. Batch testing was also conducted on the fiber fraction of effluent from a full-scale thermophilic digester. In CSTR testing, the pretreatment was conducted in a 900-mL reactor operating at 68 °C and a 3-day HRT followed by the main 2.4-liter (working volume) reactor operating at 55 °C and a 12 day HRT. The 3-liter control

reactor was a single reactor with a 15 day HRT. Both reactors had an organic loading rate of 9 g-VS d^{-1}. The feedstock for CSTR testing was whole manure. In batch tests, a 12-24% improvement was observed for vials inoculated with the hyperthermophilic strains in comparison with control vials that received autoclaved (dead) inocula. In CSTR testing, a 93% increase in methane yield was observed in the pretreatment reactor. At the end of the experiment, the overall methane yield in the two-stage setup was 5-6% higher than prior to inoculation and 9-10% higher than the single-stage control reactor. Interestingly, the pretreatment reactor showed a marked decline in methane output starting on day 47 of the test, immediately suggesting washout of the hyperthermophilic strain from the pretreatment reactor. The ability of the introduced strain to survive, compete, and thrive will be a crucial issue for all microbial-based bioaugmentation schemes.

1.5. Research Objectives

In virtually all the research conducted on ammonia inhibition in which the ammonia concentration is enhanced above the background ammonia concentration of the wastewater, NH_4Cl has been used to raise the ammonia level. One of the objectives of this study was to look at ammonia inhibition in manure substrates in which urine and the urea contained therein was used to enhance the ammonia concentration. The results could then be used to determine whether swine waste treatment systems that separate urine from feces would be advantageous to thermophilic anaerobic digesters that treat the feces only. If ammonia inhibition does impact the performance of anaerobic digesters utilizing mixtures of feces and

urine, another objective was to determine if the microbial consortium can be adapted to elevated concentrations of urine.

Another objective of the research described herein was to evaluate the use of sludge from dissolved air flotation (DAF) wastewater treatment systems used in animal processing facilities as a potential substrate for anaerobic digestion. DAF sludge is a high-lipid substrate; hydrolysis of the triglycerides in DAF sludge is likely to produce potentially inhibitory levels of long chain fatty acids (LCFA) in an anaerobic digester. Therefore, the feasibility of stably producing methane from the anaerobic co-digestion of DAF sludge and swine manure (feces only) was explored. Continuous testing was conducted to determine steady-state gas production and other performance parameters. Batch testing was conducted to help deduce the kinetics of microorganism growth on the DAF sludge/manure mixture.

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CHAPTER 2. IMPLICATIONS OF URINE TO FECES RATIO IN THE THERMOPHILIC ANAEROBIC DIGESTION OF SWINE WASTE[5]

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2.1. Abstract

Thermophilic anaerobic digestion of swine manure represents a potential waste treatment technology to address environmental concerns such as odor emissions and removal of pathogenic microorganisms. However, there are concerns relative to the stability of this process when swine manure is the sole substrate. In this study the potential of biogas production from swine manure as the sole substrate under thermophilic (50°C) conditions was investigated in the laboratory to determine whether separation of urine and feces as part of the waste collection process would benefit anaerobic digestion. Effluent from a continuously stirred tank reactor (CSTR) was used as the inoculum for batch tests in which the substrate contained three different concentrations of urine (urine-free, as-excreted urine:feces ratio and double the as-excreted urine:feces ratio). Inocula were acclimated to these same urine:feces ratios to determine methane production. Results show that both urine-free and as-excreted substrates were not inhibitory to anaerobic inocula. Anaerobic microorganisms can be readily acclimated to substrate with double the as-excreted urine concentration, which contained nitrogen concentrations up to 7.20 g/L. Cumulative methane production reached similar levels in the batch tests regardless of the substrate urine concentration.

Keywords: Anaerobic digestion; Inhibition; Feces; Swine waste; Thermophilic; Urine;

2.2. Introduction

Anaerobic treatment of animal manures for fuel recovery as biogas has been extensively investigated during the previous two decades (Steinsberger and Shih, 1984; Safley and Westerman, 1990; Badger et al., 1995). Interest in this technology for confined animal feeding operations (CAFOs) has again increased due to its perceived potential to enhance manure nutrient recovery, reduce greenhouse gas emissions, kill pathogenic microorganisms, and reduce odor emissions.

Temperatures utilized for anaerobic digestion of animal manure substrates include psychrophilic (15-20°C), mesophilic (25-40°C), and thermophilic (45-60°C) ranges. Benefits of the digestion process at higher temperatures include increased rates of substrate catabolism (Varel et al., 1977; Huang and Shih, 1981) and pathogen control (Shih, 1987). However, the effects of temperature and substrate loading concentrations on anaerobic digestion and digester performance and stability have been shown to be highly variable (Chen and Day, 1986). This is especially evident in relation to the anaerobic digestion of swine manure at thermophilic temperatures. Swine waste often has very high total ammonia nitrogen concentrations due to the presence of ammonia as well as protein and urea that readily release ammonia upon anaerobic treatment (Hansen et al., 1998; Liu and Sung, 2002). The concentration of free ammonia in the swine manure digester medium, which increases at higher digester temperatures, has been reported to be a primary factor associated with digester instability (Hobson and Shaw, 1976). It has been reported that the total ammonia nitrogen (TAN) concentration that caused a 50% reduction in methane production (I_{50}) from

swine waste ranged from 1.1 to 6 g/L (Hansen et al., 1998; 1999; Hashimoto, 1983; 1984). The significant difference in inhibiting ammonia concentration can be attributed to the differences in substrates, inocula, and environmental conditions (temperature, pH) (Hashimoto, 1986; Angelidaki and Ahring, 1993). Some research has also indicated that digester instability may be reduced or eliminated under appropriate environmental acclimation conditions at thermophilic temperatures (Hashimoto, 1983).

Although commercial-scale anaerobic digesters are successfully processing swine manure under thermophilic conditions in some parts of the world (Tafdrup, 1997), the swine manure in these digesters is often co-digested with other waste substrates which may increase the carbon/nitrogen (C/N) ratio. The construction and operation of thermophilic anaerobic digesters at new or existing CAFOs may require significant capital costs and/or engineering retrofit and many CAFO operations considering this technology may not be able to efficiently incorporate waste substrates other than swine manure into the on-site digesters. In order to utilize swine waste as a single substrate for anaerobic digestion, the inhibition caused by ammonia should be fully investigated. Although extensive research has been conducted on ammonia inhibition, the high concentration of ammonia was inevitably established through addition of ammonia salts. It is unclear whether the presence of urine significantly affects thermophilic anaerobic digestion of swine waste and whether appropriate acclimation of anaerobic microorganisms can reduce the inhibition caused by ammonia. Some waste treatments currently being studied include separation of feces and urine as part of the manure collection process (Elmer et al., 2001). If the anaerobic microorganisms cannot

be readily acclimated to pure swine waste substrate, then separation of feces and urine prior to treatment becomes much more attractive. In this scenario, the feces would be anaerobically digested while the urine would be subjected to some other form of treatment. Therefore, the objectives of this study were to (1) determine the feasibility of biogas production using swine waste (feces and urine) as the sole substrate under thermophilic (50°C) anaerobic conditions; (2) determine the effect of adaptation to elevated concentrations of urine on the thermophilic anaerobic digestion of swine waste.

2.3. Methodology

2.3.1. Swine Waste

Feces and urine were collected separately from a hog barn at the Swine Education Unit of the Lake Wheeler Road Field Laboratory of North Carolina State University (Raleigh, NC). The pigs were confined in group pens and fed a ration containing 69.2% ground corn, 10.5% soybean meal, 15% soy hulls, 2.2% dicalcium phosphate, 0.3% ground limestone, 0.5% iodized salt, 0.25% vitamin premix, and 0.5% trace minerals. Antibiotics were not used in the ration. The feces were mixed with deionized water and agitated vigorously with a commercial laboratory blender (21/800EG, John Morris Scientific, Australia). To facilitate flow of substrates, suspension of the digester contents and effluent flow, the mixture was passed through a coarse mesh to remove gross solids. The total solids (TS) content was maintained at 5% throughout the experimental process by adding additional deionized water when urine-free feed was prepared. To make urine-containing feed, freshly

collected urine was also supplied to establish the appropriate urine:feces ratio while maintaining the same TS value, using deionized water as necessary. The raw swine waste was stored at -20°C before use. Waste characteristics are shown in Table 1.

2.3.2. Continuously Stirred Tank Reactor (CSTR) Operation

Swine waste digestion and thermophilic inocula cultivation were conducted in a 14 L (10 L working volume) CSTR digester (Bioflo 110, New Brunswick, NJ) (Figure 1). Thawed raw swine waste was processed into 5% TS feed and stored in a refrigerator (4ºC). The feed was stirred constantly with a magnetic stirring bar before being fed to the digester by a peristaltic pump (Cole Parmer, Vernon Hills, IL). The digester was inoculated with 1 L sludge obtained from an on-site lagoon treating wastewater generated from daily flushing of under-slat pits (Lake Wheeler Road Field Laboratory Swine Education Unit, Raleigh, NC). The digester contents were agitated at a constant rate of 150 rpm. The hydraulic retention time (HRT) of the digester was 10 days throughout the experimental period. Temperature of the digester was maintained at 55ºC by circulating water in the jacket surrounding the reactor. A wet tip gas meter (invented by Dr. R. E. Speece and available at http://wettipgasmeter.com) was used to quantify biogas production. Water in the gas meter was saturated with NaCl and pH was maintained below 2 to minimize solubilization of CO_2. The gas meter is a volumetric device -- in position 1, gas from the digester displaces water in one of two contiguous chambers until the buoyancy of the gas in that chamber causes the chamber assembly to tilt into position 2, releasing the gas in chamber 1 and orienting the chamber assembly in such a way that gas is now collected in chamber 2. Each tip is counted

by an electromagnetic sensor. The gas meter was calibrated by repeatedly injecting a known quantity of gas into the meter with a syringe and relating the volume of gas injected to the number of tips.

To test the effect of acclimation to elevated ammonia concentration, urine was added to the feces feed stored in the refrigerator. The mixture of feces and urine was fed to the CSTR digester to establish two other urine concentrations in addition to the baseline case of urine-free substrate -- a urine: feces ratio consistent with as-excreted swine waste (1x-urine substrate) and a urine:feces ratio double that of as-excreted swine waste (2x-urine substrate) while maintaining the other operating conditions as described before. The urine:feces (dry) (wt/wt) ratio in as-excreted waste was selected as 6.52 (Moeser et al., 2003). When daily gas production and pH reached a steady-state, defined by constant effluent pH and coefficient of variation (CV) of daily biogas production lower than 5% for more than five consecutive days, inocula were obtained from the CSTR digester to conduct batch inhibition tests.

2.3.3. Batch Tests

Three sets of batch tests were conducted in which effluent collected from the CSTR digester at steady-state was used as inocula. The inocula were referred as urine-free, 1x-urine, and 2x-urine inocula when urine free, 1x-urine, and 2x-urine substrates were used. In each batch test, inoculum was mixed with swine waste substrate with different urine concentrations in a 500 mL medium bottle. Table 2 shows the composition of the batch test samples. Two controls were used for each inoculum tested, one with 1x-urine substrate only (substrate control) and one with inoculum only (inoculum control), to determine their

respective gas production potential. Due to the strong buffer capacity of swine waste (Sommer and Husted, 1995), pH could be maintained between 7.0 and 7.2 without extra adjustment. Nitrogen gas was used to flush the bottle headspace before they were connected to a liquid displacement gas measuring device. Samples were incubated at 50°C in a temperature-controlled waterbath. To remove CO_2 in the biogas, a base trap containing 10 mL NaOH (5 N) was placed in each bottle. Gas analysis by gas chromatography (GC) showed that the CO_2 concentration was below 3% when the NaOH trap was replaced every two days. Gas production was recorded until cessation of gas production, which was approximately 10 days. All samples were tested in duplicate. To examine the effects of acclimation, similar batch test procedures were followed to measure the methane production for 1x-urine inoculum and 2x-urine inoculum, respectively. Effluent from the CSTR was used as inoculum for these tests after the CSTR was adapted to 1x-urine and 2x-urine substrates for a minimum of 30 days (3 HRTs).

2.3.4. Analytical Methods

Total solids, volatile solids (VS), fixed solids (FS), chemical oxygen demand (COD), soluble chemical oxygen demand (SCOD), pH, total Kjeldahl nitrogen (TKN), ammonia-N, and total phosphorus (TP) were analyzed according to Standard Methods (APHA, 1998). Biogas composition was determined by sampling reactor headspace. A Shimadzu GC 15A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 3 m × 3 mm (10 ft × 0.13 in.) stainless-steel column (100/120 Carbosieve S-II packing, Supelco, Bellefonte, Pennsylvania) and a thermal conductivity detector was used.

2.3.5. Data Analysis

Growth rates were calculated in order to determine quantitative differences between the various inocula/substrate combinations. Assuming that at initial test conditions, the substrate concentration greatly exceeds its half-saturation constant, the net specific growth rate can be expressed as:

$$\mu_{net} = \frac{1}{X}\frac{dX}{dt} \quad (1)$$

where μ_{net} = net specific growth rate (h^{-1}); X = concentration of active biomass (g/L); t = time (h). Assuming that the gas production rate is proportional to the concentration of active biomass, then:

$$\frac{dM}{dt} = \beta X \quad (2)$$

where M = methane production (mmoles); β = proportionality constant relating methane production rate to active biomass concentration, (mmole/h)/(g/L). Combining equations (1) and (2), μ_{net} can be expressed as a function of the gas production rate:

$$\mu_{net} = \frac{\beta}{\frac{dM}{dt}} \times \frac{d}{dt}\left(\frac{1}{\beta}\frac{dM}{dt}\right) \quad (3)$$

Integrating this equation using the initial condition of $dM/dt = (dM/dt)_0$ at t = 0 gives:

$$\ln\left(\frac{dM}{dt}\right) = \ln\left(\frac{dM}{dt}\right)_0 + \mu_{net} t \quad (4)$$

Thus μ_{net} can be calculated from a plot of ln(dM/dt) as a function of time during the exponential phase of gas production. The exponential phase was chosen as the first 72 hours of the test by examining the cumulative gas production curves.

For comparison, the gas production rate was also modeled using the modified Gompertz equation that accounts for an initial lag phase as well as the reduction in growth rate associated with the depletion of substrate. This equation has been modified (Lay et al., 1998) such that the equation is expressed in terms of parameters readily measured in batch culture experiments – namely cumulative methane production:

$$M = P \times \exp(-\exp(\frac{R_m \times e}{P}(\lambda - t) + 1)) \quad (5)$$

where P = methane production potential (mmole), R_m = maximum methane production rate (mmole/h); e = exp(1) and λ = lag phase (h). R_m is related to the maximum growth rate by the following equation:

$$R_m = \frac{R_g}{(Y_1 / Y_2)} \quad (6)$$

where R_g = maximum growth rate (g/h); Y_1 = growth yield coefficient, active biomass produced per gram of substrate utilized (g/g); Y_2 = methane yield coefficient, methane produced per gram of substrate utilized (mmoles/g). It should be noted that R_g, with units of g/h is not a specific growth rate like μ_{net}, which has the units of h^{-1}. Thus in this model, R_m cannot be related directly to the specific growth rate because the active biomass concentration is not modeled. Under the conditions of these batch tests, however, R_m is a good relative measure of the vigor of the culture.

Differences between treatments were evaluated by performing analyses of variance (ANOVA) ($p < 0.05$). All statistical tests were performed by SAS 8.0 software (SAS Institute Inc., Cary, NC).

2.4. Results and Discussion

2.4.1. Characteristics of Swine Waste

Table 1 shows the characteristics of the swine waste used in this study. Since as-excreted feces, which have a TS content of 15-20%, are rarely used directly for anaerobic digestion (Sutton et al., 1983, Chynoweth et al., 1998), diluted swine feces with TS of 5% were characterized. The ratio of ammonia-N to TKN in feces and urine averaged 0.15 and 0.12, respectively, indicating that the majority of the nitrogen excreted from pigs is in the form of uric acid in the urine and organic-N in the feces. Literature data show large variations in ammonia-N/TKN ratios which ranged from 0.16 to 0.8 for feces and 0.09 to 0.4 for urine (Sutton et al., 1983, Panetta et al., 2005). The discrepancy could be attributed to differences in diet, waste management system, and environmental conditions (temperature, pH) (Clanton et al., 1991). In addition, the swine manure used in this study was freshly collected and kept at -20°C during storage. The absence of the activity of microbial urease enzymes, which are ubiquitous in the environment and can convert urea into ammonia, also contributed to the low ammonia content in the swine waste studied.

Feces contributed approximately 90% of the COD value of the swine waste. The percentage was calculated based on the assumption that the urine:feces (dry) (wt/wt) ratio is

6.52 in as-excreted feces (Moeser et al., 2003). This finding was comparable to that obtained by Powers and van Horn (2000), who reported that the ratio of fecal to urine COD was 6.5-8.0.

2.4.2. Performance of CSTR Digester

Figure 2 shows the performance of the CSTR digester including pH and daily biogas production over the course of the experiment. Initially, the digester received urine-free substrate. The daily biogas yield averaged 1.41±0.01 L/L/day (liters of biogas per liter of working digester volume per day) and the methane content in the biogas was approximately 76%. The daily biogas yield is lower than the value (1.80 ± 0.12 L CH_4/L/day) reported previously by Hashimoto (1983). The low biogas production can be attributed to two reasons. Firstly, the feces used in this study have somewhat higher unbiodegradable fraction (FS) compared with other swine waste sources (Boopathy, 1998; Pagilla et al., 2000). Approximately 36% of the TS is non-digestible as calculated from Table 1 (FS/TS×100). Secondly, anaerobic digestion is a complex process that involves the synergistic action of hydrolytic, acidogenic, acetogenic, and methanogenic microorganisms. In methanogenesis, over 50 species of methanogens (*Methanobacteria, Methanococci*, and *Methanopyri*) maybe involved in methane production (Gerardi, 2003). Although separation and identification of the microorganisms was beyond the scope of this study, the consortia of microorganisms present in the inoculum could constrain the final constia of microorganisms in the digester and therefore impact gas production.

To evaluate the effect of acclimation, the feed was increased to the 1x-urine concentration at day 25. While biogas yield and biogas methane content (1.39±0.02 L/L/day and 74.5%) did not decrease dramatically, the variability in daily gas production increased markedly. By day 35, the oscillations in gas production dampened out and steady state was regained. Batch tests using inoculum acclimated to 1x-urine substrate were initiated following this return to steady-state. On day 64, the urine concentration was increased to the 2x-urine concentration. As can be seen in Figure 2c, this change caused a substantial drop in gas production from approximately 1.32 to 0.88 L/L/day (day 67). Similarly, pH and methane content in biogas decreased to 6.8 and 62.5%, respectively, indicating that the anaerobic microorganisms were inhibited by the elevated concentration of urine.

Depending on diet, swine excrete approximately 60-80% of their nitrogen in urine and 20-40% in feces (van Horn et al., 1996). Around 60-75% of the excreted nitrogen would be in urea or uric acid in the urine component (Panetta et al., 2005). Although the urine used in this study had comparatively lower ammonia concentration, ammonia content in the digester was expected to increase significantly when urine was introduced into the digester as microbial ureases in feces can readily convert urea and uric acid into ammonia. Ammonia inhibition has been widely reported as the leading cause of reactor upset and/or failure in anaerobic treatment of animal waste (Hansen et al., 1998; Liu and Sung, 2002). Between the two forms of ammonia nitrogen (NH_4^+ and NH_3), free ammonia has been suggested to be the main cause of inhibition, causing a proton imbalance, an increase in maintenance energy requirements, and inhibition of a specific enzyme reaction (Sprott and Patel, 1986; Wittmann

et al., 1995; Gallert et al., 1998). Since the experiments were performed at thermophilic temperatures (50°C), the inhibition caused by ammonia was expected to be more severe compared with that at mesophilic temperatures (Kroeker et al., 1979) because of the increase in free ammonia with temperature. By day 77, the average daily biogas production returned to a similar level as when urine-free swine waste was used as substrate, but fluctuations continued until finally dampening out at which time the last set of batch tests was conducted using 2x-urine inocula.

2.4.3. Batch Test Results

Cumulative methane production for all batch tests is shown in Figures 3-5. The changes in pH and VS reduction during batch testing are summarized in Table 3. In Figure 3, urine-free inoculum was used. No gas production was observed for the substrate control or inoculum control, indicating that swine waste was the only substrate available in this experiment and that the digestion of swine waste was performed solely by anaerobic inocula collected from the CSTR digester. The cumulative methane production for the urine-free substrate was not significantly different from that for the 1x-urine substrate ($p = 0.76$), suggesting that the ammonia concentration in the as-excreted swine waste did not have an inhibitory effect on the anaerobic digestion of swine waste. However, the methane production was inhibited for the 2x-urine substrate, as indicated by the significantly lower ($p = 0.00$) methane production (Figure 3) as well as VS reduction ($p = 0.01$). The pH of all reactors was between 7.6-7.7, which can be attributed to the strong buffering capacity of swine waste. The 1x-urine inoculum was used for the data presented in Figure 4, which again

shows that cumulative methane production is not significantly different for the urine-free and 1x-urine substrates ($p = 0.27$). But for the 2x-urine substrate, methane production was inhibited significantly ($p = 0.00$). In Figure 5, the 2x-urine inoculum used for these batch tests was effluent from the CSTR after operating for approximately 25 days on feedstock containing 2x-urine. Both cumulative methane production ($p = 0.70$) and VS reduction ($p = 0.15$) were not significantly different for the three substrates regardless of the urine:feces ratio (Figure 5 and Table 3), indicating that the anaerobic microorganisms were acclimated to the higher ammonia concentration.

Microorganisms are considered "acclimated" when they become more tolerant to inhibitory substances than those of the same origin which have not been exposed to the same inhibitory substances (Kugelman and McCarty, 1964; Sung and Liu, 2003). Acclimation of microorganisms to ammonia enables them to retain activity at concentrations far exceeding the initial inhibitory concentrations (Kroeker et al., 1979; Angelidaki and Ahring, 1993). It has been reported that while unacclimated methanogens failed to produce methane at 1.9-2 g N/L, the methane production resumed at 11 g N/L after acclimation (Koster and Lettinga, 1988). The increase in tolerance to inhibitory substances has been attributed to internal changes in the anaerobic microorganisms or to a shift in anaerobic population (Zeeman et al., 1985). Short (three weeks) acclimation periods were sufficient for conferring tolerance of the tested nitrogen concentrations. This can probably be attributed to the fact that the inocula used in this study were collected from an anaerobic lagoon treating swine wasterwaters and had been exposed to a certain concentration of ammonia. Presumably, as the TS

concentration of the feedstock rises, a point will be reached where the microorganisms can no longer adapt to the higher nitrogen concentrations resulting from increased strength of the waste. Determination of this point, however, was beyond the scope of this investigation.

Specific growth rates were computed as described above and the results are shown in Table 4. These quantitative results are consistent with observations of the cumulative methane production (Figures 3-5). For inocula acclimated to urine-free and 1x-urine substrates, the specific growth rates on 2x-urine substrate were less than zero, indicating that the death rate may have exceeded the growth rate. Gas production virtually ceased for a 24-hour period, which can be attributed to the inhibitory effect of high concentrations of urine. For 2x-urine inocula, the growth rates were not significantly different when grown on urine-free, 1x-urine, and 2x-urine substrates ($p = 0.65$). This result indicates that after acclimation, the microorganisms can retain comparatively similar methanogenic activities when TKN reached 7.20 g/L.

As stated above, a modified Gompertz model was used to analyze the data, because it can account for the lag phase and for the depletion of substrate. Figure 6 compares the actual cumulative methane production with the predictions of the modified Gompertz model and of the exponential model for one inoculum/substrate combination (2x-urine acclimated inoculum grown on urine-free substrate). As expected, the exponential model begins to depart from the actual data as the substrate is depleted. The exponential model also diverges from the actual methane accumulation in the first few hours of the experiment due to a lag in gas production. As Figure 6 shows, the modified Gompertz model fits the data

extraordinarily well throughout the experiment. Figure 7 shows the maximum methane production rate (R_m) for the various inoculum/substrate combinations. R_m is one of three parameters in the Gompertz model that is adjusted to minimize the sum of squares for the best possible fit of the actual cumulative methane production. R_m appears to be a sensitive indicator of the degree of acclimation of the inoculum to the substrate nitrogen concentration. The one anomaly in the data is the relatively low value of R_m for the 1x-urine acclimated inoculum grown on 1x-urine substrate. Apparently, the 1x-urine acclimated inoculum was not in fact fully acclimated to the 1x-urine feed it was grown on in the CSTR prior to the batch experiments. It is worth noting that while the modified Gompertz model appears to be a precise indicator of reductions in methane production rates in the presence of inhibitory substances, R_m will be positive even in cultures where the death rate exceeds the growth rate ($\mu_{net} < 0$), since a dying population will still produce methane and exhibit a maximum methane production rate. Thus R_m is not a useful indicator of the sustainability of a given set of inoculum/substrate conditions.

2.5. Conclusions

- Anaerobic digestion of swine feces with no urine, as-excreted urine, and double as-excreted urine content at thermophilic condition (50°C) was performed in a CSTR. The biogas yield from urine-free feces was 1.41±0.01 L/L/day . The relatively lower yield can be attributed to the differences in swine waste compositions as well as inoculum used.

- Anaerobic microorganisms can be readily acclimated to nitrogen concentrations commensurate with two times the as-excreted urine:feces ratio for swine waste when the TS concentration in the waste is approximately 5%. Short (25 days) acclimation periods were sufficient for conferring tolerance of the tested nitrogen concentrations, which was probably due to their previous exposure to a certain concentration of ammonia.
- The results of these tests have implications for swine waste treatment technologies. If the anaerobic microorganisms can be readily acclimated to nitrogen concentrations commensurate with as-excreted urine:feces ratios, then separating urine and feces and utilizing only the feces as feedstock for anaerobic digestion is not necessary. It should be noted that there may be other motivations for separating the urine and feces as part of manure collection (Koger et al., 2003), but unless highly concentrated waste is digested, separation is not necessary for anaerobic digestion.

2.6. References

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Table 1. Characteristics of Swine Waste[a]

Parameters	Feces	Urine
TS (g/L)	50.3-54.3	0.5-0.7
VS (g/L)	33.6-36.2	0.3-0.5
FS (g/L)	17.7-19.5	0.1-0.2
COD (g/L)	72.9-75.0	21.8-26.1[b]
SCOD (g/L)	9.6-10.6	20.6-25.2[b]
pH	7.2-7.4	7.4-7.5
TKN (g/L)	2.81-3.82	5.82-6.69
ammonia-N (g/L)	0.38-0.45	0.72-0.83
TP (g/L)	1.38-1.65	0.17-0.19

[a] swine waste was collected in May, 2004.

[b] COD values reported here were conducted in the Biological and Agricultural Engineering Environmental Analysis Laboratory. Assuming that the TKN is in the form of ammonia nitrogen, these COD values are consistent with nitrification of the ammonia nitrogen to nitrate, which would explain the dramatic difference between volatile solids and COD in the urine.

Table 2. Composition of Batch Test Samples

Components	Substrates			Substrate control	Inoculum control
	Urine-free	1x-urine	2x-urine		
Urine (g)	0	83.0	166.0	83.0	0
Feces, grams (g, dry basis)	12.7	12.7	12.7	12.7	0
D. I. water (g)	216.0	133.0	50.0	133.0	0
Inocula (g) [a]	45.0	45.0	45.0	0	45.0
Total sample charge (g)	274.0	274.0	274.0	229.0	45.0
TKN (g/L) [b]	3.15	5.18	7.20	5.42	3.83
Ammonia-N (g/L) [b]	0.55	0.92	1.28	0.97	0.67

[a] three sets of batch tests were conducted with urine-free, 1x-urine, and 2x-urine inocula.
[b] values were measured at the beginning of the batch tests.

Table 3. pH and VS Reduction of Batch Test Samples[a]

Inocula	Parameters	Substrates		
		urine-free	1x-urine	2x-urine
urine-free	final pH	7.77 (0.06) A [b]	7.67 (0.18) A	7.64 (0.02) A
	VS reduction (%)	24.29 (2.02) A	22.53 (1.17) A	11.19 (2.02) B
1x-urine	final pH	7.61 (0.04) A	7.58 (0.09) A	7.66 (0.09) A
	VS reduction (%)	20.41 (0.11) A	19.63 (2.10) A	14.61 (0.23) B
2x-urine	final pH	7.75 (0.19) A	7.75 (0.12) A	7.69 (0.19) A
	VS reduction (%)	24.74 (1.55) A	24.35 (2.02) A	26.23 (1.17) A

[a] values in parentheses are standard deviations.
[b] values in rows followed by the same capital letter are not statistically different ($p > 0.05$), see text for p values.

Table 4. Specific Growth Rates (μ_{net}, h^{-1}) for Various Inoculum/Substrate Combinations

Inocula	Substrates		
	urine-free (TKN = 3.15 g/L)	1x-urine (TKN = 5.18 g/L)	2x-urine (TKN = 7.20 g/L)
urine-free	0.0070 (0.0010)	0.0115 (0.0026)	-0.0041 [a] (0.0011)
1x-urine	0.0105 (0.0036)	0.0028 (0.0021)	-0.0032 [a] (0.0001)
2x-urine	0.0109 (0.0022)	0.0121 (0.0018)	0.0113 (0.0017)

[a] in both replicates for these inocula/substrate combinations, gas production virtually ceased for a 24-hour period, leading to an exponential curve with a growth rate < 0.

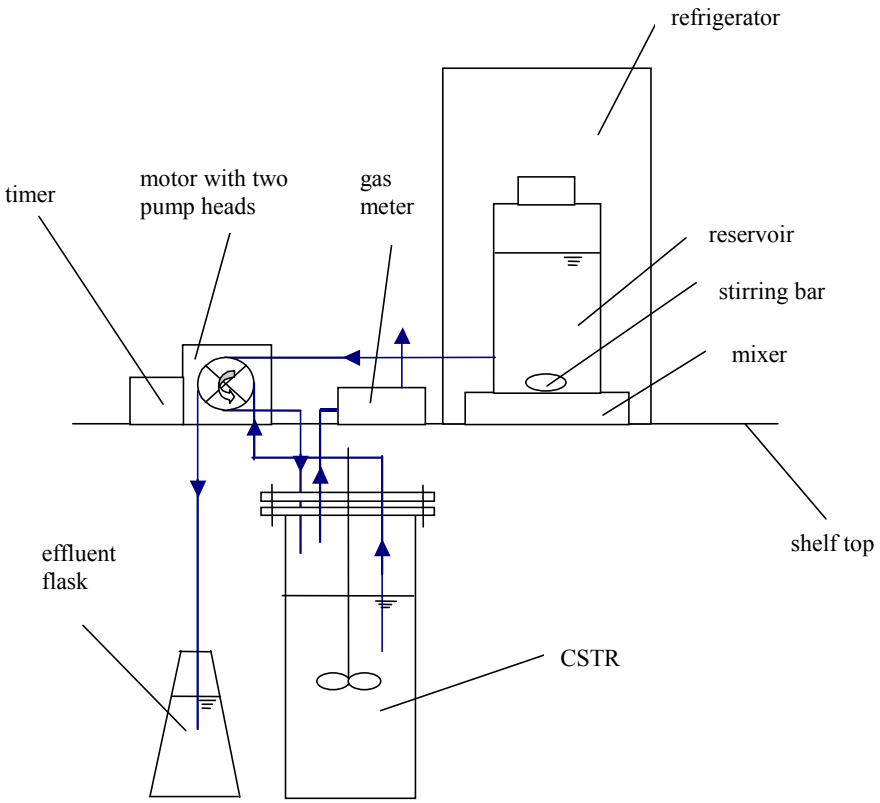

Figure 1. Thermophilic Anaerobic Digester Setup.

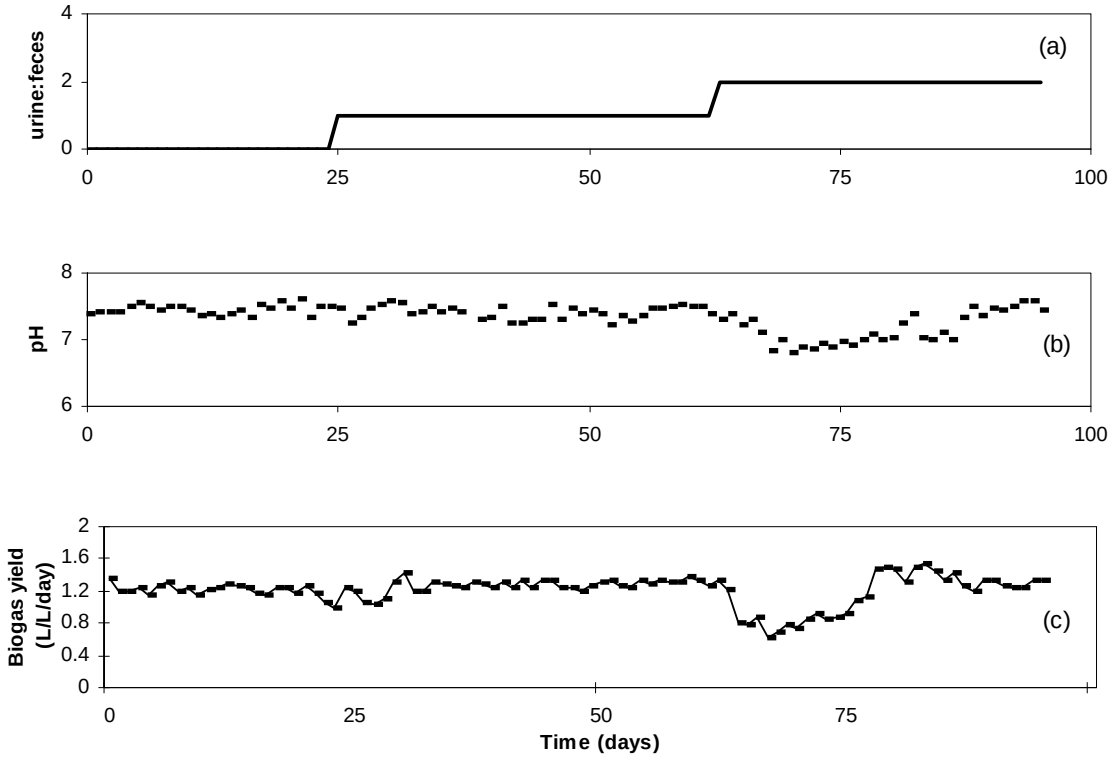

Figure 2. Operation and Performance of the CSTR Digester Treating Swine Waste: (a) Urine:Feces Ratio (Relative to As-Excreted Urine:Feces Ratio); (b) pH of Digester Contents; (c) Biogas Yield.

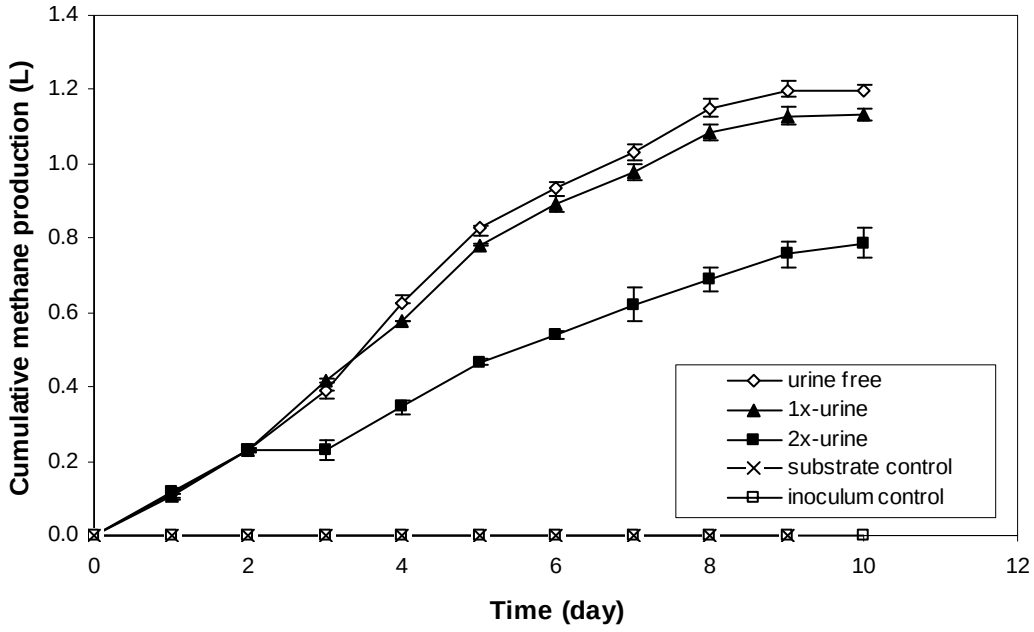

Figure 3. Effect of Urine Content on Methane Production during Anaerobic Digestion of Swine Waste Using Urine-Free Inocula.

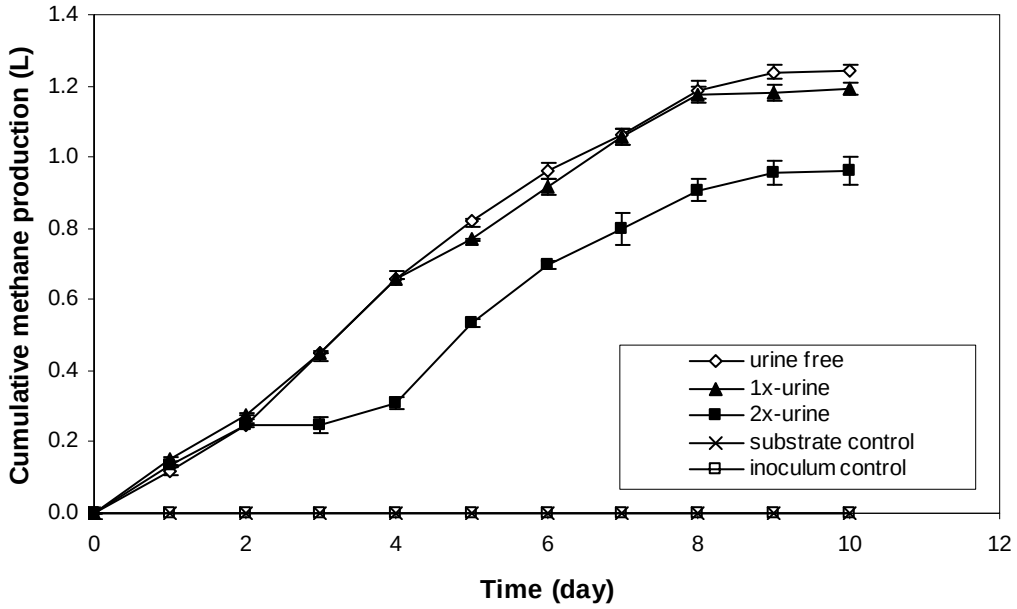

Figure 4. Effect of Urine Content on Methane Production during Anaerobic Digestion of Swine Waste Using 1x-Urine Inocula.

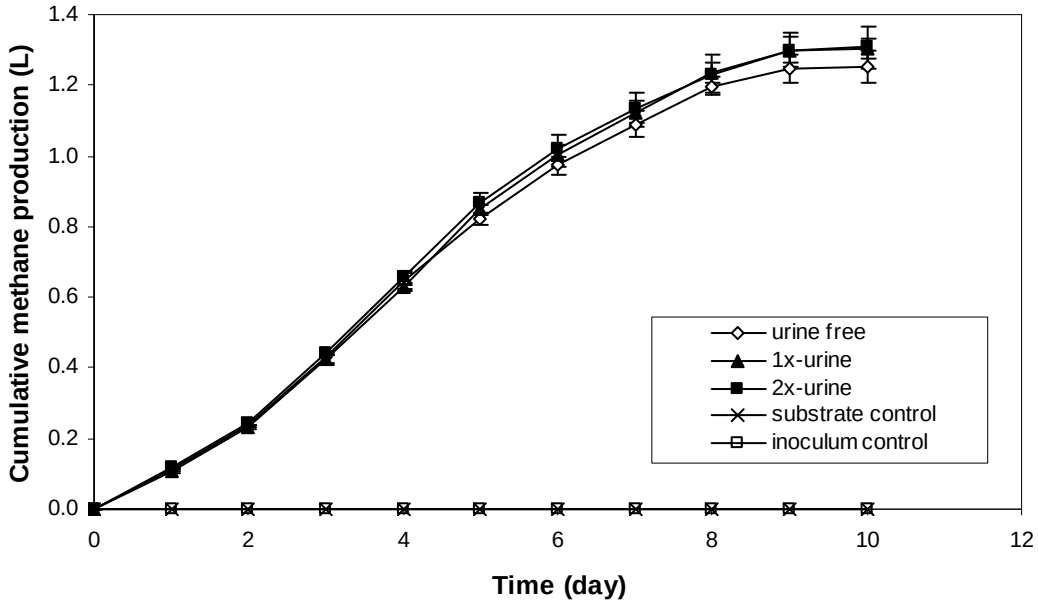

Figure 5. Effect of Urine Content on Methane Production during Anaerobic Digestion of Swine Waste Using 2x-Urine Inocula.

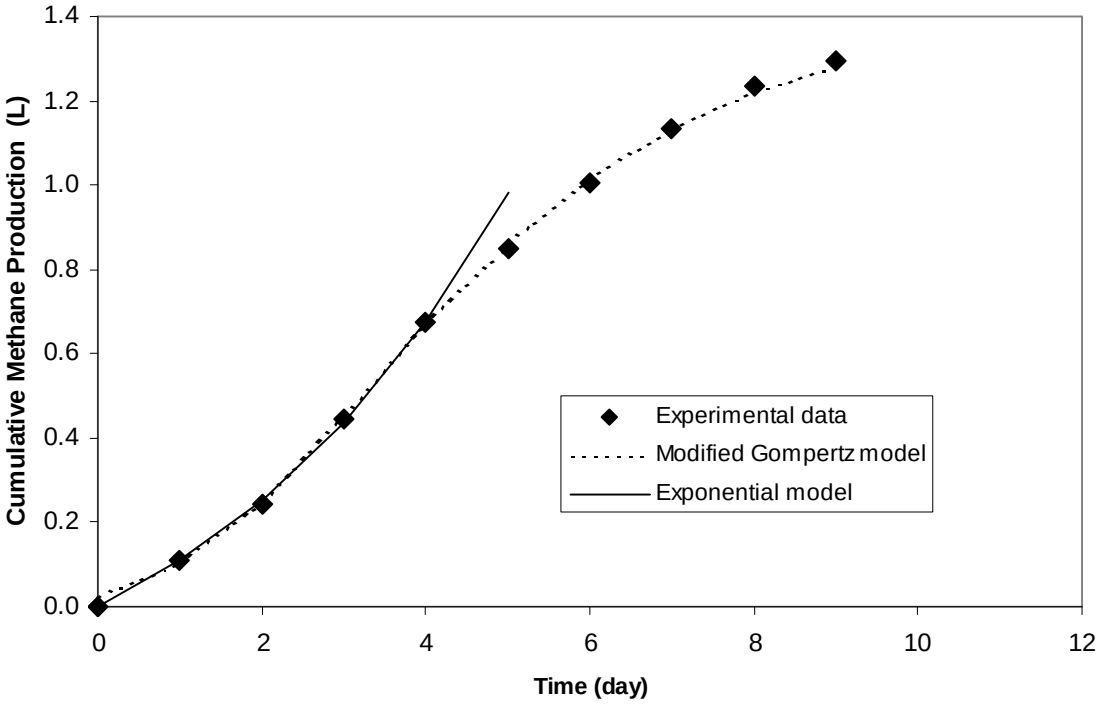

Figure 6. Actual Cumulative Methane Production (mmoles) along with Modified Gompertz and Exponential Models for 2x-Urine Acclimated Inoculum Grown on Urine-Free Substrate.

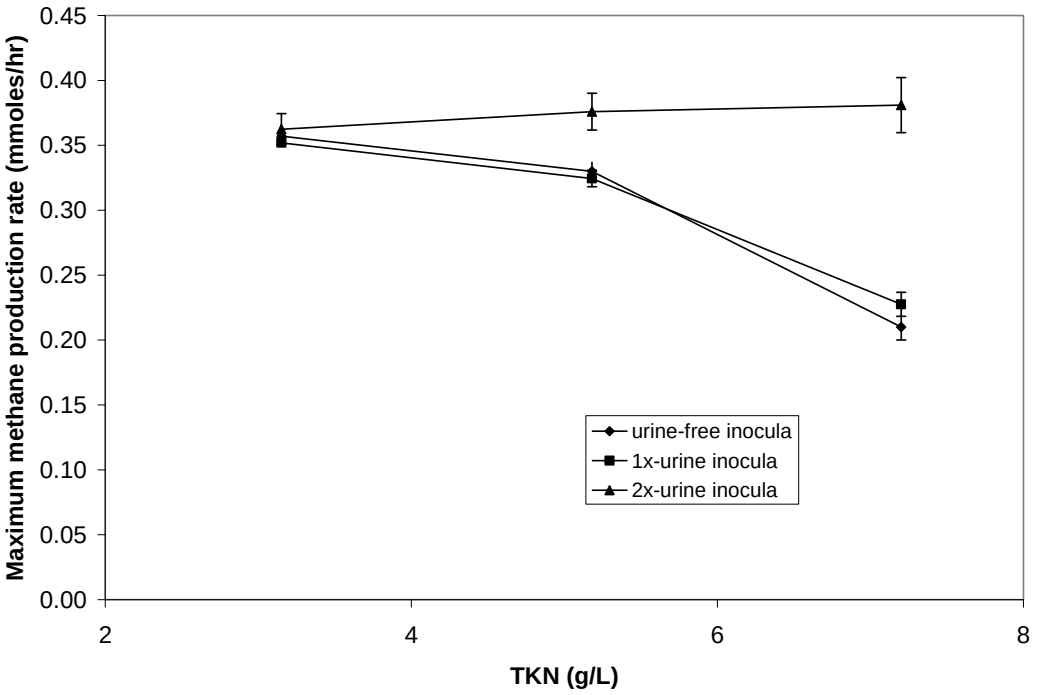

Figure 7. Maximum Methane Production Rate (mmoles/hour) as a Function of TKN (g/L) for Various Inocula/Substrate Combinations.

CHAPTER 3. STABLE THERMOPHILIC ANAEROBIC DIGESTION OF DISSOLVED AIR FLOTATION (DAF) SLUDGE BY CO-DIGESTION WITH SWINE MANURE[6]

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3.1. Abstract

Environmentally sound treatment of by-products in a value-adding process is an ongoing challenge in animal agriculture. The sludge produced as a result of the dissolved air flotation (DAF) wastewater treatment process in swine processing facilities is one such low-value residue. The objective of this study was to determine the fundamental performance parameters for thermophilic anaerobic digestion of DAF sludge. Testing in a semi-continuous stirred tank reactor and in batch reactors was conducted to determine the kinetics of degradation and biogas yield. Stable operation could not be achieved using pure DAF sludge as a substrate, possibly due to inhibition by long chain fatty acids or to nutrient deficiencies. However, in a 1:1 ratio (w/w, dry basis) with swine manure, operation was both stable and productive. In the semi-continuous stirred reactor at 54.5°C, a hydraulic residence time of 10 days, and an organic loading rate of 4.68 gVS/day/L, the methane production rate was 2.19 L/L/day and the specific methane production rate was 0.47 L/gVS (fed). Maximum specific methanogenic activity (SMA) in batch testing was 0.15 mmolesCH_4 hr^{-1} gVS^{-1} at a substrate concentration of 6.9 gVS $liter^{-1}$. Higher substrate concentrations cause an initial lag in methane production, possibly due to long chain fatty acid or nitrogen inhibition.

Keywords: anaerobic digestion; manure; thermophilic; dissolved air flotation

3.2. Introduction

In animal processing facilities, large amounts of wastewater are generated from the cleaning of carcasses, equipment and floors, as well as from other processing and hygienic operations (Environment Canada, 1981). Following primary screening, minute particles of fat, blood and meat remain in the wastewater (Carr et al., 1988). Dissolved air flotation (DAF) is a treatment process often used to remove this insoluble particulate matter from the wastewater, typically to reduce the biological oxygen demand (BOD), suspended solids (SS), and fats, oils and grease (FOG) load of the wastewater to avoid surcharges when discharging to municipal wastewater treatment plants (Westerman et al., 1989). In a DAF system, pressurized air is injected into the wastewater treatment tank, resulting in fine air bubbles that attach to particulate matter and rise to the surface, forming a sludge. Often chemical or polymer-based flocculants are added to enhance the process. The DAF sludge is typically skimmed off the top of the treatment tank and dewatered in a belt press or by other means (Westerman, et al., 1989). DAF sludge is characterized by high concentrations of FOG (Ritter, 1985) and is typically rendered or land applied, but its high energy content makes it a potential candidate for anaerobic digestion.

Digestion of wastes with high FOG concentrations, however, can be challenging. FOG increases the tendency for a scum layer to form on the digester's liquid surface (Halalsheh, 2005). Scum layer formation is related to the poor solubility of FOG at typical digester temperatures and the tendency to form a separate phase above the aqueous digester contents. One strategy for improving the degradability of FOG is to add a surfactant to the

digester to emulsify the FOG and increase its susceptibility to microbial attack (Nakhla et al, 2003). A second strategy, utilized for this work, is to operate the digester under thermophilic conditions; at higher temperatures, FOG tends to be more soluble, thereby enhancing its biodegradability. Another challenge in digesting feedstocks with high FOG concentrations is the toxicity of long chain fatty acids (LCFA) released from the hydrolysis of triglycerides. LCFA show acute toxicity towards the anaerobic consortium by adsorption to the cell wall/membrane, leading to interference with transport or protective functions (Rinzema et al., 1994). According to Hwu and Lettinga (1997), LCFA were more inhibitory to thermophilic anaerobes than to mesophilic anaerobes, perhaps due to differences in cell wall structure and composition. Angelidaki and Ahring (1992) showed that relatively low levels of oleate and stearate inhibited the metabolism of butyrate, propionate and acetate in manure-fed digesters. In batch operation, the presence of LCFA increased the lag phase, thereby causing periods of inactivity in the digester. Angelidaki and Ahring (1995) have shown, however, that it is possible to enrich the LCFA degrading members of a thermophilic anaerobic community in order to achieve higher levels of LCFA feed rates. Other studies based on the degradation of oleic acid in an anaerobic fixed-bed reactor showed that acclimation improved the resistance of the biofilm to the presence of oleate and improved the biodegradation capacity compared to the biofilm formed in the absence of lipids (Alves et al., 2001a; 2001b).

Co-digestion of different types of agricultural waste can be a means of mitigating the deficiencies of a particular feedstock such as DAF. One example of where co-digestion is beneficial is in balancing the carbon:nitrogen (C/N) ratio of the digester feed. Other

examples would be where a secondary feedstock might improve microbial nutrition, provide pH buffering capacity, or lower the concentration of a toxic or inhibitory compound in the primary feedstock. Due to the siting of animal processing facilities near concentrated livestock rearing facilities, animal manure is often a waste of environmental concern in the same regions where DAF sludge is plentiful.

The objective of this study was to determine operating conditions for stable anaerobic digestion of dissolved air flotation (DAF) sludge from an animal processing facility. A second goal was to determine key performance parameters, such as methane production rate ($L\ L^{-1}\ d^{-1}$), specific methane production rate ($L\ gVSfed^{-1}$) and specific methanogenic activity ($mmoles\ hour^{-1}\ gVS^{-1}$), to enable design of anaerobic digesters using DAF sludge as a feedstock.

3.3. Materials and Methods

3.3.1. Dissolved Air Flotation (DAF) Sludge

The DAF sludge was obtained from a swine processing facility in North Carolina. This plant uses a DAF process as part of its wastewater treatment system to reduce the BOD of discharged wastewater. Skimmings from the DAF unit are dewatered in a belt press, creating a sludge cake from which samples were collected for tests described herein. The DAF sludge was stored at -20°C prior to use. Table 1 shows a chemical analysis of the DAF sludge used in these experiments, along with analyses of DAF sludge reported in the literature for comparison. The composition varies significantly between DAF sludges,

possibly due to the type of animal being processed (swine vs. poultry processing), location of the facilities, or different operational procedures. Note, however, that all DAF sludges in Table 1 have high FOG content.

3.3.2. Swine Waste

Only feces (no urine) were used for these tests. Fresh feces were collected from a slatted-floor hog barn at the Swine Education Unit of the Lake Wheeler Road Field Laboratory of North Carolina State University (Raleigh, NC), as described previously (Creamer et al, 2008). The raw swine waste was stored at -20°C prior to use. Table 1 shows an analysis of the manure used in these experiments.

3.3.3. Continuously Stirred Tank Reactor (CSTR) Operation

Continuous testing was conducted in a 14 L (10-liter working volume) CSTR digester (Bioflo 110, New Brunswick, NJ) (Figure 1). Prior to operation on the DAF sludge/manure mixture, the digester operated on a manure (feces-only) feedstock for at least 12 months; the original inoculum for the digester came from the Lake Wheeler Road Field Laboratory Swine Education Unit, Raleigh, NC (Creamer et al, 2008). The semi-continuous stirred reactor was fed 1 kg of substrate once per day. The raw feedstocks (as-received DAF sludge and feces) were thawed in a 4ºC refrigerator. Measurements of moisture contents of the feedstocks prior to freezing were used to determine the amount of each raw feedstock required so that the feedstock ratio in the substrate was 1:1 DAF sludge:manure (w/w, dry basis). De-ionized water was then added so that the final total solids concentration would be 5%. Measurement

of the raw feedstock moisture contents showed that the actual ratio of DAF sludge:manure was 1.16:1 (w/w, dry basis), and that the actual total solids level was 5.52%. The mixture was then agitated until homogenous with a commercial laboratory blender (21/800EG, John Morris Scientific, Australia). Just prior to adding fresh substrate, 1 kg of digester contents were removed by siphoning through an access port in the top of the reactor. The fresh substrate was then added with the aid of a funnel, also through an access port in the top of the reactor. The digester was operated on the DAF sludge/manure mixture until steady-state operation was achieved, defined by constant effluent pH and coefficient of variation of daily biogas production lower than 5% for more than 5 consecutive days. Approximately ninety-days were required for the system to reach steady-state.

Mixing for the semi-continuous stirred reactor was provided by the Bioflo 110's built-in agitator. Agitator rotation speed was set to 150 rpm. All CSTR testing was conducted at a hydraulic retention time (HRT) of 10 days, achieved through manual siphoning of one liter of effluent each day followed by addition of one liter of the DAF sludge/manure feedstock, giving a feed rate of 4.68 gVS/day/L (11.24 gCOD/day/L). The digester is equipped with an external circulating water jacket that allowed maintenance of a constant temperature of 54.5°C inside the digester. Gas production was measured with a wet tip meter (invented by Dr. R. E. Speece and available at http://wettipgasmeter.com). CO_2 absorption in the gas meter was deliberately minimized through saturation with NaCl and maintenance of a low pH (<2). The gas meter is a volumetric device -- in position 1, gas from the digester displaces water in one of two contiguous chambers until the buoyancy of

the gas in that chamber causes the chamber assembly to tilt into position 2, releasing the gas in chamber 1 and orienting the chamber assembly in such a way that gas is now collected in chamber 2. Each tip is counted by an electromagnetic sensor. The gas meter was calibrated by repeatedly injecting a known quantity of gas into the meter with a syringe and relating the volume of gas injected to the number of tips.

3.3.4. Batch Tests

Batch tests were conducted at three feed substrate concentrations in order to gain insight into growth rates/kinetics. The nominal feed substrate concentrations were 5%, 7.5% and 10% TS (the feed substrate was mixed with the inoculum to give the final TS concentrations shown in Table 2). Effluent collected from the CSTR digester at steady-state was used as inocula. In each batch test, 270 grams of inoculum was mixed with 30 ml of the DAF sludge/manure mixture in a 500 mL medium bottle. The high ratio of inoculum to feedstock was chosen so that the inoculum experienced similar conditions to those found in the CSTR. Earlier batch and continuous testing (data not shown) showed that the microbial community was very sensitive to changes in organic loading rate and feed volatile solids concentration. Two controls were used for these tests, a substrate-only control and an inoculum-only control. The purpose of the inoculum-only control was to determine the background biogas production from the residual substrate in the inoculum, thereby enabling an estimate of biogas production from fresh substrate only. Table 2 shows the composition of the batch test samples.

Due to the strong buffering capacity of swine waste (Sommer and Husted, 1995), pH could be maintained between 7.0 and 7.2 without adjustment. Nitrogen gas was used to flush the bottle headspace before the medium bottles were connected to a liquid displacement gas measuring device. Samples were incubated at 54.5°C in a temperature-controlled water bath. Gas production was recorded until cessation. For each treatment condition, two replicates were used.

3.3.5. Analytical Methods and Data Analysis

Total solids (TS), volatile solids (VS), pH, chemical oxygen demand (COD), total organic carbon (TOC), and ortho-phosphorus were analyzed by the Biological and Agricultural Engineering Department's Environmental Analysis Lab at NC State University in accordance with Standard Methods (APHA, 1998). With the exception of FOG, all other analyses reported in Table 1 were performed by the Agronomic Services Division of the North Carolina Department of Agriculture & Consumer Services, also in accordance with Standard Methods (APHA, 1998). FOG was measured using a Soxhlet extraction apparatus in accordance with Method 5520 in Standard Methods (APHA, 1998). As recommended in Method 5520, the solvent used for the extraction was n-hexane.

Biogas composition was determined by sampling reactor headspace for both the CSTR and batch digesters. A Shimadzu GC 15A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 3 m × 3 mm (10 ft × 0.13 in.) stainless-steel column (100/120 Carbosieve S-II packing, Supelco, Bellefonte, Pennsylvania) and a thermal conductivity detector was used.

The method of Sørensen and Ahring (1993) was used to measure the specific methanogenic activity (SMA) of the batch cultures. Consistent with Sorensen and Ahring, the cumulative methane production was measured as described above over the initial, linear period of methane production in the batch reactors. Linear methane accumulation was observed over the first 21 hours of batch operation. The accumulated methane was divided by the volatile solids content of the inoculum, which serves as a proxy for active biomass in the batch reactors. The results were calculated as an average of the two replicate batch tests conducted for each treatment.

3.4. Results and Discussion

3.4.1. Continuous Testing

Initially, the CSTR digester was operated on 100% DAF sludge at a nominal feed concentration of 5% TS and a 10-day HRT (organic loading rate of 5.24 gVS/day/L of digester working volume). Operation under these conditions was highly unstable, characterized by rapid and irreversible drops in pH. Attempts were made to buffer the digester with lime but stable operation was still difficult (data not shown). It was postulated that poor operation was not entirely due to the lack of buffering capacity of the DAF sludge, but also due to the deficiency of key elements in DAF sludge. Gerardi (2003) gives recommended levels of key microbial nutrients as a percentage of the COD concentration. The concentration of phosphorus in DAF sludge, for example, is only one-fifth the level

recommended by Gerardi. The iron concentration in DAF sludge may also be too low to support robust microbial growth; it is only one-fourth the level recommended by Gerardi.

To overcome the apparent shortcomings of DAF sludge, swine feces were used as a co-substrate in subsequent testing in a nominal 1:1 ratio (dry basis). Addition of manure provides several potential benefits as compared to a DAF sludge-only feedstock. One immediate and measurable benefit was the greatly improved buffering capacity. With the addition of manure, rapid pH swings were completely eliminated without the need for lime. Another potential benefit of manure is improved microbial nutrition. As shown in Table 1, manure contains levels of certain key nutrients that may be deficient in a feedstock based on DAF sludge alone. In addition, the impact of potential toxic or inhibitory compounds in the DAF sludge, such as LCFA would be mitigated by dilution of DAF sludge with manure. Results of continuous and batch tests of the DAF sludge/manure mixture are described below.

Table 3 shows the results of the CSTR testing (first row of data), which represents an average of data taken on a daily basis over a nine day period following the achievement of steady-state operation, as defined above. For comparison, data from the literature for the thermophilic anaerobic digestion of manure-only feedstocks is also listed. As can be seen in Table 3, the performance of the DAF sludge/manure mixture at a 10 day HRT is as good as or better than any manure-only feedstock shown, from the standpoint of biogas production per day per unit working volume, biogas methane content, and biogas production per unit of volatile solids fed. It must be noted that the results from this study cannot be definitively

compared to the other results in Table 3 because in no case are the operating conditions exactly the same. The measured parameters, including hydraulic residence time and organic loading rate will have a strong influence on methane production and methane concentration. Substrate manure type (for example, dairy versus swine manure) will also have a significant effect on performance as will unmeasured parameters such as particle size and animal diet. Nonetheless, a superior performance with DAF sludge as a co-feedstock is not surprising, given the high FOG content. As can be deduced from Table 1, the COD of DAF sludge is 36% greater than the COD of manure on a dry basis. The higher FOG content and the higher COD suggest a higher methane producing potential, which provides an incentive for maximizing the ratio of DAF sludge to manure in the feedstock mixture, subject to the constraints due to microbial nutrition and pH buffering. Further testing in this area is warranted.

3.4.2. Batch Testing

As mentioned above, batch testing was conducted to give greater insight into the growth kinetics of the thermophilic microbial community on the DAF sludge/manure mixture. Of particular interest was the response of the microbes to increased substrate concentration. Substrate concentration is especially important for batch reactors and plug flow reactors, since higher substrate concentration translates directly into reduced reactor size, which in turn reduces the capital costs of the digester. Earlier testing (data not shown) indicated that the inoculum used for batch testing was very sensitive to sudden changes in

substrate concentration. For this reason, a high inoculum to substrate ratio was chosen for these batch tests.

Methane production from the batch tests, shown as cumulative methane production (liters, STP) divided by the initial fresh substrate (grams), is shown in Figure 2. Two replicates were used for each treatment in the batch test; shown are the average values for the two replicates. Note that this is the cumulative net methane production from fresh substrate only; the methane production from the inoculum-only control (which was quite modest) was subtracted from the total methane production, consistent with the methods of Sørensen and Ahring (1993). Methane production during the first 21 hours was highly linear (R^2>0.99).

For the treatment with the highest fresh substrate concentration, not only did methane production lag behind the other treatments, but the yield of methane per gram of volatile solids (fresh substrate) was lower. The lag in methane production can be seen clearly upon determination of SMA for the three batch treatments. As shown in Figure 3, SMA increases as the substrate concentration is increased from 4.6 to 6.9 g/L. But as the concentration is increased further to 9.2 g/L, SMA decreases to about the same level as SMA at 4.6 g/L, indicating inhibition associated with the substrate. Without further testing, it is not possible to determine the inhibitory compound, but a leading candidate would be the long-chain fatty acids (LCFA) present in the DAF sludge (Angelidaki and Ahring, 1992), as discussed above. It is worth noting, however, that the concentration of fats, oils and greases (FOG) introduced through the DAF-containing feedstock is much higher than the concentration of fatty acids in fresh manure and anaerobic lagoon sludge from a swine production facility in North

Carolina, as measured by Loughrin and Szogi (2006). They found maximum values of 47.7 μg of fatty acids per gram of (dry) manure solids and 4.0 μg of fatty acids per gram of (dry) anaerobic lagoon sludge. In the present study, the lowest initial level of FOG in batch testing was 49 mg of FOG per gram of dry solids in the batch reactor, corresponding to a concentration of 1.5 g/L. The composition of the FOG extracted from the DAF sludge was not analyzed, but based on the source of the material and the process from which it is derived, a high proportion of LCFA would be expected.

Among the other measured components of manure and DAF sludge, nitrogen is another inhibitory candidate. From Tables 1 and 2, the nitrogen (TKN) contribution of fresh substrate can be calculated; the contribution of fresh substrate TKN to the overall TKN was 1,584 mg/L and 3,171 mg/L at the low and high fresh substrate concentrations, respectively. The balance of TKN in batch tests was introduced with the inoculum. From sampling of the digester effluent used as the inoculum, the contribution of inoculum TKN was 1,544 mg/L (nitrogen in the inoculum was 65.5% mineralized to NH_4), bringing the total TKN in the high substrate treatment to 4,715 mg/L. If the nitrogen measured as TKN is in organic form, it will not contribute to inhibition. But mineralization of organic nitrogen in organic feedstocks can be rapid at thermophilic temperatures. Nakashimada et al (2008) found that over 50% of the organic nitrogen in waste activated sludge was mineralized in 1.33 days at 55°C and Bujoczek et al (2000) found overall mineralization rates to be as high as 80.3% in anaerobically digested chicken manure. With fast and efficient mineralization, ammonia

levels in the high substrate treatment could easily exceed the 3,000 mg/L level that Hashimoto (1983) found inhibitory for the thermophilic digestion of swine waste.

3.5. Conclusions

Because of its high fats, oil and grease (FOG) content, dissolved air flotation (DAF) sludge from animal processing facilities is a potent feedstock for anaerobic digestion. High FOG content feedstocks, however, require special attention when used in anaerobic digesters, because of scum formation, phase separation, and inhibition by long chain fatty acids. We have shown that in a 1:1 ratio (w/w, dry basis) with manure, a thermophilic anaerobic digester can operate stably and productively on DAF sludge. The manure provides buffering capacity and may also provide nutrients that are deficient in DAF sludge. It seems likely that in a commercial application of the anaerobic digestion of DAF sludge, a swine processing facility would want to minimize the amount of co-feedstock used (unless, unlike manure, it is readily available at the processing facility). Therefore, additional work should focus on the minimum amount of manure necessary for stable operation of the digester.

3.6. Acknowledgements

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Table 1. Manure and DAF Sludge Analyses

	Swine Manure	Swine DAF Sludge	Poultry DAF sludge	Poultry DAF sludge	Poultry DAF sludge
pH	6.4	4.9	5.9	6.5	5.5
VS[a]	71.8%	95.8%	95.3%	80.8%	n/a
FOG[a]	4.0%[b]	54.3%	48.9%	25.6%	45.3%
	All values below are in mg/L adjusted to a 5% TS concentration				
COD	104,704	142,518	44,891	68,588	95,667
TOC	n/a	33,035	15,584	n/a	n/a
TKN	1,059	1,976	2,584	2,433	2,750
NH_4-N	300	69	7	1,663	n/a
NO_3-N	2	1	3	<11	n/a
Total P	1,375	125	569	1,045	336
Ortho-P	n/a	64	62	904	n/a
Cl	76	32	95	n/a	n/a
Na	155	40	32	n/a	n/a
K	501	39	33	n/a	229
Ca	2,659	389	46	n/a	n/a
Mg	551	27	15	n/a	n/a
Cu	13.7	3.9	14.6	n/a	2.7
Zn	133.2	18.2	7.7	n/a	14.0
Fe	177.6	70.7	38.3	n/a	n/a
Mn	45.1	2.5	1.3	n/a	n/a
Ni	0.6	0.4	1.6	n/a	n/a
Cd	0.1	0.1	0.3	n/a	0.1
S	148.0	134.1	n/a	n/a	n/a
Reference	This study	This study	Westerman et al., 1989	Ritter, 1985	Carr, 1988

[a] values are reported as a percentage (%) of total solids (TS).
[b] swine waste FOG content was not measured in this study; value shown is from Angelidaki and Ahring (1997)

Table 2. Conditions for Batch Tests

	Treatment 1	Treatment 2	Treatment 3	Control 1 (feed only)	Control 2 (inoculum only)
TS (%)	3.1	3.3	3.6	10.7	2.8
DAF sludge:manure ratio (dry basis)	1.16	1.16	1.16	1.16	
DAF sludge in substrate (g/L)	27.4	41.2	54.9	54.9	
Manure in substrate (g/L)	23.7	35.5	47.3	47.3	
Substrate (g, wet basis)	30	30	30	30	
Inoculum (g, wet basis)	270	270	270	0	270
Total batch charge (g, wet basis)	300	300	300	30	270
Initial VS[a], g/L	4.58	6.87	9.16	91.62	0

[a] The initial VS reported here is from the fresh substrate only. The inoculum also contributes VS to the Total Batch Charge, but these volatile solids are not considered substrate – they represent microbial biomass plus recalcitrant volatile solids that have remained undigested after 10 days in the CSTR.

Table 3. Performance of Thermophilic CSTR Digesters on DAF Sludge/Manure

Feed	Working volume, L	HRT days	Temp °C	Organic loading rate gVS/day/L	VS conc. in the feed, gVS/L	Methane produced, L/gVSfed	Methane produced, L/L/day	Methane content of biogas, %	References
DAF/ swine	10	10	54.5	4.7	46.8	0.47	2.19	71	This study
swine	3	15	55.0	3.4	50.4	0.43	1.45	61	Hashimoto, 1983
swine	3	10	55.0	5.0	50.4	0.36	1.80	61	Hashimoto, 1983
swine	3	5	55.0	10.1	50.4	0.31	3.12	61	Hashimoto, 1983
swine	3	15	55.0	3.0	45.0	0.07	0.20	51	Hansen et al., 1998
dairy	3	15	55.0	3.5	52.0	0.15	0.50	58	Lo et al., 1985
dairy	3	10	55.0	5.2	52.0	0.12	0.62	60	Lo et al., 1985
dairy	3	4	55.0	13.0	52.0	0.07	0.94	56	Lo et al., 1985
dairy/ beef	3	20	60.0	3.0	60.0	0.23	0.70	58	Mackie and Bryant, 1995

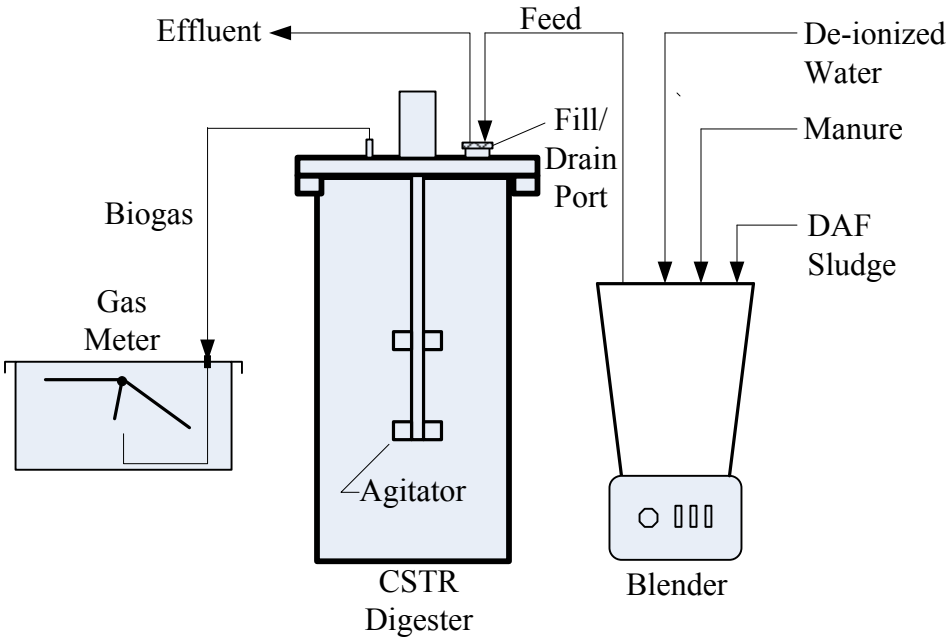

Figure 1. CSTR Digester Test Setup

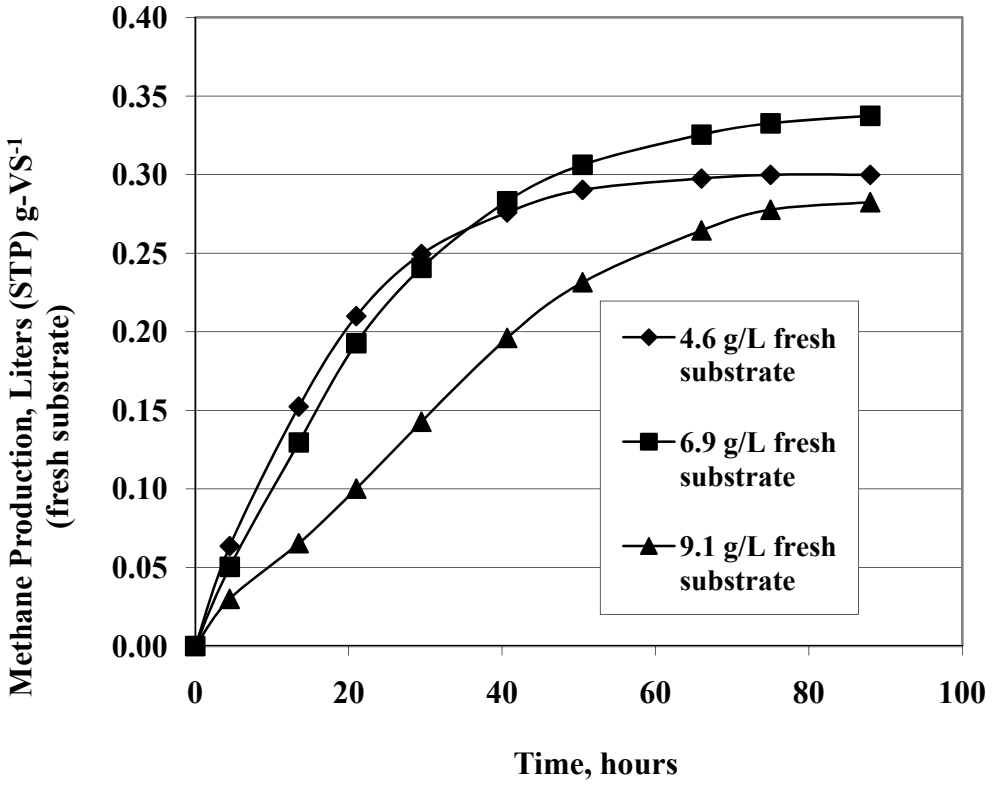

Figure 2. Cumulative Methane Production for Batch Digester Tests

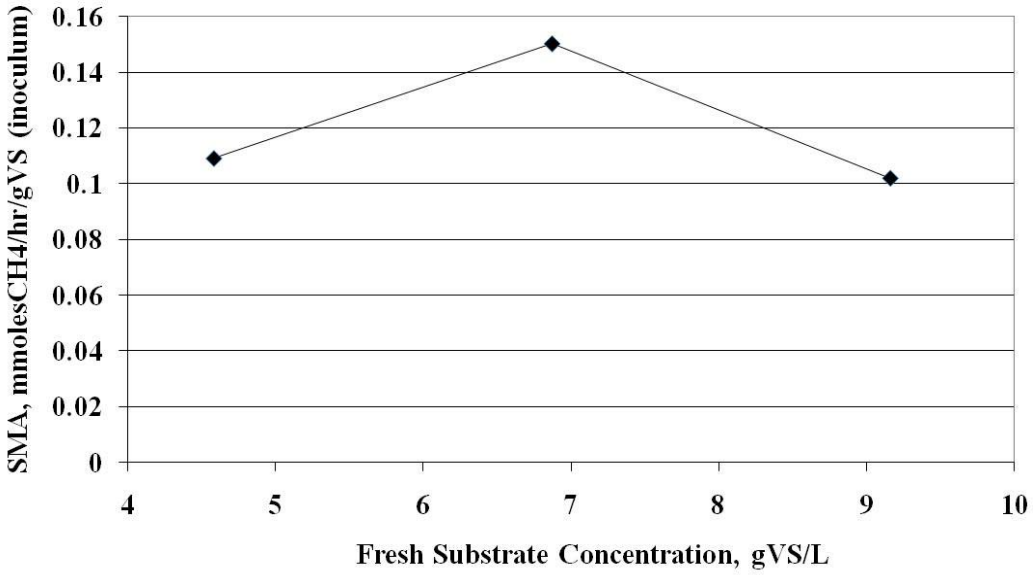

Figure 3. Specific Methanogenic Activity (SMA) for Batch Digester Tests

CHAPTER 4. CONCLUSIONS AND FUTURE RESEARCH RECOMMENDATIONS

4.1. Conclusions

Anaerobic digestion of swine feces with no urine, as-excreted urine, and double as-excreted urine content at thermophilic condition (50°C) was performed in a CSTR. The biogas yield from urine-free feces was 1.41±0.01 L/L/day . The relatively lower yield can be attributed to the differences in swine waste compositions as well as inoculum used.

Anaerobic microorganisms can be readily acclimated to nitrogen concentrations commensurate with two times the as-excreted urine:feces ratio for swine waste when the TS concentration in the waste is approximately 5%. Short (25 days) acclimation periods were sufficient for conferring tolerance of the tested nitrogen concentrations, which was probably due to their previous exposure to a certain concentration of ammonia.

The results of these tests have implications for swine waste treatment technologies. If the anaerobic microorganisms can be readily acclimated to nitrogen concentrations commensurate with as-excreted urine:feces ratios, then separating urine and feces and utilizing only the feces as feedstock for anaerobic digestion is not necessary. It should be noted that there may be other motivations for separating the urine and feces as part of manure collection (Koger et al., 2003), but unless highly concentrated waste is digested, separation is not necessary for anaerobic digestion.

Because of its high fats, oil and grease (FOG) content, dissolved air flotation (DAF) sludge from animal processing facilities is a potent feedstock for anaerobic digestion. High FOG content feedstocks, however, require special attention when used in anaerobic digesters, because of scum formation, phase separation, and inhibition by long chain fatty acids. We have shown that in a 1:1 ratio (dry weight basis) with manure, a thermophilic anaerobic digester can operate stably and productively on DAF sludge. The manure provides buffering capacity and may also provide nutrients that are deficient in DAF sludge.

4.2. Future Research Recommendations

Although the research described herein shows that a thermophilic anaerobic digester utilizing a 50:50 (w/w) mixture of DAF sludge and manure can operate stably and productively, in commercial applications it may be desirable to minimize the amount of manure used in a DAF:manure mixture. An animal processing facility is unlikely to want to import manure from surrounding farms in order to operate a digester on site. On the other hand, the amount of DAF sludge produced at an animal processing facility would most likely overwhelm a farm-scale digester, should the DAF sludge be exported. Thus an animal processing facility may want to minimize the amount of manure imported or find another nutrient rich substrate already available on-site to co-digest with DAF sludge, such as stomach contents.

Another research area that was not addressed herein but has substantial potential to improve methane production rate and specific methane yield in digesters is pretreatment and bioaugmentation. Undigested lignocellulosic fibers, for example, represent a currently

underutilized component of anaerobic digester feedstocks. Extensive research is currently underway to develop pretreatment technology designed to modify the structure of biomass to improve its accessibility to enzymes for the purpose of cellulosic ethanol production (Mosier et al. 2005). This research is directly relevant to those working in the field of anaerobic digestion. The pretreatment technologies being developed will also have a direct impact on the accessibility of lignocellulosic substrates by anaerobic microbes in a digester. Therefore, anaerobic digester researchers should monitor closely these developments and apply the learnings to pretreatment of anaerobic digester substrates.

Efforts to use microorganisms to enhance anaerobic digestion have been sporadically attempted for many years. While the impact of the introduced microorganism on the digester gross performance was measured, understanding how it worked or whether it even survived in the digester could not be determined. With increasingly sophisticated molecular and genetic tools, a much more systematic approach to the introduction of bioaugmenting microbes can be undertaken. One significant challenge will be creating an environment in which the introduced microbe can thrive given that in normal digester operation, microbes are introduced continually with the feedstock. It may be necessary to examine ways to suppress the background microbial consortia in the feedstock to allow the introduced microbe to survive.

Enzymes may have utility in improving anaerobic digester performance. A range of enzyme activities can, in theory, enhance bioavailability of digester substrates. Of particular

interest are cellulases and hemicellulases, which could enable methane production from the underutilized fiber fraction of digester substrates.

4.3. References

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple, and M. Ladisch. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96 (6):673-686.

APPENDICES

A.1. Raw Data, Urine-Feces Batch Testing

Raw Gas Production Using Inoculum Adapted to Urine:Free Substrate, mL									
Day		T1, R1	T1,R2	T2, R1	T2,R2	T3, R1	T3,R2	Control A	Control B
0	Start of Experiment	50	50	50	50	50	50	50	50
		50	50	50	50	50	50	50	50
		0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0
1	Initial Reading	160	165	170	165	155	160	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	110	115	120	115	105	110	0	0
	mmol CH4	4.57	4.78	4.99	4.78	4.36	4.57	0.00	0.00
2	Initial Reading	170	165	160	165	170	175	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	120	115	110	115	120	125	0	0
	mmol CH4	4.99	4.78	4.57	4.78	4.99	5.20	0.00	0.00
3	Initial Reading	205	210	155	140	250	225	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	155	160	105	90	200	175	0	0
	mmol CH4	6.44	6.65	4.36	3.74	8.31	7.27	0.00	0.00
4	Initial Reading	290	290	180	150	190	225	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	240	240	130	100	140	175	0	0
	mmol CH4	9.98	9.98	5.40	4.16	5.82	7.27	0.00	0.00
5	Initial Reading	250	255	175	170	255	260	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	200	205	125	120	205	210	0	0
	mmol CH4	8.31	8.52	5.20	4.99	8.52	8.73	0.00	0.00
6	Initial Reading	150	155	105	135	165	155	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	100	105	55	85	115	105	0	0
	mmol CH4	4.16	4.36	2.29	3.53	4.78	4.36	0.00	0.00
7	Initial Reading	150	150	130	140	120	150	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	100	100	80	90	70	100	0	0
	mmol CH4	4.16	4.16	3.33	3.74	2.91	4.16	0.00	0.00
8	Initial Reading	165	170	120	115	150	165	50	50
	Final Reading	50	50	50	50	50	50	50	50

	Biogas Released	115	120	70	65	100	115	0	0
	mmol CH4	4.78	4.99	2.91	2.70	4.16	4.78	0.00	0.00
9	Initial Reading	90	105	115	120	105	85	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	40	55	65	70	55	35	0	0
	mmol CH4	1.66	2.29	2.70	2.91	2.29	1.45	0.00	0.00
10	Initial Reading	50	50	85	75	50	55	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	0	0	35	25	0	5	0	0
	mmol CH4	0.00	0.00	1.45	1.04	0.00	0.21	0.00	0.00

T1 = Treatment 1, feces only

T2 = Treatment 2, feces plus 2x as-excreted ratio of urine:feces

T3 = Treatment 3, feces plus 1x as-excreted ratio of urine:feces

R1 = Replicate 1

R2 = Replicate 2

Control A = as-excreted substrate only, no inoculum

Control B = inoculum only, no substrate

Raw Gas Production Using Inoculum Adapted to 1x Urine:Feces Substrate, mL									
Day		T1, R1	T1,R2	T2, R1	T2,R2	T3, R1	T3,R2	Control A	Control B
0	Start of Experiment	50	50	50	50	50	50	50	50
		50	50	50	50	50	50	50	50
		0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0
1	Initial Reading	160	180	185	180	200	205	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	110	130	135	130	150	155	0	0
	mmol CH4	4.57	5.40	5.61	5.40	6.23	6.44	0.00	0.00
2	Initial Reading	180	180	160	170	175	175	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	130	130	110	120	125	125	0	0
	mmol CH4	5.40	5.40	4.57	4.99	5.20	5.20	0.00	0.00
3	Initial Reading	255	240	50	50	210	240	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	205	190	0	0	160	190	0	0
	mmol CH4	8.52	7.90	0.00	0.00	6.65	7.90	0.00	0.00
4	Initial Reading	250	275	115	110	260	250	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	200	225	65	60	210	200	0	0
	mmol CH4	8.31	9.35	2.70	2.49	8.73	8.31	0.00	0.00
5	Initial Reading	225	200	275	270	165	155	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	175	150	225	220	115	105	0	0
	mmol CH4	7.27	6.23	9.35	9.14	4.78	4.36	0.00	0.00
6	Initial Reading	175	200	227	200	200	200	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	125	150	177	150	150	150	0	0
	mmol CH4	5.20	6.23	7.36	6.23	6.23	6.23	0.00	0.00
7	Initial Reading	150	150	150	150	165	214	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	100	100	100	100	115	164	0	0
	mmol CH4	4.16	4.16	4.16	4.16	4.78	6.82	0.00	0.00
8	Initial Reading	175	180	160	160	180	160	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	125	130	110	110	130	110	0	0
	mmol CH4	5.20	5.40	4.57	4.57	5.40	4.57	0.00	0.00

9	Initial Reading	100	100	100	100	50	55	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	50	50	50	50	0	5	0	0
	mmol CH4	2.08	2.08	2.08	2.08	0.00	0.21	0.00	0.00
10	Initial Reading	60	50	50	60	60	65	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	10	0	0	10	10	15	0	0
	mmol CH4	0.42	0.00	0.00	0.42	0.42	0.62	0.00	0.00

T1 = Treatment 1, feces only

T2 = Treatment 2, feces plus 2x as-excreted ratio of urine:feces

T3 = Treatment 3, feces plus 1x as-excreted ratio of urine:feces

R1 = Replicate 1

R2 = Replicate 2

Control A = as-excreted substrate only, no inoculum

Control B = inoculum only, no substrate

Raw Gas Production Using Inoculum Adapted to 2x Urine:Feces Substrate, mL									
Day		T1, R1	T1,R2	T2, R1	T2,R2	T3, R1	T3,R2	Control A	Control B
0	Start of Experiment	50	50	50	50	50	50	50	50
		50	50	50	50	50	50	50	50
		0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0
1	Initial Reading	160	165	170	165	155	160	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	110	115	120	115	105	110	0	0
	mmol CH4	4.57	4.78	4.99	4.78	4.36	4.57	0.00	0.00
2	Initial Reading	180	175	180	170	180	170	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	130	125	130	120	130	120	0	0
	mmol CH4	5.40	5.20	5.40	4.99	5.40	4.99	0.00	0.00
3	Initial Reading	250	230	245	255	250	235	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	200	180	195	205	200	185	0	0
	mmol CH4	8.31	7.48	8.11	8.52	8.31	7.69	0.00	0.00
4	Initial Reading	275	255	250	275	250	260	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	225	205	200	225	200	210	0	0
	mmol CH4	9.35	8.52	8.31	9.35	8.31	8.73	0.00	0.00
5	Initial Reading	220	235	250	270	255	285	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	170	185	200	220	205	235	0	0
	mmol CH4	7.07	7.69	8.31	9.14	8.52	9.77	0.00	0.00
6	Initial Reading	205	195	200	215	190	215	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	155	145	150	165	140	165	0	0
	mmol CH4	6.44	6.03	6.23	6.86	5.82	6.86	0.00	0.00
7	Initial Reading	175	160	160	165	165	170	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	125	110	110	115	115	120	0	0
	mmol CH4	5.20	4.57	4.57	4.78	4.78	4.99	0.00	0.00
8	Initial Reading	150	165	140	155	170	160	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	100	115	90	105	120	110	0	0
	mmol CH4	4.16	4.78	3.74	4.36	4.99	4.57	0.00	0.00

9	Initial Reading	110	90	120	115	110	120	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	60	40	70	65	60	70	0	0
	mmol CH4	2.49	1.66	2.91	2.70	2.49	2.91	0.00	0.00
10	Initial Reading	60	50	50	65	60	50	50	50
	Final Reading	50	50	50	50	50	50	50	50
	Biogas Released	10	0	0	15	10	0	0	0
	mmol CH4	0.42	0.00	0.00	0.62	0.42	0.00	0.00	0.00

T1 = Treatment 1, feces only

T2 = Treatment 2, feces plus 2x as-excreted ratio of urine:feces

T3 = Treatment 3, feces plus 1x as-excreted ratio of urine:feces

R1 = Replicate 1

R2 = Replicate 2

Control A = as-excreted substrate only, no inoculum

Control B = inoculum only, no substrate

A.2. Raw Data, DAF Sludge Batch Tests

Raw Biogas Production, mL									
Date	**Hours**	**T1R1**	**T1R2**	**T2R1**	**T2R2**	**T3R1**	**T3R2**	**C1**	**C2**
5/27/07 6:00 PM	0	0	0	0	0	0	0	0	0
5/27/07 10:30 PM	5	205	200	225	230	190	200	10	70
5/28/07 7:30 AM	14	250	220	305	290	190	205	5	50
5/28/07 3:00 PM	21	160	130	230	215	160	180	10	25
5/28/07 11:30 PM	29	95	90	160	160	180	195	5	10
5/29/07 10:40 AM	41	55	55	135	130	210	235	5	0
5/29/07 8:30 PM	50	30	30	70	75	145	150	5	0
5/30/07 12:00 PM	66	15	15	55	65	145	130	0	0
5/30/07 9:00 PM	75	5	5	20	25	55	55	10	0
5/31/07 10:00 AM	88	0	0	20	10	25	15	0	0
Net Biogas Production from Fresh Substrate (minus inoculum control), mL									
Date	**Hours**	**T1R1**	**T1R2**	**T2R1**	**T2R2**	**T3R1**	**T3R2**	**C1**	**C2**
5/27/07 6:00 PM	0	0	0	0	0	0	0	0	0
5/27/07 10:30 PM	5	135	130	155	160	120	130	-60	0
5/28/07 7:30 AM	14	200	170	255	240	140	155	-45	0
5/28/07 3:00 PM	21	135	105	205	190	135	155	-15	0
5/28/07 11:30 PM	29	85	80	150	150	170	185	-5	0
5/29/07 10:40 AM	41	55	55	135	130	210	235	5	0
5/29/07 8:30 PM	50	30	30	70	75	145	150	5	0
5/30/07 12:00 PM	66	15	15	55	65	145	130	0	0
5/30/07 9:00 PM	75	5	5	20	25	55	55	10	0
5/31/07 10:00 AM	88	0	0	20	10	25	15	0	0
Cumulative Net Biogas Production from Fresh Substrate, mL									
Date	**Hours**	**T1R1**	**T1R2**	**T2R1**	**T2R2**	**T3R1**	**T3R2**	**C1**	**C2**
5/27/07 6:00 PM	0	0	0	0	0	0	0	0	0
5/27/07 10:30 PM	5	135	130	155	160	120	130	-60	0
5/28/07 7:30 AM	14	335	300	410	400	260	285	-105	0
5/28/07 3:00 PM	21	470	405	615	590	395	440	-120	0
5/28/07 11:30 PM	29	555	485	765	740	565	625	-125	0
5/29/07 10:40 AM	41	610	540	900	870	775	860	-120	0
5/29/07 8:30 PM	50	640	570	970	945	920	1010	-115	0
5/30/07 12:00 PM	66	655	585	1025	1010	1065	1140	-115	0
5/30/07 9:00 PM	75	660	590	1045	1035	1120	1195	-105	0
5/31/07 10:00 AM	88	660	590	1065	1045	1145	1210	-105	0

Averaged Cumulative Net Methane Production from Fresh Substrate (STP), mL				
5/27/07 6:00 PM	0	0	0	0
5/27/07 10:30 PM	5	87	104	82
5/28/07 7:30 AM	14	209	267	180
5/28/07 3:00 PM	21	288	397	275
5/28/07 11:30 PM	29	343	496	392
5/29/07 10:40 AM	41	379	583	539
5/29/07 8:30 PM	50	399	631	636
5/30/07 12:00 PM	66	409	671	727
5/30/07 9:00 PM	75	412	686	763
5/31/07 10:00 AM	88	412	696	776
Averaged Cumulative Net Methane Production (in Liters) from Fresh Substrate (STP) per Unit of Fresh Substrate (grams)				
5/27/07 6:00 PM	0	0.00	0.00	0.00
5/27/07 10:30 PM	5	0.06	0.05	0.03
5/28/07 7:30 AM	14	0.15	0.13	0.07
5/28/07 3:00 PM	21	0.21	0.19	0.10
5/28/07 11:30 PM	29	0.25	0.24	0.14
5/29/07 10:40 AM	41	0.28	0.28	0.20
5/29/07 8:30 PM	50	0.29	0.31	0.23
5/30/07 12:00 PM	66	0.30	0.33	0.26
5/30/07 9:00 PM	75	0.30	0.33	0.28
5/31/07 10:00 AM	88	0.30	0.34	0.28