ABSTRACT

Title of Dissertation: **INCREASING ANAEROBIC DIGESTION ADOPTION THROUGH NOVEL INOCULUM PRESERVATION AND UNDERSTANDING THE EFFECT OF WASTE STREAM DYNAMICS**

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This research aimed to decrease barriers to anaerobic digestion (AD) adoption through: 1) preservation of AD inoculum, 2) iron addition to improve biogas quality, 3) sulfur addition to increase potential waste streams treated, and 4) pretreatment methods for municipal solid waste (MSW) to increase methane (CH_4) production from AD.

Preservation of AD inoculum with 10% skim milk exhibited complete CH_4 recovery, while 10% glycerol and 10% glycerol/skim milk mixture yielded 76% and 4% CH_4 recovery, respectively. The inoculum growth phase before preservation (mid-exponential or stationary growth phase) did not significantly affect CH_4

recovery. The study showed that inoculum can be preserved via lyophilization with a 10% skim milk cryoprotectant and reactivated for food waste digestion.

Iron addition to dairy manure at 20 and 50 mM resulted in significant reductions of hydrogen sulfide (H_2S) in the biogas (38 to 100% and 89 to 96%, respectively), with $FeCl_2$ and $FeSO_4$ additions yielding the highest H_2S removal. However, $FeCl_2$ and $FeSO_4$ resulted in significant CH_4 reduction, while 50 mM Fe_2O_3 addition did not reduce CH_4 and decreased H_2S concentration below the minimum requirement for biogas use in engine generation sets.

Addition of more than 2 mM sulfate (SO_4^{2-}) to dairy manure significantly decreased CH_4 production by 21 to 65%, while elemental sulfur (S^0) additions above 5 mM resulted in 26 to 63% reduction in CH_4. K_2SO_4 and S^0 addition greater than 5 mM resulted in significant increases in H_2S, while $FeSO_4$ reduced H_2S by 44 to 96%. The SO_4^{2-} additions were successfully treated, with a 48 to 95% decrease in SO_4^{2-}, showing that dairy manure AD was able to treat high SO_4^{2-} waste without fouling the AD system.

MSW thermal pretreatment at 66, 77, and 99 °C resulted in no significant difference in CH_4 production (241 to 277 mL CH_4/g COD). Two pretreatments prior to AD did result in increased CH_4 production: 1) washed and thermally treated MSW with 45 mM NaOH addition, and 2) pressing unwashed, thermally treated MSW. Use of AD to treat MSW wastewater with only thermal pretreatment of 66 °C would result in plant energy cost savings of $339,000.

INCREASING ANAEROBIC DIGESTION ADOPTION THROUGH NOVEL INOCULUM PRESERVATION AND UNDERSTANDING THE EFFECT OF WASTE STREAM DYNAMICS

by

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Dedication

I would like to dedicate this to my parents, Judy and Stanley Yarberry and my husband, Dustin Poppendieck. Thank you for helping me become the best version of myself.

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1 Introduction

1.1 Rational for Conducting the Research

In the last few decades, there has been an increase in global awareness of the need to reduce anthropogenic greenhouse gas (GHG) emissions. In the Paris Agreement of 2015, countries agreed to keep global temperature rise in this century to only 2 °C from pre-industrial levels. In order to prevent temperature rise, countries should focus on reducing GHG emissions from the largest contributing sectors, which includes the energy sector (60% of global GHG emissions) (UN, 2015), the livestock sector (14.5% of global GHG emissions) (FAO, 2017), and the waste sector (3% of global GHG emissions) (Bogner et al., 2008). Potential pathways for GHG emissions from these sectors is to move away from the use of fossil fuel based energy and transportation fuels, reduce the amount of methane (CH_4) emitted to the atmosphere from livestock manure management (13% of US agricultural sector emissions) , and reduce the organics going to landfills responsible for production of CH_4 rich landfill gas (86% of US waste sector emission) (EPA, 2016).

Use of AD for treatment of livestock manure, food waste, and municipal solid waste (MSW) directly reduces GHG emissions from the agricultural and waste sectors, while offsetting GHG emissions from fossil fuel derived energy production in the energy sector. Anaerobic digestion (AD) is a microbial process that breaks down organic waste in an oxygen-free environment and results in both pollution reduction and energy recovery from multiple waste streams. Biogas produced from anaerobic

digesters is rich in methane (CH_4) and can be used to generate heat and/or electricity that can be used at the biogas production location, or transferred to the grid.

The goal of this research was to decrease the barriers to AD adoption by: 1) providing preserved inoculum as a startup seed for AD, thus, decreasing the cost of transportation and increasing the locations for startup of new AD, 2) enhancing the quality of the biogas from dairy manure digestion, and 3) increasing the use of AD for the treatment of waste streams not typically considered. The work investigated improving AD using three different waste sources: food waste, dairy manure, and wastewater from municipal solid waste pretreatment. The objectives of this study were to: 1) determine an effective mechanism to preserve AD inoculum for quick reactivation to be used as a microbial starter for AD systems of food waste; 2) quantify the changes in CH_4 and hydrogen sulfide (H_2S) production when different forms of iron and sulfur are added to dairy manure digestion; and 3) determine pretreatment procedures for MSW (prior to ethanol fermentation) that will increase CH_4 production during AD of the resulting pretreatment wastewater.

1.2 Inoculum Preservation

Preservation of bacteria for storage and use has been tested and utilized since the beginning of the 20th century. Pure microbial cultures have been preserved by freezing, dry desiccation, vacuum-drying, spray-drying, fluidized bed drying, and freeze-drying (lyophilization) (Santivarangknaetal et al., 2007; Krall et al., 2011). Of these methods, lyophilization is the preferred long-term preservation method, as the resulting cultures are easy to transport and there is little to no cost for culture

maintenance (Prakash et al., 2012). Lyophilization is a three-step process: freezing, sublimation, and desorption. During freezing, the sample is subjected to cryogenic temperatures turning the accessible water in the sample and within the cell interior to ice. The ice is then directly converted to vapor during the sublimation step. After sublimation, however, there is still a significant amount of unfrozen water bound to the sample, which is removed via desorption (Perry, 1995).

There are several processes to take into consideration prior to freezing the microbial consortia in order to optimize microbial viability: incubation media, growth phase, cell concentration, and cryoprotectant use. The incubation media for the inoculum should consist of compatible substrates that are optimized for both heterotrophic and methanogenic communities. The addition of a nutrient media during incubation is important because if media is only added during the drying process, there is not enough time for nutrient assimilation (Santivarangknaetal et al., 2007). After nutrients are assimilated, increase in cell viability has resulted from harvesting cells during the stationary growth phase (Prakash et al., 2012). It was hypothesized that during the stationary phase, the microbial community has a greater tolerance to dehydration due to depletion of nutrients, which prepares them for the harsh conditions of being freeze-dried. Another factor that has shown to correlate positively with viable cell recovery is an initial cell concentration above 10^8 cells/mL, which can be achieved through microfiltration or centrifugation at 8,000 rpm for 20 minutes (Cleland et al., 2004; Morgan et al., 2006; Liu, 2008).

After the cells have been concentrated, a cryoprotectant should be added to the sample before freeze-drying to prevent cryo-injuries and cell death from osmotic

imbalance caused by the exposure to the extremely low temperatures during lyophilization (Prakash et al., 2012). Protective substances, such as sugars, alcohols, amino acids, and complex reagents, such as skim milk, provide relief during drying (sublimation). The relief is provided by either replacement of the water in the lipid head groups of the cell, thus preventing leakage from the cell membrane after rehydration, or by scavenging the free-radicals (Morgan et al., 2006; Santivarangknaetal et al., 2007). The appropriate choice of a cryoprotectant and preservation method; however, has been found to be inoculum-specific (Castro et al., 2002; Santivarangknaetal et al., 2007; Morgan et al., 2006). Rothrock et al. (2011) investigated the effectiveness of skim milk media and glycerol as cryoprotectants for anammox bacteria and found that glycerol had no effect, but the cultures were able to survive four months of storage after being preserved in skim milk media. Additionally, Cleland et al. (2004) found a pure methanogenic culture experienced a 2 to 4 log loss of cell viability after long term storage using glycine betaine and sucrose/bovine serum albumin as cryoprotectants.

Once the inoculum has been fed, protected, and preserved, the final step is rehydration. The rehydration media should be complex in order to help promote the repair of damaged cells and provide key nutrients that may be depleted in the inoculum. It has been reported that using the same medium for rehydration and cryopreservation increases viability (Morgan et al., 2006). Also, using lower temperatures for rehydration (15-25 °C) instead of the higher operation temperatures of the inoculum has been shown to increase viability after rehydration (Morgan et al., 2006).

Previous studies concerning microbial preservation have focused on refining preservation methods for pure cultures. Studies that have investigated the use of preservation on microbial consortia have mostly been on aerobic consortia, with limited research on anaerobic consortia. This research investigated the appropriate cryoprotectant and inoculum growth phase prior to preservation for the microbial consortia in AD inoculum. The determination of an AD inoculum preservation method is the first step in the development of an AD 'starter kit' that can increase use of AD in remote locations.

1.3 Iron Addition to AD for Biogas Improvement

In order to protect electricity generators from harmful contaminants in biogas both physical/chemical and biological gas treatment processes have been developed. Physical/chemical methods include catalytic purification, adsorption, scrubbing, membrane separation, and condensation. Biological methods include biofilters, bio-scrubbers, and bio-trickling filters. In-situ methods for removal of H_2S from biogas have also been utilized, with iron salt addition being the preferred chemical due to its low cost (Romero-Güiza et al., 2016).

Iron addition to AD can be used to control H_2S production through microbial and abiotic physical/chemical methods. Iron is a macro- and micro-nutrient that serves similar physiological functions for both sulfate reducing bacteria (SRB) and methanogens and can also act as an electron donor for both types of bacteria (Zhang et al., 2011). When Fe(III) was added to anoxic paddy soils with an abundance of electron donors, sulfate reduction was maintained, but methanogenesis was inhibited.

The CH_4 inhibition coincided with a hydrogen (H_2) concentration below the usable threshold for methanogens, which indicates that SRB can deplete the electron donors needed for methanogens (Achtnich et al., 1995). However, decreased H_2S production in biogas when iron is added can also be due to physical/chemical interactions between iron and aqueous sulfides. Physical/chemical interactions between iron and dissolved sulfides occur through precipitation as metal sulfides (Zhang et al., 2008). Sulfide species in AD are primarily present either as $H_2S_{(aq)}$ or HS^-, as the pH range is typically between 6 and 8 during digestion. When ZVI was added to an upflow anaerobic sludge blanket (UASB), sulfate reduction increased, but H_2S production decreased because the ZVI acted as a buffering agent, changing the dominant dissolved sulfide form from $H_2S_{(aq)}$ to HS^- (Zhang et al., 2011). The mechanism of sulfide removal by Fe(II) is precipitation as ferrous sulfide (FeS), while Fe(III) removes sulfide by oxidizing it to elemental sulfur and concurrently being reduced to Fe(II), which further removes sulfides via precipitation.

There are mixed findings on whether Fe(II) or Fe(III) is more effective in precipitating sulfides. Dezham et al. (1988) found that $FeCl_2$ and $FeCl_3$ dosing of wastewater as it entered a wastewater treatment plant (WWTP) was effective in reducing the H_2S concentration to 300 ppm, but that less $FeCl_2$ was required, resulting in cost savings. When $FeCl_3$ was added to an AD in a WWTP, it was rapidly reduced to Fe(II) and subsequently formed FeS with available sulfides in the system. However when $FeCl_3$ was added to the wastewater prior to AD, the Fe(III) did not reduce to Fe(II) as rapidly or as completely as it did when added to the AD, indicating that pre-existing Fe(III) up-stream from AD is possibly in a form unavailable for

chemical reduction (Haaning Nielsen et al., 2005). Zhang et al. (2011) found that FeS precipitation by Fe(II) only accounted for 2.21% of the total sulfur removal when ZVI was added to an UASB, indicating that precipitation was not responsible for the increased reactor performance.

While iron compounds have been shown to consistently reduce H_2S concentrations in biogas, previous studies on the effects of iron on CH_4 production have been mixed. Increased CH_4 production has resulted from Fe(III) addition to anaerobic sludge and wetland and rice paddy sediments in the form of hematite (Chen et al., 2014; Kato et al., 2012), goethite (Tan et al., 2015) and $FeCl_3$ (Peng et al., 2014; Raju et al., 1991). However, when hematite, magnetite, and ferrihydrite were added to rice paddy sediments, ferrihydrite had negligible effects or suppressed CH_4 production, while hematite and magnetite resulted in an increase in CH_4. The increased CH_4 production with the semi-conductive Fe(III)-oxide species was proposed to be a result of the *Geobacter* spp. using the Fe(III)-oxide as an electrode through which electrons can be passed to methanogens (Kato et al., 2012). Roden (2003) found that CH_4 production was inhibited when hematite, goethite and amorphous hydrous ferric oxide were added to freshwater wetland sediments due to a diversion of electron flow from methanogenesis to Fe(III) reduction, which is in agreement with the results of Lovley and Phillips (1986) and Tan et al. (2015).

Fe(III)-oxides have also been shown to decrease CH_4 production in sediments. Bond and Lovely (2002) investigated the potential for Fe(III)-oxide reduction by a variety of pure methanogenic cultures and determined that insoluble Fe(III)-oxide and extracellular quinones can be reduced by most methanogenic cultures through the use

of a hydrogenase enzyme. This study was in partial agreement with the study by van Bodegom et al. (2004), which found that in pure cultures exposed to Fe(III) in the form of $Fe(OH)_3$, only hydrogenotrophic methanogens were able to reduce Fe(III) to Fe(II).

While previous studies have indicated that iron will dependably reduce H_2S concentrations, the amount and mechanism of reduction is highly dependent on the oxidation state of the iron being used. The effects of iron on CH_4 production are not as consistent and are dependent on the iron oxidation state, the microbial source, and substrate being utilized. When CH_4 production was suppressed, the system typically had lower levels of electron donors (more dilute substrate). This study investigated the effects of the addition of multiple iron compounds at three oxidation states to dairy manure digestion on CH_4 and H_2S production and methanogen and SRB numbers. It is possible that when utilizing manure as a substrate, the diversity of methanogenic species coupled with the heightened concentrations of electron donors compared to sediments can provide conditions favorable for methane enrichment through iron addition.

1.4 Effects of Sulfate and Elemental Sulfur Addition on Anaerobic Digestion

When sulfate (SO_4^{2-}) is available in a digester, the acidogenic, acetogenic and methanogenic microorganisms compete with SRB for available electron sources. Within a digester, SRB can outcompete methanogens for available substrates, as SO_4^{2-} reduction is more energetically favorable. Manipulation of the chemical oxygen demand: sulfate ($COD:SO_4^{2-}$) ratio in digesters has shown to impact the biogas

production, with lower ratios typically, but not always, resulting in higher sulfide production and less methane production (Hulshoff Pol et al., 1998). Theoretically, a COD:SO_4^{2-} ratio less than 0.67 indicates an excess of SO_4^{2-}, meaning that all COD removal in an AD could theoretically be achieved through sulfate reduction. A COD:SO_4^{2-} greater than 0.67 indicates that the influent is SO_4^{2-} limited and methanogens can use the extra substrate for complete COD removal (Patidar and Tare, 2004).

The effect of the COD:SO_4^{2-} ratio on SO_4^{2-} reduction and CH_4 production has been investigated in attached film anaerobic wastewater treatment (Chou et al., 2008; de Smul et al., 1999; McCartney and Oleszkiewicz, 1993; O'Flaherty et al., 1998). Chou et al. (2008) found that when the COD:SO_4^{2-} ratio varied from 0.5 to 3.0, when the influent COD:SO_4^{2-} ratio was above 1.3 the methanogens were able to out-compete the SRB. McCartney and Oleszkiewicz (1993) found that a COD:SO_4^{2-} ratio less than 1.6 g/g resulted in sulfate reduction which increased H_2S production, but a ratio of 3.7 g/g did not show significant sulfate reduction, and thus, reduced H_2S production, when using lactate as an AD substrate.

While previous studies have indicated that low COD:SO_4^{2-} ratios in fixed film reactors treating SO_4^{2-} rich waste have enabled methanogens to out compete SRB, in practice, anaerobic treatment is typically sucessful when the COD:SO_4^{2-} ratio is grater than 10 (Hulshoff Pol et al., 1998). In an UASB, when SO_4^{2-} was added to yield a COD:SO_4^{2-} of 4.5, the COD removal decreased from 85.1% to 58.2% and CH_4 production from 11 L/d to 5.6 L/d, compared to the control reactor (Zhang et al., 2011). Patidar and Tare (2004) found that addition of SO_4^{2-} to three different reactor

types: an UASB, an anaerobic batch reactor and a hybrid anaerobic baffled reactor, resulting in a COD:SO_4^{2-} of 6.9-7.0, inhibited CH_4 production and COD removal when compared to the systems without SO_4^{2-} addition.

The results of these studies indicate that when altering the COD:SO_4^{2-} ratio, it is possible to decrease sulfate reduction, but only within a small range, and outside of that range CH_4 production and COD removal may be inhibited. In this study, a wide range of COD:SO_4^{2-} ratios was investigated through the addition of elemental sulfur and SO_4^{2-} to determine the effects of sulfur addition on digestion of separated dairy manure.

1.5 MSW Pretreatment

Research on AD of municipal solid waste (MSW) has mainly been focused on the organic fraction of MSW and the optimal digester design, pre-treatment methods, operational conditions, and co-digestion options. For this research, the waste product being digested is a by-product of the pretreatment of MSW for cellulosic ethanol production. In the cellulosic ethanol preparation process, the large recyclables are separated out, and the remaining organics, paper waste and miscellaneous plastics, glass, and inorganics in the waste stream are pulped in a high pressure pulper. The solids from the pulper are further separated into the remaining recyclables and organic biomass, which is mostly paper and food waste. The pulped biomass is then pretreated in a washing tunnel with water. After pretreatment, the solids are sent to hydrolysis and fermentation to produce ethanol and the liquid fraction is utilized in AD.

1.5.1 Thermal Pretreatment

Heat treatment of solids breaks down solids and solubilizes organics, making them more available for digestion (Barlindhaug and Odegaard, 1996; Bougrier et al., 2006; Bougrier et al., 2008; Gianico et al., 2013; Kim et al., 2003; Wang et al., 1997). It has been shown that the amount of COD solubilized in waste activated sludge (WAS) increased linearly with an increase in treatment temperature from 20°C to 210°C, with thermal treatments being applied using an autoclave for 30 minutes (Bougrier et al., 2008). Interestingly, even though thermal pretreatment resulted in increased solubility of organics in WAS, it increased the mean particle size, indicating the creation of chemical bonds (Bougrier et al., 2006).

When using thermal pre-treatment as a mechanism to increase solubility of organics for increased waste stabilization and energy recovery, the cost and energy input requirements should not exceed the benefits of the pretreatment. Wang et al. (1997) determined that lower temperature pretreatment on continuous anaerobic digestion of WAS was more cost effective and operationally more convenient. The lower temperature pretreatments (60°C-100°C) resulted in an increase in CH_4 generation from 30-52%. When 70°C was used as the pretreatment temperature for secondary and primary sludge, which was digested under both mesophilic and thermophilic conditions, CH_4 production increased for all conditions with the highest increase seen with secondary sludge digested under mesophilic conditions (43 to 145%, depending on temperature exposure duration) (Gavala et al., 2003). Differences in CH_4 production rate for primary and secondary sludge was attributed to the compositional differences of the organics present in the sludge, with primary

sludge having a higher carbohydrate composition than secondary sludge (Gavala et al., 2003; Wilson and Novak, 2009).

Fewer studies on thermal pretreatment have been done on MSW, with most of the focus on the organic fraction and food waste. Qiao et al. (2011) investigated the effects of hydrothermal pretreatment at 170°C on cow manure, swine manure, sludge, fruit and vegetable waste and food waste. At 170°C, biogas increased for all pretreated wastes, except for food waste, and CH_4 production increased for all pretreated wastes, except for food waste and cow manure. The decrease in CH_4 production by thermally pretreated cow manure was attributed to a low protein content of the waste (Qiao et al., 2011). Similarly, when Liu et al. (2012) thermally pretreated kitchen waste, vegetable/fruit residue and WAS at 175°C, CH_4 potential increased by 34.8% for WAS, but decreased by 7.9% and 11.7% for kitchen waste and vegetable/fruit residue, respectively. The decrease in CH_4 in the food waste was attributed to melanoidin production, due to the Maillard reaction between carbohydrates and amines. When Komemoto et al. (2009) exposed food waste, representative of actual food waste in Japan, to a 22-day thermal pretreatment fermentation at temperatures of 15, 25, 35, 45, 55 and 65°C, the mesophilic temperatures of 35°C and 45°C had solubilization rates of 70 and 72.7%, respectively, which were due to microbial processes. Thermophilic temperatures of 55°C and 65°C resulted in solubilization rates of 56.1 and 45.9%, respectively, which were due to physiochemical degradation (Komemoto et al., 2009). However, when waste representative of kitchen waste in Taiwan was thermally pretreated at 37, 50 or

60°C, the most effective temperature was 60°C, achieving a hydrolytic efficiency of 27.3% (Kuo and Cheng, 2007).

Previous studies indicate that not only is the selection of temperature an important parameter, but the type of organics in the waste will also impact the efficiency of the thermal pretreatment on organics solubilization and biogas production. The temperatures used in this study were between 66 and 99°C, which have been shown to be effective in COD solubilization of WAS (Bougrier et al., 2008).

1.5.2 Alkaline Pretreatment

Alkaline treatment of solid waste, sludge and slaughterhouse waste has been shown to increase soluble COD (sCOD) concentrations and decrease the average particle size of the waste (Hamzawi et al., 1998; Kim et al., 2003; Li et al., 2012; Lin et al., 1997; Lin et al., 2009; Masse et al., 2001). The availability of hydroxide ions (OH^-) in alkaline conditions results in the breakdown of proteins and generation of free amino acids, enhancing protein solubilization and subsequent soluble protein degradation (Dahiya et al., 2015). Kim et al. (2003) found that when various alkaline agents were used as a pretreatment for WAS, NaOH outperformed the other agents (KOH, $Mg(OH)_2$ and $Ca(OH)_2$), and monobasic agents resulted in higher solubilization percentages than dibasic additions. Similarly, when MSW was soaked in five different treatments: water, a dilute NaOH solution, press water from the dilute NaOH solution, cellulase treatment, and a dilute lime solution; the dilute NaOH solution produced the highest CH_4 yield (Ghosh et al., 2000). The optimal dose of

NaOH when pretreating WAS was determined to be 7 g/L (0.18 M) when the tested dose range was from 0-21 g/L (0-0.53 M), which increased the soluble protein concentration by 98% (Kim et al., 2003). Previous studies have indicated that of potential alkaline agents, NaOH is the most effective at decreasing particle size and solubilizing organics.

When utilizing NaOH for lignocellulosic wastes, it has been found that NaOH and other alkaline treatments actually delignify the biomass, increasing the porosity of the biomass by breaking the ester bonds that cross-link lignin and xylan (Silverstein et al., 2007; Zhao et al., 2008). The alkaline treatment also disrupts the cell wall of the lignocellulosic substance by dissolving the hemicellulose and lignin through hydrolysis of the uronic and acetic acid esters, which decreases the crystallinity of the cellulose (Gáspár et al., 2007). When using a 10% NaOH solution (2.5 mM) to crofton weed stem ratio of 6:1 at 110°C, Zhao et al. (2008) found that 30.25% of the carbohydrates in the crofton weed stem dissolved. Silverstein et al. (2007) determined that 0.5% NaOH (125 mM) is too low to affect delignification of cotton stalks for treatment times up to 90 minutes and temperatures as high as 121°C in the autoclave. However, concentrations as low as 1% NaOH (250 mM) had a significant impact on delignification of cotton stalks, with lignin reduction increasing linearly with concentration of NaOH (Silverstein et al., 2007). In this research, delignification is not desirable prior to solids being hydrolyzed for the cellulosic ethanol process.

Caution should be taken when adding alkaline pretreatment to sludge due to the increase in pH, which can disrupt digester stabilization. Li et al. (2012) found that if

the pH was higher than 8, a lag-phase of 3 days developed, and above a pH of 9.33 the reactor failed. Conversely, when Lin et al. (2009) added NaOH to pulp and paper sludge, the alkalinity of the seed and sludge was able to overcome even the highest dose of NaOH at 8g NaOH/100 g TS sludge, with the pH remaining between 7.7 and 8.7.

1.5.3 Surfactant Pretreatment

Surfactants are amphiphilic chemicals containing both hydrophobic and hydrophilic moieties, which make them unique in their ability to enhance the water solubility of organic pollutants in soils, especially organics with hydrophobic tendencies (Mao et al., 2015). The main types of surfactants are anionic, cationic, nonionic and zwitterionic, all of which have been used for soil remediation (Mao et al., 2015; Paria, 2008). Cationic surfactants have been used historically for antimicrobial purposes and are the most toxic of surfactant types, followed by anionic surfactants, with nonionic surfactants being considered non-toxic (Katsoyannos et al., 2012; Mao et al., 2015).

Solubilization during surfactant-enhanced soil washing of hydrophobic organic pollutants occurs above the critical micelle concentration increasing the partition of pollutants in the aqueous phase via micelle formation (Mao et al., 2015). When four nonionic surfactants: Span 20, Tween™ 61, Tween™ 81 and Tween™ 85, and two nonionic surfactant blends: Tween™ 21/Span 20 and Tween™ 20/Span 20 were investigated for their effectiveness in extracting proteins using a micro-emulsion liquid membrane extraction, Tween™ 85 was the most successful surfactant. A weak

electrostatic protein-surfactant interaction was responsible for the protein extraction by the Tween™ 85 micro-emulsion, however when the pH was dropped below 5.2, the surfactant lost its ability to extract the proteins (Vasudevan and Wiencek, 1996). Katsoyannos et al. (2012) investigated the suitability of four nonionic surfactants for the separation of phenols and carotenoids from olive mill wastewater: Tween™ 20, Tween™ 80, Span 20 and PEG 400 using cloud point extraction. Tween™ 80 was the most successful, with 96.4% total phenol recovery and 64.3% total carotenoid recovery.

Because surfactants alter interfacial behavior by decreasing interfacial tensions and change the way other molecules behave at interfaces and in solution, they may cause membrane disruption and cell lysis (Van Hamme et al., 2006). Few studies have investigated the effects of surfactants on AD. Linear alkylbenzene sulfonates (LAS) are the most common anionic surfactant used as detergents. As a result, LAS are commonly found in WWTP sludge (Gavala and Ahring, 2002). Gavala and Ahring (2002) found that LAS in WWTP sludge inhibits the acetogenic and methanogenic microbes in AD by preventing the transport of nutrients and/or substrate into the cells. Garcia et al. (2000) investigated the effects of the two most commonly used cationic surfactants: DHTDMAC and esterquats on AD. The two esterquats tested were between 70% and 100% biodegradable in AD and the DHTDMAC was 0% biodegradable. In toxicity tests, the DHTDMAC was inhibitory at a concentration of 200 mg C/L, but below that no inhibitory effects were observed and the esterquats did not inhibit AD at all. The two esterquats tested resulted in increased biogas production, indicating that the microbes can utilize the esterquats as

a carbon source (Garcia et al., 2000). Yeh et al. (1998) examined 16 surfactants and their effect on methanogenesis: 14 nonionic surfactants from two main nonionic categories: polyoxyethylene alcohols and polyoxyethylene sorbitan fatty acid esters, and two surfactants known to be inhibitory: an octylphenol ethoxylate and an anionic surfactant. Of all the surfactants tested, the Tween™ surfactants were the only surfactants that did not inhibit the total amount of CH_4 produced and actually accelerated the rate of CH_4 production in cultures fed with lactate.

In previous studies, surfactants have been used to either remediate hazardous waste from soils or as a less toxic option for molecular extractions. In this study, the capability of the surfactant to alter the aqueous environment and to enhance the solubility of more hydrophobic organics was utilized with MSW and compared to other pretreatment options as a means to increase the soluble organics content of the MSW pretreatment wastewater for AD.

1.5.4 Ultrasonic Pretreatment

Ultrasonic pretreatment has shown to increase soluble COD and CH_4 production when used as a pretreatment of WAS digested under both mesophilic and thermophilic conditions (Gianico et al., 2015). Ultrasound causes cavitation in a solution through pressure waves, which can lead to cell wall disassembly (Chu et al., 2002). Ultrasonic pre-treatment for two hours at 42 kHz was able to solubilize 18.4% of the total COD in WAS (Kim et al., 2003). When a high performance ultrasound reactor specifically designed for sludge treatment was utilized at a frequency of 31

kHz in flow-through mode set to 64 seconds, Tiehm et al. (1997) observed an increase in volatile solids reduction of 9%.

Weak ultrasound pretreatment refers to sonic energy unable to disrupt the floc structure of WAS. Chu et al. (2002) applied weak ultrasound to 150 mL samples at a frequency of 20 kHz and a power of 0.33 W/mL for a duration of 20 minutes. The weak ultrasonic treatment increased CH_4 yield by 104%. Similarly, at an operating frequency of 20 kHz and WAS sample volume of 0.5 L, Bougrier et al. (2006) found that 15% of the COD was solubilized and mean particle size decreased by 70%. The solubilized COD during sonication was readily biodegradable due to the increased availability of particulates to microorganisms, resulting in a 33% increase in CH_4 production. When pulp mill sludge was exposed to a high intensity probe contained within a continuous flow cell with a frequency of 20 Hz and a specific energy of 1.5 kWh/kg TSS sludge, sonication lead to a 69% increase in sCOD and an average of 30% increase in CH_4 production (Elliott and Mahmood, 2012). Salsabil et al. (2010) found a 97.7% increase in soluble proteins and 33% increase in soluble carbohydrates when sonication was applied to WWTP sludge at 20 kHz and a power of 1 W/mL sludge.

Unlike previous studies, this study investigated the ability of an ultrasonic bath to break down organics in MSW submerged in water at a 1:5 solids:water ratio. The waste stream in this study was less fluid and homogeneous than the WAS studied previously and the effectiveness of ultrasonic treatment on solubilizing the organics was unknown. Ultrasonic pretreatment is energy intensive, and under higher frequencies and specific power deliveries may actually increase the solubilization of

carbohydrates over other pretreatments, such as low temperature pretreatments (Salsabil et al., 2010). In order for sonication to be a viable pretreatment the CH_4 increase has to be high enough to off-set the cost of the treatment, while keeping the percentage of carbohydrates solubilized low.

1.5.5 Press Pretreatment

Pressing is a mechanical technique that has been commonly used to extract oil for biodiesel production. Depending on the type of press employed, oil extraction from feedstocks varies from 60% to 80% of the available oil (Atabani et al., 2012). Pressing is also used to process olive oil and sugar pulp, and results in waste products high in organics. Few studies have investigated pressing as a pretreatment option for AD substrates.

Fantozzi and Buratti (2011) took samples of the organic fraction of MSW and squeezed the waste using a machine press, then performed anaerobic biogasification potential (ABP) tests on the squeezed organic fraction of MSW slurry and the original, un-squeezed waste. The un-squeezed waste ABP test was not inoculated, so there was not a comparison of the increase in CH_4 produced due to only utilizing the squeezed fraction. The squeezed fraction had a pH range of 3.30 to 4.53, which caused the normalized cumulative CH_4 production to be low at 35 mL/g VS added (Fantozzi and Buratti, 2011). Another study investigated the difference in CH_4 production between the organic fraction of MSW, press water from a mash-separator, and biowaste suspension. Biowaste suspension is a result of biowaste being density separated in liquid suspension of digester effluent supernatant and rain water, where

the heavy materials settle out and the lighter inorganic materials float to the top; the remaining suspension had a 90% moisture content. The biowaste suspension and press water had high concentrations of easily degradable substances as evidenced by the CH_4 production rates in the first three days, with press water producing approximately 24% more CH_4. The untreated organic fraction of MSW was slower to digest, but produced the highest amount of CH_4 at 550 mL/g VS added (Nayono et al., 2010a). In a separate study, Nayono et al. (2010b) focused on the press water of the organic fraction of MSW and reported that the high sCOD of press water accelerated the acidification process, which was evidenced by high total volatile fatty acid concentrations of 9.51 g/L. After seven days, CH_4 production from press water was complete with a cumulative CH_4 production of 540 mL/g VS added.

Unlike in previous studies, the MSW in this study was a mixture of organics and plastics, papers, and glass remaining after sorting. While other studies have observed an improvement in digestion using the press water from the organic fraction of MSW, the benefits of pressing a mixed waste stream have not been reported.

1.6 Objectives

There were four main objectives addressed in this research to meet the goal of decreasing the barriers to AD adoption:

Objective 1: Determine the effect of lyophilizing inoculum on CH_4 production using: 1) three inocula, 2) two inoculum to substrate ratios (ISR), 3) two cryoprotectants, and 4) two inoculum growth phases.

Objective 2: Determine the effect of four iron compounds: zero valent iron (ZVI), iron(III)-oxide (Fe_2O_3), ferrous chloride ($FeCl_2$), and iron sulfate ($FeSO_4$), on CH_4 and H_2S production, the methanogenic and SRB community numbers, and the nutrient composition of the digestate after digestion.

Objective 3: Determine the effects of SO_4^{2-} and S^0 addition on CH_4 and H_2S production and SO_4^{2-} reduction when using separated dairy manure as an organic substrate for SRB from AD inoculum acclimated to dairy manure as the substrate.

Objective 3: Determine the MSW pretreatment method that solubilizes organics and maximizes CH_4 production from AD of the resulting pretreatment wash water.

2 Effect of Anaerobic Digester Inoculum Preservation on Methane Recovery for Startup of Remote Digesters

2.1 Introduction

Finding a suitable microbial seed source, i.e. inoculum, for anaerobic digestion (AD) start-up can be difficult (Ghanimeh et al., 2012; Kobayashi et al., 2009; Suwannoppadol et al., 2011), with the lag phase, methane (CH_4) production, and degree of waste stabilization highly dependent on the amount and type of inoculum used (Elbeshbishy et al., 2012; Forster-Carneiro et al., 2007; Pandey et al., 2011). Each AD inoculum source contains a different microbial community balance based on unique conditions and stressors during incubation. The most appropriate microbial seed for AD is inoculum from an existing digester treating the same type of substrate and/or exposed to similar conditions. If established inoculum is unavailable, fresh animal manure from ruminants can be a viable option, but will result in a longer start-up period (El-Fadel et al., 2013). Recommendations for the initial inoculum volume range from 10 to 60% of the reactor volume (Angelidaki et al., 2006; El-mashad et al., 2003; Ike et al., 2010). For full-scale reactors, this could result in introducing more than 1,000 m^3 of inoculum, a costly and possibly infeasible logistics feat depending on geographical location. However, if inoculum were available in a dehydrated, preserved form that could be rehydrated on-site with minimal additional start-up time, compared to fresh inoculum, startup of AD in remote or new locations could become more feasible.

Studies have evaluated inoculum preservation via drying (Agrawal et al., 1997; Massalha et al., 2015), vacuum evaporation (Li et al., 2014; Massalha et al., 2014), refrigeration, freezing, and freeze-drying (Castro et al., 2002; Colleran et al., 1992), with mixed success. Dried sewage sludge (Agrawal et al., 1997) and granular sludge (Massalha et al., 2015) displayed higher stability and resistance to biomass washout with increases in organic loading rates compared to fresh granular inoculum. Sludge inoculum that was vacuum evaporated had a CH_4 recovery of 76 and 84% after two or four months of storage, respectively, compared to the fresh inoculum (Li et al., 2014). Castro et al. (2002) determined that the CH_4 recovery of freeze-dried (lyophilized) inoculum in trehalose or glucose was 17 and 0% of the original inoculum, respectively. While previous studies have focused on the impacts of different preservation conditions on a single inoculum source, no study has compared the impacts of inoculum preservation using inoculum from multiple anaerobic digesters sources.

Pure microbial cultures have been successfully preserved via desiccation for long-term storage using foam drying, spray drying, fluidized bed drying, and lyophilization. Lyophilization is the most common method due to low maintenance and ease of transporting the resulting cultures (Morgan et al., 2006; Prakash et al., 2013). Parameters that impact the success of microbial preservation of pure cultures include the cryoprotectant utilized (Costa et al., 2000; Heylen et al., 2012), initial cell concentration (Morgan et al., 2006), and growth phase of the microorganisms when preserved (Corcoran et al., 2004). Additionally, Elbeshbishy et al. (2012) determined that residual biodegradable organics in the inoculum can influence CH_4 production

from inoculum seed and should be removed prior to incubation. Anderson et al. (2012) determined that when *M. barkeri* cells were preserved, harvesting stationary and late-exponential phase cells resulted in better recovery than cells harvested at mid-exponential phase. While impacts of preservation parameters have been studied individually using pure cultures, there has not been a systematic investigation using heterogeneous AD inocula.

The objective of this study was to determine a suitable inoculum source and preservation methodology for startup of AD systems treating food waste. In order to determine a preservation methodology, effects of the inoculum to substrate ratio (ISR), inoculum cell concentration, cryoprotectant, and inoculum growth phase on preservation were determined. This effort will allow the startup of digesters where inoculum sources are not readily available, such as in disaster relief and refugee camps. In such scenarios, crowded conditions and lack of infrastructure can lead to human and environmental health risks. Implementation of portable AD solutions can provide significant improvements in health and quality of life, and the success of such systems can be maximized if inoculum sources are delivered in a pre-seeded commercial system.

2.2 Methods

2.2.1 Inocula and Substrate Utilized

Fresh inoculum samples were collected from three sources: 1) dairy manure digestate (DAIRY) collected from a mesophilic AD receiving the liquid fraction of dairy manure from 100 dairy cows, 2) the effluent of a covered lagoon (CO-DIG) AD

receiving flushed dairy manure from 550 cows and 2% food waste (by volume), and 3) primary sludge digestate from a municipal wastewater treatment plant (WWTP) digester. The inocula were stored at 4 °C for one day to three weeks before use.

2.2.2 Biochemical Methane Potential (BMP) Test

Anaerobic incubations were conducted using the biochemical methane potential (BMP) protocol developed by Owen et al. (1979) and adapted by Moody et al. (2011) to determine the CH_4 production potential of a defined food waste substrate over a period of 45 days. The food waste substrate mixture represents the mixture used in an AD located at Tyndall Air Force Base, FL and was designed to represent food waste typical of a disaster relief camp or expeditionary base camp. The food waste recipe consisted of two loaves of white bread (567 g), two cans of pork and beans (32 oz), and 8 servings of potato flakes (176 g), all homogenized in a blender prior to loading. The characteristics of the three inocula and the food waste substrate are presented in Table 2.1.

The digestion vessels were 250 mL bottles with a liquid volume of 200 mL and a headspace volume of 50 mL. Prior to incubation, the BMP bottles were flushed with 70:30 N_2:CO_2 for 3 minutes to ensure anaerobic conditions. The bottles were then capped with rubber septa and placed on a shaker (New Brunswick™ innOva® 2300, Hamburg, Germany) at 110 rpm in a temperature controlled incubator at 35 °C.

Biogas production was quantified volumetrically using a glass, gas-tight syringe equilibrated to atmospheric pressure. Biogas samples were analyzed for CH_4 composition using a gas chromatograph (Agilent Technologies, Inc.; Shanghai China;

model 7890 A) with a thermal conductivity detector at 250°C, a HP-Plot Q capillary column (Agilent J&W; USA), helium as the carrier gas at 8.6 ml/min, and the oven operated at 60°C for 2 min and subsequently ramped at 30°C/min to 240°C. Biogas production and CH_4 content was measured daily during the first week of the experiment, approximately every other day the following week, and then bi-weekly for the remainder of the BMP experiments, with measurement frequency based on the quantity of biogas produced.

To account for biogas production from residual biodegradable material in the inoculum, inoculum controls were incubated using the same conditions for each inoculum source and sampled simultaneously to allow for subtraction of biogas production not attributed to the food waste substrate. The CH_4 produced from these controls was subtracted from the CH_4 produced by the treatments using similarly treated inoculum based on the quantity of VS in the inoculum added to each treatment.

2.2.3 Preservation by Lyophilization

To evaluate the effect of preservation on CH_4 production, the inoculum was preserved via lyophilization. The preservation process included pelletizing the inoculum cells via centrifugation (12,000 x *g*, 10 min), pouring off the supernatant, and resuspending the cell pellet through low speed vortexing in a cryoprotectant (2.5 mL cryoprotectant/g wet weight inoculum pellet). After resuspension in cryoprotectant, samples were frozen at -20 °C and lyophilized for 48 hours (Labconco 6+, Kansas City, MO). Lyophilized samples were capped and stored at 4 °C for 3

weeks prior to being resuspended for BMP testing. Prior to BMP testing, lyophilized pellets were resuspended in 30 mL of nutrient buffer solution, as detailed in (Moody et al., 2011) and loaded into the BMP bottles as preserved inoculum.

2.2.4 BMP1: Effect of Inoculum Source, Inoculum Pretreatment, and Preservation

The first experiment (BMP1) was designed to determine the effect of preservation through lyophilization on CH_4 production using three AD inoculum sources with or without inoculum pretreatment. The three inoculum sources were tested using preserved inoculum at two inoculum to substrate ratios (ISRs). The loading ratios utilized in BMP1 were determined from a preliminary study that investigated the effect of ISR on CH_4 production from non-preserved inoculum for each of the three inoculum sources (Supplemental Table A 1). The results of the preliminary study determined that CH_4 production response is linear with respect to ISR within an ISR range of 0.67:1 to 10:1. ISRs chosen in this study (2:1 and 4:1) were within the linear range found in the previous study (Supplementary Figure A 1).

2.2.4.1 *Inoculum Pretreatment*

A pretreatment process was used to remove the soluble organics and alkalinity inherent within each inoculum source so that effects of preservation on CH_4 production were not confounded by physical/chemical properties in the inocula. The pretreatment process was also designed to increase the cell concentration within the inocula, which has shown to be beneficial in previous preservation studies (Costa et al., 2000; Morgan et al., 2006). Soluble organics and alkalinity within each inocula

were removed via centrifugation (12,000 x *g*, 10 min), pouring off the supernatant, and resuspending the cell pellet through low speed vortexing in 30 mL of 0.05 M phosphate buffered saline (PBS) (pH 7.1). The resuspended cells were concentrated a second time by centrifugation prior to resuspension in skim milk media (2.5 mL skim milk/g wet weight inoculum pellet), freezing at -20 °C, and lyophilization. Skim milk was chosen as the cryoprotectant for BMP1 based on previous studies (Abadias et al., 2001; Morgan et al., 2006). Pretreatment was only tested in BMP1 and not utilized prior to lyophilization in subsequent BMPs.

2.2.4.2 *Experimental Design*

In order to determine if inoculum pretreatment was a beneficial step prior to preservation each inoculum source was preserved with pretreatment and without pretreatment at a 2:1 inoculum to substrate ratio (ISR). Food waste substrate addition was based on volatile solids (VS) concentration. In addition to the 2:1 ISR analyses, a 4:1 ISR was tested for each inoculum source to determine the effect of increasing inoculum mass on CH_4 production from preserved inoculum. Specific VS mass additions of inoculum, food waste, and cryoprotectant for each triplicate treatment are shown in Table 2.2.

The ISRs in BMP1 were calculated based on the VS of the inoculum and food waste added and did not account for the VS of the remaining skim milk in the samples. The reported CH_4 values were, however, normalized by both the VS of the food waste and the calculated VS of remaining skim milk.

2.2.5 BMP2: Evaluation of Different Cryoprotectants on CH_4 Yield

The second BMP experiment (BMP2) was conducted to determine the most effective cryoprotectant to add prior to lyophilization. In BMP2, only the CODIG inoculum was utilized. The CODIG inoculum was pelletized as described in Section 2.3, and resuspended in one of three cryoprotectants: 10% skim milk media (w/v), 10% glycerol (v/v), or 10% glycerol mixed with 10% skim milk media (v/v). The resuspended pellet was either not preserved (NP) and transferred to a BMP bottle (BMP2-NP), or preserved (P) in a wide mouthed glass jar as outlined in Section 2.3 (BMP2-P). Both the preserved and not preserved inoculum were resuspended in 200 mL of nutrient buffer solution in triplicate BMP bottles, with the inoculum, food waste, and cryoprotectant VS mass additions shown in Table 2.3.

2.2.6 BMP3: Effect of Growth Phase on CH_4 Yield

The third experiment (BMP3) sought to determine the effects of inoculum cell growth phase prior to preservation on CH_4 yield and lag phase after inoculum reactivation. Initially, 24 BMP bottles were loaded with food waste and pelletized CO-DIG inoculum, with three additional inoculum-only controls. The growth phase was monitored using the daily cumulative CH_4 production curve. On Day 7, there were 12 BMP bottles removed from the incubator to represent mid-exponential phase treatments (BMP3-E). On Day 11, the other 12 BMP bottles were removed from the incubator to represent stationary phase treatments (BMP3-S). At the time of removal (either mid-exponential or stationary phase), the contents of each BMP bottle were pelletized and weighed. All of the pellets from each bottle were re-suspended in 10%

skim milk. Pellets from six bottles (three from each phase removal) were not preserved (NP) and loaded back into BMP bottles with nutrient buffer to a final volume of either 140 mL (E) or 120 mL (S) and incubated at 35°C, with only the 10% skim milk cryoprotectant added (no food waste) (BMP3-NP-SM). Six of the NP bottles (three from each phase removal) received additional food waste (BMP3-NP-SM+FW). Six BMP bottles were preserved (P) with skim milk (from each phase extraction) by freezing at -20 °C and lyophilization, with three bottles receiving additional food waste after preservation (BMP3-P-SM+FW) and three bottles not receiving any additional food waste (BMP3-P-SM). Inoculum, food waste, and cryoprotectant VS mass addition for each triplicate treatment are shown in Table 2.4.

The addition of 10% skim milk only (SM) and skim milk with additional food waste (SM + FW) after removal was designed to represent two scenarios of inoculum resuspension in the field: resuspend and wait to add food waste until CH_4 production is stable (SM) or add food waste at the time of inoculum resuspension (SM + FW).

2.2.7 Analytical Methods

The inoculum, food waste, and cryoprotectant treatments were characterized with respect to pH, total chemical oxygen demand (COD), and total and volatile solids (TS, VS) (Table 2.1). The pH was determined with an Accumet AB 15 pH meter. Total solids (APHA Method 2540B) and volatile solids (APHA Method 2540E) were determined using the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). HACH method 8000 was used to determine COD with HACH high range COD vials (20 to 1,500 mg/L).

2.2.8 Statistical Analyses

The experimental design for BMP1 was a completely randomized design of 27 experimental units (BMP bottles) with each treatment conducted in triplicate. Comparisons were made within each inoculum source between samples that were loaded with and without pretreated inoculum at the 2:1 ISR and between samples that were loaded with pretreated inoculum at the 2:1 and 4:1 ISRs. Significant differences in CH_4 yields were determined using a two-sample t-test. Methane yields between the three inocula at the two ISRs were statistically compared using a single factor ANOVA followed by Tukey-Kramer's post hoc test.

The second and third experiments (BMP2-NP, BMP2-P, BMP3-NP, and BMP3-P) each consisted of two completely randomized designs containing 15 experimental units (BMP bottles), each treatment conducted in triplicate. Statistical comparisons were made between BMP2-NP and BMP2-P treatments using a two sample t-test. In order to compare CH_4 production from each treatment, a single factor ANOVA followed by Tukey-Kramer's post hoc test was employed.

All statistical testing was conducted in SAS with proc t-test for the t-tests and proc mixed for ANOVA comparisons. The level of significance was held at 0.05 for all statistical analyses and reported values are given as means with standard errors.

Table 2.1: Inocula, substrate, and cryoprotectant characteristics, with average ± standard error of triplicate samples shown.

Parameter	**WWTP Inoculum**	**DAIRY Inoculum**	**CO-DIG Inoculum**	**Food Waste Substrate**	**10% Skim Milk**	**10% Glycerol**	**10% Glycerol/ 10% Skim**
TS (mg/g)	107 ± 0.4[a]	122 ± 6[a]	76.7 ± 1.9[a]	508 ± 3	95.9 ± 0.2	110 ± 4	189 ± 0.1
VS (mg/g)	68.8 ± 0.4[a]	94.5 ± 4.5[a]	64.7 ± 2.5[a]	485 ± 3	88.1 ± 0.3	110 ± 4	182 ± 0.2
COD (g/L)	26.0 ± 0.3[b]	16.7 ± 0.8[b]	2.93 ± 0.09[b]	612 ± 96	117 ± 0.1	148 ± 2	122 ± 3
pH	7.76	7.65	7.85	ND[c]	6.67	3.75	6.60

[a] TS and VS were conducted on inoculum cell pellets
[b] COD was conducted on liquid inoculum
[c] pH of food waste not determined

2.3 Results and Discussion

2.3.1 BMP1: Inoculum Pretreatment and Inoculum Source

2.3.1.1 *Inoculum Source*

The effect of preservation on CH_4 production from three inocula was determined in BMP1 at two different ISRs (2:1 and 4:1), with pretreatment of the three inocula tested at the 2:1 ISR. There was no significant difference in CH_4 production between the three inocula at the 2:1 (p-values: 0.993 to 0.999) or the 4:1 (p-values: 0.392 to 0.950) ISRs (Table 2.2). Methane production for the three inocula ranged from 141 to 146 mL CH_4/g VS at the 2:1 ISR, with a significant increase of 57% and 52% (225 and 218 mL CH_4/g VS) at the 4:1 ISR for the DAIRY and CODIG inocula (p-values: 0.005 and 0.042, respectively). There was no significant difference in CH_4 production between the 2:1 and 4:1 ISRs for the WWTP inoculum (p-value 0.084).

The results of BMP1 indicate that preservation using lyophilization with skim milk as a cryoprotectant can be achieved with multiple anaerobic digestion inoculum sources. Based on results from previous studies indicating that WWTP inoculum outperformed inoculum from food waste digesters and lagoon digestate (Elbeshbishy et al., 2012; Pandey et al., 2011), it was expected that the WWTP inoculum in this study would produce higher amounts of CH_4 than the other two inoculum sources. The similarity in performance among the three inoculum sources after preservation in this study indicates that lyophilization may cause a similar shift in the microbial communities within each inoculum source and select for similar microorganisms.

In this study, CH_4 production was 57 and 55% higher at the 4:1 ISR than the 2:1 ISR for the DAIRY and CODIG inocula without preservation, respectively, as was expected. The increase in CH_4 production at the higher ISR was most likely due to the increase in methanogens in the system. While previous studies were mixed on the impact of ISR on CH_4 production (Liu et al., 2009; Raposo et al., 2006), no previous study has investigated the impact of ISR of preserved inoculum on CH_4 production. In this study, it was shown that the average increase of 56% in CH4 from the preserved inoculum at the 4:1 ISR compared to the 2:1 ISR for the DAIRY and CODIG inocula, was higher than the increase in the non-preserved DAIRY samples and lower than the non-preserved CODIG indicating that preservation impacted the two inoculum differently.

The CH_4 production from the three inocula at the 4:1 ISR after preservation was within the range reported in a review on AD of solid organic waste of 200 to 850 mL CH_4/g VS (Khalid et al., 2011). The lower CH_4 values from the 2:1 ISR could be explained by inoculum VS concentrations of 1.05 g VS inoculum/L for WWTP and DAIRY inocula and 0.554 g VS inoculum/L for the CODIG inoculum that were below reported optimal batch study ranges of 2.1 to 37.2 g VS inoculum/L (Raposo et al., 2011). Although, inoculum VS concentrations tested in a preliminary study below the optimal inoculum VS range reported by Raposo et al. (2006) produced CH_4 values within the published range (Khalid et al., 2011) (Supplementary Table A 1).

Having a preservation methodology that is applicable across multiple inoculum sources, representing wastewater treatment plant digesters, manure digesters, and manure/food waste co-digestion vessels, shows the inherent ability of multiple types

of inocula from well-established digesters to be preserved and digest food waste successfully after preservation. The extra pre-treatment step was not shown to significantly increase CH_4 production, and, thus, was not used in subsequent BMPs. The results show the potential of using preserved AD inoculum for AD start-up or re-activation after stress events in remote locations through use of lyophilization.

Table 2.2: Average cumulative CH_4 production and VS (g) added of preserved inoculum, skim milk media, and food waste to each treatment for BMP1 at a 2:1 and 4:1 ISR utilizing pretreated preserved inoculum and three inoculum sources. Uppercase superscripts for CH_4 indicate significant differences between treatments.

Parameter	WWTP 2:1	WWTP 4:1	DAIRY 2:1	DAIRY 4:1	CODIG 2:1	CODIG 4:1
VS Inoculum[a] (g)	0.210 ± 0.001	0.421 ± 0.002	0.209 ± 0.001	0.420 ± 0.001	0.111 ± 0.0001	0.222 ± 0.001
VS Skim Milk Media (g)	0.168 ± 0.001	0.337 ± 0.001	0.119 ± 0.0004	0.238 ± 0.001	0.119 ± 0.0001	0.239 ± 0.001
VS Food Waste (g)	0.104	0.104	0.106	0.104	0.0545	0.0556
CH_4 (mL/g VS)	146 ± 12^{B}	194 ± 17^{AB}	143 ± 11^{B}	225 ± 10^{A}	141 ± 20^{B}	218 ± 17^{A}

[a] All treatments were diluted to 200 mL total volume with nutrient buffer solution

2.3.1.2 *Inoculum Pretreatment*

In BMP1, the effect of inoculum pretreatment on CH_4 production after sample preservation was tested (Figure 2.1). Contrary to what was expected, there was no significant difference in CH_4 production between the inoculum that did and did not receive pretreatment (p-values: 0.129 to 0.647 for all inocula). In previous studies, it was shown that concentrating the cells prior to lyophilization increased viability after reactivation (Costa et al., 2000; Morgan et al., 2006), with the concentration of cells from 10^8 to 10^{10} cell/mL being beneficial for activity recovery (Costa et al., 2000; Morgan et al., 2006). It is possible that above a certain cell concentration threshold additional concentration is not beneficial for activity recovery. Typical AD inoculum is usually well above the published beneficial range at 10^{16} cells/mL without concentrating (Amani et al., 2010), which would explain the lack of benefit from concentration in this study. Even though concentrating the anaerobic digestion inoculum did not increase CH_4 production from inoculum in this study, centrifuging the inoculum prior to lyophilizing does remove the bulk moisture from the inoculum prior to lyophilizing resulting in less time required for lyophilization.

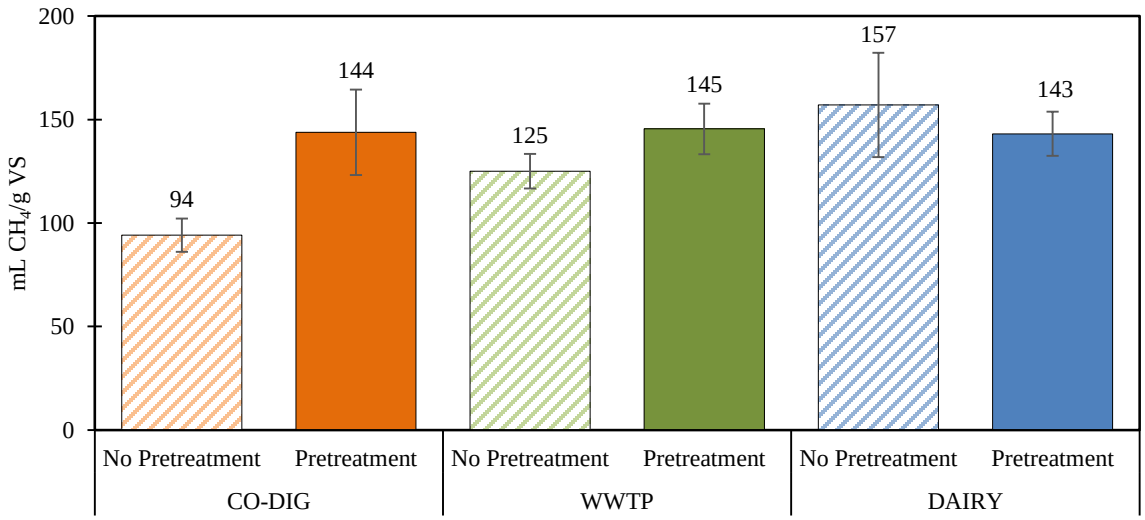

Figure 2.1: Comparison of the cumulative CH_4 production from the three inocula at a 2:1 ISR for treatments that either utilized pelletized and washed inoculum (pretreatment) or received no inoculum pretreatment (no pretreatment). Treatments were conducted in triplicate BMP bottles, with error bars representing standard error.

2.3.2 BMP2: Cryoprotectant Effect

In BMP2, the effect of cryoprotectant use on preservation success was evaluated using the CO-DIG inoculum (Table 2.3). Preservation success was determined using two metrics: percent of CH_4 production recovered after preservation and increase in lag phase after preservation (Figure 2.2). Skim milk media produced 3- and 13-fold more CH_4 than the glycerol and glycerol/skim milk treatments in BMP2-NP, respectively, and 4- and 300-fold more than the glycerol and glycerol/skim milk treatments in BMP2-P, respectively (p-values < 0.0001, Table 2.3); indicating that the addition of skim milk is beneficial for CH_4 production. Even though the addition of skim milk was beneficial for overall CH_4 production compared to glycerol and the glycerol/skim milk combination, there was no significant

difference in CH_4 production between BMP2-NP and BMP2-P for any of the cryoprotectant treatments (Table 2.3), indicating that the choice of cryoprotectant and the starting conditions had a larger effect on CH_4 production than the actual preservation.

The enhancement of CH_4 in the skim milk treatment in this study may be due to the benefits of skim milk as a cryoprotectant for methanogens over glycerol. Studies have found conflicting results when using glycerol as a cryoprotectant for anaerobic digestion inoculum (Castro et al., 2002; Colleran et al., 1992). While Colleran et al. (1992) observed 64 to 84.6% methanogenic activity recoveries using 10% glycerol as a cryoprotectant prior to lyophilization, which is in agreement with the CH_4 production recovery using glycerol in this study (76%); Castro et al. (2002) found that 10% glycerol resulted in a complete loss of specific methanogenic activity when utilized as a cryoprotectant for anaerobic lagoon sludge.

In this study, glycerol was utilized as a cryoprotectant due to its reported use as a frequently used cryoprotectant for microorganisms (Prakash et al., 2013). However, the two glycerol treatments in this study resulted in significantly less CH_4 production after preservation compared to skim milk. Glycerol is a cell penetrating protectant that binds the intracellular water preventing excessive dehydration and formation of ice crystals within the cell during freezing (Saarela et al., 2005). Depending on the cell, glycerol can be slow to penetrate requiring low dosing at high concentrations in order to avoid osmotic shock (Mortain-Bertrand et al., 1996). In this study, the glycerol was added at a 10% concentration to resuspend the cell pellet prior to freezing, which may not have allowed for cell penetration. Furthermore, archaea (the

domain in which methanogens are classified) have a different cell envelope than bacteria, which lacks a general cell wall polymer making them insensitive to antibiotics that target bacterial cell walls (Albers and Meyer, 2011). The cell wall of *Methanosaeta concilii*, the only mesophilic species in its genus and commonly found in anaerobic digestion inoculum (Karakashev et al., 2005; Rocheleau et al., 1999), is a tubular paracrystalline proteinaceous sheath that exhibits very low porosity (Albers and Meyer, 2011). The combination of archaeal cell envelope insensitivity to penetration and low porosity could render glycerol ineffective as a cryoprotectant for methanogens.

Conversely, skim milk is an extracellular cryoprotectant that forms a viscous layer on the cell surface (Saarela et al., 2005), with the proteins in skim milk providing a protective coating on the cell wall proteins and the calcium yielding increased survival rate after freeze-drying (Li et al., 2011). It has been suggested that the solids in skim milk prevent cellular injuries via cell membrane constituent stabilization (Li et al., 2011). The cryoprotective effects of skim milk are potentially beneficial to the entire microbial consortia within anaerobic digestion inoculum because it is a non-penetrating cryoprotectant; whereas glycerol may only be beneficial for the bacterial portion of the inoculum.

While 10% glycerol/10% skim milk resulted in 41.3% recovery of *Candida sake* when 10% skim milk was used as a rehydration media in Abadias et al. (2001), in this study the addition of 10% glycerol/10% skim milk resulted in reduced CH_4 production for both not preserved and preserved samples. The decrease in CH_4 production from the glycerol/skim milk treatment in this study could be due to an

overloading of organics. The glycerol/skim milk treatment received 1.6- to 1.9-fold more VS as substrate in BMP2-NP and 1.7- to 1.8-fold more VS as substrate in BMP2-P than the glycerol and skim milk treatments, respectively. The pH of the glycerol/skim milk treatment decreased from low initial pH condition (6.76) to 5.28 during the course of BMP2-NP, and from 7.17 to 5.02 during BMP2-P, indicating the build-up of acids in the system. It is also possible that the combination of glycerol and skim milk provided an added beneficial cryoprotectant effect during lyophilization of the acidogens in the inoculum. Acidogens may have a higher capacity for glycerol penetration allowing for intercellular protection as well as extracellular protection from the skim milk, resulting in higher acids production in the system. However, the glycerol/skim milk cryoprotectant may not have been as beneficial for the methanogens, resulting in acid accumulation and a decrease in post-BMP pH.

Inoculum preservation through lyophilization increased the lag phase in BMP2-P compared to BMP2-NP, with an 8-day lag phase for the skim milk treatment, 22 days for the glycerol, and 45 days for the glycerol/skim milk treatment (Figure 2.2). In BMP1-NP, CH_4 production for all treatments began on Day 1 with no lag phase. For the NP skim milk treatment, 29% of the total cumulative CH_4 was produced from Days 1 to 8, while in BMP2-P, only 10% of the total CH_4 production occurred prior to Day 8, with the majority of CH_4 production (64%) occurring between Day 8 and Day 21. For the glycerol treatment, in BMP2-NP, 93% of the total CH_4 production occurred before Day 22, while in BMP2-P, only 1% of the total CH_4 was produced prior to Day 22. The lag phase for the glycerol/skim milk treatment in BMP2-P lasted the duration of the BMP test.

The lag phase of the skim milk treatment in this study was 8 days, which is similar to the 12-day lag phase in CH_4 production experienced when Massalha et al. (2014) preserved granular sludge without a cryoprotectant using aerobic oven drying or anaerobic vacuum drying. In this study, glycerol had a negative effect on the inoculum, while skim milk likely served as both a cryoprotectant and a beneficial substrate during digestion. The results of this experiment indicate that AD inoculum can be preserved with skim milk and completely recover the ability to produce CH_4 with minimal increases to AD startup time.

Table 2.3: Average cumulative CH_4 production and VS (g) added of inoculum, skim milk media, and food waste to each treatment for not-preserved (BMP2-NP) and preserved (BMP2-P) treatments. Superscripts for CH_4 production represent significant differences in CH_4 yield within NP (bolded and underlined) and P treatments of all cryoprotectants. Treatments were conducted in triplicate with ± standard error shown.

Parameter	**10% Skim Milk Media**		**10% Glycerol**		**10% Glycerol/10% Skim Milk**	
	BMP2-NP	**BMP2-P**	**BMP2-NP**	**BMP2-P**	**BMP2-NP**	**BMP2-P**
VS Inoculum (g)	0.460 ± 0.003	0.497 ± 0.011	0.443 ± 0.005	0.454 ± 0.004	0.443 ± 0.021	0.424 ± 0.007
VS Skim Milk Media (g)	1.05 ± 0.01	1.07 ± 0.09	1.25 ± 0.01	1.17 ± 0.03	2.12 ± 0.10	2.03 ± 0.03
VS Food Waste (g)	0.115	0.124	0.111	0.113	0.111	0.106
CH_4 (mL/g VS)	227 ± 5$^{\mathbf{\underline{A}}}$	234 ± 15^{A}	84.2 ± 5.5$^{\mathbf{\underline{B}}}$	64.3 ± 1.4^{B}	18.0 ± 12.3$^{\mathbf{\underline{C}}}$	0.782 ± 0.494^{C}
p-value[a]	0.720		0.060		0.296	

[a] Statistical comparisons are between NP and P samples for each cryoprotectant.

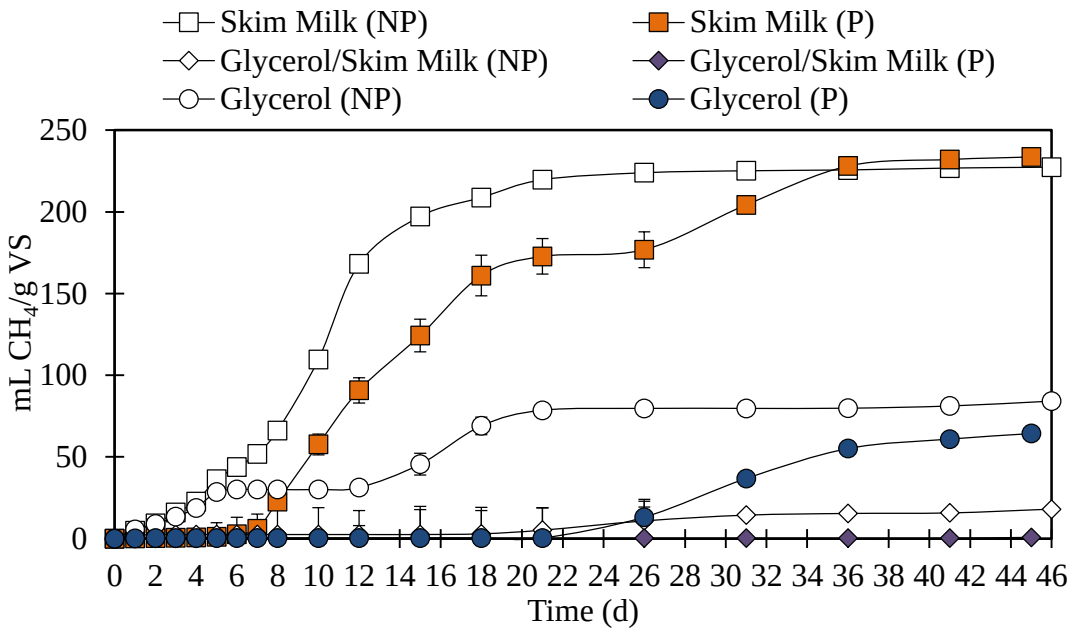

Figure 2.2: Comparison of the lag phases between non-preserved (NP) and preserved (P) treatments in BMP2 when using skim milk, glycerol, and glycerol/skim milk as cryoprotectants. Treatments were conducted in triplicate, with error bars representing standard error.

2.3.3 BMP3: Growth Phase Effect

In order to establish the optimal growth phase of the inoculum cells prior to preservation, the CO-DIG inoculum was lyophilized at the mid-exponential and stationary phases. Preservation had no significant impact on CH_4 production from the treatments preserved during mid-exponential phase, regardless of substrate addition, with similar CH_4 production after preservation compared to treatments not preserved (p-values: 0.228 and 0.786) (Table 2.4; Figure 2.3). Unlike the treatments preserved during mid-exponential phase, substrate addition did have an impact on recovery of CH_4 production after preservation for treatments preserved during the stationary phase. There was no significant difference in CH_4 production between BMP3-NP and

BMP3-P from the stationary phase treatment with food waste added after preservation (p-value = 0.145); however, the stationary phase treatment with no food waste added had 7% less CH_4 after preservation (p-value = 0.032). These results indicate that food waste can be added during the reactivation phase, without impacting CH_4 production; regardless of growth phase prior to preservation. However, when food waste was not added during resuspension, inoculum preserved during the mid-exponential phase outperformed inoculum preserved during the stationary phase. Comparing all preserved samples, there was no significant difference in CH_4 production after preservation among the treatments, regardless of growth phase or food waste addition (p-values: 0.123 to 1.00) (Table 2.4). The lag phase was between six and seven days for all treatments after preservation compared to the not preserved samples, which was consistent with the lag phase from BMP2-P (Figure 2.3).

It was expected in this study that lyophilization during the stationary phase would increase the tolerance of the inoculum to environmental stressors, such as preservation as observed in Anderson et al. (2012). However, in this study, the only treatment that did not recover CH_4 production after preservation was the stationary phase treatment with no food waste added. Corcoran et al. (2004) found that the survival of a human-derived probiotic strain (*Lactobacillus rhamnosus*) was highest when using 20% skim milk and stationary-phase cells, but with a 10% skim milk/10% polydextrose cryoprotectant the exponential-phase cells were higher than stationary-phase cells; indicating a potential relationship between cryoprotectant and growth phase. It is possible in this study that the proteins in skim milk media were better incorporated into the cell wall proteins of mid-exponential phase cells due to the

increased growth rate at the time of preservation compared to the stationary phase cells allowing for complete recovery without the addition of food waste. Whereas the stationary phase preserved inoculum required an additional substrate in order to overcome the effects of lyophilization. The discrepancy between the two stationary phase treatments could also be explained by the inherent variability in preservation of heterogeneous cultures, which will have an impact on the effect of cell growth phase on recovery after preservation.

The results from this study indicate that the inoculum should be preserved during mid-exponential growth phase and food waste can be added immediately upon resuspension in the field, but stationary phase extraction of the inoculum prior to preservation is also acceptable. Use of preserved inoculum will decrease transportation costs and increase the number of potential locations for new digesters, with a minimal increase in startup time and no loss in CH_4 production capability shown in the results.

Table 2.4: Average cumulative CH_4 production and VS (g) added of inoculum, skim milk and food waste for BMP3-NP and BMP3-P. Uppercase superscripts represent significant differences in CH_4 yield within the not-preserved (NP) and preserved (P) treatments. Treatments were conducted in triplicate with ± standard error shown.

Parameter	**Mid-Exponential SM[a]**		**Mid-Exponential SM+FW[b]**		**Stationary SM[a]**		**Stationary SM+FW[b]**	
	BMP3-NP	**BMP3-P**	**BMP3-NP**	**BMP3-P**	**BMP3-NP**	**BMP3-P**	**BMP3-NP**	**BMP3-P**
VS Inoculum (g)	0.398 ± 0.012	0.403 ± 0.004	0.405 ± 0.018	0.405 ± 0.016	0.348 ± 0.007	0.356 ± 0.005	0.350 ± 0.003	0.346 ± 0.004
VS Skim Milk Media (g)	0.967 ± 0.017	0.980 ± 0.005	0.985 ± 0.025	0.984 ± 0.022	0.845 ± 0.009	0.864 ± 0.006	0.850 ± 0.004	0.841 ± 0.006
VS Food Waste (g)	NA	NA	0.101	0.101	NA	NA	0.087	0.087
CH_4 (mL/g VS)	474 ± 9[A]	472 ± 1[A]	492 ± 12[A]	472 ± 3[A]	498 ± 6[A]	461 ± 10[A]	509 ± 8[A]	486 ± 10[A]
p-value[c]	0.786		0.228		0.032		0.145	
Percent CH_4 Recovery[d]	100% ± 8%		96% ± 12%		93% ± 6%		96% ± 8%	

[a] Treatment received 10% skim milk media (SM) as a cryoprotectant and no additional food waste (FW).
[b] Treatment received 10% skim milk media (SM) as a cryoprotectant and additional food waste (FW).
[c] Significant differences between NP and P treatments of similar growth phase removal and substrate additon.
[d] Percent CH_4 recovery between NP and P treatments of similar growth phase removal and substrate addition

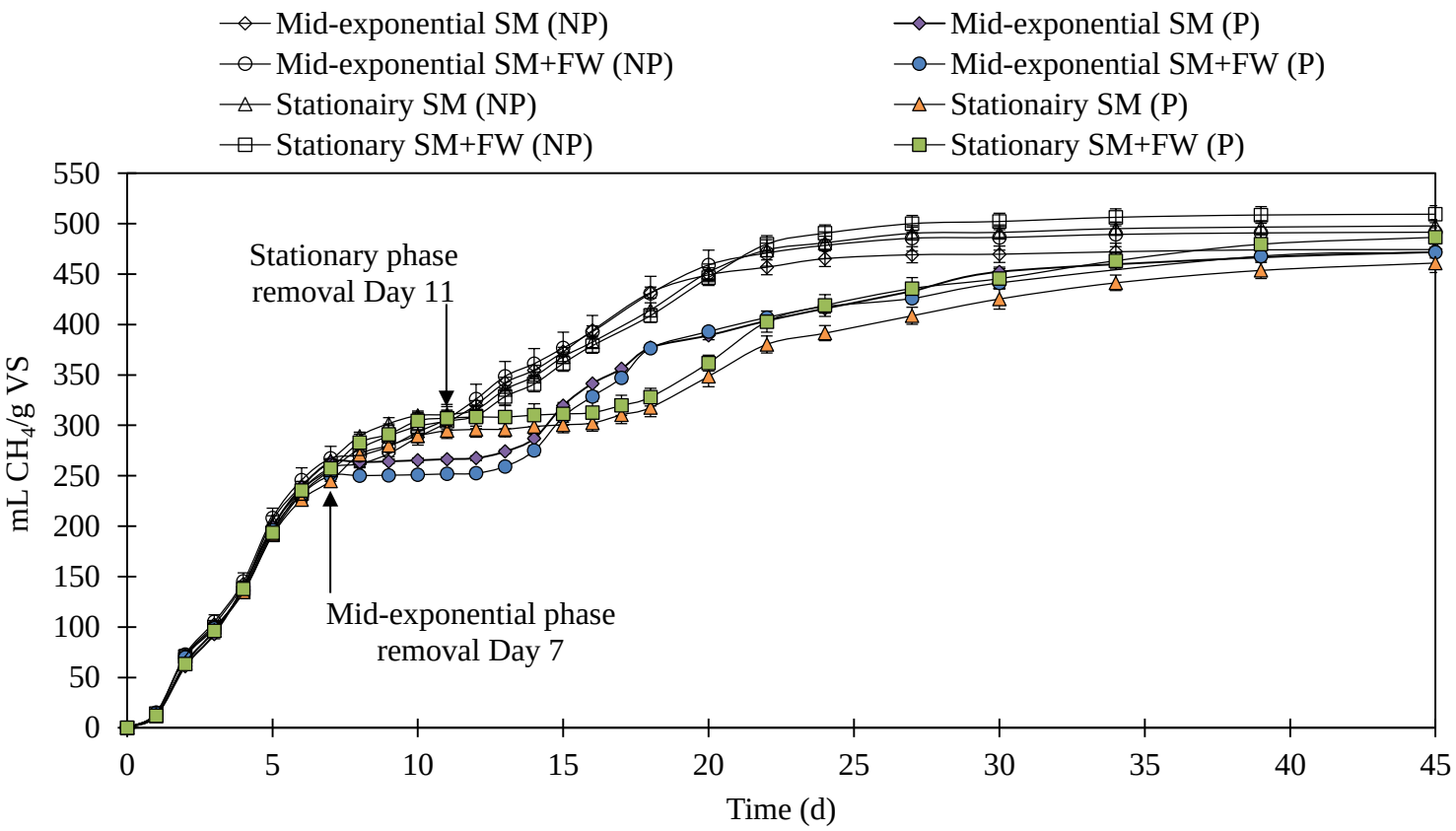

Figure 2.3: Cumulative CH_4 production from BMP3-NP and BMP3-P. Treatments with inoculum cells preserved during mid-exponential phase were removed at Day 7, and treatments with inoculum cells preserved during stationary phase were removed at Day 11. Lag phase during was six to seven days for all preserved samples. Treatments were conducted in triplicate with error bars indicating standard error.

2.4 Conclusions

This study demonstrated the impact of lyophilizing AD inoculum under different preservation conditions on CH_4 production using BMP testing with food waste as the substrate. Three inoculum sources were all successfully preserved via lyophilization with post-preservation CH_4 production similar to non-preserved samples. Food waste can be added immediately during resuspension from inoculum extracted at the mid-exponential or stationary growth phase and preserved via lyophilization in 10% skim milk and freezing at -20 °C. When starting the digester, inoculum should be resuspended and added to the food waste at quantities that will yield ISRs > 2:1 for the optimal CH_4 production.

3 Investigation of Iron Addition on the Biological Methane Potential and Methanogen and Sulfate Reducing Bacteria Numbers for Improvement of Anaerobic Digester Biogas

3.1 Introduction

Manure management currently accounts for 13% of the greenhouse gas emissions (GHG) from the agricultural sector, which is equivalent to 1.1% of the total US GHG emissions (EPA, 2016). Use of anaerobic digesters (AD) as a manure management option by all US dairies with liquid manure management systems and more than 500 cows could result in an 85% reduction in methane (CH_4) from dairy manure management, while also offsetting an additional 17.6 Tg CO_2 equivalents from energy production (Owen and Silver, 2015).

In order for the energy in biogas to be utilized in energy conversion technologies, hydrogen sulfide (H_2S) in the biogas must be reduced to levels below 300 to 500 ppm. Hydrogen sulfide in biogas when combusted produces SO_2, a regulated air pollutant that contributes to acid rain. Combustion of biogas containing high concentrations of H_2S also causes corrosion, which damages energy co-generation equipment and other technologies used for energy conversion. Removal of H_2S from biogas has been studied extensively and includes a number of physical/chemical and biological strategies (Kapdi et al., 2005; Krayzelova et al., 2015; Osorio and Torres, 2009). However, in order for a H_2S removal technology to be utilized on the farm scale, where there are not employees whose sole responsibility is AD operation and maintenance, the technology needs to be low maintenance and accessible. One of the drawbacks to AD on farms is the high capital cost

of the electric generation sets (EGS), which can be inflated further by expensive biogas scrubbing technologies. This study sought to determine an appropriate in-situ desulfurization method for dairy manure digesters that would reduce H_2S concentrations below the requirement for EGS while simultaneously not affecting the CH_4 production.

The removal of sulfides using iron salts dosing is a relatively common practice at wastewater treatment plants for reduction of odors and corrosion in sewer systems (Firer et al., 2008; Zhang et al., 2008). Of the potential chemicals used for in-situ H2S removal in sewers, iron salts are used often due to the relatively low cost compared to other methods and specificity for sulfide removal (Firer et al., 2008). In addition to being used in sewage dosing, iron salts have also been used as an in-situ method for reduction of H_2S from AD biogas (Dezham et al., 1988; Jiang et al., 2017; Liu et al., 2015). However, the choice of iron salt has been largely debated with respect to effectiveness in decreasing H_2S from the biogas.

The mechanism of sulfide removal by iron depends on the oxidation state. Zero valent iron (ZVI) undergoes corrosion induced oxidation to Fe(II), resulting in an increase in alkalinity, which could potentially increase the pH, shifting the more prevalent form of aqueous sulfides from H_2S to HS^-. The production of Fe(II) then removes dissolved sulfides via precipitation as ferrous sulfide (FeS), while Fe(III) removes sulfides via oxidation to elemental sulfur and is concurrently reduced to Fe(II) further removing sulfides via precipitation (Zhang et al., 2008). Addition of ZVI to an upflow anaerobic sludge blanket (UASB) reactor resulted in an increase in sulfate reduction with a decrease in H_2S in the biogas (Zhang et al., 2011). The authors attributed the decrease in H_2S to corrosion induced oxidation of ZVI increasing the alkalinity and

keeping the dissolved sulfides in the HS^- form rather than precipitation by Fe(II) resulting from ZVI oxidation. Dezham et al. (1988) found that $FeCl_2$ and $FeCl_3$ both effectively reduced H_2S concentrations in the digester biogas to 300 ppm; however, less $FeCl_2$ was required for equivalent reduction, resulting in cost savings. When five different iron compounds: Fe_2O_3, $FeCl_2$, $FeCl_3$, $FeSO_4$, and $Fe(OH)_3$ were added to chicken manure at concentrations between 0 and 32 mM, there was no significant difference in performance between the $FeCl_3$, $FeCl_3$, and $Fe(OH)_3$, with an increase in dosage concentration increasing the desulfurization rate (Jiang et al., 2017). In general, the choice of iron salt has been highly dependent on the substrate being digested; however, an investigation of which iron salt is most effective for H_2S removal from dairy manure AD biogas has not yet been conducted.

While iron dosing in wastewater treatment plants has been used primarily for H_2S reduction, research on the effects of iron on CH_4 production from AD has been increasing (Carpenter et al., 2015; Romero-Güiza et al., 2016; Zhang et al., 2011). Increased methane production in anaerobic sludge resulting from ZVI addition has been attributed to methanogens utilizing ZVI as an electron donor via corrosion-induced H_2 production (Wu et al., 2015; Yang et al., 2013). Whereas nanoscale zero valent iron (nZVI) has shown to both hinder (Yang et al., 2013) and improve (Suanon et al., 2016) CH_4 production, depending on the dose and substrate. Peng et al. (2014) attributed the increase in CH_4 to the necessity of iron as a trace element when 0.617 and 1.54 mM $FeCl_3$ were added to cyanobacterial biomass. Preeti Rao and Seenayya (1994) investigated the effects of 20 and 50 mM additions of $FeSO_4$ on cow dung and poultry litter digestion and found that the optimal concentration for increasing methane production was 20 mM, while

Jiang et al. (2017) observed no significant difference in CH_4 production from chicken manure when 0 to 32 mM $FeSO_4$ (and four other iron compounds) was added to batch reactors. Even though efforts to determine the effects of iron addition on CH_4 production from AD have increased, the majority of research has been conducted using synthetic wastewater. Research on CH_4 production from iron addition to dairy manure digesters has been limited to a single iron compound at multiple concentrations. Because the effects of iron on biogas quality are highly dependent upon the substrate and iron compound being used, a systematic study investigating multiple iron compounds at varying concentrations on dairy manure digestion would be beneficial.

Methane production in AD is ultimately dependent on the activity of the methanogens in the system. In anaerobic systems, methanogens compete with sulfate reducing bacteria (SRB) and iron reducing bacteria (IRB) for substrate. Competition between these three groups of microorganisms is controlled by the availability and concentration of electron donors and preferred terminal electron acceptors in the system, with Fe(III) > SO_4^{2-} > CO_2 in order of terminal electron acceptor preference. Kato et al. (2012) attributed the increase in CH_4 production resulting from the addition of semi-conductive Fe(III)-oxide species to rice paddy sediment to *Geobacter* spp. using the Fe(III)-oxide as an electrode through which to pass electrons to methanogens. However, other studies have indicated a decrease in CH_4 production with the addition of Fe(III)-oxides due to a diversion of electron flow from methanogenesis to Fe(III) reduction by methanogens (Bond and Lovely, 2002; Roden, 2003; Tan et al., 2015). Most studies investigating the addition of iron to anaerobic systems have evaluated the impact of iron on methanogens; however, few studies have determined the effect of iron addition on the

relationship between sulfate reducing bacteria (SRB), which are a prevalent competitor for electron donors with methanogens, and methanogens in AD systems.

While it is possible that iron addition to dairy manure AD will increase the biogas quality by precipitating out H_2S and potentially increasing the CH_4 production, the benefits of AD on farms is not limited to biogas production. The digestate from dairy manure AD is used as a soil amendment due to the mineralization of nutrients during the AD process (Möller and Müller, 2012). Iron addition to AD has shown to precipitate out phosphates (Cheng et al., 2015; Zhang, 2012), which could reduce the benefits of use of digestate as a soil amendment. Studies that have evaluated the impact of iron addition on AD have been focused on the beneficial impacts on biogas quality, but have not investigated the effects of iron phosphate complexes on digestate nutrient quality.

The objective of this study was to determine the effect of four iron compounds: ZVI, Fe_2O_3, $FeCl_2$, and $FeSO_4$, on CH_4 and H_2S production, the methanogenic and sulfate reducing bacterial community numbers, and the water soluble nutrient composition of the digestate after digestion. It was hypothesized that the addition of iron to dairy manure would increase the CH_4 production and decrease the H_2S production compared to digestion of un-amended dairy manure. The existence of a H_2S removal technology with limited technological requirements should be more accessible to farmers looking to utilize the biogas produced from their digesters without the hindrance of H_2S fouling their generators.

3.2 Material and Methods

Biochemical methane potential (BMP) tests were conducted in order to evaluate the effects of iron addition on AD of the liquid fraction of solids-separated dairy manure.

3.2.1 Inoculum and Substrate Utilized

Inoculum and separated manure were collected in five-gallon buckets from the USDA Beltsville Agricultural Research Center (BARC) dairy farm, located in Beltsville, Maryland. The inoculum was piped from the center of a full scale anaerobic digester receiving separated dairy manure. Directly after collection, inoculum and manure were stored at 4 °C until use in experiment.

3.2.2 Experimental Design

Two BMP tests were conducted with four iron treatments: iron(III)-oxide (Fe_2O_3), iron(II) sulfate ($FeSO_4$), iron(II) chloride ($FeCl_2$), and ZVI at iron concentrations of 20 mM (BMP1) and 50 mM (BMP2). All iron compounds were analytical grade from Fisher Scientific. The iron treatments were added to 50 mL of deionized water in a 250-mL glass bottle and mixed. The same volume of inoculum (111 mL in BMP1 and 121 mL in BMP2) and manure (39 mL in BMP1 and 29 mL in BMP2) was added to each treatment at a 2:1 inoculum to manure ratio, by volatile solids (VS). In addition to the four iron treatments, there was an un-amended manure treatment containing manure, inoculum, and deionized water and an inoculum-only control, which contained inoculum and deionized water. In BMP1 (20 mM iron addition) each treatment was conducted in triplicate; however, in BMP2 (50 mM iron addition), the treatments were conducted in duplicate.

3.2.3 Biochemical Methane Potential (BMP) Test

Anaerobic incubation was conducted using a BMP protocol developed by Owen et al. (1979) and adapted by Moody et al. (2011). BMP experiments were used to determine the energy production potential, defined as CH_4 production, and the H_2S concentration of the biogas. The BMP tests were conducted until the CH_4 production in each treatment stabilized. Prior to incubation, the BMP bottles were flushed with 70:30 N_2:CO_2 for three minutes to ensure anaerobic conditions. The bottles were then capped with a rubber septum and placed on a shaker (New Brunswick™ innOva® 2300, Hamburg, Germany) at 110 rpm in a temperature controlled incubator at 35 °C.

Biogas production was quantified volumetrically using a glass, gas-tight syringe equilibrated to atmospheric pressure. Biogas samples were analyzed for CH_4 and H_2S composition using a gas chromatograph (Agilent Technologies, Inc.; Shanghai China; model 7890 A) with a thermal conductivity detector at 250°C, a HP-Plot Q capillary column (Agilent J&W; USA), helium as the carrier gas at 8.6 ml/min, and the oven operated at 60°C for 2 min and subsequently ramped at 30°C/min to 240°C. Biogas production and CH_4 content was measured daily during the first week of the experiment, approximately every other day the following week, and then as needed for the remainder of the BMP, with measurement frequency based on the quantity of biogas produced.

To account for biogas production from residual biodegradable material in the inoculum, inoculum controls were incubated using the same conditions for each inoculum source and sampled simultaneously to allow for subtraction of CH_4 production not attributed to the manure. Methane production was then normalized by the mass of VS in

the manure added to each treatment to determine the efficiency of the inoculum to utilize the organics in the treatments.

3.2.4 Analytical Methods

The inoculum and manure total and volatile solids were characterized prior to BMP loading. Samples in BMP1 and BMP2 were analyzed for pH, total chemical oxygen demand (COD), soluble chemical oxygen demand (sCOD), and volatile fatty acids (VFAs) pre- and post-digestion. Total solids (TS) (APHA Method 2540B) and VS (APHA Method 2540E) were determined using the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). The pH was determined with an Accumet AB 15 pH meter. HACH method 8000 was used to determine COD and sCOD, with samples for sCOD filtered through 1.5 μm nitrocellulose membrane filter.

For measurement of VFAs (acetic, propionic, n-butyric, and n-valeric acids), samples were acidified with 5N sulfuric acid to pH < 2 (diluted by ≤ 10%) and filtered to 0.22 μm through a nitrocellulose membrane filter. Acidified and filtered samples were then analyzed with a gas chromatograph (Agilent Technologies, Inc.; Shanghai China; model 7890 A) with a flame ionization detector (FID) at 300°C. The column was a DB-FFAP capillary column (Agilent J&W; USA) with helium as the carrier gas at 1.80 ml/min, an injection temperature of 250°C, and the oven operated at 100°C for 2 min then subsequently ramped at 10°C/min for a total run time of 10 min. Volatile fatty acid concentrations were converted to COD using the following conversion factors: 1.07 for acetic acid, 1.51 for propionic acid, 1.82 for butyric acid, and 2.04 for valeric acid (Yuan et al., 2011).

In BMP2, pre- and post-digestion samples were also analyzed for water soluble SO_4^{2-} and phosphate (PO_4^{3-}) and pre-digestion samples were analyzed for total kjeldahl nitrogen and phosphorus (TKN and TKP). Water soluble SO_4^{2-} and PO_4^{3-} were analyzed on samples filtered through 0.22 µm nitrocellulose membrane filters on an 850 Professional IC Autosampler (Metrohm USA, Inc., Riverview, FL) with a METROSEP A Supp 5-150/4.0 separation column and 20 µL injection. A Lachat (QuickChem 8500 Series 2 FIA Automated Ion Analyzer) was used to determine ammonia, TKN, and TKP. Before analysis using Lachat QuikChem Method 10-107-06-2-O, ammonia samples were acidified to a pH < 2 with 5 N H_2SO_4, then filtered through 0.45 µm nitrocellulose membranes. Samples analyzed for TKN (QuikChem Method 13-107-06-2-D) and TKP (13-115-01-1-B for TKP) went through a kjeldahl digestion with concentrated H_2SO_4 and $CuSO_4$*$5H_20$ before being analyzed.

3.2.5 DNA Extraction

For each BMP experiment, 5-mL samples were collected in a sterile 15-mL centrifuge tube from each of the treatments prior to and at the end of the BMP test and placed in a -20 °C freezer (1-month) before being transferred on ice to a -80 °C freezer. In BMP1, one pre-digestion sample per treatment was collected, with triplicate samples collected post-digestion. In BMP2, triplicate pre-digestion samples were collected and duplicate samples were collected post-digestion.

DNA samples were extracted using the PowerFecal® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The amount of DNA was quantified using a Qubit 2.0 Fluorometer (Life Technologies, Grand Islane, NY, USA).

3.2.6 Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR was used to estimate the population size of methanogens and dissimilatory sulfate reducing bacteria. The abundance of two functional genes: methyl coenzyme M reductace (*mcr*A) and dissimilatory sulfate reductase (*dsr*A) was estimated from preserved BMP samples using qPCR. A detailed description of plasmid standard construction was previously outlined in (Prasse et al., 2015). Standards and extracts were conducted in triplicate 20 µM reaction mixtures containing 10.0 µL of KiCqStart SybrGreen qPCR readymix with ROX (Sigma, St. Louis, MO), 0.5 µM final concentration of the forward and reverse primers, and 5 ng of template DNA for functional gene quantification. All reactions were conducted on the StepOne Plus real-time PCR instrument (Applied Biosystems, Foster City, CA).

Data were extracted from runs with standard curve R^2 values of > 0.97, efficiency values between 97 and 100%, and a single dominant peak in dissociation curves (Yarwood et al., 2010). In order to relativize standard plasmid curves for sample-specific inhibition, a soil standard dilution series was used (Hargreaves et al., 2013). The soil dilution series was then diluted to 1.07 ng/µL for *mcr*A genes and to 2.5 ng/µL for *dsr*A genes, with a 10-fold dilution series conducted following the gene specific conditions. Specific primers, thermocycler conditions, number of cycles, and efficiencies for the functional genes used in this study are detailed in Table 3.1

Table 3.1: Primers, reaction conditions, and efficiencies for quantitative PCR

Target	**Gene**	**Pure Culture**	**Primers**	**Reference**	**Thermocycler Conditions (Acquisition Step Bolded)**	**Number of Cycles**	**Plasmid standard and soil correction efficiency (%) r^2 values**
*mcr*A	Methyl coenzyme M reductase	*Methanococcus maripaludis*	mcrA_1035F mcrA_1530R	(Prasse et al., 2015)	95 °C for 5 min 95 °C for 30 s / 56 °C for 45 s / 72 °C for 60 s / **80 °C for 10 s**	1 40	97, 100, 97, 97 soil = 71% All r^2 > 97%
*dsr*A	Dissimilatory sulfate reductase	*Desulfovibrio vulgaris*	dsr-1F dsr-500R	(Wilms et al., 2007)	94 °C for 3 min 94 °C for 10 s / 58 °C for 20 s / 72 °C for 30 s / **82 °C for 20 s**	1 50	100, 100, 98, 95 soil = 132% All r^2 > 99%

3.2.7 Statistical Analysis

Statistical analysis was conducted using SAS 9.3 (SAS, Cary, NC). Each experiment was a single factor, completely randomized design with 18 experimental units (BMP bottles). One-way ANOVA analysis was performed to assess significant differences within treatments between cumulative CH_4 and H_2S, water quality, and microbial data at each sampling time. Pearson correlation analysis was utilized for functional relationships between *mcrA* gene copy numbers and cumulative CH_4 production, *dsrA* gene copy numbers and cumulative H_2S production, and correlations between *mcrA* and *dsrA* gene copy numbers in each BMP. The level of significance was held at an alpha of 0.05.

3.3 Results

3.3.1 Characteristics of Manure and Inoculum and BMP Results

In BMP1, the inoculum and manure contained 61% and 72% VS, respectively. In BMP2, there was 63% and 75% VS in the inoculum and manure, respectively. The effect of iron addition on pH was determined in BMP1 and BMP2. The post-digestion pH values in BMP1 and BMP2 ranged from 7.09 to 7.39 and 6.82 to 7.47, respectively (Table 3.2). All treatments maintained pH within the ideal pH range for methanogenesis of 6.5 to 8.2 (Lee et al., 2009).

Table 3.2: pH measurements for BMP1 and BMP2. In BMP1, treatments were conducted in triplicate and in BMP2, post-digestion treatments were conducted in duplicate with ± standard error shown.

	20 mM Iron		**50 mM Iron**	
	pH		**pH**	
Treatment	*Initial*	*Final*	*Initial*	*Final*
Manure	7.46 ± 0.02	7.33 ± 0.003	7.30 ± 0.01	7.43 ± 0.04
ZVI	7.63 ± 0.01	7.35 ± 0.003	7.37 ± 0.02	7.47 ± 0.02
Fe_2O_3	7.56 ± 0.03	7.34 ± 0.00	7.33 ± 0.01	7.42 ± 0.01
$FeCl_2$	7.05 ± 0.003	7.09 ± 0.01	6.74 ± 0.03	6.82 ± 0.02
$FeSO_4$	7.15 ± 0.03	7.16 ± 0.01	6.92 ± 0.003	7.04 ± 0.02

3.3.1.1 *Effect of Iron Addition on Methane Production*

The effect of iron addition on CH_4 production was determined in BMP1 by adding 20 and 50 mM ZVI, $FeCl_2$, $FeSO_4$, and Fe_2O_3 to manure in a BMP test. There was no significant difference in CH_4 production between the un-amended manure treatment (315 mL CH_4/g VS added), the ZVI treatment (318 mL CH_4/g VS added), and the Fe_2O_3 treatment (292 mL CH_4/g VS added) (p-values: 0.133 to 0.997) (Figure 3.1 and Table 3.3). Compared to the un-amended manure treatment, CH_4 production significantly decreased by 20% for $FeCl_2$ (253 mL CH_4/g VS added) (p-value = 0.001) and 29% for $FeSO_4$ (224 mL CH_4/g VS added) (p-values < 0.0001).

Similar trends were observed when the concentration of iron was increased from 20 mM to 50 mM in BMP2, with no significant difference in CH_4 production between the un-amended manure treatment (320 mL CH_4/g VS manure), ZVI treatment (310 mL CH_4/g VS manure), and Fe_2O_3 treatment (277 mL CH_4/g VS manure) (p-values: 0.356 to 0.989) (Figure 3.1 and Table 3.3). While the addition of $FeCl_2$ (225 mL CH_4/g VS added) significantly decreased CH_4 production by 30% (p-value = 0.030), there was no

significant difference in CH_4 production between the Fe_2O_3 and the $FeCl_2$ treatments (p-value = 0.227). The addition of $FeSO_4$ (171 mL CH_4/g VS added), significantly decreased CH_4 production by 47% compared to the un-amended manure treatment (p-value = 0.004).

3.3.1.2 *Effect of Iron Addition on H_2S production*

Compared to the un-amended manure treatment, addition of iron to dairy manure resulted in decreased H_2S production (Figure 3.1 and Table 3.3). In BMP1, the $FeCl_2$ and $FeSO_4$ treatments reduced H_2S below the method detection limit for the duration of the BMP. When compared to the un-amended manure treatment (1.53 mL H_2S/g VS), the ZVI (0.940 mL H_2S/g VS), Fe_2O_3 (0.520 mL H_2S/g VS), $FeCl_2$ (below detection limit), and $FeSO_4$ (below detection limit) treatments reduced cumulative H_2S production by 38%, 66%, 100%, and 100%, respectively (p-values < 0.0001) (Figure 1). The average H_2S concentration over the 19-day BMP for the un-amended manure treatment (2,360 ppm H_2S) was 1.7-fold higher than the ZVI treatment (1,400 ppm H_2S) (p-value < 0.0001). The ZVI treatment average daily H_2S concentration was 2.4-fold higher than the Fe_2O_3 treatment (582 ppm H_2S) (p-value = 0.0003).

Similar to BMP1, in BMP2 all iron treatments significantly reduced cumulative H_2S concentrations below the un-amended manure treatment (Figure 3.1 and Table 3.3). There was no significant difference in cumulative H_2S production between the ZVI (0.331 mL H_2S/g VS), Fe_2O_3 (0.230 mL H_2S/g VS), $FeCl_2$ (0.107 mL H_2S/g VS), and $FeSO_4$ (0.112 mL H_2S/g VS) treatments (p-values: 0.158 to 1.00), which significantly reduced cumulative H_2S concentrations by 89%, 92%, 96%, and 96% (p-values < 0.0001) compared to the un-amended manure treatment (2.92 mL H_2S/g VS). The Average H_2S

production over the 30-day BMP from the un-amended manure (3,540 ppm H_2S) was 13- to 31-fold higher than the ZVI (272 ppm H_2S), Fe_2O_3 (204 ppm H_2S), $FeSO_4$ (134 ppm H_2S), and $FeCl_2$ (114 ppm H_2S) treatments, with no significant difference in average H_2S production between iron treatments (p-values: 0.290 to 0.998).

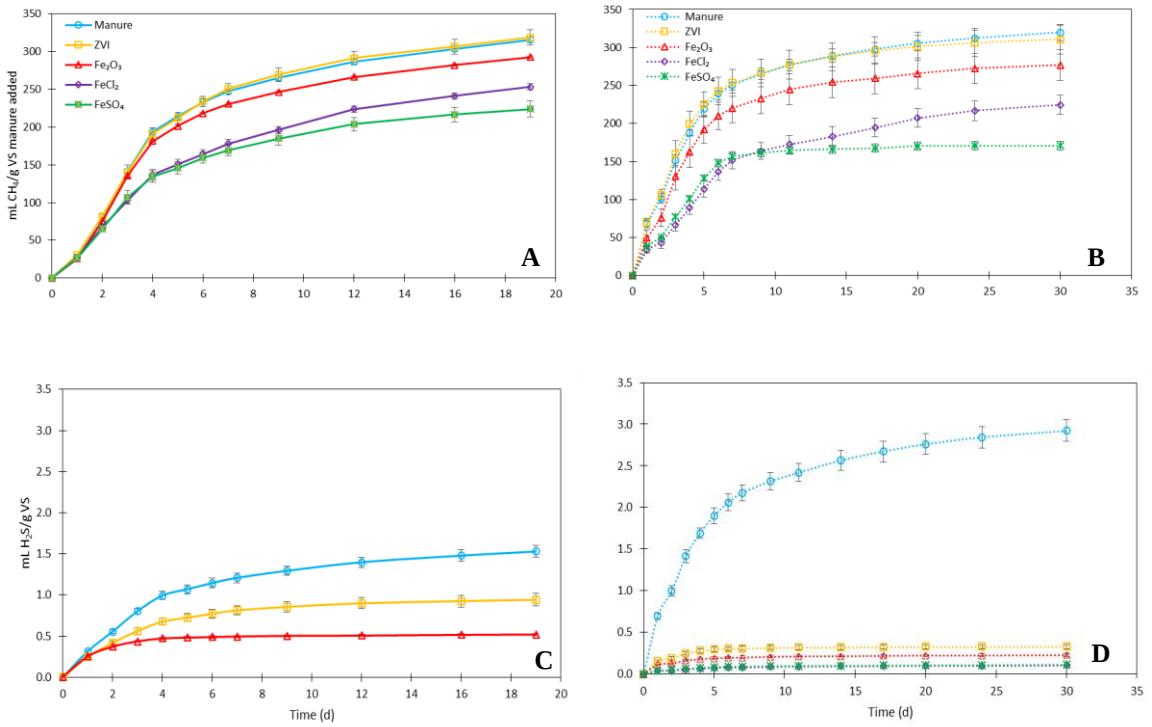

Figure 3.1: Average cumulative CH_4 production in A) BMP1 and B) BMP2 and average cumulative H_2S production in C) BMP1 and D) BMP2. In BMP1, $FeCl_2$ and $FeSO_4$ reduced H_2S below the detection limit of 5 ppm and are not shown in C. Treatments in BMP1 were conducted in triplicate and treatments in BMP2 were conducted in duplicate, error bars represent standard error.

Table 3.3: Cumulative CH_4 and H_2S production and average H_2S concentration for the 20 and 50 mM iron addition. Treatments were conducted in triplicate with ± standard error shown.

	20 mM			50 mM		
Treatment	**CH_4 (mL/g VS)**	**H_2S (mL/g VS)**	**H_2S (ppm)**	**CH_4 (mL/g VS)**	**H_2S (mL/g VS)**	**H_2S (ppm)**
Manure	315 ± 4	1.53 ± 0.07	2360 ± 130	320 ± 11	2.92 ± 0.13	3540 ± 100
ZVI	318 ± 10	0.942 ± 0.077	1400 ± 130	310 ± 19	0.331± 0.024	272 ± 15
Fe_2O_3	292 ± 2	0.519 ± 0.014	582 ±16	277 ± 20	0.230 ± 0.014	204 ± 10
$FeSO_4$	224 ± 10	b.d.l[1]	b.d.l[1]	171 ± 6	0.112 ± 0.003	134 ± 1
$FeCl_2$	253 ± 4	b.d.l[1]	b.d.l[1]	225 ± 13	0.106 ± 0.002	114 ± 0.1

[1] Below the detection limit, which is 5 ppm.

3.3.1.3 *Effect of Iron Addition on COD, sCOD, and VFA Transformations*

The effect of iron addition on COD and sCOD removal and TVFA concentrations was determined by adding 20 and 50 mM ZVI, $FeCl_2$, $FeSO_4$, and Fe_2O_3 to manure in BMP tests. Total COD concentrations in BMP1 pre-digestion ranged from 21.9 to 23.5 g COD/L and post-digestion ranged from 18.3 to 20.1 g COD/L (Table 3.4). There was no significant difference in COD removal between the un-amended manure treatment (31%) and the Fe_2O_3 (19%), $FeCl_2$ (11%), and ZVI (15%) treatments (p-values: 0.066 to 0.416). COD removal in the $FeSO_4$ treatment was significantly less (8%) than the un-amended manure treatment (p-value = 0.028). Soluble COD concentrations in BMP1 ranged from 3.30 to 4.30 g COD/L in pre-digestion samples and from 2.04 to 2.59 g COD/L in post-digestion samples. There was no significant difference in sCOD reduction between the un-amended manure treatment and the iron treatments with sCOD removals from 35 to 40% (p-values: 0.758 to 01.00) (Figure 3.2).

In BMP2, total COD concentrations in pre-digestion samples ranged from 20.0 to 22.3 g COD/L and from 16.0 to 19.9 g COD/L in post-digestion samples (Table 3.4). There was no significant difference in COD removal between the un-amended manure control and the iron treatments (p-values: 0.526 to 0.991), with COD removal from 6% to 28%. Soluble COD concentrations ranged from 4.07 to 4.94 mg COD/L in pre-digestion samples and from 2.21 to 2.88 mg COD/L in post-digestion samples. There was no significant difference in sCOD removal between the un-amended manure treatment and the iron treatments (p-values: 0.067 to 0.800), with sCOD removal from 30% to 54% (Figure 3.2).

Volatile fatty acids in BMP1 treatments were completely utilized during digestion. The pre-digestion TVFA concentrations ranged from 692 to 782 mg COD/L (Figure 3.2), with no significant difference between the treatments (p-values from 0.941 to 1.00). Acetic acid was the most abundant acid in the un-amended manure and iron treatments, comprising 65% to 72% of the TVFA. Propionic (26% to 28%) was the second most abundant VFA in treatments, followed by valeric (2% to 5%) then butyric (0% to 3). In BMP2, pre-digestion TVFA concentrations ranged from 1,220 to 1,650 mg COD/L (Figure 3.2), with no significant difference between treatments (p-values: 0.108 to 0.999). The composition of TVFAs in pre-digestion samples ranged from 50 to 57% acetic, 19 to 21% propionic, 14 to 20% butyric, and 10-13% valeric. During digestion, 100% of the propionic, butyric, and valeric acids were utilized. There was no significant difference in post-digestion acetic acid concentrations between treatments, with concentrations between 280 and 349 mg COD/L. Percent reduction of TVFA ranged from 73 to 79%, with 49 to 64% reductions in acetic acid.

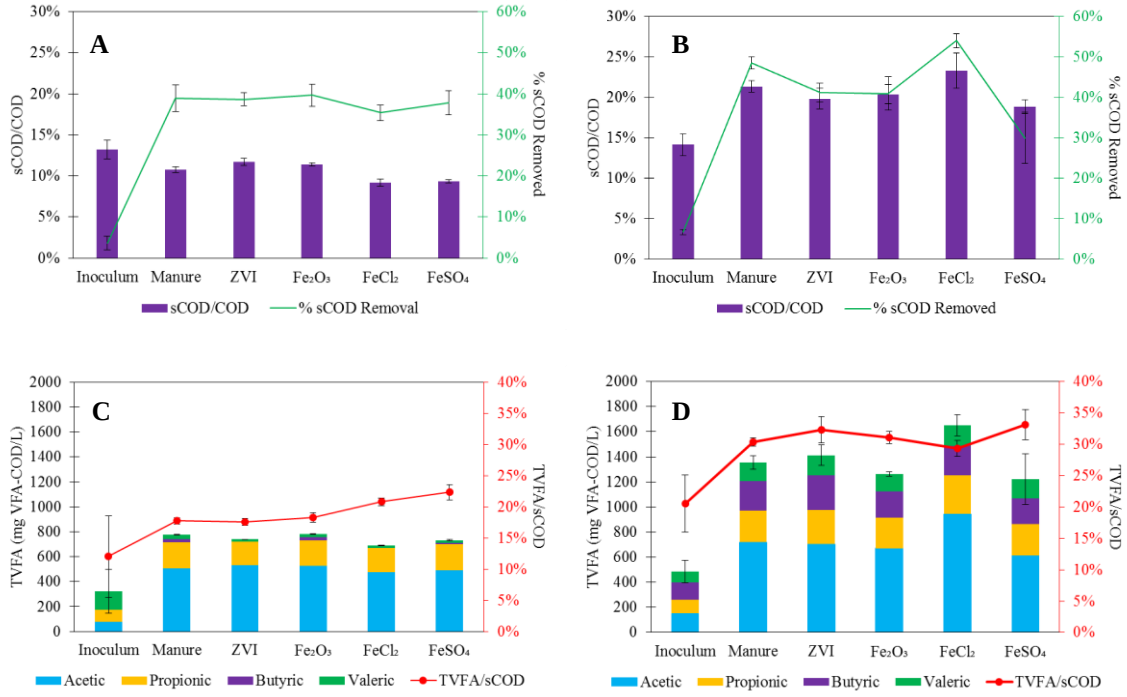

Figure 3.2: sCOD removal efficiency and sCOD/COD ratio from A) BMP1 and B) BMP2 as well as total volatile fatty acid concentrations as the sum of acetic, propionic, butyric, and valeric acid concentrations, converted to COD concentration units and TVFA/sCOD ratios from C) BMP1 and D) BMP2.Treatments in BMP1 were conducted in triplicate and BMP2 were conducted in duplicate with error bars representing ± standard error

Table 3.4: COD and sCOD of the un-amended manure treatment and the iron treatments from the 20 mM and 50 mM iron addition experiments pre- and post-digestion. In BMP1, treatments were conducted in triplicate and in BMP2, post-digestion treatments were conducted in duplicate with ± standard error shown.

	20 mM Iron Addition				**50 mM Iron Addition**			
	COD (g/L)		**sCOD (g/L)**		**COD (g/L)**		**sCOD (g/L)**	
Treatment	*Initial*	*Final*	*Initial*	*Final*	*Initial*	*Final*	*Initial*	*Final*
Manure	24.9 ± 0.5	17.3 ± 0.5	4.39 ± 0.12	2.68 ± 0.05	20.9 ± 0.2	19.4 ± 1.9	4.46 ± 0.18	2.21 ± 0.04
ZVI	22.1 ± 0.8	18.8 ± 0.6	4.22 ± 0.13	2.58 ± 0.01	22.3 ± 1.7	16.0 ± 0.4	4.37 ± 0.06	2.60 ± 0.15
Fe_2O_3	22.7 ± 0.2	18.3 ± 1.2	4.30 ± 0.17	2.59 ± 0.02	20.0 ± 0.7	17.1 ± 1.1	4.07 ± 0.16	2.34 ± 0.03
$FeCl_2$	23.5 ± 0.5	20.7 ± 0.8	3.33 ± 0.09	2.15 ± 0.05	21.4 ± 1.2	18.0 ± 0.1	4.94 ± 0.29	2.40 ± 0.07
$FeSO_4$	21.9 ± 0.2	20.1 ± 0.4	3.30 ± 0.18	2.04 ± 0.02	22.1 ± 1.4	19.9 ± 0.7	4.16 ± 0.14	2.89 ± 0.10

3.3.2 Effect of Iron Addition on Methanogen and Sulfate Reducing Bacteria Numbers

3.3.2.1 *Methanogenic Numbers*

The effect of iron addition on methanogen (*mcrA*) gene copy numbers was determined for 20 and 50 mM iron additions to manure in BMP tests. All treatments in BMP1 pre- and post-digestion samples contained between 10^9 to 10^{10} copies of *mcrA* genes/g of sample (Figure 3.3). Significant differences between pre-digestion BMP1 treatments could not be determined due to only having one sample per treatment; however, post-digestion, there was no significant difference in *mcrA* gene copy numbers between the un-amended manure, ZVI, and Fe_2O_3 treatments (p-values: 0.067 to 1.00). The $FeCl_2$ and $FeSO_4$ treatments contained 17% and 51% less *mcrA* gene copy numbers than the un-amended manure treatment, respectively (p-values = 0.025 and 0.050, respectively). After digestion, the *mcrA* gene copy numbers increased in all treatments between 1.1- and 3.2-fold, with the highest increase in the inoculum-only control of 9.5-fold. There was no significant correlation between cumulative CH_4 production and *mcrA* gene copy numbers post-digestion in BMP1, with a r value of 0.416 (p-value = 0.123) (Figure 3.4).

In BMP2, pre- and post-digestion samples of each treatment contained between 10^9 and 10^{10} copies of *mcrA* genes/g of sample (Figure 3.3). There was no significant difference in pre-digestion *mcrA* gene copy numbers between the un-amended manure treatment and the iron treatments (p-values: 0.779 to 0.999). Post-digestion, there was no significant difference in *mcrA* gene copy numbers between the un-amended manure

treatment and the iron treatments (p-values from 0.155 to 0.999). During digestion, the *mcrA* gene copy numbers increased between 1.1- and 2.8-fold for the inoculum only, un-amended manure, Fe_2O_3, $FeCl_2$, and $FeSO_4$ treatments; however, addition of ZVI resulted in a decrease in *mcrA* gene copy numbers of 7%. There was no significant correlation between cumulative CH_4 production and *mcrA* gene copy numbers post-digestion in BMP2, with a r value of 0.470 (p-value = 0.170) (Figure 3.4).

3.3.2.2 *Sulfate Reducing Bacteria Numbers*

The effect of iron addition on SRB (*dsrA*) gene copy numbers was determined for 20 and 50 mM iron additions to manure in BMP tests. Pre-digestion BMP1 treatments contained between 10^4 and 10^7 copies of *dsrA* genes/g of sample and post-digestion samples contained between 10^6 and 10^8 copies of *dsrA* genes/g of sample (Figure 3.3). Significant differences between pre-digestion BMP1 treatments could not be determined due to only having one sample per treatment; however, there was no significant difference between the treatments in post-digestion samples (p-values: 0.860 to 1.00). After digestion, *dsrA* gene copy numbers increased 10-, 2.2-, 2.3-, and 1.3-fold in the un-amended manure, ZVI, Fe_2O_3, and $FeSO_4$ treatments with the highest increase in *dsrA* copy numbers of 50-fold from the inoculum control. Unlike the other treatments, the $FeCl_2$ treatment decreased *dsrA* copy numbers by 23%. There was no significant correlation between cumulative H_2S production and *dsrA* gene copy numbers post-digestion in BMP1, with a r value of 0.477 (p-value = 0.099) (Figure 3.4).

In BMP2, there was no significant difference in *dsrA* gene copy numbers in pre-digestion samples (p-values: 0.247 to 1.00) with concentrations of 10^6 copies of *dsrA*/g sample. Post-digestion gene copy numbers ranged from 10^6 to 10^7 copies *dsrA*/g sample,

with no significant difference between treatments (p-values: 0.066 to 1.00) (Figure 3.3). Similar trends were observed in BMP2 as in BMP1 with regards to *dsrA* gene copy number increase. The inoculum control, un-amended manure, ZVI, Fe_2O_3, and $FeSO_4$ treatments all increased *dsrA* gene copy numbers 1.3- to 3.6-fold (p-values: 0.088 to 0.884) and the $FeCl_2$ treatment decreased *dsrA* gene copy numbers by 14% (p-value = 0.709). There was no significant correlation between cumulative H_2S production and *dsrA* gene copy numbers post-digestion in BMP2 with a r value of 0.477 (p-value = 0.282) (Figure 3.4).

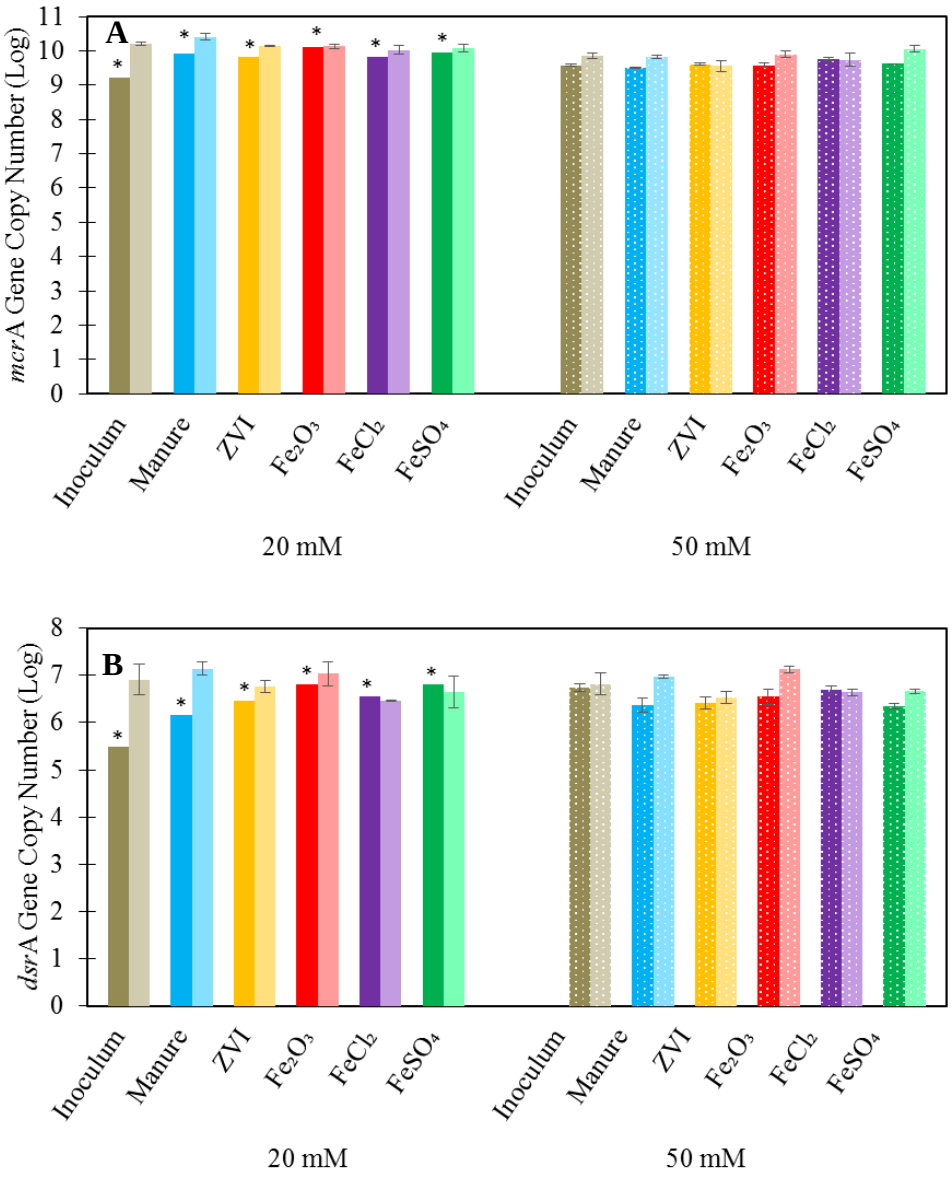

Figure 3.3: A) Log *msr*A and B) log *dsr*A gene copy numbers pre- (dark) and post-digestion (light) from BMP1 (20 mM) and BMP2 (50 mM). Treatments for pre-BMP1 (*) were not replicated, post-BMP1 and pre-BMP2 were conducted in triplicate and post-BMP2 was conducted in duplicate with error bars representing ± standard error.

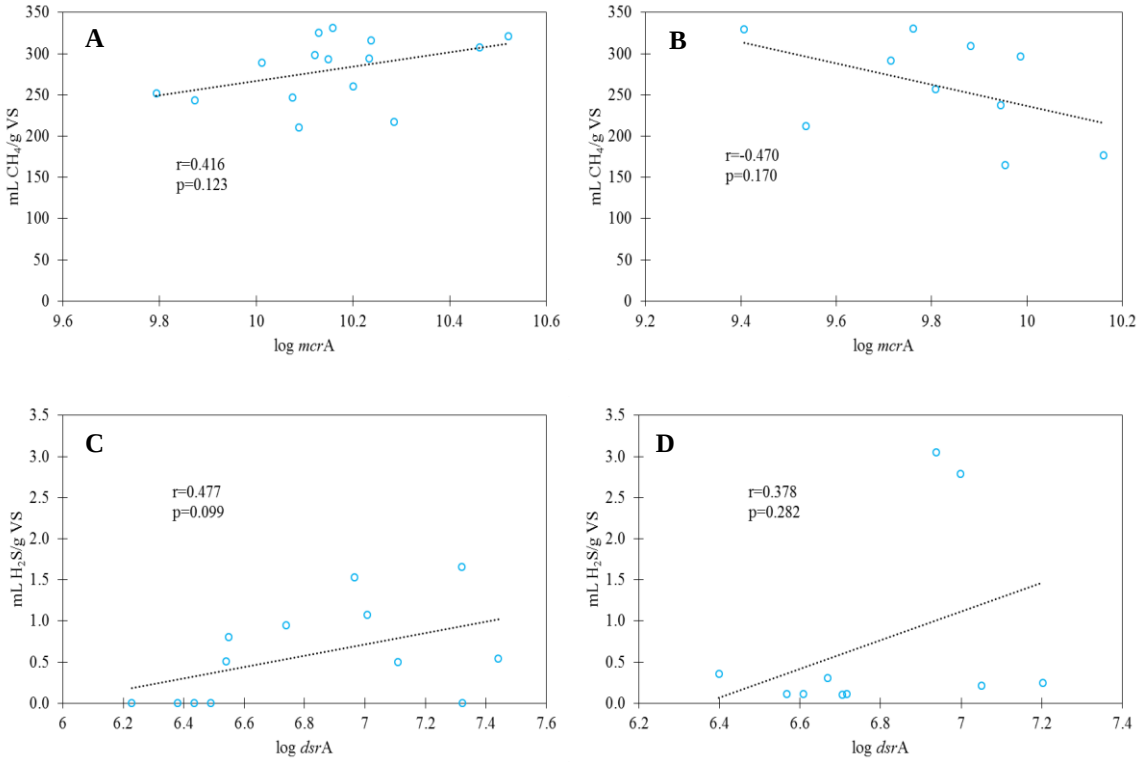

Figure 3.4: Correlations between log mcrA numbers in post-digestion treatments and cumulative CH_4 produced for A) BMP1 and C) BMP2 and correlations between log dsrA numbers in post-digestion treatments and cumulative H_2S production in C) BMP1 and D) BMP2. Data includes un-amended manure treatment and iron treatments.

3.3.2.3 *mcrA vs dsrA*

There was a significant positive correlation between *mcrA* gene copy numbers and *dsrA* gene copy numbers in post-digestion BMP1 samples, with a r value of 0.617 (p-value = 0.025). In BMP2 post-digestion samples, *mcrA* gene copy numbers and *dsrA* gene copy numbers were not significantly correlated (r value = 0.045, p-value = 0.902) (Figure 3.5).

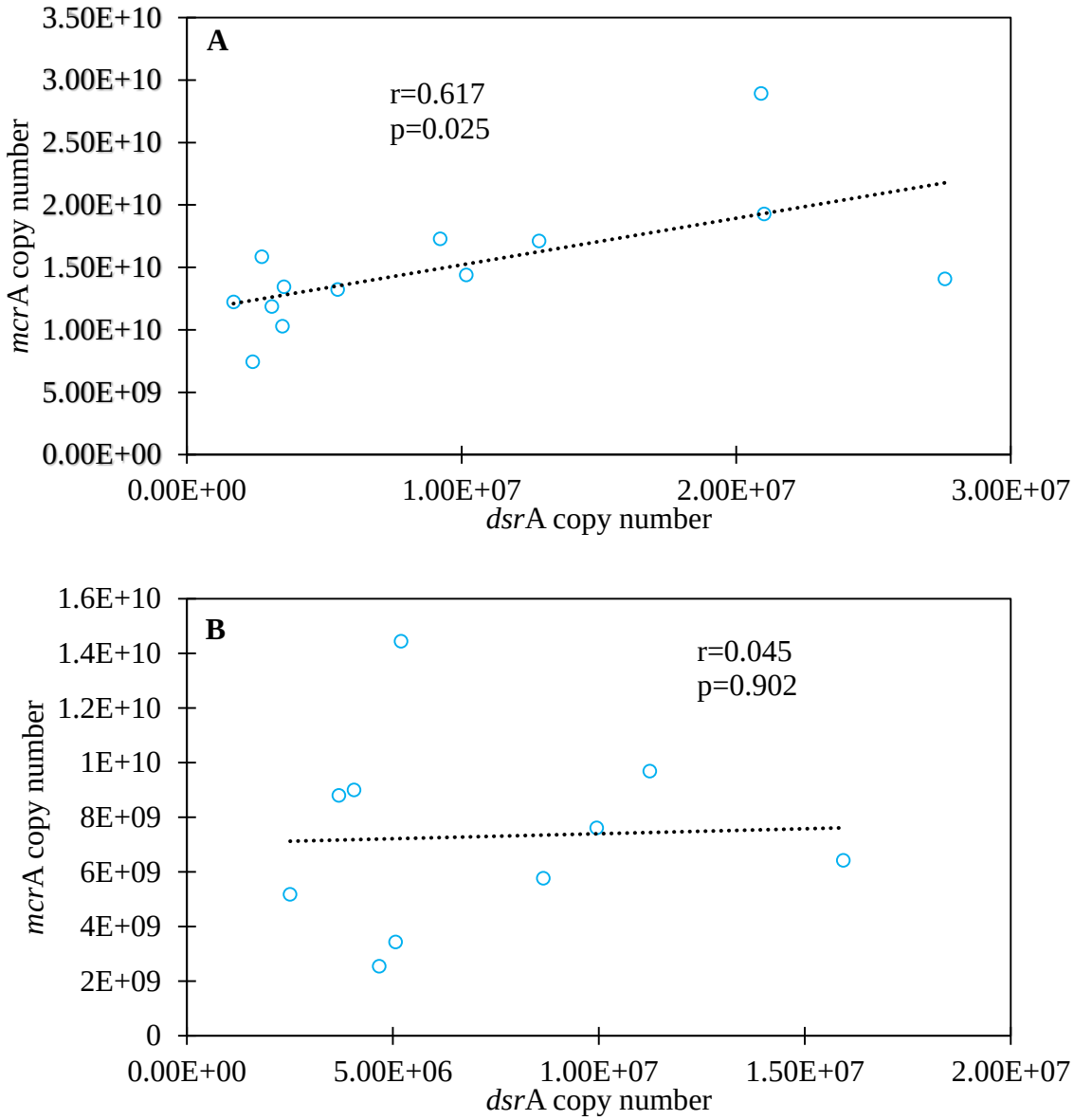

Figure 3.5: Correlation between mcrA and dsrA gene copy numbers in A) BMP1 and B) BMP2. Correlations include un-amended manure treatment and iron treatments. In BMP1, treatments were conducted in triplicate and in BMP2, treatments were in duplicate.

3.3.3 Effect of Iron Addition on Nutrients

The effect of iron addition on nutrient contents in the samples was determined for the 50 mM iron addition BMP test (Table 3.5). There was no significant difference in ammonium (NH_4^+) concentrations between the un-amended manure and iron treatments in post-digestion samples (p-values: 0.254 to 0.936), with increases in NH_4^+ between pre- and post-digestion from 1% to 17%. The $FeCl_2$ and $FeSO_4$ reduced water soluble PO_4^{3-} concentrations below the detection limit for the duration of the BMP, while the un-amended manure, ZVI, and Fe_2O_3 treatments resulted in an increase in PO_4^{3-} concentrations of 4-, 3-, and 2-fold, respectively. Compared to the un-amended manure treatment, the Fe_2O_3 and ZVI treatments significantly decreased post-digestion water soluble PO_4^{3-} concentrations by 54% and 69% (p-values < 0.001), with the ZVI treatment containing 48% less PO_4^{3-} than the Fe_2O_3 treatment (p-value = 0.0001). The reported PO_4^{3-} data from this study is lower than was determined in separated liquid fraction of dairy manure collected from the same facility (Table 3.5), likely due to sample preparation methods used in this lab, which will be further explored.

Table 3.5: Nutrient concentrations from BMP2. Pre-digestion samples were conducted in triplicate and post-digestion samples were conducted in duplicate, with ± standard error shown. Superscripts indicate significant differences between treatments within columns.

	PO_4^{3-} (mg P/L)		**NH_4^+ (mg N/L)**		**TKN (mg N/L)**	**TKP (mg P/L)**
Treatment	*Initial*	*Final*	*Initial*	*Final*	*Initial*	*Initial*
Manure	8.85 ± 1.75[a]	34.8 ± 0.5[a]	1,280 ± 22[a]	1,420 ± 27[a]	1,900 ± 11[a]	322 ± 3[a]
ZVI	3.45 ± 0.69[b]	10.8 ± 0.4[c]	1,330 ± 16[a]	1,470 ± 50[a]	1,930 ± 16[a]	336 ± 5[a]
Fe_2O_3	8.84 ± 0.79[a]	16.0 ± 0.8[b]	1,280 ± 22[a]	1,290 ± 25[a]	1,928 ± 9[a]	325 ± 1[a]
$FeSO_4$	bdl[1]	bdl[1]	1,190 ± 32[a]	1,390 ± 34[a]	1,940 ± 9[a]	322 ± 2[a]
$FeCl_2$	bdl[1]	bdl[1]	1,300 ± 40[a]	1,370 ± 29[a]	2,180 ± 89[a]	366 ± 15[a]

[1] Below the detection limit

3.4 Discussion

While it was expected that addition of iron would increase CH_4 production in the treatments due to an increase in the addition of a trace nutrient necessary for methanogenesis (Demirel and Scherer, 2011), addition of 20 and 50 mM of ZVI and Fe_2O_3 to manure did not significantly impact the CH_4 production compared to the un-amended manure treatment, and the $FeCl_2$ and $FeSO_4$ treatments significantly reduced CH_4 production. Although iron addition has commonly resulted in an increase in CH_4 production, increases in CH_4 were accompanied by a concurrent increase in methanogen numbers in samples with additional iron (Preeti Rao and Seenayya, 1994; Yang et al., 2013). When decreases in CH_4 were reported, the decrease coincided with cell damage due to iron exposure (Wu et al., 2015) or lower methanogen numbers compared to the control (Yang et al., 2013). Similarly, in BMP1 of this study, the reduction in CH_4 compared to the un-amended manure control due to $FeCl_2$ and $FeSO_4$ addition was most likely due to a decrease in methanogenic activity as indicated by the significant decrease in methanogen numbers in post-digestion Fe(II) samples compared to the un-amended

manure control. In BMP2, however, there was no significant difference in methanogen numbers between the un-amended manure treatment and the $FeCl_2$ and $FeSO_4$ treatments in pre- or post-digestion samples. While the decreased methanogen numbers in BMP1 agrees with the decrease in CH_4, the method for quantifying methanogen numbers in this study does not rely on microbial activity. Therefore, the lack of significant difference in methanogen numbers in BMP2 does not reflect the activity of the methanogens in the samples (Alvarado et al., 2014), as can be seen by the lack of significant correlation between CH_4 production and methanogen numbers in the samples for both BMP1 and BMP2. Other studies have reported a lack of correlation between mcrA gene copy numbers and CH_4 production, concluding that gene copy numbers are only indicative of the total quantity of methanogens, not the community and can also include the presence of dormant or lysed cells (Freitag and Prosser, 2009; Witarsa et al., 2016).

There was also no significant correlation between H_2S and SRB gene copy numbers. The lack of functional correlation between SRB and H_2S production in this study indicates that chemical, not biological, mechanisms were responsible for H_2S reduction by the iron treatments. As was expected, in BMP1, the $FeCl_2$ and $FeSO_4$ treatments removed significantly higher amounts of H_2S than the insoluble ZVI and Fe_2O_3 treatments. Theoretically, with complete solubility in the sample, the 20 mM $FeCl_2$ and $FeSO_4$ iron additions should result in 4 mmol of Fe^{2+} available for H_2S precipitation as FeS (Haaning Nielsen et al., 2005). However, based on reported values of Fe^{2+} in anaerobic samples containing hematite (Chen et al., 2014) and ZVI (Zhang et al., 2011), the addition of 20 mM of Fe_2O_3 and ZVI in this study would have resulted in reductive dissolution of 0.017 and 0.019 mmol of Fe^{2+}, respectively (Table B 3). The theoretical

decreased availability of Fe^{2+} in the Fe_2O_3 and ZVI treatments compared to that in the $FeCl_2$ and $FeSO_4$ treatments would result in significantly less H_2S precipitation, which was observed in this experiment. While the theoretical amount of H_2S precipitated was within 6% of the observed amount for the ZVI treatment and was 77-fold less than the observed amount for the $FeCl_2$ and $FeSO_4$, the observed H_2S removal by Fe_2O_3 was 98% higher than the theoretical calculation (Table B 3). One explanation for the higher observed H_2S removal is that there was a higher amount of Fe^{2+} reductively dissolved from hematite in this study than was reported by Chen et al. (2014), which was used for theoretical calculations in this study. Potential for increased Fe^{2+} concentrations in this study from Fe_2O^3 could be due to differences in the surface area of Fe_2O_3 used or in the microbial populations and redox potential between studies. Another possibility for the increase in observed H_2S removal is that the SRB in the Fe_2O_3 treatment were preferentially reducing Fe^{3+} over sulfate, thus decreasing the amount of H_2S formation in the sample (Zhang et al., 2013). While 20 mM iron was not sufficient to provide adequate Fe^{2+} concentrations in the ZVI and Fe_2O_3 treatments for similar H_2S reduction compared to the $FeCl_2$ and $FeSO_4$ treatments, there was no significant difference in H_2S removal between iron treatments in BMP2. Additionally, in BMP2, the theoretically calculated Fe^{2+} in all iron treatments was between 1.02- to 210-fold higher than the observed H_2S removal, indicating that H_2S removal in BMP2 was likely due to precipitation by solubilized Fe^{2+} for all treatments (Table B 3).

It was expected that SRB and methanogens would compete for the same substrates, which would lead to an inverse relationship between methanogens and SRB; however, contrary to what was expected, there was a significant correlation between the post-

digestion methanogen and SRB numbers in BMP1 of this study. Shu et al. (2015) also observed a positive correlation between SRB and methanogens and attributed the correlation to the potential for the SRB to utilize propionate over organics preferred by methanogens. While the positive correlation in BMP1 also indicates that the addition of each iron compound had a similar effect on methanogens and SRB, there was no correlation between SRB and methanogen numbers in BMP2. It is more likely that in BMP1, both methanogens and SRB became substrate limited at the end of the BMP, as indicated by a complete reduction in TVFAs. However, in BMP2, acetic acid was the only remaining VFA in the samples after digestion, indicating that while methanogens that utilize acetic acid had substrate remaining; the propionate degrading SRB could have become substrate limited, resulting in a lack of correlation in SRB and methanogen numbers.

Addition of 50 mM iron, regardless of compound, significantly decreased PO_4^{3-} concentrations compared to the un-amended manure control in post-digestion samples, as was expected (Roussel and Carliell-Marquet, 2016). There was likely a preferential removal of sulfides prior to PO_4^{3-} precipitation, as vivianite, in the anaerobic treatment, which resulted in similar H_2S removal with 50 mM iron addition but varying concentrations of Fe^{2+} remaining for PO_4^{3-} precipitation based on the iron compound added (Roussel and Carliell-Marquet, 2016). Theoretically, after accounting for H_2S precipitation, the Fe_2O_3 and ZVI treatments contained between three and four orders of magnitude less Fe^{2+} for PO_4^{3-} precipitation than the $FeCl_2$ and $FeSO_4$ treatments (calculated based on the reductive Fe^{2+} dissolution from the Fe_2O_3 and ZVI treatments possible (Chen et al., 2014; Zhang et al., 2011) (Table B 4). The trends in theoretically

calculated PO_4^{3-} precipitation were in agreement with the observed post-digestion PO_4^{3-} concentrations, with Fe_2O_3 treatment > ZVI treatment > $FeCl_2$ and $FeSO_4$ treatments (Table 3.5).

Typically, after digestion, the digestate is either directly spread on fields as manure, or treated via solid-liquid separation before being land applied (Möller and Müller, 2012). However, potential drawbacks to land application of digestate include ammonia volatilization and nutrient runoff (Nkoa, 2013). While the addition of iron did not reduce the NH_4^+ concentration compared to the un-amended manure treatment in this study, water soluble PO_4^{3-} in post-digestion samples was significantly reduced by 54 to 100%, indicating that iron addition could provide a partial solution to the environmental issue of nutrient runoff via the precipitation of PO_4^{3-} to vivianite (Roussel and Carliell-Marquet, 2016). Additionally, application of iron-containing digestate can increase the soil phosphorus storage capacity in soils providing additional sites for phosphorus storage, decreasing the loss of phosphorus from soils to surface waters (Lu et al., 2012).

While the decrease of water soluble PO_4^{3-} is beneficial from an environmental standpoint, the removal of PO_4^{3-} ions from digestate decreases the immediate availability of phosphorus to plants, as plants only absorb the inorganic ionized forms of phosphorus (Holford, 1997). Even though iron reduces the water extractable portion of bioavailable phosphorus, Bachmann et al. (2016) reported that 70 to 90% of total phosphorus content of digestate from manure digestion was in a bioavailable form, indicating abundance of remaining phosphorus for plant uptake in iron-amended digestate, while possibly binding the more mobile PO_4^{3-} ions in the digestate into iron precipitates.

3.5 Conclusions

In this study, the effects of iron addition to dairy manure on the biogas quality, methanogen and SRB numbers, and plant-available nutrient content were determined using BMP testing. While the addition of $FeCl_2$ and $FeSO_4$ resulted in 96% to 100% removal of H_2S from the biogas, CH_4 production was also significantly reduced, decreasing the energy content of the biogas. Addition of $FeCl_2$ and $FeSO_4$ also resulted in significant decrease PO_4^{3-}, which reduces the quality of the digestate as a soil amendment. The addition of Fe_2O_3 and ZVI had no significant effect on CH_4 production and the Fe_2O_3 resulted in 66% and 92% higher H_2S removal than the ZVI in BMP1 and BMP2, respectively. The minimum average H_2S concentration of 204 ppm in BMP2 in the Fe_2O_3 treatment is below the corrosion requirement for most engine generation equipment (300-500 ppm). While the Fe_2O_3 treatment did result in a significant reduction in PO_4^{3-} concentrations post-digestion compared to the manure treatment, it yielded the smallest PO_4^{3-} reductions of the iron treatments, with 48% more PO_4^{3-} than the ZVI treatment. The results of this study indicate that 50 mM Fe_2O_3 is the most appropriate iron addition for improvement of biogas quality and increased PO_4^{3-} precipitation leading to possible decreased runoff of PO_4^{3-} to adjacent waterways from addition of digestate to soils.

4 Dairy Manure as Substrate for Anaerobic Digestion of High Sulfate Wastewaters

4.1 Introduction

High sulfur waste streams are produced from the mining industry (Akcil and Koldas, 2006; Cocos et al., 2002) and various industrial processes (Hao et al., 2014; Hulshoff Pol et al., 1998). Treatment of these waste streams to reduce sulfate (SO_4^{2-}) prior to migration into the groundwater flow system or discharge to surface waters is necessary. Both chemical and biological methods have been used for remediation of high SO_4^{2-} waste streams. Biological treatment of high SO_4^{2-} waste streams is dependent upon the use of anaerobic sulfate reducing bacteria (SRB), which reduce SO_4^{2-} to sulfides that can subsequently precipitate the metal ions also known to be prevalent in such waste streams (Gibert et al., 2004). Choice of biotechnology, SRB source, and carbon addition all impact SO_4^{2-} removal in high SO_4^{2-} waste streams (Hao et al., 2014).

Typical biotechnologies that are employed for treatment of high SO_4^{2-} waste streams include treatment wetlands, permeable reactive barriers, denitrifying sulfide removal processes, and anaerobic digestion (AD). Anaerobic digestion configurations most commonly utilized for high sulfate wastewaters include two-phase reactors: a separate acidogenic sulfate removal reactor coupled with a methanogenic reactor (Wei et al., 2007; Zhang et al., 2013), or an up flow anaerobic sludge blanket (UASB) reactor (Liu et al., 2015; Lu et al., 2016; Patidar and Tare, 2004; Zhang et al., 2011). One of the benefits of using AD for SO_4^{2-} treatment over the other biotechnologies is the potential for high energy biogas production; however, competition of SRB with methanogens

could decrease the suitability of the biogas for use in electrical generators without further treatment.

When SO_4^{2-} is available as an electron acceptor in a digester, the acidogenic, acetogenic and methanogenic microorganisms compete with sulfate reducing bacteria (SRB) for available electron donors. Within a digester, SRB can outcompete methanogens for available substrates, as sulfate reduction is more energetically favorable. Manipulation of the chemical oxygen demand to SO_4^{2-} ($COD:SO_4^{2-}$) ratio in digesters has shown to impact the biogas production, with lower ratios typically, but not always, resulting in higher sulfide production and less methane (CH_4) production (Hulshoff Pol et al., 1998). Theoretically, a $COD:SO_4^{2-}$ ratio less than 0.67 indicates an excess of SO_4^{2-}, meaning that all COD removal in an AD could be achieved through SO_4^{2-} reduction. A $COD:SO_4^{2-}$ greater than 0.67 indicates that the influent is SO_4^{2-} limited and methanogens can use the extra substrate for CH_4 production and COD removal (Patidar and Tare, 2004).

While previous studies have indicated that low $COD:SO_4^{2-}$ ratios in fixed film reactors treating sulfate rich waste have enabled methanogens to outcompete SRB (Chou et al., 2008; de Smul et al., 1999; McCartney and Oleszkiewicz, 1993; O'Flaherty et al., 1998), in practice, anaerobic treatment is typically sucessful when the $COD:SO_4^{2-}$ ratio is grater than 10 (Hulshoff Pol et al., 1998). In an UASB reactor treating synthetic wastewater, a decrease in the $COD:SO_4^{2-}$ ratio from 16.7 to 4.5 resulted in decreased COD removal from 85.1% to 58.2%, increased effluent undissociated hydrogen sulfide (H_2S) production from 15 mg/L to 70 mg/L, and decreased CH_4 production from 11 L/d to 5.6 L/d (Zhang et al., 2011). Patidar and Tare (2004) found that SO_4^{2-} addition to

synthetic jaggery wastewater in three different reactor types: an UASB, an anaerobic batch reactor, and a hybrid anaerobic baffled reactor, resulting in a COD:SO_4^{2-} of 6.9-7.0, inhibited CH_4 production and COD removal while increasing H_2S concentrations when compared to the systems without SO_4^{2-} addition. The use of AD for treating synthetic substrates high in SO_4^{2-} has resulted in a decrease in CH_4 production due to sulfide toxicity (Hulshoff Pol et al., 1998; McCartney and Oleszkiewicz, 1993); however, most high SO_4^{2-} waste streams are also high in heavy metals, which precipitate out the sulfides in the digester. Previous studies have determined how increasing SO_4^{2-} concentrations in the influent of a reactor degrading synthetic wastewater impacts digestion under a range of COD:SO_4^{2-} ratios; however, few studies have determined how the addition of SO_4^{2-} to a complex organic, such as dairy manure, changes the outputs of AD compared to the baseline conditions with no SO_4^{2-} additon.

Research has been conducted to determine beneficial organic substrates for SO_4^{2-} reduction by SRB from natural systems in order to increase the efficiency and economic feasibility of biological SO_4^{2-} reduction using agricultural and food processing wastes (Choudhary and Sheoran, 2012; Gibert et al., 2004; Hao et al., 2014). However, investigation of a beneficial substrate for SO_4^{2-} reduction in AD has not been the focus of previous studies. Gibert et al. (2004) determined that sheep manure was the most successful organic substrate in promoting sulfidogenesis with > 99% SO_4^{2-} removal when compared to compost, poultry manure, and oak leaf, and using creek sediment as a SRB source. Similarly, Choudhary and Sheoran (2012) found that cow, goat, or buffalo manure were more promising substrates when used as a single substrate for SRB incubated from cow manure when compared to more cellulosic substrates including

woodchips, sawdust and millet fodder. When sawdust, poultry manure, and cow manure were utilized as substrates for SRB isolated from urban soil in batch tests, poultry manure provided the most suitable organic material for SRB utilization with SO_4^{2-} reduction of 79% (Zhang and Wang, 2014). Previous studies have thus shown that animal manure is the preferred organic material for SRB harvested from various natural environments, but none of the studies have coupled the use of separated dairy manure as a substrate for SRB in an AD using inoculum acclimated to dairy manure.

The objectives of this study were to determine the effects of SO_4^{2-} and S^0 addition on CH_4 and H_2S production and SO_4^{2-} reduction when using separated dairy manure as an organic substrate for SRB in AD inoculum acclimated to dairy manure substrate. The use of AD for treatment of high SO_4^{2-} waste can potentially provide energy recovery in the form of biogas. The effects of sulfide toxicity were also examined by the addition of SO_4^{2-} with and without ferrous iron. It was hypothesized that the addition of K_2SO_4 would result in decreased CH_4 production compared to addition of $FeSO_4$ at the same molar concentration of SO_4^{2-} due to decreased H_2S toxicity via sulfide precipitation by Fe^{2+}. In this study, a biochemical methane potential (BMP) test was used to determine the impacts on CH_4 and H_2S production and SO_4^{2-} reduction when two different SO_4^{2-} salts (potassium sulfate (K_2SO_4) and iron sulfate ($FeSO_4$)) and elemental sulfur (S^0) were added to separated dairy manure at varying COD: SO_4^{2-} ratios.

4.2 Material and Methods

4.2.1 Inoculum and Substrate Utilized

Inoculum and separated manure were collected in five-gallon buckets from the USDA Beltsville Agricultural Research Center (BARC) dairy farm, located in Beltsville, Maryland, USA. The inoculum was piped from the center of a full-scale AD treating separated dairy manure. Inoculum and manure were stored at 4 °C directly after collection until use in biochemical methane potential (BMP) test.

4.2.2 Experimental Design

Two BMP tests were conducted to determine the effects of SO_4^{2-} addition on batch digestions with separated dairy manure as the substrate. All chemicals were of analytical grade and were supplied by Fisher Scientific. In BMP1, K_2SO_4 was added at varying masses to achieve a range of COD: SO_4^{2-} ratios surrounding 10, and in BMP2, $FeSO_4$ and elemental sulfur (S^0) were added to determine the differences in SO_4^{2-} versus S^0 addition on biogas and water quality (Table 4.1). The purpose of adding S^0 to the dairy manure was to separate out the effects of S^0 versus SO_4^{2-} on digestion parameters. It should be noted that adding S^0 did not decrease the COD:SO_4^{2-} ratio since in anaerobic environments S^0 is not chemically or biologically oxidized to SO_4^{2-}. Each SO_4^{2-} treatment was conducted in triplicate, with the SO_4^{2-} salt or S^0 added to 50 mL of deionized water in a 250-mL glass bottle and mixed. Inoculum and manure were added to each treatment at a 2:1 inoculum to manure ratio by volatile solids (VS). A manure treatment without the addition of SO_4^{2-} and an inoculum control were also tested. In BMP1, each treatment contained 50 mL of deionized water, 29 mL of manure (1.00 g VS), and 121 mL of

inoculum (2.01 g VS). In BMP2, each treatment contained 50 mL of deionized water, 31 mL manure (1.19 g VS), and 119 mL of inoculum (2.37 g VS). The inoculum control did not contain manure in either BMP test.

4.2.3 Biochemical Methane Potential (BMP) Test

Anaerobic incubation was conducted using a BMP protocol developed by Owen et al. (1979) and adapted by Moody et al. (2011). BMP experiments were used to determine the energy production potential, defined as CH_4 production, and H_2S concentration of the biogas. The BMP tests were conducted until the CH_4 production in each treatment stabilized. Prior to incubation, the filled BMP bottles were flushed with 70:30 N_2:CO_2 for 3 minutes to ensure anaerobic conditions. The bottles were then capped with a rubber septum and placed on a shaker (New Brunswick™ innOva® 2300, Hamburg, Germany) at 110 rpm in a temperature controlled incubator at 35 °C.

Biogas production was quantified volumetrically using a glass, gas-tight syringe equilibrated to atmospheric pressure. Biogas samples were analyzed for CH_4 and H_2S composition using a gas chromatograph (Agilent Technologies, Inc.; Shanghai China; model 7890 A), with a thermal conductivity detector at 250°C, a HP-Plot Q capillary column (Agilent J&W; USA), helium as the carrier gas at 8.6 ml/min, and the oven operated at 60°C for 2 min and subsequently ramped at 30°C/min to 240°C. Biogas production and CH_4 and H_2S content were measured daily during the first week of the experiment, approximately every other day the following week, and then as needed for the remainder of the BMP experiments, with measurement frequency based on the quantity of biogas produced.

To account for biogas production from residual biodegradable material in the inoculum, inoculum controls were incubated using the same conditions for each inoculum source and sampled simultaneously to allow for subtraction of biogas production not attributed to the manure. Methane production was then normalized by the mass of VS of manure added to each treatment to determine the efficiency of the inoculum to utilize the organics in the treatments.

4.2.4 Analytical Methods

The total and volatile solids of the inoculum and manure were characterized prior to BMP loading. Samples were analyzed for pH, oxidation reduction potential (ORP), soluble chemical oxygen demand (sCOD) and sulfate (SO_4^{2-}). The pH was determined with an Accumet AB 15 pH meter. The ORP was determined using a YSI Quatro Professional meter with a 1002 ORP probe. Total solids (TS) (APHA Method 2540B) and VS (APHA Method 2540E) were determined using the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). HACH method 8000 was used to determine sCOD and samples for sCOD were filtered through 0.45 µm nitrocellulose membrane filter. Sulfate was analyzed on samples filtered through 0.22 µm nitrocellulose membrane filters on an 850 Professional IC Autosampler (Metrohm USA, Inc., Riverview, FL) with a METROSEP A Supp 5-150/4.0 separation column and 20 µL injection.

4.2.5 Statistical Analysis

Statistical analysis was conducted using SAS 9.3 (SAS, Cary, NC). Each experiment was a single factor, completely randomized design. One-way ANOVA analysis was

performed to assess significant differences between cumulative CH_4 and H_2S production between treatments and differences between water quality parameters for all treatments at the two sampling points (pre- and post-digestion). Pearson correlation analysis was utilized for correlations between COD:SO_4^{2-} ratio in treatments and CH_4 production, H_2S production, sCOD removal, and SO_4^{2-} removal. The level of significance was held at an alpha of 0.05.

Table 4.1: Sulfate and elemental sulfur additions for BMP1 and BMP2.

Treatment	**COD:SO_4^{2-}**	**Influent Sulfate (mg SO_4^{2-}/L)**
	BMP1	
Inoculum Control	NA	40
Manure	278	75
31 mM K_2SO_4	6.40	3020
27 mM K_2SO_4	7.44	2620
21 mM $FeSO_4$	10.7	2060
21 mM K_2SO_4	10.2	2030
18 mM K_2SO_4	13.6	1770
16 mM K_2SO_4	13.7	1510
	BMP2	
Inoculum Control	NA	30
Manure Control	346	77
50 mM $FeSO_4$	5.59	5,140
20 mM $FeSO_4$	12.0	2110
5 mM $FeSO_4$	72.0	519
2 mM $FeSO_4$	86.0	288
50 mM S^0	129	203
20 mM S^0	343	79
5 mM S^0	449	68
2 mM S^0	484	62

4.3 Results

4.3.1 Effects of SO_4^{2-} and S^0 Addition on Biogas Quality

Addition of K_2SO_4 at all SO_4^{2-} concentrations significantly decreased CH_4 production from 29% to 37% compared to the un-amended dairy manure treatment (p-values: 0.001 to 0.007) (Figure 4.1 and Table 4.2). There was no significant difference in CH_4 production among the K_2SO_4 treatments, with CH_4 production ranging from 201 to 227 mL CH_4/g VS (p-values: 0.748 to 1.00). The addition of 21 mM $FeSO_4$ yielded a similar CH_4 reduction as the 21 mM K_2SO_4 treatment (p-value = 1.00), producing 34% less CH_4 than the un-amended manure treatment (p-values = 0.002).

There was no significant difference between the 2 mM $FeSO_4$ (247 mL CH_4/g VS) and S^0 (246 mL CH_4/g VS) treatments and the un-amended manure treatment (286 mL CH_4/g VS) (p-values < 0.001 to 0.067) (Figure 4.1 and Table 4.2). However, the 5 mM $FeSO_4$ treatment (227 mL CH_4/g VS) produced 11% less CH_4 than the 5 mM S^0 treatment (255 mL CH_4/g VS) (p-value = 0.333), with no significant difference between the 5 mM S^0 treatment and the un-amended manure treatment (p-value = 0.247). Both the 20 mM and 50 mM $FeSO_4$ and S^0 treatments significantly reduced CH_4 production by 26 to 65% compared to the un-amended manure treatment (p-values < 0.001), with methane production between 99.3 and 212 mL CH_4/g VS. The 20 mM $FeSO_4$ treatment produced 23% less CH_4 than the 20 mM S^0 treatment (p-value = 0.012), however there was no significant difference between the two 50 mM sulfur additions (p-value = 0.999).

As expected, SO_4^{2-} addition in the form of K_2SO_4 significantly increased H_2S production 3.4- to 3.7-fold with respect to the un-amended manure treatment (p-values <

0.0001). The 21 mM $FeSO_4$ treatment significantly reduced H_2S production by 96% compared to the un-amended manure treatment (p-value = 0.002) (Figure 4.2 and Table 4.2).

While the 20 mM and 50 mM S^0 treatments significantly increased H_2S production compared to the un-amended manure treatment, 12- and 28-fold, respectively (p-values < 0.0001), the 5 and 2 mM S^0 treatments resulted in no significant difference in H_2S production compared to the un-amended manure treatment (p-values = 0.116 and 0.949, respectively). The $FeSO_4$ treatments all produced significantly less H_2S than the un-amended manure treatment, with the decrease in H_2S production ranging from 44% to 96% (p-values < 0.0001) (Figure 4.2 and Table 4.2).

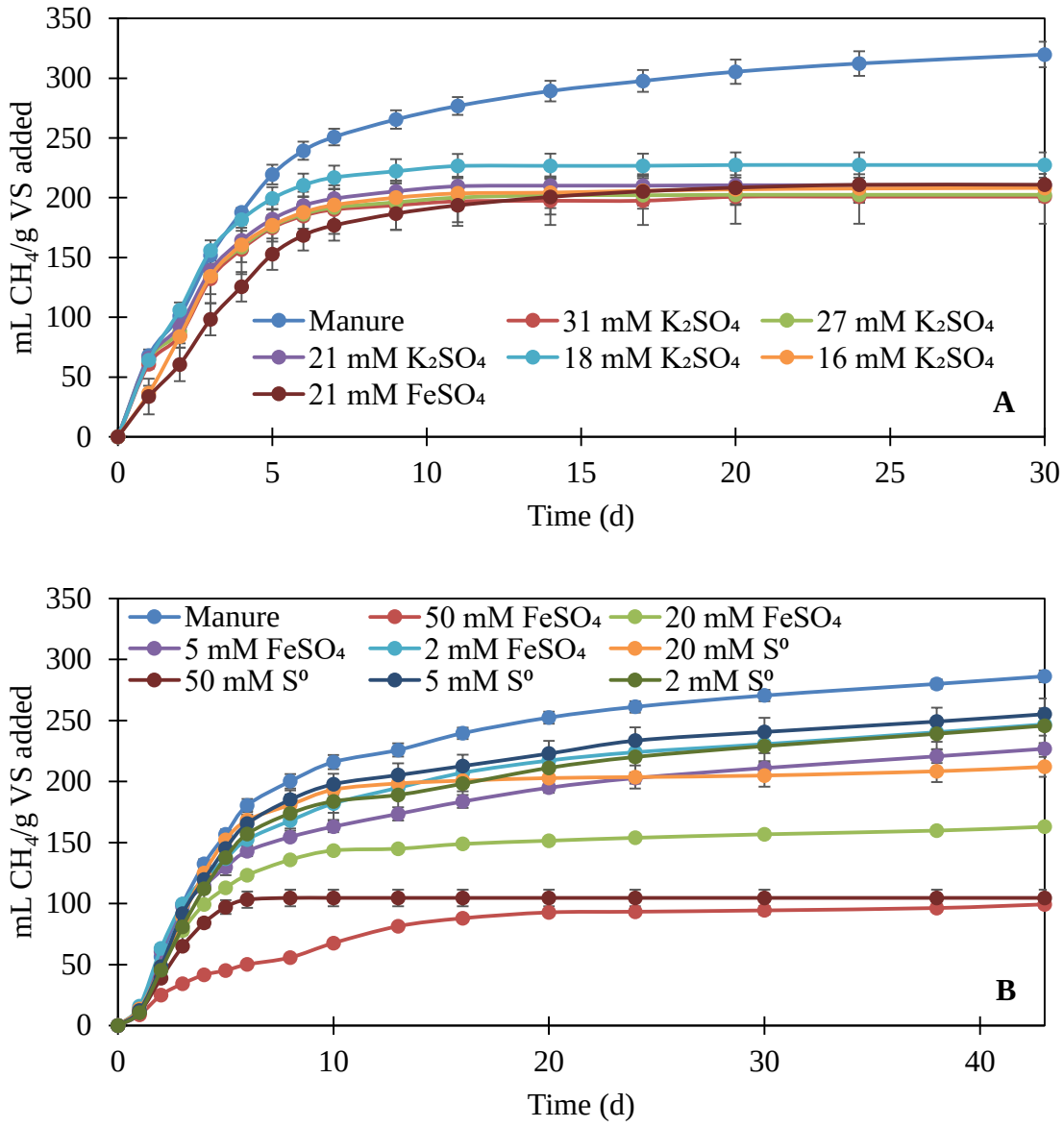

Figure 4.1: Cumulative CH_4 production in A) BMP1 and B) BMP2. Treatments were conducted in triplicate with error bars representing ± standard error.

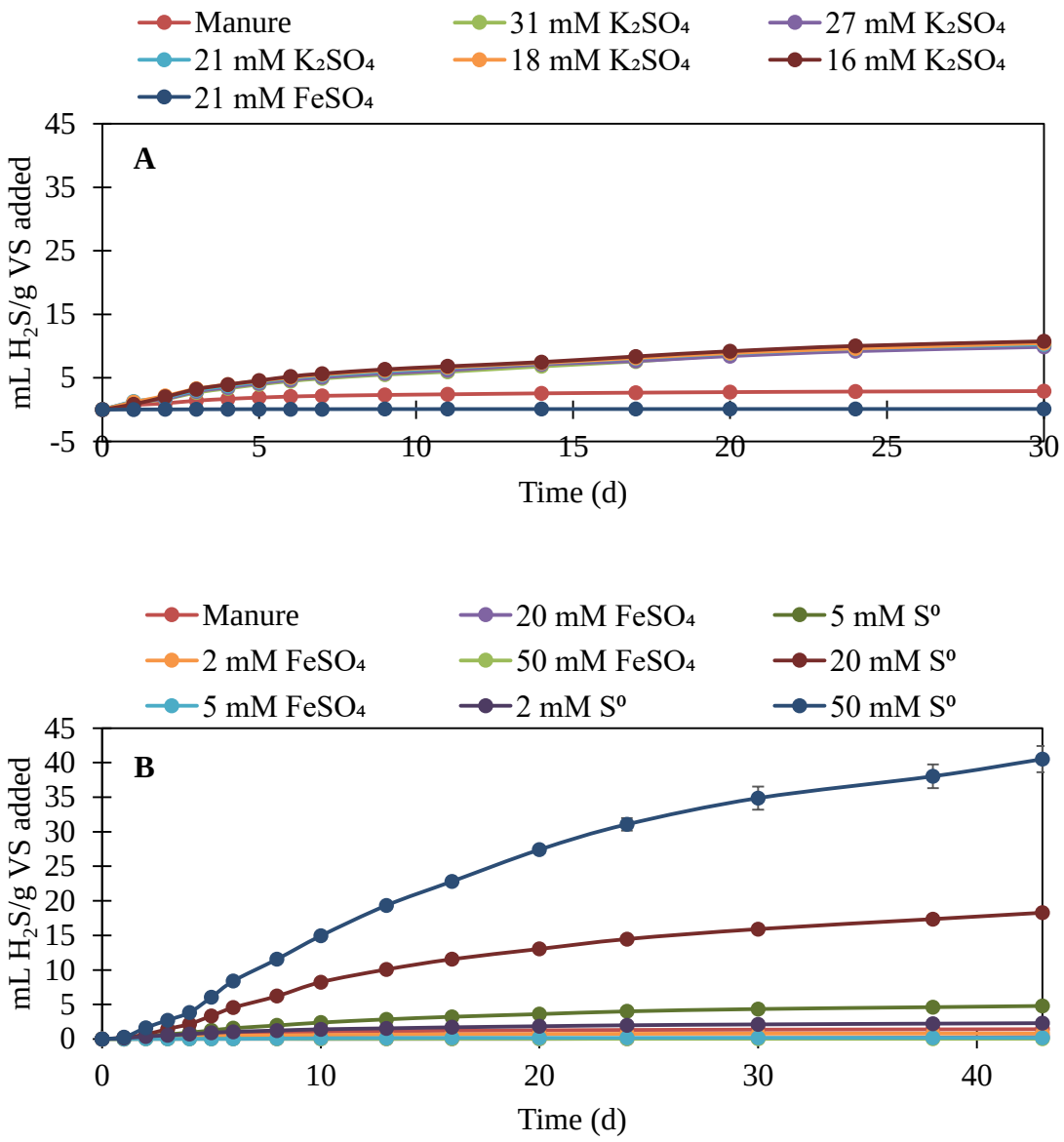

Figure 4.2: Cumulative H_2S production in A) BMP1 and B) BMP2. Treatments were conducted in triplicate with ± standard error shown.

Table 4.2: Average cumulative CH_4 and H_2S production from BMP1 and BMP2. Treatments were conducted in triplicate with ± standard error shown.

Treatment	CH_4 (mL/g VS)	H_2S (mL/g VS)
	BMP1	
Manure	320 ± 11	2.92 ± 0.13
31 mM K_2SO_4	201 ± 23	10.1 ± 0.7
27 mM K_2SO_4	202 ± 4	9.88 ± 0.27
21 mM K_2SO_4	210 ± 3	10.3 ± 0.25
18 mM K_2SO_4	227 ± 11	10.5 ± 0.33
16 mM K_2SO_4	208 ± 12	10.8 ± 0.08
21 mM $FeSO_4$	211 ± 14	0.109 ± 0.0005
	BMP2	
Manure	286 ± 5	1.42 ± 0.03
50 mM $FeSO_4$	99.3 ± 3.0	0.054 ± 0.002
20 mM $FeSO_4$	163 ± 1	0.308 ± 0.011
5 mM $FeSO_4$	227 ± 4	0.181 ± 0.004
2 mM $FeSO_4$	247 ± 9	0.852 ± 0.017
50 mM S^0	104 ± 7	40.5 ± 1.9
20 mM S^0	212 ± 8	18.3 ± 0.4
5 mM S^0	255 ± 13	4.83 ± 0.03
2 mM S^0	246 ± 14	2.30 ± 0.22

4.3.2 Effect of SO_4^{2-} and S^0 Addition on pH, ORP, and sCOD and SO_4^{2-} Removal

In BMP1, the post-digestion pH of the un-amended manure, K_2SO_4, and $FeSO_4$ treatments was between 7.26 and 7.48 and in BMP2, the pH of the un-amended manure, $FeSO_4$ and S^0 treatments was between 7.00 and 7.35. The pH of all samples was within the ideal pH range for methanogenesis of 6.5 to 8.2 (Lee et al., 2009). The ORP in BMP1

post-digestion samples was between -32 and -385 mV and in BMP2 post-digestion samples ranged from -332 to -426 mV (Table 4.3 and Table 4.4).

There was no significant difference in pre- (p-values from 0.218 to 1.00) or post-digestion (p-values: 0.061 to 1.00) sCOD concentrations between all of the treatments, indicating that the addition of SO_4^{2-} in the form of K_2SO_4 and $FeSO_4$ did not significantly affect the removal of sCOD when compared to the un-amended manure treatment. The sCOD removal for the K_2SO_4 treatments ranged from 38% to 45%, with the un-amended manure treatment having the highest sCOD removal (51%) and the 21 mM $FeSO_4$ treatment yielding the least sCOD removal (24%) (Table 4.3).

The SO_4^{2-} removal for the K_2SO_4 treatments was between 53% and 93%, while the un-amended manure treatment yielded a SO_4^{2-} removal efficiency of 86%. Interestingly, the 21 mM $FeSO_4$ treatment yielded 2-fold less SO_4^{2-} removal than the 21 mM K_2SO_4 treatment (Table 4.3).

As expected, there was no significant difference in pre-digestion sCOD concentrations among all treatments (p-values from 0.516 to 1.00). However, the sCOD concentration in the 50 mM S^0 treatment post-digestion was significantly higher than the other treatments (p-values < 0.001), yielding 27% sCOD removal. The 50 mM $FeSO_4$ and 20 mM S^0 treatments yielded significantly higher post-digestion sCOD values than the un-amended manure treatment (p-values = 0.001 and 0.038, respectively), with sCOD removals of 64% and 47%, respectively. There was no significant difference in post-digestion sCOD concentrations between the 2, 5, and 20 mM $FeSO_4$, 2 and 5 S^0, and un-amended manure treatments (p-values from 0.202 to 1.00), with sCOD reductions from 54% to 69% (Table 4.4).

The SO_4^{2-} reduction for treatments with 2, 5, 20, and 50 mM $FeSO_4$ ranged from 48% to 95%. In the S^0 treatments, SO_4^{2-} reductions ranged from 87% to 91%, which were all higher than the SO_4^{2-} removal for the un-amended manure treatment of 82% (Table 4.4).

Table 4.3: Average pre- and post-digestion pH and ORP and sCOD and SO_4^{2-} removal from BMP1. Treatments were conducted in triplicate and ± standard is shown.

	pH		**ORP (mV)**		**sCOD**	**SO_4^{2-}**
Treatment	*Initial*	*Final*	*Initial*	*Final*	**Removal**	**Removal**
Manure	7.30 ± 0.01	7.43 ± 0.04	-164 ± 11	-198 ± 0.5	51%	87%
31 mM K_2SO_4	7.43 ± 0.02	7.43 ± 0.01	-171 ± 11	-385 ± 14	38%	53%
27 mM K_2SO_4	7.36 ± 0.01	7.47 ± 0.01	-174 ± 3	-383 ± 5	42%	69%
21 mM K_2SO_4	7.32 ± 0.03	7.45 ± 0.02	-163 ± 10	-350 ± 5	45%	80%
18 mM K_2SO_4	7.37 ± 0.01	7.46 ± 0.02	-175 ± 6	-337 ± 1	43%	93%
16 mM K_2SO_4	7.34 ± 0.02	7.46 ± 0.02	-300 ± 29	-333 ± 2	43%	93%
21 mM $FeSO_4$	7.21 ± 0.01	7.26 ± 0.02	-224 ± 8	-32.0 ± 5.7	24%	40%

Table 4.4: Average pre- and post-digestion pH and ORP and sCOD and SO_4^{2-} removal from BMP2. Treatments were conducted in triplicate and ± standard is shown.

Treatment	pH		ORP		sCOD Removal	SO_4^{2-} Removal
	Initial	Final	Initial	Final		
Manure	7.49 ± 0.01	7.31 ± 0.01	-67.3 ± 35.6	-354 ± 9	65%	81%
2 mM $FeSO_4$	7.43 ± 0.01	7.30 ± 0.01	-69.7 ± 11.5	-341 ± 18	62%	95%
5 mM $FeSO_4$	7.34 ± 0.01	7.31 ± 0.01	-164 ± 15	-351 ± 5	61%	93%
20 mM $FeSO_4$	7.17 ± 0.02	7.18 ± 0.03	-186 ± 13	-384 ± 5	64%	82%
50 mM $FeSO_4$	7.01 ± 0.01	7.00 ± 0.00	-79.3 ± 7.4	-332 ± 8	54%	48%
2 mM S^0	7.48 ± 0.01	7.30 ± 0.03	-35.7 ± 11.1	-384 ± 4	64%	87%
5 mM S^0	7.49 ± 0.02	7.31 ± 0.02	-62.3 ± 40.9	-394 ± 7	69%	88%
20 mM S^0	7.50 ± 0.02	7.23 ± 0.03	-105 ± 60	-425 ± 5	47%	87%
50 mM S^0	7.52 ± 0.03	7.12 ± 0.01	-41.7 ± 8.4	-426 ± 2	27%	91%

4.3.3 Effect of COD:SO_4^{2-} Ratio on CH_4 and H_2S Production and sCOD and SO_4^{2-} reduction

The data from both BMP1 and BMP2 were concatenated and correlation between the COD:SO_4^{2-} ratio of each treatment and resulting cumulative CH_4 and H_2S production and sCOD and SO_4^{2-} reduction were determined (Figure 4.3). The correlation between COD:SO_4^{2-} and H_2S was conducted without the $FeSO_4$ treatments due to the precipitation of H_2S by ferrous iron.

There was a significant weak positive correlation between COD:SO_4^{2-} ratio on CH_4 production, with a Pearson correlation coefficient (r) of 0.497 (p-value = 0.0004). There was no significant correlation between COD:SO_4^{2-} ratio and H_2S production with a r of -0.288 (p-value = 0.110). There was also a significant weak positive correlation between COD:SO_4^{2-} ratio and sCOD removal and SO_4^{2-} removal, with r-values of 0.382 and 0.348, respectively (p-values = 0.008 and 0.017).

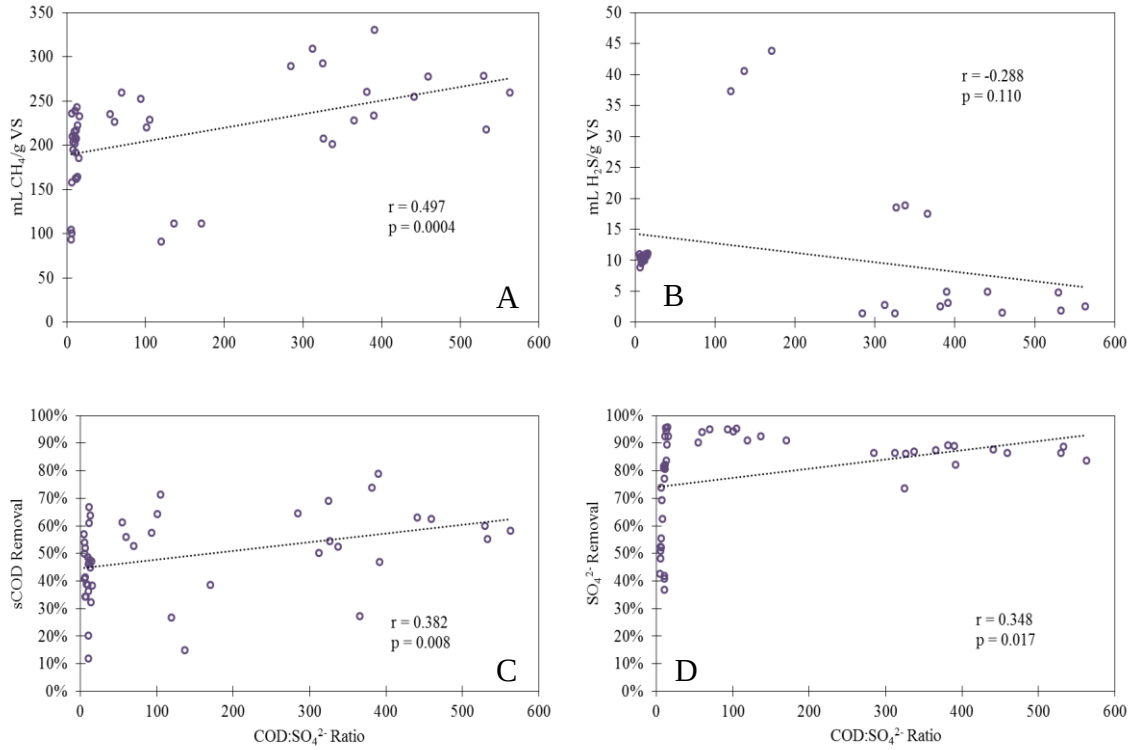

Figure 4.3: Correlation between COD:SO_4^{2-} ratios and A) cumulative normalized CH_4 production, B) cumulative normalized H_2S prodution, C) sCOD removal, and D) SO_4^{2-} removal. Correlations between COD:SO_4^{2-} ratios and CH_4 production, sCOD removal, and SO_4^{2-} removal were made using data from all treatments in BMP1 and BMP2; however, H_2S correlation was conducted with data from un-ammended manure, K_2SO_4, and S^0 treatments in BMP1 and BMP2.

4.4 Discussion

In this study, the effects of SO_4^{2-} and S^0 on dairy manure AD were determined using BMP tests. Unlike in other studies that evaluated the effect of SO_4^{2-} addition on digestion parameters using synthetic wastewaters and SO_4^{2-} addition to achieve a set range of COD:SO_4^{2-} ratios below 20, this study started with a complex substrate already being treated with AD and evaluated the effect of SO_4^{2-} and S^0 addition on AD. When 2 mM $FeSO_4$ and 2 and 5 mM S^0 were added to separated dairy manure, there was no

significant difference in CH_4 production or sCOD removal, with an increase in SO_4^{2-} removal from 81% in the un-amended manure treatment to 95% in the $FeSO_4$ treatment. It is likely that the addition SO_4^{2-} up to 288 mg SO_4^{2-}/L acts as a primer for SRB, increasing SO_4^{2-} reduction from electron donors not utilized by methanogens, which was also observed when 300 mg SO_4^{2-}/L was added to a substrate containing coffee grounds, coffee liquid, milk waste and dewatered WAS in an anaerobic membrane bioreactor (AnMBR) (Li et al., 2015). The SRB in the AnMBR degraded the propionic acid in the reactor and did not inhibit CH_4 production or COD removal (Li et al., 2015).

When SO_4^2 concentrations were increased above 2 mM (using both $FeSO_4$ and K_2SO_4) and S^0 concentrations were increased above 5 mM, CH_4 production decreased significantly compared to the un-amended manure treatment, as was expected. Similar observations in CH_4 reduction due to decreased COD:SO_4^{2-} ratio have been observed at COD:SO_4^{2-} ratios below 16.7 (Liu et al., 2015; Lu et al., 2016), which is within the range of the K_2SO_4 treatments and the 20 mM and 50 mM $FeSO_4$ treatments in this study. Previous studies have attributed the decrease in CH_4 production to sulfide toxicity resulting from unionized H_2S permeating through the cell membranes interfering with the sulfur metabolism (Hulshoff Pol et al., 1998). However, in this study, CH_4 reduction was observed in treatments with and without ferrous iron, which was not expected. Because Fe^{2+} addition precipitates out aqueous H_2S as FeS (Zhang et al., 2011), it was expected that CH_4 production would be higher in SO_4^{2-} treatments without ferrous iron. This result indicates that the resulting decrease in CH_4 in $FeSO_4$ treatments was more likely due to the organics in the manure being utilized as electron donors by SRB over methanogens rather than H_2S toxicity. While methanogens were found to utilize the majority of

electrons at COD:SO_4^{2-} ratios above one in a study by Lu et al. (2016); electron utilization by SRB increased from 5.2% to approximately 30% with decrease in COD:SO_4^{2-} ratios from 10 to 2. While there was no significant correlation between H_2S production and COD:SO_4^{2-} ratio in this study, as SO_4^{2-} concentrations increased, H_2S concentrations also increased indicating a shift in electron utilization towards SRB.

As was expected, $FeSO_4$ addition to manure resulted in significant reduction in H_2S concentrations in the biogas from 44% to 96% compared to the un-amended manure treatment due to the precipitation of H_2S by ferrous iron. The K_2SO_4 treatments increased H_2S production from 3.4- to 3.7-fold compared to the manure treatment was also not expected due to the increase in SO_4^{2-} in a system that is not substrate limited (Dar et al., 2008). Lu et al. (2016) attributed the increase in H_2S resulting from a decrease in COD:SO42- ratio to the elevated influent SO_4^{2-} concentration, thus increasing the SRB numbers in the reactor. While in this study SRB numbers were not quantified, it is likely that an increase in the preferred electron acceptor for SRB resulted in favorable conditions for SRB number increases.

The increase in H_2S in this study by 12- and 28-fold from the 20 mM and 50 mM S^0 treatments, respectively, was not expected. The increase in H_2S resulting from S^0 addition indicates that SRB in the inoculum were capable of utilizing S^0 as an electron acceptor. While S^0 has been shown to be one of the common electron acceptors for most SRB species (Hao et al., 2014), S^0 reduction in AD has not been discussed. In this study, if SO_4^{2-} were considered the only valid electron acceptor for SRB, the H_2S production in the S^0 treatments would comprise 99% to 623% of the total SO_4^{2-} removed on a molar basis (Table C 3). However, H_2S produced by the S^0 treatments comprises only 20% to

28% of the added S^0 in each treatment, indicating that S^0 is being utilized by SRB as an electron acceptor in the presence of SO_4^{2-}(Table C 3).

SO_4^{2-} removal in this study was between 40% and 95% for all treatments, which is in agreement with SO_4^{2-} removal in other studies using biological treatment for high SO_4^{2-} wastewater treatment (Liu et al., 2015; Zhang and Wang, 2014). It was expected that as SO_4^{2-} concentrations increased, there would be an increase in SO_4^{2-} reduction; however, in this study SO_4^{2-} reduction generally decreased with increased influent SO_4^{2-} concentrations. This trend is most likely due to the SRB becoming substrate limited as SO_4^{2-} increased, illustrated by the similarity in the mass of sulfate removed in each treatment (Table C 1).With influent SO_4^{2-} concentrations of 288 mg SO_4^{2-}/L, which is similar to concentrations used by Hughes and Gray (2013) when simulating acid mine drainage, CH_4 production from AD was maintained compared to the un-amended manure treatment. Even though CH_4 was reduced upon further increase in SO_4^{2-} concentrations, SO_4^{2-} was still removed indicating that high SO_4^{2-} wastewater treatment can be achieved when added to dairy manure digestion process.

4.5 Conclusions

This study determined dairy manure AD was a potential biological treatment for high SO_4^{2-} wastewater by increasing SO_4^{2-} concentrations in BMP treatments. The results of this study indicate that dairy manure is an appropriate substrate for SO_4^{2-} reduction of high SO_4^{2-} wastewaters, with the potential to reduce SO_4^{2-} concentrations up to 95% while providing methane for energy use. At a SO_4^{2-} concentration of 288 mg SO_4^{2-}/L, CH_4 production was maintained at levels produced by the un-amended manure treatment

and the addition of ferrous iron in the feed decreases the H_2S concentrations in the biogas while providing SO_4^{2-} reductions greater than 93%. $SO4^{2-}$ removal greater than 40% was achieved by all treatments with continued, albeit reduced, production of CH_4, indicating that AD can be used to treat high sulfate wastewaters with the benefit of energy recovery.

5 Pretreatment of MSW for Anaerobic Digestion of the Solubilized Organics in a Waste Stream from a Cellulosic Ethanol Plant

5.1 Introduction

As the world shifts from using fossil fuel based transportation fuels, which have led to an accumulation of CO_2 in the atmosphere, to cleaner burning fuels, such as bioethanol; there is higher scrutiny over the sources utilized for ethanol production. The biomass utilized to produce bioethanol can be divided into first and second generation sources: first generation refers to non-lignocellulosic crops specifically grown for bioethanol production and second generation includes agricultural and municipal waste products as well as lignocellulosic energy crops. Bioethanol produced from second generation biomass is called cellulosic ethanol. Typical second generation biomass considered for cellulosic ethanol production include corn stover, wheat straw, sugar cane bagasse, and woody biomass (Hu et al., 2017; Kim and Kim, 2014); with few studies investigating the potential for use of municipal solid waste (MSW).

Collection and transportation of agricultural biomasses used for cellulosic ethanol production has proven difficult with most of the biomass sources not in proximity to living centers, thus increasing distribution costs of the final ethanol product (Voith, 2009). Unlike agricultural biomasses, MSW is a resource that is already collected and transported, is produced in the same location as the ethanol consumers, and is a low-to-no cost biomass. There is also concern that supply of second generation biomass will fall short of cellulosic ethanol demand; however, in a life cycle analysis Chester and Martin (2009) determined that between 1.0 and 1.5 billion gallons of ethanol per year could be

produced using the organics that are discarded to landfills in California, which was higher than the demand of 900 million gallons per year.

In order to utilize MSW as a biomass in cellulosic ethanol, the organics need to be separated and pretreated. Pretreatment processes utilized for conversion of cellulosic biomass to ethanol are designed to alter the biomass size, structure, and chemical composition to facilitate the hydrolysis of carbohydrates to fermentable sugars (Zheng et al., 2009). Pretreatment processes can be mechanical, thermal, chemical, and biological (Taherzadeh and Karimi, 2008). Li et al. (2007) subjected a selection of biodegradable MSW to 15 different pre-hydrolysis treatments including: dilute acids, steam treatment, and microwave treatment, with 1% sulfuric acid pretreatment followed by steam treatment of 121 °C yielding the most glucose. Similarly, Tian et al. (2013) used a dilute acid-steam process (1% phosphoric acid and steam at 180 °C) for sugar cane bagasse prior to hydrolysis with cellulase. Other common pretreatment methods for cellulosic biomass include wet oxidation (Torry-Smith et al., 2003), steam explosion (Hu et al., 2017), and thermal soaking (Kaparaju et al., 2009). While other studies have investigated the effects of different pretreatment mechanisms on agriculturally derived cellulosic biomass, none of these pretreatment methods have been optimized for use with MSW as the biomass for cellulosic ethanol.

Economic analyses of cellulosic ethanol production have determined that pretreatment of biomass is responsible for approximately 20% of total cellulosic ethanol costs (Yang and Wyman, 2008). Methods to decrease the cost of pretreatment include: limited chemical requirement, limited particle size reduction, reduction in pretreatment wastewater conditioning, and minimal heat and power requirements (Yang and Wyman,

2008). The integration of anaerobic digestion, a waste conversion technology that produces a biogas rich in methane (CH_4), for cellulosic ethanol wastewater treatment can reduce the overall operation costs of cellulosic ethanol; offsetting heat and power requirements from the pretreatment process.

Use of AD for the treatment of cellulosic ethanol wastewater has been investigated sparingly as a way to recover energy from as well as treat the wastewater before it exits a plant (Hu et al., 2017; Kaparaju et al., 2009; Uellendahl and Ahring, 2010). Even though process water from lignocellulose-based ethanol production can contain high concentrations of inhibitory compounds and organics, Torry-Smith et al. (2003) observed that AD is capable of removing 79% to 100% of inhibitory compounds in fermented wheat straw supernatant without process complications. Digestion of stillage collected from the distillation of fermented sugar cane bagasse produced 200 mL CH_4/g VS, which would provide an estimated 62% of energy consumed during distillation (Tian et al., 2013). Rabelo et al. (2011) found that an increase in sugar cane bagasse solids being pretreated resulted in an increase in CH_4 from AD of the pretreated liquor. However, in a techno-economic analysis of sugar cane bagasse to cellulosic ethanol process, Barta et al. (2010) determined that while AD of the entire stillage stream produced more biogas than AD of the liquid fraction, the increased biogas production from the whole stillage did not result in overall cost savings due to the high cost of AD required for whole stillage treatment (Barta et al., 2010). While previous studies on AD of cellulosic ethanol waste streams have focused on the stillage and pretreatment waste from agricultural by-products, few studies have evaluated AD of the pretreatment wastewater from MSW.

While the pretreatment of MSW for cellulosic ethanol production has not been studied extensively, studies investigating pretreatment methods to solubilize the organic fraction of MSW for AD are more common (Taherzadeh and Karimi, 2008). Although, studies on pretreatment methods for the organic fraction of MSW typically investigate one pretreatment method under varying conditions, not multiple methods on a single substrate. The benefit of this study is that multiple pretreatment methods were conducted on the same substrate under similar conditions, allowing a direct comparison between pretreatment methods. Pretreatment methods considered in this study included: hydrothermal, alkaline, surfactant, pressing, and water solubilization.

The use of hydrothermal and alkaline pretreatments was based on commonality between use for MSW pretreatment prior to AD and use for cellulosic biomass pretreatment prior to cellulosic ethanol production. Liquid hot water treatment, or pulping, is a hydrothermal pretreatment applied to lignocellulosic biomass that utilizes water under high pressure to penetrate the biomass, hydrate cellulose, and remove hemicellulose and part of the lignin (Taherzadeh and Karimi, 2008). Thermal treatments for MSW have resulted in solubilization of proteins and increased removal of particulate carbohydrates (Jain et al., 2015). Alkali pretreatment results in increased specific surface area, removing lignin and part of the hemicellulose from lignocellulosic biomass through solvation and saphonication (Jain et al., 2015; Taherzadeh and Karimi, 2008). Alkali pretreatment is preferred in AD over acid pretreatments due to the addition of alkalinity, which provides buffering during the acidogenic phase (Jain et al., 2015).

While surfactants are not typically used for MSW or cellulosic biomass pretreatment, they have been shown to enhance the water solubility of organic pollutants

in soils, especially organics with hydrophobic tendencies (Mao et al., 2015). Surfactant addition is also beneficial for the hydrolysis step during cellulosic ethanol production. Typically during hydrolysis, cellulose irreversibly binds to the surface of cellulose, decreasing the enzymatic activity; however, addition of surfactants modifies the cellulose surface property, minimizing the irreversible binding of cellulase on cellulose (Sun and Cheng, 2002). Previous studies have demonstrated the ability of AD to treat surfactant-containing waste; however, no study has used surfactants as a MSW pretreatment method prior to AD.

Pressing is a mechanical technique that has been commonly used to extract oil for biodiesel production. Pressing has also been utilized to release the soluble organics from the liquid fraction of the organic fraction of MSW. Nayono et al. (2010b) observed a high methane potential from press water from source separated organic fraction of MSW coupled with AD process stability at high loading rates. While pressing is a common method for biodiesel production, it has been used sparingly as a pretreatment method to solubilize organics from MSW for AD.

The objective of this study was to determine a pretreatment method for MSW resulting in a wastewater that maximizes CH_4 from AD. In this study, MSW obtained from a cellulosic ethanol pilot plant in Lawrenceville, VA was used as the sole substrate for pretreatment using different physical, chemical, and thermal pretreatment methods. The methane potential of the pretreatment wastewater was determined using a biochemical methane potential (BMP) test. In addition to methane potential, the solubilization of organics and VFA composition of the resulting wastewater was determined. The results of this study were then used to calculate the theoretical energy

offset from the use of AD at a cellulosic ethanol plant receiving MSW as the biomass source.

5.2 Material and Methods

In this study, MSW collected from Fiberight's pilot cellulosic ethanol plant (Figure 5.1) was pretreated using thermal, chemical, and physical methods. The resulting wastewater from the pretreatment processes was digested in a BMP test to determine the pretreatment method that results in the highest CH_4 yield. The thermal treatment was conducted at Fiberight's pilot plant in a pulper and the chemical and physical pretreatments were conducted on pulped MSW at a lab scale.

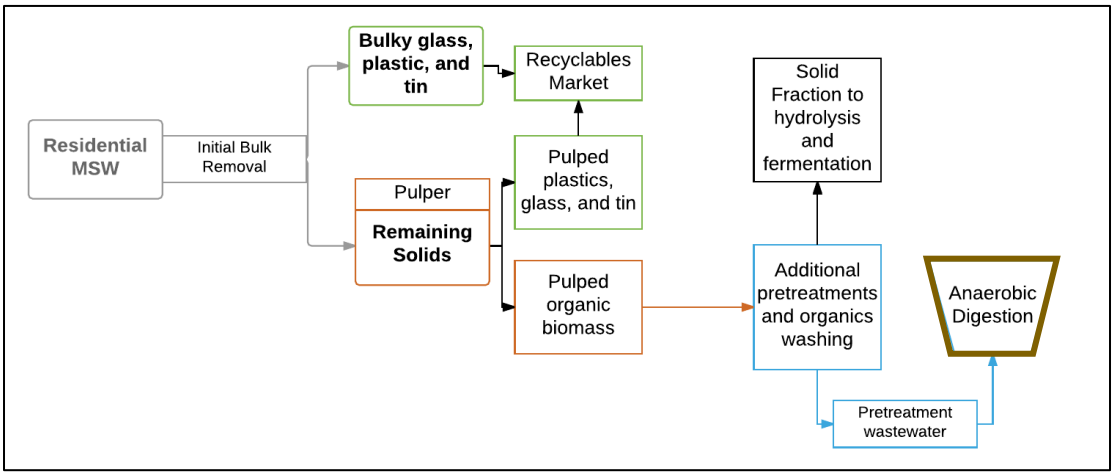

Figure 5.1: Process diagram for cellulosic ethanol production at Fiberight pilot plant.

5.2.1 Thermal Pretreatment

Residential MSW collected from the population of Lawrenceville, VA was mechanically sorted on site at Fiberight's pilot-scale cellulosic ethanol plant to separate

large bulky items. Batches of the remaining solids were hydrothermally treated via pulping (orange boxes Figure 5.1). Specifically, the separated MSW was placed into a pulper with a charge of heated water and pulped at three different temperatures for one-hour (Table 5.1). Three five-gallon buckets of MSW pulped at each of the temperatures (66, 77, and 99 °C) were collected and transported to University of Maryland in College Park, MD. The pulped MSW solids were stored at 4 °C until utilized for testing in a biochemical methane potential (BMP) test.

5.2.2 Pilot Scale Washing of Thermally Treated MSW

In order to determine the effects of pulping temperature on CH_4 production in AD, the pulped solids were washed in a pilot-scale washer at 20 ºC at a solids to water wash ratio of 1:5 by volume for one-hour (blue boxes Figure 5.1). Solids from each pulping condition (Table 5.1) were washed in triplicate by placing 2.4 gallons of pulped solids into the washer with 12 gallons of tap water. The washer agitated the solids in the water for one-hour. After each washing, three one-liter grab samples were collected in Nalgene® bottles after passing through a 20 mesh sieve. To get representative samples one sample was collected at the start of washer drainage, one after five gallons had drained, and one after another five gallons had drained from the washer. Samples were stored at 4 °C until loaded into a BMP test.

Table 5.1: Pulping conditions

Treatment	Mass MSW Pulped (kg)	Charge Water Temperature (°C)	Biomass Exit Temperature (°C)	Biomass Exit Moisture Content (%)
Low	406	66	63	66.9
Medium	488	77	56	73.1
High	474	99	66	68.5

5.2.3 Chemical and Physical Pretreatments

The chemical and physical pretreatments were all conducted at a lab scale on MSW thermally treated at 77 °C (Table 5.2). Similar to the thermal treatments, the physical and chemical treatments were conducted in triplicate at a 1:5 solids:water wash ratio (blue boxes Figure 5.1), with the exception of the press only treatment. In order to compare the thermal treatment to the treatments with additional physical and chemical treatments, a lab scale version of the 77 °C thermal treatment was conducted. For the lab scale 77 °C thermal treatment, or water only treatment, 0.5 L of MSW pulped at 77 °C was added to 2.5 L of tap water in a 2.5-gallon bucket and mixed at 80 rpm on a shaker for one-hour. The mixture was poured through a 20 mesh sieve and the wastewater was collected in one-liter Nalgene® bottles and stored at 4 °C until BMP testing.

5.2.3.1 *NaOH Pretreatment*

Two NaOH concentrations (45 mM and 250 mM) were utilized in this study to determine the effects of alkaline pretreatment on solubilization and digestibility of organics in the wastewater. Specifically, a 5 M stock NaOH solution was made by dissolving 200 g NaOH (Fisher Scientific Certified ACS Grade) into deionized water in a 1-L volumetric flask. NaOH stock solution was then serial diluted to a volume of 2.5 L to

yield 45 mM and 250 mM NaOH concentrations. The pulped MSW was then added to the 2.5 L 45 and 250 mM NaOH solutions and mixed on a shaker at 80 rpm for one-hour in a 2.5-gallon bucket. The mixture was poured through a 20 mesh sieve and the wastewater was collected in one-liter Nalgene® bottles and stored at 4 °C until used as a substrate in a BMP test.

5.2.3.2 *Tween™ 85 Pretreatment*

In order to determine the effects of surfactant pretreatment on solubilization and digestibility of organics, three concentrations of Tween™ 85 (Acros Organics) were employed: 0.001%, 0.1%, and 10% (volume Tween™ 85/volume water). The concentrated Tween™ 85™ was added to the 2.5 L of water in a 2.5-gallon bucket and mixed until in solution prior to addition of the MSW. The pulped MSW was then added to the Tween™ 85 solution and mixed at 80 RPM for one-hour on a shaker. The mixture was poured through a 20 mesh sieve and the wastewater was collected in one-liter Nalgene® bottles and stored at 4 °C until used as a substrate in a BMP test.

5.2.3.3 *Sonication Pretreatment*

A Branson ultrasonic cleaner (Bransonic 12, 40 kHz) was used to sonicate 0.1 L of pulped MSW in 0.5 L water. The mixture was indirectly sonicated in a beaker. The liquid in the sonication tank was above the liquid line in the beaker during the one-hour treatment. The mixture was poured through a 20 mesh sieve and the wastewater was collected in one-liter Nalgene® bottles and stored at 4 °C until used as a substrate in a BMP test.

5.2.3.4 *Press pretreatments*

The pulped MSW was placed into the basket of a 1.3 L aluminum/stainless steel fruit press and pressed until the liquid fraction of the solids was no longer draining from the basket. In order to collect enough press water from the solids, each press only treatment contained press water from 2 L of MSW. On average, 2 L of MSW yielded 0.3 L of press water. The press water was collected in 500 mL Nalgene® bottles and stored at 4 °C until used as a substrate in the BMP test.

For the water then press treatment, 0.5 L of pulped MSW were washed in 2.5 L of water in a 2.5-gallon bucket. The wastewater was poured through a 20 mesh sieve and collected in a 2.5-gallon bucket. The solids were then pressed in the fruit press and the press water was collected in the same 2.5-gallon bucket as the wastewater. The wastewater was stored in one-liter Nalgene® bottles at 4 °C until used as a substrate in a BMP test.

Table 5.2: Chemical and physical pretreatment methods applied to MSW pulped at 77 °C. Pretreatments were conducted in triplicate with ± standard error shown.

Pretreatment	Solids Mass (g)	Volume Solids (L)	Volume Water (L)
45 mM NaOH	278 ± 13	0.5	2.5
250 mM NaOH	258 ± 10	0.5	2.5
0.001% Tween™ 85	254 ± 21	0.5	2.5
0.1% Tween™ 85	239 ± 4	0.5	2.5
10% Tween™ 85	289 ± 6	0.5	2.5
Sonication	59 ± 4	0.1	0.5
Press Only	1,160 ± 86	2	NA
Water then Press	259 ± 7	0.5	2.5
Water Only	278 ± 4	0.5	2.5

5.2.4 Inoculum

The inoculum in this study was a granular inoculum collected from a pilot scale upflow anaerobic sludge blanket (UASB) at Fiberight's pilot plant in Lawrenceville, VA (brown box Figure 5.1). The inoculum was collected in 2.5 L buckets and placed on ice during transport to the University of Maryland in College Park, MD. The inoculum was stored at 4 °C until utilized in BMP tests.

5.2.5 Experimental Design

The wastewater from the thermal, physical, and chemical pretreatments (Table 5.1 and Table 5.2) was added to the granular inoculum at a 2:1 inoculum to substrate ratio by volatile solids (Table 5.3). The two NaOH treatments were loaded into six BMP bottles each and three of the six bottles were neutralized with 5N H_2SO_4. Treatments were loaded in triplicate, resulting in 36 treatments and 3 inoculum controls.

Table 5.3: Average mass of wastewater and inoculum VS added to each BMP treatment. The total volume of each bottle was 200 mL, with the exception of the press only treatment, which had 100 mL.

Treatment	VS Inoculum (g)	VS Wastewater (g)
Inoculum Only	9.85	NA
66 °C	0.688 ± 0.038	0.344 ± 0.019
77 °C	0.742 ± 0.089	0.371 ± 0.045
99 °C	0.852 ± 0.017	0.426 ± 0.008
45 mM NaOH	0.705 ± 0.060	0.352 ± 0.030
250 mM NaOH	0.773 ± 0.061	0.387 ± 0.030
0.001% Tween™ 85	0.583 ± 0.045	0.291 ± 0.022
0.1% Tween™ 85	0.650 ± 0.087	0.325 ± 0.044
10% Tween™ 85	4.30 ± 0.04	2.15 ± 0.02
Sonication	0.565 ± 0.054	0.282 ± 0.027
Press Only	2.28 ± 0.01	1.12 ± 0.02
Water then Press	0.664 ± 0.044	0.332 ± 0.022
Water Only	0.644 ± 0.032	0.322 ± 0.016

5.2.6 Biochemical Methane Potential (BMP) Test

Anaerobic incubation was conducted using a BMP protocol developed by Owen et al. (1979) and adapted by Moody et al. (2011). BMP experiments were used to determine the energy production potential, defined CH_4 production, of the biogas. The BMP tests were conducted until the CH_4 production in each treatment stabilized. The digestion vessels were 250-mL bottles with a liquid volume of 200 mL and a headspace volume of 50 mL. Prior to incubation, the filled BMP bottles were flushed with 70:30 N_2:CO_2 for three minutes to ensure anaerobic conditions. The bottles were then capped with a rubber septum and placed on a shaker (New Brunswick™ innOva® 2300, Hamburg, Germany) at 110 rpm in a temperature controlled incubator at 35 °C.

Biogas production was quantified volumetrically using a glass, gas-tight syringe equilibrated to atmospheric pressure. Biogas samples were analyzed for CH_4 composition using a gas chromatograph (Agilent Technologies, Inc.; Shanghai China; model 7890 A) with a thermal conductivity detector at 250°C. The column was a HP-Plot Q capillary column (Agilent J&W; USA) using helium (He) as the carrier gas at 8.6 ml/min with the oven operated at 60°C for 2 min and subsequently ramped at 30°C/min to 240°C. Biogas production and CH_4 content were measured daily during the first week of the experiment, approximately every other day the following week, and then as needed for the remainder of the BMP experiments, with measurement frequency based on the quantity of biogas produced.

To account for biogas production from residual biodegradable material in the inoculum, inoculum controls were incubated using the same conditions for each inoculum source and sampled simultaneously to allow for subtraction of biogas production not attributed to the wastewater substrate. Methane production was normalized:

1. By the mass of chemical oxygen demand (COD) in the wastewater added to each BMP bottle, to determine the efficiency of the inoculum to utilize the organics in the treatments. The organics added to each bottle included the COD from the solubilized MSW and, for the surfactant treatments, the COD also of the surfactant (Tween™ 85) used in the pretreatment.

2. By the mass of MSW that was washed for each treatment. For this normalization the organic contribution of the Tween™ 85 surfactants was adjusted by multiplying the mL CH_4/g COD of wastewater added to the BMP

bottle by the g COD corrected for surfactant divide by the mass of MSW treated (Table D 1).

5.2.7 Analytical Methods

The inoculum and wastewater total and volatile solids were characterized prior to BMP loading. Wastewater samples were analyzed for COD, soluble chemical oxygen demand (sCOD), and volatile fatty acids (VFAs). Total solids (TS) (APHA Method 2540B) and VS (APHA Method 2540E) were determined using the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). HACH method 8000 was used to determine COD and sCOD, samples for sCOD were filtered through 0.45 µm nitrocellulose membrane filter. The COD in the surfactant treatments was reported as COD resulting from the surfactant subtracted, which was used for the CH_4 production normalization by g MSW. The addition of the surfactant to the 10% Tween™ 85 treatment decreased the sCOD, as the surfactant likely adsorbed to the insoluble organics in the wastewater, which were removed via filtration; therefore, the sCOD of the 10% Tween™ 85 treatment was not reported.

For measurement of VFAs (acetic, propionic, n-butyric, and n-valeric acids), samples were acidified with 5N sulfuric acid to pH < 2 (diluted by ≤ 10%) and filtered to 0.22 µm through a nitrocellulose membrane filter before analysis via a gas chromatograph (Agilent Technologies, Inc.; Shanghai China; model 7890 A). The gas chromatograph used a flame ionization detector (FID) at 300°C, a DB-FFAP capillary column (Agilent J&W; USA), He as the carrier gas at 1.80 ml/min, injection temperature of 250°C, and the oven was operated at 100°C for 2 min. After 2 min. the oven was subsequently ramped at

10°C/min for a total run time of 10 min. Volatile fatty acid concentrations were converted to COD using the following conversion factors: 1.07 for acetic acid, 1.51 for propionic acid, 1.82 for butyric acid, and 2.04 for valeric acid (Yuan et al., 2011).

5.2.8 Statistical Analysis

Statistical analysis was conducted using SAS 9.3 (SAS, Cary, NC). The experiment was a single factor, completely randomized design with 39 experimental units (BMP bottles). One-way ANOVA analysis was performed to assess significant differences between cumulative CH_4 production among treatments, and differences in COD, sCOD, and VFA concentrations for all wastewater samples. The level of significance was held at an alpha of 0.05.

5.3 Results

In order to determine the most appropriate pretreatment method for MSW as the first process in cellulosic ethanol production three pulping temperatures were utilized. Then, subsequent chemical and physical pretreatment methods were conducted on the MSW pulped at 77 °C.

5.3.1 Effect of Thermal Pretreatment on CH_4 Production, Organics Transformation, and TVFAs

The effect of pulping temperature on CH_4 production, organics transformation, and TVFAs was determined by digesting wastewater resulting from washing pulped MSW at a 1:5 solids:water ratio (Table 5.4 and Figure 5.2). There was no significant difference in CH_4 production normalized by COD from the three thermal treatments tested (66, 77, and

99 °C), which ranged from 241 to 277 mL CH_4/g COD (p-values: 0.700 to 0.960). There was also no significant difference between CH_4 production from the thermal treatments when normalized by mass of MSW washed per treatment, which ranged from 7.85 to 11.6 mL CH_4/g MSW washed (p-values: 0.065 to 0.476) (Table 5.4).

There was no significant difference in COD produced by the three thermal treatments when normalized by COD or by mass of MSW washed (p-values: 0.711 to 0.978 and 0.505 to 0.996, respectively). COD concentrations for the three treatments ranged from 3,000 to 3,800 mg COD/L wastewater and 32.4 to 42.9 mg COD/g MSW washed. There was also no significant difference in sCOD concentrations in the wastewater from the thermally treated solids, with values ranging from 1,800 to 2,300 mg COD/L wastewater (p-values: 0.446 to 0.945) and 19.3 to 26.8 mg COD/g MSW washed (p-values: 0.196 to 0.895). The thermal treatments solubilized between 59% and 65% COD (Table 5.4).

There was no significant difference in TVFA concentrations between the thermal treatments with concentrations ranging from 1,470 to 1,790 mg VFA-COD/L wastewater (p-values: 0.451 to 0.994) (Figure 5.2). When normalized by mass of MSW washed, the TVFA concentrations ranged from 16.7 to 21.3 mg VFA-COD/g MSW washed with no significant difference between thermal treatments (p-values: 0.417 to 0.980).

Table 5.4: Average CH_4 production, COD and sCOD concentrations and sCOD/COD for the thermal treatments. Treatments were conducted in triplicate with standard error shown.

Treatment	mL CH4/g COD	mL CH4/g MSW	mg COD/L	mg COD/g MSW	mg sCOD/L	mg sCOD/g MSW	sCOD/COD (%)
66 °C	253 ± 44	10.0 ± 0.3	3,830 ± 1,160	42.9 ± 9.9	2,170 ± 80	25.1 ± 0.8	65 ± 14
77 °C	241 ± 21	7.85 ± 1.24	2,980 ± 470	32.4 ± 3.9	1,790 ± 420	19.3 ± 3.6	59 ± 7
99 °C	277 ± 23	11.6 ± 1.0	3,620 ± 280	42.1 ± 2.3	2,290 ± 190	26.8 ± 2.7	65 ± 9

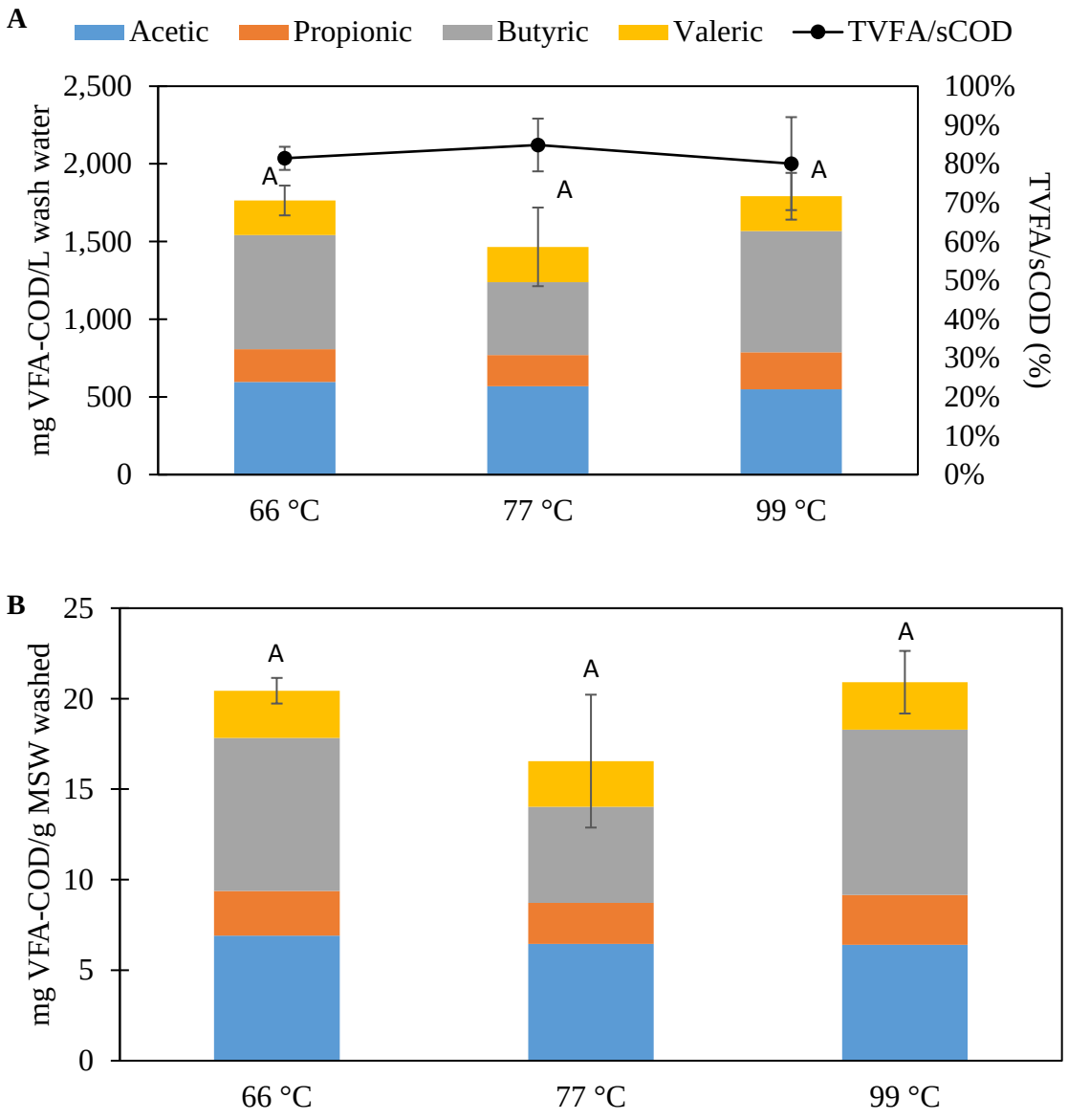

Figure 5.2: TVFA concentrations as COD in the wastewater from MSW pulped at 66 °C, 77 °C, and 99 °C A) per volume of wastewater produced with TVFA/sCOD and B) per mass of MSW washed. Treatments were conducted in triplicate with error bars representing ± standard error. Letters above the error bars indicate significant differences between treatments.

5.3.2 Pretreatments Administered to the MSW Thermally Treated at 77 °C

The effect of additional chemical and physical pretreatments administered to the MSW on CH_4 production was determined by pretreating MSW pulped at 77°C (Figure 5.3). When normalizing by COD in the wastewater added to each treatment, the press only treatment yielded the highest CH_4 production, which was only significantly higher than the 10% Tween™ 85, 45 mM NaOH neutralized, and 250 mM NaOH neutralized treatments by 70%, 204%, and 222%, respectively (p-values: <0.001 to 0.008).

The effect of the pretreatments on CH_4 production per mass of MSW treated was also determined, with the 45 mM NaOH treatment (10.0 mL CH_4/g MSW) producing 1.4- to 3.0- fold more CH_4 than the other treatments. While there was no significant difference in CH_4 production between the 45 mM NaOH treatment, the 10% and 0.1% Tween™ 85, and the water then press treatments (p-values: 0.068 to 0.212), the 45 mM NaOH treatment did yield significantly higher CH_4 production than the other remaining treatments (p-values: < 0.001 to 0.024) (Figure 5.3). The 250 mM NaOH treatment that was not neutralized prior to digestion failed to produce any CH_4 for the duration of the BMP. The CH_4 production from the Tween™ 85 treatments were corrected for the CH_4 produced from the additional organics in the surfactant, as stated in Section 5.2.6 and detailed in Table D 1. The CH_4 production from the 0.001, 0.1, and 10% Tween™ 85 treatment wastewater was 0.1, 12 and 91% higher due to the additional organics in the Tween™ 85 (Figure D 1), compared to the CH_4 production after surfactant COD taken into account (Figure 5.3).

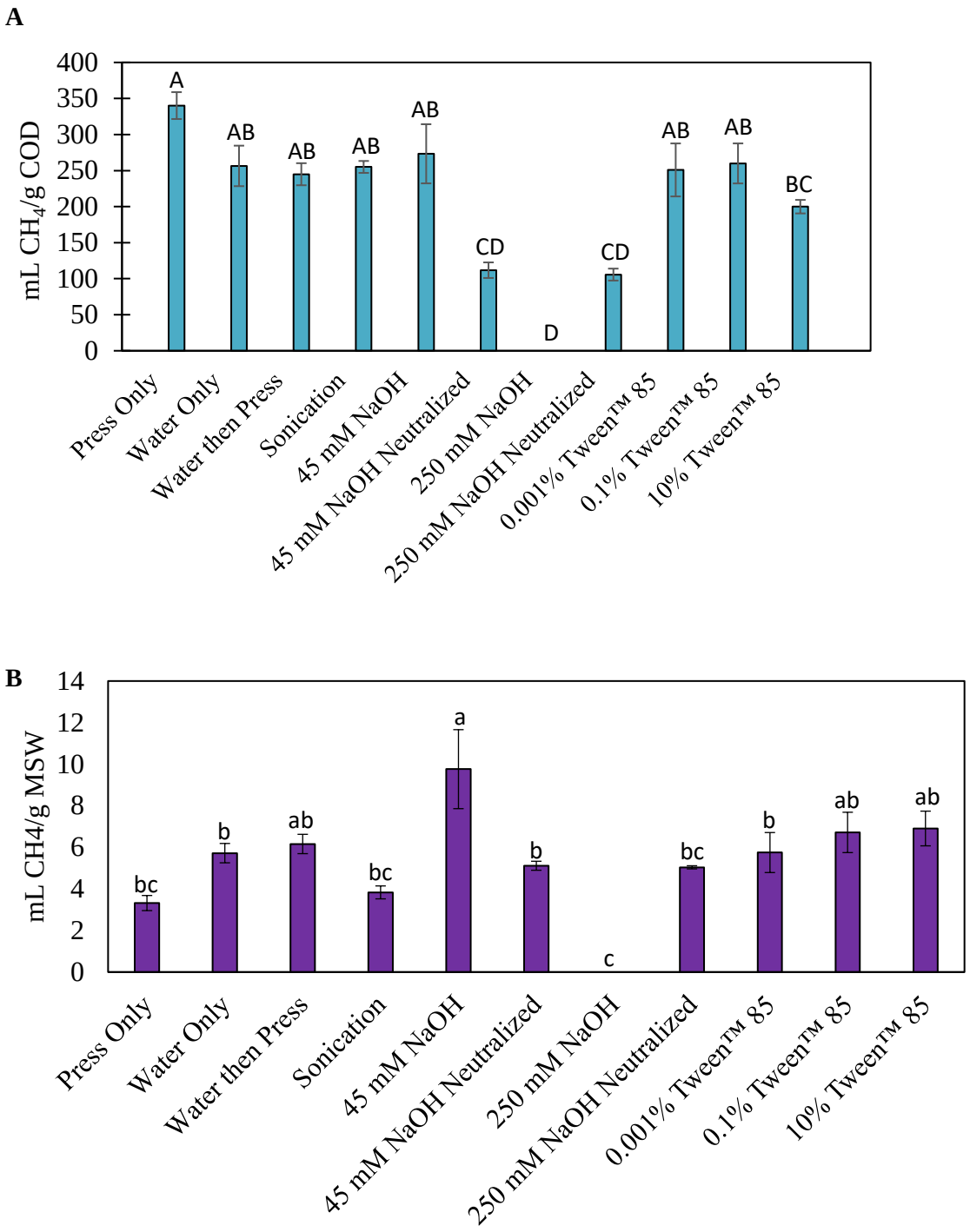

Figure 5.3: Cumulative CH_4 production from physical and chemical pretreatments normalized by A) g COD wastewater added to each treatment BMP bottle, and B) g MSW with the COD of surfactant accounted for in Tween™ 85 treatment normalization.

There was a significant increase in available organics (COD) of 10- to 22-fold in the wastewater from the press only treatment compared to wastewater from the other treatments (p-values < 0.001), with no significant difference in organics among the other chemical or physical treatments (Table 5.5). Similarly, the press only treatment increased soluble organics in the wastewater 10- to 38-fold compared to the sCOD concentrations in the other pretreatments (p-values < 0.001), with no significant difference in sCOD concentrations among the other treatments.

When COD was normalized by the mass of MSW washed, the 45 mM NaOH treatment increased organics in the wastewater 1.03- to 3.71-fold compared to the other treatments, with a significant increase in COD concentration over the water only, sonication, and press only treatments (p values: <0.001 to 0.042) (Table 5.5). The effect of pretreatment methods on soluble organic concentrations was similar to the effects on total organics. Addition of 45 and 250 mM NaOH to MSW increased soluble organics in the wastewater between 1.5- and 4.0-fold compared to the other pretreatment methods (p-values: <0.001 to 0.018). Unlike with COD, the soluble organics in the water only, 0.001% and 0.1% Tween™ 85, and water then press treatments were significantly higher than in the press only and sonication treatments (p-values: 0.023 to 0.005).

Table 5.5: Average COD and sCOD concentrations from chemical and physical pretreatments administered to MSW thermally treated at 77 °C. Treatments conducted in triplicate with standard error shown. Superscripts indicate significant difference between treatments within columns.

Treatment	mg COD/L[1]	mg COD/g MSW[1]	mg sCOD/L[1]	mg sCOD/g MSW[1]	sCOD/COD (%)[3]
Press Only	38,600 ± 2,400[a]	9.83 ± 0.67[d]	23,800 ± 3,100[a]	5.96 ± 0.38[c]	61 ± 5
Water Only	2,530 ± 270[b]	22.7 ± 2.3[bcd]	1,630 ± 50[b]	14.7 ± 0.5[b]	66 ± 6
Water then Press	2,620 ± 240[b]	25.3 ± 2.3[abc]	1,360 ± 150[b]	13.2 ± 1.6[b]	52 ± 4
Sonication	1,750 ± 100[b]	15.0 ± 0.7[cd]	629 ± 195[b]	5.53 ± 1.96[c]	36 ± 11
45 mM NaOH	3,760 ± 60[b]	36.5 ± 0.8[a]	2,280 ± 160[b]	22.1 ± 1.3[a]	60 ± 4
250 mM NaOH	3,900 ± 320[b]	35.4 ± 4.2[ab]	2,460 ± 60[b]	22.2 ± 1.1[a]	64 ± 4
0.001 % Tween™ 85	2,390 ± 500[b]	23.5 ± 4.6[abc]	1,470 ± 270[b]	14.3 ± 1.8[b]	62 ± 10
0.1% Tween™ 85	2,480 ± 320[b]	25.8 ± 2.9[abc]	1,270 ± 110[b]	13.3 ± 1.0[b]	51 ± 2
10% Tween™ 85	3,960 ± 250[b]	34.3 ± 2.8[ab]	n.d.[2]	n.d.[2]	n.d.[2]

[1] The COD from the surfactant added to the Tween™ 85 treatments was subtracted from the COD in the wastewater to reflect COD due to organics in MSW
[2] The sCOD from the surfactant added to the 10% Tween™ 85 treatments was not able to be subtracted due to potential for Tween™ 85 sorption to organic particles removed during sample filtration.
[3] Calculated using g COD/L

When normalized by volume of wastewater, the TVFA concentrations from the press only treatment were 11- to 52-fold higher than the other treatments (p-values < 0.001). There was no significant difference in average TVFA concentrations between the other treatments, with concentrations ranging from 242 to 1,160 mg TVFA-COD/L wastewater (p-values: 0.981 to 1.00). When normalized by mass of MSW washed, the 0.1% Tween™ 85 treatment contained the highest TVFA concentration, which was only significantly higher than the press only and sonication treatments by 3- and 5- fold, respectively (p-values = 0.022 and 0.003, respectively) (Figure 5.4).

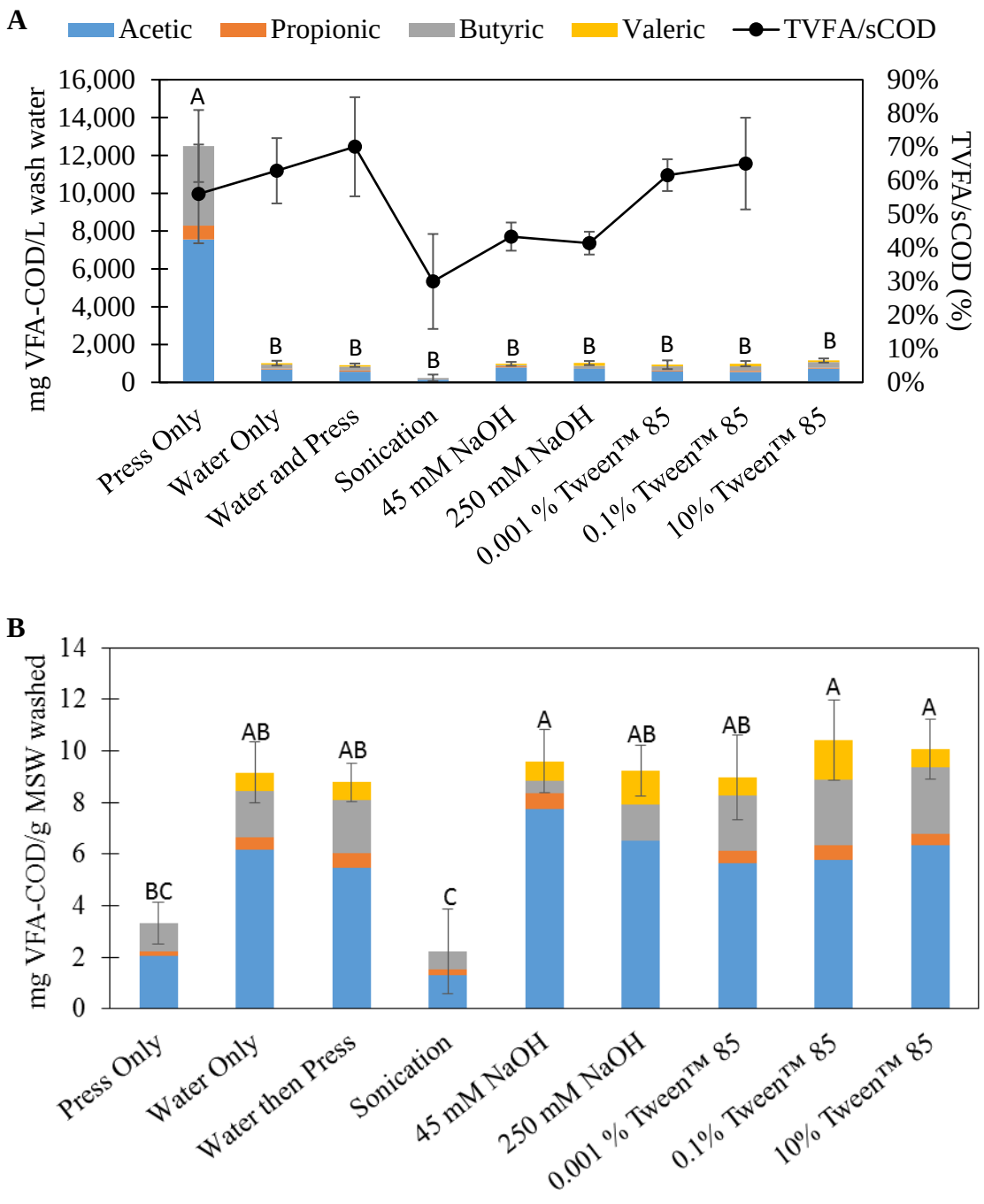

Figure 5.4: TVFA concentrations in the wastewater from pretreatments administered to MSW pulped at 77 °C A) per volume of wastewater produced with TVFA/sCOD, and B) per mass of MSW washed. Treatments were conducted in triplicate with error bars representing ± standard error. Letters above the error bars indicate significant differences between treatments.

5.4 Discussion

5.4.1 Thermal Pretreatments

It was expected that the increase in pulping temperature from 66 to 99 °C would result in an increase in CH_4 production from the wastewater captured from the washed and pulped solids due to the potential for increased organics solubilization (Bougrier et al., 2008). However, there was no significant difference in CH_4 production, COD concentrations (Table 5.4), or TVFA concentrations (Figure 5.2) between the wastewater from MSW pulped at 66, 77, and 99 °C and washed at a 1:5 solids:water ratio. Previous studies reported similar results, concluding that at temperatures between 50 and 100 °C the organic matter was not effectively disintegrating or transforming into small molecules (Jin et al., 2016; Kuglarz et al., 2013). However, Appels et al. (2010) observed COD solubilization from WAS significantly increased with an increase in temperature between 70 and 90 °C, concluding the increase in solubilization was attributed to disruption of chemical bonds in cell walls and membranes by thermal treatment. In this study, there was not a high microbial concentration in the substrate being pretreated (MSW), which could have limited the beneficial effect of excess temperature on organics solubilization and CH_4 production. The similarity in CH_4 production between the MSW pulped at 66, 77, and 99 °C in this study is due to the similarity in COD, sCOD and TVFA concentrations between the three treatments. The results of this study indicate that pulping at 66 °C is sufficient to solubilize organics from MSW, which saves on energy costs compared to increased temperature pulping. It is possible that the organics in the MSW processed could have been effectively solubilized at lower temperatures; however,

this study was limited to the three temperatures tested. It would be beneficial for future studies to investigate lower pulping temperatures to determine the optimal temperature for CH_4 production of the pretreatment wastewater.

5.4.2 Physical Chemical Treatment: CH_4 Normalized by g COD Added

It was expected that the addition of chemical pretreatments to the thermally treated MSW would result in increased CH_4 production from the resulting wastewater due to a beneficial increase in organics solubilization (Kim et al., 2003), which was not observed. It is possible that the addition of physical and chemical treatments to the pulped MSW were not beneficial for CH_4 production due to the efficiency of the thermal treatment to solubilize the available organics in the MSW, which was observed by Bougrier et al. (2006), who concluded that thermal pretreatment was the most efficient pretreatment method when compared to sonication and ozonation. This result suggests that the agitation of the pulped MSW in water was sufficient to solubilize the biodegradable organics in MSW that are efficiently converted to CH_4 in the digestion process. Additional pretreatments do not provide an additive benefit of increased biodegradable organics in the wastewater. With the exception of the press only treatment, no other pretreatment increased organics concentrations beyond the water only control, further indicating that there was no benefit to additional pretreatments.

The increase of COD and sCOD in the press only treatment was expected due to the lack of dilution from not washing the MSW at a 1:5 solids to water ratio and an increase in mass of MSW pressed per treatment compared to the MSW washed in the other pretreatments. In this study, the press only treatment yielded the highest CH_4

production when normalized by the COD added to the treatments, which was similar (14% higher) to CH_4 production resulting from pressing the organic fraction of MSW in Nayono et al. (2010b), but was not significantly higher than the water only treatment in this study. The benefit of utilizing the press only treatment instead of the water only treatment, with a solids to water wash ratio of 1:5, is that there is less wastewater to treat, resulting in a smaller AD with a lower capital cost and potential savings in water and energy use during the MSW pretreatment process.

It was expected that the sonication treatment would provide higher solubilization of the organics in the pulped MSW due to the addition of cavitation as a solubilizing mechanism (Elliott and Mahmood, 2012). However, the sonication treatment did not increase the sCOD concentration beyond the water only treatment and resulted in significantly less TVFAs than all but the press only treatment. While the sonication treatment in this study yielded less TVFAs than the other treatments, which make up a portion of the soluble organics, the CH_4 production, a measure of organics biodegradability, was not significantly different than the water only treatment. This result indicates that additional pretreatment of thermally treated MSW by sonication did not enhance solubilization of biodegradable organics compared to washing the thermally treated MSW.

While no other studies have utilized a surfactant as a pretreatment method for organics solubilization prior to AD, the expectations were that surfactant would yield an increase in CH_4 production due the mechanisms that surfactants employ for carbohydrate and protein extraction (Vasudevan and Wiencek, 1996). However, there was no significant increase in CH_4 production from the addition of Tween™ 85 in this study

compared to the water only treatment, with the 10% Tween™ 85 treatment in this study producing within 14% of CH_4 observed from bioethanol effluent fermented in media containing Tween™ 80 (Torry-Smith et al., 2003). The results indicate that the addition of Tween™ 85 to the cellulosic ethanol production, which may benefit the hydrolysis and fermentation steps, will not hinder AD or significantly reduce CH_4 production compared to the water only treatment of pulped MSW. However, the additional organics inherent in the surfactant do not increase overall CH_4 production efficiency (normalized by g COD), indicating that the removal of the surfactant prior to AD for reuse in MSW pretreatment could be beneficial from a cost and biogas prospective.

5.4.3 Physical Chemical Treatment: CH4 Normalized by g MSW Treated

When the COD of the surfactant was taken into account and the treatments were normalized by the mass of MSW treated; however, the addition of both physical and chemical pretreatments to the MSW pulped at 77 °C enhanced CH_4 production compared to the water only treatment. The 45 mM NaOH produced significantly more CH_4 than all treatments except the 0.1% and 10% Tween™ 85 and the water then press treatments. It was expected that the addition of NaOH to the thermally treated solids would result in higher CH_4 production due to the enhancement in the mechanisms responsible for solubilizing the organics from the MSW (Kim et al., 2003). The significant increase in soluble organics resulting from the addition of both 45 and 250 mM NaOH treatments over the other physical and chemical treatments indicates that the alkaline treatment is breaking down organics in the MSW that are not able to be broken down by thermal treatment alone. Thermal treatments at lower temperatures have been shown to

deflocculate macromolecules and increase the solubilization of proteins, while alkaline pretreatments can induce the swelling of solids, which increases the surface area of the solids and breakdown of acids and esters (Ariunbaatar et al., 2014). Similar to this study, Rafique et al. (2010) concluded that the combination of thermal and chemical pretreatments provides additive enhancement of CH_4 production over thermal and chemical treatments alone. Even though this study attempted to remove the effects of surfactant COD on CH_4 production, the method used was purely theoretical. Future studies on the impact of surfactant addition for CH_4 production should include a treatment with surfactant as the sole organics source.

The significance of implementing an AD to treat wastewater from the pretreatment process at a MSW to cellulosic ethanol production plant is savings in energy costs due to CH_4 production. As an example, an ethanol plant producing one million gallons of ethanol would require 1.79×10^7 MJ of energy for the distillation of ethanol (Tian et al., 2013). In order to produce one million gallons of ethanol, 63,000 tons of MSW would be required (Malo de Molina Melendez, 2013). If the pretreatment method for MSW being prepared for cellulosic ethanol conversion was pulping at 66 °C followed by a solids to water wash ratio of 1:5, the AD process would produce 2.03×10^7 MJ of energy, which is 113% of the energy requirement of the plant. Even though this analysis could underestimate the energy required to operate an ethanol plant producing one million gallons of ethanol, the production of energy by AD would provide 2.82×10^6 kWh of electricity using a generator efficiency of 50%, a CH_4 conversion to MJ of 31.46 MJ/m^3 CH_4, and a kWh/MJ of 0.278 (Nayono et al., 2010b). Production of 2.82×10^6 kWh of electricity would save the plant approximately $339,000 dollars in electricity costs for

production of 1 million gallons of ethanol (or $0.34 per gallon of ethanol, assuming an electrical cost of $0.12/kWh).

5.5 Conclusions

The results of this study indicate that pulping the MSW at 66 °C is sufficient to maximize CH_4 production compared to the other thermal treatments investigated. However, further testing on lower pulping temperatures would be beneficial for optimization of temperature requirements for maximized CH_4 production from AD of thermal pretreatment wastewater. The conclusions regarding further pretreatment of the thermally treated MSW depend on the CH_4 production normalization and how the surfactant COD is taken into account. If the surfactant was not taken into account and CH_4 was normalized by g COD, then the alkaline, surfactant, and press treatments used in this study in addition to the thermal pretreatment at 77 °C yielded no significant increase in CH_4 production, illustrating that extra processing and chemical addition is neither beneficial nor necessary. However, if the surfactant is taken into account and CH_4 is normalized by g MSW washed in each treatment, then the addition of a 45 mM NaOH wash after pulping the MSW at 77 °C results in a significant increase in CH_4 production over the thermal treatment washed in water. This result indicates that addition of a 45 mM NaOH wash to the MSW pretreatment may be beneficial; however, an economic analysis should be conducted to determine whether the additional CH_4 production is sufficient to justify the continual purchase of NaOH. While the 10% Tween™ 85 addition increased COD and sCOD concentrations, the increased organics concentrations did not result in an increase in CH_4 production, indicating that Tween™ 85 is not

beneficial for AD; nor is it detrimental. If Tween™ 85 is a potential benefit for the cellulosic ethanol production, then the choice to use Tween™ 85 will not inhibit biogas production when compared to washing the pulped solids at a 1:5 solids:water ratio. If it is desirable to save in water usage at the plant, then not washing the pulped solids and investing in a mechanical press would yield higher COD concentrations and CH_4 production, while allowing for a smaller, less capital intense, AD. If the 66 °C pulping temperature was utilized with a 1:5 solids to water ratio at an ethanol plant processing MSW for cellulosic ethanol production, the plant could save $339,000 dollars a year on electricity costs per million gallons of ethanol produced.

6 Conclusions

6.1 Summary

One of the themes to emerge from this analysis was that even though AD has been used for centuries, there are still research advances that can increase the breadth of AD application and the efficiency of AD biogas production.

One hindrance to AD adoption in remote locations is the inability to secure well established inoculum for timely AD startup. The findings suggest that it is possible for AD inoculum to be preserved using lyophilization in 10% skim milk media. The resulting preserved inoculum can startup a new AD, but there will be an increase in the lag phase of eight days before the biogas production stabilizes compared to fresh inoculum. The findings imply that there is potential to use preserved inoculum for AD startup in locations where transport of fresh, acclimated inoculum is infeasible due to either transportation cost or distance.

Another drawback to the use of AD is that the biogas, while rich in CH_4, also contains H_2S. Hydrogen sulfide is corrosive to metallic components of energy conversion technologies, and once combusted produces SO_2, a regulated air quality contaminant that contributes to the formation of acid rain. While many technologies exist that remove H_2S from the biogas, these technologies are typically cost prohibitive on a farm-scale and require a skilled operator to keep them maintained. The results of this work indicate that in-situ addition of Fe_2O_3, an iron(III)-oxide, to dairy manure digestion might reduce maintenance compared to other H_2S removal technologies and can reduce H_2S concentration in biogas by as much as 92%. The resulting H_2S levels were within

acceptable levels for combustion in engine generation sets (300 to 500 ppm). The implications of this research are that iron can be used as a low-tech in-situ method for H_2S reduction from dairy manure digestion while maintaining the energy content of the biogas.

Finally, this work sought to apply AD to waste from sectors that do not typically utilize AD. The two waste streams incorporated into this work were dairy manure as a substrate for sulfate reduction of high SO_4^{2-} wastewater and wash water from MSW being processed for cellulosic ethanol production.

Through the evaluation of addition of SO_4^{2-} and S^0 to dairy manure, it was determined that wastewaters high in SO_4^{2-} decrease the energy content (CH_4) of AD biogas. Even though biogas energy content was reduced by SO_4^{2-} addition to dairy manure, this study showed AD is a valid method for the reduction of SO_4^{2-}. Anaerobic digestion has the added benefit of CH_4 production that other biological SO_4^{2-} treatment technologies cannot offer.

Application of AD to the wastewater and process waters from cellulosic ethanol plants provides energy necessary to make cellulosic ethanol plants economically competitive. The findings in this work indicate that application of thermal pretreatment of MSW at 66 °C is sufficient to increase CH_4 production from MSW wash water, with no additional pretreatments necessary. Use of the CH_4 produced from treatment of the wash water could potentially provide 113% of the plant energy requirements, thus decreasing the overall cost of cellulosic ethanol production.

The goal of this research was to investigate ways decrease the barriers to AD adoption. This effort shows that through innovative pretreatment, preservation and

optimization techniques AD can be applied to a wider variety of problems and situations than are currently in practice.

6.2 Limitations

This work was performed at the lab scale in batch reactors using BMP tests. While BMPs provide a method for comparison of effects resulting from different perturbations to a system and the potential for a substrate to be utilized by a specific inoculum, inferences drawn from results of BMP studies may not necessarily be applicable to larger systems.

There were also limitations in the data collected for each of the chapters that would have provided insight into mechanisms that were not fully understood. In chapter 2 of this work was limited to CH_4 production recovery as an indicator that the microbial community in the AD inoculum survived. Actual microbial data would enhance this study and allow for a broader understanding of how preservation impacted the microbial communities. Microbial data is currently being processed and analyzed, but was not able to make it into the current study. The work in Chapter 3 included quantifying methanogen and SRB population size, but did not offer any information about the actual methanogenic or SRB communities. Not being able to determine if iron additions actually shifted the methanogenic and SRB communities weakened the discussion of the impacts of iron on the microbial population. Similarly, in Chapter4, the discussion of substrate utilization by SRB and the impact of sulfate addition on SRB was strictly hypothetical due to a lack of microbial data. In Chapter 4, there were limitations on the volume of samples that could be processes in the amount of time allotted, so the physical and

chemical pretreatments were limited to the 77 °C thermal temperature, even though the 66 °C thermal treatment ended up being the most cost effective treatment.

6.3 Future Work

In the current work, AD inoculum was successfully preserved and reactivated with 100% recovery of CH_4 production in a BMP test. The microbial data for the inoculum source comparison study is currently being processed. Analysis of the microbial data will answer the question of how microbial populations shifted after preservation, not just methanogens. The microbial data will also provide information about whether the same microbial shifts occurred in the three different inocula tested.

There is also hopes to expand the batch laboratory-scale preservation experiments to the pilot-scale. Increase in scale will provide more reliable knowledge on how much inoculum will need to be preserved to start up a full-scale reactor and the implications of using preserved inoculum on startup time.

Further research into the use of biogas from AD as cooking fuel is also of interest. In developing countries, AD is used for waste treatment and the biogas is used as a cooking fuel, replacing fossil fuel derived gasses such as propane. However, the H_2S in the biogas being utilized for cooking is not always scrubbed, which could be a health hazard due to the impact on indoor air quality. Inhalation of biogas containing H_2S, as well as SO_2 resulting from H_2S combustion, can lead to upper respiratory irritation and wheezing (Yeatts et al., 2012). Few studies have investigated the effects of utilizing AD biogas for cooking on indoor air quality compared to the use of propane or even biomass

burning. If findings indicate that H_2S is an issue, the results from the current work could be utilized to implement low-cost in-situ H_2S remediation.

Appendices

Appendix A: Supplementary Material for Microbial Preservation (Chapter 2)

Table A 1: Average cumulative CH_4 production and inoculum loading (g VS) for a biochemical methane potential test conducted using inoculum from three anaerobic digesters. The results are shown for five inoculum to substrate ratios (ISRs) without preservation. Treatments were conducted in triplicate with ± standard error shown. Uppercase superscripts indicate significant differences between treatments.

Inoculum Source	ISR[a]	Inoculum VS Concentration (g VS/L)	Methane Production (mL CH_4/g VS)
CO-DIG	0.67:1	0.350 ± 0.0001	97.1 ± 10.7^{C}
CO-DIG	1:1	0.525 ± 0.0002	199 ± 20^{C}
CO-DIG	2:1	1.05 ± 0.0003	274 ± 45^{C}
CO-DIG	4:1	2.10 ± 0.004	640 ± 84^{B}
CO-DIG	10:1	5.26 ± 0.04	$1{,}840 \pm 180^{A}$
WWTP	0.67:1	0.350 ± 0.0001	79.0 ± 5.8^{C}
WWTP	1:1	0.525 ± 0.0002	101 ± 4^{C}
WWTP	2:1	1.05 ± 0.0003	147 ± 15^{C}
WWTP	4:1	2.10 ± 0.004	216 ± 26^{C}
WWTP	10:1	5.26 ± 0.04	589 ± 86^{B}
DAIRY	0.67:1	0.350 ± 0.0001	95.3 ± 7.1^{C}
DAIRY	1:1	0.525 ± 0.0002	81.4 ± 9.9^{C}
DAIRY	2:1	1.05 ± 0.0003	169 ± 13^{C}
DAIRY	4:1	2.10 ± 0.004	228 ± 13^{C}
DAIRY	10:1	5.26 ± 0.04	684 ± 36^{B}

[a] ISR calculated using g VS of inoculum and g VS food waste added, food waste was held constant across all treatments at 0.533 g VS/L.

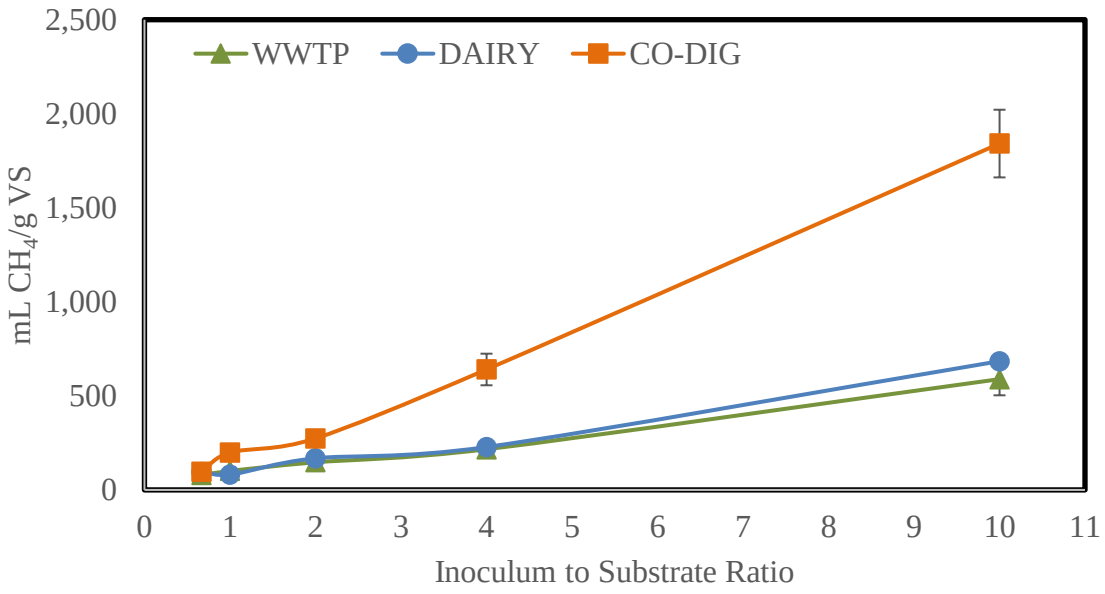

Figure A 1: Cumulative CH_4 production as a function of inoculum to substrate ratio for the three inocula tested without preservation. Treatments were conducted in triplicate and standard error is shown.

Appendix B: Supplemental Calculations for Iron and H_2S removal (Chapter 3)

H_2S Removal and Theoretical FeS Precipitation in the 20 and 50 mM Experiments

In order to determine the quantity of H_2S removed from the iron treatments in BMP1 and BMP2, the ideal gas law was used at 25 °C and atmospheric pressure:

$$n = \frac{PV}{RT},$$

Where: P = 1 atm, T = 298.15 K, R = universal gas constant, 0.0821 L-atm/mol-K, and V = Cumulative H_2S volumes over the duration of the BMP (L). The amount of H_2S removed for each iron treatment was determined by subtracting the mol H_2S produced from each of the iron treatments from the H_2S produced in the un-amended (Table B 1 and Table B 2).

Table B 1: Observed H_2S removed from 20 mM iron treatments compared to the un-amended manure control.

Treatment	mL H_2S	L H_2S	mol H_2S produced	mmol H_2S removed compared to manure
Manure	1.28E+00	1.28E-03	5.22E-05	NA
Fe_2O_3	4.34E-01	4.34E-04	1.77E-05	3.45E-02
$FeSO_4$	0.00E+00	0.00E+00	0.00E+00	5.22E-02
$FeCl_2$	0.00E+00	0.00E+00	0.00E+00	5.22E-02
ZVI	7.88E-01	7.88E-04	3.22E-05	2.00E-02

Table B 2: Observed H_2S removed from 50 mM iron treatments compared to the un-amended manure control.

Treatment	mL H_2S	L H_2S	mol H_2S produced	mmol H_2S removed compared to manure
Manure	2.93E+00	2.93E-03	1.20E-04	
Fe_2O_3	2.30E-01	2.30E-04	9.41E-06	4.28E-02
$FeSO_4$	1.12E-01	1.12E-04	4.58E-06	4.77E-02
$FeCl_2$	1.07E-01	1.07E-04	4.36E-06	4.79E-02
ZVI	3.32E-01	3.32E-04	1.36E-05	3.87E-02

In order to calculate the theoretical amount of H_2S that was precipitated out due to iron addition, the mechanism for H_2S precipitation was assumed to be driven by Fe^{2+} precipitation as FeS in the treatments. Aqueous Fe^{2+} concentrations were not measured in this study. Instead, the percent of total hematite (Fe_2O_3) added to digested samples resulting in aqueous Fe^{2+} dissolution in anaerobic samples measured by Chen et al. (2014) of 0.44% was used to calculate the reductive dissolution of Fe_2O_3 in this study. Similarly, the percent of total ZVI added to reactors in Zhang et al. (2011) that resulted in aqueous Fe^{2+} concentrations in the samples of 0.47% was used to calculate the Fe^{2+} that was solubilized from ZVI in this study. The mass of Fe^{2+} in the $FeCl_2$ and $FeSO_4$ samples in this study was assumed to be the total amount of Fe added to those treatments, as both chemicals were added to the treatments below their aqueous solubility masses.

The precipitation of FeS by Fe^{2+} and H_2S is governed by the following equation (Zhang et al., 2008):

$$Fe^{2+} + HS^- \xrightarrow{yields} FeS + H^+ \, , \Delta G = -21.0 \frac{KJ}{mol\ HS^-}$$

At a 1:1 molar ratio, the amount of sulfides precipitated out will be equal to the amount of aqueous Fe^{2+} in the system (Table B 3).

Table B 3: Theoretical and observed sulfide removal in 20 and 50 mM Iron treatments

	20 mM Iron[a]		**50 mM Iron[a]**	
Treatment	Theoretical aqueous Fe^{2+} (mmol)[b]	H_2S removed compared to manure (mmol)	Theoretical aqueous Fe^{2+} (mmol)[b]	H_2S removed compared to manure (mmol)
ZVI[b]	1.89E-02	2.00E-02	4.73E-02	3.87E-02
Fe_2O_3[c]	1.74E-02	3.45E-02	4.35E-02	4.28E-02
$FeCl_2$	4.00E+00	5.22E-02	1.00E+01	4.79E-02
$FeSO_4$	4.00E+00	5.22E-02	1.00E+01	4.77E-02

[a] Total volume of all bottles was 0.2 L
[b] Millimoles aqueous Fe^{2+} assumed to equal theoretical millimoles of sulfide precipitated
[c] Fe^{2+} concentration = 0.47% * 20 mM * 0.2 L (Zhang et al., 2008)
[d] Fe^{2+} concentration = 0.44% * 20 mM * 0.2 L (Chen et al., 2014)

Theoretical and Observed PO_4^{3-} Precipitation in the 50 mM Experiment

In order to determine the PO_4^{3-} precipitated out as vivianite in the 50 mM experiment, the remaining Fe^{2+} in the samples after preferential sulfide precipitation was determined by subtracting the observed H2S removed in the system from the theoretical Fe^{2+} in the system. Precipitation of PO_4^{3-} as vivianite is governed by the following equation:

$$3\ Fe^{2+} + 4\ PO_4^{3-} \xrightarrow{yields} Fe_3(PO_4)_2$$

At a 3:2 molar ratio, the amount of PO_4^{3-} precipitated out will be equal to 2/3 the Fe^{2+} remaining in the system after sulfide precipitation (Table B 4). The observed PO_4^{3-} removal was determined using the values measured in this study and were the difference between PO_4^{3-} produced in the manure treatment and the PO_4^{3-} produced in the iron treatments.

Table B 4: Theoretical and observed PO_4^{3-} removal in the 50 mM iron experiment.

Treatment	Theoretical aqueous Fe^{2+} (mmol)	H_2S removed compared to manure (mmol)	Amount of Fe^{2+} left for PO_4^{3-} precipitation (mmol)	Theoretical PO_4^{3-} precipitated (mmol)	Observed PO_4^{3-} removal (mmol)
ZVI	4.73E-02	3.87E-02	8.66E-03	5.77E-03	3.59E-01
Fe_2O_3*	4.35E-02	4.28E-02	6.83E-04	4.55E-04	2.81E-01
$FeCl_2$	1.00E+01	4.79E-02	9.95E+00	6.63E+00	5.20E-01
$FeSO_4$	1.00E+01	4.77E-02	9.95E+00	6.63E+00	5.20E-01

Visual MINTEQ Fe^{2+} Speciation Modeling in the 50 mM Experiment

As a quick investigation into how Fe^{2+} would speciate in the anaerobic systems in this study, Visual MINTEQ was used. While Visual MINTEQ is an equilibrium model, it was used as quick look at Fe^{2+} speciation using the macro-conditions in this experiment. Conditions that were selected included: pH = 6.92, the Eh = 8, the redox couple HS^- /SO_4^{2-} was selected, and the temperature = 35 °C. The concentrations of measured organics and inorganics from the experiment added to the model are in Table B 5, with model output in Table B 6 and Table B 7. The model predicted that the precipitated PO43- was in the form vivianite, with a total predicted concentration of 4.84E-04 mol/kg.

Table B 5: Total initial concentration of components included in Visual MINTEQ model

Component	Total Concentration (mM)
H+	0
E-	0
Fe2+	50
PO43-	0.969
SO42-	51
Acetate-	9.55
Butyrate-	1.27
Dissolved Organic Carbon	346
Dissolved Organic Matter	0
Propionate-	2.25
Valerate-	0.72
HS-	0

Table B 6: Concentrations of aqueous inorganic species and percent distribution among dissolved and adsorbed species.

Component	Total Component Concentration	% of total concentration	Species name
SO_4^{2-}	2.82E-02	55.5	SO_4^{2-}
		44.5	$FeSO_4$ (aq)
Valerate$^-$	7.15E-04	99.4	Valerate$^-$
		0.6	H-Valerate (aq)
Fe^{2+}	2.41E-02	49.7	Fe^{2+}
		3.5	Fe-Acetate$^+$
		0.2	$FeOH^+$
		46.7	$FeSO_4$ (aq)
Dissolved organic Matter	2.90E-02	97.2	DOM1
		2.8	H DOM1
Propionate$^-$	2.23E-03	99.3	Propionate$^-$
		0.7	H-Propionate (aq)
Acetate$^-$	7.83E-03	82.0	Acetate$^-$
		0.4	H-Acetate (aq)
		17.6	Fe-Acetate$^+$
Butyrate$^-$	1.26E-03	99.4	Butyrate$^-$
		0.6	H-Butyrate (aq)
DOC (Gaussian DOM)	3.46E-01	100.0	DOC (Gaussian DOM)
HS^-	1.21E-32	0.0	HS-1
		0.0	H2S (aq)
		100.0	FeHS+
PO_4^{3-}	1.84E-15	4.8	HPO4-2
		4.1	H2PO4-
		22.0	FeH2PO4+
		69.1	FeHPO4 (aq)

Table B 7: Distribution of components between dissolved and precipitated phases

Component	Total dissolved (mM)	% dissolved	Total precipitated (mmol/kg)	% precipitated
Fe^{2+}	4.85E-02	97.1	1.45E-03	2.91
PO_4^{3-}	2.44E-09	0	9.69E-04	100

Appendix C: Supplemental SO_4^{2-} Removal and H_2S Calculations for Chapter 4

SO_4^{2-} Removal in BMP1 and BMP2

Table C 1: Mass of SO_4^{2-} removed in BMP1 for K_2SO_4, $FeSO_4$, and manure treatments

Treatment	SO_4^{2-} in pre-digestion samples (mg/L)	SO_4^{2-} in post-digestion samples (mg/L)	SO_4^{2-} in pre-digestion samples (mg)	SO_4^{2-} in post-digestion samples (mg)	SO_4^{2-} removed (mg)	mol S removed
31 mM K_2SO_4	3024	1425	605	285	320	3.33E-03
27 mM K_2SO_4	2615	812	523	162.5	360	3.75E-03
21 mM K_2SO_4	2028	409.0	406	81.80	323.8	3.37E-03
18 mM K_2SO_4	1766	123.8	353.1	24.76	328.4	3.42E-03
16 mM K_2SO_4	1514	99.0	302.9	19.80	283.1	2.95E-03
21 mM $FeSO_4$	2059.3	1240.2	411.9	248.05	163.8	1.71E-03
Manure	75.2	9.50	15.0	1.90	13.1	1.37E-04

Table C 2: Mass of SO_4^{2-} removed in BMP2 for $FeSO_4$, S^0, and manure treatments

Treatment	SO_4^{2-} in pre-digestion samples (mg/L)	SO_4^{2-} in post-digestion samples (mg/L)	SO_4^{2-} in pre-digestion samples (mg)[1]	SO_4^{2-} in post-digestion samples (mg)[1]	SO_4^{2-} removed (mg)	mol S removed[2]
50 mM $FeSO_4$	5142	2690	1028	538	490	5.10E-03
20 mM $FeSO_4$	2110	373	422	74.6	347	3.62E-03
5 mM $FeSO_4$	519	37.6	104	7.53	96.3	1.00E-03
2 mM $FeSO_4$	288	14.5	57.7	2.91	54.7	5.70E-04
50 mM S^0	203	17.4	40.6	3.48	37.1	3.86E-04
20 mM S^0	78.7	10.3	15.7	2.06	13.7	1.42E-04
5 mM S^0	67.9	8.38	13.6	1.68	11.9	1.24E-04
2 mM S^0	62.0	7.88	12.4	1.58	10.8	1.13E-04
Manure	77.5	14.3	15.5	2.87	12.6	1.31E-04

[1] Total volume of each bottle was 0.2 L

[2] Calculated based on m.w of SO_4^{2-}=96.06 g/mol and 1 mol SO_4^{2-}/mol S

H_2S Production from S^0 treatments in BMP2

The moles H_2S produced by the S^0 treatments were calculated using the method outlined in Appendix B (Table C 3). Then the percent of H_2S production accounted for by both SO_4^{2-} reduction (Table C 2) and S^0 addition was calculated on a molar basis (Table C 3).

Table C 3: Molar quantity of H_2S produced in S^0 treatments from BMP2

Treatment	H_2S (mL)	H_2S (L)	H_2S (mol)	S^0 added (mol)	SO_4^{2-} removed as H_2S (%)	H_2S from S^0 reduction (%)
50 mM S^0	48.1	4.81E-02	1.96E-03	1.00E-02	508	20%
20 mM S^0	21.7	2.17E-02	8.86E-04	4.00E-02	623	22%
5 mM S^0	5.7	5.68E-03	2.32E-04	1.00E-03	187	23%
2 mM S^0	2.7	2.73E-03	1.12E-04	4.00E-04	99	28%

Appendix D: Supplemental Calculations for Chapter 5

Tween™ 85 Surfactant CH_4 Normalization

In order to separate out the CH_4 produced from the organics in the surfactant and the CH_4 produced by the organics solubilized from the MSW in the Tween™ 85 treatments, the CH_4 production normalized by the total COD in the wastewater was multiplied by a COD correction factor.

For the COD correction factor, the g COD of the surfactant added to the 0.001, 0.1, and 10% Tween™ 85 treatments was subtracted from the total g COD in the wastewater, which contained the COD from the surfactant and the COD from the solubilized MSW. The remaining g COD was assumed to be the g COD of the solubilized MSW. Then, the g COD of the solubilized MSW from the Tween™ 85 treatments was divided by the g MSW that was treated giving g COD of solubilized MSW/g MSW treated. This correction factor was multiplied by the mL CH_4/g COD wastewater to provide a proxy for CH_4 production in the Tween™ 85 treatments due to organics solubilized from the MSW (Table D 1).

Table D 1: COD in Tween™ 85 treatments corrected for COD from Tween™ 85 addition

Treatment	Total COD in wastewater (g/L)	Tween™ 85/treatment (mL)	Tween™ 85/treatment (L)	Total volume of wastewater (L)	COD Tween™ 85 (g)	COD wastewater (g)	COD from MSW (g)	COD MSW (g /L)	MSW treatment density (g/L)	COD correction Factor (g COD/g MSW)
0.001 % v/v Tween™ 85	1.60	3.50E-03	3.50E-06	2.5	7.44E-03	4.00	3.99	1.60	87.4	1.83E-02
0.001 % v/v Tween™ 85	2.27	4.64E-03	4.64E-06	2.5	9.88E-03	5.67	5.66	2.26	116	1.95E-02
0.001 % v/v Tween™ 85	3.30	4.05E-03	4.05E-06	2.5	8.60E-03	8.25	8.24	3.30	101	3.26E-02
0.1 % v/v Tween™ 85	2.33	3.75E-01	3.75E-04	2.5	7.97E-01	5.83	5.04	2.01	93.7	2.15E-02
0.1 % v/v Tween™ 85	2.63	3.75E-01	3.75E-04	2.5	7.97E-01	6.58	5.79	2.31	93.7	2.47E-02
0.1 % v/v Tween™ 85	3.43	3.96E-01	3.96E-04	2.5	8.42E-01	8.58	7.74	3.10	99.0	3.13E-02
10 % v/v Tween™ 85	43.9	46.6	4.66E-02	2.5	99.1	110	10.6	4.25	117	3.65E-02
10 % v/v Tween™ 85	41.9	44.4	4.44E-02	2.5	94.3	105	10.4	4.17	111	3.76E-02
10 % v/v Tween™ 85	44.1	47.8	4.78E-02	2.5	102	110	8.62	3.45	120	2.88E-02

CH_4 from Tween™ 85 Treatments Normalized by g MSW Without COD Correction

As a comparison, the CH_4 produced in the Tween™ 85 treatments was normalized by the g MSW without the correction factor (Figure D 1)

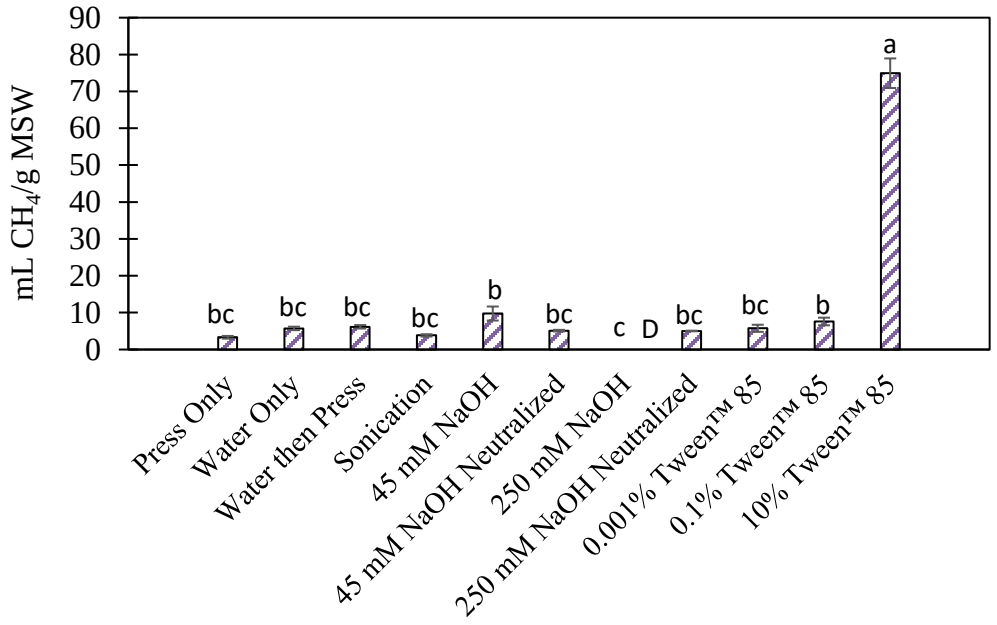

Figure D 1: The cumulative CH_4 production from pretreatments normalized by g MSW without accounting for COD of Tween™ 85 in the Tween™ 85 treatments. Treatments were conducted in triplicate with ± standard error shown. Lowercase letters represent significant differences among treatments

Detailed Calculations for Energy Savings from Digestion of MSW wash water from Anaerobic Digestion

Data for this calculation were based on:

- Abengoa Bioenergy's production estimate that 25,000 tons of MSW will produce 1.5 million liters of bioethanol for use as fuel (Malo de Molina Melendez, 2013).

- The cellulosic ethanol plant energy requirements for distillation of one million gallons of ethanol of **1.79 x 10^7 MJ** (Tian et al., 2013).

First the mass of MSW required to generate one million gallons of ethanol was determined:

$$\left(\frac{25{,}000\ tons\ MSW}{1{,}500{,}000\ Liters\ Ethanol}\right)\left(\frac{2{,}000\ lbs}{ton}\right)\left(\frac{Liter}{0.264\ gal}\right)\left(\frac{453.6\ grams}{1\ lb}\right)(1{,}000{,}000\ gallons\ of\ ethanol)$$

$$= \mathbf{5.72\ x\ 10^{10}\ g\ MSW}$$

Then data from this study were used to determine the theoretical energy production from AD of the MSW thermally treated at 66 °C with subsequent washing at 1:5 solids:water ratio.

- Volume of wastewater generated per mass of MSW pretreated: 0.012 L wastewater/g MSW (This study)
- COD of wastewater: 3.83 g COD/L wastewater (This study)
- CH_4 production from the 66 °C thermal treatment: 253 mL CH_4/g COD

$$(5.72\ x\ 10^{10} g\ MSW)\left(\frac{0.012\ L\ wastewater}{g\ MSW}\right)\left(\frac{3.83\ g\ COD}{L\ wastewater}\right)\left(\frac{253\ mL\ CH_4}{g\ COD}\right)\left(\frac{L}{1000\ mL}\right)\left(\frac{m^3}{1000\ L}\right)$$

$$= \mathbf{6.45\ x\ 10^5\ m^3 CH_4}$$

Finally, energy conversions were used to determine the energy and cost savings from utilizing AD at the cellulosic ethanol plant to treat the wastewater from MSW pretreatment:

- CH_4 conversion to MJ of 31.46 MJ/m^3 CH_4, and a kWh/MJ of 0.278 (Nayono et al., 2010b)
- Engine generation efficiency of 50%
- Electricity cost of $0.12/kWh

$$(6.45\ x\ 10^5\ m^3CH_4)\left(\frac{31.46\ MJ}{m^3CH_4}\right) = \mathbf{2.03\ x\ 10^7\ MJ}$$

$$(2.03\ x\ 10^7\ MJ)(50\%)\left(\frac{0.278\ kWh}{MJ}\right)\left(\frac{\$0.12}{kWh}\right) = \$\mathbf{339,000\ savings}$$

Bibliography

1. Abadias, M., Teixido, N., Usall, J., Benabarre, A., Vinas, L., 2001. Viability, efficacy, and storage stability of freeze-dried biocontrol agent candida sake using different protective and rehydration media. J. Food Prot. 64(6), 856-861.
2. Achtnich, C., Bak, F., Conrad, R., 1995. Competition for electron donors among nitrate reducers, ferric iron reducers, sulfate reducers, and methanogens in anoxic paddy soil. Biology and Fertility of Soils. 19, 65-72.
3. Agrawal, L.K., Jayadevan, J.Y., Harada, H., Nakamura, K., 1997. Utilization of dried pelletized anaerobic sludge for anaerobic treatment of wastewater. J. Ferment. and Bioeng. 83(1), 91-95.
4. Akcil, A., Koldas, S., 2006. Acid mine drainage (amd): Causes, treatment and case studies. Journal of Cleaner Production. 14(12-13), 1139-1145.
5. Albers, S.V., Meyer, B.H., 2011. The archaeal cell envelope. Nat Rev Microbiol. 9(6), 414-26.
6. Alvarado, A., Montanez-Hernandez, L.E., Palacio-Molina, S.L., Oropeza-Navarro, R., Luevanos-Escareno, M.P., Balagurusamy, N., 2014. Microbial trophic interactions and mcra gene expression in monitoring of anaerobic digesters. Front Microbiol. 5, 597.
7. Amani, T., Nosrati, M., Sreekrishnan, T.R., 2010. Anaerobic digestion from the viewpoint of microbiological, chemical, and operational aspects — a review. Environ. Rev. 18, 255-278.
8. Anderson, K.L., Apolinario, E.E., Sowers, K.R., 2012. Desiccation as a long-term survival mechanism for the archaeon methanosarcina barkeri. Appl. Environ. Microbiol. 78(5), 1473-9.
9. Angelidaki, I., Chen, X., Cui, J., Kaparaju, P., Ellegaard, L., 2006. Thermophilic anaerobic digestion of source-sorted organic fraction of household municipal solid waste: Start-up procedure for continuously stirred tank reactor. Water Res. 40, 2621-8.
10. APHA, 2005. Standard methods for the examination of water and wastewater. 21st ed. American Public Health Association, Washington, D.C. USA. :.
11. Appels, L., Degreve, J., Van der Bruggen, B., Van Impe, J., Dewil, R., 2010. Influence of low temperature thermal pre-treatment on sludge solubilisation, heavy metal release and anaerobic digestion. Bioresour Technol. 101(15), 5743-8.
12. Ariunbaatar, J., Panico, A., Esposito, G., Pirozzi, F., Lens, P.N.L., 2014. Pretreatment methods to enhance anaerobic digestion of organic solid waste. Applied Energy. 123, 143-156.
13. Atabani, A.E., Silitonga, A.S., Badruddin, I.A., Mahlia, T.M.I., Masjuki, H.H., Mekhilef, S., 2012. A comprehensive review on biodiesel as an alternative energy resource and its characteristics. Renewable and Sustainable Energy Reviews. 16(4), 2070-2093.
14. Bachmann, S., Uptmoor, R., Eichler-Löbermann, B., 2016. Phosphorus distribution and availability in untreated and mechanically separated biogas digestates. Scientia Agricola. 73(1), 9-17.

15. Barlindhaug, J., Odegaard, H., 1996. Thermal hydrolysis for the production of carbon source for denitrification. Water Science & Technology. 34(1-2), 371-378.
16. Barta, Z., Reczey, K., Zacchi, G., 2010. Techno-economic evaluation of stillage treatment with anaerobic digestion in a softwood-toethanol process. Biotechnology for Biofuels. 3(21), 1-11.
17. Bogner, J., Pipatti, R., Hashimoto, S., Diaz, C., Mareckova, K., Diaz, L., Kjeldsen, P., Monni, S., Faaij, A., Gao, Q., Zhang, T., Ahmed, M.A., Sutamihardja, R.T., Gregory, R., Intergovernmental Panel on Climate Change Working, G., III, 2008. Mitigation of global greenhouse gas emissions from waste: Conclusions and strategies from the intergovernmental panel on climate change (ipcc) fourth assessment report. Working group iii (mitigation). Waste Manag Res. 26(1), 11-32.
18. Bond, D.R., Lovely, D.R., 2002. Reduction of fe(iii) oxide by methanogens in the presence and absence of extracellular quinones. Environmental Microbiology. 4(2), 115-124.
19. Bougrier, C., Albasi, C., Delgenès, J.P., Carrère, H., 2006. Effect of ultrasonic, thermal and ozone pre-treatments on waste activated sludge solubilisation and anaerobic biodegradability. Chemical Engineering and Processing: Process Intensification. 45(8), 711-718.
20. Bougrier, C., Delgenès, J.P., Carrère, H., 2008. Effects of thermal treatments on five different waste activated sludge samples solubilisation, physical properties and anaerobic digestion. Chemical Engineering Journal. 139(2), 236-244.
21. Carpenter, A.W., Laughton, S.N., Wiesner, M.R., 2015. Enhanced biogas production from nanoscale zero valent iron-amended anaerobic bioreactors. Environ Eng Sci. 32(8), 647-655.
22. Castro, H., Queirolo, M., Quevedo, M., Muxi, L., 2002. Preservation methods for the storage of anaerobic sludges. Biotechnol. Lett. 24, 329-333.
23. Chen, T.H., Wang, J., Zhou, Y.F., Yue, Z.B., Xie, Q.Q., Pan, M., 2014. Synthetic effect between iron oxide and sulfate mineral on the anaerobic transformation of organic substance. Bioresour Technol. 151, 1-5.
24. Cheng, X., Chen, B., Cui, Y., Sun, D., Wang, X., 2015. Iron(iii) reduction-induced phosphate precipitation during anaerobic digestion of waste activated sludge. Separation and Purification Technology. 143, 6-11.
25. Chester, M., Martin, e., 2009. Cellulosic ethanol from municipal solid waste: A case study of the economic, energy, and greenhouse gas impacts in california. Environmental Science & Technology. 43(14), 5183-5189.
26. Chou, H.-H., Huang, J.-S., Chen, W.-G., Ohara, R., 2008. Competitive reaction kinetics of sulfate-reducing bacteria and methanogenic bacteria in anaerobic filters. Bioresource Technology. 99, 8061-8067.
27. Choudhary, R.P., Sheoran, A.S., 2012. Performance of single substrate in sulphate reducing bioreactor for the treatment of acid mine drainage. Minerals Engineering. 39, 29-35.
28. Chu, C.P., Lee, D.J., Chang, B.-V., You, C.S., Tay, J.H., 2002. "Weak" ultrasonic pre-treatment on anaerobic digestion of flocclated activated biosolids. Water Research. 36, 2681-2688.

29. Cocos, I.A., Zagury, G.J., Clement, B., Samson, R., 2002. Multiple factor design for reactive mixture selection for use in reactive walls in mine drainage treatment. Water Research. 32, 167-177.
30. Colleran, E., Concannon, F., Golden, T., Geoghegan, F., Crumlish, B., Killilea, E., Henry, M., Coates, J., 1992. Use of methanogenic activity tests to characterize anaerobic sludges, screen for anaerobic biodegradability, and determine toxicity thresholds aganist individual anaerobic trophic groups and species. Wat. Sci. Tech. 25(7), 31-40.
31. Corcoran, B.M., Ross, R.P., Fitzgerald, G.F., Stanton, C., 2004. Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. J. Appl. Microbiol. 96, 1024-39.
32. Costa, E., Usall, J., Teixido, N., Garcia, N., Vinas, I., 2000. Effect of protective agents, rehydration media and initial cell concentration on viability of pantoea agglomerans strain cpa-2 subjected to freeze-drying. J. Appl. Microbiol. 89, 793-800.
33. Dahiya, S., Sarkar, O., Swamy, Y.V., Mohan, S.V., 2015. Acidogenic fermentation of food waste for volatile fatty acid production with co-generation of biohydrogen. Bioresour Technol. 182, 103-13.
34. Dar, S.A., Kleerebezem, R., Stams, A.J., Kuenen, J.G., Muyzer, G., 2008. Competition and coexistence of sulfate-reducing bacteria, acetogens and methanogens in a lab-scale anaerobic bioreactor as affected by changing substrate to sulfate ratio. Appl Microbiol Biotechnol. 78(6), 1045-55.
35. de Smul, A., Goethals, L., Verstraete, W., 1999. Effect of cod to sulphate ratio and temperature in expanded-granular-sludge-blanket reactors for sulphate reduction. Process Biochemistry. 34, 407-416.
36. Demirel, B., Scherer, P., 2011. Trace element requirements of agricultural biogas digesters during biological conversion of renewable biomass to methane. Biomass and Bioenergy. 35(3), 992-998.
37. Dezham, P., Rosenblum, E., Jenkins, D., 1988. Digester gas h2s control using iron salts. Water Environment Federation. 60(4), 514-517.
38. El-Fadel, M., Saikaly, P., Ghanimeh, S., 2013. Startup and stability of thermophilic anaerobic digestion of ofmsw. Crit. Rev. Environ. Sci. Technol. 43(24), 2685-2721.
39. El-mashad, H.M., Zeeman, G., van Loon, W.K.P., Bot, G.P.A., Lettinga, G., 2003. Anaerobic digestion of solid animal waste in an accumulation system at mesophilic and thermophilic conditions, start up. Wat. Sci. Tech. 48(4), 217-220.
40. Elbeshbishy, E., Nakhla, G., Hafez, H., 2012. Biochemical methane potential (bmp) of food waste and primary sludge: Influence of inoculum pre-incubation and inoculum source. Bioresour. Technol. 110, 18-25.
41. Elliott, A., Mahmood, T., 2012. Comparison of mechanical pretreatment methods for the enhancement of anaerobic digestion of pulp and paper waste activated sludge. Water Environment Research. 84(6), 497-505.
42. EPA. 2016. Inventory of u.S. Greenhouse gas emissions and sinks: 1990-2014.

43. Fantozzi, F., Buratti, C., 2011. Anaerobic digestion of mechanically treated ofmsw: Experimental data on biogas/methane production and residues characterization. Bioresour Technol. 102(19), 8885-92.
44. FAO. 2017. Key facts and findings, by the numbers: Ghg emissions by livestock, Vol. 2017, Food and Agriculture Organization of the United Nations.
45. Firer, D., Friedler, E., Lahav, O., 2008. Control of sulfide in sewer systems by dosage of iron salts: Comparison between theoretical and experimental results, and practical implications. Sci Total Environ. 392(1), 145-56.
46. Forster-Carneiro, T., Perez, M., Romero, L.I., Sales, D., 2007. Dry-thermophilic anaerobic digestion of organic fraction of the municipal solid waste: Focusing on the inoculum sources. Bioresour. Technol. 98, 3195-3203.
47. Freitag, T.E., Prosser, J.I., 2009. Correlation of methane production and functional gene transcriptional activity in a peat soil. Appl Environ Microbiol. 75(21), 6679-87.
48. Garcia, M.T., Campos, E., Sanchez-Leal, J., Ribosa, I., 2000. Anaerobic degradation and toxicity of commercial cationic surfactants in anaerobic screening tests. Chemosphere. 41, 705-710.
49. Gáspár, M., Kálmán, G., Réczey, K., 2007. Corn fiber as a raw material for hemicellulose and ethanol production. Process Biochemistry. 42(7), 1135-1139.
50. Gavala, H.N., Ahring, B.K., 2002. Inhibition of the anaerobic digestion process by linear alkylbenzene sulfonates. Bidegradation. 13, 201-209.
51. Gavala, H.N., Yenal, U., Skiadas, I.V., Westermann, P., Ahring, B.K., 2003. Mesophilic and thermophilic anaerobic digestion of primary and secondary sludge. Effect of pre-treatment at elevated temperature. Water Research. 37(19), 4561-4572.
52. Ghanimeh, S., El Fadel, M., Saikaly, P., 2012. Mixing effect on thermophilic anaerobic digestion of source-sorted organic fraction of municipal solid waste. Bioresour. Technol. 117, 63-71.
53. Ghosh, S., Henry, M.P., Sajjad, A., Mensinger, M.C., Arora, J.L., 2000. Pilot-scale gasification of municipal solid wastes by high-rate and two-phase anaerobic digestion (tpad). Water Science and Technology. 41(3), 101-110.
54. Gianico, A., Braguglia, C.M., Cesarini, R., Mininni, G., 2013. Reduced temperature hydrolysis at 134 c before thermophilic anaerobic digestion of waste activated sludge at increasing organic load. Bioresource Technology. 143, 96-103.
55. Gianico, A., Braguglia, C.M., Gallipoli, A., Mininni, G., 2015. Innovative two-stage mesophilic/thermophilic anaerobic degradation of sonicated sludge: Performances and energy balance. Environmental Science and Pollution Research. 22(10), 7248-7256.
56. Gibert, O., de Pablo, J., Luis Cortina, J., Ayora, C., 2004. Chemical characterisation of natural organic substrates for biological mitigation of acid mine drainage. Water Res. 38(19), 4186-96.
57. Haaning Nielsen, A., Lens, P., Vollertsen, J., Hvitved-Jacobsen, T., 2005. Sulfide-iron interactions in domestic wastewater from a gravity sewer. Water Res. 39(12), 2747-55.

58. Hamzawi, N., Kennedy, K.J., McLean, D.D., 1998. Anaerobic digestion of co-mingled municipal solid waste and sewage sludge. Water Science & Technology. 38(2), 127-132.
59. Hao, T.W., Xiang, P.Y., Mackey, H.R., Chi, K., Lu, H., Chui, H.K., van Loosdrecht, M.C., Chen, G.H., 2014. A review of biological sulfate conversions in wastewater treatment. Water Res. 65, 1-21.
60. Hargreaves, S.K., Roberto, A.A., Hofmockel, K.S., 2013. Reaction- and sample-specific inhibition affect standardization of qpcr assays of soil bacterial communities. Soil Biology and Biochemistry. 59, 89-97.
61. Heylen, K., Ettwig, K., Hu, Z., Jetten, M., Kartal, B., 2012. Rapid and simple cryopreservation of anaerobic ammonium-oxidizing bacteria. Appl. Environ. Microbiol. 78(8), 3010-3.
62. Holford, I.C.R., 1997. Soil phosphorus: Its measurement, and its uptake by plants. Australian Journal of Soil Research. 35, 227-39.
63. Hu, Q., Fan, L., Gao, D., 2017. Pilot-scale investigation on the treatment of cellulosic ethanol biorefinery wastewater. Chemical Engineering Journal. 309, 409-416.
64. Hughes, T.A., Gray, N.F., 2013. Co-treatment of acid mine drainage with munivipal wastewater: Performance evaluation. Environ Sci Pollut Res. 20, 7863-7877.
65. Hulshoff Pol, L.W., lens, P.N.L., Stams, A.J.M., Lettinga, G., 1998. Anaerobic treatment of sulfate-rich wastewaters. Biodegradation. 9, 213-224.
66. Ike, M., Inoue, D., Miyano, T., Liu, T.T., Sei, K., Soda, S., Kadoshin, S., 2010. Microbial population dynamics during startup of a full-scale anaerobic digester treating industrial food waste in kyoto eco-energy project. Bioresour. Technol. 101, 3952-7.
67. Jain, S., Jain, S., Wolf, I.T., Lee, J., Tong, Y.W., 2015. A comprehensive review on operating parameters and different pretreatment methodologies for anaerobic digestion of municipal solid waste. Renewable and Sustainable Energy Reviews. 52, 142-154.
68. Jiang, H., Li, T., Stinner, W., Nie, H., Ding, J., Zhou, H., 2017. Selection of in-situ desulfurizers for chicken manure biogas and prediction of dosage. Polish Journal of Environmental Studies. 26(1), 155-161.
69. Jin, Y., Li, Y., Li, J., 2016. Influence of thermal pretreatment on physical and chemical properties of kitchen waste and the efficiency of anaerobic digestion. J Environ Manage. 180, 291-300.
70. Kaparaju, P., Serrano, M., Thomsen, A.B., Kongjan, P., Angelidaki, I., 2009. Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresour Technol. 100(9), 2562-8.
71. Kapdi, S.S., Vijay, V.K., Rajesh, S.K., Prasad, R., 2005. Biogas scrubbing, compression and storage: Perspective and prospectus in indian context. Renewable Energy. 30(8), 1195-1202.
72. Karakashev, D., Batstone, D.J., Angelidaki, I., 2005. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Appl Environ Microbiol. 71(1), 331-8.

73. Kato, S., Hashimoto, K., Watanabe, K., 2012. Methanogenesis facilitated by electric syntrophy via (semi)conductive iron-oxide minerals. Environ Microbiol. 14(7), 1646-54.
74. Katsoyannos, E., Gortzi, O., Chatzilazarou, A., Athanasiadis, V., Tsaknis, J., Lalas, S., 2012. Evaluation of the suitability of low hazard surfactants for the separation of phenols and carotenoids from red-flesh orange juice and olive mill wastewater using cloud point extraction. J Sep Sci. 35(19), 2665-70.
75. Khalid, A., Arshad, M., Anjum, M., Mahmood, T., Dawson, L., 2011. The anaerobic digestion of solid organic waste. Waste. Manag. 31, 1737-44.
76. Kim, J., Park, C., Kim, T.-H., Lee, M., Kim, S., Kim, S.-W., Lee, J., 2003. Effects of various pretreatments for enhanced anaerobic digestion with waste activated sludge. Journal of Bioscience and Bioengineering. 95(3), 271-275.
77. Kim, T.H., Kim, T.H., 2014. Overview of technical barriers and implementation of cellulosic ethanol in the u.S. Energy. 66, 13-19.
78. Kobayashi, T., Yasuda, D., Li, Y.Y., Kubota, K., Harada, H., Yu, H.Q., 2009. Characterization of start-up performance and archaeal community shifts during anaerobic self-degradation of waste-activated sludge. Bioresour. Technol. 100, 4981-8.
79. Komemoto, K., Lim, Y.G., Nagao, N., Onoue, Y., Niwa, C., Toda, T., 2009. Effect of temperature on vfa's and biogas production in anaerobic solubilization of food waste. Waste Manag. 29(12), 2950-5.
80. Krayzelova, L., Bartacek, J., Díaz, I., Jeison, D., Volcke, E.I.P., Jenicek, P., 2015. Microaeration for hydrogen sulfide removal during anaerobic treatment: A review. Reviews in Environmental Science and Bio/Technology. 14(4), 703-725.
81. Kuglarz, M., Karakashev, D., Angelidaki, I., 2013. Microwave and thermal pretreatment as methods for increasing the biogas potential of secondary sludge from municipal wastewater treatment plants. Bioresour Technol. 134, 290-7.
82. Kuo, W.C., Cheng, K.Y., 2007. Use of respirometer in evaluation of process and toxicity of thermophilic anaerobic digestion for treating kitchen waste. Bioresour Technol. 98(9), 1805-11.
83. Lee, D.H., Behera, S.K., Kim, J.W., Park, H.S., 2009. Methane production potential of leachate generated from korean food waste recycling facilities: A lab-scale study. Waste Manag. 29(2), 876-82.
84. Li, A., Antizar-Ladislao, B., Khraisheh, M., 2007. Bioconversion of municipal solid waste to glucose for bio-ethanol production. Bioprocess Biosyst Eng. 30(3), 189-96.
85. Li, B., Tian, F., Liu, X., Zhao, J., Zhang, H., Chen, W., 2011. Effects of cryoprotectants on viability of lactobacillus reuteri cicc6226. Appl Microbiol Biotechnol. 92(3), 609-16.
86. Li, H., Li, C., Liu, W., Zou, S., 2012. Optimized alkaline pretreatment of sludge before anaerobic digestion. Bioresour Technol. 123, 189-94.
87. Li, J., Zicari, S.M., Cui, Z., Zhang, R., 2014. Processing anaerobic sludge for extended storage as anaerobic digester inoculum. Bioresour. Technol. 166, 201-10.

88. Li, Q., Li, Y.Y., Qiao, W., Wang, X., Takayanagi, K., 2015. Sulfate addition as an effective method to improve methane fermentation performance and propionate degradation in thermophilic anaerobic co-digestion of coffee grounds, milk and waste activated sludge with anmbr. Bioresour Technol. 185, 308-15.
89. Lin, J.-G., Chang, C.-N., Chang, S.-C., 1997. Enhancement of anaerobic digestion of waste activated sludge by alkaline solubilization. Bioresource Technology. 62, 85-90.
90. Lin, Y., Wang, D., Wu, S., Wang, C., 2009. Alkali pretreatment enhances biogas production in the anaerobic digestion of pulp and paper sludge. J Hazard Mater. 170(1), 366-73.
91. Liu, G., Zhang, R., El-Mashad, H.M., Dong, R., 2009. Effect of feed to inoculum ratios on biogas yields of food and green wastes. Bioresour Technol. 100(21), 5103-8.
92. Liu, X., Wang, W., Gao, X., Zhou, Y., Shen, R., 2012. Effect of thermal pretreatment on the physical and chemical properties of municipal biomass waste. Waste Manag. 32(2), 249-55.
93. Liu, Y., Zhang, Y., Ni, B.J., 2015. Zero valent iron simultaneously enhances methane production and sulfate reduction in anaerobic granular sludge reactors. Water Res. 75, 292-300.
94. Lovley, D.R., Phillips, J.P., 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Applied and Environmental Microbiology. 51(4), 683-689.
95. Lu, Q., He, Z.L., Stoffella, P.J., 2012. Land application of biosolids in the USA: A review. Applied and Environmental Soil Science. 2012, 1-11.
96. Lu, X., Zhen, G., Ni, J., Hojo, T., Kubota, K., Li, Y.Y., 2016. Effect of influent cod/so4(2-) ratios on biodegradation behaviors of starch wastewater in an upflow anaerobic sludge blanket (uasb) reactor. Bioresour Technol. 214, 175-83.
97. Malo de Molina Melendez, P. 2013. Abengoa inaugurates its first demonstration plant using waste-to-bioruels (w2b) technology, Vol. 2017, Abengoa Bioenergy, S.A.
98. Mao, X., Jiang, R., Xiao, W., Yu, J., 2015. Use of surfactants for the remediation of contaminated soils: A review. J Hazard Mater. 285, 419-35.
99. Massalha, N., Brenner, A., Sheindorf, C., Sabbah, I., 2015. Application of immobilized and granular dried anaerobic biomass for stabilizing and increasing anaerobic bio-systems tolerance for high organic loads and phenol shocks. Bioresour. Technol. 197, 106-12.
100. Massalha, N., Brenner, A., Sheindorf, C., Sabbah, I., 2014. The effect of anaerobic biomass drying and exposure to air on their recovery and evolution. Water Res. 63, 42-51.
101. Masse, L., Kennedy, K.J., Chou, S., 2001. Testing of alkaline and enzymatic hydrolysis pretreatments for fat particles in slaughterhouse wastewater. Bioresource Technology. 77, 145-155.
102. McCartney, D.M., Oleszkiewicz, J.A., 1993. Competition between methanogens and sulfate reducers: Effect of cod:Sulfate ratio and acclimation. Water Environment Research. 65(5), 655-664.

103. Möller, K., Müller, T., 2012. Effects of anaerobic digestion on digestate nutrient availability and crop growth: A review. Engineering in Life Sciences. 12(3), 242-257.
104. Moody, L.B., Burns, R.T., Bishop, G., Sell, S.T., Spajic, R., 2011. Using biochemical methane potential assays to aid in co-substrate selection for co-digestion. Appl. Eng. in Agric. 27(3), 433-439.
105. Morgan, C.A., Herman, N., White, P.A., Vesey, G., 2006. Preservation of micro-organisms by drying; a review. J. of Microbiol. Methods. 66, 183-193.
106. Mortain-Bertrand, A., Etchart, F., de Boucaud, M.-T., 1996. A method for the cryoconservation of dunaliella salina (chlorophyceae): Effect of glycerol and cold adaptation. J. Phycol. 32, 346-352.
107. Nayono, S.E., Gallert, C., Winter, J., 2010a. Co-digestion of press water and food waste in a biowaste digester for improvement of biogas production. Bioresour Technol. 101(18), 6998-7004.
108. Nayono, S.E., Winter, J., Gallert, C., 2010b. Anaerobic digestion of pressed off leachate from the organic fraction of municipal solid waste. Waste Manag. 30(10), 1828-33.
109. Nkoa, R., 2013. Agricultural benefits and environmental risks of soil fertilization with anaerobic digestates: A review. Agronomy for Sustainable Development. 34(2), 473-492.
110. O'Flaherty, V., Lens, P., Leahy, B., Colleran, E., 1998. Long-term competition between sulphate-reducing and methane-producing bacteria during full-scale anaerobic treatment of citric acid production wastewater. Water Resources. 32(3), 815-825.
111. Osorio, F., Torres, J.C., 2009. Biogas purification from anaerobic digestion in a wastewater treatment plant for biofuel production. Renewable Energy. 34(10), 2164-2171.
112. Owen, J.J., Silver, W.L., 2015. Greenhouse gas emissions from dairy manure management: A review of field-based studies. Glob Chang Biol. 21(2), 550-65.
113. Owen, W.F., Stuckey, D.C., Healy, J., J. B., Young, L.Y., McCarty, P.L., 1979. Bioassay for monitoring biochemical methane potential an anaerobic toxicity. Water Res. 13, 485-492.
114. Pandey, P.K., Ndegwa, P.M., Soupir, M.L., Alldredge, J.R., Pitts, M.J., 2011. Efficacies of inocula on the startup of anaerobic reactors treating dairy manure under stirred and unstirred conditions. Biomass and Bioenergy. 35, 2705-2720.
115. Paria, S., 2008. Surfactant-enhanced remediation of organic contaminated soil and water. Adv Colloid Interface Sci. 138(1), 24-58.
116. Patidar, S.K., Tare, V., 2004. Effect of micro-nutrients in anaerobic degradation of sulfate laden organics. Canadian Journal of Civil Engineering. 31, 420-431.
117. Peng, S.-C., Xue, J., Shi, C.-B., Wang, J., Chen, T.-H., Yue, Z.-B., 2014. Iron-enhanced anaerobic digestion of cyanobacterial biomass from lake chao. Fuel. 117, 1-4.
118. Prakash, O., Nimonkar, Y., Shouche, Y.S., 2013. Practice and prospects of microbial preservation. FEMS Microbiol. Lett. 339, 1-9.

119. Prasse, C.E., Baldwin, A.H., Yarwood, S.A., 2015. Site history and edaphic features override the influence of plant species on microbial communities in restored tidal freshwater wetlands. Appl Environ Microbiol. 81(10), 3482-91.
120. Preeti Rao, P., Seenayya, G., 1994. Improvement of methanogenesis from cow dung and poultry litter waste digesters by addition of iron. World Journal of Microbiology & Biotechnology. 10, 211-214.
121. Qiao, W., Yan, X., Ye, J., Sun, Y., Wang, W., Zhang, Z., 2011. Evaluation of biogas production from different biomass wastes with/without hydrothermal pretreatment. Renewable Energy. 36(12), 3313-3318.
122. Rabelo, S.C., Carrere, H., Maciel Filho, R., Costa, A.C., 2011. Production of bioethanol, methane and heat from sugarcane bagasse in a biorefinery concept. Bioresour Technol. 102(17), 7887-95.
123. Rafique, R., Poulsen, T.G., Nizami, A.-S., Asam, Z.-u.-Z., Murphy, J.D., Kiely, G., 2010. Effect of thermal, chemical and thermo-chemical pre-treatments to enhance methane production. Energy. 35(12), 4556-4561.
124. Raju, N.R., Sumithra Devi, S., Nand, K., 1991. Influence of trace elements on biogas production from mango processing waste in 1.5 m3 kvlc digesters. Biotechnology Letters. 13(6), 461-464.
125. Raposo, F., Banks, C.J., Siegert, I., Heaven, S., Borja, R., 2006. Influence of inoculum to substrate ratio on the biochemical methane potential of maize in batch tests. Process Biochemistry. 41(6), 1444-1450.
126. Raposo, F., De la Rubia, M.A., Fernández-Cegrí, V., Borja, R., 2011. Anaerobic digestion of solid organic substrates in batch mode: An overview relating to methane yields and experimental procedures. Renew. Sustain. Energy Rev. 16, 861-877.
127. Rocheleau, S., Greer, C.W., Lawrence, J.R., Christiane, C., Laramee, L., Guiot, S.R., 1999. Differentiation of methanosaeta concilii and methanosarcina barkeri in anaerobic mesophilic granular sludge by fluorescent in situ hybridization and confocal scanning laser microscopy. Applied and Environmental Microbiology. 65(5), 2222-2229.
128. Roden, E.E., 2003. Diversion of electron flow from methanogenesis to crystalline fe(iii) oxide reduction in carbon-limited cultures of wetland sediment microorganisms. Applied and Environmental Microbiology. 69(9), 5702-5706.
129. Romero-Güiza, M.S., Vila, J., Mata-Alvarez, J., Chimenos, J.M., Astals, S., 2016. The role of additives on anaerobic digestion: A review. Renewable and Sustainable Energy Reviews. 58, 1486-1499.
130. Roussel, J., Carliell-Marquet, C., 2016. Significance of vivianite precipitation on the mobility of iron in anaerobically digested sludge. Frontiers in Environmental Science. 4.
131. Saarela, M., Virkajarvi, I., Alakomi, H.L., Mattila-Sandholm, T., Vaari, A., Suomalainen, T., Matto, J., 2005. Influence of fermentation time, cryoprotectant and neutralization of cell concentrate on freeze-drying survival, storage stability, and acid and bile exposure of bifidobacterium animalis ssp. Lactis cells produced without milk-based ingredients. J Appl Microbiol. 99(6), 1330-9.

132. Salsabil, M.R., Laurent, J., Casellas, M., Dagot, C., 2010. Techno-economic evaluation of thermal treatment, ozonation and sonication for the reduction of wastewater biomass volume before aerobic or anaerobic digestion. J Hazard Mater. 174(1-3), 323-33.
133. Shu, D., He, Y., Yue, H., Wang, Q., 2015. Microbial structures and community functions of anaerobic sludge in six full-scale wastewater treatment plants as revealed by 454 high-throughput pyrosequencing. Bioresour Technol. 186, 163-72.
134. Silverstein, R.A., Chen, Y., Sharma-Shivappa, R.R., Boyette, M.D., Osborne, J., 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresour Technol. 98(16), 3000-11.
135. Suanon, F., Sun, Q., Mama, D., Li, J., Dimon, B., Yu, C.P., 2016. Effect of nanoscale zero-valent iron and magnetite (fe3o4) on the fate of metals during anaerobic digestion of sludge. Water Res. 88, 897-903.
136. Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresource Technology. 83, 1-11.
137. Suwannoppadol, S., Ho, G., Cord-Ruwisch, R., 2011. Rapid start-up of thermophilic anaerobic digestion with the turf fraction of msw as inoculum. Bioresour. Technol. 102, 7762-7.
138. Taherzadeh, M.J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. Int J Mol Sci. 9(9), 1621-51.
139. Tan, J., Wang, J., Xue, J., Liu, S.-Y., Peng, S.-C., Ma, D., Chen, T.-H., Yue, Z., 2015. Methane production and microbial community analysis in the goethite facilitated anaerobic reactors using algal biomass. Fuel. 145, 196-201.
140. Tian, Z., Mohan, G.R., Ingram, L., Pullammanappallil, P., 2013. Anaerobic digestion for treatment of stillage from cellulosic bioethanol production. Bioresour Technol. 144, 387-95.
141. Tiehm, A., Nickel, K., Neis, U., 1997. The use of ultrasound to accelerate the anaerobic digestion of sewage sludge. Water Science and Technology. 36(11), 121-128.
142. Torry-Smith, M., Sommer, P., Ahring, B.K., 2003. Purification of bioethanol effluent in an uasb reactor system with simultaneous biogas formation. Biotechnol Bioeng. 84(1), 7-12.
143. Uellendahl, H., Ahring, B.K., 2010. Anaerobic digestion as final step of a cellulosic ethanol biorefinery: Biogas production from fermentation effluent in a uasb reactor-pilot-scale results. Biotechnol Bioeng. 107(1), 59-64.
144. UN. 2015. Sustainable development goals: 17 goals to transform our world, Vol. 2017, United Nations.
145. van Bodegom, P.M., Scholten, J.C., Stams, A.J., 2004. Direct inhibition of methanogenesis by ferric iron. FEMS Microbiol Ecol. 49(2), 261-8.
146. Van Hamme, J.D., Singh, A., Ward, O.P., 2006. Physiological aspects. Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. Biotechnol Adv. 24(6), 604-20.

147. Vasudevan, M., Wiencek, J.M., 1996. Mechanism of the extraction of proteins into tween 85 nonionic microemulsions. Industrial & Engineering Chemistry Research. 35f, 1085-1089.
148. Voith, M., 2009. Cellulosic scale-up: Doe-backed ethanol producers encounter difficulties converting waste streams to commercially viable fuel. Chem. Eng. News. 87(17), 10-13.
149. Wang, Q., Noguchi, C., Hara, Y., Sharon, C., Kakimoto, K., Kato, Y., 1997. Studies on anaerobic digestion mechanism: Influence of pretreatment temperature on biodegradation of waste activated sludge. Environmental Technology. 18(10), 999-1008.
150. Wei, C.-h., Wang, W.-x., Deng, Z.-y., Wu, C.-f., 2007. Characteristics of high-sulfate wastewater treatment by two-phase anaerobic digestion process with jet-loop anaerobic fluidized bed. Journal of Environmental Sciences. 19(3), 264-270.
151. Wilms, R., Sass, H., Kopke, B., Cypionka, H., Engelen, B., 2007. Methane and sulfate profiles within the subsurface of a tidal flat are reflected by the distribution of sulfate-reducing bacteria and methanogenic archaea. FEMS Microbiol Ecol. 59(3), 611-21.
152. Wilson, C.A., Novak, J.T., 2009. Hydrolysis of macromolecular components of primary and secondary wastewater sludge by thermal hydrolytic pretreatment. Water Res. 43(18), 4489-98.
153. Witarsa, F., Lansing, S., Yarwood, S., Gonzalez Mateu, M., 2016. Incubation of innovative methanogenic communities to seed anaerobic digesters. Appl Microbiol Biotechnol. 100(22), 9795-9806.
154. Wu, D., Zheng, S., Ding, A., Sun, G., Yang, M., 2015. Performance of a zero valent iron-based anaerobic system in swine wastewater treatment. J Hazard Mater. 286, 1-6.
155. Yang, B., Wyman, C.E., 2008. Pretreatment: The key to unlocking low-cost cellulosic ethanol. Biofuels, Bioproducts and Biorefining. 2(1), 26-40.
156. Yang, Y., Guo, J., Hu, Z., 2013. Impact of nano zero valent iron (nzvi) on methanogenic activity and population dynamics in anaerobic digestion. Water Res. 47(17), 6790-800.
157. Yarwood, S.A., Bottomley, P.J., Myrold, D.D., 2010. Soil microbial communities associated with douglas-fir and red alder stands at high- and low-productivity forest sites in oregon, USA. Microb Ecol. 60(3), 606-17.
158. Yeatts, K.B., El-Sadig, M., Leith, D., Kalsbeek, W., Al-Maskari, F., Couper, D., Funk, W.E., Zoubeidi, T., Chan, R.L., Trent, C.B., Davidson, C.A., Boundy, M.G., Kassab, M.M., Hasan, M.Y., Rusyn, I., Gibson, J.M., Olshan, A.F., 2012. Indoor air pollutants and health in the united arab emirates. Environ Health Perspect. 120(5), 687-94.
159. Yeh, D.H., Pennell, K.D., Pavlostathis, S.G., 1998. Toxicity and biodegradability screening of nonionic surfactants using sediment-derived methanogenic consortia. Water Science & Technology. 38(7), 55-62.
160. Yuan, Q., Sparling, R., Oleszkiewicz, J.A., 2011. Vfa generation from waste activated sludge: Effect of temperature and mixing. Chemosphere. 82(4), 603-7.

161. Zhang, J., Zhang, Y., Chang, J., Quan, X., Li, Q., 2013. Biological sulfate reduction in the acidogenic phase of anaerobic digestion under dissimilatory fe (iii)--reducing conditions. Water Res. 47(6), 2033-40.
162. Zhang, J., Zhang, Y., Quan, X., Liu, Y., An, X., Chen, S., Zhao, H., 2011. Bioaugmentation and functional partitioning in a zero valent iron-anaerobic reactor for sulfate-containing wastewater treatment. Chemical Engineering Journal. 174(1), 159-165.
163. Zhang, L., De Schryver, P., De Gusseme, B., De Muynck, W., Boon, N., Verstraete, W., 2008. Chemical and biological technologies for hydrogen sulfide emission control in sewer systems: A review. Water Res. 42(1-2), 1-12.
164. Zhang, M., Wang, H., 2014. Organic wastes as carbon sources to promote sulfate reducing bacterial activity for biological remediation of acid mine drainage. Minerals Engineering. 69, 81-90.
165. Zhang, X., 2012. Factors influencing iron reduction–induced phosphorus precipitation. Environmental Engineering Science. 29(6), 511-519.
166. Zhao, X., Zhang, L., Liu, D., 2008. Comparative study on chemical pretreatment methods for improving enzymatic digestibility of crofton weed stem. Bioresour Technol. 99(9), 3729-36.
167. Zheng, Y., Zhongli, P., Zhang, R., 2009. Overview of biomass pretreatment for cellulosic ethanol production. International Journal of Agricultural and Biological Engineering. 2(3), 51-68.