ANAEROBIC DIGESTION: SULFATE AND ACETOCLASTIC METHANOGENESIS

SULFATE REDUCING BACTERIA AND ACETOCLASTIC METHANOGENS FOR PROCESS INTENSIFICATION OF ANAEROBIC DIGESTION

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Abstract

Anaerobic digestion (AD) is an essential process in wastewater treatment to stabilize waste organic solids and produce biogas. This research is comprised of two projects in the discipline of anaerobic digestion. First, the effect of high sulfate concentration on anaerobic digestion of wastewater sludge was investigated. Secondly, the performance of acetoclastic methanogens *Methanosaeta* spp. and *Methanosarcina* spp. were investigated under intensified AD operation conditions (i.e., elevated acetate concentrations, vigorous mixing, etc.). In the sulfate experiments, the cumulative biogas and methane production decreased linearly with increasing initial sulfate doses (0 – 3,300 mg S L^{-1}) and the correlation between the sulfate dose and methane production was verified with theoretical predictions, indicating complete reduction of sulfate to sulfide in AD. The examined sulfate concentrations resulted in no clear negative effects on the COD (chemical oxygen demand) removal or VSS (volatile suspended solids) destruction of the wastewater sludge, indicating that previous findings on sulfide toxicity might have been attributed to potential COD overestimation of digested sludge with high levels of sulfide. To avoid potential misinterpretation of AD performance on sulfide toxicity effects, we proposed a new method for COD correction for digested sludge. In the second project focused on acetoclastic methanogens, vigorous mixing conditions substantially decreased *Methanosarcina* spp. growth and methane production, and the decreased methanogenesis was more pronounced at higher acetate concentrations. *Methanosarcina* spp. prefer to grow in clusters and the vigorous mixing can disrupt cluster formation; as a result, reduced chances for cluster formation limited the growth of *Methanosarcina* spp.. While *Methanosarcina* spp. growth and methane production increased with the increasing acetate concentration, *Methanosaeta* spp. growth was unaffected by the examined vigorous mixing and soluble substrate conditions with negligible relative growth. Thus, rapid enrichment of *Methanosarcina* spp. is critical for successful operation intensified of AD processes under high organic loading conditions.

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List of Abbreviations

AD	Anaerobic digestion
(T)WAS	(Thickened) Waste activated sludge
SRT	Solids retention time
VFA	Volatile fatty acid
LCFA	Long chain fatty acid
spp.	Species
COD	Chemical oxygen demand
sCOD	Soluble chemical oxygen demand
SRB	Sulfate reducing bacteria
FOG	Fats, oil and grease
BMP	Biochemical methane potential
TSS	Total suspended solids
VSS	Volatile suspended solids
ICP-OES	Inductive coupled plasma optical emission spectrometer
qPCR	Quantitative polymerase chain reaction
DNA	Deoxyribonucleic acid
DI	De-ionized
WWTP	Wastewater treatment plant
WEF	Water Environment Foundation

Declaration of Academic Achievement

Thesis includes two chapters that are intended for publication in peer-reviewed scientific journals (Chapter 2 and 3). I will be the primary author on both articles, my supervisor Dr. Younggy Kim will be the second author. My contributions to the work include literature review, conducting experiments, data analysis and manuscript writing. My supervisor provided guidance, support, training, and expertise during every stage of the work.

1 Introduction

1.1 Anaerobic Digestion

1.1.1 Background on Anaerobic Digestion

Anaerobic digestion (AD) is a biological treatment process that allows for energy recovery from organic wastes that would have otherwise contributed to pollution. In the AD process a complex bacterial and archaeal community converts organic substrate into biogas, which is composed of methane (a source of renewable energy, ~70%) and carbon dioxide (~30%) [1]. It is important to understand and improve the AD process in the field of wastewater treatment engineering. A major type of waste regularly used as substrate for AD is sewage sludge, commonly known as waste activated sludge (WAS), a by-product of wastewater treatment processes. The main goals of the anaerobic digestion of WAS are volatile solids reduction, destruction of human pathogens, and methane production [2]. The energy recovery from the methane produced can be used to offset operating costs of sludge treatment in an environmentally friendly and sustainable way.

Recently there has been growing interest by both industry and academia to improve the AD process [3]–[7]. Typical anaerobic digesters require minimum solid retention times (SRT) of 15 days, and many have 20 to 25-day SRTs to ensure the retention of key microorganisms [1]. Additionally, anaerobic digesters are typically operated at either mesophilic (35 to 40 °C) or thermophilic (50 to 55 °C) temperatures [8], which means operating (heating) costs are quite substantial [15]. WAS management and final disposal accounts for around 50% of the total operating costs for wastewater treatment facilities

[1], largely due to high AD operating costs. The need for large digesters and constant heat input present cost barriers preventing more widespread implementation of AD technology. However, governments around the world are starting to enforce stricter regulations on sludge disposal, leading to a need to improve and reduce costs in AD [9], [10]. In 2022 many Canadian provinces are banning landfilling and incineration: two major alternative biosolids stabilization processes that have more harmful environmental impacts then AD.

There has been increasing interest in using other forms of biomass as substrate for anaerobic digestion, such as yard waste, manure, food waste (thus diverting it from a landfill) and energy crops [8]. These wastes can be used as the sole substrate for digesters, but more commonly a process called co-digestion is used, in which WAS is mixed with the alternative substrate and they are fed to the digester together [11]. The use of co-digestion leads to more complex substrate composition and thus can lead to more strain on the microbial communities responsible for a successful digestion. Co-digestion is more widespread as process intensification efforts in AD increase. With process intensification (typically pre-treatment of TWAS) more room is available in existing digesters, thus allowing for additional substrate to be fed alongside the pre-treated wastewater sludge.

AD is a complex biological process that requires many different groups of microorganisms to work together to ensure process stability. Thus, there are many possible avenues for process disruption, inhibition and failure. AD is sensitive to toxicants, including free ammonia, volatile fatty acids (VFAs), sulfate/sulfide, heavy

metal ions, and certain organics. [12]–[14] The reported concentration of each of these substances that can cause process inhibition or failure greatly varies in the literature, but it is widely agreed that increased concentrations of these compounds in digesters are detrimental to performance. Sulfate/sulfide inhibition was a major topic of this study and will be discussed in much more detail in proceeding sections of this report.

1.1.2 Biological Reactions in Anaerobic Digestion

Anaerobic Digestion is a complex and multistep biological process driven by numerous microorganisms; however it can be explained generally as a three-step process. These steps are hydrolysis, acidogenesis and methanogenesis [15].

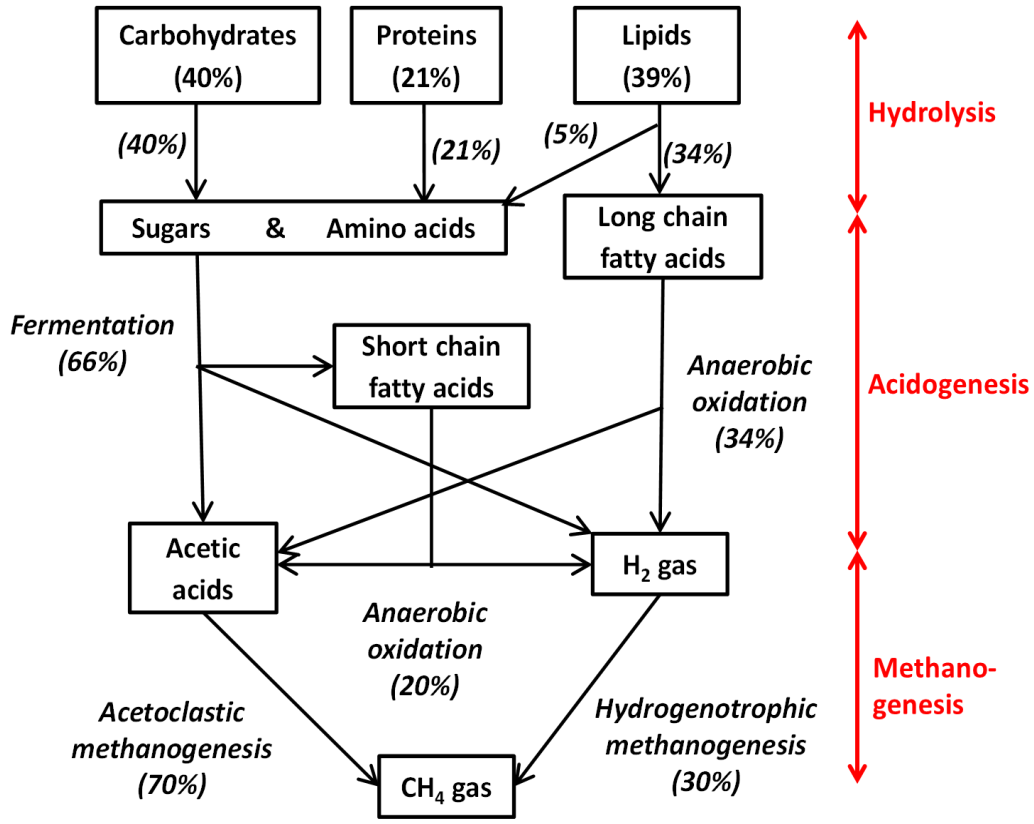

Figure 1.1. High-level overview of the Anaerobic Digestion Process.

In the hydrolysis step particulate organics (carbohydrates, lipids and proteins) are solubilized by extracellular enzymes and turned into amino acids, sugars, long-chain fatty acids (LCFAs), etc. [16]. The standard measurement for quantifying the organic content of the substrate is chemical oxygen demand (COD). During the hydrolysis step the fraction of particulate COD decreases while the fraction of soluble COD (sCOD) increases [15]. Hydrolysis is generally considered rate limiting due to complex nature of particulate substrates (i.e., WAS) [1] and the rate of hydrolysis rate is a function of substrate type, pH, temperature and residence time [17]. To accelerate the rate of hydrolysis, pre-treatment is sometimes employed (physical, chemical, thermal or biological) [18].

In the acidogenesis step the monomers created in hydrolysis step are converted into VFAs, alcohols and CO_2/H_2 [1]. The acidogenesis reactions are rapid as the microbial communities that support these reactions are diverse [15]. The long-chain fatty acids are degraded to acetic acid. During acidogenesis the production of VFAs can lead to pH decreases if there is not sufficient alkalinity present or supplied to the digester. These pH decreases can significantly reduce the activity of microorganisms in the digester which leads to process disruption/failure.

In the methanogenesis step methanogenic archaea metabolize acetate and H_2/CO_2 to produce methane. Methane production is the mechanism by which COD is removed in AD. Methanogenesis is considered to be rate limiting in cases where the substrate has undergone pre-treatment to enhance hydrolysis. The two main groups of methanogens are the acetoclastic methanogens and the hydrogenotrophic methanogens. Hydrogenotrophic

methanogens convert H_2 and CO_2 to methane and they are genetically diverse, making them insensitive to operating conditions. Acetoclastic methanogens are divided into only two genera, *Methanosaeta* spp. and *Methanosarcina* spp. [19]. *Methanosarcina* spp. and *Methanosaeta* spp. will be discussed in more detailed in proceeding sections of this report.

The temperature of the anaerobic digester, the influent substrate and the retention time are three important parameters for determining performance and stability of anaerobic digestion process. They influence the microbial community structure, biochemical conversion pathways, kinetics and thermodynamic balance of biochemical reactions and the stoichiometry of products formed. If these parameters are not chosen correctly accumulation of inhibitory substances is possible because non-ideal conditions can cause the formation and consumption reactions of these substances to occur at different rates. These problems are observed more in thermophilic (high temperature) systems, when the substrate is high in protein (leads to NH_3 accumulation) or lipids (leads to long-chain fatty acid accumulation), or when the retention time is too low for methanogenesis to occur which results in fatty acid accumulation in the digester effluent [15].

1.2 Literature Review: Sulfate effect on Anaerobic Digestion

Sulfate is a widely used chemical in various industries, including steel processing, fertilizer production, pulp and paper, food processing, mining and petroleum refining; as a result, it can be found at high levels in wastewater and thus in wastewater sludge fed to anaerobic digesters. Sulfate levels in industrial wastewater (and consequently wastewater sludge) can range between 0.2 g L^{-1} (pulp and paper mills) to 20 g L^{-1} (mining), and even

as high as 284 g L^{-1} in the chemical industry [20]. It is generally agreed that high sulfate concentration in AD substrate is detrimental to the performance of anaerobic digesters, due to two types of sulfate inhibition [21]. The primary inhibition occurs due to sulfate used as the terminal electron acceptor by sulfate reducing bacteria (SRB) [13], [14]. With a large amount of sulfate, SRB compete with other anaerobic bacteria and archaea for available organic substrates. The secondary inhibition is induced by the toxic effects of sulfide on anaerobic bacteria and archaea [13], [14] as SRB convert sulfate into sulfide in AD.

In anaerobic conditions sulfate is reduced to sulfide by sulfate-reducing bacteria that compete with methanogens for hydrogen and acetate [12], [14], [22]–[25]. The sulfate reducing reactions are energetically more favourable than methanogenic reactions [24], [26] due to the strong oxidizing capacity of sulfate as a terminal electron acceptor, resulting in decreased methanogenic activity. This is commonly referred to as primary inhibition [12] although it is a competition (rather than an inhibition) between methanogens and SRB [14]. Primary inhibition is problematic as it can greatly reduce the bioenergy recovery (i.e. reduced methane production) from the AD process if the COD-to-sulfate ratio in the feed is low. Primary inhibition is a well-known phenomenon and the stoichiometric relationships governing the conversion of organics to sulfide are presented here. Equation 1.1 shows the reaction driven by SRB, sulfate reduction to sulfide. The amount of the electrons consumed in this reaction can be converted to the equivalent amount of oxygen demand using equation 1.2.

$$SO_4^{2-} + 10H^+ + 8e^- \rightarrow H_2S + 4H_2O \quad (1.1)$$

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \quad (1.2)$$

Dividing the number of electrons consumed in sulfate reduction by the number of electrons consumed in oxygen reduction shows that 2 mols of oxygen is required to oxidize the same amount of COD as one mole of sulfate. Converted to a mass basis this relationship is 0.67 g-COD oxidized per g-SO_4^{2-} reduced. While the relationship between COD and sulfate is well understood, there are no studies that verify that this relationships can be used for quantitative prediction of the primary inhibition (i.e. reduction in methane production) based on sulfate concentration.

The toxic effect of sulfide is referred to as the secondary inhibition [12]. Although the occurrence of sulfide toxicity is broadly accepted in the literature, the inhibition concentration has been reported in a wide range of 0.1-1.4 g L^{-1} as dissolved sulfide [4, 6], while the Water Environment Foundation (WEF) reports sulfide concentrations of only 200 mg/L are strongly inhibitory to AD processes. [27] This wide range indicates limited understanding of the secondary inhibition of sulfide in anaerobic wastewater and sludge treatment. An oft-cited reason for the wide range of inhibition concentrations is the acclimatization of anaerobic communities to sulfide. Anaerobic communities can be extremely diverse and there are some reported cases of improved performance at sulfate-reducing conditions due to sulfide acclimatization [12], [13]. However, O'Flaherty et al. found that anaerobic sludges acclimated to sulfate-reducing conditions do not resist sulfide toxicity more effectively than non-acclimated sludges [28], which directly contradicts the aforementioned studies.

Substrate type/complexity is a factor that may affect an anaerobic communities' adaptability to sulfate reducing conditions and sulfide toxicity. Maillacheruvu et al. found that the choice of substrate has an effect on performance in sulfate reducing conditions [29]. Polprasert and Haas found that changing the ratio of glucose/acetate under sulfate reducing conditions effected COD removal and methane production. They observed that the sulfide inhibition affects acidogenesis the most, while not having a strong affect on the methanogenic step [30]. This has been further shown by Cetecioglu et al. who found that the digestion of simple substrates such as acetate, propionate and butyrate using sulfate acclimated sludge were unaffected by COD/SO_4^{2-} ratios as low as 0.5 g:g, which indicates that sulfide inhibition had no effect on the acetogens and methanogens in those cultures [31]. However, other studies report methanogens are the most inhibited by sulfide toxicity [33, 34].

In addition to toxicity, residual sulfide in digested sludge samples can result in an overestimation in COD (chemical oxygen demand) measurement due to oxidation of sulfide in COD vials. Consequently, overestimated COD of the digested sludge can lead to a potential misinterpretation as sulfide toxic effects (i.e., secondary inhibition) with inhibited COD removal in AD.

1.3 Literature Review: Acetoclastic Methanogenesis

The process intensification of AD has been an important research and development goal in wastewater treatment engineering. AD is a complex biological process that requires many different groups of microorganisms to work together to ensure process stability. Methanogenesis and hydrolysis are typically the rate limiting steps of AD, but process

intensification research has successfully developed methods to overcome the slow rate of hydrolysis through substrate pre-treatment (physical, chemical, thermal or biological) [18]. Thus, process intensification by improving methanogenesis is increasingly important. The two main groups of methanogenic archaea responsible for methanogenesis are the acetoclastic methanogens and the hydrogenotrophic methanogens [15]. Hydrogenotrophic methanogens convert hydrogen and carbon dioxide to methane and they are robust and diverse, making hydrogenotrophic methanogenesis insensitive to operating conditions. Acetoclastic methanogens are divided into only two genera, *Methanosaeta* spp. and *Methanosarcina* spp., and with the limited diversity, they are less robust to changes in operating conditions [19].

Methanosarcina spp. are more robust than *Methanosaeta* spp., they can survive a wider pH-range, double the concentration of ammonia-nitrogen and 5-times the concentration of acetate that *Methanosaeta* spp. can [19]. However, *Methanosarcina* spp. is expected to dominate in anaerobic environments when acetate concentrations are above 250-500 mg-COD/L, a threshold that is not typically exceeded in municipal anaerobic digesters. *Methanosaeta* spp. are dominant in most anaerobic digesters due to them having a higher affinity for acetate then *Methanosarcina* spp., with reported half saturation coefficients for *Methanosaeta* spp. and *Methanosarcina* spp. of 10-50 mg-COD/L and 200-280 mg-COD/L respectively [34]. *Methanosaeta* spp.'s higher affinity for acetate allows it to grow and effectively convert acetate to methane even at very low acetate concentrations. The disadvantage is that these digesters typically have retention times of over 20 days, due to the slow growth rate of *Methanosaeta* spp. [19]. *Methanosaeta* spp. have doubling

times on the order of 4-6 days, whereas *Methanosarcina* spp. have higher growth rates and doubling times of 1-1.2 days [19].

Methanosarcina spp. have a preference to grow in clusters, and some previous studies suggest that high shear forces disrupt *Methanosarcina* spp. growth and reduce methane production in AD [35]–[38]. However, one study described the opposite effect and found that *Methanosarcina* spp. abundance increased as the mixing intensity increased in a digester treating animal manure. [39] A study by Liu et al. found that increasing the solids concentration in a digester from 10% to 19% decreased the abundance of *Methanosarcina* spp. by 44% [40]. Based on the fact that both mixing and high solids concentrations can disrupt cluster formation, the lack of opportunity for cluster formation may be a limiting factor for the growth of *Methanosarcina* spp..

Methanosarcina spp.'s high growth rate and ability to handle stressors (pH change, ammonia, high VFA's) is appealing for process intensification applications and for treating high-COD substrate. Selective enrichment of *Methanosarcina* spp. should allow for high-rate methane production when compared to typical digesters. This is especially true considering the recent developments in hydrolysis pre-treatments, which rapidly convert particulate COD in the substrate to soluble COD [2], [41], [42]. These treatments result in high soluble substrate concentrations in the digesters, a condition that is theoretically well-suited for *Methanosarcina* spp. growth. Cell yield is low in AD so nutrient supplementation is typically unnecessary when treating complex waste streams [15]. However, high-COD and low-solids waste streams (characteristics of some effluents from hydrolysis pre-treatments) can be nutrient deficient and Conklin et al. found that in

these cases nickel and cobalt supplementation up to 6 μM and 2.4 μM respectively may be necessary for healthy methanogenesis [34]. The recommended concentration of nickel is well below the inhibitory concentration reported by the WEF of 34 μM [27].

Bioaugmentation of failing anaerobic digesters due to high VFA concentrations with *Methanosarcina* spp. has been proposed by De Vrieze et al [36] as a means to restore digester performance. If an inoculum enriched with *Methanosarcina* spp. is added to a failing digester, it may help in one of two ways. Firstly, the *Methanosarcina* spp. added can grow quickly and the digester will recover, now with a methanogenic community dominated by *Methanosarcina* spp.. In the second case, the *Methanosarcina* spp. will grow quickly and reduce the VFA concentrations down to a level where *Methanosaeta* spp. is able to regain dominance, and the digester will then continue operation as a *Methanosaeta* spp. dominated digester. Both outcomes are acceptable as both result in restored operation.

The type of substrate being digested influences the methanogenic community. Kurade et al examined the co-digestion of fats, oil and grease (FOG) with thickened sludge. [43] They found that the addition of FOG, a high carbon substrate that leads to VFA production and pH decreases, was initially detrimental to digester performance vs. a thickened sludge control. After 60 days of digestion the methanogen community in the co-digester had transitioned from being 94% *Methanosaeta* spp. and 0.52% *Methanosarcina* spp., to being 95% *Methanosarcina* spp. and 1% *Methanosaeta* spp. This microbial community adaption led to a 217% increase in methane production in the FOG co-digester vs. the thickened sludge control. This result supports the bioaugmentation

idea proposed by De Vrieze et al. (2012) [36]. If *Methanosarcina* spp.-rich inoculum had been added to the co-digester at the time of the FOG addition it is likely that the microbial adaption and subsequent enhanced digestion would have started sooner then the 60 days it took in this case with no bioaugmentation.

The above scenarios are examples in which the dominance of *Methanosarcina* spp. can be beneficial for digester performance. These examples highlight the importance of understanding the conditions at which *Methanosarcina* spp. can thrive, so that their rapid growth and robustness can be utilized in a variety of process intensification scenarios.

1.4 Research Objectives

1.4.1 Research Objectives in the SRB Project (Thesis Chapter 2)

Considering the limited understandings on sulfate/sulfide chemistry in AD operation and analysis, three research objectives are proposed and investigated in this thesis.

1. The first objective was to quantify the impact of primary inhibition on methane production and biogas composition as there are no known systematic studies that verified the well-known stoichiometric relationships governing the conversion of organics to methane and sulfide. Validated relationships can be used as a rapid and accurate model to predict methane loss due to the competition between SRB and methanogens in anaerobic digesters.
2. The second objective was to confirm the relative insignificance of secondary inhibition on organics removal and investigate if perceived secondary inhibition can be attributed to overestimation of effluent COD due to aqueous sulfide.

3. The third objective was to suggest a new COD correction method in AD operation with wastewater sludge containing sulfate to eliminate the possibility of misinterpretation of the effluent COD concentration.

1.4.2 Research Objectives in the Acetoclastic Methanogenesis Project (Thesis Chapter 3)

Considering the promising potential for process intensification using *Methanosarcina* and limited understanding of the effects of vigorous mixing on competition between acetoclastic methanogens, this study investigated the effect of mixing, initial acetate concentration, initial seed VSS, and Ni/Co supplementation on acetoclastic methanogen growth and methane production in AD. The four specific research objectives of this study are:

1. Firstly to investigate the effect of vigorous mixing conditions on the growth and activity of acetoclastic methanogens under process intensification conditions (e.g., high soluble organic substrate conditions).
2. Secondly, to examine the high soluble substrate conditions in terms of toxicity of high acetate concentration and competition between *Methanosaeta* spp. and *Methanosarcina* spp.
3. Thirdly, to determine if additional metallic minerals (i.e., Ni and Co) are necessary in AD with process intensification scenarios where acetoclastic methanogenesis governs the overall rate of AD (i.e., the role of other anaerobic microbes are relatively unimportant).

4. Finally, to determine the effect of particle concentration on acetoclastic methanogen growth (because AD reactors with process intensification are often designed and operated at high solids concentration).

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2 Importance of chemical oxygen demand correction in anaerobic digestion of sulfate-rich wastewater sludge

Abstract

Anaerobic digestion (AD) is used to stabilize organic solids and produce biogas by biological reactions. In wastewater sludge digestion, the presence of sulfate negatively affects the biological reactions through two types of inhibition. Here we investigated and quantified the effect of high sulfate concentration on anaerobic digestion of wastewater sludge using lab-scale batch experiments at 40 °C. In the experiment with thickened secondary sludge from a municipal wastewater treatment plant, the cumulative biogas and methane production decreased with increasing initial sulfate doses (0 – 3,300 mg S L^{-1}). The correlation between the sulfate dose and methane production was consistent with theoretical predictions, indicating rapid and complete reduction of sulfate to sulfide. The theoretical prediction method can be used to simulate biogas yield and composition in AD applications. The carbon dioxide content in the biogas decreased linearly with the increasing sulfate dose. The examined high sulfate concentrations resulted in no clear negative effects on the COD (chemical oxygen demand) removal or VSS destruction of the wastewater sludge, indicating negligible inhibition by sulfide toxicity. Even considering ferrous sulfide precipitation, which can control sulfide toxicity, it was determined the residual sulfide concentration was higher than reported concentrations that trigger process inhibition. We confirmed the importance of COD correction in AD of wastewater sludge with high sulfate levels because COD of digested sludge without the correction can be misinterpreted as sulfide toxicity

2.1 Introduction

Anaerobic digestion (AD) is a biological treatment process used to stabilize organic solids and produce biogas [1]. The biogas produced contains methane, a common source of renewable energy, and thus there are strong industrial and academic interests in optimizing the anaerobic conversion of organic solids (including wastewater sludge) to methane [2]. AD is also used in conjunction with municipal wastewater treatment processes to reduce the volatile and organic content of the wastewater sludge produced in the processes [3]. In wastewater sludge, there are a number of contaminants and inhibitors that have detrimental effects on biological reactions in AD. These include organic toxicants (e.g., chlorophenols and long chain fatty acids) and inorganic inhibitors (e.g., sulfate, free ammonia, and heavy metals) [4]–[7]. Among these chemicals, sulfate is known to decrease the biogas production and its by-product sulfide slows down the overall rate of biological reactions in AD. Sulfate is a widely used chemical in various industries, including steel processing, fertilizer production, pulp and paper, food processing, mining and petroleum refining; as a result, it can be found at high levels in wastewater and thus in wastewater sludge fed to anaerobic digesters. Sulfate levels in industrial wastewater (and consequently wastewater sludge) can range between 0.2 g L^{-1} (pulp and paper mills) to 20 g L^{-1} (mining), and even as high as 284 g L^{-1} in the chemical industry [8]. This study investigated the effect of sulfate on biogas production and composition and proposed a new protocol for estimating AD treatability of wastewater sludge containing high levels of sulfate.

The concentration of sulfate in wastewater sludge is an important consideration because sulfate acts as an electron acceptor in anaerobic environments [1]. The presence of sulfate induces unwanted biological reactions in AD. For instance, sulfate is reduced to sulfide by sulfate-reducing bacteria (SRB) that compete with methanogens for hydrogen and acetate [5], [6], [9]–[12]. The sulfate reducing reactions are energetically more favourable than methanogenic reactions [11], [13] due to the strong oxidizing capacity of sulfate as a terminal electron acceptor, resulting in decreased methanogenic activity in AD. This decreased methanogenesis is referred to as primary inhibition [5] although it is a competition (rather than an inhibition) between methanogens and SRB [6]. Primary inhibition can greatly reduce the bioenergy recovery (i.e. reduced methane production) from organic waste especially when the COD-to-sulfate ratio in the feed is low. Primary inhibition is a well-known phenomenon and stoichiometric relationships governing the conversion of organics to methane and sulfide are well understood. However, there were no systematic studies that verified relationships for quantitative prediction of the primary inhibition based on sulfate concentration. Validated relationships will be used as a rapid and accurate model to predict methane loss due to the competition between SRB and methanogens in anaerobic digesters.

In addition to the primary inhibition, sulfide produced by SRB is known to be toxic towards anaerobic microorganisms in AD [4]–[7] and this toxic effect of sulfide is referred to as the secondary inhibition [5]. Although the occurrence of sulfide toxicity is broadly accepted in the literature, the inhibition concentration has been reported in a wide range of 0.1-1.4 g L^{-1} as dissolved sulfide [4], [6]. This wide range of the inhibition

concentration indicates limited understanding of the secondary inhibition of sulfide in anaerobic wastewater and sludge treatment. In addition, residual sulfide in digested sludge samples can result in an overestimation in COD measurement due to oxidation of sulfide in COD vials. Consequently, overestimated COD of the digested sludge can lead to a potential misinterpretation as sulfide toxic effects (i.e., secondary inhibition) with inhibited COD removal in AD. Considering these limited understandings on sulfate/sulfide chemistry in AD operation and analysis, this study focused on the following four specific research objectives: (1) accurate quantification of the primary inhibition on methane production and biogas composition; (2) confirmation of the relative insignificance of secondary inhibition; and (3) suggestion of a new COD correction method in AD operation with wastewater sludge containing sulfate.

2.2 Materials and Methods

2.2.1 Biochemical methane potential tests

The effect of high initial sulfate concentrations on the anaerobic digestibility of wastewater sludge samples was examined through employing lab-scale batch experiments [14]. The experiments were carried out for 16 days in duplicate using 160 mL serum bottles at 40°C (75L Gravity Convection Incubator, VWR, USA). In the experiment, the substrate was thickened secondary sludge and the seed was digested wastewater sludge in anaerobic digesters. Both the substrate and seed were collected from a local municipal wastewater treatment facility (Woodward Wastewater Treatment Plant, Hamilton, Ontario). Their detailed characteristics are summarized in Table 2.1. Note that the substrate and seed sludges were kept at 4°C until it was used in the experiments.

Table 2.1. Characterization of the substrate and seed sludges used in the experiments. The substrate sludge is thickened waste activated sludge obtained from a local WWTP. The seed sludge was digestate obtained from the same WWTP.

Parameters	Substrate (65 mL)	Seed (40 mL)
pH	7.1	7.3
TSS (g L^{-1})	27.3 ± 0.5	33.2 ± 1.2
VSS (g L^{-1})	21.2 ± 0.4	17.5 ± 0.7
Total COD (g L^{-1})	36.8 ± 1.7	29.8 ± 1.9
Soluble COD (g L^{-1})	2.02 ± 0.01	1.50 ± 0.01
Ferric Iron (g L^{-1})	1.54 ± 0.08	N/A
Conductivity (mS cm^{-1})	4.72	10.23

The values proceeding the ± symbol represent one standard deviation (n = 2).

To maintain a stable pH condition during the experiment, 6.7 g L^{-1} of $NaHCO_3$ was added to keep alkalinity at 4 g L^{-1} as $CaCO_3$ as described in the previous study [14]. To ensure that biological reactions (including SRB reactions) are not limited by nutrients, trace minerals and vitamins (prepared in accordance to Cheng et al. [15]) were supplied in the serum bottles as recommended by Angelidaki et al. [14]. Three different sulfate concentrations were examined by adding Na_2SO_4 in the serum bottles (Table 2.2). The substrate wastewater sludge (65 mL) and seed sludge (40 mL) were mixed and introduced into the serum bottles before the bottles were purged with nitrogen for about 25 seconds, sealed with a rubber septum and aluminum crimp and placed in an incubator. A blank test

was conducted only with the seed sludge (40 mL) and deionized water (65 mL). All experimental results shown in this study were adjusted with the blank test results.

Table 2.2. Sulfate doses and corresponding sulfate-to-COD ratios.

Initial SO_4^{2-} Concentration ($g\ L^{-1}$ as S)	Sulfate-to-COD ratio (g-COD*: g-COD)
0	0
0.83	0.0739
1.67	0.148
3.33	0.296

*Sulfate concentration is written as an oxygen demand by converting the amount of the electrons released in sulfate reduction to the equivalent amount of oxygen demand (Eqs. 3 and 5)

2.2.2 Experimental sample analysis

The experimental samples were analyzed for total suspended solids (TSS), volatile suspended solids (VSS), and total and soluble chemical oxygen demand (COD) according to the standard methods [16]. The soluble fraction of the sludge sample was obtained by centrifuging 25 mL of the sample at 9000 RPM for 10 min and then filtering the centrate using a filter with a pore size of 0.45 μm (Whatman Grade 934-AH, GE Healthcare, USA). The sample pH was measured using a pH meter and probe (SevenMulti, Mettler Toledo, Switzerland). Sulfate in the digested sludge sample at the end of the experiment was not detectable (TNT 864 Vial, HACH, USA), indicating complete reduction of sulfate during the experimental test period.

The iron content in the substrate wastewater sludge was measured using an inductively coupled plasma – optical emission spectrometer (ICP-OES) (Vista-Pro, Agilent Technologies, USA). Prior to the ICP-OES analysis, 30 mL of substrate wastewater sludge was acidified with 90 mL of 70% w/w nitric acid for ~5 days to dissolve iron compounds in precipitant forms (e.g., FeS). The acidified sample was filtered using a syringe filter with a pore size of 0.45 μm (25 mm syringe filter with polysulfone membrane, VWR International, USA).

2.2.3 COD Correction for Sulfide

The measured COD results were corrected by subtracting overestimated oxygen demand contributed by sulfide in the COD vial. In a separate experiment, sulfide was confirmed to react with dichromate in the COD measurement (Eq. 2.1). The measured COD of a 1,000 mg S L^{-1} solution (2,438 mg Na_2S L^{-1}) was 773 ± 7 mg COD L^{-1} (n = 2), which is 94 ± 1% of the theoretical oxygen demand for sulfide oxidation (819 mg COD L^{-1}) by Eq. 2.2.

$$4Cr_2O_7^{2-} + 3H_2S + 26H^+ \rightarrow 8Cr^{3+} + 3SO_4^{2-} + 16H_2O \quad (2.1)$$

$$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+ \quad (2.2)$$

Consequently, sulfide in the sludge samples in our experiment resulted in an overestimation of COD, requiring a correction. In this correction, sulfate added in the serum bottles was assumed to be fully converted to sulfide. All COD analysis results are shown both as measured and corrected values.

2.2.4 Biogas analysis

Biogas production from the serum bottle was measured periodically over the course of the 16-day test period using an air-tight glass syringe (50 ml, Dyna Medical Corporation, Canada). The biogas composition was determined in thermal conductivity detector-gas chromatography (SRI 8610C, SRI Instruments, USA) using a molecular sieve column (ShinCarbon ST 19808, Restek, USA).

2.3 Results and Discussion

2.3.1 Effect of Sulfate on Biogas Production

The cumulative biogas and methane production decreased in the experiments with the increasing initial sulfate dose (Figure 2.1). This finding can be explained by the primary inhibition caused by the high sulfate concentrations [5]. The reduced methane production can be attributed to sulfate consumed as an electron acceptor by SRB that compete for acetic acid and hydrogen gas with methanogens. SRB are known to have more favourable kinetics for their growth and substrate utilization than methanogenic archaea [11], [17]; thus, the reduction reaction of sulfate was expected to occur early in the experiment. This rapid growth of SRB explains the decreased rate of methane production until 6 days (i.e., the decreased slope of methane production in Figure 2.1A) for the increased sulfate doses. In the later stage of the test (i.e., 10 days and thereafter), however, the slope became insensitive to the sulfate dose, implying that the sulfate added was rapidly consumed by SRB in less than 10 days.

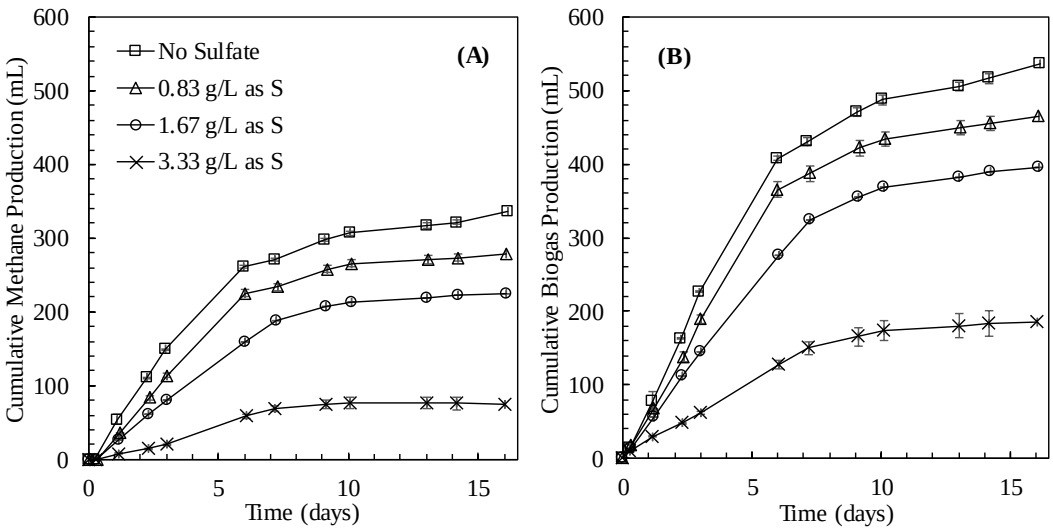

Figure 2.1. (A) Cumulative methane production and (B) biogas production in the experiments (corrected for 1 atm and 0°C). The error bars represent twice the standard deviation of the duplicated experiments (n = 2).

2.3.2 Prediction of methane loss by primary inhibition

The effect of sulfate on methane production was precisely estimated by theoretical predictions (Figure 2.2). In AD, sulfate is expected to be reduced to elemental sulfur (S^0) or sulfide (H_2S). The two prediction lines were prepared based on two expected reactions driven by SRB: sulfate reduction to sulfide (Eq. 2.3); and to sulfur (Eq. 2.4). The amount of the electrons released was then converted to the equivalent amount of oxygen demand (Eq. 2.5).

$$SO_4^{2-} + 10H^+ + 8e^- \rightarrow H_2S + 4H_2O \quad (2.3)$$

$$SO_4^{2-} + 8H^+ + 6e^- \rightarrow S^0 + 4H_2O \quad (2.4)$$

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \quad (2.5)$$

For instance, in Figure 2.2, the dotted line represents the theoretical estimation for the methane loss written in COD due to the primary inhibition where sulfate is reduced to sulfide by SRB. Similarly, the dashed line shows the methane loss due to sulfate reduction to elemental sulfur. This theoretical estimation for the degree of the primary inhibition was consistent with the experimental results as the 95% confidence interval about the slope of the experimental data contains the slope of the line representing sulfate conversion to sulfide. This finding indicates that the majority of the dosed sulfate was completely reduced to sulfide rather than elemental sulfur (Figure 2.2).

The stoichiometry of the complete reduction of sulfate to sulfide (Figure 2.2) was consistent with experimental results for methane loss. These consistent results suggest that methane loss can be solely attributed to the activity of SRB competing for biodegradable substrate (primary inhibition) rather than the sulfide-induced secondary inhibition for the tested amount of sulfate (up to 3.33 g S L^{-1} or 0.3 g SO_4^{2-} $gCOD^{-1}$).

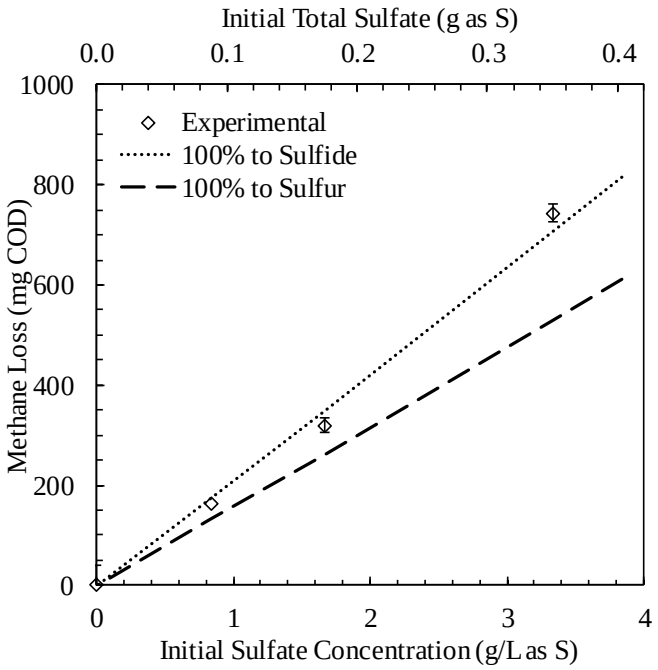

Figure 2.2. Methane loss due to the primary inhibition of sulfate. The methane loss is written in COD ($CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$). The dotted line (100% to Sulfide) assumes complete reduction of sulfate to sulfide; and the dashed line (100% to Sulfur) denotes partial sulfate reduction to elemental sulfur. For the experimental results, the error bars represent two times the standard deviation of the duplicated serum bottles (n = 2).

2.3.3 Effect of sulfate reduction on CO_2 in biogas

The carbon dioxide content in the biogas decreased linearly with the increasing sulfate dose in the experiment (Figure 2.3). The decrease in the CO_2 content is consistent with a previous study where the CO_2 production rate dropped substantially by ~65% with a sulfate concentration of 500 mg L^{-1} [18]. However, the reduced CO_2 production with activated SRB still remains unexplained.

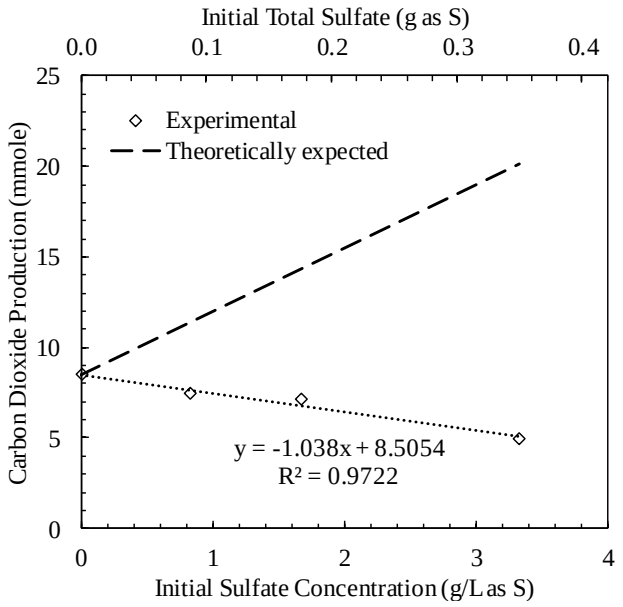

Figure 2.3. Effect of sulfate on carbon dioxide content in biogas.

SRB compete against methanogens in AD; thus, changes in the CO_2 content in the biogas can theoretically be predicted by comparing the activity of SRB and methanogens. For instance, acetoclastic methanogenesis creates one mole of CO_2 (Eq. 2.6) while 2 moles of CO_2 are generated by acetate-oxidizing SRB (Eq. 2.7), indicating that the addition of sulfate to the serum bottles should have increased the CO_2 content in the biogas. Similarly, hydrogenotrophic methanogenesis consumes one mole of CO_2 (Eq. 2.8) while the redox reaction by H_2-oxidizing SRB does not affect CO_2 production (Eq. 2.9). Thus, the competition between H_2-oxidizing SRB and hydrogenotrophic methanogens is also expected to increase the CO_2 content in the biogas. Regardless of the substrate (hydrogen or acetate), one extra mole of carbon dioxide was expected to be produced for each mole of sulfide produced in the place of methane as shown in Figure 2.3 (dashed line for theoretical prediction).

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (2.6)$$

$$CH_3COOH + SO_4^{2-} \rightarrow S^{2-} + 2CO_2 + 2H_2O \quad (2.7)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (2.8)$$

$$4H_2 + SO_4^{2-} \rightarrow S^{2-} + 4H_2O \quad (2.9)$$

This inconsistency between the theoretical estimation and experimental result can be explained by relatively high cell yield for SRB [8], [19]. Assuming that the SRB cell yield is responsible for the smaller CO_2 production than the theoretical estimation (Figure 2.3), the net yield coefficient of SRB is estimated to be 0.465 g VSS g COD^{-1}. Note that $C_5H_7O_2N$ was assumed for cell VSS and then normalized by the amount of sulfate written in g COD. The SRB yield coefficient reported in literature is 0.3 g VSS g COD^{-1} (0.2 g VSS g $SO_4^{2-\ -1}$) [20], [21], which is slightly smaller than the estimated yield coefficient (0.465 g VSS g COD^{-1}). Compared to SRB, methanogens have relatively negligible cell yield (0.019 g VSS g COD^{-1} for acetoclastic methanogens and 0.056 g VSS g COD^{-1} for hydrogenotrophic methanogens) [22]. Carbon is a major component of bacterial cells [23] and thus the lower CO_2 production than the estimation (Figure 2.3) can be explained by SRB growth.

2.3.4 COD and VSS removal

The COD and VSS analysis results confirmed that sulfide (generated as a by-product of sulfate reduction) did not inhibit other biological reactions; that is, secondary inhibition was not observed in this study. The relatively high initial sulfate concentrations (Table 2.2) resulted in no clear negative effects on the COD removal in the experiment as the corrected COD was always 40% to 43% regardless of the sulfate doses (Figure 2.4A).

Also, the VSS destruction of waste activated sludge was not strongly affected by the initial sulfate concentration (Figure 2.4B). The VSS removal ranged between 34% and 38% with sulfate while the control experiment (no sulfate) resulted in 36% VSS removal. Thus, it was concluded that SRB do not play a governing role in COD and VSS destruction in anaerobic digestion for the examined sulfate concentration (up to 3.33 g S L^{-1}) or sulfate-to-COD ratio (up to 0.45 g S g COD^{-1}).

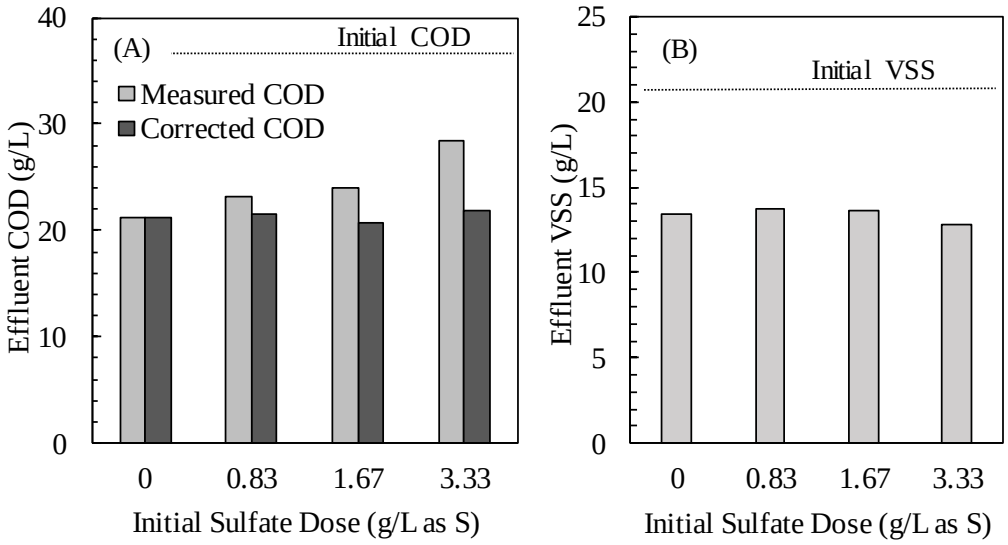

Figure 2.4. Effect of sulfate on (A) COD removal and (B) VSS removal.

The COD and VSS analysis results in this study are contradictory with previous studies where the secondary inhibition significantly lowered the COD and VSS removal [18]. Since sulfide inhibition was not observed in these experiments; it could be claimed that secondary inhibition does not occur in the anaerobic digestion of thickened WAS. However, the response of an anaerobic community to sulfide concentration is case-dependent, as anaerobic communities can be substantially diverse and acclimate to sulfide

[4], [5]. It is possible that anaerobic communities typically seen in the digestion of waste activated sludge have a high tolerance for sulfide, but further study would need to be conducted to determine this.

2.3.5 Sulfide Toxicity

In our experimental work, the secondary inhibition (i.e., sulfide toxicity) was not observed as the discrepancies in methane production for the different sulfate conditions were accurately predicted by the primary inhibition (Figure 2.2). With secondary inhibition, there would have been an additional decrease in methane production with the increasing sulfate dose. Furthermore, the majority of the methane production (>90%) was completed in 10 days regardless of the sulfate doses, indicating that high sulfide concentrations did not delay methane production compared to the experiment with no or lower sulfate doses. Another evident indication of the absence of secondary inhibition was the similar VSS and corrected-COD destruction for the examined sulfate dosages, including the control test (Figure 2.4).

Ferric iron (Fe^{3+}) in the waste activated sludge can be an explanation for the absence of the sulfide toxicity in the experiment. Ferric iron was used for phosphorus removal in the wastewater treatment facility where the substrate waste activated sludge was collected. Ferric iron (Fe^{3+}) in anaerobic digesters is reduced to ferrous iron (Fe^{2+}) which forms an insoluble and non-toxic precipitate with sulfide [23]–[25] (Eq. 2.10). As a result, the effective sulfide concentration could have been low, thus negating or reducing secondary inhibition during the experiment.

$$Fe^{2+} + S^{2-} \rightarrow FeS \downarrow \quad (2.10)$$

The iron concentration in the substrate waste activated sludge was found to be 1.54 ± 0.08 g L^{-1} (27.4 ± 1.4 mM, n = 3) from the ICP analysis. We assumed that all of the iron reacted with sulfide although the majority of Fe^{2+} was expected to react with water to form $FeOH_{2(s)}$ [26]. With this conservative assumption, the remaining sulfide concentration would be decreased by 22% from 3.33 to 2.61 g S L^{-1} for the experiment with the highest sulfate dose. This decreased concentration (the minimum residual sulfide concentration due to FeS precipitation) is still higher than the reported sulfide concentrations that trigger secondary inhibition [4], [6]. Thus, our experimental results imply that secondary inhibition can be negligible in AD even at relatively high sulfate concentration (2.61 g S L^{-1}).

Many studies on secondary inhibition were conducted with pure or enriched cultures of specific microorganisms (*Methanosarcina barkeri*, *Methanosarcina mazei*) fed with synthetic substrates (ethanol, glucose, propionate, acetate, butyrate, H_2/CO_2) [7], [27]–[29]. These studies effectively isolated the toxic effect of sulfide on a certain step of the anaerobic process or microbial species, but the experimental conditions cannot represent anaerobic digestions treating municipal wastewater sludge. Other studies that report evidence of sulfide toxicity in anaerobic wastewater treatment of saline wastewater, vinasse, and starch wastewater [30]–[33], were conducted with liquid wastewater with negligible concentrations of suspended solids. Therefore, the lack of sulfide toxicity observed in our study can be explained by diverse microbial communities and relatively high suspended solids conditions. The literature reports a wide range of sulfide

concentrations that trigger toxicity in anaerobic environments (50 – 1500 mg-S/L) [4], [5], [7], [18], [27]–[29], indicating that the toxic effect can vary substantially under different conditions. Thus, secondary inhibition (i.e., sulfide toxicity) can be negligible depending on the AD operation conditions (e.g., high suspended solids and enhanced microbial diversity). In addition, as previous studies did not reflect the effect of sulfide on COD measurement [18], [33], a potential overestimation of effluent COD may have caused relatively low COD removal efficiencies [18], which can be misinterpreted as the secondary inhibition. Considering negligible effects of secondary inhibition in anaerobic digestion of WAS, co-digestion with high sulfate biosolids (e.g., food processing waste) can be a viable strategy to mitigate the effect of sulfide toxicity on the digestion of high sulfate waste streams.

2.4 Conclusions

The primary inhibition was accurately predicted based on the initial sulfate concentration in the substrate sludge. We also confirmed that methane loss can be solely attributed to the activity of SRB in the primary inhibition, not the sulfide-induced secondary inhibition for the examined amount of sulfate (up to 3.33 g S L^{-1} or 0.3 g SO_4^{2-} $gCOD^{-1}$). The stoichiometry of the complete reduction of sulfate to sulfide (0.99 moles of CH_4 reduction per mole of SO_4^{2-}) allowed a reliable prediction on how the initial amount of sulfate affects methane yield in AD. As the sulfate reduction occurs in an early stage of AD (in 9 days or earlier), this prediction method can be used to estimate biogas yield and composition in AD operation and numerical model applications on feed wastewater sludge treatability. Using such numerical tools for estimating losses in energy recovery,

operators can determine if actions (e.g., $CaSO_4$ precipitation [34]) have to be taken to maintain methane production.

The CO_2 content in the biogas decreased linearly with the increasing initial sulfate concentration although the CO_2 production in AD is theoretically expected to increase with the sulfate reduction reactions. The high cell yield of SRB compared to methanogens is thought to decrease the CO_2 production as H_2-oxidizing SRB grow autotrophically with CO_2 as the carbon source. Our experimental results with the reduced CO_2 content with sulfate are consistent with previous studies [18], [19], [32]. Based on our experimental result, a linear relationship was established between CO_2 in the biogas and sulfate dose ($y = -1.038x$, where y is CO_2 in mmol and x is sulfate as g S L^{-1}).

The COD and VSS destruction were not inhibited by the high initial sulfate concentrations, implying insignificance of the secondary inhibition for the examined sulfate doses. The lack of secondary inhibition observed in this study was unexpected as the majority of previous studies concluded that secondary inhibition was a direct cause of AD process failures. [4]–[7] It is possible that uncorrected effluent COD can be misinterpreted as secondary inhibition because sulfide oxidation in COD vials result in a substantial overestimation of the effluent COD. Thus, the importance of the COD correction must be emphasised for anaerobic treatment of wastewater or organic waste containing high levels of sulfate.

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3 Enrichment of acetoclastic methanogens for process intensification of anaerobic digestion

Abstract

Anaerobic digestion (AD) is a biological treatment process in which a complex bacterial and archaeal community converts organic waste to methane (renewable energy). Process intensification in AD involves combining high solids loading rates with hydrolysis pre-treatment for rapid solubilization, leading to elevated acetate concentrations and the acetoclastic methanogenesis step becoming rate-limiting. Here we investigated the effect of vigorous mixing, high acetate concentrations (1,000 – 3,000 mg-COD/L), initial seed concentration, and nickel and cobalt supplementation on enrichment of two acetoclastic methanogens (*Methanosaeta* spp. and *Methanosarcina* spp.) Vigorous mixing conditions substantially decreased *Methanosarcina* spp. growth and methane production, and the decreased methanogenesis was more pronounced at higher acetate concentrations. Increasing initial seed concentration (325 mg-VSS/L – 2875 mg-VSS/L) decreased *Methanosarcina* spp. growth. Based on *Methanosarcina* spp.'s preference to grow in clusters and that both mixing and high solids concentrations can disrupt cluster formation, lack of opportunity for cluster formation may limit the growth of *Methanosarcina* spp.. *Methanosarcina* spp. growth and methane production increased with the increasing acetate concentration while *Methanosaeta* spp. growth was negligible and unaffected by the examined vigorous mixing condition and acetate concentrations. Thus, enrichment of *Methanosarcina* spp. (rather than *Methanosaeta* spp.) is critical for rapid acetoclastic methanogenesis in intensified AD operation under high organic concentration. While

supplementation of nickel and cobalt is widely emphasized in process intensification of AD, relatively low doses of 0.05 μM for nickel and 0.02 μM for cobalt were sufficient for enrichment of *Methanosarcina* spp. and stable methanogenesis.

3.1 Introduction

Anaerobic digestion (AD) is a biological treatment process that allows for energy recovery in the form of methane from organic waste. In the AD process a complex bacterial and archaeal community converts organic substrate into stable biosolids and biogas, which is composed of methane (~70%) and carbon dioxide (~30%) [1]. Thus, anaerobic digestion is used for organic biosolid stabilization (i.e. volatile fatty acid (VFA) removal), inactivation of human pathogens, and energy recovery [2]. The process intensification of AD has been an important research and development goal in wastewater treatment engineering. AD is a complex biological process that requires many different groups of microorganisms to work together to ensure process stability. The biological reactions in AD can be succinctly summarized into three steps: hydrolysis, acidogenesis/fermentation and methanogenesis. Methanogenesis and hydrolysis are typically the rate limiting steps of AD, but process intensification research has successfully developed methods to overcome the slow rate of hydrolysis through substrate pre-treatment (physical, chemical, thermal or biological) [3]. Thus, process intensification by improving methanogenesis is increasingly important. The two main groups of methanogenic archaea responsible for methanogenesis are the acetoclastic methanogens and the hydrogenotrophic methanogens [1]. Hydrogenotrophic methanogens convert hydrogen and carbon dioxide to methane and they are robust and diverse, making

hydrogenotrophic methanogenesis insensitive to operating conditions. Acetoclastic methanogens are divided into only two genera, *Methanosaeta* and *Methanosarcina*, and with the limited diversity, they are less robust to changes in operating conditions [4].

Methanosarcina spp. are more robust methanogen's than *Methanosaeta* spp., it can survive a wider pH-range, double the concentration of ammonia-nitrogen and 5-times the concentration of acetate that *Methanosaeta* spp. can [4]. However, *Methanosarcina* spp. is expected to dominate in anaerobic environments when acetate concentrations are above 250-500 mg-COD/L, a threshold that is not typically exceeded in municipal anaerobic digesters. *Methanosaeta* spp. are dominant in most anaerobic digesters due their higher affinity for acetate then *Methanosarcina* spp., with reported half saturation coefficients for *Methanosaeta* spp. and *Methanosarcina* spp. of 10-50 mg-COD/L and 200-280 mg-COD/L respectively [5]. *Methanosaeta* spp.'s higher affinity for acetate allows them to grow and effectively convert acetate to methane even at very low acetate concentrations. The disadvantage is that digesters in which *Methanosaeta* spp. are dominant typically have retention times of over 20 days, due to the slow growth rate of *Methanosaeta* spp. [4]. *Methanosaeta* spp. have doubling times on the order of 4-6 days, whereas *Methanosarcina* spp. have a higher growth rate and doubling times of 1-1.2 days [4].

Methanosarcina spp. have a preference to grow in clusters, and some previous studies suggest that high shear forces disrupt *Methanosarcina* spp. growth and reduce methane production in AD [6]–[9]. However, one study described the opposite effect and found that *Methanosarcina* spp. abundance increased as the mixing intensity increased in a digester treating animal manure [10]. A study by Liu et al. found that increasing the solids

concentration in a digester from 10% to 19% decreased the abundance of *Methanosarcina* spp. by 44% [11]. Based on the fact that both mixing and high solids concentrations can disrupt cluster formation, the lack of opportunity for cluster formation may be a limiting factor for the growth of *Methanosarcina* spp..

Methanosarcina spp.'s high growth rate and ability to handle stressors (pH change, ammonia, high VFA's) is appealing for process intensification applications and for treating high-COD substrate. Selective enrichment of *Methanosarcina* spp. should allow for high-rate methane production when compared to typical digesters. This is especially true considering the recent developments in hydrolysis pre-treatments, which rapidly convert particulate COD in the substrate to soluble COD [2], [12], [13]. These treatments result in high soluble substrate concentrations in the digesters, a condition that is theoretically well-suited for *Methanosarcina* spp. growth. Cell yield is low in AD so nutrient supplementation is typically unnecessary when treating complex waste streams [1]. However, high-COD and low-solids waste streams (characteristics of some effluents from hydrolysis pre-treatments) can be nutrient deficient and Conklin et al. found that in these cases nickel and cobalt supplementation may be necessary for healthy methanogenesis [5].

Considering the promising potential for process intensification using *Methanosarcina* spp. and limited understanding of the effects of vigorous mixing on competition between acetoclastic methanogens this study investigated the effect of mixing and initial acetate concentration on methane production, acetoclastic methanogen growth and final solids concentration via batch tests. Following that, the effect of initial seed volatile suspended

solids (VSS) and acetate concentration on acetoclastic methanogen (particularly *Methanosarcina* spp.) enrichment was examined using batch experiments carried out over three feeding cycles. The specific research objectives are to: (1) investigate the effect of vigorous mixing conditions on the growth and activity of acetoclastic methanogens under process intensification conditions (e.g., high soluble organic substrate conditions); (2) examine the high soluble substrate conditions in terms of toxicity of high acetate concentration and competition between *Methanosaeta* spp. and *Methanosarcina* spp.; (3) determine if additional metallic minerals (i.e., Ni and Co) are necessary in AD with process intensification scenarios where acetoclastic methanogenesis governs the overall rate of AD (i.e., the role of other anaerobic microbes are relatively unimportant); and (4) determine effect of particle concentration on acetoclastic methanogen growth (because AD reactors with process intensification are often designed and operated at high solids concentration).

3.2 Materials and Methods

3.2.1 Batch experiments

The 3 batch tests were carried out using 160 mL serum bottles at 37.5°C. In the experiment, the substrate was sodium acetate (1367 mg-$NaCH_3COO$/L for COD_0 of 1,000 mg/L; 2050 mg-$NaCH_3COO$/L for COD_0 of 1,500 mg/L; 4100 mg-$NaCH_3COO$/L for COD_0 of 3,000 mg/L). The seed was digested sludge from a lab-scale anaerobic digester (retention time = 21 d; continuous fed-batch operation with thickened waste activated sludge from local municipal wastewater treatment plant). Two different seed concentrations were examined (Table 3.1). Note that the residual soluble COD in the seed

sludge was 33 mg/L which is negligible compared to added acetate as substrate. Thus, acetate was considered as the sole substrate for microbial growth in the experiment. To maintain a stable pH condition of 7.2 – 7.3 during the experiments, phosphate ($NaHPO_4$ 4.22 mM; NaH_2PO_4 5.78 mM) was added with trace minerals and vitamins (Cheng et al. [14], Angelidaki et al. [15]). The residual nickel and cobalt concentrations from the seed reactor in Batch Test's I and III were 0.05 μM and 0.02 μM respectively. In Batch Test II, 6 μM of nickel and 2.4 μM of cobalt was added to the reactors to examine the effect of supplementation of the metals on the methanogenic growth (Conklin et al. [5]). The serum bottles were purged with nitrogen for about 25 seconds, sealed with a rubber septum and aluminum crimp and placed in the incubator at 37.5°C (75L Gravity Convection Incubator, VWR, USA). A blank test was conducted only with the seed sludge (no acetate substrate) and the methane production in the experiments was adjusted with the blank test results. The reactors were prepared in two groups: one group without a mixing condition and the other group with vigorous mixing using magnetic stirrers (Table 3.2).

Table 3.1. Initial composition of the serum bottles used in the batch experiments. All of TSS, VSS, and particulate COD were contributed by the seed.

Parameters	Batch Tests I and II	Batch Test III
pH	7.2	7.3
TSS (mg L^{-1})	400 ± 9	1086 ± 20
VSS (mg L^{-1})	221 ± 10	706 ± 0.4
Particulate COD (mg L^{-1})	313 ± 5	1016 ± 6
Duration (days)	14	8

The values proceeding the ± symbol represent one times the standard deviation of duplicated analyses.

Table 3.2. Summary of the experimental conditions of the three batch tests. The word to the left of the hyphen in the condition row indicates the acetate concentration level (High or Low) and the word to the right of the hyphen indicates if the experiment was vigorously mixed (Mix or Still). These naming conventions will be used for the remainder of the report.

Condition	Batch Test I	Batch Test II	Batch Test III
High-Mix	COD_0 = 3000 mg/L Vigorous mixing No Ni/Co supplement	COD_0 = 3000 mg/L Vigorous mixing Added Ni/Co supplement	COD_0 = 3000 mg/L Vigorous mixing No Ni/Co supplement
Low-Mix	COD_0 = 1500 mg/L Vigorous mixing No Ni/Co supplement	COD_0 = 1500 mg/L Vigorous mixing Added Ni/Co supplement	COD_0 = 1000 mg/L Vigorous mixing No Ni/Co supplement
High-Still	COD_0 = 3000 mg/L No mixing No Ni/Co supplement	COD_0 = 3000 mg/L No mixing Added Ni/Co supplement	COD_0 = 3000 mg/L No mixing No Ni/Co supplement
Low-Still	COD_0 = 1500 mg/L No mixing No Ni/Co supplement	COD_0 = 1500 mg/L No mixing Added Ni/Co supplement	COD_0 = 1000 mg/L No mixing No Ni/Co supplement

3.2.2 Three-cycle enrichment experiments

Six reactors were prepared using 160 mL serum bottles with effective initial volumes of 100 mL. In the experiment, the substrate was sodium acetate (2050 mg-$NaCH_3COO$/L for COD_0 of 1,500 mg/L; 4100 mg-$NaCH_3COO$/L for COD_0 of 3,000 mg/L). The seed was digested sludge from a lab-scale anaerobic digester (retention time = 21 d; continuous fed-batch operation with thickened waste activated sludge and sodium acetate from local municipal wastewater treatment plant). Three different seed concentrations and two different target acetate concentrations were examined (Table 3.3). The experiments were carried out for three, three-to-four-day cycles and concentrated acetate was added for each cycle to maintain the target acetate concentrations. For each cycle, approximately 5 mL of sample was taken from the bottles for the solids and COD analyses. The residual soluble COD in the seed sludge at the beginning of cycle one was negligible compared to added acetate as substrate. Thus, acetate was considered as the sole substrate for microbial growth in the experiment. To ensure biological reactions were not limited by nutrients trace minerals and vitamins were added to the serum bottles (Cheng et al. [14], Angelidaki et al. [15]). The serum bottles were purged with nitrogen for about 25 seconds, sealed with a rubber septum and aluminum crimp and placed in the incubator at 37.5°C (75L Gravity Convection Incubator, VWR, USA).

Table 3.3. Summary of the experimental conditions for the continuous batch tests. The naming conventions in the brackets under the continuous batch test condition column will be used for the remainder of the report.

Test Condition	Initial seed	Initial Acetate (Each Cycle)
High Seed-High Acetate (**HS-HA**)	2875 mg-VSS/L	3,000 mg-COD/L
High Seed-Low Acetate (**HS-LA**)	2875 mg-VSS/L	1,500 mg-COD/L
Middle Seed-High Acetate (**MS-HA**)	1300 mg-VSS/L	3,000 mg-COD/L
Middle Seed-Low Acetate (**MS-LA**)	1300 mg-VSS/L	1,500 mg-COD/L
Low Seed-High Acetate (**LS-HA**)	320 mg-VSS/L	3,000 mg-COD/L
Low Seed-Low Acetate (**LS-LA**)	320 mg-VSS/L	1,500 mg-COD/L

3.2.3 Experimental sample analysis

The experimental samples were analyzed for total suspended solids (TSS), volatile suspended solids (VSS), and total and soluble chemical oxygen demand (COD) according to the standard methods [16]. The soluble fraction of the sample was obtained via filtering using a syringe filter with a pore size of 0.45 μm (25 mm syringe filter with polysulfone membrane, VWR International, USA). The sample pH was measured using a pH meter and probe (SevenMulti, Mettler Toledo, Switzerland). For the batch experiment, pH in all reactors were maintained neutral between 7.2-7.6 and for the three-cycle experiment pH stayed mostly constant at ~7.8.

3.2.4 DNA extraction and qPCR analysis

The initial and final samples from Batch Test III and the three-cycle experiments were analyzed in qPCR to quantify 16S-rDNA of *Methanosarcina* spp. and *Methanosaeta* spp.. The primers used are summarized in Table 3.4. Prior to the qPCR analysis a DNA extraction was performed to prepare the samples for downstream analysis. Firstly, samples were sonicated for 20 minutes and then diluted to the target VSS of

approximately 5 mgVSS/L using sterilized DI water. The diluted samples were then sonicated for another 20 minutes. Following sonication 1 mL of the samples was centrifuged for 15 minutes at 12,000 rpm, then 800 µL of supernatant was removed and 800 µL of sterilized DI water was added before centrifuging for 3 more minutes at 12,000 rpm. After centrifugation 800 µL of supernatant was removed and 200 µL of InstaGene (Bio-Rad, California, USA) was added to the pellet. The samples were then incubated (DRB 200, HACH, USA) at 56 °C for 30 minutes, then vortexed for 10 seconds and incubated at 100 °C for 10 minutes and vortexed again. Finally the samples were centrifuged at 12,000 rpm for 3 minutes, and the resulting supernatant was used as DNA templates for qPCR.

Table 3.4. Primers used in the qPCR analysis

Target Microorganism	Primer	Sequence	Reference
Methanosarcina spp.	MB1b	CGGTTTGGTCAGTCCTCCGG	[17]
	SAR835r	AGACACGGTCGCGCCATGCCT	
Methanosaeta spp.	Msta 571F	TAAAGGGTCTGTAGCCGGCC	[18]
	Msta 927R	CCCGCCAATTCCTTTAAGTTT	

PCR reactions were carried out in 96-well microplates (BIO-RAD, California, USA) with a reaction volume of 20 µL. A single reaction contained 10 µL of iTaq Universal SYBR Green Supermix (BIO-RAD, California, USA), 1 µL of the microorganism-specific forward primer, 1 µL of the microorganism-specific reverse primer, 2 µL of sterilized DI water and 6 µL of the DNA template. PCR thermal cycling consisted of 50 cycles at 94 °C for 5 s, and 60 °C for 30 s. A melt curve was constructed after initial cycling by

incrementing by 0.5 °C from 65 °C to 95 °C, for 5 s at each increment. A CFX96 Touch Real-Time PCR (Bio-Rad, California, USA) was used to perform the qPCR procedure and data processing was completed using software provided by Bio-Rad. The threshold cycle (C_T) value obtained via the data processing software was converted to the growth factor (how many times larger the final microorganism population is than the initial) using Equation 3.1.

$$Growth\ Factor = 2^{C_{T\,Initial} - C_{T\,Final}} \tag{3.1}$$

3.2.5 Biogas analysis for batch experiments

In the batch experiments biogas production from the serum bottle was measured periodically over the course of the test period using an air-tight glass syringe (50 ml, Dyna Medical Corporation, Canada). The biogas composition was determined for methane production in thermal conductivity detector-gas chromatography (SRI 8610C, SRI Instruments, USA) using a molecular sieve column (ShinCarbon ST 19808, Restek, USA).

3.3 Results and Discussion

3.3.1 Methane production and methanogen growth in the batch tests

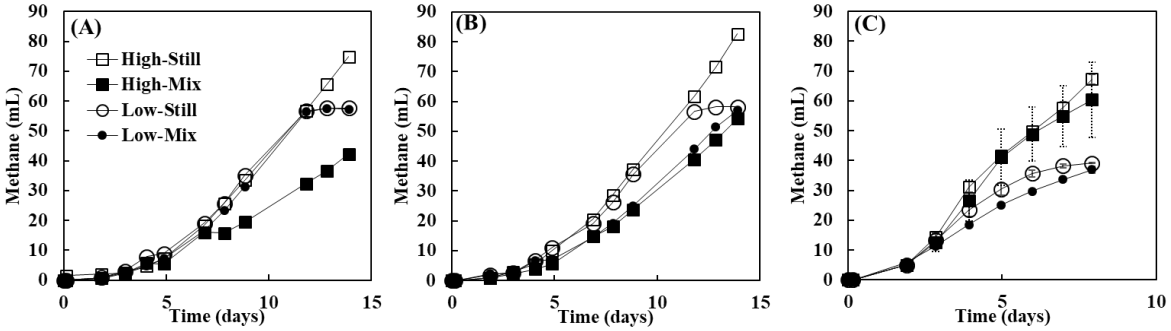


Figure 3.1. Cumulative methane production over the course of the batch tests. (A) shows the results of batch test I, (B) shows the results of batch test II and (C) shows the duplicate results from batch test III, where error bars represent one standard deviation (n=2).

A clear detrimental effect was observed on methane production in the mixed reactors and this effect was more pronounced at the higher acetate concentrations (higher COD_0). In every test (except for Low-Still and Low-Mix in Batch Test I), the mixing condition led to a visible decrease in the rate of methanogenesis at the same acetate concentrations (Figure 3.1). The High-Still condition always resulted in the highest methane production rate in each test. However, the High-Mix condition led to the lowest methanogenesis rate in Batch Tests I and II (Figures 3.1A and 3.1B). For Batch Test III, the outperformance of High-Still (vs. High-Mix) was not pronounced compared to Batch Tests I and II possibly due to the higher initial seed concentration (Table 3.1). Higher seed concentration results in a greater number of methanogens initially, making the methane production result relatively insensitive to the mixing conditions. It should also be emphasized that the lower acetate concentration conditions (Low-Still vs. Low-Mix) were not as strongly affected by mixing as the higher acetate concentration conditions (High-Still vs. High-Mix). This

trend in methane production was more pronounced for Batch Tests I and II, implying the importance of the initial seed concentration.

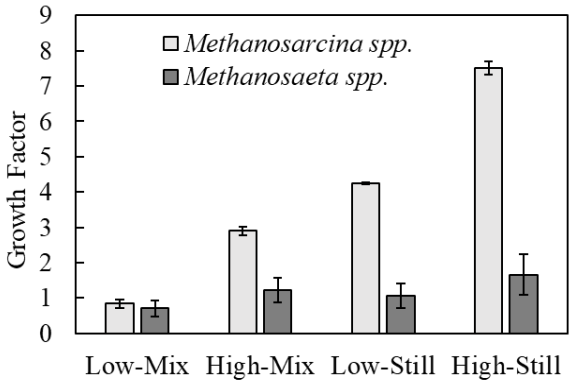


Figure 3.2. Relative acetoclastic methanogen growth vs. the initial population in the seed over the 8 days of Batch Test III. A value of 1 for the growth factor (y-axis) means the methanogen population did not change while a growth factor of 2 means that the population doubled. The error bars represent one times the standard deviation of duplicated experiments.

The overall acetoclastic methanogen growth was greater at the still conditions and at higher acetate concentrations. (Figure 3.2). The growth factor for *Methanosarcina* spp. indicated that the hydrodynamic condition of the reactor (Mix vs. Still) has a more dominant effect on the growth of *Methanosarcina* spp. than the initial acetate concentration (High vs. Low). *Methanosarcina* spp. are known to grow in clusters or flocs [9]; as a result, their growth was slowed by the vigorous mixing condition (Figure 3.2).

The change in acetate concentration significantly affected the growth of *Methanosarcina* spp.. Increasing the acetate concentration from 1000 to 3000 mg-COD/L at both the mixed and still conditions caused clear and reproducible increases in *Methanosarcina* spp. growth. This result agrees with the generally observed dominance of *Methanosarcina* spp. at high acetate concentrations compared to *Methanosaeta* spp. [4],

[5]. The results also suggested an interaction effect between mixing and acetate concentration. At the high acetate condition mixing decreased *Methanosarcina* spp. growth by a factor of 2.6 whereas at the low acetate condition mixing decreased *Methanosarcina* spp. growth by a factor of 5.0.

Based on the observed growth factor (Figure 3.2), the methane production by *Methanosaeta* spp. was considered to be negligible, meaning that the majority of the methane production in Batch Test III (Figure 3.1C) was driven by the growth of *Methanosarcina* spp. At the 3000 mg-COD/L concentration mixing has a strong negative effect on the rate of methanogenesis (Figure 3.1). *Methanosarcina* spp. are expected to dominate at this concentration as *Methanosaeta* spp. do not grow well at high acetate concentrations. Thus the slow methanogenesis at this condition can be explained by the negative impact of mixing on *Methanosarcina* spp.. The lower acetate concentration conditions were not as strongly affected by mixing as the higher acetate concentration conditions. *Methanosaeta* spp. is more competitive at these conditions, so the less pronounced effect of mixing at low concentrations can be explained by mixing's weak influence on *Methanosaeta* spp. growth (Figure 3.2).

3.3.2 VSS and particulate COD in the batch tests

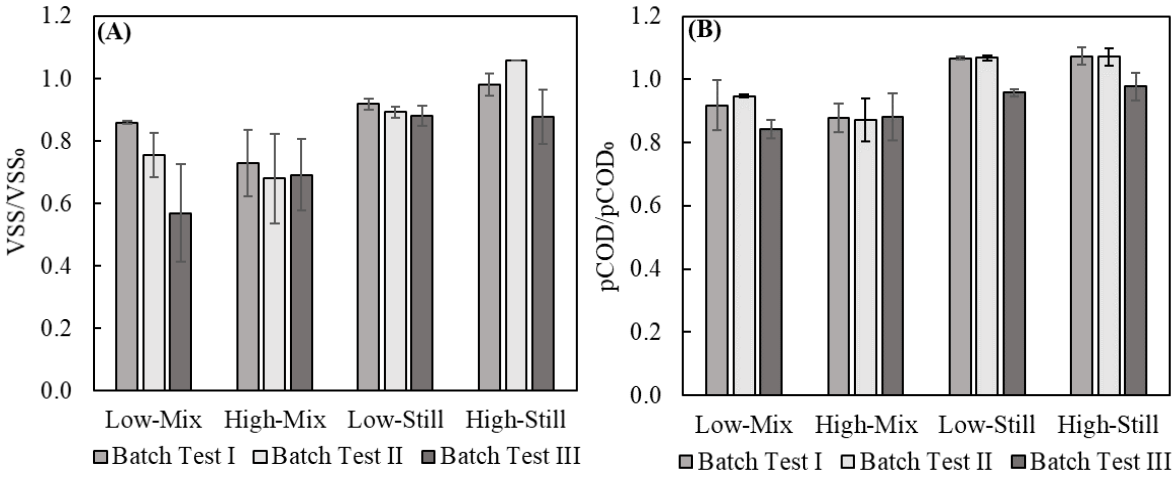
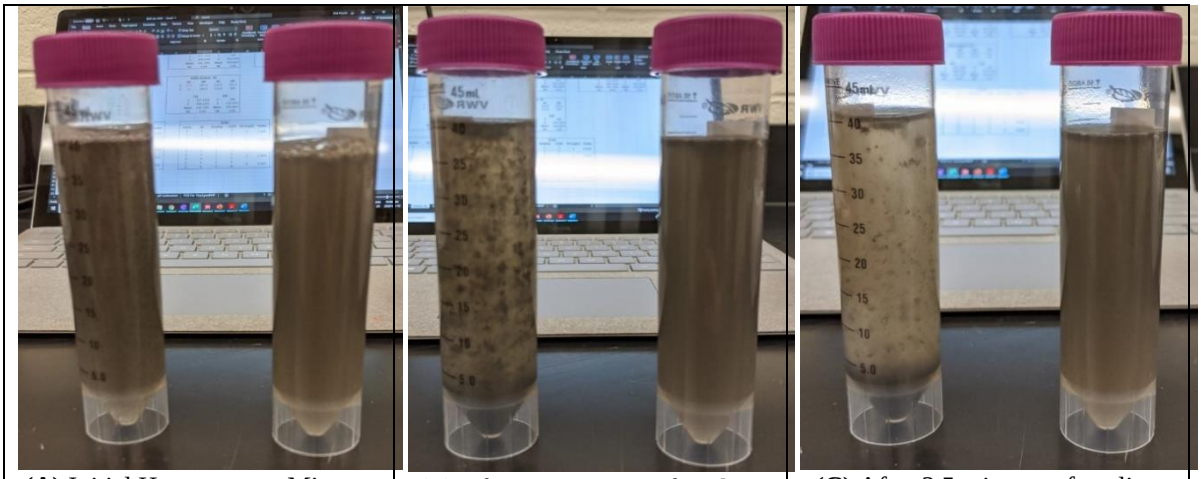


Figure 3.3. The relative VSS and particulate COD from the batch tests normalized by the initial concentrations. (A) shows the relative VSS and (B) shows the relative particulate COD for each of the batch tests. In all cases the error bars represent one standard deviation (n=2).

The final concentrations of VSS and particulate COD were lower for the mixed conditions than for the still conditions in all three batch tests (Figure 3.3). The trend in *Methanosarcina* spp. growth found via qPCR (Figure 3.2) is reflected in the VSS and particulate COD results. Note that the experiment was conducted with acetate as the sole substrate, allowing only acetoclastic methanogenic growth in the reactors. The qPCR results show much greater *Methanosarcina* spp. growth in the still reactors and the VSS and particulate COD at the still conditions is higher as well. This implies that *Methanosarcina* spp. was the dominant methanogen in these experiments because *Methanosaeta* spp. growth was negligible in all experiments.



(A) Initial Homogenous Mixture | **(B)** After 1.6 minutes of settling | **(C)** After 3.5 minutes of settling

Figure 3.4. Settling comparison between still (left) and mixed (right) effluent from the first test at 0, 1.6 and 3.5 minutes of settling.

A settling comparison test was performed by allowing homogenous mixtures from a mixed reactor and a still reactor to settle over time. Comparison between the homogenous mixture and 1.6 minutes of settling revealed that the sludge from the still sample was unstable rapidly forming large and visible flocs in only 1.6 min (Figures 3.4A and 3.4B). As a result, the settled sludge layer was observed near the bottom of the sample tube in 3.5 min while the mixed effluent was visually unchanged from the homogenous condition (Figure 3.4C).

3.3.3 The effect of trace metal supplementation on the batch tests

The initial nickel and cobalt concentrations in Batch Test's I and III were 0.05 μM and 0.02 μM respectively. Nutrient addition in AD is typically unnecessary however process intensification scenarios with higher than typical expected methanogen growth may necessitate supplementing certain nutrients [1], so in Batch Test II 6 μM of nickel and 2.4 μM of cobalt were added to the reactors. The nickel and cobalt supplementation did not result in significant increases in methane production when comparing Batch Test I and

Batch Test II (Figure 3.1A and 3.1B) Similarly, the final concentrations of VSS and particulate COD in Batch Test I and II were comparable, indicating nickel and cobalt supplementation did not improve methanogen growth. Thus nickel and cobalt concentrations of 0.05 μM and 0.02 μM respectively are sufficiently high and provide non-limiting growth conditions for acetoclastic methanogenesis in process intensified high-substrate conditions up to 3000 mg-COD/L.

3.3.4 Methanogen growth in the three-cycle enrichment experiments

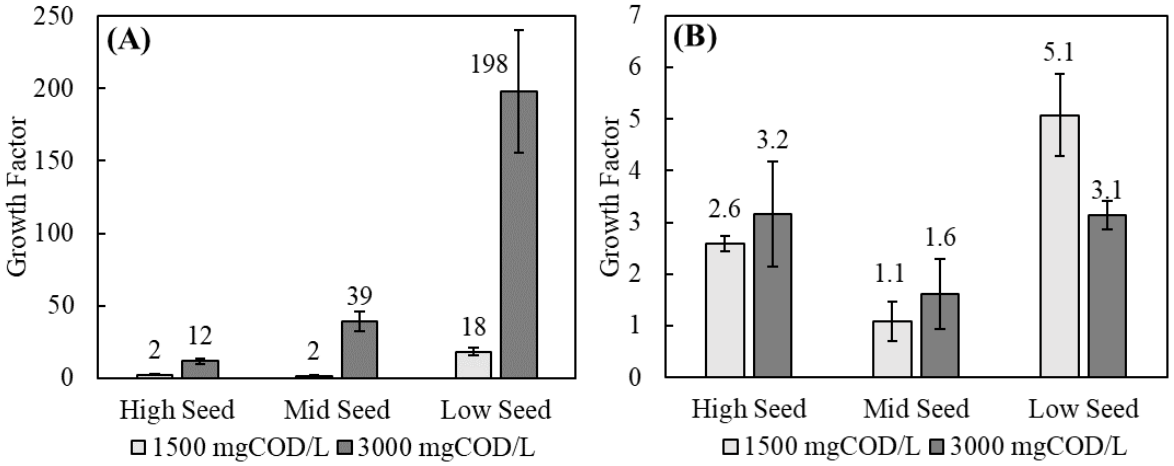


Figure 3.5. Relative acetoclastic methanogen growth vs. the initial population in the seed at the end of the third cycle of the continuous methanogen enrichment experiment. (A) Growth factor for *Methanosarcina* spp. and (B) Growth factor for *Methanosaeta* spp. A growth factor (y-axis) of 1 is the initial condition for both methanogens. The error bars represent one standard deviation (n=2).

The *Methanosarcina* spp. growth was higher at the 3,000 mg-COD/L acetate concentration and increased with the decreasing initial seed amount, with a maximum growth of about 198 times the initial population over the 3 cycles of the LS-HA operation (Figure 3.5A). *Methanosarcina* spp.'s preference for high acetate concentrations and the abundance of substrate at the 3,000 mg-COD/L acetate concentration allowed its population to significantly multiply vs. the 1,500 mg-COD/L condition. The growth

factor was significantly small at the low acetate condition (LA = 1,500 mg-COD/L) compared to high acetate (HA) conditions (HA = 3,000 mg-COD/L) for all the three seed conditions (HS, MS, LS).

The strong effect of initial seed concentration on the *Methanosarcina* spp. growth can be partially explained by the nature of the growth factor calculation, which measures multiplication of the original population and not changes in the absolute value of the microorganisms. Because there was a lower concentration of *Methanosarcina* spp. at the low seed condition, equivalent increases in the absolute number of microorganisms result in a higher growth factor compared to the middle or high seed conditions. Correcting the growth factor by normalizing to the initial VSS at the high seed condition resulted in adjusted growth factors of 11.6, 19.5 and 24.8 times the initial population of *Methanosarcina* spp. for the HS, MS and LS conditions. Thus, the absolute growth of *Methanosarcina* spp. increased at lower seed concentrations. *Methanosarcina* spp. prefers to grow in flocs and clusters [8], [9] and the high initial VSS (seed) concentration may have disrupted cluster formation thus inhibiting the growth of *Methanosarcina* spp.. This agrees with the results of Liu et al. who found that increasing the solids concentration in a digester from 10% to 19% decreased the abundance of *Methanosarcina* spp. by 44% [11]. This is similar to the growth factor results from the batch experiments where the vigorous mixing condition disrupted cluster formation, slowing the multiplication of *Methanosarcina* spp..

The *Methanosaeta* spp. growth was low for all the seed and acetate conditions compared to the growth of *Methanosarcina* spp.; as a result, the highest growth factor was only 5.1

for the LS-LA operation. (Figure 3.5B) A possible reason that the growth was low is that the initial seed came from a WAS-fed digester that likely had a relatively high *Methanosaeta* spp. population vs. *Methanosarcina* spp. population [4], thus concentration increases of the same absolute quantity represent less multiplications of the *Methanosaeta* spp. population vs. the *Methanosarcina* spp. one. A second reason for the low *Methanosaeta* spp. growth is the competition with *Methanosarcina* spp.. *Methanosarcina* spp. is expected to dominate in anaerobic environments with acetate concentrations above 250-500 mg-COD/L, [7] a threshold that was always exceeded in these experiments. (Figure 3.6).

3.3.5 sCOD Removal in the three-cycle enrichment experiments

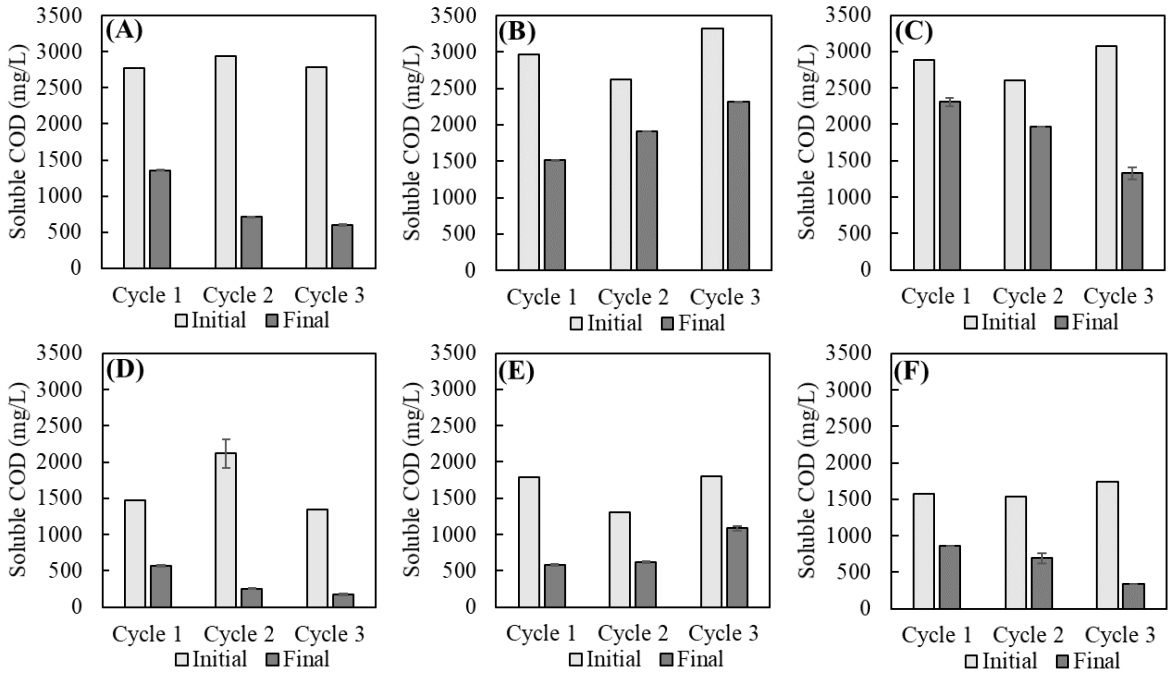


Figure 3.6. Initial and final sCOD for the 3 cycles of the methanogen enrichment experiment. (A) is the HS-HA condition, (B) is the MS-HA condition, (C) is the LS-HA condition, (D) is the HS-LA condition, (E) is the MS-LA condition and (F) is the LS-LA condition. The error bars represent one standard deviation (n=2).

Soluble COD removal was consistently observed over the course of the 3 repeated cycles for all test conditions (Figure 3.6). Note that the only soluble substrate present in the reactors was acetate; thus, the stable soluble COD removal is clear evidence of acetoclastic methanogen activity. The soluble COD removal generally increased at later cycles, with the exception being the middle seed (MS) condition where acetate removal did not increase from cycle one to two at either acetate concentration. An increase in soluble COD removal was anticipated, as the methanogen (particularly *Methanosarcina* spp.) population in the reactors increased over the course of the cycles. (Figure 5) The high seed reactors showed an improved capacity for acetate removal between cycles one and two, but removal remained stable between cycles two and three. In contrast, the low seed reactors exhibited noticeable improvements in acetate removal capacity for each cycle. This implies that the soluble COD removal was limited by the low initial seed concentration and that greater methanogen growth needed to occur in the low solid's reactors than the high seed reactors to achieve similar soluble COD removal.

3.3.6 VSS changes and decay in the three-cycle enrichment experiments

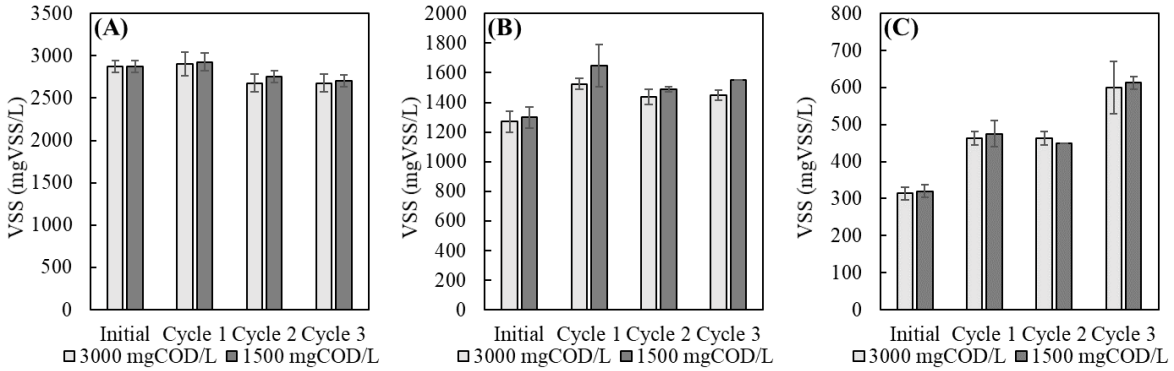


Figure 3.7. Initial VSS and the VSS after each cycle for the 3 cycles of the methanogen enrichment experiment. (A) is the HS condition, (B) is the MS condition and (C) is the LS condition. The error bars represent one standard deviation (n=2).

Distinctly different trends were observed in the VSS concentrations over the course of the three cycles at the different initial seed concentrations. However, the acetate concentration did not have a dominant effect on VSS for a given initial seed concentration, the VSS at 3,000 mg-COD/L and 1,500 mg-COD/L was similar in all cases (Figure 3.7). At the HS condition VSS decay dominated over VSS growth, evidenced by the decreasing trend from cycle 1 to 3 (Figure 3.7A). An initial increase in VSS concentration was observed at the MS condition, followed by a slight decay and then a "flattening out" (Figure 3.7B). A clear increasing trend in VSS concentration was observed at the LS condition (Figure 3.7C). The observed effect was driven by the balance between the decay of non-methanogenic microorganisms (because they are unable to grow on acetate, the sole substrate in these experiments) and the growth of *Methanosarcina* spp.. Microorganism decay is considered linearly proportional to microorganism concentration [1] and thus it was expected that the non-methanogenic microorganisms at the high seed condition would initially decay twice as fast as at the middle seed condition, and eight times as fast as at the low seed condition. As initial seed concentration increased, the effect of the decay of non-methanogenic microorganisms lowering VSS became more pronounced and the effect of the growth of *Methanosarcina* spp. raising VSS became less pronounced.

3.4 Conclusions

Vigorous mixing conditions had a negative impact on *Methanosarcina* spp. growth while *Methanosaeta* spp. growth was not significant in the batch tests. The growth factor for *Methanosarcina* spp. ranged from 0.8 to 7.5 while the growth factor for *Methanosaeta*

spp. had a smaller range of 0.7 to 1.6. Based on the observed growth factors the methane production by *Methanosaeta* spp. was considered to be negligible, meaning that the majority of the methane production was driven by the growth of *Methanosarcina* spp.. Methane production was lower at the mixed conditions compared to the still conditions in the batch tests. The negative effect was more pronounced at higher acetate concentrations compared to lower ones. This was due to mixing disrupting *Methanosarcina* spp.'s ideal growth condition, which is to grow in clusters.

The final VSS and particulate COD was lower in the mixed bottles than the still ones. This effect can be linked to *Methanosarcina* spp. growth, as the limited growth at mixing conditions meant a decreased cell mass at those conditions, leading to lower VSS and particulate COD. In high-rate anaerobic treatments on soluble substrate, vigorous mixing should be avoided to maximize methane production and enrich for *Methanosarcina* spp.. This could be problematic for process intensification as typically high-rate digesters have high-solids concentrations and thus require strong mixing. Multiple stage digestion in which the hydrolysis pre-treatment and methane production steps are performed in separate stages with different mixing conditions could solve this issue.

The growth factors for *Methanosarcina* spp. from all the experiments indicated that greater *Methanosarcina* spp. growth occurred at the high acetate concentration conditions compared to the lower ones (up to 20 times greater). This was expected due to the high substrate concentrations used (high acetate concentrations can inhibit *Methanosaeta* spp. growth) and because *Methanosarcina* spp. have doubling times of 1-1.2 days whereas *Methanosaeta* spp. have doubling times of 4-6 days. This allowed *Methanosarcina* spp. to

multiply quickly. This result strongly suggests that *Methanosarcina* spp. would be the dominant acetoclastic methanogen in process intensification scenarios following hydrolysis pre-treatment, as the soluble substrate concentrations are high.

In the three-cycle test the observed growth factors indicated that the LS-HA condition had the highest relative and absolute growth. *Methanosarcina* spp. prefers to grow in flocs and clusters and the high initial seed at the HS and MS conditions may have disrupted cluster formation (similar to mixing) thus inhibiting the growth of *Methanosarcina* spp.. The *Methanosaeta* spp. growth was low for all the conditions examined and no trends between *Methanosaeta* spp. growth and seed concentration was observed.

As initial seed concentration increased, the effect of the decay of non-methanogenic microorganisms lowering VSS became more pronounced and the effect of the growth of *Methanosarcina* spp. raising VSS became less pronounced. The growth factor and VSS results suggest that when starting up anaerobic reactors for treatment of high-COD soluble substrate (i.e. for process intensification), using a low (<400 mg-VSS/L) concentration of seed might lead to more effective long-term treatment as there is more absolute growth at these conditions. It could also lead to a more efficient start-up, as the decay of non-methanogenic organisms from high seed concentrations could cause process disruptions (i.e. ammonia release from dead cells can cause toxicity and pH change).

The nickel and cobalt supplementation in Batch Test II did not significantly impact the results. Thus nickel and cobalt concentrations of 0.05 μM and 0.02 μM respectively are

sufficiently high and provide non-limiting growth conditions for acetoclastic methanogenesis in process intensified high-substrate conditions up to 3000 mg-COD/L.

3.5 References

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4 Conclusions and suggestions for future work

4.1 Conclusions

4.1.1 Anaerobic digestion of sulfate rich wastewater sludge

The primary inhibition was accurately predicted based on the initial sulfate concentration in the substrate sludge. It was also confirmed that methane loss can be solely attributed to the activity of SRB in the primary inhibition, not the sulfide-induced secondary inhibition for the examined amounts of sulfate (up to 3.33 g S L^{-1} or 0.3 g SO_4^{2-} $gCOD^{-1}$). The stoichiometry of the complete reduction of sulfate to sulfide (0.99 moles of CH_4 reduction per mole of SO_4^{2-}) allowed a reliable prediction on how the initial amount of sulfate affects methane yield in AD. As the sulfate reduction occurs in an early stage of AD (in 9 days or earlier), this prediction method can be used to estimate biogas yield and composition in AD operation and numerical model applications on feed wastewater sludge treatability. Using such numerical tools for estimating losses in energy recovery, operators can determine if actions, such as $CaSO_4$ precipitation, have to be taken to maintain methane production.

The CO_2 content in the biogas decreased linearly with the increasing initial sulfate concentration although the CO_2 production in AD is theoretically expected to increase with the sulfate reduction reactions. The high cell yield of SRB compared to methanogens is thought to decrease the CO_2 production as H_2-oxidizing SRB grow autotrophically with CO_2 as the carbon source. The reduction in CO_2 content with sulfate is consistent with previous studies. Based on the experimental results, a linear relationship was established

between CO_2 in the biogas and sulfate dose ($y = –1.038x$, where y is CO_2 in mmol and x is sulfate as g S L^{-1}).

The COD and VSS destruction were not inhibited by the well-known toxic effect of sulfide, implying insignificance of the secondary inhibition for the examined sulfate doses. The lack of secondary inhibition observed in this study was unexpected as the majority of previous studies concluded that secondary inhibition was a direct cause of AD process failures. Considering the negligible effects of secondary inhibition in anaerobic digestion of WAS found in this study along with the wide range of sulfide concentrations that trigger toxicity reported in literature it is clear that the toxic effect of sulfide can vary substantially under different conditions. Thus, co-digestion of WAS with high sulfate biosolids (e.g., food processing waste) could be a viable strategy to mitigate the effect of sulfide toxicity on the digestion of high sulfate waste streams.

It is possible that uncorrected effluent COD can be misinterpreted as secondary inhibition because sulfide oxidation in COD vials result in a substantial overestimation of the effluent COD. Thus, the importance of the COD correction must be emphasised for anaerobic treatment of wastewater or organic waste containing high levels of sulfate.

4.1.2 Enrichment of acetoclastic methanogens for process intensification of anaerobic digestion

Vigorous mixing conditions had a negative impact on *Methanosarcina* spp. growth while *Methanosaeta* spp. growth was not significant in the batch tests. The growth factor for *Methanosarcina* spp. ranged up to 7.5 while the growth factor for *Methanosaeta* spp. was always less than 1.6. Based on the observed growth factors the methane production by

Methanosaeta spp. was considered to be negligible, meaning that the majority of the methane production was driven by the growth of *Methanosarcina* spp.. Methane production was lower at the mixed conditions compared to the still conditions in the batch tests. This negative effect of vigorous mixing was more pronounced at higher acetate concentrations compared to lower ones. The mixing conditions were considered to prevent *Methanosarcina* cells from forming clusters. Note that *Methanosarcina* spp. grows more rapidly when they form large flocs and clusters.

The final VSS and particulate COD was lower in the mixed bottles than the still ones. This effect can be linked to *Methanosarcina* spp. growth, as the limited growth at mixing conditions meant a decreased cell mass at those conditions, leading to lower VSS and particulate COD. In high-rate anaerobic treatments on soluble substrate, vigorous mixing should be avoided to maximize methane production and enrich for *Methanosarcina* spp.. This could be problematic for process intensification as typically high-rate digesters have high-solids concentrations and thus require strong mixing. Multiple stage digestion in which the hydrolysis pre-treatment and methane production steps are performed in separate stages with different mixing conditions could solve this issue.

The growth factors for *Methanosarcina* spp. from all the experiments indicated that greater *Methanosarcina* spp. growth occurred at the high acetate concentration conditions compared to the lower ones (up to 20 times greater). This was expected due to the high substrate concentrations used (high acetate concentrations can inhibit *Methanosaeta* spp. growth) and because *Methanosarcina* spp. have doubling times of ~1 day whereas *Methanosaeta* spp. have doubling times of 4-6 days. This result suggests that

Methanosarcina spp. would be the dominant acetoclastic methanogen in process intensification scenarios following hydrolysis pre-treatment, as the soluble substrate concentrations are high.

In the three-cycle test the observed growth factors indicated that the LS-HA condition had the highest relative and absolute growth. *Methanosarcina* spp. prefers to grow in flocs and clusters and the high initial seed at the HS and MS conditions may have disrupted cluster formation (similar to mixing) thus inhibiting the growth of *Methanosarcina* spp.. The *Methanosaeta* spp. growth was low for all the conditions examined and no trends between *Methanosaeta* spp. growth and seed concentration was observed. As initial seed concentration increased, the effect of the decay of non-methanogenic microorganisms lowering VSS became more pronounced and the effect of the growth of *Methanosarcina* spp. raising VSS became less pronounced. The growth factor and VSS results suggest that when starting up anaerobic reactors for treatment of high-COD soluble substrate (i.e. for process intensification), using a low (<400 mg-VSS/L) concentration of seed might lead to more effective long-term treatment as there is more absolute growth at these conditions.

The nickel and cobalt supplementation in Batch Test II did not significantly impact the results. Nutrient supplementation is typically only necessary when treating low-solids substrate. While the substrate in this test was soluble, the seed came from a high-solids reactor and contained sufficient concentrations of nickel and cobalt. If the three-cycle tests had been carried out for more cycles it is possible that supplementation would have been necessary.

4.2 Suggestions for future work

4.2.1 Anaerobic digestion of sulfate rich wastewater sludge

The primary recommendation for future work on this topic is to repeat the experiments described in the study to guarantee the results are reproducible. Similar experiments could also be run at higher sulfate dosages to investigate if a COD to sulfate ratio greater than the limiting ratio of 0.67 gCOD:gSO_4^{2-} exists that triggers secondary inhibition. If the experiments were to be repeated, adding molecular analysis such as qPCR would provide valuable insights. Daily sampling for qPCR would allow the researcher to track the growth of SRB over the course of the experiment and verify that the majority of sulfate reduction (and thus SRB growth) occurs at the beginning of the digestion period, as indicated by the methane production results in our experiments. Furthermore, by examining the growth in the SRB population over the course of the test a stronger conclusion may be able to be reached regarding the reduced CO_2 production in high sulfate conditions.

Similar experiments in which WAS is mixed with sulfate-rich organic substrates (such as food processing waste) would be beneficial to examine if co-digestion is a viable strategy to mitigate the effects of secondary inhibition on the digestion of high sulfate waste streams. Low-solids waste streams with high sulfate concentrations would be the most interesting co-substrates, as sulfide toxicity has been found to negatively impact the digestion of these substrates.

4.2.2 Enrichment of acetoclastic methanogens for process intensification of anaerobic digestion

Future experiments on this topic should be motivated by applicability to AD process intensification. The batch experiments could be repeated with greater control over the mixing conditions. If mechanical mixers with fine controls are used a correlation between mixing intensity and methanogen growth could be obtained. These experiments could also be performed at a large range of different acetate concentrations and initial seed concentrations to evaluate the effects of those factors on methanogen growth and methane production and examine how all three factors interact. A statistical "Design of Experiments" approach could be used to maximize the amount of information obtained from the experiments.

Similar experiments to the three-cycle experiment could be performed; however they should be extended with more cycles and more qPCR sampling to monitor the transient methanogen growth. The extended amount of cycles would allow the researcher to investigate how long it takes for a maximum *Methanosarcina* spp. or *Methanosaeta* spp. concentration to be reached. If bacteria primers are available, qPCR could also be used to monitor the decrease in the non-methanogenic microorganism population over the course of the cycles.

Finally, similar experiments should be performed using effluent of hydrolysis pre-treatment processes as substrate instead of sodium acetate. The results can be used to optimize the design of process intensified digesters based on which pre-treatment strategy produces effluent that permits the greatest *Methanosarcina* spp. growth and methane

production. Once the optimal combination of pre-treatment strategy, mixing condition and initial seed concentration is determined from lab-scale batch tests, continuous flow lab-scale tests and pilot-scale demonstrations should be developed.

Appendix A: Normalized Methane Production Figures

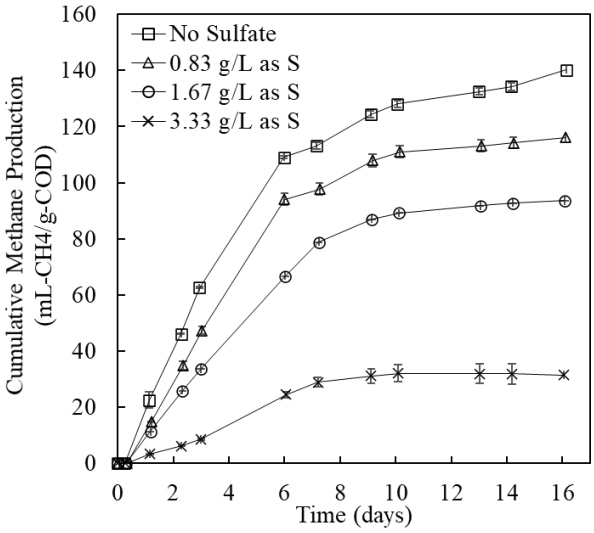

Figure A.1. Cumulative methane production written in mL-CH_4/g-COD in the experiments from chapter 2 (Figure 2.1) of this thesis (corrected for 1 atm and 0°C). The theoretical maximum methane production (100% biodegradable substrate) is 350 mL-CH_4/g-COD. The error bars represent twice the standard deviation of the duplicated experiments (n = 2).

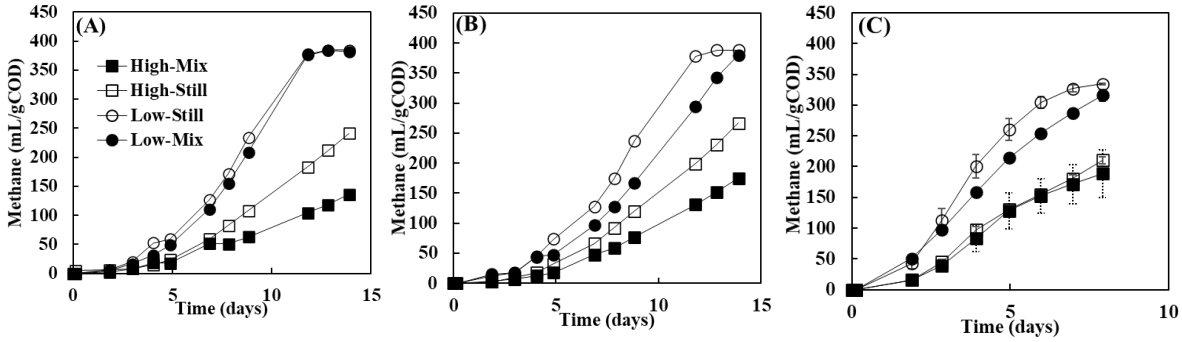

Figure A.2. Cumulative methane production written in mL-CH_4/g-COD over the course of the three batch tests from chapter 3 (Figure 3.1) of this thesis. (A) shows the results of batch test I, (B) shows the results of batch test II and (C) shows the duplicate results from batch test III. The theoretical maximum methane production (100% biodegradable substrate) is 400 mL-CH_4/g-COD. The error bars represent one standard deviation (n=2).