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Effects of Testing Time on Anaerobic Toxicity Assessment

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EFFECTS OF TESTING TIME ON ANAEROBIC TOXICITY ASSESSMENT

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ABSTRACT

Anaerobic digestion has been widely used for wastewater treatment for decades. Several Anaerobic Toxicity tests have been developed to screen wastewater for toxicity and assess the treatability of such toxicants. However, past research and reviews on Biochemical Methane Potential (BMP) test and Anaerobic Toxicity Assay (ATA) were considered in this study.

The experimental tests included ammonium-nitrogen and sodium chloride toxicity tests with varied concentration and solid retention time. These tests were designed to demonstrate the impact of ammonia- nitrogen and sodium chloride on the biological degradation process. The anaerobic test set-up includes bench-scale test reactors and respirometers used for measuring gas production volume and rate.

The experimental data using the gas production volumes and rates were plotted and analyzed. The hourly rate of gas production significantly decreases before the daily production volume decreases. Also, the gas production volume curves showed little or no toxicity when the gas production rates showed toxicity and acclimation.

In conclusion, the hourly production rate curves will indicate toxicity faster and extensively than the cumulative daily production volumes curves, thereby reducing testing time.

This thesis is approved for recommendation

to the Graduate Council.

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DEDICATION

This thesis is dedicated to my family and friends for their encouragement, financial and emotional support, and to Dr. Young and Anne at the laboratory for their technical support and help in the completion of this project.

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1.0 INTRODUCTION

Anaerobic digestion is without doubt one of the most efficient waste and wastewater treatment process, and it has been widely used for the treatment of municipal sludge, organic industrial wastes and agricultural wastes (Parkin and Miller 1983). It is a treatment process that compensates operational usage of fossil fuels by the production of methane during the treatment process (Chen et al. 2008). Many of the products/chemicals in waste and wastewater are toxic substances. A toxic substance is any chemical or mixture that may be harmful to the environment and to human health if inhaled, swallowed, or absorbed. Toxic substances in wastewater are mostly synthetic which means they are man-made (EPA 2012). Since toxic substances often occur at substantial concentrations in wastewater and sludge, they are found to be the leading cause of failure and upset in anaerobic digester or treatment process.

Although many researchers have investigated the toxicity level in anaerobic digestion (McCarty and McKinney 1961; McCarty 1964; Van Velsen 1979; Koster and Lettinga 1988; Hansen et al. 1998; Gallert et al. 1998) and the effects of toxicity on methanogensis, the range of values differ under various experimental conditions. The variations might be due to complexity and mechanism of anaerobic treatment process such as antagonism, synergism, acclimation and the complex compounds formed (Chen et al. 2008). However, toxicity in anaerobic processes can be measured and prevented.

Several anaerobic toxicity assessment procedures have been developed to test the treatability of industrial effluents from anaerobic sludge digesters because a universal monitoring device for toxicity testing is unlikely. A greater number of recent studies have dealt with the use of Biochemical Methane Potential assay and Anaerobic Toxicity Assay for

toxicity screening and assessment. Hungate (1969) developed Biochemical Methane Potential (BMP) assay and Anaerobic Toxicity Assay (ATA) for toxicity and degradability of organic matter which were modified by Miller and Wolin (1974) and Owen et al. (1979).

This report provides detailed descriptions and analyses of past and present studies on the anaerobic digestion from selective articles and books; identification of factors affecting anaerobic toxicity assessments, methods and determination of acute and chronic toxicity, analysis of methane production curve, and acclimation/testing time. The methods used for conducting ammonium-nitrogen and sodium chloride toxicity tests were described and experimental data were analysed and plotted. The effect of ammonia-nitrogen and sodium chloride on anaerobic biodegradation and the relationship between daily gas production volume (cumulative data every 24 hours) and the gas production rate were observed. Conclusions were reached from the interpretations and observations made from the gas production curve and the effect of testing time (hourly and daily) on anaerobic toxicity assessments were determined.

2.0 LITERATURE REVIEW

2.1 ANAEROBIC DIGESTION

Anaerobic digestion has been used for decades in treating organic matter in wastewater. It involves the degradation and stabilization of organic materials under anaerobic conditions by microorganisms resulting in production of biogas, which is a mixture of carbon dioxide, methane (Bordac and Young 1998) and microbial biomass (Kelleher et al. 2000). Degradation is achieved by specialized groups of microorganisms in the absence of oxygen. The fundamentals of anaerobic treatment are biodegradation reaction, biological growth, toxicity, temperature, Solid Retention Time (SRT) and other factors affecting performance. Several important biodegradation reactions take place simultaneously during anaerobic treatment process (Figure 1). Complex organic materials are converted first to simpler organics, which in turn are converted to organic acids and hydrogen.

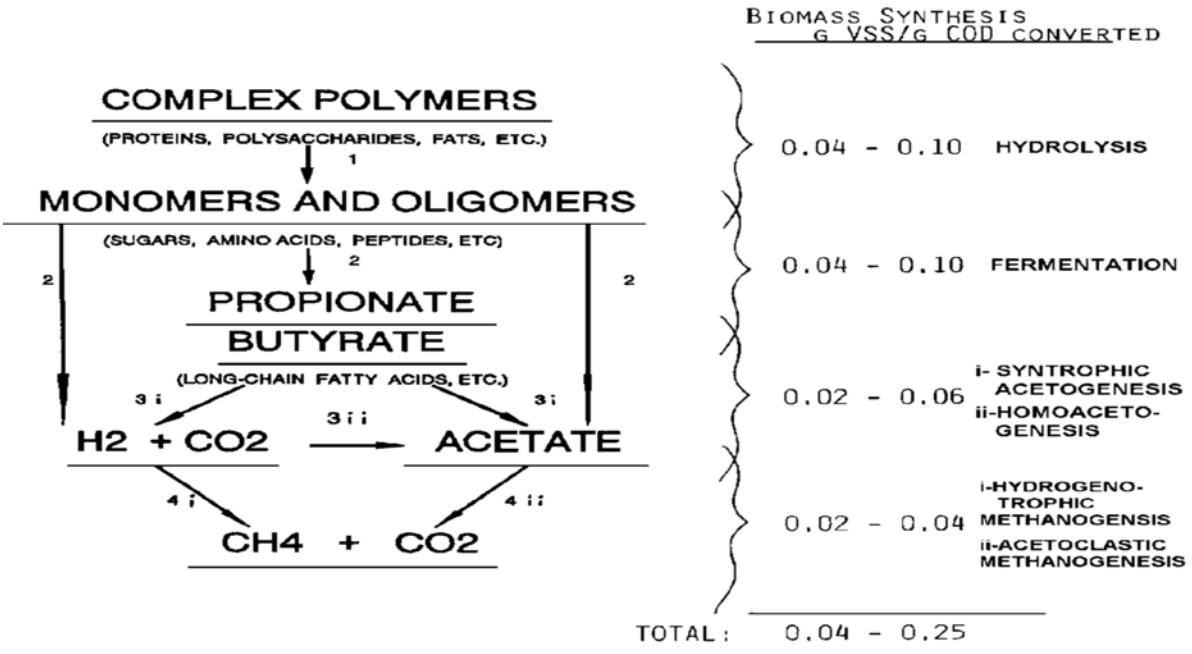

Figure 1: Anaerobic Biodegradation Reactions

(Young and Cowan 2004)

The organic acids and hydrogen are converted to methane by specialized microorganisms known as methanogens. All reactions must work together; otherwise overall treatment will be adversely affected.

Toxicity or inhibition is a major fundamental of anaerobic treatment. It affects the maximum substrate uptake (g COD/g VSS/d) and half-saturation coefficients (g COD/L) of kinetic models as well as reduces COD removal efficiency and loading rate capacity. Thus, a substance or water constituent can be inhibitory or toxic when it decreases the microbial population or bacterial growth rate. The decrease in microbial activity results in difficulty in controlling anaerobic process and imbalance in the anaerobic system, causing a decrease in pH (Jeris and Kugelman 1985; Bajpai and Iannotti 1988) due to the increase in volatile acids. Toxic substances or compounds affect the metabolic enzymes of the microorganisms group, the energy metabolism by uncoupling of growth and ATP production, and the cell membranes by creating intracellular changes of the pH (Gallert et al. 1998). However, some toxic chemicals can be accommodated during anaerobic treatment of industrial wastewaters because toxicants like formaldehyde, acrylate, chloroform, trichloroethylene, and cyanide can be biodegradable.

Favorable anaerobic conditions are required in the removal of toxicants including low toxicant concentration compared to IC_{50} (50% inhibition) concentration, biodegradable toxicant under the treatment conditions and acclimation of the biomass to some toxicants. Therefore, anaerobic toxicity assessment is important for the investigation of toxic substances that affect biological processes because of the increasing number of biogas plants and complexity of the water/wastewater sector.

2.2 ANAEROBIC TOXICITY ASSESSMENT

Several works of literature have proposed different laboratory methods for measuring the potential methane production and toxicity under anaerobic conditions (Hungate 1969; Miller and Wolin 1974; Owen et al. 1979; Hansen et al. 2004; Schievano et al. 2008, 2009a). This led to the development of the Biochemical Methane Potential (BMP) test and Anaerobic Toxicity Assay (ATA) (Tothill and Turner 1996).

ATA and BMP tests can be used individually or collectively to define the toxicity of pollutants in anaerobic applications and to measure methane/gas production by evacuation of small volume increment through a specially designed flow monitoring device as a respirometer (Young and Cowan 2004). For complex toxicity, a bioassay screening tool like the respirometer can be used for aerobic or anaerobic process in measuring gas production and toxicity (Speece 2008). The first anaerobic respirometer toxicity test was developed and conducted by Owen et al. (1979) for assessing organic constituent's inhibition and biodegradability (James et al. 1990; Young and Cowan 2004). The respirometer measures oxygen uptake or gas production, analyses substrate conversion and formation of by-product, evaluates biomass production and the toxicity of waste water and specific chemicals (Young and Cowan 2004). Also, the progress of the reaction is monitored, methane production rates and biomass activity are tracked, and the data is stored by computerized monitoring schemes (Speece 2008).

The respirometer tests run as batch assays in serum bottles and methane/gas production is monitored by passing the gas through a CO_2 scrubber. Most Anaerobic Toxicity tests are commonly conducted in batch modes where organic and inorganic energy substrates are added to the serum bottles along with adequate nutrients, minerals, buffer to control the pH, and microorganism seed culture (inoculum) at the beginning of the test.

2.2.1 ANAEROBIC TOXICITY ASSAY (ATA)

ATA (Owen et al. 1979; Demirer and Speece, 1998) is the correlative measure of potential toxicity of a wastewater or chemical sample plus a biodegradable organic substrate (ethanol, acetate, benzoate etc.) with an anaerobic biomass contained within a sealed vessel developed by the McCarty's group (Owen et al. 1979). Also, ATA is required to analyse the impact of specific chemicals on anaerobic processes. Biodegradable organic pollutants like acrylic acid, acrolein, lipids, and chlorinated organics when above certain concentrations, manifest inhibition (Speece 2008).

The procedure involves the addition of 20 – 50 mL of wastewater sample and biodegradable organic substrates to anaerobic cultures in a 200 - 500 mL sealed serum bottle or 2-L laboratory reactors in the absence of oxygen. The biodegradable substrate concentration may range from 0 in the culture blank to 2 - 4 times the diluted wastewater concentration in other reactors (Young and Cowan 2004). Furthermore, the full impact of toxicity cannot be easily evaluated by the ATA for wastewater sample with diluted inoculum. Dilution of the wastewater must be considered during the interpretation of the ATA data except when dewatered granular sludge is used. To avoid substrate limitation, ATA is conducted in the presence of excess substrate added to the initial mixture in the serum bottles. Also, the biomass evaluated is placed in the serum bottles and gassed with 50% carbon-dioxide (CO_2) and 50% methane (CH_4).

The initial gas production rate is of primary interest in the ATA (Speece 2008). Most importantly, if the waste water sample contains toxic substances, will toxicity reflect in the reduced initial rate of the gas production to volume of waste water added. For proper evaluation of toxicity, ATA assays are conducted using batch reactors with a range of wastewater containing biomass inoculum that allows the manifestation of reduced methane/gas production

rate (Speece 2008). At maximum metabolic rate, biomass activity does not depend on the substrate concentration and any reduction in gas production rate would relate to toxicity in the wastewater sample.

2.2.2 BIOCHEMICAL METHANE POTENTIAL (BMP) TEST

BMP test is the correlative measure of anaerobic biodegradation developed by Hungate (1969), and modified by the McCarty's group (Owen et al. 1979; Chynoweth et al. 1993; Hansen et al. 2004). It is a powerful test conducted to assess the performance and treatability of wastes in anaerobic digester (Speece 1996; Bishop et al. 2009). The test is a simple method used to evaluate the conversion rate of waste to methane (Gunaseelan 1997), determine the potential of anaerobic process efficiency, measure the residual organic pollutants, and determine the non-biodegradable substances after treatment (Speece 2008). BMP tests involve exposing anaerobic cultures to wastewater constituents and recording the amount of methane or total biogas produced (Owen et al. 1979; Angelidaki et al. 2009; Young 2006).

BMP test is usually performed along with Biochemical Oxygen Demand (BOD) assay, although regulatory requirements might specify only BOD test. Since BMP test measures only the effective biodegradable fraction of the organic matter, the parameters considered are both quantitative and qualitative aspects of a substrate (Schievano et al. 2011). Similarly, BMP assay measures organic pollutants biodegradation rate under anaerobic conditions that a BOD assay would measure under aerobic conditions (Hansen et al. 2004). The advantage of BMP test is the estimation of certain kinetic parameters (Donoso-Bravo et al. 2011) and its applicability to all kinds of wastewater (Lesteur et al. 2010).

The procedure involves the addition of an effluent sample to a 20 – 50 mL inoculum from an anaerobic culture in a 200 - 500 mL serum bottles or 2-L laboratory reactors (Young and Tabak 1991, 1993). The headspace in the serum bottle is purged with gas at 30 – 50% CO_2 composition for pH control, nitrogen gas (N_2) or methane (CH_4) (Speece 2008). The serum bottles are incubated at 35^oC and gas production, usually methane, are measured by inserting a hypodermic needle connected to a calibrated fluid reservoir, through the serum cap over periods ranging from 1 to 60 days (Robinson and Tiedje 1983), with or without the additions of chemicals or wastewater samples (Young and Cowan 2004). According to the stoichiometric relationship, 395 mL of CH_4 production is equivalent to 1 gram COD at the temperature of 35^oC use for the determination of COD reduction in liquid phase (Speece 2008).

BMP assays use different sources of anaerobic biomass which include a laboratory maintained biomass and an inoculum from an anaerobic digester of a municipal wastewater treatment plant. To assess the ability for sustained biodegradation, the modern BMP test was developed with the addition of multiple substrates that are not easily degradable (Benjamin et al 1984) over a period of 3 to 60 days (Young and Tabak 1993). For a BMP test, the biomass must be acclimated to the pollutants. Therefore, care must be taken to run a control with only an anaerobic inoculum and insure adequate time /acclimation for the biomass to metabolize the pollutant (Speece 2008). In addition, care must be taken in overlooking toxicity when initially characterizing the anaerobic treatability assessment of wastewaters because excessive time may be required to reach the ultimate BMP.

2.2.3 DIFFERENCES AND SIMILARITIES BETWEEN BMP AND ATA

ATA and BMP assays are different in several ways. ATA are used for the preliminary estimate of any toxicity inherent in wastewater while BMP tests give a preliminary estimate of the potential amount of COD bio-transformed into methane (Young and Cowan 2004). ATA is flooded with acetate or other simple substrate while BMP test is not. ATA uses easily degradable compound to determine toxicity by comparing the methane production rate with concentration of the test compound while BMP test uses no added biodegradable compound and the methane production rate is a direct measure of the biodegradation potential of the organic compounds in the test sample (Benjamin et al. 1984).

In terms of initial and total gas production, the initial gas production rate is important in ATA while the total gas production volume is indicative of the BMP. Sell et al. (2011) analyzed the ability of BMP assay to predict methane production of full scale anaerobic digester and compared it to the methane production for different biomass using a known degradable feedstock (Moody et al. 2011a). The BMP data revealed that the enzyme processing wastewater was ideal for dilution while ATA data revealed toxicity at low inclusion rate due to high ammonia concentration in the wastewater (Sell et al. 2011).

On the other hand, both BMP and ATA are used to determine the potential mixture of substrates necessary for the characterization of hydraulic retention time (Owen et al. 1979), organic loading rate and methane yield by relating laboratory scale test result to full scale anaerobic digesters (Wilkie et al. 2004; Sell et al. 2010). Sometimes the biomass used in ATA and BMP test has the ability to acclimate to the toxic substance. The length of time required for a microbial population to acclimate to a particular toxic compound is determined by the two methods (Benjamin et al. 1984).

Sell et al. (2010) performed BMP assays in triplicate using modified international standards ISO 117 34 (1995) and ATA in triplicate using the modified International standards ISO 13641-1 (2003) at different dilution ratios to analyze materials of unknown toxicity. Effects of toxicants were calculated using the ratio of all % inclusion yields (toxicants combined with degradable substrate and inoculum in seven mass concentrations) to the control. Methane yield was determined using the linear portion of methane production curve. Methane production decreased as the ratio of test substrate to degradable substrate increased. Multiple procedures such as substrate characterization, methane production and toxicity analysis, mixture combination based on design criteria, substrate selection and analysis were recommended for the design and operation of full scale anaerobic digesters.

However, there is advantage in the combined use of BMP assay and ATA. BMP tests determine substrate that inhibits methane production from the mixture of known substrates and test substrates. As a result, BMP tests have the potential of high dilution of substrate inhibition which can be overcome by ATA (Moody et al. 2009) using limited substrate in batch system, which does not represent the operational condition of typical anaerobic digesters (Sell et al. 2010).

2.3 FACTORS AFFECTING ANAEROBIC TOXICITY ASSESSMENT

A range of values is reported in the literature for anaerobic toxicity assessments and the variations are often disturbing. The variations might be due to experimental errors or factors that cause greater difference like the loading rate of the system, biomass inventory, and the effects of acclimation and nutrient insufficiency. Anaerobic Toxicity Assessment is sensitive to test

parameters (substrate, inoculum) which depend on operating conditions like inoculum/biomass inventory, temperature, loading rate, acclimation, pH and size of particle (Lesteur et al. 2010).

2.3.1 INOCULUM/BIOMASS INVENTORY

With anaerobic treatment of wastewater, there is need for high biomass concentration to compensate for lower biodegradation rate of recalcitrant substances and prolong cellular residence times (Speece 1996). For anaerobic toxicity test, low inoculum affects biogas production. Also, the substrate/inoculum ratio affects kinetics (Gunaseelan 1995; Raposo et al. 2006) and lag phase time but not ultimate methane production (Lesteur et al. 2010). At high substrate/inoculum ratio, lag time is shorter (Chen and Hashimoto 1996).

King et al. (2011) performed two sets of 35^{o}C BMP assays based on established procedures (Owen et al. 1979) which comprises three batches. Each batch was conducted in triplicate using substrate alone, biomass alone, and biomass with substrate. Biogas production was monitored daily to determine the initial rate of methane production. The initial rates of methane production increased across substrate alone, biomass alone and substrate plus biomass assays with increasing temperature. In conclusion, the initial rate of methane production was highest in assay with the biomass plus substrate at 35^{o} C.

2.3.2 TEMPERATURE

Temperature affects the rate of biomass metabolism activity and biodegradation. Batch assays for different anaerobic sludge were used to determine acute toxicity of oleic acid (monounsaturated fatty acid found naturally in many plant and animal products) at different

temperature (30^{o}C, 40^{o}C & 55^{o}C). It was discovered that oleate was over 12-fold more toxic at thermophilic conditions than mesophilic conditions (Hwu and Lettigna 1997).

Zupancic and Jemec (2010) conducted BMP assays to determine potential biogas production with different substrate and varied organic load at two temperatures (37^{o}C and 55^{o}C) using inoculum collected from a local municipal wastewater treatment plant. Due to different substrates and temperatures, significant differences were expected in anaerobic biodegradability. However, the differences were due to different inoculum employed at different temperature. It was concluded that BMP assay provides the first possible estimate of the substrate anaerobic degradation to determine the necessity of further experiments.

Biodegradation of organic matter is faster at thermophilic conditions (55^{o}C) than mesophilic conditions due to higher microbial metabolic activity (Fountoulakis et al 2008) but BMPs should be conducted under mesophilic conditions at 35^{o}C (Lesteur et al. 2010; Danoso-Bravo et al. 2011).

2.3.3 LOADING RATE

In developing toxicity data, the primary substrate should be tested over a range of concentrations. Low concentrations of toxic substances in a wastewater may be readily degraded whereas higher concentration may be toxic to the metabolism. An additional toxicant may have a negligible impact when the unit biomass metabolism rate is below the maximum potential capacity. As a result, toxicity tests are set up in a way that only one factor (toxicant concentration) is controlling and all other factors are not rate-limiting. Consideration should be given to gathering and interpreting toxicity data under substrate limiting condition since the IC_{50} toxicant is higher under a non-limiting substrate conditions (Speece 2008).

Eskicioglu et al. (2011) used BMP assay to study anaerobic biodegradability of whole stillage under both mesophilic (35 ± 2 ^{o}C) and thermophilic (55 ± 2 ^{o}C) conditions. No significant lag phase was experienced at the beginning of the experiment under specific organic loading of 0.27 – 4.20 g TCOD/g VS but degradation was completed at high organic loading of 0.051 g TCOD/L - 4.20 g TCOD/g VS. From the thermophilic BMP, higher overall biogas production was observed because of the additional biogas from the thermophilic inoculum. Also, the study emphasized that BMP assay does reflect the conditions of a full scale anaerobic digester and it is a cheaper and effective way of testing methane potential using the Owen et al. (1979) method.

2.3.4 ACCLIMATION

Other researchers (Yang et al. 1979, 1980; Parkin and Speece 1982) have studied and reported the response and recovery of methanogens to numerous organic and inorganic toxicants**.** When using toxicity tests for industrial wastewater, the biomass must be allowed adequate acclimation time to metabolize the pollutants (Speece 2008). The acclimation of anaerobic biomass to toxicants can be realized by first exposing the biomass to low concentrations, before ramping up the concentrations to the target range of prototype. This procedure for biomass acclimation is developed under favourable conditions with the concentration of biodegradation toxicants in the reactor maintained at a lower level than that in the wastewater sample (Speece 2008). If the toxicants can be degraded into methane after some acclimation period, a decrease in initial gas production rate or a lag time period indicates toxicity (Benjamin et al. 1984).

Biomass has the capability to acclimate to some toxicants, such that the same concentration of toxicants can be inhibitory to an un-acclimated biomass causing cessation in

metabolic activity while its shows no reduction in metabolic rate for an acclimated biomass. As a result, the concentration at first observed negative impact known as the threshold toxicity concentration of some toxicants can be increased as much as ten-fold with adequate acclimation (Speece 2008).

2.3.5 OTHER FACTORS

Other factors that affect anaerobic toxicity assessments include pH and size of particle. A low pH due to a lower temperature, results in an increase of Volatile Fatty Acids (VFA) in an anaerobic reactor while high pH due to a higher temperature, produces free ammonia which is toxic to methanogens (Young and Cowan 2004). Therefore, a buffer is required to maintain neutral pH during BMP test (Lesteur et al. 2010). In addition, the size of particles affects kinetics and lowers the hydrolysis rate (Lesteur et al. 2010) due to variations in the available specific surface area of the substrate. Decreasing the size of substrate particle containing high fiber level improves gas production and decreases digestion time (Palmowski and Muller 2000).

2.4 ACUTE AND CHRONIC TOXICITY

Acute toxicity (EC_{50} or IC_{50}) expresses the effective concentration that produces a 50% decrease in the activity of the microorganisms (biomass) exposed to the toxic substance or effluent for 48 hours. Acute toxicity is a result of sudden exposure of un-acclimated biomass to high levels of a toxicant because the biomass has not developed enough enzyme systems to adjust or adapt to the imposed stress.

Chronic toxicity (IC_{25}) tests allow the assessment of more subtle adverse effects like reduction in reproduction or growth of biomass exposed to toxic substance or effluent over 48 hours (Chaparro and Pires 2011). Chronic toxicity occurs as a result of gradual and prolonged exposure to toxic substances which give the biomass an opportunity to develop the enzyme system (acclimation) to degrade the toxicant. In some situations, biomass may adapt to toxicants without degradation.

Anaerobic toxicity assessments are useful as a screening procedure for preliminary assessment of toxicant effect and the determination of acute and chronic toxicity as well as biological degradability (Benjamin et al. 1984). The full impacts of toxicity can be muted by the dilution of the sample with the inoculum (Young and Cowan 2004).

Several publications have reported BMP values for a variety of wastewaters from different sources. If a toxicant inhibits biomass activity by 50% (IC_{50} concentration), then the biomass needed to treat a given amount of pollutant should be doubled to prevent a progressive rise in effluent concentration. All toxicants manifest toxicity relative to their concentrations (Speece 2008). IC_{50} concentration of a variety of organic chemicals to un-acclimated biomass was determined by Blum and Speece (1991) and the selected toxicity data from the literature can be viewed in Speece (2008).

In addition, Chen et al. (2003) evaluated the chronic and acute toxicity of sodium to methanogens under thermophilic conditions. The biomass was acclimated to increasing sodium concentration of 4.1, 7.1, and 10.0 g/L using duplicate anaerobic toxicity assays to evaluate the chronic toxicity while un-acclimated biomass was used in a series of batch anaerobic toxicity assays to determine the acute toxicity. Methanogens had minimum adaptation time at high sodium concentrations and sufficient adaptation time with increasing sodium concentration.

Moreover, the batch ATAs simulated acute toxicity and showed a steep decline in the methanogenic activity. Even with the decrease in methanogenic activity, the methanogens acclimated to the higher concentrations of sodium with time, without deterioration in system performance with respect to COD removal and methane production. The acclimated methanogens seemed to function well at sodium concentrations lethal (LC_{50}) to microorganisms in the batch tests. The effect of chloride during the tests was not mentioned

From the findings, it was concluded that the acute and chronic toxicity can be caused by biodegradable fraction of organic compounds (Chaparro et al. 2010).Therefore, acute and chronic toxicity using ATA and BMP assays can be observed or determined from the methane production curve at various testing time.

2.4.1 METHANE PRODUCTION CURVE

Anaerobic respirometers are used to monitor the methane production. The pattern of methane production due to the relationship between methane production and kinetics control in anaerobic digestion determines the mechanism of toxicity (Young 2006). The biogas/methane production curves have various patterns depending on substrate biodegradation and production of toxic substances which controls the kinetics of anaerobic digestion.

The cumulative methane production for the control is plotted as a straight line having a slope equal to the rate of daily methane production or hourly methane production expressed as mL/g COD_{added}. If the cumulative methane production curve for the test waste coincides with the control curve, then the test waste is biodegradable. If the slope is less than that of control, then the relative biodegradability of wastewater is the ratio of the slope of the test waste curve to

control substrate curve (Young and Cowan 2004). A decrease in slope indicates the occurrence of threshold toxicity caused by constituents in the wastewater.

Labatut et al. (2011) used BMP assays to determine the bio-methane potential of more than 30 substrates. It was determined that the rate and extent of methane production were reduced when the required enzyme for a particular substrate's degradation was present in the mixture. The curves aid in identifying anaerobic degradation issues. A steep curve indicated easily degradable substrate while a curve with an initial low slope, then an increase in slope indicated high inhibition during the first days of BMP test. The inhibition was due to the attainment of the biomass homeostatic threshold which altered thermodynamic balance and inhibited the metabolic reaction. However, when the thermodynamic conditions changes, it means acclimation occurred. To ensure ideal anaerobic conditions and prevent inhibition during BMP test, appropriate substrates and biomass combination were used under ideal environmental conditions especially substrate and biomass concentrations below inhibitory/toxic levels.

2.4.2 TESTING TIME

Data collection for anaerobic toxicity test requires precision and accuracy, and significant time commitment to measure the biogas or methane production (Gayle et al. 2010). Long acclimation time (1-2 months) tests are required to provide a large range of data for the estimation of methane production (Labatut et al. 2011). Toxicity tests may be extended for 30 or 60 days to accommodate acclimation by toxicants or pollutants in some industrial waste water due to the lower growth rate of anaerobic microbes. The 30-day BMP is a first cut evaluation of the amount of methane produced from the conversion or biodegradation of the pollutant in a wastewater sample. An evaluation less than 30 days will not indicate key considerations such as

biofilm or granule development, net biomass yield, kinetic coefficients, trace metal supplementation requirements, and acclimation characteristics of biomass. However, assay durations less than 30 days can be valuable when considering the suitability of anaerobic treatment for a given wastewater (Speece 2008). Besides, short term ATA and BMP tests are needed for preliminary design of anaerobic digesters and measurements of toxicity. Some industries monitor toxicity by holding wastewater in a separate vessel (toxicity assays) for 24 hours to determine the toxicity conditions before it is routed to the microbial treatment process (Speece 2008). A 24 or 48 hour routine test is required to measure an acute toxicity. Therefore, methods of reducing testing time of anaerobic toxicity assessment are necessary.

Although the effect of product inhibition on reaction kinetics is difficult to prevent, the adequate digestion time allows the stabilization of substrate degradation resulting in maximum methane production represented by threshold limit to methane production curve. For instance, Sell et al. (2011) performed two sets of BMP tests. The first BMP test provided estimates of methane production rate while the second test was performed to extend the range for methane production estimate. The testing time for the second BMP test was selected based on the inability of the start-up or first BMP test to predict daily methane production rate during the duration (105 days) of the study. The early methane production rate was above the predicted maximum value due to degradation of inoculum remnants. BMP measures the maximum methane production volume per gram of Volatile Solid (VS). BMP tests also can identify microbial inhibition, overloading and acclimation.

Past research showed that the initial and cumulative volume of methane production of an anaerobic toxicity assessment can be measured hourly (Young et al. 1997; Young 2001; Cho et al. 2004) or daily (Lesteur et al. 2010). The initial rate techniques for predicting anaerobic

digester operation was studied by Danoso-Bravo et al. (2011). Batch experiments in triplicate were run and biogas production was measured manually. The initial specific methane production rates were calculated from the batch experiment conducted to assess five substrate concentrations. The measurement of the initial methane production rate involved the evaluation of substrate depletion and product build-up. The initial slope of the biogas or methane production curve is proportional to the biomass concentration while the maximum slope between the biogas production and time (Illanes 2008) estimates the initial rate. After three days of operation, the cumulative methane production graph showed increasing methane production rate at increasing initial substrate concentration and decreasing linear fed/inoculum (F/I) ratio resulting in biomass surplus for organic matter degradation at low F/I. The study concluded that the 3 days test duration instead of the normal 20 days made the method easier to implement on a full scale operation, even though the loading rate, temperature and inhibition problems could not be predicted. An adequate range of linear F/I ratio was determined using different biomass concentration in anaerobic batch tests at constant substrate concentration, to achieve a linear biogas or methane concentration versus time achieved within a reasonable testing time (Illanes 2008), allows for proper dilution of the biomass concentration and evaluation of the effect of substrate concentration (Danoso-Bravo et al. 2011).

For hourly methane production rate curves, Pearson et al. (1980) showed the effect of time and toxicant concentration under shock loading on anaerobic gasification rate by presenting the hourly rates of gas production per unit volume of sludge with peak rate after 4 or 5 days and declination after 14 days. The authors conducted batch tests using four toxicants plus a control in a 25-sludge bottle setup. The bottles were mixed and one third of the content removed and replaced with fresh raw wastewater twice each week at a temperature range of 9^{o}C to 29^{o}C. The

gas production rates of the anaerobic test were computed by plotting the hourly gas production per unit volume of sludge versus temperature. The graph showed a decrease in gas production rate with increasing dose of toxicant.

The fact that BMP tests are conducted in batches and measure biogas production, makes it difficult to relate their results to anaerobic digester that operate in continuous mode (Donoso-Bravo et al. 2011). Introduction of new wastewater material into a bio-reactor will affect the microbial population and decrease the methane yield. Thus, in order to increase the testing time for BMP assessment, faster method for analyzing the chemical composition of a waste needs to be determined (Lesteur et al. 2010).

In conclusion, the above review highlighted past research on anaerobic digestion and toxicity assessment which includes a detailed description of anaerobic digestion, anaerobic toxicity assessment methods (BMP and ATA which are good indicators for assessing toxicity of chemicals that might affect anaerobic performance adversely); factors that affects anaerobic toxicity assessment; and Acute and Chronic toxicity; methane production curve; and testing time. Since accurate sampling and spanning of the testing time are necessary during the anaerobic assessment of some industrial waste water, the toxicity test is monitored daily by observing the daily cumulative gas production volume and the hourly gas production rate during 24 hours (hourly rate). A fact about anaerobic toxicity assessment is that important information required for the operation and treatment of toxicity can be overlooked by assays with daily cumulative gas production volume and long testing time.

Also, the correlation of anaerobic toxicity assessment for different testing time can be useful in extrapolating literature data or substituting different toxicity test (Blum and Speece 1991). Due to the fact that characterizing organic matter (biodegradable and non-biodegradable

fraction) and assessing anaerobic toxicity using standard test methods (Owen et al. 1979) is very time consuming, more effort should be given to determine the effect of testing time on anaerobic toxicity assessments. There are variations in experimental data and results, and no studies evaluating the effect of testing time (hourly and daily) on anaerobic toxicity assessments for the determination of toxicity and its effects on anaerobic treatment were found in literature.

Therefore, the objective of the current study was to show that the reduction in rate of biogas production is a better measure of toxic impact than a reduction in daily biogas production volume that is a measure of gas production rate is better than gas production volume.

3.0 METHODOLOGY

The experimental plan for the current anaerobic toxicity test was divided into two groups; ammonium-nitrogen toxicity tests and sodium chloride toxicity tests. Ammonium-nitrogen is a common toxic substance while sodium chloride (salts) is occasionally present or introduced in municipal and industrial wastewater. Based on the past research and present wastewater treatment operation these two products or chemicals were used as test chemicals for this research. These tests were designed to demonstrate whether ammonium-nitrogen had an impact on the biological degradation process, evaluate the effect of solid retention time on ammonium-nitrogen toxicity, and the impact of sodium chloride on gas production rate. Tests were conducted using bench-scale anaerobic test reactors for dosing the toxicants and anaerobic respirometers were used to measure gas production volume and rate.

3.1 ANAEROBIC TEST

The anaerobic test apparatus consisted of eight 500 ml serum bottles each containing 40 mL of anaerobic seed culture at 2,500mg/L VSS suspended in a 400 mL Nutrient/Mineral/Buffer solution (NMB). The seed culture was obtained from an industrial anaerobic reactor or digester. The anaerobic test was set up as described in Appendix A. The NMB solution was prepared by adding 20 mL Min I, Min II and Nutrient solutions from Appendix B and 6 g of $NaHCO_3$ per liter of distilled water. Feedstock organic solutions were prepared as described in Appendix B. Both acetate and ethanol were used as substrates for the tests. All feedstock solutions were prepared fresh before each test series and refrigerated to prevent deterioration during the testing.

The test bottles where then placed on an eight-position variable speed magnetic stirrer and mixed at approximately 250 rpm to distribute the feed wastewater, chemical additives, and

biomass evenly throughout the reactor. The bottles were capped with a septum and connected to a Challenge Environmental Systems bubble cell respirometer. A 20 mL syringe containing potassium hydroxide (KOH) pellets and anhydrous calcium sulfate was used to remove carbon dioxide and water vapor from the gas before the flow was measured. The respirometer was configured to measure and record the gas production of each individual reactor.

The reactors were fed acetate feedstock for several days to stabilize each culture and verify acceptable gas production. After stabilization, the pH of the mixed liquor was checked. If the pH was 0.5 below or above 7.0, it was adjusted to 7.0 by adding 2 - 10 mL of potassium hydroxide (KOH) or hydrochloric acid (HCl) respectively. The test temperature was 35^{o}C.

During each test run the mixed liquor in the bottles was sampled weekly using a syringe. These samples were tested for total and volatile suspended solids (TSS and VSS), soluble COD (sCOD), pH, (NH_3 + NH_4^+), and the values were recorded. The TSS and VSS tests were conducted according to the methods described in Standard Methods (American Public Health Association, American Water Works Association, Water Environment Federation, 1995). The sCOD and (NH_3 + NH_4^+) tests were conducted using HACH test kits (HACH Company) and the pH was measured using a calibrated electronic pH meter (Accumet AB15 Fisher Scientific).

3.1.1 AMMONIUM-NITROGEN TOXICITY TEST AT VARIED CONCENTRATION

The objective of this test series was to determine the impact of ammonium-nitrogen on anaerobic degradation. For the ammonium-nitrogen toxicity test, 6 mL of stabilization acetic acid was fed daily to each reactor as a biodegradable substrate for 2 to 3 days to give a COD dose of 2,400 mg/L/day. Following stabilization, the reactors where switched to the test feed (see Appendix C.1 for composition) and ammonium-nitrogen dosing schedule (Table 1). Acetate

feedstock (without base) was added as an organic substrate and ammonium-nitrogen feedstock was added at different volumes to respective test bottles.

Then, ammonium-nitrogen feedstock was fed daily for seven days to the respective test reactors at 0 (Control), 2, 5, 15, 20 and 25 mg/L/day to give a 200, 500, 1500, 2000 and 2500 mg N/L. In this manner, the cumulative dose of ammonium-nitrogen increased with time of operation**.**

Table 1: Ammonia-Nitrogen toxicity test feeding and dosage schedule at varied concentration.

Reactor	Feed	Toxicants
1,2	6 mL acetic acid feedstock	None
3	6 mL acetic acid feedstock	2 mL NH_4-N feedstock
4	6 mL acetic acid feedstock	5 mL NH_4-N feedstock
5	6 mL acetic acid feedstock	15 mL NH_4-N feedstock
6	6 mL acetic acid feedstock	20 mL NH_4-N feedstock
7	6 mL acetic acid feedstock	25 mL NH_4-N feedstock

3.1.2 AMMONIUM-NITROGEN TOXICITY TEST AT VARIED SOLID RETENTION TIME

Following the initial setup of the ammonium-nitrogen test, the bottles were cleaned and the test was setup and run again using a different procedure for the ammonium-nitrogen toxicity test with variable solids retention time (Table 2). The different solid retention times were

achieved and controlled daily by adding the same defined amount of ammonium-nitrogen to the reactors after removing a defined amount of the mixed liquor.

For the first 3 days, acetic acid feed stock without base was added to the test bottles for stabilization of the culture. Following stabilization, the reactors were switched to the test feed (see Appendix C.2 for composition) and ammonium-nitrogen dosing schedule (Table 2). An acetate control feed stock of pH 4.1 was added to the control reactors while ammonium acetate feedstock at pH 4.0 was added at different volumes to respective test chemicals rectors. To maintain different solids retention time, 25 mL, 17 ml, 11 mL of mixed liquor was wasted daily from respective bottles by using a syringe. An acetic acid-based feedstock having a COD of 10,000 mg/L was then fed at a rate of 25, 17, and 11 mL/day to give respective doses of 500, 340 and 220 mg (250, 170, and 110 mg COD/day).

Table 2: Ammonia-Nitrogen toxicity test feeding and dosage schedule at varied Solid Retention Time.

Reactor	Waste	SRT (days)	Toxicants
1,2	25 mL	20	25 mL acetate feedstock
3,4	25 mL	20	25 mL ammonium acetate feedstock
5,6	17 mL	30	17 mL ammonium acetate feedstock
7,8	11 mL	45	11 mL ammonium acetate feedstock

The bottles were stabilized for 2 to 3 days prior to testing using an acetate feedstock. Then, acetate feedstock was added to the control reactors while ammonium acetate feedstock was added at different volumes to respective test bottles. The mixed liquor in these bottles was also tested weekly and VSS, TSS, sCOD, pH, and (NH_3 + NH_4^+-N) were measured and recorded.

3.1.3 SODIUM CHLORIDE TOXICITY TEST

The objective of this test series was to evaluate the impact of sodium chloride on gas production rate. The setup was similar to the ammonium-nitrogen toxicity test but with different feedstock. For this test, ethanol was used as substrate. For the first 3 days, 6 mL (1200 mg COD) of the ethanol feedstock containing 200,000 mg COD/L was added to the test bottles for stabilization of the culture. Following stabilization, the reactors where switched to the test feed (see Appendix C.3 for composition) and sodium chloride dosing schedule (Table 3). 6 ml of ethanol feed stock was added to the reactors daily. Also, two different doses of sodium chloride containing 200g and 1,500 g COD/L were added to the reactors. The daily cumulative sodium chloride dose added to reactor 1, 2, 3, 4, 5, 6, 7 and 8 are 0, 6400, 9800, 13200, 16600, 20000, 23400 and 28800 mg/L NaCl respectively (Table 3).

Table 3: Sodium Chloride toxicity test feeding and dosage schedule at varied concentration.

Reactor	Feed (Ethanol Feedstock)	Toxicants		
		First Dose (Sodium chloride feedstock I)	Second Dose (Sodium chloride feedstock II)	Cumulative sodium concentration per day
1	6 mL	0 mL	0 mL	0 mg/L
2	6 mL	1 mL	2 mL	6,400 mg/L
3	6 mL	2 mL	3 mL	9,800 mg/L
4	6 mL	3 mL	4 mL	13,200 mg/L
5	6 mL	4 mL	5 mL	6,600 mg/L
6	6 mL	5 mL	6 mL	20,000 mg/L
7	6 mL	6 mL	7 mL	23,400 mg/L
8	6 mL	11 mL	8 mL	28,800 mg/L

The feedstock was refrigerated to prevent degradation of the compound during the test program, after culture was stabilized, the pH was checked and if below 6.5, the pH of the feedstock was readjusted to 6.5. The mixed liquor in these bottles was also tested weekly and VSS, TSS, sCOD, pH, and (NH_3+ NH_4^+-N) were measured and recorded.

4.0 RESULTS

After conducting the three sets of anaerobic toxicity tests, the experimental data were downloaded from the computer system connected to the anaerobic respirometer system and analyzed with Microsoft Excel. The measured values of gas production in mL gas/g COD versus time (days), hourly gas production rate in mL/hour versus time, gas production as a percent of control versus time, and the gas production rate versus cumulative dose of toxicants were plotted using line graphs and interpreted for each toxicity test.

4.1 AMMONIUM–NITROGEN TOXICITY TEST WITH VARIABLE CONCENTRATIONS

The first set of toxicity tests was conducted to determine the impact of ammonia-nitrogen on anaerobic degradation and to determine the concentration at which ammonium-nitrogen toxicity was observed. The reactors were first stabilized by feeding 6 mL of acetic acid feedstock fed daily for 3 days at a dose of 2,400 mg COD/L/ day plus sufficient ammonium-nitrogen feedstock to give concentrations of 200, 500, 1,000, 1,500, 2,000 and 2,500 mg N/L/day, respectively.

The data were analyzed and plotted as gas production volumes in milliliters per gram COD applied as shown in Figure 2. Initially, the cumulative gas production volumes for the reactors receiving ammonium-nitrogen coincided with that of the control reactor at values between 500 and 600 mL/g COD. Starting on day 4, the reactors receiving the 2,000 and 2,500 mg/L NH_4-N feedstock began to show a decrease in gas production rates – as indicated by the reduced slope of the gas production curves – but only around 15% reduction in daily volumes (Figure 2).

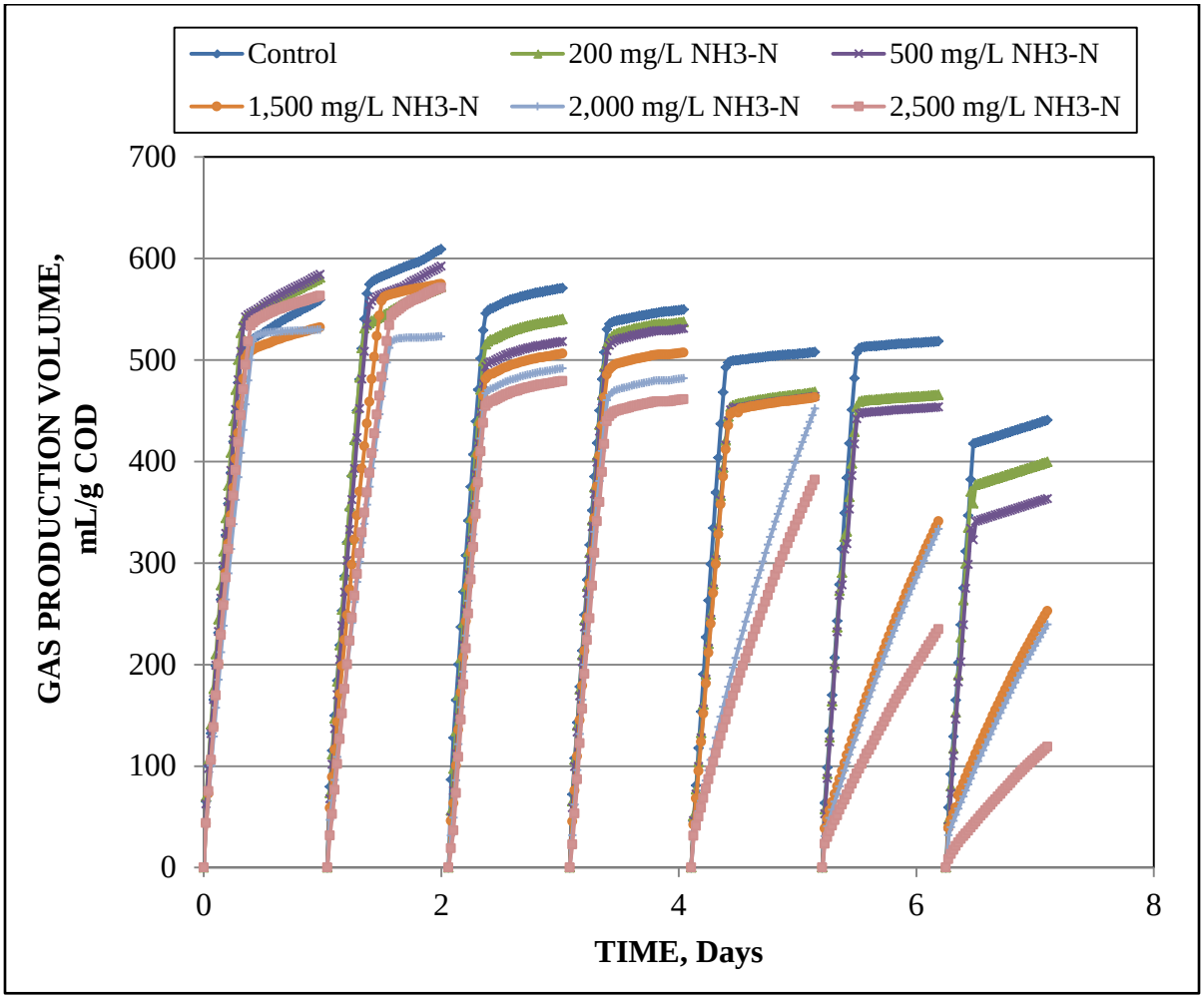

Figure 2: Daily gas production volumes at varied ammonium-nitrogen concentration.

Figure 3 shows the hourly gas production rates for the test reactors. Initially, hourly rate of gas production coincided with that for the control reactor. Rates began to decrease dramatically after the fourth day in reactors receiving the highest ammonium-N dose rates.

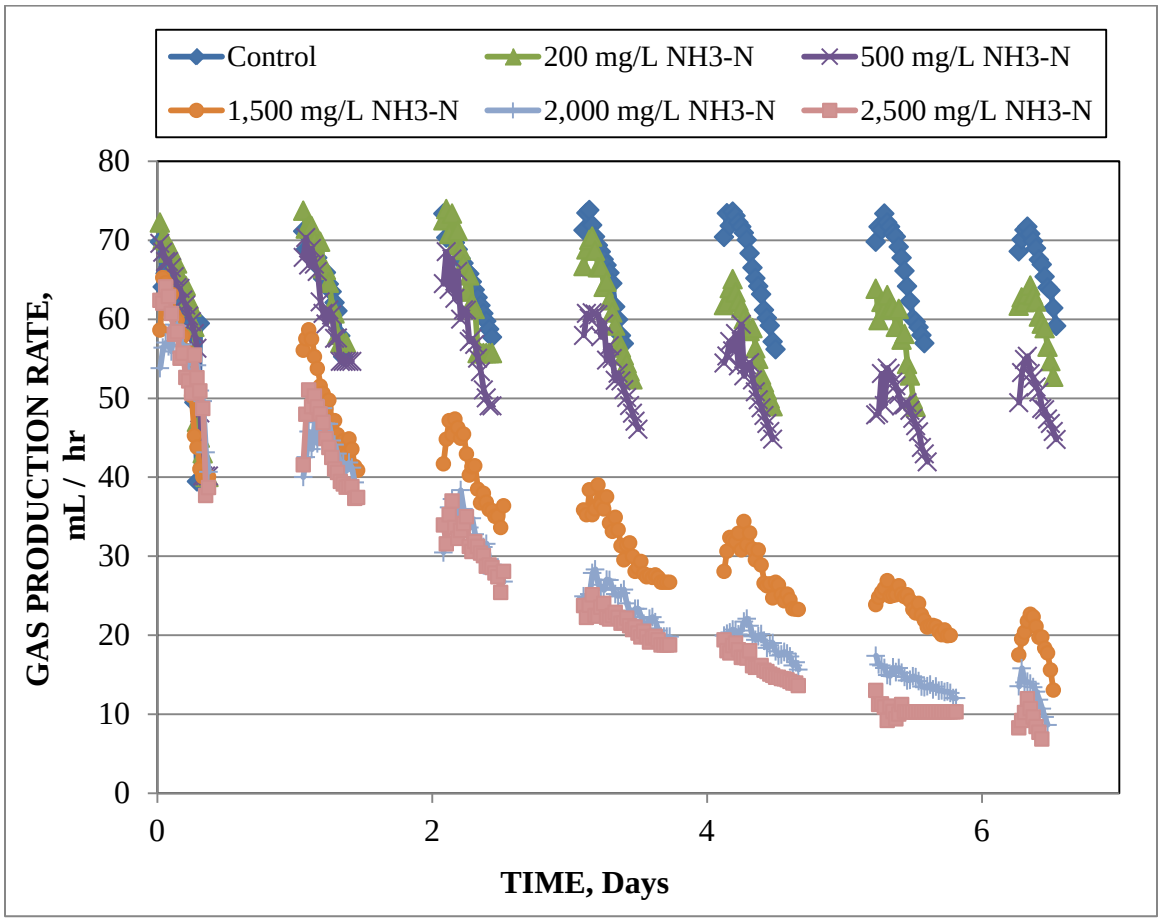

Figure 3: Hourly gas production rates at varied ammonium-nitrogen concentration.

The cumulative gas production volumes for the reactors as a percentage of the control are shown in Figure 4. This graph shows that the gas production volumes for reactors receiving NH_4-N decreased with time relative to that for the control reactor. Percentage of gas produced relative to that of the control for reactors receiving the 200, 500, 1,500, 2,000 and 2,500 mg/L NH_4-N daily doses were about 101, 98, 94, 91 and 87% respectively on day 1 which decreased to 85, 77, 54, 51 and 23% after 7 days.

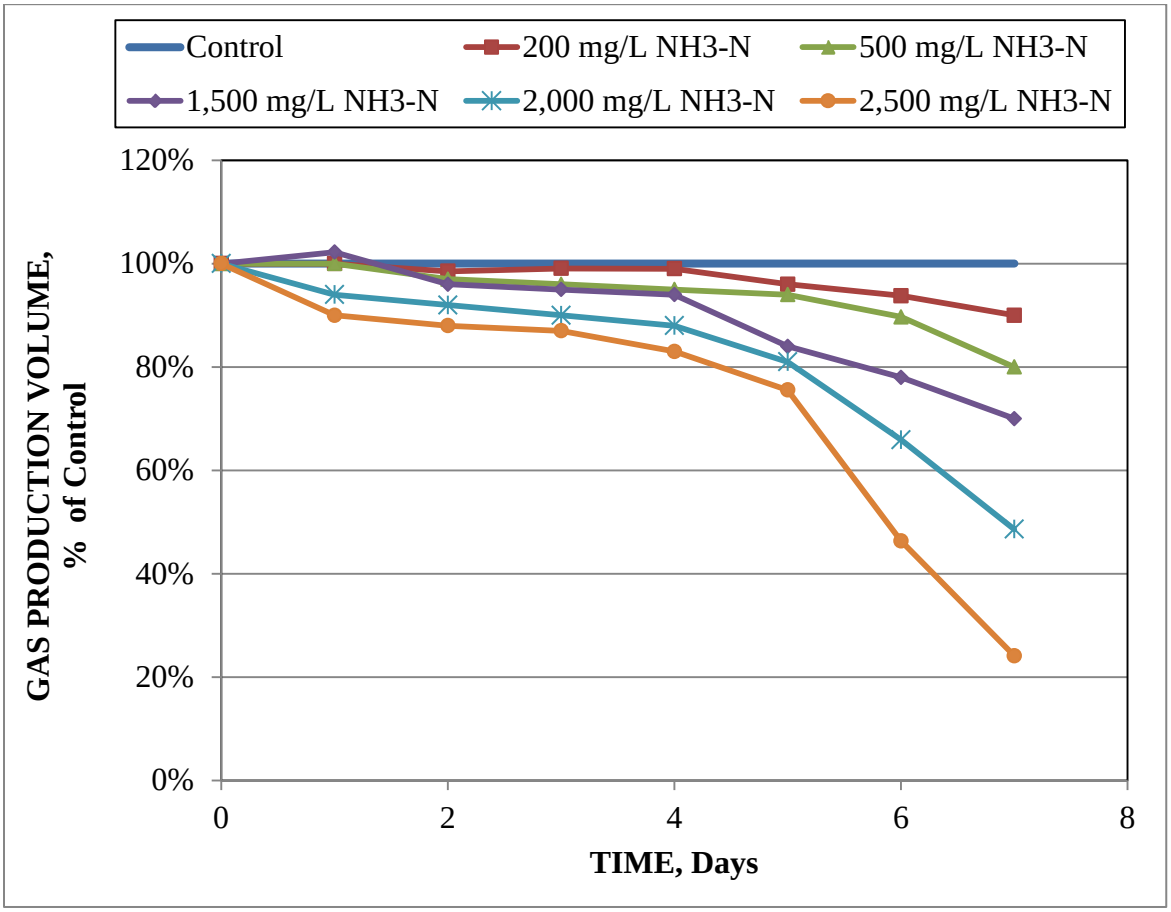

Figure 4: Cumulative gas production volumes expressed as a percentage of that for the control reactor at varied ammonium-nitrogen concentration.

However, the maximum hourly gas production rates for the reactors receiving NH_4-N decreased with time relative to that for the control reactor (Figure 5).

Percentage of gas produced relative to that of the control for reactors receiving the 200, 500, 1,500, 2,000 and 2,500 mg/L NH_4-N daily doses were about 101, 93, 89, 87 and 86% respectively on day which decreased to 78, 76, 45, 27 and 18 % after 7 days.

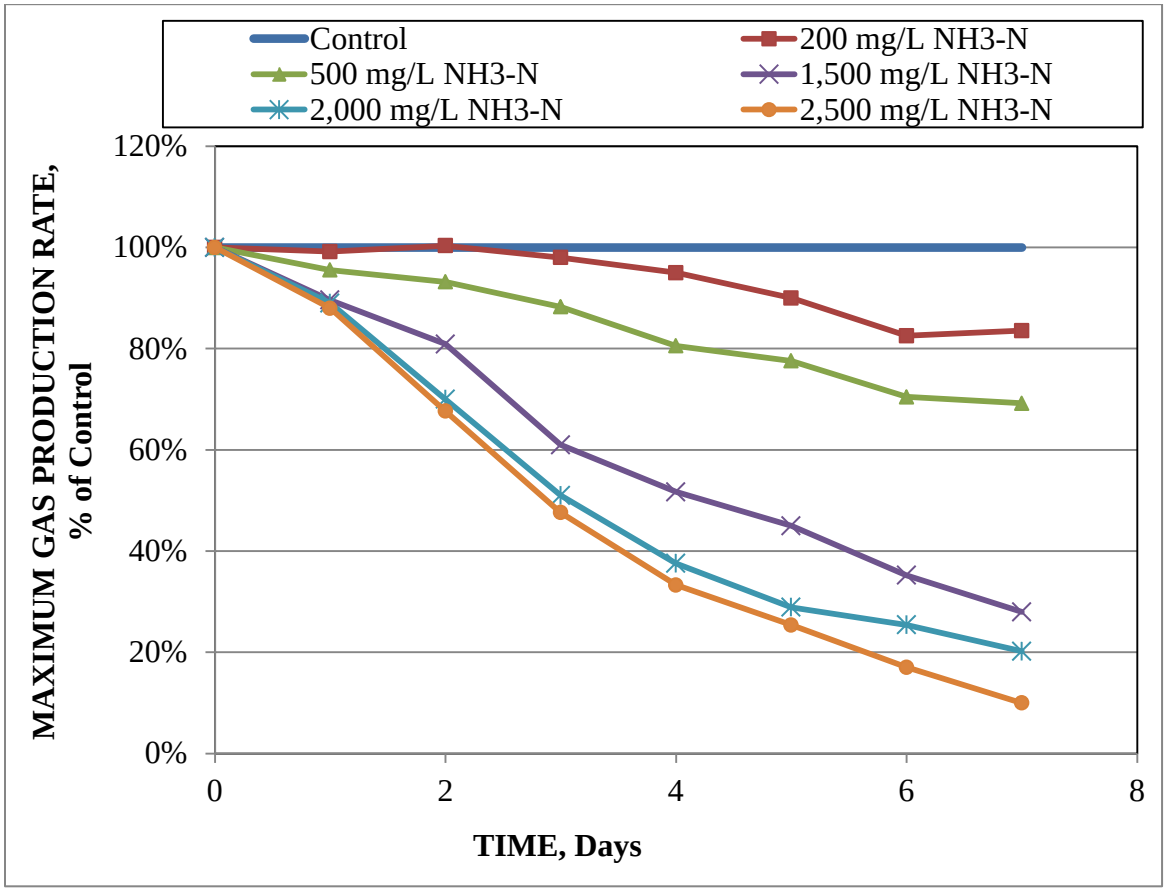

Figure 5: Maximum hourly gas production rates expressed as a percentage of that for the control reactor at varied ammonium-nitrogen concentration.

Figure 6 shows the cumulative gas production volumes versus cumulative ammonium-nitrogen dose. The gas production volumes relative to the control decreased with increasing cumulative ammonium-nitrogen concentration.

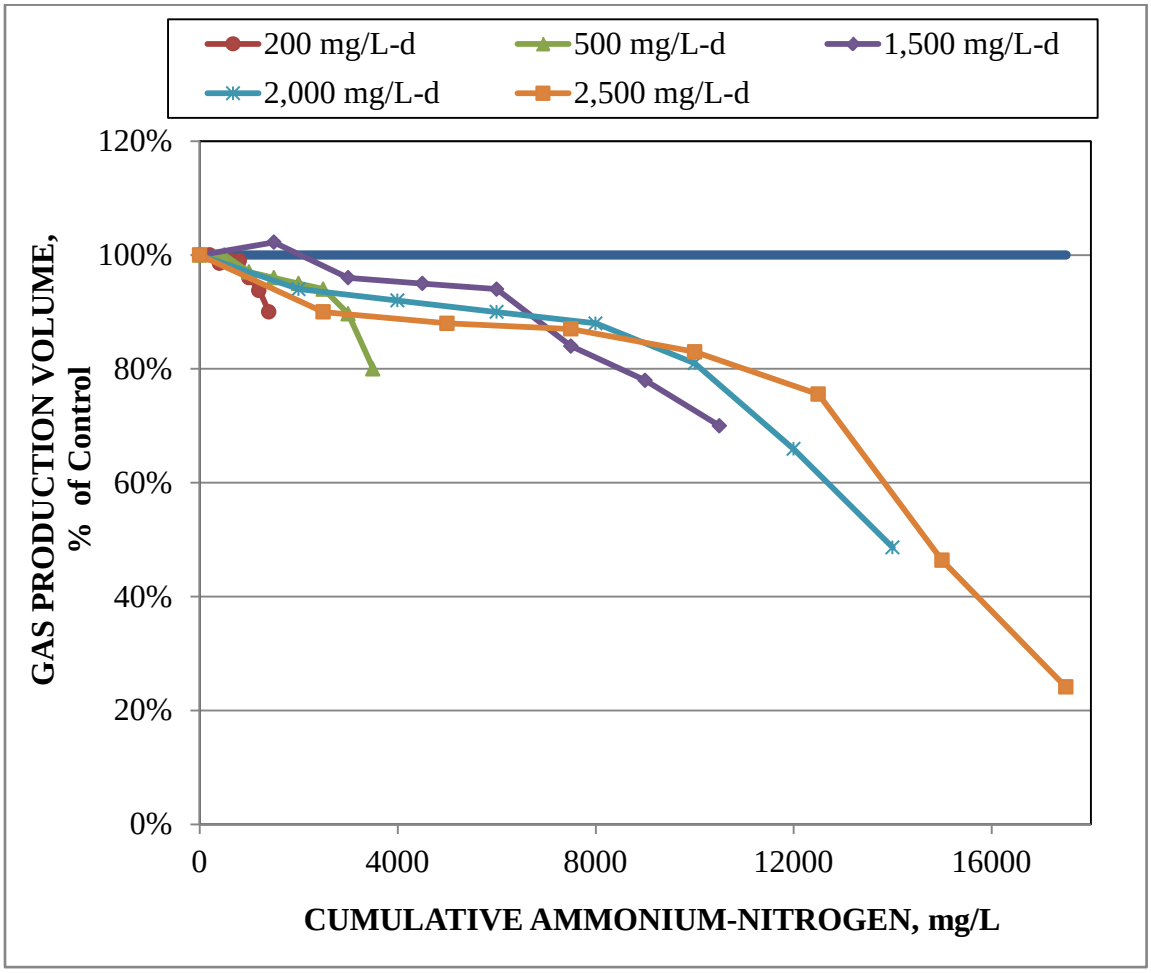

Figure 6: Gas production volumes expressed as a percentage of that for the control reactor at varied ammonium-nitrogen concentration.

Figure 7 shows that maximum gas production rates relative to the control decreased with increasing cumulative ammonium-nitrogen concentration. The fact that the relationship between gas production rates and cumulative ammonium-nitrogen addition was the same for all dose rates indicates that the microorganisms did not acclimate to the ammonium-N regardless of the dose rate.

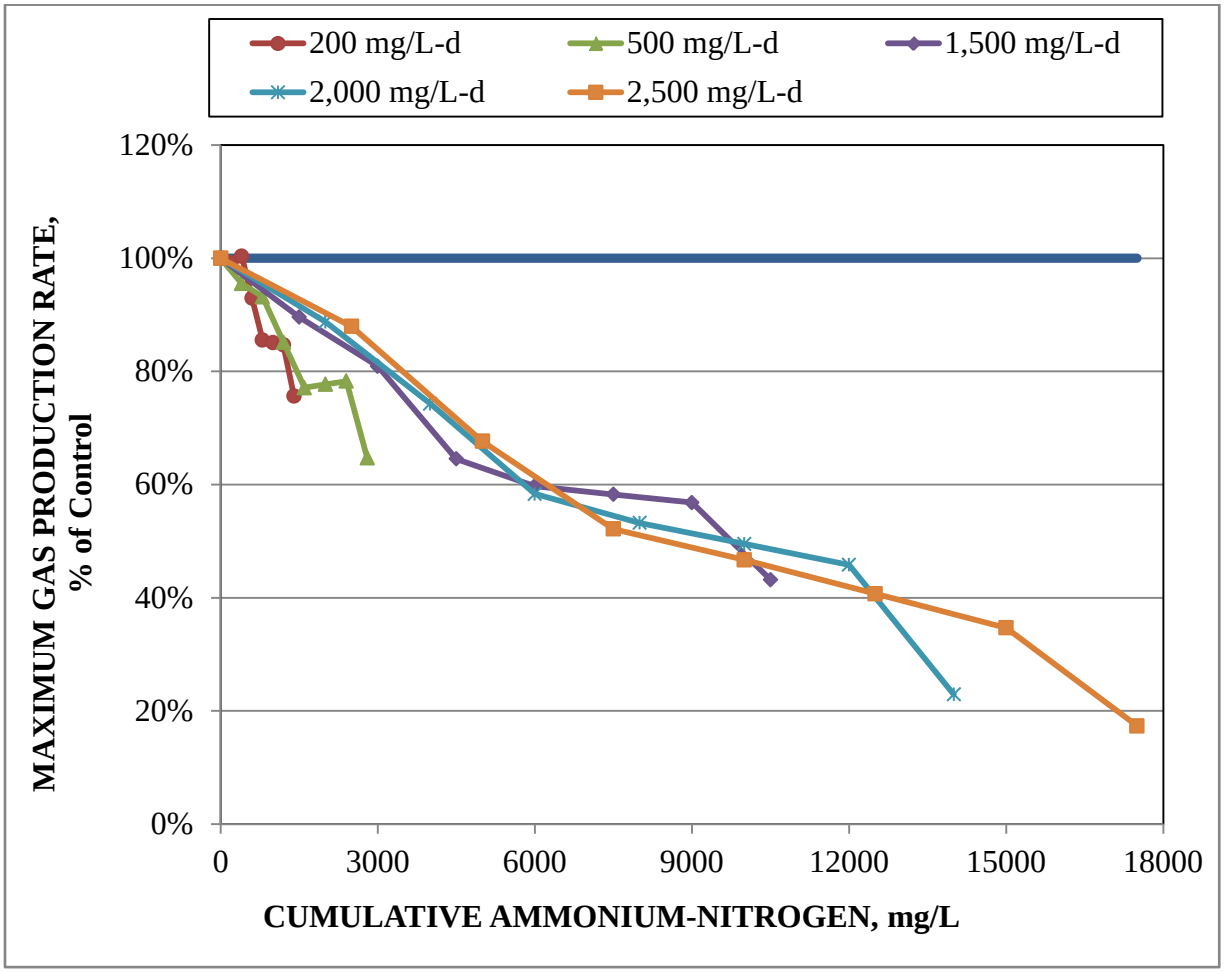

Figure 7: Maximum gas production rates expressed as a percentage of that for the control reactor at varied ammonium-nitrogen concentration.

Comparing the graphs of the daily gas production volumes (Figure 6) with hourly gas production rates (Figure 7) from the ammonia toxicity tests with varied NH_4-N concentration showed that the hourly rate of gas production decreased before the daily gas production volume decreased. Therefore, the hourly gas production rate graphs were adversely impacted significantly before a reduction in the daily gas production volume.

4.2 AMMONIUM–NITROGEN TOXICITY TEST WITH VARIABLE SOLID RETENTION TIME

A second set of anaerobic tests was conducted to determine the impact of Ammonium-nitrogen on biodegradation at different Solid Retention Time (SRT). The test setup was similar to the first ammonia toxicity test. In this case, 6 mL of acetic acid feedstock was added to the test bottles for 3 days at a dose rate of 2,400 mg/L/day for stabilization. Then 25 mL of the contents of the control reactor and 25, 17 ml, 11 mL of the contents of respective test reactors were removed daily and replaced with equal volumes of fresh acetate feedstock at 20,000 mg COD/L. The test reactors were also dosed with 500, 340 and 220 mg/L NH_3-N/day to the 20-d, 30-d and 45-d SRT reactors, respectively.

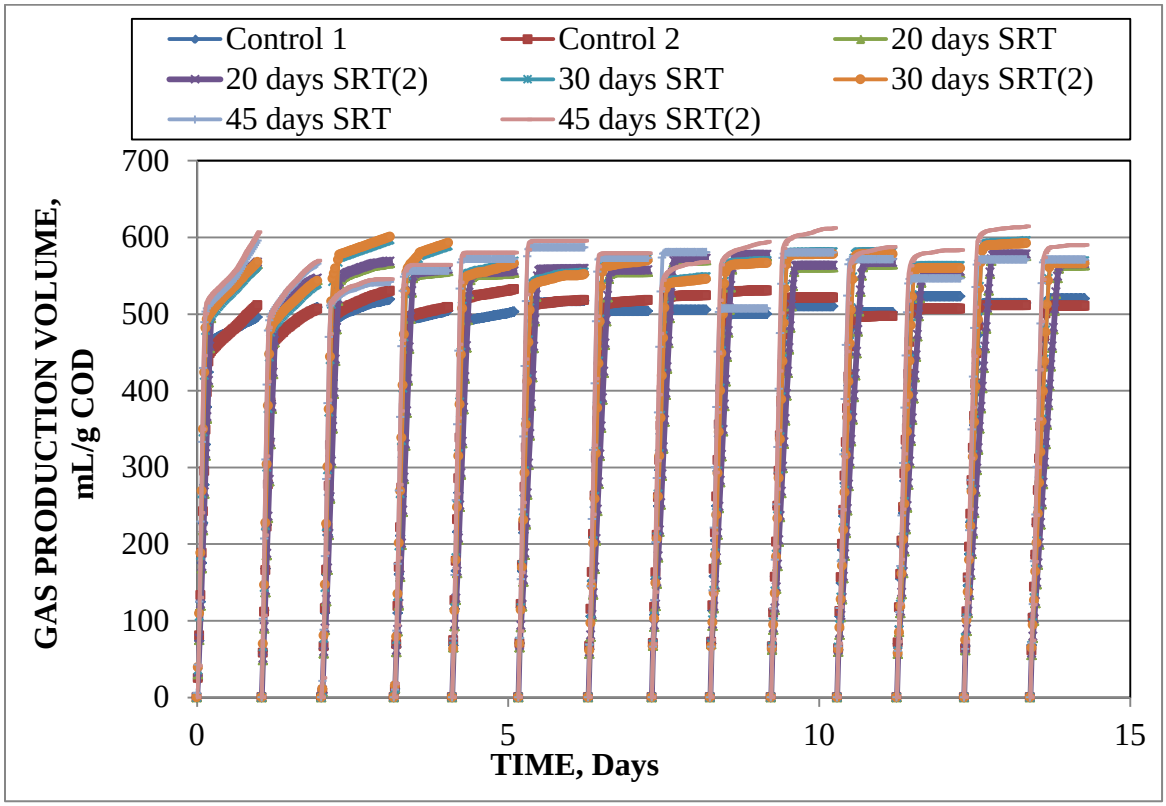

Figure 8: Daily gas production volumes for the ammonium-nitrogen toxicity test at different SRT.

Figure 8 shows the daily gas production volumes at solid retention times of 20, 30 and 45 days. The maximum gas production volumes were between 500 and 600 mL/g COD. There were no significant changes in gas production volumes for the test reactors relative to that for the control.

Figure 9 shows the hourly gas production rates during the test. The rates decreased with increasing time for each reactor. The maximum gas production rate was about 230 ml gas/ hour at 45 days-SRT with a 50% decrease by the end of the test.

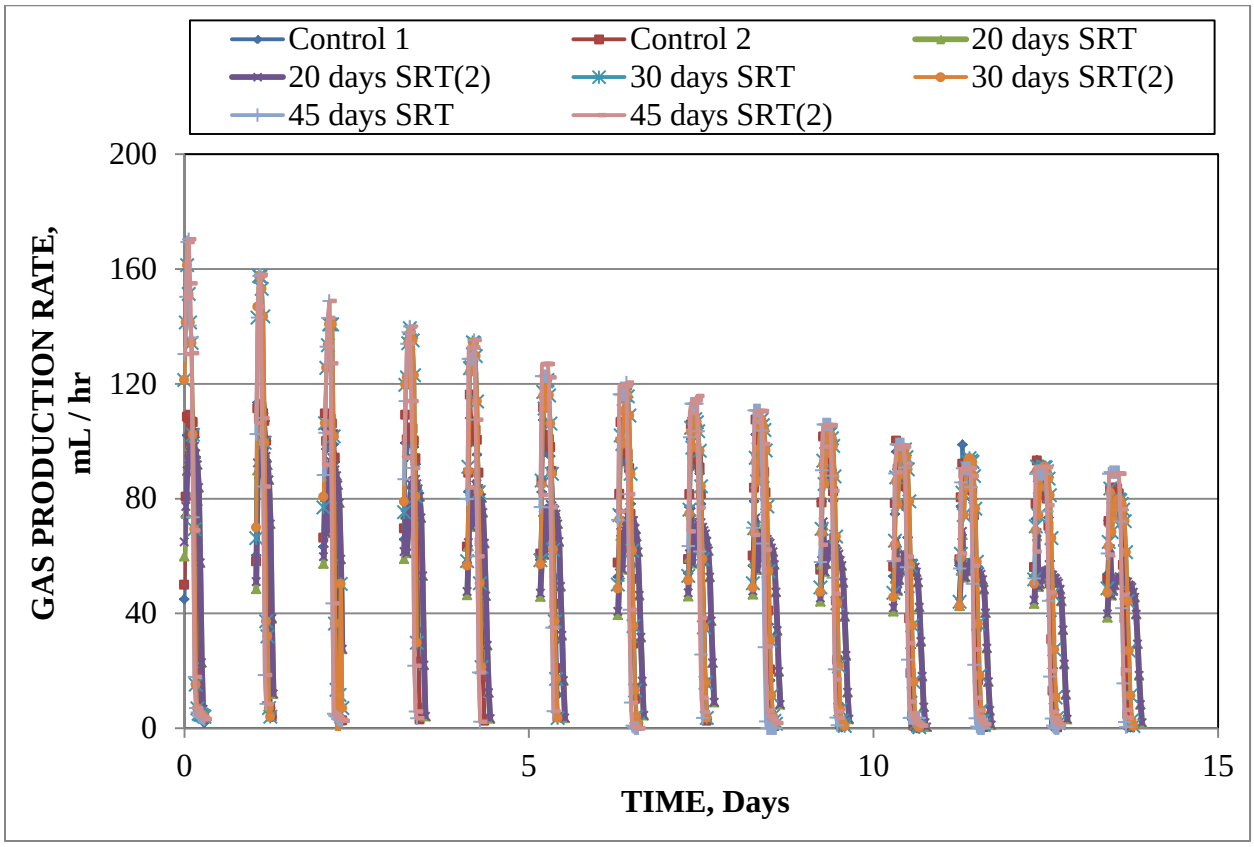

Figure 9: Hourly gas production rates during ammonium-nitrogen test at different SRT.

The daily gas production volumes for the test reactors expressed as a percentage of that for the control reactor remained relatively constant over time but decreased with decreasing SRT

(Figure 10). The maximum hourly gas production volumes of the 20, 30 and 45 days-SRT reactors were above the control values which do not indicate toxicity.

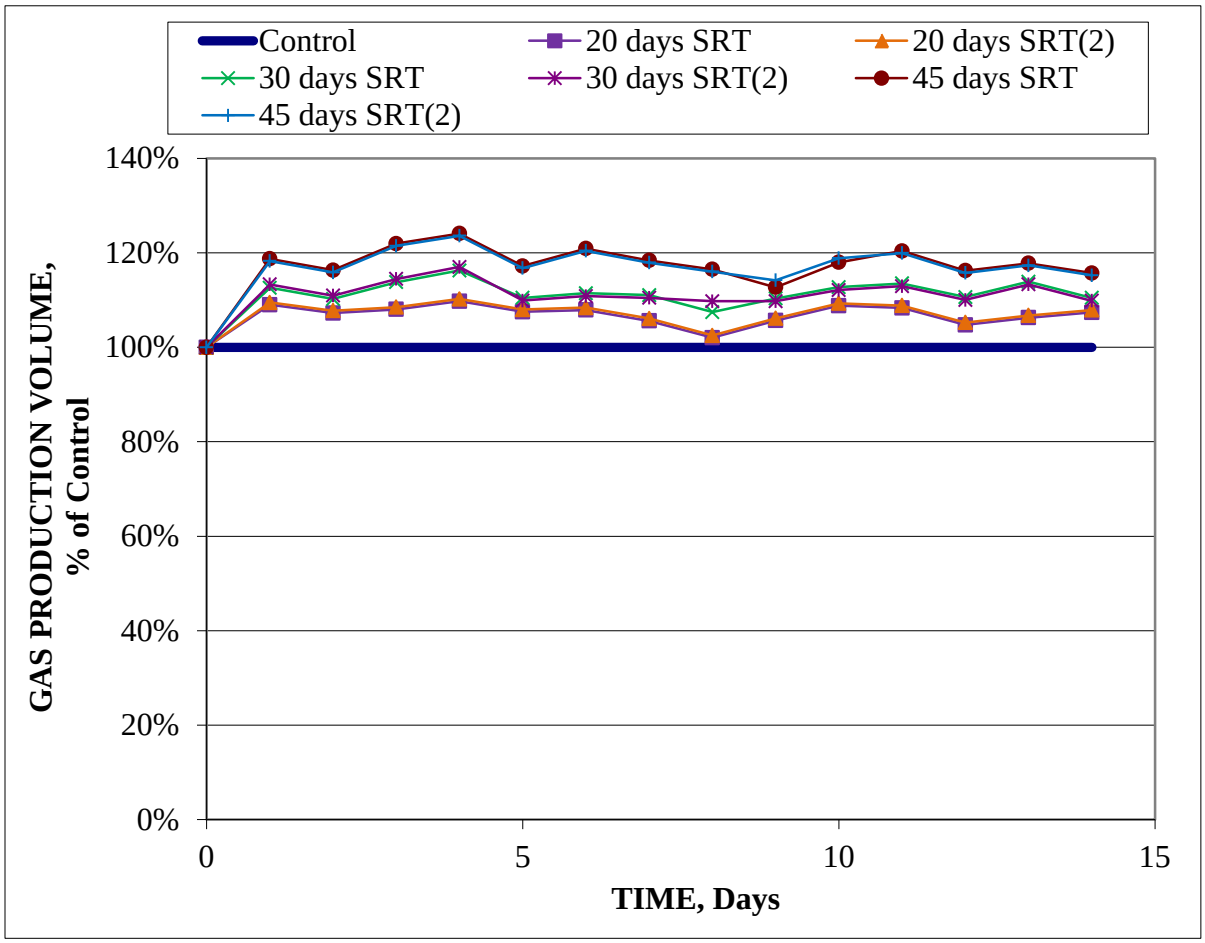

Figure 10: Cumulative gas production volumes expressed as a percentage of that for the control reactor at different SRT.

The maximum hourly gas production rates for the test reactors, expressed as a percentage of that for the control reactor however decreased with increasing testing time (Figure 11). Also, the percentage gas production rate decreased with decreasing solids retention time. The maximum hourly gas production rates of the 20 day-SRT reactors were below that of the control while the rates of the 45 day-SRT reactors were above the control respectively. Initially, the

maximum hourly gas production rates of the 30 day-SRT reactors were above the control values but eventually decrease below the control values.

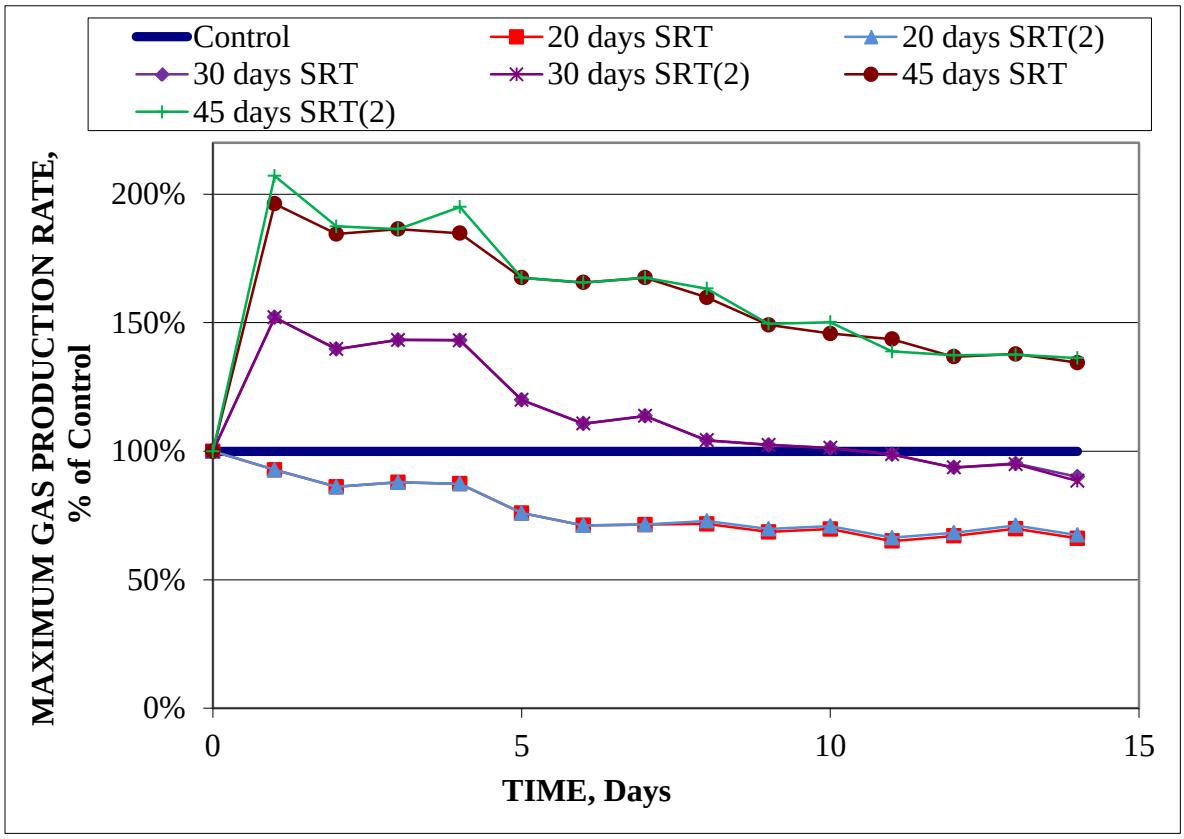

Figure 11: Maximum hourly gas production rates expressed as a percentage of that for the control reactor at different SRT.

Daily gas production volumes versus cumulative ammonium-nitrogen are shown in Figure 12. The percentage gas production volumes for the 20 day-SRT reactors were above that of the control while the 30 and 45 day-SRT reactors were below that of the control which contradicts past research that gas production increases with increasing SRT. However, in all cases, the gas production as a percentage of the control decreased with increasing cumulative ammonium-nitrogen indicating only slight toxicity.

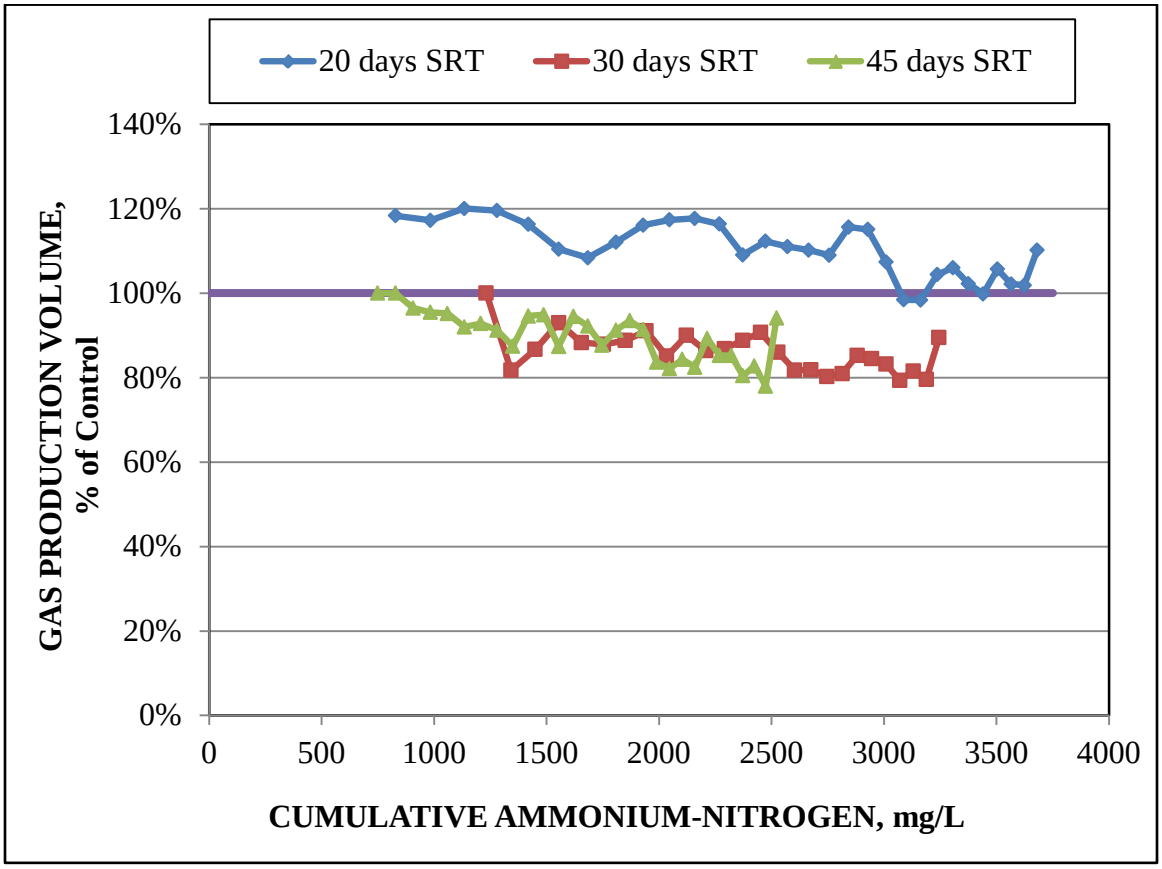

Figure 12: Gas production volumes expressed as a percentage of that for the control reactor at different SRT.

Figure 13 shows gas production rates for the test reactors expressed as a percentage of that for the control reactors versus cumulative ammonium-nitrogen concentrations. These data show a gradual decrease, followed by a rise in the gas production rates with time and increasing ammonium-nitrogen concentration. The observed reduction in relative gas production rate starting around 1000 mg/L ammonium-nitrogen inhibition by ammonium-nitrogen and the increasing values starting around 2,500 mg/L cumulative ammonium-N suggests recovery from toxic impact.

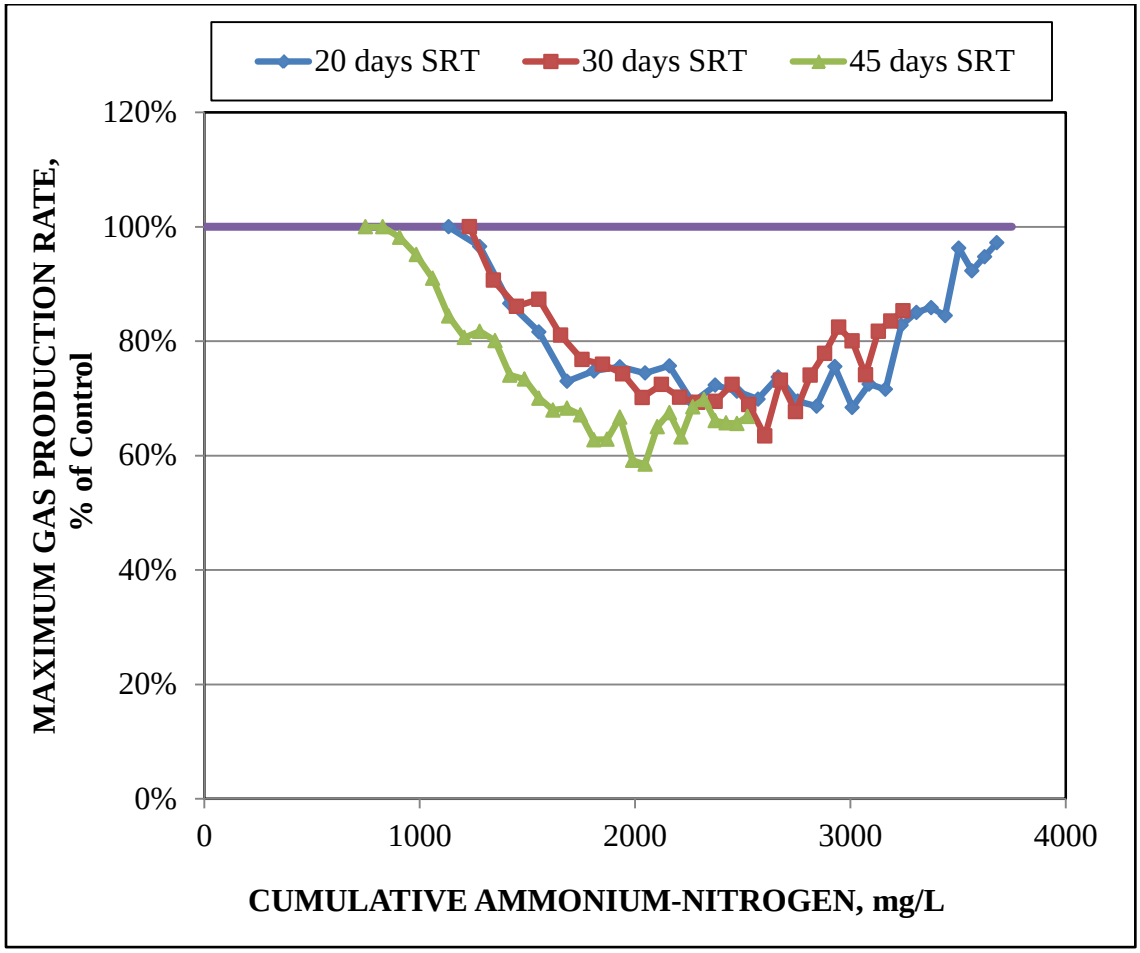

Figure 13: Maximum gas production rates expressed as a percentage of that for the control reactor at different SRT.

Comparing the gas production volumes graphs with the gas production rates graphs for the ammonia toxicity test at different SRT showed that the hourly gas production rates showed a decrease before the daily volume showed a decrease.

4.3 SODIUM CHLORIDE TOXICITY TEST

The third set of tests was conducted to determine the impact of sodium chloride on anaerobic degradation**.** For this test, 6 mL (400 g COD/L) of ethanol feedstock was added to the test bottles for 3 days to allow stabilization of the culture. After the third day, 6 ml of ethanol feed stock as well as 0, 6,400, 9800, 13,200, 16,600, 20,000, 23,400 and 28,800 mg/L of sodium chloride feedstock I and/or II were added to reactor 1, 2, 3, 4, 5, 6, 7 and 8 respectively.

Methane production when using ethanol as an organic substrate has two stages of reaction. The first stage is the related to acetate and hydrogen conversion while the second stage is related to acetate only conversion. The two reactions are due to the manner in which ethanol is degraded by anaerobic microorganisms. Ethanol reacts through fermentation to produce acetate and hydrogen gas. The acetate and hydrogen are converted to methane by different anaerobic microorganisms.

$$C_2H_5OH + H_2O \longrightarrow CH_3COO^- + H^+ + 2H_2 \qquad (4.1)$$

After the test, the data were analyzed and the results were plotted as presented in Figures 14 and 15. Figure 14 shows daily gas production volumes versus time. The initial daily gas production volumes were similar for all reactors for the first two days. Starting on day 3, the gas production rate, as indicated by the slope of the methane production curves, gradually decreased with increasing dose of sodium chloride. However, the daily gas production volumes remained essentially constant through day 6.

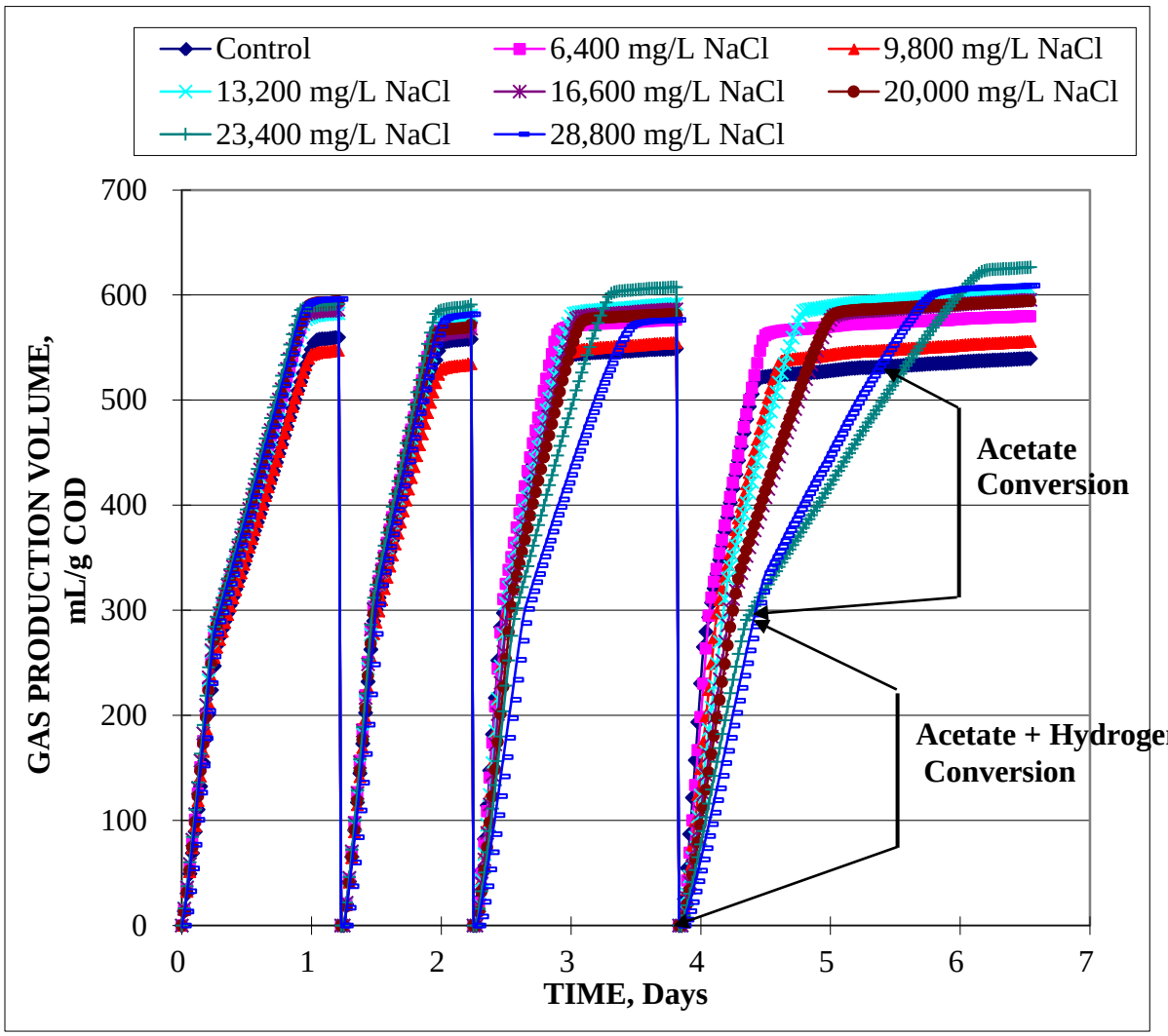

Figure 14: Daily gas production volumes at varied sodium chloride concentration.

Figure 15 shows the hourly gas production rates for the reactors. For the two conversion stages, the impact of sodium chloride on biodegradation could be observed from the initial and hourly gas production rate. In this case, the hourly rates for both the acetate only and acetate plus hydrogen conversion reactions began to decrease dramatically on the third dose.

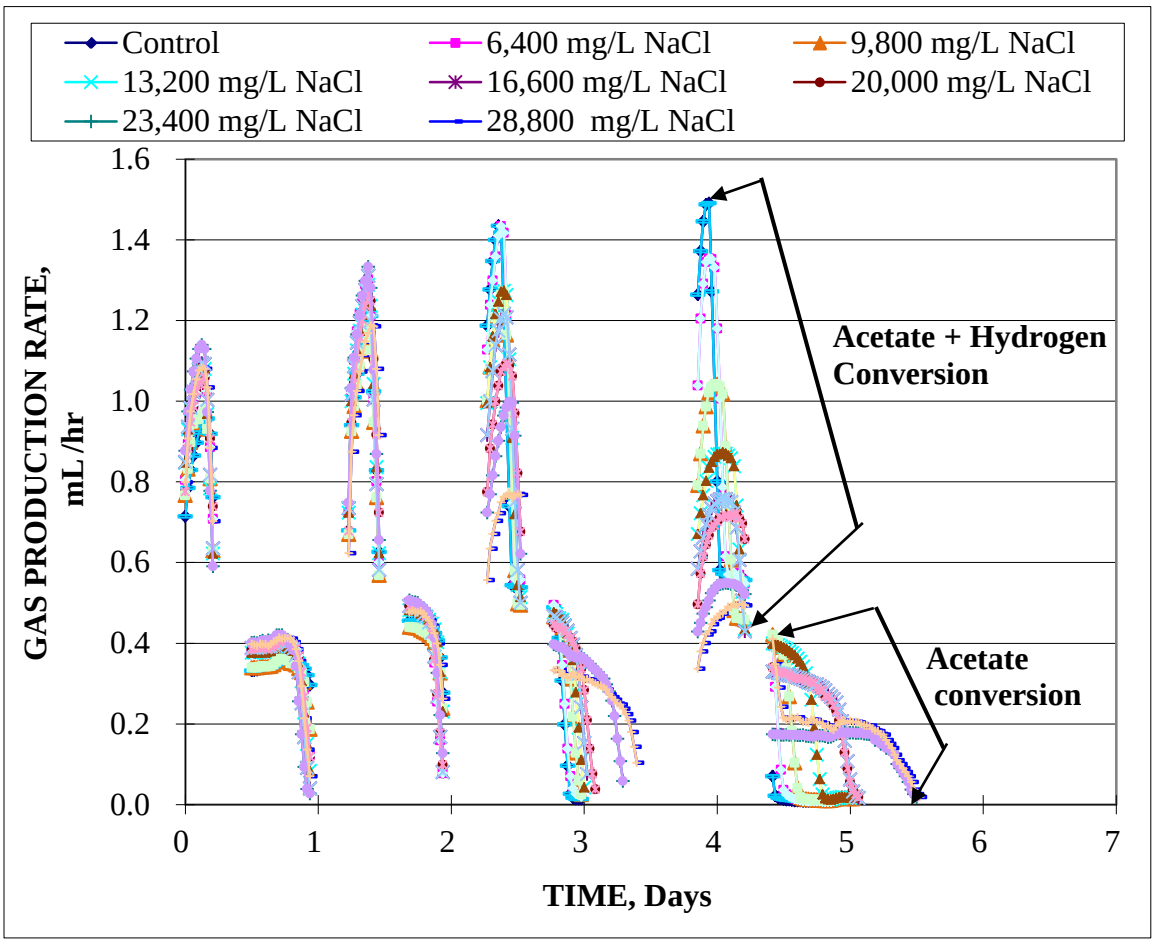

Figure 15: Hourly gas production rates at varied sodium chloride concentration.

The gas production rates dropped by 50% as the daily dose of sodium chloride increased from 0 to 28,800 mg/L. Also, the hourly gas production rate was higher during the acetate hydrogen conversion stage than the acetate only conversion stage.

In addition, the cumulative gas production volumes expressed as a percentage of that for the control reactor (Figure 16) indicated that the percentage volume of gas produced increased from that for the control reactor as time progressed. This rise in the percentage production rate indicates little or no toxicity with increasing sodium chloride dose.

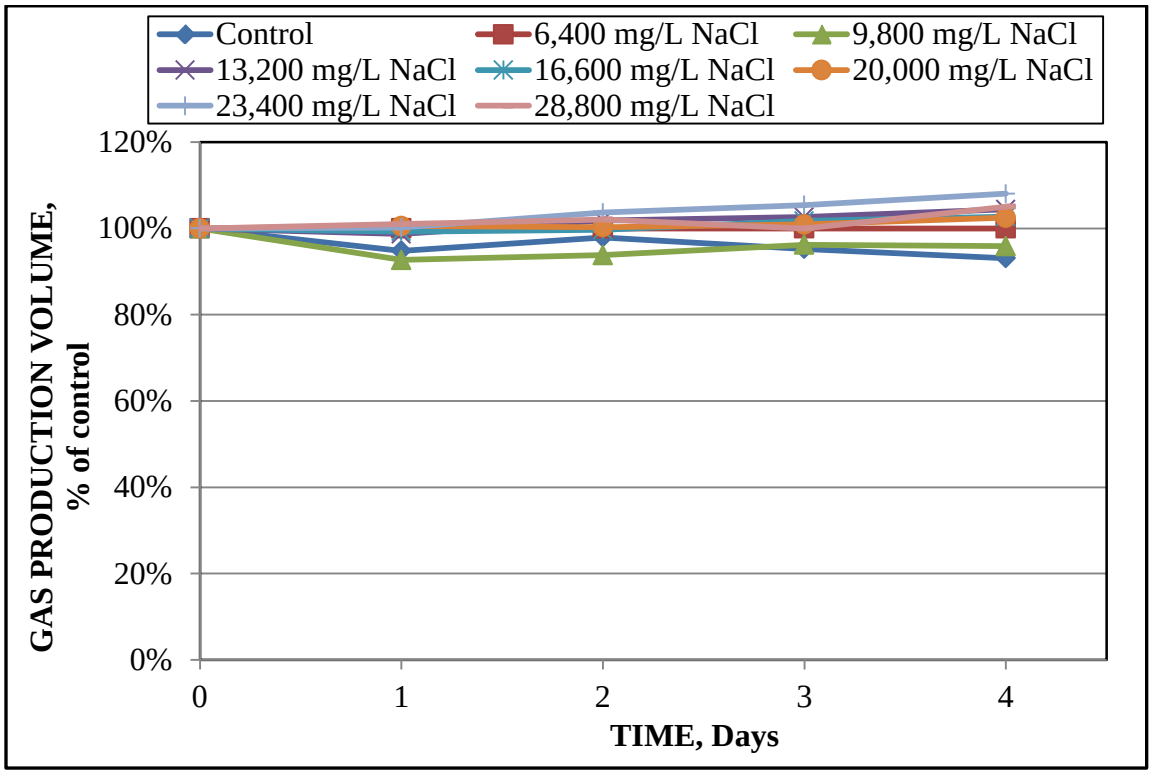

Figure 16: Cumulative gas production volumes expressed as a percentage of that for the control reactor at varied sodium chloride concentration.

However, the maximum hourly gas production rates expressed as a percentage of that for the control reactor indicated that the rate of gas production reactors receiving sodium chloride decreased from that for the control reactor as time progressed (Figure 17 and 18). This drop in the percentage production rate indicates increasing toxicity with increasing sodium chloride dose. Initially, the gas production rates for the test reactors were above the control but dropped below that of the control after 2 days. The initial increase is due to phenomena known as simulation. During this period, the biomass growth and activity increases with increasing test chemicals concentration until a threshold level is reached. At this level, the test chemicals become toxic to the biomass and inhibit the treatment process. The percentage gas rates showed the same threshold toxicity regardless of the dose rate excluding 6,400 mg/L.

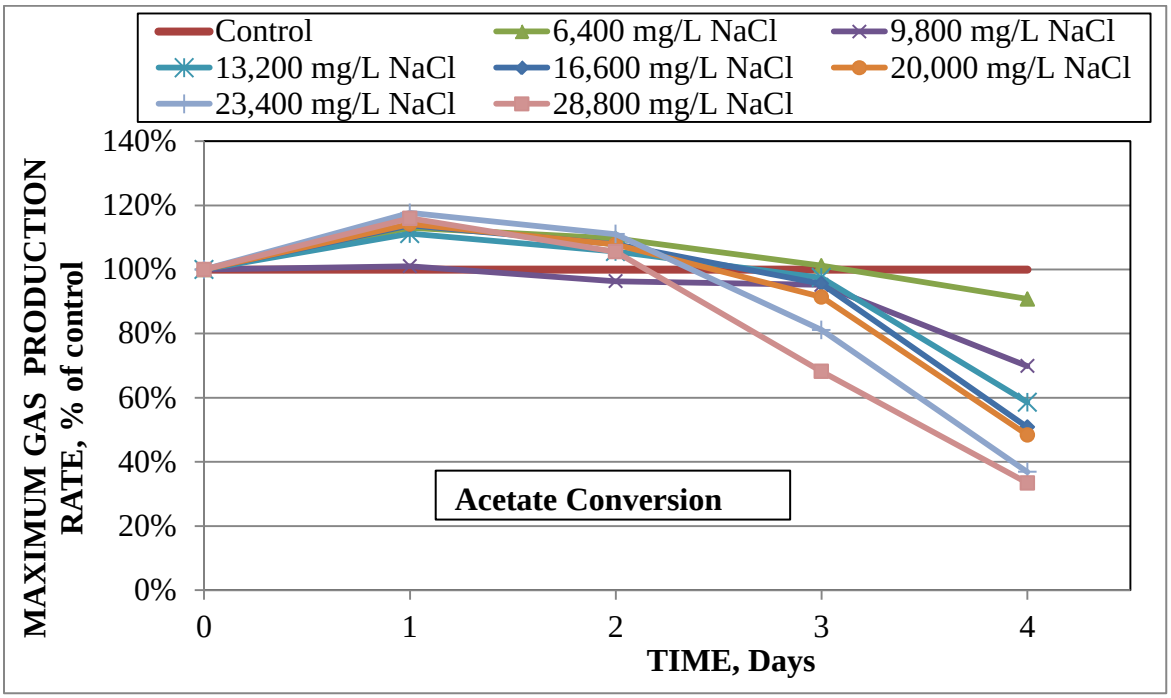

Figure 17: Maximum hourly gas production rates expressed as a percentage of that for the control reactor at varied sodium chloride concentration (Acetate Conversion).

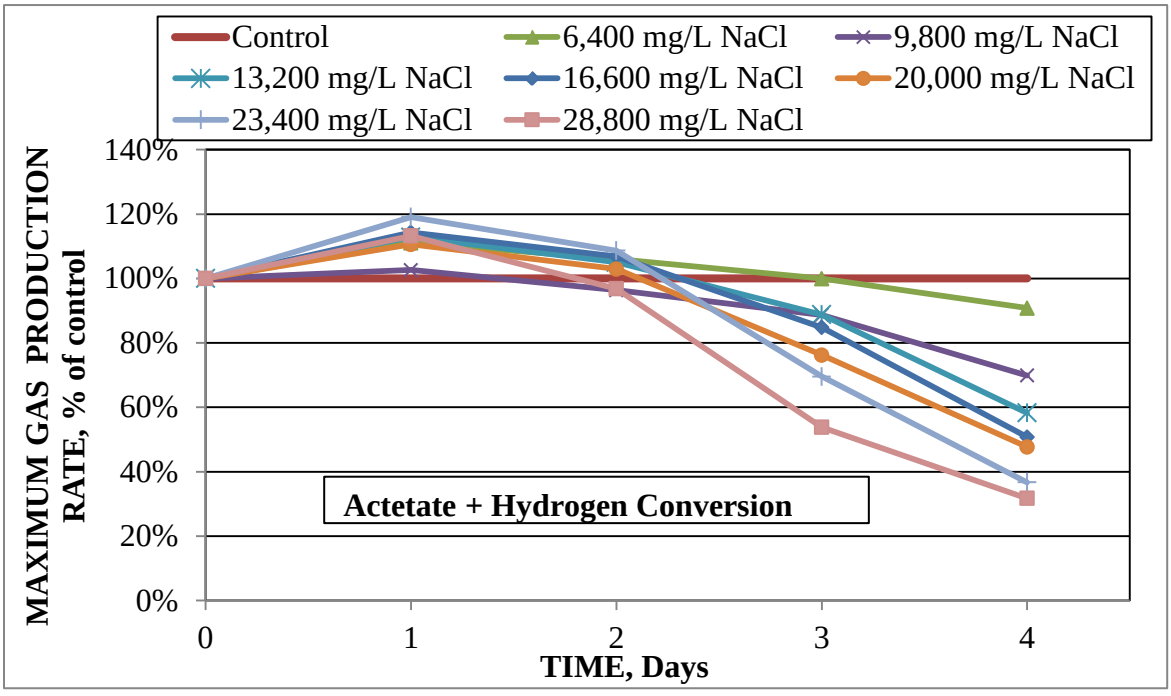

Figure 18: Maximum hourly gas production rates expressed as a percentage of that for the control reactor at varied sodium chloride concentration (Acetate + Hydrogen Conversion).

For practical purposes, sodium chloride had similar effect on the gas production rates with time during the acetate only conversion and acetate plus hydrogen conversion stages.

Figure 19 shows that the cumulative gas production volumes as a percentage of control increased with increasing dose of sodium chloride. An increase in cumulative gas production volume was observed with increasing cumulative sodium chloride and dose rate. The gas production volumes were greater than 100% of the control excluding that of the 6,400 mg/l reactor which drops below the control which implies that one or more constituent of the sodium chloride formulation stimulated the growth of gas forming microorganisms.

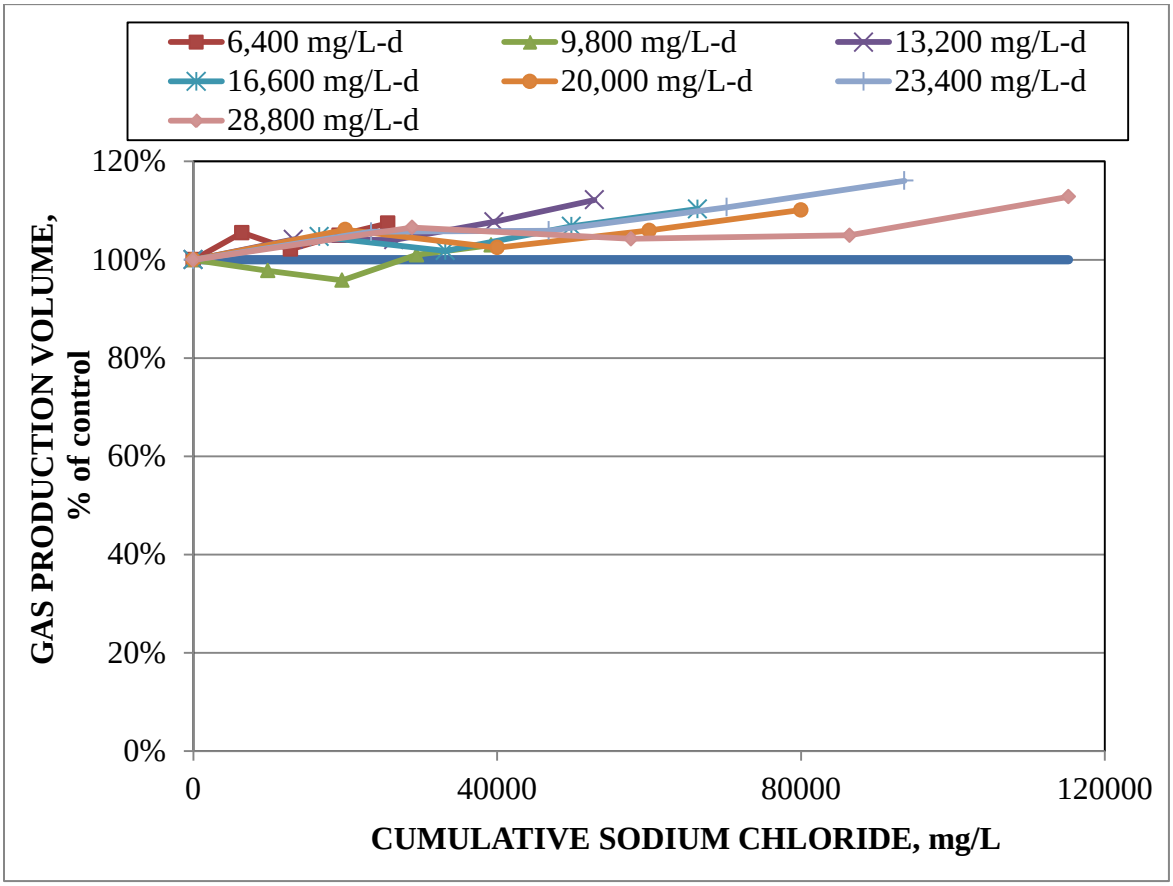

Figure 19: Gas production volumes expressed as a percentage of that for the control reactor at varied sodium chloride concentration.

The hourly gas production rates expressed as a percentage of that for the control reactor initially increased with increasing dose of sodium chloride (Figure 20 and 21). A reduction in hourly gas production rate was observed in all reactors after various cumulative sodium chloride doses. This observation implies that the sodium chloride stimulated the growth of gas forming microorganisms before the threshold toxic level was reached. From Figure 20 and 21, the sodium chloride had similar effect on the gas production rates relative to that of control as the cumulative sodium chloride in the test bottles increased during the acetate only conversion and acetate plus hydrogen conversion stages.

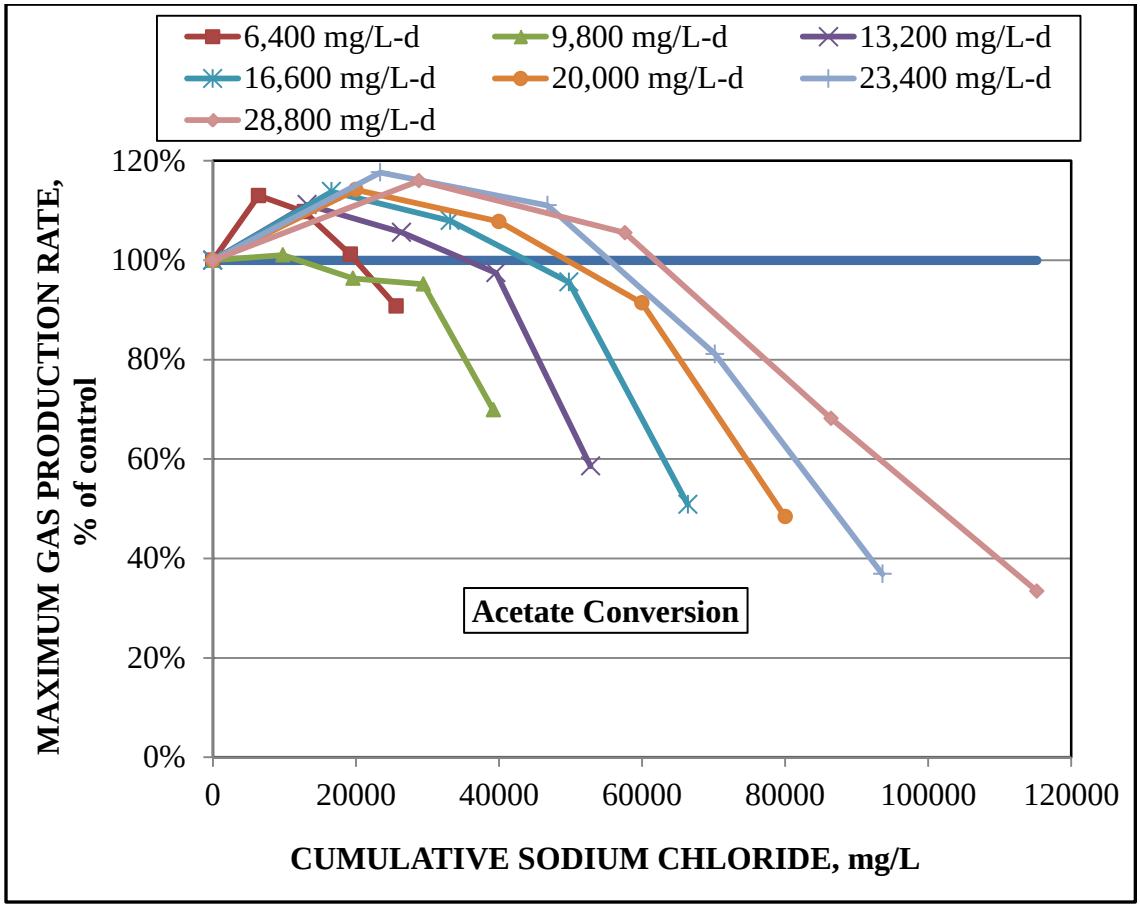

Figure 20: Maximum gas production rates expressed as a percentage of that for the control reactor at varied sodium chloride concentration (Acetate Conversion).

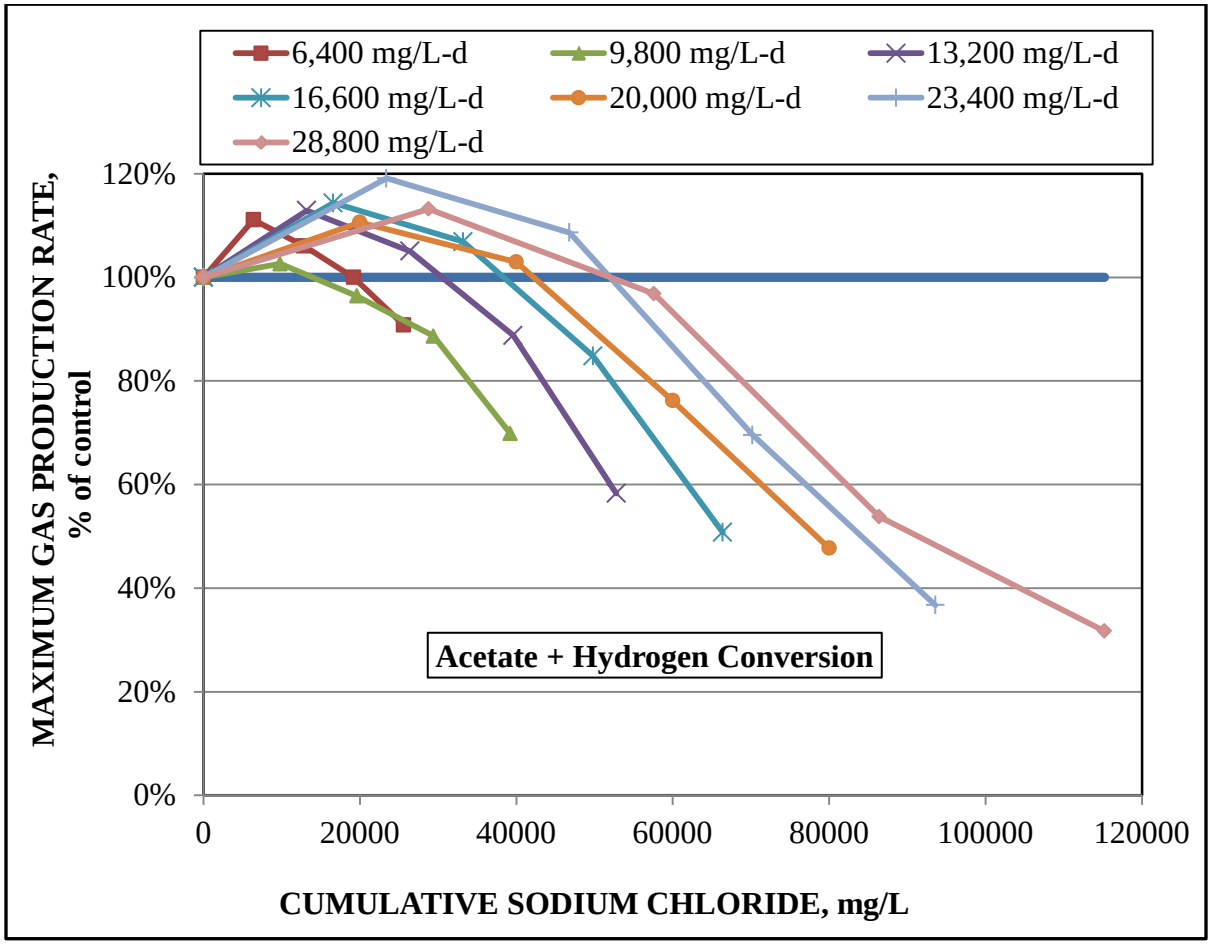

Figure 21: Maximum gas production rates expressed as a percentage of that for the control reactor at varied sodium chloride concentration (Acetate + Hydrogen Conversion).

Finally, gas production volumes versus gas production rates for the sodium chloride test showed different effects at varied dosing rates. The gas production volume showed little or no toxicity while the gas production rates showed increasing toxicity with increasing cumulative concentrations.

5.0 DISCUSSION OF RESULTS

The current toxicity tests have provided valuable insight into the impact of ammonium-nitrogen and sodium chloride toxicity on anaerobic biodegradation as well as the impact of solids retention time on ammonium-nitrogen toxicity. The relationship between ammonium-nitrogen toxicity at varied concentration and SRT; and the inhibitory impact of sodium chloride on biodegradation has not been demonstrated previously in literature or previous research. The results of these research experiments help to explain the effect of the ammonium-nitrogen and sodium chloride at various loading rates on anaerobic biodegradation; show the effect solids retention time on ammonium-nitrogen toxicity; and the relationship between the hourly rate and daily volume of gas production for the tests.

For the ammonium-nitrogen toxicity test at varied concentration, the impact of the ammonium-nitrogen at varied concentration on biodegradation was more evident in the hourly gas production rate measurements than in the daily gas production volumes. In all cases, the hourly gas production rates showed a significant decrease with increasing ammonium-nitrogen concentration and time before the daily gas production volume decreased. Also, the decrease in hourly gas production rate was more significant compared to the daily cumulative production volume when a threshold toxic dose was achieved (Figure 2 and 3). This process was observed to be in proportion to the ammonium-nitrogen feed rate.

In addition, the ammonium-nitrogen toxicity test at varied solid retention times showed that the longer the SRT, the higher the gas production rates. The cumulative gas production volumes for the reactors after 24 hours were almost the same but hourly gas production rates during the 24 hours showed a significant decrease with increasing solids retention time and testing time. The daily gas production volumes showed little or no toxicity while the hourly gas

production rates showed a reduction due to toxicity. The observed reduction in gas production rates in the different SRT reactors compared to the control reactor may be due to the increase in the inhibitory effect of ammonium-nitrogen on the biomass at the threshold level or stimulated the growth of gas forming microorganisms before the toxic threshold was reached. In this case, a solids retention time below 30 days caused a measureable decline in gas production volumes after only seven days of feeding. Comparing gas production volumes to gas production rates at different SRTs revealed that the hourly gas production rates decreased at lower ammonium-nitrogen concentrations than daily volumes.

Similar to the ammonium-nitrogen toxicity test, the sodium chloride toxicity test showed significant decreases in hourly gas production rates during 24-hrs feed cycles as compared to the daily cumulative gas production volumes. Since the hydrogenotrophic and acetoclastic methanogenesis occur simultaneously, the presence of hydrogen gas doubled the gas production rate during the early stages of the reaction. A comparison of the volumes and rates as a percentage of the control reactor showed relatively stable daily gas production volumes but decreasing hourly gas production rates with increasing testing time and cumulative sodium chloride concentration. Therefore, gas production rates show toxic impacts well before they are seen in the daily gas production volumes.

In as much as these anaerobic toxicity tests have provided useful information and relationships between ammonium-nitrogen concentration and loading rate, ammonium-nitrogen toxicity and SRT, as well as sodium chloride concentration and loading rate on anaerobic treatment process performance, environmental engineers may use this information to support the design and operation of anaerobic wastewater treatment plants that are potentially impacted by

these toxicants. Also, wastewater plant operators should consider longer SRT for waste water with high ammonia content and use gas production rates curves to determine toxicity quicker.

The ammonium-nitrogen and sodium chloride toxicity tests were beneficial in measuring the gas production at short intervals (using a respirometer). The impact of these toxicants on gas production or COD conversion by biomass was more evident in the hourly production rates curve during 24 hours than in the cumulative production volumes curve after every 24 hours. An area for possible future research lies in determining the impact of different concentration of compounds like sodium chloride at different SRT and the impact of feed schedule and dosing rate on process configuration.

6.0 CONCLUSIONS

The test program described in this report yielded the following conclusions:

1. Ammonium-nitrogen toxicity test proved that the levels of ammonium-nitrogen loading that could be sustained indefinitely were relatively high (below 1500mg N/L). At a dosing rate equal or greater than 1,500 mg/L-d, ammonium–nitrogen would cause a measureable and sustained decrease in gas production rate with a resulting increase in soluble COD after only three days of feeding. In addition, the gas production rates as a percentage of control showed significant decrease relative to that control before the gas production volumes.
2. The ammonium-nitrogen toxicity test at varied solid retention times showed the daily cumulative gas production volumes were the same at all solids retention time. However, the hourly gas production rate increased with increasing SRT and decreased with increasing time.
3. Gas production rates expressed as a percentage of a control versus the cumulative ammonium-nitrogen concentration showed that the microorganisms acclimated to the ammonium–nitrogen at cumulative ammonium-nitrogen concentration of about 1200, 1300, 1000 mg/L at 20, 30 and 45 days-SRT respectively.
4. Sodium chloride exhibited a significant dose rate–cumulative concentration impact by day 3 at a concentration greater than 13,200 mg/L. The culture seemed to be adversely affected more by cumulative concentration when operating at higher dose rates.
5. From the three test series, toxicity was seen in hourly gas production rates at substantially lower cumulative toxicant concentrations than were seen in daily gas production volumes.

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APPENDICES

APPENDIX A: ANAEROBIC TEST SETUP

1. Clean the test bottles and relate parts using conventional laboratory cleaners (such as Alconox^{TM}). Rinse thoroughly. (Note: Use of dichromate cleaning solution is not recommended).
2. Insert a Telfon^{TM}-coated magnetic stirring bar in each bottle (35-mm magnets seem to work best for bottle sizes ranging from 250 to 500 mL).
3. Flush each bottle using nitrogen gas, a mixture of nitrogen-CO_2, or methane-CO_2, depending on test objectives.
4. Transfer test culture from an anaerobic digester or laboratory-scale reactor to each test vessel using anoxic conditions to prevent oxygen contact with the test culture. (Note: The culture volume ideally should represent 75 to 80 percent of the reactor volume to minimize the effect of pressure and temperature variations).
5. Place the screw cap with inserts butyl rubber septum on each test bottle. Tighten snugly to seal. (Note: To minimize the chance of leaks, use a new septum for each test).
6. Add desired nutrients and/or synthetic substrates through the septum using a syringe.
7. Place test bottles on the MS8-300 magnetic stirring base in a constant temperature room, incubator chamber, or water bath (35 mm stir bars have been frond to work best when using 250 and 500 mL serum bottles). Adjust the stirring rate to provide good mixing action. (Note: The temperature of the test culture should be within $\pm 1^{o}$C of the test temperature before beginning the test).
8. Vent the test bottles by briefly inserting a clean 20”-gage needle through the septum. This venting prevents blowing cell fluid from the cell due to gas buildup in the bottle during set up.

9. Insert the control needle attached to the tubing connected to a flow measuring cell into the septum of the test bottle. The needle should be inserted through the outer edge of the septum (between the edge of the cap and the thin ring on the septum) and at an angle of about 60 degrees (from horizontal) to reduce needle strain on the septum. Be sure that water is removed from all tubing. Also, 20”-gage needles must be used for all tests. Otherwise, the calibration and stability of the system may be affected. For most dependable results, use a new needle for each test.
10. Reset the cell counters and timers from the Control Screen and initiate data acquisition by clicking on the **Start** button.

The Challenge AER-200 Respirometer manual (Unpublished)

APPENDIX B: ANAEROBIC EXPERIMENTAL FEEDSTOCKS

This section contains the composition of all of the feeds and additives used in the anaerobic tests.

B.1 20,000 mg/ L COD ACETIC ACID STABILIZATION FEEDSTOCK

Preparing acetic acid stabilization feedstock, 95 mL of glacial acetic acid is added to 250 mL distilled water. KOH of 30% concentration is used to neutralize the mixture to a pH of 5.5. The volume is made up to 500 mL with distilled water to achieve a COD of 200 mg for the acetic acid.

B.2 20,000 mg/ L COD ACETIC ACID STABILIZATION FEEDSTOCK (Without base)

For the acetate Control feedstock, 95 mL glacial acetic acid was added to 2500 mL distilled water and then the volume was made up to 500mL without neutralizing to achieve a 200 mg COD/mL acetic acid.

B.3 20,000 mg/L COD ACETATE CONTROL FEEDSTOCK

For the acetate Control feedstock, 18 mL glacial acetic acid was added to 500 mL NMB (Mineral I, Mineral II, and Nutrient solution described in Appendix A.3, A.4, and A.6) solution. Then, 3.5g KOH (Fisher Scientific) pellets was slowly added and mixed to give 3,000 mg/L alkalinity. Volume is made up to 1,000 mL using distilled water to achieve mixture COD of 20,000 mg/L.

B.4 200,000 mg/L COD AMMONIUM-NITROGEN FEEDSTOCK

This feedstock was prepared by adding 96g of ammonium chloride to 300mL distilled water. Then, KOH of 30% concentration is added to neutralize and raise the mixture to a pH of 7.0 the volume is made up to 500ml with distilled water without overheating. This solution will contain 50 mg NH_4-N/mL.

B.5 20,000 mg/L COD AMMONIUM ACETATE ACID FEEDSTOCK

20 g of ammonium chloride (NH_4Cl) is added to 500 mL distilled water. 18 mL glacial acetic acid is slowly added to this solution. Next, 3.5 g KOH pellets was added slowly while mixing to give 3,000 mg/L alkalinity. The volume is made up to 1,000 mL using distilled water to achieve a COD of 20,000 mg. The pH of the solution is checked and recorded

B.6 20,000 mg/L COD AMMONIUM ACETATE FEEDSTOCK

17.5 g of $CH_3CO_2NH_4$ (ammonium acetate) was added to 500 mL distilled water. Then, 18 mL glacial acetic acid is added slowly to the mixture. The volume is made up to 1,000 mL using distilled water to achieve a COD of 20,000 mg COD/L. The pH of the solution is checked and recorded.

B.7 400,000 mg/L COD ETHANOL STABILIZATION FEEDSTOCK

244mL of ethanol was added to 500 mL NMB. The volume is made up to 1,000 mL using distilled water to achieve a COD of 400,000 mg COD/L. The pH of the solution is checked and recorded.

B.8 200,000 mg/L COD SODIUM CHLORIDE FEEDSTOCK I

200g of NaCl (sodium chloride) was added to 500 mL distilled water. The volume is made up to 1,000 mL using distilled water to achieve a COD of 200,000 mg COD/L. The pH of the solution is checked and recorded.

B.9 1,500,000 mg/L COD SODIUM CHLORIDE FEEDSTOCK II

150g of NaCl (sodium chloride) was added to 100 mL distilled water. The volume is made up to 1,000 mL using distilled water to achieve a COD of 1,500,000 mg COD/L. The pH of the solution is checked and recorded

B.10 NUTRIENT/MINERAL/BUFFER (NMB) SOLUTIONS FOR ANAEROBIC TREATBILITY TESTING

The NMB water is a mixture of mineral base I, mineral base II, nutrient base and buffer base to

B.10.1 MINERAL BASE I

The following compounds was added to 800 mL of reagent quality water and then diluted to 1.0 L. (Note: Most of these minerals already may be present in municipal wastewater. This mixture may form a light precipitate and should be agitated vigorously before transferring)

$CoCl_2 \bullet 6H_2O$	0.25	g	(0.062 mg Co/mL)
$Na_2MoO_4 \bullet 2H_2O$	0.005	g	(0.0020 mg Mo/mL)
$FeCl_2 \bullet 4H_2O$	4.0	g	(1.126 mg Fe/mL)
$NiCl_2 \bullet 6H_2O$	0.025	g	(0.0062 mg Ni/mL)
$MnCl_2 \bullet 4H_2O$	0.05	g	(0.0139 mg Mn/mL)

Na_2SeO_4	0.025	g	(0.0104 mg Se/mL)
H_3BO_3	0.025	g	(0.0044 mg B/mL)
$CuCl_2 \bullet 2H_2O$	0.007	g	(0.0026 mg Cu/mL)
$ZnCl_2$	0.02	g	(0.0119 mg Zn/mL)

B.10.2 MINERAL BASE II

The following compounds was dissolved in 800 mL of distilled water and then diluted to 1.0 L

(Note: Most of these minerals already may be present in municipal wastewaters).

$CaCl_2$	15	g	(5.4 mg Ca/mL)
$MgCl_2 \bullet 6H_2O$	20	g	(2.36 mg Mg/mL)

B.10.3 NUTRIENT BASE

The following compounds was dissolved in 800 mL of distilled water, neutralized to pH 7 using 50% NaOH and then diluted to 1.0 L.

(Note: Sufficient nitrogen and sulfate may be present in municipal wastewater)

NH_4Cl	53	g	(13.9 mg N/mL)
KH_2PO_4	50	g	(11.4 mg P/mL)
Na_2SO_4	30	g	(6.76 mg S/mL)

B.10.4 BUFFER BASE

The following compound was dissolved in 800 mL of distilled water and then diluted to 1.0 L

$NaHCO_3$	60	g

B.10.5 NMB WATER

The following solutions should be added to 800 mL of reagent grade water and then diluted to 1 L.

Mineral I Solution	20mL/L
Mineral II Solution	20mL/L
Nutrient Solution	20mL/L
$NaHCO_3$	4 g/L

APPENDIX C: TEST PLANS

This section contains the test plans for each of the tests run during this experiment.

APPENDIX C.1: AMMONIUM-NITROGEN TOXICITY TEST AT VARIED CONCENTRATION

1. Obtain a 2-L sample of active mixed liquor from an operating anaerobic treatment plant, either full-scale or lab-scale. Measure sCOD, TSS, VSS, sNH3-N, sP, tP, total TKN.
2. Prepare ammonium-nitrogen feedstock by adding 96 g NH4Cl to 300 mL distilled water. This material may not dissolve until neutralization. Use 30% KOH to raise the pH to 7.0. Make up the volume to 1000 mL using distilled water. Watch the temperature to make sure it does not overheat. This solution contains 50 mg NH4-N/mL.
3. Prepare nutrient/mineral/buffer (NMB) water by adding 20 mL Min I, Min II and Nutrient solutions from Appendix A and 4 g $NaHCO_3$ per liter of distilled water.
4. Prepare acetic acid stabilization feedstock: Add 95 mL glacial acetic acid to 250 mL distilled water. Neutralized to pH = 5.5 using 30% KOH. Make volume to 500 mL using distilled water (= 200 mg COD/mL acetic acid).
5. Prepare acetic acid test feedstock: Add 95 mL glacial acetic acid to 250 mL distilled water. Do not neutralize. Make volume to 500 mL using distilled water (= 200 mg COD/mL acetic acid).
6. Anaerobic toxicity tests
   A. Set up 8, 700 mL serum bottles with 500 mL culture volume to give 5,000 mg VSS/L. Use NMB water for dilution.
   B. Stabilize reactors for 2 to 3 days by feeding 6 mL/d stabilization acetate feedstock (Step 4) to give a COD/VSS ratio = 0.5 (10 mL acetate solution/4000 mgVSS = 0.5). Let reactions go to completion before adding subsequent doses.
   C. Use the stabilization data to calculate biomass activity.

D. After stabilization is compete, feed the test reactors ammonia (Step 2) and un-neutralized acetate (Step 4) feedstocks as follows:

R1: Control with 6 mL acetic acid feedstock only, fed daily (F/M = 0.5 g/g)

R2: Control with 6 mL acetic acid feedstock only, fed daily

R3: Control with 2 mL NH_4-N feedstock + 6 mL acetic acid feedstock daily (200mg N/L).

R4: Control with 5 mL NH_4-N feedstock + 6 mL acetic acid feedstock daily (500mg N/L).

R5: Control with 15 mL NH_4-N feedstock + 6 mL acetic acid feedstock daily (1500 mg N/L).

R6: Control with 20 mL NH_4-N feedstock + 6 mL acetic acid feedstock daily (2,000 mg N/L).

R7: Control with 25 mL NH_4-N feedstock + 6 mL acetic acid feedstock daily (2,500 mg N/L).

7. Check and record pH daily before feeding and adjust to 7.0 as necessary using KOH or HCL.
8. Measure gas production on each bottle using respirometers.
9. Run tests for at least 10 days or until the gas production rate in the least-affected reactor is 50% of that for the Control.

APPENDIX C.2: AMMONIUM-NITROGEN TOXICITY TEST AT VARIED SOLIDS RETENTION TIME

1. The objective of this test series is to evaluate the impact of solids retention time on ammonium-nitrogen toxicity.
2. Obtain a sample of active mixed liquor from an operating anaerobic treatment plant, either full-scale or lab-scale. Measure sCOD, TSS, VSS, sNH_3-N, sP, tP, total TKN.
3. Prepare nutrient/mineral/buffer (NMB) water by adding 20 mL Min I, Min II and Nutrient solutions from Appendix A and 6 g $NaHCO_3$ per liter of distilled water.
4. Prepare acetic acid stabilization feedstock: Add 95 mL glacial acetic acid to 250 mL distilled water. Neutralize to pH = 5.5 using 30% KOH. Make volume to 1000 mL using distilled water (100 mg COD/mL acetic acid).
5. Prepare acetate control feedstock: Slowly add 18 mL glacial acetic acid to 500 mL NMB solution. Slowly add 3.5 g KOH pellets while mixing to give 3,000 mg/L alkalinity. Make volume to 1,000 mL using distilled water (20,000 mg/L COD). Check and record pH of this solution.
6. Prepare ammonium acetate acid test feedstock: Add 20 g NH_4Cl to 500 mL distilled water. Mix until it is dissolved. Slowly add 18 mL glacial acetic acid to this solution. Slowly add 3.5 g KOH pellets while mixing to give 3,000 mg/L alkalinity. Make volume to 1,000 mL using distilled water (20,000 mg/L COD). Check and record pH of this solution.
7. Anaerobic toxicity tests
   A. Set up 8, 700 mL serum bottles with 500 mL culture volume to give 2,500 mg VSS/L. Use NMB water for dilution.

B. Stabilize reactors for 2 to 3 days by feeding 6 mL/d stabilization acetate feedstock (Step 4) to give a COD/VSS ratio = 0.48 (1200 mg acetate COD/2500 mg VSS = 0.48). Let reactions go to completion before adding subsequent doses.

C. After stabilization is compete, feed the test reactors ammonium acetate feedstock (Step 4) feedstocks as follows:

R1, R2: Control 1 & 2: Waste 25 mL mixed liquor and feed 25 mL acetate Feedstock (Step 5)

R3, R4: 20-d SRT: Waste 25 mL mixed liquor and feed 25 mL ammonium acetate feedstock (Step 6)

R5, R6: 30-d SRT: Waste 17 mL mixed liquor and feed 17 mL ammonium acetate feedstock (Step 6)

R7, R8: 45-d SRT: Waste 11 mL mixed liquor and feed 11 mL ammonium acetate feedstock (Step 6).

8. Check pH daily before feeding and adjust to 7.0 as necessary using KOH or HCL.

9. Measure gas production on each bottle using respirometers.

10. Run tests for at least 10 days or until the gas production rate in the least-affected reactor is 50% of that for the Control.

APPENDIX C.3: SODIUM CHLORIDE TOXICITY TEST

1. Obtain a 2-L sample of active mixed liquor from an operating anaerobic treatment plant, either full-scale or lab-scale. Measure sCOD, TSS, VSS, sNH3-N, sP, tP, total TKN.
2. Prepare nutrient/mineral/buffer (NMB) water by adding 20 mL Min I, Min II and Nutrient solutions from Appendix A and 4 g NaHCO3 per liter of distilled water.
3. Prepare ethanol stabilization feedstock: Add 244 mL of ethanol to 500mL of NMB water. The COD of this solution should be 400,000 mg/L.
4. Prepare sodium chloride feedstock I: Add 200 g of NaCl to 500 mL distilled water. The volume is made up to 1000 mL using distilled water to achieve a COD of 200,000 mg/L.
5. Prepare sodium chloride feedstock II: Add 150 g of NaCl to 100 mL distilled water. The volume is made up to 1000 mL using distilled water to achieve a COD of 1,500,000 mg/L.
6. Anaerobic toxicity tests
   A. Set up 8, 700 mL serum bottles with 500 mL culture volume to give 4,800 mg VSS/L. Use NMB water for dilution.
   B. Stabilize reactors for 2 to 3 days by feeding 6 mL/d stabilization ethanol feedstock (Step 4) to give a COD/VSS ratio = 0.61 (10 mL acetate solution/4800 mgVSS = 0.61). Let reactions go to completion before adding subsequent doses.
   C. After stabilization is compete, feed the test reactors sodium chloride (Step 2) and un-neutralized ethanol (Step 4) feedstocks as follows:

      R1: Control with 6 mL ethanol feedstock only, fed daily (F/M = 0.5 g/g)

      R2: Control with 1 mL NaCl feedstock I + 2 mL NaCl feedstock II + 6 mL ethanol feedstock daily (6,400 mg NaCl/L).

R3: Control with 2 mL NaCl feedstock I + 3 mL NaCl feedstock II + 6 mL ethanol feedstock daily (9,800 mg NaCl/L).

R4: Control with 3 mL NaCl feedstock I + 4 mL NaCl feedstock II + 6 mL ethanol feedstock daily (13,200 mg NaCl/L).

R5: Control with 4 mL NaCl feedstock I + 5 mL NaCl feedstock II + 6 mL ethanol feedstock daily (16,600 mg NaCl/L).

R6: Control with 5 mL NaCl feedstock I + 6 mL NaCl feedstock II + 6 mL ethanol feedstock daily (20,000 mg NaCl/L).

R7: Control with 6 mL NaCl feedstock I + 7 mL NaCl feedstock II + 6 mL ethanol feedstock daily (23,400 mg NaCl/L).

R8: Control with 11 mL NaCl feedstock I + 8 mL NaCl feedstock II + 6 mL ethanol feedstock daily (28,8 00 mg NaCl/L).

Check and record pH daily before feeding and adjust to 7.0 as necessary using KOH or HCL.

7. Measure gas production on each bottle using respirometers.
8. Run tests for at least 10 days or until the gas production rate in the least-affected reactor is 50% of that for the Control.