UNESCO-IHE
Institute for Water Education

Assessment of free nitrous acid and hydrogen sulfide as alternative pre-treatment techniques to enhance methane production from waste activated sludge

Moses Nyakana
Student Number: 44426
MSc Thesis UWS-SE 2017-08

Assessment of free nitrous acid and hydrogen sulfide as alternative pre-treatment techniques to enhance methane production from waste activated sludge

Master of Science Thesis
by
Moses Nyakana

Supervisors
Prof. Jules van Lier

Mentors
Dr. Carlos M. Lopez Vasques, PhD
Dr. Francisco J. Rubio Rincon, PhD

Examination committee
Dr. Carlos M. Lopez Vasques, PhD
Dr. Peter Vander Steen, PhD
Prof. Jules van Lier, PhD

This research is done for the partial fulfilment of requirements for the Master of Science degree at the UNESCO-IHE Institute for Water Education, Delft, the Netherlands

Delft
March 2017

Abstract

Anaerobic digestion of Waste Activated Sludge (WAS) presents a sustainable and cost efficient way of dealing with excess sludge generated by Conventional Activated Sludge Systems. However, the hydrolysis of suspended matter and complex organic solids limits the methanogenic process, a bottleneck that can be overcome by efficient pre-treatment of WAS.

Recirculated Activated Sludge (RAS) and anaerobically digested sludge from Harnaschpolder wastewater treatment plant were used as substrate and inoculum respectively. The RAS was centrifuged to attain thickener conditions and pre-treatment with 0.04 mg HNO_2-N/gVS, 0.06 mg HNO_2-N/gVS, 4.54 mg H_2S/gVS, 9.09 mg H_2S/gVS, 16.63 mg H_2S/gVS and 18.18 mg H_2S/gVS at a temperature of 25˚C and pH 5.8 for 24 hours was done alongside a control with no FNA or sulfide pre-treatment at the same temperature and pH. The changes in sludge characteristics following pre-treatment were measured and the Biochemical Methane Potential (BMP) was assessed.

Free Nitrous Acid pre-treatment of RAS with 0.04 mg HNO_2-N/gVS and 0.06 mg HNO_2-N/gVS resulted into decreased COD solubilization with 53% and 31% less COD respectively relative to the control. Protein hydrolysis was severely inhibited while 85% and 80% less VFA was formed compared to the control for 0.04 mg HNO_2-N/gVS and 0.06 mg HNO_2-N/gVS pre-treatment respectively. Methane production was also significantly reduced compared to the control with 0.04 mg HNO_2-N/gVS and 0.06 mg HNO_2-N/gVS pre-treatment achieving 16% and 29% less methane respectively. The hallmark of sulfide pre-treatment was enhanced hydrolysis and acidogenesis. COD solubilization was improved by 54%, 34%, 17% and 20% relative to the control for 4.54 mg H_2S/gVS, 9.09 mg H_2S/gVS, 16.63 mg H_2S/gVS and 18.18 mg H_2S/gVS pre-treatment respectively. VFA formation was significantly enhanced by sulfide pre-treatment of WAS with 4.54 mg H_2S/gVS realizing 22%, 26% and 30% more total VFA, acetic acid and propionic acid than the control. The 9.09 mg H_2S/gVS pre-treatment showed no difference with the control in total VFA but 19% more propionic acid was realized. Pre-treatment with 16.63 mg H_2S/gVS relative to the control achieved 6%, 12% and 19% more total VFA, acetic acid and propionic acid respectively. The 18.18 mg H_2S/gVS pre-treatment comparative to the control achieved 6%, 5% and 22% more total VFA, acetic acid and propionic acid. The paradox of this improved hydrolysis and acidogenesis was an inverse production of methane relative to the control by 3-12% across the sulphide pre-treatment range.

The observed Sulfide enhancement of hydrolysis requires further study on how it can be translated into improved methane production while further insight on FNA pre-treatment inhibition of hydrolysis and methanogenesis has been provided by the research.

Key words: WAS, Anaerobic digestion, Hydrolysis, pre-treatment and methane production

Acknowledgement

I would like first to appreciate God for taking care of things I can never have control of. My trust in you abounds.

I am thankful to the Rotary Club of Mbarara for giving me the opportunity to pursue my career as a Sanitary engineer. I am humbled by your humanity. To my host chancellor Ir. Kees de Groot and your wife Julie, thank you for the immense support and encouragement.

I recognise the support of the study team: My supervisor, mentors, the lab team and study partners at Delfluent. Dr. Carlos Lopez, your guidance and encouragement made me understand the true values of a great teacher. Thank you for the patience, humility and hope when everything seemed to be crumbling. To Dr. Francisco Rubio, thank you for the time to guide me even at your busiest moment of your study. To Prof. Jules van Lier, thank you for the insight into the study at the earliest moment, indeed the bugs agreed earlier on what, when and how to eat! To Mr. Berend Lolkema, thank you for being the lab Superman and putting a smile on my face when the going was quite hard. To Eng. Paul Weij and Ir. Rogier van Kempen, your support is highly appreciated.

My sincere appreciation goes to Mr. Isaac Ongora for the encouragement on sanitary work. I did not see myself growing from an operator to a Sanitary engineer. I am glad our lives crossed paths. To Mr. Paul Coope, Mr. Frank Hedley and Dr. Francois Talbot, you inspired me a lot to go forward in Sanitary engineering and I have learned greatly from you.

To my mum Restie, thank you for believing in me and inspiring my life. It's impossible to find a better mum! To Brenda, thank you for the patience, love and encouragement. To my boy Sanders, when you are grown you will understand that a man ought to leave town sometime.

To all my friends at UNESCO-IHE, thank you for being there for me. To Roberto, thank you for teaching me how to hide in a glass house, I will try it sometime! To Dalva, you have always been helpful. To Mulbah, we had a great time.

Last but not least, I appreciate the entire Sanitary engineering class 2015-2017. We have shared the best and worst moments together. We have endured together and persevered to stay afloat in the great sewer of learning and finally we see the light together. I appreciate the presence and help of everyone during the study period. Always follow excellence and success shall find you!

Table of Contents

List of Figures

List of Tables

List of Equations

Abbreviations

AD	Anaerobic digestion
AMPTS	Automatic Methane Potential Test System
AOB	Ammonia oxidizing bacteria
BMP	Biochemical methane potential
BSA	Bis (trimethylsilyl) acetamide
CAS	Conventional Activated Sludge
COD	Chemical Oxygen Demand
FEEM	Fluorescence Excitation Emission Matrix
FNA	Free Nitrous Acid
HRT	Hydraulic Retention Time
NOB	Nitrite-oxidizing bacteria
PAO	Phosphate-accumulating organisms
RAS	Recirculated Activated Sludge
SBR	Sequential Batch Reactor
SCFA	Short Chain Fatty Acids
SCOD	Soluble Chemical Oxygen Demand
SKN	Soluble Khedjal nitrogen
SRB	Sulphate Reducing Bacteria
SRT	Sludge Retention Time
TCOD	Total Chemical Oxygen Demand
TOC	Total dissolved organic Carbon
VFA	Volatile Fatty Acid
VS	Volatile Solids
VS	Volatile Solids
WAS	Waste activated Sludge

CHAPTER 1

Introduction

1.1. Background

Conventional activated sludge (CAS) processes which are widely used in biological wastewater treatment generate large amounts of waste activated sludge (WAS) after conversion of the organics influent in wastewater. A typical value of about 20kg/capital/year of sludge is generated (Bundgaard and Saabye, 1992). The application of the European council directive 91/271/EEC aimed at enhancing nutrient removal results in extended sludge retention times leading to increased WAS generation (Kelessidis and Stasinakis, 2012). Thus sludge presents serious management issues and 60% of the overall wastewater treatment costs are due to sludge treatment and disposal (Canales, et al., 1994). These high costs of treatment and disposal are related to the WAS characteristics such as: having a very high moisture content, slow biodegradation rates, poor dewaterability, instability, odorous, and with a high pathogen content. The aerobic process in CAS is also highly energy intensive to provide the required oxygen to run the plant. For instance the mean daily carbonaceous oxygen demand per Kg COD load on the reactor is 0.635kgO_2 /Kg COD for settled water (van Lier, et al., 2008). Thence CAS biological treatment is not sustainable due to the environmental detriments, handling costs and public health hazards.

Nevertheless, raw wastewater influent has the potential to be transformed into energy with 13.5MJ CH_4 energy/kg COD degraded which is equivalent to 1.5 kWh electricity at 40% electric conversion efficiency (van Lier, et al., 2008). This energy can be used to offset energy requirements for aeration, mixing and digester heating. Primary sedimentation provides more readily biodegradable organics that would be anaerobically digested to provide more biogas, but the need for organic matter as a substrate for biological nutrient removal bacteria limits its use.

Anaerobic core treatment has been used to evade the menace of the vast quantities of WAS generated and also to produce biogas. Anaerobic digestion takes place in sequential steps of hydrolysis, acidogenesis, acetogenesis and methanogenesis but hydrolysis has been observed to be the rate limiting step in WAS digestion (Appels, et al., 2008, Shimizu, et al., 1993). Thus current and future scientific research aims at development of cost efficient and sustainable pre-treatment applications that increase the hydrolysis rate. Particularly, thermal, chemical and

mechanical pre-treatments are currently being researched and industrially being applied (Appels, et al., 2008, Carballa, et al., 2011, Carrère, et al., 2010, Foladori, et al., 2010, Lissens, et al., 2004). The destruction of cells and/or extra polymeric substances (EPS) followed by the release of intracellular and extracellular constituents to the aqueous phase provides easily biodegradable substances in anaerobic digestion that enhances biogas production (Appels, et al., 2008, Carrère, et al., 2010, Foladori, et al., 2010). For example pre-treatment of WAS at 175˚C for 60 minutes was observed to increase biogas by 42% (Stuckey and McCarty, 1978). However, these techniques are affected by increased energy consumption, higher capital and operation costs, formation of refractory compounds, and environmental issues (Stamatelatou and Tsagarakis, 2015).

The energy factory concept proposed by the Foundation for Applied Water Research (STOWA) further invigorates growth of alternative pre-treatment techniques that that exploit local pre-existing conditions to enhance biogas production to provide opportunities and sustainable solutions for making WWTPs energy neutral (Roeleveld, et al., 2010). For example, the anaerobic digester liquor has a high concentration of ammonia and hydrogen sulphide which can provide cost efficient chemicals for enhancing hydrolysis. Ammonia can be transformed to nitrous acid, a novel pre-treatment of WAS that shows a six fold increase in COD solubilisation after 24 hour treatment with 2.13mg HNO_2 –N/L and increasing methane production by 27% (Wang, et al., 2013).

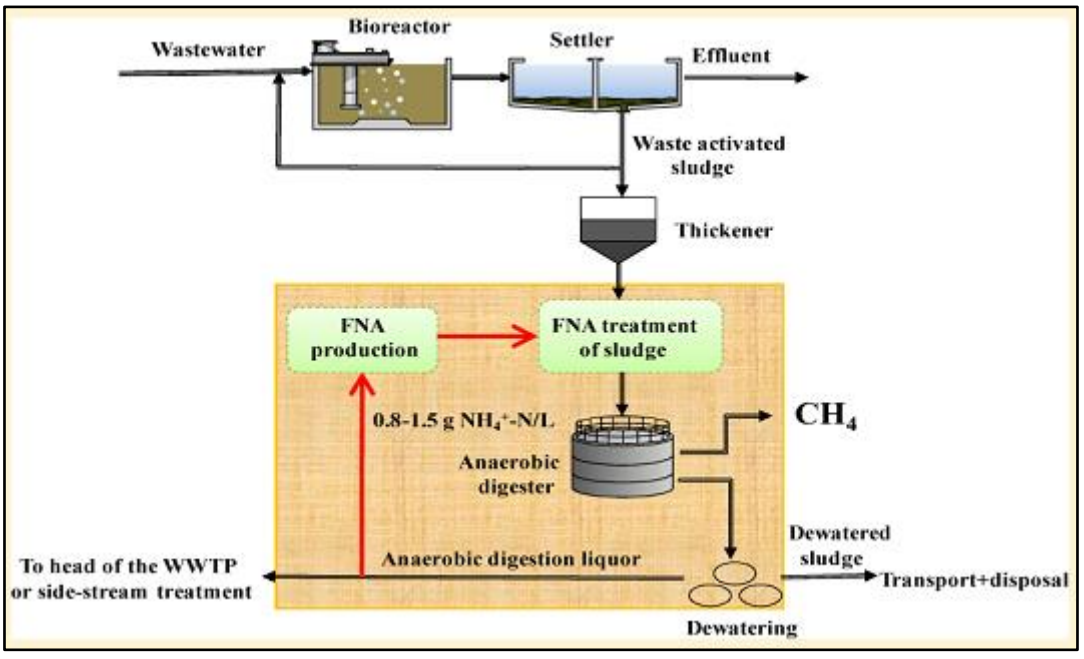


Figure 1: FNA production and pre-treatment schematic

Figure 1(Wang, et al., 2013); shows a schematic of how the anaerobic digestion liquor could be used to produce FNA.

Hydrogen sulfide has been observed to cause inhibition and toxicity of a broad range of wastewater treatment organisms present in WAS and anaerobic digesters including phosphate accumulating organisms, nitrifiers, denitrifiers, methanogens and sulphate reducing bacteria (Bejarano Ortiz, et al., 2013, Jiang, et al., 2011b, Koster, et al., 1986b, Saad, et al., 2013). The toxicity mechanism of WAS microorganisms is due to the non-dissociated hydrogen sulphide freely diffusing across cell membranes and causing protein denaturation and interfering with assimilatory metabolism of Sulphur with concentrations as low as 0.002-0.003mol/L H_2S being toxic to microorganisms at pH 6.8-7.2 (Boe and Angelidaki, 2006, Chen, et al., 2008). It is hypothesized that since both FNA and sulfide show toxicity to activated sludge, sulfide could also be used to induce improvement in WAS degradation as a pre-treatment to enhance biogas production in anaerobic digestion

1.2. Problem statement

Hydrolysis is the limiting step during the anaerobic digestion process of suspended matter and organic solids. Low biodegradability and consequential low methane yield is brought about by the low biodegradability of bacterial cell walls and extracellular polymeric substances often requiring longer hydrolysis time, bigger anaerobic digester volumes and thus increasing cost of digester construction. Thus, there is a need to increase the hydrolysis rate in anaerobic digesters. While methods based on change of pH or temperature had proven to increase the hydrolysis rate, their application increases the operational cost via chemical addition or energy requirements. Thus, alternative solutions as the use of free nitrous acid (FNA) or sulphide are promising, but their functionality still needs to be assessed. This research focussed on the assessment of a range concentration of FNA (0.04-0.06 mg HNO_2-N/gVS) and sulphide (4.54-18.18 mgH_2S/gVS) in the possible increase of hydrolysis rate.

1.3. Research aim and objective

The research aim and objective was to validate the use of FNA as an effective alternative pre-treatment method to increase biogas production and also study the potential use of sulfide as an alternative pre-treatment technique.

1.4. Research question

To what extent can the use of FNA and sulfide as WAS pre-treatment improve biogas production in comparison to conventional anaerobic digestion of WAS?

1.5. Research hypothesis

We hypothesized that pre-treatment of WAS with FNA and sulfide increases the hydrolysis and acidogenesis thus increasing the soluble biodegradable organics for anaerobic digestion that

would improve biogas production from WAS. This is based on their toxicity towards activated sludge. Non dissociated Hydrogen sulfide freely diffuses across cell membranes causing protein denaturation and interfering with cell metabolism. FNA can exert a protonophore uncoupling effect based on the equilibrium that exists between nitrite and un-dissociated FNA. Both effects can lead to increased lability of activated sludge cells to hydrolytic enzymes thus releasing readily biodegradable intracellular components into solution.

CHAPTER 2

Literature review

2.1. Anaerobic digestion

2.1.1. Process description and General Principles

According to van Lier, et al. (2008), anaerobic digestion is a fermentative process where organic matter is degraded and biogas consisting mainly of methane and CO_2 is produced. Furthermore, anaerobic digestion is considered to be one of the earliest technologies for wastewater stabilization introduced at the end of the 19th century with applications for human wastewater treatment in septic tanks, slurry treatment in digesters and sewage sludge in municipal treatment plants (van Lier, et al., 2001). Anaerobic digestion technology has matured to provide optimized wastewater treatment plant (WWTP) costs, environmental foot print and is an integral part of a modern WWTP (Appels, et al., 2008). The drivers of anaerobic digestion are having 90% sludge reduction, 90% WWTP footprint reduction for expanded sludge bed systems, smaller reactor volumes with high COD loading rates of 20-35kg COD per m^3 of reactor per day and its ability to produce about 13.5MJ CH_4 energy/kg COD degraded which is equivalent to 1.5kWh electricity at 40% electric conversion efficiency (van Lier, et al., 2008).

Anaerobic digestion of organic matter and the consequential methane production occurs in four sequential steps namely: hydrolysis, acidogenesis, acetogenesis and methanogenesis (*Figure 2: internet source*). Since there are four complex processes in the anaerobic digestion process, the step resulting into process failure under imposed kinetic stress is the rate limiting step (Aslanzadeh, 2014). For this matter, the kinetic stress refers to a constantly reducing value of the solids retention time to the less than critical value that will ultimately lead to a washout of microorganisms (Pavlostathis and Giraldo-Gomez, 1991). In the digestion of complex organic matter substrates like WAS, hydrolysis is reported as the rate limiting step (Aquino and Stuckey, 2008, Ghyoot and Verstraete, 1997, Qasim, 1998, Tiehm, et al., 2001, Wang, et al., 1999), whereas methanogenesis is the rate limiting step for easily biodegradable non-complex substrates.

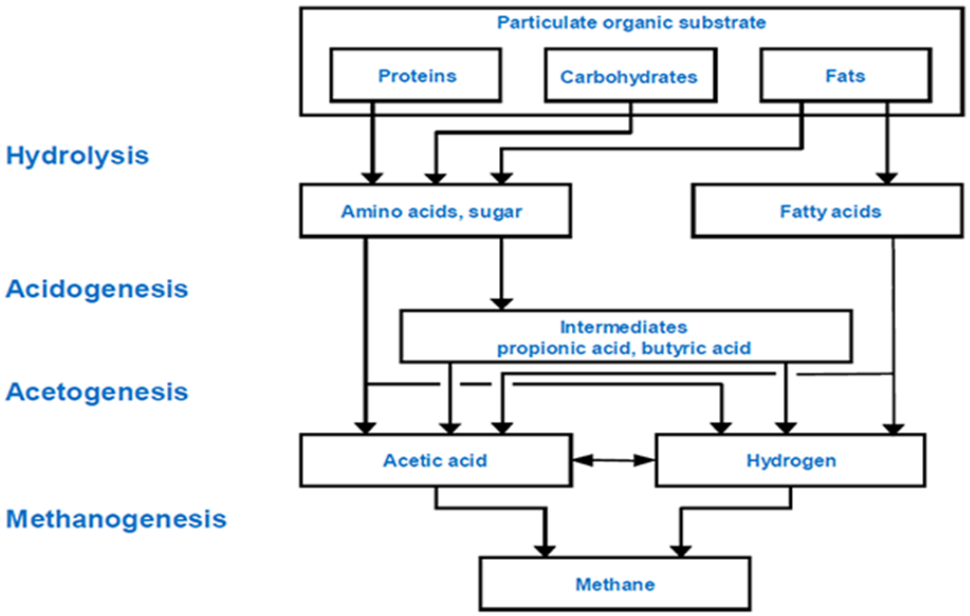


Figure 2: Anaerobic digestion process flow diagram

Hydrolysis

This is the initial step of anaerobic digestion, entailing an enzyme mediated transformation of insoluble, complex, high molecular weight compounds such as lipids, polysaccharides, proteins, fats, nucleic acids et cetera into less complex, soluble compounds that are permeable across cell walls of fermentative bacteria for their use as a carbon source. Thus, simpler compounds such as monosaccharides, amino acids, and other simpler organic compound substrates are a by-product of this extracellular enzymatic activity coordinated by a group of microorganisms referred to as saccharolytic and proteolytic if degrading polysaccharides or proteins respectively. Thus enzymatic degradation of complex organic matter is enzymatic specific and the rate of decomposition during hydrolysis is dependent on the inherent complexity of the substrate. For example, the transformation of cellulose and hemicellulose generally is more gradual than the decomposition of proteins (Schnurer and Jarvis, 2010). WAS contains cells and/or extra polymeric substances that need to be slowly degraded into intracellular and extracellular constituents that are finally released into the aqueous phase providing easily biodegradable substances that are utilized in the acidogenesis phase (van Lier, et al., 2008). *Table 1*(van Lier, et al., 2008); shows the sludge hydrolytic enzymes, their specific substrates and the hydrolysis products.

Table 1: Hydrolytic enzymes and their by-products

Enzymes	Substrates	Degradation products
Proteinase	Proteins	Amino acids
Cellulase	Cellulose	Cellobiose and glucose
Hemicellulase	Hemicellulose	Sugars (glucose, xylose and arabinose).
Amylase	Starch	Glucose
Lipase	Lipids	Fatty acids and glycerol
Pectinase	Pectin	Sugars (galactose, arabinose and polygalacticuronic acid)

Acidogenesis

The monomer substrates produced in the hydrolysis process step are intracellularly consumed by facultative and obligatory anaerobic bacteria referred to as acidogens to provide short chain organic acids like butyric acids, propionic acids, acetic acids, alcohol, hydrogen, carbon dioxide and ammonia according to van Lier, et al. (2008). The microorganisms mediating acidogenesis consist of hydrolytic and non-hydrolytic microorganisms of which 1% of all known bacteria are facultative fermenters. Therefore, facultative bacteria can be classified as acidifying or acidogenic microorganisms (van Lier, et al., 2008).

According to van Lier, et al. (2008), acidogenesis is the most rapid step of the anaerobic digestion process with the ΔG°' of acidifying reactions being highest consequently resulting into extreme bacterial growth rates of up to 20 times higher compared to methanogens. Acidogenic bacterial yields and conversion rates that are five times higher than methanogens result in anaerobic digester souring where instantaneous pH drops occur when anaerobic digesters are overloaded or fed by perturbing toxic influents.

Table 2: Acidogenic reactions with sucrose as substrate, free energy ΔG°' at 25°C.

Reactions	ΔG°' (kJ/mol)
$C_{12}H_{22}O_{12} + 9H_20 \rightarrow 4CH_3COO^- + 4HCO_3^- + 8H^+ + 8H_2$	-457.5
$C_{12}H_{22}O_{12} + 5H_20 \rightarrow 2CH_3CH_2CH_2COO^- + 4HCO_3^- + 6H^+ + 4H_2$	-554.1
$C_{12}H_{22}O_{12} + 3H_20 \rightarrow 2CH_3COO^- + 2CH_3CH_2COO^- + 2HCO_3^- + 6H^+ + 2H_2$	-610.5

Thus, the partial pressure of hydrogen present in the system influences the type of end products. For example according to van Lier, et al. (2008) in *Table 2*, the ΔG°' of less energetic acidogenic reactions with sucrose as the substrate is a factor of the hydrogen concentration of the system. For instance, if the hydrogen concentration is low because of its removal by hydrogen scavengers like methanogens, acetate would result as the main end product but if the hydrogen concentration is higher as a consequence of methanogenesis retardation, highly reduced byproducts such as propionate and butyrate or even lactate and alcohols would be produced (van Lier, et al., 2008).

These produced acids consume alkalinity, consequently increasing the concentration of non-associated VFAs and thence exacerbating the inhibition of methanogens. The inhibition of methanogens therefore results into quicker accumulation of VFAs and more pH drops that favor the proliferation of acidifiers at the expense of methanogens.

Amino acid conversion is characteristic of the Stickland reaction, whereby amino acids anaerobic oxidation de-ammonification also yields VFAs and hydrogen, but with the synergetic reductive de-ammonification of other amino acids, the produced hydrogen is consumed (van

Lier, et al., 2008). These reactions produce NH_3 which is a proton acceptor, consequently leading to a pH increase with no net proton production and the system is buffered from pH drops (van Lier, et al., 2008).

Acetogenesis

The products of the acidogenic phase, except acetate are further transformed to acetate, hydrogen gas, carbon dioxide and new cell materials by acetogens. The acetogenesis step mainly utilizes propionate and butyrate as the major substrates but lactate, ethanol, methanol, hydrogen and carbon dioxide can (homo) acetogenically be converted to acetate.

The strong symbiotic relationships between the H_2 producing acetogenic bacteria and the hydrogen consuming methanogenic bacteria governs the H_2 level in the anaerobic digestion reactor. Thus in stabilized anaerobic digestion environments, the hydrogen partial pressure is preserved at a low level by the effective uptake of methanogens and sulphate reducing bacteria.

Equation 1: Stoichiometric conversion reaction of propionate

$$\Delta G' = G^{\circ\,\prime} + RT\ ln\frac{[Acetate].[carbon\ dioxide]\cdot[hydrogen]^3}{[propionate]}$$

From the *Equation 1*(van Lier, et al., 2008); the accumulation of H_2 would inhibit the metabolism of the acetogenic bacteria which are obligate hydrogen producers. The coupling of the production and usage of H_2 is referred to as interspecies hydrogen transfer and thus synergetic collaboration between acetogens and methanogens ensures the partial pressure of H_2 is maintained between 10^{-4} to 10^{-6} atm in properly functional methane producing anaerobic reactors (van Lier, et al., 2008). This microbial collaboration between H_2 producing microorganisms proliferating exclusively in the existence of H_2 consuming microorganisms is called syntrophic association and is very important for the proper functioning of the anaerobic digesters in producing methane (van Lier, et al., 2008).

Methanogenesis

Methanogenesis is the final stage of the entire anaerobic conversion of organic matter to methane and carbon dioxide and is mediated by methanogenic archea under strict anaerobic conditions. Basically, carbon dioxide is reduced by archea using hydrogen as an electron donor with a decarboxylation of acetate to form methane. Methanogenic archea have a narrow substrate spectrum with substrates such as acetate, methylamines, methanol, formate, and H_2/CO_2 or CO being utilized (van Lier, et al., 2008). According to van Lier, et al. (2008), the methanogenesis process is four times slower compared to acidogenesis based on conversion rates of 13 and 3 g COD/ g VSS d^{-1} . Based on the fore described interactions between the anaerobic digestion microorganisms, the methanogenesis phase is extremely sensitive to the predecessor phases.

2.2. Factors Affecting anaerobic digestion

The anaerobic environment consists of complex chemical environment with various parameters in interplay thus affecting the rate at which the previously described steps of the digestion process proceed. These parameters include pH, alkalinity, retention times and temperature.

2.2.1. Alkalinity, pH and Volatile acids/ alkalinity ratio

The anaerobic groups of organisms coordinating the different steps have varying optimum pH thus pH is inherently a very important factor for anaerobic digestion. Methanogens show extreme sensitivity to pH with optimum ranges between 6.5-7.2 (Boe and Angelidaki, 2006, Turovskiy and Mathai, 2006). However a wider optimum pH range of 4.0-8.5 exists for fermentative bacteria, thus less sensitivity to pH (Hwang, et al., 2004) with acetic acid and butyric acid as prevailing products at lower pH while acetic and propionic acid are dominantly produced at pH 8 (Boe and Angelidaki, 2006).

The pH of the anaerobic digester is dependent on the production of volatile fatty acids and the activity of methanogens. VFAs lower the pH while alkalinity from the methanogens in the form of CO_2, HCO_3^- and NH_3 have a counter effect (Turovskiy and Mathai, 2006). Thus the system's overall pH is a factor of CO_2 concentration in the gas phase and the HCO_3^- alkalinity in the liquid phase (Appels, et al., 2008, Turovskiy and Mathai, 2006). Liquid phase HCO_3^- alkalinity increase while CO_2 concentration remaining stable in the gas phase results into increased digester pH (Turovskiy and Mathai, 2006). It is thus imperative to have a buffering capacity of 70meq $CaCO_3$/L or a bicarbonate/VFA ratio of at least 1.4: 1 to have a stable and well buffered anaerobic digestion process (STORA, 1985).

2.2.2. Temperature

The operation of anaerobic digesters is at mesophilic ranges (30-40˚C) or moderate thermophilic (50-60˚C) to provide optimal temperature ranges for the different groups of anaerobic microorganisms (Ahring, 1994, van Lier, 1996). Research by Lepistö and Rintala (1996) showed operation is possible at 80˚C. Thermophilic conditions in sewage treatment have advantages of increasing solubility of organic compounds, increased chemical and biological rates and pathogen inactivation (Boe and Angelidaki, 2006, Rehm, et al., 2000), but this is overshadowed by digester instability (van Lier, et al., 1993). Ranges of 50-60˚C show stable digestion and efficient performance as observed in mesophilic digestion (van Lier, et al., 1993). Furthermore, the concentration of ammonia is higher in thermophilic conditions with pronounced toxicity according to van Lier, et al. (2001), but the increasing pK_a of VFA further exacerbates the problem (Boe and Angelidaki, 2006). Acetotrophic methanogens show sensitivity to increasing temperature. In addition, degradation of propionate and butyrate is sensitive to temperature above 70˚C with this temperature significantly affecting the partial

pressure of H_2 in the digester hence favoring the kinetics of syntrophic metabolism (Appels, et al., 2008). Endergonic reactions (under standard conditions) like breakdown of propionate into acetate, CO_2, H_2 would be energetically more favorable while exergonic reactions like hydrogentrophic methanogenesis would be less favored at high temperature (Rehm, et al., 2000).

2.2.3. Solids Retention Time

The average time spent by solids in the anaerobic digester is the solids retention time (SRT) while the hydraulic retention time (HRT) is the average time the liquid sludge spends in the reactor and WAS digestion entails a onetime feed and withdrawal thus the SRT and the HRT are the same (Appels, et al., 2008).

Anaerobic digestion sequential steps are directly related to the SRT since a decrease in SRT decreases the extent of the reaction and the reverse is true. Withdraw of sludge at any time results in a fraction of the bacterial population being removed and thus requirements for cellular growth to compensate for the biomass removal to ensure steady state and prevent process failure (Turovskiy and Mathai, 2006).

Based on laboratory studies in a (semi-) continuous stirred reactor (CSTR), SRT of less than 5 days are insufficient for stable digestion since VFA concentration is increasing due to methanogenic bacteria washout (Appels, et al., 2008). It has also been observed that VFA concentrations will remain persistently high in the first 5-8 days since there is incomplete breakdown of compounds especially lipids. Stable digestion will only be achieved at 8-10days with low VFA concentrations and lipid breakdown beginning. Thus digestion will only be stable after 10 days (Appels, et al., 2008).

The SRT is the prime design criterion of anaerobic digesters such that a minimum value of maintaining the methanogenic conversion capacity of the sludge is attained (van Lier, et al., 2008). It should always be more than three times the doubling time of the biomass controlling the rate limiting step and will never be below 30 days in plant application (van Lier, et al., 2008). It is dependent on the sludge temperature, influent suspended solids concentration, rate of solids digestion in the reactor, growth and decay of the anaerobic biomass, sludge retention in the settler and the frequency of withdrawal of excess sludge (van Lier, et al., 2008). The SRT can be calculated by the formula in *Equation 2* (van Lier, et al., 2008):

Equation 2: Calculation of SRT.

$$SRT = \frac{Xreactor.Vreactor}{Qeff.Xeff + Qexcess\ sludge.Xexcess\ sludge}$$

2.3. Factors inhibiting anaerobic digestion

Anaerobic digestion inhibition or toxic compounds can either be directly fed in as digester substrate or generated in substrate transformation processes within the digester leading to process failure.

2.3.1. Ammonia

Ammonia is a by-product of degradation of nitrogenous matter usually in form of proteins and urea (Boe and Angelidaki, 2006, Chen, et al., 2008) with ammonium (NH_4^+) and free ammonia (NH_3) mostly present. Free ammonia shows higher toxicity than ammonium because it can penetrate the cell membrane (Chen, et al., 2008, Sung and Liu, 2003) causing proton imbalance and potassium deficiency (Chen, et al., 2008). Hansen, et al. (1998) further describes the factors on which free ammonia depends on as total ammonia concentration, temperature and pH.

Despite higher temperature having a positive effect on microbial growth rate, the free ammonia concentration increases and inhibition is more exacerbated in thermophilic digestion (Hansen, et al., 1998, Liu and Sung, 2002, van Lier, et al., 2001).

The increase in pH exacerbates toxicity because of a shift in the ratio of $[NH_3]/[NH_4^+]$; this instability favours an increase in VFA which brings about a decrease in pH and the free ammonia concentration drops again (Chen, et al., 2008). Thus the process is stabilized but methane yield is often reduced (Hansen, et al., 1998, Sung and Liu, 2003).

Despite ammonia concentrations less than 200 mg N/L being a beneficial nitrogen source to anaerobic microorganisms(Sung and Liu, 2003), free ammonia concentrations of 560-568 mg NH_3-N/L can potentiate a 50% methanogenic activity inhibition at thermophilic conditions with a pH of 7.6 (Sung and Liu, 2003).

According to Chen, et al. (2008), differential susceptibility to free ammonia is exhibited by the acetogens and methanogens with the latter being less tolerant. Chen, et al. (2008), increased the ammonia concentration exposure to 4051-5734 mgNH_3-N/L for acetogens and methanogens, the latter lost 56.1% activity while the former were not affected. However, acclimatization of methanogens to ammonia inhibition was also reported by Chen, et al. (2008) and it was attributed to shift in the methanogenic population or internal changes in predominant methanogenic species.

2.3.2. Sulfide

Sulphate is one of the common constituents of wastewater influent. In the anaerobic environment, sulphate is used as an electron acceptor using COD as the electron donor thence reduced to sulphide by sulphate reducing bacteria (SRB) (Boe and Angelidaki, 2006, Chen, et

al., 2008, van Lier, et al., 2008). In the anaerobic digester, two forms of inhibition are shown by SRB; the primary competitive inhibition and secondary toxicity related to the sulfide production.

Primary competitive inhibition is a consequence of the wider substrate spectrum of SRB where substrates such as H_2 , formate, acetate, methanol, pyruvate, propionate, butyrate, higher and branched fatty acids, lactate, ethanol and higher alcohols, fumarate, succinate, malate and aromatic compounds are utilized by SRB (Colleran, et al., 1995). This is at the disadvantage of acetogenic and methanogenic bacteria which require the same substrates synthesized by fermentative bacteria (Appels, et al., 2008). According to van Lier, et al. (2008), the sulfide production pathway is favored by a COD/SO_4^{2-} ratio of 0.67, which theoretically implies that there is enough sulphate available for SRB to beat the competition. However, a COD/SO_4^{2-} ratio exceeding 0.67 enables both sulphate reduction and methanogenesis to simultaneously occur (van Lier, et al., 2008). Further studies have suggested that extremely high concentrations of sulphate can initiate population shifts in the digester from hydrogentrophic methanogens to hydrogentrophic sulphate reducers due to a more favorable Ks for the latter (Boe and Angelidaki, 2006, Chen, et al., 2008). Chen, et al. (2008), further suggests that higher temperatures favour methanogens to SRB since methanogen populations are more dominant at thermophilic temperatures. In anaerobic digestion, the natural presence of propionate potentiates the activities of SRB, achieving stable sulphate reduction with a resultant decrease in the amount of methane production (Brand, et al., 2014). Brand, et al. (2014) further assets that the acetate content of VFA will favor methanogenic activity while propionate potentiates SRB activity leading to an eventual out competition of methanogens by SRB. The metabolic activity of SRB is not limited to the presence of an electron acceptor since they are able to proliferate by fermentative/ acetogenic reaction as described by Widdel, et al. (1988) and syntrophic associations with Hydrogenotrophic methanogenic bacteria (Stefanie, et al., 1994, Widdel, et al., 1988, Wu, et al., 1991) have also been described. Furthermore, the sulphide production is more toxic/inhibitory to methanogens than SRB(Brand, et al., 2014). Therefore, the end product of the anaerobic mineralization process as sulfide or methane is dependent on the sequel of the competition between SRB and methanogenic bacteria. Sulfate rich wastewater streams will therefore demerit the economic attractiveness of anaerobic wastewater treatment in terms of methane production per unit of organic matter and also distort the overall energy balance of the process, moreover affecting the quality of the effluent and biogas since sulfide will be present in both which is rather a costly venture in terms of further treatment (Visser, 1995). However, despite the negative effect on the methane production, sulfate reduction in conjunction with methanogenesis enables the removal of oxidized sulfur compounds from wastewater and allows the precipitation of heavy metals that would otherwise present potential toxicity to the anaerobic digestion process (Visser, 1995). Further trade-offs in sulphate reduction are the removal of xenobiotics like naphthalene and TNT (Widdel, 1988, Zhang and Bennett, 2005) and; fecal coliforms reduction (Abdeen, et al., 2010).

Secondary toxicity of both methanogens and SRB is due to the formed sulfide. The physiological mechanisms of this toxicity is described by several scholars as being due to the free diffusion of the non-dissociated sulphide (dihydrogen sulphide) across the cell membrane achieving pH reduction (Comeau, et al., 1986, Koster, et al., 1986b), protein denaturation and interfering with the assimilatory metabolism of Sulphur and other key micronutrients (Bejarano Ortiz, et al., 2013, Boe and Angelidaki, 2006, Chen, et al., 2008). There is also reported interference of acetyl Coenzyme A and M through formation of sulfide linkages. Acetyl CoA pathway for carbon dioxide fixation is utilized by both SRB and methanogenic bacteria (Stouthamer, 1988) and this explains the toxicity effect on both. The chemical and physical equilibrium determines the concentration of undissociated sulfide in anaerobic digesters:

$$H_2S\ (l) \rightleftarrows H_2S\ (g)$$

$$H_2S\ (l) \rightleftarrows HS^- + H^+$$

At 18˚C, the *PKa* value of the dissociation equilibrium of sulfide is about 7.04 (Weast, 1976) and this implies small pH changes in the pH range of 6-8 have a significant effect on the concentration of sulfide. Also since the gas-liquid distribution coefficient for sulfide is about 2.27 at 30˚C (Wilhelm, et al., 1977), the sulfide concentration in the liquid phase can remarkably be lower.

Several scholars have described the effect of sulfide on the anaerobic digestion process as early as the 50s (Aulenbach and Heukelekian, 1955, Bannink and Muller, 1951, Butlin, et al., 1956, Rudolfs and Amberg, 1952). However, the limitation of these studies is that they did not consider sulfide speciation and effects based on pH making them less beneficial in describing the actual effect of sulfide in reactors. pH has received more attention in more recent studies whilst describing inhibitory effects of sulfide in anaerobic reactors. For example Koster, et al. (1986a) describes inhibition caused by sulfide at high pH (7-8) to be significantly higher than in the lower pH range of 6.7-7.2 because in the pH range of 6.7-7.2, the inhibition is based on the H_2S concentration whilst at higher pH (7-8), it is a factor of total sulfide concentrations. Koster, et al. (1986a) reported 50% inhibition values at 250 mg H_2S/L at pH 6.2-7.2 and 825 mg/L of total sulfide at pH 7-8. Literature further reviewed shows that sulfide inhibition is pH and temperature specific for different sludge types and substrates. *Table 3* with excerpts from (Visser, 1995), outlines sulfide H_2S concentrations causing 50% inhibition.

Table 3: Reported sulfide concentrations causing 50% inhibition

Biomass	Substrate	Temperature (˚C)	pH	H_2S (mg/L)	Reference
Methanogenesis					
Suspended sludge	acetate	Not reported	Not reported	50	(Kroiss and Plahl-Wabnegg, 1983)

Biomass	Substrate	Temperature (˚C)	pH	H_2S (mg/L)	Reference
Suspended sludge	Distillery wastewater	37	7.0-7.2	130	(Karhadkar, et al., 1987)
Suspended sludge	acetate	35	6.5-7.4 7.7-7.9	125 100	(Oleszkiewicz, et al., 1989)
Suspended sludge	lactate	35	7 8	100 100	(McCartney and Oleszkiewicz, 1993)
Granular sludge	acetate	30	6.2-6.4 7.0-7.2 7.8-8.0	246 252 90	(Koster, et al., 1986a)
Substrate specific inhibition					
Granular sludge	Propionate	30	7.0-7.5	140	(Rinzema and Lettinga, 1988)
Granular sludge	Propionate	35	6.5-7.4 7.7-7.9	100 60	(Oleszkiewicz, et al., 1989)
Suspended sludge	Butyrate	35	6.5-7.4 7.7-7.9	235 >200	(Oleszkiewicz, et al., 1989)
Suspended sludge	Lactate	35	6.5-7.4 7.7-7.9	320 390	(Oleszkiewicz, et al., 1989)

2.3.3. Heavy metal toxicity, sulfide and trace metal bioavailability.

According to Mudhoo and Kumar (2013), heavy metals affect biochemical reactions that take place during anaerobic digestion. These effects can be stimulatory, inhibitory or toxic. Trace metals are needed to maintain microbial metabolism and growth (Fermoso, et al., 2009) and their absence in sufficient quantities negatively impacts on anaerobic microorganisms activity in bioreactors (Fermoso, et al., 2008). Zinc, cobalt, nickel, copper, selenium, chromium, molybdenum, tungsten, manganese or iodine are referred to as trace metals since they are required in small amounts to catalyze various enzymatic anaerobic reactions and transformations (Oleszkiewicz and Sharma, 1990, Zandvoort, et al., 2006).

Heavy metals inhibition of the anaerobic digestion process is associated with blocking of enzyme function in excess. Nies (1999) described heavy metal toxicity as non-specific and reversible and less frequently as competitive if competing with the substrate. This latter form

of inhibition has been described by Oleszkiewicz and Sharma (1990) as dependent on the affinity of the metal and the enzyme and on their relative concentrations.

Van der Veen, et al. (2007) described the bioavailability and mobility of essential trace metals to be controlled by sulfide chemistry in UASB reactors. Based on stability constants proposed by Martell and Smith (1989), metal ions in anaerobic wastewater environments will precipitate with sulfide, carbonate and phosphate. Fermoso, et al. (2009) describes metal sulfide precipitation to be the most important process. Furthermore, Van der Veen, et al. (2007) asserts that sulphide's role in metal fixation in anaerobic granules is supported by the high acid volatile sulfide and metal content in Oxidizable fractions present in UASB systems.

Since metal sulphides have extremely low solubility products according to Martell and Smith (1989) , this may limit the bioavailability of these metals to the methanogenic consortia in anaerobic systems. Gonzalez-Gil, et al. (2003), further suggests that ageing of sulfidic precipitation that occurs in sludge during reactor operation may lower the dissolution rates thereby lowering the metal bioavailability.

Therefore, sulfide presence in an anaerobic reactor is not only significant due to the formation of metal precipitates, but also in the accumulation of dissolved metal complexes and this could have ramifications on the entire anaerobic digestion process if no trace metal supplementation in the right doses occurs.

2.4. Anaerobic digestion of waste activated sludge

2.4.1. Process description

Anaerobic digestion of WAS follows similar anaerobic digestion steps of hydrolysis, acidogenesis, acetogenesis and methanogenesis. Due to the complexity of the organic substrates, hydrolysis is the rate limiting step in the anaerobic digestion of WAS. Prior to digestion, WAS is usually thickened by gravity thickeners, centrifuges, floatation or belt filtration thereby achieving about a third of its initial volume (Appels, et al., 2008).

WAS is anaerobically digested to achieve volume reduction by reducing the water content, stabilization, meeting environmental disposal acceptance regulation and the production of biogas (Appels, et al., 2008). Despite the fore mentioned incentives of the process, WAS digestion has inevitable limitations including: partial digestion of the organic fraction; slow reaction rates with associated large digester volumes and elevated costs; vulnerability of the digestion process to toxicity and; poor supernatant quality production.

In practice, WAS digestion is performed under mesophilic (30-38˚C) and thermophilic (50-57˚C) conditions. Thermophilic digestion is faster due to increased biochemical reaction rates as a result of increased temperature. In addition, thermophilic digestion enables increased solids

reduction, improved sludge dewatering and pathogen inactivation but is disadvantaged by higher energy demands, poor quality supernatants, higher odor potential and increased instability of process. Thermophilic digestion is unstable because thermophilic bacteria are much more sensitive to temperature fluctuations compared to mesophilic bacteria. Several types reactor types are used for anaerobic digestion of WAS but commonly, the standard-rate (cold), high rate and 2 stage digesters are used (Appels, et al., 2008, Boe and Angelidaki, 2006).

2.4.2. Pre-treatment processes in anaerobic digestion

Despite the advantages of anaerobic digestion, the application of anaerobic digestion to WAS is limited by very long retention times (20-30 days), and an entire low degradation efficiency achieving only 30-50% of the organic dry solids (Appels, et al., 2008). These limiting factors are generally a consequence of the hydrolysis phase according to Tiehm, et al. (2001). Inherently, WAS contains bacterial cell walls and extra polymeric substances that need to be raptured resulting in the release of easily accessible and readily biodegradable organic materials for the acidogenic micro-organisms (Appels, et al., 2008). The increased resistance to biodegradation has indeed confirmed the hydrolysis step as rate limiting and thus necessitating the use of various sludge disintegration pre-treatments aimed at disintegrating the cell wall and releasing previously inaccessible intracellular components into solution and by-passing the rate- limiting hydrolysis stage (Appels, et al., 2008). These pre-treatment methods include: pre-acidification; thermal; mechanical and chemical pre-treatment.

Pre-acidifiers

Pre-acidification pre-treatment entails an engineered phase separation of the anaerobic digestion process whereby a smaller pre-acidification reactor achieves fermentation and hydrolysis by optimizing the environmental conditions for the proliferation of acidogens whilst restricting the growth of methanogens. These environmental conditions include pH of 6.5 and below, short retention times and increased temperature. This promotes increased hydrolysis and VFA formation prior to transfer of the formed products to a normal digester where methanogens are favoured to complete the transformation of these pre-formed products to methane (Lettinga and Pol, 1991).

Thermal Pre-treatment

Thermal pre-treatment has been employed for so many years to improve sludge degradability by breaking down chemical bonds of the cell wall and membrane thus making proteins accessible for degradation unlike carbohydrates and lipids which are easily accessible and degradable to microorganisms. Several scholars have concluded that the temperature and duration of pre-treatment is dependent on the nature of the sludge but in practice, optimum temperature range of 160˚C-180˚C for 30-60 minutes treatment with pressures ranging from 600-2500kpa (Lu, et al., 2008, Weemaes and Verstraete, 1998). Generally WAS thermal pre-treatment considerably increases methane production for mesophilic anaerobic digesters but not

significantly for thermophilic digesters because the latter are already efficient at VSS reduction and methane production(Appels, et al., 2008). The formation of toxic refractory compounds at temperatures higher than 170-190˚C has been reported with decreased sludge biodegradability yet higher solubilisation efficiencies have been achieved thus lowering biogas production (Bougrier, et al., 2007). Thermal pre-treatment requires approximately 700kJ/m^3, a considerable amount of energy to increase the sludge temperature at the expense of the biogas produced (Zhang, et al., 2010).

Mechanical pre-treatment

Mechanical pre-treatment achieves solubilisation of particulate matter in liquid phase with various techniques including ultra-sonication, grinding and high pressure homogenization. The overall aim of these methods is to increase the degradability of organic matter through bacterial wall lysis and floc disruption (Appels, et al., 2008, van Lier, et al., 2001).

Ultra-sonication gives efficient sludge disintegration, with improved biodegradability, increase in methane percentage in biogas, reduction in anaerobic digestion time and reduction in sludge volume (Appels, et al., 2008, Tiehm, et al., 2001). The mechanisms of ultrasonic treatment are based on cavitation at low frequencies and chemical reactions due to formation of OH^-, H_20 and H^+ radicals at high frequency (Carrère, et al., 2010). According to (Wang, et al., 1999), the specific energy applied and the sonication time affect the efficiency of the sonication process in increasing methane production.

Grinding uses a wet milling technique where small beads are used to rapture cell walls. The study of Baier and Schmidheiny (1997) reported that bead size is critical for sludge disintegration with a small diameter of 0.2-0.25mm having the best performance.

High pressure homogenization employs sludge compression to 60MPA (Harrison, 1991), followed by decompression through a valve at high speed against a compaction ring resulting in turbulence, cavitation and shear stress resulting in cell disintegration (Appels, et al., 2008).

Chemical pre-treatment

Chemical pre-treatment aims at hydrolysing the bacterial cell walls and membranes thereby increasing solubility of organic matter (Appels, et al., 2008) that was previously inaccessible to anaerobic digester microorganisms. The major chemical pre-treatment methods are: acid and alkaline pre-treatment and, oxidative methods.

Acid and alkaline pre-treatment involves either addition of acids or bases to solubilise the sludge. These methods avoid the use of high temperatures since they are effective at moderate or ambient temperatures. However, extreme pH levels of sludge need to be neutralized before

anaerobic digestion thus making it costly and less frequently applied in anaerobic digestion of WAS (Appels, et al., 2008).

Oxidative methods include ozonation and pero-oxidation. Ozone is a strong oxidant that is applied to achieve the destruction of bacterial cell walls. A major drawback of ozonation is the need to lower the pH of sludge to 3 and thus would need neutralization after (Appels, et al., 2008). Pero-oxidants such as peroxymonosulphate (POMS) and dimethyl-dioxirane (DMDO) avoids the lowering of pH whilst significantly increasing the biogas production during anaerobic digestion of WAS (Appels, et al., 2008).

2.5. Alternative Pre-treatment techniques

2.5.1. FNA pre-treatment of WAS

The application of FNA as a pre-treatment agent on WAS is increasingly being studied by several scholars under different conditions to elucidate the effect of the pretreatment towards overcoming barriers of limited hydrolysis, low availability of readily biodegradable organics and reduced accessibility of organic substrates to microorganisms involved in the anaerobic digestion process. By doing assays of the increased presence of hydrolysis and acidification products, protein digestion products and measuring the increase of biogas production after the FNA pretreatment, significant information required for its use as an alternative pretreatment is increasingly being gained.

The drivers for consideration of using FNA are its ability to be produced from the high concentration of ammonia naturally present in the anaerobic digestion liquor in quantities sufficient enough for industrial applications thus making it a cost efficient and environmentally friendly pre-treatment technique (Zhou, et al., 2011).

Temperature, pH and nitrite concentration as a factor in free nitrous acid production.

Anthonisen, et al. (1976), observed that the formation of FNA was dependent on the temperature, pH and nitrite concentration. *Equations 3(Wang, et al., 2013)* and *4(Anthonisen, et al., 1976)* describe the relationship of FNA formation:

Equation 3: Calculation of nitrite concentration

$$SN - NO_2^- = K_a \, x10^{pH}$$

Equation 4: Calculation of acid 1onization constant of FNA

$$K_a = e^{-2300/(273+^{\circ}C)}$$

Thus, based on the *Equations 3* and *4*, an increase in pH and temperature necessitates a higher concentration of nitrite to produce FNA.

FNA and enhancing biogas production

Li, et al. (2016), explored the use of free nitrous acid in stimulating short chain fatty acids (SCFA) from waste activated sludge. It should be noted that short chain fatty acids are hydrolysis products that are readily anaerobically biodegradable for biogas production. The effect of FNA exposure on the net generation of SCFA was 170.6, 195.7, 151.6 and 125.2 mg COD/g VSS in WAS fermentation reactors exposed to 0.9, 1.8, 2.7 and 3.6 mg $HN0_2$ –N/L respectively with the blank generating only 52 mg COD/ g VSS at the 12th day of fermentation. Thus 3.7 times higher SCFA was generated at an FNA concentration of 1.8 mg $HN0_2$ –N/L compared to the blank. However, a reduction of SCFA generation was observed beyond 1.8 mg $HN0_2$ –N/L and this was ascribed to the toxic effects of higher FNA concentrations on the enzymes responsible for acetogenesis and hydrolysis according to Pijuan, et al. (2012) and Wang, et al. (2014b). An analysis by Zhao, et al. (2016) realized 325 mg COD/g VSS as SCFA with 1.54 mg $HN0_2$ –N/L FNA but a negative correlation was obtained with concentrations beyond that which further justifies that increased concentrations are toxic to hydrolysis and acetogenesis.

Furthermore, the observations of Li, et al. (2016) showed increased extra polymeric substances (EPS) disruption and lysis of cell walls of activated sludge at an FNA concentration of 1.8 mg $HN0_2$–N/L at 10 hours exposure with increased soluble COD, soluble proteins and soluble carbohydrates as indicators. In this study, 1.8 mg $HN0_2$–N/L FNA caused 252% protein, 211% carbohydrate and 225% COD increase in solution while VSS reduction was 123% more than the blank. Thus, more soluble substrates for subsequent hydrolysis and acidification were released. The expressed solubilization at 1.8 mg $HN0_2$–N/L FNA was 2458 mg/l proteins and 651 mg/l carbohydrates on day 4 of the test compared to 552 mg/l proteins and 124mg/l carbohydrates in the blank. However, Zhao, et al. (2016) reported 854 mg/l soluble proteins and 165 mg/l soluble polysaccharides at 1.54 mg $HN0_2$–N/L FNA.

To further illustrate the significant hydrolysis and acidification FNA concentration at 1.8 mg $HN0_2$ –N/L achieved, synthetic wastewater containing Bis-(trimethylsilyl) acetamide (BSA), dextran, L-alanine and glucose respectively achieved higher hydrolysis rates of 60.5%, 73.5%, 72.6% and 85.6% compared to 38.4%, 53.%, 59.1% and 63.7% in blank respectively on day 3 of the test (Li, et al., 2016).

According to Luo, et al. (2015), an increased soluble NH_4^+-N concentration is an indicator of increased protein hydrolysis. An increase in soluble NH_4^+-N concentration was observed in the study of Li, et al. (2016) from day 0-6 of the fermentation time. Furthermore, PO_4^{3-} -P release was observed with concentrations of 104.2, 112.1, 96.6 and 81.2 mg/l on day 6 when FNA concentration was 0.9, 1.8, 2.7 and 3.6 mg $HN0_2$–N/L respectively compared to the blank with

55.1 mg/L of PO_4^{3-} -P. These observations were consistent with the high hydrolysis rate of FNA pre-treatment. However, NH_4-N and PO_4^{3-}-P concentrations decreased beyond an FNA concentration exposure of 1.8 mg $HN0_2$–N/L likely due to increased toxicity of major enzymes in hydrolysis and acidification as proposed by Wang, et al. (2013) and (Pijuan, et al., 2012)

Nevertheless, Li, et al. (2016) observed an inhibition of methanogenesis in their study. The SCFAs, generated from hydrolysis were not consumed by methanogens because of addition of nitrite to the anaerobic digesters which changed conditions from strict anaerobic to anoxic conditions during WAS fermentation. Also the pH drop from 7 to 5.2 was not favorable for methanogens which operate at an optimum of pH 7. This pH drop did not affect acidogens according to Wang, et al. (2013) and Wang, et al. (2014a) emphasizes that nitrite itself suppresses methanogens. Thus continued activity of acidogens further soured the reactor.

Wang, et al. (2013) explored the effect of exposing waste activated sludge on increasing soluble COD and methane production with increasing FNA concentrations of 0, 0.36, 0.71, 1.07, 1.42, 1.78 and 2.13 mg HNO_2/L at a stable pH of 5.5 and temperature of 25˚C in contact batch tests for 24 hours and subsequent biochemical methane potential (BMP) tests for 44 days. It was observed that the highest increase in soluble COD was at an FNA concentration of 2.13 mg HNO_2/L specifically 0.16 mg COD/mg VS compared to the control with 0.025mg COD/mg VS which is six times more, thus hydrolysis was increased. The study reported a substantial increase in soluble KN, soluble proteins and soluble polysaccharides in comparison to the control with no FNA exposure. However, there was a general decreasing trend of NH_4^+-N and VFA with increased FNA concentration (Wang, et al., 2013) which was in agreement with the observations of Pijuan, et al. (2012) and was attributed to the inhibitory or toxic effects of FNA on sludge hydrolytic and acidogenic enzymes (Pijuan, et al., 2012).

Increased FNA concentrations showed an increasing trend of methane production with the highest concentration of 2.13 mg HNO_2/L ranking first after 15 days (Wang, et al., 2013). Furthermore, model based analysis indicated that both hydrolysis and methane potential were improved by 50% (from 0.16 to 0.25 d^{-1}) and 27% (201 to 255 L CH_4/Kg VS added) respectively.

On the contrary, Zhang, et al. (2016) reported a minimal increase in release of readily biodegradable substances to the soluble phase with FNA pre-treatment of primary sludge resulting into a 7% decrease of BMP compared to un-pretreated primary sludge. This outcome was attributed to the macromolecular composition of primary sludge being mainly fatty acids rather than proteins that constitute WAS predominantly yet FNA achieves increased biodegradation through protein deaminative polymerization (Zhang, et al., 2016). However, Dunn and Schmidt (1922) observed that the rate of deamination of several substituted amino acids with FNA decreases with increasing distance between the amino and the carboxyl group. This implies that the deaminative effect will vary from one type of amino acid to another.

Further contradictory reports of FNA increasing methane production were observed by Jiang, et al. (2011a) where 0.09 mg-N/L as FNA after 6 hour exposure to anaerobic sewer biofilms adequately reduced methane production.

Wang, et al. (2014b) explored an alternative approach of FNA pre-treatment using heat in combination with FNA to compare solubilization and methane potentials achieved with only FNA treatment with a control experiment having no heat or FNA. In the control experiment, soluble COD increased by only around 0.06 mg COD/mg VS while with the FNA exposed experiment by 0.11-0.13 mg COD/mg VS which is about 2.2 times more (Wang, et al., 2014b). Heat pre-treatment only had soluble COD increasing by 0.14-0.26 mg COD/mg VS which is 4.3 fold more than the control while the combined heat and FNA pre-treatment had soluble COD increase by 0.16-0.28 mg COD/mg VS which is 4.7 times more than the control and 0.02mg COD/mg VS higher than FNA pre-treatment alone.

There was a similar trend in increase of soluble Khedjal nitrogen (SKN), soluble proteins and soluble polysaccharides with both Wang, et al. (2013) and (Wang, et al., 2014b). Strikingly, both Wang, et al. (2013) and (Wang, et al., 2014b) showed a decreasing trend of NH_4^+-N and was attributed to the effect of FNA and heat deactivation of hydrolysis and acetogenesis enzymes according to Pijuan, et al. (2012). In Wang, et al. (2014b), methane production was highest in combined FNA and heat pre-treatment compared to the control or FNA treatment thus reemphasizing the assertion of Paul and Liu (2012) that thermal energy could not transform the refractory materials. Thus FNA helps in changing the chemical structure of these refractory materials into anaerobically biodegradable materials according to (Wang, et al., 2014b) and other prior studies have supported the same idea (Halliwell, et al., 1992, Horton and Philips, 1973, Lewis and Updegraff, 1923, Rowe, et al., 1979, Yoon, et al., 2006).

Pijuan, et al. (2012) investigated the effect of 2.02 mg HNO_2/L FNA on aerobic biodegradability of the FNA exposed sludge and found out that approximately 50% and 90% of the FNA treated sludge was biodegradable after 6 and 14 days aerobic digestion respectively. The control experiment with no FNA exposure to sludge showed no detectable degradation on the 6th day, but only 40% degradation on the 14th day. However, the digestion was done aerobically and tests to show increased hydrolysis were limited to only oxygen uptake to calculate the COD provided by the FNA addition. 391 mg COD compared to -48.6 mg COD (control experiment) was provided after FNA pre-treatment. Pijuan, et al. (2012) further recommended the use of FNA to provide a source of readily biodegradable COD to anaerobic digestion processes thereby enhancing biogas production and reducing hydraulic retention times thus need for smaller anaerobic digesters which is in agreement with the assertions of Carrère, et al. (2010).

Table 4: Effective FNA concentration and WAS effect

Study	Effective FNA concentration mg HNO_2 -N/L	Effect
Li, et al. (2016)	1.8	Increased hydrolysis, increased solubilization, increased cell lysis and EPS disintegration
Zhao, et al. (2016)	1.54	Increased hydrolysis, increased solubilization, increased cell lysis and EPS disintegration
Zhao, et al. (2015)	1.54	Increased hydrolysis, increased acidification, shortened fermentation time
Wang, et al. (2013)	2.13	Increased hydrolysis, increased solubilization, and increased biogas production
Wang, et al. (2014b)	1.43	Increased hydrolysis, increased solubilization, and increased biogas production
Pijuan, et al. (2012)*	2.02	Increased biodegradability (loss of MLVSS, inorganic nitrogen production and oxygen consumption)

*Aerobic digestion

It is important to note that there are differing effective concentrations of FNA and differing effects on WAS characteristics towards enhancing increase in biogas production (*Table 4*). This can be attributed to different sludge characteristics that could be influenced by operational processes and environmental conditions. Also the experiment setup could have affected the outcome of these studies. Reporting of effective concentrations as biomass based concentrations such as mg FNA/gVS would make comparability possible. *Table 5* shows the sludge characteristics of the studies previously reviewed in the literature above.

Table 5: Sludge characteristic differences

Parameter	(Wang, et al., 2014b)	(Wang, et al., 2013)	(Li, et al., 2016)	(Zhao, et al., 2016)	(Zhao, et al., 2015)
SRT (d)	15	15	20	15	20
TS (g/L)	48.7	42.6	13.4±0.3	13.8 ± 0.208(TSS)	11.8 ± 0.8(TSS)
VS (g/L)	39.4	33.7	10.2±0.2	10.12 ± 0.145(VSS)	9.9 ± 0.4(VSS)
TCOD (g/L)	59.9	54.1	15.2±0.3	14.15 ± 0.22	12.1 ± 1.2
SCOD (g/L)	1.94	0.65	0.18+0.016	0.22 ± 0.02	
PH	6.64	6.4	6.8±0.1	6.8 ± 0.1	6.8 ± 0.1

FNA inhibition/ toxicity

FNA has been observed to cause inhibitory/ toxic effects to different WAS microorganisms including nitrifiers, denitrifiers, phosphate accumulating organisms (PAOs) and Annamox. For example Anthonisen, et al. (1976) observed an inhibitory effect of nitrifiers to be depending on pH, temperature and nitrite concentration and based on the equilibrium that exists between nitrite and the unassociated FNA.

This broad range of inhibition occurs under different thresholds of FNA between groups of microorganisms and under different environmental conditions, process factors and depends also on acclimation of microorganisms to nitrite. Beccari, et al. (1983) observed that the biomass concentration alongside pH and nitrite concentration governed the inhibitory effect of FNA against WAS microorganisms. However, this inhibition has been observed in laboratory scale reactors and may not necessarily occur in full scale reactors. For example Burrell, et al. (1999) and Daims, et al. (2001) observed that *Nitrobacter* is commonly found in lab scale reactors while *Nitrospira* is the dominant microorganism in full scale reactors. *Table 6* below with excerpts from (Zhou, et al., 2011) shows the FNA thresholds on a broad range of common microbial consortiums in wastewater treatment plants.

Table 6: Comparative Inhibition thresholds of FNA

Reference	Culture	FNA(100%) inhibition Mg HNO_2 –N/L	FNA(50%) inhibition Mg HNO_2 –N/L
Hellinga, et al. (1999)	AOB	Not reported	0.2
Vadivelu, et al. (2006b)	NOB	0.023	0.0175
Ma, et al. (2010)	Denitrifiers	0.2	0.025
Strous, et al. (1999)	Annamox	0.006	
Pijuan, et al. (2010)	PAO ($An0_2$-Ox)	0.004	0.0005
Zhou, et al. (2010)	PAO ($An0_2$-Ax)	0.037	0.01
Ye, et al. (2010)	GAO ($An0_2$-Ox)	0.02(70%)	0.01
Zhou, et al. (2008)	N_2O reduction (PAO AnO_2-Ax)	0.004	0.0007
Klüber and Conrad (1998)	Methanogens	0.0003	0.00015

FNA inhibitory mechanisms

FNA inhibition and toxicity affects several metabolic processes like oxygen uptake, cellular membrane active transport and oxidative phosphorylation (Zhou, et al., 2011). Furthermore, energy consuming anabolic processes are more predisposed to FNA inhibition than catabolic processes involved in ATP production, however both can be affected (Pijuan, et al., 2010, Vadivelu, et al., 2006a, Ye, et al., 2010, Zhou, et al., 2010). Thus three mechanisms namely protonophore uncoupling, enzymatic effect and nitric oxide inhibition have been supported (Almeida, et al., 1995, Baumann, et al., 1997, Carlsson, et al., 2001, Hinze and Holzer, 1986, Mortensen, et al., 2008, O'Leary and Solberg, 1976, Park, 1993, Rake and Eagon, 1980, Rasmussen, et al., 2005, Schulthess, et al., 1995, Sijbesma, et al., 1996, Zhou, et al., 2011).

2.5.2. Can sulfide be an alternative pre-treatment agent?

Sulfide exposure to WAS microorganisms has been shown to be inhibitory to nitrifiers and phosphate accumulating organisms (PAOs) and Annamox (*Table 7 and 8: with excerpts from (Bejarano Ortiz, et al., 2013)*). These studies have observed that cell metabolism has been affected. However, these studies do not show the effect on cell viability, increased hydrolysis and provision of readily biodegradable organic matter that can be utilized in anaerobic digestion process to increase biogas production.

Nielsen and Keiding (1998), observed a weakening of the floc strength leading to an increased shear sensitivity of the flocs consequently leading to bacterial organic colloids and dissolved extracellular polymeric substances (EPS) being released from the activated sludge flocs. This floc disintegration led to the dissolution of up to 10% of the total organic matter.

Since sulphide like FNA inhibits activated sludge microorganisms, could it be a potential pre-treatment agent?

Table 7: Sulfide inhibition of PAO

Reference	Concentration (mg S/L)	Effect
Rincon et al.(2016)	22	50% decrease in anaerobic P-release 81% decrease in aerobic P-uptake
	48 115	0% aerobic P-uptake
	189	P-release instead of uptake aerobically
Saad, et al. (2013)	60	50% decrease in aerobic acetate uptake
		55% decrease in anaerobic P-release

Table 8: Sulfide inhibition of nitrifiers

Reference	Sulfide concentration (mg/L)	inoculum	Main effects
Beristain-Cardoso, et al. (2009)	1.7-1.8	Nitrifying biofilm (3.5g VSS L^{-1} ± 0.5g VSS L^{-1})	Decrease in specific rates of NH_4^+ consumption and $N0_3^-$ formation
Erguder, et al. (2008)	1.3-82.5	Nitrifying sludge 2.7±0.06 gVSS L^{-1}	Increase in the NO_2^- -N to (NO_2^--N +NO_3^--N) accumulation ratio
Sears, et al. (2004)	0.5-3.6	Nitrifying sludge 1 gVSS L^{-1}	Decline in the volumetric NH_4^+ consumption
Æsøy, et al. (1998)	0.5-206 0.5-2.6	Not reported	Decline in the volumetric NH_4^+ consumption
Becker, et al. (1997)	323	Mixed population of nitrifying and denitrifying bacteria	Decrease in the NH_4^+ consumption efficiency
Joye and Hollibaugh (1995)	1.9 or 3.2	Estuarine sediments	Decrease in the specific rates of NO_2^- and NO_3^- formation
Martienssen, et al. (1995)	7.2	Not reported	Decrease in the NH_4^+ consumption efficiency
Bentzen, et al. (1995)	2.0-5.4	Not reported	Decrease in the NH_4^+ consumption efficiency

2.6. Summary of literature review

It is imperative to say that most studies have focused on the inhibition/toxicity of FNA and sulphide on a broad range of microorganisms rather than how this is translated into hydrolysis. Also the sensitivity of particular groups of microorganisms differs since different thresholds of FNA and sulphide concentrations have been reported. The difference in experimental setups and methodology further complicates the situation while different sludge characteristics and process operations may influence the outcome of the study observations.

The studies done on nitrite/ FNA do not clearly elucidate what is exactly responsible for the inhibition/toxicity since both species seem to be implicated in the toxicity. However, Anthonisen, et al. (1976) describes the occurrence of FNA higher concentration at lower pH. Furthermore, there seems to be no single inhibitory/toxic mechanism for FNA and thus several studies agree that a combination of uncoupling, enzymatic inhibition and NO formation could explain the toxicity effects. Toxicity of methanogens to nitrite is well documented (Banihani, et al., 2009, Baumann, et al., 1997, Klüber and Conrad, 1998, Li, et al., 2016, O'Reilly and

Colleran, 2005, Schulthess, et al., 1995), but most studies describing FNA enhancement of methane production do not show the nitrite carry over to the anaerobic digester and the effects observed. Thus further research showing which particular mechanism is responsible for inhibition/ toxicity of a particular group of microorganisms and particularly toxicity/inhibition of methanogens due to nitrite after FNA pre-treatment is required.

To date, no study has yet observed the effect of sulfide on the hydrolysis and subsequent increased methane production. It is also imperative to study if toxicity will result into increased hydrolysis and provision of readily biodegradable organics that can be anaerobically digested to improve biogas production since the products of pre-treatment may be refractory.

Therefore, this raises the following questions:

1. Does inhibition/ toxicity translate into hydrolysis with readily biodegradable products?

2. Concerning the effect of exposure time to both FNA and sulfide, what is the required exposure time to initiate lysis of the microorganisms in waste activated sludge? Is prior acclimation of the sludge to nitrite and sulfide important in limiting toxicity and subsequent hydrolysis of the microorganisms to produce readily biodegradable products that can be anaerobically digested to produce more biogas?
3. Concerning the concentration of FNA being low at higher temperature yet reaction rates increase with increasing temperature: would the application of higher concentrations of nitrite to increase the concentration of FNA at higher temperature be feasible and cost friendly?
4. Which is the most potent intoxicant to waste activated sludge that would provide higher solubilization and methane production between FNA and sulfide?

Therefore, to do this study, it is important to hypothesize that the observed toxicity/inhibition translates into hydrolysis with readily biodegradable organics that would increase biogas production. However, the study will need to rule out the questions raised above.

CHAPTER 3

Research Methodology

This chapter describes the general research methodology applied to achieve the research study objectives. An outline of the batch experiments on pre-treatment of the activated sludge and anaerobic biochemical methane potential set up is given.

3.1. General research Approach

Batch experimental work in terms of pre-treatment and BMP was phased in 3 stages. *Figure 3* shows an outline of the 3 phases

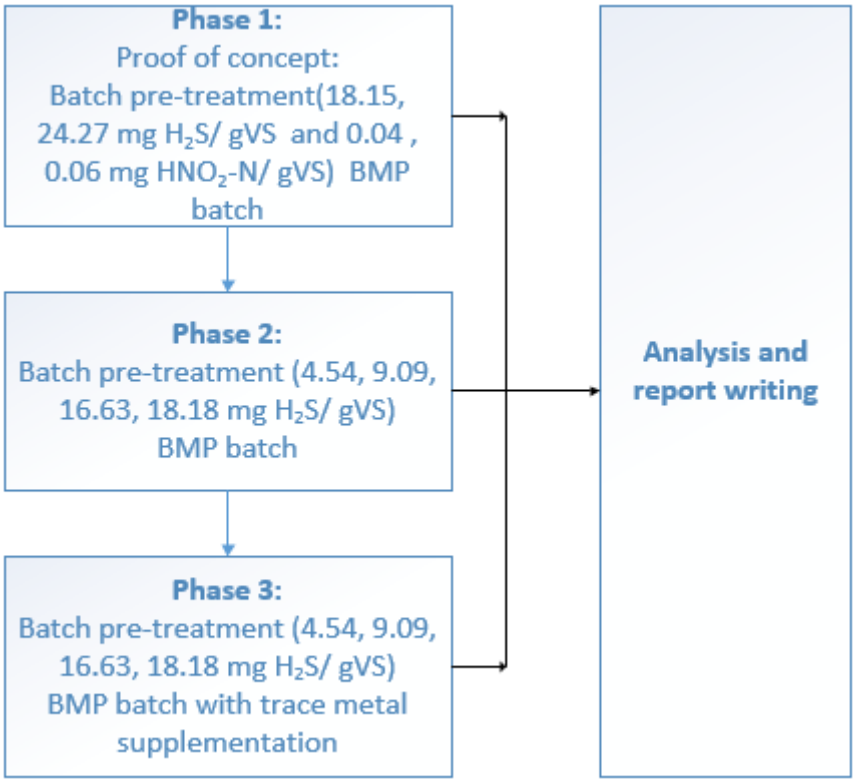


Figure 3: Outline of research activities

An initial proof of concept phase was performed to access the possibility of enhancing sludge biodegradability with both sulfide and FNA concentrations and subsequent anaerobic digestion considering the fact that sulfide and nitrite both present challenges in anaerobic digestion both as inhibitors and nitrite also being an alternative electron acceptor that consumes organic matter.

In the second phase of the study, sulfide concentrations in the range of 4.54 to 18.18 mg H_2S/ gVS were used for the pre-treatment of activated sludge before it was further anaerobically digested and the methane production measured. This range of concentrations used enabled us to access the effects of increasing sulfide concentration on biodegradability and methane production.

Sulphide is documented to lead to precipitation of key trace elements. In order to ensure bioavailability of trace metals being sufficient enough during the methanogenesis period, the inoculum was supplemented with trace metals in the third phase, while maintaining the activated sludge pre-treatment with sulphide in the range of 4.54 to 18.18 mg H_2S/ gVS. Also importantly, the pre-treated activated sludge was rid of any sulfide traces at pH of 7.6 by N_2 splurging to avoid any related toxicity.

CHAPTER 4

Materials and methods

4.1. Inoculum and Recirculated Activated Sludge (RAS)

4.1.1. Inoculum

The inoculum for the BMP test was harvested from the mesophilic anaerobic digester of Harnaschpolder wastewater treatment plant treating both primary and secondary sludge. Harnaschpolder wastewater treatment plant receives a mixed wastewater influent of both domestic and industrial wastewater. The treatment plant has a treatment capacity of 1.3 million population equivalents and utilizes both primary and secondary settling of sludge. The sampled inoculum was collected in 20L plastic buckets, kept at 30˚C temperature for 30 days to ensure complete conversion of all residual substrate to biogas and cellular maintenance. Buckets were momentarily opened daily to let off any accumulated biogas. After thirty days, the inoculum was then transferred to a cold storage room where it was maintained at 4˚C until required for the BMP tests. At the time of BMP testing, the inoculum was brought to a room controlled at 30˚C for 7 days and the characteristics of total solids, volatile solids, soluble COD, dissolved organic carbon and pH tested in triplicate before use for the BMP test. The same inoculum was used for all the 3 phases of the study.

4.1.2. Substrate

Since the waste activated sludge was mixed with primary sludge and magnesium hydroxide at the time of thickening in plant operations, the substrate of choice was taken from the return activated sludge (RAS) stream of the treatment plant. To achieve thickener conditions, the RAS was concentrated to approximately 57.10 gVS/L by letting to stand and settle out in a 4˚C cold room, the supernatant decanted off while the sludge further centrifuged at 4800 rpm for 10 minutes with a Rotina 380 benchtop centrifuge (Germany). The supernatant was decanted and some kept for future sludge dilution in pre-treatment. The centrifuge pellet was used as the substrate for pre-treatment after initial testing for TS and VS. Soluble baseline characteristics of this substrate were measured after filtering the supernatant through a 0.45µm pore filter.

4.2. Stock nitrite and sulfide

4.2.1. Stock nitrite

A standardized stock nitrite solution of 10.20 g/l was prepared from potassium nitrite salt. The calculation for achieving the required FNA concentration was based on *Equations 3 and 4* (Anthonisen, et al., 1976) and aliquots of nitrite were taken from this stock to the substrate for pre-treatment.

4.2.2. Stock sulfide

A fresh 0.1M S^{2-} solution was prepared by dissolving 6g of 35% sodium sulfite salt in 250ml of 0.1M sodium hydroxide solution. An aliquot of this stock was introduced to the substrate sludge at the time of pre-treatment simultaneously with an equivalent 0.5M hydrochloric acid aliquot to offset the sodium hydroxide pH to enable the speciation of sulfide at a buffered pH of 5.8.

4.3. RAS pre-treatment with sulfide and FNA

4.3.1. Phase 1: FNA pre-treatment

The pre-treatment step (enhancement of hydrolysis) was carried out in 600 mL closed bottles with a net working volume of 500mL. In order to mimic the thickening process of the sludge, RAS was centrifuged at 4800 rpm up to a concentration of 56.80 gVS/L. 193.66 mL of concentrate RAS was added to each of the 2 bottles. To the first bottle (0.04 mg HNO_2-N/gVS pre-treatment), 268.58 mL of the activated sludge supernatant was added while to the second bottle (0.06 mg HNO_2-N/gVS pre-treatment), 262.21 mL of the same was added. pH was adjusted to 5.8 by the addition of 0.5M HCl and phosphate buffer solution (PBS) to both bottles followed by the addition of 260.10 mg NO_2^--N/L and 390.15 mg NO_2^--N/L from the 10200 mg NO_2^--N/L stock solution to bottle 1 and 2 resulting into FNA concentrations of 0.04 mg HNO_2-N/gVS and 0.06 mg HNO_2-N/gVS respectively with an initial VS concentration of 22 gVS/L in each bottle. Both bottles were securely closed, placed on reciprocating shakers at 140rpm in a 25°C temperature controlled incubator for 24 hours. *Table 9* summarises this experimental procedure.

Table 9: Experimental setup of FNA pre-treatment in a batch reactor in phase 1

Pre-treatment parameter	Value	Value	Unit
VS of RAS after centrifuging	56.8	56.8	gVS/L
Final bottle TS after pre-treatment	22	22	gVS/L
Net bottle volume after pre-treatment	500	500	mL
Volume of sludge for pre-treatment	193.66	193.66	mL

Pre-treatment parameter	Value	Value	Unit
FNA pre-treatment ratio Wang, et al. (2013)	0.04	0.06	mg $HN0_2$-N/gVS
Temperature of pre-treatment	25	25	˚C
Ka	4.45E-04	4.45E-04	
pH of pre-treatment	5.8	5.8	
Required FNA concentration	0.93	1.39	mg HNO_2-N/L
SNO_2^--N	260.10	390.15	mg/L
Stock solution concentration	10200	10200	mg NO_2^--N/L
Volume of stock solution to be added	12.75	19.12	mL
Total PBS volume	25	25	mL
KH_2PO_4 to K_2HPO_4 ratio	0.91:0.08	0.91:0.08	
Volume of KH_2PO_4	22.87	22.87	mL
Volume of K_2HPO_4	2.12	2.12	mL
Volume of RAS supernatant	268.58	262.21	mL

4.3.2. Phase 1: Sulfide pre-treatment

Sulfide pre-treatment to enhance hydrolysis was carried out in two 600mL closed bottles with a net working volume of 500 mL. The WWTP thickening process of the sludge was mimicked by RAS centrifugation at 4800 rpm up to a concentration of 56.80 gVS/L. 193.66 mL of concentrate RAS was added to each of the 2 bottles. To the first bottle, 206.46mL of RAS supernatant was added while to the second bottle 181.22 mL of RAS supernatant was added. pH was adjusted to 5.8 by the addition of 0.5M HCl and phosphate buffer solution (PBS) to both bottles. To the first bottle, 62.39mL of sulfide stock at a concentration of 0.1M S^{2-} was added simultaneously with 12.47mL of 0.5M HCl resulting into a sulfide concentration of 18.15 mg H_2S/g VS with an initial VS concentration of 22 gVS/L. To the second bottle, 83.42mL of sulfide stock at a concentration of 0.1M S^{2-} was added simultaneously with 16.68mL of 0.5M HCl achieving a sulfide concentration of 24.27 mg H_2S/g VS with an initial VS concentration of 22 gVS/L. Both bottles were securely closed, placed on reciprocating shakers at 140rpm in a 25˚C temperature controlled incubator for 24 hours. *Table 10* summarises this experimental procedure.

Table 10: Experimental setup of sulfide pre-treatment in a batch reactor in phase 1

Pre-treatment parameter	0 mg H_2S/gVS	18.15 mg H_2S/gVS	24.27 mg H_2S/gVS
VS of RAS after centrifuging (g/L)	56.80	56.80	56.80
Final bottle VS at pre-treatment (g/L)	22.00	22.00	22.00

Pre-treatment parameter	0 mg H_2S/gVS	18.15 mg H_2S/gVS	24.27 mg H_2S/gVS
Net bottle volume after pre-treatment (mL)	500.00	500.00	500.00
Volume of RAS for pre-treatment (mL)	193.66	193.66	193.66
Sulfide ratio based on Rubio-Rincón, et al. (2016)	Not applicable	18.15	24.27
Sulfide concentration in 0.1M stock (mg/L)	Not applicable	3200.00	3200.00
Volume of sulphide for pre-treatment (mL)	Not applicable	62.39	83.42
Volume of 0.5M HCL for neutralization of sodium hydroxide (ml)	Not applicable	12.47	16.68
Total PBS volume (ml)	25.00	25.00	25.00
KH_2PO_4 to K_2HPO_4 ratio	0.91:0.08	0.91:0.08	0.91:0.08
Volume of KH_2PO_4 (ml)	22.87	22.87	22.87
Volume of K_2HPO_4 (ml)	2.12	2.12	2.12
Volume of RAS supernatant (mL)	281.33	206.46	181.22

4.3.3. Phase 1: Control of pre-treatment

The pre-treatment in phase 1 had a control experiment performed alongside with similar characteristics of temperature and pH but with no chemical pre-treatment as sulfide or FNA. A 600mL closed bottle with a net working volume of 500 mL was used. The thickening process of the sludge in waste water plants was achieved by RAS was centrifugation at 4800 rpm up to a concentration of 56.80 gVS/L, and 193.66 mL of concentrate RAS was added. 281.33mL of RAS supernatant was added to the bottle and pH was adjusted to 5.8 by the addition of 0.5M HCl and phosphate buffer solution (PBS) achieving a VS concentration of 22 gVS/L. The control bottle reactor was securely closed, placed on reciprocating shaker at 140rpm in a 25°C temperature controlled incubator for 24 hours. *Table 10* summarises this experimental procedure with volumes.

4.3.4. Phase 2: Sulphide pre-treatment

To study the effects of 4.54-18.18 mg H_2S/gVS pre-treatment on hydrolysis of the RAS, the experiment was carried out in four 600mL closed bottles with a net working volume of 500 mL. Thickening conditions were mimicked by RAS centrifugation at 4800 rpm up to a concentration of 50.10 gVS/L. Of this concentrated RAS, 219.56 mL was added to each of the 4 bottles. A specific volume of RAS supernatant as: 236.91mL; 217.94mL; 186.81mL; and 180.44mL was

added to bottles 1, 2, 3 and 4 respectively, and pH adjusted to 5.8 by the addition of 0.5M HCl and phosphate buffer solution (PBS). To bottle 1, 15.62 mL of 0.1M S^{2-} stock solution was added simultaneously with 3.13 mL of 0.5M HCl. To bottle 2, 31.24 mL of 0.1M S^{2-} stock solution was added simultaneously with 6.25 mL of 0.5M HCl. To bottle 3, 57.18 mL of 0.1M S^{2-} stock solution was added simultaneously with 11.44mL of 0.5M HCl. To bottle 4, 62.49mL of 0.1M S^{2-} stock solution was added simultaneously with 12.50 mL of 0.5M HCl. Sulfide concentrations of 4.54, 9.09, 16.63 and 18.18 mg H_2S/gVS were achieved in bottle 1, 2, 3, and 4 respectively with all bottles at a VS concentration of 22 gVS/L. All bottles were securely closed, placed on reciprocating shakers at 140rpm in a 25°C temperature controlled incubator for 24 hours. *Table 11* summarises this experimental procedure.

4.3.5. Phase 2: Control of pre-treatment

A control experiment with the same concentrated RAS and similar characteristics of temperature and pH but with no chemical pre-treatment as sulfide was performed in phase 2. A 600mL closed bottle with a net working volume of 500 mL was used. In order to mimic the thickening process of the sludge, RAS was centrifuged at 4800 rpm up to a concentration of 50.10 gVS/L, and 219.56 mL of concentrate RAS was added. 255.43 mL of RAS supernatant was added to the bottle and pH was adjusted to 5.8 by the addition of 0.5M HCl and phosphate buffer solution (PBS) achieving a VS concentration of 22 gVS/L. The control bottle reactor was securely closed, placed on reciprocating shaker at 140rpm in a 25°C temperature controlled incubator for 24 hours. *Table 11* summarises this experimental procedure.

Table 11: Experimental setup of sulfide pre-treatment in a batch reactor in phase 2

Pre-treatment parameter	**0.00 mg H_2S/gVS**	**4.54 mg H_2S/gVS**	**9.09 mg H_2S/gVS**	**16.63 mg H_2S/gVS**	**18.18 mg H_2S/gVS**
VS of RAS after centrifuging (g/L)	50.10	50.10	50.10	50.10	50.10
Final bottle VS at pre-treatment (g/L)	22.00	22.00	22.00	22.00	22.00
Net bottle volume after pre-treatment (mL)	500.00	500.00	500.00	500.00	500.00
Volume of RAS for pre-treatment (mL)	219.56	219.56	219.56	219.56	219.56
Sulfide ratio based on Rubio-Rincón, et al. (2016)	Not applicable	4.54	9.09	16.63	18.18
Sulfide concentration in 0.1M stock (mg/L)	Not applicable	3200.00	3200.00	3200.00	3200.00
Volume of sulphide for pre-treatment (mL)	Not applicable	15.62	31.24	57.18	62.49
Volume of 0.5M HCL for neutralization of sodium hydroxide (ml)	Not applicable	3.12	6.24	11.43	12.49
Total PBS volume (mL)	25.00	25.00	25.00	25.00	25.00
KH_2PO4 to K_2HPO_4 ratio	0.91:0.08	0.91:0.08	0.91:0.08	0.91:0.08	0.91:0.08
Volume of KH_2PO_4 (mL)	22.87	22.87	22.87	22.87	22.87
Volume of K_2HPO_4 (mL)	2.12	2.12	2.12	2.12	2.12
Volume of RAS supernatant (mL)	255.43	236.91	217.94	186.81	180.44

4.3.6. Phase 3: Sulphide pre-treatment

The phase 3 experiments were carried out to assess the effect of 4.54-18.18 mg H_2S/gVS pre-treatment on sludge hydrolysis. However, this experiment differs from phase 2 by the stripping of any trace sulphide from sludge before anaerobic batch BMP at pH 7.6 which was presumed not of any effect on the VFA. In addition, phase 3 ensured that sulfide precipitation of vital trace elements necessary for anaerobic digestion did not occur by the supplementation of inoculum with trace metals. The pre-treatment step with was performed in four 600mL closed bottles with a net working volume of 500 mL. RAS thickening was achieved by centrifuging at 4800 rpm up to a concentration of 64.40 gVS/L. Of this RAS concentrate, 170.80 mL was dispensed to each of the 4 bottles. Specific RAS supernatant volumes as 285.44mL, 266.69 mL, 235.56 mL, and 229.19 mL were aliquoted to bottles 1, 2, 3 and 4 respectively, and pH was adjusted to 5.8 by the addition of 0.5M HCl and phosphate buffer solution (PBS). To bottle

1, 15.62 mL of 0.1M S^{2-} stock solution was added simultaneously with 3.13mL of 0.5M HCl. To bottle 2, 31.24mL of 0.1M S^{2-} stock solution was added simultaneously with 6.25 mL of 0.5M HCl. To bottle 3, 57.18 mL of 0.1M S^{2-} stock solution was added simultaneously with 11.44mL of 0.5M HCl. To bottle 4, 62.49mL of 0.1M S^{2-} stock solution was added simultaneously with 12.50 mL of 0.5M HCl. Sulfide concentrations of 4.54, 9.09, 16.63 and 18.18 mg H2S/gVS were achieved in bottle 1, 2, 3, and 4 respectively with all bottles at a VS concentration of 22 gVS/L. All bottles were securely closed, placed on reciprocating shakers at 140rpm in a 25°C temperature controlled incubator for 24 hours. *Table 12* summarises this experimental procedure. After the 24h pre-treatment step, samples for the pre-treatment outcome were taken off while the sludge for the batch BMP was brought to pH 7.6 and N_2 gas splurged in the sludge and bottle head space to get rid of the remnant sulfide.

4.3.7. Phase 3: Control of experiment

The pre-treatment in phase 3 had a control experiment performed alongside with similar characteristics of temperature and pH but with no chemical pre-treatment as sulfide. A 600mL closed bottle with a net working volume of 500 mL was used. The previously thickened RAS (170.80 mL), at a concentration of 64.40 gVS/L was dispensed into the bottle. A 304.19 mL aliquot of RAS supernatant was added to the bottle and pH adjusted to 5.8 by the addition of 0.5M HCl and phosphate buffer solution (PBS) to achieve a VS concentration of 22 gVS/L. The control bottle reactor was securely closed, placed on a reciprocating shaker at 140rpm in a 25°C temperature controlled incubator for 24 hours. A similar N2 splurging procedure at pH 7.6 was done to the control despite no sulfide addition to ensure comparability. *Table 12* summarises this experimental procedure.

For all pre-treatment in phase 1-3, both a calculated and measured VS were used. The calculated VS was fixed at 22g/l to enable us calculate the amount of RAS to add to the 500ml reactor bottle. *Equation 5* was used to calculate for the fixed RAS volumes in the different experimental phases.

Equation 5: Calculation of volume of centrifuged RAS for pre-treatment

$$Volume\ of\ RAS = \frac{Final\ botle\ VS * Net\ Volume\ of\ botle\ after\ pre-treatment}{VS\ after\ Centrifugation\ Thickening}$$

Thus:

$$Volume\ of\ RAS = \frac{22\ (\frac{gVS}{l}) * 500\ (ml)}{VS\ after\ centrifugation\ thickening\ (g/l)}$$

After obtaining the volume of RAS to add to the five reactor bottles of each pre-treatment phase, and since all other chemical pre-treatment volumes were known, the remaining volume was topped up to 500ml by addition of the RAS supernatant earlier obtained in centrifugation

thickening. After mixing the sludge and pre-treatment chemicals, the actual TS and VS measurement were done according to standard methods to confirm the calculated VS.

Table 12: Experimental setup of sulfide pre-treatment in a batch reactor in phase 3

Pre-treatment parameter	**0.00 mg H_2S/gVS**	**4.54 mg H_2S/gVS**	**9.09 mg H_2S/gVS**	**16.63 mg H_2S/gVS**	**18.18 mg H_2S/gVS**
VS of RAS after centrifuging (g/L)	64.40	64.40	64.40	64.40	64.40
Final bottle VS at pre-treatment (g/L)	22.00	22.00	22.00	22.00	22.00
Net bottle volume after pre-treatment (mL)	500.00	500.00	500.00	500.00	500.00
Volume of RAS for pre-treatment (mL)	170.80	170.80	170.80	170.80	170.80
Sulfide ratio based on Rubio-Rincón, et al. (2016)	Not applicable	4.545	9.09	16.63	18.18
Sulfide concentration in 0.1M stock (mg/L)	Not applicable	3200	3200.00	3200.00	3200.00
Volume of sulphide for pre-treatment (mL)	Not applicable	15.62	31.24	57.18	62.49
Volume of 0.5M HCL for neutralization of sodium hydroxide (mL)	Not applicable	3.12	6.24	11.43	12.49
Total PBS volume (mL)	25.00	25.00	25.00	25.00	25.00
KH_2PO_4 to K_2HPO_4 ratio	0.91:0.08	0.91:0.08	0.91:0.08	0.91:0.08	0.91:0.08
Volume of KH_2PO_4 (mL)	22.87	22.87	22.87	22.87	22.87
Volume of K_2HPO_4 (mL)	2.12	2.12	2.12	2.12	2.12
Volume of RAS supernatant (mL)	304.19	285.44	266.69	235.56	229.19

4.4. Biochemical methane potential tests

4.4.1. Phase 1 BMP

Fifteen glass bottle AMPTS reactors cleaned and dried were used for the test performance in triplicate for the control and the four sulphide pre-treated RAS samples. 300 mL of the inoculum at a VS concentration of 16.6 ± 0.4 gVS/L and pH 7.2 were added to each of the 15 bottles. 100 mL of the substrate after pre-treatment were added to the bottle reactors containing the inoculum and for each pre-treatment concentration including the control, this was done in triplicate. By dropwise addition of 0.5M HCl, the inoculum–substrate mixture was adjusted to

pH 7.2. Bottles were then closed with exception of the gas inlet and outlet. Homogeneous mixing was done and the head space of the bottle reactors was purged with N_2 gas for 2 minutes through the gas inlet while the gas outlet was connected to a gas-trap. After no more bubbling observed in the gas-trap after N_2 splurging, both the inlet and outlet were plugged to maintain the anaerobic conditions created and reactor bottles placed in a 30˚C water bath. The gas outlet was momentarily opened and connected to a gas scrubbing solution of 12% sodium hydroxide solution with a colour indicator for detecting scrubber solution saturation with CO_2 and H_2S. Continuous mixing was started and the biogas over the digestion period was logged until there was no significant increase (not more than 1% increase for 3 consecutive days). The data collected in triplicate was corrected for outliers by a Dixon statistical test as recommended by Holliger, et al. (2016) and average accumulation used for reporting average collection. *Table 13* shows the experimental set-up outline of the BMP in phase 1.

Table 13: Experimental setup of BMP phase 1

Test	Volume of anaerobic Inoculum (ml)	Volume of RAS pre-treated (ml)	Start pH	Temperature (˚C)	Continuous Mixing regime (%)
Control	300.00	100.00	7.20	30.00	80.00
0.04 mg HNO_2-N/gVS	300.00	100.00	7.20	30.00	80.00
0.06 mg HNO_2-N/gVS	300.00	100.00	7.20	30.00	80.00
18.15 mg H_2S/gVS	300.00	100.00	7.20	30.00	80.00
24.27 mg H_2S/gVS	300.00	100.00	7.20	30.00	80.00

4.4.2. Phase 2 BMP

The BMP was performed in fifteen clean and dry glass bottle AMPTS reactors. A 290 mL aliquot of the inoculum at a VS concentration of 16.6 ± 0.4 gVS/L and pH 7.2 was dispensed to each of the 15 bottles. A fixed 90 mL of each of the substrate including the control after pre-treatment was dispensed in triplicate to the bottle reactors containing the inoculum. The VS characteristics of the substrate with standard deviation were 19.66± 0.25, 19.66± 0.90, 20.26± 0.66, 21.6± 1.02 and 19.2± 0.1 gVS/L for the control, 4.54 mg H_2S/gVS, 9.09 mg H_2S/gVS, 16.63 mg H_2S/gVS and 18.18mg H_2S/gVS pre-treated substrate all at pH 5.8. To ensure pH stability, 20 mL of phosphate buffer solution at pH 7.6 were added and homogeneously mixed and by dropwise addition of 0.5M HCl, the inoculum–substrate mixture was adjusted to pH 7.6. Bottles were then closed with exception of the gas inlet and outlet. The head space of the bottle reactors was purged with N_2 gas for 2 minutes through the gas inlet while the gas outlet was connected to a gas-trap. Both the gas inlet and outlet were plugged to maintain the anaerobic conditions created after no more observed bubbling in the gas-trap and reactor bottles placed in

a 35˚C water bath. The gas outlet was momentarily opened and connected to a gas scrubbing solution of 12% sodium hydroxide solution with a colour indicator for detecting scrubber solution saturation with CO_2 and H_2S. Continuous mixing was started and the methane accumulation over the digestion period was logged until there was no significant increase (not more than 1% increase for 3 consecutive days). The data collected in triplicate was corrected for outliers by a Dixon statistical test as recommended by Holliger, et al. (2016) and average accumulation used for reporting average collection. *Table 14* shows the experimental set-up outline of the BMP in phase 2.

Table 14 : Experimental setup of BMP phase 2

Test	Volume of anaerobic Inoculum (mL)	Volume of RAS pre-treated (mL)	Volume of pH 7.6 buffer	Start pH	Temperature (˚C)	Continuous Mixing regime (%)
0 mg H_2S/gVS (Control)	290.00	90.00	20.00	7.60	35.00	80.00
4.54 mg H_2S/gVS	290.00	90.00	20.00	7.60	35.00	80.00
9.09 mg H_2S/gVS	290.00	90.00	20.00	7.60	35.00	80.00
16.63 mg H_2S/gVS	290.00	90.00	20.00	7.60	35.00	80.00
18.18mg H_2S/gVS	290.00	90.00	20.00	7.60	35.00	80.00

4.4.3. BMP phase 3

The phase 3 BMP was performed after stripping of any remnant sulfide both in the pre-treated sludge medium or head space of the AMPTS reactors. The experiment was done in triplicate for the five sludge samples as control, 4.54 mg H_2S/gVS, 9.09 mg H_2S/gVS, 16.63 mg H_2S/gVS and 18.18mg H_2S/gVS. Substrate aliquots of 90 mL in triplicate were dispensed into AMPTS bottle reactors. The VS characteristics of the substrate with standard deviation were 19.33± 0.25, 20.66± 1.15, 22± 0, 20± 0 and 20± 0 gVS/L for the control, 4.54 mg H_2S/gVS, 9.09 mg H_2S/gVS, 16.63 mg H_2S/gVS and 18.18mg H_2S/gVS pre-treated substrates at pH 5.8. Fixed aliquots of 20 mL phosphate buffer solution at pH 7.6 were added and homogeneously mixed followed by 20 mL of the trace element solution to each of the fifteen bottles and uniformly mixed. The pH of each bottle was tested and found to be 6.5±0.2 and 290 mL of the inoculum at a VS concentration of 16.6 ± 0.4 gVS/L and pH 7.2 were added to each of the AMPTS reactor

bottles. The inoculum-substrate mixture was mixed homogeneously with a pH probe and thereafter tested for pH which was averagely at 7.2±0.2 for all bottles and adjusted to 7.6 by dropwise addition of 0.5M HCl. To test for BMP start conditions, 20 mL of the above mixture was taken off each bottle to measure BMP beginning conditions and bottles were then closed with exception of the gas inlet and outlet. The head space of the bottle reactors was purged with N_2 gas for 2 minutes through the gas inlet while the gas outlet was connected to a gas-trap to ensure complete expulsion of all previously present gases. After no more gas exiting the gas-trap as bubbles, both the inlet and outlet were plugged to maintain the anaerobic conditions created and reactor bottles placed in a 35˚C water bath. The gas outlet was momentarily opened and connected to a gas scrubbing solution of 12% sodium hydroxide solution with a colour indicator for detecting scrubber solution saturation with CO_2 and H_2S. Continuous mixing was started and the methane accumulation over the digestion period was logged until there was no significant increase (not more than 1% increase for 3 consecutive days). The data collected in triplicate was corrected for outliers by a Dixon statistical test as recommended by Holliger, et al. (2016) and average accumulation used for reporting average collection. *Table 15* shows the experimental set-up outline of the BMP in phase 3 while *Table 16* shows the trace metal composition based on (Moody, et al., 2009).

Table 15: Experimental setup of BMP phase 3

Test	Volume of anaerobic Inoculum (mL)	Volume of RAS pre-treated (mL)	Volume of trace element solution (mL)	Volume of pH 7.6 buffer	Start pH	Temperature (˚C)	Continuous Mixing regime (%)
0 mg H_2S/gVS (Control)	290.00	90.00	20.00	20.00	7.60	35.00	80.00
4.54 mg H_2S/gVS	290.00	90.00	20.00	20.00	7.60	35.00	80.00
9.09 mg H2S/gVS	290.00	90.00	20.00	20.00	7.60	35.00	80.00
16.63 mg H2S/gVS	290.00	90.00	20.00	20.00	7.60	35.00	80.00
18.18mg H2S/gVS	290.00	90.00	20.00	20.00	7.60	35.00	80.00

Table 16: Trace element supplementation solution composition

Trace element	Concentration (g/L)
Boric acid	0.05
Manganous chloride	0.50
Zinc chloride	0.05

Trace element	Concentration (g/L)
Ammonium molybdate	0.05
Cobalt chloride	2.00
EDTA	1.00
Calcium chloride	0.038
Nickelous chloride	0.142
Sodium selenite	0.123
Iron (11) chloride	10.00

4.5. Measurement of physical and chemical properties

4.5.1. Soluble fraction of ammonium, COD, nitrite and sulfide

In this thesis, the soluble fraction of any parameter measurement refers to a sample prepared by centrifugation of 200mL of the sample followed by filtration of the supernatant with a 0.45µm pore acetate filter. This filter was used to eliminate solids and colloids which do not form part of the readily biodegradable components and also to remove particles that would interfere with spectrophotometric measurements. The filter was first rinsed with 10 mL of demineralised water and the first 10mL of filtered sample were discarded. Analysis of these parameters was done according to APHA 1998. Method calibration for ammonium, COD, nitrite and sulfide is shown in the *Appendix A, B, D and E* respectively.

4.5.2. Total dissolved organic carbon

Total dissolved organic carbon of 0.45µm filtered Samples was measured by using Schimadzu TOC –L series analyser (Japan).

4.5.3. Fluorescence excitation and emission matrix (FEEM)

The FEEM spectra was measured by Horiba Jobin Yvon flouromax-3 spectrophotometer (USA) to analyse the different types of organic matter in the wastewater samples before pre-treatment and after pre-treatment. Comparability of these measurements was obtained by diluting all samples to 1 mg/l of the earlier measured dissolved organic carbon using milliQ water.
The quality control of the measurement of the FEEM spectra was obtained by verification of the performance of the 150 watt ozone-free xenon arc lamp first, followed by correction of the Raman peak.

The 3D analysis range of wavelength used was: Excitation 240-450nm (in 10nm increment intervals) and emission 290-500nm (in 2nm increment intervals). The test samples EEMs were corrected to remove Raman scatter peaks of MilliQ were used as a blank.

To categorize the EEM contours as dissolved organic fractions of fulvic like, humic like and protein materials, the EEM contours were plotted using *Matlab* software with a code specific

for the analysis. Images of the contour plots were analysed based on (Leenheer and Croué, 2003) as shown in *Table 17* below.

Table 17: Fluorescence emission spectra of major dissolved organics in wastewater

Component	Excitation range (nm)	Emission range (nm)
Fulvic/ Humic	330-350	420-480
Humic like	250-320	380-480
Protein like	270-280	300-350

Table with excerpt from (Leenheer and Croué, 2003)

4.5.4. Volatile fatty acids measurement

Volatile fatty acids as acetate, propionic acid, butyric acid, isobutyric acid and valeric acid were measured with a Varian 430GC (USA) equipped with a splitter injection (200˚C), a WCOT fused silica column (105˚C) and coupled to a FID detector (300˚C). VFA was measured under standardized conditions in an acidic environment of about 2% formic acid with a 25 meter WAX 58 FFAP CB column and FID detector within 5 minutes. Isovaleric acid at a concentration of 50 mg/L was used as an internal standard based on its being negligibly produced after pre-treatment. Detection of VFA is based on peaks developed at specific times with acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid at elution times of 1.5, 1.9, 2.1, 2.8, 3.3 and 4.5 minutes respectively. Quality control of measurements was based on instrument calibration with standards of the volatile fatty acids to be analysed and interpretation of results based on the same calibration line for each set of measurement done (*Appendix C*).

4.5.5. Gas content measurement

The gas composition of the biogas sampled from the head space of the BMP reactors was measured by gas chromatography using a Scion 456-GC (UK) chromatography equipment that is equipped with a TCD detector and uses helium gas as a carrier. Detection of gases is based on the peaks formed at different elution times with N_2 at 0.8 minutes, CH_4 at 0.9 minutes and CO_2 at 1.1 minutes. Quantification of gases was based on calibration by standard gases introduced into the gas chromatography machine and calibration lines generated. These are shown in *Appendix* F.

CHAPTER 5

Results and discussion

5.1. Phase 1

5.1.1. Phase 1 ammonium results

The baseline ammonium concentration was measured in the supernatant fluid of the RAS and assessment of the changes in the batch pre-treatment of the control, and RAS samples pre-treated with 18.15 mg H_2S/gVS, 24.27 mg H_2S/gVS, 0.04 mg HNO_2-N/gVS, and 0.06 mg HNO_2-N/gVS after 24h and pH 5.8 were done. The change in ammonium indicator is an indicator of organic matter mineralization of the RAS and protein hydrolysis according to Luo, et al. (2015). *Appendix A-1* outlines the concentration of ammonium measured at the end of pre-treatment. The baseline ammonium concentration in the un-pre-treated RAS was 3.48 mg NH_4^+-N/L. According to Luo et al., (2015), during the anaerobic digestion process ammonia can be correlated with protein hydrolysis. There was no considerable difference regarding the change in the ammonia concentration for the sulphide pre-treated samples with sulphide with respect to the control test (6.7±0.7 mg NH_4-N/gVS). Interestingly, a lower ammonia concentration of 3.4±0.2 mg NH_4-N/gVS was observed in the pre-treated samples with FNA. This, suggests that the protein hydrolysis was not enhanced by the addition of sulphide but probably inhibited by the addition of FNA (*Figure 4*). According to Pijuan, et al. (2012) the addition of FNA could have inhibited the protease enzyme needed for the hydrolysis process, resulting in a lower ammonia concentration as it was observed. The studies performed by Wang, et al. (2013) showed a decreasing trend in ammonium although increase in the concentration of soluble proteins was reported. Though, this is still unclear regarding the discrepancy of both results (ammonium and proteins), a low ammonia concentration would indicate that the degree of biodegradability in the subsequent anaerobic digestion is reduced.

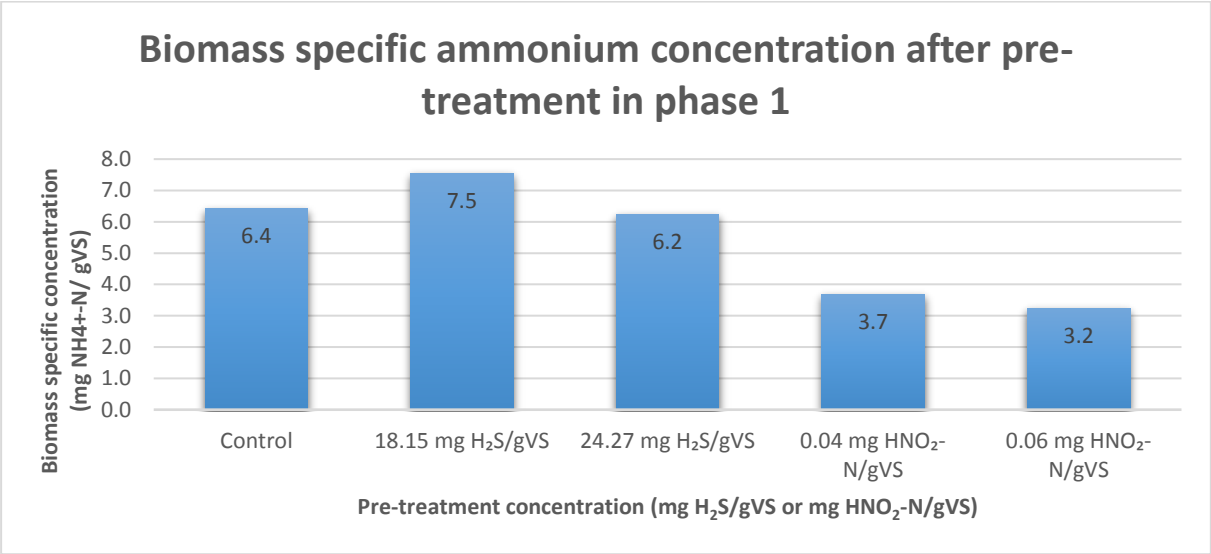


Figure 4: Trend of biomass specific ammonium concentration after pre-treatment phase 1.

5.1.2. Phase 1 FEEM analysis

To confirm the findings of ammonium test as an indicator of protein hydrolysis, FEEM analysis was done for the phase 1 pre-treatment with all samples diluted first to 1 mg/L of total organic carbon.

Figure 5 shows the FEEM result of ultra-pure water with no visible fluorescence for the protein-like fluorescence (Ex 270-280nm; Em 300-350nm), Humic like fluorescence (Ex 250-320nm; Em 380-480nm) or Fulvic-like fluorescence (Ex 330-350nm; Em 420-480nm). This serves as a reference point for no observable fluorescent organic matter and also as a quality check of the experiment. All tested samples were at the same concentration of dissolved organic matter with the ultra-pure water sample and images are presented on the same fluorescence scale. The images interpretation for all samples is based on (Ahmad and Reynolds, 1995, Lai, et al., 2011, Leenheer and Croué, 2003) with the reference Ex and Em wavelengths described above.

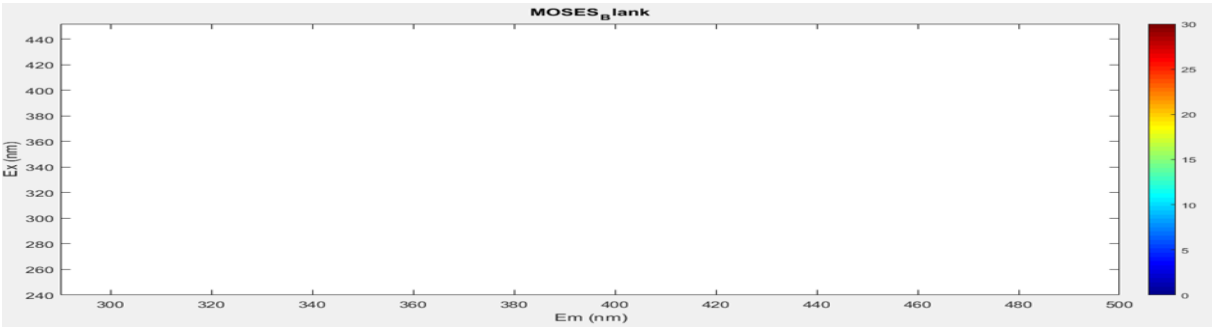


Figure 5: FEEM results of a blank sample (MilliQ)

The protein like fluorescence (PLF) peak is significantly visible for the control implying protein hydrolysis took place in the absence of any chemical pre-treatment (*Figure 6*). Also this confirms the presence of amino acids that can be acidogenically converted to VFA and H_2 by

anaerobic oxidation in typical Stickland reactions (van Lier, et al., 2008). Also the absence of humic like and fulvic substances rules out their inhibiting effect on enzymatic anaerobic hydrolysis (Fernandes, et al., 2015).

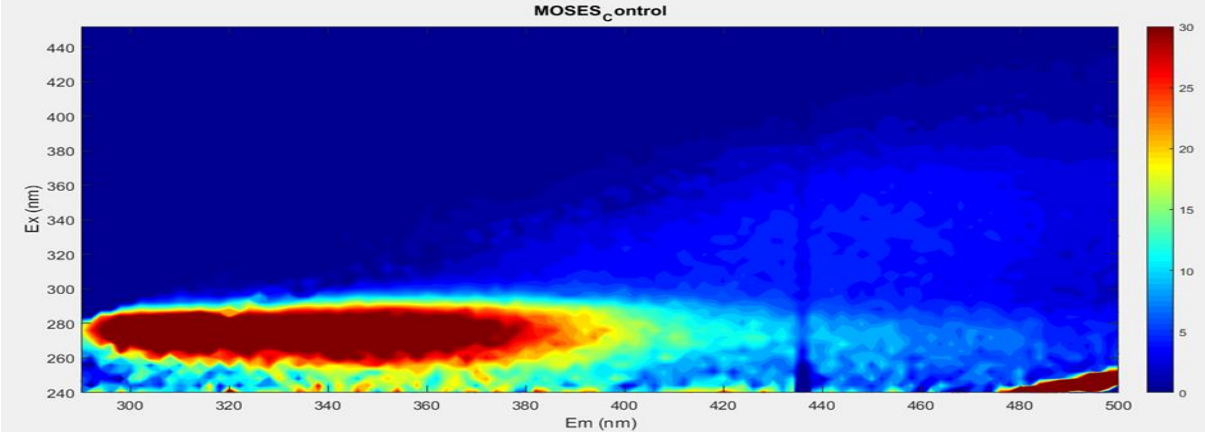


Figure 6: FEEM results of the control in phase 1

The PLF is also clearly visible for the 15.15 mg H_2S/gVS pre-treated activated sludge (*Figure 7*) implying that no protein hydrolysis inhibition occurred. However, since the test is qualitatively indicative it cannot be used to compare with the control in terms of protein concentration. The presence of soluble proteins is indicative of readily biodegradable matter for anaerobic acidogenic conversion to VFA and H_2 (van Lier, et al., 2008). The pre-treatment according to Fernandes, et al. (2015), does not introduce fulvic and humic like acids that would be inhibitory to enzymatic anaerobic hydrolysis since their fluorescence is not detected.

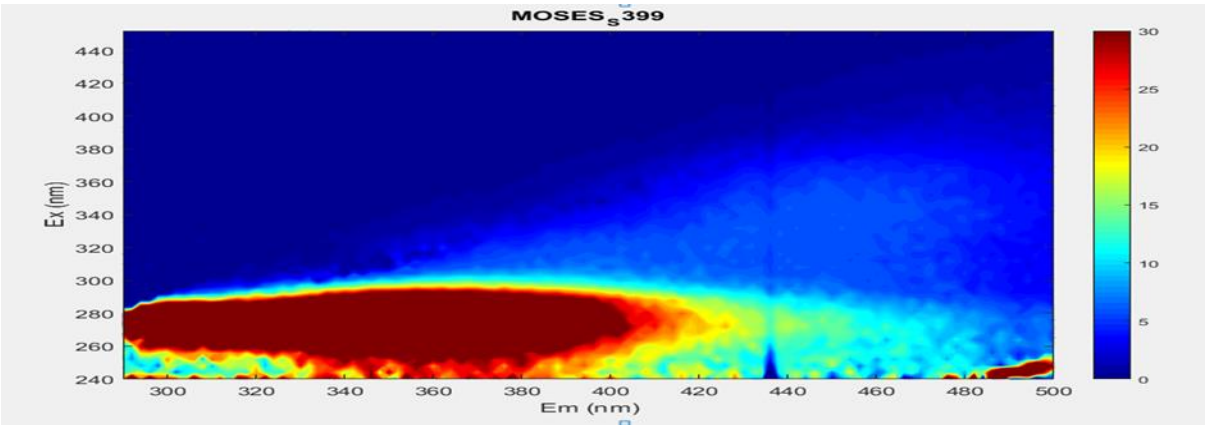


Figure 7: FEEM results of the 15.15mg H_2S/gVS pre-treated activated sludge in phase 1

Similarly, the 24.27 mg H_2S/gVS pre-treatment FEEM analysis (*Figure 8*) shows a clear band of protein fluorescence with no fulvic and humic like acids detected. Thus protein hydrolysis inhibition did not occur but the test is limited to being indicative thus we cannot access the hydrolysis enhancement.

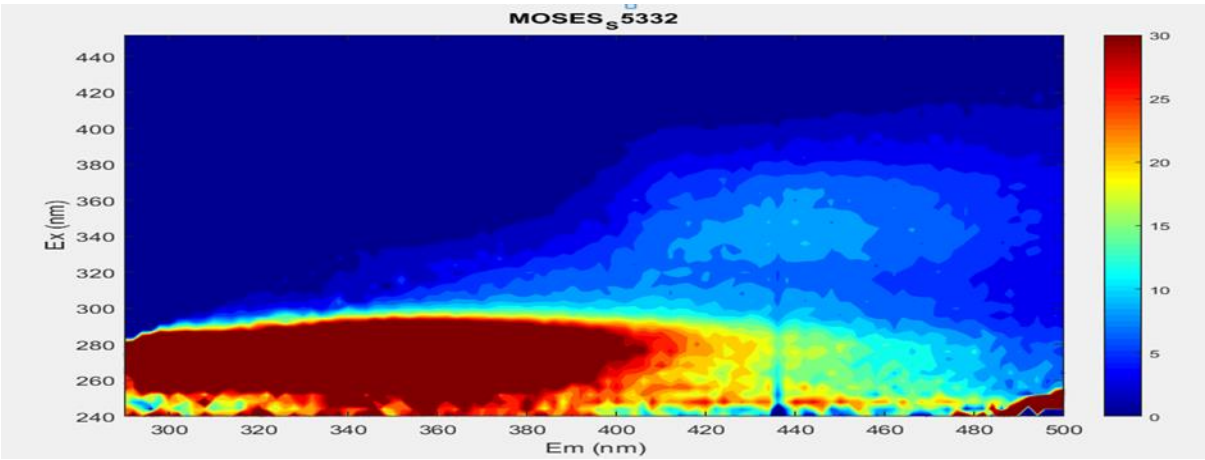


Figure 8: FEEM results of 24.27 mg H_2S pre-treated activated sludge in phase 1

Both the 0.04 mg HNO_2/gVS and 0.06 mg HNO_2/gVS pre-treatment had no protein fluorescence observed in the FEEM analysis (*Figure 9 and 10*) indicating inhibition of protein hydrolysis. According to Pijuan, et al. (2012), FNA could potentially inhibit protease enzyme and other hydrolytic enzymes. These observations are in contrast with the observations of Wang, et al. (2013) where a similar FNA concentrations (on mg HNO_2/gVS) achieved enhanced proteinolysis compared to the control.

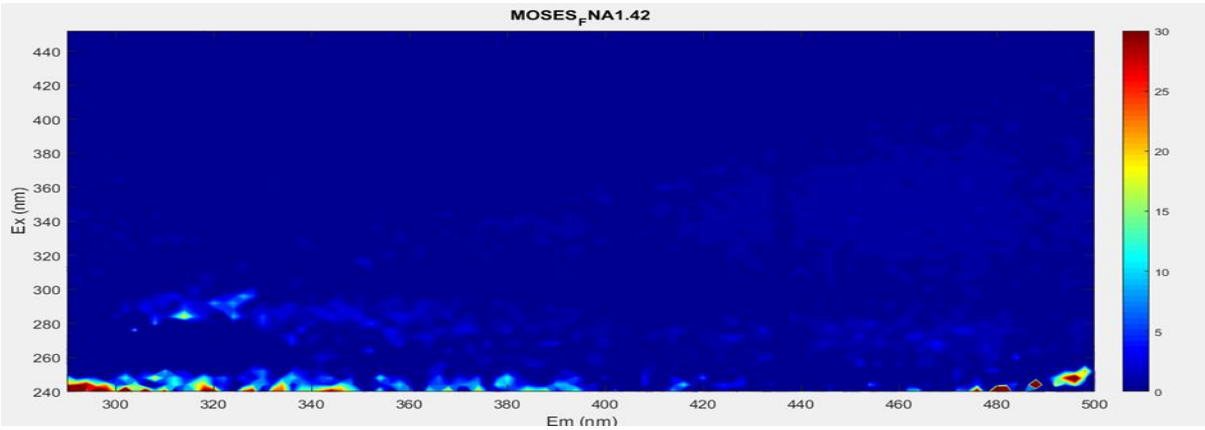


Figure 9: FEEM results of 0.04 mg HNO_2-N /gVS pre-treated activated sludge in phase 1

The FEEM analysis further corroborates the ammonia results which were 50% and 42% lower in comparison to the control for 0.06 mg HNO_2-N/ gVS and 0.04 mg HNO_2-N /gVS pre-treated activated sludge.

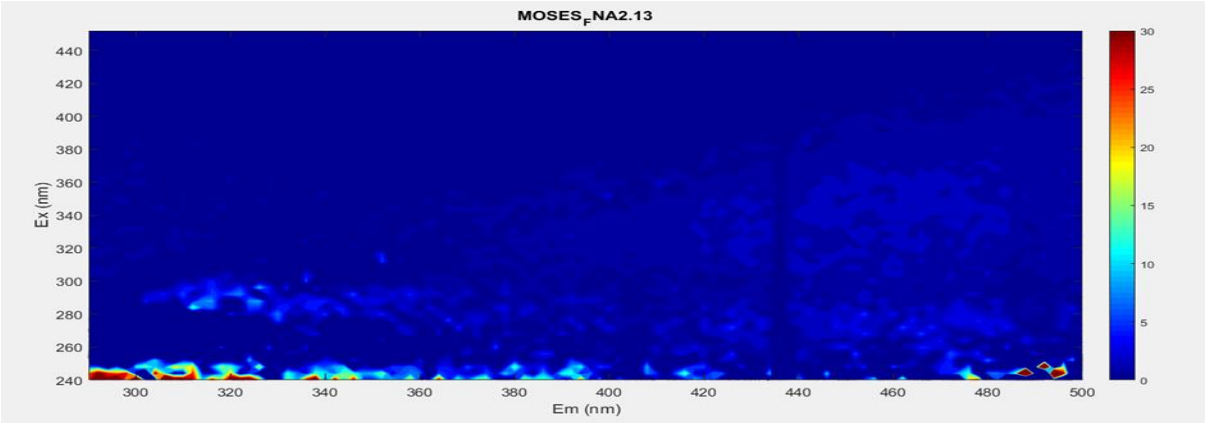


Figure 10: FEEM results of 0.06 mg HNO_2-N /gVS pre-treated activated sludge in phase 1

5.1.3. Phase 1 SCOD results

The baseline soluble COD concentration was measured in the supernatant fluid of the RAS and assessment of the changes in the batch pre-treatment of the control, and RAS samples pre-treated with 18.15 mg H_2S/gVS, 24.27 mg H_2S/ gVS, 0.04 mg HNO_2-N/gVS, and 0.06 mg HNO_2-N/gVS after 24h and pH 5.8 were done. The phase 1 RAS supernatant SCOD was 127 mg/l while chemical pretreatment with sulfide and FNA concentrations achieved the following results (*Table 18*) after pre-treatment.

Table 18: SCOD results after pre-treatment in phase 1

Sample	Actual Concentration (mg/l)	SCOD (mg/gVS)	Percentage decline in SCOD compared to control (%)
Control	2625.0	119.32	Not applicable
0.04 mg HNO_2-N/gVS	1235.0	56.14	53.0
0.06 mg HNO_2-N/gVS	1820.0	82.73	31.0
18.15 mg H_2S/gVS	2340.0	106.36	11.0
24.27 mg H_2S/gVS	1635.0	74.32	38.0

The result indicates a significant reduction in hydrolysis for all pre-treatment concentrations showing a 53%, 38%, 31% and 11% reduction in SCOD for pre-treatment of activated sludge with 0.04 mg HNO_2-N/gVS, 24.27 mg H_2S/gVS, 0.06 mg HNO_2-N/gVS and 18.15 mg H_2S/gVS respectively in comparison to the Control. Concerning FNA pre-treatment, our results

differ with Wang, et al. (2013) where a significant increase in COD solubilization was observed. Nitrite at such a concentration could probably be toxic or inhibitory to enzymatic pathways of hydrolysis. With regards to sulfide, Grady Jr, et al. (2011) described sulfide to affect activated sludge physiology inclusive of hydrolysis at concentrations exceeding 100 mg H_2S/L. In comparison with the concentrations used in this study, they highly exceed this threshold and thus inhibition of hydrolysis would possibly occur.

5.1.4. Phase 1 Total organic carbon

The analysis of total dissolved organic carbon realized the following outcome after pre-treatment with FNA and sulphide alongside the control as shown in *Table 19.*

Table 19: Total dissolved organic carbon results phase 1

Sample	NPOC TOC (mg/L)	Increase in TOC compared to control (%)
Control	424.50	Not applicable
0.04 mg HNO_2-N/gVS	1223.40	188.0
0.06 mg HNO_2-N/gVS	1494.90	252.0
18.15 mg H_2S/gVS	588.30	39.0
24.27 mg H_2S/gVS	742.65	75.0
RAS supernatant unpre-treated	71.21	Not applicable

A significant increase in total dissolved organic carbon compared to the control was observed as 252%, 188%, 75%, and 39% for 0.06 mg HNO_2-N/gVS, 0.04 mg HNO_2-N/gVS, 24.27 mg H_2S/gVS and 18.15 mg H_2S/gVS pre-treatment respectively.

According to van Lier, et al. (2008) this test is certainly not a direct indicator of the amount of biodegradable organic matter present in solution for transformation into methane or use in cell synthesis. However, this is indicative of the stress exerted on activated sludge flocs and the dissolved organic carbon is expected to increase upon lysis of cellular walls as observed by Nielsen and Keiding (1998) where bacterial organic colloids and soluble EPS is released from the cells. Nielsen and Keiding (1998) reported a 10% increase in dissolved organic matter for sulphide exposure to activated sludge, however our method of pre-treatment was different and greater increments of organic carbon at 39% and 75% were observed for 18.15 mg H_2S/gVS and 24.27 mg H_2S/gVS pre-treated RAS respectively. Also comparing these TOC results to the soluble COD results obtained, we note that there was a decrease in COD solubilization yet the TOC is increasing significantly. van Lier, et al. (2008) asserted that the most reliable information concerning the organic matter concentration can be obtained from the COD test not TOC. Therefore, TOC measurements in determining the degree of hydrolysis after pre-treatment are less useful.

5.1.5. Phase 1 VFA results

Post pre-treatment in phase 1, the volatile fatty acids generated were measured by gas chromatography and the results are shown in *Table 20* while *Figure 11* shows the graphical trend of biomass specific VFA.

Table 20: VFA results after sulfide and FNA pre-treatment of activated sludge phase1

VFA	Control	0.04mg HNO_2-N/gVS	0.06 mg HNO_2-N/gVS	18.15 mg H_2S/gVS	24.27 mg H_2S/gVS
Acetic acid (mg/L)	430.40	95.18	119.05	535.34	376.06
Propionic acid (mg/L)	236.87	36.71	51.63	317.54	189.37
Isobutyric acid (mg/L)	218.71	6.16	7.95	37.33	19.30
Butyric acid (mg/L)	129.52	15.31	22.46	95.74	67.78
Valeric acid (mg/L)	61.13	6.04	9.63	69.06	48.78
Total (mg/L)	1076.63	159.40	210.72	1055.01	701.30

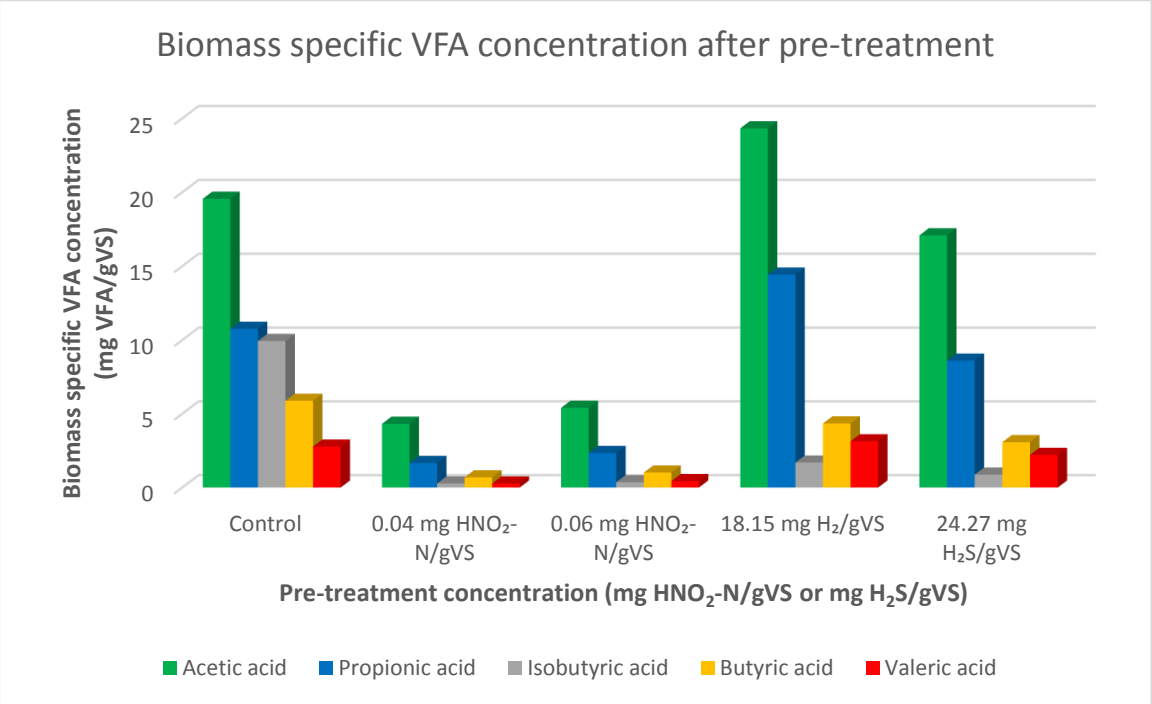


Figure 11: Biomass specific VFA concentration after pre-treatment in phase 1

Generally, pre-treatment in this phase did not enhance VFA formation. The control achieved the highest acidogenesis while a decline of 85%, 80%, 35% and 2% as total VFA was observed for 0.04 mg HNO_2-N/gVS, 0.06 mg HNO_2-N/gVS, 24.27 mg H_2S/gVS and 18.15 mg H_2S/gVS activated sludge pre-treatment. However, 18.15 mg H_2S/gVS pre-treatment had 24% and 34%

greater acetic acid and propionic acid formation respectively in comparison to the control despite the 2% less on the total VFA. This is a substantial increase in the major VFA required for methanogenesis. Concerning FNA pre-treatment, Wang, et al. (2013) observed the same effect with increasing sulfide concentrations. This decline in VFA can be attributed to the inhibitory or toxicity of FNA to sludge hydrolytic enzymes or enzymes in the acidogenic pathway (Pijuan, et al., 2012). The decline in acidogenesis with 24.27 mg H_2S/gVS pre-treatment is probably also an effect of inhibition on sludge hydrolytic and acidogenic enzymes since concentrations of sulphide above 100 mg H_2S/L have previously been described to be inhibitory to enzymatic functions in activated sludge (Grady Jr, et al., 2011).

5.1.6. Residual nitrite and sulfide after pre-treatment phase 1

The residual nitrite after pre-treatment with free nitrous acid was measured and compared to the applied concentration. *Table 21* shows the measured concentration and the calculated percentage change.

Table 21: Change in nitrite concentration between start and end of pre-treatment phase 1

Sample	Concentration of nitrite applied (mg NO_2^--N/L)	Concentration of nitrite after pre-treatment (mg NO_2^--N/L)	Percentage nitrite removal (%)
Control	0	7.76	Not applicable
0.04 mg HNO_2-N/gVS	260.10	224.57	13.60
0.06 mg HNO_2-N/gVS	390.15	367.85	5.70

The low efficiency of nitrite removal shows inhibition of denitrification in activated sludge. According to Schulthess, et al. (1995), saturation of activated sludge with nitrite results into the accumulation of nitric oxide which inhibits nitrite reductase enzyme. Baumann, et al. (1997), further clarifies that in the presence of FNA, the nitrite reductase concentration is up to 15 fold less in activated sludge, an inhibition due to inactivation or conformational changes of the enzyme induced by FNA.

The activated sludge pre-treated with free nitrous acid was consequently diluted four times with anaerobic digested sludge as inoculum for the BMP batch anaerobic digestion. This would imply approximately 56.00 mg NO_2^--N/L and 92.00 mg NO_2^--N/L was transferred to the anaerobic digestion for 0.04 mg HNO_2-N/gVS and 0.06 mg HNO_2-N/gVS pre-treatment respectively. Li, et al. (2016) asserted that the change of anaerobic conditions to anoxic conditions is inhibitory to methanogens and creates a lag phase of recovery. Banihani, et al. (2009) reported the 50% inhibitory concentration of nitrite to aceticlastic and hydrogenotrophic

methanogens as 0.83 and 0.38 mg NO_2^--N/L with the final methane yield being inversely proportional to the added nitrite. Klüber and Conrad (1998), asserted that the inhibition of nitrite on methanogenesis both irreversibly and reversibly depended on both the type of methanogens and the nitrite concentration. For example, *Methanosarcina bakeri* activity was reduced to 50% by 0.7 mg NO_2^--N/L while the same effect was observed in *Methanobacterium bryantii* at a nitrite concentration of 14 mg NO_2^--N/L, with 80% inhibition of methane production of mixed methanogenic cultures at 50 mg NO_2^--N/L (Klüber and Conrad, 1998). However, O'Reilly and Colleran (2005) observed a complete recovery of methanogens to nitrite exposure in the range of 5-150 mg NO_2^--N/L but the lag phase of recovery increased with concentration. The implication of this high nitrite concentration is a probable toxicity/ inhibition of both FNA pre-treated activated sludge substrate and possible longer lags of recovery.

Table 22: Change in sulfide concentration between start and end of pre-treatment phase 1

Sample	Concentration applied (mg H_2S/l)	Concentration after pre-treatment (mg S^{2-}/L)	Percentage sulfide removal (%)
18.15 mg H_2S/gVS	399.30	0.07	99.98
24.27 mg H_2S/gVS	533.94	0.09	99.98

According to Wilhelm, et al. (1977), the liquid phase sulfide concentration can considerably be lower around 30℃. The remnant soluble sulfide (*Table 22*) would probably not initiate secondary toxicity (Koster, et al., 1986a, Rinzema and Lettinga, 1988). However, other sulphur species were not determined yet they could provide competition between methanogens and Sulphate reducing bacteria for intermediate substrates like propionate, butyrate and molecular hydrogen (Visser, 1995). Besides the substrate competition, the reduction of these sulphur species presents secondary toxicity especially to methanogens since they are more susceptible to the formed sulfide according to (Brand, et al., 2014).

5.1.7. Anaerobic batch BMP results in phase 1

The gas content for the control and the pre-treated activated sludge was analysed by gas chromatography after taking gas samples from the headspace of the reactor bottles. Sulfide pre-treated samples and control had CO_2 and methane detected while FNA pre-treated activated sludge gas samples had N_2, CO_2 and methane detected. The 0.04 mg HNO_2-N/gVS pre-treated activated sludge was composed of 57.58% CH_4, 24.72% N_2, and 17.70% CO_2 while the 0.06 mg HNO_2-N/gVS pre-treated sludge was composed of 51.47% CH_4, 28.88% N_2 and 19.65% CO_2. However, Zhang and Verstraete (2001), reported a biogas composition of 54% N_2, 10% CO_2 and 36% CH_4 for wastewater containing nitrite treated in an expanded granular sludge bed. According to the experimental setup, the CO_2 would be scrubbed by the 12% NaOH solution

but the N_2 would be logged as methane by the methane counter. Thus based on the results from the gas analysis as 24.72% N_2 and 28.88% N_2 for 0.04 mg HNO_2-N/gVS and 0.06 mg HNO_2-N/gVS pre-treated sludge respectively, the total cumulative biogas values were corrected for this N_2. The methane content of the whole experimental setup is presented in *Table 23*.

Table 23: Methane content of activated sludge in phase 1 pre-treatment

Sample	Methane content (%)
18.15 mg H_2S/gVS	65.33
24.27 mg H_2S/gVS	64.62
0.04 mg HNO_2-N/gVS	57.58
0.06 mg HNO_2-N/gVS	51.47
Control	65.31

The gas accumulation as biogas for FNA pre-treated sludge and as methane for sulfide pre-treated sludge and control is presented in *Figure 12* (data shown in *Appendix G-1*) while after a calculated correction for the N_2 gas (data in *Appendix G-2*), *Figure 13* shows the methane accumulation after the BMP test.

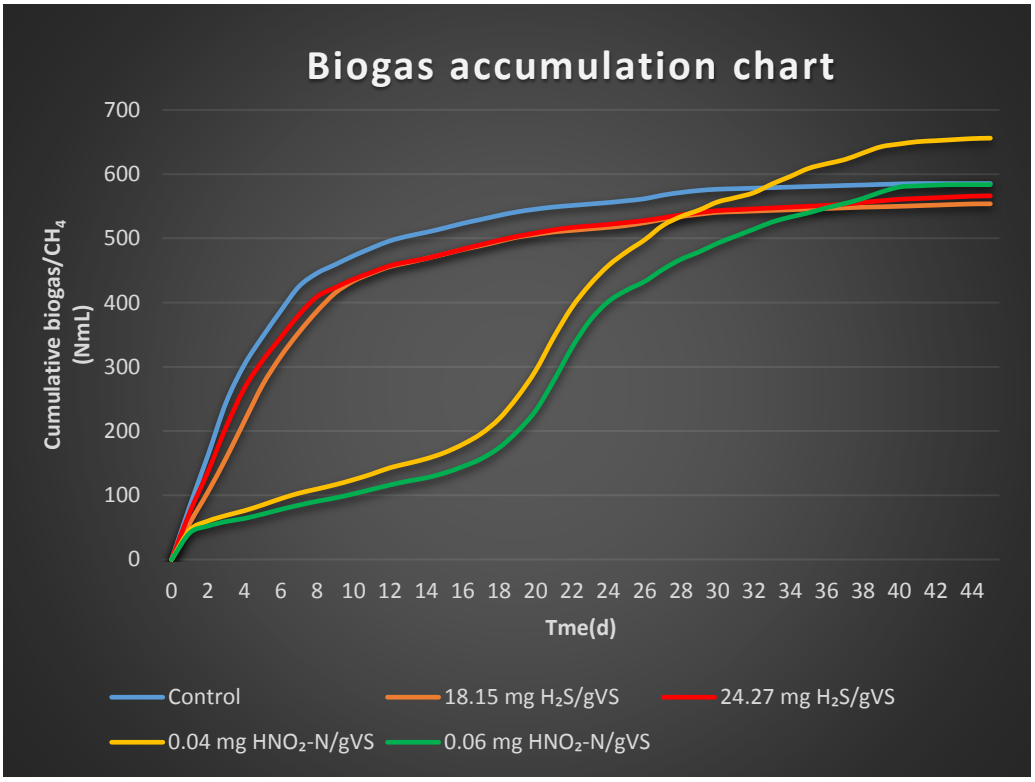


Figure 12: Biogas (FNA) and methane accumulation for BMP phase 1

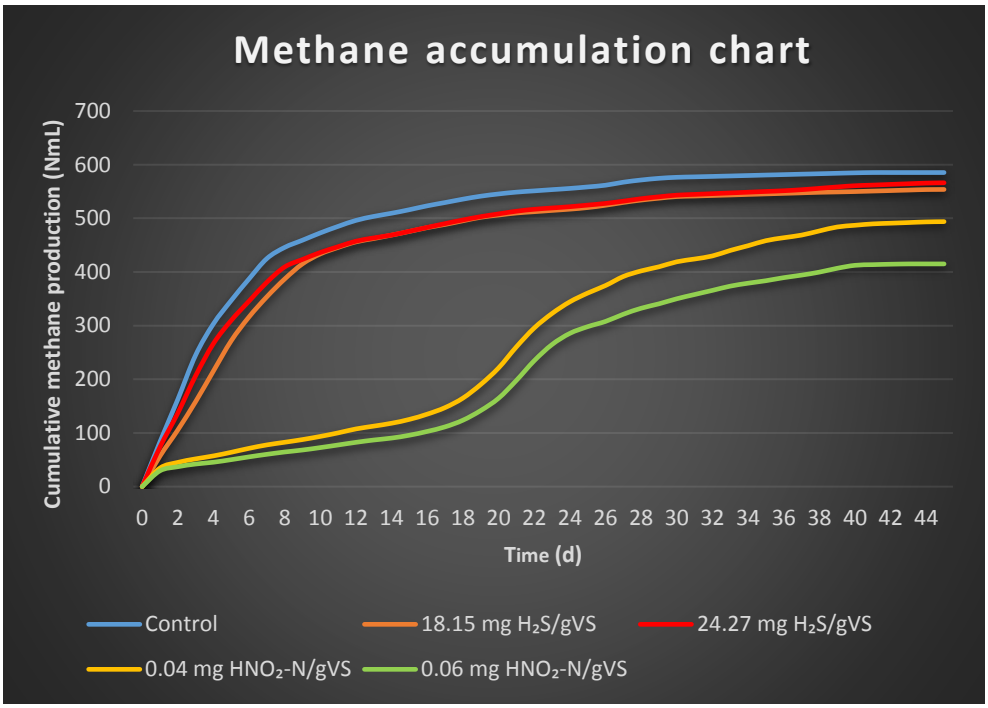


Figure 13: methane accumulation for BMP phase 1

The average gas flow rates of this BMP experiment were also logged and *Figure 14*(data in *Appendix G-3*) presents this.

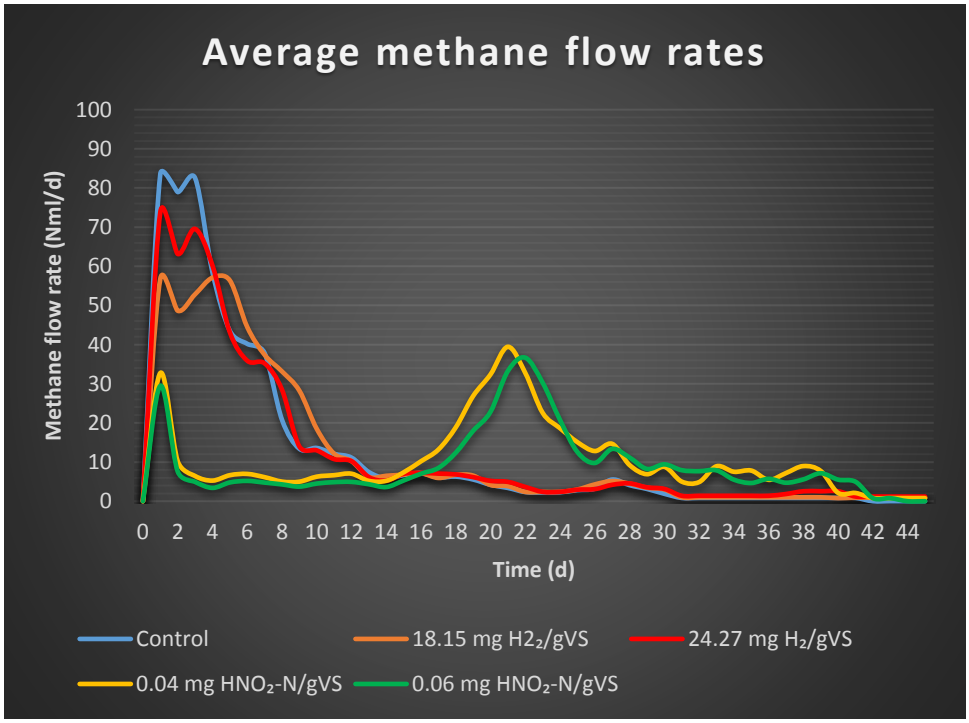


Figure 14: Average biogas (FNA)/ methane flow rates for BMP phase 1

Based on the data analyzed after the BMP, it was observed that the control test performed better than the pre-treated samples. *Table 24* shows the average cumulative methane gas at the end of

digestion time and the percentage change in methane production due to pre-treatment compared to the control (extracted from *Appendix G-2*).

Table 24: Summary of cumulative methane production and percentage change of methane accumulation in sulfide/FNA pre-treated RAS compared to control in phase 1.

Test	Volume methane	Change due to pretreatment (%)
Control	585.40	Not applicable
0.04 mg HNO_2-N/gVS	493.80	-15.60
0.06 mg HNO_2-N/gVS	415.00	-29.10
18.15 mg H_2S/gVS	553.80	-5.40
24.27 mg H_2S/gVS	566.30	-3.30

Considering FNA pre-treatment, the methane production rates at the beginning were lower for all FNA pre-treated samples because the VFA and SCOD was low after pre-treatment. Methane production showed a steep reduction on the first day and a lag of fifteen days is observed where the average gas production is 8.50NmL/d and 6.50NmL/d for 0.04 mg HNO_2-N/gVS and 0.06 mg HNO_2-N/gVS pretreated activated sludge respectively compared to average methane production rates of 31.00NmL/d in the control (*Appendix G-3*). The FNA pre-treated activated sludge gas production rates were four and five fold less for 0.04 mg HNO_2-N/gVS and 0.06 mg HNO_2-N/gVS respectively compared to the control. During these 15 days, the activities of methanogens are seriously inhibited while the acidogens remain metabolically active. However, pH conditions during this period were not analyzed because reactor bottle opening could certainly introduce oxygen that would change the experiment conditions.

The initial inhibition is attributed to nitrite and during this period methanogenesis is inhibited while denitrification occurs. Nitrite presence in the reactor presents anoxic conditions yet strict anaerobic conditions are required for methanogenesis. Methanogenesis resumes at the end of 15 days when the nitrite is depleted and average methane production rates of 22.0 NmL/day and 20.0 NmL/day for 0.04 mg HNO_2-N/gVS and 0.06 mg HNO_2-N/gVS pretreated activated sludge respectively were maintained up to the 25^{th} day of digestion after which production rates drop significantly due to the depletion of readily biodegradable substrates. Also noteworthy, is the erratic flow rates after the 25^{th} day probably due to both substrate depletion and residual nitrite. These assertions are in agreement with the observations of Li, et al. (2016) and Zhang and Verstraete (2001). On the contrary, Wang, et al. (2013) asserted that the 75 mgNO_2^--N introduced into the BMP test after 0.06 mg HNO_2-N/gVS pre-treatment did not have an outstanding effect on the inoculum activity and that N_2 was only detected on the first day of digestion with no observed lag and complete nitrite removal resulting into 30% increase in methane production. Our study as opposed to Wang, et al. (2013) show that the 56 and 96 mgNO_2^--N introduced into BMP for the 0.04 mg HNO_2-N/gVS and 0.06 mg HNO_2-N/gVS pretreated activated sludge respectively was only reduced to 4.9 and 13.04 mgNO_2^--N accordingly at the end of the 45 digestion days with a methane production reduction of 16% and 19% respectively. FNA pre-treatment reduction of methane production by 1-7% was also

reported by Zhang, et al. (2016), with significant lag phases as the pre-treatment concentration increased but, attributed this declining effect on primary sludge characteristics whilst recommending FNA to be suitable for WAS. However, our results suggest that the demerits of FNA pre-treatment are associated with substrate hydrolysis inhibition at pre-treatment, nitrite toxicity of the inoculum and organic matter consumption to facilitate denitrification. The lag phase due to nitrite inhibition is further described by O'Reilly and Colleran (2005) while van Lier, et al. (2008) also postulated that in case of presence of excessive concentrations of organic electron acceptors such as nitrite, their complete removal will first occur at the expense of organic matter consumption thus reducing the methane production. These observations indicate that free nitrous acid is not an efficient pre-treatment agent and nitrite transfer to anaerobic digestion will perform as an alternative electron acceptor scavenging on organic matter and also presenting reversible inhibition to methanogens.

Sulfide pre-treated activated sludge produced lower methane volume than the control in the BMP test. Preliminary testing for COD solubilization (hydrolysis) and acidogenesis after pre-treatment showed that the 24.27 mg H_2S/gVS pre-treated activated sludge was substantially inhibited in terms of hydrolysis and acidogenesis compared to the control and 18.15 mg H_2S/gVS pre-treatment.

The 18.15 mg H_2S/gVS RAS pre-treatment resulted into a 10.90% less COD solubilization parallel to the control implying that sludge hydrolytic enzymes were inhibited. Also a 2.00% decrease in VFA formation compared to the control was observed after pre-treatment suggesting an inhibition of the acidogenesis pathway. However, important to note is that despite a 2% decrease in total VFA production relative to the control, the 18.15 mg H_2S/gVS RAS pre-treatment had 24% and 34% more acetic and propionic acid production respectively. Despite this acidogenesis enhancement, the 18.15 mg H_2S/gVS RAS pre-treatment produced less methane than the 24.27 mg H_2S/gVS pre-treated activated sludge. Brand, et al. (2014), previously asserted that methanogens were adequately outcompeted by methanogens in the presence of a mixed substrate feed of acetate and propionate. This could have probably been the case of the 18.15 mg H_2S/gVS RAS anaerobic digestion. The succumbing to SRB reduction pathway not only utilizes organic matter intended for methanogenesis but introduces secondary toxicity of methanogens to sulfide. Methane production rates of the 18.15 mg H_2S/gVS pre-treated RAS dropped from 56.80NmL/d on the first day to 48.65NmL/d unlike the control and 24.27 mg H_2S/gVS pre-treated activated sludge which proceeded with high rates of 78.95NmL/d and 69.55NmL/d respectively within the same period of time. Also, according to Visser (1995), any Sulphur species could have subjected methanogens to primary competition from sulphate reducing bacteria for anaerobic intermediate substrates such as propionate and molecular hydrogen thus a low methane output for both sulphide pre-treated activated sludge. However, due to technical limitations, Sulphur species other than Sulfide were not determined in the wastewater before and after pre-treatment. Van den Brand, et al. (2015), previously reported the presence of approximately 92 mg SO_4^{2-}/L and SRB present in Harnaschpolder

WWTP influent, but this can only be predictive on our part since we did not determine current influent concentrations of SO_4^{2}-. The presence of SRB in the anaerobic digester can also lead to the consumption of propionate in the absence of SO_4^{2-} as observed by Wu, et al. (1991), another probable explanation for the low methanogenesis of the 18.15 mg H_2S/gVS pre-treated activated sludge. The 24.27 mg H_2S/gVS RAS pre-treatment resulted into greater inhibition of both hydrolysis and acidogenesis showing 37.70% less COD solubilization and 34.90% less VFA formation which explains the low methane production in comparison to the control.

5.2. Phase 2 results

Despite phase 1 not achieving the research main objective of improving methane production through FNA and sulphide pre-treatment, phase 2 proceeded to test hydrolysis, acidogenesis and methanogenesis with a range of sulfide pre-treatment concentrations (4.5-18.18 mg H_2S/gVS). This was partly because the concentrations previously used were at the high end of normal plant-wide concentrations yet acetate and propionate production was enhanced with 18.15 mg H_2S/gVS pre-treatment and the highest methane reduction was just a marginal 5%. The first phase was also characterized of method testing for pre-treatment, technical challenges in Sulfide spiked samples and ascertaining feasible test methods to obtain reliable results. FNA pre-treatment being clearly to no avail in the anaerobic digestion cycle was disregarded.

5.2.1. Ammonium results

Baseline measurement of the soluble ammonium concentration at the time of RAS sampling was 3.53 mg NH_4^+-N/L. Ammonium concentration for sulfide pre-treated RAS and the control was done and a trend generated. *Table 25* below shows the outcome of the ammonium testing per gram VS (22.00g/l) of RAS.

Table 25: Ammonium results after pre-treatment in phase 2

Sample	Ammonium concentration average (mg NH_4^+-N/L)	Biomass specific ammonium concentration (mg NH4+-N/gVS)	Change in ammonium compared to control (%)
0 mg H_2S/gVS	215.04	9.80	Reference concentration
4.54 mg H_2S/gVS	211.16	9.60	-2.00
9.09mg H_2S/ gVS	185.72	8.30	-15.00
16.63 mg H_2S/gVS	174.94	8.00	-18.00
18.18 mg H_2S/gVS	176.23	8.00	-18.00

A trend of the ammonium results following pre-treatment was generated and is expressed by *Figure 15* below.

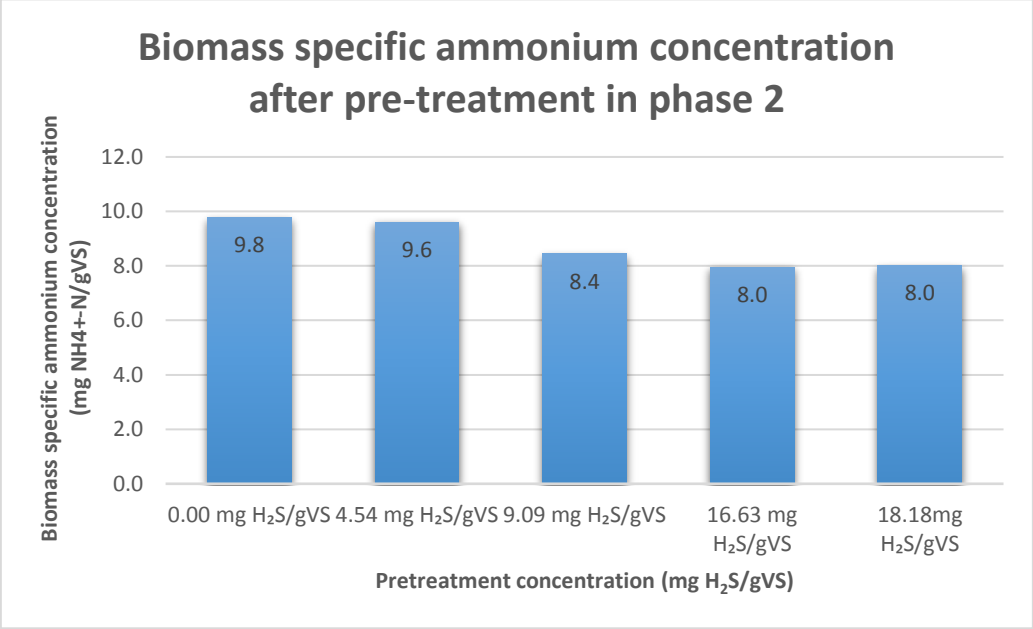


Figure 15: Trend of biomass specific ammonium concentration after pre-treatment phase 2.

The decrease in ammonium formation with increasing sulfide concentration during pre-treatment is significant (2-18%) across the pre-treatment range and this is probably indicative of an increasing inhibition of protein hydrolysis through enzymatic inhibition of protein enzymes like carboxypeptidase as reported by Smith (2009).

5.2.2. Phase 2 SCOD results

The baseline RAS supernatant SCOD before pre-treatment was 335 mg SCOD/L. RAS pretreatment with 0 mg H_2S/gVS, 4.54 mg H_2S/gVS, 9.09 mg H_2S/gVS, 16.63 mg H_2S/gVS and 18.18mg H_2S/gVS achieved COD solubilization presented in *Table 26* as absolute concentrations in mg/L and as biomass specific values (mg COD/gVS).

Table 26: SCOD concentration after pre-treatment phase 2

Sample	Actual Concentration (mg SCOD/l)	Biomass specific SCOD (mg SCOD/gVS)	Change in SCOD compared to control after pre-treatment (%)
0 mg H_2S/gVS	2125.00	96.60	Reference concentration
4.54 mg H_2S/gVS	2325.00	105.70	9.00
9.09 mg H_2S/gVS	2325.00	105.70	9.00
16.63 mg H_2S/gVS	2075.00	94.30	-2.00
18.18mg H_2S/gVS	2391.67	108.70	13.00

Figure 16 is a plot of the biomass specific SCOD values against the pre-treatment concentrations.

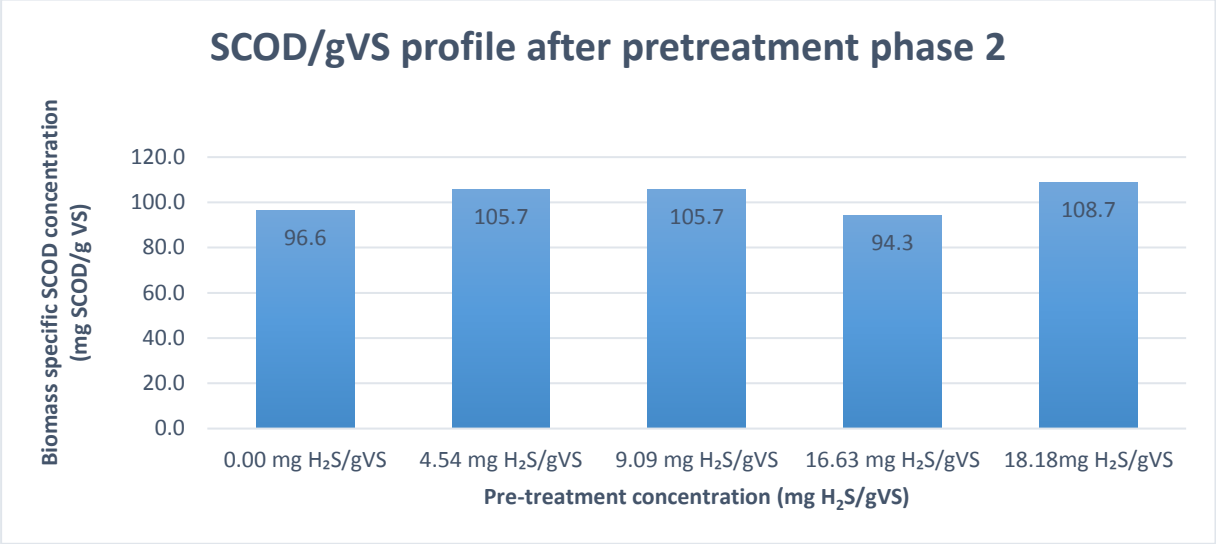


Figure 16: Trend of COD solubilization after pre-treatment phase 2.

Based on the percentage change in the soluble COD in comparison to the control, there was a slight increase in solubilization of COD and hydrolysis. The average increase in COD was observed to be 10.3±2.3% with the exception of 16.63 mg H2S/gVS pre-treatment. The sulphide composition of these samples before COD analysis was observed to be less than 0.01 mg H_2S/L but any other inorganic species of sulfur could quantitatively be oxidized and this would show an increase in soluble COD (Apha, 1998). However, these species of Sulphur were not quantified due to unavailable testing facilities in the study period.

5.2.3. Phase 2 Total organic carbon

The analysis of total dissolved organic carbon realized the following outcome after pre-treatment with sulphide alongside the control as shown in *Table 27.*

Table 27: Total dissolved organic carbon after pre-treatment phase 2.

Sample	NPOC TOC (mg/l)	Change in TOC compared to the control (%)
0.00 mg H_2S/gVS	768.60	Reference concentration
4.54 mg H_2S/gVS	969.80	26.00
9.09 mg H_2S/gVS	1037.40	35.00
16.63 mg H_2S/gVS	804.00	5.00
18.18 mg H_2S/gVS	727.90	-5.00
RAS supernatant	80.40	Not applicable

There is an observed increase in the TOC as the pre-treatment concentration increases from 4.54-9.09 mg H_2S/gVS and declining dramatically thereafter. The probable cause of decline in

TOC beyond 9.09 mg H_2S/gVS pre-treatment is however unknown but probably related to increasing sulphide toxicity to hydrolysis enzymes. Also, these results differ from the previous phase where the increase in TOC was sustained at higher concentration of sulphide pre-treatment and this could be attributed to the difference in the RAS batches probably due to plant operations. However, the applicability of TOC in determining the hydrolysis achieved is less useful (van Lier, et al., 2008). Nielsen and Keiding (1998) describes this increase in TOC as indicative of increased stress on activated sludge flocs resulting into increased EPS solubilization and probably cellular disintegration, however the formed products are not described and thus of limited significance in methane volume prediction since not all soluble organic compounds are biodegradable.

5.2.4. Phase 2 VFA results

Post pre-treatment in phase 2, the volatile fatty acids generated were measured by gas chromatography and the results are shown in *Table 28* while *Figure 17* shows the graphical trend of biomass specific VFA.

Table 28: VFA results after pre-treatment phase 2

VFA	0 mg H_2S/gVS	4.54mg H_2S/gVS	9.09 mg H_2S/gVS	16.63 mg H_2S/gVS	18.18mg H_2S/gVS
Acetic acid (mg/l)	492.62	630.61	610.31	532.18	332.12
Propionic acid (mg/l)	282.38	259.85	343.94	333.72	197.86
Isobutyric acid (mg/l)	75.21	46.08	70.72	62.59	34.18
Butyric acid (mg/l)	153.04	92.82	127.57	100.54	54.37
Valeric acid (mg/l)	51.56	30.30	47.06	49.04	28.20
Total (mg/l)	1054.81	1059.66	1199.60	1078.08	646.73

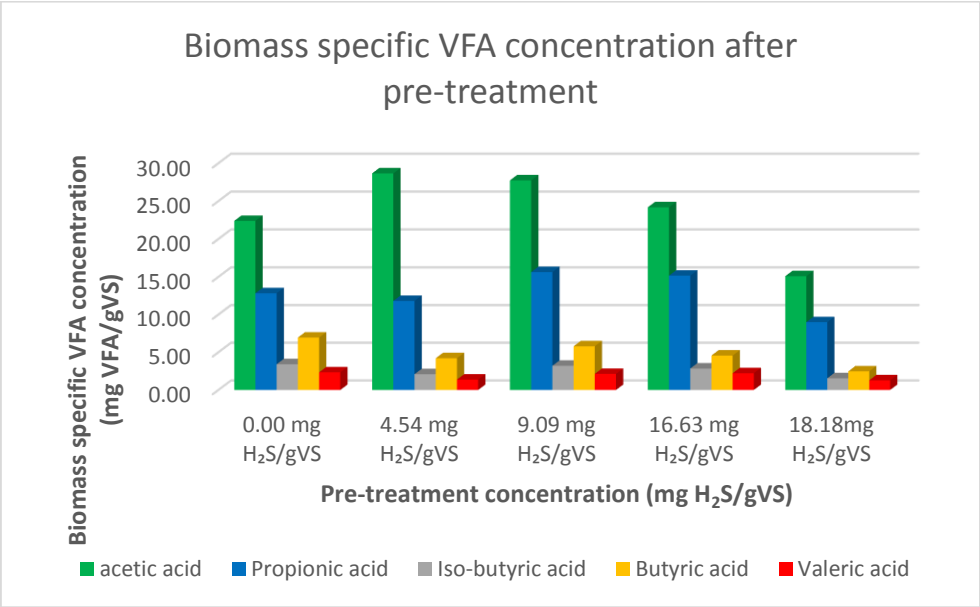


Figure 17: Trend of VFA production after pre-treatment phase 2.

The total VFA is marginally increased across the Sulfide pre-treatment range of 4.54-16.63 mgH_2S/gVS relative to the control yet a 39% decrease is observed for 18.18 mgH_2S/gVS. This decline at higher sulfide concentration could possibly be due to sulphide inducing inhibition of hydrolysis and acidogenesis. The increase in acetic acid concentration relative to the control is significant with 28%, 24% and 8% more acetic acid formed for 4.54 mgH_2S/gVS, 9.09 mgH_2S/gVS and 16.63 mgH_2S/gVS. Propionic acid is also markedly increased relative to the control by 22% and 18% for the 9.09 mgH_2S/gVS and 16.63 mgH_2S/gVS pre-treatment yet a decline by 8% and 30% was observed for 4.54 mgH_2S/gVS and 18.18 mgH2S/gVS pre-treatment. Therefore considering the increase in acetic and propionic acids, hydrolysis and acidogenesis was enhanced by sulfide pre-treatment within the range of 4.54-16.63 mgH_2S/gVS. However, fatty acids with longer carbon chains than propionic acid were not enhanced by the pre-treatment but their relevance in methane production is negligible (Novak and Carlson, 1970, van Lier, et al., 2008).

5.2.5. Residual sulfide after pre-treatment phase 2

After pre-treatment in phase 2, the residual sulfide was analyzed as an indicator of toxicity expected on methanogens. *Table 29* shows the results obtained.

Table 29: Sulphide concentration after pre-treatment phase 2.

Sample	Applied concentration at pre-treatment (mg H_2S/L)	Actual concentration (mg S^{2-}/L)	Sulfide removal efficiency (%)
4.54 mg H_2S/gVS	100.00	0.06	99.93
9.09 mg H_2S/gVS	200.00	0.04	99.98

Sample	Applied concentration at pre-treatment (mg H_2S/L)	Actual concentration (mg S^{2-}/L)	Sulfide removal efficiency (%)
16.63mg H_2S/gVS	300.00	0.06	99.99
18.18mg H_2S/gVS	400.00	0.11	99.97
0.00 mg H_2S/gVS	0.00	0.01	Not applicable

Testing for sulfide was in the liquid phase around that 30˚C and according to Wilhelm, et al. (1977), the concentration can remarkably be lower at a gas liquid distribution coefficient of 2.27. Also metal ion precipitation can lower the soluble phase sulfide concentration measured according to Martell and Smith (1989). The soluble sulfide presence after pre-treatment is negligible to inhibit anaerobic digestion based on studies by Koster, et al. (1986a) and Rinzema and Lettinga (1988). However, the creation of any other Sulphur species will certainly negatively impact methane production through primary competition for substrates between methanogens and substrate reducing bacteria (Visser, 1995) and their reduction will generate sulfide that is toxic to methanogens. However, only sulfide was quantified due to testing limitations.

5.2.6. Anaerobic batch BMP results in phase 2

While the BMP was on going, the gas content for the control and the pre-treated activated sludge was analysed by gas chromatography after taking gas samples from the headspace of the reactor bottles. *Appendix F-2* shows the measurement calibration. Sulfide pre-treated samples and control had CO_2 and methane detected but since the 12% sodium hydroxide scrubbing solution absorbed the CO_2 , it is not reflected in the cumulative methane volume logged. *Table 30* shows the methane content of the sulfide pre-treated samples and the control.

Table 30: Methane content of biogas in BMP phase 2

Sample	% Methane
0 mg H_2S/gVS	54.00
4.54 mg H_2S/gVS	53.00
9.09 mg H_2S/gVS	53.00
16.63 mg H_2S/gVS	53.00
18.18mg H_2S/gVS	56.00

According to Darnell and Jefferson (1994), the methane content of biogas will vary between 50-65% but van Lier, et al. (2008) specifies that this will depend on the COD/TOC ratio. However, the average methane composition of all samples is 53.8±1.30% and therefore the differences negligible despite varying COD/TOC ratios.

The gas accumulation as methane for sulfide pre-treated activated sludge and control activated sludge is presented in *Figure 18* (Data in *Appendix G-4*) while *Figure 19* (data in *Appendix G-5*) shows the methane flow rates for the 36 digestion days.

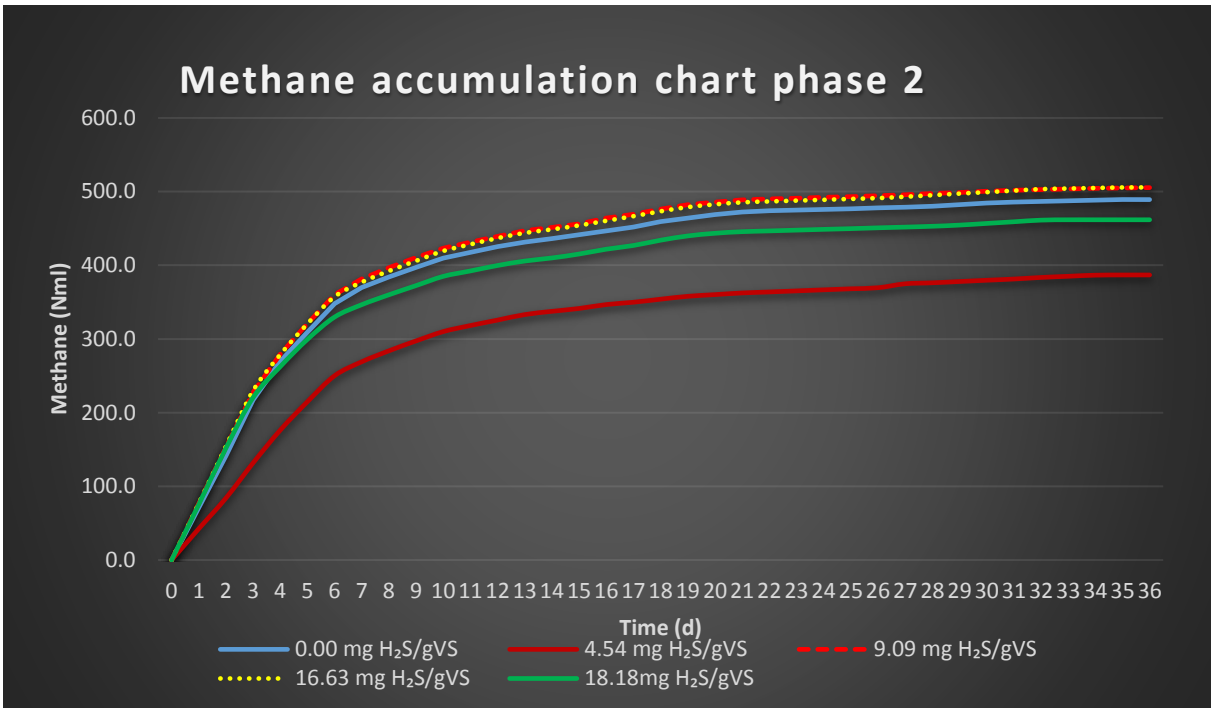


Figure 18: Cumulative methane production in BMP phase 2.

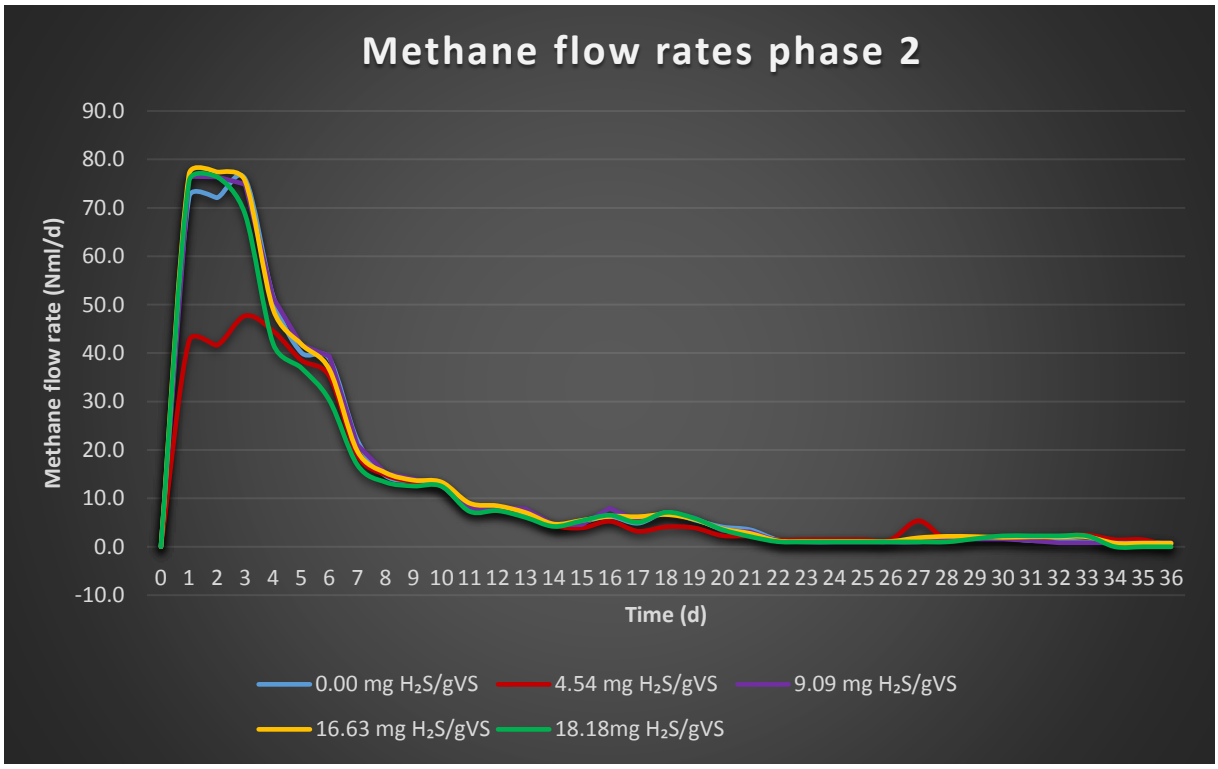


Figure 19: Methane flow rates in BMP phase 2.

Based on the data analyzed after the BMP, it was observed that the 9.09 mg H_2S/ gVS and 16.63 mg H_2S/g VS pre-treated activated sludge performed better than the control sample by 3.30% and 3.40% respectively while the 4.54 mg H_2S/g VS and 18.18mg H_2S/gVS produced 20.90 % and 5.60% respectively less methane compared to the control. *Table 31* presents the gas accumulation averages corrected by a Dixon outlier test and the percentage increase/ decline in methane production due to pre-treatment in comparison to the control.

Table 31: Summary of cumulative methane production and percentage change of methane accumulation in sulfide pre-treated RAS compared to control in phase 2.

Test	Volume methane (NmL)	Change due to pretreatment (%)
0 mg H_2S/gVS	752.70	N/A
4.54 mg H_2S/gVS	662.10	-20.90
9.09 mg H_2S/gVS	707.85	3.30
16.63 mg H_2S/gVS	730.80	3.40
18.18mg H_2S/gVS	692.00	-5.60

The methane increase is not significant in relation to the acetic and propionic acid increase and this may be attributed to Sulfur species created during pre-treatment and thereafter reduced in the anaerobic digestion by SRB (Visser, 1995). SRB utilizes anaerobic substrate intermediates to reduce these compounds at the expense of methanogens. According to Brand, et al. (2014), mixed substrate feed of acetate and propionate also makes methanogens lose competition to

SRB with a consequential reduction in methane production especially in the presence of Sulphur species that are finally reduced to sulphide. Van den Brand, et al. (2015) further observed that SO_4^{2-} and SRB are naturally present in influent of Harnaschpolder WWTP due to industrial effluents and closer proximity of the sewer to the sea, however the current situation was not verified to relate it to this result. Nevertheless, consumption of propionate is also probable in the absence of sulphate as reported by (Wu, et al., 1991) since SRB are naturally present in anaerobic conditions. Therefore, it is probable that both primary inhibition of methanogens due to substrate competition and secondary toxicity due to sulphide occurred leading to the observations in the BMP. Another probable factor leading to no significant increase in the methane volume was assumed to be sulfide precipitation of vital trace elements required for methanogenesis (Fermoso, et al., 2008) which the next phase proceeded to rule out.

5.3. Phase 3 results

5.3.1. Ammonium results

In this phase ammonium changes were tracked between the beginning of pre-treatment and the end of pre-treatment and results are presented in *Table 32.*

Table 32: Change in ammonium concentration due to pre-treatment phase 3.

Sample	At start of pre-treatment (mg NH_4^+-N/ g VS)	At end of pre-treatment (mg NH_4^+-N/ g VS)	Change in Ammonium concentration (mg NH_4^+-N/ g VS)
0.00 mgH_2S/gVS	2.60	9.80	7.20
4.54 mgH_2S/gVS	2.70	9.60	6.90
9.09 mgH_2S/gVS	1.70	8.40	6.80
16.63 mgH_2S/gVS	2.50	8.00	5.40
18.18 mgH_2S/gVS	2.40	8.00	5.60

The increase in ammonium from start to end of pre-treatment is presented graphically in *Figure 20*. The control achieved the highest change in ammonium while a decreasing trend with increasing sulfide pre-treatment was observed with 4.20%, 5.60%, 25.00% and 22.20% less ammonium change seen in 4.54 mg H_2S/gVS, 9.09 mg H_2S/gVS, 16.63 mg H_2S/gVS and 18.18mg H_2S/gVS pre-treatment respectively compared to the control.

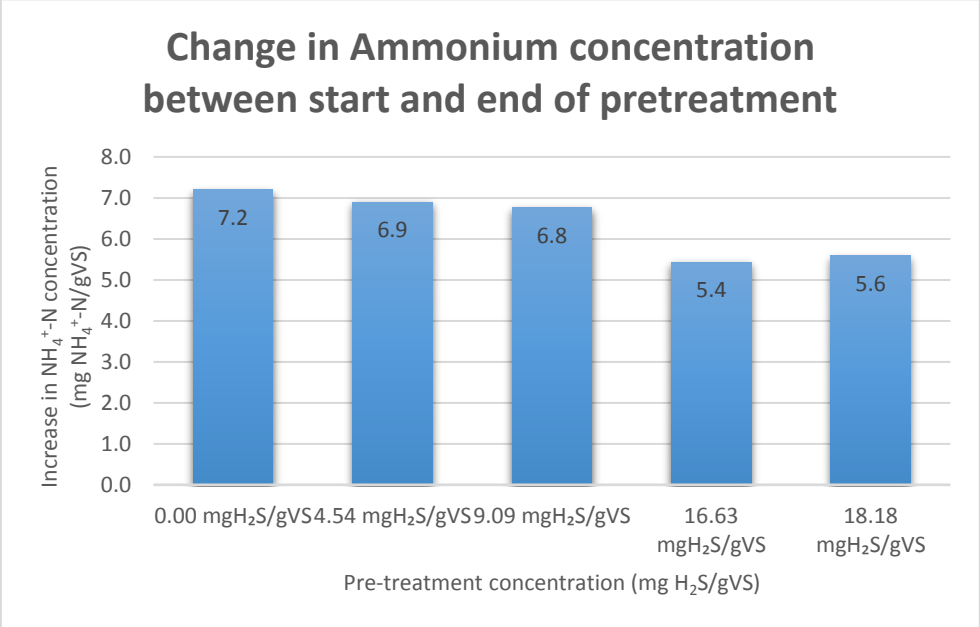


Figure 20: Increase in ammonium concentration between start and end of pre-treatment phase 3.

This decreasing ammonium concentration presents a probable progressive decline in protein hydrolysis due to inhibition of specific hydrolysis enzymes. Sulfide at a concentration of 0.037M has previously been described by Smith (2009) to be inhibitory to carboxypeptidase by binding to the magnesium ion which is essential to its function.

5.3.2. Phase 3 SCOD results

In this phase COD solubilization changes were tracked between the beginning of pre-treatment and the end of pre-treatment and results are presented in *Table 33*.

Table 33: Change in SCOD concentration due to pre-treatment phase 3.

Sample	At start of pre-treatment (mg SCOD/ gVS)	At end of pre-treatment (mg SCOD/ gVS)	Change in SCOD (mg SCOD/ gVS)
0 mg H_2S/gVS	97.00	179.00	82.00
4.54 mg H_2S/gVS	47.00	174.00	126.00
9.09 mg H_2S/gVS	31.00	141.00	110.00
16.63 mg H_2S/gVS	49.00	142.00	93.00
18.18mg H_2S/gVS	58.00	156.00	98.00

The increase in SCOD between start and end of pre-treatment is presented graphically in *Figure 21*.

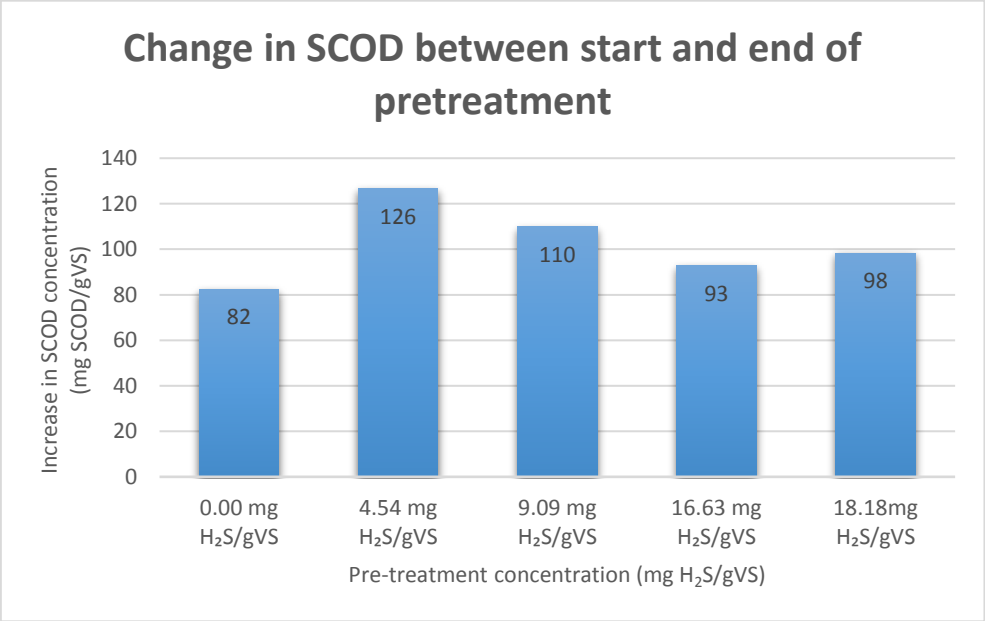


Figure 21: Increase in SCOD concentration between start and end of pre-treatment phase 3.

Based on the SCOD results, 54%, 34%, 17% and 20% more SCOD than the control was obtained due to pre-treatment with 4.54 mg H_2S/gVS, 9.09 mg H_2S/gVS, 16.63 mg H_2S/gVS and 18.18mg H_2S/gVS respectively. H_2S concentration was measured before the COD test in the same samples and determined to be 0.52, 14.01, 39.08, 88.47 and 107.66 mg H_2S/L for the control, 4.54 mg H_2S/gVS, 9.09 mg H_2S/gVS, 16.63 mg H_2S/gVS and 18.18mg H_2S/gVS pre-treated activated sludge respectively at the start of pre-treatment. The presence of sulphide could lead to a significant increase in COD due to its oxidation in the closed reflux test, however at the start the control with the lowest sulphide concentration is observed to have almost twice

the COD concentration of the samples with Sulfide. Thus the results reliability could have least been affected by sulfide presence. At the end of pre-treatment 0.57, 0.51, 0.66, 0.75 and 0.81 mg H_2S/L was measured in the control, 4.54 mg H_2S/gVS, 9.09 mg H_2S/gVS, 16.63 mg H_2S/gVS and 18.18mg H_2S/gVS pre-treated activated sludge respectively. Besides a lower sulfide concentration in the samples, the increase in COD was significant. This shows an increase in hydrolysis but the misgivings of sulfide interference of the test were ruled out by higher VFA relative to the control, which is also indicative of improved hydrolysis.

5.3.3. Phase 3 TOC results

The analysis of total dissolved organic carbon realized the following outcome between the start and end of pre-treatment with sulphide alongside the control as shown in *Table 34.*

Table 34: TOC change between start and end of pre-treatment phase 3.

Sample	TOC at start of pre-treatment (mg TOC/gVS)	TOC at end of pre-treatment (mg TOC/gVS)	Increase in TOC (mg TOC/gVS)
0.00 mg H_2S/gVS	14.11	51.23	37.12
4.54 mg H_2S/gVS	17.44	50.23	32.79
9.09 mg H_2S/gVS	10.63	44.40	33.77
16.63 mg H_2S/gVS	15.08	61.00	45.92
18.18mg H_2S/gVS	14.93	54.64	39.71

The increase in TOC between the start and end of pre-treatment is presented graphically in *Figure 22.* It is observed that the order of TOC increase in descending order is 45.92, 39.71, 37.12, 33.77 and 32.79 mg TOC/gVS for 16.63 mg H_2S/gVS, 18.18mg H_2S/gVS, 0.00 mg H_2S/gVS, 9.09 mg H_2S/gVS and 4.54 mg H_2S/gVS respectively.

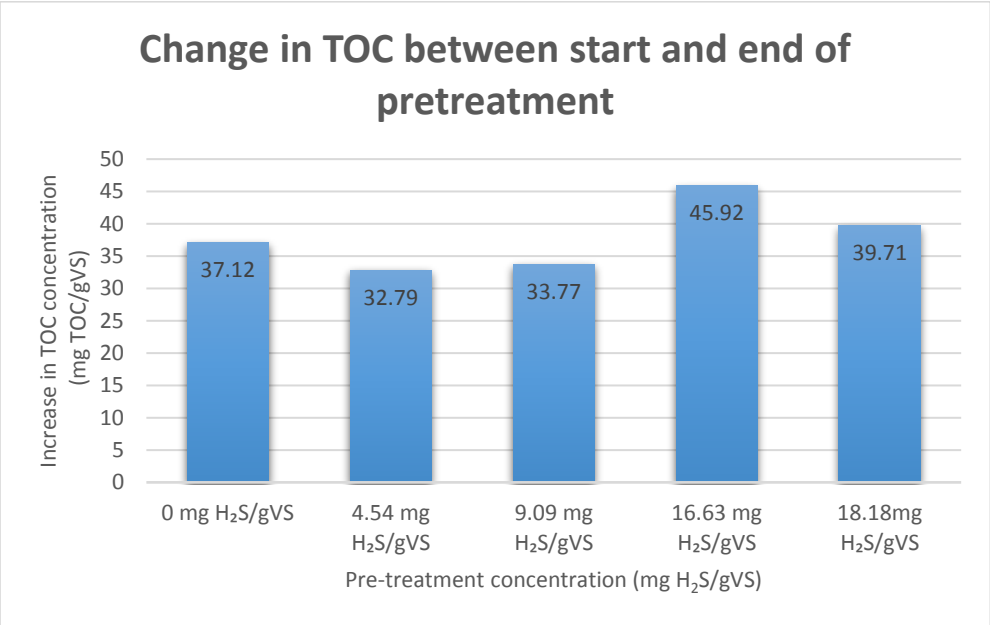


Figure 22: Increase in TOC due to pre-treatment phase 3.

Based on these results, it is not possible to predict the relative amount of methane to be produced when the mean oxidation state of all the compounds formed is unknown and the specific products formed are also not known (van Lier, et al., 2008). Nielsen and Keiding (1998) describes this increase in TOC as indicative of increased stress on activated sludge flocs resulting into increased EPS solubilization and probably cellular disintegration, however the formed products are not described and thus of limited significance in methane production prediction. Therefore the TOC result is of limited significance in describing hydrolysis.

5.3.4. Phase 3 VFA results

The changes in VFA accumulation were monitored between the start and end of pre-treatment. *Table 35* shows the change in VFA between start and end of pre-treatment as acetic, propionic, isobutyric, butyric, valeric acid and as total VFA.

Table 35: Increase in VFA due to pre-treatment phase 3.

VFA	0.00 mg H_2S/gVS	4.54 mg H_2S/gVS	9.09 mg H_2S/gVS	16.63 mg H_2S/gVS	18.18mg H_2S/gVS
Acetic acid	42.0	53.0	43.0	47.0	44.0
Propionic acid	27.0	35.0	32.0	32.0	33.0
Isobutyric acid	3.0	5.0	4.0	5.0	4.0
Butyric acid	12.0	11.0	7.0	6.0	7.0
Valeric acid	3.0	4.0	3.0	3.0	4.0
Total VFA	87.0	106.0	87.0	92.0	92.0

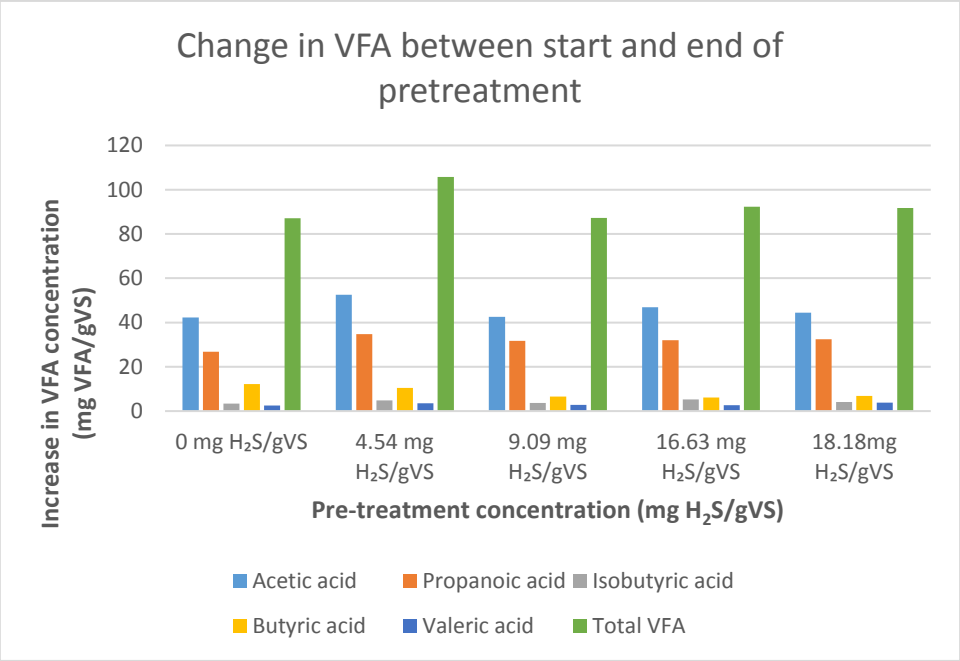


Figure 23: increase in VFA due to pre-treatment phase 3.

The increase in VFA is noteworthy for acetic and propionic acid across the entire sulfide pre-treatment range. The highest VFA production relative to control was observed in 4.54 mg H_2S/gVS pre-treatment with 26%, 30%, 67% and 22% for acetic acid, propionic acid, isobutyric acid and total VFA. This increase is significant and can cause significant methane production leaving other factors constant. Second in order of VFA production was 16.63 mg H_2S/gVS pre-treated activated sludge. Relative to the control, 12%, 19%, 67% and 6% more acetic acid, propionic acid, isobutyric acid and total VFA was produced in this pre-treatment. The 18.18mg H_2S/gVS pre-treatment was third in order of VFA production. Relative to the control, 5%, 22%, 33% and 6% more acetic acid, propionic acid, isobutyric acid and total VFA was produced in this pre-treatment. Despite the increase of VFA at the start, the anaerobic phase is expected to proceed with hydrolysis, acidogenesis, and acetogenesis thus more methanogenic intermediates will be produced and probably not based on the pre-treatment order because other factors like the SRB reduction of Sulfur species can be both competitive and toxic to the detriment of methanogenesis according to (van Lier, et al., 2008, Visser, 1995). However, since we did not determine the presence of other sulphur species, these are merely probable assumptions. Therefore this increase will certainly reduce the bottlenecks of hydrolysis as the rate-limiting step (van Lier, et al., 2008).

5.3.5. Residual sulfide after pre-treatment phase 3

The residual sulfide concentration after pre-treatment was measured to rule out secondary toxicity of methanogens due to sulfide introduction at the start of anaerobic digestion. *Table 36*

shows the remnant sulfide after pre-treatment and stripping out of Sulfide from RAS at pH 7.6 by use of N2 gas at the end of pre-treatment. Besides, Sulphide concentration in the liquid phase can remarkably be lower at 30˚C testing temperature according to Wilhelm, et al. (1977). The remnant sulfide would not be indicative of any potential secondary toxicity (Koster, et al., 1986a, Rinzema and Lettinga, 1988). However, the unquantified Sulfur species generated during pre-treatment and originally present in the sampled activated sludge could provide primary competition for substrates such as propionic acid, butyrate and molecular hydrogen with SRB reduction and consequential sulfide toxicity to the detriment of methanogenesis. (Visser, 1995).

Table 36: Remnant sulfide after pre-treatment phase 3.

Sample	**Applied concentration at pre-treatment (mg H_2S/L)**	**Actual concentration (mg S^{2-}/L)**	**Sulfide removal efficiency (%)**
4.54 mg H_2S/gVS	100.00	0.51	99.49
9.09 mg H_2S/gVS	200.00	0.66	99.67
16.63 mg H_2S/gVS	300.00	0.75	99.75
18.18mg H_2S/gVS	400.00	0.81	99.79
0.00 mg H_2S/gVS	0.00	0.57	Not applicable

5.3.6. Anaerobic batch BMP results in phase 3

The peculiarity of the BMP phase 3 to that of phase 2 is the supplementation with trace elements to avoid any limitations to methanogenesis by sulphide precipitation of trace metals. This makes the phase's results more reliable to describe the outcome of the generally improved hydrolysis products to anaerobic digestion and consequential methane production.

While the BMP was on going, the gas content for the control and the pre-treated activated sludge was analyzed by gas chromatography after taking gas samples from the headspace of the reactor bottles. *Appendix F-3* shows the measurement calibration. Sulfide pre-treated samples and control had CO_2 and methane detected but since the 12% sodium hydroxide scrubbing solution absorbed the H_2S and CO_2, it is not reflected in the cumulative methane volume logged. *Table 37* shows the methane content of the sulfide pre-treated samples and the control.

Table 37: methane content of biogas in BMP phase 3.

Sample	**Methane (%)**
0 mg H_2S/gVS	55.39
4.54 mg H_2S/gVS	51.99
9.09 mg H_2S/gVS	55.57
16.63 mg H_2S/gVS	54.52
18.18mg H_2S/gVS	53.59

No significant differences were observed for the methane composition of biogas for the Sulfide pre-treatment relative to the control and average composition of methane is 54.21±1.47%.

The accumulated methane gas for the entire digestion period is presented in *Figure 24* as an average with the triplicate measurements corrected of outliers by a Dixon statistical test (data in *Appendix G-6*).. The gas flow rates in the same digestion period are further presented in *Figure 25* (data in *Appendix G-7*).

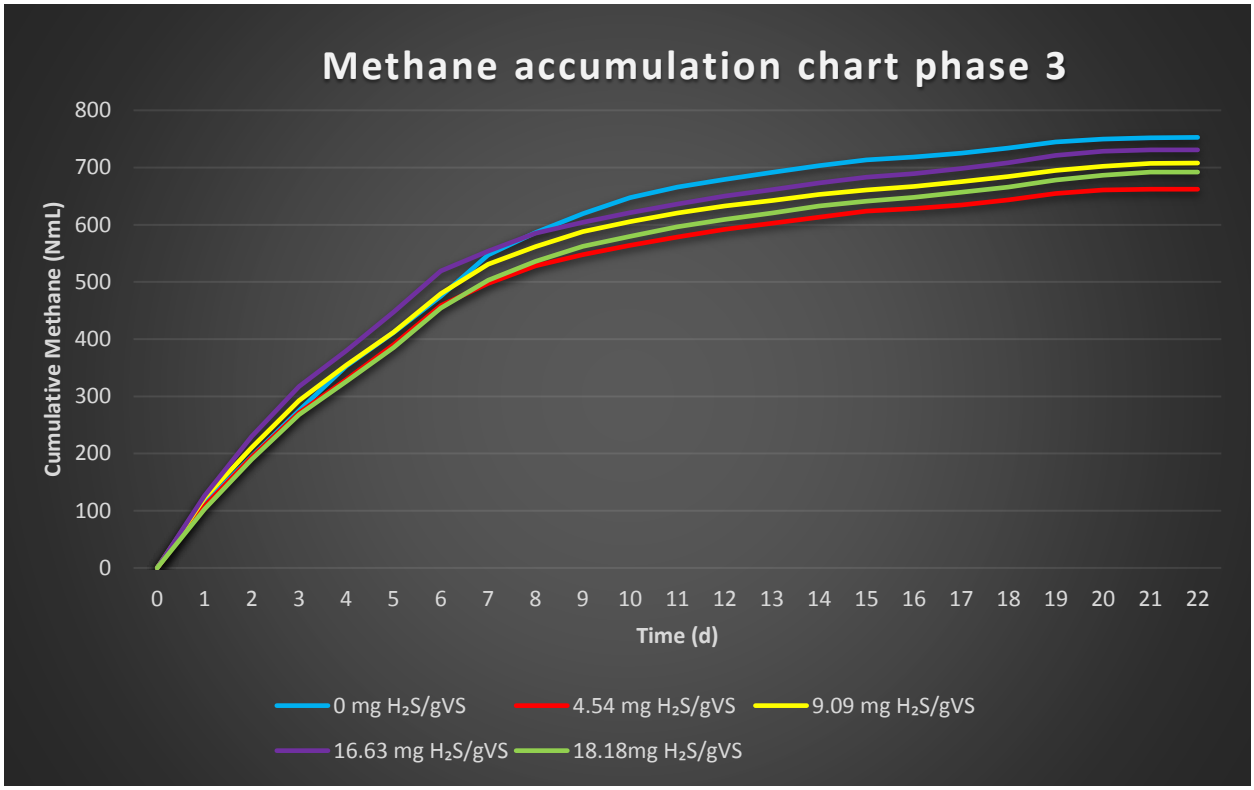


Figure 24: Cumulative methane production in BMP phase 3.

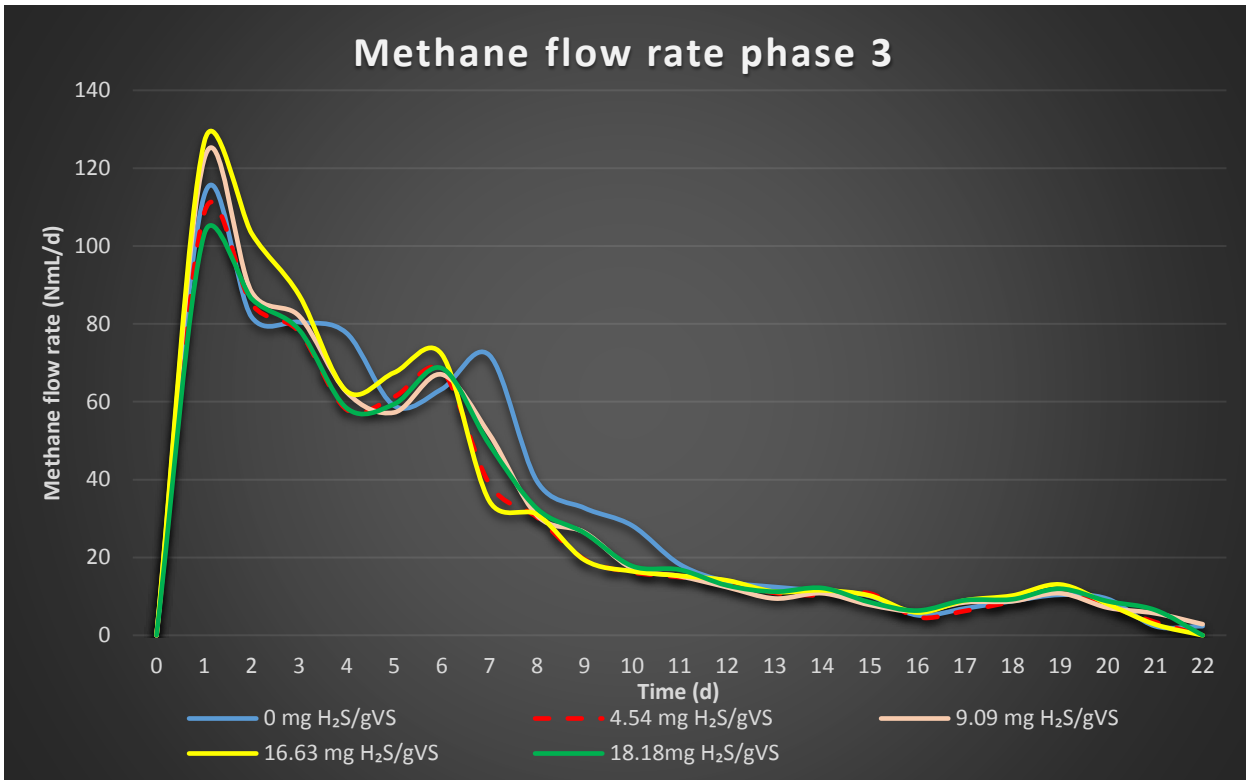


Figure 25: Methane flow rates in BMP phase 3.

Based on the data analyzed after the BMP, it was observed that the control activated sludge performed better than the pre-treated samples. *Table 38* shows the average cumulative methane gas at the end of digestion time and the percentage change in methane production due to pre-treatment compared to the control.

Table 38: Summary of cumulative methane production and percentage change of methane accumulation in sulfide pre-treated RAS compared to control in phase 3.

Test	Volume methane	Change due to pretreatment (%)
0 mg H_2S/gVS	752.70	Reference point
4.54 mg H_2S/gVS	662.10	-12.03
9.09 mg H_2S/gVS	707.85	-5.96
16.63 mg H_2S/gVS	730.80	-2.91
18.18mg H_2S/gVS	692	-8.06

Methane production due to Sulfide pre-treatment showed a 3-12% decrease (irrespective of pre-treatment concentration) relative to the control. The methane production was disproportionate to the hydrolysis increase observed at the end of pre-treatment and the prevention of trace metal precipitation did not aid Sulphide pre-treated samples to produce more methane than the control. The 4.54 mg H_2S/gVS pre-treatment presents a peculiar case of improved hydrolysis not being utilized for methanogenesis. Despite the 4.54 mg H_2S/gVS pre-treatment achieving 54%, 26%, 30%, 67% and 22% more SCOD, acetic acid, propionic acid, isobutyric acid and total VFA respectively relative to the control, it had the least accumulation of methane with

12% less methane compared to the control. The closest to the control in terms of methane production was the 16.63 mg H_2S/gVS pre-treatment, which was in the lead of methane production for the first 3 digestion days and thereafter methane production rates dropped. Such a peculiarity of anaerobic digestion where improved hydrolysis does not translate into improved methanogenesis can probably be explained in terms of the presence of alternative electron acceptors that consume intermediate anaerobic substrates and also toxicity. The probable source of competition is by SRB reducing any sulfur species created in pre-treatment or originally present in the sample since no N_2, a gaseous by-product of nitrate/nitrite was detected in the gas chromatography test. According to Colleran, et al. (1995), SRB have a wider spectrum of substrates thus possibly utilizing them for their activities instead of methanogenesis. Brand, et al. (2014) asserts that propionate favors SRB activity at the expense of methanogens and that obviously results in secondary sulfide toxicity. Wu, et al. (1991) has previously described the consumption of propionate in the absence of electron acceptors by *Desulfobulbus*-like bacteria. Brand, et al. (2014) further observed that in the presence of mixed substrate as acetate and propionate, SRB effectively outcompete and suppress methanogens if sulphate is present as an electron acceptor while Van den Brand, et al. (2015) reported a high prevalence of SRB in activated sludge from Harnaschpolder WWTP as result of it being present in the influent together with SO_4^{2-}. van Lier, et al. (2008) asserted that in the presence of SO_4^{2-} , its reduction by SRB is inevitable no matter the interventions to steer the competition in single reactor settings. Our study never determined sulphate in the sampled Harnaschpolder RAS or after pre-treatment due to testing limitations and thus cannot verify this as the cause of the low methanogenesis but would be probably. This study's trend of reduction of methanogenesis appears closely related to the concentration of propionic acid increase relative to control at the end of pre-treatment, for example 4.54 mg H_2S/gVS had 30% extra propionic acid (-12% CH_4); 18.18 mg H_2S/gVS 22% propionic acid (-8% CH_4) and; 9.09 mg H_2S/gVS 18% propionic acid (-6% CH_4). At the end of the digestion, pH was tested and found to be favorable for methanogenesis at 7.37±0.03, 7.24±0.04, 7.18±0.03, 7.21±0.01 and 7.13±0.01 for the control, 4.54, 9.09, 16.63 and 18.15 mg H_2S/gVS pre-treated sludge.

The actual determination of the cause of the disproportionate methane production to hydrolysis can be determined by the measurement of SO_4^{2-}, HS^- , $S_2O_3^{2-}$, S and H_2S present at sampling of activated sludge and after pre-treatment. Tests in continuation of this entail actual determination of Sulfide production alongside the methane and appropriate mass balances to account for the SCOD and VFA consumption. More specialized tests like the Fluorescent in situ hybridization would be applicable in determining the anaerobic species as SRB.

CHAPTER 6

Conclusions and recommendations

Anaerobic digestion of waste activated sludge perpetually remains a complex and sensitive process requiring sufficient understanding of process behavior and operational competency. The development of cost efficient and sustainable pre-treatment techniques exploiting local pre-existing conditions lies at the heart of overcoming impediments to hydrolysis of suspended matter and organic solids, consequently increasing the methane production, off- setting operation costs and transforming WWTPs to energy factories.

The study evaluated the use of FNA, as a chemical pre-treatment to increase hydrolysis and consequently improve methanogenesis, however neither hydrolysis, nor methanogenesis was improved at pre-treatment concentrations of 0.04 mg HNO_2-N/gVS and 0.06 mg HNO_2-N/gVS. Nitrite is in fact both an alternative electron acceptor and a potent reversible inhibitor of methanogens and its carryover to anaerobic digestion after pre-treatment reduced rather than enhanced methane production. We recommend collaborative studies to be initiated with scholars reporting to the contrary that FNA pre-treatment is useful in improving methane production since that is rather fictitious based on our observations. Further recommendations are made to the mode of reporting FNA concentrations applied as mg HNO_2-N/gVS rather than mg HNO_2-N since the latter is contributory to the conundrum of inconsistent effects of FNA on WAS biodegradability and methane production being reported by different researchers. WAS characteristics will obviously differ but a biomass specific concentration of FNA pre-treatment will make comparability of different studies possible. Our findings further reaffirm the need for experimental evaluation of proposed process adjustments and reported technologies to improve methane production before plant wide application.

Sulfide pre-treatment of activated sludge at concentrations of 4.54-18.18 mg H_2S/gVS showed a significant increase in hydrolysis by the increased COD solubilization and VFA formation as acetic and propionic acid . However, methane production decreased considerably yet the study did not establish the cause. A recommendation is made to do thorough evaluation of Sulphur compounds in the sampled waste activated sludge and after its pre-treatment. Anaerobic digester testing for the readily biodegradable organic matter transformation and consumption with the specific microbial consortium associations is recommended to debunk the misalignment between the observed increased hydrolysis and methanogenesis reduction.

References

Abdeen S, Di W, Hui L, Chen G-H, van Loosdrecht MC (2010) Fecal coliform removal in a sulfate reduction, autotrophic denitrification and nitrification integrated (SANI) process for saline sewage treatment. Water science and technology 62: 2564-2570

Æsøy A, Ødegaard H, Bentzen G (1998) The effect of sulphide and organic matter on the nitrification activity in a biofilm process. Water Science and Technology 37: 115-122

Ahmad S, Reynolds D (1995) Synchronous fluorescence spectroscopy of wastewater and some potential constituents. Water research 29: 1599-1602

Ahring BK (1994) Status on science and application of thermophilic anaerobic digestion. Water Science and Technology 30: 241-249

Almeida J, Julio S, Reis M, Carrondo M (1995) Nitrite inhibition of denitrification by Pseudomonas fluorescens. Biotechnology and bioengineering 46: 194-201

Anthonisen A, Loehr R, Prakasam T, Srinath E (1976) Inhibition of nitrification by ammonia and nitrous acid. Journal (Water Pollution Control Federation): 835-852

Apha A (1998) Wpcf. Standard methods for the examination of water and wastewater 20

Appels L, Baeyens J, Degrève J, Dewil R (2008) Principles and potential of the anaerobic digestion of waste-activated sludge. Progress in Energy and Combustion Science 34: 755-781 DOI http://dx.doi.org/10.1016/j.pecs.2008.06.002

Aquino SF, Stuckey DC (2008) Integrated model of the production of soluble microbial products (SMP) and extracellular polymeric substances (EPS) in anaerobic chemostats during transient conditions. Biochemical Engineering Journal 38: 138-146

Aslanzadeh S (2014) Pretreatment of cellulosic waste and high rate biogas production. School of engineering, University of Borås

Aulenbach DB, Heukelekian H (1955) Transformation and effects of reduced sulfur compounds in sludge digestion. Sewage and Industrial Wastes: 1147-1159

Baier U, Schmidheiny P (1997) Enhanced anaerobic degradation of mechanically disintegrated sludge. Water Science and Technology 36: 137-143

Banihani Q, Sierra-Alvarez R, Field JA (2009) Nitrate and nitrite inhibition of methanogenesis during denitrification in granular biofilms and digested domestic sludges. Biodegradation 20: 801-812

Bannink H, Muller F (1951) On the sensitivity of the methane fermentation of calcium acetate towards some toxic substances. Antonie van Leeuwenhoek 17: 171-182

Baumann B, van der Meer JR, Snozzi M, Zehnder AJ (1997) Inhibition of denitrification activity but not of mRNA induction in Paracoccus denitrificans by nitrite at a suboptimal pH. Antonie van Leeuwenhoek 72: 183-189

Beccari M, Passino R, Ramadori R, Tandoi V (1983) Kinetics of dissimilatory nitrate and nitrite reduction in suspended growth culture. Journal (Water Pollution Control Federation): 58-64

Becker M, Martienssen M, Fuhrmann B, Spohn U (1997) Analyzing nitrogen and sulphide elimination from leachate by coulometric continuous flow titration. Acta biotechnologica 17: 39-50

Bejarano Ortiz DI, Thalasso F, Cuervo López FdM, Texier A-C (2013) Inhibitory effect of sulfide on the nitrifying respiratory process. Journal of Chemical Technology & Biotechnology 88: 1344-1349 DOI 10.1002/jctb.3982

Bentzen G, Smith A, Bennett D, Webster N, Reinholt F, Sletholt E, Hobson J (1995) Controlled dosing of nitrate for prevention of H2S in a sewer network and the effects on the subsequent treatment processes. Water Science and Technology 31: 293-302

Beristain-Cardoso R, Texier A-C, Razo-Flores E, Méndez-Pampín R, Gómez J (2009) Biotransformation of aromatic compounds from wastewaters containing N and/or S, by nitrification/denitrification: a review. Reviews in Environmental Science and Bio/Technology 8: 325-342

Boe K, Angelidaki I (2006) Online monitoring and control of the biogas process. Technical University of DenmarkDanmarks Tekniske Universitet, Department of Systems BiologyInstitut for Systembiologi

Bougrier C, Delgenes J, Carrere H (2007) Impacts of thermal pre-treatments on the semi-continuous anaerobic digestion of waste activated sludge. Biochemical Engineering Journal 34: 20-27

Brand T, Roest K, Brdjanovic D, Chen G, Loosdrecht M (2014) Influence of acetate and propionate on sulphate-reducing bacteria activity. Journal of applied microbiology 117: 1839-1847

Bundgaard E, Saabye A (1992) State of the art on sewage sludge: environmental aspects and regulations of common sludge disposal methodsINTERNATIONAL EXHIBITION CONGRESS OF SOLID WASTE (1992: Madrid) Annals Madrid: ISWA.

Burrell P, Keller J, Blackall LL (1999) Characterisation of the bacterial consortium involved in nitrite oxidation in activated sludge. Water science and technology 39: 45-52

Butlin K, SELWYN SC, Wakerley D (1956) SULPHIDE PRODUCTION FROM SULPHATE-ENRICHED SEWAGE SLUDGES. Journal of Applied Microbiology 19: 3-15

Canales A, Pareilleux A, Rols JL, Goma Gr, Huyard A (1994) Decreased sludge production strategy for domestic wastewater treatment. Water Science and Technology 30: 97-106

Carballa M, Duran C, Hospido A (2011) Should we pretreat solid waste prior to anaerobic digestion? An assessment of its environmental cost. Environmental science & technology 45: 10306-10314

Carlsson S, Wiklund N, Engstrand L, Weitzberg E, Lundberg J (2001) Effects of pH, nitrite, and ascorbic acid on nonenzymatic nitric oxide generation and bacterial growth in urine. Nitric oxide 5: 580-586

Carrère H, Dumas C, Battimelli A, Batstone D, Delgenès J, Steyer J, Ferrer I (2010) Pretreatment methods to improve sludge anaerobic degradability: a review. Journal of hazardous materials 183: 1-15

Chen Y, Cheng JJ, Creamer KS (2008) Inhibition of anaerobic digestion process: a review. Bioresource technology 99: 4044-4064

Colleran E, Finnegan S, Lens P (1995) Anaerobic treatment of sulphate-containing waste streams. Antonie van Leeuwenhoek 67: 29-46

Comeau Y, Hall K, Hancock R, Oldham W (1986) Biochemical model for enhanced biological phosphorus removal. Water Research 20: 1511-1521

Daims H, Nielsen JL, Nielsen PH, Schleifer K-H, Wagner M (2001) In Situ Characterization ofNitrospira-Like Nitrite-Oxidizing Bacteria Active in Wastewater Treatment Plants. Applied and environmental microbiology 67: 5273-5284

Darnell J, Jefferson M (1994) New renewable energy resources: a guide to the future. World Energy Council, London, England

Dunn MS, Schmidt CL (1922) The influence of position and of temperature upon the reaction of aliphatic amino nitrogen with nitrous acid. Journal of Biological Chemistry 53: 401-410

Erguder TH, Boon N, Vlaeminck SE, Verstraete W (2008) Partial nitrification achieved by pulse sulfide doses in a sequential batch reactor. Environmental science & technology 42: 8715-8720

Fermoso FG, Bartacek J, Jansen S, Lens PN (2009) Metal supplementation to UASB bioreactors: from cell-metal interactions to full-scale application. Science of the total environment 407: 3652-3667

Fermoso FG, Collins G, Bartacek J, O'Flaherty V, Lens P (2008) Acidification of methanol-fed anaerobic granular sludge bioreactors by cobalt deprivation: Induction and microbial community dynamics. Biotechnology and bioengineering 99: 49-58

Fernandes TV, van Lier JB, Zeeman G (2015) Humic acid-like and fulvic acid-like inhibition on the hydrolysis of cellulose and tributyrin. BioEnergy Research 8: 821-831

Foladori P, Andreottola G, Ziglio G (2010) Sludge reduction technologies in wastewater treatment plants IWA publishing

Ghyoot W, Verstraete W (1997) Anaerobic digestion of primary sludge from chemical pre-precipitation. Water Science and Technology 36: 357-365

Gonzalez-Gil G, Jansen S, Zandvoort MH, van Leeuwen HP (2003) Effect of yeast extract on speciation and bioavailability of nickel and cobalt in anaerobic bioreactors. Biotechnology and Bioengineering 82: 134-142

Grady Jr CL, Daigger GT, Love NG, Filipe CD (2011) Biological wastewater treatment CRC press

Halliwell B, Hu M-L, Louie S, Duvall TR, Tarkington BK, Motchnik P, Cross CE (1992) Interaction of nitrogen dioxide with human plasma antioxidant depletion and oxidative damage. FEBS letters 313: 62-66

Hansen KH, Angelidaki I, Ahring BK (1998) Anaerobic digestion of swine manure: inhibition by ammonia. Water research 32: 5-12

Harrison ST (1991) Bacterial cell disruption: a key unit operation in the recovery of intracellular products. Biotechnology Advances 9: 217-240

Hellinga C, Van Loosdrecht M, Heijnen J (1999) Model based design of a novel process for nitrogen removal from concentrated flows. Mathematical and computer modelling of dynamical systems 5: 351-371

Hinze H, Holzer H (1986) Analysis of the energy metabolism after incubation of Saccharomyces cerevisiae with sulfite or nitrite. Archives of microbiology 145: 27-31

Holliger C, Alves M, Andrade D, Angelidaki I, Astals S, Baier U, Bougrier C, Buffière P, Carballa M, de Wilde V (2016) Towards a standardization of biomethane potential tests. Water Science and Technology: wst2016336

Horton D, Philips KD (1973) The nitrous acid deamination of glycosides and acetates of 2-amino-2-deoxy-D-glucose. Carbohydrate Research 30: 367-374

Hwang MH, Jang NJ, Hyun SH, Kim IS (2004) Anaerobic bio-hydrogen production from ethanol fermentation: the role of pH. Journal of Biotechnology 111: 297-309

Jiang G, Gutierrez O, Sharma KR, Keller J, Yuan Z (2011a) Optimization of intermittent, simultaneous dosage of nitrite and hydrochloric acid to control sulfide and methane productions in sewers. Water research 45: 6163-6172

Jiang G, Gutierrez O, Yuan Z (2011b) The strong biocidal effect of free nitrous acid on anaerobic sewer biofilms. Water Research 45: 3735-3743 DOI http://dx.doi.org/10.1016/j.watres.2011.04.026

Joye SB, Hollibaugh JT (1995) Influence of sulfide inhibition of nitrification on nitrogen regeneration in sediments. Science 270: 623

Karhadkar P, Audic J-M, Faup G, Khanna P (1987) Sulfide and sulfate inhibition of methanogenesis. Water Research 21: 1061-1066

Kelessidis A, Stasinakis AS (2012) Comparative study of the methods used for treatment and final disposal of sewage sludge in European countries. Waste management 32: 1186-1195

Klüber HD, Conrad R (1998) Inhibitory effects of nitrate, nitrite, NO and N2O on methanogenesis by Methanosarcina barkeri and Methanobacterium bryantii. FEMS Microbiology Ecology 25: 331-339

Koster I, Rinzema A, De Vegt A, Lettinga G (1986a) Sulfide inhibition of the methanogenic activity of granular sludge at various pH-levels. Water Research 20: 1561-1567

Koster IW, Rinzema A, de Vegt AL, Lettinga G (1986b) Sulfide inhibition of the methanogenic activity of granular sludge at various pH-levels. Water Research 20: 1561-1567 DOI http://dx.doi.org/10.1016/0043-1354(86)90121-1

Kroiss H, Plahl-Wabnegg F (1983) Sulfide toxicity with anaerobic waste water treatmentAnaerobic waste water treatment European symposium, pp. 72-85.

Lai TM, Shin J-K, Hur J (2011) Estimating the biodegradability of treated sewage samples using synchronous fluorescence spectra. Sensors 11: 7382-7394

Leenheer JA, Croué J-P (2003) Peer Reviewed: Characterizing Aquatic Dissolved Organic Matter. Environmental Science & Technology 37: 18A-26A DOI 10.1021/es032333c

Lepistö R, Rintala J (1996) Conversion of volatile fatty acids in an extreme thermophilic (76–80° C) upflow anaerobic sludgeblanket reactor. Bioresource technology 56: 221-227

Lettinga G, Pol LH (1991) UASB-process design for various types of wastewaters. Water science and technology 24: 87-107

Lewis HB, Updegraff H (1923) The reaction between proteins and nitrous acid. The tyrosine content of deaminized casein. Journal of Biological Chemistry 56: 405-414

Li X, Zhao J, Wang D, Yang Q, Xu Q, Deng Y, Yang W, Zeng G (2016) An efficient and green pretreatment to stimulate short-chain fatty acids production from waste activated sludge anaerobic fermentation using free nitrous acid. Chemosphere 144: 160-167

Lissens G, Thomsen AB, De Baere L, Verstraete W, Ahring BK (2004) Thermal wet oxidation improves anaerobic biodegradability of raw and digested biowaste. Environmental science & technology 38: 3418-3424

Liu T, Sung S (2002) Ammonia inhibition on thermophilic aceticlastic methanogens. Water Science and Technology 45: 113-120

Lu J, Gavala HN, Skiadas IV, Mladenovska Z, Ahring BK (2008) Improving anaerobic sewage sludge digestion by implementation of a hyper-thermophilic prehydrolysis step. Journal of Environmental Management 88: 881-889

Luo J, Feng L, Chen Y, Sun H, Shen Q, Li X, Chen H (2015) Alkyl polyglucose enhancing propionic acid enriched short-chain fatty acids production during anaerobic treatment of waste activated sludge and mechanisms. Water research 73: 332-341

Ma J, Yang Q, Wang S, Wang L, Takigawa A, Peng Y (2010) Effect of free nitrous acid as inhibitors on nitrate reduction by a biological nutrient removal sludge. Journal of hazardous materials 175: 518-523

Martell A, Smith R (1989) Other Organic Ligands. Critical Stability Constants, vol. 3 Plenum, New York.
Martienssen M, Schulze R, Simon J (1995) Capacities and limits of three different technologies for the biological treatment of leachate from solid waste landfill sites. Acta biotechnologica 15: 269-276
McCartney DM, Oleszkiewicz JA (1993) Competition between methanogens and sulfate reducers: effect of COD: sulfate ratio and acclimation. Water Environment Research 65: 655-664
Moody L, Burns R, Wu-Haan W, Spajic R (2009) Use of biochemical methane potential (BMP) assays for predicting and enhancing anaerobic digester performanceProceedings of the 44th Croatian and the 4th International Symposium on Agriculture, Opatija, Croatia, 16-20 February 2009.
Mortensen HD, Jacobsen T, Koch AG, Arneborg N (2008) Intracellular pH homeostasis plays a role in the tolerance of Debaryomyces hansenii and Candida zeylanoides to acidified nitrite. Applied and environmental microbiology 74: 4835-4840
Mudhoo A, Kumar S (2013) Effects of heavy metals as stress factors on anaerobic digestion processes and biogas production from biomass. International Journal of Environmental Science and Technology 10: 1383-1398
Nielsen PH, Keiding K (1998) Disintegration of activated sludge flocs in presence of sulfide. Water Research 32: 313-320
Nies DH (1999) Microbial heavy-metal resistance. Applied microbiology and biotechnology 51: 730-750
Novak JT, Carlson DA (1970) The kinetics of anaerobic long chain fatty acid degradation. Journal (Water Pollution Control Federation): 1932-1943
O'Leary V, Solberg M (1976) Effect of sodium nitrite inhibition on intracellular thiol groups and on the activity of certain glycolytic enzymes in Clostridium perfringens. Applied and environmental microbiology 31: 208-212
O'Reilly C, Colleran E (2005) Toxicity of nitrite toward mesophilic and thermophilic sulphate-reducing, methanogenic and syntrophic populations in anaerobic sludge. Journal of Industrial Microbiology and Biotechnology 32: 46-52
Oleszkiewicz J, Marstaller T, McCartney D (1989) Effects of pH on sulfide toxicity to anaerobic processes. Environmental Technology 10: 815-822
Oleszkiewicz J, Sharma V (1990) Stimulation and inhibition of anaerobic processes by heavy metals—a review. Biological Wastes 31: 45-67
Park J-W (1993) S-Nitrosylation of sulfhydryl groups in albumin by nitrosating agents. Archives of Pharmacal Research 16: 1-5
Paul E, Liu Y (2012) Biological sludge minimization and biomaterials/bioenergy recovery technologies Wiley Online Library
Pavlostathis S, Giraldo-Gomez E (1991) Kinetics of anaerobic treatment: a critical review. Critical Reviews in Environmental Science and Technology 21: 411-490
Pijuan M, Wang Q, Ye L, Yuan Z (2012) Improving secondary sludge biodegradability using free nitrous acid treatment. Bioresource Technology 116: 92-98 DOI http://dx.doi.org/10.1016/j.biortech.2012.04.016
Pijuan M, Ye L, Yuan Z (2010) Free nitrous acid inhibition on the aerobic metabolism of poly-phosphate accumulating organisms. Water research 44: 6063-6072
Qasim SR (1998) Wastewater treatment plants: planning, design, and operation CRC Press

Rake JB, Eagon R (1980) Inhibition, but not uncoupling, of respiratory energy coupling of three bacterial species by nitrite. Journal of bacteriology 144: 975-982
Rasmussen T, Brittain T, Berks BC, Watmough NJ, Thomson AJ (2005) Formation of a cytochrome c–nitrous oxide reductase complex is obligatory for N 2 O reduction by Paracoccus pantotrophus. Dalton Transactions: 3501-3506
Rehm H, Reed G, Puhler A, Stadler P (2000) Biotechnology, vol. 11A: Environmental processes I. Wiley, New York
Rinzema A, Lettinga G (1988) The effect of sulphide on the anaerobic degradation of propionate. Environmental Technology 9: 83-88
Roeleveld P, Roorda J, Schaafsma M (2010) NEWs: the Dutch roadmap for the WWTP of 2030 STOWA
Rowe J, Yarbrough J, Rake J, Eagon R (1979) Nitrite inhibition of aerobic bacteria. Current Microbiology 2: 51-54
Rubio-Rincón F, Lopez-Vazquez C, Welles L, van Loosdrecht M, Brdjanovic D (2016) Sulphide effects on the physiology of Candidatus Accumulibacter phosphatis type I. Applied microbiology and biotechnology: 1-12
Rudolfs W, Amberg HR (1952) White Water Treatment: II. Effect of Sulfides on Digestion. Sewage and Industrial Wastes: 1278-1287
Saad S, Welles L, Lopez-Vazquez C, van Loosdrecht M, Brdjanovic D (2013) Sulfide effects on the anaerobic kinetics of phosphorus-accumulating organisms preparation.
Schnurer A, Jarvis A (2010) Microbiological handbook for biogas plants. Swedish Waste Management U 2009: 1-74
Schulthess R, Kühni M, Gujer W (1995) Release of nitric and nitrous oxides from denitrifying activated sludge. Water research 29: 215-226
Sears K, Alleman J, Barnard J, Oleszkiewicz J (2004) Impacts of reduced sulfur components on active and resting ammonia oxidizers. Journal of Industrial Microbiology and Biotechnology 31: 369-378
Shimizu T, Kudo K, Nasu Y (1993) Anaerobic waste-activated sludge digestion–a bioconversion mechanism and kinetic model. Biotechnology and Bioengineering 41: 1082-1091
Sijbesma WF, Almeida JS, Reis MA, Santos H (1996) Uncoupling effect of nitrite during denitrification by Pseudomonas fluorescens: An in vivo 31P-NMR study. Biotechnology and bioengineering 52: 176-182
Smith EL (2009) The specificity of certain peptidases. Advances in Enzymology, edit by FF Nord 11: 191-257
Stamatelatou K, Tsagarakis KP (2015) Sewage Treatment Plants: Economic Evaluation of Innovative Technologies for Energy Efficiency IWA Publishing
Stefanie JOE, Visser A, Pol LWH, Stams AJ (1994) Sulfate reduction in methanogenic bioreactors. FEMS Microbiology Reviews 15: 119-136
Stouthamer A (1988) Bioenergetics and yields with electron acceptors other than oxygen Marcel Dekker, NY.
Strous M, Kuenen JG, Jetten MS (1999) Key physiology of anaerobic ammonium oxidation. Applied and environmental microbiology 65: 3248-3250
Stuckey DC, McCarty PL (1978) Thermochemical pretreatment of nitrogenous materials to increase methane yield; Stanford Univ., CA
Sung S, Liu T (2003) Ammonia inhibition on thermophilic anaerobic digestion. Chemosphere 53: 43-52

Tiehm A, Nickel K, Zellhorn M, Neis U (2001) Ultrasonic waste activated sludge disintegration for improving anaerobic stabilization. Water research 35: 2003-2009
Turovskiy IS, Mathai P (2006) Wastewater sludge processing John Wiley & Sons
Vadivelu VM, Keller J, Yuan Z (2006a) Effect of free ammonia and free nitrous acid concentration on the anabolic and catabolic processes of an enriched Nitrosomonas culture. Biotechnology and bioengineering 95: 830-839
Vadivelu VM, Yuan Z, Fux C, Keller J (2006b) The inhibitory effects of free nitrous acid on the energy generation and growth processes of an enriched Nitrobacter culture. Environmental science & technology 40: 4442-4448
Van den Brand T, Roest K, Chen G, Brdjanovic D, Van Loosdrecht M (2015) Occurrence and activity of sulphate reducing bacteria in aerobic activated sludge systems. World Journal of Microbiology and Biotechnology 31: 507-516
Van der Veen A, Fermoso FG, Lens P (2007) Bonding Form Analysis of Metals and Sulfur Fractionation in Methanol-Grown Anaerobic Granular Sludge. Engineering in Life Sciences 7: 480-489
van Lier JB (1996) Limitations of thermophilic anaerobic wastewater treatment and the consequences for process design. Antonie van Leeuwenhoek 69: 1-14
van Lier JB, Hulsbeek J, Stams AJ, Lettinga G (1993) Temperature susceptibility of thermophilic methanogenic sludge: implications for reactor start-up and operation. Bioresource technology 43: 227-235
van Lier JB, Mahmoud N, Zeeman G (2008) Anaerobic wastewater treatment. biological wastewater treatment, principles, modelling and design: 415-456
van Lier JB, Tilche A, Ahring BK, Macarie H, Moletta R, Dohanyos M, Pol LW, Lens P, Verstraete W, Management Committee of the IWAADSG (2001) New perspectives in anaerobic digestion. Water science and technology : a journal of the International Association on Water Pollution Research 43: 1-18
Visser A (1995) The anaerobic treatment of sulfate containing wastewater. Visser
Wang B, Wang S, Li B, Peng C, Peng Y (2014a) Integrating waste activated sludge (WAS) acidification with denitrification by adding nitrite (NO 2−). Biomass and Bioenergy 67: 460-465
Wang Q, Jiang G, Ye L, Yuan Z (2014b) Enhancing methane production from waste activated sludge using combined free nitrous acid and heat pre-treatment. Water Research 63: 71-80 DOI http://dx.doi.org/10.1016/j.watres.2014.06.010
Wang Q, Kuninobu M, Kakimoto K, Hiroaki I, Kato Y (1999) Upgrading of anaerobic digestion of waste activated sludge by ultrasonic pretreatment. Bioresource Technology 68: 309-313
Wang Q, Ye L, Jiang G, Jensen PD, Batstone DJ, Yuan Z (2013) Free nitrous acid (FNA)-based pretreatment enhances methane production from waste activated sludge. Environmental science & technology 47: 11897-11904
Weast R (1976) Handbook of Chemistry and Phystcs, 56th Edn CRC Press Cleveiand.
Weemaes MP, Verstraete WH (1998) Evaluation of current wet sludge disintegration techniques. Journal of Chemical Technology and Biotechnology 73: 83-92
Widdel F (1988) Microbiology and ecology of sulfate-and sulfur-reducing bacteria. Biology of Anaerobic Microorganisms: 469-585
Widdel F, Rouviere P, Wolfe R (1988) Classification of secondary alcohol-utilizing methanogens including a new thermophilic isolate. Archives of microbiology 150: 477-481

Wilhelm E, Battino R, Wilcock RJ (1977) Low-pressure solubility of gases in liquid water. Chemical reviews 77: 219-262

Wu W-m, Hickey R, Zeikus J (1991) Characterization of metabolic performance of methanogenic granules treating brewery wastewater: role of sulfate-reducing bacteria. Applied and environmental microbiology 57: 3438-3449

Ye L, Pijuan M, Yuan Z (2010) The effect of free nitrous acid on the anabolic and catabolic processes of glycogen accumulating organisms. Water research 44: 2901-2909

Yoon SS, Coakley R, Lau GW, Lymar SV, Gaston B, Karabulut AC, Hennigan RF, Hwang S-H, Buettner G, Schurr MJ (2006) Anaerobic killing of mucoid Pseudomonas aeruginosa by acidified nitrite derivatives under cystic fibrosis airway conditions. The Journal of clinical investigation 116: 436-446

Zandvoort M, Van Hullebusch E, Fermoso FG, Lens P (2006) Trace metals in anaerobic granular sludge reactors: bioavailability and dosing strategies. Engineering in Life Sciences 6: 293-301

Zhang C, Bennett GN (2005) Biodegradation of xenobiotics by anaerobic bacteria. Applied Microbiology and Biotechnology 67: 600-618

Zhang D, Chen Y, Zhao Y, Zhu X (2010) New sludge pretreatment method to improve methane production in waste activated sludge digestion. Environmental science & technology 44: 4802-4808

Zhang D, Verstraete W (2001) The anaerobic treatment of nitrite containing wastewater using an expanded granular sludge bed (EGSB) reactor. Environmental technology 22: 905-913

Zhang T, Wang Q, Ye L, Yuan Z (2016) Effect of free nitrous acid pre-treatment on primary sludge biodegradability and its implications. Chemical Engineering Journal 290: 31-36 DOI http://dx.doi.org/10.1016/j.cej.2016.01.028

Zhao J, Liu Y, Ni B, Wang Q, Wang D, Yang Q, Sun Y, Zeng G, Li X (2016) Combined Effect of Free Nitrous Acid Pretreatment and Sodium Dodecylbenzene Sulfonate on Short-Chain Fatty Acid Production from Waste Activated Sludge. Sci Rep 6: 21622 DOI 10.1038/srep21622

Zhao J, Wang D, Li X, Yang Q, Chen H, Zhong Y, Zeng G (2015) Free nitrous acid serving as a pretreatment method for alkaline fermentation to enhance short-chain fatty acid production from waste activated sludge. Water Research 78: 111-120 DOI http://dx.doi.org/10.1016/j.watres.2015.04.012

Zhou Y, Ganda L, Lim M, Yuan Z, Kjelleberg S, Ng WJ (2010) Free nitrous acid (FNA) inhibition on denitrifying poly-phosphate accumulating organisms (DPAOs). Applied microbiology and biotechnology 88: 359-369

Zhou Y, Oehmen A, Lim M, Vadivelu V, Ng WJ (2011) The role of nitrite and free nitrous acid (FNA) in wastewater treatment plants. Water research 45: 4672-4682

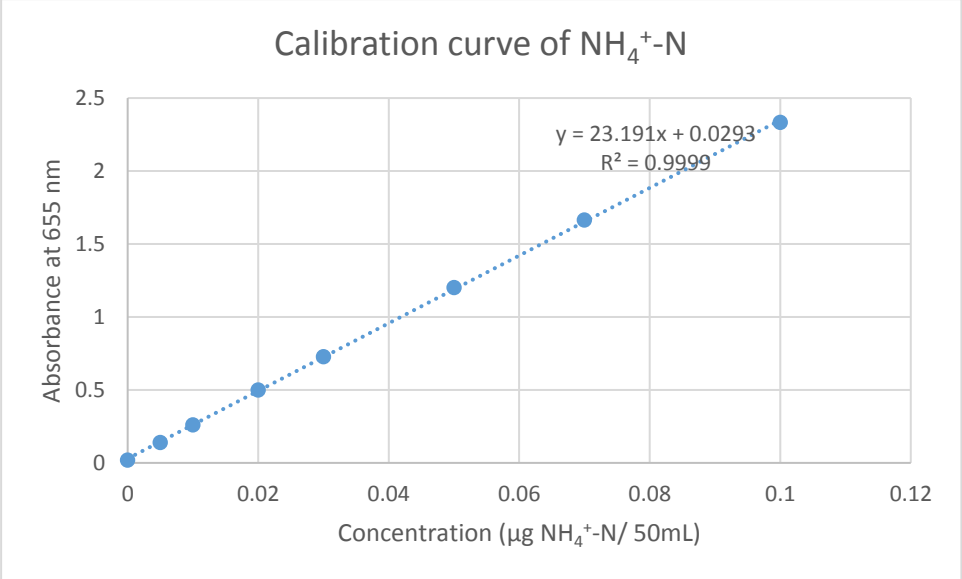
Zhou Y, Pijuan M, Zeng RJ, Yuan Z (2008) Free nitrous acid inhibition on nitrous oxide reduction by a denitrifying-enhanced biological phosphorus removal sludge. Environmental science & technology 42: 8260-8265

Internet source: https://www.slideshare.net/JephAnil/seminar-ppt-by-jeph

http://www.water-technology.net/projects/reading_sewage/reading_sewage2.html: Cover picture.

Appendix

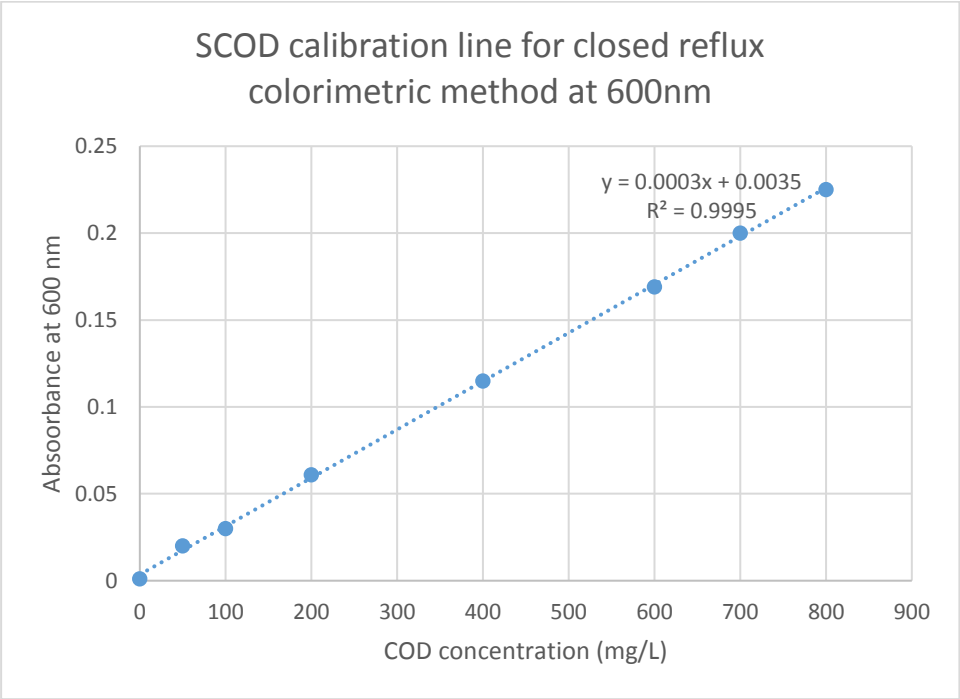
Appendix A: Ammonium Calibration Line



Appendix A-1: Ammonium results phase 1 Pre-treatment

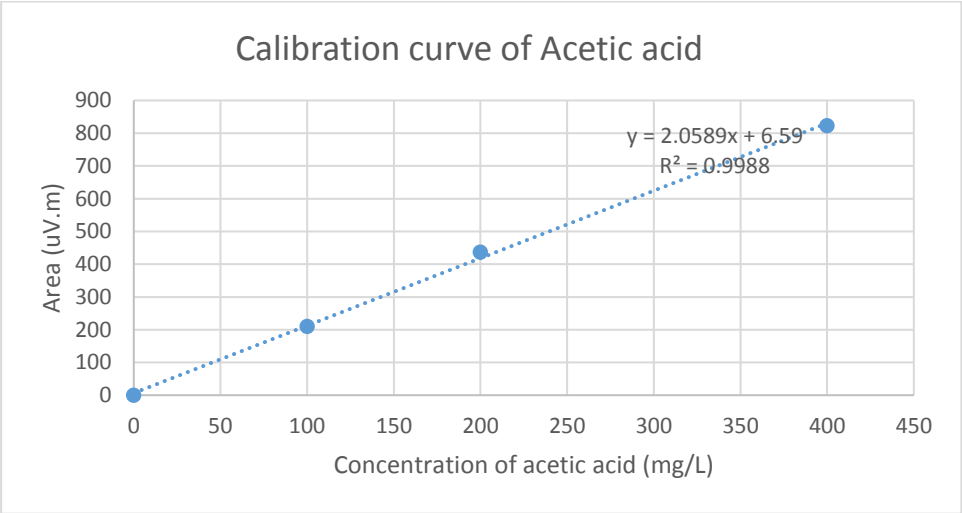
sample	**concentration (mg NH_4^+-N/L)**	**Biomass specific concentration (mg NH_4^+-N/gVS)**
Control	141.30	6.42
18.15 mg H_2S/gVS	165.88	7.54
24.27 mg H_2S/gVS	137.42	6.25
0.04 mg HNO_2-N/gVS	80.94	3.68
0.06 mg HNO_2-N/gVS	71.02	3.23

Appendix B: SCOD calibration line

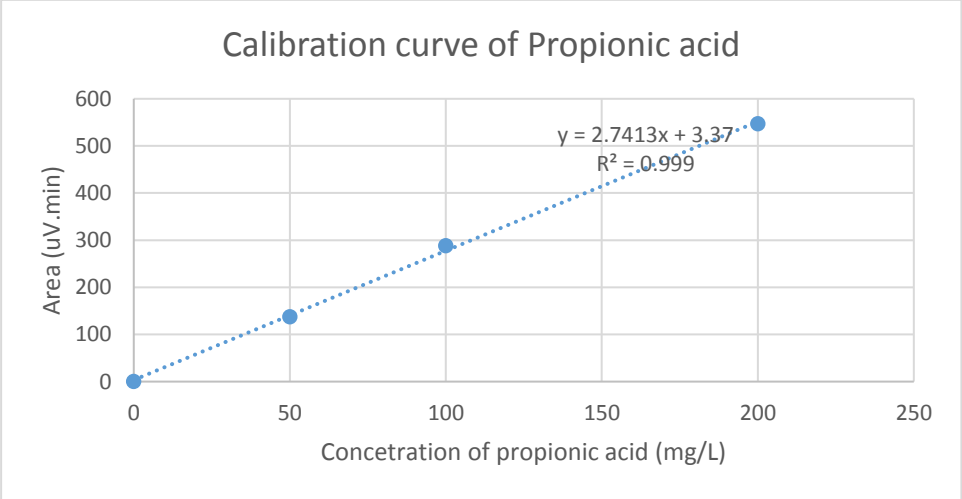


Appendix C: VFA calibration lines

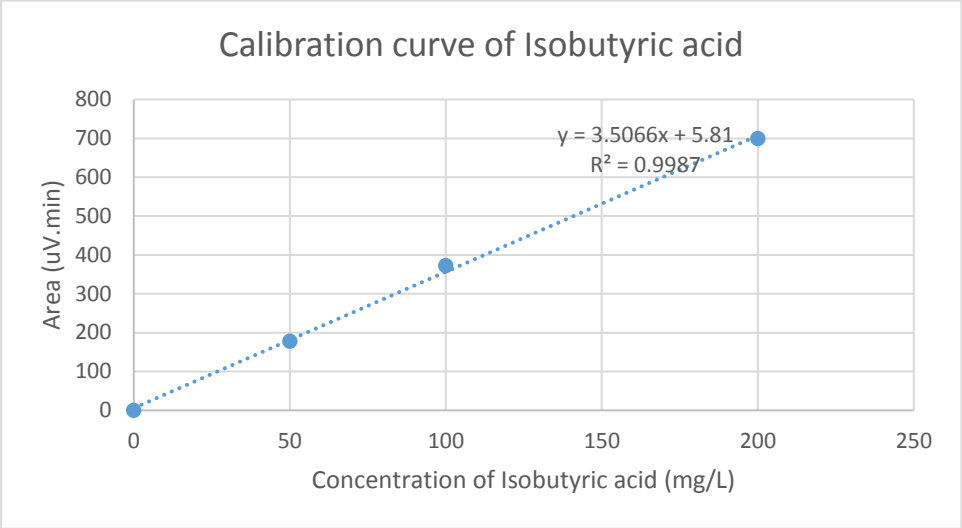
Appendix C-1: Acetic acid calibration line



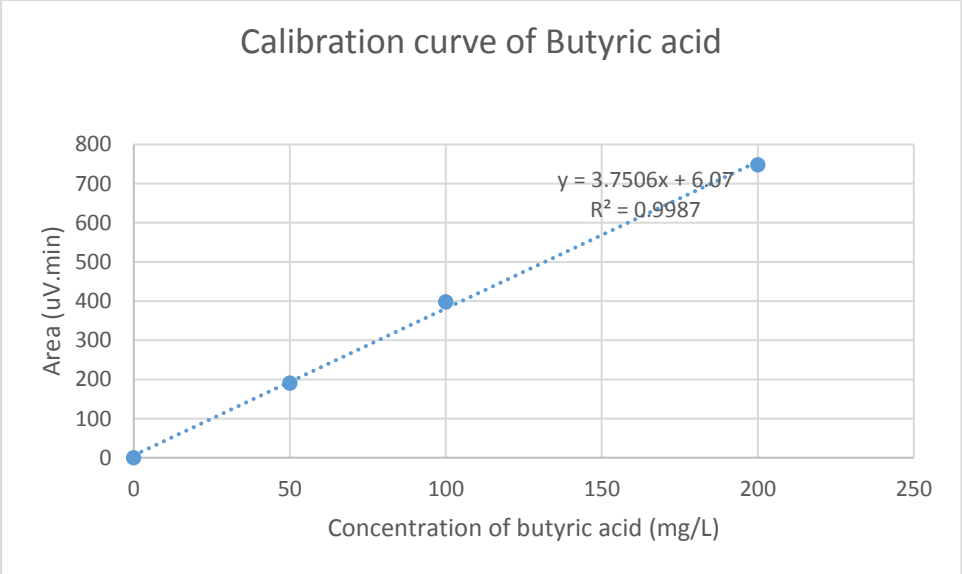
Appendix C-2: Propionic acid calibration line



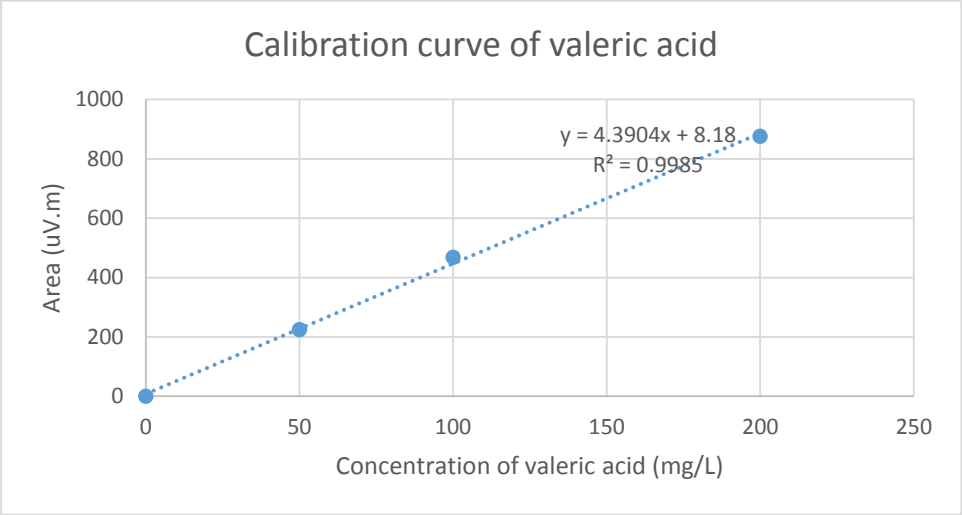
Appendix C-3: Isobutyric acid calibration line



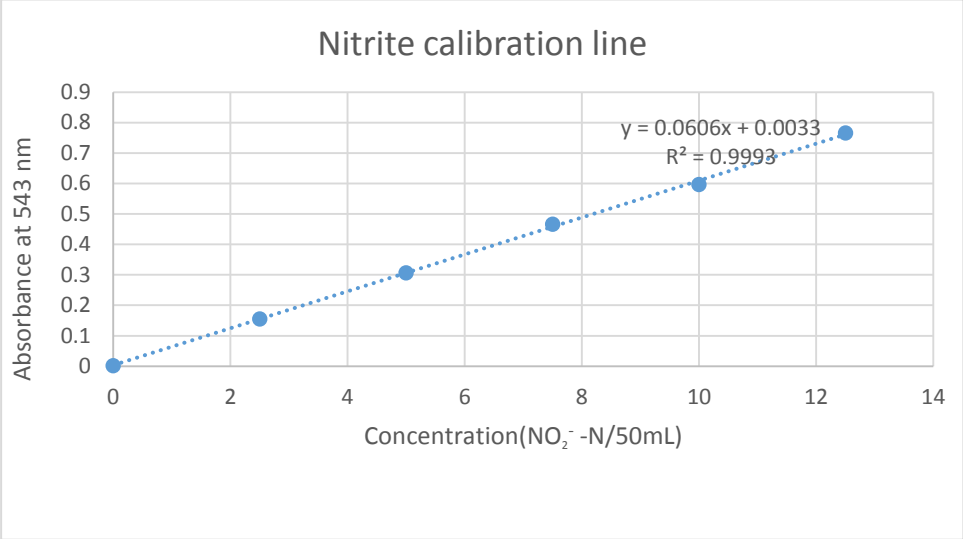
Appendix C-4: Calibration line of butyric acid



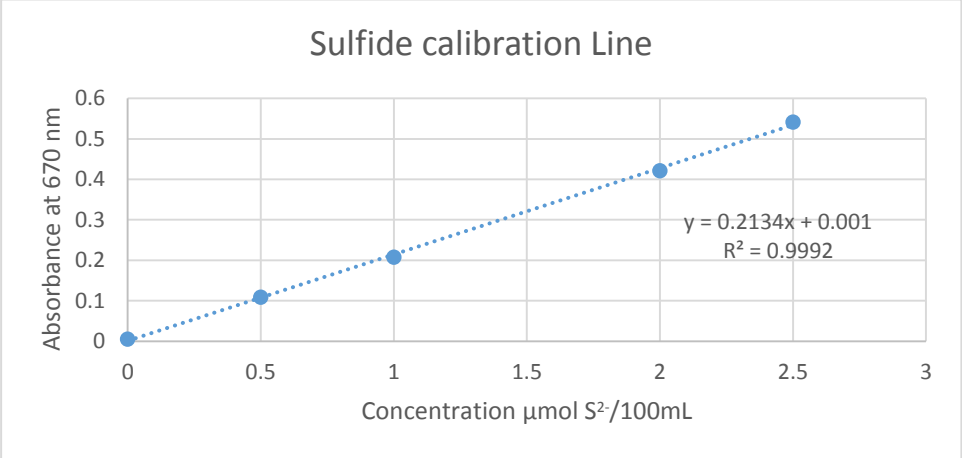
Appendix C-5: Calibration line of Valeric acid



Appendix D: Calibration line of Nitrite

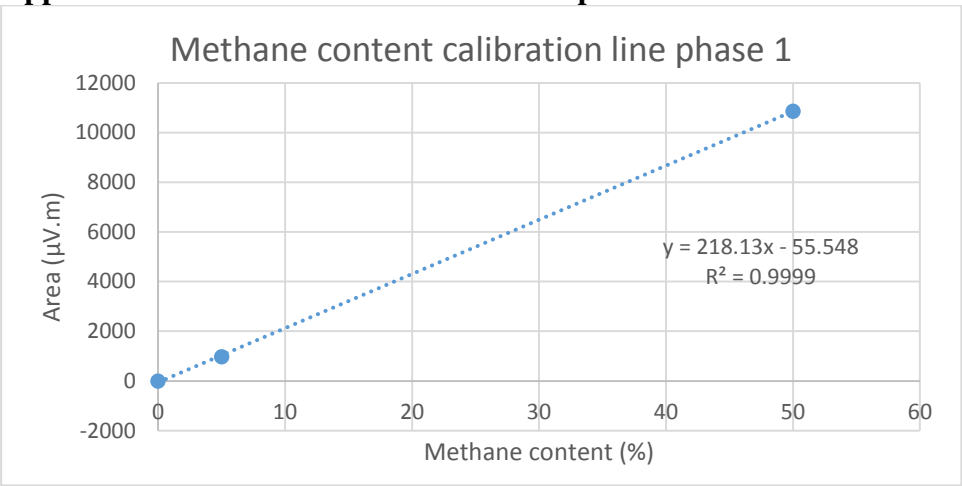


Appendix E: Sulfide calibration line

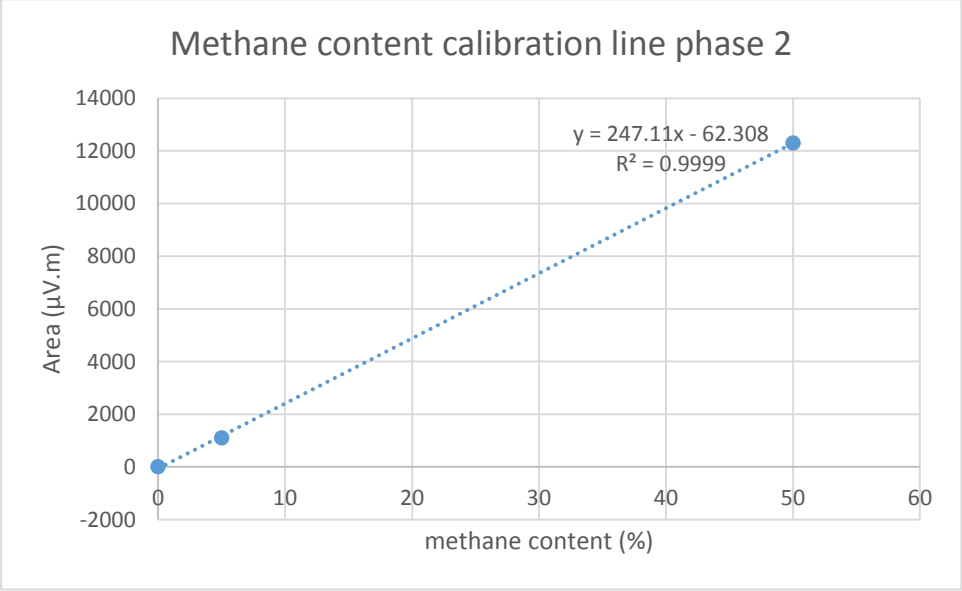


Appendix F: Methane calibration line

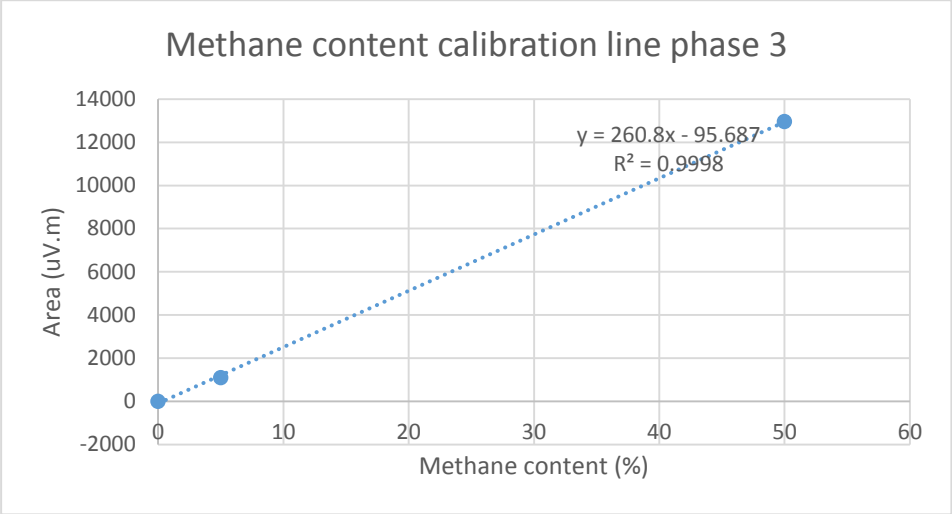
Appendix F-1: Methane calibration curve phase 1



Appendix F-2: Methane calibration curve phase 2



Appendix F-3: Methane calibration curve phase 3



Appendix G: BMP data

Appendix G-1: Average cumulative biogas production phase 1 (not corrected for N_2 gas from FNA)

Day	Control	18.15 mg H_2S/gVS	24.27 mg H_2S/gVS	0.042 mg HNO_2-N/gVS	0.06 mg HNO_2-N/gVS
0	0	0	0	0	0
1	83.55	56.80	73.80	45.90	41.45
2	162.5	105.50	136.95	59.95	52.15
3	245	158.35	206.50	68.65	59.10
4	303.45	215.45	266.65	76.00	63.90
5	347	271.85	309.80	85.00	70.65
6	387.25	316.35	345.80	94.75	77.95
7	425.10	353.80	380.95	103.25	84.65
8	445.90	386.95	409.45	109.8	90.70
9	459.30	415.20	423.40	116.5	95.90
10	472.90	433.70	436.40	124.3	102.25
11	485.05	445.80	447.15	133.05	109.10
12	496.20	456.10	457.40	142.85	116.05
13	503.65	462.35	463.55	149.75	122.15
14	509.50	468.80	468.90	156.9	127.25
15	516.15	475.60	475.90	166.45	134.70
16	523.35	482.70	483.15	179.40	144.65
17	529.45	488.65	490.20	195.70	156.65
18	535.75	495.40	497.00	219.15	174.10
19	541.3	501.95	503.15	252.90	199.40
20	545.45	506.10	508.30	293.80	231.55
21	548.85	509.90	513.25	345.95	278.35
22	551.20	512.35	516.90	392.70	329.90
23	553.50	514.75	519.30	428.60	372.40
24	555.75	517.10	521.70	457.50	401.60
25	558.55	520.05	524.70	479.15	419.20
26	561.75	524.4	527.80	497.75	432.95
27	567.35	529.55	532.00	520.15	451.75
28	571.45	534.05	536.60	534.05	467.50
29	574.60	537.50	540.15	544.20	479.10
30	576.50	540.55	543.25	556.6	492.35
31	577.35	541.55	544.60	563.50	503.50
32	578.15	542.55	546.00	571.10	514.30
33	579.00	543.50	547.35	584.70	525.40
34	579.85	544.50	548.70	596.40	533.15
35	580.70	545.50	550.10	608.90	539.65

Day	Control	18.15 mg H_2S/gVS	24.27 mg H_2S/gVS	0.042 mg HNO_2-N/gVS	0.06 mg HNO_2-N/gVS
36	581.50	546.50	551.45	616.20	547.70
37	582.35	547.50	553.35	622.70	554.30
38	583.15	548.50	555.90	632.95	562.15
39	584.00	549.15	558.50	642.75	572.20
40	584.80	549.90	560.80	646.95	580.00
41	585.35	550.85	562.00	650.50	581.75
42	585.35	551.75	563.25	652.10	582.90
43	585.35	552.70	564.45	653.75	583.65
44	585.35	553.60	565.65	655.35	583.65
45	585.35	553.80	566.25	656.05	583.65

Appendix G-2: Average cumulative methane production phase 1

Day	Control	18.15 mg H_2S/gVS	24.27 mg H_2S/gVS	0.04 mg HNO_2-N/gVS	0.06 mg HNO_2-N/gVS
0	0	0	0	0	0
1	83.55	56.80	73.80	34.55	29.47
2	162.50	105.50	136.95	45.13	37.08
3	245.00	158.35	206.50	51.67	42.02
4	303.45	215.45	266.65	57.21	45.43
5	347.00	271.85	309.80	63.98	50.23
6	387.25	316.35	345.80	71.32	55.43
7	425.10	353.80	380.95	77.72	60.19
8	445.90	386.95	409.45	82.65	64.49
9	459.30	415.20	423.40	87.70	68.19
10	472.90	433.70	436.40	93.57	72.70
11	485.05	445.8	447.15	100.16	77.58
12	496.20	456.10	457.40	107.53	82.52
13	503.65	462.35	463.55	112.73	86.86
14	509.50	468.80	468.90	118.11	90.48
15	516.15	475.60	475.90	125.30	95.78
16	523.35	482.70	483.15	135.05	102.86
17	529.45	488.65	490.20	147.32	111.39
18	535.75	495.40	497.00	164.97	123.80
19	541.30	501.95	503.15	190.38	141.79
20	545.45	506.10	508.30	221.17	164.65
21	548.85	509.9	513.25	260.43	197.93
22	551.20	512.35	516.90	295.62	234.59
23	553.50	514.75	519.3	322.65	264.81
24	555.75	517.10	521.70	344.40	285.57
25	558.55	520.05	524.70	360.70	298.09
26	561.75	524.40	527.80	374.70	307.87

Day	Control	18.15 mg H_2S/gVS	24.27 mg H_2S/gVS	0.04 mg HNO_2-N/gVS	0.06 mg HNO_2-N/gVS
27	567.35	529.55	532.00	391.56	321.23
28	571.45	534.05	536.60	402.03	332.43
29	574.60	537.50	540.15	409.67	340.68
30	576.50	540.55	543.25	419.00	350.11
31	577.35	541.55	544.60	424.20	358.03
32	578.15	542.55	546.00	429.92	365.71
33	579.00	543.50	547.35	440.16	373.61
34	579.85	544.50	548.70	448.96	379.12
35	580.70	545.50	550.10	458.37	383.74
36	581.50	546.50	551.45	463.87	389.46
37	582.35	547.50	553.35	468.76	394.16
38	583.15	548.50	555.90	476.48	399.74
39	584.00	549.15	558.50	483.86	406.89
40	584.80	549.90	560.80	487.02	412.43
41	585.35	550.85	562.00	489.69	413.68
42	585.35	551.75	563.25	490.90	414.50
43	585.35	552.70	564.45	492.14	415.03
44	585.35	553.60	565.65	493.34	415.03
45	585.35	553.80	566.25	493.87	415.03

Appendix G-3: Average Methane flow rates phase 1

Day	Control	18.15 mg H_2S/gVS	24.27 mg H_2S/gVS	0.04 mg HNO_2-N/gVS	0.06 mg HNO_2-N/gVS
0	0	0	0	0.00	0.00
1	83.55	56.80	73.80	32.75	29.48
2	78.95	48.65	63.15	10.24	7.61
3	82.55	52.85	69.55	6.45	4.94
4	58.45	57.10	60.20	5.27	3.41
5	43.55	56.40	43.15	6.65	4.80
6	40.20	44.50	35.90	6.98	5.19
7	37.85	37.40	35.20	6.15	4.76
8	20.80	33.15	28.50	5.02	4.30
9	13.40	28.25	14.00	4.99	3.73
10	13.60	18.50	13.00	6.27	4.52
11	12.10	12.15	10.75	6.65	4.87
12	11.15	10.25	10.25	7.03	4.94
13	7.45	6.20	6.15	5.19	4.34
14	5.85	6.50	5.30	5.22	3.63
15	6.70	6.80	7.00	7.38	5.30
16	7.20	7.15	7.25	10.21	7.04

Day	Control	18.15 mg H_2S/gVS	24.27 mg H_2S/gVS	0.04 mg HNO_2-N/gVS	0.06 mg HNO_2-N/gVS
17	6.10	5.95	7.05	13.20	8.50
18	6.30	6.75	6.85	18.87	12.41
19	5.55	6.55	6.15	26.87	17.99
20	4.20	4.15	5.20	32.40	22.86
21	3.40	3.85	4.95	39.45	33.28
22	2.35	2.40	3.65	32.75	36.66
23	2.25	2.40	2.40	22.56	30.26
24	2.30	2.40	2.40	18.64	20.73
25	2.85	3.00	2.95	15.08	12.55
26	3.20	4.30	3.10	12.82	9.78
27	5.55	5.15	4.20	14.65	13.37
28	4.10	4.50	4.55	9.23	11.16
29	3.15	3.45	3.55	6.90	8.25
30	1.90	3.00	3.15	8.78	9.42
31	0.85	1.00	1.40	5.02	7.93
32	0.85	1.00	1.40	4.89	7.68
33	0.85	1.00	1.40	8.93	7.89
34	0.85	1.00	1.40	7.53	5.51
35	0.85	1.00	1.40	7.83	4.66
36	0.85	1.00	1.40	5.47	5.72
37	0.85	1.00	1.85	7.20	4.73
38	0.85	1.00	2.55	8.98	5.58
39	0.85	1.00	2.55	7.73	7.15
40	0.85	0.80	2.55	2.11	5.51
41	0.85	0.90	1.20	2.11	4.98
42	0	0.90	1.20	0.80	0.78
43	0	0.90	1.20	0.80	0.78
44	0	0.90	1.20	0.80	0.00
45	0	0.90	1.20	0.80	0.00

Appendix G-4: Phase 2 BMP Methane accumulation

Day	0 mg H_2S/gVS	4.54 mg H_2S/gVS	9.09 mg H_2S/gVS	16.63 mg H_2S/gVS	18.18mg H_2S/gVS
0	0.00	0.00	0.00	0.00	0.0
1	72.20	42.50	76.50	76.90	75.50
2	144.30	84.10	152.85	154.30	151.90
3	220.30	131.80	227.75	229.90	220.60
4	272.00	176.40	279.25	278.80	262.50
5	312.10	214.90	320.90	320.70	299.50
6	351.00	250.20	360.30	357.30	329.80

Day	0 mg H_2S/gVS	4.54 mg H_2S/gVS	9.09 mg H_2S/gVS	16.63 mg H_2S/gVS	18.18mg H_2S/gVS
7	372.90	269.10	381.40	377.10	346.60
8	387.60	284.00	396.65	392.30	360.00
9	401.20	297.40	410.55	406.10	372.50
10	413.70	310.10	423.40	419.40	385.00
11	421.50	318.30	431.25	428.40	392.40
12	429.70	325.70	439.35	436.90	399.80
13	436.10	333.10	447.15	443.90	405.80
14	440.70	337.50	451.65	448.70	410.00
15	445.80	341.40	456.30	454.10	415.30
16	452.00	346.70	464.20	460.40	421.90
17	456.90	349.90	469.30	466.60	426.90
18	464.00	354.00	476.40	473.30	434.10
19	469.50	357.90	482.45	479.00	439.90
20	473.70	360.20	485.80	482.70	443.50
21	477.20	362.50	488.85	485.40	445.60
22	478.60	363.80	490.00	486.70	446.70
23	479.60	365.30	491.10	487.80	447.70
24	480.60	366.70	492.25	488.90	448.80
25	481.60	368.10	493.35	490.10	449.80
26	482.60	369.60	494.45	491.20	450.90
27	483.60	374.90	495.70	493.00	451.90
28	485.00	376.20	497.35	495.10	453.00
29	486.08	378.00	499.00	497.30	454.60
30	488.70	379.70	500.65	499.30	456.80
31	490.00	381.40	501.85	501.30	459.10
32	491.00	383.50	502.65	503.40	461.30
33	492.00	385.10	503.45	504.20	461.80
34	493.00	386.60	504.25	504.90	461.80
35	493.90	386.90	505.05	505.70	461.80
36	494.00	386.90	505.40	505.70	461.80

Appendix G-5: Phase 2 Methane flow rates

Day	0 mg H_2S/gVS	4.54 mg H_2S/gVS	9.09 mg H_2S/gVS	16.63 mg H_2S/gVS	18.18mg H_2S/gVS
0	0.00	0.00	0.00	0.00	0.00
1	72.20	42.50	76.50	76.90	75.50
2	72.10	41.70	76.40	77.40	76.40
3	76.00	47.70	74.85	75.60	68.70
4	51.70	44.60	51.45	49.00	41.90
5	40.10	38.60	41.70	41.90	37.00

Day	0 mg H_2S/gVS	4.54 mg H_2S/gVS	9.09 mg H_2S/gVS	16.63 mg H_2S/gVS	18.18mg H_2S/gVS
6	38.90	35.30	39.40	36.70	30.30
7	22.00	19.00	21.05	19.80	16.90
8	14.70	14.90	15.25	15.30	13.40
9	13.60	13.50	13.95	13.80	12.60
10	12.50	12.70	12.80	13.40	12.50
11	7.70	8.20	7.80	9.00	7.30
12	8.20	7.50	8.15	8.50	7.50
13	6.40	7.40	7.85	7.10	6.10
14	4.60	4.50	4.50	4.70	4.20
15	5.20	3.90	4.600	5.50	5.40
16	6.20	5.30	7.95	6.30	6.50
17	4.90	3.20	5.15	6.20	5.10
18	7.10	4.10	7.05	6.70	7.10
19	5.60	3.90	6.05	5.70	5.90
20	4.10	2.30	3.40	3.70	3.60
21	3.50	2.20	3.05	2.80	2.10
22	1.40	1.40	1.10	1.20	1.10
23	1.00	1.50	1.10	1.10	1.00
24	1.00	1.50	1.10	1.10	1.00
25	1.00	1.50	1.10	1.10	1.00
26	1.00	1.50	1.10	1.10	1.00
27	1.00	5.40	1.20	1.90	1.00
28	1.40	1.40	1.60	2.20	1.00
29	1.80	1.80	1.60	2.10	1.70
30	1.80	1.80	1.60	2.00	2.30
31	1.30	1.80	1.30	2.10	2.30
32	1.30	2.20	0.80	2.10	2.30
33	1.00	2.50	0.80	2.10	2.30
34	1.00	1.50	0.80	0.80	0.00
35	1.00	1.50	0.80	0.80	0.00
36	0.50	0.00	0.80	0.80	0.00

Appendix G-6: Phase 3 methane accumulation data

Day	0 mg H_2S/gVS	4.54 mg H_2S/gVS	9.09 mg H_2S/gVS	16.63 mg H_2S/gVS	18.18mg H_2S/gVS
0	0.00	0.00	0.00	0.00	0.00
1	112.85	108.35	122.20	126.50	102.90
2	194.60	193.40	210.45	229.80	189.35
3	275.10	271.40	292.40	317.15	267.75
4	352.65	329.40	354.85	379.80	326.15

5	411.65	390.65	412.20	447.30	385.55
6	474.85	458.70	479.15	519.40	454.10
7	546.85	497.55	530.80	553.85	503.15
8	586.45	527.90	561.60	585.00	535.70
9	619.15	547.45	588.05	604.40	562.00
10	647.35	563.65	605.30	620.85	579.80
11	665.55	578.60	620.50	636.15	596.60
12	679.35	591.80	632.85	650.25	609.40
13	691.70	602.75	642.30	661.45	620.60
14	703.15	613.00	653.05	673.00	632.75
15	713.20	623.70	660.85	683.20	641.30
16	718.40	628.40	667.00	689.05	647.65
17	725.30	634.65	675.45	698.00	656.70
18	734.15	643.25	684.25	708.20	665.90
19	744.55	654.50	695.00	721.25	677.85
20	749.8	660.95	702.05	728.45	686.60
21	752.15	662.10	707.00	730.80	692.00
22	752.70	662.10	707.85	730.80	692.00

Appendix G-7: Phase 3 Methane flow rates

Day	0 mg H_2S/gVS	4.54 mg H_2S/gVS	9.09 mg H_2S/gVS	16.63 mg H_2S/gVS	18.18mg H_2S/gVS
0	0.00	0.00	0.00	0.00	0.00
1	112.85	108.35	122.20	126.50	102.90
2	81.75	85.05	88.20	103.25	86.45
3	80.45	77.95	82.00	87.35	78.45
4	77.60	57.95	62.45	62.70	58.35
5	58.95	61.30	57.25	67.50	59.40
6	63.25	68.05	67.00	72.15	68.6
7	71.95	38.90	51.65	34.45	49.00
8	39.60	30.35	30.80	31.10	32.55
9	32.70	19.50	26.45	19.35	26.30
10	28.20	16.20	17.20	16.45	17.80
11	18.25	14.95	15.20	15.30	16.85
12	13.80	13.25	12.35	14.10	12.80
13	12.40	10.90	9.45	11.20	11.20
14	11.40	10.25	10.80	11.55	12.15
15	10.10	10.75	7.80	10.00	8.50
16	5.15	4.65	6.15	5.85	6.35
17	6.90	6.25	8.45	8.95	9.00
18	8.80	8.65	8.75	10.20	9.25

19	10.40	11.20	10.80	13.05	11.95
20	9.35	7.60	7.10	8.05	8.75
21	2.35	3.40	5.65	2.80	6.50
22	2.35	0.0	2.85	0.00	0.00