

## ABSTRACT

Title of Thesis: EFFECTIVENESS ASSESSMENT OF FNA IN  
IMPROVING SOLUBILIZATION AND  
CONTROLLING PROBLEMATIC  
MICROORGANISMS IN PRE-TREATMENT OF FOOD  
WASTE

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and Environmental Engineering

Anaerobic digestion (AD) is a sustainable waste-to-energy method for converting organic waste to methane. Various pre-treatment of food waste (FW) with free nitrous acid (FNA;  $pK_a=3.25$ ) were used to stabilize FW and mitigate obstacles (odor in FW storage and pathogenic bacteria) in AD. FNA concentrations ranging from 0-5 mg-N/L were tested in this study at pH ranging from 3-5.5, and volatile solids (VS) content of 16.8-135 g/L. Solubilization of organic material, inhibition of N-related biological processes, and control of problematic microorganisms, including sulfate-reducing bacteria (SRB) and pathogenic bacteria, were compared with the groups without FNA addition. At the tested pH (3, 4, and 5) and concentrations of FNA (0.3-5.0 mg-N/L), FNA addition showed inhibition to N-related biological processes contributing to a consistent FNA concentration over the 72 hours pre-treatment. However, it did not enhance the

solubilization of organic materials compared with control group without FNA addition. The FNA concentration affected the bacterial inhibition of SRB. As the concentration of FNA increased from 0.3-5.0 mg-N/L, so did SRB inhibition. Similarly, increasing FNA concentration resulted in a faster reduction (2.5 logs within 4 hours) in pathogenic bacteria compared with the group without FNA addition. Overall, a shorter pre-treatment time (<24 hours) is recommended for using low FNA concentration (1.5 mg N/L) and high solids content (> 80.57 g /L), as the effective FNA concentration cannot be maintained for longer due to  $\text{NO}_2^-$  and bioavailable organic carbon consumption by N-related or other microorganisms that remain active or recoverable under the added FNA concentration.

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CONTROLLING PROBLEMATIC MICROORGANISMS IN PRE-TREATMENT OF  
FOOD WASTE

by

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## Dedication

I would like to thank my family for their endless support, love, and encouragement. I could not be where I am today if not for my parents and sister, who constantly remind me to do anything I set my mind to. I would also like to thank Erik for constantly cheering me on.

Finally, I would like to thank my peers and mentors in the ENCE department. Patricia, Liu, Khashayar, Xiaojue, Rhuizhe, thank you for being good friends and helping me learn.

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Table of Contents	
Dedication.....	ii
Acknowledgements .....	iii
List of Tables .....	vii
List of Figures.....	viii
List of Abbreviations .....	x
Chapter 1: Introduction.....	1
1.1 Food Waste in Landfills .....	1
1.2 Anaerobic Digestion.....	3
1.3 Pre-treatment of Substrate for Anaerobic Digestion .....	8
1.3.1 Biological Pre-treatments .....	8
1.3.2 Mechanical Pre-treatments .....	9
1.3.3 Thermal Pre-treatments .....	9
1.3.4 Chemical Pre-treatments.....	10
1.3.5 Combined Pre-treatments .....	11
1.4 Free Nitrous Acids.....	12
1.5 Brief Summary of Previous Works .....	19
1.6 Research Objectives. ....	20
Chapter 2: Effectiveness of FNA Pre-Treatment in Stabilizing FW .....	21
2.1 Introduction .....	21
2.2 Materials and Methods .....	21
2.2.1 Food Waste .....	21
2.2.2 Experimental Design for FNA Pre-Treatment.....	22

2.2.3 Analysis .....	25
2.3 Results and Discussion .....	26
2.3.1 pH Change .....	26
2.3.2 Effective FNA concentration .....	27
2.3.3 Solubilization of FW .....	30
2.4 Conclusion .....	33
Chapter 3: Effectiveness of FNA Pre-treatment at Different FNA Dosing Ratios (mg FNA/g VS).....	35
3.1 Previous Work .....	35
3.2 Materials and Methods .....	36
3.2.1 Experimental Design .....	36
3.1.3 Analysis .....	37
3.2 Results and Discussion .....	38
3.2.1 Solids Contents and FNA:VS ratios .....	38
3.2.2 pH and Effective FNA Concentration .....	40
3.2.3 Solubilization of FW .....	46
3.3 Conclusion .....	47
Chapter 4: Inhibition of H <sub>2</sub> S Production and Reduction in Pathogenic Bacteria by FNA .....	48
4.1 Background Information.....	48
4.2 Materials and Methods .....	49
4.2.1 FW and WW Slurry.....	49
4.2.2 Experimental Design .....	50

4.2.3 Analytical Methods.....	52
4.2.4 Statistical Analysis .....	53
4.3 Results and Discussion.....	53
4.3.1 Sulfate Reducing Bacteria .....	53
4.3.2 Pathogenic bacteria.....	56
4.4 Conclusion.....	58
Chapter 5: Conclusion .....	60
Appendix A. Summary of Previous Works.....	62
FW only .....	62
Varying Mixture Ratios (SS:FW).....	64
Varying FNA Concentration .....	67
References .....	69

## List of Tables

Table 1. Synthetic FW recipe. The categories are listed as a total value, and the ingredients in each category are further broken down by percent of wet weight of the ingredient to the total weight of the synthetic FW. The recipe was adapted from the literature and from previous works (Buzby, Farah-Wells et al., 2014; Liu, 2021). ....	22
Table 2. Physical and chemical characteristics of synthetic FW. Measurements were conducted in triplicate, and standard deviation values are presented where available. ....	23
Table 3. Treatment groups for pH vs FNA experiment.....	24
Table 4. TS and VS of FW (high solids) at different mixing ratios with SS (low solids). As the percent of FW increases in the experimental group, so does the quantity of TS and VS. ....	36
Table 5. Experimental conditions for the different mixtures. The groups are named after their FW content (%v/v). The remainder of the treatment group is composed of FNA solution. ....	37
Table 6. Initial conditions in each reactor for the experiment testing FNA solution volume to volume of FW. Tests were conducted in triplicate, and the standard deviation is presented in the column next to each average value.....	38
Table 7. TS and VS values of treatment groups. ....	39
Table 8. Experimental setup for studying the effects of FNA (5 mg N/L) on reducing pathogen bacteria during the pre-treatment of FW at pH 5.5.....	51
Table 9. Experimental setup for studying the effects of various FNA concentrations on SRB during the pre-treatment of FW at pH 4.....	52
Table 10. Summary of previous experiments.....	63

## List of Figures

Figure 1. Processes involved in AD. The inputs for each step are in green, while the products of each step are in blue. ....	4
Figure 2. Summary of benefits of using AD for organic waste management (Wilkie, 2005).....	5
Figure 3. Anaerobic biodegradability of various components of FW. Figure created with BioRender.com (Steffen, Szolar et al., 1998).....	7
Figure 4. The proposed mechanisms by which FNA can interact with the cell membrane of bacterial cells, and lead to cell lysis. Figure created with BioRender.com (Noyes, W.A, 1943; Collins, 1971; Pine, S.H, 1987; Williams, 2004; Chislett, Guo et al., 2020). ....	14
Figure 5. A summary of the proposed used of FNA application to FW and SS for AD. Figure created with BioRender.com (Menon and Rao, 2012; Bai, Ghasemi Naghdi et al., 2014; Bai Xue, Bai et al., 2016; Parthiba Karthikeyan, Trably et al., 2018; Chislett, Guo et al., 2020). ....	18
Figure 6. Proposed interaction of FNA with each component of FW. Figure created with Biorender.com (Menon and Rao, 2012; Bai, Ghasemi Naghdi et al., 2014; Bai, Lant et al., 2016; Parthiba Karthikeyan, Trably et al., 2018; Chislett, Guo et al., 2020). ....	19
Figure 7. (A) Measured pH value at 24-hour sampling points. (B) Calculated FNA concentration (mg-N/ L), calculated using $\text{NO}_2^-$ measurements. * Concentration of $\text{NO}_2^-$ was below the detection limit of 0.05 mg $\text{NO}_2^-$ /L. Where available, error bars show the standard deviation of triplicate tests.....	27
Figure 8. $\text{NO}_2^-$ concentration in each reactor. The starting concentration was marked as the added $\text{NO}_2^-$ . * Concentration of $\text{NO}_2^-$ was below the detection limit of 0.05 mg $\text{NO}_2^-$ /L. Where available, error bars show the standard deviation of triplicate tests.....	28
Figure 9. $\text{NO}_3^-$ accumulation in the system. * $p < 0.001$ . Where available, error bars show the standard deviation of triplicate tests.....	30
Figure 10. Evidence of nitrogen gas production in the headspace of the reactors. Where available, error bars show the standard deviation of triplicate tests. ....	31
Figure 11. sCOD in each treatment group at 72 hours. The values were normalized per g VS to make direct comparisons between treatment groups. Where available, error bars show the standard deviation of triplicate tests. ....	32
Figure 12. (A) sCOD fluctuations with treatment time during the pre-treatment of FW with 5 mg FNA-N/L by adjusting pH to 5 at 35°C for 72 hours. (B) Solubilization of proteins and polysaccharides. The increase and decrease in sCOD, sP, and sPS follow similar trends. The solids content for this FW was $137 \pm 0.85$ g/L VS. Where available, error bars show the standard deviation of duplicate tests (Liu, 2021).....	33
Figure 13. (A) The TS of the reactor was divided by the total TS of the original synthetic FW. (B) The VS of the reactor was divided by the total VS of the original synthetic FW. In both cases, TS and VS, the resulting fraction is consistent with each treatment group's designed TS and VS content. The 90% FW groups with and without FNA deviate from the designed solid content. Where available, error bars show the standard deviation of triplicate tests.....	39

Figure 14. (A) pH of each treatment group as time progresses. (B) Calculated FNA concentration calculated using the pH and  $\text{NO}_2^-$  measurements. (C) Calculated FNA concentration per g VS. Where available, error bars show the standard deviation of triplicate tests. ....41

Figure 15. Nitrogen speciation in the treatment groups at each sampling time. \*Total nitrite nitrogen =  $\text{NO}_2^-$ -N + FNA-N (mg N/L). ....44

Figure 16.  $\text{NH}_3$  concentration was calculated from the ammonia measurement. Where available, error bars show the standard deviation of triplicate tests. ....45

Figure 17. sCOD oxygen demand per gram VS of FW. Where available, error bars show the standard deviation of triplicate tests. ....46

Figure 18. The calculated FNA concentration during Experiment 4. FNA concentration after 24 hours averaged  $0.013 \pm 0.001$  mg FNA-N/L. Where available, error bars show the standard deviation of triplicate tests. ....54

Figure 19. (A)  $\text{SO}_4^{2-}$  concentrations during experiment 4. (B).  $\text{H}_2\text{S}$  concentration during experiment 4. ....55

Figure 20. (A) Total coliforms in the treatment groups. (B) Total E. coli in the treatment groups. In both graphs, a dotted line marks the upper limit of detection. A dashed line marks the lower limit of detection. The detection limits of the graphs may be different from those of Fig. 21, as they are reported as microorganisms per g VS. The solids content here is 91.4 g /L VS. Additionally, the measurements that are marked at the upper limit of detection can be either at the limit or at a much higher population number. It is not possible to discern how much above higher than the upper detection limit this value may be. ....57

Figure 21. (A) Total Coliform bacteria. (B) Total E. coli. In both graphs, the maximum detection limit of the test is denoted with a dotted line. The minimum detection limit is denoted with a dashed line. The detection limits of the graphs may be different from those of Fig. 20, as they are reported as microorganisms per g VS. The solids content here is 78.8 g /L VS. ....58

Figure 22. Solids reductions during preliminary BMP test. (A) TS reduction in the different treatment groups. (B) VS reduction in the different treatment groups. The FNA treatment groups show a greater reduction in solids than the control groups. ...64

Figure 23. The percent of FW increases in each bottle from left to right. As the volume of the FW in the solution increases, the mixture's turbidity and viscosity increase. This increase in viscosity may lead to a decrease in the effectiveness of the FNA pre-treatment .....65

Figure 24. Solubilization of proteins and polysaccharides in each treatment ratio. And the corresponding solubilization of COD. ....66

Figure 25. Solubilization of organic material. Higher FNA concentrations produced higher increases in COD solubilization. ....68

## List of Abbreviations

AcoD	Anaerobic co-digestion
AD	Anaerobic digestion
AOB	Ammonia oxidizing bacteria
BMP	Biochemical methane potential
COD	Chemical oxygen demand
EPS	Extracellular polymeric substances
FNA	Free nitrous acids
FW	Food waste
GHG	Green house gas
LCFA	Long-chain fatty acids
MCE	Mushroom compost extracts
MPN	Most probable number
MSW	Municipal solid waste
NOB	Nitrite oxidizing bacteria
sCOD	Soluble chemical oxygen demand
sP	Soluble proteins
sPS	Soluble polysaccharides
SRB	Sulfate reducing bacteria
SS	Sewage sludge
tCOD	Total chemical oxygen demand
TS	Total solids
VFA	Volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids
WAS	Waste activated sludge
WW	Wastewater
WWTP	Wastewater treatment plant

## **Chapter 1: Introduction**

### **1.1 Food Waste in Landfills**

Food waste (FW) is any food product or food derivative part of food production and consumption (Okazaki, Turn et al., 2008). FW can be produced at various points in the food supply chain, such as the hospitality industry, industrial food preparation, agricultural sources, and households (Ho and Chu, 2019). Furthermore, FW can be categorized as pre-consumer and post-consumer products. Pre-consumption FW refers to the FW generated during food preparation, such as eggshells, vegetable peels, and bones. Meanwhile, post-consumption FW refers to raw or cooked uneaten food left after human consumption (Ho and Chu, 2019). It is important to note that frying oil and mineral oils are considered hazardous wastes for the hospitality industry and are typically disposed of through other waste management strategies (Pirani and Arafat, 2014).

The FW that ends in landfills poses environmental concerns due to its production of pollution and odors, which affects both ecosystem health and neighboring communities. In 2018, FW made up 21.6 % (63.13 million tons) of municipal solid waste (MSW) produced in the United States. Of these 63.13 million tons of FW, 55.9% (35.28 million tons) were landfilled, 28.1% (17.71 million tons) were managed as animal feed, bio-based products, and other methods like anaerobic digestion (AD). Furthermore, 11.9% (7.55 million tons) were combusted with energy recovery, and 4.1% (2.59 million tons) were composted (EPA, 2020).

Environmental problems of FW in landfills include greenhouse gas (GHGs) emissions such as carbon dioxide (CO<sub>2</sub>) and methane (CH<sub>4</sub>), which significantly contribute to climate change. Likewise, they pose environmental, economic, and social problems (Reichert, Small et al., 1992; Kharlamova, Mada et al., 2016). Under anaerobic conditions, hydrolytic microorganisms are capable of transforming complex organic substances (e.g. carbohydrates, lipids, and proteins) into monomers (amino acids, sugars, and fatty acids), which microbial communities can further consume with the end products of CO<sub>2</sub> and CH<sub>4</sub> (Bingemer and Crutzen, 1987). In 2019, in the United States, the production at landfills represented the third-largest source of CH<sub>4</sub> emissions (approximately 2.524x10<sup>11</sup> lbs CO<sub>2</sub> eq) (Reichert, Small et al., 1992; Kharlamova, Mada et al., 2016; EPA, 2021).

Better waste management can address environmental justice concerns. Communities located near landfills are more likely to be exposed to landfill gas and leachate thus placing them more at risk for the negative health effects of landfill exposure (Danthurebandara, Passel et al., 2013). For example, a case study of the Thohoyandou Landfill located in Limpopo Province, South Africa, reported that participants living closer to the landfill were more likely to report illnesses such as flu, eye irritation, and weakness of the body than those living far from the landfill (Njoku, Edokpayi et al., 2019). Environmental justice, pollution, and negative health effects are also a concern in the United States, where poor people and people of color are disproportionately affected by environmental inequality (Cannon, 2020).

Diversion of FW away from landfills has been considered a sustainable resource that produces biofuels, biogas, biomethane, biofertilizers, and other value-added chemicals (Talan, Tiwari et al., 2021). Reducing the volume of FW sent to landfills can prevent some of the adverse environmental effects of landfills, reduce the scarcity of land concerns, and save money for municipalities (in 2019, the United States' national average MSW landfill tip fee was USD 53.36/ton (ERF, 2019; Talan, Tiwari et al., 2021).

## **1.2 Anaerobic Digestion**

AD of FW is a promising method of waste management and has been widely used to treat organic solid waste like wastewater (WW), sewage sludge (SS), and animal manure (Xu, Li et al., 2018). FW is a suitable feedstock for AD due to its high organic content (19–346 g/L) and biodegradability (Yasin, Mumtaz et al., 2013; Talan, Tiwari et al., 2021). Biogas production through AD can be a profitable method of producing a renewable source of energy (Blokhina, Prochnow et al., 2011).

As shown in Fig. 1, AD is a four-step process: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Xiong, Yu et al., 2019). Each stage consumes the products of its predecessors and is associated with different functional microorganisms. Methanogens are responsible for the last step, where hydrogen and acetic acid are converted to CH<sub>4</sub> and CO<sub>2</sub>.

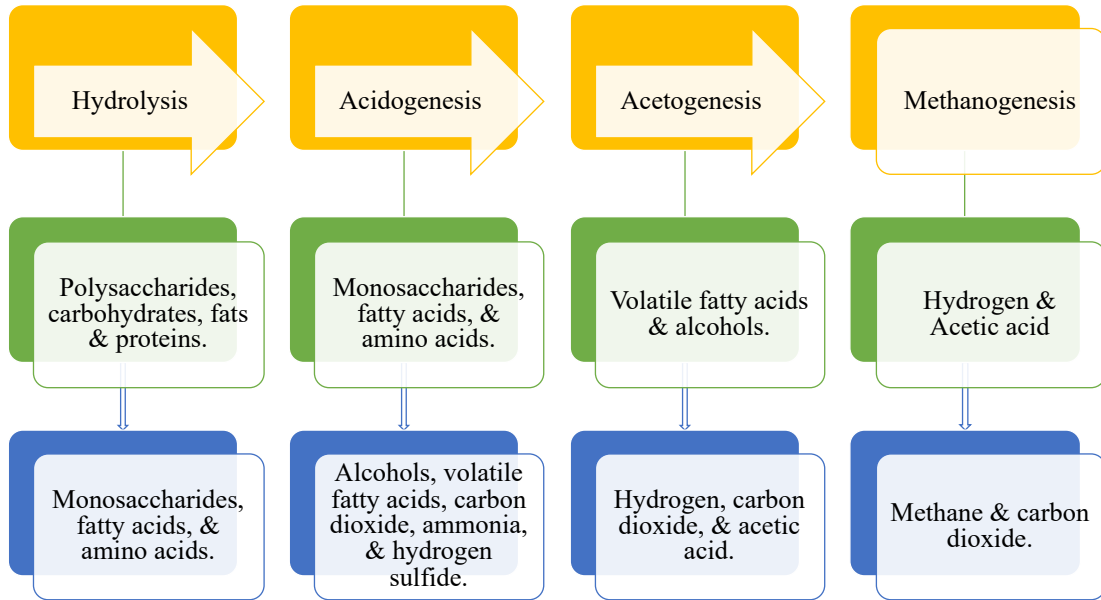


Figure 1. Processes involved in AD. The inputs for each step are in green, while the products of each step are in blue.

As summarized in Fig. 2, the benefits of AD include: 1) AD of organic wastes reduces their solid and chemical oxygen demand (COD) strength; 2) the effluent of AD can be used as a nutrient-rich fertilizer; and 3) the produced CH<sub>4</sub> can be captured as a renewable source of energy (Wilkie, 2005). AD of FW can also provide energy generation. A 2013 study in Australia found that total FW generated across multiple sites in Australia could generate 5.58x10<sup>8</sup> m<sup>3</sup> of CH<sub>4</sub> (energy equivalent of 20.3x10<sup>15</sup> J) (Lou, Nair et al., 2013). AD with energy recovery methods can reduce some of these GHG emissions. A study of disposal of organic waste in Madison, Wisconsin, USA found that co-digestion of organic waste with SS could reduce 189 kg CO<sub>2</sub>-eq/ton of GHG from being released into the atmosphere (Yoshida, Gable et al., 2012). Similarly, co-digestion with industrial waste could reduce 159 kg CO<sub>2</sub>-eq/ton, windrow composting 81.5 kg CO<sub>2</sub>-eq/ton, and high solids AD 46.0 kg CO<sub>2</sub>-eq/ton (Yoshida, Gable et al., 2012).

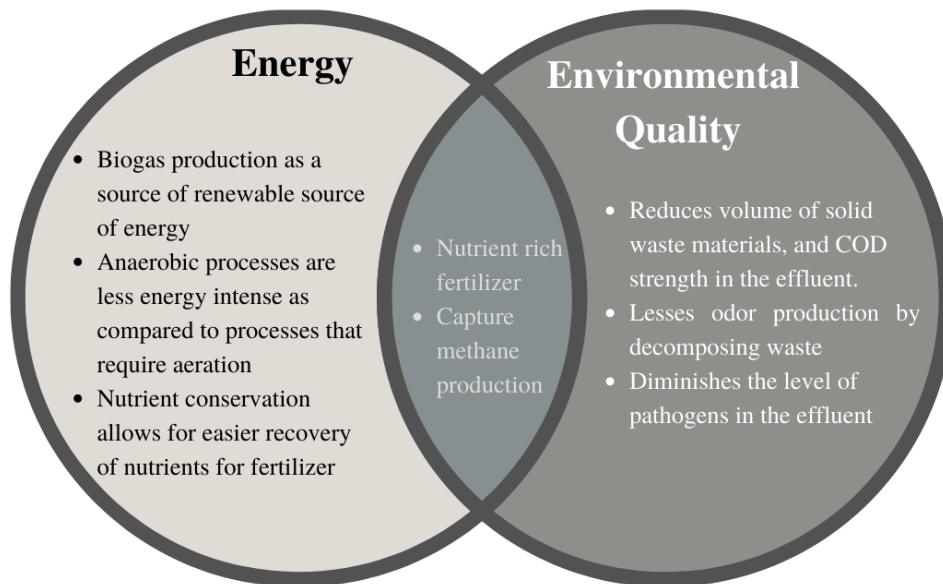


Figure 2. Summary of benefits of using AD for organic waste management (Wilkie, 2005).

Although it is beneficial, AD is not flawless. Challenges to AD include the accumulation of volatile fatty acids (VFA) and other metabolic intermediate products from the decomposition of organic substrates, which can inhibit key microbial activities, thus causing low CH<sub>4</sub> yield or foaming (Fagbohunge, Herbert et al., 2017; Xu, Li et al., 2018). An accumulation of VFA can cause the system to acidify, thus inhibiting CH<sub>4</sub> production (Chen, Cheng et al., 2008). Furthermore, the buildup of hydrogen sulfide (H<sub>2</sub>S) and ammonia (NH<sub>3</sub>) can cause microbial inhibition (Yuan and Zhu, 2016). Finally, the proliferation of long-chain fatty acids (LCFA) can cause the AD system to foam (Xu, Li et al., 2018).

An AD must operate under optimal conditions for the substrate it is handling. The C/N ratio and the organic loading rate can affect the success or failure of the system

(Grimberg, Hilderbrandt et al., 2015). Additionally, incomplete digestion in AD due to refractory and complex components in feed, short treatment time, odor and health risks caused by undesired biological processes can hinder the efficiency and implementation of AD in practice.

FW is a combination of both easy and hard biodegradable organic carbon, so an AD system must be calibrated to handle this type of substrate. Fig. 3 organizes the types of materials found in FW according to their ease of anaerobic biodegradability (Steffen, Szolar et al., 1998). When FW is used for AD, different types of carbon will be consumed at different rates. The readily biodegradable carbon sources will be consumed by the microbial community faster than less biodegradable components.

Not only do the operating parameters of an AD system have to be specific to the type of substrate it is handling, but also, the operating condition of the AD system should balance the various reactions responsible for AD. As Fig. 1 depicts, AD is a multifaceted set of reactions that relies on a varied microbial community (Calderon, Duan et al., 2021). Complex organics like carbohydrates, lipids, and proteins in FW cannot directly enter the cellular membrane and are not bioavailable for consumption (Tomei, Braguglia et al., 2009). Therefore, hydrolytic bacteria secrete extracellular enzymes to break down the polysaccharides, carbohydrates, fats, and proteins into monosaccharides, fatty acids, and amino acids, which are easier to consume (Meegoda, Li et al., 2018). However, hydrolysis is often cited as the rate-limiting step of the reaction (Calderon, Duan et al., 2021).

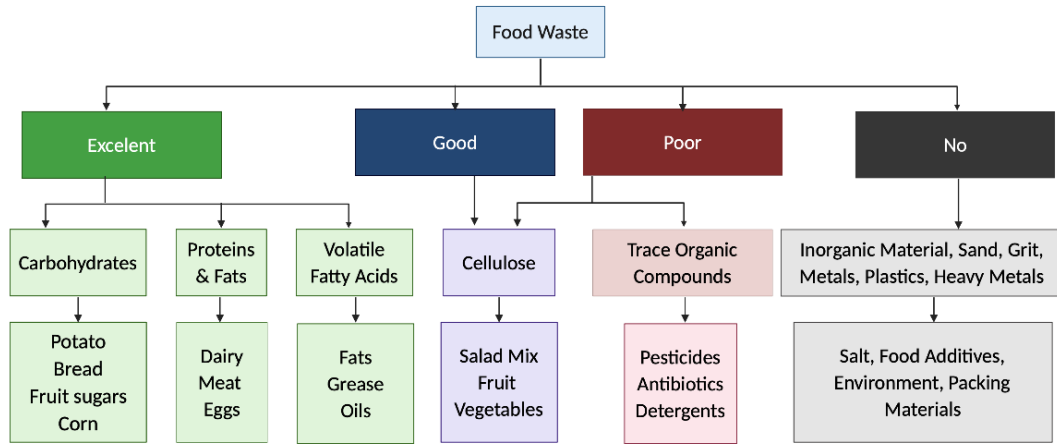


Figure 3. Anaerobic biodegradability of various components of FW. Figure created with BioRender.com (Steffen, Szolar et al., 1998).

The rate of biodegradation of the organic material depends on the rate of hydrolysis. Carbohydrates have the highest reported hydrolysis rate (0.025-0.200 day<sup>-1</sup>), followed by proteins (0.015-0.075 day<sup>-1</sup>), and the lowest hydrolysis is reported in lipids (0.005-0.010 day<sup>-1</sup>) (Christ, Wilderer et al., 2000). Different combinations of carbohydrates, proteins, and lipids can impact the specific methane yield of FW. For example, ice cream and used oil are high in lipid content, and readily biodegradable carbohydrates have the highest reported specific methane yield, 502.3, and 648.5 mL CH<sub>4</sub>/ g volatile solid (VS), respectively (Labatut, Angenent et al., 2011). In Fig. 3 food like potatoes, meat, and fats are classified as having excellent anaerobic biodegradability, because they can be easily consumed by microorganisms.

Meanwhile, fruits and vegetables have a high lignocellulosic fraction and low lipids content, thus having lower specific methane yield (Xu, Li et al., 2018). For example, cabbage has a specific methane yield of 256.6 mL CH<sub>4</sub>/ g VS (Labatut, Angenent et al., 2011). The structure of lignocellulosic biomass, like the one found in fruits and

vegetables, is composed of cellulose, hemicelluloses, and lignin and is assembled in a naturally recalcitrant structure enzymatic degradation (Zoghلامي and Paës, 2019). In Fig. 3, cellulose is classified as having good or poor anaerobic digestion because while cellulose can be easier to degrade, it is not always accessible due to its location in cells. Consequently, enhancing the rate of hydrolysis can aid in fully decomposing complex organic compounds during the designed AD reaction time and increase CH<sub>4</sub> production. For this purpose, different pre-treatments methods have been explored.

### **1.3 Pre-treatment of Substrate for Anaerobic Digestion**

Pre-treatments aim to change the characteristics and structure of the biomass thus making hard to degrade components like cellulose available for enzymatic degradation (Zhao, Zhang et al., 2012). Pre-treatments used to enhance AD can be categorized as biological, mechanical, thermal, and chemical. Many of the pre-pretreatments used combine one or more of the different types. Examples of pre-treatments are listed and discussed in the following section.

#### **1.3.1 Biological Pre-treatments**

Biological pre-treatments aim to augment the microbial communities responsible for AD and promote their activities via enzyme addition. For example, the microbial consortium BYND-9 was used to enhance the AD of corn stover (Zhao, Xu et al., 2019). The microbial consortium increased CH<sub>4</sub> yield by 62.85% at the peak phase compared to the untreated stover. In another work, the AD of pulp and paper sludge with monosodium glutamate waste liquor was pre-treated with active and inactive

mushroom compost extracts (MCE) (Lin, Wang et al., 2010). Active MCE showed better CH<sub>4</sub> production, VS destruction, and soluble chemical oxygen demand (sCOD) removal than inactive MCE. The optimal concentration of 250 A.U/g VS<sub>sludge</sub> active MCE increased CH<sub>4</sub> production by 34% compared to the untreated group.

### **1.3.2 Mechanical Pre-treatments**

Mechanical pre-treatments aim to enhance AD by rupturing cellular membranes and increasing the substrate's solubility by altering the particle size. For example, waste activated sludge (WAS) was treated by jetting and colliding it to a collision plate at 30 bar, allowing a decrease in digester sludge retention time from 13 to 6 days (Nah, Kang et al., 2000). Similarly, grinding the substrate to a particle size below 0.38 mm was reported to allow the AD of *Hybrid Pennisetum* to have enhanced CH<sub>4</sub> production to 291.9 L CH<sub>4</sub>/ kg VS compared to the untreated sample with the CH<sub>4</sub> production of 244 L CH<sub>4</sub>/ kg VS (Kang, Zhang et al., 2019).

### **1.3.3 Thermal Pre-treatments**

Thermal pre-treatments have added benefits of pathogen removal, improved dewaterability, and reduced digestate viscosity (Ariunbaatar, Panico et al., 2014). One example of thermal pre-treatment includes the treatment of WAS with temperature-phased AD. A thermophilic-mesophilic system achieved higher VS destruction and enhanced solubilization of organic material compared to a mesophilic-mesophilic system (Ge, Jensen et al., 2011). The substrate was pre-treated for two days at either thermophilic (50-70 °C) or mesophilic (35 °C) temperatures. The pre-treated substrate

was then fed into the primary mesophilic AD digester. Improved performance of the thermophilic-mesophilic system was attributed to increased hydrolysis rates. The thermophilic-mesophilic system achieved 41% VS destruction when the pre-treatment temperature was 60 °C and 48% VS destruction when the pre-treatment temperature was 70 °C. Meanwhile, the mesophilic temperatures achieved 37 % VS destruction (Ge, Jensen et al., 2011). A positive correlation between temperature and increases in sCOD was noted. For example, thermal pre-treatment of FW at 100°C for 30 minutes increased COD solubilization by 43.41% and CH<sub>4</sub> yield in AD by 23.68%, compared with the control group (Gnaoui, Karouach et al., 2020).

#### **1.3.4 Chemical Pre-treatments**

Chemical pre-treatments normally include the acidification and basification of substrates. For example, the organic fraction of MSW was treated with lime (Ca(OH)<sub>2</sub>) at concentrations varying from 40 to 100 mEq/L (López Torres and Espinosa Lloréns, 2008). The optimal treatment, 62.0 mEq Ca(OH)<sub>2</sub>/L applied for 6 hours, achieved 11.5% of the COD to solubilize. Furthermore, the highest CH<sub>4</sub> yield under AD of the pre-treated waste was 150 L CH<sub>4</sub>/kg VS (172.0% of the control). Other literature has explored an acidic pH as pre-treatment. Devlin et al. (2011) studied the effects of acidic pre-treatment using hydrochloric acid (HCl) at pH values of 1-6. A pH of 2 was the most effective in promoting dewaterability of the sludge and CH<sub>4</sub> yield (Devlin, Esteves et al., 2011).

### 1.3.5 Combined Pre-treatments

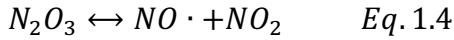
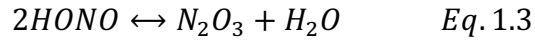
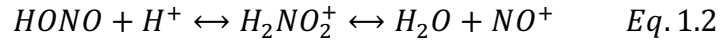
Different combinations of pre-treatment technologies have also been studied and introduced, particularly as treatments for refractory components. Such is the case for lignocellulosic feedstocks, which are often difficult to digest. For example, alkaline pre-treatment with sodium hydroxide (NaOH) was used to improve the solubilization and biodegradability (Pellera, Santori et al., 2016). The use of NaOH had a greater effect on solubilization than temperature alone. At room temperature, low doses of NaOH (0.25 mmol/g VS) were most effective at increasing CH<sub>4</sub> production potential (199 mL CH<sub>4</sub>/g VS<sub>added</sub>) compared with 196 mL CH<sub>4</sub>/g VS<sub>added</sub> in control. Meanwhile, a 22% higher CH<sub>4</sub> yield (242 mL CH<sub>4</sub>/g VS<sub>added</sub>) was achieved at a higher dose of NaOH (1 mmol NaOH/ g VS) at temperatures of 90 °C. Similarly, 6 hours at room temperature and 4 hours at 90 °C were the optimal treatment times. Additionally, alkaline (pH=10) and acidic (pH=2 and 5) treatments have been used to enhance the dissolution of WAS prior to AD (Tulun and Bilgin, 2019). The ratio of sCOD to total COD (tCOD) was used to measure the effectiveness of the pre-treatment. At pH=10, 10 minutes of treatment time, and 60 °C, the cumulative biochemical methane potential (BMP) was 47.7 mL CH<sub>4</sub>/ g VSS. While at pH=5, 15 minutes of treatment time, and 40 °C, the cumulative BMP was 12.3 mL CH<sub>4</sub>/ g VSS. The pH 2 treatment groups had the same or lower values than the control group, 105.1 and 90.5 mL CH<sub>4</sub>/g VSS at 50–60 °C.

Unfortunately, many pre-treatments mentioned above are cost and/or energy-intensive and may have negative environmental consequences due to unstainable and caustic materials. A life cycle assessment of various pre-treatments (pressurize-depressurize,

chemical, thermal, and ozonation) found that pressurize-depressurize and chemical pre-treatments could be recommended due to their beneficial net environmental performance. Meanwhile, thermal and ozonation pre-treatments require further optimization of energy use to reduce their environmental impacts (Carballa, Duran et al., 2011). A more sustainable alternative is needed to enhance AD's performance while minimizing the environmental impacts. In previous literature, free nitrous acid (FNA) was tested to pre-treat WAS and showed promising results in enhancing biogas yield in AD (Vadivelu, Keller et al., 2006; Zahedi, Icaran et al., 2016; Calderon, Duan et al., 2021). Furthermore, FNA can be recovered from ammonium-rich waste streams (e.g., the side stream of wastewater treatment plants (WWTP)) through partial nitrification and may be a method for stabilizing FW prior to AD with high sustainability.

#### **1.4 Free Nitrous Acids**

FNA (HONO) is the protonated form of nitrite ( $\text{NO}_2^-$ ), and a weak monobasic acid ( $\text{pK}_a=3.16$  at  $25^\circ\text{C}$ ) (da Silva, Kennedy et al., 2006). When FNA dissociates in acidic aqueous solutions (pH 5-7), it forms multiple species via the steps shown in Eq. 1.1-1.4, including nitrogen dioxide ( $\text{NO}_2$ ), nitrosonium cation ( $\text{NO}^+$ ), dinitrogen trioxide ( $\text{N}_2\text{O}_3$ ), and nitric oxide radical ( $\text{NO}\cdot$ ) (Oldreive and Rice-Evans, 2001; Takahama and Hirota, 2012). Its dissociation products, multiple reactive oxygen species, and reactive nitrogen species intermediates have shown evidence of biocidal properties (Duan, Gao et al., 2020).



Pre-treatment of AD feedstock with FNA is hypothesized to enhance the hydrolysis rate in AD by disintegrating the extracellular polymeric substances (EPS) and cellular membranes of bacteria. FNA was previously used as a pre-treatment method for alkaline fermentation to enhance short-chain fatty acid production from WAS (Zhao, Wang et al., 2015). The FNA pre-treatment showed accelerated disruption of the EPS and the cell envelope and enhanced the production of short-chain fatty acids. FNA at a concentration of 1.54 mg-N/L for 2 days achieved 370.1 mg COD/g VSS, which was 4.7 times more than the control and 1.5 times than that of the pH-only application. FNA pre-treatment has also been used for enhancing lipid extraction from algae used in biodiesel production (Bai, Ghasemi Naghdi et al., 2014). FNA concentrations up to 2.19 mg-N/L disrupted the algal cell envelope, with disruption increasing with FNA concentration and time of application. FNA treatment allowed the materials inside the cells to be more bioavailable.

Chislett, Guo et al., (2020) hypothesized that FNA causes cell lysis by bonding with the molecules of the cellular envelope in bacteria. In the study, molecules representative of the cell envelope (lypopolysaccharides, lipoproteins, peptidoglycans, and o-specific side chains) were treated with 6.09 mg FNA-N/L for 24 hours at a pH

of 5.0. FNA was able to break down the selected molecules via two proposed pathways: oxidative reactions with the nitrogen radicals ( $\cdot\text{NO}_2$  and  $\cdot\text{NO}$ ), and electrophilic substitution with  $\text{NO}^+$  as the reactive electrophile (Chislett, Guo et al., 2020). Fig. 4 summarizes these interactions. Furthermore, FNA can interact with functional groups like carboxylic acids, amines, alcohols, phenols and benzene rings, which may be found in cellular envelopes (Noyes, W.A, 1943; Collins, 1971; Pine, S.H, 1987; Williams, 2004; Chislett, Guo et al., 2020). Fig. 4 also lists the reactions with the functional groups found in the cell envelope of bacterial cells.

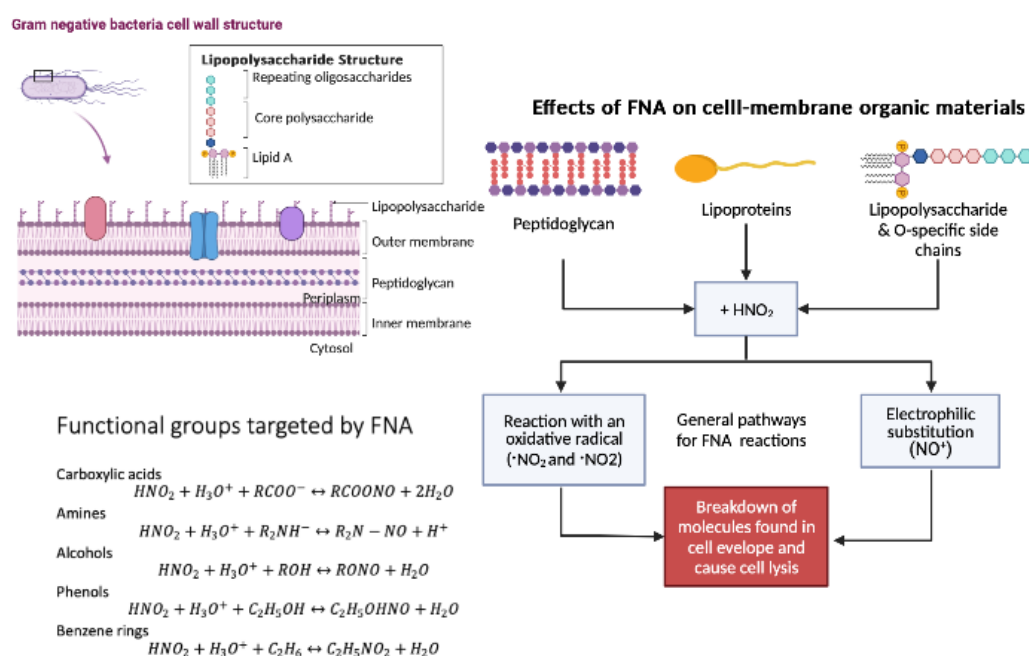


Figure 4. The proposed mechanisms by which FNA can interact with the cell membrane of bacterial cells, and lead to cell lysis. Figure created with BioRender.com (Noyes, W.A, 1943; Collins, 1971; Pine, S.H, 1987; Williams, 2004; Chislett, Guo et al., 2020).

Other works have studied the effects of heat combined with FNA pre-treatment at pH ranging from 5-6.5 and found that combining FNA with heat effectively enhances anaerobic CH<sub>4</sub> production (Wang, Jiang et al., 2014). Furthermore, FNA provided more

positive effects than acidification (Wang, Ye et al., 2013). WAS at a pH of 5.5 was treated with FNA at concentrations ranging from 0-2.13 mg FNA-N/L for 24 hours. Solubilization of WAS with FNA addition was 6 times that of the acidification-only group (no FNA addition). The highest FNA concentration, 2.13 mg FNA-N/L, had a COD content of 0.16 mg COD /mg VS, while the control group had 0.025 mg COD/mg VS.

FNA has also been used for other WW management applications. Inhibition of sulfate-reducing bacteria (SRB) in the presence of  $\text{NO}_2^-$ /FNA has been widely reported and applied in practice to prevent the corrosion and odor problems caused by the accumulation of  $\text{H}_2\text{S}$  in the sewer system (Duan, Gao et al., 2020; Zhong, Shi et al., 2020). When exposed to 4.0  $\mu\text{g}$  FNA-N/L for 1 hour, the SRB, e.g., *Desulfovibrio vulgaris* Hildenborough, experienced lowered ribosome activity and protein production and showed signs of oxidative stress. Furthermore, sulfate reduction and lactate oxidation coupled with ATP generation was suppressed, leading to energy starvation (Gao, Ho et al., 2016). The effects of FNA on *D. vulgaris* demonstrated an opportunity to use FNA as an antimicrobial agent for improved control of sewer corrosion and odor management. Six-month field studies have also shown that intermittent dosing of FNA or FNA combined with  $\text{H}_2\text{O}_2$  can disrupt sewer biofilms and be a cost-effective method for  $\text{H}_2\text{S}$  control in rising main sewers (Jiang, Keating et al., 2013). Furthermore, FNA at a concentration of 0.26 mg-N/L was dosed from a pumping station for 8 or 24 hours, and the dissolved sulfide concentration was measured downstream of the application.

The results showed that sulfide production was reduced by more than 80% in 10 days following each dose (Jiang, Keating et al., 2013).

The biocidal and inhibitory properties of FNA to broad microorganisms have also been noted. During the AD of WAS, FNA pre-treatment at concentrations of 0.52-1.43 mg-N/L and temperatures of 25 °C and 35°C achieved a 0.4-1.3 log killing in Fecal Coliform populations (Wang, Jiang et al., 2014). The reduction of pathogenic bacteria from the effluent of AD allows it to be used for Class A biosolids, a type of biosolids where the presence of pathogens has been reduced below detectable levels, and can be bagged and sold to the public if other requirements are met (National Research Council, 2002).

Similarly, thickened WAS pre-treated with FNA at concentrations of 4.9–6.1 mg-N/L for 24 hours, at a pH of 5.0, and temperatures of 22-30 °C saw a decrease in the most probable number (MPN) of Fecal Coliform populations during the pre-treatment stage. After AD, the untreated thickened WAS had a final fecal coliform concentration of 7 (log MPN/ g total solids (TS)). As a comparison, the treated thickened WAS had a final concentration of 4 (log MPN/ g TS) (Meng, Duan et al., 2020). Lastly, FNA application at concentrations of 0.5–2.0 mg N/L at treatment times of 8-48 hours caused a reduction in the viable fraction and biological activity of the biomass in secondary sludge, which was to be used for AD. After AD, the FNA treatment group saw a 90% reduction in biomass compared with a 41% reduction in the untreated group (Pijuan, Wang et al., 2012).

The farming and algal biofuel industries also take advantage of FNA pre-treatments. Pig manure has been co-digested with pre-treated algae (Astals, Musenze et al., 2015). Increased synergistic effects were reported when raw algae were co-digested with pig manure. CH<sub>4</sub> production increased from 163-245 L CH<sub>4</sub> /kg VS. FNA pre-treatment at concentrations of 0.3 g-N/L was used to release proteins in the algae and to improve lipid extraction yield from 0.14 to 0.19 kg lipids/kg VS. In the AD of algae, FNA pre-treatment at 2.31 mg-N/L increased CH<sub>4</sub> yield by 51% (from 161 to 250 L CH<sub>4</sub>/kg VS) (Bai Xue, Bai et al., 2016). Meanwhile, the solubilization of algal biomass increased up to 5.7 times with FNA pre-treatment.

A considerable amount of research has been conducted on the use of FNA in WWTP. Partly because FNA is readily available and accessible, as it can be recovered from AD digestate that contains high ammonium (0.8-1.2 g NH<sub>4</sub><sup>+</sup>-N/L) at WWTP, thus making FNA economically feasible (Duan, Gao et al., 2020). A feasibility study of the ability to produce FNA from the anaerobic sludge digestion liquor found more than 90% conversion of the ammonia (0.8 and 1.0 g NH<sub>4</sub><sup>+</sup>-N/L) to NO<sub>2</sub><sup>-</sup> (Law, Ye et al., 2015). Then acidification of this effluent led to the conversion of NO<sub>2</sub><sup>-</sup> to FNA. Therefore, making the recovery of FNA from WW feasible.

Analysis of the literature has demonstrated a knowledge gap in applying FNA pre-treatment to FW prior to AD. Considering that FNA pre-treatment has successfully enhanced CH<sub>4</sub> production and solubilized organic materials in other organic-rich

substrates (WAS, algae, and pig manure), it is relevant to understand the possibility of using FNA to enhance the biodegradability and stabilization of FW. The proposed uses of FNA application to FW and SS are summarized in Fig. 5.

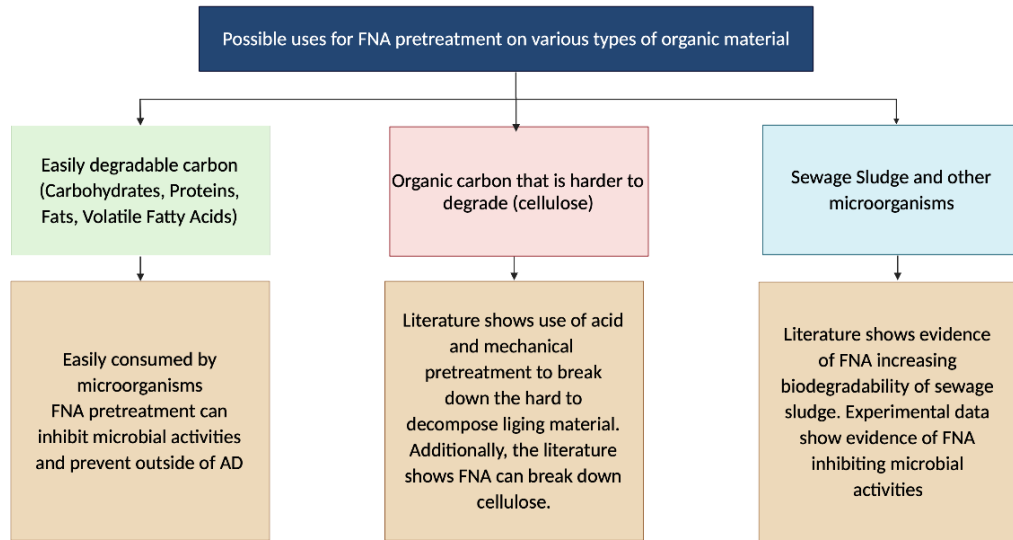


Figure 5. A summary of the proposed used of FNA application to FW and SS for AD. Figure created with BioRender.com (Menon and Rao, 2012; Bai, Ghasemi Naghdi et al., 2014; Bai Xue, Bai et al., 2016; Parthiba Karthikeyan, Trably et al., 2018; Chislett, Guo et al., 2020).

Due to the cellular composition of FW (animal, plant, and some bacterial cells), it is expected that the positive effects of FNA pretreatment will be less than those reported for SS and WAS. The solubilization of organic material and reported cell lysis in FW may not be as strong as that of WAS because SS has greater microbial biomass than FW. However, FNA pretreatment may be able to stabilize and preserve the organic carbon found in FW from consumption by the microbial community. Therefore solving one of the challenges of FW use in AD, the preservation of organic material prior to AD (Parthiba Karthikeyan, Trably et al., 2018). Additionally, FNA pre-treatment can be combined with mechanical and thermal pre-treatments to further break down the

recalcitrant organic materials in plant cells. Finally, FNA can inhibit troublesome microorganisms like SRB, pathogens, and bacteria responsible for N-related bioprocesses. The proposed effects of FNA pre-treatment on each component of FW is summarized in Fig. 6.


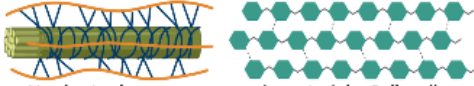


Food Waste Component	Proposed Effect of FNA Pre-treatment
 <p data-bbox="324 688 836 745">Readily biodegradable components: carbohydrates, proteins, fats, volatile fatty acids</p>	<p data-bbox="836 609 1347 661">Conservation of readily biodegradable carbon by inhibiting the consumption by the microbial community.</p>
 <p data-bbox="324 871 836 913">Harder to decompose organic materials: Cell wall components of plant cells (Cellulose, hemicellulose, lignin)</p>	<p data-bbox="836 787 1347 913">FNA will not be able to solubilize recalcitrant plant cell wall materials like lignin. Instead, these can be processed by mechanical pretreatment (blending to a small pore size and acidification). The simple cellulose can be broken down by FNA pre-treatment</p>
 <p data-bbox="324 1113 836 1186">Microbial community: pathogenic bacteria, sulfate reducing bacteria, and bacteria responsible for n-related bioprocesses.</p>	<p data-bbox="836 1018 1347 1113">FNA pretreatment will cause inhibition of the microbial community. The decomposition product of FNA will cause cell lysis by interacting with components of the cell envelope of bacterial cells.</p> 

Figure 6. Proposed interaction of FNA with each component of FW. Figure created with Biorender.com (Menon and Rao, 2012; Bai, Ghasemi Naghdi et al., 2014; Bai, Lant et al., 2016; Parthiba Karthikeyan, Trably et al., 2018; Chislett, Guo et al., 2020).

## 1.5 Brief Summary of Previous Works

This thesis is the continuation of previous work on the effectiveness of FNA in improving the biodigestibility of mixed feedstock (FW and SS) prior to anaerobic co-digestion (AcoD). The conclusions from previous work include (Liu, 2021):

1. FNA application in the tested concentration of 5.0 mg FNA-N/L is sufficient for improving the digestibility of the FW. The mixing conditions (continuous

vs. intermittent) during the pre-treatment did not show a further improvement of the biogas yield during AD.

2. Mixing FW with SS can provide increased buffer capacity of the system. FNA addition (5.0 mg FNA-N/L) to the mixture of FW and SS at the ratios of 75:25, 50:50, and 25:75 (% w/w VS) can increase the solubilization of organic material, including soluble proteins (sP) and soluble polysaccharides (sPS).
3. When SS and FW are treated at a ratio of 50:50 (%w/w VS), increasing FNA concentration (from 0.3 to 5.0 mg FNA-N/L) provided greater solubilization of COD.

### **1.6 Research Objectives.**

In this work, the goal was to advance the understanding of optimal conditions to use FNA pre-treatment for improving the quality of a selected FW mix prior to AD. In addition, the effectiveness of FNA addition on inhibiting or inactivating problematic microorganisms, including SRB and pathogens, was evaluated. This research was conducted by fulfilling the following objectives.

Objective 1: Differentiate the effects of acidification from the addition of FNA.

Objective 2: Find an optimal dosing ratio of FNA in pre-treating FW in terms of mg FNA to g VS of FW.

Objective 3: Understand the effects of FNA pre-treatment on problematic microorganisms in FW, including SRB and pathogens.

## **Chapter 2: Effectiveness of FNA Pre-Treatment in Stabilizing FW**

### **2.1 Introduction**

This experiment explores Objective 1 of the thesis, to differentiate the effects of acidification from those of FNA application for FW pre-treatment. As the FNA concentration is pH-dependent, the FNA application to FW needs to be evaluated by combining it with acid treatment. Combining an acidic pH with FNA pre-treatment allows higher concentrations of FNA to be achieved with a less added of  $\text{NO}_2^-$ .

### **2.2 Materials and Methods**

#### **2.2.1 Food Waste**

The synthetic FW recipe used for this experiment emulates the types of losses at the consumer level in the United States (Buzby, Farah-Wells et al., 2014). Table 1 lists the individual components in the recipe.

Synthetic FW was prepared by blending all the ingredients in a Waring Xtreme 3.5hp Hi-Power Blender for 2-3 minutes. DI water was added to facilitate the blending process. The homogenous FW slurry was passed through a #10 sieve (2 mm) to remove large particles. The FW was stored at  $-20\text{ }^\circ\text{C}$  until use.

The synthetic FW was characterized by measuring TS, VS, sCOD, tCOD, sPS, sP. Concentrations of  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , and  $\text{SO}_4^{2-}$  in the aqueous phase, and the total composition of organic carbon and nitrogen in the solid phase were also analyzed. The results are summarized in Table 2.

Table 1. Synthetic FW recipe. The categories are listed as a total value, and the ingredients in each category are further broken down by percent of wet weight of the ingredient to the total weight of the synthetic FW. The recipe was adapted from the literature and from previous works (Buzby, Farah-Wells et al., 2014; Liu, 2021).

Category	Category Total	Ingredients	Individual Percent
Fruit & Vegetable	73.2%	Apple peel	2.13%
		Apple cores	4.11%
		Banana peels	9.00%
		Banana fruit	1.91%
		Orange peels	5.87%
		Orange fruit	3.14%
		Potato peels	9.00%
		Salad mix	9.45%
		Cooked potato	9.00%
		Cooked corn with cob	9.00%
		Carrot	9.00%
		Canned white beans	1.61%
		Rice	1.25%
Cereal & Grain	4.99%	Corn cereal	1.25%
		Cooked pasta	1.25%
		Cooked oatmeal	1.25%
Bakery	6.52%	White bread	3.26%
		Chocolate chip cookie	3.26%
		Cooked canned beef	1.40%
Meat & Egg	8.46%	Cooked canned chicken	1.69%
		Cooked canned tuna	1.99%
		Cooked Egg	1.69%
Dairy	1.95%	Eggshells	1.69%
		Whole milk	0.65%
		Cheese	0.65%
Ground Coffee	4.86%	Butter	0.65%
		Brewed ground coffee	4.86%

### 2.2.2 Experimental Design for FNA Pre-Treatment

Three acidic pH levels (3, 4, and 5) and 1.5 mg N/L of FNA were chosen to evaluate the effects of acidification and FNA pre-treatment on pH and corresponding FNA concentration, N-related biological processes, solubilization of COD, and SRB inhibition. Table 3 summarizes the experimental conditions adopted in this experiment.

The concentration of FNA is calculated with Eq. 2.1 (Wang, Ye et al., 2013).

$$[FNA] = \frac{S_{NO_2^- - N}}{Ka * 10^{pH}} \quad \text{Eq. 2.1}$$

Where  $S_{NO_2^- - N}$  is the concentration of soluble  $NO_2^-$  added to the system in (mg  $NO_2^-$ -N/L). And  $Ka = e^{-2300/(273+T)}$ , a function of temperature T (°C)

*Table 2. Physical and chemical characteristics of synthetic FW. Measurements were conducted in triplicate, and standard deviation values are presented where available.*

Category	Value
TS (g TS/g wet FW)	0.183 ± 0.008
VS (g VS/ g wet FW)	0.175 ± 0.008
sCOD (mg COD/ g VS)	577 ± 27
tCOD (mg COD / g VS)	1351 ± 56
sPS (mg sPS / g VS)	283 ± 72
sP (ug sP/ g VS)	144 ± 6
Nitrite (ug $NO_2^-$ /g VS)	< D.L.
Nitrate (ug $NO_3^-$ /g VS)	2.42 ± 0.153
Sulfate (ug $SO_4^{2-}$ /g VS)	4.41 ± 0.198
Elemental Organic Carbon (% dry weight)	41.1±0.3
Elemental Organic Nitrogen (% dry weight)	9.58±0.05

*D.L.: value was below the detection limit of 0.05 mg  $NO_2^-$ /L.*

FNA concentration of 1.5 mg FNA-N/L was selected based on our previous study using mixed feed (FW and SS) summarized in Appendix A (Liu, 2021). In the previous work, compared with the highest performance (in terms of sCOD increase) observed in the treatment group with 5.0 mg FNA-N/L, 1.07 mg FNA-N/L used less  $NO_2^-$  addition and showed a good improvement of sCOD (increase by 87% after 72 hr pre-treatment at pH 5 compared with the control group). Similarly, 2.13 mg-N/L increased sCOD by 187% after 72 hr pre-treatment at pH 5. An FNA concentration in the middle of these values was chosen for this experiment. Furthermore, the surveyed literature reports 1.5 mg N/L FNA on the lower end of the concentration range, exhibiting microbial

inhibition effects and disinfection properties (Zhou, Oehmen et al., 2011; Zahedi, Icaran et al., 2016; Zahedi, Romero-Güiza et al., 2018).

Serum bottles with working volume of 77 mL were used to prepare the experiment in triplicate. Each treatment group was prepared in bulk and then evenly distributed into three replicates. Wet FW thawed from stored FW at -20 °C was weighed and mixed with NaNO<sub>2</sub> stock solution (or the same volume of DI water), followed by pH adjustment with 2 M HCl or 1 M NaOH to achieve the designed concentration shown in Table 3. The bottles were sealed with a rubber septum and flushed with helium for 3-5 mins to create an oxygen-limiting condition. They were then wrapped in tinfoil to create a dark environment and placed in an orbital shaker at 35 °C for 72 hours. Samples were taken every 24 hours for 72 hours (0, 24, 48, and 72 hours) to measure pH. Nitrogen gas accumulation in the headspace of the anaerobic media bottle was sampled at various times throughout the day. At 0 and 72 hours, sCOD, TS, and VS measurements were taken.

*Table 3. Treatment groups for pH vs FNA experiment.*

Conditions	Reactors					
	pH=3	pH=4	pH=5	pH=3 & FNA	pH=4 & FNA	pH=5 & FNA
pH	3	4	5	3	4	5
Nitrite Concentration (mg-N/L)	0	0	0	0.869	8.69	86.9
FNA Concentration (mg-N /L)	0	0	0	1.5	1.5	1.5
Ratio of FNA to solids (mg FNA-N/g VS)	0	0	0	0.129	0.129	0.129
Temperature	35 °C					
RPM	160					
Light Condition	Dark					

### 2.2.3 Analysis

TS and VS measurements were conducted according to standard methods (APHA, 1995). For measurements of sCOD,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , sP, and sPS, samples were filtered with a disposable syringe filter (0.45  $\mu\text{m}$  pore size). sCOD and tCOD were measured using the dichromate method in Hach COD analyzer (Loveland, CO, USA) following the approved EPA method (APHA, 2018). The concentration of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  was measured via ion chromatography using Dionex™ IonPac™ AS22 Fast IC Column (4x150mm) in Dionex ICS-1100 (Thermo Fisher Scientific Inc, USA). pH was measured with an Orion Dual Star pH Meter (Thermo Fisher Scientific Inc, USA). Nitrogen gas accumulation was measured via Gas Chromatography using Agilent 19019P-MS4 HP-Plot Molesieve column (30x320  $\mu\text{m}$  x12 $\mu\text{m}$ ) in Agilent 6890 Series GC system (Santa Clara, USA).

sPS were measured via spectrophotometry using the anthrone reagent method with glucose as the standard (Dreywood, 1946; Raunkjær, Hvitved-Jacobsen et al., 1994). sP were measured using colorimetry employing a Pierce BCA Protein Assay kit (Thermo Fisher Scientific Inc, USA). An Albumin standard was used, and the method followed was the bicinchoninic acid method (Smith, Krohn et al., 1985).

The elemental organic carbon and nitrogen in the solid phase were measured with a LECO CN628 Elemental Analyzer (LECO CN628 Carbon/Nitrogen Determinator). The FW samples were prepared by drying in the oven at 105 °C for one hour, ground up with a mortar and pestle, and passed through a 0.5 mm sieve. The powdered FW was combusted in the LECO. Carbon dioxide was measured by infrared detection, and

nitrogen gas was measured by thermal conductivity (Nelson and Sommers, 1983; Campbell and Plank, 1992).

#### **2.2.3.1 Statistical analysis**

A Student's T-test assuming a two-tailed distribution and equal variance was used to test the statistical significance of the results. The statistical analysis was done using IBM SPSS (version 28.0.0.0 (190)).

### **2.3 Results and Discussion**

#### **2.3.1 pH Change**

The pH of the treatment groups was measured every 24 hours. As shown in Fig. 7 (A), although pH adjustment was applied in different groups to achieve the initial pH of 3, 4, and 5, respectively, similar pH was observed in all groups after 24 hours pre-treatment, with the averaged pH of  $3.81 \pm 0.32$  at 72 hours. This result was consistent with our previous experiment in which FW treated with 5 mg FNA-N/L and a starting pH of 5 showed a final pH of 3.9 after 72 hours pre-treatment (Liu, 2021), indicating a buffer capacity of FW during the experiment period (72 hours). For comparison, pH of 6.01 was observed in SS, while pH of  $5.02 \pm 0.16$  was detected in the mixture of FW and SS (Liu, 2021).

The acidification of the FW can be attributed to the production of VFAs by hydrolysis, acidogenesis, and acidification that remained active under the tested FNA concentration or recovered from inhibition after failure to maintain effective FNA concentration through pre-treatment time (72 hours). Similar trends have been reported

in the literature (Jiang, Zhang et al., 2013). A study reported the effects of pH, temperature, and organic loading rate on the production of VFAs production from FW (Jiang, Zhang et al., 2013). The uncontrolled pH group with a starting pH of  $4.59 \pm 0.17$  dropped to pH of 3.0 after 8 days of operation at 35 °C.

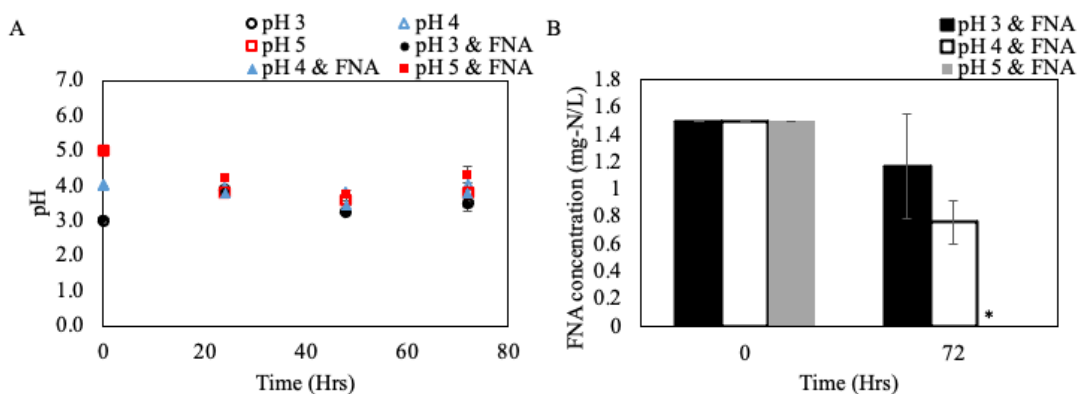


Figure 7. (A) Measured pH value at 24-hour sampling points. (B) Calculated FNA concentration (mg-N/L), calculated using  $\text{NO}_2^-$  measurements. \* Concentration of  $\text{NO}_2^-$  was below the detection limit of 0.05 mg  $\text{NO}_2^-$ /L. Where available, error bars show the standard deviation of triplicate tests.

### 2.3.2 Effective FNA concentration

According to Eq. 2.1, FNA concentration is dependent on the pH and  $\text{NO}_2^-$  concentration. The acidification of the system and  $\text{NO}_2^-$  consumption can alter the effective FNA in the system. Fig. 7 (B) depicts the FNA concentration calculated using measured  $\text{NO}_2^-$  concentration and pH at the end of the 72-hour pre-treatment. The measured  $\text{NO}_2^-$  concentrations before and after the pre-treatment are depicted in Fig.8.

Fig. 8 Depicts the added  $\text{NO}_2^-$  at 0 hours, and the final concentration of  $\text{NO}_2^-$  in each of the treatment groups. The presence of  $\text{NO}_2^-$  in the groups without FNA addition might be due to coffee grounds, vegetables like root vegetables (carrots), and green vegetables (lettuce and spinach) releasing  $\text{NO}_2^-$  and  $\text{NO}_3^-$  as they decompose (Hord,

Tang et al., 2009; Talan, Tiwari et al., 2021). There was no significant change in  $\text{NO}_2^-$  concentration observed in the pH 3 group with FNA ( $p>0.05$ ) after 72 hours of pre-treatment. As a comparison, the concentration of  $\text{NO}_2^-$  in pH 4 + FNA group decreased from 8.70 mg/L to 1.27 mg/L at the end of pre-treatment ( $p<0.001$ ). At 72 hours of pre-treatment in group pH 5 with FNA,  $\text{NO}_2^-$  concentration was below the detection limit of 0.05 mg  $\text{NO}_2^-$ /L, resulting in no effective FNA concentration (Fig. 7 (B)).

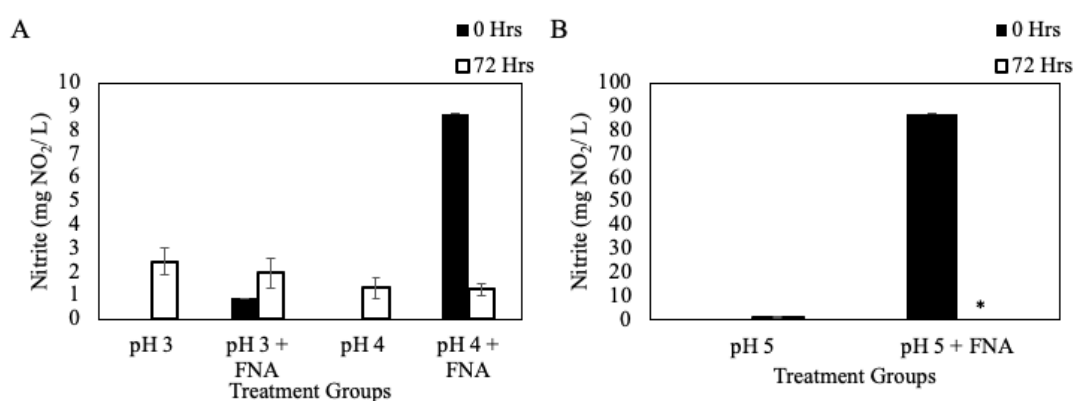


Figure 8.  $\text{NO}_2^-$  concentration in each reactor. The starting concentration was marked as the added  $\text{NO}_2^-$ . \* Concentration of  $\text{NO}_2^-$  was below the detection limit of 0.05 mg  $\text{NO}_2^-$ /L. Where available, error bars show the standard deviation of triplicate tests.

$\text{NO}_2^-$  loss can be attributed to the second step of denitrification (Eq. 2.2) (EPA, 2007).



Under anoxic conditions denitrifying microorganisms consume  $\text{NO}_2^-$  and produce  $\text{N}_2$  gas (EPA, 2007). The stable or reduction of  $\text{NO}_2^-$  concentrations at the tested pH can be explained by the combined inhibitory effects of pH and FNA/  $\text{NO}_2^-$  on denitrification. As reported by Bremner et al., (2009), decreasing pH from neutral (7-8) to acidic (5.8) levels can result in a slower denitrification rate. However, denitrification can be still detected at pH of 4.1, while complete inhibition obtained at

pH of 3.6 (Bremner and Shaw, 1958). This is consistent with our findings that the  $\text{NO}_2^-$  concentration was not detectable at higher pH (5), but it was at pH of 4 and 3.

The reduction in FNA concentration can impact the solubilization of organic material, microbial inhibition, and its biocidal effects (further discussed in Chapter 4). Previous experiments and the sCOD data (discussed in the following section) support the idea that the consumption of  $\text{NO}_2^-$  limits the effects of FNA pre-treatment. When a mixture of SS and FW mixed at a ratio of 50:50 (% w/w VS), was treated with varying concentrations of FNA (0.00, 0.30, 1.07, 2.13, 5.00 mg FNA-N/L) at pH 5.5 and 35 °C for 72 hours, the solubilization of sCOD increased with increasing FNA concentration. At 72 hours, application of 1.07 mg-N/L decreased the sCOD from  $2.96 \pm 0.22$  mg COD/g VS to  $2.00 \pm 0.11$  mg COD/ g VS. Meanwhile, 2.13 mg -N/L increased the sCOD from  $2.96 \pm 0.22$  mg COD/g VS to  $3.06 \pm 0.10$  mg COD/g VS. A higher initial concentration of FNA may last longer, solubilize more organic material, and inhibit COD consumption by microorganisms. The bacterial inhibition can be removed once the  $\text{NO}_2^-$  is consumed and FNA concentration drops below inhibitory levels (Jiang, Gutierrez et al., 2011).

$\text{NO}_2^-$  consumption can be caused by the denitrification bacteria that remain active under the tested condition (EPA, 2007). Fig. 9 indicated the presence of  $158 \pm 1.91$  mg/L  $\text{NO}_3^-$  at the beginning of the experiment. After the 72 hours pre-treatment with the oxygen-limiting condition,  $\text{NO}_3^-$  concentrations in all groups decreased, indicating the denitrification activity in the reactor. The denitrification activity during the pre-

treatment is supported by increasing  $N_2$  content detected in all reactors (shown in Fig. 10). When looking at the results in the groups with initial pH of 4 and 5, significant differences in  $NO_3^-$  concentrations were observed between the groups with and without FNA. The  $NO_3^-$  consumption in the groups without FNA at pH 4 and 5 is greater than in the groups with FNA addition ( $p < 0.001$ ). This is indicative that FNA addition will inhibit the activities of denitrifiers more than acidification alone at higher pH (4 and 5). However, this is not the case for pH 3 with and without FNA. The acidification to pH 3 is sufficiently effective and FNA addition (1.5 mg FNA-N/L) will not further improve it.

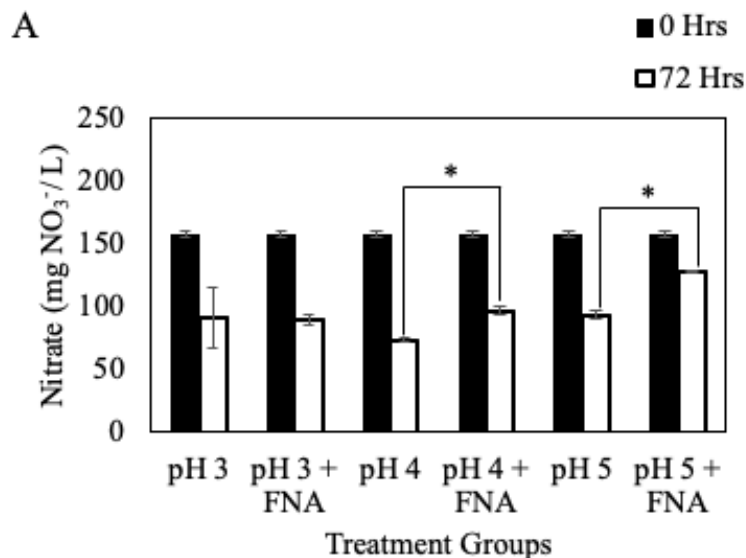


Figure 9.  $NO_3^-$  accumulation in the system. \*  $p < 0.001$ . Where available, error bars show the standard deviation of triplicate tests.

### 2.3.3 Solubilization of FW

sCOD has been used to measure the biodegradability of organic waste (Pham, Brar et al., 2009). Fig. 10 depicts the sCOD at the end of the 72 hours pre-treatment. There were no significant increases in pH 3 and pH 4 groups. pH 5 groups with and without

FNA saw an increase in sCOD, which was not significant ( $p>0.05$ ). Additionally, there was neither a statistically significant difference between the pH control groups and FNA application, nor when pH levels (with and without FNA) were compared to each other ( $p>0.05$ ). One potential reason was that the failure of maintaining a stable FNA concentration after 24 hours at a high solids contents ( $150 \pm 8.83$  g/L VS) in the system diminished the positive effects of FNA. Another reason could be that the selected FNA concentration was insufficient for solubilizing organic material. In comparison, when a higher concentration of FNA (5 mg-N/L) was used to treat FW ( $137 \pm 0.85$  g/L VS) there was a significant increase in sCOD from a starting concentration of  $279 \pm 10.3$  mg COD/g VS to  $691 \pm 26.7$  mg COD/g VS at 72 hours (Liu, 2021).

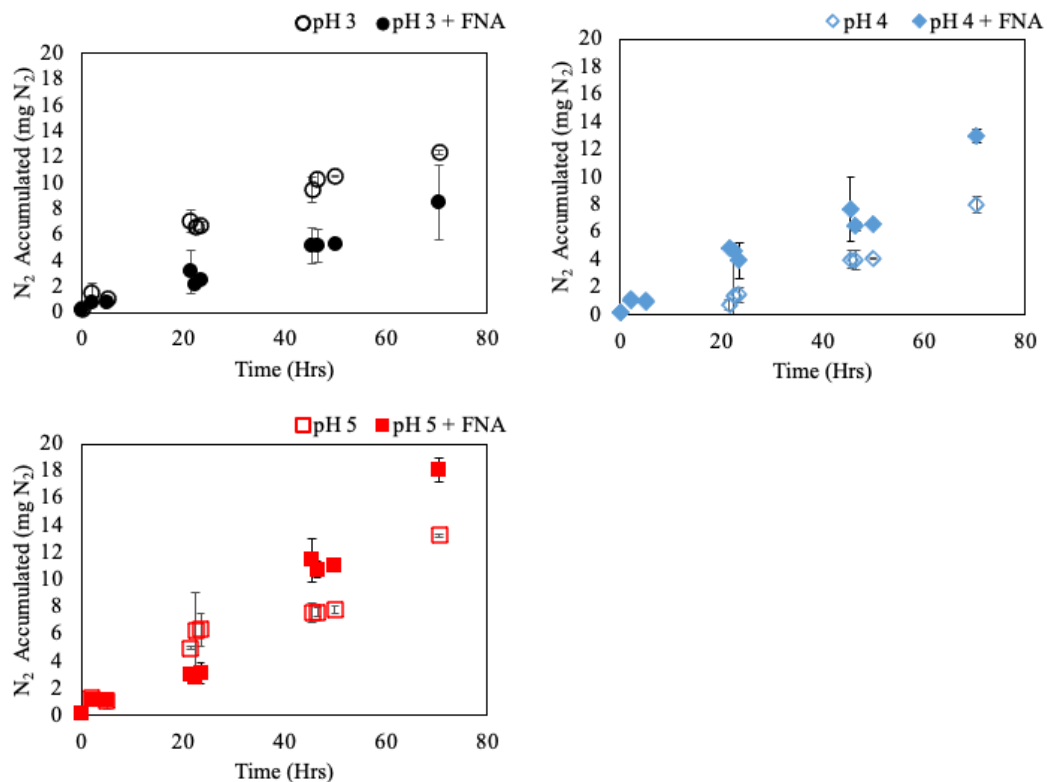


Figure 10. Evidence of nitrogen gas production in the headspace of the reactors. Where available, error bars show the standard deviation of triplicate tests.

In this work, the lack of significant sCOD consumption demonstrated in Fig. 11 indicates that the addition of 1.5 mg-N/L FNA combined with the acidification to pH 3,4, and 5 is sufficient for inhibiting microbial activities which may consume the soluble organic carbon.

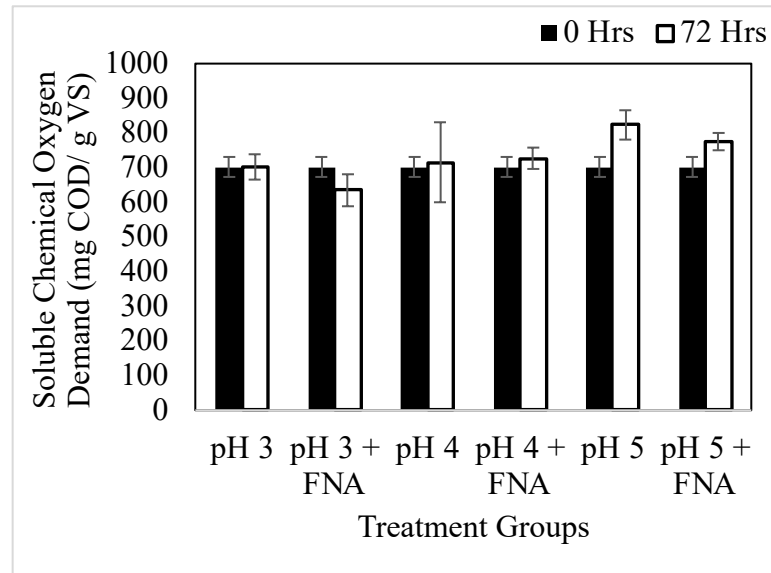


Figure 11. sCOD in each treatment group at 72 hours. The values were normalized per g VS to make direct comparisons between treatment groups. Where available, error bars show the standard deviation of triplicate tests.

In previous work, when FW was treated with 5 mg-N/L at 24 hours, the sCOD content increased to  $641 \pm 15.4$  mg COD/g VS, while at 48 hours it was  $543 \pm 7.2$  mg COD/g VS indicating consumption of organic material by the microbial community (Liu, 2021). More information about this experiment can be found in Appendix A. Summary of Previous Works. The fluctuations of sCOD concentration depicted in Fig. 12 (A) indicates that the increase in sCOD majorly occurred in first 24 hours pre-treatment. Although the details of how FNA affected COD at a shorter pre-treatment time (within 0-24 hours) are missing from the current experiment, the result are consistent with our

previous study using mixed FW and SS as feed, suggesting that a shorter pre-treatment might be sufficient and extending pre-treatment time will not further improve the solubilization of FW due to the consumption of FNA.

Additionally, in Fig. 12 (B) the solubilization of proteins and polysaccharides is presented. The increase in sP and sPS further confirm the ability of 5.0 mg FNA-N/L to solubilize organic material in FW. Complex sP and sPS are the substrate for the beginning stages of AD, so an increase in their concentration may lead to greater biogas production (Bingemer and Crutzen, 1987).

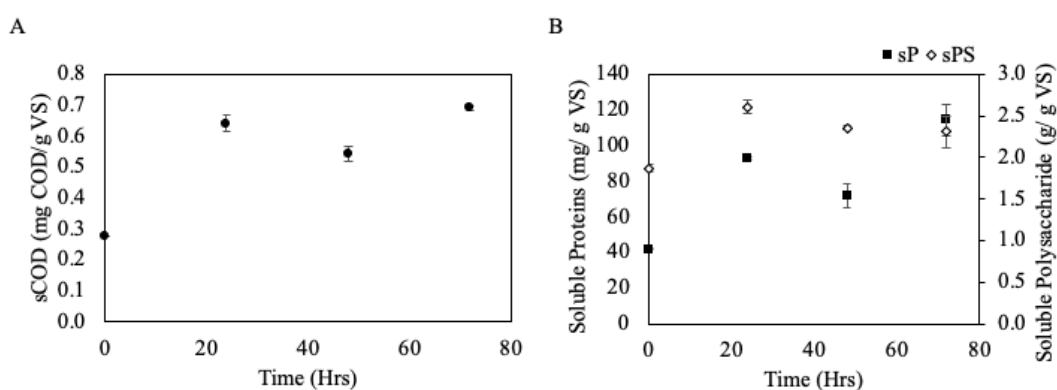


Figure 12. (A) sCOD fluctuations with treatment time during the pre-treatment of FW with 5 mg FNA-N/L by adjusting pH to 5 at 35°C for 72 hours. (B) Solubilization of proteins and polysaccharides. The increase and decrease in sCOD, sP, and sPS follow similar trends. The solids content for this FW was  $137 \pm 0.85$  g/L VS. Where available, error bars show the standard deviation of duplicate tests (Liu, 2021).

## 2.4 Conclusion

The selection of FNA concentration plays a significant role in promoting the quality of FW in terms of digestibility and microbial inhibition (e.g., denitrification). With 72 hr treatment time, the selected concentration of 1.5 mg FNA-N/L did not show significant improvement compared with acidification treatment using pH control. Additionally,

starting pH of the treatment can influence the inhibition of denitrifying organism, and the consumption of  $\text{NO}_2^-$ . A more acidic pH (3) will have greater inhibition of denitrifying organisms and thus allow the concentration of FNA to be more stable throughout the pre-treatment. A less acidic pH (4, and 5) will not completely inhibit denitrifying organism leading to consumption of  $\text{NO}_2^-$ .

However, it should be noted that the selected FNA (1.5 mg N/L) might be effective at a shorter duration (<24 hours) when sufficient inhibition is maintained (added  $\text{NO}_2^-$  is not consumed). Another source of improvement for the treatment could be the optimization of the dose of FNA in FW (in terms of mg FW per mg VS) in the pre-treatment, which was explored in the following chapter.

## **Chapter 3: Effectiveness of FNA Pre-treatment at Different FNA Dosing Ratios (mg FNA/g VS)**

### **3.1 Previous Work**

Previous work in the co-digestion of FW and SS illustrated that the effectiveness of the FNA pre-treatment in stabilizing FW might be impacted by both concentrations of FNA (mg FNA-N/L) and the amount of FW (g VS/L) that is treated (Liu, 2021). Other aspects to consider are the mass transfer limitations on the enzyme activity. A FW mixture with higher solids and higher viscosities may have limited mass transfer. In a study identifying the effect of mass transfer limitation on decreased yield in enzymatic hydrolysis of lignocellulose, it was noted that an increase in solids also increased the mass transfer limitations (Du, Cao et al., 2017). Furthermore, high solids content and/or viscosity can cause mixing difficulty, resulting in an inhomogeneous system. When the solids content was high, 15% dry matter, cellulose conversion was improved by increasing shaking speed. This was not the case for lower solids content (10% dry matter). A well-mixed system will allow enzyme reactions to be free of inhibition caused by areas of low enzyme or substrate concentration and will allow for increased contact between the enzymes and the substrate (Jørgensen, Vibe-Pedersen et al., 2007).

In the FNA pre-pretreatment process, for a selected FNA concentration, as solids content increases, the ratio of added FNA to VS decreased (mg FNA per g VS). The treatment ratios with a larger amount of VS will have less mg FNA-N per g VS. This relationship is detailed in Table 4. This will also affect the ability of FNA to degrade organic material and its biocidal and inhibitory effects. For example, a FW mixture

with low solids content will have higher mg FNA per g VS than a FW mixture with a higher solids content.

*Table 4. TS and VS of FW (high solids) at different mixing ratios with SS (low solids). As the percent of FW increases in the experimental group, so does the quantity of TS and VS.*

Ratio of SS:FW (%,VS)	TS (g/ L)	VS (g/ L)	mg FNA-N/ g VS
100:0	3.74	1.94	2.58
75:25	5.08	3.13	1.60
50:50	10.5	7.50	0.62
25:75	22.1	19.1	0.26
0:100	148	137	0.04

To further improve FNA pre-treatment of FW, this experiment was conducted to assess the relationship between the mass of VS treated per mg of FNA, and the effectiveness of the FNA in stabilizing FW.

## 3.2 Materials and Methods

### 3.2.1 Experimental Design

The FW was prepared in the same manner as outlined in Chapter 2. It was then combined with DI water and FNA solution to achieve the desired FNA:VS ratios (0.089, 0.035, 0.019, 0.012, and 0.011 mg FNA-N/g VS) with the FNA concentration of 1.5 mg N/L. The reactors were then named by their FW content (10% FW, 25% FW, 50% FW, 75% FW, and 90% FW (%v/v)). The remainder of the group was FNA solution (10% FW = 10% FW and 90% FNA solution). The control group was composed of 90% FW (% v/v) without FNA; instead, DI water was added to achieve an equal volume. These mixtures of FW and FNA solution were prepared in bulk and split into three replicates with equal volume (159 mL). The flasks were flushed with

N<sub>2</sub> for 4 mins to create an oxygen-deficient environment and sealed using parafilm. Then, the flasks were placed in an orbital shaker (160 rpm) and dark environment at 35 °C for 72 hours. Table 5 outlines the experimental conditions and other specifications.

*Table 5. Experimental conditions for the different mixtures. The groups are named after their FW content (%v/v). The remainder of the treatment group is composed of FNA solution.*

Mixture composition	10% FW	25% FW	50% FW	75% FW	90% FW	90% FW No FNA
pH				5		
Treatment duration (hours)				72		
Temperature °C				35		
RPM				160		
Nitrite concentration in the reactor (mg NO <sub>2</sub> -N/L)			87.0			0
FNA concentration in the reactor (mg FNA-N/L)			1.52			0
VS concentration (g/L)	16.8 ± 1.26	43.3 ± 1.14	80.6 ± 7.44	122 ± 0.74	133 ± 3.68	135 ± 7.68
FNA/VS ratio (mg FNA-N/g VS)	0.089	0.035	0.019	0.012	0.011	0.000

### 3.1.3 Analysis

Samples were collected at 0, 24, 48, and 72 hours. Concentrations of NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, ammonium (NH<sub>4</sub><sup>+</sup>), pH, sCOD were measured at each sampling point (0, 2, 7, 23, 48, and 72 hours). TS and VS analyses were only conducted at the beginning and final sampling points (72 Hours). NO<sub>2</sub><sup>-</sup> measurements were supplemented with analysis conducted with a nutrient analyzer model AQ300 by Seal analytical (Mequon,

Wisconsin, USA).  $\text{NH}_4^+$  was measured via Hach analyzer probe (Loveland, CO, USA).

Other analyses were carried out in the same manner as outlined in Chapter 2.

### 3.1.3.1 Statistical Analysis

A Student's T-test assuming a two-tailed distribution and equal variance was used to test the statistical significance of the results. The statistical analysis was done using IBM SPSS (version 28.0.0.0 (190)).

## 3.2 Results and Discussion

### 3.2.1 Solids Contents and FNA:VS ratios

Initial conditions in the reactors are depicted in Table 6.

Table 6. Initial conditions in each reactor for the experiment testing FNA solution volume to volume of FW. Tests were conducted in triplicate, and the standard deviation is presented in the column next to each average value.

	10% FW	25% FW	50% FW	75% FW	90% FW	90% FW No FNA
pH	4.96 ± 0	5.02 ± 0	5.01 ± 0	5.02 ± 0	5.02 ± 0	5.00 ± 0
Nitrite (mg $\text{NO}_2^-$ /g VS)	23.7 ± 2.14	6.49 ± 0.20	3.53 ± 0.62	2.32 ± 0.18	1.60 ± 0.06	0.03 ± 0.00
Nitrate (mg $\text{NO}_3^-$ /g VS)	< D.L *	0.14 ± 0.02	0.57 ± 0.19	1.04 ± 0.06	0.80 ± 0.02	0.48 ± 0.04
Sulfate (mg $\text{SO}_4^{2-}$ /g VS)	1.08 ± 0.07	0.74 ± 0.02	0.42 ± 0.37	1.19 ± 0.04	0.89 ± 0.02	0.83 ± 0.07
Ammonia (mg $\text{NH}_4^+$ /g VS)	1.23 ± 0.10	1.12 ± 0.05	1.25 ± 0.14	1.31 ± 0.04	1.48 ± 0.03	1.50 ± 0.05
Hydrogen Sulfide (ug $\text{H}_2\text{S}$ /g VS)	n.d.	0.41 ± 0.004	2.02 ± 0.18	3.26 ± 0.02	5.78 ± 0.16	9.57 ± 0.52
sCOD (mg COD/g VS)	531 ± 44	508 ± 0	564 ± 42	409 ± 50	656 ± 22	628 ± 35

D.L. \*This value was below the limit of detection of 0.113 mg  $\text{NO}_3^-$ /L.

n.d not detected

Solids contents in the treatment groups were measured and expressed in TS and VS. As the percentage of FW in the treatment group increases, so do the TS and VS values. These values are summarized in Table 7.

Table 7. TS and VS values of treatment groups.

Group name	TS (g/L)	VS (g/L)	Ratio of mg FNA-N/ g VS
10% FW	18.2 ± 1.47	16.8 ± 1.26	0.089
25% FW	47.1 ± 1.72	43.3 ± 1.14	0.035
50% FW	89.1 ± 13.3	80.6 ± 7.44	0.019
75% FW	135 ± 1.42	123 ± 0.74	0.012
90% FW	146 ± 4.15	133 ± 3.68	0.011
90% No FNA	149 ± 8.36	135 ± 7.68	0.000

The ratio of solids content in the treatment group as a fraction of the undiluted FW was graphed to examine if the desired concentrations of VS in different flasks were successfully achieved. Fig. 13 shows that the fraction of TS and VS in the treatment groups correlates to their percentage of FW to FNA solution. The dilution of FW with water successfully decreased the volatile content to the desired concentration.

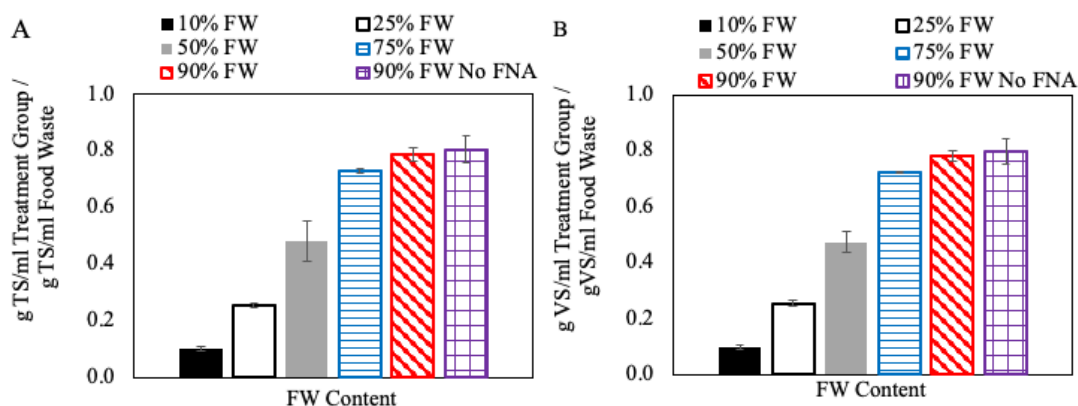


Figure 13. (A) The TS of the reactor was divided by the total TS of the original synthetic FW. (B) The VS of the reactor was divided by the total VS of the original synthetic FW. In both cases, TS and VS, the resulting fraction is consistent with each treatment group's designed TS and VS content. The 90% FW groups with and without FNA deviate from the designed solid content. Where available, error bars show the standard deviation of triplicate tests.

The lower solid content will allow for greater mass transfer, increased homogeneity of the system, and decreased viscosity. Achieving greater homogeneity in the system is desirable for various reasons. Firstly, it will allow representative sampling to occur. It is difficult to obtain a representative sample when the reactor has high viscosity and high solids content. Secondly, it will allow the treatment to be effective across the entire reactor volume. In a reactor with high solids content, the mass transfer limitation might be considerable and play a role in the treatment process to lower the effectiveness of FNA.

### **3.2.2 pH and Effective FNA Concentration**

Fig. 14 (A) shows that the FW became more acidic in all treatment groups. From an average starting pH of  $5.01 \pm 0.02$  the final pH at 72 hours decreased to  $3.80 \pm 0.28$ . In Chapter 2, the final pH for all the treatment groups was  $3.81 \pm 0.32$ . Furthermore, at a starting pH of 5, there was a significant difference in the final pH when FNA was added ( $p=0.025$ ). pH 5 group acidified to  $3.79 \pm 0.12$ , while pH 5 + FNA acidified to  $4.32 \pm 0.23$ . When comparing the final pH of the 90% FNA group ( $3.70 \pm 0.01$ ) with the 90% No FNA ( $3.99 \pm 0.04$ ), the difference in final pH was also significant ( $p < 0.001$ ). This would indicate that the addition of 1.5 mg-N/L of FNA can impact the reactions that lead to the acidification of the system, particularly when the solids content ranges from (135-152 g VS/L). There were no clear trends regarding solids content and its effect on buffer capacity.

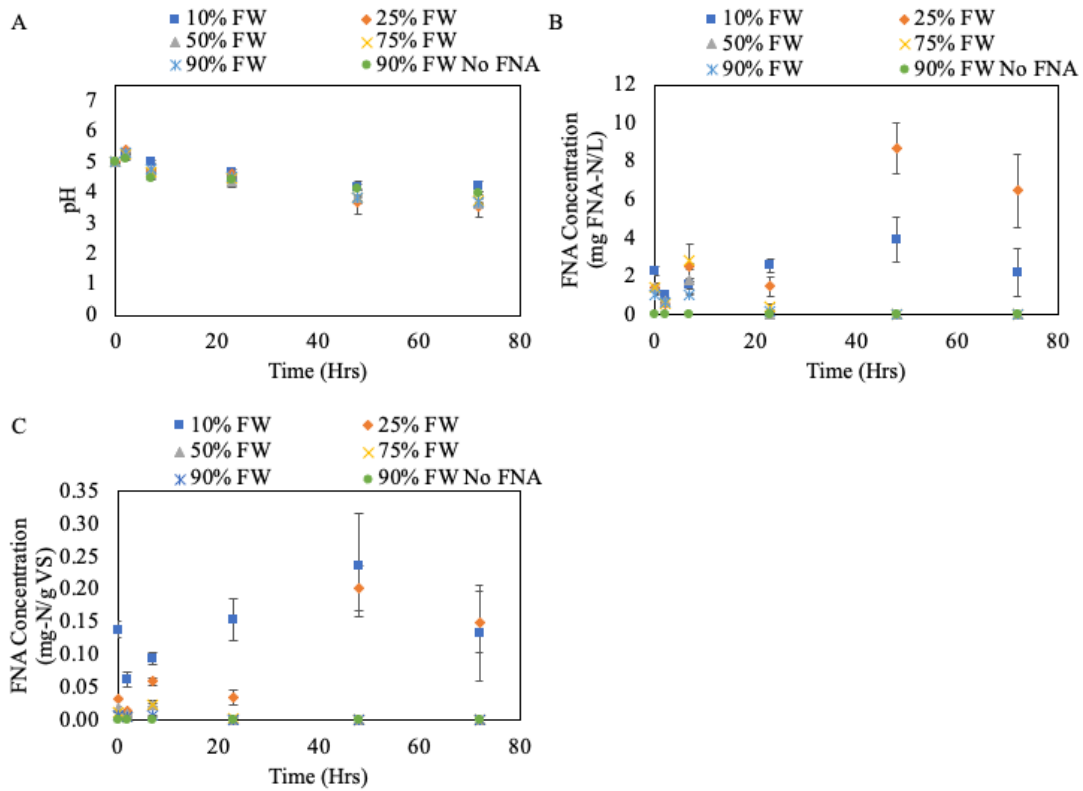


Figure 14. (A) pH of each treatment group as time progresses. (B) Calculated FNA concentration calculated using the pH and  $\text{NO}_2^-$  measurements. (C) Calculated FNA concentration per g VS. Where available, error bars show the standard deviation of triplicate tests.

Fig. 14 (B) depicts the calculated FNA concentration using the measured  $\text{NO}_2^-$  concentration and pH at each sampling time. The FNA concentration increased after 48 hours in the 10% FW and 25% FW groups. High FNA concentration was observed in the groups with lower solids content, with the highest FNA concentration calculated at 48 hours in the group of 25% FW followed by the group of 10% FW. The difference between these two groups at 48 and 72 hours was statistically significant ( $p=0.010$ , and  $p=0.031$ , respectively). As a comparison, effective FNA concentration in the groups 50% FW, 75% FW, and 90% FW started decreasing after 24 hours and became non-detected after 48 hours.

The effective FNA concentration decreasing after 24 hours is consistent with the findings in Chapter 2. When the ratio of FNA to solids content was 0.129 mg-N/g VS, the  $\text{NO}_2^-$  concentration was not detectable at 72 hours of pre-treatment. A shorter pre-treatment time, less than 48 hours, may experience less  $\text{NO}_2^-$  consumption. Fig. 14 (C) shows FNA:FW ratio in the unit of mg FNA to g VS at different pre-treatment times. FNA:FW ratio decreased with  $\text{NO}_2^-$  consumption. At pH of 5, the added  $\text{NO}_2^-$  was observed to be consumed after 48 hours, if the mg FNA-N/g VS is less than 0.035 mg-N/g VS. A higher effective FNA concentration in the groups with lower solids contents supported our hypothesis that reducing the solids content in the pre-treatment will maintain the designed FNA concentration for longer.

After 48 hours, the FNA concentration in the 50-90% FW treatment groups drops below 0.22 mg FNA-N/L to an average of  $0.024 \pm 0.008$  mg FNA-N/L, resulting in a lower inhibition potential to the microbial processes in the reactor. The potential consumption of  $\text{NO}_2^-$  caused by denitrification in the pre-treatment has been discussed in the previous chapter. Other N-related biological processes, such as nitrification, was also possible as the reactors were oxygen-limited, not fully anaerobic. The literature shows evidence of some species of ammonia oxidizing bacteria (AOB) having resistance FNA concentrations less than 0.42 mg-N/L (Laloo, Wei et al., 2018). Therefore, AOB may carry out the nitrification seen in treatment groups (50-90% FW). Meanwhile, nitrite-oxidizing bacteria (NOB) are inhibited by increasing FNA concentrations (Ma, Yang et al., 2017). At a pH of 5.0, acidification provides some inhibition, but increasing concentration from 0-4 mg-N/L can also further inhibit NOB

(Zheng, Wang et al., 2021). Furthermore, when applied to activated sludge FNA concentrations greater than 1 mg-N/L can reduce the number of active NOB by around two orders of magnitude in activated sludge (Zheng, Wang et al., 2021). At 24 hours, in reactors containing 50%, 75% and 90% FW, the concentrations of FNA dropped below the reported inhibitory levels (1 mg-N/L) to an average of  $0.22 \pm 0.19$  mg-N/L. After 24 hours, the FNA concentration is insufficient to inhibit NOB, which may lead to their re-activation.

Our results also demonstrate that inhibition of NOB can be affected by the solids content. The rate of  $\text{NO}_2^-$  consumption during the first 24 hours was calculated by using the slope of the linear trend line. The 10% FW group had the lowest rate of consumption (6.07 mg  $\text{NO}_2^-$ /L hr, while 50% FW had the greatest rate of consumption at 11.8 mg  $\text{NO}_2^-$ /L hr. 25% FW had a rate of consumption of 8.17 mg  $\text{NO}_2^-$ /L hr, 75%FW had a rate of 10.3 mg  $\text{NO}_2^-$ /L hr, and 90% FW had 9.42 mg  $\text{NO}_2^-$ /L hr. The 10% FW and 25% FW groups demonstrate that the reaction consuming  $\text{NO}_2^-$  was inhibited more than in other groups. After 24 hours, the rate of consumption in groups 50-90% FW changes, as most of the  $\text{NO}_2^-$  has been consumed.

Although the rate of denitrification  $\text{NO}_2^-$  in the 10% was the slowest, it did not lead to the highest final nitrogen concentration. This experiment was carried out in Erlenmeyer flasks with parafilm covering the entrance, so  $\text{N}_2$  gas measurements were not taken. It was only possible to evaluate the rate of denitrification from the consumption of  $\text{NO}_2^-$  not from the  $\text{N}_2$  production. The total final nitrogen concentration in the 10% group is

the smallest. This can be related to the  $\text{NH}_4^+$  released from FW. As demonstrated in Fig. 15, as the FW content increases (lower FNA:FW ratio), so does the concentration of  $\text{NH}_4^+$ . This indicates that the  $\text{NH}_4^+$  is sourced from the FW. The increased  $\text{NH}_4^+$  concentration in groups (50-90% FW) can come from higher concentrations of N-containing components in FW. For example proteins can decompose and accumulate  $\text{NH}_4^+$  (Yenigün and Demirel, 2013).

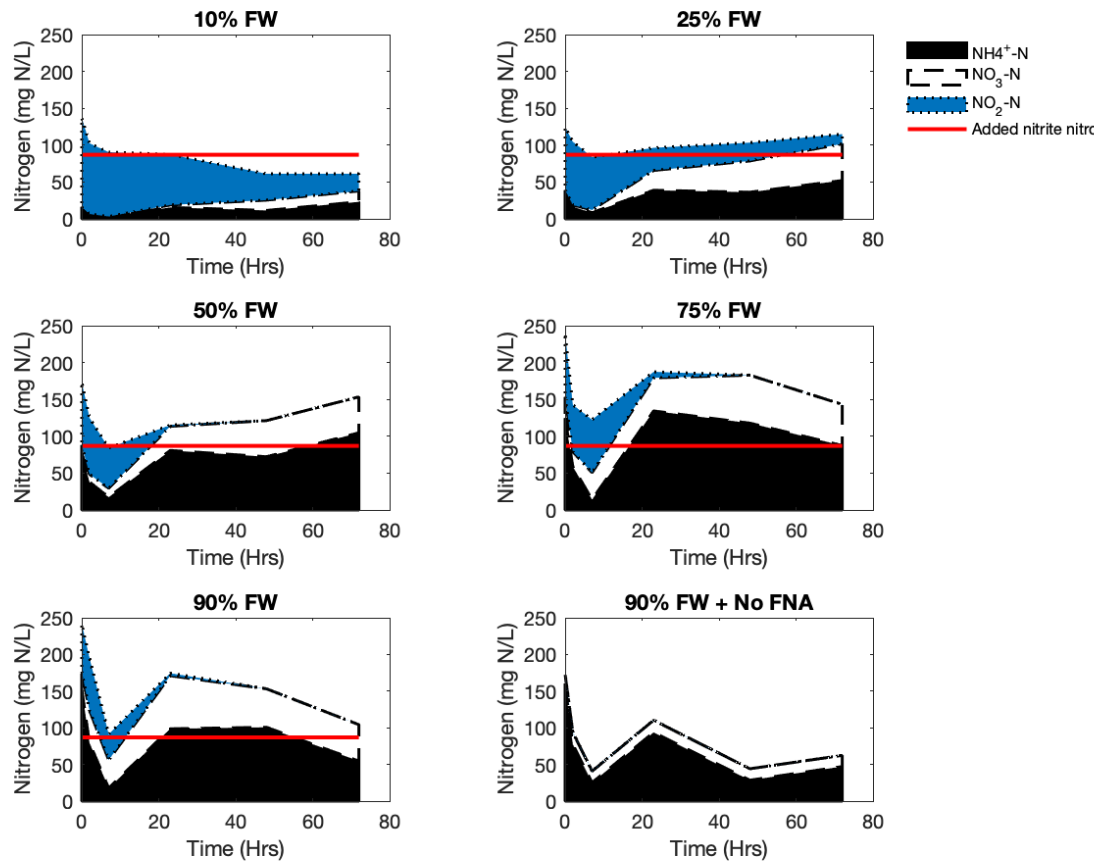


Figure 15. Nitrogen speciation in the treatment groups at each sampling time. \*Total nitrite nitrogen =  $\text{NO}_2^-\text{-N}$  + FNA-N (mg N/L).

Our  $\text{NH}_4^+$  measurements correlated with the volume of FW in each reactor. Higher percentages of FW lead to a higher concentration of proteins and, in turn, increased

$\text{NH}_4^+$  concentrations, depicted in Fig. 15. When the 10% and the 25% FW groups are compared, the difference in  $\text{NH}_4^+$  concentration is significant ( $p < 0.001$ ). However, when the 50% FW is compared to the groups with 75% FW and 90% FW, the difference is not significant ( $p > 0.05$ ). This indicates that  $\text{NH}_4^+$  content is affected when solids content is lower than  $80.6 \pm 7.44$  g VS/L.

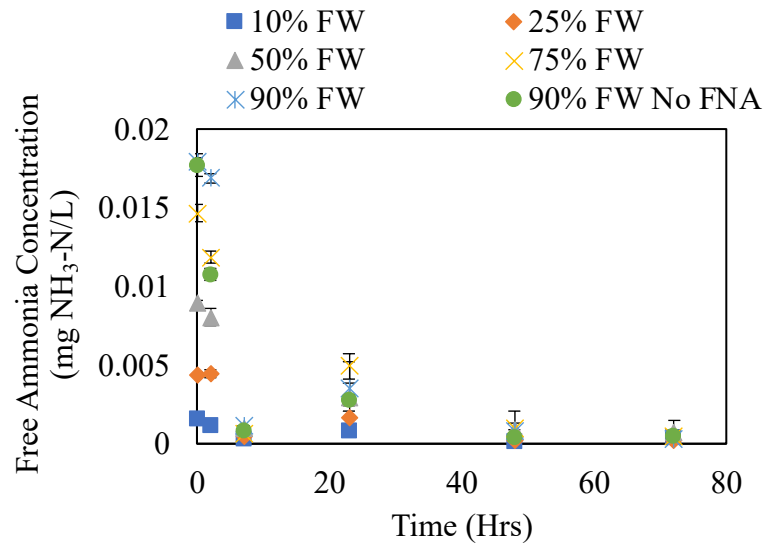


Figure 16.  $\text{NH}_3$  concentration was calculated from the ammonia measurement. Where available, error bars show the standard deviation of triplicate tests.

There is no evidence of free ammonia inhibition ( $\text{NH}_3$ ), which can be calculated from the total ammonia concentration, the ionization constant ( $pK_a$ ) and the pH value, Eq.

3.1. The  $pK_a$  can be calculated using from Eq. 3.2 (Emerson, Russo et al., 1975).

$$\text{NH}_3 = \text{total Ammonia as N} \left( \frac{\text{mg}}{\text{L}} \right) \left( \frac{100}{1 + 10^{pK_a - \text{pH}}} \right) \quad \text{Eq. 3.1}$$

$$pK_a = 0.09018 + \left( \frac{2729.92}{T} \right) \quad \text{Eq. 3.2}$$

$\text{NH}_3$  at concentrations between 10-150 mg/L were reported to show inhibition of *Nitrosomonads*, and concentrations between 0.1-1.0 mg/L can inhibit *Nitrobacter* (Anthonisen, Loehr et al., 1976).  $\text{NH}_3$  concentrations were calculated and are presented

in Fig. 16. The  $\text{NH}_3$  concentrations are not near the reported levels that would cause inhibition of AOB bacteria like *Nitrobacter* and *Nitrosomonads*. The levels of  $\text{NH}_3$  are not inhibitory in our reactors.

### 3.2.3 Solubilization of FW

The sCOD concentrations in the reactors over the 72 hours pre-treatment were presented in Fig. 17, with the similar trends observed in our previous work (Liu, 2021). The microorganisms may consume solubilized organic material, thus leading to a decrease in sCOD. Other literature has also correlated a decrease in sCOD measurements as an indicator of microorganisms consuming the organic matter (Cappelletti, Reginatto et al., 2011).

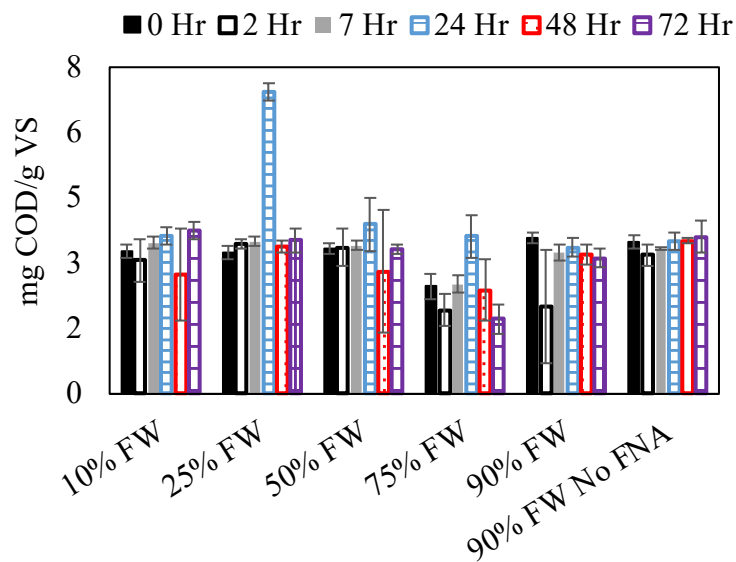


Figure 17. sCOD oxygen demand per gram VS of FW. Where available, error bars show the standard deviation of triplicate tests.

The control group performed like the rest of the groups. This would indicate that 1.5 mg FNA/L did not provide increased solubilization of organic material. This matches

the results of 1.5 mg FNA/L applied to FW at various pH levels (3, 4, and 5) in Chapter 2.

### **3.3 Conclusion**

The calculated  $\text{NH}_3$  concentrations were not in the inhibitory range reported by the literature. This would mean microbial inhibition in the system was caused by FNA rather than  $\text{NH}_3$ . A lower solids content provided an effective FNA concentration for a more extended period than higher solids contents by inhibiting the  $\text{NO}_2^-$  consumers in the reactors. The enhancement of solubilization of organic material using 1.5 mg FNA/L at different solids contents was not significant. Combining the N and sCOD data, it indicated that the selected FNA concentration was effective to inhibit the N-related biological processes but not sufficient to cause lysis of cells for improving sCOD.

## **Chapter 4: Inhibition of H<sub>2</sub>S Production and Reduction in Pathogenic Bacteria by FNA**

### **4.1 Background Information**

The presence of SRB in FW can cause problems for various reasons. FW contains sulfur compounds that can be released during fermentation (Saral, Demir et al., 2009; Fang, Yang et al., 2012; Liu, Lu et al., 2018). In food, sulfur is found in amino acids like methionine (C<sub>5</sub>H<sub>11</sub>NO<sub>2</sub>S) and cysteine (C<sub>3</sub>H<sub>7</sub>NO<sub>2</sub>S) (Doleman, Grisar et al., 2017). Foods containing these amino acids include broccoli, cabbage and turnip rape, bread, potato products, nuts, popcorn, and coffee (Mussinan, Keelan et al., 1994). These sulfur compounds can become H<sub>2</sub>S by the microbial interaction of SRB (Muyzer and Stams, 2008; Liu, Lu et al., 2018). Fermentative bacteria can turn amino acids, sugars, and long-chain fatty acids into acetate, propionate, butyrate, lactate, and hydrogen. These fermentation products can then be consumed by SRB (Muyzer and Stams, 2008).

FW's improper storage or handling can lead to an unpleasant rotten egg odor (Henry and Gehr, 1980). The unpleasant odor produced by sulfur compounds can also be a problem for FW that is sent to landfills (Fang, Yang et al., 2012). Additionally, SRB can outcompete methanogenic archaea for substrates like hydrogen, formate, and acetate (Plugge, Zhang et al., 2011). Therefore, studying the inhibition of SRB by FNA is an important topic.

In addition to SRB, pathogen bacteria in post-consumer FW is also problematic during the transportation, storage, and disposal process, causing health risks and concerns to the operators and future use. For instance, with high pathogen bacteria concentration,

the effluent after AD treatment cannot be directly used as fertilizer in agriculture sectors (Wang, Jiang et al., 2014). FW in MSW has been reported to have a *Salmonella* pathogenic load of  $9.80 \times 10^{-4}$  organisms/g and a fecal coliform load of  $2.24 \times 10^8$  organisms/g (Gerba, Tamimi et al., 2011). Therefore, reducing the pathogen content of FW can be advantageous in producing a higher quality product and protecting operators from health risks.

FNA also has biocidal effects on pathogenic bacteria (Zhou, Oehmen et al., 2011). For example, at pH 5, FNA concentrations of 2.2, 4.4, and 7.2 mg N/L applied for 24 hours showed a substantial reduction of pathogen levels in thickened WAS. The initial concentration of 5.74 Log (MPN/ g TS) was reduced to 3.70 Log (MPN/ g TS), 3.73 Log (MPN/ g TS), and 3.58 Log (MPN/ g TS), for each concentration, respectively.

There is a knowledge gap on FNA pre-treatment of FW to reduce SRB activity and pathogen numbers. Therefore, in this work, we collected data on the effects of FNA concentration on the inhibition of the production of H<sub>2</sub>S (Experiment 4). Additionally, we compared the effects of acidification and FNA application on pathogen kill (Experiments 3 and 4).

## **4.2 Materials and Methods**

### **4.2.1 FW and WW Slurry**

Synthetic FW was created according to the recipe outlined in Chapter 2. Because the synthetic FW was prepared in the laboratory under relatively sterile conditions, where

all instruments used were cleaned to laboratory standards, and the ingredients used were from new, unopened packages, it contained relatively low pathogen bacteria (below detection limit) (Tampio, Ervasti et al., 2015). Therefore, WW collected from a local WWTP in Damascus, Maryland, was used to spike the synthetic FW for providing bacteria to synthetic FW to mimic the microbial concentrations in real post-consumer FW. The solids content of added WW to FW was negligible because the TS of WW was  $0.67 \pm 0.02$  mg TS/g WW, while the VS value was  $0.33 \pm 0.01$  mg VS/g WW. TS and VS values were calculated according to standard methods (APHA, 1995). The FW and WW slurry was created by mixing FW with WW (93.3: 6.7 %, v/v), which was determined in a preliminary test to obtain a detectable initial microbial population ( $>590400$  MPN/ g FW) of total coliforms and *E. coli*.

## **4.2.2 Experimental Design**

### **4.2.2.1 Experiment 3**

To test the effect of FNA application on pathogenic bacteria in the FW mixed with WW three treatment groups were created: pH adjustment group, FNA treatment group, and control group. The control and pH adjustment groups had ratio of 75:25 slurry to DI water (% , v/v). The FNA treatment group had a concentration of 5 mg FNA-N/L and was mixed with FNA solution at a ratio of 75:25 slurry to FNA solution ratio (% , v/v). The preparation of each treatment bottle is outlined in Table 8.

FNA was added to the system with a stock solution made of  $\text{NaNO}_2$ . The concentration of FNA was calculated with Eq. 2.1. Each treatment group was made in bulk and divided into three replicates in anaerobic media bottles of equal volume (30 mL). 90

mL of extra slurry of the control group was used for the initial measurements. The headspace of all bottles was sealed with rubber caps flushed with helium for 5 mins to create an oxygen-limiting condition. The bottles were incubated in a dark environment at 35°C and mixed at 160 rpm in an orbital shaker for 72 hours.

*Table 8. Experimental setup for studying the effects of FNA (5 mg N/L) on reducing pathogen bacteria during the pre-treatment of FW at pH 5.5.*

	Control Group	FNA Treatment	pH Adjustment
TS (g /L)		95.7	
VS (g/L)		91.4	
FNA (mg-N/L)	0	5.00	0
NO <sub>2</sub> <sup>-</sup> (mg-N/L)	0	903	0
Initial pH	5.96*	5.5	5.5

*\*pH of the control group was measured and left unadjusted.*

#### **4.2.2.2 Experiment 4**

A mixture of 50% FW and 50% FNA solution (% v/v) was treated for 72 hours at various FNA concentrations, including 0.30, 0.90, 1.52, 2.13, and 5.00 mg N/L, while the group without the addition of nitrite (FNA=0 mg N/L) was used as control. Each treatment group was prepared in bulk, and then split into three replicates of equal volume (185 mL). The initial pH was adjusted to 4 using 1 M NaOH/HCl, and the prepared bottles were placed in an orbital shaker at 35° C and 155 rpm. These conditions are summarized in Table 9.

Table 9. Experimental setup for studying the effects of various FNA concentrations on SRB during the pre-treatment of FW at pH 4.

	0 FNA	0.3 FNA	0.9 FNA	1.5 FNA	2 FNA	5.0 FNA
Mass of wet FW (g)			133			
Total Volume (mL)			563			
Initial pH			4			
VS (g VS/L)			78.8 ± 1.33			
Designed FNA concentration (mg -N/L)	0.00	0.30	0.90	1.52	2.13	5.00
Designed NO <sub>2</sub> <sup>-</sup> concentration (mg-N/L)	0.00	1.74	5.17	8.66	12.2	28.6
Ratio of FNA to g VS (mg FNA/g vs)	0	0.004	0.011	0.020	0.027	0.062

### 4.2.3 Analytical Methods

For Experiment 3, total coliform counts were sampled at 0, 4, 8, 24, and 72 hours.

During Experiment 4, SO<sub>4</sub><sup>2-</sup> and H<sub>2</sub>S samples were taken at 0, 4, 24, and 72 Hours. For bacterial counts, concentrations of 0.3 and 0.9 mg FNA-N/L were only sampled at 0, 4, and 24 hours; no measurement was taken at 72 hours. Other concentrations (0.0, 1.52, 2.13, and 5.00) were sampled at sampling times 0, 4, 24, and 72 Hours.

For measuring the aqueous concentration of SO<sub>4</sub><sup>2-</sup> and H<sub>2</sub>S samples were filtered with a disposable syringe filter (0.45 um pore size) prior to analysis. The concentration of SO<sub>4</sub><sup>2-</sup> in the liquid phase was measured via ion chromatography using Dionex™ IonPac™ AS22 Fast IC Column (4x150mm) in Dionex ICS-1100 manufactured by Thermo Fisher Scientific Inc, MA, USA. Aqueous H<sub>2</sub>S was determined via the methylene blue method (Reese, Finneran et al., 2011).

Total coliform and *E. coli* microbes are not all pathogenic, but they can indicate the presence of more harmful organisms (Sigler and Bauder, 2010). Therefore, their numbers were enumerated with AOAC official method 20005.03 (International Organisation for the Standardisation, 1983) by following the manufacturer's instruction with the SimPlate® Total Coliform and *E. coli* Color Indicator (CEC-CI) (BioControl Systems, Inc, WA, USA). A peptone salt solution was used as the dilution medium (Feldsine, Lienau et al., 2019). The negative control used 5 grams of DI water, while the positive control was used to measure the initial concentration in the WW that was used to spike the FW.

#### **4.2.4 Statistical Analysis**

A Student's T-test assuming a two-tailed distribution and equal variance was used to test the statistical significance of the results. The statistical analysis was done using IBM SPSS (version 28.0.0.0 (190)).

### **4.3 Results and Discussion**

#### **4.3.1 Sulfate Reducing Bacteria**

The calculated FNA concentration for Experiment 4 is depicted in Fig. 18. As in previous experiments, most of the  $\text{NO}_2^-$  was consumed in the first 24 hours of the pre-treatment. At 24 hours, the average  $\text{NO}_2^-$  concentration was  $0.012 \pm 0.005$  mg-N/L. At a pH of 4, the added  $\text{NO}_2^-$  is lower than that at pH 5, which accounts for the lower concentration of  $\text{NO}_2^-$  as the reaction progresses. For comparison, at pH of 4 and 5

there is a significant difference in the FNA concentration at 24 hours when starting FNA concentration was 1.52 mg FNA-N/L ( $p=0.047$ ). At starting pH of 5, a solids content of 80.6 g VS/L, and an initial concentration of 1.52 mg FNA-N/L at 24 hours the average FNA concentration was  $0.097 \pm 0.046$  mg FNA-N/L. Meanwhile, at a starting pH of 4, a solids content of 78.8 g VS/L, and an initial concentration of 1.52 mg FNA-N/L at 24 hours, the average FNA concentration was  $0.009 \pm 0.003$  mg FNA-N/L. A higher pH (5 vs 4) allows the FNA concentration to be higher at 24 hours due to the increased  $\text{NO}_2^-$  concentration required.

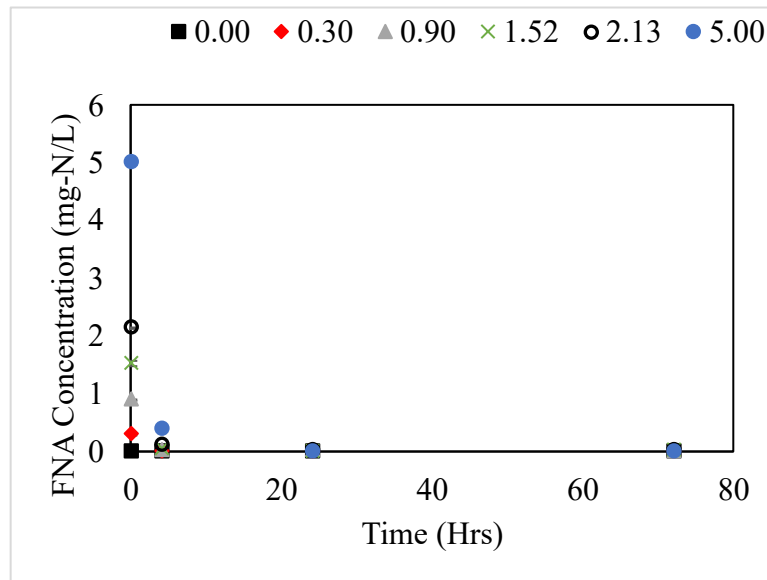


Figure 18. The calculated FNA concentration during Experiment 4. FNA concentration after 24 hours averaged  $0.013 \pm 0.001$  mg FNA-N/L. Where available, error bars show the standard deviation of triplicate tests.

Fig. 19 (A) shows the concentration of  $\text{SO}_4^{2-}$  during Experiment 4. Meanwhile, Fig. 19 (B) shows the production of  $\text{H}_2\text{S}$  during Experiment 4. During the first 4 hours, the level of inhibition corresponds with the concentration of FNA. Initial concentrations of 1.52, 2.13, and 5.00 mg FNA-N/L provided greater inhibition than initial concentrations of 0.3 and 0.9 mg FNA-N/L. This is further supported by the findings

of Jiang, Gutierrez et al., (2011), who reported that the level of the biocidal effect has a much stronger correlation with the FNA concentration rather than  $\text{NO}_2^-$  concentration or pH level. and therefore, suggesting that FNA is the actual biocidal agent when applied at pH of 5-7 (Jiang, Gutierrez et al., 2011).

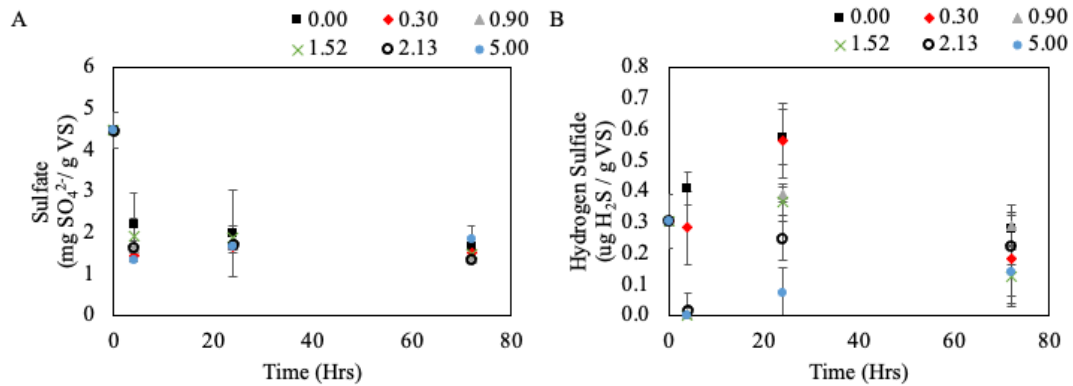


Figure 19. (A)  $\text{SO}_4^{2-}$  concentrations during experiment 4. (B).  $\text{H}_2\text{S}$  concentration during experiment 4.

A higher pH may allow FNA to be the inhibitory mechanism rather than acidification. In a study evaluating the resilience of sulfate-reducing granular sludge against temperature, pH, oxygen,  $\text{NO}_2^-$ , and FNA it was determined that the concentration of FNA must be high (2.3 mg N/L), and the pH must be moderately acidic, 5, in order to provide continuous biocidal effects to sulfate-reducing granular sludge (Hao, Mackey et al., 2016). Similarly, anaerobic biofilms experienced FNA's biocidal effects at concentrations of 0.2–0.3 mg N/L with an exposure time longer than 6 hours (Jiang and Yuan, 2013).

Initial concentrations ranging from 1.52-5.00 at pH 4 effectively inhibit SRB activities during the first 24 hours of pre-treatment. After 24 hours,  $\text{NO}_2^-$  consumption diminishes

the treatment's effectiveness. Other works have also cited that  $\text{NO}_2^-$  consumption provided lesser biocidal effects as time progressed (Ye, Pijuan et al., 2010).

#### **4.3.2 Pathogenic bacteria**

The biocidal properties of acidification are slower than those of FNA. Fig 20 depicts the total coliform and *E. coli* population trends during Experiment 3. The addition of 5.0 mg-N/L FNA provided faster pathogen kill (1.1 log reduction of total coliforms within 4 hours) than pH adjustment group and control the control group. FNA application also provided faster *E. coli* kill than the pH adjustment and control groups,  $0.463 \pm 0.135$  logs in 4 hours and  $1.56 \pm 0.514$  logs in 24 hours. This reduction in total coliform agrees with previous literature. Meng, Duan et al.,(2020) saw a decrease in the MPN of Fecal Coliform in thickened WAS treated for 24 hours at an FNA concentration of 4.9–6.1 mg N/L and a pH of 5.0 (Meng, Duan et al., 2020). Unfortunately, the quantity of log reduction was not reported in their report. Meanwhile, the acidification of the system provides pathogen kill after 24 hours of pre-treatment.

Although *E. coli* has a defense mechanism against low pH levels (Lund, Tramonti et al., 2014), the acidic pH caused by the decomposition of FW, may have contributed to the decrease in pathogen populations of the control and pH adjustment groups. As the pH of the slurry mixture decreases, the internal pH of *E. coli* will also decrease due to the accumulated organic acids lowering the intracellular pH by transporting protons into the cytoplasm (Hirshfield, Terzulli et al., 2003). Furthermore, internal pH below

7.2 has also been noted to cause lower rates of glycolysis in *E. coli* (Ugurbil, Rottenberg et al., 1978). It will be harder for *E. coli* to maintain its optimal internal pH in acidic environments. At 72 hours, the pH treatment and control groups have an environmental pH of  $3.52 \pm 0.06$ , exhibiting biocidal effects through acidification.

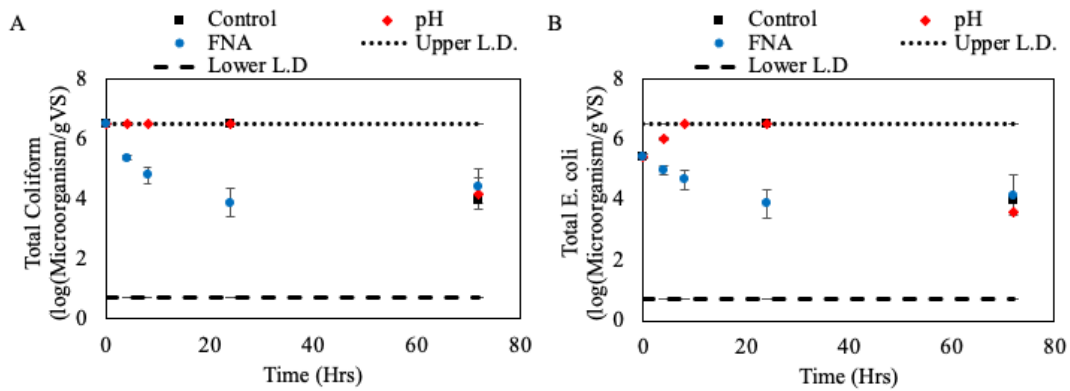


Figure 20. (A) Total coliforms in the treatment groups. (B) Total *E. coli* in the treatment groups. In both graphs, a dotted line marks the upper limit of detection. A dashed line marks the lower limit of detection. The detection limits of the graphs may be different from those of Fig. 21, as they are reported as microorganisms per g VS. The solids content here is 91.4 g/L VS. Additionally, the measurements that are marked at the upper limit of detection can be either at the limit or at a much higher population number. It is not possible to discern how much above higher than the upper detection limit this value may be.

Fig. 21 demonstrates that the biocidal effect provided by acidification to a pH below 4 is supported by Experiment 4. The acidification of the system to a starting pH of 4 and a final pH of  $3.56 \pm 0.08$  was sufficient to reduce the number of total coliforms by 2.5 logs at 72 hours.

The higher concentrations of FNA provided greater bacterial reductions (coliform) at 4 hours of pre-treatment;  $2.52 \pm 0.00$  logs (5 mg-N/L),  $1.97 \pm 0.348$  logs (2.13 mg-N/L),  $1.49 \pm 0.247$  logs (1.52 mg-N/L),  $1.78 \pm 0.357$  logs (0.90 mg-N/L),  $0.962 \pm 0.270$  logs (0.30 mg-N/L) and  $0.506 \pm 0.104$  logs (0.0 mg-N/l). At 24 hours of pre-treatment,

the bacterial populations were at the lower limit of detection (<1169 microorganisms/g VS). Similarly, in the first 4 hours, the reduction in the population of E. coli also increased with starting FNA concentration.  $1.52 \pm 0.145$  log reduction by 5.0 mg-N/L,  $1.28 \pm 0.114$  (2.13 mg-N/L),  $0.775 \pm 0.246$  (1.52 mg-N/L),  $0.917 \pm 0.215$  (0.90 mg-N/L),  $0.584 \pm 0.399$  (0.30 mg-N/L),  $0.354 \pm 0.143$  (0.0 mg-N/L) At a starting pH of 4, the ideal treatment time is less than 24 hours. The acidification of the system at pH 4 also showed a reduction in pathogenic bacteria, which is different from the results in the acidification group of the previous experiment (Fig. 20) at pH 5.5. Such difference between the acidification groups indicated that a lower pH (4) can provide faster pathogen reduction than that at a higher pH (5.5).

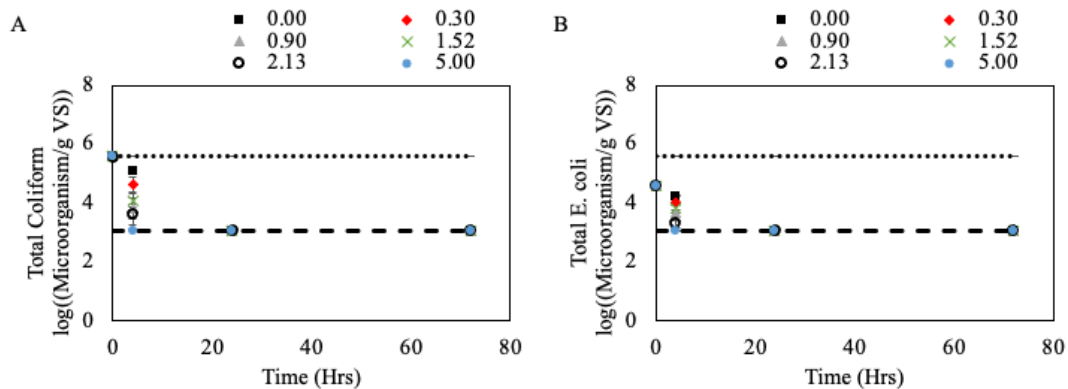


Figure 21. (A) Total Coliform bacteria. (B) Total E. coli. In both graphs, the maximum detection limit of the test is denoted with a dotted line. The minimum detection limit is denoted with a dashed line. The detection limits of the graphs may be different from those of Fig. 20, as they are reported as microorganisms per g VS. The solids content here is 78.8 g /L VS.

#### 4.4 Conclusion

Higher concentrations of FNA provide more effective inhibition of biological sulfate reduction. Inhibition can be achieved at pH 4 if the starting concentration of FNA is higher than 0.30 mg FNA-N/L. At 72 hours, the  $\text{NO}_2^-$  consumption leads to decreased

inhibition, regardless of starting FNA concentration, which may lead to a recovery of sulfate reduction activities. This may indicate the need for a shorter pre-treatment time, less than 24 hours. Similarly, FNA addition provides faster biocidal effects to pathogenic bacteria like *E. coli* than acidification alone, within 4 hours vs 24 hours. The biocidal effects of FNA are also dependent on the starting concentration of the solution. At a range from 0-5 mg FNA-N/L, higher concentrations of FNA provide greater pathogen reduction in a shorter time.

## **Chapter 5: Conclusion**

The effectiveness of FNA application in improving the digestibility of FW and controlling problematic microorganisms were studied in this research. The results indicated that the effectiveness of FNA varies under different treatment conditions, including solids contents, FNA concentration, and initial pH.

The application of FNA at 1.5 mg-N/L did not have increased solubilization of organic material. However, there was no consumption of the organic material, under the tested pH of 3, 4, and 5. Such result indicated that 1.5 mg-N/L combined with acidification to pH 3, 4, and 5 is sufficient to inhibit the consumption of organic materials by microbial communities.

N-related bioprocesses can also be inhibited by FNA application and acidification. The effectiveness of FNA application on inhibiting N-related bioprocesses depends on solid content. Under more acidic conditions (pH 3) moderate FNA application (1.5 mg-N/L) can maintain effective concentrations of FNA for a longer period because the acidic pH inhibit  $\text{NO}_2^-$  consuming organisms. Meanwhile, a low solids content (16.8-43.3 g/L VS) and moderate FNA application can also maintain more consistent FNA concentrations.

FNA pre-treatment showed an effective control in SBR and pathogens with the optimal treatment condition determined: low total solids content (16.8-43.3 g/L VS), pH 4-5.5, FNA concentration (1.5-5.0), and treatment time (<24 Hours). At a lower pH level (pH

< 4), the acidic environment can effectively inactivate the pathogen without the assistance of FNA.

FNA addition enabled faster biocidal effects to pathogenic bacteria than acidification alone. A reduction (>99.9%) in pathogenic bacteria can be achieved in a shorter pre-treatment time (<24 hours) with FNA addition, while the groups without FNA addition can take up to 72 hours.

Finally, high solids content can reduce the effectiveness of FNA pre-treatment due to mass transfer limitations. With the solid content of  $150 \pm 8.83$  g/L VS, a moderate concentration of FNA (1.5 mg FNA-N/L) does not improve solubilization of FW than acidification alone at all tested pHs (3,4, and 5). As a comparison at a lower solids content, (< 80.6 g VS/L), an FNA concentration of 1.5 mg FNA-N/L can provide a consistent FNA concentration and inhibition of the activities of microbial organisms. At a higher solids content, 80.6–133 g VS/L, the concentration of FNA decreases due to the consumption by microbial organisms.

Future work needs to explore the effects of FNA on the solubilization of organic material, like VFA, sP, and sPS. Studies in the types of organic material that is being solubilized can help determine the mechanism of action for FNA pre-treatment in FW. Additionally, an FNA concentration that can inhibit microbial activities and increase organic materials' solubilization should be further assessed and determined.

## **Appendix A. Summary of Previous Works**

These experiments were carried out at 35 °C and used the same recipe for synthetic FW as outlined in Chapter 2. Table 10 summarizes the experimental designs of previous works.

### **FW only**

FW was treated in two reactors R2 & R3 with FNA at a concentration of 5.27 mg-N/L by adjusting pH to 5 at 35°C for 28 days. The control group R1 without FNA. The effect of the mixing conditions was also examined (R2: continuous mixing at 200 rpm; R1 & R3: manual mixing for 1 min at sample collection). Weekly changes in pH, NH<sub>4</sub><sup>+</sup>, TS, VS, sCOD, sP, sPS were monitored

Results showed a more stable pH in R2 and R3 than in R1. On day 28, the sCOD in R2 increased by  $43.3 \pm 7.5$  %. Meanwhile, on day 14, the COD of R1 increased by  $10.9 \pm 0.2$ %, and R3 by  $14.3 \pm 0.1$ %. R1 and R3 saw a decrease in sCOD after 14 days. From a starting value of 26.5 g/L sP, and 64.3 g/L sP, the sP and sPS in R1 showed a decrease to 17.3 g/L sP, and 44.2 g/L sPS. The release of sP and sPS from the cells in FW was enhanced by FNA addition in R2 and R3. R2 increased (by 5% for sP and 25% for sPS), and R3 increased (by 48% for sP and 30% for sPS).

The FW was used for a preliminary BMP test at the end of the pre-treatment. The average CH<sub>4</sub> production in BC (inoculum only) was subtracted from other groups to correct the results for presenting the total CH<sub>4</sub> production from FW digestion. The biogas yield was the highest in R3 (483 NmL CH<sub>4</sub> /g VS) and R2 (481 NmL CH<sub>4</sub> /g VS). The total CH<sub>4</sub> production in R3 was 446 NmL CH<sub>4</sub> /g VS. Additionally, the rate

of initial CH<sub>4</sub> production during the first 48 hours was: 121 NmL (R1), 104 NmL (R2), 142 NmL (R3), indicating that a prolonged AD treatment might be necessary.

Table 10. Summary of previous experiments

Experiments	Reactor	Pre-treatment Duration	FW (mL)	SS (mL)	Total vol.* (mL)	TS (g/L)	VS (g/L)	Designed FNA (mg N/L)
FW only	R1	28 days	200	0	240	206 ± 0.7	204 ± 0.0	0
	R2					193 ± 19.2	185 ± 27.5	5.27
	R3					166 ± 18.2	156 ± 13.5	5.27
Varying mixture ratios (SS:FW)	R_100:0	72 hrs	0.0	150	155	3.7 ± 0.3	1.9 ± 0.3	5.00
	R_75:25		0.7	149	155	5.1 ± 0.1	3.1 ± 0.1	5.00
	R_50:50		2.0	148	155	10.4 ± 0.2	8 ± 0.1	5.00
	R_25:100		5.8	144	155	22.1 ± 0.2	19.1 ± 0.1	5.00
	R_0:100		150	0	155	149 ± 0.4	138 ± 0.8	5.00
Varying FNA conc.	R_0 <sup>##</sup>	72 hrs	4.3	146	165	5.95 ± 0.06	5.61 ± 0.07	0
	R_0.3							0.3
	R_1.07							1.07
	R_2.13							2.13
	R_5							5

\*, MilliQ water was added to the reactor to meet the designed total volume.

This experiment showed promising results for using FNA to enhance the biogas yield of FW treatment in AD, and to reduce the solids content. The solids reduction is in Fig. 22. The mixing condition did not show a further improvement of the biogas yield, indicating that a simple application of FNA in the feed storage tank can be sufficient for achieving the enhancement..

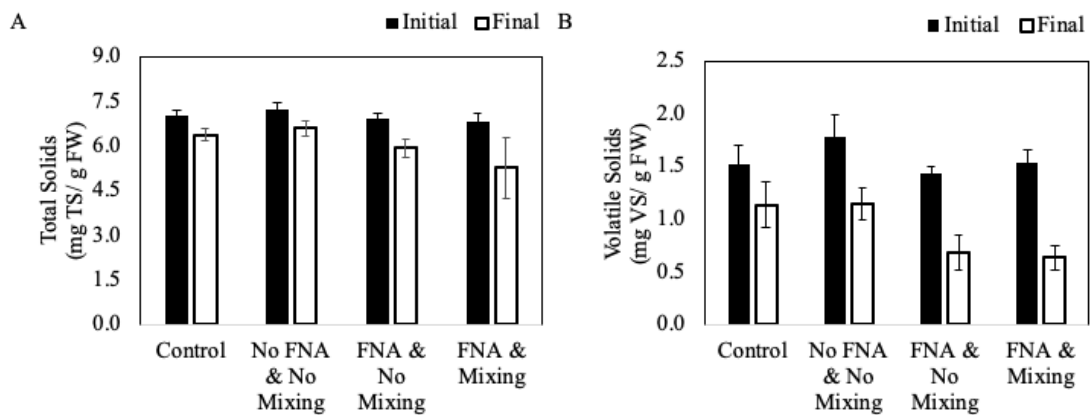


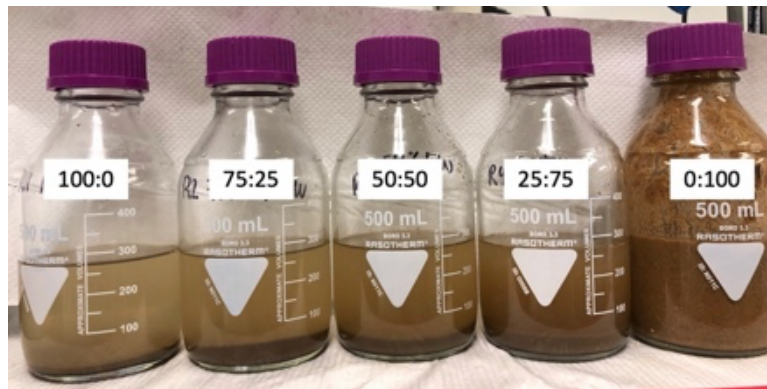
Figure 22. Solids reductions during preliminary BMP test. (A) TS reduction in the different treatment groups. (B) VS reduction in the different treatment groups. The FNA treatment groups show a greater reduction in solids than the control groups.

### Varying Mixture Ratios (SS:FW)

This work assesses the effectiveness of FNA in improving feed quality for AcoD. A mixture of synthetic FW and SS collected from local WWTP was prepared at five ratios (100:0, 75:25, 50:50, 25:75, and 0:100, % w/w VS) and treated with 5 mg FNA-N/L by adjusting pH to 5 at 35°C for 72 hours, with 155 rpm. Daily changes in pH, NH<sub>4</sub><sup>+</sup>, TS, VS, sCOD, sP, sPS were monitored.

Each reactor had a different solids content. This meant that even if equal volumes of FNA solution were added, the ratio of mg FNA/ g VS in each reactor was different. It

was suggested that as the TS and VS increase in each reactor, the effectiveness of FNA may decrease in efficacy due to increased mixture viscosity. This increase in viscosity and turbidity can be observed in Fig. 23. Similar work has been conducted with other pre-treatment technologies, like  $\beta$ -Cyclodextrins and alkaline pre-treatment, showing this was a relevant topic (Yang, Liu et al., 2016).



*Figure 23. The percent of FW increases in each bottle from left to right. As the volume of the FW in the solution increases, the mixture's turbidity and viscosity increase. This increase in viscosity may lead to a decrease in the effectiveness of the FNA pre-treatment*

The mixed feedstock showed a higher buffer capacity and maintained a more stable pH ( $5.02 \pm 0.16$ ) at 72 hours pre-treatment, compared with decreasing (FW pH=3.9) and increasing (SS pH=6.01) pH for single feedstock. A higher buffer resistance to pH change can be achieved by mixing FW with SS.

At 72 hours, sCOD increased in all reactors,  $66.7 \pm 6.7\%$  (R\_100:0),  $20.0 \pm 9.4\%$  (R\_75:25),  $16.2 \pm 13.5\%$  (R\_50:50),  $38.6 \pm 7.4$  (R\_25:75),  $147 \pm 9.6$  (R\_0:100). R\_100:0, R\_75:25, R\_50:50 show decreasing solubilization of COD as the volume of FW (% VS). This trend changes when there is more FW than SS in the mixture (R\_25:75, and R\_0:100). The increase in sPS and sP produced by the FNA pre-

treatment corresponds with literature (Wang, Ye et al., 2013). For example, FNA treatment at a concentration of 0.52-1.43 mg FNA-N/L combined with heat treatment between 35-70 °C can enhance EPS destruction and solubilization of organic material like sPS and sP (Wang, Jiang et al., 2014).

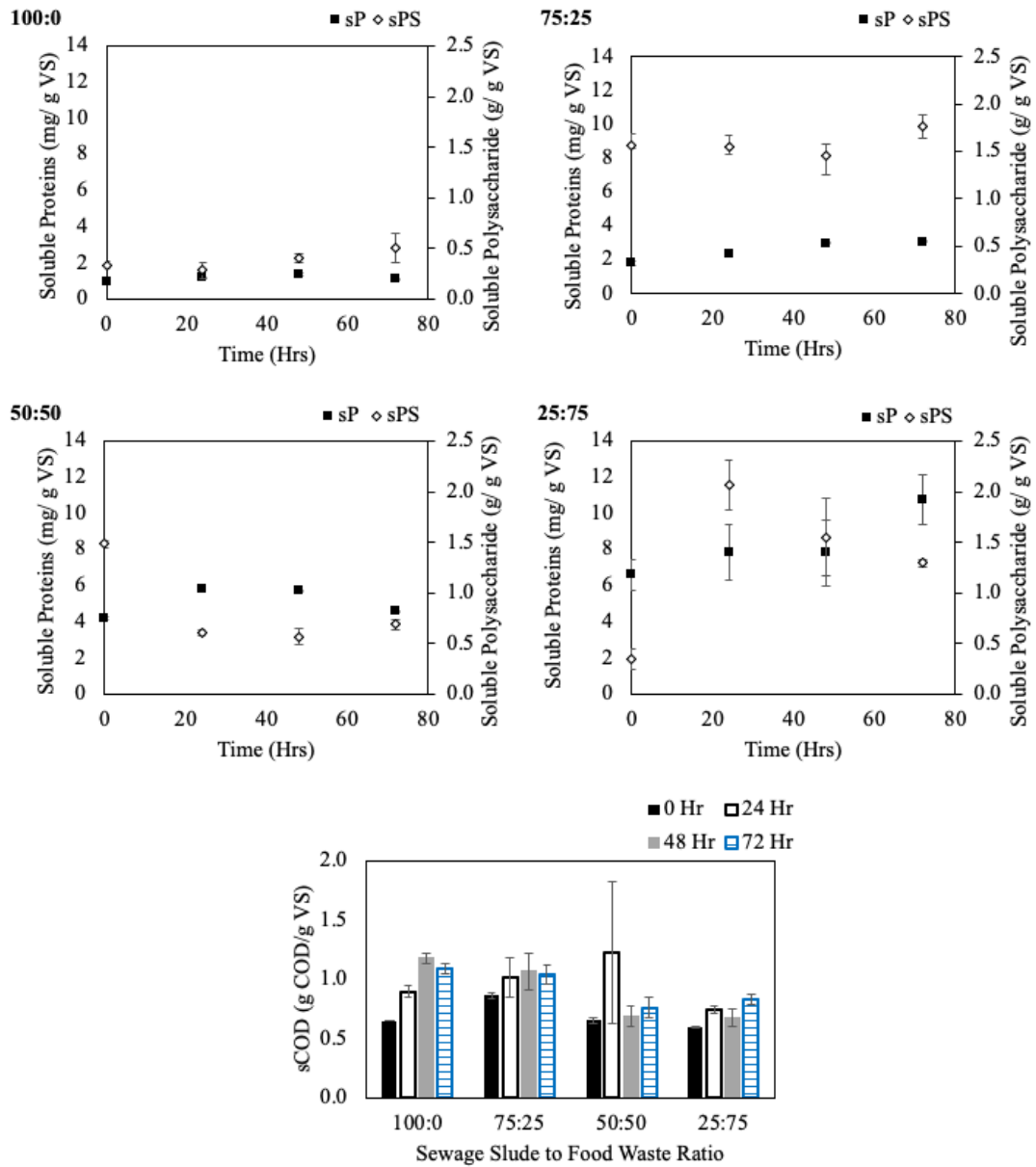


Figure 24. Solubilization of proteins and polysaccharides in each treatment ratio. And the corresponding solubilization of COD.

Different activities in the microbial communities, and their COD consumption may cause varying trends. The sP and sPS in all reactors increased with treatment duration. These are depicted in Fig. 24. These results showed improved solubilization of organic material found in SS when combined with FW. FNA pre-treatment cannot reduce the solids content as TS and VS were stable in all reactors.

Prabhu and Mutnuri 2016, reported that the optimal ratio of SS to FW for the highest biogas production during AcoD to be 2:1. Ratios of 80:20 (VS) and 50:50 (VS) SS to FW have also been explored (Kim, Han et al. 2003). As the experimental results and the literature show, there is no clear consensus of the ideal ratio that should be used to enhance co-digestion. The literature neither decides on the parameter (TS, volume, or C/N ratio) that should be used to measure the ratio, nor how to define the ratio (Mehariya, Patel et al., 2018). However, there are benefits of co-digestion of substrates, such as increased biogas yield during the co-digestion of cotton stalk and swine manure (Cheng and Zhong, 2014).

### **Varying FNA Concentration**

After selecting a mixture ratio of 50:50 % w/w VS, varying concentrations of FNA (0.00 (R\_0), 0.30 (R\_0.3), 1.07 (R\_1.07), 2.13 (R\_2.13), 5.00 (R\_5) mg FNA-N/L) at pH 5.5, 35°C for 72 hours were tested. The purpose was to explore the relationship between increasing concentration and solubilization of organic material. The results indicated that increasing FNA concentration provided greater solubilization of COD

when treatment time was longer than 24 hours. At 72 hours, the sCOD of R5 increased by  $165 \pm 42.3\%$ , and R\_2.13 increased by  $3.3 \pm 3.5\%$ . Meanwhile, R\_1.07 decreased by  $-32.8 \pm 3.9\%$ , R\_0.3 decreased by  $-48.6 \pm 0.6\%$ , and R\_0 decreased by  $-64.0 \pm 4\%$ . Increasing FNA concentration provided greater solubilization of organic material. These changes are depicted in Fig. 25.

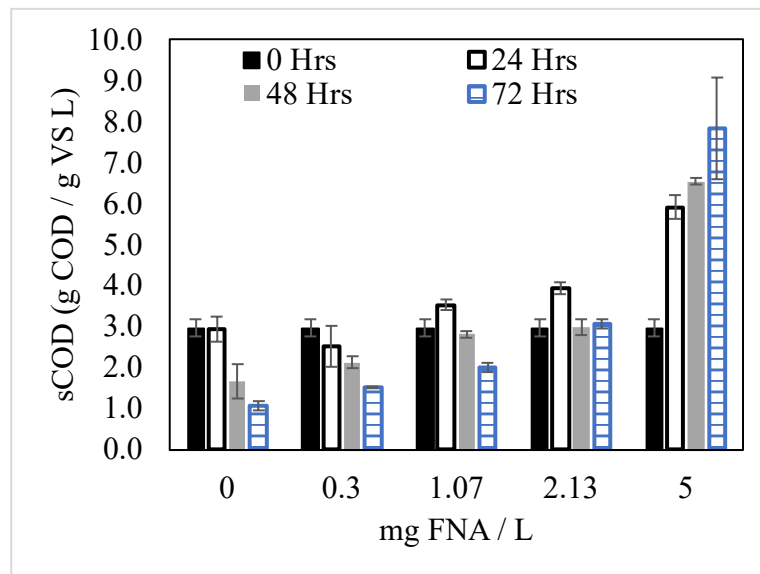


Figure 25. Solubilization of organic material. Higher FNA concentrations produced higher increases in COD solubilization.

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