NITRIFICATION PROCESS IN INTEGRATED FIXED-FILM ACTIVATED SLUDGE (IFFAS) SYSTEM

by

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Abstract

The ammonia released from wastewater is a key factor in detrimental environment concerns such as eutrophication. Nitrogenous waste is most commonly removed from wastewater through biological means, typically involving the use of ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), and denitrifying bacteria (DEN). This study looked at alternative nitrification processes, including anaerobic ammonia oxidiation (anammox) reactions that may be innately present in an integrated fixed-film activated sludge (IFFAS). Due to the unique characteristics of IFFAS configuration, the biofilm component allowed for anoxic zones, as well as higher biomass retention, both factors favouring the growth of anammox bacteria. One of the main goals of this thesis project was to look for the presence of anammox bacteria, which could be a group of microorganism that is involved in removal of ammonia and nitrite, in addition to the conventional nitrifiers found in the IFFAS system.

Through the use of batch experiments, the different forms of nitrogen in the microbial nitrogen cycle (ammonia, nitrite, and nitrate) were measured over the course of the IFFAS reaction period, under aerated and non-aerated conditions. Subsequently, fluorescence *in-situ* hybridization (FISH) techniques were utilized to confirm anammox presence. Although, initial data from the batch experiment was strongly suggestive of anaerobic ammonia oxidation in the IFFAS system, the FISH experiments did not detect anammox bacteria. Incorporating data from a parallel experiment, it was possible that a different group of bacteria, known as *Rhodanobacter*, may be responsible for the AOB to NOB imbalance rather than anammox bacteria. Certain species of *Rhodanobacter* are able to utilize nitrite and nitrate as oxidizers in the denitrification process under anoxic conditions, which may be present in the biofilms of the IFFAS system. Therefore, it is possible that *Rhodanobacter* is involved in the removal of any excess nitrite produced by the higher levels of AOB in the IFFAS reactors.

An interesting observation obtained early on in this study showed a synergistic effect between the floc and biofilm components of the IFFAS system, where higher ammonia removal was seen at the end of the 6 hour reaction cycle in the IFFAS combined floc and biofilm reactor, compared to the individual IFFAS floc and the IFAS biofilm reactor. This observation was replicated in a triplicate of non-aerated batch experiments; however, the combined floc and biofilm did not yield significantly higher ammonia removal than the floc and biofilm individually ($p > 0.05$).

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List of Abbreviations

AMO –Ammonia monooxygenase

Anammox –Anaerobic ammonia oxidization

AOA –Ammonia oxidizing archaea

AOB –Ammonia oxidizing bacteria

ATP –Adenosine triphosphate

BOD –Biological oxygen demand

CAS –Conventional activated sludge

CSLM –Confocal scanning laser microscope

DEN –Denitrifying bacteria

DNA –Deoxyribonucleic acid

DGGE –Denaturing gradient gel electrophoresis

EPS –Extracellular polymeric substances

EUB –Eubacteria

FISH –Fluorescent *in-situ* hybridization

HAO –Hydroxylamine oxidoreductase

IC –Ion chromatography

IFFAS –Integrated fixed-film activated sludge

MBR –Membrane bioreactor

MLSS –Mixed liquor suspended solids

MPN –Most probable number

NOB –Nitrite oxidizing bacteria

PCR –Polymerase chain reaction

RAS –Recycled activated sludge

RBC –Rotating biological contactors

rRNA –Ribosomal ribonucleic acid

SBR –Sequential batch reactor

SRT –Sludge retention time

SVI –Sludge volume index

TKN –Total Kjeldahl nitrogen

WWTP –Wastewater treatment plant

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Chapter 1 General Introduction and Literature Review

The study of water has always been of great interest to humans throughout history, from the disinfection of drinking water, necessary for basic survival, to the treatment of wastewater, crucial to sanitation and protection of the environment. Humans, as a species, take full advantage of all the resources provided on this planet, and as a result, also produce a significant amount of waste, both solid and liquid. It can be argued that the responsible disposal of waste is part of the foundation for a civilized society. As early as 2500 BC, the Mesopotamian Empire has utilized brick sewage systems for their waste disposal, evident through archeological studies (Gray, 1940). Prior to having basic understanding of microorganisms and their presence in wastewater, sewage and other human wastes were simply expelled into larger water bodies; unfortunately, these water bodies were typically also used as the drinking water sources. In the mid-1800s, a British physician by the name of John Snow theorized an infectious agent from contaminated raw sewage that was responsible for the transmission of deaths in London at the time (Bitton, 1994). Robert Koch discovered the bacterium *Vibrio cholera*, which was later understood to be the microorganism responsible for the deaths observed by Snow in London (Reidl and Klose, 2002). Through research in the field of microbiology and the improved understanding of wastewater compositions, these early scientists paved the way for a more civilized society, in which wastewater treatment is regarded as normality, and eventually leading to legislations for the construction of wastewater treatment plants. The conventional method of biological wastewater treatment, known as conventional activated sludge (CAS), has been in place for a century now, the general concept of which remains intact even to this day. The wastewater community is celebrating the 100th year anniversary for CAS at the time of this writing. Although the study into the history of wastewater may not be directly related to the research undertaken in this thesis

project, it is important to understand the desire shared by generations of scientists, as was in the case of this research, to study the composition of wastewater and its treatment, and thus, allow for innovation.

One of the major environmental concerns with the release of untreated effluents into water bodies is the risk of water pollution through a phenomenon known as eutrophication. Eutrophication is an increase in the rate of nutrient supply to an aquatic ecosystem (Nixon, 1995), which often leads to the overgrowth of aquatic plants and planktonic species (Smith *et al.*, 1999). Wastewater is nutrient rich in composition, a concept that was not lost to the ancient Greeks; even as early as 300 BC, the Greeks built public latrines, which delivered wastewater through networks of sewage systems to crops and orchards outside of the city as a source of nourishment (Henze, 2008). The understanding of the relationship between nutrient limitation and the growth of plants originated from the works of German chemist, Justus von Liebig, in a theory known as Liebig's Law of Minimum (De Baar, 1994); it has since been determined that specific nutrients, such as phosphorous and nitrogen, are central limiting factors to the growth of plants, both terrestrial (Schlesinger, 1991) and aquatic (Vollenweider, 1968). Eutrophication, one of the most common and widespread issues with water quality in the US and other countries (Carpenter *et al*., 1998), has a variety of negative consequences on marine ecosystems; some examples of the negative effects include: elevation in pH, depletion of dissolved oxygen in water column, shifts in composition of organisms, increased fish kills (Smith, 1998), and toxicity associated with blue-green algal blooms (Skulberg *et al*., 1984). The production of organic matter in phytoplankton is a photosynthetic process that requires the conversion of inorganic nutrients, most of which are in excess in water bodies. The nutrients that are limiting towards the needs of phytoplankton are nitrogen and phosphorous (Vollenweider, 1968), appearing in natural water bodies at micro-molar levels; it is the addition of these inorganic nutrients from raw wastewater that allows for the

overgrowth of phytoplankton and aquatic plants. Depending on the nutrient that is limiting in the water, the emphasis of eutrophication can alternative between decreasing the levels of nitrogen and phosphorous. Typically, excessive phosphorous is the cause of eutrophication in freshwaters, such as lakes and rivers; whereas, excessive nitrogen causes eutrophication in the ocean (Correll, 1998; Ryther and Dunstan, 1971).

The drivers for present wastewater treatment processes focus on many of the problems that are associated with the drastic increase in population growth. In comparison to the early 1900, around the time when CAS treatment process was first introduced, the global population growth rate has doubled from 0.6% to 1.2% per year in the year 2010; the population has increased from 2.5 to 6.1 billion people around the world in just 50 years, from the year 1950 to 2000 (Cleland, 2013). The staggering change in population brings with it an increased demand on the existing infrastructure. The wastewater researchers need to adapt to the demands that come with population growth and increased waste production; it becomes apparent that there is dire need to increase the capacity of existing infrastructure. It is also possible to build more treatment plants to allow for increased demand, but that would result in high building costs and issues with space limitation for those treatment plants. By examining different methods of wastewater treatment, including the utilization of integrated systems of flocs and biofilms, studying the characteristics of granules, or the incorporation of novel wastewater bacteria, it may be possible to expand the capacity of present wastewater treatment plants (WWTPs) without the need for major infrastructural modifications. Along with the increase in global population, the energy needs also have to be addressed. The rise in consumption and demand for energy is regarded as one of the major problems in the world today (Sen, 2004). Innovators in different fields are coming up with new ways to produce energy, as well as, methods of reducing energy costs; wastewater research is no different. For example, developments in the anaerobic treatment

facilities may greatly reduce the costs associated with powering the aerators, which in some cases contribute to the majority of the operational costs of treatment plants (Gander *et al.*, 2000). There are studies that have also shown the ability of wastewater sludge to generate biofuel, through the production of biogas (methane and hydrogen) (Angenent *et al.*, 2004), which can help WWTPs achieve net energy neutrality. As the commodity of fresh potable water is scarce in certain parts of the world, with the increase in population there will only be a stronger need for water repurposing. The development of new wastewater treatment techniques like membrane bioreactors (MBRs) may play a key role in the future of wastewater treatment, as the reactor utilizes membrane to produce effluents lacking pathogenic microorganisms (Gander *et al.*, 2000). Much of these drivers for future developments in wastewater research have also played a key role in the direction of this thesis research, which will be apparent by the end of this chapter.

1.1 Microbial Nitrogen Cycle

The focus of the research in this thesis is associated with the removal of nitrogen from wastewater; however, to understand the methods of treatment, a basic understanding of the microbial nitrogen cycle is necessary. Nitrogen can be found in many different oxidation states and chemical compounds, and is the basic building foundation of proteins, essential to life on Earth (Canfield *et al.*, 2010). The majority of nitrogen, roughly 70%, on the planet exists in its most stable form, as gaseous triple-bonded dinitrogen. This stable form of nitrogen is unable to be utilized by most living organisms as substrate, thus requires the aid of microbes to allow for its conversion into more readily accessible compounds, such as ammonia, for assimilation; ammonia is immensely important in the maintenance of biological productivity (Falkowski *et al.*, 2008). Within the nitrogen cycle, there are five different types of nitrogen transformation, including: fixation, the conversion of nitrogen gas into ammonia; assimilation, the utilization of

ammonia/nitrate to produce protein; mineralization, the breakdown of proteins into amino acids and ammonia; nitrification, the oxidation of ammonia to nitrate; and denitrification, the reduction of nitrate to nitrogen gas (Canfield *et al.*, 2010).

Specialized nitrogen-fixing bacteria thrive symbiotically with plants, usually found in root nodules of legumes, as is the case with the group of soil bacteria known as rhizobia, typically belonging to the α-Proteobacteria subclass with discoveries in the β subclass (Moulin *et al.*, 2001). It has been found that within most of the bacterial phyla, there can be representatives that possess properties of nitrogen fixation, as well as in methanogenic Archaea (Young, 1992). Typically in nature, ammonia is produced through nitrogen fixation and mineralization processes, however, anthropogenic means of producing ammonia for agricultural and industrial purposes, after the industrial revolution, have dramatically increased the levels of ammonia deposition in the environment (Grennfelt and Hultberg, 1986). Much like the other factors associated with population growth, the increase in ammonia production for fertilizers results in placing higher demand on the treatment infrastructure for industrial WWTPs.

It should be emphasized, once more, the possible threat waste ammonia nitrogen may have on the eutrophication of water resources; however, as with most phenomena in nature, there is an opposing force that returns the ammonia back to its elementary di-molecular state. The most widely studied method of ammonia removal is made up of nitrification and denitrification. Nitrification is described in the literature as two separate aerobic processes, involving the use of specialized chemolithoautotrophic prokaryotes, known as nitrifiers, in sequential oxidation reactions, converting ammonia to nitrite, and nitrite subsequently to nitrate (original work by Winogradsky, 1890; recent review by De Boer and Kowalchuk, 2001); denitrification reaction

involves anaerobic reduction, utilizing heterotrophs to convert nitrate into nitrogen gas (Mateju *et al.*, 1992).

1.1.1 Ammonia Oxidizing Bacteria (AOB)

Ammonia oxidation is the first step of nitrification, which involves the conversion of ammonia to nitrite; this oxidation reaction is carried out by a group of chemolithoautotrophic microorganisms known as ammonia oxidizing bacteria (AOB). Ammonia can exist in both its neutral, NH_3, and cationic form, NH_4^+, referred to as ammonium; however, it is important to note that AOB use nitrogen in the form of ammonia, and not ammonium (Suzuki *et al.*, 1974). AOB are typically obligate aerobes; however, studies have shown that there are species which are able to survive in anoxic environments (Bodelier *et al.*, 1996). Of the sixteen genera of AOB that have been isolated, two monophyletic groups, determined through comparative 16S rRNA sequence analysis, separate the species into *β*- and *γ-Proteobacteria* (Head *et al.*, 1993); *Nitrosomonas, Nitrosospira, Nitrosovibrio, Nitrosolobus*, and *Nitrosococcus mobilis* are found under the beta-subclass; while, *Nitrosococcus oceanus and Nitrosococcus halophilus* belong to the gamma-subclass (Nielsen *et al.*, 2009). The most commonly found, and extensively studied genus of AOB is *Nitrosomonas,* with the species *N. europaea, N. eutropha, N. mobilis,* and *N. oligotropha* being the most abundant in WWTPs (Nielsen *et al.*, 2009). *N. oligotropha* can be the most dominant species, as the name suggests, if the concentration of ammonia is limited (Bollmann *et al.*, 2002); detection of these organisms using specific oligonucleotide probe, Cluster6a192, can provide insight on the type of nutrient condition the system is operating under. The rest of the commonly observed *Nitrosomonas* species can be detected using NEU oligonucleotide probes; the use of Nso1225 detects all known AOB, thus is frequently used in parallel with NEU and Cluster6a192 probes (Nielsen *et al.*, 2009).

The overall simplified reaction for ammonia oxidation is as follows, where oxygen acts as the electron acceptor (oxidizer), and thus removes electrons from the ammonia:

$$NH_3 + O_2 => NO_2^- \quad [1]$$

The actual chemical transformation of ammonia nitrogen by AOB is more complicated and can be described as follows (Kowalchuk and Stephen, 2001):

$$2H^+ + NH_3 + 2e^- + O_2 => NH_2OH + H_2O \quad [2]$$

$$NH_2OH + H_2O => HNO_2 + 4e^- + 4H^+ \quad [3]$$

The first component of the oxidation reaction is catalyzed by the membrane-bound, ammonia monooxygenase (AMO) enzyme; while, the latter half of the reaction is catalyzed by a periplasm-associated enzyme, known as hydroxylamine oxidoreductase (HAO) (Prosser, 1986; Bodelier *et al.*, 1996). The net electron production from the overall reaction process is used to drive the proton motive force through the electron transport chain, the purpose of which is to create expendable energy resources in the form of adenosine triphosphate (ATP), important for cellular growth and maintenance. The nitrite, an anion, can form nitrous acid when interacting with the free hydrogen ions produced at the end of the reaction, which is the reasons why the nitrification process is acidic in nature.

1.1.2 Nitrite Oxidizing Bacteria (NOB)

Nitrite, which is produced by AOB, is subsequently employed by nitrite oxidizing bacteria (NOB) during the second stage in the nitrification process, converting nitrite into nitrate through an aerobic oxidation process. NOB are typically found in close proximity to AOB due to their mutualistic relationship (Maixner *et al.*, 2006); nitrite oxidizers, much like AOB, can be difficult to study due to their slow growth rates; in fact, the most abundant genus of NOB in WWTP, *Nitrospira* (Juretschko *et al.*, 1998; Schramm *et al.*, 1998), has not been isolated in a

laboratory setting, with only one example of high enrichment achieved (Spieck *et al*., 2006). At one time in history, it was widely believed that *Nitrobacter*, a very well-studied *α-Proteobacterial* NOB, was the dominant force behind nitrite oxidization in WWTPs (Bock and Koops, 1992). It was through the use of molecular biological techniques, including PCR and oligonucleotide probes, which revealed a lack of *Nitrobacter* within activated sludge and biofilms (Wagner *et al.*, 1996), with the exception of two biofilm plants (Mobarry *et al.*, 1996; Schramm *et al*., 1996). In one study, complete nitrification was detected in a bioreactor, however, instead of the expected *Nitrosomonas* and *Nitrobacter* spp., *Nitrospira* and *Nitrosospira* spp were found to be the dominant genera (Schramm *et al.*, 1998). This finding completely revolutionized the understanding of nitrification at the time. It was observed that only in bioreactors that have exceptionally high levels of nitrite, would one expect to see the population of *Nitrobacter*, adapted to thriving in high nitrite environments, dominate over *Nitrospira* (Daims *et al.*, 2001).

NOB are split up into two main distinct genera, *Nitrospira* and *Nitrobacter*. *Nitrospira* can be subdivided into four sublineages, and although sublineage I, II, and IV can be found in WWTPs, the most commonly detected *Nitrospira* is of sublineage I (Nielsen *et al.*, 2009). Conveniently, all four sublineages of *Nitrospira* can be detected simultaneously, through oligonucleotide hybridization techniques, using a combination of Ntspa712 and Ntspa662 FISH probes. There are four common *Nitrobacter* species in WWTPs: *Nitrobacter alkalicus, Nitrobacter winogradskyi, Nitrobacter vulgaris,* and *Nitrobacter hamburgensis*; all of which can be detected through the use of NIT3 oligonucleotide FISH probes. A novel β-*Proteobacterial* NOB, *Nitrotoga arctica* (Alawi *et al.*, 2007), was discovered in recent years, prompting the question of how many other types of NOB are in the WWTPs, waiting to be discovered.

1.1.3 Denitrifying Bacteria (DEN)

Denitrification is the third step in the conversion of ammonia into nitrogen gas; typically conducted by facultative anaerobic heterotrophs, represented by over 50 different genera (Kowalchuk and Stephen, 2001), with organisms in different kingdoms including *Bacteria, Archaea,* and *Eukarya* (Risgaard-Petersen *et al*., 2006). Most DEN are classified as facultative anaerobes that undergo reduction reactions of nitrate/nitrite when there is a lack of oxygen in the environment; in addition, most DEN can also perform aerobic respiration under aerobic conditions. The classification of DEN has also been observed to extend into autotrophs (Nielsen *et al.*, 2009). Under anoxic conditions, the DEN are able to undergo respiration by using nitrite or nitrate as the terminal electron acceptor, in place of oxygen, for the production of ATP, which is used for cellular growth and maintenance (Mateju *et al*., 1992). The goal of denitrification is to reduce nitrate completely into the stable form of nitrogen gas, however, to do so, the process needs to be untaken in a series of reactions. The outline of the reaction pathway is as follows:

$$NO_3^- \Rightarrow NO_2^- \Rightarrow NO + N_2O_{byproduct} \Rightarrow N_2 \qquad [4]$$

Each reaction step is catalyzed by their respective enzymes, nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (Hochstein and Tomlinson, 1988). Nitrate reductase is a membrane-bound enzyme, which produces a proton motive force during the dissimilatory reaction of nitrate into nitrite, important for the conservation of energy in certain bacteria (Mateju *et al.*, 1992). Nitrite reduction utilizes two nitrite reductase enzymes, separately characterized by having a copper center and two hemes. These nitrite reducing enzymes can be found in the membrane and the cytoplasm, producing primarily nitric oxide, but as well as nitrous oxide. Both the nitric oxide and nitrous oxide can be further reduced to nitrogen gas with their respective reductase enzymes. Nitrous oxide is a potent greenhouse gas, and as one of the intermediate of the denitrification process, DEN has been shown to contribute to the greenhouse

gas effect and global warming (Lashof and Ahuja, 1990), as well as the destruction of ozone layer (Waibel *et al*., 1999).

The more easily cultured DEN, such as *Pseudomonas, Bacillus*, and *Alcaligenes*, were first identified, and widely believed to be the dominant genera in activated sludge (Nielsen *et al.*, 2009). Based on several molecular studies on WWTPs, it was discovered that DEN belonging to families, *Comamonadaceae* and *Rhodocyclaceae*, were the most widely observed; the *β-Proteobacterial* genus, *Azoarus*, identified to be the dominant organism responsible for denitrification (Juretschko *et al.*, 2002).

1.1.4 Anaerobic Ammonia Oxidizing Bacteria (Anammox)

The largest contributor to the production of atmospheric nitrogen gas from ammonia is a process known as anaerobic ammonia oxidation. Two-thirds of the total nitrogen gas production in the world is produced from ocean sediments, while the water columns produce the other one-third (Codispoti *et al*., 2001). Sediments in seas deeper than 150 meters have been estimated to make up 53% of all sedimentary nitrogen gas production (Middelburg *et al.*, 1996), and anammox could be responsible for more than half of that production; since more than 87% of the world's ocean is deeper than 1000 meters, it may be safe to assume that anammox could be responsible for carrying out at least one-third of global sedimentary nitrogen removal (Dalsgaard *et al.*, 2005). A bold estimate from the literature suggested that anammox can be the source of one-third to one-half of the global removal of fixed nitrogen from marine environments (Devol, 2003; Dalsgaard *et al.*, 2005).

The concept of oxidation of ammonia through an anoxic process was first proposed in 1941, which was hypothesized at the time to be a possible source of nitrogen gas in the sea (Hamm and Thompson, 1941). The theory re-emerged in 1965, when oceanographers discovered low levels of ammonium in anoxic basins, and attributed this to the possibility of anaerobic microbial activities (Richards, 1965). Those ideas proved to be only theories, at a time where the only prevalent explanation for the removal of ammonia in anoxic conditions involved heterotrophic denitrification. It was not until almost the twenty-first century, when a discovery was made in a wastewater treatment plant that provided novel evidence for the anammox reaction (Mulder *et al.*, 1995). Mulder *et al.* (1995) were unable to differentiate the cause of the reaction to be biological or chemical in nature; part of the failure was associated with the lack of success in identification and cultivation of the organism of interest. The actual discovery of the bacterial group responsible for the anammox reaction came about in 1999; the first bacteria of which was named *Candidatus* Brocadia anammoxidans and determined to be related to *Planctomycetales* through comparative 16S rRNA studies after PCR amplification of DNA from enriched samples (Strous *et al.,* 1999); the findings of which were later confirmed in the anoxic water columns of the Black Sea (Kuypers *et al*., 2003). The study of anammox can be tricky, in the sense that, these are very slow growing bacteria, with doubling time of roughly nine days under optimal conditions (Strous *et al.*, 1999). Strous *et al.* (1999) were only able to cultivate a 70% pure culture initially; subsequent gradient Percoll centrifugation techniques allowed for an enrichment of 99.6% pure culture. In fact, due to complications involved in the isolation of anammox bacteria, there are still no pure cultures of anammox species available. It is possible to study anammox bacteria on a molecular level through the development of 16S rRNA oligonucleotide probes for fluorescence *in-situ* hybridization experiments.

While under the classification group of *Planctomycetes*, anammox can be divided into three genera, *Brocadia, Kuenenia,* and *Scalindua* (Schmid *et al*., 2003); in addition, there are two subspecies of *Scalindua* (Kuypers *et al*., 2003). The defining cellular characteristic of bacteria belonging to the anammox group is the possession of anammoxosome, a membrane-bound organelle (Jetten *et al*., 2001; Lindsay *et al*., 2001); in fact, this unique organelle is one of the methods of identifying anammox organisms, as it forms a hole-like appearance within the cells, producing the characteristic "doughnut" shape of anammox bacteria observed under the microscope. Covering the anammoxosome compartment is a tight intercellular membrane made up of ladderane lipids (Damste *et al*., 2002), providing a crucial barrier to the explosively-reactive intermediates produced within the organelle, such as hydrazine (van Niftrik *et al*., 2004). These ladderlane lipids are unique in nature, as they are the first natural compound with very strained linearly concatenated cyclobutane moieties (Sinninghe Damaste, 2005). It is also the embedment of those reaction enzymes in the membrane that allows for the oxidation of ammonium, with nitrite as the oxidizer (Dalsgaard and Thamdrup, 2002); and, more importantly, for the microbes, it is the protons produced from the reaction that undergo proton motive force across the membrane, thus producing energy yielding ATP molecules.

Much interest has been drawn towards the study of anammox bacteria and their role in the wastewater treatment, owing to the many advantages the bacteria group has over the more conventional pairing of nitrifiers and denitrifiers. Instead of requiring separate reaction conditions (aerobic for nitrifiers, and anoxic for denitrifiers), anammox is able to convert ammonia directly to nitrogen gas under only one set of condition (anoxic). As discussed previously, all the reactions from ammonia to nitrogen gas occurs neatly within the organelle compartment, thus no intermediate products, including the powerful greenhouse gas nitrous oxide, are made in the process (Kampschreur *et al*., 2008). In addition, the lack of denitrification also cuts down the

operational costs as the anammox process does not require the addition of carbon sources, which is necessary to serve as electron donor in the denitrification process (Siegrist *et al.*, 2008). Favouring an anaerobic environment, anammox reactions do not require aeration that is usually paired with aerobic systems, thus making it a more cost effective choice. Due to high energy demands around the world, a shift of wastewater treatment strategy from aerobic configuration into anaerobic can help alleviate some pressure from the energy grid. Their anaerobic nature, however, may also play a negative role in wastewater treatment systems. It is controversial how much oxygen is required to inhibit anammox reactions; oxygen as low as 1.1 µM has been shown to completely inhibit anammox reaction (Strous *et al.*, 1997), while some studies claim that anammox bacteria can withstand 30 minutes intervals of oxygen input at 2-3 mg O_2/L (Siegrist *et al.*, 2008). Additional benefits of running bioreactors enriched with anammox bacteria include higher nitrogen removal rates during wastewater treatment, and lower production of undesirable waste sludge (Duan *et al*., 2012; Tsushima *et al.*, 2007). The higher nitrogen removal rates with anammox enriched treatment setups allow for an increase in treatment capacity without having to modify infrastructure, which is ideal with increasing demand due to population growth. In the developing field of wastewater research, it appears that anammox, along with its many benefits, may be the new frontier in wastewater treatment.

1.1.5 Ammonia Oxidizing Archaea (AOA)

The relatively recent discovery of anammox is not the only exciting finding in the wastewater field within past decades; the discovery of the genes encoding ammonia oxidation within *Crenarchaeota* (Venter *et al*., 2004) created an emerging paradigm in the study and understanding of the microbial nitrogen cycle. Not a different species of bacteria, or genus, or even family, but rather the concept of an entirely different kingdom that plays a vital role in the

transformation of nitrogen, and therefore, a new area of research in wastewater treatment. It was not long ago that microbiologists widely accepted that Archaea only thrived as extremophiles, living in harsh environmental conditions; however, it was discovered, in the past couple of decades, that marine *Crenarchaeota*, not only lives in cold oxic ocean waters, but in fact, dominates the mesopelagic zones of the Pacific Ocean (Fuhrman *et al*., 1992; Karner *et al.*, 2001).

Venter *et al.* (2004) found genes resembling ammonia monooxygenase (amoA) on an archaeal-associated scaffold during a metagenomic study of seawater; the implication being, that one of the most abundant group of microorganism, the mesophilic *Crenarchaeota*, is able to undergo ammonia oxidation. This theory was solidified by the laboratory cultivation of the ammonia-oxidizing *Crenarchaea*, *Nitrosopumilus maritimus*, identified through comparative sequence analysis of 16S rRNA genes and electron microscopy studies (Konneke *et al*., 2005). Upon examining the subunits, a, b, and c, of the ammonia monooxygenase enzyme in the test sample, the researchers were able to confirm similarity of the genetic sequences within the ammonia-oxidizing crenarchaea in the study in comparison to previously published environmental sequences (Venter *et al*., 2004); in addition, the test samples of *Nitrosopumilus maritimus* were able to oxidize ammonia in the absence of organic carbon (Konneke *et al*., 2005).

A study performed on *Crenarchaeota* in the North Atlantic inferred that AOA are much more abundant than their bacterial counterpart, with copy numbers of the amoA gene at 10-1000 times that of *β-Proteobacterial* amoA (Wuchter *et al.*, 2006). With such great variance in the types of AOA and their impressive numeration, the increasingly more stringent effluent treatment

of WWTP may very likely depend on these mesophilic *Crenarchaeaota*; in fact, there have already been detections of AOA within wastewater treatment bioreactors (Park *et al.*, 2006).

1.2 Wastewater Treatment Configuration

Wastewater can be simply defined as water compromised through anthropogenic means, encompassing anything from household dirty-water discharge, to large-scale industrial effluents. Most commonly found compounds in domestic wastewater are proteins, carbohydrates, fats and oils, urea, trace organics, and other pollutants, the majority of which stems from human and animal excreta, and grey water (Kamma *et al.*, 1994). WWTPs function to remove solid undissolved waste from the wastewater, as well as dissolved organic and inorganic compounds, including nitrogen and phosphorous.

When wastewater enters a CAS treatment plant, a four-step process is typically undertaken: preliminary treatment to remove debris and larger material; primary treatment to physically screen and sediment out smaller matter that is not dissolved or in suspension; secondary, otherwise referred to as biological, treatment to remove dissolved organic and inorganic compounds; and finally, an advanced tertiary treatment step that further removes organics or specific toxic substances. Typically, tertiary steps are only applied to treatments that specifically require higher stringency of effluents or the removal of target chemical compounds from industrial treatment plants, such as dyes from pulp and paper mills (Thompson *et al.*, 2001). Tertiary steps can range from membrane processes like ultrafiltration, which utilizes selectively permeable membranes, or physico-chemical processes that remove toxic materials from the effluents, such as ozonation and coagulation. While all of the steps are important for proper reduction of water pollutants, the biological step is of the most interest to this current project, as it

involves nitrogen transformation and ammonia removal. The most common method of eliminating the dissolved organic, and inorganic pollutants, is through the use of bacteria and other microorganisms that feed on said pollutants. WWTPs "house" large amounts of microorganisms that convert the nutrients, undesirable to the ecological health of receiving waters, into biomass, as the microorganisms thrive and grow using the wastewater. Depending on the setup of the treatment plant, the microorganisms can either be used in their flocculated form, or in communities found within biofilms.

The structure and morphology of biologically active units in wastewater treatment plants can be observed to be in two general formations, flocculants and biofilms. There are other types of biologically active units that can be categorized under biofilm, such as granules, which are suspended biofilms. Flocculated particles, henceforth referred to as flocs, are free-floating microscopic structures involved in the transportation and settling of contaminants in water systems; and are typically composed of more than two primary particles (Droppo and Ongley, 1994; Liss *et al.*, 1996). In addition to the primary particles, there are extrapolymeric substances (EPS) within the flocs that interact with bacterial cells to help the process of flocculation (Morgan *et al.*, 1990; Wilen *et al.*, 2000). EPS is composed of high-molecular weight cellular secretions produced from the lysis of cells or the hydrolysis of macromolecules (Sheng *et al.*, 2010), as well as, the absorption of organic matter directly from the wastewater (Liu and Fang, 2003). The specific composition of EPS has been observed to be heterogeneous, with the major constituents of EPS to include carbohydrates, proteins, humic substances; other components can include lipids, nucleic acids, uronic acids, and inorganic compounds (Frolund *et al.*, 1996; Sheng *et al.*, 2010).

Biofilms, on the other hand, are a collection of bacterial cells, in the formation of an enclosed unit, adhering to a wetted surfaced (Eighmy *et al.*, 1983); bacteria in biofilms produce and embed within the complex matrix of EPS (Bridier *et al.*, 2011; Simoes *et al.*, 2010). The EPS matrix plays an important role in the protection of the bacterial communities from biocides like chlorine or peroxide compounds. EPS has been shown to cause difficulty associated with biocide penetration into interior regions of biofilm (De Beer *et al.*, 1994; Jang *et al.*, 2006), thus protecting the bacterial communities found in those regions. Biocides tend to be highly reactive in nature, thus chemically interact with the organic matter from EPS such as proteins, nucleic acids, carbohydrates, instead of reacting with the microorganisms (Lambert and Johnston, 2001). Bacterial enzymes such as catalase can be found in the EPS matrix, which act as neutralizing agents for biocides like peroxide (Stewart *et al*., 2000). Finally, the electrostatic interaction between EPS and oppositely charged biocides can also play a role in hindering the penetration ability of those biocides (Guiot *et al*., 2002). Wastewater researchers have carefully studied both types of microbial structures, and have tried to simulate these conditions in the bioreactors. Depending on the type of bioreactor, flocs, biofilms, or a combination of both can be utilized to treat the wastewater.

1.2.1 Conventional Activated Sludge (CAS) System

The most commonly practised wastewater treatment setup is the CAS system, which is an excellent example of floc utilization in a WWTP setup. The concept and configuration of the CAS system is standard wastewater knowledge, and can be found in many wastewater textbooks including Bitton (1994), contents of which will be summarized as follows:

The first step of CAS involves the primary clarifier tank that raw sewage is pumped into, which allows for the setting of undissolved organic matter. The effluent from the primary clarifier tank is subsequently pumped into an aeration tank, and mixed aerobically with return activated sludge (RAS), which is recycled sludge from the end process of the CAS system, forming a mixture known as mixed-liquor. It is the return of biomass through the RAS that makes the sludge "activated", thus coining the term conventional activated sludge. The use of mechanical or diffused aeration techniques is crucial to the aerobic autotrophic oxidation and heterotrophic respiration reactions. After aeration, the mixed liquor is then channeled into a final clarifier tank for the sedimentation process for the separation of the sludge from the treated wastewater, the final effluent, which can then be released into the receiving waters. The sludge from the final clarifier tank is separated into RAS, which returns to the aeration tank to feed on the next batch of wastewater nutrient pollutants and undergo biomass growth; while the rest of the sludge is disposed of through digestion processes. Due to the slower growth rates of nitrifiers in comparison to the heterotrophs, which are responsible for organic reduction, there can be many different ways a nitrogen removal system is set up in a CAS plant. For wastewater that contain higher biological oxygen demand (BOD) to total Kjeldahl nitrogen (TKN) ratios, or more simply put, water that contains more carbon than nitrogen compounds, a combined carbon-oxidation nitrification process can be utilized; for wastewaters that contain higher TKN, and require a greater population of the slower-growing nitrifiers, a two-stage nitrification setup is typically undertaken, which requires an initial BOD removal, prior to the use of nitrifiers in the second stage. The entire CAS process can be further complicated when accounting for denitrification. Due to the anaerobic operating conditions required by denitrifiers, one of two setups may be put in place: single sludge system allows wastewater to pass through a series of aerobic and anaerobic tanks, without settling until the final clarifier tank, allowing for microorganisms to undergo nitrification and denitrification; whereas, a multi-sludge system is characterized by three separate

tanks, each with their own clarifier tanks. The first tank in the multi-sludge system is usually aerobic carbonaceous oxidation; second tank favours aerobic nitrification; and the final tank is anoxic favouring denitrification.

As a floc-based system, the smooth operation of CAS plants relies heavily on the floc properties within the system. Proper settling of activated sludge flocs in the secondary clarifier tank is crucial to the efficiency of WWTPs (Martins *et al.*, 2004). The WWTPs run into problems with biomass loss when the secondary clarifier tanks are unable to adequately perform due to low settleability; releasing large volumes of microorganisms into the receiving waters is also a concern, as well as, having less RAS, thus contributing to an overall lower mixed-liquor suspended solids (MLSS). MLSS is the total organic and mineral suspended solids in the reactor tank, and is very important towards having the proper biomass density that allows for adequate organic/inorganic reduction. The ability of microbes to aggregate in flocs is vital to achieving low turbidity in the clarifier and higher effluent quality (Sheng *et al.*, 2010). The cations within EPS play a key role in flocculation through double layer compression (Liu *et al.*, 2007) and ion bridging (Nguyen *et al.*, 2007); in fact, the addition of multivalent cations can be used to improve flocculation of sludge (Higgins *et al.*, 2004). The composition of EPS can also play a role in flocculation. The removal of surface proteins have been shown to result in deflocculation, whereas, the loss of carbohydrates has less of an impact on the flocs (Higgins and Novak, 1997). Depending on characteristics such floc size, morphology, and density, the rate at which sludge settles can also be varied; sludge volume index (SVI) is the volume occupied by one gram of sludge, a value used to determine the sludge settleability (Bitton, 1994). The presence of polymers, produced by microorganisms during the stationary phase, contributes to the formation of flocs in activated sludge; thus good settling occurs when bacteria are at stationary phase; a higher SRT can also be indicative of good settling, as the older sludge age allows for the

collection of more polymers over time (Chao and Keinath, 1979). Sudden changes in the physical parameters, absence of nutrients, and the availability of toxicants can be associated with partial deflocculation, and result in poor settling (Chudoba, 1985). The overgrowth of filamentous bacteria, referred to as bulking, in WWTPs is another common cause of slow settling in activated sludge systems (Martins *et al.*, 2004). In fact, the presence of filamentous bacteria, as low as 1-20% of the total sludge volume, can be enough to cause bulking (Palm *et al.*, 1980).

The simplicity and effectiveness of the CAS system has allowed it to withstand the test of time over the past century, and is still widely implemented around the world; however, with increasingly stringent environmental policies, the need for infrastructure renewal due to increased capacities at the existing WWTPs, and the many floc-related issues found in CAS plants, it warrants the exploration into novel treatment setups like the integrated fixed-film activated sludge (IFFAS) system.

1.2.2 Integrated Fixed-Film Activated Sludge (IFFAS) System

Biofilm wastewater treatment systems are not new to the field of wastewater; in fact, much like the CAS, systems featuring biofilms can be traced back to a century ago (Atkinson, 1975), but only since the 1980s have scientists actually studied the biofilm system (Lazarova and Manem, 1995). The trickling filters are well-known biofilm-dependent systems, introduced in 1893, and are one of the earliest systems for biological waste treatment (Sawyer, 1944). Wastewater is added uniformly over reactors with media that support biofilm growth, such as stones, wood, or plastics; with the help of gravity, the effluent trickles through the media, and the nutrients are consumed by the microorganisms within the biofilms. Alternatively, the rotating

biological contactors (RBC) is a process introduced in the 1920s in Germany, which feature rollers or disks that are 40% submerged in wastewater, and through their rotation, the microorganisms in the biofilms reduce nutrients in the wastewater; the rotation allows for oxygen transfer and provides shear force for biofilm sloughing (Weng and Molof, 1974). Fluidized bed reactors, utilized in WWTPs since 1983, consist of heavy particles (sand) covered in biofilm growth, which maintained fluidized state through the upward flow of water. The heavy particles not only allow for rapid settling, but the large surface area helps achieve high biomass concentration, thus providing greater treatment capacity (Heijnen *et al.*, 1989). Due to the unique setup of fluidized bed reactors, new infrastructure must be constructed to accommodate for the different reactor setup of the existing CAS systems; the requirements of the liquid circulation has high energy consumptions, thus affecting the operational costs.

Of the various biofilm processes, an IFFAS system is a relatively new process that incorporates suspended bacterial growth with biofilms grown on solid media of suspended plastic pieces or fixed synthetic mesh (Kim *et al.*, 2010). The media carriers can be found in a variety of materials and shapes: wheel-shaped polyethylene pieces (Stricker *et al.*, 2009), floating sponges (Masterson *et al.*, 2004), ringlace media (Sen *et al.*, 1995), porous glass/ plastic beads (Andreottola *et al.*, 2000), and even cigarette filter rods have been employed (Sabzali *et al.*, 2013). The modification of a CAS treatment plant into one of IFFAS configuration is relatively cheap and simple to implement, as the infrastructure is the same, with the exception of media carriers, and the necessary meshing to retain the carriers (Randal and Sen, 1996). Studies have shown that IFFAS systems allow for a greater sludge age, which is ideal as a niche for slow-growing microorganisms, such as nitrifiers, to develop, and allow for increased concentration of bacterial growth (Randal and Sen, 1996). In fact, the addition of attached-biofilm to a CAS system has been shown to induce higher nitrification and denitrification rates per unit volume

than the CAS system alone, with the most visible difference observed at lower operating temperatures (Randal and Sen, 1996). The notable differences at low temperatures may be attributed to the operating conditions of conventional floc-dependent systems, which exhibit lower HRT and limited clarifier capacity at those temperature; while, conventional systems may require the expansion and construction of additional basins and clarifiers to maintain a stringent level of nitrification, the higher biomass concentration found in the IFFAS systems can prove sufficient (Randal and Sen, 1996). This becomes very important when adapting to the higher level of demand for greater treatment capacity, one of the main drivers of recent wastewater research. Depending on the media used for biofilm growth, the comparative success of nitrification has been shown to vary. With ringlace media, under optimal conditions, the level of nitrification can reach 136 percent of the controlled samples that lack media; the sponge media has been observed to be at 143 percent of the control under optimal conditions (Sen *et al.*, 1995).

Stratification of biomass in biofilm allows for anoxic regions if the film is thick enough to prevent thorough oxygen diffusion; oxygen has been shown to be depleted in regions of the biofilm deeper than 200 µm from the surface (Santegoeds *et al.*, 1998). Anoxic waters typically have no dissolved oxygen content or a very low level (concentrations of less than 0.5 mg/L) (UGSG, 2014). While the anoxic zones might not be favourable to the growth of oxygen-dependent organisms such as AOB and NOB, the presence of these anoxic zones can help support DEN, and possibly, anammox bacteria. Denitrification, a process that typically takes place under anaerobic conditions, can be introduced in aerobic setups with the addition of media for biofilm growth (Sen *et al.*, 1995). Having the ability to carry not only nitrifiers, but DEN, allows for the system to undergo complete nitrogen removal, without the need to implement a multiple tank system. In one study, the DEN were identified to be in micro-clusters within the biofilm, and the authors attributed the microbial makeup to partial oxygen penetration (Basuvaraj *et al.*, 2012).

The exact reason behind the differences seen between IFFAS and CAS are not entirely clear. The different sludge retention times (Randal and Sen, 1996), as well as different oxygen penetration profile (Santegoeds *et al.*, 1998) between biofilms and flocs, can account for the variation in the microbial populations found in their respective niches. The bacterial communities within flocs and biofilms of a full-scale IFFAS system have been analyzed using a high-throughput pyrosequencing technique. The floc community was found to be relatively similar to the previously published CAS bacterial composition, with the majority of the organisms being *Beta-*, *Alpha-*, and *Gammaproteobacteria*, and *Bacteroidetes*; the IFFAS biofilm, on the other hand, revealed a higher dominance in *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* (Kwon *et al.*, 2010). Anaerobic bacteria, such as *Lactobacillus*, were found exclusively in the attached microbial samples, which again solidified the theory that anoxic zones within the biofilms support anaerobic organisms (Kwon *et al.*, 2010). The physicochemical properties have also been shown to be different between biofilms and flocs, with the flocs having a higher negative surface charge, hydrophobicity, and overall EPS content than biofilms (Basuvaraj *et al.*, 2012). The type of EPS contents found within flocs also differed to that of the biofilms in IFFAS systems, with protein contents dominating in the flocs, and equal proportions of protein and polysaccharides in the biofilms (Basuvaraj *et al.*, 2012). The different characteristics and microbial makeup of the flocs and biofilms within IFFAS systems may contribute to its effectiveness, in comparison to a system composed entirely of flocs, aspects of which were studied in the experiments within this project.

1.3 Microbial Identification Techniques

Wastewater research is a multidisciplinary area, which involves knowledge from civil engineering, in the construction and maintenance of the infrastructure; chemical engineering, in the transformation processes involved in nutrient removal; as well as, microbiology, examining

the biological treatment system relating to the microbial composition. A vital component of wastewater research is the identification and classification of microorganisms within the WWTPs. Through examining the microbial compositions within the flocs and biofilms, engineering problems such as sludge bulking can be better understood, and means of reversing the issue can be found through studying the growth requirements of filamentous bacteria for example. Molecular biological techniques used to identify common nitrifiers and denitrifiers can be utilized to monitor and maintain the parameters required for a properly functioning nitrogen removal setup. Those same techniques can also be useful in discovering novel microorganisms like anammox bacteria, which can play an important role in the future direction of wastewater treatment. In this section, the different molecular techniques involved in the identification, quantification, and analysis of microorganisms will be explored.

The classic method of identifying and quantifying microorganisms is through culture-dependent techniques, followed by differentiation through physiological and biochemical means. A common method utilizes selective media, which allows for the growth of specific microorganisms, typically those of interest to the study, and extrapolates the numeration through counting techniques involving serial dilution (Belser and Schmidt, 1978 a). This is referred to as the most-probable number (MPN). There are a variety of tests that can be performed following selective plating, including morphological characterization through phase-contrast microscopes, Gram-staining, and oxidase testing (Kampfer *et al.*, 1996). Prior to having the technology for molecular studies, the culture-dependent counting techniques sufficed. There are a number of limitations to using culture-dependent techniques, which make it especially difficult to study wastewater microorganisms. For example, a variety of wastewater microbes have extremely slow growth rates, including nitrifers like AOB, which can require incubation periods of up to several months (Wagner *et al.*, 1995; Matulevich *et al.*, 1975), thus increasing the experimentation times

to unrealistically long durations. Viability is another concern when it comes to culture-dependent techniques, as certain species of bacteria, while active and viable in the environment, may enter dormancy (Porter *et al.*, 1995; Xu *et al*., 1982), thus creates a problem of underestimation in the number of organism. In certain cases, the dormant organisms may fail, entirely, to be cultivated in laboratory states (MacDonald, 1986). A simple wastewater study comparing the cell counts from molecular techniques to culture-dependent techniques revealed that colony counts from media plates were 20 to 100 times lower; the different types of nutrient composition for the media plates also resulted in 10 fold difference (Kampfer *et al.*, 1996). It is now widely accepted that the vast majority of prokaryotes are not able to be cultured in a laboratory through standard methods (Schleifer, 2004). The task to locate and isolate specific species of microorganisms within the heterogeneous mixture in flocs and biofilms can be daunting when attempting to use only cultivation-dependent procedures. There are faster culture-dependent methods that have been developed since, including phenotypic fingerprinting, which can be useful to differentiate heterotrophic microbial communities through the use of large variations in carbon substrates on microplates (Victorio *et al.*, 1996).

The development of molecular biological techniques changed the way microbiologists studied microorganisms. The use of fluorescent antibodies has been tested in the past to visualize and enumerate wastewater bacteria at the early stages of molecular study (Wagner *et al.*, 1995; Ward and Perry, 1980). Antibodies are protein complexes that are part of the immune system, which target foreign substances such as proteins, nucleoproteins, or polysaccharides, also known as antigens. Depending on whether the immunofluorescence is direct or indirect, the procedure will vary. The direct immunofluorescence technique utilizes antibodies with fluorescent labels that target specific antigens, which will then be analyzed using epi-fluorescence microscope or CLSM to target the specific fluorescence wavelengths of those antibody labels; the indirect

method of immunofluorescence is more sensitive, as the fluorescent tags are only found on the secondary antibodies, of which, multiple copies can bind to one primary, unlabeled antibody that target the desired antigen (Odell and Cook, 2013). There are certain disadvantages of using antibodies in the study of nitrifiers in wastewater, as it had been shown that there can be a high variety of diversity seen in the serotypes of ammonia-oxidizers (Belser and Schmidt, 1978 b), thus making it difficult to select adequate antibodies to allow for accurate quantification of organisms of interest. For example, in a species of bacteria, there may be many different strains which may all produce antigens with different variable regions, thus prevent the specific binding of antibodies to the antigens from the various serotypes. The fluorescent antibodies have also been shown to bind to EPS (Swerinski *et al.*, 1985), thus hindering the ability to identify cells from the background signals.

The use of PCR-based methods to quantify organisms in the environmental samples is a relatively faster and better option for detecting specific organisms in wastewater. The most commonly used PCR technique involves the use of denaturing gradient gel electrophoresis (DGGE) to study the diversity of complex microbial systems (Muyzer *et al.*, 1993). The process begins with PCR amplification of 16S rDNA fragments and subsequent running of the samples through polyacrylamide gels with linearly increasing gradient of denaturants. The DNA fragments contain melting domains, or lengths of nucleotides with the same base-pairing, and it is the variation in the melting domains that determine the position along the polyacrylamide gel at which the specific DNA fragment will begin to denature and melt (Fischer and Lerman, 1979; Fischer and Lerman, 1983). The mobility of partially-denatured DNA is slower than the natural helical structure, thus allowing for separation of different DNA sequences as they partially melt throughout the gel. To prevent the complete dissociation of the two DNA strands, a GC clamp, sequence of guanines and cytosines, is typically attached to the 5'-end of the DNA fragments

(Sheffield *et al.*, 1989). The different bands on the polyacrylamide gels after DGGE can be used to analyze the genetic diversity of the sample community, and subsequent hybridization of DGGE blots with specifically-labelled oligonucleotide probes can help identify organisms of interest. Analyses of excised bands from the DGGE gels can also be used in sequencing experiments to determine the genetic makeup of specific bands, and allow for the development of a thorough genetic profile of the entire community within the test sample (Amann *et al.*, 1992; Muyzer *et al.*, 1993). Although PCR can be an effective method to study different microbial species in wastewater, its main limitation in the quantification of the different organisms is in the theory behind the molecular technique. Instead of determining the number of cells for a specific organism, quantitative PCR is only able to measure the copy numbers of marker genes, thus introduces biases involved in nucleic acid extraction (Nielsen *et al.*, 2009). During nucleic acid extraction procedure, the DNA from microorganisms of interest is collected through the lysis of those microorganisms; however, there is also extracellular DNA, possibly from dead microorganisms, which will be extracted as well. These extracellular DNA may result in the overestimation of the number of live microorganisms in the sample.

Not disregarding the usefulness of the previously discussed molecular techniques, many of which can be used together, precision nucleotide-hybridization techniques of detection may be more favourable in the identification and quantification of wastewater microbes. The development of fluorescence *in-situ* hybridization (FISH) technique and its application in the wastewater sector has been immensely helpful towards the study and characterization of microbial wastewater species. FISH utilizes fluorescently labeled oligonucleotide probes that target specific regions of the bacterial rRNA, genetic ribosomal molecules that are distributed throughout prokaryotic cells, and contain highly conserved, as well as, variable sequences (DeLong *et al.*, 1989; Wagner *et al.*, 2003). Depending on the level at which the sequence is

conserved, the phylogeny can be determined at varying depths (Amann *et al.*, 1995). As FISH is utilized frequently throughout this project, a detailed protocol can be found in the methodology section; however, the concept of FISH can be described as follows.

To study a specific group of microorganisms, at whichever phylogeny, the conserved rRNA sequence needs to be first determined, and a specific FISH oligonucleotide probe, complementary to the conserved rRNA sequence, needs to be designed. There have been hundreds of published rRNA-targeted sequences that can be utilized to design oligonucleotide probes used in FISH (Loy *et al.*, 2003), which historically are tagged with fluorochromes for visualization under epi-fluorescence microscopes and confocal laser scanning microscope (CLSM) (Giovanonni *et al.*, 1988); currently, the probes can be customized with a variety of fluorochromes, such as, 5(6)-Carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS), Cy3, and Cy5, each with their unique excitation and emission wavelengths (Daims *et al.*, 2005). Due to the differences in the excitation and emission wavelengths of the fluorochromes, multiple FISH probes, each with their unique fluorochromes, can be used in one hybridization experiment to allow for increased reliability in the data produced. For example, a eubacteria probe with FLUOS cytochrome can be used in parallel with an AOB probe, tagged using Cy3 cytochrome; only the cells that respond to both probes, observed through the layering of their respective CLSM imaging, can be confidently described as an AOB, since AOB fits under the umbrella of eubacteria. The multiple probe approach filters out unspecific binding, in which the oligonucleotide probes hybrid to microbial cells that are not the target organism. After a specific oligonucleotide probe is designed and tagged with fluorochrome, it can then be applied to a heterogeneous population of microorganisms, as one would expect to find in wastewater sludge or biofilms; if the reaction conditions are optimal, ideal hybridization temperature and predetermined chemical stringencies of substrates, the probes are in theory supposed to bind to

ribosomal rRNA molecules within the cells. Slides with the hybridization are viewed through epi-fluorescence or CLSM using the correct excitation and emission wavelengths, and the organisms of interest can then be visualized.

Compared to some of the previously mentioned microbial identification techniques, FISH is much faster than culture-dependent methods, with the ability to produce results within hours of sampling; able to identify diverse serotypes, unlike fluorescent antibodies; and enumerates the bacterial cells based on the number of organisms visualized, rather than genomic estimation, such as PCR-based techniques, and thus is free of certain nucleic acid extraction biases. One additional feature of FISH is the fact that the procedure is performed *in-situ*, which means that the structure and layout of the cells are not disrupted during the hybridization; it can be immensely helpful to visualize flocs and biofilms in native composition setup, as the localization of specific organisms can provide useful insight into the relationships and behaviours of microbial structures. However, there are certain drawbacks to the FISH technique as well. In order to perform FISH, the specific rRNA sequences need to be made available for probes to be produced, the information of which may not be readily available. Sometimes the general probes used during FISH may not pick up on novel species of bacteria, thus may cause underestimation or incomplete data. The microscopy aspect of FISH is dependent on images from microscopy slides, which may not be representative of the entire population within the sample. The point of this section is not to endorse FISH as the "silver bullet" in microbial ecology research, but rather, to point out some of the characteristic of the molecular technique that can be appealing to wastewater research, such as this thesis project.

There are, of course, many more molecular biological techniques that can be used to study microorganisms in the field of wastewater research. For example, DNA microarrays and chip devices are used in wastewater research to rapidly identify microorganisms within

wastewater samples. Similar to other molecular hybridization techniques like FISH, DNA microarrays depend on the hybridization reaction of oligonucleotide probes to rRNA of specific microorganisms (Kelly *et al.*, 2005); the difference is in the number of probes that can be applied. High-density DNA microarray can contain up to 10^6 test sites (Heller, 2002). More recently, second-generation high-throughput sequencing, known as pyrosequencing, has been utilized in wastewater research to provide complete descriptions of microbial diversity through the study of its metagenome (Hu *et al.*, 2012). Current research undertaken in the lab has revealed very interesting data through 16S RNA gene sequencing and DGGE, which may shape the understanding of granule formation (Aqeel *et al.*, 2014). It is through the development and advancement in the various methods of molecular techniques that have paved the future for new innovations in the field of wastewater treatment. By analyzing the different components of WWTP, such as the microbial makeup within the reaction tanks, a more comprehensive understanding to the treatment process can be achieved. The utilization of molecular identification methodologies can allow for the discovery of novel organisms with enhanced nutrient removal abilities, such as anammox bacteria (Duan *et al.*, 2012), which will be vital to the increasing demand on treatment capacity. The purpose of wastewater research is not solely based upon the prevention of water-related environmental issues such as eutrophication, the field is moving towards the production of useful compounds in the form of biofuel, bioplastics, and even biochemicals (Angenent *et al.*, 2004; Chakravarty *et al.*, 2010). Sometimes it is as simple as combining two, already known, methods of wastewater treatment, such as the use of anammox bacteria within IFFAS treatment setup that may help contribute to enhance the treatment capacity of existing infrastructures, and reduction of greenhouse gas production from the wastewater sector.

1.4 Thesis Objectives

Many of the drivers, discussed previously, in the field of wastewater research are also the motivating factors for this thesis project. The need for infrastructure renewal due to the higher demands of the growing population has resulted in the necessity for increasing the treatment capacity of older WWTPs. The integrated system of fixed-film and activated sludge has been shown in the literature to be better at performing nitrogen removal in comparison to the conventional activated sludge systems (Randal and Sen, 1996). As the IFFAS system is currently studied in the lab, its potential as the operational setup for growing anammox bacteria was thus realized. Due to the presence of anoxic regions within the biofilm component of the IFFAS, along with the ability to provide a niche for slow growing bacteria, the IFFAS system appears ideal for anammox bacteria growth. In theory, utilizing anammox within IFFAS systems should provide a novel setup of wastewater treatment that would boast enhanced nutrient removal, with decreased sludge production, the prevention of greenhouse gas emission, and decreased energy usage associated with aeration.

The idea for the thesis initiated from an observed difference in the relative levels of AOB to NOB in IFFAS system, with the level of AOB being higher than NOB in a system that allows for complete ammonia removal (Basuvaraj *et al.*, 2012). Specific research questions were thus sparked, in an attempt to determine whether microorganisms, not categorized under AOB or NOB, may be involved in the process of nitrogen transformation within IFFAS systems. With the understanding that anammox bacteria oxidize ammonia with nitrite into nitrogen gas under anoxic conditions, theoretically found in substratum regions of IFFAS biofilms, it was hypothesized that anammox was involved in the nitrification process in the IFFAS system.

1. The primary thesis objective is to determine whether anammox bacteria is the microbial factor that is causing the imbalance of AOB to NOB observed in the IFFAS reactors. If anammox bacteria can be found to be naturally occurring in the biofilms of IFFAS, a solid foundation for future research in the combination of the two wastewater treatment methods can be put in place.
2. A secondary objective of this thesis is to examine the relative contribution of the floc and biofilm of the IFFAS system in the nitrogen removal process.

Various batch experiments can be used to track the nitrogen profiles of the IFFAS system over its reaction period, which can provide insight into the existence of any anoxic processes occurring within the IFFAS system; the batch experiments can also separate the contributions of the floc and biofilm in relation to their ability to remove ammonia. Subsequent FISH studies can further determine, with specificity and reliability, whether anammox bacteria play a natural role within the IFFAS system.

Chapter 2 Experimental Design

2.1 Introduction

Much of the previous work in the lab has been dedicated towards studying an IFFAS system (Basuvaraj *et al.*, 2012; Aqeel *et al.*, 2013). In particular, the researchers have experimented with different operating conditions of the IFFAS, and the effect it has on floc formation, EPS, and microbial diversity. The IFFAS system is run as a sequential batch reactor (SBR), and compared to the CAS SBR that is set up to run in parallel. There are a variety of reasons why a wastewater bioreactor would be selected to run in a SBR format. Apart from the fact that the lab has been previously equipped with SBR reactors, thus making it the rational choice of setup; SBR reactors are easy to control and modify. Since the reactors are fed periodically based on a predetermined feeding schedule, the wastewater influent for the system can be produced synthetically on a daily basis, thus allowing for a high degree of control and consistency. It has been known since the 1980s that methanogens, acidifying bacteria, nitrifying bacteria, and denitrifying bacteria are all able to undergo granulation when they are under starvation-induced stress (Kjelleberg and Hermansson, 1984; Van Benthum *et al.*, 1996). The SBR utilizes successive cycles of biological treatment, which contain starvation periods vital to the promotion of aerobic granulation (Tay *et al.*, 2001). The formation of granules is a beneficial addition to wastewater treatment systems, such as the IFFAS system, due to the enhanced settling abilities of the sludge, higher biomass retention, as well as being able to withstand higher organic loading (Tay *et al.*, 2001). By modifying the operating conditions of the SBR system, there could be a selection for fast settling flocs, and enrichment of slower growing microorganisms (Strous *et al.*, 1998), which is ideal for the promotion of nitrifiers and possibly anammox bacteria. The CAS SBR system was seeded with mixed-liquor from a local CAS WWTP, and even though, it is no longer considered a CAS operation due to the selectivity involved with SBR, it resembles a plug

flow CAS reactor, thus henceforth will be referred to simply as CAS. Having a side-by-side comparison of the IFFAS and CAS, the research group is thus able to study the differences between the two systems, from differences in physicochemical properties to microbial communities.

Of the previous research undertaken in the lab, one interesting finding sparked the concept that initiated this project. When studying the IFFAS AOB and NOB populations, it was observed that the AOB relative abundance to eubacteria (EUB) was much higher than its NOB counterpart (Basuvaraj *et al.*, 2012). In theory, if ammonia is completely removed from the wastewater, microorganisms, other than NOB, would likely have had a hand in the removal of nitrite produced by the larger population of AOB. The IFFAS system is known to be well-suited to culture slow-growing bacteria (Randal and Sen, 1996), due to the long solids retention time, in addition to SBR's ability to enrich slow-growing bacteria (Strous *et al.*, 1998), the combination setup of IFFAS SBR is an ideal environment for slow-growing microorganisms such as anammox bacteria. The anoxic niche created by IFFAS biofilms can also be utilized by anammox bacteria (Santegoeds *et al.*, 1998; Sen *et al.*, 1995), which is an obligate anaerobe. Other than having suitable niches for anammox bacterial growth, the ability for anammox bacteria to utilize both ammonia and nitrite as its substrate for energy production is also of interest, pertaining to the dilemma of AOB to NOB imbalance (Dalsgaard and Thamdrup, 2002). Anammox bacteria fits nicely within the project hypothesis as the secondary microbial factor involved in nitrite removal from the system, thus may help explain the previously observed lower NOB to AOB ratio in IFFAS system.

The main thesis objective is to determine whether anammox bacteria may be the secondary microbial factor within the IFFAS system that allows for a lower NOB level in

comparison to AOB, while demonstrating complete nitrogen removal; and subsequently, if the organism of interest is not anammox bacteria, then exploration into the microbial community can help identify and determine the nature of the secondary nitrifying microbial factor. By using oxygen-limiting batch experiments, simulation of one reaction cycle from the IFFAS SBR system, within enclosed reaction bottles, can be compared to that of the CAS system. If the IFFAS setup is able to remove ammonia without the presence of oxygen, while the CAS is unable, then it may help reveal the possibility of anaerobic ammonia oxidation occurrence within the IFFAS system. Once the anoxic process is revealed within the IFFAS system, a more direct approach can be applied, in which oligonucleotide probes are used to identify and locate anammox bacteria within the system using the FISH technique. By studying the microbial community within the IFFAS system and identifying the presence of anammox bacteria, a better understanding of this relatively novel integrated wastewater treatment system can be achieved.

The secondary objective of the thesis project is to study the relative contribution of the floc and biofilm of the IFFAS system, and the respective microbial composition. Much like the molecular technique of FISH applied to the anammox study, similar studies will be performed using common AOB and NOB probes in the IFFAS system to study their composition and identify the genus levels of those organisms within the IFFAS system in the lab. The ability of the IFFAS system to undergo nitrification is also compared, within this study, to their CAS counterpart; using batch experiment, run under similar aerobic conditions as the bench-scale setups, the ammonia removal patterns between the two different systems can be compared, as well as, the variations in the nitrite and nitrate levels over the course of a reaction cycle. Due to the high degree of experimental control associated with batch experiments, the different components of the IFFAS system can also be separately studied, each with their own batch reaction. New insight can be revealed when studying ammonia removal abilities of the IFFAS

floc, IFFAS biofilm, and the standard IFFAS system of combined floc and biofilm. The microbial niche, either floc, biofilm, or both, responsible for any anaerobic ammonia removal reactions can be isolated in this study, through the use of non-aerated batch reaction. In addition, it would be interesting to look at how a combined floc and biofilm system that makes up an IFFAS bioreactor differ in their ability to remove ammonia in comparison to the separate individual components.

The IFFAS system has been shown to allow for higher nitrification and denitrification rates per unit volume when compared to a completely floc-derived system such as CAS, especially evident at colder temperatures (Randal and Sen, 1996; Sen *et al.,* 1995). This is one of the reasons why this particular wastewater treatment setup is being studied in the lab, and the rationality behind applying the IFFAS in colder climate countries like Canada. The simplicity in which a CAS setup can be converted into an IFFAS is also appealing, as the infrastructure can remain the same, with just the addition of IFFAS media carriers for the biofilm growth (Randal and Sen, 1996). There are already IFFAS treatment plants implemented around Canada, including one relatively close to the lab, in the city of Peterborough, Ontario; however, the combination of an IFFAS system with enriched anammox bacteria has not been described in the literature. The benefits of having an anammox enriched treatment system include: decreased expenses towards aeration, as they thrive in an oxygen-free environment; lower greenhouse gas production, typically associated with nitrous oxide production with denitrification (Kampschreur *et al.,* 2008); and higher nitrogen removal rates while producing less waste sludge (Duan *et al.,* 2012; Tsushima *et al*., 2007). To enrich an IFFAS system with anammox bacteria, in theory, combines the benefits of both setups, which can create exciting possibilities for the future of wastewater treatment; however, the first step begins with identifying whether anammox bacteria have the innate ability to grow within IFFAS systems, one of the main drivers of this thesis project.

2.2 Materials and Methods

2.2.1 Sequential Batch Reactor (SBR) Setup

The experiments that were part of the thesis project relied heavily on a fully-functioning SBR system, which was run primarily for the purpose of a different project in the laboratory. As the reactor was shared throughout this project, as other studies not part of this thesis project also required biomass from the reactors, the setup and conditions of the reactors varied over the course of the thesis.

There were four parallel SBRs, which provided the environment for microbial growth. The microbial seed came primarily from the Cataraqui Bay Wastewater Treatment Plant, exclusively run as a CAS system to treat the municipal wastewater from the city of Kingston in Ontario, Canada.

The main composition of the synthetic feed was consistent throughout the project with the only changes being the carbon source. The molar ratio of chemical oxygen demand : nitrogen : phosphorous was 100 : 5 : 1. The carbon source varied between solely 300 mg/L glucose, and a mix of 150 mg/L glucose and 150 mg/L sodium acetate. The nitrogen and phosphorous sources were ammonium chloride and potassium phosphate, respectively. The concentration of micronutrients were: 5.07 mg/L $MgSO_4$; 2.49 mg/L $FeSO_4$; 1.26 mg/L $Na_2MoO_4 \cdot 2H_2O$; 0.31 mg/L $CuSO_4$; 0.44 mg/L $ZnSO_4 \cdot 7H_2O$; 0.25 mg/L NaCl; 0.43 mg/L $CaSO_4 \cdot 2H_2O$; 0.41 mg/L $CoCl_2 \cdot 6H_2O$; and 2.49 mg/L $FeSO_4$ (Liao *et al.*, 2001). The synthetic feed was prepared fresh daily and the reactor is kept at pH 7. All chemicals were standard reagent grade, ordered from Sigma Aldrich and Fisher Scientific (Canada).

The SBR reactors had 2 L capacity each, which were filled with 1.5 L of fresh feed every 6 hours. The feeding schedule varied between 4 hours and 6 hours, but predominately, the reactors were run under 6 hour cycles. Of the 6 hours, the time for each step was designated as follows: 15 minutes filling; 5 hours mixing with aeration; 30 minutes settling; and 15 minutes draining with the drain points of the reactors set to 0.5 L.

The reactors usually contained one CAS and three IFFAS bioreactors. The IFFAS initially contain about 50 plastic wheel-shaped media carriers (Infilco Degremont Inc., USA) in each reactor, but the number of carriers decreased over time as they were sacrificed to measure biomass and for the batch experiments.

2.2.2 Batch Experiment

When studying a complex system such as those found in wastewater treatment, it is important to do experiments with controlled variables to better understand all the different components present in the system. The use of bench-scale SBRs allows for a consistent nutrient ratio in the feed, as well as controlled temperature and operational setup, thus providing an environment to study large-scale wastewater treatment plants on the laboratory scale. As controlled as bench-scale SBRs can be, it may be difficult to break down different components of IFFAS systems and study them individually. For example, in an IFFAS SBR system, both the flocs and biofilms are grown in the same reactor, thus it is difficult to study their individual ammonia removal abilities. The bottle batch experiments designed in this project overcome the previously stated restraints and allow for an in-depth look at the different components of IFFAS system both individually and together. The bottle batch experiments also provide a simple way to

test the flocs and biofilms under non-aerated conditions, which is important for identifying anammox reactions.

Experimental setup

The bottle batch experiment compares the removal of ammonia between CAS floc, IFFAS flocs, IFFAS biofilm, and the standard IFFAS setup with both floc and biofilm. In order to compare the ammonia removal ability of the different bacterial communities found in each of the setups, the initial biomass had to be standardized between all the bottle reactions to eliminate the amount of biomass as the factor for differences in ammonia removal. It is difficult to keep the overall biomass found in the bioreactors constant across different sets of experiments, as the biomass on the biofilms are constantly changing. To ensure that the biomass is the normalized within the experiment, the number of biofilms used in the biofilm batch reactions was first determined and set to be four media carriers. Subsequently, the biomass in the floc batch reactions (IFFAS floc and CAS floc) can be calculated based on the MLSS of the bench reactors at the time of the experiment, and the amount of biomass added to their respective batch reactions will equal the amount of biomass present in four media carriers. This method, although will not provide the same overall biomass from one set of experiment to the next, within each set of experiment, the amount of biomass within each batch reactors will be constant.

First step was to determine the amount of flocs to add to each batch reaction based on the biomass of carrier media biofilm. The method of removing biofilm biomass varied over the course of the project, from physically removing the biofilm using scalpel to the more accurate method that was later implemented, described as follows. A media carrier was rinsed with distilled water to remove any unbound biomass, and dried in the oven at 100 degrees Celsius for

an hour and weighed. A solution of NaOH was then used to remove all biomass from the media carrier, which was subsequently dried again in the oven for 20 minutes. The media carrier that has been removed of its biomass was weighed a second time, and the difference compared to the first time was the calculated biomass from one media carrier. This method was an accurate way to determine the biofilm biomass; however, it does require the sacrifice of one media carrier during the measurement. The amount of biomass added to the CAS floc and IFFAS floc reactions were dependent on their respective MLSS. The method of determining the MLSS was simply the collection of 10 mL mixed liquor from the SBR reactors, followed by filtration the biomass and drying in the oven at 100 degrees Celsius for an hour. The filter and biomass were then weighed and calculated as the mass in grams per litre. Once the MLSS was determined, the volume of flocs could be calculated to equal the biomass of four media carrier biofilms. The bottle reaction for the standard IFFAS setup with both flocs and biofilms contains two media carriers and volume of IFFAS floc equaling the biomass of two media carriers. Before the addition of the biomass, 200 mL aliquots of synthetic wastewater feed were added to each reaction container. As the MLSS varied between the CAS and IFFAS reactors, the volume of mix liquor that got added to each reaction was different. To allow for constant initial ammonia concentration, the ammonia chloride was added separately from the rest of the synthetic wastewater feed, with different amounts in each reaction bottle to account for the variation in overall reaction volume. When all the necessary preparation and addition of synthetic feed had been completed, the biomasses were then added to each respective batch reaction, and shaken at about 160 rpm. At time points of 0, 0.5, 1, 2, 3, and 6 hours, 10 mL samples from each batch reaction were extracted using a syringe, filtered through 0.45 µm syringe filter to remove biomass, and stored in -20 degrees Celsius freezer. When performing the non-aerated experiments, the bottles were sealed with folding skirt rubber stoppers to prevent the exchange of oxygen; needles were thus used to pierce the caps for sample collection.

Ammonia auto-analyzer

To test for the levels of ammonia, samples were thawed and run through a continuous flow ammonium auto-analyzer system (SEAL Bran + Luebbe Auto-Analyzer III system, USA), utilizing colorimetric assay as a means to detect levels of nitrogen in the form of ammonia. The methodology for this experiment was based on the protocol developed by Queen's Analytical Services Unit, which was derived from the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Colorimetric techniques used to detect ammonia concentrations utilize Berthelot reaction, which is the reaction between ammonium salts and sodium phenoxide (Hinds and Lowe, 1980). The addition of sodium hypochlorite subsequently causes the formation of a green-compound, the colour of which is enhanced by sodium nitroprusside. Finally, to prevent the precipitation reactions, it is necessary to add an EDTA solution. The auto-analyzer system contains six components, including the autosampler, the pump, the manifold, the colorimeter, the computer, and the printer. The autosampler contains a test tube rack for the calibrants and quality control, as well as two sampling trays that can carry up to 120 samples. The calibrants are made up of eight ammonia standard solutions with increasing concentrations of 0, 0.1, 0.2, 0.3, 0.5, 1.0, 1.5, and 2.0 ppm of ammonia; the quality control tube is made up of a separately prepared ammonia standard from a different stock solution at the concentration of 1.0 ppm. Due to the maximum concentration of the calibration curve being 2.0 ppm, the samples need to be diluted with distilled/deionized H_2O accordingly to prevent exceeding the maximum. When properly diluted, the samples can be added to sampling tray in the order that is specified on the setup window on the computer. Before running the samples, three reagents need to be prepared and pumped into the auto-analyzer system. Alkaline phenol, for the Berthelot reaction, was made by adding 21.7 mL liquefied phenol, 8.5 g NaOH, and distilled/deionized H_2O to a final volume of 250 mL. Sodium hypochlorite solution was made by adding distilled/deionized

H_2O to 45 mL hypochlorite to a final volume of 100 mL, followed by the addition of 1 g NaOH. EDTA solution was made up of 8 g di-sodium EDTA, 0.1 g NaOH, 0.045 g sodium nitroprusside, 0.75 mL Triton, and ddH2O to a final volume of 250 mL. After the reagents, the calibrants, and the sample dilutions have been prepared, the auto-analyzer then automatically samples and detects the levels of ammonia. All chemicals ordered from Fisher Scientific (Canada). The levels of ammonia were compared between the different batch reactions, and t-tests were performed to look for statistical significance.

Ion Chromatography (IC)

The IC machinery (Dionex HPLC system -ICS 3000, USA) functions through the detection of conductivity using an anion exchange resin column, which separate the different anions at various retention times. It can be used to detect chlorine, nitrite, nitrate, sulphate, bromide, fluoride, bromate, and phosphate. The batch experiment samples were tested with IC to detect the changing levels of nitrite and nitrate, which were determined through comparative methods with a standard concentration series, ranging from 0.1 to 50 ppm, of sodium nitrite and sodium nitrate, respectively. After dilution and loading of the samples into the autosampler, the remainder of the test was automated through the IC machine, specifics of which can be found in the Dionex HPLC manual on the company website.

2.2.3 Molecular Method

Samples of biofilms and flocs were fixed with 4% paraformaldehyde solution at 4 degrees Celsius for 3-12 hours (Nielsen *et al.*, 2009). After washing the biomass with PBS solution, they were then stored in 1 volume of PBS to 1 volume of 100% ethanol at -20 degrees

Celsius. Alternatively, if the target organisms are Gram-positive in nature, those organisms can also be fixed by storing in 50% ethanol.

Fluorescently-labelled oligonucleotide probe stock solutions were prepared to a concentration of 100 µg/L and store in -20 degrees Celsius freezer until use. The flocs and biofilms to be tested were added to 10-well slides and diluted so only a few flocs were made available per well. The slides were left to sit at room temperature for 50 minutes to completely dry, and a 0.1% agarose solution was added to each well to coat the biomass. The slides were then immediately submerged in an alcohol dehydration series containing 50%, 70%, and 100% ethanol, in that order respectively for three minutes each. The slides were left to dry at room temperature and the hybridization buffer, composed of different ratios of 5M NaCl, 1M Tris-HCl, formamide, 10% SDS and distilled/deionized H_2O, was prepared at the stringency appropriate for the probes being tested, found in Table 1. The formamide serves to interfere with hydrogen bonds that stabilize nucleic acid duplexes, the correct stringency of which is vital to the proper hybridization of specific probes (Daims *et al.*, 2005). The same effect can be achieved by lowering the salt contents and increasing the temperature, however, the low salt contents will hinder the kinetics of the hybridization reaction, as salts are important to the formation of DNA-RNA heteroduplexes (Daims *et al*., 2005). Some probes are more lenient towards the level of formamide stringencies, thus are able to complete hybridization under a range of formamide concentrations; the stringencies of these probes are typically selected to favour more stringent probes when paired together in multiple probe experiments. After the slides dried, 10 µL of the hybridization buffer were added to each well, followed by 1µL stock solution of each probe and its associated competitor probes, a list of which can be found in Table 2. Probes were ordered from Life Technologies, Canada.

Table 1. Composition of hybridization buffers employed for FISH (Nielsen *et al.*, 2009).

	Hybridization Stringencies										
	0%	**5%**	**10%**	**15%**	**20%**	**25%**	**30%**	**35%**	**40%**	**45%**	**50%**
	Volume (µL)										
5M NaCl	180	180	180	180	180	180	180	180	180	180	180
1M Tris-HCl	20	20	20	20	20	20	20	20	20	20	20
H2O	799	749	699	649	599	549	499	449	399	349	299
Formamide	0	50	100	150	200	250	300	350	400	450	500
10% SDS	1	1	1	1	1	1	1	1	1	1	1

Table 2. Oligonucleotide probe list (Nielsen *et al.*, 2009)

Probe Name	Target organism	Sequence (5'-3')	Formamide %	Reference
EUB338 I	Eubacteria	GCT GCC TCC CGT AGG AGT	0-60	(Daims *et al.*, 1999)
EUB338 II	Eubacteria	GCA GCC ACC CGT AGG TGT	0-60	(Daims *et al.*, 1999)
EUB338 III	Eubacteria	GCT GCC ACC CGT AGG TGT	0-60	(Daims *et al.*, 1999)
NSO1225	Betaproteobacterial AOB	CGC CAT TGT ATT ACG TGT GA	35	(Mobarry *et al.*, 1996)
NEU	*Nitrosomonas* spp.	CCC CTC TGC TGC ACT CTA	35, 40	(Wagner *et al.*, 1995)
NEUcomp	Competitor probe	TTC CAT CCC CCT CTG CCG	35, 40	(Wagner *et al.*, 1995)
CLUSTER6A192	*Nitrosomonas oligotropha* lineage	CTT TCG ATC CCC TAC TTT CC	35	(Adamczyk *et al*., 2003)
CLUSTERcomp	Competitor probe	CTT TCG ATC CCC TGC TTC C	35	(Adamczyk *et al*., 2003)
NTSPA712	Phylum *Nitrospirae*	CGC CTT CGC CAC CGG CCT TCC	35, 50	(Daims *et al.*, 2001)
NTSPA712comp	Competitor probe	CGC CTT CGC CAC CGG TGT TCC	35, 50	(Daims *et al.*, 2001)
NTSPA662	Genus *Nitrospira*	GGA ATT CCG CGC TCC TCT	35	(Daims *et al.*, 2001)
NTSPA662comp	Competitor probe	GGA ATT CCG CTC TCC TCT	35	(Daims *et al.*, 2001)
NIT3	Genus *Nitrobacter*	CCT GTG CTC CAT GCT CCG	35, 40	(Wagner *et al.*, 1996)
NIT3comp	Competitor probe	CCT GTG CTC CAG GCT CCG	35, 40	(Wagner *et al.*, 1996)
Amx368	All anammox bacteria	CCT TTC GGG CAT TGC GAA	15	(Schmid *et al*., 2003)
Pla46	All *Planctomycetes*	GAC TTG CAT GCC TAA TCC	30	(Neef *et al.*, 1998)
Rhodano227	*Rhodanobacter thiooxydans*	TCG CAC ATC GGT TCG TCC TGC	30	(van den Heuvel *et al.*, 2010)

The hybridization chamber was made with 50 mL conical tubes and paper towel. A piece of paper towel around triple the size of a conical tube was folded three times, so that it will fit snuggly within the tube. The remaining hybridization buffer was then pipetted onto the paper towel within the conical tube, and the microscope slide was rested on top of the paper towel, with the hybridized side facing upwards and away from the paper towel. The cap was screwed back onto the conical tube and the hybridization chamber was left in the oven for a minimum of 1.5 hours at 46 degrees Celsius, but could stay in the oven for overnight hybridization. After hybridization, 50 mL of wash buffer were prepared composing of different ratios of 1M Tris-HCl, 10% SDS, 5M NaCl, 0.5M EDTA, and distilled/deionized H2O.The stringency of the wash buffer should be the same as the hybridization buffer; the exact composition of the wash buffer can be found in Table 3. Prior to the washing step, the wash buffer should be pre-warmed to 48 degrees Celsius. The slides were added to the wash buffer for ten minutes, rinsed with cold distilled water, and pressure dried. Finally, embedding medium and cover slip were added for confocal imaging. The hybridized slides were imaged using either Zeiss or Leica confocal scanning laser microscopes (CSLM) and analyzed for the presence of populations of interest.

Table 3. Composition of wash buffers employed for FISH (Nielsen *et al.*, 2009).

	Hybridization Stringencies										
	0%	**5%**	**10%**	**15%**	**20%**	**25%**	**30%**	**35%**	**40%**	**45%**	**50%**
	Volume (mL)										
5M NaCl	9.00	6.30	4.50	3.18	2.15	1.49	1.02	0.70	0.46	0.30	0.18
1M Tris-HCl	1	1	1	1	1	1	1	1	1	1	1
0.5M EDTA	0	0	0	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5
H2O	to 50	to 50	to 50	to 50	to 50	to 50	to 50	to 50	to 50	to 50	to 50

Chapter 3 Results and Discussion

3.1 Results

3.1.1 Relative contributions of floc and biofilm in the IFFAS system

The first component of the thesis project was to look for the presence of anaerobic ammonia oxidation in the bench-scale IFFAS reactors. Batch experiments were set up to determine whether there would be any signs of anaerobic oxidation reaction in selected biomass communities. The amount of biomass was the same in all four of the batch reactions; however, the overall biomass varies from one set of experiment to the next. As the biomass was normalized between the individual batch reaction bottles within each set of experiments, any difference observed in ammonia removal abilities should be attributed to the differences in microbial communities. The results from the initial batch experiment (Figure 1) showed minor levels of ammonia oxidation in CAS flocs and IFFAS floc under non-aerated conditions, as the percent of ammonia removed was relatively low during the six hour period at 7.91% and 7.80% respectively. The IFFAS biofilm allowed for 31.56% ammonia removal in the six hours and the IFFAS combined floc and biofilms demonstrated the highest ammonia removal at 50.25%. If there were to be any anoxic reactions for ammonia oxidation, it was hypothesized to be observed in the biofilms, which was observed in this initial experiment. Interestingly, the highest ammonia removal was observed in the IFFAS combined reaction, which was simulating the conditions of an actual IFFAS system. Note that media feed and the biomass did not take up the entire volume of the batch reaction bottles, thus a headspace (~50 mL) of air was left captured within the closed system of the reaction bottles. The dissolved oxygen levels within the media solutions of the batch reactions were not measured in the batch experiments; it was hypothesized based on the

amount of biomass added to each reaction that over the course of the six hour reaction, any free oxygen species would have been utilized by the bacteria within the biomass.

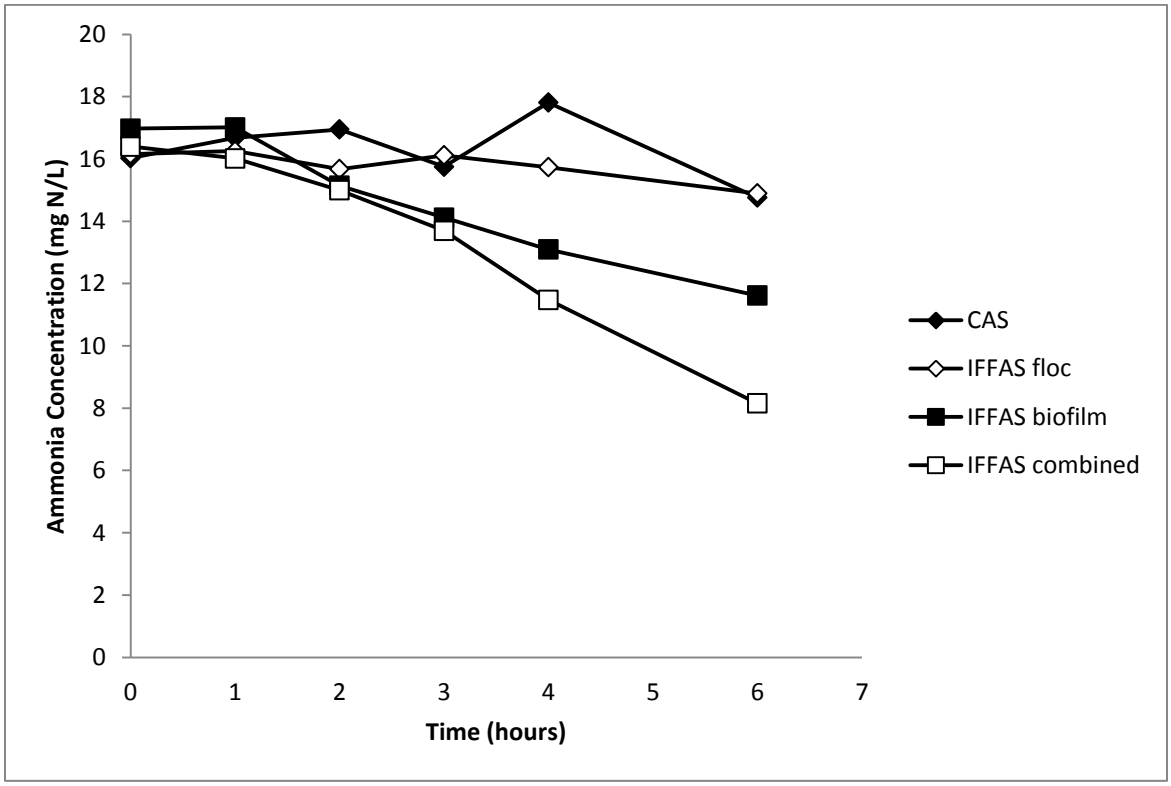

Figure 1. Ammonia removal (mg N/L) observed in CAS, IFFAS floc, IFFAS biofilm, and IFFAS combined flocs and biofilm for initial batch experiment (6 hours) run under non-aerated conditions.

The non-aerated batch experiment was repeated in triplicate and the average ammonia concentrations during the six hour period were shown in (Figure 2) with standard deviations represented in error bars. Unlike the initial data, the results from the three sets of batch experiments run under oxygen limitation showed a relatively linear decrease of ammonia concentration in all four of the batch reactions. The results suggested either anaerobic ammonia oxidation occurrence in both flocs and biofilms of the IFFAS system as well as the flocs of the CAS system, or the possibility that the biomass in the reactions were not deprived of oxygen

during the six hour non-aerated reaction period. Of the three IFFAS batch reactions, the average ammonia removal of the IFFAS combined setup was the highest at 77.51%, the IFFAS biofilm in the middle at 67.67%, and the IFFAS flocs being the lowest at 53.74%. This trend was replicative of that found in the preliminary batch data, which was suggestive of floc and biofilm synergy. However, when statistical analysis was performed on the data, it appears that IFFAS combined did not have significantly lower ammonia concentrations after six hours ($p > 0.05$), thus the synergy found in the initial batch experiment could not be replicated. The ammonia concentrations after six hours were not significantly different between the CAS and the any of the IFFAS setups ($p > 0.05$).

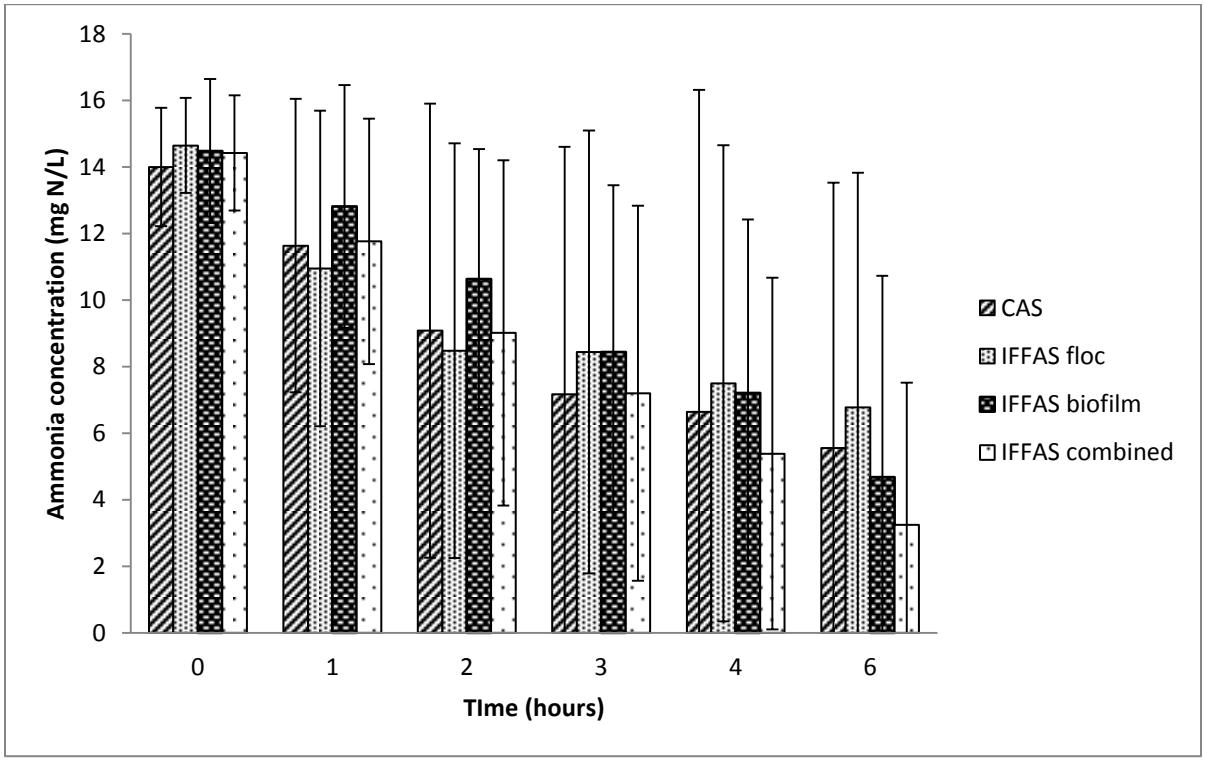

Figure 2. Ammonia removal (mg N/L) observed in CAS, IFFAS floc, IFFAS biofilm, and IFFAS combined flocs and biofilm for batch experiment (6 hours) run under non-aerated conditions. All data are presented in triplicate with standard deviations shown with error bars.

The nitrite and nitrate concentrations were also tested during the non-aerated batch experiments. The levels of nitrite were found to be zero for CAS, IFFAS floc, IFFAS biofilm, and the IFFAS combined floc and biofilm over the six hour period. The levels of nitrate were relatively constant, varying around 1 ppm over the six hour period.

After analyzing the results from the first stages of experiments, the decision was made to move on forward with the FISH experiments and the batch experiments were to be revisited after confirmation of anammox bacteria using FISH, results of which can be found in the next section.

The batch studies were replicated after the FISH experiments, with the parameters changed to include aeration of the reactors to study the AOB and NOB species present in the IFFAS system. The aerobic ammonia removal for CAS, IFFAS floc, IFFAS biofilm, and IFFAS combined floc and biofilm was observed in four separate aerated batch experiments (Figure 3). All four batch reaction setups were able to remove ammonia over the period of six hours; however, there were no significant difference in the ammonia concentrations after the six hour reaction period between any of the batch reactors ($p > 0.05$). There was no synergistic effect observed in the IFFAS combined setup.

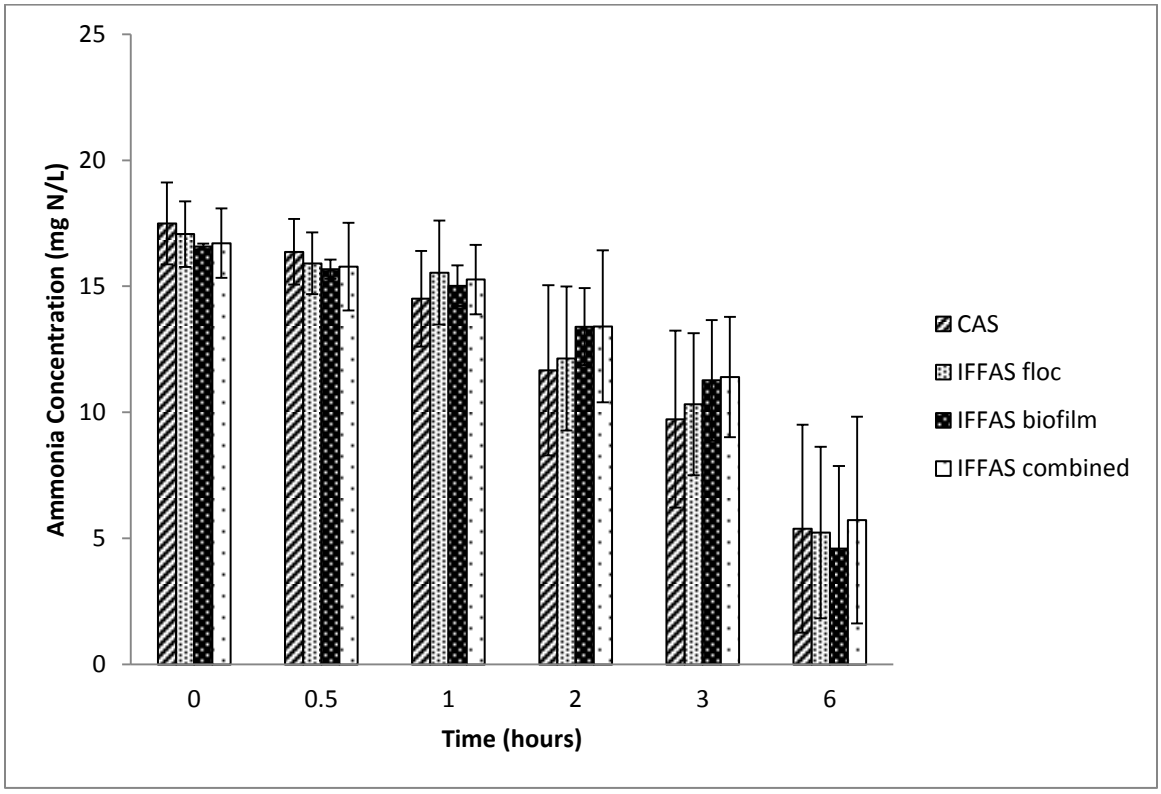

Figure 3. Ammonia removal (mg N/L) observed in CAS, IFFAS floc, IFFAS biofilm, and IFFAS combined flocs and biofilm for batch experiment (6 hours) run under aerated conditions. All data collected from four replicates with standard deviations shown with error bars.

The nitrite and nitrate levels were collected during three of the aerated batch experiments run for six hour periods and used to compare the differences between the CAS floc, IFFAS floc, IFFAS biofilm, and IFFAS combined floc and biofilm (Figure 4 and Figure 5). For the nitrite concentrations, all the samples appear to display increasing trends over the six hours; while, the nitrate levels start with a slight decrease, followed by increase at around 1 hour period in all of the samples. The biofilm samples for both nitrite and nitrate were observed to be much lower than the other samples.

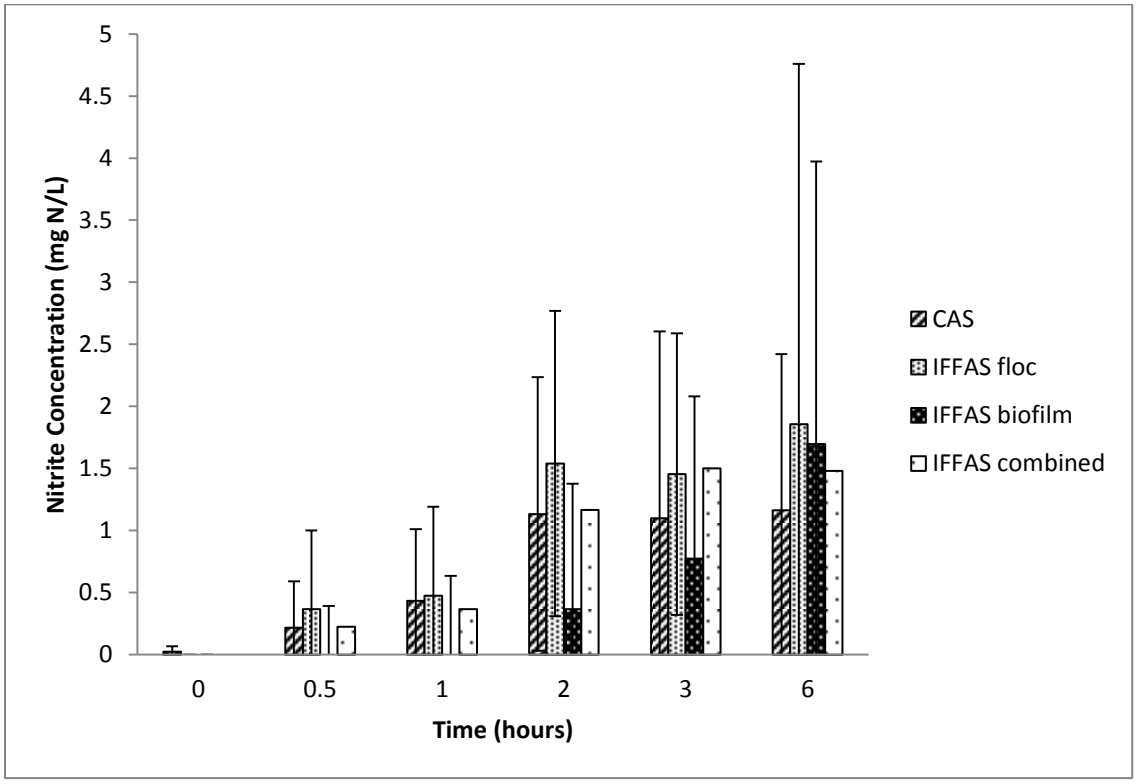

Figure 4. Nitrite concentrations (mg N/L) observed in CAS, IFFAS floc, IFFAS biofilm, and IFFAS combined flocs and biofilm for aerated batch experiment (6 hours). All data collected in triplicate with standard deviations shown with error bars.

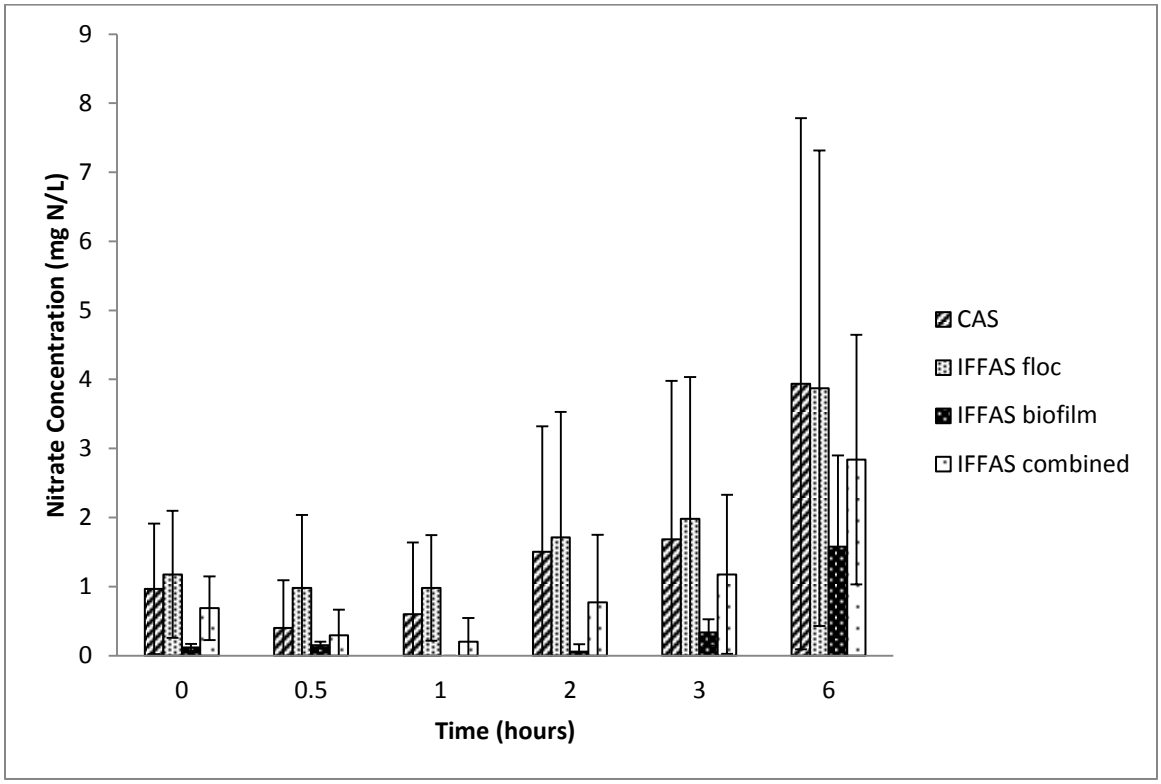

Figure 5. Nitrate concentrations (mg N/L) observed in CAS, IFFAS floc, IFFAS biofilm, and IFFAS combined flocs and biofilm for aerated batch experiment (6 hours). All data collected in triplicate with standard deviations shown with error bars.

3.1.2 Identification of key nitrogen-removal organisms

With the initial purpose of looking for anammox bacteria in the IFFAS system, amx368 and pla46 FISH probes were used to detect the presence of target species. The results from at least triplicate studies showed no signs of anammox bacteria in the IFFAS system run during this thesis. The pla46 probe, targeting all planktomycetes was confirmed to be working properly with positive controls of anammox bacteria as was seen in Figure 6; the amx368 probe did not undergo the positive control test as the positive samples of anammox were not available at the time of experiment. The pla46 probe was able to be activated by select aggregates of bacteria, as shown

in Figure 7; however, the inconsistency of the structure and physical appearance in comparison to published literature (Nielsen *et al.*, 2009), the positive control sample (Figure 6), and expert consultation from Markus Schmid and Holger Daims strongly suggested that the signals from pla46 FISH probes did not correspond to anammox species.

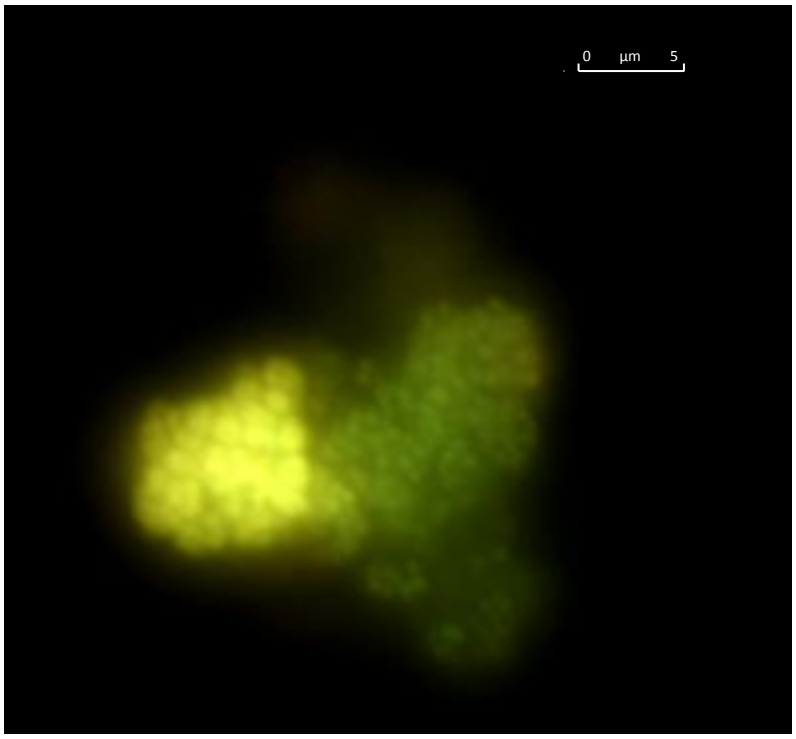

Figure 6. CLSM image of anammox bacteria cluster from positive control sample visualized through FISH. Anammox bacteria imaged in yellow with overlap of pla46 (orange) and eub (green) oligonucleotide probes.

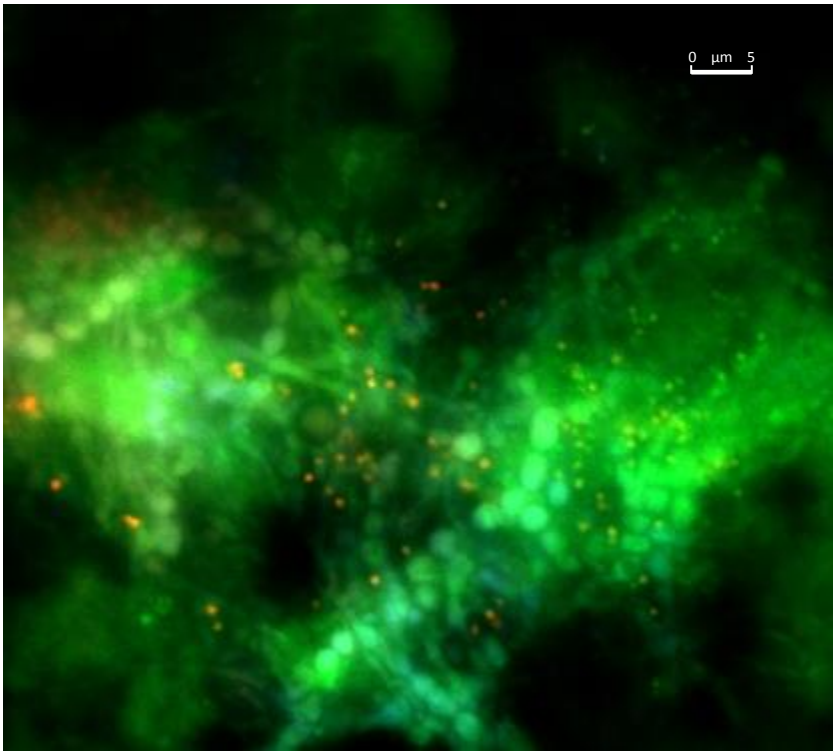

Figure 7. Epi-fluorescent image of planktomycetes from IFFAS biofilm sample visualized through FISH. Planktomycetes imaged in orange with overlap of pla46 (orange) and eub (green) oligonucleotide probes.

Subsequent experiments were conducted on the IFFAS system to look for the presence of more common microbial species such as ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Upon the use of AOB FISH probe mix containing nso1225, neu, cluster6a192, and necessary competitor probes, the results showed positive signal for AOB and NOB (Figure 8). To confirm the presence of target organisms, the multiple probe approach was used in which several probes with different signature regions and fluorescent labels targeting the same group of microbe were tested to increase the confidence in the observed signals. The different AOB probes used showed congruency in their results, especially evident with the cluster6a192 probe that detects *Nitrosomonas oligotropha* (Figure 9). The signals from the NOB probes, while strong and overlapped with eub probe signals, were not able to show consistency when testing with multiple

NOB probes with different signature regions and fluorescence as well as lacking distinctive NOB floc structures (Figure 10), thus unable to confirm presence with certainty. Subsequently, a second set of experiments showed similar results with clear presence of AOB and questionable NOB detection, again due to morphology of aggregates (Figure 11).

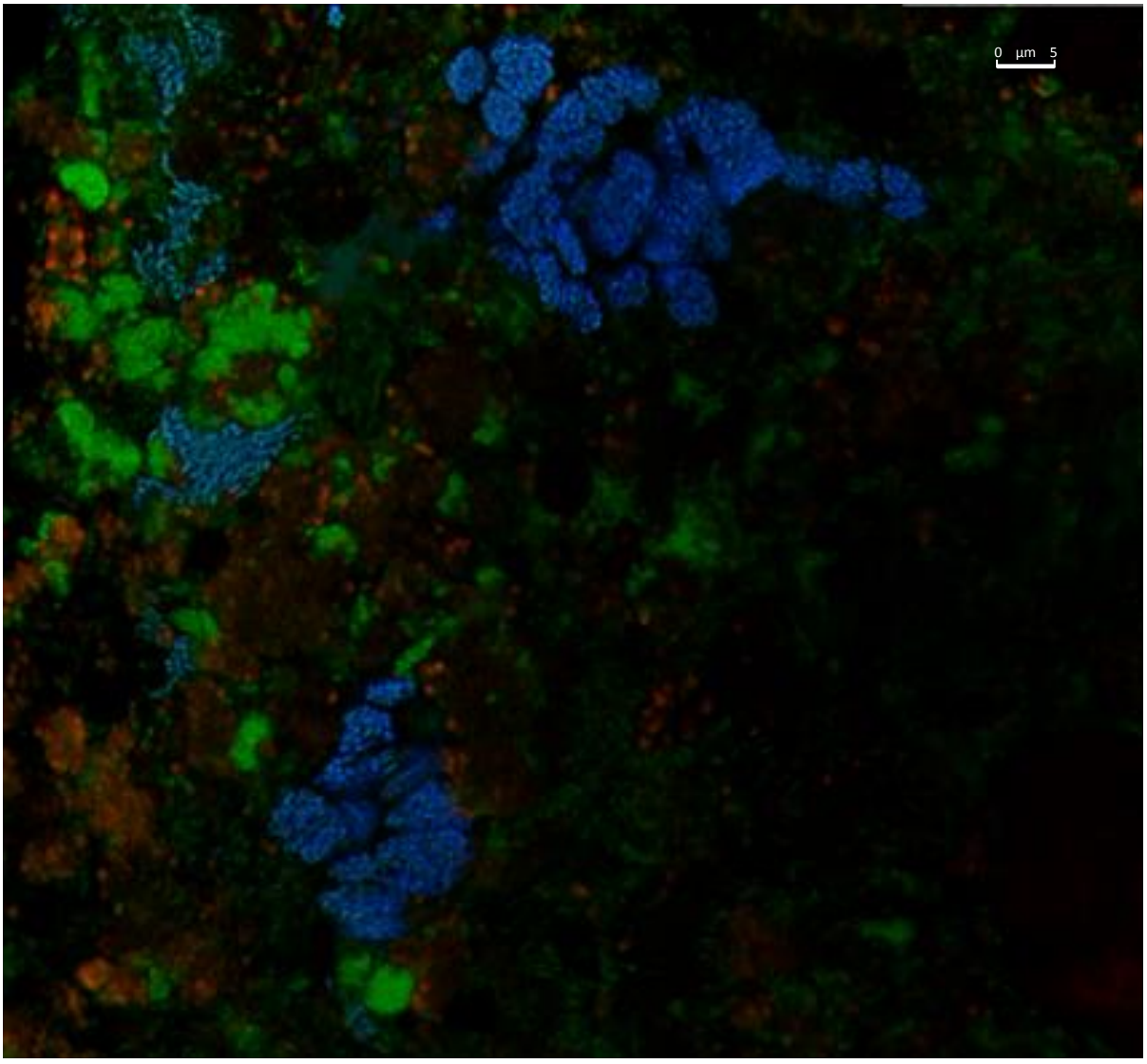

Figure 8. CLSM image of AOB and NOB clusters from IFFAS biofilm sample visualized through FISH. AOB imaged in blue, NOB imaged in red, and eubacteria imaged in green.

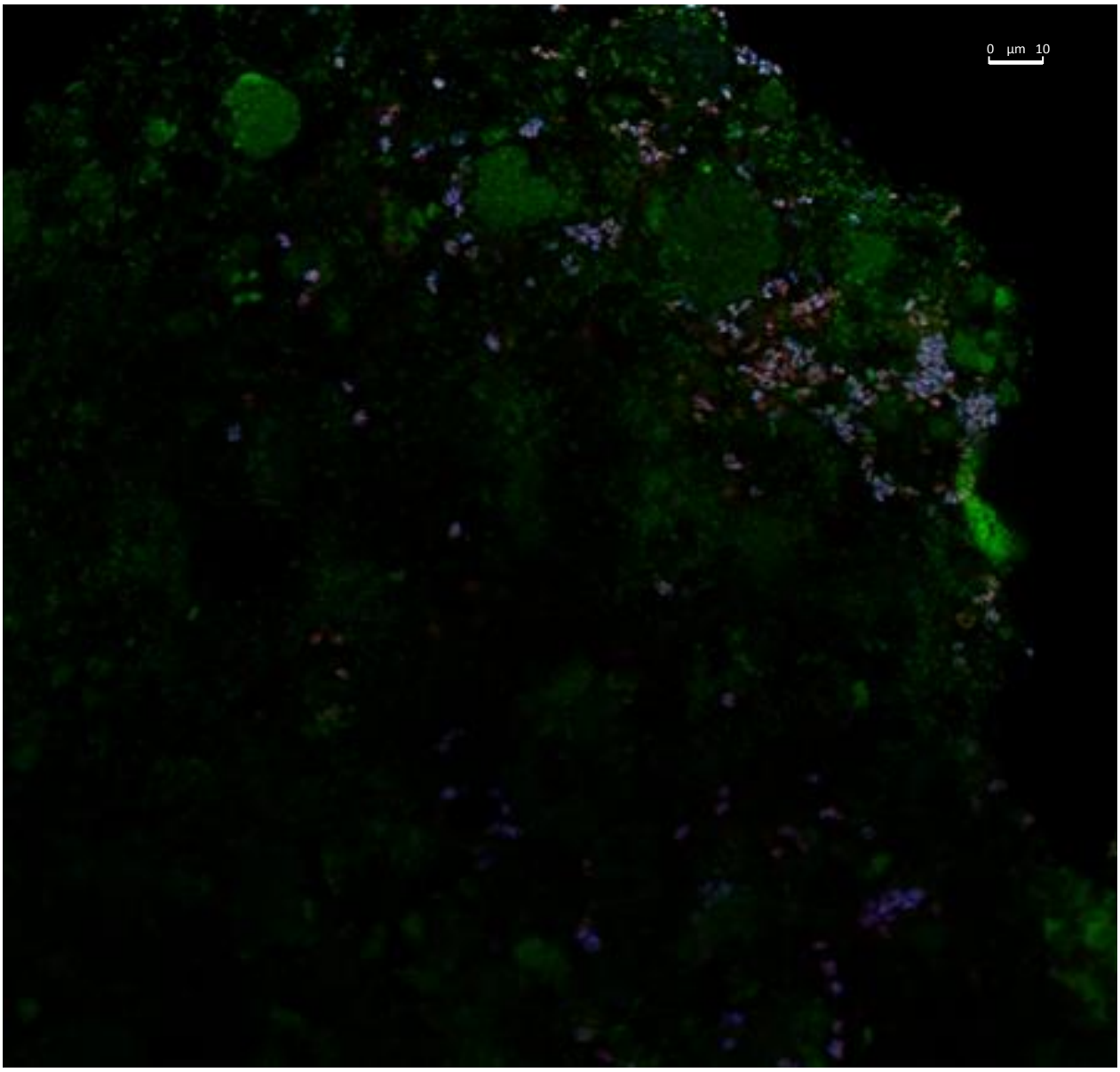

Figure 9. CLSM image of *Nitrosomonas oligotropha* clusters from IFFAS biofilm sample visualized through FISH. *N. oligotropha* imaged in white with overlap of cluster6a192 (red), AOB probe set (blue), and eub (green) oligonucleotide probes.

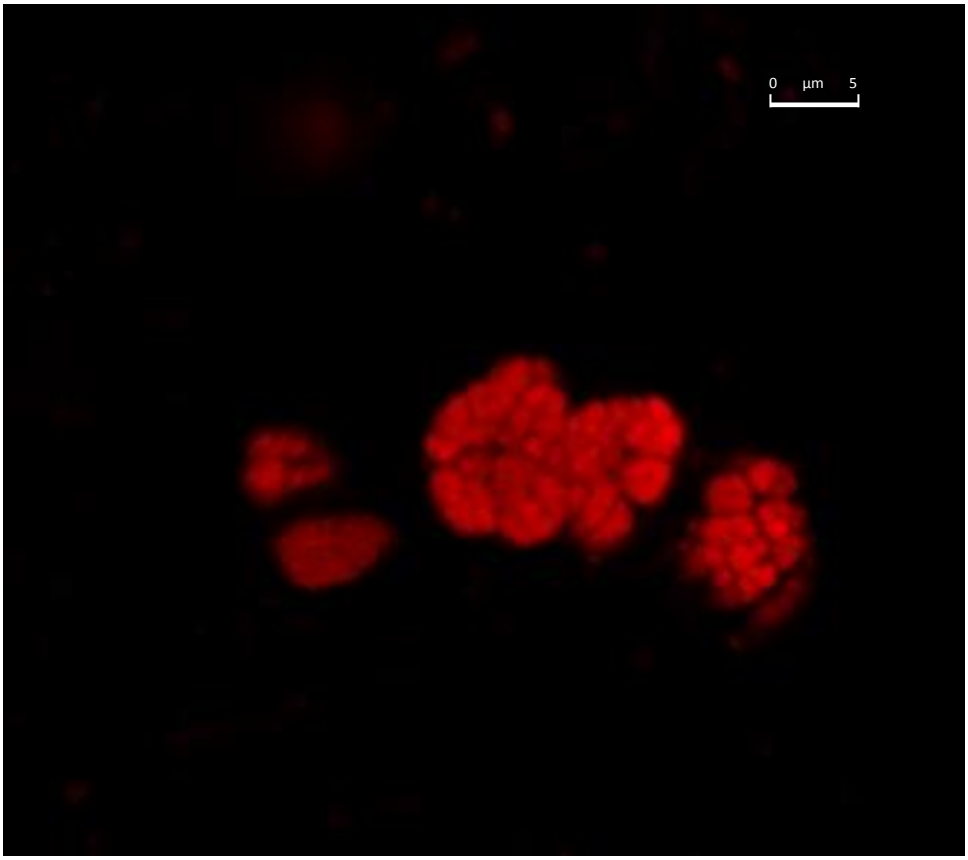

Figure 10. CLSM image of NOB cluster from positive control sample visualized through FISH. NOB imaged in red with set of NOB oligonucleotide probes.

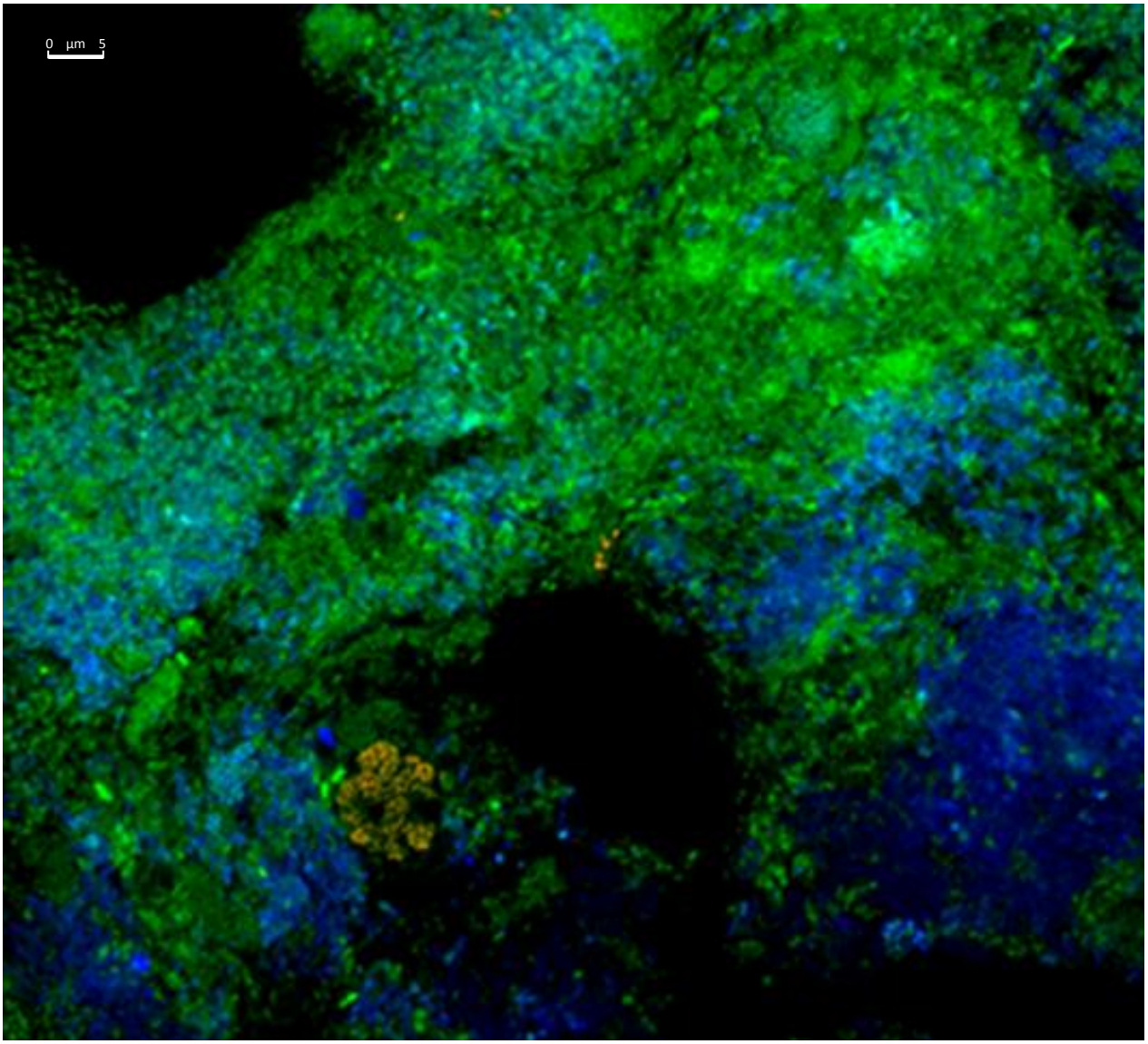

Figure 11. CLSM image of AOB and NOB clusters from IFFAS biofilm sample visualized through FISH. AOB imaged in orange with overlap of AOB (red) and eubacteria (green) oligonucleotide probes; NOB imaged in teal with overlap of NOB (blue) and eubacteria (green) oligonucleotide probes.

Due to the inability to detect anammox and the uncertainty behind the NOB presence, the secondary nitrite removal from the IFFAS system required an alternate explanation. A parallel study done on the IFFAS system in the lab revealed the metagenomic data, which will not be

discussed in the thesis, that support the claims of *Rhodanobacter* presence at the time of the sample fixation for the FISH studies. The results from the subsequent experiments using rhodano227 FISH probe did not yield any clear detection of said organism.

3.2 Discussion

The main objective of this thesis project was to gain a better understanding of the IFFAS system, through the examination of possible secondary microbial factors involved in the nitrification process in the system. In a previous study performed by Basuvaraj *et al.* (2012), the level of ammonia oxidizers and nitrite oxidizers relative abundance to eubacteria revealed an imbalance, in which the AOB levels were higher than NOB levels. The same observation has been noted in other literature with regards to IFFAS system, which the authors suggested may be associated with a lack of sensitivity in the characterisation methods for NOB (gene probes), or that NOB has a higher activity than AOB (Germain *et al.*, 2007). The hypothesis posed by the thesis suggested the possibility of alternate groups of non-AOB/NOB present in the IFFAS reactor that could account for the low AOB to NOB ratio, whilst allowing for complete ammonia removal.

Anammox bacteria are the hypothesized group of microorganism responsible for the difference in AOB and NOB levels observed in the past. Anammox bacteria are able to undergo ammonia oxidation reactions while under anoxic conditions, but more importantly, they have been found to utilize both ammonia and nitrite as substrates (Dalsgaard and Thamdrup, 2002). Due to the unique characteristics of the IFFAS system, such as their ability to allow for higher solids retention time and the presence of anoxic zones in the substratum layers of the biofilms, the conditions are ideal for slow anaerobic growth of anammox (Randal and Sen, 1996; Strous *et al.*, 1998; Santegoeds *et al.*, 1998; Sen *et al.*, 1995). In order to detect the presence of anammox in

the IFFAS system, a batch experiment was designed to allow for the study of ammonia removal in the different microbial components of the IFFAS system under non-aerated conditions. In theory, only the batch reactors containing anammox bacteria should be able to remove ammonia once the reactors becomes anoxic.

The initial batch experiment was set up to run without aeration in a closed system for six hours to study the changes in ammonia concentrations in batch reactions containing CAS floc, IFFAS floc, IFFAS biofilm, and IFFAS combined floc and biofilm. The data revealed relatively low levels of ammonia removal in the CAS and IFFAS floc reactions, at 7.91% and 7.80% respectively after six hours (Figure 1). The IFFAS biofilm was better than both floc setups, removing 31.56% of the initial ammonia; however, the surprise was the IFFAS combined setup that allowed for a 50.25% ammonia removal. The results correspond to the hypothesis that there is the possibility of anammox bacteria present in the IFFAS systems, and specifically in the biofilms, where the conditions are more favourable for slow-growing anaerobes. More interestingly was the observation that the IFFAS combined setup fared better than a system exclusively composed of IFFAS biofilms. Based on this set of data alone, it suggests the possibility of a synergy between flocs and film, an observation that has not been published in previous literature. The synergy may be specific to a set of microorganisms that thrive separately in floc and biofilm, which during an interaction, might have a mutualistic relationship in the way they remove ammonia. For example, an AOB bacterium that may be inhibited by nitrite during ammonia oxidation, which requires a NOB bacterium to clear out the nitrite and prevent its accumulation. If that AOB bacterium is found in the floc while the NOB is found in the biofilm, then it can account for why a synergistic effect may be observed in the presence of floc and biofilm.

The non-aerated batch experiment was repeated in triplicate and the results were averaged with standard deviations shown in Figure 2. The large standard deviations are due to the variations in the operational parameters, including difficulties to establish constant initial ammonia concentrations and the differences in overall biomass from one experiment to the next. The biomass is kept consistent between the different reaction bottles during each experiment, so the total biomass of the CAS floc is the same as the total biomass of the IFFAS floc, which contains the same amount of biomass as a set number of media carriers for the biofilms batch reaction. As the experiment dates vary, the total biomass found on the set number of media carriers will vary as well, which dictates the amount of floc biomass that is added to their respective reaction bottles. As there is variation with the amount of biomass, the level of ammonia removal is not expected to stay constant, thus explaining the high standard deviation observed; however, the key component to take away from the data is the overall trend, which shows almost linear decreases of ammonia in all four experimental setups over the six hour period. The results from this set of experiments are not consistent with the data presented in the preliminary batch study, which showed only ammonia removal in the IFFAS biofilm and IFFAS combined during the non-aerated six hour period. When comparing the different IFFAS setups with the CAS, it was shown that there are no significant differences in the concentration of ammonia after six hour period ($p > 0.05$). When interpreting the data objectively, there presents two possible explanations as to why these sets of non-aerated batch experiments observed ammonia removal in the flocs of CAS and IFFAS. The first explanation is simply that there may be anammox reactions occurring in all four test bottles; however, it is unlikely for there to be anammox bacteria in the flocs of CAS and IFFAS, as the sources from which the floc samples were obtained have aeration setups. The biofilms utilized were also collected from aerated setups, but it is possible for there to be anoxic regions in biofilms at certain thickness, even if the biofilms were run under aerated setups. The second explanation may involve the experimental

setup of the batch bottles, which as mentioned previously contain some headspace, as well as the likelihood of dissolved oxygen present in the media feed. There is no way of known how much dissolved oxygen were available in the batch reactions as the dissolved oxygen was not measured during the course of the batch experiments. The way the experiments were designed should allow for the removal of oxygen through metabolic reactions from the biomass that would in theory slowly transform the reactor environment into one of oxygen-limiting and perhaps even anoxic. The removal of oxygen from the closed system was central to the design of the experiment as a lack of oxygen is required to separate the aerobic ammonia oxidation from that of the anoxic. If the second, and more likely explanation, were to be true, then the experiment itself would be unable to differentiate between aerobic and anaerobic ammonia oxidation. When looking at possible synergy between the flocs and biofilm components of the IFFAS system, which was observed in the initial experiment, the IFFAS combined set-up did not have significantly lower ammonia concentration at six hours in comparison to the IFFAS floc and the IFFAS biofilm ($p > 0.05$).

Nitrite and nitrate data showed that they were almost nonexistent in the non-aerated reactions, which introduces the question as to what microbial factors are allowing for ammonia to be removed without detecting increases of nitrite and nitrate. Again, when analyzing the data objectively, two possible explanations come to mind. First being the presence of bacteria that are able to utilize ammonia, nitrite, and nitrate as substrates, or able to convert ammonia into a nitrogen species that is not nitrite or nitrate. This explanation is along the lines of the hypothesized anammox bacteria, which is able to utilize ammonia and nitrite to directly produce nitrogen gas without by-products. If the bacteria are utilizing nitrite and nitrate, it may be possible that the nitrite and nitrate were removed too rapidly to be detected. The second explanation for this phenomenon is the possibility that the ammonia underwent anabolic reactions for cellular

growth in the form of assimilation, rather than catabolic oxidation reactions; this would explain why all four samples were able to remove ammonia without aeration, as well as, the lack of nitrite and nitrate production.

To examine whether there is a presence of anammox bacteria, a more straight forward approach was taken. The use of fluorescently-labelled molecular probes can be used to directly bind to the ribosomal RNA of target organisms, thus the technique known as FISH was deployed next.

The FISH probe amx368 targets all anammox bacteria, thus was the first probe chosen for the microbial identification component of this project. When used along with eub probes that target all eubacteria, specific overlapping signals from the amx368 and the eub probes, in theory, can be observed using confocal scanning laser microscope (CSLM). The amx368 probe was unable to produce any verifiable signal, a difficulty that arose from not knowing whether the probe is malfunctioning or whether there lacked anammox bacteria in the tested samples. Through collaboration with the University of Vienna (Department of Microbial Ecology), positive control samples of anammox were made available. Due to the lack of amx368 probe at the facilities in Vienna, a broader pla46 probe was used instead, which targets all *planktomycetes*, a group of bacteria that encompasses anammox. The pla46 probe was verified to be functioning against the positive control sample of anammox that was tested. When performing FISH on the fixed samples from the IFFAS reactors using the pla46 probes, the resultant images showed some speckled signals (Figure 7), but nothing that resembled the clusters of anammox cells that were shown in the positive control (Figure 6), as well as lacking in the characteristic "doughnut" shape of the individual anammox cells. Based on expert consultation from two of the professors at the University of Vienna specializing in FISH and anammox, Dr. Markus Schmid and Dr. Holger

Daims, the likelihood that signals are from anammox bacteria is relatively low. Although low, it is possible that there are undiscovered anammox species out there which may not respond to the general FISH probes used for detection. Another possibility is that the samples hybridized on the microscopy slides did not contain anammox bacteria, while those bacteria may have been hidden in other samples not examined. It may also be possible that, although, the conditions may have been adequate for anammox growth within the IFFAS biofilms, a lack of anammox bacteria in the original seed used to start up the reactors can be the reason that no anammox was grown over the course of the study. In fact, a parallel study looking at the metagenome of the IFFAS reactors did not detect anammox in the seed samples. Upon determining that IFFAS reactors did not have anammox bacteria, the next step is to continue searching for the bacterial group responsible for the ammonia removal without the production of nitrite and nitrate that was observed in the oxygen-limiting batch experiments. Before looking for the culprit behind the enigma, FISH experiments were done on the IFFAS samples to look for common nitrifiers to verify that the system is running normally, in addition, any absence can also help clarify the picture.

The AOB probes showed positive signals, verified using the multiple probe approach (Figure 8). The use of different probes to target the same group of bacteria, not only verified the presence of AOB, but more specifically revealed that the majority of the AOB in the IFFAS system belonged to a species known as *Nitrosomonas oligotropha* (Figure 9). The presence of *N. oligotropha* is suggestive of a reactor run under low ammonia levels (Bollmann *et al.*, 2002), which might help explain the lack of anammox bacteria within the sample, as they require higher ammonia substrates levels. The level of ammonia substrates required for anammox growth have varied in the literature, from 50-150 mg N/L to 30-50 mg N/L; both of which are higher than the influent levels utilized in this study (~15 mg N/L) (Fernandez *et al.*, 2012). One study looking at the short-term effects of ammonia loading on anammox bacteria showed inhibitory anammox

activity at ammonium concentrations higher than 70 mg/L of influent ammonium nitrogen; however, the study was still able to see anammox activity at concentrations higher than 700 mg/L of influent ammonium nitrogen. Although the study only looked at the higher thresholds of ammonium, it shows how well anammox is able to withstand high ammonium loading, which suggests that anammox bacteria prefers overall higher ammonia loading. The possible inadequate ammonia loading may also explain the lack of anammox observed in the IFFAS reactor. The NOB probes were able to produce some signal that overlapped with the EUB probes; however, they were not able to withstand the multiple probe test and they lacked the characteristic "cauliflower" cluster shapes, visualized in a positive control sample in Figure 10. The AOB and NOB FISH experiments were replicated at the lab back at Queen's University. Much like the previous experiments, the AOB were very easily detected, while the NOB presence was still questionable (Figure 11). If the IFFAS system did not possess NOB or anammox, based on the FISH data, then results are still unable to account for the removal of ammonia with a lack of nitrite buildup.

A decision was made to go back and revisit the batch experiments, but perform them under aerated condition. Upon examining four replicate sets of data, all four reaction samples were able to undergo ammonia oxidation in the six hours; the term oxidation was used instead of removal because the nitrite levels gradually increased over the 6 hour period (Figure 4), and the nitrate levels were observed to increase after two hours (Figure 5). This is strongly suggestive of both AOB and NOB presence, as the AOB oxidize ammonia into nitrite, thus explaining the nitrite increase; and the NOB oxidize the newly produced nitrite substrate into nitrate, thus increasing the nitrate levels. Previously, the FISH experiments were unable to determine NOB presence with confidence; however, when paired with the batch experiment data, it seems more likely that there are in fact NOB within the system. The biofilm samples were observed to

undergo the same trends in nitrite and nitrate changes as the other samples, however, at much lower concentrations. The lower nitrite and nitrate levels with biofilm samples may be attributed to the difference in biomass age, between the biofilm and the flocs. When a reactor is starting up, the seed flocs are obtained from the CAS treatment plant, which have mixed liquor with a certain sludge age. The IFFAS media carriers, on which the biofilms grow, are typically added to these already aged sludge flocs, and over time, the biofilms slowly mature. Depending on the time at which the samples are taken for the batch experiments, the age of the biofilms may vary, and the age of the flocs are different than the biofilm as well. Another possible explanation for the lower concentrations of nitrite and nitrate observed in the biofilm samples may be, simply, that the biofilms contain less nitrifiers than the flocs, which has been observed before in an IFFAS system (Li *et al.*, 2012).

When the batch experiments were performed with aeration, ammonia removal was observed in all the batch reaction bottles, without any significant difference in the ammonia concentrations at six hours ($p > 0.05$). The combined IFFAS floc and biofilm batch reaction did not provide significantly lower levels of ammonia at the end of the six hour reaction in comparison to the IFFAS floc and the IFFAS biofilm separately ($p > 0.05$). This suggests that there is in fact no synergy observed between the floc and biofilm components of the IFFAS system, and that the microbial community in each component contribute the same amount of ammonia removal.

Chapter 4 Conclusion

The hypothesis that started the thesis project revolved around the idea that anammox bacteria were involved in the nitrification process found within the IFFAS system. The innate existence of these anammox organisms was believed to have played a role in the imbalance of AOB to NOB ratio, previously observed in the IFFAS system (Basuvaraj, 2012). Over the course of the thesis, the following two objectives were undertaken: a) identify whether anammox bacteria is the secondary microbial factor associated with the low NOB levels previously seen; b) study the relative contribution of the floc and biofilm components of the IFFAS system in the nitrogen removal process.

The initial batch experiment, run under non-aerated conditions, provided hopeful data towards the possibility of having anaerobic ammonia oxidation within the biofilm communities of the IFFAS system. Ammonia was observed to decrease much more in the IFFAS biofilm sample, and the IFFAS combined biofilm and floc sample (Figure 1). Understanding the possibility of having anoxic zone within the stratification of biofilms (Santegoeds *et al.*, 1998; Basuvaraj *et al.*, 2012), as well as, the ability of the IFFAS system to favour slow growing bacteria (Randal and Sen, 1996), the initial data from the batch experiment showed promise for anammox bacteria presence. In addition, the initial batch experiment also revealed a synergy between the floc and biofilm of the IFFAS system (Figure 1). Even though the biomass in each batch reaction was consistent, the IFFAS combined setup with both floc and biofilm demonstrated better ammonia removal abilities than the IFFAS floc, and IFFAS biofilm independently. If the mechanism of IFFAS system does provide synergy, the reactor kinetics of the IFFAS system would then need to be revisited, implications of which could have a staggering influence on the way integrated

systems are studied and characterized in the future. Unfortunately, all the subsequent replicates of the batch experiments (both non-aerated and aerated) were unable to show any synergy, as the ammonia concentrations in the combined IFFAS batch reaction were not significantly lower than the individual components of the IFFAS system after six hours of reaction ($p > 0.05$) (Figure 2 and Figure 3). The lack of synergy suggested that the microbial contributions of the two components of the IFFAS system, the floc and biofilm, were the same in terms of ammonia removal.

The primary objective of the study was to identify whether anammox bacteria are present in the IFFAS system. The most direct approach was through the use of FISH probes, designed specifically to bind to anammox organisms. The FISH technique can be difficult to master, and without proper guidance from skilled researchers, it may be challenging to determine whether signals observed are legitimate (not created due to unspecific binding) or simply background noise. I was fortunate enough to be introduced to two of the world's leading researchers in the field of microbial ecology at the University of Vienna, Dr. Holger Daims and Dr. Markus Schmid. As one can quickly realize from taking a glance at the reference section of this thesis, both professors have been extensively quoted throughout this work, with reference to FISH and anammox. Dr. Daims was one of the main authors in the fundamental textbook on FISH techniques, and Dr. Schmid was part of the team that produced ground-breaking discoveries in new species of anammox bacteria. Clearly, both are authorities in their respective fields. The long-winded introduction for Dr. Daims and Dr. Schmid serves to present their pedigrees, as well as, the fact that if they cannot help determine the presence of anammox in the IFFAS reactors, then it is extremely likely that anammox bacteria are simply not in the system. Through the use of amx368 and pla46 FISH probes, it became apparent that there are no anammox bacteria present in our IFFAS system, at the time of biomass sampling. The data proved to be very demoralizing, as

the main hypothesis, for the imbalance in AOB to NOB ratio in IFFAS system, was determined to be null. However, just because there is a lack of evidence for anammox presence in the IFFAS reactors at the time of experimentation does not mean that anammox bacteria are not naturally found in other IFFAS setups. The seed used to start up the IFFAS reactors at the lab did not contain any anammox organisms, based on the parallel study looking at the metagenome of the IFFAS reactor. Even though the conditions within the IFFAS systems may be suitable for the growth of anammox bacteria, it would not have been possible to locate said microorganisms. For future directions, it would be very interesting to see how an IFFAS system reacts to the introduction of anammox enriched sludge seed. Not only will it be important to identify anammox bacteria within the seed, before starting up the reactors, it may be helpful to prepare the seeds in conditions more favourable for anammox bacteria growth. For example, provide external nitrite sources, and higher ammonia loading, as well as, providing an anoxic condition for the anammox to grow. Molecular techniques like FISH can be used to quantify and track the growth, or decline, of the anammox enriched seed over time.

The FISH techniques, honed under careful supervision in Vienna, are some of the most valuable components of this entire thesis project. FISH was used with frequency in this project, including the use of a variety of different AOB and NOB probes, to observe the existence of other common nitrifiers within the IFFAS system. AOB were detected in all of the IFFAS samples, verified through the use of multiple probe approach; in fact, with the aid of species specific probe, the dominate AOB in the system were found to be *Nitrosomonas oligotropha* (Figure 9). NOB, although detected in the IFFAS samples using FISH, proved to be more difficult to verify using the multiple probe approach; however, when paired with aerated batch experiment data, its presence in the IFFAS system has increased in confidence. In fact, the batch experiments, when run aerobically, showed ammonia removal in both CAS and IFFAS systems; as well as, increases

in nitrite and nitrate levels, over the reaction period. Although lacking in exciting discoveries, the aerated batch and the FISH experiments demonstrated that the IFFAS systems are running under normal nitrogen-removal conditions in our lab. The finding that *N. oligotropha* was the dominate AOB in our bench-scale IFFAS system is suggestive of insufficient ammonia loading on the system, as that specific species of AOB, as the name suggests, favours oligotrophic nutrient conditions (Bollmann *et al.,* 2002).

The microorganism of interest, which may have been involved in the utilization of excess nitrite formed from the higher AOB to NOB communities, has thus far been determined not to be under the classification of the anammox group, and its identity is still uncertain. During the process of this thesis project, a parallel study on the IFFAS system was undertaken in the lab, studying the microbial distribution of the system using metagenomics. The results of that study will not be discussed in this thesis, with the exception of an organism, revealed in the metagenome as *Rhodanobacter*. *Rhodanobacter* is a genus of Gram-negative, non-spore-forming bacteria within the family *Xanthomonadales*, classified under *γ-Proteobacteria* (Nalin *et al.*, 1999). *Rhodanobacter* is typically studied in soils, as it is a soil denitrifier; as far as the research conducted in this thesis has found, it has not been observed or studied in wastewater setups. A study running SBR bioreactors using soil slurry as the inoculum observed denitrification with nitrate reduction and nitrous oxidize production for about a year while running at pH 4 (van den Heuvel *et al.*, 2010). FISH analysis and clone libraries performed in that study found *Rhodanobacter*-like bacterium to constitute up to 70% of the total community. Due to the lack of pH control at the beginning of this thesis project, the pH of the IFFAS SBR reactors were as low as 4. It is possible that the nitrite removal from the IFFAS reactor may have been associated with *Rhodanobacter* bacteria, which can help explain the initial AOB to NOB ratio phenomenon, as *Rhodanobacter* may be the secondary microbial factor. Although the specific substrates for

different species of *Rhodanobacter* may vary, there are certain species of the bacteria that have been classified as facultative anaerobes with the ability to grow on substrates of nitrate, nitrite and nitrous oxide (Prakash *et al*., 2012). With the emphasis on the former two substrates, measured in the IFFAS reactors belonging to the thesis project, the possibility of *Rhodanobacter* utilizing the nitrite produced from AOB and the subsequent nitrate production is not a far stretch. Although, as mentioned previously, not all *Rhodanobacter* bacteria utilize nitrite as a substrate, but its abundance in the IFFAS reactor, revealed from the metagenomics, paired with the search for a secondary microbial factor helping with nitrite removal, warrants a closer look at the organism. As *Rhodanobacter* has only been studied in soil innoculums, its presence in a wastewater setup is novel. The only paper in the literature, with published FISH probe sequences, used the probes to locate a specific species of *Rhodanobacter,* known as *Rhodanobacter thiooxydens* (van den Heuvel *et al.*, 2010). Unfortunately, the metagenomic data did not provide the species level information for the *Rhodanbacter*, so it was decided to try the rhodano227 probe, featured in the previous paper. The probe proved to be unsuccessful at detecting *Rhodanobacter* within the IFFAS samples, possibly due to difference in species. One future direction of research can be to study *Rhodanobacter* within IFFAS reactors and the way in which their unique microbial characteristics may attribute to the nitrification process. It may be useful to gather genomic data on the specific species of *Rhodanobacter* present in our IFFAS reactor to design FISH probes, which can be utilized to confirm their presence and abundance, as well as, reveal their niche through co-localization studies.

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