SIDE-STREAM TREATMENT OF ANAEROBIC DIGESTER FILTRATE BY ANAEROBIC AMMONIA OXIDATION

by

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ABSTRACT

Anaerobic Ammonia Oxidation (Anammox) has become an important topic in environmental microbiology and engineering in the last 15 years. The application of Anammox in wastewater treatment provides many beneficial advantages over traditional nitrogen removal processes, particularly in treating ammonium-rich waste streams.

In this study, the Anammox process was applied to a fed-batch reactor to treat raw digester filtrate from a local treatment plant. During initial treatment, the filtrate was diluted and an external nitrite source was supplemented. After reaching stable removal, a partial-nitritation (PN) reactor was started-up and fed with the same raw filtrate (undiluted). The effluent from the PN reactor was then fed directly to the Anammox (in place of diluted filtrate). A very long solids retention time (SRT) of 200 days was maintained throughout the study via manual wasting and decanting in order to produce very little sludge and still maintain efficient nitrogen removal. Sequence analysis and fluorescence in-situ hybridization (FISH) were performed on the biomass communities from both reactors. Automated ribosomal intergenic spacer analysis (ARISA) was also conducted on the Anammox biomass throughout the study period.

The reactor operated at a moderate loading rate (average 0.33±0.03 with a max of 0.4 g N $(L\ day)^{-1}$) comparable with many other fed-batch reactors in literature. It also achieved significant N removal (average of 82±4%) and specific removal rates (average 0.28±0.05 with max of 0.35 g N $(g\ VSS\ day)^{-1}$) likewise comparable with similar studies

despite maintaining a very long SRT. Sequence analysis and FISH showed that *K. stuttgartiensis* dominated the enriched Anammox community (approximately 65% of the biomass) along with several unidentified, but seemingly enriched, potential Anammox strains. ARISA analysis of the Anammox community showed no noticeable shift in the community profile despite the change in feed composition during the study period. It has been found in other studies that the species *K. stuttgartiensis* is capable of dissimilatory nitrate reduction to ammonium (DNRA), which would give it a selective advantage in conditions created by maintaining a long SRT.

Ammonia oxidizing bacteria (AOBs) of the *N. europaea* lineage dominated the community in the PN reactor, agreeing with literature showing that lineage to dominate in oxygen-limited, ammonium-rich conditions.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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CHAPTER I

INTRODUCTION

Current Filtrate Management Challenges

The use of anaerobic sludge digestion for the management of biosolids is a popular practice among municipal wastewater treatment plants around the world. The attractiveness is due to a reduction of solid waste, up to 50% (Metcalf and Eddy, 2003), and the energy-savings associated with harvesting and combusting biogas to cogenerate heat and power (CHP), which can be utilized on-site to heat and provide electricity to the facility and other treatment unit processes. Anaerobic digestion can also be used to reduce pathogens, yielding land-applicable biosolids (Iranpour *et al.*, 2006), which can be sold as fertilizer to further subsidize operational costs. Yet, despite these benefits, the liquid effluent from anaerobic digesters is very rich in ammonium nitrogen.

Anaerobic digesters typically produce filtrate with an ammonium concentration of about 1000 mg $(L)^{-1}$ NH_4-N (Dapena-Mora *et al.*, 2004b). Often, the ammonium-rich filtrate is recycled back to the head of the plant for treatment. Yet, the recycled filtrate can increase the ammonium loading to the treatment train by as much as 30%, despite the very small contribution of recycled flow (about 1%) (Lackner *et al.*, 2008).

Ammonium is a common pollutant with a significant oxygen demand (up to 4.57 g O_2 (g NH_4^+-N)$^{-1}$) and toxic to aquatic life. It is often regulated in wastewater discharge and is traditionally treated by means of nitrification in which ammonium is oxidized to

nitrite and nitrate, which process requires the addition of oxygen. The nitrate produced from nitrification is also commonly regulated, particularly because it contributes to eutrophication. Likewise, when receiving waters join potential drinking water sources, nitrates and nitrites may be restricted to prevent methemoglobinemia in infants. Nitrate is traditionally removed via denitrification, which reduces nitrate to dinitrogen gas and typically requires readily biodegradable organic substrate (rbCOD).

The recycled nitrogen load from filtrate will increase the aeration requirement to achieve conventional nitrification and the addition of a supplementary rbCOD to carry out denitrification, if effluent standards require the removal of nitrogen. The increased oxygen requirements and additional organics contribute significantly to facility energy consumption and operational costs. Also, in climates with cold seasons, when the specific removal rates slow down, the additional ammonium load can potentially cause the treatment plant to fail to meet the NPDES permit limit.

Introduction to Anammox

Discovery and development

Anaerobic ammonia oxidation (Anammox) was originally theorized using thermodynamic equations to explain observed soluble inorganic nitrogen losses in the biologically deprived (in terms of available carbon and nutrients) water column of our oceans (Broda, 1977). He proposed that "lithotrophs missing in nature" were carrying out denitrification with ammonium as the electron donor. The term Anammox, however, was not coined until the 1990s, when microbes were experimentally identified in a denitrifying fluidized bed reactor, in which ammonium was being oxidized in an anoxic environment, producing dinitrogen gas (Mulder *et al.*, 1995; van de Graaf *et al.*, 1995).

Subsequent studies showed that nitrite was the primary electron acceptor instead of oxygen (van de Graaf *et al.*, 1995). Equation 1 represents the observed stoichiometry of the reaction including the consumption of bicarbonate and production of biomass (Strous *et al.*, 1998). Based on this stoichiometry, the Anammox process can theoretically achieve 89% N removal. However, this can vary depending on the ratio of available nitrite and ammonium, as well as whether any nitrate is reduced (Caffaz *et al.*, 2006).

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow$$
$$0.066CH_2O_{0.5}N_{0.15} + 1.02N_2 + 0.26NO_3^- + 2.03H_2O \quad (1)$$

Since discovery, the investigation of the Anammox process has led to its uncovering in many diverse environments. It has been found in marine environments, such as the Black Sea (Kuypers *et al.*, 2003), as well as freshwater environments, such as Lake Tanganyika in Tanzania (Schubert *et al.*, 2006). Sediments, such as those in Chesapeake Bay, have also been found to contain Anammox bacteria (Rich *et al.*, 2008). Anammox activity has even been reported in constructed wetlands (Paredes *et al.*, 2007) as well as multiple wastewater and leachate treatment facilities (Dong and Tollner, 2003; Egli *et al.*, 2001; Fujii *et al.*, 2002; Helmer-Madhok *et al.*, 2002; López *et al.*, 2008; Pynaert *et al.*, 2003; Schmid *et al.*, 2003; Tal *et al.*, 2003; Toh and Ashbolt, 2002). In fact, it is hypothesized that up to 50% of the atmospheric nitrogen is a result of widespread Anammox activity (Dalsgaard *et al.*, 2005; Kuypers *et al.*, 2003; Schmid *et al.*, 2007; Strous and Jetten, 2004).

Phylogeny

Because Anammox bacteria are strict anaerobes and autotrophic and have long doubling times, growth in pure culture has not yet been possible (Kuenen, 2008; Tsushima *et al.*, 2007b). However, a considerable amount of information has been determined about the phylogeny, ultrastructure and function in lab-scale studies. Anammox bacteria form a deep-branching clade within the phylum planctomycetes (Schmid *et al.*, 2005; Schmid *et al.*, 2007; Strous *et al.*, 1999b) and belong to the order Planctomycetales and family Anammoxiceae (Ping, 2009). So far, five genera of Anammox bacteria have been identified: "*Candidatus Kuenenia*" (Schmid *et al.*, 2000), "*Candidatus Brocadia*" (Kartal *et al.*, 2004; Kuenen and Jetten, 2001) "*Candidatus Anammoxoglobus*" (Kartal *et al.*, 2007b), "*Candidatus Scalindua*" (Schmid *et al.*, 2003) and most recently, "*Candidatus Jettenia*" (Quan *et al.*, 2008). Research shows that *Candidatus Brocadia* and *Candidatus Kuenenia* are most commonly found in wastewater treatment plants and Anammox bioreactors (Kuenen, 2008; Schmid *et al.*, 2000), as is the more recently discovered *Candidatus Jettenia asiatica* (Quan *et al.*, 2008). *Candidatus Anammoxoglobus propionicus* thrives in enrichments containing ammonium, nitrite and propionate and has a competitive niche in such environments (Kartal *et al.*, 2007b). "*Candidatus Scalindua,*" on the other hand, is mostly found in natural habitats such as marine sediments and areas with minimal oxygen (Kuenen, 2008; Kuypers *et al.*, 2003; Schmid *et al.*, 2003; Schmid *et al.*, 2007). However, strains of these Anammox communities differ among the various cultures (Mohan *et al.*, 2004), and are typically found in mixed cultures (Dalsgaard *et al.*, 2003; Dalsgaard *et al.*, 2005; Kuypers *et al.*,

2003; Risgaard-Petersen *et al.*, 2003; Rysgaard *et al.*, 2004; Thamdrup and Dalsgaard, 2002; van de Graaf *et al.*, 1997).

The evolutionary distances between the different species discovered is quite large (< 85% similarity in the 16S rRNA gene), yet they all share very similar physiologies, ultrastructure and metabolism, suggesting the likelihood of an early, rapid evolutionary change (Jetten *et al.*, 2005a; Kuenen, 2008).

Cellular characteristics

Anammox bacteria have several unique cellular characteristics. For instance, unlike most bacteria, as members of the planctomycetales order, they lack peptidoglycan and have a proteinaceous cell wall (König *et al.*, 1984; Liesack *et al.*, 1986; Strous *et al.*, 2006). They have no outer membrane and two inner membranes (Lindsay *et al.*, 2001; Strous *et al.*, 1999b). A third membrane surrounds an anammoxosome "organelle," which is unique to Anammox bacteria (van Niftrik *et al.*, 2008b). Anammox membrane lipids are also unique, as they comprise a combination of ether-linked (typical of archaea) and ester-linked (typical of bacteria and eukarya) membrane lipids (van Niftrik *et al.*, 2008a). Likewise, most of the lipids contain ladderane moieties (Sinninghe Damste *et al.*, 2002; Sinninghe Damste *et al.*, 2005). These ladderane lipid structures (built from concatenated cyclobutane rings) have only been found in Anammox bacteria and seem to render the anammoxosome less permeable to the toxic hydrazine, which is formed within the organelle as a metabolic intermediate (Boumann *et al.*, 2006; Sinninghe Damste *et al.*, 2005). Another unique feature of these cultures is their distinctive red color, resulting from high concentrations of cytochrome *c*, which is a component of the nitrite reductase (NirS) found in the anammoxosome (van Niftrik *et al.*, 2008a; van Niftrik *et al.*, 2008b).

Metabolic pathways

The pathway for energy metabolism in Anammox (see Figure 1), which is energetically favorable to nitrification/denitrification processes (Jetten *et al.*, 2001), includes the reduction of nitrite to hydroxylamine by hydroxylamine oxidoreductase, which is combined with ammonium by a membrane bound enzyme complex to form hydrazine. The hydrazine is oxidized by the same hydroxylamine oxidoreductase-like (HAO) enzyme, similar to that found in aerobic ammonia-oxidizing bacteria, forming dinitrogen gas and free electrons to reduce more nitrite (Jetten *et al.*, 1998) as well as promote ATP generation (van Niftrik *et al.*, 2008a; van Niftrik *et al.*, 2008b).

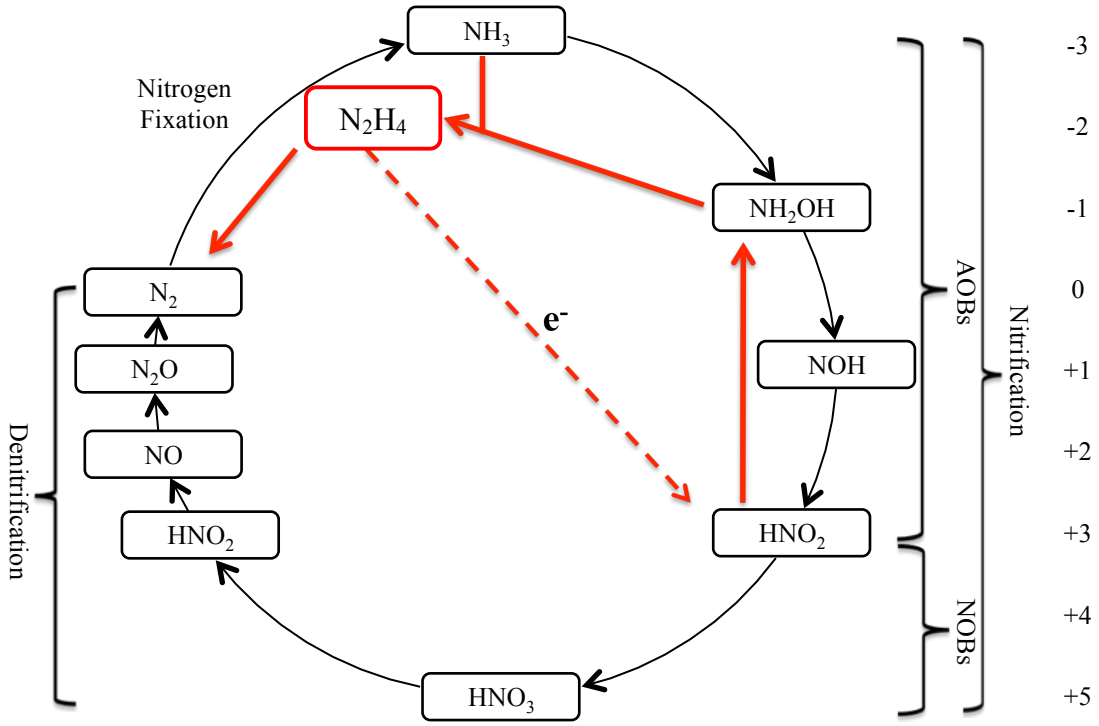

Figure 1. Nitrogen cycle involved in tradition N removal (black) and Anammox (red) [adapted from Ahn, 2006]

With the discovery of an analogue of nitric-oxide producing nitrite reductase (NirS) in the *Candidatus* Kuenenia stuttgartiensis genome, possible variations of the originally proposed pathway have been investigated. One such variation is nitrite first being reduced to nitric-oxide by nitrite reductase (NirS) (Strous *et al.*, 2006; van Niftrik *et al.*, 2008b). Since nitric-oxide is a radical, its "direct attack of ammonium" and subsequent uptake of three more electrons would yield hydrazine, via the enzyme hydrazine hydrolase (van Niftrik *et al.*, 2008b), forming another possible metabolic pathway (Kuenen, 2008; Strous *et al.*, 2006).

Both pathways would result in the production of hydrazine, which has been directly detected in previous studies (Jetten *et al.*, 1998). The energy-rich hydrazine can donate its electrons to produce reduced ferredoxin (Kuenen, 2008), and is oxidized to dinitrogen gas by hydrazine/hydroxylamine oxidoreductase, an octaheme cytochrome *c*, also found in the anammoxosome (Schalk *et al.*, 2000; Shimamura *et al.*, 2007). The four electrons derived from this oxidation are transferred from ferredoxin to soluble cytochrome *c* electron carriers (Cirpus *et al.*, 2005; Huston *et al.*, 2007) and finally to nitrite reductase and hydrazine hydrolase, building up the proton motive force, as mentioned earlier, to synthesize ATP (van Niftrik *et al.*, 2008a; van Niftrik *et al.*, 2008b).

However, the versatility of Anammox itself was yet expanded further by studies that showed certain Anammox strains capable of dissimilatory nitrate reduction to ammonium (DNRA), which is a secondary metabolic process found among sulfate reducing bacteria (Rysgaard *et al.*, 1996). DNRA converts nitrate (which is a product of Anammox) into more ammonium and/or nitrite that can then be converted to dinitrogen gas via Anammox (Kartal *et al.*, 2007a; Kuypers *et al.*, 2005; Risgaard-Petersen *et al.*,

2003). Even though Anammox are considered autotrophic, the DNRA process includes organic acid oxidation which serves as the electron donor for the nitrate reduction and which forms carbon dioxide (Guven *et al.*, 2005). Thus, DNRA offers a competitive advantage for certain strains of Anammox to produce their own substrates (ammonium, nitrite and carbon dioxide) from nitrate and organics (Kartal *et al.*, 2007a). Overall, the result is greater production of dinitrogen gas from nitrate via a pathway different than conventional denitrification. Although some studies suggest that this process is insignificant compared to denitrification (Risgaard-Petersen *et al.*, 2003), others suggest it could be significant (Trimmer *et al.*, 2003). One Anammox strain in particular, *K. stuttgartiensis*, is shown to be capable of DNRA (Kartal *et al.*, 2007a).

Growth conditions and inhibition

Anammox processes are temperature-dependent. Bioreactors are typically optimally operated at temperatures of approximately 30-37°C (Jetten *et al.*, 1998). However, in marine sediments, where *Candidatus Scalindua* is important in the nitrogen cycle, optimal Anammox activity occurs at temperatures ranging from 12°C to 15°C and decreases sharply above 25°C (Hietanen and Kuparinen, 2008).

Although Anammox are also sensitive to pH, activity is detectable in a pH range between 6.4 and 8.3 (Schmidt *et al.*, 2002), with an optimum pH value between 7.5 and 8.2 and inhibition of Anammox activity at values greater than 8.5 (López *et al.*, 2008). Van de Graaf *et al.* (1996) found that if the pH regulation failed in a bioreactor, N_2O was formed and disturbed the system. If the condition is kept anoxic by flushing with Ar/CO_2 (95/5%), the CO_2 present in the gas can be sufficient, if controlled, to buffer the solution

to a pH between 7.0 and 8.0 (Jetten *et al.*, 2005b). However, if the pH is not buffered, the pH diverges away from an unstable neutral point and must be controlled by other means.

Anammox bacteria are typically strict anaerobes and in bioreactors are inhibited by low amounts of oxygen (less than 1 μM) (Jetten *et al.*, 2005a; Strous *et al.*, 1997b), although marine Anammox processes do not seem to be constrained to fully anoxic environments (Jensen *et al.*, 2008). Inhibition by dissolved oxygen in bioreactors can be overcome by simply purging with Argon or Nitrogen to restore anaerobic conditions.

The most notable inhibitory compound of the Anammox process is elevated concentrations of nitrite. Nitrite concentrations greater than 10 mM (Strous *et al.*, 1998) or 50-150 mg N $(L)^{-1}$ (Strous *et al.*, 1999a) in cultures not acclimated to converting such high concentrations will inhibit Anammox activity. However, this inhibition can also be overcome with the addition of either hydroxylamine or hydrazine, both of which are process intermediates (Strous *et al.*, 1999a).

Other compounds that have been found to be inhibitory to Anammox in some studies include acetylene, high phosphate concentrations (Dapena-Mora *et al.*, 2007; van de Graaf *et al.*, 1996), a high salt concentration (Dapena-Mora *et al.*, 2007), as well as alcohols such as methanol (Guven *et al.*, 2005; Jensen *et al.*, 2007). A high organic content (C/N >2) is also found to be inhibitory due to competition with heterotrophs (Lackner *et al.*, 2008). Acetate can actually increase activity at low concentration but begins to be inhibitory above 10mM (Dapena-Mora *et al.*, 2007; van de Graaf *et al.*, 1996). Sulfide is also shown to be inhibitory in most studies (Dapena-Mora *et al.*, 2007; Jensen *et al.*, 2008), although it has also been found that sulfide can stimulate Anammox activity when using nitrate as the electron donor, suggesting the nitrate could be reduced

to nitrite by sulfide (van de Graaf *et al.*, 1996). Ammonium and nitrate have no effect on Anammox activity (Strous *et al.*, 1999a), nor does chlorine seem to have any effect (van de Graaf *et al.*, 1996).

Application of Anammox in Treating Ammonium-rich Wastewater

The growing understanding of the Anammox process and its advantages over conventional nitrification and denitrification has led to its incorporation into full-scale wastewater treatment in several European countries as a supplemental and/or alternative nitrogen removal process. The first full-scale reactor went online in 2002 in Rotterdam, Netherlands followed by several more in other parts of Europe (van der Star *et al.*, 2007). To date, there are no full-scale Anammox reactors operating in the United States.

In applying the Anammox process to ammonium-rich wastewater treatment, two main process designs have been developed: The two-reactor Anammox application (i.e. SHARON-Anammox or PN-Anammox) process and the one-reactor Anammox application (i.e. CANON) process, with acronyms developed for different variations of each (Li, 2008; van der Star *et al.*, 2007; Wett, 2007). Both processes have been investigated and found to work successfully, although they each have distinct benefits and challenges (Schmidt *et al.*, 2003).

Primary advantages and disadvantages

The primary advantages of Anammox over traditional nitrification and denitrification are that the bacteria responsible are autotrophic and strict anaerobes. As a result, no aeration or rbCOD addition is required, resulting in considerable cost savings (Jetten *et al.*, 2001). This is particularly useful for waste streams that are rich in

ammonium and have low COD concentrations, such as digester filtrate. While treating such streams with traditional nitrification and denitrification would require substantial aeration and rbCOD supplementation, the low COD/N ratio found in anaerobic digester filtrate is actually favorable for the Anammox process (Lackner *et al.*, 2008).

Anammox bacteria are also very slow growers; about an order of magnitude slower than Nitrifiers, and with lower yield (Table 1). Although it can also be considered a disadvantage, due to potentially longer start-up periods for full-scale application, slow growth rates are usually compensated with higher substrate utilization rates (Kieling *et al.*, 2007). Also, slow growth and low yield results in minimal sludge production (van Dongen *et al.*, 2001), which will require less solids handling and disposal, further reducing operational costs.

Altogether, no aeration or additional rbCOD requirement, and less sludge production are significant operational advantages (Caffaz *et al.*, 2006; Fux *et al.*, 2002; van Dongen *et al.*, 2001). Some studies claim the Anammox process can reduce operating costs as much as 90% when compared to recycling filtrate back to the main treatment train to undergo traditional nitrogen removal processes (Jetten *et al.*, 2001).

Table 1: Growth characteristics of nitrifiers and Anammox
[as reported by Jetten and Strous (Jetten *et al.*, 2001)]

Characteristic	**Nitrifiers**	**Anammox**
Max specific growth rate, μ_m $(h)^{-1}$	0.04	0.003
Doubling time, t_d (days)	0.73	10.6
Yield, $Y_{X/N}$ (mol (mol C)$^{-1}$)	0.08	0.07

Since the Anammox reaction requires an approximate 1:1 to 1.7:1 ratio of nitrite-N to ammonium-N, its application in treating ammonium-rich wastewater requires the partial conversion of ammonium to nitrite (partial-nitritation). Yet, only some of the ammonium must be converted to nitrite and no conversion of nitrite to nitrate is required, so the amount of oxygen required is as much as 60% less than that required for traditional nitrification (van Dongen *et al.*, 2001).

SHARON-Anammox application

The SHARON-Anammox application (see Figure 2), was developed on principles of the SHARON (single reactor high activity ammonia removal over nitrite) process, originally intended for streamlined ammonia nitrogen removal (ammonia oxidation and denitrification) via nitrite (Hellinga *et al.*, 1998). SHARON includes converting all ammonium to nitrite in an aerobic reactor and inhibiting the nitrite oxidizing bacteria (NOBs), to prevent the conversion of nitrite to nitrate. In a subsequent anaerobic reactor, denitrifiers convert the nitrite from the first reactor into nitrogen gas (Ahn, 2006). This process removes the conversion to and from nitrate, reducing the oxygen requirement, but still requires all the ammonium be converted to nitrite. By altering the process in the first reactor, so that only about half the ammonium is converted to nitrite, the second reactor can be replaced with Anammox. By replacing denitrification, associated with the original SHARON process, with Anammox, no organic carbon is needed (van Dongen *et al.*, 2001). This combination has been called several different names, but is generally referred to as the SHARON-Anammox process or partial-nitritation (PN) – Anammox process (van Dongen *et al.*, 2001).

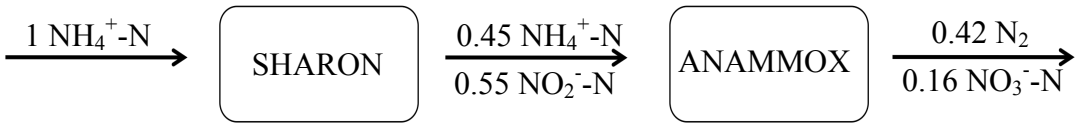

Figure 2: Simple schematic of SHARON-Anammox process
[adapted from Schmidt et al., 2003]

The SHARON-Anammox (PN-Anammox) process is what is being used in the WWTP Dokhaven, Rotterdam, NL, as well as several other full-scale treatment plants in Europe (van der Star *et al.*, 2007). It allows for little process control, high loading rates, low oxygen requirement in the first reactor, and little pH control (van Dongen *et al.*, 2001). Using two separate reactors also allows less risk of Anammox inhibition by toxic compounds in influent (filtrate) (Vazquez-Padin *et al.*, 2009a). Since anaerobic digester filtrate generally has plentiful bicarbonate and is typically alkaline, the free ammonia concentration inhibits NOBs, which are more sensitive to free ammonia than ammonia oxidizing bacteria (AOBs) (Anthonisen *et al.*, 1976). Likewise, since only partial ammonium oxidation is required, the bicarbonate in filtrate is usually plentiful enough to buffer the pH change associated with ammonium oxidation (van Dongen *et al.*, 2001). Plus, less aeration is required by using partial-nitritation than even the conventional SHARON-denitrification process (van Dongen *et al.*, 2001). Plus, oxygen-limiting conditions helps to inhibit NOBs, which have lower oxygen affinity than AOBs (Vazquez-Padin *et al.*, 2009a; Wiesmann, 1994).

The first reactor can be run with or without solids retention. Some studies have run without solids retention, such that solids retention time (SRT) equals hydraulic retention time (HRT), which is one of the distinctions of the original SHARON process (van Dongen *et al.*, 2001). Other studies that implemented solids retention of various

degrees (Fux and Siegrist, 2004; Vazquez-Padin *et al.*, 2009a), still achieved successful partial-nitritation. Overall, a short SRT seems to be less important than a short HRT in preventing nitrite-oxidation. A short HRT (~1 day) as well as mesophilic temperatures (~30 °C) are typically required in order to give enough advantage to AOBs to inhibit the conversion of nitrite to nitrate (Wilsenach and van Loosdrecht, 2006), although some studies have investigated the possibility of operation at room temperature (Vazquez-Padin *et al.*, 2009a).

The ratios of nitrite to ammonium provided by the first reactor (partial-nitritation) will influence the Anammox reactor (Dapena-Mora *et al.*, 2006). Thus, the previously mentioned measures are necessary to allow nitrite to accumulate in the partial-nitritation reactor to the appropriate ratio. Likewise, if too much nitrite is produced, raw filtrate can be added directly to the second stage (Anammox) to prevent nitrite accumulation in the Anammox reactor (Fux *et al.*, 2002).

CANON application

The CANON (Completely Autotrophic Nitrogen removal Over Nitrite) application was developed specifically for the Anammox process (Jetten *et al.*, 2001). The primary distinction of the CANON process is the use of a single reactor with a mixed culture to provide both partial-nitritation and anaerobic ammonia oxidation (Anammox) (see Figure 3). The ideology is similar to the SHARON-Anammox process in that the same reactions are taking place and NOBs are inhibited. However, the CANON process requires solids retention (SRT ≠ HRT) (Vazquez-Padin *et al.*, 2009a), and both aerobic and anaerobic (oxygen-limiting) conditions must exist within the biomass in the reactor, (Jetten *et al.*, 2001).

Figure 3: Simple schematic of CANON process
[adapted from Schmidt et al., 2003]

In order for the CANON application to work, Anammox bacteria must grow in symbiosis with the AOBs. The Anammox grow inside the granules surrounded by AOBs on the outside, which provides AOBs with ammonium and oxygen, and provides Anammox with ammonium and the nitrite produced, along with anoxic conditions (Nielsen *et al.*, 2005; Vazquez-Padin *et al.*, 2009a). The aeration of the reactor can be done in cycles (Third *et al.*, 2005) or at a constant rate (Sliekers *et al.*, 2003) to provide just enough oxygen for the AOBs to convert about half of the ammonium to nitrite, similar to the SHARON or partial-nitritation reactor, and the Anammox simultaneously converting the remaining ammonium and nitrite to nitrogen gas (Sliekers *et al.*, 2003; Vazquez-Padin *et al.*, 2009a).

The CANON process is already being used in full-scale treatment of anaerobic digester filtrate at WWTP Strass, Austria as well as in Hattingen, Germany (Vazquez-Padin *et al.*, 2009a). One of the main advantages of the CANON process is the use of a single reactor, which reduces the footprint and initial capital cost. Another benefit of the single reactor CANON application is that the NOBs are automatically inhibited as a result of having to compete with AOBs for limited oxygen and with Anammox for nitrite (Sliekers *et al.*, 2003; Vazquez-Padin *et al.*, 2009a). Because of this added advantage of AOBs over NOBs, the CANON process has been shown to work effectively at room temperature, which further removes heating costs normally associate with the SHARON

process (Vazquez-Padin *et al.*, 2009a). Although the nitrogen removal rates vary more widely among the CANON-based studies than SHARON-Anammox studies (Ahn, 2006), a recent study analyzing both the SHARON-Anammox and CANON processes showed the CANON process to achieve greater overall nitrogen removal rates (Vazquez-Padin *et al.*, 2009a).

The challenges encountered with the CANON application include the potential inhibition of Anammox if exposed to oxygen and balancing the activity of the mixed culture of AOBs and Anammox (Sliekers *et al.*, 2003; Vazquez-Padin *et al.*, 2009a). The CANON process is subject to sensitive operational characteristics in terms of dissolved oxygen, nitrogen load, biomass thickness and temperature (Ahn, 2006). Startup can be complicated and lengthy as a result of the time required to attain enriched Anammox within biomass granules and achieve steady partial-nitritation and Anammox activity, since both Nitrifiers and Anammox are very slow growers (Third *et al.*, 2005). A common start-up method is to gradually decrease the oxygen in a nitrifying reactor until it is low enough to inoculate with Anammox (Vazquez-Padin *et al.*, 2009a; Vazquez-Padin *et al.*, 2009b). Start-up might include several phases of enriching the AOB community in addition to jump starting and stabilizing Anammox activity to get both processes working effectively (Third *et al.*, 2005).

Reactor designs

Anammox studies have been conducted in both suspended growth and attached growth reactors including batch reactors (Strous *et al.*, 1998; van de Graaf *et al.*, 1995), fluidized bed reactors (Mulder *et al.*, 1995; Strous *et al.*, 1997a; van de Graaf *et al.*, 1996), fixed bed reactors (Strous *et al.*, 1997a; Zhang *et al.*, 2005), gas lift reactors

(Dapena-Mora *et al.*, 2004b; Sliekers *et al.*, 2003), upflow sludge blanket reactors (Jin *et al.*, 2008b), and membrane bioreactors (Trigo *et al.*, 2006). Suspended growth reactors select for the well-settling biomass and wash out the undesired suspended solids, promoting the retention of slow growing Anammox granules (Dapena-Mora *et al.*, 2004a; Kartal *et al.*, 2008; Kieling *et al.*, 2007; van der Star *et al.*, 2007). These well settling granules are ubiquitous among Anammox and are formed with extracellular polymeric substances (EPS) (Kartal *et al.*, 2008). Fixed film or biolfilm reactors promote the growth of *Nitrosomonas*-like aerobic ammonia-oxidizing bacteria on the surface, consuming any remaining oxygen and producing nitrite, creating a suitable environment for the Anammox beneath (Tsushima *et al.*, 2007a). The support material, such as in an upflow biofilter (a specific type of biofilm reactor), can also promote retention of slow growing Anammox (Furukawa *et al.*, 2003). However, when used in pre-Anammox (partial-nitritation) treatment, the very long SRT can result in accumulation of nitrate in the Anammox influent (Fux and Siegrist, 2004).

Batch tests are limited in analyzing Anammox due to nitrite loading limitation, because nitrite cannot be loaded in high concentrations without inhibiting the Anammox (Strous *et al.*, 1998; Strous *et al.*, 1999a). Traditional sequencing batch reactors (SBRs) are similarly limited, although some studies have controlled the exchange volume to achieve exceptional removal rates (Fux *et al.*, 2002). When operated as a fed-batch reactor, the continuous or semicontinuous nitrite loading and consumption prevents inhibitory concentrations (Kuenen, 2008; López *et al.*, 2008).

Studies show gas-uplift reactors to withstand greater loading rates than batch reactors (Dapena-Mora *et al.*, 2004b; Sliekers *et al.*, 2003), so long as shear stress is

managed properly (Arrojo *et al.*, 2008); so does the upflow biofilter (Jin *et al.*, 2008a). Upflow anaerobic sludge blanket reactors appear more robust than both sequencing batch reactors and fixed bed reactors (Jin *et al.*, 2008b). However, generally speaking, sequencing batch reactors offer several advantages in analyzing Anammox communities and determining operational parameters, including simpler set-up, reliable operation over long periods, better biomass retention, simpler mass balance, more homogeneous mixture and easier scale-up (Strous *et al.*, 1998).

Start-up strategies

Start-up of Anammox reactors has several potential complications such as long start-up times, biomass washout, and nitrite accumulation. Several start-up strategies have been used to overcome the complications and reach steady-state performance, yet a stable start up strategy is not well defined (Kieling *et al.*, 2007). Start-up and/or enrichment of Anammox often take a long time due to slow growth rate compared to traditional activated sludge or nitrifying reactors (Third *et al.*, 2005; Trigo *et al.*, 2006; Zheng *et al.*, 2004). Biomass is usually completely retained in the reactor during start-up in order to prevent washout of slow-growing Anammox (Third *et al.*, 2005; Trigo *et al.*, 2006). Likewise, loading rates must be controlled in order to prevent nitrite accumulation (Third *et al.*, 2005).

The control strategies depend on the substrate source. For Anammox studies using synthetic feed solution, control of loading rate is relatively simple (López *et al.*, 2008). Studies involving the treatment of actual filtrate or leachate (very high ammonium concentrations) may require a method, such as dilution, to reduce the concentration during start-up in order to regulate loading rates (Caffaz *et al.*, 2006; Ruscalleda *et al.*,

2008; Vazquez-Padin *et al.*, 2009a). Another option is to start with synthetic feed then switch to actual filtrate (Caffaz *et al.*, 2006; Dapena-Mora *et al.*, 2006).

Start-up strategies are different for SHARON-Anammox (Hwang *et al.*, 2005) as opposed to CANON (Vazquez-Padin *et al.*, 2009b) applications in terms of treating actual ammonium-rich wastewater. For studies using SHARON (PN), it may be necessary to provide an alternative form of nitrite initially during start-up of the PN reactor. One way to do this is to add sodium nitrite in proportion to ammonium (Caffaz *et al.*, 2006; Hwang *et al.*, 2005). Another method is to add nitrate initially, allowing any denitrifiers in the inoculum sludge to reduce it to nitrite (Third *et al.*, 2005).

To retain biomass during start-up, different reactor types offer different amounts of retention efficiency. Membrane bioreactors (Trigo *et al.*, 2006) and SBRs (Dapena-Mora *et al.*, 2004a), in particular, can have excellent retention; yet some studies have used alternative measures to retain biomass in the system, such as an external settling device (Third *et al.*, 2005). However, if start-up involves enriching Anammox from activated sludge (Chamchoi and Nitisoravut, 2007; Zheng *et al.*, 2004) or digester sludge (Jianlong and Jing, 2005), some washout may be helpful in removing undesired communities (Kieling *et al.*, 2007).

Solids retention time

Solids retention is a major component of the Anammox process even after start-up, since Anammox are very slow growers (van Dongen *et al.*, 2001). Studies involving Anammox processes have a large degree of variability of operating solid retention times (SRT) (see Table 2), which are significantly longer for Anammox processes than for either heterotrophic or nitrification processes.

Table 2: SRT values reported in studies involving Anammox reactors

Study	SRT (days)
Dapena-Mora et al. 2004a	35-130
van der Star et. al. 2007	45-160
Chamchoi et al. 2007	42
Vazquez-Padin et al. 2009a	30
Dosta et al. 2008	150
Park et al. 2010	25
Vazquez-Padin et al. 2009b	40-150
Wett et al. 2007	30

A very long SRT would reduce sludge production but also results in lower consumption rates and greater cell decay due to substrate limitation and unstable metabolism (Shuler and Kargi, 2002). In the case of Anammox, it can also allow heterotrophic denitrification, since dead cells provide organic matter that heterotrophic denitrifying bacteria can use with both nitrite and nitrate as electron acceptors for denitrification (Kieling *et al.*, 2007). Denitrifiers not only coexist with Anammox, but can also compete with Anammox (Kindaichi *et al.*, 2004; Kindaichi *et al.*, 2007; Lackner *et al.*, 2008; Mohan *et al.*, 2004). However, some strains have also been found to carry out (along with Anammox) dissimilatory nitrate reduction to ammonium (DNRA), which involves the oxidation of organics to CO_2 and reduction of nitrate to nitrite and ammonium. Thus, if the enriched culture is capable of DNRA, it could potentially use the organic matter from cell decay and available nitrate from Anammox to provide itself

with additional ammonium, nitrite and CO_2 (Guven *et al.*, 2005; Kartal *et al.*, 2007a; Kuypers *et al.*, 2005; Risgaard-Petersen *et al.*, 2003; Trimmer *et al.*, 2003).

Research Hypothesis and Objectives

Hypothesis

A suspended-growth fed-batch Anammox reactor, kept at a very long SRT (200 days), can maintain efficient nitrogen removal at a loading rate comparable with similar studies.

Objectives

Objective A. Start-up Anammox reactor and reach steady-state conditions at moderate loading rate using diluted filtrate from local POTW, supplemented with sodium nitrite.

Objective B. Maintain SRT of 200 days by careful, manual decanting and wasting.

Objective C. Take regular measurements of biomass concentration in reactor, as well as nitrogen concentrations in Anammox feed and effluent to track N removal (in terms of Ammonium, Nitrite and Nitrate) and specific N removal (in terms of VSS).

Objective D. After Anammox reactor is running at steady-state, start-up partial-nitritation (PN) reactor feeding with raw filtrate (from same location), and begin using partial-nitritation effluent as feed for Anammox. Continue to dilute and supplement sodium nitrite to Anammox feed as needed to maintain appropriate ratios until PN reactor reaches full performance.

Objective E. Perform automated ribosomal intergenic spacer analysis **(**ARISA) on Anammox biomass samples taken before PN effluent is used, during stabilization with PN effluent, and after reaching steady-state with only PN effluent fed to Anammox to analyze potential shift in community.

Objective F. After both reactors have maintained long-term steady-state performance, conduct cloning and sequencing as well as fluorescence in-situ hybridization (FISH), to analyze enriched community within both reactors. Compare with ARISA results.

CHAPTER II

MATERIALS AND METHODS

Anammox Reactor Operation

A 5.0 L semicontinuously fed sequencing batch reactor (fed-batch reactor) was maintained to achieve simultaneous ammonium and nitrite removal (Anammox) (see Figure 4). The FBR was inoculated with Anammox biomass received from the City College of New York (Civil Engineering Department). Anaerobic digester filtrate, used as feed, was regularly acquired from the belt press of a local wastewater treatment plant (CVWRF, SLC, UT). This provided the nutrients required for bacterial growth as well as a high initial concentration of ammonium. During start-up, and phase 1, the reactor was fed with diluted filtrate supplemented with sodium nitrite. After maintaining steady-state for 60 days, phase 2 was initiated in which a preliminary step of biological partial-nitritation of the raw filtrate was included to achieve an approximate 1.2:1 ratio of nitrite-N to ammonium-N and to remove any BOD. This ratio was chosen in order to make nitrite the limiting substrate, in order to prevent nitrite accumulation, which leads to Anammox inhibition (Strous *et al.*, 1999a).

The FBR was operated with two cycles per batch, yielding an HRT of 4 days. Each cycle included 2.5 L of feed being added over a 48 h period (52 mL $(h)^{-1}$) to reach a volume of 5.0 L, concluded by 30 min of settling and manually decanting of 2.5 L. Due to the large well-settling Anammox granules (Kartal *et al.*, 2008)., careful decanting

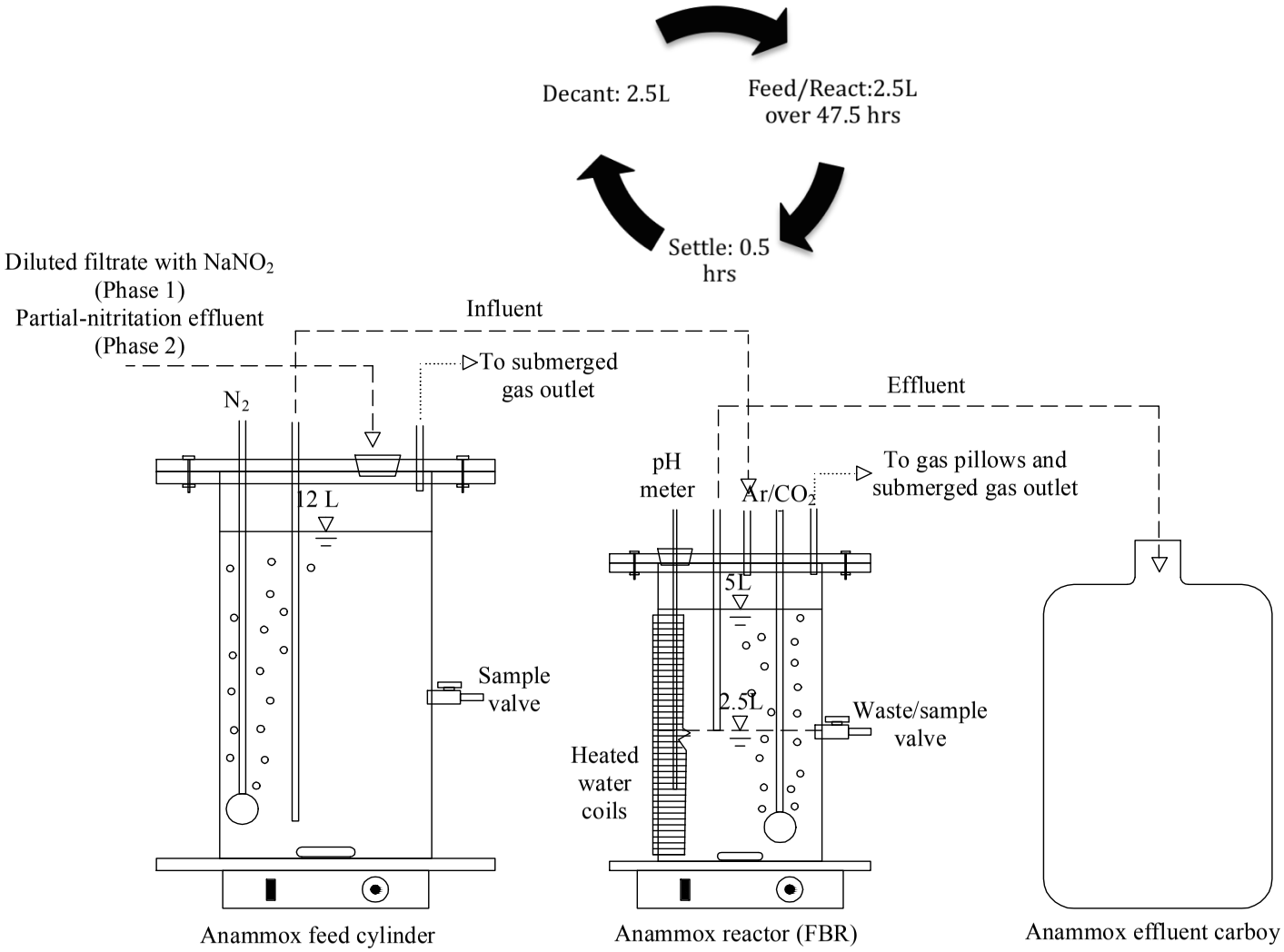

Figure 4: Schematic of Anammox reactor

and visual inspection ensured negligible biomass loss in effluent. A pH of 7.8±0.3 was maintained via bicarbonate addition to the feed and by the slow purging with a 95% Argon and 5% CO_2 mixture (5- 60 mL $(min)^{-1}$) depending on pH), also ensuring anaerobic conditions. The feed pump was automated with electronic timers (ChronTrol, San Diego, CA). The SRT in the FBR was maintained at 200 days by wasting mixed liquor from the FBR at the end of each cycle, prior to settling. The reactor was kept at 32° C using a water bath with warm water circulating in tubes around the reactor. Anaerobic conditions were maintained by keeping the reactor sealed and gas outlet tubing submerged, and by purging the feed with nitrogen gas.

Partial-nitritation Reactor Operation

During phase 2, a 2.0 L sequencing batch reactor (SBR) was incorporated to achieve partial-nitritation of the anaerobic digester filtrate (see Figure 5). The SBR was seeded with biomass from a nitrification SBR (Racz *et al.*, 2010), kept in an anoxic holding tank during phase 1. The partial-nitritation (PN) reactor was operated on a 12-h cycle with two cycles per batch (HRT of 1 day). Each cycle consisted of the addition of 1 L of raw filtrate, 12 h of aerobic reaction, concluded by 30 min of settling and decanting of 1 L. Dissolved oxygen was maintained at 1.5±0.5 mg $(L)^{-1}$ by aeration at approximately 2.5 liters per minute (LPM) with an aquarium pump. All pumps were automated with electronic timers (ChronTrol, San Diego, CA).

Biomass was retained in the PN reactor by placing the decant tube at a depth corresponding to the settled volume of biomass required to carry out the desired partial-nitritation and allowing any growth above that depth to be removed with the supernatant at the end of each cycle. The reactor temperature was maintained at ~ 32° C using a

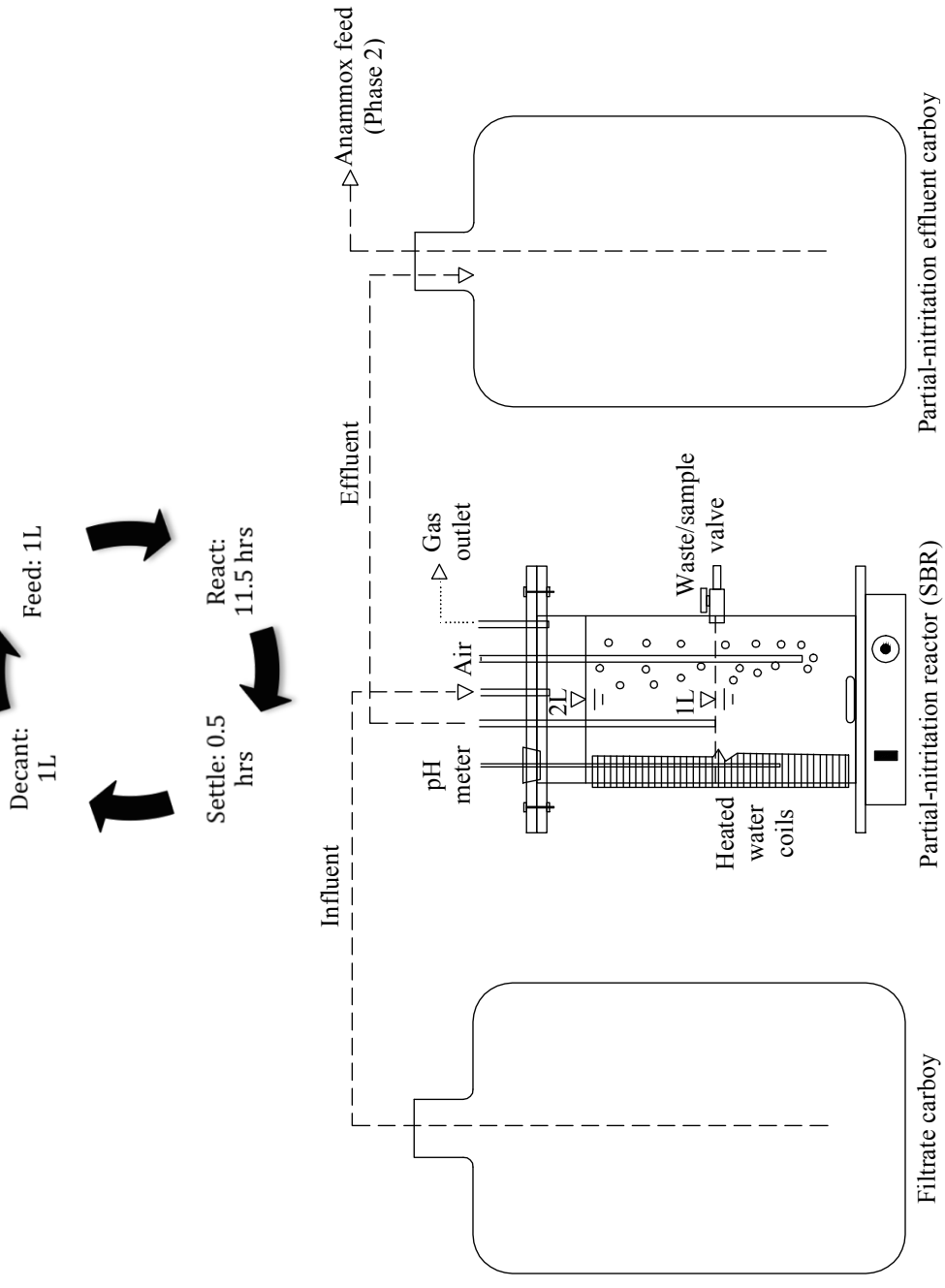

Figure 5: Schematic of partial-nitritation reactor

circulating water bath. The bicarbonate present in the raw filtrate primarily buffered the pH at 8.0±0.5, but a small amount of base and a pH controller (Cole Parmer Instrumentation Company, Vernon Hills, Illinois) was used as contingency. The short HRT, mesophilic temperature, alkaline pH, high-ammonium levels and low DO suppressed nitrite-oxidizing activity and allowed nitrite to accumulate (Vazquez-Padin *et al.*, 2009a). Prior to being used as Anammox feed, effluent from the partial-nitritation reactor was stored for several days in 4° C and nitrogen compound concentrations were measured just in case the partial-nitritation reactor were to over-produce nitrite, which could be inhibitory to the Anammox reactor.

Sample Collection and Analytical Methods

Effluent samples were routinely collected, filtered (0.45 μm) and analyzed at the end of cycles. Anammox feed (partial-nitritation effluent) measurements were conducted in duplicates (one initial and one after any required ratio adjustments) to assure proper feeding ratios and prevent nitrite accumulation in the Anammox reactor. Chemical oxygen demand (COD), ammonia (NH_3-N), nitrate (NO_3^--N), and nitrite (NO_2^--N) were quantified using HACH methods 8000, 10031 (Salicylate method), 10020 (Chromotropic Acid method), and 8153 (Ferrous Sulfate method), respectively.

Mixed liquor samples were collected via valves located midheight on the bioreactors. The valve was opened and flushed briefly to remove any accumulated biomass. The mixed liquor solids concentrations were determined as total suspended solids (TSS) and as volatile suspended solids (VSS), according to Standard Methods (Clesceri *et al.*, 1996).

Phylogenetic Analysis

Phylogenetic analysis was conducted on biomass from both reactors. Cloning and sequencing along with FISH and quantification were conducted on biomass from both reactors while running at steady-state. ARISA was conducted on DNA samples extracted from the Anammox biomass collected at various times during phases 1 and 2.

DNA extraction

DNA was extracted from 1mL mixed liquor collected from the reactors using UltraClean Soil DNA kit (MoBio Laboratories, Solana Beach, CA) according to the manufacturer's protocol. The extraction was verified in 1% (w/v) agarose gel after staining with ethydium bromide.

Anammox cloning and sequencing

PCR-based Anammox-specific 16S rDNA amplification. Using the extracted DNA from biomass in the Anammox reactor, the 16S rDNA region was amplified using universal primers, 8f and 1492r, the product of which was used for nested PCR targeting regions specific for subgroups or genera of Anammox bacteria. PCR reaction volume of 25 µL included 12.5 µL 2X Mastermix (Promega M750B), 0.1 mg $(ml)^{-1}$ BSA, 2.0 µL of DNA template and 1.0 µL of each primer. The final volume (25 µL) was reached by adding nuclease free water. The target genera and specific primer sequences used in amplification are listed in Table 3.

The reaction mixes were placed in a gradient thermal cycler (Eppendorf, Hamburg, Germany) for target region amplification. The thermal cycle program included initial denaturing time of 4 min at 94 °C, followed by 30 cycles of amplification. Each

Table 3: 16S rDNA primer sets used to target specific Anammox genera
[Primer Sequences were obtained from Amano et al.]
(Amano *et al.*, 2007)

Target Genus/Genera	**Primer**	**Sequence (5'-3')**	**Specificity**
Kuenenia and/or Brocadia	AMX 368F	TTCGCAATGCCCGAAAGG	All Anammox genera except *Anammoxoglobus*
	AMX 820R	AAAACCCCTCTACTTAGTGCCC	*Candidatus Kuenenia/Brocadia*
Scalindua	AMX 368F	TTCGCAATGCCCGAAAGG	All Anammox genera except *Anammoxoglobus*
	BS 820R	TAATTCCCTCTACTTAGTGCCC	*Candidatus Scalindua*
All Anammox belonging to Planctomycetes	Pla 46F	GGATTAGGCATGCAAGTC	All Planctomycetes
	AMX 1480R	TACGACTTAGTCCTCCTCAC	All known Anammox

cycle consisted of denaturing at 94 °C for 30 sec, followed by annealing at either 50 or 56 °C (56 °C for *Kuenenia/Brocadia* target genera and 50 °C for others) for 30 sec and finished with elongation for 1 min at 72 °C. A final elongation step of 7 min at 72 °C was used to finish any incomplete elongations. The size of the amplicons was verified on 1% agarose gel running against a 1kb DNA ladder (Fermentas). After gel electrophoresis, the PCR products were purified using QIAQuick PCR purification kit (Qiagen Inc., Valencia, CA).

Generation of Anammox-specific 16S rDNA clone libraries and sequencing. The purified PCR products were ligated to a pCR®4-TOPO® (Invitrogen, CA) vector, and chemically competent *E. coli* cells were then transformed with ligated product following manufacturer's protocol. Plasmid DNA from the clones was extracted using the Zyppy™ Plasmid Miniprep Kit (Zymo Research, CA). To conduct sequencing, 1μL of the plasmid DNA was used as template with universal primers M13F (5'-GTAAAACGACGGCCAG-3') for target genera Kuenenia/Brocadia and Scalindua, and EUB 338F (5'-ACTCCTACGGGAGGCAGC-3') for target genera including all Anammox. Cycle sequencing using ABI 3130 DNA sequencer (Applied Biosystems, Foster City, CA) was performed at the University of Utah Core Facilities.

Partial-nitritation cloning and sequencing

PCR-based amoA gene DNA amplification. From the extracted DNA of the biomass from the partial-nitritation reactor, the amoA rDNA gene was amplified using universal primers, 1F (5'-GGGGTTTCTACTGGTGGT-3') and 2R (5'-CCCCTCKGSAAAGCCTTCTTC-3'). PCR reaction volume of 25 μL included 12.5 μL

2X Mastermix (Promega M750B), 0.1 mg $(ml)^{-1}$ BSA, 2.0 µL of DNA template and 1.0 µL of each primer. The final volume (25 µL) was reached by adding nuclease free water.

The reaction mixes were placed in a gradient thermal cycler (Eppendorf, Hamburg, Germany) for target region amplification. The thermal cycle program included initial denaturing time of 4 min at 94 °C, followed by 30 cycles of amplification. Each cycle consisted of denaturing at 94 °C for 30 sec, followed by annealing at 56°C for 30 sec and finished with elongation for 1 min at 72 °C. A final elongation step of 7 min at 72 °C was used to finish any incomplete elongations. The size of the amplicons was verified on 1% agarose gel running against a 100bp DNA ladder (Fermentas). After gel electrophoresis, the PCR products were purified using QIAQuick PCR purification kit (Qiagen Inc., Valencia, CA).

Generation of amoA gene DNA clone libraries and sequencing. The purified PCR products were ligated to a pCR®4-TOPO® (Invitrogen, CA) vector, and chemically competent *E. coli* cells were then transformed with ligated product following manufacturer's protocol. Plasmid DNA from the clones was extracted using the Zyppy™ Plasmid Miniprep Kit (Zymo Research, CA). To conduct sequencing, 1µL of the plasmid DNA was used as template along with the universal primer 1F (5'-GGGGTTTCTACTGGTGGT-3') for cycle sequencing using ABI 3130 DNA sequencer (Applied Biosystems, Foster City, CA) at the University of Utah Core Facilities.

Sequence data analysis

Sequences obtained from the clone libraries were compared with other identified species/ sequences using NCBI-BLAST 2.2.12 program. Reference sequences were then aligned with and trimmed to an appropriate common length. MEGA software version 4.0

(Tamura *et al.*, 2007) was used to align sequences of the recovered clones with other published sequences and to construct phylogenetic trees using the maximum likelihood method. Bootstrap values were based on 100 trials.

FISH and quantification

Biomass was taken from both reactors and analyzed separately using FISH. The biomass was washed twice in PBS, purged and fixed in 4% (v/v) paraformaldehyde solution for 45 min. Following fixation, the cells were filtered, washed with distilled and deionized water, and placed on a gelatin-coated glass slide. The cells on the slide were then hybridized with 200 mL of (40%) formamide hybridization buffer and 7.5 mL of probe solution (12.5 mM). The probe used to hybridize with biomass from the Anammox reactor was AMX 820 labeled with Cy3, along with 40% hybridization buffer, as found in a study by Schmid and colleagues (Schmid *et al.*, 2005). The probe used to hybridize with biomass from the partial-nitritation reactor was Nm1 labeled with Cy3, along with 35% hybridization buffer. The fixed cells were allowed to hybridize at 46 °C for 12–16 h after which the cells were washed 3-4 times in wash buffer (40% for AMX820 and 35% for Nm1) and then incubated for 20 min at 48 °C. The slide was then removed and washed 7-9 times with 4 °C distilled and deionized (DI) water and allowed to dry at room temperature in the dark.

Following hybridization with the respective probe, the sample was stained with 100 mL of 4', 6'-diamidino-2-phenylindole (DAPI) (5 mg $(ml)^{-1}$) for 5 min in the dark as counterstain to visualize nontarget cells. The slides were again washed with 4 °C DI water and allowed to air dry. The cells were viewed under epifluorescence microscope (Olympus BX51) equipped with a halogen lamp and CCD camera (Olympus DP71).

Communities targeted by the probes were quantified using imageJ. The background interference was removed by adjusting the brightness and contrast. Then, RGB analysis was done on overlaid images (Cy3 and DAPI). Quantification was based on percentage of red and green light over RGB light.

ARISA analysis on Anammox population

Automated Ribosomal Intergenic Spacer Analysis (ARISA) was done using PCR to amplify the 16S to 23S intergenic spacer regions, in the rRNA operons of the Anammox reactor population during phases 1 and 2 (Fisher and Triplett, 1999). The primers used were 1406F (5' -TGYACACACCGCCCGT- 3', labeled with HEX) and 23SR (5' -GGGTTBCCCCATTCRG- 3'). The thermal profile was as follows: denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 sec, and elongation at 72°C for 2 min, with a polishing steps at 72°C for 2 min. Aliquots (2 mL) of 2x diluted PCR products were mixed with 0.5 mL of ROX-labeled GENEFLOt 625 internal length standard (CHIMERx, Milwaukee, WI) and 10mL of formamide. Samples were processed with an ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA) at the University of Utah Core Facilities and analyzed using the GeneScan software (Applied Biosystems, Foster City, CA) version 2.6.

CHAPTER III

RESULTS AND DISCUSSION

Anammox Reactor Performance

Figure 6 illustrates the Anammox reactor performance over a 167-day period (days 40-207). Start-up of the reactor took approximately 40 days (days 1-39) to reach ~80% removal of influent total inorganic nitrogen (TIN - referred to in this and other similar studies as N). The Anammox reactor was fed with diluted filtrate and supplemented with sodium nitrite during days 40-100 (phase 1). During days 100-207 (phase 2), partial-nitritation was used as a preliminary step to Anammox.

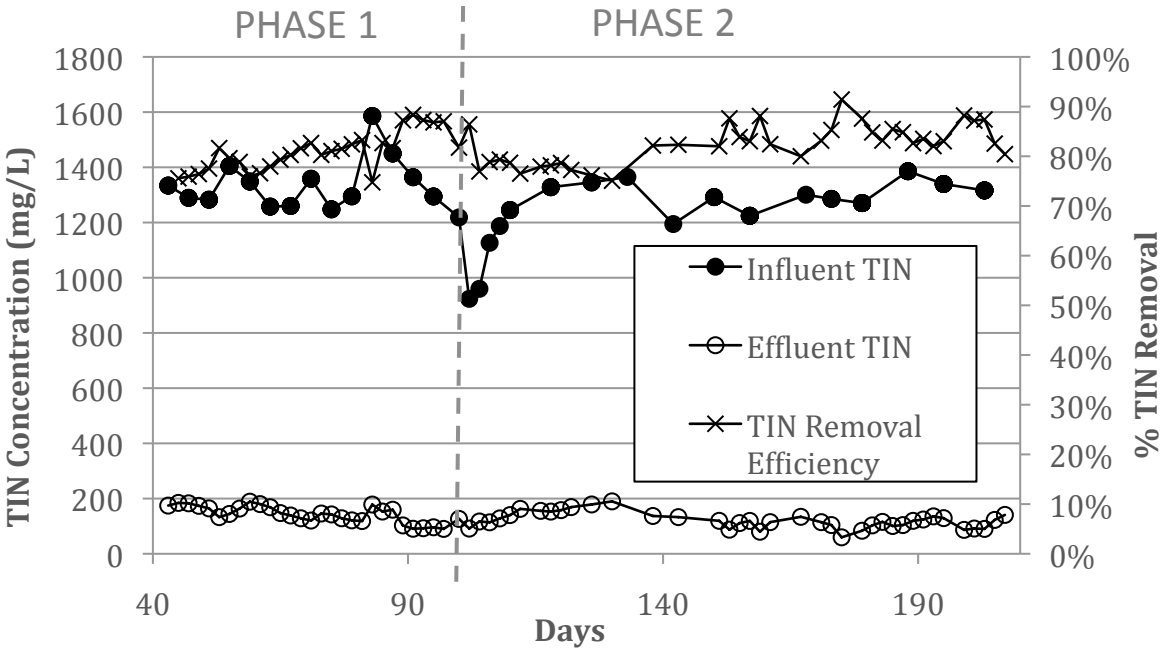

Figure 6: Graph of Anammox reactor performance

Around day 100, the Anammox process became inhibited and nitrite accumulated. The cause is likely due to solvents from the new feed cylinder, which was built and put in place at that time (and only allowed to dry for one night). To mitigate nitrite accumulation, the feed was diluted and loading slowed, as well as 0.1mM of hydrazine added to restore reactor activity (Strous *et al.*, 1999a; Third *et al.*, 2005). The reactor returned to normal activity in approximately 5 days.

The overall nitrogen loading rate to the Anammox was 0.33±0.03 g N $(L\text{-day})^{-1}$ with a maximum of 0.4 g N $(L\text{-day})^{-1}$. Average VSS concentration was 1109±189 mg VSS $(L)^{-1}$ and average overall N removal efficiency was 82±4%. The average specific removal was 0.28±0.05 g N $(g\ VSS\text{-day})^{-1}$ with a maximum of 0.35 g N $(g\ VSS\text{-day})^{-1}$ over phases 1 and 2. Table 4 shows a comparison of the max loading and specific removal rate, as well as average removal efficiency, with other studies using fed-batch Anammox reactors.

The average Anammox influent concentration over the entire 167 days period was 593±55 mg $(L)^{-1}$ NH_4^+-N, 698±57 mg $(L)^{-1}$ NO_2-N, and 8±8 mg $(L)^{-1}$ NO_3-N, with an average nitrite to ammonium ratio of 1.18±0.1 g NO_2-N $(g\ NH_4^+\text{-N})^{-1}$. The average Anammox effluent was 60±23, 5±5, and 66±21 mg $(L)^{-1}$ as NH_4^+-N, NO_2-N, and NO_3-N, respectively. Ammonium was always found in the effluent because nitrite-limitation was used as a process control, to prevent nitrite accumulation in the Anammox reactor. The average removal ratio of nitrite and ammonium was approximately 1.31±0.13, which agrees closely with Strous' empirical equation (Strous *et al.*, 1998).

The ratio of nitrate produced to ammonium consumed was approximately 0.11±0.05, less than the ratio of 0.26 reported by Strous (Strous *et al.*, 1998). This was

Table 4: Loading and specific removal rate comparison with other Anammox studies that used fed-batch reactors

Feed	**Max Loading Rate (g N (L-day)$^{-1}$)**	**Max Specific Removal (g N (g VSS-day)$^{-1}$)**	**Average Removal Efficiency (%)**	**Reactor Temp (C°)**	**Source**
Synthetic	0.3	0.35		30	Arrojo et al. 2008
Synthetic	0.3	0.4		15-30	Dosta et al. 2008
PN effluent	0.28	0.28	69-80	20-30	Vazquez-Padin et al. 2009a
PN effluent	0.4	0.35	82	32	This Study
PN effluent	0.53		87	36	Ruscalleda et al. 2008
PN effluent	0.7	0.45	68	35	Dapena-Mora et al. 2006

expected as a result of running at a long SRT (which should produce organic acids via cell decay). The nitrate and organic acids being used in denitrification and/or DNRA, are evidenced by consistent mild reductions of COD seen in periodic COD measurements. This is also supported by many studies showing Anammox to coexist with denitrifiers (Dalsgaard *et al.*, 2003; Dalsgaard *et al.*, 2005; Kuypers *et al.*, 2003; Risgaard-Petersen *et al.*, 2003; Rysgaard *et al.*, 2004; Thamdrup and Dalsgaard, 2002; van de Graaf *et al.*, 1997), as well as studies confirming some Anammox strains carrying out nitrate reduction (DNRA), and organic acid oxidation, to produce nitrite, ammonium and carbon dioxide (substrates of the Anammox process) (Guven *et al.*, 2005; Kartal *et al.*, 2007a).

Partial-nitritation Reactor Performance

During the approximately 45-day start-up period (100-145) of the partial-nitritation reactor, process controls were optimized, including aeration rate and HRT. Also, some sodium nitrite was added to the effluent until the partial-nitritation reactor reached steady-state production of a ratio of approximately 1.2:1 nitrite-N to ammonium-N, needed for Anammox feed. The average filtrate concentration during the start-up period was 1230±61 mg $(L)^{-1}$ NH_4^+-N and average VSS in the PN reactor was 1171±156 mg $(L)^{-1}$. The average effluent concentrations during start-up were 331±165 mg $(L)^{-1}$ NO_2-N, 861±159 mg $(L)^{-1}$ NH_4^+-N, and 38±15 mg $(L)^{-1}$ NO_3-N.

During days 146 – 207, when the PN reactor ran at steady-state, the average filtrate concentration was 1334±69 mg $(L)^{-1}$ NH_4^+-N and the average VSS was 2070±259 mg $(L)^{-1}$. Effluent concentrations at steady-state were 704±50 mg $(L)^{-1}$ NO_2-N, 607±76 mg $(L)^{-1}$ NH_4^+-N, and 23±11 mg $(L)^{-1}$ NO_3-N with an average nitrite to ammonium ratio

of approximately 1.18:1 g NO_2-N (g NH_4^+-N)$^{-1}$, which is suitable for Anammox feed and promotes nitrite-limiting conditions, preventing nitrite accumulation in the Anammox.

Microbial Ecology of Anammox Bacteria

Cloning and sequence analysis results were obtained and compared with FISH results to qualitatively and quantitatively analyze the Anammox biomass community in the Anammox reactor.

Phylogenetic classification based on Anammox-specific 16S rDNA regions

Figure 7 represents the overall phylogeny of Anammox in the reactor. Based on a ≥ 99% homology, approximately 53% of the clones obtained using primer pair Pla 46F and AMX 1480R (specific to all known Anammox bacteria) were homologous to *Kuenenia stuttgartiensis* (CT573071). All the clones obtained using primer pair AMX 368F and AMX 820R (specific to *Candidatus Kuenenia/Brocadia*) were homologous to *Candidatus Kuenenia*, suggesting the likelihood of a complete absence of *Brocadia*. Although specific primers were used to target genera *Scalindua*, they were not found to be present in the clone library. Likewise, the genera *Candidatus Jettenia* and *Candidatus Anammoxoglobus* were not found.

The apparent enrichment of *K. stuttgartiensis* in the Anammox population can be attributed to many months of steady-state performance. It is also supported by many other sources that found a dominance of *Candidatus Kuenenia* in enriched Anammox communities within lab-scale and full-scale bioreactors (Dosta *et al.*, 2008; Hwang *et al.*, 2005; Schmid *et al.*, 2005; Strous *et al.*, 2006).

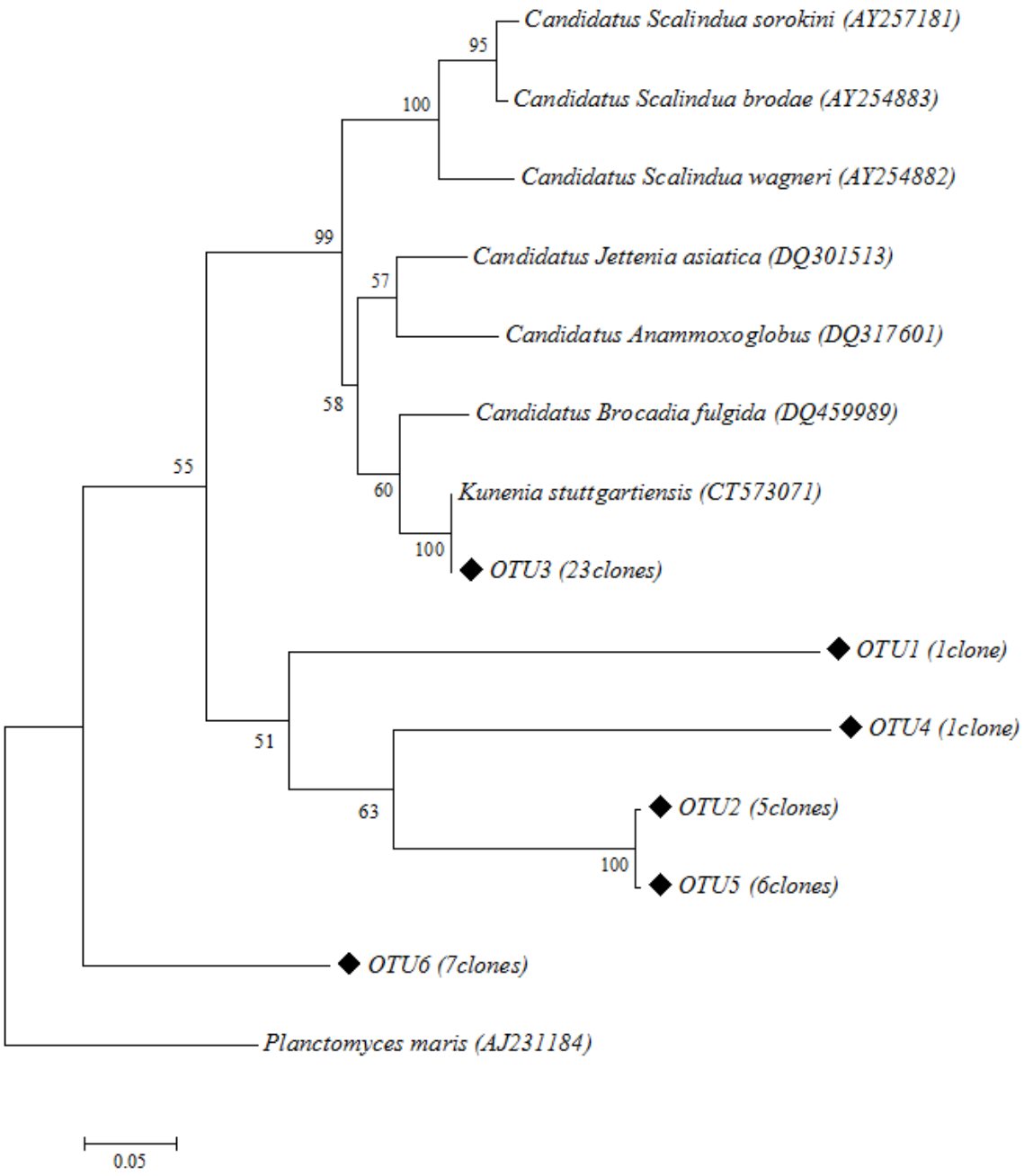

Figure 7: Phylogenetic tree of Anammox community

Table 5 shows a comparison among other Anammox studies using fed-batch reactors. The comparisons are grouped according to the reported genus or genera enriched. Studies with positive hybridization of probes specific to certain strains or positive sequence homology to a certain strain were designated as containing that strain, while those that only report positive hybridization with general probes (i.e. AMX 820), with no other analyses conducted, were designated as containing the respective genus or genera (i.e. *Candidatus Kuenenia* and/or *Brocadia*).

The strain *K. stuttgartiensis* is reported to be capable of DNRA and organic oxidation (Kartal *et al.*, 2007a) which coincides with the Anammox reactor performance, finding that nitrate in the effluent was much less than the stoichiometric ratio reported by Strous (Strous *et al.*, 1998). Since the loading rate throughout the entire study was modest, and the very long SRT would result in some cell decay (organic addition), the dominance of *K. stuttgartiensis* seems to be the result of a selective advantage over other Anammox communities in the reactor by its diverse metabolic capabilities; it has an alternative metabolic pathway, DNRA, which can be used to reduce nitrate (produced by the Anammox process) to nitrite and/or ammonium via organic oxidation, producing more substrates necessary for the Anammox process (Kartal *et al.*, 2007a).

Besides *Candidatus Kuenenia*, five other operational taxonomic units (OTUs) homologous to uncultured and/or unclassified Planctomycetes or Anammox bacteria were found within the clone library. It is interesting to note that, even though these OTUs do not match with published Anammox strains, there is significant enrichment even among them, evidenced by the few number of OTUs and the fact that most of these OTUs have multiple clones (see Figure 7).

Table 5: Comparison of dominant strains in various Anammox studies that used fed-batch reactors

Configuration	Feed Type	Dominant Genus/Species	Source
Anammox	Synthetic	*K. stuttgartiensis*	Arrojo et al. 2008
Anammox	Synthetic	*K. stuttgartiensis*	Dosta et al. 2008
PN-Anammox	PN effluent	*K. stuttgartiensis*	This Study
PN-Anammox	PN effluent	*Candidatus Kuenenia and/or Brocadia*	Dapena-Mora et al. 2006
CANON	Filtrate	*Candidatus Kuenenia and/or Brocadia*	Vazquez-Padin et al. 2009a
Anammox	Synthetic	*Candidatus Kuenenia and/or Brocadia*	Jin et al. 2008a
CANON	Synthetic	*B. Anammoxidans*	Third et al. 2005
Anammox	Synthetic	*B. Anammoxidans*	Lopez et al. 2008
Anammox	Synthetic + propionate	*Candidatus Anammoxoglobus propionicus*	Kartal et al. 2007a

Anammox bacteria are a relatively recent discovery and new species/genus level diversity is still being discovered (Quan *et al.*, 2008; Schmid *et al.*, 2005); so it is very possible that the other unidentified OTUs could also be somewhat-enriched, novel strains carrying out Anammox, especially since cloning and sequencing was done with Anammox specific primers. However, further studies involving quantification and targeting genes specific to the Anammox process would be required to positively affirm the presence of novel group(s) of Anammox in the bioreactor.

FISH analysis and quantification

Figure 8 shows overlaid micrographs obtained from FISH performed on mixed liquor samples of the Anammox reactor. The results show a definite presence and likely dominance of Anammox bacteria in the reactor biomass. The probe used, AMX 820 (Cy3), was specific to *Candidatus Kuenenia* and/or *Candidatus Brocadia*. Based on the sequence analysis, the stained biomass (Cy3) is probably almost entirely *K. stuttgartiensis*. Quantification of that community showed their dominance within Anammox reactor to be approximately 65% of the biomass. Due to the specificity of the probe, and the observation of other (likely-Anammox) OTUs, it is likely that other Anammox strains are also present, but not shown (Cy3 labeled). It is also likely that much of the bacteria stained blue (DAPI) are denitrifiers, since occasional COD measurements in the feed and effluent consistently showed mild COD decreases in the Anammox reactor, and since Anammox bacteria have been consistently shown to coexist with denitrifiers (Ruscalleda *et al.*, 2008).

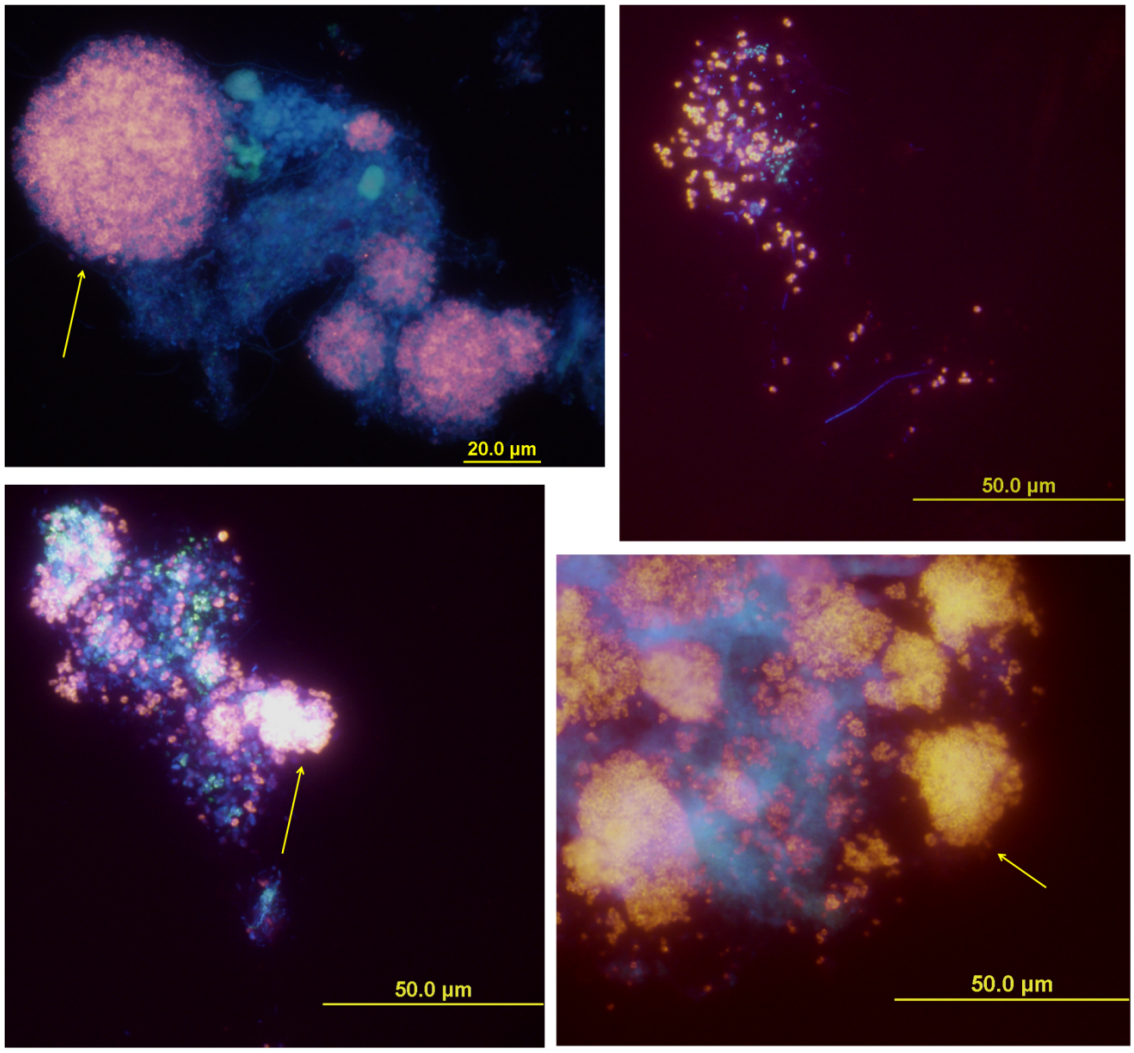

Figure 8: Micrographs obtained from FISH analysis of biomass
from Anammox reactor using AMX820 probe

Microbial Ecology of Ammonia-oxidizing Bacteria

Cloning and sequence analysis results were obtained and compared with FISH results to qualitatively and quantitatively analyze the ammonia-oxidizing bacteria (AOB) community in the partial-nitritation reactor.

Phylogenetic classification based on amoA gene DNA regions

Figure 9 shows the sequence analysis results for the *amoA*-based clone library (AOBs in the PN reactor). Although none of the clones matched exactly with published sequences, all OTUs did fall under *N. europaea* lineage and none under *N. oligotropha* lineage. This agrees with many other studies that used partial-nitritation reactors, which report a large degree of dominance of the *N. europaea* lineage due to oxygen-limiting and concentrated ammonium conditions, characteristic of partial-nitritation processes (Nielsen *et al.*, 2005; Otawa *et al.*, 2006; Park *et al.*, 2010; Pynaert *et al.*, 2003; Quan *et al.*, 2008).

FISH analysis and quantification

Figure 10 shows overlaid micrographs obtained from FISH performed on mixed liquor samples of the partial-nitritation reactor. The results show a definite presence and likely dominance of AOB bacteria in the reactor biomass. Based on the sequence analysis, the AOB community is probably almost entirely of the *N. europaea* lineage. Quantification of the hybridized AOB community suggests its dominance within the partial-nitritation reactor to be approximately 62% of the community. However, the probe used for FISH (Nm1) hybridizes to a conserved region for *Nitrosomonas halophila* (Park *et al.*, 2010) and potentially other related strains. So, it is very likely that AOB

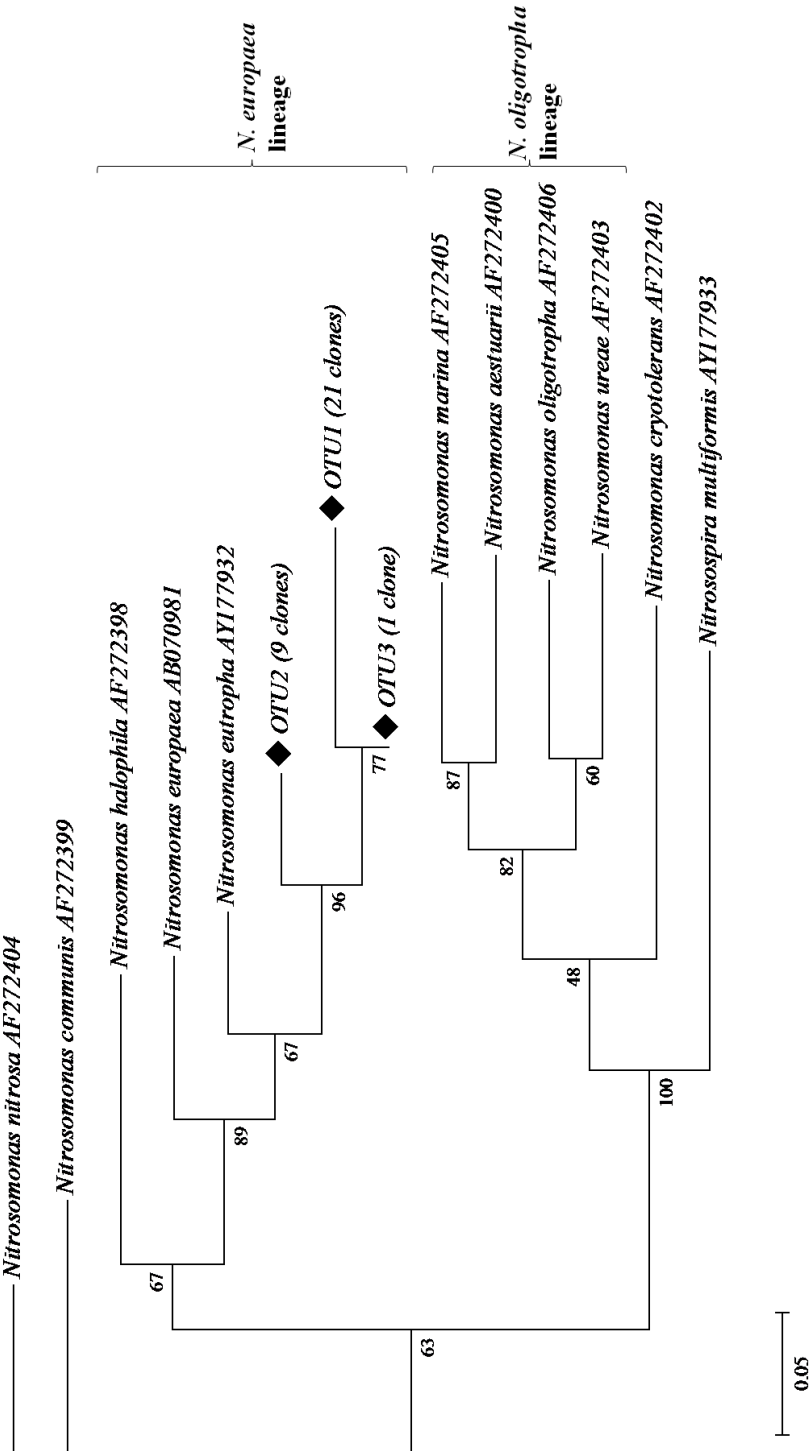

Figure 9: Phylogenetic tree of AOB community from partial-nitritation reactor

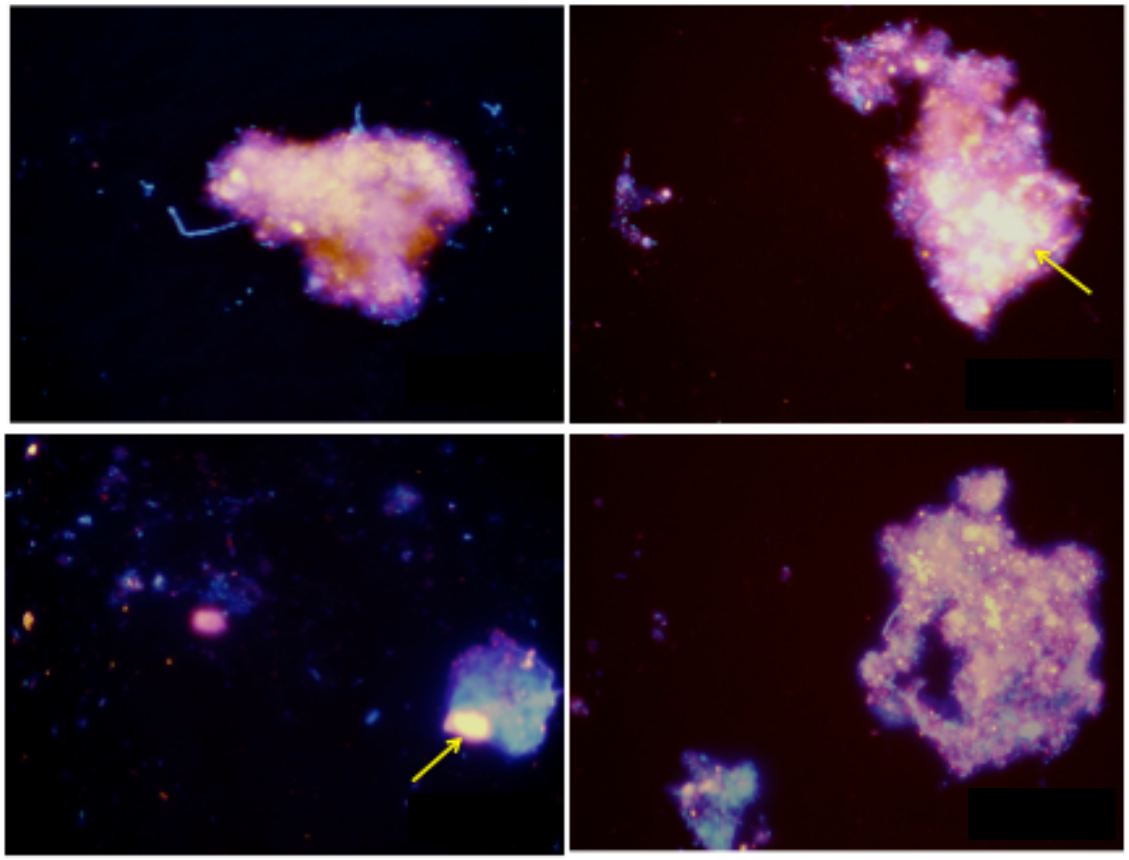

Figure 10: Micrographs obtained from FISH analysis of biomass from the partial-nitritation reactor using Nm1 probe

dominance in the overall community is much greater, since many other strains within the *N. europaea* lineage exist besides *N. halophila* and sequencing results showed OTUs outside of the *N. halophila* strain. It is also likely that some of the bacteria stained blue (DAPI) are heterotrophic in nature due to the semi-aerobic environment and consistent presence of COD in the filtrate.

ARISA Analysis of Anammox Population

ARISA results for days 95-207, with snapshots at days 95, 146 and 207, respectively, are represented in Figure 11. The continual enrichment of the dominant culture can be seen by the gradual reduction of competing populations (smaller peaks). The first ARISA (day 95) was just prior to the start of partial-nitritation reactor.

Although the ARISA results are not qualitative, they do show the dominance of a particular group within the reactor. Using NCBI to isolate of the intergenic spacer region of the published partial-genome for *Kuenenia stuttgartiensis*, correlating with the primer set used, yields a length of approximately 800 base pairs, correlating with the dominant peak in the ARISA profiles and supporting the findings from the Sequences analysis and FISH results.

The dominance of *K. stuttgartiens*is is over the entire period despite the shift from diluted filtrate with sodium nitrite to concentrated filtrate pretreated with partial-nitritation. Thus, the shift from phase 1 to phase 2 (around day 100) does not seem to have had any effect on the community other than sustaining the enrichment of the already dominant group. This may be a result of the filtrate being from the same source during both phases, such that most of macro- and micronutrients would have been similar in the feed, even though the concentrations were different due to dilution (phase 1) and nitrite

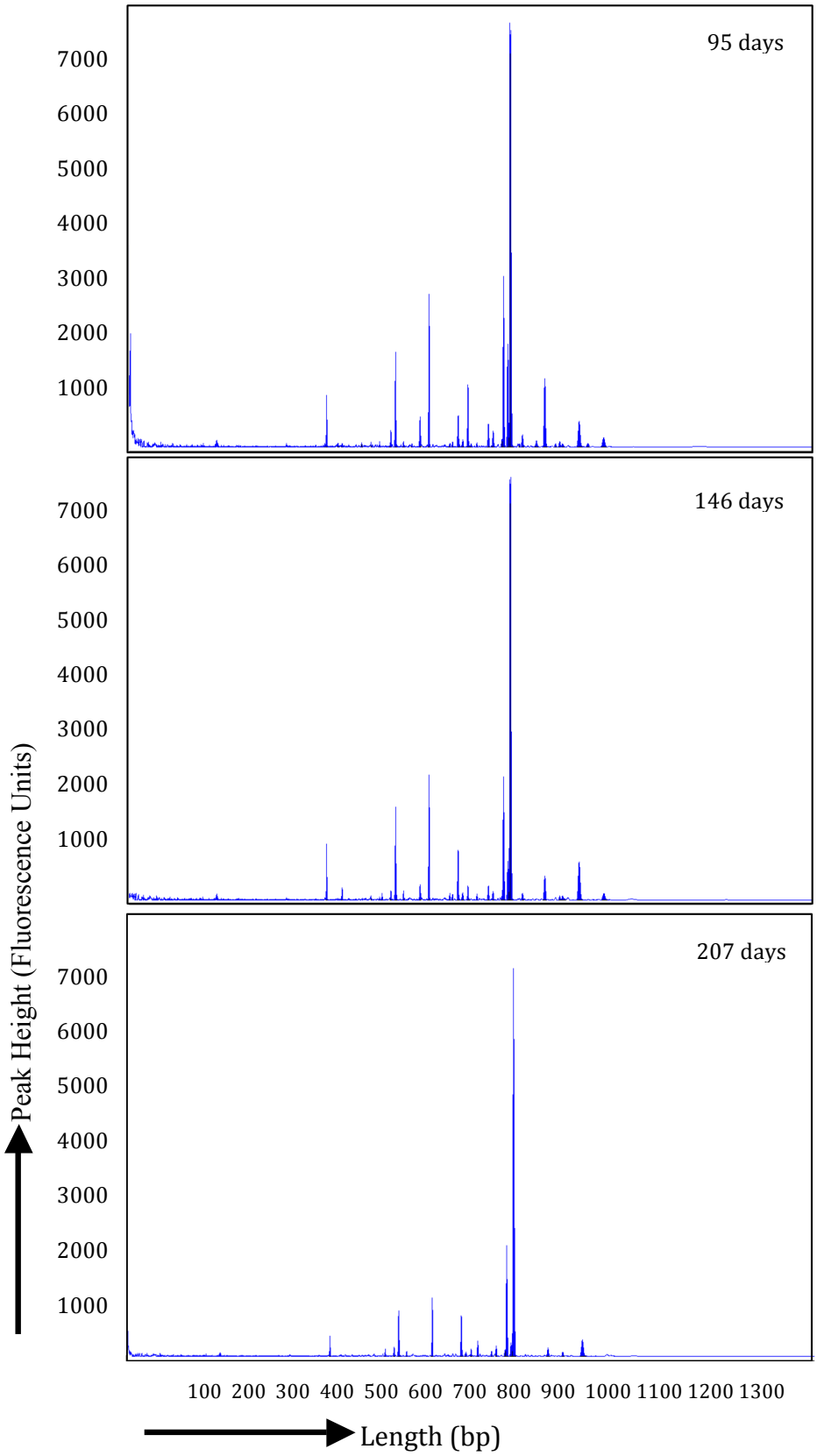

Figure 11. ARISA profile of Anammox community over study period

was supplied by two different means during each phase. Further investigation would add an interesting perspective to the finding that community enrichment is dependant on feed composition and concentration (Park *et al.*, 2010). It may also be that the selective advantage of *K. stuttgartiensis* throughout the entire study, as described previously, played a greater role in supporting its enrichment than the filtrate composition. However, further analysis is needed to make any direct statements as to which factors controlled.

CHAPTER IV

CONCLUSIONS

Conclusions from Study

This study simulated a side-stream PN-Anammox process to treat filtrate at a very long SRT. Phylogenetic results were used to analyze the enriched communities, the degree of enrichment and whether shifting the feed caused a shift in the Anammox population. According to the results found, the following conclusions can be made:

- The suspended-growth fed-batch Anammox reactor, fed with partial-nitritation effluent, kept at a moderate loading rate comparable with other fed-batch reactor studies and a very long SRT (200 days), reached steady-state performance and achieved an average N removal of 82% (≥ 80%), a maximum specific removal rate comparable with other similar studies, and little sludge production.
- Maintaining a very long SRT and moderate loading rate resulted in selective enrichment of an Anammox community that is capable of DNRA (*K. stuttgartiensis*), which has an advantage in substrate-limiting conditions, due to its ability to utilize available nitrate and organics (from cell decay) to produce substrates for its primary metabolism (Anammox).
- Shifting the Anammox feed from diluted filtrate and sodium-nitrite to undiluted filtrate with nitrite provided strictly via partial-nitritation caused no noticeable

shift in the Anammox population, only continued enrichment (dominance of *K. stuttgartiensis* and reduction of competing communities).

- The partial-nitritation (PN) reactor community became enriched with AOBs within the *N. Europaea* lineage, which according to other literature is due to oxygen limitation and high ammonium concentrations in partial-nitritation reactors treating digester filtrate or a similarly ammonium-rich wastewater.

The exact reason(s) for the lack of shift in the Anammox population seen throughout the study, despite change in the feed characteristics, would require further analysis, including repeating the shift under higher loading or shorter SRT (non-substrate-limiting) conditions. Likewise, further studies are needed to positively confirm novelty of the unidentified Anammox OTUs, and to clarify their role in the Anammox community.

Applying the long SRT to full-scale treatment would likely be successful, especially since full-scale applications typically run at higher loading rates. The potential substrate-limiting conditions and endogenous decay that may result from the long SRT could facilitate the consumption of available nitrate by Anammox capable of DNRA. These could compete with denitrifiers for nitrate and organics and result in less biomass production while still achieving efficient nitrogen removal.

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