SUSPENDED GROWTH AND ATTACHED GROWTH ANAMMOX FOR NITROGEN REMOVAL FROM DIFFERENT WASTE STREAMS- PROCESS STARTUP AND PERFORMANCE

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

The partial nitritation/anammox (PN/A) process, although found to be an energy and cost-effective process, is not well understood yet. This study was carried out to provide a better understanding of PN/A reactors with suspended and attached growth configurations for treating different waste streams that have potential stress factors. Two PN/A reactors with different configurations were successfully initiated to investigate the difference of suspended growth reactor (SR) and attached growth reactor (AR) in nitrogen removal and the overall microbial composition. During the 300 days of operation, both reactors showed a similar nitrogen removal rate at 35 ℃ and 21 ℃, and harbored similar communities dominated mainly by three phyla: *Chloroflexi*, *Planctomycetes*, and *Proteobacteria*. To further study the external stress effect on the PN/A performance, the suspended growth reactor was kept at 35 ℃ to 21 ℃ and finally at 13 ℃.. It was confirmed that lower temperature or sulfide content as low as 5 $mg_S\ L^{-1}$ could eliminate both *Nitrosomonas europaea* related ammonium oxidizing bacteria (AOB) and Ca. Brocadia sp. affiliated anammox bacteria (AMX). The activity of AOB was inversely correlated with *amoA* gene expressions. Just the opposite was found with the *hzsA* gene expression since it correlated well with the activity of AMX.

Additionally, anammox process was applied to treat poststruvite precipitated urine in two-stage and single-stage systems. It was found that coupling the struvite precipitation and PN/A process, 99% recovery of phosphorus and up to 80% removal of

nitrogen could be achieved. Compared to the two-stage system, the single-stage reactor had a lower nitrogen removal rate. Also, a pilot-scale PN/A reactor was designed and fabricated to treat reject water in a 300 gal sequencing batch reactor at room temperature. The reactor was successfully started and was able to remove 0.164 ± 0.086 $kg_N kg_{VSS}^{-1} d^{-1}$, indicating a relatively high bacterial activity at room temperature.

In conclusion, this study evaluated the feasibility and sustainability of the PN/A system in treating different waste streams containing high ammonium. It provided a better understanding of startup and operation strategies for the full-scale installations of anammox in wastewater treatment plants.

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CHAPTER 1

INTRODUCTION

The nitrogen cycle in the ecosystem

Nitrogen is crucial for any life on earth as it is a component in all amino acids, incorporated into proteins, and it is present in the bases that make upnucleic acids, such as DNA and RNA (Cornell et al., 1995; Tamm, 1991). Basically, nitrogen can be converted between its various forms (e.g., NH_4^+, N_2, N_2O, NO, NO_2^-, NO_3^-) through chemical, physical, and biological processes. Figure 1.1 illustrates the fate of the main nitrogenous compounds in natural systems.

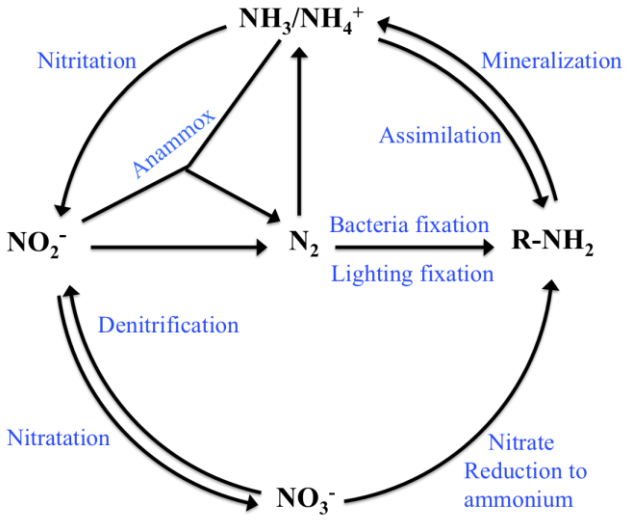

Figure 1.1 Nitrogen cycle in ecosystem.

The overall nitrogen is balanced by physical and biochemical reactions. However, over the last few decades, with the increase in global population and rapid industrialization, humans have played a significantly increased role in transforming the global nitrogen cycle. They have caused a cascade of environmental problems, including increased fresh water nitrate levels as well as increased nitrous oxide production (Galloway et al., 2008; Camargo et al., 2005; Mosier, et al., 1998; Ravishankara et al., 2009). Fortunately, a significant amount of human-involved reactive nitrogen ends up as nonpoint source pollution converted to nitrogen gas. Additionally, another portion of the nitrogen, found in point source pollution, is removed in municipal wastewater treatment facilities.

Nitrogen in wastewater treatment plants

Generally, municipal wastewater treatment plants (WWTPs) receive nitrogen at 30-200 $mg_N\ L^{-1}$ (Czerwionka et al., 2012; Rosenberger et al., 2002), of which ~80% comes from human urine (Wilsenach et al., 2003). The nitrogen in the influent exists primary as organic-N, NH_4^+-N, and NO_3^--N. During preliminary and primary treatment, most of the particular nitrogen is settled with the sludge. A series of nitrogen-related reactions happen in the biological process of removing nitrogen from wastewater, including: (a) hydrolysis, organic nitrogen is converted to ammonium; (b) nitrification, ammonium is oxidized to nitrate by nitrifers; (c) assimilation, ammonia is consumed by heterotrophs for the cell growth; and (d) denitrification, nitrate is reduced to nitrogen gas by denitrifying bacteria if there is an anoxic zone. As a result, most nitrogen is present either in sludge as the cell component or in the secondary effluent as nitrate.

The excess sludge produced is usually anaerobically digested to reduce its volume. In this process, the organic nitrogen is converted to ammonium. The digested biomass is then taken to a belt press to further concentrate the biomass for further handling. The black liquid, which comes out of the belt press, is commonly referred as centrate, filtrate, or reject water. Together with the supernatant from the anaerobic digester, this black liquor is very rich in ammonia which can range anywhere from 500 to 1500 $mg_N L^{-1}$ (Hellinga et al., 1998). The actual amount depends upon the handling practices at the treatment plant. To remove the ammonium, the reject water is traditionally recycled back to the head of the plant, as shown in Figure 1.2. This combined flow, although it is a small flow (<1% of total volumetric loading), can be responsible for up to 30% of the total ammonia nitrogen load to the treatment plant (Lackner et al., 2008).

Due to the potential of increased eutrophication and toxicity to water life by nitrogen species, the growing public concern for environmental protection has increased in the last few decades. The concern has generated much stricter discharge standards, with more efforts to achieve better effluent quality, especially for nitrogen.

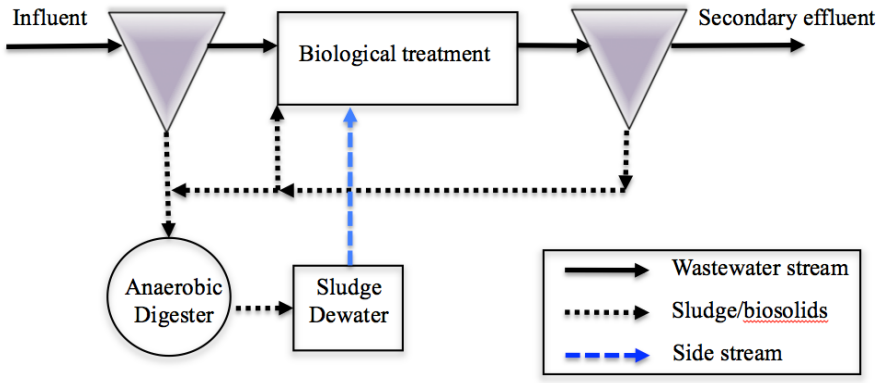

Figure 1.2 The fate of nitrogen in traditional municipal wastewater treatment plants with anaerobic digester.

Hence, each mg L^{-1} of total nitrogen (TN) present in the influent to treatment plants has operational and cost implications for wastewater treatment plant operators. As a result, the research towards the sidestream N removal is more frequently accepted as it can improve the quality of discharge and reduce the operational costs without the need for expansion of current construction volume (van Loosdrecht and Salem, 2006).

Biological nitrogen removal (BNR) methods

Nitrification and denitrification

Conventionally, ammonium in wastewater is removed by sequential nitrification and denitrification, which was first applied in the 1960s (Johnson and Schroepfer, 1964; Wiesmann, 1994). During the nitrification process, ammonium is oxidized to nitrite by ammonium-oxidizing bacteria (AOB) (Equation (1)), and nitrite is further oxidized to nitrate by nitrite-oxidizing bacteria (NOB) (Equation (2)). Nitrification is pH-sensitive with the optimal pH range of 7.5-8, and rates decline significantly at pH values below 6.8 (Feng et al., 2010; Park et al., 2003).

$$55NH_4^+ + 76O_2 + 109HCO_3^- \rightarrow C_5H_7NO_2 + 54NO_2^- + 591H_2O + 104H_2CO_3 \quad (1)$$

$$400NO_2^- + NH_4^+ + 195O_2 + HCO_3^- + 4H_2CO_3 \rightarrow C_5H_7NO_2 + 400NO_3^- + 3H_2O \quad (2)$$

Where $C_5H_7NO_2$ refers to the cells of AOB and NOB reproduced. In the next step, known asdenitrification process, the NO_3^- is reduced to N_2 gas by denitrifiers under anoxic conditions with a suitable electron donor. Although autotrophic and heterotrophic denitrifiers have been discovered, the heterotrophic denitrification is widely used in WWTPs for nitrogen removal. The equation is shown below (Ahn, 2006):

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7O_2N + 0.47N_2 + 1.68H_2O + HCO_3^- \quad (3)$$

Combining nitrification and denitrification with methanol as the carbon source for denitrifying bacteria, produces the following reaction (Mateju et al., 1992):

$$NH_4^+ + 1.856O_2 + 1.058CH_3OH + 0.103HCO_3^- \rightarrow 0.457N_2 + 0.907H^+ + 0.744CO_2 + 2.452H_2O + 0.421CH_{1.4}O_{0.4}N_{0.2} \quad (4)$$

As the most traditional BNR, nitrification and denitrification processes have been widely applied in engineered wastewater treatment systems for targeted water quality improvement (Grady et al., 2011; Lu et al., 2014). However, the high energy demand and the potential of nitrous oxide (N_2O) emission have forced engineers searching for a more energy-effective and environmentally friendly BNR (Anderson et al., 1986; Wrage et al., 2001; Zumft, 1997).

Nitritation and denitritation

In recent years, a short cut for nitrogen removal based on traditional nitrification and denitrification has been developed: nitritation and denitritation (Hellinga et al., 1998; Surmacz-Górska et al., 1997). Denitritation refers to the process that nitrite, instead of nitrate, is used as the electron acceptor by denitrifiers, shown in Equation (5) (Ahn, 2006).

$$NO_2^- + 0.53CH_3OH + 0.67H_2CO_3 \rightarrow 0.04C_5H_7O_2N + 0.48N_2 + 1.23H_2O + HCO_3^- \quad (5)$$

Compared to conventional nitrification and denitrification processes, the sequential nitritation and denitritation requires 25% less oxygen, 40% less organic carbon and produces less surplus sludge (Surmacz-Górska et al., 1997). This process is usually

used for nitrogen removal from high ammonium content wastewater (NH_4^+-N =100 ~ 5000 $mg_N L^{-1}$), such as reject water, piggery manure, landfill leachate, and industrial wastewater (Daverey et al., 2013; Kotay et al., 2012; Liang et al., 2008; Magrí et al., 2013; van Hulle et al., 2010).

In this process, to avoid nitrite being further oxidized to nitrate, NOB activity is suppressed in several ways: (a) the combination of moderately high temperature (>26 ℃) and short solids retention time (SRT) to promote the growth of AOB and the wash-out of NOB (Allferi et al., 2002; Hellinga et al., 1998; Yang et al., 2007); (b) maintain a low DO concentration below 0.4 mg/L or 5% of air saturation as NOB have lower affinity for oxygen than AOB (Ciudad et al., 2006; Münch et al., 1996); (c) raise NH_4^+ or NO_2^- concentration as they can inhibit the growth of NOB (Schmidt et al., 2003).

Partial nitritation-anammox

The advent of anaerobic ammonium oxidation (anammox) in the 1990s has opened up a better understanding of the global nitrogen cycle and provided a new pathway for nitrogen removal for wastewater practitioners. It is hypothesized that up to 50% of the atmospheric nitrogen resulted from the anammox activity (Dalsgaard et al., 2005; Kuypers et al, 2003; Schmid et al., 2007; Strous and Jetten, 2004).

The anammox process is mediated by anammox bacteria (AMX), which can directly use NH_4^+as electron donor and NO_2^-mainly as an electron acceptor with inorganic carbon as the carbon source under anaerobic conditions. The stoichiometric reaction was developed by Strous et al. (1999) and shown in Equation (6).

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15} \quad (6)$$

Recently, Lotti et al. (2014) modified the equation for a more detailed stoichiometry with more stable conditions, asdescribed in Equation (7)

$$NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0.986N_2 + 0.161NO_3^- + 0.071CH_{1.74}O_{0.31}N_{0.20} + 2.002H_2O \quad (7)$$

To combine the partial nitritation and anammox processes into one process, it would be shown as below:

$$NH_4^+ + 0.792O_2 + 0.080HCO_3^- \rightarrow 0.435N_2 + 0.111NO_3^- + 1.46H_2O + 1.029H^+ + 0.052CH_{1.4}O_{0.4}N_{0.2} + 0.028CH_2O_{0.5}N_{0.15} \quad (8)$$

Compared to conventional nitrification and denitrification processes, the combined partial nitritation and anammox process has an excellent capacity to remove nitrogen without the addition of costly organic carbon source. The process requires considerably less oxygen, resulting in significant cost savings (Jetten et al., 2001). It is reported that the wastewater treatment plant in Strass (Austria) was able to decrease the energy consumption from 2.66 to 1.50 KWh per kg N removed by using the partial nitritation/anammox process (Schaubroeck et al., 2015). The theoretical energy demand and sludge production for the three BNR are listed in Table 1.1.

Table 1.1 Comparison of the three BNR methods (adapted from Ma et al., 2016).

Energy	**Unit**	**Nitrification+ denitrification**	**Nitritation+ denitritation**	**PN/A**
O_2 demand	kg O_2/kg NH_4-N	4.57	3.43	1.95
Alkalinity demand	kg $CaCO_3$/kg NH_4-N	7.07	N/A	4.6
BOD demand	kg BOD/kg NH_4-N	3.71	2.30	0
Biomass yield	kg DS/kg NH_4-N	0.8	0.5	0.1

Configuration of anammox-based system

Based on the number of bioreactors for the anammox-based system, the installation could be divided into two-stage and single-stage (see Figure 1.3). In a two-stage configuration, sequential partial nitritation and the anammox take place in separate bioreactors (Figure 1.3(a)) whereas in a single stage process, these two processes occur in the same reactor (Figure 1.3(b)). The two-stage process includes oxidizing about 55% of the ammonium to nitrite and inhibiting the activity of NOB in an aerobic reactor and then converting ammonium and nitrite to nitrogen gas in another anaerobic reactor. Using two separate reactors allows less risk of anammox inhibition by toxic compounds in the influent, especially for the case of industrial high ammonium waste streams (Vazquez-Padin et al., 2009).

In the early stage, most of the anammox implementations were applied in two-stage (Lackner et al., 2014). With the knowledge and experience gained in the past few years, focus has shifted mainly to the single-stage system. In the single-stage process, partial nitritation and anammox happens in one reactor, usually given the name as "PN/A" (Partial Nitritation/Anammox), "deammonification", "CANON" (Completely Autotrophic Nitrogen removal Over Nitrite) or "SNAP" (Single-stage Nitrogen removal using Anammox and Partial nitritation).

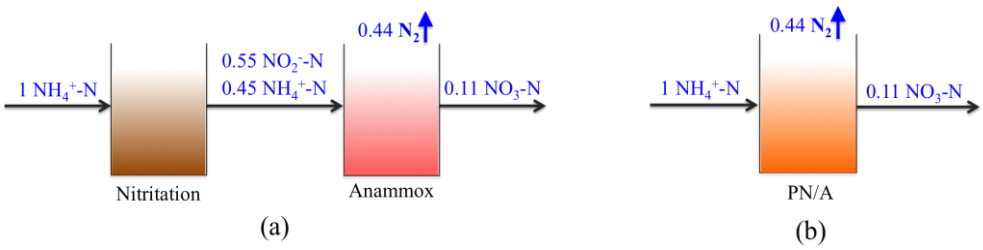

Figure 1.3 Schematic of (a) two-stage and (b) single-stage anammox system.

The single stage system has a small footprint and requires less capital investment cost. However, challenges associated with controlling ammonia oxidation (e.g., minimizing nitrite accumulation), separating anammox biomass from the nitrifying biomass during biomass wastage and in general the overall system stability still exist.

Based on the characteristics of the biomass growth, the PN/A application can also be divided into suspended growth (Figure 1.4(a)), attached growth (Figure 1.4(b) and (c)) and granular system (Figure 1.4(d)).

In the suspended growth system, AMX growsmainly as granules and AOB grows mainly as flocs, and live in symbiosis with each other (Jetten et al., 2001). This configuration is usually operated in a sequencing batch reactor (SBR) mode, and used often in current full-scale implementations (Lackner et al., 2014). As one of the suspended growth PN/A, the DEMON® configuration was first installed in Strass, Austria in 2004, to remove ammonium from sidestream reject water. The first PN/A installation in the United States was also DEMON® in Hampton Roads Sanitation District' (HRSD), York River, VA. So far, DEMON® has been the most popular PN/A configuration around the world.

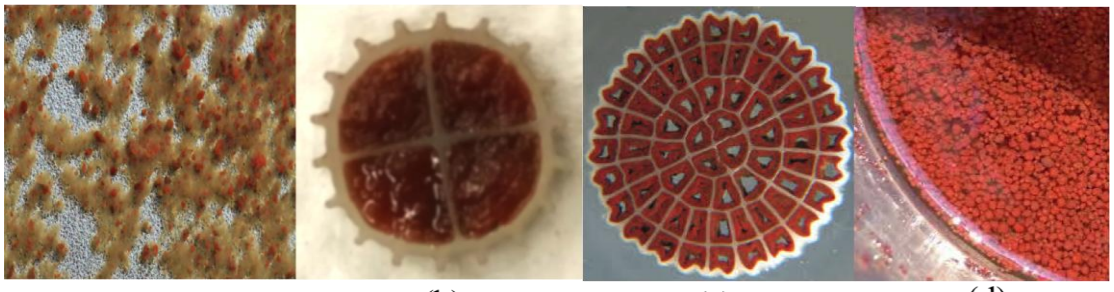

Figure 1.4 The different biomass growth configurations for PN/A reactors: (a) suspended growth; (b) attached growth with K1 media; (c) attached growth with K5 media; and (d) granular biomass.

The attached growth system refers to biomass on the surface of carrier material; such as polyurethane, polypropylene, or membrane surfaces (Abma et al., 2007). The typical attached growth for the PN/A process is the moving bed biofilm reactor (MBBR) with Kaldnes^{TM} carriers. The specific surface area is 500 m^2/m^3 for Kaldnes^{TM} K1 media (see Figure 1.4(b)), 800 m^2/m^3 for Kaldnes^{TM} K5 media (see Figure 1.4(c)). This design enables the biofilm to be protected by the biocarriers, resulting lower sensitivity to external stress factors and better recovery from shock loading. A popular attached growth applied in nitrogen removal is ANITAMox^{TM}, which was first implemented in 2011 in Malmö, Sweden by Veolia and AnoxKaldnes. The so-called BioFarm that they developed serves not only for treating reject water, but also for growing carriers as seed material for other installations (Lackner et al., 2014).

In a granular sludge type system, the biomass forms dense and well-settling granules (shown in Figure 1.4(d)). The main advantage of the granular system is the higher volumetric loading rate due to the large surface area. The effective surface of a granular reactor can be up to 3000 m^2/m^3, compared to 800 m^2/m^3 of MBBR (Abma et al., 2007; Thole et al., 2005). Also, the mixing of the reactor content is easier because the mixing is not disturbed by the carrier media. The granular biomass is dispersed more easily, avoiding sulfide formation in dead zones (Abma et al., 2007). Current granular systems have been mainly developed by Paques. Since 2006, Paques has been designing single-stage granular reactors with the majority of the systems applied for industrial wastewater treatment. Table 1.2 displays the advantages and disadvantages of the suspended growth, attached growth and Paques' granular anammox systems.

Table 1.2 Comparison of different reactor configurations for partial nitritation/anammox. process.

PN/A configurations	**Advantages**	**Disadvantages**
Suspended growth	− Conventional − Common (inoculum more available)	− Usually low sludge settling ability − Need additional AOB/AMX separation facilities − High chance of losing biomass
Attached growth	− No need for a clarifier or biomass recirculation − Low sludge production − Efficient retaining of AMX in the system − Easy to operate and simple design − Low maintenance required − No problem clogging	− Longer start up period may be required − Expensive seeded biocarriers
Granules	− High volumetric loading − No need for a clarifier if SBR is used − Higher biomass retention − No sludge return − Higher settling ability	− More complex operation − Extensive shear stress may damage granules − Long start up period

Anammox application status

In the past decade, more than 100 full-scale anammox-based reactors have been developed around the world (mainly in Europe), with increased interest about side-stream treatment implementation appearing in North America (Lackner et al., 2014). In the U.S., the first full-scale PN/A reactor was developed in HRSD York River Treatment Plant, in Seaford, Virginia, 2012. After that, several full-scale reactors have been either constructed or operating in Virginia, Florida, and Pennsylvania as well as some pilot-scale reactors that now exist innumerous states. So far, the PN/A process has been primarily applied or researched as a sidestream treatment option to treat the reject water from the anaerobic digester. Other waste streams containing relatively high concentrations of ammonium, such as landfill leachate, distillery wastewaters, and livestock manureare also reported to be treated by the PN/A system (see Table 1.3).

Table 1.3 Typical full-scale PN/A installation treating different waste streams (Adapted from Lackner et al., 2014).

Location	**Configuration**	**Year**	**Waste stream**	**Reactor volume, m^3**	**NH_4^+-N content, mg/L**	**Designed loading, kg N/m^3-day**
Strass, AT	DEMON®	2004	Reject water	500	1844±92	0.6
Thun, CH	DEMON®	2008	Reject water	606	--	0.67
Binzhou, CN	ANAMMOX®Paques	2009	Yeast	500	300~800	2
Shaoxing, CN	ANAMMOX®Paques	2011	Distillery	560	--	2
Trento, IT	DEMON®	2011	Leachate	--	--	--
Malmö, SE	ANITA MOX™	2011	Reject water	4×50	800~1200	--
York River, US	DEMON®	2012	Reject water	--	900~1000	0.5
Son, NL	ANAMMOX®Paques	2013	Meat processing	3000	--	2
James River, US	ANITA MOX™	2013	Reject water	378	800~1200	0.7

The PN/A process is becoming more familiar since several full- and pilot-scale installations have been developed in Europe and China. Despite significant advances in PN/A research, there is still a lack of confidence among wastewater practitioners, especially in the United States. Certain challenges still exist, which leave some practitioners concern, but this will be discussed later.

Furthermore, from a fundamental point of view, the application of PN/A has been limited to the treatment of concentrated reject water with little or no consideration of the process as a mainstream treatment scheme or for other concentrated waste streams such as diluted urine. Hence, a better understanding of the microorganism involved in the process can positively influence more wide spread application of this energy-efficient system.

Characteristics of AOB and AMX

Characteristics of AOB

As the first step in the process of ammonium conversion, ammonia oxidation, which is driven by AOB plays an essential role in the PN/A process. AOB can be distinguished by the cell morphologies and Gram-negative multilayered cell walls, and motile using flagella (Ge et al., 2015). To date, five genera of nitrifiers including *Nitrosomonas*, *Nitrosospira*, *Nitrosolobus*, *Nitrovibrio*, and *Nitrosococcus* are found responsible for ammonium oxidation. Among them, only *Nitrosococcus* was located within the γ-subclass of *Proteobacteria* and the rest were grouped in the β-subclass (Fiencke et al., 2005; Junier et al., 2010; Koops and Pommerening-Röser, 2001; Pukhold et al., 2000; Woese et al., 1984). Among the five genera, *Nitrosomonas* and *Nitrosospira*

have been considered the most important bacteria for aerobic ammonium oxidation in most wastewater treatment plants (Aoi et al., 2000; Coskuner and Curtis, 2002; Siripong and Rittmann, 2007). In an ammonium-rich system (such as activated sludge), a biofilm reactor or an oxygen-limited system, *Nitrosomonas europaea* is reported to be favored more often than other AOB (Schramm et al., 1998; Park and Noguera, 2004). This is due to *Nitrosomonas europaea'*s lower affinity to ammonia and higher affinity to DO (Pommerening-Röser and Koops, 1996; Park and Noguera, 2006). Previous research also showed that the *Nitrosomonas* dominated culture had increased resistance to cold temperature as compared to other AOB (Ducey et al., 2009). The physiological characteristics of *Nitrosomonas* and *Nitrosospira* can be found in Table 1.4. Aerobic ammonia oxidation is a complex process that requires several enzymes, proteins and the presence of oxygen.

Table 1.4 Physiological characteristics of the two typical genera of AOB: *Nitrosomonas* and *Nitrosospira*, and two genera of AMX: *Brocadia* and *Kuenenia*.

Physiological characteristics	**AOB**		**AMX**	
	Nitrosomonas	***Nitrosospira***	***Brocadia***	***Kuenenia***
Growth temperature, ℃	20-30	4-37	20-45	25-37
pH range	6.5-9c	5-9	6.7-8.8	6.5-9.0
Growth rate, h^{-1}	0.039-0.043	N/A	0.0027-0.0041	0.0026-0.0035
Doubling time, day	0.33-0.5	N/A	7-10.7	8-11
Km for O_2, mg/L	0.22-0.56	N/A	N/A	N/A
Km for NH_4^+/NH_3, mg N/L	5.88-46.20 (NH_4^+) 0.17-0.84 (NH_3)	1.96(NH_4^+) 0.01-0.15(NH_3)	<0.07(*B.anammoxidans*) 0.392,0.056(*B. sinica*)	0.0028-0.042
Km for NO_2^-, mg N/L	N/A	N/A.	<0.07(*B.anammoxidans*) 1.2,0.056(*B. sinica*)	N/A

Basically, it can be divided into two main steps: (1) ammonia is oxidized to hydroxylamine by ammonia monooxygenase (AMO), shown in Equations (9), and (10). Hydroxylamine is converted to nitrite by hydroxylamine oxidoreductase (HAO), as shown in Equation 10):

$$NH_3 + O_2 + 2H^+ \xrightarrow{AMO} NH_2OH + H_2O \quad (9)$$

$$NH_4OH + H_2O \xrightarrow{HAO} NO_2^- + 5H^+ + 4e^- \quad (10)$$

AMO is a membrane-bound hetero-trimetric copper enzyme, with broad substrate ranges (Semrau et al., 1995). AMO is coded by three subunit genes: amoA, amoB and amoC (Junier et al., 2010), of which only some of the amoA gene is expressed for the enzyme synthesis (Fukushima et al., 2012; Kuo et al., 2010). *Nitrosonomas europaea* has two copies of the *amoA* gene (McTavish et al., 1993), either of which could support the growth if there is a disruption of the other copy (Hommes et al., 1998). This system would allow the bacteria to synthesize a large amount of AMO in a short time (Bollmann and Laanbroek, 2001; Hommes et al., 1998), and thus maintain a relatively high activity under unfavorable conditions. Different from AMO, HAO is located in the periplasm and has multi-c-heme and homotrimer subunits (Arp et al., 2002). It is coded by the gene cluster of *hao*, which reveals a highly conserved gene encoding protein (Bergmann et al., 2005). To identify AOB by the polymerase chain reaction (PCR)- based method, the 16S ribosomal RNA (16S rRNA) and *amoA* functional gene are mostly used (Kowalchuk et al., 1997; Rotthauwe et al., 1997). The primers generally used are listed in Table 1.5. The *hao* gene is not often used as a biomarker, mainly due to the lack of sufficient sequence information of the gene (Schmid et al., 2008).

Table 1.5 Common primers used to target AOB and AMX.

Target	Primers	Sequences (5'-3')	References
amoA gene	amoA_1F	GGGGTTTCTACTGGTGGT	Rotthauwe et al., 1997
	amoA_2R	CCCCTCKGSAAAGCCTTCTTC	
hzsA gene	hzsA_1597F	WTYGGKTATCARTATGTAG	Harhangi et al., 2012
	hzsA_1857R	AAABGGYGAATCATARTGGC	
AOB 16S rRNA gene	CTO 189fA/B	GGAGRAAAGCAGGGGATCG	Hermansson et al., 2001
	CTO 189fC	GGAGGAAAGTAGGGGATCG	
	RT1r	CGTCCTCTCAGACCARCTACTG	
AMX 16S rRNA gene	Pla46F	GGATTAGGCATGCAAGTC	Neef et al., 1998; van der Star et al., 2007
	AMX667R	ACCAGAAGTTCCACTCTC	

Characteristics of AMX

The bacteria that involved in the anammox process belong to the bacterial phylum *Planctomycetes*. Because anammox bacteria are anaerobes and autotrophic, they have long doubling time of 8~12 days (Kartal et al, 2012; Kuenen, 2008; van der Star et al., 2007) and growth in pure culture has not yet been possible (Tsushima et al., 2007).

To date, five genera of anammox bacteria have been described. They are: *Candidatus Kuenenia*, *Candidatus Brocadia*, *Candidatus Anammoxoglobus*, *Candidatus Scalindua*, and *Candidatus Jettania* (Jetten et al., 2009). The genome of 13 species have been isolated: *Kuenenia: K.stuttgartiensis* (Strous et al., 2006), *Brocadia: B. anammoxidans; B. fulgida; B. sinica; B. caroliniensis* (Kartal et al., 2008; Oshiki et al., 2011; Rothrock et al., 2011; Strous et al., 1999); *Anammoxoglobus: A. propionicus* (Kartal et al., 2007)*; Scalindua; S. brodae; S. sorokinii; S. wagneri; S. marina* (Brandsma et al., 2011; Schmid et al., 2003; Woebken et al., 2008), and *Jettania: J. asiatica; J. caeni; J. moscovienalis* (Hira et al. 2012; Nikolaev et al., 2015; Quan et al., 2008). Those

species are widely found in many diverse environments, including marine sediments (Kuyper et al., 2003), freshwater environments (Rich et al., 2008; Schubert et al., 2006), constructed wetlands (Paredes et al, 2007) and wastewater treatment plants (Dong and Tollner, 2003; Egli et al., 2001; Fujii et al., 2002; Tal et al., 2003; Toh and Ashbolt, 2002). The species *Candidatus Brocadia* and *Candidatus Kuenenia* are the most studied and the most commonly found organisms in wastewater plants as well as in full-scale anammox reactors (Kuenen, 2008).

Table 1.4 listed the physiological characteristics of *Brocadia* and *Kuenenia*. Despite those similar features illustrated, there are significant differences between the two species. *Kuenenia* has a lower ammonium oxidation activity of 26.5 nmol min^{-1} $mg_{protein}^{-1}$, compared to 55 nmol min^{-1} $mg_{protein}^{-1}$ for *Brocadia* (Kartal et al., 2013). Besides, *Kuenenia* has a higher tolerance to nitrite and less inhibited by phosphate (Khin and Annachhatre, 2004).

The whole biological process is mediated by NO and N_2H_4 under several enzymes, (1) NO_2^- is reduced to NO by nitrite reductase (NirS), (2) and NO are converted to N_2H_4 by hydrazine synthase (HZS), and (3) N_2H_4 is oxidized to N_2 by hydrazine oxidoreductase (HDH). Equations (11), (12), and (13) found below show the reactions catalyzed by these three enzymes.

$$NO_2^- + 2H^+ + e^- \xrightarrow{NirS} NO + H_2O \quad (11)$$

$$NO + NH_4^+ + 2H^+ + 3e^- \xrightarrow{HZS} N_2H_4 + H_2O \quad (12)$$

$$N_2H_4 \xrightarrow{HDH} N_2 + 4H^+ + 4e^- \quad (13)$$

The analysis of *Kuenenia stuttgartiensis* genome showed that HZS makes up about 20% of the protein complement (Kartal et al., 2012). As isolated, the enzyme

complex is not very active, and appreciable activity of 20 nmol/hr-mg of protein is only obtained when the hydrazine synthesis and hydrazine oxidation are coupled (Kartal et al., 2012). The in vivo analysis showed that HZS is a very slow enzyme, which possibly explains the slow growth rate of anammox bacteria (Kartal et a., 2011). The HZS protein encoded by *hzsC*, *hzsB*, *hzsA* gene cluster was successfully purified and used as a very specific biomarker for anammox bacteria as the gene cluster is found to be very unique (Harhangi et al., 2012).

As for *nirS* in *K. stuttgartiensis* genome, they are hardly expressed at the transcriptional level and are barely detectable in the proteome (Kartal et al., 2011). However, in striking contrast, *Nir*S is one of the most abundant proteins in *Scalindua* (Ma et al., 2016). It is doubted that another enzyme rather than *NirS* in *K. stuttgartiensis* accounts for nitrite reduction (Kartal et al., 2012). Besides, 30% of the protein complement was heme c proteins, which is bright red and makes the anammox biomass reddish. The enzymes involved are summarized and listed in Figure 1.5.

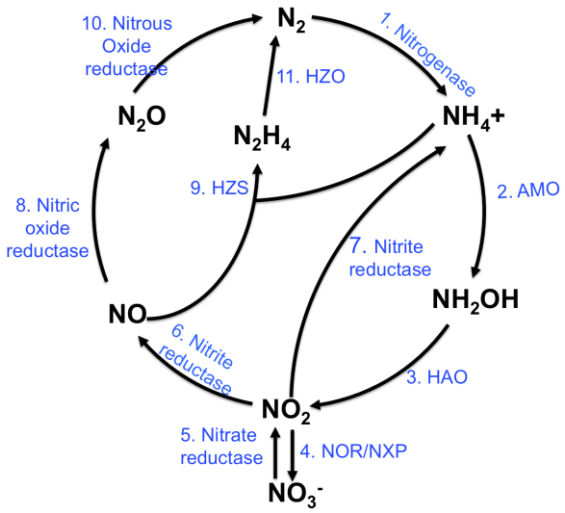

Figure 1.5 Enzymes involves in biological nitrogen cycle.

Factors impact PN/A system

Like all biological processes, certain conditions need to be controlled for the PN/A process to occur effectively. The most important parameters include dissolved oxygen, pH, substrate concentrations, solid retention time management, temperature, organics and other possible constituents in wastewater.

Dissolved oxygen (DO)

In the early stage, anammox bacteria were considered as strictly anaerobes, whose activity would be inhibited when DO was present (van de Graaf et al., 1996; Tsushima et al., 2007). Hence, most of the anammox installations were in two-stage systems, with the anammox bioreactors sealed to avoid any inhibition by oxygen (Fux et al., 2002; Jetten et al., 2001; Tsushima et al., 2007; van der Star et al., 2007; van Dongen et al., 2001). With more knowledge of anammox, it was found that AMX could survive and work together with AOB in one reactor (Joss et al., 2009; Szatkowska et al., 2007; Third et al., 2005; Vlaeminck et al., 2009; Wang et al., 2010). It is assumed that this is due to the structure of the sludge aggregates/biofilm in which the outer layer depletes the oxygen for nitritation and the inner part remains anaerobic (Gong et al., 2007; Joss et al., 2009; Liu et al., 2008). Liu et al. (2008) found that the anammox aggregates gradually adapted to the DO concentration up to 8 mg L^{-1} by a step-wise culturing, and the maximum anammox activity was very decreased in small amount after long-term oxygen exposure.

Generally, DO is introduced either by continuous or intermittent aeration. In continuous aeration, (at least theoretically), any nitrite produced by AOB should be consumed by AMX immediately. The activity of AOB and AMX is supposed to be equal.

Otherwise, nitrite would be accumulated and cause inhibition to the anammox bacteria. The principal advantages of continuous aeration consist of (1) simplicity, (2) better monitoring of reactor and (3) eventual higher performance (Joss et al., 2009).

In intermittent aeration, the aerators are kept sequentially on and off to provide the aerobic condition for AOB to produce NO_2^-, and then the anoxic condition for AMX to consume NO_2^- to N_2. This aeration method allows extra time for anammox to be active, preventing the accumulation of nitrite. In the DEMON® SBR system, the aeration facilities are controlled by a timer for ON/OFF controlling of 8~12 min ON and 2~20 min OFF to maintain a mean DO of 0.2~0.3 mg L^{-1} (Lackner et al., 2014).

pH

pH in the PN/A system influences the levels of free ammonia (FA) and free nitrous acid (FNA) (Jin et al., 2012; Peng and Zhu, 2006), as shown in the equations.

$$NH_4^+ + OH^- \rightarrow NH_3(FA) + H_2O \quad (14)$$

$$HNO_2(FNA) \rightarrow NO_2^- + H^+ \quad (15)$$

A higher pH is conductive to the formation of FA, which can diffuse easily into the cells, change the intracellular pH and the transmembrane potential, and cause cell death in the worst case (Jin et al., 2012; Martinelle et al., 1996). Waki et al. (2007) found that FA level of 13~90 mg_N L^{-1} could be toxic to anammox organisms. Similarly, a 50% reduction of specific anammox activity (SAA) was observed at the FA concentration of 38 mg_N L^{-1} in a short term test and when FA level above 35~40 mg_N L^{-1}, the biomass removal efficiency decreased to zero in the long term operation (Fernández et al., 2012). Although served as substrate for AOB (Suzuki et al., 1974), FA is also reported to inhibit

AOB activity in the range of 10~150 mg NH_3-N/L (Kim et al., 2006)

In contrast, the lower pH favors the production of FNA, which is reported to have impact on numerous metabolic processes, including the active transport of substrate across the cell membrane, substrate uptakeand the energy-consuming anabolic process (Tallec et al., 2006; Vadivelu et al., 2006a; Vadivelu et al., 2006b; Ye et al., 2010; Zhang et al., 2010; Zhou et al., 2010). Previous research showed that the *Brocadia anammoxidans* dominated culture was completely inhibited by FNA at a very low concentration of 6.0×10^{-3} mg_N L^{-1} (Strous et al., 1999), and *Kuenenia stuttgartiensis* culture lost its activity at FNA of 0.04 mg_N L^{-1} (Egli et al., 2001). As for AOB, 0.42~1.72 mg_N L^{-1} had resulted in 50% reduction of their activity (Anthonisen et al., 1976; Fux and Siegrist, 2004; Hellinga et al., 1999; Stein and Arp, 1998; Tan et al., 2008; Tora et al., 2010; Vadivelu et al., 2006a). Thus, in the PN/A system, the pH is controlled in the median range of 6.5~8 to maintain a higher bacterial activity (Lackner et al., 2014).

Substrate concentration

The ammonium concentration up to 1000 mg_N L^{-1} did not bring about any negative effect on the PN/A process (Strous et al., 1999). Actually, it was widely accepted that it is the FA (NH_3) rather than ammonium (NH_4^+) is responsible for the suppression of the bacteria activity (Dapena-Mora et al., 2007; Fernández et al., 2012; Strous et al., 1999).

A crucial parameter for the PN/A process is the nitrite concentration. Nitrite is an essential substrate but also inhibitory to the reaction. Previous studies showed the threshold NO_2^--N concentration span over a wide range of 5 ~280 mg_N L^{-1} under different

experimental condition and operation mode (Jin et al., 2012). Strous et al. (1999) reported that NO_2^- above 100 mg_N L^{-1} completely inhibited the anammox process. A 50% inhibition of the anammox process at 350 mg_N L^{-1} NO_2^- was shown by Dapena-Mora et al. (2007). Also, Fux et al. (2003) found that long-term exposure to a nitrite level of 40 mg_N L^{-1} over several days caused an irreversible inactivation of AMX. However, Lotti et al. (2012) observed a full recovery of activity after removing the nitrite from the system. It is quite possible that the nitrite effect depends on the exposure time, the type of biomass, the operational condition, temperature, reactor configuration, and HRT. In engineering, a typical DEMON® plant keeps nitrite below 5 mg_N L^{-1} to avoid any inhibition to anammox (Wett, 2007a).

Temperature

The maximum activity of AMX was observed between 35~40 ℃ (Dosta et al., 2008; Oshili et al., 2011; Strous et al., 1999) and the temperature for AOB was 28 ℃ (Alawi et al., 2007). For AMX, temperature above 45 ℃ causes an irreversible decrease in anammox activity due to cell lysis, while a temperature below 15 ℃ could bring an unstable performance (Dosta et al., 2008). Although some cold adapted AOB, such as *N. cryotolerans* was able to grow even at -5 ℃ (Alawi et al., 2007; Jone et al., 1988), the majority of AOB could be inhibited when temperature decreased. Based on the physiology of AOB and AMX, PN/A reactors are generally operated at the mesophilic temperature of 25~35 ℃ (Lotti et al., 2015; van Hulle et al., 2010). The relatively higher temperature not only favors the activity of slow growing anammox bacteria but also assists AOB to out-compete the nitrite oxidizing bacteria (NOB). On the other hand,

higher temperature brings up the operation cost and thus limits much of the application.

Despite the reported decrease in activity at lower temperatures (<25 ℃) (Dosta et al., 2008; Vázquez-Padín et al., 2011), anammox bacteria have been found to thrive at low temperature (<10 ℃) in natural system such as marine sediments (Van de Vossenberg et al., 2008). This indicates that there is a possibility of developing anammox process for wastewater treatment even at low temperature. Hu et al., (2013) corroborated this finding when observing a shift of optimal temperature from 35 ℃ to 25 ℃ for a "CandidatusBrocadiafulgida"-like strain in a batch PN/A bioreactor. A similar study by Gilbert et al., (2014) and Persson et al., (2014) on the potential of biofilm media tolerance to temperatures as lower as 10 ℃ showed that although the removal efficiency of the reactors decreased, the stable anammox activity kept the reactor performance stable throughout. Furthermore, Gilbert et al. (2015) observed that the temperature coefficient was slightly decreased in 4 different PN/A reactor configurations after 25 weeks of adaption. This indicates that it is feasible to adapt the biomass in PN/A system to cold temperature overthe course of months or years. However, in real world application, the cold time would only last for several months, which may not be able to support the long "adaption" process. Hence, a better understanding of the PN/A biomass related to both cold temperature and shortening the adaption process may boost the wide application of PN/A for nitrogen removal.

Solid retention time (SRT) management

The maximum growth rate of anammox bacteria is $0.0027h^{-1}$, which is 14 times slower than the growth rate of AOB. To maintain a specific portion of AMX in the

biomass, it is important to retain AMX in the system and wash-out of any other faster grow bacteria at the same time. It is recommended that in PN/A system, the SRT for AOB be kept for 3 days and for AMX maintained 30 days (Wett et al., 2010a). In the suspended growth system, the SRT management is generally fulfilled by a special designed hydrocyclone (Wett et al., 2007b; Wett et al., 2010b), which can separate AOB from anammox granules by their difference in particle size. Yet, this hydrocyclone is usually applied in large-scale wastewater treatment plants. For some small-scale plants, a hydrocyclone is neither economically feasible nor applicable. Thus, more separation methods need to be introduced to favor the development of one-stage PN/A systems for small-scale plants.

Organics

Both AOB and AMX are chemoautotrophic microorganisms that use CO_2 as the only carbon source. The concentration of inorganic carbon is particularly important for efficient nitrogen removal in the PN/A system. However, organic carbon is widely considered to have adverse effects on the two autotrophic bacteria (Chamchoi et al., 2008; Tang et al., 2010; van de Graaf et al., 1996). First, organics can foster the growth of heterotrophic bacteria, which compete with AOB for oxygen and with AMX for nitrite. Moreover, the fast-growing heterotrophs may lead to a higher sludge production, which may result in the anammox biomass loss. Chamchoi et al. (2008) reported that AMX were outcompeted by heterotrophs when the COD/N ratio in the influent was higher than 2.0 g_{COD} g_N^{-1}. Second, specific organic compounds can be directly toxic to AOB and AMX. For example, it is found that AMX isolated from marine sediment would lose their

activity in 3-4 mmol $L^{-}1$ of methanol (Jensen et al., 2007). Antibiotics in sewage treatment plants also demonstrated to have impact on the anammox process (Fernandez et al., 2009; van de Graaf et al., 1995). Third, some AMX are reported to be able to perform dissimilatory nitrate reduction to ammonium (DNRA) with the presence of fatty acids (Güven et al., 2005; Kartal et al., 2007). As a result, the anammox activity is lower, causing a decrease in nitrogen removal performance. In general, the PN/A process has been primarily applied to high-strength nitrogenous wastewater with very low biodegradable organic carbon content (less than 0.5 g_{COD} g_N^{-1}) (Wett et al., 2007a). However, Jenni et al. (2014) successfully increased the influent COD/N ratio to 1.4 g_{COD} g_N^{-1} gradually by adding acetate and found that the nitrogen removal efficiency of the PN/A reactor was increased from ~85% to ~95%. Therefore, it can be concluded that AMX can coexist with heterotrophs at an elevated COD/N ratio if a sufficient high SRT is maintained.

Other possible constituents in wastewater

Sulfide is often present in anaerobic digestion supernatant by the reduction of SO_4^{2-} (Dapena-Mora et al., 2007). This biological process mainly occurs in biofilm system or anaerobic conditions, which is characterized by slow flow rates or insufficient aeration at relatively high temperature (Hvitved-Jacobsen et al., 2000; Nielsen et al., 1992; Zhang et al., 2008). H_2S, instead of S^{2-}, is toxic to bacterial cells since it can diffuse into the cell membrane (Turman and Cork, 1988). Once inside the cytoplasm, H_2S may cause the denaturing of native proteins (Conn et al., 1987), bringing negative deterioration to the bacterial activity.

By 2014, reject waters presumably containing high S^{2-} had led to a loss/reduction in performance at three full-scale PN/A applications (Lackner et al., 2014). Sulfide in different wastewater has been reported varied from 0.03 to 25 $mg_S\ L^{-1}$ elsewhere (Bulc, 2006; Padival et al., 1995; Rodriguez-Gomez et al., 2005; Tomar and Abdullah, 1994; USEPA, 1992). The concentration of sulfide as low as 1.0 $mg_S\ L^{-1}$ might be partial or completely inhibit the ammonium oxidizing bacteria (Beccari et al., 1980; Erguder et al., 2008; Sears et al., 2004).

As for the sulfide impact on AMX, Jin et al. (2013) found that the short and long-term effects of sulfide on anmmox biomass were distinctive: the SAA was halved at an sulfide level of 32 $mg_S\ L^{-1}$ within 13 days, while only 17.2% reduction during 18 days with 40 $mg_S\ L^{-1}$. Dapena-Mora et al. (2007) reported that 160 $mg_S\ L^{-1}$ led to a complete inhibition of anammox activity. In contrast, van de Graaf et al. (1996) observed that the sulfide could favor the anammox activity. This suggests that the characteristics of the anammox-involved system under sulfide still needs more research.

Research objectives

The study was carried out with the main objective to provide a better understanding of PN/A reactors with suspended and attached growth configurations for treating different waste streams that have potential stress factors. The finding would eventually help process engineers to develop a more reliable, robust and energy efficient startup and operation strategies for PN/A installations. Based on my literature review of the current application status of anammox in the U.S and the challenges identified for the application, I have formulated the following objectives.

Objective 1

The first objective is to study the total nitrogen removal potential in laboratory scale PN/A reactors with different configurations using real reject water.

Task 1: Start up of suspended growth and attached growth single stage PN/A reactors.

Task 2: Evaluate the reactor performance in terms of nitrogen removal.

Objective 2

Study the microbial ecologies in lab scale suspended and attached growth reactors.

Task 3: investigate the overall microbial ecologis in both reactors using 16S rRNA amplicon sequencing.

Objective 3

The third objective is to evaluate the effect of different environmental stress factors that can cause upsets and, study the gene expression.

Task 4: Study the effect of temperature and toxic loading on the reactor performances.

Task 5: Investigate the ex-situ bacterial activity impact by the stress factors.

Task 6: Monitor gene expressions during the stress tests.

Objective 4

The forth objective is to investigate the feasibility that apply PN/A reactor for urine treatment.

Task 7: Start up two-stage anammox reactors and a single stage PN/A reactor to treat poststruvite precipitated urine and monitor the reactors performance.

Objective 5

With lessons learned from objective 1, the last objective is to design, fabricate and run a pilot-scale PN/A reactor in the field.

Task 8: Design and fabricate a pilot scale PN/A reactor at North Davis Sewer District in Utah.

Task 9: Optimize the pilot reactor performance at room temperature.

CHAPTER 2

COMPARISON OF MICROBIAL COMMUNITIES IN SUSPENDED GROWTH AND ATTACHED GROWTH PARTIAL NITRITATION/ANAMMOX SYSTEM UNDER DIFFERENT TEMPERATURES

Introduction

Anaerobic ammonia oxidation (anammox) is a shortcut in the nitrogen cycle to convert dissolved ammonium to dinitrogen gas with nitrite as an electron acceptor (Strous et al., 1999). Anammox is considered an energy efficient and costeffective alternative to conventional biological nitrogen removal (BNR). Compared to traditional nitrification-denitrification, anammox-based BNR processes involve no external organic carbon source, offer approximately 60% less oxygen demand, and yield much less biomass (Strous et al., 1999; Third et al., 2005). Anammox is generally coupled with aerobic partial oxidation of ammonium to nitrite (called nitritation) and these two processes are collectively known as partial nitritation-anammox, PN/A, or deammonification configuration.

The PN/A process is mainly driven by two groups of prokaryotes: (a) ammonium oxidizing bacteria (AOB) and (b) anammox bacteria (AMX). First, AOB oxidize ammonium (NH_4^+) to nitrite (NO_2^-) in the presence of dissolved oxygen (DO). As of the writing of this report, five recognized genera of AOB in two phylogenetically distinct

groups have been reported: *Nitrosomonas (e.g., N. eutropha*, *N. europaea*), *Nitrosospira*, *Nitrosovibrio*, *Nitrosolobus*, and *Nitrosococcus* (Junier et al., 2010). AMX are able to produce N_2 gas from NO_2^- and NH_4^+under anoxic conditions. Similar to AOB, five genera of AMX have been reported: *Brocadia*, *Scalindua*, *Kuenenia*, *Jettenia*, and *Anammoxoglobus* (Strous et al., 1999). All five bacteria were found in freshwater systems and wastewater treatment plants except *Scalindua*, which was enriched from marine sediments (Kartal et al., 2012). As none of these species have been isolated into a pure culture, the genome information and growth physiology are still under way.

In PN/A systems, other phenotypes such as nitrite oxidizing bacteria (NOB) may exist and compete with AMX for nitrite. Typical are *Nitrobacter* and *Nitrospira* (Persson et al., 2014). To outcompete the NOB in the system, PN/A reactors are generally operated at temperatures higher than 25℃ to maintain a relatively higher activity of AMX. Besides NOB, other heterotrophs widely found in these systems include *Ignavibacteriacea* (Gonzalez-Martinea et al., 2014; Zhang et al., 2014), *Anaerolinea* (Chu et al., 2015; Gilbert et al., 2014), and denitrifiers *Rhodocyclacea*, *Comamondadacea* (Gilbert et al., 2014).

So far, more than a hundred PN/A installations have been commissioned globally (Lackner et al., 2014). In recent years, there has been a trend toward moving from two-stage (i.e., nitritation and anammox happening in separate bioreactors) to single-stage (i.e., nitritation and anammox simultaneously work in one reactor) as the single-stage PN/A configurations offer reduced footprints and better operational control. In applying the PN/A process to WWTPs, the two most commonly installed reactor configurations are suspended growth (e. g., DEMON®) and attached growth (e. g., Anita^{T}Mox). The

spatial distributions and the activity of AOB and anammox bacteria in the suspended growth and attached growth have been investigated and reported: AOB are active in the outer oxic region of biofilms or aggregates while AMX are protected in the inner anoxic region (Chu et al., 2015; Gilbert et al., 2013; Persson et al., 2014; Vlaeminck et al., 2010).

Although so many installations have been supplemented, it is still unclear how the reactor configuration and temperature impact the selection of anammox species. Not only that, but how does reactor configuration and temperature impact the flanking species involved in the nitrogen transformation in the PN/A system and is that impact different in suspended growth and attached growth PN/A systems operated under similar conditions? From a practical perspective, knowledge of the overall microbial community structure in suspended and attached growth PN/A systems at different temperatureswill contribute towards the performance optimization and process stability under temperature change. Thus, the overall objective of this research was to explore the effect of temperature on the microorganism hierarchy within PN/A systems in order to facilitate the development of intricate process optimization procedures for wastewater engineers.

Methods and materials

Suspended and attached growth reactor startup and operation

A 9 L suspended growth PN/A rector (hereafter called the SR reactor) was inoculated with biomass collected from a single-stage DEMON® in HRSD York River Treatment Plant (Seaford, VA) to maintain the initial MLSS of 3000 mg L^{-1}. Anaerobic digester filtrate from a local wastewater treatment plant (North Davis Sewer District, Syracuse, UT) was used as the feed solution for the reactor. NH_4^+ concentration in the

filtrate was 406±23 mg-N L^{-1}. Dissolved Oxygen (DO) concentration was maintained below 0.4 mg L^{-1} by intermittent aeration with an aquarium pump. The reactor was operated in batch mode with four batches per day, yielding an HRT of 2 days in the beginning anddecreasing to 0.5 days at steadystate. Each batch included 10 min of feeding, 4 h of reaction, 20 min of final aeration, 20 min of settling, and 10 min of decanting. The 4-h reaction period contained 16 aeration cycles consisting of 10 min of aerobic conditions and 5 min of anoxic conditions. The SR was maintained at 35℃ for 122 days by wrapping the reactor with silicone heating tape (BriskHeat, Columbus, OH). Afterwards, the heating tape was removed to let the reactor sit at room temperature of 21℃ for 183 more days.

Along with the suspended growth PN/A reactor, an attached growth PN/A reactor (hereafter called the AR reactor) was started and operated with a working volume of 10.5 L. The AR was filled with round bioballs (CoraLife Bio Ball Aquarium Filter) as the biofilm media. 50% of the bioballs were grown with fresh media and the rest with anammox biofilm. The bioballs with anammox biofilm were obtained from an ongoing attached growth anammox reactor in the lab. In order to form the nitrifying biofilm and to accelerate the startup process, suspended nitrifying biomass from a partial nitritation reactor (Kotay et al., 2013) was added to the reactor to allow the nitrifying film to form on the bioballs. The AR reactor with anammox biofilm containing bioballs and the nitrifying biomass was allowed to stand for 7~10 days. Thereafter, the AR reactor was flushed with real centrate to remove the suspended biomass.

Each bioball has a diameter of 2.54 cm and a surface area of 88.9 cm^2. In the beginning, diluted filtrate was fed to the AR to thicken the biofilm growing on the

bioballs and let the microbial community form biofilm on the fresh attached growth media. The nitrogen loading was slowly increased depending on the reactor performance. Similar to the SR, AR was also operated with four batches every day. The AR was operated with intermittent aeration for about half a month. However, it was found that the NO_2^- concentration after 10 min of aeration was consistently lower than 1 mg-N L^{-1}even when the DO in the AR was maintained at a levelsimilar to the DO in the SR. Following this observation, continuous aeration was applied to the AR PN/A reactor and the settling phase was removed to maximize the reaction time. As a result, each 6-h cycle in the AR consisted of 10 min of feeding at the beginning and 10 min of decanting at the end with continuous aeration in between. The AR was operated at 35 ℃ for 133 days and then at 21 ℃ for 172 days.

Analysis methods

Influent and effluent samples were collected regularly and filtered with 0.45 μM Millipore membrane filter paper for nitrogen species analysis. NH_4^+-N in the influent and NH_4^+-N, NO_2^--N, and NO_3^--N in the effluent were monitored. NO_2^--N and NO_3^--N were analyzed using Ion Chromatography while NH_4^+-N was measured usingHigh Range Ammonia Nitrogen via the AmVer™ Salicylate Test N Tube.

DNA extraction and amplicon sequencing

For the SR, mixed liquor samples were harvested and used for DNA extraction. For the AR, 10 bioballs were randomly collected and carefully washed twice with deionized water. Biomass was carefully scrapped off using autoclaved pipette tips and

was resuspended in deionized water. The biomass was then vortexed for at least 5 min until the big flocs were dispersed evenly. DNA was extracted from the biomass using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) as described in the manufacturer's protocol. The nucleotide concentration was examined on a Nanodrop spectrophotometer (Nanodrop Technologies Inc., USA). Then the DNA concentration was normalized to ~ 20 ng μL^{-1} and was sent to Research and Testing laboratory, TX, for Miseq Illumina sequencing. The primers used for 16S rRNA V4 region amplification were 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′).

The raw reads obtained were interleaved using PEAR Illumina paired-end read merger (Zhang et al., 2013). Low quality sequences with Phred Scores below 25 were removed through an internally developed quality-trimming algorithm. After being trimmed for quality, reads were sorted by length from longest to shortest. Prefix de-replication and clustering at 4% divergence was performed using USEARCH algorithm (Edgar, 2010), wherein sequences shorter than 100bps were ignored, with no minimum cluster size to allow singleton clusters in the output. All clusters containing < 2 members were removed from the datasets. The UPARSE OTU selection algorithm (Edgar, 2013) was used to classify the large number of clusters into OTUs. Chimera check was performed on each selected OTU using UCHIME (Edgar et al., 2011). In order to determine the taxonomic information, the sequences were run through the USEARCH global alignment program (Bokulich et al., 2014), wherein quality filtered reads were mapped to their corresponding nonchimeric clusters. Output generated was then assigned to a taxonomic profile using an internally developed script.

Results and discussion

Performance of SR and AR at 35 ℃

The operation of the SR can be divided into two phases based on the temperature maintained in this reactor. As illustrated in Figure 2.1(a) and Table 2.1, during Phase I, where the temperature was maintained at 35 ℃, the HRT was decreased from 2 days to 1day and finally to 0.5 day. This adjustment was fulfilled via increasing the influent/effluent volume that was loaded and drawn out of the reactor. At the same time, aeration was gradually increased to improve the AOB activity and eventually to provide more NO_2^- for anammox.

It was noticed that NH_4^+ in the effluent significantly increased while the NO_2^- stayed below 20 mg-N L^{-1} with a maximum concentration of 19.8 mg-N L^{-1}on 110^{th} day. Such a buildup of NO_2^- might have occurred due to the high AOB activity. Based on the fact that AOB and NOB mainly grow as tiny flocs while AMX tend to grow as big granules, resulting different settling abilities (Hubaux et al., 2015; Nielsen et al., 2005; Vlaeminck et al., 2010), a shorter settling time of 5 min during each batch was applied to improve the wash-out of nitrifiers. From the 57^{th} day, the NH_4^+concentration in the final started to increase. After checking the pH, alkalinity in the influent, and the aeration pump, it was determined that the reactor was alkalinity limited. Thereafter, an equivalent amount of $CaCO_3$ (added as $NaHCO_3$) was provided in the influent. Following this change, the NH_4^+-N removal efficiency increased from 56.5% at day 58 to 82.4% at day 72. The inadequacy of alkalinity occurred during the gradual increase in the removal rate to 0.49 kg-N m^{-3} d^{-1} with a pH of 7.8. It is not yet known if the higher pH was due to reduced alkalinity or reduced metabolic activity of microbes in the system.

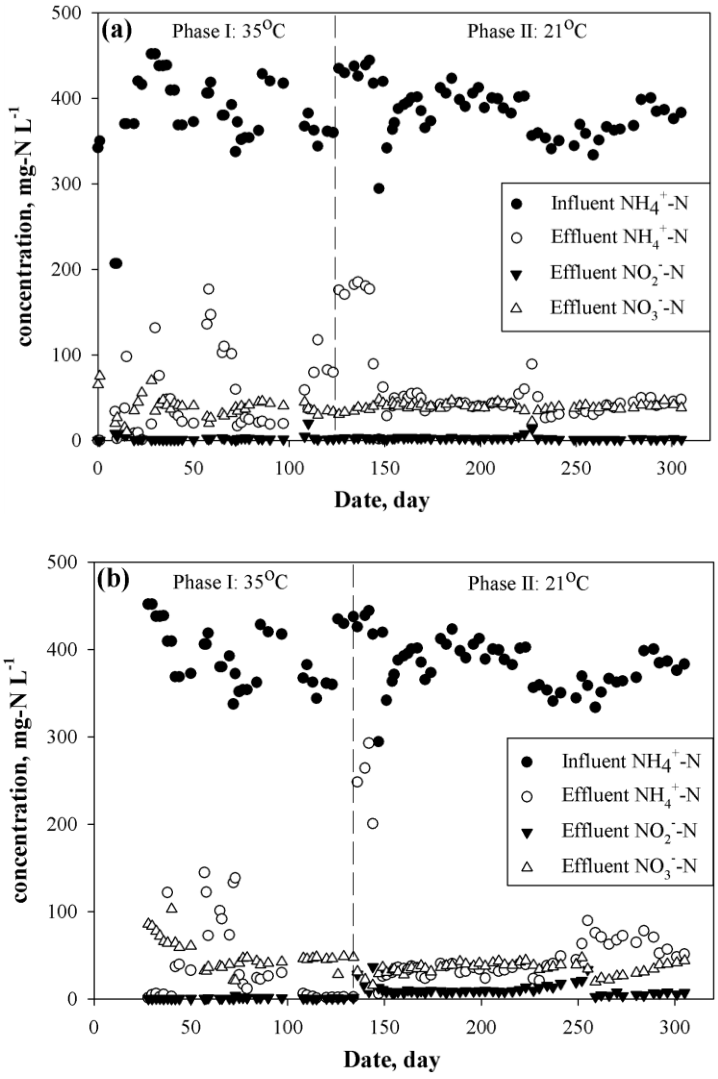

Figure 2.1 Variation of influent and effluent concentrations of ammonium, nitrite and nitrate for SR (a) and AR (b) during the study period.

The operation of the AR can also be divided into two phases based on the temperature gradients provided to the PN/A system (see Figure 2.1(b)). During the initial 27 days, to allow development and growth of biofilm on the surface of media, the reactor was operated manually and aeration was adjusted frequently (Data not shown). From the 28th day, the reactor was fed with the same reject water as SR. Similar to the SR, NH_4^+ in the effluent was increased when the HRT was decreased on days 38 and 57.

Table 2.1 Performance and operation of SR and AR.

Parameters	SR performance				AR performance			
	Phase I			Phase II	Phase I			Phase II
	HRT=2 days	HRT=1 day	HRT=0.5 day	HRT=0.5 day	HRT=2 day	HRT=1 day	HRT=0.5 day	HRT=0.5 day
Temperature	35	35	35	21	35	35	35	21
TIN loading, kg N/m3-day	0.17±0.040	0.41± 0.034	0.76±0.055	0.77±0.061	0.22±0.003	0.39±0.021	0.77±0.063	0.77±0.057
TIN removal rate, kg N/m3-day	0.14±0.038	0.32±0.024	0.54±0.093	0.57±0.061	0.18±0.003	0.27±0.029	0.60±0.111	0.56±0.103
TIN removal efficiency, %	81.5±7.10	78.4±6.1	71.7±10.7	74.4±9.0	82.1±1.6	69.9±8.6	77.9±11.7	73.7±13.2
NO_3-N/NH_4-N	0.12±0.058	0.12±0.017	0.11±0.015	0.12±0.013	0.17±0.015	0.20±0.032	0.12±0.018	0.11±0.019

The details of the AR's performance are also listed in Table 2.1. When the AR was operated at 35 ℃, NO_2^- in the effluent never exceeded 5 mg-N L^{-1}. The ratio of NO_3^--N production to NH_4^+-N depletion was 0.17, which is higher than the reported ratio of 0.11 in single-stage PN/A systems (Strous et al., 1999). This might be due to the exist of NOB in the AR, where a long solid retention time (SRT) was maintained, resulting an inadequate wash-out of NOB from the system. Moreover, the continuous aeration strategy may also have favored the growth of NOB. Due to the limitation of available alkalinity on the 72nd day the temperature shock was delayed till a steady state was reached.

Performance of SR and AR at 21 ℃

At the 123rd day, a temperature shock was provided to the SR reactor by reducing the temperature to 21 ℃. Following this, the NH_4^+ in the effluent increased significantly, while NO_2^- and NO_3^- kept stable, indicating significantly decreased AOB activityin the SR PN/A system.

To investigate the AMX activity change, ex-situ tests were performed in triplicate under anaerobic conditions. The results indicated a decrease in specific activity of AMX by ~40% during the temperature shock from 35 ℃ to 21 ℃ (Raw data not shown). This could have been due to (1) temperature shock to AMX bacteria and (2) limited availability of NO_2^-due to suppression of AOB activity. This suggests that NH_4^+ removal rate could be further improved if AOB activity is improved. During the next 20 days of the acclimatization stage, the average TIN removal was 0.46 kg-N m^{-3} d^{-1}. By the 144th day, the NH_4^+concentration in the effluent slowly dropped to 89.2 mg-N L^{-1}, yielding a removal of 0.57 kg-N m^{-3} d^{-1}.

Afterwards, the reactor performance was stable with an average TIN removal rate of 0.59 kg-N m^{-3} d^{-1}, indicating a successful adaptation to room temperature by the microbial communities performing PN/A in the SR.

In contrast to the SR, NO_2^- accumulation and NO_3^- depletion in the effluent was observed in the AR for couples of days right after the decrease in temperature. The nitrite and ammonium accumulation indicated that both AOB and AMX activity decreased due to cold temperature shock. The build down of NO_3^- in the effluent was resulted from the decreased activity of AMX, where $NO_3^-$was produced via oxidizing NO_2^- for biomass synthesis (kartal et al., 2012). The AR reactor recovered from the cold temperature shock in just 10 days (Day 135 to day 144). The TIN removal rate increased from 0.24 kg-N $m^{-3}d^{-1}$at the 136th day to, past day 147, a constant TIN removal of 0.58 kg-N m^{-3} d^{-1} with 89% of NH_4^+ removal efficiency was consistently recorded in the AR PN/A reactor. This result indicatesthat the PN/A system can be operated at room temperature with high removal efficiency.

Microbial communities analyzed by amplicon sequencing

The primer set 515F/806R targeting the V4 hypervariable region of the 16S rRNA gene yields accurate phylogenetic information and has few biases against any bacterial taxa (Bergmann et al., 2011). So far, these primers have been used to reveal considerably accurate microbial community structure in PN/A reactors (Gilbert et al., 2014). The Illumina sequencing (Illumina, CA) yielded a total of 16463 to 19798 short reads for 4 samples (Table 2.2). In order to compare the species abundance between both samples, OTUs were picked to reflect the bacterial species richness. The species richness ranged from 344 to 398. Shannon, chao1, and Simpson indices were calculated to evaluate the diversity of the species at different temperatures in both reactors. From Table 2.2, it could be concluded that the bacterial diversity in both reactors increased with decreasingtemperature.

In order to evaluate whether the produced data was enough to cover all species as well as to represent species abundance, rarefaction curves were plotted. The rarefaction curves are plotted in Figure 2.2. The nearly asymptotic nature of the curves as the number of reads on the x-axis increased demonstrate that the number of reads obtained for all samples from sequencing provided sufficient coverage of the overall microbial communities.

Table 2.2 Summary of sequencing processing and results.

Sample Name	**Total sequences**	**OTU counts**	**Shannon index**	**Chao1**	**Simpson index**
SR-35	19798	344	2.60	350.89	0.119
SR-21	15549	367	2.98	382.35	0.075
AR-35	18929	357	2.24	407.38	0.248
AR-21	16463	398	2.49	364.15	0.168

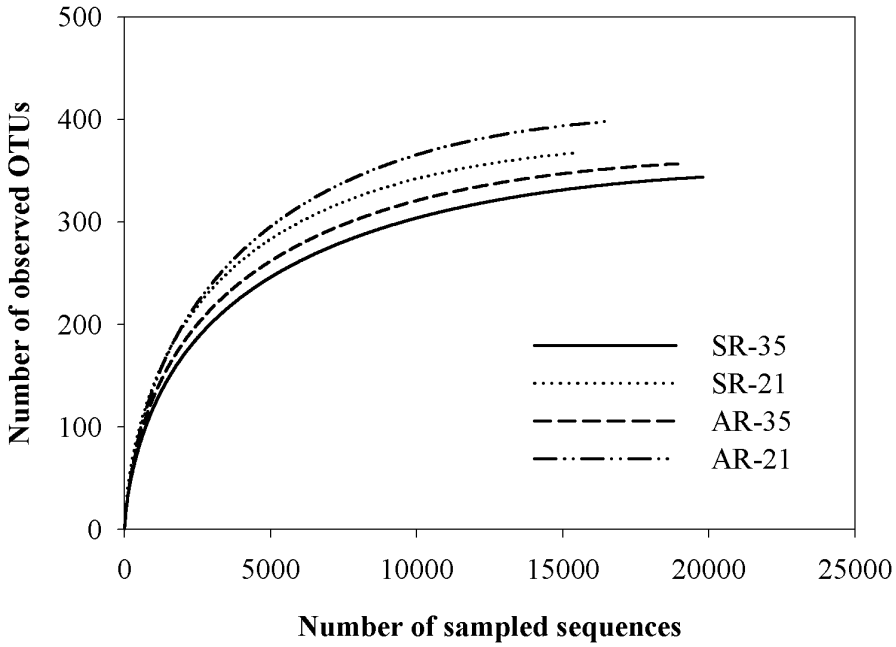

Figure 2.2 Rarefaction curves of the sequencing results from two reactors (SR and AR) under 35 ℃ and 21 ℃.

The microbial community results are illustrated in Figure 2.3 for both reactors at different temperatures. The dominant phylum noticed were *Chloroflexi, Planctomycetes, Bacteroidetes, Verrucomicrobia, Acidobacteria,* and *Proteobacteria* for all four samples. In general, the percentage of *Chloroflexi* decreased in both reactors when the temperature decreased from 35 ℃ and 21 ℃. For the SR, the relative abundance of *Chloroflexi* and *Planctomycetes* decreased from 31.4% to 25.0% and from 22.2% to 15.1%, respectively. On the other hand, in the SR reactor the relative abundance of *Proteobacteria* increased from 22.9% to 29.3%, *Bacteroidetes* from 3.0% to 9.1%, and *Verrucomicrobia* from 0.19% to 1.1%. In the case of AR, when it was operated at 35 ℃, the most abundant species were found to be the *Chloroflexi*, which account for 54.1% of the total reads. Surprisingly, the abundance of *Planctomycetes* was only accounted for 16.4% in the AR.

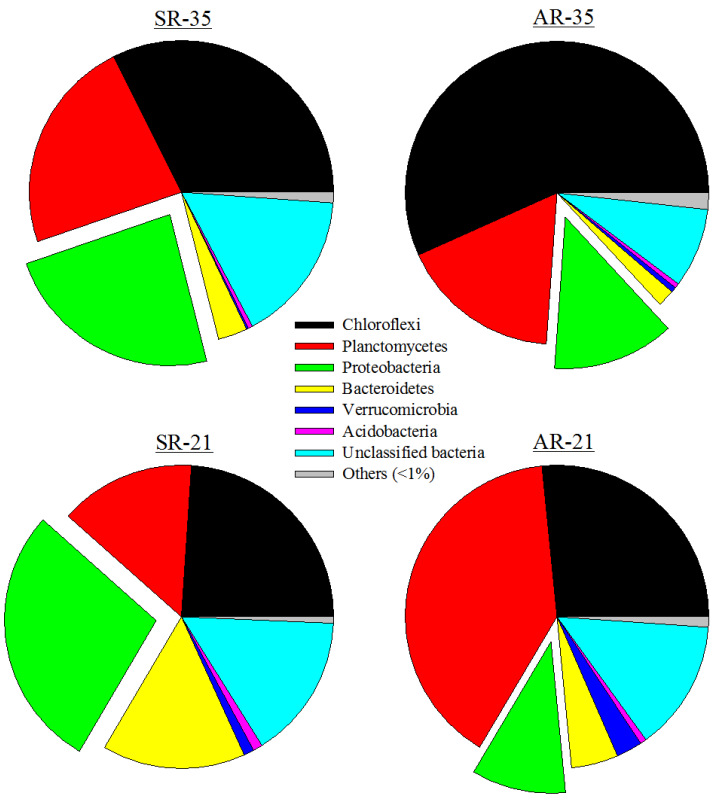

Figure 2.3 Relative abundance of different phyla in the two reactors (SR and AR) operated under 35 ℃ and 21 ℃.

As mentioned previously, during ~300 days of operation, the SR and AR were able to remove (a) 0.54 ±0.093 and 0.60 ±0.111 kg-N m^{-3} d^{-1}, respectively, at 35 ℃ and (b) 0.57 ±0.061 and 0.56 ±0.103 kg-N m^{-3} d^{-1}, respectively, at 21 ℃. The similar performance of SR and AR might be a result of the similar microbial communities, represented in both reactors by three phyla: *Planctomycetes*, *Chloroflexi*, and *Proteobacteria*. Additionally, the morphology of suspended growth granules/flocs and attached growth biofilms in PN/A system have been reported to be similar: the internal AMX zone shielded by an outer AOB rim (Chu et al., 2015; Gilbert et al., 2013; Persson et al., 2014; Vlaeminck et al., 2010). This morphological structure ensures minimum exposure to DO for AMX enabling them to be active even under aerobic conditions. In this research, AR was

operated under continuously aerated conditions, showing that the "protection" of AMX is more effective in attached growth PN/A configurations.

Temperatures and configures impact *Planctomycetes*

In this study, a single species of AMX, *Brocadia fulgida*, accounted for more than 99% of all species under *Planctomycetes* for all four samples. Thus, the relative abundance of *Planctomycetes* could be used as the indicator of *Brocadia fulgida*. As shown in Figure 2.3, with the decrease in temperature, the amount of *Brocadia* in the SR decreased from 18.7% to 10.8% in contrast to the AR wherein *Brocadia* grew from 13.2% to 33.7%. It is worth mentioning that the two reactors were seeded with different sources of biomass, while the dominant AMX species for both reactor configurations were *Candidatus* Brocadia fulgida. This suggested that *Ca.* B fulgida might be selective over other anammox species in single-stage systems fed with the same feed. Corroborating our finding, similar results have been also reported by other researchers where these researchers concluded that *Brocadia* sp. adapt better in engineered ecosystems because of their lower affinity for ammonium nitrite and higher tolerance to DO with higher growth rate (Hendrickx et al., 2014). Moreover, *Brocadia* sp. are able to thrive at elevated COD concentrations and can coexist with heterotrophic bacteria (Kartal et al., 2008).

Temperatures and configurations impact *Proteobacteria*

Various orders of species under *Proteobacteria* were detected as *Bukholderiales*, *Nitrosomonadales*, *Rhodocyclales*, and a group of uncultured gamma *Proteobacteria*.

Figure 2.4 shows how different *Proteobacteria* species occupied the phylum in both the reactors.

When operated at 35 ℃, *Nitrosomonas europaea*, classified under *Nitrosomonadales*, was found to be the responsible AOB in both the SR (3.15% of total reads) and the AR (3.41% of total reads). However, when the temperature was decreased to 21 ℃, another *Nitrosomonas* sp. was detected together with *N. europaea* for aerobic ammonium oxidation. This might indicate that the emerging *Nitrosomonas* sp. is more tolerant to lower temperature although more evidence is needed for a definitive conclusion. *Bukholderiales* and *Rhodocyclales* were regarded as heterotrophs able to denitrify nitrate to nitrogen gas under anoxic condition with an organic carbon source. The presence of denitrifying bacteria in both the SR and AR might be due to the organic carbon present in the influent and the organics produced from decay of cells.

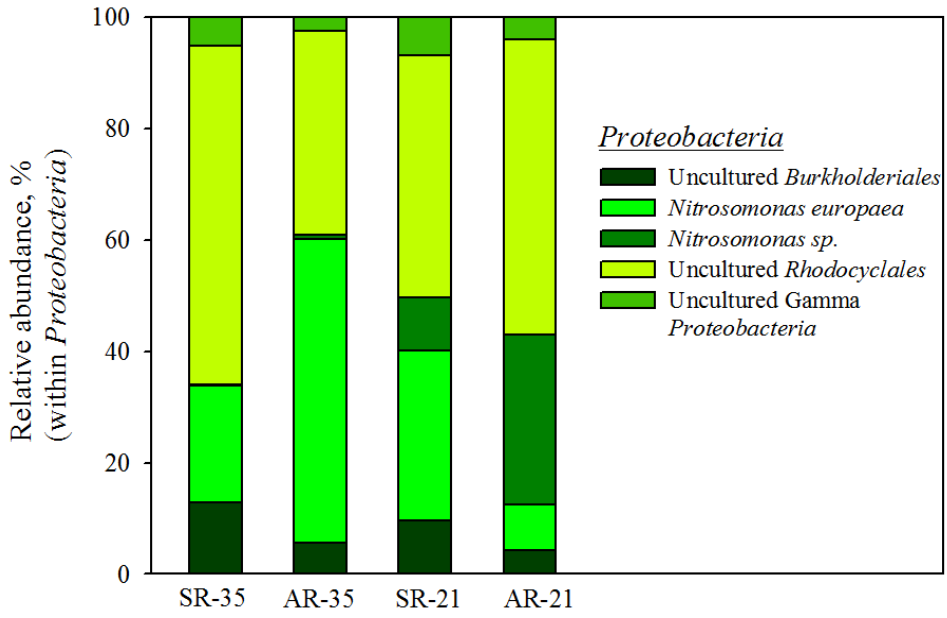

Figure 2.4 Relative abundance of the different species within *Proteobacteria*.

In the biofilm system, *Nitrosomonas*, which have low affinity constant for NH_3, might have a growth advantage over other AOB (Karkman et al., 2011). Moreover, previous studies showed an increase in nitrifying biofilm thicknesses with exposure to cold temperatures in a nitritation system (Bjornberg et al., 2009; Delatolla et al., 2012). In this case, the total *Nitrosomonas* population did not increase significantly in the AR over the 300 days of operation. It can be concluded that in PN/A systems, where AMX grow inside the floc or the granule/biofilm and AOB grow outside, the thickness of the outer layer of AOB was constant due to the shear force of air flow. In SR, the doubling of *Nitrosomonas* population and the decrease of *Brocadia* possibly hints that separating the excess AOB from the system via shortening the settling time may not be efficient. The expanding of AOB population in the reactors could work over the short term (e.g., the 300 days in this research) without causing any nitrite accumulation. However, in full-scale applications, which generally operate over multiple years, a more efficient sludge separation method (i.e., hydrocyclone) is necessary. Besides, DO can be another factor controlling the process as, by limiting the ammonium oxidation rate, ensures a balance in the activity of AOB with AMX.

With the decrease in temperature, an increase was observed for a different *Nitrosomonas* sp. Similar results have been reported by Gilbert et al. (2014) highlighting the coexistence of *Nitrosomonas* sp. JL21 with *N. europaea* when the temperature of a PN/A reactor gradually decreased from 20 ℃ to 10 ℃. This indicated that *N. europaea* might be less tolerant to cold condition. The emerging *Nitrosomonas sp*. is in the same branch as *N. nitrosa,* which has an advantage in environments with fluctuating oxygen levels or during the absence of oxygen (Limpiyakorn et al., 2005).

Temperatures and configurations impact *Chloroflexi* and other heterotrophs

Under the phylum *Chloroflexi* ahighly dominant species of uncultured *Anaerolineales* was detected ranging from 83.1%~89.6% in both reactors with the remainder being unknown *Chloroflexi* species. The uncultured *Anaerolineales* found in this study was closely related (100% homology) to an uncultured *Chloroflexi* which was isolated from an anammox biofilm reactor reported by Kindaichi et al. (2012). So far, *Chloroflexi* has been reported in many PN/A bioreactors, including suspended growth systems (Chu et al., 2015; Gonzalez-Martinez et al., 2014), and attached growth systems (Gilbert et al., 2014; Park et al., 2010). The species classified under the phyla *Chloroflexi* belonged to the order *Anaerolineales.* These heterotrophic species are known to be thermophilic with a relatively slow growth rate and a need to associate with other microbes for efficient growth (Yamada and Sekiguchi, 2009).

Based on the fact that in any bioreactor, including PN/A systems, soluble microbial products (SMP) and extracellular polymeric substances (EPS) are inevitable (Laspidou and Rittmann, 2002), the presence of *Anaerolineales* is not a surprise. The carbon source for species belonging to phyla *Chloroflexi* was probably from the decay of bacterial cell materials in the system (Ni et al., 2012; Kindaichi et al., 2012). A decline in abundance of *Anaerolineales* species for both the reactors was observed as the result of the low temperature. In the SR *Anaerolineales* decreased from 27.25% to 22.35% and in the AR *Anaerolineales* declined from 49.97% to 22.17%, suggesting this uncultured *Anaerolineales* are sensitive to lower temperature. Compared to suspended growth, the attached growth system maintains an extremely long SRT, thus, the higher decay rate of microorganisms accelerates the growth of *Chloroflexi*. This may explain why the highest

abundance of *Chloroflexi* was found in the AR when operated at 35℃. At room temperature, the growth of the thermophilic bacteria was limited by the lower temperature, which resulted in the decrease of relative abundance of *Chloroflexi* in both the SR and AR at low temperatures. Furthermore, *Chloroflexi* has been reported to be the backbone for the three-dimensional microbial aggregates called flocs and to reinforce the granule structure in suspended growth systems (Cho et al., 2010). *Chloroflexi*are also involved in spatial organization in the biofilm of attached growth systems (Botchkova et al., 2014). Therefore, it could be inferred that at the beginning of the operation, the relatively high temperature favored the growth of *Chloroflexi*, which, in turn, was involved in the growth of biofilm in the AR.Overall, irrespective of whether AR or SR, *Chloroflexi* were the species responsible for creating the structure in the PN/A system. However, the role of *Chloroflexi* on the ammonium removal rate of the reactors remains to be explored.

Besides *Chloroflexi*, other microbes present in the PN/A reactors also compete for SMP and EPS. These microbes include *Denitratisoma* and *Ignavibacterium*. *Denitratisoma* are denitrifiers able to use complex organics as their carbon source to reduce nitrate to nitrogen gas. However, the ratio of produced NO_3^- to removed NH_4^+in both reactors did not show any remarkable sign of heterotrophic denitrification. The reported value of 0.11 for molar ratio of NO_3^- production to NH_4^+ depletion was recorded and no NOB were detected in either system. *Ignavibacterium* albumhas been proposed as a facultative anaerobe, moderately thermophilic, and a heterotrophic fermenter (Liu et al., 2012). Moreover, this bacterium has been reported carrying the gene *NifAH*, which encodes for the key enzyme responsible for dissimilatory nitrate reduction to ammonia (DNRA) (NCBI 2014 gene IDs for *A. thermophile nrfA, nrfH*: 10172349, 10171709;

Ignavibacterium album strain JCM 16511 NC_017464 *nrf*A: NC_017464 region: 957373-958887) (Liu *et al.,* 2012). This finding suggested this bacterium has the potential to perform DNRA when an appropriatecondition is provided (i. e., absence of oxygen, and available nitrate and organic substrates (Sgouridis et al., 2011)). To confirm the metabolic pathway of *Ignavibacterium*, further research is needed.

Summary

This research provided a detailed insight into the effect of temperature on microbial community structure for different PN/A reactor configurations.

The suspended growth (SR) and attached growth (AR) systems had similar microbial communities enabling them to perform a similar high nitrogen removal during the 300 days of operation at 35 ℃ and 21 ℃.

In supplement PN/A systems for nitrogen removal in wastewater treatment plants, operating at a relatively higher temperature in the startup process would allow the growth of *Chloroflexi*, which could help construct the backbone structure for all the communities and accelerate the startup process.

In the long run, the nitrogen removal rate in the PN/A systems did not deteriorate when the operation temperature decreased to 21 ℃. In fact, both the reactors adapted to the lower temperature within 2 weeks, showing that in full-scale application it is feasible to bring down the bioreactor temperature to reduce the operation cost.

When the temperature shifted from 35 ℃ to 21 ℃, the AOB communities in both the AR and SR shifted from a single phenotype of *N. eurapaea* to a mixed community of *N. europaea* and another *Nitrosomonas* sp. As for ananmmox related bacteria, Brocadia

fulgida were always the dominant species in both the SR and AR regardless of the temperature.

CHAPTER 3

EFFECT OF TEMPERATURE VARIATIONS AND SULFIDE TOXICITY ON THE MICROBIAL ECOLOGY AND KEY FUNCTIONAL GENES IN A PARTIAL NITRITATION-ANAMMOX REACTOR

Introduction

The wastewater industry is poised for significant innovation driven by the desires for resource recovery, energy neutrality, smaller carbon footprints, and reduced greenhouse gas emissions in wastewater treatment plants. Due to the potential of increased eutrophication and toxicity to water life, the growing public concern for environmental protection has pushed traditional wastewater treatment plants (WWTPs) to emphasize the need to remove carbon, nitrogen, and phosphorus. This approach has resulted in energy reserve depletion, production of waste activated sludge, and greenhouse gas (GHG) emissions (Wang et al., 2015). In just the last five years, municipal wastewater utilities in North America have accelerated their adoption of partial nitritation/anammox (PN/A) as an energy efficient process and as a viable means to economically remove nitrogen from the liquor generated during dewatering of anaerobically digested sludge. Efforts are now in place to evaluate the feasibility of PN/A systems for mainstream applications.

As a promising answer to the global nutrient challenge, PN/A systems have

provided a much needed innovation in wastewater treatment. Still, how these systems will respond to external perturbations such as temperature variations and changing feed composition is not fully understood. The success of a PN/A system depends upon a perfect synergy between ammonia oxidizing (AOB) and anammox (AMX) bacteria (Figure 3.1), which may notably be impacted by temperature decreases and presence of inhibitors in the wastewater.

Based on the physiology of AOB and AMX, PN/A reactors are generally operated at mesophilic temperatures of about 25 to 35 ℃ (Lotti et al., 2015; van Hulle et al., 2010). The relatively higher temperature not only favors the activity of slow-growing AMX bacteria but also assists AOB to outcompete the nitrite oxidizing bacteria (NOB) in the PN/A reactors. Despite the reported decrease in activity at lower temperatures (<25 ℃) (Dosta et al., 2008; Vázquez-Padín et al., 2011), it is a fact that in natural ecosystems, especially in marine environments, anammox bacteria have been found to thrive at low temperatures (<10 ℃) (van de Vossenberg et al., 2008), indicating the possibility of developing an anammox process for wastewater treatment even at low temperatures.

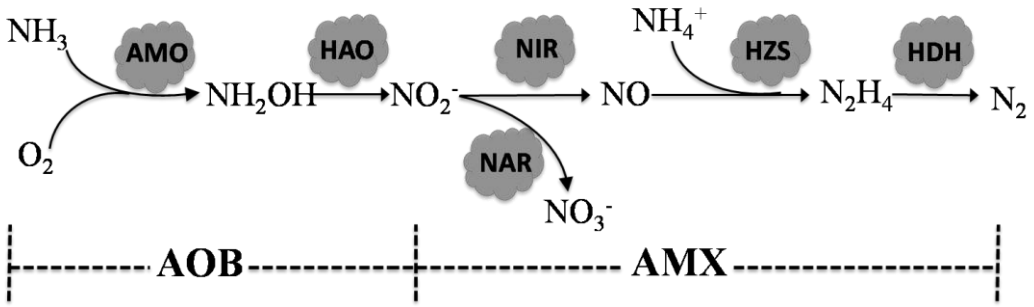

Figure 3.1 Nitrogen-related metabolic pathways in PN/A process fulfilled by AOB and AMX. AMO, Ammonia monooxygenase; HAO, Hydroxylamine oxidoreductase; NIR, nitrite reductase; NAR, Nitrate reductase; HZS, Hydrazine synthesis; HDH, hydrazine dehydrogenase.

Corroborating this finding, Hu et al. (2013) observed that the optimal temperature for biomass in a PN/A system shifted from 35 ℃ to 25 ℃ when operated at 12 ℃ for more than 300 days. Similar studies by Gilbert et al. (2014) and Persson et al. (2014) on the potential for biofilm media tolerance of temperatures as low as 10 ℃ showed that although the removal efficiency of the reactors decreased, stable anammox activity kept the reactor performance consistent throughout. Furthermore, Gilbert et al. (2015) observed a slight decrease in the temperature coefficient for four different lab-scale PN/A reactor configurations after 25 weeks adaptation, indicating that it is feasible to adapt the biomass in a PN/A system to cold temperatures over the course of months or years. However, in real world applications, the cold period would only last for several months, which may not be long enough to support an adaptation process. In almost all of these efforts the system performance was reported in terms of removal efficiencies and activity coefficients with little or no information on the functional genetic activation of key populations involved.

The wastewater constituents can also affect PN/A system performance. Sulfide is of concern. This constituent is generally present in anaerobic environments, and thus can interfere with the whole microbiology and chemistry of PN/A systems. Sulfide toxicity to the nitrifying population is well documented (Esoy et al., 1998). Yet the information on the effect of sulfide on anammox populations is scarce. More importantly, how sulfide affects the synergy between AOB and anammox bacteria is not known. Sulfide is toxic at higher concentrations but can serve as an electron donor for denitrification at lower concentrations (Chen et al., 2008; Yin et al., 2015). At low sulfide concentrations (threshold unknown), autotrophic denitrification can take place and can compete with

anammox bacteria for the available nitrite (Chen et al., 2008). An increased sulfide to nitrate ratio can also promulgate dissimilatory nitrite reduction to ammonium (DNRA), resulting in diverting nitrite from the PN/A pathway.

Although there have been few studies focusing on the effect of temperature on PN/A systems and very little on sulfide, not much has been reported on how these external perturbations affect the synergism between AOB and AMX bacteria at a functional gene level. A mechanistic study involving the responsible functional genes is missing. This current study was conducted to facilitate the development of a better understanding of the effect of temperature and sulfide on the efficiency and stability of the PN/A process in terms of activities and key functional gene expressions. The information presented in this manuscript will provide parameters for developing definite guidelines for wastewater practitioners, as well as help them in understanding upsets in the PN/A process.

Material and methods

Reactor operation

A 9 L PN/A reactor was inoculated with biomass obtained from the Hampton Roads Sanitation District (HRSD) York River DEMON® reactor (Seaford, VA) and maintained with an initial concentration of mixed liquor suspended solids (MLSS) of 3000 mg/L. Anaerobic digester filtrate from a local municipal wastewater treatment plant, Central Valley Water Reclamation Facility (CVWRF, Salt Lake City, UT), was fed to the PN/A reactor. The dissolved oxygen (DO) concentration was maintained below 0.2 mg O_2/L by constantly bubbling air in the mixed liquor using an aquarium pump. The reactor

was operated at two batches per day, with 2.25 L of fresh filtrate fed to the reactor in the beginning and 2.25 L drawn out at the end of each batch (i.e., 25% volume exchange ratio). This resulted in a 2-day hydraulic retention time (HRT). The settling time was initially set at 10 min to allow most of the AMX granules to settle and to wash out a part of the flocculent biomass which mostly contained AOB.

Temperature changes

The reactor was operated at 35±2 ℃ and kept at this value for 74 days using a circulating water bath. Thereafter, the temperature was dropped to 21±2 ℃ in 18 h and run at this temperature for 27 days. A further drop in temperature was done to 13±2 ℃ for 15 days. A thermometer was kept inside the reactor to monitor the actual temperature of the mixed liquor.

Sulfide toxicity tests

Serum bottles were used to evaluate the short-term effect of sulfide stress on AOB and AMX. The sulfide inhibition was evaluated by measuring the maximum bacterial activity at different sulfide doses.

To evaluate sulfide inhibition on AMX, 50 mL of biomass from the ongoing PN/A reactor was transferred to three sets of 70 mL serum bottles. One of these bottles was used as a control (i.e., no sulfide spike) and the second and third bottles were supplemented with 5 mg/l S^{2-} (low dose) and 30 mg/l S^{2-} (high dose) in the form of $Na_2S{\cdot}9H_2O$, respectively. All three batches were run in triplicate and supplemented with predetermined concentrations of ammonium and nitrite. pH was adjusted in the median

range of 7.5~8. Anoxic conditions were ensured through beginning by purging the mixed liquor in the serum bottles with nitrogen gas for 10 min. The bottles were capped airtight using a butyl rubber cap. The bottles were incubated at 35^{o}C on a shaker to ensure optimum conditions. Mixed liquor samples were collected at t = 0 h and t = 3 h (at the end of the test) to measure AMX activity in terms of ammonia oxidation.

For sulfide inhibition tests on AOB, the same protocol used for AMX was employed except that the bottles were supplemented with ammonium and kept open to the atmosphere to ensure aerobic conditions. Air was not bubbled in the mixed liquor so as not to cause a loss of sulfide through stripping. Mixed liquor samples were collected after every hour from the beginning of the test.

Mixed liquor samples from both AOB and AMX tests were also stored in RNA later for further use in functional gene expression analysis during each batch test.

AOB and AMX activity tests at different temperatures

AOB and AMX activities at different temperatures were measured in separate batch reactors with 50 mL of biomass taken from the main 9 L PN/A reactor. This was primarily due to distinctly separate AOB and AMX activities under truly aerobic and anoxic conditions as the main 9 L reactor was continuously aerated and open to the atmosphere. For the batch activity measurements during temperature changes, biomass was harvested from the reactor at day 50, 98, and 116 when the reactor was being operated at 35±2 ℃, 21±2 ℃, and 12±2 ℃, respectively. The biomass was washed twice, spiked with 50 mL of feed containing either NH_4^+-N (for AOB activity) or a mixture of NH_4^+-N and NO_2^--N (for AMX activity).

In order to determine the AOB activity, the biomass was spiked with diluted and 0.22 μm filtered raw filtrate to obtain a final NH_4^+ concentration of ~50 $mg_N L^{-1}$. The mixed liquor was sparged with air to provide DO as well as to keep the biomass suspended.

To determine the maximum specific activity of anammox, the washed biomass was spiked with the effluent collected from an in-house partial nitrification reactor described elsewhere (Kotay et al., 2013). This partial nitrification reactor is also fed with same filtrate the 9 L PN/A reactor was receiving. The serum bottles for AMX activity tests were sealed and purged with N_2 for 10 min with the aim to remove residual DO.

All activity tests were done in triplicate and at the same temperatures under which the PN/A reactor was operated by placing the batch reactors in water baths of the selected temperatures. Sodium bicarbonate was added to the serum bottles to buffer the pH during the tests, and pH was checked at both the beginning and the end to make sure the reactions were under median condition (pH = 7~8). Mixed liquor samples were also stored in RNA later for further use in functional gene expression analysis during each batch test.

Analysis of bacterial community compositions

Biomass was collected from the reactor at day 50, 98, and 116, respectively, and the genomic DNA was extracted from biomass using PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) as described in the manufacture's instruction. The nucleotide concentration was examined by a Nanodrop spectrophotometer (Nanodrop Technologies Inc., USA). The genomic DNA (~20 ng μL^{-1}) was sent to Research and Testing

laboratory, TX, for amplicon sequencing. The primers used for 16S rRNA V4 region amplification were 515F and 806R as detailed in Chapter 2 using MiseqIllumina sequencer.

The raw reads obtained were interleaved using PEAR Illumina paired-end read merger (Zhang et al., 2014). Low quality sequences with Phred Score below 25 were removed through an internally developed quality-trimming algorithm. Postquality trimming the reads were sorted by length from longest to shortest. Prefix de-replication and clustering at 4% divergence was performed using USEARCH algorithm (Edgar, 2010), wherein sequences shorter than 100bps were ignored, with no minimum cluster size to allow singleton clusters in the output. All clusters containing <2 members were removed from the datasets. UPARSE operational taxonomic units (OTU) selection algorithm (Edgar, 2013) was used to classify the large number of clusters into OTUs. Chimera check was performed on each selected OTU using UCHIME (Edgar et al., 2011). In order to determine the taxonomic information, the sequences were run through the USEARCH global alignment program (Bokulich et al., 2014), wherein quality filtered reads were mapped to their corresponding nonchimeric clusters. Output generated was then assigned taxonomic profile using an internally developed script. In order to compare the species abundance between the samples collected at different temperatures, Shannon, Chao1, and Simpson indices were calculated using EstimateS (Colwell, 2000).

Functional gene expression analysis

The key functional genes expressed were also quantified at different temperatures using total RNA extraction followed by reverse transcription and quantitative PCR. As

shown in Figure 3.1, a network of functional genes is involved in the complete execution of the PN/A process. In the ammonia oxidation pathway, amoA catalyzes the first step of ammonium oxidation and has been used as a universal biomarker to track the ecology of AOB (Rotthauwe et al., 1997). In anammox metabolism, the hzs gene cluster is responsible for the synthesis of hydrazine from ammonium and nitric oxide (Strous et al., 2006). This gene cluster is not present in other nitrogen cycling bacteria and, as a result, hzs is a very good biomarker for anammox bacteria (Harhangi et al., 2011). Therefore, ammonium monooxygenase (*amoA*) for AOB (Rotthauwe et al., 1997; Purkhold et al., 2000) and hydrazine synthase (*hzs*) for AMX are used as key biomarkers to study the diversity of these bacteria (Harhangi et al., 2011).

Total RNA was extracted from ~0.7 g of the biomass (same date as DNA extraction and activity test) by means of a PureLink RNA mini kit (Life technology, NY). Genomic DNA was removed by an on-column PureLink DNase set (Life technologies, NY). The quantity and quality of RNA were checked on a bioanalyzer (Agilent, USA). Reverse transcription (RT) was conducted using SuperScript VILO^{TM} cDNA synthesis kit (Life technology, NY) by adding an equal amount of 1.25 μg RNA. Quantitative real-time PCR (qPCR) was done to quantify the 16S rRNA and *amoA* genes of AOB, and the 16S rRNA and *hzsA* genes of AMX using different primer sets, listed in Table 1.5 (in Chapter 1).

Other analytical methods

As the concentrations of NO_2^- and NO_3^- in the influent of the PN/A reactor were negligible (< 5 $mg_N\,L^{-1}$) compared with the high content of NH_4^+ (900~1200 $mg_N\,L^{-1}$),

only the NH_4^+ concentration was monitored in the influent. For the effluent, NH_4^+, NO_2^-, and NO_3^- were monitored three times each week. NO_2^- and NO_3^- were analyzed by ion chromatography (chromatograph model, supplier), while NH_4^+ was measured using a HACH kit. The concentrations of total and volatile suspended solids were analyzed using standard methods (APHA, 1998).

Results

Impact of temperature on the PN/A reactor performance

The PN/A reactor was started at 35±2 ℃ and operated at this temperature until a steady state in terms of percentage total nitrogen removal was accomplished. The average NH_4^+ concentration in the influent to the PN/A reactor was 1195±68 $mg_N\ L^{-1}$ and the pH ranged from 7.5 to 8.3. The average NH_4^+-N load to the reactor was calculated to be 0.60±0.03 $kg_N\ m^{-3}\ d^{-1}$. The average MLSS and MLVSS in the reactor were 2310±700 mg L^{-1} and 1890±500 mg L^{-1}, respectively, over the course of operation during temperature changes and sulfide inhibition tests.

The reactor performance in terms of different nitrogen species is depicted in Figure 3.2. Over the 120 days period, the reactor operation could be divided into 3 phases with each phase characterized by the operating temperature of the reactor (Figure 3.2). In Phase I, the temperature was kept at 35±2 ℃, which represents a temperature at which most PN/A configurations are generally operated. During the first 74 days in Phase I, the reactor was able to remove NH_4^+ at a loading rate of 0.53±0.05 $kg_N\ m^{-3}\ d^{-1}$. A NO_2^--N accumulation up to 50.5 mg L^{-1} was observed by the end of day 20. In Phase II, the PN/A reactor was operated at 21±2 ℃.

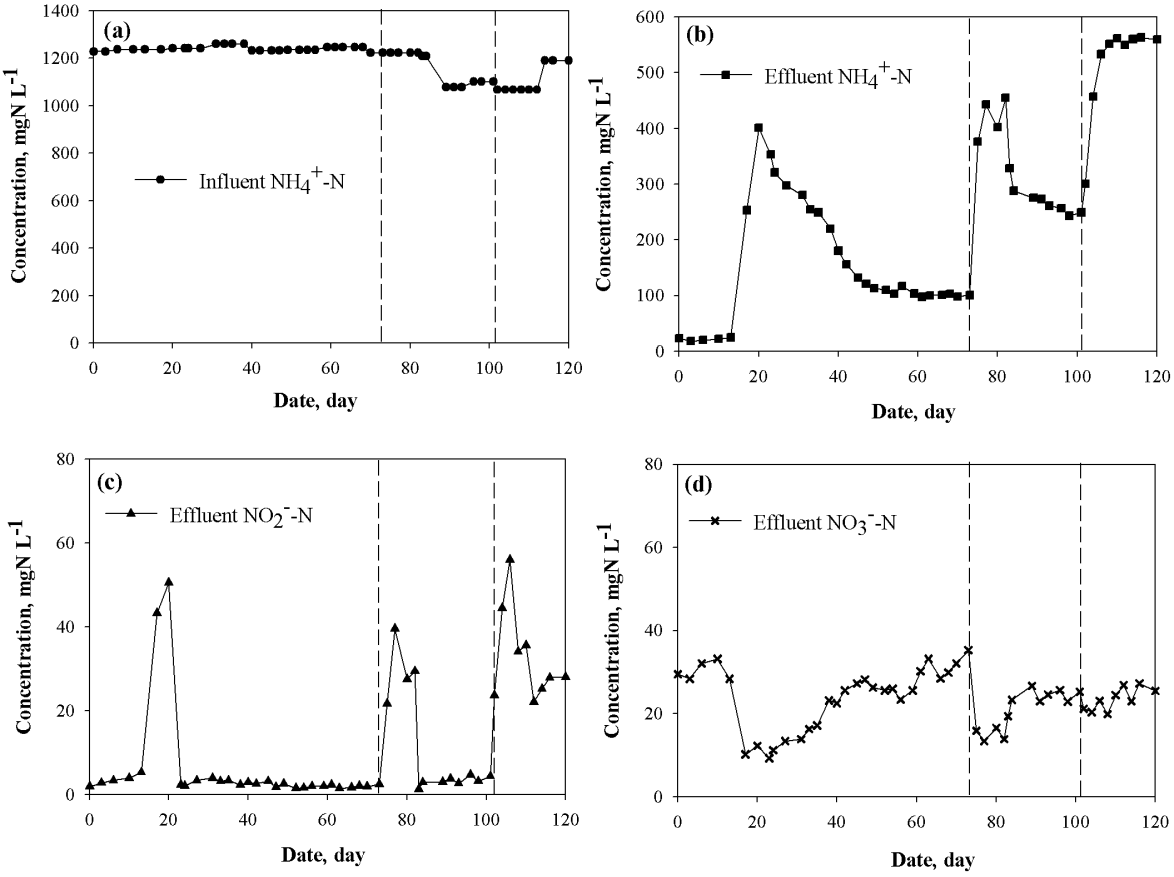

Figure 3.2 Nitrogen concentration characteristics of the PN/A reactor along the decreasing operational temperatures delineated. Phase I: 33-35 ℃, Phase II: 21-23 ℃, and Phase III: 13-15 ℃ for (a) influent ammonium (NH_4^+), (b) effluent NH_4^+, (c) effluent nitrite (NO_2^-) and (d) effluent nitrate (NO_3^-). Decreases of reactor efficiency are characterized by (transient) unfavorable accumulation of ammonium (NH_4^+) and nitrite (NO_2^-) in the effluent.

The temperature drop was accomplished by removing the circulating water bath and letting the reactor run at room temperature. As a consequence, the total NH_4^+ removal rate dropped from 0.53 ± 0.05 $kg_N\, m^{-3}\, d^{-1}$ to 0.36 $kg_N m^{-3}\, d^{-1}$ by day 77. A gradual recovery from the cold shock was observed thereafter. On day 102, the temperature of the reactor was further decreased to 13 ± 2 ℃ and, as a result, the NH_4^+ removal dropped significantly; NO_2^- accumulation up to 56 mg L^{-1} was recorded in the reactor.

To protect AMX bacteria from high NO_2^--N toxicity, the air flowrate was

decreased. Despite the air flow adjustment, the NO_2^-concentration in the reactor remained high (> 20 mg L^{-1}) and the reactor was deemed unstable at this stage.

AOB and AMX activities and gene expressions at different temperatures

Figure 3.3 summarizes the activities of AOB and AMX measured ex-situ at different temperaturesin aerobic and anoxic serum bottles, respectively. These biomass specific activities (expressed as g_N g_{VSS}^{-1} d^{-1}) were calculated by dividing the measured volumetric rates of NH_4^+-N disappearance by the concentration of biomass (expressed as MLVSS) present in the serum bottles.

When the PN/A reactor was operated at 35±2 ℃, the activities of AOB and AMX were 0.405±0.020 and 0.387±0.070 g_N g_{VSS}^{-1} d^{-1}, respectively (Figure 3.3).

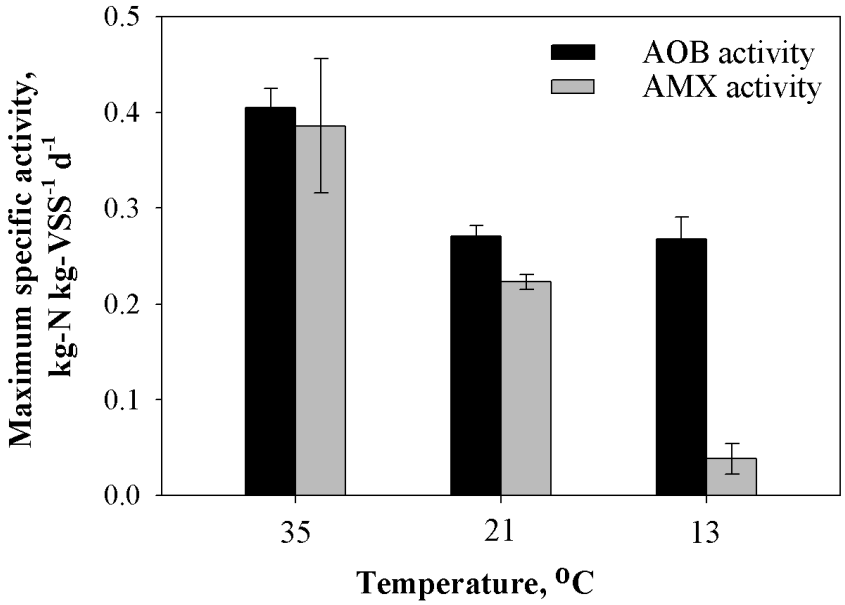

Figure 3.3 Changes in average biomass specific activities of aerobic (AOB) and anaerobic (AMX) ammonium-oxidizing bacteria along the decreasing operational temperatures delineated by Phase I: 33-35 ℃, Phase II: 21-23 ℃, and Phase III: 13-15 ℃.

The $NO3^-$-N concentration in the mixed liquor always remained less than 0.1 mg/L (data not shown) during the 3 h of aerobic conditions, the AOB activity batch tests indicating a negligible oxidation of nitrite into nitrate. When the temperature was dropped to 21 °±2 ℃, both AOB and AMX activities decreased to 0.271±0.011 and 0.223 ± 0.080 $g_N\ g_{VSS}^{-1}\ d^{-1}$ resulting in 33% and 42% of activity losses versus operation at 35 ℃, respectively. To our surprise, no significant difference in AOB activity was observed when temperature further dropped to 13 ℃±2 ℃. In contrast, AMX activity significantly dropped to 0.039±0.016 $g_N\ g_{VSS}^{-1}\ d^{-1}$ (i.e., 90% of activity loss versus operation at 35 ℃). The expressed abundances of *amo*A and *hzs*A were calculated based on the comparative C_T method, which is also known as $2^{-\Delta\Delta CT}$ method developed by Schmittgen et al. (2008) as detailed in Equation (16) hereafter:

$$\text{fold change} = 2^{-[(\text{Ct1 of target}-\text{Ct1 of reference})-(\text{Ct0 of target}-\text{Ct0 of reference})]} \quad (16)$$

where C_T is the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. The reference gene is the internal control gene, which is expressed steadily under all conditions without any dependency on external environmental changes. In this research, the 16S rRNA gene of AMX and AOB was chosen as the reference gene. Further, the expressed gene abundances at lower temperatures were normalized to the gene abundance at 35±2 ℃ which means the expressions at 35±2 ℃ were treated as 100% for comparison purposes. Expression of the *amoA* and *hzs* functional genes was measured along with the changes in bacterial activities of AOB and AMX under the different temperatures (Figure 3.4). AOB displayed antagonist trends of biomass specific activities and *amoA* gene expression under the temperature constrain.

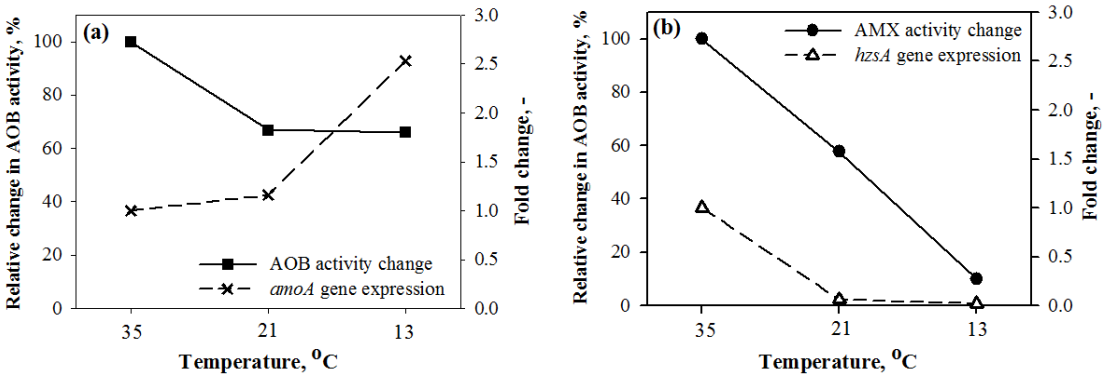

Figure 3.4 Relationship between bacterial activity and functional gene expression of aerobic (AOB, a) and anaerobic (AMX, b) ammonium-oxidizing bacteria under decreasing operational temperatures.

When the temperature was decreased from 35±2 ℃ to 21±2 ℃, the AOB activity decreased 3-fold while the *amoA* gene expression slightly increased 1.2-fold (Figure 3.4(a)). The expression of the *amoA* gene was even more pronounced (2.5-fold) when the temperature was further dropped to 13±2 ℃.

In contrast, AMX activities and *hzsA* gene expressions concomitantly decreased with lower temperatures (Figure 3.4(b)).The increase in *amoA* gene expression with decreasing temperature is intriguing. However, a close search in the available literature related to nitrification inhibition suggests that AOB tend to overexpress the *amoA* gene under stressful conditions. For example, Park and Ely (2009) demonstrated overexpression of the *amoA* gene in *Nitrosomonas europaea* under small concentrations of cyanide. Likewise, Radniecki et al., (2009) also recorded overexpression of the *amoA* gene in the presence of zinc chloride. These researchers hypothesized that de novo synthesis of the *amoA* gene is needed to overcome external stress (Radniecki et al., 2009). In light of these recent findings, it is not surprising to observe overexpression of the *amoA* gene at reduced temperatures.

Composition of the overall microbial community

Before the PN/A reactor was subjected to temperature changes and sulfide toxicity, the overall bacterial community was studied using 16S rRNA gene targeted high throughput amplicon sequencing. Since temperature changes and sulfide toxicity tests were made on rather short term (<1 SRT), it was assumed that the overall microbial community would be fairly constant over these changes. Nevertheless, samples for high throughput amplicon sequencing were collected at different temperatures.

Gradual changes in overall bacterial community composition were identified by computation of diversity indices at operational taxonomic unit (OTU) level. The decrease in temperature from 35℃ to 13℃ resulted in a drop in the number of OTUs detected in the amplicon sequencing dataset from 441 to 166, which is also expressed by the chao1 richness values of 445 and 167, respectively (Table 3.1). The Shannon H' index displayed an only slight decrease in α-diversity from 2.67 to 2.38. Moreover, the chao1 values were almost identical to the OTU counts, indicating a sufficient depth of sequencing to cover the whole diversity. Analysis at phylum level did not display significant changes over the 120 days of operation of the PN/A reactor under decreasing temperatures (Figure 3.5(a)).

Table 3.1 Summary of sequencing processing and results for the samples at different temperature.

Temperature	**OTU counts**	**Shannon index**	**Chao1**	**Simpson index**
35	441	2.67	444.75	0.184
21	286	2.43	293.23	0.224
13	166	2.38	166.48	0.239

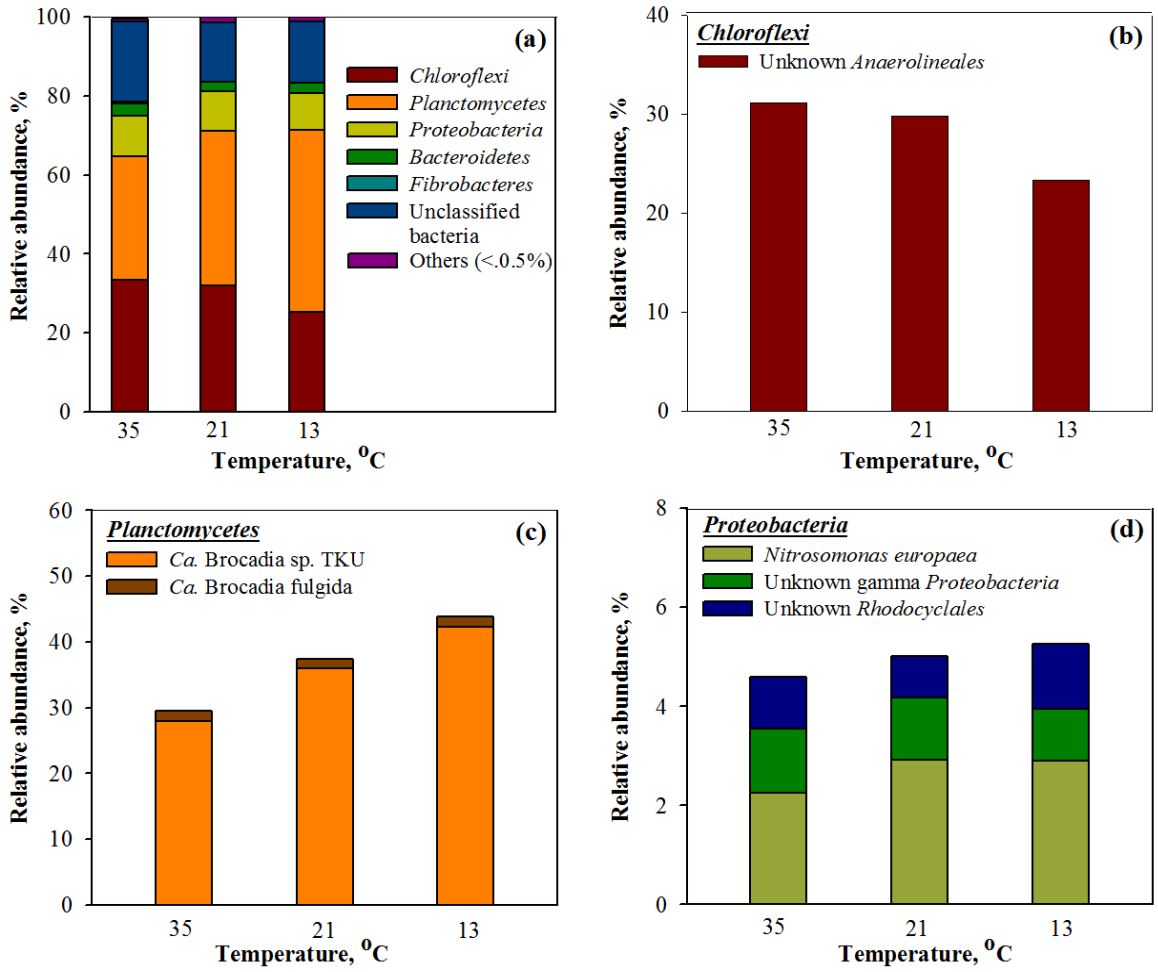

Figure 3.5 Composition and gradual changes of the bacterial community of the PN/A reactor along the decreasing operational temperatures: (a) overall composition at phylum level; (b) dominant genus under the phylum of *Chloroflexi* affiliating with unknown *Anaerolineales*; (c) the dominant species under the phylum of *Plantomycetes* affiliate with the anammox genus "*Ca.* Brocadia"; and (d) the dominant species under the phylum of *Proteobacteria* affiliates with AOB related to *Nitrosomonas europaea*. Phyla with read abundances less than 0.5% were summed together and are represented as "others" in Panel "a" in Figure 3.5(a).

The predominant phyla affiliated with *Planctomycetes* (from 31.3 to 46.1% of relative abundance), C*hloroflexi* (33.3 to 25.2%), and *Proteobacteria* (10.2 to 9.5%). Apart from these three abundant phyla, accompanying phyla affiliated with *Bacteroidetes* (3.2 to 2.4%), and *Fibrobacteres* (0.5 to 0%).

At genus level, unknown *Anaerolineales* accounted for almost 93~94% of the phylum of *Chloroflexi* under all temperatures (Figure 3.5(b)). More than 99% of the bacteria under the phylum of *Planctomycetes* were identified as "*Ca.* Brocadia*"* (Figure 3.5(c)). The dominant proteobacterium that was most likely responsible for nitritation in the reactor was closely related to the genus *Nitrosomonas europaea*, which accounted for 2.2% of the total reads at 35℃, 2.9% at 21℃, and 2.9% at 13℃. Besides the genus *Nitrosomonas*, other organisms affiliating with the betaproteobacterial order of *Rhodocyclales* (possibly comprises denitrifiers) and gammaproteobacterial order of *Chromatiales* (purple sulfur bacteria) were also detected under the phylum *Proteobacteria*.

Impact of sulfide toxicity on the gene expression

Activities of both AOB and AMX were severely inhibited by sulfide and reflected a decreasing trend with increasing concentrations of sulfide (Figure 3.6). When spiked with 5 mg_S L^{-1} of S^{2-}, the ammonium oxidation rate mediated by AOB decreased by 19% from 0.150 ± 0.011 kg_N kg_{VSS}^{-1} d^{-1} to 0.122 ± 0.011 kg_N kg_{VSS}^{-1} d^{-1}. The AOB activity dropped remarkably to 0.021 ± 0.008 kg_N kg_{VSS}^{-1} d^{-1} at a sulfide concentration of 30 mg_S L^{-1}. The sulfide toxicity affected AMX performance in a negative way as well; a nearly 40% reduction in AMX activity was recorded at 5 $mg_S L^{-1}$of S^{2-} from 0.140 ± 0.014 kg_N

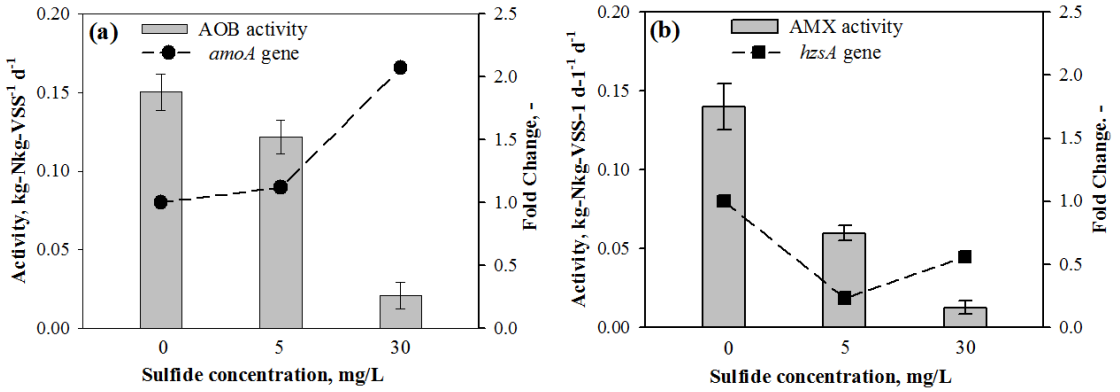

Figure 3.6 Impact of increasing sulfide concentrations on the transcriptional response of the two key functional genes of AOB and AMX in relationship with the activity of these target bacterial populations: (a) AOB activity and amoA gene expression; (b) AMX activity and *hzsA* gene expression.

kg_{VSS}^{-1} d^{-1} to 0.060 ± 0.005 kg_N kg_{VSS}^{-1} d^{-1}. Hence, AMX bacteria responded more negatively than AOB at mg_S L^{-1} of S^{2-}. For AOB, the patterns of *amoA* gene expression under increasing sulfide concentrations (Figure 3.6) were similar to those obtained under temperature decrease. *amoA* gene expression was indeed triggered (1.12-fold at 5 mg_S L^{-1} dosage and 2.07 at 30 mg_S L^{-1} dosage) by increasing sulfide concentrations. For AMX bacteria, the *hzs* gene expression dropped under sulfide toxicity similarly to the way it did under decreasing temperatures.

Discussion

Temperature effect on reactor and gene expressions

Theoretically, in continuously aerated sequencing batch PN/A reactors, any nitrite produced by AOB should be consumed by anammox bacteria immediately. In other words, the activity of AOB and of AMX are supposed to balance each other to obtain complete ammonium transformation to nitrogen gas. Either fading of AMX activity or flourishing of AOB activity will cause nitrite accumulation, which can in turn elevate the

inhibition to AMX (Dapena-Mora et al., 2007; Lotti et al., 2012; Strous et al., 1999). In this study, nitrite accumulation was observed in all three phases. First, it happened in the beginning of 35±2 ℃. Afterwards, nitrite accumulated when the reactor temperature was changed from 35±2 ℃ to 21±2 ℃ and from 21±2 ℃ to 13±2 ℃, showing that the low temperatures affect AMX more than AOB.

The reactor performance in terms of nitrogen removal is shown in Figure 3.2. The reactor performance was steady at 35±2 ℃ except a onetime nitrite accumulation around day 17 of the reactor operation. The NH_4-N concentration in the effluent also increased, indicating inhibition of AMX bacteria by the accumulated nitrite (Egli et al., 2001; Jung et al., 2007; Lotti et al., 2012; Strous et al., 1999). Considering that AOB may have outgrown AMX bacteria, a shorter settling time (5 min) at the end of each cycle was applied to encourage the washout of AOB from the reactor. In the meantime, the aeration rate was also decreased to prevent the overactivity of AOB. As seen in Figure 3.2, the NH_4^+-N in the final effluent gradually decreased without any further accumulation of NO_2^--N, resulting in an increased removal of total inorganic nitrogen (TIN). Once the reactor operation was adjusted in terms of controlling the growth of AOB by adjusting the aeration, no nitrite accumulation occurred. Thereafter, the average NO_2^--N concentration in the final effluent was 2.4±0.7 mg L^{-1}. The average NH_4^+-N removal during this initial period at 35±2 ℃ was 88.4±4.8 %.

On day 74, the temperature in the reactor was dropped to 21±2 ℃ by removing the hot water circulating jacket. The average NH_4^+-N removal over the period of operation at 21±2 ℃ dropped to 72.4±5.1%. Likewise, the reactor performance in terms of NH_4^+-N removal further dropped to 46±6.7% when the operating temperature was reduced from

21±2 ℃ to13±2 ℃. So far, many studies have reported that the optimal temperature for most described AOB is ~28 ℃ and the ammonium oxidation rate is related to temperature (Alawi et al., 2007). Downing and Hopwood (1964) found that the ammonium oxidation rate doubled as temperature increased by 10 ℃. Similar results were obtained by Wang and Yang (2004), who observed the ammonium oxidation rate increased by a factor of 4.5 when temperature was increased from 12 ℃ to 30 ℃. As for the AOB response to temperature downshift, Hu et al. (2013) reported that the AOB activity in a PN/A bioreactor doubled when temperature dropped from 25 ℃ to 12 ℃ over the course of 24 days. Similarly, Guo et al. (2010) found that the low temperature did not deteriorate the ammonium oxidation when the aeration control was adjusted.

In this study, the ex-situ test showed that the activity of AOB was dropped by 33% from 35 ℃ to 21 ℃, while only 1% from 21 ℃ to 13 ℃. The similarity of activity at 21 ℃ and 13 ℃ might be due to the bacteria adapting to the temperature drop, which refers to the process that bacteria go through—a transient arrest of cell growth termed an "acclimation phase"—and after this phase, cells become adapted to the lower temperature and resume growth but at a slower rate (Barria et al., 2013; Phadtare, 2004).

As nitrite accumulated when temperature decreased, it could be concluded that the acclimation phase for AOB is much shorter than AMX. In other words, when temperature decreased, both the activity of AOB and anammox decreased, however, the downshift of AOB activity was much lower and shorter than anammox, resulting in the buildup of nitrite. The reason of the short adaptation may be explained by the expression of the *amoA* gene, which was increased by 2.53-fold when the reactor was operated at 13 ℃. A similar result was reported by Radniecki et al. (2009) that when loading 30 $\mu mol \cdot L^{-1}$ of

zinc to a continuously cultured *Nitrosomonas europaea*, the ammonium oxidation rate reduced slightly while the *amoA* gene increased significantly as response. Our results are in agreement with those obtained by Radniecki et al. (2009) in which case AMO activity decreased under external stresses but the *amoA* gene expression increased.

Previous research showed the specific anammox activities for "*Ca.* Brocadia"at 20 and 10 ℃ have amounted to the 39% and 14%, respectively, of the activity measured at the reference temperature of 30 ℃ (Lotti et al., 2014). In this study, the anammox activities at 21 and 13 ℃ were the 58% and 10%, respectively, of the activity measured at the reference temperature of 35 ℃. The extremely low activity at 13 ℃ may be due to the cold shock generated over a period of only 2 weeks, which may not be enough for a successful adaptation of the anammox biomass to low temperature. This contrasts with previous research where AMX bacteria were subjected to low temperatures for several months (Gilbert et al., 2014; Hu et al., 2013; Persson et al., 2014). Moreover, for the PN/A reactor at 13 ℃, the average nitrogen removal rate was 0.22 $kg_N\ m^{-3}\ d^{-1}$, indicating that AMX, although performing an extremely low ammonium oxidation rate, can be active at this low temperature. Adaptation of AMX to cold conditions might then be foreseen when operating the PN/A system at this condition for longer period of time.

Ali et al. (2014) have reported a correlation of the transcription level of *hzsA* to the activity of AMX with R^2=0.8319 and that this functional gene can be used as a genetic marker to evaluate the ammonium oxidation rate. However, in this study, the fold change of *hzsA* gene expression was not strictly proportional to the activity of AMX. This may be due to the complex nitrogen metabolic pathway in AMX, where other enzymes such as nitrite reductase (*nirS*) and hydrazine dehydrogenase (*hdh*) also play an essential

role in the process. Moreover, because the *hdh* and *nirS* genes can be present in other bacteria, the primers targeting these gene are much less specific (Harhangi et al., 2012; Li et al., 2010; Schmid et al., 2008), it was impossible to decide to which level the previous reactions mediated by these gene encoded enzymes affects the *hzsA* gene expression.

The fold change of the *hzsA* gene expressed at 21 ℃ was only 0.06-fold while the ex-situ test showed 58% of activity comparing to those at 35 ℃. The variation in gene expression and activity might be the result of the presence of other genes that encode for different enzymes to catalyze the nitrogen transformation instead of *hzsA*. Nevertheless, this assumption needs to be confirmed through whole community transcriptomics analyses in the future.

Temperature effect on microbial community

In this study, the main species for aerobic ammonium oxidation was *Nitrosomonas europaea*, agreeing with many other researchers on PN/A systems (Furukawa et al., 2006; Park et al., 2015; Vlaeminck et al., 2010). *Nitrosomonas europaea* was reported to be dominant in ammonium-rich systems, such as activated sludge or biofilm reactors (Schramm et al., 1998), and in oxygen-limited systems (Park et al., 2004). Previous research also showed that the *Nitrosomonas* dominated culture had increased resistance to cold temperatures compared to other AOB (Ducey et al., 2009). The AMX found in the study were affiliated to "*Ca.* Brocadia sp.", which increased from 27.9% to 42.3% during the 120 days' operation. It was clear that the low temperature did not select for other "cold tolerant" anammox species than "*Ca.* Brocadia". Linking with other research on PN/A at low temperatures, "*Ca.* Brocadia sp." were the most reported

responsible for anaerobic ammonium removal (Gilbert et al., 2014; Gilbert et al., 2015; Hendrickx et al., 2014; Hu et al., 2012; Winkler et al., 2012), which might suggest that the genus "*Ca.* Brocadia" has a competitive advantage over other AMX at lower temperature.

According to amplicon sequencing results, members of the phylum of *Chloroflexi* were present at a relatively high amount, which agrees with other research on PN/A systems (Gilbert et al., 2014). *Chloroflexi* in suspended growth reactors have been reported as a backbone for the three-dimensional microbial aggregates called flocs and as reinforcement of the granule structure (Bjornsson et al., 2002; Bossier and Verstraete, 1996; Cho et al., 2010). It is still uncertain whether the abundance of *Chloroflexi* has any effect on the performance of the reactor in PN/A systems. In this research, the population of *Chloroflexi* in the reactor was decreased when temperature was dropped to 13 ℃. The negative growth may be due to the physiology of the bacteria, which are mostly thermophiles. Moreover, the carbon source for *Chloroflexi* in the PN/A system is from decay of bacteria (Kindaichi et al, 2012). Under 13 ℃, the cell decay rate in the reactor might get slower, which possibly limited the growth of *Chloroflexi.*

Impact of sulfide toxicity

During anaerobic digestion, SO_4^{2-} is often reduced to S^{2-}, resulting in inhibition to bacteria in the downstream biological treatment units (Dapena-Mora et al., 2007; Jin et al., 2012). By 2014, observation of the reject water presumably containing high S^{2-} had led to a loss/reduction in performance at three full-scale PN/A applications (Lackner et al., 2014). So the evaluation of the S^{2-} effect on AOB and AMX is very important.

It is generally accepted that H_2S, instead of S^{2-}, is more toxic to bacterial cells since it can diffuse into the cell membrane (Beauchamp et al., 1984). Once inside the cytoplasm, H_2S may cause denaturing of native proteins (Tursman and Cork, 1989), thus bringing negative deterioration to the bacterial activity. Similar to what was observed under cold temperatures, the expression of *amoA* genes by *Nitrosomonas* increased, and the transcriptional level of *amoA* was correlated with the concentration spike of S^{2-}. This may suggest that *amoA* may be an attractive candidate stress gene to monitor S^{2-} toxicity in AOB. However, the long-term effects of sulfide on AOB need to be studied where the "adaption" is considered for bacterial activity and gene expression.

So far, several groups have studied the effect of sulfide inhibition on AMX (Dapena-Mora et al., 2007; Jin et al., 2012; Kalyuzhnyi et al., 2006; van de Graaf et al., 1996) with adverse results. Jin et al. (2013) found that the short- and long-term effects of sulfide on anmmox biomass were distinctive: the SAA was halved at a sulfide level of 32 mg S/L within 13 days while only 17.2% reduction within 18 days by 40 $mg_S\ L^{-1}$. Dapena-Mora et al. (2007) reported that 160 $mg_S\ L^{-1}$ led to a complete inhibition of anammox activity, in contrast to van de Graaf et al. (1996) who observed that the sulfide could favor anammox activity. It is still unknown what parameters of operation are related to sulfide effects, either negative or positive. In our case, the S^{2-} concentration as low as 5 $mg_S\ L^{-1}$ brought down the activity of AMX to 40%. Surprisingly, the *hzsA* gene expressed at 30 mg/L of S^{2-} was higher than that at 5 mg/L. It was not clear whether this unexpected higher transcriptional level of *hzsA* was due to the oxidation of sulfide driven by nitrate (Jin et al., 2013).

Summary

This study highlighted the effects of temperature and sulfide toxicity on the activities and functional gene expression of aerobic and anaerobic ammonium oxidizing bacteria involved in PN/A:

- PN/A system performance deteriorated by temperatures lower than 20 ℃ and in the presence of sulfide in concentrations as low as 5 $mg_S\ L^{-1}$. These conditions are expected for installations for nitrogen removal from reject water in municipal wastewater treatment plants.
- AMX activity of "*Ca.* Brocadia sp." was positively correlated with the expression of the hydrazine synthase (*hzsA*) gene, while AOB activity of *Nitrosomonas europaea* was inversely correlated with *amoA* gene expression. The gene transcripts of *amoA* can therefore not be used alone to assess the active role of AOB in ammonia oxidation such as under stress conditions.
- Although the activities decreased significantly, the residual activity of AMX bacteria at lower temperature may indicate that a longer acclimation time could result in substantial AMX performance under psychrophilic conditions.
- On top of nitrifying *Proteobacteria* and anammox *Plantomycetes*, *Chloroflexi* was detected as a dominant phylum whose abundance decreased with decreasing temperature. Future efforts targeted at understanding PN/A system performance and stability under different operational regimes may also focus on identifying the putative role of this flanking population. Such molecular efforts should further include analyses of complete functional gene networks for a broader picture on microbial community efficiency in the PN/A context.

CHAPTER 4

THE FEASIBILITY OF APPLYING PARTIAL NITRITATION/ANAMMOX (PN/A) FOR URINE TREATMENT

Introduction

Human urine is the most nutrient-rich component in municipalliquid waste. About 80% of the nitrogen (N), 70% of the potassium (K) and up to 50% of the total phosphate (P) in domestic wastewater come from urine (Kujawa-Roeleveld and Zeeman, 2006; Larsen and Gujer 1996; Larsen and Lienert 2004). The main nutrients of concern are P and N, and the presence of both in surface waters causes eutrophication (Conley et al., 2009). Nutrient-rich human urine has gained increasing attention as a potential renewable fertilizer since phosphate, one of the key components in commercially available fertilizers, comes from limiting and fast depleting resource (i.e., minerals) (Smil, 2000; Wilsenach et al., 2007). As a result, source separation and management of urine involving nutrient recovery and treatment are increasingly becoming attractive.

Human urine has been used as fertilizers in developing countries for centuries (Makaya et al., 2014). Storage is the easiest and cheapest sanitation method commonly used to eliminate active pathogens (Maurer et al., 2006a; Santo et al., 2004). During the storage, ubiquitously present bacterial enzyme urease hydrolyzes urea ($CO(NH_2)_2$) to produce ammonia (NH_3/NH_4^+) and bicarbonate (HCO_3^-), raising the pH from neutral to 9.

The pH, storage time and temperature are important in controlling growth of pathogenic organisms (Höglund et al., 1998,; 1999,; 2000). Alternatively, primary fertilizer components of P and N can be recovered in the form of struvite ($MgNH_4PO_4 \bullet 6H_2O$) (Udert et al., 2003a). The stored urine has an average pH of 9 and has been shown to provide the optimum chemical conditions for struvite precipitation (Adnan et al., 2004; Johnston and Richards, 2003; Ronteltap et al., 2007; Udert et al., 2003b; Wu et al., 2005). Struvite recovered from human urine has been used as an effective fertilizer in many cases (Etter et al., 2011; Karak and Bhattacharyya, 2011; Mihelcic et al., 2011).

Based on urine composition data, P: N molar ratio in human urine is 1: 29~40 (Jonsson et al., 1997; Kirchmann and Perttersson, 1994; Maurer et al., 2006a; Udert et al., 2003a). After P and N recovery from urine in the form of struvite, it stills contains 95-98% of NH_3/NH_4^+-N, which needs to be further treated. Physicochemical processes such as ammonia stripping may be used to remove NH_3 from wastewater. However, it can be costly (Behrendt et al., 2002; USEPA, 2000) and unstable, which may yield high ammonia in the effluent (Maurer et al., 2006). Alternatively, coupled partial nitritation and anaerobic ammonia oxidation are an attractive alternative for nitrogen management in urine due to theirpotential to handle high concentrations of ammonia with low energy needs (Strous et al., 2006; Wilsenach and van Loosdrecht, 2004).

As introduced in Chapter 1, the PN/A system is comprised of two biological processes: (1) partial oxidation of ammonia to nitrite by AOB and (2) anaerobic oxidation of ammonium and nitrite to nitrogen gas by AMX. The system can take place in two separate tanks (i.e., two stage process) or in one tank (i.e., single stage PN/A process). De Graaf et al. (2011) reported up to 89% N removal from black water using a two-stage

process. By applying a single-stage configuration, 76% N removal from digested black water and 78% N removal from diluted human urine have been reported (Vlaeminch et al., 2009; Udert et al., 2003a).

Coupled nutrient recovery and nitrogen management provides an excellent option for the management of source separated urine, and fits very well in the changing paradigm of municipal wastewater treatment in which case, efforts are in place to make municipal wastewater resource and energy positive. However, to the best of our knowledge, few studies have been carried out to study the feasibility of applying anammox process for the treatment of source-separated urine after struvite precipitation. In this study, synthetic human urine was used to investigate the nitrogen removal coupled with phosphate recovery in two reactor configurations: two-stage or single stage PN/A process shown in Figure 4.1.

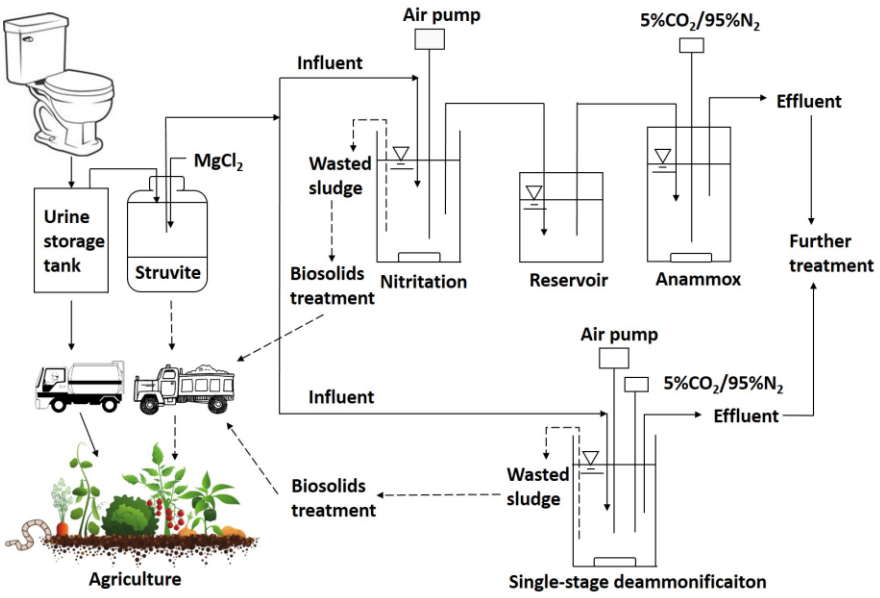

Figure 4.1 Overall urine seperation and treatment train.

Materials and methods

Synthetic urine preparation

Hydrolyzed and non-hydrolyzed synthetic human urine solutions were prepared and used depending on the experiments. Hydrolyzed urine did not have urea but ammonia and bicarbonate, while nonhydrolyzed urine still contained urea. The hydrolyzed urine solution contained (per L): 0.65g , 9.13g , 4.6g NaCl, 2.3g $NaSO_4$, 1.1 g creatinine, 1.6g KCl, 38g NH_4Cl, 0.02g $Na_2(COO)_2$, 4.2g KH_2PO_4, 8.4g $NaHCO_3$, and were pH 9.0 adjusted with 10N NaOH. The nonhydrolyzed version contained all compounds listed above except 37 g of NH_4Cl and all $NaHCO_3$ were replaced with 25g of urea, without $Na_3C_6H_5O_7$. The synthetic urine formula used in this study was based on the recipe reported by Griffiths et al. (1976). All chemicals were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Aldrich (St. Louis, MO) unless stated otherwise.

Struvite precipitation

Struvite precipitation optimization studies were conducted in duplicate in 50 mL centrifuge tubes with 1) hydrolyzed synthetic urine at pH 7 and 2) hydrolyzed urine at pH 9 (pH adjusted with 10N NaOH). Three different concentrations were used to enable Mg:P ratios of 1:1, 1.5:1 and 2:1. The experiments were performed at room temperature and shaken at 200 rpm for 3h to precipitate struvite according to Wilsenach et al. (2007).

Two-stage partial nitritaion-anammox reactor operation

The system consisted of two 5 L plexiglass reactors: a nitritation reactor and an anammox reactor. The working volume of each reactor was 4 L. The reactors were fed

with the poststruvite hydrolyzed synthetic urine solution (Figure 4.1). The nitritation and anammox biomasses were seeded from on-going partial nitrification and anammox reactors, respectively (Kotay et al., 2013). Both the reactors were operated for 100 days. Because the poststruvite hydrolyzed urine solution contained a high concentration of NH_4^+ (9500 mg_N L^{-1}), NH_4^+ loading was increased in steps as summarized in Table 4.1. The cycle of the nitritation sequencing batch reactor (SBR) started with feeding, followed by 11.5 h of aerobic reaction and 0.5 h of settling and decanting. The temperature of this reactor was maintained at approximately 30 ℃ by using a heating pad (Grainger Industrial Supply, Salt Lake City, UT).

The effluent from the nitritation reactor was automatically transferred to an anammox semicontinuously-fedbatch reactor (FBR) (operated at room temperature) through an intermediate collection vessel (Figure 4.1). Diluted poststruvite-nitritation hydrolyzed urine solution was used as the influent to protect anammox culture from NO_2^- toxicity. Table 4.1 shows the detailed operation of the anammox FBR. More anammox biomass was added on day 11. The anammox FBR cycle included 22 h of stepwise feeding followed by 0.5 h of settling and decanting. Anammox FBR was purged with a 95% N_2 and 5% CO_2 mixture (5 mL min^{-1}) for 10 min every 2 h to maintain the pH below 8 as well as to ensure truly anaerobic conditions. Nitritation rate and TIN removal rate in the nitritation reactor and anammox FBR were estimated as mg_N L^{-1} d-1 based on equations (17) and (18):

$$\text{Nitritation rate} = \frac{NH_{4\,\text{influent}}^{+} - NO_{2\,\text{effluent}}^{-} - NO_{3\,\text{effluent}}^{-}}{\text{HRT}} \quad (17)$$

$$\text{TIN removal rate} = \frac{TIN_{\text{influent}} - TIN_{\text{effluent}}}{\text{HRT}} \quad (18)$$

Table 4.1 Operational condition of two-stage and single-stage PN/A reactors.

	Two-stage					Single-stage		
Periods	**Time (days)**	**Nitritation SBR**			**Anammox FBR**	**Time (days)**	**PN/A**	
		Influent NH_4^+-N (mg/L)	**HRT/ SRT (days)**	**Effluent**	**Influent**	**HRT**	**Influent NH_4^+-N (mg/L)**	**HRT/ SRT (days)**
Phase I	0-10	643±25.4	2/10	No dilution		4	40**-54	293±21.2	2/35
Phase II	11-47	985±30.6	2/15	No dilution		4	55-69	397±14.8	
Phase III	48-100	2580±188	2/15	4X dilution		2	70-121	512±18.9	

*This is the NH_4-N concentration usually found in stored urine from collection systems (Feng et al., 2008; Kirchmann and Pettersson, 1994; Udert et al., 2003a).
** The first 39 days, the reactor was optimizing to reach the stable nitrogen removal condition.

PN/A reactor operation

A 5 L plexiglass reactor was seeded from the PN/A reactor described in Chapter 2. The reactor was fed with poststruvite hydrolyzed urine solution and was operated for over 120 days. The amount of NH_4^+-N loading was gradually increased as described in Table 4.1 as the solution contained high NH_4^+-N and had pH as high as 9, which could bring inhibition to the bacteria as described in Chapter 1.

The reactor was operated at room temperature. Dissolved oxygen (DO) was adjusted to approximately 0.5 mg L^{-1} by constantly bubbling air. The reactor cycle consisted of a filling phase, 44 subcycles (6 min aeration and 9 min anoxic condition by purging 4 min 95% N_2 and 5% CO_2 mixture (5.8 ml min^{-1})), 20 min final aeration, 20 min settling and decanting. Equation (3) above was also used for calculating the TIN mass removal rate in this system.

Magnesium inhibition test

Ex-situ tests were done to investigate if the residual Mg^{2+} after struvite precipitation has any effect on AOB or AMX activity. Similar to the test described in Chapter 3, biomass from the reactor was harvested and prepared in triplicate bottles supplemented with ammonium and nitrite as well as different concentration of Mg^{2+} ranged from 0 mg L^{-1} and 360 mg L^{-1} in the form of $MgCl_2$, respectively. pH was adjusted in the median range of 7.5~8. Anoxic conditions were ensured by purging nitrogen gas at the beginning for 10 min in the mixed liquor directly in serum bottles incubated at 35^{o}C. Mixed liquor samples were collected every 40 min and filtered using 0.45 um Millipore filter paper to measure AMX activity in terms of nitrogen concentration.

For magnesium effect tests on AOBs, the same protocol used for AMX was employed except that the bottles were supplemented with ammonium and kept open to the atmosphere to ensure aerobic conditions.

Chemical analysis

All liquid samples were filtered with a 0.45 m membrane filter and analyzed immediately. Chemical oxygen demand (COD), ammonia (NH_4^+-N), nitrate (NO_3-N), nitrite (NO_2-N), and dissolved phosphorus (PO_4^{3-}P) were quantified using HACH methods 8000, 10031 (Salicylate method), 1002 (Chromotropic acid method), 8153 (Ferrous sulfate method), and 8048 (Ascorbic acid method), respectively. The mixed liquor solids concentration was determined as total suspended solids (TSS) and as volatile suspended solids (VSS), both were measured with standard methods (APHA, 1998).

Results

Struvite precipitation

Optimization of struvite crystallization was performed to determine the minimum Mg dose to achieve maximum phosphorous recovery from the hydrolyzed synthetic urine. Three different doses were tested at a Mg:P molar ratio of 1:1, 1.5:1, and 2:1. Figure 4.2 shows over 99% P-removal was achieved when the Mg to P ratio was greater than 1.5. Struvite crystal is known to be least soluble when pH between 8.5 and 10.7.[54] During the optimization study, two different pH levels were tested: hydrolyzed urine without any pH adjustment (i.e., pH of 7) and the one withadditional NaOH giving it a pH of 9. As expected, the maximum struvite formation (i.e., the maximum P-removal) was observed at pH 9. Nearly all of the P (99.2 ± 0.65%) and approximately 5% (5.46 ± 0.34%) of total NH_4^+-N present were recovered as struvite from the synthetic urine solution. This suggests that there was plenty of NH4+-N remaining in struvite recovered synthetic urine.

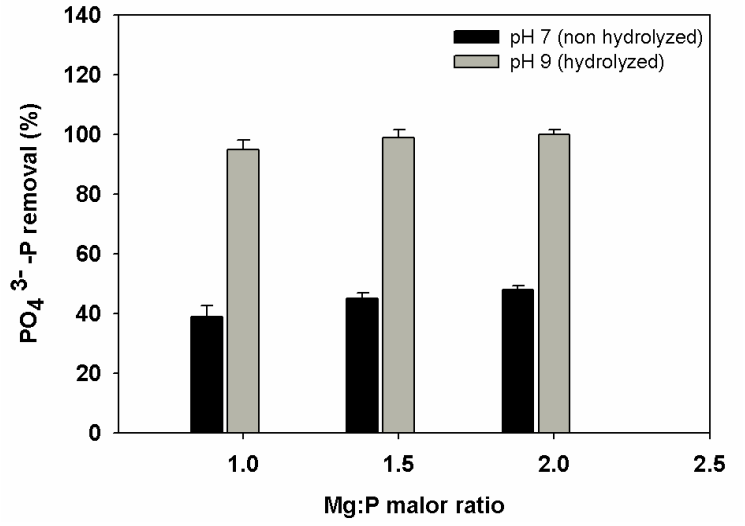

Figure 4.2 Result of struvite precipitation.

Magnesium effect on AOB and AMX

As illustrated in Figure 4.3, up to 360 mg_{Mg} L^{-1} of Mg^{2+} did not have significant effect on the AOB activity. As for the anammox process, trace amounts of Mg^{2+}(48 mg_{Mg} L^{-1}) could favor the ammonium oxidation rate by 16.7%, while the highest dose of Mg^{2+} at 360 mg_{Mg} L^{-1}L showed slight negative effect of 8.8% on the AMX activity. The results demonstrated that the residual Mg^{2+} in the urine after struvite precipitation should have little impact on the following two-stage or single-stage PN/A process.

Two-stage system performance

Figure 4.4(a) shows the performance of the partial nitritation SBR. Diluted synthetic urine was used to start the partial nitritation reactor and the concentration of NH_4^+-N in the influent was slowly increased to acclimatize the biomass in this SBR. The effluent NO_3-N concentration increased when the influent NH_4^+-N loading increased and 46.9 ±5.3 mg L^{-1}, 82.78 ±13.22 mg L^{-1}, 133 ±9.74 mg L^{-1} NO_3-N were recorded in the final effluent in Phase I, II, and III, respectively (Figure 4.4(a)).

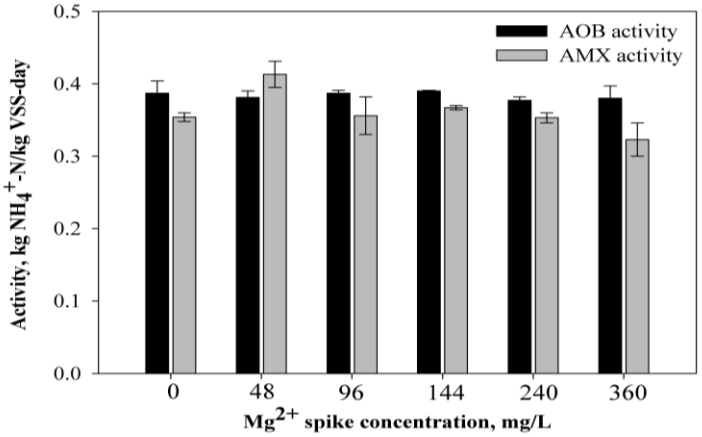

Figure 4.3 Magnesium supplement on the effect of AOB and AMX.

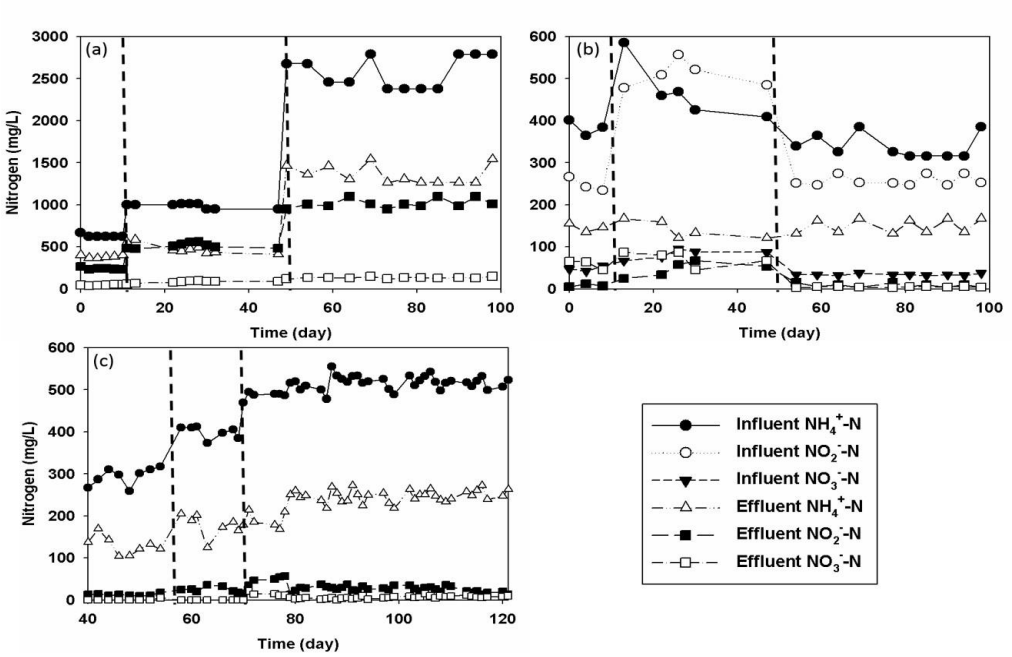

Figure 4.4 Reactor performance in (a) nitritation (b) anammox and (c) PN/A reactors.

NO_3-N in the effluent did not exceed 10% of the influent total inorganic nitrogen (TIN) in all phases (Figure 4.4(a)), which was similar to previous studies (Udert et al., 2003b; Udert et al., 2012). The COD removal efficiency was 50% in the partial nitrification reactor. Higher DO concentrations and longer SRT were maintained during Phases II and III, resulting in higher nitritation rates (Table 4.1). TSS/VSS in the nitritation reactor during PhasesI and III were $1475 \pm 59/1242 \pm 74$ mg L^{-1} and $2107 \pm 198/1683 \pm 237$ mg L^{-1}, respectively. During each cycle, as previous studies observed (Udert et al., 2003a), ammonia oxidation stopped when the pH progressively decreased to 5.4.

Figure 4.4(b) illustrates the inorganic nitrogen species in the anammox FBR. As the reactor was fed with the effluent from PN-SBR, the operation could also be divided into three phases.

During Phase I, the influentratio of NO_2-N to NH_4^+-N was 0.62, which was much lower than the reported ratio of 1.146 (Lotti et al., 2014), resulting in NO_2-N limitation in the reactor and a TIN mass removal rate of117 ± 6.59 mg/L during this phase. During the second phase, as the ratio of NO_2-N to NH_4^+-N in the influent increased significantly to 1.09 due to efficient nitrification in the PN-SBR, the average removal rate was 211 ± 12.7 mg_N L^{-1}, suggesting that the nitrogen removal rate could be further improved if the PN-SBR is provided with the appropriate concentrations of NO_2^--N and NH_4^+-N. In Phase III, the operation was changed (Table 4.1) from an HRT of 4 days to 2 days while simultaneously decreasing the influent TIN to anammox-FBR. In this period, very limited NO_2-N (8.2 ± 4.4 mg L^{-1}) and NO_3-N (4.6 ± 1.2 mg L^{-1}) levels were observed in the effluent of the anammox-FBR, resulting in a higher average TIN removal rate of 241 ± 13.3 mg L^{-1}. The ratios of produced NO_3-N to removed NH_4^+-N were much lower than 0.26:1. NO_3-N was reduced 11% and 57% during Phase II and III, respectively. The effluent pH of the anammox FBR was 7, and the COD removal efficiency was 18%.

PN/A reactor performance

Figure 4.4(c) showed the performance of the single-stage PN/A SBR. The post-struvite hydrolyzed influent provided several challenges, such as high pH and a faster growth rate for AOB over anammox resulting in nitrite accumulation. During the first 40 days, operational conditions (i.e. HRT and the aeration duration in each cycle) were adjusted to optimize the system and to maintain stable nitrogen removal. The effluent NO_2-N in Phase I was predominantly below 15 mg L^{-1}. When NH_4-N loading was increased in Phase II, increased TIN removal was observed (Figure 4.4 (c) and Table 4.2).

Table 4.2 The nitritation activity and anammox activity in both two-stage and single-stage reactors.

	Two-stage						Single-stage	
Periods	Nitritation SBR		Anammox FBR				SBR	
	NO_2-N: NH_4-N	Nitritation rate	NO_2-N: NH_4-N	NO_3-N: NH_4-N	TIN rem.	TIN mass rem.	TIN rem.	TIN mass rem.
	effluent ratio	mg/L/day	Ratio rem.	Ratio prod.	%	mg/L/day	%	mg/L/day
Phase I	0.62±0.04	146±5.93	1.01±0.05	0.04±0.07	68.9±1.58%	117±6.59	51.3±8.01	75.6±14.2
Phase II	1.09±0.12	301±19.4	1.43±0.20	-0.52±0.08	79.5±0.56%	211±12.7	49.2±4.26	97.8±5.01
Phase III	0.75±0.08	574±27.0	1.33±0.19	-0.16±0.02	76.7±2.35%	241±13.3	46.5±2.80	119±6.17

Meanwhile, the NO_2-N concentration in the effluent also increased to 25.6±6.8 mg L^{-1}. However, in Phase III, with increased ammonium loading, NO_2-N was accumulated up to 57 mg L^{-1} at Day 78. In order to prevent further nitrite accumulation, 500 mL of mixed liquor from the reactor was removed as waste sludge and more anammox biomass was added. At the same time, the 20 min of final aeration at end of the batch was replaced by anoxic mixing to further reduce the nitrite level before being discharged as effluent.

From day 79 to 121, less NO_2-N (26.1±10.5 mg L^{-1}) was produced. A stable TIN removal of 119±6.17 g_N m^{-3} d^{-1} was achieved in this phase. During the overall operational period, the ratio of average produced NO_3-N to removed NH_4^+-N was 0.02±0.02, indicating a successful suppression of nitrite oxidation to nitrate.

In this system, as AOB are proton producers and anammox are proton consumers, pH was buffered by the two bacteria from 8.5 at the beginning to 6.5 at the end of the cycle, and the COD removal efficiency was 86%. TSS/VSS in the anammox FBR and single-stage combined SBR were 1575±104/1232±98 mg L^{-1} and 2016±219/1629±322 mg L^{-1}, respectively.

Discussion

The recovery of useful chemicals from human urine has been drawing increased attention over the last few decades. The world's phosphorus sources are being depleted at an alarming rate. We will run out of known phosphorus reserves in around 80 years if the current consumption levels are maintained (Schroder et al., 2010). Source separated urine treatment offers not only renewable P and N resources for sustainable fertilizer production, but also causes reductions in domestic WWTP operational costs due todecreases of 80% in N and 60% in P loadings (Kujawa-Roeleveld and Zeeman, 2006; Larsen et al., 2004).

Struvite precipitation

Urine originated struvite is a slow release fertilizer and has no heavy metal contaminations (Ghosh et al., 1996; Li and Zhao, 2003; Liu et al., 2011). Typically, the pH of stored urine is around 9.0. This is the pH at which struvite is the least soluble in water, allowing the maximum amount of struvite to be precipitated (Nelson et al., 2003). While struvite precipitation from urine is not new, the feasibility of applying anammox for the poststruvite formation has not well known. In this study, greater than 99% of the P in the synthetic urine was recovered as struvite, suggesting that urine originated struvite could be a better candidate than other nutrients removal methods.

N-removal efficiency

Overall, the two-stage PN/A configuration showed greater TIN mass removal rate than those for the single-stage alternative (Table 4.2). There were more operational

challenges with the single-stage system than the two-stage system. In the single-stage reactor, pH fluctuated between 6.4 and 8.5 whereas the optimum pH for single-stage PN/A systems has been reported as between 7 to 7.3 (Wett, 2007a). pH stress to AOB and anammox cultures were noticed in the single-stage, while no such problem was noticed in the two-stage system even though both systems lacked any external pH control systems. In the single-stage PN/A it was also challenging to achieve an ideal ratio of AOB and anammox bacteria, which is one of the critical parameters for successful operation of a single-stage PN/A system (Wet et al., 2010). AOB over growth was noted and accumulation of nitrite (NO_2^-) during Phase II in the single-stage system took place. NO_2^- produced by AOB (i.e., nitritation), is a substrate but also an inhibitor of anammox (Dapena-More et al., 2007; Huang et al., 2011). Therefore, more AOB culture was wasted to increase TIN removal, resulting a decrease of the SRT for AOB (Figure 4.4). This issue was also not observed in the two-stage system.

Another important parameter for successful TIN removal in PN/A systems is the COD:N ratio. Previous studies showed that a COD:N ratio higher than 0.5 could interfere negatively with the anammox by stimulating heterotrophic denitrification and influencing the SRT of the anammox biomass(Chamchoi et al., 2008; Dapena-Mora et al., 2007; Molinuevo et al., 2009; Udert et al., 2008). The influent COD: N ratio in a single-stage system was 0.27 and a stable TIN removal was observed. But inhibition of anammox activities was found when the organics in the influent was higher than 300 mg L^{-1} as COD (Breisha, 2010; Chamchoi et al., 2008). In the two-stage system, 50% of the COD removal happened in the aerobic nitritation reactor. The COD:N ratio of the influent to the anammox reactor was 0.14, which is much lower than that in the single-stage PN/A

system. TIN removal rate in the two-stage system was higher than that in the single-stage system.

In both systems, lower nitrate remained in the effluent than stoichiometrically expected (Figure 4.4). This suggests that occurrence of denitrification in parallel to anammox is due to the presence of COD in the urine. In the anammox FBR of the two-stage configuration, the ratio of NO_3^- -N produced to NH_4^+-N consumed (Table 4.2) was lower than the stoichiometrically expected value of 0.26 (Strous et al., 1999), suggesting dissimilative nitrate reduction to ammonia (DNRA) might be happening in addition to anammox (Burgmann et al., 2011; Huang et al., 2011; Kotay et al., 2013).

The results and observations suggested that the two-stage system yielded higher TIN removal efficiency with less operational challenges. However, the TIN mass removal rate found for the two-stage system was less than the similar study with 700 $mg_N\ L^{-1}\ d^{-1}$ (Vlaeminch et al., 2009). The performance of the two-stage system constructed in this study should be improved by maintaining pH around 7.5 (Uder and Wachter, 2012), feeding appropriate ammonia (Burns, 1996; Feng et al., 2008), adding more alkalinity (Sun et al., 2012; Udert et al., 2003b), and/or increasing SRT in the anoxic anammox reactor (Feng et al., 2008; Udert et al., 2003a)

Summary

This study investigated the feasibility of combining chemical and biological processes to recover phosphorus and remove nitrogen from synthetic urine. Both two-stage and single stage partial nitritation/anammox systems were studied in term of nitrogen removal. The results showed that the recovery efficiency of PO_4^{3-}P using struvite

precipitation was greater than 95% when the molar ratio of Mg:P was higher than 1:1. The residual Mg^{2+} in the poststruvite urine may not have any significant deteriorate to the following biological nitrogen removal.

Successful treatment of poststruvite hydrolyzed urine solution was achieved in both two-stage and single-stage PN/A systems. The two-stage system, with less COD and pH impact to the anammox bacteria, was able to remove up to 80% of the total inorganic nitrogen from urine. As for the single-stage PN/A reactor, the nitrogen removal efficiency was lower than the two-stage system. Although COD directly loading to the single-stage reactor brought operational challegen, it enabled the growth of heterotrophs, which brought down the nitrate production level in the reactor. This research demonstrated that coupling struvite precipitation and PN/A could be an energy and cost effective alternative for urine treatment.

Moreover, as nitrate is one of the end products for anammox process, a relatively high content of nitrate together with the untreated ammonium in the effluent may cause environmental problems. To eliminate the residual nitrogen, additional physical and chemical treatment will be necessary.

CHAPTER 5

EXPLORING THE PARTIAL-NITRITATION ANAMMOX PROCESS IN NORTH DAVIS SEWER DISTRICT

Introduction

The North Davis Sewer District (NDSD) is located near the shoreline of the Great Salt Lake in Syracuse, Utah. The treatment plant is designed to treat 34 million gallons per day (MGD) of local municipal wastewater and discharge the treated water to the Great Salt Lake. The particular plant was designed for total volatile solids (TSS) and biological BOD removal utilizing biotowers followed by an activated sludge solids contact process as illustrated in Figure 5.1. Before discharge, the secondary effluent is disinfected in chlorine contact basins. The excess sludge produced is decomposed and stabilized during anaerobic digestion, and subsequently dewatered by belt filtering. The reject water is recycled to the equalization basin to settle down the inorganic solids, and then it is combined with the raw influent for further treatment. The anaerobic digestion of sludge also provides heat and electricity from the cogeneration of biogas, followed by production of compost for agriculture. According to the data provided by NDSD, the plant received an average daily flow of 20.2 $\pm$ 0.5 MGD over the courseof year 2013. As shown in Figure 5.2(a), ammonium is the predominate form of dissolved nitrogen found in raw municipal wastewater, which was 20.0 $\pm$ 1.8 $mg_N\ L^{-1}$ in that year.

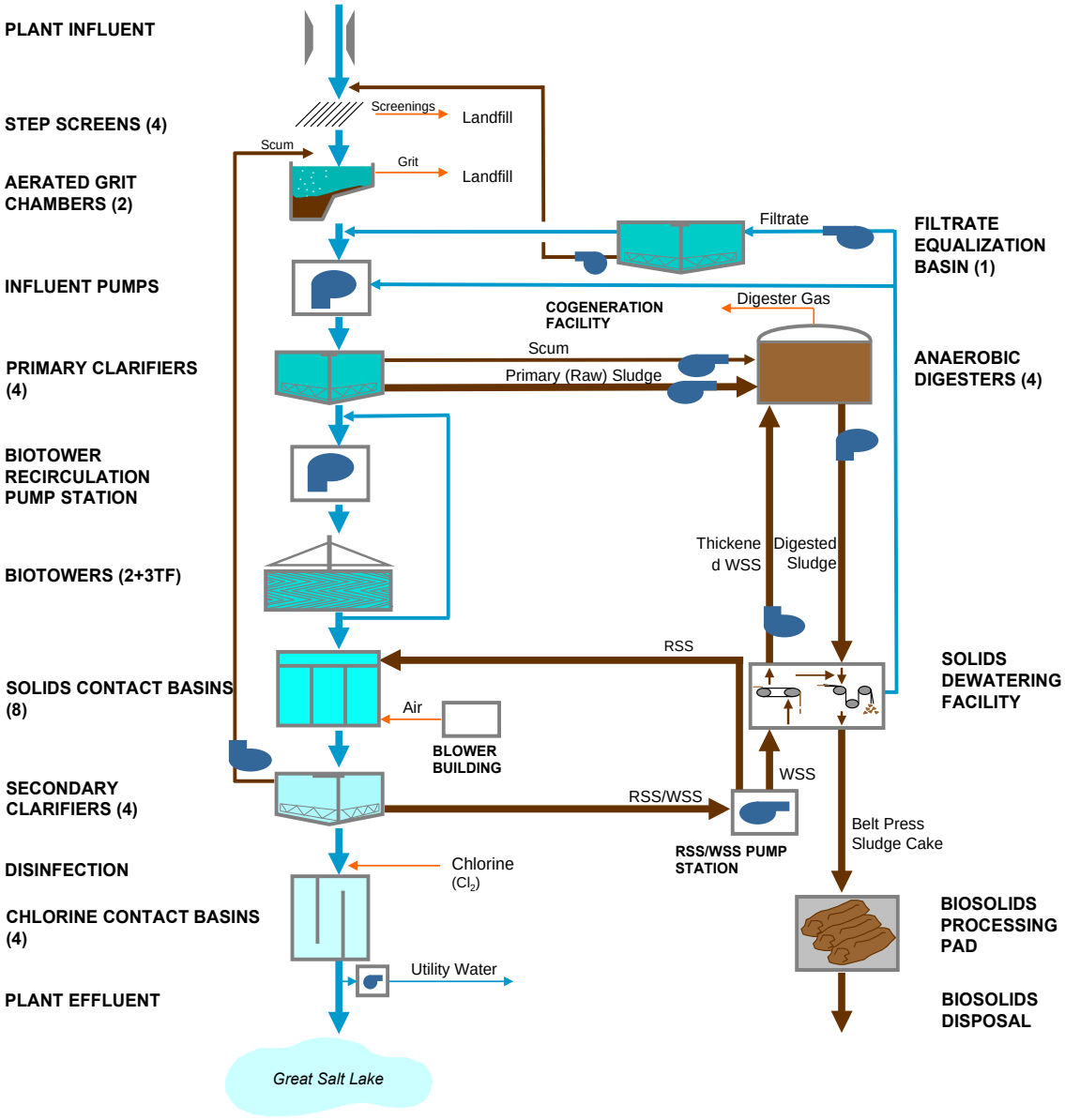

Figure 5.1 NDSD process flow diagram (North Davis Sewer District © 2016).

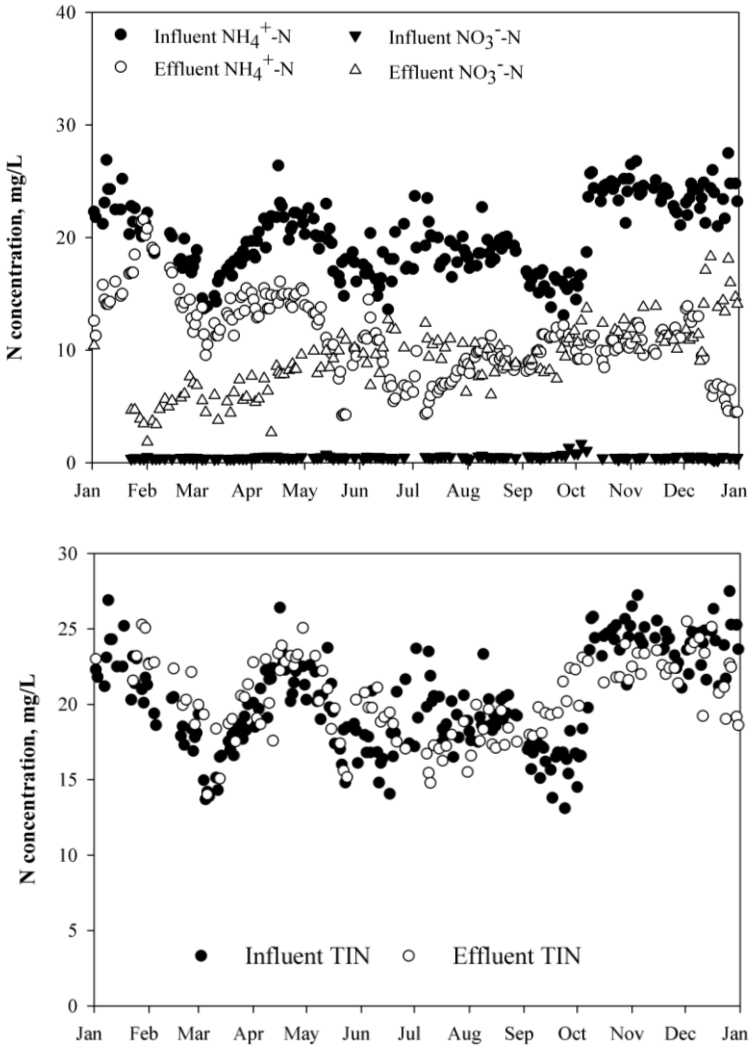

Figure 5.2 The nitrogen removal performance of North Davis Sewer District during 2013. It shows (a) influent and effluent ammonium/nitrate; and (b) influent and effluent TIN.

The nitrate in the influent was 0.5 ± 0.1 mg_N L^{-1}. The ammonium and nitrate concentration in the secondary effluent were 11.3 ± 1.9 mg_N L^{-1} and 9.1 ± 1.8 mg_N L^{-1}, respectively. The average total inorganic nitrogen (TIN) in the effluent was 20.5 ± 1.9 mg_N L^{-1}, indicating a negative TIN removal from the treatment plant (see Figure 5.2(b)). In 2014, there were no nitrogen discharge standards for the plant, buta stricter regulation was expected to be applied in the near future.

Giving the fact that ammonium is extremely toxic in comparison to nitrate, the

facility has begun experimenting with a “reaeration” (i.e., add more air) process in the solids contact basin aiming to provide additional nitrification to reduce the amount of NH_4^+ in the final effluent.

The electronic consumption was raised as the result of increased aeration, due to which, NDSD started looking into options for a cost effective method to reduce the ammonium content before discharge. By looking at the nitrogen source loaded to the plant, it was found that 24 ± 7% of ammonium loading to the treatment came from the reject water. As mentioned earlier, the reject water is produced from the dewatering of digested sludge. During 2013, 139,700±27,200 gallons per day (GPD) of the reject water were produced with an average of NH_4^+ concentration of 406 ±23 $mg_N\ L^{-1}$. Therefore, in attempt to solve the problems occurring at NDSD, applying a small sidestream nitrogen removal reactor would be more efficient as apposed to upgrading the current mainstream biotowers or solid contact basins.

As discussed in Chapter 1, the partial nitritation/anammox (PN/A) process is more energy-efficient compared to the conventional nitrification/denitrification or nitritation/denitritation for ammonium removal. It is estimated that by applying a sidestream PN/A bioreactor, approximately 40~50% of the total electrical consumption can be eliminated (Siegrist et al. 2008). Although the suspended growth PN/A process has been applied in several plants in the United States with an elevated temperature around 25~30 ℃, successful start-up strategies at room temperature have not been well understood. Based on the lab-scale reactor startup and operation, we demonstrated that the PN/A system could be operated at room temperature with a relatively high nitrogen removal rate. Therefore the purpose of this pilot scale study was to (1) identify any

possible operation issues that the suspended growth PN/A system may experience, (2) to develop solutions that can be applied to rectify or prevent these issues, and (3) to help to develop full-scale start-up and operation strategies at room temperature.

Reactor start-up and operation

Preparation: Reactor design

The pilot reactor's potential total volume was 300 gal, so the working volume was designed to be 250 gal with intermittent aeration. For the operation design, a strategy similar to the suspended growth reactor described in Chapter 2 would be applied. Considering that the raw filtrate has high content of inorganic solids, an equalization tank was installed for processing the reject water before goinginto the pilot reactor. The equalization tank was 1000 gallons with a liquid outlet on the top of the tank to capture filtrate and a sludge outlet at the bottom to remove the settled solids.

Before starting the reactor, several hydraulic tests were done using sludge from the anaerobic digester and mixed with tap water to check whether all of the components worked as anticipated. The flow rate of influent and effluent pumps were adjusted, based on which the running duration of each pump was decided. Also, the flow rate of aeration pump was set to ensure that the dissolved oxygen inside the reactor was below 0.4 mg L^{-1}.

Stage I: Inoculum enriching

The seed biomass came from the Hampton Roads Sanitation District's (HRSD) York River Treatment Plant which applied DEMON® for their sidestream nitrogen removal. At first, ~20 gal of mixed liquor was delivered to NDSD. The TSS of the mixed

liquor was about 2000 mg L^{-1}. When dumped into the reactor, the results showed that MLSS = 340 mg $L^{-1}$and MLVSS = 167 mg L^{-1}.

Since the biomass concentration was too low, more biomass was required. During the following 2 weeks, another 20 gal of concentrated biomass was delivered and added to the reactor, resulting in a MLSS of 1700 mg $L^{-1}$and MLVSS of 730 mg L^{-1}. At this time, a regular monitoring of the reactor was done (Shown in Table 5.1). During this phase, HRT of the reactor was kept at 1.67 days.

The batch begins at 11:00am and continuously run 15 min of aeration followed by 30 min of anoxic mixing, until 10: 40am of the next day. After that, a short settling was applied to allow most of the biomass goes to the bottom of the reactor. Then the effluent pump withdrawed 150 gal of treated wastewater followed by feding 150 gal of filtrate.

Table 5.1 The pilot-scale PN/A reactor start-up and operation details.

	Stage Name	**Date**	**Major accomplishments**	**MLSS/ MLVSS, mg L^{-1}**	**HRT, d**
Preparation	Reactor design	2/1/2014-7/27/14	– Reactor design and modification – Hydraulic test	--	--
Stage I	Inoculum enriching	7/28/14-8/12/14	– Adding seed – Activity test	340/167~ 1700/730	1.67
Stage II	Reactor operation	8/13/14-11/15/14	– DO controlling – Performance monitoring	1700/730~ 1340/570	3.33

Stage II: Reactor operation

During stage II, HRT was maintained at an average of 3.33 days to enrich the biomass and avoid any loss with effluent. All the pumps and the air blower were controlled using an Easy-Set Logic^{TM} timer (Orbit, Bountiful, UT). Additionally,in case any manual operation was necessary, a controlling panel was installed over the timer. DO in the reactor was kept below 0.3mg L^{-1} during the aeration stage and < 0.05 mg L^{-1} during the anoxic mixing period to prevent nitrite accumulation. It was noted that the minimum detection of the DO probe was 0.04 mg L^{-1}. The pH in the reactor was not controlled but it was always monitored to be in the median range of 7.6 to 7.9. Since the reactor was kept inside, theambient temperature was at ~20 ℃. Figure 5.3(a) illustrates the different components in the pilot-scale PN/A reactor and (b) demonstrates the flow of the sidestream treatment.

Chemical analysis

Mixed liquor samples were taken at the beginning and the end of each batch to monitor the inorganic nitrogen concentration. NH_4^+-N, NO_2^--N, and NO_3^--N were analyzed by TNT 832, TNT 840, and TNT 835 (HACH, Loveland, CO). TSS/VSS of the mixed liquor was measured according to the standard methods (APHA, 1995)

In-situ batch test

To investigate the activity of AOB, an in-situ batch test was performed during Phase I and Phase II by aerating for several hours and monitoring the NH_4^+ and NO_2^- concentration hourly until the NO_2^- was above 25 mg_N L^{-1}.

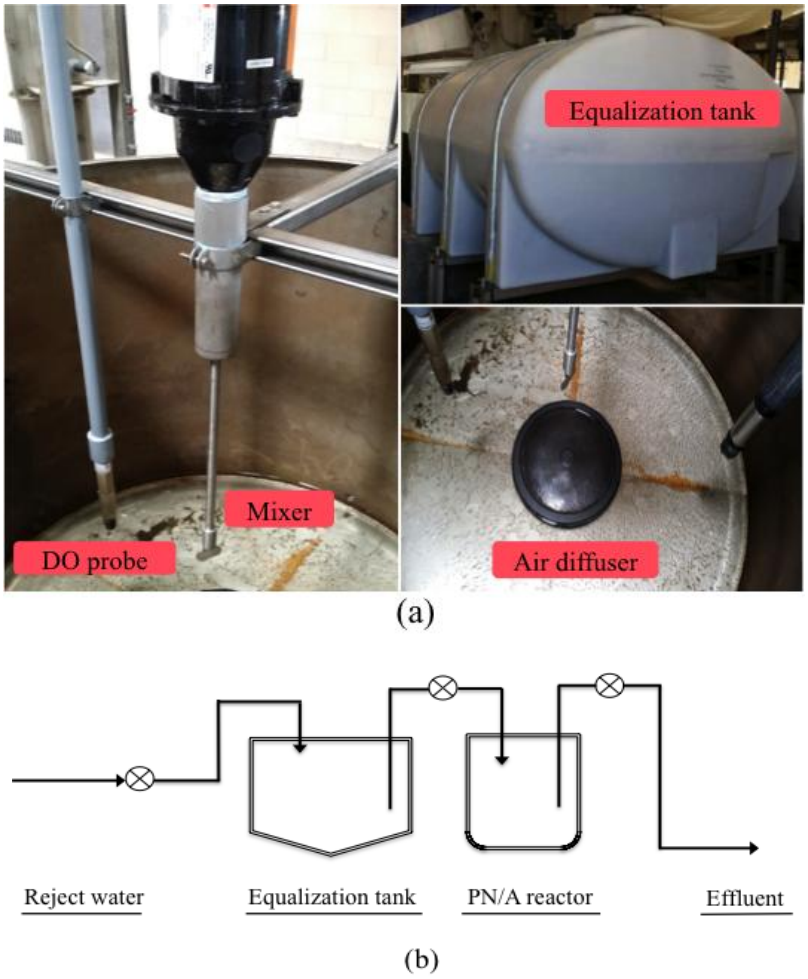

Figure 5.3 The design and application of pilot-scale PN/A reactor in North Davis Sewer District.

Subsequently, to evaluate the in-situ AMX activity, the pilot reactor was maintained anoxic for hours with subsequently measuring the NH_4^+-N and NO_2^--N content every two hours until NO_2^- below 5 mg_N L^{-1} was achieved.

Results

MLSS and MLVSS

In the beginning of stage II, the MLSS = 1700 mg L^{-1} and MLVSS = 730 mg L^{-1} in the pilot reactor. After about one month of operation, the MLSS reduced to 950 mg L^{-1}

and MLVSS to 470 mg L^{-1}, indicating a continuous loss of biomass from the reactor. By careful observation of the settling and decanting phases for several days, it was found that dozens of small bubbles occasionally came out from the sludge bed when the biomass had settled for 10 min. This resulted in a slight mixing of biomass and the top layer of clear water, which was withdrawn out by the effluent pump. This leaded to the biomass loss.

The production of the bubbles could have been from the aerator, when a sudden shut off of the air pump caused an unbalance of pressure inside. Eventually, the air trapped inside the aerator slowly escaped. The low ratio of MLVSS/MLSS <0.5 indicated an accumulation of the inorganic solids in the reactor. This might be due to the insufficient settling in the equalization tank, where some of the solids were floating on the top.

DO profile

Generally, the concentration of DO for the suspended growth PN/A system is< 0.4 mg L^{-1} during aeration and <0.05 mg L^{-1} during anoxic mixing. However, this number is based on a high biomass concentration, which requires more oxygen. In this case, since the biomass was less, the DO was set lower than 0.3 mg L^{-1} for partial nitritation to avoid over-aeration. The final DO profile of the reactor is shown as follows in Figure 5.4. When aeration was started, DO increased quickly from 0.05 mg L^{-1} to 0.27 mg L^{-1} in 4 min, and when aeration stopped, DO decreased immediately to the minimum of 0.05 mg L^{-1}. It demonstrated a real aerobic condition of 15 min followed by an anoxic condition of 30 min.

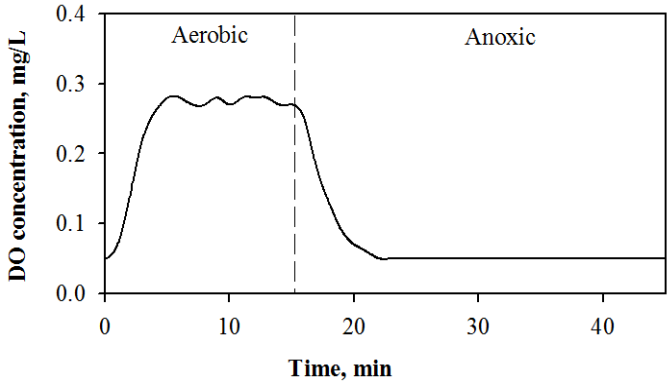

Figure 5.4 Dissolved oxygen concentration profile during one cycle.

Biomass activity and reactor performance

In stage I, when the inoculum was enriched, the in-situ activity results showed that the reactor was able to produce 0.188 $kg_{NO2\text{--}N}\ kg_{VSS}^{-1}\ d^{-1}$ at the aerobic condition, and consume 0.099 $kg_{NO2\text{-}N}\ kg_{VSS}^{-1}\ d^{-1}$ at the anoxic condition. Compared to the lab-scale reactor at start-up phase, where the values were 0.230/ 0.187 $kg_{NO2^-\text{-}N}\ kg_{VSS}^{-1}\ d^{-1}$ at 35 ℃, respectively, the biomass activity was reduced by 20~50% at room temperature. During stage II, a regular monitoring of NH_4^+, NO_2^- and NO_3^- at the beginning as well as the end of each batch was performed and is represented in Figure 5.5. The average NH_4^+-N at the beginning of batch was 362.6 ±95.9 mg L^{-1}, with 230 ±125.5 mg L^{-1}at the end of batch. The average removal efficiency was only 40.0 ±25.4%. The low efficiency value was mainly due to the low biomass concentration. So when related to the MLVSS in the reactor, an average of 0.164 ±0.086 kg of NH_4^+-N was consumed by 1 kg of VSS every day. The removal of ~0.18kg of NH_4^+-N at steady-statewas reported by others (Wett, 2007a), indicating a successful start-up of the pilot plant. It is possible that the biomass may need more time to acclimatize with the new waste stream and the different operation strategies.

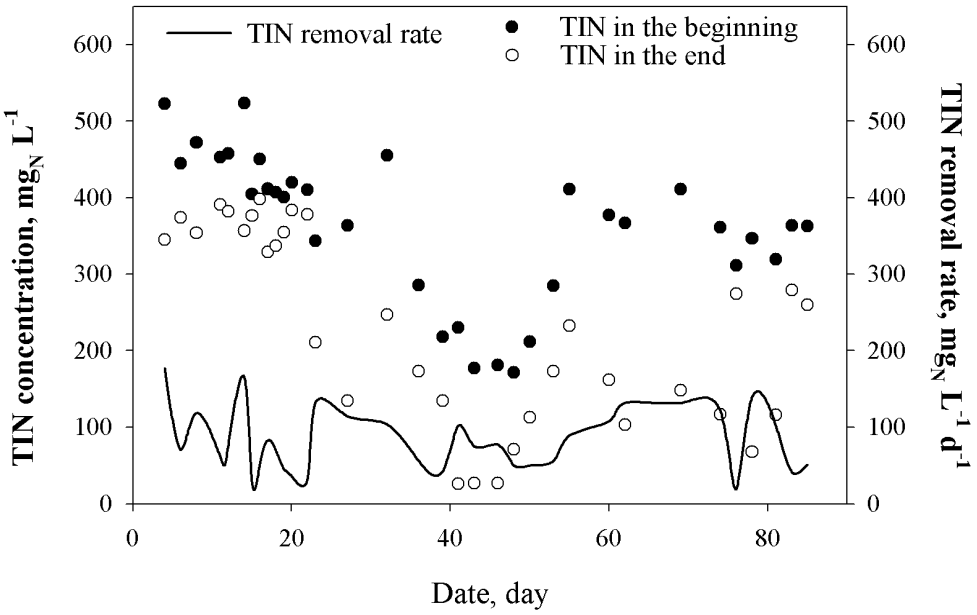

Figure 5.5 The performance of the pilot reactor during 90 days of operation.

Biomass loss

On day 92, a mechanical problem was occurred with the timer, which made the effluent pump to draw out most of the biomass when the reactor was well mixed. As a result, the mixed liquor in the reactor became almost clear.

Discussion

Due to the physiology of anammox bacteria, the start-up process for PN/A installation usually takes long time, ranging from 6 months to several years if no inoculum is provided (Ling et al., 2009; van der Start et al., 2007; Wett, 2006). However, this process can be shortened to only few weeks by adding sufficient seeding biomass. In this study, the seed obtained was very robust. The relative low nitrogen removal efficiency was mainly due to the low biomass content in the reactor. The MLSS never exceeded 2000 mg L^{-1} in relation to a regular operation at 3500~4000 mg L^{-1} (Joss et al.,

2009; Lackner et al., 2014; Vlaeminck et al., 2012; Wett et al., 2007a).

Compared to a lab-scale reactor, which needs much less seed biomass to start, the pilot or full-scale application requires large amount of the active seed. Adequate seed can considerably shorten the start-up process. Similar to our observation and finding, van der Star et al. (2007) also concluded that inoculation with large amounts of active biomass enabled a fast check of the installation. When the automation systems were functioning well, it lead to a much shorter startup period. However, obtaining sufficient inoculum is very challenging and costly due to less applications in the filed of engineering research (Guestavsson, 2012). For large-scale applications, it is common that the engineers start with a smaller scale and gradually increase the volume until enough biomass can be obtained.

In the beginning of the operation, the air flow rate was adjusted numerous times to maximize the nitrogen removal. However, the increase or decrease of air flow rates did not result in a significant DO change in the reactor, while the NO_2^--N in the effluent varied widely from 0 to 20 mg L^{-1}. Therefore, monitoring the air flow rate instead of DO concentrations could provide better and more reliable control strategies for alow DO requirement. This finding also agreed with the research of Joss et al. (2011).

The equalization tank, although it was able to settle down most of the inorganic solids from the reject water. However, a floatation of sludge on the top of the liquid level was observed. The floated sludge went to the PN/A system with the feed, resulting in a very low MLVSS/MLSS ratio in the reactor. It was reported that high TSS in the influent led to an increase in nitrate production and the growth of NOB in the system (Lackner et al., 2014). Moreover, the solids in the reactor could impact the oxygen transformation to

AOB, resulting in an unstable ammonium oxidation (Zhang et al., 2015). It is recommended that before starting a PN/A reactor, a hydraulic test should be done to investigate the settling abtility of the equalization tank and to make sure it is functioning.

During the operation stage, the effluent pump did not function well numerous times. It was found that during the decanting process, when the effluent pump withdraw all the liquid above the 100 gal level leading toair bubble trapped inside the pump, eventually reducing the withdraw rate for the effluent. As a result, more than 100 gal of treated liquid were retained in the reactor, increasing the total working volume above 250 gal. Fortunately, the malfunction of the pump did not cause any over flow. To prevent this from happening again, the effluent pipeline was modified by the staff from NDSD. For any future PN/A installation, this should be considered to be important because if the effluent pump cannot withdraw the equal amount of treated wastewater, the subsequent feeding process could lead to overflowing and loss of biomass.

For large scale PN/A installation, it is recommended that all the effluent discharge from the reactor is fulfilled by a hydrocyclone system in order to prevent the loss of biomass from the decanter. Additionally, in correlating with previous research shown in Chapter 2, we suggested that the attached growth PN/A system be more applicable especially for wastewater treatment plants with little experience on anammox process handling.

The lab-scale study indicated that the alkalinity in the filtrate was not sufficient for complete ammonium removal. In the pilot study, the low amount of biomass led to a low removal rate. The alkalinity addition was not considered in this case. However, in other PN/A applications, the decreased performance of the reactors was due to the

insufficient alkalinity and the restoring of nitrogen removal by additional alkalinity (Klein et al., 2012).

If the plant is expecting >90% nitrogen removal, the cost of inorganic carbon (e.g., soda, lime) should be taken into account when evaluating the capital and operation costs. Last but not the least, training thestaff in all wastewater treatment plants to obtain the knowledge of the PN/A system is advised.

Summary

A 250-gallon pilot-scale partial nitritation-anammox reactor was applied in the North Davis Sewer District to remove the ammonium content from reject water, which is produced by anaerobic digestion of the excess of sludge from biological treatment. Due to the low biomass concentration in the reactor, the average ammonium removal efficiency was only 40%. However, by relating the total inorganic nitrogen to the MLVSS, the reactor was able to remove 0.164 ± 0.086 kg_N $kg_{VSS}^{-1}d^{-1}$, indicating a relatively high bacterial activity at room temperature.

It is no doubt that running a pilot or full scale reactor is much more complicated than operating a lab-scale reactor. Despite the hydraulic testsdone before initializing the reactor, several mechanical issues came forward. First, the unsettled sludge floating in the equalization tank entered the reactor and could not be removed easily. Second, small bubbles from the air blower during settling and decanting phases resulted in a loss of the biomass. Third, the effluent pump malfunctioned several times, which could have caused an overflow but fortunately it did not. However, an unexpected problem occurred to the controlling timer, which enabled the effluent pump to workwhen the reactor was well

mixed. This led to a loss of most of the biomass, without which the reactor could not perform any nitrogen removal. Based on these findings, more careful considerationneeds to be taken into account while start-up and operation of a pilot or full-scale PN/A application.

CHAPTER 6

CONCLUSIONS

There is no doubt that the concern about the rising cost of stricter nitrogen removal from wastewater has drawn much attention in recent years. The anammox process has been considered as an alternative approach to treat different waste streams, such as anaerobic digestion supernatant, source separated urine, and other wastewater containing high ammonium content. However, despite a substantialamount of research on this novel technology during the last decade, there is still a lack of understanding about the reliability, stability and performance in full-scale application for this new technology. This study provides a broader understanding of the need to reduce operation costs related to temperature control and other potential stress factors in order to ensure successful implementation of the anammox process for nitrogen removal from wastewater.

The lab-scale suspended and attached growth reactors demonstrated that at 35 ℃ and 21 ℃, both reactors harboredsimilar nitrogen removal performance and similar microbial communities. They were dominated by three main phyla: *Chloroflexi*, *Planctomycetes*, and *Proteobacteria*. In order to accelerate the startup process, an elevated temperature was preferred allowing the growth of *Chloroflexi*, which could help construct the backbone structure for the bacteria in the system. At steady-state, both reactors adapted to lower temperature within 2 weeks, showing that in full-scale

application it is feasible to bring down the reactor temperature to reduce the operation cost.

The granular/flocculent biomass from the suspended growth bioreactor was studied in order to investigate the responses of AOB and AMX to low temperatures and sulfide dosage. The temperature study showed that from 35 ℃ to 21 ℃, the activity of AOB decreased by 33% while AMX activity dropped by 42%; from 21 ℃ to 13 ℃, AOB showed no significant difference in activity while AMX suffered a 90% activity loss. The *amoA* and *hzsA* genes, which were the most typical biomarkers for AOB and AMX, respectively, showed that the activity of *Nitrosomonas europaea* affiliatedAOB was inversely correlated with *amoA* gene expression. In contrast, the activity of Candidatus Brocadia related AMX was correlatedwith *hzsA* gene. Sulfide that may be present in reject water could bring negative effects on the activity of AOB and AMX, even as low as 5 mg_S L^{-1}. Thus, when applying PN/A in full-scale nitrogen removal, the content of sulfide in the influent needs to be carefully watched. Understanding how these external stress factors impact the bacteria involved in nitrogen transformation during the PN/A process is critical for developing operation strategies and for creating gene expression-based biosensors to detect these inhibitors in engineered environments.

Urine contributes less than 1% of the volume but 80% of the nitrogen in municipal wastewater. Therefore, the source separation and treatment of urine would provide more benefits for removing nutrients. Up to 99% of phosphorus and 5% of nitrogen can be recovered during struvite precipitation from the hydrolyzed urine. The residual ammonium was removed by either a two-stage system with up to 75% of efficiency or by a single-stage system with 50% efficiency. Despite the finding that COD

and high pH in the urine posts several challenges for the single-stage PN/A reactor, the nitrate production could bepotentially eliminated as the result of heterotrophic denitrification. This research demonstrated that both two-stage and single-stage anammox systems could be reliable alternatives for urine treatment.

Given the absence of anammox application in Utah, a pilot-scale reactor was started and operated in the North Davis Sewer District, Syracuse, Utah.The pilot study showed that the biomass maintained relatively high activity at room temperature demonstrating a successful startup of the PN/A reactor for reject water treatment. Moreover, the loss of biomass after 3 months operation suggested that a hydrocyclone that separates AOB from AMX is recommended for all effluent discharge,instead of liquid pumpsin the case of suspended growth PN/A systems.

This work contains information that should be helpful for engineers in the development of more reliable, robust, and energy efficient start-up and operation strategies for suspended growth and attached growth anammox systems to remove nitrogenous compounds from wastewater.

The finding would eventually help process engineers to develop a more reliable, robust and energy efficient startup and operation strategies for PN/A installations.

CHAPTER 7

SCIENTIFIC CONTRIBUTION AND DISSEMINATION OF RESULTS

Publications

Wu, S., Weissbrodt, D.G., Morgenroth, E., Goel, R. Effect of temperature decrease and sulfide toxicity on bacterial activation in a Partial Nitritation/Anammox reactor. (Submitted to Water Research)

Wu, S., Bhattacharjee, A.S., Goel, R. Comparison of microbial communities in suspended growth and attached growth Partial Nitritation/Anammox system under different tempratures. (Submitted to Bioresource Technology)

Huang, P., Mukherji. S. T., Wu, S. & Goel, R. (2016). Fate of 17β-Estradiol during synthetic urine separation and P recovery integrated with treatment using partial nitritation-anammox systems. Environmental Science & Technology. (Accepted)

Bhattacharjee, A.S., Wu, S., Lawson, C.E. Goel, R. Metagenomics reveals similar microbial community structures in suspended and attached growth of anammox reactor. (To be submitted forEnvironmental Science & Technology)

Proceedings

Wu, S., Goel, R. Comparison of attached growth and suspended growth anammox reactors for real filtrate/centrate treatment- process start-up and performance. WEFTEC. September 26th-30th 2013. Chicago, Illinois.

Wu, S., Hogset, M., Heck, P., & Goel, R. Developing Operation Strategies for Pilot Scale One-stage Deammonification Reactor in Utah North Davis Sewer District (NDSD). WEAU Annual Conference.April 30th –May 2nd 2014. St. George, Utah

Bhattacharjee, A. S., Wu, S., & Goel R. An insight into the complex microbial communities of Attached Growth and Suspended Growth Anammox Reactors for real

centrate treatment process. ASM 114th General Meeting. May 17th -20th 2014. Boston, Massachusetts.

Wu, S., Bhattacharjee, A.S., & Goel, R. Evaluation of hybrid deammonification reactor for filtrate treatment in lab-scale: Start-up and performance. WEFTEC. September 27th - October 1st 2014. New Orleans, Louisiana.

Wu, S; Goel, R. Challenges in applying deammonification reactor in wastewater treatment plant.WEAU Annual Conference.April 30th –May 2nd 2015. St. George, Utah.

Lawson, C., Wu, S., Bhattacharjee, A. S., McMohan K. D. Goel, R., & Noguera D. R. Distributed metabolism in suspended and attached growth anammox bioreactors revealed through metagenomic sequencing. Water Microbiology Conference. May 18th – 22nd 2015. Chapel Hill, North Carolina.

Wu, S., Weissbrodt, D. G., Morgenroth, E., & Goel, R. Resiliency Of Anammox Against External Stress Factors - Effect Of Temperature And Wastewater Constituents. WEFTEC. September 26th-30th 2015. Chicago, Illinois.

Lawson, C. E., Wu, S., Bhattacharjee, A. S., McMahon, K. D., Goel R., & Noguera, D. R. WEFTEC. September 26th-30th 2015. Chicago, Illinois.

Bhattacharjee, A. S., Wu, S., & Goel, R. Metagenomics Reveals Different Microbial Community Structures in Suspended and Attached Growth anammox Reactors. WEFTEC. September 26th-30th 2015. Chicago, Illinois.

Dasgupta, S., Wu, S., Goel, R. Coupling autotrophic denitrification with partial nitritation-anammox (PNA) for efficient total inorganic nitrogen removal. WEFTEC. September 26th-30th 2015. Chicago, Illinois.

REFERENCES

Adnan, A.; Dastur, M.; Mavinic, D. S.; Koch, F. A. Preliminary Investigation into Factors Affecting Controlled Struvite Crystallization at the Bench Scale. *J. Environ. Eng Sci*. **2004**, *3*(3), 195-202.

Alawi, M.; Lipski, A.; Sanders, T.; Spieck, E. Cultivation of a Novel Cold-Adapted Nitrite Oxidizing Betaproteobacterium from the Siberian Arctic. *The ISME J*. **2007**, *1*(3), 256-264.

Ali, M.; Oshiki, M.; Okabe, S. Simple, Rapid and Effective Preservation and Reactivation of Anaerobic Ammonium Oxidizing Bacterium "Candidatus Brocadia sinica". *Water Res*. **2014**, *57*, 215-222.

Allferi, P.; Valter, T.; Carmela, L. Influence of Aeration and Sludge Retention Time on Ammonium Oxidation to Nitrite and Nitrate. *Water Res*. **2002**, *36*, 2541-2546.

Anderson, I. C.; Levine, J. S. Relative Rates of Nitric Oxide and Nitrous Oxide Production by Nitrifiers, Denitrifiers, and Nitrate Respirers. *Appl. Environ. Microb*. **1986**, *51*(5), 938-945.

Arp, D. J.; Sayavedra-Soto, L. A.; Hommes, N. G. Molecular Biology and Biochemistry of Ammonia Oxidation by Nitrosomonas Europaea. *Arch. Microbiol*. **2002**, *178*(4), 250-255.

Aoi, Y.; Miyoshi, T.; Okamoto, T.; Tsuneda, S.; Hirata, A.; Kitayama, A.; Nagamune, T. Microbial Ecology of Nitrifying Bacteria in Wastewater Treatment Process Examined by Fluorescence in-situ Hybridization. *J. Biosci Bioeng*. **2000**, *90*(3), 234-240.

Barria, C.; M, Malecki, M.; Arraiano, C. M. Bacterial Adaptation to Cold. *Microbiology*. **2013**, *159*, 2437-2443.

Beauchamp, R. O.; Bus, J. S.; Popp, J. A.; Boreiko, C. J.; Andjelkovich, D. A.; Leber, P. A Critical Review of the Literature on Hydrogen Sulfide Toxicity. *CRC Critical Rev. Toxicol*. **1984**, *13*(1), 25-97.

Beccari, M.; Passino, R.; Ramadori, R.; Tandoi, V. Inhibitory Effects on Nitrification by Typical Compounds in Coke Plant Wastewaters. *Environ. Technol*. **1980**, *1*(5), 245-252.

Behrendt, J.; Arevalo, E.; Gulyas, H.; Niederste-hollenberg, J.; Niemiec, A.; Zhou, J.; Otterpohl, R. Production of Value Added Products from Separately Collected Urine.

Water Sci. Technol. **2002**, *46*, 341.

Bergmann, D. J.; Hooper, A. B.; Klotz, M. G. Structure and Sequence Conservation of hao Cluster Genes of Autotrophic Ammonia-Oxidizing Bacteria: Evidence for Their Evolutionary History. *Appl. Environ. Microb*. **2005**, *71*(9), 5371-5382.

Bergmann, G.; Bates, S.; Eilers, K.; Lauber, C.; Caporaso, J.; Walters, W. The Under-recognized Dominance of *Verrucomicrobia* in Soil Bacterial Communities. *Soil. Biol. Biochem.* **2011**, *43*, 1450–1455.

Bjornberg, C.; Lin, W.; Zimmerman, R. Effect of Temperature on Biofilm Growth Dynamics and Nitrification Kinetics in a Full-scale MBBR System. *Proc.Water Environ. Fed*. **2009**, *12*, 4407-4426.

Björnsson, L.; Hugenholtz, P.; Tyson, G. W.; Blackall, L. L. Filamentous Chloroflexi (Green Non-Sulfur Bacteria) are Abundant in Wastewater Treatment Processes with Biological Nutrient removal. *Microbiology*, **2002**, *148*(8), 2309-2318.

Bokulich, N. A.; Thorngate, J. H.; Richardson, P. M.; Mills, D. A. Microbial Biogeography of Wine Grapes is Conditioned by Cultivar, Vintage, and Climate. *P. Natl. Acad. Sci.* **2014**, *111*(1), 139-148.

Bollmann, A.; Laanbroek, H. J. Continuous Culture Enrichments of Ammonia Oxidizing Bacteria at Low Ammonium Concentrations. *FEMS Microbiol. Ecol.* **2001**, *37*(3), 211-221.

Bossier, P.; Verstraete, W. Triggers for Microbial Aggregation in Activated Sludge? *Appl. Microbiol Biotechnol.* **1996**, *45*(1-2), 1-6.

Botchkova, E. A.; Litti, Y. V.; Kuznetsov, B. B.; Nozhevnikova, A. N. Microbial Biofilms Formed in a Laboratory-Scale Anammox Bioreactor with Flexible Brush Carrier. *J. Biomater. Nanobiotechnol.* **2014**, *05*(2), 76-82.

Brandsma, J.; van de Vossenberg, J.; Risgaard-Petersen, N.; Schmid, M. C.; Engstrom, P.; Eurenius, K.; Strous, M. A Multi-proxy Study of Anaerobic Ammonium Oxidation in Marine Sediments of the Gullmar Fjord, Sweden. *Environ. Microbiol. Rep*. **2011**, *3*(3), 360-366.

Breisha, G. Z.; Winter, J. Bio-Removal of Nitrogen from Wastewaters-A review. *J. Am. Sci*. **2010**, *6*(12), 508-528.

Bulc, T. G. Long Term Performance of a Constructed Wetland for Landfill Leachate Treatment. *Ecol. Eng*. **2006**, *26*(4), 365-374.

Burgmann, H.; Jenni, S.; Vazquez, F.; Udert, K.M. Regime Shift and Microbial Dynamics in a Sequencing Batch Reactor for Nitrification and Anammox Treatment of Urine. *Appl. Environ. Microbiol*. **2011**, *77*, 5897–5907.

Camargo, J. A.; Alonso, A.; Salamanca, A. Nitrate Toxicity to Aquatic Animals: Review with New Data for Freshwater Invertebrates. *Chemosphere*. **2005**, *58*(9), 1255-1267.

Chamchoi, N.; Nitisoravut, S.; Schmidt, J. E. Inactivation of ANAMMOX Communities under Concurrent Operation of Anaerobic Ammonium Oxidation (ANAMMOX) and Denitrification. *Biores. Technol*. **2008**, *99*(9), 3331-3336.

Chen, C.; Wang, A.; Ren, N.; Kan, H.; Lee, D. J. Biological Breakdown of Denitrifying Sulfide Removal Process in High-Rate Expanded Granular Bed Reactor. *Appl. Microbiol Biotechnol*. **2008**, *81*(4), 765-770.

Cho, S.; Takahashi, Y.; Fujii, N.; Yamada, Y.; Satoh, H.; Okabe, S. Nitrogen Removal Performance and Microbial Community Analysis of an Anaerobic Up-Flow Granular Bed Anammox Reactor. *Chemosphere*. **2010**, *78*(9), 1129-1135.

Chu, Z. R.; Wang, K.; Li, X. K.; Zhu, M. T.; Yang, L.; Zhang, J. Microbial Characterization of Aggregates Within a One-Stage Nitritation–Anammox System Using High-Throughput Amplicon Sequencing. *Chem. Eng. J*. **2015**, *262*, 41-48.

Colwell, R. K. EstimateS 6.0: Statistical estimation of species richness and shared species from samples (Software and Users' Guide). 2000. http://viceroy.eeb.uconn.edu/estimates/EstimateS

Conley, D. J.; Paerl, H. W.; Howarth, R. W.; Boesch, D. F.; Seitzinger, S. P.; Havens, K. E.; Likens, G. E. Controlling Eutrophication: Nitrogen and Phosphorus. *Science*. **2009**, *323*(5917), 1014-1015.

Cornell, W. D.; Cieplak, P.; Bayly, C. I.; Gould, I. R.; Merz, K. M.; Ferguson, D. M.; Kollman, P. A. A second Generation Force Field for the Simulation of Proteins, Nucleic Acids, and Organic Molecules. *J. Am. Chem. Soc*. **1995**, *117*(19), 5179-5197.

Coskuner, G.; Curtis, T. P. In situ Characterization of Nitrifiers in an Activated Sludge Plant: Detection of Nitrobacter Spp. *J. Appl. Microbiol*. **2002**, *93*(3), 431-437.

Czerwionka, K.; Makinia, J.; Pagilla, K. R.; Stensel, H. D. Characteristics and Fate of Organic Nitrogen in Municipal Biological Nutrient Removal Wastewater Treatment Plants. *Water Res.* **2012**, *46*(7), 2057-2066.

Dapena-Mora, A.; Fernandez, I.; Campos, J. L.; Mosquera-Corral, A.; Mendez, R.; Jetten, M. S. M. Evalution of Activity and Inhibition Effects on Anammox Process by Batch Tests Based on the Nitrogen Gas Production. *Enzyme Micro. Technol*. **2007**, *40*, 859-865.

Daverey, A.; Su, S. H.; Huang, Y. T.; Chen, S. S.; Sung, S.; Lin, J. G. Partial Nitrification and Anammox Process: a Method for High Strength Optoelectronic Industrial Wastewater Treatment. *Water Res*. **2013**, *47*(9), 2929-2937.

de Graaff, M. S.; Vieno, N. M.; Kujawa-Roeleveld, K.; Zeeman, G.; Temmink, H., Buisman, C. J. N. Fate of Hormones and Pharmaceuticals during Combined Anaerobic

Treatment and Nitrogen Removal by Partial Nitritation-Anammox in Vacuum Collected Black Water. *Water Res*. **2011**, *45*, 375–383.

Delatolla, R.; Tufenkji, N.; Comeau, Y.; Gadbois, A.; Lamarre, D.; Berk, D. Effects of Long Exposure to Low Temperatures on Nitrifying Biofilm and Biomass in Wastewater Treatment. *Water Environ. Res.* **2012**, *84*(4), 328-338.

Dosta, J.; Fernandez, I.; Vazquez-Padin, J. R.; Mosquera-Corral, A.; Campos, J. L.; Mata-Alvarez, J.; Mendez, R. Short-and Long-Term Effects of Temperature on the Anammox Process. *J. Hazard. Mater*. **2008**, *154*(1), 688-693.

Downing, A. L.; Hopwood, A. P. Some Observations on The kinetics of Nitrifying Activated-Sludge Plants. *Aquatic Sci.* **1964**, *26*(2), 271-288.

Ducey, T. F.; Vanotti, M. B.; Shriner, A. D.; Szogi, A. A.; Ellison, A. Q. Characterization of a Microbial Community Capable of Nitrification at Cold Temperature. *Bioresour. Technol*. **2010**, *101*(2), 491-500.

Edgar, R. *Usearch*. Lawrence Berkeley National Laboratory (LBNL), Berkeley, CA (United States). 2010.

Edgar, R. C.; Haas, B. J.; Clemente, J. C.; Quince, C.; Knight, R. UCHIME Improves Sensitivity and Speed of Chimera Detection. *Bioinformatics* **2011**, *27*(16), 2194-2200.

Edgar, R.C. UPARSE: Highly Accurate OTU Sequences from Microbial Amplicon Reads. *Nat. Methods*. **2013**, *10*(10), 996-998.

Egli, K.; Fanger, U.; Alvarez, P. J. J.; Siegrist, H.; Van der Meer, J. R.; Zehnder, A. R. J. Enrichment and Characterization of an Anammox Bacterium from a Rotating Biological Contactor Treating Ammonium-Rich Leachate, *Arch. Microbiol*. **2001**, 175(3), 198–207.

Erguder, T. H.; Boon, N.; Vlaeminck, S. E.; Verstraete, W. Partial Nitrification Achieved by Pulse Sulfide Doses in a Sequential Batch Reactor. *Environ. Sci. Technol*. **2008**, *42*(23), 8715-8720.

Esoy, A.; Odegaard, H.; Bentzen, G. The Effect of Sulfide and the Organic Matter on the Nitrification Activity in a Biofilm Process. *Water Sci. Technol*. **1998**, *37*(1), 115-122.

Etter, B.; Tilley, E.; Khadka, R.; Udert, K. M. Low-cost Struvite Production Using Source-separated Urine in Nepal. *Water Res*. **2011**, *45*(2), 852-862.

Feng, D.; Wu Z.; Xu S. Nitrification of Human Urine for its Stabilization and Nutrient Recycling. *Bioresour. Technol*. **2008**, *99*, 6299-6304.

Feng, Y.; Yu, Y.; Duan, Q.; Tan, J.; Zhao, C. The Characteristic Research of Ammonium Removal in Grain-slag Biological Aerated Filter (BAF). *Desalination*. **2010**, *263*(1), 146-150.

Fiencke, C.; Spieck, E.; Bock, E. Nitrifying Bacteria. In *Nitrogen fixation in agriculture, forestry, ecology, and the environment* . Springer Netherlands. 2005, pp 255-276

Fukushima, T.; Wu, Y. J.; Whang, L. M. The influence of Salinity and Ammonium Levels on amoA mRNA Expression of Ammonia Oxidizing Prokaryotes. *Water Sci. Technol*. **2012**, *65*(12), 2228-2235.

Furukawa, K.; Lieu, P. K.; Tokitoh, H.; Fujii, T. Development of Single-stage Nitrogen Removal Using Anammox and Partial Nitritation (SNAP) and its Treatment Performances. *Water Sci. Technol*. **2006**, *53*(6), 83-90.

Fux, C.; Boehler, M.; Huber, P.; Brunner, I.; Siegrist, H. Biological Treatment of Ammonium-rich Wastewater by Partial Nitritation and Subsequent Anaerobic Ammonium Oxidation (Anammox) in a Pilot Plant. *J. Biotechnol*. **2002**, *99*(2002), 295-306.

Galloway, J. N.; Townsend, A. R.; Erisman, J. W.; Bekunda, M.; Cai, Z.; Freney, J. R.; Martinelli, L. A.; Seitzinger, S. P.; Sutton, M. A. Transformation of the Nitrogen Cycle: Recent Trends, Questions, and Potential Solutions. *Science*. **2008**, *320*, 889-892.

Ge, S.; Wang, S.; Yang, X.; Qiu, S.; Li, B.; Peng, Y. Detection of Nitrifiers and Evaluation of Partial Nitrification for Wastewater Treatment: A Review. *Chemosphere* **2015**, *140*, 85-98.

Gilbert, E. M.; Müller, E.; Horn, H.; Lackner, S. Microbial Activity of Suspended Biomass from a Nitritation–anammox SBR in Dependence of Operational Condition and Size Fraction. *Appl. Microbiol. Biotechnol*. **2013**, *97*(19), 8795-8804.

Gilbert, E. M.; Agrawal, S., Karst, S. M.; Horn, H.; Nielsen, P. H. (2014).Low Temperature Partial Nitritation/Anammox in a Moving Bed Biofilm Reactor Treating Low Strength Wastewater. *Environ. Sci. Techno*l. **2014**, 48, 8784-8792.

Gilbert, E. M.; Agrawal, S.; Schwartz, T.; Horn, H.; Lackner, S. Comparing Different Reactor Configurations for Partial Nitritation/Anammox at low Temperatures. *Water Res*. **2015**, *81*, 92-100.

Ghosh, G. K.; Mohan, K. S.; Sarkar, A. K. Characterization of Soil-fertilizer P Reaction Products and their Evaluation as Sources of P for Gram (Cicer arietinum L.). *Nutr. Cycl. Agroecosys*. **1996**, *46*(1), 71-79.

Gong, Z.; Yang, F.; Liu, S.; Bao, H.; Hu, S.; Furukawa, K. Feasibility of a Membrane-Aerated Biofilm Reactor to Achieve Single-stage Autotrophic Nitrogen Removal Based on Anammox. *Chemosphere* **2007**, *69*(5), 776-784.

Gonzalez-Martinez, A.; Rodriguez-Sanchez, A.; Muñoz-Palazon, B.; Garcia-Ruiz, M. J.; Osorio, F.; van Loosdrecht, M. C.; Gonzalez–Lopez, J. Microbial Community Analysis of a Full-scale DEMON Bioreactor. *Bioproc. Biosyst. Eng*. **2014**, *38*(3), 499-508.

Grady, C. L.; Daigger, G. T.; Love, N. G.; Filipe, C. D. Grady, C. P. L. *Biological wastewater treatment*. CRC press. **2011**, pp 32-44.

Griffith, D. P.; Musher, D. Á.; Itin, C. Urease: The Primary Cause of Infection-induced Urinary Stones. *Investig. Urol*. **1976**, *13*(5), 346-350.

Guest, J. S.; Skerlos, S. J.; Barnard, J. L.; Beck, M. B.; Daigger, G. T.; Hilger, H.; Mihelcic, J. R. A New Planning and Design Paradigm to Achieve Sustainable Resource Recovery from Wastewater. *Environ Sci. Technol*, **2009**, *43*(16), 6126-6130.

Guo, J.; Peng, Y.; Huang, H.; Wang, S.; Ge, S.; Zhang, J.; Wang, Z. Short-and Long-term Effects of Temperature on Partial Nitrification in a Sequencing Batch Reactor Treating Domestic Wastewater. *J. Hazard. Mater*. **2010**, *179*(1), 471-479.

Harhangi, H. R.; Roy, M. L.; Alen, T. V.; Hu, B. I.; Groen, J.; Kartal, B.; Tringe, S. G.; Quan, Z. X.; Jetten, M. S. M.; Camp, H. J. M Op Den. Hydrazine Synthase, a Unique Phylomarker with Which To Study the Presence and Biodiversity of Anammox Bacteria. *Appl Environ. Microbiol*, **2011**, *78*(3), 752-758.

Hellinga, C.; Schellen, A. A. J. C.; Mulder, J. W.; van Loosdrecht, M. C. M.; Heijnen, J. J. The Sharon Process: An Innovative Method for Nitrogen Removal From Ammonium-rich Waste Water. *Water Sci. Technol*. **1998**, *37*(9), 135-142.

Hendrickx, T. L.; Kampman, C.; Zeeman, G.; Temmink, H.; Hu, Z.; Kartal, B.; Buisman, C. J. High Specific Activity for Anammox Bacteria Enriched from Activated Sludge at 10 °C. *Bioresour. Technol*. **2014**, *163*, 214-221.

Hesselsoe, M.; Füreder, S.; Schloter, M.; Bodrossy, L.; Iversen, N.; Roslev, P.; Loy, A. (2009). Isotope Array Analysis of Rhodocyclales Uncovers Functional Redundancy and Versatility in an Activated Sludge. *The ISME J*. **2009**, *3*(12), 1349-1364.

Hira, D.; Toh, H.; Migita, C. T.; Okubo, H.; Nishiyama, T.; Hattori, M.; Fujii, T. Anammox Organism KSU-1 Expresses a NirK-type Copper-containing Nitrite Reductase Instead of a NirS-type with Cytochrome cd 1. *FEBS Letters* **2012**, *586*(11), 1658-1663.

Höglund, C.; Stenström, T. A.; Jönsson, H.; Sundin, A. Evaluation of Faecal Contamination and Microbial Die-off in Urine Separating Sewage Systems. *Water Sci. Technol*. **1998**, *38*(6), 17-25.

Höglund, C. E.; Stenström, T. A. B. Survival of Cryptosporidium Parvum Oocysts in Source Separated Human Urine. *Can. J. Microbiol*. **1999**, *45*(9), 740-746.

Höglund, C.; Vinnerås, B.; Stenström, T. A.; Jönsson, H. Variation of Chemical and Microbial Parameters in Collection and Storage Tanks for Source Separated Human Urine. *J. Environ. Sci. Heal. A*. **2000**, *35*(8), 1463-1475.

Hommes, N. G.; Sayavedra-Soto, L. A.; Arp, D. J. Mutagenesis and Expression of amo, which Codes for Ammonia Monooxygenase in Nitrosomonas Europaea. *J. Bacteriol*. **1998**, *180*(13), 3353-3359.

Hu, Z.; Lotti, T.; de Kreuk, M.; Kleerebezem, R.; van Loosdrecht, M.; Kruit, J.; Kartal, B. Nitrogen Removal by a Nitritation-anammox Bioreactor at Low Temperature. *Appl Environ. Microbiol*, **2013**, *79*(8), 2807-2812.

Huang, P.; Hogsett, M. The Robustness of ANAMMOX Communities Treating Full-Scale Sidestream Municipal Anaerobic Digester Filtrate. *Proc. Water Environ. Feder*. **2011**, *13*, 3147-3155.

Hubaux, N.; Wells, G.; Morgenroth, E. Impact of Coexistence of Flocs and Biofilm on Performance of Combined Nitritation-anammox Granular Sludge Reactors. *Water Res*. **2015**, *68*, 127-139.

Hvitved-Jacobsen, T.; Vollertsen, J.; Tanaka, N. An Integrated Aerobic/Anaerobic Approach for Prediction of Sulfide Formation in Sewers. *Water Sci. Technol*. **2000**, *41*(6), 107-115.

Jetten, M. S. M.; Wanger, M.; Fuerst, J.; van Loosdrecht, M.; Kuene, G.; Strous, M. Microbiology and Application of the Anaerobic Ammonium Oxidation ('ANAMMOX') Process. *Curr. Opin. Biotechnol*. **2001**, *12* (3), 283-288.

Jetten, M. S.; Niftrik, L. V.; Strous, M., Kartal, B.; Keltjens, J. T.; Op den Camp, H. J. Biochemistry and Molecular Biology of Anammox Bacteria. *Crit. Rev. Biochem. Molecul. Biol*. **2009**, *44*(2-3), 65-84.

Jin, R. C.; Yang, G. F.; Yu, J. J.; Zheng, P. The Inhibition of the Anammox Process: a Review. *Chem. Eng. J*. **2012**, *197*, 67-79.

Jin, R. C.; Yang, G. F.; Zhang, Q. Q.; Ma, C.; Yu, J. J.; Xing, B. S. The Effect of Sulfide Inhibition on the ANAMMOX Process. *Water Res.* **2013**, *47*(3), 1459-1469.

Johnson, W. K.; Schroepfer, G. J. Nitrogen Removal by Nitrification and Denitrification. *J. Water Pollut. Contr. Feder*. **1964**, 1015-1036.

Johnston, A. E.; Richards, I. R. Effectiveness of Different Precipitated Phosphates as Phosphorus Sources for Plants. *Soil Use Manage*. **2003**, *19*(1), 45-49.

Jönsson, H.; Stenström, T. A.; Svensson, J.; Sundin, A. Source Separated Urine-nutrient and Heavy Metal Content, Water Saving and Faecal Contamination. *Water Sci. Technol*. **1997**, *35*(9), 145-152.

Joss, A.; Salzgeber, D.; Eugster, J.; Konig, R.; Rottermann, K.; Burger, S.; Fabijan, P.; Leumann, S.; Joachim, M.; Siegrist, H. Full-scale Nitrogen Removal from Digester with Partial Nitritation and Anammox in one SBR. *Environ. Sci. Technol*. **2009**, *43*(14), 5301-5306.

Junier, P.; Molina, V.; Dorador, C.; Hadas, O.; Kim, O. S.; Junier, T.; Imhoff, J. F. Phylogenetic and Functional Marker Genes to Study Ammonia-oxidizing Microorganisms (AOM) in the Environment. *Appl. Microbiol. Biotechnol*. **2010**, *85*(3), 425-440.

Jung, J. Y.; Kang, S. H.; Chung, Y. C.; Ahn, D. H. Factors Affecting the Activity of Anammox Bacteria during Start Up in the Continuous Culture Reactor, *Water Sci. Technol*. **2007**, *55*(1–2), 459–468.

Kalyuzhnyi, S.; Gladchenko, M.; Mulder, A.; Versprille, B. DEAMOX—New Biological Nitrogen Removal Process Based on Anaerobic Ammonia Oxidation Coupled to Sulphide-driven Conversion of Nitrate into Nitrite. *Water Res*. **2006**, *40*(19), 3637-3645.

Karak, T.; Bhattacharyya, P. Human Urine as a Source of Alternative Natural Fertilizer in Agriculture: A Flight of Fancy or an Achievable Reality. *Resour. Conserv. Recycl*. **2011**, *55*(4), 400-408.

Karkman, A.; Mattila, K.; Tamminen, M.; Virta, M. Cold Temperature Decreases Bacterial Species Richness in Nitrogen-Removing Bioreactors Treating Inorganic Mine Waters. *Biotechnol. Bioeng*. **2011**, *108*(12), 2876-2883.

Kartal, B.; Rattray, J.; van Niftrik, L. A.; van de Vossenberg, J.; Schmid, M. C.; Webb, R. I.; Strous, M. Candidatus "Anammoxoglobus Propionicus": A New Propionate Oxidizing Species of Anaerobic Ammonium Oxidizing Bacteria. *System. Appl. Microbiol*. **2007**, *30*(1), 39-49.

Kartal, B.; Van Niftrik, L.; Rattray, J.; Van De Vossenberg, J. L.; Schmid, M. C.; Damsté, J. S.; Strous, M. Candidatus 'Brocadia Fulgida': An Autofluorescent Anaerobic Ammonium Oxidizing Bacterium. *FEMS Microbiol. Ecol*. **2008**, *63*(1), 46-55.

Kartal, B.; van Niftrik, L.; Keltjens, J. T.; Op den Camp, H. J.; Jetten, M. S. Anammox—Growth Physiology, Cell Biology, and Metabolism. *Adv. Microb. Physiol*. **2012**, *60*, 212-252.

Kindaichi, T.; Yuri, S.; Ozaki, N.; Ohashi, A. Ecophysiological Role and Function of Uncultured Chloroflexi in an Anammox Reactor. *Water Sci. Technol*. **2012**, *66*(12), 2556-2561.

Kirchmann, H.; Pettersson, S. Human Urine-chemical Composition and Fertilizer Use Efficiency. *Fertiliz. Res*. **1994**, *40*(2), 149-154.

Klein, A.; Ekstrom, L.; Summers, A.; Wan, J.; Wett, B.; Johnson, C.; Melcer, H. Results of the Large-Scale Pilot Investigation of the DEMON® Nitrogen Removal System in Pierce County, Washington. *Proc. Water Environ. Feder*. **2012**, *17*, 470-483.

Koops, H. P.; Pommerening-Röser, A. Distribution and Ecophysiology of the Nitrifying Bacteria Emphasizing Cultured Species. *FEMS Microbiol. Ecol*. **2001**, *37*(1), 1-9.

Kotay, S. M.; Mansell, B. L.; Hogsett, M.; Pei, H.; Goel, R. Anaerobic Ammonia Oxidation (ANAMMOX) for Side-Stream Treatment of Anaerobic Digester Filtrate Process Performance and Microbiology. *Biotechnol. Bioeng*. **2013**, *110*(4), 1180-1192.

Kowalchuk, G. A.; Stephen, J. R.; De Boer, W. I. E. T. S. E.; Prosser, J. I.; Embley, T. M.; Woldendorp, J. W. Analysis of Ammonia-Oxidizing Bacteria of the beta Subdivision of the Class Proteobacteria in Coastal Sand Dunes by Denaturing Gradient Gel Electrophoresis and Sequencing of PCR-Amplified 16S Ribosomal DNA Fragments. *Appl. Environ. Microbiol*. **1997**, *63*(4), 1489-1497.

Kuenen, J. G. Anammox Bacteria: From Discovery to Application. *Natr. Rev. Microbiol*. **2008**, *6*(4), 320-326.

Kujawa-Roeleveld, K.; Zeeman, G. Anaerobic Treatment in Decentralised and Source-Separation-based Sanitation Concepts. *Rev. Environ. Sci. Biotechnol*. **2006**, *5*(1), 115-139.

Kuo, D. H. W.; Robinson, K. G.; Layton, A. C.; Meyers, A. J.; Sayler, G. S. Transcription Levels (amoA mRNA-based) and Population Dominance (amoA gene-based) of Ammonia-oxidizing Bacteria. *J. Industr. Microbiol. Biotechnol*. **2010**, *37*(7), 751-757.

Lackner, S.; Terada, A.; Smets, B. F. Heterotrophic Activity Compromises Autotrophic Nitrogen Removal in Membrane-Aerated Biofilms: Results of a Modeling Study. *Water Res*. **2008**, *42*(4-5), 1102-1112.

Lackner, S.; Gilbert, E. M.; Vlaeminck, S. E.; Joss, A.; Horn, H.; van Loosdrecht, M. C. M. Full-scale Partial Nitritation/ Anammox Experiences-An Application Survey. *Water Res*. **2014**, *55*, 292-303.

Larsen, T. A.; Lienert, J.; Joss, A.; Siegrist, H. How to Avoid Pharmaceuticals in the Aquatic Environment. *J. Biotechnol*. **2004**, *113*(1), 295-304.

Larsen, T.A.; Gujer, W. Separate Management of Anthropogenic Nutrient Solutions (Human Urine). *Water Sci. Technol*. **1996**, *34*(3-4), 87-94.

Laspidou, C. S; Rittmann, B. E. A Unified Theory for Extracellular Polymeric Substances, Soluble Microbial Products, and Active and Inert Biomass. *Water Res*. **2002**, *36*(11), 2711-2720.

Li, M.; Hong, Y.; Klotz, M. G.; Gu, J. D. A Comparison of Primer Sets for Detecting 16S rRNA and Hydrazine Oxidoreductase Genes of Anaerobic Ammonium-Oxidizing Bacteria in Marine Sediments. *Appl. Microbiol. Biotechnol*. **2010**, *86*(2), 781-790.

Li, X. Z.; Zhao, Q. L. Recovery of Ammonium-nitrogen from Landfill Leachate as a Multi-nutrient Fertilizer. *Ecolog Eng*. **2003**, *20*(2), 171-181.

Liang, Z.; Liu, J. Landfill Leachate Treatment with a Novel Process: Anaerobic Ammonium Oxidation (Anammox) Combined with Soil Infiltration System. *J. Hazard.*

Mater. **2008**, *151*(1), 202-212.

Limpiyakorn, T.; Shinohara, Y.; Kurisu, F.; Yagi, O. Communities of Ammonia-oxidizing Bacteria in Activated Sludge of Various Sewage Treatment Plants in Tokyo. *FEMS Microbiol. Ecol*. **2005**, *54*(2), 205-217.

Liu, S.; Yang, F.; Meng, F.; Chen, H.; Gong, Z. Enhanced Anammox Consortium Activity for Nitrogen Removal: Impacts of Static Magnetic Field. *J. Biotechnol*. **2008**, *138*(3), 96-102.

Liu, Y.; Kwag, J. H.; Kim, J. H.; Ra, C. Recovery of Nitrogen and Phosphorus by Struvite Crystallization from Swine Wastewater. *Desalination*. **2011**, *277*(1), 364-369.

Liu, Z.; Frigaard, N. U.; Vogl, K.; Iino, T.; Ohkuma, M.; Overmann, J.; Bryant, D. A. (2012). Complete Genome of Ignavibacterium Album, A Metabolically Versatile, Flagellated, Facultative Anaerobe from the Phylum Chlorobi. *Front. Microbiol*. **2012**, *3*, 185

Logan, B. E.; Rabaey, K. Conversion of Wastes into Bioelectricity and Chemicals by Using Microbial Electrochemical Technologies. *Science*. **2012**, *337*(6095), 686-690.

Lotti, T.; Kleerebezem, R.; Lubello, C.; Van Loosdrecht, M. C. M. Physiological and Kinetic Characterization of a Suspended Cell Anammox Culture. *Water Res*. **2014**, *60*, 1-14.

Lotti, T.; Kleerebezem, R.; van Loosdrecht, M. C. M. Effect of Temperature Change on Anammox Activity. *Biotechnol. Bioeng*. **2015**, *112*(1), 98-103.

Loy, A.; Schulz, C.; Lücker, S.; Schöpfer-Wendels, A.; Stoecker, K.; Baranyi, C. Wagner, M. 16S rRNA Gene-based Oligonucleotide Microarray for Environmental Monitoring of the Betaproteobacterial Order "Rhodocyclales". *Appl. Environ. Microbiol*. **2005**, *71*(3), 1373-1386.

Lu, H.; Chandran, K.; Stensel, D. Microbial Ecology of Denitrification in Biological Wastewater Treatment. *Water Res*. **2014**, *64*, 237-254.

Magrí, A.; Béline, F.; Dabert, P. (2013).Feasibility and Interest of the Anammox Process as Treatment Alternative for Anaerobic Digester Supernatants in Manure Processing–An Overview. *J. Environ. Manag*. **2013**, *131*, 170-184.

Makaya, J. M.; Savadogo, A.; Somda, M. K.; Bour, J. B.; Barro, N.; Traoré, A. S. Quality of Human Urine Used as Fertilizer: Case of an Ecological Sanitation System in Ouagadougou Peri-urban Areas-Burkina Faso. *J. Environ. Protect.* **2014**, *22*(5), 467-474.

Matějů, V.; Čižinská, S.; Krejčí, J.; Janoch, T. Biological Water Denitrification—a Review. *Enzyme. Microbial Technol*. **1992**, *14*(3), 170-183.

Martinelle, K.; Westlund, A.; Häggström, L. Ammonium Ion Transport—A Cause of Cell Death. *Cytotechnology*. **1996**, *22*(1-3), 251-254.

Maurer, M.; Pronk, W.; Larsen, T. A. Treatment Processes for Source-Separated Urine. *Water Res*. **2006**, *40*, 3151–3166.

McTavish, H. J. A. F.; Fuchs, J. A.; Hooper, A. B. Sequence of the Gene Coding for Ammonia Monooxygenase in Nitrosomonas Europaea. *J Bacteriol.* **1993**, *175*(8), 2436-2444.

Mihelcic, J. R.; Fry, L. M.; Shaw, R. Global Potential of Phosphorus Recovery from Human Urine and Feces. *Chemosphere*. **2011**, *84*(6), 832-839.

Molinuevo, B., García, M. C., Karakashev, D., Angelidaki, I. Anammox for Ammonia Removal from Pig Manure Effluents: Effect of Organic Matter Content on Process Performance. *Bioresour. Technol*. **2009**, *100*(7), 2171-2175.

Mosier, A.; Kroeze, C.; Nevison, C.;Oenema, O.; Seitzinger, S.; van Cleemput, O. Closing the Global N_2O Budget: Nitrous Oxide Emissions through the Agricultural Nitrogen Cycle. *Nutr. Cycl. Agroecosys*. **1998**, *52*(2-3), 225-248.

Münch, E. V.; Lant, P.; Keller, J. Simultaneous Nitrification and Denitrification in Bench-scale Sequencing Batch Reactors. *Water Res*. **1996**. *30*(2), 277-284.

Ni, B. J.; Ruscalleda, M.; Smets, B. F. Evaluation on the Microbial Interactions of Anaerobic Ammonium Oxidizers and Heterotrophs in Anammox Biofilm. *Water Res*. **2012**, *46*(15), 4645-4652.

Nielsen, P. H.; Raunkjaer, K.; Norsker, N. H.; Jensen, N. A.; Hvitved-Jacobsen, T. Transformation of Wastewater in Sewer Systems- a Review. *Water Sci. Technol*. **1992**, *25*(6), 17-31.

Nelson, N. O.; Mikkelsen, R. L.; Hesterberg, D. L. Struvite Precipitation in Anaerobic Swine Lagoon Liquid: Effect of pH and Mg: P Ratio and Determination of Rate Constant. *Bioresour. Technol*. **2003**, *89*(3), 229-236.

Nielsen, M.; Bollmann, A.; Sliekers, O.; Jetten, M.; Schmid, M.; Strous, M.; Schmidt, I.; Larsen, L. H.; Nielsen, L. P.; Revsbech, N. P. Kinetics, Diffusional Limitation and Microscale Distribution of Chemistry and Organisms in a CANON Reactor. *FEMS Microbiol. Ecol*. **2005**, *51*(2), 247-256.

Nikolaev, Y. A.; Kozlov, M. N.; Kevbrina, M. V.; Dorofeev, A. G.; Pimenov, N. V.; Kallistova, A. Y.; Patutina, E. O. Candidatus "Jettenia moscovienalis" sp. nov., A New Species of Bacteria Carrying out Anaerobic Ammonium Oxidation. *Microbiology* **2015**, *84*(2), 256-262.

Oshiki, M.; Shimokawa, M.; Fujii, N.; Satoh, H.; Okabe, S. Physiological Characteristics of the Anaerobic Ammonium-Oxidizing Bacterium 'Candidatus Brocadia

Sinica'. *Microbiology*. **2011**, *157*(6), 1706-1713.

Padival, N. A.; Weiss, J. S.; Arnold, R. G. Control of Thiobacillus by Means of Microbial Competition: Iimplications for Corrosion of Concrete Sewers. *Water Environ. Res.* **1995**, *67*(2), 201-205.

Park, S. J.; Oh, J. W.; Yoon, T. I. The Role of Powdered Zeolite and Activated Carbon Carriers on Nitrification in Activated Sludge with Inhibitory Materials. *Proc. Biochem*. **2003**, *39*(2), 211-219.

Park, H.; Noguera, D. R. Evaluating the Effect of Dissolved Oxygen on Ammonia-Oxidizing Bacterial Communities in Activated Sludge. *Water Res*. **2004**, *38*(14), 3275-3286.

Park, H.; Rosenthal, A.; Jezek, R.; Ramalingam, K.; Fillos, J.; Chandran, K. Impact of Inocula and Growth Mode on the Molecular Microbial Ecology of Anaerobic Ammonia Oxidation (anammox) Bioreactor Communities. *Water Res*. **2010**, *44*(17), 5005-5013.

Park, H.; Sundar, S.; Ma, Y.; Chandran, K. Differentiation in the Microbial Ecology and Activity of Suspended and Attached Bacteria in A Ntritation-Anammox Process. *Biotechnol. Bioeng*. **2015**, *112*(2), 272-279.

Park, K.; Ely, R.L. Whole Genome Transcriptional and Physiological Responses of Nitrosomonas europaea to Cyanide: Identification of Cyanide Stress Response Genes. *Biotechnol. Bioeng*. **2009**, *102*(6), 1645-1653.

Peng, Y.; Zhu, G. Biological Nitrogen Removal with Nitrification and Denitrification via Nitrite Pathway. *Appl. Microbiol. Biotechnol*. **2006**, *73*(1), 15-26.

Pesson, F.; Sultana, R.; Suarez, M.; Hermansson, M.; Plaza, E.; Wilén, B. M. Structure and Composition of Biofilm Communities in a Moving Bed Biofilm Reactor for Nitritation-Anammox at Low Temperatures. *Bioresour. Technol*. **2014**, *154*, 267-273.

Phadtare, S. Recent Developments in Bacterial Cold-shock Response. *Curr. Issue. Mol. Biol*. **2004**, *6*, 125-136.

Pommerening-Röser, A.; Rath, G.; Koops, H. P. Phylogenetic Diversity within the Genus Nitrosomonas. *Syst. Appl. Microbiol*. **1996**, *19*(3), 344-351.

Purkhold, U.; Pommerening-Röser, A.; Juretschko, S.; Schmid, M. C.; Koops, H. P.; Wagner, M. Phylogeny of All Recognized Species of Ammonia Oxidizers Based on Comparative 16S rRNA and amoA Sequence Analysis: Implications for Molecular Ddiversity Surveys. *Appl. Environ. Microbiol*. **2000**, *66*(12), 5368-5382.

Quan, Z. X.; Rhee, S. K.; Zuo, J. E.; Yang, Y.; Bae, J. W.; Park, J. R.; Park, Y. H. Diversity of Ammonium-Oxidizing Bacteria in a Granular Sludge Anaerobic Ammonium-Oxidizing (Anammox) Reactor. *Environ. Microbiol*. **2008**, *10*(11), 3130-3139.

Radniecki, T. S.; Semprini, L.; Dolan, M. E. Expression of merA, amoA and hao in Continuously Cultured Nitrosomonas Europaea Cells Exposed to Zinc Chloride Additions. *Biotechnol. Bioeng*. **2009**, *102*(2), 546-553.

Ravishankara, A. R.; Daniel, D.; Portmann, R. W. Nitrous oxide (N_2O): The Dominant Ozone-Depleting Substance Emitted in the 21st Century. *Science*. **2009**, *326*(5949), 123-125.

Rodríguez-Gómez, L. E.; Delgado, S.; Álvarez, M.; Elmaleh, S. Inhibition of Sulfide Generation in a Reclaimed Wastewater Pipe by Nitrate Dosage and Denitrification kinetics. *Water Environ. Res*. **2005**, *77*(2), 193-198.

Ronteltap, M.; Maurer, M.; Gujer, W. Struvite Precipitation Tthermodynamics in Source-separated Urine. *Water Res*. **2007**, *41*, 977–984.

Rosenberger, S.; Krüger, U.; Witzig, R.; Manz, W.; Szewzyk, U.; Kraume, M. Performance of a Bioreactor with Submerged Membranes for Aerobic Treatment of Municipal Waste Water. *Water Res*. **2002**, *36*(2), 413-420.

Rothrock J, M. J.; Vanotti, M. B.; Szögi, A. A.; Gonzalez, M. C. G.; Fujii, T. Long-term Preservation of Anammox Bacteria. *Appl. Microbiol. Biotechnol*. **2011**, *92*(1), 147-157.

Rotthauwe, J. H.; Witzel, K. P.; Liesack, W. The Ammonia Monooxygenase Structural Gene amoA as a Functional Marker: Molecular Fine-scale Analysis of Natural Ammonia-Oxidizing Populations. *Appl. Environ. Microbiol*. **1997**, *63*(12), 4704-4712.

Santos, R. L. S.; Manfrinatto, J. A.; Cia, E. M. M.; Carvalho, R. B.; Quadros, K. R. S.; Alves-Filho, G.; Mazzali, M. Urine Cytology as a Screening Method for Polyoma Virus Active Infection. *Transplant. Proc.* **2004**, *36(4), 899-901*.

Schmidt, I.; Sliekers, O.; Schmid, M.; Bock, E.; Fuerst, J.; Kuenen, J. G.; Jetten, M. S. M.; Strous, M. New Concepts of Microbial Treatment Processes for the Nitrogen Removal in Wastewater. *Microbiol Rev*. **2003**, *27*, 481-492

Schmid, M. C.; Hooper, A. B.; Klotz, M. G.; Woebken, D.; Lam, P.; Kuypers, M. M.; Jetten, M. S. Environmental Detection of Octahaem Cytochrome c Hydroxylamine/hydrazine Oxidoreductase Genes of Aerobic and Anaerobic Ammonium-Oxidizing Bacteria. *Environ. Microbiol*. **2008**, *10*(11), 3140-3149.

Schmittgen, T. D.; Livak, K. J. Analyzing Real-time PCR Data by the Comparative C_T Method. *Nature Protocols*. **2008**, *3*, 1101-1128.

Schramm, V. L. Enzymatic Transition-state Analysis and Transition-State Analogs. *Methods Enzym*. **1999**, *308*, 301-355.

Schröder, J. J.; Cordell, D.; Smit, A. L.; Rosemarin, A. Sustainable Use of Phosphorus. *Wageningen: Plant Res. Int*. **2010**, *357*, 138-257

Sears, K.; Alleman, J. E.; Barnard, J. L.; Oleszkiewicz, J. A. Impacts of Reduced Sulfur Components on Active and Resting Ammonia Oxidizers. *J Industr. Microbiol. Biotechnol.* **2004**, *31*(8), 369-378.

Semrau, J. D.; Chistoserdov, A.; Lebron, J.; Costello, A.; Davagnino, J.; Kenna, E.; Lidstrom, M. E. Particulate Methane Monooxygenase Genes in Methanotrophs. *J Bacteri*. **1995**, *177*(11), 3071-3079.

Sgouridis, F.; Heppell, C.M.; Wharton, G.; Lansdown, K.; Trimmer, M. Denitrification and Dissimilatory Nitrate Reduction to Ammonium (DNRA) in a Temperate Re-connected Floodplain. *Water Res*. **2011**, *45*(16), 4909-4922.

Siripong, S.; Rittmann, B. E. Diversity Study of Nitrifying Bacteria in Full-scale Municipal Wastewater Treatment Plants. *Water Res*. **2007**, *41*(5), 1110-1120.

Smil, V. Nitrogen and Food Production: Proteins for Human Diets.*AMBIO: J Human Environ*. **2002**, *31*(2), 126-131.

Strous, M.; Heijnen, J. J.; Kuene, J. G.; Jetten, M. S. M. The Sequencing Batch Reactor as Powerful Tool for the Study of Slowly Growing Anaerobic Ammonium Oxidizing Microorganisms. *Appl. Environ. Microbiol*. **1998**, *50* (5), 589-596.

Strous, M.; Kuenen, J. G.; Jetten, M. S. Key Physiology of Anaerobic Ammonium Oxidation. *Appl. Environ. Microbiol*. **1999**, *65*(7), 3248-3250.

Strous, M.; Pelletier, E.; Mangenot, S.; Rattei, T.; Lehner, A.; Taylor, M. W.; Barbe, V. Deciphering the Evolution and Metabolism of an Anammox Bacterium from a Community Genome. *Nature* **2006**, *440*(7085), 790-794.

Sun, F. Y., Yang, Y. J., Dong, W. Y., Li, J. Granulation of Nitrifying Bacteria in a Sequencing Batch Reactor for Biological Stabilisation of Source-Separated Urine. *Appl. Biochem. Biotechnol.* **2012**, *166*, 2114–2126.

Surmacz-Górska, J.; Cichon, A.; Miksch, K. Nitrogen Removal from Wastewater with High Ammonia Nitrogen Concentration via Shorter Nitrification and Denitrification. *Water Sci. Technol*. **1997**, *36*(10), 73-78.

Szatkowska, B.; Cema, G.; Płaza, E.; Trela, J.; Hultman, B. A One-stage System with Partial Nitritation and Anammox Processes in the Moving Bed Biofilm Reactor. *Water Sci. Technol*. **2007**, *55*(8-9), 19-26.

Tamm, C. O. Introduction: Geochemical Occurrence of Nitrogen. Natural Nitrogen Cycling and Anthropogenic Nitrogen Emissions. Springer Berlin Heidelberg, 1991.

Third, K. A.; Paxman, J.; Schmid, M.; Strous, M.; Jetten, M. S. M.; Cord-Ruwisch, R. Enrichment of Anammox from Activated Sludge and its Application in the CANON Process. *Microb. Ecol*. **2005**, *49*(2), 236-244.

Tomar, M.; Abdullah, T. H. Evaluation of Chemicals to Control the Generation of Malodorous Hydrogen Sulfide in Waste Water. *Water Res*. **1994**, *28*(12), 2545-2552.

Tsushima, I.; Ogasawara, Y.; Kindaichi, T.; Satoh, H.; Okabe, S. Development of High-rate Anaerobic Ammonium Oxidizing (anammox) Biofilm Reactors. *Water Res*. **2007**, *41*(8), 1623-1634.

Tursman, J. F.; Cork, D. J. Influence of Sulfate and Sulfate-reducing Bacteria on Anaerobic Digestion Technology. *Adv. Biotechnol. Proces*. **1989**, *12*, 273-285.

Udert, K. M.; Larsen, T. A.; Biebow, M.; Gujer, W. Urea Hydrolysis and Precipitation Dynamics in a Urine-collecting System. *Water Res*. **2003a**. *37* (11), 2571-2582.

Udert, K. M.; Fux, C.; Munster, M.; Larsen, T. A.; Siegrist, H.; Gujer, W. Nitrification and Autotrophic Denitrification of Source-separated Urine. *Water Sci Technol*. **2003b**, *48* (1), 119-130.

Udert, K. M.; Kind, E.; Teunissen, M.; Jenni, S.; Larsen, T. A. Effect of Heterotrophic Growth on Nitritation/Anammox in a Single Sequencing Batch Reactor. *Water Sci. Technol*. **2008**, 58, 277–8.

Udert, K. M.; Wächter, M. Complete Nutrient Recovery from Source-separated Urine by Nitrification and Distillation. *Water Res*. **2012**, 46, 453–464.

USEPA. Detection, Control, and Correction of Hydrogen Sulfide Corrosion in Existing Wastewater System.Office of Wastewater Enforcement and Compliance, Washington, DC, 1992, 20460.

USEPA. National Air Pollution Trends, 1990–1998, USEPA. http://www.epa.gov/ttn/chief/trends/trends98/ (Accessed 2002)

van de Graaf, A. A.; de Bruijn, P.; Robertson, L. A.; Jetten, M. S.; Kuenen, J. G. Autotrophic Growth of Anaerobic Ammonium-oxidizing Micro-organisms in a Fluidized Bed Reactor. *Microbiology*. **1996**, *142*(8), 2187-2196.

van der Star, W. R.; Abma, W. R.; Blommers, D.; Mulder, J. W.; Tokutomi, T.; Strous, M.; van Loosdrecht, M. C. Startup of Reactors for Anoxic Ammonium Oxidation: Experiences from the First Full-scale Anammox Reactor in Rotterdam. *Water Res*. **2007**, *41*(18), 4149-4163.

van de Vossenberg, J.; Rattray, J. E.; Geerts, W.; Kartal, B.; Van Niftrik, L.; Van Donselaar, E. G.; Damste, J. S.; Strous, M.; Jetten, M. S. Enrichment and Characterization of Marine Anammox Bacteria Associated with Global Nitrogen Gas Production. *Environ. Microbiol*. **2008**, *10*(11), 3120-3129.

van Dongen, U.; Jetten, M. S.; van Loosdrecht, M. C. The SHARON-Anammox Process for Treatment of Ammonium Rich Wastewater. *Water Sci. Technol*. **2001**, *44*(1), 153-160.

Van Hulle, S. W.; Vandeweyer, H. J.; Meesschaert, B. D.; Vanrolleghem, P. A.; Dejans, P.; Dumoulin, A. Engineering Aspects and Practical Application of Autotrophic Nitrogen Removal from Nitrogen Rich Streams. *Chem. Eng. J*. **2010**, *162*(1), 1-20.

Van Loosdrecht, M. C. M.; Salem, S. Biological Ttreatment of Sludge Digester Lliquids. *Water Sci. Technol*. **2006**, *53*(12), 11-20.

Vazquez-Padın, J. R.; Fernandez, I.; Morales, N.; Campos, J. L.; Mosquera-Corral, A.; Méndez, R. Autotrophic Nitrogen Removal at Low Temperature. *Water Sci. Technol*. **2011**, *63*(6), 1282-1288.

Vlaeminck, S. E.; Terada, A.; Smets, B. F.; Linden, D. V. D.; Boon, N.; Verstraete, W.; Carballa, M. Nitrogen Removal from Digested Black Water by One-Stage Partial Nitritation and Anammox. *Environ. Sci. Technol*. **2009**, *43*(13), 5035-5041.

Vlaeminck, S. E.; Terada, A.; Smets, B. F.; De Clippeleir, H.; Schaubroeck, T.; Bolca, S.; Verstraete, W. Aggregate Size and Architecture Determine Microbial Activity Balance for One-stage Partial Nitritation and Anammox. *Appl. Environ. Microbiol*. **2010**, *76*(3), 900-909.

Waki, M.; Tokutomi, T.; Yokoyama, H.; Tanaka, Y. Nitrogen Removal from Animal Waste Treatment Water by Anammox Enrichment. *Bioresour. Technol*. **2010**, *98*(14), 2775-2780.

Wang, C. C.; Lee, P. H.; Kumar, M.; Huang, Y. T.; Sung, S.; Lin, J. G. Simultaneous Partial Nitrification, Anaerobic Ammonium Oxidation and Denitrification (SNAD) in a Full-Scale Landfill-Leachate Treatment Plant. *J Hazard. Mater.* **2010**, *175*(1), 622-628.

Wang. J.; Yang, N. Partial Nitrification under Limited Dissolved Oxygen Conditions. *Proc. Biochem*. **2004**, *39*(10), 1223-1229.

Wett, B. Development and Implementation of a Robust Deammonification Process. *Water Sci. Technol*. **2007a**, *56*(7), 81-88.

Wett, B.; Murthy, S.; Takács, I.; Hell, M.; Bowden, G.; Deur, A.; O'Shaughnessy, M. Key Parameters for Control of DEMON Deammonification Process. *Water Pract*. **2007b**, *1*(5), 1-11.

Wett, B.; Hell, M.; Nyhuis, G.; Puempel, T.; Takacs, I.; Murthy, S. Syntrophy of Aerobic and Anaerobic Ammonia Oxidisers. *Water Sci. Technol*. **2010a**, *61*(8), 1915-1922.

Wett, B.; Nyhuis, G.; Takács, I.;Murthy, S. Development of Enhanced Deammonification Selector. *Proc. Water Environ. Feder.* **2010b**, *10*, 5917-5926.

Wiesmann, U. Biological Nitrogen Removal from Wastewater. *Biotechnics Wastewater*. 1994, pp 113-154.

Wilsenach, J. A.; Schuurbiers, C. A. H.; van Loosdrecht, M. C. M. Phosphate and

Potassium Recovery from Source Separated Urine through Struvite Precipitation. *Water Res.* **2007**, *41*, 458–466.

Wilsenach, J. A.; van Loosdrecht, M. C. M. Impact of Separate Urine Collection on Wastewater Treatment Systems. *Water Sci. Technol*. **2003**, *48*(1), 103-110.

Wilsenach, J. A.; van Loosdrecht, M. C. M. Effects of Separate Urine Collection on Advanced Nutrient Removal Processes. *Environ. Sci. Technol*. **2004**, *38*, 1208–1215.

Winkler, M. K.; Kleerebezem, R.; Van Loosdrecht, M. C. M. Integration of Anammox into the Aerobic Granular Sludge Process for Main Stream Wastewater Treatment at Ambient Temperatures. *Water Res*. **2012**, *46*(1), 136-144.

Woebken, D.; Lam, P.; Kuypers, M. M. M.; Naqvi, S. W.; Kartal, B.; Strous, M.; Jetten, M. S. M.; Fuchs, B. M.; Amann, R. A Microdiversity Study of Anammox Bacteria Reveals a Novel Candidatus Scalindua Phylotype in Marine Oxygen Minimum Zones. *Environ. Microbiol*. **2008**, *10*, 3106-3119

Woese, C. R.; Weisburg, W. G.; Paster, B. J.; Hahn, C. M.; Tanner, R. S.; Krieg, N. R.; Stackebrandt, E. The Phylogeny of Purple Bacteria: The Beta Subdivision. *Syst. Appl. Microbiol*. **1984**, *5*(3), 327-336.

Wrage, N.;Velthof, G. L.; Van Beusichem, M. L.; Oenema, O. Role of Nitrifier Denitrification in the Production of Nitrous Oxide. *Soil Boil. Biochem*. **2001**, *33*(12), 1723-1732.

Wu, Q.; Bishop, P. L.; Keener, T. C. A Strategy for Controlling Deposition of Struvite in Municipal Wastewater Treatment Plants. *Water Environ. Res*. **2005**, *77*(2), 199-207.

Yamada, T.; Sekiguchi, Y. Cultivation of Uncultured Chloroflexi Subphyla: Significance and Ecophysiology of Formerly Uncultured Chloroflexi 'subphylum I' with Natural and Biotechnological Relevance. *Microb. Environ*. **2009**, *24*(3), 205-216.

Yang, Q.; Peng, Y.; Liu, X.; Zeng, W.; Mino, T.; Satoh, H. Nitrogen Removal via Nitrite from Municipal Wastewater at Low Temperatures Using Real-time Control to Optimize Nitrifying Communities. *Environ. Sci. Technol*. **2007**, *41*(23), 8159-8164.

Yin, S. X.; Chen, D.; Chen, L. M.; Edis, R. Dissimilatory Nitrate Reduction to Ammonium and Responsible Microorganisms in two Chinese and Australian Paddy Soils. *Soil Biol. Biochem*. **2002**, *34*(8), 1131-1137.

Zhang, Q.; Clippeleir, H. D.; Murthy, S. Efficient THP-AD Filtrate Treatment via Optimized Control Strategies in Sidestream Deammonification Reactor. *Proc. Water Environ. Feder.* **2015**.

Zhang, J.; Kobert, K.; Flouri, T.; Stamatakis, A. PEAR: A Fast and Accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **2014**, *30*(5), 614-620.

Zhang, J.; Zhou, J.; Han, Y.; Zhang, X. Start-up and Bacterial Communities of Single-Stage Nitrogen Removal Using Anammox and Partial Nitritation (SNAP) for Treatment of High Strength Ammonia Wastewater. *Bioresour. Technol*. **2014**, *169*, 652-657.

Zhang, L.; De Schryver, P.; De Gusseme, B.; De Muynck, W.; Boon, N.; Verstraete, W. Chemical and Biological Technologies for Hydrogen Sulfide Emission Control in Sewer Systems: a Review. *Water Res*. **2008**, *42*(1), 1-12.

Zumft, W. G. Cell Biology and Molecular Basis of Denitrification. *Microbiol Molecul. Biol. Rev*. **1997**, *61*(4), 533-616.