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Nitrogen removal by combined nitritation-anammox process in an upflow anaerobic sludge blanket (UASB) reactor

by

Xiaojin Li

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Co-majors: Civil Engineering (Environmental Engineering);
Biorenewable Resources & Technology

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Iowa State University
Ames, Iowa
2014

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ABSTRACT

Anaerobic ammonium oxidation (anammox) process, where anammox bacteria convert ammonium to N_2 with nitrite as the terminal electron acceptor in the absence of O_2, has been extensively studied since its discovery in the late 1990s. The combined nitritation-anammox process represents a promising innovative biological nitrogen removal technology, especially for treating wastewater with low chemical oxygen demand (COD)/ammonium ratio. Due to the low growth rates of both aerobic and anaerobic ammonium-oxidizing bacteria (i.e. AOB and anammox bacteria), efficient biomass retention is critical for successful reactor start-up and stable operation.

In this thesis, two similar laboratory-scale upflow anaerobic sludge blanket (UASB) reactors have been developed for cultivating anammox bacteria under complete absence of oxygen (UASB #1), and nitritation-anammox biomass with low oxygen levels (UASB #2), respectively. The ratio of influent NO_2^--N to NH_4^+-N was optimized to evaluate the long-term performance of the reactor. The observed NO_2^--N to NH_4^+-N ratios under different influent NO_2^--N to NH_4^+-N ratios did not agree with the proposed ratio of 1.32 (Eq. (1.3)), but showed a positive correlation with influent ratios. Synthetic wastewater with a NO_2^--N/NH_4^+-N ratio of 1.2 achieved the highest total nitrogen removal efficiency of 96-97%. The average ratio of observed NO_3^--N/NH_4^+-N was much smaller than proposed ratio of 0.256.

The nitritation-anammox reactor operated under the low oxygen level (≤0.5 mg/L) for over 250 days was able to remove more than 85% of the supplied total nitrogen loads without nitrite accumulation. The nitritation-anammox granules were successfully

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enriched with typical colors of brown-yellow, reddish, light red, red, etc., depending on the microbial community compositions. Granules with porous structures had a mean diameter of 3 mm and featured good settling ability. The microbial community compositions in UASB #2 reactor, investigated by fluorescence *in situ* hybridization (FISH), showed the coexistence of AOB and anammox bacteria in the granules. In addition, the two groups of bacteria exhibited an overlapping growth style, which can improve the availability of ammonium for anammox bacteria and facilitate the immediate consumption of the nitrite produced by AOB by anammox bacteria. FISH results also proved that most nitrite oxidizing bacteria were eliminated under high temperature and oxygen-limiting conditions.

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CHAPTER 1. INTRODUCTION

1.1 Motivation of This Study

Nutrients such as nitrogen (N) and phosphorus (P) from wastewater effluent and agriculture run-off are often directly discharged into water bodies without proper treatment. Excessive concentrations of nitrogen and phosphorus in water bodies can result in eutrophication which would exert negative impacts on environment. For example, eutrophication results in accelerated growth of algae and plankton over other more complicated plants, which leads to the depletion of dissolved oxygen, deterioration of water quality and shifts of biotic community composition (Smith et al. 1999, Wolfe and Patz 2002). Eutrophication is pervasive in the United States (U.S.), occurring in 78% of the coastal areas and 48 % of the reservoirs, lakes and streams (Galloway et al. 2003, Selman et al. 2008). Recently, the U.S. EPA nutrient pollution control strategy is urging nationwide implementation of new numeric nutrient discharge criteria. More stringent nutrient discharge regulations are likely implemented in the near future.

The energy demand of wastewater treatment plants (WWTPs) accounts for about 3% of the U.S. electrical energy load (EPA 2006). Energy costs accounts for 30% of the total operation and maintenance costs of WWTPs (Carns 2005). Meanwhile, the aeration systems generally account for the greatest portion of energy consumption of WWTPs (WEF 2009). For instance, energy cost for aeration in a conventional activated sludge treatment process typically represents 45-60% of the plant's total energy consumption (Bolles 2006). Unfortunately, upgrading existing sewage treatment facilities with conventional biological nutrient removal (BNR) systems to meet the nutrient discharge

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criteria will lead to an increase in energy consumption, an economically, environmentally and politically undesirable outcome. Traditional BNR technologies (nitrification/denitrification) either consume high amount of energy for nitrification, or require external organic carbon sources for denitrification.

Furthermore, the conventional BNR systems can release considerable amount of nitrous oxide (N_2O), accounting for about 5% of all U.S. greenhouse gas (GHG) emissions from anthropic activities (EPA 2014). Nitrous oxide is primarily produced through nitrification and denitrification processes. The U.S. emitted 5.1 teragrams of carbon dioxide equivalent (Tg CO_2 Eq., or million metric tons carbon dioxide equivalent, $MMTCO_2E$) of N_2O from wastewater treatment in 2011, which has increased by 1.6 Tg CO_2 Eq. (45.7%), compared with that of 1990 (EPA 2013). The comparative impact of N_2O on climate change is over 300 times greater than CO_2 over a 100-year period, due to its long lifespan of approximately 120 years in the atmosphere (Houghton et al. 2001).

The proposed innovative biological nitrogen removal system combined partial nitrification and anaerobic ammonium oxidation (anammox) processes, so called the nitritation-anammox process, requires only 37.5% as much oxygen as the traditional BNR technologies, eliminates additional organic carbon sources, and produces less excess sludge due to the low growth rates of autotrophic microorganisms. By replacing denitrification with the anammox process and reducing nitrification requirements, the improved system is expected to reduce both energy demand and N_2O emission. The successful development of the novel system will not only advance the knowledge in biological nitrogen removal, but also provide a sustainable, environment-friendly and

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cost-effective treatment technology for building or upgrading BNR facilities to meet the increasingly stringent nutrient discharge standards.

1.2 Background

The traditional BNR process consists of two processes: sequential aerobic autotrophic nitrification and anoxic heterotrophic denitrification (Figure 1.1). Nitrification is a process of partial oxidation of ammonium to nitrite and further oxidation of nitrite to nitrate. Ammonia oxidizing bacteria (AOB, same as nitrous bacteria) and nitrite oxidizing bacteria (NOB, same as nitric bacteria) are two groups of microorganisms responsible for partial nitrification (PN, also called nitritation, nitrosation) and complete nitrification (also known as nitration), respectively. Ammonia and nitrite oxidizers are referred to as "nitrifiers or nitrobacteria". Partial nitrification is usually considered as the rate-limiting step of nitrification.

Denitrification is a process where nitrate is reduced to nitrogen gas under anoxic conditions, passing through nitrite, nitric oxide and nitrous oxide. A wide range of bacteria called denitrifying bacteria or denitrifiers, equipped with a complete enzyme apparatus, are able to carry out the entire sequence of reactions. The reaction equations, specific enzymes and major bacteria species involved in nitrification and denitrification processes are shown in Table 1.1. The global stoichiometric equations of nitrification and denitrification are shown as well (Eq. (1.1) and Eq. (1.2)). It should be noted that all these reaction equations do not take biosynthesis into account.

N_2O emissions from the BNR systems has been studied intensively in the past years (Ahn et al. 2010, Desloover et al. 2012). AOB were reported to be capable of utilizing

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NO_2^- and subsequently NO as alternative electron acceptors, releasing N_2O and N_2 under aerobic to suboxic conditions through the nitrifier denitrification pathway, which was considered as a major contributor to N_2O emission (Kim et al. 2010, Kool et al. 2011, Wrage et al. 2001). Generally, N_2O can be produced through different pathways, including nitrification, autotrophic denitrification by AOB, and heterotrophic denitrification.

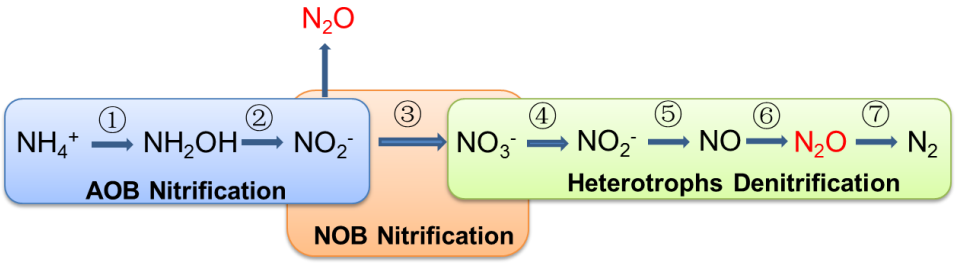

Figure 1.1. Traditional BNR process.

Table 1.1. Summary of traditional BNR process.

Step	Equation	Enzyme	Major species
①	$2NH_4^+ + O_2 \rightarrow 2NH_2OH + 2H^+$	Ammonia monooxygenase (AMO)	*Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus*
②	$2NH_2OH + 2O_2 \rightarrow 2H^+ + 2H_2O + 2NO_2^-$	Hydroxylamine dehydrogenase/ oxidoreductase (HAO)	
③	$2NO_2^- + O_2 \rightarrow 2NO_3^-$	Nitrite oxidase	*Nitrobacter*, *Nitrococcus*, *Nitrospira*, and *Nitrospina*
④	$2NO_3^- + 4H^+ + 4e^- \rightarrow 2NO_2^- + 2H_2O$	Nitrate reductase	*Pseudomonas*, *Thiobacillus*, *Paracoccus* and *Naisseria*
⑤	$2NO_2^- + 4H^+ + 2e^- \rightarrow 2NO + 2H_2O$	Nitrite reductase	
⑥	$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$	Nitric oxide reductase	
⑦	$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$	Nitrous oxide reductase	

Global equation of nitrification: $2NH_4^+ + 4O_2 \rightarrow 4H^+ + 2H_2O + 2NO_3^-$ Eq. (1.1)

Global equation of denitrification: $2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O$ Eq. (1.2)

The traditional BNR process has several disadvantages, including intensive oxygen demand for nitrification as well as the requirement of additional organic carbon sources

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for denitrification. Therefore, it is uneconomical and complicated when applied to treat high nitrogen strength wastewater with low C/N ratio.

Anammox bacteria were first discovered in a denitrifying pilot plant in 1995 by Mulder et al. (1995). During the anammox process, ammonium and nitrite are directly converted to N_2 under strictly anoxic conditions. The growth of anammox bacteria is reversibly inhibited by oxygen concentrations even below 0.5% air saturation (Strous et al. 1997a). Anammox bacteria play an important role in the marine nitrogen cycle and have been detected in various oxygen minimum zones (OMZs) and man-made ecosystems (Dalsgaard et al. 2003, Galán et al. 2009, Hu et al. 2013a, Hu et al. 2012, Kuypers et al. 2003, Pitcher et al. 2011, Schmid et al. 2007, Schubert et al. 2006, Woebken et al. 2008).

The elemental composition of protein of anammox bacteria was found to be $CH_2O_{0.5}N_{0.15}$ when the laboratory-scale reactor was operating under the steady-state condition (Strous et al. 1998). The stoichiometry of the overall anammox metabolic reaction is described in Eq. (1.3). Anammox bacteria utilize CO_2 as the sole carbon source and NO_2^- as the electron acceptor for ammonium oxidation (Mulder et al. 1995, Schmid et al. 2001). Concurrently, NO_2^- is used as the electron donor for CO_2 reduction (Kuenen 2008). The exact anammox metabolism pathway is still unclear. Hydrazine (N_2H_4) and nitric oxide (NO) are identified as two metabolic intermediates of anammox process (Kartal et al. 2011, Strous et al. 2006). It has been proposed that anammox is a three-reaction process (Eq. (1.4), Eq. (1.5), and Eq. (1.6)) (Kartal et al. 2011, Strous et al. 2006). NO_2^- is reduced by nitrite reductase (NirS) to NO, which subsequently reacts

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with NH_4^+ to form N_2H_4, catalyzed by the unique hydrazine synthase (HZS), and finally N_2H_4 is oxidized to N_2 by hydrazine dehydrogenase/oxidoreductase (HDH/HZO).

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.256NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O$$

Eq. (1.3)

$$NO_2^- + 2H^+ + e \rightarrow NO + H_2O$$

Eq. (1.4)

$$NO + NH_4^+ + 2H^+ + 3e \rightarrow N_2H_4 + H_2O$$

Eq. (1.5)

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e$$

Eq. (1.6)

1.3 Literature Review

1.3.1 Anammox process

The nitrogen removal has conventionally been achieved by the combined nitrification-denitrification process, which is energy-consuming and thus results in high operating cost. In addition, the traditional BNR process leads to high biomass production and GHG emissions, causing additional disposal costs and environmental issues.

Anammox process has been recognized as an efficient and cost-effective alternative to the traditional BNR process because it reduces oxygen (60% less) and alkalinity demands for nitrification, and it does not need external organic carbon source for denitrification. It also offers additional advantages of reducing undesirable by-products such as GHGs (e.g., N_2O, 90% less) and decreasing biomass yields (Jetten et al. 1997, Schmidt et al. 2003), resulting in significant savings in operational costs. Therefore, anammox process is also considered as an environmental friendly and sustainable process for nitrogen removal.

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It is estimated that anammox bacteria account for approximately 50% of all N_2 released into the atmosphere (Kartal et al. 2012). They are a monophyletic group of anaerobic chemoautotrophic bacteria that branch deeply in the *Planctomycetales* tree (Strous et al. 1999). To date, ten different anammox species divided over five genera have been enriched and identified. They are Candidatus *Kuenenia (K. stuttgartiensis), Brocadia (B. anammoxidans, B. fulgida,* and *B. sinica), Anammoxoglobus (A. propionicus), Jettenia (J. asiatica) and Scalindua (S. brodae, S. sorokinii, S. wagneri, and S. profunda)* (Schmid et al. 2000, Schmid et al. 2003, Strous et al. 1999, Woebken et al. 2008). They all have the taxonomical status of 'Candidatus' since none of these genera have been isolated as pure cultures (majorly due to their slow specific growth rates). The common and unique characteristic of these anammox bacteria species is the presence of a specialized organelle called anammoxosome which is surrounded by a particular lipid that contains remarkable enzyme system (Lindsay et al. 2001).

Anammox bacteria are strict anaerobes and autotrophs. As mentioned above, they grow in microbial mixtures and have not yet been isolated in pure culture (Tsushima et al. 2007a). Based on previous study, anammox bacteria are characterized with a slow growth rate and have a doubling time of approximately 11 days (Strous et al. 1998). Thus, the industrial application of anammox process has been partly impeded by the availability of anammox biomass for inoculation. Various methods have been applied to cultivate and enrich anammox biomass from different types of seed sludge, such as activated sludge (Dapena-Mora et al. 2004), nitrifying activated sludge (van der Star et al. 2007), and anaerobic sludge (Jetten et al. 2005). Reducing the washing out potential

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of anammox biomass during the reactor operation is very important, especially at the start-up stage. Enrichment culture of anammox bacteria obtained from marine sediments has also successfully been achieved in a lab-scale reactor (van de Vossenberg et al. 2008).

Previous studies have observed population shifts of anammox bacteria during the operation of anammox reactors (Kartal et al. 2007, Park et al. 2010, van der Star et al. 2007, van der Star et al. 2008a, van der Star et al. 2008b). For example, the population shifted from dominant 'Candidatus *Brocadia*' at the initial stage to 'Candidatus *Kuenenia*' under stable operating conditions in a membrane bioreactor (van der Star *et al.*, 2008a; van der Star *et al.*, 2008b). However, a population shifted from 'Candidatus *Kuenenia*' to 'Candidatus *Brocadia*' has also been observed during the long-term operation of a full-scale biofilm-based CANON (Completely Auto trophic Nitrogen removal Over Nitrite) reactor (Park et al. 2010, van der Star et al. 2007). The differences in physiological characteristics among anammox bacteria may contribute to the population shifts, such as substrate affinity constants and maximum growth rates (Oshiki et al. 2011).

During the past decade, different types of reactors have been used to enrich anammox biomass, including completely stirred tank reactor (CSTR) (Guven et al. 2004), sequencing batch reactor (SBR) (Strous et al. 1998), anammox non-woven membrane reactor (ANMR) (Ni et al. 2010c, Ni et al. 2010d), up-flow reactor (Imajo et al. 2004), fluidized bed reactors (Mulder et al. 1995, Strous et al. 1997b), fixed bed biofilm reactors (Kindaichi et al. 2007, Tsushima et al. 2007b), upflow anaerobic sludge

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blanket reactors (Ahn et al. 2004), membrane sequencing batch reactor (MSBR) (Trigo et al. 2006), rotating biological contactor (RBC) (Egli et al. 2001), gas-lift reactor (Sliekers et al. 2003), moving bed biofilm reactor (MBBR) (Winkler et al. 2012) and biofilm reactors (Gong et al. 2007, Tsushima et al. 2007b). Fast growth of anammox bacteria with a high enrichment of 97.6% has been achieved at a sludge residence time (SRT) of 12 days (van der Star et al. 2008a). Anammox process can achieve very high volumetric nitrogen removal rates up to 76 kg N/ (m^3 day) (Tang et al. 2011), indicating its potential application for treating wastewater with high ammonium strength. Performance comparisons of different anammox reactors are summarized in Table 1.3.

1.3.2 Autotrophic nitrogen removal process

Complete anaerobic treatment of domestic wastewater has the potential to achieve net energy production while meeting stringent effluent standards (McCarty et al. 2011). Since the discovery of anammox bacteria in the early 1990s, researchers have been developing more sustainable domestic wastewater treatment technologies by coupling with anammox process.

Generally, ammonium is a dominant form of nitrogen compound in most wastewater. In order to apply anammox process, part of ammonium is required to be oxidized to nitrite, and then the produced nitrite together with the remaining ammonium is converted to N_2 by anammox bacteria. Anaerobic digester followed by nitrification-anammox process can be practical and has become a promising technology. The first step is converting organic carbonaceous compounds to CH_4 in the anaerobic

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digester. Then the remaining wastewater containing majorly ammonium leftover can be removed by nitritation-anammox process (Hu et al. 2013c).

The combined nitritation–anammox process can be achieved either in two separate reactors as the SHARON (Single reactor system for High-rate Ammonium Removal Over Nitrite)–anammox process (Hellinga et al. 1998, van Dongen et al. 2001), or in a single reactor such as OLAND (Oxygen-Limited Autotrophic Nitrification–Denitrification) (Meulenberg et al. 1992), CANON process (Cho et al. 2011, Sliekers et al. 2003, Third et al. 2001), SNAP (Single-stage Nitrogen removal using Anammox and Partial nitritation) (Furukawa et al. 2006), and DEMON (the pH-controlled DEamMONification system) (Wett 2007). They are considered as the two-stage or one-stage systems, respectively. The choice of which configuration is preferable depends on the specific case, including wastewater characteristics, the existing constructions, space availability, etc. Generally, one-stage process has a lower capital cost than two-stage system because of no additional reactor requirement. But high nitrite level might be toxic to the responsible microorganisms. Two-stage system instead can be more effective, flexible and stable than one-stage process since nitritation and anammox processes can be controlled and optimized separately (Hu et al. 2013b). For instance, the inhibitory effect of O_2 on the anammox bacteria can be relieved.

Besides these systems, new and innovative processes have continued to be developed. For instance, Mulder (2007) proposed DEAMOX (DEnitrifying AMmonium OXidation) which combines anammox process with autotrophic denitrification process using sulphide as an electron donor for converting nitrate to nitrite within an anaerobic

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biofilm. A novel non-woven rotating biological contactor (NRBC) reactor has been applied to study SNAD (Simultaneous Partial nitrification, Anammox and Denitrification) process that is able to remove ammonium and COD in a single reactor (Chen et al. 2009).The SNAD process has also been investigated in a full-scale landfill-leachate treatment plant in Taiwan (Wang et al. 2010). Recently, ANME-D (ANaerobic MEthane oxidation coupled to Denitrification) with nitrite as an electron acceptor has been recognized as important pathways in the microbial nitrogen cycle (Ettwig et al. 2010, Zhu et al. 2010). The possible coexistence of anammox and nitrite-dependent anaerobic methane oxidation bacteria (n-damo, or denitrifying methanotrophic bacteria) may allow their application to wastewater containing substantial amounts of both dissolved methane and ammonium (Luesken et al. 2011).

The partial nitrification–anammox process has been successfully applied to treat sewage sludge digester liquor (van Dongen et al. 2001) and livestock manure digester liquor (Yamamoto et al. 2008, Yamamoto et al. 2011). The first full-scale granular anammox reactor in the world was implemented in 2007 at the WWTP of Waterboard Hollandse Delta in Rotterdam, Netherland (Abma et al. 2007). The up-flow anammox reactor was coupled with a SHARON reactor which provides the mixture of ammonium and nitrite (ratio is about 1:1) (van der Star et al. 2007). The full-scale application of deammonification was successfully achieved in Austria in the DEMON reactor, the sludge in which contains the mixture of red granules and brownish flocs (Innerebner et al. 2007, Wett 2006).

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The reaction equations involved in these innovative BNR processes are shown in Table 1.2.

Table 1.2. Summary of innovative BNR processes.

Process	Reaction equation
SHARON-Anammox	Nitritation: $2NH_4^+ + 1.5O_2 + 2HCO_3^- \rightarrow NH_4^+ + NO_2^- + 2CO_2 + 3H_2O$
	Anammox: $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$
	Total: $2NH_4^+ + 1.5O_2 + 2HCO_3^- \rightarrow N_2 + 2CO_2 + 5H_2O$
CANON	$NH_4^+ + 0.85O_2 \rightarrow 0.13NO_3^- + 0.435N_2 + 1.4H^+ + 1.3H_2O$
OLAND	$2NH_4^+ + 1.5O_2 \rightarrow N_2 + 2H^+ + 3H_2O$
n-damo	$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O$
DEAMOX	$NO_3^- + 0.25HS^- \rightarrow NO_2^- + 0.25SO_4^{2-} + 0.25H^+$

To date, various factors have been evaluated to achieve stable partial nitrification, such as pH (Ciudad et al. 2007, Furukawa et al. 2006, Hellinga et al. 1998, Law et al. 2011), temperature (Guo et al. 2010, Hao et al. 2002, Hellinga et al. 1998, Peng et al. 2007, Qiao et al. 2010), dissolved oxygen (DO) (Blackburne et al. 2008, Chuang et al. 2007, Guo et al. 2009), free ammonia (Peng et al. 2004, Vadivelu et al. 2007, Vadivelu et al. 2006), free nitrite (Qiao et al. 2010, Yamamoto et al. 2011) and hydroxylamine (Kindaichi et al. 2004, Schmidt et al. 2004, van der Star et al. 2008b).

Performance comparisons of different autotrophic nitrogen removal in single-stage reactors are summarized in Table 1.4.

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Table 1.3. Performance comparisons of different anammox reactors.

Reactor type	Inoculum	Operation time	T	pH	Substrate concentration NH_4^+	Substrate concentration NO_2^-	HRT	Nitrogen removal efficiency	NLR_{max}	NRR_{max}	References
		Day	°C		mg/L		h	%	kg $N/m^3/d$	kg $N/m^3/d$	
GLR	Anammox sludge	86-100	-	7.5	1545±62	-	10	42	3.7	1.5	Sliekers et al. (2003)
UASB	Anammox granules	235	37	7.5-8	100-458	100-575	<24	99.29	1.03	0.93	Ni et al. (2011)
ABF	Anammox sludge	97	37	7.2	93.3-350	40.6-330.7	0.67-3	-	19.1	11.5	Isaka et al. (2007)
ABF	Anammox sludge	446	20-22	7.2	93.3-350	40.6-330.7	0.67-3	-	~12	8.1	Isaka et al. (2007)
UFFBB	Denitrifying sludge	247	37	7-7.5	20-550	20-460	0.2-8	50-63	58.5	26	Tsushima et al. (2007b)
UAGSB	Anaerobic granular	324-330	35±1	-	70-400	70-500	1.01-10.1	-	16.4	15.4	Tang et al. (2009)
SBR	Activated sludge	365	36 ±0.3	7.2–8.7	1268	1661.4	46-86	96–99.5	1.6	1.57	Lopez et al. (2008)
SBR	Anammox sludge	400	30	7.5–8.3	700	0	-	60	0.28	1.0	Vazquez-Padin et al. (2009a)
SBR	Anammox sludge	400	18–24	7.7 ±0.2	200-250	0	-	77	-	0.36 avg.	Vazquez-Padin et al. (2009b)
SBR	Anammox granules	218	30	7.8	150	150	24	98	0.3	-	Arrojo et al. (2006)
SBR	Anammox sludge	329	33	7.8	30-300	30-300	24	99-100	0.6	-	Fernández et al. (2008)
MSBR	Anammox sludge	375	35	8	390	390	24	<80	-	0.71	Trigo et al. (2006)
CTR	Anammox sludge	340	25	-	300	300	3.9-24.3	81.3	3.6	-	Qiao et al. (2009)
UASB	Anaerobic granules	400	30	7.5	709-1027	927.8-1409		86.5-92.3	-	2.28-6.39	Imajo et al. (2004)
UASB	Aerobic granules	300	30	-	200	220	30-24	90	0.5	-	Vazquez-Padin et al. (2009a)
ANMR	Anammox sludge	260	35	7.5-8	-	-	24-60	-	1.26	1.05	Ni et al. (2010c)

NLR, nitrogen loading rate; NRR, nitrogen removal rate.

GLR, gas-lift reactor; UAGSB, upflow anammox granular sludge bed; UFFBB, up-flow fixed-bed biofilm reactor; UASB, upflow anaerobic sludge blanket; SBR, sequencing batch reactor; ABF, anaerobic biological filter; MSBR, membrane sequencing batch reactor; CTR, column type reactor; ANMR, anammox non-woven membrane reactor.

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Table 1.4. Performance comparisons of completely autotrophic nitrogen removal in single-stage reactors.

Reactor type	Inoculum	HRT	Temp.	pH	Ammonium concentration in influent	N removal efficiency	NLR	NRR	References
		h	°C		mg/L	%	kg $N/m^3/d$	kg $N/m^3/d$	
SBR	Anammox+AOB	24	30		-	36-92	0.07-0.22	0.04-0.11	Third et al. (2001)
SBR	Anammox	24	30	7.5	131	42		0.064-0.315	Sliekers et al. (2002)
SBR	Nitrifying+Anammox	12	21	7.7	180-330 200-250	78		0.36	Vazquez-Padin et al. (2009b)
MABR	Nitrifying+Anammox	-	35	7.6	200	84	0.87	0.72	Gong et al. (2008)
RBC	OLAND sludge	24	30-35	7-8	840	89±5	0.675-1.189		Pynaert et al. (2003)
RBC	OLAND sludge	32±2	26	7.9	1215 ± 54	76		0.7, max 1.3	Vlaeminck et al. (2009)
NRBC	Anammox+partial nitrifying	5-6	35	8-8.2	200	70	0.69	-	Chen et al. (2009)
Up-flow	Anaerobic granules and anoxic activated sludge	120	30		438±26	56.7		0.07	Ahn and Choi (2006)
Up-flow	Nitrifying	5	37	7.83	130-300	22.1±0.16		0.23±0.16 max. 0.57	Cho et al. (2011)
Up-flow	Anammox	3-5	37	7.6	206±28	16.6±8.9		0.35±0.19 max. 0.77	Cho et al. (2011)

OLAND sludge, aerobic nitrifiers + heterotrophic denitrifiers+ anammox biomass.

MABR, membrane aerated biofilm reactor.

RBC, rotation biological contactor.

NRBC, non-woven RBC.

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DO concentration is critical for controlling the growth rates of both AOB and NOB during the nitrification process. However, anammox bacteria are strict anaerobes and reversibly inhibited by certain oxygen levels. But AOB and anammox activities could occur under oxygen-limiting conditions (Meulenberg et al. 1992, Third et al. 2001).

Additional low concentrations of hydroxylamine (NH_2OH, 250 μM) have been shown to completely inhibit the NOB growth for more than 40 days (Kindaichi et al. 2004). The free ammonia (NH_3) concentration depends on the ammonium concentration, pH and temperature (Anthonisen et al. 1976). NH_3 has an inhibitory effect on nitrite oxidoreductase, locating on the cell membrane of NOB.

Previous studies reported anammox bacteria have an optimal pH and temperature of 7.5-8.0, 30-40 ℃, respectively (Strous et al. 1997b, Van de Graaf et al. 1996). Jetten et al. (2001) also pointed out that the optimum temperature for anammox bacteria growth was around 30 to 35 ℃. In addition, the AOB had an optimal temperature of about 28 ℃ (Alawi et al. 2007). The maximum specific growth rate of *Nitrosomonas* (AOB) surpasses that of *Nitrobactor* (NOB) at 35 ℃ (van Dongen et al. 2001). Temperatures higher than 30 ℃ and pH between 7.8-8.5 have been applied to suppress NOB activities and promote AOB growth (Bae et al. 2001, Hellinga et al. 1998). Mulder et al. (2001) have also reported that temperatures ranging between 30-40 ℃ were most suitable for partial nitrification. On the other hand, some studies suggested that NOB had higher activities than AOB at the temperature range of 10 to 15 ℃ (Yang et al. 2007).

Consider that AOB and anammox bacteria tend to grow well at higher temperatures; NOB could consume the limiting O_2 and nitrite ahead of AOB and anammox bacteria

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under low temperature (depending on their affinities for nitrite and O_2). As a result, this would lead to nitrate accumulations and even the deterioration of the system, causing violation of discharge criteria. On the other hand, AOB and anammox bacteria have abilities to thrive at the temperature below 10 ℃ in natural ecosystems (e.g. OMZs), indicating that both groups of microorganisms are capable to outcompete NOB at low temperatures.

1.4 Objective of the Research

I intended to develop a more sustainable BNR technology so called nitritation-anammox process to meet the increasingly stringent nutrient discharge standards. One of the biggest obstacles of the combined process has been the slow growth rates of the functional microorganisms. UASB reactors with very efficient biomass retention can overcome this obstacle to a certain extent. In the study, two laboratory-scale UASB reactors have been developed and operated over long periods.

Based on the previous studies, many anammox reactors did not follow the proposed anammox reaction equation. One reason is that none of anammox species have been obtained as pure cultures. The bacteria composition can be various in different reactors. Thus, experimentally verification of substrate conversion will be studied in UASB #1 reactor. The optimal influent ratio of nitrite to ammonium for anammox process will be determined in the first step. Then the long-term performance of the reactor will be evaluated by applying the optimal influent ratio. As a result, an empirical reaction equation is expected to generate based on mass balance.

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ORP is a more sensitive parameter for process control compared to pH or DO. Substrates consumptions and the shifts of aerobic/anoxic/anaerobic environments can be readily detectable by on-line ORP. To my best knowledge, the application of ORP as a monitoring or controlling parameter for developing and operating the combined nitritation-anammox process in a UASB reactor has not yet been investigated. The strategy used to develop the nitritation-anammox process was to fix ammonium concentration but gradually reduce nitrite concentration in the influent. Correspondingly, the influent ratio of NO_2^--N/NH_4^+-N decreased from 1.2 to 0. The amounts of oxygen supply were controlled and adjusted by gas flow meter according to the results of on-line ORP and DO probes.

1.5 Thesis Organization

The thesis will be divided into three parts. Part I will provide significance and motivation of this research, background information and research progress. Part II will introduce the reactor setup and operation, as well as testing methods. Part III will describe (1) the optimization and application of the influent ratio of NO_2^--N/NH_4^+-N to evaluate the substrates conversion and long-term performances in UASB #1 reactor, (2) the development of the combined nitritation-anammox process in UASB #2 reactor under low oxygen conditions using ORP and DO probes as combined monitoring tools, (3) the characteristics of granules in both UASB reactors, (4) the microbial community composition analysis of granules by FISH technique in UASB #2.

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CHAPTER 2. MATERIALS AND METHODS

2.1 Synthetic Medium

Synthetic wastewater, made according to Table 2.1 (Imajo et al. 2004), was used throughout the experiments. Ammonia and nitrite were supplemented to mineral medium as needed in the forms of $(NH_4)_2SO_4$ and $NaNO_2$, respectively. The amount of ammonia and nitrite is based on experimental design. The trace elements solution I contains (g/L): EDTA 5 and $FeSO_4$ 5. The trace elements solution II is composed of (g/L): EDTA-2Na 15, $CuSO_4 \cdot 5H_2O$ 0.25, $ZnSO_4 \cdot 7H_2O$ 0.43, $NaMoO_4 \cdot 2H_2O$ 0.22, $MnCl_2 \cdot 4H_2O$ 0.99, $NiCl_2 \cdot 6H_2O$ 0.19, $CoCl_2 \cdot 6H_2O$ 0.24, $NaSeO_4 \cdot 10H_2O$ 0.21 and H_3BO_4 0.014 (Imajo et al. 2004). The pH of the influent was around 7.3-7.5. In order to maintain a controlled DO level in the reactor, the synthetic wastewater was deoxygenated by flushing with N_2, and stored in a gas tight collapsible low density polyethylene (LDPE) container.

Table 2.1. Composition of the synthetic wastewater.

Chemicals	g/L	g/mol	mol	mM
$(NH_4)_2SO_4$	Various			
$NaNO_2$	Various			
$NaHCO_3$	0.420	83.9	0.1	5
KH_2PO_4	0.027	136	0.004	0.2
$MgSO_4$	0.059	120	0.010	0.488
$CaCl_2.2H_2O$	0.18	147	0.024	1.224
EDTA	0.005			
$FeSO_4$	0.009			
Trace elements solution I	1 mL			
Trace elements solution II	1 mL			

2.2 Reactor Setup and Operation

Previous studies have shown that UASB reactor is very suitable for the cultivation, enrichment and study of very slowly-growing anammox bacteria. Two laboratory-scale UASB reactors with working volume of 5 L (height 1.10 m, diameter 0.10 m) have been

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used for the cultivation of anammox bacteria (UASB #1) and nitritation-anammox biomass (UASB #2), respectively.

2.2.1 UASB #1 reactor

The inactive methanogenic granules and anammox granules were inoculated to start up UASB #1 (Meng 2012). It has been steadily operated for more than three years. Previous studies have proved that the inactive methanogenic granules were suitable for rapid anammox granulation at high nitrogen concentrations (Ni et al. 2010b).

The configuration of UASB reactors is shown in Figure 2.1. The reactor was fitted with an influent port (also used for effluent recirculation and oxygen delivery) at the bottom, an effluent and a recirculation ports on the top, as well as two ports for sampling water and biomass located in the middle part. To maintain suitable temperature (35±1 ˚C) for anammox bacteria growth, the warm water from a thermostat water bath was recirculated into the integrated water jacket. The glass funnel on top functioned as a three-phase separator, collecting gas produced from the reactor, allowing liquid to flow out while preventing biomass run-off. Gravels with different sizes (approximately 2, 5, and 10 mm) were placed at the bottom part of the reactor to achieve better biomass retention and water/gas distribution.

The reactor was continuously fed with synthetic wastewater by a peristaltic pump (MasterFlex, Cole-Parmer Instrument, Vernon Hills, IL, USA). The treated water was recycled back to the influent port to create proper upflow velocity. The recirculation can dilute the influent to avoid inhibition effect of high nitrite strength, keep the biomass in suspension, as well as facilitate substrates transport. All tubes and connectors were made

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of black butyl rubber or polyvinylchloride (PVC) to prevent light transmission and air permeability. The off-gas collected from the reactor was measured by a connected gas meter.

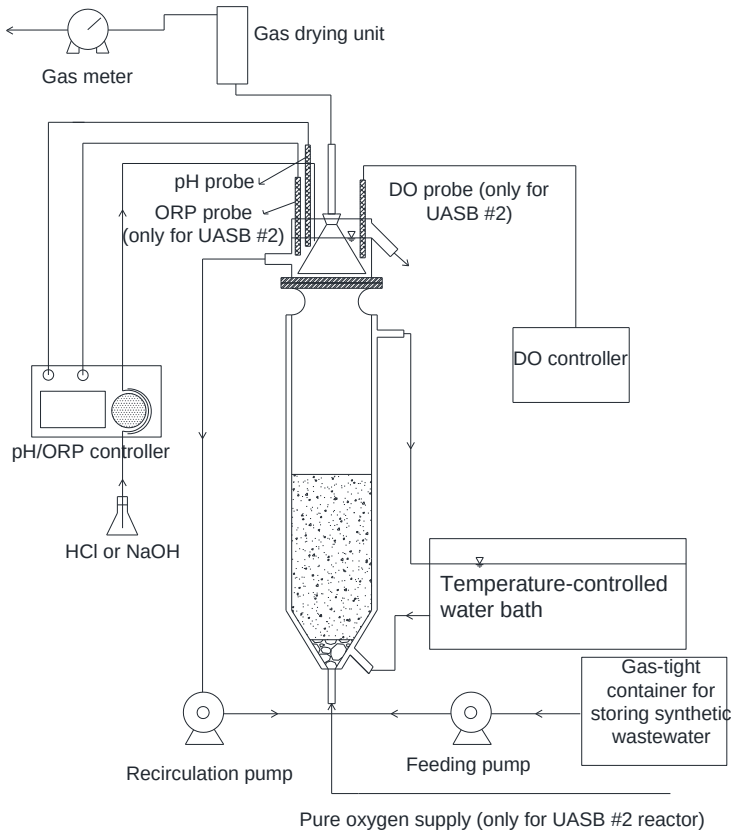

Figure 2.1. Schematic diagram of experimental setup

The reactor was continuously fed with synthetic wastewater at various nitrogen loading rates. The hydraulic retention time (HRT) was set at 1 ± 0.1 days for UASB #1. The pH inside the reactor was monitored by pH online probe coupled with a pH controller (pH 2000, New Brunswick Science, Edison, NJ, USA). The pH was adjusted and maintained at 7.6-7.8 automatically by feeding of 0.1 M hydrochloric acid (HCl) during the experimental period.

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2.2.2 UASB #2 reactor

Approximately 1.5 L of inactive methanogenic granules, 300 mL of anammox granules from UASB #1 (70 to 80% enriched) and subsequently 200 mL of aerobic activated sludge from a local municipal WWTP were used as seed sludge to inoculate UASB #2 to enrich nitritation-anammox biomass.

The UASB #2 reactor has a similar configuration with UASB #1 (Figure 2.1). The only different is that oxygen was gradually supplied in UASB #2 to stimulate the growth of AOB. Pure oxygen gas was delivered from gas cylinder. DO dosage was controlled via needle valve and gas flow meter. The online pH/ORP control system (MC125, Milwaukee Instruments, Inc., Rocky Mount, NC, USA) and online DO controller (HI 8410, Hanna Instruments Inc., Woonsocket, RI, USA) were connected to the reactor. The HRT was set as 36 h, and the temperature of the reactor was maintained at 35 ℃ through recirculation from a water bath. Since the bicarbonate in the mineral medium was insufficient to buffer the liquid in the reactor, HCl and NaOH were added when necessary to maintain a pH between the range of 7-8.

2.3 Analytical Methods

To monitor the performance of the reactor, concentrations of ammonium (NH_4^+-N), nitrite (NO_2^--N) and nitrate (NO_3^--N) in the effluent were regularly measured throughout the experiment. Ammonium concentration was measured by the standard ammonia ion selective electrode (Thermo Fisher Scientific Inc., Beverly, MA, USA) according to Standard Methods (APHA 1998). Nitrite and nitrate concentrations were determined by spectrophotometer (DR 3900, Hach Company, Loveland, CO, USA). Concentration of

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free ammonia was calculated as a function of pH, temperature and total NH_4^+-N as described previously (Anthonisen et al. 1976). Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were determined according to the standard methods (APHA 1998).

Elemental analysis of the biomass was performed using a CHNS analyzer (Vario Micro cube, Elementar Analysensysteme GmbH, Hanau, Germany). Proximate analysis was carried out using a thermogravimetric analyzer (TGA/DSC1, Mettler Toledo, Columbus, OH, USA) according to ASTM D5142 methods. Oxygen content was calculated by difference based on mass balance.

2.4 Fluorescence *in situ* Hybridization (FISH)

Spatial distribution of microorganisms in the granule was analyzed using FISH technology. FISH is a cytogenetic technique used to identify the presence of certain DNA sequences on chromosomes. FISH analysis was carried out as described previously (Kindaichi et al. 2006, Okabe et al. 1999, Tsushima et al. 2007a). During hybridization process, specific fluorescence probes bind to a particular region of a chromosome with special sequence. Matching bacteria can be observed under fluorescence microscopy, providing information on the compositions of microbial community in the granules.

Several 16S rRNA targeted-oligonucleotide probes (Sigma-Aldrich, St. Louis, MO, USA) used in this study and their hybridization conditions are listed in Table 2.2. Synthesis scale is 0.5 μmol, and formamide (FA) concentration in the hybridization buffer depends on the probe types. The probes were labeled with fluorescein isothiocyanate (FITC) or Texas RedTM (TXRD) at the 5' end. EUB388 probe was used

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to identify all bacteria (Amann et al. 1990). Amx820 probe (5'-AAAACCCCTCTACTTAGTGCCC-3') with TXRD label hybridizes specifically with Candidatus *Brocadia anammoxidans* and Candidatus *Kuenenia stuttgartiensis* (Schmid et al. 2001). FLC labeled NSE1472 and NSV443 probes were used to identify AOB, such as *Nitrosomonas europaea* and *Nitrosospira* spp., respectively (Mobarry et al. 1996). TxRd-labeled NIT3 probe (5'-CCTGTGCTCCATGCTCCG-3') is specific to *Nitrobacter* spp.

Table 2.2. List of 16S rRNA-targeted oligonucleotide probes used in this study.

Probe	Sequence (5' to 3')	FA (%)	5' Mod	Specificity	References
EUB338	GCTGCCTCCCGTAGGAGT	35	Flc	Most bacteria	Amann et al. (1990)
Amx820	AAAACCCCTCTACTTAGTGCCC	35	TxRd	Cand. ''*Brocadia anammoxidans*'' Cand. ''*Kuenenia stuttgartiensis*''	Schmid et al. (2001)
NSE1472	ACCCCAGTCATGACCCCC	50	Flc	*Nitrosomonsa* europea	Mobarry et al. (1996)
NSV443	CCGTGACCGTTTCGTTCCG	30	Flc	*Nitrosospira* spp.	Mobarry et al. (1996)
NIT3	CCTGTGCTCCATGCTCCG	40	TxRd	*Nitrobacter* spp.	Wagner et al. (1996)
ACI208	GTGCTCCCCCGCCAATTCCT	20	Flc	*Acidovorax* spp.	Raskin et al. (1994)

In general, there are three steps for FISH experiment: sample fixation, cryosectioning, and hybridization. Fresh granules were obtained from reactors and fixed in 4% paraformaldehyde solution (with buffer) at 4˚C for 3-4 hours. Cryostat microtome was used to rapidly freeze sample with Tissue-Tek® OCT (OCT stands for optimal cutting temperature) compound at -22˚C. Sections with16-20 μm thin were obtained by cryostat sectioning. Phosphate-buffered saline (1x PBS) was used to wash the sample slice. Then air dried slides were dehydrated with ethanol series 50%, 80% and 90%. Samples were hybridized with with 9 μL of hybridization buffers and 1 μL of probes ($50\,ng/\mu L$) at 40 ℃ for 1-1.5 hours. The hybridization stringency was adjusted by

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adding different amount of formamide to the hybridization buffer according to the probe types. After hybridization, the slides were washed at 48 ℃ for 5 min in washing buffer and then washed by distilled water. Finally, slides were air-dried and mounted with an anti-fading media (Fluoromount, Electron Microscopy Sciences, USA) under coverslips for microscopy observation. FICT and TRITC filters were used to obtain images of hybridized bacteria in this study.

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CHAPTER 3. RESULTS AND DISUSSION

3.1 Experimental Verification of Substrates Conversion in UASB #1 Reactor

The UASB #1 reactor has been operating for more than two years in the lab before the work presented here was performed. By inoculating a small amount of anammox biomass and strictly controlling growth conditions, the inactive methanogenic granules can be slowly converted into red anammox granules, which play a key role in the stability and efficiency of anammox reactor. The anammox granules are dark red and have irregular spheres, with an average diameter of approximately 3.8 mm.

As shown in Eq. (1.3), the proposed stoichiometric ratio of the ammonium conversion, nitrite consumption, and nitrate production is 1: 1.32: 0.256 (Strous et al. 1998). Since anammox granules have been highly enriched in UASB #1 reactor, a series of experiments were designed to verify the conversion ratio. The ratio of influent NO_2^--N/NH_4^+-N was optimized and the optimal ratio was applied throughout the experiments to evaluate long-term reactor performance.

3.1.1 Determination of the optimal ratio of influent NO_2^--N/NH_4^+-N

The optimal influent NO_2^--N/NH_4^+-N ratio was determined with NH_4^+-N fixed at 420 mg N/L (30 mM) and 490 mg/L (35 mM) (Table A.1). For the two sets of NH_4^+-N concentrations, the influent NO_2^--N/NH_4^+-N ratios were controlled at 1.31, 1.2, and 1.1, 1.31, 1.2, 1.1 and 1, respectively to study the reactor performances.

The effluent concentrations of ammonium, nitrite and nitrate are shown in Figure 3.1. Similar trends were observed for the two sets of experiments. Higher influent NO_2^--N/NH_4^+-N ratios (1.2 and 1.31) resulted in very low effluent ammonium concentrations.

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For instance, the average effluent ammonium concentrations were 0.77 mg N/L and 0.92 mg N/L when feeding 420 mg N/L and 490 mg N/L ammonium, respectively. In comparison, with lower influent NO_2^--N/NH_4^+-N (1 and 1.1), higher effluent ammonium concentrations (e.g. average of 36 ±10 mg N/L when ratio was 1) were observed possibly because of insufficient nitrite for anammox bacteria. On the other hand, nitrite accumulation occurred when influent ratio was 1.31. For Set II experiment, nitrate concentration showed a positive correlation with influent ratio (Figure 3.1B). In general, 1.2 should be considered as the optimal influent ratio of NO_2^--N/NH_4^+-N, since it yields the highest TN removal rate (on average 96%-97%).

The observed NO_2^--N/NH_4^+-N ratio and observed NO_2^--N/NH_4^+-N ratio were illustrated in Figure 3.2. The definition of these ratios is described in Eq. (3.1).

$$Influent\ ratio = \frac{influent\ NO_2^- - N}{influent\ NH_4^+ - N}$$

$$Ratio\ of\ observed\ \frac{NO_2^- - N}{NH_4^+ - N} = \frac{influent\ NO_2^- - N\ -\ effluent\ NO_2^- - N}{influent\ NH_4^+ - N\ -\ effluent\ NH_4^+ - N}$$

$$Ratio\ of\ observed\ \frac{NO_3^- - N}{NH_4^+ - N} = \frac{effluent\ NO_3^- - N}{influent\ NH_4^+ - N\ -\ effluent\ NH_4^+ - N}$$

$$Total\ nitrogen\ removal\ efficiency\% = 100 \times \left[1 - \frac{effluent\ (NH_4^+ - N + NO_2^- - N + NO_3^- - N)}{influent\ (NH_4^+ - N + NO_2^- - N)}\right]$$

Eq. (3.1)

Surprisingly, the observed NO_2^--N/NH_4^+-N ratios were in accordance with the influent NO_2^--N/NH_4^+-N ratios. For instance, the observed NO_2^--N/NH_4^+-N ratios were 1.08 and 1.13 (Figure 3.2B), slightly off the influent ratios of 1 and 1.1, respectively. The reason was that nitrite was almost completely removed but some ammonium was accumulated in the effluent. However, the observed NO_2^--N/NH_4^+-N ratios were 1.2 and

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1.3 (Figure 3.2B), which were very close to the influent ratios of 1.2 and 1.31, respectively. The similar tendency should be explained from different ways. Under influent ratio of 1.2, there were no ammonium and nitrite accumulations while both ammonium and nitrite accumulations occurred under the influent ratio of 1.3. The observed NO_2^--N/NH_4^+-N ratios were not exactly the same as proposed ratio of 1.32, possibly due to the activities of other microorganisms. For example, Meng (2012) has found that AOB can still survive in the strictly oxygen-limited anammox system.

The observed NO_3^--N/NH_4^+-N ratios were also much lower than the proposed ratio of 0.256, possibly causing by the activities of denitrifiers. Researchers have studied the co-existence of anammox and denitrification, which can refer to a review published by Kumar and Lin (2010).

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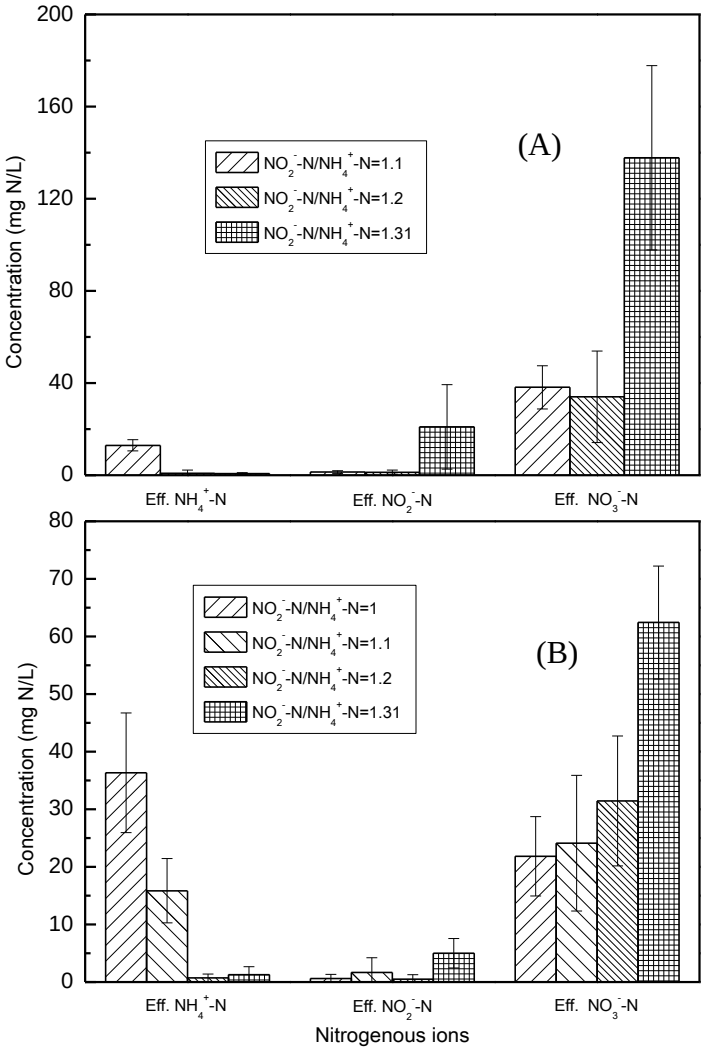

Figure 3.1. Effluent profiles under different influent NO_2^--N/NH_4^+-N ratios.

(A) 30 mM of influent NH_4^+-N. (B) 35 mM of influent NH_4^+-N

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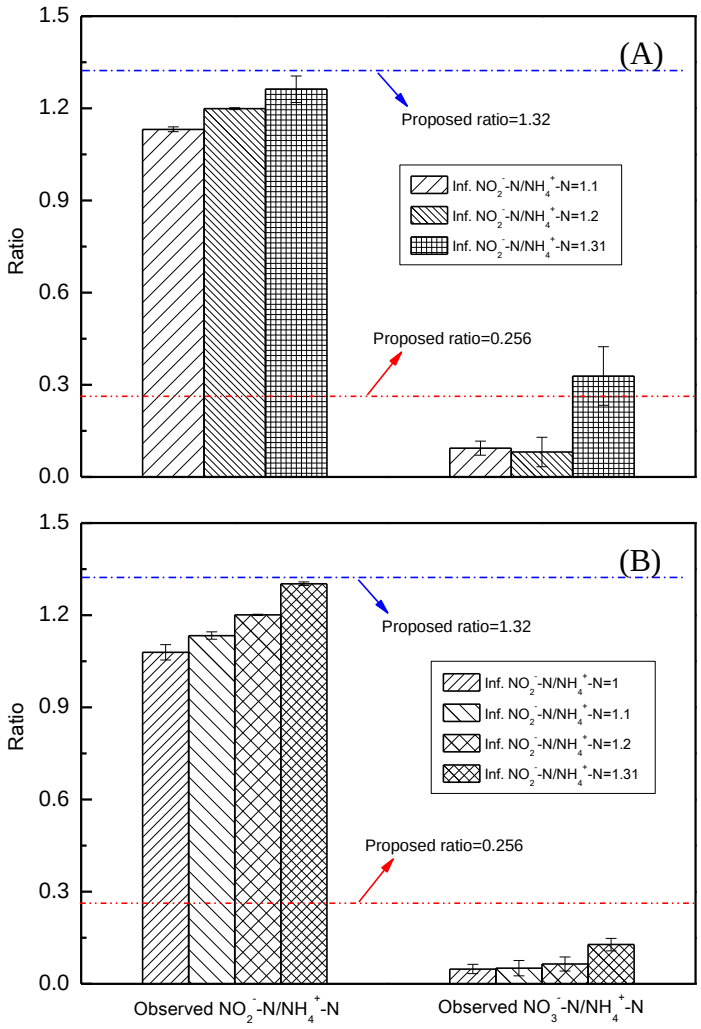

Figure 3.2. The observed NO_2^--N/NH_4^+-N ratio and observed NO_3^--N/NH_4^+-N ratio under different ratios of influent NO_2^--N/NH_4^+-N.

(A) 30 mM of influent NH_4^+-N. (B) 35 mM of influent NH_4^+-N.

3.1.2 Long-term performance under steady state conditions in anammox reactor

The long-term performance under steady state conditions of anammox reactor was investigated by two sets of experiments (Table A.2). The main difference between the two sets of experiments was the influent concentrations of ammonium and nitrite. NH_4^+-

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N loads used were 30 mM (420 mg/L, scenario I) and 35 mM (490 mg/L, scenario II), respectively. The optimal influent NO_2^--N/NH_4^+-N ratio of 1.2 was used, resulting in NO_2^--N loads of 504 mg/L and 588 mg/L, respectively.

The effluent profiles and TN removal efficiency were shown in Figure 3.3. The performance of the anammox reactor showed a high stability. Ammonium and nitrite removal efficiencies were extremely high (both were more than 99%) for both scenarios.

Growth of the anammox bacteria is associated with nitrate production, because part of the nitrite is oxidized to nitrate which serves as the terminal electron donors for cell carbon fixation (Hu et al. 2013c). It has been reported that anammox bacteria exhibited tolerance to nitrite up to 13 mM (195 mg N/L) (Egli et al. 2001). However, much higher concentrations of NO_2^- (up to 690 mg N/L) have been applied to a full-scale anammox granular reactor (Abma et al. 2007). A series of batch experiments were conducted with various initial NO_2^- concentrations to investigate effects of the NO_2^- concentration on anammox activity (Cho et al. 2010). Their results indicated that anammox granules tolerated higher NO_2^- concentration as compared with the homogenized biomass, probably because of the lower NO_2^- concentration inside the granule due to substrate diffusion limitation (Cho et al. 2010). For Scenarios I and II in UASB #1 reactor, the influent NO_2^--N concentrations were as high as 504 mg/L and 588 mg/L, respectively, but the mean effluent NO_2^--N concentrations were 1.8 mg/L and 0.26 mg/L, respectively, even sometimes below the detection limit. Thus, the inhibition effect by high concentration of NO_2^- depends on biomass characteristics and operational conditions as well as reactor types.

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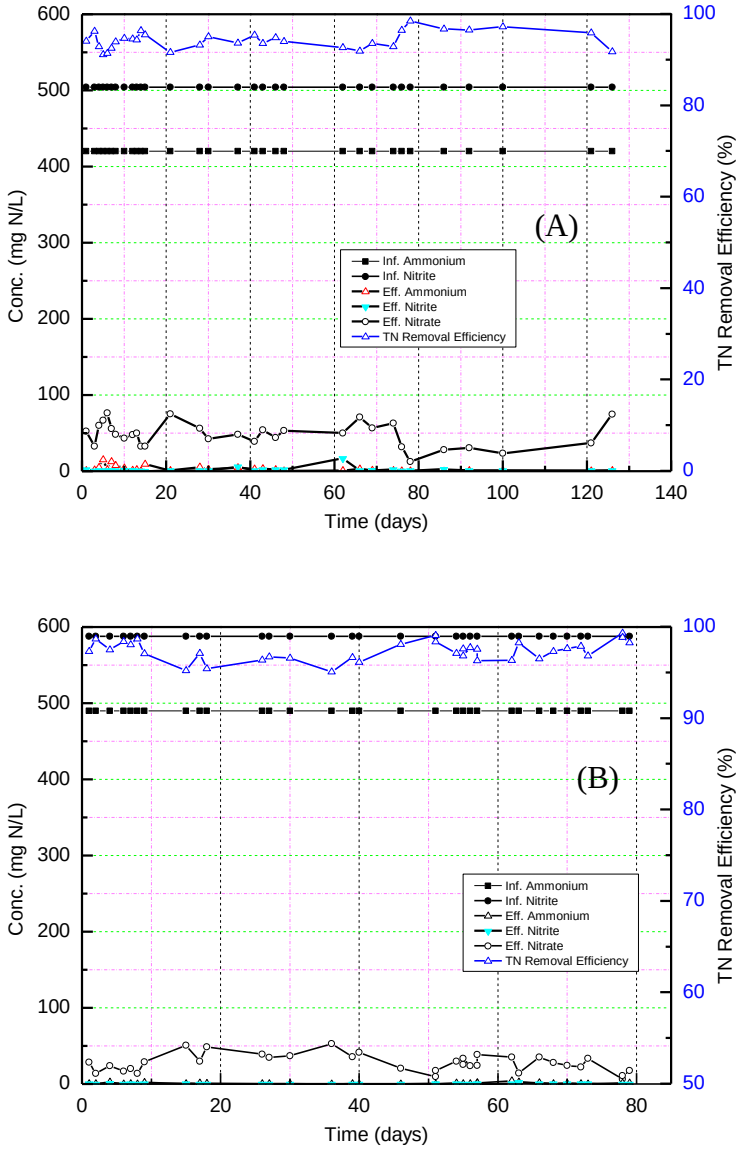

Figure 3.3. Reactor performances under steady state operating conditions.

(A) 30 mM of influent NH_4^+-N. (B) 35 mM of influent NH_4^+-N.

Since ammonium and nitrite were almost completely removed, the TN removal efficiency is closely related to nitrate production. Based on the Eq. (1.3), about 11% of the total nitrogen load is converted to nitrate. However, the concentrations of nitrate for Scenarios I and II were 48±16 mg N/L (i.e. 5.2±1.7% of TN load) and 28±12 mg N/L

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(i.e. 2.6±1.1% of TN load), respectively. Interestingly, the higher TN load rate produced less amount of nitrate. For instance, the mean TN removal efficiency of Scenario II was 97.3%, which was 3% higher than that of Scenario I.

To compare with the stoichiometry of the anammox reaction Eq. (1.3), nitrogen balances were performed in UASB #1 reactor. As shown in Figure 3.4, the mean ratio of observed NO_2^--N/NH_4^+-N for both scenarios was about 1.20, very close the ratio of NO_2^--N/NH_4^+-N in the influent. Though it is not in good agreement with proposed ratio, similar ratio has been reported by Ni et al. (2010a). However, the mean ratios of observed NO_3^--N/NH_4^+-N for scenarios I and II were 0.12 and 0.06, respectively, which are much lower than proposed ratio of 0.256 as well as that of 0.22 reported by Ni et al. (2010a). Meng (2012) reported the ratio of consumed NH_4^+-N: consumed NO_2^--N: produced NO_3^--N of 1:1.21:0.19 at steady state using the same reactor. In contrast, the stoichiometric ratio of the ammonium conversion, nitrite removal, and nitrate production was calculated as 1:1.26:0.44 (Xiong et al. 2013). The nitrate production was much higher than that in this study.

Based on the substrates conversion and mass balance, an empirical reaction equation was proposed for UASB #1 reactor without considering cell synthesis (Eq. (3.2)). Compared to Scenario II, the substrate conversion in Scenario I was in very good agreement with the proposed equation. The concentrations of produced nitrate in Scenario II were much lower than expected based on the equation.

$$NH_4^+ + 1.2NO_2^- + 0.08H^+ \rightarrow 1.04N_2 + 0.12NO_3^- + 2.04H_2O$$

Eq. (3.2)

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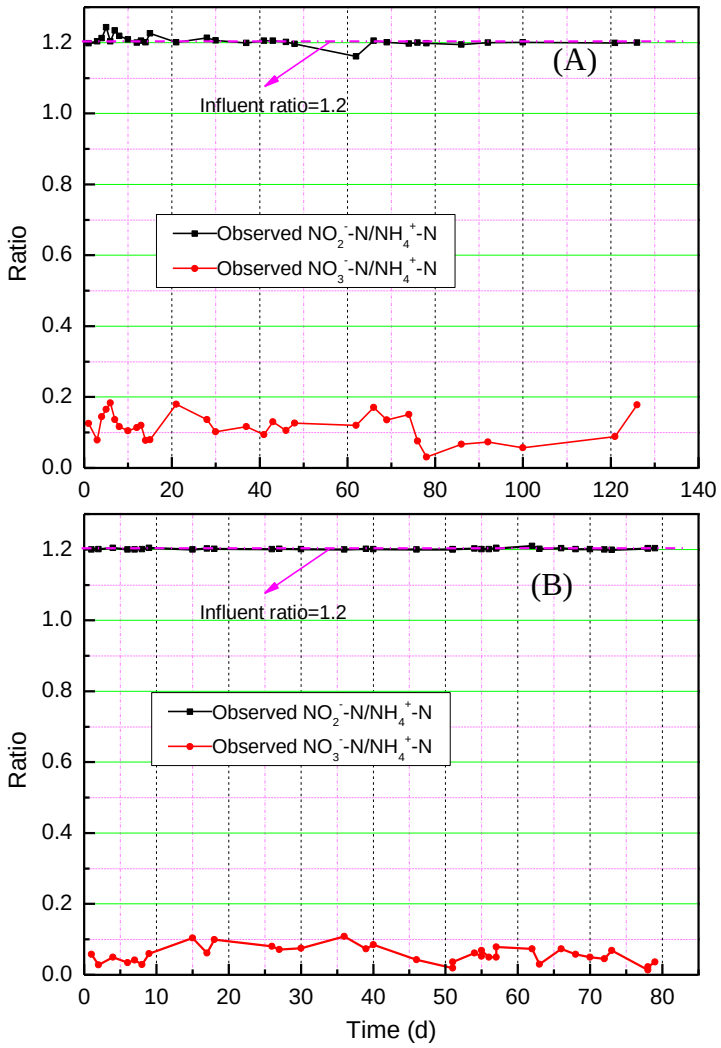

Figure 3.4. The observed NO_2^--N/NH_4^+-N ratio and observed NO_3^--N/NH_4^+-N ratio under steady state operating conditions

(A) 30 mM of influent NH_4^+-N. (B) 35 mM of influent NH_4^+-N.

3.1.3 Characteristics of the anammox granules

Hydrodynamic shear force in UASB reactors mainly results from upflow velocity and gas production (Liu and Tay 2002). Anaerobic granulation proceeded well at relatively high liquid upflow velocity (Arne Alphenaar et al. 1993), while upset

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granulation processes at weak shear force have been reported (Alves et al. 2000, O'Flaherty et al. 1997). The selection pressure theory has been used to explain the effects of upflow velocity on anaerobic granulation process (Hulshoff Pol et al. 1988). The selection pressure includes temperature, HRT, upflow velocity, substrate loading rate, pH, reactor configuration, seed sludge, etc. A relatively high liquid upflow velocity could cause washout of dispersed bacteria and those bacteria competent for aggregation would be kept in the reactor (Liu and Tay 2002).

The anammox granules play an important role in the high nitrogen removal performance of anammox reactor. The mature anammox granules had diameters typically between 1.0 and 7.0 mm (Figure B.1), with the majority (approximately 74%) having a diameter of 2.1 to 5 mm (Figure 3.5). Their porous structure can facilitate the diffusion of substrates as well as the release of N_2. The concentrations of MLSS and MLVSS of the sludge bed were 33.1 g/L and 27.6 g/L, respectively.

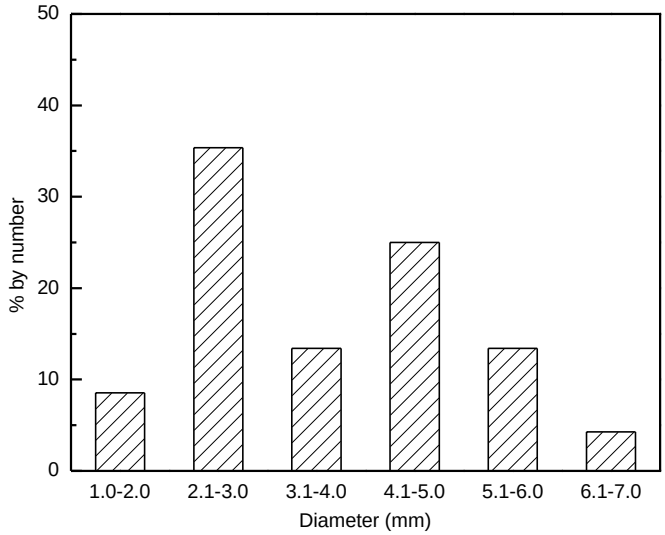

Figure 3.5. Particle size distribution of the anammox granules by number.

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The mature anammox granules settled very well at velocities ranging from 57 to 154 m/h (on average 104 m/h) , which were higher than those of the methanogenic granules and aerobic granules (e.g., 52 m/h and 86.4 m/h) (Blaszczyk et al. 1994, Shin et al. 1992). Also, the settling ability of anammox granules is much better than that of the flocculated anammox biomass. Such high settling velocities indicated that the anammox granules have a highly dense and compact structure, which benefits biomass retention in the reactor and improves effluent quality.

The upflow velocity is a very important parameter for successful operation of UASB reactor. The formation of anaerobic granules can be enhanced through a purely physical aggregation, e.g., the hydrodynamic stress by increasing the liquid upflow velocity (Liu and Tay 2002). It has been suggested that an upflow velocity of 0.6 to 0.9 m/h must be maintained to keep the sludge blanket in suspension. In addition, settling velocity was significantly improved as the upflow velocity increased, resulting in a reduction of biomass washout rate from 46% to 2% (Liu and Tay 2002). A suitable velocity of 1.2-1.5 m/h was employed in anammox reactor not only to keep the granules in suspension but also to prevent biomass washout.

The element compositions of the dry anammox granules were shown in Table 3.1. The molecular formulas of anammox granules from the reactor were different from the previously reported formulas $CH_2O_{0.5}N_{0.15}S_{0.05}$ (Strous et al. 1998) and $C_5H_{13.3}O_{3.3}N$ (i.e. $CH_{1.8}O_{0.5}N_{0.2}$) for anammox sludge (Hao 2001), possibly due to the different microbial community compositions. Elser et al. (2000) also hypothesized that the variation in cell

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elemental composition may reflect the species interactions developed in the systems under different conditions.

It has been reported that the general formula for biomass grown under carbon-limited conditions is $CH_{1.8}O_{0.5}N_{0.2}$, or $CH_{1.8}O_{0.5}N_{0.2}S_{0.002}P_{0.02}$ when sulfur and phosphorus are taken into account (Heinzle et al. 2007). Based on our results, anammox biomass has a lower H in the case of scenario II, but silmiar O and N contents when compared with above general formula. In addition, the S content in anammox biomass is significantly high though the reason is unclear.

Based on Table 3.1, the ratio of VSS/SS increased from 75% to 84% as C content increased in anammox granules, in agreement with Liu et al. (2005) who found a positive corelation between C content and the VS/TS ratio of P-accumulating microbial granules.

Table 3.1. Elemental analysis of anammox biomass.

Scenario	C, %	H, %	O, %	N, %	S, %	Molecular formula	MLVSS/MLSS
I (30 mM of inf. NH_4^+-N)	33.2±0.9	4.7±0.1	33	6.5±0.2	3.82±0.49	$CH_{1.7}O_{0.85}N_{0.15}S_{0.04}$	0.75
II (35 mM of inf. NH_4^+-N)	35.3±0.4	3.8±0.7	34.9	6.7±0.1	2.1±0.1	$CH_{1.28}O_{0.74}N_{0.16}S_{0.02}$	0.84

3.2 Development of Nitritation-Anammox Process in UASB #2 Reactor

The experimental period was divided into two phases. During phase I (330 days), anammox granules were enriched under strict DO control. The goal of phase II (>250 days) was to cultivate nitritation-anammox biomass under low oxygen conditions.

An important factor in the successful startup of anammox process is to ensure efficient biomass retention. Anammox bacteria have a natural ability to form

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biofilms (Abma et al. 2007). The biofilms can then form dense, well settling aggregates in a granular sludge type system, such as UASB. After obtaining sufficient anammox granules, oxygen gas was cautiously supplied to facilitate the growth of AOB. The hydraulic mixing conditions in the reactor was caused by effluent recirculation, oxygen gas supply and nitrogen gas production, resulting in good suspension of the biomass and dilution of high influent strength.

3.2.1 Enrichment of the anammox granules

Addition of red anammox biomass has been reported to significantly accelerate start-up and enhance reactor performance (Tang et al. 2011), which was also confirmed during the start-up of the first full-scale anammox reactor (van der Star et al. 2007). Therefore, 300 mL of the anammox granules (80-90% enriched) from UASB #1 reactor as well as the inactive methanogenic granules were used as inoculum for enrichment of anammox granules in UASB #2 reactor.

At the start-up stage, the reactor was initially fed with the synthetic wastewater with a relatively low nitrogen loading rate (NLR). Then the reactor was continuously operated for about 200 days with NH_4^+-N of 210 mg/L and NO_2^--N of 252 mg/L (ratio=1.2) as the substrates until it achieved sufficient anammox granules (data not shown). During this period, some small flocs detached from granules were washed out, and new granulation process occurred. The anammox bacteria grew and attached on the surface of granules. The color of the methanogenic granules at the bottom (approximately 30%) of sludge bed gradually turned from blackish to brownish, while

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the inoculated anammox granules remained red and became larger, suggesting that the anammox bacteria grew well in the reactor.

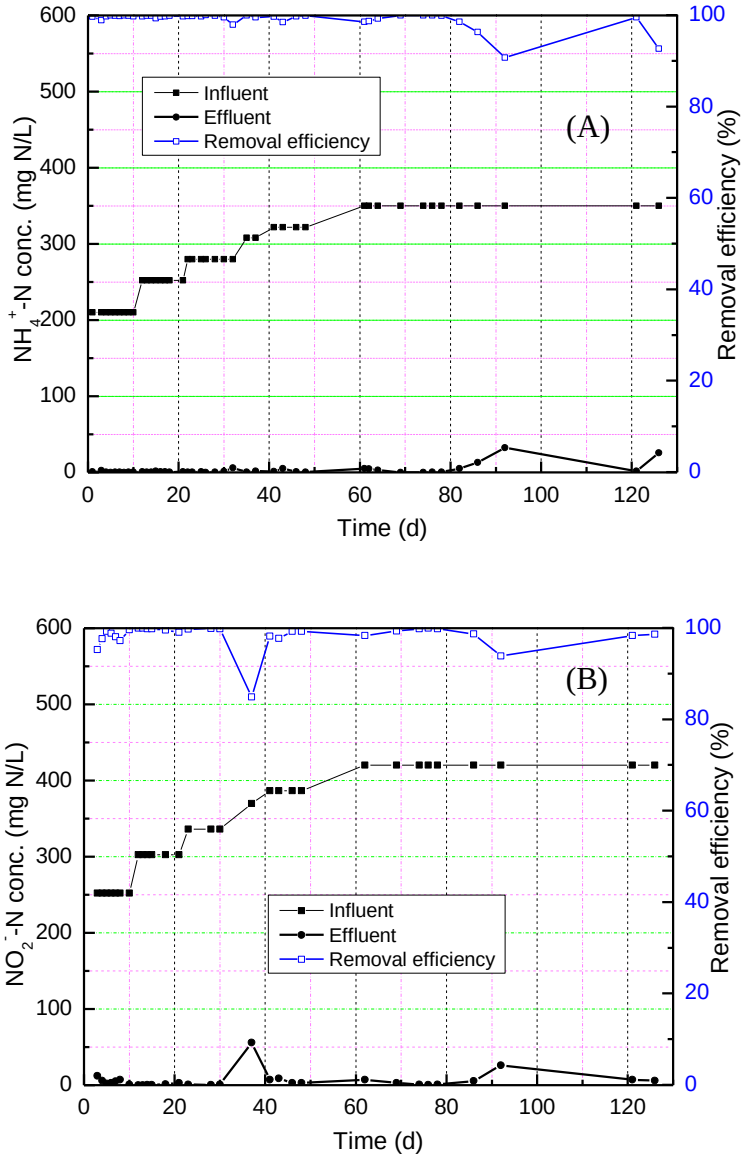

Figure 3.6. Reactor performances during Phase I.

(A) Concentration profile of NH_4^+-N. (B) Concentration profile of NO_2^--N.

Then the NLR was elevated by increasing the influent ammonium and nitrite concentrations. As a result, more granules turned from black to brown and then red

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subsequently, implying the significant enhancement in the activity of anammox bacteria. The NLR was gradually increased once the reactor exhibited stable performance. As shown in Figure 3.6, NH_4^+-N and NO_2^--N concentrations were raised up to 350 mg/L and 420 mg/L, respectively. The average NH_4^+-N, NO_2^--N and total nitrogen removal efficiencies were 98.8%, 97.9%, and 88.5%, respectively. On Day 37, nitrite accumulation was up to 56 mg N/L. However, this decrease in anammox activity was reversible.

The gas emission rate profile was depicted in Figure 3.7. The general trend was that the nitrogen gas production rate increased as the NLR increased. The mean gas emission rate was 1.8 L/d for the last 66 days when NLR was maintained at 0.51 kg/m^3/d.

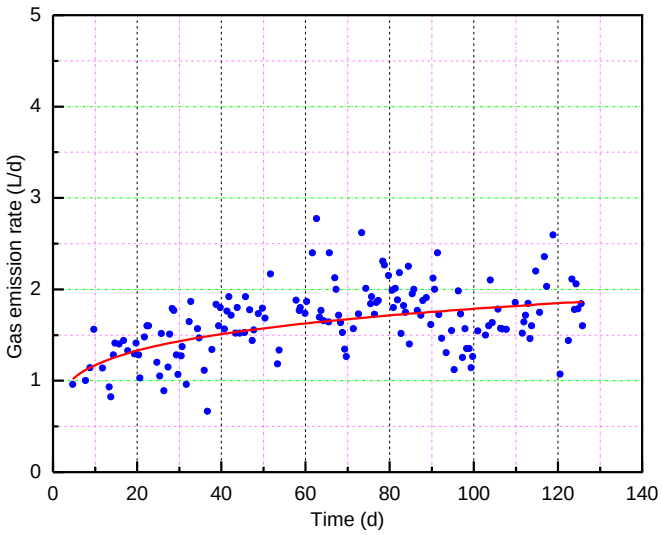

Figure 3.7. The profile of gas emission rates during Phase I.

3.2.2 Enrichment of nitritation-anammox biomass

After more than 320 days of operation in Phase I, the outstanding nitrogen removal performance has been accomplished. More brownish-red and bright-red

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anammox granules with diameters of 0.5-4.5 mm were visible at the bottom of the reactor (40-50% of sludge layer).

Nitritation-anammox process in a single reactor has been developed mostly by inoculating nitrifying sludge into anammox reactors (Sliekers et al. 2003, Third et al. 2001), and less frequently by inoculation of anammox biomass into partial nitrification reactors (Gong et al. 2008, Vazquez-Padin et al. 2009b). In this study, 200 mL of aerobic activated sludge was inoculated in the reactor, intending to provide AOB sources for developing nitritation-anammox biomass. Most of heterotrophic bacteria went through starvation when exposed in the medium without organic carbon, eventually died and were washed out of the reactor.

The TN removal performance depends on the reactor setup, operational conditions and the activities of each bacterial group. For successful start-up and operation of the combined nitritation- anammox process, AOB are supposed to convert approximately half of the supplied ammonium to nitrite under oxygen-limiting conditions. In turn, the produced nitrite together with the remaining ammonium is converted to N_2 by anammox bacteria. Nitritation a critical step because it is rate-limiting and difficult to develop and maintain suitable conditions (Cho et al. 2011). Nitrite is seldom accumulated in the reactors due to its fast conversion rate to nitrate (Satoh et al. 2003). Thus, a suitable environment must be provided to accumulate AOB but eliminate or inhibit NOB based on the differences of their specific growth rates. According to previous studies, the growth and activity of NOB can be prevented based on their lower affinities for oxygen compared to AOB as well as for nitrite compared to anammox

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bacteria (van der Star et al. 2008a). In addition, NOB can be successfully washed out at high temperatures between 30 and 40 ℃ with a short SRT of about 1.5 days (Hellinga et al. 1998).

The possible microorganisms involved in a single-stage nitritation-anammox reactor and their relationships are summarized in Table 3.2. Anammox bacteria thrive by competition as well as by delicate metabolic interactions with other organisms associated with nitrogen cycle (Kartal et al. 2012).

Table 3.2. The possible microorganisms existing in the microbial community and their relationships

Microorganisms	Relationship	Remarks
AOB vs. NOB	Competition	Competing for O_2.
	Commensalism	NOB consume nitrite produced by AOB.
AOB vs. Anammox bacteria	Competition	Competing for ammonium. AOB might compete for nitrite through nitrifier denitrification pathway.
	Commensalism	Anammox bacteria consume nitrite produced by AOB. In addition, the activity of AOB consume O_2 resulting in a suitable oxygen level for anammox bacteria. Anammox process can supply some alkalinity since nitritation process will lower pH.
NOB vs. Anammox bacteria	Competition	Competing for nitrite. NOB should be eliminated.
Denitrifier vs. Anammox bacteria	Commensalism	Denitrifier can take care of nitrate produced by anammox bacteria when organic carbons are available. It may also consume soluble microbial products (SMPs) produced during anammox metabolism and biomass decay.

The strategy used in this study was to fix ammonium concentration to be 350 mg N/L (25 mM) but gradually reduce nitrite concentration from 420 mg N/L to 0 in the influent. Correspondingly, the ratio of influent NO_2^--N/NH_4^+-N decreased from 1.2 to 0. The amounts of oxygen supply were controlled and adjusted by gas flow meter according to the results of on-line ORP and DO probes. The substrate compositions during the Phase II were shown in Table 3.3 and Table A.3 in Appendix B.

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Table 3.3. Operation time for various ratios of influent NO_2^--N/NH_4^+-N during Phase II

Influent NO_2^--N/NH_4^+-N	1.2	1.1	1	0.8	1
Operation time/day	1-14	14-27	27-70	70-94	94-114
Influent NO_2^--N/NH_4^+-N	0.9	0.8	0.7	0.6	0.5
Operation time/day	114-138	138-142	142-149	149-174	174-180
Influent NO_2^--N/NH_4^+-N	0.4	0.3	0.2	0.1	0
Operation time/day	180-203	203-218	218-237	237-247	247-

At the beginning of Phase II, the reactor was operated anaerobically for about 4 weeks. For the first two weeks, the reactor was fed with 350 mg N/L (25 mM) ammonium and 420 mg N/L (30 mM) nitrite (ratio=1.2). Within this period, anammox bacteria were responsible for most ammonium and nitrite consumptions. Since Day 27 when the ratio of influent NO_2^--N/NH_4^+-N decreased to 1, pure O_2 was introduced to the reactor to facilitate the AOB growth. Anammox bacteria consumed most of NH_4^+ when the ratio was high (ratios of 0.9 and 1). To avoid inhibition of anammox bacteria, O_2 was supplied in such a way that it was below 0.2 mg O_2/L. As the influent nitrite continued to decrease, O_2 supply was increased slowly because more ammonium was required to be converted to nitrite by AOB.

The nitrite comes from two sources, including nitrite present in the influent (except the last stage without additional nitrite) and the conversion of ammonium to nitrite by AOB. During the entire operation time at Phase II, there was no nitrite accumulation in effluent though a complete nitrite removal has not been achieved. The mean nitrite concentration in the effluent was about 1.8 mg N/L. Such a small amount of nitrite leftover may contribute to higher activity of AOB than anammox bacteria.

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On Day 70, the ratio of influent NO_2^--N/NH_4^+-N decreased from 1 to 0.8, resulting in a sudden increase of effluent ammonium (up to 108 mg N/L). Probably the abundance of AOB was insufficient to bear the shock load of ammonium at that time. After feeding the influent ratio of 1 medium, the reactor performance recovered slowly.

On day 247, the concentration of ammonium remained the same (350 mg N/L) but nitrite was no longer supplied to the reactor. After this point, the nitrite required for the anammox reaction was completely produced by the AOB. The average nitrite accumulation at this stage was 2.8 mg N/L, which was a little bit high than before. The possible reason was that AOB activity was higher than anammox bacteria because of improved oxygen supply.

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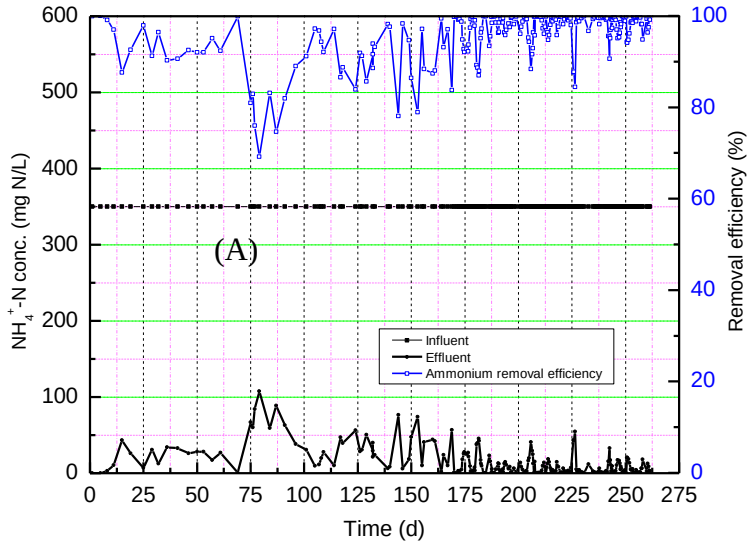

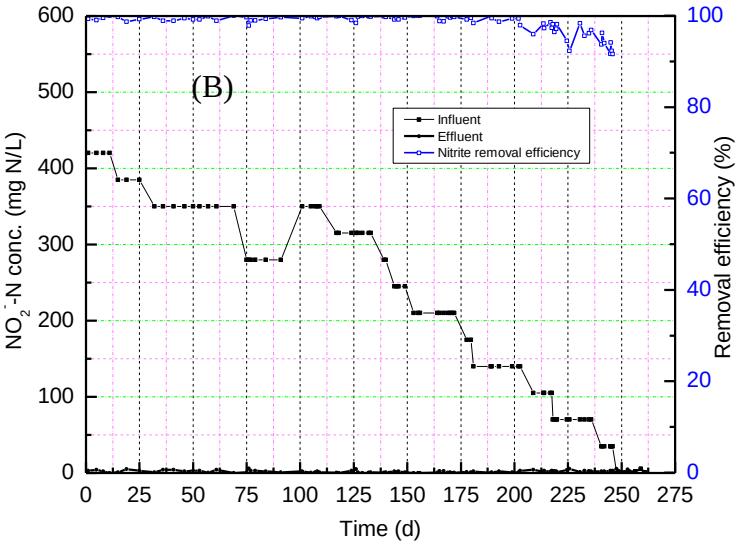

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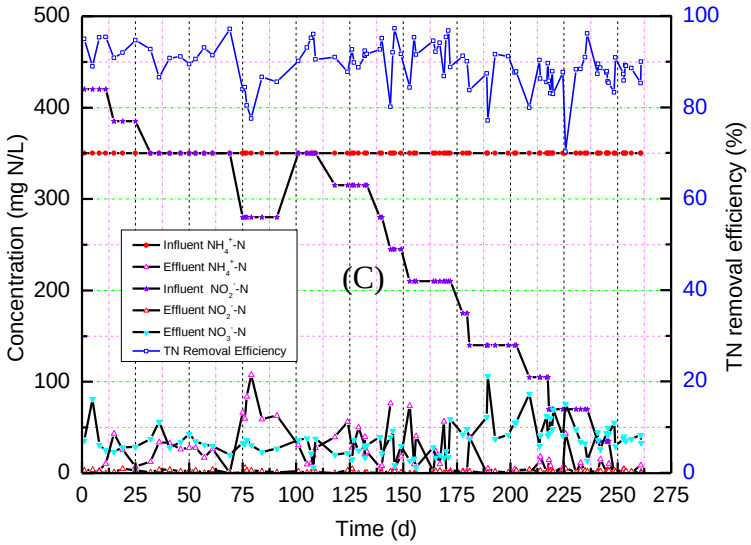

Figure 3.8. Reactor performances during Phase II.

(A) Concentration profile of NH_4^+-N. (B) Concentration profile of NO_2^--N.

(C) Concentration profiles of NH_4^+-N, NO_2^--N, NO_3^--N and TN removal efficiency.

Anammox bacteria oxidize part of nitrite to nitrate to reduce equivalents necessary for cell carbon fixation (Hu et al. 2013c). Therefore, nitrate production is observed during the growth of anammox bacteria. The control of nitrate production is very important since it affects the TN removal efficiency. Two pathways are related to nitrate conversion in the nitritation-anammox system. First, NOB can convert nitrite to nitrate if oxygen supply is more than AOB requirement. Second, denitrifying bacteria may convert nitrate to N_2 when the organic carbon sources are available (e.g., SMPs produced during cell lysis or decay of functional bacteria). Based on FISH results (as discussed below), the activities of NOB were suppressed under low oxygen condition, and therefore NOB did not likely contribute to nitrate formation.

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The mean nitrate concentration was 36±18 mg N/L. On day 191 (influent NO_2^--N=140 mg/L) and day 210 (influent NO_2^--N=105 mg/L), nitrate concentrations went up to 106.5 and 86.9 mg N/L, respectively, possibly due to NOB activity with the increased oxygen supply, which was also proven by ORP of above 180 mV (Figure 3.12).

Based on Figure 3.9, compared with the ratio of influent NO_2^--N/NH_4^+-N, the variation of the observed NO_3^--N/NH_4^+-N was relatively small, as long as the ORP/DO was maintained in the appropriate range (as discussed below). The average ratio of observed NO_3^--N/NH_4^+-N was 0.11, which was slightly smaller than the ratio of 0.13 proposed in CANON equation (Eq. (3.3)). A higher ratio of 0.18 was reported, where the influent NO_2^--N/NH_4^+-N ratio was 0.71 (Hu et al. 2013c). As mentioned above, lower nitrate production in this research might be associated with denitrification process because denitrifiers were detected by FISH (Figure 3.17).

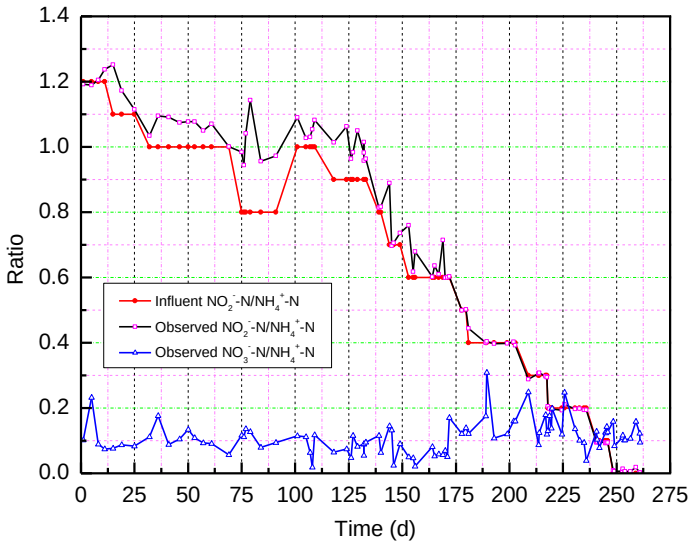

Figure 3.9. The ratios of influent NO_2^--N/NH_4^+-N, observed NO_2^--N/NH_4^+-N and observed NO_3^--N/NH_4^+-N during Phase II.

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Figure 3.10 shows the changes of total NLR and nitrogen removal rate (NRR) with time. The graph showed a progressive decrease of the loading rate and removal rate during Phase II.

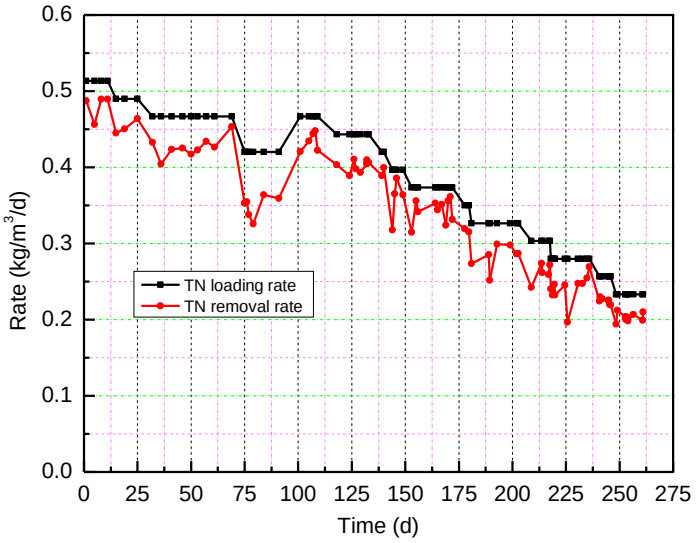

Figure 3.10. The profiles of total NLR and NRR during Phase II.

In the nitritation-anammox system, H^+ is produced during the ammonium oxidation by AOB, while H^+ is also consumed by anammox bacteria. The final effluent pH depends on their activity intensities. HCl (0.1 M) was used to adjust pH to 7.6-7.8 when the ratio of influent NO_2^--N/NH_4^+-N was 1-1.2. It was observed that much less HCl was consumed when the ratio was 1, possibly due to the balance of increased alkalinity by anammox process and decreased alkalinity by AOB. However, when the ratio of influent NO_2^--N/NH_4^+-N was reduced to 0.8, the effluent pH decreased significantly (as low as 6.52), which may inhibit the activities of AOB and anammox bacteria. Thus, starting from Day 74, NaOH with various concentrations depending on the influent substrate compositions were applied to buffer the liquid in the reactor. The consumption of base

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increased as the nitrite concentrations decreased in the influent because of high AOB activities. The effluent pH in the nitritation-anammox system will eventually decrease due to the net H^+ production, which can be seen from Eq. (3.3).

CANON: $NH_4^+ + 0.85O_2 \rightarrow 0.13NO_3^- + 0.435N_2 + 1.4H^+ + 1.3H_2O$

Eq. (3.3)

The changes of online pH values versus time were presented in Figure 3.11. Overall, the pH was successfully maintained in the range of 7-8, which was lower than the previous studies (Cho et al. 2010, Qiao et al. 2010). They suggested that high pH and NLR were required to establish partial nitrification. The pH values occasionally fell below 7 due to the depletion of NaOH solution.

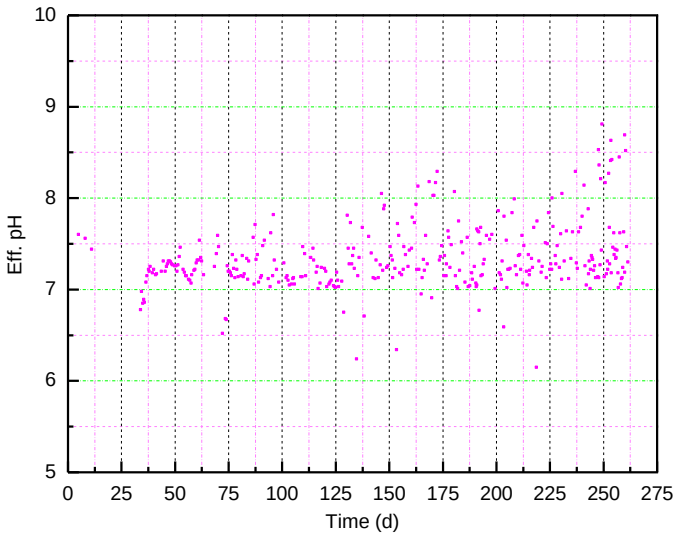

Figure 3.11. The pH profile during Phase II.

3.2.3 Application of ORP/DO as combined monitoring parameters

Because of the complex interplay between the aerobic, anoxic and anaerobic microorganisms, appropriate control of DO level in the nitritation-anammox reactor is

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very important. The oxygen supply should meet the requirement of AOB but not inhibit anammox bacteria and favor NOB growth. NOB was inhibited at DO concentrations ≤1.0 mg O_2/L (Hanaki et al. 1990, Turk and Mavinic 1989), probably because the lower growth rate of NOB than AOB at DO concentrations below 1.0 mg O_2/L (Park and Noguera 2004, Tokutomi 2004). In addition, it was reported that NOB was outcompeted at low DO concentrations (<0.5 mg O_2/L), due to their weaker affinity for oxygen as compared to AOB (Bernet et al. 2001).

The ORP has been widely studied as a parameter for online monitoring and control of BNR process since the early 1980s (Charpentier et al. 1998, Kim and Hao 2001, Li and Bishop 2002, Tanwar et al. 2008). It has been considered as a more sensitive parameter for process control compared to pH or DO due to its high signal range (Lackner and Horn 2012). For instance, the ORP has been applied to detect ammonium or nitrate depletion to control the durations of aerobic and anoxic phases in BNR systems (Akın and Ugurlu 2005, Ra et al. 2000). Researchers have suggested that certain biochemical events such as the depletion of organic carbon or ammonium under low oxygen condition, and aerobic/anoxic/anaerobic shifting environments, can be readily detectable by on-line ORP profile (Holman and Wareham 2003). In contrast, other control parameters such as the DO may only show little or no change in these situations. Therefore, online ORP could become a promising technology for the automated control of the BNR process.

Thus, the ORP may provide insight into the state of the system (e.g. aerobic, anoxic or anaerobic conditions). To my best knowledge, the application of ORP as a monitoring

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or controlling parameter for developing and operating the combined nitritation-anammox process in a UASB reactor has not yet been investigated.

The profiles of ORP, DO and oxygen flow rate during Phase II were shown in Figure 3.12. Variations in influent substrate concentrations, oxygen flow rate and effluent quality could conceivably influence the ORP profile. On days 180, 186 and 193 where ammonium levels were reduced to very low levels (below 0.6 mg N/L), abrupt increases in ORP were found. The similar observations have been reported by Holman and Wareham (2003), who suggested that the real-time ORP process control system could detect the changes in ORP slope and theoretically reduce aeration intensity at ammonium turning points, resulting in significant cost-savings on energy consumption.

Based on the results, ORP had a higher sensitivity than DO at low-oxygen levels, but there was no obvious relationship between ORP and DO, though a strong linear relationship between ORP and the log of DO was found by Ndegwa et al. (2007). During the first 240 days, most of the ORP levels fell on the range of -25--75 mV. ORP dropped to negative values when the ratio of influent NO_2^--N/NH_4^+-N reduced to 0.1 (35 mg N/L of nitrite). In contrast, DO levels were still around 0.2-0.3 mg O_2/L. However, the ORP increased to positive valves when the influent was absent of nitrite on Day 247. For the last 7 days, ORP increased rapidly (up to 260 mV) with the average oxygen supply rate of 35 L/d, leading to an excellent ammonium removal efficiency (99.5%). The increasing ORP value represents the improvement of the redox status in the reactor. Meanwhile, DO concentrations exhibited in the range of 0.2-0.4 mg O_2/L. According to

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the entire experimental period, DO level ≤0.5 mg O_2/L was recommended to startup and operate nitritation-anammox reactors.

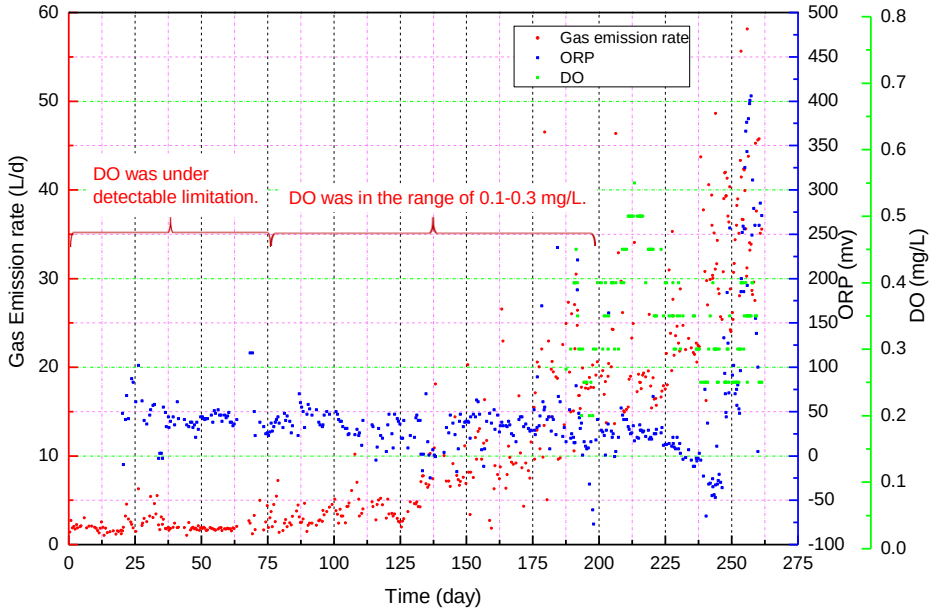

Figure 3.12. The profiles of ORP, DO and oxygen flow rate during Phase II.

3.2.4 Characteristics of nitritation-anammox granules

The nitritation-anammox granules cultivated in UASB #2 reactor had several notable features. First, the granular biomass was desirable for the substrates dispersion and gas diffusion (e.g. N_2, O_2). Second, the biomass retention in the reactor was guaranteed due to the excellent settling ability of high-density granules. Third, a high conversion rate was achieved in the granular sludge reactor due to high biomass concentration and a large surface area for mass transfer.

Up flow velocity in the reactor was controlled at 1.5-1.6 m/h to maintain the sludge layer in suspension and wash out the flocky sludge, which can promote granulation

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process. However, together with the oxygen supply, it may also cause incidental losses of AOB and anammox biomass.

There were plenty of typical red anammox granules after Phase I. However, the color of biomass has changed gradually as oxygen gas was introduced to facilitate the formation and enrichment of nitritation-anammox biomass during Phase II. Those transitions of anammox granules to AOB-anammox granules were shown in Figure B.2. In general, the biomass mixture was composed of granules with different colors. The typical red anammox granules progressively changed color from dark red to light red even to reddish because the AOB (also other bacteria) grew in the same granules after starting aeration. The similar color transition was also reported by Cho et al. (2011), while developing a simultaneous partial nitrification-anammox process in an up-flow biofilm reactor. Most importantly, more and more granules with brown-yellow color were observed. The color was recognized as the typical sign of nitrifiers (Wang et al. 2008). Alleman and Preston (1991) also pointed out that nitrifying biofilms tend to have a brown to orange-brown color, which turns reddish brown as the fraction of nitrifiers increases.

The size distribution of the granules is depicted in Figure 3.13. The mean diameter of the granules was 3 mm. Over 55% of the granules had a settling velocity over 100 m/h that was similar to the anammox granules in UASB #1 reactor. It should be mentioned that the granule samples used to determine size distribution and settling velocity were collected from the reactor at a height of 15 cm from the bottom (Figure B.3). Thus, the

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granules at the bottom of sludge bed should have even larger size and higher settling velocity.

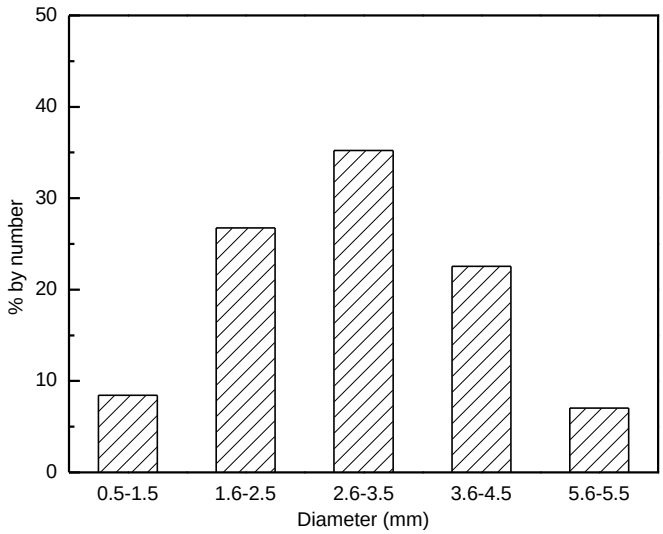

Figure 3.13. Particle size distribution of the nitritation-anammox granules by number

3.2.5 Microbial community composition of nitritation-anammox granules

Previous studies have investigated the effects of seed biomass and operating conditions on the formation and microbial population of aggregates (Vazquez-Padin et al. 2009b, Vlaeminck et al. 2010). The spatial distributions and activities of AOB and anammox bacteria in the aggregates from CANON reactor have been reported (Nielsen et al. 2005). Under oxygen-limiting conditions, researchers observed that anammox bacteria were present in the inner anoxic zone of biofilms or aggregates, while AOB survived in the outer aerobic zone (Kindaichi et al. 2007, Nielsen et al. 2005), possibly due to the DO concentration gradient created in the biofilms. Different types of microorganisms tend to predominantly grow in the specific spots with suitable conditions.

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Nitritation-anammox biomass was highly enriched in UASB #2 reactor by introducing limited amounts of oxygen. For one typical granule, anammox bacteria accounted for about 60% of total bacteria, while AOB accounted for 35%.

On day 201, granule samples were collected for analyzing microbial community composition. Figure 3.14 shows microbial community structure in FISH images using NSV443 and NSE1472 probes that target AOB, and Amx820 probe that targets anammox bacteria. As shown in those images, AOB and anammox bacteria co-existed in the same granule. Furthermore, they were overlapping each other. This is different from previous studies (Meng 2012, Okabe et al. 2011b). They found that anammox bacteria were present throughout the granules, while AOB were detected only in the granule surface and around the clusters of anammox bacteria. Though oxygen was supplied to the reactor, anammox bacteria were able to survive under the low DO conditions (below 0.5 mg/L). With such a distinctive and overlapping growth features, anammox can consume nitrite immediately as it is produced by AOB. In addition, the majority of microorganisms were observed on the outer layer of granule, where oxygen and substrates are more readily available. For instance, AOB and anammox bacteria compete for NH_4^+ since it is a co-substrate of the partial nitrification and anammox processes. The granules are porous which benefits substrate dispersion and oxygen diffusion. The spatial distributions of microorganisms would improve their competitive advantages. The hollow structure characteristic may be caused by N_2 release during anammox process. Anammox bacteria and AOB comprised approximately 90% of the population,

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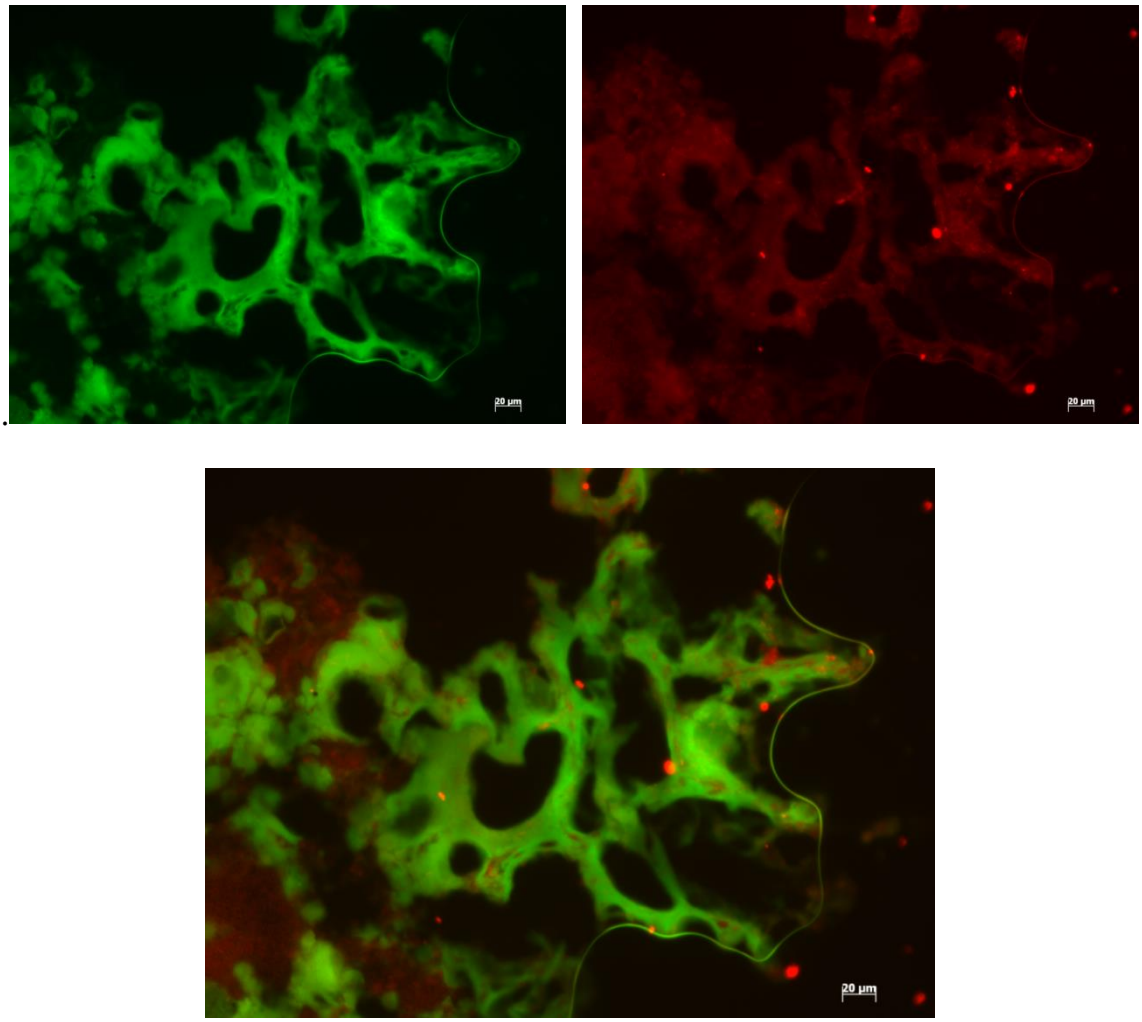
which was confirmed by counterstaining with DAPI (4, 6-diamidino-2-phenylindole) (data not shown).



(A)

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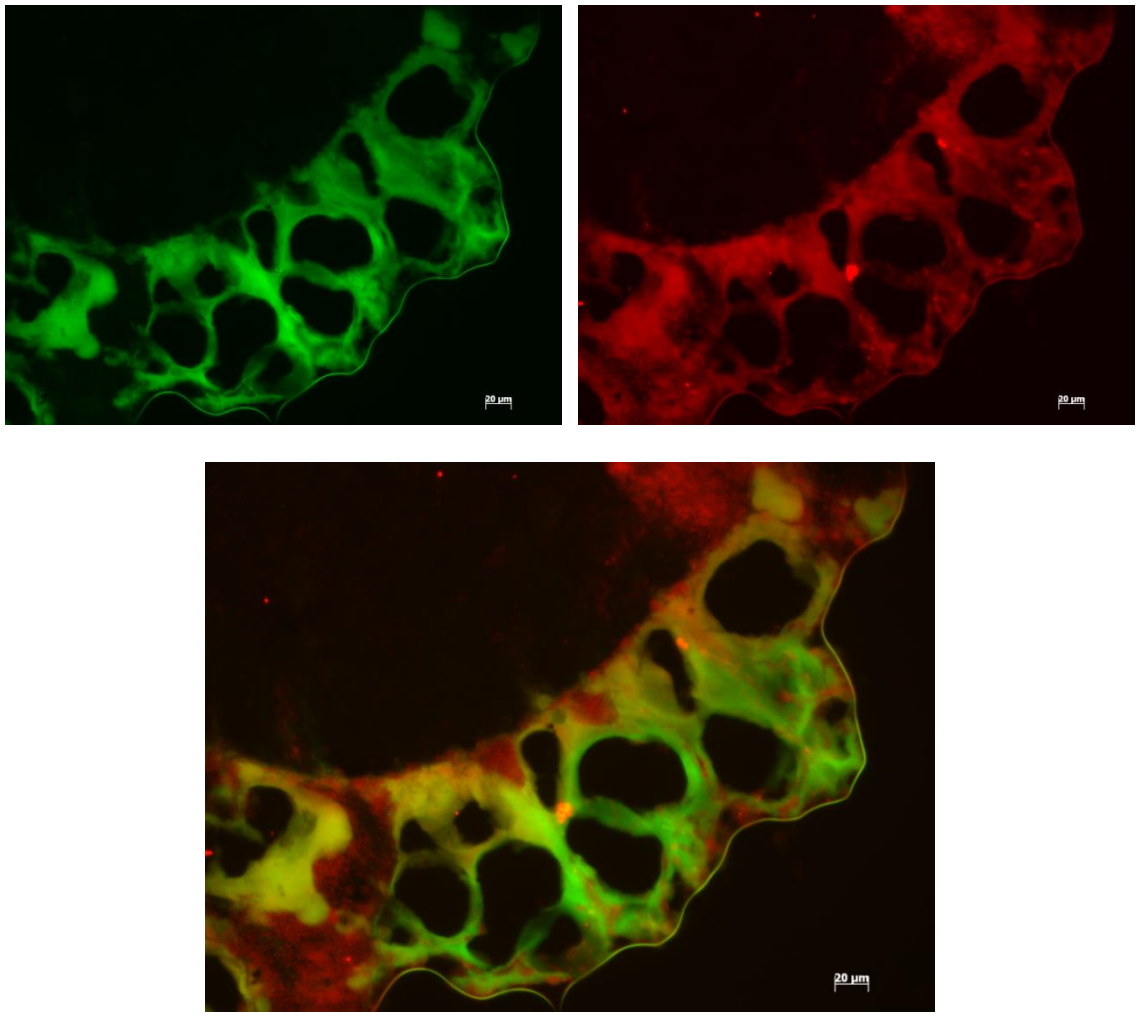
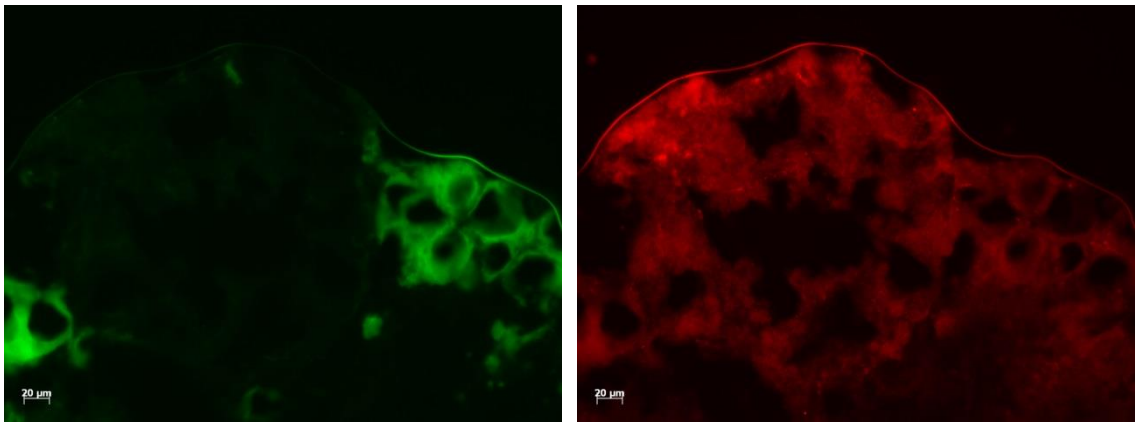


(B)



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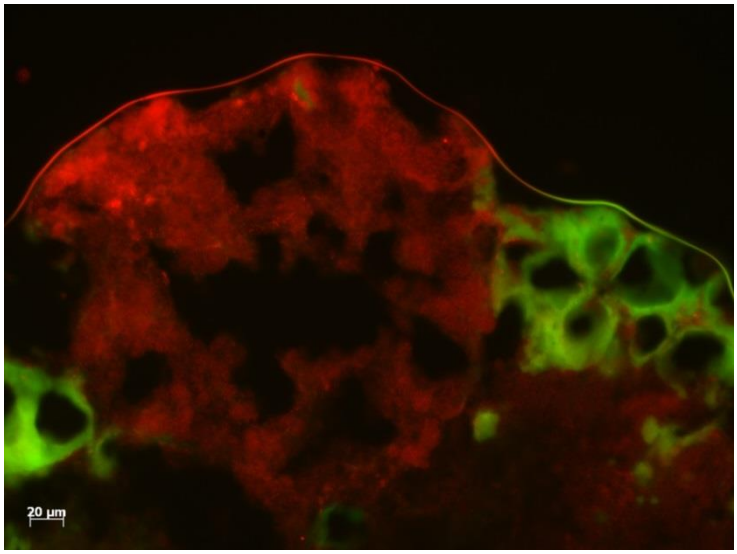
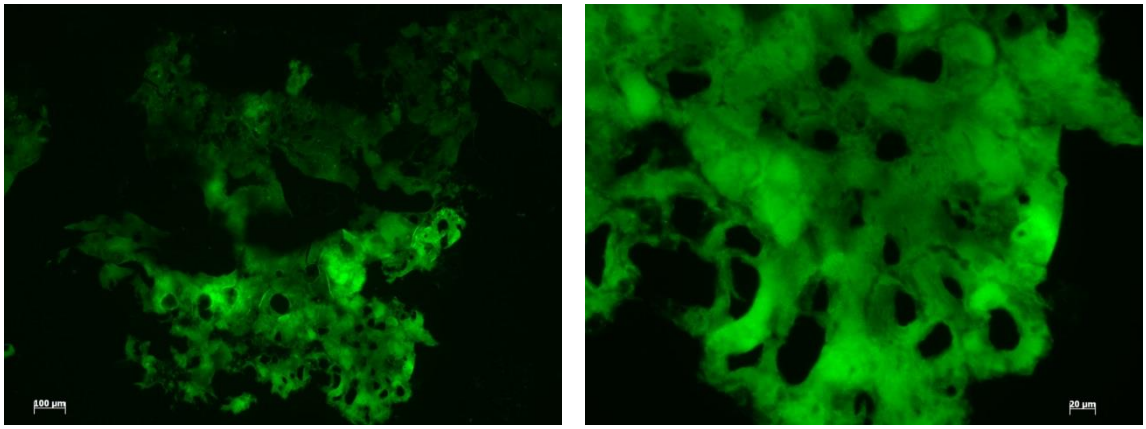


(C)

Figure 3.14. AMX820 probe targeting *Brocadia*- and *Kuenenia*- like anammox bacteria was labeled with TXRD (red). NSE1472 and NSV443 probes targeting *Nitrosomonas europaea* and *Nitrosospira* spp. were labeled with FLC (green).

(A), (B) and (C) represent the different zones in the same nitritation-anammox granule.



(A) (B)

Figure 3.15. FISH micrographs with FLC-labeled NSE1472 and NSV443.

FISH analysis of the granules from the nitritation-anammox reactor showed a clear increase of AOB population abundance after aeration.

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Figure 3.15 (A) shows that AOB were observed in a brown-yellow granule, which presumably contain abundant AOB. As expected, AOB were present throughout the sample, but more concentrated in the outer part of granule. The close-up image also showed an apparent hollow structure (Figure 3.15 (B)). The high enrichment of AOB resulted from application of high ammonium loading rate, high temperature, and absence of organic carbon in synthetic medium (Okabe et al. 2011a).

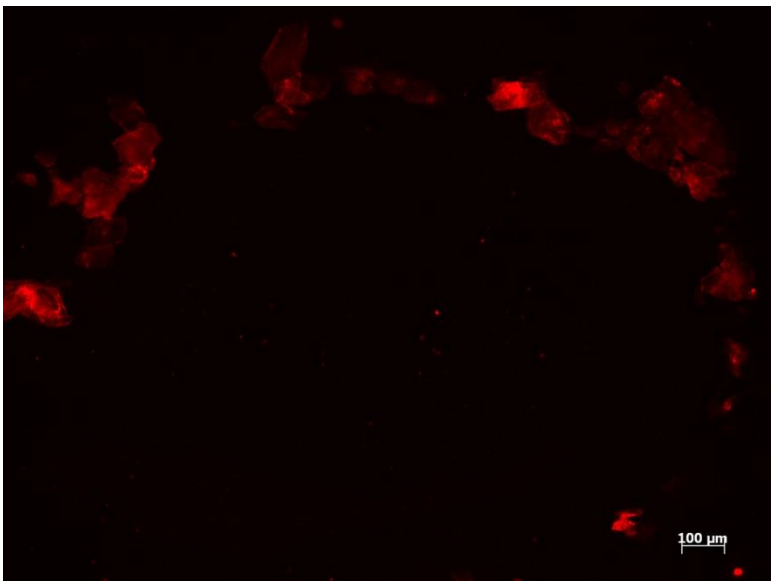

Figure 3.16. FISH micrograph with TXRD-labeled NIT3 (targeting *Nitrobacter* spp.).

NOB exhibited a lower affinity to nitrite than anammox bacteria when both nitrite and O_2 were limited. Thus, anammox bacteria over-competed NOB and consumed nitrite produced by AOB (Blackburne et al. 2008, Schramm et al. 2000). No hybridization signal was observed for most granules when *Nitrobacter*-specific probe NIT3 was used. Based on FISH results, the activity of NOB can be suppressed under low oxygen condition and therefore NOB did not likely contribute to nitrate formation. Figure 3.16

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shows the only granule sample with detectable *Nitrobacter*, which was present in the outer part of the granule, probably because oxygen is readily available. High AOB abundance in the granules without NOB accumulation was an important reason leading to stable long-term operation of the nitritation-anammox system.

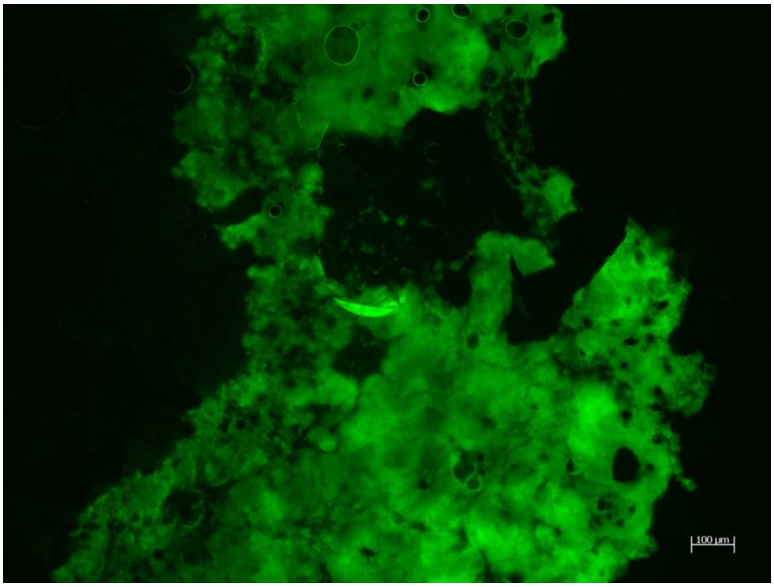

Figure 3.17. FISH micrograph with FLC-labeled ACI208 (targeting *Acidovorax spp.*).

Figure 3.17 shows a granule where denitrifying bacteria were detected. It is reasonable that denitrification process might take place in the reactor when nitrite/nitrate and organic carbon become available. Although no organic carbon source was provided in the feeding medium, cell lysis or decay may provide organic carbon as electron donor to convert nitrite or nitrate to N_2. That also partly explains the relatively low nitrate concentrations in the effluent.

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CHAPTER 4. CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

The excess nitrogen discharging into water bodies causes significant negative impacts on the environment and ecosystem, such as eutrophication, and toxicity to aquatic organisms. In contrast to conventional BNR process, the innovative autotrophic nitrogen removal process offers higher nitrogen removal efficiency, considerable savings in both energy and organic carbon consumptions, as well as the reduction of GHG emission and sludge production, especially for treating wastewater rich in ammonium but devoid of organic carbon. An efficient biomass retention is required for succeful start-up and operation of the innovative process due to the low growth rates of autotrophs (e.g., anammox bacteria, AOB). The UASB reactor provides a desirable condition for granulation, resulting in an excellent settling ability of biomass.

Two similar laboratory-scale UASB reactors have been developed for this research. The UASB #1 reactor was used to study the substrate conversion involved in the anammox process under complete absence of oxygen. The reactor was operated at 35±2 ℃ and with a hydraulic residence time (HRT) of 24 h. The ratio of influent NO_2^--N/NH_4^+-N was optimized and the optimal ratio was applied to evaluate long-term reactor performance under steady-state operating conditions. Under various ratios of influent NO_2^--N/NH_4^+-N, the ratios of observed NO_2^--N/NH_4^+-N were not in agreement with the proposed ratio of 1.32, but showed a positive correlation with influent ratios. Ammonium accumulation was observed when the reactor was fed with lower influent ratios (1 and 1.1), while nitrite and nitrate accumulations occurred when influent ratio

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was 1.31. Thus, the optimal influent ratio of NO_2^--N/NH_4^+-N in the UASB #1 reactor was 1.2, since it yielded the highest TN removal efficiency (on average 96%-97%) without accumulating any nitrogenous ions. Based on the substrates conversion and mass balance, an empirical equation which does not take account into cell synthesis was proposed as $NH_4^+ + 1.2NO_2^- + 0.08H^+ \rightarrow 1.04N_2 + 0.12NO_3^- + 2.04H_2O$ for UASB #1 reactor. The elemental formulas of the anammox granules obtained from the two operation scenarios were $CH_{1.7}O_{0.85}N_{0.15}S_{0.04}$ and $CH_{1.28}O_{0.74}N_{0.16}S_{0.02}$, which were different from the previously proposed $CH_2O_{0.5}N_{0.15}$.

The combined nitritation-anammox process was developed in a single-stage UASB #2 reactor seeded with anammox granules, inactive methanogenic granules and aerobic activated sludge. The strategy used in this study was to fix ammonium concentration to be 350 mg N/L (25 mM) but gradually reduce nitrite concentration from 420 mg N/L to 0 in the influent. Thus, the nitrogen loading rate decreased as the reduction of influent nitrite. The favorable conditions (e.g., high temperature, low oxygen level) were optimized to facilitate the synergistic effects on the growth of AOB and anammox bacteria but depress NOB activity. The start-up took longer than expected. It may be shortened by increasing oxygen level (below 0.5 mg/L) from the early stage to stimulate AOB growth. ORP was found to be a sensitive tool to monitor the oxidation-reduction conditions in the reactor.

The nitritation-anammox bioreactor was operated for over 250 days without nitrite/ammonium accumulation and was able to remove 90% of the supplied nitrogen loads. The nitritation-anammox granules were successfully enriched with different

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colors, such as brown-yellow, red, light red, reddish, etc., depending on the microbial community compositions. Granules with porous structure had a mean diameter of 3 mm and featured good settling ability (a high settling velocity of 100 m/h).

The microbial community composition in UASB #2 reactor was investigated by FISH, which showed the coexistence of AOB and anammox bacteria in the granules. The two groups of bacteria grew overlapping each other so that the nitrite produced by AOB can be consumed immediately by anammox bacteria. Most NOB were believed to be eliminated by application of high temperature and low oxygen level, which was also proved by FISH results.

4.2 Future Work

1) AOB and anammox bacteria have abilities to thrive at the temperature below 10 ℃ in the natural ecosystems (e.g. OMZs), indicating that both groups of microorganisms are capable of outcompeting NOB under low temperatures. Thus, investigation of the nitritation-anammox reactor performance under low temperatures is recommended.
2) N_2O emissions have been observed in nitritation-anammox systems under oxygen limiting-conditions. AOB were regarded as major contributors. Thus, the study of N_2O emission from the nitritation-anammox UASB reactor is needed.
3) Study of leftover nitrate removal with organic carbon oxidation by denitrification in the nitritation-anammox systems is worth to pursue since denitrifiers have been detected in the granules in this study.
4) Further studies are warranted to determine the feasibility of the application of such a process at pilot-scale and full-scale and its application to real wastewater.

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APPENDIX A. SUPPLEMENT TABLES

Table A.1. Influent substrate compositions for determination of the optimal ratio of influent ammonium-N to nitrite-N in UASB #1 reactor.

Set	Substrate	NH_4-N/NO_2-N	g/L	mmol N/L	mg N/L
I	$(NH_4)_2SO_4$		1.98	**30**	420
	$NaNO_2$	1.31	2.712	39.3	550.2
		1.2	2.484	36	504
		1.1	2.277	33	462
II	$(NH_4)_2SO_4$		2.31	**35**	490
	$NaNO_2$	1.31	3.164	45.85	641.9
		1.2	2.898	42	588
		1.1	2.657	38.5	539
		1	2.415	35	490

Table A.2. Influent substrate compositions for evaluation of the long-term performance under steady-state conditions in UASB #1 reactor.

Scenario	Period	$(NH_4)_2SO_4$	$NaNO_2$	TN Loading Rate
	days	mg N/L		$kg/m^3/d$
I	126	420	490	0.92
II	80	504	588	1.08

Table A.3. Influent substrate compositions for cultivation of the AOB and anammox bacteria in UASB #2 reactor during Phase II.

Substrate	NH_4-N/NO_2-N	g/L	mmol N/L	mg N/L
$(NH_4)_2SO_4$		1.65	**25**	350
$NaNO_2$	1.2	2.070	30	420
	1.1	1.898	27.5	385
	1	1.725	25	350
	0.9	1.553	22.5	315
	0.8	1.380	20	280
	0.7	1.208	17.5	245
	0.6	1.035	15	210
	0.5	0.863	12.5	175
	0.4	0.690	10	140
	0.3	0.518	7.5	105
	0.2	0.345	5	70
	0.1	0.173	2.5	35
	0	0.000	0	0

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APPENDIX B. SUPPLEMENT FIGURES

Figure B.1. Mature anammox granules in UASB #1 reactor on Day 66 under steady state operating condtion (Influent NH_4^+-N=35 mM).

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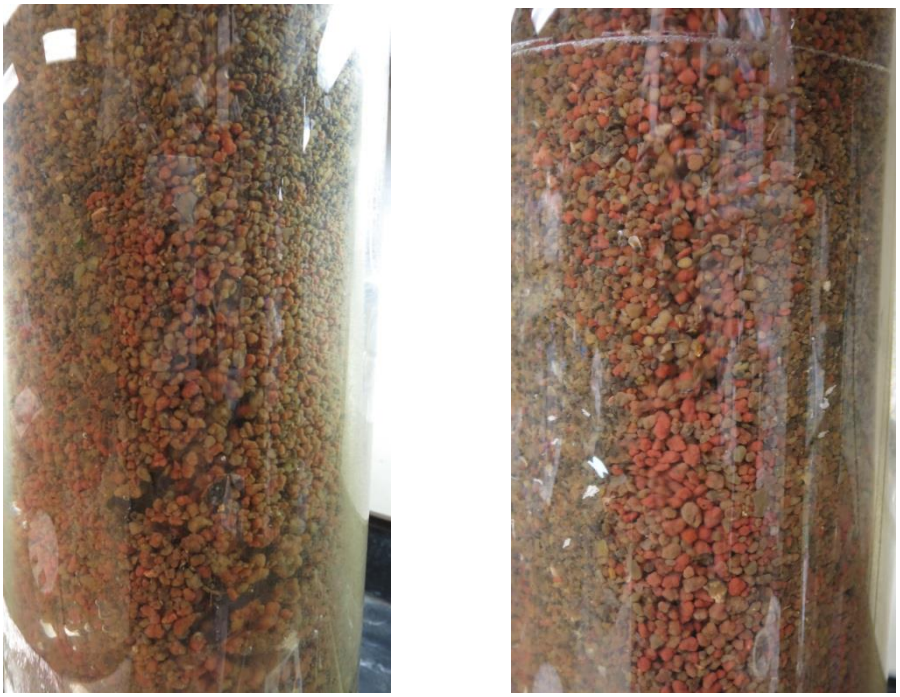
Day 76 Day 170 Day 205



Day 224 Day 246

Figure B.2. Color changes of granules in UASB #2 reactor.

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Figure B.3. Granules in UASB #2 reactor.

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APPENDIX C. LIST OF ABBREVIATIONS

Anammox	anaerobic ammonium oxidation
ANME-D	anaerobic methane oxidation coupled to denitrification
ANMR	anammox non-woven membrane reactor
AOB	aerobic ammonium-oxidizing bacteria
BNR	biological nitrogen removal
CANON	completely autotrophic nitrogen removal over nitrite
COD	chemical oxygen demand
CSTR	completely stirred tank reactor
CTR	column type reactor
DAPI	4, 6-diamidino-2-phenylindole
DEAMOX	denitrifying ammonium oxidation
DEMON	the pH-controlled deammonification system
DO	dissolved oxygen
EPA	environmental protection agency
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
GHG	greenhouse gas
GLR	gas-lift reactor
HAO	hydroxylamine oxidoreductase
HDH/HZO	hydrazine dehydrogenase/oxidoreductase
HRT	hydraulic retention time
HZS	hydrazine synthase
LDPE	low density polyethylene
MABR	membrane aerated biofilm reactor
MBBR	moving bed biofilm reactor
MLSS	mixed liquor suspended solid
MLVSS	mixed liquor volatile suspended solid
MMTCO$_2$E	million metric tons carbon dioxide equivalent

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MSBR	membrane sequencing batch reactor
n-damo	denitrifying methanotrophic bacteria
NirS	nitrite reductase
NLR	nitrogen loading rate
NOB	Nitrite-oxidizing bacteria
NRBC	non-woven rotating biological contactor
NRR	nitrogen removal rate
OCT	optimal cutting temperature
OLAND	oxygen-limited autotrophic nitrification-denitrification
OMZ	oxygen minimum zone
ORP	oxidation reduction potential
PBS	phosphate-buffered saline
PN	partial nitrification
PVC	polyvinylchloride
ROC	rotating biological contactor
SBR	sequencing batch reactor
SHARON	single reactor system for high-rate ammonium removal over nitrite
SMP	soluble microbial product
SNAD	simultaneous partial nitrification, anammox and denitrification
SNAP	single-stage nitrogen removal using anammox and partial nitritation
SRT	sludge residence time/solids retention time
TEA	terminal electron acceptor
TGA	thermogravimetric analyzer
TN	total nitrogen
UAGSB	upflow anammox granular sludge bed
UASB	upflow anaerobic sludge blanket
UFFBB	up-flow fixed-bed biofilm reactor
WWTP	wastewater treatment plant

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