2012

Nitrous oxide emission in Anammox reactor

Jun Meng
Iowa State University

Nitrous oxide emission in Anammox reactor

by

Jun Meng

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Civil Engineering (Environmental Engineering)

Program of Study Committee:

Shihwu Sung, Major Professor
James Alleman
Zhiyou Wen

Iowa State University

Ames, Iowa

2012

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I am tremendously fortunately to have had the opportunity to work with Shihwu Sung on the idea of this research and thesis. I would like to thank him and his research team for the support in the past few years. Shihwu kindly invited me to his research team when I was an undergraduate school student, and later on agreed to take me on as his graduate student, and encouraged me to immerse myself in something I had a passion for. In many ways he opened my eyes to see things that exceeded my vision. I appreciate all the opportunities he has had provided me. I could not have asked for a better mentor.

I am grateful to James Alleman and Zhiyou Wen for serving on my orals committee member. James has been a great source of encouragement before, during, and after my time at Iowa State University.

I would also like to express my gratitude to Iowa State University and Department of CCEE for all the opportunities that they have given me over the years.

I would like to thank all my wonderful people at the Microscopy and Nano Imaging Facility. I am very grateful for Tracey Pepper and Harry Horner's generous help on setting up FISH lab and hands on tutorial with great encouragement and patient advice. Also, thanks to Water Environmental Federation ISU Student Chapter, which allowed me to serve as the president in the past year. It is where I learned so much about leadership, responsibility, as well as the knowledge inside and outside of water research.

I would especially like to thank my collaborators: Kanyarat Saritpongteeraka and Shou-qing Ni.

I would also like to thank my friends outside the lab who made my life more enjoyable. Thanks to Tianming Chen and Zhiyuan Jin, who has been great friends and provided guidance and encouragement in my life. Thanks to my dear friends, Kanyarat Saritpongteeraka and Ya Channok, who provided me countless care and new perspective of life. Thanks to Brian Cheney, who showed me the purpose of life and helped me practice English so I can reach out to endless new opportunities but most importantly, to be able to finish this thesis.

Finally, I would like to thank my family, Juru Gao and Tinghui Meng, for their love and support. My parents have supported me emotionally and financially to provide steady guidance and encouragement. This dissertation is dedicated to them.

ABSTRACT

Nitrous oxide emission has been paid more attention in the past few years due to its significant greenhouse effect. Nitrous oxide is a powerful greenhouse gas and its concentration in the atmosphere kept climbing at a constant rate. Nitrous oxide emission from wastewater treatment plant is considered as a major source of anthropogenic input. Conventional nutrient removal processes such as nitrification and denitrification produce nitrous oxide as intermediate or side-product. New treatment technology-Anammox is a promising process for nitrogen removal due to its low energy consumption and high removal efficiency. While being able to significantly decrease carbon dioxide emission, its potential of eliminating nitrous oxide emission has not been studied carefully. In the study, an UASB Anammox reactor was built to investigate nitrous oxide emission source from the reactor. Shock-loading and dissolved oxygen level could affect nitrous oxide concentration in the off-gas. Enrichment of biomass under strict dissolved oxygen control significantly brought nitrous oxide production down from 400 ppm to 5 ppm. With evidence from fluorescence in situ hybridization lab, nitrifier denitrification could be the source of nitrous oxide emission. In the end of study, the average emission of nitrous oxide was only 0.07 % of recovered nitrogen.

CHAPTER 1. INTRODUCTION

1.1 Background information

Nutrient removal in wastewater before discharging to natural stream has been paid more attention in the past few decades. Discharging excess nutrient produced from human activity brings significant negative impacts, such as eutrophication in lakes, streams, rivers, and coastal areas. In wastewater, ammonia is a major nutrient that needs to be removed before effluent discharge.

Conventional biological nutrient removal (BNR) process usually consists of two steps, nitrification and denitrification. Nitrification is an ammonia oxidation process where ammonia is oxidized to nitrite and nitrate by a group of chemoautotrophic bacteria under aerobic condition. During this process, large amount of oxygen (air) is required to be introduced to the system by intensive mechanical aeration, which alone consumes a large amount of energy, accounting for 50-60% of the electricity usage by the facility. In United States, nearly 4% of the nation's electricity use goes towards moving (80%) and treating water/wastewater (EPRI, 2002). Nitrification is then followed by denitrification process where nitrate and nitrite get reduced to nitrogen gas by a large group of bacteria that uses nitrite or nitrate as alternative electron acceptors under anoxic condition. In order to achieve complete denitrification, external organic carbon (e.g., methanol) addition is commonly practiced due to low BOD/TKN ratio in most. Both nitrification and denitrification process can be costly because of intensive mechanical aeration and external carbon addition. Excessive sludge production also increases operation cost.

1.2 Greenhouse gas emission from wastewater treatment

Since greenhouse gas emissions are associated with conventional BNR process, nitrous oxide emission has been paid attention in the past few years due to its increasing role in global warming. Nitrous oxide (N_2O), a strong greenhouse gas, possesses 300 fold global warming potential compared to carbon dioxide (CO_2) based on the 100-year global warming potential (IPCC 2007). It also has long lifetime of approximately 120 years (Solomon 2007). In some treatment plants, nitrous oxide emission can reach up to 80% of the operational CO_2 footprint (Desloover 2012).

Preindustrial value of tropospheric nitrous oxide concentration was about 270 ppb. In 2007, this value has increased to about 314 ppb, indicating 0.2-0.3% increases per year (Solomon 2007). According to study provided by the United States Environmental Protection Agency (USEPA), the US nitrous oxide emissions from human sewage treatment in 1990 were estimated at 3.7 TgCO2 (teragrams of CO_2) Equivalents. In 2010, it has increased by over 30%, reached to 5.0 TgCO2 Equivalents (USEPA 2010). Data released from Intergovernmental Panel on Climate Change (IPCC) indicates that global anthropogenic nitrous oxide emission reached 17.7 Tg N $year^{-1}$ in 2004, accounting 7.9% of global anthropogenic GHG emissions (IPCC 2007).

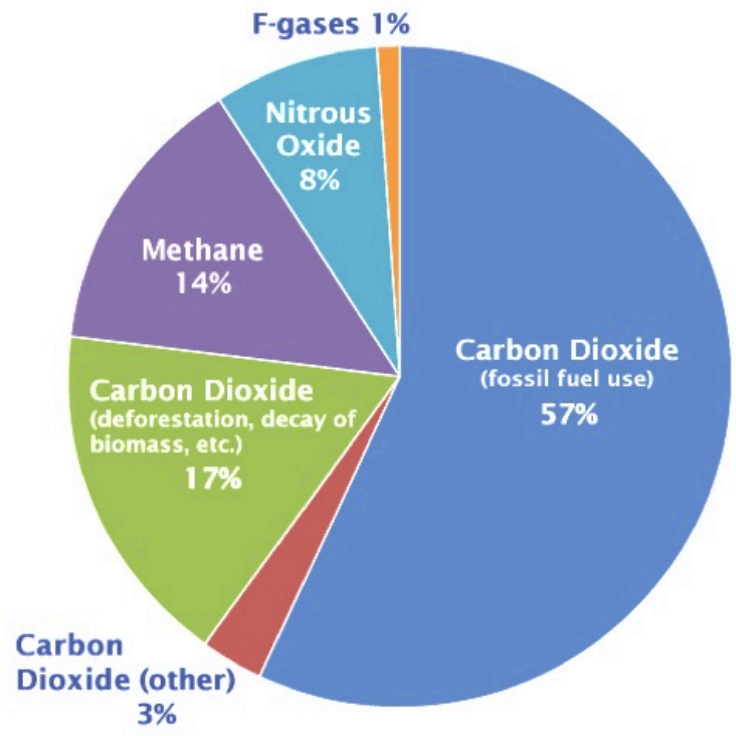

Fig.1 Global greenhouse gas emissions by gas (IPCC 2007)

According to 2010 NOAA (National Oceanic and Atmospheric Administration) Annual Greenhouse Gas Index (AGGI), of the five long-lived greenhouse gases (Fig. 1) that contribute 96% to radiative climate forcing, CO_2 and N_2O are the only ones that continue to increase at a regular rate (National Oceanic and Atmospheric Administration 2010).

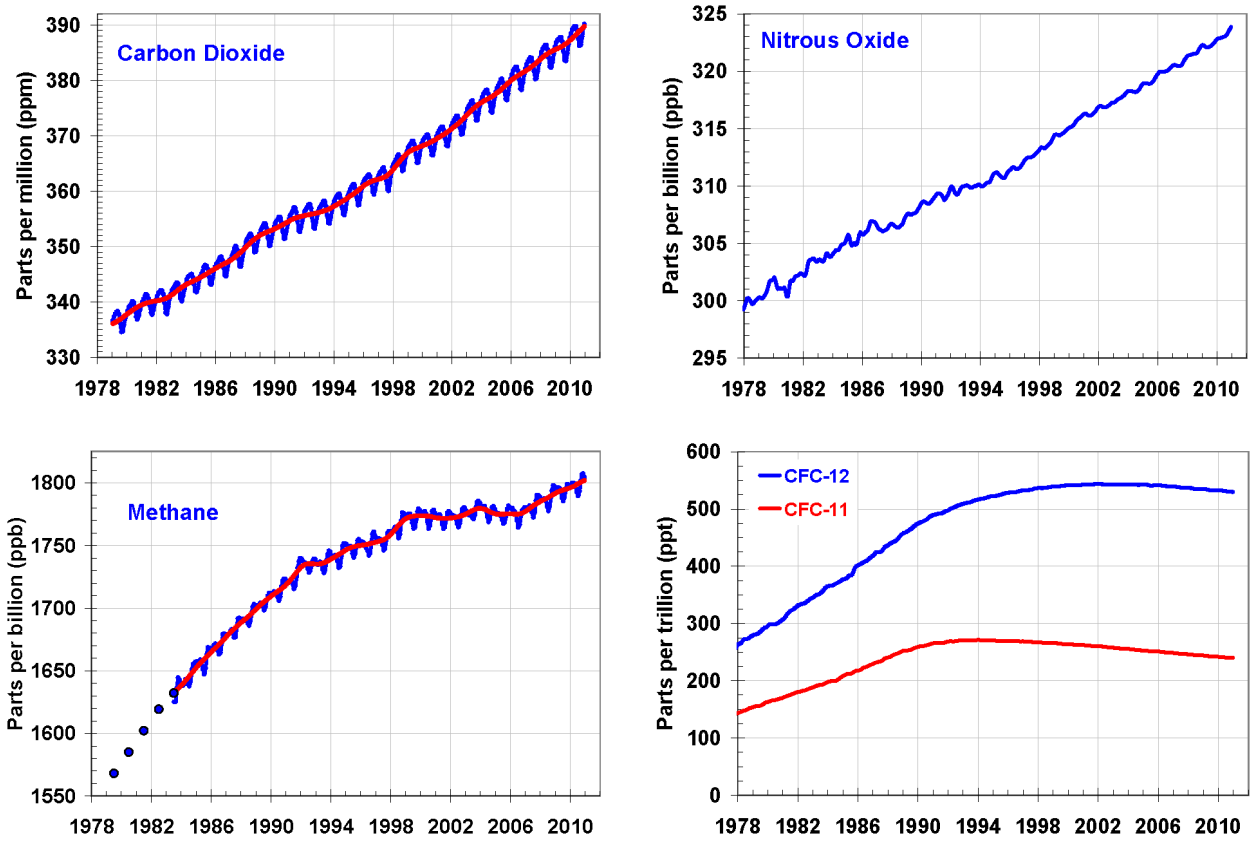

Fig. 2 Global average abundances of the major, well-mixed, long-lived greenhouse gases, including carbon dioxide, methane, nitrous oxide, CFC-12 and CFC-11 (National Oceanic and Atmospheric Administration 2010)

Since nitrous oxide has 300 fold global warming potential compared to carbon dioxide (CO_2) based on the 100-year global warming potential, the 24 ppb increase of N_2O concentration in the atmosphere from 1978 to 2010 is equivalent to about 7.2 ppm of CO_2 increase in the atmosphere, accounting 13.3% of global warming effect caused by the increase of CO_2. Therefore, N_2O emission is an important player in the global warming, and proper control of N_2O emission is necessary.

1.3 Anammox – an effective nutrient removal process with minimum environmental impact

Recently, a novel nitrogen removal process named Anaerobic Ammonium Oxidation (Anammox) has been put into practice to treat ammonium-rich wastewater. Anammox microorganisms create a shortcut in the nitrogen cycle to remove nitrogen in the wastewater. Instead of going through nitrification and denitrification, ammonium can be directly oxidized to nitrogen gas with the presence of nitrite as electron acceptor. Thanks to this new technology, intensive aeration and external organic carbon (methanol) addition in conventional BNR process can be eliminated. This process only requires the conversion of 50% of the ammonium to nitrite resulting in the reduced need for aeration, thus saving energy. Energy consumption can be reduced by 60%, resulting in significant savings in operation costs (Abma 2007). Also, Anammox is able to reduce carbon dioxide emissions by up to 90% compared to conventional nitrification/denitrification processes. It also occupies up to 50% less space and reduces aeration energy by up to 60% (Jettena 2001).

Since Anammox bypassed nitrification and denitrification route, we can expect significant less N_2O emission compared to conventional BNR process. In order to investigate possible elimination of nitrous oxide production from Anammox process, an UASB Anammox reactor was developed and cultivated to handle high nitrogen load. In the end of study nitrogen loading rate (NLR) was up to of 0.6 kg-N/d/m^3. Nitrous oxide emission was monitored along with adjustment of operating condition. Fluorescence in situ hybridization (FISH), a cytogenetic technique was used to determine microorganism composition in the Anammox granule.

CHAPTER 2. LITERATURE REVIEW

Nutrient removal of wastewater usually consists of nitrification process followed by denitrification process, which is the most common BNR process. Newer BNR process such as Sharon-Anammox has been put into practice in several places in the world, such as two full-scale plants in the Netherlands. Nitrification is the process by which ammonium (NH_4^+-N) is oxidized first to nitrite (NO_2^--N) by ammonium oxidizing bacteria (AOB), and then to nitrate (NO_3^--N) by nitrite oxidizing bacteria (NOB). Denitrification is the process by which nitrate (NO_3^--N) or nitrite (NO_2^--N) get reduced to dinitrogen gas (N_2) through series intermediate nitrogen oxide products such as NO and N_2O. Sharon process (aka. nitritation) use temperature (35˚C) and alkalinity as selection pressure for enrichment of AOB and elimination of NOB in order to achieve partial nitrification. In this process, 50% of ammonium in the wastewater gets oxidized to nitrite and no further oxidation will occur. Sharon process is then followed by Anammox process where certain groups of bacteria convert nitrite and the remaining 50% of ammonium to nitrogen gas. This sustainable process has been put in a lot of efforts worldwide by industry leaders and researchers due to its significant potential to achieve high nutrient removal capability and energy saving.

Nitrous oxide emission can be detected during nitrogen removal at WWTPs. Ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), and denitrifying microorganisms are responsible for N_2O emission (Kampschreur 2009). Various operational parameters such as dissolved oxygen (DO) concentration, pH, nitrite

concentration in both nitrification and denitrification stage and carbon availability in denitrification stage affect N_2O turnover (Okabea 2011). Anammox's environmental impact, such as nitrous oxide emission, on the other hand, has only been probed by a few numbers of researchers. Due to many advantages of Anammox process over traditional BNR process, especially when it comes to environmental impact, nitrous oxide emission needs to be studied further. From this study, we can obtain a more complete picture of the next-generation BNR process.

2.1 The complete nitrogen cycle

The nitrogen cycle was generally believed to be complete when denitrification and nitrification process was confirmed in 1882 and 1890, respectively (Strous 2004). However in 1995, Mulder *et al.*, started up a 23 L capacity fluidized bed reactor for treating bakery yeast wastewater effluent in the Netherland, and found that nitrate and ammonium disappeared at the same time in the reactor (Mulder 1995). Since nitrification and denitrification could not destruct ammonium in anoxic condition except assimilation, this finding interested researchers to do an in-depth study.

An article published by Broda *et al.* predicted that there are two lithographs missing in the nature. One of them was reported to utilize ammonium as electron donor and nitrite as electron acceptor to form nitrogen gas (Broda 1977). The difference here is that whether nitrite or nitrate was electron acceptor for the reaction. Later on, Graaf *et al.* successfully demonstrated the anaerobic ammonium oxidation using nitrite as electron acceptor not nitrate by using a fluidized bed reactor and introducing $^{15}NH_4^+$ and $^{14}NO_2^-$

as tracers (van de Graff 1995). Mulder *et al.*'s study was later proved nitrate was first reduced to nitrite and react with ammonium to proceed with Anammox process. Based on these discoveries, the nitrogen cycle was revised as shown in Fig. 3.

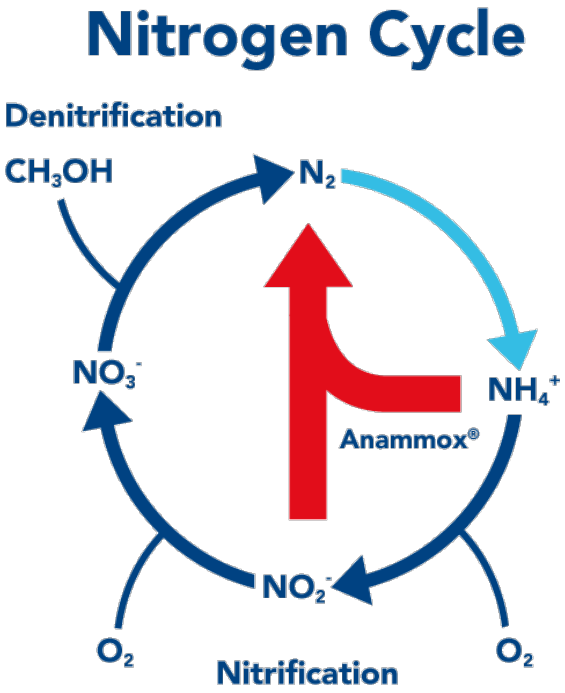

Fig. 3 Complete nitrogen cycle (PAQUES 2011)

Based on mass balance, Stous *et al.* formulated a complete metabolic equation for Anammox reaction (van de Graff 1995). $CH_2O_{0.5}N_{0.15}$ was found to be the protein content and elemental composition. The stoichiometry of Anammox is illustrated in eq. (1).

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13H^+ \rightarrow 1.02\ N_2 + 0.256\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \quad (1)$$

From the stoichiometry of Anammox, we can see that the biomass yield is very low, suggesting long cultivation/start up time and low excess sludge production. Second, the reaction use inorganic carbon source, meaning that this process absorbs carbon instead of producing carbon dioxide. Third, nitrite is not only electron accepter but also donor. Part of nitrogen in nitrite gets oxidized to nitrate to provide energy for biomass assimilation.

2.2 Nitrous oxide production from AOB organism

Complete nitrification involves Ammonia oxidizer (AOB) and nitrite oxidizer (NOB) (Bock 1986). AOB belong to the genera *Nitrosomonas*, *Nitrosococcus*, *Nitrosopira*, *Nitrosovibrio*, and *Nitrosolobus*. *Nitrobacter* is the representative of the NOB (Wrage, 2001). These nitrifying organisms are chemoautotrophs, and use carbon dioxide as their carbon source for growth. Previous research has shown that AOB can produce NO and N_2O either as a side-product in the catabolic pathway, as known as nitritation, which is the very first step towards nitrogen removal of wastewater treatment process. Nitritation (see figure below) is the partial oxidation process of ammonium (NH_4^+) or ammonia (NH_3) in the wastewater to nitrite (NO_2^-).

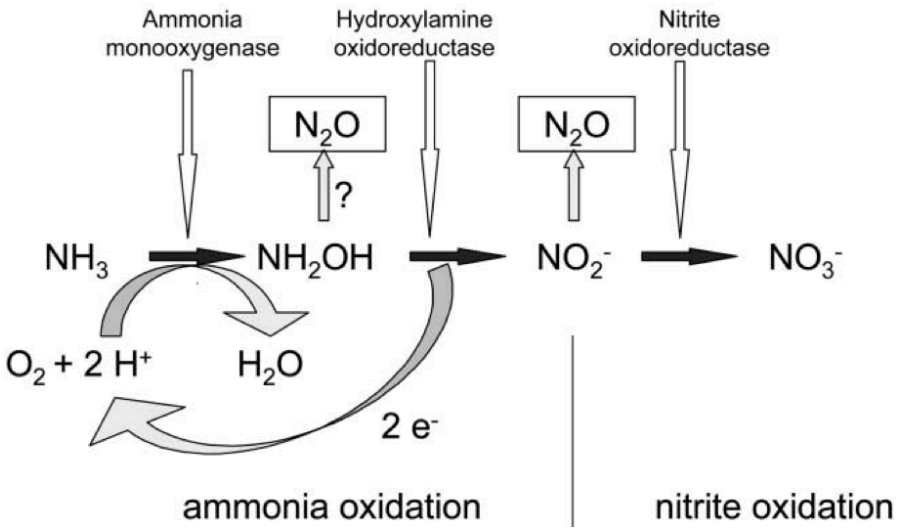

Fig. 4 Nitrification: outline of the pathway and enzymes involved (Wrage 2001)

The first intermediate in nitrification is hydroxylamine (NH_2OH). The oxidation of NH_3 to NH_2OH is catalyzed by ammonia monooxygenase (Wood 1986). Monooxygenases are enzymes that incorporate one hydroxyl group into substrates in many metabolic pathways. Fig. 4 shows that one of the atoms of O_2 is reduced by using two electrons produced from the next step, the oxidation of NH_2OH to NO_2^- (Hollocher 1981). Then, the oxidation of NH_2OH is mediated by the enzyme hydroxylamine oxidoreductase. In biochemistry, an oxidoreductase is an enzyme that catalyzes the transfer of electrons from one molecule to another. Hydroxylamine oxidoreductase is a potential source of nitrous oxide emission (Hooper 1979). Next, NOB further oxidizes NO_2^- to NO_3^- in a one-step reaction (Wrage 2001). The catalyzer involved in this reaction is hydroxylamine oxidoreductase (Nicholas 1960).

Alternately, AOB produce NO and N_2O by denitrification of nitrite with ammonia, hydrogen or pyruvate as electron donor (Colliver 2000) (Schmidt 2004). This pathway

is called nitrifier denitrification. The denitrifying pathway of AOB would yield N_2O only, and this route is often linked to low oxygen levels (Poth 1985). Under very low DO concentration, nitrifier may not be enriched, but they can at least survive since they have anaerobic metabolism (Schmid 2000). Compared to Anammox, they have more versatile metabolism. The highest anaerobic ammonium oxidizing activity of AOB is 25 times lower than that of Anammox (55 nmol NH_4^+-N (mg protein)$^{-1}$ min^{-1}) (Kuenen 2001), but enough to survive (Liu 2009).

Chemical decomposition of intermediate between NH_4^+ and NO_3^-, such as NH_2OH and NO_2^- can turn over N_2O (Wrage 2001). Formation of N_2O from incomplete oxidation of NH_2OH was also realized in the early study (Hooper 1979). Therefore, we can nitritation (partial nitrification, from NH_4^+ to NO_2^-) could also be a source of N_2O production.

2.3 Nitrous oxide production from denitrifying organism

Denitrification is a microbiological process where NO_2^- and NO_3^- get reduced to dinitrogen gas (N_2) through series intermediate nitrogen oxide products such as NO and N_2O, as they are in the catabolic respiratory pathway. It is primarily carried out by a large group of heterotrophic bacteria (such as *paracoccus denitrificans* and various *pseudomonads*) that use NO_2^- or NO_3^- as an alternative electron acceptor when oxygen concentration is low (Carlson 1983).

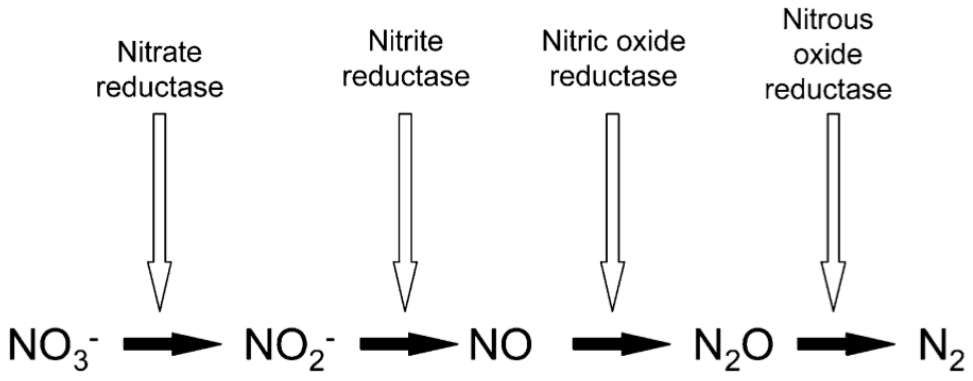

Fig. 5 Denitrification: outline of the pathway and enzymes involved (Wrage 2001)

The biological process need reduced carbon source as electron donor, such as external organic carbon (e.g., methanol), which is commonly practiced in BNR plants in order to achieve complete denitrification. Incomplete denitrification could possible yield N_2O only. Some previous studies showed that N_2O production during denitrification was only registered in the absence of dissolved organic matter and the presence of nitrite or low DO (Hanaki 1992) (Itokawa 2001).

2.4 Nitrous oxide emission from Anammox process

Anammox (anaerobic ammonium oxidization) is a recently discovered nitrogen removal process. Ammonium is oxidized directly into nitrogen gas using nitrite as an electron acceptor with stoichiometric ratio of the Anammox reaction between ammonium, nitrite, and nitrate, is 1:1.31:0.22. Most of the ammonium is converted into nitrogen gas, bypassing the oxidation process to form nitrate. Due to its autotrophic property, this process requires no external carbon source. Anammox is usually combined with partial

nitrification process which partially pre-oxidizes ammonium in the wastewater to nitrite by AOB prior to the entry of Anammox reactor. The combined process can provide a substantial reduction in energy use, which was estimated to save up to 62.5% of air supply.

Up to date, researchers rarely find Anammox bacteria yield N_2O, but it was observed in the off-gas produced from both lab scale and full scale Anammox reactors. The potential of N_2O emission by Anammox is unknown. However, low levels of NO and N_2O was detected in the off-gas from Anammox enrichment (Strous 1999). It was unclear whether it was due to Anammox or by other bacteria in the community. Research conducted by Kartal *et al.* showed that physically purified Anammox cells (purity higher than 99.9%) did not turn over N_2O (Kartal 2007).

In full-scale single-stage partial nitrification-Anammox reactor treating potato processing factory wastewater and reject water of a municipal sludge dewatering plant, N_2O production was 1.2% of the total nitrogen load (Kampschreur 2009). In this study, N_2O production was only 0.07% of the total recovered nitrogen at steady state. This result is much higher than the emission of lab-scale Anammox enrichment reactors. In a study by Strous *et al.,* the sequencing batch Anammox reactor showed 0.03-0.06% N_2O yield of the total nitrogen load (Strous, 1998). In a study by Van de Graaf *et al.*, the fluidized bed reactor (FBR) Anammox reactor showed less than 0.1% N_2O production of the total nitrogen load (van de Graaf 1997). In a study by Wyffels *et al.,* two-stage oxygen-limited autotrophic nitrification denitrification process showed less than 0.1% N_2O turnover of the total nitrogen load (Wyffels 2004).

2.5 The goal of this study

Key factors leading to N_2O emission during nitrogen removal from wastewater were reported are low dissolved oxygen (DO), presence of nitrite, low chemical oxygen demand (COD), as well as short solid retention time. In order to develop a low greenhouse gas emission nitrogen removal process, source of N_2O need to be further investigated and eventually be able to help to find best way to manage N_2O emission in the operation of wastewater treatment plants. According literature study, nitrifier denitrification could be the most probable source of N_2O emission in Anammox reactor. In order to characterize N_2O emission from Anammox granule, a lab-scale UASB reactor was developed to investigate key player in the bacteria community that are responsible for N_2O emission. During the study, Anammox biomass was enriched with synthetic wastewater and N_2O concentration in the off-gas was monitored alongside. FISH technique was used to identify bacteria and to verify hypothesis.

CHAPTER 3. MATERIAL AND METHODS

3.1 Anammox reactor

An up-flow granular-sludge Anammox reactor with working volume of 3.5 L (height: 1.10 m, diameter: 0.10 m) has been steadily operated for more than 2 years at low nitrogen load. Inactive methanogenic granules from full-scale UASB reactor (1.5 L) and Anammox sludge (50 mL) were used to start up in our previously laboratory. The very initial substrate concentration for startup was 134 mg NH_4^+–N/L and 145 mg NO_2^-–N/L. The nitrogen loading rate increased gradually from 140mg/L/d to 480 mg/L/d after 120 days of inoculation, while achieving average ammonium and nitrite removal efficiencies of 95.8 ± 1.1% and 98.8 ± 0.7%, respectively. Previous Real-time PCR showed over 67% of the cells in the red Anammox granules were Anammox bacteria (Ni 2010). Prior to this study, the reactor had been running for more than 6 months under relatively low nitrogen concentration. Ammonia and nitrite concentration were about 46.72 mg NH_4^+–N/L and 61.67 mg NO_2^-–N/L, respectively. Prior to this study, dissolved oxygen in the feed was only controlled by deoxygenating with argon gas before feeding. Synthetic wastewater container was not sealed and oxygen might be able to re-dissolve in the substrate after deoxygenating.

3.2 Reactor set up and operation

The configuration of the Anammox reactor is shown in Fig. 6. To maintain proper temperature for best Anammox growth, the integrated water jacket was connected to a

water bath allowing constant warm water (35˚C) to recirculate through the reactor. The glass funnel on top would collect gas produced from Anammox reaction and allowing liquid to flow while maintaining bio-solids in the reactor. The reactor was fitted with an influent/recirculation port on the bottom, an effluent and a recirculation port on top, as well as a sampling port in the middle section. Gravel with different sizes (2, 5, and 10 mm) was placed in the lowest portion of the reactor for better wastewater distribution and biomass retention. The reactor was continuously fed with synthetic wastewater by peristaltic pumps (MasterFlex, Cole-Parmer Instrument, Vernon Hills, IL, USA). The wastewater was stored in a gas tight collapsible LDPE container (Cole-Parmer Instrument, Vernon Hills, IL, USA), which avoids substrate from oxygen transmission from headspace to maintain a controlled DO concentration. Treated wastewater from top of the reactor was recycled back to the influent port at a ratio of 1000% based on the influent flow rate, which provides good Anammox granule expansion as well as dilution to avoid high-level nitrite inhibition (Strous 1999). All tubing used was made of black butyl rubber to prevent light transmission and air permeability. Off-gas collected from the reactor is connected to a gas meter for quantification measurement.

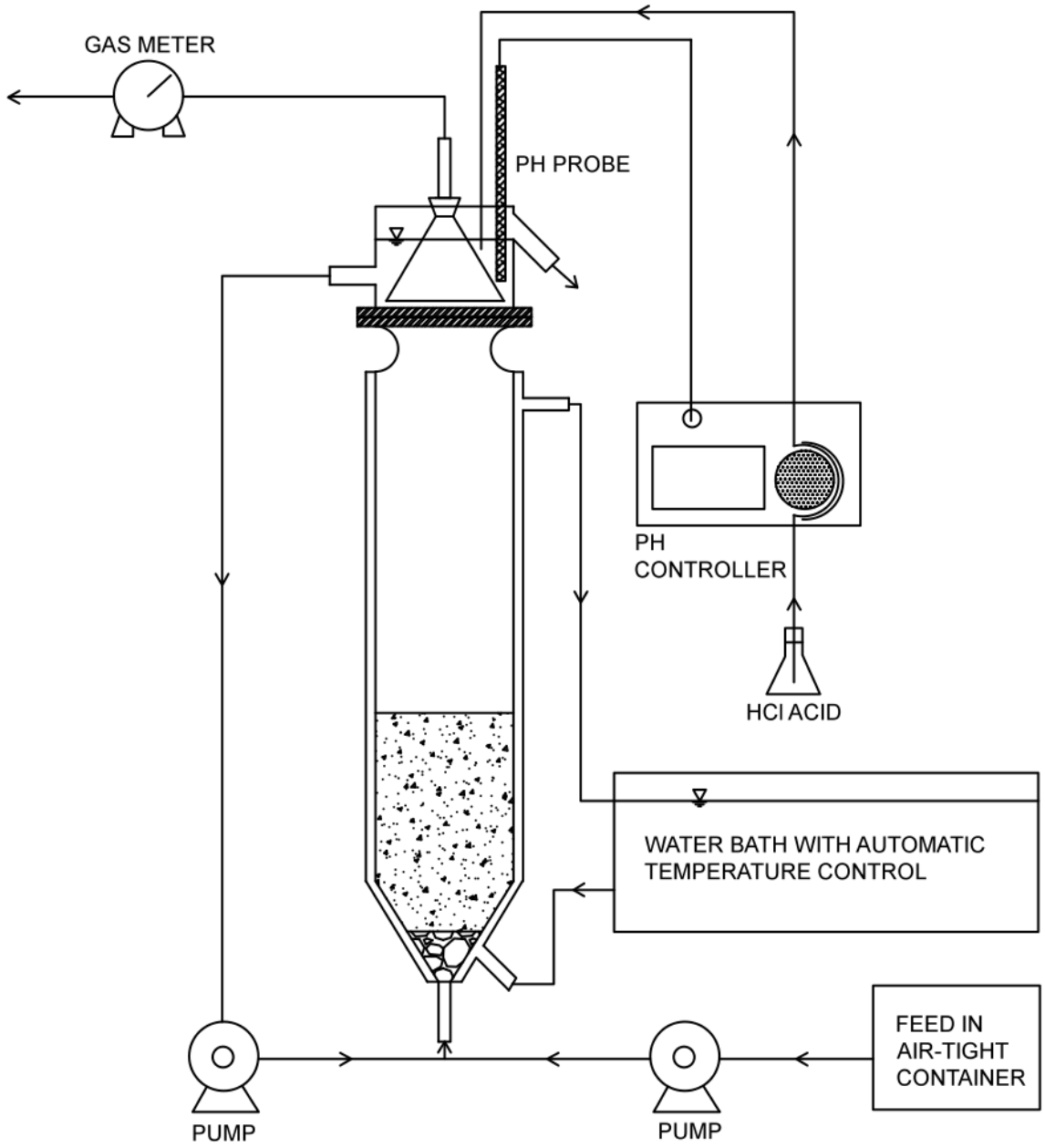

Fig. 6 Schematic diagram of experimental setup

The reactor was fed continuously with synthetic wastewater. The hydraulic retention time (HRT) was set to 1.5±0.2 days in this study. A pH controller (pH 2000, New Brunswick Science, Edison, NJ, USA) was set up to monitor the pH value inside of the

reactor and a pH of 7.8 was maintained by automatic feeding of 0.1 mole/L hydrochloric acid during the study period. The day 1 is when reactor achieved 98.6% total nitrogen removal rate.

The experiment was divided into two stages. The first stage was the enrichment of Anammox bacteria under strict DO control. During this period, nitrogen load increased dramatically once the reactor reaches optimal performance, while N_2O concentration in the off-gas was measured. In the second stage, nitrogen load was maintained at the same level while DO in the feed was altered to observe the correlation of DO and N_2O production.

3.3 Wastewater

The trace elements solution I contained (g/L): EDTA 5 and $FeSO_4$ 5. Trace elements solution II contained (g/L): EDTA 15, $ZnSO_4 \cdot 7H_2O$ 0.43, $CoCl_2 \cdot 6H_2O$ 0.24, $MnCl_2 \cdot 4H_2O$ 0.99, $CuSO_4 \cdot 5H_2O$ 0.25, $NaMoO_4 \cdot 2H_2O$ 0.22, $NiCl_2 \cdot 6H_2O$ 0.19, $NaSeO_4 \cdot 10H_2O$ 0.21 and H_3BO_4 0.014. Synthetic wastewater contained (g/L): $KHCO_3$ 0.5, KH_2PO_4 0.0272, $MgSO_4 \cdot 7H_2O$ 0.18, $CaCl_2 \cdot 2H_2O$ 0.12 and 1 mL trace elements solution I and 1 mL trace elements solution II (Imajo 2004) . The amount of ammonia and nitrite used were depended on the total nitrogen removal capacity of the running reactor and increased over time. Ammonia and nitrite were given in the form of $(NH_4)_2SO_4$ and $NaNO_2$. The wastewater solution was deoxygenated by flushing with argon gas (15 minutes, gas delivered through a porous stone sponger) and kept in a gas tight collapsible LDPE container before feeding to the reactor.

3.4 Analytical Method

Ammonium concentration was measured by ammonia-selective electrode according to Standard Methods (APHA, 1998). Nitrite and nitrate concentration were determined by spectrophotometer (DR 3900, Hach Company, Loveland, CO, USA) using corresponding powder pillow methods. The pH value is obtained via pH 2000 controller (Brunswick Scientific, Enfield, CT, USA) and pH electrode (Thermo Fisher Scientific, Waltham, MA, USA). SS and VSS were determined by the weighing method after being dried at 103–105˚C and burnt to ash at 550˚C (APHA, 1998).

The total N_2O production includes N_2O emission in the gaseous form and the N_2O dissolved in the liquid. Nitrous oxide in the headspace was measured off-line on a Tremetrics 540 gas chromatograph (Porapak Q Column 1m x 2mm i.d., nitrogen gas as carrier gas at 25mL/min, electron capture detector, temperature of injector, column, and detector were 125, 30, and 300 ˚C, respectively). The off-gas was collected using gas-tight syringe. To derive dissolved N2O concentration, the overhead space method was used. The concentration of N_2O was quantified and corrected to the concentration at standard condition for temperature (25°C) and pressure (100 kPa).

3.5 Sample fixation and cryosectioning

As described previously (Okabea 2011), fresh granular Anammox sample was obtained from reactor and fixed in 4% paraformaldehyde solution at temperature of 4˚C for 24 hours. Phosphate-buffer saline (1x PBS) was used to wash the sample before it was soaked in Tissue-Tek OCT compound overnight that allows OCT compound to infiltrate

biofilm and replace some of the water in the biomass. Cryostat Microtome was used to rapid freeze sample at -21˚C, and cryostat sectioning was then performed to obtain 15-20 μm thin sections.

3.6 Fluorescence in situ Hybridization

FISH (fluorescence in situ hybridization) is a cytogenetic technique used to identify the presence of certain DNA sequences on chromosomes. During hybridization process, fluorescence probes on bind to those chromosomes with special sequence. Using fluorescence microscopy, matching bacteria (in this study) can be observed and thus this technique is used to verify hypotheses bacteria composition in the Anammox granule.

Several 16S rRNA targeted-oligonucleotide probes (Sigma-Aldrich, St. Louis, MO, USA) were used in this study. EUB388 was used to identify all bacteria (Daims 1999). AMX820 with TXRD label was used for *Candidatus Brocadia Anammoxidan* and *Candidatus Kuenenia Stuttgartiensis* that are the common species present in Anammox reactor (Schmid 2000). NSE1472 with FLC label and NSV443 with FLC label were used for ammonium oxidizing bacteria such as *Nitrosomonas europea* and *Nitrosospira spp.*, respectively (Ohashi 1995). Synthesis scale and formamide are described in Table 1.

Table 1 - A list of 16S rRna targeted-oligonucleotide probes used in this study

Probe	Specificity	HPLC Sequence (5' to 3')	5' Mod	Synthesis Scale μmol	Formamide %	Reference
EUB338	Most bacteria	GCT GCC TCC CGT AGG AGT	Flc	0.05	35	Daims et al., 1999
AMX820	Candidatus brocadia anammoxidan Candidatus Kuenenia stuttgartiensis	AAA ACC CCT CTA CTT AGT GCC C	TxRd	0.05	35	Schmid et al., 2001
NSE1472	Nitrosomonas europea	ACC CCA GTC ATG ACC CCC	Flc	0.05	50	Mobarry et al., 1996
NSV443	Nitrosospira spp.	CCG TGA CCG TTT CGT TCC G	Flc	0.05	30	Mobarry et al., 1996
ACI208	Acidovorax spp.	CGC GCA AGG CCT TGC	Flc	0.05	20	Amann et al., 1996

Hybridization procedure

1) Fixed sample cells were spotted on coated slides and air-dried at 37°C room temperature for 5-10 min.
2) Dried slides were dehydrated with ethanol series 50%, 80% and 99%; 3 min/each, and then air dried at room temperature.
3) Sample were hybridized with oligonucleotide probes at 40°C for 60-90 min with 9 µl of hybridization buffer and 1 µl of probes (probe concentration: 50 ng/µl, or 50,000 ng/mL). Hybridization stringency was adjusted by adding formamide to hybridization buffer.
4) After hybridization, the slides were washed at 48°C for 5 min in washing buffer.
5) Washing buffer was removed with distilled water.
6) Slides were air-dried and mount with anti-fading (*Fluoromount*) for microscopy observation.

3.7 Microscope observation

Axioplan II compound research microscope was used in this study. Black and white camera was selected due to its high clarity and performance. The hybridized biomass was illuminated with light of a certain wavelength that excited fluorescence in the 16S rRNA targeted-oligonucleotide probe and it became illuminated. Two filters were used in this process. One was excitation filter, which purpose was to ensure the correct wavelength was applied to the hybridized biomass. The other one was an emission filter, which blocked excitation light source before it reached to the camera.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Enrichment of Anammox bacteria under strict DO control

The Anammox reactor was first operated effectively under low DO concentration and achieved ammonia and nitrite removal efficiency (99%). At HRT of 1.5 day, initial influent nitrogen concentration was 43 mg NH_4^+–N/L and 55 mg NO_2^-–N/L. Nitrogen concentration increased gradually to 448 mg NH_4^+–N/L and 587 mg NO_2^-–N/L after 170 days of enrichment, while achieving average ammonium and nitrite removal efficiencies were 99% and 99%, respectively.

At the beginning of the study, Anammox granule appeared to be in brownish color. After 170 days of enrichment, granule color gradually changed to reddish color (Fig. 7), which is the indication of Anammox bacteria became more dominant than before. Off-gas nitrous oxide measurement campaign was conducted during entire study using offline gas chromatography method.

The initial N_2O concentration was over 400 ppm at the beginning of the study. Since nitrification could possibly yield N_2O and dissolved oxygen inhibits Anammox activity, more strict DO control was put into place by replacing original substrate container with air-tight LDPE container started on day 10. Substrate was deoxygenated by flushing with argon gas before feeding the reactor. Since then, DO level in the feed was maintained at undetectable level.

Fig. 7 Granules in UASB Anammox reactor (diameter approximately 3 mm)

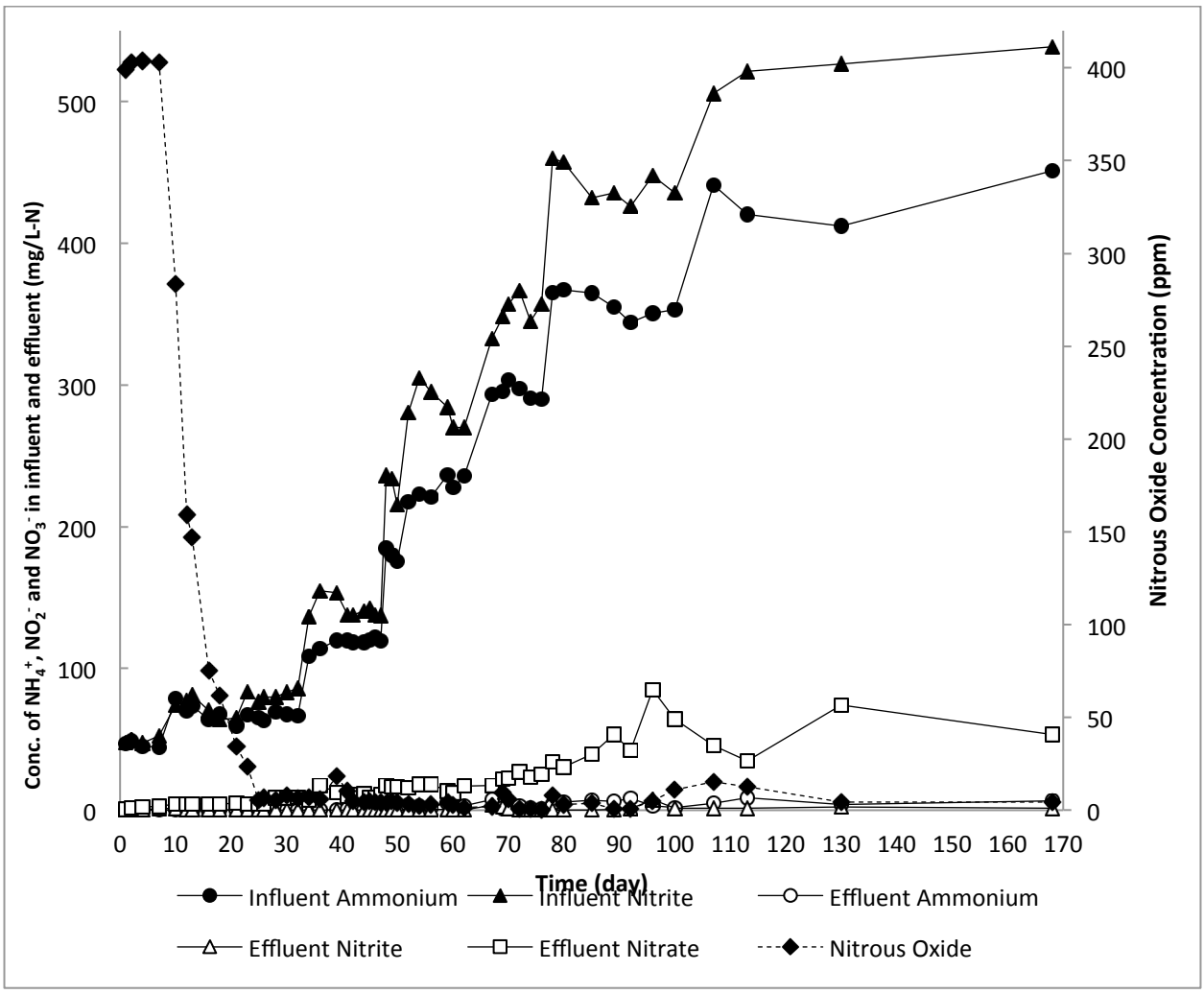

Fig. 8 The influent, effluent, and nitrous oxide profile in the UASB Anammox reactor during the first 170 days of enrichment (The left Y-axis shows the concentration of ammonium, nitrite and nitrate. The right Y-axis shows the concentration of nitrous oxide produced from reactor.)

4.2 Shock loading's effect on nitrous oxide turnover

During 170 days of continuous operation, nitrogen concentration increased gradually to 448 mg NH_4^+–N/L and 587 mg NO_2^-–N/L, while surprisingly, N_2O turnover decreased

significantly from 402.88 ppm to 4.54 ppm. Considering FISH observation (describe in section 4.5), this dramatic change of N_2O emission was may be due to DO's effect on nitrifying bacteria such as AOB. While decrease of N_2O concentration was a significant observation, it is noticed that shock loading affected N_2O production as well. During enrichment process, nitrogen loading was increased when nitrogen removal efficiency reach more than 95%. When shock loading applied, it was observed that N_2O turnover increase by up to 10 times.

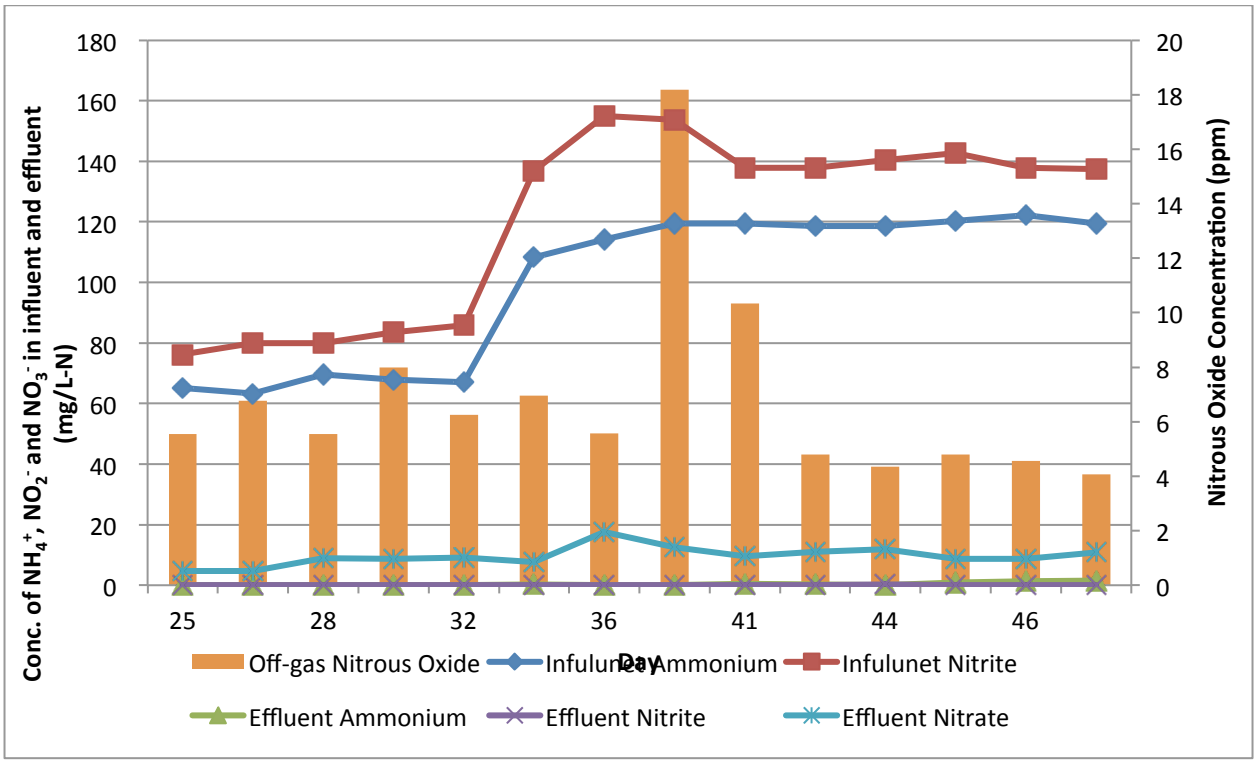

Fig. 9 Shock Loading Effect on N_2O Emission During Day 24-47

Fig. 9 shows the nitrogen profile during day 25-47. Nitrogen concentration increased from 65 mg NH_4^+–N/L and 76 mg NO_2^-–N/L to 108 mg NH_4^+–N/L 136 mg NO_2^-–N/L on day 34. This shock loading caused disturbance of the steady system. N_2O

concentration increased from about 7 ppm to peak high 18.2 ppm on day 39. However, N_2O concentration gradually decreased to original level after day 39.

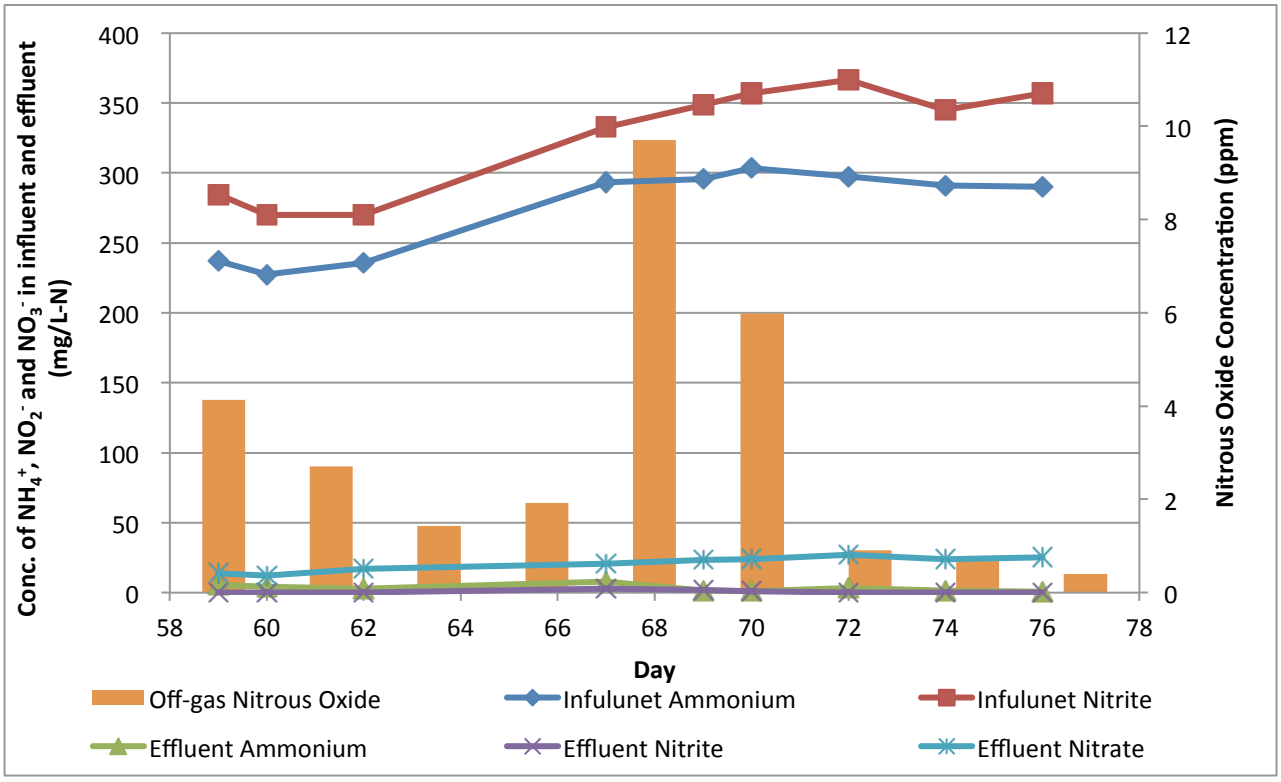

Fig. 10 Shock Loading Effect on N_2O Emission During Day 59-76

Fig. 10 shows the nitrogen profile during day 59-76. Same shock loading took effect on N_2O emission. We can see that nitrogen concentration increased from 237 mg NH_4^+–N/L and 284 mg NO_2^-–N/L to 293 mg NH_4^+–N/L 333 mg NO_2^-–N/L on the day 67. N_2O concentration increased from about 3 ppm to peak high 9.7 ppm on day 69. However, N_2O concentration gradually decreased to original level after day 69.

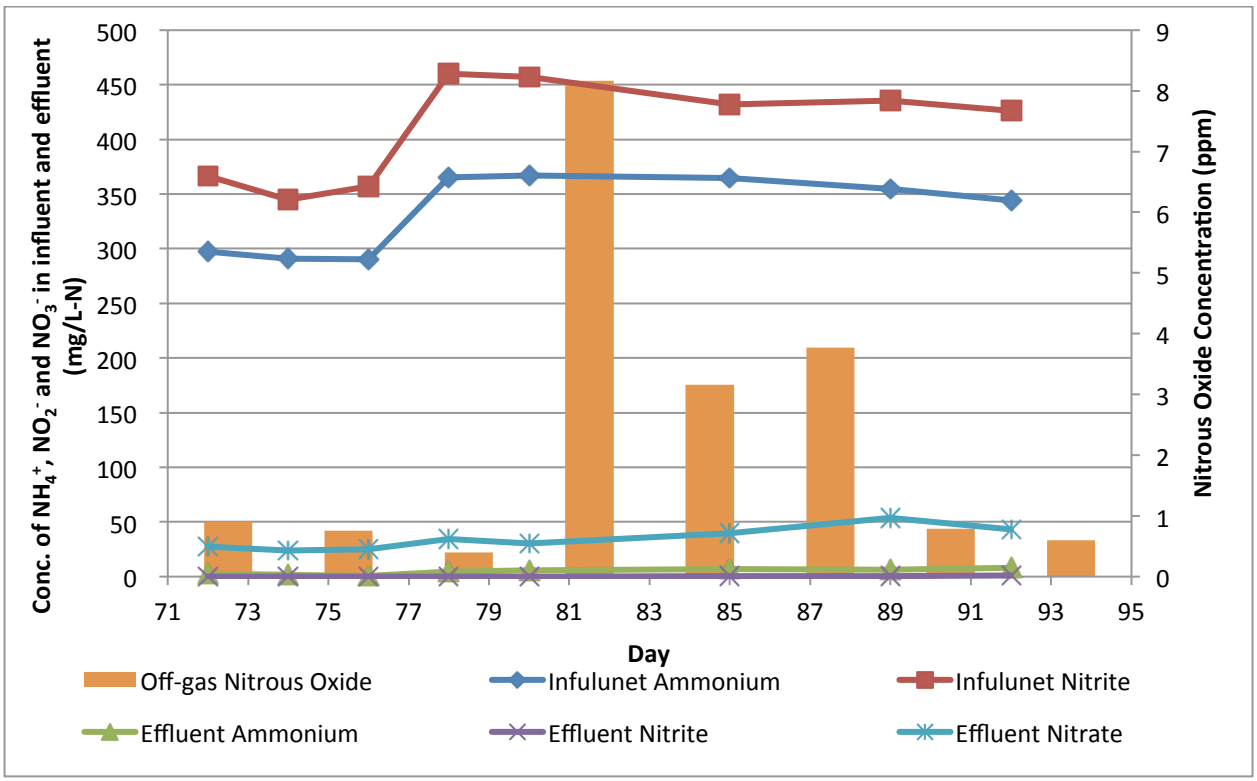

Fig. 11 Shock Loading Effect on N_2O Emission During Day 72-92

Similarly, during day 72-92, shock loading took greater effect on N_2O turnover by 10 folds (Fig. 11). On day 78, nitrogen concentration increased from 298 mg NH_4^+–N/L and 367 mg NO_2^-–N/L to 366 mg NH_4^+–N/L 460 mg NO_2^-–N/L. On the same day, nitrous oxide concentration increased significantly from about 0.7 ppm to 8.17 ppm. From above three cases we can see that shock loading brought disturbance to steady ecosystem, causing the increase of nitrous oxide turnover rate. It is also observed that Anammox reactor soon get used to new substrate concentration, reaching new steady state, where nitrous oxide concentration dropped to lower level.

4.3 Dissolved oxygen's effect on nitrous oxide emission

It is worth mentioning that the lab-scale USAB reactor was originally inoculated with Anammox sludge and inactive methanogenic granular sludge. The Anammox bacteria purity (percentage of Anammox cells in the bacteria community) in the seed sludge was less than 1%. After 170 days of continuous enrichment, it's outstanding performance on ammonia nitrogen and nitrite nitrogen removal provide us strong signal that enrichment process was successful, not to mention granule's color change from brown to red. It is reasonable to lead us to believe that the percentage of Anammox bacteria in the granule increased considerably to handle more nitrogen stress. However, we can certainly assume that there must be some other bacteria co-exist with Anammox bacteria simply because it is not a pure-culture environment. For example, nitrifier such as ammonia oxidizing bacteria (AOB) can continue to survive under low DO and low COD condition, where AOB undergo nitrifier denitrification and obtain energy source to survive. (Schmid 2000) Nitrous oxide is a major product of nitrifier denitrification. In order to prove this hypothesis, the second study about DO's influence on nitrous oxide emission was carried out. Increase DO concentration will activate AOB to perform nitrification. Since nitrous oxide is an important intermediate and byproduct, increase of nitrous oxide production can be an indication of the existence of AOB and its activity.

After 170 days of enrichment period, substrate concentration was kept at the same level to feed the reactor for another 47 days. During this period, DO concentration in the feed was adjusted to different levels and turbulence of nitrous oxide turnover was observed (F1g. 12).

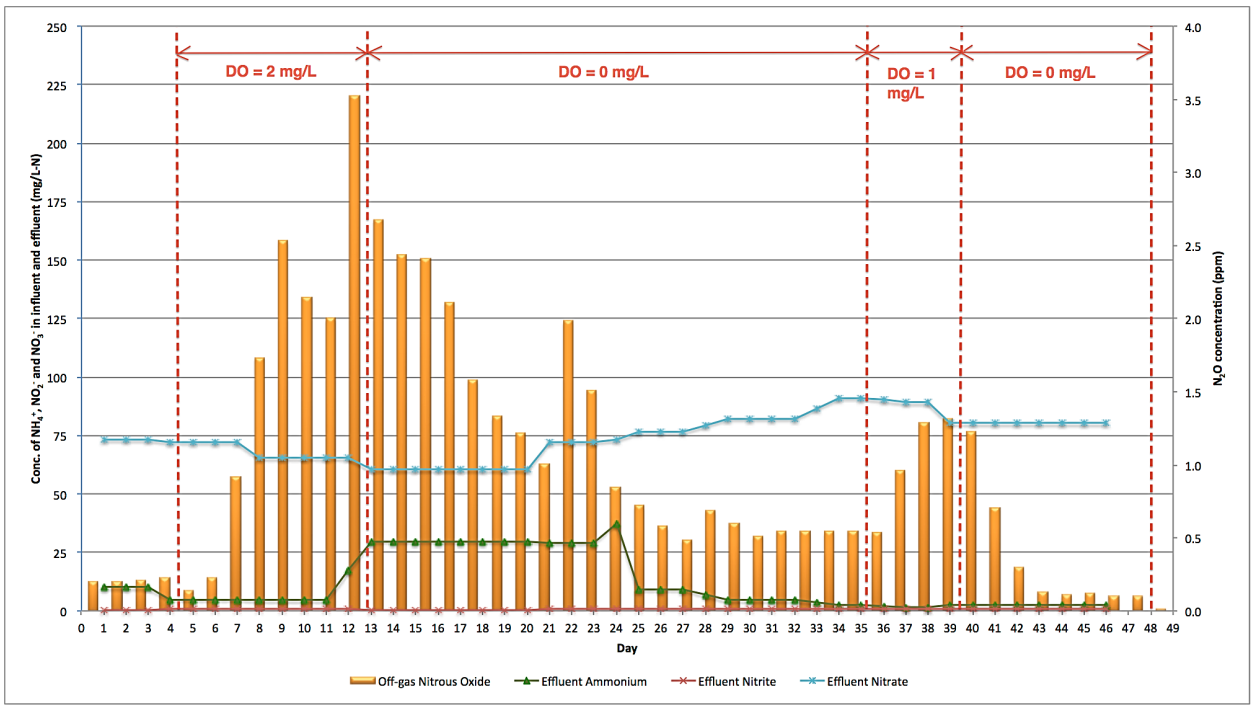

Fig. 12 Dissolved Oxygen Effect on N_2O Emission

On day 5, dissolved oxygen was introduced to substrate at level of 2 mg/L. New substrate was prepared every day to avoid DO level drop caused by possible bacteria activity inside the LDPE container before feeding the Anammox reactor. On day 6, gas chromatography showed slight off-gas nitrous oxide increase. In the next 6 days, nitrous oxide concentration kept increasing to 3.5 ppm. When compared with previous strict anaerobic condition, which nitrous oxide concentration was about 0.2 ppm, peak nitrous oxide concentration under 2 mg/L DO was 15 times more than before. Increase of effluent ammonia-nitrogen concentration was also observed on day 12. It may be caused by DO toxicity to Anammox bacteria. In order to keep reactor's good performance on nitrogen removal, reactor was flushed with argon gas on day 13, and substrate was prepared with zero DO. In the next few days, ammonia removal efficiency

didn't pick up immediately, since recovery from DO toxicity usually takes a while. However, we observed decrease of nitrous oxide concentration in the off-gas. DO's effect on reactor's nitrous oxide emission was obvious and led to believe that AOB activity was activated when DO level was raised in the feed. Nitrification took place and therefore producing nitrous oxide as a byproduct.

On day 29, ammonia concentration in the effluent decreased to 4.54 mg NH_4^+–N/L, a good ammonia nitrogen removal efficiency of 98.8% was observed, which indicate Anammox reactor was recovered from DO inhibition. A smaller amount of DO was then introduced to the feed at 1 mg/L to confirm nitrification reaction on day 36. Just as expected, again, nitrous oxide concentration gradually increased from 0.5 ppm to 1.3 ppm on day 37. Since smaller dosage of DO was utilized, DO toxicity didn't occur. On day 39, new substrate was prepared without dissolved oxygen, and decrease of nitrous oxide concentration was observed afterwards.

The above evidence indicates the possibility of Anammox bacteria co-existed with nitrifier. To further prove our hypothesis, fluorescence in situ hybridization technique was performed to identify Anammox and AOB in the granular sludge.

4.4 Using FISH and advanced microscopy technique to study Anammox granule composition

On day 274, Anammox granule sample was obtained from reactor and Cryostat Microtome was used to rapid freeze sample at -21˚C, and cryostat sectioning was then performed to obtain 15-20 μm thin sections. AMX820 with TXRD label, NSV443 with

FLC label and NSE1472 with FLC label was used in hybridization procedure. DIC, FICT, TRITC filter were applied to camera lens to obtain image of hybridized bacteria. Microscope with bright field mode was used to obtain image of granule's cross-section image and layered structure is presented in Fig. 13.

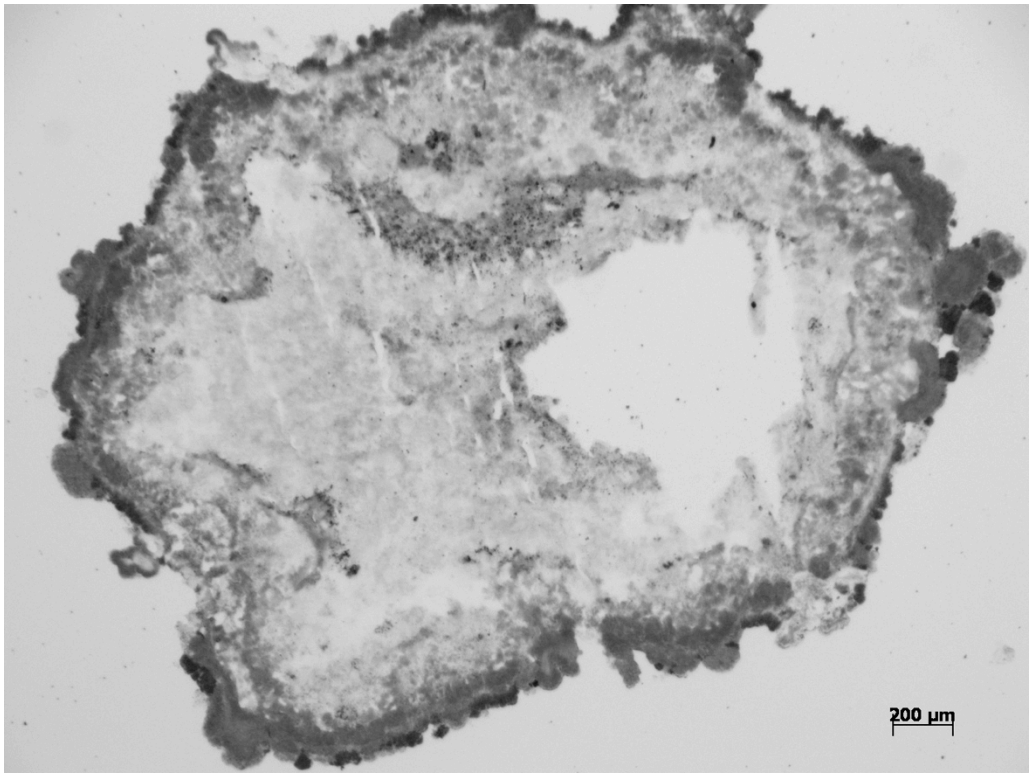

Fig. 13 Cross-section structure of Anammox granule (DIC filter)

The granule showed in the Fig. 13 has a diameter of 3 mm and appeared to be in red color. The internal structure of granule consists of different layers as seen in the figure, which is the indication of possible co-existing bacteria community. In the center of the

granule, a hollow structure was observed – it is believed formed from Anammox gas production.

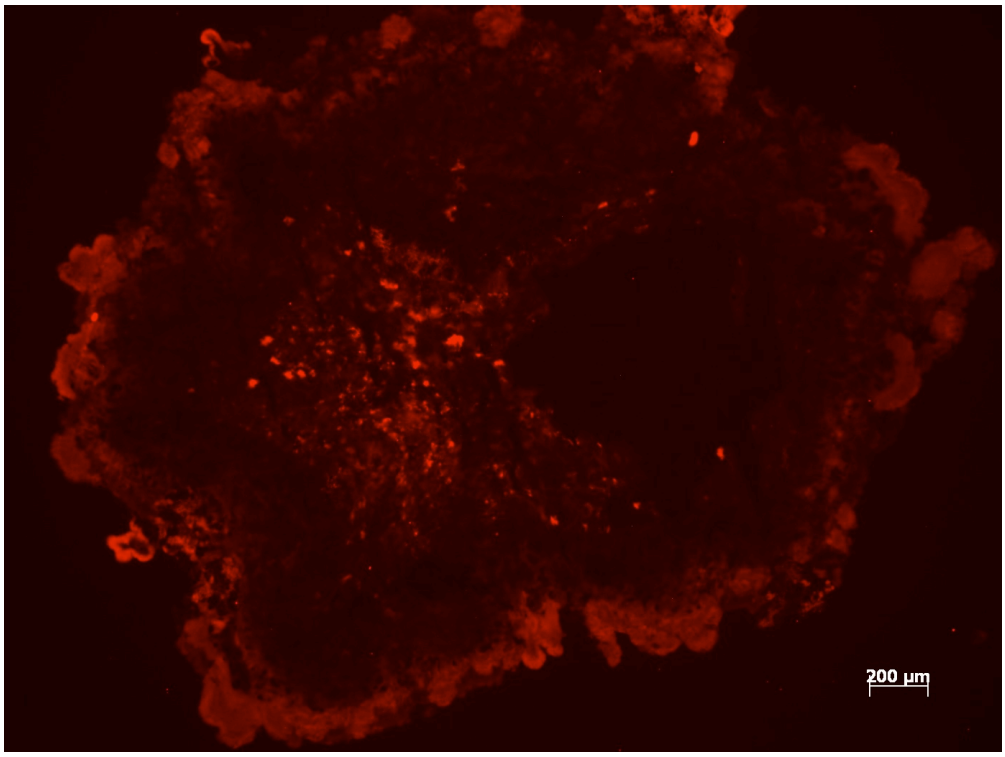

Fig. 14 Anammox bacteria (red) in cross-section (TRITC filter)

Fig. 14 shows Anammox bacteria in the granule respond to AMX820 probe. As seen in the picture, Anammox bacteria were present throughout the entire granule, but more concentrated in the inner part of granule next to the hollow area. With further zoom of, image obtained (Fig. 15) shows Anammox bacteria in the center on the granule.

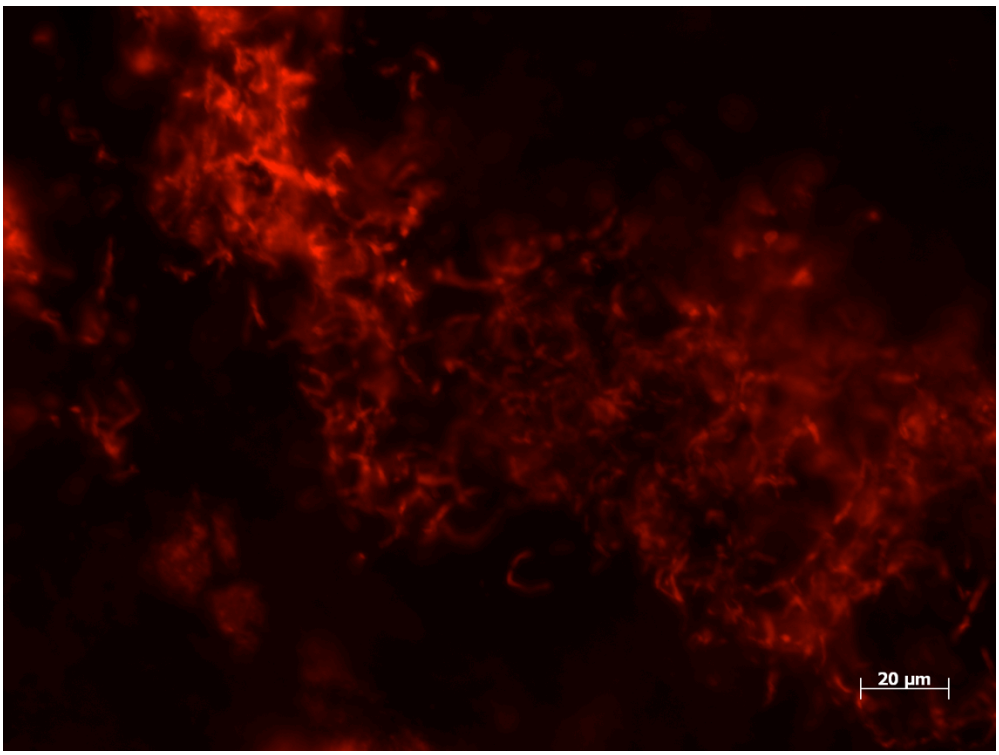

Fig. 15 Anammox bacteria located in the center part of cross-section (TRITC filter)

Fig. 16 and Fig. 17 show FISH image using NSV443 and NSE1472 probe that target AOB bacteria with FLC filter. As we can see from the image, AOB do exist in the Anammox granule. Although the system had undergone strict DO control, AOB survived under anaerobic condition. The majority of AOB appeared to be found on the surface layer of granule, where oxygen is more readily available than anywhere else. This finding is in accordance to previous paper by Okabe, published in 2011. In his study, Anammox bacteria were present throughout the granule, whereas ammonium-oxidizing bacteria (AOB) were restricted to only the granule surface. However, Okabe's reactor set-up is Sharon+Anammox process, where the leftover oxygen from Sharon process can enter the following Anammox reactor. In our case, Anammox reactor is set up under strict

oxygen control, meaning no oxygen should enter the reactor. It is quite surprising that AOB can still survive under this condition.

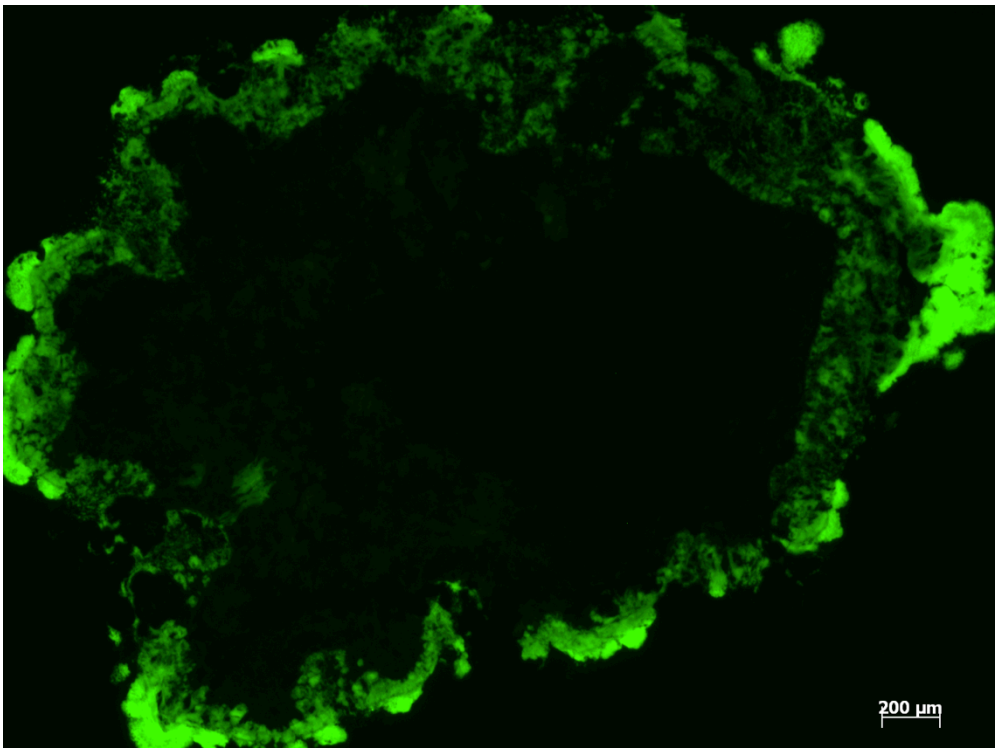

Fig. 16 Ammonium oxidizing bacteria (AOB) in the cross-section of Anammox granule (FLC filter)

According to previous studies, AOB undergoes a different pathway called nitrifier denitrification under low DO and organic carbon condition where nitrous oxide is the intermediate of denitrification process. Again, physically purified Anammox bacteria (purity more than 99.9%) do not yield nitrous oxide. (Kartal 2007) It is worth mentioning how dramatic nitrous oxide production decreased since oxygen control was

put in place. The lack of dissolved oxygen caused the halt of nitrification in the reactor - a process that produces nitrous oxide as an intermediate side product. The decline of DO forced AOB shifting to nitrifier denitrification metabolism. Due to very little carbon content was provided from the synthetic wastewater, nitrifier denitrification was the only way AOB to gain energy from and to survive. Nitrous oxide concentration was low, accounting for only 0.07% of nitrogen removal rate. However, it was an important pathway to keep them survives.

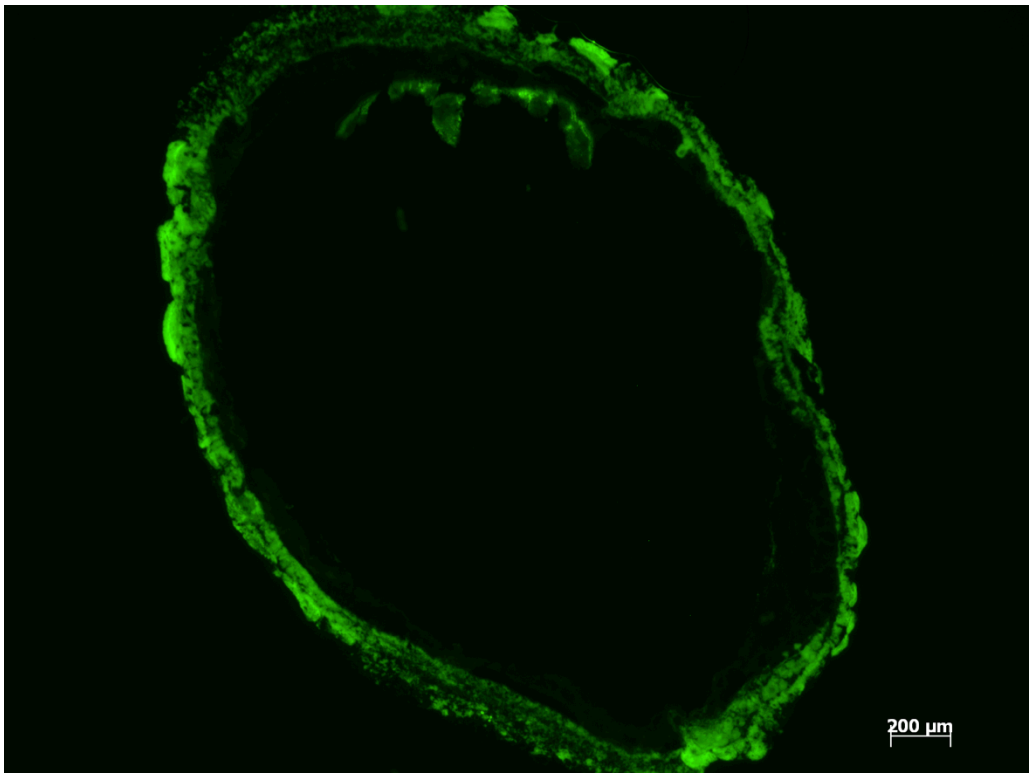

Fig. 17 Ammonium oxidizing bacteria (AOB) in the cross-section of Anammox granule (FLC filter)

In the later part of study, DO was given to the reactor in small dose, and nitrifier corresponded it with increased nitrous oxide production. AOB on the surface of the granule shifted from nitrifier denitrification metabolism to nitrification once oxygen became available. From this evidence we can conclude that nitrifier are rather flexible bacteria and have strong capability to survive under different scenarios, and they are the reason why nitrous oxide emission can be detected from granular Anammox reactor.

4.5 Stoichiometry of the Anammox process

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13H^+ \rightarrow 1.02\ N_2 + 0.256\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \quad (1)$$

According to the eq. 1, the theoretical ratio of removed NH_4^+-N: NO_2^--N: produced NO_3^--N is 1: 1.32: 0.256. In this study, at steady state, the ratio was 1: 1.21: 0.19. It was very close to theoretical ratio, but slightly off. By calculating NH_4^+-N: $NO2^-$-N ratio in the feeding substrate, error of HACH 3900 spectrophotometer was realized because the NH_4^+-N: $NO2^-$-N ratio was 1:1.18 instead of 1: 1.21. That been said, nitrite was consumed more than theoretical value. In terms of nitrate production, the ratio showed that less than theoretical amount of nitrate was detected from the effluent of the reactor. Considering FISH result, which proved AOB's existence, the variance of the ratio can be understood. When the reactor was under anaerobic condition, AOB shifted to nitrifier denitrification pathway, which consumed nitrite from feeding substrate and nitrate produced Anammox process. This is a direct evidence of AOB activity inside the reactor.

CHAPTER 5. CONCLUSION

Greenhouse gas emission has been paid more attention in the past decade. Greenhouse emission from anthropological activities has in fact, affected our environment and we have to pay the price. Lately, more attention has been put on nitrous oxide emission, which is a greenhouse gas that's 300 times more powerful compared to carbon dioxide with long lifetime. In today's world, there is the need to manage nitrous oxide emission and promote minimal nitrous oxide emission. Sewage treatment, which involved nitrogen removal from wastewater, is a big player in nitrous oxide emission from anthropological activities.

There is a need to develop more sustainable nitrogen removal process to treat wastewater that has minimum impact on environment. It has become clear that more stringent nutrient removal policy will be in place in the near future. With its substantial energy saving and ability to handle high nitrogen stress, Anammox technology is a great candidate that will fully benefit our environment and society. However, there aren't enough research has been done to investigate its nitrous oxide emission. Published works showed mixed result on this issue and that's why there was the need to carry out this research.

In this study, a lab-scale one reactor Anammox UASB reactor was developed to investigate nitrous oxide emission. The average emission of N_2O was 0.07 % of recovered nitrogen. The source of N_2O emission from Anammox granule was believed to come from AOB. When reactor was under strict anaerobic condition, AOB could survive

by shifting to nitrifier denitrification metabolism to obtain energy. The last experiment carried out by using FISH technique proved AOB's existence. The majority of AOB were located on the surface of Anammox granule. The general trend showed that the amount of N_2O emitted from the reactor is correlated to nitrogen load. The higher nitrogen concentration in the feed, the lower N_2O emission was observed. This is due to the percentage of AOB in the granule decreased while Anammox bacteria were enriched when the reactor was put under strict anaerobic condition. Surprisingly, shocking loading showed effect on N_2O emission. When shock loading was applied, N_2O concentration increased significantly. The cause of this phenomenon was unclear, but it should somehow relate to the disturbance brought by shock loading to the steady eco-system. Last, this study showed N_2O emission responded to DO concentration in the feed. When DO was present, N_2O emission increase significantly. Since AOB existed in the granule, nitrification by AOB was believed to be the cause of this phenomenon.

Based on all experiment results gathered from this study (such as shock-loading test, DO test, FISH, etc.), the nitrous oxide emission from Anammox reactor is most likely from nitrifier denitrification. Although N_2O can be observed from reactor, with strict DO control and avoiding shock loading, Anammox reactor's N_2O emission can be controlled at extremely low level. In this study, the average emission of N_2O was only 0.07 % of recovered nitrogen. With its outstanding capability for nitrogen removal and extremely low N_2O emission, Anammox was once again proved to be a "green" wastewater nutrient removal technology. It is foreseeable that with more stringent nutrient regulation putting into place, Anammox is a promising technology for future generation wastewater treatment plants.

BIBLIOGRAPHY

Abma, W.R., Schultz, C.E., Mulder, J.W., van der Star, W.R.L., Strous, M., Tokutomiand, T., van Loosdrecht, M.C.M. "Full-scale granular sludge Anammox process." *Water Science & Technology* 55, no. 8-9 (2007): 27–33.

Bock, E., Koops, H.P., Harms, H. "Cell biology of nitrifying bacteria." *Special Publications of the Society for General Microbiology*, no. 20 (1986): 17-38.

Broda, E. "Two kinds of lithotrophs missing in nature." *Zeitschrift fur allgemeine Mikrobiologie* 17 (6) (1977): 491-493.

Carlson, C. A., J. L. Ingraham. "Comparison of denitrification by Pseudomonas stutzeri, Pseudomonas aeruginosa, and Paracoccus denitrificans." *Applied Environmental Microbiology*, 1983: 1247-1253.

Colliver, B.B., Stephenson, T. "Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers." *Biotechnol. Adv.* 18 (3) (2000): 219–232.

Daims, H., Brühl, A., Amann, R., Schleifer, K.H., Wagner, M. "The Domain-specific Probe EUB338 is Insufficient for the Detection of all Bacteria: Development and Evaluation of a more Comprehensive Probe Set." *Systematic and Applied Microbiology* 22, no. 3 (1999): 434–444.

Desloover, J. "Strategies to mitigate N_2O emissions from biological nitrogen removal systems." *Current Opinion in Biotechnology* (Elsevier), 2012.

Electric Power Research Institute, Inc. (EPRI) "U.S. Electricity Consumption for Water Supply & Treatment - The Next Half Century" *Water & Sustainability*, 2002

Hanaki, K., Hong, Z., Matsuo, T. "Production of nitrous oxide gas during denitrification of wastewater ." (Water Sci. Technol.) 26 (5-6) (1992): 1027-1036.

Hollocher, T.C., Tate, M.E., Nicholas, D.J.D. "Oxidation of ammonia by Nitrosomonas europaea ." *Journal of Biological Chemistry*, no. 256 (1981): 10834-10836.

Hooper, A.B., Terry, K.R. "Hydroxylamine oxidoreductase of Nitro- somonas production of nitric oxide from hydroxylamine ." *Biochimica et Biophysica Acta* , no. 571 (1979): 12-20.

Imajo, U., Tokutomi, T., Furukawa, K. "Granulation of Anammox microorganisms in up-flow reactors." *Water Sci. Technol* 49, no. 5-6 (2004): 155–163.

IPCC. *Climate Change 2007: Synthesis Report* . Geneva: IPCC, 2007.

Itokawa, H., Hanaki, K., Matsuo, T. "Nitrous Oxide production in high-loading biological nitrogen removal process under low COD/N ratio condition." *Water Res.* 35 (3) (2001): 657-664.

Jettena, M.S.M., Wagnerc, M., Fuerstd, J., van Loosdrechtb, M., Kuenenb, G., Strousa, M. "Microbiology and application of the anaerobic ammonium oxidation (‘anammox’) process." *Current Opinion in Biotechnology* 12, no. 3 (2001): 283–288.

Kampschreur, M.J., Temminl, H., Kleerebezem, R., Jetten, M.S.M., van Loosdrecht, M.C.M. "Nitrous oxide emission during wastewater treatment." 43 (17) (2009): 4093-4103.

Kampschreur, M.J., van der Stara, W.R.L., Wieldersa, H.A., Mulderb, J.W., Jettena, M.S.M., van Loosdrechta, M.C.M. "Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment." *Water Research*, 2007.

Kartal, B., Kuypers, M.M.M., Lavik, G., Schalk, J., op den Camp, H.J.M., Jetten, M.S.M., Strous, M. "Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium ." *Environ. Microbiol.* 9 (3) (2007): 635–642.

Kuenen, J.G., Jetten, M.S.M. "Extraordinary anaerobic ammonium-oxidizing bacteria." *ASM News* 67 (2001): 456-463.

Liu, S., Yang, F., Xue, Y., Gong, Z., Chen, H., Wang, T., Su, Z. "Evaluation of oxygen adaptation and identification of functional bacteria composition for anammox

consortium in non-woven biological rotating contactor." (Bioresource Technology) 99 (2009): 8273–8279.

Mulder, A., Vandegraaf, A.A., Robertson, L.A. and Kuenen, J.G. "ANAEROBIC AMMONIUM OXIDATION DISCOVERED IN A DENITRIFYING FLUIDIZED-BED REACTOR." *Fems Microbiology Ecology* 16 (3) (1995): 177-183.

National Oceanic and Atmospheric Administration. *THE NOAA ANNUAL GREENHOUSE GAS INDEX (AGGI)*. 2010. http://www.esrl.noaa.gov/gmd/aggi/ (accessed 2012).

Ni, S.Q., Fessehaie, A., Lee, P.H., Gao, B.Y., Xu, X., Sung, S.W. "Interaction of anammox bacteria and inactive methanogenic granules under high nitrogen selective pressure." *Bioresource Technology* 101 (2010): 6910–6915.

Nicholas, D.J.D., Jones, O.T.G. "Oxidation of hydroxylamine in cell- free extracts of Nitrosomonas europaea." *Nature*, no. 185 (1960): 512-514.

Ohashi, A., Viraj de Silva, D.G., Mobarry, B., Manem, J.A., Stahl, D.A., Rittmann, B.E. "Influence of substrate C/N ratio on the structure of multi-species biofilms consisting of nitrifiers and heterotrophs." *Water Science and Technology* 32, no. 8 (1995): 75-84.

Okabea, S., Oshiki, M., Takahashia, Y., Satoha, H. "N_2O emission from a partial nitrification–anammox process and identification of a key biological process of N2O emission from anammox granules." *Water Research* 45, no. 19 (2011): 6461-6470.

PAQUES. *ANAMMOX* . 2011. http://en.paques.nl/pageid=66/ANAMMOX®.html (accessed 6 17, 2011).

Poth, M., Focht, D.D. "Nitrogen-15 kinetic analysis of nitrous oxide production by Nitrosomonas europaea: an examination of nitrifier denitrification ." *Appl. Environ. Microbiol.* 49 (5) (1985): 1134–1141.

Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., Metzger, J., Schleifer, K.H., Wagner, M. "Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation ." *Sys. Appl. Microbiol.* 23 (2000): 93-106.

Schmidt, I., van Spanning, R.J.M., Jetten, M.S.M. "Denitrification and ammonia oxidation by Nitrosomonas europaea wildtype, and NirK- and NorB-deficient mutants." *Microbiology (Reading UK)* 150 (12) (2004): 4107–4114.

Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L. In *Climate Change 2007: The Physical Science Basis.*, edited by Qin D, Manning M, Chen Z, Marquis M, Averyt AB, Tignor M, Miller HL. Solomon S, 21-92. Cambridge University Press, 2007.

Strous, M., Heijnen, J.J., Kuenen, J.G., Jetten, M.S.M. "The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms ." *Appl. Microbiol. Biotechnol.* 50 (5) (1998): 589–596.

Strous, M., Jetten, M.S.M. "Anaerobic oxidation of methane and ammonium." *Annual Review of Microbiology* 58 (2004): 99-117.

Strous, M., Kuenen, J.G., Jetten, M.S.M. "Key physiology of anaerobic ammonium oxidation." *Appl. Environ. Microbiol.* 65 (7) (1999): 3248–3250.

USEPA. *Nitrous Oxide*. 2010. http://www.epa.gov/nitrousoxide/sources.html (accessed 2012).

van de Graaf, A.A., De Bruijn, P., Robertson, L.A., Jetten, M.S.M., Kuenen, J.G. "Metabolic pathway of anaerobic ammo- nium oxidation on the basis of ^{15}N studies in a fluidized bed reactor." *Microbiology (Reading UK)* 143 (7) (1997): 2415–2421.

van de Graff, A.A., Mulder, A., Debruijn, P., Jetten, M.S.M., Robertson,L.A. and Kuenen, J.G. "anaerobic oxidation of ammonium is a biologically mediated process." *Applied and Environmental Engineering Microbiology* 61(4) (1995): 1246-1251.

Wood, P.M. "Nitri®cation as a bacterial energy source. In: Prosser, J.I. Ed.), Nitrification." *Special Publications of the Society for General Microbiology*, no. 20 (1986): 39-62.

Wrage, N., Velthof, G.L., van Beusichem, M.L., Oenema, O. "Role of nitrifier denitrification in the production of nitrous oxide." *Soil Biology & Biochemistry* (PERGAMON), no. 33 (2001): 1723-1732.

Wyffels, S., Boeckx, P., Pynaert, K., Zhang, D., Van Cleemput, O., Chen, G., Verstraete, W. "Nitrogen removal from sludge reject water by a two-stage oxygen-limited autotrophic nitrification denitrification process ." *Water Sci. Technol.* 49 (5–6) (2004): 57–64.