Nitrous oxide and nitric oxide production in partial nitrification and anaerobic ammonium oxidation granular sludge

Submitted by

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ABSTRACT

Since nitrogen (N) is an essential element to maintain life, some of its fixed forms are used as key ingredients of fertilizer to achieve high crop yields. However, N is one of the major pollutants causing many problems in the aquatic environment. For example, excess N input leads to eutrophication, in which dissolved oxygen is depleted, septic conditions are caused and odor problem arises in water bodies. Moreover, ammonia is toxic to aquatic life and nitrite and nitratecause several health effects, such as methemoglobinemia and vitamin A shortage. Therefore, stringent water quality standards for N are established to protect aquatic environment. Since municipal, industrial, and agricultural wastewaters are the major N source, N removal from the wastewaters is critical. N is generally removed from wastewaters by biological processes and a conventional biological N removal technology is a combination of nitrification and denitrification processes. However, an alternative and innovative approach, a partial nitrification (PN) process followed by an anaerobic ammonium oxidation (anammox) process (a PN-anammox process), has recently drawn attention. It has several advantages, such as no need for external carbon addition, less energy and oxygen requirement, and less sludge production. Especially, granular sludge reactors for PN and anammox processes are promising because of because of good sludge settleability, long-term retention of slow-growing bacteria in a reactor and high specific reaction rate. It is generally accepted that N removal process in a wastewater treatment system are an anthropogenic source of nitrous oxide (N_2O) and nitric oxide (NO). N_2O is an important greenhouse gas with a global warming potential of about 300 times higher than CO_2 and the major stratospheric ozone-depleting substance. NO is a highly reactive free radical and is toxic to a wide range of organisms. Besides its environmental adverse effect, a NO molecule has some ecological influences on microbial consortiaby regulating the specific microbial activities. To gain the insight into N_2O and NO production and consumption rates, their pathways, and the factors influencing them is essential to control N_2O and NO emissions from a PN-anammox process. However, very limited studies have been conducted to characterize N_2O and NO production in granular PN-anammox process. Therefore, this thesis started with estimation of N_2O emission rates and identification of a key N_2O production pathway in a granular PN reactor. Then the effects of dissolved oxygen (DO) and pH on the N_2O production rates and pathways were investigated. Furthermore, we experimentally identified NO emission pathways in anammox granules and investigated physicochemical parameters affecting the NO emissions. To achieve the objectives, we conducted batch tests, microsensor measurements, isotopomer analysis, fluorescence in situ hybridization (FISH) and microbial community structure analysis.

We investigated the emission of N_2O from a lab-scale granular sequencing batch reactor (SBR) for PN treating synthetic wastewater without organic carbon. The average N_2O emission rate from the SBR was 0.32 ± 0.17 mg-N L^{-1} h^{-1}, corresponding to the average emission of N_2O of 0.8 ± 0.4% of the incoming N load (1.5 ± 0.8% of the converted ammonia). Analysis of dynamic concentration profiles during one cycle of the SBR operation demonstrated that N_2O concentration in off-gas was the highest just after starting aeration whereas dissolved N_2O concentration in effluent gradually increased in the initial 40 min of the aeration period and decreased thereafter. Isotopomer analysis was conducted to identify the main N_2O production pathway in the reactor during one cycle. The hydroxylamine (NH_2OH) oxidation pathway accounted for 65% of the total N_2O emission in the initial phase during a cycle, whereas contribution of the NO_2^- reduction pathway to N_2O production was comparable with that of the NH_2OH oxidation pathway in the latter phase. In addition, spatial distributions of bacteria and their activities in single microbial granules taken from the reactor were determined with microsensors and by FISH. PN occurred mainly in the oxic surface layer of the granules and ammonia-oxidizing bacteria were abundant in this layer. N_2O production was also found mainly in the oxic surface layer. Based on these results, although N_2O was produced mainly via NH_2OH oxidation pathway in the autotrophic PN reactor, N_2O production mechanism is complex and could involve multiple N_2O production pathways.

Moreover, the effects of DO and pH on N_2O production rates and pathways in autotrophic PN granules were investigated at the granular level. We conducted batch experiments to investigate the effects of DO and pH on N_2O emission rates. Allylthiourea (ATU) was used to distinguish the amount of N_2O produced by nitrification (NH_2OH oxidation) and denitrification (nitrifier denitrification and heterotrophic denitrification). N_2O emission and ammonia oxidation rates increased with increasing bulk DO concentration from 0.6 to 2.3 mg L^{-1}. The inhibition tests with ATU revealed that N_2O was mainly produced via NH_2OH oxidation and DO dominantly affected this pathway. The linear correlation between N_2O emission and ammonia oxidation rates emphasized that an increase in DO concentration promoted NH_2OH oxidation and then stimulated N_2O production via NH_2OH oxidation. To investigate the effect of pH on the N_2O emission rates from the PN granules, batch tests were conducted at different pH values from 6.5 to 8.5. In contrast to the effects of DO, the change in pH affected the both N_2O production via NH_2OH oxidation and denitrification. Although the ammonia oxidation rate was unchanged in the range of pH 6.5 to 8.5, the highest N_2O emission was observed at pH 7.5. The results from microsensor measurements, FISH analysis and microbial community analysis revealed that DO and pH mainly influenced N_2O production by *Nitrosomonas europaea* and *Nitrosomonas eutropha*, in the oxic surface layer (< 200 μm) of the autotrophic PN granules. However, the

mechanisms underlying pH effect on N_2O production via NH_2OH oxidation are currently unclear. This study suggests that in situ analysis of PN granules is essential to gaining insight into N_2O emission mechanisms in a PN granule in order to establish a strategy to mitigate N_2O emissions in PN processes.

We investigated the microorganisms and pathways responsible for and the factors affecting the NO emissions from the microbial granules taken from an anammox reactor. Anammox bacteria were identified as the members of "*Candidatus* Brocadia sinica" and accounted for 88% of the total bacteria in the granules. Stable isotope-labeling studies indicated that most of N_2 was emitted by anammox bacteria, NO was produced only by nitrite reduction and the inhibitors for anammox bacteria reduced N_2 and NO emissions. Both anammox and denitrifying bacteria were responsible for NO emission from the anammox granules. The NO emitted from the anammox granules accounted for <1.1% of the total gaseous N. In situ analysis showed that the density and activity of the anammox bacteria and NO production rate were higher in the outer layer of the anammox granules. NO emissions were strongly influenced by ammonia, nitrite and pH levels. The results presented in this study are useful for strategies to control NO emissions from anammox processes.

In summary, NH_2OH oxidation contributed mainly to N_2O emission from a granular PN-SBR treating autotrophic wastewater. Nitrification occurred in the surface layer of the granules. DO and pH influenced the N_2O production rates of NH_2OH oxidation. Increase in DO concentrations increase N_2O emission from PN granules and N_2O production rate was highest at pH 7.5. Anammox and denitrifying bacteria were responsible for NO emission from the anammox granules. This study concludes that the combined use of multiple analytical techniques is indispensable to our knowledge of N_2O and NO production mechanisms in PN and anammox granules.

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Introduction

1 INTRODUCTION

1.1 THE MICROBIAL NITROGEN CYCLE

The microbial nitrogen (N) cycle is the conversion of N between the different forms by the microorganisms. N is the major constituent of the atmosphere and it is the fifth most abundant element in the solar system (Hily-Blant et al., 2013). N in the atmosphereexits mainly as dinitrogen gas (N_2), which is an inert molecule. Considerable energy is required to reduce N_2 into biochemically available ammonium (NH_4^+), which is referred to as N fixation (Figure 1.1). N fixation is considered as an essential process in many eco-environments. Fixed N is the most important form of N because it is involved in many biochemical processes and makes up the substances of life on earth. For example, N is required for all of the living organisms to live and grow because it is the essential component of proteins and nucleic acids (*i.e* DNA and RNA).Certain bacteria and archaea are capable to reduce N_2into NH_4^+ that is essential for the N supply to the biosphere.

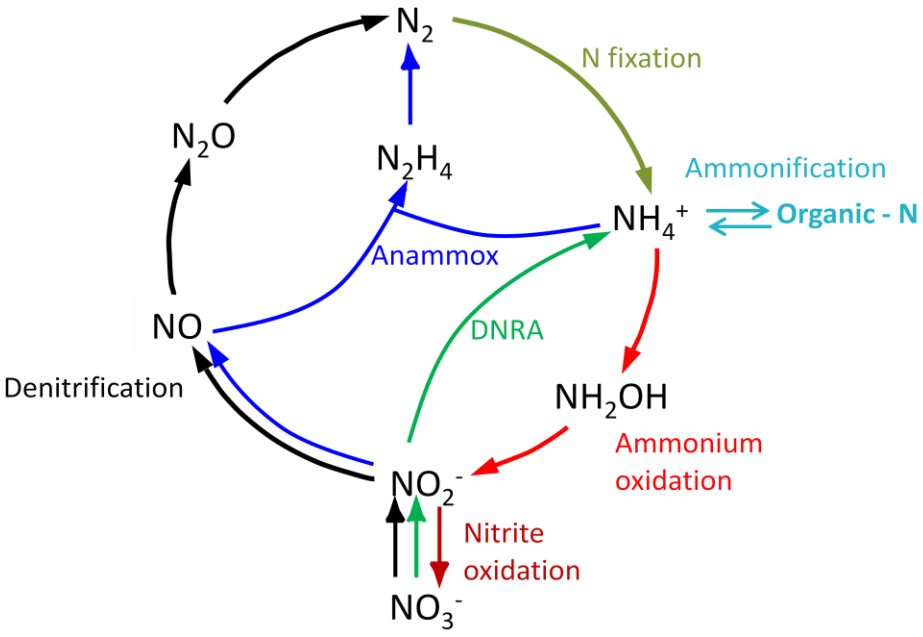

Figure 1.1: Simplified overview of the biological nitrogen cycle.

Besides its role as a nutrient, each N compound can serve either as an electron donor or acceptor in a variety of microbiological reactions because N compounds have a wide range of oxidation states from +5 to -3 (Figure 1.1). These biologically mediated N transformations lead to acceleration of the N cycle.

Once $N_2$is fixed into NH_4^+, other forms of fixed N are formed mainly by biologically mediated transformations of NH_4^+. Also, a dead plant and animal are decomposed, organic N compounds are transformed back to NH_4^+ by bacteria or fungi. This process is so-called ammonification. Oxidation of NH_4^+ generally occurs in oxic environments and it is divided into two steps, ammonia (NH_3) oxidation and nitrite (NO_2^-) oxidation. In an NH_3 oxidation process, NH_3 is oxidized to NO_2^-via hydroxylamine (NH_2OH). In a NO_2^- oxidation process, NO_2^- is oxidized to nitrate (NO_3^-). Both NH_3 and NO_2^- oxidation are usually autotrophic processes.

Denitrification is responsible for loss of fixed N to atmosphere. Mainly in the absence of oxygen (O_2), many bacterial speciesutilize NO_3^- as a terminal electron acceptor instead of O_2, produce N_2 and release N_2 back into the atmosphere. In the process, NO_3^-, NO_2^-, NO and N_2O are successively reduced to N_2. Surprisingly, with the discovery of anaerobic ammonium oxidation (anammox), it has been verified that anammox bacteria carry out anaerobic oxidation of NH_4^+ to N_2 with NO_2^- as an electron acceptor (Karat et al., 2013). In the anammox process, NO_2^- is reduced to NO and then NO and NH_4^+form hydrazine (N_2H_4), which are finally oxidized to N_2. Dissimilatory nitrate and nitrite reduction to ammonium (DNRA) is another microbial process which only occurred under the strict anaerobic conditions. It is divided into two steps: reduction of NO_3^- to NO_2^- and NO_2^- to NH_4^+.

1.2 ENVIRONMENTAL PROBLEMS WITH NITROGEN

During the last 150 years, human activities have significantly influenced the global N cycle. At present, the human activities such as the applying of fertilizers for agriculture or fossil fuel combustion for energy generation are responsible for approximately 50% of the production of fixed N (Canfield et al., 2010). Moreover, most of the anthropogenic N input is used to meet the ever growing demand for agriculture, which led to an 800% increase in the use of N fertilizer between 1960 and 2000 (Almstrand, 2012). However, the low efficiency of N fertilization causes discharging of large amount of fixed N into natural water bodies. In addition, untreated or treated domestic and industrial wastewaters are the rich source of a variety of fixed N compounds. Entering nutrients such as N and phosphorus into natural water bodies ultimately leads to nutrient pollution in water bodies. Today, N pollution is considered as one of the primary causes of eutrophication in water systems (Howarth & Marino, 2006). When N is oversupplied into

natural water bodies, excess growth of plants and algae occurs. After the decay of such plants and algae, DO decreases during the decomposition of these organic materials, resulting in hypoxia. This process is called eutrophication. Eutrophication results in the septic conditions, odor problems, incidence of fish kills in natural water bodies. In addition, NH_3causes direct toxic effects on aquatic life. Higher NO_3^-concentration in drinking water causes some serious human disease like blue baby syndrome. High NH_3 concentration in the raw water for a drinking water treatment facility requires a higher chlorine dosage to achieve free chlorine residual enough for disinfection. Gaseous N compounds such as NO, N_2O, and NO_2 have adverse effects on environment and human health, which are explained in detail in the section 1.4.2 in this thesis.

1.3 BIOLOGICAL NITROGEN REMOVAL FROM WASTEWATERS

The control of the N amount entering into water bodies is growing concern. Hence, many governments and organizations introduced stringent effluent standards for N. As a result, N removal has become common practice in many communal wastewater treatment plants.

N is removed from wastewaters by physicochemical and biological processes. The typical physicochemical N removal processes are air stripping, breakpoint chlorination and selective ion exchange (Lee et al., 2006). By air stripping of wastewater, gaseous $NH_3$is stripped out from wastewater. Even though this process is simple, high cost and expensive operational and maintenance costs are the main drawbacks. In the breakpoint chlorination process, NH_3 in wastewater can be oxidized with better control (Harp, 2003). Selective ion exchange using zeolites is another simple technologically to remove NH_3 from wastewater (Sarioglu, 2005; Bowman, 2003).

In general, N is removed from wastewater by biological processes. Because, the biological wastewater treatment process shows advantages over physiochemical N removal process such as high removal efficiency, easy process control and high process stability and reliability (Lee et al., 2006; Tchobanoglous et al., 2003).There are several processes for biological N removal. The most common approach is a combination of two-step process consisting of aerobic, autotrophic nitrification and subsequent anoxic, heterotrophic denitrification. This is also referred to as a

conventional microbial N removal process. The second alternative is a combination of aerobic partial nitrification (PN) and anaerobic anammox processes.

1.3.1 A conventional biological nitrogen removal process.

In the conventional method of microbial N removal process, N is removed based on autotrophic nitrification and heterotrophic denitrification. The autrotrophic nitrification is further split between two biological reactions, oxidation of NH_3 to NO_2^- and subsequent oxidation of $NO_2^-$to NO_3^-.

In the NH_3oxidation, autotrophic ammonia-oxidizing bacteria (AOB) oxidize NH_3 to NO_2^- via hydroxylamine (NH_2OH) under oxic conditions (Equations 1.1 and 1.2). Membrane-bound ammonia monooxygenase (Amo) and hydroxylamine oxidoreductase (Hao) are responsible for oxidation of NH_3 and NH_2OH in nitrification, respectively. Due to their slow growth rates and sensitivity to environmental factors, nitrification has often been considered as an unreliable and failure-prone process (Bellucci, et al., 2011).

$$\mathbf{NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O} \qquad \textbf{Equation 1.1}$$

$$\mathbf{NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^-} \qquad \textbf{Equation 1.2}$$

Combination of equations 1.1 and 1.2 yields equation 1.3.

$$\mathbf{NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+} \qquad \textbf{Equation 1.3}$$

In the second step of nitrification, nitrite-oxidizing bacteria (NOB) oxidize NO_2^- into NO_3^- with the membrane-bound nitrite oxidoreductase (Nxr) (Equation 1.4).

$$\mathbf{NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-} \qquad \textbf{Equation 1.4}$$

In the heterotrophic denitrification process, $NO_3^-$is reduced via several intermediates to N_2 (equations 1.5, 1.6, 1.7 and 1.8). Denitrification is mediated by several key enzymes. Membrane-bound NO_3^- reductase (Nar) involves the reduction of NO_3^- to NO_2^-. The main enzyme

responsible for reduction of $NO_2^-$to NO is NO_2^- reductase (Nir). Extensive studies on Nir enzyme revealed that some bacteria involved in denitrification has two completely different periplasmic enzymes; a heme-containing cytochrome cd1 Nir (NirS) and a copper-containing Nir (NirK) (Cutruzzola, 1999). The reductions of NO to N_2O and N_2O to N_2 in denitrification process are mediated by NO reductase (Nor) and N_2O reductase (Nos), respectively. A variety of electron donors, such as organic substances in wastewater, methanol and acetate are used in the denitrification process.

$$\mathbf{NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O} \qquad \textbf{Equation 1.5}$$

$$\mathbf{NO_2^- + 2H^+ + 2e^- \rightarrow NO + H_2O} \qquad \textbf{Equation 1.6}$$

$$\mathbf{2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O} \qquad \textbf{Equation 1.7}$$

$$\mathbf{N_2O + H^+ + e^- \rightarrow N_2 + H_2O} \qquad \textbf{Equation 1.8}$$

The entire process or parts of it can be performed by denitrifying bacteria. However, ability to denitrify is widespread among bacteria and archaea and has been observed even in some nitrifying bacteria (Bock and Wagner, 2006).

N removal via a conventional method has been modified and many advanced N removal processes such as Bardenpho process, postdenitrification, predenitrification, step feeding process and sequencing batch mode have been developed to minimize the disadvantages and limitations of a conventional method (Tchobanoglous et al. 2003; Khin and Annachhatre 2004; Dapena-Mora et al. 2004; Zhu et al. 2005, 2007a, 2007b).

1.3.2 Partial nitrification and anammox processes

Recently, N removal from wastewaters by a combination of PN and anammox processes has drawn our attention as an alternative to a conventional nitrification-denitrification process (Kampschreur, et al., 2008b; Desloover, et al., 2011; Joss et al., 2009). NH_3 is oxidized to NO_2^- by AOB with O_2 in a PN reactor and $NH_4^+$is oxidized to N_2 by NO_2^- that is produced in the preceding PN reactor. The stoichiometry of PN and anammox reactions is given by Equations 1.3 and 1.9, respectively (Feng et al., 2007).

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow$$
$$1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \qquad \textbf{Equation 1.9}$$

A stable PN that oxidizes approximately 57% of $NH_4^+$to $NO_2^-$is essential for the subsequent anammox process according to Equation 1.9. A PN-anammox process provides great advantages over conventional nitrification–denitrification processes. Conventional nitrification-denitrification processes require large amount of energy for aeration during NH_3 oxidation to NO_3^-. However, since a PN process oxidizes about half of the NH_3 in wastewater to NO_2^-, it requires significantly less amount of energy for aeration for PN reaction. In addition, conventional nitrification-denitrification processes may need addition of an organic carbon source to remove NO_3^- in denitrification process. In contrast, since both PN and anammox processes are completely autotrophic process, a PN-anammox process can be operated without carbon addition. Moreover, since both AOB and anammox bacteria have slow growth rates, the amount of excess sludge produced in a PN-anammox process is smaller than that in a conventional nitrification–denitrification process. It provides additional credit to PN-anammox process, because sludge treatment requires extra cost and energy (Kartal et al., 2010;Fux, et al., 2002).

After first full-scale anammox reactor operation in the world was started in Rotterdam, Netherland (Mulder et al., 2001), number of full-scale PN-anammox plants installation are steady increased (Lackner et al., 2014) (Figure 1.2). More than 100 full-scale PN-anammox installations are available worldwide by 2014. Table 1.1 shows details of some full-scale PN-anammox processes operated in worldwide. The majority of PN-anammox plants are installed in Europe. However, implementation of PN-anammox processes as side-stream treatment process is interested in North American region (Lackner et al., 2014). PN-anammox process treats variety of wastewaters such as rejects water, industrial wastewater from food processing industry, beverage industry, leachate, etc. In addition, some PN-anammox plants treat digested sludge liquor and wastewater from semiconductor plants. According to the comprehensive survey by Lackner et al. (2014) on full-scale PN-anammox plant installation in the world, designed N load to the plants are varied from 0.02 to 10 kg-N m^3 d^{-1}.

Table 1.1: Detail of some full-scale PN-anammox reactors operated in the world

Type of reactor	City, Country	Source of wastewater	Reactor volume (m^3)	N loading rate (kg-N m^{-3} d^{-1})
Contrinous flow Nitritation /anammox	Rotterdam, NL	Digester liquor	1500/70	14
Nitritation/anammox	Mei prefecture, JP	Semiconductor plant	50	4.4
Floc-based PN/anammox (four-stage)	Bergen op Zoom, NL	Potato processing effluent	2370/1650	0.23
PN/anammox SBR	Balingen, DE	Reject water	705	0.17
Simultaneous PN/anammox and denitrification process	Taiwan	Landfill lechate	394	0.5
One-stage PN/anammox	Weibenfels, DE	Reject water	2250	0.49
PN/anammox SBR	Breda, NL	Reject water	1000	0.99
PN/anammox SBR	St. Gallen, CH	Reject water	300	0.72
PN/anammox SBR	Pfannenstiel, CH	Reject water	320	0.23
PN/anammox biofilm reactor	Shangdong, CN	Distillery	560	2
Two-stage PN/anammox	Budrio, IT	Digestate liquor	1509	0.23
PN/anammox biofilm reactor	Minworth, UK	Reject water	1760	2.3
PN/anammox biofilm reactor	Yixing, CN	Sweetener	1600	1.4

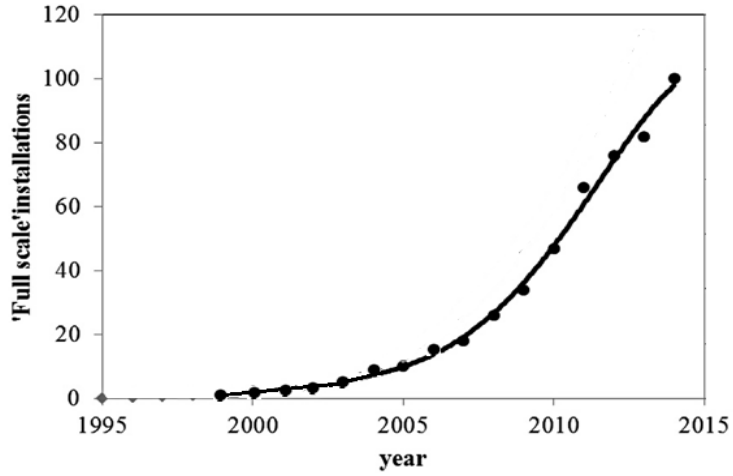

Figure 1.2: Cumulative development of full-sacel PN-anammox plants installations. (Taken from Lancker et al., 2014 and modified)

1.3.3 Granular partial nitrification reactors

Granular biomass is a compact and dense microbial biofilm or aggregate with a nearly spherical outer shape. Recently, granular PN reactors have attracted attention due to their advantages. The main advantages of granular PN processes over conventional activated sludge system are lower footprint requirements for reactor construction, higher treatment capacity (Val del Rio, 2012). Dense and compact structure of the granular biomass enhance the settleability of biomass. Therefore, wastewater treatment without secondary clarifiers can be achieved (Beun et al., 1999). Moreover, the volume required for granular sludge reactor is smaller than that in conventional activated sludge system. Because, granular sludge reactors are designed with a large hight to diameter ratio. In addition, the compact biomass in a granular reactor allows to treat wastewater with high substrate loading rates and anoxic condition which developed in inner part of the granular biomass allows to simultaneously occur different types of processes (*e.g.*, nitrification in outer oxic layer and denitrification in deeper anoxic layer) in the single reactor (Song et al., 2013). As shown in Figure 1.3, the more than 20% of full scale PN-anammox processes are operated as granular process (Lackner et al., 2014). More importantly, considering the average nitrogen-load per plant, granular systems contribute to treat most N (Figure 1.3) (Lackner et al., 2014). Therefore, for this study, our attention was attracted to nitrogen removal with PN (granular) and Anammox process.

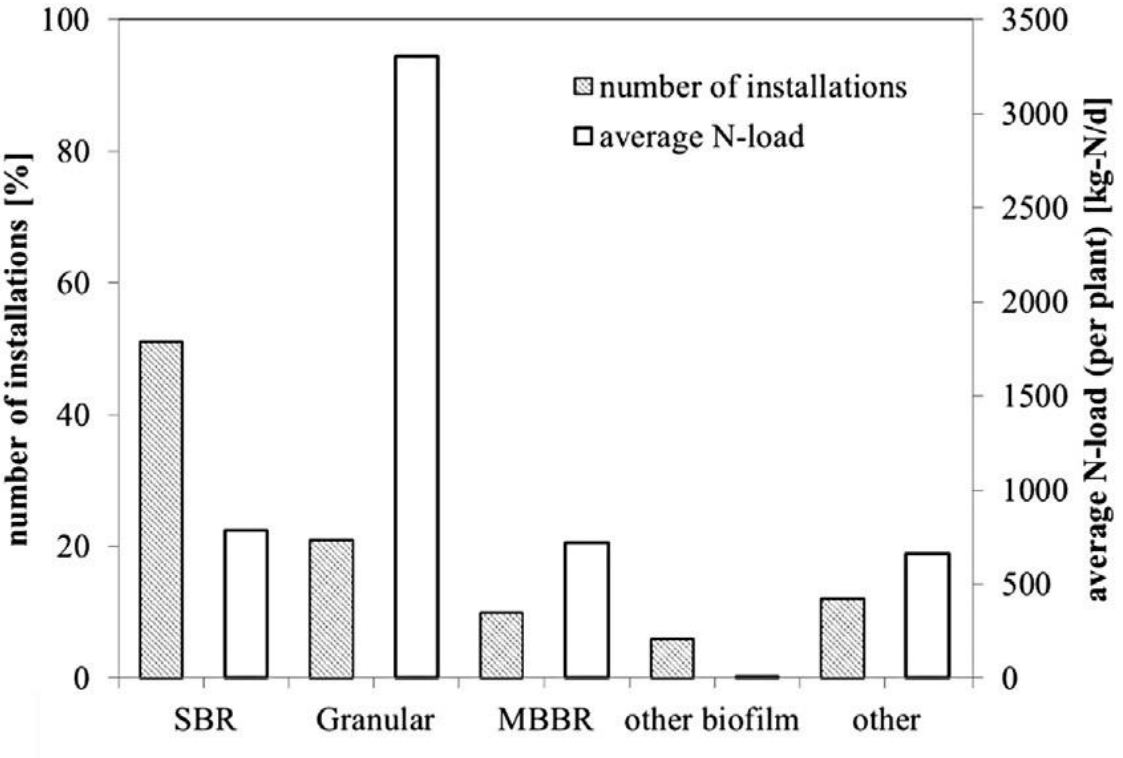

Figure 1.3: The distribution of different types of full-scale PN-anammox processes and their N loading rates (Taken and modified from Lackner et al., 2014)

1.3.4 Sequential batch reactor

The N removal system in sequencing batch reactor (SBR) is a fill and draw process and wastewater is treated in a single reactor. A one cycle of a SBR is consists of five different phases namely fill, react, settle, draw, and idle. Figure 1.4 shows a typical SBR operation system. During the fill phase, wastewater is filled to reactor. Filling is done in static, mixed or aerated condition depending on the requirement. Once, filling is completed, react phase which supplies suitable environmental condition for biological reaction (e.g. oxygenation by aeration for nitrification) is initiated. The duration of react phase depends on the quality of the effluent. When react phase is finished, treated effluent is separated by settling in the settle phase for successful separation of biomass and effluent. Then effluent is withdrawn from the reactor in draw phase. The idle phase is inserted between draw and filling. During idle phase, excess sludge is removed from the reactor. Idle phase can be eliminated by solid wasting during the reaction phase.

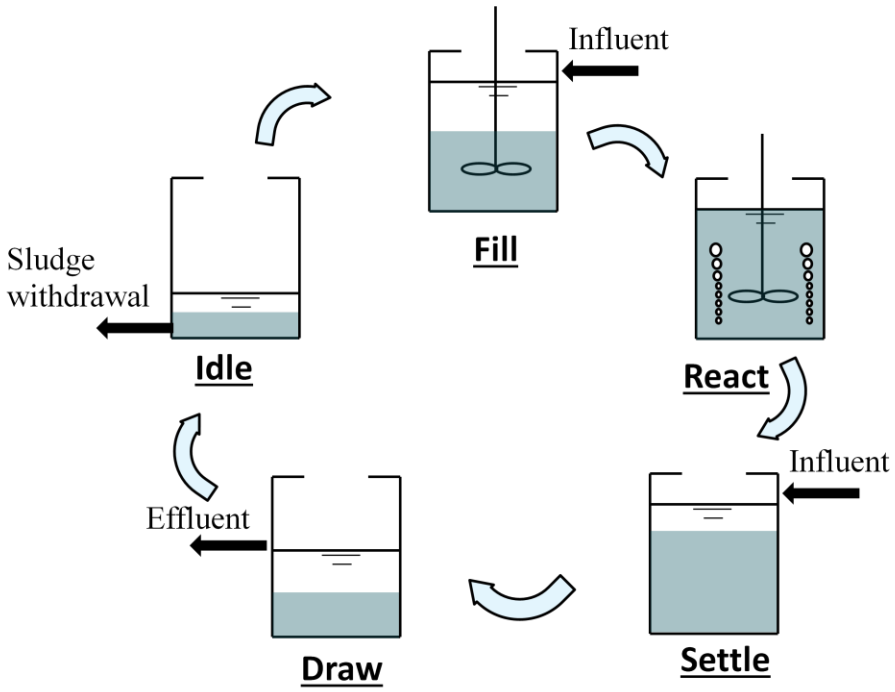

Figure 1.4: Five diffrenet phases in a typical SBR cycle

Wastewater treatment in SBR systems provides advantages over continuous flow wastewater treatment systems. Since SBR is a single reactor system, necessity of separate tank or reactors for equalization, primary clarification biological treatment, and secondary clarification can be eliminated. Another advantage of a SBR system is flexibility of operation and control. This operational flexibility provides SBRs to meet many different treatment requirements. In addition to these advantages, SBRs require minimal footprint for low capital cost due to eliminating clarifiers and other required equipments. Therefore, as shown in Figure 1.3, more than 50% of full-scale PN-anammox systems in the world are operated as SBR (Lackner et al., 2014).

1.4 N_2O AND NO PRODUCTION DURING MICROBIAL NITROGEN REMOVAL PROCESSES

1.4.1 Environmental and ecological importance of N_2O and NO

N_2O is one of the six greenhouse gases cited in the Kyoto Protocol (the Intergovernmental Panel on Climate Change (IPCC) 2013). In 2004, N_2O accounts for 6.9% of the global anthropogenic greenhouse gas emissions (EPA, 2012). N_2O is expected to be the most dominant ozone-depleting substance in the 21st century (Ravishankara et al., 2009). The atmospheric N_2O concentration has increased since 1750 from around 270 ppb to 324 ppb in 2011 (Figure 1.5,)

(IPCC 2013). The annual source of N_2O from the Earth's surface has increased by about 40 to 50% over pre-industrial levels as a result of human activity (IPCC 2007). The main anthropogenic source of N_2O is agriculture and N_2O is also produced naturally from a wide variety of biological sources in soil and water, particularly microbial action in wet tropical forests (IPCC 2013). IPCC (2013) has identified sewerage treatment as an important contributor of anthropogenic source of N_2O.

The global N_2O emission from wastewater sector is approximatly account for 2.4% of total anthropogenic N_2O emissions (EPA, 2012). N_2O emission in wastewater handling in United Kingdom (UK) accounts about 3.9% in 2009. Although the relative contribution of wastewater treatment to total anthropogenic N_2O emissions is small fraction, the N_2O emission from wastewater treatment is increased with the time (Skiba et al., 2015; EPA, 2012). For example, between 1990 and 2005, global N_2O emissions in the world from domestic wastewater treatment are increased by about 20% (EPA, 2012) and global N_2O emission are projected to be increased by approximately 22% from 2005 t0 2030. In addition, N_2O emission in wastewater sector has increased by 10% from 1999 to 2009 in UK (Skiba et al., 2015).

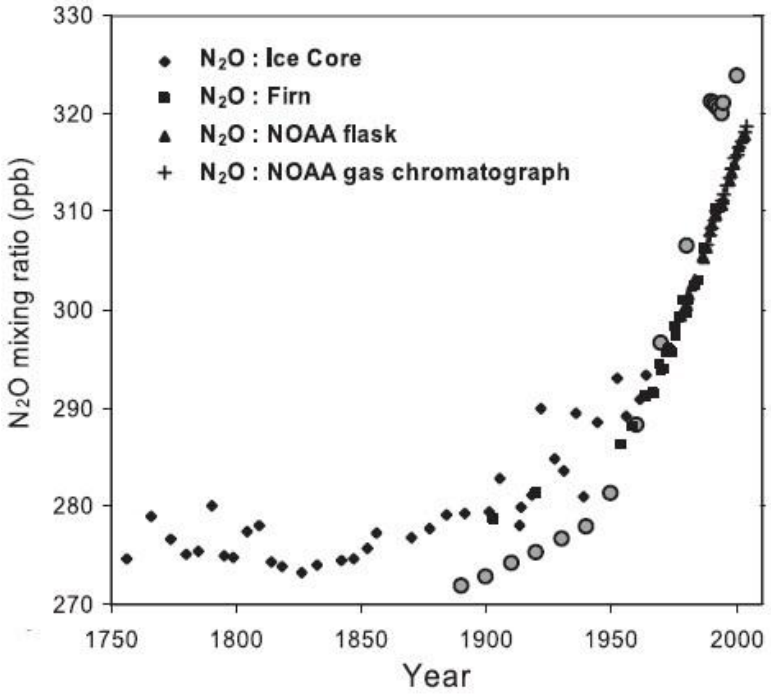

Figure 1.5: Change in N_2O abundance in the atmosphere for the last 1,000 years as determined from ice cores, firn, flask and whole air samples. NOAA is referred for National Oceanic and Atmospheric Administration (Taken from IPCC 2007and modified).

NO and nitrogen dioxide (NO_2) which is an oxidation byproduct of NO are important atmospheric trace gases and they have shown direct effect on the ozone chemistry of the atmosphere (Schreiber, et al., 2008). Due to its hydrophobicity, NO can easily diffuse over cell membranes of organisms. In addition to the reactivity of NO with proteins containing transition metals and oxygen, it has a mutagenic effect in bacteria. In addition, NO, NO_2 and N_2O gases are toxic to humans and therefore relatively low maximum 8-h exposure limits are defined by the Dutch government (MAC values, as of 1/1/2007) of 0.2, 0.2 and 83 ppm, respectively (Kampschreur, et al., 2008b). Besides the environmental effect and toxicity on human of NO, the ecological influence of NO on microbial consortia has been reported. Several studies have reported that NO serve as a toxicant for microorganisms (Wink and Mitchell, 1998; Zumff, 1997; 1993). NO regulates the specific microbial activities (Zart et al., 2000; Nadeem et al., 2013) and growth mode (Schmidt et al., 2004) of biofilm. In addition, NO can also enhance the formation (Schmidt et al., 2004) or dispersion of biofilm (Barraud et al., 2006; 2009).

1.4.2 N_2O and NO emission from wastewater treatment processes

Many studies reported that N_2O and NO emission factors (amount of N_2O or NO emitted relative to N load) for full-scale and lab-scale wastewater treatment plants substantially vary over ranges from 0 to 25% and 0 to 95%, respectively (Law, et al., 2012b; Kampschreur, et al., 2009b). Such large variations might be attributed to the different reactor configurations and operational conditions. Furthermore, this result implies that N_2O and NO can be further reduced through proper plant design and operations. N_2O and NO emissions from full-scale and lab-scale nitritation and anammox processes reported in the previous studies are summarized in Table 1.2.

In general, N_2O and NO emission levels were higher in the conventional nitrogen removal processes (<16% of incoming nitrogen) than those in nitrogen removal via anammox process (Kampschreur et al., 2009b). However, N_2O production in the PN–anammox process still needs to be reduced because N_2O emission from an energy-saving and cost-effective PN-anammox process would hamper the practical application (Okabe et al., 2011). To the best of our knowledge, very limited information on mechanisms of and strategies for reduction of N_2O and NO emission from PN-SBR processes and are available in literature.

Table 1.2: N_2O and NO emission factors reported during wastewater treatment process.

Process used in the WWTP	Type of wastewater	N_2O emission (% of N-influent)	NO emission (% of N-influent)	Referances
Nitritation-anammox	Sludge digester liquor	2.3	0.2	Kampschreur, et al., 2008b
Intermittent activated sludge	Municipal sewerage	0.47		Peu et al., 2006
Floc-based PN-anammox	Anaerobically digested industrial wastewater	5.1- 6.6		Desloover, et al., 2011
PN–SBR	Digester Liquid	0.4–0.6	Close to detection limit	Joss et al., 2009
PN SBR	Synthetic wastewater with organic carbon	5.6		Ishii et al., 2014
Lab-scale two reactor PN-anammox	Synthetic wastewater without organic carbon	4.1		Okabe et al., 2011
Full-Scale single-stage nitritation-anammox	Wastewater from a potato processing factory and the rejection water of a municipal sludge dewatering plant	1.2	0.005	Kampschreur et al., 2009a
Continuous nitrifying activated sludge	Synthetic wastewater	2.3–16		Zheng et al., 1994
Continuous anaerobic–anoxic SBR activated sludge	Synthetic wastewater	90		Zeng et al., 2003
Continuous oxic–anoxic SBR activated sludge	Synthetic wastewater	5–95		van Benthum et al.,1998
PN SBR	Synthetic wastewater	0.75		Kong et al., 2013b
Continuous oxic–anoxic activated sludge SBR	Real high strength wastewater	1–35		Osada et al., 1995

1.4.3 Analytical methods of N_2O and NO

N_2O and NO measurements in gas and liquid.

In full-scale wastewater treatment processes, N_2O and NO emitted from reactors are usually captured using closed floating chambers. Owing to lack of online measurements of N_2O and NO in the past, samples had been obtained from the head space of a reactor. In addition, floating chambers were used to collect grap samples from reactors. Although the emitted N_2O and NO can be captured through the floating chambers, dynamic changers in the N_2O and NO emission profiles cannot be captured by the grab samples (Law et al., 2012b). Therefore, online, continuous monitoring of N_2O and NO of the headspace allows for real-time monitoring of N_2O and NO emissions (Law et al., 2012b).N_2O and NO in liquid-phase have been measured by off-line grab sampling followed by gas chromatography analysis (Okabe et al., 2011; Desloover et al., 2011). Recently, dissolved N_2O and NO in liquid-phase was continuously monitored with N_2O and NO microsensors (Kampschreur, et al., 2008a; Law et al., 2011).

In-situ N_2O and NO measurements in biofilm

N_2O and NO production in wastewater treatment processes are mainly attributed to the activities of complex microbial communities (see section 1.3.2). Classical microbiological techniques to analyze microbial function and activities of such complex microbial communities like isolation and physiological characterization of microorganisms have limitations (Okabe, et al., 2004). Therefore, appropriate methods with sufficiently high spatial resolution are needed for identification and measurements of their in situ activities with relation to N_2O and NO production. Microsensors have attracted attention as a powerful and reliable tool for identification of in situ activities of complex microbial communities. The configurations of amperometric and potentiometric microsensors are shown in Figure 1.6.

An amperometric microsensor measures the current caused by electrochemical reaction of the analyte (e.g., O_2, NO and N_2O) at the tip of a microsensor. The measured current is proportional to the concentration of the analyte. The electrochemical reaction is driven by a potential difference between the working and reference electrodes (Figure 1.6) (Santegoeds, et al., 1998).

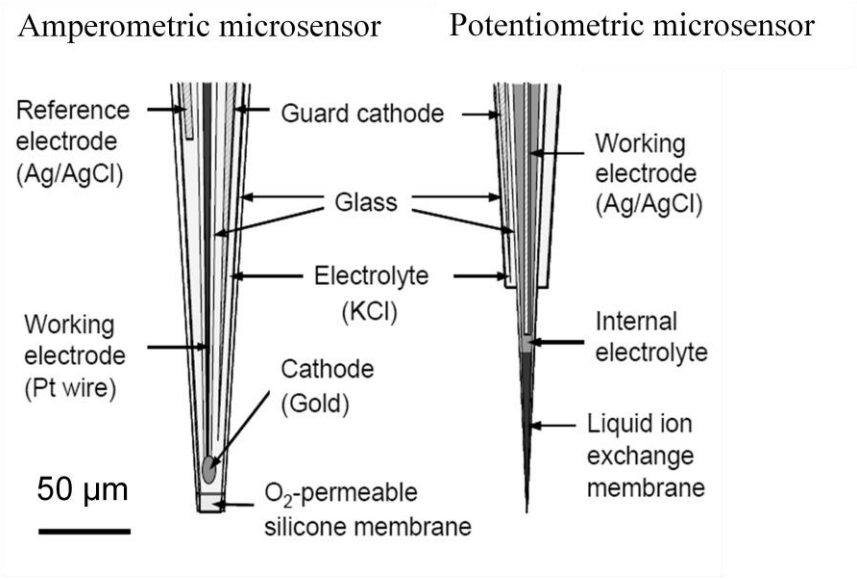

Figure 1.6: The structure of the amperometric and potentiometric (LIX) microsensors (Taken from Okabe et al., 2004 and modified).

A potentiometric microsensor is based on charge separation of an ion across a membrane, called a liquid ion exchanging (LIX) membrane. An electrical potential difference is hereby generated according to Nernst equation (Equation 1.10).

$$\Delta E = \frac{RT}{zF} ln\left(\frac{a_i}{a_e}\right) = E_o + klog(a_i) \qquad \textbf{Equation 1.10}$$

In Equation 1.10, R is the gas constant, T is the absolute temperature, Z is the charge of the target ion, F is the Faraday constant, a_i and a_e are the ion activities in the sample and in the electrolyte solution, respectively. As a_e can be considered constant, the electrical potential difference across the membrane becomes proportional to the logarithm of the ion activity in the sample. The formula can be simplified as shown above introducing a constant k and an off-set potential E_o.

The spatial resolution of a microsensor is about two times of the tip diameter of a microsensor as long as analyte consumption by the microsensor is negligible and the microsensor is small enough to cause minimum disturbance on microbial aggregate. The typical tip diameter of a microsensor applied to biofilm analysis is about 10 µm (Figure 1.6), indicating the spatial resolution of about 20 µm. This spatial resolution is good enough to characterize the concentration gradients across the biofilms, microbial mats and sediments grown in the

environment and to calculate the net areal and volumetric production and consumption rates at a certain depth or of whole microbial community (Schramm, 2003). Okabe et al. (2011) conducted microsensor measurements on anammox granule obtained from lab-scale PN-anammox reactors, demonstrating that anammox activity and N_2O production are spatially separated. Therefore, application of microsensors is very important to understand in situ microbial activities regarding N_2O and NO production with sufficient spatial resolutions.

The use of NO-reactive fluorescent indicators, which allows for bio imaging of NO with high spatial and temporal resolution in conjunction with fluorescence microscopy, was reported (Kojima et al., 1999). Probably the most successful indicator for NO is 4,5-diaminofluorescein diacetate (DAF2-DA) and 4-amino-5-methylamino- 2′,7′-difluorescein (DAF-FM). NO-reactive fluorescent indicators did not exhibit strong fluorescent signals but emitted very strong signal once the indicators combined with NO molecules (Figure 1.7) (Kartel et al., 2011). Kartel et al. (2011) (Figure 1.7B and Law et al. (2011) used these indicators to detect NO accumulation in anammox bacteria and aerobic nitrifying culture, respectively.

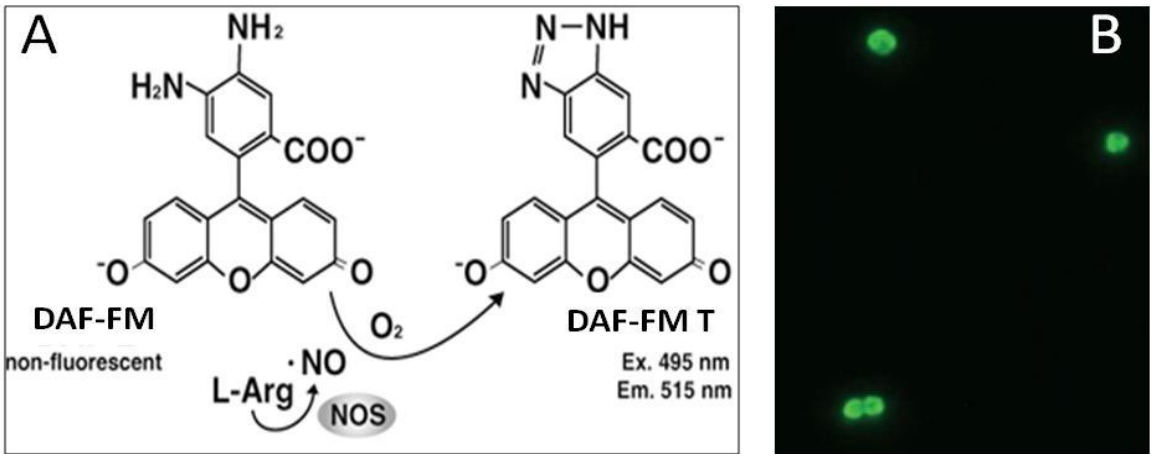

Figure 1.7: (A) Structures of fluorinated DAF-FM before and after reacting with NO molecules. (B) Epifluorescence image of DAF2-DA derivative of NO formed during conversion by anammox bacteria (Taken from Kartel et al., 2011 and modified)

1.4.4 Pathways of N_2O and NO production during wastewater treatment

N_2O and NO can be produced during wastewater treatment due to several nitrogen conversion reactions. Nitrification and denitrification, and chemodenitrification contribute on N_2O and NO

production. The key metabolic and chemical reactions involved in N_2O and NO production are reviewed in this section.

Nitrifier denitrification.

Nitrifier denitrification is pathway of nitrification, the oxidation of NO_2^- to NO, N_2O, and N_2 (Figure 1.8).The sequence of reactions is carried out by only AOB. However, only genes NirK and Nor are present in the AOB but not Nos (Casciotti and Ward, 2001). Therefore, N_2O may be the end product of the nitrifier denitrification pathway rather than N_2 (Figure 1.8). NH_2OH, hydrogen (H_2) and NH_3 can act as electron donors for nitrifier denitrification (Law et al., 2012b). N_2O production via nitrifier denitrification pathway played big role especially under anoxic and sub-oxic conditions (Kampschreur, et al., 2008a; Wrage et al., 2001). However, some recent reports have suggested that NO rather N_2O was main product of nitrifier denitrification under strict anoxic conditions (Kester et al., 1997; Yu et al., 2011).

Autotrophic ammonia oxidation

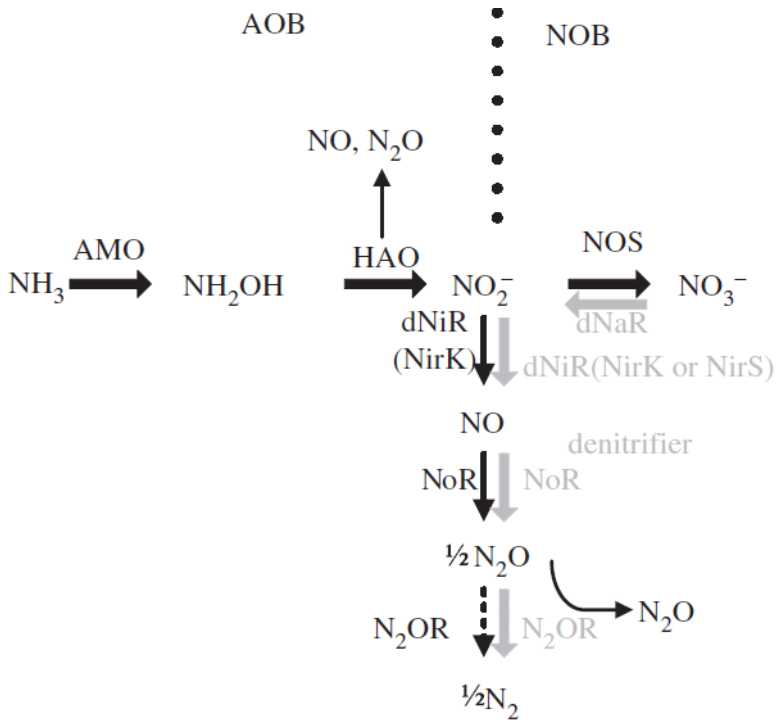

Figure 1.8: Nitrogen transformation pathways of AOB, NOB and denitrifying bacteria. Large dotted line divides AOB and NOB pathways and grey colour shows denitrifying pathway (Taken from Law et al., 2012b and modified).

As described in section 2.2.1, NH_3 oxidation to NO_2^- is consisted of two steps. $NH_3$is first converted into NH_2OH by Amo and as subsequent step NH_2OH is converted to NO_2^- by Hao. It has been reported that NH_2OH oxidation split into two reactions involves: (i) conversion of NH_2OH to nitosyl radical (NOH), and (ii) conversion of NOH to NO_2^-. N_2O (Igarashi et al., 1997) and NO can be produced as an intermediate during the enzymatic splitting of NOH and due to unstable breakdown of NOH, respectively (Poughon et al., 2001).

Heterotrophic denitrification

Heterotrophic denitrification is the reduction of nitrate, nitrite, NO, and N_2O into N_2 coupled to oxidation of organic substrates (Figure 1.8). Since N_2O and NO are intermediate of denitrification pathway, those can be produce because of incomplete denitrification (Kampschreur, et al., 2009a; Okabe et al., 2011; Itokava et al., 2001). Genes Nor and Nos have higher nitrogen turnover than Nar and Nir. This indicates that NO and N_2O could be completely reduced without occurrences of their accumulation or emissions. However, fluctuations in environmental conditions inhibit or influence the activity of N_2O reductase and NO reductase resulting of accumulation and emission of N_2O and NO due to unbalance enzyme activity (Law et al., 2012b).

Anammox process

In the anammox process, $NH_4^+$is anaerobially oxidized in to N_2 gas. Using inhibition studies and fluorescent labelling of the cells NO has been recognized as an intermediate was of metabolic pathways of anammox bacteria (Figure 1.9) (Kartel et al., 2011). Some recent studies have repoted that small amount of NO is emitted from anammox reactors. (Kampscheur et al., 2008a; 2008b; 2009a). However, it has been suggested previously that anammox bacteria do not produce N_2O under physiologically relevant conditions (Kartel et al., 2007).

Chemodenitrification

In WWTPs N_2O formation is possible by the reaction between NO_2^- and NH_2OH and NO and an N_2O and NO_2^- reduction with inorganic or organic compounds (van Cleemput, 1998). In the first reaction, NH_2OH is produced by AOB, and it is complicated to distinguished the chemical and biological N_2O production.

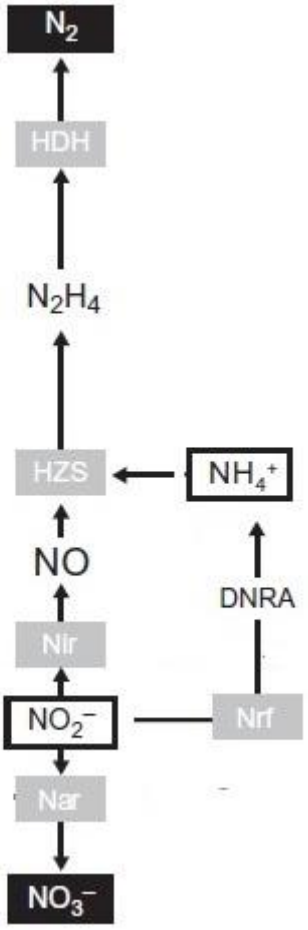

Figure 1.9: The metabolism of anammox bacteria. HDH, hydrazine dehydrogenase; HZS, hydrazine synthase; Nar, nitrite:nitrate oxidoreductase; Nir, nitrite::nitric oxide oxidoreductase; nrf, nitrite:ammonium oxidoreductaseDNRA, dissimilatory nitrite reduction to ammonium (Taken and modified from Kartel et al., 2012).

1.4.5 Factors affecting on N_2O and NO production during WW tearments.

N_2O and NO emission levels from the WWTPs are extremely variable depending on the many operational parameters such as dissolve oxygen (DO), NO_2^- concentration, carbon availability, pH, and rapid changing of process conditions (Kampschreur et al., 2009a; Okabe et al., 2011).

Dissolved oxygen concentration (DO)

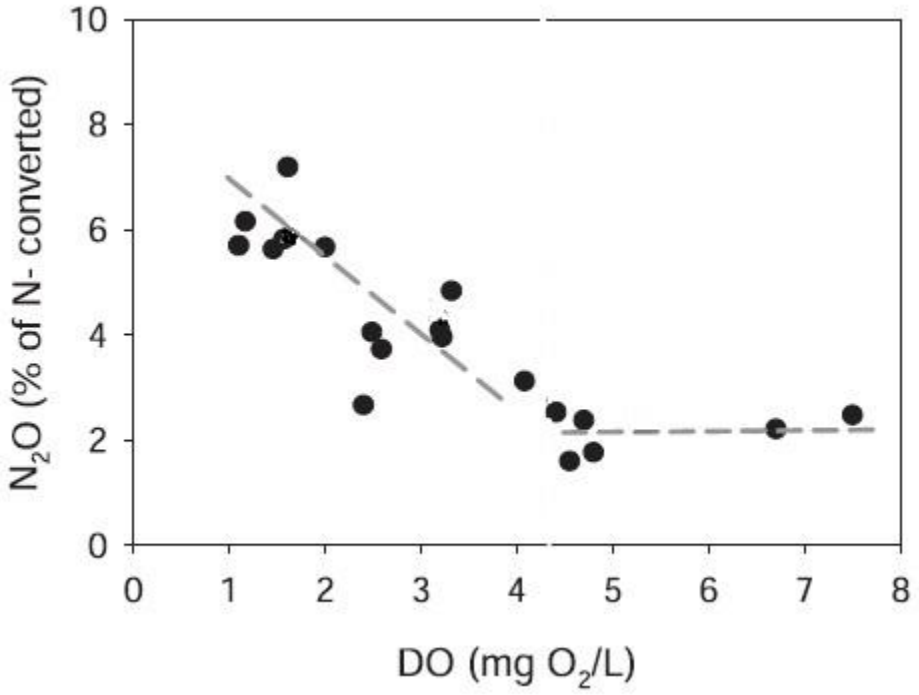

Figure 1.10: The relationship between DO concentration the N_2O emission factor (Taken and modified from Pijuan et al., 2014).

Among above-mentioned factors, DO concentration is considered as very important controlling parameter for N_2O and NO emission during both nitrification and denitrification (Zheng et al., 1994; Tallec et al., 2006; Kampschreur et al., 2008a). Oxygen limitation conditions lead to higher N_2O and NO production through nitrifier denitrification (Tallec, et al., 2006; Kampschreur, et al., 2008a). Moreover, when oxygen concentrations below1 mgL^{-1} to 10% of the N load was can correspond to N_2O production (Goreau et al., 1980). In contrast, increment of N_2O and NO emission at the high airflow rates (*i.e* high DO) was reported (Kampschreur, et al., 2008b; Law et al., 2012a; Furuya et al., 2013). Law et al. (2012a) have reported a clear exponential relationship between the biomass specific N_2O production rate and nitrification rate which resulted due to change in DO concentration of an enriched AOB culture. Moreover,Pijuan et al. (2014) reported that N_2O emissions in a continuous granular airlift nitritation reactor from ammonium-rich reject wastewater were DO dependent in the range of 1- 4.5 $mg\text{-}O_2\ L^{-1}$, and N_2O emissions increasing within this range with decreasing the DO values and at higher DO concentrations above 4.5 $mg\text{-}O_2L^{-1}$, N_2O emissions remained constant (Figure 1.10). During the denitrification process,

oxygen inhibits both synthesis and activity of denitrifying enzymes. The presence of small amount of oxygen during the denitrification leads to N_2O emission because Nos is much sensitive to DO than other enzymes (Otte et al., 1999).

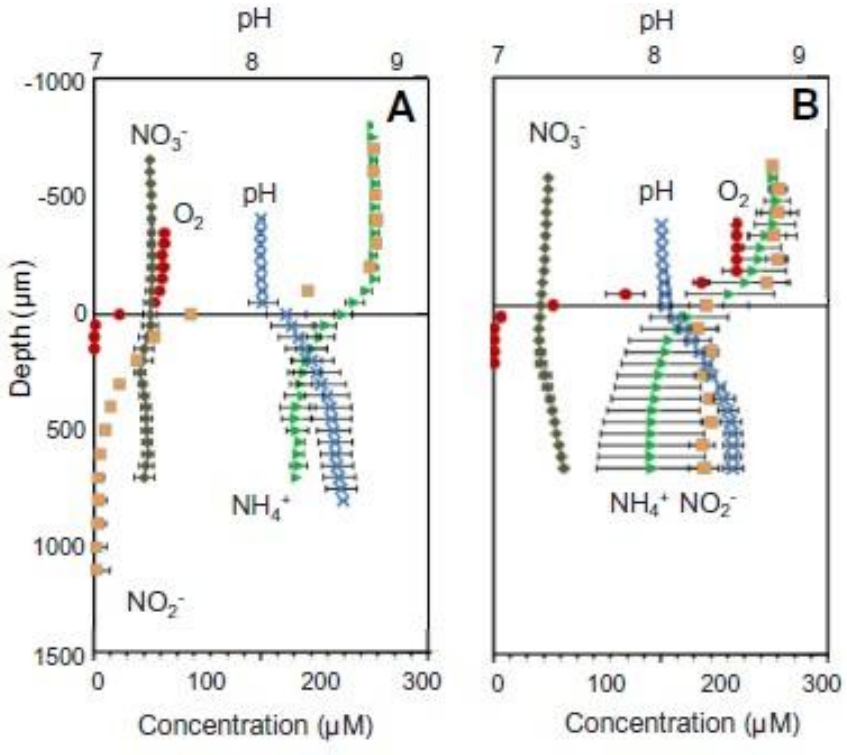

Figure 1.11: The steady state concentration profiles of NH_4^+, NO_2^-, NO_3^{-1}, O_2, and pH in the aerobic PN granules with DO levels of 2.5 mg L^{-1} (A) and 7 mg L^{-1} (B). The means ± standard deviations (n = 3) are shown (Taken and modified from Song et al., 2013).

One reason for the complexity of DO effect on N_2O emission process is probably that the physicochemical parameters in microbial aggregates (e.g., bacterial granules, biofilms and activated sludge flocs) could be different from those in bulk liquid. For example, phenomenon regarded of the effect of DO on N_2O production and its pathways from activated sludge systems may not exactly valid for granular PN systems. Because, very compact and ticker structure of granular biomasses (Li et al., 2008; Liu and Tay, 2008) may results steep gradients of DO inside the PN granules. The changes in DO concentration from 2.5 mgL^{-1} to 7.0 mgL^{-1} in the bulk liquid in a PN-SBR did not affect the DO concentrations inside the PN granules (Figure 1.11). Therefore, it is essential to measure in situ N_2O production and DO concentration in granules to better understanding of DO related N_2O emission from granular wastewater treatment processes.

Nitrite concentration

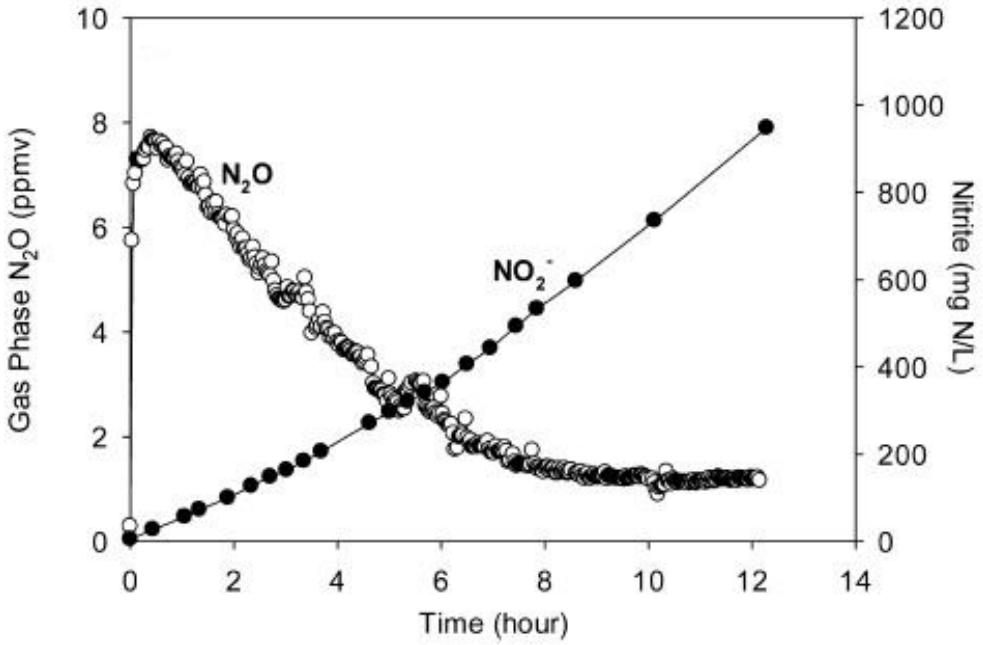

Figure 1.12: The effect of NO_2^- accumulation on the specific N_2O production rate (Taken and modified from Law et al., 2013).

It is well known that the concentration of NO_2^-leads to increase the N_2O and NO during both nitrification and denitrification. During nitrification increased NO_2^-concentration leads to production of N_2O and NO via nitrifier denitrification (Colliver and Stephenson, 2000). Furthermore, when denitrification occurs at high NO_2^- concentration causes lower denitrification rate and it results the accumulation of NO and N_2O (Schulthess et al., 1995). Kampschreur et al., (2008a) have reported that NO level in the wastewater treatment reactors has been directly depending on the NO_2^-level in the reactors. They reported that higher NO is emitted when NO_2^- concentration is increased. In addition, Tallec et al. (2006) reported a DO depended 4- to 8 fold increased N_2O emission during nitrification due to artificial NO_2^- pulses of 10 mg L^{-1}. Interestingly, in contrast, N_2O emission from an enriched AOB culture was deceased with increased in NO_2^- concentration (Figure 1.12) (Law et al., 2013).

Biodegradable organic carbon to nitrogen ratio (COD/N)

The limited availability of biodegradable carbon is a very important governing factor on NO and N_2O production during denitrification process (Chung and Chung, 2000; Schalk-Otte et al.,

2000). At law availability of carbon, various denitrifying enzymes compete for carbon resulting incomplete denitrification. COD/N of 4 is required for complete denitrification. With the availability of limited carbon sources, the Nar, Nir, Nor and Nos genes compete for electrons which results an incomplete denitrification (Law et al., 2012b). The importance of availability of carbon source has been widely reported (Itokawa et al., 2001; Kishida et al., 2004; Schalk-otte et al., 2000). For example, about 20–30% of influent N was emitted as N_2O at COD/N was lower than 3.5 of an intermittently aerated laboratory scale reactor (Itokawa et al., 2001) which agreed with the observations by Kishida et al. (2004). In addition, a study with pure culture of *A. faecalis* shows that N_2O formation increases by 32–64%, and N_2 production decreases significantly when carbon sources are limiting (Schalk-otte et al., 2000). Interestingly, when extra carbon source was supplied to avoid electron competition, formation of N_2O decreased immediately. In contrast, less N_2O generation and emission has been observed regardless of carbon deficiency in full-scale studies that only (Ahn et al., 2010).

Rapid changing in process conditions

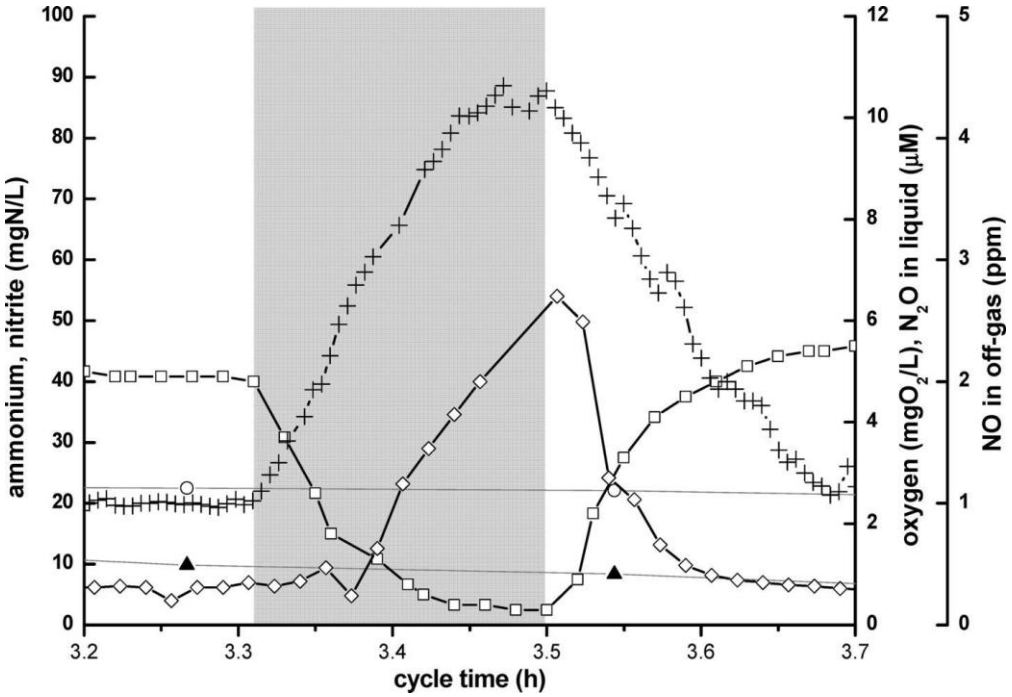

Figure 1.13: Fluctuation of NO/N_2O emissions with the change of DO concentration (the shaded area shows the period in which air was replaced by N_2). Symbols: concentration of NH_4^+ **(▲),** NO_2^- (○), DO (□), and NO (◊) and N_2O (+) (Taken and modified from Kampschreur et al., 2008a).

The rapid changing of process conditions is considered an important factor for N_2O and NO production during the wastewater treatment plants. The experiments have clearly shown that an elevated NO and N_2O emission due to rapidly changes in concentrations of NO_2^-, NH_4^+, and DO (Kampschreur, et al., 2008a; Burgess, et al., 2002;Yu et al., 2010). For examples, the transition from aerobic to anoxic conditions, N_2O and NO production rates from a nitrifying culture was increased instantly (Figure 1.13) and the NO production increased immediately with NO_2^- pulsing under both aerobic condition (Figure 1.14) (Kampschreur et al., 2008a). Yu et al. (2010) observed that N_2O was produced when pure AOB culture transits from anoxic to aerobic condition where as NO were produced when culture was switch from aerobic to anoxic condition. Probably, bacteria needs times to respond to change in environmental conditions, resulting higher emission of N_2O and NO at rapid changing of conditions in wastewater treatment processes. For example, N_2O emission by *A. faecalis was* reduced from 86% to 28% of NO_2^-converted after 10 cycles of pulse-wise addition of substrate (Schalk-Otte et al., 2000).

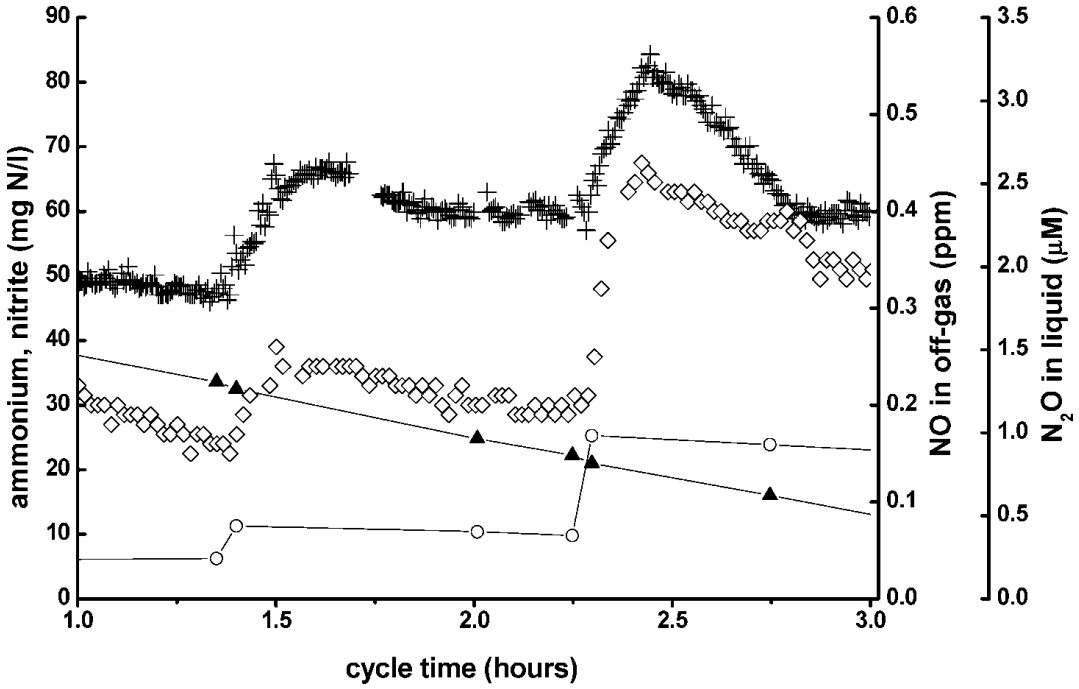

Figure 1.14: NO_2^- pulses (5 and 15 mg N NO_2^-/L) in the aerobic NH_4^+ oxidation phase cause increase in NO and N_2O emissions. Symbols: concentrations of NO in gas (◊) and N_2O (+), NH_4^+ (▲), and NO_2^- (o) in liquid (Taken from Kampschreur et al., 2008a).

pH

Most of the researchers believed that pH plays a minor or no role on N_2O and NO production from WWTPs. Because, WWTPs have being operated pH between 7.0 and 8.0 or rather stable (Kampscheur et al., 2009b). However, some studies have challenged this statement. N_2O production of an enriched AOB culture was much highest at pH 8.0 when compared with pH 6.0 and pH 9.0 (Law et al., 2011) (Figure 1.15). In addition, N_2O emission via heterotrophic denitrification increased with the decrease in pH (Okabe et al., 2011; Hanaki et al., 1992; Thoern and Sorensson, 1996). A possible explanation for pH dependent N_2O production is that the lower pH ranges might be due primarily to the interference of N_2O reductase by free nitrous acid (FNA). Because, Zhou et al. (2008) reported that 0.004 mg HNO_2-N L^{-1} of FNA completely inhibited N_2O reductase during denitrification. However, exact effect of pH on N_2O and NO production is still need to be investigated.

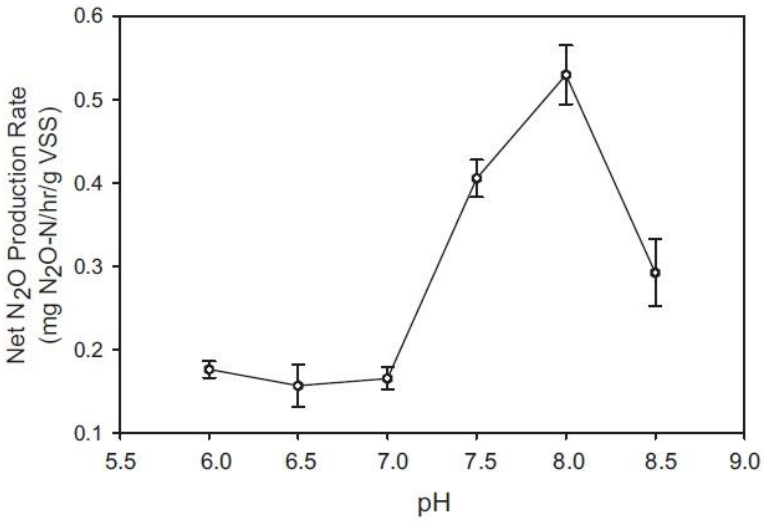

Figure 1.15: The effect of pH on the net N_2Oproduction rate (Taken and modified from Law et al., 2011).

The significant fluctuations of pH during the operations of WWTPs specially in sequencing batch reactors (SBRs) have also been reported. Song et al. (2013) reported that the pH value in a cycle of PN-SBR was fluctuated between 6.8 to 8.3 (Figure 1.16) indicating that pH changes in SBR are significant. Therefore, pH effect on N_2O production in SBR should be further investigated.

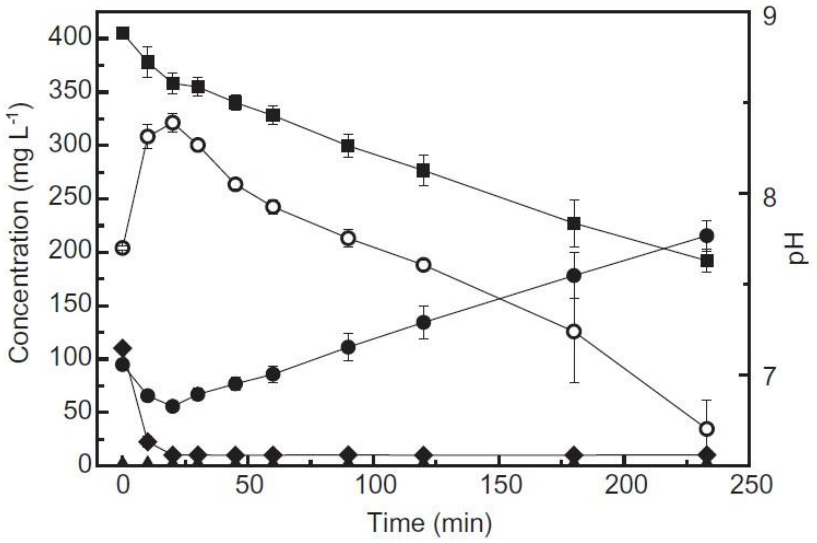

Figure 1.16: Time course of the concentrations of N compounds, TOC, and pH in the PN aerobic granule reactor during one cycle condition in a steady state. Measurements were made in three successive cycles, and the means and standard deviations are shown. Legend: NH_4^+ (■), $NO2^-$ (●), NO_3^- (▲), TOC (♦) concentrations, and pH (○) with an air flow of 1 L min L^{-1} (Taken and modified from Song et al., 2013).

Other factors

Moreover, many other minor factors such as solid retention time, toxic compounds, and temperatures are also considered as controlling factors of N_2O and NO emission from WWTPs (Kampschreur et al., 2009b).

1.5 AIMS OF THIS STUDY

This main objective of this study is to use of multiple analytical techniques is indispensable to our knowledge of N_2O and NO production mechanisms during N removal via PN-SBR and anammox granules. PN-SBR and anammox process was selected for our study due to it is advantages over conventional nitrogen removal process, and popularity of granular PN-annamox processes in the world. The key prerequisites to control N_2O emission from PN process is to identifying the N_2O production mechanisms and the influencing factors on the N_2O emission and its production pathways. Since most of the PN reactors implemented in the world is in granular and SBR mode, the specific aims were first, to denitrifying the mechanisms of N_2O production in granular-SBR PN reactor. Identification of key N_2O production pathway is a challenge, because physiochemical condition in PN-SBR is dynamic. In second, investigation of effect of DO and

pH as important parameters on N_2O production rates and its pathways in granular PN reactor; and third to investigate the NO emission pathways, influencing factors from annular anammox process. Because, although NO emission from anammox reactor was reported, no study was done to investigate these details. These objectives can contribute in understanding the N_2O and NO production mechanisms and influence of some important environmental condition on N_2O and NO production from granular PN and anammox process. Ultimately, the finding of this study may be used as manage the N_2O and NO production during biological nitrogen removal.

1.6 ORGANIZATION OF THIS THESIS

The organization of the dissertation is as follows. The thesis consists of five chapters. Chapter one describes the background of the study and provides a detailed literature review pertaining to the research area. It will provide the reader an impression regarding the need of this kind of study. Chapter 2 discusses the results of N_2O produced and its production pathways in an autotrophic granular PN-SBR. In this study, a lab-scale PN-SBR was operated and N_2O emission from the reactor was determined with an on-line monitoring system and source of N_2O was determined using polyphase experiments. Chapter 3 presents the results related to identify the effects of DO concentration and pH on N_2O production pathways and rates of autotrophic PN granules taken from the autotrophic PN-SBR and identify the microbial community structure in the PN granules. Results and findings obtained from the experiments are discussed in detail. Chapter 4 presents the results related to NO production from anammox granules and responsible microbial group of NO productions. Anammox granules were taken from granular column reactor and batch experiments and insitu measurements of NO production were carried out. Results and findings obtained from the experiments are discussed in detail. Last chapter, Chapter 5 concludes this thesis, highlighting the main contributions of this research along with some recommendations on future research directions.

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Chapter 2

Source identification of nitrous oxide on autotrophic partial nitrification in a granular sludge reactor

2 SOURCE IDENTIFICATION OF NITROUS OXIDE ON AUTOTROPHIC PARTIAL NITRIFICATION IN A GRANULAR SLUDGE REACTOR

2.1 INTRODUCTION

Nitrous oxide (N_2O) emissions draw attention since N_2O is expected to be a major stratospheric ozone-depleting substance in the future (Ravishankara et al., 2009) and is an important greenhouse gas with a global warming potential of about 300 times higher than CO_2 (Desloover et al., 2012; IPCC, 2007). It is generally accepted that nitrogen removal processes in a wastewater treatment system are an anthropogenic source of N_2O (Desloover et al., 2012). Conventionally, biological nitrogen removal is achieved by a combination of nitrification and denitrification processes. In contrast, an alternative and innovative approach is the use of a partial nitrification (PN) process followed by an anaerobic ammonium oxidation (anammox) process (PN-anammox process), which has several advantages, such as no need for external carbon addition, less energy and oxygen requirement, and less sludge production (van Dongen et al., 2001; Kartal et al., 2010). The PN-anammox process is applicable to reject water (Desloover et al., 2011; Kampschreur et al., 2008; 2009a; Joss et al., 2009; Okabe et al., 2011b), landfill leachate(Wang et al., 2010), and wastewater from semiconductor factory (Tokutomi et al., 2011).N_2O emission from PN-anammox processes, especially from the PN process, has been reported (Desloover et al., 2011; Kampschreur et al., 2008; Okabe et al., 2011b). Especially, a granular sludge reactor for PN process draws attention because of high specific nitrification rate, efficient biomass retention and excellent settleability. There are three main microbial pathways involved in N_2O production.

During nitrification, it is produced from hydroxylamine (NH_2OH) as a side product of the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) (Poughon et al., 2001; Hooper and Terry, 1979). During denitrification, N_2O is produced as an intermediate during reduction of nitrate (NO_3^-) to N_2 by heterotrophic denitrifiers (Lu and Chandran, 2010; Schmidt et al., 2004). Some ammonia-oxidizing bacteria (AOB) reduce NO_2^- to N_2O or N_2 through a process called nitrifier denitrification (Tallect et al., 2006; Wrage et al., 2001; Colliver and Stephenson, 2000). Many studies have been conducted to estimate N_2O emission rate of PN processes (Kong et al., 2013a; Kong et al., 2013b; Okabe et al., 2011b; Law et al., 2011; Desloover et al., 2011; de Graff et al., 2010; Kampscheur et al., 2008). In contrast, there are few studies on N_2O production pathways.

Nitrifier denitrification was the key biological pathway of N_2O production in an intermittently aerated sequencing batch biofilm reactor for PN treating synthetic ammonium-rich wastewater (Kong et al., 2013b) while NH_2OH oxidation pathway was the main source of N_2O in a sequencing batch reactor (SBR) for PN (PN-SBR) (Law et al., 2011; Yang et al., 2009). To determine which pathway is responsible for N_2O production in a wastewater treatment process is still challenging, because a variety of operational parameters (concentrations and loading rates of nitrogenous compounds, dissolved oxygen (DO) and organic carbon, pH, a ratio of organic carbon and nitrogenous compounds (COD/N) and temperature) influence N_2O production in a PN process (Tallec et al., 2006; Kampschreur et al., 2009b; Desloover et al., 2012; Wunderlin et al., 2012; Law et al., 2011). Furthermore, their temporal changes also affect N_2O production.

Analyses of the intermolecular distributions of ^{15}N in N_2O (isotopomers) are regarded as useful parameters to infer the predominant N_2O production pathway (Wunderlin et al., 2013; Sutka et al., 2006; Toyoda et al., 2005; 2011). Isotopomer ratios (site-specific N isotope ratios in asymmetric molecules of NNO) give us qualitative information on N_2O production and consumption pathways. Toyoda et al. (2011) and Wunderlin et al. (2013) conducted isotopomer analysis and distinguished N_2O produced during NH_2OH oxidation from N_2O produced during NO_2^- reduction in wastewater treatment processes. However, N_2O production pathways in a PN-SBR have not been investigated by isotopomer analysis. In a PN-SBR, temporal changes in the operational parameters (DO, NH_4^+ and NO_2^- concentrations and pH level) are more significant than conventional activated sludge processes, which likely play an important role in N_2O production pathways.

In this study, source of N_2O produced in an autotrophic granular PN-SBR was investigated. A lab-scale PN-SBR was operated and N_2O emission from the reactor was determined with an on-line monitoring system. Dissolved N_2O in the reactor was monitored with a microsensor for N_2O. N_2O, DO, pH, NH_4^+, NO_2^- and NO_3^- concentrations in the PN-SBR for one cycle were continuously monitored. We measured temporal changes in intermolecular ^{15}N-site preference (SP) in N_2O in the PN-SBR for one cycle. In addition, the spatial distribution of N_2O, DO, pH, NH_4^+, NO_2^- and NO_3^- in he PN granules were determined with the microsensors to estimate net production and consumption rates of N_2O, NH_4^+ and NO_2^- in single granules. The spatial distribution of AOB and other bacteria in the PN granules was determined by FISH. The

combination of microsensor measurements and FISH analysis allows us to deduce function of AOB.

Finally, these results were compared and we discussed the source of N_2O in the PN-SBR. Concentrations in the PN-SBR for one cycle were continuously monitored. We measured temporal changes in intermolecular ^{15}N-site preference (SP) in N_2O in the PN-SBR for one cycle. In addition, the spatial distribution of N_2O, DO, pH, NH_4^+, NO_2^- and NO_3^- in the PN granules were determined with the microsensors to estimate net production and consumption rates of N_2O, NH_4^+ and NO_2^- in single granules. The spatial distribution of AOB and other bacteria in the PN granules was determined by FISH. The combination of microsensor measurements and FISH analysis allows us to deduce function of AOB. Finally, these results were compared and we discussed the source of N_2O in the PN-SBR.

2.2 MATERIALS AND METHODS

2.2.1 Operation of a lab-scale autotrophic PN-SBR

A lab-scale autotrophic PN-SBR with working volume of 2.0 L was operated. The reactor was inoculated with 0.3 L of PN granules (3-5 mm in diameter), which was obtained from the PN reactor operated in our laboratory (Okabe et al., 2011b). One cycle of the reactor operation was 4 h. It consisted of feeding of a synthetic wastewater (3 min), aeration (232 min), settling of the granules (3 min), and discharging of treated wastewater (2 min). The composition of a synthetic wastewater was as follows: $(NH_4)_2SO_4$ (1650 mg L^{-1}), $KHCO_3$ (3300 mg L^{-1}), $CaCl_2 \cdot 2H_2O$ (135 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (300 mg L^{-1}), and KH_2PO_4 (22 mg L^{-1}). Trace element solutions I and II were prepared and added as described by van de Graaf et al. (1996). The influent pH was adjusted to 7.7 ± 0.1. The hydraulic retention time (HRT) of the PN reactor was fixed at 8 h. The airflow rate was changed according to reactor performance until the PN process became stable. After the PN process became stable, airflow rate was fixed at 0.2 L min^{-1}.

2.2.2 Water and gas analyses

The PN reactor performance was determined by collecting grab samples of influent and effluent at arbitrary time intervals during the operation. NH_4^+, NO_2^- and NO_3^- concentrations in the influent and effluent were measured by using ion-exchange chromatography (DX-100, DIONEX,

CA., USA) with an IonPac CS3 cation column and IonPac AS9 anion column after filtration with a 0.45-μm pore size membrane (ADVANTEC, Tokyo, Japan). The N_2O concentrations in the off-gas from the reactor were determined with a 1412 Photo acoustic Field Gas-Monitor (INNOVA, Copenhagen, Denmark). Grab samples were taken from 115 min to 125 min during 4-h cycles. For batch tests, the N_2O concentrations in the off-gas were determined once every minute. The dissolved N_2O (D-N_2O) concentration in the effluent of the reactor was measured with a N_2O microsensor (Unisense, Aarhus, Denmark). N_2O emission rate into the headspace of the PN-SBR was calculated by multiplying the N_2O concentration in the off-gas by gas emission rate, and D-N_2O discharge rate into the effluent of the PN-SBR was calculated by multiplying the D-N_2O concentration in the effluent by hydraulic flow rate.

2.2.3 Isotopomer analysis

Isotopomer ratios (δ) in N_2O in the off-gas from the PN reactor were measured to identify N_2O production pathway. The notations of isotopomer ratios are shown below.

$$\delta^{15}N^{\alpha} = ({}^{15}R^{\alpha}_{sample} - {}^{15}R^{\alpha}_{standard}) / {}^{15}R^{\alpha}_{standard} \quad \textbf{Equation 2.1}$$

$$\delta^{15}N^{\beta} = ({}^{15}R^{\beta}_{sample} - {}^{15}R^{\beta}_{standard}) / {}^{15}R^{\beta}_{standard} \quad \textbf{Equation 2.2}$$

Where, ${}^{15}R^{\alpha}$ and ${}^{15}R^{\beta}$ donates ${}^{14}N^{15}N^{16}O/{}^{14}N^{14}N^{16}O$ and ${}^{15}N^{14}N^{16}O/{}^{14}N^{14}N^{16}O$, respectively, for samples and standards (atmospheric N_2). Here, we define a certain parameter called ^{15}N-site preference (SP) as an illustrative parameter of intermolecular distribution of ^{15}N that was defined as follows (Toyoda et al., 2005; 2011).

$$\textbf{}^{15}\textbf{N-site preference (SP)} = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta} \quad \textbf{Equation 2.3}$$

The off-gas samples of the PN reactor were collected into evacuated 50 mL glass bottles at arbitrary time intervals in the aeration phase. The isotopomer ratios of the collected gas samples were measured on an isotope-ratio monitoring mass spectrometer (MAT 252; Thermo Fisher Scientific K.K, Yokohama, Japan) using an online analytical system at the Tokyo Institute of Technology, Japan (Toyoda et al., 2005; 2009; 2011). The precision of the isotopomer ratios were typically better than 0.5‰ for $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$. Characteristic SP values of 33‰ and 0‰ for

NH_2OH oxidation and NO_2^- reduction (nitrifier denitrification and heterotrophic bacterial denitrification), respectively, which were estimated in specific pure cultures, were used for estimation of the contribution to each process (Sutka et al., 2006). Approximate contributions of NH_2OH oxidation and NO_2^- reduction to N_2O production were estimated by assuming that each process is linearly proportional to the SP value using the following equation:

Contribution of NH_2OH oxidation (%) = SP/(SP for NH_2OH oxidation - SP for NO_2^- reduction) × 100 = SP/33 × 100 **Equation 3.4**

Contribution of NO_2^- reduction (%) = 100 - contribution of NH_2OH oxidation **Equation 3.5**

2.2.4 Microsensor measurements

The steady-state concentration profiles of DO, N_2O, NH_4^+, NO_2^-, NO_3^- and pH in the PN granules were measured in a synthetic medium for microsensor measurements with microsensors. DO and N_2O microsensors were purchased from Unisense (Aarhus, Denmark). LIX-type NH_4^+, NO_2^-, NO_3^- and pH microsensors were constructed in our laboratory as described by de Beer et al. (1997) and calibrated and used according to a protocol reported by Okabe et al. (1999a). The synthetic medium for the microsensor measurements of DO, N_2O and pH was as follows (mg L^{-1}): NaH_2PO_4 (19), $(NH_4)_2SO_4$ (990), $NaHCO_3$ (2770), $NaNO_2$ (690), $MgSO_4 \cdot 7H_2O$ (300), $CaCl_2 \cdot 2H_2O$ (135) and trace element solution I and II (van der Graaf et al., 1996). Trace element solution I contained EDTA (5 g L^{-1}) and $FeSO_4$ (5 g L^{-1}), and trace element solution I1 contained EDTA (15 g L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.43 g L^{-1}), $CoC1_2 \cdot 6H_2O$ (0.24 g L^{-1}), $MnC1_2 \cdot 4H_2O$ (0.99 g L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.25 g L^{-1}), $NaMoO_4 \cdot 2H_2O$ (0.22 g L^{-1}), $NiC1_2 \cdot 6H_2O$ (0.19 g L^{-1}), $NaSeO_4 \cdot 10H_2O$ (0.21 g L^{-1}), and H_3BO_4 (0.014 g L^{-1}).pH was adjusted to 7.5. For NH_4^+, NO_2^- and NO_3^- concentration measurements, the concentration of the species to be measured was adjusted to 250 μM, 250μM and 50μM, respectively. The PN granules with diameters of 2 to 3 mm were sampled from the reactor at 120 min after aeration was started and positioned with five insect needles in the flow chamber (2.0 L) that was filled with the synthetic medium. DO concentration in the medium was controlled at the required value by continuous bubbling with N_2 gas (99.9%) and/or atmospheric air, which also provided sufficient mixing of the medium. The granules were acclimated in the medium at least 3 h to ensure that steady-state

profiles were obtained. At least five concentration profiles of each species were measured in different granules taken in one cycle. The concentration profiles were determined in five cycles. Net volumetric production rates of N_2O, NH_4^+ and NO_2^- in the granules were estimated from the averaged steady-state concentration profiles by using Fick's second law of diffusion as previously described by Santegoeds et al. (1999). Diffusion coefficients of 1.38×10^{-5} cm^2 s^{-1}, 1.25×10^{-5} cm^2 s^{-1} and 2.10×10^{-5} cm^2 s^{-1}were used for NH_4^+, NO_2^- and N_2O, respectively, at 25°C for the calculation of net volumetric production rates (Okabe et al., 2011b).

2.2.5 Fluorescence in situ hybridization (FISH)

Ten granules were taken from the reactor in a cycle at 120 min after aeration was started. The sampling was conducted in five cycles from day 100 to day 200. The granule samples were fixed in 4% (w/v) paraformaldehyde solution at 4°C for 24 h, washed three times with phosphate-buffer saline (PBS; 10mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2), and embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) at -30°C overnight to infiltrate the OCT compound into granules. 20-μm-thick vertical thin sections were prepared by using a cryostat (Reichert-Jung Cryocut 1800, Leica, Bensheim, Germany). FISH was performed as previously described by Okabe et al. (1999b). The 16S rRNA-targeted probes used in our present study were as follows; Mixture of EUB, EUBII, and EUBIII probes (Daims et al., 1999) in an equimolar for all bacteria and Nso1225 probe (Mobarry et al., 1996) for betaproteobacterial ammonia-oxidizing bacteria. Hybridized samples were observed using a model LSM510 confocal laser-scanning microscope (CLSM, Carl Zeiss, Oberkochen, Germany) equipped with an Ar ion laser (458 and 488 nm) and two He-Ne ion lasers (543 and 633 nm).

2.3 RESULTS AND DISCUSSION

2.3.1 Reactor performance

Figure 2.1 shows concentrations of NH_4^+, NO_2^-, NO_3^- and D-N_2O in the influent and the effluent, N_2O concentrations in the off-gas, and N_2O emission rates into the headspace and D-N_2O discharge rate into the effluent of the PN-SBR. The reactor was operated at an average nitrogen loading rate (NLR) (± standard deviation) of 43 ± 2.7 mg-N L^{-1} h^{-1}. In the initial stage of the reactor operation, airflow rate was adjusted to achieve stable NO_2^- production. The stable PN was achieved at day 30 and the airflow rate was fixed at 0.2 L min^{-1}. The average

concentration of NH_4^+ in the influent was 350 ± 21 mg-N (Figure 2.1A). The average NH_4^+ and NO_2^- concentrations in the effluent were 168 ± 18 mg-N L^{-1} and 182 ± 29 mg-N L^{-1},

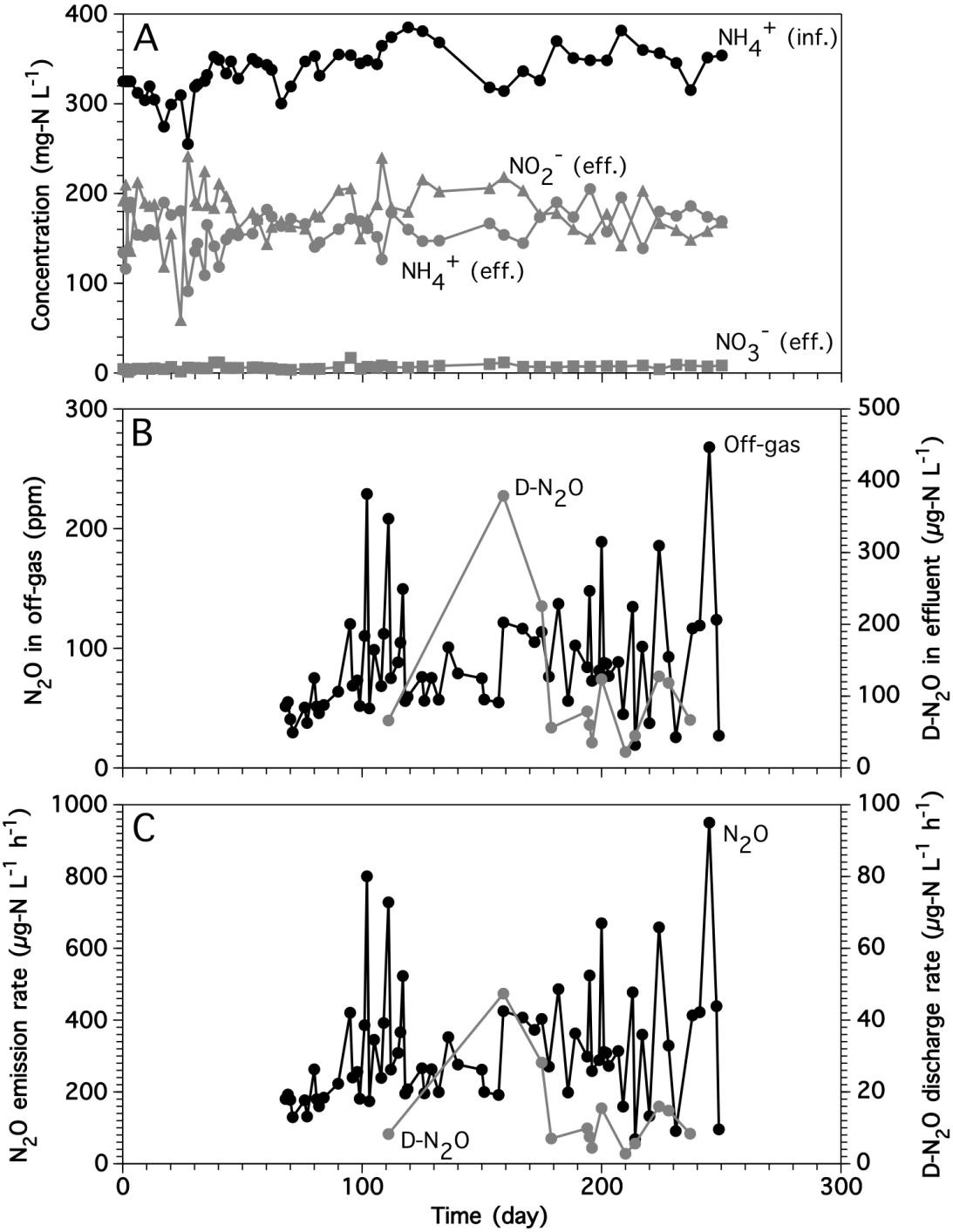

Figure 2.1: (A) Concentrations of NH_4^+ in the influent, and NH_4^+, NO_2^- and NO_3^- in the effluent of the PN reactor. (B) N_2O concentration in the off-gas and D-N_2O concentration in the effluent. (C) N_2O emission rates into the headspace and into the effluent.

respectively. Approximately 50% of the influent $NH_4^+$was converted to NO_2^- with the NH_4^+ oxidation rate of 22 ± 2.9 mg-N L^{-1} h^{-1}, indicating that a favorable NH_4^+/NO_2^- ratio for anammox process was achieved. NO_3^- concentration in the effluent was 0.5 ± 0.1 mM.

N_2O concentrations in the off-gas and the effluent of the PN reactor were measured (Figure 2.1B). The N_2O concentration in the off-gas varied from 30 ppm to 230 ppm (89 ±48 ppm on average). D-N_2O concentration in the effluent varied between 14 µg-N L^{-1} and 420 µg-N L^{-1}. Fluctuation of N_2O concentrations in the off-gas and the liquid phase might be due to fluctuation of airflow and hydraulic flow rates.

The N_2O emission and D-N_2O discharge rates from the PN-SBR are shown in Figure 2.1C. The N_2O emission rate from the PN reactor was 0.67 ± 0.34 mg-N h^{-1} per reactor and 0.32 ± 0.17 mg-N L^{-1} h^{-1} as specific rate. Fluctuation of the N_2O emission might be due to fluctuation of airflow and hydraulic flow rates, followed by the change in microbial activities of production or consumption of N_2O. A large portion (more than 96%) of N_2O produced in the PN reactor was evolved to the headspace by aeration. The average ratio of N_2O production rate to NLR was 0.8 ± 0.4% (or 1.5 ± 0.8% of the converted NH_4^+ in the PN reactor).

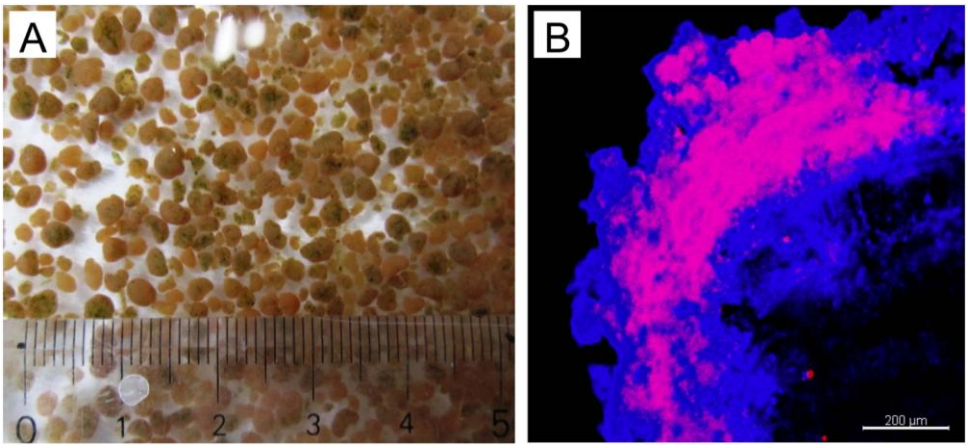

Figure 2.2: (A) An image of the PN granules. (B) Confocal laser scanning microscopeimages of thin cross-section of the PN granules showing in situ spatial distribution of AOB (magenta) and other bacteria (blue) after fluorescence in situ hybridization with Cy5-labeled EUB338 mix probe and TRITC-labeled Nso1225 probe.

Figure 2.2A shows an image of the PN granules. The average diameter and the settling velocity of PN granules were approximately 2 mm and 160 cm min^{-1}, respectively. FISH, using a TRITC-labeled Nso1225 probe and a Cy5-labeled EUB338 mix probe, revealed that the outer layer (ca. 600 μm thick) was dominated by bacteria and AOB were found in the upper 400 μm (Figure 2.2B). The probe specific for anammox bacteria was not applied.

Table 2.1: Summary of the ratios of N_2O production rate to nitrogen loading rate and parameters affecting the N_2O production rate in PN reactors

Type of reactor	NLR[a] (mmol-N/L/d)	N_2O emission factor[b] (%)	DO(mg/)	pH	Ref.
A lab-scale PN SBR	71 ± 7	0.8 ± 0.4	2.0 ± 0.3	7.4 – 7.8	This study
A full-scale nitritation CFR	46.3	0.85	2.5		Kampscheur et al. 2008
A single-stage PN-anammox reactor	71.4	0.6	5.0		Kampscheur et al. 2009a
A lab-scale PN CFR	37.1	0.3 – 1.3	4.1 ± 0.73	6.8 ± 0.33	de Graff et al. 2010
A lab-scale PN SBR	89.3	0.75	1.3	7.1 – 7.5	Kong et al., 2013b
A lab-scale PN SBR	214	0.4	1.0	6 – 7.5	Kong et al., 2013a
A lab-scale PN SBR	571	0.28	0.5 – 0.8	6.4 ± 0.05	Law et al., 2011
A lab-scale PN CFR	179	2.0 ± 0.8	< 2.0		Okabe et al., 2011b
A full-scale PN SBR	14.7	2.55 – 3.3	0.75 ± 0.05	7.5 ± 0.1	Desloover et al., 2011

[a] nitrogen loading rate.

[b] The N_2O emission factor was calculated to divide N_2O production rate (μmol-N L^{-1} h^{-1}) by NLR.

SBR: Sequencing batch reactor.

CFR: Continuous flow reactor.

The ratios of N_2O production rate to NLR and the parameters affecting the N_2O production rate in the PN-SBR were compared with those reported in the previous studies (Table 2.1). The ratio of N_2O production rate to NLR in this study (0.8%) was in the same order (between 0.28% and 0.85%) of the other reactors. The ratios of a lab-scale column biofilm reactor (Okabe et al.,

2011b) and a full scale floc based sequential PN reactor (Desloover et al., 2011) were higher than the other reactors. The variation in N_2O emission in previous studies (Table 2.1) is attributed to a complicated pathway of biological and chemical N_2O production, for example, NH_2OH oxidation by AOB, NO reduction by heterotrophic bacteria and AOB, and chemodenitrification (Poughon et al., 2001; Lu and Chandran, 2010; Wrage et al., 2001; van Cleemput, 1998) and consumption (N_2O reduction by heterotrophic bacteria and AOB (Pan et al., 2012; Schmidt et al., 2004)). Therefore, it was obvious that difference in operational parameters (DO concentration, NH_4^+ and NO_2^- concentration, COD/N ratio, NH_4^+ loading rate, and pH) of the PN reactors could strongly affect them (Kampschreur et al., 2009a; Law et al., 2011; Burgess et al., 2002).

2.3.2 Dynamic N_2O emission in one cycle of the PN-SBR operation

The dynamics of N_2O, NH_4^+, NO_2^-, NO_3^- and DO concentrations and pH level in one cycle of the PN-SBR are shown in Figure 2.3. In the settling period (-7 min to -4 min) both N_2O and D-N_2O concentrations decreased due to gas-liquid equilibrium and dilution of off-gas with air (an insertion panel in Figure 2.3A). In the discharging (-4 min to -2 min) and feeding (-2 min to 0 min) periods, N_2O concentrations in off-gas further decreased due to dilution with air. In contrast, D-N_2O concentration in the bulk liquid increased in the feeding period. N_2O accumulation in the settling granular sludge bed was experimentally confirmed by a N_2O microsensor measurement (Figure 2.4). Subsequently, N_2O concentration in off-gas suddenly increased just after starting the aeration due to release of N_2O from the bulk liquid and decreased over the operation. D-N_2O concentration was gradually increased in the initial 40 min of the aeration period and was decreased thereafter. Thus, the net N_2O production rate was higher in the initial phase of aeration period. These trends were reproducible. We measured these concentrations in five cycles and found the same trend of changes in them. However, the level of N_2O was different between each test, which agreed with the result shown in Figure 2.3C. Dynamics of N_2O emission from the PN-SBR suggests that the continuous measurement of N_2O in off-gas is necessary for reliable estimation of N_2O emission rate from a PN-SBR.

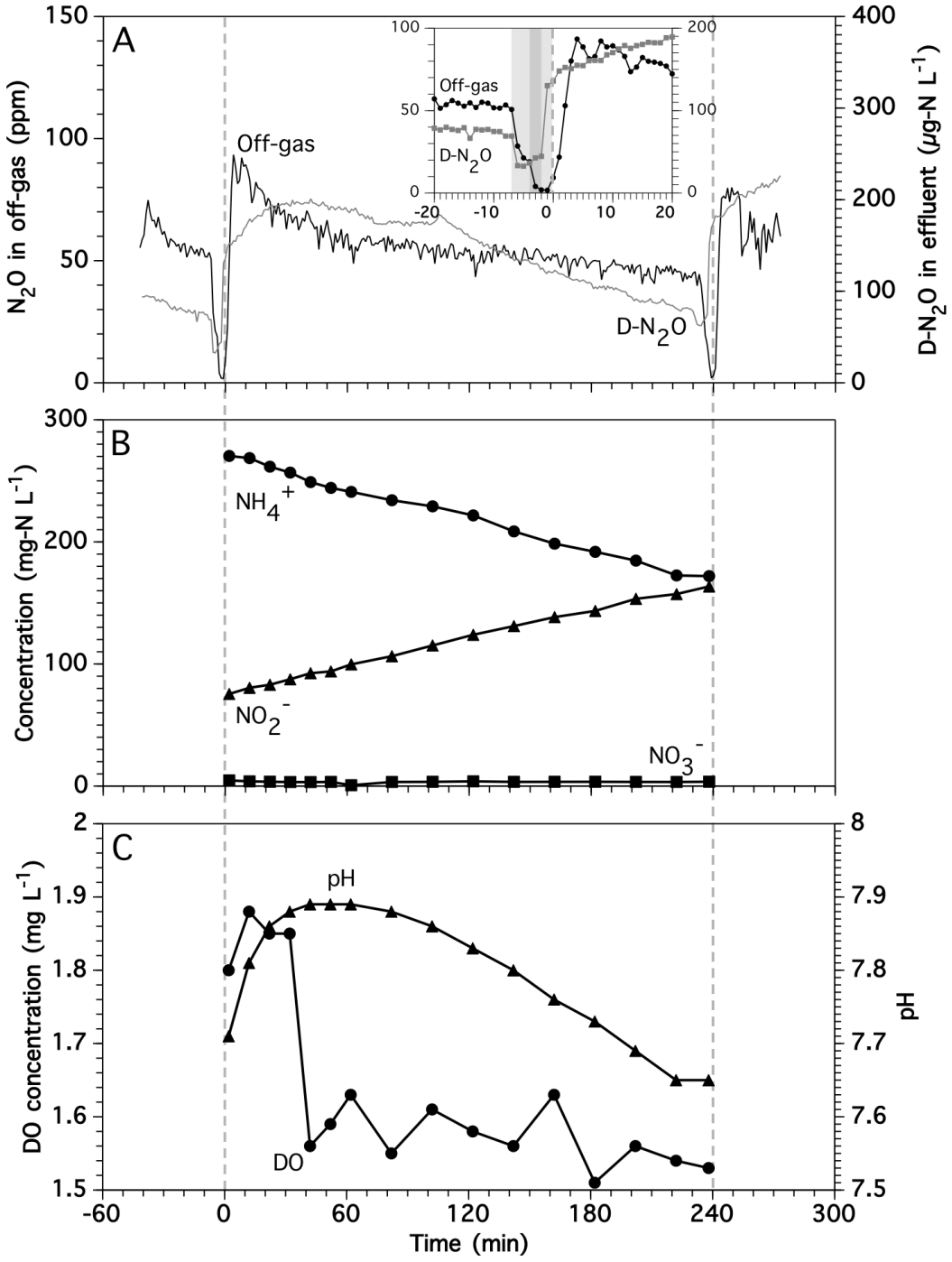

Figure 2.3: The concentration profiles of (A) N_2O in the off-gas and D-N_2O, (B) NH_4^+, NO_2^- and NO_3^-, and (C) DO and pH during one typical cycle of the sequencing batch reactor operation. Inset of panel A shows the concentration profiles of N_2O in the off-gas and D-N_2O from -20min to +20 min. A one cycle of the reactor operation was 4 h and aeration was started at 0 min.

Dynamics of N_2O emission from the PN-SBR suggests that the continuous measurement of N_2O in off-gas is necessary for reliable estimation of N_2O emission rate from a PN-SBR. Dynamics of N_2O emission in our reactor (Figure 2.3A) might be attributed to perturbation of the operating conditions, such as DO and pH (Figure 2.3C). N_2O emission rate was also high in the initial phase of aeration period of a lab-scale PN-SBR (Kong et al., 2013a). Difference of N_2O sampling methods (e.g., timing and amount of a sample) among studies reported in Table 2.1 might result in difference of N_2O emission rates.

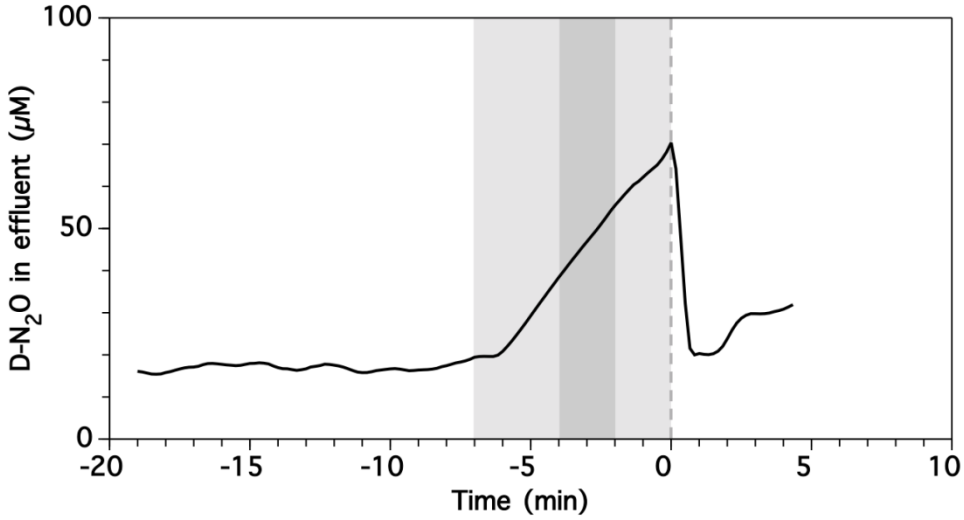

Figure 2.4: The profile of D-N_2O concentration in the PN reactor during the settling (-7 min to -4 min), discharging (-4 min to -2 min) and feeding (-2 min to 0 min) periods.

2.3.3 N_2O isotopomer analysis

The $\delta^{15}N^{\alpha}$, $\delta^{18}O$, and calculated SP value for the off-gas samples collected at different stages of the aeration period in the PN-SBR are shown in Figure 2.5A. The SP values ranged from 23‰ to 16‰. No significant increase in $\delta^{18}O$ in the remaining N_2O indicates that contribution of N_2O reduction to $N_2$was not strongly occurred in the reactor (van Groenigen et al., 2005; Schmidt et al., 2004). Production of N_2 as estimated based on the N balance calculation was 3.5 ± 4.7%, which might not be strong enough to influence the $\delta^{18}O$ values and the SP value. Therefore, approximate contributions of NH_2OH oxidation and NO_2^- reduction (nitrifier denitrification and heterotrophic bacterial denitrification) to N_2O production were estimated by assuming that each process is linearly proportional to the SP value (Figure 2.5B). The result indicates that N_2O was

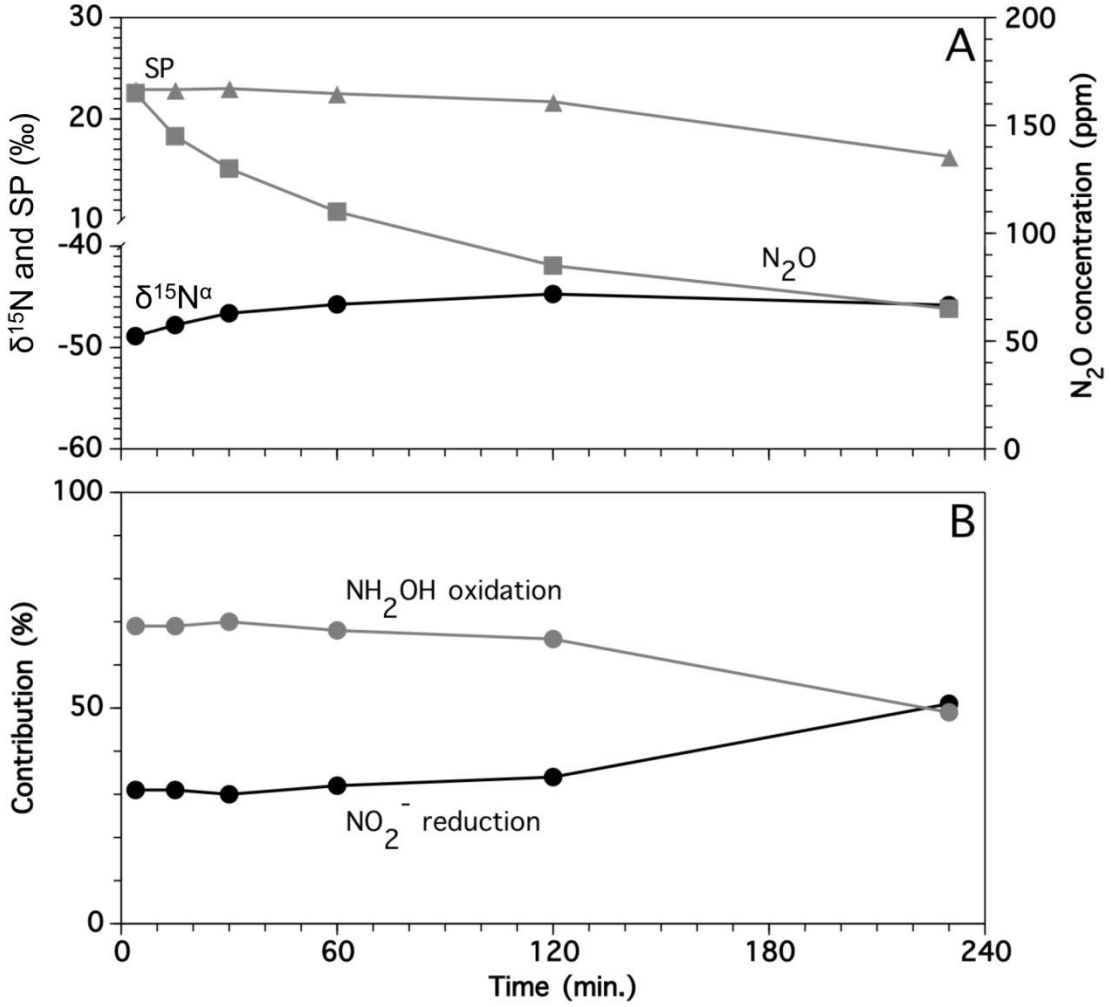

Figure 2.5: (A) N_2O concentration, isotopomer ratios and SP values in off-gas at each sampling time over one cycle.(B) Contribution of NH_2OH oxidation and NO_2^- reduction pathways to N_2O emission from the PN reactor.

produced in the PN-SBR by combination of NH_2OH oxidation and NO_2^- reduction pathways. Within initial 60 min of the aeration phase, about 70% of the totally produced N_2O was produced via the NH_2OH oxidation pathway (Figure 2.5B). After 60 min, the contribution of the NH_2OH oxidation pathway to the total N_2O production gradually decreased. At the end of the aeration phase, the contribution of the NH_2OH oxidation pathway was comparable with that of the NO_2^- reduction pathway. To the best of our knowledge, this is the first study to distinguish contribution of nitrification and denitrification processes to N_2O production pathways in an autotrophic granular PN-SBR.

Higher contribution of NH_2OH oxidation on N_2O production within the initial 60 min is due to sudden fluctuation in DO and NH_4^+ concentrations and pH level. Wunderlin et al. (2012) reported that N_2O production by NH_2OH oxidation pathway was favored at high NH_4^+ and low NO_2^- concentrations, in contrast, the contribution of nitrifier denitrification increased under the condition of higher NO_2^- and lower NH_4^+ concentrations. In addition, N_2O production was only observed during recovery to aerobic conditions after a period of anoxia in chemostat cultures of model nitrifying bacteria (Yu et al., 2010), and N_2O production rates of the AOB enriched culture were increased with increases in pH and NH_4^+ oxidation rate (Law et al., 2011; 2012). Increase in the contribution of NO_2^- reduction pathway might be due to relative enhancement of denitrification caused by increase in NO_2^- concentration and decrease in NH_4^+ concentration in the reactor (Wunderlin et al., 2012). The contribution of the NH_2OH oxidation pathway to the total N_2O production was about 65% throughout one cycle, indicating that the NH_2OH oxidation pathway was the key pathway of N_2O production in the autotrophic PN-SBR. In the latter phase the N_2O production via the NO_2^- reduction pathway was comparable with that via the NH_2OH oxidation pathway.

In contrast, in the previous studies to investigate N_2O production pathways in NH_4^+ oxidation process in wastewater treatments by isotopomer analysis, NO_2^- reduction contributed to N_2O production greater than NH_2OH oxidation did (Wunderlin et al., 2013; Toyoda et al., 2011). It might be because operational parameters affect N_2O production pathways. In the present study, isotopomer analysis was conducted in only one cycle attributed to perturbation of the operating conditions, such as DO and pH (Figure 2.3C).

Reproducibility of the trend of shift in N_2O production pathways should be confirmed in the future study. N_2O production pathways have also been investigated with the use of mathematical models (Ni et al., 2013; Law et al., 2012) and by addition of substrates (NH_2OH or NO_2^-) (Wunderlin et al., 2012; Yang et al., 2009). In contrast to this method, isotopomer analysis can reveal the N_2O production pathways directly and quantitatively with high reliability. However, there are some limitations to isotopomer analysis. For example, it cannot distinguish N_2O production by heterotrophic denitrification from that by nitrifier denitrification. For more specific and quantitative identification of N_2O source in a PN-SBR, other analytical methods

(e.g., functional gene expression analysis (Philippot and Hallin, 2005)) have to be combined with isotopomer analysis.

2.3.4 Spatial distributions of bacteria and their activities in single PN granules

The steady-state concentration profiles of DO, pH, NH_4^+, NO_2^-, NO_3^-, and N_2O in the PN granules were measured (Figure 2.6A under the typical operational conditions of the PN-SBR and the spatial distributions of net volumetric production rates of NH_4^+, NO_2^- and N_2O were calculated (Figure 2.6B). N_2O was detected throughout the granule and the net N_2O production rate was higher in the oxic layer (within 300 μm) of the granules (Figure 2.6B). NH_4^+ consumption and NO_2^- production were found mainly in the oxic surface layer without a significant production or consumption of NO_3^-, demonstrating that partial nitrification occurred efficiently in the PN granules. Moreover, FISH results revealed that AOB were abundant in the outermost layer of the granules (Figure 2.2B). These results reflect that AOB might be responsible for N_2O production in the PN granules. Unfortunately, based on the microsensor measurements we cannot conclude that the NH_2OH oxidation by AOB was the main N_2O production pathway rather than NO_2^- reduction by AOB and/or heterotrophic denitrifiers, as could be demonstrated by isotopomer analysis.

Less but detectable N_2O production probably by heterotrophic denitrifiers in the deeper anoxic parts of the granules were found (Figure 2.6B), which could be expected by isotopomer analysis. Although the PN-SBR operated without an external organic carbon supply, it has been hypothesized that heterotrophic bacteria scavenge organic matter derived from biomass decay and substrate metabolism of nitrifying bacteria (Okabe et al., 2005). In addition, under the limited availability of biodegradable carbon, N_2O can be produced due to the incomplete denitrification process and/or endogenous denitrification (Chung and Chung, 2000; Itokawa et al., 2001). As a result, the microsensor measurements revealed that the N_2O production mechanisms in the PN granules involve multiple N_2O production pathways, because there were steep vertical gradients of physicochemical parameters in the PN granules.

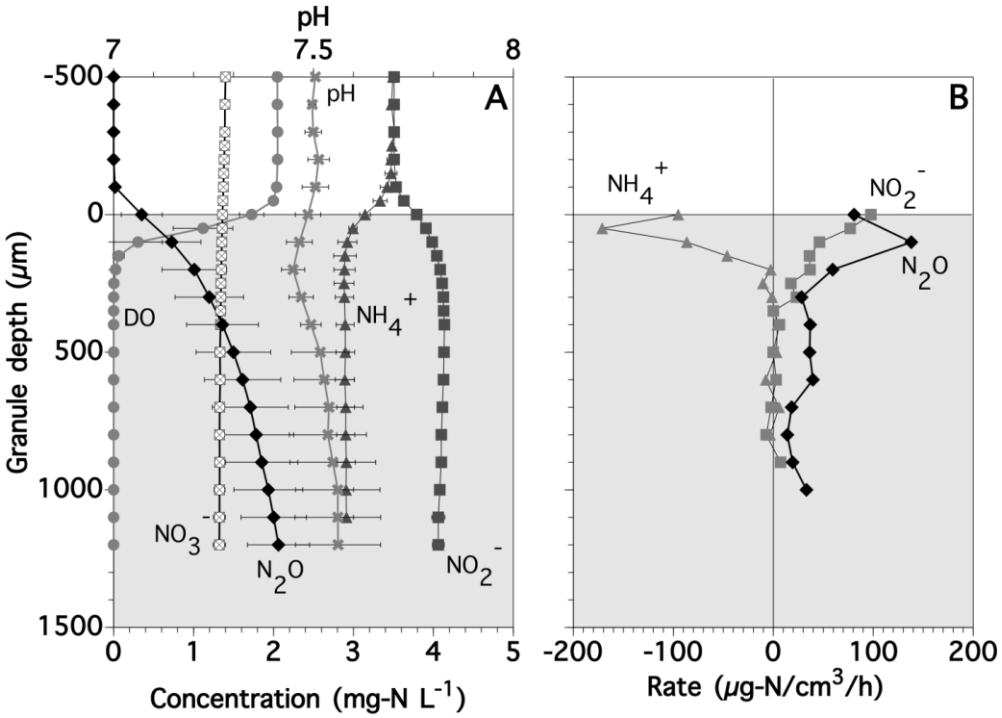

Figure 2.6: (A) Steady-state concentration profiles of DO, pH, NH_4^+, NO_2^-, NO_3^-, and N_2Oin the PN granules. (B) Net volumetric production rates of NH_4^+, NO_2^- and N_2O in the PN granules. Positive and negative values indicate production and consumption rates, respectively.

2.4 CONCLUSIONS

A lab-scale sequencing batch reactor for partial nitrification treating synthetic wastewater without organic carbon was operated to identify source of N_2O in an autotrophic partial nitrification reactor. The average N_2O emission rate from the reactor was 0.32 ± 0.17 mg-N L^{-1} h^{-1} and the average emission of N_2O was 0.8 ± 0.4% of the incoming nitrogen load. N_2O emission rate and N_2O production pathways were dynamic during one cycle of the sequencing batch reactor operation; N_2O emission rate was high in the initial phase of the aeration period, where hydroxylamine oxidation pathway accounted for 65% of the total N_2O production. The active N_2O production as well as partial nitrification was found in the oxic surface layer of the granule, where ammonia-oxidizing bacteria were abundant. Based on all experimental results (including isotopomer analysis, microelectrode and FISH), although N_2O was produced mainly via NH_2OH oxidation pathway in the autotrophic partial nitrification reactor, N_2O production mechanisms were complex and could involve multiple N_2O production pathways.

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Chapter 3

Effects of dissolved oxygen and pH on nitrous oxide production rates in autotrophic partial nitrification granules

3 EFFECTS OF DISSOLVED OXYGEN AND PH ON NITROUS OXIDE PRODUCTION RATES IN AUTOTROPHIC PARTIAL NITRIFICATION GRANULES

3.1 INTRODUCTION

Nitrogen removal from wastewater via a combination of partial nitrification (PN) and anaerobic ammonium oxidation (anammox) processes has recently drawn attention as an alternative to conventional nitrification-denitrification processes (Castro-Barros et al., 2015; Cho et al., 2011; Kampschreur et al., 2008;Okabe et al., 2011a). The advantages of the PN-anammox process over conventional processes include lack of a need for external organic carbon, low oxygen demand, small sludge production, and low emissions of carbon dioxide (CO_2) (Kartal et al., 2010). Therefore, this technology enables low cost removal of nitrogen from wastewater while using little energy (Kartal et al., 2010). In addition, use of granular sludge and biofilm for PN instead of activated sludge flocs has attracted attention because of good sludge settleability, long-term retention of slow-growing nitrifying bacteria in a reactor and high specific nitrification rate (Zheng et al., 2005).

Nitrous oxide (N_2O) can be produced in PN granular sludge processes. N_2O emissions were higher in a PN reactor than a full nitrifying reactor (Wei et al., 2014), probably owing to high nitrite (NO_2^-) concentrations and dissolved oxygen (DO)-limited conditions (Castro-Barros et al., 2015; Ishii et al., 2014; Pijuan et al., 2014; Rathnayake et al., 2013; Wei et al., 2014). N_2O is an important greenhouse gas that has a global warming potential about 300 times greater than that of CO_2 and therefore poses a significant threat to the ozone layer (Desloover et al., 2012). Anthropogenically emitted N_2O has both chemical (combustion, incineration and industrial) and biological (agricultural soils, manure storage tanks and nitrogenous solid and liquid waste treatment) origins (IPCC, 2007). During biological nitrogen removal processes in wastewater treatment, N_2O can be produced via three primary microbiological pathways.

(1) As a byproduct of nitrification: During this process, nitrosyl radical (NOH) is produced as an intermediate during oxidation of hydroxylamine (NH_2OH) into NO_2^-(Igarashi et al., 1997). Next, NOH is biologically transformed into NO and then N_2O, or chemically broken down to N_2O (Poughon et al., 2001).

(2) As an intermediate during denitrification (*i.e.*, reduction of NO_2^- to N_2) by heterotrophic bacteria (Lu and Chandran, 2010).

(3) By ammonia-oxidizing bacteria (AOB) (*i.e.*, nitrifier denitrification) under high NO_2^- or oxygen limiting conditions (Wrage et al., 2001).

The N_2O emission factors range from 1.5% of the ammonium (NH_4^+) oxidized (Rathnayake et al. 2013) to 19% of the nitrogen oxidized (Pijuan et al., 2014). Such variety in N_2O emission rates (NERs) could be explained by the different responses of N_2O producing reactions (e.g., NH_2OH oxidation, nitrifier denitrification and heterotrophic denitrification) to physicochemical parameters such as DO, NH_4^+ and NO_2^- concentrations, pH, temperature and chemical oxygen demand (COD)/nitrogen ratio (Kampschreur et al., 2009; Okabe et al., 2011b). Accordingly, many studies have been conducted to investigate the effects of these parameters on N_2O production pathways and emission rates during PN processes (Law et al., 2013, 2012, 2011; Pijuan et al., 2014; Wang et al., 2014). Some studies have shown that N_2O emissions increased with decreasing DO concentration in a nitritation reactor receiving anaerobic sludge digestion liquor (Wang et al., 2014) and in a full-scale two-reactor PN–anammox process (Kampscheur et al., 2008). In contrast, other studies suggested that N_2O emissions increased with increasing DO concentrations in an AOB culture in a nitritation system fed with synthetic anaerobic digester liquor (Law et al., 2012) and pilot-scale anaerobic-anoxic-oxic (A2O) reactors (Furuya et al., 2013). Moreover, N_2O emissions were strongly dependent on pH in a highly enriched culture of AOB in a nitritation system fed with synthetic anaerobic digester liquor (Law et al., 2011). However, the effects of physicochemical parameters on N_2O emissions from PN granular sludge processes have not been extensively investigated (Castro-Barros et al., 2015; Pijuan et al., 2014).

In granular sludge, a steep concentration gradient is created towards the deeper part of the granule because the microbial density of granules is very high (Okabe et al., 2011b). As a result, the physicochemical parameters in the deeper parts are significantly different from the bulk liquid (Rathnayake et al., 2013; Song et al., 2013) and stratified distribution of microorganisms is created over a granule (Ishii et al., 2014). This can make the N_2O production and consumption processes in a granule more complicated and consequently might be responsible for various NERs among PN granular sludge processes. However, no studies have examined the effects of physicochemical parameters on N_2O production in a PN granule. Accordingly, it is essential to

investigate the N_2O production pathways and N_2O production rate *in situ* relative to local physicochemical conditions and microbial community structures in a granule to enhance understanding of N_2O emission mechanisms and then establish a strategy to mitigate N_2O emissions in PN processes. Therefore, the present study was conducted to reveal (i) the effects of DO concentration and pH on N_2O production pathways and NERs of autotrophic PN granules taken from the autotrophic PN-sequencing batch reactor (SBR) (Rathnayake et al., 2013), (ii) the microbial community structure in PN granules, and (iii) the spatial distribution of the *in situ* N_2O production rate in PN granules. Based on these results, the effects of DO and pH on N_2O emission pathways and rates in the PN reactor were explained by the microbial community structure and the *in situ* N_2O production rate in the PN granules.

3.2 MATERIAL AND METHODS

3.2.1 Batch experiments

Batch experiments were carried out using PN granules sampled from previously developed autotrophic PN-SBR (Rathnayake et al., 2013) to investigate the effects of DO concentration and pH on N_2O emissions from PN granules. All batch experiments were conducted in a laboratory bench-scale reactor with a working volume of 1.0 L and a headspace of 0.25 L. This batch reactor had the same configuration as the parent reactor except for the volume (Figure 3.1). The synthetic medium for the batch experiments contained the same ingredients used in the autotrophic PN-SBR (Rathnayake et al., 2013), except for the concentrations of $(NH_4)_2SO_4$ (1050 mg L^{-1}, corresponding to 220 mg-N L^{-1}) and $NaNO_2$ (610 mg L^{-1}, corresponding to 124 mg-N L^{-1}). The NH_4^+ and NO_2^- concentrations corresponded to those in the parent PN-SBR at 2 h after aeration started. A grab sample of the PN granules (3.0 g-volatile suspended solids (VSS)) was taken from the parent PN-SBR at 2 h after aeration started, washed three times with synthetic medium without NH_4^+ and NO_2^-, then re-suspended in 1.0 L of medium.

To investigate the effects of DO concentration on the NERof PN granules, batch tests were conducted at various DO concentrations (0.6, 1.1, 1.7 and 2.3 mg L^{-1}) and a fixed pH of 7.5 ± 0.05. DO was manually controlled by changing the ratio of N_2 gas and atmospheric air while the total gas flow rate was maintained at 150 ± 10 mL min^{-1}. To investigate the effects of pH on the NER of the PN granules, batch tests were conducted at different pH values (6.5, 7.0, 7.5, 8.0 and

8.5) while the DO concentration and the gas flow rate were fixed at 1.3 ± 0.1 mg L^{-1} and 150 ± 10 mL min^{-1}, respectively. These DO concentrations and pH values for the batch tests were adopted based on the measured DO concentrations (0.8–2.5 mg L^{-1}) and pH values (6.3–8.5) in the parent PN-SBR. Each batch test was performed in triplicate. DO and N_2O concentrations and pH were continuously monitored until the N_2O emissions reached steady state (less than 40 min), which was defined as the point at which fluctuation in N_2O concentration for 5 min became less than 10% of the total change in N_2O concentration in a batch test. The bulk liquid samples in the batch reactor were collected at arbitrary time intervals and filtered using a 0.45-µm pore nitrocellulose membrane (Advantec, Tokyo, Japan). Concentrations of NH_4^+, NO_2^-, nitrate (NO_3^-) and DO and the pH of the liquid samples and N_2O concentration in the gaseous samples were measured as described by Rathnayake et al. (2013). pH was controlled by adding HCl (0.5 M) or NaOH (0.5 M).

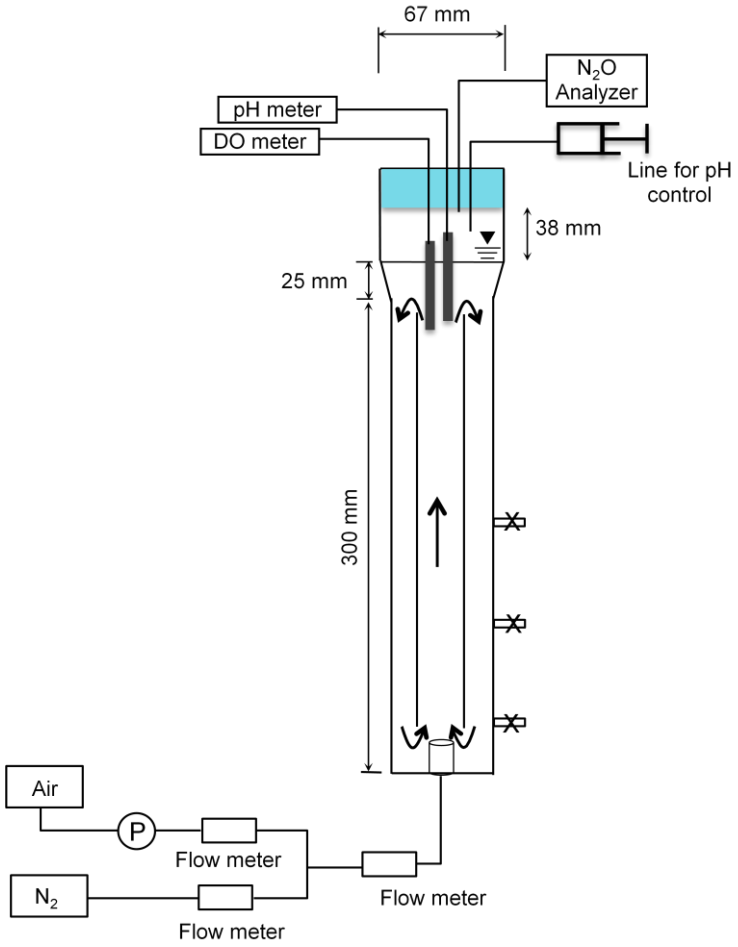

Figure 3.1: Schematic diagram of a bench-scale batch reactor. Liquid is circulated by air through an internal cylindrical plastic tube.

We used allylthiourea (ATU) to distinguish the amount of N_2O produced by denitrification (nitrifier denitrification and heterotrophic denitrification) from that generated by nitrification (NH_2OH oxidation) in the batch experiments (Tallec et al., 2006). ATU inhibits aerobic oxidation of ammonia to NH_2OH by AOB. We confirmed in advance that 10 mg-ATU L^{-1} completely inhibited the ammonia oxidation of our PN granules. N_2O produced in the batch test without ATU was defined as that produced via both nitrification and denitrification, whereas N_2O produced in the batch test with ATU was considered to be produced by denitrification alone. N_2O produced via nitrification was subsequently calculated by subtracting the amount produced by denitrification from that generated via both nitrification and denitrification. NER (mg-N g-VSS^{-1} h^{-1}) was calculated by multiplying the N_2O concentration in the off-gas by the gas flow rate and then dividing the product by the mass of the granules (g-VSS). The NH_4^+oxidation rate (AOR) was calculated based on changes in NH_4^+ concentrations in a batch test and the mass of granules (g-VSS).

3.2.2 Microbial community structure in the PN granules

Microbial community structure was analyzed by 16S rRNA gene amplicon deep sequencing conducted using the MiSeq technology (Illumina, Hayward, CA). Briefly, genomic DNA was extracted from the PN granules (n=3) using a PowerSoil DNA Isolation kit (MoBio Technologies, Carlsbad, CA). The partial 16S rRNA gene sequences including the V3 and V4 regions were then amplified using primers Bakt_341F and Bakt_805R (Herlemann et al., 2011) withIllumina overhang adaptor sequences attached to their 5′ ends. The reaction mixture (25μL) contained 1×KAPA HiFi HotStart ReadyMix (Kapa Biosystems), 0.2 μM of each primer, and 2μL of template cDNA. Polymerase chain reaction (PCR) was performed using a Veriti thermal cycler (Applied Biosystems,Foster City, CA)to subject the samples to the following conditions: initial annealing at 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s.Twenty-five cycles of PCR were confirmed to benecessary and sufficient to reach the log-linear phase based on quantitative PCR analysis conducted using a KAPA SYBR Fast qPCR kit (Kapa Biosystems) and the Bakt_341F and Bakt_805R primers. The amplicons with Illumina overhang adaptor sequences were then purified using a LaboPass PCR Purification Kit (COSMO Genetech, Seoul, Korea), after which they were quantified with PicoGreen dsDNA quantification reagent (Molecular Probes, Eugene, OR, USA). The Illumina sequencing adaptor

and index tag sequences were added to the amplicons using a Nextera XT Index kit (Illumina) according to the manufacturer's instructions,after which the resulting amplicons were purified using an AMPure XP kit (Beckman Coulter). Amplicons from triplicate samples were pooled in equal amounts and then purified again using a GeneRead Size Selection Kit (Qiagen).The resulting DNA was mixed with Phi X control DNA and employed as a template forpaired-end sequencing using the MiSeq Reagent Kit v2 (500 cycles) and a MiSeq sequencer (Illumina). Sequence reads from triplicate samples were analyzed using QIIME 1.8.0 (Caporaso et al., 2010) with the Silva 119 database. Representative sequences related to AOB were used to construct a phylogenetic tree based on the maximum-likelihood method using MEGA version 5 (Tamura et al., 2007).

3.2.3 Spatial distributions of AOB and N_2O producing activities in the single PN granules

The spatial distributions of betaproteobacterial AOB in single PN granules were determined by FISH (Rathnayake et al., 2013). Additionally, the steady-state concentration profiles of DO, N_2O and pH in the single PN granules were measured with microsensors at different DO concentrations or pH (Rathnayake et al., 2013). The granules were acclimated in the synthetic medium for the batch experiment at least 1 h before microsensor measurements to ensure that steady-state profiles were obtained. Physicochemical parameters of the medium were almost unchanged during the microsensor measurements. The net volumetric emission rates of N_2O ($R(N_2O)$) in the single granules were estimated from the concentration profile. The spatial distributions of betaproteobacterial AOB in single PN granules were determined by fluorescence in situ hybridization (FISH) (Rathnayake et al., 2013). Additionally, the steady-state concentration profiles of DO, N_2O and pH in the single PN granules were measured with microsensors at different DO concentrations or pH (Rathnayake et al., 2013). The granules were acclimated in the synthetic medium used for the batch experiment at least 1 h before microsensor measurements to ensure that steady-state profiles were obtained. Physicochemical parameters of the medium were almost unchanged during the microsensor measurements. The net volumetric emission rates of N_2O ($R(N_2O)$) in the single granules were estimated from the concentration profile as previously described by Santegoeds et al. (1999).

3.3 RESULTS AND DISCUSSION

3.3.1 Effect of DO concentration on N_2O emission from the PN granules

An increase in DO concentration from 1.1 mg L^{-1} to 2.3 mg L^{-1} at 20 min triggered an immediate increase in N_2O concentration in the off-gas, and the N_2O concentration stabilized after 30min (Figure 3.2A). The N_2O concentration decreased rapidly to the original concentration in response to decreasing DO concentration (2.3 mg L^{-1} to 1.1 mg L^{-1}). These results indicate that the impact of DO concentration on N_2O emissions from the PN granules was rapid and reversible. This DO-dependent N_2O emission phenomenon was highly reproducible (data not shown).

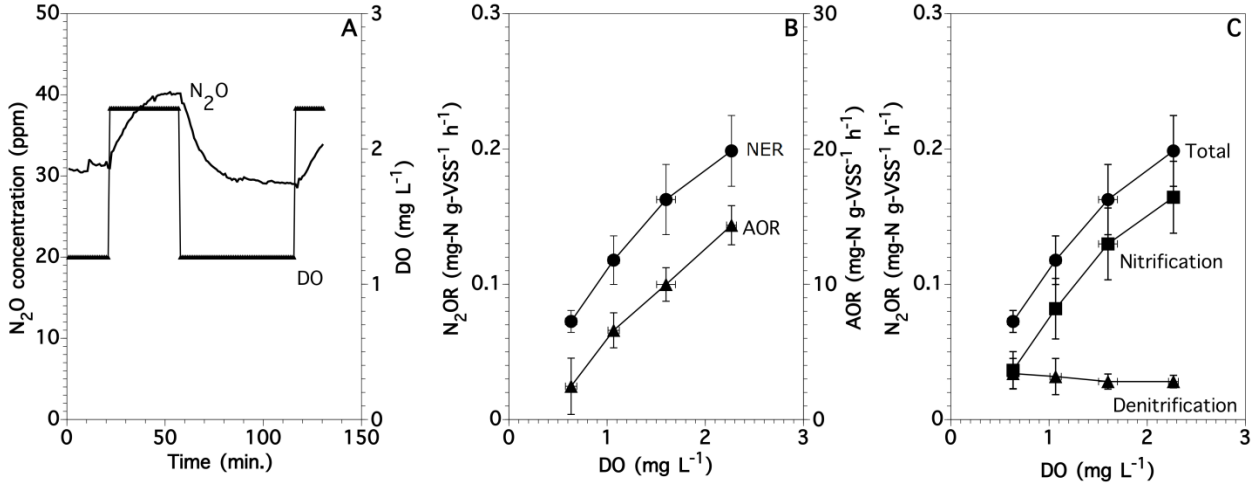

Figure 3.2: (A) Time-course profiles of DO concentration in the bulk liquid and N_2O concentration in the off-gas. (B) Effects of DO on N_2O production rate (NER) and ammonia oxidation rate (AOR). (C) The total NER and NERs via nitrification and denitrification at a variety of DO concentrations.

The effects of DO concentration on NER and AOR in the PN batch reactor were investigated at pH 7.5 based on the results of the batch tests described above. The AOR and NER increased linearly as DO concentration increased (Figure 3.2B). It should be noted that the relative fraction of N_2O emissions over the oxidized ammonia decreased from 2.9% (0.07 mg-N/2.4 mg-N) at 0.6 mg-DO L^{-1} to 1.4% (0.20 mg-N/14 mg-N) at 2.3 mg-DO L^{-1}.

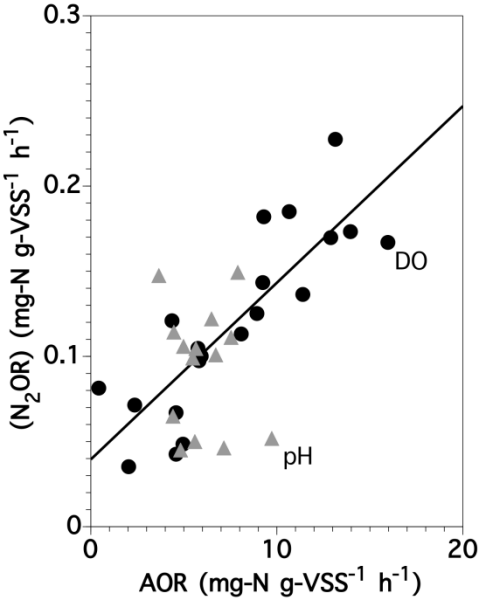

Figure 3.3: Correlation between ammonia oxidation rate (AOR) and N_2O production rate (NER) at a variety of DO concentrations and pH levels. The solid line indicates linear regression of the DO concentrations, which was given by the following equation: $y = 0.010x + 0.040$ ($r^2 = 0.71$).

The effects of DO concentration on N_2O production pathways (NH_2OH oxidation or denitrification (*i.e.*, nitrifier and heterotrophic denitrification)) in the PN granules were investigated using ATU as an inhibitor for aerobic ammonia oxidation. When the DO concentration was 0.6 mg L^{-1}, NER via nitrification was comparable to that via denitrification (Figure 3.2C). However, the NER via nitrification increased as the DO concentration increased. In contrast, the NER via denitrification was almost unchanged with increasing DO concentration. Plotting the NERs obtained from all batch tests against the corresponding AOR revealed a positive correlation ($r^2 = 0.71$) between these two parameters at AOR ranging from 0 to 20 mg-N g-VSS^{-1} h^{-1}, suggesting that N_2O was produced via ammonium oxidation (Figure 3.3). These results agreed with those of a previous study of PN-SBR from which the PN granule samples were taken in this study that showed NH_2OH oxidation was responsible for up to 70% of the total N_2O emissions (Rathnayake et al., 2013).

3.3.2 Effect of pH on NER of the PN granules culture

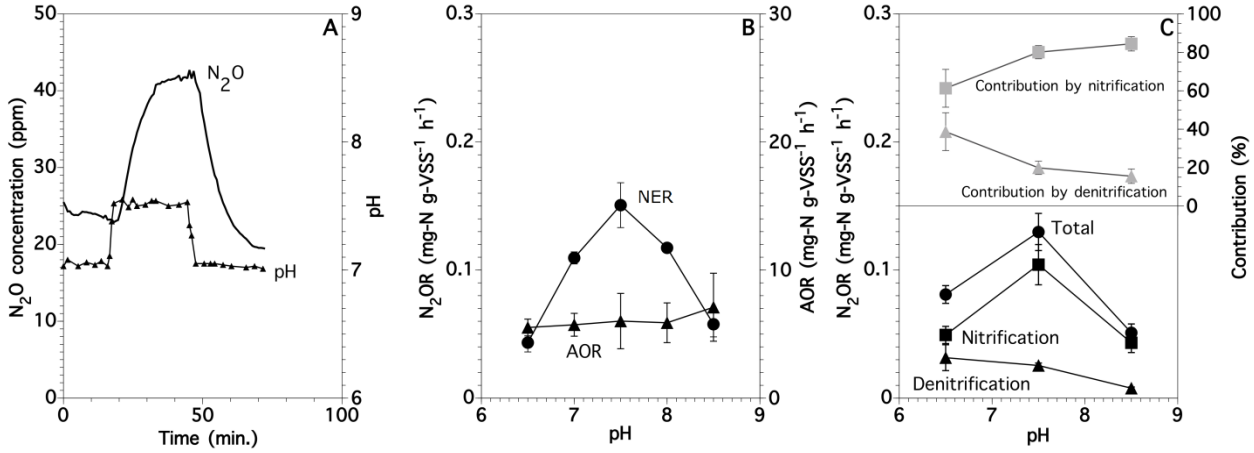

Figure 3.4: (A) Time-course profiles of pH in the bulk liquid and N_2O concentration in the off-gas. (B) Effects of pH on the N_2O production rate (NER) and ammonia oxidation rate (AOR) at a variety of pH levels.(C) The total NER and NERs via nitrification and denitrification, and the contribution of nitrification and denitrification to N_2O production at a variety of pH levels.

An increase in pH from 7.0 to 7.5 triggered an increase in the N_2O concentration of the off-gas with a 3 min time lag. The N_2O concentration reached a steady state N_2O concentration of 42 ppm at pH 7.5 within 30 min (Figure 3.4A). The N_2O concentration decreased in response to a decrease in pH from 7.5 to 7.0, reaching a steady state N_2O concentration of 20 ppm within 25 min. These findings indicate that the effects of changes in pH on N_2O emissions were reversible. More time was required to reach steady state in the experiments when pH was changed than when DO was changed.

The effects of pH on AOR and NER were investigated at DO 1.1 mg L^{-1} based on the results from the batch tests described above. The NER at pH 7.5 (0.15 ± 0.02 mg-N g-VSS^{-1} h^{-1}) was highest between pH 6.5 and 8.5 (Figure 3.4B). The relative fraction of N_2O emissions over the oxidized ammonia was also higher at pH 7.5 (2.5%) than at pH 6.5 (0.80%) and 8.5 (0.80%). In contrast, AOR were relatively constant between pH 6.5 and 8.5. Thus, there was no trend between AOR and NER (Figure 3.3).

The effects of pH on the N_2O production pathways in the PN granules were further investigated using 10 mg L^{-1} of ATU as an inhibitor for aerobic ammonia oxidation. The NERs via

nitrification were higher than those via denitrification at all tested pH values (Figure 3.4C). Moreover, these values were highest at pH 7.5 (0.10 ± 0.02 mg-N^{-1} g-VSS^{-1} h^{-1}), which accounted for 80% of the total N_2O emissions (Figure 3.4C). The NER via denitrification decreased as pH increased. Specifically, the NER via denitrification at pH 8.5 (0.008 ± 0.001 mg-N g-VSS^{-1} h^{-1}) was four times lower than that at pH 6.5 (0.032 ± 0.01 mg-N^{-1} g-VSS^{-1} h^{-1}). Thus, the contribution of nitrification to the total N_2O emissions further increased to 85% at pH 8.5.

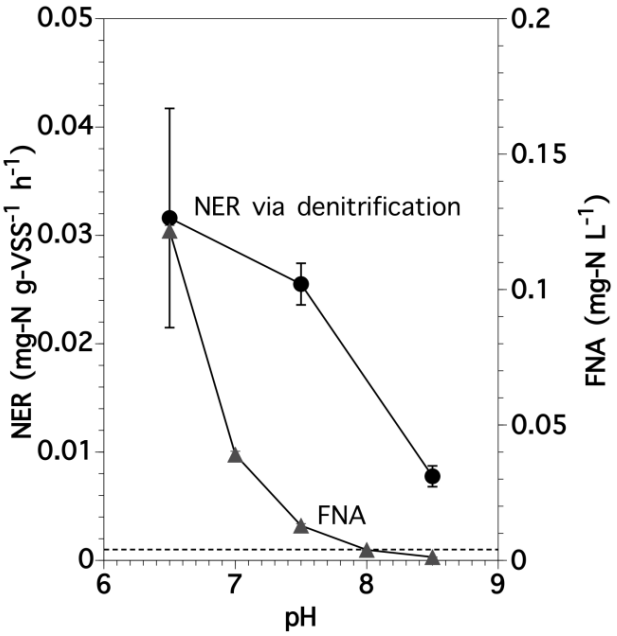

Figure 3.5: N_2O production rate (NER) and FNA concentration at various pH values. The dashed line indicates an FNA concentration of 0.004 mg-N L^{-1}.

Similarly, previous studies demonstrated that N_2O emissions via heterotrophic denitrification increased as pH decreased (Okabe et al., 2011b). The higher N_2O emissions at lower pH ranges might be due to interference with N_2O reductase by free nitrous acid (FNA).Zhou et al. (2008) reported that 0.004 mg HNO_2-N L^{-1} of FNA completely inhibited N_2O reductase during denitrification.FNA concentration is a function of nitrite concentration, pH and temperature as described by the following equation (Qiao et al., 2010):

$$\mathrm{FNA}\left(\mathrm{mg\,HNO_2-N\,L^{-1}}\right)=\frac{\left[\mathrm{NO_2^-}-\mathrm{N}\right]}{e^{\left[\frac{-2300}{(273+t)}\right]}\times 10^{\mathrm{pH}}}$$

Equation 3.1

FNA concentrations in the bulk liquid of the batch experiments were calculated to be 0.12 and 0.013 mg HNO_2-N L^{-1} at pH 6.5 and 7.5, respectively, using the above equation (Figure 3.3). These concentrations were high enough to completely inhibit N_2O reductase (Zhou et al., 2008). Increasing the pH led to decreased FNA concentrations (Figure 3.3), and consequently an increase in N_2O reducing activity, resulting in a decrease in NER via denitrification (Figure 3.4C).

3.3.3 Bacterial community structure, their spatial distribution and in situ N_2O production rates in the PN granules.

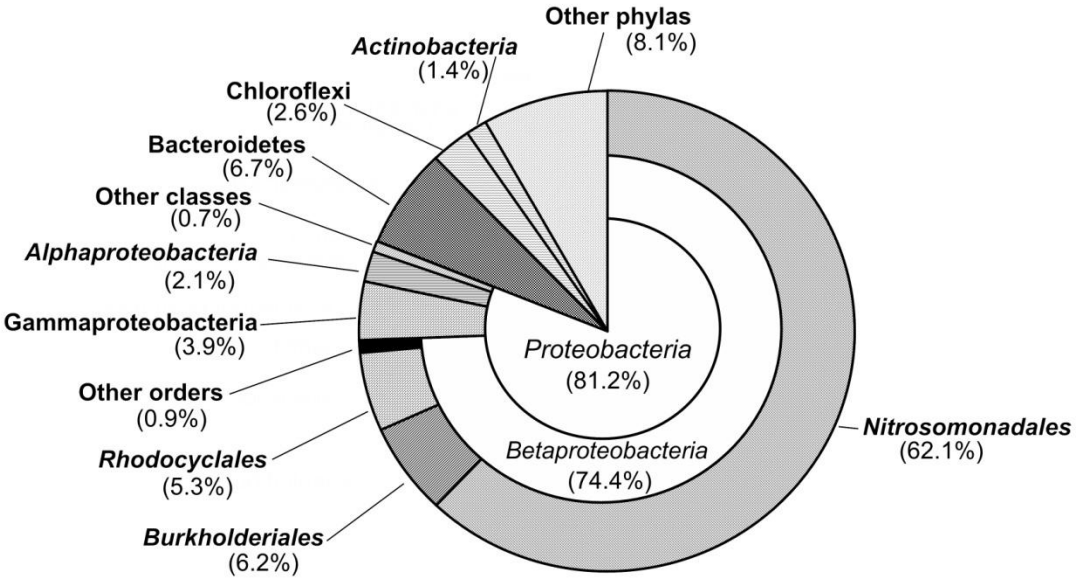

Figure 3.6: (A) Microbial community composition of aerobic PN granules based on 16S rRNA gene sequences obtained by MiSeq sequencing. The inner, middle, and outer pie charts show the community composition at the phylum, order, and class levels, respectively.

The 16S rRNA gene deep sequencing analysis was conducted to examine the bacterial community structure in the PN granules, and a total of 290157 sequences was obtained from triplicate samples (Table 3.1). Microbial community structures were very similar among triplicate samples. AOB belonging to the bacterial order *Nitrosomonadales* were detected in high abundance (62.1 ± 5.6%) in autotrophic PN granules (Figure 3.6), in which an operational taxonomic unit (OTU), PN1_7035 sequence (99.4% similar to *Nitrosomonas europaea* ATCC 19718 (GenBank accession NC_004757)), accounted for 60.5 ± 2.0% (Figure 3.7). Another OTU, PN1_5365 (99.4% similar to the Bac02f3clone retrieved from heterotrophic PN granules

by Song et al., 2013), accounted for 20.5 ± 2.1%. These betaproteobacterial AOB were primarily detected in the oxic surface layer of the PN granules using a Nso1225 probe (Figure 3.8). Similar to the microbial community in the heterotrophic PN granules (Song et al., 2013), 16S rRNA gene sequences of potential denitrifiers such as *Burkholderiales* and *Rhodocyclales* were also detected, but their abundances were low (11.5%).

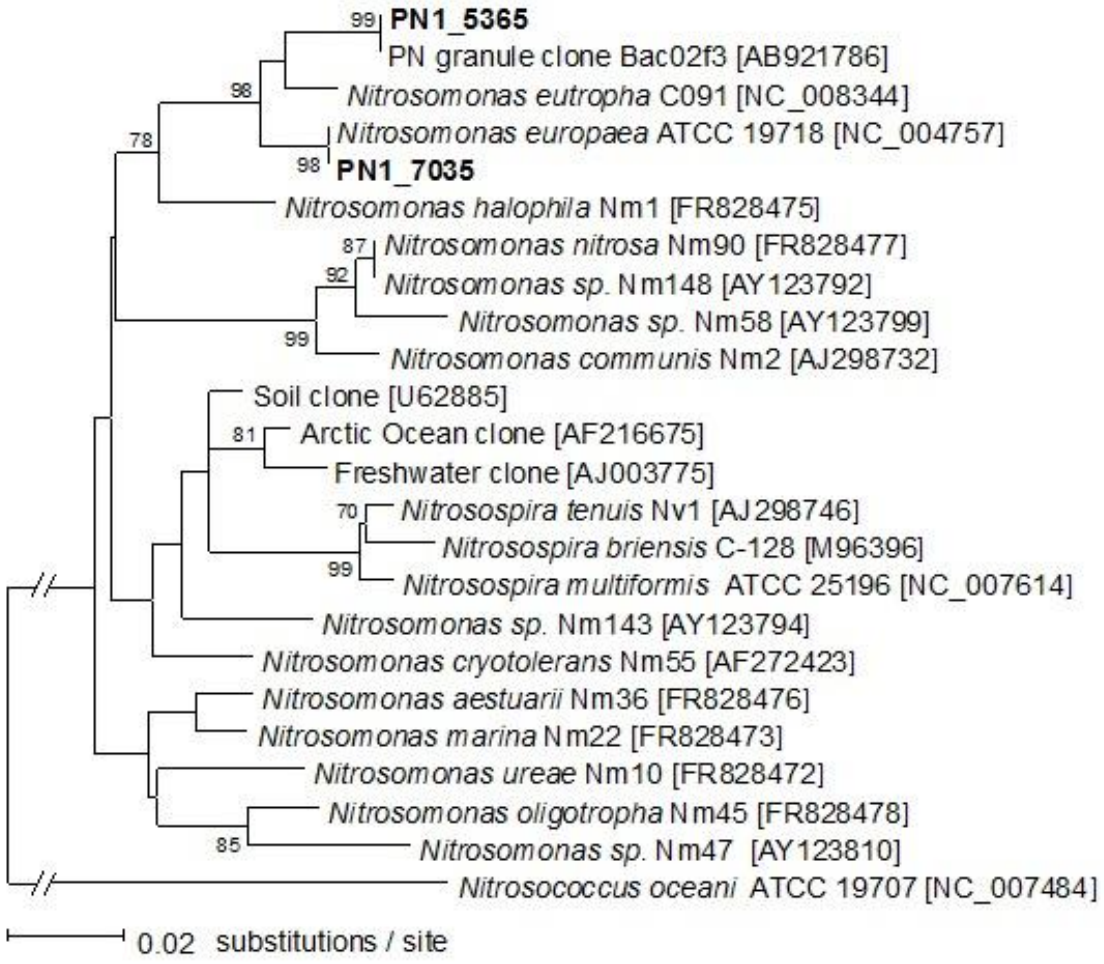

Figure 3.7: Phylogenetic relationship between the representative AOB-related sequences obtained in this study (shown in bold) and other AOB. The phylogenetic tree was constructed by the maximum-likelihood method using the 16S rRNA gene from *Nitrosococcus oceani* [NC_007484] as an outgroup for the tree. The accession numbers of the reference strains in the DDBJ/EMBL/GenBank databases are shown in brackets. The bootstrap values (>50%) derived from 1,000 replicates are indicated next to the branches.

Table 3.1: Similarity of microbial community compositions among triplicate samples, as determined by MiSeq 16S rRNA gene deep sequencing.

	Rep 1		Rep 2		Rep 3	
	Number	(%)	Number	(%)	Number	(%)
Bacteria	122108	100.0	53287	100.0	114762	100.0
Proteobacteria	102755	84.2	41456	77.8	93393	81.4
Betaproteobacteria	95894	78.5	37619	70.6	85040	74.1
Nitrosomonadales	83356	68.3	30546	57.3	69621	60.7
PN1_7035	52276	42.8	18194	34.1	41156	35.9
PN1_5365	15036	12.3	6696	12.6	14904	13.0
Burkholderiales	5910	4.8	3793	7.1	6936	6.0
Rhodocyclales	5615	4.6	2789	5.2	7484	6.5

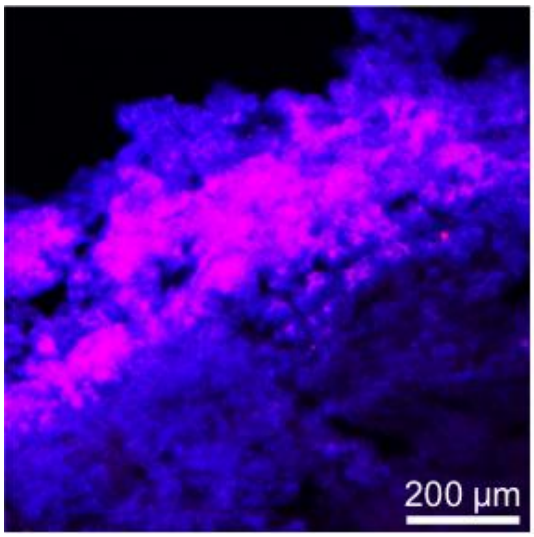

Figure 3.8: Confocal laser-scanning microscope images of thin cross-section of a PN granule showing *in situ* spatial distribution of AOB (magenta) and other bacteria (blue) after fluorescence in situ hybridization using a Cy5-labeled EUB338 mix probe and TRITC-labeled Nso1225 probe. (For interpretation of the colors in this figure, see the web version of this article)

3.3.4 Microsensor measurements

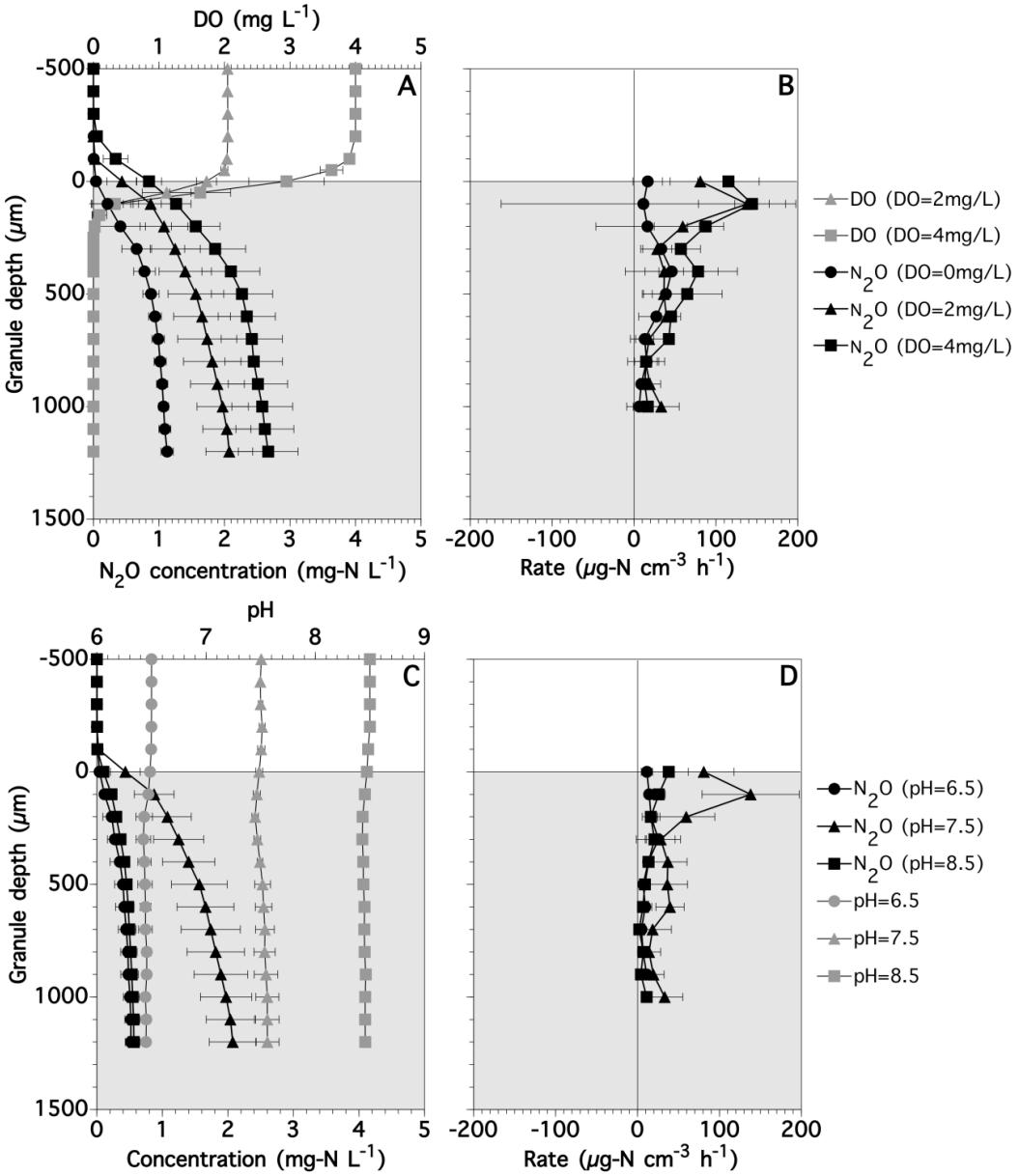

Figure 3.9: (A) Steady-state concentration profiles of N_2O in PN granules at DO concentrations of 0 (●), 2 (▲), and 4 mg/L (■), and DO concentration profiles at DO concentrations of 2 (gray triangle) and 4 mg/L (gray square). (B) Net volumetric production rates of N_2O ($R(N_2O)$) in the single PN granules at DO concentrations of 0 (●), 2 (▲), and 4 mg/L (■). Positive and negative values indicate production and consumption rates, respectively. (C) Steady-state concentration profiles of N_2O (black points) and pH (gray points) profiles in the PN granules at pH 6.5 (●), 7.5 (▲) and 8.5 (■) in the bulk liquid. (D) $R(N_2O)$ in the single PN granules at pH 6.5 (●), 7.5 (▲), and 8.5 (■). Positive and negative values indicate production and consumption rates, respectively

The *in situ*N_2O and DO concentrations and R(N_2O) in PN granules were measured at different DO concentrations and pH levels using microsensors (Figure 3.8). Regardless of the bulk DO concentrations (2 or 4 mg L^{-1}), oxic layers were limited to the upper 200 μm of the granules (Figure 3.9A), which agrees with the results of previous reports (Okabe et al., 1999,Song et al., 2013). The R(N_2O) calculated from the N_2O concentration profiles clearly indicated that N_2O production mainly occurs in the oxic layer (upper 200 μm) (Figure 3.9B and D). As the bulk DO concentrations increased, the N_2O production rates and the N_2O concentration inside the granules increased, mainly in the surface oxic layer.

These results indicated that increasing DO concentrations in the bulk liquid primarily stimulated N_2O production in the surface oxic layer. The FISH images and analysis of bacterial community structure showed that AOB, mainly *Nitrosomonas europaea*, were predominant in this layer. These findings indicated that N_2O might be produced by nitrification in the PN granules and that increases in DO in the bulk liquid predominantly stimulated N_2O production via nitrification in the surface oxic layer among the three N_2O production pathways. These *in situ* results clearly explained those of the batch tests (Figures 3.2 and 3.3), suggesting the importance of *in situ* analyses of PN granules to elucidate N_2O emission mechanisms in a PN process. In contrast to the autotrophic PN granules, N_2O was produced by heterotrophic denitrifiers in the deeper anoxic parts(>500 μm) of the heterotrophic PN granules, and the contribution of nitrification and nitrifier denitrification to N_2O emissions was relatively small (Ishii et al., 2014). Although we could not distinguish nitrifier denitrification from heterotrophic denitrification in the present study, N_2O production in the anoxic zones of the granules (Figure 3.9B) might be attributed to heterotrophic denitrification because N_2O is not produced via nitrifier denitrification under anoxic conditions (Kester et al., 1997; Yu et al. 2011). This may also have occurred because ammonium oxidation (*i.e.*, NH_2OH oxidation) by AOB does not occur under anoxic conditions and the density of AOB was very low in the anoxic zones (Rathnayake et al., 2013).

Higher *in situ* N_2O production was observed in the surface oxic layer of granules at pH 7.5 than at pH 6.5 and 8.5 (Figure 3.9D), which is consistent with the results of the batch experiments (Figure 3.4). The pH decreased slightly in the surface oxic layer owing to active ammonium oxidation (Figure 3.9C). Unlike the results of DO, the change in pH of the bulk liquid resulted in changes in pH throughout the granule. Therefore, changes in pH might affect N_2O production by

both nitrification in the outer parts and denitrification in the inner parts of the granule (Figure 3.4).

N_2O production pathways and rates would be affected by other physicochemical parameters such as NO_2^- concentration (Kampschreur et al., 2008; Law et al., 2013), NH_4^+ loading rate (Ni and Yuan, 2013), and COD/N ratio (Kampschreur et al., 2009). Therefore, further studies are required to determine the effects of NO_2^- and NH_4^+ concentrations and COD/N ratio on N_2O production mechanisms.

3.4 CONCLUSIONS

N_2O was mainly produced as a byproduct of nitrification by AOB belonging to the bacterial order *Nitrosomonadales* in the oxic surface layer of the autotrophic PN granules; therefore, increases in the bulk DO concentration stimulated ammonia oxidation and subsequently N_2O production. In PN granular sludge processes,N_2O is produced in microbial aggregates in which local physicochemical conditions differ greatly from those in bulk liquid. Therefore, it is essential to analyze *in situ* N_2O production and microbial community structures in relation to local physicochemical conditions to gain insight into N_2O emission mechanisms in PN processes.

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Chapter 4

Experimental evidence for nitric oxide emission from microbial granules by anaerobic ammonia oxidizing bacteria

4 EXPERIMENTAL EVIDENCE FOR NITRIC OXIDE EMISSIONFROM ANAEROBIC AMMONIA-OXIDIZING BACTERIAL GRANULES.

4.1 INTRODUCTION

Anaerobic ammonium oxidization (anammox) is the process performed by anammox bacteria in which ammonium (NH_4^+) is anaerobically oxidized with nitrite (NO_2^-) as electron acceptor to produce nitrogen gas (Mulder et al., 1995). Anammox bacteria are obligate anaerobic and chemoautotrophic bacteria affiliated with a monophyletic group in the phylum *Planctomycetes* (Strous et al., 1999). Recently, nitrogen removal from wastewaters by anammox processes has drawn attention due to its remarkable advantages over conventional nitrification–denitrification process, such as no requirement for external organic carbon, low oxygen demand, and small sludge production (Kartel et al., 2010; Jetten et al., 1997; Schmidt et al., 2003).

Some studies have reported the emission of nitric oxide (NO) in anammox processes (Kampscheur et al., 2008a; 2008b; Lotti et al., 2014), which 0.003 – 0.03% of nitrogen load of the anammox processes was emitted as NO. In addition, NO emissions from anammox granules (Carvajal-Arroyo et al. 2014) have been reported. NO is an important atmospheric trace gas, which has a direct effect on the ozone chemistry of the atmosphere (Crutzen, 1979). NO is a free radical and toxic to a wide range of organisms (Kampschreur 2008b; Zumft, 1993, 1997; Wink and Mtchell, 1998; Choi et al., 2006). Considering the toxicity of NO to humans, the Dutch government (MAC values, as of 1/1/2007) defined the relatively low maximum 8-h exposure limit (0.2 ppm). NO molecules have ecological influences on microbial consortia by regulating the specific microbial activities (Zart et al., 2000; Nadeem et al., 2013) and growth mode of biofilms (Schmidt et al., 2004), and enhancing the formation (Schmidt et al., 2004) and dispersion of bacterial biofilms (Barraud et al., 2006; 2009).

In an anammox biofilm or granule, NO can be produced via mainly four microbiological pathways. Firstly, NO is produced as an intermediate of the metabolic pathways of anammox bacteria (Kartel et al., 2011). Secondly, NO is produced in a nitrification process where nitrosyl radical (NOH) can be produced as an intermediate in oxidation of NH_2OH (Igarashi et al., 1997) as a side product during hydroxylamine (NH_2OH) oxidation into nitrite (NO_2^-) (Poughon et al., 2001) and the unstable NOH is biologically transformed to NO. Coexistence of aerobic ammonia oxidizing-bacteria (AOB) that oxidized ammonia by consuming trace amount of oxygen in the

surface of anammox granules was reported (Kindaichi et al., 2007; Tsushima et al., 1997a; 1997b; Okabe et al., 2011b). Thirdly, AOB can produce NO to reduce NO_2^- at higher NO_2^- concentration or under oxygen limiting condition (Wrage et al., 2001), which is well known as nitrifier denitrification. Furthermore, NO is produced as an intermediate during denitrification (i.e., reduction of NO_2^- to N_2) by heterotrophic bacteria (Zumff, 1997). The heterotrophic bacteria coexisted in anammox granules (Kindhaichi et al., 2007; Tsusima et al., 1997a; 1997b; Okabe et al., 2011b).

So far, only one study has revealed the mechanism of NO emissions from anammox granules (Carvajal-Arroyo et al. 2014). This is probably due to difficulty of detection of NO that is highly reactive with oxygen. Although NO concentrations were determined in off-gas of anammox reactors in the previous studies (Kampscheur et al., 2008a; 2009; 2008b), it is unclear which microorganisms and pathways are responsible for NO emissions in an anammox process. Consequently, the objective of this study was to identify NO emission pathways in anammox granules taken from a lab-scale granular anammox reactor and to investigate physicochemical parameters affecting the NO emissions.

4.2 MATERIALS AND METHODS

4.2.1 Anammox Reactor

A lab-scale up-flow column reactor (220 mL) (Bio column reactor, Fujisaki, Osaka, Japan) was operated at 37°C as previously described (Tsushima et al., 2007a). Anammox granules (3-5 mm in diameter) obtained from another anammox reactor (Okabe et al., 2011a) were inoculated with 70% (v/v) packing ratio, and the column reactor was operated with continuous supply of effluents discharged from a partial nitrification (PN) reactor (Rathnayake et al., 2013). Hydraulic retention time was set at 1.1 h. The total nitrogen (NH_4^+, NO_2^- and NO_3^-) removal efficiency (T-NRE) was calculated by dividing the total nitrogen removal rate (T-NRR) by total nitrogen loading rate (T-NLR).

4.2.2 Batch experiments

Standard anaerobic techniques were employed in an anaerobic chamber (Coy Laboratory Products, Grass Lake Charter Township, MI, USA) where oxygen concentration was maintained

at lower than 1 ppm (Ito et al., 2002; Oshiki et al., 2013). Anammox granules (7.5 to 25 mg-BSA) were harvested from the reactor, washed three times with 15 mL of an anoxic inorganic nutrient medium and homogenized with a mortar pestle style tissue homogenizer (Thomas Scientific, Swedesboro, NJ, USA). The inorganic nutrient medium (pH 7.2-7.5) consisted of $MgSO_4 \cdot 7H_2O$ (0.24 mM), $CaCl_2$ (0.47 mM), KH_2PO_4 (0.18 mM), $KHCO_3$ (1.0 mM), and 0.5 mL L^{-1} of trace element solutions I and II (van de Graaf et al., 1996). Oxygen was removed by purging N_2 gas for 30 min. The homogenized biomass was re-suspended in the medium at final concentrations of 0.15 to 0.5 mg-BSA mL^{-1}. The vial was sealed with a butyl rubber stopper and an aluminum cap. The headspace was replaced 3 times with He gas (>99.99995%) by vacuuming (2 min) and purging (1 min) and then the vial were incubated at 37°C under a dark condition. $^{14}NH_4^+$ and $^{15}NO_2^-$ were added to the vials at the final concentration of 5 mM, and the vials were incubated for 12 h. All of the above steps except exchange of gas were carried out in the anaerobic chamber. The concentrations of NH_4^+, NO_2^- and NO_3^- in the liquid phase and of $^{29}N_2$, $^{30}N_2$, ^{30}NO, and ^{31}NO in the headspace were determined in time course. $^{29}N_2$ is produced via $^{14}NH_4^+$ oxidation with $^{15}NO_2^-$ exclusively by anammox bacteria whereas $^{30}N_2$ is produced by heterotrophic bacteria. ^{30}NO is produced via $^{14}NH_4^+$ oxidation by AOB whereas ^{31}NO is produced via $^{15}NO_2^-$ reduction by anammox bacteria, heterotrophic denitrifiers and AOB via nitrifier denitrification.

Furthermore, the batch experiments with or without inhibitors of anammox were conducted to identify ^{31}NO production pathways. During this series of batch tests, biomass was incubated with under different conditions (Table 4.1). As positive control, $^{14}NH_4^+$ and $^{15}NO_2^-$were added to the vials at the final concentration of 2.5 mM. In parallel, biomass was incubated with (1) $^{14}NH_4^+$ (2.5 mM), $^{15}NO_2^-$ (2.5 mM), and methanol (1 mM), (2) $^{15}NO_2^-$ (2.5 mM), $^{15}NO_2^-$ (20 mM) (3) $^{14}NH_4^+$ (2.5 mM), $^{15}NO_2^-$ (2.5 mM), and penicillin G (500 mgL^{-1}). In addition, autoclaved biomass was incubated with $^{14}NH_4^+$ (2.5 mM), $^{15}NO_2^-$ (2.5 mM) as negative control. All the types of microorganisms are supposed to be active in the positive control vials. Most of the bacteria (*i.e* AOB and heterotrophic denitrifiers) except anammox bacteria are suppose to be in active in the vials which incubated with penicillin G (Okabe et al, 2011b). Activity of anammox bacteria in the vials with methanol addition is expected to be significantly smaller than the positive control.

Table 4.1: Experimental conditions for the batch experiments with or without inhibitors of anammox were conducted to identify ^{31}NO production pathways

	Sunstrate added (initial conc, (mM)	Addictive (Initial conc.)	Inhibitory effects
# I (Positive control)	$^{14}NH_4^+$ (2.5 mM) $^{15}NO_2^-$ (2.5 mM)	–	All the bacteria are active
# II	$^{14}NH_4^+$ (2.5 mM) $^{15}NO_2^-$ (2.5 mM)	Methanol (1.0 mM)	Anammox bacteria are inhibited
# III	$^{14}NH_4^+$ (2.5 mM) $^{15}NO_2^-$ (20 mM)	–	Favorable for heterotrophic denitrifiers
# IV	$^{14}NH_4^+$ (2.5 mM) $^{15}NO_2^-$ (2.5 mM	Penicillin G (500 mg L^{-1})	Most of the heterotrophic denitrifiers and AOB were inhibited
# V	$^{14}NH_4^+$ (2.5 mM) $^{15}NO_2^-$ (2.5 mM	Autoclaved biomass	No biological activity

Influences of pH on $^{29}N_2$ and ^{31}NO emissions were examined in the range of pH 6.0 to 9.0. During batch tests to investigate the influences of pH on $^{29}N_2$ and ^{31}NO emissions, biomass was incubated with $^{14}NH_4^+$ and $^{15}NO_2^-$ at the final concentration of 2.5 mM. pH was adjusted by adding 2N H_2SO_4 or 1N NaOH. Influence of NH_4^+ and NO_2^- concentrations on $^{29}N_2$ and ^{31}NO emission was examined at NH_4^+ and NO_2^-concentrations of 2 to 30 mM. Biomass-specific gas ($^{29}N_2$ and ^{31}NO) emission rates in the batch vials were calculated based on the changes in gas concentrations in the headspace of the batch vial during the initial active gas emission phase of the batch test and the mass of protein in biomass (mg-BSA).

4.2.3 Spatial distributions of bacteria and their activities in single anammox granules

The spatial distributions of total and anammox bacteria in single anammox granules were determined by fluorescence in situ hybridization (FISH) (Okabe et al., 2011b). The relative

abundance of the anammox bacteria in the single granule was determined by dividing the area of anammox bacteria by that of total bacteria. The steady-state concentration profiles of NH_4^+, NO_2^-, NO_3^-, pH, N_2O, and NO in the single granules were determined with microsensors (Rathnayake et al., 2013). The synthetic medium for the microsensor measurements consisted of $(NH_4)_2SO_4$ (2.25 mM), $NaNO_2$ (4.5 mM), and inorganic salts (Rathnayake et al., 2013). The granules with the diameter in a range of 2–3 mm were acclimated in 2.5 L of the medium under anoxic condition at least 3 h before microsensor measurements to ensure that steady-state profiles were obtained. Physicochemical parameters of the medium were almost unchanged during the microsensor measurements. Net volumetric emission rates of NO, N_2O, NH_4^+ and NO_2^- in the single granules were estimated from the measured concentration profiles (Okabe et al., 1999). Diffusion coefficients of 1.38×10^{-5}, 1.25×10^{-5}, 2.10×10^{-5}, and 2.21×10^{-5} cm^2 s^{-1} for NH_4^+, NO_2^-, N_2O, and NO, respectively, at 25°C were used to calculate the net volumetric production rates (Okabe et al., 2011b; Satoh et al., 2007; Schreiber et al. 2009).

4.2.4 DAF-FM DA dye staining

Spatial accumulation of NO molecules in anammox granules was investigated by staining the granules with 4-amino-5-methylamino- 2′ ,7′ -difluorescein (DAF-FM DA) that reacts with NO to form fluorescent product and can be observed by fluorescence microscopy (Kojima et al., 1999). Intact anammox granules were incubated in an anoxic batch vial prepared in the same procedure as described in the batch experiment section. NH_4^+ and NO_2^-were added to the vial at a final concentration of 4.5 mM, and the granules were incubated at 37°C under a dark condition. After 3 h of pre-incubation, DAF-FM DA (Sekisui medical, Tokyo, Japan) was supplemented at a final concentration of 10 μM, and the granules were further incubated for 30 min. The granules were also incubated in the medium without NH_4^+ and NO_2^-addition as a negative control. The stained granules were washed three times with sterile phosphate buffer solution embedded into Tissue-Tec OCT compound (Sakura Finetek, Torrance, CA), and then sliced into 10-μm-thick specimens with a cryostat CM 1510S (Leica, Bensheim, Germany). The specimens were placed on a gelatin-coated microscopic slide (Cellline Associates) and dried in the dark. Green fluorescence of DAF-FM DA was observed using a model LSM510 confocal laser-scanning microscope (CLSM, Carl Zeiss, Oberkochen, Germany).

4.2.5 Chemical analysis

Protein concentration in the biomass was determined with an RC DC Protein Assay kit (Bio-Rad) as described previously (Oshiki et al., 2013). NH_4^+, NO_2^- and NO_3^- concentrations were determined by ion chromatography as described previously (Rathnayake et al., 2013). Concentrations of ^{15}N-labeled gaseous compound (i.e., $^{29}N_2$, $^{30}N_2$, ^{30}NO and ^{31}NO) were determined by gas chromatography mass spectrometry (GC-MS) analysis as described previously (Oshiki et al., 2013). Fifty microliter of headspace gas was collected with a gas-tight glass syringe (VICI AG International, Schenkon, Switzerland), immediately injected to a GCMS-QP2010 SE gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a CP-Pora BOND Q fused silica capillary column (Agilent Technologies, Santa Clara, CA), and m/z = 29, 30 and 31 were monitored in scan mode for determination of $^{29}N_2$, $^{30}N_2{}^{30}NO$, and ^{31}NO, respectively.

4.3 RESULTS AND DISCUSSIONS

4.3.1 Reactor performance

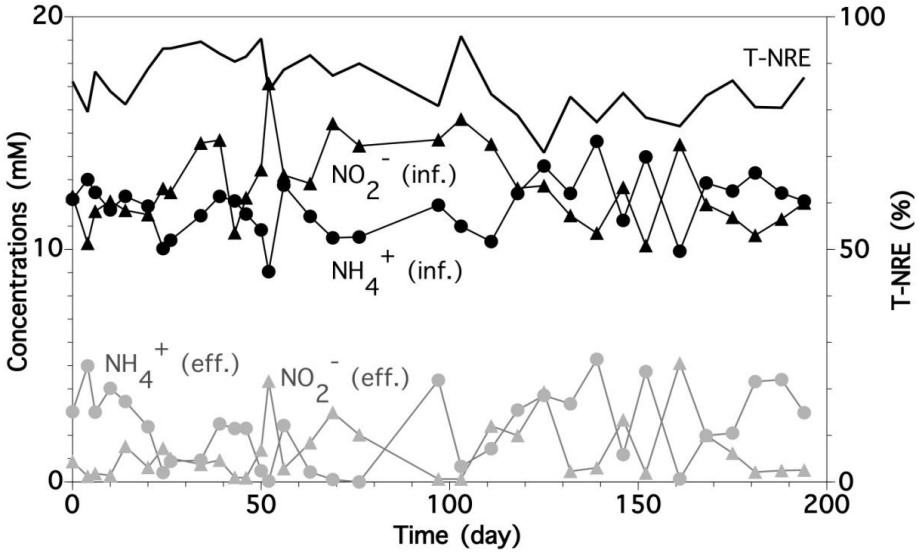

Figure 4.1: Concentrations of NH_4^+ and NO_2^- in influents and effluents, total nitrogen removal (TNR) efficiency of the anammox reactor.

NH_4^+ and NO_2^- concentrations in the influent and the effluent and T-NRE of the reactor are shown in Figure 4.1. Average NH_4^+ and NO_2^- concentrations (± standard deviation) in the influent provided from a PN reactor were 12.1 ± 1.5 and 12.6 ± 1.7 mM, respectively. Average NO_3^- concentrations in the influent were < 0.6 mM. Thus, the reactor was operated at an average

T-NLR of 18.0 ± 6.6 mmol L^{-1} h^{-1}. Average NH_4^+, NO_2^- and NO_3^- concentrations in the effluent were 2.6 ± 1.7, 1.2 ± 1.2 and 2.0 ± 0.6 mM, respectively. . The stoichiometric ratio of NH_4^+ consumption: NO_2^- consumption: NO_3^- production in the reactor was 1.0: 1.19: 0.14, that is close to the previously determined anammox stoichiometry (1.0: 1.15: 0.16) (Lotti et al., 2014)T-NRE and T-NRR were calculated to be 84.5 ± 7.8% and 14.2 ± 5.9 mmol L^{-1} h^{-1}, respectively. pH in the influent ranged from 7.1 to 7.8 and pH increased 8.3 ± 0.2 in the effluent, due to consumption of protons by anammox reaction. The average N_2O emission rate was 45 ± 61 μmol L^{-1} h^{-1}, which accounted for 0.0025% of the T-NLR. ^{30}NO concentrations in the grab off-gas from the reactor was measured by using GC-MS. However, we GC-MS measurement could not detect any ^{30}NO emission from our anammox reactor.

4.3.2 Bacterial community structure and phylogenetic analysis

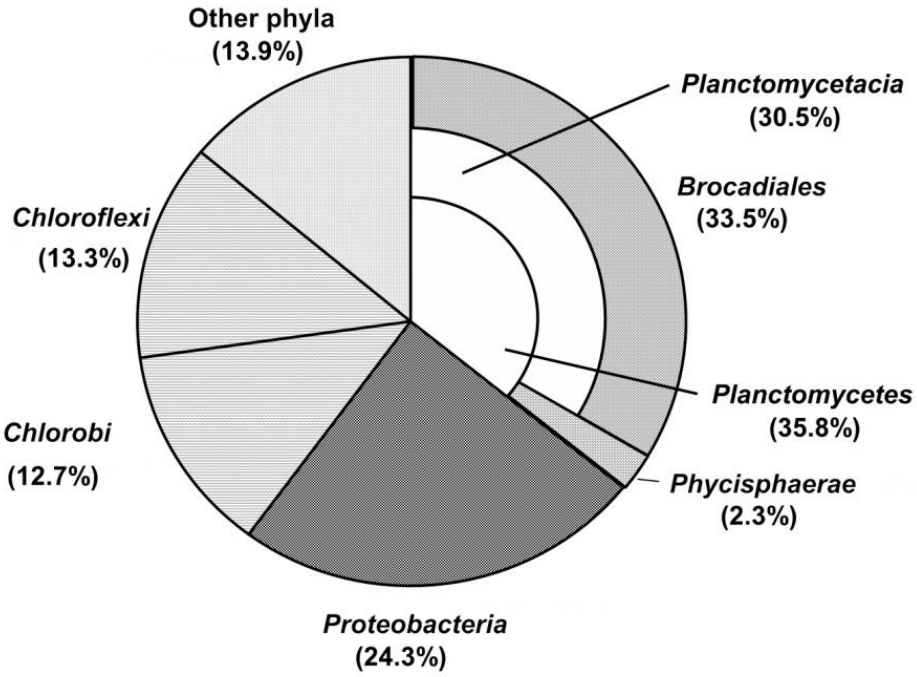

Figure 4.2: Microbial community composition of the anammox based on the 16S rRNA gene sequences obtained by MiSeq sequencing technology. The inner, middle, and outer pie charts show the community composition at the phylum, order, and class levels, respectively.

16S rRNA gene sequence library analysis was conducted to examine the bacterial community structure in the anammox granules. Various types of bacteria were identified based on the 16S rRNA gene deep sequencing analysis (Figure 4.2). Sequences related to *Brocadiales* were

present in high abundance (33.5 ± 6.4%) in our anammox granules. These findings suggested that *Brocadiales* was highly enriched in our anammox granules. We also detected 16S rRNA gene sequences from potential denitrifiers such as *Burkholderiales* (1.77 ± 0.68%) and *Rhodocyclales* (16.7 ± 6.8%), and *Nitrosomonadales* (0.27 ± 0.12%) although their proportions were smaller (Table 4.2). Bioreactor clone which represent the anammox bacteria sequence in our reactor and '*Ca.* Brocadia sinica' shared 99% 16S rRNA gene sequence similarity (Figure 4.3), which indicates that the anammox bacterium represented by the nucleotide sequence Bioreactor clone is closely related to the group ''*Ca.* Brocadia sinica''.

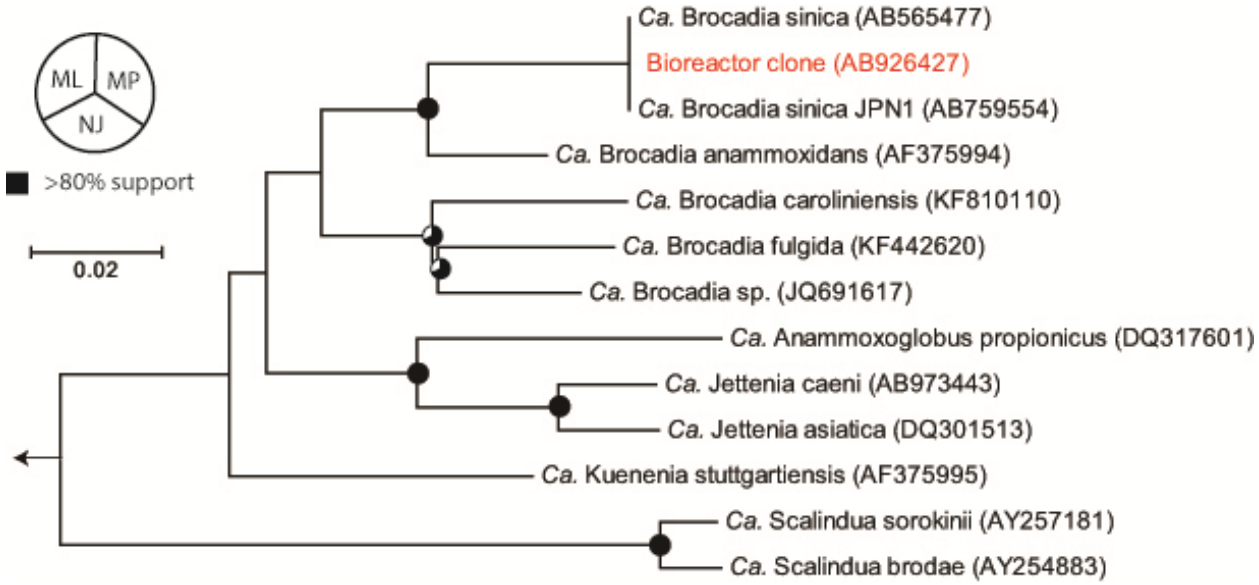

Figure 4.3: Phylogenetic relationship between the representative anammox-related sequences obtained in this study (shown in red) and other anammox based on the 16S rRNA gene sequences. The phylogenetic tree was constructed by the maximum-likelihood method (ML), most probable method (MP) and neighbour joining (NJ) method. The accession numbers of the reference strains in the DDBJ/EMBL/GenBank databases are shown in brackets**.** The bootstrap values (>50%) derived from 1,000 replicates are indicated next to the branches.

Table 4.2: The microbial community structure in anammox granules

	Relative abundance (%)
Bacteria	100 ± 0
Planctomycetes	35.8 ± 6.6
Planctomycetacia	33.5 ± 6.4
Brocadiales	33.5 ± 6.4
Proteobacteria	24.3 ± 4.5
Betaproteobacteria	19.4 ± 6.0
Rhodocyclales	16.7 ± 6.8
Burkholderiales	1.77 ± 0.68
Nitrosomonadales	0.27 ± 0.12
Gammaproteobacteria	2.47 ± 1.27
Alphaproteobacteria	2.10 ± 0.44
Deltaproteobacteria	0.3 ± 0.1
Chlorobi	12.7 ± 2.0
Chloroflexi	13.3± 5.8
Armatimonadetes	6.9 ± 1.7

4.3.3 Batch experiments

The time-course changes in $^{29}N_2$ and ^{31}NO concentrations in the gas phase and $^{15}NO_2^-$ and NH_4^+ concentrations in the liquid phase of the batch test vial are shown in Figure 4.4. Biomass amount in the vial was 16.5 mg-BSA. $^{14}NH_4^+$ and $^{15}NO_2^-$ concentrations decreased from 191 μmol vial^{-1} (4.8 mM) and 221 μmol vial^{-1} (5.5 mM) to 97 μmol vial^{-1} (2.4 mM) and 93 μmol vial^{-1} (2.3 mM) for 12 h, respectively, under anoxic condition.$^{29}N_2$ and $^{30}N_2$were produced to 133 μmol vial^{-1} and 7.7 μmol vial^{-1}, respectively, while NH_4^+ and $^{15}NO_2^-$ were consumed. NO_3^- concentration slightly increased from 1.1 μmol vial^{-1} to 5.9 μmol vial^{-1}. ^{31}NO was accumulated until 8 h and thereafter became constant. A ratio of ^{31}NO emission to $^{29}N_2$ emission for 8 h was 0.0007. ^{30}NO was not detected in 12 h of incubation.

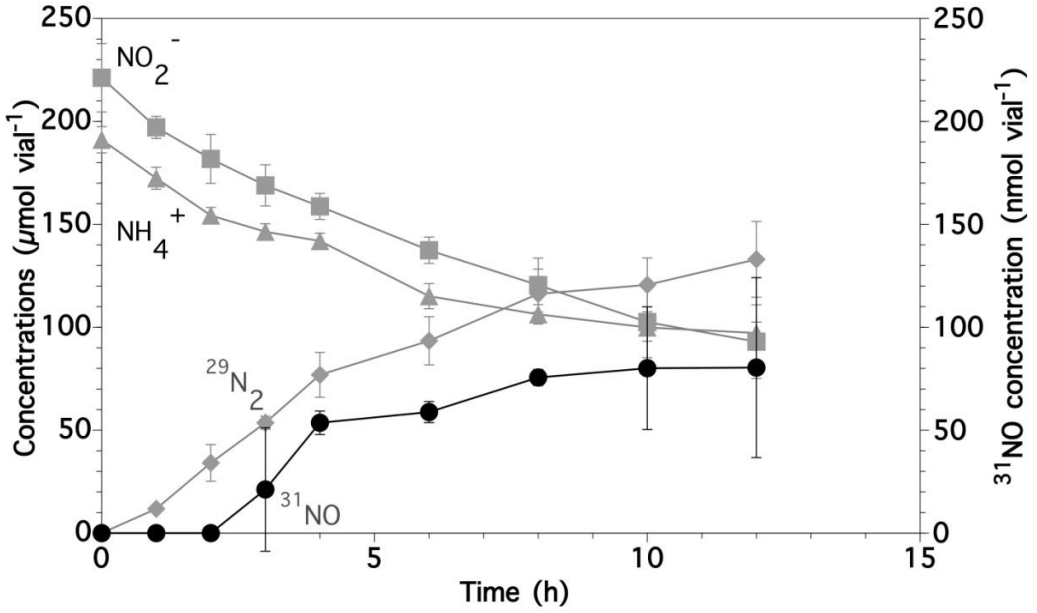

Figure 4.4: Typical time-course changes in $^{29}N_2$ and ^{31}NO concentrations in the gas phase and $^{15}NO_2^-$ and $^{14}NH_4^+$ concentrations in the liquid phase of the batch test vial during incubation of anammox granules. The initial $^{14}NH_4^+$ and $^{15}NO_2^-$ concentrations were 5 mM. Error bars indicated standard deviations obtained from triplicate vials (n = 3).

The biomass-specific $^{29}N_2$ and ^{31}NO emission rates determined from the batch experiments without or with inhibitors are shown in Figure 4.5A. The $^{29}N_2$ and the ^{31}NO emission rates were calculated to be 0.41 ± 0.16 μmol mg-BSA^{-1} h^{-1} and 51.6 ± 11.9 nmol mg-BSA^{-1} h^{-1} under the positive control condition (I in Figure 4.5A)

When the granules were incubated with methanol (II in Figure 4.5A) the $^{29}N_2$ emission rates were reduced significantly, and the ^{31}NO production rate was not significantly affected as compared with those under the positive control condition. In contrast, the incubation with penicillin G (IV in Figure 4.5A) did not have statistically significant effect on the $^{29}N_2$ and the ^{31}NO emission rates. Neither $^{29}N_2$ nor ^{31}NO was produced from the autoclaved granules prepared as the negative control (V in Figure 4.5). As to NO production, ^{31}NO was not produced from autoclaved biomass, indicating that the NO was produced by biological reaction. No ^{30}NO was not detected in all batch tests further indicated that contribution of NH_2OH oxidation by AOB and NOB on NO emission in anammox granules was negligible. It is known that penicillin G is not active against anammox bacteria but inhibit the activity of most of the bacteria (Okabe et al., 2011b) and methanol inhibit the activity of anammox bacteria immediately and

irreversibly(Güven et al., 2005). Therefore, it is likely that both anammox and heterotrophic denitrifying bacteria responsible for the ^{31}NO produced in the anammox granules.

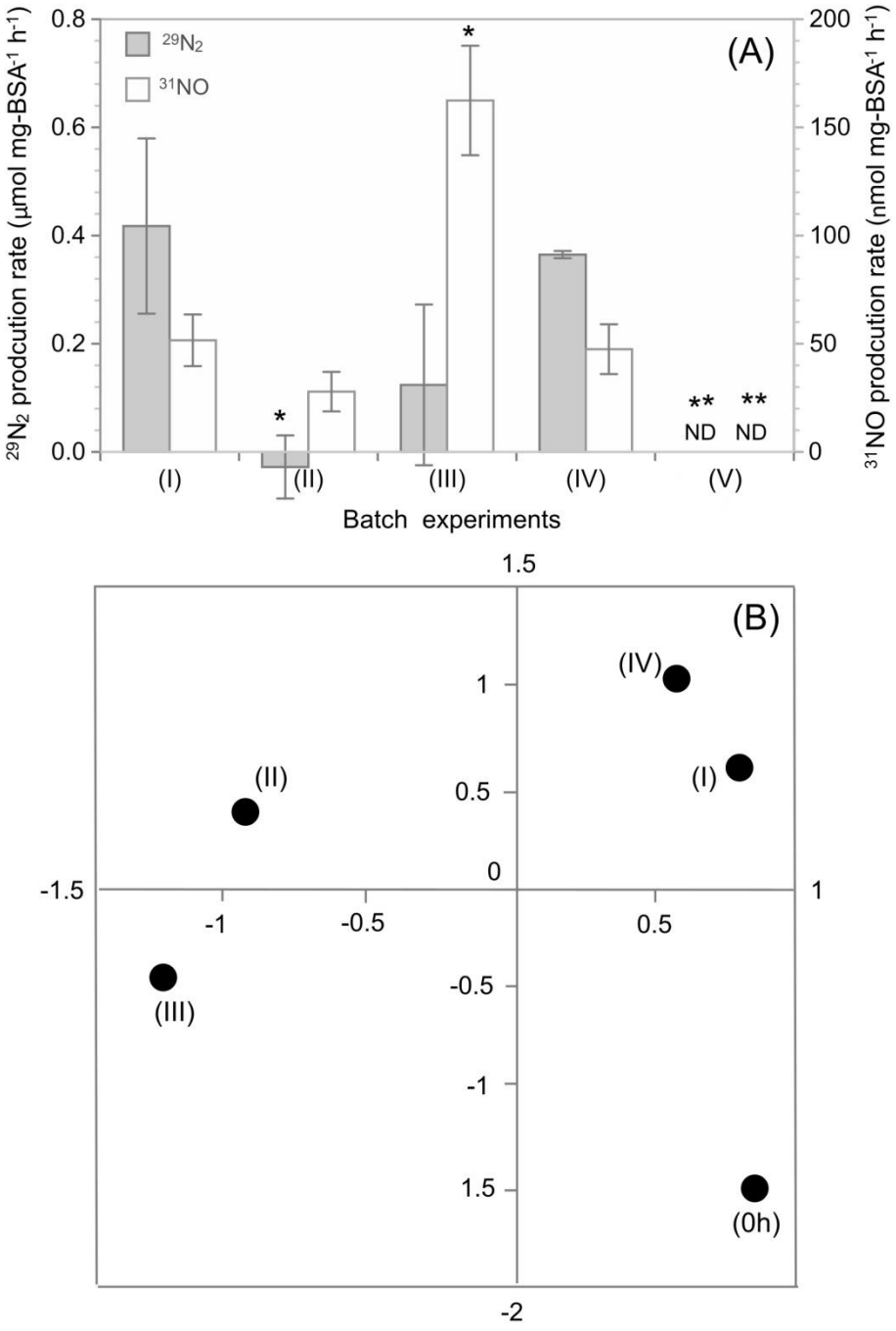

Figure 4.5: (A) Biomass-specific $^{29}N_2$ and ^{31}NO emission rates determined in batch culture experiments. (B) The results of principle component (PCA) analysis of trancriptome of "*Ca. Brocadia sinica*".The culture conditions were summarized in Table 4.1. (n = 3; *P < 0.01, **P < 0.001, Student's t-test; error bars, standard deviations). ND; not detected

Transcriptome analysis was performed to identify the genes associated with NO production from anammox granules. Total RNA was extracted from anammox granules, reverse-transcribed to cDNA, and nucleic acid sequences were determined using illumina Miseq. Transcriptomes of "*Ca.* Brocadia sinica" were further analyzed by principle component (PCA) analysis (Figure 4.5B). The PCA analysis showed that the transcriptome from anammox granules (^{14}NH4+, ^{15}NO2^{-}, each 2 mM) incubated with (IV in Figure 4.5B) or without penicillin G (I in Figure 4.5 were grouped into the same cluster. Penicillin G is a strong inhibitor of most heterotrophs, while the "*Ca.* Brocadia sinica" is insensitive to the penicillin G (Oshiki et al., 2013; Okabe et al., 2011). Therefore, the outcome of PCR analysis indicates that the transcriptomes were mostly attributed to "*Ca.* Brocadia sinica"; i.e., Remarkable difference in transcriptome must appear if our transcriptomes contains large amounts of transcripts derived from heterotrophs. The supplementation of methanol and increase of $^{15}NO_2^-$ concentration from 2 to 20 mM resulted in remarkable alteration of the transcriptomes (Figure 4.5B). Because ^{31}NO production was more prominent in the presence of 20 mM $^{15}NO_2^-$ (Figure 4.5A), the transcripts induced under the condition probably participated to the ^{15}NO production. Under the presence of methanol, anammox activity of "*Ca.* Brocadia sinica" was completely compromised, while the anammox granule produced significant amounts of ^{31}NO (II in Figure 4.5A). On the other hand, increase of $^{15}NO_2^-$ concentration from 2 to 20 mM resulted in 2-folds increase of ^{31}NO production rates although anammox activity was almost unchanged (III in Figure 4.5A). Those outcomes suggest that ^{15}NO produced under these two conditions was mostly synthesized from nitrite reduction by denitrifiers but not from the anammox process of "*Ca.* Brocadia sinica". The hypothesis was supported from abundances of NirS and NirK transcripts; the transcripts were 2-times more abundant in the anammox granule incubated with 20 mM $^{15}NO_2^-$ (Table 4.4). Additionally, our FISH analysis demonstrated that nitrifiers and heterotrophs are coexisting in anammox granules of "*Ca.* Brocadia sinica". Denitrifying bacteria might reduce $^{15}NO_2^-$ to ^{31}NO by using organic matter derived from anammox bacteria as an electron donor (Kindaichi et al., 2004).

Table 4.3: Transcriptome of genes involved in nitrogen transformation by "*Candidatus Brocadia sinica*"; Sequence reads were mapped to the "*Ca.* Brocadia sinica" genome (GenBank accession number BAFN01000001-BAFN01000003). Abbreviation of locus tag; BROSI_. Hdh/Hao; hydrazine/hydroxylamine dehydrogenase, Nrf; pentahaem cytochrome c nitrite reductase, Nxr; nitrite/nitrate oxidoreductase, Hzs; hydrazine synthase. ND; not detected

Gene	locus_tag	Fragments per kilobase per million fragments				
		(I)	(II)	(III)	(IV)	0 h
Hdh/Hao	A0131	1749	1067	1048	1667	2198
	A0501	1484	841	746	1373	1368
	A0718	259	298	264	223	183
	A1461	602	461	448	528	486
	A2345	2.1	7.5	22	4	3
	A2485	118	179	154	109	151
	A2677	5499	2735	2182	5130	6147
	A2680	98	52	60	58	89
	A3534	125	208	196	111	103
	A3864	3056	2388	1491	4009	1774
NrfA	A3711	465	460	326	498	472
NxrA	A0941	2028	1220	1018	1943	2005
NxrB	A0944	1293	835	692	1184	1594
NorV	A3434	238	195	165	201	102
HzsB	A0631	ND	ND	ND	ND	ND
	A2674	ND	ND	ND	ND	ND
HzsG	A0630	207	172	179	192	509
	A2675	304	347	538	251	855
HzsA	A0629	ND	ND	ND	ND	ND
	A2676	ND	ND	ND	ND	ND

Table 4.4: Relative abundances of genes involved in nitrogen transformation; Two replicate libraries (n=1 and 2) were prepared from each samples for transcriptome analysis. Orthologues of each genes were examined by blastn analysis using nucleic acid sequences deposited in fungene database (http://fungene.cme.msu.edu) as reference database. Relative abundances = blast hits / total reads. NarG; nitrate reductase, Nxr; nitrite/nitrate oxidoreductase, NirS; cytochrome cd_1-type NO-forming nitrite reductase, NirK; copper-containing NO-forming nitrite reductase, NorB: nitric oxide reductase, NosZ; nitrous oxide reductase, and AmoA; ammonia monooxygenase. ND; not detected.

Sample ID		Total reads	Relative abundances (%) NarG	NxrB	NirS/NirK	NorB	NosZ	AmoA
(I)	n=1	3286146	0.036	0.0013	0.0068	0.0067	0.0450	0.000091
	n=2	3272284	0.037	0.0013	0.0067	0.0068	0.0450	0.000092
(II)	n=1	2957548	0.032	0.0021	0.0072	0.0045	0.0330	0.000034
	n=2	2949506	0.033	0.0021	0.0073	0.0045	0.0340	0.000034
(III)	n=1	881305	0.031	0.0024	0.0092	0.0031	0.0090	0.000230
	n=2	879816	0.032	0.0023	0.0093	0.0027	0.0090	0.000230
(IV)	n=1	2397357	0.037	0.0014	0.0068	0.0041	0.0360	ND
	n=2	2387587	0.037	0.0014	0.0066	0.0040	0.0350	ND
0 h	n=1	911834	0.049	0.0021	0.0073	0.0020	0.0110	0.000110
	n=2	910567	0.048	0.0021	0.0074	0.0020	0.0110	0.000110

However, most of the induced transcripts of "*Ca.* Brocadia sinica" were annotated as hypothetical protein, and biological function could not be deduced by analysis of metabolic pathway in KEGG database (http://www.genome.jp/kegg/). In addition to the BROSI_A2345 gene, transcripts of NirS and NirK encoding cytochrome *cd1*-type and copper-containing NO-forming nitrite reductase, respectively, were also more abundant in the transcriptome of anammox granules incubated with 20 mM $^{15}NO_2^-$ as determined by blast analysis using fungene database (http://fungene.cme.msu.edu) (Table 4.4). Because the NirS and nirK are not located in the "*Ca.* Brocadia sinica" genome (Oshiki et al., 2015), those transcripts were probably derived from coexisting denitrifiers. Among a core gene set involved in nitrogen transformation of "*Ca.*

Brocadia sinica", the BROSI_A2345 gene encoding hydrazine dehydrogenase (Hdh) was 10-times more abundant.

The involvement of "*Ca.* Brocadia sinica" in the ^{31}NO production still cannot be excluded, because 31NO was produced in the batch vial where denitrifiers are inhibited (IV in Figure 4.5) and specific functions of genes induced by increasing $^{15}NO_2^-$ concentration could not be identified. The authors recently demonstrated $^{15}NO_2^-$ reduction to ^{31}NO by cell-free extracts of "*Ca.* Brocadia sinica", but the responsible nitrite reductase is yet to be identified (Oshiki et al., under reviewing). Obviously, other study identifying NO-forming nitrite reductase in anammox granules is required in future. It is notable that abundances of the hdh transcript increased 10 folds by increasing $^{15}NO_2^-$ concentration. The Hdh is an orthologue of Hao encoding hydroxylamine dehydrogenase, and the "*Ca.* Brocadia sinica" genome encodes 10 copies of Hdh/Hao (Oshiki et al., 2015). Probably, those anammox bacterial hdh/hao genes have different physiological functions; *i.e.*, 1) hydrazine oxidation to N_2 gas by Hdh, 2) hydroxylamine oxidation to NO by Hao, and 3) NO reduction to hydroxylamine by Hao (4). NO_2^- reduction to NO by anammox bacterial Hdh has not been demonstrated, which remains to be explored.

NO has been detected in the off-gas of anammox reactors (Kampschreur et al., 2008a; 2008b; 2009), anammox culture inhibited with acetylene (Kartal et al., 2011), anammox granular sludge incubated with NO_2^- (Carvajal-Arroyo et al., 2014) and a highly enriched anammox microbial community (Lotti et al., 2014). However, the mechanism of NO emission in anammox granules is unclear. NO is toxic to organisms and it has a significant effect on the environment as described in Introduction section. In addition, NO is toxic to microorganisms so that NO suppresses growth of bacteria (Choi et al., 2006), and regulates the specific microbial activities (Zart et al., 2000; Nadeem et al., 2013) and growth mode of the biofilms (Schmidt et al., 2004), and enhances the formation (Schmidt et al., 2004) and dispersion of biofilms (Barraud et al., 2006; 2009). In addition, NO functions as a signaling molecule in bacteria (Barraud et al., 2006; Vollack and Zumft , 2001; Zumft, 2002**).**

The effects of NH_4^+ and NO_2^- concentrations and pH values on biomass-specific $^{29}N_2$ and ^{31}NO emission rates were investigated (Figure 4.6). When NH_4^+ concentrations were below 5 mM, specific $^{29}N_2$ emission rates were approximately 0.3 μmol mg-BSA^{-1} h^{-1} (Figure 4.6A). The rates

decreased as NH_4^+ concentrations increased. Specific ^{31}NO emission rates at > 20 mM of NH_4^+ concentrations were about twice as high as those at < 10 mM of NH_4^+ concentrations.

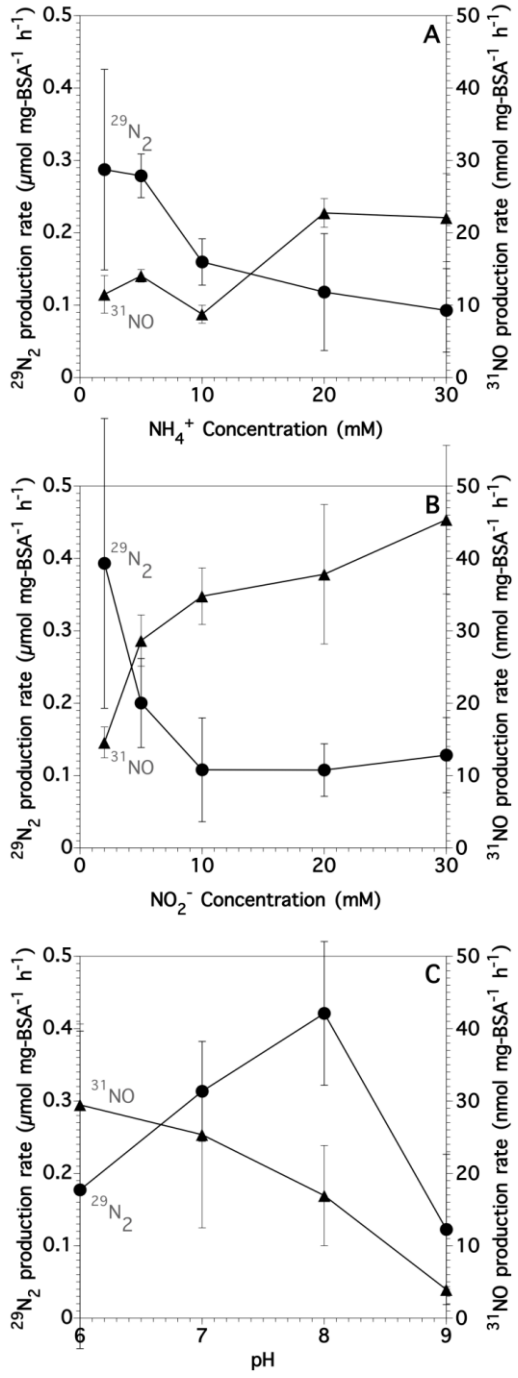

Figure 4.6: Effects of NH_4^+(A) and NO_2^- (B) concentrations and pH values (C) on NO emissions from anammox granules. The concentration of $^{15}NO_2^-$ in experiment (A) and $^{14}NH_4^+$ in experiment (B), and $^{15}NO_2^-$ and $^{14}NH_4^+$ concentrations in experiment (C) was 2.5 mM, 2.5 mM, and 2.5 mM and 2.5 mM, respectively.

Increase in $^{15}NO_2^-$ concentration resulted in decrease in specific $^{29}N_2$ emission rates and stimulation of ^{31}NO emission at < 10 mM of NO_2^- concentrations (Figure 4.6B). When the granules were incubated in a range of pH 6–9 (Figure 4.6C), specific $^{29}N_2$ emission rate was highest at pH 8 whereas specific ^{31}NO emission rates decreased with increasing pH. Higher NO_2^- concentration increased NO concentrations in the off-gas of a single-stage nitritation-anammox reactor (Kampscheur et al., 2009) and in headspace of an anammox granular culture (Carvajal-Arroyo et al., 2014), which agreed with our results (Figure 4.6B). The increase in NO production with elevated NO_2^- concentration may occur due to interruption of the metabolic steps following NO_2^- reduction (Carvajal-Arroyo et al. 2014), intracellular imbalance between the production and consumption of electron equivalents (Perez-Garcia et al., 2014), and increase in the contribution of heterotrophic denitrifies. The 50% inhibitory concentrations for "*Ca.* Brocadia sinica" for NO_2^- was 16 mM (Oshiki et al., 2011). Therefore, the higher NO emissions and lower anammox activities at higher NO_2^- concentrations were probably due to the interruption of the metabolic steps following NO_2^- reduction, that is, synthesis of N_2H_2, and or generation of N_2 gas (Carvajal-Arroyo et al. 2014). Recently, Perez-Garcia et al. (2014) reported that NO production by *N. europaea* primarily resulted from an intracellular imbalance between the production and consumption of electron equivalents. Similarity, we can hypothesize that observed high NO production at higher NO_2^- concentration may probably due to the excess electron production under large NH_4^+ / NO_2^-. Moreover, increase in the contribution of heterotrophic denitrifies to NO production at higher NO_2^- cannot be negated. Because, transcriptome level analysis revealed that denitrifier's Nir gene was highly expressed at high NO_2^- concentrations (Table 4.4).

Increase in NO production with lowering of pH was observed. Carvajal-Arroyo et al. (2014b) recently reported that anammox granules which dominating genus *Borcadia* emitted more NO at lower pH, which agreed with the results in the present study (Figure 4.6C). They clearly concluded that addition of substrates (*i.e* NO_2^- and NH4+) into resting (*i.e* no anammox reaction was occurred) anammox cells inhibited by NO_2^- and/or pH at acidic conditions. Carvajal-Arroyo et al. (2014b) further hypothesized the occurrence of NO_2^- inhibition of anammox bacteria due to accumulation of $NO_2^{-\ in}$ the sensitive area of the anammox cells (eg. iboplasms, anammoxosome). Since, the anammox biomass used in our experiments was in resting condition before addition the substrate, the addition of substrate may cause partial NO_2^- inhibition under acidic condition resulting high NO emission at low pH values.

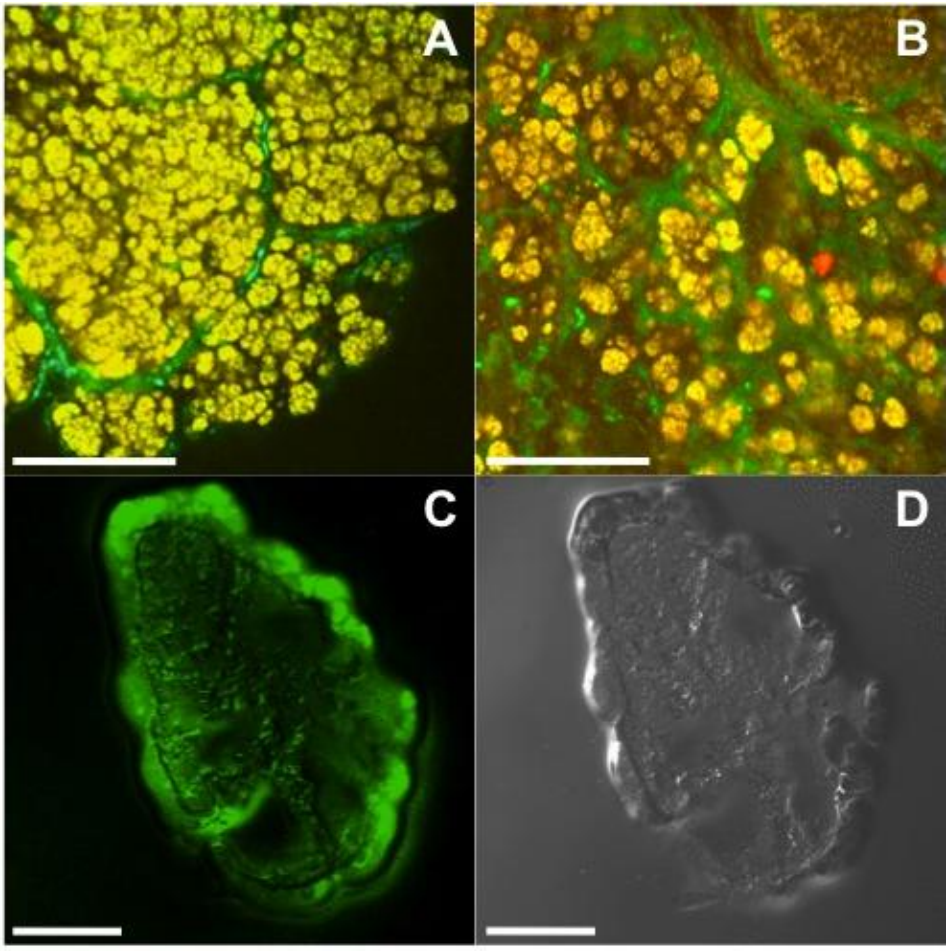

Figure 4.7: (A) Confocal laser scanning microscope images of a thin cross-section of the outer surface of the anammox granules showing in situ spatial distribution of anammox bacteria (yellow) and other bacteria (green) after fluorescence in situ hybridization. (B) Coexistence of anammox bacteria (yellow) and other bacteria (green)in the inner part of granule.(C) An epifluorescence image of DAF-FM derivative of NO accumulation in an entire granule, showing high NO accumulation in the outer surface.(D) A phase contrast image corresponding to panel C. The scale bars indicate 20 μm in panels A and B and 100 μm in panels C and D, respectively.

4.3.4 Spatial distribution of microorganisms and their activities

The cells of "*Ca*. Brocadia sinica" proliferated in the anammox granules as micro colonies (Figure 4.7A and Figure 4.7B). The micro colonies distributed densely in the surface layer (Figure 4.7A) while they were less dense in the inner parts of the granules (Figure 4.7B). In-situ detection of NO molecules in the anammox granules was carried out by DAF-FM DA staining and fluorescence microscopy. Green fluorescence derived from the DAF-FM DA was detected

from the surface layer of the anammox granules (Figure 4.7C and 4.7D), indicating the NO molecules accumulated. No fluorescence was detected when the anammox granules were incubated under the same condition without addition of NH_4^+ and NO_2^- (data not shown), suggesting the DAF-FM DA reacted with NO molecules produced in the anammox granules.

The steady state concentration profiles of NH_4^+, NO_2^-, NO_3^-, pH, N_2O, and NO in the anammox granules were determined with microsensors (Figure 4.8A and Figure 4.8C), based on which the spatial distributions of the volumetric production rates of nitrogenous compounds were calculated (Figure 4.8B and Figure 4.8D). NH_4^+ and NO_2^- concentrations decreased to a depth of 700 μm under the realistic measuring condition (*i.e* average reactor operational condition) while NO_3^- concentration slightly increased (Figure 4.8A). Simultaneous NH_4^+ and NO_2^- consumption, indicating anammox reaction, was mainly detected in the upper 300 μm (Figure 4.8B).

NO was mainly produced in the upper 200 μm under realistic measuring condition, in contrast, N_2O was mainly produced below a depth of 300 μm (Figure 4.8D). When the granules were incubated in the media with methanol (*i.e*. II in Figure 4.8C and Figure 4.8D), NO concentrations and NO production rates in the granules decreased about half of that under the positive control condition. The presence of penicillin G (*i.e*. IV in Figure 4.8C and Figure 4.8D) did not significant affect these values. NO concentrations in the granules, which were autoclaved or treated with carboxy-PTIO, were below detection limit (3 nM) (date is not shown).

The maximum NO concentration in the anammox granule (2.7 μM) in our present study was comparable to that in a nitrifying biofilm (2.3 μM) (Schreiber et al., 2008) whereas it was one order of magnitude higher than those in autotrophic nitrifying biofilm (0.35 μM) (Schreiber et al., 2009), in dental biofilm (0.2 μM) (Schreiber et al., 2011), and in PN granules (0.15 μM) obtained from the PN reactor that connected to our anammox reactor (data ia not shown). Since NO concentrations in the anammox granules in the present study (up to 2.7 μM) were higher than those in nitrifying and dental biofilms (Schreiber et al., 2009; Schreiber et al., 2011), the potential influence of NO on microorganisms in the anammox granules should be investigated in the future study.

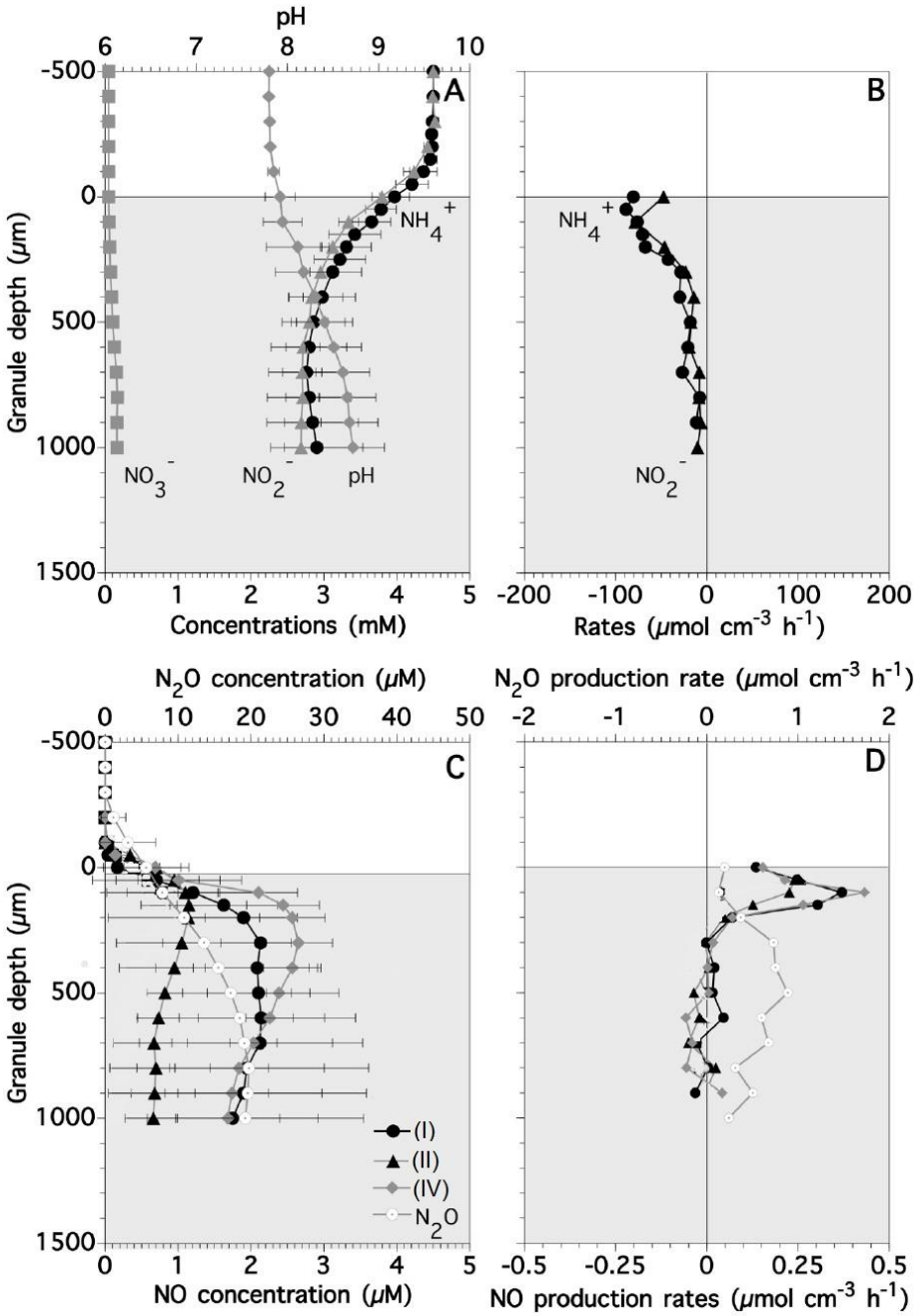

Figure 4.8: (A) Steady-state concentration profiles of pH, NH_4^+, NO_2^-, and NO_3^- in single anammox granules. (B) Net volumetric production rates ofNH_4^+ and NO_2^- that are calculated from concentration profiles in panel A. (C) Steady-state concentration profiles of NO and N_2O in the single anammox granules. Legends (I), (II), and (IV), represent steady-state concentration profiles of NO corresponding to conditions described in Table 4. 1. (D) Net volumetric production rates of NO and N_2O that are calculated from concentration profiles in panel C. Positive and negative values indicate production and consumption rates, respectively. Error bars indicated standard deviations obtained from triplicate vials (n = 3).

Interestingly, the zones of NO production and anammox was spatially separated from N_2O production zone in the anammox granules (Figure 4.8D), suggesting that anammox bacteria may significantly contributed to NO production but not to N_2O production, and NO was transferred from the upper layer to the inner layer in which other bacteria produced N_2O from NO. It is known that anammox bacteria do not produce N_2O under physiologically relevant conditions (Kartel et al., 2007). The lower net NO production rate in the inner parts of the anammox granules might be attributed to higher activity of denitrifiers, lower NO_2^- concentrations and higher pH values (Figure 4.6C and Figure 4.8D).

To the best of our knowledge, this is the first report demonstrating the bacterial species responsible for NO emissions, NO accumulation inside an anammox granule and the effects of physicochemical parameters on NO production. Since NO concentrations in the anammox granules in the present study (up to 2.7 μM) were higher than those in other types of biofilms (Schreiber et al., 2009; Schreiber et al., 2011).

4.4 CONCLUSIONS

The microorganisms and pathways responsible for and the factors affecting on the NO emissions from the anammox granule in a lab-scale column anammox reactor was investigated. The active NO production as well as anammox activity was found in the surface layer of the granule, where Anammox bacteria bacteria were abundant. Base on the polyphasic experiments, it was likely that both "*Candidatus* Brocadia sinica" and heterotrophic denitrifiers contributed in NO production in the anammox granules. However, the key genes involving the NO production in anammox granules could not recognized. Ammonium, nitrite and pH levels significantly influenced the NO production in the anammox granules.

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Chapter 5

Conclusions and Recommendations

5 CONCLUSIONS AND RECOMMENDATIONS

The main aim of this research was to investigate the N_2O and NO production from the nitrogen removal processes via partial nitrification (PN) and anaerobic ammonium oxidation (anammox) process.

The emission of N_2O from a lab-scale sequencing batch partial nitrification reactor treating synthetic wastewater without organic carbon was therefore investigated in this study. The average emission rate of N_2O from the reactor was 0.8 ± 0.4 mol-% of the incoming nitrogen load. Analysis of dynamic concentration profiles during one cycle of the sequencing batch reactor operation demonstrated that both N_2O concentrations in off-gas and effluent were high in the initial phase in one cycle. Isotopomer analysis revealed that the hydroxylamine (NH_2OH) oxidation pathway accounted for 65% of the total N_2O production in the initial phase in one cycle, whereas contribution of the NO_2^- reduction pathway to N_2O production was comparable with that of the NH_2OH oxidation pathway in the latter phase. In addition, spatial distributions of bacteria and their activities in single microbial granules taken from the reactor were determined with microsensors and by in situ hybridization. Partial nitrification occurred mainly in the oxic surface layer of the granules and ammonia-oxidizing bacteria were abundant in this layer. N_2O production was also found mainly in the oxic surface layer.

The effects of dissolved oxygen (DO) and pH on N_2O production rates and pathways in autotrophic PN granules were investigated at the granular level. Polyphasic analysis revealed that N_2O was primarily produced by the betaproteobacterial ammonia-oxidizing bacteria, mainly *Nitrosomonas europaea*, in the oxic surface layer (< 200 μm) of the autotrophic PN granules. Therefore, N_2O production increased significantly with increasing bulk DO concentration owing to activation of the ammonia (*i.e.*, hydroxylamine) oxidation rate (AOR) in this oxic layer. Since the relative fraction of N_2O emissions over the oxidized ammonia decreased from 2.9% at 0.6 mg-DO L^{-1} to 1.4% at 2.3 mg-DO L^{-1}, the operating of PN reactor at high AOR will reduced the total N_2O emission in the PN-SBR. Although the AOR was unchanged in the range of pH 6.5 to 8.5, the highest N_2O emission was observed at pH 7.5. Therefore, N_2O from PN reactor can be significantly minimized by avoiding the pH around 7.5 in the reactor. The results of our present

study suggest us that DO and pH are two of most important parameters to control N_2O emission from PN reactors and engineers should develop operational strategies based on our findings.

NO emission from wastewater treatment processes has great impacts on human health, microorganisms and the environment. Although, NO emissions from anammox processes have been reported, the knowledge about responsible microorganisms and pathways the factors affecting for the NO emissions from anammox granules is limited. Therefore, we investigated the microorganisms and pathways responsible for and the factors affecting the NO emissions from the microbial granules taken from an anammox reactor. Anammox bacteria were identified as the members of "*Candidatus* Brocadia sinica" and they were enriched in the granules. According to the results of inhibition batch tests and trancriptome analysis, it was likely that both anammox and heterotrophic denitrifiers contributed in NO production in the anammox granules. In situ analyses showed that the density and activity of the anammox bacteria and NO production rate were higher in the outer layer of the anammox granule. Ammonium, nitrite and pH levels significantly affected the NO production rates in the anammox granules. The results like presented in this study are useful for strategies to control NO emissions from anammox processes. Since the detected NO concentration in the anammox granules was higher as 2.7 μM, the role of NO molecule in the anammox granules should be deeply investigated in the future researches.

Applying of multiple analytical techniques very indispensable to our knowledge of complicated N_2O and NO production mechanisms in PN and anammox granules.