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Muhammad Ali, Rathnayake M.L.D. Rathnayake, Lei Zhang, Satoshi Ishii, Tomonori Kindaichi, Hisashi Satoh, Sakae Toyoda, Naohiro Yoshida, Satoshi Okabe

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N$_2$O production of a single-stage nitritation-anammox reactor

**Nitritation-anammox granular biomass**

**Oxic zone**
- Contribution: 70%

**Anoxic zone**
- Contribution: 30%

**N$_2$O isotopomer analysis**
- $SP = \delta^{15}N^\alpha - \delta^{15}N^\beta$

**N$_2$O production pathways**
- N$_2$O production of a single-stage nitritation-anammox reactor
- Nitritation-anammox granular biomass
- Anoxic zone: Contribution: 30%
- Oxic zone: Contribution: 70%
- A single-stage nitritation-anammox SBR

**Contribution:**
- NH$_4^+$ + NO$_2^-$ contribution: 50%
- NO$_2^-$ reduction by nitrifiers & heterotrophs: Contribution: 50%
- NH$_2$OH oxidation: Contribution: 50%
- N$_2$O production pathways: Contribution: 30%
Source identification of nitrous oxide emission pathways from a single-stage nitritation-anammox granular reactor

By

Muhammad Ali a,b, Rathnayake M.L.D. Rathnayakea,c, Lei Zhanga, Satoshi Ishiia,d, Tomonori Kindaichie, Hisashi Satoha, Sake Toyodaf, Naohiro Yoshida and Satoshi Okabea,*

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a Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, North 13, West-8, Sapporo, Hokkaido 060-8628, Japan. b King Abdullah University of Science and Technology (KAUST), Water Desalination and Reuse Center (WDRC), Biological and Environmental Science & Engineering (BESE), Thuwal 23955-6900, Saudi Arabia. c Department of Civil Engineering, Faculty of Engineering, University of Peradeniya, Peradeniya, 20400, Sri Lanka. d Department of soil, water and climate, University of Minnesota, 258 Borlaug Hall, 1991 Upper Buford Circle, St. Paul, MN 55108, USA. e Department of Civil and Environmental Engineering, Graduate School of Engineering, Hiroshima University, 1-4-1 Kagamiyama, Higashihiroshima, Hiroshima 739-8527, Japan. f Environmental Science and Technology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8502, Japan.

*Corresponding author: Satoshi Okabe, North 13, West-8, Sapporo, Hokkaido 060-8628, Phone & Fax: +81-(0)11-706-6266, E-mail: sokabe@eng.hokudai.ac.jp.
Abstract

Nitrous oxide (N\textsubscript{2}O) production pathway in a signal-stage nitritation-anammox sequencing batch reactor (SBR) was investigated based on a multilateral approach including real-time N\textsubscript{2}O monitoring, N\textsubscript{2}O isotopic composition analysis, and in-situ analyses of spatial distribution of N\textsubscript{2}O production rate and microbial populations in granular biomass. N\textsubscript{2}O emission rate was high in the initial phase of the operation cycle and gradually decreased with decreasing NH\textsubscript{4}+ concentration. The average emission of N\textsubscript{2}O was 0.98 ± 0.42% and 1.35 ± 0.72% of the incoming nitrogen load and removed nitrogen, respectively. The N\textsubscript{2}O isotopic composition analysis revealed that N\textsubscript{2}O was produced via NH\textsubscript{2}OH oxidation and NO\textsubscript{2}− reduction pathways equally, although there is an unknown influence from N\textsubscript{2}O reduction and/or anammox N\textsubscript{2}O production. However, the N\textsubscript{2}O isotopomer analysis could not discriminate the relative contribution of nitrifier denitrification and heterotrophic denitrification in the NO\textsubscript{2}− reduction pathway. Various in-situ techniques (e.g. microsensor measurements and FISH (fluorescent in-situ hybridization) analysis) were therefore applied to further identify N\textsubscript{2}O producers. Microsensor measurements revealed that approximately 70% of N\textsubscript{2}O was produced in the oxic surface zone, where nitrifiers were predominantly localized. Thus, NH\textsubscript{2}OH oxidation and NO\textsubscript{2}− reduction by nitrifiers (nitrifier-denitrification) could be responsible for the N\textsubscript{2}O production in the oxic zone. The rest of N\textsubscript{2}O (ca. 30%) was produced in the anammox bacteria-dominated anoxic zone, probably suggesting that NO\textsubscript{2}− reduction by coexisting putative heterotrophic denitrifiers and some other unknown pathway(s) including the possibility of anammox process account for the anaerobic N\textsubscript{2}O production. Further study is required to identify the anaerobic N\textsubscript{2}O production pathways. Our multilateral approach can be useful to quantitatively examine the relative contributions of N\textsubscript{2}O production pathways. Good understanding of the key N\textsubscript{2}O production pathways is essential to establish a strategy to mitigate N\textsubscript{2}O emission from biological nitrogen removal processes.
1 Introduction

Nitrous oxide (N\textsubscript{2}O) emission from wastewaters is a growing concern and accounts for about 6% of N\textsubscript{2}O global emission from all sources (the sixth largest contributor) (Ciais et al., 2013). During wastewater treatment, N\textsubscript{2}O is mainly released from biological nitrogen removal systems. Large variations in the N\textsubscript{2}O emissions were reported from biological nitrogen removal processes at bench-scale (0–95% of nitrogen load) and full-scale wastewater treatment plants (0–14.6% of nitrogen load) (Kampschreur et al., 2009b). Conventionally, nitrification and denitrification (N&DN) process was employed for nitrogen removal from wastewaters. However, anaerobic ammonium oxidation (anammox) process has potential to transform nitrogen removal from wastewaters. Nitritation and anammox processes have been used as an alternative treatment process for NH\textsubscript{4}+-rich wastewater streams (such as digester liquor) and considered as more economical and environmental friendly due to lower oxygen requirement, no external carbon source demand and less sludge production (Kuenen, 2008; Mulder et al., 1995; Okabe et al., 2011a). There are presently more than 110 full-scale anammox-based treatment plants worldwide and about 90% of the plants are being operated as single-stage systems (nitritation and anammox in one reactor) (Ali and Okabe, 2015; Lackner et al., 2014).

Extensive studies were conducted to study N\textsubscript{2}O emission dynamics and patterns from both two-stage and single-stage nitritation-anammox processes (Table 1). There are three scientifically known biological pathways of N\textsubscript{2}O production in wastewater treatment systems: hydroxylamine (NH\textsubscript{2}OH) oxidation during nitrification (NH\textsubscript{4}+ → NH\textsubscript{2}OH → NOH → N\textsubscript{2}O) (Law et al., 2012; Wunderlin et al., 2012) and NO\textsubscript{2}− reduction by nitrifiers (nitrifier-denitrification) (Colliver and Stephenson, 2000; Wrage et al., 2001) and by denitrifiers (heterotrophic denitrification) (Lu and Chandran, 2010). Though not conclusive, possibility of low-level N\textsubscript{2}O production by anammox bacteria was also stated previously by some researchers (Harris et al., 2015; Lotti et al., 2014). Although relatively low N\textsubscript{2}O productions (averagely
0.27% of the incoming nitrogen load) were reported for anammox reactors (Desloover et al., 2012; Kampschreur et al., 2008; Okabe et al., 2011a), it was confirmed that anammox reaction was not a main source of the \( \text{N}_2\text{O} \) emission (Okabe et al., 2011a). For two-stage nitritation-anammox processes, it was reported that \( \text{NH}_2\text{OH} \) oxidation pathway accounted for approximately 65% of total \( \text{N}_2\text{O} \) production from partial nitrification (PN) reactor fed with \( \text{NH}_4^+ \)-rich inorganic synthetic wastewater (Rathnayake et al., 2013). The source of \( \text{N}_2\text{O} \) was different when a PN reactor was fed with \( \text{NH}_4^+ \)-rich organic synthetic wastewater (Ishii et al., 2014). In their study, heterotrophic denitrification was a main source of \( \text{N}_2\text{O} \) emission from the PN reactor (70-80% of total \( \text{N}_2\text{O} \) production). For single-stage nitritation-anammox processes, the information related to \( \text{N}_2\text{O} \) production pathways is very limited. The major pathways of \( \text{N}_2\text{O} \) production in a pilot-scale PN-anammox sequencing batch reactor (SBR) were investigated using online isotopic analysis of off-gas \( \text{N}_2\text{O} \) with quantum cascade laser absorption spectroscopy (QCLAS) (Harris et al., 2015). In this study, \( \text{N}_2\text{O} \) emissions increased at high DO concentrations and it was concluded that this increase in \( \text{N}_2\text{O} \) was due to enhanced nitrifier denitrification based on \( \text{N}_2\text{O} \) isotopic site preference (SP) measurement. However, the SP value was much higher (up to 40‰) than previously reported in wastewater treatment systems, though higher SP values (up to 40‰) were observed in soil environments (Toyoda et al., 2011b). Therefore, more studies are needed to fully understand \( \text{N}_2\text{O} \) production pathways in single-stage nitritation-anammox systems.

In order to identify the sources of \( \text{N}_2\text{O} \) production in a single-stage nitritation-anammox process, a lab-scale sequential batch reactor (SBR) was operated and \( \text{N}_2\text{O} \) production was monitored at a reactor and microbial community (i.e., granule) level. Microbial community structure was analyzed based on 16S rRNA gene sequences using next-generation sequencing (Illumina MiSeq), and then the spatial distribution and abundance of important N-cycle stakeholders, ammonium-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and anammox bacteria, in granules were analyzed by fluorescence \textit{in-situ} hybridization (FISH). The
microbial community distribution was related to in-situ $\text{N}_2\text{O}$ production as measured by microsensors. Furthermore, temporal changes in intermolecular $^{15}\text{N}$-site preference (SP) of $\text{N}_2\text{O}$ in off-gas were measured along with temporal changes in $\text{NH}_4^+$, $\text{NO}_2^-$, $\text{NO}_3^-$, DO and pH during reactor operational cycles. Finally, all data were integrated to identify the source of produced $\text{N}_2\text{O}$ in the single-stage nitritation-anammox granular reactor.

2 Materials and methods

2.1 Reactor establishment and operation

A 2-L lab-scale sequential batch reactor (SBR) (height of 1000 mm and inner diameter of 58 mm) was operated continuously at 37°C (Fig. 1S). The volumetric exchange ratio was 50% and the hydraulic retention time was fixed at 0.5 day. The reactor was configured at 6 hr cycle: 5 min for feeding, 345 min for aeration, 5 min for settling and 5 min was allocated for effluent withdrawal. A programmable relay (ZEN-10C1AR-A-V2, Omron, Japan) was used to control the actuations of the air and water pumps, and regulated the different phases of the operational cycle. Air was supplied at a flow rate of 150 ml min$^{-1}$ by a diaphragm pump (Laboport N86, KNF, Japan). A ceramic air diffuser located at the bottom of the reactor was used for formation of small bubbles. DO and pH of the reactor was about 1.0 mg L$^{-1}$ and 7.6, respectively. Reactor was inoculated with nitrifying biomass (3 g-volatile suspended solid (VSS) L$^{-1}$) taken from a PN reactor (Rathnayake et al., 2013) and anammox biomass (5 g-VSS L$^{-1}$) taken from an up flow column reactor (Tsushima et al., 2007). The reactor was fed with synthetic wastewater contained: $\text{NH}_4^+$ (about 300 mg-N L$^{-1}$), $\text{CaCl}_2$ 100 mg L$^{-1}$, $\text{MgSO}_4$ 300 mg L$^{-1}$, $\text{KH}_2\text{PO}_4$ 30 mg L$^{-1}$, $\text{KHCO}_3$ 1500 mg L$^{-1}$ and trace element solutions (van de Graaf et al., 1995). At steady state operation, an average granular size was $2.42\pm1.48$ mm (ca. 70% of granules have diameters between 1.5 – 3.5 mm).

2.2 Nitrous oxide measurement
Real-time N\textsubscript{2}O concentrations in the off-gas from the reactor were measured with a photo acoustic field gas monitoring devise (1412, INNOVA, Copenhagen, Denmark). In addition, N\textsubscript{2}O concentrations in the representative off-gas samples were measured using a gas chromatography equipped with electron capture detector (GC-2014, Shimadzu, Kyoto, Japan). The effluent water samples were collected and analyzed by N\textsubscript{2}O headspace technique as described previously (Elkins, 1980; Okabe et al., 2011a).

2.3 Next generation sequencing

16S rRNA gene amplicon deep sequencing was conducted using the MiSeq technology (Illumina, Hayward, CA) to analysis microbial community structure. Genomic DNA was extracted from the nitritation-anammox 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\textunderscore 341F and Bakt\textunderscore 805R (Herlemann et al., 2011) with Illumina overhang adaptor sequences attached to their 50 ends (Table S1) (Rathnayake et al., 2015). Twenty-five cycles of PCR were confirmed to be necessary 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\textunderscore 341F and Bakt\textunderscore 805R primers. The purified DNA was mixed with Phi X control DNA and employed as a template for paired-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.

2.4 Fluorescence in-situ hybridization

Granular biomass harvested from the reactor was fixed with 4% (w/v) paraformaldehyde in phosphate-buffer saline at 4ºC for 6 hr. Thin sections (10 \textmu m in thickness) of biomass were pasted on teflon coated glass slides and hybridization was performed using hybridization probes NSO190, Ntspa662 and AMX 820 to identify AOB, NOB and anammox bacterial population,
respectively as described previously (Okabe et al., 1999). Another hybridization was performed using AMX 820 and mix of GNSB941 and CFX1223 (Kindaichi et al., 2012) to detect abundance and spatial distribution of anammox and Chloroflexi bacterial cells, respectively. The probes were labelled with Cy3 (or Alexa Fluor 555), Alexa Fluor 488, or Alexa Fluor 647 at the 5' end. Hybridized samples were observed with a LSM700 confocal laser-scanning microscope equipped with diode lasers (488, 555, and 639 nm) (Carl Zeiss, Oberkochen, Germany).

2.5 Microelectrode measurement

The steady state concentration micro-profiles of NH₄⁺, NO₂⁻, and NO₃⁻ were measured using liquid ion exchanger (LIX) type microelectrodes (de Beer et al., 1997). The sizes of NH₄⁺, pH and NO₃⁻ specific microelectrode tips were between 5 – 10 µm and the sizes of NO₂⁻ specific microelectrode tips were between 10 – 15 µm in diameter. Micro-profiles of DO and N₂O were measured using DO (OX-25, Unisense, Aarhus, Denmark) and N₂O (N₂O-25, Unisense, Aarhus, Denmark) microsensors, respectively. The microelectrodes were prepared, calibrated, and operated as previously described (Okabe et al., 1999; Satoh et al., 2007). Briefly, granular biomass (2 - 3 mm diameter size) were harvested from the nitritation-anammox reactor, fixed with insect needles in a flow chamber (2.5 L) and pre-incubated for 3 hr before microelectrode measurements to ensure that steady-state profiles were obtained. Microelectrodes were inserted almost perpendicular to the surface of the biomass. At least three profiles were measured for each chemical species and separate granule was used for each measurement. Measurement step size was 100 µm; however, smaller step size (50 µm) was chosen where fine resolution measurements were desired. Furthermore, net volumetric N₂O consumption or production rates were estimated from the respective concentration profiles by using the Fick’s second law of diffusion (Lorenzen et al., 1998) and Microsoft EXCEL add-ins solver were applied for the calculations as described previously (Ali et al., 2015).

2.6 N₂O isotopomer analysis
The off-gas samples were collected in gas sampling bag (1LA-6SN, Analytic-barrier, Tokyo, Japan) equipped with turning screw open/close valve at 6, 30, 60, 120, 240 and 345 min of cycle operation during aeration phase from three different operation cycles. Later, the gas samples were injected into evacuated glass serum vials (120 mL, Nichiden-Rika Glass, Tokyo, Japan) sealed with a rubber stopper and aluminum cap. Subsequently, isotopomer ratios (δ) of N₂O in the off-gas samples were measured using gas chromatograph isotope ratio monitoring mass spectrometry (GC-IRMS, MAT252 system, thermos Fisher Scientific K.K., Yokohama, Japan) as described elsewhere (Toyoda et al., 2011b, 2005). Site-specific nitrogen isotope analysis in N₂O was conducted using ion detectors, which were modified to allow mass analysis of the fragment ions of N₂O (NO⁺) containing N atoms in the center positions of N₂O molecules, whereas the bulk nitrogen and oxygen isotope ratio were determined from molecular ions (Toyoda and Yoshida, 1999), detailed calculations were shown in the supplemental information.

2.7 Chemical Analyses

Influent and effluent samples were collected and concentrations of NH₄⁺, NO₂⁻ and NO₃⁻ were determined using ion chromatographs (IC-2010, TOSOH, Tokyo, Japan) equipped with TSKgel IC-Anion HS or TSKgel IC-Cation columns (TOSOH, Tokyo, Japan) after filtration using 0.45 µm-pore-size membranes (Okabe et al., 2011b). DO was measured using a DO meter (DO-5Z, KRK, Tokyo, Japan). Biomass concentration was determined as protein concentration using DC Protein Assay Kit (Bio-Rad, Tokyo, Japan) and bovine serum albumin as protein standard as previously described (Ali et al., 2014). Conversion of protein concentration to VSS concentration was performed with conversion coefficient 1.64 (Oshiki et al., 2011).

3 Results

3.1 Reactor performance and efficiency
After about six-month operation, the stable nitrogen removal rate (NRR) of 0.4 - 0.6 kg-N m$^{-3}$ d$^{-1}$ was achieved under the nitrogen loading rate (NLR) of 0.5 - 0.8 kg-N m$^{-3}$ d$^{-1}$, corresponding to the total nitrogen removal rate of about 80% (Fig. 1). However, it should be noted that relatively high concentrations (around 65 mg L$^{-1}$) of NO$_3^-$, an end-product of anammox process, remained in the effluent, indicating that complete nitrification (oxidation of NH$_4^+$ to NO$_2^-$ and further to NO$_3^-$) occurred to some extent. In contrast, the effluent NO$_2^-$ concentration was almost zero during the operation. The relative nitrite oxidation rate (NOR), ammonium oxidation rate (AOR) and total nitrogen removal rate by anammox bacteria (ANR) were calculated based on nitrogen balance and the stoichiometry of the anammox process (Vázquez-Padín et al., 2009) (Fig. 1C). Detailed calculations of AOR, NOR and ANR were presented in supplemental information. The mean AOR, NOR and ANR were ascertained about 0.31, 0.07 and 0.50 kg-N m$^{-3}$ d$^{-1}$, respectively, suggesting that NOR accounted for 14% of the mean ANR. The biomass concentration in the reactor was stable at around 8 g-VSS L$^{-1}$ during the operation.

3.2 Cyclic emission of N$_2$O

Dissolved N$_2$O concentrations in the reactor effluent and gaseous N$_2$O concentrations in the off-gas were varied from 0.4 to 240 ppm and from 4.4 to 67 µM, respectively (Fig. S2). The average N$_2$O emission was recorded as 0.98 ± 0.42% of the incoming nitrogen load (1.35 ± 0.72% of the removed nitrogen), which was comparable with the values reported in the previous studies (Table 1). At the beginning of aeration (operation cycle), N$_2$O emission rate was very high and then decreased at the end of aeration (Fig. 2). This trend was reproducible (Fig. S3) and consist with the previous studies of lab-scale PN reactors (Kong et al., 2013a; Rathnayake et al., 2013). The average N$_2$O emission rate was measured as 0.26 ± 0.08 mg-N L$^{-1}$ h$^{-1}$. The dynamics of N$_2$O, DO, NH$_4^+$, NO$_2^-$, and NO$_3^-$ concentrations in one operation cycle were measured three times and the averages were shown in Fig. 2. Only NH$_4^+$ concentration rapidly
increased due to the feed of fresh medium and gradually decreased with time. DO and pH were in the range of 0.8 to 1.3 mg-O$_2$ L$^{-1}$ and 7.5 to 7.9, respectively.

### 3.3 Microbial community structure

Bacterial community structure and spatial distribution of AOB, NOB, anammox bacteria and others in the granules were analyzed by the 16S rRNA gene deep sequencing and FISH analysis. A total of 29,900 sequence reads per sample were obtained from triplicate samples. Microbial community structures were very similar among triplicate samples. The Miseq analysis revealed that anammox bacteria belonging to the order *Brocadiales* were identified as the sole anammox bacteria in the granules (12.5 ± 0.6%). AOB belonging to the order *Nitrosomonadales* (5.0 ± 0.4%) and NOB belonging to the phylum *Nitrospirae* (12.1 ± 0.3%) were also detected in the granules (Fig. 3).

The spatial distribution of AOB, NOB and anammox bacteria in the nitritation-anammox granular biomass was analyzed in detail by FISH. The NSO190-stained *betaproteobacterial* AOB were primarily detected in the outermost surface layer of the granules, and Ntspa662-stained NOB cells were detected underneath the AOB cells layer (Fig. 4). This AOB and NOB cells layer was rather thin (only about 50 - 100 µm). Amx820-stained anammox bacteria dominated inside of granules (underneath the AOB and NOB cells layer). Clear stratified spatial distribution of aerobic AOB and NOB and anammox bacteria was observed.

Deep sequencing of 16S rRNA gene analysis revealed that the most frequently detected sequence was the Phylum *Cholorflexi* (33.1 ± 0.2%), which has often been observed in anammox reactors fed with synthetic nutrient medium without organic carbon compounds (Kindaichi et al., 2012; Miura et al., 2007). It is speculated that *Cholorflexi* act as a scavenger of organic matter derived from anammox bacterial cells (Miura and Okabe, 2008). However, FISH analysis revealed that the abundance of GNSB 941 and CFS 1223-stained *Cholorflexi* was very low as compared to anammox bacteria cells (Fig. 4S), and they were detected only in the
anammox bacteria dominated zone. This discrepancy is probably due to PCR amplification bias (Ross et al., 2013) and difference in 16S rRNA gene copy numbers; Chloroflexi species contain up to five 16S rRNA genes per genome (Schirrmeister et al., 2012) whereas anammox bacteria (e.g., Ca. Brocadia) has only one 16S rRNA genes per genome (Oshiki et al., 2015).

3.4 In-situ microbial activity

The steady-state concentration profiles of DO, NH$_4^+$, NO$_2^-$, and NO$_3^-$ in the granular biomass harvested from a single-stage nitritation-anammox reactor were measured under the condition representing the end of operation cycle (i.e., NH$_4^+$ concentration <50 mg L$^{-1}$) (Fig. 5). DO was consumed rapidly to below 0.1 mg L$^{-1}$ at ca.100 µm from the surface of the granule (Fig. 5A). The net volumetric O$_2$ consumption rate was observed down to 250 µm depth where simultaneous consumptions of NH$_4^+$ and NO$_2^-$ and production of NO$_3^-$ were observed. No net NO$_2^-$ production was observed in the oxic and anoxic zones, indicating that NO$_2^-$ consumption by NOB and anammox bacteria was faster than generation by AOB in the granule. Anammox activity (i.e., nitrogen removal performance) was limited by NO$_2^-$ availability; because NH$_4^+$ was still plenty inside granules but no anammox reaction occurred. The ratios of consumed NO$_2^-$ / consumed NH$_4^+$ and produced NO$_3^-$ / consumed NH$_4^+$ were 1.21 and 0.34 in the oxic zone (0-250 µm) and 0.95 and 0.29 in the anoxic zone (250-500 µm), respectively (Table S2). This indicated the simultaneous occurrence of (partial) nitrification and anammox (Fig. 5C).

Under low NH$_4^+$ condition (<50 mg L$^{-1}$), in-situ N$_2$O production was low, whereas high N$_2$O productions were observed under high NH$_4^+$ condition (150 mg L$^{-1}$) (Fig. 6A). This observation was in line with the time-course change in N$_2$O concentration (Fig. 2). The net volumetric N$_2$O production rates calculated from the average concentration profiles revealed that approximately 70% of total N$_2$O was produced in the oxic zone (Fig. 6B and 6C). These results suggest that nitrifiers, predominantly present in the outer layer of granules, were mainly responsible for N$_2$O production in the oxic zone. Heterotrophic bacteria could be partly responsible for the N$_2$O production at the oxic-anoxic interface of the granules (below 100 µm) where anammox
bacteria were predominantly present. It should be noted that N$_2$O production was very dynamic and highly influenced by many operating parameters such as pH, DO, NH$_4^+$ and NO$_2^-$ concentrations (Domingo-Félez et al., 2014; Joss et al., 2011; Rathnayake et al., 2015). This is probably the reason that N$_2$O microprofiles have relatively large standard deviations.

3.5 N$_2$O isotopomer analysis

The $\delta^{15}$N$^{\text{bulk}}$, $\delta^{18}$O, and SP values were measured for N$_2$O molecules collected from the off-gas samples at different stages of the operation cycle (Fig. 7A). Off-gas sample was also collected at 345 min, however, N$_2$O isotopic composition could not be measured due to very low N$_2$O concentration. The bulk isotopic composition ($\delta^{15}$N$^{\text{bulk}}$) refers to the average value of $\delta^{15}$N$^\alpha$ and $\delta^{15}$N$^\beta$ estimated from equation 1 and 2 (shown in supplemental information), respectively. The SP values were relatively constant (15 - 19‰) throughout the cycle. Reduction of N$_2$O by heterotrophic denitrification increases the SP value of the remaining N$_2$O, which hinders from partitioning different production pathways. To overcome these difficulties, combination of site preference data with simultaneous measurements of $\delta^{18}$O and $\delta^{15}$N$^{\text{bulk}}$ can help to constrain the effect of N$_2$O reduction by heterotrophic denitrification (Toyoda et al., 2011b). The shifts in SP due to N$_2$O reduction can be qualitatively evaluated based on the relationships between $\delta^{18}$O and $\delta^{15}$N$^{\text{bulk}}$, and $\delta^{18}$O and $\delta^{15}$N$^\alpha$ (Jinuntuya-Nortman et al., 2008; Ostrom et al., 2007). A line with a slope of 2.5 for the plot of $\delta^{18}$O versus $\delta^{15}$N$^{\text{bulk}}$ and 1.7 for the plot of $\delta^{18}$O versus $\delta^{15}$N$^\alpha$ was regarded as an indicative of N$_2$O reduction. Based on this, slopes greater than one for both the relationships were suggested as a criterion to identify the influence of N$_2$O reduction on SP (Ostrom et al., 2007). However, in our study, the slopes of lines relating $\delta^{18}$O and $\delta^{15}$N$^{\text{bulk}}$, and $\delta^{18}$O and $\delta^{15}$N$^\alpha$ were 0.14 and 0.15, respectively, suggesting that the influence of N$_2$O reduction on SP value was not significant in this study (Fig. S5).

Therefore, relative contributions of NH$_2$OH oxidation and NO$_2^-$ reduction (nitrifier-denitrification and/or heterotrophic denitrification) to N$_2$O production were calculated as
described in supplemental information. The relative contributions of NH$_2$OH oxidation and NO$_2^-$ reduction were calculated to be 52.1 ± 2.3% and 47.9 ± 2.3%, respectively (Fig. 7B), suggesting that their contributions were roughly equal.

4 Discussion

Better understanding of the pathways and influencing factors for formation and emission of N$_2$O is essential to propose effective mitigation strategies. N$_2$O isotopomer analysis is a powerful tool to distinguish whether N$_2$O originates from NH$_2$OH oxidation and/or NO$_2^-$ reduction (nitrifier denitrification and heterotrophic denitrification). Previously, isotopic composition of N$_2$O produced by pure cultures was investigated; the following SP values were reported: 1) -10.7 ± 2.9 to 0.1 ± 1.7‰ for nitrifier-denitrification by AOB; 2) 30.8 ± 5.9 to 36.3 ± 2.4‰ for NH$_2$OH oxidation; and 3) -5 to 0‰ for N$_2$O production by heterotrophic denitrification (Sutka et al., 2006; Toyoda et al., 2011b).

In addition, many field studies were conducted for measurement of isotopic composition of N$_2$O (Koba et al., 2012; Yamagishi et al., 2007) including a municipal wastewater treatment plant (Toyoda et al., 2011a). For example, NH$_2$OH oxidation pathway accounted for 65% of the total N$_2$O production from a PN reactor fed with synthetic inorganic wastewater (Rathnayake et al., 2013). In contrast, heterotrophic denitrification contributed 70 – 80% in production of N$_2$O from a PN reactor fed with synthetic wastewater containing organic carbon (Ishii et al., 2014). Later, this technique was applied to a pilot-scale nitritation-anammox reactor to optimize the reactor operation (Harris et al., 2015). However, N$_2$O production pathway could not be identified due to unexpected high SP values that have not been observed in wastewater treatment processes so far, suggesting as yet unknown N$_2$O production pathways including the possibility of anammox process.
There are several limitations of N\textsubscript{2}O isotopomer analysis. For example, N\textsubscript{2}O reduction increases the SP value; therefore, isotopomer analysis alone may underestimate the contribution of NO\textsubscript{2}\textsuperscript{-} reduction pathway, when N\textsubscript{2}O reduction is intense (Ostrom et al., 2007). Secondly, it is not possible to distinguish the relative contributions of nitrifier denitrification and heterotrophic denitrification because both pathways occur through NO\textsubscript{2}\textsuperscript{-} reduction (Toyoda et al., 2011a). The first problem can be solved with simultaneous measurements of $\delta^{18}$O and $\delta^{15}$N\textsubscript{bulk} as discussed above in section 3.5. In order to overcome the second problem, several techniques (e.g. microsensor measurements, FISH and microbial community analysis) must be combined together to relate the spatial distributions of N\textsubscript{2}O production rate (function) and microbial populations in nitritation-anammox granules.

For microsensor measurements, it should be noted that the microprofiles presented in this study were artificial profiles that were not actually occurring under reactor conditions because, for example, the reactor hydrodynamic was different. In addition, since these were net concentration profiles as a net result of consumption and production, NO\textsubscript{2}\textsuperscript{-} consumption and production rates could not be precisely determined from these profiles. Second, the spatial resolution of microelectrode measurements is considered to be about 2-3 times of the tip diameters of microelectrodes (i.e., about 30 - 50 $\mu$m in this study), a strict comparison with FISH results would have limitations.

Microsensor results revealed that more than 70% of the total N\textsubscript{2}O production occurred in the outer oxic zone of the granules, where nitrifiers were predominantly present (Fig. 4 and Fig. 5). Whereas, the rest of N\textsubscript{2}O (about 30%) was produced in the anoxic zone, where anammox bacteria and putative heterotrophic denitrifiers mainly inhabited, suggesting anammox, heterotrophic denitrification and/or some other unknown pathway(s) contributed to the N\textsubscript{2}O emission in this zone, as previously reported (Harris et al., 2015). Furthermore, the oxic-anoxic interface was vertically moved and variable, which was highly dependent on various operational
parameters, *i.e.* bulk DO concentration, microbial activity, spatial distribution of microbes in granules, surface heterogeneity, and granule size (Domingo-Félez et al., 2014; Rathnayake et al., 2015).

In this study, NH$_2$OH oxidation and NO$_2^-$ reduction pathways contributed roughly equal in N$_2$O production. This result was in good agreement with the previous observation implying that about 70% of N$_2$O produced in the oxic zone could be attributed to NH$_2$OH oxidation and NO$_2^-$ reduction by nitrifiers, and the rest of N$_2$O produced in the anoxic zone could be mediated by NO$_2^-$ reduction by heterotrophic denitrifiers. However, some other unknown pathway(s) might be involved in the N$_2$O production such as anammox process (Harris et al., 2015; Wunderlin et al., 2013a). Further study is absolutely necessary to validate the possibility.

Under high NH$_4^+$ concentration, N$_2$O production also increased. It was reported that N$_2$O production via NH$_2$OH pathway was favored at high NH$_3$ and low NO$_2^-$ concentrations (Wunderlin et al., 2013a). Similar N$_2$O emission pattern was reported earlier in a PN reactor, where the contribution of NH$_2$OH oxidation pathway was relatively higher at high NH$_4^+$ concentration (Rathnayake et al., 2013). Biotic N$_2$O production by NH$_2$OH oxidation can be minimized by keeping the concentration of NH$_4^+$ relatively low (*e.g.* by intermittent dosing of wastewater). On the other hand, N$_2$O production by nitrifier-denitrification is particularly promoted under oxygen-limiting and completely anoxic conditions (Kampschreur et al., 2009b).

The single-stage nitritation-anammox reactor was operated under NO$_2^-$-limiting condition in this study (Fig. 1). In order to improve the nitrogen removal performance (anammox activity), more NO$_2^-$ must be supplied to the anammox zone. The controllable variable would be DO concentration, which must be optimized to maximize the NH$_4^+$ oxidation to NO$_2^-$ without unwanted NO$_2^-$ oxidation to NO$_3^-$ by NOB and inhibition of anammox activity (Harris et al., 2015; Joss et al., 2011; Wunderlin et al., 2013b). Since ammonium oxidation to nitrite
(nitritation) is highly dependent on DO concentration (Vázquez-Padín et al., 2010), adequate DO control is essential for the single-stage nitritation-anammox reaction.

In this study, DO concentration was maintained at around 1.0 mg-O$_2$ L$^{-1}$, resulting in a clear vertical stratification of microbial populations; aerobic AOB were restricted to a thin outer shell (ca. 100 µm) while anammox bacteria were present in the inner anoxic zone of the granular biomass (Fig. 4). Active nitrogen transformation occurred within outer 500 µm (Fig. 5). The thickness of active zone was dependent on the bulk DO and NO$_2^-$ concentrations (Nielsen et al., 2005). The volumetric conversion rate of ammonium was very high in the outer layer (100 µm) of the granular biomass to maintain syntrophy with anammox bacteria and to some extend with NOB (Fig. 5C). Nitrate production was observed in the oxic zone (0-250 µm) with an average ratio of produced NO$_3^-$ to consumed NH$_4^+$ of about 0.11, indicating occurrence of complete nitrification by AOB and NOB (Fig. 4 and Table S2). The stoichiometric ratio of produced NO$_3^-$ to consumed NH$_4^+$ in the anoxic zone (below 250 µm) was 0.28, which was close to an anammox stoichiometry (Strous et al., 1998).

Application of single-stage nitritation-anammox reactor configuration is more popular as compared to two-stage systems (Lackner et al., 2014). When comparing N$_2$O emissions from single-stage systems with two-stage systems, N$_2$O emission was generally lower in single-stage systems than the two-stage systems (Table 1). In two-stage nitritation-anammox systems, average N$_2$O emission were previously reported as 2.5% (0.8 – 6.1%) and 0.27% (0.1 – 0.6%) of NLR, respectively. In this study, the average emission of N$_2$O was ascertained as 1.35 ± 0.72% of the incoming nitrogen loading rate, which was comparable with the previous reported values for single-stage nitritation-anammox systems (0.1 – 3.0%).

5 Conclusions
In this study, N\textsubscript{2}O emission dynamics and sources of a lab-scale single-stage nitritation-anammox sequencing batch reactor (SBR) were investigated by applying microscale \textit{in-situ} analyses (microsensor measurements and FISH) and reactor level analyses (N\textsubscript{2}O monitoring and isotopomer analysis). The following conclusions are drawn:

- The average N\textsubscript{2}O emission was 1.35 ± 0.72 % of the removed nitrogen (0.98 ± 0.42% of the incoming nitrogen load)
- N\textsubscript{2}O isotopomer analysis revealed that the contributions of NH\textsubscript{2}OH oxidation and NO\textsubscript{2} reduction pathways to N\textsubscript{2}O production were roughly equal, although there is an unknown influence from N\textsubscript{2}O reduction and/or anammox N\textsubscript{2}O production.
- Microsensor measurements and FISH analysis revealed that about 70% of N\textsubscript{2}O was produced in the oxic zone where nitrifiers mainly inhabited, and thus probably through NH\textsubscript{2}OH oxidation and autotrophic NO\textsubscript{2} reduction pathways. The rest of N\textsubscript{2}O (ca. 30%) was produced in the anammox bacteria-dominated anoxic zone, thus possibly mediated by anammox, heterotrophic denitrification and/or other unknown pathway(s). Further study is required to identify the N\textsubscript{2}O emission source in the anoxic zone.

This study provided important information for establishing appropriate strategies to mitigate N\textsubscript{2}O emission from autotrophic biological nitrogen removal systems, which is essential to reduce the carbon footprint of wastewater treatment plants. Identifying N\textsubscript{2}O emission source allow us to achieve specific design and operational conditions of single-stage nitritation-anammox systems.

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References


292–303.


Figure 1: Performance of a single-stage nitritation-anammox reactor; A) Influent ammonium (filled circle) concentration and effluent ammonium (empty circle), nitrite (filled diamond) and nitrate (filled square) concentrations measured during the period of study after optimization of the reactor; B) Nitrogen loading (filled circle) and removal (empty circle) rate of the reactor during investigation; C) Activities of AOB (AOR, empty circle), NOB (NOR, filled diamond) and anammox bacteria (ANR, empty square) were calculated based on nitrogen balance and the stoichiometry of the anammox process.
Figure 2: A) The concentrations of NH$_4^+$ (filled circle), NO$_2^-$ (filled triangle), NO$_3^-$ (filled square) and N$_2$O (open circle) during an operation cycle of the reactor. B) Cyclic pattern of DO and pH during an operation cycle. Duration of reactor operation cycle was 360 min starting from 0 min: 5, 345, 5 and 5 min were allocated for feeding, aeration, settling and effluent withdrawal. Exchange ratio was 50%, resulting in hydraulic retention time of 12 hour. Error bars represent the range of standard deviation (SD) among triplicate samples collected from three different operation cycles.
Figure 3: Microbial community composition of nitritation-anammox granular biomass based on 16S rRNA gene sequences using next-generation sequencing (Illumina MiSeq). The inner, middle and outer pie charts show the community composition at the phylum, order and class levels, respectively. Values shown are average of triplicate biomass samples.
Figure 4: Confocal laser scanning microscope images of thin cross-section of the granular biomass from the nitritation-anammox reactor: These images are showing in-situ spatial organization of ammonia-oxidizing bacteria (AOB, green), nitrite-oxidizing bacteria (NOB, red) and anammox bacteria (blue). Fluorescence in-situ hybridization was performed with Alexa488-labeled Nso190 probe (green) for AOB and Cy3-labeled Ntspa662 (red) for NOB and Alexa647-labeled Amx820 probe (blue) for anammox bacteria. Dashed lines squares shown in Panel A & B indicating location of the displayed images in Panel C & D, respectively. Scale bars represent 50 and 10 µm for Panel A & B and Panel C & D, respectively.
Figure 5: Steady state concentration profiles of NH$_4^+$ (circle), NO$_2^-$ (diamond), NO$_3^-$ nitrate (square) (A) and DO (diamond) (B) in granular biomass of nitritation-anammox reactor. Dashed line represents a liquid-granule interface. Three profiles were measured for each chemical species using three granules. Error bars indicate the range of standard deviations (SD) derived from triplicate measurements. Grey bars are showing spatial distributions of the net volumetric consumption rates of DO, calculated from the average DO concentration profile using Microsoft EXCEL 2013 add-ins solver. (C) Spatial distributions of net volumetric consumption rates of NH$_4^+$ (red bars) and NO$_2^-$ (blue bars) and net volumetric production rates NO$_3^-$ (green bars) were calculated from the corresponding average concentration profiles using Microsoft EXCEL 2013 add-ins solver.
Figure 6: Steady state concentration profiles of N\textsubscript{2}O in the granular biomass harvested from the nitritation-anammox reactor (A). N\textsubscript{2}O concentration profiles were measured under two different operating conditions mimicking the start (filled circle) and end (empty circle) of operation cycle. The start and end of operation cycle refers to high NH\textsubscript{4}\textsuperscript{+} concentration (150 mg-N L\textsuperscript{-1}) and low NH\textsubscript{4}\textsuperscript{+} concentration (25 mg-N L\textsuperscript{-1}), respectively. Rest of the operating parameters i.e. substrate concentrations, pH, temperature and DO were kept the same in both incubations. Dashed line represents a liquid-solid interface of granular biomass. Four profiles were measured for each operating condition using different granules. B) Spatial distributions of net volumetric production rates of N\textsubscript{2}O were calculated from the average concentration profiles of N\textsubscript{2}O at the start (filled bars) and end (empty bars) of operation cycle using Microsoft EXCEL 2013 add-ins solver. Positive and negative values indicate the consumption and production rates, respectively. C) Cumulative N\textsubscript{2}O production rate in percentage. Gray shaded area representing as observed anoxic zone in granular biomass. Dashed line represents a liquid-solid interface of granular biomass.
Figure 7: A) N$_2$O isotopomer ratios and SP values in off-gas at each sampling time over one operation cycle. B) Contribution of NH$_2$OH oxidation and NO$_2^-$ reduction pathways to N$_2$O emission from the nitritation-anammox reactor. Gas samples were also collected at 345 min, however isotopic composition could not be accurately measured due to very low N$_2$O concentration in off-gas. Error bars indicate the range of standard deviation (SD) from triplicate samples collected from three different operating cycles.
**Table 1:** Nitrous oxide production rate observed in various studies during biological nitrogen removal process

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Reactor Scale</th>
<th>Reactor Type</th>
<th>Nitrogen loading rate (NLR) [kg-N m$^{-3}$ d$^{-1}$]</th>
<th>Nitrogen removal rate (NRR) [NLR]</th>
<th>The ratio of N$_2$O production to NLR [%]</th>
<th>The ratio of N$_2$O production to NRR</th>
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<td>(Kong et al., 2013b)</td>
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<td>Reactor Scale</td>
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<td>0.5-0.8</td>
<td>0.4-0.6</td>
<td>0.98 ± 0.42</td>
<td>1.35 ± 0.72</td>
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</table>

PN; partial nitrification reactor

N&A; single-stage nitritation-anammox reactor

\(^a\) The NRR values reported for PN reactors are NH\(_4^+\) to NO\(_2^-\) conversion rates for the respective reactor

\(^b\) For all PN reactors, ratios of N\(_2\)O production rate to conversion rate of NH\(_4^+\) to NO\(_2^-\) are reported here.
Highlights

1. $\text{N}_2\text{O}$ production pathways were studied by $\text{N}_2\text{O}$ isotopic, FISH, and microsensor analyses.
2. Ca. 70% of $\text{N}_2\text{O}$ was produced in nitrifiers-dominated oxic surface zone of granules.
3. The rest of $\text{N}_2\text{O}$ (30%) was produced in the anammox bacteria-dominated anoxic zone.
4. Overall, $\text{N}_2\text{O}$ was produced via $\text{NH}_2\text{OH}$ oxidation and $\text{NO}_2^-$ reduction pathways equally.