Source identification of nitrous oxide emission pathways from a single-stage nitrification-anammox granular reactor

By

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2 **Abstract**

3 Nitrous oxide (N₂O) production pathway in a signal-stage nitrification-anammox sequencing
4 batch reactor (SBR) was investigated based on a multilateral approach including real-time N₂O
5 monitoring, N₂O isotopic composition analysis, and *in-situ* analyses of spatial distribution of
6 N₂O production rate and microbial populations in granular biomass. N₂O emission rate was high
7 in the initial phase of the operation cycle and gradually decreased with decreasing NH₄⁺
8 concentration. The average emission of N₂O was 0.98 ± 0.42% and 1.35 ± 0.72% of the
9 incoming nitrogen load and removed nitrogen, respectively. The N₂O isotopic composition
10 analysis revealed that N₂O was produced via NH₂OH oxidation and NO₂⁻ reduction pathways
11 equally, although there is an unknown influence from N₂O reduction and/or anammox N₂O
12 production. However, the N₂O isotopomer analysis could not discriminate the relative
13 contribution of nitrifier denitrification and heterotrophic denitrification in the NO₂⁻ reduction
14 pathway. Various *in-situ* techniques (e.g. microsensor measurements and FISH (fluorescent *in-*
15 *situ* hybridization) analysis) were therefore applied to further identify N₂O producers.
16 Microsensor measurements revealed that approximately 70% of N₂O was produced in the oxic
17 surface zone, where nitrifiers were predominantly localized. Thus, NH₂OH oxidation and NO₂
18 reduction by nitrifiers (nitrifier-denitrification) could be responsible for the N₂O production in
19 the oxic zone. The rest of N₂O (ca. 30%) was produced in the anammox bacteria-dominated
20 anoxic zone, probably suggesting that NO₂⁻ reduction by coexisting putative heterotrophic
21 denitrifiers and some other unknown pathway(s) including the possibility of anammox process
22 account for the anaerobic N₂O production. Further study is required to identify the anaerobic
23 N₂O production pathways. Our multilateral approach can be useful to quantitatively examine the
24 relative contributions of N₂O production pathways. Good understanding of the key N₂O
25 production pathways is essential to establish a strategy to mitigate N₂O emission from
26 biological nitrogen removal processes.

27

28 **1 Introduction**

29 Nitrous oxide (N₂O) emission from wastewaters is a growing concern and accounts for
30 about 6% of N₂O global emission from all sources (the sixth largest contributor) (Ciais et al.,
31 2013). During wastewater treatment, N₂O is mainly released from biological nitrogen removal
32 systems. Large variations in the N₂O emissions were reported from biological nitrogen removal
33 processes at bench-scale (0–95% of nitrogen load) and full-scale wastewater treatment plants
34 (0–14.6% of nitrogen load) (Kampschreur et al., 2009b). Conventionally, nitrification and
35 denitrification (N&DN) process was employed for nitrogen removal from wastewaters.
36 However, anaerobic ammonium oxidation (anammox) process has potential to transform
37 nitrogen removal from wastewaters. Nitritation and anammox processes have been used as an
38 alternative treatment process for NH₄⁺-rich wastewater streams (such as digester liquor) and
39 considered as more economical and environmental friendly due to lower oxygen requirement,
40 no external carbon source demand and less sludge production (Kuenen, 2008; Mulder et al.,
41 1995; Okabe et al., 2011a). There are presently more than 110 full-scale anammox-based
42 treatment plants worldwide and about 90% of the plants are being operated as single-stage
43 systems (nitritation and anammox in one reactor) (Ali and Okabe, 2015; Lackner et al., 2014).

44 Extensive studies were conducted to study N₂O emission dynamics and patterns from both
45 two-stage and single-stage nitritation-anammox processes (**Table 1**). There are three
46 scientifically known biological pathways of N₂O production in wastewater treatment systems:
47 hydroxylamine (NH₂OH) oxidation during nitrification (NH₄⁺ → NH₂OH → NOH → N₂O)
48 (Law et al., 2012; Wunderlin et al., 2012) and NO₂⁻ reduction by nitrifiers (nitrifier-
49 denitrification) (Colliver and Stephenson, 2000; Wrage et al., 2001) and by denitrifiers
50 (heterotrophic denitrification) (Lu and Chandran, 2010). Though not conclusive, possibility of
51 low-level N₂O production by anammox bacteria was also stated previously by some researchers
52 (Harris et al., 2015; Lotti et al., 2014). Although relatively low N₂O 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 N_2O emission (Okabe et al., 2011a). For two-stage nitrification-anammox processes, it was reported that NH_2OH oxidation pathway accounted for approximately 65% of total N_2O production from partial nitrification (PN) reactor fed with NH_4^+ -rich inorganic synthetic wastewater (Rathnayake et al., 2013). The source of N_2O was different when a PN reactor was fed with NH_4^+ -rich organic synthetic wastewater (Ishii et al., 2014). In their study, heterotrophic denitrification was a main source of N_2O emission from the PN reactor (70-80% of total N_2O production). For single-stage nitrification-anammox processes, the information related to N_2O production pathways is very limited. The major pathways of N_2O production in a pilot-scale PN-anammox sequencing batch reactor (SBR) were investigated using online isotopic analysis of off-gas N_2O with quantum cascade laser absorption spectroscopy (QCLAS) (Harris et al., 2015). In this study, N_2O emissions increased at high DO concentrations and it was concluded that this increase in N_2O was due to enhanced nitrifier denitrification based on N_2O 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 N_2O production pathways in single-stage nitrification-anammox systems.

In order to identify the sources of N_2O production in a single-stage nitrification-anammox process, a lab-scale sequential batch reactor (SBR) was operated and N_2O 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 *in-situ* hybridization (FISH). The

79 microbial community distribution was related to *in-situ* N₂O production as measured by
80 microsensors. Furthermore, temporal changes in intermolecular ¹⁵N-site preference (SP) of N₂O
81 in off-gas were measured along with temporal changes in NH₄⁺, NO₂⁻, NO₃⁻, DO and pH during
82 reactor operational cycles. Finally, all data were integrated to identify the source of produced
83 N₂O in the single-stage nitrification-anammox granular reactor.

84

85 **2 Materials and methods**

86 **2.1 Reactor establishment and operation**

87 A 2-L lab-scale sequential batch reactor (SBR) (height of 1000 mm and inner diameter
88 of 58 mm) was operated continuously at 37°C (**Fig. 1S**). The volumetric exchange ratio was
89 50% and the hydraulic retention time was fixed at 0.5 day. The reactor was configured at 6 hr
90 cycle: 5 min for feeding, 345 min for aeration, 5 min for settling and 5 min was allocated for
91 effluent withdrawal. A programmable relay (ZEN-10C1AR-A-V2, Omron, Japan) was used to
92 control the actuations of the air and water pumps, and regulated the different phases of the
93 operational cycle. Air was supplied at a flow rate of 150 ml min⁻¹ by a diaphragm pump
94 (Laboport N86, KNF, Japan). A ceramic air diffuser located at the bottom of the reactor was
95 used for formation of small bubbles. DO and pH of the reactor was about 1.0 mg L⁻¹ and 7.6,
96 respectively. Reactor was inoculated with nitrifying biomass (3 g-volatile suspended solid
97 (VSS) L⁻¹ taken from a PN reactor (Rathnayake et al., 2013) and anammox biomass (5 g-VSS L⁻¹
98 ¹) taken from an up flow column reactor (Tsushima et al., 2007). The reactor was fed with
99 synthetic wastewater contained; NH₄⁺ (about 300 mg-N L⁻¹), CaCl₂ 100 mg L⁻¹, MgSO₄ 300 mg
100 L⁻¹, KH₂PO₄ 30 mg L⁻¹, KHCO₃ 1500 mg L⁻¹ and trace element solutions (van de Graaf et al.,
101 1995). At steady state operation, an average granular size was 2.42±1.48 mm (ca. 70% of
102 granules have diameters between 1.5 – 3.5 mm).

103 **2.2 Nitrous oxide measurement**

104 Real-time N₂O concentrations in the off-gas from the reactor were measured with a
105 photo acoustic field gas monitoring devise (1412, INNOVA, Copenhagen, Denmark). In
106 addition, N₂O concentrations in the representative off-gas samples were measured using a gas
107 chromatography equipped with electron capture detector (GC-2014, Shimadzu, Kyoto, Japan).
108 The effluent water samples were collected and analyzed by N₂O headspace technique as
109 described previously (Elkins, 1980; Okabe et al., 2011a).

110 **2.3 Next generation sequencing**

111 16S rRNA gene amplicon deep sequencing was conducted using the MiSeq technology
112 (Illumina, Hayward, CA) to analysis microbial community structure. Genomic DNA was
113 extracted from the nitrification-anammox granules (n = 3) using a PowerSoil DNA Isolation kit
114 (MoBio Technologies, Carlsbad, CA). The partial 16S rRNA gene sequences including the V3
115 and V4 regions were then amplified using primers Bakt_341F and Bakt_805R (Herlemann et al.,
116 2011) with Illumina overhang adaptor sequences attached to their 50 ends (**Table S1**)
117 (Rathnayake et al., 2015). Twenty-five cycles of PCR were confirmed to be necessary and
118 sufficient to reach the log-linear phase based on quantitative PCR analysis conducted using a
119 KAPA SYBR Fast qPCR kit (Kapa Biosystems) and the Bakt_341F and Bakt_805R primers.
120 The purified DNA was mixed with Phi X control DNA and employed as a template for paired-
121 end sequencing using the MiSeq Reagent Kit v2 (500 cycles) and a MiSeq sequencer (Illumina).
122 Sequence reads from triplicate samples were analyzed using QIIME 1.8.0 (Caporaso et al.,
123 2010) with the Silva 119 database.

124 **2.4 Fluorescence in-situ hybridization**

125 Granular biomass harvested from the reactor was fixed with 4% (w/v) paraformaldehyde
126 in phosphate-buffer saline at 4°C for 6 hr. Thin sections (10 µm in thickness) of biomass were
127 pasted on teflon coated glass slides and hybridization was performed using hybridization probes
128 NSO190, Ntspa662 and AMX 820 to identify AOB, NOB and anammox bacterial population,

129 respectively as described previously (Okabe et al., 1999). Another hybridization was performed
130 using AMX 820 and mix of GNSB941 and CFX1223 (Kindaichi et al., 2012) to detect
131 abundance and spatial distribution of anammox and *Chloroflexi* bacterial cells, respectively. The
132 probes were labelled with Cy3 (or Alexa Fluor 555), Alexa Fluor 488, or Alexa Fluor 647 at the
133 5' end. Hybridized samples were observed with a LSM700 confocal laser-scanning microscope
134 equipped with diode lasers (488, 555, and 639 nm) (Carl Zeiss, Oberkochen, Germany).

135 **2.5 Microelectrode measurement**

136 The steady state concentration micro-profiles of NH_4^+ , NO_2^- , and NO_3^- were measured
137 using liquid ion exchanger (LIX) type microelectrodes (de Beer et al., 1997). The sizes of NH_4^+ ,
138 pH and NO_3^- specific microelectrode tips were between 5 – 10 μm and the sizes of NO_2^- specific
139 microelectrode tips were between 10 – 15 μm in diameter. Micro-profiles of DO and N_2O were
140 measured using DO (OX-25, Unisense, Aarhus, Denmark) and N_2O (N_2O -25, Unisense, Aarhus,
141 Denmark) microsensors, respectively. The microelectrodes were prepared, calibrated, and
142 operated as previously described (Okabe et al., 1999; Satoh et al., 2007). Briefly, granular
143 biomass (2 - 3 mm diameter size) were harvested from the nitrification-anammox reactor, fixed
144 with insect needles in a flow chamber (2.5 L) and pre-incubated for 3 hr before microelectrode
145 measurements to ensure that steady-state profiles were obtained. Microelectrodes were inserted
146 almost perpendicular to the surface of the biomass. At least three profiles were measured for
147 each chemical species and separate granule was used for each measurement. Measurement step
148 size was 100 μm ; however, smaller step size (50 μm) was chosen where fine resolution
149 measurements were desired. Furthermore, net volumetric N_2O consumption or production rates
150 were estimated from the respective concentration profiles by using the Fick's second law of
151 diffusion (Lorenzen et al., 1998) and Microsoft EXCEL add-ins solver were applied for the
152 calculations as described previously (Ali et al., 2015).

153 **2.6 N_2O isotopomer analysis**

154 The off-gas samples were collected in gas sampling bag (1LA-6SN, Analytic-barrier,
155 Tokyo, Japan) equipped with turning screw open/close valve at 6, 30, 60, 120, 240 and 345 min
156 of cycle operation during aeration phase from three different operation cycles. Later, the gas
157 samples were injected into evacuated glass serum vials (120 mL, Nichiden-Rika Glass, Tokyo,
158 Japan) sealed with a rubber stopper and aluminum cap. Subsequently, isotopomer ratios (δ) of
159 N_2O in the off-gas samples were measured using gas chromatograph isotope ratio monitoring
160 mass spectrometry (GC-IRMS, MAT252 system, thermos Fisher Scientific K.K., Yokohama,
161 Japan) as described elsewhere (Toyoda et al., 2011b, 2005). Site-specific nitrogen isotope
162 analysis in N_2O was conducted using ion detectors, which were modified to allow mass analysis
163 of the fragment ions of N_2O (NO^+) containing N atoms in the center positions of N_2O molecules,
164 whereas the bulk nitrogen and oxygen isotope ratio were determined from molecular ions
165 (Toyoda and Yoshida, 1999), detailed calculations were shown in the supplemental information.

166 2.7 Chemical Analyses

167 Influent and effluent samples were collected and concentrations of NH_4^+ , NO_2^- and NO_3^-
168 were determined using ion chromatographs (IC-2010, TOSOH, Tokyo, Japan) equipped with
169 TSKgel IC-Anion HS or TSKgel IC-Cation columns (TOSOH, Tokyo, Japan) after filtration
170 using 0.45 μm -pore-size membranes (Okabe et al., 2011b). DO was measured using a DO meter
171 (DO-5Z, KKK, Tokyo, Japan). Biomass concentration was determined as protein concentration
172 using DC Protein Assay Kit (Bio-Rad, Tokyo, Japan) and bovine serum albumin as protein
173 standard as previously described (Ali et al., 2014). Conversion of protein concentration to VSS
174 concentration was performed with conversion coefficient 1.64 (Oshiki et al., 2011).

175

176 3 Results

177 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 NO₃⁻, an end-product of anammox process, remained in the effluent, indicating that complete nitrification (oxidation of NH₄⁺ to NO₂⁻ and further to NO₃⁻) occurred to some extent. In contrast, the effluent NO₂⁻ 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 N₂O

Dissolved N₂O concentrations in the reactor effluent and gaseous N₂O 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 N₂O 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), N₂O 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 N₂O emission rate was measured as 0.26 ± 0.08 mg-N L⁻¹ h⁻¹. The dynamics of N₂O, DO, NH₄⁺, NO₂⁻, and NO₃⁻ concentrations in one operation cycle were measured three times and the averages were shown in **Fig. 2**. Only NH₄⁺ concentration rapidly

203 increased due to the feed of fresh medium and gradually decreased with time. DO and pH were
204 in the range of 0.8 to 1.3 mg-O₂ L⁻¹ and 7.5 to 7.9, respectively.

205 3.3 *Microbial community structure*

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

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

221 Deep sequencing of 16S rRNA gene analysis revealed that the most frequently detected
222 sequence was the Phylum *Chloroflexi* ($33.1 \pm 0.2\%$), which has often been observed in
223 anammox reactors fed with synthetic nutrient medium without organic carbon compounds
224 (Kindaichi et al., 2012; Miura et al., 2007). It is speculated that *Chloroflexi* act as a scavenger of
225 organic matter derived from anammox bacterial cells (Miura and Okabe, 2008). However, FISH
226 analysis revealed that the abundance of GNSB 941 and CFS 1223-stained *Chloroflexi* was very
227 low as compared to anammox bacteria cells (**Fig. 4S**), and they were detected only in the

228 anammox bacteria dominated zone. This discrepancy is probably due to PCR amplification bias
229 (Ross et al., 2013) and difference in 16S rRNA gene copy numbers; *Chloroflexi* species contain
230 up to five 16S rRNA genes per genome (Schirrmeister et al., 2012) whereas anammox bacteria
231 (e.g., *Ca. Brocadia*) has only one 16S rRNA genes per genome (Oshiki et al., 2015).

232 **3.4 In-situ microbial activity**

233 The steady-state concentration profiles of DO, NH_4^+ , NO_2^- , and NO_3^- in the granular
234 biomass harvested from a single-stage nitrification-anammox reactor were measured under the
235 condition representing the end of operation cycle (i.e., NH_4^+ concentration $<50 \text{ mg L}^{-1}$) (**Fig. 5**).
236 DO was consumed rapidly to below 0.1 mg L^{-1} at ca. $100 \mu\text{m}$ from the surface of the granule
237 (**Fig. 5A**). The net volumetric O_2 consumption rate was observed down to $250 \mu\text{m}$ depth where
238 simultaneous consumptions of NH_4^+ and NO_2^- and production of NO_3^- were observed. No net
239 NO_2^- production was observed in the oxic and anoxic zones, indicating that NO_2^- consumption
240 by NOB and anammox bacteria was faster than generation by AOB in the granule. Anammox
241 activity (i.e., nitrogen removal performance) was limited by NO_2^- availability; because NH_4^+
242 was still plenty inside granules but no anammox reaction occurred. The ratios of consumed
243 NO_2^- / consumed NH_4^+ and produced NO_3^- / consumed NH_4^+ were 1.21 and 0.34 in the oxic
244 zone (0- $250 \mu\text{m}$) and 0.95 and 0.29 in the anoxic zone ($250\text{-}500 \mu\text{m}$), respectively (**Table S2**).
245 This indicated the simultaneous occurrence of (partial) nitrification and anammox (**Fig. 5C**).
246 Under low NH_4^+ condition ($<50 \text{ mg L}^{-1}$), *in-situ* N_2O production was low, whereas high N_2O
247 productions were observed under high NH_4^+ condition (150 mg L^{-1}) (**Fig. 6A**). This observation
248 was in line with the time-course change in N_2O concentration (**Fig. 2**). The net volumetric N_2O
249 production rates calculated from the average concentration profiles revealed that approximately
250 70% of total N_2O was produced in the oxic zone (**Fig. 6B and 6C**). These results suggest that
251 nitrifiers, predominantly present in the outer layer of granules, were mainly responsible for N_2O
252 production in the oxic zone. Heterotrophic bacteria could be partly responsible for the N_2O
253 production at the oxic-anoxic interface of the granules (below $100 \mu\text{m}$) where anammox

254 bacteria were predominantly present. It should be noted that N₂O production was very dynamic
255 and highly influenced by many operating parameters such as pH, DO, NH₄⁺ and NO₂⁻
256 concentrations (Domingo-Félez et al., 2014; Joss et al., 2011; Rathnayake et al., 2015). This is
257 probably the reason that N₂O microprofiles have relatively large standard deviations.

258 3.5 N₂O isotopomer analysis

259 The $\delta^{15}\text{N}^{\text{bulk}}$, $\delta^{18}\text{O}$, and SP values were measured for N₂O molecules collected from the
260 off-gas samples at different stages of the operation cycle (**Fig. 7A**). Off-gas sample was also
261 collected at 345 min, however, N₂O isotopic composition could not be measured due to very
262 low N₂O concentration. The bulk isotopic composition ($\delta^{15}\text{N}^{\text{bulk}}$) refers to the average value of
263 $\delta^{15}\text{N}^{\alpha}$ and $\delta^{15}\text{N}^{\beta}$ estimated from equation 1 and 2 (shown in supplemental information),
264 respectively. The SP values were relatively constant (15 - 19‰) throughout the cycle.
265 Reduction of N₂O by heterotrophic denitrification increases the SP value of the remaining N₂O,
266 which hinders from partitioning different production pathways. To overcome these difficulties,
267 combination of site preference data with simultaneous measurements of $\delta^{18}\text{O}$ and $\delta^{15}\text{N}^{\text{bulk}}$ can
268 help to constrain the effect of N₂O reduction by heterotrophic denitrification (Toyoda et al.,
269 2011b). The shifts in SP due to N₂O reduction can be qualitatively evaluated based on the
270 relationships between $\delta^{18}\text{O}$ and $\delta^{15}\text{N}^{\text{bulk}}$, and $\delta^{18}\text{O}$ and $\delta^{15}\text{N}^{\alpha}$ (Jinuntuya-Nortman et al., 2008;
271 Ostrom et al., 2007). A line with a slope of 2.5 for the plot of $\delta^{18}\text{O}$ versus $\delta^{15}\text{N}^{\text{bulk}}$ and 1.7 for
272 the plot of $\delta^{18}\text{O}$ versus $\delta^{15}\text{N}^{\alpha}$ was regarded as an indicative of N₂O reduction. Based on this,
273 slopes greater than one for both the relationships were suggested as a criterion to identify the
274 influence of N₂O reduction on SP (Ostrom et al., 2007). However, in our study, the slopes of
275 lines relating $\delta^{18}\text{O}$ and $\delta^{15}\text{N}^{\text{bulk}}$, and $\delta^{18}\text{O}$ and $\delta^{15}\text{N}^{\alpha}$ were 0.14 and 0.15, respectively, suggesting
276 that the influence of N₂O reduction on SP value was not significant in this study (**Fig. S5**).
277 Therefore, relative contributions of NH₂OH oxidation and NO₂⁻ reduction (nitrifier-
278 denitrification and/or heterotrophic denitrification) to N₂O production were calculated as

described in supplemental information. The relative contributions of NH_2OH oxidation and NO_2^- reduction were calculated to be $52.1 \pm 2.3\%$ and $47.9 \pm 2.3\%$, 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_2O is essential to propose effective mitigation strategies. N_2O isotopomer analysis is a powerful tool to distinguish whether N_2O originates from NH_2OH oxidation and/or NO_2^- reduction (nitrifier denitrification and heterotrophic denitrification). Previously, isotopic composition of N_2O produced by pure cultures was investigated; the following SP values were reported: 1) -10.7 ± 2.9 to $0.1 \pm 1.7\text{‰}$ for nitrifier-denitrification by AOB; 2) 30.8 ± 5.9 to $36.3 \pm 2.4\text{‰}$ for NH_2OH oxidation; and 3) -5 to 0‰ for N_2O 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_2O (Koba et al., 2012; Yamagishi et al., 2007) including a municipal wastewater treatment plant (Toyoda et al., 2011a). For example, NH_2OH oxidation pathway accounted for 65% of the total N_2O 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_2O from a PN reactor fed with synthetic wastewater containing organic carbon (Ishii et al., 2014). Later, this technique was applied to a pilot-scale nitrification-anammox reactor to optimize the reactor operation (Harris et al., 2015). However, N_2O 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_2O production pathways including the possibility of anammox process.

303 There are several limitations of N₂O isotopomer analysis. For example, N₂O reduction
304 increases the SP value; therefore, isotopomer analysis alone may underestimate the contribution
305 of NO₂⁻ reduction pathway, when N₂O reduction is intense (Ostrom et al., 2007). Secondly, it is
306 not possible to distinguish the relative contributions of nitrifier denitrification and heterotrophic
307 denitrification because both pathways occur through NO₂⁻ reduction (Toyoda et al., 2011a). The
308 first problem can be solved with simultaneous measurements of δ¹⁸O and δ¹⁵N^{bulk} as discussed
309 above in section 3.5. In order to overcome the second problem, several techniques (*e.g.*
310 microsensor measurements, FISH and microbial community analysis) must be combined
311 together to relate the spatial distributions of N₂O production rate (function) and microbial
312 populations in nitrification-anammox granules.

313 For microsensor measurements, it should be noted that the microprofiles presented in this
314 study were artificial profiles that were not actually occurring under reactor conditions because,
315 for example, the reactor hydrodynamic was different. In addition, since these were net
316 concentration profiles as a net result of consumption and production, NO₂⁻ consumption and
317 production rates could not be precisely determined from these profiles. Second, the spatial
318 resolution of microelectrode measurements is considered to be about 2-3 times of the tip
319 diameters of microelectrodes (*i.e.*, about 30 - 50 μm in this study), a strict comparison with
320 FISH results would have limitations.

321 Microsensor results revealed that more than 70% of the total N₂O production occurred in
322 the outer oxic zone of the granules, where nitrifiers were predominantly present (**Fig. 4** and **Fig.**
323 **5**). Whereas, the rest of N₂O (about 30%) was produced in the anoxic zone, where anammox
324 bacteria and putative heterotrophic denitrifiers mainly inhabited, suggesting anammox,
325 heterotrophic denitrification and/or some other unknown pathway(s) contributed to the N₂O
326 emission in this zone, as previously reported (Harris et al., 2015). Furthermore, the oxic-anoxic
327 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_2OH oxidation and NO_2^- reduction pathways contributed roughly equal in N_2O production. This result was in good agreement with the previous observation implying that about 70% of N_2O produced in the oxic zone could be attributed to NH_2OH oxidation and NO_2^- reduction by nitrifiers, and the rest of N_2O 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_2O 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_2O production also increased. It was reported that N_2O production via NH_2OH pathway was favored at high NH_3 and low NO_2^- concentrations (Wunderlin et al., 2013a). Similar N_2O emission pattern was reported earlier in a PN reactor, where the contribution of NH_2OH oxidation pathway was relatively higher at high NH_4^+ concentration (Rathnayake et al., 2013). Biotic N_2O production by NH_2OH 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_2O production by nitrifier-denitrification is particularly promoted under oxygen-limiting and completely anoxic conditions (Kampschreur et al., 2009b).

The single-stage nitrification-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

352 (nitrification) is highly dependent on DO concentration (Vázquez-Padín et al., 2010), adequate
353 DO control is essential for the single-stage nitrification-anammox reaction.

354 In this study, DO concentration was maintained at around 1.0 mg-O₂ L⁻¹, resulting in a
355 clear vertical stratification of microbial populations; aerobic AOB were restricted to a thin outer
356 shell (ca. 100 µm) while anammox bacteria were present in the inner anoxic zone of the
357 granular biomass (**Fig. 4**). Active nitrogen transformation occurred within outer 500 µm (**Fig. 5**).
358 The thickness of active zone was dependent on the bulk DO and NO₂⁻ concentrations (Nielsen et
359 al., 2005). The volumetric conversion rate of ammonium was very high in the outer layer (100
360 µm) of the granular biomass to maintain syntrophy with anammox bacteria and to some extent
361 with NOB (**Fig. 5C**). Nitrate production was observed in the oxic zone (0-250 µm) with an
362 average ratio of produced NO₃⁻ to consumed NH₄⁺ of about 0.11, indicating occurrence of
363 complete nitrification by AOB and NOB (**Fig. 4 and Table S2**). The stoichiometric ratio of
364 produced NO₃⁻ to consumed NH₄⁺ in the anoxic zone (below 250 µm) was 0.28, which was
365 close to an anammox stoichiometry (Strous et al., 1998).

366 Application of single-stage nitrification-anammox reactor configuration is more popular as
367 compared to two-stage systems (Lackner et al., 2014). When comparing N₂O emissions from
368 single-stage systems with two-stage systems, N₂O emission was generally lower in single-stage
369 systems than the two-stage systems (**Table 1**). In two-stage nitrification-anammox systems,
370 average N₂O emission were previously reported as 2.5% (0.8 – 6.1%) and 0.27% (0.1 – 0.6%)
371 of NLR, respectively. In this study, the average emission of N₂O was ascertained as 1.35 ±
372 0.72% of the incoming nitrogen loading rate, which was comparable with the previous reported
373 values for single-stage nitrification-anammox systems (0.1 – 3.0%).

374

375 5 Conclusions

376 In this study, N₂O emission dynamics and sources of a lab-scale single-stage nitrification-
377 anammox sequencing batch reactor (SBR) were investigated by applying microscale *in-situ*
378 analyses (microsensor measurements and FISH) and reactor level analyses (N₂O monitoring and
379 isotopomer analysis). The following conclusions are drawn:

- 380 ▪ The average N₂O emission was 1.35 ± 0.72 % of the removed nitrogen ($0.98 \pm 0.42\%$ of
381 the incoming nitrogen load)
- 382 ▪ N₂O isotopomer analysis revealed that the contributions of NH₂OH oxidation and NO₂⁻
383 reduction pathways to N₂O production were roughly equal, although there is an
384 unknown influence from N₂O reduction and/or anammox N₂O production.
- 385 ▪ Microsensor measurements and FISH analysis revealed that about 70% of N₂O was
386 produced in the oxic zone where nitrifiers mainly inhabited, and thus probably through
387 NH₂OH oxidation and autotrophic NO₂⁻ reduction pathways. The rest of N₂O (ca. 30%)
388 was produced in the anammox bacteria-dominated anoxic zone, thus possibly mediated
389 by anammox, heterotrophic denitrification and/or other unknown pathway(s). Further
390 study is required to identify the N₂O emission source in the anoxic zone.

391 This study provided important information for establishing appropriate strategies to mitigate
392 N₂O emission from autotrophic biological nitrogen removal systems, which is essential to
393 reduce the carbon footprint of wastewater treatment plants. Identifying N₂O emission source
394 allow us to achieve specific design and operational conditions of single-stage nitrification-
395 anammox systems.

396

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405

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615 **Figure 1:** Performance of a single-stage nitrification-anammox reactor; **A)** Influent ammonium
 616 (filled circle) concentration and effluent ammonium (empty circle), nitrite (filled diamond) and
 617 nitrate (filled square) concentrations measured during the period of study after optimization of
 618 the reactor; **B)** Nitrogen loading (filled circle) and removal (empty circle) rate of the reactor
 619 during investigation; **C)** Activities of AOB (AOR, empty circle), NOB (NOR, filled diamond)
 620 and anammox bacteria (ANR, empty square) were calculated based on nitrogen balance and the
 621 stoichiometry of the anammox process.

622

623 **Figure 2:** A) The concentrations of NH_4^+ (filled circle), NO_2^- (filled triangle), NO_3^- (filled
 624 square) and N_2O (open circle) during an operation cycle of the reactor. B) Cyclic pattern of DO
 625 and pH during an operation cycle. Duration of reactor operation cycle was 360 min starting
 626 from 0 min: 5, 345, 5 and 5 min were allocated for feeding, aeration, settling and effluent
 627 withdrawal. Exchange ratio was 50%, resulting in hydraulic retention time of 12 hour. Error
 628 bars represent the range of standard deviation (SD) among triplicate samples collected from
 629 three different operation cycles.

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Figure 4: Confocal laser scanning microscope images of thin cross-section of the granular biomass from the nitrification-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 nitrification-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_2O in the granular biomass harvested from the nitrification-anammox reactor (A). N_2O 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_4^+ concentration (150 mg-N L^{-1}) and low NH_4^+ concentration (25 mg-N L^{-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_2O were calculated from the average concentration profiles of N_2O 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_2O 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.

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668 **Figure 7: A)** N_2O isotopomer ratios and SP values in off-gas at each sampling time over one
 669 operation cycle. **B)** Contribution of NH_2OH oxidation and NO_2^- reduction pathways to N_2O
 670 emission from the nitrification-anammox reactor. Gas samples were also collected at 345 min,
 671 however isotopic composition could not be accurately measured due to very low N_2O
 672 concentration in off-gas. Error bars indicate the range of standard deviation (SD) from triplicate
 673 samples collected from three different operating cycles.

674 **Table 1:** Nitrous oxide production rate observed in various studies during biological nitrogen removal process

S. No.	Reactor Scale	Reactor Type	Nitrogen loading rate (NLR)	Nitrogen removal rate (NRR)	The ratio of N ₂ O production to NLR	The ratio of N ₂ O production to NRR	Reference
			[kg-N m ⁻³ d ⁻¹]			[%]	
1	Full-scale	PN	0.65	0.34	1.7	3.3	(Kampschreur et al., 2008)
2	Lab-scale	PN	0.52	0.31	1.9 ± 0.53	3.22	(Graaff et al., 2010)
3	Lab-scale	PN	1.25	0.63	1.50 ± 0.22	3	(Kong et al., 2013b)
4	Lab-scale	PN	3	1.47	0.80 ± 0.19	1.63	(Kong et al., 2013a)
5	Lab-scale	PN	2.5	1.15 ± 0.35	4.0 ± 1.5	9.6 ± 3.2	(Okabe et al., 2011a)
6	Full-scale	PN	0.18-0.22	0.08-0.10	5.1-6.6	11.5-15.5	(Desloover et al., 2011)
7	Pilot-scale	PN	0.85	0.83	6.1±2.2	6.22	(Rodriguez-Caballero and Pijuan, 2013)
8	Lab-scale	PN	0.994	0.5	0.8 ± 0.4	1.5 ± 0.8	(Rathnayake et al., 2013)
9	Lab-scale	PN	8	4.4	0.55	1.0 ± 0.1	(Law et al., 2011)
10	Lab-scale	Aerobic PN Granules	1.22	0.64	5.6	10.7	(Ishii et al., 2014)
11	Full-scale	Anammox	11.47	9.67	0.6	0.68	(Kampschreur et al., 2008)
12	Lab-scale	Anammox	11.6±1.2	7.5±1.2	0.1 ± 0.07	0.14±0.09	(Okabe et al., 2011a)
13	Full-scale	Anammox	0.27	0.26	0.1	0.1	(Desloover et al., 2011)
14	Full-scale	N&A	2	1.48	1.2	1.67	(Kampschreur et al., 2009a)
15	Lab-scale	N&A	0.1315	0.11	0.1	0.11	(Sliekers et al., 2002)
16	Lab-scale	N&A	0.30-0.74	0.25-0.61	0.4-2	0.48-2.44	(Yang et al., 2013)
17	Lab-scale	N&A	0.025	0.021	2.45	2.4	(Hu et al., 2013)
18	Lab-scale	N&A	0.69	0.48	0.28-1.06	0.41-1.53	(Xiao et al., 2013)
19	Full-scale	N&A	1.5	1.35	0.8	0.89	(Joss et al., 2009)

S. No.	Reactor Scale	Reactor Type	Nitrogen loading rate (NLR)	Nitrogen removal rate (NRR)	The ratio of N ₂ O production to NLR	The ratio of N ₂ O production to NRR	Reference
			[kg-N m ⁻³ d ⁻¹]			[%]	
20	Pilot-scale	N&A	0.58-2.7	0.47-1.31	3.0-6.4	11.1-19.1	(Clippeleir et al., 2013)
21	Full-scale	N&A	1.76	1.31	2	2.69	(Castro-Barros et al., 2015)
22	Lab-scale	N&A	0.75	0.66	1.2-6.0	1.7-7.0	(Domingo-Félez et al., 2014)
23	Lab-scale	N&A	0.5-0.8	0.4-0.6	0.98 ± 0.42	1.35 ± 0.72	This study

PN; partial nitrification reactor

N&A; single-stage nitrification-anammox reactor

^a The NRR values reported for PN reactors are NH₄⁺ to NO₂⁻ conversion rates for the respective reactor

^b For all PN reactors, ratios of N₂O production rate to conversion rate of NH₄⁺ to NO₂⁻ are reported here.

N₂O production of a single-stage nitrification-anammox reactor

A single-stage nitrification-anammox SBR

Nitrification-anammox granular biomass

Oxic zone

Contribution: 70%

Anoxic zone

Contribution: 30%

N₂ ← **Anammox bacteria**

$$\text{NH}_4^+ + \text{NO}_2^-$$

AOB

$$\text{NH}_4^+ \rightarrow \text{NH}_2\text{OH}$$

NH₂OH oxidation

Contribution: 50%

NOH

$$\text{N}_2\text{O}$$

N₂O isotopomer analysis

$$\underline{SP = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta}}$$

**NO₂⁻ reduction
by nitrifiers & heterotrophs**
Contribution: 50%

N₂O production pathways