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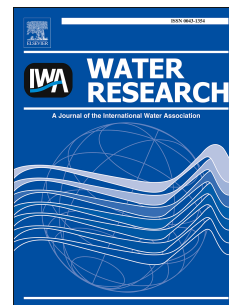
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# Accepted Manuscript

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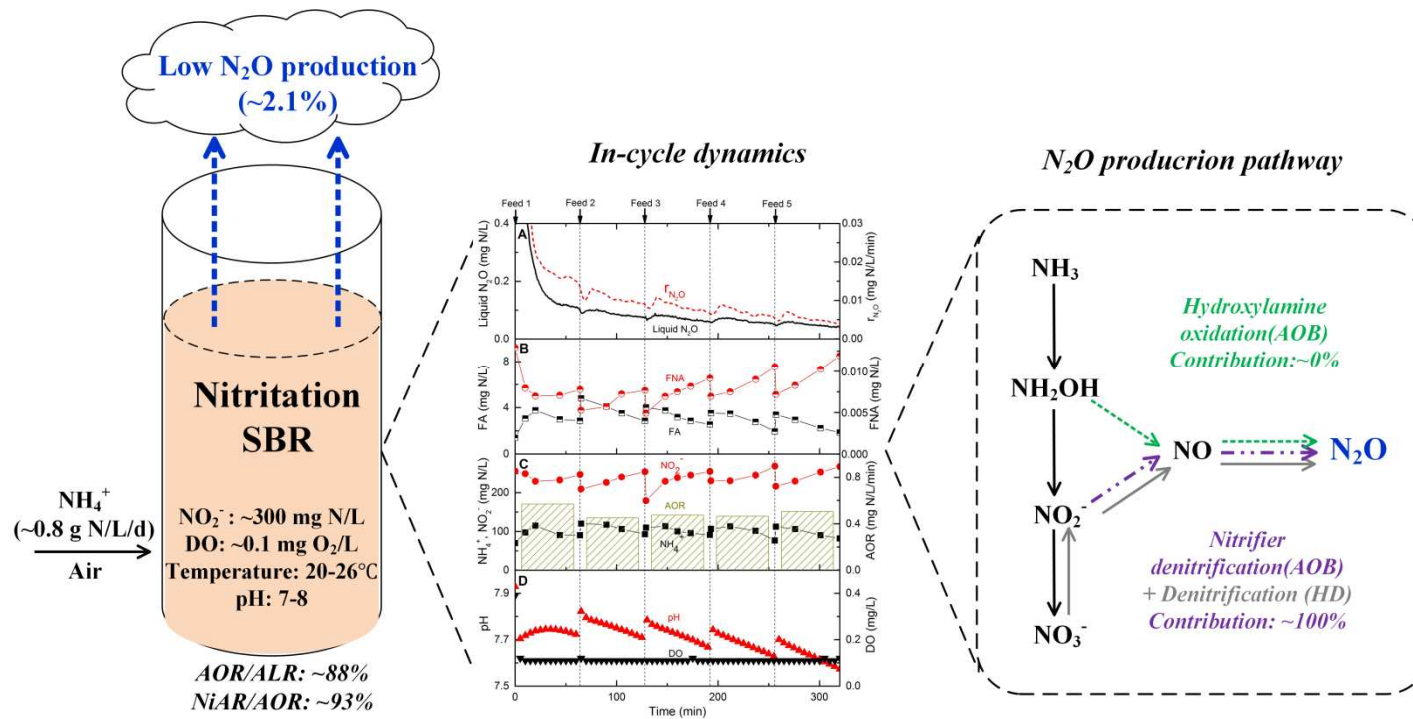
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**Low nitrous oxide production through nitrifier-denitrification in  
intermittent-feed high-rate nitrification reactors**

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## Abstract

Nitrous oxide (N<sub>2</sub>O) production from autotrophic nitrogen conversion processes, especially nitrification systems, can be significant, requires understanding and calls for mitigation. In this study, the rates and pathways of N<sub>2</sub>O production were quantified in two lab-scale sequencing batch reactors operated with intermittent feeding and demonstrating long-term and high-rate nitrification. The resulting reactor biomass was highly enriched in ammonia-oxidizing bacteria, and converted  $\sim 93 \pm 14\%$  of the oxidized ammonium to nitrite. The low DO set-point combined with intermittent feeding was sufficient to maintain high nitrification efficiency and high nitrification rates at 20-26 °C over a period of  $\sim 300$  days. Even at the high nitrification efficiencies, net N<sub>2</sub>O production was low ( $\sim 2\%$  of the oxidized ammonium). Net N<sub>2</sub>O production rates transiently increased with a rise in pH after each feeding, suggesting a potential effect of pH on N<sub>2</sub>O production. In situ application of <sup>15</sup>N labeled substrates revealed nitrifier denitrification as the dominant pathway of N<sub>2</sub>O production. Our study highlights operational conditions that minimize N<sub>2</sub>O emission from two-stage autotrophic nitrogen removal systems.

**Keywords:** Nitrous oxide; Nitrification; Ammonia-oxidizing bacteria; Intermittent feeding; pH; Nitrifier denitrification

## 1. Introduction

Autotrophic nitrogen removal by combined partial nitrification (PN, aerobic ammonium ( $\text{NH}_4^+$ ) oxidation to nitrite ( $\text{NO}_2^-$ )) and anammox (anaerobic  $\text{NH}_4^+$  oxidation with  $\text{NO}_2^-$  to dinitrogen gas ( $\text{N}_2$ )) is being implemented as an energy and resource-efficient process compared to traditional nitrification and heterotrophic denitrification process (Siegrist et al., 2008; Wett et al., 2013). Autotrophic nitrogen removal can be achieved either in one- or two-stage systems. Although the two-stage process requires higher investment costs related to the construction, this configuration allows for coordination and optimization of the individual conversion stages (Desloover et al., 2011). The PN-anammox process offers a promising alternative for nitrogen removal that meets both lower energy consumption, mainly due to lower aeration need, and lower carbon footprint emission without requirement for external carbon addition (Kartal et al., 2010). Nitrification can be achieved by manipulating operation parameters, such as low dissolved oxygen (DO) and high  $\text{NH}_4^+$  loadings, that are favorable for ammonia-oxidizing bacteria (AOB) over nitrite-oxidizing bacteria (NOB) (Blackburne et al., 2008; Vadivelu et al., 2007). However, low DO and high  $\text{NH}_4^+$  as well as high accumulation of  $\text{NO}_2^-$  produced by AOB in two-stage systems may promote accumulation and emission of nitrous oxide ( $\text{N}_2\text{O}$ ) (Kampschreur et al., 2008; Kim et al., 2010; Mampaey et al., 2016; Peng et al., 2015, 2014; Tallec et al., 2006).

The ongoing accumulation of  $\text{N}_2\text{O}$  in the atmosphere (~0.3% per year) is of great concern because it contributes to global warming ( $\text{N}_2\text{O}$  has a ca. 300 times higher global warming potential than  $\text{CO}_2$ ) and the destruction of stratospheric ozone (IPCC, 2013; Stokal and Kroeze, 2014). Indeed, documented  $\text{N}_2\text{O}$  emissions of up to 17% of the  $\text{NH}_4^+$  oxidized from both lab-scale and full-scale PN reactors have been higher compared to measurements from conventional nitrification-denitrification processes (Desloover et al., 2011; Gao et al., 2016; Kong et al., 2013; Lv et al., 2016; Mampaey et al., 2016). The variation in  $\text{N}_2\text{O}$  emissions might be explained by the different

responses of N<sub>2</sub>O production and consumption pathways to different operation strategies (e.g. feeding and aeration pattern) and parameters ( e.g. NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup>, DO and pH) (Burgess et al., 2002; Domingo-Félez et al., 2014; Law et al., 2011; Rathnayake et al., 2015; Schneider et al., 2014). There are two main pathways involved in N<sub>2</sub>O produced by AOB: (a) the reduction of NO<sub>2</sub><sup>-</sup> to N<sub>2</sub>O via nitric oxide (NO), known as nitrifier denitrification (ND) (Ishii et al., 2014; Kim et al., 2010; Wrage et al., 2001) and (b) N<sub>2</sub>O as a side product during incomplete oxidation of hydroxylamine (NH<sub>2</sub>OH) to NO<sub>2</sub><sup>-</sup> (Law et al., 2012; Poughon et al., 2001; Tallec et al., 2006), known as hydroxylamine oxidation. Furthermore, denitrifying bacteria can be as important as AOB in the production of N<sub>2</sub>O under very low C/N conditions (Domingo-Félez et al., 2017). During heterotrophic denitrification (HD), N<sub>2</sub>O is an obligate intermediate and is produced during incomplete denitrification. The exact biological pathways and environmental controls of N<sub>2</sub>O production in two-staged autotrophic nitrogen removal systems still remains to be quantified (Ishii et al., 2014; Law et al., 2012; Terada et al., 2017). A better quantitative understanding of the mechanisms for N<sub>2</sub>O production is crucial to develop novel strategies or new designs to mitigate N<sub>2</sub>O.

The principle goal of this study was to investigate N<sub>2</sub>O dynamics and determine N<sub>2</sub>O production pathways in two intermittently-fed lab-scale sequencing batch reactors (SBRs) with high nitrification performance. This was achieved by N<sub>2</sub>O online measurements and *in situ* applications of <sup>15</sup>N labeled NH<sub>4</sub><sup>+</sup> or NO<sub>2</sub><sup>-</sup> followed by monitoring of <sup>15</sup>N labeled and unlabeled products. In addition, the nitrification performance was assessed during the ~300 days of operation.

## 76 2. Materials and methods

### 77 2.1. Setup and operation of sequencing batch reactors (SBRs)

#### 78 2.1.1 Reactor description and operation

79 Two SBRs (R1 and R2) with a working volume of 5L were used (Fig. S1, Support information). Air  
80 supply was introduced by a bubble air diffuser and continuous mixing was provided with a  
81 magnetic stirrer during the reaction and feeding phase. Air supply, mixing, and actuation of pumps  
82 for fill and discharge were controlled by a programmable power strip EG-PM2-LAN (Gembird  
83 Software Ltd., Almere, Netherlands).

84 R1 and R2 were operated as duplicates for 121 days, stopped for 170 days, where the biomass was  
85 stored separately at 4 °C, and restarted for another 172 days. The operation period can be divided  
86 into two phases: phase 1 (day 0–121) and phase 2 (day 291–463). The  $\text{NH}_4^+$  and oxygen loading  
87 were the two manipulative variables to sustain a low NOB/AOB activity. To recover biomass  
88 activity after storage and maintain high  $\text{NO}_2^-$  accumulation, excess  $\text{NH}_4^+$  and oxygen limitation  
89 were set by stepwise increasing the ammonium loading rate (ALR) and air flow rate from 0.29 to  
90 0.79 g N/L/d and 0.2 to 0.55 L/min, respectively (Table S1).

91 A 6-h working cycle was applied over the entire experiment. One cycle consisted of 320 min  
92 reaction phase including five consecutive intervals of 1 minute feeding followed by a 63 minutes  
93 inter-feed period, 30 min settling phase, 5 min decanting phase and 5 min idle phase. The  
94 volumetric exchange ratio (VER) was 50%, resulting in a hydraulic retention time (HRT) of 12h.  
95 The sludge retention time (SRT) was controlled at 20 days by wasting sludge at the end of reaction  
96 phase. The reactors were operated at room temperature (20–26 °C) and without pH control.



### 2.1.2. Seed sludge and synthetic wastewater

The seeding sludge, originated from the return activated sludge stream at Mølleåværket WWTP (Lyngby, Denmark), was pre-cultivated and then inoculated into two SBRs.

Ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ) was the only nitrogen source in the synthetic wastewater while  $\text{NH}_4\text{HCO}_3$  and sodium bicarbonate ( $\text{NaHCO}_3$ ) provided the inorganic carbon. The composition of trace chemicals (van de Graaf et al., 1996) was: 169.7 mg/L  $\text{KH}_2\text{PO}_4$ , 751.1 mg/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 451.6 mg/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 5 mg/L EDTA, 5 mg/L  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and trace element solution of 1mL/L. The trace element solution contained 0.43 mg/L  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.24mg/L  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.99mg/L  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.25mg/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.22mg/L  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.19mg/L  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  and 0.21mg/L  $\text{NaSeO}_4 \cdot 10\text{H}_2\text{O}$ .

### 2.2. $\text{N}_2\text{O}$ measurement

Liquid phase  $\text{N}_2\text{O}$  was analyzed by a  $\text{N}_2\text{O}$ -R Clark-type microsensor (UNISENSE A/S, Århus, Denmark) and data was logged every 30s. Off-gas  $\text{N}_2\text{O}$  concentration was measured during phase 2 and logged on a minute basis (Teledyne API, San Diego, USA) to compare liquid and off-gas  $\text{N}_2\text{O}$  dynamics. As the reactors were not completely gas-tight during the periodic off-gas  $\text{N}_2\text{O}$  measurements, the liquid phase  $\text{N}_2\text{O}$  concentrations were used for the quantification of  $\text{N}_2\text{O}$  emission rates.

Net  $\text{N}_2\text{O}$  production and emission rates were calculated from the following equations:

$$\text{Instantaneous net } \text{N}_2\text{O} \text{ production rate, } r_{\text{N}_2\text{O}_i} = \frac{\Delta \text{N}_2\text{O}_i}{\Delta t} + k_L a_{\text{N}_2\text{O}_i} \cdot \text{N}_2\text{O}_i \quad \text{Eq. 1}$$

$$\text{Daily averaged net } \text{N}_2\text{O} \text{ production rate, } R_{\text{N}_2\text{O}} = \sum (r_{\text{N}_2\text{O}_i} \cdot \Delta t) \times 4 \frac{\text{cycle}}{\text{day}} \quad \text{Eq. 2}$$

Where  $r_{\text{N}_2\text{O}_i}$  is the instantaneous net  $\text{N}_2\text{O}$  production rate at time  $i$ ,  $\frac{\Delta \text{N}_2\text{O}_i}{\Delta t}$  is the differential term of liquid concentration at time  $i$ , and  $k_L a_{\text{N}_2\text{O}_i} \cdot \text{N}_2\text{O}_i$  is the stripping rate at time  $i$ , which equals the

emission rate. The  $\text{N}_2\text{O}$  volumetric mass transfer coefficient ( $k_{\text{LaN}_2\text{O}}$ ) was determined experimentally at different volume/flow rates scenarios (Domingo-Félez et al., 2014) (Table S2). The net  $\text{N}_2\text{O}$  produced per  $\text{NH}_4^+$  oxidized ( $\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$ , %) and the specific net  $\text{N}_2\text{O}$  production rate ( $\text{N}_2\text{OR}$ , mg N/g VSS/d) were calculated from the daily averaged net  $\text{N}_2\text{O}$  production rate (Eq. 2).

### 2.3. DNA extraction and qPCR

Biomass samples were collected periodically from SBRs and centrifuged at 10,000 rpm for 5 min. Pellets were stored at  $-80^\circ\text{C}$  until DNA extraction. DNA was extracted by FastDNA™ SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA), according to the manufacturer's instructions. The quantity and quality of the extracted DNA was measured and checked by its 260/280 ratio with a NanoDrop (ThermoFisher Scientific, Rockwood, TN, USA), and was stored at  $-20^\circ\text{C}$  until further processing within a couple of weeks. qPCR was carried out on all the extracted DNA samples to determine the relative abundance of ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (*Nitrobacter* NOB, *Nitrospira* NOB), anammox (AnAOB) and denitrifying bacteria, based on appropriate 16S rRNA targets and functional genes. Details on the procedure can be found in Terada et al. (2010). Primers and conditions used in various genes detection are listed in Table S3. All samples, including control reactions without template DNAs, were measured in duplicates.

### 2.4. $^{15}\text{N}$ additions and analysis

A  $^{15}\text{N}$  experiment was designed to identify the microbial sources of  $\text{N}_2\text{O}$  production during operation of the nitrification SBRs (day 106 to 111). The  $^{15}\text{N}$ -labeled nitrogen compounds ( $>98\%$   $^{15}\text{N}$ ; Sigma-Aldrich) were added together with the second feed during the same cycle on different days (Table S4).

141 The resulting  $^{15}\text{N}$  mole fractions of the nitrogen pools was 17-18% for  $^{15}\text{NH}_4^+$  and 11-13 % for  
 142  $^{15}\text{NO}_2^-$ , as determined from the isotopic  $^{15}\text{N}$  and total concentrations after additions. Reactor liquid  
 143 (12 ml) was sampled every 10 minutes after tracer additions until the fourth feed of the cycle. For  
 144 isotopic analysis of  $\text{N}_2\text{O}$  and  $\text{N}_2$ , 3-mL and 6-mL Exetainer vials, respectively, prefilled with 100  $\mu\text{L}$   
 145 of 50% (w/v)  $\text{ZnCl}_2$  to stop microbial activity, were filled completely and immediately screw-  
 146 capped with butyl rubber septa. Previous experiments had shown that  $\text{ZnCl}_2$  efficiently quenched N  
 147 transformations in this biomass (data not shown). The rest of the sample was filtered (0.22  $\mu\text{m}$ ) and  
 148 frozen immediately for later analyses of nutrients and isotopic composition of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$  and  
 149 nitrate ( $\text{NO}_3^-$ ).

150 Just before isotopic analysis of  $\text{N}_2\text{O}$  and  $\text{N}_2$ , 1 and 1.5 ml of water was removed with a syringe and  
 151 needle through the septum of the 3-mL and 6-mL Exetainer vials, respectively, while replacing the  
 152 volumes with helium. The isotopic composition and concentration of  $\text{N}_2\text{O}$  and  $\text{N}_2$  were determined  
 153 using a gas chromatograph-isotope ratio mass spectrometer (Thermo Electron, Delta V advantage  
 154 system) by injecting 1-mL and 200- $\mu\text{L}$  samples of headspace directly from the Exetainer vials  
 155 (Dalsgaard et al., 2012). The N-isotopic composition of  $\text{NH}_4^+$  was analyzed after conversion to  $\text{N}_2$   
 156 with hypobromite (Warembourg, 1993).  $^{15}\text{NO}_2^-$  was converted to  $\text{N}_2$  with sulfamic acid (Füssel et  
 157 al., 2012), while  $^{15}\text{NO}_3^-$  was analyzed, after removal of any  $^{15}\text{NO}_2^-$  with sulfamic acid, by cadmium  
 158 reduction followed by conversion of the  $\text{NO}_2^-$  product to  $\text{N}_2$  with sulfamic acid (McIlvin and  
 159 Altabet, 2005).

160 Rates of  $^{15}\text{N}$ -labeled  $\text{N}_2\text{O}$  and  $\text{N}_2$  production were calculated from the measured excess  
 161 concentrations of  $^{14}\text{N}^{15}\text{NO}$ ,  $^{15}\text{N}^{15}\text{NO}$ ,  $^{14}\text{N}^{15}\text{N}$ , and  $^{15}\text{N}^{15}\text{N}$  and the  $k_{\text{La}}$  for  $\text{N}_2\text{O}$  and  $\text{N}_2$ , respectively,  
 162 similar to the calculations for bulk net  $\text{N}_2\text{O}$  production rate described above.

163 The total conversion of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  to the gaseous products, irrespective of the pathway, was  
 164 determined by division of the rate of  $^{15}\text{N}$ -labeled gas production ( $^{15}\text{N}-\text{N}_2\text{O} = ^{14}\text{N}^{15}\text{NO} + 2 \times$

$^{15}\text{N}^{15}\text{NO}$ ;  $^{15}\text{N}-\text{N}_2 = ^{14}\text{N}^{15}\text{N} + 2 \times ^{15}\text{N}^{15}\text{N}$ ) by the labeling fraction  $F$  of the substrate ( $F_A = [^{15}\text{NH}_4^+] \times [\text{NH}_4^+]^{-1}$  and  $F_N = [^{15}\text{NO}_2^-] \times [\text{NO}_2^-]^{-1}$ ), e.g.:

$$\text{Rate}(\text{NH}_4^+ \rightarrow \text{N}_2\text{O}) = \text{Rate}(^{15}\text{NH}_4^+ \rightarrow ^{15}\text{N}-\text{N}_2\text{O}) \times F_A^{-1} \quad \text{Eq. 3}$$

Production of  $\text{N}_2\text{O}$  through denitrification in the  $^{15}\text{NO}_2^-$  experiments was calculated in two ways (Eq. 4 and 5), both based on the principle of random nitrogen isotope pairing (Nielsen, 1992) and resting on the assumption that denitrification is the only source of double-labeled products with  $^{15}\text{NO}_2^-$ . Here, Eq. 4 represents a rate based on  $\text{NO}_2^-$  in the bulk liquid only, with a known  $F_N$ , and Eq. 5 represents a situation where  $F_N$  at the site of reaction may differ from that in the bulk liquid and is instead estimated from the ratio of  $^{15}\text{N}^{15}\text{NO}$  production to  $^{14}\text{N}^{15}\text{NO}$  production,  $R_{46}$ :

$$\text{Denitrification}_{\text{N}_2\text{O}, \text{ bulk}} = \text{Rate}(^{15}\text{N}^{15}\text{NO}) \times F_A^{-2} \quad \text{Eq. 4}$$

$$\text{Denitrification}_{\text{N}_2\text{O}, \text{ coupled}} = \text{Rate}(^{15}\text{N}^{15}\text{NO}) \times (2R_{46} \times [1 + 2R_{46}]^{-1})^{-2} \quad \text{Eq. 5}$$

## 2.5. Analytical methods

Liquid effluent samples were filtered through 0.45  $\mu\text{m}$  pore size filters before nitrogen species analysis.  $\text{NH}_4^+$  and  $\text{NO}_2^-$  were measured colorimetrically according to Bower and Holm-Hansen (1980) and Grasshoff (1999) respectively, while  $\text{NO}_3^-$  was analyzed by autoanalyzer (AutoAnalyzer 3, SEAL Analytical) with the cadmium-reduction method (Armstrong et al., 1967; Grasshoff, 1999). Reactor performance was described by computing the observed ammonium oxidizing rate (AOR, mg N/L/d), nitrite accumulation rate (NiAR, mg N/L/d), nitrate accumulation rate (NaAR, mg N/L/d) (Eq. S2-4). Free ammonia (FA) and free nitrous acid (FNA) concentration were calculated following Anthonisen et al. (1976) (Eq. S5-6). Mixed liquid suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were measured following standard methods (APHA, 1998). DO and pH were monitored continuously (WTW GmbH, Weilheim, Germany).

### 3. Results

#### 3.1. Reactor performance

##### 3.1.1. Nitritation performance

Both reactors were operated towards high nitritation performance, and displayed stable  $\text{NH}_4^+$  removal at the end of phase 1 (day 78–121) and phase 2 (day 291–463) (Fig. 1). At the loading of 0.57 g N/L/d at the end of phase 1, the average ammonium oxidizing efficiency (AOR/ALR) was  $83 \pm 12\%$  (average  $\pm$  standard deviation) and  $90 \pm 11\%$  for R1 and R2, respectively. With stepwise increases in loading from 0.29 to 0.79 g N/L/d during phase 2, the average AOR/ALR remained relatively stable at  $86 \pm 11\%$  (R1) and  $88 \pm 8\%$  (R2) during phase 2, except for a  $\sim 19\%$  decline in the final days of the reactors (Fig. 1). There was high  $\text{NO}_2^-$  accumulation at the end of phase 1 and throughout phase 2, maintaining average nitrite accumulation efficiency (NiAR/AOR) of  $92 \pm 17\%$  and  $93 \pm 14\%$  in R1 and R2, respectively.  $\text{NO}_3^-$  accumulated at low concentrations throughout the whole operation period (Fig. 1). Nitrate accumulation efficiency (NaAR/AOR) in R1 and R2 was maintained at  $11 \pm 9\%$  and  $14 \pm 8\%$  respectively, indicating low NOB activity.

##### 3.1.2. In-cycle dynamics of nitrogen species, DO and pH

The reactors were operated with five intermittent feedings, without on-line pH control, and pH slightly decreased from 7.85 to 7.55 within a cycle (Fig. 2). pH transiently increased after each feeding due to the bicarbonate and phosphate content of the influent. During the inter-feed periods, pH decreased due to proton release during nitritation. DO concentrations were close to the limit of quantification of 0.1 mg/L during the reaction phase (Fig. 2).  $\text{NH}_4^+$  concentration increased at each feeding while  $\text{NO}_2^-$  concentration decreased due to dilution. Concentrations of FA and FNA varied between 1.39 to 4.79 mg N/L and 0.005 to 0.013 mg N/L, respectively, reflecting the changes in  $\text{NH}_4^+$  and  $\text{NO}_2^-$  concentrations at different pH (Fig. 2). During the inter-feed periods, AOR was relatively constant with an average value of  $0.49 \pm 0.04$  mg N/L/min (Fig. 2).

## 211 3.2. N<sub>2</sub>O production

### 212 3.2.1. Overall N<sub>2</sub>O production

213 During the end of phase 1, the average net N<sub>2</sub>O produced per NH<sub>4</sub><sup>+</sup> oxidized ( $\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$ ) in R1  
 214 and R2 was  $0.6 \pm 0.2\%$  and  $0.8 \pm 0.3\%$  respectively; while it was  $2.0 \pm 1.0\%$  and  $2.1 \pm 0.7\%$  during  
 215 phase 2 (Table 1). The liquid N<sub>2</sub>O concentrations as well as  $\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$  increased during phase 2  
 216 (Fig. 3 and Table 1) in two reactors. The differences in the specific net N<sub>2</sub>O production rate (N<sub>2</sub>OR)  
 217 between the two reactors were likely due to the differences in MLVSS concentrations. Furthermore,  
 218 each inter-feed period did not contribute equally to the total N<sub>2</sub>O production of a cycle. N<sub>2</sub>O gas  
 219 escaping after feed 1, ranging between 23 to 41% in both reactors during two phases, was  
 220 considerable higher compared to the emissions following the other feeds (Table 1).

### 221 3.2.2. N<sub>2</sub>O dynamics during intermittent feedings

222 The patterns of liquid N<sub>2</sub>O concentration profiles over the reaction phase were very reproducible  
 223 during the whole period for both reactors (Fig. 2 and 3). In-cycle N<sub>2</sub>O profiles had the following  
 224 pattern: after the settling phase from the previous cycle, an initial maximum in N<sub>2</sub>O concentration  
 225 occurred when the first feed initiated, after which the concentration declined until the next feeding;  
 226 another four smaller peaks in N<sub>2</sub>O concentration were observed in the subsequent feedings. N<sub>2</sub>O  
 227 concentration reached minimum values in the inter-feed periods but with concentrations higher than  
 228 the detection limit of the sensor. Thus, based on liquid N<sub>2</sub>O concentrations there was always a  
 229 positive net production of N<sub>2</sub>O in both reactors, with rates ( $r_{\text{N}_2\text{O}_i}$ ) increasing after each feeding and  
 230 decreasing during inter-feed periods (Fig. 3). Off-gas N<sub>2</sub>O profiles followed the same trends during  
 231 the reaction phase.

### 3.3. Microbial community composition dynamics

The optimization of the reactor operation during phase 1 caused clear shifts in the microbial community, as indicated by qPCR analysis using relevant primers (Fig. 4). The microbial community composition was similar between the two reactors. The relative abundance of *Nitrobacter* spp. decreased at the end of phase 1, where *Nitrobacter* spp. was 2–3 orders of magnitude higher than *Nitrospira* spp. Both *Nitrobacter* spp. and *Nitrospira* spp remained very low throughout phase 2. Both 16S rRNA gene and *nxrA* targeted NOB quantifications were consistent in phase 2 (Fig. 4 and S2). The overall reduction in NOB relative abundance was mirrored by a significant increase in AOB numbers, as reflected by both the 16S rRNA gene and *amoA* targeted quantifications (Fig. 4 and S2). AOB remained dominant in both reactors throughout the operation period. The relative abundance of AnAOB, based on 16S rRNA gene quantification, was low but existent ( $0.96 \pm 0.01\%$  and  $1.94 \pm 0.01\%$  in R1 and R2, respectively). The ratio of *nirS* plus *nirK* over *nosZ*-targeted quantifications was far above 1 (Fig. S2).

### 3.4. N<sub>2</sub>O production pathway

In incubations with <sup>15</sup>N-labeled substrates, the label was transferred to both N<sub>2</sub>O and N<sub>2</sub> within 2–3 minutes of addition, irrespective of whether <sup>15</sup>N was added as <sup>15</sup>NO<sub>2</sub><sup>−</sup> or <sup>15</sup>NH<sub>4</sub><sup>+</sup> (Fig. 5). The dynamics of <sup>15</sup>N-N<sub>2</sub>O mirrored those of bulk N<sub>2</sub>O, and N<sub>2</sub>O was the dominating product in <sup>15</sup>NO<sub>2</sub><sup>−</sup> incubations accounting for 57–58% of the labeled N<sub>2</sub>O + N<sub>2</sub> in both feedings, while it only accounted for 17–23% with <sup>15</sup>NH<sub>4</sub><sup>+</sup>. The production of N<sub>2</sub> was also highly dynamic, showing an even steeper rise after feeding than for N<sub>2</sub>O. The production of <sup>15</sup>N-N<sub>2</sub>O from <sup>15</sup>NO<sub>2</sub><sup>−</sup> corresponded to a total conversion of NO<sub>2</sub><sup>−</sup> to N<sub>2</sub>O of 5.7–9.9 μg N/g VSS/min, which was not significantly different from the total net N<sub>2</sub>O production (Table 2), implying that NO<sub>2</sub><sup>−</sup> was the main source of N<sub>2</sub>O in the incubations.

255 There was no detectable production of  $^{15}\text{NH}_4^+$  in the incubations with  $^{15}\text{NO}_2^-$  (data not shown),  
 256 which implies that all  $^{15}\text{N-N}_2\text{O}$  and  $^{15}\text{N-N}_2$  in these incubations was formed exclusively through  
 257 reductive pathways, i.e., not via dissimilatory nitrate/nitrite reduction to ammonium (DNRA) and  
 258 subsequent oxidation of  $\text{NH}_4^+$ .

259 Indeed, the relative production of  $^{14}\text{N}^{15}\text{NO}$  and  $^{15}\text{N}^{15}\text{NO}$  from  $^{15}\text{NO}_2^-$  (Fig. 5) was close to that  
 260 expected from denitrification with random isotope pairing (either heterotrophic or nitrifier  
 261 denitrification). Thus, the production of  $\text{N}_2\text{O}$  through denitrification (calculated by Eq. 4)  
 262 corresponded to 80% and 77% of total net  $\text{N}_2\text{O}$  production from  $\text{NO}_2^-$  (the  $\text{NO}_2^-$ -to- $\text{N}_2\text{O}$  conversion  
 263 rates calculated by Eq. 3) on average for feed 2 and 3, respectively (Table 2). The remaining 20–  
 264 23% of  $\text{NO}_2^-$ -derived  $\text{N}_2\text{O}$  corresponds to a surplus of  $^{14}\text{N}^{15}\text{NO}$  relative to the prediction from  
 265 random isotope pairing from the bulk  $\text{NO}_2^-$  pool, and therefore indicates pairing of N from this pool  
 266 with N from a second source of unlabeled N. The surplus of  $^{14}\text{N}^{15}\text{NO}$  may arise if the labeling  
 267 fraction of  $\text{NO}_2^-$ ,  $F_N$ , in the immediate vicinity of the nitrite reductase enzymes is lower than the  
 268 bulk  $F_N$  value used for the calculations (Eq. 4), e.g., because of dilution with unlabeled  $\text{NO}_2^-$  from  
 269 nitrification maintained by diffusional gradients either intracellularly or within microaggregates. This  
 270 is reflected in the  $\text{N}_2\text{O}$  production calculated by Eq. 5, which derives  $F_N$  at the site of  $\text{NO}_2^-$   
 271 reduction from the relative production of  $^{14}\text{N}^{15}\text{NO}$  and  $^{15}\text{N}^{15}\text{NO}$ . Thus, assuming that all conversion  
 272 of  $\text{NO}_2^-$  to  $\text{N}_2\text{O}$  occurred through a denitrification pathway, total  $\text{N}_2\text{O}$  production was calculated  
 273 based on the relative production of  $^{14}\text{N}^{15}\text{NO}$  and  $^{15}\text{N}^{15}\text{NO}$  (Nielsen, 1992), yielding rates that  
 274 exceeded the  $\text{NO}_2^-$ -to- $\text{N}_2\text{O}$  conversion rates by 24–31% (Table 2).

275 The production of  $\text{N}_2\text{O}$  from  $\text{NH}_4^+$ , determined in incubations with  $^{15}\text{NH}_4^+$  showed very similar  
 276 temporal dynamics as  $\text{N}_2\text{O}$  production from  $\text{NO}_2^-$  (Fig. 5). After the 2<sup>nd</sup> feed, the production from  
 277  $\text{NH}_4^+$  corresponded, on average, to 42% of the production from  $\text{NO}_2^-$  (Table 2). This fraction  
 278 increased to 58% after the 3<sup>rd</sup> feed, which is explained by the accumulation of  $^{15}\text{NO}_2^-$  and the



279 resulting increasing contribution of  $^{15}\text{N}_2\text{O}$  from denitrification, as also reflected in the higher  
 280 concentrations of  $^{15}\text{N}-\text{N}_2\text{O}$  reached after the 3<sup>rd</sup> feed relative to the 2<sup>nd</sup> (Fig. 5). The amount of  $^{15}\text{N}-$   
 281  $\text{N}_2\text{O}$  produced from  $^{15}\text{NH}_4^+$  via nitrification, mixing of the formed  $^{15}\text{NO}_2^-$  with the bulk  $\text{NO}_2^-$  pool,  
 282 and subsequent denitrification, was estimated for each reactor based on the rates of  $\text{N}_2\text{O}$  production  
 283 determined in the  $^{15}\text{NO}_2^-$  incubations in the same reactor and the  $F_N$  values (data not shown) from  
 284 the  $^{15}\text{NH}_4^+$  incubations (Eq. 3). These calculations indicated that 25% and 49% of  $\text{N}_2\text{O}$  production  
 285 determined with  $^{15}\text{NH}_4^+$  occurred via bulk  $\text{NO}_2^-$  after feed 2 and 3, respectively. The  $^{15}\text{NH}_4^+$ -based  
 286  $\text{N}_2\text{O}$  production that was not attributable to this route averaged  $2.6 \mu\text{g N/g VSS/min}$  after both  
 287 feedings, corresponding to 25% of the combined  $\text{N}_2\text{O}$  production detected with  $^{15}\text{NO}_2^-$  and  $^{15}\text{NH}_4^+$   
 288 (Table 2), and the sum of this rate and the production of  $\text{N}_2\text{O}$  from  $\text{NO}_2^-$  matched the estimated  $\text{N}_2\text{O}$   
 289 production from denitrification closely ( $7.7$  vs.  $7.3 \mu\text{g N/g VSS/min}$  and  $12.1$  vs.  $12.5 \mu\text{g N/g}$   
 290  $\text{VSS/min}$  for R1 and R2, respectively). The contribution of the hydroxylamine oxidation pathway to  
 291  $\text{N}_2\text{O}$  production did *not* increase immediately after the addition of  $\text{NH}_4^+$ , as the production ratio  
 292 between  $^{15}\text{N}^{15}\text{NO}$  and  $^{15}\text{N}^{14}\text{NO}$  did not change significantly over time after feed 2 and 3. Thus, the  
 293  $^{15}\text{NO}_2^-$  and  $^{15}\text{NH}_4^+$  in combination support a denitrification pathway as the main and possibly sole  
 294 source of  $\text{N}_2\text{O}$  in this SBR system.

295 In the  $^{15}\text{NO}_2^-$  incubations, the relative abundance of single and double-labeled  $\text{N}_2$  ( $^{14}\text{N}^{15}\text{N}$  and  
 296  $^{15}\text{N}^{15}\text{N}$ ) differed markedly from that of  $\text{N}_2\text{O}$ , with  $^{15}\text{N}^{15}\text{N}$  accounting for  $\leq 0.5\%$  of the labeled  $\text{N}_2$   
 297 compared a contribution of  $\sim 5\%$  from  $^{15}\text{N}^{15}\text{NO}$  to labeled  $\text{N}_2\text{O}$  (Fig. 5). This pointed towards  
 298 another  $\text{N}_2$  source than denitrification. The total  $\text{N}_2$  production rate from  $\text{NO}_2^-$  (Eq. 3) was  $4.4 \pm 0.9$   
 299 and  $6.4 \pm 0.8 \mu\text{g N/g VSS/min}$  for R1 and R2, respectively. Substantially higher  $\text{N}_2$  production rates  
 300 were obtained for the  $^{15}\text{NH}_4^+$  than with  $^{15}\text{NO}_2^-$ :  $10.2 \pm 3.5$  and  $21 \pm 0.8 \mu\text{g N/g VSS/min}$  for R1 and  
 301 R2, respectively. Correction of these rates for  $^{15}\text{N}-\text{N}_2$  produced from the accumulating  $^{15}\text{NO}_2^-$

(performed similarly as for the  $\text{N}_2\text{O}$  production rates from  $^{15}\text{NH}_4^+$ ) only reduced these rates slightly to  $9.4 \pm 3.5$  and  $19.7 \pm 1.5$   $\mu\text{g N/g VSS/min}$ , respectively.

## 4. Discussion

### 4.1. Mechanisms to achieve high and stable nitrification performance

Two SBRs were operated for approximately 300 days with high  $\text{NO}_2^-$  accumulation and no significant production of  $\text{NO}_3^-$ , which indicates that NOB were successfully outcompeted by AOB (Fig. 1). The suppression of NOB and enrichment of AOB was verified by an average AOB/NOB ratio of  $>200$  at the end of phase 1 and during phase 2 (Fig. 4). Various parameters such as DO, FA, FNA, temperature and feeding strategy have been reported to affect the selective enrichment of AOB over NOB (Blackburne et al., 2008; Hellenga et al., 1998; Liu and Wang, 2014; Vadivelu et al., 2007; Yang et al., 2013).

Oxygen limitation is a critical factor to achieve and maintain high nitrification performance. AOB are postulated to outcompete NOB at low DO concentrations due to the higher oxygen affinity of AOB than NOB (Blackburne et al., 2008; Wiesmann, 1994). DO below 1.0 mg/L was previously reported to inhibit the growth of NOB and instead enhance the growth of AOB, resulting nitrite accumulation (Sinha and Annachhatre, 2007; Tokutomi, 2004). For instance, stable nitrite accumulation efficiency (NiAR/AOR) of 70% and 85% is achieved at DO of 0.1 mg/L and 0.5–1.0 mg/L, respectively (Gao et al., 2016; Guo et al., 2013). As the DO level in our two nitrification SBRs was  $\leq 0.1$  mg/L, oxygen limitation is an important factor for NOB inhibition at the end of phase 1 and throughout phase 2, where high nitrification efficiencies of  $92 \pm 17\%$  (R1) and  $93 \pm 14\%$  (R2) were maintained (Fig. 1).

Among other factors, FA and FNA are commonly selected as the key parameters to achieve high nitritation because of the different impacts on AOB and NOB (Anthonisen et al., 1976; Brockmann and Morgenroth, 2010; Vadivelu et al., 2007; Yamamoto et al., 2008). Many studies have reported FA and FNA concentrations that might inhibit NOB growth and trigger AOB proliferation; however, the critical values reported in these studies were variable (Anthonisen et al., 1976; Bae et al., 2001; Vadivelu et al., 2007). Regarding FA, NOB has been found to be inhibited at concentrations ranging from 0.1 to 1 mg N/L, while AOB was inhibited at 10-150 mg N/L (Anthonisen et al., 1976). This agrees with a recent study by Vadivelu and coworkers (2007), where NOB activity was totally inhibited by 6.0 mg N/L and AOB activity was unaffected at up to 16 mg N/L. The increase in FA concentration by a factor of ~5 from phase 1 I to phase 1 II and 2, where the FA concentration was  $3.1 \pm 0.8$  mg N/L, could be the reason for a decrease in nitrate accumulation, especially in R1 (Fig. 1 and 2). However, FA did not fully inhibit the activity of NOB at any time in our study. Also, within the observed FA concentration, FA likely had no effect on the activity of AOB.

It has been reported that NOB activity was inhibited by FNA at concentrations between 0.02 and 0.2 mg N/L (Hellinga et al., 1998; Vadivelu et al., 2007). Compared to these studies, FNA at  $0.008 \pm 0.002$  mg  $\text{NO}_2^-$ -N/L was too low to have a negative effect on NOB activity (Fig. 2). Throughout the whole SBR operation period, AOR correlated positively with  $\text{NO}_2^-$  concentrations, reaching the maximum (0.8 g N/L/d) at 323 mg N/L (Fig. S3). Hence, no evidence of  $\text{NO}_2^-$  inhibition was obtained. The observed increase in AOR with increasing  $\text{NO}_2^-$  concentration agrees with a previous study with mixed microbial communities, showing high ammonium oxidation to  $\text{NO}_2^-$  (150–160 mg  $\text{NO}_2^-$ -N/h/g VSS) at  $\text{NO}_2^-$  concentrations up to 1000 mg N/L (Law et al., 2013). Nevertheless, the calculated FNA concentrations in this study (ca. 0.008 mg  $\text{HNO}_2^-$ -N/L) remain much below reported inhibitor concentrations (FNA of 0.1 mg/L) (Hiatt and Grady, 2008).

Temperature is another parameter that can affect the relative competitiveness of AOB over NOB. NOB were outcompeted by AOB at moderate temperatures (20-26 °C), resulting in high nitrification efficiency from day 78 onwards (Fig. 1). This finding contrasts with the general assumption of high temperatures (30-35 °C) are needed for selective removal of NOB over AOB (Hellenga et al., 1998; Yang et al., 2007).

It is often difficult to maintain stable nitrification over the long-term period even in successfully established nitrification systems (Bernet et al., 2001; Fux et al., 2004; Villaverde et al., 2000; Yang et al., 2013). For instance, Villaverde and coworkers (2000) obtained high NiAR/AOR of 65% in submerged nitrifying biofilters, however, after 6 months NOB became acclimated to high FA and NiAR/AOR decreased to 30%. Moreover, Bernet and coworkers (2001) observed a transition from stable nitrification in a two-stage PN-anammox process for more than 100 days to complete nitrification within 2 days caused by a transient increase of DO. Here, SBRs were operated for ~300 days with high nitrification efficiency and high AOB abundance accompanied by low  $\text{NO}_3^-$  accumulation and low NOB abundance. We speculate that using intermittent feeding together with low DO set-points successfully enabled long-term high nitrification performance in the two SBR reactors. While long-term high-rate nitrification has not been reported yet in intermittently fed SBRs, high nitrite accumulation (NiAR/AOR) of 85% and >95% was previously reported for 150 and 174 days, respectively, in step-feed A/O SBRs (Lemaire et al., 2008; Yang et al., 2007). Hence, low DO control and intermittent feeding appear key operational strategies to obtain continuous NOB suppression at suboptimal temperatures.

#### 4.2. Low $\text{N}_2\text{O}$ production

The net  $\text{N}_2\text{O}$  produced per  $\text{NH}_4^+$  oxidized ( $\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$ ) and the specific net  $\text{N}_2\text{O}$  production rate ( $\text{N}_2\text{OR}$ ) of the two nitrification SBRs were compared to previously reported values together with the identification of reactor types, operation strategies, performance and AOB presence (Table S5). The

372 average net N<sub>2</sub>O production in phase 2 increased to  $2.0 \pm 1.0\%$  and  $2.1 \pm 0.7\%$  of the NH<sub>4</sub><sup>+</sup> oxidized  
 373 in R1 and R2, respectively, while the average specific net N<sub>2</sub>O production rate was  $8.4 \pm 3.5$  and  
 374  $10.2 \pm 3.5$  mg N/g VSS/d in R1 and R2, respectively (Table 1 and S5). The net N<sub>2</sub>O production in  
 375 both reactors corresponded well with the genetic potential for N<sub>2</sub>O production, as the ratio of *nirS*  
 376 plus *nirK* over *nosZ*-targeted genes was far above 1 (Fig. S2). The higher N<sub>2</sub>O production in phase  
 377 2 compared to phase 1 is puzzling as it cannot be explained by higher AOR (Table 1). We speculate  
 378 that the long-term operation under elevated NO<sub>2</sub><sup>-</sup> may have selected for new microbes with higher  
 379 expression of the nitrifier-denitrification pathway or the cultured microbes adapted to higher NO<sub>2</sub><sup>-</sup>,  
 380 resulting in higher expression of the pathway, and with that higher N<sub>2</sub>O production. This theory,  
 381 however, calls for deeper analysis of the microbial community than obtained with qPCR.

382 The N<sub>2</sub>O production factors of ~2% are in the low range of previous reports for both lab-scale and  
 383 full-scale PN systems, ranging between 1–17% (Table S5). Our study is the first study to measure  
 384 low N<sub>2</sub>O emissions at very high nitrification efficiencies. Low DO (0.35 mg/L) and high NO<sub>2</sub><sup>-</sup>  
 385 conditions (10 – 50 mg N/L) boost N<sub>2</sub>O production (Peng et al., 2015, 2014). Measured N<sub>2</sub>O  
 386 emissions are lower compared to other lab-scale PN SBRs operated under low DO and high NO<sub>2</sub><sup>-</sup>  
 387 conditions (N<sub>2</sub>O emissions of 17%) (Gao et al., 2016; Lv et al., 2016). With the intermittent feeding  
 388 strategy at low DO, we force relatively low ammonia oxidation rates (Fig. 2, Table 1), which has  
 389 previously been shown to decrease N<sub>2</sub>O emissions from autotrophic nitrogen removal systems  
 390 (Domingo-Félez et al., 2014; Law et al., 2011). Law and coworkers (2011) found that a decline in  
 391 feeding rate from 1 L/2.5 min to 1 L/25 min during the reaction phase lead to a substantial reduction  
 392 in N<sub>2</sub>O production without affecting the nitrification performance. Instead of reducing the feeding rate,  
 393 our nitrification reactors were operated with five intermittent feedings within a cycle. This step-feed  
 394 strategy has previously been suggested as an effective optimization approach to reduce N<sub>2</sub>O

emissions from SBRs (Mavrovas, 2014; Yang et al., 2009, 2013). Therefore, we postulate that intermittent feeding is the cause for the low  $\text{N}_2\text{O}$  emission from high-performance nitrification system.

#### 4.3. Potential pH effect on in-cycle $\text{N}_2\text{O}$ production dynamics

Distinctive  $\text{N}_2\text{O}$  production profiles were observed within the representative cycles (Fig. 2 and 3). The maximum net  $\text{N}_2\text{O}$  production and the subsequent decrease after the first feed has also been described in various studies (Ali et al., 2016; Itokawa et al., 2001; Kampschreur et al., 2008; Mampaey et al., 2016; Rodriguez-Caballero and Pijuan, 2013). Rodriguez-Caballero and Pijuan (2013) showed that 60% of the total  $\text{N}_2\text{O}$  production occurred during the settling phase in their lab-scale PN SBR, while 70% of the quantified  $\text{N}_2\text{O}$  emission was attributed to the anoxic  $\text{N}_2\text{O}$  formation in a full-scale PN SHARON reactor (Mampaey et al., 2016). Tentative liquid  $\text{N}_2\text{O}$  measurements indicated that  $\text{N}_2\text{O}$  accumulated during the non-aerated settling phase (data not shown). Denitrification might be responsible for this  $\text{N}_2\text{O}$  accumulation during the settling phase, which is then released at the onset of aeration (Itokawa et al., 2001). The genetic potential for  $\text{N}_2\text{O}$  production by denitrifiers was present through the high relative abundance of *nirS* (Fig. S2).

A potential effect of pH on  $\text{N}_2\text{O}$  production during the reaction phase was indicated by the transiently increase in net  $\text{N}_2\text{O}$  production rates with the rise in pH after each feeding pulse (Fig. 2 and 3). There was no obvious changes in DO, and although  $\text{NH}_4^+$  and FA increased transiently after each feeding, FA was always in excess compared to the  $K_m$  value of 0.0075 mg/L for AOB, and therefore AOR remained unaffected (Fig. 2) (Hiatt and Grady, 2008). Thus, pH appears the only potential variable affecting in-cycle  $\text{N}_2\text{O}$  dynamics. Only few studies have been able to isolate the effect of pH on  $\text{N}_2\text{O}$  production from the variations in FA and FNA, and the reported effect of pH on  $\text{N}_2\text{O}$  production differ. In contrast to our results, Law and coworkers (2011) obtained highest  $\text{N}_2\text{O}$  and AOR at pH 8 in the investigated pH range of 6.0–8.5, independently from FA and FNA concentrations, suggesting that an increase in ammonium oxidation activity might promote  $\text{N}_2\text{O}$

production. Oppositely, Rathnayake et al. (2015) observed highest N<sub>2</sub>O emission at pH 7.5 in PN granules, although AOR was unchanged between pH 6.5 and 8.5. Further research is needed to resolve whether the pH effect on N<sub>2</sub>O production is direct or indirect.

#### 4.4. N<sub>2</sub>O production pathway

The experiments with <sup>15</sup>N labeled substrates point to nitrifier denitrification as the dominant source of N<sub>2</sub>O in the SBR nitrification systems. A denitrification-type process rather than a direct production of N<sub>2</sub>O from ammonium oxidation via hydroxylamine was demonstrated by more than 3 times higher rates of N<sub>2</sub>O production from NO<sub>2</sub><sup>-</sup> than from NH<sub>4</sub><sup>+</sup>, when <sup>15</sup>NH<sub>4</sub><sup>+</sup>-derived rates were corrected for accumulation of <sup>15</sup>NO<sub>2</sub><sup>-</sup> (Table 2). Moreover, isotope pairing calculations showed that NO<sub>2</sub><sup>-</sup> during its reduction to N<sub>2</sub>O was mixed with nitrogen from an unlabeled source. In the nitrification-dominated system, NH<sub>4</sub><sup>+</sup> is the most obvious candidate, and indeed, the production rate of N<sub>2</sub>O from NH<sub>4</sub><sup>+</sup> that did not go via bulk NO<sub>2</sub><sup>-</sup> closely matched the difference between total and bulk NO<sub>2</sub><sup>-</sup>-dependent denitrification. We therefore hypothesize that essentially all N<sub>2</sub>O was produced through nitrifier-denitrification with part of the newly-formed NO<sub>2</sub><sup>-</sup> shunted directly to reduction either intracellularly or within cellular aggregates before it could mix completely with NO<sub>2</sub><sup>-</sup> in the bulk liquid. Alternatively, the combination of N from NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> could occur at the level of NO if this compound is a free intermediate during ammonium oxidation (Stein, 2011). The <sup>15</sup>N-labeling technique in itself cannot distinguish nitrifier denitrification from heterotrophic denitrification. However, several pieces of evidence point to the former process. Firstly, the stimulation of N<sub>2</sub>O production by each NH<sub>4</sub><sup>+</sup> feeding points to NH<sub>4</sub><sup>+</sup> dependence rather than heterotrophy. Secondly, there is no convincing evidence for heterotrophic N<sub>2</sub> production: (a) The rate of N<sub>2</sub>O production exceeds the rate of N<sub>2</sub> production from NO<sub>2</sub><sup>-</sup> whereas N<sub>2</sub>O is generally a minor byproduct of heterotrophic denitrification (Betlach and Tiedje, 1981); (b) the dynamics of N<sub>2</sub> and N<sub>2</sub>O production are out of phase with the peak in N<sub>2</sub> preceding that of N<sub>2</sub>O, where the opposite



would be expected during heterotrophic denitrification (e.g., Jensen et al., 2009), and (c) the very low ratio of  $^{15}\text{N}^{15}\text{N}$  to  $^{14}\text{N}^{15}\text{N}$ , differing markedly from the  $^{15}\text{N}^{15}\text{NO}:$  $^{14}\text{N}^{15}\text{NO}$  ratio in  $\text{N}_2\text{O}$ , suggests that  $\text{N}_2$  production from  $\text{NO}_2^-$  is mainly due to another process, possibly anammox.

The complete dominance of nitrifier-denitrification as source of  $\text{N}_2\text{O}$  is in general agreement with the understanding that this process is favored by low DO and high  $\text{NO}_2^-$  levels (e.g., Colliver and Stephenson, 2000; Kampschreur et al., 2008; Peng et al., 2015; Tallec et al., 2006). The high rates of  $\text{N}_2$  production observed in the  $^{15}\text{NH}_4^+$  incubations, relative to both  $\text{N}_2\text{O}$  production in the same experiment and to  $\text{N}_2$  production with  $^{15}\text{NO}_2^-$ , suggests an involvement of anammox. Only a small part of the  $\text{N}_2$  produced with  $^{15}\text{NH}_4^+$  could be explained with oxidation to  $\text{NO}_2^-$  and subsequent reduction, which means that  $\text{NH}_4^+$  appeared to be converted directly from  $\text{NH}_4^+$  to  $\text{N}_2$ . As  $\text{N}_2$  production has not been documented in aerobic ammonium oxidizers, this suggests the involvement of anammox bacteria, which were indeed detected in the biomass (Fig. 4) in low abundance. As anammox represents a 1:1 pairing of N from  $\text{NH}_4^+$  and  $\text{NO}_2^-$ , similar rates of  $\text{N}_2$  production should, however, be obtained with additions of  $^{15}\text{NH}_4^+$  and  $^{15}\text{NO}_2^-$  (van de Graaf et al., 1995), whereas we observed ~2.5-fold higher production from  $^{15}\text{NH}_4^+$  than from  $^{15}\text{NO}_2^-$ . Potential explanations for the imbalance in rates are either a close coupling of nitrification and anammox, which would require a physical association of anammox bacteria and ammonium oxidizers, or variation in anammox rates between the two series of experiments, which were conducted 5 days apart. The resolution of these issues is, however, beyond the scope of this study.

## 5. Conclusion

Two lab-scale intermittently-fed nitrification SBRs were operated to investigate  $\text{N}_2\text{O}$  dynamics and identify  $\text{N}_2\text{O}$  production pathways.



- High nitrification performance with  $\sim 93 \pm 14\%$  of the oxidized  $\text{NH}_4^+$  converted to  $\text{NO}_2^-$  was achieved in intermittently-fed SBRs at 20-26°C for  $\sim 300$  days.
  - The averaged net  $\text{N}_2\text{O}$  production factor of  $2.1 \pm 0.7\%$  is in the low range: Operation with intermittent feeding may be an effective approach to minimize  $\text{N}_2\text{O}$  emissions from nitrification systems.
  - Increased net  $\text{N}_2\text{O}$  production rate was observed with pH increase after each feeding. Further investigations are required to identify the exact mechanisms of the pH effect on enzymes, pathways and bacteria involved in  $\text{N}_2\text{O}$  production.
  - Nitrifier denitrification was the dominant source of  $\text{N}_2\text{O}$ .
- This study has demonstrated operational conditions (low dissolved oxygen and intermittent feeding) that achieve high-rate and long-term nitrification under normal temperature, which could enlarge the applicability of the nitrification process in WWTPs. The relatively low  $\text{N}_2\text{O}$  production at high nitrification efficiencies reduces the growing concern of  $\text{N}_2\text{O}$  production from autotrophic nitrogen processes in WWTPs. The identification of nitrifier denitrification as the main pathway of  $\text{N}_2\text{O}$  emissions will open up for more focused strategies to lower the  $\text{N}_2\text{O}$  footprint even more in nitrification systems.

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Table 1. Overview of AOR, N<sub>2</sub>OR and  $\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$  in R1 and R2 during phase 1 and 2. The net N<sub>2</sub>O produced during each feed is stated as the percentage of total net N<sub>2</sub>O production during the entire cycle.

	R1		R2	
	Phase 1 (Day 106–112)	Phase 2 (Day 395–451)	Phase 1 (Day 106–112)	Phase 2 (Day 397–463)
AOR (g N/L/d)	0.5 ± 0.05	0.60 ± 0.05	0.5 ± 0.02	0.76 ± 0.06
AOR (g N/g VSS/d)	1.04 ± 0.11	0.46 ± 0.09	1.78 ± 0.08	0.5 ± 0.02
N <sub>2</sub> OR (mg N/g VSS/d)	5.9 ± 1.8	8.4 ± 3.5	16.0 ± 5.9	10.2 ± 3.5
$\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$ (%)	0.6 ± 0.2	2.0 ± 1.0	0.8 ± 0.3	2.1 ± 0.7
Feed 1 (%)	23 ± 5	41 ± 9	30 ± 5	27 ± 5
Feed 2 (%)	22 ± 1	14 ± 2	21 ± 2	17 ± 2
Feed 3 (%)	19 ± 1	15 ± 2	18 ± 2	18 ± 2
Feed 4 (%)	17 ± 2	16 ± 2	16 ± 2	19 ± 1
Feed 5 (%)	18 ± 3	15 ± 4	15 ± 2	21 ± 5
# cycles	n=22	n=23	n=22	n=20

Table 2. Summary of net N<sub>2</sub>O production rates during the <sup>15</sup>N experiment (μg N/g VSS/min). Bulk N<sub>2</sub>O production was based on liquid N<sub>2</sub>O concentrations, measured with microsensors, while N<sub>2</sub>O source partitioning is based on isotope additions

Days of operation	R1				R2			
	<sup>15</sup> NO <sub>2</sub> <sup>-</sup> additions				<sup>15</sup> NO <sub>2</sub> <sup>-</sup> additions			
	110		111		110		111	
	Feed 2	Feed 3	Feed 2	Feed 3	Feed 2	Feed 3	Feed 2	Feed 3
Bulk N <sub>2</sub> O production rate	4.7	4.7	6.9	7.1	12	13	10	9.3
N <sub>2</sub> O production rate from NO <sub>2</sub> <sup>-</sup> (Eq. 3)	5.7	6.9	6.8	5.8	9.4	8.1	9.9	8.7
N <sub>2</sub> O production from bulk NO <sub>2</sub> <sup>-</sup> through ND (Eq. 4)	4.9	6.2	6.2	4.6	6.6	5.1	7.3	6.5
Total N <sub>2</sub> O production through ND (Eq. 5)	6.7	7.6	7.4	7.4	13	13	13	11
Days of operation	<sup>15</sup> NH <sub>4</sub> <sup>+</sup> additions				<sup>15</sup> NH <sub>4</sub> <sup>+</sup> additions			
	106		107		106		107	
	Feed 2	Feed 3	Feed 2	Feed 3	Feed 2	Feed 3	Feed 2	Feed 3
	Feed 2	Feed 3	Feed 2	Feed 3	Feed 2	Feed 3	Feed 2	Feed 3
Bulk N <sub>2</sub> O production rate	6.1	5.0	5.5	5.3	13	14	11	13
N <sub>2</sub> O production from NH <sub>4</sub> <sup>+</sup> (Eq. 3)	2.1	3.6	1.9	3.1	5.2	6.7	4.9	6.4
N <sub>2</sub> O production via bulk NO <sub>2</sub> <sup>-</sup>	0.49	1.8	0.70	1.8	0.82	2.4	1.5	3.4
N <sub>2</sub> O production not via bulk NO <sub>2</sub> <sup>-</sup>	1.6	1.8	1.2	1.3	4.4	4.3	3.4	3.0



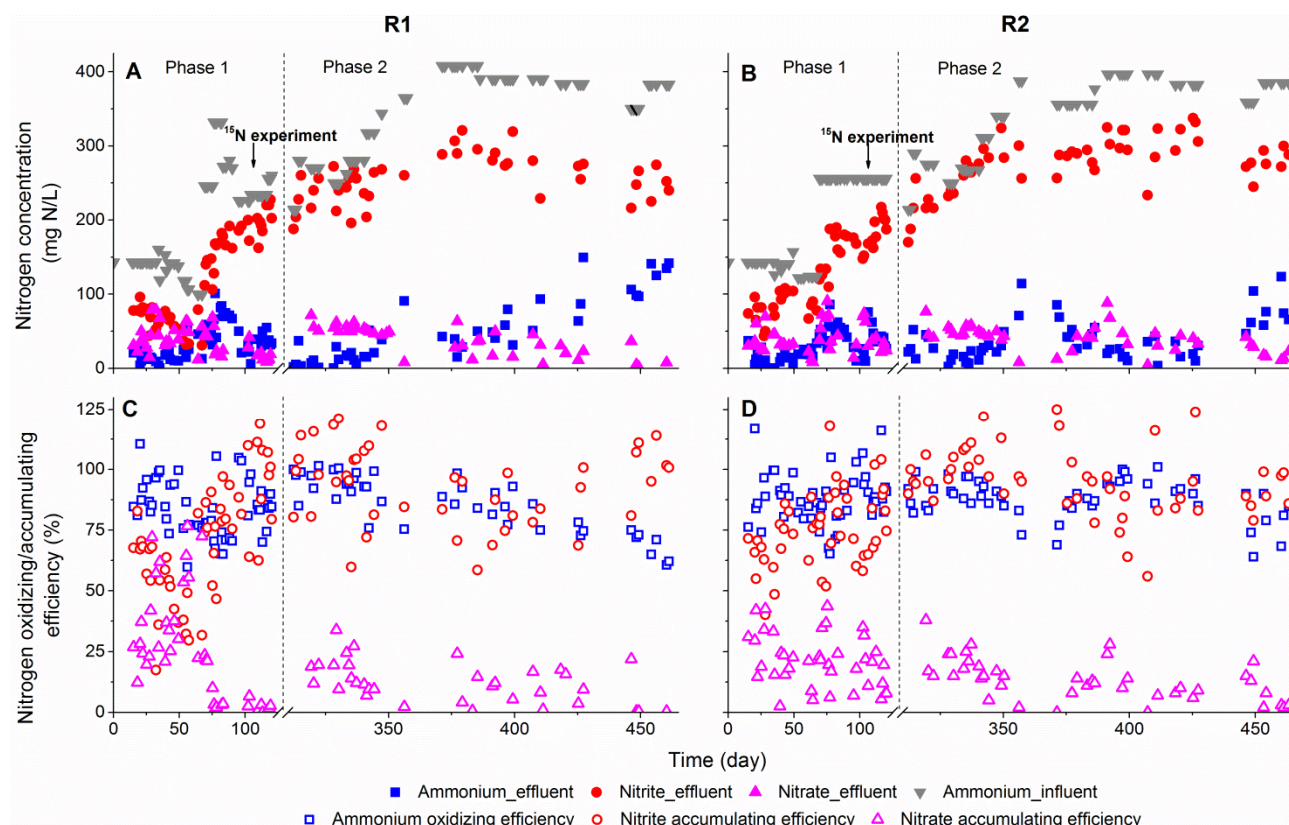


Fig. 1. Nitritation performance in R1 (A, C) and R2 (B, D) throughout the operational period. (A, B) Nitrogen concentrations (ammonium, nitrite and nitrate in effluent, ammonium in influent). (C, D) Nitrogen conversion efficiency (ammonium oxidizing efficiency (AOR/ALR), nitrite accumulation efficiency (NiAR/AOR), nitrate accumulation efficiency (NaAR/AOR)). The break at the X-axis represents a period of 170 days, when the reactors were stopped and biomass was stored at 4 °C.



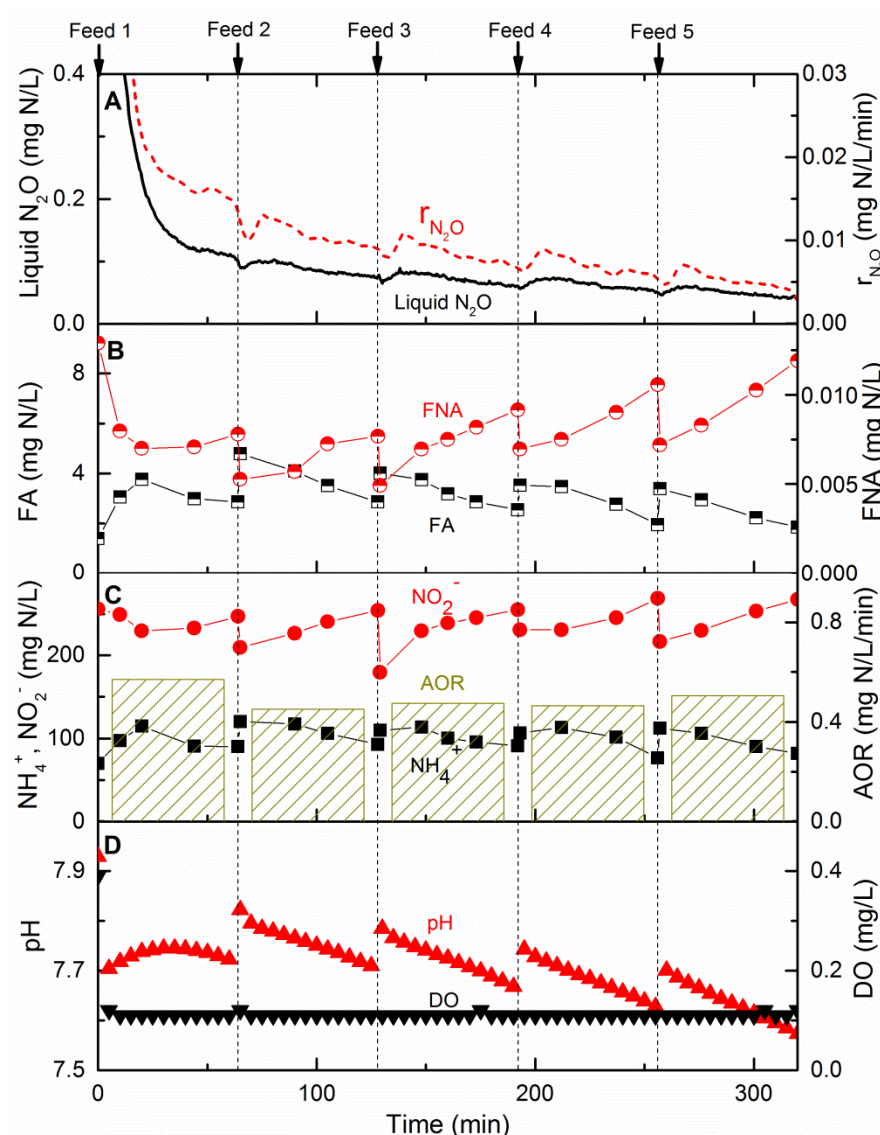


Fig. 2. In-cycle profiles of nitrogen species, pH, DO and  $N_2O$  in R1 (day 397). (A) Liquid  $N_2O$  concentrations and net  $N_2O$  production rates. (B, C) Bulk liquid nitrogen species ( $NO_2^-$  and  $NH_4^+$ ), calculated free nitrous acid (FNA), free ammonia (FA) and ammonium oxidizing rates (AORs). (D) pH and DO.

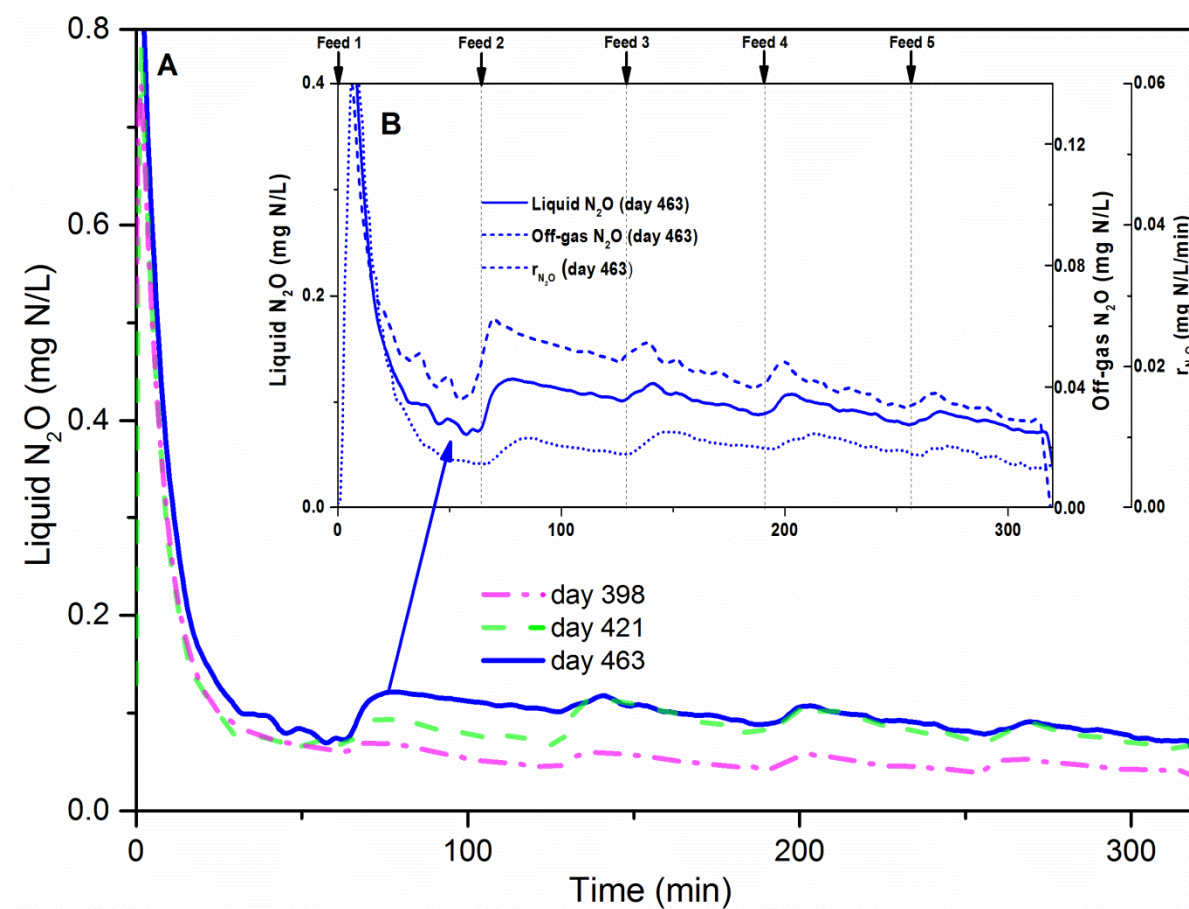


Fig. 3. (A) Profiles of liquid  $N_2O$  concentrations in one cycle in R2 on day 398, 421 and 463. (B) Profiles of liquid and off-gas  $N_2O$  concentrations and calculated net  $N_2O$  production rates in one cycle in R2 on day 463.

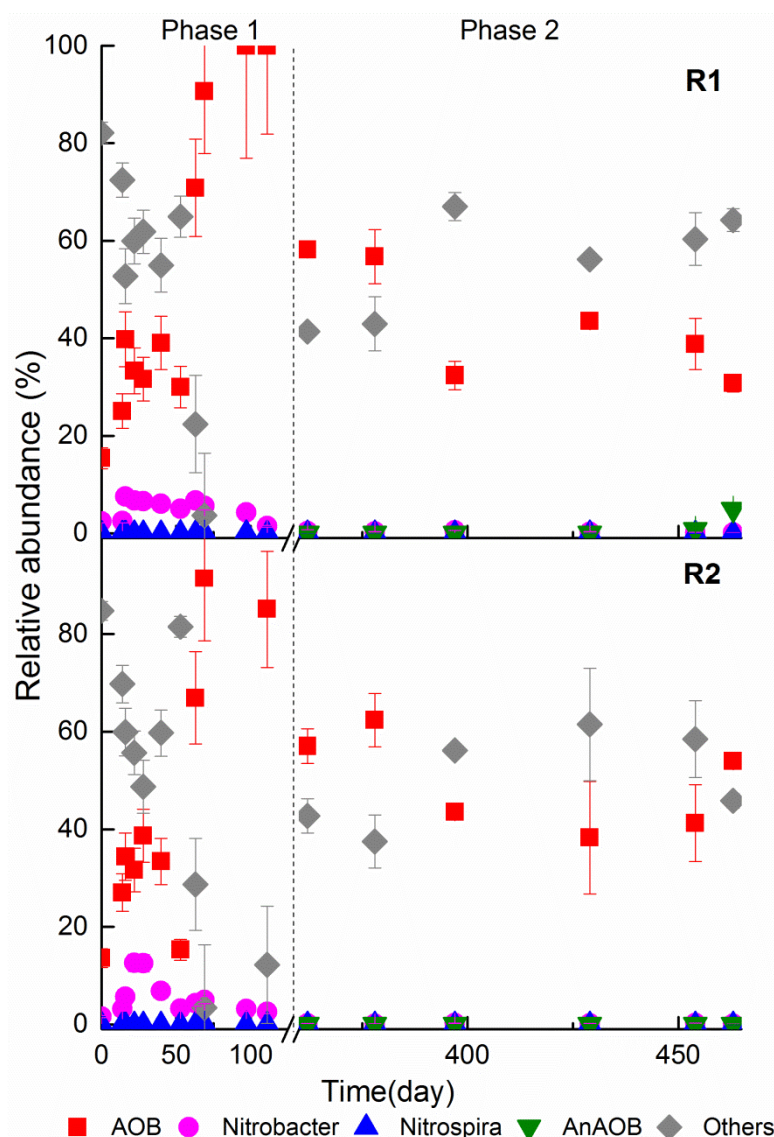


Fig. 4. Relative abundances of AOB, NOB, AnAOB and other bacteria in R1 and R2 over time based on qPCR of 16S rRNA genes. Error bars indicate standard deviations of duplicate measurements.

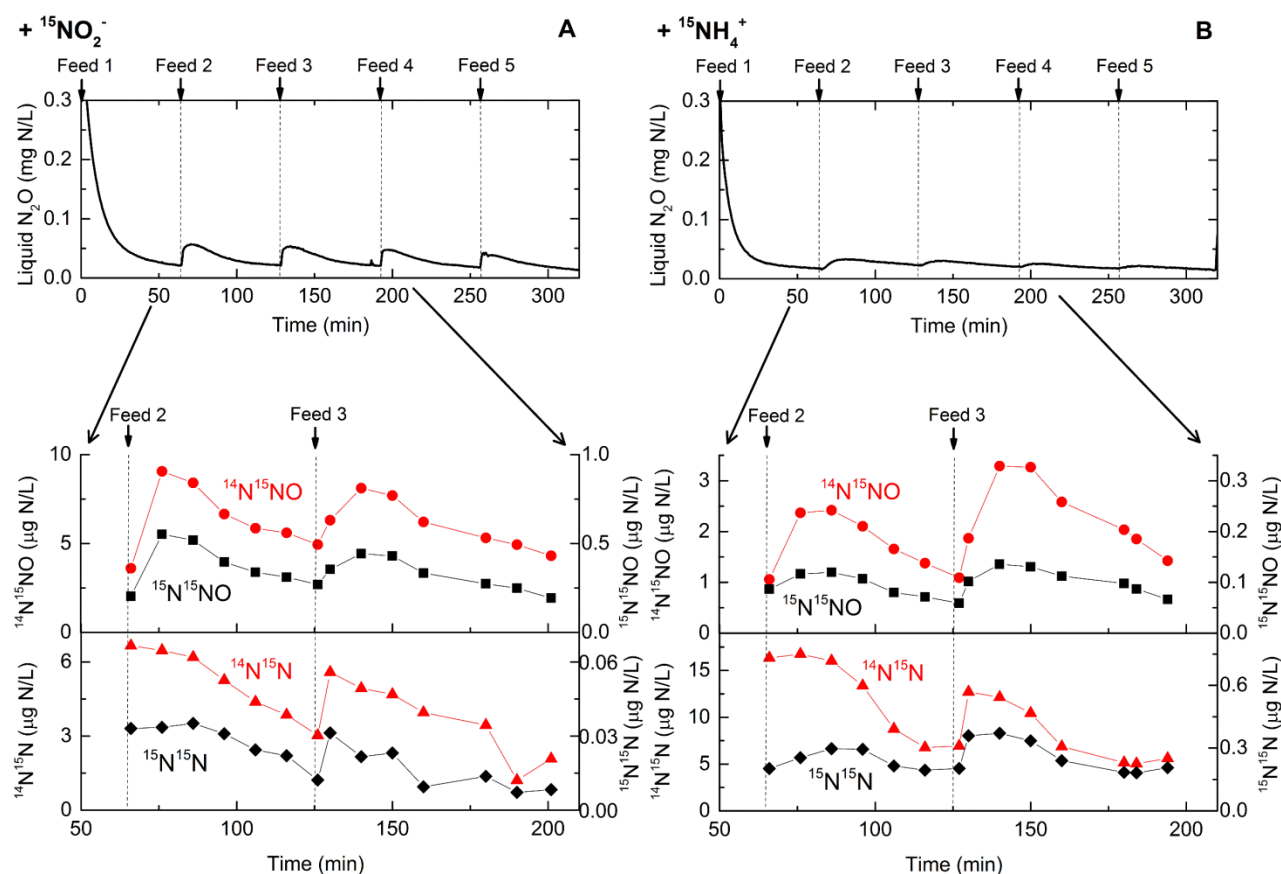


Fig. 5. Plots of bulk liquid  $\text{N}_2\text{O}$  concentrations versus time during the reaction phase of one cycle (upper panels) and isotopically labeled  $\text{N}_2\text{O}$  and  $\text{N}_2$  concentrations versus time for feed 2 and 3 (lower panels) in Reactor 1.  $^{15}\text{NO}_2^-$  spikes were performed at 111 days of operation (A) and  $^{15}\text{NH}_4^+$  spikes at 107 days of operation (B).

## Highlights

- Long-term high nitrification performance was achieved in intermittently-fed SBRs.
- Net  $\text{N}_2\text{O}$  production was, on average, 2.1% of the oxidized ammonium.
- Intermittent feeding appears an effective approach to mitigate  $\text{N}_2\text{O}$  emission.
- pH has a potential stimulatory effect on  $\text{N}_2\text{O}$  production.
- Nitrifier denitrification was the dominant source of  $\text{N}_2\text{O}$  production.