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Low $N_2O$ production ($\sim 2.1\%$)

Nitritation SBR

$NH_4^+$ ($\sim 0.8$ g N/L)
Air

$NO_2^-$: $\sim 300$ mg N/L
DO: $\sim 0.1$ mg O$_2$/L
Temperature: 20-26°C
pH: 7-8

$AOR/ALR: \sim 88\%$
$NiAR/AOR: \sim 93\%$

In-cycle dynamics

$N_2O$ production pathway

Hydroxylamine oxidation (AOB)
Contribution: $\sim 8\%$

Nitrifier denitrification (NOB)
Contribution: $\sim 100\%$
Low nitrous oxide production through nitrifier-denitrification in intermittent-feed high-rate nitritation reactors

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Abstract

Nitrous oxide (N₂O) production from autotrophic nitrogen conversion processes, especially nitritation systems, can be significant, requires understanding and calls for mitigation. In this study, the rates and pathways of N₂O production were quantified in two lab-scale sequencing batch reactors operated with intermittent feeding and demonstrating long-term and high-rate nitritation. The resulting reactor biomass was highly enriched in ammonia-oxidizing bacteria, and converted ~93 ± 14% of the oxidized ammonium to nitrite. The low DO set-point combined with intermittent feeding was sufficient to maintain high nitritation efficiency and high nitritation rates at 20-26 °C over a period of ~300 days. Even at the high nitritation efficiencies, net N₂O production was low (~2% of the oxidized ammonium). Net N₂O production rates transiently increased with a rise in pH after each feeding, suggesting a potential effect of pH on N₂O production. In situ application of ¹⁵N labeled substrates revealed nitrifier denitrification as the dominant pathway of N₂O production. Our study highlights operational conditions that minimize N₂O emission from two-stage autotrophic nitrogen removal systems.

Keywords: Nitrous oxide; Nitritation; Ammonia-oxidizing bacteria; Intermittent feeding; pH; Nitrifier denitrification
1. Introduction

Autotrophic nitrogen removal by combined partial nitritation (PN, aerobic ammonium (NH$_4^+$) oxidation to nitrite (NO$_2^-$)) and anammox (anaerobic NH$_4^+$ oxidation with NO$_2^-$ to dinitrogen gas (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). Nitritation can be achieved by manipulating operation parameters, such as low dissolved oxygen (DO) and high 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 NH$_4^+$ as well as high accumulation of NO$_2^-$ produced by AOB in two-stage systems may promote accumulation and emission of nitrous oxide (N$_2$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 N$_2$O in the atmosphere (~0.3% per year) is of great concern because it contributes to global warming (N$_2$O has a ca. 300 times higher global warming potential than CO$_2$) and the destruction of stratospheric ozone (IPCC, 2013; Strokal and Kroeze, 2014). Indeed, documented N$_2$O emissions of up to 17% of the 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 N$_2$O emissions might be explained by the different
responses of $N_2O$ production and consumption pathways to different operation strategies (e.g. feeding and aeration pattern) and parameters (e.g. $NH_4^+$, $NO_2^-$, 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 $N_2O$ produced by AOB: (a) the reduction of $NO_2^-$ to $N_2O$ via nitric oxide (NO), known as nitrifier denitrification (ND) (Ishii et al., 2014; Kim et al., 2010; Wrage et al., 2001) and (b) $N_2O$ as a side product during incomplete oxidation of hydroxylamine (NH$_2$OH) to $NO_2^-$ (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 $N_2O$ under very low C/N conditions (Domingo-Félez et al., 2017). During heterotrophic denitrification (HD), $N_2O$ is an obligate intermediate and is produced during incomplete denitrification. The exact biological pathways and environmental controls of $N_2O$ 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 $N_2O$ production is crucial to develop novel strategies or new designs to mitigate $N_2O$.

The principle goal of this study was to investigate $N_2O$ dynamics and determine $N_2O$ production pathways in two intermittently-fed lab-scale sequencing batch reactors (SBRs) with high nitritation performance. This was achieved by $N_2O$ online measurements and in situ applications of $^{15}N$ labeled $NH_4^+$ or $NO_2^-$ followed by monitoring of $^{15}N$ labeled and unlabeled products. In addition, the nitritation performance was assessed during the ~300 days of operation.
2. Materials and methods

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

2.1.1 Reactor description and operation

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

R1 and R2 were operated as duplicates for 121 days, stopped for 170 days, where the biomass was stored separately at 4 °C, and restarted for another 172 days. The operation period can be divided into two phases: phase 1 (day 0–121) and phase 2 (day 291–463). The NH$_4^+$ and oxygen loading were the two manipulative variables to sustain a low NOB/AOB activity. To recover biomass activity after storage and maintain high NO$_3^-$ accumulation, excess NH$_4^+$ and oxygen limitation were set by stepwise increasing the ammonium loading rate (ALR) and air flow rate from 0.29 to 0.79 g N/L/d and 0.2 to 0.55 L/min, respectively (Table S1).

A 6-h working cycle was applied over the entire experiment. One cycle consisted of 320 min reaction phase including five consecutive intervals of 1 minute feeding followed by a 63 minutes inter-feed period, 30 min settling phase, 5 min decanting phase and 5 min idle phase. The volumetric exchange ratio (VER) was 50%, resulting in a hydraulic retention time (HRT) of 12h. The sludge retention time (SRT) was controlled at 20 days by wasting sludge at the end of reaction 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 (NH$_4$HCO$_3$) was the only nitrogen source in the synthetic wastewater while NH$_4$HCO$_3$ and sodium bicarbonate (NaHCO$_3$) provided the inorganic carbon. The composition of trace chemicals (van de Graaf et al., 1996) was: 169.7 mg/L KH$_2$PO$_4$, 751.1 mg/L MgSO$_4$·7H$_2$O, 451.6 mg/L CaCl$_2$·2H$_2$O, 5 mg/L EDTA, 5 mg/L FeSO$_4$·7H$_2$O and trace element solution of 1mL/L. The trace element solution contained 0.43 mg/L ZnSO$_4$·7H$_2$O, 0.24mg/L CoCl$_2$·6H$_2$O, 0.99mg/L MnCl$_2$·4H$_2$O, 0.25mg/L CuSO$_4$·5H$_2$O, 0.22mg/L NaMoO$_4$·2H$_2$O, 0.19mg/L NiCl$_2$·6H$_2$O and 0.21mg/L NaSeO$_4$·10H$_2$O.

2.2. N$_2$O measurement

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

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

\[
\text{Instantaneous net } N_2 \text{O production rate, } r_{N_2O_i} = \frac{\Delta N_{2O_i}}{\Delta t} + k_L a_{N_2O_i} N_{2O_i}
\]

\[
\text{Daily averaged net } N_2 \text{O production rate, } R_{N_2O} = \sum (r_{N_2O_i} \Delta t) \times 4 \frac{\text{cycle}}{\text{day}}
\]

Where \( r_{N_2O_i} \) is the instantaneous net N$_2$O production rate at time i, \( \frac{\Delta N_{2O_i}}{\Delta t} \) is the differential term of liquid concentration at time i, and \( k_L a_{N_2O_i} N_{2O_i} \) is the stripping rate at time i, which equals the
emission rate. The N$_2$O volumetric mass transfer coefficient ($k_{L,a}$N$_2$O) was determined experimentally at different volume/flow rates scenarios (Domingo-Félez et al., 2014) (Table S2). The net N$_2$O produced per NH$_4^+$ oxidized ($\Delta$N$_2$O$/\Delta$NH$_4^+$, %) and the specific net N$_2$O production rate (N$_2$OR, mg N/g VSS/d) were calculated from the daily averaged net N$_2$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 °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 °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. $^{15}$N additions and analysis

A $^{15}$N experiment was designed to identify the microbial sources of N$_2$O production during operation of the nitritation SBRs (day 106 to 111). The $^{15}$N-labeled nitrogen compounds (>98% $^{15}$N; Sigma-Aldrich) were added together with the second feed during the same cycle on different days (Table S4).
The resulting $^{15}$N mole fractions of the nitrogen pools was 17-18% for $^{15}$NH$_4^+$ and 11-13% for $^{15}$NO$_2^-$, as determined from the isotopic $^{15}$N and total concentrations after additions. Reactor liquid (12 ml) was sampled every 10 minutes after tracer additions until the fourth feed of the cycle. For isotopic analysis of N$_2$O and N$_2$, 3-mL and 6-mL Exetainer vials, respectively, prefilled with 100 µL of 50% (w/v) ZnCl$_2$ to stop microbial activity, were filled completely and immediately screw-capped with butyl rubber septa. Previous experiments had shown that ZnCl$_2$ efficiently quenched N transformations in this biomass (data not shown). The rest of the sample was filtered (0.22 µm) and frozen immediately for later analyses of nutrients and isotopic composition of NH$_4^+$, NO$_2^-$ and nitrate (NO$_3^-$).

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

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

The total conversion of NH$_4^+$ and NO$_2^-$ to the gaseous products, irrespective of the pathway, was determined by division of the rate of $^{15}$N-labeled gas production ($^{15}$N-N$_2$O = $^{14}$N$^{15}$NO + 2 x
\[ ^{15}\text{N}^{15}\text{NO}_2; ^{15}\text{N}-\text{N}_2 = ^{14}\text{N}^{15}\text{N} + 2 \times ^{15}\text{N}^{15}\text{N} \] by the labeling fraction \( F \) of the substrate \( (F_A = [^{15}\text{NH}_4^+] x [\text{NH}_4^+])^{-1} \) and \( F_N = [^{15}\text{NO}_2^-] x [\text{NO}_2^-]^{-1} \), e.g.:

\[
\text{Rate}(\text{NH}_4^+ \rightarrow \text{N}_2\text{O}) = \text{Rate}(^{15}\text{NH}_4^+ \rightarrow ^{15}\text{N}-\text{N}_2\text{O}) \times F_A^{-1}
\]

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, \( R_{46} \):

\[
\text{Denitrification}_{\text{N}_2\text{O, bulk}} = \text{Rate}(^{15}\text{N}^{15}\text{NO}) \times F_A^{-2}
\]

Eq. 4

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

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 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 ± 12% (average ± standard deviation) and 90 ± 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 ± 11% (R1) and 88 ± 8% (R2) during phase 2, except for a ~19% decline in the final days of the reactors (Fig. 1). There was high NO$_2^-$ accumulation at the end of phase 1 and throughout phase 2, maintaining average nitrite accumulation efficiency (NiAR/AOR) of 92 ± 17% and 93 ± 14% in R1 and R2, respectively. 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 ± 9% and 14 ± 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). NH$_4^+$ concentration increased at each feeding while 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 NH$_4^+$ and NO$_2^-$ concentrations at different pH (Fig. 2). During the inter-feed periods, AOR was relatively constant with an average value of 0.49 ± 0.04 mg N/L/min (Fig. 2).
3.2. N2O production

3.2.1. Overall N2O production

During the end of phase 1, the average net N2O produced per NH4+ oxidized (ΔN2O/ΔNH4+) in R1 and R2 was 0.6 ± 0.2% and 0.8 ± 0.3% respectively; while it was 2.0 ± 1.0% and 2.1 ± 0.7% during phase 2 (Table 1). The liquid N2O concentrations as well as ΔN2O/ΔNH4+ increased during phase 2 (Fig. 3 and Table 1) in two reactors. The differences in the specific net N2O production rate (N2OR) between the two reactors were likely due to the differences in MLVSS concentrations. Furthermore, each inter-feed period did not contribute equally to the total N2O production of a cycle. N2O gas escaping after feed 1, ranging between 23 to 41% in both reactors during two phases, was considerable higher compared to the emissions following the other feeds (Table 1).

3.2.2. N2O dynamics during intermittent feedings

The patterns of liquid N2O concentration profiles over the reaction phase were very reproducible during the whole period for both reactors (Fig. 2 and 3). In-cycle N2O profiles had the following pattern: after the settling phase from the previous cycle, an initial maximum in N2O concentration occurred when the first feed initiated, after which the concentration declined until the next feeding; another four smaller peaks in N2O concentration were observed in the subsequent feedings. N2O concentration reached minimum values in the inter-feed periods but with concentrations higher than the detection limit of the sensor. Thus, based on liquid N2O concentrations there was always a positive net production of N2O in both reactors, with rates (rN2O) increasing after each feeding and decreasing during inter-feed periods (Fig. 3). Off-gas N2O profiles followed the same trends during 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 ± 0.01% and 1.94 ± 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. N₂O production pathway

In incubations with ¹⁵N-labeled substrates, the label was transferred to both N₂O and N₂ within 2–3 minutes of addition, irrespective of whether ¹⁵N was added as ¹⁵NO₂⁻ or ¹⁵NH₄⁺ (Fig. 5). The dynamics of ¹⁵N-N₂O mirrored those of bulk N₂O, and N₂O was the dominating product in ¹⁵NO₂⁻ incubations accounting for 57–58% of the labeled N₂O + N₂ in both feedings, while it only accounted for 17–23% with ¹⁵NH₄⁺. The production of N₂ was also highly dynamic, showing an even steeper rise after feeding than for N₂O. The production of ¹⁵N-N₂O from ¹⁵NO₂⁻ corresponded to a total conversion of NO₂⁻ to N₂O of 5.7–9.9 µg N/g VSS/min, which was not significantly different from the total net N₂O production (Table 2), implying that NO₂⁻ was the main source of N₂O in the incubations.
There was no detectable production of $^{15}$NH$_4^+$ in the incubations with $^{15}$NO$_2^-$ (data not shown), which implies that all $^{15}$N-N$_2$O and $^{15}$N-N$_2$ in these incubations was formed exclusively through reductive pathways, i.e., not via dissimilatory nitrate/nitrite reduction to ammonium (DNRA) and subsequent oxidation of NH$_4^+$. Indeed, the relative production of $^{14}$N$^{15}$NO and $^{15}$N$^{15}$NO from $^{15}$NO$_2^-$ (Fig. 5) was close to that expected from denitrification with random isotope pairing (either heterotrophic or nitrifier denitrification). Thus, the production of N$_2$O through denitrification (calculated by Eq. 4) corresponded to 80% and 77% of total net N$_2$O production from NO$_2^-$ (the NO$_2^-$-to-N$_2$O conversion rates calculated by Eq. 3) on average for feed 2 and 3, respectively (Table 2). The remaining 20–23% of NO$_2^-$-derived N$_2$O corresponds to a surplus of $^{14}$N$^{15}$NO relative to the prediction from random isotope pairing from the bulk NO$_2^-$ pool, and therefore indicates pairing of N from this pool with N from a second source of unlabeled N. The surplus of $^{14}$N$^{15}$NO may arise if the labeling fraction of NO$_2^-$, $F_N$, in the immediate vicinity of the nitrite reductase enzymes is lower than the bulk $F_N$ value used for the calculations (Eq. 4), e.g., because of dilution with unlabeled NO$_2^-$ from nitritation maintained by diffusional gradients either intracellularly or within microaggregates. This is reflected in the N$_2$O production calculated by Eq. 5, which derives $F_N$ at the site of NO$_2^- $ reduction from the relative production of $^{14}$N$^{15}$NO and $^{15}$N$^{15}$NO. Thus, assuming that all conversion of NO$_2^-$ to N$_2$O occurred through a denitrification pathway, total N$_2$O production was calculated based on the relative production of $^{14}$N$^{15}$NO and $^{15}$N$^{15}$NO (Nielsen, 1992), yielding rates that exceeded the NO$_2^-$-to-N$_2$O conversion rates by 24–31% (Table 2).

The production of N$_2$O from NH$_4^+$, determined in incubations with $^{15}$NH$_4^+$ showed very similar temporal dynamics as N$_2$O production from NO$_2^-$ (Fig. 5). After the 2$^\text{nd}$ feed, the production from NH$_4^+$ corresponded, on average, to 42% of the production from NO$_2^-$ (Table 2). This fraction increased to 58% after the 3$^\text{rd}$ feed, which is explained by the accumulation of $^{15}$NO$_2^-$ and the
resulting increasing contribution of $^{15}\text{N}_2\text{O}$ from denitrification, as also reflected in the higher
concentrations of $^{15}\text{N}$-$\text{N}_2\text{O}$ reached after the 3$^{rd}$ feed relative to the 2$^{nd}$ (Fig. 5). The amount of $^{15}\text{N}$-$\text{N}_2\text{O}$ produced from $^{15}\text{NH}_4^+$ via nitritation, mixing of the formed $^{15}\text{NO}_2^-$ with the bulk $\text{NO}_2^-$ pool, and subsequent denitrification, was estimated for each reactor based on the rates of $\text{N}_2\text{O}$ production determined in the $^{15}\text{NO}_2^-$ incubations in the same reactor and the $F_N$ values (data not shown) from the $^{15}\text{NH}_4^+$ incubations (Eq. 3). These calculations indicated that 25% and 49% of $\text{N}_2\text{O}$ production determined with $^{15}\text{NH}_4^+$ occurred via bulk $\text{NO}_2^-$ after feed 2 and 3, respectively. The $^{15}\text{NH}_4^+$-based $\text{N}_2\text{O}$ production that was not attributable to this route averaged 2.6 µg N/g VSS/min after both feedings, corresponding to 25% of the combined $\text{N}_2\text{O}$ production detected with $^{15}\text{NO}_2^-$ and $^{15}\text{NH}_4^+$ (Table 2), and the sum of this rate and the production of $\text{N}_2\text{O}$ from $\text{NO}_2^-$ matched the estimated $\text{N}_2\text{O}$ production from denitrification closely (7.7 vs. 7.3 µg N/g VSS/min and 12.1 vs. 12.5 µg N/g VSS/min for R1 and R2, respectively). The contribution of the hydroxylamine oxidation pathway to $\text{N}_2\text{O}$ production did not increase immediately after the addition of $\text{NH}_4^+$, as the production ratio 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 $^{15}\text{NO}_2^-$ and $^{15}\text{NH}_4^+$ in combination support a denitrification pathway as the main and possibly sole source of $\text{N}_2\text{O}$ in this SBR system.

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 $^{15}\text{N}^{15}\text{N}$) differed markedly from that of $\text{N}_2\text{O}$, with $^{15}\text{N}^{15}\text{N}$ accounting for ≤0.5% of the labeled $\text{N}_2$ compared a contribution of ~5% from $^{15}\text{N}^{15}\text{NO}$ to labeled $\text{N}_2\text{O}$ (Fig. 5). This pointed towards another $\text{N}_2$ source than denitrification. The total $\text{N}_2$ production rate from $\text{NO}_2^-$ (Eq. 3) was 4.4 ± 0.9 and 6.4 ± 0.8 µg N/g VSS/min for R1 and R2, respectively. Substantially higher $\text{N}_2$ production rates were obtained for the $^{15}\text{NH}_4^+$ than with $^{15}\text{NO}_2^-$: 10.2 ± 3.5 and 21 ± 0.8 µg N/g VSS/min for R1 and 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 N$_2$O production rates from $^{15}$NH$_4^+$) only reduced these rates slightly to 9.4 ± 3.5 and 19.7 ± 1.5 µg N/g VSS/min, respectively.

4. Discussion

4.1. Mechanisms to achieve high and stable nitritation performance

Two SBRs were operated for approximately 300 days with high NO$_2^-$ accumulation and no significant production of 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; Hellinga 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 nitritation 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 in 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 nitritation SBRs was ≤ 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 nitritation efficiencies of 92 ± 17% (R1) and 93 ± 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 I to phase II and 2, where the FA concentration was 3.1 ± 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 ± 0.002 mg 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 NO$_2^-$ concentrations, reaching the maximum (0.8 g N/L/d) at 323 mg N/L (Fig. S3). Hence, no evidence of NO$_2^-$ inhibition was obtained. The observed increase in AOR with increasing NO$_2^-$ concentration agrees with a previous study with mixed microbial communities, showing high ammonium oxidation to NO$_2^-$ (150–160 mg NO$_2^-$N/h/g VSS) at 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 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 nitritation 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 (Hellinga et al., 1998; Yang et al., 2007).

It is often difficult to maintain stable nitritation over the long-term period even in successfully established nitritation 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 nitritation 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 nitritation efficiency and high AOB abundance accompanied by low NO\textsubscript{3}\textsuperscript{-} accumulation and low NOB abundance. We speculate that using intermittent feeding together with low DO set-points successfully enabled long-term high nitritation performance in the two SBR reactors. While long-term high-rate nitritation 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 N\textsubscript{2}O production

The net N\textsubscript{2}O produced per NH\textsubscript{4}\textsuperscript{+} oxidized (\(\Delta\text{N}_2\text{O}/\Delta\text{NH}_4\textsuperscript{+}\)) and the specific net N\textsubscript{2}O production rate (N\textsubscript{2}OR) of the two nitritation SBRs were compared to previously reported values together with the identification of reactor types, operation strategies, performance and AOB presence (Table S5). The
average net N₂O production in phase 2 increased to 2.0 ± 1.0% and 2.1 ± 0.7% of the NH₄⁺ oxidized in R1 and R2, respectively, while the average specific net N₂O production rate was 8.4 ± 3.5 and 10.2 ± 3.5 mg N/g VSS/d in R1 and R2, respectively (Table 1 and S5). The net N₂O production in both reactors corresponded well with the genetic potential for N₂O production, as the ratio of nirS plus nirK over nosZ-targeted genes was far above 1 (Fig. S2). The higher N₂O production in phase 2 compared to phase 1 is puzzling as it cannot be explained by higher AOR (Table 1). We speculate that the long-term operation under elevated NO₂⁻ may have selected for new microbes with higher expression of the nitrifier-denitrification pathway or the cultured microbes adapted to higher NO₂⁻, resulting in higher expression of the pathway, and with that higher N₂O production. This theory, however, calls for deeper analysis of the microbial community than obtained with qPCR.

The N₂O production factors of ~2% are in the low range of previous reports for both lab-scale and full-scale PN systems, ranging between 1–17% (Table S5). Our study is the first study to measure low N₂O emissions at very high nitritation efficiencies. Low DO (0.35 mg/L) and high NO₂⁻ conditions (10 – 50 mg N/L) boost N₂O production (Peng et al., 2015, 2014). Measured N₂O emissions are lower compared to other lab-scale PN SBRs operated under low DO and high NO₂⁻ conditions (N₂O emissions of 17%) (Gao et al., 2016; Lv et al., 2016). With the intermittent feeding strategy at low DO, we force relatively low ammonia oxidation rates (Fig. 2, Table 1), which has previously been shown to decrease N₂O emissions from autotrophic nitrogen removal systems (Domingo-Félez et al., 2014; Law et al., 2011). Law and coworkers (2011) found that a decline in feeding rate from 1 L/2.5 min to 1 L/25 min during the reaction phase lead to a substantial reduction in N₂O production without affecting the nitritation performance. Instead of reducing the feeding rate, our nitritation reactors were operated with five intermittent feedings within a cycle. This step-feed strategy has previously been suggested as an effective optimization approach to reduce N₂O
emissions from SBRs (Mavrovas, 2014; Yang et al., 2009, 2013). Therefore, we postulate that intermittent feeding is the cause for the low N\textsubscript{2}O emission from high-performance nitritation system.

4.3. Potential pH effect on in-cycle N\textsubscript{2}O production dynamics

Distinctive N\textsubscript{2}O production profiles were observed within the representative cycles (Fig. 2 and 3). The maximum net N\textsubscript{2}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 N\textsubscript{2}O production occurred during the settling phase in their lab-scale PN SBR, while 70% of the quantified N\textsubscript{2}O emission was attributed to the anoxic N\textsubscript{2}O formation in a full-scale PN SHARON reactor (Mampaey et al., 2016). Tentative liquid N\textsubscript{2}O measurements indicated that N\textsubscript{2}O accumulated during the non-aerated settling phase (data not shown). Denitrification might be responsible for this N\textsubscript{2}O accumulation during the settling phase, which is then released at the onset of aeration (Itokawa et al., 2001). The genetic potential for N\textsubscript{2}O production by denitrifiers was present through the high relative abundance of nir\textsubscript{S} (Fig. S2).

A potential effect of pH on N\textsubscript{2}O production during the reaction phase was indicated by the transiently increase in net N\textsubscript{2}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 NH\textsubscript{4}\textsuperscript{+} and FA increased transiently after each feeding, FA was always in excess compared to the K\textsubscript{m} value of 0.0075 mg/L for AOB, and therefore AOR remained unaffected (Fig. 2) (Hiatta and Grady, 2008). Thus, pH appears the only potential variable affecting in-cycle N\textsubscript{2}O dynamics. Only few studies have been able to isolate the effect of pH on N\textsubscript{2}O production from the variations in FA and FNA, and the reported effect of pH on N\textsubscript{2}O production differ. In contrast to our results, Law and coworkers (2011) obtained highest N\textsubscript{2}OR 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 N\textsubscript{2}O
production. Oppositely, Rathnayake et al. (2015) observed highest N$_2$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$_2$O production is direct or indirect.

4.4. N$_2$O production pathway

The experiments with $^{15}$N labeled substrates point to nitrifier denitrification as the dominant source of N$_2$O in the SBR nitritation systems. A denitrification-type process rather than a direct production of N$_2$O from ammonium oxidation via hydroxylamine was demonstrated by more than 3 times higher rates of N$_2$O production from NO$_2^-$ than from NH$_4^+$, when $^{15}$NH$_4^+$-derived rates were corrected for accumulation of $^{15}$NO$_2^-$ (Table 2). Moreover, isotope pairing calculations showed that NO$_2^-$ during its reduction to N$_2$O was mixed with nitrogen from an unlabeled source. In the nitritation-dominated system, NH$_4^+$ is the most obvious candidate, and indeed, the production rate of N$_2$O from NH$_4^+$ that did not go via bulk NO$_2^-$ closely matched the difference between total and bulk NO$_2^-$-dependent denitrification. We therefore hypothesize that essentially all N$_2$O was produced through nitrifier-denitrification with part of the newly-formed NO$_2^-$ shunted directly to reduction either intracellularly or within cellular aggregates before it could mix completely with NO$_2^-$ in the bulk liquid. Alternatively, the combination of N from NH$_4^+$ and NO$_2^-$ could occur at the level of NO if this compound is a free intermediate during ammonium oxidation (Stein, 2011).

The $^{15}$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$_2$O production by each NH$_4^+$ feeding points to NH$_4^+$ dependence rather than heterotrophy. Secondly, there is no convincing evidence for heterotrophic N$_2$ production: (a) The rate of N$_2$O production exceeds the rate of N$_2$ production from NO$_2^-$ whereas N$_2$O is generally a minor byproduct of heterotrophic denitrification (Betlach and Tiedje, 1981); (b) the dynamics of N$_2$ and N$_2$O production are out of phase with the peak in N$_2$ preceding that of N$_2$O, where the opposite
would be expected during heterotrophic denitrification (e.g., Jensen et al., 2009), and (c) the very low ratio of $^{15}$N$^{15}$N to $^{14}$N$^{15}$N, differing markedly from the $^{15}$N$^{15}$NO:$^{14}$N$^{15}$NO ratio in N$_2$O, suggests that N$_2$ production from NO$_2^-$ is mainly due to another process, possibly anammox.

The complete dominance of nitrifier-denitrification as source of N$_2$O is in general agreement with the understanding that this process is favored by low DO and high 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 N$_2$ production observed in the $^{15}$NH$_4^+$ incubations, relative to both N$_2$O production in the same experiment and to N$_2$ production with $^{15}$NO$_2^-$, suggests an involvement of anammox. Only a small part of the N$_2$ produced with $^{15}$NH$_4^+$ could be explained with oxidation to NO$_2^-$ and subsequent reduction, which means that NH$_4^+$ appeared to be converted directly from NH$_4^+$ to N$_2$. As 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 NH$_4^+$ and NO$_2^-$, similar rates of N$_2$ production should, however, be obtained with additions of $^{15}$NH$_4^+$ and $^{15}$NO$_2^-$ (van de Graaf et al., 1995), whereas we observed ~2.5-fold higher production from $^{15}$NH$_4^+$ than from $^{15}$NO$_2^-$. Potential explanations for the imbalance in rates are either a close coupling of nitritation 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 nitritation SBRs were operated to investigate N$_2$O dynamics and identify N$_2$O production pathways.
• High nitritation performance with ~93 ± 14% of the oxidized NH$_4^+$ converted to NO$_2^-$ was achieved in intermittently-fed SBRs at 20-26°C for ~300 days.

• The averaged net N$_2$O production factor of 2.1 ± 0.7% is in the low range: Operation with intermittent feeding may be an effective approach to minimize N$_2$O emissions from nitritation systems.

• Increased net N$_2$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 N$_2$O production.

• Nitrifier denitrification was the dominant source of N$_2$O.

This study has demonstrated operational conditions (low dissolved oxygen and intermittent feeding) that achieve high-rate and long-term nitritation under normal temperature, which could enlarge the applicability of the nitritation process in WWTPs. The relatively low N$_2$O production at high nitritation efficiencies reduces the growing concern of N$_2$O production from autotrophic nitrogen processes in WWTPs. The identification of nitrifier denitrification as the main pathway of N$_2$O emissions will open up for more focused strategies to lower the N$_2$O footprint even more in nitritation systems.

Acknowledgements

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

Water Res. 102, 147–157.


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Table 1. Overview of AOR, N\textsubscript{2}O\textsubscript{R} and \(\Delta\text{N}_{2}\text{O}/\Delta\text{NH}_4^{+}\) in R1 and R2 during phase 1 and 2. The net N\textsubscript{2}O produced during each feed is stated as the percentage of total net N\textsubscript{2}O production during the entire cycle.

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th></th>
<th>R2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phase 1 (Day 106–112)</td>
<td>Phase 2 (Day 395–451)</td>
<td>Phase 1 (Day 106–112)</td>
<td>Phase 2 (Day 397–463)</td>
</tr>
<tr>
<td>AOR (g N/L/d)</td>
<td>0.5 ± 0.05</td>
<td>0.6 ± 0.05</td>
<td>0.5 ± 0.02</td>
<td>0.76 ± 0.06</td>
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<tr>
<td>AOR (g N/g VSS/d)</td>
<td>1.04 ± 0.11</td>
<td>0.46 ± 0.09</td>
<td>1.78 ± 0.08</td>
<td>0.5 ± 0.02</td>
</tr>
<tr>
<td>N\textsubscript{2}OR (mg N/g VSS/d)</td>
<td>5.9 ± 1.8</td>
<td>8.4 ± 3.5</td>
<td>16.0 ± 5.9</td>
<td>10.2 ± 3.5</td>
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<tr>
<td>(\Delta\text{N}_{2}\text{O}/\Delta\text{NH}_4^{+}) (%)</td>
<td>0.6 ± 0.2</td>
<td>2.0 ± 1.0</td>
<td>0.8 ± 0.3</td>
<td>2.1 ± 0.7</td>
</tr>
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<td>Feed 1 (%)</td>
<td>23 ± 5</td>
<td>41 ± 9</td>
<td>30 ± 5</td>
<td>27 ± 5</td>
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<td>Feed 2 (%)</td>
<td>22 ± 1</td>
<td>14 ± 2</td>
<td>21 ± 2</td>
<td>17 ± 2</td>
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<td>Feed 3 (%)</td>
<td>19 ± 1</td>
<td>15 ± 2</td>
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<td>Feed 4 (%)</td>
<td>17 ± 2</td>
<td>16 ± 2</td>
<td>16 ± 2</td>
<td>19 ± 1</td>
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<td>Feed 5 (%)</td>
<td>18 ± 3</td>
<td>15 ± 4</td>
<td>15 ± 2</td>
<td>21 ± 5</td>
</tr>
<tr>
<td># cycles</td>
<td>n=22</td>
<td>n=23</td>
<td>n=22</td>
<td>n=20</td>
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</table>
Table 2. Summary of net N\textsubscript{2}O production rates during the $^{15}$N experiment (µg N/g VSS/min). Bulk N\textsubscript{2}O production was based on liquid N\textsubscript{2}O concentrations, measured with microsensors, while N\textsubscript{2}O source partitioning is based on isotope additions.

<table>
<thead>
<tr>
<th>Days of operation</th>
<th>R1 $^{15}$NO\textsubscript{2} additions</th>
<th>R2 $^{15}$NO\textsubscript{2} additions</th>
<th>R1 $^{15}$NH\textsubscript{4}\textsuperscript{+} additions</th>
<th>R2 $^{15}$NH\textsubscript{4}\textsuperscript{+} additions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>110 Feed 2 Feed 3 Feed 2 Feed 3</td>
<td>110 Feed 2 Feed 3 Feed 2 Feed 3</td>
<td>106 Feed 2 Feed 3 Feed 2 Feed 3</td>
<td>106 Feed 2 Feed 3 Feed 2 Feed 3</td>
</tr>
<tr>
<td>Bulk N\textsubscript{2}O production rate</td>
<td>4.7 4.7 6.9 7.1</td>
<td>12 13 10 9.3</td>
<td>6.1 5.0 5.5 5.3</td>
<td>13 14 11 13</td>
</tr>
<tr>
<td>N\textsubscript{2}O production from NO\textsubscript{2}\textsuperscript{-} (Eq. 3)</td>
<td>5.7 6.9 6.8 5.8</td>
<td>9.4 8.1 9.9 8.7</td>
<td>2.1 3.6 1.9 3.1</td>
<td>5.2 6.7 4.9 6.4</td>
</tr>
<tr>
<td>N\textsubscript{2}O production from bulk NO\textsubscript{2}\textsuperscript{-} through ND (Eq. 4)</td>
<td>4.9 6.2 6.2 4.6</td>
<td>6.6 5.1 7.3 6.5</td>
<td>0.49 1.8 0.70 1.8</td>
<td>0.82 2.4 1.5 3.4</td>
</tr>
<tr>
<td>Total N\textsubscript{2}O production through ND (Eq. 5)</td>
<td>6.7 7.6 7.4 7.4</td>
<td>13 13 13 11</td>
<td>1.6 1.8 1.2 1.3</td>
<td>4.4 4.3 3.4 3.0</td>
</tr>
</tbody>
</table>

*Note: R1 and R2 are two different treatment reactors.*
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 N₂O in R1 (day 397). (A) Liquid N₂O concentrations and net N₂O production rates. (B, C) Bulk liquid nitrogen species (NO₂⁻ and NH₄⁺), calculated free nitrous acid (FNA), free ammonia (FA) and ammonium oxidizing rates (AORs). (D) pH and DO.
Fig. 3. (A) Profiles of liquid $\text{N}_2\text{O}$ concentrations in one cycle in R2 on day 398, 421 and 463. (B) Profiles of liquid and off-gas $\text{N}_2\text{O}$ concentrations and calculated net $\text{N}_2\text{O}$ 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 N$_2$O concentrations versus time during the reaction phase of one cycle (upper panels) and isotopically labeled N$_2$O and N$_2$ concentrations versus time for feed 2 and 3 (lower panels) in Reactor 1. $^{15}$NO$_2^-$ spikes were performed at 111 days of operation (A) and $^{15}$NH$_4^+$ spikes at 107 days of operation (B).
Highlights

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