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**The effect of pH on N₂O production in intermittently-fed nitritation
reactors**

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Abstract

The effect of pH on nitrous oxide (N₂O) production rates was quantified in an intermittently-fed lab-scale sequencing batch reactor performing high-rate nitrification. N₂O and other nitrogen (N) species (e.g. ammonium (NH₄⁺), nitrite, hydroxylamine and nitric oxide) were monitored to identify in-cycle dynamics and determine N conversion rates at controlled pH set-points (6.5, 7, 7.5, 8 and 8.5). Operational conditions and microbial compositions remained similar during long-term reactor-scale pH campaigns. The specific ammonium removal rates and nitrite accumulation rates varied little with varying pH levels ($p > 0.05$). The specific net N₂O production rates and net N₂O yield of NH₄⁺ removed ($\Delta N_2O / \Delta NH_4^+$) increased up to seven-fold from pH 6.5 to 8, and decreased slightly with further pH increase to 8.5 ($p < 0.05$). Best-fit model simulations predicted nitrifier denitrification as the dominant N₂O production pathway ($\geq 87\%$ of total net N₂O production) at all examined pH. Our study highlights the effect of pH on biologically mediated N₂O emissions in nitrogen removal systems and its importance in the design of N₂O mitigation strategies.

Keywords: Nitrous oxide; pH effect; Ammonia oxidizing bacteria; Nitrification; Nitrifier denitrification

1. Introduction

Nitrous oxide (N_2O) is emitted during biological nitrogen removal (BNR) in wastewater treatment plants (WWTPs). This has raised great concerns due to the large radioactive forcing properties (ca. 300 times higher global warming potential than carbon dioxide for a 100-year timescale) and stratospheric ozone depletion potential of N_2O (IPCC, 2013; Ravishankara et al., 2009). Nitrous oxide emissions in lab-scale and full-scale BNR systems were reported to account for up to 17% of the ammonium (NH_4^+) removed (Desloover et al., 2011; Gao et al., 2017). Consequently, efficient operational strategies are required in order to minimize N_2O emissions from BNR systems.

Ammonia oxidizing bacteria (AOB) are commonly identified as major contributors to the N_2O production in BNR systems, especially in nitrification reactors (Kampschreur et al., 2008a; Law et al., 2012). AOB are chemolithoautotrophs that conduct the oxidation of ammonia (NH_3) via hydroxylamine (NH_2OH) to nitrite (NO_2^-) catalyzed by ammonia monooxygenase (AMO) and hydroxylamine dehydrogenase (HAO), respectively. More recently, *in vitro* experiments have revealed that HAO oxidizes NH_2OH to nitric oxide (NO) and then NO reacts with O_2 to form NO_2^- under aerobic conditions (Caranto and Lancaster, 2017). N_2O is produced by AOB via one or more of the three pathways:

- Nitrifier nitrification (NN) (also called NH_2OH oxidation): N_2O is produced during incomplete oxidation of NH_2OH to NO_2^- by HAO, through intermediates of NO/HNO that can be further chemically or biologically converted to N_2O (Caranto and Lancaster, 2017; Law et al., 2012). A recent study revealed that the cytochrome (cyt) P460 in *Nitrosomona europaea* also contributes to NO and N_2O emissions by interacting with ferric nitric oxide complex (Caranto et al., 2016).
- Nitrifier denitrification (ND): This pathway involves the reduction of NO_2^- to N_2O catalyzed by nitrite reductase (NIR) and nitric oxide reductase (NOR) (Wrage et al., 2001).

- Abiotic reactions: Several chemical reactions involving the nitrogen intermediates (e.g. NH_2OH) during nitrification process are known to produce N_2O , yet the contribution of abiotic N_2O emissions was not highlighted until recently (Schreiber et al., 2012; Soler-Jofra et al., 2018, 2016; Su et al., 2019; Terada et al., 2017).

In addition, N_2O is accumulated during heterotrophic denitrification (HD) under limited organic carbon or in the presence of O_2 (Chung and Chung, 2000; Wunderlin et al., 2012). The contribution of HD pathway to total net N_2O production is often considered negligible in nitrification systems (Ishii et al., 2014; Wunderlin et al., 2012). Furthermore, heterotrophic denitrifying bacteria have the genetic potential (i.e. nitrous oxide reductase (NOS) encoded by *nosZ* clade I & II) to reduce N_2O to N_2 and with that act as a N_2O sink.

In order to prevent N_2O emissions from nitrification reactors, optimal operational conditions have been explored - by investigating the effect of factors like NH_4^+ , NO_2^- , DO and pH levels - that minimize net N_2O production by AOB and denitrifiers (Law et al., 2011, 2013; Schneider et al., 2014). Among these parameters, pH can have a significant effect on the net N_2O production by AOB and denitrifiers for the following reasons. First, pH would influence the dissociation equilibriums of NH_4^+ versus NH_3 (free ammonia, FA) and NO_2^- versus HNO_2 (free nitrous acid, FNA). An increasing pH shifts NH_4^+ to FA, which is the true substrate for AOB. High concentrations of FA (10-150 mg N/L) can, however, inhibit AOB (Anthonisen et al., 1976; Vadivelu et al., 2007). Conversely, acidic pH increases the FNA concentration, which is inhibitory to AOB at 0.6 mg N/L (Park et al., 2010). Second, pH has also been suggested to affect the conversion rates of enzymes involved in N_2O production (AMO, HAO, NIR and NOR) and consumption (NOS) (Blum et al., 2018; Illanes et al., 2008). Thus pH may imbalance the enzymatic reaction steps that could lead to an accumulation of intermediates, like NH_2OH and NO, followed by biological or chemical N_2O production (Blum et al., 2018). However, the knowledge on the

effect of pH on enzymatic activity is incomplete, partly due to involvement of different enzymes and multiple pathways, and partly due to the direct and indirect effects of pH on various central processes (pH-dependency of the true substrate molecule, signaling or transcriptional and post-transcriptional phenomena) in bacterial cells (Blum et al., 2018; Law et al., 2011). The application of mechanistic modeling will contribute to unraveling the effect of pH on N₂O production pathways and the reduction of N₂O emissions through pH set-point management.

In a previous study, we observed the simultaneous increase in N₂O production rate and pH during intermittent feeding of two lab-scale high-performing nitrification reactors ($93 \pm 14\%$ of the removed NH₄⁺ to NO₂⁻) (Su et al., 2017). This observation suggested an effect of pH on N₂O production by AOB through the ND pathway, and the potential to set pH to manage N₂O emissions from two-stage PNA systems (Su et al., 2017). Here, we report a detailed investigation of the effect of pH set-points, normally found in BNR systems (Henze and Comeau, 2008), on N₂O production in lab-scale high-performing nitrification reactors. The principal goal of this study was to quantify the effect of pH (6.5, 7, 7.5, 8 and 8.5) on net N₂O production rate and to predict N₂O production pathways in a highly AOB-dominated community. Nitrogen (N) species (NH₄⁺, NO₂⁻, NO₃⁻, NH₂OH, NO and N₂O) were monitored to identify in-cycle dynamics and determine N conversion rates at different pH set-points. A mechanistic N₂O model (Domingo-Félez and Smets, 2016) describing all four aforementioned pathways (i.e. NN, ND, HD and abiotic reactions - NDHA) was applied to predict N₂O production rates of each pathway and their corresponding contribution to N₂O emissions at different pH.

2. Materials and methods

2.1. Reactor description and operation

Two lab-scale sequencing batch reactors (SBRs) (R1 and R2) were operated as duplicates and fed with synthetic medium, containing ammonium bicarbonate (350 ± 20 mg N/L (average $\pm$ standard deviation)), sodium bicarbonate and trace chemicals (van de Graaf et al., 1996). No external organic carbon was added during the operation period, while organic carbon was added to off-line bath tests to quantify potential heterotrophic N_2O production and consumption rates (SI, Section 6). The hydraulic and sludge retention times were 12 hours and 20 days, respectively, resulting in an ammonium loading rate (ALR) of 0.7 ± 0.03 g N/L/d. A 6-h working cycle consisted of a reaction phase (320 min, including 5 consecutive intervals of 1 min feeding followed by 63 min inter-feed period), settling phase (30 min), decanting phase (5 min) and idle phase (5 min). During the reaction phase, aeration was supplied with a constant air flow rate of 0.55 L/min, resulting in measured DO concentrations between 0.3 and 0.8 mg O_2 /L. The reactors were operated at room temperature (20-26 °C). During baseline operation, without any pH control in the reactor, pH values ranged between 7.4 and 7.9. Before onset of the pH experiment (see below), the reactor biomass was highly enriched in AOB and converted $93 \pm 14\%$ of the removed NH_4^+ to NO_2^- (Su et al., 2017). The operation at a low DO set-point and with intermittent feeding was sufficient to maintain high-rate and long-term nitrification over a period of 600 days (Su et al., 2017). The details of reactor design, operation and performance are described in Su et al. (2017).

2.2. Overview of the pH experiment

The pH experiment was conducted over 80 days (Support Information (SI), Fig. S1). pH was controlled at five different values (pH = 6.5, 7, 7.5, 8 and 8.5) in one of two parallel reactors (named R2). An online controller (HACH, Loveland, USA) was used to control pH automatically by adding 0.5 M NaHCO_3 and/or 0.5 M HCl . The reactor (R2) was operated at each pH value for 3-

9 days to reach pseudo-steady state after a pH change (Fig. S1). Before and after each pH change, the reactor was operated without pH control (varying between 7.4 and 7.9) for at least 4 days (this is named baseline (B), B1-B5 in chronological order) (Fig. S1). Before the baseline operation, biomass from reactor R1 and R2 was mixed and distributed equally into the two reactors. This experimental design allowed us to maintain similar volatile suspended solid (VSS) concentrations during the experimental period, to recover any activity loss by the microorganisms after potential pH shocks, and to avoid cumulative impacts on microbial activities and composition from previous pH changes.

Sampling of the reactor was conducted 1, 2, 5, 15, 20 min after the feed-spike within the reaction phase and twice during the settling phase on Day 3 or 5 after a pH change and during baseline operation (without pH control) (Fig. S1, Table S1). These samples were analyzed for bulk NH_4^+ , NO_2^- , NO_3^- and NH_2OH , while NO (liquid phase) and N_2O (liquid & off-gas phase) were continuously monitored. Biomass samples were taken before and at the end of the cycle to analyze VSS concentrations, particle size distribution (PSD) and to quantify microbial community compositions using qPCR.

2.3. Data analytical methods

Bulk samples were collected and filtered through 0.22 μm pore size filters for NH_4^+ , NO_2^- , NO_3^- and NH_2OH analyses. NH_4^+ , NO_2^- and NO_3^- concentrations were determined colorimetrically by a continuous-flow auto-analyzer (SKALAR San⁺⁺, Netherlands). Suspended solids (SS) and VSS were assayed following standard methods (APHA, 1998). Particle size distribution was measured with a Mastersizer 2000 laser diffractometer (Malvern Instruments Ltd., Malvern, UK). DO and pH were monitored continuously (WTW GmbH, Weilheim, Germany).

Hydroxylamine was measured spectrophotometrically after reaction with 8-quinolinol to form stable 5, 8-quinolinequinone-5-(8-hydroxy-5-quinolyimide) (SI, Section 1) (Frear and Burrell, 1955). To prevent the oxidation of NH_2OH , 0.2 mL of 0.1 g/mL sulfamic acid was added immediately after filtration of the collected sample. The addition of sulfamic acid ensured NH_2OH stability by lowering pH in the sample and avoided the reaction of NH_2OH with NO_2^- (233 ± 39 mg N- NO_2^- /L in this study) (Soler-Jofra et al., 2016). Additionally, 3.5 mg N/L NH_2OH stock solution was added in one of triplicate bulk samples as the internal standard to verify the measuring accuracy. Recovery efficiency of $93 \pm 6\%$ of the added internal standard ($n=12$) indicated that the adopted pretreatment and measurement procedures were applicable and precise for NH_2OH quantification of our samples.

Liquid N_2O and NO were monitored online by N_2O -R and NO-500 Clark-type microsensors (UNISENSE A/S, Århus, Denmark), respectively, with data logged every 20s. The pH sensitivity of N_2O sensor was below 0.2% of signal changes and the signal was also not interfered by DO level and stir intensity (SI, Section 2). Off-gas N_2O concentration was measured continuously and logged on a minute basis (Teledyne API, San Diego, USA) to compare with liquid N_2O dynamics. The liquid phase concentrations of N_2O and NO were used for the quantification of the net production rates of N_2O and NO , respectively.

The instantaneous or averaged ammonium removal rate ($r_{\text{NH}_4^+}$ or ARR), nitrite accumulation rate ($r_{\text{NO}_2^-}$ or NiAR), nitrate accumulation rate ($r_{\text{NO}_3^-}$ or NaAR) and hydroxylamine accumulation rate ($r_{\text{NH}_2\text{OH}}$ or NhAR) were determined based on the changes in concentrations (mg N/L) over time (min or d) ($n \geq 3$), and the rates were further normalized to VSS concentrations (g VSS/L) (Eq. S1-7). Concentrations of FA and FNA (mg N/L) were calculated based on Eq. S8-9. The instantaneous net N_2O production rate ($r_{\text{N}_2\text{O}}$, mg N/L/min) was calculated from the N_2O accumulation rate in the liquid phase and the N_2O stripping rate estimated using a volumetric N_2O mass transfer coefficient

($k_{L\text{a}_{\text{N}_2\text{O}}}$, min^{-1}) (Eq. S10). The daily average N_2O production rate (N_2OR , mg N/L/d) was calculated by multiplying the calculated net N_2O production rate and the known 4 cycles per day (Eq. S11). The net N_2O produced per NH_4^+ removed ($\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$, %) and the specific net N_2O production rate (mg N/g VSS/d) were calculated from the daily averaged net N_2O production rate (Eq. S11). All calculation procedures for NO were the same as N_2O .

2.4. DNA extraction and qPCR

Biomass samples were centrifuged at 10,000 rpm for 5 min and stored at -80°C until DNA extraction. DNA was extracted using FastDNATM 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 stored at -80°C until qPCR analysis (Roche LightCycler 96, Mannheim, Germany). The gene copy numbers of AOB, nitrite-oxidizing bacteria (NOB) (*Nitrobacter* and *Nitrospira*), anaerobic ammonia oxidizing bacteria (AnAOB) and denitrifiers were determined based on appropriate 16S rRNA targets, following the procedure for qPCR as described elsewhere (Terada et al., 2010). Primers and conditions used in various genes detection are listed in Table S2. All samples, including control reactions without template DNAs, were measured in duplicates.

2.5. Modeling N_2O production rates and pathway contributions at different pH levels

A mechanistic N_2O model was calibrated by experimental ARR and N_2OR at different pH set-points and baseline conditions, and was evaluated to predict the corresponding N_2O production pathways. The detailed model calibration procedure, process matrices, and model parameters are listed in SI, Section 4. The ASM-based NDHA model describes the NN, ND and HD pathways, and had been calibrated via off-line extant respirometric assays using the same biomass (Domingo-Félez et al., 2017). The model considers unionized species as AOB substrates (NH_3 and HNO_2), and the

inhibitory effect of pH was described with substrate inhibition coefficients ($K_{AOB.I.NH_3}$, $K_{AOB.I.HNO_2}$). The effect of pH on enzymatic activity (i.e., values of maximum AMO/HAO/NIR/NOR/NOS-mediated reaction rate) was not considered due to the lack of knowledge and higher model complexity (2 parameters) compared to one substrate inhibition constant (Blum et al., 2018; Law et al., 2011). Based on the low abiotic N_2O production rates measured in additional batch tests, the abiotic contribution was not considered in the model structure (SI, Section 5). A step-wise procedure was followed to calibrate the model: (1) estimate the biomass composition and ARR at steady-state, (2) fit the O_2 consumption and ARR, (3) fit the N_2OR and $\Delta N_2O/\Delta NH_4^+$ (Fig. S7). The calibrated model was then applied to estimate the contribution of individual N_2O pathways to the total net N_2O production at different pH set-points (Fig. S7). A global sensitivity analysis (GSA, SRC method) was performed at each model calibration step to rank and select the most sensitive parameters for ARR and N_2OR , which were then estimated to fit experimental ARR and N_2OR , at different pH set-points and under baseline conditions (Campolongo and Saltelli, 1997) (SI, Section 4, Fig. S7, Fig. S9). The root mean squared error (RMSE) was the objective function representing the quality of the model fit, which was evaluated via correlation coefficients (R^2) and F-test (hypothesis of linear regression with simultaneous unit slope and zero intercept). Simulations were implemented in the Matlab-Simulink environment (The MathWorks, Natick, MA), and the SBR model was based on Valverde-Pérez et al. (2016).

3. Results

3.1. Reactor performance

3.1.1. Operational conditions and nitrification efficiency

While pH varied between 7.4 and 7.9 during baseline operation without pH control, it was precisely maintained at the targeted set-point during controlled pH campaigns (Fig. 1-A). DO concentrations

were nearly constant within a cycle and similar at different pH levels (0.67 ± 0.10 mg O₂/L), with exceptions at pH 6.5 and pH 8, when DO increased to 2.2 ± 0.5 mg O₂/L and decreased to 0.2 ± 0.03 mg O₂/L, respectively (Fig. 1-A). The biomass in reactor was flocculent (Fig. S2), the VSS was 0.5 ± 0.1 g/L and similar average particle size of 205 ± 29 μ m was maintained during the experimental period (Fig. 1-B). Bulk NH₄⁺ and NO₂⁻ concentrations were 74 ± 39 mg N/L and ± 39 mg N/L, respectively, resulting in FA and FNA concentrations in the range of 0.4-15 and 0.0013-0.07 mg N/L, respectively. Measured NH₂OH concentrations were around 0.05 ± 0.01 mg N/L and NO₃⁻ remained below 10 mg N/L (Fig. 1-D, E). Under similar NH₄⁺ loadings of 0.7 ± 0.03 g N/L/d, ammonium removal efficiency (ARR/ALR) decreased from 93% at pH 8 to 53% at pH 6.5. Conversely, the reactor displayed stable nitrite accumulation efficiency (NiAR/ARR) above 90% regardless of pH changes (Fig. 1-C). Under baseline operation, reactor performance was allowed to recover with an average ARR/ALR and NiAR/ARR of $84 \pm 6\%$ and $92 \pm 2\%$, respectively (data not shown).

3.1.2. In-cycle dynamics and conversion rates of N-species

Very reproducible patterns of in-cycle dynamics of N-species were observed at different pH scenarios (Fig. 2, Fig. S4). DO concentrations were almost stable during the reaction phase except for a transient and small increase (ca. 17%) after each feeding due to the higher DO in the influent (Fig. 2-C, Fig. S4-C, Fig. S5). NH₄⁺ concentration increased by approximately 50% after each feeding while NO₂⁻ concentration decreased due to dilution. NH₂OH was always detected; its concentration increased rapidly after feeding and remained almost constant during inter-feed periods (Fig. 2-B, Fig. S4-B). Dissolved and gaseous N₂O concentrations peaked after each feeding and then declined until the next feeding during reaction phase, and increased continuously during settling phase (Fig. 2-A, Fig. S4-A). Compared to significant changes of N₂O within the cycle, NO concentrations were fairly steady during reaction phase, and started to increase when aeration

stopped. Based on bulk concentrations, in-cycle conversion rates of N-species were calculated (Fig. 2-D, Fig. S4-D). $r_{\text{NH}_4^+}$, $r_{\text{NH}_2\text{OH}}$ and $r_{\text{NO}_2^-}$ peaked transiently after each feeding (2-4 times higher, $p < 0.05$) and returned to nearly constant values during the inter-feed period. There was always a positive net production of NO and N₂O over a cycle: r_{NO} remained unchanged ($p > 0.05$) and close to zero, while $r_{\text{N}_2\text{O}}$ increased after each feeding and decreased during the inter-feed period ($p < 0.05$) (Fig. 2-D, Fig. S4-D). During baseline operation without any pH control, pH transiently increased after each feeding, resulting in more significant increases in FA and N₂O concentrations than under controlled pH scenarios (Fig. S4, Fig. S5).

3.1.3. N flux during feed and inter-feed period within the cycle

By normalizing the amount of produced NH₂OH, NO₂⁻, N₂O and NO (mg N) to the amount of NH₄⁺ removed (mg N), the mass balance during feed and inter-feed period at different pH values and baselines was calculated (Fig. 3). Approximately 70% of the removed NH₄⁺ was converted to NO₂⁻ during feed period while it was ca. 93% during inter-feed period. Furthermore, NH₂OH production accounted for 0.16-0.63% of the removed NH₄⁺ after feedings while it was barely observed during inter-feed period. Despite the observed peaks of N₂O and $r_{\text{N}_2\text{O}}$ after feedings, a larger fraction of the NH₄⁺ removed was converted to N₂O during inter-feed period.

3.1.4. Microbial community composition dynamics

The imposition of varying pH conditions did not cause apparent shifts in the overall microbial community, as indicated by qPCR analysis (Fig. 4). AOB dominated the microbial community, as measured both through 16S rRNA and functional gene (*amoA*, *hao* and *nirK*) targeted analyses. The NOB gene copy number was 3 orders of magnitude lower than that of AOB, among which *Nitrobacter* spp. was 1-10 times higher than *Nitrospira* spp (Fig. 4-A). *hzsA* targeted quantifications were 4-5 orders of magnitude lower than functional genes of other bacteria, indicating a very low

AnAOB presence in the microbial community. The detection of *nirS/nirK* and *nosZ* genes indicated the presence of heterotrophic denitrifiers and their potential contribution to N₂O production and consumption (Fig. 4-B).

3.2. N₂O production

The specific ARR remained nearly constant at 1.2 ± 0.2 g N/g VSS/d across the examined pH range ($p > 0.05$) (Fig. 5). No significant changes of NiAR and NOR with pH were observed ($p > 0.05$), whilst NhAR decreased as pH increased from 6.5-8 and then increased slightly at pH 8.5 ($p < 0.05$). Conversely, the specific N₂OR and $\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$ increased with pH from 6.5 to 8, decreased slightly when pH was further increased to 8.5 ($p < 0.05$) (Fig. 5). A maximum value of 0.08 ± 0.01 g N/g VSS/d and $7.0 \pm 1.3\%$ were measured for the specific N₂OR and $\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$, respectively, at pH 8. The specific ARR under baseline operation was consistent at 1.2 ± 0.1 g N/g VSS/d (Fig. S3-B). However, the specific N₂OR dropped by almost half towards the end of baseline operation, which was associated with (and probably caused by) higher DO concentrations in the reactor (see explanation in discussion) (Fig. S3-A, C).

3.3. Model-based estimation of ARR and N₂OR at varying pH set-points

The model was first evaluated with default parameters at baseline conditions (B1), and long-term simulations (200 cycles) were run to obtain a steady-state biomass composition and an accurate prediction of biomass concentration (VSS) (Fig. S8). Only the most sensitive parameter for ARR, the maximum AMO-specific reaction rate was estimated ($\mu_{\text{AOB.AMO}} = 0.26 \text{ d}^{-1}$), resulting in a good ARR and VSS fit (Fig. S9-A). However, the ARR could not describe low and high pH set-points datasets, and hence the second most sensitive parameter, the NH₃ affinity constant ($K_{\text{AOB.NH}_3}$) at low pH (6.5, 7), and NH₃ inhibition affinity constant ($K_{\text{AOB.I.NH}_3}$) at high pH (8, 8.5) were estimated ($K_{\text{AOB.NH}_3} = 0.28 \text{ mg N/L}$, $K_{\text{AOB.I.NH}_3} = 38.5 \text{ mg N/L}$). The average modeling errors for ARR and

DO at the pH scenarios and baseline conditions were below 10% ($R^2 = 0.82$ and 0.91 , respectively, F-test = 1), indicating that the model captured the effect of pH on ARR (Fig. S10, Fig. S11). In the next step, $\Delta N_2O/\Delta NH_4^+$ was also fitted across pH scenarios and baseline conditions ($R^2 = 0.85$ and 0.80 , respectively, F-test = 1) (Fig. S10, Fig. S11). Based on the GSA results for N_2O (Fig. S9-B), the top three most sensitive parameters were selected: the maximum NO_2^- reduction factor ($\eta_{NIR} = 0.32$), the O_2 inhibition constant of NO_2^- reduction ($K_{AOB.I.O_2} = 0.20$ mg/L) and the NH_2OH affinity constant during NO reduction ($K_{AOB.NH_2OH.ND} = 0.16$ mg N/L). The pH-dependency of N_2O production was captured by the HNO_2 affinity constant at high pH set-points (8.5) ($K_{AOB.HNO_2.ND} = 0.00014$ mg N/L), and the HNO_2 inhibition constant at low pH set-points (6.5 and 7) for N_2OR ($K_{AOB.I.HNO_2.ND} = 0.017$ mg N/L) (Fig. S9-B). The model predicted an increase in N_2OR and $\Delta N_2O/\Delta NH_4^+$ as pH increased from 6.5 to 8 and a slight decrease at pH 8.5 (Fig. 5-B, Table S3). At all tested pH levels, N_2OR_{ND} dominated other pathways, contributing to 87-96% of the total net N_2O production in the reactor (Fig. 5-B, Table S3). The simulated best-fit contributions of N_2O pathways did not change significantly across the tested pH range: N_2OR_{NN} followed a similar trend as N_2OR_{ND} , and net N_2O production rates via HD pathway were insignificant compared to the total net N_2OR ($\leq 3\%$).

4. Discussion

4.1. Insights of in-cycle N dynamics and fluxes

NH_2OH is a known intermediate during NH_3 oxidization by AOBs. Due to its short-live and high reactivity, the measurement and role of NH_2OH in N_2O production are limited (Soler-Jofra et al., 2018, 2016). NH_2OH concentrations was measured at 0.05 ± 0.01 mg N/L, consistent with earlier values in lab-scale PN reactors (0.03-0.11 mg N/L) (Kinh et al., 2017; Soler-Jofra et al., 2016). Bulk liquid NH_2OH concentrations are the result of the balance between production associated with

305 biological NH_3 oxidation and consumption from conversion of NH_2OH to NO_2^- or to N_2O , either
306 biologically or chemically. Even low NH_2OH concentrations are still compatible with high $r_{\text{N}_2\text{O}}$
307 since the turnover of NH_2OH may be high (Fig. 2, Fig. 5, Fig. S4) (Soler-Jofra et al., 2016). As a
308 potential toxic intermediate and the primary energy yielding substrate during NH_3 oxidation,
309 NH_2OH typically did not accumulate except immediately after feedings, when transiently short-
310 term elevated $r_{\text{NH}_4^+}$ were measured (Fig. 2, Fig. S4). This finding is consistent with Yu et al. (2017)
311 and Liu et al. (2017), who observed an immediate accumulation of NH_2OH after the activation of
312 NH_3 oxidation during the anoxic-to-oxic transition. Moreover, a 0.3-0.7% of instantaneous
313 $\text{NH}_2\text{OH}:\text{NO}_2^-$ accumulation ratio measured after feeding in our experiment is comparable with the
314 value reported for AOB pure cultures (0.1-0.6 %) (Liu et al., 2017) (Fig. 3).

315 The observation that NO increased during non-aerated settling phase but was nearly constant during
316 aerated reaction phase is in agreement with previous studies on pure AOB cultures or partial
317 nitrification-anammox reactors (Domingo-Félez et al., 2014; Kampschreur et al., 2008a; Yu et al.,
318 2010; Yu and Chandran, 2010). Under anoxic conditions, NO is suspected to be generated via ND,
319 NN and HD pathways (Caranto et al., 2016; Yu et al., 2018; Yu and Chandran, 2010). Since over-
320 expressed NIR and under-expressed AMO, HAO and NOR have been observed in *N. europaea*
321 cultures under anoxic conditions (Yu et al., 2010), NO could be produced via ND process by using
322 NO_2^- as an electron acceptor. HAO or cyt P460 mediated NH_2OH oxidation was also regarded as an
323 importance source of NO after imposing anoxic conditions (Caranto et al., 2016; Yu et al., 2018),
324 verified by ca. 20% drop of NH_2OH concentrations during settling phase. In addition, heterotrophic
325 denitrifiers might accumulate NO and N_2O under anoxic conditions due to the limited organic
326 carbon availability (Chung and Chung, 2000). A possible explanation for substantial reduction of
327 NO concentrations upon recovery to aerated reaction phase is that the presence of O_2 would inhibit
328 both ND and HD pathway and also decrease NO production via NN pathway as NO can rapidly

react with O_2 to form NO_2^- instead of N_2O . The three biological NO production rates are described in the N_2O model structure considered in this study. The dynamics of NO accumulation within a cycle was not concurrent with any of the other measured N species (Fig. 2, Fig. S4). This could be explained by different expression patterns of the four genes (*amoA*, *hao*, *nirK* and *norB*) involved in N transformations of AOB (Yu et al., 2018, 2010). The different transcription of sequential enzymatic steps in the AOB metabolism was probably due to in-cycle physicochemical dynamics, such as DO changes in the aerated and non-aerated phase and increasing NH_4^+ /FA/pH after each feeding.

The accumulation of N_2O during the settling phase was subsequently stripped at the onset of aeration of the following cycle, resulting in the maximum N_2O emission after the first feed (Fig. 2, Fig. S4) (Su et al., 2017). For the other four N_2O peaks within the cycle, changes in pH, DO, NH_4^+ and FA after each feeding pulse could be the possible triggers. Compared to changes in pH and DO, there were more obvious increases of NH_4^+ (ca. 50%) and FA (ca. 80%) after each feeding (Fig. S5). High FA availability might lead to high metabolic rates during periods of high N flux, as reflected by transient peaks of $r_{NH_4^+}$, r_{NH_2OH} and $r_{NO_2^-}$ (Fig. 2-D, Fig. S4-D). Thus, more substrates (e.g. NH_2OH and NO_2^-) and electrons (produced by NH_2OH oxidization) were available to potentially enhance N_2O productions after feedings.

4.2. The effect of pH on reactor performance

The operational conditions (DO and NH_4^+ concentrations of 0.3-0.8 mg O_2 /L and 74 ± 39 mg N/L, respectively and nitrification performance (specific ARR of 1.2 ± 0.2 g N/g VSS/d and ca. 90% of the removed NH_4^+ converted to NO_2^-) was comparable to other nitrification reactors (Fig. 1) (Desloover et al., 2011; Kampschreur et al., 2008b; Kinh et al., 2017; Rathnayake et al., 2015). In full-scale WWTPs, AOB can grow across a wide range of pH with optima at 7.4-8.2 (Blum et al., 2018). While the activity of AOB has been reported to increase with increasing pH (Nicol et al., 2008), we

observed nearly constant specific ARR_s across the examined pH range. Furthermore, the relative abundances of AOB and functional genes (*amoA* and *hao*) remained stable throughout the experimental period (Fig. 4), as expected due to the applied experimental strategy. Therefore, the experimental data in this study was not biased by the transient effect of previous pH changes on microbial composition. With respect to substrate deficiency and inhibition, FA at 0.4-15 mg NH₃-N/L was in excess compared to the estimated K_{AOB,FA} value (0.28 mg NH₃-N/L); concentrations of FA and FNA (0.0013-0.07 mg HNO₂-N/L) remained below the inhibitory concentrations, i.e. 38.5 mg NH₃-N/L and 0.017 mg HNO₂-N/L, respectively, except at high pH set-points (8.5) and at low pH set-points (6.5 and 7), when the highest FA and FNA occurred. The unchanged AOB activities under pH fluctuations suggest either rapid selection or fast adaptation of predominant AOB species. The predominant AOB species in microbial community might be shifted to alleviate the effect of pH shocks (Kinh et al., 2017). Alternatively, microorganisms could gradually acclimate to pH shocks (Kinh et al., 2017), since controlled pH campaigns were conducted under long-term (3-9 days) reactor operation. Further investigations through high-throughput sequencing and functional gene transcription analysis would help verify these interpretations.

In this study, pH showed a major effect on N₂O production: the specific N₂OR and ΔN₂O/ΔNH₄⁺ increased with pH from 6.5 to 8 and decreased at pH 8.5 (Fig. 5). Our results were consistent with previous reports: Rathnayake et al. (2015) and Kinh et al. (2017) observed unchanged ARR between pH 6.5 and 8.5 in PN reactors but highest N₂O emission at pH 7.5 and 7, respectively; Law and coworkers (2011) reported highest N₂OR and ARR at pH 8 (pH 6.0–8.5) and a linear relationship between them, suggesting that the increasing NH₃ oxidation activity might promote N₂O production. In contrast, Lv et al. (2016) found that ARR and N₂OR decreased with increasing initial pH from 7.5 to 8.5 in a PN reactor. HD was identified as the dominant pathway and its contribution to total N₂O emissions decreased from 69 % at pH 7.5 to 40% at pH 8.5, probably

because of increasing N₂O consumption caused by high NOS activity at alkaline pH (Lv et al., 2016). Different observations on pH effects are probably due to different predominant N₂O production pathway, i.e. ND in our study, and HD in Lv et al. (2016), which responded differently to pH changes. Compared to the short-term batch tests or transient pH changes in previous studies (where responses were measured minutes or hours afterwards), here we imposed longer-term reactor-scale pH campaigns (i.e. the reactor was operated at each pH value for 3-9 days). Hence, in this study microorganisms may have acclimated to new pH changes and reach stable nitrification activities. In summary, according to the pH effect on N₂O production observed in this study, operating nitrification systems at slightly acidic or neutral pH (which still permit sufficient microbial activity) can reduce N₂O production by up to seven-fold. The results obtained in this study are representative for AOB-enriched nitrification systems. The effects of pH on net N₂O production will depend on the microbial compositions in other systems (e.g. activated sludge), where heterotrophic denitrification might become an important pathway for N₂O accumulation. However, the potential application of pH control for N₂O mitigation in full-scale WWTPs remains to be verified and combined with economic and environmental assessment.

4.3. The effect of pH on N₂O production pathways

Even without any addition of organic carbon, heterotrophic denitrifiers could still thrive dependent on biomass decay and affect both N₂O production and consumption in nitrification systems (Shen et al., 2015; Wunderlin et al., 2012). In additional batch tests with excess organic carbon (SI, Section 6), the maximum N₂O consumption rate was detected under anoxic conditions at 0.04 ± 0.01 g N/g VSS/d, while N₂O consumption was not observed in the presence of O₂ (DO > 0.05 mg O₂/L) (Fig. S12). Hence, considering the limitation of organic carbon, oxygen inhibition and lagging recovery during transient oxic to anoxic (20 minutes lag off), N₂O consumption was insignificant compared to total net N₂O produced within the whole cycle. The model prediction further confirmed that N₂O

consumption only accounted for $2 \pm 0.4\%$ of total net N_2O production. With respect to N_2O production by heterotrophic denitrifiers, very low net N_2O production rate ($0.004 \text{ g N/g VSS/d}$) was measured under $0.3 \pm 0.1 \text{ mg O}_2/\text{L}$ and $300 \text{ mg NO}_2^- \text{-N/L}$ in off-line batch tests (Fig. S13). Therefore, based on experimental and modeling results we conclude that HD was a minor source of N_2O during aerated reaction phase ($0.67 \pm 0.10 \text{ mg O}_2/\text{L}$). In consideration of low DO ($< 0.1 \text{ mg O}_2/\text{L}$) and high NO_2^- ($233 \pm 39 \text{ mg N/L}$) during the anoxic settling phase, HD was speculated to be a dominant N_2O source (Itokawa et al., 2001; Ma et al., 2017). Two orders of magnitude higher heterotrophic N_2O production rate has been observed in anoxic conditions than in oxic incubations ($\geq 0.2 \text{ mg O}_2/\text{L}$) in the nitrification–anammox biomass (Ma et al., 2017). However, previous studies could not elaborate on the individual contribution of heterotrophic denitrification solely with bulk N_2O measurements due to the co-occurrence of both pathways under similar NO_2^- and DO thus leading to possible interferences between autotrophic and heterotrophic N_2O production in mixed culture biomass (Itokawa et al., 2001; Shen et al., 2015; Wu et al., 2014).

Since intermediates of the nitrification process (e.g. NH_2OH) may engage in chemical reactions to generate N_2O , the contribution of abiotic N_2O production requires to be carefully examined. In a series of abiotic batch tests without biomass under different pH campaigns (SI, Section 5), the highest N_2O production rates were measured for the reaction of NH_2OH with HNO_2 and acidic pH was found to strongly stimulate N_2O production from this reaction (Table S6) (Su et al., 2019). The estimated abiotic N_2O production rates was 1-5 orders of magnitude lower than total net N_2O production rates in the reactor across the examined pH range (Table S6). Correspondingly, the abiotic N_2O production was estimated to be a minor source ($< 3\%$ of total net N_2O production) in the nitrification reactor across pH 6.5-8 and would only become significant at extremely acidic pH (≤ 5) (Table S6) (Su et al., 2019). In contrast, previous studies concluded that both abiotic and biotic pathways contribute in a comparable degree to N_2O emissions (at pH 7) (Harper et al., 2015; Soler-

425 Jofra et al., 2018, 2016; Terada et al., 2017). The significance of abiotic N₂O production in
 426 nitrification systems has been overestimated in recent studies (Su et al., 2019).

427 The NDHA model predicted the complete dominance of the ND pathway, which is consistent with
 428 the result from our previous study, as revealed via *in situ* ¹⁵N labeled analysis (Su et al., 2017).
 429 While the ND pathway is favored by low DO and high NO₂⁻ levels (Kampschreur et al., 2008a;
 430 Peng et al., 2015), model simulations points towards HNO₂ instead of NO₂⁻ as the ND substrate
 431 since NO₂⁻ concentrations were in similar range at different pH set-points and baselines (233 ± 39
 432 mg N/L) (Fig. 1). Furthermore, a HNO₂ inhibition was added to the model (K_{AOB.I.HNO2.ND}), which
 433 had been observed on a similar nitrifying biomass when exposed to high NO₂⁻ concentrations (Law
 434 et al., 2013). The model pointed to ND pathway as the primary contributor for increasing N₂O
 435 production at higher pH, with N₂OR_{ND} 5-10 times higher at pH 8 than at pH 6.5 (Fig. 5-B, Table
 436 S3). The ND pathway was strongly stimulated at pH 8 due to the lowest DO (0.2 ± 0.03 mg O₂/L)
 437 in the reactor, and was significantly inhibited at pH 6.5 due to the highest DO (2.2 ± 0.5 mg O₂/L)
 438 far above K_{AOB.I.O2} (0.2 mg O₂/L). Modeling results showed the dominant effect of pH over DO:
 439 compared to FA and FNA inhibition, the DO limitation had a smaller impact on sARR; the overall
 440 trend of pH effect on the modeled ΔN₂O/ΔNH₄⁺ did not change when DO was evaluated at the
 441 average value of 0.67 mg O₂/L at all pH set-points (Fig. S11). The reduced N₂O production at pH
 442 8.5 is associated with limiting concentrations of electron donor caused by relatively lower ARR,
 443 and with limiting electron acceptor, HNO₂ (close to K_{AOB.HNO2.ND}) caused by low acid dissociation
 444 constant. In addition, the model also captured the 65% lower N₂OR at B4 than B1, and predicted
 445 two-fold lower N₂OR via ND pathway at B4 caused by higher DO condition (0.8 ± 0.2 mg O₂/L)
 446 (Fig. S11-B, Table S3). Further investigations through the use of stable isotope labeling will
 447 confirm the modeling prediction on N₂O production pathways. Collectively, modeling results
 448 demonstrated that pH is a key factor influencing N₂O production by affecting substrate availability

and inhibition (i.e. NH_3 and HNO_2). The effect of pH on maximum conversion rates of enzymes was not considered in the model. The activities of involved enzymes are assumed to be pH dependent, such as alkaline pH optima for AMO (7.5), HAO (8.5) and NOS (7-8), and acidic pH optima for NIR (5.6-6.7) and NOR (5-6), respectively (Blum et al., 2018). Despite of this, the NDHA model still show a good description of experimental data, indicating that the effect of pH on enzyme activities might not be particularly significant.

5. Conclusion

We present a comprehensive study on the effect of pH (6.5-8.5) on N_2O production in an AOB-enriched nitrification system. The specific ARR and NiAR remained nearly constant with pH ($p > 0.05$), while the specific N_2OR and $\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$ increased with pH from 6.5 to 8 and decreased slightly at pH 8.5 ($p < 0.05$). The modeling results indicated that the ND pathway dominated the NN and HD pathways at all examined pH, contributing to 87-96% of total net N_2O production. This study suggests that process models are valuable tools to design N_2O mitigation strategies and future N_2O model studies requires to consider the pH effect on dissociation equilibriums of substrates driving N_2O production: e.g. $\text{NH}_3/\text{NH}_4^+$, $\text{NO}_2^-/\text{HNO}_2$.

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Fig. 1. Overview of reactor performance during the pH experiment. (A) Measured pH and DO averaged over one cycle and all cycles. (B) VSS and PSD of biomass. (C) Nitrogen conversion efficiency (ARR/ALR, NiAR/ARR and NaAR/ARR). (D) Calculated FA and FNA within one cycle and all cycles. (E) Bulk concentrations of NH_4^+ , NO_2^- , NO_3^- and NH_2OH . Each data point represents the average of all measurements at the same pH level ($n > 6$). Error bars indicate standard deviations of measurements. Abbreviations: particle size distribution (PSD), dissolved oxygen (DO), volatile suspended solid (VSS), ammonium loading rate (ALR), ammonium removal rate (ARR), nitrite accumulation rate (NiAR), nitrate accumulation rate (NaAR), free ammonia (FA), free nitrous acid (FNA).

Fig. 2. In-cycle dynamics and conversion rates of N species at pH 8 (1) and 7 (2). (A) Liquid & off-gas N_2O and liquid NO concentrations. (B) Bulk concentrations of NH_4^+ , NO_2^- and NH_2OH . (C) pH, DO, calculated FA and FNA. (D) Conversion rates of N species ($r_{\text{NH}_4^+}$, $r_{\text{NO}_2^-}$, $r_{\text{NH}_2\text{OH}}$, r_{NO} and $r_{\text{N}_2\text{O}}$). No significant NO_3^- concentrations were measured (<10 mg N/L). The calculation of conversion rates is based on in-cycle concentrations of N species; each point represents the slope of 2-3 concentration points within a certain time period. Abbreviations: free ammonia (FA), free nitrous acid (FNA).

Fig. 3. N mass balance during feed and inter-feed period at different pH set-points and baselines. No significant NO_3^- concentrations were measured (<10 mg N/L). The calculation of average and standard deviation was based on data points during feed 2-5 ($n=4$).

Fig. 4. Gene copy numbers pr. g VSS of SSU rRNA (A) and functional genes (B) during pH experiment (n=2-10). Abbreviations: ammonia oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) *Nitrobacter* and *Nitrospira*, ammonia monooxygenase (amo), hydroxylamine dehydrogenase (hao), hydrazine synthase (hzs), nitrite reductase (nir) and nitrous oxide reductase (nos).

Fig. 5. The experimentally measured and model predicted specific N conversion rates (A) and net N₂O yield of NH₄⁺ removed ($\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$) (B) at different pH set-points (n=5-29). The data of B1 and B4 are shown in Fig. S11. Abbreviations: ammonium removal rate (ARR), nitrite accumulation rate (NiAR), hydroxylamine accumulation rate (NhAR), net NO production rate (NOR), net N₂O production rate (N₂OR), nitrifier denitrification (ND), nitrifier nitrification (NN) and heterotrophic denitrification (HD).

Support information for:

The effect of pH on N₂O production in intermittently-fed nitritation reactors

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13 Fig. S1. Overview of pH experiment. The experiment period: 25th September, 2017 – 12nd
 14 December, 2017. Sampling campaigns were conducted on Day 3 or 5 after a pH change when the
 15 reactor (R2) reached pseudo-steady state.

16

17 Fig. S2. Microscopic images of flocculent biomass.

Fig. S3. Dissolved oxygen (DO), specific ammonium removal rate (ARR) and net N₂O production rate (N₂OR) during pH campaigns (pH 6.5 to 8.5) and baseline operation (B1-5).

22 (1) pH 6.5

23
24 (2) pH 7.5

25
26
27

28 (3) pH 8.5

29

30 (4) Baseline 1

31

32

33

(5) Baseline 4

Fig. S4. In-cycle dynamics and conversion rates of N species at pH 6.5 (1), pH 7.5 (2), pH 8.5 (3), baseline 1 (4) and baseline 4 (5). (A) Liquid & off-gas N_2O and liquid NO concentrations. (B) Bulk concentrations of NH_4^+ , NO_2^- and NH_2OH . (C) pH, DO, calculated FA and FNA. (D) Conversion rates of N species (NH_4^+ , NO_2^- , NH_2OH , NO and N_2O). The calculation of conversion rates is based on in-cycle concentrations of N species; each point represents the slope of 2-3 concentration points within a certain time period. No significant NO_3^- concentrations were detected (<10 mg N/L), thereby it was not shown in the figure. Abbreviations: free ammonia (FA), free nitrous acid (FNA).

Fig. S5. Relative changes of NH_4^+ , FA, pH and DO concentrations after feedings during controlled pH campaigns and baseline operation. Relative changes are calculated as the difference between concentrations just before and 2 minutes after the feeding. The average and standard deviation of values was calculated based on feed 2-5 within the cycle at different pH set-points and baselines.

48 Table S1. In-cycle sampling schedule during controlled pH campaigns and baseline operation.

Phase	Reactor volume(L)	Time	Sampling				
		min	NH ₄ ⁺ , NO ₂ ⁻ , NO ₃ ⁻ (mL) ^a	NH ₂ OH (mL) ^b	DNA (mL) ^c	VSS/PSD (mL)	Total (mL)
Previous cycle	5	-40	1.4	1.6		20	23
Feed 1	2.5 → 3	0-1					
Inter-feed 1	3	2	1.4	1.6			3
		6	1.4	1.6			3
		16	1.4	1.6			3
		26	1.4	1.6			3
		41	1.4	1.6			3
		63	1.4	1.6			3
Feed 2	3 → 3.5	64-65					
Inter-feed 2	3.5	67	1.4	1.6			3
		70	1.4	1.6			3
		75	1.4	1.6			3
		80	1.4	1.6			3
		90	1.4	1.6	3		6
		105	1.4	1.6			3
		127	1.4	1.6			3
Feed 3	3.5 → 4	128-129					
Inter-feed 3	4	131	1.4	1.6			3
		134	1.4	1.6			3
		139	1.4	1.6			3
		144	1.4	1.6			3
		154	1.4	1.6			3
		169	1.4	1.6			3
		191	1.4	1.6			3
Feed 4	4 → 4.5	192-193					
Inter-feed 4	4.5	198	1.4	1.6			3
		208	1.4	1.6			3
		218	1.4	1.6			3
		233	1.4	1.6			3
		255	1.4	1.6			3
Feed 5	4.5 → 5	256-257					
Inter-feed 5	5	262	1.4	1.6			3
		272	1.4	1.6			3
		282	1.4	1.6			3
		297	1.4	1.6			3
		319	1.4	1.6			3
Settling	5	325	1.4	1.6			3
		340	1.4	1.6		20	23

^a Samples were filtered through 0.22 µm-sized filters and stored at -20 °C.

^b 0.2 mL 0.1 g/L sulfamic acid was added after filtration of the collected sample, and measured immediately.

^c DNA samples were centrifuged at 10,000 rpm for 5 min and stored -80 °C before extraction.

52 Table S2. Primers and conditions used for the quantification of bacterial numbers and specific genes
53 by qPCR.

Target Organism	Primers	Sequence (5'-3')	Annealing Temperature	Reference
All Bacteria	1055f	ATG GCT GTC GTC AGC T	55	(Ferris et al., 1996; Lane, 1991)
	1392r	ACG GGC GGT GTG TAC		
β-proteo- bacterial	CTO189fa/b	GGA GRA AAG CAG GGG ATC G	60	(Hermansson and Lindgren, 2001; Kowalchuk et al., 1997)
	CTO189fc	GGA GGA AAG TAG GGG ATC G		
AOB	RT1r	CGT CCT CTC AGA CCA RCT ACT G		
NOB <i>Nitrobacter</i>	FGPS872f	CTA AAA CTC AAA GGA ATT GA	50	(Degrange and Bardin, 1995)
	FGP1269r	TTT TTT GAG ATT TGC TAG		
NOB <i>Nitrospira</i>	Nspra675f	GCG GTG AAA TGC GTA GAK ATC G	58	(Graham et al., 2007)
	Nspra746r	TCA GCG TCA GRW AYG TTC CAG AG		
Anammox	Amx809f	GCC GTA AAC GAT GGG CAC T	67	(Tsushima et al., 2007)
	Amx1066r	AACGTCTCACGACACGAGCTG		
amoA	amoA 1f	GGG GTT TCT ACT GGT GGT	56	(Rotthauwe et al., 1997)
	amoA 2r	CCC CTC KGS AAA GCC TTC TTC		
hao	HAO1Fq	TGAGCCAGTCCAACGTGCAT	59	(Yu et al., 2010)
	HAO1Rq	AAGGCAACAACCCTGCCTCA		
nirS	Cd3aF	GTS AAC GTS AAG GAR ACS GG	45	(Throback et al., 2004)
	R3cd	GAS TTC GGR TGS GTC TTG A		
nirK	F1aCu	ATC ATG GTS CTG CCG CG	57	(Hallin and Lindgren, 1999)
	R3cu	GCC TCG ATC AGR TTG TGG TT		
nosZ cI	nosZ2F	CGC RAC GGC AAS AAG GTS MSS GT	60	(Henry et al., 2006)
	nosZ2R	CAK RTG CAK SGC RTG GCA GAA		
nosZ cII	nosZ-II-F	CTI GGI CCI YTK CAY AC	54	(Juhanson et al., 2017)
	nosZ-II-R	GCI GAR CAR AAI TCB GTR C		
hzsA	hzsA_1597F	TATCAGTATGTAG	54	(Harhangi et al., 2012)

hzsA_1857R

AAAGGGTGAATCATAATGGC

54 * *Nitrospira* and *Nitrosomonas* species belong to the beta-proteobacteria. The *amoA* primers do not detect
55 comammox *Nitrospira*, however, the *Nitrospira* specific 16S rRNA primers would detect comammox *Nitrospira*.

Section 1 - NH₂OH analysis

The NH₂OH concentration was measured following the protocol proposed by Frear and Burrell (1955) and Soler-Jofra et al. (2016):

1. Add 1.6 mL of the filtered sample containing NH₂OH in the range of 0-2.1 mg N/L to 5 mL test tube, follow with 0.2 mL of 0.1g/mL sulfamic acid to and mix.
2. Add 1 mL of 0.05 M phosphate buffer, 0.2 mL of 12 wt% trichloroacetic acid and 1 mL of 1% 8-quinolinol (w/v), then swirl gently.
3. Add add 1.0 mL of 1.0 M sodium carbonate solution, shake vigorously.
4. Heat 1 min at 100 °C in a water bath and cool for 15 min, and then read in spectrophotometer at 705 µm.
5. Carry out simultaneously a blank by replacing the sample volume by the same volume of demineralized.

Section 2 - Measurement of pH, oxygen and stirring sensitivity of N₂O sensor

The pH sensitivity of N₂O sensor was measured under stepwise increases or decreases of pH values by sequentially spiking alkali or acid (Fig. S6). The signal changes in response to pH changes were below 0.2%, indicating a low pH sensitivity of the N₂O sensor. In addition, we performed tests at different DO levels and stir intensities by spiking oxygen saturated liquid and changing stirring, rates, respectively. No significant changes in N₂O signal were observed.

Fig. S6. The sensitivity of N₂O sensor towards pH changes.

Section 3 - Calculation of nitrogen conversion rates

Nitrogen conversion rates

Instantaneous ammonium removal rate ($r_{NH_4^+}$), nitrite accumulation rate ($r_{NO_2^-}$), nitrate accumulation rate ($r_{NO_3^-}$) and hydroxylamine accumulation rate (r_{NH_2OH}) (mg N/L/min) within the cycle were determined through linear regression of the measured concentration profiles of NH_4^+ , NO_2^- , NO_3^- and NH_2OH (mg N/L):

$$\text{Instantaneous } r_{NH_4^+} = \frac{\Delta NH_4^+ - N_i}{\Delta t} \quad \text{Eq. S1}$$

$$\text{Instantaneous } r_{NO_2^-} = \frac{\Delta NO_2^- - N_i}{\Delta t} \quad \text{Eq. S2}$$

$$\text{Instantaneous } r_{NO_3^-} = \frac{\Delta NO_3^- - N_i}{\Delta t} \quad \text{Eq. S3}$$

$$\text{Instantaneous } r_{NH_2OH} = \frac{\Delta NH_2OH - N_i}{\Delta t} \quad \text{Eq. S4}$$

Where $\frac{\Delta NH_4^+ / NO_2^- / NO_3^- / NH_2OH - N_i}{\Delta t}$ is the differential term of liquid concentration at time i.

Daily averaged ammonium removal rate (ARR), nitrite accumulation rate (NiAR), nitrate accumulation rate (NaAR) and hydroxylamine accumulation rate (NhAR) (mg N/L/d) were calculated based on following equations:

$$\begin{aligned} \text{Daily averaged ARR} = & \left([NH_4^+ - N]_{\text{time 1}} \cdot V_{\text{time 1}} + [NH_4^+ - N]_{\text{inf}} \cdot V_{\text{inf}} - [NH_4^+ - N]_{\text{time 2}} \cdot V_{\text{time 2}} \right) \\ & \cdot 4 \frac{\text{cycle}}{\text{day}} \left(\frac{\Delta \text{mg } NH_4^+ - N \text{ in reactor}}{\text{day}} \right) \end{aligned} \quad \text{Eq. S5}$$

$$\text{Daily averaged NiAR} = ([NO_2^- - N]_{\text{time 1}} \cdot V_{\text{time 1}} + [NO_2^- - N]_{\text{inf}} \cdot V_{\text{inf}} - [NO_2^- - N]_{\text{time 2}} \cdot V_{\text{time 2}})$$

$$\cdot 4 \frac{\text{cycle}}{\text{day}} \left(\frac{\Delta \text{mg NO}_2^- \text{-N in reactor}}{\text{day}} \right) \quad \text{Eq. S6}$$

$$\text{Daily averaged NaAR} = ([\text{NO}_3^- \text{-N}]_{\text{time 1}} \cdot V_{\text{time 1}} + [\text{NO}_3^- \text{-N}]_{\text{inf}} \cdot V_{\text{inf}} - [\text{NO}_3^- \text{-N}]_{\text{time 2}} \cdot V_{\text{time 2}})$$

$$\cdot 4 \frac{\text{cycle}}{\text{day}} \left(\frac{\Delta \text{mg NO}_3^- \text{-N in reactor}}{\text{day}} \right) \quad \text{Eq. S7}$$

Where time 1 is the start of reaction phase, time 2 is the end of reaction phase, inf is the influent fed to the reactor, $V_{\text{time 1}}$ is the volume in the reactor at time 1 (2.5 L), $V_{\text{time 2}}$ is the volume in the reactor at time 2 (5 L), and V_{inf} is the volume of influent fed during the whole cycle (2.5 L).

Free ammonia (FA) and free nitrous acid (FNA)

FA (mg N/L) and FNA (mg N/L) were calculated according to Eq. S8 and S9, respectively.

$$\text{FA} = \frac{K_{a,\text{NH}_3} \cdot [\text{NH}_4^+ \text{-N}]}{10^{-\text{pH}}} \quad \text{Eq. S8}$$

$$\text{FNA} = \frac{10^{-\text{pH}} \cdot [\text{NO}_2^- \text{-N}]}{K_{a,\text{HNO}_2}} \quad \text{Eq. S9}$$

Where K_{a,NH_3} and K_{a,HNO_2} are dissociation equilibrium constants of FA and FNA, respectively, which are determined by the temperature (0.000562 and 562.34 $\mu\text{mol/L}$ (33°C, 1atm)).

Net N₂O production rate

Net N₂O production rates were calculated from the following equations:

$$\text{Instantaneous net N}_2\text{O production rate, } r_{\text{N}_2\text{O}_i} = \frac{\Delta \text{N}_2\text{O}_i}{\Delta t} + k_L a_{\text{N}_2\text{O}_i} \cdot \text{N}_2\text{O}_i \quad \text{Eq. S10}$$

$$\text{Daily averaged net N}_2\text{O production rate, N}_2\text{OR} = \sum (\text{N}_2\text{OR}_i \cdot \Delta t) \times 4 \frac{\text{cycle}}{\text{day}} \quad \text{Eq. S11}$$

Where $r_{\text{N}_2\text{O}_i}$ is the instantaneous net N₂O 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

113 emission rate. The N₂O volumetric mass transfer coefficient ($k_L a_{N_2O}$, min⁻¹) was determined
114 experimentally at different volume/flow rates scenarios as described elsewhere (Su et al., 2017).

Section 4 - NDHA model

The NDHA model considers N_2O production by AOB via nitrifier nitrification (NN) and nitrifier denitrification (ND) and by heterotrophic denitrifiers (HD) (Domingo-Félez and Smets, 2016). NN and ND pathway are distinguished by different O_2 and HNO_2 dependencies of their NO production pathways. NO and N_2O are produced as side products during incomplete oxidation of NH_2OH to NO_2^- in NN, while they are produced by the reduction of HNO_2 in ND. NN pathway tends to be favored at higher DO and NH_4^+ concentrations while ND is promoted under low DO, high NO_2^- , and low pH conditions. The NDHA model includes a four-step denitrification process, considering individual reaction kinetics, inhibition and substrate affinities of every step. The process matrices and model parameters are listed in Table S4 and S5, respectively.

The model calibration procedure followed a 4-step procedure (Fig. S7). First, with a long-term dataset the biomass composition and volatile suspended solid (VSS) concentration were estimated. Second, with pH set-point datasets O_2 -consumption and ARR were fitted. Third, N_2O production rates were fitted by estimating parameters associated to N_2O production but not to ARR. Last, the contribution of individual N_2O pathways to the total N_2O production was calculated.

Fig. S7. Model calibration procedure of the NDHA model to predict ARR and N₂OR at different pH set-points.

The biomass composition was obtained from long-term simulations ($t = 50$ days, 200 cycles) performed at baseline operating conditions (Fig. 1 and Fig. S4) with pH, k_{La} , and SRT used as model inputs. The steady-state solution of the biomass composition and VSS concentration were independent of the initial AOB composition (20-40-60-75%) ($< 1\%$ variation of ΔNH_4^+ effluent and ΔSS concentration during 1 SRT), and the steady-state biomass composition was considered the same for all pH set-point experiments (63% AOB/Active Biomass, 80% Active Biomass/Particulate COD, $VSS/SS = 0.90$) (Fig. S8).

The analytical error of the experimental data used was considered during model simulations (initial NH_4^+ , initial NO_x^- , influent NH_4^+ , flow rate, STD = 5%; VSS, STD = 1.5%) was propagated to the model outputs and evaluated after 3 cycles, yielding an average NH_4^+ removal uncertainty of 13% (300 MonteCarlo simulations).

A global sensitivity analysis (GSA, SRC method) was performed at different pH set-points and baseline conditions to study the effect of pH on the ARR and N_2OR (Fig. S9). The SRC method was used, and a regression over 0.8 was considered as a threshold for accurate linearization, achieved for 300 MonteCarlo simulations, with uniform and uncorrelated parameter variations over the default parameter value, following Sin et al. (2009). The linearization was performed at 400 time points within the simulation period and regression results averaged.

ARR prediction

The most sensitive parameter for ARR was the maximum specific AMO-mediated reaction rate ($\mu_{\text{AOB.AMO}}$), followed by NH_3 affinity constant ($K_{\text{AOB.NH}_3}$) at low pH set-points (6.5 and 7) and NH_3 inhibition affinity constant ($K_{\text{AOB.I.NH}_3}$) at high pH set-points (8 and 8.5) (Fig. S9-A). As the model with default parameters failed to describe ARR at low and high pH set-points, two substrate affinity constants were estimated ($K_{\text{NH}_3} = 0.28 \text{ mg N/L}$, $K_{\text{I.NH}_3} = 38.5 \text{ mg N/L}$). The average ARR and DO modeling errors at different pH set-points and baselines (B1 and B4) were below 10% ($R^2 = 0.82$ and 0.91, respectively, F-test = 1) (Fig. S10-A, B), indicating a good model description of the pH effect on ARR (Fig. S11-A). Additionally, because the model could not predict the highest ARR at the significantly lower DO at pH 8 (Fig. 1, 5), the ARR at pH 8 was evaluated at the average DO of the other pH set-points ($\text{DO} = 0.67 \text{ mg/L}$) to avoid a possible error of DO measurement (Fig. S11, red bar). Consequently, the ARR fit with corrected DO improved from 27% error to 9%.

N_2OR prediction

The most sensitive parameters for N₂OR were the maximum NO₂⁻ reduction factor (η_{NIR}), O₂ inhibition constant of NO₂⁻ reduction ($K_{\text{AOB.I.O}_2}$), NH₂OH affinity constant during NO reduction ($K_{\text{AOB.NH}_2\text{OH.ND}}$). The effect of pH on N₂O production was observed on two parameters: HNO₂ affinity constant ($K_{\text{AOB.HNO}_2.\text{ND}}$) at high pH set-points (8.5) and HNO₂ inhibition constant ($K_{\text{AOB.I.HNO}_2.\text{ND}}$) at low pH set-points (6.5 and 7) (Fig. S9-B). The model-simulated N₂OR and $\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$ also fitted the experimental values across pH levels and baseline conditions ($R^2 = 0.85$ and 0.80 , respectively, F-test = 1) (Fig. S10-C, D, S11-B). Model-predicted specific N₂O production rates of each pathway and relative contributions to total N₂O production at different pH campaigns and baseline operation are presented in Table S3.

Fig. S8. Long-term simulations (200 cycles) of VSS (in different initial AOB fractions) and NH_4^+ concentrations in effluent during B1.

Fig. S9. Parameter sensitivity during model prediction of ammonium removal rate (ARR) (A) and N₂O production rate (N₂OR) (B).

Fig. S10. Evaluation of model response: experimental and model predicted specific ammonium removal rate (sARR) (A), DO (B), specific N₂O production rate (sN₂OR) (C) and $\Delta\text{N}_2\text{O}/\Delta\text{NH}_4^+$ (N₂O_EF) (D).

Fig. S11. Comparisons of experimental and model predicted specific ammonium removal rate (sARR) (A), $\Delta N_2O/\Delta NH_4^+$ (B) and in-cycle dynamics of NH_4^+ , NO_2^- and N_2O (C). Red squares in panel (A) and (B) indicate sARR and $\Delta N_2O/\Delta NH_4^+$ calculated based on corrected DO at 0.67 mg/L. Black triangles in panel (A) indicate sARR calculated according to the AOB proportion in the biomass. Dark blue, red and pink lines in panel (C) represent best fit, while light lines represent 95% confidence intervals from analytical error propagation.

195 Table S3. Model-predicted specific N₂O production rates (N₂OR) of each pathway, and relative contributions to total N₂O production at
196 different pH set-points and baseline operation. Standard deviation is calculated from evaluation of 10 consecutive cycles.

pH	NN		ND		HD		HD consumption	
	N ₂ OR (mgN/gVSS/d)	Contribution (%)	N ₂ OR (mgN/gVSS/d)	Contribution (%)	N ₂ OR (mgN/gVSS/d)	Contribution (%)	N ₂ OR (mgN/gVSS/d)	Contribution (%)
6.5	0.56 ± 0.13	7 ± 2	6.61 ± 1.11	87 ± 15	0.38 ± 0.13	5 ± 2	0.10	1.4
7	0.87 ± 0.17	3 ± 1	30.57 ± 2.87	95 ± 8	0.70 ± 0.13	2 ± 0	0.57	1.8
7.5	0.90 ± 0.09	2 ± 0	46.40 ± 0.65	96 ± 1	1.19 ± 0.04	2 ± 0	0.86	1.8
8	1.14 ± 0.12	2 ± 0	62.87 ± 7.44	93 ± 10	3.87 ± 0.44	6 ± 1	1.81	2.7
8.5	0.80 ± 0.20	2 ± 1	35.56 ± 5.15	95 ± 12	1.02 ± 0.14	3 ± 0	0.72	1.9
B1	0.97 ± 0.18	2 ± 0	49.60 ± 2.35	96 ± 4	1.34 ± 0.11	3 ± 0	0.93	1.8
B4	1.06 ± 0.46	3 ± 1	35.10 ± 4.07	95 ± 11	0.69 ± 0.32	2 ± 1	0.59	1.6

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198 Table S4. Stoichiometric matrix and process rates of NDHA model.

Component ▶ Process ▼	1 S_S	2 S_{O_2}	3 S_{NH_3}	4 S_{NH_2OH}	5 S_{HNO_2}	6 S_{NO_3}	7 S_{NO}	8 S_{N_2O}	9 S_{N_2}	11 $X_{B, AOB}$	12 $X_{B, NOB}$	13 $X_{B, H}$	14 $X_{B, An}$	15 X_S	16 X_I
AOB growth															
Aerobic_AMO 1		-1.14	-1	1											
Aerobic_HAO* 2			$-i_{NXB}$	$-\frac{1}{Y_{AOB}}$			$\frac{1}{Y_{AOB}}$			1					
Aerobic_HAO 3		$-\frac{(2.29 - Y_{AOB})}{Y_{AOB}}$	$-i_{NXB}$	$-\frac{1}{Y_{AOB}}$	$\frac{1}{Y_{AOB}}$					1					
Anox_A_NIR 4				-1	-3		4								
Anox_A_NOR 5				-1			-2	3							
NOB growth															
Aer_NOB_growth 6		$-\frac{(1.14 - Y_{NOB})}{Y_{NOB}}$	$-i_{NXB}$		$-\frac{1}{Y_{NOB}}$	$\frac{1}{Y_{NOB}}$					1				
HB growth															
Aerobic_H_growth 7	$-\frac{1}{Y_{HB}}$	$-\frac{(1 - Y_{HB})}{Y_{HB}}$	$-i_{NXB}$									1			
Anox_H_NAR 8	$-\frac{1}{Y_{HB}}$		$-i_{NXB}$		$\left(\frac{1 - Y_{HB}}{1.14 \cdot Y_{HB}}\right)$	$-\left(\frac{1 - Y_{HB}}{1.14 \cdot Y_{HB}}\right)$						1			
Anox_H_NIR 9	$-\frac{1}{Y_{HB}}$		$-i_{NXB}$		$-\left(\frac{1 - Y_{HB}}{0.57 \cdot Y_{HB}}\right)$		$\left(\frac{1 - Y_{HB}}{0.57 \cdot Y_{HB}}\right)$					1			
Anox_H_NOR 10	$-\frac{1}{Y_{HB}}$		$-i_{NXB}$				$-\left(\frac{1 - Y_{HB}}{0.57 \cdot Y_{HB}}\right)$	$\left(\frac{1 - Y_{HB}}{0.57 \cdot Y_{HB}}\right)$				1			
Anox_H_NOS 11	$-\frac{1}{Y_{HB}}$		$-i_{NXB}$				$-\left(\frac{1 - Y_{HB}}{0.57 \cdot Y_{HB}}\right)$	$\left(\frac{1 - Y_{HB}}{0.57 \cdot Y_{HB}}\right)$				1			
AnAOB growth															
Anox_AnAOB_growth 12			$-i_{NXB} - \frac{1}{Y_{AnAOB}}$		$-\frac{1.52 + 1}{Y_{AnAOB}}$	1.52							1		
Lysis															
AOB 13			$i_{NOB} \cdot f_{li} \cdot i_{NOB} \cdot (1 - f_{li}) \cdot i_{NXS}$							-1				$1 - f_{li}$	f_{li}
NOB 14			$i_{NOB} \cdot f_{li} \cdot i_{NOB} \cdot (1 - f_{li}) \cdot i_{NXS}$								-1			$1 - f_{li}$	f_{li}
HB 15			$i_{NOB} \cdot f_{li} \cdot i_{NOB} \cdot (1 - f_{li}) \cdot i_{NXS}$									-1		$1 - f_{li}$	f_{li}
AnAOB 16			$i_{NOB} \cdot f_{li} \cdot i_{NOB} \cdot (1 - f_{li}) \cdot i_{NXS}$										-1	$1 - f_{li}$	f_{li}
Hydrolysis															
Aerobic 17	1		i_{NXS}											-1	
Anoxic 18	1		i_{NXS}											-1	
Anaerobic 19	1		i_{NXS}											-1	

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Process ▼		Process Rate (g·m ⁻³ ·min ⁻¹)
AOB growth		
Aerobic_AMO	1	$\mu_{AOB}^{AOB} \cdot \frac{S_{O_2}}{S_{O_2} + K_{O_2,AMO}^{AOB}} \cdot \frac{S_{NH_3}}{S_{NH_3} + K_{NH_3}^{AOB} + S_{NH_3}^2 / K_{I,NH_3}^{AOB}} \cdot \frac{K_{I,HNO_2}^{AOB}}{S_{HNO_2} + K_{I,HNO_2}^{AOB}} \cdot X_{AOB}$
Aerobic_HAO*	2	$\mu_{HAO}^{AOB} \cdot \varepsilon_{AOB} \cdot \frac{S_{NH_3OH}}{S_{NH_3OH} + K_{NH_3OH}^{AOB}} \cdot X_{AOB}$
Aerobic_HAO	3	$\mu_{HAO}^{AOB} \cdot (1 - \varepsilon_{AOB}) \cdot \frac{S_{O_2}}{S_{O_2} + K_{O_2,HAO}^{AOB}} \cdot \frac{S_{NH_3OH}}{S_{NH_3OH} + K_{NH_3OH}^{AOB}} \cdot X_{AOB}$
Anox_A_NIR	4	$\mu_{HAO}^{AOB} \cdot \eta_{NIR} \cdot \frac{K_{I,O_2}^{AOB}}{S_{O_2} + K_{I,O_2}^{AOB}} \cdot \frac{S_{NH_3OH}}{S_{NH_3OH} + K_{NH_3OH}^{AOB}} \cdot \frac{S_{HNO_2}}{S_{HNO_2} + K_{HNO_2}^{AOB} + S_{HNO_2}^2 / K_{I,HNO_2,ND}^{AOB}} \cdot X_{AOB}$
Anox_A_NOR	5	$\mu_{HAO}^{AOB} \cdot \eta_{NOR} \cdot \frac{S_{NH_3OH}}{S_{NH_3OH} + K_{NH_3OH}^{AOB}} \cdot \frac{S_{NO}}{S_{NO} + K_{NO,ND}^{AOB}} \cdot X_{AOB}$
NOB growth		
Aer_NOB_growth	6	$\mu_{NOB}^{NOB} \cdot \frac{S_{O_2}}{S_{O_2} + K_{O_2}^{NOB}} \cdot \frac{S_{HNO_2}}{S_{HNO_2} + K_{HNO_2}^{NOB} + S_{HNO_2}^2 / K_{I,HNO_2}^{NOB}} \cdot \frac{K_{I,NH_3}^{NOB}}{S_{NH_3} + K_{I,NH_3}^{NOB}} \cdot X_{NOB}$
HB growth		
Aerobic_HB_growth	7	$\mu_{HB}^{HB} \cdot \frac{S_{O_2}}{S_{O_2} + K_{O_2}^{HB}} \cdot \frac{S_{NH_4}}{S_{NH_4} + K_{NH_4}^{HB}} \cdot \frac{S_S}{S_S + K_S^{HB}} \cdot X_{HB}$
Anox_HB_NAR	8	$\mu_{NAR}^{HB} \cdot \eta_{HD} \cdot \frac{K_{I,O_2,NAR}^{HB}}{S_{O_2} + K_{I,O_2,NAR}^{HB}} \cdot \frac{S_S}{S_S + K_S^{HB}} \cdot \frac{S_{NH_4}}{S_{NH_4} + K_{NH_4}^{HB}} \cdot \frac{S_{NO_2}}{S_{NO_2} + K_{NO_2}^{HB}} \cdot X_{HB}$
Anox_HB_NIR	9	$\mu_{NIR}^{HB} \cdot \eta_{HD} \cdot \frac{K_{I,O_2,NIR}^{HB}}{S_{O_2} + K_{I,O_2,NIR}^{HB}} \cdot \frac{S_{NO}}{S_{NO} + K_{NO,NIR}^{HB}} \cdot \frac{S_{NH_4}}{S_{NH_4} + K_{NH_4}^{HB}} \cdot \frac{S_{NO_2}}{S_{NO_2} + K_{NO_2}^{HB}} \cdot X_{HB}$
Anox_HB_NOR	10	$\mu_{NOR}^{HB} \cdot \eta_{HD} \cdot \frac{K_{I,O_2,NOR}^{HB}}{S_{O_2} + K_{I,O_2,NOR}^{HB}} \cdot \frac{S_{NH_4}}{S_{NH_4} + K_{NH_4}^{HB}} \cdot \frac{S_{NO}}{S_{NO} + K_{NO,NOR}^{HB} + S_{NO}^2 / K_{I,NOR,NOR}^{HB}} \cdot X_{HB}$
Anox_HB_NOS	11	$\mu_{NOS}^{HB} \cdot f(pH) \cdot \eta_{HD} \cdot \frac{K_{I,O_2,NOS}^{HB}}{S_{O_2} + K_{I,O_2,NOS}^{HB}} \cdot \frac{K_{I,NO,NOS}^{HB}}{S_{NO} + K_{I,NO,NOS}^{HB}} \cdot \frac{S_S}{S_S + K_S^{HB}} \cdot \frac{S_{NH_4}}{S_{NH_4} + K_{NH_4}^{HB}} \cdot \frac{S_{N_2O}}{S_{N_2O} + K_{N_2O}^{HB}} \cdot X_{HB}$
AnAOB growth		
Anox_AnAOB_growth	12	$\mu_{AnAOB}^{AnAOB} \cdot \frac{S_{NH_3}}{S_{NH_3} + K_{NH_3}^{AnAOB}} \cdot \frac{S_{HNO_2}}{S_{HNO_2} + K_{HNO_2}^{AnAOB}} \cdot \frac{K_{I,O_2}^{AnAOB}}{S_{O_2} + K_{I,O_2}^{AnAOB}} \cdot X_{AnAOB}$
Lysis		
AOB	13	$b_{AOB} \cdot \left(\frac{S_{O_2}}{S_{O_2} + K_{O_2,b}} + \eta_{b,anox} \cdot \frac{K_{O_2,b}}{K_{O_2,b} + S_{O_2}} \cdot \frac{S_{NO_2}}{K_{NO_2} + S_{NO_2}} + \eta_{b,anox} \cdot \frac{K_{O_2,b}}{K_{O_2,b} + S_{O_2}} \cdot \frac{K_{NO_2}}{K_{NO_2} + S_{NO_2}} \right) \cdot X_{AOB}$
NOB	14	$b_{NOB} \cdot (...) \cdot X_{NOB}$
HB	15	$b_{HB} \cdot (...) \cdot X_{HB}$
AnAOB	16	$b_{AnAOB} \cdot (...) \cdot X_{AnAOB}$
Hydrolysis		
Aerobic	17	$k_H \cdot \frac{X_S / X_{BH}}{K_X + X_S / X_{BH}} \cdot \frac{S_{O_2}}{K_{O_2}^{HB} + S_{O_2}} \cdot X_{HB}$
Anoxic	18	$k_H \cdot \eta_{ANOX} \cdot \frac{X_S / X_{BH}}{K_X + X_S / X_{BH}} \cdot \frac{K_{O_2}^{HB}}{K_{O_2}^{HB} + S_{O_2}} \cdot \frac{S_{NO_2}}{K_{NO_2}^{HB} + S_{NO_2}} \cdot X_{HB}$
Anaerobic	19	$k_H \cdot \eta_{AN} \cdot \frac{X_S / X_{BH}}{K_X + X_S / X_{BH}} \cdot \frac{K_{O_2}^{HB}}{K_{O_2}^{HB} + S_{O_2}} \cdot \frac{K_{NO_3}^{HB}}{K_{NO_3}^{HB} + S_{NO_3}} \cdot X_{HB}$

201 Table S5. Parameters applied in NDHA model.

Parameter	Definition	Value	Units	Ref.
AOB				
$K_{AOB.NH_2OH}$	S_{NH_2OH} affinity for AOB	0.3	mgN/L	(1)
$K_{AOB.NH_2OH.ND}$	S_{NH_2OH} affinity for AOB during NO reduction	0.159	mgN/L	(t.s.)
$K_{AOB.NH_3}$	S_{NH_3} affinity for AOB	0.277	mgN/L	(t.s.)
$K_{AOB.NO.ND}$	S_{NO} affinity for AOB	0.004	mgN/L	(1)
$K_{AOB.HNO_2.ND}$	S_{HNO_2} affinity for ND, AOB	0.137	μ gN/L	(t.s.)
$K_{AOB.i.HNO_2.ND}$	S_{HNO_2} inhibition constant for ND, AOB	0.0167	mgN/L	(t.s.)
$K_{AOB.O_2.AMO}$	S_{O_2} AMO-mediated affinity constant	0.05	mgO ₂ /L	(1)
$K_{AOB.O_2.HAO}$	S_{O_2} HAO-mediated affinity constant	0.3	mgO ₂ /L	(1)
$K_{AOB.O_2.i}$	S_{O_2} inhibition constant for AOB	0.199	mgO ₂ /L	(t.s.)
$K_{AOB.i.NH_3}$	S_{NH_3} inhibition constant for AOB	38.45	mgN/L	(t.s.)
$K_{AOB.i.HNO_2}$	S_{HNO_2} inhibition constant for AOB	0.59	mgN/L	(6)
ε_{AOB}	Reduction factor for HAO-mediated maximum reaction rate	0.000483	(-)	(1)
η_{NIR}	Anoxic reduction factor for NO ₂ reduction	0.358	(-)	(t.s.)
η_{NOR}	Reduction factor for NO reduction	0.15	(-)	(1)
$\mu_{AOB.AMO}$	Maximum AMO-mediated reaction rate	0.26	1/d	(t.s.)
$\mu_{AOB.HAO}$	Maximum HAO-mediated reaction rate	0.78	1/d	(2)
b_{AOB}	Endogenous decay rate for AOB	0.096	1/d	(2)
Y_{AOB}	Yield coefficient for AOB	0.18	mgCOD/mgN	(2)
NOB				
$K_{NOB.HNO_2}$	S_{HNO_2} affinity for NOB	0.1	μ gN/L	(3)
$K_{NOB.O_2}$	S_{O_2} affinity constant for NOB	1.2	mgO ₂ /L	(2)
$K_{NOB.i.NH_3}$	S_{NH_3} inhibition constant for NOB	0.2	mgN/L	(3)
$K_{NOB.i.HNO_2}$	S_{HNO_2} inhibition constant for NOB	0.04	mgN/L	(3)
μ_{NOB}	Maximum NOB growth	0.78	1/d	(2)
b_{NOB}	Endogenous decay rate for NOB	0.096	1/d	(2)
Y_{NOB}	Yield coefficient for NOB	0.06	mgCOD/mgN	(2)
Others				
f_{XI}	Fraction of inerts in biomass	0.08	(-)	(2)
i_{NXB}	Nitrogen content of biomass	0.086	mgN/mgCOD	(2)
i_{NXI}	Nitrogen content of inerts	0.02	mgN/mgCOD	(2)
i_{NXS}	Nitrogen content of particulate	0.06	mgN/mgCOD	(2)
$\eta_{b,anox}$	Anoxic reduction factor of endogenous decay	0.4	(-)	(3)
$\eta_{b,anaer}$	Anaerobic reduction factor of endogenous decay	0.4	(-)	(3)
$K_{O_2.b}$	S_{O_2} affinity constant of endogenous decay	0.2	mgO ₂ /L	(3)
K_{NOx}	$S_{NO_2}+S_{NO_3}$ affinity constant of endogenous decay	0.2	mgN/L	(3)
k_H	Hydrolysis rate	2.21	1/d	(3)
K_X	Affinity constant for hydrolysis	0.15	mgCOD/mgCOI	(3)
$\eta_{h,anox}$	Anoxic hydrolysis factor	0.4	(-)	(3)
$\eta_{h,anaer}$	Anaerobic hydrolysis factor	0.4	(-)	(3)

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Parameter	Definition	Value	Units	Ref.
HB				
$K_{HB.NH4}$	S_{NH4} affinity constant for HB	0.01	mgN/L	(3)
$K_{HB.NO3}$	S_{NO3} affinity constant for HB	0.2	mgN/L	(3)
$K_{HB.NO2}$	S_{NO2} affinity constant for HB	0.2	mgN/L	(3)
$K_{HB.NO}$	S_{NO} affinity constant for HB	0.05	mgN/L	(3)
$K_{HB.N2O}$	S_{N2O} affinity constant for HB	0.05	mgN/L	(3)
$K_{HB.S}$	S_S affinity constant for HB	20	mgCOD/L	(3)
$K_{HB.S.NAR}$	S_S affinity constant for S_{NO3} reduction	20	mgCOD/L	(3)
$K_{HB.S.NIR}$	S_S affinity constant for S_{NO2} reduction	20	mgCOD/L	(3)
$K_{HB.S.NOR}$	S_S affinity constant for S_{NO} reduction	20	mgCOD/L	(3)
$K_{HB.S.NOS}$	S_S affinity constant for S_{N2O} reduction	40	mgCOD/L	(3)
$K_{HB.O2}$	S_{O2} affinity constant for HB	0.1	mgO2/L	(3)
$K_{HB.O2.i.NAR}$	S_{O2} inhibition constant for S_{NO3} reduction	0.1	mgO2/L	(3)
$K_{HB.O2.i.NIR}$	S_{O2} inhibition constant for S_{NO2} reduction	0.1	mgO2/L	(3)
$K_{HB.O2.i.NOR}$	S_{O2} inhibition constant for S_{NO} reduction	0.1	mgO2/L	(3)
$K_{HB.O2.i.NOS}$	S_{O2} inhibition constant for S_{N2O} reduction	0.1	mgO2/L	(3)
$K_{HB.NO.i.NIR}$	S_{NO} inhibition constant for S_{NO2} reduction	0.5	mgN/L	(3)
$K_{HB.NO.i.NOR}$	S_{NO} inhibition constant for S_{NO} reduction	0.3	mgN/L	(3)
$K_{HB.NO.i.NOS}$	S_{NO} inhibition constant for S_{N2O} reduction	0.075	mgN/L	(3)
μ_{HB}	Maximum HB growth rate	6.24	1/d	(3)
$\mu_{HB.NAR}$	Maximum $NO3$ -reduction reaction rate	1.75	1/d	(3)
$\mu_{HB.NIR}$	Maximum $NO2$ -reduction reaction rate	1	1/d	(3)
$\mu_{HB.NOR}$	Maximum NO -reduction reaction rate	2.18	1/d	(3)
$\mu_{HB.NOS}$	Maximum $N2O$ -reduction reaction rate	2.18	1/d	(3)
η_{HD}	Reduction factor for HB denitrification	1	(-)	(3)
b_{HB}	Endogenous decay rate for HB	0.41	1/d	(2)
Y_{HB}	Yield coefficient for HB	0.6	mgCOD/mgCOD	(2)
AnAOB				
$K_{AnAOB.HNO2}$	S_{HNO2} affinity for AnAOB	0.0028	μ gN/L	(4)
$K_{AnAOB.NH3}$	S_{NH3} affinity for AnAOB	0.0012	mgN/L	(4)
$K_{AnAOB.i.O2}$	S_{O2} inhibition constant for AnAOB	0.01	mgO2/L	(4)
μ_{AnAOB}	Maximum AnAOB growth	0.028	1/d	(5)
b_{AnAOB}	Endogenous decay rate for AnAOB	0.001	1/d	(5)
Y_{AnAOB}	Yield coefficient for AnAOB	0.16	mgCOD/mgN	(4)

(1) Domingo-Felez et al. 2017, (2) Henze 2000, (3) Hiatt-Grady 2008, (4) Strous 1998, (5) Hao 2002, (6) Park 2009

(t.s.) this study

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Section 5 – Abiotic batch tests

Reactive intermediates during the nitrification processes (e.g. NH_2OH) may engage in chemical reactions that yield N_2O , especially in the presence of trace metals (e.g. iron) (Heil et al., 2016). The dominant chemical reactions, environmentally relevant to nitrification reactor conditions, that yield N_2O are (Schreiber et al., 2012): (1) The oxidation of NH_2OH by HNO_2 ($\text{NH}_2\text{OH} + \text{HNO}_2 \rightarrow \text{N}_2\text{O} + 2\text{H}_2\text{O}$); The oxidation of NH_2OH by O_2 ($2\text{NH}_2\text{OH} + \text{O}_2 \rightarrow \text{N}_2\text{O} + 3\text{H}_2\text{O}$); (3) The disproportionation of NH_2OH ($4\text{NH}_2\text{OH} \rightarrow 2\text{NH}_3 + \text{N}_2\text{O} + 3\text{H}_2\text{O}$); (4) The reduction of HNO_2 by Fe^{2+} ($\text{HNO}_2 + 2\text{Fe}^{2+} + 2\text{H}^+ \rightarrow 2\text{Fe}^{3+} + 0.5\text{N}_2\text{O} + 1.5\text{H}_2\text{O}$); (5) The oxidation of NH_2OH by Fe^{3+} ($4\text{Fe}^{3+} + 2\text{NH}_2\text{OH} \rightarrow 4\text{Fe}^{2+} + \text{N}_2\text{O} + \text{H}_2\text{O} + 4\text{H}^+$). Here, abiotic batch tests (without biomass) were performed to quantify N_2O production rates through these chemical reactions under similar operational conditions in our nitrification reactors during pH experiments (i.e. 17.8 mM NO_2^- , 0.07 mM NH_2OH , 0.5 mM FeSO_4 and 0.5 mM FeCl_3) (Su et al., 2019). The tests were conducted in a 0.4 L jacketed glass vessel at room temperature (24-26°C) in deionized water (diH_2O) or synthetic medium under high DO (8-8.4 mg O_2/L) or low DO (< ca.1 mg O_2/L) conditions. The composition of synthetic medium was the same as used for the reactor influent.

The highest N_2O production rates were measured for the reaction of NH_2OH with HNO_2 , followed by HNO_2 reduction by Fe^{2+} , NH_2OH oxidation by Fe^{3+} , and finally NH_2OH disproportionation and/or oxidation by O_2 . The estimated abiotic N_2O production rates was 1-5 orders of magnitude lower than overall N_2O production rates in the reactor across the examined pH range (Table S6). Correspondingly, the abiotic contributions accounted for less than 3% of total N_2O production and varied with pH, increasing from 0.025% at pH = 8 to 2.6% at pH = 6.5 for the reaction of NH_2OH with HNO_2 .

227 Table S6. The contribution of abiotic reactions to overall N₂O production in nitrification reactors at different pH set-points

Total N ₂ O production in nitrification reactors					Abiotic N ₂ O production			
Experimental condition				Measured total N ₂ O production rates (mg N/L/h)	Considered abiotic reactions	Method	Estimated abiotic N ₂ O production rates (mg N/L/h)	Fraction of abiotic pathway to total N ₂ O production (%)
pH	NH ₂ OH (mg N/L)	HNO ₂ (mg N/L)	NO ₂ ⁻ (mg N/L)					
6.5	0.062 ± 0.008	0.065 ± 0.012	163.3 ± 25.5	0.16 ± 0.06	NH ₂ OH oxidation by HNO ₂	Abiotic batch tests without biomass	4.1 ± 1.0 × 10 ⁻³	2.6 ± 1.2
					NH ₂ OH disproportionation and/or oxidation by O ₂		4.4 ± 1.0 × 10 ⁻⁵	0.028 ± 0.013
					HNO ₂ reduction by Fe ²⁺		4.9 × 10 ⁻⁵	0.031
7.0	0.058 ± 0.017	0.030 ± 0.002	233.1 ± 11.9	0.56 ± 0.28	NH ₂ OH oxidation by HNO ₂	Abiotic batch tests without biomass	8.2 ± 2.5 × 10 ⁻⁴	0.15 ± 0.087
8.0	0.052 ± 0.014	0.0038 ± 0.0001	282.9 ± 2.0	1.97 ± 0.37	NH ₂ OH oxidation by HNO ₂		4.9 × 10 ⁻⁴	0.025 ± 0.0047
					NH ₂ OH disproportionation and/or oxidation by O ₂		6.0 ± 1.7 × 10 ⁻⁶	0.00031 ± 0.00010
					HNO ₂ reduction by Fe ²⁺	1.6 × 10 ⁻⁵	0.0008	

Section 6 - Batch tests on N₂O consumption and production by heterotrophic denitrifiers

Off-line batch tests were performed to measure potential N₂O production and consumption rates of denitrifiers in the biomass under different DO conditions. Biomass samples were collected and centrifuged at 3600 g for 5 min, washed and re-suspended in nitrogen-free mineral medium 3 times to eliminate any soluble substrate. The VSS concentrations (gVSS/L) of biomass sample were measured prior to the initiation of the tests. Tests were performed in parallel at 25°C in two 400 mL jacketed glass vessels completely filled with biomass and sealed with the insertion of DO, pH and N₂O sensors for online monitors. Stock solutions of N₂O and oxygen (O₂) were obtained by sparging ≥ 99.998% N₂O gas and pure oxygen (Sigma-Aldrich), respectively, in deionized water. N₂O and O₂ were then added to the vessels to measure N₂O consumption rates of denitrifiers under different DO levels. For the detection of N₂O production rates by heterotrophic denitrifiers, 2.5 g N/L of stock NaNO₂ solution was added to reach final NO₂⁻ concentration of 300 mg N/L. Sodium acetate (C₃H₅NaO₂) was supplied in stoichiometric excess as the organic carbon source during batch tests. Instantaneous N₂O consumption and production rates (mg N/L/min) were determined through linear regression of the measured concentration profiles of N₂O (mg N/L). The specific N₂O consumption and production rates (g N/g VSS/d) were calculated and normalized to the VSS concentration.

Fig. S12. N₂O consumption rates by heterotrophic denitrifiers under oxic and anoxic conditions.

Fig. S13. N₂O production rates by heterotrophic denitrifiers at 300 mg NO₂⁻-N/L, 0.3 ± 0.1 mg O₂/L, pH 7.8.

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