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Published in:
Water Research

Link to article, DOI:
[10.1016/j.watres.2018.06.025](https://doi.org/10.1016/j.watres.2018.06.025)

Publication date:
2018

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Chen, X., Yuan, Z., & Ni, B-J. (2018). Nitrite accumulation inside sludge flocs significantly influencing nitrous oxide production by ammonium-oxidizing bacteria. *Water Research*, 143, 99-108.
<https://doi.org/10.1016/j.watres.2018.06.025>

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Accepted Manuscript

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PII: S0043-1354(18)30475-5

DOI: [10.1016/j.watres.2018.06.025](https://doi.org/10.1016/j.watres.2018.06.025)

Reference: WR 13852

To appear in: *Water Research*

Received Date: 23 January 2018

Revised Date: 12 June 2018

Accepted Date: 13 June 2018

Please cite this article as: Chen, X., Yuan, Z., Ni, B.-J., Nitrite accumulation inside sludge flocs significantly influencing nitrous oxide production by ammonium-oxidizing bacteria, *Water Research* (2018), doi: 10.1016/j.watres.2018.06.025.

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Graphical Abstract

**Nitrite Accumulation inside Sludge Flocs Significantly Influencing Nitrous Oxide
Production by Ammonium-Oxidizing Bacteria**

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ABSTRACT

This work aims to clarify the role of potential nitrite (NO_2^-) accumulation inside sludge flocs in N_2O production by ammonium-oxidizing bacteria (AOB) at different dissolved oxygen (DO) levels with focus on the conditions of no significant bulk NO_2^- accumulation ($<0.2 \text{ mg N/L}$). To this end, an augmented nitrifying sludge with much higher abundance of nitrite-oxidizing bacteria (NOB) than AOB was enriched and then used for systematically designed batch tests, which targeted a range of DO levels from 0 to $3.0 \text{ mg O}_2/\text{L}$ at a fixed ammonium concentration of 10 mg N/L . A two-pathway N_2O model was applied to facilitate the interpretation of batch experimental data, thus shedding light on the relationships between N_2O production pathways and key process parameters (i.e., DO and NO_2^- accumulation inside sludge flocs). The results demonstrated (i) the biomass specific N_2O production rate firstly increased and then decreased with DO, with the maximum value of $3.03 \pm 0.05 \text{ mg N/h/g VSS}$ obtained at DO level of $0.75 \text{ mg O}_2/\text{L}$, (ii) the AOB denitrification pathway for N_2O production was dominant (98.0%) at all DO levels tested even without significant bulk NO_2^- accumulation ($<0.2 \text{ mg N/L}$) observed in the system, but its contribution decreased with DO, (iii) DO had a positive impact on the hydroxylamine pathway for N_2O production which therefore increased with DO, and (iv) the nitrite accumulation existed inside the sludge flocs and induced significant N_2O production from the AOB denitrification pathway.

Keywords: Nitrous oxide (N_2O), nitrite accumulation, dissolved oxygen, production pathway, mathematical modelling

1. INTRODUCTION

Wastewater treatment plants (WWTPs) have been identified as a significant source of nitrous oxide (N_2O). N_2O is not only a significant greenhouse gas with a global warming effect 300 times stronger than carbon dioxide but also a major scavenger of stratospheric ozone leading to ozone layer depletion (IPCC 2013, Ravishankara et al. 2009, Strokal and Kroeze 2014). During biological nitrogen removal from wastewater, nitrification, which requires oxygen as the electron acceptor to convert ammonium (NH_4^+) to nitrite (NO_2^-) via hydroxylamine (NH_2OH) as an intermediate, is mediated by ammonium-oxidizing bacteria (AOB) and regarded as the major contributor to N_2O production (Kampschreur et al. 2009, Law et al. 2012). Two main pathways are involved in N_2O production by AOB: (i) N_2O as a by-product of incomplete oxidation of NH_2OH to NO_2^- , called the NH_2OH pathway and (ii) N_2O as the final product of AOB denitrification with NO_2^- as the terminal electron acceptor, known as the AOB denitrification pathway (Chandran et al. 2011). When present, ammonium-oxidizing archaea (AOA) might also contribute to N_2O production (Hu et al. 2015). Different responses of N_2O contributing pathways to different operational conditions and process parameters have been reported to be responsible for the wide variation of N_2O emission factors recorded (Massara et al. 2017, Su et al. 2017). Of all process parameters, dissolved oxygen (DO) and NO_2^- have been identified as two key ones influencing N_2O production by AOB during nitrification. However, consensus has not yet been reached in terms of the effects of DO and NO_2^- on N_2O production by AOB.

In general, both full-scale and lab-scale studies have presented a positive relationship between N_2O production by AOB and NO_2^- concentration (Foley et al. 2010, Gong et al. 2012, Kim et al. 2010, Shen et al. 2014, Shiskowski and Mavinic 2006, Wunderlin et al. 2012), which might be

explained by the favored AOB denitrification pathway at higher NO_2^- concentration (Peng et al. 2015). On the contrary, Law et al. (2013) observed an inhibitory effect of NO_2^- on N_2O production by AOB in a nitrification system at NO_2^- concentration of higher than 50 mg N/L. This phenomenon might be the result of the enhanced NH_2OH pathway and the decreased contribution of the AOB denitrification pathway for N_2O production at elevated NO_2^- concentration, which was supported by a modelling study by Ni et al. (2014). Similarly, some studies have reported the increased N_2O production with decreased DO concentration due to the enhanced AOB denitrification pathway under oxygen limiting conditions (Kampschreur et al. 2008, Peng et al. 2014). In contrast, different correlations between N_2O production and DO concentration have been discovered by other studies (Tallec et al. 2006, Yang et al. 2009). The discrepancy might be related to the facilitated N_2O production via the NH_2OH pathway under aerobic conditions when DO concentration was high (Chandran et al. 2011).

As discussed by Peng et al. (2014), the common presence of heterotrophic bacteria (HB) complicates the accurate deduction of the N_2O production profile by AOB. The metabolisms of HB involve N_2O as an obligate intermediate, with its production and consumption also affected by both DO and NO_2^- (e.g., Wunderlin et al. (2012), Yang et al. (2009)). Moreover, difficulty remains in separating the effect of DO from that of NO_2^- , considering their close relationship in the metabolisms of AOB. For example, when investigating the impact of DO on N_2O production by AOB, variation of DO concentration usually brings about variable NO_2^- accumulation, thus incurring an accompanying effect of NO_2^- . By adding extra sludge enriched with nitrite-oxidizing bacteria (NOB) to nitrifying sludge (consisting of both AOB and NOB) to consume NO_2^- produced by AOB, Peng et al. (2014) attempted to single out the sole effect of DO on N_2O

production by AOB. However, the interference of NO_2^- could not be completely ruled out in view of the low but still apparent NO_2^- accumulation (1.5~2.0 mg N/L) observed in the bulk liquid, especially given the low NO_2^- affinity constant for the AOB denitrification pathway (e.g., 0.14 mg N/L in Ni et al. (2011)). Besides, as microbial activities mainly take place inside the nitrifying sludge flocs, there usually exists substrate concentration gradient within the domain of certain floc sizes. Therefore, the concentration of NO_2^- produced by AOB inside the nitrifying sludge flocs might be higher than that measured in the bulk liquid. This means that NO_2^- might still exert an impact on N_2O production even in the absence of significant NO_2^- detected in the bulk liquid. To the best of our knowledge, there has been no study reported looking into whether the potential nitrite accumulation inside nitrifying sludge flocs, if any, would affect N_2O production by AOB. This overlook of the role of potential nitrite accumulation in floccular structure in N_2O production is mainly due to the generally low nitrite accumulation potentially present in floccular structure when compared with granular structure.

Therefore, this work aims to clarify the role of potential nitrite accumulation inside sludge flocs in N_2O production by AOB at different DO levels for the first time, focusing on the conditions of no significant bulk NO_2^- accumulation (<0.2 mg N/L). To get rid of NO_2^- accumulation in the bulk liquid when varying DO, an augmented nitrifying culture comprising mainly NOB with much lesser coexistence of AOB in same flocs was enriched. With the potential contribution of HB to either consumption or production of N_2O being identified, batch tests were then conducted to quantify N_2O production at different DO levels using the augmented nitrifying sludge. To determine the nitrite content potentially accumulating inside the nitrifying sludge flocs, high-output sonication was employed to treat samples collected during batch tests in order to release

nitrite from the flocs. Mathematical modelling was also applied to gain insight into the contributions of the two known N_2O production pathways by AOB. This work is expected to shed more light on the N_2O production dynamics in activated sludge flocs, which remain as the main form of sludge adopted at WWTPs for nitrogen removal technologies, such as nitrification and denitrification which has been reported to exhibit significant N_2O emissions (Massara et al. 2017).

2. MATERIALS AND METHODS

2.1. Enrichment of Augmented Nitrifying Culture

A sequencing batch reactor (SBR) seeded with sludge collected from Luggage Point WWTP in Brisbane, Australia was operated in the lab with temperature controlled at 22.0 – 23.0 °C. The SBR had a working volume of 8 L and was operated with a cycle time of 6 h consisting of 260 min aerobic feeding, 25 min aeration, 0.5 min sludge wasting, 50 min settling and 24.5 min decanting. The 260 min aerobic feeding period was further divided into 52 cycles and each cycle consisted of 1 min feeding with aeration and 4 min aeration without feeding. The purpose of this setup was to avoid potential nitrite inhibition during the enrichment process. 2 L of synthetic wastewater was fed to the SBR during each 6 h cycle, resulting in a hydraulic retention time (HRT) of 24 h. By wasting 80 mL of mixed sludge during the 0.5 min sludge wasting period, the solids retention time (SRT) was kept around 13 days. With the programmed logic controller (PLC) installed, DO in the SBR, which was continuously monitored online with a miniCHEM DO meter, was controlled between 2.5 and 3.0 mg O_2 /L during the feeding and aerobic phases using compressed air. pH was measured on a miniCHEM pH meter and regulated between 7.4

and 7.6 during the feeding and aerobic phases by dosing 1M NaHCO_3 or 1M HCl through a small peristaltic pump.

To obtain the augmented nitrifying sludge with much higher abundance of NOB than AOB, the feeding concentration of NO_2^- in the form of NaNO_2 was subject to stepwise increase from 250 mg N/L to 1250 mg N/L while that of NH_4^+ in the form of NH_4HCO_3 was fixed at 250 mg N/L. This decreasing $\text{NH}_4^+\text{-N}/\text{NO}_2^-\text{-N}$ ratio from 250:500 (i.e., 1:2) to 250:1500 (i.e., 1:6) in the feed ($\text{NO}_2^-\text{-N}$ included that produced by AOB) significantly favored the growth of NOB over AOB in the nitrifying sludge. In addition to NaNO_2 and NH_4HCO_3 , the synthetic wastewater fed to the SBR also contained (per liter) 2.0 g NaHCO_3 , 1.3 g K_2HPO_4 and 1.3 g KH_2PO_4 as well as 2 mL of trace element solution with composition specified in Peng et al. (2014).

At the time when the batch tests detailed in the next section were conducted, the SBR was already operated in steady state for more than 2 months, as evidenced by 100% conversion of feeding NO_2^- and NH_4^+ to NO_3^- at the end of each cycle as well as the stable mixed liquor volatile suspended solids (MLVSS) concentration of 1523 ± 40 mg/L ($n = 11$) in the SBR. Both the settling velocity index (~ 50) and morphology (i.e., tawny flocs with size D_{50} of 114 μm) of the augmented nitrifying sludge flocs were determined to be consistent with the typical activated sludge flocs. The dominance of NOB over AOB in the augmented nitrifying sludge was confirmed by fluorescence in-situ hybridization (FISH) analysis (see Figure S1 in the supporting information (SI)) which was conducted according to the procedure applied by Law et al. (2011). The biovolume fraction of the bacteria of interest was determined by analyzing 10 FISH images using DAIME version 1.3 (Daims et al. 2001) which returns mean percentages with standard

deviations. The FISH analysis indicated that $35 \pm 3\%$ of the EUBMix probe targeted cells bound to NSO1225 probe, representing *beta-proteobacterial* AOB including *Nitrospira*, *Nitrosococcus* and *Nitrosomonas* genera, while $52 \pm 5\%$ of the EUBMix probe targeted cells bound to NIT3 probe, specific for NOB belonging to *Nitrobacter* genus. NOB belonging to *Nitrospira* genus was indicated to be absent or of extremely low abundance, considering the fact that no signals were detected for Ntspa662 and Ntspa712 probes. The 16S rRNA gene pyrosequencing analysis was also conducted. The DNA of the augmented nitrifying sludge was extracted using a FastDNA SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instruction. The extracted DNA sample was sent to Australian Centre for Ecogenomics (ACE) for 16S Amplicon sequencing by Illumina Miseq Platform. The result was analyzed according to Hülsemann et al. (2016) and showed an AOB (*Nitrosomonas*):NOB (*Nitrobacter*) ratio of 1:2.3 in terms of operational taxonomic unit (OTU), which was comparable to the FISH result. The absence of *Nitrospira* detected by pyrosequencing was also consistent with the FISH analysis.

2.2. Batch Experiments

To clarify the role of potential nitrite accumulation inside sludge flocs in N_2O production by AOB at different DO levels without significant bulk NO_2^- accumulation ($<0.2 \text{ mg N/L}$), nine sets of triplicate batch tests were carried out, targeting nine DO levels, i.e., 0, 0.2, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 and $3.0 \text{ mg O}_2/\text{L}$. For each batch test, 0.16 L of mixed liquor was withdrawn from the SBR and diluted to 1 L using decant from the SBR, thus rendering a final concentration of mixed liquor suspended solids (MLSS) of 320 mg/L and its volatile fraction (i.e., MLVSS) of 295 mg/L . The diluted mixed liquor was adjusted to pH of 7.5 and placed in a 1.3 L batch reactor with a

sealable lid on a magnetic stirrer (200 rpm) at 22.0 – 23.0 °C. A miniCHEM DO meter and a miniCHEM pH meter were mounted in the batch reactor, enabling continuous online monitoring of DO and pH, respectively. During the entire experiment, DO concentration was controlled at the designed level with a gas mixture of N₂ and air. The N₂ flow rate and air flow rate were adjusted using two mass flow controllers (Smart-Trak 50 series, Sierra) with the total gas flow rate being controlled constantly at 0.5 L/min: each change in the N₂ flow rate was compensated for by an equivalent change in the air flow rate. pH was maintained at 7.5 by dosing 0.1 M HCl manually using a dropper seamlessly attached to the batch reactor.

Each batch test lasted for 60 min including 10 min control phase and 50 min experimental phase. The same DO level was applied to both phases. In the control phase, no NH₄⁺ was added and any N₂O production could therefore be attributed to heterotrophic activity. In the following experimental phase of all batch tests, the NH₄⁺ concentration was controlled at 10 mg N/L through manually adding a stock solution of 22.6 g/L NH₄HCO₃ and 24 g/L NaHCO₃ at an interval of 10 min, with amount determined according to the method described in Peng et al. (2014). During each test, 7.5 mL mixed liquor sample was taken every 10 min using a syringe. 5.0 mL of the mixed liquor sample was immediately filtered through disposable Millipore filter (0.22 µm pore size). Half of the 5.0 mL filtered sample was stored for liquid phase N₂O measurement using a gas chromatograph (Agilent GC7890A, Agilent Technologies, Santa Clara, California, USA) while the other half for bulk NO₂⁻, NH₄⁺ and NO₃⁻ analyses. The remaining 2.5 mL of the mixed liquor sample was firstly treated with sonication using Branson Sonifier 250 at the maximum amplitude output of as high as 100% for 3 min consistently to release substances accumulating inside sludge flocs to the liquid phase (Mozo et al. 2016, Salsabil et al. 2009). The

effectiveness of this sonication setup was proved by the change in the particle size distribution D50 measured using the Malvern Mastersizer/E, which significantly decreased from 114 μm to 10 μm after high-output sonication treatment. The treated sample was then filtered through disposable Millipore filter (0.22 μm pore size) to measure the post-sonication NO_2^- , NH_4^+ and NO_3^- concentrations. During the entire batch test with a constant gas flow rate of 0.5 L/min, N_2O concentration in the gas phase of the batch reactor was recorded consistently with a URAS 26 infrared photometer (Advance Optima Continuous Gas Analyzer AO2020 series, ABB) following the instruction reported by Peng et al. (2014). At the end of each batch test, 20 mL mixed liquor sample was taken in triplicate from the batch reactor to determine the concentrations of MLSS and MLVSS.

To evaluate the possible N_2O consumption by HB, four N_2O consumption tests were also conducted at DO levels of 0.5, 1.0, 2.0 and 3.0 mg O_2/L using the same diluted augmented nitrifying sludge. The headspace in the batch reactor was removed by adding the same diluted augmented nitrifying sludge, and the URAS 26 infrared photometer was disconnected. Aeration was stopped after DO reached the designed level, and 1.5 mL of saturated N_2O solution was added into the batch reactor. Afterwards the batch reactor was immediately sealed. Samples were taken every 10 min and immediately filtered for measurement of N_2O concentration in the liquid phase. Then N_2O consumption rate at each DO level was determined from the corresponding N_2O concentration profile measured.

2.3. Calculations

The biomass specific ammonium oxidation rate (AOR), biomass specific N_2O production rate (N_2OR) and N_2O emission factor (i.e., the ratio between the N_2O nitrogen emitted and the ammonium nitrogen converted) were calculated for each batch test. The N_2O nitrogen emitted was quantified as the integral of N_2O emission rate calculated by multiplying the gas phase N_2O concentration measured on the URAS 26 infrared photometer and the known gas flow rate over time. It should be noted that only steady-state AOR, N_2OR and N_2O emission factor during the second half of the 60 min testing period were accounted for in this work. In view of the nearly constant NH_4^+ concentration during each batch test achieved by periodical dosing of NH_4HCO_3 , the converted NH_4^+ for AOR was calculated according to the exact same amount of NH_4^+ added during each 10 min period. The calculation of N_2OR was based on the accumulative amount of N_2O emitted during the 30 min steady state. The biomass specific AOR ($\text{mg NH}_4^+\text{-N/h/g VSS}$) and biomass-specific N_2OR ($\text{mg N}_2\text{O-N/h/g VSS}$) were calculated by normalizing the AOR and N_2OR data with the MLVSS concentration of the diluted augmented nitrifying sludge. The N_2O emission factor was calculated as the ratio between N_2OR and AOR obtained for each batch test.

2.4. Chemical Analyses

NO_2^- , NH_4^+ and NO_3^- concentrations were analysed using a Lachat QuikChem8000 Flow Injection Analyser (Lachat Instrument, Milwaukee). MLSS and MLVSS concentrations were measured according to the standard methods (APHA 1998).

2.5. Model-Based Interpretation

The two-pathway N_2O model proposed by Ni et al. (2014), which links the oxidation and reduction processes through a pool of reduced form (M_{red}) and oxidized form (M_{ox}) of electron

carriers, was employed in this work to interpret the experimental data. In the model, both the AOB denitrification pathway and the NH_2OH pathway consume M_{red} and generate the equivalent level of M_{ox} . Though other processes such as ammonium oxidation, NH_2OH oxidation and O_2 reduction complicate the turnover between M_{red} and M_{ox} , the total level of electron carriers is assumed to remain constant. Additionally, an oxygen inhibition term was included to consider the impact of DO on the AOB denitrification pathway (Ni et al. 2011, Pocquet et al. 2016). The soluble and particulate model components are listed in Table S1 in the SI, while the stoichiometric/kinetic matrix and the parameters of the two-pathway N_2O model are summarized in Tables S2 and S3, respectively. The augmented nitrifying sludge flocs were modelled as a floc-based particle system in consideration of inside substrate diffusion using the AQUASIM software package (Reichert 1998). Similar to Volcke et al. (2010), flocs were modelled to be spherical in shape, i.e., as particles. Only radial diffusion transport described by Fick's law was considered, and all relevant diffusion coefficients were taken from Sabba et al. (2015). The purpose of this modelling setup, different from Peng et al. (2014), was to enable the investigation into the role of potential nitrite accumulation inside sludge flocs in N_2O production by AOB. A similar particle-based modelling setup has been applied to simulate activated sludge flocs considering their structural characteristics as aggregates of microbial origin (Li and Bishop 2003a, Li and Bishop 2003b) so that microenvironments inside sludge flocs could be described. A particle size distribution was avoided in this work to reduce the complexity of numerical computations whilst retaining a reasonably good understanding of the system (Ni et al. 2009). Therefore, following the method of Ni et al. (2009), the number of the augmented nitrifying sludge flocs in the reactor was calculated, assuming a uniform particle size of $114\text{ }\mu\text{m}$ (i.e., D_{50}) and a density of 50000 mg COD/L (Koch et al. 2000).

262

263 For the modelling, the biomass concentrations of AOB and NOB in the augmented nitrifying
264 sludge were determined based on the measured MLVSS concentrations together with the relative
265 biomass abundance quantified by 16S rRNA gene pyrosequencing (i.e., AOB:NOB ratio of
266 1:2.3). Following the common modelling practice, a conversion factor of 1.42 (i.e., ratio between
267 COD and VSS) was used to determine biomass concentration as COD (Contreras et al. 2002). In
268 view of the incapability of the model configured with parameters directly taken from reported
269 literature (see Table S3 in the SI) to describe the batch experimental data in this work (data not
270 shown), a model calibration was imperative to obtain a reliable model for understanding the N₂O
271 production by AOB in the augmented nitrifying sludge of this work. Prior to model calibration, a
272 sensitivity analysis was firstly conducted using the AQUASIM built-in algorithms to locate the
273 most important parameter(s) which could be reliably estimated under the batch experimental
274 conditions. The “absolute-relative” sensitivity function was used in this work, and the base
275 values of parameters and initial conditions were set according to the literature reported values
276 (see Table S3 in the SI) and the specific batch experimental settings used for model calibration.
277 The sensitive parameter(s) identified by the sensitivity analysis was then calibrated using the
278 batch tests at DO levels of 0.2, 0.75, 1.0, 2.0 and 3.0 mg O₂/L. Model validation was then
279 conducted using batch tests at DO levels of 0.5, 1.5 and 2.5 mg O₂/L. For the model
280 calibration/validation process, the profiles of NO₂⁻, NH₄⁺, DO, and N₂O were used at the same
281 time. Parameter estimation and parameter uncertainty evaluation, with 95% confidence, were
282 conducted according to Batstone et al. (2003). The confidence regions for parameter
283 combinations were determined using a modified version of AQUASIM 2.1 d (Ge et al. 2010).
284 Finally, the developed model was utilized to obtain insight into the role of potential nitrite

accumulation inside sludge flocs in regulating the two pathways for N_2O production by AOB under different DO concentration conditions.

3. RESULTS

3.1. N_2O production at different DO levels without significant bulk nitrite accumulation

As shown in Figure 1, experimental data for all batch tests in this work exhibited similar trends. No N_2O was detected in both gas and liquid phases during the first 10 min control phase, indicating the negligible N_2O production contribution of heterotrophic denitrification due to the unavailability of organic carbon source. The potential N_2O consumption by HB was also ruled out by N_2O consumption control tests with results shown in Figure S2. Therefore, any N_2O change in the following phase of batch tests would be solely ascribed to the activities of AOB. Applicable to both gas phase and liquid phase data, N_2O concentration reached steady-state level in about 10 min after dosage of 10 mg N/L of NH_4^+ under all DO concentration conditions studied except for DO level of 3.0 mg O_2/L (i.e., Figure 1H). As illustrated in Figure 1, NH_4^+ concentration remained relatively constant at 10 mg N/L due to the precise periodic dosing of NH_4HCO_3 , DO was well controlled at designated levels, and NO_3^- concentration maintained around 1500 mg N/L, which was consistent with the expectation in the cycle effluent of the SBR. NO_2^- concentration for all DO levels studied was lower than 0.2 mg N/L (see Figure 1), indicating the realization of removing NO_2^- accumulation in the bulk liquid.

Figure 2 shows the biomass specific AOR and N_2OR as well as N_2O emission factor calculated based on the batch experimental results presented in Figure 1. Biomass specific AOR increased with DO but reached a steady level when DO exceeded 2.5 mg O_2/L (Figure 2A), resulting from

the limited conversion capacity of AOB in the augmented nitrifying sludge. Biomass specific N_2OR first increased with DO, peaked at DO of 0.75 mg O_2/L with a value of 3.03 ± 0.05 mg N/h/g VSS, and thereafter kept decreasing until DO reached the maximum studied level of 3.0 mg O_2/L (Figure 2B). As demonstrated in Figure 2C, the correlation between biomass specific N_2OR and biomass specific AOR was positive when biomass specific AOR was below 22.0 ± 1.2 mg N/h/g VSS, above which biomass specific N_2OR was negatively correlated to biomass specific AOR. The N_2O emission factor in Figure 2D exhibited a negative relationship with DO and gradually decreased from $18.6 \pm 0.5\%$ at the lowest non-zero DO level of 0.2 mg O_2/L to $4.9 \pm 0.3\%$ at the maximum DO level of 3.0 mg O_2/L .

3.2. Model-based analysis of batch test data to infer N_2O production pathways

All the model parameters in Table S3 in the SI were assessed in the sensitivity analysis, with the N_2O production at five DO levels (i.e., 0.2, 0.75, 1.0, 2.0 and 3.0 mg O_2/L) being the model output. Figure S3 in the SI indicates the sensitivity of the N_2O production to the top six most sensitive model parameters. Among all the parameters, the specific maximum nitrite reduction rate $r_{NO_2,red}^{AOB}$ was found to exert the most determinant impact on the N_2O production by AOB under the batch experimental conditions in this work. Although not the most sensitive one (see Figure S3), the oxygen inhibition constant K_{I,O_2}^{AOB} should also be evaluated, considering its first appearance in the electron carriers-based N_2O model. Therefore, $r_{NO_2,red}^{AOB}$ and K_{I,O_2}^{AOB} were chosen to be evaluated in the model calibration and validation process using the batch experimental data obtained in this work. Literature reported values were directly adopted for the remaining parameters in the following modelling process.

The model calibration and validation process was successfully carried out (see Figure S4 in the SI). As presented in Figures S3A, S3C, S3D, S3F and S3H which show the model calibration results at DO levels of 0.2, 0.75, 1.0, 2.0 and 3.0 mg O₂/L, respectively, the model captured all the variation profiles including N₂O, DO, NH₄⁺ and NO₂⁻. The good match proved the validity of the calibrated model. The best fit was obtained when $r_{NO_2,red}^{AOB}$ equaled 0.52 ± 0.02 mmol/mmol-COD/h and K_{I,O_2}^{AOB} equaled 0.016 ± 0.001 mmol/L. Figure S5 in the SI shows the 95% confidence region for $r_{NO_2,red}^{AOB}$ and K_{I,O_2}^{AOB} together with their uncorrelated confidence intervals obtained during the model calibration process. The uncorrelated confidence intervals of both parameters were relatively small and mostly covered by the correlated confidence region, indicating a good level of reliability and identifiability of the estimated parameter values. The validity of parameters and structure of the developed model was further supported by the good model validation results in Figures S3B, S3E and S3G corresponding to DO levels of 0.5, 1.5 and 2.5 mg O₂/L, respectively.

As shown in Figure 3, the developed model could capture the experimentally measured DO-dependent N₂O_R to a large degree ($R^2=0.88$). The maximum relative error at DO of 3.0 mg O₂/L was < 20%, which was acceptable in terms of model predictions. In virtue of this, the developed model was used to deduce the contributions of the two pathways to the N₂O production under different DO concentration conditions. The model predicted results were incorporated into Figure 3. Albeit exhibiting a decreasing trend, the AOB denitrification pathway was dominating (>98.0%) under all the DO concentration conditions studied in this work, even though there was no significant bulk NO₂⁻ accumulation (<0.2 mg N/L) in the system. Therefore, the response of the AOB denitrification pathway to DO was responsible for the trend observed in Figure 2B. In

contrast, the contribution of the hydroxylamine pathway to the N_2O production was minor at all DO levels and increased slightly but consistently from 0.1% at DO of 0.2 mg O_2/L to 1.8% at DO of 3.0 mg O_2/L .

3.3. Nitrite accumulation inside sludge flocs potentially regulating N_2O production pathways

Albeit the low bulk NO_2^- concentration (<0.2 mg N/L) observed under all DO concentration conditions (see Figure 1), the mixed liquid at steady state underwent an evident increase in NO_2^- concentration from 0.18 ± 0.03 to 0.51 ± 0.05 mg NO_2^- -N/L after high-output sonication treatment, as shown in Figure 4. However, no change in bulk NO_2^- concentration was observed for mixed liquid samples taken right after the NH_4^+ stock solution was added at 10 min (data was not shown but post-sonication NO_2^- concentration remained around 0), suggesting no direct NO_2^- release due to initial NO_2^- presence in the floc structure and/or cell lysis. Therefore, this increase in the low bulk NO_2^- concentration after high-output sonication treatment implied a significant NO_2^- accumulation inside the nitrifying sludge flocs despite the trace NO_2^- detected in the bulk liquid outside the sludge flocs. As NO_2^- is a key factor/precursor regulating the N_2O production from the AOB denitrification pathway, NO_2^- accumulation inside the sludge flocs might favor the AOB denitrification pathway, which would result in the trends obtained in this work.

Figure 5 delineates the model predicted distribution of N_2O production pathways in the representative floc structure considering a thickness of 57 μm (i.e., half of D50 measured). The NO_2^- induced AOB denitrification pathway in the floc was uneven across thickness. At low DO level of 0.2 mg O_2/L , the rate of the AOB denitrification pathway was high in the middle part of

floc and low at the core and surface of the floc. However, at DO level higher than 0.2 mg O₂/L, the rate of the AOB denitrification pathway was highest at the core of floc (i.e., thickness 0 in Figure 5) and decreased gradually towards the floc surface. The overall N₂O production rate of the AOB denitrification pathway first increased and then decreased with DO, consistent with the finding in Figures 2B and 4. In contrast, the hydroxylamine pathway did not change significantly across thickness and kept increasing with DO level but at rates much lower than the AOB denitrification pathway under all DO concentration conditions studied.

4. DISCUSSION

In nitrifying sludge, microbial activities mainly take place inside flocs. Intermediates are produced inside flocs and then diffuse into the bulk liquid, leading to possible substrate concentration gradient. Therefore, the concentration of NO₂⁻ produced by AOB inside the nitrifying sludge flocs might be higher than that measured in the bulk liquid, resulting in N₂O production even in the absence of significant NO₂⁻ detected in the bulk liquid. However, to date no work has been done to elucidate the role of the potential nitrite accumulation inside sludge flocs in N₂O production by AOB at different DO levels, due to the interferences of either heterotrophic contribution in the sludge (Tallec et al. 2006, Wunderlin et al. 2012, Yang et al. 2009) or accompanying NO₂⁻ accumulation in bulk liquid (e.g., 1.5~2.0 mg NO₂⁻-N/L in Peng et al. (2014)). To deal with these two interferences, an augmented nitrifying sludge with higher abundance of NOB than AOB in sludge flocs was enriched in this work. Through applying this augmented nitrifying sludge, the interference from heterotrophic denitrification was eliminated, which was supported by both the control phase data in Figure 1 and the results of N₂O consumption control tests in Figure S2. The existence of substrate concentration gradient was

verified by Figure S6 in the SI which showed the model predicted profiles in the representative sludge floc at bulk DO concentration of 2.0 mg O₂/L. The NO₂⁻ accumulation in the bulk liquid at all DO levels studied in this work was well controlled at very low levels (<0.2 mg NO₂⁻-N/L in Figures 1 and 4), thus minimizing the interference from accompanying significant bulk NO₂⁻ accumulation. The results demonstrated that the AOB denitrification pathway for N₂O production was dominant (98.0%) at all DO levels tested even without significant bulk NO₂⁻ accumulation (<0.2 mg N/L) observed in the system, which was in fact due to the nitrite accumulation which existed inside the sludge flocs and therefore induced significant N₂O production from the AOB denitrification pathway.

The maximum specific AOR in this work (~35 mg N/h/g VSS in Figure 2A) was lower than the one obtained in Peng et al. (2014) (~74 mg N/h/g VSS). One reason could be the lower abundance of AOB (i.e., 35 ± 3% based on the FISH analysis) in the augmented nitrifying sludge used in this work. The trends in Figures 2B and 2C revealed that DO level higher than 0.75 mg O₂/L would exhibit an inhibitive effect on the N₂O production by AOB, irrespective of higher biomass specific AOR induced. The DO level for maximum N₂O production located in this work (i.e., 0.75 mg O₂/L) was close to the reported DO level of 0.85 – 1.0 mg O₂/L by some studies of similar interest (Peng et al. 2015, Tallec et al. 2006, Yang et al. 2009). Although the relationship between N₂O production by AOB and DO was similar, the bulk NO₂⁻ concentration in this work (i.e., 0.18 ± 0.03 mg NO₂⁻-N/L) was much lower than Peng et al. (2015) which dosed external NO₂⁻ to maintain a constant level of 3 – 50 mg NO₂⁻-N/L in the bulk liquid. As presented in Figure 4, a significant increase from 0.18 ± 0.03 to 0.51 ± 0.05 mg NO₂⁻-N/L was observed in the bulk NO₂⁻ concentration after high-output sonication was applied, implying a significant

level of NO_2^- accumulation in the nitrifying sludge flocs. The NO_2^- accumulated in the sludge flocs could explain the model predicted dominant role of the AOB denitrification pathway in N_2O production by AOB in Figure 3, even though the bulk NO_2^- concentration was extremely low. The noticeable discrepancy between experimentally observed and model predicted N_2O at DO concentrations of 2.5 and 3.0 $\text{mg O}_2/\text{L}$ in Figure 3 might be due to the possible existence of other minor N_2O contributing pathways at relatively high DO concentrations such as abiotic N_2O production (Liu et al. 2017) and AOA associated N_2O production pathway (Hu et al. 2015). However, the overall good match between experimentally observed and model predicted data (as evidenced by the correlation factor of 0.98 and R^2 of 0.88) together with the dominance of AOB and NOB in the augmented nitrifying sludge confirmed the validity of AOB associated N_2O dynamics reported in this work. Given that the bulk NO_2^- concentrations before and after sonication were low and similar at different DO levels (see Figure 4), the differences in N_2O production in different batch tests of this work could still be reliably attributed to DO variation. As indicated in Figure 3, the AOB denitrification pathway contributed the most (>98.0%) to N_2O production under the DO concentration conditions studied in this work. Therefore, the shift in the biomass specific N_2O with DO in Figure 3 to great extent profiled the DO-dependent rate of the AOB denitrification pathway. Although the rate of the AOB denitrification was stimulated at low DO levels but got inhibited after DO exceeded 0.75 $\text{mg O}_2/\text{L}$, its contribution to N_2O production decreased consistently with DO, due to the increased contribution of the hydroxylamine pathway on which DO had a positive effect (see Figure 3). Though the positive relationship between N_2O production from the hydroxylamine pathway and DO was commensurate with Peng et al. (2015) and Peng et al. (2014), the contribution was much lower in this work (maximum share of only 1.8% at the highest studied DO level of 3.0 $\text{mg O}_2/\text{L}$).

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447 The impact of DO on the N₂O production observed in Figure 3 could also be explained using
 448 model kinetics. The calibrated oxygen inhibition constant for the AOB denitrification pathway
 449 K_{I,O_2}^{AOB} (i.e., 0.016 mmol/L or 0.51 mg O₂/L) is relatively small, meaning high level of DO (e.g.,
 450 0.75 mg O₂/L in this work) would definitely inhibit N₂O production by the AOB denitrification
 451 pathway. When the DO inhibition was not significant (e.g., at DO level of 0.2 or 0.5 mg O₂/L),
 452 the elevation in DO would enhance NH₄⁺ and hence NH₂OH oxidation, thus rendering more
 453 electron supply for both pathways. Consequently, the rates of both pathways increased. In
 454 addition to the direct inhibition of DO on the microbial activity via the AOB denitrification
 455 pathway, the negative impact of DO level of higher than 0.75 mg O₂/L on N₂O production by the
 456 AOB denitrification pathway would be intensified considering the one order of magnitude
 457 difference in M_{red} affinity constants for oxygen reduction $K_{Mred,3}$ (i.e., 0.78 mmol/mmol-COD)
 458 and NO₂⁻ reduction $K_{Mred,4}$ (i.e., 2.1 mmol/mmol-COD). K_{Mred} represents the affinity of the
 459 corresponding reduction reaction to M_{red}, with lower value indicating a higher affinity and thus
 460 a higher ability to compete for electrons. Therefore, on the premise of the comparable specific
 461 maximum rates for oxygen reduction $r_{O_2,red}^{AOB}$ (i.e., 0.54 mmol/mmol-COD/h) and NO₂⁻ reduction
 462 $r_{NO_2,red}^{AOB}$ (i.e., 0.52 mmol/mmol-COD/h), increased AOR with higher DO would render more
 463 electrons for oxygen reduction to H₂O and less for the AOB denitrification pathway for N₂O
 464 production. This explanation agreed well with the assumption made by Li et al. (2015). As M_{red}
 465 affinity constant for NO reduction $K_{Mred,2}$ (i.e., 1.1×10^{-4} mmol/mmol-COD) is four orders of
 466 magnitude lower than that for nitrite reduction $K_{Mred,4}$ (i.e., 2.1 mmol/mmol-COD), the NH₂OH
 467 pathway has a higher ability than the AOB denitrification pathway to compete for electrons for
 468 N₂O production. Therefore, the adversely affected N₂O production by the AOB denitrification

pathway at DO level of higher than 0.75 mg O₂/L would divert more electrons for N₂O production by the NH₂OH pathway, leading to the increased N₂O production by the NH₂OH pathway in Figure 3. However, as the calibrated value of specific maximum nitrite reduction rate $r_{NO_2,red}^{AOB}$ (i.e., 0.52 mmol/mmol-COD/h) is three orders of magnitude higher than the value of the specific maximum NO reduction rate $r_{NO,red}^{AOB}$ (i.e., 1.8×10^{-4} mmol/mmol-COD/h), the AOB denitrification pathway was still prevailing (>98.0%) under the DO concentration conditions studied in this work (see Figure 3).

This finding pertaining to N₂OR was significantly different from Peng et al. (2014) reporting a positive relationship between N₂OR and DO which might be ascribed to the difference in microbial and floc structure as well as dominant N₂O production pathway(s) involved. Despite the discrepancy in N₂OR, the negative dependence of the overall N₂O emission factor on DO and the magnitude of N₂O emission factor at corresponding DO level in Figure 2D were still commensurate with Peng et al. (2014). Overall, a moderate DO level (e.g., 3.0 mg O₂/L in this work) is needed in order to maximize the capacity of AOB whilst reducing N₂O production by AOB.

This work also confirmed the necessity of modelling the nitrifying sludge flocs as a particle system when considering intermediates (e.g., NO₂⁻ and NH₂OH), especially at low concentrations. More insight into the true niche where microbial activities take place could be only revealed when treating the nitrifying sludge flocs as a particle system. As demonstrated in Figure 4, even though bulk nitrite concentration was low, nitrite accumulation because of microbial activities as well as substrate diffusion existed inside the sludge flocs. It was this part

of nitrite accumulation inside the sludge flocs that induced stratification of N_2O production by the AOB denitrification pathway in Figure 5 and hence the overall N_2O production trend in Figure 3. However, this stratified phenomenon couldn't be captured if the sludge flocs were modelled as a completely mixed suspended system, where uniform concentration was assumed and N_2O production due to nitrite accumulation inside the nitrifying sludge flocs might be overlooked. From the perspective of practical application, the significant role of nitrite accumulation inside the sludge flocs in N_2O production revealed in this work underpinned the coupling of nitrification with denitrification or anaerobic ammonium oxidation (anammox) in one-stage systems. Despite the well-known advantages in reduced energy demands, this advanced nitrogen removal technology would realize in-situ nitrite consumption inside the sludge flocs and thus reduce N_2O production/emission. A modelling study by Sabba et al. (2015) also suggested that N_2O production from nitrifying biofilms could be significantly greater than from suspended growth systems under similar conditions, mainly due to the important role of intermediate diffusion in the attached form. The consideration of the nitrifying sludge flocs as a particle system with substrate diffusion in the N_2O pathway model would be of particular interest when it comes to developing viable control technologies for reducing N_2O production/emission in nitrogen removal systems, as most reported model-based studies focusing on N_2O control (such as Behera et al. (2015) and Boiocchi et al. (2017)) didn't take into account the role of floccular structure of nitrifying sludge.

It should be highlighted that the obtained results with respect to the impact of potential nitrite accumulation inside sludge flocs on N_2O production could be generalized considering the comparable physicochemical properties of the augmented nitrifying sludge flocs to the typical

activated sludge flocs. It is also acknowledged that the contributions of the two N_2O production pathways were only calculated using mathematical modelling in this work. Though direct quantification using isotopic analysis is possible, further improvement in the accuracy and reliability of this technology is needed (Duan et al. 2017).

5. CONCLUSIONS

This work clarified the role of potential NO_2^- accumulation inside sludge flocs in N_2O production by AOB at different DO levels with focus on the conditions of no significant bulk NO_2^- accumulation (<0.2 mg N/L). The biomass specific N_2O production rate firstly increased and then decreased with DO, with the maximum value of 3.03 ± 0.05 mg N/h/g VSS obtained at DO level of 0.75 mg O_2 /L. The AOB denitrification pathway for N_2O production was dominant (98.0%) at all DO levels tested even without significant bulk NO_2^- accumulation (<0.2 mg N/L) observed in the system, but its contribution decreased with DO. DO had a positive impact on the hydroxylamine pathway for N_2O production which therefore increased with DO. The nitrite accumulation existed inside the sludge flocs and induced significant N_2O production from the AOB denitrification pathway. The findings not only highlighted the importance of achieving in-situ NO_2^- consumption inside sludge flocs in reducing N_2O production/emission, but also underpinned the necessity of considering the floccular sludge structure in the model when developing N_2O control strategies.

ACKNOWLEDGEMENTS

This study was supported by the Australian Research Council (ARC) through Future Fellowship FT160100195. The authors acknowledge the assistance of Haoran Duan and Jiaoyang Pu at The University of Queensland in the FISH and pyrosequencing analyses.

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Figure 1. Levels of gas phase N_2O , liquid phase N_2O , NH_4^+ , NO_2^- and NO_3^- in batch tests at DO concentrations of (A) 0.2 mg O_2 /L, (B) 0.5 mg O_2 /L, (C) 0.75 mg O_2 /L, (D) 1.0 mg O_2 /L, (E) 1.5 mg O_2 /L, (F) 2.0 mg O_2 /L, (G) 2.5 mg O_2 /L and (H) 3.0 mg O_2 /L.

Figure 2. Relationships between (A) AOR and DO, (B) N₂OR and DO, (C) N₂OR and AOR and (D) N₂O emission factor and DO detected in batch tests (error bars stand for standard deviation, n=3).

Figure 3. Experimentally observed and model predicted N_2OR as well as model predicted contribution of the two pathways to N_2O production at different DO concentrations.

Figure 4. Bulk NO_2^- concentration before and after sonication at different DO levels.

Figure 5. Model predicted distribution of N_2O production rates in the representative sludge floc.

Highlights

- Study on effect of DO on N_2O production by AOB without significant bulk NO_2^- accumulation.
- Evaluation and application of a two-pathway model to distinguish N_2O production.
- DO stimulated NH_2OH pathway but AOB denitrification pathway dominated (98%).
- NO_2^- accumulation inside sludge flocs significantly affected N_2O production by AOB.