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Nitrous Oxide Production in Autotrophic Nitrogen Removal Granular Sludge: A Modeling Study

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ABSTRACT

The sustainability of autotrophic granular system performing partial nitration and anaerobic ammonium oxidation (Anammox) for complete nitrogen removal is impaired by the production of nitrous oxide (N2O). A systematic analysis of the pathways and affecting parameters is therefore required for developing N2O mitigation strategies. To this end, a mathematical model capable of describing different N2O production pathways was defined in this work by synthesizing relevant mechanisms of ammonium-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), heterotrophic bacteria (HB), and Anammox bacteria. With the model validity reliably tested and verified using two independent sets of experimental data from two different autotrophic nitrogen removal biofilm/granular systems, the defined model was applied to reveal the underlying mechanisms of N2O production in the granular structure as well as the impacts of operating conditions on N2O production. The results show that: 1) in the aerobic zone close to the granule surface where AOB contribute to N2O production through both the AOB denitrification pathway and the NH2OH pathway, the co-

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occurring HB consume $N_2O$ produced by AOB but indirectly enhance the $N_2O$ production through providing NO from $NO_2^-$ reduction for the NH$_2$OH pathway, 2) the inner anoxic zone of granules with the dominance of Anammox bacteria acts as a sink for $NO_2^-$ diffusing from the outer aerobic zone and therefore reduces $N_2O$ production from the AOB denitrification pathway, 3) operating parameters including bulk DO, influent NH$_4^+$, and granule size affect the $N_2O$ production in the granules mainly through regulating the NH$_2$OH pathway of AOB, accounting for 34%–58% of $N_2O$ turnover, and 4) the competition between the NH$_2$OH pathway and heterotrophic denitrification for nitric oxide leads to the positive role of HB in reducing $N_2O$ production in the autotrophic nitrogen removal granules, which could be further enhanced with the presence of a proper level of influent organics.

**Graphical Abstract**

The sustainability of autotrophic granular system performing partial nitration and anaerobic ammonium oxidation (Anammox) for complete nitrogen removal is impaired by the production of nitrous oxide ($N_2O$). A systematic analysis of the pathways and affecting parameters is therefore required for developing $N_2O$ mitigation strategies.

**Keywords**: Nitrous oxide ($N_2O$); granule; anaerobic ammonium oxidation (Anammox); partial nitritation; mathematical modeling

1. **INTRODUCTION**

Compared to the conventional nitrification and denitrification process, the autotrophic nitrogen removal through combining partial nitritation with anaerobic ammonium oxidation (Anammox) represents a more sustainable alternative due to its less requirement for aeration, lower production of sludge, and saving of organic carbon for energy recovery (Jetten et al. This article is protected by copyright. All rights reserved.
An application survey by Lackner et al. (2014) reported over 100 full-scale installations worldwide performing partial nitriation/Anammox (PN/A), around 90% of which were operated as single-stage systems. Nevertheless, studies have documented various N\textsubscript{2}O emissions from PN/A systems of different scales (Ali et al. 2016; Castro-Barros et al. 2015; Domingo-Félez et al. 2014; Joss et al. 2009; Sliekers et al. 2002). N\textsubscript{2}O is not only a potent greenhouse gas with a global warming effect 265 times stronger than carbon dioxide but also a major scavenger of stratospheric ozone leading to ozone layer depletion (IPCC 2014; Ravishankara et al. 2009). It is generally recognized that Anammox bacteria don’t produce N\textsubscript{2}O (Kartal et al. 2006); N\textsubscript{2}O is therefore not specifically included in the stoichiometry of Anammox process. Thus, autotrophic nitrifiers, i.e., ammonium-oxidizing bacteria (AOB), have been regarded as the main contributors to the N\textsubscript{2}O production in autotrophic PN/A systems through either the hydroxylamine (NH\textsubscript{2}OH) pathway or the AOB denitrification pathway or both. Heterotrophic bacteria (HB) might grow on microbial decay products under inorganic influent conditions and therefore sustain in autotrophic nitrogen removal systems (Lotti et al. 2014; Mozumder et al. 2014; Ni et al. 2012). Despite the proposed positive contribution to nitrogen removal through reducing nitrate produced by Anammox bacteria, the presence of HB might also play a significant role in regulating the N\textsubscript{2}O production in autotrophic nitrogen removal systems, as N\textsubscript{2}O is an obligate intermediate of the sequential heterotrophic denitrification process (Hanaki et al. 1992).

Relative to biomass of suspended growth, compact sludge aggregates in the form of granules are particularly suitable to obtain the coexistence of AOB and Anammox bacteria in single-stage autotrophic nitrogen removal system. The DO concentration gradient in granules enables the creation of spatially separated aerobic and anoxic zones for AOB and Anammox bacteria, respectively. Moreover, the large accumulation of microbial decay products in the granular structure (Matsumoto et al. 2009) might be conducive to the proliferation of HB.
However, among the numerous studies reported so far, limited efforts have been dedicated to investigating the N$_2$O production in single-stage autotrophic granular systems. Ali et al. (2016) applied the isotopic composition analysis to identify the N$_2$O production pathways in a single-stage PN/A granular reactor. However, the relative contribution of HB denitrification to N$_2$O production could not be distinguished from that of the AOB denitrification pathway. The complicated interactions between different N$_2$O production pathways mediated by various microorganisms place the foremost difficulty in both qualitatively and quantitatively analyzing the N$_2$O production in autotrophic granular systems solely through experimentation.

Mathematical modeling can serve as a powerful tool for gaining an in-depth, microscale understanding of the rather complicated processes associated with N$_2$O production. Lu et al. (2018) extended the Activated Sludge Model No. 1 (ASM1) to decipher the N$_2$O production pathways in an autotrophic granular sequencing batch reactor. However, the usage of a homogeneous model that lumped granular structure into apparent substrate affinities might overlook the important role of granular structure characterized by substrate concentration gradients and microbial stratification in the underlying N$_2$O production mechanisms. In fact, previous model-based studies have revealed that the consideration of analogous biofilms as either suspended or attached growth in the model did generate distinctly different N$_2$O production characteristics for both nitrifying biofilms (Sabba et al. 2015) and denitrifying biofilms (Sabba et al. 2017). Although Van Hulle et al. (2012) applied a one-dimensional biofilm model to study the N$_2$O emissions during autotrophic nitrogen removal in a granular system, the NH$_2$OH pathway of AOB and the contribution of HB were overlooked. In view of the above, more efforts are still needed to systematically and comprehensively explore the N$_2$O productions in autotrophic nitrogen removal granular systems.

This work therefore aims to further understand the mechanisms of N$_2$O production in autotrophic nitrogen removal granules. To this end, a mathematical model capable of describing relevant mechanisms in autotrophic nitrogen removal systems was firstly defined.
The validity of the defined model was then tested and verified using two independent sets of literature reported experimental data; one was taken from an analogous autotrophic nitrogen removal biofilm reactor while the other from an enriched granular reactor. The verified model was coupled with relevant mass transfer terms in the modelling platform to simulate an autotrophic granular system in continuous mode. Extensive simulations were lastly performed to disclose the underlying mechanisms of N₂O formation and reduction inside the autotrophic granules as well as the impacts of operating conditions on the N₂O production and pathway differentiation in the autotrophic nitrogen removal granular system.

2. MATERIALS AND METHODS

2.1. Model Description

As presented in Fig. 1, the mathematical model in this work synthesizes relevant mechanisms of functional microorganisms in an autotrophic nitrogen removal system including AOB, nitrite oxidizing bacteria (NOB), HB, and Anammox bacteria. The detailed process reactions of AOB and HB as N₂O mediators are included in the supporting information (SI). The three pathways considered for N₂O production in this work include 1) N₂O as a by-product of incomplete oxidation of NH₂OH to NO₂⁻ via NO by AOB (i.e., the NH₂OH pathway), 2) N₂O as the final product of AOB denitrification (i.e., the AOB denitrification pathway), and 3) N₂O as an intermediate of sequential reduction of oxidized nitrogen oxides to N₂ by HB. In the defined model, NO reduction to N₂O in the NH₂OH pathway is coupled with NH₂OH oxidation to NO₂⁻, while direct NO₂⁻ reduction to N₂O in the AOB denitrification pathway is accompanied by NH₂OH oxidation. This setup of AOB-associated N₂O production pathways is consistent with Pocquet et al. (2016) and has been proved capable of reliably describing and predicting N₂O production dynamics when AOB played a significant role (Ni et al. 2011; Ni et al. 2013; Pocquet et al. 2016).
To reduce the model complexity whilst maintaining the high model validity, ionized NH$_4^+$ and NO$_2^-$ which could be directly and reliably measured (instead of unionized NH$_3$ and HNO$_2$) are assumed to be the substrate for AOB and NOB, respectively, in the model. In addition, the possible inhibition of NH$_3$ and HNO$_2$ on the activities of AOB and NOB are neglected, as their concentrations are usually relatively low compared to their inhibitory levels (Pocquet et al. 2016). However, similar to Chen et al. (2018) and Ni et al. (2011), the inhibition of DO on the AOB denitrification pathway is accounted for in the model. The aerobic reaction of NOB and the anoxic reaction of Anammox bacteria are described by following the generally accepted modelling practice. The Activated Sludge Model for Nitrogen (ASMN) (Hiatt and Grady 2008) is adopted to describe the mechanisms of HB, including the sequential denitrification from NO$_3^-$ to NO$_2^-$, NO, N$_2$O and finally to N$_2$ under anoxic conditions and the anabolism for biomass growth under aerobic conditions. The decay process for each microbial species is modelled to generate inert and slowly biodegradable organics. The slowly biodegradable organics is subject to hydrolysis to yield readily biodegradable organics, thus making it possible for HB to grow in an autotrophic nitrogen removal system without external input of organics.

In summary, the defined model contains 9 soluble components and 6 solid components, as listed in Table S1 in the SI. The stoichiometric relationships of all components that conform to mass balance are presented in Table S2 (SI). Except for the decay processes which are represented by the first-order equation with respect to biomass, kinetic control of all microbial reactions, as shown in Table S3 (SI), is described by the Monod equation. The model parameters as well as their values, units, and sources are summarized in Table S4 (SI).

2.2.Experimental Data for Testing Model Validity

Two independent experimental datasets previously reported in Wang et al. (2017) and Lu et al. (2018) that studied N$_2$O production dynamics in autotrophic nitrogen removal
biofilm/granular systems under different operational conditions were used to test the validity of the defined model.

Dataset 1 (Wang et al. 2017): In order to obtain one-stage completely autotrophic nitrogen removal, Wang et al. (2017) applied a 15-L steady-state sequencing batch biofilm reactor (SBBR) with polyacrylonitrile-activity carbon fiber (PAN-ACF) to support the attached growth of biomass. The SBBR was fed with inorganic synthetic wastewater containing 200 g NH$_4^+$-N/m$^3$ and operated in 24-h cycles, consisting of 5-min feeding phase, 23-h reacting phase with aeration and mixing, 50-min settling phase, and 5-min decanting phase. The data for model testing captured bihourly variations of NH$_4^+$, NO$_2^-$, NO$_3^-$, and N$_2$O in the SBBR for 22 hours during a typical operational cycle with DO controlled at 2.00 ± 0.20 g/m$^3$. The applicability of these reported data for testing the defined model could be justified by the following two facts: 1) the operational data clearly demonstrated the SBBR’s capability of achieving completely autotrophic nitrogen removal and 2) the microbial community analysis directly revealed the coexistence of AOB, NOB, HB, and Anammox bacteria in the biofilm (Wang et al. 2017).

Dataset 2 (Lu et al. 2018): The N$_2$O production behavior was observed in a 10-L bench-scale plexiglass sequencing batch reactor (SBR) with biomass retained in the form of granules. The SBR was fed with inorganic synthetic wastewater containing a mixture of 140 g NH$_4^+$-N/m$^3$ and 182 g NO$_2^-$-N/m$^3$ and operated in 3-h cycles, consisting of 30-min feeding phase, 2-h mixing phase, 20-min settling phase, and 10-min decanting phase withdrawing an aliquot of 2.5 L. The data for model testing described the dynamic variations of NH$_4^+$, NO$_2^-$, NO$_3^-$, and N$_2$O in the SBR for the first 2.5 hours of an operational cycle as a batch test, the low DO of which was derived from atmospheric reoxygenation. The detected microbial diversity and the clearly documented activities of AOB, NOB, HB, and Anammox bacteria in the granules proved the applicability of these reported data for testing the defined model.

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2.3. Model Testing and Simulation Approach

The same set of model parameters was used throughout to test the validity of the defined model as well as for all the following simulations. The SBBR was represented as a one-dimensional planar biofilm reactor compartment while the granular SBR was modelled as a one-dimensional spherical biofilm reactor compartment in AQUASIM (Reichert 1998). Following the practice proposed by Wanner and Morgenroth (2004), the stoichiometric and kinetic relationships between components of the defined model were constructed in the biofilm reactor compartment to simulate the autotrophic nitrogen removal with involvement of \( \text{N}_2\text{O} \) production mediated by the cohort of AOB, NOB, HB, and Anammox bacteria. Diffusion of soluble components in biofilm matrix was set with the introduction of reduced diffusion coefficients in water, which are provided in Table S5 (SI).

Model testing using Dataset 1 reported in Wang et al. (2017) was performed as a two-step process to verify the defined model. In the first step, the biofilm area was adjusted to fit the cycle data of \( \text{NH}_4^+ \), \( \text{NO}_2^- \), and \( \text{NO}_3^- \) by assuming a biomass density of 100000 g-COD/m\(^3\), a biofilm porosity of 0.75 (Koch et al. 2000), and an average biofilm thickness of 800 µm (Wang et al. 2017). The biomass composition was set according to the relative abundance of species in the microbial community analysis recorded in Wang et al. (2017), i.e., AOB of 7.77%, NOB of 6.91%, Anammox of 5.44%, and HB of 7.73%, with the remaining biomass (72.15%) being recognized as the sum of slowly biodegradable and inert organics. In the second step, instead of \( \text{N}_2\text{O} \) concentration, the accumulative \( \text{N}_2\text{O} \) production, indicative of total \( \text{N}_2\text{O} \) productivity, was compared with the \( \text{N}_2\text{O} \) predicted by the model configured in the first step without further adjustment. The reason for such a two-step model evaluation process was mainly due to the unavailability of direct measurement of biofilm dimensions, thus posing the foremost difficulty for the accurate representation of such a SBBR in the model. However, the validity of the defined model in terms of the intrinsic structure regulating the relationship between nitrogen removal capacity and \( \text{N}_2\text{O} \) production dynamics would still be
guaranteed, provided that a good match between experimental data and model prediction could be obtained simultaneously for both steps.

Model testing using Dataset 2 reported in Lu et al. (2018) was conducted by applying all NH$_4^+$, NO$_2^-$, NO$_3^-$, and N$_2$O profiles simultaneously to further verify the defined model. Assuming a biomass density of 100000 g-COD/m$^3$ and a biofilm porosity of 0.75 (Koch et al. 2000), the number of granules in the SBR was calculated based on the measured median granule size (D$_{50}$ = 728 µm) and the mixed liquor volatile suspended solids (MLVSS) concentration (2399 g/m$^3$) reported in Lu et al. (2018). Similar to the aforementioned handling, the biomass composition was decided by reference to the experimentally quantified abundance of microbial species in consideration of slowly biodegradable and inert organics. Mass transfer of N$_2$O from the liquid phase of the SBR to the atmosphere was modelled through a gas-liquid mass transfer equation mediated by the mass transfer coefficient $K_L a$ and the Henry's law constant $H$ (Schulthess and Gujer 1996), i.e., $R_{N2O} = K_L a_{N2O}(S_{N2O} - \frac{S_{N2O,air}}{H_{N2O}})$, where $K_L a_{N2O}$ was assumed at 2 d$^{-1}$, $S_{N2O,air}$ was 0.0003 g-N/m$^3$, and $H_{N2O}$ was 1.9 (Foley et al. 2010). If a good agreement between experimental data and model prediction could still be achieved, the validity of the defined model would be regarded as high enough to reliably support the subsequent model-based evaluations.

2.4. Simulations for Revealing Mechanisms of N$_2$O Production and Its Affecting Factors

Different simulation scenarios (cf. Table S5 in the SI) were performed using the tested model to explore the N$_2$O production in an autotrophic nitrogen removal granular system, which was modelled to be operated in continuous mode and composed of spherical granules with a uniform size in AQUASIM (Reichert 1998). The base scenario (Scenario 0) probed into the mechanisms of N$_2$O formation and reduction in autotrophic nitrogen removal granules and assumed 1) an average granule size (radius) of 500 µm, 2) an influent NH$_4^+$ concentration of 500 g-N/m$^3$, 3) a constant bulk DO concentration of 0.25 g/m$^3$, and 4) an influent COD.
concentration of 0. Other 4 scenarios were then implemented to investigate the impacts of operating conditions on the N₂O production in the autotrophic granular system. Specifically, Scenarios 1 to 3 focused on bulk DO concentration of 0.10 – 0.50 g/m³, influent NH₄⁺ concentration of 50 – 1000 g-N/m³, and granule size (radius) of 50 – 1000 µm, respectively. Considering that the potential existence of some biodegradable organics in wastewater might affect the growth of HB and therefore alter the overall microbial community structure, Scenario 4 was designed to assess the impact of influent organics concentration of 0 – 100 g-COD/m³ on the autotrophic granular system.

For all simulation scenarios, a total system volume of 1 m³ (including 0.2 m³ of granules and 0.8 m³ of bulk liquid) and an inflow rate of 1 m³/d was considered, giving rise to a hydraulic retention time of 1 day. The number of granules was calculated by dividing the total volume of granules by the volume of each granule. Assuming the same biomass density and porosity as used in model testing, the total solid concentration in the autotrophic granular system was 5000 g-COD/m³ for all simulation scenarios. The setting of diffusion of soluble components in the granular structure was in line with model testing, and zero-flux of soluble components was set at the granule center. Similar to Sabba et al. (2015), the mass transfer resistance through the liquid boundary layer was neglected for all soluble components; the concentration at the granule surface was equal to that in the bulk liquid. For the convenience of data calculation and the consistency in results interpretation, the N₂O produced was assumed to leave the continuously-operated autotrophic granular reactor in the effluent; no liquid-to-gas transfer of N₂O was considered in all the model simulations. This setting was acceptable in view of the essential N₂O production inside the granules and the minor impact of potential liquid-to-gas transfer of N₂O (in the form of a zero and non-zero mass transfer coefficient for N₂O) on the N₂O production factor (i.e., a relative change of < 5%) of the studied autotrophic granular system requiring a relatively low DO level, as demonstrated in Figure S1 in the SI.

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For each simulation scenario, the initial concentrations of all 9 soluble components in the autotrophic granules as well as the bulk liquid were assumed to be zero, while each of AOB, NOB, HB, Anammox bacteria, and abiotic organics (inert organics (50%) + slowly biodegradable organics (50%)) was assumed to take up 20% of the initial solid COD in the granules. The steady-state granule size was controlled by the surface detachment velocity without considering re-attachment of detached solids. All simulations assumed an initial granule radius of 50 μm and were run to reach steady-state conditions indicated by constant effluent concentrations, N₂O production, granule size, and microbial composition.

3. RESULTS

3.1. Testing Model Predictive Capability with Experimental Data

At the first step of the model testing process using Dataset 1, the biofilm area was adjusted to 1.4 m², which rendered a good match (R²=0.95) between the model predicted and experimentally measured data in terms of NH₄⁺ consumption, NO₃⁻ formation, and NO₂⁻ accumulation, as illustrated in Fig. 2A. On top of the satisfactory result obtained at the first step, the defined model was able to predict the accumulative N₂O production during a typical operational cycle to a high degree (R²=0.99) in the second step, as evidenced by Fig. 2B. A good performance was also acquired for model testing with Dataset 2 (Fig. 3). The defined model was able to capture not only the bulk transformations of NH₄⁺, NO₃⁻, and NO₂⁻ (R²=0.92) in Fig. 3A but also the trace profile of N₂O (R²=0.97) in the granular reactor. With these, the defined model has been proved capable of describing the relationship between nitrogen removal capacity and N₂O production dynamics in the autotrophic nitrogen removal biofilm/granular systems involving AOB, NOB, HB, and Anammox bacteria simultaneously. Therefore, the defined model could be reliably applied in the following simulations to assess N₂O production in the autotrophic granular system in a holistic manner.
3.2. Mechanisms of N₂O Production inside Autotrophic Granules

This section explores the mechanisms of N₂O production inside autotrophic granules under the conditions of Scenario 0 (Table S5 in the SI), which gives a total nitrogen (TN) removal efficiency (i.e., ratio between N₂ produced and influent NH₄⁺) of 90.3% with an accompanying net N₂O production factor (i.e., ratio of net N₂O produced to total NH₄⁺ removed) of 1.7%. Granule radius-wise profiles are presented in Fig. 4, where “r” denotes the rate of individual process listed in Table S3 (SI) while “R” indicates the net rate of certain soluble nitrogenous component taking into account all relevant processes that produce or consume it. A positive “R” means net production while a negative “R” represents net consumption.

Overall, steady-state microbial stratification (Fig. 4A) and substrate concentration gradients (Fig. 4B) are observed in the representative autotrophic granule. AOB and HB only exist at the granule surface, and their fractions decrease towards the interior of the granule from 500 µm to 400 µm. By comparison, the fraction of Anammox bacteria peaks at 400 µm and drops consistently towards both the granule core (0 µm) and the bulk liquid (500 µm). NOB are absent in the entire range of granule radius. Due to the efficient conversion of slowly biodegradable organics (Xₛ) by HB at the granule surface, Xₛ is not present between 400 µm and 500 µm, while inert organics (Xᵢ) is immanent across the granule radius as a result of the persistent biomass decay processes. Different from the nearly constant concentrations of NO₃⁻, NH₂OH, NO, and N₂O in the representative autotrophic granule, the profiles of soluble components in Fig. 4B clearly exhibit the decreasing trends of NH₄⁺ and NO₂⁻ from the bulk liquid to the granule core and the quick consumption of DO at the granule surface from 500 µm to 400 µm, well in line with the profiles of AOB and HB in Fig. 4A.

As shown in Fig. 4C, the process rates of NH₄⁺ oxidation to NH₂OH (r₁), NH₂OH oxidation to NO (r₂), NO oxidation to NO₂⁻ (r₃), NO reduction to N₂O (r₄), and NO₂⁻...
reduction to N\textsubscript{2}O (r5) by AOB all exhibit decreasing trends in the granule from 500 \, \mu\text{m} to 400 \, \mu\text{m}. Similar to Sabba et al. (2015) investigating N\textsubscript{2}O production from nitrifying biofilms, NH\textsubscript{2}OH produced in the outer layer of the granule diffuses into the inner layer which serves as a sink for NH\textsubscript{2}OH, making r2 exceed r1 therein. Consequently, NH\textsubscript{2}OH is lost in the outer granule while there is gain of NH\textsubscript{2}OH in the inner granule, as reflected in the positive net NH\textsubscript{2}OH conversion rate ($R_{\text{NH2OH}}^{AOB}$) between 480 \, \mu\text{m} and 500 \, \mu\text{m} and the negative $R_{\text{NH2OH}}^{AOB}$ between 400 \, \mu\text{m} and 480 \, \mu\text{m} (Fig. 4D). Both the NH\textsubscript{2}OH pathway (r4) and the AOB denitrification pathway (r5) contribute to the steady-state N\textsubscript{2}O production by AOB in the granule (i.e., $R_{N2O}^{AOB}$ in Fig. 4D). Fig. 4E illustrates the process rates of aerobic growth (r6), anoxic NO\textsubscript{3}\textsuperscript{-} reduction to NO\textsubscript{2}\textsuperscript{-} (r7), anoxic NO\textsubscript{2}\textsuperscript{-} reduction to NO (r8), anoxic NO reduction to N\textsubscript{2}O (r9), and anoxic N\textsubscript{2}O reduction to N\textsubscript{2} (r10) by HB in the representative granule. Compared to other processes, the significantly higher value of r6 indicates the usage of the majority of hydrolyzed biodegradable organics (S\textsubscript{S}) as the substrate to sustain the aerobic growth instead of the anoxic denitrifying activities of HB. The slight shift of the maximum r7, r8, r9, and r10 from the granule surface at 500 \, \mu\text{m} where HB biomass is highest to the granule radius at 490 \, \mu\text{m} is due to the inhibition by DO that is maximum at the granule surface. Resulting from the competition between the NH\textsubscript{2}OH pathway of AOB and HB denitrification for NO, the rate of anoxic NO reduction to N\textsubscript{2}O (r9) is much lower than r8 and r10 (Fig. 4E), leading to the positive net NO conversion rate ($R_{NO}^{HB}$) and the negative net N\textsubscript{2}O conversion rate ($R_{N2O}^{HB}$) in the granule (Fig. 4F). The process rates of NO\textsubscript{2}\textsuperscript{-} reduction (r11) and NH\textsubscript{4}\textsuperscript{+} oxidation (r12) by Anammox bacteria in Fig. 4G follow the same distribution pattern of Anammox bacteria in the representative granule (Fig. 4A). The inexistence of NOB throughout the granule radius corresponds to the null process rate of NO\textsubscript{2}\textsuperscript{-} oxidation by NOB (r13), as shown in Fig. 4G.

The collective contributions of AOB, NOB, HB, and Anammox bacteria to the conversion rates of NH\textsubscript{4}\textsuperscript{+} ($R_{NH4}^{NH}$), NO\textsubscript{2}\textsuperscript{-} ($R_{NO2}$), NO\textsubscript{3}\textsuperscript{-} ($R_{NO3}$), NH\textsubscript{2}OH ($R_{NH2OH}$), NO ($R_{NO}$),
and N₂O (\(R_{N2O}\)) are displayed in Fig. 4H. N₂O production only takes place at the granule surface between 400 µm and 500 µm. Both HB and Anammox bacteria exert direct and/or indirect impact on N₂O production in the granule. The low consumption of NO₂⁻ by HB in the granule (i.e., the slightly negative \(R_{NO2}^{HB}\) in Fig. 4F) manifests the negligible impact of HB on the AOB denitrification pathway for N₂O production (i.e., r5 in Fig. 4C), which is dependent on the availability of NO₂⁻. Since \(R_{NO}\) in Fig. 4H is around 0, the net NO production by HB (i.e., the positive \(R_{NO}^{HB}\) in Fig. 4F) is compensated by the net NO consumption by AOB (i.e., the negative \(R_{NO}^{AOB}\) in Fig. 4D). Therefore, the NO produced by HB is mainly directed to favor the N₂O production by AOB through the NH₂OH pathway (i.e., r4 in Fig. 4C). In this sense, HB play an indirect role in affecting the NH₂OH pathway of AOB for N₂O production in the autotrophic granule. Moreover, the direct consumption of N₂O by HB (i.e., the negative \(R_{N2O}^{HB}\) in Fig. 4F) results in the lower overall \(R_{N2O}\) (Fig. 4H) than the net N₂O production rate of AOB (i.e., \(R_{N2O}^{AOB}\) in Fig. 4D). As the influent is lacking in NO₂⁻, Anammox bacteria gain access to NO₂⁻ produced by AOB through its diffusion from the outer granule to the inner granule. The NO₂⁻ consumption by Anammox bacteria in the inner granule not only facilitates the autotrophic nitrogen removal but also lowers the availability of NO₂⁻ for N₂O production through the AOB denitrification pathway (i.e., r5 in Fig. 4C). The relatively high conversion rates of NH₄⁺ and NO₂⁻ as shown in Fig. 4H give rise to the stratification of NH₄⁺ and NO₂⁻ inside the autotrophic granule, whereas the nearly even distribution of NO₃⁻, NH₂OH, NO, and N₂O in Fig. 4B is mainly determined by their diffusivities in presence of relatively low conversion rates.

### 3.3 Impacts of Operating Parameters on N₂O production in Autotrophic Granule

This section examines the effects of bulk DO concentration (Scenario 1), influent NH₄⁺ concentration (Scenario 2), granule size (radius) (Scenario 3), and influent organics concentration (Scenario 4) on the N₂O production in the autotrophic nitrogen removal process.
granular system. To this end, steady-state TN removal efficiency is correlated with the corresponding microbial community structure in the granules, while steady-state net N₂O production factor is analyzed in reference to the accompanying contribution differentiation of N₂O production pathways. The simulated results are compiled in Fig. 5.

### 3.3.1. Impact of bulk DO

**Nitrogen removal.** As shown in Fig. 5A, a low bulk DO of 0.10 g/m³ limits oxygen supply for AOB to oxidize influent NH₄⁺ to NO₂⁻ for Anammox bacteria; most NH₄⁺ is left untreated in the effluent. As a result, a low TN removal efficiency of 34.1% is mediated by AOB (7.3%), HB (2.2%), and Anammox bacteria (90.5%) in the granules. The increase in bulk DO to 0.20 g/m³ enhances partial nitritation by AOB, thus creating more NO₂⁻ for Anammox bacteria. Therefore, the active biomass fraction of Anammox bacteria increases to 91.1% while that of AOB remains around 7.3%. The active biomass fraction of HB drops to 1.5% due to their simultaneous competitions with AOB and Anammox bacteria for oxygen and NO₂⁻, respectively. The resulting TN removal efficiency reaches 86.4% at bulk DO of 0.20 g/m³ (Fig. 5A). A higher bulk DO of around 0.25 g/m³ leads to nearly complete utilization of influent NH₄⁺ and intensifies the competition between AOB and Anammox bacteria for the influent NH₄⁺ supply. Consequently, the active biomass fraction of AOB drops to 6.8% while that of Anammox bacteria and HB increases to 91.3% and 1.9%, respectively, giving rise to the peak TN removal efficiency of 90.3% (Fig. 5A). Further increase in bulk DO to the maximum studied in this work (i.e., 0.50 g/m³) pushes AOB towards full nitritation and generates an unbalanced, excessive NO₂⁻/NH₄⁺ ratio for Anammox bacteria. In consequence, as depicted in Fig. 5A, the active biomass fraction of Anammox bacteria decreases while those of AOB and HB increase, leading to the descending TN removal efficiency.

**N₂O production.** The increasing N₂O production factor from 1.5% at bulk DO of 0.10 g/m³ to 2.5% at bulk DO of 0.20 g/m³ (Fig. 5B) is in line with the increasing activity of AOB.
in NH$_4^+$ oxidation. Further increase in bulk DO firstly causes an abrupt drop of N$_2$O production factor to 1.7% at bulk DO of 0.25 g/m$^3$ and then leads to the gradual decrease in N$_2$O production factor, which reaches 1.3% at bulk DO of 0.50 g/m$^3$. As indicated in Fig. 5B, the increase in bulk DO from 0.10 g/m$^3$ to 0.20 g/m$^3$ motivates N$_2$O production from both the AOB denitrification pathway and the NH$_2$OH pathway while diminishing the contribution of HB denitrification. Further increase in bulk DO till 0.50 g/m$^3$ brings no significant change to the contribution of the AOB denitrification pathway. By contrast, the positive contribution (i.e., production) of the NH$_2$OH pathway decreases consistently, compensated by the increasing negative contribution (i.e., consumption) of HB denitrification to N$_2$O turnover (Fig. 5B).

### 3.3.2. Impact of influent NH$_4^+$

**Nitrogen removal.** A too low influent NH$_4^+$ concentration represents limited NH$_4^+$ supply for Anammox bacteria to react with NO$_2^-$ produced by AOB while a too high NH$_4^+$ concentration brings about insufficient NO$_2^-$ production by AOB for Anammox bacteria to oxidize NH$_4^+$. Therefore, as demonstrated in Fig. 5C, an increasing influent NH$_4^+$ concentration from 50 g-N/m$^3$ to 200 g-N/m$^3$ increases the active biomass fraction of Anammox bacteria whilst decreasing those of AOB and HB, thus elevating the TN removal efficiency. At the influent NH$_4^+$ concentration of 300 g-N/m$^3$, the TN removal efficiency reaches the peak of 90.6% with AOB (6.0%), HB (2.0%), and Anammox bacteria (92.0%) coexisting in the granules. Further increase in the influent NH$_4^+$ concentration till 800 g-N/m$^3$ slightly promotes the active biomass fraction of AOB but demotes those of HB and Anammox bacteria in the granules. Despite the relatively stable abundance of AOB (7.1%), HB (1.7%), and Anammox bacteria (91.2%) in the granules at the influent NH$_4^+$ concentration of higher than 800 g-N/m$^3$, a downward trend is still observed for the TN removal efficiency in Fig. 5C.

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**N₂O production.** At the influent NH₄⁺ concentration of lower than 300 g-N/m³, N₂O production factor is low and fluctuates around 1.0%, with the AOB denitrification pathway, the NH₂OH pathway, and HB denitrification each accounting for around 30%, 35%, and -35% of N₂O turnover (Fig. 5D). At the influent NH₄⁺ concentration of 300 g-N/m³ where maximum TN removal efficiency of 90.6% is obtained, the N₂O production factor is minimum with a value of 0.9%. An influent NH₄⁺ concentration higher than 300 g-N/m³ favors the NH₂OH pathway while suppressing the contribution of HB denitrification to N₂O turnover. By comparison, the significant increase in the influent NH₄⁺ concentration from 300 g-N/m³ to 1000 g-N/m³ only gives rise to slight decrease in the contribution of the AOB denitrification pathway to N₂O turnover from 27.5% to 26.1% (Fig. 5D).

### 3.3.3. Impact of granule size

**Nitrogen removal.** A small granule radius of not higher than 100 µm enables oxygen penetration and therefore fails to create anoxic zone for Anammox bacteria, resulting in the dominance of AOB (~80%) and HB (~20%) in the granules and the trace TN removal (~1.3%) (Fig. 5E). When the granule radius is 200 µm, Anammox bacteria appear in the granules and cooperate with AOB and HB for nitrogen removal, corresponding to the TN removal efficiency of 35.6%. Granules with greater radius firstly increase and then decrease the TN removal efficiency, with the maximum value of 90.3% obtained at the granule radius of 500 µm (Fig. 5E). The increasing TN removal efficiency from 200 µm to 500 µm is because of the higher abundance of Anammox bacteria in the granules. In contrast, the decreasing TN removal efficiency when the granule radius exceeds 500 µm is the result of the limitation of NO₂⁻ diffusing from the surface to the inner layer of the granules, which pushes Anammox bacteria away from the granule core and makes the inner layer of the granules inactive. Therefore, with the increasing granule radius from 500 µm to 1000 µm, the active biomass fractions of AOB and HB gradually increase while that of Anammox bacteria slightly decreases, as described in Fig. 5E.

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**N₂O production.** The increasing N₂O production factor from 1.0% to 1.5% when the granule radius increases from 50 µm to 100 µm is ascribed to the lowered negative contribution of HB denitrification to N₂O turnover (Fig. 5F), on top of the trace nitrogen removal performance due to the absence of Anammox bacteria. With the increasing presence of Anammox bacteria and the decreasing relative abundance of AOB and HB in the granules, the N₂O production factor slightly increases from 1.5% at the granule radius of 200 µm to 1.7% at the granule radius of 500 µm. Regarding N₂O turnover, the contribution of the NH₂OH pathway increases while those of the AOB denitrification pathway and HB denitrification decrease. Further increase in the granule radius to 1000 µm firstly leads to the enhanced NH₂OH pathway and then favors the N₂O consumption by HB. As a result, the N₂O production factor approaches the maximum of 2.5% at the granule radius of around 700 µm and is subject to insignificant change thereafter (Fig. 5F).

### 3.3.4. Impact of influent organics

**Nitrogen removal.** Different from the aforementioned simulated results of Scenarios 1 to 3, the granular system exhibits a much simpler trend when facing the influent organics (Scenario 4). As shown in Fig. 5G, the increasing organics in the influent stimulates the growth of HB in the granules and favors the heterotrophic denitrification process. Consequently, despite the decreasing abundance of AOB and Anammox bacteria, the TN removal increases slightly but consistently from 90.3% to 92.0% with the influent organics concentration within the studied range from 0 to 100 g-COD/m³.

**N₂O production.** The simulated results in Fig. 5H clearly indicate the positive role of the influent organics in reducing the N₂O production in the granular system. With the increase in the influent organics concentration from 0 to 100 g-COD/m³, the N₂O production factor drops consistently from 1.7% to 0.2%. Both the contributions of the AOB denitrification pathway...
and the NH$_2$OH pathway to N$_2$O turnover decrease, while the N$_2$O consumption by HB is reinforced.

4. DISCUSSION

A better understanding of the pathways and affecting parameters formulates the basis for the development of reliable N$_2$O mitigation strategies for the single-stage autotrophic nitrogen removal granular system. Steady-state simulated results in Fig. 4A demonstrate clear microbial distribution in the granules. Similar trends have been documented in both experimental (Ali et al. 2016) and simulation studies (Liu et al. 2017; Mozumder et al. 2014) on granule-based systems for autotrophic nitrogen removal. The accompanying concentration profiles of soluble components in the granules (Fig. 4B) are consistent with previous experimental measurements (Ali et al. 2016; Nielsen et al. 2005; Vázquez-Padin et al. 2010; Wang et al. 2017) and simulated results (Liu et al. 2017). The distinct concentration gradients of DO, NH$_4^+$, and NO$_2^-$ are due to their relatively high conversion rates in the granules (Fig. 4H), whereas the nearly even distribution of NO$_3^-$, NH$_2$OH, NO, and N$_2$O across the granule radius is regulated by their diffusivities in presence of relatively low conversion rates (Fig. 4H). On this basis, this work systematically explores the mechanisms of N$_2$O production inside the autotrophic granules with the spatially separated aerobic and anoxic zones. Firstly, N$_2$O production is shown to only take place in the aerobic zone at the granule surface, with simultaneous contributions of both the AOB denitrification pathway and the NH$_2$OH pathway. Secondly, though favoring the in-situ consumption of N$_2$O produced by AOB, the co-occurrence of HB in the aerobic zone is found to generate NO and thus indirectly enhance the N$_2$O production by AOB through the NH$_2$OH pathway. The fact that both AOB and HB are located close to the granule surface facilitates the diffusion of N$_2$O from the granules into the bulk liquid. Therefore, the autotrophic granular system in this work might produce more N$_2$O than the membrane-aerated biofilm system with counter diffusing fluxes of oxygen and ammonium, where N$_2$O production has been shown to take place at the biofilm base (Ni and This article is protected by copyright. All rights reserved.)
Yuan 2013). Thirdly, the inner anoxic zone is discovered to act as a sink for NO$_2^-$ diffusing from the outer aerobic zone and therefore reduce N$_2$O production through the AOB denitrification pathway. The multispecies nature of autotrophic granule complicates the mechanisms associated with N$_2$O production, which are significantly different from those reported for single-species nitrifying biofilms (Sabba et al. 2015) and denitrifying biofilms (Sabba et al. 2017).

Analyses on the impacts of operating parameters in Fig. 5 manifest the possibility of achieving high-level nitrogen removal whilst mitigating N$_2$O production in the granular system by 1) controlling bulk DO, influent NH$_4^+$, and granule size at proper levels, or 2) providing organic influent. NOB will be washed out of the granules due to their simultaneous competitions with AOB for oxygen and/or with HB and Anammox bacteria for nitrite. Consistent with Hao et al. (2002) and Liu et al. (2017), the increase in bulk DO firstly increases the aerobic ammonium oxidation and then inhibits the activity of Anammox bacteria (Fig. 5A). On the contrary, the increasing influent NH$_4^+$ concentration firstly improves the nitrogen removal by enhancing the Anammox process and then impede the nitrogen removal because of limited oxygen supply (Fig. 5C). A too small granule size fails to provide the anoxic zone for Anammox bacteria while a too big granule size reduces the active biomass volume due to diffusion limitation in the granules (Fig. 5E). A proper level of influent organics benefits the nitrogen removal through enhancing the heterotrophic denitrification process present in the granules (Fig. 5G).

The N$_2$O production factor obtained in this work (0.2%-2.7%) is well in the range for single-stage autotrophic nitrogen removal systems reported Ali et al. (2016). Through analyzing the correlation between the contribution of each N$_2$O production pathway and the N$_2$O production factor under inorganic influent conditions (Scenarios 1, 2, and 3), it is found that the trends of N$_2$O production factor in Figs. 4B, 4D, and 4F are mainly mediated by the NH$_2$OH pathway considering its highest positive contribution to N$_2$O turnover (34%-58%).
and its highest correlation coefficient of >0.95 with the N₂O production factor. The AOB denitrification pathway also contributes significantly to N₂O production and accounts for 17%~31% of N₂O turnover. The HB denitrification consistently helps consume N₂O produced by AOB under the simulated conditions in this work. This is due to the competition between the NH₂OH pathway and HB denitrification for intermediate NO, which makes the N₂O formation rate from NO lower than the N₂O reduction rate by HB. The close interaction between the NH₂OH pathway and HB denitrification in regulating N₂O production is proved by their high correlation (i.e., 0.78 for Scenario 1 and 0.99 for Scenarios 2 and 3) as well as the very low NO concentration in the representative granule in Fig. 4B. Despite the minor role in affecting the nitrogen removal (Mozumder et al. 2014), HB growing on microbial decay products have been shown in this work to contribute significantly to reducing N₂O production in the autotrophic granular system. As shown in Fig. 5H, the positive role of HB in mitigating N₂O production will be enhanced by introducing a proper amount of organics in the influent, which therefore lowers the contributions of the AOB related pathways. From the perspective of practical application, the organics, if not already present in the influent wastewater, could originate from external addition to the granular system.

5. CONCLUSIONS

This work investigated the underlying mechanisms of N₂O production as well as the impacts of operating conditions on N₂O production in autotrophic nitrogen removal granules. The main conclusions are:

- In the aerobic zone close to the granule surface where AOB contribute to N₂O production through both the AOB denitrification pathway and the NH₂OH pathway, the co-occurring HB consume N₂O produced by AOB but indirectly enhance the N₂O production through the NH₂OH pathway.
• The inner anoxic zone of granules with the dominance of Anammox bacteria acts as a sink for NO$_2^-$ diffusing from the outer aerobic zone and therefore reduces N$_2$O production from the AOB denitrification pathway.

• Operating parameters including bulk DO, influent NH$_4^+$, and granule size affect the N$_2$O production in the granules mainly through regulating the NH$_2$OH pathway of AOB, accounting for 34%~58% of N$_2$O turnover.

• The competition between the NH$_2$OH pathway and heterotrophic denitrification for nitric oxide leads to the positive role of HB in reducing N$_2$O production in the autotrophic nitrogen removal granules, which could be further enhanced with the presence of a proper level of influent organics.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.
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Figures

Fig. 1. Schematic of the model for autotrophic nitrogen removal involving the mechanisms of AOB, NOB, HB, and Anammox bacteria.

Fig. 2. Model evaluation results based on experimental cycle data of (A) \( \text{NH}_4^+ \), \( \text{NO}_2^- \) and \( \text{NO}_3^- \), and (B) \( \text{N}_2\text{O} \) reported in Wang et al. (2017).
Fig. 3. Model evaluation results based on experimental batch data of (A) NH$_4^+$, NO$_2^-$ and NO$_3^-$, and (B) N$_2$O reported in Lu et al. (2018).
Fig. 4. Profiles of component fractions/concentrations and process/conversion rates in a representative granule: (A) active particulate components, (B) soluble components, (C) process rates of AOB, (D) conversion rates of nitrogenous components by AOB, (E) process rates of HB, (F) conversion rates of nitrogenous components by HB, (G) process rates of Anammox and NOB, and (H) overall conversion rates of nitrogenous components.
Fig. 5. Effects of (A and B) bulk DO, (C and D) influent NH$_4^+$, (E and F) granule size, and (G and H) influent organics on active biomass fraction and TN removal performance as well as net N$_2$O production factor and contribution differentiation of N$_2$O production pathways.