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Published in:
Biotechnology and Bioengineering

Link to article, DOI:
10.1002/bit.26062

Publication date:
2017

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Heterotrophs are key contributors to nitrous oxide production in mixed liquor under low C-to-N ratios during nitrification – batch experiments and modelling

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Running title: N₂O production in nitrifying batch experiments: heterotrophic and autotrophic contributions.
Abstract

Nitrous oxide (N₂O), a by-product of biological nitrogen removal during wastewater treatment, is produced by ammonia-oxidizing bacteria (AOB) and heterotrophic denitrifying bacteria (HB). Mathematical models are used to predict N₂O emissions, often including AOB as the main N₂O producer. Several model structures have been proposed without consensus calibration procedures. Here, we present a new experimental design that we used to calibrate AOB-driven N₂O dynamics of a mixed culture. Even though AOB activity was favoured with respect to HB, oxygen uptake rates indicated HB activity. Hence, rigorous experimental design for calibration of autotrophic N₂O production from mixed cultures is essential. The proposed N₂O production pathways were examined using five alternative process models confronted with experimental data inferred. Individually, the autotrophic and heterotrophic denitrification pathway could describe the observed data. In the best-fit model, which combined two denitrification pathways, the heterotrophic contribution to N₂O production was stronger than the autotrophic. Importantly, the individual contribution of autotrophic and heterotrophic to the total N₂O pool could not be unambiguously elucidated solely based on bulk N₂O measurements. NO data availability will increase the practical identifiability of N₂O production pathways.

Keywords: Nitrous oxide, Batch, Nitrification, Denitrification, Model
1. Introduction

Nitrous oxide (N\textsubscript{2}O) is known as both a stratospheric ozone depleter and a greenhouse gas with 300 times higher radiative forcing than carbon dioxide (Stocker et al., 2013). N\textsubscript{2}O is emitted during biological nitrogen removal and its emission factors are highly variable between wastewater treatment plants (WWTPs) (0.01-3.3% N\textsubscript{2}O\textsubscript{emitted}/TN\textsubscript{removed}) (Ahn et al., 2010). Moreover, the carbon footprint of a WWTP is highly sensitive to N\textsubscript{2}O emissions (Gustavsson and Tumlin, 2013), as an N\textsubscript{2}O emission factor of 1% can increase its carbon footprint by 50% (Monteith et al., 2005).

N\textsubscript{2}O is biologically produced during wastewater treatment by ammonium oxidizing bacteria (AOB) and heterotrophic denitrifying bacteria (HB). AOB can produce N\textsubscript{2}O as a by-product of hydroxylamine oxidation (NH\textsubscript{2}OH) or by nitrite (NO\textsubscript{2}\textsuperscript{-}) reduction. As an obligate intermediate during nitrate (NO\textsubscript{3}\textsuperscript{-}) reduction, N\textsubscript{2}O can also be produced by HB (Law et al., 2012). The three pathways are commonly known as nitrifier nitrification (NN), nitrifier denitrification (ND) and heterotrophic denitrification (HD), respectively.

Certain wastewater constituents such as dissolved oxygen (DO) and NO\textsubscript{2}\textsuperscript{-} have been identified as key variables affecting N\textsubscript{2}O dynamics (Kampschreur et al., 2009; Schreiber et al., 2012). However, other variables such as inorganic carbon content, known to affect nitrification rates (Jiang et al., 2015; Torà et al., 2010), have shown contradictory results with respect to N\textsubscript{2}O (Khunjar et al., 2011; Peng et al., 2015a). Hence, the metabolic regulation of N\textsubscript{2}O production is still under study (Perez-Garcia et al., 2014). Identifying the individual contribution of each pathway is critical for the design of N\textsubscript{2}O mitigation strategies.

One way to elaborate on the individual contributions of the pathways is through N\textsubscript{2}O process models. Several N\textsubscript{2}O models have been proposed for one or two of the aforementioned N\textsubscript{2}O production pathways (Guo and Vanrolleghem, 2013; Ni et al.,
2013a) with the final goal of mitigating its emissions. Models vary based on the true
substrate considered for AOB (NH$_3$ vs. NH$_4^+$), a reaction’s electron donor, or whether
substrate inhibition is considered (Pan et al., 2013; Spérandio et al., 2016). How to
mathematically describe these effects will impact the structural identifiability of model
parameters (Dochain and Vanrolleghem, 2001).

Calibration of N$_2$O models typically rely on the same data series as N-removing models
(DO, NH$_4^+$, NO$_2^-$, NO$_3^-$, COD) and additionally N$_2$O (Guo and Vanrolleghem, 2013; Ni
et al., 2011). The type and quality of experimental data will affect the practical
identifiability of model parameters (Dochain and Vanrolleghem, 2001). Literature for
N$_2$O-associated parameters shows large variability for similar processes. For example,
the AOB affinity for NO$_2^-$ during autotrophic denitrification in nitrifying biomass has
been reported from 0.14 to 8 mgN/L (Kampschreur et al., 2007; Schreiber, 2009).
Similarly, for the same model, a wide range of autotrophic NO affinity constants has
been used, from 0.004 to 1 mgN/L (Mampaey et al., 2013; Spérandio et al., 2016).
Variations can arise from considering different microbial communities, model
assumptions, quality of data or the calibration procedure selected.

Depending on the system, AOB or HB have been considered to be the main contributor
to the total N$_2$O production (Itokawa et al., 2001; Ni et al., 2013a). ND and HD occur
under similar DO and NO$_2^-$ concentrations, thus leading to possible interferences
between autotrophic and heterotrophic N$_2$O production (Shen et al., 2015; Wu et al.,
2014). However, under certain operating conditions, the contribution of a pathway can
be considered negligible, thus allowing for more accurate model calibrations.
Experiments can be therefore specifically designed to study the autotrophic contribution
to the total N$_2$O production pool from mixed liquor biomass. Nitric oxide (NO) is the
direct precursor of N$_2$O for the three pathways, and even though it is included in most
N$_2$O models (Ni et al., 2011; Ni et al., 2014) few studies have focused on quantifying and describing NO emissions (Kampschreur et al., 2007; Schreiber et al., 2009), which has been shown to be a useful tool to calibrate N$_2$O models (Pocquet et al., 2016).

In this study, we assess to what extent batch experiments – designed to assess N$_2$O dynamics under nitrifying conditions from a mixed culture biomass from a typical BNR plant – allow for calibration of N$_2$O models. Specifically, without assuming prior knowledge of the main N$_2$O producing pathway, our objective was to:

- Identify what model structures are capable of describing N$_2$O production of mixed liquor during batch tests at varying substrate concentrations.
- Quantify the individual contribution of the main biological N$_2$O-producing pathways to the total modelled N$_2$O production.
- Elucidate challenges encountered during calibration of N$_2$O models with combined pathways.

2. Materials and Methods

2.1. Batch reactor configuration.

Batch experiments were performed in a 3L PYREX glass vessel (Bellco Glass Inc., USA), with 4 side ports used for pH, DO and N$_2$O microsensors, and inflow/outflow gas (Supporting Information (SI), Figure S1). The inlet and outlet gas flow was set at 60 mL/min with gas flow meters. Oxic and anoxic conditions in the reactor were obtained by air and N$_2$ supplied through a bubble diffuser. Aeration and mixing were controlled using a Labview (National Instruments, Austin, USA) routine. The DO and temperature data, (CellOx 325, WTW, Germany) and pH (SenTix41, WTW, Germany) was continuously logged at 0.017 Hz. Liquid N$_2$O concentrations were measured with Clark-type microsensors (N2O-R, Unisense A/S, Aarhus, Denmark). Gaseous N$_2$O concentrations were measured with an infrared gas analyzer (T320, Teledyne, USA).
Photometric test kits were used to analyse N-substrates (1.14752, 1.09713, 1.14776, Merck KGaA, Darmstadt, Germany). Biomass content (MLSS, MLVSS) was measured in triplicates according to APHA (APHA et al., 1999). Alkalinity was measured by titration after addition of sulphuric acid (APHA et al., 1999).

2.2. Batch tests.

Mixed liquor from a full-scale wastewater treatment plant (Lynetten, Copenhagen, Denmark) was sampled over a period of three months (May-July 2012). Mixed liquor was aerated overnight and the biomass concentration adjusted to 2-3 gVSS/L with aerated clarified wastewater before experiments. After two days of experimentation the biomass was discarded to prevent significant changes in biomass composition (Torà et al., 2010). The biomass composition was calculated thermodynamically (SI_1). Biomass samples for DNA extraction were taken for every new experiment (n = 8). Details on the qPCR quantification procedure can be found elsewhere (Terada et al., 2010) (SI_2).

Two sets of experiments were performed while aeration was kept constant. Instantaneous extant substrate loadings of 1-3 mgN/gVSS were designed to mimic typical plant loading conditions, which produce a representative description of the parent system (Ellis et al., 1996). In the first set of experiments (i) solely NH$_4^+$ was spiked at incremental concentrations (1-8mgN/L). NH$_4^+$ removal was monitored off-line via liquid analysis and online by observing DO drops (Table SII). In the second set of experiments (ii), again NH$_4^+$ spikes (3-5mgN/L) were made and when nearing NH$_4^+$ depletion a NO$_2^-$ or NO$_3^-$ spike (2mgN/L) was made, monitoring responses in liquid and gas phase. Experiments allowed for nitrogenous concentration changes at both high and low DO concentrations (DO = 6.5 – 0.2 mg/L), providing useful information regarding substrate affinities and growth rates and covering a wide range of potential N$_2$O
producing scenarios. Experiments were conducted and repeated the day after on consecutive weeks.

Heterotrophic activity was monitored during an anoxic experiment (iii) where $N_2$ was supplied instead of air under $NO_3^-$ excess and no organic carbon addition. $NO_3^-$ reduction was assumed to occur fed on hydrolysed products originated from biomass decay as no organic substrate was added. Simultaneously, $NH_4^+$ would be released and accumulate in the bulk phase.

To determine $N_2O$ and $O_2$ mass transfer coefficients, stripping and reoxygenating experiments (iv) were performed separately at the same batch conditions in preaerated clarified wastewater (Eq. 1) (Garcia-Ochoa and Gomez, 2009). Liquid phase $N_2O$ measurements were used to estimate net $N_2O$ production rates as previously described (Domingo-Félez et al., 2014) (Eq. 2).

\[
N_2O_{liq}(t) = N_2O_{liq}(t=0) \cdot e^{(-k_{LaN2O} \cdot t)} \quad (mgN/L) \quad (Eq. 1)
\]

\[
N_2O \text{ Prod. Rate}_i = \frac{\Delta N_2O_{liq,i}}{\Delta t} + k_{LaN_2O} \cdot N_2O_{liq,i} \quad (mgN/L \cdot min) \quad (Eq. 2)
\]

2.3. Model description and calibration: $NH_4^+$, $NO_2^-$, $NO_3^-$, DO.

$NH_4^+$ to $NO_3^-$ conversion was described by a 2-step nitrification model (Table SIII). First, AOB oxidize $NH_4^+$ to $NH_2OH$ followed by its oxidation to $NO_2^-$. Subsequently NOB oxidize $NO_2^-$ to $NO_3^-$. Heterotrophic denitrification was included as a 4-step process with $NO_2^-$, NO and $N_2O$ as intermediates (Hiatt and Grady, 2008). Hydrolysis of particulates and ammonification were simplified into one hydrolytic process following biomass decay as no particulate N or soluble organic N data was available at the beginning of the experiments (Table SIV). Rates were not dependent on inorganic carbon as it was in excess during the experiments (5.8-6.0 mM HCO$_3^-$).
The simulation model was implemented in AQUASIM 2.1 (Reichert, 1998).

The objective of the following calibration procedure was to fit DO, NH$_4^+$, NO$_2^-$ and NO$_3^-$ data. First, physico-chemical parameters ($k_L$) were estimated from experiments (iv). Second, nitrification was evaluated by experiments (i) and (ii). The measured OUR$_{\text{max}}$ were used to estimate the NH$_4^+$ affinity ($K_{\text{NH}_4}^{\text{AOB}}$), and the NH$_4^+$ oxidation rates at varying DO to estimate the DO affinity ($K_{\text{O}_2}^{\text{AMO}}$) (SI_3). Then, oxic hydrolysis was evaluated against heterotrophic aerobic growth in experiments (i) and (ii) when reduced nitrogenous species were absent. Anoxic hydrolysis was assessed under anoxic conditions in experiment (iii). Finally, maximum growth rates ($\mu_{\text{AMO}}^{\text{AMO}}$, $\mu_{\text{NOB}}$) were estimated from NH$_4^+$ removal followed by NO$_2^-$ removal and NO$_3^-$ accumulation from experiments (ii). The rest of parameter values describing nitrification and denitrification were taken from published literature (Table SV). The biomass composition was modelled throughout the experiments to account for decay processes.

After good fits of DO and profiles of NH$_4^+$, NO$_2^-$ and NO$_3^-$ were achieved, the N$_2$O producing model structures (Tables S4) were calibrated.

2.4. Model description and calibration: N$_2$O.

The objective of implementing different N$_2$O model structures was to investigate what model structure, with accepted parameters, can describe the experimental data. Two model structures for AOB driven N$_2$O production were evaluated. The nitrifier denitrification (ND) pathway considers the consecutive reduction of NO$_2^-$ to NO and N$_2$O as two processes. The model structure chosen in this study considers DO inhibition, and NH$_2$OH is modelled as the electron donor (Ni et al., 2011). The nitrifier nitrification (NN) pathway considers a 2-step NH$_2$OH oxidation over NO to NO$_2^-$. A
Fraction of NO is reduced to N$_2$O with NH$_2$OH as the electron donor independent of DO levels (Ni et al., 2013a). Finally, N$_2$O can also be produced as an intermediate of heterotrophic denitrification in the 4-step model (HD) (Hiatt and Grady, 2008). Every step in the HD pathway considers independently easily biodegradable organic substrate as electron donor coupled with DO and NO inhibitions. Parameter values from two different denitrifying activated sludge systems (SRT = 3 and 10 days) (Hiatt and Grady, 2008; Schultess et al., 1994) have been used regularly to describe HD (Table SVI). Because the aim of the experiments was to study the autotrophic N$_2$O production, both parameter subsets were considered throughout the study to avoid biases from the possible heterotrophic contribution: HD$_a$ and HD$_b$.

Five different AOB-HB pathway combinations were tested to evaluate what model structures best describe the experimental N$_2$O data (Table I). Three scenarios consider a single N$_2$O production pathway: in scenarios NN and ND only nitrifier nitrification or nitrifier denitrification produce N$_2$O, while HD is modelled as a 2-step denitrification directly reducing NO$_2^-$ to N$_2$ (i.e. no chance of heterotrophic N$_2$O production). Scenario HD considers only N$_2$O production through a 4-step denitrification process. Two scenarios, NN-HD and ND-HD, consider the combination of an autotrophic (either nitrifying nitrification or denitrification) with the heterotrophic pathway (Ni et al., 2011; Ni et al., 2013a). Differently from other comparative studies both autotrophic and heterotrophic pathways are considered without any prior assumption of the main producer (Spérandio et al., 2016). A multiple-pathway AOB model was not considered as the assumptions for the ND pathway make it incompatible with the 4-step denitrification model (Pocquet et al., 2016). The continuity for all the model structures was numerically evaluated following Hauduc et al. (2010) (Hauduc et al., 2010).
For each pathway, only certain parameters are specific to describe N₂O production. For the AOB-associated pathways (NN, ND), only parameters not affecting directly NO₂⁻ production were first considered: ηAOB and Kₐ⁰₈ for NN and ηAOB, Kₐ₀₈, Kₐ₀₂ and Kₐ₀₂ for ND (Table III). The high number of parameters describing each denitrification step (5) does not allow individual parameter estimation. Consequently, a sensitivity analysis based on the relative-relative function was used to avoid calibration of insensitive parameters in the three pathways. During calibration, the lower and upper limits were set to ± 50% from their original literature values.

Parameter estimation was performed by minimizing the sum of the squared errors weighted by their standard deviations. The likelihood measured of each fit was evaluated following Mannina et al. (2011), where an overall model efficiency (Eᵣ) value of 1 corresponds to a perfect fit and tends zero for large errors (Eq. 3) (Mannina et al., 2011), where αᵢ corresponds to each data series and Mᵢ,ᵢ and Oᵢ,ᵢ to modelled and observed points.

\[
Eᵣ = \frac{1}{\sum n} \sum^n \alpha_i L(\theta_i / Y_i) = \frac{1}{N} \sum^n \alpha_i \cdot \exp \left( -\frac{\left( \sum (Mᵢ,ᵢ - Oᵢ,ᵢ)^2 \right)}{\sum (\alpha_i)^2} \right) \]  
(Eq. 3)

In addition, the RMSE was calculated. The contribution of each individual process to the N₂O and NO concentration at any time was calculated by multiplying each process rate (Pᵢ) with its stoichiometric coefficient (vᵢ,ᵢ). The sum of all terms corresponds to the net production/consumption of the state variable (Sᵢ) (Eq. 4).

\[
S_{net,prod,i} = \sum_i (P_i \cdot vᵢ,ᵢ) \]  
(Eq. 4)

Uncertainty analysis was done following Sin et al. (2010) by randomly sampling Kₐ₀₂ and Kₐ₀₂ (0.02 ± 90% mgN/L).

3. Results
3.1. Oxygen uptake and hydrolysis during autotrophic batch experiments.

Experiments (i) and (ii) started with NH$_4^+$ and DO excess, reaching first DO followed by NH$_4^+$ limitation. DO reached limiting but never truly anoxic conditions (0.2-0.4 mg DO/L). NO$_2^-$ accumulated shortly and was consumed simultaneously with NH$_4^+$ until depletion, upon which the DO concentration rapidly increased to pre-spike levels. NO$_3^-$ accumulated to levels similar to the NH$_4^+$ added, indicating complete nitrification of NH$_4^+$ (Figure 1, left).

Because of the low amount of substrate added a simplified model structure not including biomass growth was first considered. However, in the absence of NH$_4^+$ or NO$_2^-$ and at constant aeration DO never reached saturation, indicating an additional oxygen uptake process (Figure 1, right). Thus the model had to include processes producing biodegradable carbon from biomass decay. As no other organic source was present, the heterotrophic aerobic growth was responsible for the continuous oxygen uptake. Hence, hydrolysis affects DO availability even during short batch tests.

Under anoxic conditions hydrolytic processes also release biodegradable carbon and NH$_4^+$. Experimental and modelling results from the anoxic experiment (iii) showed agreement of ammonification and NO$_3^-$ reduction (Figure S2).

3.2. N$_2$O production during autotrophic batch experiments.

During experiments (i), after NH$_4^+$ spikes N$_2$O increased slowly at high DO and sharply when reaching DO < 0.5 mg/L, and decreasing after NH$_4^+$ depletion and consequent DO increase (Figure S3). Experiments (ii) were used to investigate the effect of DO, followed by NO$_2^-$ or NO$_3^-$ addition, on N$_2$O production during NH$_4^+$ oxidation. After adding NH$_4^+$, N$_2$O concentration gradually increased until DO became limiting, which
rapidly increased its production (Figure 2A, time < 20 min). A NO$_3^-$ spike added to promote heterotrophic denitrification during DO limiting conditions did not increase the net N$_2$O production compared to a sole NH$_4^+$ spike (Figure 2B). On the other hand, NO$_2^-$ addition at low oxygen concentrations and in the presence of NH$_4^+$ drastically increased the N$_2$O production (Figure 2C). These results are in agreement with literature where NO$_2^-$ showed a larger impact on N$_2$O production compared to NO$_3^-$ under endogenous conditions (Wu et al., 2014). The net N$_2$O produced after an NH$_4^+$ (or NH$_4^+$ followed by NO$_3^-$) spike was approximately 0.9% of the nitrogen oxidized, while 1.9% of the nitrogen oxidized was converted to N$_2$O when NH$_4^+$ was spiked followed by NO$_2^-$.

3.3. Model calibration for oxygen and nitrogenous substrates.

The objective of the calibration was to obtain a set of parameters that could describe the NH$_4^+$, NO$_2^-$, NO$_3^-$ and DO profiles before simulating the associated N$_2$O production.

The nitrifying fraction of the mixed liquor was calculated from thermodynamics to be 4.1% AOB and 1.8% NOB of the active biomass (SI_1). These results are in agreement with FISH results from other Danish wastewater treatment plants with the same configuration (AOB = 3-5%, NOB = 2.5-3%) (Mielczarek, 2012). Moreover, 16S rRNA-based qPCR quantification of dominant AOB and NOB taxa over 11 weeks showed no variation of the nitrifying community (78 ± 5% AOB/(AOB+NOB), n = 8).

NOB affinity constants differ significantly between species (Nowka et al., 2014), thus NOB affinities were considered as those of Nitrospira spp. (Manser et al., 2005) (Nitrospira spp. 92 ± 3% relative abundance in comparison to 8 ± 3% of Nitrobacter spp.). Results from experiments (i) allowed for estimation of the DO affinity for the first
nitrification step ($K_{O2_{AMO}}^{AOB} = 0.4$ mg/L), and the NH$_4^+$ affinity ($K_{NH4}^{AOB} 0.25$ mgN/L) (Figure S4). The model could describe hydrolysis and ammonification with default parameter values (Figure S2). Finally, autotrophic maximum specific growth rates ($\mu_{AMO}^{AOB}$, $\mu_{NOB}$) were estimated with low uncertainty (Table II). After model calibration a good individual fitting of DO, NH$_4^+$, NO$_2^-$ and NO$_3^-$ was obtained ($R^2 > 0.97$, $n > 30$) (Figure 1, left).

3.4. Modelling N$_2$O production from mixed cultures in autotrophic batch tests.

We analysed the capabilities of the model structures considered (NN, ND, HD, NN-HD, ND-HD) to describe experiments (ii). For each of the five models the best-fit residuals of the N$_2$O-associated parameter subsets are shown in Table III. Results for the models with the HD_a parameter subset are described below.

(NN): The nitrifying nitrification pathway (NN) describes N$_2$O production as a fraction of the oxidized NH$_4^+$. The NN model does not consider an effect of NO$_2^-$ on the N$_2$O produced, and it cannot predict the net N$_2$O production increase after NO$_2^-$ addition (Figure 2C). The best-fit obtained clearly did not follow the observed N$_2$O data (Figure 3) ($E_{NN} = 0.83$).

(ND): The nitrifying denitrification pathway (ND) could describe the observed N$_2$O responses to substrate concentration changes ($E_{ND} = 0.98$). The best-fit parameter subset increased the NO$_2^-$ and NO reduction processes with a higher anoxic reduction factor (Table III). The sensitivity of N$_2$O production to NO$_2^-$ can be described with a low NO$_2^-$ affinity (Figure 3).

(HD): Heterotrophic denitrification processes were limited by the organic substrate ($S_S$) and DO inhibited. However, an adequate fit could be obtained ($E_{HD} = 0.98$). Compared
to the initial parameter values the NOR process increased its rate compared to NIR and NOS, indicating a faster NO-to-N₂O turnover (higher $\mu_{\text{NOR}}$, $K_{\text{NOR,}1,\text{O₂}}^{\text{HB}}$, lower $K_{\text{NOR,}5}^{\text{HB}}$).

*(NN – HD):* The NN-HD model considered the simultaneous NN and HD associated $N_2O$ production. The best fit of the NN-HD model ($E_{\text{NN-HD}} = 0.97$) was obtained when the NN contribution to the total N₂O pool was the lowest. This result is in agreement with the fact that NN-associated N₂O production could not describe the data while HD-associated could ($E_{\text{NN}} = 0.83$ vs. $E_{\text{HD}} = 0.98$). Nonetheless, the best-fit was slightly worse than the HD model and better than the NN (Figure 3).

*(ND – HD):* In the ND-HD model the autotrophic and heterotrophic denitrification pathways were considered and yielded the best fit ($E_{\text{ND-HD}} = 0.99$). The observed oxygen-inhibited and NO₂⁻-associated N₂O production could be best described by two independent reductive processes.

The N₂O production rates associated to excess DO were much lower, and lasted shorter periods than N₂O production under DO-limiting conditions (Figure 2). For this reason, models containing one or two denitrification pathways (ND, HD, NN-HD, ND-HD) yielded a better fit than the one associated only with NH₄⁺ oxidation (NN). Hence, models containing at least one denitrification pathway obtained very similar fits but suggested different N₂O pathway contributions ($N_2O_{\text{ND}}$, $N_2O_{\text{HD}} = 0\text{-}100\%$) (Figure 3, Figure S5).

**3.5. Influence of HD on N₂O modelling results.**

The best N₂O fit was obtained when two simultaneous denitrification processes were considered (ND-HD) regardless of the HD parameter subset chosen (Table III, Table SVII). Even though the total N₂O production was described equally well by ND-HD_a
and ND-HD_b, other model outputs showed very different results (Table IV).

Surprisingly, HD was suggested as the main contributor to the total N\textsubscript{2}O pool: 96% N\textsubscript{2}O\textsubscript{HD_a}/N\textsubscript{2}O\textsubscript{TOT} and 61% N\textsubscript{2}O\textsubscript{HD_b}/N\textsubscript{2}O\textsubscript{TOT}. The total NO emitted predicted by the ND-HD models also showed significant differences (0.2 and 10.5% NO/N\textsubscript{2}O for ND-HD\textsubscript{a} and ND-HD\textsubscript{b}). Hence, the model could describe the total N\textsubscript{2}O production but neither the individual N\textsubscript{2}O pathway contribution nor NO emissions.

4. Discussion

4.1. Predicting capabilities of N\textsubscript{2}O model structures.

The best-fit obtained for the N\textsubscript{2}O profiles in experiments (ii) varied considerably among the models considered. However, because of the low N\textsubscript{2}O emission factor, all the N\textsubscript{2}O models in this study could describe NH\textsubscript{4}\textsuperscript{+}, NO\textsubscript{2}\textsuperscript{-}, NO\textsubscript{3}\textsuperscript{-} and DO profiles.

Single pathways

In the NN model, N\textsubscript{2}O production is directly linked to NH\textsubscript{2}OH oxidation. The initial N\textsubscript{2}O production after an NH\textsubscript{4}\textsuperscript{+} spike can be described by a high concentration of electron donors and electron acceptors (Figure 2, t < 20 min). Even though the NN model could not predict the observed N\textsubscript{2}O production at limiting DO and as a response to NO\textsubscript{2}\textsuperscript{-} changes (Figure 2C), it was suitable for non-limiting DO conditions (Ni et al., 2013b; Peng et al., 2015b). The ND model captured the observed N\textsubscript{2}O data, suggesting complete autotrophic N\textsubscript{2}O production. The larger production of N\textsubscript{2}O at low DO and high NO\textsubscript{2}\textsuperscript{-} was captured by changes in oxygen inhibition (K\textsubscript{A0B}\textsubscript{4O2}) and NO\textsubscript{2}\textsuperscript{-} affinity (K\textsubscript{A0B}\textsubscript{NO2}) from their literature values.

Interestingly, the HD model also captured the N\textsubscript{2}O produced suggesting complete heterotrophic N\textsubscript{2}O production. Even at conditions of minimum C/N and in the presence of inhibitory DO concentrations for heterotrophic denitrification the best-fit obtained for
the ND and HD models were similar ($E_i = 0.98$). It should be highlighted that not considering hydrolysis, the only carbon source in these experiments, would have neglected the possible heterotrophic contribution.

**Combined pathways**

In the NN-HD model, the best-fit results suggest a high HD ($N_2O_{HD} = 90\%$) and small NN ($N_2O_{NN} = 10\%$) contribution to the total $N_2O$ pool as the NN pathway is independent of $NO_2^-$ levels. Both autotrophic and heterotrophic pathways consider $N_2O$ production from NO reduction, thus allowing NN-associated $N_2O$ production to occur even at low DO regardless of NO’s producer. The predictions obtained using the ND-HD model yielded the best fit ($E_i > 0.99$) by combining two denitrification pathways and suggested a very low autotrophic contribution ($N_2O_{ND} = 4\%$). Shen et al. (2014) also suggested that $N_2O$ production during nitrification could be significantly affected by the microbial competition with heterotrophic activity (Shen et al., 2015). As two denitrification processes, ND and HD have similar affinities for N-substrate and DO. Moreover, the organic carbon limitation of heterotrophs under low C/N is counteracted by a larger fraction of the microbial community in mixed liquor. ND and HD can therefore co-occur at similar conditions and rates, which difficult the identifiability of individual pathways solely with bulk $N_2O$ measurements.

Hence, one cannot ignore heterotrophic contribution to $N_2O$ even during a short batch test where the only carbon source was released from hydrolysis of decay products. This is illustrated by two different combined ND-HD models that could best describe the observed data with parameter values within literature range.

Spérandio et al. (2016) compared five $N_2O$ models (HD + NN or ND) to four long-term dataseries (Spérandio et al., 2016). The relative contribution of autotrophs (ND) and
heterotrophs (HD) to the total N₂O production was calculated for a full-scale UCT process. For every 3 units of N₂O produced by the ND pathway 2 were consumed by HD, highlighting the importance of including the HD under AOB-driven N₂O production.

The better performance of multiple-pathway models suggests that new and more complex models will be necessary to predict N₂O emissions from dynamic systems (Spérandio et al., 2016). Considering additional pathways increases their fitting capabilities but, as highlighted in this study, our understanding of simple models is still limited. Moreover, overparameterization might compromise the precision and identifiability of complex models, which has not been critically addressed yet. This will support the model discrimination procedure towards developing a new biologically congruent N₂O model.

4.2. Limitations of modelling combined N₂O production pathways from bulk N₂O measurements.

The aim of modelling biological N₂O production during wastewater treatment operations is to mitigate its emissions by understanding how operating conditions relate to N₂O production. The desired mitigation strategies of N₂O models are specific to the main producing pathway. If the production of each pathway is accounted for individually we can better understand the relevant N₂O producing processes (Ni et al., 2014). However, because no direct pathway measurements are possible, model predictions are considered instead. N₂O models are usually calibrated with N₂O bulk measurements (liquid or gas phase), from which the contribution of each pathway is calculated (Guo and Vanrolleghem, 2013; Ni et al., 2014). The uncertainty associated to
model predictions can be calculated by mapping input uncertainty (error in parameter estimates) onto model outputs.

The high variability found in N₂O model parameters was studied in the ND-HD model by varying one parameter commonly fixed (K^{KNO}_{AOB}, K^{KNO}_{HB}) within literature range (Hiatt and Grady, 2008; Spérandio et al., 2016). Because the total N₂O production is not sensitive to these parameters (data not shown) no effect is seen in the model output for experiments (ii) (Figure 4, Figure S6). However, variables such as the autotrophic N₂O contribution or the total NO production can vary significantly (Figure 4A,B). These results indicate that fixing K^{KNO} values from literature values can lower model predicting capabilities for individual N₂O pathway contributions based on calibrations from N₂O bulk measurements.

NO plays an important role in N₂O production as its precursor in every production pathway (HD, ND, NN) and can, under certain conditions, contribute more than N₂O to the nitrogen loss (Castro-Barros et al., 2016). In experiments (ii), measuring NO would help to elucidate the main NO and N₂O production pathways by not lumping NO_{2} and NO reduction processes, an assumption made by new N₂O models (Ni et al., 2014; Pocquet et al., 2016). For a combination of K^{KNO}_{AOB} and K^{KNO}_{HB} values the model output for NO and N₂O is shown in Figure 5. The total error of N₂O production, shown as RMSE, does not vary regardless of the K^{KNO}_{AOB}-K^{KNO}_{HB} values (Figure 5A). On the other hand, both the contribution of the autotrophic pathway (Figure 5B) and the total NO produced (Figure 5C) vary significantly (1-56% N₂O_{AOB}/N₂O_{TOT}, 0.2-4.0% NO/N₂O). Thus, because NO is more sensitive to K^{KNO} than N₂O is, NO data availability will increase the identifiability of K^{KNO}_{AOB}-K^{KNO}_{HB}. Consequently, the contribution of each N₂O production pathway can be estimated more accurately. This is in agreement with the suggestion of
Spérandio et al. (2016) of using the ratio NO/N₂O as a parameter for model discrimination (Spérandio et al., 2016).

5. Conclusions

In this work, N₂O production from nitrifying batch experiments with mixed liquor was studied experimentally and compared to predictions by five model structures. Contrary to our hypothesis even under very low C/N conditions heterotrophic activity was found comparable to autotrophic nitrification activity in terms of N₂O production. Interestingly, process models accounting for heterotrophic and autotrophic denitrification pathways could describe total N₂O profiles only slightly better than single-pathway denitrification models. In a conventional N-removing system, where heterotrophs are more abundant than autotrophs, different combinations of denitrification N₂O-producing pathways could describe the observed biological N₂O production. Thus, based on N₂O bulk measurements from mixed liquor, models cannot unambiguously elucidate the contribution of each N₂O production pathway due to parameter uncertainty.

Acknowledgements

This research was funded by the Danish Agency for Science, Technology and Innovation through the Research Project LaGas (12-132633). The authors have no conflict to declare.
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Heterotrophs are key contributors to nitrous oxide production in mixed liquor under low C-to-N ratios during nitrification – batch experiments and modelling

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Figure 3 – Experimental and best-fit simulations of N$_2$O concentrations during experiments (i). Individual pathways: HD, ND, NN (left); and combined pathways: ND-HD, NN-HD (right). Parameter subset HD_a.

Figure 4 – Modelling results for ND-HD_a best-fit parameters in experiment (ii) (Table III). 250 K$_{NO(AOB,HB)}$ pairs of values sampled randomly in the range 0.02 ± 90% mgN/L. Total contribution (black) and decomposed HD (red) and ND (blue) individual contributions and to the N$_2$O pool (left). Total NO production (right). Dashed lines correspond to the 95% percentiles.

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(A) N$_2$O fit (RMSE), (B) autotrophic contribution to the total N$_2$O pool, (C) NO/N$_2$O produced.
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Parameter subset HD_a.

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Heterotrophs are key contributors to nitrous oxide production in mixed liquor under low C-to-N ratios during nitrification – batch experiments and modelling

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Running title: N₂O production in nitrifying batch experiments: heterotrophic and autotrophic contributions.
### Table I – Combination of N₂O-producing model structures considered.

<table>
<thead>
<tr>
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<tbody>
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<td>NN</td>
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</tr>
<tr>
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<td></td>
<td>✓</td>
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<tr>
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<td></td>
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<td>✓ 4 step (a / b)</td>
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<tr>
<td>ND-HD</td>
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<td>✓ 4 step (a / b)</td>
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Heterotrophic denitrification (HD) is modelled with two different parameter subsets (a) and (b).
Table II – Best-fit parameter estimates during NH$_4^+$, NO$_2^-$, NO$_3^-$ and DO calibration.

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>Best-fit$_a$</th>
<th>Best-fit$_b$</th>
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<tbody>
<tr>
<td>$u_{AMO}$ (h$^{-1}$)</td>
<td>0.205</td>
<td>$0.182 \pm 0.0019$</td>
<td>$0.187 \pm 0.0023$</td>
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<tr>
<td>$u_{NOB}$ (h$^{-1}$)</td>
<td>0.060</td>
<td>$0.015 \pm 0.0001$</td>
<td>$0.015 \pm 0.0001$</td>
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<tr>
<td>Correlation</td>
<td>0.51</td>
<td>0.55</td>
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</table>
Table III – Best-fit estimates of N2O-related parameters for each model structure considered (HD_a).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NN</th>
<th>ND</th>
<th>HD</th>
<th>NN-HD</th>
<th>ND-HD</th>
<th>Lit. Range</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \eta_{\text{AOB}} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Anoxic reduction factor</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( K_{\text{AOB NO}_2} )</td>
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<td></td>
</tr>
<tr>
<td>( K_{\text{AOB} \text{ O}_2} )</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>( K_{\text{HB} \text{ O}_2 \text{ NIR}} )</td>
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<td></td>
</tr>
<tr>
<td>( K_{\text{HB} \text{ O}_2 \text{ NOR}} )</td>
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<tr>
<td>( K_{\text{HB} \text{ S NIR}} )</td>
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<tr>
<td>( K_{\text{HB} \text{ S NOR}} )</td>
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<tr>
<td>( K_{\text{HB} \text{ S NOS}} )</td>
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<tr>
<td>Max. NO(_2) reduction rate</td>
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<tr>
<td>Max. NO reduction rate</td>
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<td></td>
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<tr>
<td>Max. N(_2)O reduction rate</td>
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<td></td>
</tr>
<tr>
<td>( K_{\text{HB} \text{ S NIR}} )</td>
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<td></td>
</tr>
<tr>
<td>( K_{\text{HB} \text{ S NOR}} )</td>
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<td></td>
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<tr>
<td>( K_{\text{HB} \text{ S NOS}} )</td>
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<tr>
<td>( K_{\text{HB S NIR}} )</td>
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<tr>
<td>( K_{\text{HB S NOR}} )</td>
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</tr>
<tr>
<td>( K_{\text{HB S NOS}} )</td>
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</tbody>
</table>

| Best-fit | \( E_{\text{N2O}} \) |      |      |      |       |           |            |
|          | RMSE            |      |      |      |       |           |            |
|          | 0.83           | 0.98 | 0.98 | 0.97 | 0.99 |            |            |
|          | 0.022          | 0.012 | 0.013 | 0.014 | 0.010 |           |            |

Table IV – Modelling results for the ND-HD model.

<table>
<thead>
<tr>
<th></th>
<th>ND-HD_a</th>
<th>ND-HD_b</th>
</tr>
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<tbody>
<tr>
<td>$E_i$</td>
<td>-0.993</td>
<td>0.995</td>
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<tr>
<td>$N_2O_{AOB}/TOT$</td>
<td>4</td>
<td>39</td>
</tr>
<tr>
<td>$NO/N_2O$</td>
<td>0.2</td>
<td>10.5</td>
</tr>
<tr>
<td>$NO_{AOB}/TOT$</td>
<td>67</td>
<td>37</td>
</tr>
<tr>
<td>$N_2$</td>
<td>0.19</td>
<td>0.39</td>
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