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Title

Modelling N₂O dynamics of activated sludge biomass: uncertainty analysis and pathway contributions

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Abstract

Nitrous oxide (N_2O) is a potent greenhouse gas emitted during biological wastewater treatment. A pseudo-mechanistic model describing three biological pathways for nitric oxide (NO) and N_2O production was calibrated for mixed culture biomass from an activated sludge process using laboratory-scale experiments.

The model (NDHA) comprehensively describes N_2O producing pathways by both autotrophic ammonium oxidizing bacteria and heterotrophic bacteria. Extant respirometric assays and anaerobic batch experiments were designed to calibrate endogenous and exogenous processes (heterotrophic denitrification and autotrophic ammonium/nitrite oxidation) together with the associated net N_2O production. Ten parameters describing heterotrophic processes and seven for autotrophic processes were accurately estimated (variance/mean < 25%). The model predicted accurately NO and N_2O dynamics at varying dissolved oxygen, ammonium and nitrite levels, and was validated against an independent set of experiments with the same biomass.

In aerobic ammonium oxidation experiments the nitrifier denitrification and heterotrophic denitrification estimated pathway contributions increased at high nitrite and low oxygen concentrations; while the nitrifier nitrification pathway showed the largest contribution at high dissolved oxygen levels. The uncertainty of N_2O emissions during model calibration is commonly overlooked, which limits the confidence of model-based mitigation strategies. Here we show that the precision of the estimated parameters resulted in a low uncertainty of the N_2O emission factors during aerobic ammonium oxidation at $\text{DO} \approx 0.5 \text{ mg/L}$ ($1.2 \pm 0.1\%$) and $\text{DO} \approx 2.0 \text{ mg/L}$ ($4.6 \pm 0.6\%$).

Highlights

- Respirometric assays allow the calibration of a model describing N₂O production.
- NH₄⁺ oxidation-driven N₂O production increased at low DO and presence of NO₂⁻.
- N₂O emission factors of the calibrated model showed low uncertainty ($\leq 12\%$).

Keywords: Nitrous oxide, Activated Sludge, Modelling, Uncertainty, Respirometry

1. Introduction

Nitrous oxide (N_2O) is a greenhouse gas emitted at wastewater treatment plants. During biological nitrogen removal N_2O is mainly emitted from aerated zones or during aerated periods due to physical stripping [1,2]. N_2O is produced biologically by two microbial guilds: autotrophic ammonia oxidizing bacteria (AOB) and heterotrophic denitrifying bacteria (HB). AOB aerobically oxidize ammonium (NH_4^+) into nitrite (NO_2^-) via hydroxylamine (NH_2OH). Pure culture studies of model AOBs have shown that a side-reaction of hydroxylamine oxidation to nitrite (HAO-mediated) can produce nitric oxide (NO) and N_2O regardless of dissolved oxygen (DO) levels [3,4]. Also, direct N_2O production from hydroxylamine oxidation catalysed by the enzyme cytochrome (cyt) P460 was recently documented [5]. At low DO concentrations AOBs use nitrite as terminal electron acceptor producing NO, further reduced to N_2O [6–8]. Heterotrophic denitrification of nitrate (NO_3^-) to dinitrogen gas (N_2) is a 4-step process via NO_2^- , NO and N_2O [9].

AOB and HB have been suggested as equal contributors to N_2O production during traditional nitrification and denitrification processes [10–13]. However, in-situ quantification of AOB and HB contributions via nitrogen and oxygen isotopic signatures is rare in full-scale systems compared to lab-scale [14,15]. To gain insights on the process contributions bulk N_2O emissions are correlated to operational parameters, or by multiple linear regression to operational factors [16]. In nitrogen removing systems N_2O production is associated to high NH_4^+ and NO_2^- , and low DO concentrations [17–19]. The NH_4^+ load and influent NH_4^+ concentration have been correlated to N_2O emissions from aerobic zones operating at high DO concentrations [10,20]. As the direct precursor of N_2O in most of the biological pathways, NO has shown the highest correlations with N_2O but NO is often not measured [18,19,21].

Mechanistic models for N-removal have been extended to include N_2O and NO production processes, which allow the prediction of individual pathway contributions. Three biological N_2O production pathways are considered to co-occur: the nitrifier nitrification (NN), the nitrifier denitrification (ND) and the heterotrophic denitrification (HD) pathway. Models considering the three pathways have better predictive capabilities than two pathway models [22], but the mathematical description of N_2O production (i.e. number of processes and variables) varies between model structures. For example, several models describe the ND pathway as direct NO_2^- reduction to N_2O [23,24], not considering NO an intermediate of autotrophic denitrification [3,8]. Hence, NO model predictions would overestimate other NO-producing pathways to fit NO measurements (e.g. NN, HD). Most N_2O models associate the AOB-driven NH_2OH oxidation reaction to obligate DO reduction [23,24] even though O_2 is not required as electron acceptor, and anoxic NH_2OH oxidation yields N_2O [4,25]. The NDHA model considers the three biological pathways for NO and N_2O production and abiotic contribution, DO-independent NH_2OH oxidation, and was previously calibrated for an AOB-enriched biomass [25].

Validation of N₂O model response typically relies on regression coefficients between data and simulation (e.g. R²). However, more rigorous analysis of residuals is required to validate the model response [26]: the performance of two models cannot be compared via visual inspection [24] or regression coefficients, which do not identify structural deficiencies [27]. The frequency of data acquisition from online sensors (e.g. DO, N₂O) impacts the calibration results of N₂O models. Methods used to calculate confidence regions estimated biokinetic parameters often rely on structural assumptions of the residuals, but are often overlooked: gaussian distribution and interdependency at different lag times [28]. Testing the model response can avoid over interpretation of the calibration datasets and parameter uncertainty underestimation (e.g. variance/mean $\ll$ 0.001% [29]). The carbon footprint of wastewater systems is very sensitive to N₂O emissions [30] and precise predictions are desired. Yet, the uncertainty of N₂O emissions associated to parameter estimation is barely studied [25,31]. Here, we aim at calibrating the NDHA model for a mixed culture biomass from a full-scale wastewater treatment plant via respirometric assays to assess the uncertainty of N₂O emissions.

Objectives

- Quantify N₂O dynamics from AS biomass via extant respirometric assays.
- Calibrate the NDHA model to describe N-removing processes and N₂O production of the AS biomass and assess the accuracy of estimated parameters.
- Evaluate the predictive ability of the calibrated model against a different AS biomass dataset.
- Quantify the uncertainty of N₂O emissions during aerobic NH₄⁺ removal.

2. Materials and Methods

2.1. Extant respirometric assays

Mixed liquor from a full-scale phase-isolated activated sludge (AS) wastewater treatment plant (700,000 PE Lynetten, Copenhagen, Denmark) was sampled and aerated overnight; it is here referred to as the AS biomass. To prevent significant changes in biomass composition biomass was discarded, and new samples taken, after two days of experimentation [32]. Biomass samples were harvested by centrifugation at 4700 rpm for 3 min, washed and resuspended in nitrogen-free mineral medium (modified after Graaf et al., 1996) three times to eliminate any soluble substrates. Assays were performed in parallel at 25°C in two 400-mL jacketed glass vessels completely filled with biomass and sealed with the insertion of a Clark-type polarographic DO electrode (YSI Model 5331, Yellow Springs, OH). Biomass samples were saturated with air, pure oxygen or dinitrogen gas prior to the initiation of the assays. A decrease in the DO level in the vessel due to substrate oxidation was measured by the DO probe and continuously acquired (0.2 Hz) by a personal computer interfaced to a DO monitor (YSI Model 5300, Yellow Springs, OH) by a multi-channel data acquisition device (LabPC+, National Instruments, Austin, TX). pH was also monitored (WTW GmbH,

Weilheim, Germany) and liquid NO and N₂O concentrations were measured with Clark-type microsensors (NO-500, N₂O-R, Unisense A/S, Aarhus, Denmark). Stock solutions for all the reagents were prepared from high-purity chemicals for NH₄HCO₃, NH₂OH·HCl, NaNO₂, HCOONa, C₂H₃NaO₂, C₃H₅NaO₂ (Sigma Aldrich) and by initially sparging ≥ 99.998%, 99.5% gas N₂O and O₂ (Sigma-Aldrich, AGA). Photometric test kits were used to analyse N species (1.14752, 1.09713, 1.14776, Merck KGaA, Darmstadt, Germany). Biomass content (MLSS, MLVSS) was measured in duplicates according to APHA [34] and estimates of nitrifying fraction were made from stoichiometric principles [35].

2.2. Experimental Design

The experiments were designed to obtain informative data on N₂O dynamics from mixed liquor biomass. Respirometric approaches were taken (on-line, high-rate O₂ and N₂O measurements) as they are more suited for accurate parameter estimation in comparison to substrate depletion experiments [36]. The kinetics of the oxidation of the primary N-species (NH₄⁺ and NO₂⁻) were individually and step-wise measured via extant respirometry at varying DO concentrations, while simultaneously measuring N₂O. Batch experiments were conducted to measure the heterotrophic and abiotic contributions to total N₂O production (**Table 1**). During anoxic experiments processes were measured both in absence and presence of supplied organic carbon (mixture of formate + acetate + propionate) under NO_x⁻ excess. With no organic carbon supply NO_x⁻ reduction was assumed supported by hydrolysis products originated from biomass decay (**S-0**). A scenario (e.g. (Scenario C)) was defined as a group of experiments with the same primary N-species added by pulses (**Table 1**). At least duplicate experiments were performed for each N-species. The oxygen consumption rate was the additive effect of several independent oxygen consumption processes, potentially including endogenous respiration, NO₂⁻ respiration, and NH₄⁺-respiration (**S-I**). By sequentially following the respirometric response from more to less oxidized N-species (i.e., first NO₂⁻, then NH₄⁺) the identifiability of nitrification kinetic parameters increases [37].

Table 1 – Experimental design for respirometric assays (Scenarios A-C) and for model validation (Scenarios D-E).

Scenario	Oxygen level	Species added	Species monitored	Targeted Processes
(A)	Anoxic	COD+NO ₃ ⁻ / COD+NO ₂ ⁻ / COD+N ₂ O	NO ₃ ⁻ , NO ₂ ⁻ , N ₂ O, NH ₄ ⁺	Heterotrophic denitrification, hydrolysis
	Non-aerated: from excess DO (air-sat) into anoxia dynamically	COD	DO	Biomass decay, hydrolysis
(B)	Anoxic	NO ₃ ⁻ / NO ₂ ⁻	N ₂ O, NO	HB-driven NO/N ₂ O dynamics
(C)	Non-aerated: from excess DO (O ₂ -sat) into anoxia dynamically	NH ₄ ⁺ / NH ₂ OH / NO ₂ ⁻	DO, N ₂ O, NO	NH ₄ ⁺ , NO ₂ ⁻ removal AOB/HB-driven NO/N ₂ O dynamics
(D)	Constant aeration (high and low DO)	NH ₄ ⁺	DO, N ₂ O, NH ₄ ⁺ , NO ₂ ⁻	NH ₄ ⁺ , NO ₂ ⁻ removal, N ₂ O dynamics

(E)	Constant aeration (high and low DO)	$\text{NH}_4^+ / \text{NO}_2^- / \text{NO}_3^-$	DO, N_2O , NH_4^+ , NO_2^-	NH_4^+ , NO_2^- removal, N_2O dynamics
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2.3. Dataset for model evaluation

Separate batch experiments were executed in a 3-L lab-scale reactor with mixed liquor biomass from the same WWTP (Lynetten, Copenhagen, Denmark): (D) Four batch tests received sequential increasing NH_4^+ pulses while subject to constant aeration (2.3, 3.6, 4.7, 5.5 mgN/L); (E) three batch tests received a singular NH_4^+ pulse (4 mg NH_4^+ -N/L) followed by addition of either NO_2^- or NO_3^- when reaching low DO concentrations. The dataset comprises online DO, pH and N_2O liquid measurements together with grab samples for NH_4^+ and NO_2^- . Details of these experiments have been reported before [35].

2.4. Model description

The NDHA model was proposed to describe NO/ N_2O dynamics under a variety of conditions for biomass containing both autotrophic (ammonium and nitrite oxidizing bacteria) and heterotrophic fractions [38]. Shortly, it considers N_2O formation from two autotrophic and one heterotrophic biological pathway, plus abiotic N_2O formation based on recent findings [39]. Unlike any other model, NDHA qualitatively captures NO and N_2O profiles observed at both high and low DO. The observed N_2O production during NH_4^+ oxidation at high DO is described by the Nitrifier Nitrification pathway (NN) [20], where ε_{AOB} describes the fraction of NH_2OH converted to NO instead of NO_2^- . The Autotrophic Denitrification pathway (ND) captures the increasing NO production at low DO and in the presence of NO_2^- , where η_{NIR} describes the NO_2^- reduction to NO inhibited by DO. The higher N_2O yield (N_2O produced / N-species consumed) from NH_2OH oxidation, as observed for pure AOB cultures and mixed communities, is also captured by the model as NH_2OH is the electron donor in both NN and ND pathways, following the oxygen-independent NH_2OH oxidation driven by HAO [3,5,25,40]. The two autotrophic pathways produce NO, which is reduced to N_2O in a last combined step, where η_{NOR} describes the autotrophic NO reduction to N_2O . A 4-step heterotrophic denitrification model was considered based on earlier reports [41]. Individual process rates and inhibition/substrate coefficients were used as suggested for systems with low substrate accumulation. Here we aim to calibrate the NDHA model for AS biomass (S-I).

2.5. Sensitivity analysis and uncertainty evaluation

A global sensitivity analysis (GSA) was performed to identify the most determinant parameters for model outputs via Monte Carlo simulations. Uncertainty from model parameters is propagated as 10-25-50% uniform variations from their default value to model outputs [42] (S-II). Latin hypercube sampling (LHS) was used to cover the parameter space and the Standardized Regression Coefficient method was used to calculate the sensitivity measure β_i , which indicates the effect of the parameter on the corresponding model

output (convergence found for 1200 samples, β_2 threshold > 0.7) [43]. The duration of every experiment was discretized in 400 time steps, and the GSA run at each point, obtaining a dynamic profile of global sensitivity metrics.

The effect of the estimated uncertainty in kinetic parameters on the model output was evaluated by Monte Carlo simulations. Parameter values were sampled via LHS ($n = 500$) for two cases: (1) from literature as in the GSA, and (2), from the distributions obtained after parameter estimation. Model simulations were performed in the Matlab environment (The Mathworks Inc., Natick, USA).

2.6. Parameter estimation procedure

The objective of the parameter estimation procedure was to sequentially estimate kinetic parameters that describe: (1) rates of hydrolysis, heterotrophic denitrification and heterotrophic oxygen consumption; (2) dynamics of nitrogen oxides (including NO and N₂O) under anoxia and endogenous conditions; (3) NO₂⁻ oxidation; (4) NH₄⁺ oxidation; (5) N₂O production associated with NH₄⁺ oxidation (S-0). The error function for problem minimization was defined as:

$$\text{RMNSE} = \sum_k^m \sum_j^n \frac{\text{RMSE}_j}{\bar{y}_{\text{obs},j}}; \quad \text{RMSE}_j = \sqrt{\frac{\sum_i^p (y_{\text{sim},i} - y_{\text{obs},i})^2}{p}}$$

Where m is the number of experiments in one scenario (e.g. 2 experiments where NH₄⁺ is added: Scenario C), n the number of data series in one experiment (e.g. NO, N₂O), p the number of experimental points in each data series, $y_{\text{sim},i}$ the model prediction and $y_{\text{obs},i}$ the experimental data at time i . A global search algorithm (Global Search) was used over a wide parameter space, followed by a local minimization algorithm (Pattern Search). Parameters describing the elemental biomass composition (e.g. i_{NXB}), yield and temperature coefficients were fixed at default values and not subject to estimation. Newly estimated parameters were fixed at their best-fit estimate for the following parameter estimation step.

2.7. Validation of model response and parameter estimates

To test the validity of the model response (i.e., the adequacy of model to predict the observed data points) the interdependency of residuals ($y_{\text{sim},i} - y_{\text{obs},i}$) was analysed by autocorrelation for different lag times [28]. The quality of the model fit was evaluated via correlation coefficients (R^2) and more rigorously by an F-distribution test, with the hypothesis of a linear regression with simultaneous unit slope and zero intercept [27]. The identifiability of a parameter subset K was evaluated by a collinearity index (γ_K), which quantifies the near-linear dependence of local sensitivity functions. A collinearity index higher than 15 indicates a

poorly identifiable parameter subset K [44]. Approximate confidence regions were calculated following J_{crit}

$$= J_{opt} \left(1 + \frac{p}{N_{data} - p} F_{\alpha, p, N_{data} - p} \right) [45].$$

2.8. Case study: nitrification/denitrification cycle

To study the sensitivity of N_2O and NO emissions during NH_4^+ removal with AS biomass a nitrification/denitrification cycle in a sequencing-batch reactor was simulated (**S-II**). Initially, NH_3 was added as a pulse (30 mgN_{tot}/L), consumed during 2 h of constant aeration, followed by 0.5 h anoxic period with excess carbon dosage (200 $mgCOD/L$). To investigate the effect of DO concentration on N_2O emissions the aeration rates ($k_L a_{O_2}$) were adjusted to attain DO levels of approximately 0.5 and 2 mg/L respectively. The biomass content was set to 4 $gTSS/L$ and stripping for N_2O and NO was calculated from diffusivity corrections [46]. A global sensitivity analysis was performed when removal rates were stable within one cycle (SRC method). After validating the model, the same nitrification/denitrification cycle was re-evaluated to study the pathway contributions and the uncertainty of N_2O emissions associated to kinetic parameters.

3. Results

3.1. Sensitivity analysis on the nitrification/denitrification case study

Simulation results for the nitrification/denitrification case study were used to investigate the main processes driving N_2O production. The majority of N_2O was produced and emitted during the aerobic part of the cycle at both low and high aeration rates, when NH_4^+ oxidation occurs. The highest ranked parameters were associated to DO and NO_2^- sensitivity. The GSA ranking showed that at high DO the two most sensitive parameters correspond to AOB, followed by NOB and heterotrophic NO_2^- reduction. At low DO all three microbial groups share high sensitivity (**S-II**). Overall, the results highlight the importance of interactions between AOB, NOB, and HB, on the N_2O production from AS biomass during aerobic NH_4^+ oxidation. After identifying the key parameters activity tests can be designed accordingly.

3.2. Experimental results

Biomass activity tests

During Scenario A experiments heterotrophic denitrification and hydrolysis of biomass decay products were monitored. Under anoxic conditions the hydrolytic processes released NH_4^+ into the bulk (0.16 $mgNH_4^+-N/gVSS/h$). Simultaneously, biodegradable carbon was released and heterotrophic denitrification of NO_3^- , NO_2^- and N_2O was measured individually at excess electron acceptor concentrations (1.5, 2.5 and 4.7 $mgN/gVSS/h$ respectively) (**Figure 1A**). The specific denitrification rates were significantly higher in the

presence of excess electron donor (mix of C-sources) compared to endogenous conditions (7, 6.2 and 12 mgN/gVSS/h). The maximum N_2O reduction rate varied 3-fold in the pH range 6.5 - 9, with a maximum at around pH = 8 (**Figure 1B**). Under endogenous aerobic conditions, a positive oxygen uptake rate was constantly measured, reflecting respiration of the biodegradable carbon and NH_4^+ released during hydrolysis (**S-V**). Addition of external biodegradable C-source increased the oxygen consumption rate compared to endogenous conditions (35 vs 4.5-7 mgCOD/gVSS/h).

Scenario C experiments were designed to study oxygen uptake associated with nitrification and started at excess DO ($> 30\text{mg/L}$). The oxygen uptake showed a dynamic response, increasing from a positive baseline up to a maximum rate after the N pulse, and decreasing until either the N-species (NH_4^+ , NO_2^-) or DO reached limiting conditions ($\text{OUR}_{\text{max,NH}_4} = 31 \text{ mgO}_2/\text{gVSS.h}$, $\text{OUR}_{\text{max,NO}_2} = 18 \text{ mgO}_2/\text{gVSS.h}$) (**Figure 1C,D**).

Figure 1 – Experimental and modelling results for heterotrophic processes (A, Scenario A), Anoxic N_2O reduction dependency on pH (B, Scenario A), Aerobic NO_2^- oxidation (C, Scenario C), Aerobic NH_4^+ oxidation (D, Scenario C). Experimental data (markers), best-fit model simulations (solid lines), 95% confidence intervals (dashed lines).

N_2O dynamics during respirometric assays

During Scenario B experiments under anoxic conditions, NO and N₂O transiently accumulated after NO₂⁻ and NO₃⁻ pulses (S-V), but did not accumulate after sole NH₄⁺ addition. After a NO₃⁻ pulse NO concentration increased until reaching a maximum, followed by a steady decrease. The same trend was observed for N₂O but delayed with respect to NO; N₂O accumulation stopped after NO disappeared, followed by a continuous decrease. Similar NO and N₂O dynamics was observed after NO₂⁻ addition (**Figure 2A,B**), indicating NO as N₂O precursor during heterotrophic denitrification.

In Scenario C experiments neither NO nor N₂O accumulated in the bulk during NO₂⁻ oxidation (**Figure 1C**). NH₄⁺ oxidation lead to a low NO accumulation compared to the simultaneous and higher N₂O increase (**Figure 1D, 2C**). The sole addition of NH₂OH, an intermediate of NH₄⁺ oxidation to NO₂⁻, yielded the highest NO and N₂O accumulation rates (N₂O accumulation rates $\approx 0 - 0.05 - 0.2$ mgN/gVSS/h for NO₂⁻, NH₄⁺ and NH₂OH) (**Figure 2C**). Irrespective of the N-species being oxidized, at the onset of anoxia NO and N₂O concentrations increased. First NO, and then N₂O, reached a maximum followed by a steady decrease, in a similar pattern to that observed after NO₂⁻ or NO₃⁻ pulses in anoxic experiments (Scenario B). The NO and N₂O decrease indicate net consumption rates, but simultaneous production/consumption could still occur.

Figure 2 – Experimental and modelling results obtained during parameter estimation (A, Scenario A; B, Scenario B; C, Scenario C) and after parameter estimation (D, Scenario C). NO and N₂O dynamics after anoxic NO₂⁻ pulse under endogenous conditions (A). N₂O consumption in the absence and presence of DO (DO pulses at t = 13, 21 min) (B). Oxygen consumption, NO and N₂O dynamics after NH₄⁺ pulse addition (t = 10 min) (C). Oxygen consumption and N₂O dynamics after NH₄⁺ pulse addition (D). Experimental data (markers), best-fit simulations (solid lines), 95% confidence intervals for the uncertainty of all model parameters (dashed lines).

3.3. Modelling results

The applicability of the NDHA model to describe the observed data was examined. Because GSA results highlighted the interactions between HB, NOB and AOB during N₂O production a step-by-step calibration procedure was followed that included all sensitive processes. First, the release of NH₄⁺ and biodegradable organic carbon was studied under aerobic and anoxic conditions. The model simulated the anoxic NH₄⁺ release from hydrolysis ($R^2 = 0.97$), and μ_{HB} was estimated to fit the oxygen uptake measured during Scenario A experiments (**Table 2**). Then, the maximum denitrification rates on NO₃⁻ and NO₂⁻ (μ_{NAR} , μ_{NIR}) were estimated from experiments in Scenario A ($R^2 > 0.96$). With the N₂O dataset the pH-dependency of the N₂O reduction was fitted using a sinusoidal model [47] (w_{NOS} , $pH_{opt.NOS}$) while the substrate affinity for N₂O reduction ($K_{HB.N2O}$) could be estimated from the N₂O-limited part of individual experiments from Scenario A ($R^2 > 0.98$). The NO and N₂O concentrations from experiments in Scenario B, under endogenous respiration, were sensitive to changes in the affinity to biodegradable carbon for NO₂⁻, NO and N₂O reduction (**S-III**). Hence, the NO and N₂O datasets were used to identify three of the affinities that were within the top sensitive parameters ($K_{HB.S.NIR}$, $K_{HB.S.NOR}$, $K_{HB.S.NOS}$, $\gamma < 15$, $R^2 = 0.94$).

The oxygen consumption rate is a measured variable in all experiments from Scenario C, and in the NDHA model structure it is the sum of several processes: nitrification, nitrification, heterotrophic aerobic growth, decay and hydrolysis. The experimental design allowed the independent and sequential estimation of the interferences of these processes on AOB activity (**Table 2**). First, the substrate affinity and maximum growth rate of NO₂⁻ oxidation ($K_{NOB.HNO2}$, μ_{NOB}) were estimated ($\gamma < 7$). Then, NH₄⁺ oxidation was described by estimating the substrate affinity and maximum growth rate of the first nitrification step ($K_{AOB.NH3}$, $\mu_{AOB.AMO}$). The NDHA model considers NH₃ as true substrate for AOB, and at lower pH levels NH₃ oxidation slows down, increasing the information content of the experiment compared to the same NH₄⁺-N pulse at higher pH. Hence, the ammonia affinity for AOB ($K_{AOB.NH3} = 7 \mu\text{gN/L}$) could be estimated from NH₄⁺ pulses (2-3 mgN/L) at low pH as the DO sensitivity to changes in $K_{AOB.NH3}$ increases at lower pH levels (**S-III**).

Table 2 – Best-fit values for the parameters estimated (at 25 °C).

Parameter	Units	Value	Scenario	Parameter	Units	Value	Scenario	Parameter	Units	Value	Scenario
$pH_{opt,nos}$					mgCO ₂ /L						
Z	(-)	7.9 ± 0.1	(A)	$K_{HB,S,NIR}$	D/L	4.3 ± 0.69	(B)	μ_{NOB}	d ⁻¹	1.51 ± 0.07	(C)
$w_{nos,Z}$	(-)	2.2 ± 0.2	(A)	$K_{HB,S,NO}$	D/L	5.3 ± 0.83	(B)	μ_{HB}	d ⁻¹	7.23 ± 0.16	(A)
K_{HB,N_2O}	mgN/L	0.078 ± 0.020	(A)	$K_{HB,S,NO}$	D/L	4.1 ± 0.40	(B)	ϵ_{AOB}	(-)	0.0031 ± 0.0001	(C)
$\mu_{HB,NAR}$	d ⁻¹	1.71 ± 0.11	(A)	$K_{AOB,NH}$		7.00 ± 1.17	(C)	η_{NIR}	(-)	0.22 ± 0.01	(C)
$\mu_{HB,NIR}$	d ⁻¹	1.11 ± 0.07	(A)	$K_{NOB,HN}$		0.027 ± 0.006	(C)	η_{NOR}	(-)	0.36 ± 0.02	(C)
$\mu_{HB,NOS}$	d ⁻¹	1.17 ± 0.02	(A)	$\mu_{AOB,AM}$		0.86 ± 0.02	(C)				

Single NH_2OH pulses were not considered for parameter estimation as the electron flow during NH_2OH oxidation in the AOB metabolism differs from that during NH_4^+ oxidation (two out of the four electrons are shuttled back from HAO to AMO). Hence, NH_2OH pulses are not representative of NH_4^+ -oxidation driven N_2O production. Also, the contribution of NH_2OH to abiotic $\text{NO}/\text{N}_2\text{O}$ production was not significant after NH_2OH pulses for experiments in Scenario C (S-V).

The analysis of residuals from best-fit simulations after parameter estimation with the complete datasets showed a high autocorrelation of the residuals for some experiments. The DO and N_2O datasets were down-sampled by lowering the data acquisition frequency down to non-autocorrelated values [25,28]. In Scenario C, the DO dataset used to estimate the parameter subset $K_{AOB.NH_3}$ and $\mu_{AOB.AMO}$ was down-sampled thirteen-fold to correct autocorrelation (from 1230 to 96 data points). With the updated dataset the best-fit estimates did not change significantly (0.7 and 4.2% variation between datasets), but the uncertainty increased almost four-fold (S-IV).

3.4. Model validation

The predictive ability of the calibrated NDHA model was evaluated on a different set of batch experiments where the AS biomass from the same WWTP was subject to varying N pulses under constant aeration [35]. The model was evaluated with the calibrated parameter set in Scenarios D and E against DO, NH_4^+ , NO_2^- and N_2O data. Only the oxygen mass transfer coefficient ($k_L a_{O_2}$) was tuned as the aeration in the respirometric assays from Scenarios A, B, C was null. Overall, the model captured the trends of DO, main N-species and liquid N_2O without modifying any parameter value (R^2_{avg} for DO = 0.98; NH_4^+ = 0.99; NO_2^- = 0.84; N_2O = 0.80), and only the N_2O residuals ($y_{\text{sim},i} - y_{\text{obs},i}$) did not pass the more rigorous F-distribution test ($F_{\text{N}_2\text{O}} = 0$) (Figure 4).

Figure 4 –Model validation results from Scenario D. Effect of NO_3^- pulse (A1,A2) and NO_2^- pulse (B1,B2) during aerobic NH_4^+ oxidation. Main substrates: DO, NH_4^+ , NO_2^- (A1, B1), N_2O and N_2O pathway contributions (A2, B2).

Experimental results (markers), best-fit simulations (black lines), 95% confidence intervals (red lines). Nitrifier nitrification (cyan), nitrifier denitrification (blue), and heterotrophic denitrification (black) pathway contributions; mean (solid lines) and 95% confidence intervals (dashed lines). Confidence intervals of uncertainty from the estimated parameters only (Table 2).

The model predicts a higher N_2O fraction with increasing NH_4^+ pulses as NH_4^+ oxidation occurs at low DO for longer periods, thus promoting the contribution of denitrification pathways (**Figure 5**). In terms of pathway contributions to the total N_2O pool, the model predicts that after addition of an NH_4^+ pulse at high DO the NN pathway has the largest contribution (**Figure 4**). When NO_2^- accumulates in the bulk and DO reaches low concentrations ($\text{DO} < 0.5 \text{ mg/L}$), or when NO_2^- is spiked, the ND and HD contributions increase. Even though the model predicts a positive HD pathway contribution to the N_2O pool (no net N_2O consumption) it was significantly lower than the ND pathway (**Figure 5**).

Scen.	Pulse (mgN/L)			N_2O emission factor $\text{N}_2\text{O} / \text{NH}_{4,\text{rem}}$	N_2O pathway contribution		
	NH_4^+	NO_2^-	NO_3^-		NN (%)	ND (%)	HD (%)
D-1	2.3			0.6%	57%	33%	10%
D-2	3.6			1.7%	22%	57%	21%
D-3	4.7			2.5%	19%	60%	21%
D-4	5.5			3.2%	13%	64%	23%
E-1	3.9			0.8%	43%	44%	13%
E-2	4.0		2.0	0.8%	41%	47%	12%
E-3	4.1	2.5		1.7%	17%	66%	17%

Figure 5 – Model evaluation results for continuously aerated batch tests for different NH_4^+ , NO_2^- , NO_3^- pulse additions: N_2O emission factor and N_2O pathway contributions.

3.5. Model evaluation and uncertainty of N_2O emissions during N-removal

N_2O emissions from AS biomass during aerobic NH_4^+ oxidation simulations with best-fit estimate parameters were predicted for a wider range of DO (0.2 – 4 mg/L) and NO_2^- (0 – 1.4 mgN/L) at excess NH_4^+ . The N_2O emission factor and individual pathway contributions to the total N_2O pool at pseudo-steady state are shown in **Figure 6**. The NN pathway contributes most at the lowest NO_2^- and highest DO (98%), and the least at high NO_2^- and low DO (3%). The ND and HD pathways showed similar trends and opposite compared to the NN pathway, with maximum contributions of 72% and 43% respectively. The N_2O emission factor ranged between 0.45 and 12.3% with a very sharp increase towards low DO and high NO_2^- . For example, at DO = 2 mg/L, with increasing NO_2^- concentrations the emission factor increased from 0.45 to 1.26%.

Figure 6 – Model evaluation at varying NO_2^- and DO concentrations during excess NH_4^+ removal (pH = 7.2). From left to right: Pathway contributions to total N_2O pool NN, ND, HD; N_2O emission factor.

The uncertainty in prediction of N-removing processes propagates into prediction of N_2O production, increasing the uncertainty of N_2O emissions compared to main N-species (**Figure 4**). Here, the precision of the biological parameter estimates from respirometric assays the uncertainty of N_2O emissions in the nitrification/denitrification case study (low and high DO) was evaluated [42]. Two cases were analysed: the calibrated parameter subset with the estimated uncertainty from Table 2, and using uncertainty classes as a reference (**S-II**). By comparing the two cases the uncertainty calculated from Table 2 was only 28% of that simulated with the reference. The calibrated NDHA model predicted for low and high DO an N_2O emission factor of $4.6 \pm 0.6\%$ and $1.2 \pm 0.1\%$, which corresponds to low coefficients of variation of the emission factors (9 and 12%) (**Figure 7**). Similarly, the uncertainty of the contributions from each biological pathway was low, indicating precise parameter estimates ($\text{CV}_{\text{NN}} = 8\%$, $\text{CV}_{\text{ND}} = 6\%$, $\text{CV}_{\text{HD}} = 19\%$).

4. Discussion

4.1. N-dynamics in respirometric assays: experimental and modelling results

Under endogenous conditions the lowest denitrification rate corresponded to NO_3^- reduction, followed by NO_2^- and N_2O reductions (**Figure 1**). A limiting electron flow could explain these differences as more reduced NO_x^- compounds require fewer electrons to produce N_2 . To avoid a biased denitrification rate due to a single carbon source [48], a mixture of three carbon sources was considered representative of a complex wastewater (20% formate, 40% acetate, 40% propionate COD-based) as the estimated μ_{HB} value deviated 21% from the original literature value (5.2 vs. 6.2 d^{-1}) [41]. Overall, the denitrification rates observed were in the low range of literature values (**S-V**). The maximum reduction rate corresponded to NO , which agrees with the low NO accumulation levels observed during denitrification, followed by NO_3^- , N_2O and NO_2^- . The differences in the carbon usage by each reduction step can be linked to the high modularity of the heterotrophic denitrifying community [49]. The highest affinity for the electron donor corresponds to N_2O , and lowers for NO_2^- , NO_3^- and NO , similarly to other calibrated denitrification models [50]. After model fitting, the reduction factors for the denitrification steps compared to the aerobic growth rate are similar to reported values.

N_2O is typically found at very low concentrations due to stripping and/or simultaneous production/consumption processes. Hence, the only biological N_2O consumption process could be isolated by only providing N_2O at high concentrations as the only N-source ($> 1\text{mgN/L}$). pH changes significantly affected the maximum N_2O consumption rate, in a similar fashion to that found by Pan *et al.*, (2012) for a methanol-fed denitrifying culture, and for a pure culture of *Paracoccus denitrificans* [52]. By including a sinusoidal function the model captured this trend (**Figure 1B**). The substrate affinity was independently estimated as $\mu_{HB,NOS}$ had been previously fixed ($K_{HB,N_2O} = 0.078\text{mgN/L}$) and, differently from Pan *et al.*, (2012), the individual K_{HB,N_2O} values estimated from each experiment did not correlate with pH ($R^2 = 0.06$, $n = 12$, **S-IV**). Liquid N_2O concentrations are commonly measured at similar or lower values than K_{HB,N_2O} , indicating that N_2O consumption is a substrate limited process and specific experiments must be designed to estimate it [19,53].

AOB-driven N_2O production is described by three processes whose maximum specific rates are reduction factors for NH_2OH oxidation ($\mu_{AOB,HAO}$). The three reduction factors could be estimated: ε_{AOB} (NN), η_{NIR} (ND) and η_{NOR} (NN + ND). Direct comparison of the estimated values to other studies is not possible due to differences in model structure. However, ε_{AOB} (0.0031) and η_{NIR} (0.22) were in the same range of other reduction factors from 2-pathway AOB models ($\eta_{NN} = 0.0007 - 0.12$, $\eta_{NIR} = 0.03 - 0.48$) (**S-I**).

Moreover, the respirometric experimental design benefited from NO measurements compared to only N_2O measurements as it allowed: (1) identification of specific parameters (e.g. $K_{HB,S,NIR}$) and (2) lowering the estimated uncertainty of ε_{AOB} and η_{NOR} 4 and 9-fold respectively (**S-IV-1**).

4.2. Applicability of the NDHA model to mixed liquor biomass: pathway contributions and uncertainty analysis.

The nitrification/denitrification cycle was re-evaluated with the newly estimated parameters to study the pathway contributions and the uncertainty associated to N_2O emissions. Almost all the N_2O was emitted during the aerated phase, as during the anoxic phase N_2O was consumed with excess COD. The simulated cycle at low DO yielded a higher N_2O emission factor as compared to that at higher DO (4.6 and 1.2% respectively), in agreement with other SND systems [13,54]. The N_2O emission factors are comparable with those reported by Wunderlin *et al.* (2012) after NH_4^+ pulses at the same DO levels (3.8 and 2%). Also, in a full-scale activated sludge plant the emission factor varied between 0.69 and 3.5% for a range of DO and NO_2^- conditions [56].

Figure 7 – Nitrification/Denitrification case study after model calibration: N_2O emission factor (left) and N_2O and NO pathway contribution (right). The error bars correspond to the standard deviation of the uncertainty from estimated parameters: (top bar) in this study (Table 2), (bottom bar) default, $n = 500$.

The contribution of the NN pathway increased with DO, while the two denitrification pathways, ND and HD, decreased (**Figure 7**). For both DO levels the ND contribution was significantly larger than the HD contribution (64 vs 17%, 42 vs 7% respectively). Similarly, in other modelling studies an anaerobic/oxic/anoxic process showed that at $DO < 1$ mg/L the ND pathway was also the main contributor to the total N_2O pool, followed by NN and HD [57]. However, based on experimental observations other studies predicted a higher HD contribution compared to ND [11,55]. Separating the ND and HD contributions from NH_4^+ oxidation processes with AS biomass is not trivial [35]. Even at minimum C/N ratios the high heterotrophic abundance compensates for the low electron donor concentrations, yielding similar ND and HD rates, both increasing with NO_2^- and low DO concentrations. Here, as compared to other

studies, the step-wise and independent calibration of the three pathways provides more confidence in the model simulations.

During the past years sequential N₂O dynamic modelling efforts have focused on improving model structures to capture the variety of N₂O production phenomena and rates observed during wastewater treatment operations. From single to double AOB pathways, and in combination with HB, N₂O models have increased in complexity (i.e. more processes and parameters) and improved in their best-fit capabilities. The confidence of model predictions is addressed in WWTP models to assess the risk of excessive effluent nutrient concentrations, but has not been studied for N₂O models. In our previous study for an AOB-enriched biomass, the calibrated NDHA model showed an 8% variation of the N₂O emission factor when only the estimated parameters were considered, but 40% when all the model parameters were considered uncertain [25]. In this study, the uncertainty was 11% and 38% respectively, which would propagate to the carbon-footprint uncertainty of the process. While these results highlight the importance of evaluating the uncertainty of biological parameter estimates in N₂O emissions, only two other studies are available [25,31]. We believe that future comparison of best-fit simulations and uncertainty results (e.g. 95% CI) will improve calibration procedures for N₂O models.

4.3. Biological realism of N-dynamics during N₂O model calibration

4.3.1. Role of nitric oxide on N₂O models

Models with similar N₂O pathway structures cannot be discriminated only with N₂O datasets, and additional measurements such as NO can improve model discrimination [58]. However, only a few N₂O models have been tested against NO [22]. In this study net NO and N₂O production from NH₄⁺ oxidation under aerobic conditions was significantly lower than at the onset of anoxia (**Figure 2**), as was also reported for pure AOB cultures and nitrifying systems [3,19,55]. The higher anoxic rates can be explained by the transient accumulation of NH₂OH, which has been suggested to act as electron donor for NO₂⁻ reduction to N₂O in a two-step process over NO [7,8,59]. The accumulation of NO at low DO and anoxic conditions was also confirmed by an increase in the transcription of the *nirK* gene in a *N. europaea* pure culture [6], which highlights the importance of N₂O models including NO as an intermediate of the ND pathway. Still, some N₂O models simplify the ND pathway by direct NO₂⁻ reduction to N₂O [23,24], while in the HD pathway NO₂⁻ is reduced to N₂O over NO [31]. These model structures would associate NO production during NH₄⁺ removal at low DO fully to the HD pathway (**Figure 2**), therefore underestimating the contribution of the ND pathway.

The ratio between the substrate affinity of NO reductases, $K_{AOB.NO}/K_{HB.NO}$, is an important parameter of N₂O models as it can shift the predicted contributions of the ND and HD pathways for the same overall N₂O fit [35]. However, direct estimation of NO affinity is difficult due to cellular toxicity [60]. In N₂O models K_{NO}

values are typically assumed [41,61] and, when estimated, no identifiability metrics are reported [62]. This could partially explain the high variability of reported NO affinities and $K_{AOB,NO}/K_{HB,NO}$ ratios considered in literature ($K_{HB,NO} = 0.00015\text{--}0.05$ mgN/L and $K_{AOB,NO}$, $0.004\text{--}1$ mgN/L, $K_{AOB,NO}/K_{HB,NO} = 1 - 56$) [22,41,50,62–64]. For example, in the study by Wang *et al.* (2016) the HD pathway has an NO affinity over 50 times higher than the NN pathway. The HD pathway could theoretically uptake NO produced by the NN pathway at a much higher rate and could underestimate the NN contribution to the total N_2O pool. Based on current lack of knowledge and to avoid a preferential NO-consumption/ N_2O -production pathway the NO affinity of AOB and HB was set equal.

4.3.2. Role of hydroxylamine on N_2O models

NH_2OH is an important intermediate in the 2-step NH_4^+ oxidation to NO_2^- as the electron source for the metabolism of AOB. Low NH_2OH bulk concentrations have been reported in the liquid phase of pure AOB cultures and nitrifying systems ($NH_2OH < 0.2$ mgN/L) [39,65,66], indicating a quick turnover of NH_2OH . N_2O models have adopted the 2-step NH_4^+ oxidation where NH_2OH is the direct substrate for NO and N_2O production [20,23,24,50,57]. However, the accuracy of NH_2OH predictions is not addressed. For more accurate NH_2OH predictions a faster HAO process compared to AMO is necessary as it prevents high NH_2OH accumulations [25]. This is in agreement with the calibrated NDHA model where differently from other models, $\mu_{HAO} > \mu_{AMO}$ and $K_{AOB,NH_2OH} < K_{AOB,NH_4}$ ($K_{AOB,NH_2OH} = 0.2$ mgN/L this study, $0.7 - 2.4$ mgN/L [20,23,24,50,57]). The NDHA model predicted average NH_2OH concentrations of 0.15 ± 0.09 mgN/L in Scenario C experiments and 0.13 ± 0.04 mgN/L in Scenario D experiments, in close agreement with reported NH_2OH concentrations.

The higher N_2O yield observed after NH_2OH pulses compared to other N-species under aerobic conditions is in agreement with other studies (≈ 0.05 and 0.2 mg N_2O -N/gVSS/h for NH_4^+ and NH_2OH) [3,40,55,67]. The NDHA model structure can describe N_2O production associated to: aerobic NH_4^+ oxidation (S-I, Processes 1-5), aerobic NH_2OH oxidation (Processes 3-5), and anoxic NH_2OH oxidation (Processes 2, 5). However, the metabolism of AOB during aerobic NH_2OH oxidation in the absence of NH_4^+ is not representative of wastewater treatment operations: when AMO is not active higher electron availability from NH_2OH oxidation would lead to higher N_2O yields from NO and NO_2^- reduction. Hence, experiments with solely aerobic NH_2OH additions should not be used to calibrate the N_2O models describing the NN and ND pathways. Other N_2O pathways, such as the associated to Cyt P460, could be responsible for the high N_2O production observed during NH_2OH oxidation [5].

The NDHA model does not describe individually all the possible co-occurring N_2O pathways in the AOB metabolism, but captures the relevant biological N_2O production associated to wastewater treatment conditions in the NN and ND processes, where NH_4^+ is the N source.

5. Conclusions

- A global sensitivity analysis-based experimental design allowed the calibration of an N₂O model during nitrification and denitrification via respirometric techniques.
- The model considering three biological N₂O production pathways was calibrated and predicted the NO and N₂O dynamics at varying NH₄⁺, NO₂⁻ and DO levels. Parameters from hydrolysis, heterotrophic denitrification, nitrite and ammonia oxidation were identified.
- The calibrated model response indicated autocorrelation of residuals, which was corrected by decreasing the frequency of data acquisition. Yet, a total of 17 parameters could be identified with low confidence intervals (CV < 25%).
- The uncertainty of N₂O model predictions was evaluated during aerobic NH₄⁺ oxidation at two different DO levels showing low uncertainty of the N₂O emission factors (CV ≤ 12 %).
- Biological N₂O consumption was highly dependent on pH, described with a sinusoidal function, which helps associating N₂O dynamics to both changes in N₂O production and N₂O consumption.

Software availability

The MATLAB/SIMULINK code containing the implementation of the model is free upon request to the corresponding author.

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Mixed Liquor WWTP → Lab-scale Respirometry → Model Calibration / Validation → N₂O Pathway contribution?