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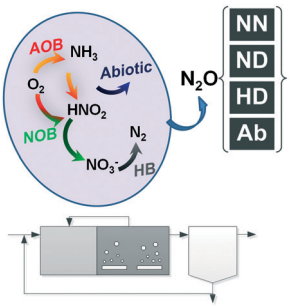

A consilience model to describe N_2O production during biological N removal

C. Domingo-Félez and B. F. Smets*

A mathematical model congruent with the current understanding of the biological processes occurring during wastewater treatment operations is proposed.

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Nitrous oxide (N_2O), a potent greenhouse gas, is produced during biological nitrogen conversion in wastewater treatment operations. Complex mechanisms underlie N_2O production by autotrophic and heterotrophic organisms, which continue to be unravelled. Mathematical models that describe nitric oxide (NO) and N_2O dynamics have been proposed. Here, a first comprehensive model that considers all relevant NO and N_2O production and consumption mechanisms is proposed. The model describes autotrophic NO production by ammonia oxidizing bacteria associated with ammonia oxidation and with nitrite reduction, followed by NO reduction to N_2O. It also considers NO and N_2O as intermediates in heterotrophic denitrification in a 4-step model. Three biological NO and N_2O production pathways are accounted for, improving the capabilities of existing models while not increasing their complexity. Abiotic contributions from NH_2OH and HNO_2 reactions are also included. The model structure can theoretically predict NO and N_2O emissions under a wide range of operating conditions and will help develop mitigation strategies.

Water impact

Wastewater treatment operations are anthropogenic sources of nitrous oxide (N_2O), a potent greenhouse gas and ozone depleting compound. While energy efficiency has been the recent focus of technology development in wastewater management, the carbon footprint of a wastewater treatment plant is utmost sensitive to its N_2O emissions. Informed by a review of known biological and chemical N_2O producing mechanisms, an improved mathematical model structure that may help the development of N_2O mitigation strategies for full-scale treatment operations is proposed.

1. Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas emitted from wastewater treatment processes during biological nitrogen conversions. Due to its high radiative forcing, the carbon footprint of wastewater treatment plants (WWTPs) is highly sensitive to N_2O emissions,[1] which vary largely between WWTPs.[2] Biologically mediated, N_2O can be produced during nitrification and exists as an obligate intermediate during denitrification.[3] The mechanisms and regulations of N_2O production in these processes are still under investigation, and identification and better understanding of the key variables driving N_2O production are necessary.

With the final goal of mitigating N_2O emissions, mathematical models are useful tools to translate our understanding of biological phenomena into equations and predictions. Models must be developed by identifying, combining and translating into mathematical equations the key processes and influencing variables that govern N_2O dynamics.

The first models that described autotrophic N_2O production considered only one of two pathways, either the nitrifier nitrification (NN) or the nitrifier denitrification (ND) pathway. Each pathway was modelled with different levels of complexity affecting the number of considered variables and substrate or inhibition dependencies.[4] However, the range of applicability of single pathway models is narrow.[5] Newly developed models consider both nitrifier pathways, better capturing the state of knowledge on mechanisms. However, the simplification proposed to one of the N_2O pathways might not always be true, thus limiting their applicability.[6,7]

In combination with N_2O production, physicochemical processes transfer N_2O from the liquid to the gas phase resulting in actual N_2O emissions. Mass-transfer processes are relatively well studied, and our emphasis here is on the production processes.[8] A comprehensive model structure should be capable of describing N_2O production under a wide range of operating conditions. By increasing the model complexity with additional components and parameters,

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model predictions can be more accurate. However, model over-parameterization challenges the calibration process and increases parameter identifiability problems. The large variability of reported model parameters in N_2O models is likely an indicator of the limited structural and practical identifiability of the models. For example, reported substrate affinity constants for nitrite (NO_2^-) and nitric oxide (NO) reduction in current N_2O models range across almost two orders of magnitude (Table S1†). Assessing calibration results helps one to discriminate between models by comparing parameter identifiability or prediction uncertainty.[9] It is therefore necessary to obtain simple, yet sufficiently complete, model structures that capture the fundamental mechanisms of N_2O during wastewater treatment operations.

The aims of this communication are (i) to identify key processes and variables driving N_2O production during N removal and (ii) to propose a simple yet comprehensive model structure capable of describing reported N_2O observations. The model should increase the applicability of existing N_2O models and be consistent with current knowledge on N_2O production mechanisms.

2. N_2O production during wastewater treatment operations

Biological nitrogen removal typically is a two-step process where nitrifying bacteria oxidize ammonia (NH_3) to nitrogen oxides (NO_x^-), followed by anoxic NO_x^- reduction to dinitrogen gas (N_2) with organic matter (COD) as an electron source usually by heterotrophic denitrifying bacteria. N_2O can be produced by ammonia oxidizing bacteria (AOB) and archaea during oxidation of ammonia to nitrite (NO_2^- or, more correctly, nitrous acid (HNO_2)) and by heterotrophic bacteria (HB) as an obligate intermediate of denitrification. We do not discuss the scenario of completely autotrophic N removal which would involve a combination of aerobic and anaerobic ammonium oxidation (anammox) as anammox bacteria have no known N_2O production mechanisms.

Autotrophic N_2O production

The oxidation of NH_3 with molecular oxygen to hydroxylamine (NH_2OH) by ammonia monooxygenase requires two electrons. These electrons are supplied by the subsequent oxidation of NH_2OH to HNO_2 consuming molecular water, which releases four electrons, while oxygen is reduced in the terminal oxidase. Aerobic NH_2OH oxidation is therefore the electron-yielding process for AOB growth[10,11] and essential for energy production.

AOB can produce N_2O from the incomplete oxidation of NH_2OH to HNO_2 *via* NO or to its reduced form HNO.[12] This process is referred to as nitrifier nitrification (NN),[13] recently shown to be uncoupled from HNO_2 production.[14] In addition, AOB have a denitrifying functionality, where a set of NO_2^-- and NO-reducing enzymes (NIR, NOR) can result in N_2O production termed nitrifier denitrification (ND) (this has been confirmed by genomic analysis of *Nitrosomonas europaea*[15]). Under low dissolved oxygen (DO) conditions, HNO_2 is reduced to N_2O *via* NO in the presence of an electron donor such as NH_2OH.[11,16,17] DO differently affects the expression of NIR and NOR enzymes. NO production, regulated by NIRK, is favoured under anoxic conditions,[18–21] while NORB activity is upregulated under oxic conditions.[22] Moreover, the enzymology of AOB suggests the presence of additional NO reducing catalytic units similar to the NOR cluster such as the CYT554.[23,24]

Varying DO levels are common during wastewater treatment operations which, together with dynamic HNO_2 concentrations, can lead to imbalances in NO and N_2O emissions.[21,25] Thus, process conditions can switch the dominant AOB-associated N_2O production pathway between NN and ND.

pH levels have two distinct effects on autotrophic N_2O production. First, on the enzymatic level, maximum activities have been described as pH-dependent.[26] Second, the true substrates available for AOB enzymes AMO and NIR are NH_3 and HNO_2. The actual concentrations of these species are in a pH-dependent equilibrium with their ionized counterparts NH_4^+ and NO_2^- (ref. 27) (pK_{a,HNO_2} = 3.25, pK_{a,NH_4^+} = 9.25, 25 °C (ref. 28)).

Inorganic carbon (IC) is the carbon source subject to C fixation during AOB growth. At limited IC availability, NH_3 is oxidized at a lower rate due to increased cellular maintenance energy demand, with a simultaneous decrease in N_2O production.[29] However, at the same NH_3 oxidation rates, low IC levels increase the fraction of N_2O produced.[30] Depending on the nitrogen removal system, wastewaters can have varying IC levels. Due to the heterotrophic oxidization of the organic content of conventional urban wastewater, IC is typically in excess for autotrophic growth, but high N-strength wastewaters with a lower C/N ratio may result in IC limited AOB growth.[31]

Heterotrophic N_2O production

Under DO limited conditions, canonical denitrifiers respire NO_3^-, NO_2^-, NO and N_2O anaerobically, catalysed by enzymes encoded by *nar*, *nir*, *nor*, and *nosZ* genes. Heterotrophic denitrifiers constitute a highly modular microbiome with very different distributions of denitrifying genes.[32] Cellular co-occurrence of *nar*, *nir* and *nor* genes without *nosZ* would yield a net N_2O producer, while non-denitrifier N_2O reducers carrying an atypical *nosZ* have been identified and may act as N_2O sinks.[33] The potential of a heterotrophic community to serve as a N_2O source or sink may be governed by the diversity and relative abundance of the *nosZ* gene with respect to *nar*, *nir* and *nor* genes.[33,34]

The rate of NO^- reduction has been suggested as inhibited by products in the respiratory chain, such as NO_3^- reduction would be influenced by the concentration of further terminal electron acceptors and the number of other reductases.[35] In the presence of both N_2O and NO_2^-, the N_2O reductase

competes with NO_2^- reductase for electrons from the reduced cytochrome c.[36] In addition, the four enzymes responsible for denitrification may compete for electrons with cytochrome oxidases, where O_2 is reduced. The reversible inhibitory effect of DO on NO_x^- reduction is similar for each step.[36,37] N_2O reduction is the most sensitive step towards DO, and its inhibition will promote N_2O accumulation compared to the other N species.[38]

A limited flow of electron donors (as provided by the external chemical oxygen demand, COD) can also slow down NO_x^- reduction rates. Therefore, N_2O accumulation may result due to a reduced N_2O reduction rate due to a lower electron affinity compared to previous reduction steps. Consequently, side stream processes, characterized by high N content and low COD content, are potential hotspots for heterotrophic N_2O production.[3]

Moreover, the activities of enzymes encoded by the *nir*, *nor* and *nosZ* genes, located in the periplasm, are pH-dependent, with different optima for each denitrification step.[39] Thus, pH will have a direct effect on the concentration of intermediates. Specifically for N_2O, high and low pH values promote its consumption and accumulation, respectively.[40]

Abiotic N_2O production

Two chemical reactions driven by NH_2OH (ref. 41) can occur at relevant rates under wastewater treatment conditions.[42,43]

$$4NH_2OH \rightarrow N_2O + 2NH_3 + 3H_2O \quad (1)$$

$$NH_2OH + HNO_2 \rightarrow N_2O + 2H_2O \quad (2)$$

NH_2OH can decompose to N_2O at high pH (eqn (1); the acidic form NH_3OH^+ is more stable,[44] pK_a = 5.9 at 25 °C). In the second reaction, an N–N linkage is formed by N-nitrosation of NH_2OH, a nucleophile, with a nitrosating agent, HNO_2, at low pH[45] (eqn (2)). Thus, independently from the main driving process (*e.g.* nitrification or denitrification) and the environmental conditions (*e.g.* aerobic or anaerobic), biotically-driven (as NH_2OH is biotically produced) abiotic N_2O production is possible in WWTPs.

While previously considered to be insignificant, NH_2OH concentrations from highly N-loaded wastewaters can be substantial (0.03–0.11 mg N L^{-1}),[46] and abiotic N_2O production may have been underestimated.[47] For example, a nitritation reactor treating reject water (high AOB activity and NO_2^- accumulation) had a 1.1% abiotic N_2O emission factor.[46]

3. Modelling N_2O dynamics

With the final purpose of mitigating N_2O emissions, it is critical to accurately quantify the contribution of individual N_2O production and consumption pathways to the total N_2O pool. Process models are useful tools for this purpose, and several models have been proposed for each of the aforementioned biological N_2O production pathways.[48] Models vary based on the number of processes and variables considered and on the mathematical description of the process rates.

AOB driven N_2O models

Initially, single-pathway models were proposed describing either the NN or the ND pathway. The main difference between models is with regards to the stoichiometric coefficients, the number of considered substrates, the identity of the direct electron donor, and the inclusion or absence of substrate inhibition. Initial models described NO and N_2O production as directly dependent on NH_4^+, DO and NO_2^- levels.[49,50] In subsequent models, NH_2OH was considered an intermediate of NH_3 oxidation, allowing the NN pathway to be modelled as a fraction of NH_2OH oxidation to NO_2^-, either *via* NOH (ref. 51) or *via* NO.[52] In the ND pathway, NH_2OH acts as an electron donor for the consecutive reduction of NO_2^- to N_2O *via* NO.[53] To increase their predicting capabilities, newer models consider unionized species as the true substrates (NH_3–HNO_2 *vs.* NH_4^+–NO_2^-) and more complex functions are included in the process rates, resulting in more model parameters.[54] However, N_2O dynamics cannot be captured with single-pathway models, and recent models that combine the NN and ND pathways provide better descriptions of N_2O production than single-pathway models.[6,7]

The two-pathway AOB model by Pocquet *et al.*[7] considers NH_3 and HNO_2 as substrates and NH_2OH as the electron donor for both NO and HNO_2 reduction to N_2O in the NN and ND pathways, respectively. NO is formed from NH_2OH oxidation, and HNO_2 is formed from subsequent NO oxidation: in other words, all NH_2OH is first converted to NO, which is considered as a substrate for subsequent oxidation to HNO_2. In this model, NH_2OH oxidation to NO is modelled as consuming oxygen to maintain COD mass balance continuity, but this is in contradiction with the fact that no oxygen is actually consumed in this reaction.[11,16] Hence, the Pocquet model implies that NH_2OH oxidation is only feasible under aerobic conditions. The ND pathway is described as a one-step process wherein HNO_2 is reduced directly to N_2O, and the intermediate NO is ignored. Ignoring NO is necessary in the Pocquet model for mathematical reasons: the formed NO in the ND pathway would be a substrate in the NN pathway and be oxidized to HNO_2, which in turn could be reduced to NO in the ND pathway. Ignoring NO as an intermediate in the ND pathway is not in agreement with reality but avoids a futile NO cycling between NN and ND pathways.

In a different approach, global cellular oxidation (electron generating) and reduction (electron consuming) reactions in AOB are linked by a common pool of electron carriers, represented by one model component.[6] This model aggregates all intracellular electron carriers into one component, which cannot be experimentally quantified. In this model, NH_2OH and NO oxidation compete for oxidized electron carriers as cosubstrates and produce reduced

electron carriers. The reduction reactions of O_2, O_2/NH_4^+, NO and NO_2^- compete for the reduced carriers, which are transformed back to their oxidized forms.[6] Oxidative and reductive processes are uncoupled, and competition is described with specific kinetic parameters. Similarly to the previously described two-pathway model, in the ND pathway, a one-step reduction of NO_2^- to N_2O is included.

The two-pathway AOB models are adequate in predicting a shift in NN and ND contributions to the total N_2O production at different DO and NO_2^- concentrations. However, these models would not describe the increased NO emissions at low DO and high NO_2^- levels as observed in several nitrifying systems.[18,19,25,55,56] Hence, ND-associated NO production would be wrongly attributed to the NN pathway, overestimating the NN contribution to total N_2O production. As NO is the direct precursor of N_2O, and its emissions can be measured, it would seem preferable to retain NO in any model expressions. Experimental data on NO could then help assess and validate proposed mechanisms and model structures.

HB driven N_2O models

Two approaches have been widely used to model heterotrophic denitrification. In the electron competition approach, a model component describing a common pool of electrons, originating from carbon oxidation, exists for which the four enzymes in the denitrification respiratory pathway compete.[39] In the direct approach, no internal pool of electrons are considered, as carbon oxidation is assumed to provide a non-limiting supply of electrons to all denitrification enzymes.[57] Both approaches describe the electron donor and acceptor limitations with a specific Monod dependency for each denitrification step.[57,58] The known oxygen inhibition of the HD pathway has been described by either a single inhibition constant or a specific oxygen inhibition constant for each denitrification step.[53,57]

Even though the indirect approach has been heralded as superior as it can potentially describe more data sets, information about newly proposed reaction kinetics is not available in the literature.[59]

The direct HD modelling approach adequately predicts COD and nitrogen removal for systems with low intermediate accumulation (NO_2^-, N_2O)[48] but might be inadequate for systems with high intermediate accumulation levels.

Abiotic N_2O models

Systems treating high-strength wastewaters are particularly prone to chemical production of N_2O due to high AOB activity and associated high NH_2OH concentrations.[60] However, only one model has considered abiotic contribution together with biologically-driven N_2O production (ND and HD pathways).[47] The abiotic contribution was modelled with no pH dependency as a second order reaction for NH_2OH and NO_2^-, limiting the applicability to conditions of constant pH (eqn (2)).

4. Model development (NDHA)

An improved model including all the relevant mechanisms responsible for N_2O production during biological N removal is proposed (Table S2†). The NDHA model considers N_2O production from the three known biological pathways (NDHA) as well as abiotic production (NDHA) (Fig. 1). By explicitly considering NO as the direct precursor of N_2O production, three distinct biological NO production pathways can be identified while only including quantifiable state variables.

Different from current AOB driven models, the two autotrophic pathways are distinguished by two NO-producing processes with different DO and HNO_2 dependencies. The simplification of current AOB models that ignore NO as an intermediate during ND-driven N_2O production is solved: NO is an intermediate of both the NN and the ND pathways. A single autotrophic N_2O-producing process accounts for the combined NO reduction. Heterotrophic denitrification is described as a 4-step process, and two chemical reactions, which involve NH_2OH and HNO_2, describe the abiotic N_2O production.

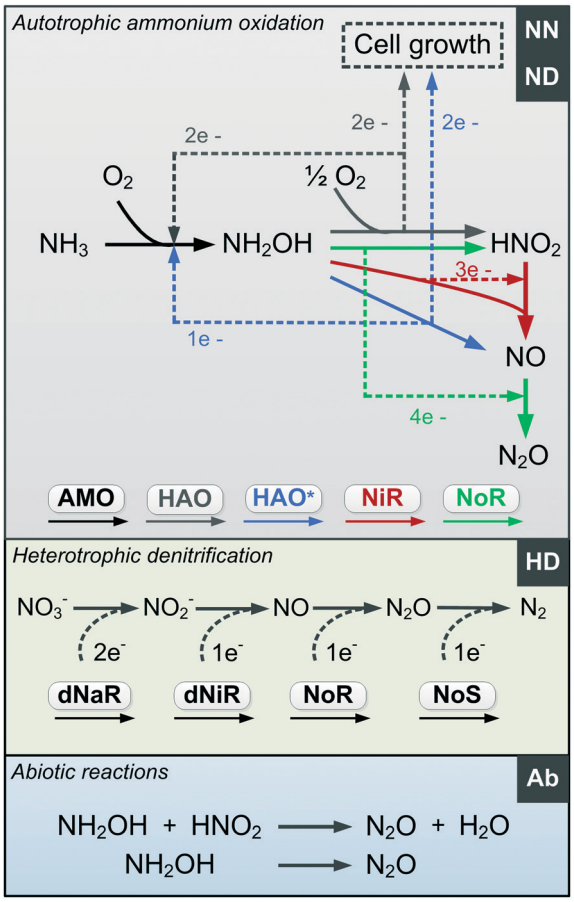

Fig. 1 Diagram of the proposed N_2O-producing mechanisms occurring during N removal: nitrifier nitrification, nitrifier denitrification, heterotrophic denitrification and abiotic pathways (NDHA).

Nitrifier nitrification (NN)

The first process considers NH_3 oxidation to NH_2OH (P1) (Table S3†). NH_2OH can be oxidized incompletely to NO_{NN} (P2) – a secondary catalyzed reaction of HAO – or completely to HNO_2 – the primary catalysed reaction of HAO – in the presence of DO (P3). The effect of IC limitation on NH_3 oxidation is described by a Monod dependency.[61] In the NN pathway (P2), NH_2OH reacts with H_2O;[62] the NN process is, therefore, indirectly dependent on the NH_3 oxidation rate, reducing the DO dependency only to P1. The fraction of NH_2OH oxidized *via* the NN pathway is described by the factor ε.

P1 – AMO: $NH_3 + O_2 \rightarrow NH_2OH$

$$\mu_{AMO}^{AOB} \cdot \frac{S_{O_2}}{S_{O_2} + K_{O_2\_AMO}^{AOB}} \cdot \frac{S_{NH_3}}{S_{NH_3} + K_{NH_3}^{AOB}} \cdot \frac{S_{IC}}{S_{IC} + K_{IC}^{AOB}} \cdot X_{AOB}$$

P2 – HAO*: $NH_2OH \rightarrow NO_{NN}$

$$\mu_{HAO}^{AOB} \cdot \varepsilon \cdot \frac{S_{NH_2OH}}{S_{NH_2OH} + K_{NH_2OH}^{AOB}} \cdot \frac{S_{IC}}{S_{IC} + K_{IC}^{AOB}} \cdot X_{AOB}$$

P3 – HAO: $NH_2OH + 0.5O_2 \rightarrow HNO_2 + H_2O$

$$\mu_{HAO}^{AOB} \cdot (1-\varepsilon) \cdot \frac{S_{O_2}}{S_{O_2} + K_{O_2\_HAO}^{AOB}} \cdot \frac{S_{NH_2OH}}{S_{NH_2OH} + K_{NH_2OH}^{AOB}} \cdot \frac{S_{IC}}{S_{IC} + K_{IC}^{AOB}} \cdot X_{AOB}$$

Nitrifier denitrification (ND)

In the ND pathway, HNO_2 denitrification to NO_{ND} is negatively affected by DO (P4).

Different from other two-pathway AOB models, N_2O production from its precursor (NO) is described by one process (P5) as there is no evidence of different NO reduction mechanisms within individual cells.[23] The NN and ND pathways are, therefore, mainly described by two NO-producing processes with different DO and HNO_2 dependencies. These dependencies govern the shift between pathways.[24,25] N_2O_{NN} production is enhanced at high NH_3 and DO levels, while N_2O_{ND} increases at low DO and high HNO_2 levels. By considering NH_2OH as an electron donor of both NO and HNO_2 reduction, the model minimizes the number of model components and fewer parameters are necessary to describe the electron competition (Table S4†).

The NO/N_2O ratio can be used to help elucidate the individual contribution of each pathway during model calibration.[7] An advantage of the proposed model is the uncoupling of the NN- and ND-driven NO production, which allows for a more biologically congruent estimate of NO/N_2O.

P4 – NIR: $3HNO_2 + NH_2OH \rightarrow 4NO_{ND}$

$$\mu_{HAO}^{AOB} \cdot \eta_{NIR} \cdot \frac{K_{i\_O_2}^{AOB}}{S_{O_2} + K_{i\_O_2}^{AOB}} \cdot \frac{S_{NH_2OH}}{S_{NH_2OH} + K_{NH_2OH\_ND}^{AOB}} \cdot \frac{S_{HNO_2}}{S_{HNO_2} + K_{HNO_2}^{AOB}} \cdot X_{AOB}$$

P5 – NOR: $2(NO_{ND} + NO_{NN}) + NH_2OH \rightarrow 1.5N_2O$

$$\mu_{HAO}^{AOB} \cdot \eta_{NOR} \cdot \frac{S_{NH_2OH}}{S_{NH_2OH} + K_{NH_2OH}^{AOB}} \cdot \frac{S_{NO}}{S_{NO} + K_{NO}^{AOB}} \cdot X_{AOB}$$

Heterotrophic denitrification (HD)

A four-step complete denitrification is considered following the ASM-N model.[57] Individual reaction kinetics (pH-dependent), inhibition and substrate affinities are considered for every step as recently suggested for systems with low intermediate accumulation.[48] Moreover, because of its wide applicability, the direct approach has been extended to new denitrification models coupled with phosphorus removal.[63]

P6 – HD: $NO_{x,oxidized} + COD \rightarrow NO_{x,reduced}$

$$\mu_{i\,(pH)}^{HB} \cdot \frac{K_{i\_O_2\_NO_{x,i}}^{HB}}{S_{O_2} + K_{i\_O_2\_NO_{x,i}}^{HB}} \cdot \frac{S_S}{S_S + K_{S\_NO_{x,i}}^{HB}} \cdot \frac{S_{NH_4}}{S_{NH_4} + K_{NH_4}^{HB}} \cdot \frac{S_{NO_{x,i}}}{S_{NO_{x,i}} + K_{NO_{x,i}}^{HB}} \cdot X_{HB}\,\pi$$

Heterotrophic consumption and autotrophic production of N_2O can occur simultaneously, at different rates, throughout wastewater treatment operations. Ignoring heterotrophic N_2O consumption can underestimate the autotrophic production. Thus, an N_2O model should always include compatible structures for both the autotrophic and the heterotrophic pathways.[64]

Abiotic (Ab)

Two biologically-driven abiotic N_2O production processes are considered (P7). Nitrification produces NH_2OH which can form HNO.[65] HNO dimerizes *via* $H_2N_2O_2$ to N_2O and H_2O (eqn (1)). Nitrosation of NH_2OH (eqn (2)) with HNO_2 has also been postulated as a relevant reaction in partial nitrification reactors.[46] Reaction rates are modelled with pH dependent second order kinetics.

P7 – Abiotic: $NH_2OH \rightarrow N_2O$; $NH_2OH + HNO_2 \rightarrow N_2O$

$$(k_{Abiotic\_1} \cdot S_{NH_2OH} \cdot f(pH)); (k_{Abiotic\_2} \cdot S_{NH_2OH} \cdot S_{HNO_2})$$

Model predictions for every pathway are pH-dependent, due to either substrate speciation or an enzymatic effect on the maximum specific growth rate. Implicit pH calculations also allow for estimations of IC and therefore limitations on AOB growth.[66] Aerobic growth of nitrite oxidizing bacteria on FNA and that of heterotrophs on soluble COD are also included.

Table 1 Main differences between two-pathway AOB models for N_2O production

	Pocquet *et al.* (2016)	Ni *et al.* (2014)	NDHA
NH_2OH oxidation: steps	2-step process to HNO_2 *via* NO	2-step process to HNO_2 *via* NO	2 processes: to NO and to HNO_2
NH_2OH oxidation: e-acceptor	NH_2OH and NO oxidation require O_2	Requires O_2, NO_2^- or NO reduction	HNO_2 production requires O_2, NO does not
NH_2OH oxidation: anoxic	Not possible	Possible (produces HNO_2)	Possible (produces N_2O)
Direct substrate for HNO_2 production	NO	NO	NH_2OH
Denitrifying NO production	Not considered	Not considered	Considered
NO-producing pathways	NN	NN	NN and ND
N_2O-producing pathways	NN and ND	NN and ND	NN and ND
pH-dependent substrate	Yes	No	Yes
Additional state variables	No	Yes	No
Model parameters (processes)	13(5)	18(6)	13(5)

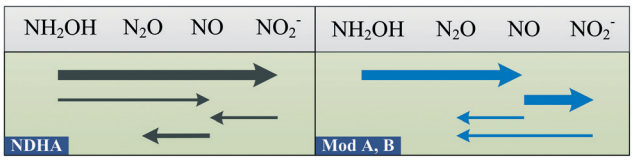

Fig. 2 Schematic comparison of the reactions involved in two-pathway AOB models for N_2O production. The arrow widths represent typical reaction rates. Model A[7] and Model B.[6]

5. Discussion

Current two-pathway AOB models share the same nitrogenous substrates and reactions to describe one NO and two N_2O-producing processes.[6,7] The proposed NDHA model adds the denitrification contribution to the NO pool that could not be considered in current models (Table 1).

The NN pathway is based on NO produced during NH_2OH oxidation. Differently from Pocquet *et al.*[7] and in agreement with Ni *et al.*,[6] the production of NO_{NN} in the NDHA model does not require the presence of oxygen.

Until now, models have considered HNO_2, coupled with an electron donor, as the direct precursor of N_2O for the ND pathway.[6,7] However, ND-associated NO reduction is not always faster than that of HNO_2, leading to NO accumulation.[18,19,25,55,56] In the NDHA model, this assumption is resolved and NO_{ND} is produced from HNO_2 reduction (Fig. 2). Whether the source of NO is NH_2OH oxidation or HNO_2 reduction will determine the contribution of each autotrophic pathway to N_2O production, NN or ND, respectively.

Although oxidation and reduction processes are not uncoupled in the NDHA model, the competition for electrons is represented by NH_2OH, the common electron donor: HNO_2, NO and DO compete for NH_2OH instead of reduced electron carriers.

Because of the structural assumption of the current AOB models, NO-associated N_2O production is only related to the NN pathway. As well as for the ND pathway, this assumption should be extrapolated to the HD pathway to avoid the NO exchange (simultaneous oxidation–reduction). Consequently, during model calibration, any possible ND or HD contributions to total NO would be falsely associated with the NN pathway. The NDHA model can describe more NO/N_2O pathways with the same or fewer parameters than the other models (Table 1).

The same N_2O net production rate can result from different individual N_2O production/consumption rates. Thus, together with total N_2O production, correctly predicting the individual contribution of each pathway is key for N_2O models. For example, the mitigation strategy of an autotrophic system with a small N_2O sink capacity will differ from that of mixed liquor with a higher N_2O consuming capacity.

Advances on N_2O models have led to more complete structures that can potentially describe any N_2O dynamics data set. However, the structural identifiability of none of the N_2O models has ever been assessed, and parameter identifiability analysis, if conducted, is limited to confidence interval depiction. Not all the model parameters are usually estimated from the available data as practical identifiability problems arise due to overparameterization of activated sludge models (ASM).[67] Model discrimination studies should therefore critically address calibration results as well as structural limitations. Best-fit parameter estimates provide little information and need to be supplemented with additional metrics (correlation matrix, sensitivity functions, analysis of residuals, estimation biases, *etc.*) in future model comparisons.

Additional complexity could be added, if necessary, to capture transient phenomena, relevant for systems with dynamic conditions. For example, the physiological state of the biomass can directly affect cellular activity and has been included in denitrifying models.[38,68] The high modularity of heterotrophic organisms, lumped into individual parameters for each denitrifying step, could be described by distinct microbial subpopulations and would yield more accurate kinetic parameters.[69] However, it is typically out of the scope of ASM models.

6. Conclusions

A mathematical model structure that describes N_2O production during biological nitrogen removal is proposed. Three biological pathways, two autotrophic and one heterotrophic,

are coupled with abiotic processes. Consistent with experimental studies, the model considers NO as the direct precursor of N_2O in *all* three biologically-driven pathways. This model can describe all relevant NO and N_2O production pathways with fewer parameters than other proposed models. A simplified and biologically congruent model will help develop mitigation strategies during wastewater treatment operations.

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