A consilience model to describe N2O production during biological N removal

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A consilience model to describe N₂O 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$_2$O), a potent greenhouse gas, is produced during biological nitrogen conversion in wastewater treatment operations. Complex mechanisms underlie N$_2$O production by autotrophic and heterotrophic organisms, which continue to be unraveled. Mathematical models that describe nitric oxide (NO) and N$_2$O dynamics have been proposed. Here, a first comprehensive model that considers all relevant NO and N$_2$O 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$_2$O. It also considers NO and N$_2$O as intermediates in heterotrophic denitrification in a 4-step model. Three biological NO and N$_2$O production pathways are accounted for, improving the capabilities of existing models while not increasing their complexity. Abiotic contributions from NH$_2$OH and HNO$_2$ reactions are also included. The model structure can theoretically predict NO and N$_2$O emissions under a wide range of operating conditions and will help develop mitigation strategies.

1. Introduction

Nitrous oxide (N$_2$O) 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$_2$O emissions,¹ which vary largely between WWTPs.² Biologically mediated, N$_2$O can be produced during nitrification and exists as an obligate intermediate during denitrification.³ The mechanisms and regulations of N$_2$O production in these processes are still under investigation, and identification and better understanding of the key variables driving N$_2$O production are necessary.

Water impact

Wastewater treatment operations are anthropogenic sources of nitrous oxide (N$_2$O), 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$_2$O emissions. Informed by a review of known biological and chemical N$_2$O producing mechanisms, an improved mathematical model structure that may help the development of N$_2$O mitigation strategies for full-scale treatment operations is proposed.

With the final goal of mitigating N$_2$O 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$_2$O dynamics.

The first models that described autotrophic N$_2$O 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$_2$O pathways might not always be true, thus limiting their applicability.⁶,⁷

In combination with N$_2$O production, physicochemical processes transfer N$_2$O from the liquid to the gas phase resulting in actual N$_2$O 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$_2$O production under a wide range of operating conditions. By increasing the model complexity with additional components and parameters,
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 monoxygenase 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 denitritification (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 NOR 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) (\( pK_{a,HNO_2} = 3.25 \), \( pK_{a,NH_4^+} = 9.25, 25^\circ C \) (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 oxidation 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 \( NO_2^- \) 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\textsubscript{2} \textsuperscript{-} reductase for electrons from the reduced cytochrome c\textsuperscript{36}. In addition, the four enzymes responsible for denitrification may compete for electrons with cytochrome oxidases, where O\textsubscript{2} is reduced. The reversible inhibitory effect of DO on NO\textsubscript{2} \textsuperscript{-} reduction is similar for each step\textsuperscript{36,37}. N\textsubscript{2}O reduction is the most sensitive step towards DO, and its inhibition will promote N\textsubscript{2}O accumulation compared to the other N species\textsuperscript{38}.

A limited flow of electron donors (as provided by the external chemical oxygen demand, COD) can also slow down NO\textsubscript{2} \textsuperscript{-} reduction rates. Therefore, N\textsubscript{2}O accumulation may result due to a reduced N\textsubscript{2}O 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\textsubscript{2}O production\textsuperscript{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\textsuperscript{39}. Thus, pH will have a direct effect on the concentration of intermediates. Specifically for N\textsubscript{2}O, high and low pH values promote its consumption and accumulation, respectively\textsuperscript{40}.

### Abiotic N\textsubscript{2}O Production

Two chemical reactions driven by NH\textsubscript{2}OH (ref. 41) can occur at relevant rates under wastewater treatment conditions\textsuperscript{42,43}.

\[ 4\text{NH}_2\text{OH} \rightarrow \text{N}_2\text{O} + 2\text{NH}_3 + 3\text{H}_2\text{O} \] \hspace{1cm} (1)

\[ \text{NH}_2\text{OH} + \text{HNO}_2 \rightarrow \text{N}_2\text{O} + 2\text{H}_2\text{O} \] \hspace{1cm} (2)

NH\textsubscript{2}OH can decompose to N\textsubscript{2}O at high pH (eqn (1); the acidic form NH\textsubscript{3}OH\textsuperscript{+} is more stable\textsuperscript{44}, pK\textsubscript{a} = 5.9 at 25 °C). In the second reaction, an N-N linkage is formed by N-nitrosation of NH\textsubscript{2}OH, a nucleophile, with a nitrosating agent, HNO\textsubscript{2}, at low pH\textsuperscript{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\textsubscript{2}OH is biotically produced) abiotic N\textsubscript{2}O production is possible in WWTPs.

While previously considered to be insignificant, NH\textsubscript{2}OH concentrations from highly N-loaded wastewaters can be substantial (0.03–0.11 mg N L\textsuperscript{-1})\textsuperscript{46} and abiotic N\textsubscript{2}O production may have been underestimated\textsuperscript{47}. For example, a nitrification reactor treating reject water (high AOB activity and NO\textsubscript{2} \textsuperscript{-} accumulation) had a 1.1% abiotic N\textsubscript{2}O emission factor\textsuperscript{46}.

### 3. Modelling N\textsubscript{2}O Dynamics

With the final purpose of mitigating N\textsubscript{2}O emissions, it is critical to accurately quantify the contribution of individual N\textsubscript{2}O production and consumption pathways to the total N\textsubscript{2}O pool. Process models are useful tools for this purpose, and several models have been proposed for each of the aforementioned biological N\textsubscript{2}O production pathways\textsuperscript{48}. Models vary based on the number of processes and variables considered and on the mathematical description of the process rates.

#### AOB driven N\textsubscript{2}O 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\textsubscript{2}O production as directly dependent on NH\textsubscript{4} \textsuperscript{+}, DO and NO\textsubscript{2} \textsuperscript{-} levels\textsuperscript{49,50}. In subsequent models, NH\textsubscript{2}OH was considered an intermediate of NH\textsubscript{3} oxidation, allowing the NN pathway to be modelled as a fraction of NH\textsubscript{4}OH oxidation to NO\textsubscript{2} \textsuperscript{-}, either via NOH (ref. 51) or via NO\textsubscript{3} \textsuperscript{-}\textsuperscript{52}. In the ND pathway, NH\textsubscript{2}OH acts as an electron donor for the consecutive reduction of NO\textsubscript{2} \textsuperscript{-} to N\textsubscript{2}O via NO\textsubscript{3} \textsuperscript{-}\textsuperscript{53}.

To increase their predicting capabilities, newer models consider unionized species as the true substrates [NH\textsubscript{3} \textsuperscript{-}, HNO\textsubscript{2} vs. NH\textsubscript{4} \textsuperscript{+}, NO\textsubscript{2} \textsuperscript{-}] and more complex functions are included in the process rates, resulting in more model parameters\textsuperscript{54}. However, N\textsubscript{2}O dynamics cannot be captured with single-pathway models, and recent models that combine the NN and ND pathways provide better descriptions of N\textsubscript{2}O production than single-pathway models\textsuperscript{6,7}.

The two-pathway AOB model by Pocquet et al\textsuperscript{7} considers NH\textsubscript{3} and HNO\textsubscript{2} as substrates and NH\textsubscript{2}OH as the electron donor for both NO and HNO\textsubscript{2} reduction to N\textsubscript{2}O in the NN and ND pathways, respectively. NO is formed from NH\textsubscript{2}OH oxidation, and HNO\textsubscript{2} is formed from subsequent NO oxidation: in other words, all NH\textsubscript{2}OH is first converted to NO, which is considered as a substrate for subsequent oxidation to HNO\textsubscript{2}. In this model, NH\textsubscript{2}OH 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\textsuperscript{15,16}. Hence, the Pocquet model implies that NH\textsubscript{2}OH oxidation is only feasible under aerobic conditions. The ND pathway is described as a one-step process wherein HNO\textsubscript{2} is reduced directly to N\textsubscript{2}O, 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\textsubscript{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\textsuperscript{6}. This model aggregates all intracellular electron carriers into one component, which cannot be experimentally quantified. In this model, NH\textsubscript{4}OH 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.\textsuperscript{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$_2$O is included.

The two-pathway AOB models are adequate in predicting a shift in NN and ND contributions to the total N$_2$O 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.\textsuperscript{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$_2$O production. As NO is the direct precursor of N$_2$O, 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$_2$O 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.\textsuperscript{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.\textsuperscript{57} Both approaches describe the electron donor and acceptor limitations with a specific Monod dependency for each denitrification step.\textsuperscript{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.\textsuperscript{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.\textsuperscript{59}

The direct HD modelling approach adequately predicts COD and nitrogen removal for systems with low intermediate accumulation (NO$_2^-$, N$_2$O)\textsuperscript{18} but might be inadequate for systems with high intermediate accumulation levels.

**Abiotic N$_2$O models**

Systems treating high-strength wastewaters are particularly prone to chemical production of N$_2$O due to high AOB activity and associated high NH$_2$OH concentrations.\textsuperscript{60} However, only one model has considered abiotic contribution together with biologically-driven N$_2$O production (ND and HD pathways).\textsuperscript{47} The abiotic contribution was modelled with no pH dependency as a second order reaction for NH$_2$OH and NO$_2^-$, limiting the applicability to conditions of constant pH (eqn (2)).

**Fig. 1** Diagram of the proposed N$_2$O-producing mechanisms occurring during N removal: nitrifier nitrification, nitrifier denitrification, heterotrophic denitrification and abiotic pathways (NDHA).
Nitrifier nitrification (NN)
The first process considers NH₃ oxidation to NH₂OH (P1) (Table S3†). NH₂OH can be oxidized incompletely to NONN (P2) – a secondary catalyzed reaction of HAO – or completely to HNO₂ – the primary catalyzed reaction of HAO – in the presence of DO (P3). The effect of IC limitation on NH₃ oxidation is described by a Monod dependency.61 In the NN pathway (P2), NH₂OH reacts with H₂O;62 the NN process is, therefore, indirectly dependent on the NH₃ oxidation rate, reducing the DO dependency only to P1. The fraction of NH₂OH oxidized via the NN pathway is described by the factor ε.

P1 – AMO: NH₃ + O₂ → NH₂OH

P2 – HAO*: NH₂OH → NONN

P3 – HAO: NH₂OH + 0.5O₂ → HNO₂ + H₂O

Nitrifier denitrification (ND)
In the ND pathway, HNO₂ denitrification to NOND is negatively affected by DO (P4).

Different from other two-pathway AOB models, N₂O 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 HNO₂ dependencies. These dependencies govern the shift between pathways.24,25 N₂O₄N production is enhanced at high NH₃ and DO levels, while N₂O₄ND increases at low DO and high HNO₂ levels. By considering NH₂OH as an electron donor of both NO and HNO₂ reduction, the model minimizes the number of model components and fewer parameters are necessary to describe the electron competition (Table S4†).

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: NOx,oxidized + COD → NOx,Reduced

Heterotrophic consumption and autotrophic production of N₂O can occur simultaneously, at different rates, throughout wastewater treatment operations. Ignoring heterotrophic N₂O consumption can underestimate the autotrophic production. Thus, an N₂O model should always include compatible structures for both the autotrophic and the heterotrophic pathways.64

Abiotic (Ab)
Two biologically-driven abiotic N₂O production processes are considered (P7). Nitrification produces NH₂OH which can form HNO.65 HNO dimerizes via H₂N₂O₂ to N₂O and H₂O (eqn (1)). Nitrosation of NH₂OH (eqn (2)) with HNO₂ has also been postulated as a relevant reaction in partial nitrification reactors.46 Reaction rates are modelled with pH dependant second order kinetics.

P7 – Abiotic: NH₂OH → N₂O; NH₂OH + HNO₂ → N₂O

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.
5. Discussion

Current two-pathway AOB models share the same nitrogenous substrates and reactions to describe one NO and two N₂O-producing processes. 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 NH₂OH oxidation. Differently from Pocquet et al. and in agreement with Ni et al., the production of NO₃⁻, NO₂⁻, or NO₃⁻ in the NDHA model does not require the presence of oxygen.

Until now, models have considered HNO₂, coupled with an electron donor, as the direct precursor of N₂O for the ND pathway. However, ND-associated NO reduction is not always faster than that of HNO₂, leading to NO accumulation. In the NDHA model, this assumption is resolved and NO₃⁻ is produced from HNO₂ reduction (Fig. 2). Whether the source of NO is NH₂OH oxidation or HNO₂ reduction will determine the contribution of each autotrophic pathway to N₂O production, NN or ND, respectively.

Although oxidation and reduction processes are not uncoupled in the NDHA model, the competition for electrons is represented by NH₂OH, the common electron donor: HNO₂, NO and DO compete for NH₂OH instead of reduced electron carriers.

Because of the structural assumption of the current AOB models, NO-associated N₂O 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/N₂O pathways with the same or fewer parameters than the other models (Table 1).

The same N₂O net production rate can result from different individual N₂O production/consumption rates. Thus, together with total N₂O production, correctly predicting the individual contribution of each pathway is key for N₂O models. For example, the mitigation strategy of an autotrophic system with a small N₂O sink capacity will differ from that of mixed liquor with a higher N₂O consuming capacity.

Advances on N₂O models have led to more complete structures that can potentially describe any N₂O dynamics data set. However, the structural identifiability of none of the N₂O 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). 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. 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. However, it is typically out of the scope of ASM models.

6. Conclusions

A mathematical model structure that describes N₂O 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$_2$O in all three biologically-driven pathways. This model can describe all relevant NO and N$_2$O 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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