Establishment and calibration of consensus process model for nitrous oxide dynamics in water quality engineering

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Preface

This thesis is based on the work carried out at the Department of Environmental Engineering at the Technical University of Denmark from January 2014 to April 2017. This thesis was prepared as part of the LaGas project (http://www.lagas.dk). The research was performed under the main supervision of Professor Barth F. Smets (DTU Environment) and co-supervision of Associate Professor Benedek Gy. Plósz (DTU Environment) and Associate Professor Gürkan Sin (DTU Chemical and Biochemical Engineering).

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-V**.

I **Domingo-Félez, C.**, Pellicer-Nàcher, C., Petersen, M. S., Jensen, M. M., Plósz, B. G., Smets, B.F. 2017. Heterotrophs are key contributors to nitrous oxide production in activated sludge under low C-to-N ratios during nitrification – Batch experiments and modeling. *Biotechnology and Bioengineering*, **114**, 132-140.

II **Domingo-Félez, C.**, Smets, B.F. 2016. A consilience model to describe N2O production during biological N removal. *Environmental Science: Water Research and Technology*, **6**, 923-930.

III **Domingo-Félez, C.**, Calderó-Pascual, M., Sin, G., Plósz, B. G., Smets, B.F. 2017. Calibration of the comprehensive NDHA-N2O dynamics model for nitrifier-enriched biomass using targeted respirometric assays. *Submitted*

IV **Domingo-Félez, C.**, Smets, B.F. 2017. Application of the NDHA model to describe N2O dynamics in activated sludge mixed culture biomass. *Manuscript in preparation*.

V **Domingo-Félez, C.**, Smets, B.F. 2017. Modelling electron competition in a mixed denitrifying microbial community with different carbon sources through an electric circuit analogy. *Manuscript in preparation*.

In addition, the following authored or co-authored publications, not included in this thesis, were also concluded during this PhD study:

- **Domingo-Félez, C.**, Mutlu, A. G., Jensen, M. M., Smets, B. F. (2014). Aeration strategies to mitigate nitrous oxide emissions from single-stage nitritation/anammox reactors. *Environmental Science and Technology*. **(48**) 15: 8679-8687.

- Ma, Y., **Domingo-Félez, C.**, Plósz, B. G., Smets, B. F. (*2017*). Suppression of nitrite-oxidizing bacteria in intermittently membrane-aerated biofilms: a model-based explanation. DOI: 10.1021/acs.est.7b00463. *Accepted in Environmental Science and Technology*.

- Su, Q., Ma, C., **Domingo-Félez, C.**, Kiil, A.S., Thamdrup, B., Jensen, M.M., Smets, B. F. (*2017*). Low nitrous oxide production through nitrifier-denitrification in intermittent-feed high-rate high performance nitritation reactors. *Under revision for Water Research*.

- Blum, J. M., Su, Q., Ma, Y., Valverde-Pérez, B., **Domingo-Félez, C.**, Jensen, M. M, Smets, B. F. (*2017*). The pH dependency of N-converting enzymatic processes, pathways and microbes: effect on net-N2O production. *Submitted*.

This PhD study also contributed to international conferences with the following proceeding papers:

- **Domingo-Félez, C.**, Smets, B. F. Critical assessment of a novel N_2O model. N2O Expert Meeting and Workshop. Bochum (Germany). 21st – 22nd September 2016. Oral presentation.
- **Domingo-Félez, C.**, Valverde-Pérez, B., Plósz, B. G., Sin, G., Smets, B. F. Towards an optimal experimental design for N2O model calibration during biological nitrogen removal. 5th IWA/WEF Wastewater Treatment Modelling Seminar (WWTmod2016). Annecy (France). 2nd- 6th April 2016. Poster presentation.
- **Domingo-Félez, C.**, Pellicer-Nàcher, C., Petersen, M. S., González-Combarros, R., Jensen, M. M., Sin, G., Smets, B. F. Challenges encountered calibrating N_2O dynamics from mixed cultures. International Conference on Nitrogen (ICON4). Edmonton (Canada). University of Alberta. June 29th-July 2nd 2015. Poster presentation.

- **Domingo-Félez, C.**, Plósz, B. G., Sin, G., Smets, B. F. N_2O and NO dynamics in AOB-enriched and mixed-culture biomass: Experimental Observations and Model Calibration. Fifth International Conference on Nitrification and Related Processes (ICoN5). Vienna (Austria). 23-27 July 2017. Accepted Abstract.
- Ma, Y., **Domingo-Félez, C.**, Plósz, B. G., Smets, B. F. Suppression of nitrite-oxidizing bacteria in intermittently aerated biofilm reactors: a model-based explanation. IWA Microbial Ecology in Water Engineering & Biofilms. 4-7th September 2016. Copenhagen (Denmark). Poster presentation.
- Smets, B. F., Pellicer-Nàcher, C., **Domingo-Félez, C.**, Jensen, M. M., Ramin, E., Plósz, B. G., Sin, G., Gernaey, K., V., Modelling N_2O dynamics in the engineered cycle: Evaluation of alternate model structures. Spa (Belgium). 4th IWA/WEF Wastewater Treatment Modelling Seminar. 30 March – 2 April 2014. Poster presentation. Proceedings p. 343-346.
- **Domingo-Félez, C.**, Calderó-Pascual, M., Sin, G., Plósz, B. G., Smets, B. F. Calibration of the NDHA N_2O model via respirometric assays. Frontiers International Conference on Wastewater Treatment. 21-24th May, Palermo (Italy). Poster flash presentation.
- Ma, Y., **Domingo-Félez, C.**, Smets, B. F. N_2O Production in Membrane-aerated Nitrifying Biofilms: Experimentation and Modelling. Frontiers International Conference on Wastewater Treatment. 21-24th May, Palermo (Italy). Poster flash presentation.
- Ma, Y., **Domingo-Félez, C.**, Piscedda, A., Smets, B. F. Investigating Intermittent Aeration in Membrane-Aerated Nitrifying Biofilm Reactors. IWA 10th International Conference on Biofilm Reactors on, Dublin (Ireland) 9-12th May 2017. Oral presentation.
- Morset, M., Valverde-Pérez, B., Blum, J. M., **Domingo-Félez, C.**, Mauricio-Iglesias, M., Smets, B. F. N_2O emissions from a single-stage partial nitritation/anammox granule-based reactor – a model based assessment. IWA 10th International Conference on Biofilm Reactors on, Dublin (Ireland) 9-12th May 2017. Poster presentation.
- Ekström, S., **Domingo-Félez, C.**, Jensen, M. M., Gustavsson, D. J. I., Persson , F., Jansen J. L. C., Smets, B. F. Influence of aeration strategy on N_2O emissions from a pilot-scale mainstream anammox process. NORDIWA 2015 – 14th Nordic Wastewater Conference (November, 2015, Bergen, Norway). Oral presentation.

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To everyone in the Metlab group for the very nice work atmosphere. What started as a six-months Erasmus has become a six years long experience. Specially thanks to Carles and Gizem for your enthusiasm and hard work that helped me before I started this PhD. To those who were here when I arrived, or graduated and left, or stayed, or will graduate soon, thanks for the good times inside and outside DTU: Uli, Arda, Katerina, Arnaud, Marlene, Lene, Sara, Marta, Vaibhav, Bas, Jane, Qingxian, Yunjie, Jan, Borja, Alex, Dorka, Fabio, Elena, Elham, Katerina…

To *my* students: Ilias, Rosana, Anne-Sofie, María, Andrea and Martin, thanks for your hard work and for dealing with my, sometimes, excessive rambling about N_2O. Thanks also to the students during the TA hours with whom I also learnt and to Mathias and Marlene for the much appreciated help with the Danish translation.

To the cake club, for showing that sometimes a small effort pays off when you need it. To the Friday bar for the much needed breaks, especially when it was not Friday (Carson, Pauly, Flo, Klaus, Pedram, Camilla, Bentje, etc.) and to the rest of friends from Zaragoza, Muniesa, RI, etc.

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Be curious and doubt.

Summary

Research on biological nitrogen removal (BNR) in wastewater treatment plants (WWTP) has historically focused on achieving good effluent quality, with more recent attention to energy savings and carbon dioxide (CO_2) footprints. Novel processes and operating conditions are being implemented that enhance cost and energy efficiency in BNR, while maintaining effluent quality. Now, increasing attention is placed on direct emissions of nitrous oxide (N_2O) as by-product of BNR; N_2O is a greenhouse gas (GHG) with a high warming potential and also an ozone depleting chemical compound.

Several N_2O production pathways have been identified from pure culture studies, while mechanisms are still being unravelled. Heterotrophic bacteria (HB) and ammonium oxidizing bacteria (AOB) are well known to produce N_2O. However, the effect of environmental factors on N_2O production is not yet well understood. Current process modelling efforts aim to reproduce experimental data with mathematical equations, structuring our understanding of the system. Various mechanistic models with different structures describing N_2O production have been proposed, but no consensus exists between researchers. Hence, the existing plant-wide GHG models still lack a complete biological process model that can be integrated in a methodology that assesses N_2O emissions and their impact on overall plant performance.

A mathematical model structure that describes N_2O production during biological nitrogen removal is proposed. Two autotrophic and one heterotrophic biological pathways are coupled with abiotic processes. The model stoichiometry and process rates synthesize a comprehensive literature review on the metabolism of microbes involved in nitrogen removal. The proposed model can describe all relevant NO and N_2O production pathways with fewer parameters than present in other proposed models.

A novel experimental design based on the developed model and on extant respirometric techniques is introduced. Monitoring dissolved oxygen and N_2O allowed the isolation of individual processes and the estimation of parameters associated to oxygen consumption (endogenous activity, nitrite and ammonium oxidation) and N_2O production (NN, ND and HD pathway contributions).

To estimate parameters of the N_2O model a rigorous procedure is presented as a case study. The calibrated model predicts the NO and N_2O dynamics at varying ammonium, nitrite and dissolved oxygen levels in two independent

systems: (a) an AOB-enriched biomass and (b) activated sludge (AS) mixed liquor biomass. A total of ten (a) and seventeen (b) parameters are identified with high accuracy (coefficients of variation < 25%). The critical validation of the model response and the estimated parameter values represent a novel and rigorous tool for N_2O modelling studies. For the first time, uncertainty associated with parameter estimation from N_2O models is reported, this procedure is recommended to be included with best-fit simulations.

Additionally, modelling electron competition in heterotrophic processes is explored via an analogy to current intensity through resistors in electric circuits. While further model validation is required, this approach captured the electron competition during denitrification for four different carbon sources.

Overall, a combination of modelling and experimental efforts to study N_2O dynamics was successfully implemented. Results represent a step forward in the development of consensus process model for N_2O emissions in WQE processes.

Dansk sammenfatning

Forskning i biologisk kvælstoffjernelse på spildevandsrensningsanlæg har historisk fokuseret på at opnå en god udledningskvalitet, mens opmærksomheden de seneste år er blevet rettet mod energibesparelser og CO_2-udslip. Nye processer og driftsforhold, der nedsætter omkostninger og øger energieffektiviteten for biologisk kvælstoffjernelse implementeres samtidig med at udledningskvaliteten fastholdes. Senest er der kommet øget opmærksomhed på direkte emissioner af dinitrogenoxid (N2O), også kaldt lattergas, som er et biprodukt af biologisk kvælstoffjernelse. Lattergas er en drivhusgas med et højt drivhusgaspotentiale og en ozonnedbrydende kemisk forbindelse.

Flere bakterielle processer for lattergasproduktion er blevet identificeret ved hjælp af studier af rene kulturer, mens mekanismerne bag lattergasproduktionen stadig undersøges. Både heterotrofe denitrificerende bakterier og ammoniak-oxiderende bakterier producerer lattergas. Men man ved endnu meget lidt om hvilke faktorer, der regulerer lattergasproduktionen. Igangværende forskning inden for procesmodellering forsøger at reproducere eksperimentelle data med matematiske ligninger og derved strukturere vores forståelse af systemet. Forskellige mekanistiske modeller med forskellige strukturer der beskriver lattergasproduktion har tidligere været foreslået, men der er ingen konsensus imellem forskere. Derfor mangler de eksisterende drivhusgasemissionsmodeller for hele renseanlægget stadig en komplet biologisk procesmodel, som kan integreres på en måde der giver mulighed for at vurdere lattergasemissioner og indvirkningen af disse på den samlede anlægspræstation.

Her foreslås en matematisk modelstruktur, der beskriver lattergasproduktionen under biologisk kvælstoffjernelse. To autotrofe og en heterotrof biologisk reaktionsvej er koblet sammen med abiotiske processer. Modellens støkiometri og reaktionsrater udspringer fra et omfattende litteraturstudie i de mikroorganismers metabolisme, der er involveret i fjernelse af nitrogen. Modellen kan beskrive alle relevante kvælstofoxid- og lattergas-produktionsveje med færre parametre end i tidligere publicerede modeller.

I afhandlingen introduceres også et nyt eksperimentelt design baseret på den udviklede model og på eksisterende respirometriske teknikker. Målinger af opløst oxygen og lattergas gjorde det muligt at isolere individuelle processer og estimering af parametre forbundet med iltforbrug (endogen aktivitet, ni-

trit- og ammonium-oxidation) og lattergasproduktion (bidrag fra NN-, ND- og HD-productionsveje).

For at estimere parametre i lattergas modellerne, fremlægges en stringent procedure som et case study. Den kalibrerede model forudsiger dynamikken af kvæfstofoxid- og lattergas-akkumulering ved forskellige niveauer af ammonium-, nitrit- og opløst oxygen i to uafhængige systemer: (a) en beriget ammoniak-oxiderende biomasse og (b) aktiveret slam biomasse. I alt blev ti (a) og sytten (b) parametre identificeret med høj nøjagtighed (variationskoefficienter <25%). Den kritiske validering af modeludkastet og de estimerede parameterværdier repræsenterer et nyt og stringent redskab til lattergas modelleringsstudier. For første gang rapporteres usikkerheden i forbindelse med parametervurdering fra lattergas-modeller. Det anbefales at tilføje denne fremgangsmetode til best-fit simuleringsprocedurer.

Derudover undersøges modellering af konkurrencen om elektroner imellem de heterotrofe processer analogt til strøm-intensiteten gennem modstande i elektriske kredsløb. Mens yderligere validering af modellen er påkrævet, fangede fremgangsmåden den elektronkonkurrence, der forekommer når denitrificerende bakterier oxiderer fire forskellige kulstofkilder.

Samlet set blev en kombination af modellering og forsøg med formålet at studere N2O-dynamik succesfuldt gennemført. Resultaterne er et skridt fremad i udviklingen af en konsensus procesmodel for lattergasemissioner i ingeniørmæssige vandkvalitets-processer.

Table of contents

Abbreviations

ACF	Autocorrelation function
AMO	Ammonia monooxygenase
AOB	Aerobic ammonia oxidizing bacteria
AS	Activated sludge
ASM	Activated sludge model
BNR	Biological nitrogen removal
COD	Chemical oxygen demand
CV	Coefficient of variation
FIM	Fisher information matrix
GHG	Greenhouse gas
GSA	Global sensitivity analysis
HAO	Hydroxylamine oxydoreductase
HB	Heterotrophic bacteria
HD	Heterotrophic denitrification
ICE	Indirect coupling of electrons
LHS	Latin hypercube sampling
LSA	Local sensitivity analysis
MLR	Multiple linear regression
NAR	Nitrate reductase
ND	Nitrifier denitrification
NIR	Nitrite reductase
NN	Nitrifier nitrification
NOB	Aerobic nitrite oxidizing bacteria
NOR	Nitric oxide reductase
NOS	Nitrous oxide reductase
RMSE	Root mean squared error
SMN	Stoichiometric metabolic network
SRC	Standardized regression coefficient
VSS	Volatile suspended solids
WQE	Water quality engineering
WWTP	Wastewater treatment plant
DO	Dissolved oxygen
HNO_2	Free nitrous acid
NH_2OH	Hydroxylamine
NH_3	Ammonia
NH_4^+	Ammonium
N_2	Dinitrogen gas
NO	Nitric oxide
N_2O	Nitrous oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NO_x^-	Nitrogen oxides
O_2	Molecular oxygen

1 Introduction

1.1 Background and motivation of the study

Nitrous oxide (N_2O) is a stratospheric ozone depleter and a greenhouse gas (GHG), recently identified as the most important threat to the ozone layer of the 21st century (Ravishankara *et al.*, 2009). The global warming potential of N_2O is 300 times higher than that of CO_2 due to its long residence time in the atmosphere (Stocker *et al.*, 2013).

In the anthropogenic water cycle N_2O emissions can contribute up to 26% of the GHG footprint (Desloover *et al.*, 2012), and specifically during sewage treatment accounts for 3.2% of the total N_2O global emission rates (Mosier *et al.*, 1999). The objective of wastewater treatment is of sanitary purposes, reducing the number of pathogens present in wastewater. However, still 47% of wastewater produced in manufacturing and domestic sectors is untreated (Stocker *et al.*, 2013). Hence, global N_2O emissions may be enhanced by the increasing wastewater treatment loadings.

The carbon footprint of full-scale wastewater treatment plants (WWTPs) consists of direct emissions of GHG (e.g. methane, nitrous oxide), energy consumption, use of chemicals, etc. The study of Scandinavian municipal WWTPs indicated that the most important contributions corresponded to the direct GHG emissions and energy categories (Gustavsson and Tumlin, 2013). Overall, while energy neutral and energy self-sufficient WWTPs exist (Yan *et al.*, 2017), carbon neutral WWTPs are still lacking in the literature (Gustavsson and Tumlin, 2013).

A high variability in N_2O emissions exists and emission factors are not representative for individual process configurations (Ahn *et al.*, 2010). The impact assessment of N_2O emissions from the nitrogenous liquid waste should be thus addressed at a local level.

Intensive on-site measurements together with accurate measurement protocols have been reported as an alternative to estimate N_2O emissions (Chandran, 2011). Mechanistic models have also been suggested to predict N_2O emissions from plant-wide systems and incorporated during control strategies (Snip *et al.*, 2014). However, poor knowledge of key processes driving N_2O production and lack of consensus on how to model the producing pathways has impeded the implementation of plant-wide GHG models (Desloover *et al.*, 2012). Models have increased their predictive capabilities,

but convergence towards a consilience N_2O model has not been achieved yet (Mannina *et al.*, 2016).

Compared to full-scale systems, the differences in formation mechanisms and kinetics between biomass cultures can be studied in lab-scale reactors or targeted experiments as they offer more controlled environments. Model development can also benefit from recent advances on microbial metabolism and analytical measurements (e.g. pure culture studies, quantification of microbial communities, isotopic portioning, microelectrodes, etc.). Therefore, a better understanding of the biological factors that control N_2O production and consumption will improve the mathematical prediction of new N_2O process models.

Additionally, the high parameter variability of reported N_2O models highlights possible model limitations to address regulation of multiple pathways, microbial population switches, or hydrodynamic heterogeneities (Manser *et al.*, 2005; Spérandio *et al.*, 2016). The confidence of model predictions is critical when comparing the performance of N_2O models during the development of mitigation strategies as the carbon footprint is highly sensitive to N_2O emissions (Gustavsson and Tumlin, 2013). Moreover, as an end-product of nitrogen removal N_2O predictions are greatly affected by the uncertainty of primary N-substrates (e.g. NH_4^+, NO_2^-, etc.). The quality of the calibration results is commonly addressed in environmental models (Bennett *et al.*, 2013) but has not been studied for N_2O emissions. Hence, rigorous methods for N_2O model response evaluation will benefit model discrimination procedures, and improve mitigation strategies (Belia *et al.*, 2009).

1.2 Aim of the thesis

This thesis is embedded in a project (LaGas) that focuses on untangling the factors driving N_2O production from wastewater treatment. LaGas is a multi-disciplinary project in which this thesis aims to contribute by building and validating a consensus mechanistic process model for N_2O dynamics for water quality engineering use. This thesis represents the modelling link between lab-scale stable isotope techniques and intensive full-scale measuring campaigns.

In this thesis, a state-of-the-art overview of the pathways driving N_2O production during BNR is exposed, current N_2O modelling approaches are discussed and a consilience model is proposed. An overview of the research approach followed in this thesis is shown in **Figure 1.1**.

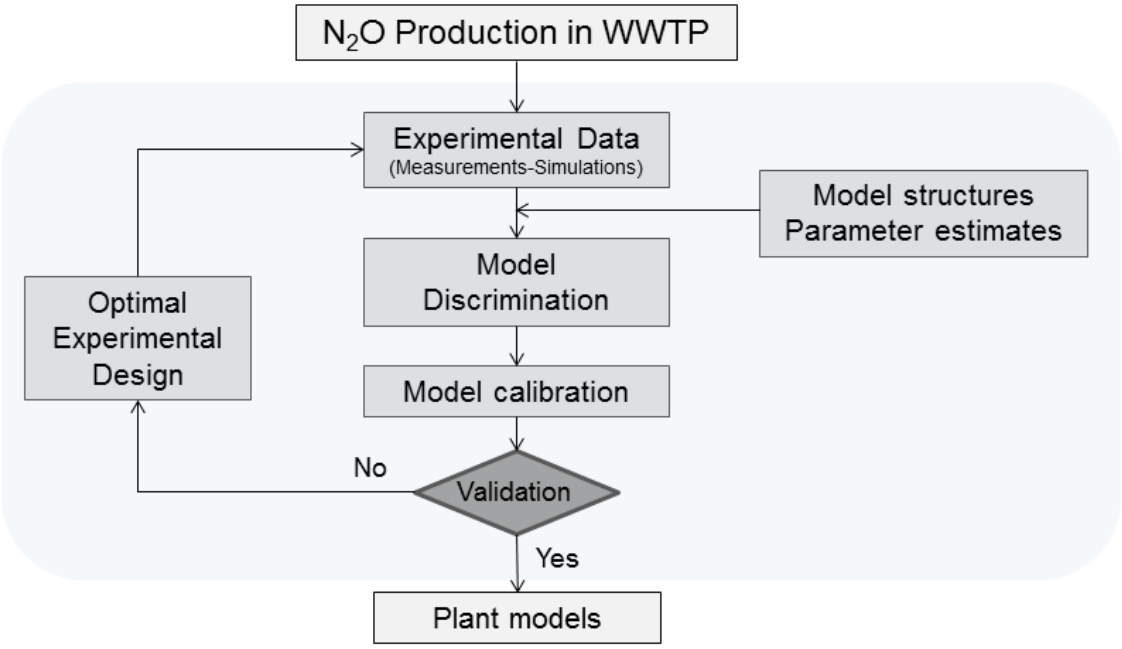

Figure 1.1. Overview of the research approach in this thesis.

The objectives of the thesis are:

• Critically review N_2O models to evaluate the prediction accuracy and assess structural limitations. (**Paper I**).

• Develop a consilience N_2O model structure capable of describing the known biological and abiotic pathways relevant for water quality engineering processes (**Paper II**).

• Design of lab-scale experiments to accurately obtain parameters describing N_2O production (**Paper III** and **IV**).

• Validate the estimated model parameters by assessing the model response and the parameter values (**Paper III** and **IV**).

• Analyse the predictive capabilities and precision of validated process models (**Paper III, IV** and **V**).

• Explore a modelling approach that describes electron competition; specifically applied for heterotrophic processes (**Paper V**).

2 Nitrous oxide production during biological nitrogen removal

2.1 Biological nitrogen removing organisms

The microbiome of wastewater treatment plants is a complex community comprised mostly of bacteria, and to a lesser extent, archaea. A large assumption is that all sewage treatment microbial communities will have roughly similar community compositions. The number of bacteria in activated sludge is estimated to be in the range of 1–10 x 10^{12}/g VSS (Nielsen and Nielsen, 2002), 80% of which are typically active. Chemoorganoheterotrophs are the most abundant populations in activated sludge, belonging to Alpha-, Beta-, Gamma-, Delta- and Actinobacteria. These microbes are capable of nitrogen removal, iron reduction, sulfate reduction, phosphate and glycogen accumulation, among other functions. In the next sections the microbial communities involved in nitrogen removal as well as the biochemical processes they mediate will be discussed in more detail (**Figure 2.1**).

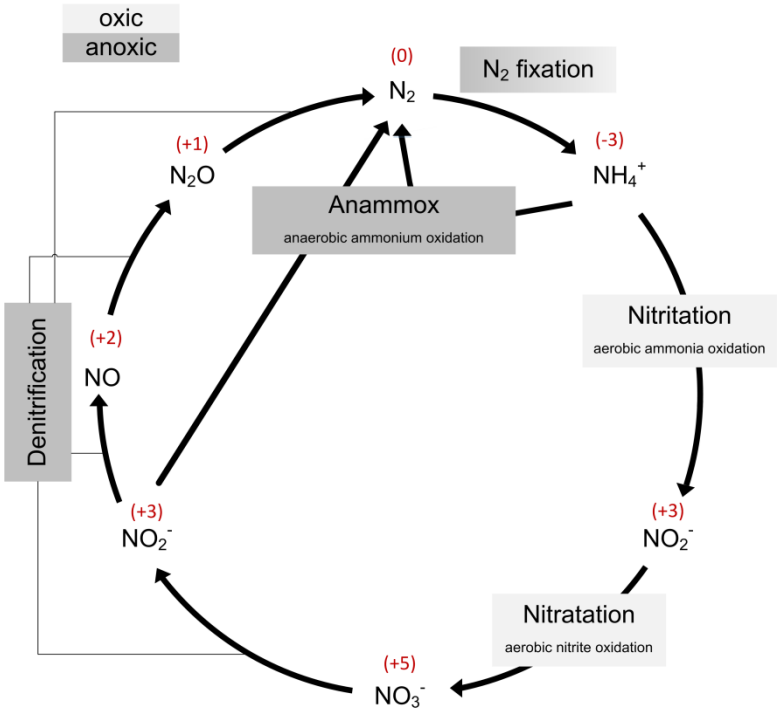

Figure 2.1. Simplified nitrogen cycle and relevant biological transformations.

2.1.1 Aerobic ammonia oxidizing bacteria

Aerobic ammonia oxidizing bacteria (AOB) are chemolithoautotrophic Proteobacteria (i.e., they use inorganic energy sources). AOB obtain energy from the oxidation of ammonia (NH_3) to nitrite (NO_2^-) with molecular oxygen (O_2) as electron acceptor (**2.1**). The oxidation of NH_3 with oxygen to hydroxylamine (NH_2OH) is an endergonic process catalysed by the ammonia monooxygenase (AMO) (**2.2**) (Sayavedra-Soto *et al.*, 1996). This step requires two electrons, supplied by the subsequent NH_2OH oxidation to NO_2^-, catalysed by the enzyme hydroxylamine oxidoreductase (HAO) (Böttcher and Koops, 1994; de Bruijn *et al.*, 1995) (**Figure 2.2**). NH_2OH oxidation releases four electrons, two sustain NH_3 oxidation and the other two are utilized for anabolic processes (**2.3**) (Vajrala *et al.*, 2013). While carbon dioxide (CO_2) is the preferred carbon source incorporated during growth, the metabolism of AOB is more versatile and they can also incorporate and obtain energy from small organic substrates (Daims and Wagner, 2010).

$$NH_3 + 0.0496CO_2 + 1.44O_2 \rightarrow 0.01C_5H_7O_2N + 0.99NO_2^- + 0.97H_2O + 0.99H^+ \quad (\mathbf{2.1})$$

$$NH_3 + O_2 + 2H^+ + 2e^- \xrightarrow{AMO} NH_2OH + H_2O \quad (\mathbf{2.2})$$

$$NH_2OH + H_2O + 2e^- \xrightarrow{HAO} HNO_2 + 4H^+ + 4e^- \quad (\mathbf{2.3})$$

In addition, AOB have a denitrifying functionality where NO_2^- can be used as electron acceptor at low dissolved oxygen (DO) conditions. NH_2OH oxidation provides the electrons for the sequential NO_2^- reduction to nitrous oxide (N_2O) via nitric oxide (NO) (Poth and Focht, 1985). This process is encoded by a set of NO_2^-- and NO-reducing enzymes (NIR, NOR) and is termed nitrifier denitrification (ND). AOB can also produce N_2O from the incomplete oxidation of NH_2OH to HNO_2 via NO, or its reduced form HNO (Hooper and Terry, 1979). This process is referred to as nitrifier nitrification (NN) (Zhu *et al.*, 2013) associated N_2O production. The enzymology of AOB suggests the presence of alternate N_2O producing pathways such as one mediated by CYT554 which possesses a NO reducing catalytic units similar to the NOR cluster (Upadhyay *et al.*, 2006; Kozlowski *et al.*, 2014). Recently, a direct enzymatic conversion of NH_2OH to N_2O mediated by CYTP460 was also demonstrated (Caranto *et al.*, 2016). DO differently affects the transcription and expression of NIR and NOR enzymes. NO production, regulated by NirK, would be favoured under anoxic conditions (Kester, 1997; Perez-

Garcia *et al.*, 2014), while NorB activity would be upregulated under oxic conditions (Yu and Chandran, 2010).

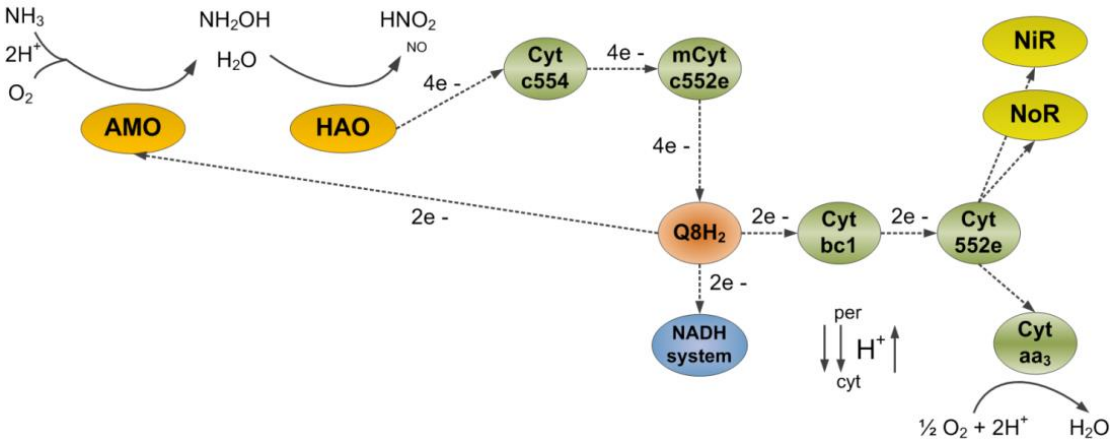

Figure 2.2. Simplified NH_3 oxidation to HNO_2 by AOB, main intermediates, electron flow and enzymatic sites.

The majority of AOB species belong to the Betaproteobacteria class (*Nitrosomonas*, *Nitrosospira*) while two known species belong to the Gammaproteobacteria (*Nitrosococcus halophilus* and *N. oceani*). Different sub-lineages of the genus *Nitrosomonas* are frequently detected by 16S rRNA and amoA sequence analysis in wastewater treatment plants (Nielsen *et al.*, 2010; Purkhold *et al.*, 2000). *Nitrosomonas europaea*, or *Nitrosomonas eutropha* adapt to higher ammonia concentrations compared to *Nitrosomonas oligotropha*. Diversity varies between systems, with some being dominated by one species and others, where ammonium concentrations vary over a wide range, by several species (Daims and Wagner, 2010).

Ecologically, *Nitrosomonas* cells have a higher specific growth rate than *Nitrosospira* species and a lower substrate affinity, suggesting a better adaptation to systems with high substrate as wastewater treatment plants (Schramm *et al.*, 1999; Terada *et al.*, 2013). The NH_4^+ and NH_2OH aerobic oxidation by AOB pure cultures (*N. europaea*, *N. communis*, and *N. multiformis* among others) revealed different physiological responses of NO and N_2O production (Kozlowski *et al.*, 2016).

2.1.2 Aerobic nitrite oxidizing bacteria

Some nitrite oxidizing bacteria (NOB) belong to the Alphaproteobacteria class (e.g. *Nitrobacter* spp,), the Betaproteobacteria (e.g. *Nitrotoga* spp.) oth-

ers to the Nitrospira phylum (e.g. *Nitrospira* spp.) and recently some Chloroflexi (e.g. *Nitrolanceta* spp.) were discovered (Sorokin *et al.*, 2012). They are also more physiologically diverse than AOB, not all NOB being chemolithoautotrophs (Madigan *et al.*, 2010). NOB obtain energy from the oxidation of NO_2^- to nitrate (NO_3^-) catalysed by nitrite oxidoreductase (NXR) using water as oxygen source (**2.4**). Molecular oxygen is reduced with the electrons released during NO_2^- oxidation in a cytochrome *aa3*-type terminal oxidase (Daims and Wagner, 2010). NOB genera *Nitrobacter* and *Nitrospira* can also grow mixotrophically on small organic compounds in the absence of NO_2^-.

$$NO_2^- + H_2O \xrightarrow{NXR} NO_3^- + 2H^+ + 2e^- \qquad (\mathbf{2.4})$$

Four genera comprise the best studied NOB: *Nitrobacter*, *Nitrospira*, *Nitrococcus*, and *Nitrospina*. In wastewater treatment operations *Nitrobacter*-like bacteria were considered the dominating species, but recent microbial characterization of activated sludge systems and biofilms showed a wider distribution of *Nitrospira* (Nielsen *et al.*, 2010) and in some cases *Nitrotoga* seems dominant (Lücker *et al.*, 2015).

From pure culture studies *Nitrobacter* spp. are considered r-strategist, being outcompeted by *Nitrospira* spp. at low substrate concentrations, K-strategists (Nowka *et al.*, 2014). Coexistence of *Nitrobacter* and *Nitrospira* has been observed in highly-loaded nitrifying reactors, but *Nitrospira* seems to outcompete *Nitrobacter* at low-load activated sludge systems (**Paper IV**).

N_2O is not part of the metabolism of NOB, but they possess a NirK gene responsible for NO_2^- reduction to NO (Perez-Garcia *et al.*, 2016a). Hence, indirectly, NOB play an important role on N_2O emissions from wastewater treatment operations. Indeed, by consuming NO_2^-, a possible substrate for N_2O production by AOB, NOB can act as an indirect N_2O mitigator in nitrifying systems.

2.1.3 Denitrifying bacteria

Denitrifying bacteria are commonly heterotrophs which at low oxygen tension can use nitrate, nitrite, nitric oxide and nitrous oxide as electron acceptors in their respiratory metabolism (**2.5**). Most denitrifiers can also respire organic carbon with oxygen as electron acceptor. Denitrifiers of the Betaproteobacteria class belong to the genera *Curvibacter*, *Thaurea*, *Azoarcus*, *Zoo-*

gloea and *Accumulibacter* (Daims and Wagner, 2010). In addition, chemolitotrophic denitrifers exist, that use compounds such as elemental sulfur, sulphide, or hydrogen as electron donor (Berks *et al.*, 1995); they will not be discussed here as they would not dominate typical water quality engineering systems.

$$NO_3^- + 1.08CH_2OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7O_2N + 0.47N_2 + 1.68H_2O + HCO_3^- \quad (\mathbf{2.5})$$

$$NO_3^- \xrightarrow{NAR} NO_2^- \xrightarrow{NIR} NO \xrightarrow{NOR} N_2O \xrightarrow{NOS} N_2 \quad (\mathbf{2.6})$$

The four-step reduction is carried out by the NAR, NIR, NOR and NOS enzymes (**2.6**); NAR is a membrane-bound enzyme while NIR, NOR and NOS are located in the periplasm (Berks *et al.*, 1995) (**Figure 2.3**). Heterotrophic denitrifiers possess a highly modular microbiome with very different distribution of denitrifying genes (Graf *et al.*, 2014). Co-occurrence of NAR, NIR and NOR enzymes without NOS would yield a net N_2O producer, while non-denitrifier N_2O reducers carrying an atypical *nosZ* gene have been identified and may act as N_2O sinks (Jones *et al.*, 2014). Moreover, the reduction of N_2O also occurs in some non-denitrifying bacteria (Domeignoz-Horta *et al.*, 2016). The potential of an heterotrophic community to serve as 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 (Sanford *et al.*, 2012; Jones *et al.*, 2014). In WWTP removing phosphorus and nitrogen biologically some Phosphate-Accumulating Organisms (PAO) also act as denitrifiers (Ekama and Wentzel, 1999).

The electrons released from carbon oxidation are distributed through the respiratory electron transport chain, to the ubiquinol pool and circulated to two branches: nitrate reductase and cytochrome *c* (Richardson *et al.*, 2009). Both branches have been shown to compete for a limited flow of electrons from NADH and succinate dehydrogenases (Kucera *et al.*, 1983). Similarly, nitrite and nitrous oxide reductases compete for electrons from the reduced cytochrome c (Alefounder *et al.*, 1983). Thus, the reduction rate of individual nitrogen oxide are influenced by the presence of other terminal acceptors (Kucera *et al.*, 1983). The reversible inhibitory effect of DO on NO_x^- reduction is similar for each step (Alefounder *et al.*, 1983; Richardson *et al.*, 2009). N_2O reduction is the most sensitive step towards DO, and under low DO N_2O accumulation is promoted compared to the other N-species (Wild *et al.*, 1994). The activity of enzymes encoded by the *nir*, *nor* and *nosZ* genes,

located in the periplasm, are pH-dependent, with different optima for each denitrification step (Thomsen *et al.*, 1994).

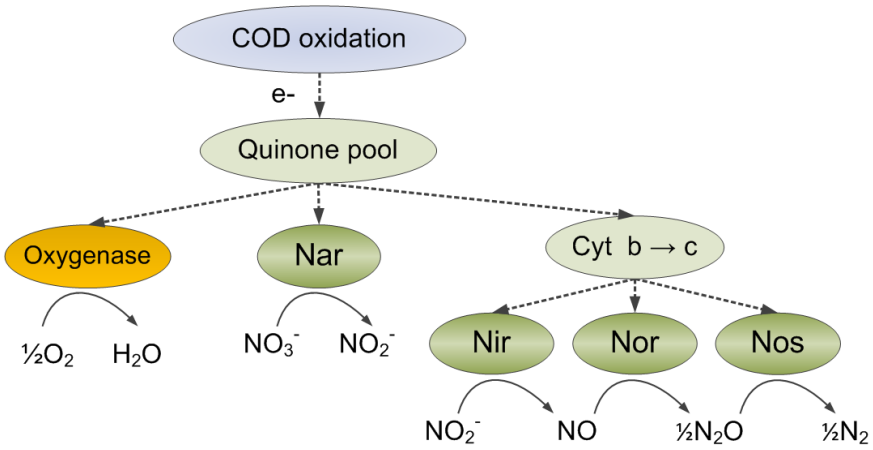

Figure 2.3. Diagram of canonical electron transport system in denitrification.

External carbon sources such as methanol, ethanol or acetate are commonly added to wastewater to enhance denitrification and improve nitrogen removal (Mokhayeri *et al.*, 2009). The denitrification rates and yield vary significantly based on the carbon source used, which has been proposed as the controlling factor for the community function and structure (Lu *et al.*, 2014). The metabolic pathways to oxidize each carbon source are different (Madigan *et al.*, 2010), and thus, dosage of a specific carbon source can shape the microbial community (Hallin *et al.*, 2006). Acetate-fed enriched for members of the *Comamonadaceae* and *Rhodocyclaceae* family, while methanol-fed enriched for members of the *Methylophilaceae* (Osaka *et al.*, 2006). Methanol oxidizers typically represent a small fraction of the complex denitrifying guild in wastewater treatment plants but increase after an adaptation period (Ginige *et al.*, 2004; Lu *et al.*, 2014).

2.1.4 Anaerobic ammonium oxidizing bacteria

Theoretical calculations predicted the existence lithotrophs that could oxidize NH_4^+ to N_2 with NO_3^- or O_2 as electron acceptors (Broda, 1977). Anaerobic ammonium oxidizing, "anammox" bacteria, are chemolithoautotrophs that obtain energy from the anaerobic oxidation of NH_4^+ with NO_2^- as electron acceptor and fix inorganic carbon (**2.7**) (Strous and Heijnen, 1998).

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}O_{0.15} + 2.03H_2O \quad (\mathbf{2.7})$$

Anammox bacteria comprise five genera *Ca. Anammoxoglobus*, *Ca. Brocadia*, *Ca. Jettenia*, *Ca. Kuenenia*, and *Ca. Scalindua*, all belonging to the *Planctomycetes* phylum. Previously considered as slow growers (doubling time of 10-15 days), it was recently shown to grow much faster (2.1 – 3.9 days) (Zhang *et al.*, 2017). N_2O is not part of the metabolism of Anammox bacteria, but intermediates of N_2O production pathways such as NO_2^- and NO, are part of the metabolism of Anammox. Hence, as well as NOB, Anammox play an important role on N_2O emissions from wastewater treatment operations.

2.1.5 Recent discoveries in the nitrogen cycle

Thermodynamic calculations predicted the existence of complete nitrifying organisms, capable of oxidizing ammonium into nitrate (Costa *et al.*, 2006). Recently, "comammox" organisms (completely ammonium oxidizers) have been discovered, reshaping our understanding of the nitrogen cycle (van Kessel *et al.*, 2015; Daims *et al.*, 2015; Palomo *et al.*, 2016). The abundance of comammox in wastewater treatment plants is significantly lower than AOB, and thus, this study will solely focus on AOB as aerobic ammonium oxidizers (Chao *et al.*, 2016).

2.1.6 Abiotic reactions

Earlier studies on abiotic N_2O production have highlighted the importance of two chemical reactions driven by NH_2OH (Heil *et al.*, 2014) that can occur at relevant rates under wastewater treatment conditions.

$$4NH_2OH \rightarrow N_2O + 2NH_3 + 3H_2O \quad (\mathbf{2.8})$$

$$NH_2OH + HNO_2 \rightarrow N_2O + 2H_2O \quad (\mathbf{2.9})$$

NH_2OH can decompose to N_2O at high pH (**2.8**, (Feelisch and Stamler, 1996); its acidic form NH_3OH^+ is more stable (Liu *et al.*, 2014) (pKa = 5.9, 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 (Spott *et al.*, 2011) (**2.9**, (Döring and Gehlen, 1961)). Thus, independently from the main driving process (e.g., nitrification or denitrification) and the environmental condi-

tions (e.g., aerobic or anaerobic), biotically-driven (because it requires NH_2OH) abiotic N_2O production is possible in WWTP.

Previously considered as low, NH_2OH concentrations from highly N-loaded wastewaters can be higher than expected (0.03-0.11 mgN/L) (Soler-Jofra *et al.*, 2016), highlighting a possible underestimation of the abiotic N_2O production (Harper *et al.*, 2015). For example, a nitritating reactor for reject water (high AOB activity and NO_2^- accumulation) estimated a 1.1% abiotic emission factor driven by NH_4^+ oxidation (Soler-Jofra *et al.*, 2016).

Nitrate or nitrite reduction coupled with Fe(II) oxidation was also proposed as abiotic contributor to NO and N_2O production under anoxia at high nitrite levels in wastewater treatment systems (**2.10**, **2.11**) (Kampschreur *et al.*, 2011).

$$NO_2^- + Fe^{2+} + 2H^+ \rightarrow NO + Fe^{3+} + H_2O \qquad \textbf{(2.10)}$$

$$NO + Fe^{2+} + 1H^+ \rightarrow Fe^{3+} + 0.5N_2O + 0.5H_2O \qquad \textbf{(2.11)}$$

The observations hinted to a role for iron oxidation coupled to nitrite reduction from mixed liquor because of its considerable iron reducing activity. However, the presence or absence of Fe(II) or Fe(III) did not affect aerobic abiotic N_2O production (Terada *et al.*, 2017; Soler-Jofra *et al.*, 2016). For more details on abiotic N_2O production the reader is referred to (Zhu-Barker *et al.*, 2015).

2.2 Nitrogen removal in wastewater treatment and nitrous oxide emissions

Sewage treatment contributes to 3.2% of the anthropogenic N_2O emissions, but can triplicate if manure, landfill leacheates and industrial nitrogenous effluents are included (Desloover *et al.*, 2012). The carbon footprint of a WWTP is highly sensitive to N_2O emissions (Gustavsson and Tumlin, 2013), where an N_2O emission factor of 1% increases the carbon footprint by 50% (Monteith *et al.*, 2005), reaching up to 83% of the operational CO_2 footprint of a Biological Nitrogen Removal (BNR) plant (Desloover *et al.*, 2011).

All of the BNR processes include an aerobic zone in which biological nitrification occurs. Some anoxic volume or time must also be included to provide biological denitrification to complete the objective of total nitrogen removal. Biological nitrification/denitrification is the most common treatment in WWTP due to its high efficiency, stability and reliability. Energy savings are linked to economic savings, and hence, processes that reduce the high use of energy in aeration are considered as attractive alternatives to actual BNR processes. Short-cut nitritation-denitritation, the combination of nitritation and anammox in single or two-stage systems are such alternatives with lower energy demands (Joss *et al.*, 2011). However, a trade-off seems to exist between aeration costs and reduced N_2O emissions (Ahn *et al.*, 2011).

N_2O mitigation strategies have been proposed based on intensive measurement campaigns (Desloover *et al.*, 2012; Foley *et al.*, 2010), but N_2O emissions are highly variable even for similar processes (0.001 – 25.3% N_2O emitted/N-load). A ranking of BNR technologies based on the potential N_2O risk cannot be established because of the yet unknown high variability of reported N_2O emissions (Desloover *et al.*, 2012; Kampschreur *et al.*, 2008a). Key variables such as low dissolved oxygen or high nitrite accumulation have been identified as potential hotspots for N_2O emissions in BNR processes (Sun *et al.*, 2015; Kampschreur *et al.*, 2009b).

2.3 Regulation of nitrous oxide production in wastewater treatment

In nitrogen removing systems N_2O production has been associated to several variables and operational parameters. Suggestions on how to fine-tune these variables has been applied to manage N_2O emissions using a black-box approach (**Figure 2.4**) (Brotto *et al.*, 2015; Kampschreur *et al.*, 2009a; Park *et al.*, 2000). These methods rely on obtaining a better understanding of N_2O emissions by means of correlation analysis: what variables trigger N_2O emissions?

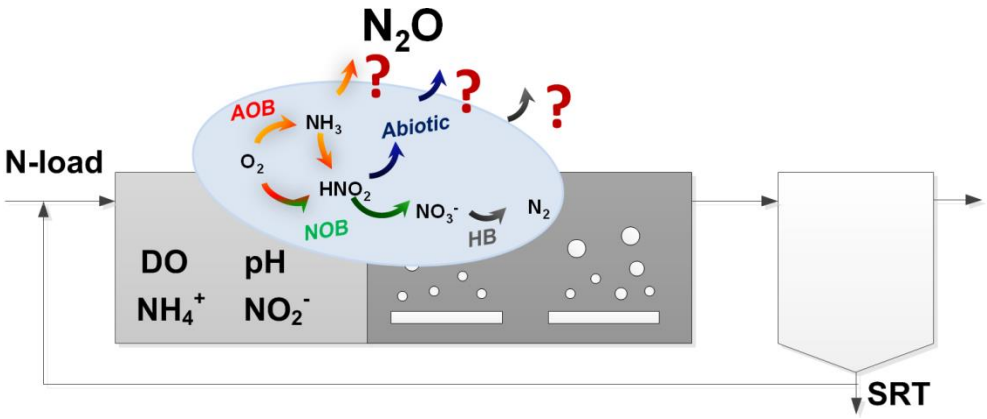

Figure 2.4. Nitrous oxide emission during biological nitrogen removal.

The NH_4^+ load and influent NH_4^+ concentration have been correlated to N_2O emissions from aerobic zones operating at high dissolved oxygen (DO) concentrations (Lotito *et al.*, 2012; Ni *et al.*, 2013b). At low DO NH_4^+ is oxidized at a lower rate but a higher fraction is converted to N_2O (Burgess *et al.*, 2002; Li and Wu, 2014) (**Figure 2.5**). The aeration strategy, i.e. aeration rate and frequency of aeration, also impact N_2O emission (Yu *et al.*, 2010; Domingo-Félez *et al.*, 2014; Kampschreur *et al.*, 2008a).

NO_2^- accumulation has also lead to higher N_2O emissions in N-removing systems (Wang *et al.*, 2016b; Kampschreur *et al.*, 2008b) (**Paper I**). As the direct precursor of N_2O in most of the biological pathways, NO has shown the highest correlations with N_2O (Kampschreur *et al.*, 2008b; Wang *et al.*, 2016b; Domingo-Félez *et al.*, 2014).

pH levels have two distinct effects on N_2O production. First, on the enzymatic level, maximum activities have been described as pH-dependent (Park *et al.*, 2007) (**Figure 2.5**). Second, the availability of the true substrates for

AOB and NOB are assumed to be NH_3 and HNO_2 respectively; the actual concentrations of these species are in a pH-dependent equilibrium with their ionized counterparts NH_4^+ and NO_2^- (Udert *et al.*, 2005) (pKa_{HNO2} = 3.25, pKa_{NH4+} = 9.25, 25 C (Lide, 2009)). Acidification enhanced the N_2O yield of *Nitrosospira*-dominated community, suggested due to the hybrid N_2O-forming reaction of NH_2OH and HNO_2 (Frame *et al.*, 2017).

Inorganic carbon (IC) is fixed to form cellular carbon during AOB growth. At limiting IC availability, NH_3 is oxidized at a lower rate due to increased cellular maintenance energy demand, which decreases the overall N_2O produced (Jiang *et al.*, 2015). However, under the same NH_3 oxidation rates, IC-limitation increases the fraction of N_2O produced (Mellbye *et al.*, 2016). Depending on the nitrogen removal system, wastewaters can have varying IC levels.

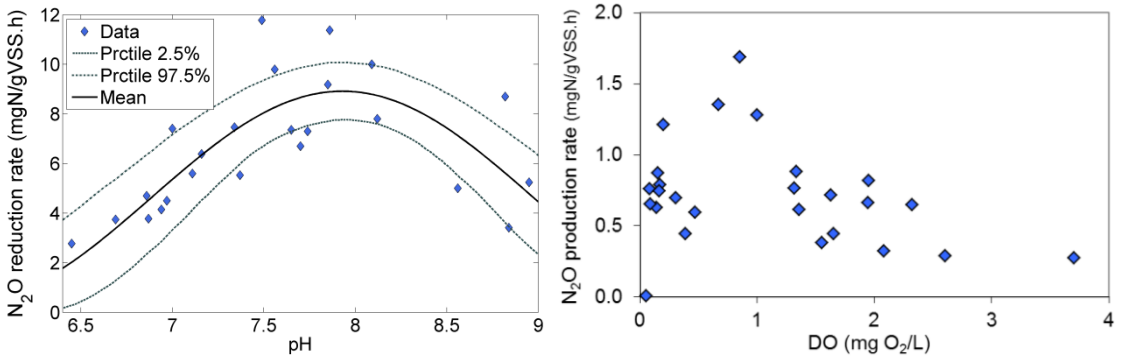

Figure 2.5. Left: Nitrous oxide consumption dependency on pH (**Paper IV**). Right: Net production rates at varying dissolved oxygen concentrations from mixed liquor biomass. (Unpublished data).

The heterotrophically-oxidized organic content of conventional urban wastewater typically produces excess IC for autotrophic growth, but high N-strength wastewaters with a lower C/N ratio, may result in IC limited AOB growth (Panwivia *et al.*, 2014).

Operational parameters and wastewater characteristics have also shown to affect N_2O emissions. A limited flow of electron donors (COD) due to a low carbon-to-nitrogen ratio of the incoming wastewater can also slow down NO_x^- reduction rates. Therefore, N_2O production can be enhanced by a lower N_2O reduction rate compared to previous steps because of the lower electron affinity. Consequently, side stream processes, characterized by a high N and low COD content, are potential hotspots for heterotrophic N_2O production (Kampschreur *et al.*, 2009b; Yang *et al.*, 2009; Hu *et al.*, 2013). Additionally,

N_2O consumption is the most sensitive denitrification step to the presence of DO and thus, N_2O can be released in the presence of low DO concentrations (Richardson *et al.*, 2009).

Other operational parameters such as the solids retention time (SRT) have shown increasing N_2O emission factors for low SRT values (Li and Wu, 2014; Lotito *et al.*, 2012). Seasonal variations of N_2O emissions have been observed and associated to temperature changes that affect the microbial populations involved in nitrogen removal (Wang *et al.*, 2014, 2016b).

In biofilms the spatial distribution of microbial communities and mass transfer limitations are linked by chemical gradients (Manser *et al.*, 2005; Picioreanu *et al.*, 2016). Biofilms showed a lower emission factor compared to suspended-growth systems with smaller particle size (Park *et al.*, 2000). For example, in partial nitritation/anammox suspended granules, anammox are located in the inner anoxic layers, acting as a NO_2^- sinks and thus, reducing the risk of N_2O production. Other parameters affecting N_2O production in suspended and biofilm wastewater treatment operations have been recently reviewed (Todt and Dörsch, 2016; Massara et al., 2017).

3 Modelling nitrous oxide emissions during WQE

3.1 Modelling biological nutrient removal

Models are simplifications of reality that describe, through mathematical equations, a system of interest. The purpose of the model also defines the scope and detail that model predictions should achieve. For example, in wastewater treatment applications models have been used to develop control strategies, to evaluate new plant designs or to support management decisions (Henze *et al.*, 2008). The modelling objectives tend to align with regulatory discharge wastewater characteristics (e.g. particulate, organic carbon, and nutrient content of effluents).

A wastewater treatment process model typically comprises a variety of different components: influent characterisation model, hydraulic process model, sedimentation model and reaction model (**Figure 3.1**).

Specifically, the reaction model integrates the hydrodynamic mixing model and the biological model. The first one considers the model components and flow through the reactor volume, ideally as a Completely-Stirred Tank Reactor (CSTR), Plug-Flow Reactor (PFR), or a combination of ideal reactors. The focus of this thesis is on the biological model that describes the conversions of state variables.

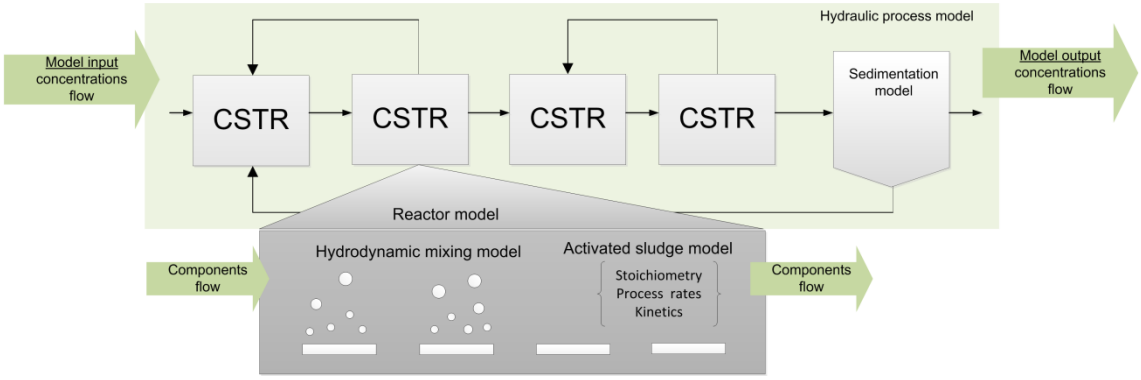

Figure 3.1. Representation of a complete wastewater treatment plant model (Modified from (Meijer, 2004)).

3.2 ASM-based models for nutrient removal

The increasing metabolic understanding of nutrient removal can be described with mathematical equations and has been successfully used to predict the fate of C, N and P in wastewater treatment operations (Henze *et al.*, 2000). The Activated Sludge Model (ASM) No. 1, No.2, and No. 2d are grey-box models where different microbial guilds present in the activated sludge and their specific functionality are incorporated in a so-called population-based model. ASM1, 2, and 2d consider the microbes as a black box and do not take into account intracellular processes. However, new ASM-based extensions incorporate metabolic process descriptions that result in bigger and more complex models (Snip *et al.*, 2014).

A generic mathematical model (e.g. ASM-based) can be described by the following equations:

$$\frac{d\underline{x}}{dt} = f(t, \underline{x}, \underline{u}, \underline{\theta})$$

$$\underline{x}(t_0) = \underline{x}_0$$

$$\underline{y} = g(\underline{x}(t))$$

Where t is time, $\underline{x}$ are the state variables ($\underline{x}_0$ the initial states), $\underline{u}$ the input variables, $\underline{\theta}$ the input parameters and $\underline{y}$ the output variables. Underscored symbols correspond to vector variables. The partial differential equation describes the substrate utilization and dynamic accumulation. The general rate expression for compound S_i that is affected by multiple processes P_j is described by ρ_j (**Table 3.1**).

Table 3.1. Stoichiometric matrix for a two-process and three-component model.

Processes P_j ↓ Components S_i →	S_1	S_2	X_1	Process Rate (ρ_j)
P_1	$\nu_{1,1}$	$\nu_{1,2}$	$\nu_{1,3}$	$f(\theta_1, \theta_2, S_1, S_2, X_1)$
P_2	$\nu_{2,1}$		$\nu_{2,3}$	$f(\theta_3, X_1)$
Parameters: Kinetic $(\theta_1, \theta_2, \theta_3)$ Stoichiometric $\nu_{j,i}$	Dissolved substrate 1 (-)	Dissolved substrate 2 (-)	Particulate biomass 1 (-)	Observed transformation rate $r_i = \sum_j \rho_j \cdot \nu_{j,i}$

The process rate is described by model components and kinetic parameters. The mass balance for a compound corresponds to the observed transformation rate r_i, and the rates are coupled through conservation relations (stoichiometry).

3.3 Nitrous oxide models

Mathematical models can be useful tools to predict N_2O emissions and thus, help develop mitigation strategies to reduce the carbon footprint of wastewater treatment operations. N_2O models are developed as extensions from existing models for N-removal. Additional state variables, process rates and parameters increase the complexity of N_2O models conventional N-removal models.

Models vary based on the number of processes and/or variables considered in N_2O production and the relationships of their mathematical rates (Liu *et al.*, 2016; Perez-Garcia *et al.*, 2014; Pocquet *et al.*, 2016).

In empirical models N_2O emissions and nitrogen removal rates are fit to operational factors (e.g. pH value, temperature, feeding and aeration strategy, etc.) via multiple linear regression models (MLR) (Leix *et al.*, 2017; Liu *et al.*, 2016). The specific effects and combined influences are then used to find conditions for N_2O mitigation.

Of increasing complexity, Stoichiometric Metabolic Network (SMN) models make use of the increasing knowledge on metabolic engineering to describe microbial interactions (Perez-Garcia *et al.*, 2016b). N_2O production from nitrification by *N. europaea* at steady state was described with a SMN model containing 44 metabolites and 49 stoichiometric reactions (Perez-Garcia *et al.*, 2014). For wastewater treatment purposes ASM-based models are widely used, and many N_2O extensions have been proposed (Ni *et al.*, 2011, 2014; Pocquet *et al.*, 2016; Guo and Vanrolleghem, 2014; Hiatt and Grady, 2008). The ASM-based models differ on the biological description and the number of N_2O pathways, which are always significantly lower than for SMN models (6-7 metabolites, 5-6 reactions) (**Paper II**). Control strategies based on N_2O predictions are being developed for the reduction of N_2O emissions (Boiocchi *et al.*, 2016).

3.3.1 Autotrophic models

Initially, single-pathway models were proposed describing/capturing either the NN or ND pathway. The main differences between models regards the stoichiometric coefficients, the number of substrates considered, the identity

of the 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 (Kampschreur *et al.*, 2007; Schreiber, 2009). 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 (Law *et al.*, 2012) or via NO (Ni *et al.*, 2013a) (**Figure 3.2, A**). In the ND pathway NH_2OH acts as electron donor for the consecutive reduction of NO_2^- to N_2O via NO (Ni *et al.*, 2011) (**Figure 3.2, B**). However, N_2O dynamics cannot be captured with single-pathway models, and recent models combining the NN and ND pathways provide better descriptions of N_2O production than single-pathway models (Ni *et al.*, 2014; Pocquet *et al.*, 2016; Ding *et al.*, 2016) (**Figure 3.2, C, D, E**).

In a novel approach, global cellular oxidation (electron generating) and reduction (electron consuming) reactions are linked by a common pool of electron carriers, represented by one model component. This model aggregates all intracellular electron carriers such as cytochromes and ubiquinone into one component that cannot be directly quantified (Kim *et al.*, 2010). Oxidative and reductive processes are therefore uncoupled and competition is described with specific kinetic parameters (Ni *et al.*, 2014).

The two-pathway AOB models are adequate in predicting a shift in NN and ND contributions to 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 observed in several nitrifying systems (Chandran *et al.*, 2011; Kester, 1997; Rodriguez-Caballero and Pijuan, 2013).

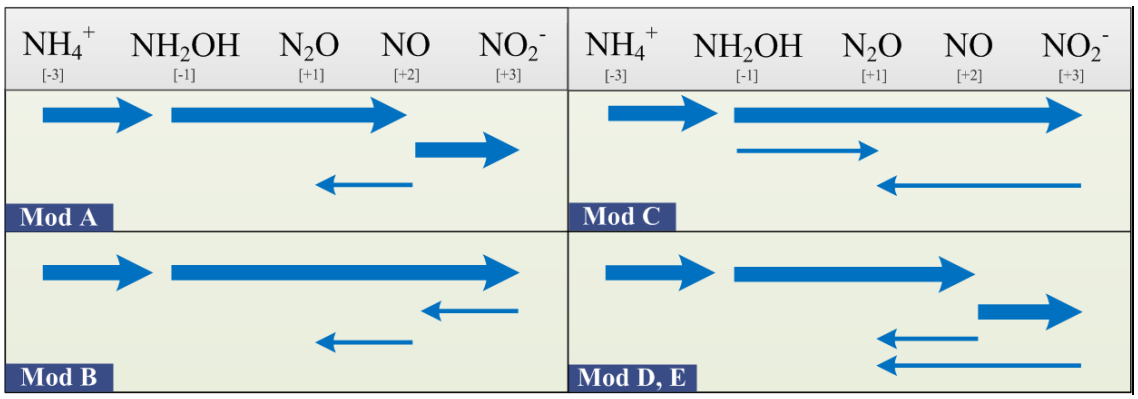

Figure 3.2. Comparison of the reactions involved in autotrophic models for N_2O production. The arrow widths represent typical reaction rates. Mod A (Ni *et al.*, 2013b), Mod B (Ni *et al.*, 2011), Mod C (Ding *et al.*, 2016), Mod D (Pocquet *et al.*, 2016), Mode E (Ni *et al.*, 2014).

3.3.2 Heterotrophic denitrification models

The first kinetic model describing heterotrophic denitrification was based on pure cultures and described each denitrification step according to the Michaelis-Menten equation (Betlach and Tiedje, 1981). This approach considers every reduction rate independent from each other and has been widely used (Schulthess *et al.*, 1995; Hiatt and Grady, 2008). Wild *et al.*, (1994) explicitly calculated the concentration of enzymes to describe the delay in denitrification and N_2O accumulation after aerobic growth, which was recently updated to the four steps (Zheng and Doskey, 2015). However, these models are limited to the assumption that carbon oxidation supplies all the electrons necessary for the four denitrification steps. Hence, only nitrogenous species limit denitrification rates under excess organic carbon conditions (Pan *et al.*, 2015).

Differently, branched models reflect the modularity of the electron transport chain (Richardson *et al.*, 2009). Grant and Pattey, (1999) developed a model where a maximum electron supply is distributed among electron acceptors, with preference given to the most oxidized compounds in a feed-back redox control ('*inhibition by product via respiratory chain'*). A different approach considered a double branch with common electron mobile carriers and described the accumulation of intermediates, but was not validated experimentally (Thomsen *et al.*, 1994). Almeida *et al.* (1997) proposed an analogy between an electric circuit and the electron flow through the cell membrane. The model was validated with experimental results from two pure culture studies where NO_2^- (*Pseudomonas fluorescens*), and NO_2^- and N_2O (*Paracoccus denitrificans*) accumulated. The indirect coupling of electrons approach (ICE) calculates the concentration of internal electron carriers, uncoupling the carbon oxidation and denitrification processes at the cost of higher complexity (Pan *et al.*, 2013).

Even though the indirect approach has been heralded as superior as it can potentially describe all experimental observations (Pan *et al.*, 2015) more information about reaction kinetics is required. The direct approach can predict COD and nitrogen removal for systems with low intermediates accumulation (NO_2^-, N_2O) (Ni and Yuan, 2015) but might be inadequate for systems with high accumulation levels.

Heterotrophic denitrification: competitive electron distribution

The direct approach first developed by (Betlach and Tiedje, 1981) for denitrification is widely used in ASM-based models and hence will be used here. However, other approaches exist, such as the indirect modelling of carbon and nitrogen removal (Thomsen *et al.*, 1994; Pan *et al.*, 2013; Almeida *et al.*, 1997). The modelling approach presented by (Almeida *et al.*, 1997) is explored here.

A model describing 4-step denitrification and aerobic organic carbon removal is developed based on the analogy between electron competition during denitrification and electron distribution in electric circuits (**Figure 3.3, M1**).

A potential (E) is created by the presence of an electron donor/acceptor pair. The reaction rate is kinetically analogous to the current intensity (i_i) through a resistor. The resistance depends on the concentration of the substrate (Monod kinetics, $K_{S,i}$) and a minimum resistance (**3.1**) at substrate (S_i) limiting conditions the resistance (r_i) is infinite and no current flows, while at excess substrate the resistance becomes minimal, with value (R_i).

$$r_i = R_i \cdot \frac{(S_i + K_{S,i})}{S_i} \qquad \left[E \cdot \frac{mgN}{gVSS} \cdot h\right] \qquad (\mathbf{3.1})$$

Following the conservation of potential (**3.2**) and conservation of charge (**3.3**), the current through any resistor can be calculated. Thus, for any branched model the electron distribution from common pools (e.g. quinones, cytochromes) to several electron acceptors can be calculated.

$$E = E_{COD} + E_{NOx} \qquad (\mathbf{3.2})$$

$$i_{COD} = \sum i_{NOX} \qquad (\mathbf{3.3})$$

$$Rate_{NIR} = E_{tot} / \left[r_{COD} \cdot \left(r_{NIR} \cdot \left(\frac{1}{r_{AER}} + \frac{1}{r_{NAR}} + \frac{1}{r_{NIR}} + \frac{1}{r_{NOR}} + \frac{1}{r_{NOS}} \right) + r_{NIR} \right) \right] \cdot X_{HB} \quad (\mathbf{3.4})$$

Compared to the original model structure by (Almeida *et al.*, 1997) two new electron distribution analogies considering additional processes are implemented: a one-branch model where all the reduction steps compete for electrons from a common source, and a two-branch model that resembles more precisely the intracellular electron distribution at a cost of an additional parameter (**Figure 3.3**). The model uses fewer parameters compared to existing state-of-the art denitrification models (Pan *et al.*, 2013). Model fitting is performed with data obtained from batch experiments with mixed denitrifying

communities for a combination of nitrogen oxides and for four different carbon sources in excess: methanol, ethanol, acetate and a carbon mixture.

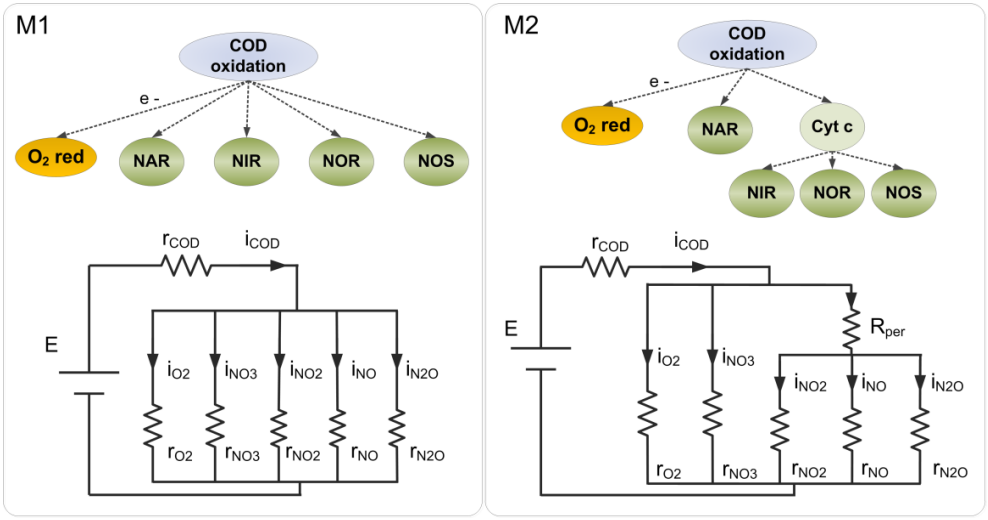

Figure 3.3. Simplified electron distribution in heterotrophic respiration and corresponding electric circuit analogy: one-branch model (M1), two-branch model (M2). (**Paper V**).

The model successfully describes the competition for electrons during batch experiments at excess substrate concentrations for a combination of nitrogen oxides (**Figure 3.4**). The total electron consumption rate predicted was not additive as non-competitive models suggest (Hiatt and Grady, 2008), and was distributed differently among the four denitrification steps (**Figure 3.4**).

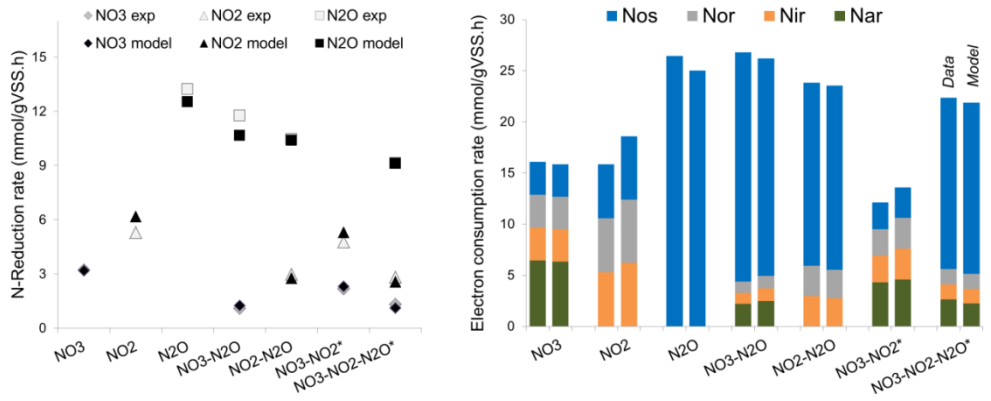

Figure 3.4. Left: Experimental (grey) and simulated (black) denitrification rates. Right: Electron consumption rates by NO_3^- reduction (Nar – green), NO_2^- reduction (Nir – orange), NO reduction (Nor – grey) and N_2O reduction (Nos – blue) in Methanol-fed denitrifying experiments. Experimental (left bar) and modelling results (right bar). (* not used during calibration). (**Paper V**).

Among the four carbon sources evaluated calibration results indicate faster specific denitrification rates for methanol compared to any of the other carbon sources ($R_{NAR/NIR/NOS,MeOH} < R_{NAR/NIR/NOS,Acet,EtOH,C\text{-}mix}$) (**Table 3.2**).

Table 3.2. Best-fit parameters for denitrification batches: Methanol, Acetate, Ethanol, C-mix. (**Paper V**).

	Methanol	Acetate	Ethanol	C-mix
R_{NAR}	5.6	11.8	8.2	8.9
R_{NIR}	5.0	9.2	44.3	25.9
R_{NOS}	0.6	15.0	7.6	11.1

In the scenarios evaluated in this study - excess electron donor (methanol) and electron acceptor - a simpler model such as M1 performs better than the ASM-ICE model. Further evaluation under a wider range of operation conditions (e.g. different carbon loadings) will benefit model discrimination between M1 and ASM-ICE. Overall, a different modelling approach for denitrification was explored but further validation is required.

3.4 NDHA model

An ASM-based model structure that describes N_2O production during biological nitrogen removal is proposed. The model builds on existing structures for nitrogen removal and expands the number of processes to describe N_2O dynamics. The model intends to answer the limitations of existing N_2O models. For example, a better understanding of the AOB pathways would help identify operating conditions affecting N_2O production and improve the accuracy of N_2O predictions (Mannina *et al.*, 2016).

Theoretically, the model describes all relevant NO and N_2O production pathways with fewer parameters than other proposed models. The NDHA model comprises the three known biological pathways (NDHA) as well as abiotic production (NDHA) (**Figure 3.5**, **Table 3.3**).

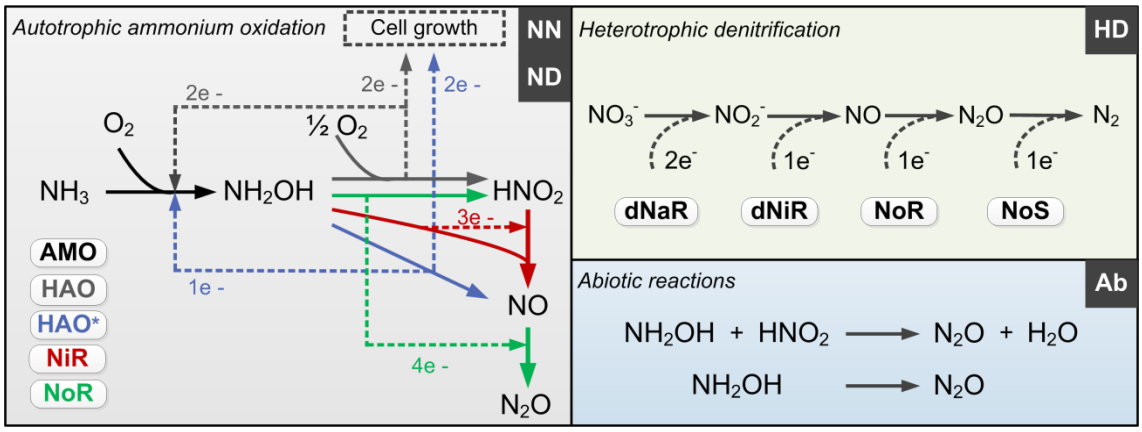

Figure 3.5. Diagram of the proposed N_2O-producing mechanisms occurring during N-removal: nitrifier nitrification, nitrifier denitrification, heterotrophic denitrification and abiotic pathways (NDHA). (Adapted from **Paper II**).

Nitrifier Nitrification (NN): The first process considers NH_3 oxidation to NH_2OH (P1). NH_2OH can be oxidized incompletely to NO_{NN} (P2) or completely to HNO_2 in the presence of DO (P3). The effect of IC limitation on NH_3 oxidation is described by a Monod dependency (Guisasola *et al.*, 2007). The NN process is 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_{AOB}.

P1. AMO $\quad NH_3 + O_2 \rightarrow NH_2OH$

$$\mu_{AMO}^{AOB} \cdot \frac{S_{O2}}{S_{O2} + K_{O2\_AMO}^{AOB}} \cdot \frac{S_{NH3}}{S_{NH3} + K_{NH3}^{AOB}} \cdot \frac{S_{IC}}{S_{IC} + K_{IC}^{AOB}} \cdot X_{AOB}$$

P2. HAO* $NH_2OH \rightarrow NO_{NN}$

$$\mu_{HAO}^{AOB} \cdot \varepsilon_{AOB} \cdot \frac{S_{NH2OH}}{S_{NH2OH} + K_{NH2OH}^{AOB}} \cdot \frac{S_{IC}}{S_{IC} + K_{IC}^{AOB}} \cdot X_{AOB}$$

P3. HAO $NH_2OH + 0.5\ O_2 \rightarrow HNO_2 + H_2O$

$$\mu_{HAO}^{AOB} \cdot (1-\varepsilon_{AOB}) \cdot \frac{S_{O2}}{S_{O2} + K_{O2\_HAO}^{AOB}} \cdot \frac{S_{NH2OH}}{S_{NH2OH} + K_{NH2OH}^{AOB}} \cdot \frac{S_{IC}}{S_{IC} + K_{IC}^{AOB}} \cdot X_{AOB}$$

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 (Upadhyay *et al.*, 2006). The NN and ND pathways are differentiated by two NO-producing processes with different DO and HNO_2 dependencies. These dependencies govern the shift between pathways (Chandran *et al.*, 2011; Kozlowski *et al.*, 2014). 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.

The NO/N_2O ratio can be used to help elucidate the individual contribution of each pathway during model calibration (Pocquet *et al.*, 2016). An advantage of the proposed model is uncoupling the NN- and ND-driven NO production, which allows for a more biologically congruent estimate of NO/N_2O.

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

$$\mu_{HAO}^{AOB} \cdot \eta_{NIR} \cdot \frac{K_{i\_O2}^{AOB}}{S_{O2} + K_{i\_O2}^{AOB}} \cdot \frac{S_{NH2OH}}{S_{NH2OH} + K_{NH2OH\_ND}^{AOB}} \cdot \frac{S_{HNO2}}{S_{HNO2} + K_{HNO2}^{AOB}} \cdot X_{AOB}$$

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

$$\mu_{HAO}^{AOB} \cdot \eta_{NOR} \cdot \frac{S_{NH2OH}}{S_{NH2OH} + K_{NH2OH\_ND}^{AOB}} \cdot \frac{S_{NO}}{S_{NO} + K_{NO\_ND}^{AOB}} \cdot X_{AOB}$$

Heterotrophic denitrification (HD):

Because of the wide applicability of the direct approach a four-step complete denitrification is used following the ASM-N model (Hiatt and Grady, 2008). The indirect coupling approach was not considered because of its limited application (only one full-scale study has been reported (Wang *et al.*, 2016a)), and hence limited information about reaction kinetics. Moreover, the ASM-N model has also been extended coupled with phosphorus removal (Liu *et al.*,

2015). In the four-step denitrification model individual reaction kinetics (pH-dependent), inhibition and substrate affinities are considered for every step as recently suggested for systems with low intermediates accumulation (Ni and Yuan, 2015).

P8-11. HD $NO_{X,oxidized} + COD \rightarrow NO_{X,reduced}$

$$\mu_{i\,(pH)}^{HB} \cdot \frac{K_{i\_O2\_NOx,i}^{HB}}{S_{O2} + K_{i\_O2\_NOx,i}^{HB}} \cdot \frac{S_S}{S_S + K_{S\_NOx,i}^{HB}} \cdot \frac{S_{NH4}}{S_{NH4} + K_{NH4}^{HB}} \cdot \frac{S_{NOx,i}}{S_{NOx,i} + K_{NOx,i}^{HB}} \cdot X_{HB}$$

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 heterotrophic pathways (**Paper I**).

Abiotic (Ab): Two biologically-driven abiotic N_2O production processes are considered (P7). Nitrification produces NH_2OH, which is oxidized to HNO_2, while also forming HNO (Igarashi *et al.*, 1997). HNO dimerizes via $H_2N_2O_2$ to N_2O and H_2O. Nitrosation of NH_2OH with HNO_2 has also been postulated as a relevant reaction in partial nitrification reactors (Soler-Jofra *et al.*, 2016). Reactions rates are modelled with pH dependent second order kinetics. A model combined for the first time the abiotic reaction between NH_2OH and HNO_2 together with the ND pathway (Harper *et al.*, 2015). Nitritation reactors with high NH_4^+ removal rates or low pH that lead to higher NH_2OH and HNO_2 accumulations could thus be relevant sources of simultaneous abiotic and biotic N_2O production.

P. Abiotic $NH_2OH \rightarrow N_2O$

$NH_2OH + HNO_2 \rightarrow N_2O$

$$\left(k_{Abiotic\_1} \cdot S_{NH2OH} \cdot f(pH)\right);\ \left(k_{Abiotic\_2} \cdot S_{NH2OH} \cdot S_{HNO2}\right)$$

Model predictions for every pathway are pH-dependent, either due to substrate speciation or to an enzymatic effect on the maximum growth rate. Aerobic growth of nitrite oxidizing bacteria on HNO_2 and heterotrophs on soluble COD are also included.

In the NDHA model the assumption that there is no ND-associated NO production is resolved and NO_{ND} is produced from HNO_2 reduction as experimentally observed (Rodriguez-Caballero and Pijuan, 2013; Chandran *et al.*, 2011; Kester, 1997; Wang *et al.*, 2016b; Domingo-Félez *et al.*, 2014).

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 (**Figure 3.6**). 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.

The same net N_2O 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.

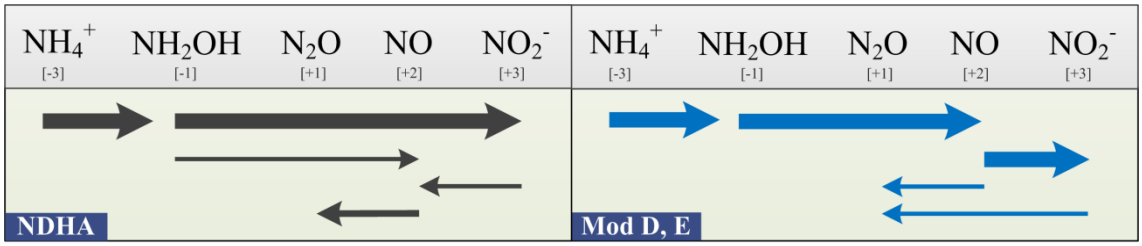

Figure 3.6. Schematic comparison of the reactions involved in two-pathway autotrophic models for N_2O production. Arrow widths represent typical reaction rates. Model D (Pocquet *et al.*, 2016), Model E (Ni *et al.*, 2014). (Adapted from **Paper II**).

Table 3.3. Gujer matrix for the NDHA model (**Paper IV**):

Component ▸ Process ▼		1 S_S	2 S_{O2}	3 S_{NH3}	4 S_{NH2OH}	5 S_{HNO2}	6 S_{NO3}	7 S_{NO}	8 S_{N2O}	9 S_{N2}	11 $X_{B,AOB}$	12 $X_{B,NOB}$	13 $X_{B,H}$	14 X_S	15 X_I
AOB growth															
Aerobic_AMO	1		-1.14	-1	1										
Aerobic_HAO*	2			$-i_{NXB}$	$-\frac{1}{Y_{AOB}}$			$\frac{1}{Y_{AOB}}$			1				
Aerobic_HAO	3		$-\left(\frac{2.29-Y_{AOB}}{Y_{AOB}}\right)$	$-i_{NXB}$	$-\frac{1}{Y_{AOB}}$	$\frac{1}{Y_{AOB}}$					1				
Anox_A_NIR	4				-1	-3		4							
Anox_A_NOR	5				-1			-2	3						
NOB growth															
Aer_NOB_growth	6		$-\left(\frac{1.14-Y_{NOB}}{Y_{NOB}}\right)$	$-i_{NXB}$		$-\frac{1}{Y_{NOB}}$	$\frac{1}{Y_{NOB}}$					1			
HB growth															
Aerobic_H_growth	7	$-\frac{1}{Y_{HB}}$	$-\left(\frac{1-Y_{HB}}{Y_{HB}}\right)$	$-i_{NXB}$									1		
Anox_H_NAR	8	$-\frac{1}{Y_{HB}}$		$-i_{NXB}$		$\left(\frac{1-Y_{HB}}{1.14\cdot Y_{HB}}\right)$	$-\left(\frac{1-Y_{HB}}{1.14\cdot Y_{HB}}\right)$						1		
Anox_H_NIR	9	$-\frac{1}{Y_{HB}}$		$-i_{NXB}$		$-\left(\frac{1-Y_{HB}}{0.57\cdot Y_{HB}}\right)$		$\left(\frac{1-Y_{HB}}{0.57\cdot Y_{HB}}\right)$					1		
Anox_H_NOR	10	$-\frac{1}{Y_{HB}}$		$-i_{NXB}$				$-\left(\frac{1-Y_{HB}}{0.57\cdot Y_{HB}}\right)$	$\left(\frac{1-Y_{HB}}{0.57\cdot Y_{HB}}\right)$				1		
Anox_H_NOS	11	$-\frac{1}{Y_{HB}}$		$-i_{NXB}$					$-\left(\frac{1-Y_{HB}}{0.57\cdot Y_{HB}}\right)$	$\left(\frac{1-Y_{HB}}{0.57\cdot Y_{HB}}\right)$			1		
Lysis															
AOB	12			i_{NXB}-f_I.i_{NXI}-(1-f_I).i_{NXS}							-1			1-f_{XI}	f_{XI}
NOB	13			i_{NXB}-f_I.i_{NXI}-(1-f_I).i_{NXS}								-1		1-f_{XI}	f_{XI}
HB	14			i_{NXB}-f_I.i_{NXI}-(1-f_I).i_{NXS}									-1	1-f_{XI}	f_{XI}
Hydrolysis															
Aerobic	15	1		i_{NXS}										-1	
Anoxic	16	1		i_{NXS}										-1	
Anaerobic	17	1		i_{NXS}										-1	

Process ▼		Process Rate ($g \cdot m^{-3} \cdot min^{-1}$)
AOB growth		
Aerobic_AMO	1	$\mu_{AMO}^{AOB} \cdot \frac{S_{O2}}{S_{O2} + K_{O2\_AMO}^{AOB}} \cdot \frac{S_{NH3}}{S_{NH3} + K_{NH3}^{AOB} + S_{NH3}^2 / K_{i\_NH3}^{AOB}} \cdot \frac{K_{i\_HNO2}^{AOB}}{S_{HNO2} + K_{i\_HNO2}^{AOB}} \cdot X_{AOB}$
Aerobic_HAO*	2	$\mu_{HAO}^{AOB} \cdot \varepsilon_{AOB} \cdot \frac{S_{NH2OH}}{S_{NH2OH} + K_{NH2OH}^{AOB}} \cdot X_{AOB}$
Aerobic_HAO	3	$\mu_{HAO}^{AOB} \cdot (1 - \varepsilon_{AOB}) \cdot \frac{S_{O2}}{S_{O2} + K_{O2\_HAO}^{AOB}} \cdot \frac{S_{NH2OH}}{S_{NH2OH} + K_{NH2OH}^{AOB}} \cdot X_{AOB}$
Anox_A_NIR	4	$\mu_{HAO}^{AOB} \cdot \eta_{NIR} \cdot \frac{K_{i\_O2}^{AOB}}{S_{O2} + K_{i\_O2}^{AOB}} \cdot \frac{S_{NH2OH}}{S_{NH2OH} + K_{NH2OH\_ND}^{AOB}} \cdot \frac{S_{HNO2}}{S_{HNO2} + K_{HNO2}^{AOB}} \cdot X_{AOB}$
Anox_A_NOR	5	$\mu_{HAO}^{AOB} \cdot \eta_{NOR} \cdot \frac{S_{NH2OH}}{S_{NH2OH} + K_{NH2OH\_ND}^{AOB}} \cdot \frac{S_{NO}}{S_{NO} + K_{NO\_ND}^{AOB}} \cdot X_{AOB}$
NOB growth		
Aer_NOB_growth	6	$\mu_{NOB} \cdot \frac{S_{O2}}{S_{O2} + K_{O2}^{NOB}} \cdot \frac{S_{HNO2}}{S_{HNO2} + K_{HNO2}^{NOB} + S_{HNO2}^2 / K_{i\_HNO2}^{NOB}} \cdot \frac{K_{i\_NH3}^{NOB}}{S_{NH3} + K_{i\_NH3}^{NOB}} \cdot X_{NOB}$
HB growth		
Aerobic_HB_growth	7	$\mu_{HB} \cdot \frac{S_{O2}}{S_{O2} + K_{O2}^{HB}} \cdot \frac{S_{NH4}}{S_{NH4} + K_{NH4}^{HB}} \cdot \frac{S_S}{S_S + K_S^{HB}} \cdot X_{HB}$
Anox_HB_NAR	8	$\mu_{NAR}^{HB} \cdot \eta_{HD} \cdot \frac{K_{i\_O2\_NAR}^{HB}}{S_{O2} + K_{i\_O2\_NAR}^{HB}} \cdot \frac{S_S}{S_S + K_{S\_NAR}^{HB}} \cdot \frac{S_{NH4}}{S_{NH4} + K_{NH4}^{HB}} \cdot \frac{S_{NO3}}{S_{NO3} + K_{NO3}^{HB}} \cdot X_{HB}$
Anox_HB_NIR	9	$\mu_{NIR}^{HB} \cdot \eta_{HD} \cdot \frac{K_{i\_O2\_NIR}^{HB}}{S_{O2} + K_{i\_O2\_NIR}^{HB}} \cdot \frac{K_{i\_NO2\_NIR}^{HB}}{S_{NO} + K_{i\_NO2\_NIR}^{HB}} \cdot \frac{S_S}{S_S + K_{S\_NIR}^{HB}} \cdot \frac{S_{NH4}}{S_{NH4} + K_{NH4}^{HB}} \cdot \frac{S_{NO2}}{S_{NO2} + K_{NO2}^{HB}} \cdot X_{HB}$
Anox_HB_NOR	10	$\mu_{NOR}^{HB} \cdot \eta_{HD} \cdot \frac{K_{i\_O2\_NOR}^{HB}}{S_{O2} + K_{i\_O2\_NOR}^{HB}} \cdot \frac{K_{i\_NO2\_NOR}^{HB}}{S_{NO} + K_{i\_NO2\_NOR}^{HB}} \cdot \frac{S_S}{S_S + K_{S\_NOR}^{HB}} \cdot \frac{S_{NH4}}{S_{NH4} + K_{NH4}^{HB}} \cdot \frac{S_{NO}}{S_{NO} + K_{NO}^{HB}} \cdot X_{HB}$
Anox_HB_NOS	11	$\mu_{NOS}^{HB} \cdot f(pH) \cdot \eta_{HD} \cdot \frac{K_{i\_O2\_NOS}^{HB}}{S_{O2} + K_{i\_O2\_NOS}^{HB}} \cdot \frac{K_{i\_NO2\_NOS}^{HB}}{S_{NO} + K_{i\_NO2\_NOS}^{HB}} \cdot \frac{S_S}{S_S + K_{S\_NOS}^{HB}} \cdot \frac{S_{NH4}}{S_{NH4} + K_{NH4}^{HB}} \cdot \frac{S_{N2O}}{S_{N2O} + K_{N2O}^{HB}} \cdot X_{HB}$
Lysis		
AOB	12	$b_{AOB} \cdot \left(\frac{S_{O2}}{S_{O2} + K_{O2\_b}} + \eta_{b,anox} \cdot \frac{K_{O2\_b}}{K_{O2\_b} + S_{O2}} \cdot \frac{S_{NOx}}{K_{NO3}^{HB} + S_{NOx}} + \eta_{b,anaer} \cdot \frac{K_{O2\_b}}{K_{O2\_b} + S_{O2}} \cdot \frac{K_{NO3}^{HB}}{K_{NO3}^{HB} + S_{NOx}} \right) \cdot X_{AOB}$
NOB	13	$b_{NOB} \cdot (...) \cdot X_{NOB}$
HB	14	$b_{HB} \cdot (...) \cdot X_{HB}$
Hydrolysis		
Aerobic	15	$k_H \cdot \frac{X_S / X_{BH}}{K_X + X_S / X_{BH}} \cdot \frac{S_{O2}}{K_{O2}^{HB} + S_{O2}} \cdot X_{HB}$
Anoxic	16	$k_H \cdot \eta_{ANOX} \cdot \frac{X_S / X_{BH}}{K_X + X_S / X_{BH}} \cdot \frac{K_{O2}^{HB}}{K_{O2}^{HB} + S_{O2}} \cdot \frac{S_{NOx-}}{K_{NO3}^{HB} + S_{NOx-}} \cdot X_{HB}$
Anaerobic	17	$k_H \cdot \eta_{AN} \cdot \frac{X_S / X_{BH}}{K_X + X_S / X_{BH}} \cdot \frac{K_{O2}^{HB}}{K_{O2}^{HB} + S_{O2}} \cdot \frac{K_{NO3}^{HB}}{K_{NO3}^{HB} + S_{NOx-}} \cdot X_{HB}$

4 Experimental design and parameter estimation

4.1 Monitoring nitrous oxide production for model calibration

N_2O is highly soluble in water, over 20 times more than O_2 at 20 C, leading to potentially high N_2O bulk concentrations. Yet, at ambient atmospheric N_2O gas concentration (330 ppb, (Stocker *et al.*, 2013)) the equilibrium aqueous concentration is 0.27 μgN/L. The biological production is in equilibrium with physico-chemical processes such as abiotic reactions and physical stripping due to liquid-gas partitioning.

In wastewater treatment applications N_2O can be monitored in both liquid and gas phase. Gas phase measurements are preferred over liquid as N_2O emissions can be directly calculated. However, under low stripping conditions (e.g. mechanical mixing and no aeration) no information is obtained. Liquid phase N_2O measurements are correlated with N_2O emissions via a volumetric mass transfer coefficient (k_La_{N2O} [d^{-1}]) that can be experimentally determined (Domingo-Félez *et al.*, 2014). Hence, liquid N_2O measurements provide qualitatively richer information on the net production dynamics compared to gas phase measurements.

Reactor configurations

Datasets for N_2O model calibration need to capture the range of operating conditions in which the model will be used. This information can be either directly obtained from the daily reactor performance (Ding *et al.*, 2016) or by conducting targeted experiments (Yang *et al.*, 2009).

Long-term measuring campaigns from full-scale systems provide valuable information on diurnal and seasonal variations (Daelman *et al.*, 2013; Wang *et al.*, 2016b; Spérandio *et al.*, 2016). The hydrodynamic model is, however, as important as the biological model, which increases the model complexity (Ye *et al.*, 2014). The reactor configuration (i.e. SBR, CSTR) and operating conditions (i.e. feeding and aeration strategies) will also impact the information content of the dataset. In a SBR cycle the system undergoes a wide range of concentrations provide compared to a CSTR, where the information content depends on the influent characteristics (Pocquet *et al.*, 2016; Ni *et al.*, 2013b).

Lab-scale systems allow for more controlled environments and more degrees of freedom in the experimental design. However, limitations exist on the representability of lab-scale data on full-scale data (Sin *et al.*, 2005). For example, transient phases or mass transfer limitations can hamper the transferability of information from the lab-scale to the full-scale.

Datasets

N_2O models are extensions of existing model structures describing nitrogen transformations. Consequently, the calibration of N_2O models requires datasets of the primary substrates (i.e. DO, NH_4^+, NO_2^- , etc.) and additional N_2O measurements (liquid and mass transfer coefficients, or gas phase). The number, the amount and the quality the dataset will pose a direct impact on the calibration results (Brockmann *et al.*, 2008; Dochain and Vanrolleghem, 2001). Quantification of N_2O production intermediates such as NO is not common despite its potential role in model discrimination studies because of its low bulk accumulation (Kampschreur *et al.*, 2008b; Yu *et al.*, 2010; Wang *et al.*, 2016b; Pocquet *et al.*, 2016). Similarly, NH_2OH is rarely quantified and the liquid accumulation is reported low (< 0.1mgN/L) (Yu and Chandran, 2010; Soler-Jofra *et al.*, 2016).

Respirometry

Respirometry is an experimental protocol for estimating metabolic rates by measuring consumption of oxygen (or potentially other terminal electron acceptor). The acquisition of DO data relies on high frequency and high sensitive liquid oxygen measurements, allowing automated and continuous measurements (**Figure 4.1**). The burden of chemical-specific analyses (e.g. NH_4^+, NO_2^-) associated to substrate depletion tests is alleviated (Chandran *et al.*, 2008). Respirometric tests are best for the determination of extant kinetic parameters, which are representative of the existing condition of the biomass (Ellis *et al.*, 1996), and have been applied to characterize aerobic degradation processes in activated sludge (Vanrolleghem *et al.*, 1999). Aerobic carbon degradation (Gernaey et al., 2002) and nitrification processes have been interpreted and optimized via respirometry (Chandran and Smets, 2005). The N_2O and NO response of several pure cultures of AOB during NH_3 and NH_2OH oxidation has also been determined via microrespirometric assays (Kozlowski *et al.*, 2016).

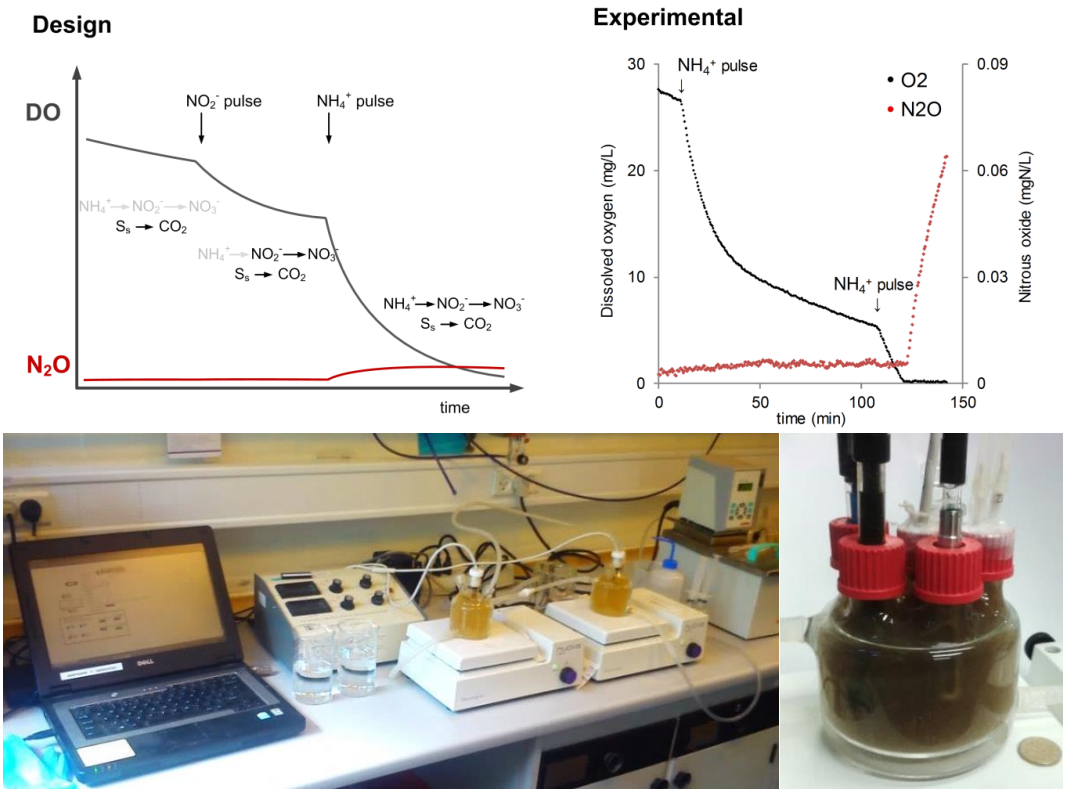

Figure 4.1. Top: Schematic of a respirometric assay to estimate nitrification kinetics: left, design; right, experimental DO and liquid N_2O concentrations for two consecutive NH_4^+ pulses. Bottom: Experimental setup used for respirometric assays. (**Paper III, IV**).

Experimental design

In the initial experimental design of this study the regulation of N_2O production – effect of DO, NO_x^-, etc. – allows evaluating the performance of existing N_2O model structures (**Figure 4.1**). Mostly, parameters associated to N_2O production are estimated and the capabilities of model structure are assessed based on best-fit simulations (**Paper I**) (Ni *et al.*, 2013c; Ding *et al.*, 2016). However, the experimental design indicates that N_2O emissions are also sensitive to parameters indirectly related to N_2O production (e.g. μ_{NOB}, k_H). Hence, the following experimental design aims at obtaining accurate parameter estimates that will reduce the uncertainty of N_2O emissions.

Specific respirometric assays are designed to estimate parameters from the NDHA model structure. Parameter estimates should reflect *in situ* microbial activity (extant) and minimize Monod parameter correlation. Designs consider a low initial substrate-to-initial biomass concentration (S_0/X_0) but sufficiently high initial substrate-to-substrate affinity (S_0/K_S) (Huang *et al.*, 2014).

During respirometric assays the electron donor consumption (e.g. NH_4^+) is measured indirectly by tracking electron acceptor depletion (DO). Simultaneously, the set up can monitor online other variables of interest (e.g. N_2O, NO, pH). Datasets for the NDHA model calibration are obtained from respirometric assays and, for those under anoxic conditions, substrate depletion experiments (**Table 4.1**).

Table 4.1. Experimental design for respirometric assays (shaded corresponds to anoxic experiments)

Spikes	Targeted processes	N_2O pathways
NH_4^+	NH_4^+ removal by AOB	NN, ND
NH_2OH	NH_2OH removal by AOB	NN, ND
NO_2^-	NO_2^- removal by NOB	HD
NH_4^+, NH_2OH, NO_2^-	AOB-driven N_2O production	NN, ND
N_2O, NO_2^-	HB-driven N_2O production	HD

A lab-scale respirometer (400-mL) was designed to continuously monitor DO consumption rates. The vessel geometry allows the continuous monitoring of DO, pH, NO and N_2O, and the collection of grab samples (**Figure 4.1**). Parallel assays are performed at 25°C in jacketed glass vessels completely filled with biomass and sealed with the insertion of the following sensors: Clark-type polarographic DO electrode, liquid NO, liquid N_2O and pH. In the respirometric assays two types of biomass representative of wastewater treatment systems are studied in Respirom_PN, Respirom_ML:

Mixed liquor - Mixed liquor derived from a full-scale phase-isolated activated sludge wastewater treatment plant (700,000 PE Lynetten, Copenhagen, Denmark). Quantitative polymerase chain reaction (qPCR) was used to enumerate the quantities of AOB, *Nitrobacter spp.* and *Nitrospira spp.*, targeting the 16S gene (*Nitrospira spp.* 92 ± 3% relative abundance in comparison to 8 ± 3% of *Nitrobacter spp*., and AOB:NOB = 3:1). Details on the qPCR protocol can be found in (Terada *et al.*, 2010).

Nitritating enrichment - A lab-scale nitrifying sequencing batch reactor (5 L) enrichment from an AS mixed liquor sample with NH_4^+ as the only nutrient was maintained at oxygen-limited conditions. NH_4^+ removal was 82 ± 14%, and nitritation efficiency ($NO_2^-/NH_4^+{}_{removed}$) at 85 ± 24%. The biomass composition, based on 16 rRNA targeted qPCR analysis had a dominance of AOB over NOB (30:1). Among NOB species, and differently from the mixed liquor biomass, *Nitrobacter spp.* dominates over *Nitrospira spp*. (≈ 700:1).

Differences in relative abundance of NOB species are in accordance with their substrate affinity, where *Nitrobacter spp.* dominate over *Nitrospira spp.* in high NO_2^- environments (NO_2^- > 100 and < 1 mgN/L in nitritating enrichment and mixed liquor respectively) (Nowka *et al.*, 2014).

	Respirom_PN	Respirom_ML	Respirom_ML_aer		Batch_HD*
Aeration mode	Preaerated → anoxia	Preaerated → anoxia	Continuous	Continuous	-
Substrate addition	Pulse	Pulse	Pulse	Pulse (excess)	Pulse (excess)
Biomass	AOB-enriched	Mixed Liquor	Mixed Liquor	Mixed Liquor	Mixed denitrifying
Reactor volume	400 mL	400 mL	400 mL	3 L	330 mL
Used in	Paper III	Paper IV		Paper I	Paper V

* from Ribera-Guardia *et al.* (2014)

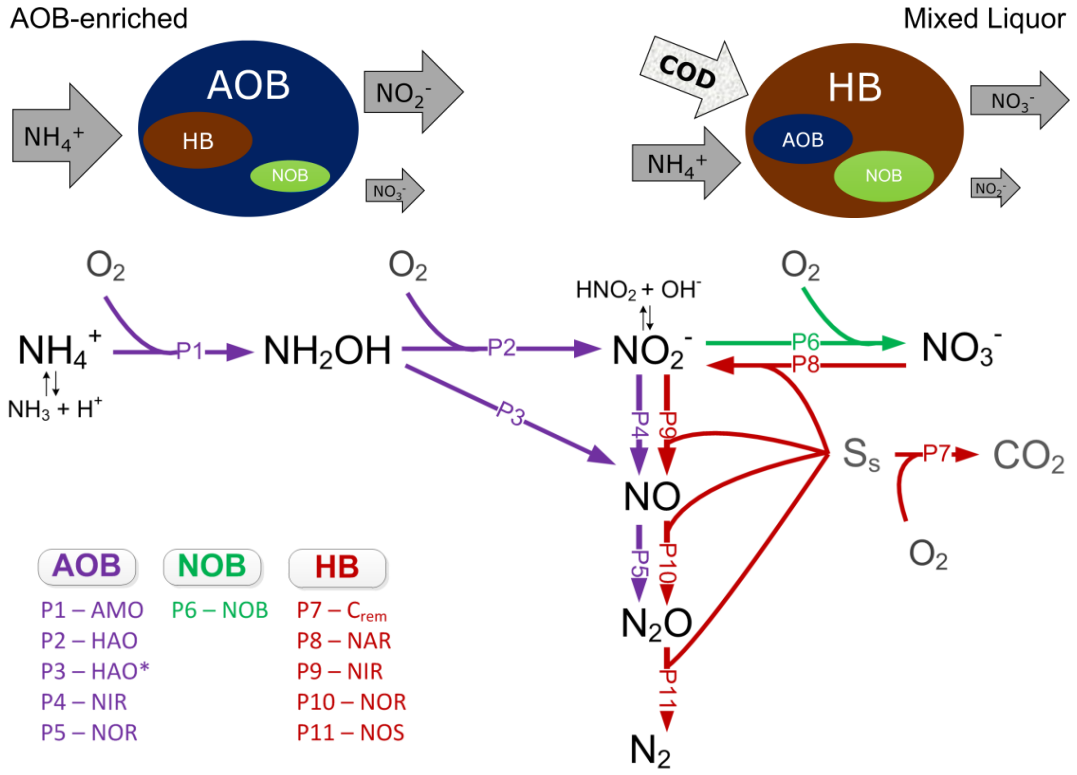

Figure 4.2. Top: Characteristics of the experimental designs used. Middle: Diagram of the microbial composition for the two different biomasses studied. Bottom: Main substrates and processes from a nitrogen removing community (**Paper I, III, IV, V**).

The kinetics of the oxidation of the primary N-substrates (NH_4^+ and NO_2^-) are individually and step-wise measured via extant respirometry at varying DO concentrations (Chandran and Smets, 2005). The purpose is to predict the fate of the primary N-substrates based on the specific oxygen-consuming rate. If a model captures accurately the relevant oxygen-consuming processes, then DO and the primary N-substrates are predicted accurately too. By sequentially adding substrate pulses from oxidized to reduced form (endogenous →

$NO_2^- \rightarrow NH_2OH \rightarrow NH_4^+$), based on the NDHA model structure the individual rates can be isolated (**Figure 4.2**, P1-P11). In all experiments, even prior to any substrate spikes, oxygen consumption is always positive and proportional to the biomass concentration due to endogenous respiration.

Based on the overall good fit of model predictions and experimental data the NDHA model describes the dynamics of the measured DO and N-species for the AOB-enriched and mixed liquor biomass ($R^2 > 0.99$ and 0.94 respectively) (**Figure 4.3**). Best-fit parameter estimates are estimated at high accuracy: coefficients of variation are below 7% for the AOB-enriched and below 25% for the mixed liquor and the collinearity indices below 15, as suggested for identifiable subsets (Brun *et al.*, 2002) (**Table 4.2**). The high correlation observed between $\mu_{AOB.AMO}$-$K_{AOB.NH3}$ and μ_{NOB}-$K_{NOB.HNO2}$ ($\rho > 0.80$) typically occurs for Monod-type kinetics but it does not affect their identifiability.

In sum, the respirometric experimental design can be used to precisely identify and calibrate the primary substrate dynamics of the NDHA model based on the DO profiles.

Table 4.2. Estimated NDHA model parameters from DO datasets (estimated at 20 C) (**Paper III, IV**).

Respirom_PN		
Parameter	**Unit**	**Value**
$\mu_{AOB.AMO}$	d^{-1}	0.49 ± 0.01
μ_{NOB}	d^{-1}	0.67 ± 0.07
k_H	d^{-1}	2.01 ± 0.02
$K_{AOB.NH3}$	mgN/L	0.12 ± 0.005
$K_{AOB.O2.AMO}$	mgO2/L	0.23 ± 0.02

Respirom_ML		
Parameter	**Unit**	**Value**
$\mu_{AOB.AMO}$	d^{-1}	0.49 ± 0.01
μ_{NOB}	d^{-1}	1.04 ± 0.05
μ_{HB}	d^{-1}	5.15 ± 0.11
$K_{AOB.NH3}$	µgN/L	7.00 ± 1.17
$K_{NOB.HNO2}$	µgN/L	0.027 ± 0.006

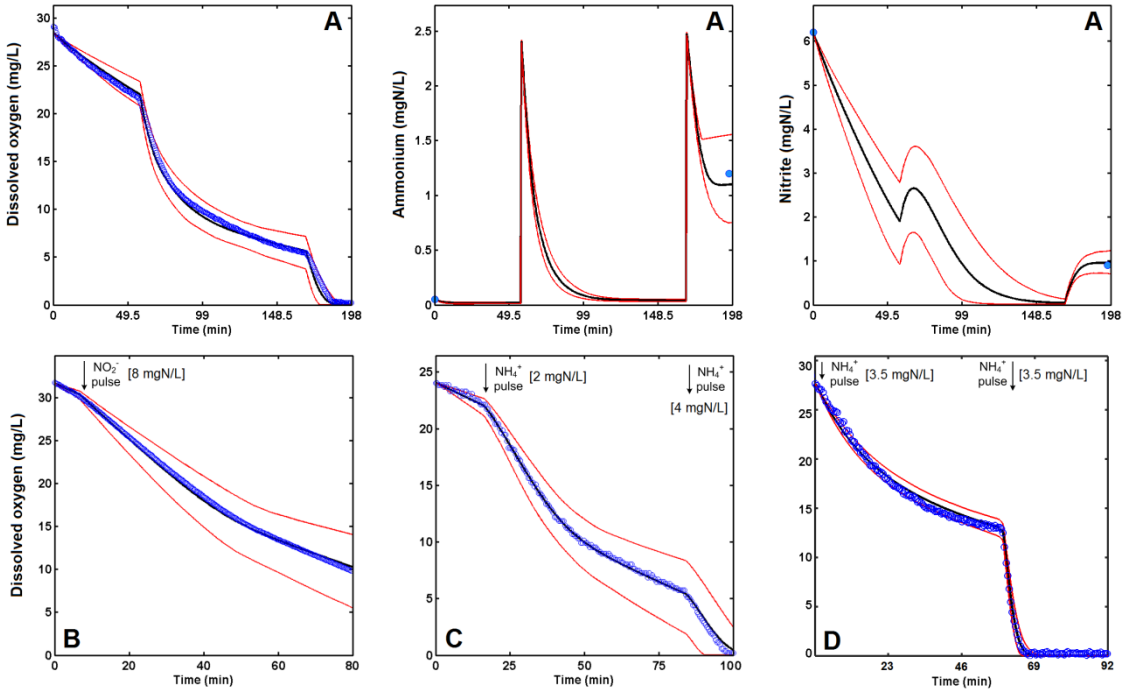

Figure 4.3. Experimental DO, NH_4^+ and NO_2^- (blue markers) and model predictions (black line best-fit, red lines 95% CI) for the DO calibration from respirometric assays. (A) DO, NH_4^+ and NO_2^- concentrations. (B), (C), (D), DO concentrations after pulse additions (D: pH changed from 7 to 8 before the second NH_4^+ pulse). Respirom_PN (A, D), Respirom_ML (B, C). (**Paper III, IV**).

Abiotic N_2O production

To study the effect of HNO_2, NH_2OH and pH on abiotic N_2O production a factorial experimental design is constructed (**Table 4.3**). Results show that in the absence of NO_2^-, NH_2OH-driven abiotic N_2O production only occurs at very high pH (≥ 8.7) (**Figure 4.4**). Coupling HNO_2 and NH_2OH produces N_2O at high pH (≥ 8) and high NH_2OH (≥ 0.5 mgN/L). Therefore high NO_2^- and NH_2OH concentrations are necessary, outside the range of typical wastewater systems (pH > 8.4, NO_2^- > 500 mgN/L, NH_2OH ≥ 0.5 mgN/L).

Table 4.3. Factorial experimental design to study abiotic N_2O production. (Unpublished data).

HNO_2 (µgN/L)	0	0.2	2	20	100
NH_2OH (mgN/L)	0	0.05	0.2	0.5	2
pH	6.5	7.25	8	8.7	9.4

Overall, the substrate concentrations necessary to produce N_2O abiotically are outside the range of the experiments design to calibrate the NDHA model: high pH, NO_2^- and NH_2OH.

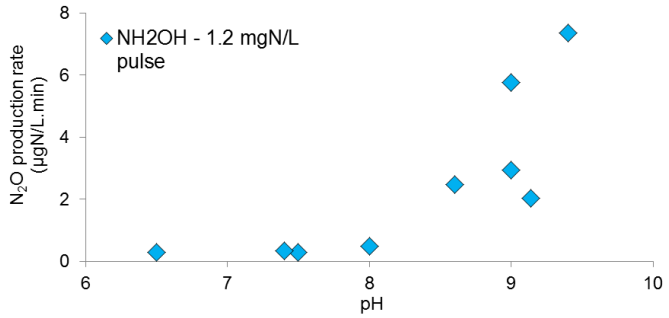

Figure 4.4. Abiotic N_2O production rates for NH_2OH pulses (1.2 mgN/L) at varying pH. (Unpublished data).

4.2 Parameter estimation and model evaluation

The objective of the experimental studies is to obtain informative N_2O datasets that allows the estimation of parameters associated to N_2O production with the NDHA model. The ability of the results obtained from lab-scale experimentation to predict full-scale processes remains to be validated.

Systematic calibration protocols for activated sludge models are applied to wastewater treatment operations. Numerous experimental methodologies and calibration approaches exist with varying degrees of automatization and requirements (e.g. influent fractionation, parameter subset selection, parameter estimation procedure, etc.) (Corominas et al., 2011; Mannina et al., 2011; Sin et al., 2008). Deterministic methods have been commonly used, but with increasing computational power Bayesian methodologies are being proposed to activated sludge models (Sharifi *et al.*, 2014; Martin and Ayesa, 2010).

However, N_2O modelling studies still lack fundamental process understanding and have not been integrated in calibration protocols yet. While some N_2O models have reported a calibration framework (Guo and Vanrolleghem, 2014), in most N_2O models the parameter estimation procedures are often ill-described, with little information about each step. For example, the parameter subset selection procedure is sometimes not addressed.

N_2O modelling efforts currently focus on evaluating the capabilities of model structures to describe N_2O production with best-fit simulations (Ni *et al.*, 2013c; Spérandio *et al.*, 2016) (**Paper I**). However, the quality of the N_2O calibration results has not been analysed further in-depth as occurs for other environmental models (Bennett *et al.*, 2013). Hence, more rigorous tools for model response evaluation will become more important to discriminate between N_2O models, especially for models with similar best-fit predictions (Lang *et al.*, 2017).

The focus of this study is on the parameter estimation procedure and validation of the model response and the estimated parameter values (**Figure 4.5**). The methods presented represent a rigorous tool that will benefit N_2O model discrimination procedures.

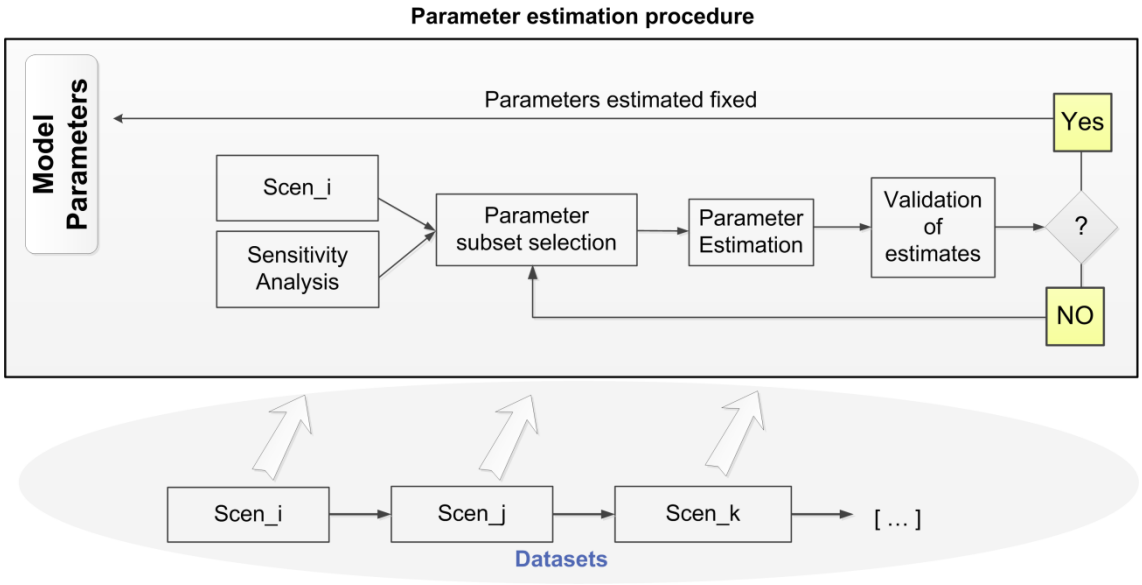

Figure 4.5. Parameter estimation procedure. (**Paper III, IV**).

Parameter subset selection

The objective of this step is to select the parameters to be estimated from a given scenario. Sensitivity analysis techniques identify those parameters where a change in value leads to a large variation in model output. High sensitivity is a necessary, but not sufficient, condition for a parameter to be identified (Dochain and Vanrolleghem, 2001). Local sensitivity analysis (LSA) analyses the model response to individual parameter changes and have been applied to N_2O model calibrations (Pocquet *et al.*, 2016; Spérandio *et al.*, 2016). The drawback of LSA rankings is that results depend on the parameter values and do not capture parameter interactions, for which global sensitivity analysis is required (GSA) (Brun *et al.*, 2001; Sweetapple *et al.*, 2013). For GHG emissions, GSA methods are preferred over LSA despite the higher computational costs (Sweetapple *et al.*, 2013; Boiocchi *et al.*, 2017; Mannina and Cosenza, 2015). GSA is beneficial to identify sensitive parameters, but more importantly, to identify what parameters cannot be estimated to fix their values. Hence, the Standardized Regression Coefficient (SRC) method is used to identify non-sensitive parameters and fix them to their default value (**Figure 4.6**). Dynamic and averaged results are combined as a screening method to manually select the top sensitive parameters that are considered for estimation (Machado *et al.*, 2009). Among these parameters subsets of different size and combination of parameters are considered. Metrics such as RDE (Machado *et al.*, 2009) and modE are used to quantify the information con-

tent of a dataset and elucidate what parameter subset should be estimated (**Paper III**).

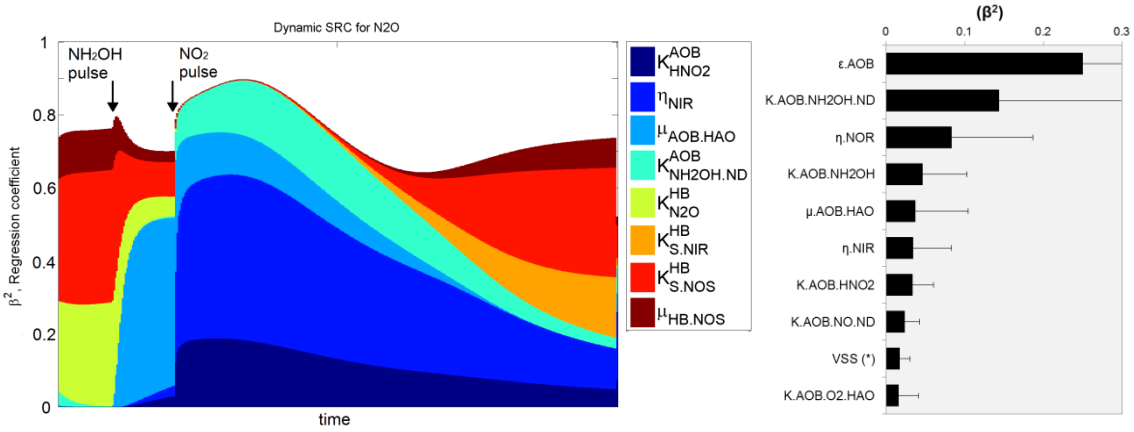

Figure 4.6. Global sensitivity analysis for N_2O, an example from Respirom_PN (**Figure 4.1**). Left: Dynamic N_2O sensitivity (β^2) of an experiment targeting ND-associated parameters where NH_2OH and NO_2^- were spiked. The sensitivity of $\mu_{AOB.HAO}$ increases after the NH_2OH pulse and the sensitivity η_{NIR} and $K_{AOB.HNO2}$ increased after the NO_2^- pulse. Right: Averaged sensitivity during an NH_4^+ oxidation experiment targeting NN-associated parameters. The three parameters to which liquid N_2O concentrations are most sensitive to: ε_{AOB}, $K_{AOB.NH2OH}$ and η_{NOR}. (**Paper III**).

Parameter estimation

The objective function for the minimization problem is defined as:

$$\mathrm{RMNSE} = \sum_{k}^{m}\sum_{j}^{n}\frac{\mathrm{RMSE_j}}{\bar{y}_{obs,j}}; \qquad \mathrm{RMSE_j} = \sqrt{\frac{\sum_i^p\left(y_{sim,i} - y_{obs,i}\right)^2}{p}}$$

Where *m* is the number of experiments in one scenario (e.g. 2 NH_4^+ experiments in Scenario (C)), *n* the number of data series in one experiment (e.g. NO, N_2O), *p* experimental points of each data series, $y_{sim,i}$ the model prediction and $y_{obs,i}$ the experimental data at time *i*. As the dimensions of the minimization problem increase (i.e. number of parameters) the convergence of the algorithm to a minimum becomes more computationally demanding. Additionally, single search algorithms might not find the global minimum among multiple minima (Nelder and Mead, 1965). Thus, to avoid finding a local minimum, global and multiple largely-bounded local optimization algorithms are used (Wágner *et al.*, 2016).

Validation of model response

Previous N_2O models describe the overall fit and capabilities based on the visual inspection or regression of model simulations and experimental data (Lang *et al.*, 2017; Pan *et al.*, 2015; Ding *et al.*, 2016). For example, the performance of two models cannot be compared via visual inspection (Ni *et al.*, 2014) or regression coefficients (R^2), which do not identify structural deficiencies unless combined with quantitative metrics such as RMSE (Haefner, 2005). A more rigorous analysis of residuals (e.g. Gaussian distributions, autocorrelation functions (ACF), F-test, etc.) is required to validate the model response (Bennett *et al.*, 2013). In this study the F-test is used as it can identify a deficient model fit despite a visually good fit and high degree of correlation ($R^2 > 0.99$).

Validation of parameter values

By addressing the practical identifiability of newly estimated parameters model calibrations and experimental designs can be compared to discriminate between N_2O models. What is the confidence in the reported best-fit parameter values? Approximate confidence regions can be calculated with different methods. Based on the error function and the size of the parameter subset and dataset (Beale, 1960), or considering the Fisher Information Matrix (FIM) as a lower bound for the variance matrix (Dochain and Vanrolleghem, 2001). The FIM summarises the information concerning the model parameters gained from an experiment:

$$\mathrm{FIM} = \sum_{i=1}^{N} Y_p\left(t_i, \underline{\theta}\right)^T \underline{Q_i} Y_p\left(t_i, \underline{\theta}\right)$$

where Y_p is the output sensitivity function with respect to the parameters θ and $\underline{Q_i}$ the weighting matrix, typically selected as the inverse of the error covariance matrix. This method is widely used, but assumes a linear approximation of the state variables with respect to the parameters, which might not apply to non-linear systems. The bootstrap method analyses the system properties by using repeated simulations, like a Monte-Carlo method, and has been successfully applied in those cases (Joshi *et al.*, 2006). Hence, if the confidence intervals of the parameter estimates are determined more accurately the 95% confidence intervals of the state variables will be calculated more precisely.

In N_2O model evaluation studies the parameter variance and correlation matrix, indicators of the confidence that can be given to a value, are not typically reported, which complicates the comparison between studies (Spérandio *et al.*, 2016; Ding *et al.*, 2016; Pocquet *et al.*, 2016; Kim *et al.*, 2017) (**Paper I**). Sometimes overlooked, methods used to calculate confidence intervals for parameter estimates often rely on structural assumption of the residuals. Here, to improve these limitations the gaussian distribution (Kolmogorov-Smirnov test 95%) and the interdependency of residuals at different lag times are analysed and minimized when possible (Lilliefors, 1967; Cierkens *et al.*, 2012) (**Figure 4.7**). The autocorrelation of residuals is minimized by reducing the data acquisition frequency, which increased the confidence interval of the estimated parameters (**Figure 4.7**). Testing the model response can avoid over interpretation of the dataset and uncertainty underestimation (variance/mean ≪ 0.001% (Peng *et al.*, 2015)).

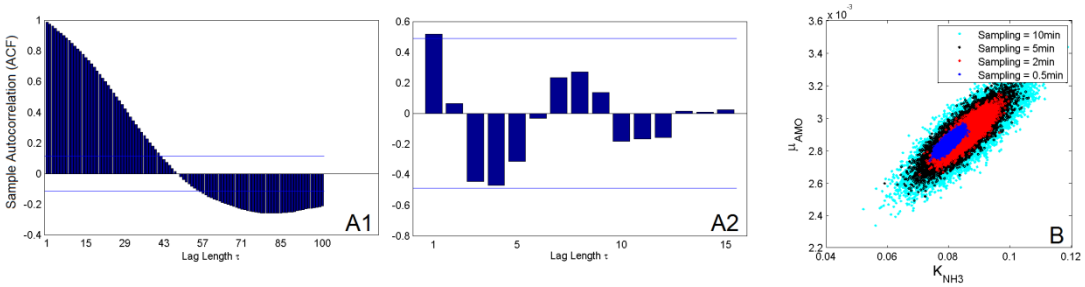

Figure 4.7. Autocorrelation of DO residuals for increasing time lags (τ) from an experiment used to estimate parameters associated with NH_4^+ oxidation ($K_{AOB.NH3}$, $\mu_{AOB.AMO}$). A1: Residuals from the original dataset. A2: residuals from the downsampled dataset. B: Pairwise samples from the estimated multivariate normal distribution for $K_{AOB.NH3}$ (mgN/L) and $\mu_{AOB.AMO}$ (min^{-1}) for sampling rates of 0.5 (blue), 2 (red), 5 (black) and 10 (cyan) minutes. (**Paper III**).

Uncertainty propagation

The uncertainty obtained during parameter estimation can be used to build confidence intervals in model predictions (Neumann and Gujer, 2008; Belia *et al.*, 2009).

The precision, or width of the confidence interval, associated to N_2O emissions will be a key factor to consider when comparing the performance of N_2O models during the development of mitigation strategies. Specifically, the carbon footprint of wastewater systems is very sensitive to N_2O emissions (Gustavsson and Tumlin, 2013) and precise predictions are desired. Yet, the

uncertainty of N_2O emissions associated to parameter estimation has never been studied.

Here, the uncertainty from the parameter estimation results is evaluated via Monte-Carlo simulations. The reliability of predictive distributions (95% confidence intervals) is used to validate the model response as suggested by (Jin *et al.*, 2010). Parameter values were sampled via Latin Hypercube Sampling (LHS, n = 500) for two cases: (1) from literature following (Sin *et al.*, 2009), and (2), compared to the distributions obtained after parameter estimation. As an example, in **Paper IV**, the uncertainty of N_2O and NO emissions during excess NH_4^+ oxidation at two different DO levels (0.5, 2.0 mg/L) is described. The calibrated NDHA model predicts for low and high DO, an N_2O emission factor of 4.6 ± 0.6 % and 1.2 ± 0.1% (case (2)), which corresponds to low coefficients of variation (9 and 12%). However, when the uncertainty is propagated based on the reference case (1) the confidence intervals are 360% larger. These results highlight the importance of evaluating the uncertainty of parameter estimates in N_2O emissions, but unfortunately cannot be compared to other N_2O modelling studies.

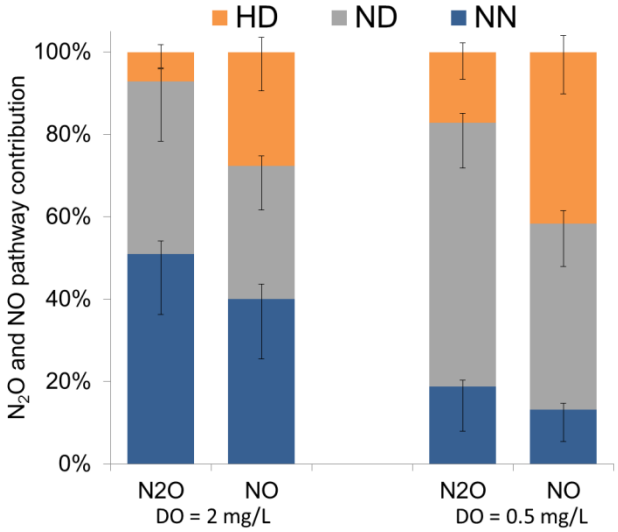

Figure 4.8. Nitrous oxide (N_2O) and nitric oxide (NO) pathway contribution during NH_4^+ oxidation at low and high DO for the calibrated NDHA model for mixed liquor biomass. The standard deviations correspond to uncertainty from estimated parameters: (top) from Respirom_ML, (bottom) default by classes following (Sin *et al.*, 2009) (n = 500). (**Paper IV**).

In this work, mathematical models and calculations were implemented in the Matlab-Simulink environment (The MathWorks, Natick, MA) (**Paper III**, **IV** and **V**) and in Aquasim (Reichert, 1998) (**Paper I**).

5 Model evaluation

5.1 Case 1: AOB-enriched biomass

A novel experimental design to calibrate N_2O models through extant respirometry is evaluated on an AOB-enriched biomass.

Nitrous oxide production: Experimental and modelling results

Aerobic NH_4^+-oxidation products, NH_2OH and NO_2^- are responsible for the higher N_2O production rate at the onset of anoxia and not NH_4^+ itself, which requires molecular O_2 for its oxidation (Sayavedra-Soto *et al.*, 1996). The higher N_2O yield of nitrifying biomass and pure cultures fed on NH_2OH compared to NH_4^+ observed has been already reported (de Bruijn *et al.*, 1995; Kim *et al.*, 2010; Kozlowski *et al.*, 2016). However, even under anoxic conditions the sole presence of NH_2OH also yields a large amount of N_2O, recently suggested as a new N_2O producing pathway by (cyt) P460 (Caranto *et al.*, 2016). The addition of an electron donor like NO_2^- further increases N_2O production, highlighting the role of the primary N-substrates on N_2O dynamics, especially of NH_2OH (**Figure 5.1**). Based on the model structure of other two-pathway models for AOB none can predict the observed N_2O dynamics: while in certain models NH_2OH does not react under anoxic conditions (Ding *et al.*, 2016; Pocquet *et al.*, 2016), in other NH_2OH reacts producing both N_2O and HNO_2 (Ni *et al.*, 2014).

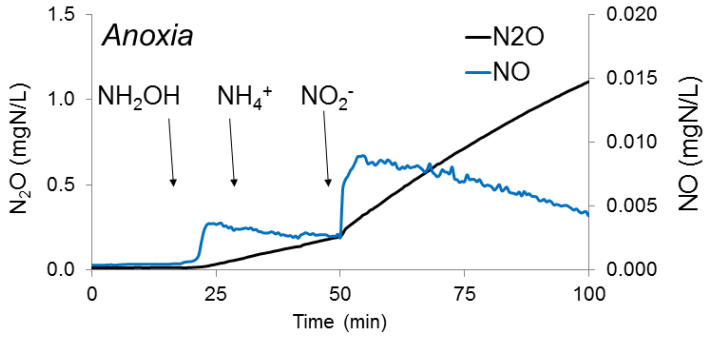

Figure 5.1. Experimental N_2O (black) and NO (blue) liquid concentrations after NH_2OH, NH_4^+ and NO_2^- pulses added under anoxia for the AOB-enriched biomass. (Unpublished data).

Parameter estimation to fit the N_2O datasets is performed after the NDHA model showed a good fit for DO and hence for the main N-substrates (NH_4^+,

NO_2^-, NO_3^-). The sequence in which the N_2O-associated parameters are estimated targeted each N_2O production pathway as follows: under anoxia and no electron donors for AOB (e.g. NH_2OH) the contributions of NN and ND are null and hence HD-associated parameters can be estimated independently, and the new estimated parameters fixed. During NH_4^+ oxidation experiments at high DO levels the ND and HD contributions are minimal, as both are inhibited by DO, and NN-associated parameters can be estimated and fixed. Finally, the ND contribution is estimated from NH_4^+ and NH_2OH oxidation experiments at low DO.

Specifically for the AOB contribution, N_2O production observed from NH_4^+ oxidation at high DO is used to calibrate the NN pathway. Then, experiments designed to reach anoxia at varying HNO_2 concentrations are used to estimate parameters associated to the ND pathway, as they are the most sensitive. After parameter estimation the NDHA model describes the N_2O production dynamics and yield observed in the calibration datasets (F-test = 1). After parameter estimation the 95% predictive distribution for liquid N_2O narrows by 58% from the reference uncertainty scenario (Sin *et al.*, 2009). The model is then validated on three batches with lower HNO_2 and with higher NH_2OH pulses. The average Janus coefficient is 1.57 and R^2 is 0.985, indicating a good validation (**Figure 5.2**, bottom). Hence, the NDHA model can describe the N_2O production rates at a range of DO and HNO_2 concentrations. For more details see **Paper III**.

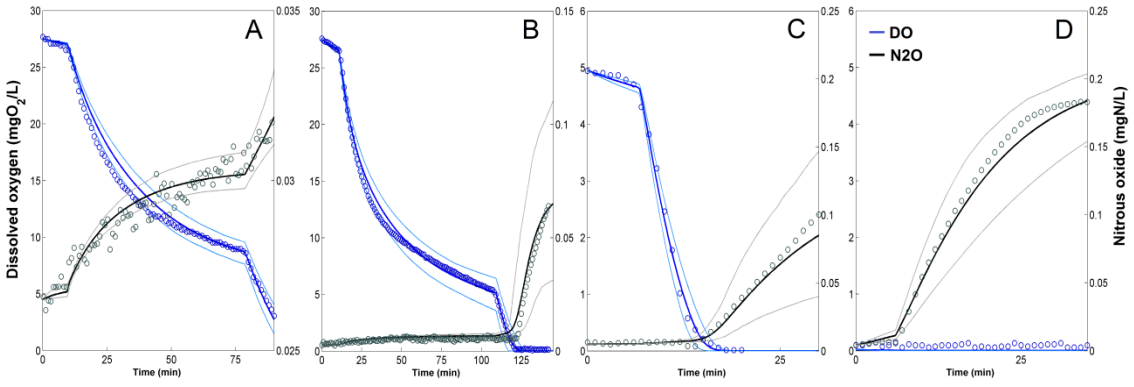

Figure 5.2. Experimental (markers) and model predictions (dark lines – best-fit, light lines - 95% CI) for the experiments from Respirom_PN. From left to right: (A) Aerobic NH_4^+ pulses, (B) Aerobic → anoxic NH_4^+ pulses, (C) Aerobic NH_4^+ pulse, (D) Anoxic NH_2OH pulse. (**Paper III**).

Model evaluation

Evaluations of the NDHA model at varying DO and HNO_2 concentrations at pH = 7.5 are performed to study the variability of N_2O emissions at a wider range of operating conditions (**Figure 5.3**). The model predicts the largest N_2O emission at the lowest DO and high HNO_2; and the lowest N_2O emission at the highest DO and lowest HNO_2. This relationship has been described by other two-pathway models, where ND was the main contributor to the N_2O emission factor during NH_4^+ oxidation (Pocquet *et al.*, 2016; Ni *et al.*, 2014). The contribution of the NN pathway is maximal when HNO_2 is not present and decreased with increasing HNO_2. On the other hand, the ND contribution follows opposite trends, indicating a shift between autotrophic pathways driven by HNO_2 and DO. The HD contribution is maximal at low DO and high HNO_2 but at low levels.

The uncertainty of the N_2O emission factor is, in average, only 25% of that predicted with the reference case. Taken together, the N_2O production observed in all the scenarios can only be potentially described by the NDHA model structure compared to other N_2O models (Ni *et al.*, 2014; Pocquet *et al.*, 2016; Ding *et al.*, 2016) (**Paper II**) (**Figure 3.6**). Additionally, the estimated parameters from respirometric assays decrease significantly the uncertainty of N_2O emissions.

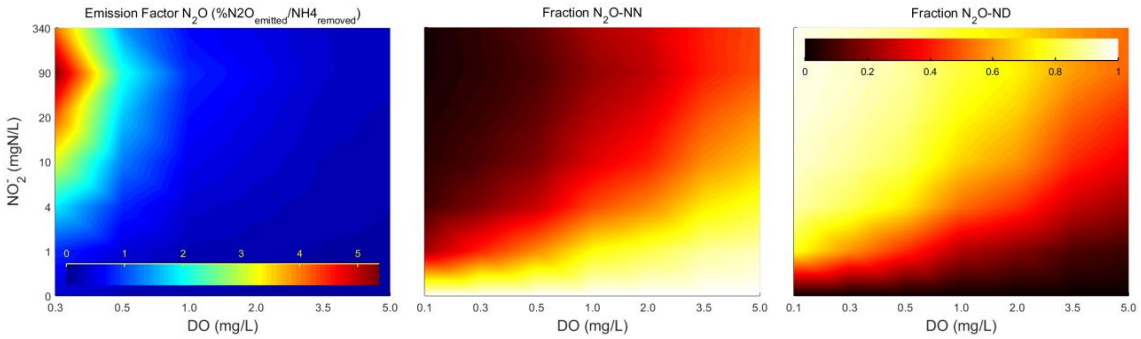

Figure 5.3. NDHA model simulations with best-fit parameters: N_2O emissions (% $N_2O_{emitted}/NH_4^+{}_{removed}$) and NN (middle) and ND (right) pathway contributions. The contribution of the HD pathway is not shown, maximum 0.02. NH_4^+ oxidation by AOB-enriched biomass at constant DO (0.1 - 0.3 - 0.5 - 1.0 - 2.0 - 3.5 - 5.0 mg/L), and NO_2^- (0 – 1 – 4 – 10 – 20 – 90 – 340 mgN/L). (**Paper III**).

5.2 Case 2: Mixed liquor biomass

A nitrification/denitrification case study is used to investigate, with default parameter values, the main processes driving N_2O production and sources of uncertainty during aerobic NH_4^+ removal. The majority of N_2O is emitted during the aerobic part of the cycle, when NH_4^+ oxidation occurs. The GSA ranking shows that up to four of the ten most sensitive parameters for N_2O and NO liquid concentrations correspond to AOB, and the rest to NOB and HB. These results highlight the importance of NOB and HB together with AOB on the N_2O production from a mixed culture biomass during NH_4^+ oxidation. The experimental design developed that targets sources of uncertainty for N_2O emission predictions should include NOB and HB processes.

Nitrous oxide production: Experimental and modelling results

Irrespectively of the N-substrate being oxidized, at the onset of anoxia NO and N_2O concentrations increase. First NO, and then N_2O, reach a maximum followed by a steady decrease, indicating net N_2O consumption.

In this study, the HD contribution is estimated first as no electron donors for AOB are present (addition of N_2O, NO_2^-, NO_3^- or soluble organic carbon). Hence, ten parameters associated to hydrolysis of particulates, heterotrophic denitrification and organic carbon removal are estimated. Of special interest, three parameters associated to N_2O consumption: two describing the pH dependence of the maximum reduction rate (w_{nosZ}, $pH_{opt.nosZ}$) and the substrate affinity for N_2O ($K_{HB.N2O}$) (**Figure 5.4**, **Table 5.1**). Similarly to **Paper III**, the contribution of the NN pathway is estimated next during NH_4^+ oxidation at high DO, followed by the ND.

Table 5.1. Selected NDHA model parameters estimated from N_2O and NO datasets. (**Paper III, IV**).

Respirom_PN

Parameter	Unit	Value
ε_{AOB}	(-)	0.48 ± 0.005 ($x10^{-3}$)
η_{NOR}	(-)	0.16 ± 0.005
$K_{AOB.NH2OH.ND}$	mgN/L	0.25 ± 0.005
$K_{AOB.HNO2}$	µgN/L	0.67 ± 0.03

Respirom_ML

Parameter	Unit	Value
ε_{AOB}	(-)	0.0031 ± 0.0001
η_{NOR}	(-)	0.36 ± 0.02
η_{NIR}	(-)	0.22 ± 0.01
$pH_{opt.nosZ}$	(-)	7.9 ± 0.1
w_{nosZ}	(-)	2.2 ± 0.2
$K_{HB.N2O}$	mgN/L	0.078 ± 0.020

Based on the overall good fit of model predictions and experimental data the NDHA model describes the dynamics of the measured DO and N-species ($R^2 \geq 0.94$). A total of 17 parameters are estimated with bounded approximate

confidence regions indicating good identifiability (CV < 25%). For more details see **Paper IV**.

The predictive ability of the calibrated NDHA model is evaluated on a set of batch experiments when mixed liquor biomass from the same WWTP had been subject to varying N pulses at constant aeration (For details see **Paper I**).

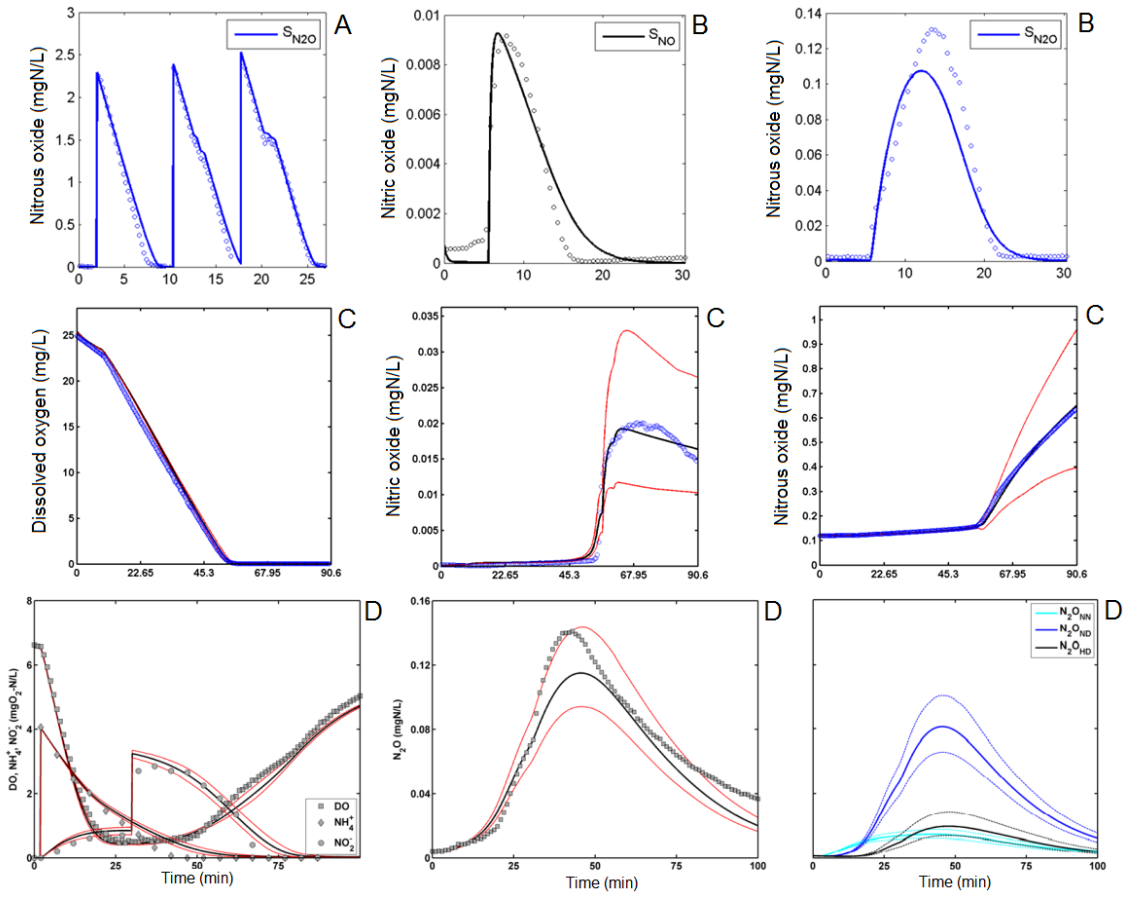

Figure 5.4. Experimental and modelling results obtained during parameter estimation. N_2O consumption profile after DO pulses (t = 13, 21 min) (A). NO and N_2O production after anoxic NO_2^- pulse under endogenous conditions (B). Oxygen consumption, NO and N_2O accumulation rates after NH_4^+ pulse addition (t = 10 min) (C). Model evaluation results for mixed liquor biomass: Effect of NO_2^- pulse (t_{pulse} = 30 min) (D). (**Paper IV**).

Overall, the model captures the trends of DO, main N-substrates and liquid N_2O without any parameter modification (R^2_{avg} for DO = 0.98; NH_4^+ = 0.99; NO_2^- = 0.84; N_2O = 0.80). Higher NH_4^+ pulses yield more N_2O as more NH_4^+ oxidation occurs at low DO, thus promoting the contribution of denitrifica-

tion pathways. Addition of a NO_2^- pulse increases the fraction of N_2O produced compared to a NO_3^- pulse or no pulse (**Figure 5.4, D**).

Model evaluation

NH_4^+ oxidation simulations with best-fit estimate parameters are run for a wider range of DO (0.2 – 4 mg/L) and NO_2^- (0 – 1.4 mgN/L), representative of full-scale system where the biomass originates. The N_2O emission factor and individual pathway contributions to the total N_2O pool at pseudo-steady state are shown in **Figure 5.5**. The simulated NH_4^+ oxidation at low DO yields a higher N_2O emission factor as compared to that at higher DO (4.6 and 1.2% respectively), in agreement with other nitrification/denitrification systems (Hu *et al.*, 2010; Tallec *et al.*, 2006) and comparable with those reported by (Wunderlin *et al.*, 2012). 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 show similar trends with maximum contributions of 72% and 43% respectively, but opposite compared to the NN pathway.

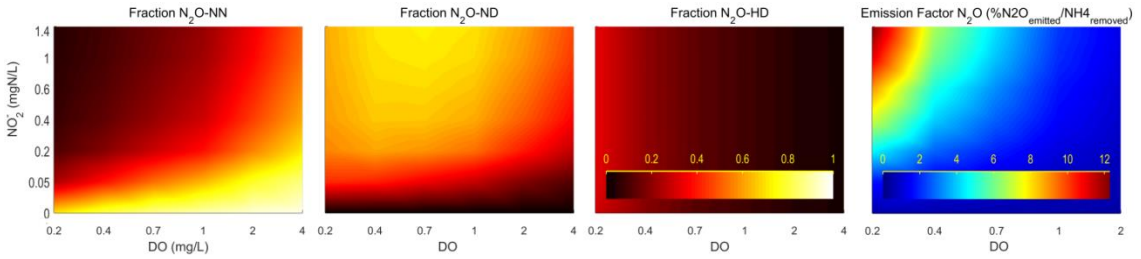

Figure 5.5. Model evaluation at varying NO_2^- and DO concentrations during excess NH_4^+ removal (pH = 7.2). From left to right: Pathway contributions to total N_2O pool NN, ND, HD; N_2O emission factor. (**Paper IV**).

The different microbial community composition between AOB-enriched and mixed liquor biomass poses a significant effect on the associated N_2O production during NH_4^+ removal. In the mixed liquor biomass the NO_2^- sink is much larger due to a higher NOB biomass fraction, and hence, a higher N_2O emission factor is expected from the AOB-enriched biomass. If N_2O is produced during anoxic periods, or transiency into anoxia, it accumulates in the liquid phase and can be stripped at the onset of aeration. In this scenario the mixed liquor biomass also offers an advantage with respect to the AOB-enriched biomass as the heterotrophic fraction of the biomass will act as an

N_2O sink even in the absence of additional organic carbon (**Figure 5.7**). Assuming a constant autotrophic N_2O production, the observed or net N_2O production from mixed liquor biomass is expected to be lower as the heterotrophic biomass can mask autotrophically-driven N_2O production (**Figure 5.7**).

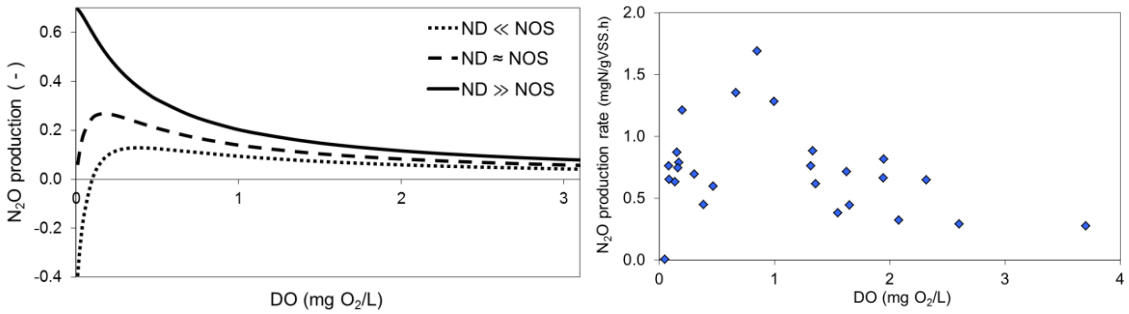

Figure 5.7. Left: Theoretical model evaluations for mixed microbial communities: AOB ≫ HB (solid line), AOB ≈ HB (dashed line), and AOB ≪ HB (dotted line). Right: net N_2O production rates observed during NH_4^+ oxidation at constant DO. Mixed liquor biomass from Respirom_ML_aer (**Figure 4.2.**).

Role of hydroxylamine on nitrous oxide models

Low NH_2OH bulk concentrations were reported for AOB pure cultures and nitrifying systems (NH_2OH < 0.1mgN/L) (Soler-Jofra *et al.*, 2016; Yu and Chandran, 2010), indicating a quick turnover of NH_2OH. However, NH_2OH predictions from N_2O models are not verified and would overestimate NH_2OH equilibrium concentrations during NH_4^+ oxidation ($\mu_{AMO} \geq \mu_{HAO}$, $K_{AOB.NH2OH} \approx K_{AOB.NH4}$ = 0.7 – 2.4mgN/L) (Pocquet *et al.*, 2016; Ding *et al.*, 2016; Ni *et al.*, 2011, 2013b, 2014). Here, a faster HAO process compared to AMO prevents high NH_2OH accumulations and is therefore necessary for more accurate NH_2OH predictions. This is in agreement with the calibrated NDHA model where $\mu_{AMO} < \mu_{HAO}$ and $K_{AOB.NH2OH} < K_{AOB.NH4}$, being $K_{AOB.NH2OH}$ = 0.2 mgN/L the lowest value reported in N_2O models. Simulation results of a biofilm system also calculated overestimation of NH_2OH release from other N_2O models, where 27% of the NH_3 oxidized accumulated as NH_2OH (Todt and Dörsch, 2016).

Experimental and modelling results suggest that N_2O pathways such as the associated to cyt P460, could be responsible for the high N_2O production observed during aerobic NH_2OH oxidation (Kozlowski *et al.*, 2016). The NDHA model might not individually describe all the co-occurring N_2O pathways in the AOB metabolism. However, N_2O production associated to

wastewater treatment conditions is successfully captured by lumping pathways into the NN and ND processes.

Role of nitric oxide on nitrous oxide models

NO and N_2O production from NH_4^+ oxidation under aerobic conditions is significantly lower than at low oxygen tension, as reported for AOB pure cultures (Kozlowski *et al.*, 2016). In nitrifying systems NO and N_2O production was also triggered by NO_2^- and anoxic conditions (Kampschreur *et al.*, 2008b; Wunderlin *et al.*, 2012). Modelling results show that the higher anoxic rates can be explained by the transient accumulation of NH_2OH which, under anoxia, has been suggested to act as electron donor for NO_2^- reduction to N_2O in a two-step process over NO (de Bruijn *et al.*, 1995; Poth and Focht, 1985; Yu and Chandran, 2010; Kester, 1997).

The ratio between the substrate affinity of NO reductases, $K_{AOB.NO}$ / $K_{HB.NO}$, is an important parameter of N_2O models as it can shift the contributions of the ND and HD pathways for the same overall N_2O fit (**Paper I**). However, direct estimation of NO affinity is difficult due to its toxicity (Schulthess and Gujer, 1996). In N_2O models K_{NO} values are typically assumed (Pan *et al.*, 2013; Hiatt and Grady, 2008) and highly variable ($K_{HB.NO}$ = 0.00015-0.05 mgN/L and $K_{AOB.NO}$, 0.004-0.1mgN/L, $K_{AOB.NO}$ / $K_{HB.NO}$ = 1 - 56) (Wang *et al.*, 2016a; Hiatt and Grady, 2008; Spérandio *et al.*, 2016; Schreiber *et al.*, 2009; Ni *et al.*, 2011). For example, in the study by (Wang *et al.*, 2016a) the HD pathway has an NO affinity over 50 times higher than the NN pathway. The HD pathway could, in theory, uptake NO produced by the NN pathway at a much higher rate and underestimate the NN contribution to the total N_2O pool. Hence, based on current knowledge and to avoid a preferential NO-consumption/N_2O-production pathway the NO affinity ratio between AOB and HB is set to one (**Figure 4.8**).

6 Conclusions

The main findings of this thesis are:

- In microbial communities from conventional biological nitrogen removal systems heterotrophs are more abundant than autotrophs and heterotrophic activity should not be neglected even under very low carbon-to-nitrogen conditions. Hence, in mixed microbial communities the heterotrophic contribution to N_2O production should always be considered.
- A consilient 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 three biologically-driven pathways. This model can describe all relevant NO and N_2O production pathways with fewer parameters than other proposed models.
- An experimental design to estimate N_2O model parameters through extant respirometry is developed and applied to two different biomass types: AOB-enriched and Activated Sludge mixed liquor communities. The experimental design allowed the isolation of individual process rates and the estimation of parameters associated with oxygen consumption (endogenous, nitrite and ammonium oxidation) and N_2O production (NN, ND and HD pathway contributions). In respirometric and batch assays N_2O and NO production increased during ammonium oxidation under low dissolved oxygen concentrations and the presence of nitrite.
- The model predicted the NO and N_2O dynamics at varying ammonium, nitrite and dissolved oxygen levels from two independent systems: (a) an AOB-enriched biomass and (b) Activated Sluge mixed liquor biomass. A total of ten (a) and seventeen (b) parameters were identified with high accuracy (coefficients of variation < 25%).
- A rigorous procedure to estimate parameters associated to N_2O models is presented. Moreover, the critical validation of the model response and the estimated parameter values will benefit N_2O model discrimination procedures.
- As an end-product in the metabolism of aerobic ammonium oxidizers and obligate intermediate of heterotrophic denitrifiers, the uncertainty of nitrogenous substrates (e.g. ammonium, nitrite, etc.) propagates to N_2O

predictions. Hence, N_2O model predictions should be described by best-fit N_2O predictions together with uncertainty metrics (e.g. confidence intervals).

- A model describing organic carbon oxidation and four-step denitrification through electron competition using fewer parameters than other models is proposed. The model describes reaction rates as analogy to current intensity through resistors in electric circuits. The model describes the electron competition during the reduction rates of single and most of the combined nitrogen oxides for four different carbon sources. Further validation under different carbon and nitrogen loadings needs to be explored.

7 Future perspectives

In this study N_2O datasets for parameter estimation relied on online sensors for bulk measurements. The model can then predict the contribution of each pathway to the total N_2O pool. Other analytical techniques such as stable isotope labelling (^{15}N, ^{18}O or isotopic signatures) could be performed simultaneously to validate or correct predictions regarding pathway contributions.

The applicability of the proposed model could be extended to continuous or full-scale systems. However, for the purpose of model discrimination and model development lab-scale systems with defined controlled environments are preferred. For example, biochemical gradients exist along the bioaggregates in biofilm configurations where pH changes from the bulk to the inner layer of the aggregate. Hence, biofilm models should consider explicit pH calculations as N_2O formation ($K_{AOB.HNO2}$) and consumption ($pH_{opt.nosZ}$) are pH-dependent. It was shown that heterotrophs are ubiquitous, even if supported by biomass decay products and thus, should always be considered in N_2O models.

The role of NH_2OH as electron donor in the AOB metabolism remains to be untangled as new N_2O producing pathways are discovered (Caranto *et al.*, 2016). NH_2OH might not be the direct electron donor for NO_2^- and NO reduction (cytochromes) and the simplification of our assumption vs the use of lumped set of electron carriers (Ni *et al.*, 2014) deserves further examination.

As more datasets are being retrieved, direct comparison of the benefits of more complex mechanistic models compared to empirical approaches could be studied (Leix *et al.*, 2017).

Another suite of questions are: How marginal is the benefit of including more species that share function but differ in their kinetic parameters? (e.g. *Nitrospira spp.*, *Nitrobacter spp.*). How complex do N_2O models need to be to capture N_2O emissions with a given accuracy and precision? Mechanistic models have focused on *accuracy*; and the *precision* example shown in this study can be considered as a reference for further comparisons.

Additionally, it is recommended for N_2O modelling studies to recognize and quantify uncertainties associated to N_2O emissions together with best-fit simulations and parameter identifiability metrics. If the uncertainty of N_2O predictions from parameter variance is identified (i.e. certain parameters carry most of the uncertainty) then Optimal Experimental Design (OED) tech-

niques can help reducing it (Munack and Posten, 1989). OED criteria have been successfully applied to batch experiments with important improvements in parameter confidences.

In this work an example methodology is proposed, but other modelling frameworks such as a Bayesian hierarchical approach that considers a probabilistic parameter estimation could be used. It provides identifiability and sensitivity metrics and has also been applied for the estimation of activated sludge process parameters (Sharifi *et al.*, 2014; Cox, 2004). A substantial limitation compared to the method presented here is the higher computational cost (number of simulations). On the other hand, if the parameter subset to consider for estimation could be minimized, the complexity of the multidimensional problem would decrease significantly.

Finally, in the next step the model is ready to be used for plant-wide applications. While some parameters will certainly need to be estimated (after the mass balance for solids the maximum growth rates will probably differ), the parameter set reported here should describe the kinetics of full-scale systems.

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9 Papers

I **Domingo-Félez, C.**, Pellicer-Nàcher, C., Petersen, M. S., Jensen, M. M., Plósz, B. G., Smets, B.F. 2017. Heterotrophs are key contributors to nitrous oxide production in activated sludge under low C-to-N ratios during nitrification – Batch experiments and modeling. *Biotechnology and Bioengineering*, **114**, 132-140.

II **Domingo-Félez, C.**, Smets, B.F. 2016. A consilience model to describe N2O production during biological N removal. *Environmental Science: Water Research and Technology*, **6**, 923-930.

III **Domingo-Félez, C.**, Calderó-Pascual, M., Sin, G., Plósz, B. G., Smets, B.F. 2017. Calibration of the comprehensive NDHA-N2O dynamics model for nitrifier-enriched biomass using targeted respirometric assays. *Submitted*

IV **Domingo-Félez, C.**, Smets, B.F. 2017. Application of the NDHA model to describe N2O dynamics in activated sludge mixed culture biomass. *Manuscript in preparation*.

V **Domingo-Félez, C.**, Smets, B.F. 2017. Modelling electron competition in a mixed denitrifying microbial community with different carbon sources through an electric circuit analogy. *Manuscript in preparation*.