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Blum, Jan-Michael

Publication date:
2018

Document Version
Publisher's PDF, also known as Version of record

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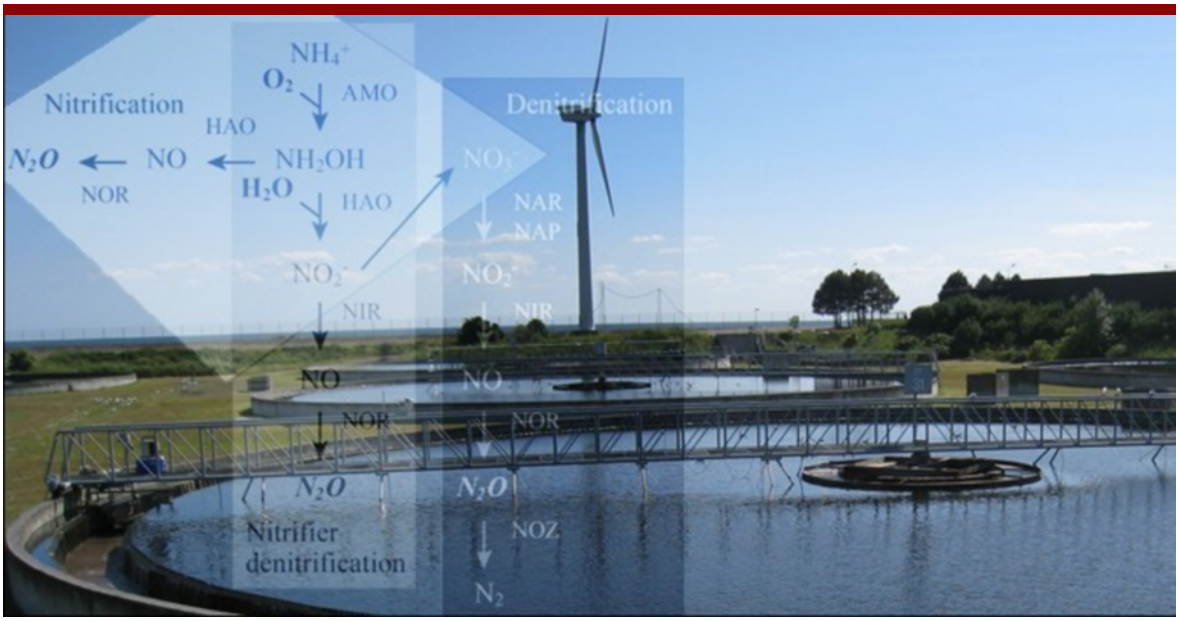

Jan-Michael Blum
PhD Thesis
June 2018

DTU Environment
Department of Environmental Engineering

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Address: DTU Environment
Department of Environmental Engineering
Technical University of Denmark
Miljoevej, building 113
2800 Kgs. Lyngby
Denmark

Phone reception: +45 4525 1600
Fax: +45 4593 2850

Homepage: http://www.env.dtu.dk
E-mail: reception@env.dtu.dk

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Preface

The presented thesis is based on the work carried out at the Technical University of Denmark, Department of Environmental Engineering from March 2015 to April 2018. The work was performed in the N_2OMAN project, which was funded by The Danish Council for Independent Research Technology and Production Sciences (FTP) [Project N2Oman, File No. 1335-00100B]. The research was supervised by Professor Barth F. Smets and Marlene Mark Jensen, PhD.

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-IV**.

I **Blum, J.-M.**, Jensen, M.M., Smets, B.F. Nitrous oxide production in intermittently aerated Partial Nitritation- Anammox reactor: oxic N_2O production dominates and relates with ammonia removal rate. *Chemical Engineering Journal 335, 458-466 (2018).*

II **Blum, J-M.**, Palomo, A., Jensen, M.M., Smets, B.F. Intermittent aeration regimes as tool to manipulate bio-granule size and engineer microbial community composition in Partial Nitritation-Anammox reactors.

III **Blum, J.-M.**, Su, Q., Ma, Y., Valverde-Pérez, B., Domingo-Félez, C., Jensen, M.M., Smets, B.F. The pH dependency of N-converting enzymatic processes, pathways and microbes: effect on net-N_2O production. *Environmental Microbiology (2018).*

IV **Blum, J.-M.**, Jensen, M.M., Smets, B.F. Nitrous oxide production in intermittently aerated Partial Nitritation-Anammox reactor: alkaline pH fosters N_2O consumption and reduces overall emissions.

In this online version of the thesis, paper **I-VI** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

This PhD study also contributed to national and international conferences with the following proceedings and conference papers:

- **Blum, J.-M.,** Smets, B.F. Changes in intermittent aeration regimes are effective tools to manage bio-granule size and microbial communities in partial nitritation-anammox SBRs. 11th Annual Meeting of Danish Water Forum 2017, Copenhagen, Denmark. Oral presentation.
- **Blum, J.-M.,** Jensen, M.M., Smets, B.F. Intermittent aeration regimes are effective tools to manage size of bio-granules and microbial communities in PN/A SBRs. 10th International Conference on Biofilm Reactors 2017, Dublin, Ireland. Oral presentation.
- **Blum, J-M.**, Smets, B.F. Where does N_2O from Partial Nitritation-Anammox processes come from? 12th Annual Meeting of Danish Water Forum 2018, Copenhagen, Denmark. Oral presentation.
- **Blum, J.-M.**, Smets, B.F. Microbial granulation management: Simple changes in reactor operation enable control of granular properties and the engineering of microbial communities in wastewater applications. The Danish Microbiological Society Annual Congress 2016, Copenhagen, Denmark. Poster presentation.
- **Blum, J.-M.**, Jensen, M.M., Smets, B.F. N_2O production and mitigation in the Partial Nitritation-Anammox process. Nordic Wastewater Conference 2017, Aarhus, Denmark. Poster presentation.
- **Blum, J.-M.**, Smets, B.F. pH dependent release of NO from hydroxylamine oxidoreductase – a model proposal. Anammox Workshop at MEWE and Biofilms IWA Specialist Conference 2016, Copenhagen, Denmark. Oral presentation.
- **Blum, J.**-M., Smets, B.F. pH dependent release of NO from hydroxylamine oxidoreductase – a model proposal. Nitrous oxide emissions from biological wastewater treatment Expert Meeting and Workshop 2016, Bochum, Germany. Oral presentation.

Acknowledgements

I want to thank my supervisors Barth F. Smets and Marlene Mark Jensen for providing me with the opportunity, the environment and the professional help to perform the research that led to this PhD thesis. Their guidance, valuable feedback and openness to letting me explore new paths enabled me to grow as an environmental engineer and independent researcher.

Many thanks to Carlos Domingo-Félez in particular, who was a mentor in the beginning and a sparring-partner at a later stage of the project. He shared his valuable experience at many occasions and his deep knowledge about the topic allowed fruitful and rewarding discussions.

I also want to thank the other members of the Metlab research group, particularly Arnaud Dechesne for teaching me the method of FISH-microscopy, Alejandro Palomo-González and Jane Fowler for their support in molecular biology and bioinformatics and Bastiaan Cockx for new insights into biogranules. Many thanks to Qingxian Su, Yunjie Ma and Sike Wang, who not only worked on related projects so that an exchange of knowledge was common, but they simply contributed to a pleasant working atmosphere in the laboratory and office. Thanks to Joanna Cieslukowska, who conducted her Master's thesis under my supervision, for her enthusiasm, pragmatism and hard work.

A special thanks to the hidden champions of the department without whom the project and daily life would simply be impossible: among them the technicians Lene Kirstejn Jensen, Flemming Møller, Sinh Hy Nguyen and Malene Grønvold for their excellent work, René Leps from IT, who prevented even a single equipment failure, virus infection or any loss of data in the entire time of the project, and representative for the administration Anne Harsting, Charlotte Lind, Camilla Bitsch, Kim Ryberg, Christina Pannach and Thomas Højlund Christensen for their continuous support.

I want to thank Maajke Hoekstra (TU Delft) and Michele Laureni (EAWAG) for an open exchange during international conferences and meetings.

Finally, many thanks to all, who accompanied me on this journey: colleagues, Friday bar participants, cake club members and my family and friends for making the last years an unforgettable experience.

Summary

Urban wastewater treatment plants are designed to remove pathogens and pollutants from wastewater in order to provide sanitation and to protect receiving water bodies from eutrophication. Reactive nitrogen, mainly in the form of ammonium, is one of the components in wastewater that is converted to dinitrogen gas during treatment. The Partial Nitritation-Anammox process (PNA) uses the capacity of autotrophic aerobic and anaerobic ammonia oxidizing bacteria (AOB and AnAOB) to perform this task. The process is mainly applied to treat ammonium-rich wastewater streams with low concentrations of organic carbon. Its advantages over conventional nitrogen removal technology include lower energy needs for aeration, no addition of organic carbon and reduced sludge production. However, emissions of the greenhouse gas nitrous oxide (N_2O), that are commonly measured during process operation, have the potential to determine the CO_2 footprint of PNA, thus off-setting its environmental benefits. Consequently, strategies to mitigate N_2O emissions from PNA are needed.

In the presented Ph.D. study a laboratory scale PNA model system was investigated to infer beneficial modes of process operation for high rate PNA with simultaneously low N_2O emissions. Dynamics of N_2O emission, production and consumption reactions were quantified to identify correlations between N_2O and process parameters, a method to engineer the microbial community by aeration patterns was tested and a N_2O mitigation strategy based on pH set-points was evaluated. The concept of Microbial Resource Management (MRM) was adopted to steer the fraction of AOB and AnAOB in bio-granules and to manipulate the activity of enzymes relevant for N_2O metabolism. Both, microorganisms and enzymes were considered as microbial resources.

The characterization of the system revealed that net N_2O production in aerated phases primarily contributed to overall N_2O emissions (80-100%). The remaining N_2O was produced during non-aerated phases, leading to distinct N_2O emission peaks at the onset of aerated phases. Net N_2O production rates in aerated phases were positively related with the specific ammonia removal rate, while during non-aerated phases net N_2O production rates were positively correlated with the nitrite concentration (NO_2^-). Operation of PNA at reduced specific ammonia removal rates is, therefore, a feasible strategy to mitigate N_2O emissions.

However, when high ammonium removal rates shall be maintained, an increased NO_2^- sink capacity by a relatively larger fraction of AnAOB has the potential to reduce N_2O production. Changes of the intermittent aeration regime were applied to engineer the microbial community. Indeed, longer aerated phases were feasible to increase the size of bio-granules by approx. two-fold, which resulted in an increase of the AnAOB/AOB ratio from 0.4 up to 1.4. However, an effect of median granule size and granule size distribution on process performance was not detectable, since periodic non-aerated phases in intermittently aerated PNA had stronger regulating effects on NO_2^- accumulation, than the granule size distribution had. Consequently, the granule size distribution was less relevant for stable process performance in intermittently aerated PNA systems, than for continuously aerated systems.

Net N_2O production is the difference between N_2O production and consumption. Therefore, increased N_2O consumption rates reduce net N_2O production, also when production is not altered. A comprehensive review, synthesizing current knowledge on the effect of pH on enzymes involved in nitrogen conversions, suggested a N_2O mitigation strategy based on pH set-points. Indeed, pH exerted a strong effect on N_2O emissions, when emission factors (% $N_2O\text{-}N_{produced}/NH_4^+\text{-}N_{removed}$) increased with pH from 2.5% at pH 6.5 to 3.3% at pH 7.0 and 7.5, before substantially decreasing to 1.6% at pH 8.0. The cause for low net N_2O production rates at alkaline pH was assigned to high N_2O reduction rates by the enzyme nitrous oxide reductase, which became relevant at pH > 7.5. Based on the results obtained in the Ph.D. study, operation of Partial Nitritation-Anammox at relatively long aerated phases, low specific ammonia removal rates and pH 8.0 is suggested to reduce N_2O emissions from the process.

Dansk sammenfatning

Spildevandsrensningsanlæg er designet til at fjerne patogener og forurenende stoffer fra spildevand for at sikre hygiejne og beskytte vandmiljøet mod eutrofiering. Reaktivt kvælstof, hovedsageligt i form af ammonium, er en af bestanddelene i spildevand, der omdannes til frit kvælstof. I processen med delvis nitrifikation og efterfølgende anammox (PNA på engelsk) udnyttes kapaciteten af autotrofe aerobe og anaerobe ammonium oxiderende bakterier (AOB og AnAOB) til behandlinge af spildevandstrømme med høje ammonium koncentrationer og lave koncentrationer af organisk kulstof. I forhold til konventionel kvælstoffjernelse er denne proces fordelagtig pga. et lavere energibehov til beluftning, intet behov for tilsætning af organisk kulstof og reduceret slamproduktion. Emissioner af drivhusgassen lattergas (N_2O), der ofte dannes under procesdrift, kan have stor betydning for CO_2-fodaftrykket under PNA og dermed ophæve de miljømæssige fordele. Der er derfor et behov for forskellige strategier til at reducere N_2O emissioner fra PNA.

I denne Ph.D. afhandling undersøges et laboratorie-baseret PNA system for at opnå en PNA proces drift med høj aktivitet og samtidig lave N_2O emissioner. Lattergas emissioner samt produktion og forbrug af næringssalte blev kvantificeret for at identificere sammenhænge mellem N_2O og procesparametre. Endvidere blev en metode til at konstruere det mikrobielle samfund vha. luftningsmønstre testet, samt blev et tiltag til nedbringelse af N_2O emission baseret på pH værdier vurderet. Konceptet ”Microbial Resource Management” (MRM) blev brugt til at styre fraktionen af AOB og AnAOB i biogranulater og til at manipulere aktiviteten af enzymer, der er relevante for N_2O omsætningen. Både mikroorganismer og enzymer blev betragtet som mikrobielle ressourcer.

Karakteriseringen af PNA systemet viste at netto N_2O produktion i beluftede faser udgjorde det meste af den samlede N_2O emission (80-100%). Det resterende N_2O blev dannet i de faser, hvor der ingen beluftning var. Sidstnævnte førte til en stigning i N_2O emissionen lige efter beluftningen blev startet igen. Der var en positiv sammenhæng imellem netto N_2O produktionsrater og specifikke ammoniakfjernelsesrater under beluftning, mens netto N_2O-produktionsrater var positivt korreleret med nitritkoncentrationen (NO_2^-) i de faser, hvor beluftningen var slukket. Styring af PNA proces ved reducerede specifikke ammoniakfjernelsesrater er derfor en mulig strategi til at reducere frigivelsen af N_2O.

Hvis høje ammoniumfjernelsesrater skal opretholdes har en forøget NO_2^- reduktionskapacitet, igennem en relativt større brøkdel af AnAOB, potentialet til at reducere N_2O produktionen. Ændringer af det intermitterende beluftningsregime blev anvendt til at konstruere det mikrobielle samfund. Faktisk var det med længere beluftningsfaser muligt at forøge størrelsen af bio-granulaterne med ca. en faktor 2, hvilket resulterede i en stigning i forholdet imellem AnAOB og AOB fra 0,4 op til 1,4. En effekt af median granulestørrelse og granulstørrelsesfordeling på procesydelse var imidlertid ikke påviselig, da periodiske faser uden beluftning havde en stærkere regulerende virkning på NO_2^--akkumulering end granulstørrelsesfordelingen. Således var granulat-størrelsesfordelingen mindre relevant for stabil procesydeevne i PNA systemer med intermitterende beluftning end det er i systemer med konstant beluftning

Da netto N_2O produktion er forskellen mellem N_2O produktion og reduktion, vil højere N_2O reduktionsrater i forhold til N_2O produktion reducere netto N_2O produktionen. Efter en omfattende opsummering af relevante publikationer, der sammenfatter den nuværende viden om effekten af pH på enzymer involveret i omdannelser af kvælstofforbindelser blev endnu et tiltag til reduktion af N_2O emission udarbejdet. Under en test af den udarbejdede teori viste det sig at pH havde en stærk virkning på N_2O emissioner, idet emissionsfaktoren ($\%N_2O\text{-}N_{produceret}/NH_4^+\text{-}N_{fjernet}$) steg fra 2,5% ved pH 6,5 til 3,3% ved pH 7,0 og 7,5, før den igen faldt væsentligt til 1,6% ved pH 8,0. Årsagen til de lave N_2O produktionsrater ved basisk pH kan skyldes de høje N_2O-reduktionsrater ved hjælp af enzymet N_2O-reduktase, som bliver relevant ved pH > 7,5. Baseret på de opnåede resultater i ph.d. afhandlingen, foreslås en styring med forholdsvis lange beluftningsfaser, lave specifikke ammoniakfjernelsesrater og en pH på 8,0 som effektive handlingsplaner til at nedbringe frigivelsen af N_2O fra PNA processen.

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1 Introduction

1.1 Background

1.1.1 The relevance of sustainable urban wastewater treatment

2009 marked the year, when for the first time in human history more people lived in urban, than in rural areas (UN-HABITAT, 2008). According to the United Nations the trend of urbanization continues and by the year 2050 around 70% of the world's population is expected to live in cities. Coupled with an ongoing growth of world population, we will witness the emergence of mega-cities and gigantic urban clusters. These urban areas will produce large quantities of wastewater. Already today cities are hubs of wastewater streams that, if not properly treated before discharge, constitute severe environmental and sanitary burdens for receiving water bodies and the cities themselves (UN-HABITAT, 2008). The discharge of excess nutrients causes, among others, eutrophication in receiving habitats that leads to algae blooms with subsequent oxygen depletion and loss of marine life (Commission of the European Communities, 1999). The efficient treatment of urban wastewater streams is therefore paramount to enable a sustainable balance between urban areas and the environment they rely on. Sustainable urban wastewater treatment, therefore, contributes to the fulfillment of the United Nations Sustainability Development Goals (SDG) 6: Clean Water and Sanitation, SDG 11: Sustainable Cities and Communities and SDG 14: Life below Water by removing environmentally hazardous nutrients from water streams, while simultaneously reducing the carbon footprint of the process, especially by saving fossil fuel based energy and by avoiding the emission of potent greenhouse gases (United Nations, 2015).

Urban wastewater contains nutrients, like carbon (mainly in the form of organic carbon), phosphorus (mainly in the form of phosphate) and nitrogen (mainly in the form of ammonium). Modern wastewater treatment plants (WWTPs) are able to extract the majority of carbon from the water and convert it into biogas. The biogas is either used for electricity and heat production on site or is introduced to the urban energy grid for further usage. The technology allows WWTPs to transform from energy consumers to energy producers. By doing so their carbon footprint is substantially reduced, as not only the supply of external power becomes needless, but renewable energy is introduced to the urban grid, which makes the use of non-renewable energy at other places expendable. The phosphorus in urban wastewater can also be

recovered to a large extent, for instance in the form of struvite or as organic fertilizer that is reintroduced to the agricultural value chain. However, no economically attractive form of nitrogen recovery has been established to date.

1.1.2 Removal of nitrogen from urban wastewater: the Partial Nitritation-Anammox Process

The current narrative is to convert environmentally reactive and water soluble nitrogen species (mainly ammonium (NH_4^+)), to environmentally inert dinitrogen gas (N_2). By reintroducing N_2 into the atmosphere the geochemical nitrogen cycle is closed. Biological processes have proven successful for the removal of NH_4^+ from urban wastewater and constitute the dominant form of N-removal technology to date. Among available technology conventional nitrification-denitrification is the most applied process. Nitrification-denitrification comprises two unit operations and exploits the capacity of bacteria to first convert NH_4^+ into nitrate (NO_3^-) under oxic conditions and subsequently to reduce NO_3^- to N_2 under anoxic conditions. The process provides efficient NH_4^+ to N_2 conversion, but is relatively energy and therefore cost and CO_{2eq} intensive. The addition of organic carbon from external sources is often necessary to achieve good performance, which adds another cost factor.

In the last decade significant reduction of costs has been achieved by the development of the Partial Nitritation-Anammox process (PNA). Like nitrification-denitrification, PNA converts NH_4^+ into N_2, but exploits different microbial pathways. PNA consists of two biological conversion steps: i) during Partial Nitritation (PN) approximately half of the influent NH_4^+ is converted to nitrite (NO_2^-) by aerobic ammonia oxidizing bacteria (AOB)(Third et al., 2001). Molecular oxygen is required for this step:

$$1\ NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O \qquad (1)$$

During the second step, i.e. anaerobic ammonia oxidation (anammox), the remaining NH_4^+ is, together with NO_2^-, converted to N_2 by anaerobic ammonia oxidizing bacteria (AnAOB)(Strous, 2000). Molecular oxygen is not required for anammox and in fact inhibits it:

$$1\ NH_4^+ + 1.3\ NO_2^- \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2\ H_2O \qquad (2)$$

Equations (1) and (2) result in the overall stoichiometry of PNA (Third et al., 2001):

$$1\ NH_4^+ + 0.85\ O_2 \rightarrow 0.435\ N_2 + 0.13\ NO_3^- + 1.3\ H_2O + 1.4\ H^+ \qquad (3)$$

The main aspect that made PNA gain market share is of economical nature. PNA requires approx. 60% less oxygen, than nitrification-denitrification to remove the same amount of NH_4^+ and hence uses less energy for aeration: on the one hand, oxidation of NO_2^- to NO_3^- is omitted during PNA, on the other hand AOB and AnAOB are autotrophs, i.e. they only require inorganic carbon as carbon source (Van Hulle et al., 2010). Thus, no addition of external organic carbon, like methanol or acetate, is required, which saves operational costs. The reasons why PNA has not replaced nitrification-denitrification processes include that stable operation of PNA is currently mainly achieved at elevated temperatures – although "cold" anammox is under development – and that organic carbon in the wastewater perturbs process performance (Xu et al., 2015). Therefore, PNA is nowadays mainly applied at mesophilic temperatures for the treatment of NH_4^+-rich wastewater streams with low COD/N ratios (Lackner et al., 2014). These conditions apply, for example, to water streams from sludge digestion dewatering processes.

1.1.3 Nitrous oxide offsets environmental benefits of PNA

PNA is an attractive process for wastewater treatment utilities, if not emissions of the greenhouse gas nitrous oxide (N_2O) had the potential to cancel out its environmental benefits (Schaubroeck et al., 2015). Nitrous oxide is a greenhouse gas with a large global warming potential (GWP_{100} = 298) and a long life time in the atmosphere (121 years)(IPCC, 2013). A GWP_{100} of 298 means that 1 kg of N_2O has a similar potential for global warming as 298 kg of carbon dioxide (CO_2) have. Besides it greenhouse effect, N_2O also contributes to the depletion of stratospheric ozone, when the products of photocatalytic N_2O cleavage NO and NO_2 degrade ozone to molecular oxygen (Ravishankara et al., 2009). Due to its large GWP_{100} already small amounts of N_2O emitted can determine the carbon footprint of PNA and negatively affect its environmental impact (Kampschreur et al., 2009). Naturally, process operators seek to minimize emissions.

N_2O is the result of biological activity and accumulates, when N_2O production rates exceed consumption rates (Schreiber et al., 2012). Since, in principal, both production and consumption reactions exist in PNA, the detected net N_2O production is the result of imbalances between the two, both on a temporal and spatial scale:

$$\text{Net } N_2O \text{ production} = N_2O \text{ production} - N_2O \text{ consumption}$$

Therefore, the question arises how production and/or consumption rates can be managed in order to reduce net N_2O production rates.

1.1.4 Microbial Resource Management as a path forward to low N_2O emissions

The goal of environmental biotechnology is to "develop, use and regulate biological systems for remediation of contaminated environments (land, air, water)" (according to International Society for Environmental Biotechnology). A tool for wastewater engineers and operators to achieve this goal is to design and set a certain "operational space" that provides an environment for favorable microorganisms to accumulate. Certain microorganisms are desired, as they carry genes for specific enzymes. The enzymes then catalyze a chemical conversion reaction at increased efficiency, e.g. conversion of NH_4^+ to N_2. In other words: engineers provide an environment that enriches favorable microorganisms, which with their traits catalyze a chemical conversion reaction that fulfills an engineering target in a way superior to other treatment technology (positive cost-effectiveness analysis). The "operational space" can be manipulated by a variety of parameters, e.g. choice of reactor type, nutrient loading rates, sludge retention time, aeration rate and pattern, shear stress, etc. (Van Hulle et al., 2010).

The concept of Microbial Resource Management (MRM), as an analogue to human resource management, describes a framework for environmental biotechnology to "reveal strategies to obtain and maintain a highly performant microbial community" (Verstraete et al., 2007). MRM has been applied to PNA to enable engineers and operators to improve process performance by evaluating inputs, catalysts and outputs (Vlaeminck et al., 2012). In respect to N_2O emissions from PNA, the MRM framework highlights the relevance of the balance between AOB and AnAOB activity, the size of granules and low fluctuations of DO and substrate concentrations (Vlaeminck et al., 2012). However, the MRM does, to date, not fully consider the potential of the heterotrophic community, particularly for N_2O consumption. Only recently it became clear that the genetic potential for N_2O consumption is widely spread in heterotrophic bacteria and that suitable candidates for N_2O mitigation strategies may be among them (Jones et al., 2013). Furthermore, the MRM describes catalysts on a cellular level, but does not include the effect of process parameters on certain enzymatic activities. Enzymatic catalysis is discussed in this text and enzymes are understood as microbial resources. Including them in MRM may be a valuable addition to the framework.

1.1.5 The backbone of Partial Nitritation-Anammox: the microbial community

The microbial community of PNA systems is complex and commonly comprises hundreds of different taxa (often named as operational taxonomic units (OTUs)(Wagner et al., 2002; Weissbrodt et al., 2014). Among others, their abundance and competition for substrates and electron acceptors and their inhabitation of different niches within bio-granules is relevant for stable process operation and net N_2O production. It is feasible to cluster different OTUs in functional guilds, out of which four different ones are most relevant for PNA systems: aerobic ammonia oxidizing bacteria (AOB), anaerobic ammonia oxidizing bacteria (AnAOB), nitrite oxidizing bacteria (NOB) and heterotrophic denitrifying bacteria (HD). For ideal PNA only AOB and AnAOB are required, as they catalyze partial nitritation and anammox (see 1.1.2). NOB are undesired, as they compete with AOB for oxygen and with AnAOB for NO_2^-. An effective suppression of NOB is crucial for stable process performance and is commonly well achieved in PNA systems (Pérez et al., 2014; Vlaeminck et al., 2012). The production of extracellular polymeric substances (EPS) and organic decay products by AOB, AnAOB and other bacteria provides HD with substrate and enables them to commonly comprise a substantial fraction of the biomass, even in autotrophically fed systems (Ali et al., 2016)(Paper I). Heterotrophic denitrifiers can be tolerated in PNA systems to some extent, but need to be monitored as they can constitute sources of N_2O, but – under the right conditions - also consume N_2O.

Aerobic ammonia oxidizing bacteria

Aerobic ammonia oxidizing bacteria (AOB) are chemolithotrophs and mainly affiliate with *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosococcus* genera, with *Nitrosomonas* and *Nitrosospira* as the most dominant AOB in WWTPs (Chu et al., 2015; Kowalchuk and Stephen, 2001). The common model describes that electrons for the generation of the proton motive force are derived from the sequential oxidation of ammonia (NH_3) to hydroxylamine (NH_2OH) and then to NO_2^-:

$$NH_3 + 0.0496\ CO_2 + 1.44\ O_2 \rightarrow$$

$$0.01\ C_5H_7O_2N + 0.99\ NO_2^- + 0.97\ H_2O + 0.99\ H^+ \quad (4)$$

$$NH_3 + O_2 + 2\ H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (5)$$

$$NH_2OH + H_2O + 2\ e^- \rightarrow HNO_2 + 4\ H^+ + 4\ e^- \quad (6)$$

These reactions are catalyzed by the enzymes ammonia monooxygenase (AMO) and hydroxylamine dehydrogenase (HAO), respectively (Schreiber et al., 2012). The process is termed nitritation. However, AOB can also reduce NO_2^- via NO to N_2O through the nitrifier denitrification pathway. The reduction of NO_2^- is catalyzed by the enzymes nitrite reductase (NIR) and nitric oxide reductase (NOR), respectively (Wrage et al., 2001). Nitrifier denitrification occurs particularly at low dissolved oxygen (DO) concentrations.

Hydroxylamine oxidation and nitrifier denitrification occur in the context of the hydroxylamine-ubiquinone-redox module (HURM). The HURM describes the flow of energy and reductant in AOB (Kozlowski et al., 2016)(Fig. 1). The oxidation of NH_2OH by HAO drives the nitritation pathway, when the oxidation of NH_2OH yields $4e^-$ from which $2e^-$ are required by the ammonia monooxygenase to perform the initial step of oxygen reduction to H_2O. Soluble cytochromes play a central role in the HURM. Cytochromes c_{554} and c_{M552} serve as electron carriers between HAO and the ubiquinone-ubiquinol pool for the generation of the proton motive force, whereas c_{552} appears to have a central role in supplying NIR and NOR with electrons for the reduction of NO_2^- and NO. The HURM is subject of ongoing research and mechanisms that regulate the flux of electrons are being unraveled. For example, the protein nitrosocyanin has been assigned a role in regulating, whether electrons are loaded to soluble or membrane bound cytochromes, thus determining their flux towards either nitrifier denitrification or the generation of the proton motive force (Stein et al., 2013). Further knowledge about the HURM will contribute to understanding and modelling N_2O production by AOB.

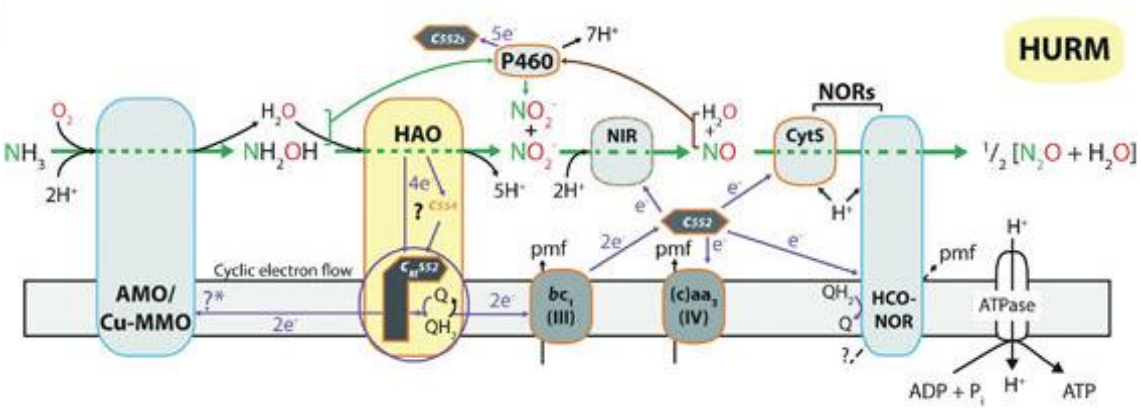


Fig. 1 - Pathways of N-oxide transformation in ammonia-oxidizing bacteria [figure from (Kozlowski et al., 2016)].

Anaerobic ammonia oxidizing bacteria

Anaerobic ammonia oxidizing bacteria (AnAOB) are chemolitotrophs and sequentially oxidize NH_4^+ and NO_2^- to N_2 via NO and hydrazine (N_2H_4). The reactions are catalyzed by the enzymes nitrite reductase (NIR), hydrazine synthase (HZS) and hydrazine dehydrogenase (HDH)(Kartal et al., 2011):

$$NH_4^+ + NO_2^- \rightarrow N_2 + H_2O \quad (7)$$

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad (8)$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \quad (9)$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \quad (10)$$

AnAOB in wastewater treatment systems mainly affiliate with the genera *Kuenia*, *Brocadia*, *Anammoxoglobus* and *Jettenia* (Kartal et al., 2013). The organisms possess a unique cell compartment: the anammoxosome (Niftrik et al., 2004)(Fig. 2). The anammoxosome is located in the cytoplasm and occupies a large volume of the cells (van Niftrik et al., 2008). It is separated from the cytoplasm by a single bi-layer membrane that contains ladderane lipids. These lipids make the membrane particularly dense, which for instance reduces passive proton diffusion through the membrane (Sinninghe Damsté et al., 2005, 2002). The anammox catabolism occurs in the anammoxosome and the proton motive force is created over the anammoxosome membrane (Kartal et al., 2011). AnAOB are not considered to directly produce or consume N_2O, yet the fact that NO is an obligate intermediate in anammox, may lead to NO related N_2O production by other organisms or side-reactions (Lotti et al., 2014).

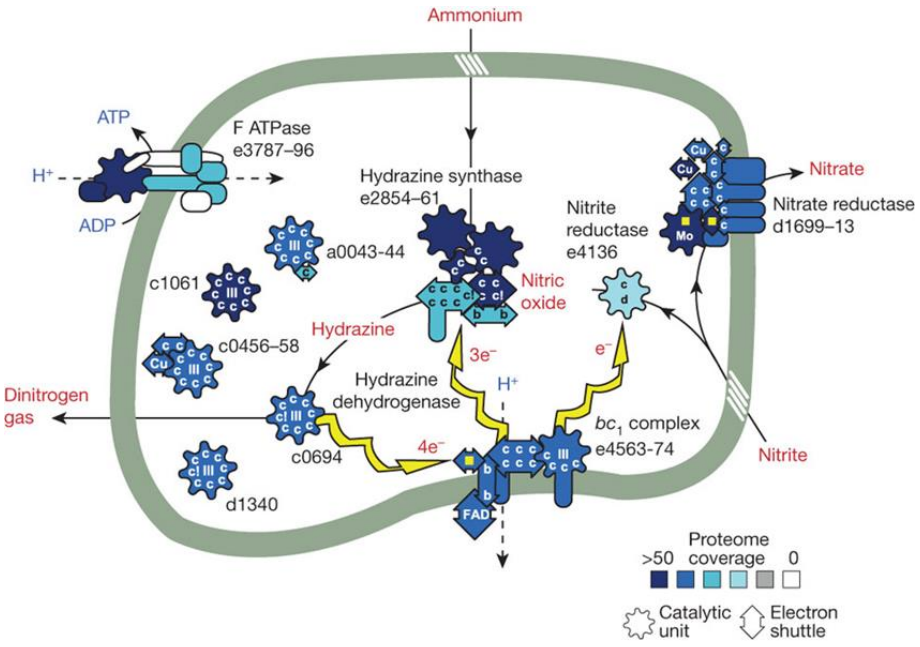


Fig. 2 - Biochemical pathway and enzymatic machinery of *K. stuttgartiensis* [figure from (Kartal et al., 2011)].

Nitrite oxidizing bacteria

Nitrite oxidizing bacteria (NOB) are chemolithotrophs and obtain their energy from the oxidation of NO_2^- to NO_3^-. The reaction is catalyzed by the enzyme nitrite oxidoreducates (NXR)(Schreiber et al., 2012)(Fig. 3):

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (11)$$

The capacity to use NO_2^- as an electron donor is found in different bacterial lineages, i.e. *Nitrobacter*, *Nitricoccus*, *Nitrispina*, *Nitrispira* and *Nitrotoga* (Daims et al., 2001). NOB are undesired in PNA systems, as they compete with AOB for O_2 and with AnAOB for NO_2^-. High NOB activity is associated with lower process performance. When NOB oxidize NO_2^- aerobically to NO_3^-, both a higher total oxygen load is required to provide AnAOB with sufficient amounts of NO_2^-, and more NO_3^- is produced, which is considered a waste product of PNA. It is therefore in the interest of process operators to suppress NOB activity to obtain close to ideal process stoichiometry. NOB suppression has received much attention in research and two factors appear to govern successful suppression of NOB: inhibition by NH_3 and periodic exposure to anoxic phases (Pérez et al., 2014). In practice a combination of both has been demonstrated to suppress NOB activity efficiently and de facto remove NOB stoichiometry from overall process performance, although a complete elimination of NOB from microbial communities has not been achieved (Domingo-Félez et al., 2014).

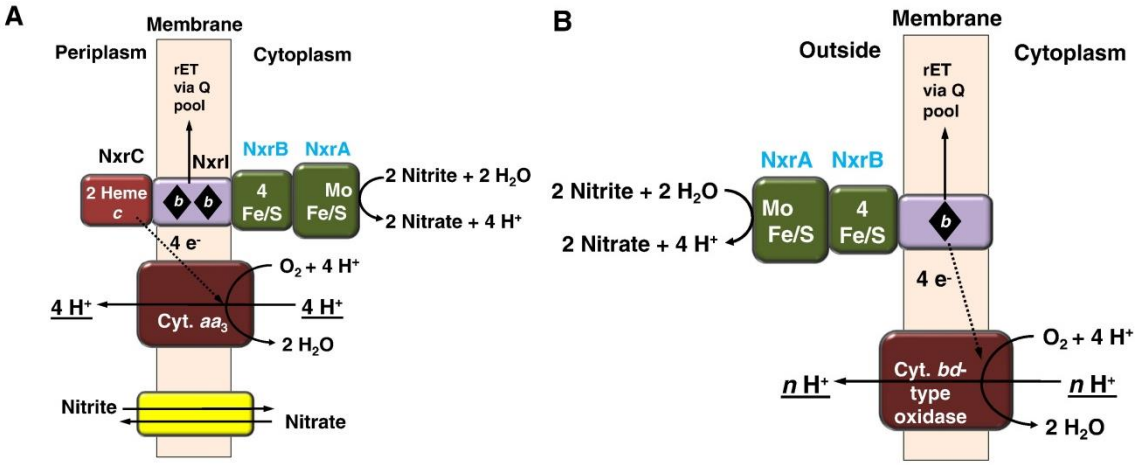


Figure 3 - Model of ETCs in different nitrite-oxidizing bacteria. A. Bacteria of the genus *Nitrobacter* (phylum Proteobacteria). B. Bacteria of the genus *Nitrospira* (phylum Nitrospirae)[figure from (Simon and Klotz, 2013)].

Denitrifying bacteria

Denitrifying bacteria are a diverse group of microorganisms that can be found in a broad range of environments, including soils and aquatic habitats (Knowles, 1982). Denitrifiers are capable of denitrification, which describes the sequential reduction of NO_3^- → NO_2^- → NO → N_2O → N_2 (Zumft, 1997). The complete denitrification pathway requires the enzymes nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR)(Schreiber et al., 2012). The nitrogen oxides serve as acceptors for electrons that are commonly obtained from heterotrophic metabolism. Heterotrophic denitrifiers (HD) are also capable of oxygen respiration and the availability of molecular oxygen affects the balance between denitrification and aerobic respiration, although evidence emerges that both pathways can in fact function simultaneously (Chen and Strous, 2013). The presence of extracellular polymeric substances (EPS) and decay products in PNA biomass commonly feeds a rich community of heterotrophic denitrifying bacteria, even in purely autotrophically fed systems (Paper II). HD do not contribute to PNA stoichiometry, but offer potential routes to remove by- and waste products of the process, namely N_2O and NO_3^-. However, not all HD possess the genetic potential to conduct complete denitrification (Graf et al., 2014). When the organisms lack N_2OR or the enzyme's activity is inhibited by other means, they can also become sources of N_2O. Accordingly, the management of the heterotrophic denitrifying community in PNA biomass becomes relevant for net N_2O production.

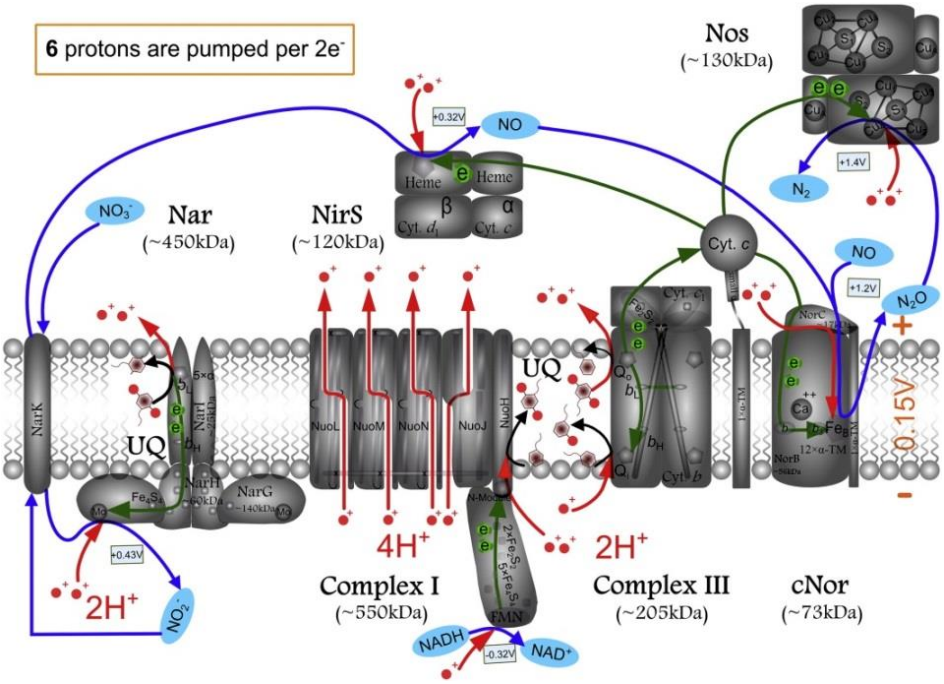


Figure 4 - The canonical respiratory chain of denitrification [figure from (Chen and Strous, 2013)].

1.1.6 Bio-granules

The biomass of PNA systems grows as biofilm, when microorganisms are either attached to surfaces, e.g. carrier material, or form bio-granules. Bio-granules can be described as "compact and dense aggregates of microbial origin with an approximately spherical external appearance that do not coagulate under reduced hydrodynamic shear and settle significantly faster than conventional activated sludge flocs." (Lemaire et al., 2008). From an engineering perspective bio-granules have two main advantages over floccular biomass: a) they settle fast and b) stratification of redox zones enables the simultaneous activity of aerobic and anaerobic microorganisms. The relatively high density and the spherical shape of bio-granules lead to high settling velocities of bio-granules, e.g. 18-40 m/h (Ni et al., 2009). An efficient separation of the biomass from the water phase is important to retain slow growing organisms like AnAOB in the system and to reduce idling time of bioreactors (Van Hulle et al., 2010). Secondly, bio-granules are co-diffusional biofilms and steep concentration profiles, e.g. of DO, in the biofilm are common (Nerenberg, 2016). In PNA granules, AOB are found at the surface of bio-granules, where they rapidly consume DO from the bulk liquid within the top 100 µm of biofilm (Fig. 5)(Paper II). The absence of DO deeper in the biofilm creates anoxic zones in which anaerobic bacteria like AnAOB and HD can grow. This redox stratification enables the co-existence of AOB and AnAOB and is a corner stone of single-stage PNA (Vlaeminck et al., 2010).

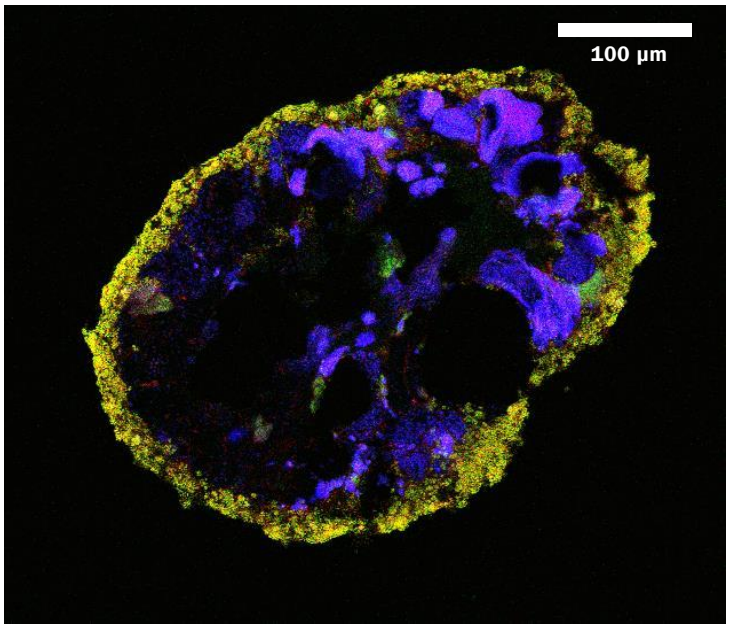


Figure 5 – FISH image of cryo-sectioned bio-granule (Paper II). AOB (green) accumulate at surface, while AnAOB (blue) grow in deeper layers of biofilm.

1.2 Research objectives and thesis structure

The research question of the PhD project was, whether the management of microbial resources in a Partial Nitritation-Anammox system could be achieved by means of targeted changes in reactor operation and whether these changes were suitable to mitigate N_2O emissions. The constraint was that the daily volumetric NH_4^+ removal rate of the system remained unchanged. In order to achieve the objectives, the N_2O production and emission dynamics of the studied system were characterized, methods to engineer the microbial community were tested and a N_2O control strategy was evaluated. The tasks were approached by:

- Identification and quantification of main sources of N_2O during Partial Nitritation-Anammox in order to focus mitigation efforts effectively (Paper I)
    - Monitor process performance and N_2O dynamics at high temporal resolution
    - Quantify contribution of oxic and anoxic N_2O production to overall emission
    - Identify correlations between process parameters and net N_2O production

- Investigation of how granule size and microbial community can be manipulated by changes of reactor operation (Paper II)
    - Test, whether aeration strategies are suitable tools to manipulate the bio-granule size distribution
    - Document changes of the microbial community composition at different granule sizes
    - Investigate, whether differences in granule size affect process performance, including N_2O emission.

- Evaluation of pH as key parameter to mitigate N_2O emissions, while maintaining process performance (Paper III & IV)
    - Examine and synthesize the state of knowledge on the effect of pH on microorganisms, pathways and enzymes involved in nitrogen conversion reactions and deduct pH optima for process operation and N_2O mitigation.
    - Document process performance, N_2O emissions and N_2O consumption rates from reactors that are operated at different pH set-points.
    - Interpret findings in respect to predicted effect of pH on N_2O production and consumption dynamics in PNA

1.3 Subject of investigation and methods

Laboratory scale PNA systems under well-defined conditions were investigated to address the research question. Applied equipment and modes of reactor operation during different measurement campaigns are summarized below; details are given in Paper I, Paper II and Paper IV.

1.3.1 Sequencing batch reactor and modes of operation

Two identical 4L sequencing batch reactors (SBR) were operated during the PhD project after a protocol that had previously been shown to be suitable for stable PNA operation (Mutlu et al., 2013). **Standard operation** was defined with a SBR cycle time of 8 hours, with an influent phase of 10 minutes, a reaction phase of 450 minutes, a settling phase of 13.5 minutes and an effluent phase of 6.5 minutes. During the reaction phase aerated and non-aerated phases alternated 15 times, each phase lasting for 15 minutes, which resulted in a total time of aeration and non-aeration of 225 minutes each per cycle (see Fig. 6). Additionally to standard operation, three other modes of operation were applied, when required for experimental set-up:

1. Standard operation
2. Continuous gas stripping
3. Long aerated phases
4. Short SBR cycle

The intermittent aeration regime of the standard operation was substituted with a **"continuous gas stripping regime"**, when instead of switching off aeration during non-aerated phases, N_2 gas was supplied at the same gas flow rate during non-aerated phases. That way N_2O was constantly stripped from the liquid phase during the reaction phase, which allowed a continuous analysis by the off-gas N_2O analyser.

The length of aerated and non-aerated phases was altered during **"Long aerated phase"-operation**. Instead of 15 aerated and non-aerated phases during standard operation, 10 aerated and non-aerated phases were applied to the reaction phase. As the time for each phase was extended from 15 to 22.5 minutes, the total time of aerated and non-aerated phases did not change. Thus, only the frequency of aerated and non-aerated phases changed from 15 to 10 over a SBR cycle, but not the total time of aeration.

A **"Short SBR cycle"** was operated at one third of standard operation: the total cycle time was 160 minutes (=1/3 of 480 min), aeration frequency was 5

(=1/3 of 15) and the N-load was 100 mg NH_4^+-N/L*cycle (=1/3 of 300 mg NH_4^+-N/L*cycle). Per day, the total N-load and performance did not change between standard and short cycle SBR operation (3 vs. 9 SBR cycles per day had the same NH_4^+-removal capacity).

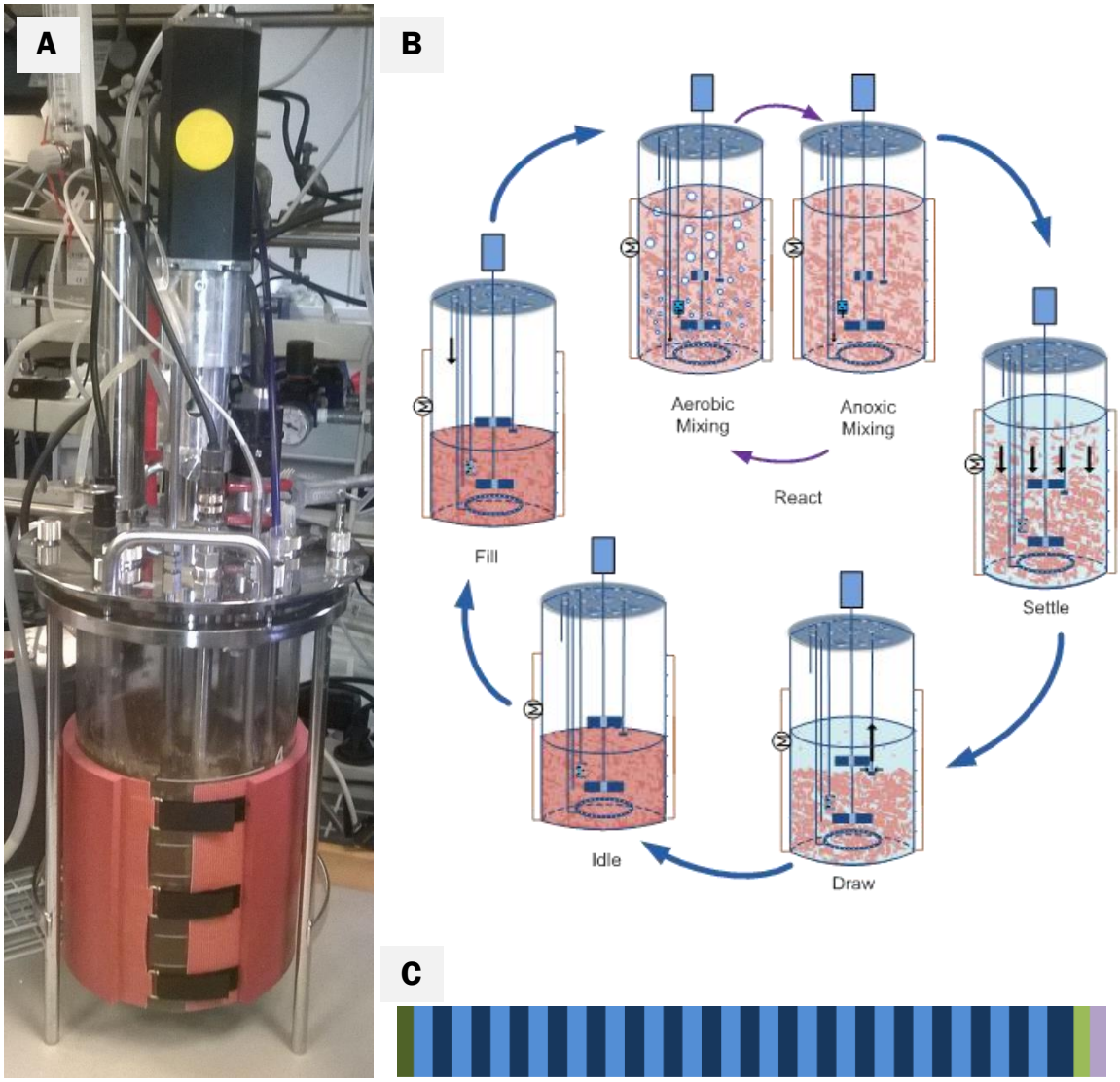


Figure 6 – Sequencing batch reactor. (A) 4 L reactor (Biostat A Plus, Sartorius) with mounted motor and heating blanket. (B) Phases of a SBR cycle: Filling, reaction with intermittent aeration, settling, drawing of effluent. (C) Timeline of SBR cycle: dark green (filling phase), light blue (aerated phase), dark blue (non-aerated phase), light green (settling) and grey (effluent withdrawal). (B) and (C) adapted from (Mutlu, 2015).

1.3.2 Monitoring of nitrogen species

NH_4^+-N, NO_3^--N and NO_2^--N

Ammonium and nitrate were measured *in situ* every minute by ion-selective electrodes. Nitrite concentrations were determined by absorbance measurements from grab samples by a colorimetric assay (details in Paper I).

Off-gas N_2O, $N_2O_{(aq)}$-N and NO-N

A gas filter correlation N_2O analyser collected off-gas from the reactor headspace at a constant flow rate and logged N_2O concentrations every minute as ppm N_2O. The concentration of N_2O-$N_{(aq)}$ and NO-$N_{(aq)}$ were determined every 30 seconds *in situ* by micro-sensors (details in Paper I). The set-up allowed the monitoring of the N-species at high temporal resolution.

1.3.3 Microbial characterization

The abundance of different functional guilds (Total bacteria, AOB, AnAOB, *Nitrobacter* and *Nitrospira*) was quantified by quantitative polymerase chain reaction (qPCR). DNA was extracted from biomass samples with a commercially available DNA extraction kit. Furthermore, extracted DNA was subject to Illumina sequencing of the 16S rRNA gene, which enabled a phylogenetic analysis of the microbial community.

The spatial organization of microorganisms in bio-granules was assessed by Fluorescence *In Situ* Hybridization (FISH) and fluorescence microscopy of cryo-sections. FISH-probes targeted the 16s rRNA genes of AOB, AnAOB and *Eubacteria* (details in Paper II).

1.3.4 Analysis and quantification of transcripts of genes of interest

A pipeline for the analysis and quantification of gene transcripts had to be established during this PhD project. In short: DNA and RNA were extracted by a customized phenol/chloroform extraction protocol. DNA was subsequently digested by DNase and the remaining RNA was purified and concentrated by RNA adhesion columns. RNA was then transcribed into cDNA, which was subject of qPCR analysis. Genes of interested included *amoA*, *hao*, *nirS*, *nirK*, *hzsA*, *nosZ cI* and *nosZ cII* (details in Paper IV).

2 Quantification of nitrous oxide emission and net production

2.1 The difference between emission and net production – effect of gas stripping

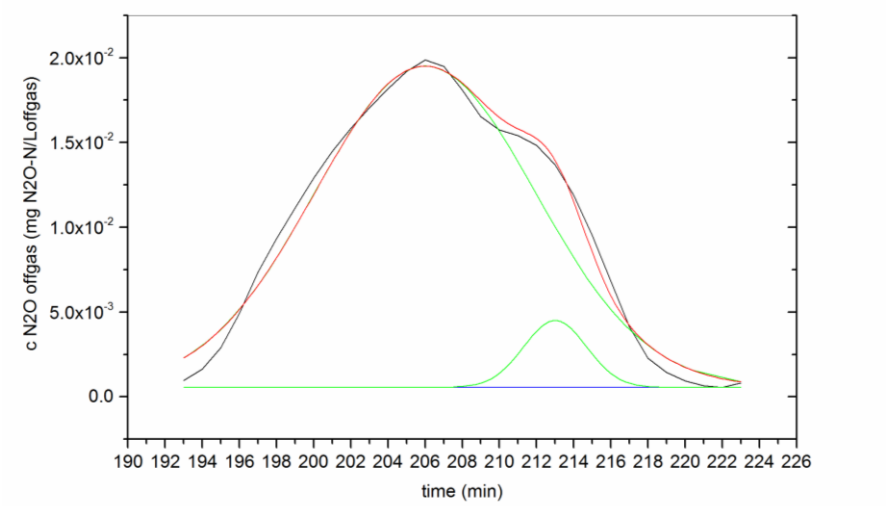
N_2O emission factors from PNA processes have been reported in a wide range of the nitrogen load: 0.1-19% N_2O-N emitted per NH_4^+-N removed ((Ali et al., 2016) and references within). Time averaged emission factors, e.g. per day or per SBR cycle, are suitable for reporting and assessing the environmental impact of N_2O emissions. However, they miss emission dynamics that hold valuable information for understanding correlations of N_2O production with other process parameters and microbial activity. Particularly in intermittently aerated systems, N_2O emissions are highly dynamic in response to the aeration pattern (Paper I).

It is important to distinguish between N_2O emission and net N_2O production, as the former is measured in the gas phase, while the latter occurs in the liquid phase. Gas stripping during aerated phases is required to transfer relevant amounts of N_2O from the water to the gas phase, thus constituting emissions (Van Hulle et al., 2010). In contrast, during non-aerated phases produced N_2O remains dissolved and accumulates in the liquid phase (Castro-Barros et al., 2015). Since N_2O production and emission are decoupled by gas stripping, N_2O emissions almost exclusively occur during aerated phases (>96%) (Paper I). However, net N_2O production can occur during both, aerated and non-aerated phases (Paper I). The transition from non-aerated to aerated phases leads to a sudden release of dissolved N_2O from the liquid phase, that shows itself in a characteristic initial emission peak at the onset of aerated phases, which is commonly observed in intermittently aerated PNA systems (Castro-Barros et al., 2015; Yang et al., 2015)(Paper I).

2.2 Peak analysis of off-gas profiles

In order to quantify net N_2O production in different phases of a SBR cycle, e.g. to distinguish between the contribution of aerated and non-aerated phases to total N_2O emission, emission profiles can be subject of peak analysis and subsequent integration. Peak analysis is a standard procedure to unravel convoluted peaks and analysis tools are readily integrated in data analysis software like OriginPro (OriginLab, 2018). In short: the algorithms apply first,

second or residual after first derivative methods to detect local maxima in the emission profiles. After the detection of individual peaks, Gauss functions were fitted to the curve until optimal convergence, which was determined by an adjusted R^2 value and the percentage of total integrated area by the model. The areas of individual peaks are then integrated, thus giving the amount of N_2O emitted per unit of time. An example is given in Fig. 7.



Fitting Results

Peak Index	Peak Type	Area Intg	FWHM	Max Height	Center Grvty	Area IntgP
1	Gauss	0.2772	13.98258	0.01893	206	94.32865
2	Gauss	0.01667	3.98356	0.00393	213	5.67135

Figure 7 – Peak analysis of N_2O emissions from an aerated/non-aerated phase pair during continuous stripping experiment (see 1.3.1). Two distinct peaks (green curves) are convoluted and form the measured off-gas N_2O profile (black curve). Red: fit to measured N_2O profile and accumulated area of identified peaks, adj. R^2 = 0.989 and integrated area of model = 99.99%.

The procedure can be directly applied to intermittent aeration off-gas profiles. However, in order to reduce masking effects of intermittent stripping, it is helpful to obtain off-gas profiles from both intermittent and continuous gas stripping experiments (Paper I). In order to reveal N_2O production during non-aerated phases, N_2O is stripped in continuous gas stripping experiments from the liquid phase with an inert gas, e.g. N_2, during non-aerated phases at the same rate as during aerated phases (Mampaey et al., 2015). The use of inert gases does not alter redox conditions in the non-aerated phases and maintains the total oxygen load. Under the assumption that stripping occurs so rapidly that $N_2O_{produced} \approx N_2O_{emitted}$, measurements from off-gas analysis can be used to quantify net N_2O production rates throughout a SBR cycle. A disadvantage of the continuous gas stripping method is that it reduces the retention time of N_2O in the liquid phase during non-aerated phases. Therefore the contribution of non-aerated phases to overall N_2O emission can be over-

estimated, since produced N_2O is directly removed from the system instead of being subject of N_2O consumption.

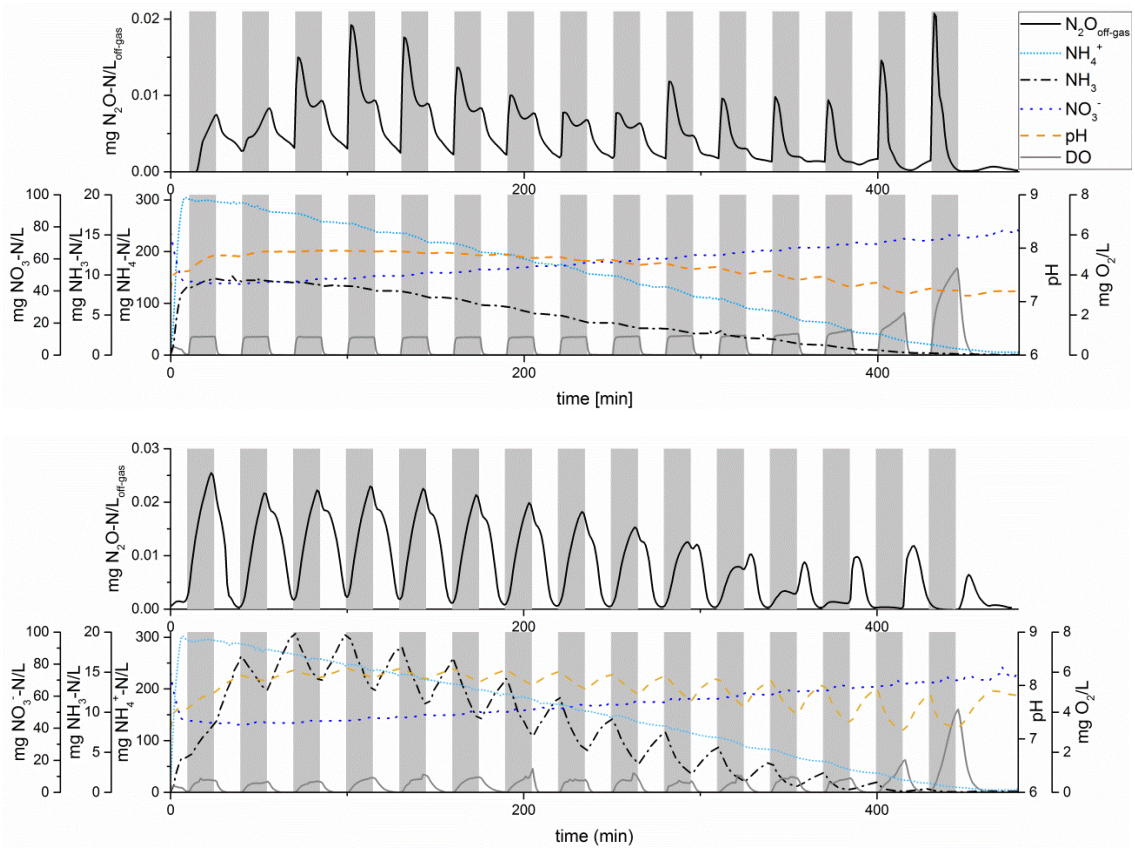


Figure 8 – Concentration profiles during the course of SBR cycles under standard operation (top) and continuous gas stripping experiment (bottom). Shaded areas represent aerated phases.

2.3 Measurements in the water phase - N_2O microsensors

N_2O can also be measured in the liquid phase by Clark-type sensors (Unisense, 2018). N_2O sensors are particularly useful to follow net N_2O production dynamics in non-aerated phases. However, also during phases of gas stripping, the instantaneous N_2O production rate, r_i, can be calculated, when the k_La value is known (Domingo-Félez et al., 2014):

$$r_i = \frac{\Delta N_2O_i}{\Delta t} + k_L a_{N_2O} * N_2O_i \quad (12)$$

$\Delta N_2O_i/\Delta t$: accumulation term estimated from linear regression
$k_La_{N2O}*N_2O_i$: stripping rate

For full-scale systems the determination of k_La can be challenging and errors may reduce the certainty of calculated rates. However, mathematical models to estimate the k_La are available and N_2O emissions calculated based on micro-sensor measurements have been demonstrated to be in good agreement with off-gas measurements (Baresel et al., 2016). The match between both methods was also demonstrated in the presented system, when both the off-gas and the $N_2O_{(aq)}$ concentration were measured in the same experiment (data in Paper I, Fig. 2). The total amount of N_2O emitted was 10.7 and 11.0 mg N_2O-N emitted per cycle, as determined by peak-analysis and micro-sensors, respectively. The resulting N_2O emission factors were 2.76% and 2.66% N_2O-N emitted per NH_4^+-N removed, respectively.

For practical reasons a combination of off-gas measurements and microsensors is advisable. On the one hand, the measurement of $N_2O_{(aq)}$ during aerated phases evaluates the assumption of off-gas analysis ($N_2O_{produced} \approx N_2O_{emitted}$) and captures N_2O consumption during non-aerated phases. On the other hand, off-gas analysis acts as a control for calculated instantaneous N_2O production rates based on dissolved N_2O.

2.4 Contribution of oxic and anoxic N_2O production to overall emission

For the studied system, the continuous gas stripping experiment clearly showed that the initial emission peaks, as observed under standard operation, were N_2O from previous non-aerated phases. N_2O production during aerated phases rather increased steadily with ongoing aeration time (compare off-gas N_2O profiles in Fig. 8). It further revealed that N_2O production during aerated phases declined with cycle time, while N_2O production during non-aerated phases was relatively constant and emerged as distinct peaks towards the end of the reaction phase (Paper I). Overall, aerated phases contributed to approx. 96% of N_2O emissions. Approx. 80% of N_2O were also produced during aerated phases, while the remaining approx. 20% were produced during non-aerated phases (Paper I). Accordingly, aerated phases were not only the dominant source of N_2O emissions, but also net N_2O production. The results of a number of studies agree qualitatively with this distribution between aerated and non-aerated phases, although the contributions were not quantified rigorously (Castro-Barros et al., 2015; Domingo-Félez et al., 2014; Yang et al., 2013). It became also clear that process operation, the accumulation of N-species (particularly of NO_2^-) and the N_2O consumption capacity of the biomass can alter the contribution of aerated and non-aerated phases to total N_2O

emissions (Paper II & Paper IV)(Domingo-Félez et al., 2014). Especially when the contribution of non-aerated phases is reduced, e.g. by enhanced N_2O consumption, aerated phases become the dominant source of N_2O emission and production (up to 100% of total N_2O emission (Paper IV)).

The specific N_2O production rate during aerated phases correlated with the specific ammonia removal rate, both in PNA systems and AOB enrichment cultures (Law et al., 2012a)(Paper I)(see

Fig. 9). The correlation points towards a dominant contribution of nitrifying bacteria and the nitrifier nitrification pathway, which is linked to NH_4^+ and NH_2OH oxidation (Ma et al., 2017). However, due to the simultaneous NO_2^- production in aerated phases, nitrifier denitrification is likely also active during aerated phases, when NH_2OH and NO_2^- diffuse into deeper, anoxic zones of the biofilm (Ali et al., 2016; Sabba et al., 2015). In contrast, during non-aerated phases the specific N_2O production rate correlated with the NO_2^- concentration (Fig. 9)(Paper I). The correlation strongly suggests that that nitrifier denitrification is the dominant N_2O production pathway upon change of oxic to anoxic conditions. The contribution of the pathway to overall N_2O production has been demonstrated to dominate over nitrifier nitrification already at low NO_2^- concentrations (≥ 2 mg NO_2^-N/L), when oxygen tensions are low (Ali et al., 2016; Ma et al., 2017; Wunderlin et al., 2013).

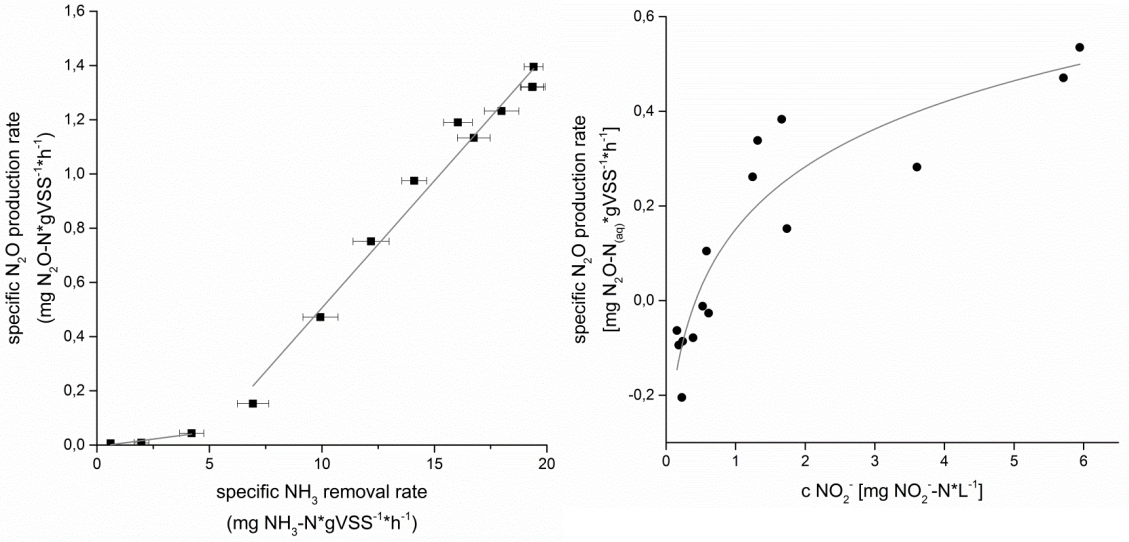


Figure 9 – Specific N_2O production rates as a function of specific NH_3 removal rates during aerated phases (left) and NO_2^- concentration during non-aerated phases (right). Details in Paper I.

3 Nitrous oxide production and consumption pathways

Nitrogen conversion reactions in PNA biomass are manifold and are catalyzed by a variety of enzymes (Fig. 10)(Paper III)(Schreiber et al., 2012). The enzymes are expressed by different functional guilds such as AOB, AnAOB, NOB and HD (see 1.1.5). N_2O production in PNA is the result of biological activity (Law et al., 2012b). Although, abiotic reactions of NH_2OH to N_2O have been described, NH_2OH itself is the result of biological NH_3 oxidation, thus making abiotic N_2O production biologically driven (Domingo-Félez and Smets, 2016; Kampschreur et al., 2009).

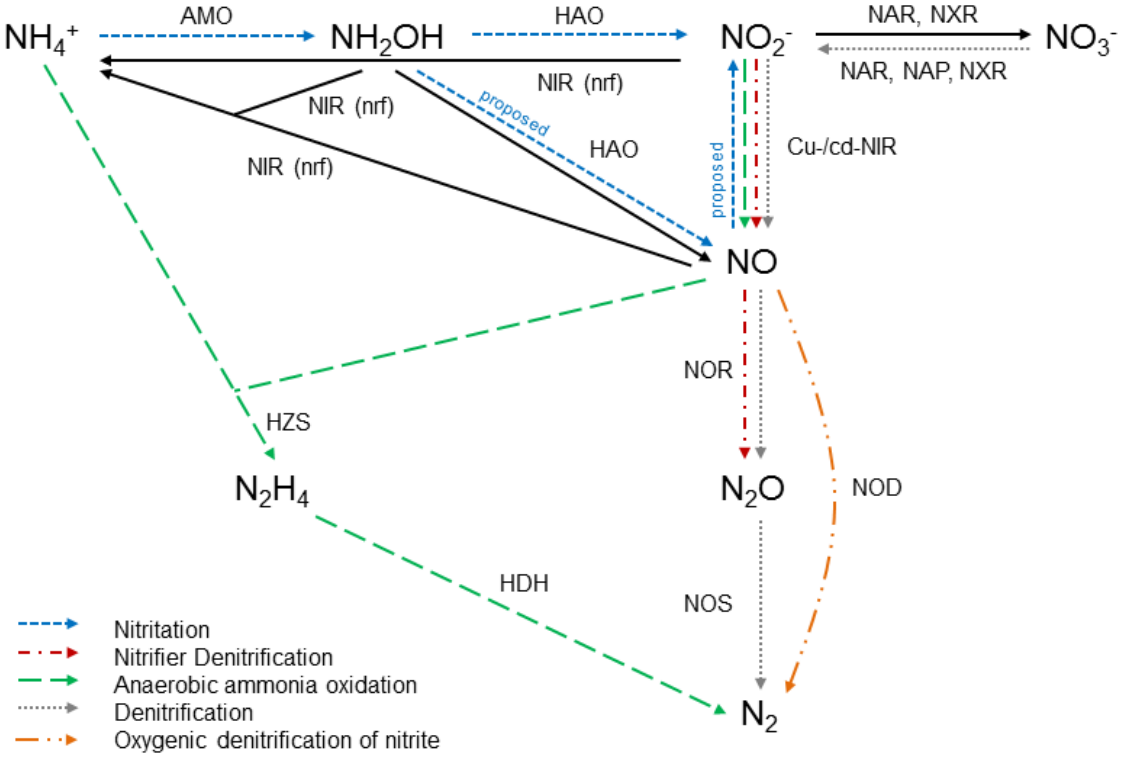


Figure 10 - Web of nitrogen conversion reactions. Enzymes involved in catalysis are ammonia monooxygenase (AMO), hydroxylamine dehydrogenase (HAO), nitratereductase (NAR), periplasmic nitrate reductase (NAP), nitrite oxidoreductase (NXR), nitrite reductase (NIR), nitric oxide reductase (NOR), nitrous oxide reductase (NOS), hydrazine synthase (HZS), hydrazine dehydrogenase (HDH) and nitric oxide dismutase (NOD) [figure from Paper III].

3.1 N_2O production by ammonia oxidizing bacteria

AOB have been identified as the main source of N_2O in PNA systems (Ali et al., 2016). The N_2O production routes in AOB are diverse and involve respiratory and dissimilatory pathways like hydroxylamine oxidation and nitrifier denitrification, but also N_2O production by for instance soluble cytochromes or the aforementioned indirect N_2O production by abiotic NH_2OH oxidation (Caranto et al., 2016; Kampschreur et al., 2009; Stein, 2010).

3.1.1 Hydroxylamine associated N_2O production pathways

A number of different N_2O production routes are associated with NH_2OH oxidation. Some involve enzymes of respiratory pathways, like hydroxylamine dehydrogenase, others are of detoxifying character and do not yield electrons for the cellular electron pool (Stein, 2010). Some convert NH_2OH directly to N_2O, while others yield NO so that N_2O production is rather the result of NO reduction by enzymes like nitric oxide reductase (NOR) or detoxification mechanisms that involve, e.g. flavohemoglobins, flavorubredoxins or cytP_{460} (Caranto et al., 2016; Stein, 2010). The enzyme hydroxylamine dehydrogenase (HAO) plays a central role in N_2O production by AOB, as it controls the availability of NH_2OH and, thus, the downstream NH_2OH associated N_2O production routes (Paper III).

HAO is a homotrimer that is located in the periplasm of AOB, such as *Nitrosomonas europaea*. In each monomer a P_{460} haem constitutes the catalytic site (Fig. 11). The P_{460} is accessible for the substrate NH_2OH through a pocket that enables exchange with the bulk liquid. NH_2OH directly binds to the catalytic iron ion (Igarashi et al., 1997). The extraction of electrons from NH_2OH proceeds stepwise via intermediate P_{460}-NO complexes (Ford and Lorkovic, 2002; Kostera et al., 2010)(Fig. 11). Extracted electrons are then transferred via a series of hemes to electron carriers, such as cytochrome c_{554}, and leave the enzymatic complex (Igarashi et al., 1997).

It is currently under debate, whether NO is the main product of HAO or rather a by-product of unbalanced oxidation. Although *in vitro* experiments with *Nitrosomonas europaea* demonstrated that NO was the primary product of HAO (Caranto and Lancaster, 2017), the bias of *in vitro* experiments and their relevance for enzymes under physiological conditions receives increasing criticism (García-Contreras et al., 2012; van Eunen and Bakker, 2014).

Generally, the idea of one "true" product of HAO may be misleading. Instead, the NO/NO_2^- product ratio of the enzyme may rather be the result of an interplay between substrate and electron carrier concentrations, the catalytic turnover and efflux rates of electrons from the enzymatic complex to receiving electron carriers. Therefore, the concentration of NO or NO_2^- released by HAO may vary with process parameters. However, the molecular mechanisms and the connection between HAO and the electron transport chain of AOB are not fully understood. Box 1 presents evidence that in fact the retention time of electrons in HAO may determine, whether the product ratio shifts towards NO or NO_2^-, respectively.

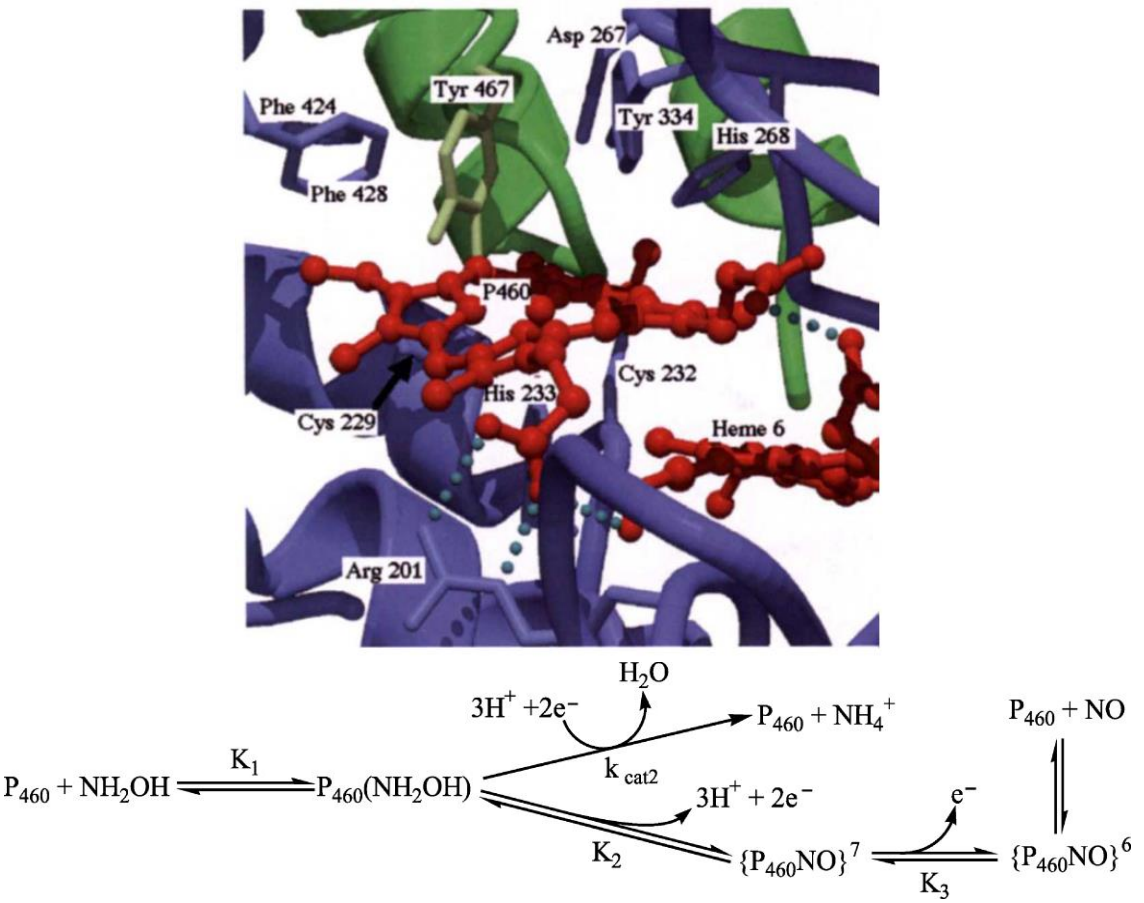


Figure 11 – Top: Schematic drawing of the catalytic pocket of hydroxylamine dehydrogenase [from (Igarashi et al., 1997), PDB entry: 1FGJ]. Bottom: Intermediate complexes during 3 e^- NH_2OH oxidation to nitric oxide [from (Kostera et al., 2010)].

Irrespective of the exact molecular mechanisms of NH_2OH oxidation, stable isotope labelling experiments demonstrated that the contribution of hydroxylamine associated N_2O production via the nitrifier nitrification (NN) pathway

increased with increasing ammonia removal rates at low oxygen concentrations (DO < 1 mg O_2/L)(Ali et al., 2016; Ma et al., 2017; Wunderlin et al., 2013). In contrast, the contribution of NN at high NH_4^+ concentrations and high oxygen concentrations (1-2 mg O_2/L) was relatively unimportant (Harris et al., 2015). Together, the findings support the hypothesis that the release of NO by HAO (with subsequent N_2O production) may be linked to the availability of oxygen and the concentration of oxidized electron carriers, such as c_{554}. However, further research is required to test this hypothesis.

3.1.2 The nitrifier denitrification pathway

Besides their nitrifying capacity, AOB can also reduce NO_2^- to N_2O via the nitrifier denitrification (ND) pathway (Wrage et al., 2001)(see Fig. 10). The enzymes involved are NIR and NOR. The electron donors for the reduction steps may be NH_2OH or the cellular pool of reduced electron carriers. Rates of ND increase with decreasing oxygen tension and increasing NO_2^- concentration (Zhu et al., 2013). The physiological role of ND is not entirely clear. However, the removal of intracellular NO_2^- to avoid its toxicity and the oxidation of reduced electron carriers under conditions of low oxygen tension are likely purposes of the pathway. The current model of electron flow in AOB describes that when electrons from NH_2OH oxidation cannot be accepted by O_2 under O_2 limited conditions, a limitation of electron flow may occur between HAO and membrane bound cytrochrome c_{M552}, or in the soluble electron carrier pool of c_{552} between cytochrome complexes C-III and C-IV (Fig. 1)(Kozlowski et al., 2016; Simon and Klotz, 2013). The activation of NIR and NOR during ND may then allow the transfer of excess electrons to NO_2^- (Kozlowski et al., 2016)(Paper III).

Box 1. Release of nitric oxide from hydroxylamine dehydrogenase – a model proposal

Simplified, the $4e^-$ that are released during the oxidation of NH_2OH to NO_2^- take the path: a) NH_2OH -> b) P_{460} -> c) hemes -> d) c_{554}; the substrate NH_2OH binds to the active site P_{460}, where it is oxidized in two sequential steps: i) NH_2OH -> (HNO) + $2H^+$ + $2e^-$ and ii) (HNO) + H_2O -> HNO_2 + $2H^+$ + $2e^-$ (Andersson and Hooper, 1983; Fernández et al., 2008). The two electrons from the first reaction are transferred to the heme subunits of the enzyme and are transported through the complex until they reach the heme 1 group. When the electrons of the first oxidation step are not immediately removed from P_{460}, P_{460} cannot accept the second pair of electrons from the second oxidation step and the intermediate complex $\{P_{460}NO\}^6$ may decompose rapidly to NO (Igarashi et al., 1997; Kostera et al., 2010). Intermediate NO complexes have been measured to dissociate from Fe^{III}(POR), similar to protoporphyrins in hemes, at rates between $0.5\text{-}0.9x10^3$ s^{-1} (Ford and Lorkovic, 2002). At the heme 1 group, the electrons are transferred to the electron acceptor cytochrome c_{554} at rates similar or higher than the reduction rate of P_{460} by NH_2OH, which indicates that the electron transfer to c_{554} is not rate limiting (Arciero et al., 1991). Each c_{554} can accept two electrons, which means that for the complete oxidation of NH_2OH with the transfer of $4e^-$, at least one exchange of c_{554} needs to occur (Arciero et al., 1991; Kurnikov et al., 2005). Therefore, the efflux rate of electrons from the enzyme is limited to the time scale of diffusion of c_{554}. The question arises, if the diffusion of c_{554} could become rate limiting. For details on the reaction kinetics of HAO the interested reader is referred to (Arciero et al., 1991; Hooper et al., 1997, 1984; Igarashi et al., 1997; Kurnikov et al., 2005).

Studies investigated how the NO/NO_2^- product ratio of HAO is regulated (Cabail and Pacheco, 2003; Kostera et al., 2010): The balance between electron in- and efflux from the enzymatic complex appears to be relevant. While studying the NO reductase activity of the electron acceptor c_{554} Upadhyay and colleagues detected a significant amount of NO species, when the ratio of HAO/cyt c_{554} was low (HAO/cyt c_{554} = 0.05)(Anup K. Upadhyay et al., 2006). When the HAO/cyt c_{554} = 1.00 more substrate (NH_2OH) was required to detect NO species. In the case of a low HAO/cyt c_{554} the authors argue that the lack of binding sites between HAO and c_{554} lead to an inefficient flux of electrons out of HAO, which promoted the release of NO species (Anup K. Upadhyay et al., 2006).

(continued on next page)

Box 1. (continued) - Release of nitric oxide from hydroxylamine dehydrogenase – a model proposal

The possibility that one c_{554} molecule remains bound to the enzyme and transfers the electrons to another c_{554} molecule is low, as the authors found that c_{554} molecules exchange electrons among each other only at a slow rate of 0.01 s^{-1}, which is low compared to the catalytic turnover of HAO (30 s^{-1}). Similarly, Perez-Garcia and colleagues discuss the balance between electron donors and acceptors as a governing factor for the release of NO from HAO (Perez-Garcia et al., 2014). In their metabolic flux model they identify the reaction at P_{460} and at the transient NO-complex (in their study termed CytP460 and HAO-hno2) to have a prominent effect on the release of NO (NO-Ex). Both studies suggest that the concentration of electron donors and electron acceptors play a role in the release of NO. In short: the electron efflux rate from P_{460} to heme6 may determine, whether NO or NO_2^- is the product of HAO (Igarashi et al., 1997)

We may, therefore, hypothesize that under low oxygen tension electron carriers like c_{554} are not rapidly enough oxidized, which results in a relatively low concentration of c_{554ox}, which in turn does not meet the demands of electron efflux from HAO and thus causes a relatively higher NO leakage from P_{460}. In contrast, at high oxygen tension the availability of c_{554ox} may be high enough to compensate increased NH_2OH oxidation rates, since a fast reduction of molecular oxygen by AMO allows a rapid oxidation of c_{554red} by the quinone pool (see also Fig. 1).

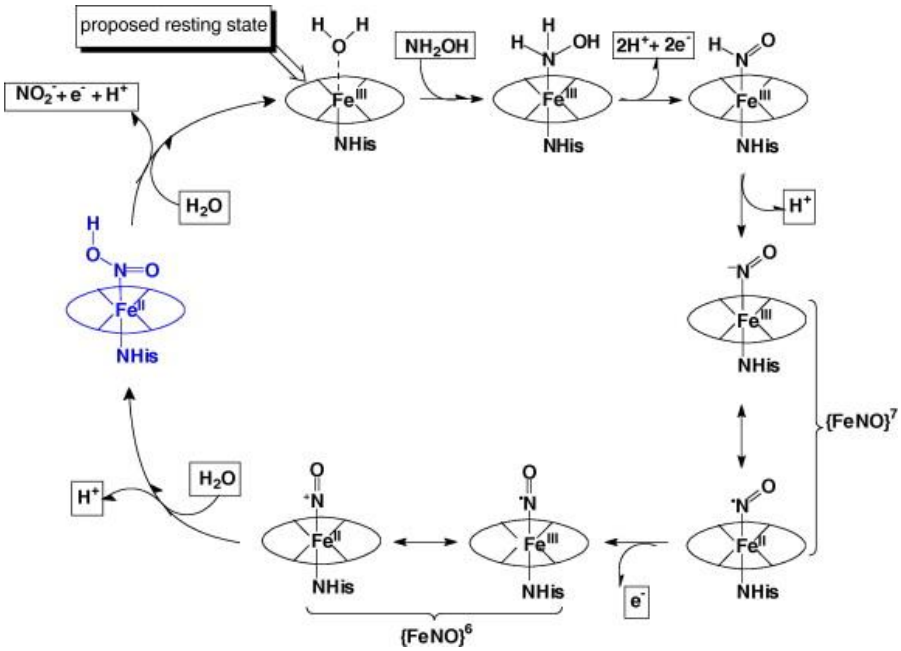


Figure B1. Putative N-containing intermediates at the P_{460} active site of hydroxylamine oxidoreductase in the conversion of NH_2OH into NO_2^- [figure from (Fernandéz et al., 2008)]

3.2 N_2O production and consumption by heterotrophic bacteria

3.2.1 Nitrous oxide reductase and (in)complete denitrification by heterotrophic denitrifiers

The last step of denitrification, the reduction of N_2O to N_2, is exergonic ($\Delta G°' = -339.5$ kJ mol^{-1}), but an activation barrier of +250 kJ mol^{-1} needs to be overcome for the reaction to occur (Jones, 1973; Zumft and Kroneck, 2006):

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \qquad (13)$$

There is only one know biological catalyst, which accelerates the reduction: nitrous oxide reductase (N_2OR). So far, two different bacterial clades I and II have been defined that host N_2OR carrying organisms. N_2OR cI and cII have slightly different structures, which may result in different electron transfer routes to different electron carriers. However, the catalytic cycle is likely the same between both clades (Figure 12)(Carreira et al., 2017).

N_2OR is a homodimer (120-160 kDa) that is located in the periplasm of mainly gram-negative bacteria. Each monomer contains two copper centers, CuA and CuZ (Carreira et al., 2017). CuA is located close to the surface of the enzymatic complex and accepts electrons from electron carriers, like c_{550}, c_{552} or pseudoazurin (Gorelsky et al., 2006; Mattila and Haltia, 2005). The electrons are then transferred to CuZ, where rapid turnover of N_2O to N_2 occurs ($k_{cat} \sim 300$ s^{-1}) (Dell'Acqua et al., 2008).

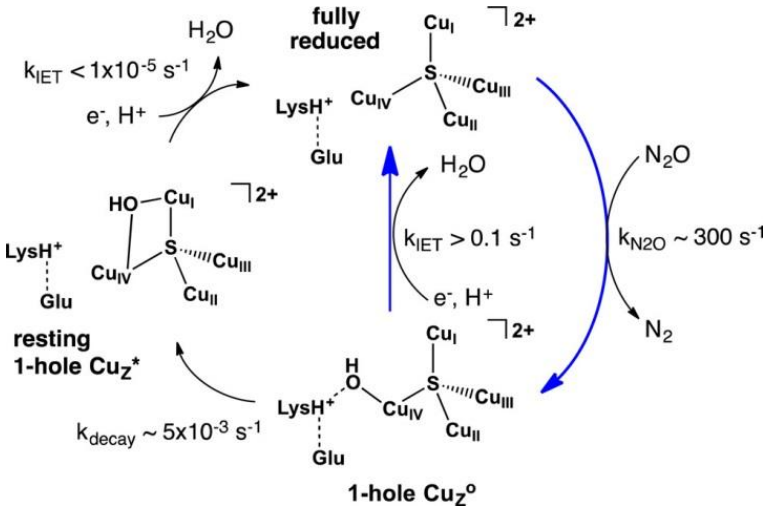


Figure 12 - Pathways of $Cu_Z°$ Formation, Reduction, and Decay to Resting 1-Hole Cu_Z* with Relevant Rates, with Blue Arrows Showing Steps Involved in Catalytic Turnover (figure from (Johnston et al., 2017)).

A prerequisite for heterotrophic denitrifiers to reduce N_2O is to possess the *nosZ* gene, which encodes N_2OR (Pauleta et al., 2013). However, not all denitrifiers possess the gene. In fact, truncated or partial denitrifying genetic potentials have been described for various microorganisms (Graf et al., 2014). The reasons for bacteria to not have the full genetic potential for denitrification are under debate (Roco et al., 2017) and niche differentiation may play a role (Graf et al., 2014; Jones and Hallin, 2010). Almost all combinations of truncated denitrifying potential have been described: organisms that only possess *nirK*, *nirS*, *nor* or *nosZ*, or different combinations of them, e.g. *nirK-nor*, *nirS-nor*, *nirK-nosZ*, *nirS-nosZ*, *nor-nosZ*, *nirK-nirS* or *nirK-nor-nosZ* (Graf et al., 2014). In respect to net N_2O production this becomes relevant, when organisms lack the *nosZ* gene and thus become N_2O producers or, in contrast, only possess *nosZ* and thus become potential sinks of N_2O. Consequently, the manipulation and management of the relative abundance of *nosZ* carrying organisms has an effect on the N_2O:N_2 product ratio of denitrifying microbial communities (Philippot et al., 2011).

For Microbial Resource Management two questions arise:

a) Which environments foster the occurrence of *nosZ* carrying microorganisms?

b) Which operational conditions favor N_2OR activity?

In order to find answers to the first question, one must assume that a correlation between the occurrence of different denitrifying genes and the environmental conditions exists. That has recently been demonstrated by an intergenomic comparison of denitrifying bacteria from various environments (Graf et al., 2014). Generally, wastewater treatment plants appear to provide a good environment for complete denitrifiers and *nosZ* carrying bacteria. *nirK/S-nor-nosZ* genomes were significantly overrepresented, when compared to other environments, like soils, fresh water or animal hosts. Particularly a positive correlation of *nirS* with *nosZ* indicated that *nirS* denitrifiers are more likely to perform complete denitrification, than *nirK* denitrifiers are (Graf et al., 2014). Wastewater treatment plants with their maximal achievable water content, as compared to for instance soils, appear to already constitute a *nirS* selecting environment, which also increases the abundance of *nosZ* (Petersen et al., 2012). The management of the abundance of *nosZ* in a microbial community may, therefore, rather be driven by a selection for *nirS* over *nirK*, than for *nosZ* itself. However, environmental factors that select *nirS* over *nirK* denitrifiers are not well understood. Frequently changing conditions in an envi-

ronment may give advantages for *nirK* carrying organisms (Petersen et al., 2012). Interestingly, microorganisms that only carried *nosZ* were overrepresented in marine habitats (Graf et al., 2014).

A variety of organisms exist that possess the *nosZ* gene, but that are not classified as denitrifiers (Jones et al., 2013). These organisms are clustered in clade II of *nosZ* phylogeny and often affiliate with Bacteriodetes, Gemmatimonadetes and Deltaproteobacteria (Hallin et al., 2018). Research is only beginning to investigate their potential for N_2O mitigation. Although N_2O as a sole electron acceptor is sufficient for growth, an enrichment of clade II *nosZ* carrying organisms in complex microbial communities may be challenging, as they appear to have lower maximum growth rates and lower N_2O affinities, than clade I *nosZ* carrying organisms, although contrasting findings have been reported (Conthe et al., 2018; Yoon et al., 2016). Future research will show if a selection for clade II nosZ can increase N_2O consumption rates in microbial communities.

3.2.2 Enhancement of N_2OR activity

The presence of *nosZ* in a denitrifying community is one prerequisite for N_2O reduction. However, N_2OR activity is also affected, when the transcription, translation, the assembly of the functional enzyme or the activity of N_2OR are inhibited. Concentrations of O_2, NO and Cu-ions are the main controlling factors of *nosZ* expression, when the presence of NO and Cu-ions triggers expression, but elevated O_2 concentrations suppress it (see (Spiro, 2012; Sullivan et al., 2013) for details). NO, O_2 and Cu-ions do not affect the transcription of *nosZ* directly, but regulate the transcription or activity of transcription regulating proteins. Notably, no regulating mechanism based on N_2O concentration has been found (Spiro, 2012).

Once expressed, the assembly and activity of N_2OR is mainly impeded by the availability of copper ions and pH. Each N_2OR homodimer requires 12 Cu ions for the successful formation of its copper-centers CuA and CuZ (Pauleta et al., 2013). A low availability of Cu ions may therefore hamper the successful assembly of N_2OR and reduce N_2O reduction rates (Richardson et al., 2009). The pH exerts a post-translational effect on N_2OR (Liu et al., 2014). Low pH appears to prevent the successful assembly of the enzyme, although the mechanism is not fully understood. Already assembled N_2OR was active over a wide range of pH also at pH, when an assembly of the enzyme was not successful (Liu et al., 2014).

The activity of fully assembled N_2OR is also affected by pH (Paper III). Two augmenting effects of pH constrain the enzyme's pH optimum to approx. pH 7-8. At more acidic pH ($4 < pH < 8$) the electron transfer rate from CuA to CuZ is rate limiting due to conformational changes of CuA (Gorelsky et al., 2006). At more alkaline pH ($pH > 8.9$) N_2OR activity is also reduced. The deprotonation of a lysine residue (lys397) in CuZ at alkaline pH likely disables the residue to stabilize the $Cu_Z°$ intermediate during the catalytic cycle of N_2O reduction, which may result in a loss of catalytic turnover (Johnston et al., 2017). In alignment with the considerations of the effect of pH on the molecular level, N_2O consumption rate measurements of PNA biomass at different pH set-points demonstrated an increase of N_2O consumption rates with pH (Fig. 13)(Paper IV).

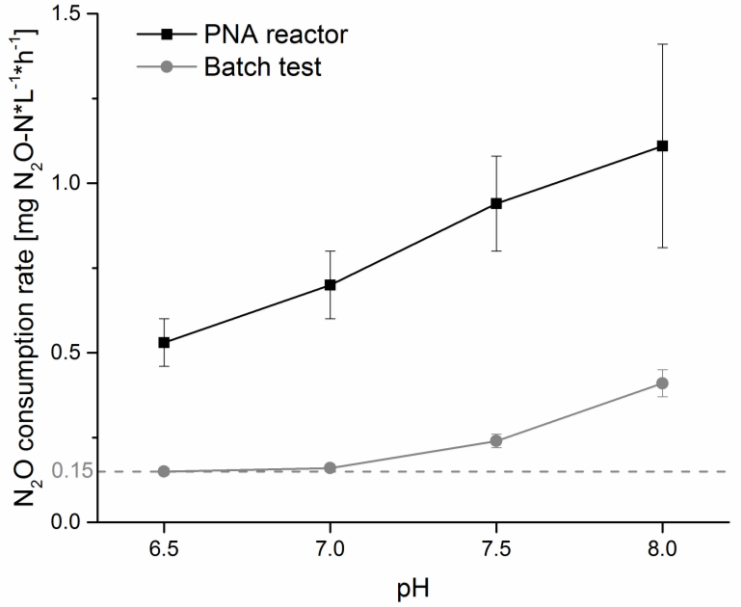


Figure 13 – N_2O consumption rate of biomass from Partial Nitritation-Anammox reactor at different pH (Paper IV). Dotted line: threshold detection level in control experiment. Grey line: consumption rate in batch experiments. Black line: consumption rates inferred from in situ $N_2O(aq)$ profiles from PNA SBR at different pH set-points. Differences to batch experiment is caused by different biomass concentrations.

3.3 Balancing N_2O production and consumption pathways

Net N_2O production is the result of unbalanced fluxes through the N-cycling network. Three strategies appear most promising for balancing N_2O production and consumption rates and thus mitigate N_2O emissions:

1. Reduce specific AOR to avoid accumulation of NH_2OH and NO_2^- and to reduce N_2O production by NH_2OH oxidation pathway
2. Increase sink of NO_2^- to reduce N_2O production by nitrifier denitrification
3. Increase N_2O consumption rate by *nosZ* carrying organisms

Some of the strategies have been demonstrated to have a positive effect on N_2O mitigation (Vlaeminck et al., 2012), others are tested and refined in this project (mainly Paper II & Paper III). In general the approaches to fulfill said tasks can be categorized in:

- Microbial community engineering: Change ratio of functional guilds based on the assumption that more microorganisms that carry a certain gene lead to higher numbers of enzymes, which in turn leads to enhancement of a certain metabolic route over another, e.g. higher number of gene copies of *nosZ* in community leads to larger N_2O consumption rates.
- Enzyme activity management: Change enzyme activity by providing certain environmental conditions based on the assumption that the amount of microorganisms that carry a specific is not directly correlated with the amount of active enzymes, but that the environment determines enzyme activity and fluxes through metabolic pathways, e.g. increase N_2O consumption rate by providing optimal environmental conditions for N_2OR (here change of pH).

In the following, chapter 4 explores microbial community engineering, while chapter 5 focuses on enzyme activity management.

4 Bio-granules as sinks and sources of nitrous oxide

4.1 Bio-granule architecture & optimal granule size

The substrates NH_4^+ and DO are supplied in the bulk liquid of granular PNA systems and diffuse from the same side into the biofilm. Therefore, bio-granules are co-diffusional biofilms (Fig. 14.A), as opposed to counter-diffusional biofilms, when for example DO is supplied at the bottom of a biofilm through membranes (Nerenberg, 2016). In co-diffusional systems, NH_4^+ and DO concentrations are high on the surface of granules and cause AOB to accumulate in the outer layer of the biofilm (Fig. 14.B). Due to the metabolic activity of AOB, NH_4^+ and DO concentrations decline with depth. While NH_4^+ is commonly present in abundance and diffuses deep into the granule, DO is rapidly consumed and commonly depleted in the outer 100 µm (Vázquez-Padín et al., 2010). In deeper layers of the biofilm anoxic conditions prevail. NO_2^- that is produced by AOB diffuses a) into the bulk liquid and b) into deeper layers of the biofilm, where it becomes available for AnAOB.

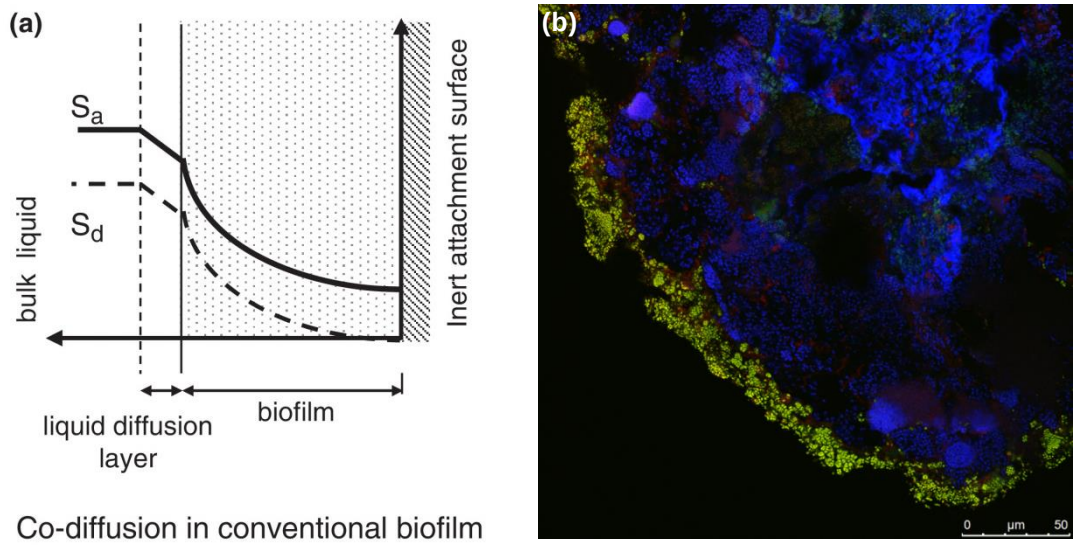


Figure 14 - (a) Substrate gradients in a co-diffusional, conventional biofilm. The bold line is the substrate released by the reactive surface (electron acceptor, S_a, in this case), while the dashed line indicates the substrate from the bulk liquid (electron donor, S_d, in this case)(Figure from (Nerenberg, 2016)). (b) FISH image of sectioned bio-granule. AOB (green) accumulate at the rim of the biofilm, while AnAOB (blue) occur at deeper layers.

Granules are of spherical shape and the change of surface to volume ratio with granule diameter can be approximated by 3/r (r = radius), i.e. larger granules have a smaller surface to volume ratio. For the same biomass concentration in a reactor, the surface area that is exposed to the bulk liquid is therefore relatively smaller for large granules, than for small ones. Consequently, the ammonia surface load is larger in larger granules, which causes the oxygen penetration depth to decrease to commonly only 20 µm (Vázquez-Padín et al., 2010; Volcke et al., 2012)(Fig. 14.B). In contrast, the anoxic volume increases with granule size. Thus, granules of different size provide different volumetric fractions of oxic and anoxic environments for aerobic and anaerobic microorganisms. Accordingly, the AOB/AnAOB ratio decreases with increasing granule radius (Paper II): small granules are dominated by AOB and constitute NO_2^- sources, balanced NO_2^- production and consumption occurs at medium granule diameters and a large fraction of AnAOB in large granules leads to net NO_2^- consumption (Vlaeminck et al., 2010). The granule size and abundance of different microorganisms therefore becomes relevant for stable process performance and low NO_2^- accumulation (Volcke et al., 2010). An 'optimal' granule size depends on various factors, like volumetric NH_4^+ loading rates, bulk DO concentrations and required effluent limits and therefore is specific for specific systems (Volcke et al., 2012, 2010).

4.2 Selection of granules with favourable size

Granules can be separated from floccular sludge granules by short settling times, when slow settling flocs are discharged with the effluent, while faster settling granules remain in the reactor. However, separation of differently sized granules by wash-out is often insufficient, as differences in density and settling velocities between small and large granules are low (Milferstedt et al., 2017). A simple way of selecting large granules over small ones is by sieving. Alternatively, granules can be separated based on settling velocity, when faster settling granules (usually also the larger granules) form the bottom of a sludge bed, while slow settling granules (usually small granules) gather on top, once mixing is stopped. The small granules can then be removed mechanically (Winkler et al., 2011). In full-scale systems a more viable method is the separation of granules by means of centrifugal force in for instance hydro cyclones (Wett et al., 2010). All methods described rely on physical or mechanical separation and require that, at given process conditions, granules mature to or beyond the desired granule size, when given suf-

ficient time. However, granules do not always grow extensively (Paper I and Paper II). A potential explanation is that nutrient limitation constraints granule size and therefore dictates the ratio of functional microbial guilds. In PNA systems NO_2^- has been demonstrated to be – depending on process operation – already depleted at low biofilm depth and was therefore hypothesised to limit growth of AnAOB at deeper layers (Vázquez-Padín et al., 2010). NO_2^- limitations may especially occur in intermittently aerated PNA systems with high aeration frequency and present a constraint for *in situ* granule size management (Domingo-Félez et al., 2014).

4.2.1 Intermittent aeration regimes as tools to manipulate granule size and microbial community

The transient accumulation of NO_2^- in the presented PNA system was low (< 2 mg NO_2^-N/L). The assumption that AnAOB in deeper layers of the biogranules were NO_2^- limited would explain an observed steady and small median particle diameter of approx. 200 µm (Paper I). In order to accumulate more NO_2^- in the bulk liquid, the positive net NO_2^- production rate in aerated phases was used (Paper I). Indeed, an extension of aerated phases resulted in an accumulation of NO_2^- to higher concentrations of approx. 5 mg NO_2^--N/L and appeared as a feasible strategy to increase granule size (Table 1 and Fig. 15)(Paper II). It was further hypothesized that a lower AOB/AnAOB ratio in larger granules would constitute a larger NO_2^- sink capacity, thus increase the relative flux of NO_2^- to AnAOB, instead of the nitrifier denitrification pathway of AOB. In short: larger were granules were hypothesized to result in lower N_2O production by nitrifier denitrification. With increasing granule size the AOB/AnAOB ratio declined as expected from approx. 3:1 in granules with a median particle diameter of 230 µm to approx. 1:1 and 1:2 at 391 µm and 1020 µm, respectively (Fig. 16)(Paper II). Fluorescence *In Situ* Hybridization (FISH) microscopy revealed that AnAOB were present in the deep layers of the granules and that the larger granule diameter was not caused by accumulation of inert material in the granule core (Fig. 17). Aeration regimes therefore appear as feasible tools manipulate the granule size and thus the relative abundance of different microbial guilds in PNA bioreactors.

Table 1 – Diameters of granules in sequencing batch reactors under standard (R1) and long aeration phase operation (R2) at beginning (day 0) and end of experiment (day 67). d0.5: median particle diameter, d0.1: 10th percentile, d0.9: 90th percentile, d0.1-d0.9: difference between d0.1 and d0.9 holds information about span of particle size distribution.

	Day 0				Day 67			
	d0.1	d0.5	d0.9	d0.1-d0.9	d0.1	d0.5	d0.9	d0.1-d0.9
R1	168	310	556	388	103	230	490	387
R2	168	300	531	363	123	391	1020	897

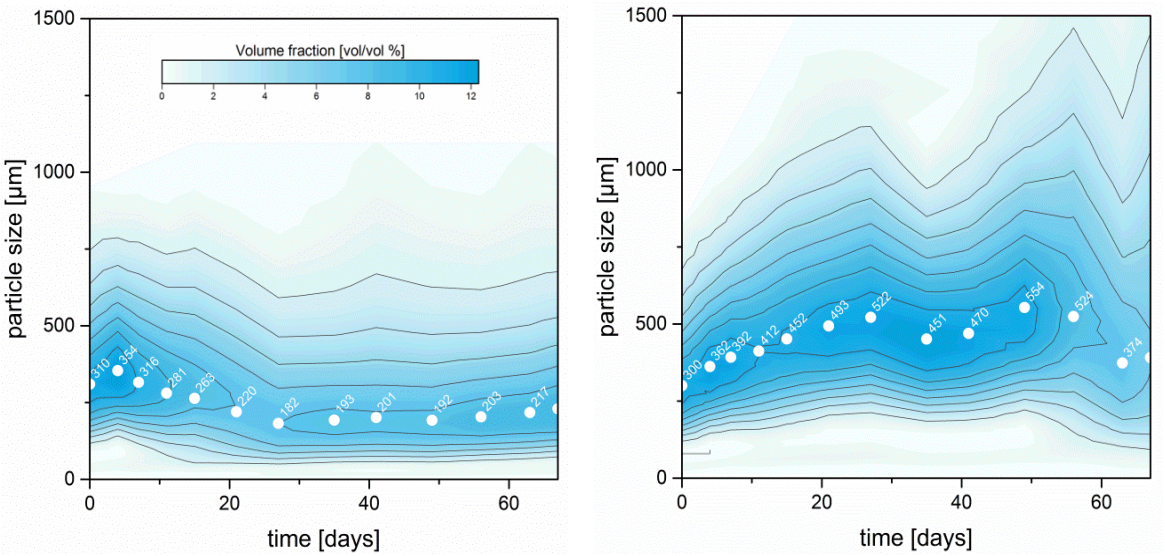


Figure 15 – Change of particle size and particle size distribution of R1 (left) and R2 (right) over 67 days. White dots: median particle size. Black lines: border of size classes. Shades of blue: volumetric fraction of class.

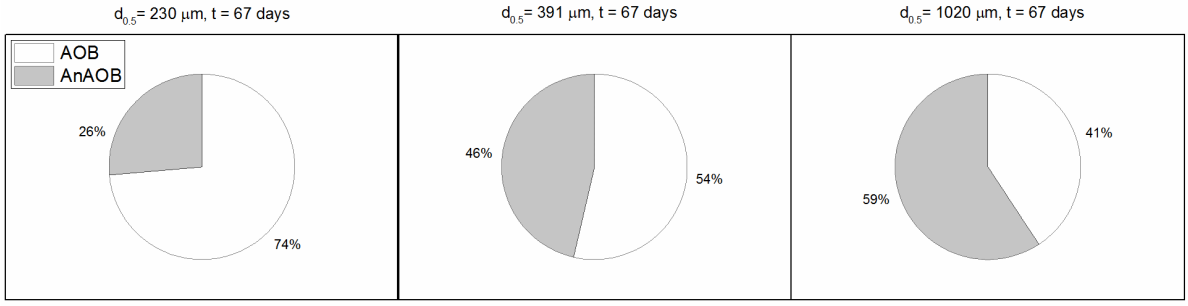


Figure 16 – Relative ratio of AOB and AnAOB in biogranules of different sizes as quantified by qPCR. Left: granules from R1, middle: granules from R2, right: particularly large granules from R2.

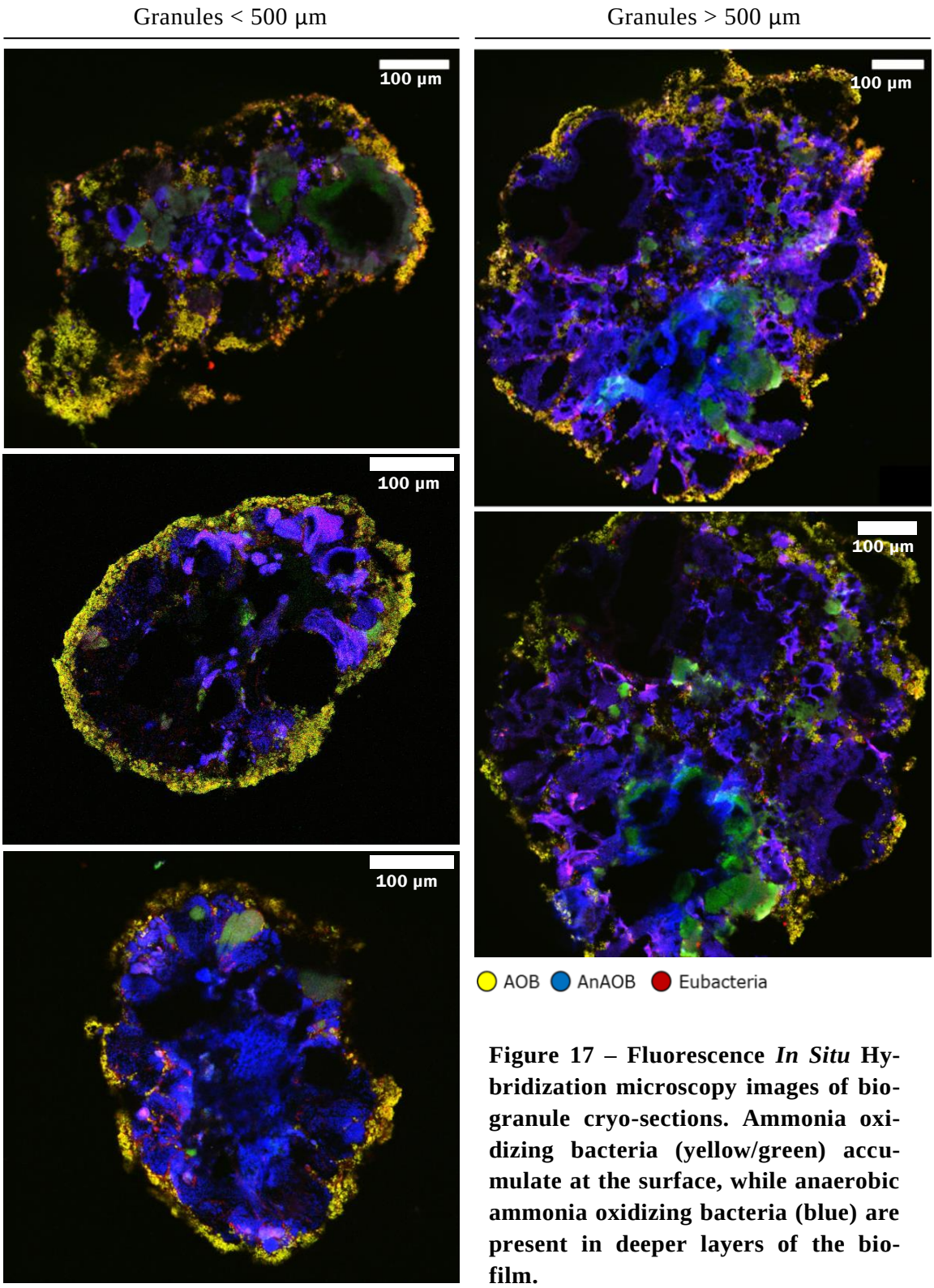


Figure 17 – Fluorescence *In Situ* Hybridization microscopy images of biogranule cryo-sections. Ammonia oxidizing bacteria (yellow/green) accumulate at the surface, while anaerobic ammonia oxidizing bacteria (blue) are present in deeper layers of the biofilm.

4.3 The heterotrophic community of PNA bio-granules

The heterotrophic community of PNA granules has generally not been in the focus of investigations, particularly in respect to their metabolic function, as heterotrophs do not comprise the microbial functional guild of primary interest. Accordingly, the N_2O sink capacity in bio-granules has not been assessed by, for instance, the quantification of *nosZ* genes. However, a number of different heterotrophic bacteria have repeatedly been documented in PNA biomass, particularly bacteria belonging to the Phyla *Chloroflexi*, *Chlorobi*, *Firmicutes* and *Bacteroidetes* (Ali et al., 2016; Chu et al., 2015; Kindaichi et al., 2012; Rodriguez-Sanchez et al., 2016). Heterotrophic bacteria live on soluble microbial products and extracellular polymeric substrates released by AOB, AnAOB, heterotrophic bacteria or decay, even in autotrophically fed PNA systems. Particularly *Chloroflexi* can degrade complex organic molecules like polysaccharides and proteins and utilize them for growth (Kindaichi et al., 2012). Furthermore, important functions in the aggregation process and a contribution to the structural integrity of bio-granules have been assigned to *Chloroflexi* due to their filamentous morphology (Gao et al., 2011). These bacteria appear to be relevant for PNA bio-granules and may enter a direct symbiotic relationship with AOB and AnAOB in PNA biomass. Generally, *Chloroflexi*, *Chlorobi* and *Bacteriodetes* are abundant in PNA biomass and may also be relevant for N_2O consumption, since they commonly carry *nosZ clade II* genes (Ali et al., 2016; Jones et al., 2013). Preliminary analysis of amplicon sequencing indicated that the relative abundance of *nosZ* genes increased with granule size (Paper II). Furthermore, the relative abundance of different *nosZ* carrying organisms changed with granule size: while *Chloroflexi* dominated in small granules, *Chlorobi* were more abundant in larger granules (Paper II). Larger granules may therefore not only be favorable in terms of their lower AOB/AnAOB ratio, but also for a potentially higher N_2O sink capacity.

4.4 Effect of granule size on process performance and net N_2O production

It is important to differentiate between the applied aeration regimes, i.e. continuous vs. intermittent aeration, when assessing the effect of granule size on process performance. Under continuous aeration NO_2^- production and con-

sumption need to be balanced throughout operation to avoid NO_2^- in the effluent. Therefore, a minimum median granule size that provides a sufficiently low AOB/AnAOB ratio is critical for stable process performance in continuously aerated systems (Vlaeminck et al., 2010). In contrast, periodic anoxic phases in intermittently aerated systems provide a tool to remove NO_2^- during the reaction phase, even when NO_2^- production temporarily exceeds consumption (Domingo-Félez et al., 2014; Joss et al., 2009). Accordingly, effluent targets can be met also with small granules (approx. 200 μm in diameter)(Paper I). The granule size and respective AOB/AnAOB ratio may therefore be less relevant in intermittently aerated systems. This was demonstrated in the presented PNA system, when overall system performance (i.e. N-removal efficiency, $NH_4^+{}_{effluent}$, $NO_2^-{}_{effluent}$, $NO_3^-{}_{effluent}$) was virtually unaffected by granule size, although intra-cycle dynamics of N-species differed between applied aeration regimes (Paper II). From an effluent target point of view, periodic anoxic phases provide strong regulating properties that allow for a resilient NO_2^- management throughout operation and outweigh increased NO_2^- sink capacities of different granule sizes (Domingo-Félez et al., 2014)(Paper II).

The N_2O consumption rate in biomass with different granule sizes varied over time. Operation under standard conditions with relatively small granules of approx. 200 μm generally showed insufficient N_2O consumption rates to remove $N_2O_{(aq)}$ in non-aerated phases (Paper I). However, similarly sized granules displayed higher consumption rates at later experiments, when high N_2O consumption rates were also measured in biomass with both small and larger median particle size (Paper II & Paper IV). Granule size alone was not sufficient to explain varying N_2O consumption rates. Other factors, like pH and sludge age, are likely to play a role in the N_2O consumption capacity of biogranules as well.

5 Operational control strategies for mitigation of net nitrous oxide production

Operators seek to find operational strategies for PNA that emit as least N_2O as possible, while maintaining volumetric NH_4^+ removal rates. A number of process parameters have been demonstrated to exert effects on N_2O production.

5.1 Substrate concentrations

Nitric oxide is a direct precursor of N_2O and a correlation has been established (Domingo-Félez et al., 2014; Kampschreur et al., 2008). NO peaks have been described particularly during transition from oxic to anoxic phases (Perez-Garcia et al., 2014; Yu et al., 2010). Therefore, operation of PNA under continuous aeration regimes likely prevents transient accumulation of NO and thus results in lower N_2O production.

Accumulation of **nitrite** triggers N_2O production (Stein, 2010). Balancing the NO_2^- production rate by AOB, with the NO_2^- consumption rate by AnAOB is therefore key to reduce N_2O production rates. Lower NO_2^- accumulation can be achieved by reducing the ammonia removal rate, by increasing the NO_2^- sink capacity of the microbial community or by increasing the frequency of aeration in intermittently aerated systems to allow periodic removal of accumulated NO_2^- during non-aerated phases (Domingo-Félez et al., 2014).

High **ammonium** concentrations and NH_4^+ loading rates increase N_2O production by AOB, as the increased availability of substrate increases the ammonia removal rate. Operation of nitrifying systems at reduced NH_4^+ concentration, e.g. by intermittent feeding reduces N_2O production rates (Su et al., 2017). However, NH_4^+ concentrations are relevant for the suppression of NOB and operation at NH_4^+ concentrations below 8 mg NH_4^+-N/L may result in relevant activity of NOB (Pérez et al., 2014).

Low availability of **inorganic carbon** can limit the ammonia removal rate of autotrophs, thus reducing their N_2O production rate (Jiang et al., 2015). However, the relative fraction of N_2O produced per NH_4^+ removed, increases with decreasing availability of inorganic carbon (Mellbye et al., 2016). Hence, the operation of PNA under inorganic carbon limitation may only be reasonable, when a N_2O sink is present.

A high **COD/N ratio** poses challenges for PNA systems, when excess COD enables heterotrophic bacteria to outcompete AnAOB (Xu et al., 2015). For stable PNA a COD/N ratio below 0.5 has been suggested (Daigger, 2014). The presence of heterotrophic bacteria causes NO_2^- to be metabolized through denitrification instead of anammox, which can lead to increased N_2O emissions (Yan et al., 2014).

5.2 Biomass related parameters

Low **sludge retention times** lead to higher N_2O emission (Li and Wu, 2014; Lotito et al., 2012). Particularly the abundance of slow growing microorganisms like AnAOB is negatively affected by short SRTs (Winkler et al., 2011). Sufficiently long SRTs are therefore critical for accumulating a microbial community that provides adequate NO_2^- and N_2O sinks.

Since oxic N_2O production correlates with the specific ammonia removal rate (Law et al., 2012a), high **biomass concentration** could help to reduce specific N_2O production rates.

5.3 Physical parameters

N_2O production in nitrifying cultures has been measured to increase with **temperature** (Reino et al., 2017). The exact reasons remain to be determined. However, temperature likely does not exert a direct effect on the balance of NH_3 oxidation by AMO and NH_2OH oxidation by HAO that may lead to an accumulation of NH_2OH, which itself fosters N_2O production. Instead, at lower temperatures a lower substrate speciation of NH_3/NH_4^+ may decrease the NH_3 removal rate. Also, the solubility of oxygen in water is higher at lower temperatures, which may decrease N_2O production by the nitrifier denitrification pathway (Reino et al., 2017). Operation at reduced temperatures may therefore reduce the relative N_2O production of AOB, but comes at the cost of lower NH_3 removal rates. Additionally, the activity of AnAOB has been described to be substantially lower at temperatures below 15°C (Laureni et al., 2016). A potential operation of PNA at lower temperatures, therefore, leads to accumulation of NO_2^-, which disturbs process performance and triggers N_2O production (De Clippeleir et al., 2013).

The N_2O production rate of nitrifying bacteria increases with the **dissolved oxygen** concentration (Lv et al., 2016a; Peng et al., 2014). However simultaneously the fraction of N_2O emitted per NH_4^+ removed decreases (Peng et al., 2015). At elevated DO concentrations (> 1 mg O_2/L), sufficient availability

of oxygen as electron acceptor likely enables the hydroxylamine dehydrogenase to completely oxidize NH_2OH to NO_2^-, instead of NO. Efficient loading of electrons to oxygen may further prevent an activation of the nitrifier denitrification pathway (Kozlowski et al., 2016; Simon and Klotz, 2013).

5.3.1 The pH

The **effect of pH** on biological nitrogen conversion reactions is complex, as it not only affects central processes in a cell like signaling, transcriptional and post-translational phenomena, but also alters substrate speciation and enzyme activity (Paper III). A hypothesized effect of pH on net N_2O production in PNA biomass was the motivation to investigate and identify favorable pH set-points for PNA (Paper IV).

The nitrification and N_2O production rate by AOB increase with pH, likely due to a higher NH_3/NH_4^+ substrate ratio and proximity to the pH optima of AMO and HAO of 7.5 and 8.5, respectively. Despite increasing N_2O production rates with pH, operation of PNA at alkaline pH was hypothesized to result in overall lower net N_2O production, primarily due to an increased N_2O consumption activity by heterotrophic denitrifiers that compensate higher N_2O production rates by AOB (Paper III). Indeed, the N_2O emissions from the studied PNA SBR displayed a 'bell-shaped' curve as a function of pH with maximum N_2O emission factors at pH 7.0-7.5 and minima at pH 6.5 and 8.0 (Paper IV). The difference between emission factors was substantial, when at pH 8.0 only half as much N_2O was emitted, as compared to pH 7.0 and 7.5 (1.6% vs. 3.3% N_2O-N emitted per NH_4^+-N removed). Simultaneously, overall process performance was not affected by pH (Table 2).

Table 2 – Performance indicators of partial nitritation-anammox sequencing batch reactors at operation at different pH set-points.

	pH 6.5	pH 7.0	pH 7.5	pH 8.0
Total N removal efficiency [%]	94±1	92±1	94±2	93±2
NH_4^+ removed [mg NH_4^+-N/cycle]	102.2±0.4	99.6±1.8	101.1±1.4	100.1±1.7
NO_3^- produced [mg NO_3^--N/cycle]	8.5±1.0	7.7±0.5	7.1±0.7	7.3±1.8
N_2O emitted/NH_4^+ removed [%]	2.5±0.03	3.3±0.3	3.3±0.4	1.6±0.1

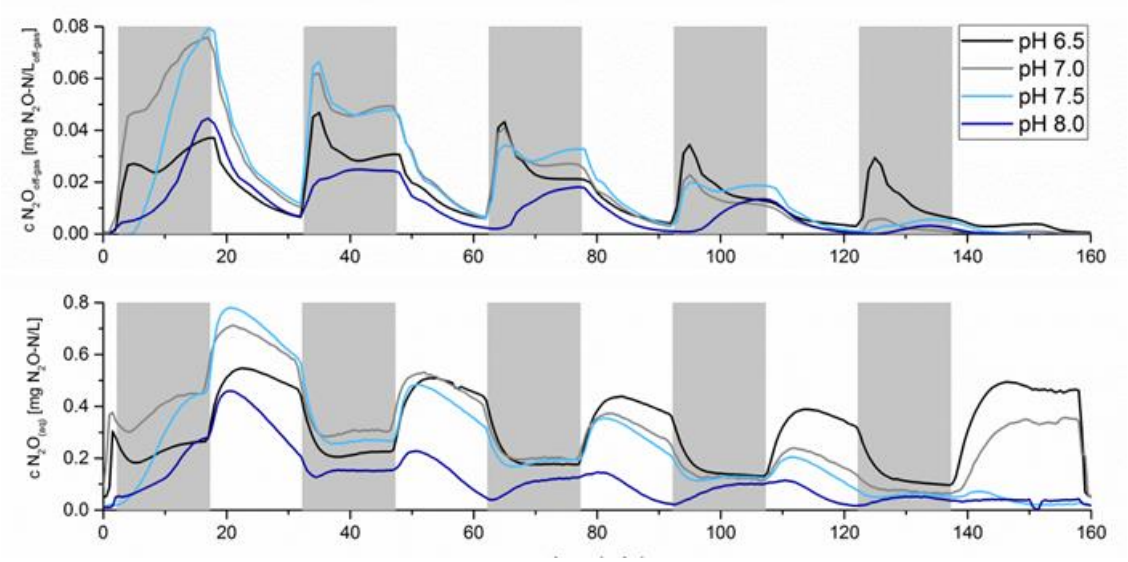


Figure 18 – off-gas N_2O (top) and $N_2O_{(aq)}$ (bottom) during SBR cycles at different pH set-points. Curves represent average concentrations (n=3). Standard deviations are depicted in Supplementary Information of Paper IV. Shaded areas represent aerated phases.

A major difference between the N_2O emission and production profiles at different pH set-points was that the N_2O consumption rate at pH 8.0 was large enough to deplete $N_2O_{(aq)}$ during non-aerated phases. That led to an exclusive contribution of aerated phases to N_2O production, which is apparent by the absence of an initial N_2O emission peak at the onset of aerated phases (Fig. 18). N_2O production during aerated phases was also lower, which indicates that N_2O consumption also occurred during aerated phases, potentially at deeper layers of the biofilm that remained anoxic irrespective of the aeration state.

Indeed, N_2O production via denitrification increases with decreasing pH (Bakken et al., 2012; Lv et al., 2016b). However, pH does not have a negative effect on transcription rates of *nosZ* (Liu et al., 2010). Rather an impaired assembly of function N_2OR enzyme seems to prevent the reduction of N_2O to N_2 under acidic conditions (Liu et al., 2014).

The pH value exerts direct effects on N_2O production in PNA systems and the correct choice of system pH appears to be suitable for the management of net N_2O production rate. A potential explanation includes that pH set-point management enables operators to synchronize fluxes through the nitrogen cycling network (Paper III & Paper IV).

6 Conclusions

The presented project investigated, whether microbial resources in an intermittently aerated Partial Nitritation-Anammox system could be managed by targeted changes of reactor operation in order to reduce the emission of the greenhouse gas N_2O from the process, while maintaining volumetric ammonium removal rates. Firstly, a thorough characterization of the investigated system defined a benchmark and identified relations between net N_2O production and process conditions. Secondly, changes in the aeration pattern were applied to engineer the ratio of functional microbial guilds in the biomass. Finally, a N_2O mitigation strategy based on pH set-points was tested. Central findings of the project are:

- The majority of net N_2O production occurred during aerated phases and positively related with the specific ammonia removal rate. Net N_2O production during non-aerated phases occurred in relation with the NO_2^- concentration.
- N_2O emissions from the system almost exclusively occurred during aerated phases, while N_2O produced in non-aerated phases remained dissolved in the liquid phase until transition to the next aerated phase.
- pH exerted a strong effect on the N_2O emission factor. Maximum N_2O emission factors of 3.3% N_2O-N emitted per NH_4^+-N removed were measured at pH 7.0-7.5, while only half as much N_2O-N was emitted at pH 8.0.
- The N_2O consumption rate increases with pH and counterbalances N_2O production rates at pH > 7.5, which resulted in reduced net N_2O production rates at alkaline conditions.
- Intermittent aeration regimes were suitable to manage the median biogranule size and engineer the ratio of AOB/AnAOB. The relative fraction of AnAOB in larger granules is larger, than in smaller granules.
- Periodic non-aerated phases during the reaction phase exerted a sufficiently strong regulating effect on NO_2^- accumulation, irrespective of the median particle size and particle size distribution. However, the genetic potential for N_2O consumption appears to increase with granule size.

- Heterotrophic bacteria play a relevant role in N_2O mitigation, as some carry the *nosZ* gene, which encodes nitrous oxide reductase. The phyla *Chloroflexi*, *Chlorobi* and *Bacteriodetes* comprise a substantial part of the heterotrophic community and include *nosZ* carrying species.
- In respect to N_2O emissions, an extension of the catalyst component in Microbial Resource Management by the effect of pH on enzyme activity will aid to better manage N_2O emissions from Partial Nitritation-Anammox systems.
- Operation of Partial Nitritation-Anammox at low specific ammonia removal rates and pH 8.0 is suggested for high NH_4^+ removal rates and low N_2O emission factors.

7 Perspectives

Net nitrous oxide production in Partial Nitritation-Anammox is the result of a complex web of nitrogen conversion reactions both on an intra-cellular level, but also as a result of the interaction of different microorganisms in a community. Particularly reactive intermediates like hydroxylamine and nitric oxide have a central role in N_2O metabolism, but are difficult to measure with high accuracy in space and time, especially in biofilm. Further research on the fate of hydroxylamine in- and outside a cell will unravel its role in the nitrifier denitrification pathway. Furthermore, multiple conversion pathways of NH_2OH towards N_2O have been described that include respiratory reactions, potential detoxifying conversion by cytochromes and abiotic reactions with other N-species or metal ions. Quantifying the contribution of the different NH_2OH removal routes would facilitate efforts to reduce its conversion to N_2O instead of NO_2^-.

In line with hydroxylamine as a substrate, the role of hydroxylamine dehydrogenase and HAO-like enzymes in the production of NO and N_2O and conditions that affect the product ratios of those enzymes requires further attention. Finding favourable conditions for these enzymes to produce less by-products has the potential to optimize the flux of N-species through the N-conversion pathways.

This study showed the potential of N_2O consumption to reduce net N_2O production. Increasing the reduction rate of nitrous oxide reductase offers a way to mitigate N_2O emissions from Partial Nitritation-Anammox. The discovery of a large diversity of microorganisms that carry the *nosZ* gene, but no other genes of the denitrification pathway, opens opportunities to increase the N_2O sink capacity of a microbial community (Jones et al., 2013). However, little is known about potential niches of these organisms (Hallin et al., 2018). The presented study suggests that granule size and sludge age may be relevant factors for increased abundance of *nosZ* carrying organisms. Further understanding of how to select these organisms will aid in enriching them in Partial Nitritation-Anammox biomass and considering them in microbial resource management.

Fragmented denitrification by heterotrophic microorganisms poses risks and opportunities for N_2O production and consumption. Further research on the occurrence of certain patterns of denitrifying genes like *nirS/K* and *nosZ* will help to select for complete over fragmented denitrification. But not only the

genetic potential, but further understanding of the regulation of transcription of the denitrification genes is relevant to avoid N_2O reduction to be inhibited. Transcript abundance and metatranscriptomics will help understand the relation between process parameters and gene expression.

The effect of pH on N_2O consumption was demonstrated and suggests process operation at alkaline pH. Long term operation of Partial Nitritation-Anammox at alkaline pH is required to document potential changes of the microbial community and the stability of process performance. That way the validity of the observed short-term benefits of alkaline pH for long-term operation can be evaluated.

The MRM framework, as a tool for engineers to optimize process performance, describes biocatalysts on the level of microorganisms. However, the effect of process conditions on the enzymatic activity is not considered in depth. Particularly in respect to N_2O, environmental conditions like pH appear to exert a strong effect on net N_2O production. An extension of the catalyst component with enzyme activity will help to refine MRM. Additionally, as research on N_2O mitigation from Partial Nitritation-Anammox systems is advancing, a maximum acceptable N_2O emission factor will provide design targets for engineers.

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9 Appendix

I **Blum, J.-M.**, Jensen, M.M., Smets, B.F. Nitrous oxide production in intermittently aerated Partial Nitritation- Anammox reactor: oxic N_2O production dominates and relates with ammonia removal rate. *Chemical Engineering Journal 335, 458-466 (2018).*

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III **Blum, J.-M.**, Su, Q., Ma, Y., Valverde-Pérez, B., Domingo-Félez, C., Jensen, M.M., Smets, B.F. The pH dependency of N-converting enzymatic processes, pathways and microbes: effect on net-N_2O production. *Environmental Microbiology (2018).*

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The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within six sections: Water Resources Engineering, Water Technology, Urban Water Systems, Residual Resource Engineering, Environmental Chemistry and Atmospheric Environment.

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Department of Environmental Engineering
Technical University of Denmark

DTU Environment
Bygningstorvet, building 115
2800 Kgs. Lyngby
Tlf. +45 4525 1600
Fax +45 4593 2850

www.env.dtu.dk