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Fate of Hydroxylamine in the Nitrogen Cycle

FATE OF HYDROXYLAMINE IN THE NITROGEN CYCLE

Dissertation

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Front & Back: Cover representing a droplet of water in the microscope, by Ada Maymó Costa and Aina Soler Jofra.

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A friend is one that knows you as you are,
understands where you have been,
accepts what you have become,
and still,
gently allows you to grow.

William Shakespeare

To my family

CONTENTS

LIST OF ABBREVIATIONS

AMO	ammonium monooxygenase
AMX	anammox - anaerobic ammonium oxidizing bacteria
AOA	ammonium oxidizing archaea
AOB	ammonium oxidizing bacteria
CMX	commamox - complete ammonium oxidizing bacteria
CSTR	continuous stirred tank reactor
Cu-ME	cooper-based enzymatic complex
Cyt P460	cytochrome P460
DEN	denitrification bacteria
DNA	deoxyribonucleic acid
DNRA	dissimilatory nitrate reduction to ammonium
DO	dissolved oxygen
E.T.C	electron transport chain
FISH	fluoresence in situ hybridization
FNA	free nitrous acid
GC	gas chromatrography
HAO	hydroxylamine oxidoreductase,
HDH	hydrazine dehydrogenase
HOX	hydroxylamine oxidase
HRT	hydraulic retention time
HZS	hydrazyne sinthase
in.	initial
max.	maximum
MBR	membrane bioreactor
Mox	oxidized electron carrier
Mred	reduced electron carrier
N	nitrogen
n.a.	not applicable
n.d.	not determined
NAR/NAP	nitrate reductase
NcyA	nitrososcyanin
NIR	nitrite reductase
NOB	nitrite oxidizing bacteria
NOO	nitric oxide reductase

NOR	nitric oxide reductase
NOS	nitrous oxide reductase reductase
NrfA	ammonia forming nitrite reductase
NXR	nitrite oxidoreductase
OD	optical density
ONR	nitrite reductse
OTU	operational taxonomic unit
PN	partal nitritation
PN/A	partial nitritation/anammox
RDR	rotating disk reactor
rRNA	ribosomal ribonucleic acid
RT	room temperature
SA	sulfamic acid
SBR	sequencial batch reactor
SHARON	single reactor high activity ammonium removal over nitrite
SI	supplementary information
SRT	sludge retention time
SSE	sum of squared errors
SVI	sludge volumetric index
TSS	total suspended solids
VSS	volatile suspended solids
WWTPs	wastewater treatment plants
εHAO	epsilonproteobacterial hydroxylamine oxidoreductase

SUMMARY

Substrates and products of the main microbial nitrogen conversions have been extensively studied for years, as well as extensively applied in engineered systems [1, 2, 3]. Even if some of the nitrogen conversions are known for more than a decade now, intermediate steps and enzymatic conversions of these microbial transformations are generally little understood and a lot of unknowns remain to be solved.

One of the least studied nitrogen compounds is hydroxylamine. Hydroxylamine is a highly reactive and toxic inorganic compound that some microorganisms use as intermediate during nitrogen conversions. The aim of this thesis was to further understand the role of hydroxylamine in the nitrogen cycle.

Current knowledge and challenges regarding the role of hydroxylamine in the nitrogen cycle were assessed in Chapter 2. Even being known to be an intermediate in aerobic ammonium oxidation by ammonium oxidizing bacteria (AOB), ammonium oxidizing archaea (AOA) and complete ammonium oxidizing bacteria (comammox), it is not yet fully clarified how hydroxylamine is transformed to nitrite. Furthermore, transient hydroxylamine accumulation has been described during ammonium oxidation, but the factors promoting its accumulations and further implications on side communities are not well known. Up until now, the impact of hydroxylamine on anaerobic ammonium oxidizing bacteria (anammox) and AOB were little studied and no general agreement on a positive or negative effect of this compound in these microbial transformations was reached. Few studies assessed, the impact of hydroxylamine on other nitrogen cycle conversions such as NOB, denitrifiers, AOA and comammox. Furthermore, part of the N_2O emissions in AOA, AOB and comammox are known to be related to hydroxylamine, but the factors promoting N_2O emissions from hydroxylamine are still to be fully understood. Thus, full of room is left to increase our knowledge regarding hydroxylamine. According to Chapter 2 the main obstacle to further advance in hydroxylamine related research is the development of a sensitive measurement method that can be easily implemented. Also, integrative approaches combining both omics and ^{15}N techniques would allow for the boosting of hydroxylamine related research, but also allow for an improved intermediate steps characterization.

As identified in Chapter 2, hydroxylamine contribution to N_2O emissions during ammonium conversion is yet to be further understood. Thus, in Chapter 3 and Chapter 4 the role of hydroxylamine in abiotic N_2O production was investigated. In Chapter 3 the abiotic reaction of hydroxylamine with free nitrous acid was identified as a potential contribution to N_2O emissions at side-stream conditions, and in a SHARON reactor. In Chapter 4 the abiotic N_2O production was assessed in a planktonic *Nitrosomonas europaea* (ATCC 19718) culture during recovery of anoxia. Biological and abiotic N_2O production rates were comparable at the conditions tested. Highlighting that when assessing biological pathways, intermediates should always be considered in the

chemical/abiotic controls. Finally, in Appendix A, a kinetic characterization of the abiotic reaction of hydroxylamine and free nitrous acid was performed. indicating that in order to minimize the abiotic reaction contribution, low pH (<7), high concentrations of nitrite (i.e. side stream conditions), low concentrations of metals (iron, specially) and high temperatures should be avoided. Overall, biological pathways would be better characterized at conditions that minimize putative abiotic transformations.

Finally, short and long term impact of hydroxylamine was assessed in anammox and in an AOB enrichment, in Chapter 5 and Chapter 6, respectively. Interestingly, when investigating the impact of hydroxylamine on the anammox enrichment in Chapter 5, hydroxylamine addition in batch mode resulted in differences on hydrazine accumulation - signature of anammox activity- depending on the substrates present during the batch. Hydrazine accumulation, when hydroxylamine was dosed, was higher with ammonium and lower with nitrite. Long term hydroxylamine feeding together with ammonium and nitrite, resulted in a decrease on the nitrate production by the anammox enrichment. Regarding the impact of hydroxylamine in AOB, dissolved oxygen was identified as the key parameter impacting hydroxylamine metabolism in Chapter 6. Higher hydroxylamine accumulation, higher N_2O production and decreased or maintained ammonium consumption were observed at low dissolved oxygen tensions, when continuous short term hydroxylamine feeding combined with an ammonium pulse was provided to the partial nitrification biomass. Contrarily, the same hydroxylamine short term feeding together with ammonium, but at higher oxygen concentrations resulted in reduced hydroxylamine accumulation and N_2O production and increased ammonium consumption. Ammonium consumption was also reduced when hydroxylamine was dosed for a long term period to an airlift reactor. However, nitrite production was maintained indicating that hydroxylamine was transformed to nitrite reducing the ammonium intake. When assessing the long term impact on the microbial community in Chapter 5 and Chapter 6 both *Ca.* Kuenenia stuttgartiensis and *Nitrosomonas* sp. were shown to be predominant within the whole operation, respectively. Consequently, there was no community shift due to continuous limited hydroxylamine supply and no putative inhibition (according also to the reported reactor operation).

Ultimately, this thesis advances in the fundamental understanding of the hydroxylamine transformations within the nitrogen cycle. Still several unknowns and challenges remain to be solved regarding hydroxylamine and the nitrogen cycle, which are discussed in Chapter 7. This thesis aims to highlight the importance of intermediate steps, and how a proper characterization of these conversion could help to widen the understanding of the microbial conversions, as well as optimize their application, accordingly.

SAMENVATTING

De substraten en producten van de microbiologische stikstofcyclus worden al lange tijd bestudeerd en gebruikt in technologische toepassingen [1, 2, 3]. Een aantal van deze reacties zijn al tientallen jaren bekend. Over de tussenproducten en enzymatische omzettingen van de microbiologische stikstofcyclus is echter veel minder bekend en van een aantal omzettingen wordt nog weinig begrepen. Eén van de minst bestudeerde stikstofverbindingen is hydroxylamine. Hydroxylamine is een zeer reactief en giftig tussenproduct bij stikstofconversies in sommige micro-organismen.

Het doel van dit proefschrift is het beter begrijpen van de rol van hydroxylamine in de stikstofcyclus. Een overzicht van de huidige kennis over de rol van hydroxylamine is te vinden in Chapter 2. Het is bekend dat het een tussenproduct is van aerobe ammonium oxidatie door ammonium oxiderende bacteriën (AOB), ammonium oxiderende archea (AOB) en volledige ammonium oxiderende bacteriën (comammox), maar het is nog onbekend hoe de conversie naar nitriet precies plaats vindt. Daarnaast is tijdelijke hydroxylamine ophoping beschreven tijdens ammonium oxidatie, maar de omstandigheden waarin dit gebeurt en de gevolgen voor microbiële populaties zijn nog niet volledig begrepen. Tot nu toe is het effect van hydroxylamine in anaerobe ammonium oxidatie (anammox) en AOB sporadisch bestudeerd en is er geen consensus over het effect van deze verbinding op microbiële omzettingen. Er zijn een klein aantal studies geweest naar het effect van hydroxylamine op omzettingen door NOB, denitrificeerders, AOA en comammox. Een deel van de lachgas emissies van AOA, AOB en comammox zijn in verband gebracht met hydroxylamine, maar de omstandigheden waarin hydroxylamine zorgt voor lachgas emissies worden nog niet volledig begrepen. Kortom, er is ruimte om de kennis van hydroxylamine te vergroten. In Chapter 2 wordt beschreven dat de grootste limitatie in het onderzoek, het ontbreken van voldoende gevoelige en makkelijk te implementeren analysemethoden is. Daarnaast kan een gecombineerde aanpak van -omics en ^{15}N technieken helpen bij het hydroxylamine onderzoek en bij het onderzoek naar andere tussenproducten.

Chapter 2 laat zien dat er over de bijdrage van hydroxylamine aan lachgas emissies tijdens ammonium oxidatie nog veel onbekend is.Chapter 3 en Chapter 4 focussen op de rol van hydroxylamine in abiotische lachgas productie. In Chapter 3 wordt het onderzoeken van de reactie van hydroxylamine met vrij waterstofnitriet in deelstroom condities en in een SHARON reactor besproken. Met als conclusie dat abiotische reacties in deze systemen een significante bijdrage leveren.

In Chapter 2 wordt de abiotische lachgasproductie onderzocht in een planktonische *Nitrosomonas europaea* (ATCC 19718) cultuur, tijdens het herstel van zuurstofloze condities. Een belangrijk resultaat hierbij is dat de biologische en abiotische omzettingen vergelijkbaar waren onder de geteste condities. Daarbij moet opgemerkt worden dat tussenproducten van biologische omzettingen niet vergeten moeten worden in chemische/abiotische controle experimenten. Aan het einde van dit

proefschrift wordt, in bijlage A (Appendix A), de abiotische reactie van hydroxylamine en waterstofnitriet beschreven. Waaruit wordt duidelijk dat, om de abiotische reactie te minimaliseren, lage pH (<7), hoge concentraties nitriet (zoals in deelstroom condities), lage concentraties van metalen (ijzer in het bijzonder) en hoge temperaturen vermeden dienen te worden. Bij het bestuderen van biologische omzettingen is het aan te raden om omstandigheden waarin abiotische omzettingen een bijdrage leveren te vermijden.

Ten slotte is het korte- en langetermijneffect van hydroxylamine in AOB en anammox bacteriën verrijkte culturen bestudeerd, beschreven in Chapter 5 en Chapter 6. In Chapter 5, bij het bestuderen van het effect van hydroxylamine op een verrijkte anammox cultuur, zorgde het toevoegen van hydroxylamine in batchexperimenten voor het ophopen van verschillende concentraties hydrazine (een teken van anammox activiteit), afhankelijk van de aanwezige substraten. Hydrazine ophoping, als gevolg van de aanwezigheid van hydroxylamine, was hoger met ammonium en lager met nitriet als substraat. Langdurige aanwezigheid van hydroxylamine met ammonium en nitriet resulteerde in verlaagde nitraatproductie in de anammox cultuur. Opgeloste zuurstof is de belangrijkste parameter met een effect op hydroxylamine omzettingen door AOB, dit wordt beschreven in Chapter 6. Verhoogde hydroxylamine concentraties, verhoogde lachgas productie en verlaagde ammonium consumptie werden gezien bij lage zuurstof concentraties, wanneer hydroxylamine voedingen gecombineerd werden met een ammonium puls, tijdens fedbatch voedingsmethoden, aan de partiële nitrificerende biomassa. Daarnaast resulteerde dezelfde hydroxylamine en ammonium voeding bij hogere zuurstof concentraties in lagere hydroxylamine concentraties, lagere lachgas productie en een verhoogde ammonium consumptie. Ammonium consumptie werd ook verlaagd als hydroxyamine voor langere termijn werd gevoed aan een airlift reactor. Nitrietvorming bleef stabiel in deze reactor, vermoedelijk werd hydroxylamine omgezet naar nitriet, met als gevolg een verminderde ammonium opname. Bij het bestuderen van de langetermijneffecten op de microbiële populatie in Chapter 5 en 6 bleek dat *Ca.* Kuenenia stuttgartiensis en *Nitrosomonas* sp. in alle monsters dominant zijn gedurende het volledige reactor bedrijf, met en zonder hydroxylamine voeding. Hieruit blijkt dat er geen verschuivingen in de microbiële samenstelling hebben plaatsgevonden door de gelimiteerde hydroxylamine concentratie (dit bleek ook uit de stabiele omzettingen in de reactor).

Aan het einde van dit proefschrift blijven er uitdagingen en hiaten in de kennis rondom de rol van hydroxylamine in de stikstofcyclus bestaan, deze worden beschreven in Chapter 7. Het doel van dit hoofdstuk is om het belang van tussenproducten onder de aandacht te brengen. Kennis van deze conversies kunnen ons helpen om microbiologische omzettingen beter te begrijpen en hun toepassingen te optimaliseren.

1

INTRODUCTION

1

1.1. THE NITROGEN CYCLE:IMPORTANCE AND CONTEXT

NITROGEN is one of the essential elements present in all living organisms together with hydrogen, carbon and oxygen. Nitrogen is mostly found in nature as nitrogen gas or as ammonia in rocks and sediments, thus it is not bioavailable for most organisms [3] (see Figure 1.1). Instead, most of the living forms rely on more easily accessible and reactive forms of nitrogen (i.e. soluble ammonia). Microbial conversions are usually responsible to make nitrogen bioavailable to other forms of living organisms. The nitrogen conversions between the different nitrogen compounds are commonly known as the nitrogen cycle [3].

However, human kind have modified this natural cycles by introducing anthropogenic nitrogen sources and conversions. The most known anthropogenic nitrogen flux and the why you might be reading this thesis today is the development of the Haber-Bosch process. This allowed for the generation of ammonium from hydrogen and dinitrogen gas at high pressures and temperatures with iron as catalyst [4, 5]. Actually, mimicking the microbial conversion process of nitrogen fixation [6], but at higher temperature and pressure. The industrial production of fertilizers dramatically increased crops productivity [5]. For instance, it was estimated that the food supply of ca. 48% of the human population was dependent on Haber-Bosch generated nitrogen by 2008 [5].

The availability of industrial produced nitrogen contributed greatly in minimizing food shortages in the last century. However, not everything related to the Haber-Bosch process had positive impact. As for instance, it also fuelled the production of explosives during the First World War [5]. Furthermore, together with the population growth other problems appeared, besides the necessity of keeping people nourished. Tons of food, animal and human waste started to be generated yearly and their treatment and correct disposal became an issue [7].

Initially, wastewater was treated by means of biological filters. Biological based activated sludge processes were introduced gradually from 1913 onwards [7]. During the first half of the 20th century rivers were responsible of part of the organic removal. It was not until the second half of the 20th century that eutrophication became an issue [7]. Briefly, eutrophication relates to huge algae blooms due to high nutrient contents (nitrogen and phosphorous mainly). This leads to oxygen depleted environments and loss of biodiversity. Thus, introducing the necessity of nitrogen and phosphorous removal from wastewater before its disposal in rivers and lakes [7].

Nowadays, strict nitrogen discharge limits exist (<10 mg-N/L according to the European Council Directive 91/271/ECC). Thus, nitrogen removal is an integral part of most wastewater treatment technologies. Furthermore, nitrogen removal is usually performed biologically, using the natural capacity of some microorganisms to transform the different forms of soluble nitrogen into dinitrogen gas [7].

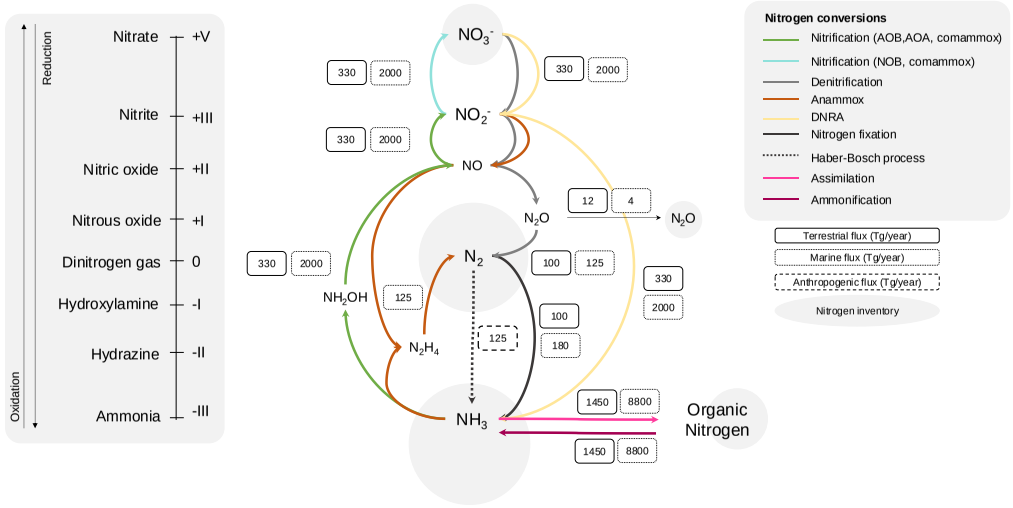

Figure 1.1: Principal conversions of the nitrogen compounds according to the nitrogen reduction state and their approximate yearly interconversions, adapted from [3]. Notice that fluxes (numbers in squares, Tg/year) are not equal between the pathways and differ a lot depending on the environment. Nitrogen inventories (grey circles) found in earth are dimensioned to the order of magnitude. NH_3 inventory is around 10^{10} Tg mainly found in rocks and sediments, so little is bioavailable . The bioavailable soluble NH3 is thought to be around 10^2-10^3 Tg. Dinitrogen gas inventory is in the order of 10^9 Tg, Nitrate and organic nitrogen around 10^5. Finally around 10^3 Tg of are found as nitrous oxide [3]. AOB stands for ammonium oxidizing bacteria, AOA for ammonium oxidizing archaea, comammox for complete ammonium oxidation bacteria, anammox for anaerobic ammonium oxidising bacteria, DNRA for dissimilatory nitrate reduction to ammonia.

1

1.2. UNDERSTANDING THE MICROBIOLOGY TO IMPROVE THE ENGINEERING OF WASTEWATER TREATMENT.

NITROGEN in wastewater is mainly present as soluble ammonium as it comes from the degradation of urea and proteins [7]. Conventional biological nitrogen removal has been based on the combination of nitrification and denitrification (Figure 1.2). In the first step, ammonium is aerobically and autotrophically transformed to nitrate by nitrifiers. Usually, ammonium is transformed to nitrite by ammonium oxidizing bacteria (AOB), and then nitrite is further transformed to nitrate by nitrite oxidizing bacteria (NOB) (Figure 1.2A). Then nitrate is further converted to dinitrogen gas anaerobically and heterotrophically by denitrifiers (Figure 1.2A). This last step usually requires of some additional organic matter as not enough COD reaches the nitrogen removal step and is needed as reducing equivalent for the reduction of nitrate to dinitrogen gas. Several configurations based on nitrification/denitrification microbial conversions have been proposed and implemented over time to optimize nitrogen removal during wastewater treatment (Figure 1.2B).

Advances in nitrogen related microbiology and new engineering solutions came up in parallel with the discovery of new microbial pathways for nitrogen removal. A clear example was the discovery of anoxic ammonium oxidation (anammox) 26 years ago [8]. Anammox bacteria are able to transform anaerobically and autotrophically ammonium and nitrite to dinitrogen gas [9]. The further understanding of the anammox bacteria metabolism in the laboratory [9, 10, 11] developed hand in hand with the anammox implementation at full scale [12, 13]. Anammox combined with a previous step of partial nitrification of ammonium to nitrite has several advantages to the conventional nitrification/denitrification processes: i) less oxygen is needed for the process, which will reduce aeration costs, ii) anammox is an autotrophic bacteria, thus no extra organic matter will be required to convert ammonium and nitrite to dinitrogen gas [12]. The partial-nitrification/anammox process has been industrially implemented in side-stream conditions (high N concentration and temperature), but it becomes a challenge at main-stream conditions (low N concentration and temperature) [14]. Next steps, for partial-nitritation/anammox implementation in the main water line relies on understanding on how NOB can be outcompeted. Thus, avoiding the oxidation of nitrite to nitrate during nitrification at low temperature and low nitrogen concentrations [14].

Step by step, the improved knowledge of nitrogen microorganisms metabolism has allowed for the development of new and optimized solutions for nitrogen removal of wastewater treatment. Up to know, several parameters have been identified to impact microbial growth and metabolism. For instance, temperature affects growth rate. pH impacts both microbial growth rate an substrates availability by impacting the chemical equilibrium (i.e. $NH_4^+ \longleftrightarrow NH_3 + H^+$, $HNO_2 \longleftrightarrow NO_2^- + H^+$ or $NH_3OH^+ \longleftrightarrow NH_2OH + H^+$). Substrates concentration and the microorganism affinity for them will also impact also growth rate, among others factors. Still the effect of intermediates of most N-cycle conversion remains elusive.

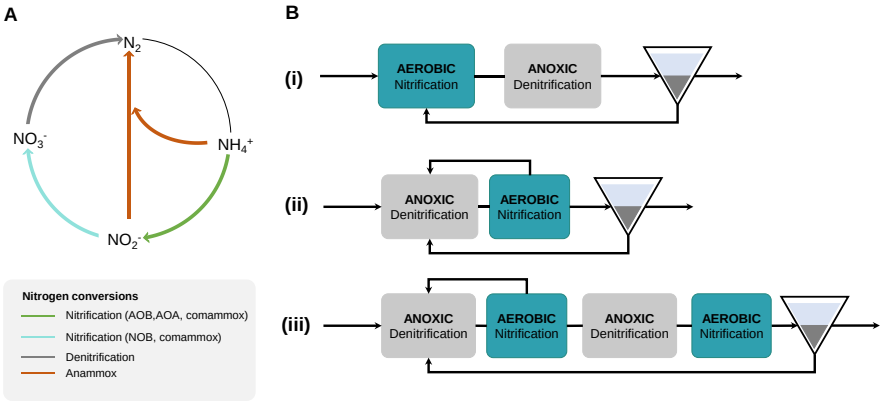

Figure 1.2: Microbial nitrogen conversions are used in engineered systems for nitrogen removal in wastewater treatment. A) Microbial conversions used in nitrogen removal in wastewater treatment B) Examples of some conventional configurations for nitrogen removal (i) Post-denitrification configuration, (ii) Modified Ludzack-Ettinger configuration, (iii) 4-stage Bardenpho configuration. Adapted from [7].

1.3. IMPORTANCE AND CHALLENGES REGARDING NITROGEN INTERMEDIATES

REGULARLY, when looking into nitrogen related processes and conversions, substrates and products are the main focus of interest. Thus, they are usually measured. However, the deeper understanding of such microorganisms, the discovery of new microbial conversions and the growing concerns about greenhouse gas emissions have brought attention to some of the intermediates involved in the nitrogen conversions.

The most studied intermediates so far, are NO and N_2O. They are intermediates in denitrification (see Figure 1.1) and also known to be emitted during nitrification [15]. However, NO and N_2O follow up during real wastewater treatment started only recently (i.e. [15] cites a first study from 1995). The increased efforts on characterizing NO/N_2O emissions is due to their contribution on greenhouse gas emissions. For instance, N_2O has ca. 300-fold time stronger warming potential than that of CO_2 [15]. Thus, understanding of the microbial pathways and factors leading to NO/N_2O emissions has been crucial to generate mitigation strategies for engineered system such as wastewater treatment.

Similarly, hydroxylamine is known to be an intermediate during aerobic ammonium

oxidation by ammonium oxidizing bacteria (AOB), ammonium oxidising archaea (AOA) and complete oxidising bacteria (comammox) (Figure 1.1) [16, 17, 1]. Hydroxylamine transiently accumulates in nitrification cultures, but the factors promoting such accumulations are unknown [18, 19, 20, 21]. Hydroxylamine is also source of NO and N_2O emissions during nitrification [22]. It was initially thought to be an intermediate in anammox, but up to date hydroxylamine role in anammox metabolism is yet not fully understood [9, 10, 23].

1.4. LOOKING INTO INTERMEDIATES SUCH AS HYDROXYLAMINE TO UNDERSTAND MICROBIAL PROCESSES: OUTLINE OF THIS THESIS

EVEN being an intermediate in different processes related to nitrogen microbial conversions (Figure 1.1). Hydroxylamine has been generally little studied and not measured during wastewater treatment related research. The aim of this thesis was to investigate how hydroxylamine could impact different microbial (and chemical) processes during nitrogen removal. Specially, highlighting the importance of this compound in different nitrogen conversions of the nitrogen cycle, as well as during N_2O emissions.

A general overview of the knowledge related to hydroxylamine and the nitrogen cycle during wastewater treatment is presented in Chapter 2. Highlighting the measurement of hydroxylamine as a big bottleneck for its study.

The impact of hydroxylamine on N_2O emissions were investigated in Chapter 3 and Chapter 4. Putative chemical conversions of intermediates during the study of biological N_2O emissions are not well assessed. Therefore, the occurrence of abiotic reactions of an intermediate such as hydroxylamine producing N_2O at conditions relevant for wastewater treatment was assessed in Chapter 3. Furthermore, the abiotic N_2O production rate from hydroxylamine and free nitrous acid was compared to the to biological N_2O emissions rate during nitrification in Chapter 4.

As hydroxylamine is known to transiently accumulate during nitrification [18, 19, 20, 21]. The mechanisms leading to hydroxylamine transient accumulation and the impact of hydroxylamine on the same AOB or neighbouring nitrogen microbial communities remain to be understood. Furthermore, the continuous limited addition of an intermediate such as hydroxylamine, could help understanding how the intermediates are transformed by the microorganisms. Even more importantly, it can give insights on how the microorganisms regulate intermediate conversions to avoid as much as possible its accumulation. Therefore, the effect of short term and long term hydroxylamine addition impacting microbial nitrogen conversions by anammox and ammonium oxidizing bacteria was investigated in Chapter 5 and Chapter 6, respectively.

A lot of unknowns and challenges regarding hydroxylamine remain to be investigated. Further steps and recommendations for future research are highlighted in Chapter 7. Overall, understanding the role and conversions of intermediate steps of the nitrogen cycle, would help on the understanding of the whole microbial conversion and related processes (specially N_2O emissions). Ultimately, allowing to design and optimized engineered processes relying on nitrogen microbial conversions.

2

HYDROXYLAMINE AND THE NITROGEN CYCLE: A REVIEW

Aina SOLER-JOFRA, Julio PÉREZ, Mark C. M. VAN LOOSDRECHT

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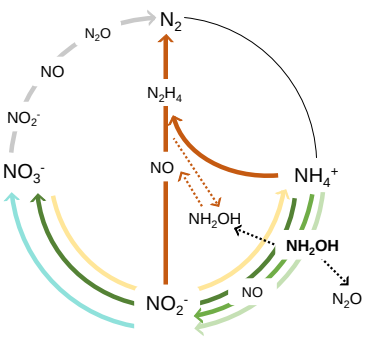
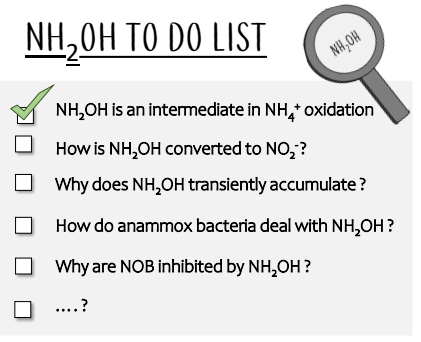
ABSTRACT

Aerobic ammonium oxidizing bacteria were first isolated more than 100 years ago and hydroxylamine is known to be an intermediate. The enzymatic steps involving hydroxylamine conversion to nitrite are still under discussion. For a long time it was assumed that hydroxylamine was directly converted to nitrite by a hydroxylamine oxidoreductase. Recent enzymatic evidences suggest that the actual product of hydroxylamine conversion is NO and a third, yet unknown, enzyme further converts NO to nitrite. More recently, ammonium oxidizing archaea and complete ammonium oxidizing bacteria were isolated and identified. Still the central nitrogen metabolism of these microorganisms presents to researchers the same puzzle: how hydroxylamine is transformed to nitrite. Nitrogen losses in the form of NO and N_2O have been identified in all three types of aerobic ammonium oxidizing microorganisms and hydroxylamine is known to play a significant role in the formation. Yet, the pathways and the factors promoting the greenhouse gas emissions are to be fully characterized. Hydroxylamine also plays a yet poorly understood role on anaerobic ammonium oxidizing bacteria and is known to inhibit nitrite oxidizing bacteria. In this review, the role of this elusive intermediate in the metabolism of different key players of the nitrogen cycle is discussed, as well as the putative importance of hydroxylamine as a key nitrogen metabolite for microbial interactions within microbial communities and engineered systems. Overall, for the first time putting together the acquired knowledge about hydroxylamine and the nitrogen cycle over the years in a review, setting potential hypothesis and highlighting possible next steps for research.



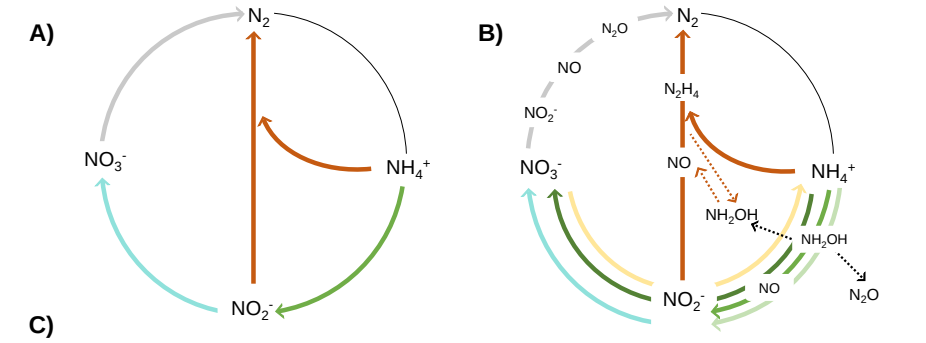


2.1. HYDROXYLAMINE IN THE NITROGEN CYCLE

NITROGEN is one of the essential elements on Earth, widely present in the environment, as well as, in living organisms. The biggest reservoir of nitrogen on Earth is dinitrogen gas (N_2), but it is too inert for most of living organisms to be incorporated into cellular structures [3]. Microorganisms have different enzymes that catalyse all sorts of nitrogen conversions, generally known as microorganisms of the nitrogen cycle. For example, nitrogen fixers are able to transform dinitrogen gas to ammonium, which makes nitrogen bioavailable to other microorganisms and other forms of life. Microbial conversions are responsible for the major fraction of the nitrogen fluxes between different nitrogen reservoirs in Earth [3]. Since the discovery of the Haber-Bosch process, which transforms dinitrogen gas to ammonium to produce fertilizers, human intervention generated lots of anthropogenic nitrogen fluxes in Earth, hampering the natural equilibrium [3]. Eutrophication of waters and increased nitrous oxide emissions are typical examples of human generated problems related to the nitrogen cycle. To mitigate such problems, engineered systems like biological wastewater treatment plants (WWTPs) are used, where ammonium dissolved in water is transformed to dinitrogen gas using different nitrogen microbial conversions [25]. Thus, detailed knowledge of the nitrogen cycle microorganisms is crucial to further understand both natural and anthropogenic nitrogen fluxes and avoid possible environmental problems. Traditionally, nitrogen cycle conversions have been classified in: assimilation, ammonification, nitrification, denitrification, anaerobic ammonium oxidation (anammox) and nitrogen fixation (see some of them in Figure 2.1 A). However, new microbial conversions have been discovered, expanding the traditional processes (Figure 2.1 B).

Hydroxylamine is an inorganic highly reactive compound that is intermediate or side metabolite in different nitrogen cycle microorganisms (Figure 2.1 B and C). Hydroxylamine impacts NO and N_2O emissions by aerobic ammonium oxidizers microorganisms. The impact of this compound in other microorganisms of the nitrogen cycle is little studied. To the best of our knowledge this review is the first to target the current knowledge about the role of hydroxylamine in the different communities of the nitrogen cycle. The review aims to assess the current state of the art on the role of hydroxylamine in the conversions by the various microbial groups participating in the nitrogen cycle, with a special focus on wastewater treatment processes. The level of understanding and research related to hydroxylamine and the microorganisms transforming nitrogen is not the same for each microbial process in the nitrogen cycle. Aerobic and anaerobic ammonium oxidizing microorganisms are presented in Section 2.2 to Section 2.5. AOB and anammox bacteria are known to harbour hydroxylamine conversion capacity in their genomic inventory. Consequently, a wide range of studies investigated the role of hydroxylamine on ammonium oxidation bacteria and anaerobic ammonium oxidizing bacteria (Section 2.2 and Section 2.5, respectively). Less information is available regarding the role of hydroxylamine in more recently discovered microorganisms such as ammonium oxidizing archaea or comammox (Section 2.3 and Section 2.4, respectively). Nitrite oxidizing bacteria are not able to transform hydroxylamine, but the inhibition of nitrite oxidizers by hydroxylamine might be of relevance when shaping nitrogen cycle communities, this is

analysed in section Section 2.6. The impact of hydroxylamine on other nitrogen cycle microorganisms, such as denitrifiers or dissimilatory nitrate reducers to ammonium (DNRA) organisms has been hardly investigated, therefore only a brief analysis was included in Section 2.7. In this section, also other microorganisms that might be relevant for wastewater treatment are mentioned. Finally, the bottlenecks for hydroxylamine measurement, its role as a putative interaction compound in microbial nitrogen cycling communities and research challenges regarding this compound are discussed in the last sections (Section 2.8 to Section 2.10).



C)

NH_2OH role	AOB	AOA	CMX	AMX	NOB	DEN	DNRA
Intermediate	yes *	yes *	yes *	under discussion	no	no	under discussion
Impact	substrate	substrate	substrate	substrate	inhibition	n.d.	n.d.
NO/N_2O emissions	yes	yes	yes	n.a.	n.a.	no	n.d.

Figure 2.1: Impact of hydroxylamine in the microorganisms of the nitrogen cycle involved in wastewater treatment. A) Simplified version of the nitrogen cycle, where only major substrates and products are represented, B) Nitrogen cycle conversions, where intermediates are depicted and the role of hydroxylamine highlighted, C) Simplified summary of the known roles of hydroxylamine in different microorganisms of the nitrogen cycle. Point style arrows indicate putative pathways or hydroxylamine interactions. * enzymes involved in the conversions are under discussion, n.d. – not determined, n.a. – not applicable, AOA - ammonium oxidizing archaea, AOB – ammonium oxidizing bacteria, NOB – nitrite oxidizing bacteria, AMX – anammox, CMX – comammox, DEN – anoxic heterotroph denitrifiers, DNRA – dissimilatory nitrate reduction to ammonium.

2.2. AMMONIUM OXIDIZING BACTERIA (AOB)

AMMONIA oxidizing bacteria (AOB) are able to transform ammonium to nitrite with oxygen as electron acceptor. These microorganisms are aerobic chemolithoautotrophic bacteria comprised in the beta and gamma subdivision of proteobacteria [26].

AOB catabolism consists of the conversion of ammonium to nitrite as electron donor and oxygen as electron acceptor as in Equation (2.1). In AOB this reaction is divided in two steps. First, the enzyme ammonia monooxygenase (AMO) catalyses the oxidation of NH_4^+ to NH_2OH (Equation (2.2)). Afterwards, hydroxylamine is further converted to

NO_2^- by hydroxylamine oxidoreductase (HAO) (Equation (2.3)) (see Figure 2.2 A). Two of the electrons generated in this last step are used by AMO to catalyse the first reaction, whereas the rest are invested in energy generation [21].

$$NH_4^+ + 1.5\,O_2 \longrightarrow NO_2^- + 2H^+ + H_2O \tag{2.1}$$

$$NH_4^+ + O_2 + 2H^+ + 2e^- \longrightarrow NH_2OH + H_2O \tag{2.2}$$

$$NH_2OH + H_2O \longrightarrow NO_2^- + 5H^+ + 4e^- \tag{2.3}$$

Recently, it was suggested that the product of HAO is NO instead of nitrite, and that NO is disproportionated abiotically or by an unknown enzyme to nitrite [27]. Thus, first ammonium is oxidized to hydroxylamine by AMO (Equation (2.2)), hydroxylamine to NO by HAO (Equation (2.4)) and NO to nitrite (Equation (2.5)) by an uncharacterized enzyme. A proteomic comparative study has recently suggested that nitrosocyanin (NcyA) was highly expressed in 3 different AOB strains, and was proposed as this third missing enzyme [28] (see Figure 2.2B). Nevertheless, the activity of HAO producing NO has only been shown in-vitro, thus further confirmation of this pathway in-vivo is needed. In-vivo metabolic studies in combination with transcriptomics/proteomics studies could shed light into another putative enzyme being able to convert hydroxylamine to nitrite.

$$NH_2OH \longrightarrow NO + 3H^+ + 3e^- \tag{2.4}$$

$$NO + H_2O \longrightarrow NO_2^- + 2H^+ + 1e^- \tag{2.5}$$

Beyond hydroxylamine as an intermediate in AOB metabolism, other putative roles of hydroxylamine are known. Hydroxylamine has been shown to transiently accumulate in AOB planktonic or mixed cultures, which might lead to interactions with other nitrogen communities. The short and long term impact of hydroxylamine has been tested in AOB without reaching definitive conclusions. Finally, it is known to be a precursor to N_2O emissions. All these roles are discussed in depth in the following sections.

2.2.1. HYDROXYLAMINE TRANSIENT ACCUMULATION EVENTS

Since hydroxylamine first mention [30] as intermediate and identification by Lees [1], it has been shown that it can transiently accumulate in the bulk liquid during cultivation at concentrations from 0.003 up to 4.3 mg-N/L (Table 2.1). These accumulations were reported in a wide variety of nitrification systems and operation conditions. For example, when performing batch tests with axenic cultures [18, 19] or nitrifying cultures [20], when AOB axenic chemostat cultures were switched from anoxic to aerobic conditions [21, 31] or when partial nitrifying reactors were operated in sequencing batch mode [32, 33]. Also hydroxylamine accumulated when a change of reactor load was imposed to a continuous partial nitrification airlift reactor leading to an increase of ammonium concentration from 2 to 25 mg-N/L [34]. Hu et al., also observed hydroxylamine accumulation in a continuous reactor when imposing a change of load, but higher load did not correlate with a change of the hydroxylamine accumulation peak [32].

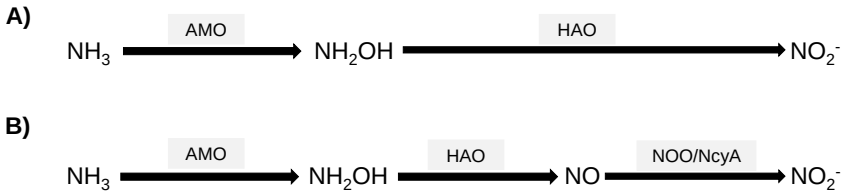


Figure 2.2: Proposed enzymes for ammonium conversion by ammonium oxidizing bacteria (AOB). A) Traditional pathway, where hydroxylamine is directly converted to nitrite. B) Alternatively, hydroxylamine is first transformed to NO and further oxidized to nitrite by a yet not fully characterized enzyme. AMO – Ammonium monooxygenase, HAO – Hydroxylamine oxidoreductase, NOO - Nitric oxide reductase, NcyA – Nitrososcyanin. Sources [29, 28].

Overall, all the mentioned experiments (Table 2.1) showed that switching AOB cells from a resting state (i.e. without/low ammonium or oxygen) to an active state (i.e. with ammonium or oxygen) triggered hydroxylamine accumulation. Consequently, hydroxylamine accumulation depended on the unbalanced coupling between the production and consumption of hydroxylamine by the designated enzymes. Thus, if AMO produces hydroxylamine faster than can be converted by HAO and other consumption reactions (or the recently proposed, still unknown enzyme [27]) are able to consume hydroxylamine, a metabolic imbalance is created, leading to the observed hydroxylamine accumulation.

Different studies point out that hydroxylamine accumulation can also be strain dependent. For instance, *Nitrosomonas europaea* and *Nitrosospira multiformis* accumulated hydroxylamine up to 11 and 31 µg-N/L, respectively [18]. In contrast, other AOB strains such as *Nitrosomonas nitrosa Nm90* and *Nitrosomonas communis* did not show hydroxylamine accumulation in batch tests with the same initial ammonium concentrations [18]. The authors attributed the differences observed for hydroxylamine accumulation levels to differences in the ammonia consumption rates. For example, *N. multiformis* showed the highest ammonium consumption rate also resulting in the highest hydroxylamine accumulation [18]. Nevertheless, *N. communis* had the fastest ammonium uptake rate but no hydroxylamine accumulation was detected, which might be due to a more efficient hydroxylamine conversion to nitrite by HAO [18].

Another study indicating that hydroxylamine accumulation is strain dependent and might also depend on the surrounding nitrogen community was performed by Stüven

Table 2.1: Hydroxylamine build-up by ammonium oxidizing bacteria. (a) Recalculated from free hydroxylamine equilibrium, (b) converted from mM, (c) converted from µM, (d) *Nitrosovibrio* alone did not accumulate hydroxylamine, PN – partial nitritation, DO – dissolved oxygen, Max. – Maximum, In. – Initial, SBR –sequential batch reactor.

Type of biomass	Type of test	Temperature (°C)	pH	DO (mg/L)	In. NH_4^+ (mg-N/L)	Max. NH_2OH (mg-N/L)	Reference
Nitrosomonas europaea	Batch	30	7.7	n.d.	7 and 28 (b)	0.006 and 0.011 (c)	[18]
Nitrosospira multiformis	Batch	30	7.7	n.d.	7 and 28 (b)	0.013 0.031 (c)	[18]
Nitrosomonas europaea	Batch	28	7.8	n.d.	140	0.003-0.015 (c)	[19]
Nitrosovibrio, Nitrobacter(d)	Batch	28	7.8	7.4.	140	0.006-0.024	[19]
Nitrosomonas europaea	Recover from anoxia	n.d.	6.8-7.4	0.5	230	0.3	[21]
Nitrosomonas europaea	Recover from anoxia	n.d.	6.8-7.4	1.5	230	0.4	[21]
Nitrosomonas europaea	Recover from anoxia	n.d.	6.8-7.4	3	230	0.35	[21]
Nitrosomonas europaea	Recover from anoxia	21	7.5		30	0.2	[31]
Nitrifying culture	Batch test	28	7.0-8.5	0.5-6	200-500	0.3-4.3 (a)	[20]
PN granular airlift	Load increase	20	7.7	3.5	2 to 22	0.06	[34]
PN sludge SBR	Two different loads	30	8	0.4-0.5	50 to 70	0.06	[32]
PN sludge SBR	Different pH set point	20-26	6.5, 7, 7.5, 8 and 8.5	0.7±0.1	74±39	0.1-0.05	[33]

and coworkers [19]. For instance, *Nitrosovibrio* did not accumulate hydroxylamine when it was cultured alone, and yet hydroxylamine accumulation (5.6-23.8 µg-N/L) occurred when it was cultured together with the NOB *Nitrobacter* [19]. Contrarily, in the same set of experiments *Nitrosomonas europaea* showed hydroxylamine accumulation (2.8-15.4 µg-N/L) when it was cultured alone, but in co-culture with *Nitrobacter* lower hydroxylamine levels where reported (5.6 to less than 2.8 µg-N/L) [19]. This is the only study that point towards a possible impact of side communities to the hydroxylamine accumulation behaviour of AOB strains. The differential behaviour related to hydroxylamine accumulation when AOB was cultured alone or together with NOB, might be due to a possible competition strategy to avoid NOB growth in the co-culture or to promote it to avoid product inhibition. Thus far, the impact of side communities on hydroxylamine accumulation has been little studied and it is still not fully understood.

The unbalance between hydroxylamine production and consumption leading to hydroxylamine accumulation might have different explanations: i) the turnover of HAO (or the enzyme responsible of hydroxylamine consumption) is smaller than the capacity of AMO to produce hydroxylamine. Genetic differences between different species in the hydroxylamine production and consumption enzymes could explain the different hydroxylamine accumulation dynamics observed between strains. ii) pH can be another factor that might cause hydroxylamine build up. As pH has an impact on both the dissociation of ammonium/hydroxylamine, as well as it has a strong impact on the enzymes rates [33]. For instance ammonium consumption is strongly impacted by acidification, while hydroxylamine oxidation is barely affected [35].This could be of importance in biofilm like systems, where strong pH gradients can occur, and pH is more acidic in the internal part of the granule [36, 37, 34, 38, 39, 40]. Future studies might shed more light on the difference in balancing the production and consumption of hydroxylamine in different AOB. For example, comparative transcriptomic/proteomic between AOB strains, studies focused on enzymatic activities and affinities comparison, or research focused on characterizing external factors promoting hydroxylamine accumulation.

2.2.2. EFFECT OF HYDROXYLAMINE DOSING IN AMMONIUM OXIDIZING BACTERIA

Several studies have investigated the effect of externally added hydroxylamine on ammonium oxidising bacteria (Table 2.2). Since hydroxylamine first discovery as intermediate of nitrification, one of the initial questions was if hydroxylamine could be used for growth. Two parallel studies have shown that different species of AOB are able to use hydroxylamine mixotrophically together with ammonium for growth [41, 42]. Both studies [41, 42] reported a higher experimental growth yield than the theoretically expected, when using a mixture of hydroxylamine and ammonium as substrate. So far, it is not fully understood why and how hydroxylamine boosts the growth. In addition, to our knowledge it is still not shown if AOB can grow on hydroxylamine as single substrate.

Other studies have focused on the short term effect of externally added hydroxylamine to ammonium oxidation using batch tests or respirometry tests. When providing ammonium to an AOB batch culture, usually there is a lag period, or so called acceleration phase, which is the time that the culture needs to switch from a slower ammonium consumption to maximum consumption rate [43]. Different studies showed that externally added hydroxylamine accelerated this initial ammonium uptake rate [43, 42, 44, 45]. The externally added hydroxylamine to a biofilm system led to disaggregation from microcolonies to scattered cells [44, 45]. Based on these results two different hypotheses on why hydroxylamine accelerates the ammonium uptake rate were proposed [43, 44, 45]: i) Hydroxylamine impacts the cell morphology, scattering the cells and having a higher cell area available, so an increased mass transfer, leading to higher ammonium uptakes, ii) Electrons obtained in the hydroxylamine transformation to nitrite are recirculated to AMO enzyme, which triggers its activity increasing ammonium consumption.

Conventional nitrogen removal in WWTPs was traditionally performed by combination of nitrification and denitrification processes. Since the discovery of anammox, the possibility to combine partial nitrification with anammox has been intensively studied. Partial nitrification-anammox technologies offer the opportunity to lower WWTPs costs. However, one of the bottlenecks for its implementation is the stable operation of partial nitrification and efficient repression of NOB [14]. Hydroxylamine has been used to recover partial nitrification in reactors where nitrate started to accumulate [46, 47, 48]. In these studies hydroxylamine dosing combined with proper reactor operation triggered the stabilization of partial nitrification. In all three studies, the activity of AOB was not hampered, whereas NOB were inhibited as nitrate stopped accumulating in the reactor [46, 47, 48]. A rapid start-up of partial nitrification reactors was also achieved by hydroxylamine addition. For example, intermittent dosing of hydroxylamine in up-flow biofilm reactor [49] or SBR [50, 51] helped to speeding up the start-up of a partial nitritation process. A stable partial nitritation was maintained only if after stopping hydroxylamine dosage, a proper reactor control was implemented [50].

Overall, hydroxylamine showed to be efficient in inhibiting nitrite oxidation to nitrate (see next section), not damaging and even promoting AOB activity in most of the studies [41, 42, 50, 51, 47, 46, 48]. However, there are other studies that claim that long term exposure to hydroxylamine hampered AOB activity [44] and even an inhibition model for AOB has been proposed [52]. In addition, the observed negative effect of

hydroxylamine on mixed cultures biofilm like structures [45, 44] will not be desirable in certain operational modes, which rely on biofilm systems (i.e. granule, biofilm carriers).

Most of the "long term" hydroxylamine studies have been performed using a pulse feeding strategy, which leads to initially high hydroxylamine concentrations (Table 2.2). Furthermore, hydroxylamine feeding was mostly added temporarily for a start-up period or to promote partial nitritation. These exposures to sudden high hydroxylamine concentrations are not likely to occur in natural environments. Thus far, the only study with continuous and limiting hydroxylamine concentration was performed by de Brujin [42] with *Nitrosomonas europaea*. Using hydroxylamine limiting conditions can help to understand the mechanisms (i.e. over or under regulation of genes/proteins) by which hydroxylamine is promoting or hampering AOB activity in nitrification environments, without the potential inhibitive effects of hydroxylamine.

Table 2.2: Short term and long-term effect of hydroxylamine addition to AOB pure cultures or nitrification systems. n.d. - not determined, SBR-sequential batch reactor, RDRs – rotating disk reactor.* Assumed that the abiotic tests were performed at the same temperature than the biological cultivations. ** 10 mg/L NH_2OH converted to mg-N/L.

Type of biomass	Type of reactor/test	Temperature (°C)	pH	NH_4^+ (mg-N/L)	NH_2OH added (mg-N/L)	Type of addition	Effect	Reference
Nitrosomonas europæa	Chemostat	30	8	280	19.6-145.6	Continuous	Growth mixotrophically on ammonium and hydroxylamine (0.34 g/mg-N-NH_2OH)	[42]
Nitrosomonas europaea ATCC29578	Shake flask	n.d	7.8	56	28	Fed-batch	Growth mixotrophically on ammonium and hydroxylamine (36 µg protein/mg-N-NH_2OH/L)	[41]
Nitrosococcus oceanus Nc.1	Shake flask,	n.d	7.8	56	28	Fed-batch	Growth mixotrophically on ammonium and hydroxylamine (20 µg protein/mg-N-NH_2OH/L)	[41]
Nitrosomonas nitrosa Nm 90	Shake flask	n.d	7.8	56	28	Fed-batch	Growth mixotrophically on ammonium and hydroxylamine (30 µg protein/ mg-N-NH_2OH/L)	[41]
Nitrosomonas europaea ATCC19718	Flasks, planktonic	25	8.3	n.a.	1.4	Fed-batch	Starvation of cells decreased NH_2OH activity but not NH_4^+.	[53]
Nitrifying culture	RDRs, biofilm	20	7.6±0.2		3.5	Continuous	Partial nitrification, inhibition NOB, Higher ammonium consumption, dense clusters to single scattered cells	[45]
Nitrifying enrichment	Respirometric tests (from a 2L SBR)	25	7.5	3-12	n.d.	Batch tests	Study of acceleration phase. Hydroxylamine shortened acceleration phase.	[43]
Full nitrification aggregates	Batch tests	23*	7.5-8	150	15	Batch	Hydroxylamine addition increased ammonium uptake rate. Impact on the aggregate size	[44]
Full nitrification aggregates	Fed-batch reactors	23*	7.5-8.8	200	10,20,40	Pulse feeding	AOB and NOB inhibition, decrease of the aggregate size	[44]
Star-up PN	UP-flow biofilm PN	35	7.8±0.1	Gradually increased.	3.5	Added continuously	Achieved PN during start-up	[49]
Full nitrifying culture to PN	SBR	25	7.8-8.2	100	2.0 **	Pulse feeding every 2 days	Full nitrification switched to partial nitritation in one week	[48]
Nitrifying enrichment	Respirometric tests (biomass from a 4L SBR)	25	7.5	8	1-3	Batch test	Model including self- inhibition of hydroxylamine in AOB	[52]
PN/AMX	SBR	33	7.9±0.2	1750-221	5,10,20	Pulse feed	Inhibition of NOB, but population recovered when dosing was stopped	[47, 46]
Start-up PN	SBR	19.5-28.2	6.7-7.9	70	5	Pulse feeding start each cycle	Both AOB activity and NOB affected. *Nitrospira* more inhibited than *Nitrobacter*	[51]
Star-up PN	SBR	25±1	n.d.	70.5±6	4.5	Pulse feeding every 24h	Both AOB activity and NOB affected. *Nitrospira* more inhibited than *Nitrobacter*	[50]

2.2.3. N_2O PRODUCTION FROM HYDROXYLAMINE

In addition to the regular ammonia oxidation metabolism, two possible pathways have been proposed in order to explain the nitrogen loss in form of nitric or nitrous oxide gasses during nitritation: i) Nitrifier denitrification, which involves the reduction of NO_2^- to NO and N_2O by a nitrite reductase (Nir) and nitric oxide reductase (Nor) respectively. It has been suggested that this pathway is predominant at low oxygen concentrations [54]. ii) Hydroxylamine oxidation that involves the oxidation of NH_2OH to NO by HAO. Then, NO can be further converted to N_2O by Nor. N_2O production through this pathway is thought to be favoured at higher O_2 concentrations [55, 56, 54] (Figure 2.3).

However, recent studies have highlighted the occurrence and contribution of other pathways to the total N_2O emissions [57, 58, 59]. Most of these new proposed pathways have hydroxylamine as substrate. Briefly, Caranto et al., showed that the cytochrome P460 of HAO can directly produce N_2O from hydroxylamine [57].

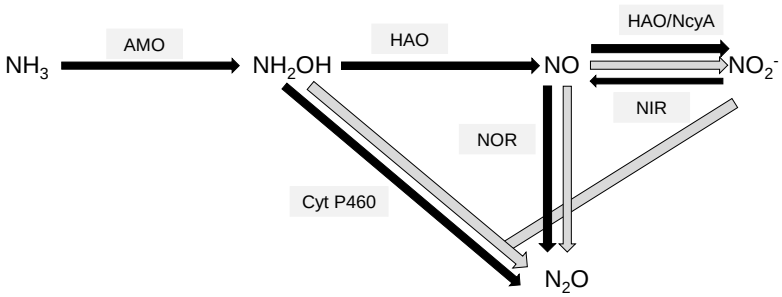


Figure 2.3: Putative N_2O emission pathways in AOB. Black arrows represent biologically mediated pathways, grey arrows represent abiotic conversions. AMO – ammonium monooxygenase, HAO – hydroxylamine oxidoreductase, NcyA - Nitrososcyanin, Cyt P460 – Cytochrome P460, NIR – nitrite reductase, NOR – NO reductase. Sources: [57, 28, 60, 59].

Besides biological conversion, hydroxylamine has been shown to react chemically by either disproportionation or with medium components such as Fe, Mn or HNO_2 resulting in N_2O formation [see [61, 62] for detailed reactions]. Two different studies showed independently the occurrence of a chemical reaction between nitrite and hydroxylamine at conditions relevant for wastewater treatment [63, 58]. Furthermore, the chemical N_2O production rate by the reaction of hydroxylamine and nitrite (or the protonated form, nitrous acid) was comparable to the biological N_2O production rates [60, 59]. Contradicting results were presented by Su and co-workers [64], proposing that abiotic reactions would only be relevant at acidic pH. All mentioned studies[64,

58, 60, 59] were performed with different medium compositions, and the impact of different compounds to the putative final reported rates is yet unknown. For instance, performing the same test with demineralized water or synthetic medium increased the hydroxylamine disproportionation by 2 to 22 fold [64]. However, abiotic tests containing both free nitrous acid and hydroxylamine were only performed with demineralized water at neutral pH [64]. At pH 8 (when free nitrous acid concentration is really low) the reaction rate increased by at least one order of magnitude when using medium instead of demineralized water [64]. Thus, from our point of view it is unclear if pH and/or medium compounds are both important when determining the chemical reaction rate. Consequently, we suggest that the kinetic characterization of hydroxylamine abiotic reactions, and the impact of different environmental conditions (i.e. trace elements concentrations, iron and others) is of relevance to understand this process properly. Even more importantly, the impact of a real wastewater matrix into such reactions is yet to be studied.

From an engineering point of view, N_2O mitigation strategies in wastewater treatment have already been implemented, even if the actual conversions behind the emissions are not fully understood [65]. To improve these strategies, a more in-depth understanding of the pathways contributing to the total N_2O emissions is needed. This would help to identify the factors promoting N_2O emissions and to include this knowledge in the design of wastewater treatment process, instead of applying mitigation strategies after operation started. The challenge is to develop a methodology that allows to identify the pathways contribution to the total N_2O emissions, as well as studying the factors impacting them. The large number of compounds and microbial groups involved combined with potential chemical conversions makes this a difficult task. For example, implementing a comprehensive approach including a combination of ^{15}N tracer studies, natural isotope signatures, modelling and transcriptomics/proteomics might be needed to be able to fully differentiate between pathways [66].

Overall, hydroxylamine has been shown for years to be a promotor of N_2O emissions, which has a 300-fold larger warming potential than that of CO_2. Thus, further understanding the factors promoting N_2O emissions from hydroxylamine, will help in the design of mitigation strategies.

2.3. AMMONIA OXIDIZING ARCHAEA (AOA)

AMMONIUM oxidizing archaea (AOA) were firstly identified using genomic tools, as the detected *amo* gene was corresponding with an archaeon scaffold [67, 68]. Later, the first isolation of an AOA microorganisms demonstrated its abilities to oxidize ammonium to nitrite [69]. AOA might play an important role in nitrification in environments such as the oceans and soils, where substrates are usually found at low concentrations and AOA high ammonium affinities allow its survival [70, 71]. Nitrifying drinking water filters are also often reported to contain AOA [72, 73, 74]. Due to their phylogenetic differences with other archaea, AOA were proposed to be classified inside a new phylum in the archaea domain; *Thaumarchaeota* [75]. Even if the first pure culture of AOA was obtained more thab 16 years ago [69], as their bacterial counterparts, its central nitrogen metabolism is still under discussion. For instance, AMO enzyme is conserved in all known AOA, but no HAO homologues have been identified [76, 29,

28, 77]. Thus, the conversion of ammonium to hydroxylamine Equation (2.2) has been proposed to be conserved and catalysed by the archaeal AMO enzyme. The further conversion of hydroxylamine to nitrite is under consideration (as it occurs with AOB and comammox). The fact that no HAO homologues have been detected in the AOA genome led to two possible central nitrogen metabolic models [28]: i) a copper complex uses NO and NH_2OH to form two molecules of nitrite, NirK enzyme is involved in the transformation of nitrite to NO (Figure 2.4A), ii) two enzymes consecutively oxidize NH_2OH to NO and NO to nitrite, proposed to be mediated by NirK or an uncharacterized copper complex (Figure 2.4B).

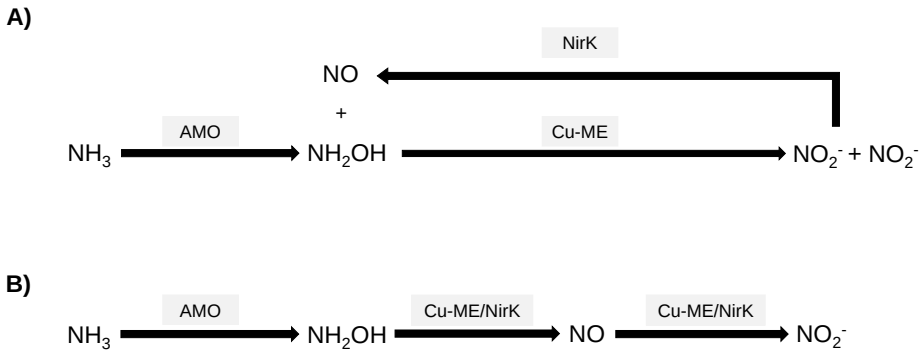


Figure 2.4: Proposed enzymes for ammonium conversion by ammonium oxidizing archea (AOA). No homologues of hydroxylamine oxidoreductase (HAO) are present in AOA, thus alternative pathways are proposed A) A cooper-based enzymatic complex (Cu-ME) is able to transform NO and hydroxylamine to form two nitrite molecules, one of this nitrite molecules is transformed back to NO by nitrite reductase (NirK) B) Alternatively, hydroxylamine is first transformed to NO and further oxidized to nitrite by a yet not fully characterized enzyme that could be either Cu-ME or NirK. Sources - [29, 28].

To the yet not fully resolved hydroxylamine to nitrite conversion pathway, it must be added the lack of cytochrome-c type proteins usually performing the electron transportation in AOB respiration. Instead, a copper based electron transport system has been postulated, as a high number of protein copper domains have been identified in the genome [70, 78].

Regarding NO and N_2O emissions AOA are not capable to perform nitrifier denitrification [79, 17], as no N_2O was formed with limited oxygen supply. Isotopic signature also suggested that the nitrogen found in N_2O comes from both ammonium and nitrite [79].Thus, N_2O production was linked to ammonium conversion, and proposed that hydroxylamine or N-intermediates abiotically react leading to N_2O [79, 17].

Due to its relatively recent identification few experiments used or measured

hydroxylamine in AOA cultures. So far, externally added hydroxylamine has been used to demonstrate its role as intermediate and postulate that hydroxylamine oxidation is coupled to ATP generation in *Nitrosopumilus maritimus* [77]. In addition, externally added hydroxylamine concentrations of 14 mg-N/L showed to completely inhibit *N. maritimus* [77], which indicates a higher sensitivity to hydroxylamine exposure of AOA to that observed in AOB (Table 2.2). Nevertheless, to our knowledge no other studies of batch or continuous exposure to externally added hydroxylamine of other AOA strain have been reported, yet. Thus, a differential behaviour of AOA strains to hydroxylamine exposure remains to be investigated.

Hydroxylamine transient accumulation has been shown to occur in AOA culture, also pointing towards a differential strain behaviour towards hydroxylamine accumulation [18]. For example, *N. gragensis* only released hydroxylamine (4.6 µg-N/L) when incubated with 28 mg-N/L ammonium, but not with 7 mg-N/L. *N. uzonensis* produced hydroxylamine with both ammonium initial concentrations of 7 and 28 mg-N/L, reached higher concentrations (4.8 µg-N/L) when incubated with the higher ammonium concentrations. Contrarily, *N. viennensis* and *Ca.* N. sp. Nd2 did not produce hydroxylamine. Thus, the hydroxylamine accumulation strain dependency observed in AOB seems to also be a differential strain trait of AOA.

Overall, the recent identification and isolation of AOA presents still unresolved questions, such as the central nitrogen metabolism, further characterization of the NO/N_2O emissions or the differential strain response to hydroxylamine accumulation and exposure.

2.4. COMPLETE AMMONIUM OXIDIZING BACTERIA (COMAMMOX)

SINCE nitrification first discovery, it was always thought that ammonium oxidation to nitrate involved a two-step microbial conversion, involving AOB and nitrite oxidizing bacteria (NOB). Complete ammonium oxidation to nitrate by a single microorganism was predicted thermodynamically possible [80], but overlooked for years until two independent studies demonstrated its existence [81, 82].

Complete ammonium oxidating (comammox) bacteria were first identified in two parallel studies demonstrating that they have all the cell machinery to oxidize ammonium to nitrite and further to nitrate [81, 82]. Mainly two reasons prevented comammox identification for so many years; i) AOB dedicated qPCR primers targeting *amo* gene were not covering the comammox *amoA* gene, due to only ca. 60% amino-acid identity, ii) 16S rRNA sequencing does not allow to distinguish comammox from NOB. Thus, comammox were usually misclassified as canonical NOB [83].

The current running hypothesis for the central metabolism of comammox is postulated to involve AMO, HAO and NXR enzymes, as copies of all the genes encoding for these enzymes have been found in the genome [81, 82]. Thus, ammonium is first transformed to hydroxylamine by AMO, hydroxylamine is further oxidized to nitrite by HAO and finally nitrite is converted to nitrate by nitrite oxidoreductase (NXR) (Figure 2.5A). As well as for AOB and AOA, the occurrence of a third intermediate step involving HAO converting NH_2OH only to NO and a further conversion of NO to

nitrite is under discussion [84] (Figure 2.5B). As discussed previously, NcyA has been hypothesised to be a lacking third enzyme for AOB . However, yet no NcyA encoding gene has been found in the available comammox genomic data [85, 84, 86]. Thus the hypothesis of comammox encoding a NO oxidoreductase in the genome has not been confirmed yet.

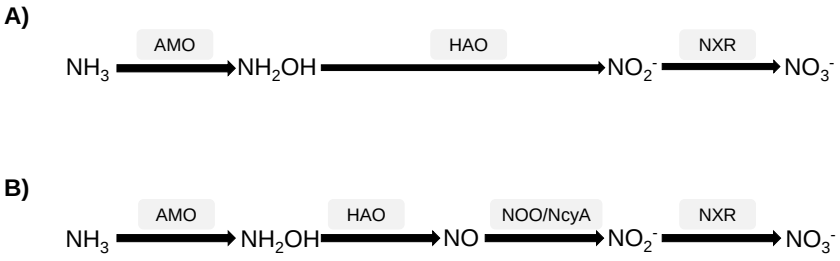


Figure 2.5: Proposed enzymes for ammonium conversion by comammox. A) Traditional pathway, where hydroxylamine is directly converted to nitrite followed by the conversion to nitrate. B) Alternatively, hydroxylamine is first transformed to NO and further oxidized to nitrite by a yet not fully characterized enzyme. AMO – Ammonium monooxygenase, HAO – Hydroxylamine oxidoreductase, NOO - Nitric oxide reductase, NcyA – Nitrososcyanin, NXR – nitrite oxidoreductase. Sources - [29, 28].

The first kinetic analysis of the first isolate *Nitrospira inopinata* [16] showed a low ammonium half-saturation coefficient (high affinity for ammonium) and a high growth yield (compared to that of AOB or AOA). This is in agreement with the theoretical higher growth yield prediction [80], as well as the comammox distribution in the environment [83]. Comammox has been detected at substrate depleted zones [83], where low ammonium affinity constant and high growth yield allows them to thrive in such minimal environments.

Due to its recent discovery little is known about the role of hydroxylamine besides being involved as intermediate in the central metabolism. Regarding hydroxylamine transient accumulation, Liu and co-workers showed hydroxylamine accumulation in batch like experiments up to 6 µg-N/L, even calculated hydroxylamine accumulation could have been up to 25 to 132 µg-N/L, depending on the initial ammonium concentration used [18]. A recent study also postulated towards abiotically formed N_2O from hydroxylamine as the main comammox emission source [84].

Overall, due to its novelty and recent discovery there is plenty of room for research to further understand the hydroxylamine role in comammox metabolism. Among others, the characterization of hydroxylamine build up in cultures, the impact of externally

added hydroxylamine in the metabolism or the involvement of hydroxylamine in N_2O emissions.

2.5. ANAEROBIC AMMONIUM OXIDIZING BACTERIA (ANAMMOX)

AMMONIUM conversion without oxygen, even predicted thermodynamically favourable [87], was thought nonexistent until the discovery of anammox [8]. Anammox bacteria are able to transform ammonium and nitrite to dinitrogen gas autotrophically and anoxically [9]. The central metabolism of anammox has been a hot topic of research since its discovery ([88, 89, 90, 10, 11], among others).

Hydroxylamine was initially hypothesized to be an intermediate of the anammox conversion, as when it was added to anammox cultures, hydrazine accumulated [9, 11]. Since then, hydroxylamine addition/hydrazine accumulation experiments have been used as characteristic activity tests to demonstrate anammox activity in enrichment cultures ([91, 9], among others). Later, NO was proposed to be intermediate of the central nitrogen metabolism instead of hydroxylamine [89]. The current hypothesis for the anammox nitrogen metabolism consists of three reactions. First, a nitrite reductase (Nir) enzyme converts nitrite to NO (Equation (2.6)). Then, NO reacts with NH_4^+ and forms hydrazine (N_2H_4) (Equation (2.7)) catalysed by hydrazine synthase (HZS). Finally, hydrazine dehydrogenase (HDS) further converts hydrazine to dinitrogen gas (Equation (2.8)) (see also Figure 2.6).

$$NO_2^- + 2H^+ + e^- \longrightarrow NO + H_2O \tag{2.6}$$

$$NO + NH_4^+ + 2H^+ + 3e^- \longrightarrow N_2H_4 + H_2O \tag{2.7}$$

$$N_2H_4 \longrightarrow N_2 + 4H^+ + 4e^- \tag{2.8}$$

2.5.1. THE YET UNKNOWN ROLE OF HYDROXYLAMINE IN ANAMMOX BACTERIA

Hydroxylamine role in the anammox metabolism is still not fully understood. For instance, not all anammox strains (i.e. *Ca.* Brocadia spp.) encode the Nir enzyme [92]. Consequently, either another enzyme, like the one encoded in the gene *kustc0458*, is doing the job [88], or hydroxylamine is involved in the pathway [90].

Another surprising and characteristic trait is that hydroxylamine oxidase (HOX), which converts hydroxylamine to NO, is one of the most highly expressed enzymes in anammox [88, 89]. Thus, anammox is investing energy and nutrients on keeping a high HOX protein content in the cell, which is puzzling, if hydroxylamine does not have an important role in the metabolism (Figure 2.6).

The only hypothesis to explain this high HOX expression, is that HZS enzyme can leak hydroxylamine and HOX is able to transform any leaking of hydroxylamine back to NO [93, 23]. Precisely, it is proposed that in HZS catalytic centre, NO is actually transformed to hydroxylamine, and hydroxylamine is reacting with ammonium to form hydrazine [93, 23]. Another explanation for this high HOX expression is that hydroxylamine might

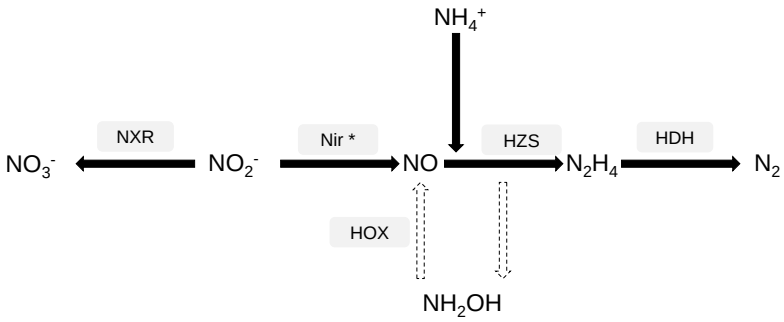


Figure 2.6: Central nitrogen metabolism of anaerobic ammonium oxidazing bacteria (anammox). * Nir is depicted here as the enzyme responsible to convert nitrite to NO, this might vary for *Ca.* Brocadia strain [90]. Also a proposed enzyme for this conversion is the one encoded in the gene *kustc0458* [88]. NXR – nitrate oxidoreductase, Nir- nitrite reductase, HZS- hydrazine synthase, HDH – hydrazine dehydrogenase, HOX- hydroxylamine oxidase. Sources- [23].

be important in anammox like environments. Overall, the role of hydroxylamine in the anammox metabolism remains as yet poorly understood.

Externally added hydroxylamine in form of batch tests had different outcomes (Table 2.3): i) When hydroxylamine was added, accumulation of hydrazine occueds, which has been used to demonstrate anammox activity [91, 9], ii) Hydroxylamine has been shown to "boost" the anammox activity [94, 95], iii) Hydroxylamine addition allowed to characterize anammox hydroxylamine metabolism [11, 96, 97]. Hydroxylamine anammox metabolism occurs via disproportionation to ammonium and dinitrogen gas (Equation (2.9)). However during this disproportionation hydrazine accumulation occurs. The accumulation is due to an imbalance between the two reactions involved in hydroxylamine disproportionation that produce (Equation (2.10)) and consume hydrazine (Equation (2.11)), respectively [96].

$$3\,NH_2OH + H^+ \longrightarrow NH_4^+ + N_2 + 3\,H_2O \tag{2.9}$$

$$NH_4^+ + 2\,NH_2OH \longrightarrow N_2H_4 + H_2O + H^+ \tag{2.10}$$

$$2\,NH_2OH + N_2H_4 + 2\,H^+ \longrightarrow 2\,NH_4^+ + N_2 + 2\,H_2O \tag{2.11}$$

Co-metabolisation of other substrates with hydroxylamine impacts its metabolism [97]. Continuous and limiting addition of hydroxylamine to anammox showed to decrease the stoichiometric nitrate needed for growth and reported no negative impact on the anammox community [97]. Thus, anammox could use hydroxylamine and survive in environments where it is present.

Table 2.3: Summary of hydroxylamine consumption experiments performed with anammox biomass. a) Units in µmol-N/vial. n.d. not determined, n.a. – not applicable, *Ca.* – *Candidatus*, Temp. – Temperature, HRT – Hydraulic retention time, CSTR – continuous stirred tank, MBR – membrane bioreactor, cons. - consumption. * Experiments performed in the optimal range for anammox growth.

Anammox specie	Type of sludge	Reactor conditions	Temp.(°C)	pH	Initial NH_4^+ (mg-N/L)	Initial NO_2^- (mg-N/L)	Initial NH_2OH (mg-N/L)	qNH_2OH cons. (mg-N/gVSS/h)	N_2H_4 peak (mg-N/L)	qN_2H_4 cons. (mg-N/gVSS/h)	Reference
n.d.	Flocs	Batch tests	30	7	112	112	42	n.d.	14.0	n.d.	[11]
Ca. Brocadia anammoxidans and stuttgarteniss	Flocs	Schott flasks	37	7	11.2	0	39.2	n.d.	8.7	n.d.	[91]
Ca. Kuenenia stuttgartensis	Granular sludge	CSTR, batch tests	37	*	28	0	7-140	4.3 – 12.7	0.4-2.7	0.2 – 1.0	[96]
Ca. Brocadia fulgida	Granular sludge	SBR, batch tests	37	*	42	0	56	n.d.	2.7	n.d.	[96]
n.d.	Granular sludge	16L reactor, batch tests	35	7.5	43.4	0	39.2	2.9	10.1	0.9	[94]
n.d.	Granular sludge	16L reactor, batch tests	35	7.5	35	30.8	0	5.6	n.a.	n.a.	[94]
Ca. Brocadia fulgida	Biofilm carriers	MBR, batch tests	25	8-8.5	84	0	14	1.5	3.4	n.d.	[95]
Ca. Brocadia sinica	Planktonic cells	MBR, batch tests	37	7.6	0	0	21	n.d.	0.1 (a)	n.d.	[90]
Ca. Kuenenia stuttgartiensis	Planktonic cells	Batch tests	30	8-8.5	0	0	22.4	75.6	2.6	4.2	[97]
Ca. Kuenenia stuttgartiensis	Planktonic cells	Batch tests	30	8-8.5	0	19.6	19.6	42	1.3	2.8	[97]
Ca. Kuenenia stuttgartiensis	Planktonic cells	Batch tests	30	8-8.5	84	19.6	29.4	112	7.2	25.8	[97]

2.6. NITRITE OXIDIZING BACTERIA (NOB)

NITRITE oxidizing bacteria perform the second step of nitrification catalysing the conversion of nitrite to nitrate with oxygen as electron acceptor. Up to date, 7 genera have been described belonging to 6 different phyla in α, β, γ Proteobacteria [98].

NOB couple the nitrite oxidation to nitrate as electron donor (Equation (2.12)) with oxygen respiration as electron acceptor (Equation (2.13)), resulting in NOB central nitrogen catabolism (Equation (2.14)). Nitrite oxidation to nitrate is catalysed by nitrite oxidoreductase (NXR), a membrane-bound enzyme that was first isolated and characterized in *Nitrobacter* by Meincke et al. [99] (Figure 2.7A).

$$NO_2^- + H_2O \longrightarrow NO_3^- + 2H^+ + 2e^- \tag{2.12}$$

$$\frac{1}{2}O_2 + 2H^+ + 2e^- \longrightarrow 2H_2O \tag{2.13}$$

$$NO_2^- + \frac{1}{2}O_2 \longrightarrow NO_3^- \tag{2.14}$$

2.6.1. HYDROXYLAMINE INHIBITS NOB

Hydroxylamine is not an intermediate in NOB metabolism, but NOB communities usually are found close to AOB, which are able to leak hydroxylamine (Table 2.1). Hydroxylamine concentrations from 0.2 to 20 mg-N/L have been reported as a potent inhibitor of NOB Table 2.4. For instance, Castignetti and Gunner (1982) [100] reported inhibition of *Nitrobacter agilis* by hydroxylamine at concentration of 5 mgNH_2OH-N/L. Stuven et al. [19] also reported hydroxylamine inhibition of *Nitrobacter* at concentrations of 1 mgNH_2OH-N/L. Later, Hao and Chen [101] demonstrated hydroxylamine inhibition in NOB by measuring nitrite build-up in complete nitrification submerged filters by the addition of 2.5-5 mgNH_2OH-N/L. Moreover, more than 30 days were needed to recover regular operation [101]. Concentration of less than 0.2 mgNH_2OH-N/L were reported to inhibit NOB by Blackburne and co-workers [102].

Another indication that supports strong NOB inhibition by hydroxylamine is the general trend observed that full nitrification fails, accumulating nitrite, when hydroxylamine is added to the system [44, 47, 48]. As discussed before, hydroxylamine external addition in nitrification systems is used to inhibit NOB either to obtain a stable partial nitrification operation or to achieve a rapid start-up of a partial nitrification reactor [50, 51, 47, 46].

However, the mechanism of hydroxylamine inhibition in NOB is still unknown (i.e. if it is affecting the gene expression, interfering with cell compounds). Some studies refer to a reversible inhibition, as far as full nitrification is restored with time after hydroxylamine exposure [51, 47, 48]. The only hypothetical theory is that the un-protonated form of hydroxylamine can diffuse through the membrane and affect the gene expression [20]. Specifically, in a later study Wang and co-workers reported a decrease in *nxrA*, a gene related with NOB [46]. Another interesting observation is that *Nitrospira* and *Nitrobacter* might be differentially inhibited by hydroxylamine, impacting more *Nitrospira* than *Nitrobacter* [51].

In full nitrification and partial nitrification processes, AOB and NOB populations are usually clustered together. Thus, understanding further the impact of hydroxylamine

on NOB activity is vital, as AOB have been shown to transiently accumulate this compound. Addition of hydroxylamine has already been shown to promote a partial nitritation system over full nitrification [47, 48]. Further understanding of this process (hydroxylamine accumulation and effect on NOB) could contribute to avoid NOB proliferation in partial nitritation anammox systems and further understand microbial community interactions.

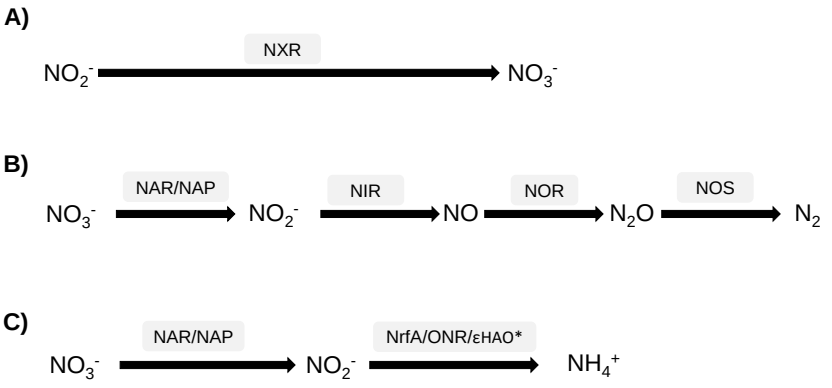


Figure 2.7: Central nitrogen metabolism of: A) Nitrite oxidizing bacteria (NOB), B) Anaerobic heterotrophic denitrifiers (DEN), C) Dissimilatory nitrate reduction to ammonium (DNRA). NXR – nitrite oxidoreductase, NAR/NAP – nitrate reductase, NIR – nitrite reductase, NOR – NO reductase, NOS – N_2O reductase, NrfA-ammonia forming nitrite reductase, ONR- nitrite reductase, ϵHAO- Epsilonproteobacterial hydroxylamine oxidoreductase.

2.7. OTHER WASTEWATER TREATMENT RELATED MICROORGANISMS AND THEIR INTERACTIONS WITH HYDROXYLAMINE

AOB, AOA, anammox and comammox are recognized consumers of hydroxylamine, and hydroxylamine metabolism has been dedicatedly studied, but still unknowns remain. The inhibition of nitrite oxidizing bacteria by hydroxylamine has been dedicatedly studied. Particularly, with the growing interest of implementing partial nitritation. Conversely, heterotrophic denitrifying bacteria play a crucial role in engineered systems such as wastewater treatment plants (WWTPs) transforming nitrate to dinitrogen gas through nitrite, NO and N_2O (see Figure 2.7B). There is only very limited information on the impact of hydroxylamine on denitrifying bacteria. A recent study showed that nitrite accumulation was favoured when dosing hydroxylamine to a complete denitrification reactor [103]. Batch tests with hydroxylamine dosing from

Table 2.4: Summary of literature reporting NOB inhibition by hydroxylamine (a) converted from mg NH_2OH/L, * referred as free hydroxylamine, OUR – oxygen uptake rate, n.d.-not determined, SBR –sequential batch reactor, FBBR-Fed-batch bioreactor, PN – Partial Nitritation.

Type of biomass	NH_2OH (mg-N/L)	Type of inhibition	Comments	References
Nitrobacter agilis	5	Irreversible	No activity was detected when the culture was transferred to fresh medium	[100]
Nitrobacter vulgaris	1	n.d.	No nitrate formation	[19]
Full nitrification culture	0.3-4.3*	n.d.	Deterioration of full nitrification, nitrite transient accumulation	[20]
Full nitrification culture (submerged fixed film)	1 - 5	Reversible	Deterioration of full nitrification, nitrite accumulation. 30 days recovery.	[101]
NOB enrichment (SBR)	0.2- 3	n.d.	Hydroxylamine decreased OUR	[102]
Full nitrification culture (FBBR)	10-40	n.d.	Deterioration of full nitrification, nitrite accumulation	[44]
Partial nitritation/anammox	4.2-8.5 (a)	Reversible	Decreased nitrate accumulation by NOB	[47]
Full nitrification culture	1-3	Non-competitive	Decreased OUR (Ki=3.233±0.093 mmol-N/L)	[52]
Start-up PN (SBR)	1.9 (a)	n.d.	*Nitrospira* more inhibited than *Nitrobacter*	[50]
Start-up PN (SBR)	2.1 (a)	Reversible	*Nitrospira* more inhibited than *Nitrobacter*, nitrate production recovered	[51]

(2-21 mg-N/L) were also performed. Nitrate consumption seemed to be promoted at low hydroxylamine concentrations dosages (2-8 mg-N/L), whereas nearly any nitrate consumption was observed during the first 20min. when hydroxylamine concentrations were higher than 14 mg-N/L. Interestingly, nitrite accumulation was always higher when hydroxylamine was added in the batch tests. Nitrate reductase (NAR) and nitrite reductase (NIR) activity were also measured after hydroxylamine exposure, showing a grater increase in NAR activity than NIR for increasing hydroxylamine doses. The difference in enzymatic activities might explain the nitrite accumulation . This was in agreement with the gene expression, as *napA* expressions was up to 2.76-fold increased when hydroxylamine was dosed [103]. Nevertheless, the impact of hydroxylamine on the subsequent steps: NO and N_2O conversions was not investigated. Also the fact that pH 9 was used in this study, might have impacted the results, as hydroxylamine is mainly unprotonated. Thus, it is able to diffuse through the membranes. Overall, more studies confirming the observed trends by [103] would be needed, specially performed at more usual pH (7-8) for wastewater treatment. Hydroxylamine usage capacity of dissimilatory nitrate reducers to ammonia (DNRA) (see Figure 2.7C) has been hypothesised based on the hydroxylamine detoxification capacity of some of their enzymes (ONR or ϵHAO) [104, 105] and hydroxylamine has been proposed as intermediate for *Nautilia profundicola* [106]. For both denitrifiers and DNRA bacteria few studies are available and no conclusions can be drawn regarding the impact of hydroxylamine.

Finally, it is worth mentioning other microorganisms that are able to use hydroxylamine and that might be involved in wastewater treatment engineered processes, even not considered directly as part of the nitrogen cycle. For instance, heterotrophic aerobic bacteria with nitrification or/and denitrification activity. The pathway for nitrification encoded in these microorganisms includes hydroxylamine as intermediate [22]. For example, *Photobacteriu* sp. [107], *Alcaligenes faecalis* [108, 109, 110], *Pseudomonas* [111] or *Enterobacter* [112]. Methanotrophs are also well known to have hydroxylamine oxidation capacity, which is involved in nitrous and nitric oxide production by those microorganisms [113, 22, 114].

Overall, hydroxylamine oxidation capacity is widespread within microorganisms of the nitrogen cycle and others involved in wastewater treatment, whereas it is also known to inhibit some of them. To achieve a comprehensive picture of its role when shaping microbial communities, further investigation on this compound in relation to the diverse N-cycle conversion is needed.

2.8. HYDROXYLAMINE PRESENCE MIGHT SHAPE MICROBIAL COMMUNITIES AND BIOFILMS DYNAMICS

ALL of the microorganisms discussed in the present review are known to be found close together in the natural environment (i.e. ocean, soils) and engineered systems where they typically grow in biofilms or aggregates (i.e. WWTPs) [3]. Thus, microbial interactions between different communities occur, and usually microbes rely on these interactions to get their substrate or to avoid product inhibition (i.e. AOB/NOB interactions).

In engineered systems, such as WWTPs, the interaction between AOB and NOB and denitrifiers have been conventionally used to remove nitrogen from wastewater. More recently, other players such as anammox, comammox or AOA have been added to the already complex community interactions. In these kind of engineered systems microorganisms are usually found forming aggregates either as activated sludge, granules or attached biofilms. As we have seen in this review and according to literature, AOB, AOA and comammox can leak hydroxylamine, and it can be related to fluctuations of substrate/oxygen. In WWTPs substrate fluctuations are usual, but also within biofilm systems strong gradients occur. Thus, fluctuations of substrate/oxygen can be enhanced by the biofilm structure (i.e. some cells that have been under starvation, receive substrates when there is an increase in the bulk liquid concentration). Consequently, hydroxylamine build up can be enhanced within a biofilm system. This fact has already been proposed by mathematical simulations [115].

Another important factor that can impact hydroxylamine accumulation and usage by microorganisms is pH. In a biofilm a pH gradient is generated with a more acidic pH in the inner core of the granule [36, 34, 38, 39, 40]. Acidic pH has been shown to strongly impact ammonium oxidation by *Nitrosomonas*, whereas hydroxylamine oxidation was barely affected [35]. This fact might favour hydroxylamine usage over ammonium by AOB in inner layers of a biofilm system.

Overall, hydroxylamine build up due to aerobic ammonium oxidizing microorganisms can have an impact in other microbial communities such as anammox, NOB, denitrifiers or the same neighbour clusters of aerobic ammonia oxidizers. Furthermore, hydroxylamine can also trigger N_2O emissions, as it has been discussed. Thus, hydroxylamine might have a yet not fully understood role when shaping microbial communities.

2.9. HYDROXYLAMINE MEASUREMENT: THE BOTTLENECK?

IN wastewater treatment related research hydroxylamine measurements are almost absent. This is due to two factors: i) hydroxylamine available measurement

techniques are laborious and really time consuming, ii) being usually an intermediate, the method should be sensitive enough for the expected low concentrations. Nevertheless, the fact that hydroxylamine is mutagenic and toxic compound for humans, microorganisms and animals has brought the need of its adequate quantitative measurement in different fields [116].

Focusing on the measurements of hydroxylamine in water samples there are mainly two extended techniques used Figure 2.8 : i) Spectrophotometric technique, based on the production of indooxine from the reaction of 8-quinolinol with hydroxylamine in presence of carbonate and ethyl alcohol, which develops a green colour [117], ii) Gas chromatography (GC) based method, which relays on the measurement of N_2O formed during the transformation of hydroxylamine to N_2O catalysed by Fe_3^+ in sealed vials [118, 119].

Both techniques have their advantages and disadvantages. Briefly, the spectrophotometric based technique can be easily implemented, as it is a reactive based methodology which needs of general present laboratory equipment (spectrophotometer, water bath and pyrex tubes). The use of a fume hood is necessary due to the toxicity of the chemicals used and it is a quite laborious method. The GC based method, has a lower detection range (ca. $>$ 0.001 mg-N/L) than that of the spectrophotometric method (ca. $>$ 0.035 mg-N/L), which might be useful for applications were concentrations of hydroxylamine are actually low. However, the need for a GC with an N_2O detection method, and ideally an autosampler, might limit its implementation in many laboratories. Also a good determination of the N_2O already present in the sample is crucial for an accurate hydroxylamine quantification [119].

Both methods are known to be impacted by interferences like pH and salinity [118]. These interferences impact a lot the N_2O recovery from hydroxylamine in the GC method, thus a good pre-treatment depending on the sample is needed [119].

Independently of the method used, pre-treatment of samples and rapid analysis is generally extensive, due to the high reactivity of hydroxylamine. For example, addition of sulfamic acid to the sample is used in both measurement techniques [119, 58] with two purposes: i) acidify the sample to stabilize hydroxylamine, and ii) remove nitrite from the sample, which has been shown to react with hydroxylamine and interferes in both methods [119, 58]. Nevertheless, time from sample collection to its analysis is still crucial, long time storage is not possible.

The complexity of hydroxylamine measurement is one of the main limitations for understanding the role of this compound in the nitrogen cycle and N_2O emissions. Thus, developing of commercial available hydroxylamine sensors might be crucial for future research. Some preliminary results on the development of a hydroxylamine sensor have been reported ([120, 121], among others). However, to our knowledge, there are no initiatives to have such sensors commercially available. If such sensors would become available, as happened for NO and N_2O sensors, or an easier measurement method technique is developed it would boost hydroxylamine related research and our understanding of the nitrogen cycle.

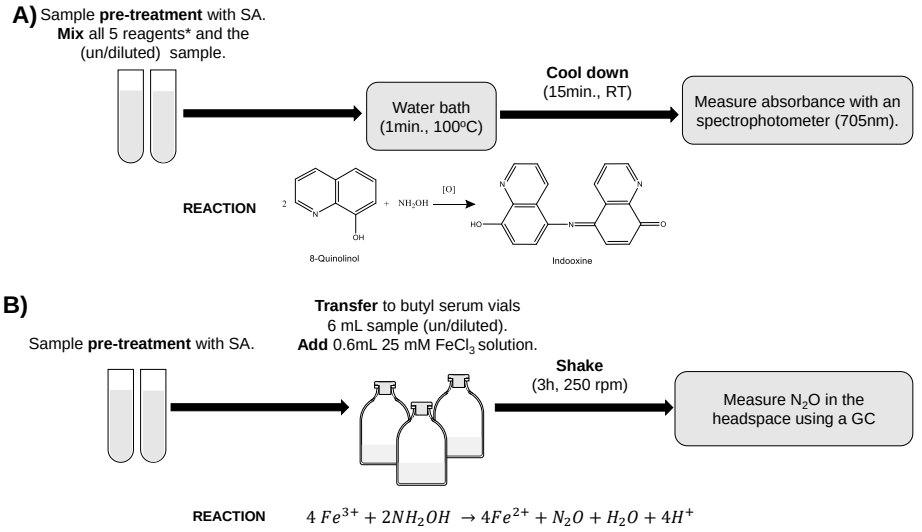


Figure 2.8: Hydroxylamine measurement techniques for hydroxylamine concentration determination in water: A) Spectrophotometric based method, B) Gas chromatography (GC) based method. Based on techniques described by [117, 119] * Reagents used are phosphate buffer solution, mili-Q water, trichloroacetic acid solution, 8-quinolinol and carbonate solutions (see [117] for exact concentrations). SA states for sulfamic acid. RT states for room temperature.

2.10. CONCLUSIONS AND FUTURE OUTLOOK

HERE the current understanding of the role of hydroxylamine in the nitrogen cycle, with special focus on the microbial communities involved in wastewater treatment has been presented. Hydroxylamine conversion is widespread within different nitrogen cycle microorganisms, whereas some are negatively impacted by it. We have highlighted that there are plenty of questions and unknowns about the role of hydroxylamine in the nitrogen cycle. The key gap of knowledge are summarized as follows:

- Clarification on how hydroxylamine is converted to nitrite by AOA, AOB and comammox is needed. So far, there are biocatalytic evidences that HAO transforms hydroxylamine to NO in AOB. AOA do not harbour HAO in their genome, thus a different transformation of hydroxylamine has been proposed. Comammox pathway for this conversion is still to be mapped. Overall, it will be interesting to assess if aerobic ammonia oxidizers have evolved differently on how to deal with the conversion of hydroxylamine to nitrite.

- Transient accumulation of hydroxylamine is usually linked to a switch from low to maximum activity (i.e., anoxic/aerobic cycles, batch tests, SBR reactors). The turnover of the differential enzymes involved in the transformation might be crucial. In addition, it seems to be a strain dependent trait.

- pH might have a crucial role on hydroxylamine usage. Firstly, because pH affects the equilibrium between the protonated and unprotonated hydroxylamine form.

Secondly, because ammonium oxidation rate is highly impacted by pH, whereas hydroxylamine oxidation to nitrite is not. Consequently, pH can be a potential contributor to hydroxylamine accumulation.

- Transient hydroxylamine accumulations seems also to be strain dependent in aerobic ammonium oxidizers, as well as it differs between AOB, AOA and comammox. Differences on enzymatic level are hypothesised to be responsible for such observations.
- Hydroxylamine is involved in N_2O emissions in AOA, AOB and comammox. Factors promoting N_2O emissions from hydroxylamine are still to be fully understood.
- Anammox is known to be able to use hydroxylamine as substrate. It is also proposed to be intermediate in *Ca.* Brocadia, whereas NO is proposed to be the intermediate in *Ca.* Kuenenia stuttgartiensis. Nevertheless, in *Ca.* Kuenenia stuttgartinesis, there is a high overexpression of HOX, an enzyme hypothesised to transform the leaking hydroxylamine from HZS to NO. Thus, the role of hydroxylamine in anammox is yet to be understood.
- pH also impacts the equilibrium between the unprotonated (free hydroxylamine) and protonated form of hydroxylamine. Thus, free hydroxylamine has the capacity to diffuse through the bacterial membranes. This is hypothesised to be the cause of NOB inhibition by hydroxylamine. However, it is yet to be demonstrated. A similar inhibition mechanism might impact denitrifiers leading to nitrite accumulation.

Overall, there are a wide range of topics to be investigated regarding hydroxylamine and the nitrogen cycle. Below we provide some crucial points and recommendations for future research, that will broaden our understanding of hydroxylamine:

- Developing an easy implemented hydroxylamine measurement technique would totally facilitate hydroxylamine related research. Either the use of sensors or an improved measuring technique that is not extremely labour intensive, would lead to widespread hydroxylamine measurements in nitrogen related research.
- Developing an integrated research approach including a combination of transcriptomics/proteomics, enzymology and ^{15}N tracer studies could help to further understand the mechanisms involved in hydroxylamine conversion and transient accumulation. Such an integrated approach will be crucial to map hydroxylamine conversion pathways, as well as studying such conversions in microbial communities.
- Mapping hydroxylamine enzymatic conversions will help also on understanding NO/N_2O production pathways. Thus, it will allow to design better mitigation strategies.

To conclude, the main focus of nitrogen cycle research has usually been the substrates and end products of the microbial conversions. More recently, due to the

urge to reduce greenhouse gas emissions, intermediates such as NO and N_2O, started to be extensively studied. The complexity of intermediate reactions and the lack of easily implemented techniques and methods for the usual low concentration measurements, results in a hard topic of study. Nevertheless the central role of hydroxylamine as intermediate in the nitrogen cycle and its relation to N_2O formation urges more attention for this compound in future research projects.

3

IMPORTANCE OF ABIOTIC HYDROXYLAMINE CONVERSION ON NITROUS OXIDE EMISSIONS DURING NITRITATION OF REJECT WATER

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ABSTRACT

The spontaneous abiotic (or chemical) reaction of hydroxylamine (NH_2OH) at low concentrations (<0.4mg−N/L*) with free nitrous acid (HNO_2) was investigated at the conditions of partial nitritation of reject water. An abiotic batch reactor test was used to quantitatively assess the kinetics of the nitrous oxide (N_2O) emission. The estimated chemical N_2O emission rate was* 0.16 mg−N/L/h. *In addition, the concentration of NH_2OH in a full scale nitritation reactor, Single reactor High Activity ammonium Removal over Nitrite (SHARON) was measured in the range ca.* 0.03-0.11 mg−N/L. *The presence of NH_2OH in the SHARON reactor together with the abiotic N_2O emissions rate (assessed in the abiotic batch reactor test) points towards a significant contribution of the abiotic N_2O emission in the full scale reactor. An equivalent emission factor (N emitted as N_2O/N oxidized in nitritation) of* 1.1 % *was estimated to be linked to the abiotic pathway, which is around one third of the total measured N_2O emission rate in the SHARON reactor.*

3.1. INTRODUCTION

HYDROXYLAMINE (NH_2OH) is an intermediate in the biological oxidation of ammonia to nitrite by ammonia-oxidizing bacteria (AOB). One of the first experimental indications that NH_2OH is an intermediate in nitrification was reported by Lees [1]. By adding NH_2OH at a very low concentration (1.5 µg−N/L) to an aerated *Nitrosomonas sp.* culture, the consumption of NH_2OH in time was measured. At pH of 8.4 and 30°C, the measured NH_2OH consumption rate was found to be even higher than that of ammonium consumption at the same conditions.

In the past, the extracellular release of NH_2OH during the oxidation of ammonia into nitrite by AOB was considered doubtful [122]. However, several publications also pointed out that NH_2OH is a measurable intermediate in the bulk liquid of reactors performing either nitritation (oxidation of ammonium into nitrite) or nitrification (oxidation of ammonium into nitrate) [20, 21, 115, 123, 124]. The values reported for the NH_2OH concentration are in the range of 0.01–1 mg−N/L. Hydroxylamine is not one of the compounds regularly followed in N_2O emission or nitrification studies neither in laboratory scale reactors nor in full scale wastewater treatment plants (WWTPs). Some of these investigations were carried out with the specific goal of clarifying the N_2O emissions pathways, quantification of rates or even setting-up mathematical models for the description of the process (among many others [125, 126, 127, 128]). In addition, several mathematical models calibrated with experimental data included NH_2OH as intermediate (for instance Ni et al. [129]), but the measurements were limited to ammonium, nitrite and N_2O, whereas actual NH_2OH concentrations were never measured.

Overall, there is a lack of knowledge regarding the formation of NH_2OH in WWTPs or laboratory reactors performing nitritation or nitrification.

Hydroxylamine and nitrite are known to be precursors for abiotic (or spontaneous) production of nitric oxide (NO) and nitrous oxide (N_2O) (see a review by Schreiber et al. [62]). Therefore, the presence of significant concentrations of NH_2OH and/or nitrite was postulated as potential environment for the chemical production of NO and N_2O in wastewater or natural aquatic ecosystems [62, 130]. The possible chemical reactions of NH_2OH and nitrite to produce nitrogen oxides are numerous and with a rather complex chemistry. Moreover, the rates can be accelerated by the presence of trace metals [62, 63]. The still reduced knowledge on the potential contribution of abiotic pathways on the overall N_2O emissions from nitrite, ammonia and NH_2OH during wastewater treatment has been previously emphasized [62, 63, 131, 132, 130, 133, 134]. Schreiber et al. [62] highlighted a few relevant environments in which the chemical production of N_2O could manifest in WWTPs, and cited as main example the nitritation of reject water in two-stage nitrogen removal systems (e.g. the Single reactor High Activity ammonium Removal over Nitrite, SHARON rector [12]). In particular, the chemical reaction between the NH_2OH and nitrite (nitrosation of NH_2OH) has been reported to produce N_2O, with overall reaction Equation (3.1) [135]:

$$NH_2OH + HNO_2 \longrightarrow N_2O + H_2O \tag{3.1}$$

The precursor of N_2O in Equation (3.1) is known to be the hyponitrous acid ($H_2N_2O_2$) – the dimer of HNO [135]. More recently, the chemical production of N_2O from NH_2OH

and nitrite has also been investigated through site-specific ^{15}N isotopic signatures [134]. However, Heil et al. [134] did not assess the kinetics of N_2O emissions, but rather focused on the isotopic signature of this reaction. They found that microbial and abiotic processes share the same intermediate steps, and therefore it was not possible to use the isotopic signature to assess the contribution of either biological or abiotic N_2O emissions.

The occurrence of Equation (3.1) during biological nitritation has been already highlighted by Harper et al. [63]. These researchers firstly analyzed the abiotic formation of N_2O and secondly used model fitting in a biological reactor to assess the contribution of the abiotic pathway to the overall N_2O production.

In this study, we investigated the chemical reaction of NH_2OH (at low concentrations) with nitrite and its potential contribution to N_2O emissions in wastewater treatment plants. To this end, some of the experiments targeted specific conditions found in nitritation reactors in two-stage nitrogen removal process applied to reject water (SHARON). This process was selected because: (i)there, the microbial growth rate of AOB is close to the maximum specific growth rate triggering NH_2OH release into the bulk liquid [21], and (ii) the high nitrite concentrations. We complemented this study with NH_2OH measurements in the full scale SHARON reactor in Rotterdam (The Netherlands), to assess the abiotic N_2O emission pathway in that type of nitrogen-converting bioreactors.

3.2. MATERIAL AND METHODS

3.2.1. ANALYSIS OF NH_2OH CONCENTRATION

THE NH_2OH concentration was measured following spectrophotometric procedure [117]: 1 mL of the sample containing hydroxylamine (range 0.00–0.25 µmol/L of hydroxylamine) was added to 1 mL of 0.05 M phosphate buffer, 0.80 mL of demineralized water, 0.2 mL of 12 wt % trichloroacetic acid, 1 mL of 1% 8-quinolinol (w/v) in ethanol and 1 mL of 1M Na_2CO_3. After shaking vigorously the mixture was heated 1 min at 100 °C in a water bath and cooled for 15 min before measuring absorbance at 705 nm (Novaspec III Amersham Biosciences). A blank was prepared by replacing the sample volume by the same volume of demineralized water.

For the samples withdrawn from the SHARON reactor, a variation of the protocol described was used, since no demineralized water was added, and 1.8 mL of sample were used instead. This was done to increase the absolute value of absorbance of samples. For more details regarding the sampling procedure in the SHARON reactor, see below the specific Section 3.2.4.

3.2.2. SMALL-SCALE REACTION TESTS

SAMPLING AND STORAGE

The NH_2OH reactivity was tested under different conditions in 2 mL Eppendorf tubes. For each test 1.6 mL of medium was mixed with 0.2 mL of a 3.5 mg−N/L hydroxylamine solution. Afterwards, 0.2 mL of 10 % sulfamic acid was added in some of the tests, as far as it is reacting 1:1 stoichiometrically with nitrite to form N_2, H_2SO_4 and H_2O [136]. The NH_2OH concentration was then immediately measured in samples with or without

sulfamic acid. After keeping different aliquots overnight at room temperature, 4 °C and −20 °C, with and without sulfamic acid, NH_2OH concentration was also measured.

The mineral medium used contained: 0.330 g/L $NaNO_2$, 0.344 g/L $(NH_4)_2SO_4$, 5 mL/L of an iron solution containing 9.14 g $FeSO_4$ and 5 g EDTA in 1 L of demineralized water, 1.25 mL/L of a magnesium solution containing 160 g $MgSO_4 \cdot 7H_2O$ in 1 L of demineralized water, 0.625 mL/L of a calcium solution containing 240 g $CaCl_2 \cdot 2H_2O$ in 1 L of demineralized water, and 2.5 mL/L of a trace element solution containing 15 g EDTA, 0.43 g $ZnSO_4 \cdot 7H_2O$, 0.24 g $CoCl_2 \cdot 6H_2O$, 1.0 g $MnCl_2 \cdot 4H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 0.22 g $(NH_4)Mo_7O_{24} \cdot 4H_2O$, 0.20 g $NiCl_2\,6H_2O$, 0.10 g $NaSeO_4$, 0.014 g H_3BO_3, 0.050 g $NaWO_4 \cdot 2H_2O$ per liter of Mili-Q water and pH adjusted to 6 with NaOH.

PRELIMINARY CHARACTERIZATION OF THE REACTION KINETICS

The NH_2OH depletion rates were measured in a set of 2 mL Eppendorf tubes at room temperature (approximately 21 °C). The above mentioned medium was added to a set of 6–7 Eppendorf tubes, then NH_2OH was added to all of them to reach a final volume of 1.8 mL. Finally 0.2 mL of 10 % sulfamic acid was added to each Eppendorf tube at a different time, in order to stop the reaction (by removing nitrite). Finally, the NH_2OH concentration remaining after different reaction times was measured. For the sample at time zero, sulfamic acid was added first followed by the medium, in order to remove nitrite before NH_2OH addition. The pH was measured before adding NH_2OH using a pH meter (827 pH lab Methrom Swiss made).

3.2.3. REACTOR SET UP FOR THE CHEMICAL REACTION AND GAS ANALYSIS

Experiments were conducted in a 1.5 L lab-scale glass fermenter, equipped with a Teflon-coated magnetic stirrer. Air was added using a gas-diffuser with the flow rates 0.316 and 0.214 L/min for batch reactor tests 1 and 2, respectively. Off-gas was measured on-line (Servomex 4900 infrared gas analyzer), allowing to follow continuously oxygen, carbon dioxide, N_2O and NO. Temperature was controlled with an external jacket and pH was followed, using a pH meter in samples without sulfamic acid, but not controlled, because it was stable. Reaction mixture contained 1.9 mL and 3.8 mL of trace element and iron solutions, respectively, both described previously. 150 mL of a 3.5 mg−N/L NH_2OH solution was added to demineralized water containing the desired amount of metals, reaching a total liquid volume of 1.5 L. Finally, solid $NaNO_2$ was added to reach desired concentration in each test Table 3.1. Samples were withdrawn from the reactor at different times using a syringe and immediately mixed with 0.2 mL of 0.1 g/mL sulfamic acid. Hydroxylamine concentration was followed in time in the samples using the procedure mentioned in Section 3.2.1.

3.2.4. MEASUREMENT OF NH_2OH CONCENTRATION ANALYSIS IN A SHARON REACTOR

Samples from the SHARON reactor in Sluisjesdijk–Dokhaven (Rotterdam, the Netherlands) were withdrawn directly from the bulk liquid at the WWTP. Following the protocol described in Section 3.2.1 and Section 3.2.2, 1 mL of 0.1 g/mL of sulfamic acid was added immediately after filtration of the collected sample through cotton and 0.45 mm filter and analyzed in less than 2 h. A fraction of the sample was not mixed with

the sulfamic acid solution to let the NH_2OH react completely with nitrite and therefore quantify the background absorbance of the supernatant. The SHARON reactor contains flocculent sludge and the process conditions are summarized in Table 3.2.

Table 3.1: Influence of conditions (pH and temperature) and free nitrous acid (HNO_2) concentration in the NH_2OH reaction rate. Free nitrous acid concentration (HNO_2) has been calculated with Equation (3.2). Initial NH_2OH concentration for each test is also provided. * A phosphate buffer solution was added.

Eppendorf	pH	T (°C)	NH_2OH (mg−N/L)	Nitrite (mg−N/L)	HNO_2 (mg−N/L)	NH_2OH depletion rate (mg−N/L/h)
1	4.3	21	0.27	54	10.0	0.56±0.09
2	5.1	21	0.35	56	1.5	0.29±0.03
3	7.6*	21	0.3	54	0.01	0.56±0.09
Batch						
1	6.2	20	0.35	135	0.38	0.2±0.02
2	7.0*	30	0.3	650	0.29	0.27±0.07

3.2.5. CALCULATIONS

From acid-base equilibrium the HNO_2 (free nitrous acid, FNA, in mg−N/L) concentration can be estimated for a given nitrite concentration (TNN, in mg−N/L), pH and temperature (T in °C) using Equation (3.2) [137]:

$$FNA = \frac{TNN}{10^{pH} \cdot \exp\left(-\frac{2300}{273+T}\right) + 1} \tag{3.2}$$

The total amount of N_2O produced (m_{N_2O}, in g−N/L) was calculated through integration in time of the curve of N_2O emission. The conversion of NH_2OH into N_2O (X_{NH_2OH/N_2O}, %) can be calculated by using the initial NH_2OH concentration ($C_{NH_2OH,ini}$, in g−N/L), the stoichiometry in Equation (3.1) and the total volume of reaction (V, in L) as in Equation (3.3):

$$X_{NH_2OH/N_2O} = \frac{\frac{m_{N_2O}}{2\,gN_2O\text{−}N_{produced}}}{V \cdot \frac{C_{NH_2OH,ini}}{1\,gNH_2OH\text{−}N_{consumed}}} \tag{3.3}$$

3.3. RESULTS AND DISCUSSION

3.3.1. FAST SCREENING OF NH_2OH REACTIVITY IN EPPENDORF TUBES

SINCE NH_2OH is known to be a very reactive short-lived compound, some preliminary tests were carried out to determine the best strategy for sample storage. To simulate a likely medium composition, the tests were carried out by using a synthetic wastewater containing ammonium, nitrite, metals and trace elements. To prevent the oxidation of NH_2OH, the addition of sulfamic acid was tested in order to remove nitrite as nitrogen gas. Nitrite and NH_2OH can react [135], and the acid reduces the pH increasing the stability of NH_2OH[138, 139]. In fact, removal of nitrite is known to improve the hydroxylamine analysis [139]. When NH_2OH was determined through the

colorimetric method immediately after the preparation of the solution (i.e. mixing the NH_2OH standard solution with the synthetic wastewater) the concentration matched the theoretical expected value (0.14±0.01 mg-N/L, Figure 3.1).

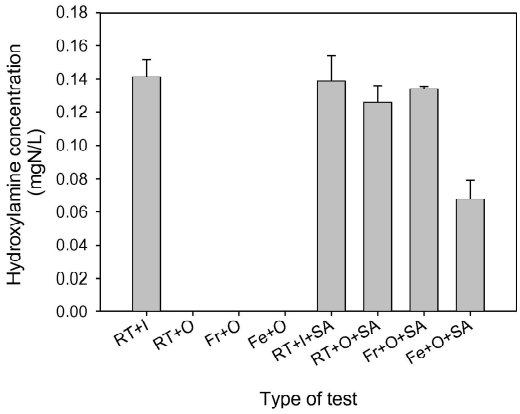

Figure 3.1: Preliminary tests establishing the sampling procedure at 0.14 mg−N/L NH_2OH in the samples: RT = room temperature; I = instantaneous measurement; O = overnight measurement; Fr = fridge storage; Fe = freezer storage; SA = sulfamic acid addition.

When repeating the same procedure but including the addition of sulfamic acid, the NH_2OH concentration was not significantly different (0.14±0.02 mg-N/L, Figure 3.1). Therefore, the sulfamic acid addition did not alter significantly the results of the analysis. When sulfamic acid was not added, the NH_2OH was found to disappear completely when the sample was kept overnight either at room temperature, at 4 °C or frozen at −20 °C.

When sulfamic acid was added, the NH_2OH concentration was rather stable overnight, showing less than 10 % decrease (Figure 3.1). Storage at 4 °C after addition of the sulfamic acid produced only a slight improvement (Figure 3.1). Storage at −20 °C was found to be not applicable because of a 50 % decrease in the concentration (Figure 3.1).

It was clear that after addition of sulfamic acid, the NH_2OH was stable for several hours, therefore the addition of sulfamic acid was adopted as a preliminary step for sample preservation. Nevertheless, the NH_2OH stability could be linked to either (i) the pH of the solution (i.e., sulfamic acid solution has a very low pH, so that the sample pH dropped from 6.76 to 1.35 after the addition of sulfamic acid), (ii) the absence of nitrite, or (iii) a combination of both.

3.3.2. CHARACTERIZATION OF THE EXTENT OF N_2O EMISSIONS THROUGH CHEMICAL REACTION

A reactor vessel was used to study the coupling of the conversion of NH_2OH and nitrite with the formation of NO and N_2O. Two different experiments were carried out.

In the first experiment, the reaction vessel contained initially NH_2OH at a concentration of ca. 0.35 mg − N/L, pH 6.2 and at 20 °C with the synthetic wastewater containing chelated iron and trace elements, but without nitrite. The reactor was aerated

Table 3.2: Influence of conditions (pH and temperature) and free nitrous acid (HNO_2) concentration in the NH_2OH reaction rate. Free nitrous acid concentration (HNO_2) has been calculated with Equation (3.2) . Initial NH_2OH concentration for each test is also provided. * A phosphate buffer solution was added. ** Performed in this study n.d. - not determined. Tests were done at pH 7 except for the abiotic test 3 that pH was not determined.

Reactor	T (°C)	NO_2^- (mg−N/L)	HNO_2 (mg−N/L)	NH_4^+ (mg−N/L)	NH_2OH (mg−N/L)	N_2O rate (mg−N/L/h)
SHARON[65]	30	650	0.13	650	0.06*	0.47
AOB,batch [128]	30	400-1000	0.07-0.18	500	n.d.	0.13
Abiotic batch [128]	n.d.	0-1000	n.d.	0	1	0
Abiotic batch [127]	20	10	0.003	0	10	0.03
Abiotic batch**	30	650	0.13	0	0.3	0.16

at 0.316 L/min. Hydroxylamine remained rather constant for more than 200 min and no significant NO or N_2O emissions were recorded (Figure 3.2 A). Therefore this indicates how NH_2OH cannot disappear at a significant rate if nitrite is not present.

After 220min, $NaNO_2$ was added to reach a nitrite concentration of 135 mg−N/L in the reactor vessel. A decrease in the NH_2OH concentration was immediately recorded and N_2O was simultaneously emitted, as detected through the gas analyzer (Figure 3.2 A). No nitric oxide (NO) emission was noticeable. Hydroxylamine decreased after nitrite addition at a rate of 0.20±0.02 mg-N/L/h (Figure 3.2 A, Table 3.1). The maximum N_2O emission rate was ca. 0.2 mg−N/h (Figure 3.2 A). Therefore this indicates that NH_2OH and nitrite react together following Equation (3.1), as expected.

In a second experiment, the reaction was carried out in the conditions commonly applied in SHARON nitritation bioreactors treating reject water (T = 30 °C, pH = 7, concentration of nitrite 650 mg−N/L, [13]) (Figure 3.2B). The reactor was aerated at 0.214 L/min. In this case, the initial abiotic reaction mixture contained synthetic wastewater, including nitrite and chelated iron, but without hydroxylamine. Neither N_2O (Figure 3.2B) nor NO emissions were detected. This showed how no significant reduction of nitrite into either NO or N_2O was happening.

After 68 min a pulse of NH_2OH was added to reach a final concentration of 0.3 mg−N/L, which resulted in immediate N_2O emission (but not NO) and decrease in NH_2OH concentration with an average conversion rate of ca. 0.27±0.07 mg-N/L/h and a maximum N_2O emission rate at ca. 0.25 mg−N/h was maintained for at least 50 min. The initial NH_2OH concentration (ca. 0.3 mg−N/L) was selected because a similar concentration was measured in a chemostat culture of *Nitrosomonas sp.* [123].

These experiments showed that only when both NH_2OH and nitrite were present, in the conditions tested, one of the reaction products was N_2O (as expected from Equation (3.1)).

The measured N_2O emission rate was integrated in time to determine the reaction yield of N_2O from NH_2OH. Theoretically, the overall reaction would require equimolecular amounts of NH_2OH and HNO_2 (see Equation (3.1)). The experimental results indicated a conversion of 22 % and 41 % of removed NH_2OH into N_2O in the first and second experiment, respectively. However, for the first experiment, the % of NH_2OH converted is underestimated, since we did not record the complete curve of

N_2O emission. The low recovery of N_2O indicates that a fraction of the reacted NH_2OH resulted in a different reaction product. Some of the NH_2OH might have reacted with one of the intermediates of Equation (3.1) (HNO, the monomer of hyponitrous acid) to produce N_2 [62, 140]:

$$NH_2OH + HNO \longrightarrow N_2 + 2H_2O \tag{3.4}$$

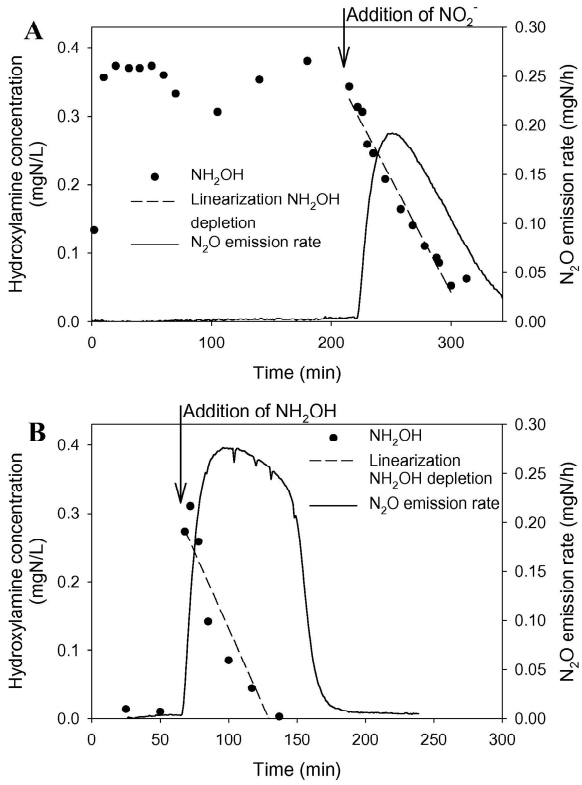

Figure 3.2: Chemical reaction of NH_2OH and nitrite in a 1.5 L batch reactor. Results of the linearization are shown in Table 3.1, as batch reactor tests 1 and 2. A) Conditions used were T = 20 °C, pH = 6.2, nitrite was added at time 220 min at a concentration of 135 mg−N/L. Air flow-rate 0.316 L/min. B) Conditions used were T = 20 °C, pH = 7, nitrite concentration 650 mg−N/L (already in the reaction vessel at time zero) and NH_2OH added at time 68 min. Air flow-rate 0.214 L/min.

Although the presence of HNO was not verified in the course of this research, Equation (3.4) could explain the gap in the nitrogen mass balances. The influence of alternative reactions of NH_2OH (e.g., disproportionation, autoxidation, oxidation by Fe^{3+}, [62]) is likely very limited at the conditions tested, because before the addition of nitrite in the first experiment, the NH_2OH concentration was stable for more than two hours (Figure 3.2A). The same reasoning would apply for the potential nitrite reactivity

to produce N_2O (e.g., nitrite reduction by Fe^{2+}, [132]) since in the second experiment no N_2O emissions were noticeable until NH_2OH was added (Figure 3.2B).

Given the measured N_2O emission rate from Equation (3.2), in case NH_2OH is leaking to the bulk liquid in the SHARON reactor, the contribution of the abiotic pathway to the total N_2O emission rate would be relevant. Therefore, the presence of NH_2OH in the SHARON reactor was also investigated (see Section 3.3.4). A systematic characterization of the effects of pH and temperature on the abiotic N_2O emission rate (i.e. the reaction kinetics) would be desirable to clarify the contribution of the abiotic pathway in other nitritation systems used in wastewater treatment.

3.3.3. FREE NITROUS ACID: THE LIMITING COMPOUND

Due to the variability found for the rate of conversion of NH_2OH, the effects of pH and temperature on the conversions were measured. Table 3.1 and Figure 3.3 give the conversion rates determined as a function of the initial free nitrous acid (HNO_2) concentration. The results indicate that the rate of reaction of NH_2OH with nitrite to produce N_2O is in fact limited by the HNO_2 concentration. Temperature and pH were not followed in the Eppendorf tube tests, making it difficult to directly link the rate to an initial HNO_2 concentration. However for the wide range of pH (4.3–7.6) tested the positive influence of lower pH on the reaction was clear.

Within the tested range of conditions, more acidic pH values would enhance the N_2O emission when NH_2OH and nitrite are present in WWTPs reactors. Higher nitrite concentrations in the wastewater would mean higher HNO_2 concentrations (at a given pH and temperature), which in turn would result in higher N_2O emissions due to the chemical reaction between NH_2OH and HNO_2. Although a pH of 7 was used here to mimic the SHARON reactor conditions, often the reactor operates at lower pH values. For example, in the pH range 6.6–6.7, as reported by Mampaey et al. [141], higher reaction rates would be expected. It is important to stress that in those tests the temperature was the same (21 °C), the concentration of nitrite was the same (55±2 mg-N/L) as well as the initial NH_2OH concentration (0.31±0.05 mg-N/L). Therefore, the change in pH was the single parameter affecting reaction rate. This was measured as NH_2OH depletion rate, which ranged from 0.02 mg−N/L/h (at pH 7.6) to 0.6 mg−N/L/h (at pH = 4.3) (Table 3.1). In fact these results (Table 3.1) are in full agreement with findings by Döring and Gehlen [135], in which the reaction rate depended more strongly on the HNO_2 concentration than on the NH_2OH concentration. The doubling of reaction rate when increasing temperature from 20 to 30 °C found by Döring and Gehlen [135] could explain the increase in NH_2OH depletion rate between the second and the first batch reactor tests (Table 3.1).

3.3.4. HYDROXYLAMINE PRESENCE IN A FULL SCALE SHARON REACTOR

The presence of NH_2OH in large scale biological nitritation reactors would allow the abiotic N_2O formation. The sampling campaign at the SHARON reactor in Rotterdam (five measurements during January and June 2015) indicated the presence of NH_2OH in the range of 0.03 to 0.011 mg−N/L (average 0.06 mg−N/L) during the aerated phase of operation. The standard deviation of the determinations based on triplicates was for all samples below 15 %, and for most of them lower than 5 %. Notably, in the range of

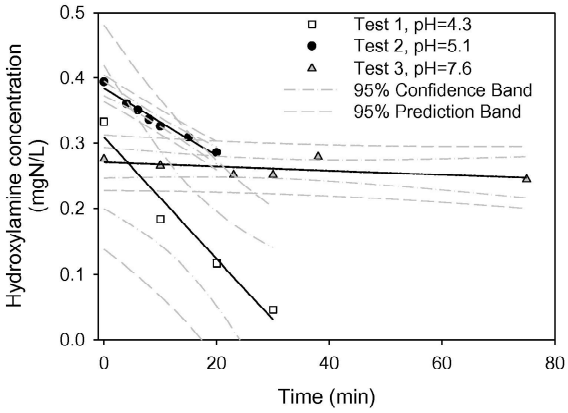

Figure 3.3: Eppendorf tube tests for determination of the NH_2OH depletion rate at different pH conditions.Corresponding to different free nitrous acid concentrations of 10 1.5 and 0.01 mg-N/L for pH 4.3, pH 5.1, pH 7.6, respectively.

NH_2OH concentrations measured in the SHARON reactor, the abiotic tests still indicated a high N_2O emission rate (see Figure 3.2B). However, the measured absorbance of the sample backgroundwas in average 76 % of that of the sample containing NH_2OH. Given the high absorbance of the sample background, a more precise analytical method would be desirable to confirm the absolute values of NH_2OH concentration here reported.

In addition to our measurements in the SHARON reactor, residual NH_2OH concentration in the bulk liquid has been reported by several researchers in similar conditions to those in the SHARON reactor [20, 21, 123, 124]. Low NH_2OH concentrations are still compatible with significant N_2O production (and emission) rates through the abiotic reaction between NH_2OH and nitrite (i.e., Equation (3.1)). In many occasions, reaction intermediates can be at low concentrations but the reaction still proceeds. For instance, nitrite is usually not measured at high concentrations in WWTPs, however nitrification of ammonium into nitrate is known to be happening.

Hydroxylamine released to the bulk liquid was detected when the growth rate of batch nitrifying cultures was close to the maximum [20, 21]. For example, hydroxylamine concentration was measured in the range of 0.03 to 0.06 mg−N/L, during the exponential growth in batch culture of *Nitrosomonas sp* [21]. Therefore, in the SHARON reactor performing partial nitritation at high specific growth rate, NH_2OH release into the bulk liquid would be expected. In that sense, the NH_2OH concentration in the flocculent sludge could locally be even higher than that measured in the bulk liquid, depending on the biomass density and the diameter of the flocs [115]. However, the gradient of NH_2OH concentration is expected to be rather low because of the small floc size. Additionally, Jiang et al. [123] measured sustained levels of NH_2OH in the bulk liquid of a *Nitrosomonas sp.* chemostat culture, in the range 0.2 to 0.3 mg−N/L. The decrease in availability of inorganic carbon (e.g. from 40 times to 4 times excess) was reported to trigger increasing NH_2OH levels [123]. The alkalinity levels of reject water (typically correlated to inorganic carbon) are not enough as to allow oxidation of all ammonium to nitrite [13]. Therefore, in SHARON reactors the availability of inorganic carbon is

reduced (although no to the level to limit the partial nitritation), which might also be a reason why sustained NH_2OH levels were detected in the bulk liquid. Also Ma et al. [124] measured concentrations of NH_2OH in the range 0.09 to 0.15 mg−N/L for a pilot scale partial nitritation/anammox reactor, with a bulk ammonium concentration of ca. 10 to 15 mg−N/L. In summary, there are several reports of NH_2OH detected at relevant concentrations in nitritation reactors, all in agreement with the results found in the present study.

The residual NH_2OH concentration in the bulk liquid will be stablished as the balance between the NH_2OH leakage rate by AOB, and the amount of NH_2OH reacting to produce N_2O (either by abiotic or biotic routes). This is why we stress that the measured NH_2OH is just the residual concentration established in the reactor, as a result of the balances between these rates: NH_2OH leakage and (abiotic and biotic) N_2O production.

3.3.5. COMPARING CHEMICAL WITH BIOLOGICAL N_2O EMISSION RATES

To put the observed abiotic conversion into the context of overall N_2O emissions from nitritation, a direct comparison to reported cases has been carried out (Table 3.2). Since the SHARON reactor was monitored in the past [65] this was the first reference case. Nevertheless,given the abundance of data reported in the literature, other key cases have been used to compare and discuss the significance of chemical N_2O emissions. The N_2O emissions rates found through the chemical reaction of NH_2OH and HNO_2 are in the same order of magnitude with those reported in the SHARON reactor and in similar type of biological lab-scale reactors (Table 3.2). This strengthened the idea that the contribution of the chemical pathway in the SHARON reactor is of major importance (as hypothesized by Schreiber et al. [62]).The rate estimated in the second batch reactor test (Figure 3.2B) was 0.16 mg−N/L/h, i.e. an N_2O emission factor (N emitted as N_2O/N oxidized in nitritation) of 1.1 % of the converted ammonium under the conditions in the SHARON reactor.

Following to our assessment of the N_2O emission rate by the abiotic pathway and given the N_2O emissions reported for the SHARON reactor (Table 3.2), we conclude that, in those conditions, both abiotic and biotic routes contribute in a comparable degree. This is in agreement with the conclusions in Harper et al. [63], who also assessed N_2O emissions in partial nitrification reactors as of hybrid origin, with contribution of both the abiotic and the biotic routes.

The direct comparison of our estimated rate for the chemical reaction with those reported for the biological conversion in an AOB batch reactor in Law et al. [128] at high nitrite (400 to 1000 mg−N/L) and high ammonium (500 mg−N/L) concentrations showed very similar values (Table 3.2). Although that study initially considered the possible abiotic production of N_2O, this was in the end ruled out based on an abiotic test with 1 mg−N/L hydroxylamine and nitrite concentrations in the range (0 to 1000 mg−N/L). However, the conditions of that test (pH and temperature) were not detailed and results were not shown. Therefore their chemical test cannot be directly compared with our results.

Wunderlin et al. [127] checked the chemical production of N_2O with NH_2OH and nitrite in a solution prepared with tap water. They assessed the abiotic contribution as very reduced, which agrees with our results since the concentration of HNO_2 was

much lower in those conditions (Table 3.2). However, the use of tap water could make a difference from our experiments, in which Fe^{2+} together with other metals were added at known concentrations.

In addition, the chemical nitrite reduction by ferrous iron Fe^{2+} was reported to be a relevant N_2O emission pathway in a full scale anammox reactor [132]. Iron is present in the reactor influent as Fe^{3+} precipitate due to dosage of $FeCl_3$ in earlier stages of the wastewater treatment plant [132]. The production of Fe^{2+} necessary for chemical N_2O formation through the nitrite reduction is thought to be mediated by biological activity. In a laboratory reactor mimicking the conditions of an anammox reactor, with Fe^{3+} and formate added, the Fe^{3+} reduction rate was 19 µmol/gDW/h [132]. Despite Fe^{2+} presence in the medium (Table 3.1,Figure 3.1 and Figure 3.2), this pathway could be neglected in our abiotic tests because no significant NO or N_2O emissions were measured before the addition of NH_2OH in the batch test No. 2 (Figure 3.2). Moreover, the Fe^{2+} is expected to be quickly oxidized in the aerobic conditions used.

Our results agree with those reported by Harper et al.[63]: the abiotic reaction might be overlooked when calibrating N_2O emission models (e.g. Ni et al. [129]; among others) and therefore the potential contribution of this pathway remains unclear for many biological systems in which partial nitritation is taking place. In addition, we would like to point out that the effects of pH and temperature regulating the concentration of HNO_2 (free nitrous acid) and the residual NH_2OH concentration in the bulk liquid are discussed and measured in the present study. It is doubtful that the high concentrations of NH_2OH (up 20 mg−N/L) used in Harper et al. [63] are present in biological systems. Moreover, the weakness of their model fitting to describe the biological tests is that NH_2OH was added to those high concentrations but its concentration was never measured.

3.3.6. PRACTICAL IMPLICATIONS FOR THE SHARON REACTOR

Given the dependence of the reaction rate on pH, N_2O emissions from the chemical pathway here described could be mitigated by increasing the pH. However, increasing the pH may have some consequences to the operation of the reactor. In the SHARON reactor nitrate production is voided by keeping a dilution rate higher than the maximum specific growth rate of nitrite-oxidizing bacteria [12]. The reactor is operated with alternating aerobic anoxic periods in such a way that a mean aerobic retention time of 1.35 days is established [141], regardless the influent flow rate. Since no pH control is applied and the alkalinity is limited, the ammonium oxidation rate is reduced by the rather low pH. Addition of base to increase the pH will therefore produce an effluent with a lower ratio NH_4^+/NO_2^-, which would not be convenient for the subsequent anammox reactor. One possibility would be to reduce the length of the aerated cycles, to decrease the ammonium oxidation rate achieved at a higher pH. In turn, the overall anoxic period will be longer and this may induce higher N_2O emissions coming from the nitrite reduction induced by the iron present in the reactor [132]. Although the chemical nitrite reduction to N_2O was indicated as significant in the anammox reactor and mediated by the anammox capacity to reduce iron, when anoxic conditions are imposed in the SHARON reactor, it is unknown if this pathway would also be of relevance.

For new installations, designing the SHARON reactor at a slightly higher (controlled)

pH of operation (i.e. assuming a higher ammonium oxidation rate) should result in a lower N_2O emission rate from the chemical reaction between NH_2OH and nitrite. Further research would be required to find out whether an optimal pH set point could be found in which N_2O emissions would be minimized.

3.4. CONCLUSIONS

IN this study a significant production of N_2O from the abiotic reaction between NH_2OH and HNO_2 was observed at process conditions in a partial nitritation reactor for reject water in a two-stage N-removal treatment. The abiotic N_2O emission rate measured at typical operating conditions reported for the SHARON reactor (T=30 °C, pH = 7 and nitrite in excess (650 mg−N/L)) was 0.16 mg−N/L/h. Furthermore, an average residual hydroxylamine concentration of 0.06 mg−N/L was measured in the SHARON reactor, which supports that the N_2O emissions in that installation can be produced from biotic and abiotic routes. Therefore the N_2O emissions in reject water treatment processes can have a biotic or abiotic production route.

4

IMPORTANCE OF HYDROXYLAMINE IN ABIOTIC N_2O PRODUCTION DURING TRANSIENT ANOXIA IN PLANKTONIC AXENIC *Nitrosomonas* CULTURES

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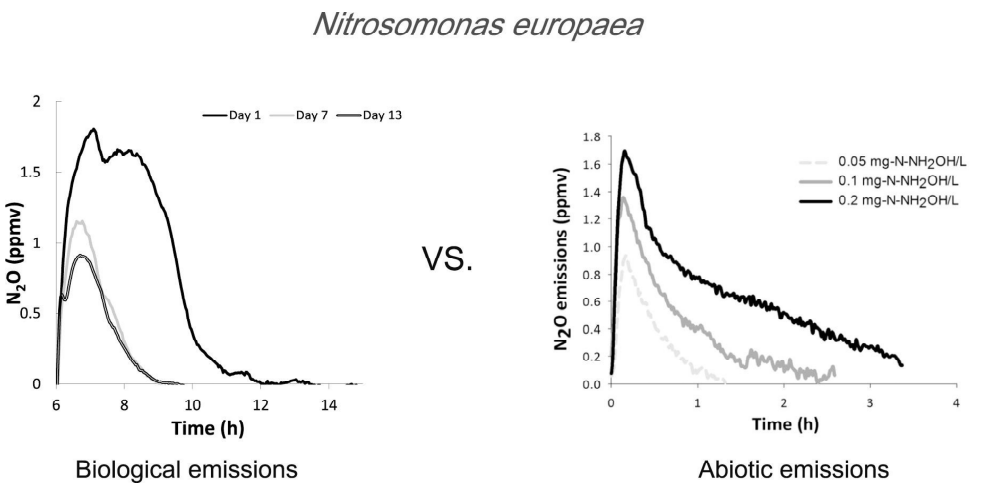
ABSTRACT

When investigating the N_2O emissions by ammonia oxidizing bacteria, research has mainly focused on identifying and quantifying the biological pathways. This work evaluated previous studies with Nitrosomonas europaea (ATCC 19718) and assessed the role of the abiotic reaction of hydroxylamine with free nitrous acid during transient anoxia. In cultivations when transient anoxia is cyclically imposed, nitrous oxide and hydroxylamine peaked every time upon recovery to aerobic conditions. When using the same culture conditions abiotically (i.e., without biomass, but adding hydroxylamine and nitrite), the volumetric N_2O emission rates were very comparable to those from the biological experiments, ranging from 0.04 to 0.08 mg−N/L/h *in both abiotic and biotic conditions. These results demonstrate that at the culture conditions tested, abiotically produced N_2O is likely the major source of emission. Therefore, for the correct investigation of the biological pathways, abiotic tests must always be performed and hydroxylamine should be added. To our knowledge there is no means to distinguish abiotic from biological N_2O production in a biological system, even using ^{15}N labelling. We suggest that the contribution of abiotic N_2O emissions can be minimized by, for example, maintaining lower nitrite concentration and higher pH.*



4.1. INTRODUCTION

NITROUSs oxide (N_2O) and nitric oxide (NO) can be emitted during nitrogen removal in lab-scale reactors and wastewater treatment plants (WWTPs). N_2O and NO are ozone depleting gases. Moreover, due to a 300-fold larger greenhouse effect capacity compared to that of CO_2, the study of N_2O emissions has increased over the past years. Main contributors to N_2O and NO emissions are both nitrification and denitrification processes [15]. Factors identified to contribute to increased emissions are: (i) low dissolved oxygen and high nitrite concentrations in both nitrification and denitrification [15], (ii) low COD/N ratio during denitrification [15], (iii) limited inorganic carbon in nitritation-anammox processes [124].

In nitrification systems ammonium oxidizing bacteria (AOB) or archaea (AOA) are reported to be the main contributors to NO/N_2O emissions [15]. AOB convert ammonium to nitrite in two steps: ammonium oxidation to hydroxylamine (NH_2OH) by ammonium monoxygenase (AMO) and further hydroxylamine oxidation to nitrite by hydroxylamine oxidoreductase (HAO). It has been recently proposed that the ammonium oxidation could also occur via a three-step reaction [27]. Experiments in vitro showed that NO is an obligate intermediate in hydroxylamine oxidation by HAO, implying that hydroxylamine is biochemically oxidized to NO which is subsequently converted in a chemical oxidation to NO_2^- [27]. In vivo studies are still needed to confirm this potential reaction mechanism. Regarding NO/N_2O production traditionally, two pathways have been described for the production of N_2O in the AOB metabolism: by hydroxylamine oxidation and by nitrifier denitrification [15, 142, 22]. However, recent studies have highlighted the complexity and the existence of multiple pathways branching from the traditional ones, with contributions by both abiotic and biological reactions [57, 17, 58, 59] (see Figure 4.1).

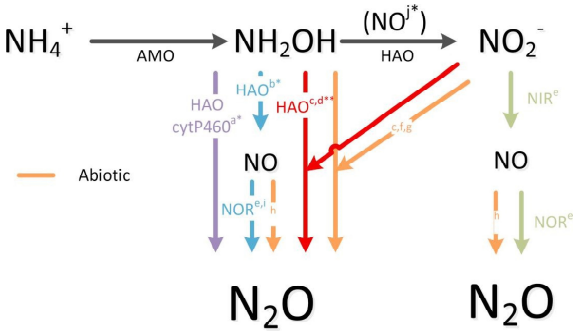


Figure 4.1: Summary of N_2O emission pathways in Ammonia Oxidizing Bacteria. Blue pathway is the tradinionally known as hydroxylamine oxidation pathway. Green pathway is the traditionally known as hydroxylamine oxidation pathway. Enzymes responsible of each pathway: AMO – Ammonia Monoxygenase, HAO – Hydroxylamine Oxidoreductase, HAO cytP460 – Cytochrome P460 of HAO, NIR - Nitrite reductase, NOR – Nitric oxide reductase. The pathways indicated by orange arrows account for putative abiotic contribution. $*$ - tested in vitro with enzymatic extracts, $**$ - proposed pathway. References a [57] ,b [55], c [59], d [143] , e [54], f [58], g [63], h [17], i [142], j [27].

Possible enzymes responsible of hydroxylamine oxidation into N_2O have been under discussion [144]. Recent studies showed a possible involvement of the cytochrome P460 from the HAO enzyme. This cytochrome can convert two NH_2OH molecules to N_2O via an iron mediated catalytic reaction [27]. Incomplete oxidation of hydroxylamine by HAO might also lead to intermediates, such as NO or HNO, that can be further converted to N_2O either by abiotic or biological reactions [17]. Finally, the importance of N_2O production from hydroxylamine and nitrite Equation (4.1) with biological and abiotic contributions in a partial nitritation system was also highlighted [59].

Regarding nitrifier denitrification, the existence of nitrite reductase (NIR) capable of converting nitrite to NO and nitric oxide reductase (NOR) catalysing the conversion of NO to N_2O have been demonstrated in AOB [54, 144]. However, not all AOB have a NOR-related enzyme encoded in the genome [17], which is also the case in some AOA [145]. Thus, abiotic conversion of NO to N_2O has also been postulated when NOR is missing in the genome [17]. Surprisingly, abiotic N_2O emissions were not considered in most of the proposed models, although chemical N_2O production is known to happen [15, 146, 61, 147]. Recent studies highlighted the contribution of abiotic N_2O emissions produced by reaction of hydroxylamine with free nitrous acid (FNA) [63, 58, 59]:

$$NH_2OH + HNO_2 \longrightarrow N_2O + H_2O \tag{4.1}$$

The chemical reaction of hydroxylamine with free nitrous acid has not been considered as a possible contributor to the overall N_2O emissions until recently [63, 58], due to several reasons. Firstly, abiotic controls are usually not executed/reported [148, 21, 125, 142, 149] and even if these controls are performed, hydroxylamine is not included in the medium [150, 151, 152, 153]. Secondly, the complicated chemistry of related N-compounds [135, 140, 122] together with the highly reactive hydroxylamine, as the intermediate that may accumulate in the bulk liquid, is not well characterized. Finally, complex engineered ecosystems are usually used to study N_2O emissions. Thus, (i) diverse AOB consortia may coexist with other potential N_2O microbial producers (e.g., heterotrophic denitrifiers) and (ii) micro-gradients of substrates and oxygen in colonies, flocs and biofilms are usually present, all resulting in a system difficult to interpret leading to underestimation of some pathways.

A better strategy to understand the basic pathways producing N_2O in AOB and the conditions affecting the extent of N_2O emissions has been the use of axenic cultures of a single representative species (e.g. *Nitrosomonas europaea*) cultivated as planktonic cells. This avoids micro-gradients of substrates and oxygen in colonies, flocs and biofilms, allowing for a more direct examination of potential N_2O pathways ([21, 125, 142, 54, 17], among others).

Consequently, more research is needed to reassess the biological pathways, while acknowledging the extent of abiotic contributions. Abiotic reaction of hydroxylamine with nitrite is likely to occur in biological systems with nitrite and hydroxylamine accumulation [146, 144, 17, 152]. Thus, part of this biologically produced hydroxylamine and free nitrous acid can abiotically react forming N_2O.

Hydroxylamine accumulation in the liquid can be the direct or indirect key factor promoting N_2O emissions. During nitrification, hydroxylamine has been reported to accumulate in the bulk liquid in the range of 0.01 to 1 mg-N-NH_2OH/L [20, 125, 123,

124, 149]. This is supposed to be happening when conditions in the reactor favours specific growth rates close to the maximum [21]. At these high conversion rates, the rate of ammonium conversion to hydroxylamine and its further oxidation to nitrite can be imbalanced, resulting in hydroxylamine accumulation. For instance, imposing transient anoxia disturbances in a continuous aerobic axenic culture of *Nitrosomonas europaea* triggered hydroxylamine build up in the bulk liquid [142, 149].

Studies of NO/N_2O production during transient anoxia are important, as most of the engineered systems are subject to sudden dissolved oxygen (DO) changes. In addition, switching conditions between aerobic and anaerobic in a single tank are imposed in conventional biological nitrogen removal processes. Both the effect of DO changes and transient anoxia on NO/N_2O emissions have been extensively studied ([150, 152, 21, 125, 142], among others). During transient anoxia NO was only produced in anaerobic conditions, and no N_2O emissions were detected [125, 142, 149]. However, immediately after recovery to aerobic conditions N_2O was emitted simultaneously with the hydroxylamine accumulation [125, 142, 149]. Yu et al. [125], could not find a correlation between N_2O emissions and gene expression, instead, they linked emissions to a metabolic shift from low to maximum specific activity. However, no abiotic controls containing both hydroxylamine and nitrite were performed in any of these studies [125, 149].

In the present study, the dynamics of N_2O emission from a *Nitrosomonas europaea* axenic culture during transient anoxia are reported, discussed and compared to abiotic tests. Culture conditions were abiotically replicated, by adding nitrite and hydroxylamine in the concentrations measured just after the switch to aerobic conditions in the biological system. The aim was to assess the consequences of transient anoxia in *N. europaea* and the contribution of abiotic emissions on N_2O formation. Furthermore, some recommendations are provided for future research in order to reduce the contribution of abiotic N_2O emissions when investigating biological production pathways in either axenic cultures or natural and engineered ecosystems.

4.2. MATERIALS AND METHODS

4.2.1. *Nitrosomonas europaea* CULTIVATION

Nitrosomonas europaea (ATCC 19718) planktonic cultures were cultivated in dark in triplicate chemostats (6L total volume, 4L operating volume, 21 °C, pH 7.5±0.1 at a dilution rate of 0.45 d^{-1} [149]. The growth medium contained 20 mM NH_4^+ and (per liter): 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 0.087 g of K_2HPO_4, 2.52 g EPPS (3-[4-(2-Hydroxyethyl)-1-piperazine] propanesulfonic acid), 1 mL of 13 % EDTA-Fe^{3+}, 1 mL of trace elements solution (10 mg of $Na_2MoO_4 \cdot 2H_2O$, 172 mg of $MnCl_2 \cdot 4H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, 0.4 mg of $CoCl_2 \cdot 6H_2O$, and 100 mL of distilled water), 0.5 mL of 0.5 % phenol red, and 0.5 mL of 2 mM $CuSO_4 \cdot 5H_2O$ [149].

In order to mimic conditions that can be found in engineered systems during biological nitrogen removal, transient anoxia was imposed and the culture adaptation was followed in time. Although not applicable across the board, the 'rule of thumb' for preliminary design of many pre-anoxic systems is 25 % anoxic, 75 % aerobic, so the transient aerobic-anoxic cycling was imposed accordingly. To impose transient anoxia,

air was substituted by filtered N_2 once per day during 6 h for 13 consecutive days (both at a flow rate of 2.7 L/min). Gaseous N_2O (gas-filter correlation, Teledyne API 320E, San Diego, CA), and NO (chemiluminescence, CLD-64, Ecophysics, Ann Arbor, MI) were measured online once every 2 min. Hydroxylamine concentration was measured spectrophotometrically [117]. More details on further reactor dynamics, physiochemical analysis and genomics/proteomics can be found in [149].

4.2.2. ABIOTIC BATCH TESTS

The conditions used for the cultivation of *N. europaea* [21, 149] were also used in abiotic tests to assess the N_2O emission rates through the reaction of hydroxylamine and free nitrous acid. In order to achieve a comparable gas composition (in ppmv), air flow to working volume ratio was maintained as in the *N. europaea* cultures [149] (0.68 / min) and as in Yu et al., [21] (0.29 / min), while the ratio headspace to total volume was also kept to 1:3. Aerobic conditions were used in all abiotic tests, as in biological systems hydroxylamine accumulation is only related to continued ammonium oxidation, which only occurs under aerobic conditions.

Reaction mixture contained 20 mM NH_4^+ and (per liter): 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 0.087 g of K_2HPO_4, 2.52 g EPPS (3-[4-(2-Hydroxyethyl)-1-piperazine] propanesulfonic acid), 1 mL of 13 % EDTA-Fe^{3+}, 1 mL of trace elements solution (10 mg of $Na_2MoO_4 \cdot 2H_2O$, 147 mg of $MnSO_4 \cdot H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, 0.482 mg of $CoSO_4 \cdot 7H_2O$, and 100 mL of distilled water), and 0.5 mL of 2 mM $CuSO_4 \cdot 5H_2O$, comparable to the medium used by [149, 125]

4.2.3. CALCULATIONS

The N_2O measurement in parts per million gas volume (ppmv) was converted to concentration of N_2O in mg-N/L gas. The N_2O emission rate (mg-N/h) was obtained from the N_2O concentration multiplied by the air flow used, followed by a normalization for the working volume to obtain the volumetric emission rate (mg-N/L/h). Details about these calculations and free nitrous acid calculations are provided in SI.

4.3. RESULTS

4.3.1. N_2O PRODUCTION DURING TRANSIENT ANOXIA IN *Nitrosomonas europaea* CULTIVATION

An axenic steady-state chemostat culture of *Nitrosomonas europaea* was exposed to transient anoxia. Full details of the experiments and discussion of transcription analysis, proteomics and metabolic network modelling can be found in [149]. To illustrate the changes during transient anoxia, the data is used here focusing on gas emissions and hydroxylamine dynamics (Figure 4.2). The culture had minimal N_2O or NO formation under steady state conditions [149]. Transient anoxia was imposed for 6 hours a day during 13 days. The nitrite concentration remained high (ca. 230 mg-N-NO_2^-/L, $18 \cdot 10^{-3}$ mg-N-FNA/L) during the whole operation [149]. The switch to aerobic conditions resulted in immediate ammonia consumption [149] and maximum hydroxylamine accumulation varied from 0.2±0.05 to 0.07±0.01 mg-N/L from day 1 to day 13 (Figure 4.2A).

NO/N_2O gas emissions were measured throughout the reactor operation. Nitric oxide (NO) production immediately increased after switching to anoxic period and then decreased gradually during the rest of the operation (Figure 4.2B). In contrast to NO, N_2O was only emitted immediately after the switch to aerobic conditions (Figure 4.2C). The highest N_2O and NO emissions were observed in the first day of transient anoxia and the concentrations in the gas reached 1.8 ppmv N_2O and 9 ppmv NO (Figure 4.2C). For hydroxylamine, accumulation in the liquid was detected at the same time with N_2O emission (Figure 4.2A). Similar with the N_2O emission pattern, maximum NH_2OH values were reached in the first day (0.2± 0.05 mg-N/L) and decreased to 0.07±0.01 mg-N/L on day 13 (Figure 4.2A). Hydroxylamine was also detected during anoxic conditions at a certain constant concentration of ca. 0.07±0.01 mg-N/L (Figure 4.2A). However, that low and steady hydroxylamine concentration could be linked to the background absorbance, due to the presence of iron ions that produce a slight yellowish tinge. This was noticed also in a previous study, where background solutions were prepared with fresh cultivation medium [58].

4.3.2. ABIOTIC N_2O PRODUCTION IN AEROBIC CONDITIONS

When replicating the *N. europaea* culture conditions without any biomass, N_2O emissions were detected immediately after the addition of hydroxylamine. N_2O concentration peaked from 0.9 to 3 ppmv depending on experimental conditions (Figure 4.3 and Table 4.1).

For the same nitrite concentration (230 mg-N-NO_2^-/L, 18· 10^{-3} mg-N-FNA/L) and medium composition, but different hydroxylamine concentrations (0.05 to 0.2 mg-N/L), the total N_2O emissions increased from 0.040±0.002 to 0.14±0.01 mg-N (Figure 4.3A, Table 4.1). The ratio of air flow to working volume was 0.68 min^{-1}, like in the biological experiments performed in this study.

For a lower air flow and working volume ratio, similar with conditions used in Yu et al., [125] the total N_2O emissions were the same for 0.1 mg-N/L of hydroxylamine concentration but 1.6 fold lower for 0.2 mg-N/L hydroxylamine (Figure 4.3B, Table 4.1).

Calculated yields assuming hydroxylamine reaction with free nitrous acid producing N_2O emissions as in Equation (4.1) ranged from 20±1 % to 40±2 % (Table 4.1), clearly indicating the presence of side reactions because the NH_2OH was completely converted at the end of experiments.

4.4. DISCUSSION

4.4.1. BIOLOGICAL N_2O SOURCES

IN the studies of Yu et al., [125, 149] (Figure 4.2, Table 4.2), N_2O emissions from *N. europaea* cultures imposed to transient anoxia were only observed under recovery to aerobic conditions. That was also the case in other studies with pure cultures where different dissolved oxygen concentrations were tested [152]. Higher N_2O emissions correlated with lower oxygen tensions [150, 152]. The increased N_2O production during recovery from anoxia was initially explained by an imbalanced metabolism during the transition from low to high cell specific activity [125]. However, a more recent study correlated the N_2O emissions to the proteomic level of cytochrome P460 [149]. On

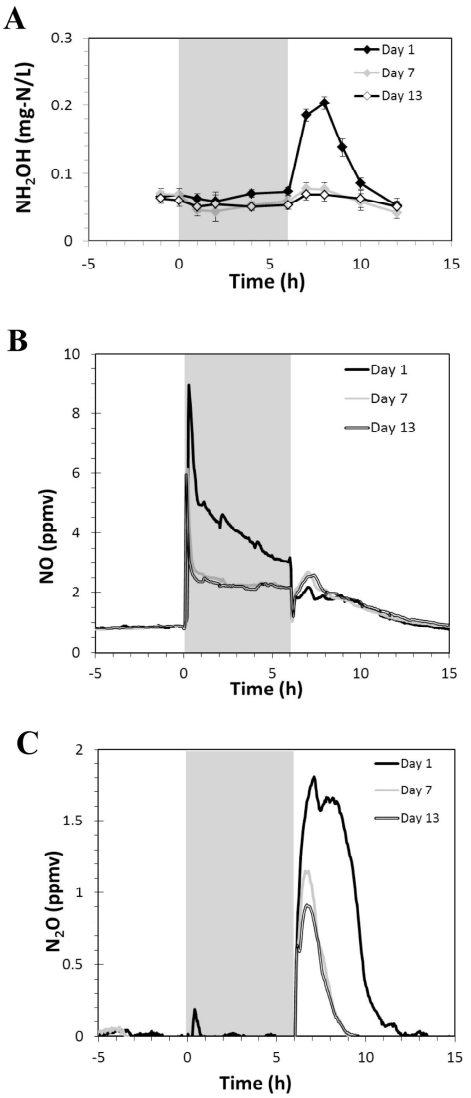


Figure 4.2: Hydroxylamine and NOx emissions during 3 different days of continuous operation where transient anoxia was imposed in a Nitrosomonas europaea axenic culture. Grey box indicates anoxic period before the transition to aerobic period. A) Hydroxylamine bulk concentration, B) NO emissions, C) N_2O emissions. Adapted from [149].

Table 4.1: Abiotic batch experiments conditions to test the impact of hydroxylamine reaction with nitrite in the emission of N_2O in pure cultures conditions. Tests were done at pH 7.41, 21 °C, 230 mg-N/L of nitrite and 1.33 L working volume. Each test was done by duplicate. $C_{NH_2OH,ini}$ states for hydroxylamine intital concentration, Q_g/V_L is the ratio of gas flow over working volume, Y_{N_2O/NH_2OH} is the yield of hydroxylamine converted to N_2O, $R_{N_2O,max}$ is the maximum N_2O emissions rate, and $r_{N_2O,max}$ is the volumetric maximum N_2O emission rate.

Batch num.	$C_{NH_2OH,ini}$ (mg-N/L)	Q_g/V_L (min^{-1})	Total N_2O (mg-N)	Y_{N_2O/NH_2OH} (%)	$R_{N_2O,max}$ (mg-N/h)	$r_{N_2O,max}$ (mg-N/L/h)
1	0.05	0.68	0.040±0.002	40±2	0.06±0.01	0.048±0.008
2	0.1	0.68	0.056±0.002	28±8	0.08±0.02	0.06±0.01
3	0.2	0.68	0.14±0.02	35±3	0.106±0.002	0.082±0.001
4	0.1	0.29	0.056±0.005	28±2	0.057±0.0005	0.0440±0.0005
5	0.2	0.29	0.083±0.004	20±1	0.080±0.002	0.065±0.002

the other hand, the model proposed in [149] could not accurately predict the N_2O emissions during the aerobic period compared to the good representation obtained for NO emissions.

Table 4.2: N_2O emissions during 3 different days in biological experiments with repeated transient anoxia conditions.Where the total N_2O refers to the N_2O emitted when recovering from transient anoxia, $R_{N_2O,max}$ is the maximum N_2O emissions rate, and $r_{N_2O,max}$ is the volumetric maximum N_2O emission rate.

Day	Total N_2O (mg-N)	$R_{N_2O,max}$ (mg-N/h)	$r_{N_2O,max}$ (mg-N/L/h)
1	1.05	0.34	0.085
7	0.29	0.21	0.053
13	0.25	0.17	0.043

One of the processes involved in nitrous oxide emissions is the nitrifier denitrification pathway. NIR and NOR have been identified as the enzymes responsible of performing successive transformation of NO_2^- into NO and then N_2O in *Nitrosomonas europaea* [131, 146, 54], (Figure 4.1). This pathway is active during anoxic and microaerobic conditions, where nitrite is used instead of oxygen as terminal electron acceptor, thus the eventually remaining oxygen can be used for ammonia oxidation. Moreover, Kozolwski et al.[54], demonstrated that a double mutant lacking NOR and NIR was unable to produce N_2O during anaerobic or hypoxic conditions. However, the same double deficient mutant did produce N_2O during aerobic conditions, thus the authors suggested the presence of other enzymes not yet characterized as a possible explanation for aerobic emissions [54]. All this indicates that the main NO emissions measured during the anoxic phase (Figure 4.2B) are likely due to nitrifier denitrification [125, 149].

However, it is surprising that in spite of NO reactivity, this was not further transformed to N_2O. Genomics and proteomics analyses suggested that long term response to cyclic transient anoxia lead to downregulation of detoxification proteins (0.6, 0.5 and 0.8 fold for CytP460, c-554 and NOR, respectively), whereas energy conversion was favored (2-fold change of AMO)[149]. Conversely, the N_2O emissions were just observed in the aerobic period (Figure 4.2C) showing that other pathway than the nitrifier denitrification was contributing to the N_2O production.

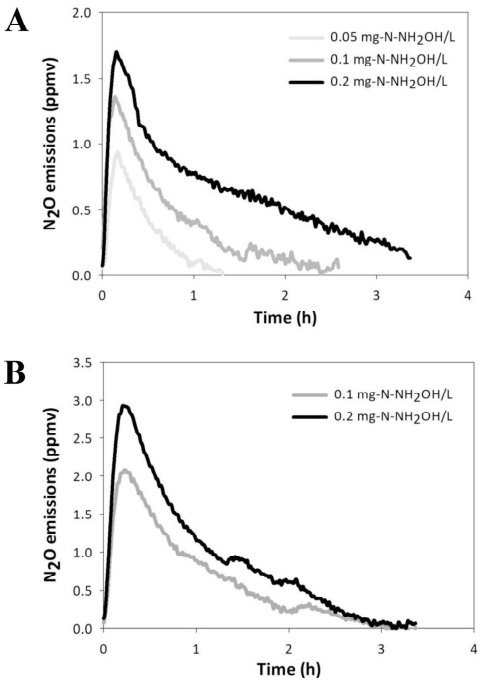


Figure 4.3: Averaged data from two abiotic duplicates replicating biological culture conditions to assess the amount of N_2O produced chemically. Experiments were abiotically performed at 21 °C, pH 7.41, 230 mg-N-NO_2^-/L, ($18{\cdot}10^{-3}$ mg-N-FNA/L). Air flow to liquid volume ratio: A) 0.69 / min, B) 0.28 / min.

N_2O emissions during aerobic conditions are mainly related to hydroxylamine oxidation pathways, and the involved enzymes are still being investigated. So far, it has been reported that enzymatic extracts of HAO were able to catalyze the production of NO and N_2O in vitro, but its potential effect in vivo remains to be assessed [55]. Recently, cytochrome P460 has been shown to catalyze N_2O production from hydroxylamine, however the experiments were performed also in vitro [57]. Finally, Terada et al. [59] highlighted the importance of the hydroxylamine reaction with nitrite either biocatalyzed or by abiotic transformations at high ammonia oxidation rates and nitrite concentrations in a partial nitritation reactor. The operation conditions were similar to those used in our study (i.e, high nitrite concentrations). The proteomic and genomic analysis performed by Yu et al. [149] revealed an adaptation of the biomass to repeated transient anoxia exposure. However, these experiments were performed in conditions where abiotic emissions can occur. In view of all these possible biological conversions, the biological contribution to N_2O emissions during the aerobic period of the *N. europaea* cultivations [125, 149] cannot be excluded.

4.4.2. IMPORTANCE OF ABIOTIC N_2O PRODUCTION IN BIOLOGICAL EXPERIMENTS WITH *N. europaea*

Most of the studies focused on the biologically emitted N_2O, even if different chemical reactions are known to produce N_2O [19, 63, 147, 61, 58]. In the present study, abiotic experiments confirmed that chemical N_2O production is possible under the conditions used for the cultivation of *N. europaea* (Figure 4.2), as well as in the conditions from a previous study [21]. The high nitrite concentration (ca. 230 mg-N/L) at pH 7.5 leads to a free nitrous acid concentration in the range of ca. 0.018 mg-N/L, which can react with hydroxylamine to produce N_2O. Hydroxylamine was added in the abiotic experiments in the concentrations measured during the axenic cultivations performed previously (Table 4.1). Similarly, other studies have also reported hydroxylamine accumulation in the liquid in the range 0.01 to 1 mg-N-NH_2OH/L [20, 21, 123, 124], and these concentrations are in agreement with those reported during the recovery from transient anoxia.

It should be noted that the hydroxylamine measured in the continuous cultivation is the result of a balance between its continuous production by ammonia oxidation and consumption by either transformation to nitrite or N_2O. This indicates that the net accumulation of hydroxylamine might be very sensitive to a small change in one of the main conversions. Thus, the amount of hydroxylamine transformed chemically to N_2O in the continuous biological experiments can be different from the residual hydroxylamine measured and therefore different from the amount used in the abiotic tests. That would explain why the total N_2O produced during the continuous biological cultivations is higher than the total N_2O produced in the abiotic batch test. A biological contribution to N_2O emissions in the continuous cultivation cannot be ruled out, because it was not possible to assess the amount of hydroxylamine chemically transformed to N_2O. A study with mixed populations of nitrifiers in aggregates performed in a partial nitrification reactor [59] found that half of the N_2O emitted was produced through the reaction of hydroxylamine and nitrite (without elucidating the proportion of abiotic and biotic contributions). However, in the present work and in

literature [21, 149], axenic cultures of planktonic cells were used, thus gradient effects due to aggregated biomass and N_2O emissions by other pathways than that of *N. europaea* can be ruled out.

In the study of Yu et al. [21], N_2O emissions in the *N. europaea* culture during transient anoxia were associated to differences in specific cell activity upon the anoxic/aerobic switch and the N_2O production was assumed to be biological. In the later work, protein analysis revealed a 0.6 fold change on cytochrome P460 protein content adaptation after 13 days imposing transient anoxia cyclcing [149]. However, the source of reducing equivalents for NO and N_2O formation from nitrite remained unknown, and it is generally assumed to be provided by an endogenous reductant pool [142]. Remarkably, with the biotic and the abiotic results reported in the present study, most of the emissions found during the biological experiments [125, 149] could be explained by the chemical transformation (i.e., volumetric rates were comparable). The unknown source of reducing equivalents (i.e., electron donor) for the nitrite reduction, claimed in [142] and [125], would be automatically hydroxylamine.

We believe that the main reason why the chemical reaction of hydroxylamine with free nitrous acid has been given little attention in the past, is that hydroxylamine has not been regularly included in control tests when trying to identify possible abiotic N_2O production. For instance, Poth et al. performed controls with heat-killed biomass incubated with ammonia and nitrite [151], Goreau et al. [150] used $HgCl_2$-killed biomass in ammonia containing flasks, Shaw et al. [153] performed non-inoculated controls in nitrite and ammonia , whereas Beaumont et al. [148] did not mention any abiotic control. Summarizing, when trying to assess abiotic N_2O production, either killed biomass or non-inoculated tests were used. The main problem is however that the medium generally used for the control tests did not contain hydroxylamine. Only Anderson et al. [154] showed that control tests containing hydroxylamine and nitrite did produce N_2O, but they ruled out the real importance of the chemical reaction based on the (wrong) assumption that hydroxylamine cannot accumulate in the biological cultures. Recently, Kozlowski et al., showed that chemical controls with hydroxylamine and nitrite resulted in lower abiotic N_2O emissions compared to those from the equivalent biological system for different AOB genera [17].

Although this seems to be in disagreement with results obtained in our study, the differences in medium composition (i.e., higher concentration of nitrite or the presence of metals) can still trigger the abiotic reaction [63, 58, 59]. Nitrite concentrations used in the chemical controls from [17] were ca. 3.5 mg-N-NO_2^-/L (7 - 23 · 10^{-5} mg-N-FNA/L), two orders of magnitude lower than those used in the current and most axenic studies [21, 125, 149, 142] during transient anoxia (ca. 230 mg-N NO_2^-/L, so ca. 18· 10^{-3} mg-N-FNA/L). Conditions used by Kozlowski et al. [17] are in agreement with the recommendations given in the present work to study N_2O emissions (i.e. low nitrite concentration, usage of planktonic and axenic cultures). Nevertheless, further research is needed to assess the contribution of abiotic hydroxylamine reaction with free nitrous acid under different cultivation conditions.

4.4.3. IMPLICATIONS AND RECOMMENDATIONS

In view of our current results, all results derived from research assuming that transient anoxia produces N_2O exclusively through biological pathways should be reconsidered. Because of the “hybrid" biotic/abiotic pathway, a last reaction step involving the fully abiotic reaction of biologically produced NH_2OH with HNO_2 to emit N_2O is possible. ^{15}N labelling can be used to follow metabolic fluxes in axenic cultures and quantify the amount of hydroxylamine transformed to N_2O, obtaining a rationing between different possible pathways, as performed by Terada et al. [59]. These experiments should also be performed in pure cultures of planktonic cells, which could provide insight in the predominant pathways at different conditions and for different AOB species. Nevertheless, with ^{15}N labelling it is not possible to differentiate between abiotic and biological production through this “hybrid" pathway, as the reaction is the same.

Other strategies could be conducted to rule out abiotic N_2O emissions, for instance, by applying low ammonia concentrations together with high dilution rates in continuous cultures, so that nitrite (and free nitrous acid) concentration remains at low levels. Similarly, a higher pH in the bulk liquid will lead to lower free nitrous acid concentrations. Besides changing the concentrations of nitrate/nitrous acid, strategies can be directed to decrease the hydroxylamine concentration in order to prevent abiotic and biological emissions. Thus, hydroxylamine measurements are essential to provide information on conditions that trigger its accumulation in the bulk. Finally, including nitrification intermediates, like hydroxylamine, when performing abiotic controls is vital to assess the impact of abiotic N_2O emissions in each culture conditions.

4.5. CONCLUSIONS

In the present work we showed that N_2O abiotic emissions from the reaction of FNA and hydroxylamine occurred at the same conditions and in comparable rates with those measured during recovery from anoxia in cultures of *Nitrosomonas europaea*. Thus, we propose that abiotic N_2O production from hydroxylamine reaction with FNA should be considered when describing the N_2O emission pathways. Additionally, to reduce the impact of the abiotic pathway on the assessment of the biologically produced N_2O, low nitrite concentration and high pH should be maintained during cultivation of AOB to reduce the amount of FNA available in the culture.

4.6. SUPPLEMENTARY INFORMATION

Supplementary information can also be found online, [60].

4.6.1. N_2O CALCULATIONS

Calculations used for conversion of ppmv to mg-N/L (Equation (4.2)) and calculation of maximum N_2O emission rates (Equation (4.3)) and maximum volumetric rate (Equation (4.4)).

$$C_{N_2O,gas} = \frac{C_{N_2O} \cdot P \cdot M_{N_2O}}{0.082 \cdot T \cdot 1000} \tag{4.2}$$

$$R_{N_2O,gas} = C_{N_2O,gas} \cdot Q_g \tag{4.3}$$

$$r_{N_2O,gas} = \frac{C_{N_2O,gas} \cdot Q_g}{V_L} \tag{4.4}$$

Where $C_{N_2O,gas}$ is the concentration of nitrogen in the gas (mg-N-N_2O/L). C_{N_2O} is the measured gas fraction by the gas analyser (ppmv). P stands for the pressure registered by the gas analyser during the measurement (atm), M_{N_2O} is the molecular mass of nitrogen per mol of compound, being 28 g-N/mol for N_2O. T is the gas temperature (K). Finally, R states for the emissions rate (mg-N/h) and r for the volumetric emission rate (mg-N/L/h), V_L is the reactor working volume (L) and Q_g is the air flow (L/h).

4.6.2. FREE NITROUS ACID CALCULATION

From acid-base equilibrium the HNO_2 (free nitrous acid, FNA, in mg−N/L) concentration can be estimated for a given nitrite concentration (TNN, in mg−N/L), pH and temperature (T in °C) using Equation (4.5) [137]:

$$FNA = \frac{TNN}{10^{pH} \cdot \exp\left(-\frac{2300}{273+T}\right) + 1} \tag{4.5}$$

5

HYDROXYLAMINE METABOLISM OF *Ca.* KEUNENIA STUTTGARTIENSIS

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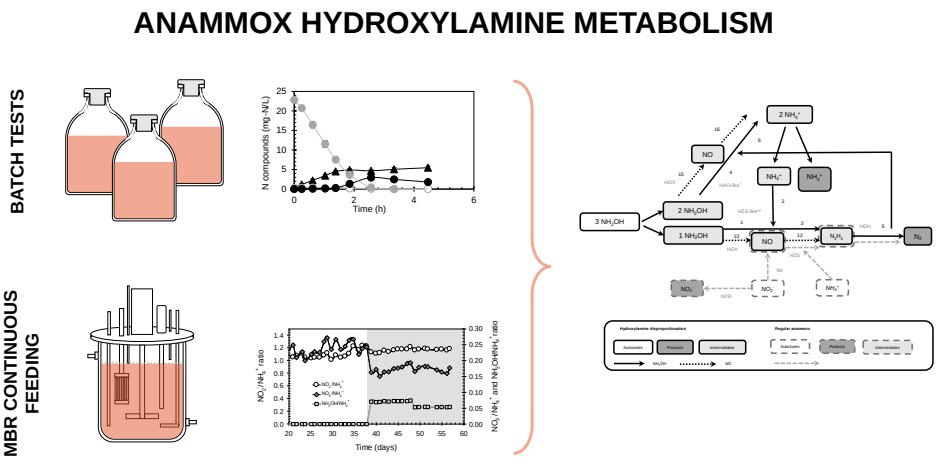
ABSTRACT

Hydroxylamine is a key intermediate in several biological reactions of the global nitrogen cycle. However, the role of hydroxylamine in anammox is still not fully understood. In this work, the impact of hydroxylamine (also in combination with other substrates) on the metabolism of a planktonic enrichment culture of the anammox species Ca. Kuenenia stuttgartiensis was studied. Anammox bacteria were observed to produce ammonium both from hydroxylamine and hydrazine, and hydroxylamine was consumed simultaneously with nitrite. Hydrazine accumulation - signature for the presence of anammox bacteria - strongly depended on the available substrates, being higher with ammonium and lower with nitrite. Furthermore, the results presented here indicate that hydrazine accumulation is not the result of the inhibition of hydrazine dehydrogenase, as commonly assumed, but the product of hydroxylamine disproportionation. All kinetic parameters for the identified reactions were estimated by mathematical modelling. Moreover, the simultaneous consumption and growth on ammonium, nitrite and hydroxylamine of anammox bacteria was demonstrated, this was accompanied by a reduction in the nitrate production. Ultimately, this study advances the fundamental understanding of the metabolic versatility of anammox bacteria, and highlights the potential role played by metabolic intermediates (i.e. hydroxylamine, hydrazine) in shaping natural and engineered microbial communities.



5.1. INTRODUCTION

ANAEROBIC ammonium oxidizing bacteria (anammox) were first reported in the 90s in a wastewater treatment plant [8]. Anammox bacteria autotrophically oxidize ammonium to dinitrogen gas with nitrite as electron-acceptor [9]. Before their discovery, even if predicted thermodynamically, ammonium activation in absence of oxygen had never been identified in nature [87]. Since then, significant efforts focused on understanding the central metabolism of anammox bacteria (e.g. [89, 90, 10, 11]).

Initially, hydroxylamine was hypothesized to be an obligate intermediate of anammox catabolism, and hydrazine was shown to accumulate when hydroxylamine was added in anammox cultures [11]. More recently, NO was proposed to be the actual intermediate in the catabolic pathway [89]. The current working hypothesis for the anammox metabolism involves three reactions. First, nitrite is converted to NO (Equation (5.1)) via a nitric oxide reductase (Nir) enzyme. Then, NO is used to activate NH_4^+ and form the N-N bond needed to produce hydrazine (N_2H_4) (Equation (5.2)) catalysed by hydrazine synthase (HZS). Finally hydrazine is further converted to dinitrogen gas (Equation (5.3)) by hydrazine dehydrogenase (HDH).

$$NO_2^- + 2H^+ + e^- \longrightarrow NO + H_2O \tag{5.1}$$

$$NO + NH_4^+ + 2H^+ + 3e^- \longrightarrow N_2H_4 + H_2O \tag{5.2}$$

$$N_2H_4 \longrightarrow N_2 + 4H^+ + 4e^- \tag{5.3}$$

However, even with NO as the central intermediate, hydroxylamine seems to play a key, yet elusive role in anammox metabolism. For example, *Ca.* Brocadia spp. strains do not encode for a Nir enzyme [92]. Thus, either *Ca.* Brocadia spp. have hydroxylamine as central intermediate as proposed by Oshiki and coworkers [90], or another enzyme (like kustc0458) rather than Nir is converting nitrite to NO [88]. Moreover, different studies showed that one of the most highly expressed enzyme in anammox bacteria is the hydroxylamine oxidase (HOX; kustc1601), which is proposed to convert hydroxylamine to NO [89, 88]. The reason for anammox bacteria to invest energy to express HOX at such high levels has been hypothesized to be the possibility to reuse any hydroxylamine leaking from HZS [93, 23]. Briefly, the actual reaction mechanisms of HZS is proposed to involve NO transformation to hydroxylamine, and ammonium and hydroxylamine reacting to form hydrazine [93, 23]. Overall, the role of hydroxylamine in anammox metabolism remains poorly understood.

In the environment where anammox bacteria thrive, free hydroxylamine has been measured [32, 18, 34, 60, 19, 64, 59, 20, 21, 125, 31]. For instance, hydroxylamine has been shown to transiently accumulate in concentrations ranging from 0.006-1 mg-N/L in different ammonium oxidizing bacteria (AOB) pure cultures [18, 19, 21, 125, 31] or mixed consortia [32, 34, 60, 64, 59, 20]. Thus, anammox exposure to external hydroxylamine cannot be ruled out, in particular in biofilm systems, where hydroxylamine can be produced by a nitrifying population and can reach higher concentrations than in the bulk liquid [115]. In these systems, hydroxylamine can be produced in the external oxic layers where AOB are present, diffuse through the biofilm and reach the anoxic (anammox bacteria) layers [115].

Batch tests with hydroxylamine addition are generally used to study the short term effects of hydroxylamine on anammox bacteria primarily to i) demonstrate anammox activity [91, 9], and ii) study the "boosting" effect of intermediates on anammox activity [94, 95]. However, to the best of our knowledge, the effect of different substrate combinations on anammox hydroxylamine consumption has not been dedicatedly studied. The only in depth study on the effect of hydroxylamine on anammox metabolism used ammonium as sole co-substrate [96].

In the present work, the impact of different combinations of substrates together with hydroxylamine on a planktonic anammox *Ca.* Kuenenia stuttgartiensis culture in batch tests was studied. Also, the long-term impacts of hydroxylamine addition were studied, and the effects on stoichiometry and microbial composition were quantified. Finally, a thermodynamic and modelling approach was developed to estimate the key kinetic parameters, and further understand the anammox metabolism with hydroxylamine as (co)substrate.

5.2. MATERIALS AND METHODS

5.2.1. BATCH TEST PREPARATION AND PROCEDURE

BIOMASS was collected from a 10L MBR highly enriched in planktonic *Ca.* Kuenenia stuttgartiensis (79±4 % as estimated by 16S rRNA gene-based amplicon sequencing analysis) (see SI) and centrifuged for 15 minutes at 4200 rpm at room temperature. Cells were re-suspended in N_2-sparged mineral medium to the desired biomass concentration. The mineral medium had the same composition as described in SI, but without ammonium or nitrite and supplemented with 1 g/L $NaHCO_3$. The pH and optical density at 660 nm (OD_{660}) were measured before aliquoting 50mL of cell suspension among 112 mL serum bottles. The optical density was correlated with dry weight (gVSS/L) (Figure 5.6). Biomass used in negative controls was boiled for 5 minutes before aliquoting. The bottles were sealed with rubber stoppers, and anoxic conditions were achieved by sparging (ca. 1 minute) and vacuuming (ca. 3 minutes) with Argon three times per bottle. Bottles were placed on a shaker (Incubator Hood TH30, Edmund Bühler GmbH, Bodelshausen, Germany) at 21 °C and 170 rpm. The pH was not adjusted, but remained between 8.0 and 8.5, within the optimal range for anammox bacteria [155]. Bottles were incubated overnight with stoichiometric concentrations of ammonium and nitrite to ensure activity.

After overnight incubation, the experiment was performed by adding a pulse of ammonium, nitrite, hydroxylamine, and/or hydrazine from anoxic stock solutions to reach the desired initial concentration in each batch test (see Table 5.1). Sampling was done over time depending on the biomass activity by removing 4 mL of cell suspension with a syringe. Samples were centrifuged for 3-5 minutes (4200 rpm, 4 °C), and the supernatant was kept for further analysis of the dissolved nitrogen compounds (see SI). Samples used for hydrazine [156] and hydroxylamine [117] determination were treated with 200 µL of a 0.1 g/mL sulfamic acid solution to remove the dissolved nitrite. Nitrite was previously shown to interfere in the hydroxylamine determination [58]. In the present study, nitrite was also shown to interfere the hydrazine measurement (see Figure 5.7). Nitrogen consumption/production rates calculations are described in SI.

Microbial community dynamics were not followed during the batch tests, but they would not be expected to shift significantly as no significant changes were observed during long term hydroxylamine feeding in a continuous reactor (see Section 5.3.2).

5.2.2. BATCH TESTS THERMODYNAMICS ANALYSIS

Based on the batch test data, the thermodynamics of the putative reactions involved in hydroxylamine consumption were studied (see Table 5.2). Reactions 3, 6, 7, 10 and 11 were originally postulated by van der Star [96]. Half reactions were derived based on N, O, H and charge balances (see example in SI, based on Kleerebezem and Van Loosdrecht 2010 [157]). The standard Gibbs energy change ΔG_R° of each reaction in Table 5.2 was calculated with the standard Gibbs energy of formation (G_R°) of each compound (Table 5.4 and Equation (5.4)). ΔG_R° was corrected with the measured concentration of each compound at each batch test time point to obtain the actual Gibbs energy change of reaction (ΔG_R^1) evolution during the batch tests (Equation (5.5) and Equation (5.6)). The aim was to detect any possible thermodynamics limitations to explain the hydrazine accumulation behaviour or the possibility to have NO as intermediate in reactions involving hydroxylamine (the approach, calculations and results are detailed in SI).

5

5.2.3. BATCH TESTS KINETIC PARAMETERS DETERMINATION

A kinetic model (Table 5.5 and Table 5.6) was proposed and adapted from van der Star [96] to obtain the kinetic parameters of the reactions involved in the batch tests. The model was implemented in Matlab R2018b and aimed to minimize the error between the experimental data and the set of differential equations proposed for each compound. As the experiments were performed in batch, the set of differential equations were equal to the results of a matrix multiplication between the proposed stoichiometric matrix and the process rate matrix (Table 5.5 and Table 5.6, respectively). A step wise approach to obtain the kinetic constants from more simple to more complex batch tests was followed (Figure 5.8). This was combined with the use of different objective functions (Equation (5.7) to Equation (5.10)) to assess if the solution of the optimization performed was independent of the objective function used (full description of the procedure is detailed in SI).

Table 5.1: Batch test conditions and specific rates measured with (if applicable) and without hydroxylamine present. Two duplicates (n=2) were performed per condition tested. ∗This rate corresponds to hydrazine consumption with hydroxylamine present.

	Initial concentrations				Specific rates with NH_2OH			Specific rates without NH_2OH			
Batch	NH_4^+	NO_2^-	NH_2OH	N_2H_4	$qNH_4^+{}_{,NH_2OH}$	$qNO_2^-{}_{,NH_2OH}$	$qNH_2OH_{,NH_2OH}$	qNH_4^+	qNO_2^-	$qN_2H_{4,production}$	$qN_2H_{4,consumption}$
	(mg-N/L)				(mg-N/gVSS/h)			(mg-N/gVSS/h)			
1	33.4 ± 0.1	16.94 ± 0.02	-	-	-	-	-	-46 ± 2	-57 ± 3	-	-
2	-	-	22.8 ± 0.1	-	18 ± 1	-	-76 ± 1	2.9 ± 0.3	-	16 ± 1	-4.3 ± 0.3
3	-	-	6.8 ± 0.1	-	9.2 ± 0.6	-	-47 ± 2	1.2 ± 0.4	-	7 ± 1	-1.9 ± 0.1
4	1.64 ± 0.03	-	7.7 ± 0.2	-	13 ± 2	-	-51 ± 3	2.78 ± 0.04	-	11 ± 1	-3.6 ± 0.1
5	2.4 ± 0.3	-	7.3 ± 0.1	-	12.3 ± 0.5	-	-47 ± 1	1.8 ± 0.2	-	5 ± 1	-2.3 ± 0.1
6	29 ± 2	-	21.9 ± 0.8	-	17 ± 2	-	-75 ± 1	3.7 ± 0.4	-	32 ± 2	-10 ± 1
7	1.1 ± 0.7	-		4.3 ± 0.1	-	-	-	3.8 ± 0.1	-	-	-6.3 ± 0.1
8	-	19.9 ± 0.3	20.1 ± 0.7	-	6.0 ± 0.8	-7.4 ± 0.9	-42 ± 6	-9 ± 1	-7.4 ± 0.9	5.9 ± 0.6	-3 ± 1
9	-	6.89 ± 0.04	7.2 ± 0.6	-	4.7 ± 0.4	-11.0 ± 0.2	-37 ± 2	-5.3 ± 0.9	-11.0 ± 0.2	2.1 ± 0.2	-0.62 ± 0.02
10	84 ± 2	19 ± 3	30 ± 1	-	-26 ± 4	-22 ± 2	-113 ± 4	-19 ± 7	-31 ± 2	15 ± 5	-26
11	52 ± 4	18.8 ± 0.1	8.7 ± 0.4	-	-31.5 ± 0.9	-17 ± 1	-39 ± 2	-26	-33 ± 1	29 ± 2	-9.8 ± 0.4
12	36 ± 4	10.79 ± 0.01	4.8 ± 0.1	-	-42 ± 2	-18.9 ± 0.4	-30.9 ± 0.2	-8.7 ± 0.4	-30.8 ± 0.3	33 ± 1	-6.6 ± 0.6
13	1.5 ± 0.3	-	8.09 ± 0.01	4.90 ± 0.01	33 ± 3	-24 ± 2 *	-60 ± 4	4.0 ± 0.1	-	-	-6.4 ± 0.2

5.2.4. REACTOR OPERATION - CONTINUOUS LONG-TERM STUDY

One litre of biomass from the same 10 L MBR used in batch tests and described in SI was used as inoculum for a 2 L MBR reactor (Figure 5.9). The HRT was kept at 2.3±0.2 d during the whole operation using a custom-made ultrafiltration membrane unit. An SRT of 8.7±1.0 d was maintained throughout the operation period by withdrawing the desired reactor content per day. Temperature was controlled at 30 °C with an external jacket, and the reactor was stirred at 170rpm. The reactor pH was controlled at 7.04±0.04 with a 53 g/L $NaHCO_3$ solution.

When hydroxylamine was continuously fed (Phase II, days 38-54 in Table 5.7), two bottles were used to avoid hydroxylamine reaction with any of the mineral medium components. The total volumetric mineral medium loads were maintained by correcting media preparation.

Samples were collected daily from the effluent line and centrifuged, supernatant was collected for further analysis of the dissolved nitrogen compounds (see SI) and treated with sulfamic acid if needed. The biomass pellets were kept at −80 °C. A fresh sample from the reactor was used to monitor daily optical density. During reactor operation volatile suspended solids (VSS) were measured periodically, and the specific correlation between OD_{660} and VSS was used for calculations (Figure 5.10). DNA samples from days 23, 33, 38, 40, 45, 50 and 55 were analysed at Novogen for 16S rRNA amplicon sequencing (see SI).

Table 5.2: Reactions involved in hydroxylamine and hydrazine disproportionation. e^- refers to electron. ΔG_R° refers to standard Gibbs free energy calculated at 25 °C, 1atm and 1M in kJ/mol referred to mol of hydroxylamine, (∗) mol NH_4^+, (∗∗) mol N_2H_4 or (∗ ∗ ∗) mol of NO reacting. Reactions 3, 6, 7 10 and 11 as proposed in the model of van der Star [96]. Half reactions were derived based on N, O, H and charge balances as in Kleerebezem and Van Loosdrecht 2010 [157].

Number	Name	Comments	Reaction	ΔG_R° (kJ/mol)
Hydroxylamine disproportionation				
1	Hydrazine production (e^- acceptor)	Half reaction	$NH_2OH + H^+ + e^- \longrightarrow 0.5\,N_2H_4 + H_2O$	-149.9
2	Hydrazine production (e^- donor)	Half reaction	$NH_4^+ \longrightarrow 0.5\,N_2H_4 + 2\,H^+ + e^-$	143.3 (*)
3	Hydrazine production in hydroxylamine disproportionation	(sum of 1 & 2)	$NH_4^+ + NH_2OH \longrightarrow N_2H_4 + H_2O + H^+$	-6.6
4	Hydrazine consumption (e^- acceptor)	Half reaction	$NH_2OH + 3\,H^+ + 2\,e^- \longrightarrow NH_4^+ + H_2O$	-293.2
5	Hydrazine consumption (e^- donor)	Half reaction	$N_2H_4 \longrightarrow N_2 + 4\,H^+ + 4\,e^-$	-127.8 (**)
6	Hydrazine consumption in hydroxylamine disproportionation	(sum of 2*4 & 5)	$2\,NH_2OH + N_2H_4 + 2\,H^+ \longrightarrow 2\,NH_4^+ + N_2 + 2\,H_2O$	-357.1
7	Hydroxylamine disproportionation	(sum of 3 & 6)	$3\,NH_2OH + H^+ \longrightarrow NH_4^+ + N_2 + 3\,H_2O$	-240.3
Hydrazine disproportionation				
8	Hydrazine disproportionation (e^- acceptor)	Half reaction	$N_2H_4 + 4\,H^+ + 2\,e^- \longrightarrow NH_4^+$	-286.6 (**)
9	Hydrazine disproportionation (e^- donor)	Half reaction	$N_2H_4 \longrightarrow 4\,H^+ + 4\,e^- + N_2$	-127.8 (**)
10	Hydrazine disproportionation	(sum of 2*8 & 9)	$3\,N_2H_4 + 4\,H^+ \longrightarrow 4\,NH_4^+ + N_2$	-233.6 (**)
11	Hydrazine consumption	(option 2 for hydrazine consumption)	$N_2H_4 + H^+ + H_2O \longrightarrow NH_4^+ + NH_2OH$	6.6 (**)
Hydroxylamine disporportionation via NO, modified reactions involving hydroxylamine				
12	Hydrazine production via NO (e^- donor)	Half reaction	$NO + 4\,H^+ + 4\,e^- \longrightarrow 0.5\,N_2H_4 + H_2O$	-259.9 (***)
13	Hydrazine production via NO (e^- acceptor)	Half reaction	$NH_2OH \longrightarrow NO + 3\,H^+ + 3\,e^-$	110
14	Overall reaction hydrazine production (e^- acceptor)	(Sum of 12 &13), same as 1	$NH_2OH + H^+ + e^- \longrightarrow 0.5\,N_2H_4 + H_2O$	-149.9
15	Hydrazine consumption via NO (e^- donor)	Half reaction	$NO + 6\,H^+ + 5\,e^- \longrightarrow NH_4^+ + H_2O +$	-403.2 (***)
16	Hydrazine consumption via NO (e^- acceptor)	Half reaction	$NH_2OH \longrightarrow NO + 3\,H^+ + 3\,e^-$	110
17	Overall reaction hydrazine consumption (e^- acceptor)	(Sum of 15 & 16), same as 4	$NH_2OH + 3\,H^+ + 2\,e^- \longrightarrow NH_4^+ + H_2O$	-293.2

5.3. RESULTS AND CONCLUSIONS

5.3.1. SHORT TERM ANAMMOX METABOLISM WITH HYDROXYLAMINE AS SUBSTRATE

ANAMMOX bacteria primarily convert ammonium and nitrite to dinitrogen gas, yet are also capable to metabolize other substrates, such as hydroxylamine, hydrazine and organic carbon [9]. In biofilm systems, hydroxylamine can leak from AOB communities [18, 64, 20], diffuse through the biofilm [115], and reach anammox bacteria in anoxic layers. Batch tests to evaluate the capacity of anammox bacteria to metabolize hydroxylamine were performed by supplying hydroxylamine together with different combination of substrates to a *Ca.* Kuenenia stuttgartiensis enrichment. The aim was to investigate if different combination of substrates impacted the conversion dynamics of the nitrogen species. Hydroxylamine concentrations used in batch are higher than those to which anammox bacteria might be exposed in nature (i.e. values of 0.006-1 mg-N/L hydroxylamine have been reported in different nitrification systems [18, 64, 20], among others), but were needed to be able to investigate the conversions.

Trends in nitrogen compounds consumption and production were independent of the initial concentrations (Table 5.1). Therefore, only tests with higher initial concentrations are discussed here (Figure 5.1), unless differently stated (Figure 5.11). The impact of substrate combinations on hydroxylamine metabolism will be discussed in the present section, whereas the impact on hydrazine accumulation will be discussed further in the next section. Positive controls to assess anammox activity with ammonium and nitrite (Figure 5.12A) were included. Data from the positive controls indicated that once nitrite was consumed, no significant changes in the ammonium concentrations were detected. Denitrifying activity was ruled out by providing nitrite as substrate (Figure 5.12D) to the anammox enrichment culture. Abiotic controls with boiled biomass and all the substrates used in the batch test were performed and did not show significant activity compared to the biological rates; for example an increase of ca. 0.1 mg-N/L of ammonium was detected in the abiotic control after ca. 7h (Figure 5.12C and E) compared to 5 mg-N/L of ammonium produced in 2h in biological tests (Figure 5.1A).

AMMONIUM PRODUCTION OCCURS FROM BOTH HYDROXYLAMINE AND HYDRAZINE

When hydroxylamine was added as the only substrate (Batches 2-5, Table 5.1), hydrazine did accumulate, as expected from previous studies [11, 96]. However, two distinct ammonium production events occurred (Figure 5.1A and Figure 5.11). The first ammonium production started as soon as hydroxylamine was added (ca. 0-2.4h in Figure 5.1A, see also Figure 5.11). When the hydroxylamine concentration became low, hydrazine started to accumulate (around 1.6h in Figure 5.1A, after ca. 15-25min. in Figure 5.11, with lower initial hydroxylamine concentration). Once hydroxylamine was depleted, a second ammonium production period started, correlating with the decrease of hydrazine (from ca. 2.4h onwards in Figure 5.1A, see also Figure 5.11). Thus, ammonium was produced both with hydroxylamine and hydrazine (Figure 5.1A) , apparently, with preference for utilization of hydroxylamine over hydrazine (Figure 5.12B).

The initial production of ammonium from hydroxylamine is in accordance with the hydroxylamine disproportionation reactions (reaction 7, Table 5.2) proposed by van

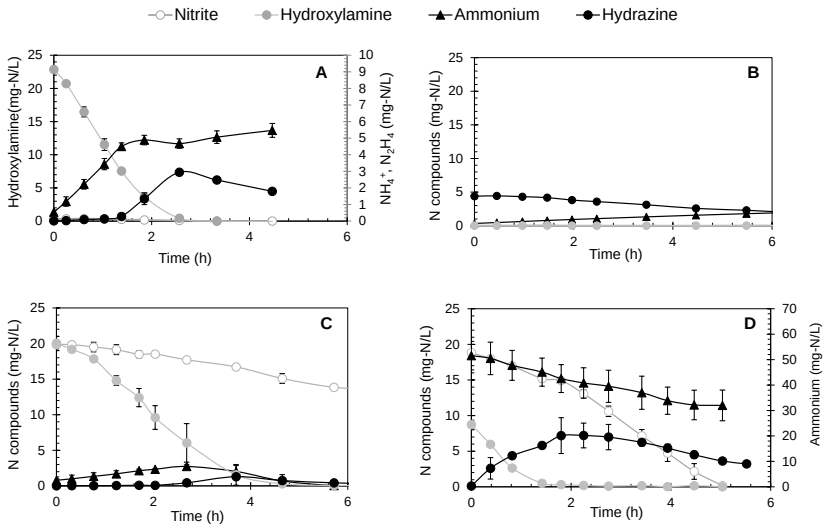


Figure 5.1: Dynamics of nitrogen compounds during anammox batch tests with different combinations of substrates: A) Hydroxylamine (Test 2 in Table 5.1) (note that ammonium and hydrazine are represented on the right axis) , B) Hydrazine (Test 7 in Table 5.1), C) Hydroxylamine and nitrite (Test 8 in Table 5.1), D) Hydroxylamine, nitrite and ammonium (Test 11 in Table 5.1) (note that ammonium is represented on the right axis). Error bars represent the standard deviation between duplicates.

de Star et al. [96]. Hydroxylamine disproportionation occurs via two intermediate reactions (reaction 3 and 6, in Table 5.2). The imbalance of reactions 3 and 6 to the observed hydrazine accumulation (see Section 5.3.1 for further details). The experimental stoichiometric ratio of consumed hydroxylamine per ammonium produced was 3.8, which is quite close to the theoretically expected of 3 for hydroxylamine disproportionation (reaction 7 in Table 5.2).

The second ammonium production from hydrazine was not observed by van der Star et al.[96], most likely obscured by the high initial ammonium concentration used in the tests (2-8 mM, 28-112 mg-N/L). The consumption of hydrazine was proposed to follow two possible reactions (reaction 10 or 11, Table 5.2) [96]. In the present study, once hydroxylamine was totally consumed, ca. 1.4 mM of ammonium were produced per mM of hydrazine consumed, close to the 1.3 theoretical ratio of hydrazine disproportionation via reaction 10 in Table 5.2 (Figure 5.1). Furthermore, hydrazine disproportionation via reaction 10 was confirmed by performing a batch test with hydrazine as the only substrate (Figure 5.1B, batch 7 in Table 5.1). 1.2 mM mole of ammonium were produced per mM of hydrazine, which is in agreement with the proposed stoichiometry of hydrazine disproportionation (reaction 10, Table 5.2) producing 1.3 moles of ammonium per mol of hydrazine. Similar stoichiometries for

ammonium produced per hydrazine consumed were previously shown in a batch test performed with only hydrazine present, but no mechanisms were proposed [11].

Based on the experimental stoichiometry, the production of ammonium from hydrazine is most likely to result from hydrazine disproportionation (as in reaction 10, Table 5.2, see also Figure 5.4, and Section 5.3.1 for a discussion on putative enzymes involved).

The occurrence of hydrazine disproportionation throughout the batch test contributed to the ammonium production when hydroxylamine is still present. Multiple confirmations were obtained from the experimental data sets and the mathematical model. For instance, when hydroxylamine and hydrazine were provided together as substrates both were consumed simultaneously (batch 13 in Table 5.1 and Figure 5.12B). Hydroxylamine was initially consumed c.a. 2.5 times faster than hydrazine (60 ± 4 and 24±2 mg-N/gVSS/h, respectively) and a transient slow down on hydrazine consumption could be observed . Furthermore, the proposed mathematical model with the parameters obtained (see Section 5.3.1 for further details) was used to compute the rates of reaction 3, 6 and 7 during a batch tests with hydroxylamine as substrate. The simulations indicate that hydrazine disproportionation is occurring throughout the batch test, even if at one order of magnitude lower rate than hydroxylamine disproportionation (see Figure 5.21).

Overall, two ammonium production events were observed in the same batch test with only hydroxylamine as substrate (test 2 to 4 in Table 5.1). When hydroxylamine is present, hydroxylamine disproportionation to ammonium and dinitrogen gas via reaction 7 is the dominant process (Table 5.2) with hydrazine disproportionation taking place at one order of magnitude lower rate (see Figure 5.21). Once hydroxylamine is consumed, the accumulated hydrazine is disproportionated to ammonium and dinitrogen gas via reaction 10 (Table 5.2).

AMMONIUM PRODUCED FROM HYDROXYLAMINE AND HYDRAZINE IS USED TO CONSUME NITRITE

To further analyse the ammonium production capacity of anammox bacteria from either hydroxylamine or hydrazine, batch tests 8 and 9 (Table 5.1) were performed. Hydroxylamine and nitrite were dosed simultaneously to assess if the ammonium production capacity from hydroxylamine and hydrazine could support nitrite consumption. As expected, the ammonium produced from hydroxylamine was used to consume nitrite (Figure 5.1C). A slight transient accumulation of hydrazine and ammonium was measured. Nitrite consumption stopped as soon as all the ammonium produced from hydroxylamine and hydrazine was consumed (Figure 5.1C or Figure 5.12F). Furthermore, the ca. 20 mg-N/L hydroxylamine consumed via the disproportionation reaction (reaction 7, Table 5.1), would lead to ca. 6.6 mg-N/L NH_4^+ which could be consumed via normal anammox metabolism. If we assume a stoichiometry close to that reported by Lotti et al. [158] of 1.146 mol NO_2^- mol NH_4^+, a maximum consumption of 7.6 mg-N/L nitrite could be converted, which is close to the experimentally observed of 6.4 mg-N/L of nitrite consumed. These observations, together with the fact that in the rest of tests (except tests 10-12 in Table 5.1, that also had nitrite) ammonium accumulated instead of being consumed, confirmed that the

substrate consumed together with nitrite was ammonium. The transient accumulation of ammonium with nitrite was shown by Hu and coworkers [94], however no hydrazine accumulation was described in those tests. Also the hydroxylamine conversion slowed down with time, maybe due to the higher concentrations used in their tests.

These results further expand the metabolic versatility of anammox bacteria: if hydroxylamine is present with nitrite only (i.e. without NH_4^+), anammox bacteria can generate ammonium from hydroxylamine and consume nitrite via the canonical anammox conversion. These results show a potential role of hydroxylamine in *Ca.* Kuenenia stuttgartinesis metabolism, in contrast to the prior unique implication of hydroxylamine in *Ca.* Brocadia sinica [90]. This situation might occur in partial nitritation/anammox (PN/A) systems when ammonium is fully depleted while residual, low concentrations of hydroxylamine might be still present, e.g. in biofilms due to gradients between microcolonies.

HYDROXYLAMINE IS CONSUMED SIMULTANEOUSLY WITH NITRITE, BUT FASTER

Batch tests were performed to assess the impact of hydroxylamine when both nitrite and ammonium were present (batches 10, 11, 12 in Table 5.1). This substrates combination is likely to occur in PN/A biofilms, although hydroxylamine concentrations might be lower than those used here. Hydroxylamine was consumed simultaneously with nitrite (Figure 5.1D). The specific hydroxylamine consumption rate (qNH_2OH) was 1.6 to 5 times higher than that of nitrite (qNO_2^-), depending on the initial hydroxylamine concentration. As soon as all hydroxylamine was depleted, a 50 % increase of nitrite consumption rate was measured (Table 5.1). Interestingly, even after hydroxylamine depletion, nitrite consumption rates remained lower than the maximal rate measured in positive controls, performed with nitrite and ammonium, usual anammox substrates (batch 1, Table 5.1). Thus, these results suggest that hydroxylamine is consumed simultaneously with nitrite, but faster than nitrite. Hydroxylamine presence together with nitrite and ammonium had a putative toxic or partially irreversible effect on nitrite consumption rates, as when hydroxylamine was fully consumed nitrite consumption rates did not reach the levels of the positive controls.

Within the range of concentrations tested, the hydroxylamine consumption rate linearly depended on the initial hydroxylamine concentration (Figure 5.2A), and was not affected by the combination of available substrates. The linear consumption of hydroxylamine observed is consistent with previously reported measurements with ammonium only [96]. In biological systems, Monod-like kinetics are usually observed for substrates consumption. For Monod kinetics to be linear, the substrate concentration has to be smaller than the half saturation coefficient. Thus, in the present study, the hydroxylamine half saturation constant would then be unexpectedly high for suspended cells (ca. $>$ 22 mg-N-NH_2OH/L, Figure 5.2A), as for example the half saturation constant for nitrite in a similar culture was 35 µg-N/L [158]. Another putative explanation for the observed linearity is the passive transport of hydroxylamine over the membrane. Hydroxylamine has a pKa of 5.9 at 25 °C [159], thus it is mostly unprotonated under the tested conditions (pH 8 and 30 °C in the batch tests). As a result, passive diffusion through the membrane, strictly depending on the difference between the bulk and cell concentration of the substrate, is likely [160].

Ultimately, the presented results further expand the known metabolic versatility of anammox bacteria. Hydroxylamine and hydrazine disproportionation were proven to occur simultaneously and, if available, hydroxylamine was shown to be consumed simultaneously and faster than nitrite as substrate.

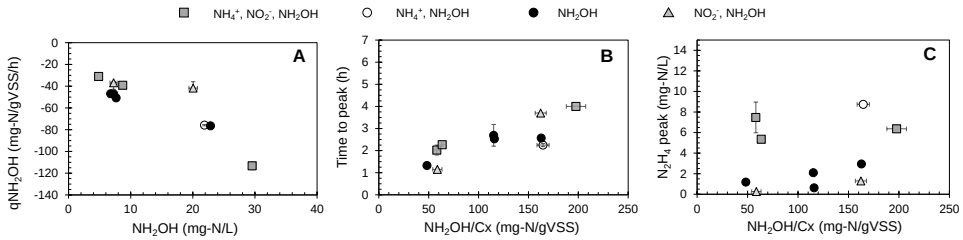


Figure 5.2: Impact of initial NH2OH concentration [NH_2OH (mg-N/L) in A] or specific initial hydroxylamine concentration [NH_2OH/Cx (mg-N/gVSS) in B and C] to: A) Initial specific hydroxylamine rate [qNH_2OH (mg-N/gVSS/L)], B) Time to reach the hydrazine peak [Time to peak (h)] and C) maximum measured hydrazine concentration [N_2H_4 peak (mg-N/L)]. Cx stands for biomass concentration (gVSS/L). Batch tests were performed with different combination of substrates: i) NH_4^+, NO_2^-, NH_2OH (squares), ii) NH_4^+, NH_2OH (empty circles), iii) NH_2OH (filled circles), iv)NO_2^-, NH_2OH (triangles). Linear dependencies between parameters are shown in A and B, independently of the combination of substrates used. Batch tests were ammonium was present (squares and empty circle) had higher N_2H_4 peak than when no ammonium was present (filled circles and triangles). Notice the Y axes are different in each figure and X axis scale is different in Figure 5.3A. Error bars represent the standard deviation between biological duplicates.

HYDROXYLAMINE DISPROPORTIONATION CONTROLS HYDRAZINE ACCUMULATION

To date, hydrazine accumulation has only been reported in the presence of hydroxylamine (e.g. [9, 89, 11]). However, hydrazine is not usually analysed in anammox systems, thus its actual concentration is unknown. To understand the mechanisms underlying hydrazine turnover, hydrazine concentration was measured during all batch tests (Table 5.1). Hydrazine transiently accumulated when hydroxylamine was added as substrate (Batches 2-6 and 8-12 in Table 5.1), and the time of the hydrazine peak depended on the initial specific hydroxylamine concentration (mg-N/gVSS, Figure 5.2B), consistently with van der Star [96].

Hydrazine transient accumulation can be explained with hydrazine disproportionation (reaction 7, Table 5.2). The disproportionation is the addition of two reactions (reactions 3 and 6, Table 5.2). Hydrazine starts accumulating when hydroxylamine concentration is low (ca. <5 mg-N/L in Figure 5.1A). Hydroxylamine concentration affects both hydrazine production (reaction 3, Table 5.2) and hydrazine consumption (reaction 6, Table 5.2), but two moles of hydroxylamine are needed for hydrazine consumption as opposed to one mole needed for its production. Thus, a lower concentration of hydroxylamine decreases the hydrazine consumption rate more than the hydrazine production rate, leading to transient hydrazine accumulation. This was further supported by the mathematical model (Figure 5.21): the hydrazine consumption rate (via reaction 6 in Table 5.2) is always lower than the hydrazine production rate (via reaction 3 in Table 5.2), resulting in hydrazine accumulation.

Originally, the accumulation of hydrazine upon addition of hydroxylamine was

ascribed to the inhibition of HDH, the enzyme responsible for the conversion of hydrazine to dinitrogen gas. This hypothesis was based on the observed in vitro inhibition of HDH by NO and hydroxylamine [161, 11]. However, our results show that HDH inhibition cannot be the explanation of hydrazine accumulation, as hydrazine did not accumulate directly after hydroxylamine addition at high initial hydroxylamine concentrations (ca. 20 mg-N/L Figure 5.1A). Instead, the hydrazine peak occurred after the consumption of more than ca. 15 mg-N/L of hydroxylamine, when hydroxylamine concentration was <5 mg-N/L. This delay was also observed by van der Star et al. [96], who initially proposed that HDH inhibition could not be the cause for hydrazine accumulation. This is contradictory with the results of Maalcke et al. [161] that showed that HDH was inhibited by NO and hydroxylamine in vitro. Similarly, Hu and co-workers [88] did not observe any sign of HDH inhibition when NO was fed continuously to an anammox culture . Overall, the HDH inhibition by hydroxylamine (and NO) demonstrated in vitro [161] was not observed to occur in vivo, meaning another process underlies the hydrazine accumulation. Instead, the imbalance of the hydrazine production rate and consumption rate (reaction 3 and 6 in Table 5.1) during hydroxylamine disproportionation is the proposed cause of hydrazine accumulation.

5

HYDRAZINE ACCUMULATION DEPENDS ON THE AVAILABLE SUBSTRATES

The wide range of combinations of added substrates used in this research showed that hydrazine accumulation is strongly impacted by the used substrate combination (Figure 5.2C). Specifically, the accumulation of hydrazine was higher when ammonium was present, while the presence of nitrite resulted in lower accumulations (Figure 5.2C). Previous research on hydrazine accumulation used only ammonium and hydroxylamine as substrate [96].

This can also be explained by the imbalance of reactions 3 and 6 during hydroxylamine disproportionation. For the hydrazine production during hydroxylamine disproportionation, ammonium is consumed (reaction 3, Table 5.2), thus higher concentration of ammonium would favour hydrazine production. Contrarily, hydrazine consumption results in ammonium production (reaction 6, Table 5.2), thus the presence of ammonium would result in this reaction being less thermodynamically favourable (see next sections).

On the other hand, nitrite decreased the hydrazine peak (Figure 5.2C). When nitrite and hydroxylamine are present as the only substrates, nitrite is consumed with the ammonium that is being produced from either hydroxylamine or hydrazine disproportionation (Figure 5.1C). Thus, nitrite presence decreases the final ammonium concentration, favouring hydrazine consumption (reaction 6, Table 5.2). When both ammonium and nitrite were present together with hydroxylamine (batches 10,11 and 12, Figure 5.1D), ammonium dominated the possible effect of nitrite, leading to high hydrazine accumulations.

Overall, we showed that hydrazine accumulation occurs in the presence of hydroxylamine, and the accumulation is promoted by ammonium and reduced by nitrite. Elucidating the role and occurrence of hydrazine accumulation in biological systems requires further full-scale experimental confirmation as measurements of hydrazine during reactor operation remain rare. Hydrazine accumulation or leakage

by anammox would be unfavourable from an energetic point of view, as hydrazine transformation to N_2 is one of the anammox electron sources (see Equation (5.3)). However, leakage of hydrazine can be a potential advantage for anammox against other direct competitors as hydrazine is toxic to other microbes of the nitrogen cycle, such as NOB [162]. Further discussion on the thermodynamics of hydrazine accumulation and the putative enzymes involved can be found in follow-up sections.

THERMODYNAMICS CANNOT EXPLAIN HYDRAZINE ACCUMULATION

To identify any possible thermodynamic limitation underlying the observed hydrazine accumulation, a thermodynamic analysis of the reactions involved (Table 5.2) was performed along concentration profiles during batch tests (Figure 5.13 and Figure 5.14).

All reactions involved in hydrazine and hydroxylamine disproportionation were thermodynamically favourable, as they had a negative Gibbs energy change of reaction (ΔG_R°), with the exception of the half reaction of ammonium conversion to hydrazine (reaction 2, Table 5.2). Even after the correction for actual batch conditions, hydrazine production from ammonium had a positive actual Gibbs energy change of reaction (ΔG_R^1) (Figure 5.3A). Even if one of the half reactions was not thermodynamically favourable (reaction 2), the overall hydrazine production (reaction 3) had negative actual Gibbs free energy. However, the hydrazine production step (reaction 3; Table 5.2), was close to the equilibrium, and batch conditions strongly impacted its overall ΔG_R^1 (Figure 5.3B). Thus, hydrazine production thermodynamics heavily depend on batch test conditions (Figure 5.3A and B). Conversely, the reactions involved in hydrazine consumption were strongly favourable (Figure 5.3C). Consequently, the thermodynamic analysis does not explain the observed accumulation of hydrazine. A kinetic limitation or and enzymatic/biological bottleneck impacting hydrazine consumption might be the explanation, as also discussed in the previous section and as shown by the mathematical model (Figure 5.21).

Impact of batch conditions, namely pH, temperature, ammonium and hydroxylamine concentration, on the potential hydrazine conversions were also investigated (Figure 5.16). Higher hydroxylamine and ammonium concentration (Figure 5.16A and B) resulted in a more favourable hydrazine production, in agreement with the experimental results (Figure 5.2C). Higher pH was also shown to make hydrazine production more thermodynamically favourable (Figure 5.16E), whereas temperature had little impact over the tested range (Figure 5.16D). In this perspective, it is worth noting that the anammoxosome - where hydrazine is being produced - is more acidic than the cytoplasm [163, 164], thus making hydrazine production less thermodynamically favourable.

THERMODYNAMICS SUGGEST THAT NO IS AN UNLIKELY INTERMEDIATE IN HYDROXYLAMINE TURNOVER

The feasibility and potential occurrence of NO as intermediate in the reactions involving hydroxylamine (reactions 12-17; Table 5.2, Figure 5.15) was analysed based on thermodynamic characteristics of the conversions. Hydroxylamine is hypothesized to be first transformed to NO, and then NO is transformed to the final product. The estimated ΔG_R° for the transformation of NO to hydrazine during batch tests was close to equilibrium, i.e. could potentially become unfavourable (positive) depending on the

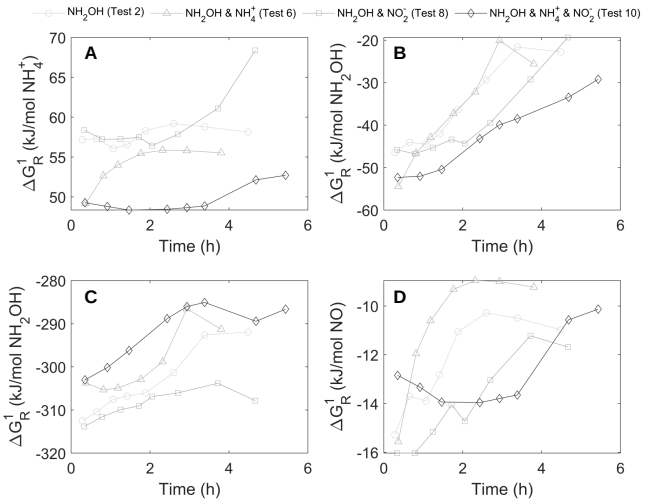


Figure 5.3: Thermodynamics of selected reactions during different batch tests (Test 2, 6, 8 and 10 of Table 5.1) A) Ammonium to hydrazine conversion electron donor (reaction 2 in Table 5.2) is the only thermodynamically positive conversion, B) Hydrazine production in hydroxylamine disproportionation (reaction 3 in Table 5.2), C) Hydrazine consumption in hydroxylamine disproportionation (reaction 6 in Table 5.2), D) NO conversion to hydrazine (reaction 12 in Table 5.2).

conditions (Figure 5.3D; reaction 12, Table 5.2). As a result, considering the previously discussed close to equilibrium reaction 3 (i.e. hydroxylamine to NO; Table 5.2), the transformation of hydroxylamine via NO would have two intermediate steps close to thermodynamic equilibrium. From a thermodynamic point of view the forward conversion is possible, but highly depending on the exact batch test conditions. The experimental evidence on the potential role of NO as intermediate in the hydroxylamine conversion would still be needed.

Ultimately, it should also be noted that a better characterization of hydroxylamine standard Gibbs energy of formation (G°_{f,NH_2OH}) value is needed for a definitive thermodynamic study. In literature, different values for G°_{f} of hydroxylamine can be found (Table 5.4), which profoundly impact the obtained results (Figure 5.17).

HYDROXYLAMINE AND HYDRAZINE DISPROPORTIONATION CAN BE EXPLAINED BY KNOWN ENZYMATIC ANAMMOX CONVERSIONS

We hypothesize that disproportionation of hydroxylamine and hydrazine might be catalysed by multiple enzymes, most of which are already characterized in anammox bacteria. For instance, hydrazine production (reaction 3 in Table 5.2) in hydroxylamine disproportionation could be catalysed by different combination of enzymes, either with NO as intermediate or not, in hydroxylamine conversions: i) NH_2OH is first transformed to NO (reaction 12, 13 and overall 14) by HOX. Then, NO and NH_4^+ could be reduced to hydrazine (reaction 2) by HZS (see Figure 5.4). ii) Alternatively, anammox bacteria encode more than 10 anammox HAO-like proteins in the genome, the function of some

of them is still unknown. Thus, it could be that one of the HAO-like proteins in *Ca.* Kuenenia could perform a similar conversion as the HZS in *Ca.* Brocadia [90]. Thus, the Brocadia-like HZS transforms directly hydroxylamine and ammonium to hydrazine, and HDH funnels hydrazine into dinitrogen gas (reaction 1, 2 leading to the overall reaction 3) (see Figure 5.4).

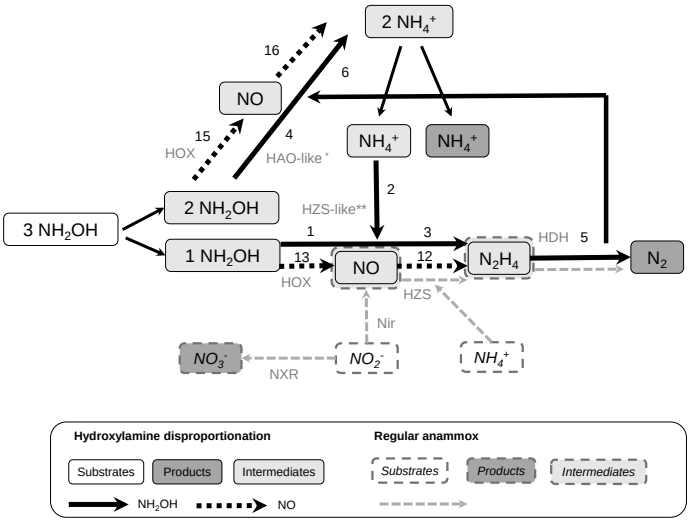


Figure 5.4: Putative enzymes involved in hydroxylamine disproportionation conversions depending on the assumed intermediates: Black lines are conversion where hydroxylamine is directly transformed to hydrazine or ammonium, black dashed lines are reactions where NO might be an intermediate. Numbers correspond to reactions in Table 5.2, where corresponding Gibbs free energy values can be found. Regular anammox metabolism is represented with grey dashed boxes and arrows. Notice that NO and N_2H_4 have both solid and dashed line boxes. ∗Anammox genome encodes more than 10 HAO-like proteins, which the function of some of them is still unknown, ∗∗HZS has been proposed to have hydroxylamine as inner intermediate [93], or in *Ca.* Brocadia it has been shown to transform hydroxylamine and ammonium to hydrazine [92, 90]. Thus, we hypothesise that there is an HZS-like protein able to transform ammonium and hydroxylamine to dinitrogen gas from the more than 10 HAO-like proteins encoded in anammox genome.

Hydrazine consumption (reaction 6) in hydroxylamine disproportionation could be a combination of a HAO-like protein reducing NH_2OH to ammonium (reaction 4) and the known activity of HDH (reaction 5) (see Figure 5.4).

Hydrazine disproportionation (reaction 10) would need HDH (reaction 9) and a dedicated enzyme to produce ammonium from hydrazine (reaction 8). An enzyme catalysing this last conversion, has not been described yet in anammox.

From the overall enzymatic conversions proposed, ammonium production from hydroxylamine and hydrazine have not been shown in anammox bacteria. However, ammonium producing activity from hydroxylamine has been hypothesized to exist based on metagenomic data from the anammox bacterium *Ca.* Scalindua profunda [165]. Furthermore, hydroxylamine conversion to ammonium has been shown in ammonium oxidizing bacteria *Nitrosomonas* [166] and enzymatic activity found in the dissimilatory nitrate reducing bacteria *Nautilia profundicola* [106], it is hypothesized

that there is an HAO-like enzyme with an ammonium producing activity from hydroxylamine. Nevertheless, to further confirm this hypothesis, transcriptomics and proteomics data would be valuable.

KINETIC MODELLING SUPPORTS THE POTENTIAL IMPACT OF HYDROXYLAMINE ON NITRITE METABOLISM

The estimation of the kinetic parameters for the discussed reactions is the prerequisite for their inclusion in mathematical models [167]. Experimental parameter determination requires highly precise measurements methods, and usually kinetic models are applied [168]. The fact that hydroxylamine is consumed via two simultaneous reactions (reactions 3 and 6, Table 5.2), and the need of both of them to describe hydrazine accumulation, makes the experimental determination of parameters impossible. Instead, a step-wise modelling approach using different optimization functions was used to assess if the available data set allows for parameter determination (see SI). To this end, the kinetic model proposed by van der Star [96] was adapted to take into account the impact of ammonium (Table 5.6). The anammox stoichiometry was also introduced in the set of reactions to model the impact of hydroxylamine addition with ammonium and nitrite (Table 5.6).

Two independent optimizations were performed to determine the kinetic parameters of hydrazine consumption (k_3 and K_{3,N_2H_4}) and anammox (k_4, K_{4,NO_2^-}, and K_{4,NH_4^+}). Independently of the used optimization function, a single set of parameters was obtained (Figure 5.18A and D, and Figure 5.19A and D). However, when hydroxylamine was the only substrate, the values obtained for the remaining kinetic parameters (k_1, K_{1,NH_2OH}, K_{1,NH_4^+}, k_2, K_{2,NH_2OH}, K_{2,N_2H_4}) depended on the objective function used (Figure 5.18B and Figure 5.19B). The set of parameter values (k1, K_{1,NH_2OH}, K_{1,NH_4^+}, k_2, K_{2,NH_2OH}, K_{2,N_2H_4}) resulting in the lowest sum of squared errors was selected (Table 5.3), and used in subsequent optimizations.

Next, parameters obtained with control (k_4,K_{4,NO_2^-}, and K_{4,NH_4^+}), hydrazine (k_3 and K_{3,N_2H_4}) and hydroxylamine tests (k_1, K_{1,NH_2OH}, K_{1,NH_4^+}, k_2, K_{2,NH_2OH}, K_{2,N_2H_4}) were used to simulate tests where ammonium, nitrite and hydroxylamine were simultaneously provided. Without any extra optimization step, the parameters previously obtained were not able to describe the experimental data. Specifically, the depletion of nitrite was predicted to be faster by the model (Figure 5.20A), suggesting a direct impact of hydroxylamine on the regular anammox metabolism of nitrite consumption. Consequently, parameters affecting the nitrite consumption (k_4, K_{4,NO_2^-}, and K_{4,NH_4^+}) were optimized using the experimental data when hydroxylamine was present, while the other parameters were kept constant (Figure 5.18C, Figure 5.19C and Figure 5.20B). The optimization resulted in ca. a 50% decrease in the specific maximum anammox rate constant (k4), and 50% increase in the nitrite half saturation coefficient (K_{4,NO_2^-}). Consistently with the experimental results, these observations further support the strong impact of hydroxylamine on the nitrite consumption by anammox bacteria.

In literature, the only available set of parameters for the reactions involving hydroxylamine [96] estimated a maximum rate constant (k) one order of magnitude smaller than the ones reported in the present study (Table 5.3). This could be explained by the use of granular biomass [96] instead of planktonic culture as done here. Part of the

Table 5.3: Kinetic parameters determined with the kinetic model. When standard deviation is given, average between the results obtained with different objective functions was performed. When no standard deviation is given, the value selected is the one that resulted in a smaller error between the model and experimental data.

Substrates	k1 (mmol/gVSS/h)	K_{1,NH_2OH} (mM)	K_{1,NH_4^+} (mM)	k_2 (mmol/gVSS/h)	K_{2,NH_2OH} (mM)	K_{2,N_2H_4} (mM)	k_3 (mmol/gVSS/h)	K_{3,N_2H_4} (mM)	k_4 (mmol/gVSS/h)	K_{4,NH_4^+} (mM)	K_{4,NO_2^-} (mM)
N_2H_4							0.285±0.007	0.030±0.003			
NH_2OH	2.8	0.16	0.057	2.8	0.0027	0.56					
$NO_2^- + NH_4^+$									4.5±0.1	0.78±0.01	0.03±0.04
$NO_2^- + NH_4^+ + NH_2OH$									2.2	1.6	0.004

biomass in the granules could be inactive leading to an apparent (slower) rate. Moreover, differences in the average growth rates between the two systems could also contribute to the differences in maximum rate constants.

5.3.2. LONG TERM CONTINUOUS EXPOSURE TO HYDROXYLAMINE REDUCES THE NO_3^- PRODUCTION

The long term effects of continuous exposure of anammox bacteria to hydroxylamine had not been studied yet. To this end, a planktonic culture of *Ca.* Kuenenia stuttgartiensis was operated in continuous mode for more than 54 days (Figure 5.5A, Figure 5.22 and Table 5.7). After the initial 20 days of stabilization, two operational phases can be distinguished: i) Phase I with ammonium and nitrite (days 20-37, Table 5.7), ii) Phase II with ammonium, nitrite and hydroxylamine (days 38-54, Table 5.7). To mimic the expected low hydroxylamine accumulation by AOB in PN/A processes, a small hydroxylamine load (ca. 26 mg-N/L/d) compared to the nitrite load (ca. 478 mg-N/L/d; Table 5.7) was chosen.

The addition of hydroxylamine did not impact the microbial community composition as revealed by 16S rRNA amplicon sequencing (Figure 5.5B). The relative abundance of *Ca.* Kuenenia stuttgartiensis remained stable at 79±4 % during 2 complete SRTs with hydroxylamine feeding. The genus *Ignavibacterium* represented the most abundant side population during the whole operation. These results indicate that simultaneous consumption of hydroxylamine, ammonium and nitrite does not impact anammox bacteria, and might even represent a competitive advantage in biofilm PN/A systems against canonical NOB, often reported to be inhibited by hydroxylamine [102, 169, 100, 101, 170, 47].

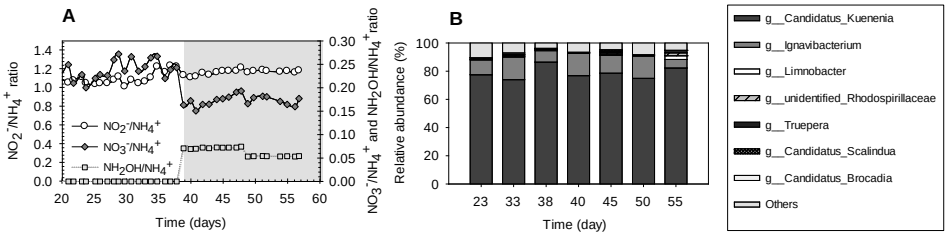


Figure 5.5: Reactor operation dynamics: A) Stochiometric ratios during reactor operation without (white background) and with hydroxylamine load (grey area). B) Microbial relative abundance based on 16S rRNA amplicon sequencing of reactor samples at different operational days.

During the whole experiment, hydrazine and hydroxylamine concentrations

remained below detection limits (Figure 5.22C). As soon as hydroxylamine was fed, a statistically significant decrease ($P \leq 0.001$, Mann-Whitney Rank Sum Test) in the nitrate production to ammonium consumption ratio was observed, from 0.24 ± 0.03 (Phase I) to 0.17±0.01 mol-NO_3^-/mol-NH_4^+ (Phase II) (Table 5.7 and Figure 5.5A). Thus, hydroxylamine reduced the formation of nitrate in the anammox conversion. It is noteworthy that the response was immediate without any visible adaptation effect. Based on the anammox biochemistry presented in this study, hydroxylamine can be metabolized via two pathways: i) NH_2OH is converted to NO and then further to N_2 via the conventional anammox metabolism in Equations (5.1) to (5.3), or ii) NH_2OH is transformed via hydroxylamine disproportionation reactions, forming NH_4^+ and N_2 as in the batch tests (reaction 7, Table 5.2). From nitrogen mass balances only, the hydroxylamine pathway could not be resolved. Given the low hydroxylamine load compared to nitrite, hydroxylamine disproportionation would have resulted in ca. 8 mg-N/L/d of extra ammonium. Such low concentrations would be masked by the high residual ammonium concentration in the reactor. Nevertheless, in a study from van De Graaf and colleagues [11] the addition of $^{15}NH_2OH$ together with unlabelled ammonium and nitrite lead to $^{30}N_2$ production, most likely resulting from the reaction of $^{15}NH_4^+$ - produced from hydroxylamine - and $^{15}NH_2OH$ itself. Thus, based on the latter study and the batch tests performed here, hydroxylamine disproportionation is likely to be the dominant pathway.

Recently, anammox bacteria were also shown to grow on only NO and ammonium, with no nitrate production [88]. These results challenged the common assumption that nitrite oxidation to nitrate is needed to provide the electrons to reduce CO_2 for biomass synthesis. Instead, the new hypothesis proposed that the high energy electrons produced during hydrazine conversion to N_2 are more likely those used in anabolism [88]. Similarly, in the present study a clear decrease in nitrate production was observed with continuous hydroxylamine feeding (Figure 5.5A). Independent of the pathway, hydroxylamine conversion releases extra high energy content electrons reducing the need for nitrite oxidation to nitrate.

This study further extends our knowledge of the metabolic versatility of anammox bacteria, demonstrating the simultaneous consumption of ammonium, nitrite and hydroxylamine by anammox bacteria. Furthermore, the reactions involved in hydroxylamine metabolism were further characterized using batch tests. This characterization resulted in the following findings: (i) ammonium can be produced from either hydroxylamine or hydrazine; and (ii) the co-metabolization of other substrates impacts hydrazine accumulation. In addition, hydrazine accumulation was analysed from a kinetics and thermodynamics point of view, further confirming that hydrazine accumulation is governed by the reactions involved in hydroxylamine disproportionation, rather than inhibition of hydrazine dehydrogenase.

Overall, this work highlights the huge metabolic versatility of anammox bacteria. This unique ability to use a broad range of substrates represents a clear competitive advantage likely underlying the ability of anammox to thrive in different environments. For example, hydroxylamine is known to be toxic for NOB, directly competing with anammox for nitrite [100, 19, 20]. From an engineering point of view, this kind of competitive advantage can be useful in systems like partial nitritation anammox, where

NOB are not desired. For example, hydroxylamine external addition has been tested to obtain a successful partial nitritation anammox process in laboratory conditions [47].

Finally, the present work sets the basis to further understand hydroxylamine metabolism by anammox bacteria. Next steps could be directed to further confirm the pathway followed by hydroxylamine in continuous operation with either ^{15}N labelling or increased loads. Doing similar experiments with other anammox bacteria species (i.e. *Ca.* Brocadia) would also help to assess putative differences between anammox species metabolism. Comparative transcriptomic or proteomic data would provide more information in the enzymes involved in the process. Overall, intermediates of the nitrogen cycle are overlooked and not studied in depth, and could be a source for not recognized conversions or interactions in microbial communities. Understanding such interactions is crucial for improving its implementation as technology.

5.4. CONCLUSIONS

THE combination of batch tests, continuous feeding, thermodynamics analysis and modelling allowed to elucidate more details about the hydroxylamine metabolism of anammox bacteria,

- Ammonium can be produced from both hydroxylamine and hydrazine. If only nitrite and hydroxylamine are available, ammonium can be produced from hydroxylamine and used for nitrite consumption.
- When hydroxylamine, ammonium and nitrite are present together in anammox batch tests, hydroxylamine and nitrite are consumed simultaneously, with hydroxylamine consumption being faster than nitrite consumption.
- Hydrazine accumulation only occurs when hydroxylamine is present and seems to be due to a biological imbalance rather than a thermodynamic limitation or enzymatic inhibition. The extent of hydrazine accumulation depends on the combination of substrates provided, i.e. promoted by ammonium and reduced by nitrite.
- Anammox bacteria can grow simultaneously with ammonium, nitrite and hydroxylamine, reducing the nitrate production.
- Anammox microbial population is not impacted by long term feeding of hydroxylamine.
- Hydroxylamine, although not a central intermediate in *Ca.* Kuenenia stuttgartiensis, might play an important role in the its metabolism if present in the environment.

5.5. SUPPLEMENTARY INFORMATION

Supplementary information can be also found online [97].

5.5.1. MATERIALS AND METHODS

SHORT TERM STUDY - BATCH TESTS

Biomass origin

A highly-enriched planktonic culture of *Ca.* Kuenenia stuttgartiensis (79 ± 4 % as estimated by 16S rRNA gene-based amplicon sequencing analysis) was used as inoculum. The culture originated from a 10 L (working volume) membrane bioreactor (MBR; pH 7, 30°C) operating at steady state with an average loading rate of ca. 380 mg-N-NO_2^-/L/d, and sparged with a gas mixture of 95-5% N_2-CO_2. Hydraulic retention time (HRT) was set to 3.0±0.5 days and solid retention time (SRT) was set to 10±1 days during the whole experimental period. Mineral medium fed to the reactor contained per liter of demineralized water: 0.91g K_2HPO_4, 0.13g KH_2PO_4, 5.0g $(NH_4)_2SO_4$, 5.2g $NaNO_2$, 1.6 mL of a trace element solution, 3.1mL of an iron solution, 0.8 mL of a magnesium solution (160g/L of $MgSO_4 \cdot 7H_2O$), 0.4 mL of a calcium solution (240 g/L of $CaCl_2 \cdot H_2O$). The trace elements solution contained per litre of mili-Q water: 19.11 g EDTA (=titriph..), 0.43 g $ZnSO_4 \cdot 7H_2O$, 0.24 g $CoCl_2 \cdot 6H_2O$, 1.0 g $MnCl_2 \cdot 4H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 0.22 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (=1.25 mM Mo), 0.20 g $NiCl_2 \cdot 6H_2O$, 0.09 g $NaSeO_3$, 0.014 g H_3BO_3, 0.054 g $Na_2WO_4 \cdot 2H_2O$. The solution was adjust to pH 6 with solid NaOH and kept in the fridge. The iron solution contained per L of mili-Q water 9.14 g $FeSO_4 \cdot 7H_2O$ and 6.37 g EDTA.

Calculations

Biomass concentration in the MBR (in gVSS/L) was correlated to OD_{660} by means of a dilution series (Figure 5.6). This correlation was used to estimate the biomass concentration in each batch test. The volumetric nitrogen conversion rates during batch tests were determined by fitting a regression line to the measured concentrations of the desired nitrogen compound in each duplicate. The average and standard deviation of the volumetric conversion rates were obtained from duplicates. The biomass specific conversion rate was subsequently calculated by dividing the volumetric conversion rate by the biomass concentration. Error propagation was used to obtain the error of the specific rate.

Physicochemical measurements

Ammonium, nitrite, and nitrate were measured using the ThermoFisher Scientific Gallery Automated Photometric Analyzer (Thermos Fisher Scientific, Waltham, USA). The nitrate measuring method available was measuring TON (Total Oxidized Nitrogen) and subtracting the measured nitrite. Due to interference of hydroxylamine on the nitrate determination when using this method, nitrate was measured when required with a commercially available Hach Lange kit.

Hydroxylamine was measured spectrophotometrically according to Frear and Burrell [117] and pre-treatment of the samples with sulfamic acid solution 0.1g/L was used to avoid reaction of hydroxylamine with nitrite as described [58]. Accordingly, 1 mL of

0.05M phosphate buffer, 1 mL of sample (diluted, if applicable), 0.8 mL of demineralised water, 0.2 mL of a 12wt% tricholoroacetic acid solution, and 1 mL of a 1% 8-quinolinol solution in ethanol were added to a 15x125 mm Pyrex tube. After mixing, 1 mL of a 1M Na_2CO_3 solution was also added. This was again shaken vigorously, before placing the tubes in a water bath (100°C) for 1 minute. After 15 minutes cooling at RT, the absorbance of the solution was measured in 1 cm cuvettes at 705 nm with the Novaspec III Amersham Biosciences. A blank for the measurement was prepared by substituting the 1 mL sample with demineralised water. The effect of sulfamic acid on the absorption was determined to be under the detection limit.

Hydrazine was measured spectrophotometrically (range 3-30µM) following the procedure of Watt and Crisp (Watt and Chrisp 1952) . Briefly, to 750 µL of sample and 200 µL of sulfamic acid solution (diluted, if applicable), 105.6 µL of a reagent solution was added (0.4 g *para*-dimethylaminobenzaldehyde dissolved in 20 mL ethanol and 2 mL concentrated hydrochloric acid). After colour development of at least 15 minutes, the absorbance of the solution was measured in 1 cm cuvettes at 458 nm with the Novaspec III Amersham Biosciences. A blank for the measurement was prepared by substituting the sample for demineralised water. Nitrite interfered the measurement (Figure 5.6), thus sulfamic acid (0.1g/L) was added to the samples. Since the used hydrazine calibration did not take into account the addition of sulfamic acid, the absorption of sulfamic acid (diluted, if applicable) was subtracted from the measured absorbance of the samples.

Dry weight (TSS) and volatile suspended solids (VSS) were determined according to standard methods [171].

Biomass correlation with OD660nm

25mL aliquots of cell suspension from the 10L MBR reactor were used to perform the correlation. Dilutions of the cell suspension with demineralized water were used to decrease the optical density of the culture. Absorbance was measured to determine the optical density of the cell suspension (at a wavelength of 660nm, OD660nm), before preforming the dry weight and volatile suspended solids determination.

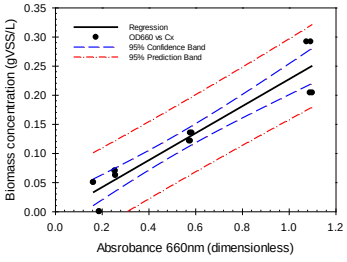


Figure 5.6: Calibration curve used for the batch test to find the correlation between the biomass concentration and the absorbance (at a wavelength of 660 nm). Linear regression resulted in y=0.232x-0.005 with an r^2 = 0.899. Where x refers to absorbance at 660nm and y biomass concentration in gVSS/L. The slope had a standard error of 0.021 gVSS/L/absorbance and the intercept and error of 0.013 gVSS/L.

Nitrite interference on hydrazine measurement

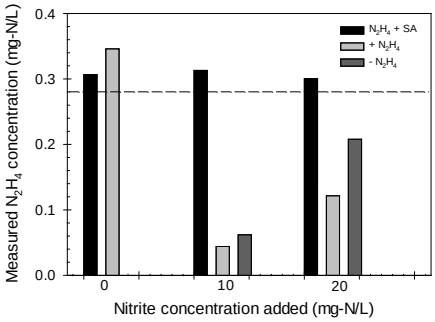


Figure 5.7: Effect of nitrite to measured hydrazine concentrations targeted to be 0.28 mg-N/L. Black bars represent hydrazine with sulfamic acid, light grey hydrazine and dark grey without hydrazine.

5

Thermodynamics analysis of batch tests

Example of half reaction derivation

Electron donor and electron acceptor half reactions are defined by first determining the oxidized form of the electron donor half reaction (D) and the reduced form of the electron acceptor half reaction (A). For example, for the hydrazine production reaction (reaction 3) in hydroxylamine disproportionation (see below), hydroxylamine is defined as electron acceptor and ammonium as electron donor.

$NH_4^+ + NH_2OH \longrightarrow N_2H_4 + H_2O + H^+$

First, the elemental balance of the central atom, in this case nitrogen is closed, using hydrazine as product:

(A) $NH_2OH \longrightarrow 0.5\,N_2H_4$

(D) $NH_4^+ \longrightarrow 0.5\,N_2\,H_4$

Then, the oxygen balance is closed using water. In the case of the donor reaction, no oxygen is involved:

(A) $NH_2OH \longrightarrow 0.5\,N_2H_4 + H_2\,O$

(D) $NH_4^+ \longrightarrow 0.5\,N_2H_4$

Next, the hydrogen balance is closed by adding protons:

(A) $NH_2OH + H^+ \longrightarrow 0.5\,N_2H_4 + H_2\,O$

(D) $NH_4^+ \longrightarrow 0.5\,N_2H_4 + 2\,H^+$

Finally, the charge balance is closed using electrons:

(A) $NH_2OH + H^+ + e^- \longrightarrow 0.5\,N_2H_4 + H_2O$

(D) $NH_4^+ \longrightarrow 0.5\,N_2H_4 + 2\,H^+ + e^-$

The hydrazine production reaction can be obtained by balancing the electrons of the donor and acceptor reaction.

General thermodynamics calculations

Standard (1 M, 1 atm) Gibbs free energy change of a reaction (ΔG°_{R}) can be calculated from the standard Gibbs energy of formation ($G^{\circ}_{f,i}$) of the substrates and products of the reaction as in Equation (5.4). Values used of for each compound can be found in Table 5.4. For hydroxylamine the G°_{f} value used was -23.4kJ/mol, if not stated otherwise. Correction by the actual environmental activities for an arbitrary reaction as in Equation (5.5) can be applied using Equation (5.6) to calculate the actual Gibbs free energy change of the reaction (ΔG^{1}_{R}) in kJ/mol. Where R is the gas constant in $8.31{\cdot}10^{-3}$ kJ $K^{-1}mol^{-1}$, T the temperature in Kelvin degrees, *a* refers to the activity of the indicated compound of a reaction R (Equation (5.5)) (with S_1, S_2, P_1 or P_2, where S refer to substrates and P the products in mol/L) and Y^{R} refers to the stochiometric coefficient of the corresponding compound S or P [157].

$$\Delta G^{0}_{R} = \sum_{i=1}^{n} Y^{R}_{i} \cdot G^{\circ}_{f,i} \tag{5.4}$$

$$S_1 + S_2 \rightarrow P_1 + P_2 \tag{5.5}$$

$$\Delta G^{1}_{R} = \Delta G^{\circ}_{R} + RT\,ln\frac{a^{Y^{R}_{P1}}_{P1} \cdot a^{Y^{R}_{P2}}_{P2}}{a^{Y^{R}_{S1}}_{S1} \cdot a^{Y^{R}_{S2}}_{S2}} \tag{5.6}$$

Table 5.4: Standard Gibbs energy of formation (G°_{f}) of the compounds.

Compound	Chemical formula	G°_{f}(kJ/mol)	Reference
Hydroxylamine	NH_2OH	-23.4, -43, 221	[96, 172, 63]
Nitric oxide	NO	86.6	[173]
Hydrazine	N_2H_4	127.8	[96]
Ammonium	NH_4^+	-79.4	[173]
Dinitrogen gas	N_2	0	[173])
Water	H_2O	-237.2	[173]
Proton	H^+	0	([173]
Electron	e^-	0	[173]

Approach and assumptions

The actual Gibbs energy change of each reaction in Table 5.2 was calculated over time and corrected with the measured concentration of each compound during the batch test. Batch conditions were set to a temperature of 30°C and pH 8. N_2 dissolved concentration was assumed equal to the difference of total nitrogen in the start of the batch test and the total nitrogen measured at each time point. Thus, it was assumed that all the missing nitrogen was being converted to dinitrogen and was kept in the liquid

phase. This was assumed for simplification and it was checked than 10 times more or less N_2 concentration in the liquid phase was not impacting the overall conclusions. NO concentration was set to $1{\cdot}10^{-12}$ M, assuming that as intermediate the actual concentrations is low and water concentration was set to 1M.

Model for kinetic parameter estimation

Stoichiometric matrix and reaction rates

Table 5.5: Stoichiometric matrix. Anammox stoichiometry was selected based on the experimental results of Table 5.7. Nitrate was not included in the model except for the anammox controls.

Process (j)	Compound (i)	NH_2OH	N_2H_4	NH_4^+	N_2	NO_2^-	NO_3^-
1	Hydrazine formation	-1	1	-1			
2	Hydrazine oxidation	-2	-1	2	1		
3	Hydrazine disproportionation		-1	4/3	1/3		
4	Anammox activity			-1	0.986	-1.1	0.24
	Units	mM	mM	mM	mM	mM	mM

Table 5.6: Process rates

j	Process	Process rate	Reference
1	Hydrazine formation	$k_1 \cdot \frac{C_{NH_2OH}}{C_{NH_2OH}+K_{1,NH_2OH}} \cdot \frac{C_{NH_4^+}}{C_{NH4}+K_{1,NH_4^+}} \cdot C_x$	Adapted from [96]
2	Hydrazine oxidation	$k_2 \cdot \frac{C_{NH_2OH}}{C_{NH_2OH}+K_{2,NH_2OH}} \cdot \frac{C_{N_2H_4}}{C_{N2H4}+K_{2,N_2H_4}} \cdot C_x$	[96]
3	Hydrazine disproportionation	$k_3 \cdot \frac{C_{N_2H_4}}{C_{N_2H_4}+K_{3,N_2H_4}} \cdot C_x$	[96]
4	Anammox activity	$k_4 \cdot \frac{C_{NO_2^-}}{C_{NO_2^-}+K_{4,NO_2^-}} \cdot \frac{C_{NH_4^+}}{C_{NH_4^+}+K_{4,NH_4^+}} \cdot C_x$	Adapted from [174]

Model approach

Kinetic parameters were obtained sequentially from less to more complex (in terms of externally provided substrates) batch tests. Different objective functions were tested for each set of optimization procedures to determine if the set of parameters obtained (the solution) was independent of the objective function used. The model was implemented in Matlab R2018b to optimize the parameters selected by minimizing the sum of the square errors between the experimental data and the model (or a variant of this method, see below Equation (5.7) to Equation (5.10)). To assess if the parameters were independent of the objective function, four different objective functions were used to minimize the error. If the parameters were different depending on the objective function used, Equation (5.7) was used with the resulting modelled data and experimental data to assess and select the parameters leading to less error (see Figure 5.8).

$$\sum_{k=1}^{l}\sum_{j=1}^{m}\sum_{i=1}^{n}\left(C_{k,j,i,experimental}-C_{k,j,i,model}\right)^2 \tag{5.7}$$

$$\sum_{k=1}^{l}\sum_{j=1}^{m}\sum_{i=1}^{n}\frac{(C_{k,j,i,experimental}-C_{k,j,i,model})^2}{\bar{C}_{k,j,experimental}} \tag{5.8}$$

$$\sum_{k=1}^{l}\sum_{j=1}^{m}\sum_{i=1}^{n}\frac{(C_{k,j,i,experimental}-C_{k,j,i,model})^2}{\max(C_{k,j,experimental})} \tag{5.9}$$

$$\sum_{k=1}^{l}\frac{1}{\max(C_{k,j,experimental})}\sum_{j=1}^{m}\sum_{i=1}^{n}(C_{k,j,i,experimental}-C_{k,j,n,model})^2 \tag{5.10}$$

Where C refers to the nitrogen compound concentration (mg-N/L) either determined experimentally or with the model. Subscripts l and k refer to the number of nitrogen compounds, m and j to number of tests used in each optimization and n and i to the number of data points of each compound. Initial values used for the optimization were 3 mM/gVSS/h, 0.01 mM, 0.01 mM, 1 mM/gVSS/h, 0.01 mM, 0.1 mM, 0.01 mM/gVSS/h, 3mM, 3mM/gVSS/h, 1 mM, 0.01 mM for k_1, K_{1,NH_2OH}, K_{1,NH_4^+}, k_2, K_{2,NH_2OH}, K_{2,N_2H_4}, k_3, K_{3,N_2H_4}, k_4, K_{4,NO_2^-}, and K_{4,NH_4^+}, respectively. For those parameters that were independent of the objective function used, selection of the initial value did not impact the final solution.

The approach to obtain the parameter sets is summarized in Figure 5.8 and explained in the following paragraphs:

1. Batch tests with N_2H_4 as substrate (Test 7 in Table 5.1) were used to obtain k_3 and K_{3,N_2H_4}.

2. Anammox positive controls with NO_2^- and NH_4^+ as substrates (Test 1 in Table 5.1)) were used to obtain parameters k_4, K_{4,NO_2^-}, K_{4,NH_4^+}, of reaction 4

3. Batch tests with NH_2OH as substrate (Tests 2 to 5 in Table 5.1)) were used to obtain parameters k_1, K_{1,NH_2OH}, K_{1,NH_4^+}, k_2, K_{2,NH_2OH}, K_{2,N_2H_4} from reactions 1 and 2 in Table 5.6. Parameters from previous optimization (point 1)) were used for reaction 3. As resulting parameters from the optimization depended on the objective function used (see Figure 5.18B), all parameters set were used and tested in further optimizations.

4. Parameters obtained previously (see points 1, 2 and 3) were used in tests with NO_2^- , NH_4^+ and NH_2OH as substrate (Test 10 to 12 in Table 5.1)) without any optimization. As nitrite consumption was not being able to be described with the current parameters (Figure 5.20A), the impact of hydroxylamine on parameters of reaction 4 was investigated. Batch tests were split in two (Figure 5.20) and different approaches were followed depending on the presence or absence of hydroxylamine:

    - Hydroxylamine present - data points where hydroxylamine was still present were used to optimize k_4, K_{4,NO_2^-}, K_{4,NH_4^+}, of reaction 4, with the rest of parameters already obtained from points a and b (see results in Table 5.3)).

Different sets obtained in point c for constant of reactions 1 and 2 were tested, and the combination of parameters leading to less sum of squared error was presented in Table 5.3 and used for Figure 5.17C, Figure 5.18C, and Figure 5.19B.

- Hydroxylamine absent - no optimization was performed and parameters from points a to c were used.

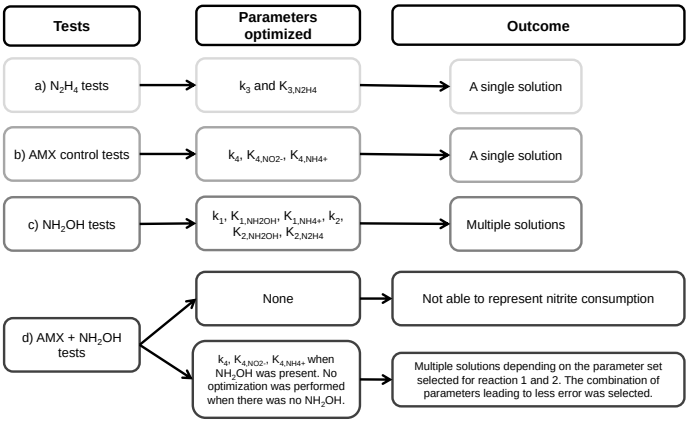


Figure 5.8: Graphical summary of the procedure used during the step wise optimization to obtain the values of the reactions parameters.

LONG TERM STUDY - CONTINUOUS HYDROXYLAMINE ADDITION

Reactor configuration

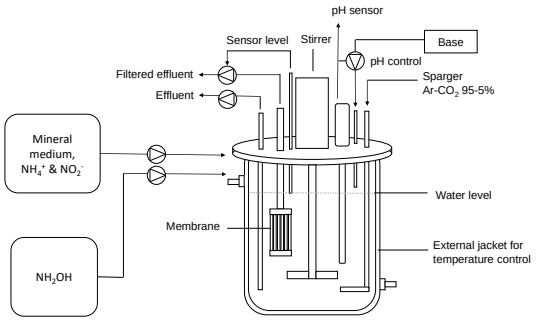


Figure 5.9: Reactor configuration. Medium was continuously fed, the reactor volume was kept constant by means of a sensor level that activates a pump, which filters the reactor content through a membrane. Solid retention time was set with a second pump that withdraws part of the reactor content.

Calculations

Mass balances during continuous operation in the 1L MBR system for any soluble nitrogen compound can be derived as in Equation (5.11).

$$V_r \frac{dC_N}{dt} = Q_1 \cdot (C_N^{out} - C_N^{in}) + Q_2 \cdot (C_N^{out} - C_N^{in}) + r_N \cdot V_r \tag{5.11}$$

where Q_1 (L/d) refers to the mineral medium containing nutrients, nitrite and ammonium and Q_2 (L/d) to the mineral medium containing hydroxylamine. Q_2 was zero during Phase I. During Phase II Q_1 and Q_2 were equal, and their sum was equal to the total Q_1 added in Phase I. C_N (mg-N/L) refers to the concentration of the desired nitrogen compound. Superscripts *in* or *out* refer to the concentration in the influent or effluent, respectively. r_N (mg-N/L/d) refers to the volumetric rate of the nitrogen compound N. V_r (L) refers to the reactor working volume. t (d) refers to time. Assuming that the accumulation term can be estimated as a difference quotient (increment between two consecutive days measurements). Production or consumption rates of any liquid nitrogen compound can be calculated using Equation (5.12).

$$r_N = \frac{\{Q_1 \cdot (C_N^{out} - C_N^{in}) + Q_2 \cdot (C_N^{out} - C_N^{in})\}}{V_r} + \frac{\Delta C_N}{\Delta t} \tag{5.12}$$

To estimate the N_2 production rate, it was assumed that all the missing nitrogen was being converted to dinitrogen gas using Equation (5.13).

$$r_{N_2} = \frac{\{Q_1 \cdot (Total_N^{out} - Total_N^{in}) + Q_2 \cdot (Total_N^{out} - Total_N^{in})\}}{V_r} + \frac{\Delta C_N}{\Delta t} \tag{5.13}$$

The ratio of nitrate produced per ammonium consumed with and without hydroxylamine was analyzed statistically using Sigma-Plot. As the two data sets to compare did not have equal variance, a Mann-Whitney Rank Sum Test Statistical analysis was applied. Obtaining that the difference in the median values between the two groups is greater than would be expected by chance. Thus, there was a statistically significant difference ($P \leq 0.001$).

Biomass correlation with OD660nm obtained during the continuous operation

Optical density was measured daily in the 1L MBR. VSS and TSS were measured occasionally, as at least 50mL (5% of the reactor content) are needed for each measurement to have duplicates and enough sensitivity in the measurement . A correlation between the biomass concentration (measured as volatile suspended solids per liter, gVSS/L) and the optical density measured at a wavelength of 660nm throughout the reactor operation was used for the estimation of the daily biomass concentration in gVSS/L.

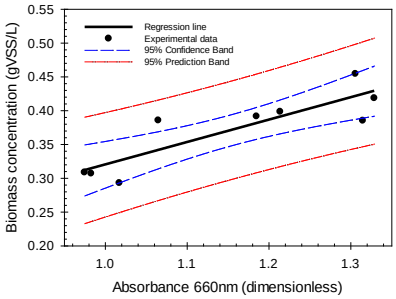


Figure 5.10: Calibration curve used for the batch test to find the correlation between the biomass concentration and the absorbance (at a wavelength of 660 nm). Linear regression resulted in y=0.331x-0.01 with an $r^2 = 0.760$. Where x refers to absorbance at 660nm and y to biomass concentration in gVSS/L. The slope had a standard error of 0.070gVSS/L/absorbance and the intercept and error of 0.082 gVSS/L.

5

16S rRNA data

DNA of the selected samples was extracted for analysis using the DNeasy UltraClean Microbial Kit (Qiagen, The Netherlands). Approximately 250 mg wet biomass was treated with 5 minutes of heat (65°C) followed by 5 minutes of bead-beating for cell disruption on a Mini-Beadbeater-24 (Biospec, U.S.A.). After extraction, the DNA was checked for quality by gel electrophorese and quantified using a Qubit 4 (Thermo Fisher Scientific, U.S.A.).

After quality control, samples were send to Novogene Ltd. (Hongkong, China) for amplicon sequencing of the V4 region of the 16S-rRNA gene (position 515-806) on a Illumina paired-end platform. After sequencing, the raw read were quality filtered, chimeric sequences were removed and OTUs were generated on the base of 97% identity. Subsequently microbial community analysis was performed by Novogene using Mothur & Qiime software (V1.7.0). For phylogenetical determination the most recent SSURef database from SILVA (`http://www.arb-silva.de/`) was used.

5.5.2. RESULTS

SHORT TERM STUDY - BATCH TESTS

Hydrazine dynamics with hydroxylamine as substrate

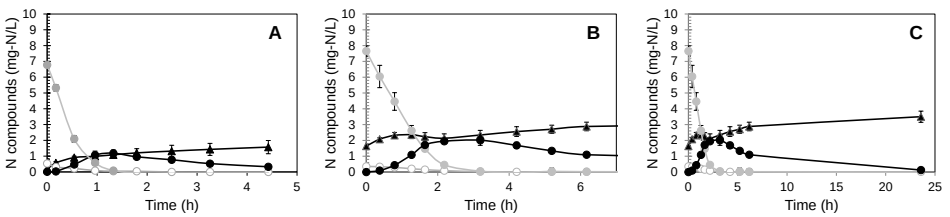


Figure 5.11: Batch tests dynamics with hydroxylamine as substrate: A) Batch test 3 in Table 5.1, B) Batch test 4 in Table1, C) Extended batch test 4 with a sample taken after 24h of the test.

Batch tests positive and negative controls

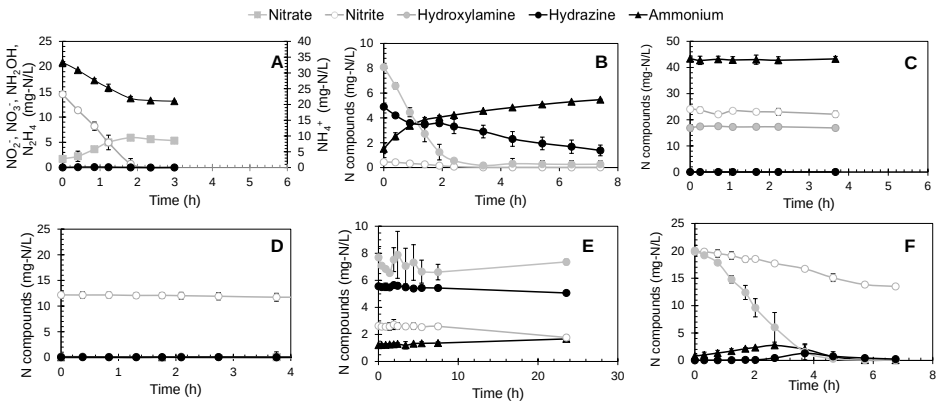


Figure 5.12: Batch tests dynamics with anammox biomass: A) Anammox activity, B) Dynamics with hydrazine and hydroxylamine, C) Negative control with boiled biomass, D) Control of activity with only nitrite as substrate, E) Negative control with boiled biomass with hydrazine and hydroxylamine, F) Nitrite and hydroxylamine as substrate (Figure 5.2C with different x-axis) Note that axes might differ between graphs.

Thermodynamic analysis of batch tests

Hydroxylamine disproportionation thermodynamics

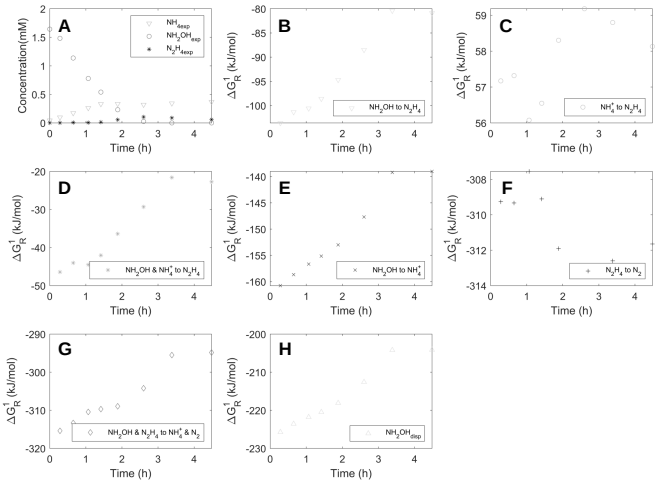


Figure 5.13: Example of thermodynamics analysis results of the reactions involved in hydroxylamine disproportionation in anammox during a batch test (Test 2, Table 5.1). A) Batch tests nitrogen concentrations,B) Hydroxylamine reaction to form hydrazine (Reaction 1, Table 5.2), C)Ammonium reaction to form hydrazine (Reaction 2, Table 5.2), D) Hydrazine production from hydroxylamine and ammonium (Reaction 3, Table 5.2), E) Hydroxylamine reaction to ammonium (Reaction 4, Table 5.2), F) Hydrazine to dinitrogen gas (Reaction 5, Table 5.2), G) Hydroxylamine and hydrazine to ammonium and dinitrogen gas (Reaction 6, Table 5.2), H) Overall hydroxylamine disproportionation (Reaction 7, Table 5.2). Note that y-axes scale differs between graphs.

Hydrazine disproportionation thermodynamics

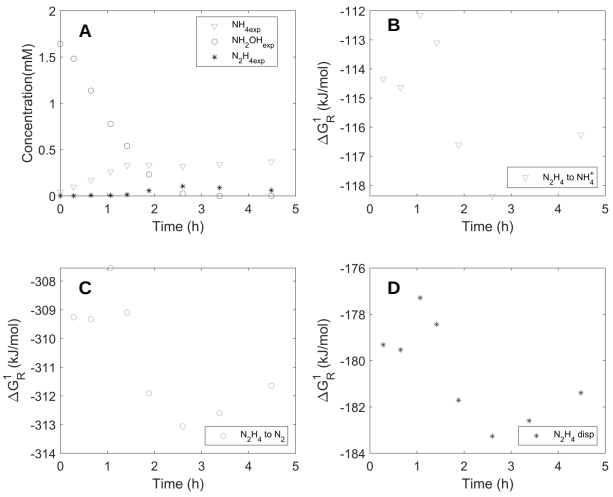


Figure 5.14: Thermodynamics of reactions involved in hydrazine disproportionation in batch tests with hydroxylamine as substrate (Test 2 of Table 5.1): A) Batch tests nitrogen concentrations, B) Hydrazine conversion to ammonium (Reaction 8, Table 5.2), C) Hydrazine conversion to dinitrogen gas (Reaction 9, Table 5.2), D) Hydrazine disproportionation (Reaction 10, Table 5.2).

Studying the feasibility of NO as intermediate in all reactions involving hydroxylamine.

When hydroxylamine is involved in nitrogen conversions the question if NO is the follow up intermediate always arises. For instance, in ammonium oxidizing bacteria (AOB) the first step of ammonium conversion to hydroxylamine is catalyzed by an ammonium monooxygenase. The subsequent step of hydroxylamine oxidation to nitrite was always thought to be a single reaction catalyzed by a hydroxylamine oxidoreductase enzyme [1]. However, the recent work of Caranto and coworkers [27] proposed that this second step is actually a two step process, where hydroxylamine is first transformed to NO, and NO subsequently oxidized to nitrite. A similar discussion applies to the central metabolism of anammox: is nitrite transformed to NO? Is hydroxylamine involved in any of the intermediate steps? What is for sure known is that anammox is able to use hydroxylamine [9, 90, 11]. The mechanisms and reactions involved in hydroxylamine transformation are not fully clear as the putative involvement of NO as intermediate is still a question [90]. For instance all the reactions proposed by van de Star [96], can also occur via NO. For example, hydroxylamine disproportionation occurs via a hydrazine production reaction followed by hydrazine consumption reaction (reaction 1 to 7 in Table 5.2). Hydroxylamine is involved in both reactions (reactions 3 and 6 in Table 5.2). Thus, it can be argued that hydroxylamine conversion in any of these reactions can occur

via NO (reactions 12 to 16 in Table 5.2). The putative thermodynamics of the occurrence of these reactions during the batch test were analyzed (Figure 5.14).

From all reactions analyzed, most of them were thermodynamically favourable at the conditions of the batch tests, even if the standard Gibbs energy change (ΔG_R°) of hydroxylamine conversion to NO at standard conditions was positive (see Table 5.2). The actual Gibbs energy change (ΔG_R^1) was close to equilibrium for the transformation of NO to hydrazine (Figure 5.14B, reaction 12 in Table 5.2). Thus, batch conditions will have a strong impact on the thermodynamic equilibrium.

Consequently, hydroxylamine transformation via NO would mean having two reactions close to the equilibrium: i) hydrazine production in hydroxylamine disproportionation (reaction 3, Table 5.2), and ii) the transformation of NO to hydrazine if hydroxylamine is transformed via NO (reaction 12, in Table 5.2). See further discussion in the main manuscript.

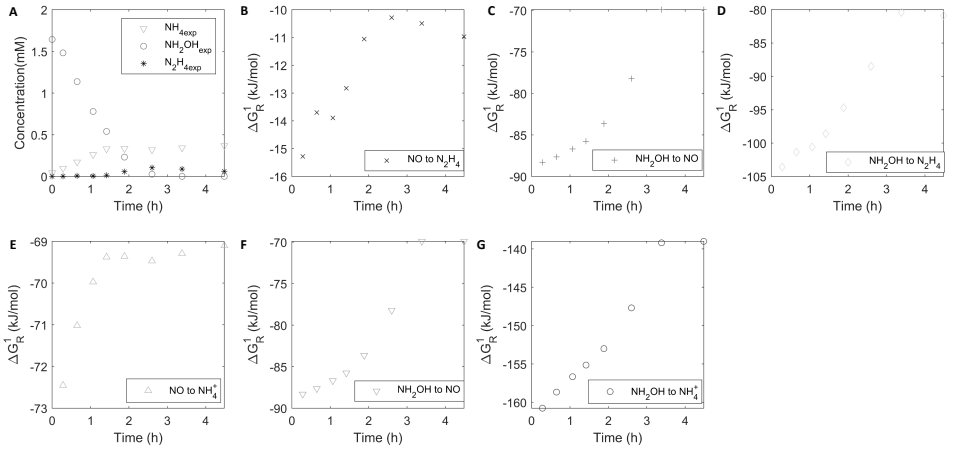


Figure 5.15: Anammox batch tests with hydroxylamine as substrate (Test 2, Table 5.1). Thermodynamics analysis of the occurrence of hydroxylamine transformation via NO. A) Batch tests nitrogen concentrations, B) NO to hydrazine (Reaction 12, Table 5.2), C) Hydroxylamine to NO (Reaction 13, Table 5.2), D) Hydroxylamine to hydrazine (Reaction 14, Table 5.2) , E) NO to ammonium (Reaction 15, Table 5.2), F) Hydroxylamine to NO (Reaction 16, Table 5.2), G) Hydroxylamine to ammonium (Reaction 17, Table 5.2).

Impact of batch test conditions on the hydrazine formation reaction

Batch test conditions, either concentration of a nitrogen compound, temperature or pH was changed within a desired range to assess what was the impact on the actual free energy Gibbs of hydrazine formation (reaction 3 in Table 5.2). The rest of the variables were kept at pH 8, 30°C, concentration of 20 mg-N-NH_4^+/L, 5 mg-N-NH_2OH/L and 1 mg-N-N_2H_4/L.

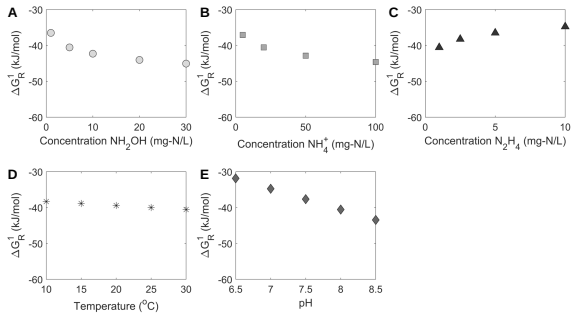


Figure 5.16: Impact of batch tests conditions on actual Gibbs energy change of reaction of hydrazine production during hydroxylamine disproportionation: A) Effect of hydroxylamine concentration, B) Effect of ammonium concentration, C) Effect of hydrazine concentration, D) Impact of temperature, E) Impact of pH.

5

Selection of free energy Gibbs of formation value of hydroxylamine strongly impacts hydrazine production reaction

In literature there are several references for the energy Gibbs of formation of hydroxylamine that strongly differ from each other. Just as examples: i) -43 kJ/mol used by Poughon [172], ii) -23.4 kJ/mol by van der Star [96] o iii) 221 kJ/mol used by Harper [63]. Furthermore, to our knowledge, the value has not been determined experimentally but rather estimated by different methods. Thus, the impact of the selection of the hydroxylamine free Gibbs energy of formation value was studied (Figure 5.15) on the close to equilibrium reaction involving hydrazine formation (reaction 3 in Table 5.2). The more negative the G_f° value of hydroxylamine was, the closer to the equilibrium was the formation of hydrazine. Thus, the thermodynamic analysis is strongly influenced by the correct determination of this value, which has not been determined experimentally yet, to our knowledge.

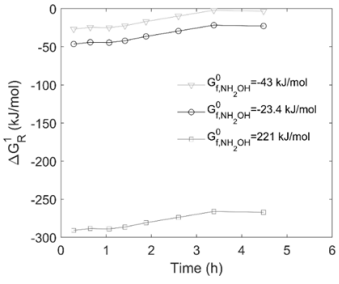


Figure 5.17: Impact of energy Gibbs of formation of hydroxylamine selection of the thermodynamics of hydrazine production reaction in an anammox batch tests with hydroxylamine as substrate (Test 2).

Model for kinetic parameter estimation -batch tests

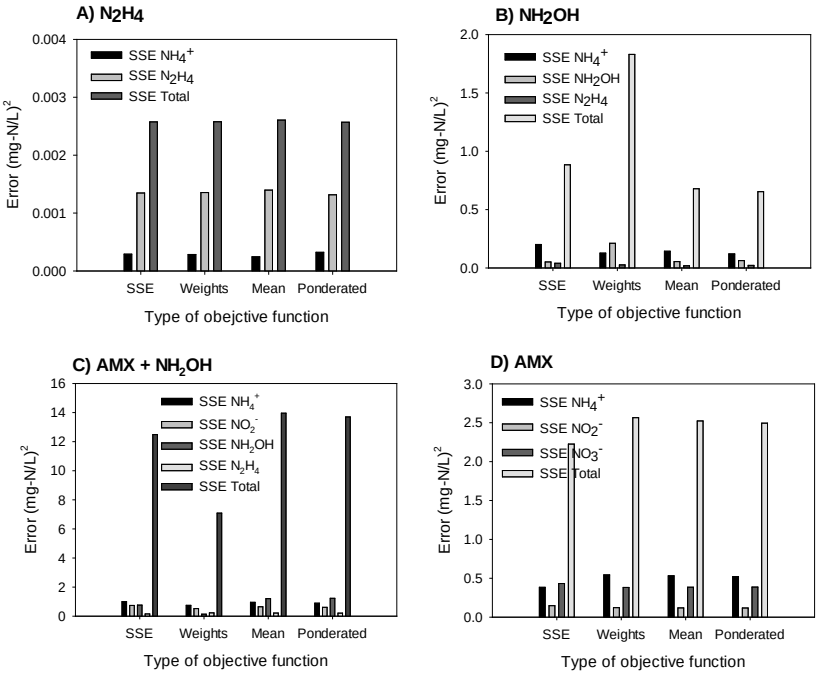


Figure 5.18: Sum of squared errors (SSE) of the model and experimental data depending on the objective function used. Each bar corresponds to the SSE of each nitrogen compound curve in all the tests used in the optimization or the total error of the estimate using the parameters obtained during the optimization with different objective functions. Parameters were estimated with batches provided with different substrates: A) Hydrazine as substrate (m=2), B) Hydroxylamine as substrate (m=6), C) AMX (anammox) substrates, so ammonium and nitrite, together with hydroxylamine (m=6) , D) AMX (anammox) controls with only nitrite and ammonium as substrates (m=4). SSE, weights, mean and ponderated as type of objective functions refers to the usage of of Equation (5.7) to Equation (5.10), respectively, for the optimization of the selected parameters.

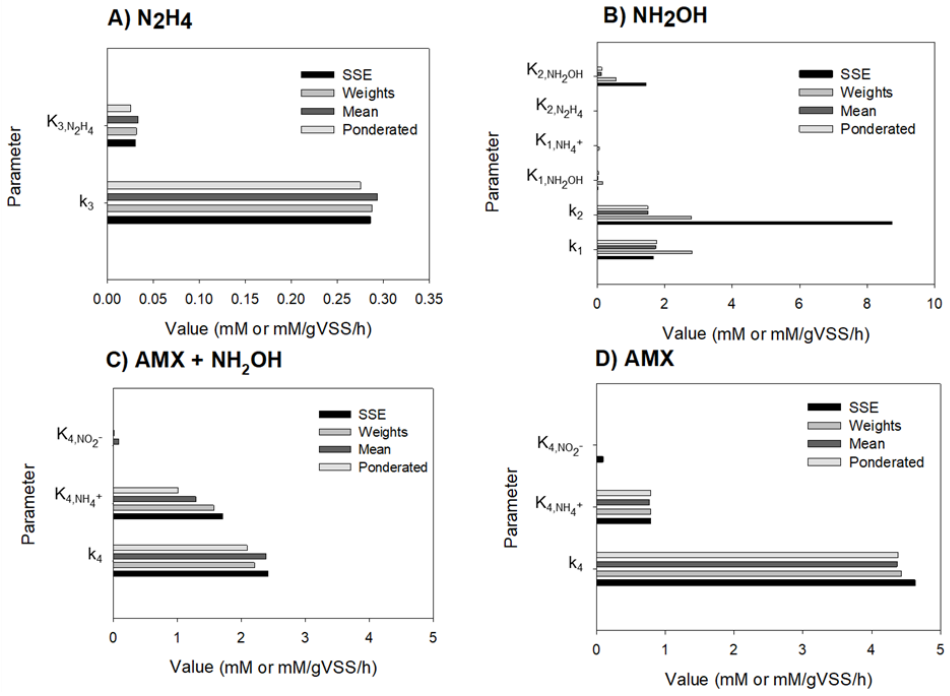


Figure 5.19: Parameters values obtained using different objective functions. Affinity constants (K) in mM and rate constants (k) in mM/gVSS/h. SSE, weights, mean and ponderated as type of objective functions refers to the usage of Equation (5.7) to Equation (5.10), respectively, for the optimization of the selected parameters. Parameters were estimated with batches provided with different substrates: A) Hydrazine as substrate (m=2), B) Hydroxylamine as substrate (m=6), C) AMX (anammox) substrates, so ammonium and nitrite, together with hydroxylamine (m=6), D) AMX (anammox) controls with only nitrite and ammonium as substrates (m=4).

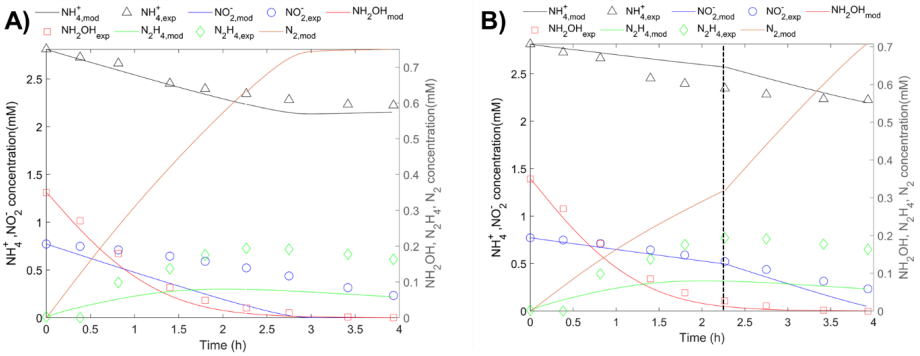


Figure 5.20: Example of modelling results with results from one duplicate of Test 12 from Table 5.1. Nitrite, ammonium and hydroxylamine were used as substrates. A) No optimization was performed and parameters obtained in previous steps were used, B) Parameters k_4, K_{4,NO_2^-} and K_{4,NH_4^+} were optimized with the data points where hydroxylamine was present (left part of the dashed black vertical line), whereas in the absence of hydroxylamine (right part of the dashed vertical line) parameters from the previous optimization steps was used.

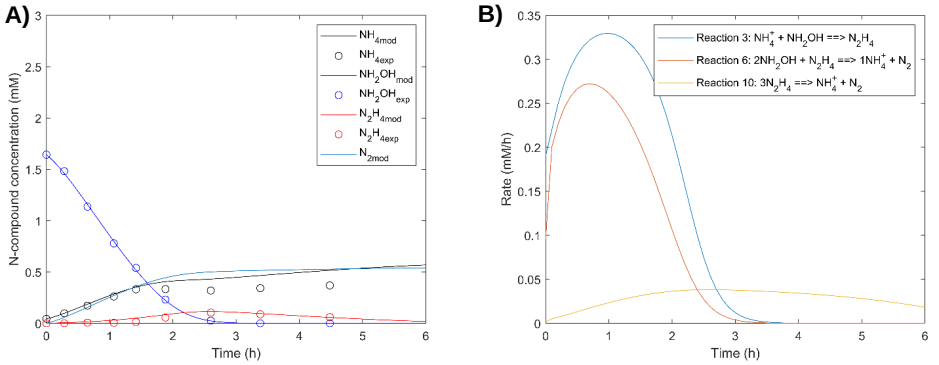


Figure 5.21: Batch test with hydroxylamine as substrate dynamics and mathematical simulation results. A) Nitrogen compounds concentrations, symbols represent experimental data points of one replicate of Test 2 in Table 5.1 and lines the mathematical model with the parameters of Table 5.3, B) Simulated rates during the batch tests of reaction 3, 6 and 10 of Table 5.2.

5.5.3. LONG TERM STUDY - CONTINUOUS HYDROXYLAMINE ADDITION

REACTOR DYNAMICS

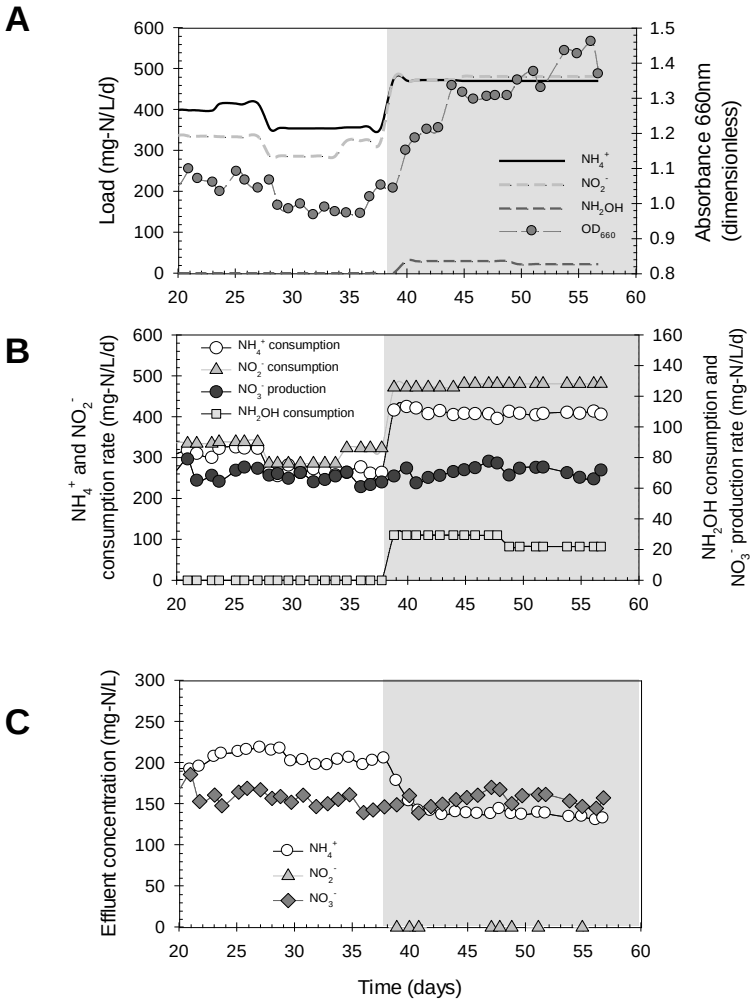


Figure 5.22: Reactor operation dynamics. Grey area indicates time were hydroxylamine feeding was performed. A) Load and biomass OD_{660nm} absorbance, B) Production and consumption rates, C) Effluent nitrogen concentrations.

Table 5.7: Average rates and stoichiometric ratios measured during the continuous operation of the anammox MBR. During Phase I of operation the reactor was fed with mineral medium containing ammonium and nitrite, while during phase II hydroxylamine was added sidewise to ammonium and nitrite. X refers to biomass and r to volumetric rates.

	Days (d)	rNH_4^+ (mg-N/L/d)	rNO_2^- (mg-N/L/d)	rNO_3^- (mg-N/L/d)	rNH_2OH (mg-N/L/d)	rX (gVSS/L/d)	rN_2 (mg-N/L/d)	NO_2^-/NH_4^+ (mol/mol)	NO_3^-/NH_4^+ (mol/mol)	N_2/NH_4^+ (mol/mol)	X/NH_4^+ (molC/mol)	NH_2OH/NH_4^+ (mol/mol)	NH_2OH/NO_3^- (mol/mol)
Phase I	20-37	286±26	314±23	68±4		0.034±0.015	533±47	1.10±0.13	0.24±0.03	0.93±0.04	0.070±0.033		
Phase II	38-54	410±6	478±4	71±4	26±4	0.053±0.011	839±5	1.18±0.02	0.17±0.01	0.99±0.09	0.076±0.014	0.06±0.01	0.4±0.1

6

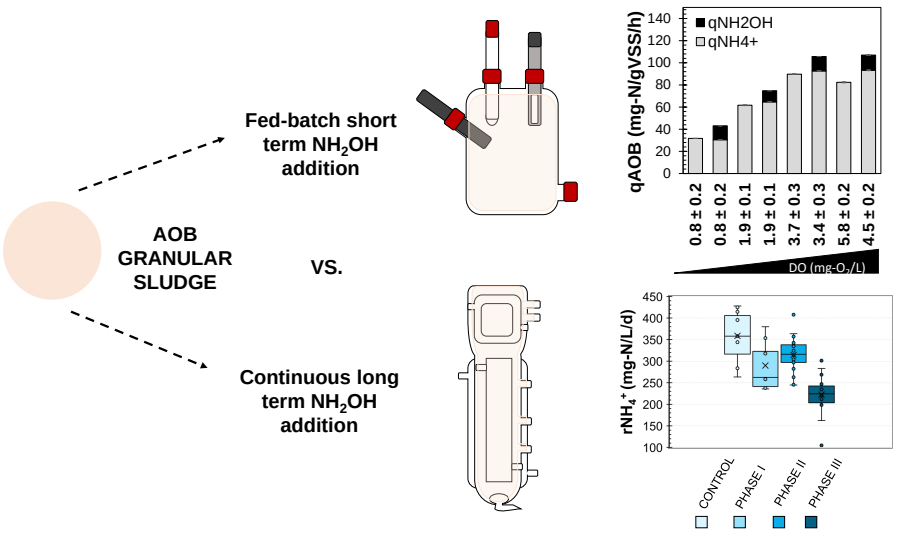
SHORT AND LONG TERM IMPACT OF CONTINUOUS HYDROXYLAMINE FEEDING IN A GRANULAR SLUDGE PARTIAL NITRITATION REACTOR

Aina SOLER-JOFRA, Lisbeth SCHMIDTCHEN, Lluc OLMO, Mark C. M.VAN LOOSDRECHT, Julio PÉREZ

This chapter has been submitted for publication as **A. Soler-Jofra**, L. Schmidtchen, Ll. Olmo, M.C.M van Loosdrecht and J. Pérez *Short and long term impact of continuous hydroxylamine feeding in a granular sludge partial nitritation reactor*

ABSTRACT

Hydroxylamine is a nitrogen intermediate of ammonium oxidizing bacteria (AOB) that can transiently accumulate during nitrification. The impact of hydroxylamine on aerobic ammonium oxidation is still obscure. In the present study the short and long term impact of hydroxylamine on partial nitritation granular sludge was investigated. Dissolved oxygen was the governing factor determining the hydroxylamine impact in short term studies with continuous hydroxylamine feeding. Continuous short term hydroxylamine feeding together with low dissolved oxygen resulted in higher hydroxylamine accumulation, higher N_2O production and decreased or maintained ammonium consumption. Instead, high dissolved oxygen reduced hydroxylamine accumulation and N_2O production and increased ammonium consumption. Long term continuous hydroxylamine feeding reduced ammonium consumption rate while the constant nitrite production rate indicated that dosed hydroxylamine was mainly transformed to nitrite. This indicates that hydroxylamine was preferred over ammonium as substrate. Nitrosomonas sp. was shown to be predominant during continuous hydroxylamine feeding while side community shifted.



6.1. INTRODUCTION

AEROBIC ammonium oxidizing bacteria (AOB) have been studied for over a century. These autotrophic organisms are able to transform ammonium to nitrite aerobically. Hydroxylamine was postulated as intermediate in ammonium oxidation by Lees and coworkers [1]. Thus, according to the current AOB central nitrogen metabolism ammonium is transformed to hydroxylamine by the enzyme ammonium monooxygenase (AMO) (see Equation (6.1)). The transformation of ammonium by AMO requires oxygen and two electrons. Then produced hydroxylamine is subsequently transformed to nitrite (see Equation (6.2)) by hydroxylamine oxidoreductase (HAO). It has also been proposed that HAO only catalyses the transformation of hydroxylamine to NO (see Equation (6.3)), while NO is further transformed to NO_2^- chemically or by a yet unidentified enzyme (Equation (6.4)) [27, 55]. Independently of the intermediate steps, the conversion of hydroxylamine to nitrite results in 4 electron being produced. It is generally accepted that two of these electrons are funnelled back to HAO, while two are dedicated to energy generation within the terminal electron acceptor [131, 105, 22]. Where, oxygen generally acts as terminal electron acceptor Equation (6.5).

$$NH_3 + O_2 + 2H^+ + 2e^- \longrightarrow NH_2OH + H_2O \tag{6.1}$$

$$NH_2OH + H_2O \longrightarrow NO_2^- + 5H^+ + 4e^- \tag{6.2}$$

$$NH_2OH \longrightarrow NO + 3H^+ + 3e^- \tag{6.3}$$

$$NO + H_2O \longrightarrow NO_2^- + 2H^+ + 1e^- \tag{6.4}$$

$$0.5O_2 + 2H^+ + 2e^- \longrightarrow H_2O \tag{6.5}$$

Two N_2O production pathways have been described in AOB. When there is not sufficient terminal electron acceptor (anoxic conditions), nitrite or hydroxylamine can act as terminal electron acceptor while generating N_2O. The use of nitrite as electron acceptor is the so called nitrifier denitrification pathway [22]. The anoxic conversion of hydroxylamine to N_2O has been recently proposed to be mediated by the cytP460 of HAO [57]. Contrarily, the hydroxylamine oxidation pathway producing N_2O is generally associated to high oxygen concentrations leading to hydroxylamine partial oxidation to NO or N_2O [146, 21]. Hydroxylamine is seldom measured or experimentally evaluated in wastewater treatment related research. Only few studies have reported transient hydroxylamine accumulation events with concentrations ranging from 0.003 up to 4.3 mg-N/L [32, 18, 58, 19, 33, 21, 31]. Generally, reported data are associated with SBR operations [32, 33], batch tests [18, 19] or switching conditions from aerobic/anoxic environments [21, 31]. The mechanisms leading to such transient hydroxylamine accumulations are not fully understood.

Different studies have investigated the impact of hydroxylamine on AOB communities, with contradictory results. For instance, Wang and co-workers proposed the use of hydroxylamine to avoid complete nitrification (considered as nitrite being further oxidized to nitrate by nitrite oxidizing bacteria) and achieved a stable partial nitritation operation [47, 46]. Contrarily, Harper and co-workers showed that hydroxylamine addition boosted ammonium consumption in the short term, but negatively impacted conversion on the long term resulting into a

disaggregation of microcolonies [44]. Most of these studies used a pulse like feeding strategy of hydroxylamine, with generally high hydroxylamine concentrations. High concentrations of hydroxylamine are not usual in natural environments and might have caused inhibitory effects, as it is a highly reactive compound. Instead, using a limited continuous feeding would limit the potential toxicity of hydroxylamine and would mimic putative environmental conditions of hydroxylamine exposure due to transient accumulation. The only study that supplied limited concentrations of hydroxylamine was by de Bruijn et al. [42] with a planktonic chemostat culture of *Nitrosomonas europaea*. In this study hydroxylamine led to a higher biomass yield as compared to growth on ammonium only. They also observed a soluble nitrogen loss that increased with increasing hydroxylamine load, as NO and/or N_2O was formed [42].

Additionally, mathematical models developed to describe NO and N_2O emissions from nitrifying sludge included the description of the hydroxylamine oxidation by declaring hydroxylamine concentration as state variable [144]. Most of the modelling calibrations to determine the kinetic parameters related to hydroxylamine oxidation were performed by fitting other measured nitrogen species, DO and/or N_2O [175, 176, 129], but without direct measurements of hydroxylamine concentration. The exception is the study by Domingo-Felez and coworkers (2017), that included hydroxylamine concentration measured data in order to estimate the parameters of hydroxylamine oxidation step and N_2O production [177].

A more comprehensive understanding of the hydroxylamine metabolism by AOB is needed since its metabolism has a complex kinetic regulation, impacting also the associated N_2O emissions. To further investigate the factors governing hydroxylamine metabolism and its regulation, short term fed-batch addition of hydroxylamine at different dissolved concentrations on a granular sludge culture performing partial nitritation was used. Continuous addition of hydroxylamine was used to study the long term impact on nitrogen conversions and microbial community dynamics. The results were analysed using a simplified kinetic model that help identifying the rate limiting steps depending on the substrate conditions and it was also used to explain the hydroxylamine accumulation dynamics. Finally, the apparent kinetic parameters related to the hydroxylamine oxidation were roughly estimated.

6.2. MATERIALS AND METHODS

6.2.1. REACTOR SET-UP AND OPERATION

TWO different airlift reactors were operated in this study: R1 with a 5L working volume and R2 with a 2.6L working volume (Figure 6.9 and Figure 6.11 and Figure 6.13). Inoculum characteristics and synthetic medium are described in SI.

The pH and the dissolved oxygen (DO) concentrations were measured using online sensors, but they were not controlled. Airflow was controlled with a rotameter (Aalborg, Denamark) in both R1 and R2. Airflow rate and influent rate were adjusted, when needed, to maintain partial nitritation (see Figure 6.9, Figure 6.11A). During R2 operation with hydroxylamine loading, air flow was maintained at 6L/h. Between days 140 and 240 of operation of R2 (Figure 6.11 and Figure 6.13), the reactor operation was maintained but not fully characterized due to impossibility to go often to the laboratory.

N_2O was measured when needed and when possible using a Clark type sensor (Unisense, Denmark).

Temperature was controlled with an external water jacket and subsequent external cryostat at ca. 22°C, when needed during R1 operation (Figure 6.9B). Temperature in R2 was only controlled during the start-up at ca. 25°C for ca. 100 days (Figure 6.11B). However, during the hydroxylamine loading experiments, temperature was not controlled but remained rather stable at ca. 22°C (Figure 6.13B).

Periodically, samples were withdrawn from the reactor for further analysis of dissolved nitrogen compounds concentrations, biomass concentration, average size distribution, settling velocity tests, FISH and 16s analysis.

6.2.2. BATCH AND FED-BATCH TESTS

To assess the short term impact of hydroxylamine, a slow feeding of hydroxylamine in ammonium oxidizing granular biomass batch tests was studied. Once ammonium was totally consumed, a fed-batch test was performed with an ammonium pulse and continuous limited hydroxylamine feeding. Granular sludge from two reactors (R1 and R2, see SI for further details) performing partial nitritation was used. The rationale behind using a continuous addition strategy for the hydroxylamine feeding was to mimic what could happen in natural environments where hydroxylamine can transiently accumulate in the bulk liquid [18, 19, 33, 31]. Instead of using pulse like additions with high initial hydroxylamine concentrations, as it has been previously done [43, 47]. The batch tests performed with biomass from R1 were performed at different days of operation and also targeting different hydroxylamine loading rates. Consequently, different dissolved oxygen concentrations were reached at each test (Table 6.1). A targeted study of the DO impact was performed with biomass from R2 and in 4 consecutive days to perform batch and fed-batch tests 6 to 9 in Table 6.1. In between experimental days the R2 biomass was aerated and left with an ammonium pulse, to avoid as much as possible starvation. Thus, the next morning the biomass was washed with free nitrogen medium and the procedure for the tests started.

To perform the tests 1L of R1 or R2 biomass was withdrawn from the reactor and washed twice with nitrogen free synthetic medium. R1 biomass was resuspended in 1L of free nitrogen medium and transferred to a 1.2L vessel to perform the batch/fed-batch tests. The final volume of the vessel used with biomass from R2 was 0.8L, as 1L vessel was used. A magnetic stirrer was used for agitation. Tests were performed at 25°C controlling the temperature with an external water bath. pH was controlled at pH 8.00±0.08 with a pH sensor (WTW-Sentix 81), and acid (1M HCl) or base (1M NaOH) addition with a microburete. An O_2 sensor (WTW-Cellox 325) and an N_2O sensor (Unisense) were used to monitor dissolved oxygen and nitrous oxide online. Compressed air or nitrogen (when needed) were sparged using a stone diffuser at a flow rate controlled with a mass flow controller (Bronckhorst F201-C).

After stabilization of all online signals (DO, pH) once pH control was started, the oxygen gas transfer coefficient (k_La) was estimated for each batch tests by stopping the air flow (or sparging with nitrogen) followed by air sparging again (see SI). Afterwards, the batch tests (B) were started by a pulse addition of ammonium to the targeted initial concentrations (Table 6.1). Once the batch was finalised, the fed-batch test was started

by combining a pulse of ammonium and fed-batch (FB) addition of hydroxylamine at the desired load with a programable microburete. A co-metabolization (COM in Table 6.1) test of hydroxylamine and ammonium was performed by pulse addition of both substrates to assess putative hydroxylamine inhibition.

Liquid samples (3-4mL) were withdrawn over time, filtered and stored in the fridge for further analysis of nitrogen compounds. For hydroxylamine determination, 1.8mL of sample was mixed with 0.2mL of sulfamic acid at 5g/mL. Biomass concentration during the test was analyzed at the end. See Physiochemical analysis section for further details. Rate calculations are described in SI.

6.2.3. DETERMINATION OF HYDROXYLAMINE KINETIC PARAMETERS FROM EXPERIMENTAL DATA

The continuous airlift reactor data was used to estimate the maximum hydroxylamine consumption rate and the half-saturation coefficient for hydroxylamine. Residual hydroxylamine concentration measured during the continuous reactor operation against total AOB consumption ($q_{NH_4^+} + q_{NH_2OH}$) was fit to a Monod equation Equation (6.6) using Sigma Plot. Total AOB consumption was used as assumed that all ammonium being transformed was being converted via hydroxylamine to nitrite. Thus, contributing to the total hydroxylamine consumption rate of the system.

$$q_{NH_2OH} = \frac{q^{max}_{NH_2OH} \cdot C_{NH_2OH}}{K_{NH_2OH} + C_{NH_2OH}} \tag{6.6}$$

6.2.4. PHYSICOCHEMICAL ANALYSIS AND MICROBIAL COMMUNITY ANALYSIS

Nitrogen compounds were analysed in liquid samples after filtration (0.22µm). Nitrite and nitrate were analysed by ionic chromatography using ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA). Ammonium was measured using a gas selective electrode (AMTAX sc, Hach Lange, Germany). When needed, ammonium, nitrite or nitrate were measured using Hach Lange kits. Hydroxylamine was measured spectrophotometrically after pre-treatment with sulfamic acid as in [117, 58]. Briefly, 1 mL of 0.05M phosphate buffer, 1 mL of sample (diluted, if applicable), 0.8 mL of demineralised water, 0.2 mL of a 12wt% trichloroacetic acid solution, and 1 mL of a 1% 8-quinolinol solution were added to Pyrex tubes. After mixing, 1 mL of a 1M Na_2CO_3 solution was also added. After mixing, the tube's content was boiled in a water bath (100°C) for 1 minute and cooled down for 15 minutes at room temperature. Finally, samples absorbances were measured at 705nm. Blank was performed by substituting the 1mL of sample for 1mL of mili-Q water. For biomass concentration determination total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to standard methods [171]. The Sludge Volume Index (SVI) of 1L of biomass was determined at 5 minutes and 30 minutes in a graduated cylinder. Volume size distribution and average particle size was determined with a laser diffraction analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK) (see Figure 6.10, Figure 6.12 and Figure 6.14). Biomass was harvested periodically for microbial community analysis by FISH and 16S rRNA sequencing as described in SI.

Table 6.1: Batch/fed-batch tests conditions performed with biomass from R1 or R2. A batch (B) tests with a pulse of ammonium was followed with a fed-batch tests (FB) with a pulse addition of ammonium and continuous hydroxylamine feeding. Temperature was 25°C and pH set point of 8 leading to an averaged pH of 8.0±0.1. DO- dissolved oxygen reached during stable phase, Max. – maximum. ΔqNH_4^+,FB/B stands for the difference on ammonium consumption rate between the fed-batch and the batch test. * Initial hydroxylamine concentration (mg-N/L).qNH_4^+, qNH_2OH and DO were used in Figure 6.1.

Test	Biomass source, day	Type of test	Air flow (mL/min)	DO mg-O_2/L	NH_4^+ (mg-N/L)	NH_2OH load (mg-N/gVSS/h)	qNH_4^+ (mg-N/gVSS/h)	qNH_2OH (mg-N/gVSS/h)	Max. NH_2OH (mg-N/L)	ΔqNH_4^+,FB/B (%)
1	R1, 99d	B	200	2.1±0.2	19.7		69.2±0.1			
1	R1, 99d	FB	200	2.0±0.1	18.2	4.6	64.4±0.1	4.6	0.03	-7
2	R1, 129d	B	200	2.6±0.1	19		58.9±0.1			
2	R1, 129d	FB	200	2.6±0.1	18.3	9.0	58.0±0.1	8.59±0.02	0.16	-1
3	R1, 144d	B	200	1.2±0.1	20.6		45.9±0.1			
3	R1, 144d	FB	200	1.0±0.5	21.1	14.2	46.3±0.3	10.87±0.04	1.38	1
4	R1, 164d	B	200	1.5±0.1	18.6		38.4±0.1			
4	R1, 164d	FB	200	1.3±0.1	21.6	6.2	26.4±0.1	5.62±0.02	0.18	-31
5	R1, 211d	B	200	3.7±0.2	15.1		25.1±0.2			
5	R1, 211d	FB	200	3.6±0.2	19.7	6.0	29.9±0.2	4.8±0.1	2.74	19
6	R2, 98d	B	400	5.8±0.2	20.8	0	82.4±0.4			
6	R2, 98d	FB	400	4.5±0.2	19.7	16.2	93.3±0.4	13.61±0.03	0.38	13
7	R2, 98d	B	100	3.7±0.3	20.1	0.0	89.8±0.2			
7	R2, 98d	FB	100	3.4±0.3	18.2	13.4	92.7±0.1	13.03±0.03	0.62	3
8	R2, 98d	B	10	0.8±0.2	18.9	0.0	31.7±0.1			
8	R2, 98d	FB	10	0.8±0.2	18.1	13.8	30.5±0.1	12.57±0.04	1.42	-4
9	R2, 98d	B	40	1.9±0.1	19.5	0.0	61.7±0.1			
9	R2, 98d	FB	40	1.9± 0.1	20.0	13.6	64.7±0.1	10.08±0.04	1.08	5
9	R2, 98d	COM	40	2.7±0.4	16.2	7*	56.7±0.1	18.40±0.08		

6.3. RESULTS

6.3.1. BATCH/FED-BATCH TESTS AS A TOOL TO ASSESS THE IMPACT OF HYDROXYLAMINE IN PARTIAL NITRITATION AEROBIC GRANULAR SLUDGE

DISSOLVED OXYGEN REGULATES AMMONIUM AND HYDROXYLAMINE CONSUMPTION AS WELL AS N_2O PRODUCTION

GRANULAR sludge from two different reactors was used for batch (B) tests with an ammonium pulse followed with fed-batch (FB) tests with the same ammonium pulse but with hydroxylamine continuous feeding (see Figure 6.1). Comparing the ammonium consumption rate results of tests with only ammonium and the tests where hydroxylamine was also continuously fed, the impact of a low (limiting) hydroxylamine concentration in the medium on the ammonium uptake rate (qNH_4^+ in Table 6.1) could be assessed. Depending on the dissolved oxygen concentration during the test two different trends where observed: i) at low DO to middle DO range (0.8 to 3.6 mg-O_2/L, being tests 1, 2, 3, 4, 7, 8 and 9 in Table 6.1), hydroxylamine dosing resulted in a slight increase (1-5%) in the ammonium uptake rate (tests 3, 7 and 9 in Table 6.1) or a 1-31% decrease in the ammonium uptake rate (tests 1, 2, 4 and 8 in Table 6.1). ii) At a high DO concentration (3.6 to 5.8 mg-O_2/L, test 5 and 6, in Table 6.1), the ammonium uptake rate increased when hydroxylamine was also dosed to the system (13 and 19% increase, respectively). Total nitrogen oxidation activity (i.e., including N-ammonium and N-hydroxylamine) was generally increased (see Figure 6.1).

Overall, with granular sludge from both R1 and R2, DO concentration was the variable impacting the most the metabolism of ammonium and hydroxylamine consumption. The effect of DO concentration can be observed specially with tests 6-9 (Figure 6.1B and Table 6.1). Dissolved oxygen (DO) strongly affected ammonium consumption rates as it ranged from ca. 30 to 93 mg-N/gVSS/h) depending on the DO. Instead, hydroxylamine consumption rate was slightly influenced being rather constant around 10-13 mg-N/gVSS/h (Table 6.1 and Figure 6.1B).

Interestingly, DO not only impacted consumption rates, but also hydroxylamine accumulation and N_2O emissions Figure 6.2, only when hydroxylamine was dosed continuously during the fed-bath tests. Lower DO led to higher hydroxylamine accumulation, as hydroxylamine accumulation went from 0.35 mg-N/L to 1.4 mg-N/L when oxygen went down from 4.5 to 0.8 mg-O_2/L (Figure 6.2A). Furthermore, lower DO also resulted in higher N_2O emissions factor. The N_2O emission factor went from 1.3-3% at DO 1.8-5 mg-O_2/L up to ca. 21% at DO of 0.8 mg-O_2/L (Figure 6.2B) when hydroxylamine was continuously added in the tests. Consequently, lower DO resulted in higher hydroxylamine accumulation and higher N_2O emissions.

As just mentioned, in some fed-batch tests hydroxylamine was accumulating up to relatively high concentrations (>1 mg-N/L see Table 6.1). To specifically investigate the effect of elevated hydroxylamine concentrations the co-metabolization of hydroxylamine and ammonium was evaluated in tests were a pulse of both hydroxylamine and ammonium where added after a nitrification batch test without hydroxylamine addition. The initial hydroxylamine concentration was 7 mg-N/L, for test 9 (see Table 6.1). Thus, hydroxylamine concentrations were higher during

the co-metabolization experiments than those observed in the tests with continuous hydroxylamine addition. The ammonium oxidation rate was 61 mg-N/gVSS/h during the batch with only ammonium and 56 mg-N/gVSS/h when hydroxylamine and ammonium were both present.

Summarising, at low DO hydroxylamine addition resulted in decreased or slightly increased ammonium consumption rate, hydroxylamine accumulation and N_2O production, whereas at high DO, hydroxylamine addition increased ammonium consumption with less hydroxylamine accumulating and less N_2O produced (Figure 6.1 and Figure 6.2).

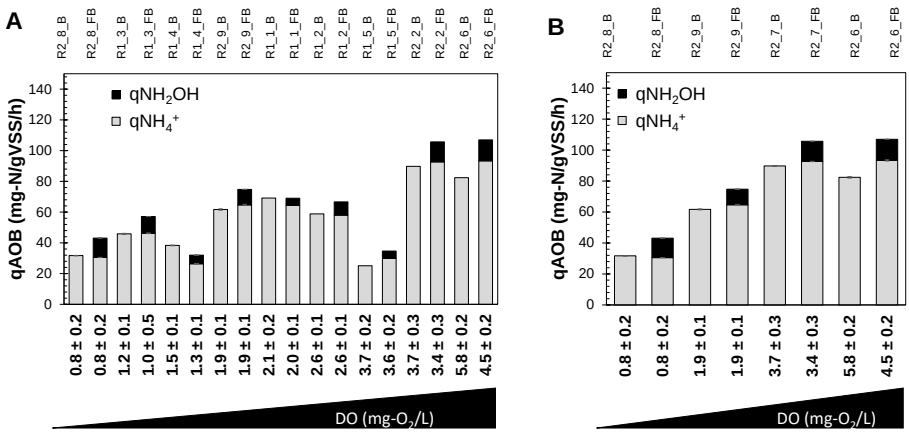


Figure 6.1: Tests results performed with biomass from either inoculation period 1 (R1) or 2 (R2) followed by the number that corresponds with test conditions in Table 1. All respirometer tests consisted of an initial batch test (B) with only ammonium followed by a fed-batch (FB) with ammonium batch addition combined with hydroxylamine fed-batch. A) Comparison of all tests performed batch and fed-batch ammonium oxidation activity specific activity (q_{AOB}), it is the addition of ammonium oxidation specific activity ($q_{NH_4^+}$) and hydroxylamine oxidation specific activity (q_{NH_2OH}) sorted by increasing conditions of oxygen. B) Comparison of batch and fed-batch tests performed with biomass from R2, sorted by increasing dissolved oxygen concentrations. Notice that between the same batch and fed-batch test, slight oxygen decrease might occur in the second test. Thus, the order of increased DO is based on the batch tests.

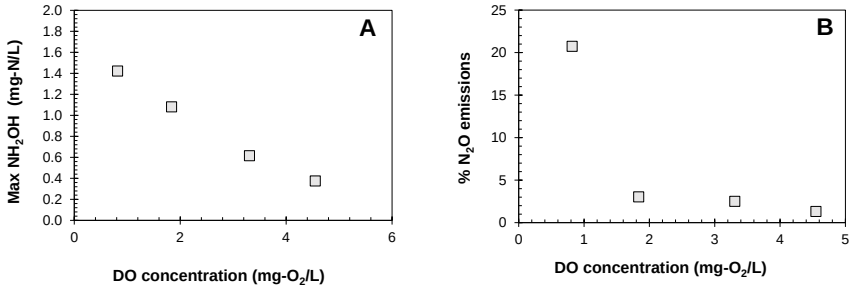


Figure 6.2: Nitrification tests with ammonium present and continuous hydroxylamine feeding with granular sludge (biomass from reactor R2). Impact of dissolved concentration on: A) Maximum accumulated hydroxylamine concentration (total amount added was between 7.4 to 3.5 mg-N/L, depending on the duration of the test) and B) N_2O emissions as percentage of ammonium conversion.

6.3.2. LONG TERM IMPACT OF HYDROXYLAMINE FEEDING TO A CONTINUOUS FLOW PARTIAL NITRITATION REACTOR

HYDROXYLAMINE IS TRANSFORMED TO NITRITE AND REDUCES AMMONIUM CONSUMPTION

A stable operating continuous flow granular sludge nitritation reactor was subjected to adding hydroxylamine at various loading rates. The impact on the nitrogen conversion processes and the microbial population dynamics were assessed. The hydroxylamine load to the granular sludge airlift reactor was gradually increased during 3 operational phases (Phase I, II and III in Figure 6.13 and Figure 6.15). The airflow was maintained stable during the whole operation, the DO gradually increased from 3.6±0.2 mg-O_2/L without hydroxylamine addition to 4.3±0.4, 4.4±0.9 and 5.2±0.5 mg-O_2/L in phase I, II and III, respectively (see Figure 6.13B and Figure 6.15B). The pH did not vary significantly during the operation (see Figure 6.13B and Figure 6.15C). Granule size and particle size distribution was stable over time (Figure 6.14) as well as biomass concentration during Phase I,II and III (see Figure 6.13C and Figure 6.15A).

Interestingly, ammonium consumption rate decreased with increasing hydroxylamine loading rate (see Figure 6.3A). Nitrite production remained the same with or without hydroxylamine. The decreased ammonium conversion and constant nitrite production indicates that the hydroxylamine conversion to nitrite compensated the decreased ammonium conversion. The stoichiometric ratio calculated from the produced nitrite over total nitrogen consumed, including NH_2OH and ammonium consumption, was close to the theoretical value of 1 (Figure 6.3D). A maximum of ca. 2-7% nitrogen was unaccounted in the nitrogen balance during the whole reactor

operation, which can be due to accumulated measurement error combined with N_2O production. For instance, occasional N_2O was detected in the liquid phase at the beginning of hydroxylamine feeding during Phase I, but decreased after few hours (see Figure 6.16A). Occasionally, N_2O peaks were detected in the liquid (<1 mg-N/L), but not in a consistent and continuous way (see Figure 6.16B). More N_2O measurements could not be performed during the rest of the phases due to technical problems with the sensor.

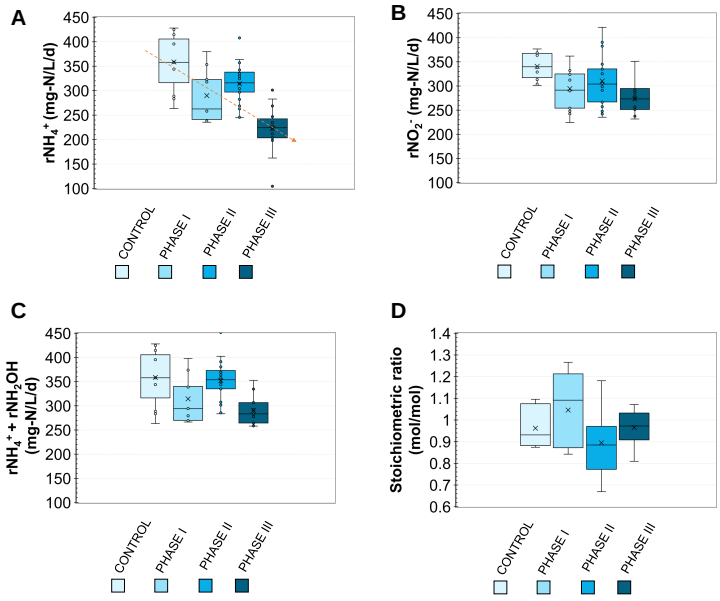


Figure 6.3: Airlift reactor conversion rates and stoichiometric ratios for different hydroxylamine loading rates. A) Ammonium consumption rate, B) Nitrite production rate, C) Sum of ammonium and hydroxylamine consumption rate, D) Stoichiometric ratio of nitrogen produced over nitrogen consumed taking into hydroxylamine consumption.

MICROBIAL COMMUNITY DYNAMICS

The microbial population dynamics during the airlift reactor operation without and with hydroxylamine feeding was investigated using FISH and 16S rRNA sequencing. FISH performed with pottered biomass indicated that the majority of the population was formed by AOB. Over time, a slight increase in NOB population and a decrease of the EUB signal was observed (Figure 6.17). When observing cryosectioned granules a very low number of NOB could be observed (Figure 6.18). The relative abundance of AOB *Nitrosomonas* sp. in the 16S rRNA analysis was stable at 42±6% of the operational taxonomic units (OTUs) over the full operational period (see blue colored bars in Figure 6.4). This is in agreement with the generally distributed hybridization of Nso190 probe in the FISH. Three different OTUs were identified as AOB *Nitrosomonas*. One of them *Nitrosomonas* sp. 2 (dark blue bars in Figure 6.4) significantly increased from the beginning of the hydroxylamine feeding from 5±2% to 13±3% in days 102-283 and days 296-332, respectively. Interestingly, no NOB OTUs were detected with 16S rRNA analysis

contradicting partially the FISH results for pottered biomass samples.

The side population, as observed by 16S rRNA sequencing, showed a considerable change when hydroxylamine was fed. For instance, *Acidovorax* went from ca. 6±1% in the control phase up to ca. 25±3% in Phase I and II (green bar in Figure 6.4). However, in Phase III it decreased again down to 14±2 % and the side population switched to Unclassified microorganisms from the family *Xanthomonadaceae*, *Thermomonas* and *Brevundimonas* (dark to light orange color bars in Figure 6.4).

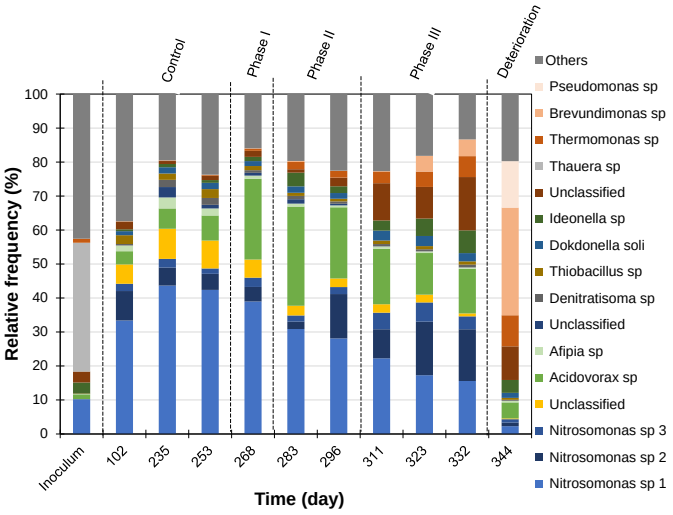


Figure 6.4: Microbial relative frequency based on 16S rRNA sequencing of the predominant OTUs during different days of the airlift reactor operation. Phase I, II and III were exposed to increasing loads of hydroxylamine. *Nitrosomonas* strains are AOB.

EXPERIMENTAL ASSESSMENT OF APPARENT HYDROXYLAMINE CONSUMPTION KINETICS

In the present work, during the continuous feeding of hydroxylamine and ammonium to the continuous flow granular sludge airlift reactor, residual hydroxylamine was measured daily for each hydroxylamine loading rate. The total hydroxylamine consumption by the reactor is derived from the inflow hydroxylamine as well as from the ammonium oxidation (as hydroxylamine is an intermediate step). The apparent half-saturation coefficient and maximum consumption rate for the granular sludge system was estimated using curve fitting of the data to a Monod Model (Figure 6.5). Maximum hydroxylamine consumption was estimated to be 359±16 mg-N/L/d (46 ± 22 mg-N/gVSS/h) and hydroxylamine half-saturation coefficient was estimated as 0.015± 0.006 mg-N/L.

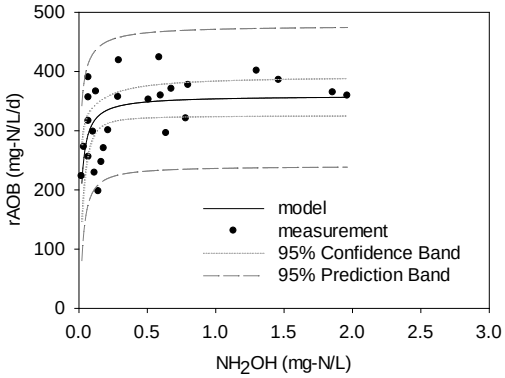


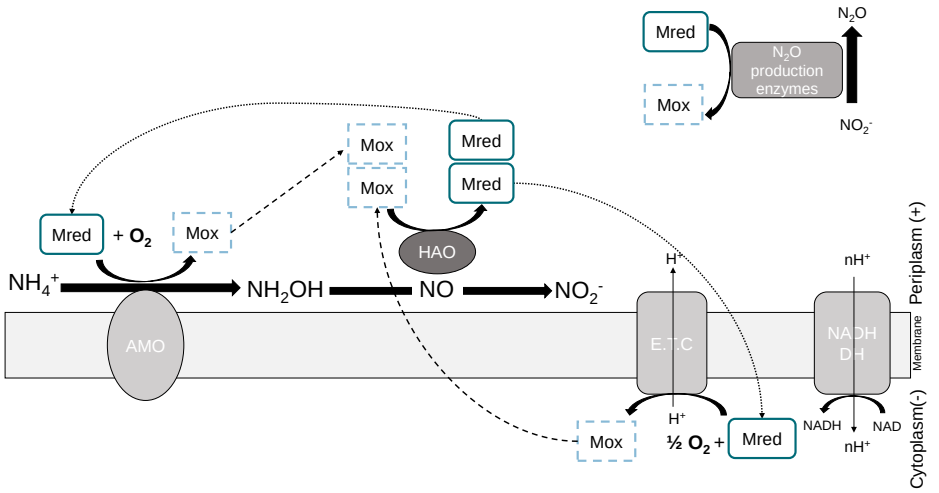
Figure 6.5: Estimations of kinetic parameters for ammonium oxidation by fitting Equation (6.6) to the data from experiments with long and short term feeding of hydroxylamine: A) Effluent residual concentration over total ammonium oxidation consumption (hydroxylamine and ammonium) during continuous long term feeding. B) Example of results B and FB.

6.4. DISCUSSION

6.4.1. DISSOLVED OXYGEN GOVERNS HYDROXYLAMINE ACCUMULATION AND N_2O PRODUCTION

THE DO concentration was identified as the main factor governing hydroxylamine accumulation and N_2O production in a nitrification process (Figure 6.1 and Figure 6.2). Additionally, the ammonium consumption rate was impacted by hydroxylamine dosage, depending on the DO concentration (Figure 6.1). In order to be able to explain the observed impact of hydroxylamine addition on ammonium oxidation, the kinetics and factors governing ammonium oxidation are crucial (Figure 6.6). Four main processes are responsible for the observed ammonia oxidation rates: 1) ammonium transformation to hydroxylamine (mediated by AMO), 2) Hydroxylamine oxidation to nitrite (mediated by HAO), 3) the terminal electron acceptor where electrons are funeled into oxygen by electron transport chain (E.T.C), and 4) Nitrifier denitrification pathway for N_2O production as a sink for Mred equivalents. These processes are all linked by the reduced (Mred) or oxidized forms of the electron carriers (Mox) (see Figure 6.6).

When both ammonium and oxygen concentrations are high, their corresponding Monod terms will tend towards one (see r_{NH_3} of process 1, and r_{O_2} of process 3 in Figure 6.6, respectively), resulting in the simplified Equation (6.7) and Equation (6.8). The availability of Mred will depend on the hydroxylamine consumption rate (r_{NH_2OH}). Consequently, r_{NH_2OH} will determine the overall ammonium consumption process velocity. In such conditions, hydroxylamine is not likely to accumulate as sufficiently Mox should be available and hydroxylamine consumption will determine both r_{NH_3} and r_{O_2} by providing them with Mred. Consequently, r^{max} of each reaction and the affinities for Mred will govern the r_{NH_3} and r_{O_2} kinetic rates (see Equation (6.7) and Equation (6.8)).



PROCESS	ENZYMES	SUBSTRATES	PRODUCTS	RATES
1. Ammonium oxidation	AMO	NH_4^+ O_2 Mred	NH_2OH Mox	$r_{NH_3} = r_{max,NH_3} \frac{C_{O_2}}{C_{O_2}+K_{O_2,NH_3}} \cdot \frac{C_{NH_3}}{C_{NH_3}+K_{NH_3}} \cdot \frac{C_{Mred}}{C_{Mred}+K_{Mred,NH_3}}$
2. Hydroxylamine oxidation	HAO	NH_2OH 2 Mox	NO_2^- (via NO) 2 Mred	$r_{NH_2OH} = r_{max,NH_2OH} \cdot \frac{C_{NH_2OH}}{C_{NH_2OH}+K_{NH_2OH}} \cdot \frac{C_{Mox}}{C_{Mox}+K_{Mox,NH_2OH}}$
3. Electron terminal acceptor	E.T.C	1/2 O_2 Mred	Mox	$r_{O_2} = r_{max,O_2} \frac{C_{O_2}}{C_{O_2}+K_{O_2}} \cdot \frac{C_{Mred}}{C_{Mred}+K_{Mred,O_2}}$
4. Nitrifier denitrification	N_2O production enzymes	NO_2^- Mred	1/2 N_2O Mox	$r_{N_2O,1} = r_{max,N_2O,2} \frac{C_{NO_2^-}}{C_{NO_2^-}+K_{NO_2^-,N_2O}} \cdot \frac{C_{Mred}}{C_{Mred}+K_{Mred,2,N_2O}}$

Figure 6.6: Schematic model of ammonium oxidation. Electron transport has been simplified by using Mred and Mox as the reduced and oxidized forms of the electron carriers, respectively. Each Mox/Mred is able to accept or donat 2 electrons. AMO – Ammonium monooxygenase, HAO – hydroxylamine oxidoreductase, E.T.C – Electron transport chain. Notice that the location of the catalytic site of AMO is not fully defined. Thus, it could also be located in the periplasm. Also as hydroxylamine oxidation can occur without ammonium, thus two Mox equivalents might come from the E.T.C..

$$r_{NH_3} = r_{NH_3}^{max} \cdot \frac{C_{Mred}}{K_{Mred,NH_3} + C_{Mred}} \tag{6.7}$$

$$r_{O_2} = r_{O_2}^{max} \cdot \frac{C_{Mred}}{K_{Mred,ETC} + C_{Mred}} \tag{6.8}$$

On the contrary, if ammonium concentration is high and dissolved oxygen concentration is low, the conversion of hydroxylamine (r_{NH_2OH} from process 2 in Figure 6.6) will be limited by the turnover of Mox, once Mred is used during ammonium consumption or the oxygen electron consumption in the terminal electron acceptor chain. In such conditions, the ammonium Monod term or r_{NH_3} will not play an important role as it will approach 1 (see r_{NH_3} of process 1 in Figure 6.6). As oxygen concentrations are low, oxygen affinities and maximum rates will now play an important role and small differences in these values will determine if r_{NH_3} or r_{O_2} is the rate limiting process (see Equation (6.9) and Equation (6.10)). Thus, hydroxylamine (and putatively other intermediates) will tend to accumulate, if the Mox needed for the reaction is not regenerated fast enough. As a strategy to avoid NH_2OH (and other intermediates) accumulation, Mred can be used to convert nitrite to N_2O and generate Mox, as it does not depend on oxygen availability.

$$r_{NH_3} = r_{NH_3}^{max} \cdot \frac{C_{O_2}}{K_{O_2,NH_3} + C_{O_2}} \tag{6.9}$$

$$r_{O_2} = r_{O_2}^{max} \cdot \frac{C_{O_2}}{K_{O_2,ETC} + C_{O_2}} \cdot \frac{C_{Mred}}{K_{Mred,ETC} + C_{Mred}} \tag{6.10}$$

Overall, ammonium oxidation is highly regulated by the availability and turnover of Mred and Mox. The affinities for substrates and the difference of maximum rates between the AMO, HAO and the E.T.C corresponding reactions might impact the process regulation (see Equation (6.6) to Equation (6.9)). Depending on the ammonium and oxygen consumption parameters, hydroxylamine accumulation will be most likely at high ammonium concentrations and limiting oxygen concentrations.

The kinetic explanation is in agreement with the experimental observations reported here. For instance, in the fed-batch tests where hydroxylamine was continuously dosed and performed at lower DO and high initial ammonium concentrations (batches 8 and 9 in Table 6.1 and Figure 6.2A) hydroxylamine accumulated the most. More N_2O was produced at lower DO concentrations (Figure 6.2B), as a strategy to use the available Mred to generate Mox without consuming oxygen. When DO was higher, less hydroxylamine accumulated and less N_2O was produced (Figure 6.2). Overall, the impact of DO on hydroxylamine accumulation shows that somehow the limiting step for the process is the Mox production (either resulting from ammonium consumption or oxygen consumption in E.T.C.).

The kinetic analysis proposed here, also easily fits with observed transient accumulation events of hydroxylamine reported in literature (without external addition of hydroxylamine). For instance, hydroxylamine has been transiently detected when switching from anoxic to aerobic conditions, in planktonic chemostat cultures of *Nitrosomonas europaea* [21, 149, 31]. At such conditions, the oxidized form of the electron carriers will be limiting at the beginning of the aeration phase, limiting the availability of Mox for hydroxylamine consumption. It makes it likely that hydroxylamine transiently accumulates until sufficient Mox is generated. Indeed N_2O peaks were detected just after the switch from anoxic to aerobic conditions in nitrification cultures

[21, 149, 31]. Consequently, N_2O was generated at the beginning of the transition to aerobic conditions to use the available Mred and convert it to Mox to generate sufficient Mox for hydroxylamine conversion. Similarly, in SBR processes with mixed cultures transient hydroxylamine production right after the start of the feeding phase has been detected [33]. A step increase in ammonium loading rate also showed to generate transient accumulation events in continuous systems [32, 34]. When a step-increase in ammonium load occurs, the system will tend to be more (kinetically) oxygen limited.

Finally, when performing batch tests with only ammonium, hydroxylamine has also been shown to accumulate [18, 19]. The initial high concentration of ammonium allowing a fast conversion can easily lead to oxygen uptake limitations and higher levels of Mred and accumulation of some hydroxylamine. Liu et al [18] measured higher hydroxylamine concentration peaks with higher initial ammonium concentrations. The higher the ammonium concentration the more likely it will be kinetically limited by oxygen. In the same study, different hydroxylamine accumulation pattern were also observed depending on the AOB/AOA and comammox strains used [18]. This would fit with the high dependency of hydroxylamine accumulation on the affinities and maximum rates of AMO and E.T.C. conversions. Thus, depending on the affinities or maximum velocities of the different AOB/AOA/comammox strains they will be able to maintain a sufficiently high Mox supply or reducing ammonium consumption to avoid hydroxylamine accumulation.

In the present study, during the batches (B) with only ammonium as substrate, no hydroxylamine accumulation was observed independent of the DO concentration imposed. It should be highlighted that the granular structure of our sludge might have masked putative transient accumulation of hydroxylamine in the inner parts of the granule, where DO concentrations are much lower than in the bulk liquid. Thus, hydroxylamine could be transformed before reaching the bulk liquid.

Overall, it should be highlighted that when having conditions with low oxygen and high ammonium concentrations, the ammonium oxidation or the consumption of oxygen in the electron transport chain could be limiting the hydroxylamine conversion step. This would favour hydroxylamine accumulation, if the oxidized electron mediators are not provided fast enough.

6.4.2. AMMONIUM CONSUMPTION IS REGULATED BASED ON HYDROXYLAMINE AND ELECTRON DONOR AVAILABILITY

The DO concentration not only triggered hydroxylamine accumulation, but also had a direct impact on the ammonium consumption rate. In the batch tests with continuous hydroxylamine feeding, ammonium consumption was increased when hydroxylamine was present at high DO (batches 5 and 6 in Figure 6.1 and Table 6.1). However, if DO was low, ammonium consumption was maintained or slightly reduced when hydroxylamine was fed (Figure 6.1, Table 6.1). The increase of ammonium consumption at high DO can be explained based on the fact that hydroxylamine conversion will be less limited by Mox (Figure 6.6). When hydroxylamine is added extra, the available Mred will increase which can boost the ammonium consumption (see Figure 6.7A).

In previous studies, the impact of hydroxylamine was only investigated during the so called “acceleration phase”, but never during continuous exposure. The

"acceleration phase" is related to the delay observed on reaching the maximum ammonium consumption capacity when adding an ammonium pulse to a nitrification system [43, 178]. The hypothesis was that the level of reduced electron mediator was initially limiting the ammonium oxidation [178]. Adding hydroxylamine in form of a pulse to an aerated system, before the addition of ammonium, reduced dramatically the acceleration phase [43]. It was hypothesised that hydroxylamine oxidation previous to the addition of ammonium increased the level of reduced electron mediator, allowing ammonium to proceed at maximal rate once it was added tot he system [43]. However, our study did not focus on the initial ammonium consumption rate as in [43, 178], but rather on the ammonium consumption rate once stable DO was reached. Our study indicates extra hydroxylamine can increase ammonium consumption when sufficient oxygen is available continuously.

When DO was low and hydroxylamine was provided, ammonium consumption was either maintained, slightly increased or reduced and hydroxylamine accumulated resulting in high N_2O formation in the fed-batch tests (Figure 6.1 and Figure 6.2). When hydroxylamine was fed continuously to the airlift reactor at different loading rates, ammonium oxidation was also reduced and DO increased gradually, indicating a decrease on the oxygen uptake rate (Figure 6.3A and SI). During the long term feeding of hydroxylamine, the DO might have been rate limiting especially in the inner parts of the granules. The conditions observed during the continuous feeding might have been comparable to the middle DO conditions of the fed-batch test experiments: low amounts of hydroxylamine accumulated and a low N_2O production was observed while ammonium consumption decreased with increasing hydroxylamine loading rates (Figure 6.3A). The range of DO triggering higher ammonium consumption rates might be impacted by other conditions. Continuous long term operation was performed at lower pH and temperature (ca. 7.6 and 22°C) than the batch tests (pH 8 and 30°C). At lower temperature and lower pH the ammonium consumption rate is decreased [179]. Consequently, maximum ammonium consumption rate will be lower and extra hydroxylamine dosing might have a different impact as limiting DO ranges will differ.

The reduction of ammonium consumption when hydroxylamine was provided and DO was kinetically limiting might be explained as follows: from a kinetic point of view other process competing for similar substrates (oxygen and Mred) are relatively faster than ammonium consumption, resulting in the reduced ammonium oxidation rate. Alternatively, when supplying extra hydroxylamine, more Mred is generated with higher DO. Thus, the affinity for Mred is less critical. If DO is not sufficiently high, the extra generated Mred is not redistributed to increase ammonium consumption. Oxygen will then limit the turnover of Mred to Mox, not providing the Mox needed for hydroxylamine conversion (Figure 6.7B).

Having a slightly faster oxygen consumption in the E.T.C than for ammonium consumption when oxygen is limiting makes sense as a strategy to avoid as much as possible intermediates accumulation (NH_2OH or NO) under oxygen limiting conditions. This is what was actually observed in the continuous airlift reactor, where ammonium consumption was reduced and dissolved oxygen in the system increased with increasing hydroxylamine loading rates (see Figure 6.3A and SI). It should be highlighted that even ammonium consumption was reduced, total nitrite production was not (Figure 6.3).

Indicating that hydroxylamine was mainly being transformed to nitrite (Figure 6.3).

With the observed increased bulk DO concentration over time, it would have been expected for AOB to use such extra DO to consume more ammonium. However, ammonium consumption rate was reduced. Suggesting that the E.T.C. rate was the main rate-governing factor in the process, as if more electrons were derived from NH_2OH there was less demand to derive electrons from full ammonium oxidation to nitrite. Consequently, reducing the ammonium consumption and also the oxygen consumption. Overall small differences between the parameters will efficiently regulate the whole process. As oxygen consumption during ammonium consumption needs to be generally faster than the E.T.C, otherwise, ammonium consumption would not take place. Understanding the coupling between each process would allow to further understand the central nitrogen metabolism regulation.

N_2O formation was detected both in the batch tests and during the continuous airlift reactor feeding (Figure 6.2 and Figure 6.17). Specifically, higher N_2O emissions were detected during the batch tests with hydroxylamine feeding at low DO concentration (Figure 6.2). At the conditions at which the tests were performed (pH 8 and 25°C and low nitritre concentrations), the abiotic reaction of hydroxylamine and free nitrous acid is not likely [58, 64]. The traditional biochemical pathway resulting in N_2O emissions when oxygen concentration is low, is called nitrifier denitrification [146]. Nitrite is reduced to N_2O to compensate limiting amounts of oxygen as terminal electron acceptor. However, in our experiments hydroxylamine accumulation might be more direct related to N_2O formation. Recently a biochemical route from hydroxylamine to N_2O was proposed to be mediated by cytP460 [57] in the absence of oxygen. At the low DO in granular sludge systems the deeper layers in the granules will be deprived of oxygen. To further confirm if either nitrite or hydroxylamine was being consumed to form N_2O, ^{15}N or/and site preference studies will be necessary.

Overall, when oxygen is high, the hydroxylamine oxidation step is in charge on regulating the whole AOB metabolism. At these conditions, if external hydroxylamine is available, ammonium consumption is boosted by the extra formation of Mred. It is interesting to realize that the 'maximal' ammonium oxidation rate can be increased in the presence of external added hydroxylamine. When oxygen is limiting, the preferential use of oxygen as terminal electron acceptor will reduce the ammonium oxidation rate, preventing accumulation of nitrification intermediates. Indirectly, hydroxylamine oxidation will be also regulated by the oxygen use in the E.T.C., if not sufficient Mox is provided hydroxylamine will accumulate. Consequently, the differences between the ammonium oxidation rate and the turnover of terminal acceptor will determine the putative accumulation of hydroxylamine.

6.4.3. MORE HYDROXYLAMINE LIMITED CONTINUOUS FEEDING STUDIES ARE NEEDED TO UNDERSTAND THE LONG TERM IMPACT OF HYDROXYLAMINE ON AMMONIUM OXIDATION

In the continuous airlift reactor hydroxylamine was efficiently transformed to nitrite, combined with a reduced ammonium oxidation. However, after ca. 3 months of operation with hydroxylamine feeding, the reactor operation deteriorated quite quickly (see Figure 6.13). Other indications of deterioration are the decreased EUB signal

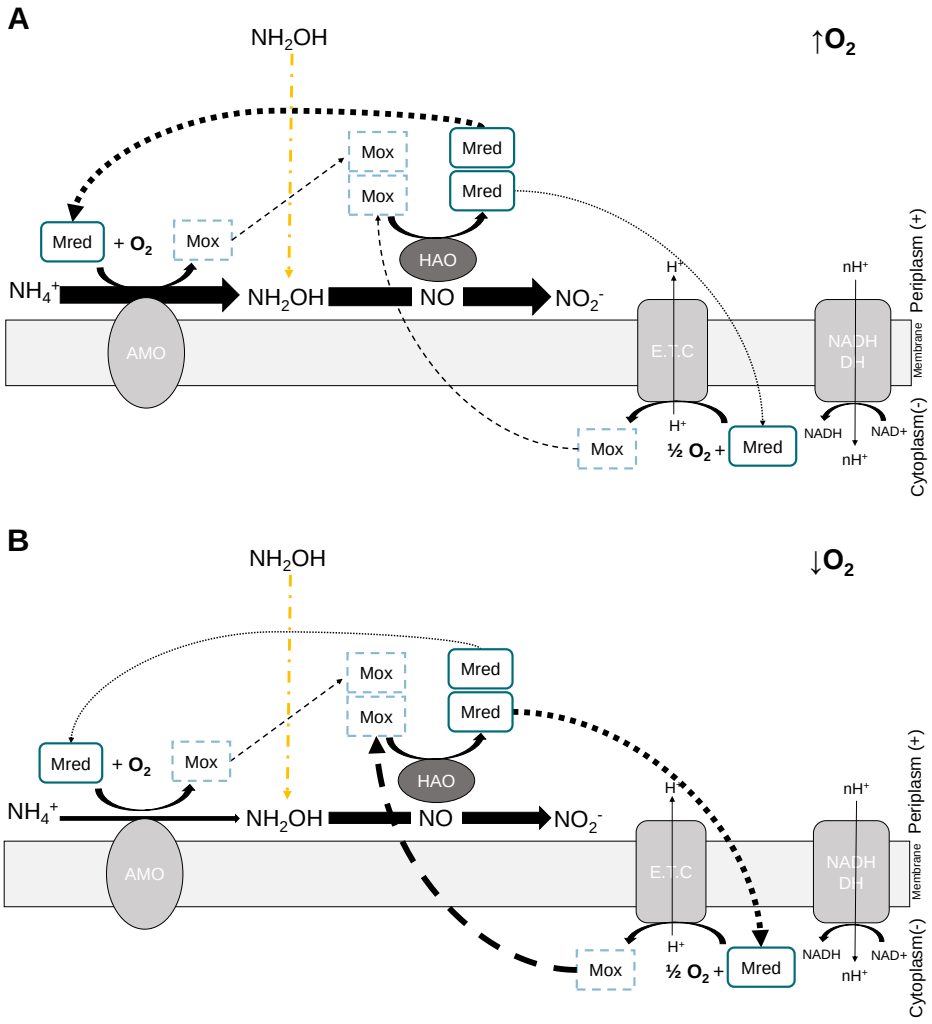


Figure 6.7: Redistribution of Mox/Mred leads to higher or lower ammonium consumption when external hydroxylamine is provided depending on the DO available on the system: A) High dissolved oxygen concentration, B) Low dissolved oxygen concentration.

observed with FISH over time, and the appearance of a mainly oligotrophic side-population (Figure 6.4 and Figure 6.17 and Figure 6.18). Different possibilities might explain this deterioration. At the working conditions (pH 7.7) hydroxylamine is mainly unprotonated (see Figure 6.19B), thus it can diffuse over the cell membrane. This gives an uncoupling effect and results in extra energy demand reducing the growth of bacteria. Another possibility was that the cell could not cope with the reduced ammonium

consumption, increased E.T.C. velocity and growth at the same time. Interestingly, the occurrence of the oligotroph community when hydroxylamine was continuosly fed to the reactor Figure 6.4 might indicate that they might be resistant to small hydroxylamine concentrations (<1 mg-N/L). Still, their activity was not directly targeted in the present work, it could be interesting to assess possible resistance and usage of hydroxylamine by these communities.

Previous studies with hydroxylamine were mainly performed by pulse like addition strategies [44, 49, 52, 47]. With these type of feedings a negative impact of hydroxylamine was reported by Harper and co-workers on the long run [44], while other studies used hydroxylamine as a strategy to promote partial nitrification over full nitrification [52, 47].

The only comparable study in terms of feeding strategies was performed by de Bruijn and co-workers [42]. A higher growth yield was reported when feeding mixotrophically ammonium and hydroxylamine than when feeding only with ammonium, and no deterioration was mentioned. Thus, indicating that *Nitrosomonas europaea* could grow mixotrophically with hydroxylamine. This would fit with the fact that the biomass concentration was maintained during the different hydroxylamine feeding (Figure 6.13C and Figure 6.15A). De Bruijn et al. also observed a bigger nitrogen imbalance [42] than observed in the present study. Their experiments were performed with planktonic cells and conditions (i.e 30% DO saturation, pH 8 and 30°C)[42] that are deviating from those in the present study (i.e. granular sludge, 22.3 ±1.2°C, pH 7.4±0.1, DO from 3.6±0.2 to 5.2±0.5 mg-O_2/L, see Figure 6.15). One putative explanation for the higher nitrogen imbalance observed by de Bruijn et al. is that at higher pH and temperature ammonium consumption is promoted (see Figure 6.20). Thus, if on top of that, an intermediate like hydroxylamine is fed, it is more likely that hydroxylamine accumulates; promoting a bigger nitrogen imbalance and the formation of N_2O emissions, as seen in the experiments of de Bruijn et al. [42]. It would be interesting to have a better characterization of NO/N_2O emissions, as due to technical problems N_2O was not followed during the whole operation with hydroxylamine.

Overall, to assess the possible long term impact of hydroxylamine feeding, transcriptomics and proteomics will be crucial to give more insight on the impact of hydroxylamine in nitrification. More studies will be needed with limiting hydroxylamine feeding to reach definitive conclusions.

6.4.4. IMPORTANCE OF THE HYDROXYLAMINE OXIDATION STEP IN MODELLING N_2O EMISSIONS

The hydroxylamine accumulation and the NO or N_2O production as a function of the oxygen concentration will be very sensitive to the kinetic parameters (half-saturation coefficient and maximum rate) governing each process step in a mathematical model. The importance of parameter estimations in model applications is crucial for a good model use and process understanding [180, 181]. The widely used Activated Sludge Models (ASM) did not include intermediate processes, such as nitrite or hydroxylamine [167]. This was justified by the main model purpose, estimating the effluent concentrations. The concentrations of intermediates of ammonium conversion to nitrate have a marginal impact on the nitrogen mass balance of a municipal wastewater treatment plant. In recent years the ASM have been extended for nitrite because of

the interest in partial nitrification/anammox process [182]. With the growing interest in greenhouse gas emissions also other intermediates in nitrogen conversion processes (NO and N_2O) became state variables in activated sludge models [144]. Parameters related to the hydroxylamine oxidation step in nitrification (r_{NH_2OH} in Figure 6.6), such as hydroxylamine half-saturation coefficient (K_{NH_2OH}) or maximum hydroxylamine oxidation rate ($r^{max}_{NH_2OH}$, K_{NH_2OH} or $r^{max}_{NH_3}$ in d^{-1} or mg-N/gVSS/h depending on the nomenclature and notation used) are usually not evaluated from dedicated experiments. Even, more importantly, the oxygen affinity of the E.T.C. during hydroxylamine oxidation as single substrate has rarely been determined. Hydroxylamine conversion related parameters have usually been obtained from multivariable optimization of a large set of parameters through ammonium, nitrite, DO and/or N_2O data fitting [175, 176]. Recently, Domingo-Felez et al. [177], presented a calibration methodology to obtain such parameters which takes into account hydroxylamine measurement data and dedicated respirometry experiments [177]. There are hardly or no good hydroxylamine conversion data with mixed cultures and only few experimental data with hydroxylamine conversion by pure cultures [42, 35, 183]. Furthermore, in view of the DO impact on ammonium and hydroxylamine oxidation rates as well as on the N_2O formation (see Figure 6.1 and Figure 6.2). Kinetic parameter determinations will be really sensible to oxygen concentrations. For instance to be able to determine maximum ammonium consumption rates one should be aware to avoid or reduce as much as possible oxygen limitations. With the hydroxylamine continuous addition experiments performed in the present study a maximum hydroxylamine conversion coefficient and apparent affinity constant were estimated (Figure 6.5). It should be noted that the parameters presented here are all apparent and performed with granular sludge. Thus, they might differ depending on the biofilm type, structure and microbial composition.

Also the inclusion of NO measurements would allow for a better understanding of the role of this other intermediate in nitrification [182, 184, 65]. NO measurements during hydroxylamine feeding might also be of interest to see if this compound transiently accumulates or is emitted when hydroxylamine is added.

6.4.5. IMPLICATION OF THE FINDINGS AND FUTURE CHALLENGES

In the present study the effect of DO on hydroxylamine by an AOB enriched granular sludge biomass was studied. One important aspect that was not investigated is the pH effect. The dissociation of nitrogen compounds has in general a strong impact on nitrification processes [137]. Hydroxylamine is a weak base (pKa value 5.94 at 25°C [159]) and will therefore change from NH_3OH^+ to NH_2OH in the physiological pH range (see Figure 6.19). In the simplified model proposed (Figure 6.6), pH can have several effects on the different reactions involved in ammonium oxidation: i) the equilibrium between protonated and unprotonated form of ammonium and hydroxylamine might impact its diffusion through the membrane, ii) Frijlink et al. [35] showed that ammonium oxidation was highly impacted by pH whereas hydroxylamine oxidation was not, reducing ammonium oxidation with lower pH iii) The difference in proton concentration between the periplasm and cytoplasm will impact the E.T.C. and potentially also the energy generation step. Besides, the intrinsic impact of pH on substrates and each conversion step rates, pH gradients usually exist in biofilm

systems and granule systems. Thus, different consumption rates might be expected depending on the location in the biofilm. This overall complexity of influence of pH on hydroxylamine conversion warrants a more detailed investigation in the future. The impact of intermediates (NO and hydroxylamine) on nitrous oxide production during nitrification can only be understood when the detailed biochemistry of these conversion has been revealed [97, 146]. Proteomics and transcriptomics techniques have emerged and strongly developed in recent years. Transcriptomics and proteomic studies in similar systems as the one studied here might help to reveal the enzymes involved in N_2O productions or the putative enzyme involved in NO conversion to nitrite (if there is one). As if forcing hydroxylamine conversion, one would expect that the enzymes related to its transformation to be up-regulated. As also discussed in the present study, the combination of limited hydroxylamine feeding with 15N and site preference techniques could help also to elucidate and further understand the fate of hydroxylamine in such systems depending on the conditions. For example, helping to reveal if it is nitrite or hydroxylamine the one being transformed to N_2O at low dissolved oxygen concentrations.

Finally, it should be highlighted that most of the wastewater treatment facilities operate at low DO concentrations, to minimise aeration energy and costs. Furthermore, fluctuations in ammonium concentrations are usually occurring [7]. These form the perfect conditions for hydroxylamine accumulation, according to what has been discussed in the present work and the transient hydroxylamine accumulations events observed in literature [18, 19, 33]. The transient accumulation that is seen in the bulk liquid is the result of consumption and production processes. Inside flocs and biofilms conditions also vary and DO is low. Thus, accumulation of hydroxylamine in the inner parts of the biofilms is likely, even due to the reactivity of hydroxylamine it cannot be measured in the bulk liquid. Consequently, the understanding of how small quantities of hydroxylamine might impact common side communities of AOB, such as NOB, anammox, DNRA, denitrifiers or neighbouring cells of AOB is important. More interestingly, the contribution of hydroxylamine to produce N_2O anoxically has still to be further investigated. Highlighting the importance of unders¬¬tanding intermediate steps to be able to understand the process as a whole.

6.5. CONCLUSIONS

OVERALL, there are still a lot of research questions related to hydroxylamine. However, in the present study limited hydroxylamine feeding allowed for a better understanding of the hydroxylamine metabolism by AOB, which could be summarized as follows:

- DO combined with high ammonium concentrations kinetically governs hydroxylamine accumulation and N_2O emissions. Lower DO leads to higher hydroxylamine accumulation and higher N_2O emissions.
- DO also determines the impact of hydroxylamine in ammonium consumption. When DO is sufficiently high, ammonium consumption can be increased, as more reducing equivalents are generated from supplemented hydroxylamine oxidation. On the contrary, when DO is low, ammonium consumption will be reduced.

- Long term hydroxylamine feeding reduced ammonium consumption, while nitrite production was maintained. Dissolved oxygen increased with increasing loading rate and that extra electron generated from hydroxylamine were allocated in the electron transport chain instead of ammonium oxidation.
- *Nitrosomonas* remained rather dominant during long term airlift reactor operation with hydroxylamine continuous feeding
- A better experimental estimation of hydroxylamine kinetic parameters will allow for improved NO/N_2O models.

6.6. SUPPLEMENTARY INFORMATION

6.6.1. MATERIALS AND METHODS

REACTOR SET-UP AND OPERATION: INOCULUM AND SYNTHETIC MEDIUM COMPOSITION

Granular biomass already performing partial nitritation was used as inoculum for the R1 operation. However, R2 inoculation was performed with a mixture of fast settling partial nitritation granular sludge and bioaugmentation with granular biomass. Biomass was obtained from a pilot scale reactor performing partial nitritation at side-stream conditions. The difference on the initial inoculum size distribution can be seen in (Figure 6.10 and Figure 6.12).

Synthetic wastewater was used, containing per litre of demineralized water: 0.73 g K_2HPO_4, 0.104 g KH_2PO_4, 1.2 g $NaHCO_3$, 0.25 mL of an iron solution and 0.12mL of a trace elements solution. $(NH_4)_2SO_4$ was used as ammonium supply at the desired concentration to achieve the targeted medium concentration (200 mg-N/L in R1 and 100-150 mg-N/L in R2, see Figure 6.9 and Figure 6.11)

Trace elements solution contained per litre of mili-Q water: 19.11 g EDTA (=titriph..), 0.43 g $ZnSO_4 \cdot 7H_2O$, 0.24 g $CoCl_2 \cdot 6H_2O$, 1.0 g $MnCl_2 \cdot 4H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 0.22 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (=1.25 mM Mo), 0.20 g $NiCl_2 \cdot 6H_2O$, 0.09 g $NaSeO_3$, 0.014 g H_3BO_3, 0.054 g $Na_2WO_4 \cdot 2H_2O$. The solution was adjust to pH 6 with solid NaOH and kept in the fridge. The iron solution contained per L of mili-Q water 9.14 g $FeSO_4 \cdot 7H_2O$ and 6.37 g EDTA.

6

RATE CALCULATIONS DURING BATCH AND FED-BATCH TESTS

To calculate consumption rates during the batch and fed-batch tests linear regression was performed with the ammonium measurement points over time. Thus, errors of the rates could be estimated using Excel Linest function. The ammonium consumption rate was calculated in two different moments during the tests: i) initial ammonium consumption rate concerning the first three ammonium time point measurements, ii) ammonium consumption rate during stable DO concentration concerning the ammonium time point measurements while DO was stabilized. Average DO concentration was calculated during the time interval that DO was stable (see Figure 6.8 in SI for an example). During the fed-batch phase, the addition of hydroxylamine over time did not represent in total more than the 5% of the batch volume, which was also compensated with the sampling over time. Thus, the liquid volume was considered constant over time. To assess the hydroxylamine rate r_{NH_2OH} in mg-N/L/h at each measurement point the general mass balance can be reordered as in Equation (6.11), assuming constant volume and that there is no effluent.

$$r_{NH_2OH} = \frac{\Delta C_{NH_2OH}}{\Delta t} - \frac{Q_{NH_2OH}}{V_r} \cdot C^{in}_{NH_2OH} \tag{6.11}$$

$\Delta\, C_{NH_2OH}$ mg-N/L being the difference in hydroxylamine concentration during two consecutive time point measurements. Δt in h being the increase of time between two consecutive measurement points. Q_{NH_2OH} in L/h being the hydroxylamine influent rate during the fed-batch tests.V_r in L is the reactor volume and $C^{in}_{NH_2OH}$ in mg-N/L is the hydroxylamine concentration in the feeding solution.

The increase or decrease of ammonium consumption (ΔqNH_4^+,FB/B in Table 6.1) when comparing the batch (B) and fed-batch (FB) was calculated using Equation (6.12).

$$\Delta q_{NH^+_{4,FB/B}} = \frac{q^+_{NH_4,FB} - q^+_{NH_4,B}}{q^+_{NH_4,B}} \cdot 100 \tag{6.12}$$

Regarding the N_2O balance in the liquid phase it can be derived as in Equation (6.13) and Equation (6.14).

$$\frac{dC_{N_2O,L}}{dt} = r_{N_2O} - T_{L\rightarrow G,N_2O} \tag{6.13}$$

$$T_{L\rightarrow G,N_2O} = k_L a_{,N_2O} \cdot (C_{N_2O,L}(t) - C^*_{N_2O,L}) \tag{6.14}$$

Where $\frac{dC_{N_2O}}{dt}$ refers to the accumulation of N_2O in the liquid phase in mg-N/L/d, rN_2O is the N_2O production rate in mg-N/L/d, and $T_{L\rightarrow G}$ is the transfer rate of N_2O from liquid to gas phase in mg-N/L/d. $C_{N_2O,L}$ refers to the concentration of N_2O in the liquid phase in mg-N/L, and k_La refers to the specific transfer coefficient of N_2O in d^{-1}. As $C^*_{N_2O,L}$ is the liquid concentration in the interphase in mg-N/L which must be in equilibrium with the partial pressure of N_2O in the gas phase, which can be calculated as in Equation (6.15).

$$C^*_{N_2O,L} = Hcp \cdot P \cdot f_{N_2O} \tag{6.15}$$

Where Hcp is the Henry constant defined via concentration in mol/L/atmN_2O, thus refers to the aqueous concentration of N_2O in the aqueous phase function of the partial pressure of N_2O in the gas phase under equilibrium. However, the fraction of N_2O in compressed air is around 310ppb [185], which is negligible. Thus, simplifying the accumulation term with increments, the rate calculation can be rearranged as in Equation (6.16).

$$r_{N_2O} = \frac{\Delta C_{N_2O}}{\Delta t} + k_L a_{,N_2O} \cdot C_{N_2O,L} \tag{6.16}$$

Then from the r_{NH_2OH} over time, total N_2O emitted can be calculated by trapezoidal integration. As there were several measurements per minute, a moving average was used to minimize the noise, specially the one due to the accumulation term.

REACTOR NITROGEN BALANCES

Mass balances during continuous operation of R1 and R2 can be derived from the mass balance in Equation (6.17) and applied to any soluble nitrogen compound.

$$V_r \cdot \frac{dC_N}{dt} = Q_1 \cdot (C^{in}_N - C^{out}_N) + Q_2 \cdot (C^{in}_N - C^{out}_N) + r_N \cdot V_r - T_{L\rightarrow G} \cdot V_r \tag{6.17}$$

Where Q_1 (L/d) refers to the mineral medium containing nutrients and ammonium and Q_2 (L/d) to the medium containing hydroxylamine. Q_2 was zero during R1 operation and the control phase of R2. During Phase II to Deterioration in R2 Q_1 and Q_2 were estimated based on the total flow rate recovered over time from the effluent

and the weight difference of the hydroxylamine medium. C_N (mg-N/L) refers to the concentration of the desired nitrogen compound. Superscripts in or out refer to the concentration in the influent or effluent, respectively. r_N (mg-N/L/d) refers to the volumetric rate of the nitrogen compound N. V_r (L) refers to the reactor working volume. t (d) refers to time. $T_{L\rightarrow G}$ (mg-N/L/d) is transfer of the soluble gases from liquid to gas phase.

For the rate calculation of nitrogen compounds that are soluble and not a gas (ammonium, nitrite and nitrate) Equation (6.18) can be used. Assuming that the accumulation term can be estimated as a difference quotient (increment between two consecutive days measurements).

$$r_N = \frac{[Q_1 \cdot (C_N^{out} - C_N^{in}) + Q_2 \cdot (C_N^{out} - C_N^{in})]}{V_r} + \frac{\Delta C_N}{\Delta t} \tag{6.18}$$

If we perform the balance for N_2O in the bulk liquid, the influent N_2O in the liquid is zero, thus the following derivation applies assuming that the accumulation term can be estimated as a difference quotient (increment between two consecutive days measurements)

$$r_N = \frac{(Q_1 + Q_2) \cdot C_N^{out}}{V_r} + \frac{\Delta C_N}{\Delta t} + T_{L\rightarrow G} \tag{6.19}$$

As in the batch tests, the reactor is sparged with air, which has a negligible concentration of N_2O. Thus, all the produced N_2O will be stripped. Consequently, the term $T_{L\rightarrow G}$ can be substituted as in Equation (6.16).

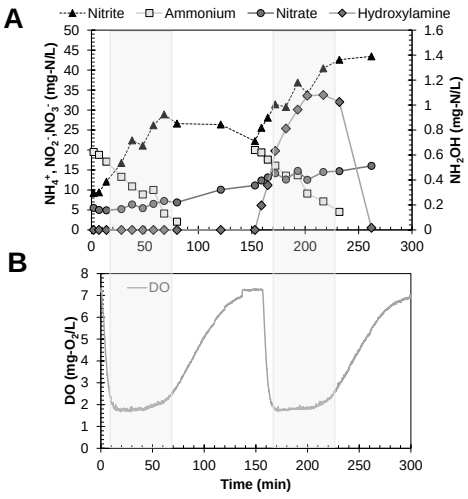


Figure 6.8: Example of time frame selection and measurement points to calculate ammonium consumption rates during stable DO in batch and fed-batch tests with data from test 9 in Table 1. The available data points falling within stable DO were used for calculations using linear regression. A) Nitrogen concentrations, B) DO concentrations.

K_LA CALCULATIONS

Oxygen mass transfer coefficient of either the reactors R1, R2 or during the respirometry tests were obtained by measuring dissolved oxygen concentration when either switching off the air or sparging with N_2 (abiotic controls) and switching back on the air supply. Oxygen concentration in the liquid was followed in time with a dissolved oxygen probe and k_La was estimated at different conditions (Table 6.2Table S1) assuming a first order differential equation (Equation (6.20)).

$$\frac{dC_{O_2}}{dt} = k_L a_{O_2} \cdot (C_{O_2,E} - C_{O_2}(t)) (Eq.S10) \tag{6.20}$$

Where, dC_{O_2}/dt (mg-O_2/L/d), refers to the accumulation of oxygen in time in the liquid phase $k_La_{O_2}$ (d^{-1}), is the oxygen mass transfer coefficient, $C_{O_2,E}$ (mg-O_2/L) refers to the constant oxygen concentration reached after starting again the aeration, is equivalent to the oxygen saturation concentration at the working conditions. C_{O2} (t) (mg-O_2/L) refers to the measured oxygen concentration at each time point (t).

Then, nitrous oxide mass transfer coefficient was estimated with N_2O and O_2 diffusivities as in Equation (6.21). Parameters used can be found in Table 6.2Table S1).

$$k_L a_{N_2O} = k_L a_{O_2} \cdot \sqrt{\frac{D_{F,N_2O}}{D_{F,O_2}}} (Eq.S11) \tag{6.21}$$

Where k_La refers to mass transfer coefficient, D_{F,O_2} and D_{F,N_2O} to oxygen and nitrous oxide diffusivities were obtained being $2.2.10^{-5}$ and $1.88.10^{-5}$ cm^2 s^{-1}, respectively [186, 187].

Table 6.2: Oxygen k_La values for batch tests performed with biomass from R2 and parameters used to estimate N_2O k_La. *Estimated from linear regression of air flow versus k_La experimental data from the rest of tests.

Test	Temperature (°C)	Air flow (L/min)	$k_La_{O_2}$ (h^{-1})	$k_La_{N_2O}$ (h^{-1})
R2,B/FB	25	10	3.2	3.0
R2,B/FB	25	40	3.9*	3.7
R2,B/FB	25	100	5.4	5.0
R2,B/FB	25	400	11.4	10.6

FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Pottered biomass and granules cryosection were used aiming to assess the quantity and location of the microbial population within the biofilm. Pottered biomass was resuspended in phosphate buffer and fixed for 1h in a 4% paraformaldehyde solution after washing 3 times with 1xPBS solution. After fixation, 3 washings with PBS were performed and stored in the freezer with a 0.6 parts of volume of ethanol. For cryosection, granules were washed in PBS for 1h and the same fixation procedure as for the pottered biomass was applied.

Before the cryosection, granules stored in the freezer were washed three times in a 0.1M phosphate buffer and resuspended in a 30% sucrose solution in 0.1M phosphate

buffer. A freeze-microtome (Leica CM3050S), freezing medium OCT (Tissue Tek®, Sakura 4583) and plastic mold (Tissue Tek Cryo mold, biopsy, 25mm x 20mm x 5mm (4557)) were used to cut the granules. The 10 µm slices containing different granule sizes per sample day were put onto pre-coated slides (Thermo Scientific® Menzel®, SuperFrost PlusTM).

Probe hybridization in both pottered and cryosectioned granules was performed by successively immersing the slides in 50%, 80% and 96% ethanol solutions for 3minutes each. After drying, two consecutive hybridization were performed at 55% formamide concentration for Nso190-Alexa594 probe, followed by a hybridization at 40% formamide concentration of EUB -Alexa488 and NOBmix-Alexa647 (see Table 6.3).

A confocal microscope (Leica TCS SP5) , equipped with Diode UV 405 nm, multilinear Argon 458, 476, 488, DPSS 561 nm, Helium-Neon 633 lasers and the subsequent required detectors were used for imaging.

Table 6.3: FISH probes used in this study

Probe	Probe mix	Dye	Sequence	Formamide (%)	Ref.
Nso-190	AOB	Alexa 594	CGA TCC CCT GCTTTT CTC C	55	[188]
EUB 338 I	EUB mix	Alexa 488	GCT GCC TCC CGT AGG AGT	40	[189, 190]
EUB 338 II	EUB mix	Alexa 488	GCA GCC ACC CGTAGG TGT	40	[189, 190]
EUB 338 II	EUB mix	Alexa 488	GCT GCC ACCCGT AGG TGT	40	[189, 190]
Nit3	NOB mix	Alexa 647	CCT GTG CTC CATGCT CCG	40	[191]
Ntspa662	NOB mix	Alexa 647	GGA ATT CCG CGCTCC TCT	40	[192]
Ntoga122b	NOB mix	Alexa 647	TCCGGGTACGTTCCGATAT	40	[193]
Nit3 competitor	NOB mix		CCT GTG CTC CAGGCT CCG	40	[191]
Ntspa662 competitor	NOB mix		GGA ATT CCG CTCTCC TCT	40	[192]
Ntoga122_1 competitor	NOB mix		TCW GGG TAC GTT CCG ATA T	40	[193]
Ntoga122_2 competitor	NOB mix		TCY GGG TAC GTT CCG ATG T	40	[193]

16S rRNA ANALYSIS

Biomass was pottered and centrifuged and stored in the freezer until further use every ca. 2 weeks. DNA from selected samples from different reactor operation days were extracted using the Soil DNA Isolation Plus KitTM (Norgen Biotek Corp, Canada) following the manufacturer protocol. Research and Testing Laboratory (Lubbock, Texas, USA) performed the DNA sequencing with an Illumina MiSeq platform. Bacterial 16S rRNA primer pair 515F-806R was used targeting the general bacterial regions V2-V4.

6.6.2. RESULTS

REACTOR OPERATION

First inoculation (R1)

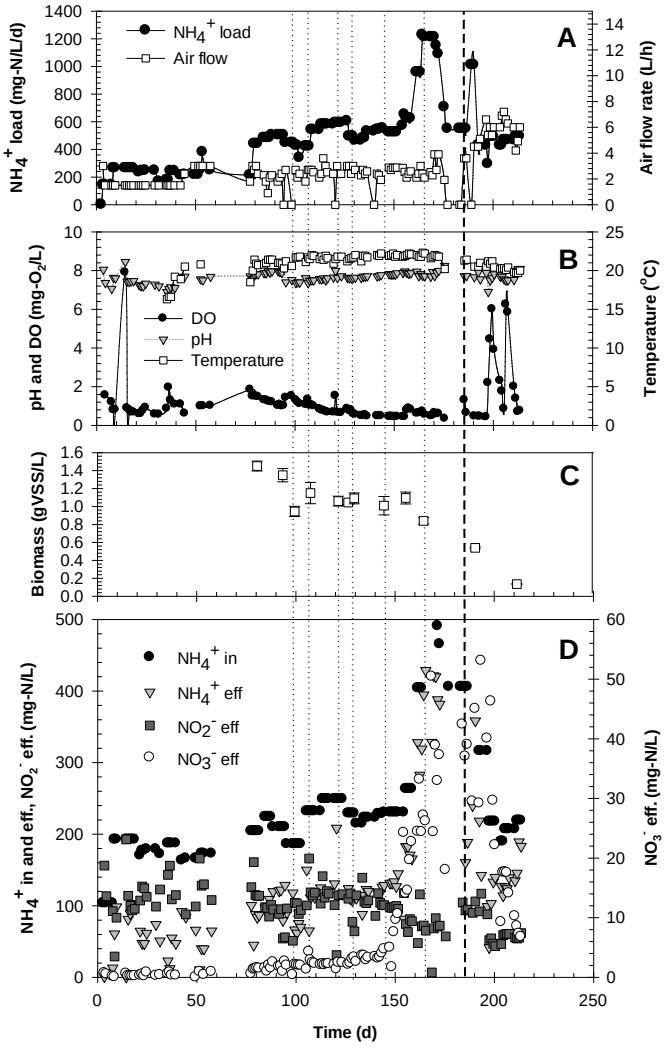


Figure 6.9: Reactor operation dynamics after first inoculation (R1): A) Ammonium load and air flow, B) Dissolved oxygen, pH and temperature, C) Biomass concentration, D) Nitrogen concentrations in the influent and effluent. Dotted line indicates a day when biomass was removed from the reactor, a respirometry tests was made and biomass was put back after the test. Dashed line indicates a day when biomass attachment on the top of the reactor started to occur daily.

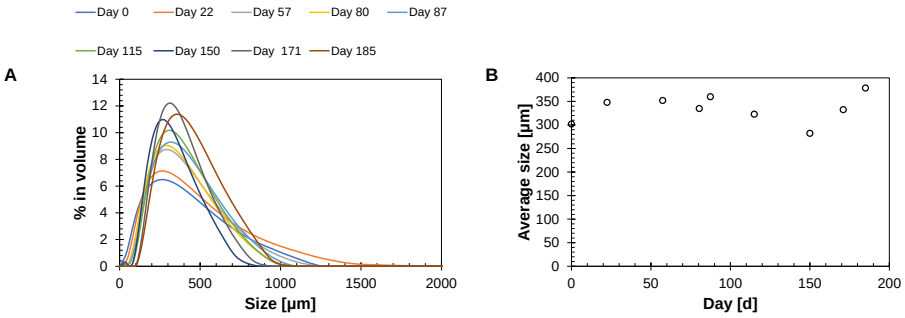


Figure 6.10: Granule morphology dynamics after first reactor inoculation (R1) during reactor operation: A) Particle size distribution, B) Average size distribution.

SECOND INOCULATION (R2 - Start-up and respirometries)

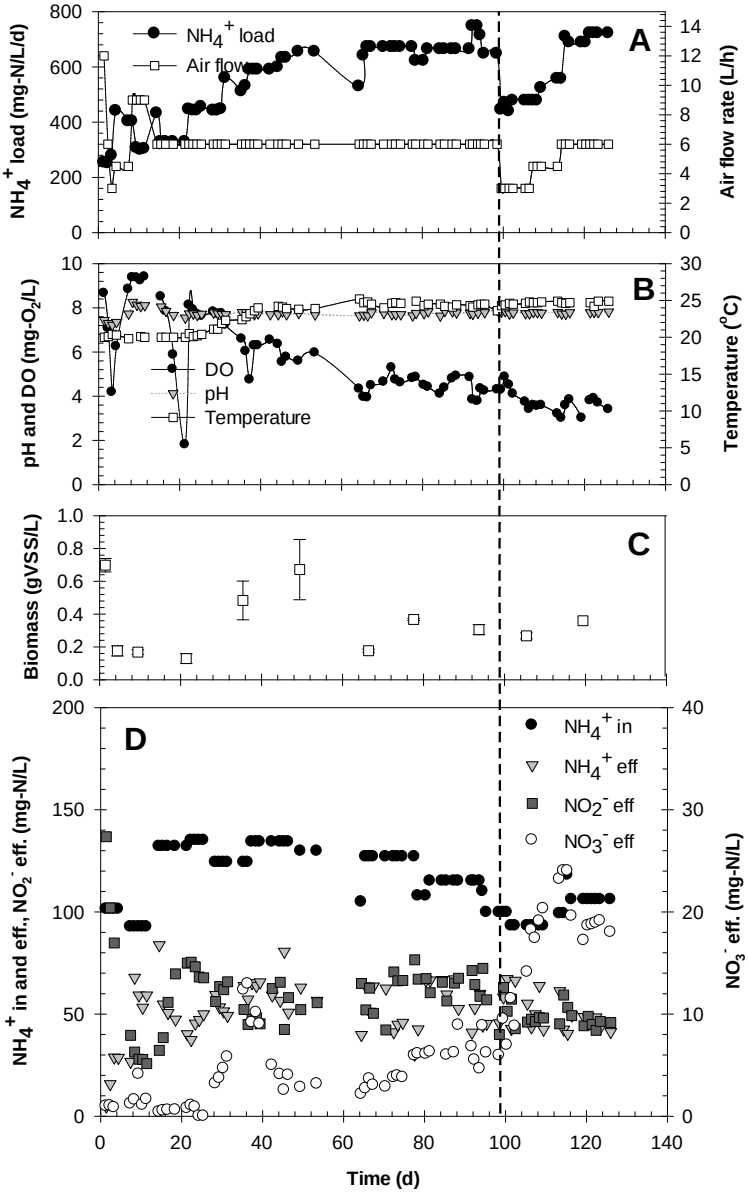


Figure 6.11: Reactor operation dynamics after 2^{nd} inoculation (R2): A) Ammonium load and air flow, B) Dissolved oxygen, pH and temperature, C) Biomass concentration, D) Nitrogen concentrations in the influent and effluent. Dashed line indicates a day when half of the biomass was removed and used for respirometry tests and not added back.

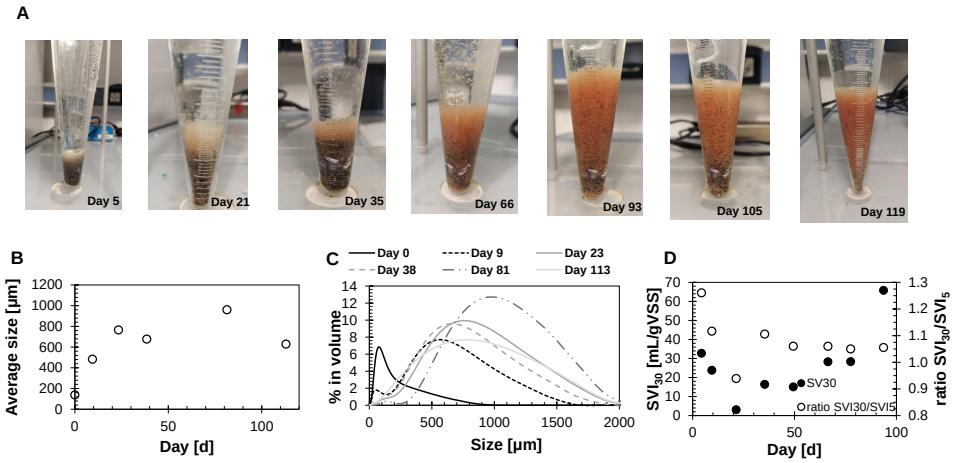


Figure 6.12: Granule morphology dynamics after second reactor inoculation (R2) during reactor operation: A) Color development, B)Average size, C) Particle size distribution, D) Settling velocity index (SVI).

SECOND INOCULATION (R2 - Hydroxylamine loading)

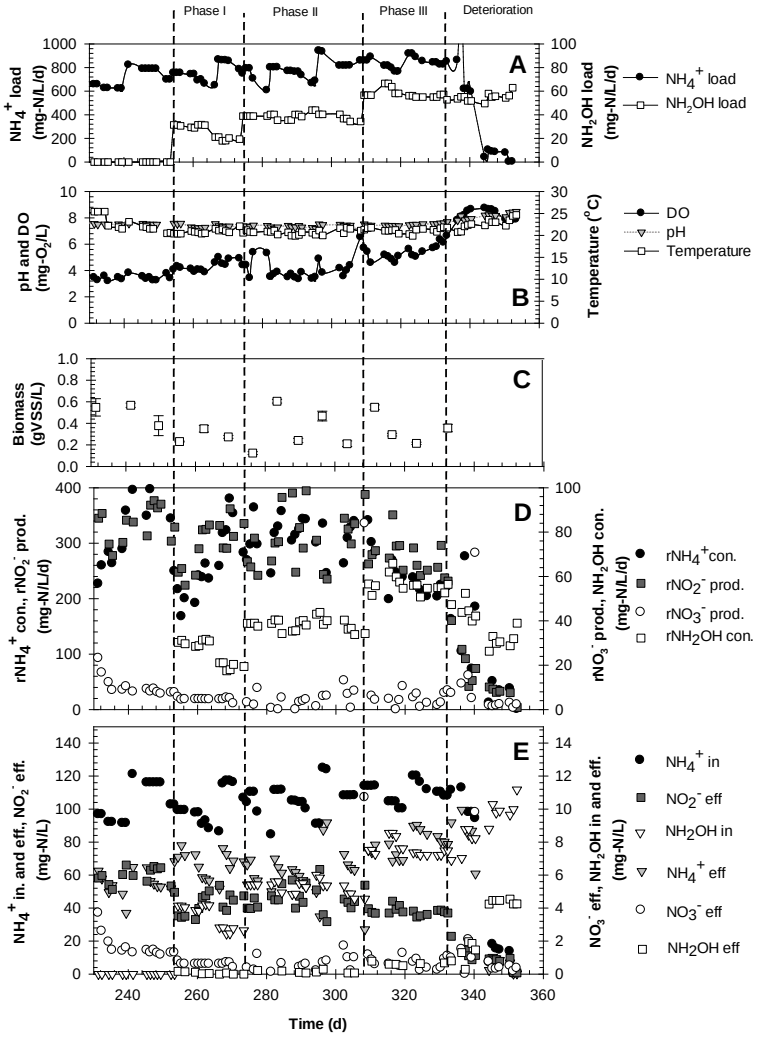


Figure 6.13: Reactor operation dynamics of R2 during different hydroxylamine loading rates: A)Ammonium and hydroxylamine volumetric rates, B)pH and dissolved oxygen (DO), C) Biomass concentration, D) Nitrogen consumption and production rates, E)Effleunt and influent concentrations.

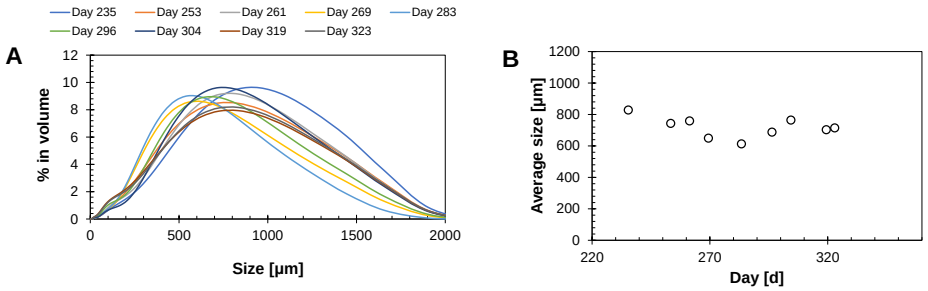


Figure 6.14: Granule morphology dynamics during R2 reactor operation with hydroxylamine loading: A) Particle size distribution, B)Average size.

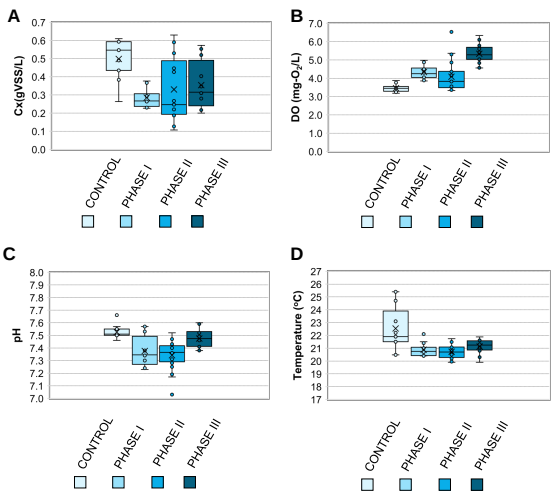


Figure 6.15: Comparison of different parameters during different hydroxylamine loads. A) Biomass concentration, B) Dissolved oxygen (DO) C)pH, D) Temperature.

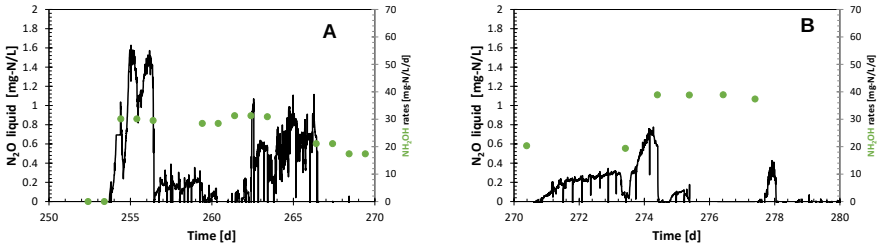


Figure 6.16: N_2O measurements in the liquid during continuous addition of hydroxylamine in the system.

MICROBIAL POPULATION DYNAMICS

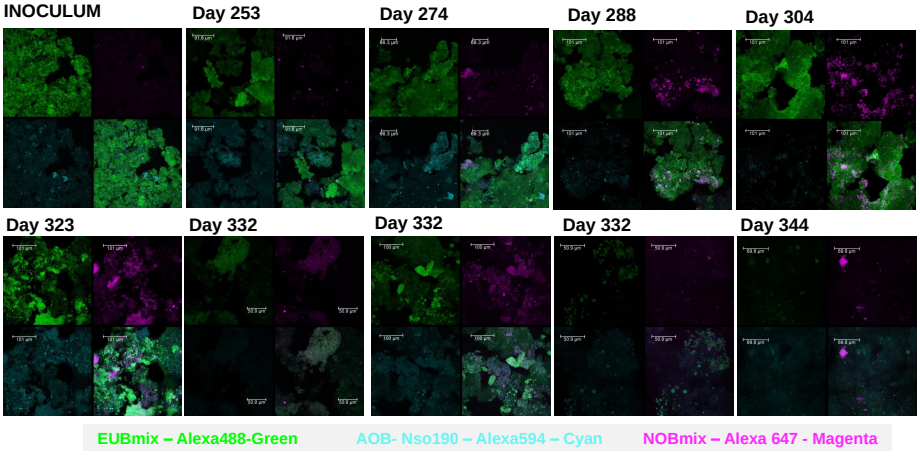


Figure 6.17: FISH results performed in R2 with pottered biomass over different operational days.

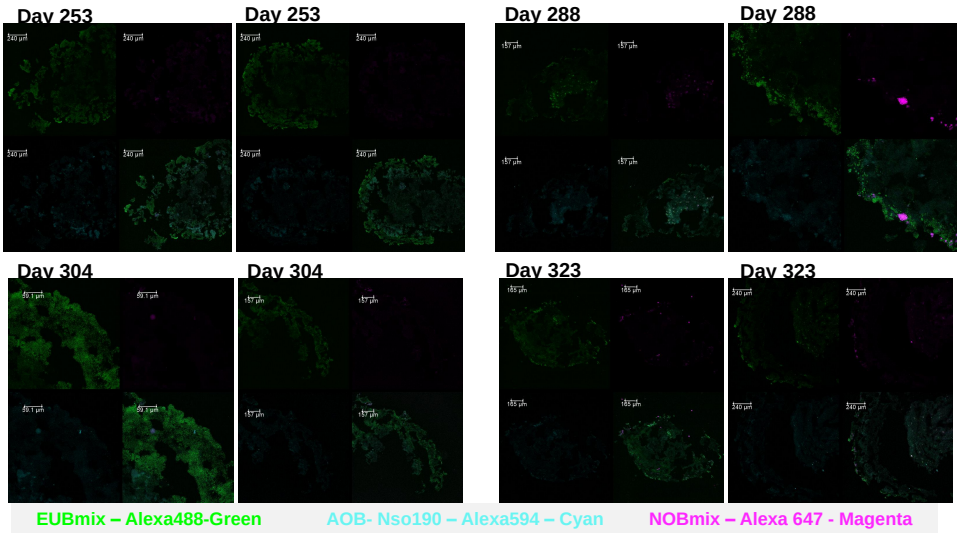


Figure 6.18: FISH results with cryosectioned biomass performed in R2 over different operational days.

6

EFFECT OF PH AND TEMPERATURE ON SUBSTRATES AND μ_{max}

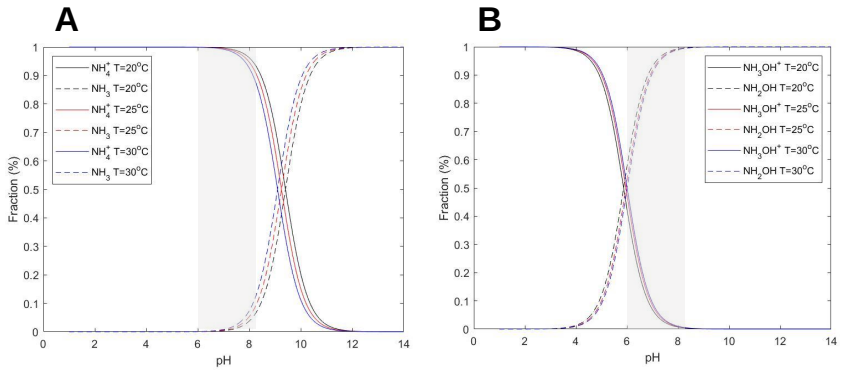


Figure 6.19: Equilibrium of the protonated and unprotonated forms of A) ammonium and B) hydroxylamine at different pH and temperature.

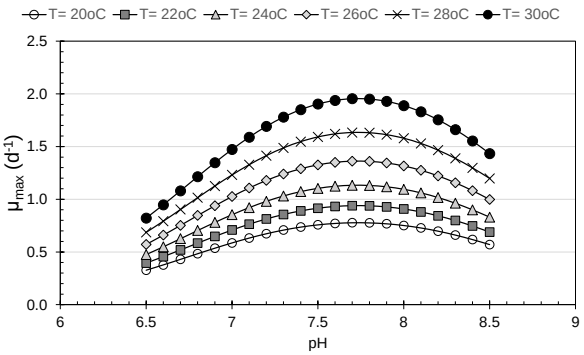


Figure 6.20: Impact of temperature and pH in the maximum ammonium oxidising bacteria growth rate according to [179].

7

Outlook

7.1. MINIMIZING ABIOTIC REACTIONS AS KEY TO UNDERSTAND THE BIOLOGICAL NITROGEN CONVERSIONS

ABIOTIC hydroxylamine conversion with free nitrous acid to N_2O is likely to occur in environments with elevated nitrite concentrations, slightly acidic pH (<7), mesophilic temperature (20-30°C) and the presence of metals in the medium (Chapter 3, Chapter 4 and Appendix A). Working at conditions that minimize the abiotic contribution when studying N_2O emissions would help in the interpretation of results.

Nevertheless, as seen in the present thesis, a wide range of factors influence hydroxylamine abiotic reaction with free nitrous acid. Interestingly, a 100% yield of conversion was never achieved in the present work or in literature [44, 64]. Indicating, that yet unidentified abiotic side-reactions might be hindering the characterization of chemical (and also biological) kinetic rates. Introducing real wastewater conditions would make it even more challenging. The potential catalysing effects of certain metals/minerals further complicates the evaluation of the chemical conversions. Still, a lot of room for the kinetic characterization of free nitrous acid abiotic reaction with hydroxylamine is left. Furthemore, the exact mechanism leading to transient hydroxylamine accumulation in biological cultures is yet to be characterized. It is important to remember that the transient hydroxylamine accumulation seen during some nitrification conditions is the result of consumption and production reactions. This further complicates the analysis of the contribution of putative abiotic conversions to the total N_2O emissions.

Not only hydroxylamine might react abiotically, but also nitric oxide (NO). The current central nitrogen model proposes that hydroxylamine is transformed to NO in AOB, AOA and comammox (see Chapter 2 and [27]). For instance, the enzymes responsible for transformation of NO to nitrite are yet to be identified. Alternatively, it is proposed that this last step could be chemically driven. Thus, not only hydroxylamine might react abiotically, but also other intermediates of the biological nitrogen cycle. Recently, the abiotic transformation of micropollutants by AOB and specifically hydroxylamine has been also highlighted [194, 31].

As a key take home message, the inclusion of abiotic controls with intermediates when assessing physiological activities might shed light into possible reactions that usually are unaccounted or attributed only to biological pathways. Specially now that abiotic reactions have been proposed to be part of the central metabolism of different microbial conversions (i.e. AOB, AOA, comammox etc). Consequently, the inclusion of abiotic controls including intermediates, will be even more important to be able to clarify and distinguish between biologically mediated reactions and chemical reactions. Thus, arising the next challenge: could natural isotopes abundance in combination with isotope labeling be implemented to distinguish between abiotic or biological conversions if the end product is the same?

7.2. HYDROXYLAMINE FEEDING TO UNDERSTAND THE NITROGEN METABOLISM REGULATIONS

IN the present thesis, short and long term hydroxylamine feeding strategies have been used to understand the metabolism rearrangement when dealing with hydroxylamine during anaerobic and aerobic ammonium oxidation (Chapter 5 and Chapter 6, see Figure 7.1).

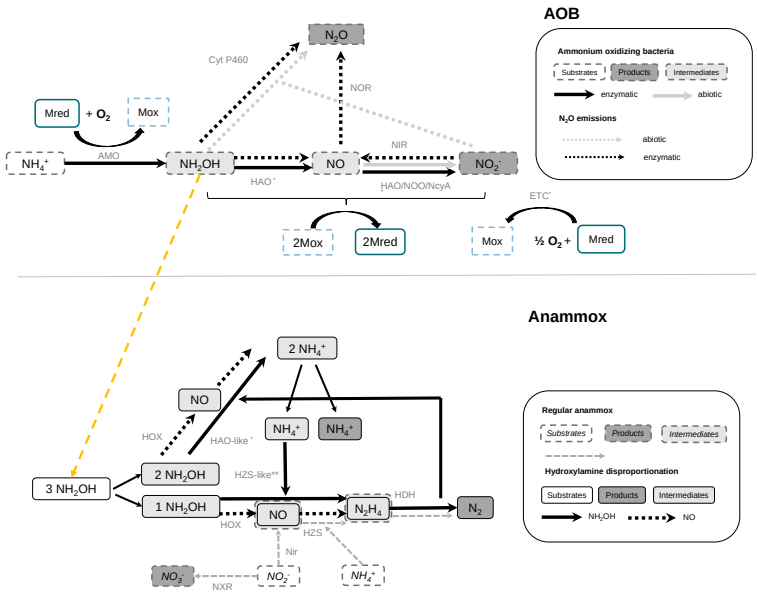


Figure 7.1: Summarized schematic model of the pathways analysed in the present thesis in ammonium oxidizing bacteria (AOB) and anaerobic ammonium oxidizing bacteria (anammox). Abiotic N_2O emissions from free nitrous acid and hydroxylamine is also depicted. Yellow dasehd line depicts putative hydroxylamine leakage from AOB that can reach anammox microbial communities. AMO – Ammonium monooxygenase, HAO – hydroxylamine oxidoreductase, ETC – Electron transport chain,HOX - hydroxylamine oxidoreductase, HZS - Hydrazine synthase, HDH - Hydrazine dehydrogenase, NcyA - Nitrosocyanine, NOO - Nitric oxide reductase, NOR - NO reductase, NIR - nitrite reductase, Cyt P460 - Cytochrome P460, NXR - Nitrite oxidoreductase.

For instance, short term hydroxylamine addition experiments performed with anammox showed that depending on the co-substrates concentrations the hydrazine accumulation was impacted (Chapter 5). Long term hydroxylamine continuous limited feeding reduced the need of nitrate production (Chapter 5) in the anabolic conversions. NO addition showed similar reduction in nitrate production [88] as when hydroxylamine continuous limited feeding was performed. The fact that less nitrate is produced during anammox growth, indicates that anammox is not dependent on the nitrate production for CO_2 fixation into biomass. Interestingly, similar experiments to those performed in the present work, or with other intermediates such as NO [88] might shed light on putative differences on the central metabolism of different anammox species (i.e.*Ca.*

Brocadia, vs. *Ca.* Keunenia).

In Chapter 6 it was shown how short and long term hydroxylamine continuous addition allowed to identify dissolved oxygen as the key substrate governing the hydroxylamine metabolism in a partial nitritation reactor. Low dissolved oxygen in combination to an ammonium pulse and continuous hydroxylamine feeding led to higher hydroxylamine accumulation and N_2O production in comparison to high DO tests. Ammonium consumption was increased when hydroxylamine was dosed continuously in combination with ammonium if DO was sufficiently high (ca. >3.6 mg-O_2/L). Both facts allowed to identify the turnover of electron carriers (Mred/Mox) as the rate limiting step when hydroxylamine was fed (see Figure 7.1).

Several transient hydroxylamine accumulation events in AOB cultures have been described in literature when switching from low to high activity (i.e. SBR operation, batch test, transient anoxia [43, 18, 19, 33], among others). In the present thesis, it was hypothesized that hydroxylamine transient accumulation was due to a transient limitation of the oxidized electron carriers equivalents (Mox). Mox are needed for hydroxylamine consumption to take place Chapter 6. However, further dedicated experiments would be needed to confirm the factors promoting hydroxylamine accumulation during ammonium oxidation. These type of experiments will be quite challenging, as small difference in the state of the cell could impact the results and the characterization, specially if performed with biofilm or sludge systems. A good strategy to characterize the factors impacting hydroxylamine accumulation experimentally, would be the use of planktonic cultures first, and then switching to more complex systems. Furthermore, the combination of limited continuous hydroxylamine feeding in combination with a detailed follow up of NO and N_2O production might be also a big possibility to identify pathways and factors promoting emissions from side metabolisms.

Finally, the characterization of the transcriptomic and proteomic response to limited continuous hydroxylamine feeding (or other intermediates), could also help in understanding the regulation of intermediates at the enzymatic level. Thus, the combined use of intermediate feeding strategies with the detection of up/down regulated pathways, could help identifying key regulatory steps and enzymes that are important to avoid intermediates accumulation. These kind of strategies might help in the future with the identification of the yet unknown putative enzymes responsible for NO transformations to nitrite in AOB, AOA and comammox (among others).

7.3. WHAT ABOUT THE OTHER MICROORGANISMS INVOLVED IN WASTEWATER TREATMENT?

THE generally widespread capacity of hydroxylamine usage among microorganisms in the wastewater treatment is at least intriguing (Chapter 2). Hydroxylamine consumption capacity is not only present in the nitrogen cycle, but also for example methanotrophs are able to use it [114, 195].

In the present thesis, when hydroxylamine was fed continuously in an anammox reactor (Chapter 5) no significant changes in the side communities were observed. Contrarily, a development of a side community of oligotrophs was detected when hydroxylamine was fed in the partial nitritation reactor (Chapter 6). Even the

conversions of this side communities were not dedicatedly studied with activity tests, it might indicate that some of the usually considered denitrifiers are at least resistant to small hydroxylamine accumulations (<1 mg-N/L) or either they thrive on the extra microbial subproducts resulting from hydroxylamine.

Transient accumulation of hydroxylamine might impact not only aerobic ammonium oxidizing bacteria, but also other microorganisms that are usually clustered together in wastewater treatment environments, soils or others. An interesting process where hydroxylamine impact could be relevant is partial nitritation anammox (PN/AMX). One of the bottlenecks for PN/A implementation at mainstream conditions is NOB proliferation. Interestingly, hydroxylamine transient accumulation by AOB could promote anammox conversion and inhibit NOB. Preliminary studies have pointed out to the usage of externally added hydroxylamine to promote PN/AMX over NOB [50, 51]. Next steps, could be directed to investigate if conditions promoting transient accumulation of hydroxylamine by AOB could be used to inhibit NOB in PN/AMX processes.

Understanding hydroxylamine impact on other microbial communities is needed: NOB, denitrifiers, DNRA, methanotrophs, etc. Even the occurrence of hydroxylamine-consuming aerobic heterotrophic bacteria or the discovery of putative microorganisms able to grow on hydroxylamine. Investigating either the capacities to resist hydroxylamine inhibition or identifying other microorganisms able to consume hydroxylamine could broaden our understanding of the metabolism of this compound in the nitrogen cycle and also unravel unidentified microbial interactions.

7.4. THE UNRESOLVED MYSTERIES SURROUNDING HYDROXYLAMINE

THE intermediate conversion steps of many nitrogen cycle metabolic pathways present challenges and unresolved questions. Focusing on hydroxylamine the mysteries and unresolved questions are wide and various (see Chapter 2). One of the key unresolved questions corresponds to the characterization of the conversion step of hydroxylamine to nitrite in AOB, AOA and comammox. Solving this puzzle might help also to further understand the N_2O emission pathways related to hydroxylamine. The experimental characterization of the factors that trigger hydroxylamine transient accumulation would also be important to investigate putative interactions between microbial communities based on hydroxylamine. Finally, a proper experimental determination of hydroxylamine energy Gibbs of formation (Chapter 5) would allow for an accurate thermodynamics analysis of putative nitrogen conversions involving hydroxylamine. Overall, to improve hydroxylamine related research, hydroxylamine measurement techniques need to be further developed Chapter 2. Ultimately, more precise and easily implemented hydroxylamine measurement techniques could allow for a more widespread measurement of this compound. The development of better measurements techniques will definitely lead to a better understanding of the role of hydroxylamine in the nitrogen cycle.

A

KINETIC CHARACTERIZATION OF ABIOTIC HYDROXYLAMINE REACTION WITH FREE NITROUS ACID TRANSFORMATION

A

A.1. INTRODUCTION

AFTER assessing in Chapter 2 and Chapter 3 that abiotic N_2O production from hydroxylamine and free nitrous acid could occur at environmental conditions relevant for wastewater treatment (pH 7 to 7.5, 20-30°C and high nitrite concentrations). Further characterization of the factors impacting the abiotic reaction of free nitrous acid and hydroxylamine were assessed with a special focus on temperature, concentration of metals and pH.

A.2. MATERIALS AND METHODS

A First screening was performed (Table A.1) and then selected conditions were tested again in duplicate (Table A.2). A change of laboratory took place between the first and second characterization (identified as old/new laboratories). Medium used was a 0.05M phosphate buffer with ratios of monobasic and dibasic salt adapted depending on the pH conditions to test (Table A.1 and Table A.2). pH was controlled with 0.5M NaOH and 0.5M HCl solution and temperature was controlled with an external jacket at the desired conditions (Table A.1 and Table A.2). The impact of metals concentrations was tested by using a mixture of a trace element solution and iron solution (described in Chapter 3, Chapter 5 and Chapter 6) at different concentrations: i) absence (identified as no metals), ii) 0.25 and 0.125 mL/L of the iron and trace elements solution, respectively (identified as metals) or iii) 2.25 mL/L or 1.25 mL/L of the iron and trace elements solution, respectively (identified as 10xmetals). Nitrite concentration mimicked side-stream conditions (i.e., wastewater resulting of the dewatering of the digested sludge) and was 600 mg-N/L in all tests. Initial hydroxylamine was targeted to 0.35 mg-N/L in all tests. Abiotic tests were performed by first addition of nitrite and the selected concentration of metals (if applicable), later a pulse of hydroxylamine hydrochloride solution was added. Samples for hydroxylamine rate determination were taken overtime, mixed with a sulfamic acid solution and measured according to Frear and Burrell [117] (see Materials and Methods in Chapter 3 and Chapter 4 for a more detailed description). N_2O in the off-gas was followed online (Servomex 4900 infrared gas analyzer). Free nitrous acid and N_2O rate was calculated as in Chapter 4. Hydroxylamine consumption rate (r_{NH_2OH}) was calculated by linear regression.

Table A.1: Abiotic batch tests experiments conditions to assess the impact of different parameters in the reaction between hydroxylamine and free nitrous acid. Initial targeted hydroxylamine concentration was 0.35 mg-N/L. Final reactor working volume was 1L. cons. – Consumption, max. – maximum, prod. – production. Kinetic constant was determined only in those tests were hydroxylamine was measurements were performed.

Test	Temp. (°C)	pH	Iron solution (mL/L)	Trace elements solution (mL/L)	Air gas flow (L/min)	FNA (mg-N/L)	$rNH_2OHcons.$ (mg-N/L)	$rN_2Omax.$ prod. (mg-N/L/h)	Yield (%)	$k_{abiotic}$ (L/mmol/h)
1.No metals, 20°C	20	6.74	0	0	0.18	0.34	-	0.053	29.0	-
2.Metals, 20°C	20	6.66	0.25	0.12	0.17	0.32	0.11	0.078	30.6	19
3. Iron, 20°C	20	6.68	0.25	0	0.212	0.33	0.082	0.057	29.4	15
4. No metals, 30°C	30	6.65	0	0	0.25	0.30	0.099	0.060	26.4	21
5. Metals, 30°C	30	6.6	0.25	0.12	0.25	0.27	0.26	0.208	33.1	58
6. 10xMetals, 30°C	30	6.64	2.5	1.2	0.23	0.27	0.53	0.373	33.7	188
7. 10xIron, 30°C	30	6.65	2.5	0	0.25	0.27	-	0.330	34.4	-
8. 10xTrace, 30°C	30	6.65	0	1.2	0.25	8.29	-	0.173	40.4	-
9. pH=5, 10xMetals, 30°C	30	5.15	2.5	1.2	0.33	1.49	-	2.5±0.5	64.4	-
10. pH=6, 10xMetals, 30°C	30	5.9	2.5	1.2	0.33	0.01	-	1.3±0.04	60.1	-
11. pH=8, 10xMetals, 30°C	30	7.9	2.5	1.2	0.33	0.34	-	0.054	19.3	-

A.3. RESULTS AND DISCUSSION

A.3.1. LOW PH AND THE PRESENCE OF METALS AS TRIGGERING FACTORS OF HYDROXYLAMINE AND FREE NITROUS ACID ABIOTIC CONVERSION

THE impact of different conditions to the abiotic transformation of hydroxylamine and free nitrous acid was assessed. Generally, N_2O production trends were comparable, but absolute values differed (see Figure A.1). Metals concentrations were identified as an important factor, as higher metals concentrations (10x metals) led to up to 6 fold higher N_2O production than when no metals were added in the test (Figure A.1A). When trying to identify whether iron or the metals present in the trace elements solution were impacting the most the abiotic rate, tests with only one of those solutions were performed (Test 7 and 8 in Table A.1 and Table A.2). Iron had a slightly higher impact on the N_2O production rate than the trace element solution (Figure A.1A).

Temperature had a big impact on the N_2O production rate, particularly if metals were present. At 30°C a 4 to 6 fold N_2O production rate increase was observed compared to tests performed at 20°C, with metals (old lab) and 10xmetals (new lab), respectively (see Figure A.1B). When metals were not present, there was not a big increase on N_2O production rate with a 10°C temperature difference (see Figure A.1B).

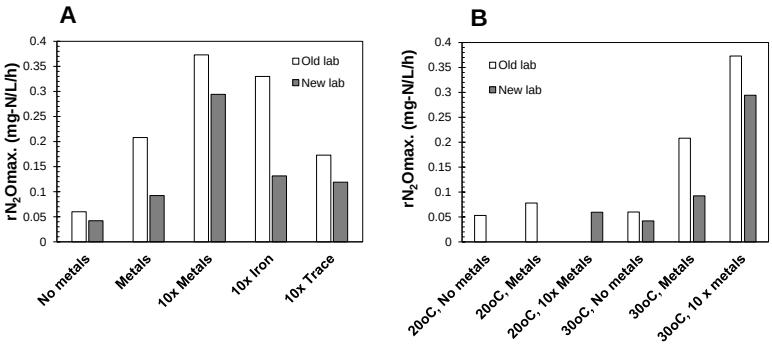


Figure A.1: Maximum reported N_2O production rate depending on the conditions tests: A) Impact of metals concentration, B) Impact of temperature and metals. Missing columns indicate tests that were not performed. Notice the slight difference on pH between tests (Table A.1 and Table A.2).

Finally, low pH was identified as the major factor triggering the production of N_2O (see Figure A.2). At lower pH (<6), constant NO emissions were also detected when nitrite was added (data not shown), due to nitrite decomposition at low pH. Small variations in pH led to a significant difference in the maximum N_2O production rate and hydroxylamine consumption rate (see Figure A.2). For example, see test 4, 5 and 6 in Table A.2. Hydroxylamine recovery yield as N_2O increased with decreasing pH, but was

never higher than 60% (Figure A.2B). Most likely, due to yet unidentified side reactions.

Volumetric N_2O rates reported here at around pH 7 (ca. 0.05 to 0.37 mg-N/L/h) depending on the metals concentration were in the range with those reported in Chapter 3 and Chapter 4 (0.2-0.25 mg-N/L/h and 0.057 to 0.1 mg-N/L/h, respectively).

The trends observed in the present section are also in agreement with those reported by Su and coworkers [64], where the pH was also identified as the main factor impacting the abiotic transformation. It was also observed a difference of 1 order of magnitude increased in the hydroxylamine conversions depending if mineral medium instead of demi water was used [64]. However, it was concluded that abiotic conversion would only be relevant at acidic pH [64]. Nevertheless, the impact of metals on the production rate was not assessed at neutral pH, only at pH 8 and at pH 4.5. Thus, taking into account that metals concentrations are way higher in wastewater treatment compared to those found in demineralized water ([196]), abiotic hydroxylamine reaction with free nitrous acid might still occur at a relevant rate at neutral pH. For instance, as it has been showed in Chapter 4 and in Figure A.1 here.

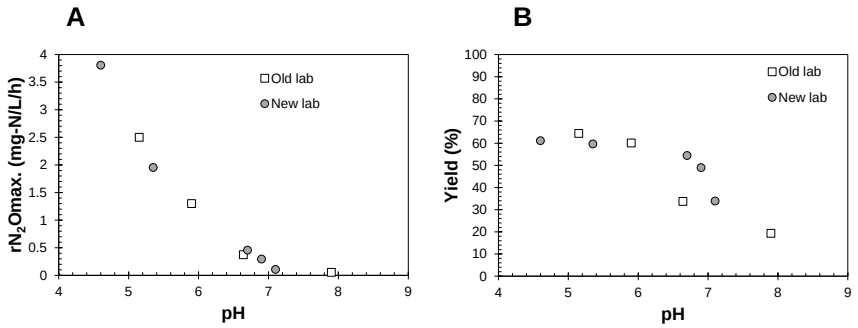


Figure A.2: Impact of pH on abiotic conversion of hydroxylamine with free nitrous acid: A) Maximum N_2O production rate and B) Reaction yield. Tests performed with 10xMetals and at 30°C.

A.3.2. pH AS THE MAIN GOVERNING FACTOR OF THE REACTION RATE

In the present study, differences were observed between tests performed at the old and new laboratory. Mainly they could be attributed to differences in the pH, as small variations impacted a lot the results (Figure A.1 and Figure A.2). Differences in the N_2O curve shape were observed (Figure A.3). Overall, resulting in the impossibility to fit a first order reaction (Equation (A.1)) to most of the characterization results performed in the new laboratory (Table A.2). Using the kinetic model different factors (i.e. gas flow rate, kLa, constant) were assessed in order to check if the plateau like curve could be

Table A.2: Abiotic batch tests experiments conditions to assess the impact of different parameters in the reaction between hydroxylamine and free nitrous acid. Initial targeted hydroxylamine concentration was 0.35 mg-N/L. Final reactor working volume was 1L. 0.4 L/min of air was used. cons. – Consumption, max. – maximum, prod. – production.

Tests	Temp. (°C)	pH	Iron solution (mL/L)	Trace elements solution (mL/L)	FNA (mg-N/L)	rNH_2OH con. (mg-N/L/h)	rN_2Omax. prod. (mg-N/L/h)	Yield (%)
1. 10xMetals, 20°C	20	7.2	2.5	1.25	0.10	0.346±0.005	0.059±0.009	41±7
2. No Metals, 30°C	30	6.9	0	0	0.15	0.201±0.005	0.042±0.007	26±11
3. Metals, 30°C	30	6.9	0.25	0.125	0.15	0.390±0.009	0.09±0.03	45±27
4. 10xMetals, 30°C	30	6.9	2.5	1.25	0.15	0.33±0.01	0.294±0.009	49±1
5. 10xMetals, 30°C	30	7.1	2.5	1.25	0.09	0.30±0.01	0.108±0.002	33.9±0.5
6. 10xMetals, 30°C	30	6.7	2.5	1.25	0.24	0.51±0.01	0.46±0.04	54±7
7. 10xIron, 30°C	30	6.9	2.5	0	0.15	0.345±0.08	0.131±0.009	50±6
8. 10xTrace, 30°C	30	6.9	0	1.25	0.15	0.528±0.005	0.119±0.004	60±6
9.pH=4.6, 10xMetals, 30°C	30	4.6	2.5	1.25	28.42	0.6±0.8	3.80±0.09	61±6
10. pH=5.4, 10xMetals, 30°C	30	5.4	2.5	1.25	5.25	0.56±0.04	1.9±0.2	60±5
11. pH=8, 10xMetals, 30°C	30	8	2.5	1.25	0.01	-	-	-

simulated, but any of the factors yielded successful results (Figure A.3B). However, the first order reaction could be fitted to the results of the characterization performed in the old laboratory (see Figure A.3A, Table A.1). It should be highlighted that the kinetic constants obtained here are higher (from 15 to 188 L/mmol/h) than those obtained by Su and coworkers (kinetic constants lower than 20 L/mmol/h at pH 6) [64]. However, kinetic constants were calculated for the experiments performed with demineralized water, temperature was between 24-26°C and initial nitrite was 249.2 mg-N/L. Thus, quite different conditions than those tested in the present section (Table A.1 and Table A.2).

$$r_{NH_2OH} = k_{NH_2OH,abiotic} \cdot C_{NH_2OH} \cdot C_{HNO_2} \tag{A.1}$$

In view of the present results and the impossibility to fit the first order reaction model to part of the results of the second characterization, it was hypothesized that a side reaction of hydroxylamine was impacting the hydroxylamine reaction emissions and recovery yield. For instance, it was known that high cupper contents were found in the water in the new laboratory, as blue crystals precipitates were formed in the influent when feeding a biological reactor. Consequently, it was assessed the impact of using mili-Q water or demi-water in the tests (with the supplement of metals), but no significant difference could be observed (data not shown). Another possibility for such different behaviour could be due to difference in free nitrous acid caused by the small differences in pH. For instance, in most of the tests performed in the second characterization free nitrous acid was lower than initial hydroxylamine (0.35 mg-N/L) (see Table A.2). Whereas in the first characterization free nitrous acid concentrations were closer to the initial hydroxylamine addition (Table A.1). Thus, it could have affected the initial maximum rate reported and the N_2O curve shape, as free nitrous acid might have been limiting in the second characterization.

Furthermore, hydroxylamine was added as hydroxylamine hydrochloride salt dissolved in water. Thus, its addition generally causes a small descent in the pH before the pH control is able to return to the set point. Consequently, small variations during addition might have caused differences in initial free nitrous acid, thus different N_2O productions rates and shapes.

No definitive explanation could be found for the differences observed during the characterizations. Consequently, highlighting the sensitivity of such abiotic reaction

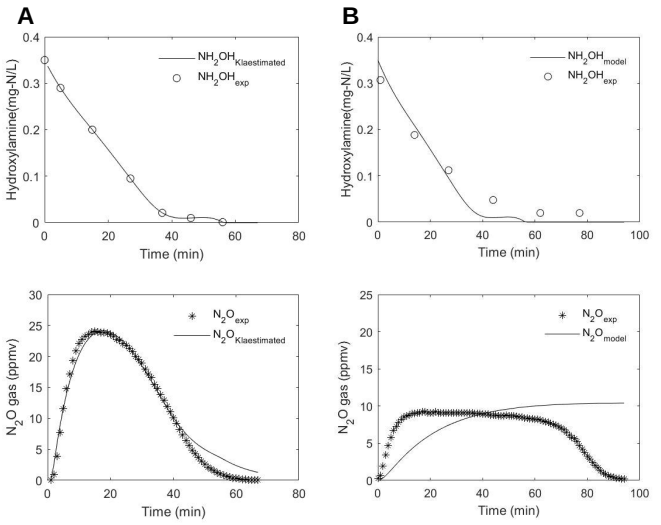


Figure A.3: Experimental and simulated results assuming a first order reaction as in Equation (A.1) of tests performed with 10xMetals and 30°C A) Test 5 in Table 1, B) Test 4 in Table 2.

to small changes. These shows the importance of characterizing the abiotic reactions with hydroxylamine in each specific set of conditions used. The characterization of such conversion with, for example, real wastewater can be an even bigger challenge.

A.4. CONCLUSIONS

OVERALL, to minimize N_2O emissions from abiotic sources low nitrite concentrations can be used, also higher pH (ca. 8) and lower temperature (ca. 20°C or less) would favour low abiotic reactions rates. Finally, the less supplemented and limited the metals are in solution, the lower the abiotic N_2O emissions would be. The results of this section confirm that side-stream conditions (high nitrite, temperature and likely high concentrations of other compounds generated during anaerobic digestion) are the perfect conditions for hydroxylamine abiotic conversions. Consequently, if the aim would be to characterize biological emissions, working at conditions that minimize abiotic transformations would be of importance. At the same time, the characterization of the chemical conversions of intermediates at the working conditions of each particular case would be crucial, in view of the sensitivity of the abiotic reactions to both pH and metals concentrations.

REFERENCES

[1] H. Lees, Hydroxylamine as an intermediate in nitrification, Nature **169**, 156 (1952).

[2] B. Kartal, J. v. Kuenen, and M. Van Loosdrecht, Sewage treatment with anammox, Science **328**, 702 (2010).

[3] M. M. Kuypers, H. K. Marchant, and B. Kartal, The microbial nitrogen-cycling network, Nature Reviews Microbiology (2018), 10.1038/nrmicro.2018.9.

[4] F. Haber and R. Le Rossignol, Uber die technische Darstellung von Ammoniak aus den Elementen, Zeitschrift fur Elektrochemie und angewandte physikalische Chemie **19**, 53 (1913).

[5] J. W. Erisman, M. A. Sutton, J. Galloway, Z. Klimont, and W. Winiwarter, How a century of ammonia synthesis changed the world, Nature Geoscience **1**, 636 (2008).

[6] J. B. Howard and D. C. Rees, Structural basis of biological nitrogen fixation, Chemical Reviews **96**, 2965 (1996).

[7] G.-H. Chen, M. C. van Loosdrecht, G. A. Ekama, and D. Brdjanovic, *Biological wastewater treatment: principles, modeling and design* (IWA Publishing, 2020).

[8] A. Mulder, A. Graaf, L. Robertson, and J. Kuenen, Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor, FEMS Microbiology Ecology **16**, 177 (1995).

[9] M. S. Jetten, M. Strous, K. T. Van de Pas-Schoonen, J. Schalk, U. G. van Dongen, A. A. van de Graaf, S. Logemann, G. Muyzer, M. C. van Loosdrecht, and J. G. Kuenen, The anaerobic oxidation of ammonium, FEMS Microbiology Reviews **22**, 421 (1998).

[10] M. Strous, J. Heijnen, J. Kuenen, and M. Jetten, The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms, Applied Microbiology and Biotechnology **50**, 589 (1998).

[11] A. A. Van De Graaf, P. De Bruijn, L. A. Robertson, M. S. Jetten, and J. G. Kuenen, Metabolic pathway of anaerobic ammonium oxidation on the basis of ^{15}N studies in a fluidized bed reactor, Microbiology **143**, 2415 (1997).

[12] C. Hellinga, A. Schellen, J. W. Mulder, M. Van Loosdrecht, and J. Heijnen, The SHARON process: an innovative method for nitrogen removal from ammonium-rich waste water, Water Science &Technology **37**, 135 (1998).

[13] U. Van Dongen, M. S. Jetten, and M. Van Loosdrecht, The SHARON®-Anammox® process for treatment of ammonium rich wastewater, Water Science and Technology **44**, 153 (2001).

[14] Y. Cao, M. C. van Loosdrecht, and G. T. Daigger, Mainstream partial nitritation–anammox in municipal wastewater treatment: status, bottlenecks, and further studies, Applied Microbiology and Biotechnology **101**, 1365 (2017).

[15] M. J. Kampschreur, H. Temmink, R. Kleerebezem, M. S. Jetten, and M. C. van Loosdrecht, Nitrous oxide emission during wastewater treatment, Water Research **43**, 4093 (2009).

[16] K. D. Kits, C. J. Sedlacek, E. V. Lebedeva, P. Han, A. Bulaev, P. Pjevac, A. Daebeler, S. Romano, M. Albertsen, and L. Y. Stein, Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle, Nature **549**, 269 (2017).

[17] J. A. Kozlowski, K. D. Kits, and L. Y. Stein, Comparison of Nitrogen Oxide Metabolism among Diverse Ammonia-Oxidizing Bacteria, Frontiers in Microbiology **7** (2016), 10.3389/fmicb.2016.01090.

[18] S. Liu, P. Han, L. Hink, J. I. Prosser, M. Wagner, and N. Bruggemann, Abiotic conversion of extracellular NH_2OH contributes to N_2O emission during ammonia oxidation, Environmental Science & Technology **51**, 13122 (2017).

[19] R. Stüven, M. Vollmer, and E. Bock, The impact of organic matter on nitric oxide formation by *Nitrosomonas europaea*, Archives Microbiology **158**, 439 (1992).

[20] L. Yang and J. Alleman, Investigation of batchwise nitrite build-up by an enriched nitrification culture, Water Science and Technology **26**, 997 (1992).

[21] R. Yu and K. Chandran, Strategies of *Nitrosomonas europaea* 19718 to counter low dissolved oxygen and high nitrite concentrations, BMC Microbiology **10**, 70 (2010).

[22] L. Y. Stein, Heterotrophic nitrification and nitrifier denitrification, in *Nitrification* (American Society of Microbiology, 2011) pp. 95–114.

[23] B. Kartal and J. T. Keltjens, Anammox biochemistry: a tale of heme c proteins, Trends in Biochemical Sciences **41**, 998 (2016).

[24] A. Soler-Jofra, J. Pérez, and M. C. van Loosdrecht, Hydroxylamine and the nitrogen cycle: a review, Water Research , 116723 (2020).

[25] M. Van Loosdrecht and M. Jetten, Microbiological conversions in nitrogen removal, Water Science and Technology **38**, 1 (1998).

[26] A. Teske, E. Alm, J. Regan, S. Toze, B. Rittmann, and D. Stahl, Evolutionary relationships among ammonia-and nitrite-oxidizing bacteria, Journal of Bacteriology **176**, 6623 (1994).

[27] J. D. Caranto and K. M. Lancaster, Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase, Proceedings of the National Academy of Sciences **114**, 8217 (2017).

[28] L. Y. Stein, Insights into the physiology of ammonia-oxidizing microorganisms, Current Opinion in Chemical Biology **49**, 9 (2019).

[29] K. M. Lancaster, J. D. Caranto, S. H. Majer, and M. A. Smith, Alternative bioenergy: updates to and challenges in nitrification metalloenzymology, Joule **2**, 421 (2018).

[30] E. Mumford, The mechanism of nitrification, in *Proceedings Chemical Society(London)*, Vol. 30 (1914) p. 36.

[31] R. Yu, O. Perez-Garcia, H. Lu, and K. Chandran, *Nitrosomonas europaea* adaptation to anoxic-oxic cycling: Insights from transcription analysis, proteomics and metabolic network modeling, Science of The Total Environment **615**, 1566 (2018).

[32] B. Hu, J. Ye, J. Zhao, X. Ding, L. Yang, and X. Tian, Characteristics of N_2O production and hydroxylamine variation in short-cut nitrification SBR process, Water Science and Technology , wst2017496 (2017).

[33] Q. Su, C. Domingo-Félez, Z. Zhang, J.-M. Blum, M. M. Jensen, and B. F. Smets, The effect of pH on N_2O production in intermittently-fed nitritation reactors, Water Research (2019), https://doi.org/10.1016/j.watres.2019.03.015.

[34] V. Poot, M. Hoekstra, M. A. Geleijnse, M. C. van Loosdrecht, and J. Pérez, Effects of the residual ammonium concentration on NOB repression during partial nitritation with granular sludge, Water Research **106**, 518 (2016).

[35] M. J. Frijlink, T. Abee, H. J. Laanbroek, W. de Boer, and W. N. Konings, The bioenergetics of ammonia and hydroxylamine oxidation in *Nitrosomonas europaea* at acid and alkaline pH, Archives of Microbiology **157**, 194 (1992).

[36] D. De Beer, J. Van Den Heuvel, and S. Ottengraf, Microelectrode measurements of the activity distribution in nitrifying bacterial aggregates, Applied and Environmental Microbiology **59**, 573 (1993).

[37] A. Gieseke, S. Tarre, M. Green, and D. De Beer, Nitrification in a biofilm at low pH values: role of in situ microenvironments and acid tolerance, Applied and Environmental Microbiology **72**, 4283 (2006).

[38] F. Schreiber, B. Loeffler, L. Polerecky, M. M. Kuypers, and D. De Beer, Mechanisms of transient nitric oxide and nitrous oxide production in a complex biofilm, The ISME journal **3**, 1301 (2009).

[39] S. Uemura, S. Suzuki, K. Abe, A. Ohashi, H. Harada, M. Ito, H. Imachi, and T. Tokutomi, Partial nitrification in an airlift activated sludge reactor with experimental and theoretical assessments of the pH gradient inside the sponge support medium, International Journal of Enviornmental Research (2011), 10.22059/IJER.2010.288.

[40] M. K. Winkler, R. Kleerebezem, J. G. Kuenen, J. Yang, and M. C. van Loosdrecht, Segregation of biomass in cyclic anaerobic/aerobic granular sludge allows the enrichment of anaerobic ammonium oxidizing bacteria at low temperatures, Environmental Science & Technology **45**, 7330 (2011).

[41] B. Böttcher and H.-P. Koops, Growth of lithotrophic ammonia-oxidizing bacteria on hydroxylamine, FEMS Microbiology Letters **122**, 263 (1994).

[42] P. de Bruijn, A. A. Van De Graaf, M. S. Jetten, L. A. Robertson, and J. G. Kuenen, Growth of *Nitrosomonas europaea* on hydroxylamine, FEMS Microbiology Letters **125**, 179 (1995).

[43] K. Chandran and B. F. Smets, Biokinetic characterization of the acceleration phase in autotrophic ammonia oxidation, Water Environment Research **80**, 732 (2008).

[44] W. F. Harper, A. Terada, F. Poly, X. Le Roux, K. Kristensen, M. Mazher, and B. F. Smets, The effect of hydroxylamine on the activity and aggregate structure of autotrophic nitrifying bioreactor cultures, Biotechnology and Bioengineering **102**, 714 (2009).

[45] T. Kindaichi, S. Okabe, H. Satoh, and Y. Watanabe, Effects of hydroxylamine on microbial community structure and function of autotrophic nitrifying biofilms determined by in situ hybridization and the use of microelectrodes, Water Science and Technology **49**, 61 (2004).

[46] Y. Wang, H. Wang, J. Zhang, L. Yao, and Y. Wei, Deciphering the evolution of the functional genes and microbial community of the combined partial nitritation-anammox process with nitrate build-up and its in situ restoration, RSC Advances **6**, 111702 (2016).

[47] Y. Wang, Y. Wang, Y. Wei, and M. Chen, In-situ restoring nitrogen removal for the combined partial nitritation-anammox process deteriorated by nitrate build-up, Biochemical Engineering Journal **98**, 127 (2015).

[48] G. Xu, X. Xu, F. Yang, S. Liu, and Y. Gao, Partial nitrification adjusted by hydroxylamine in aerobic granules under high DO and ambient temperature and subsequent Anammox for low C/N wastewater treatment, Chemical Engineering Journal **213**, 338 (2012).

[49] S. Okabe, M. Oshiki, Y. Takahashi, and H. Satoh, Development of long-term stable partial nitrification and subsequent anammox process, Bioresource Technology **102**, 6801 (2011).

[50] J. Li, L. Zhang, J. Liu, J. Lin, and Y. Peng, Hydroxylamine addition and real-time aeration control in sewage nitritation system for reduced start-up period and improved process stability, Bioresource Technology **294**, 122183 (2019).

[51] J. Li, Q. Zhang, X. Li, and Y. Peng, Rapid start-up and stable maintenance of domestic wastewater nitritation through short-term hydroxylamine addition, Bioresource Technology **278**, 468 (2019).

[52] X. Wan, P. Xiao, D. Zhang, P. Lu, Z. Yao, and Q. He, The kinetics for ammonium and nitrite oxidation under the effect of hydroxylamine, Water Science and Technology **73**, 1067 (2016).

[53] R. Wilhelm, A. Abeliovich, and A. Nejidat, Effect of long-term ammonia starvation on the oxidation of ammonia and hydroxylamine by *Nitrosomonas europaea*, The Journal of Biochemistry **124**, 811 (1998).

[54] J. A. Kozlowski, J. Price, and L. Y. Stein, Revision of N_2O-producing pathways in the ammonia-oxidizing bacterium *Nitrosomonas europaea* ATCC 19718, Applied Environmental Microbiology **80**, 4930 (2014).

[55] A. B. Hooper and K. Terry, Hydroxylamine oxidoreductase of *Nitrosomonas*: Production of nitric oxide from hydroxylamine, Biochimica et Biophysica Acta **571**, 12 (1979).

[56] M. G. Klotz and L. Y. Stein, Genomics of ammonia-oxidizing bacteria and insights into their evolution, HNO **1**, 8 (2011).

[57] J. D. Caranto, A. C. Vilbert, and K. M. Lancaster, *Nitrosomonas europaea* cytochrome P460 is a direct link between nitrification and nitrous oxide emission, Proceedings ot the National Academy of Sciences **113**, 14704 (2016).

[58] A. Soler-Jofra, B. Stevens, M. Hoekstra, C. Picioreanu, D. Sorokin, M. C. van Loosdrecht, and J. Pérez, Importance of abiotic hydroxylamine conversion on nitrous oxide emissions during nitritation of reject water, Chemical Engineering Journal **287**, 720 (2016).

[59] A. Terada, S. Sugawara, K. Hojo, Y. Takeuchi, S. Riya, W. F. Harper, T. Yamamoto, M. Kuroiwa, K. Isobe, and C. Katsuyama, Hybrid nitrous oxide production from partial nitrifying bioreactor: Hydroxylamine interactions with nitrite, Environmental Science & Technology **51**, 2748 (2017).

[60] A. Soler-Jofra, C. Picioreanu, R. Yu, K. Chandran, M. C. van Loosdrecht, and J. Pérez, Importance of hydroxylamine in abiotic N_2O production during transient anoxia in planktonic axenic *Nitrosomonas* cultures, Chemical Engineering Journal **335**, 756 (2018).

[61] J. Heil, H. Vereecken, and N. Brüggemann, A review of chemical reactions of nitrification intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil, European Journal Soil Science **67**, 23 (2015).

[62] F. Schreiber, P. Wunderlin, K. M. Udert, and G. F. Wells, Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: biological pathways, chemical reactions, and novel technologies, Frontiers in Microbiology **3**, 372 (2012).

[63] W. F. Harper, Y. Takeuchi, S. Riya, M. Hosomi, and A. Terada, Novel abiotic reactions increase nitrous oxide production during partial nitrification: Modeling and experiments, Chemical Engineering Journal **281**, 1017 (2015).

[64] Q. Su, C. Domingo-Felez, M. M. Jensen, and B. F. Smets, Abiotic nitrous oxide (N_2O) production is strongly pH dependent, but contributes little to overall N_2O emissions in biological nitrogen removal systems, Environmental Science & Technology **53**, 3508 (2019).

[65] M. J. Kampschreur, N. C. Tan, R. Kleerebezem, C. Picioreanu, M. S. Jetten, and M. C. v. Loosdrecht, Effect of dynamic process conditions on nitrogen oxides emission from a nitrifying culture, Environmental Science & Technology **42**, 429 (2008).

[66] H. Duan, L. Ye, D. Erler, B.-J. Ni, and Z. Yuan, Quantifying nitrous oxide production pathways in wastewater treatment systems using isotope technology – a critical review, Water Research **122**, 96 (2017).

[67] A. H. Treusch, S. Leininger, A. Kletzin, S. C. Schuster, H. Klenk, and C. Schleper, Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling, Environmental Microbiology **7**, 1985 (2005).

[68] J. C. Venter, K. Remington, J. F. Heidelberg, A. L. Halpern, D. Rusch, J. A. Eisen, D. Wu, I. Paulsen, K. E. Nelson, and W. Nelson, Environmental genome shotgun sequencing of the Sargasso Sea, Science **304**, 66 (2004).

[69] M. Könneke, A. E. Bernhard, R. José, C. B. Walker, J. B. Waterbury, and D. A. Stahl, Isolation of an autotrophic ammonia-oxidizing marine archaeon, Nature **437**, 543 (2005).

[70] D. A. Stahl and J. R. de la Torre, Physiology and diversity of ammonia-oxidizing archaea, Annual Review of Microbiology **66**, 83 (2012).

[71] C. Wuchter, B. Abbas, M. J. Coolen, L. Herfort, J. van Bleijswijk, P. Timmers, M. Strous, E. Teira, G. J. Herndl, and J. J. Middelburg, Archaeal nitrification in the ocean, Proceedings of the National Academy of Sciences **103**, 12317 (2006).

[72] T. H. Erguder, N. Boon, L. Wittebolle, M. Marzorati, and W. Verstraete, Environmental factors shaping the ecological niches of ammonia-oxidizing archaea, FEMS Microbiology Reviews **33**, 855 (2009).

[73] I. Kasuga, H. Nakagaki, F. Kurisu, and H. Furumai, Predominance of ammonia-oxidizing archaea on granular activated carbon used in a full-scale advanced drinking water treatment plant, Water Research **44**, 5039 (2010).

[74] P. W. Van der Wielen, S. Voost, and D. van der Kooij, Ammonia-oxidizing bacteria and archaea in groundwater treatment and drinking water distribution systems, Applied Environmental Microbiology **75**, 4687 (2009).

[75] C. Brochier-Armanet, B. Boussau, S. Gribaldo, and P. Forterre, Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the *Thaumarchaeota*, Nature Reviews Microbiology **6**, 245 (2008).

[76] R. Hatzenpichler, Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea, Applied Environmental Microbiology **78**, 7501 (2012).

[77] N. Vajrala, W. Martens-Habbena, L. A. Sayavedra-Soto, A. Schauer, P. J. Bottomley, D. A. Stahl, and D. J. Arp, Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea, Proceedings of the National Academy of Sciences **110**, 1006 (2013).

[78] C. Walker, J. De La Torre, M. Klotz, H. Urakawa, N. Pinel, D. Arp, C. Brochier-Armanet, P. Chain, P. Chan, and A. Gollabgir, *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea, Proceedings of the National Academy of Sciences **107**, 8818 (2010).

[79] M. Stieglmeier, M. Mooshammer, B. Kitzler, W. Wanek, S. Zechmeister-Boltenstern, A. Richter, and C. Schleper, Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea, The ISME journal **8**, 1135 (2014).

[80] E. Costa, J. Pérez, and J.-U. Kreft, Why is metabolic labour divided in nitrification?, Trends in Microbiology **14**, 213 (2006).

[81] H. Daims, E. V. Lebedeva, P. Pjevac, P. Han, C. Herbold, M. Albertsen, N. Jehmlich, M. Palatinszky, J. Vierheilig, and A. Bulaev, Complete nitrification by *Nitrospira* bacteria, Nature **528**, 504 (2015).

[82] M. A. Van Kessel, D. R. Speth, M. Albertsen, P. H. Nielsen, H. J. O. den Camp, B. Kartal, M. S. Jetten, and S. Lücker, Complete nitrification by a single microorganism, Nature **528**, 555 (2015).

[83] C. E. Lawson and S. Lücker, Complete ammonia oxidation: an important control on nitrification in engineered ecosystems?, Current Opinion in Biotechnology **50**, 158 (2018).

[84] K. D. Kits, M.-Y. Jung, J. Vierheilig, P. Pjevac, C. J. Sedlacek, S. Liu, C. Herbold, L. Y. Stein, A. Richter, and H. Wissel, Low yield and abiotic origin of N_2O formed by the complete nitrifier *Nitrospira inopinata*, Nature Communications **10**, 1836 (2019).

[85] P. Y. Camejo, J. Santo Domingo, K. D. McMahon, and D. R. Noguera, Genome-enabled insights into the ecophysiology of the comammox bacterium "*Candidatus* Nitrospira nitrosa", MSystems **2**, e00059 (2017).

[86] A. Palomo, A. G. Pedersen, S. J. Fowler, A. Dechesne, T. Sicheritz-Pontén, and B. F. Smets, Comparative genomics sheds light on niche differentiation and the evolutionary history of comammox *Nitrospira*, The ISME journal **12**, 1779 (2018).

[87] E. Broda, Two kinds of lithotrophs missing in nature, Zeitschrift für allgemeine Mikrobiologie **17**, 491 (1977).

[88] Z. Hu, H. J. Wessels, T. van Alen, M. S. Jetten, and B. Kartal, Nitric oxide-dependent anaerobic ammonium oxidation, Nature Communications **10** (2019), https://doi.org/10.1038/s41467-019-09268-w.

[89] B. Kartal, W. J. Maalcke, N. M. de Almeida, I. Cirpus, J. Gloerich, W. Geerts, H. J. O. den Camp, H. R. Harhangi, E. M. Janssen-Megens, and K.-J. Francoijs, Molecular mechanism of anaerobic ammonium oxidation, Nature **479**, 127 (2011).

[90] M. Oshiki, M. Ali, K. Shinyako-Hata, H. Satoh, and S. Okabe, Hydroxylamine-dependent anaerobic ammonium oxidation (anammox) by "*Candidatus* Brocadia sinica", Environmental Microbiology (2016), 10.1111/1462-2920.13355.

[91] K. Egli, U. Fanger, P. J. Alvarez, H. Siegrist, J. R. van der Meer, and A. J. Zehnder, Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate, Archives of Microbiology **175**, 198 (2001).

[92] M. Oshiki, K. Shinyako-Hata, H. Satoh, and S. Okabe, Draft genome sequence of an anaerobic ammonium-oxidizing bacterium,"*Candidatus* Brocadia sinica", Genome Announcments **3**, e00267 (2015).

[93] A. Dietl, C. Ferousi, W. J. Maalcke, A. Menzel, S. de Vries, J. T. Keltjens, M. S. Jetten, B. Kartal, and T. R. Barends, The inner workings of the hydrazine synthase multiprotein complex, Nature **527**, 394 (2015).

[94] A. Hu, P. Zheng, Q. Mahmood, L. Zhang, L. Shen, and S. Ding, Characteristics of nitrogenous substrate conversion by anammox enrichment, Bioresource Technology **102**, 536 (2011).

[95] I. Zekker, K. Kroon, E. Rikmann, T. Tenno, M. Tomingas, P. Vabamäe, S. E. Vlaeminck, and T. Tenno, Accelerating effect of hydroxylamine and hydrazine on nitrogen removal rate in moving bed biofilm reactor, Biodegradation **23**, 739 (2012).

[96] W. R. Van Der Star, A. I. Miclea, U. G. Van Dongen, G. Muyzer, C. Picioreanu, and M. C. Van Loosdrecht, The membrane bioreactor: a novel tool to grow anammox bacteria as free cells, Biotechnology and Bioengineering **101**, 286 (2008).

[97] A. Soler-Jofra, M. Laureni, M. Warmerdam, J. Pérez, and M. C. van Loosdrecht, Hydroxylamine metabolism of *Ca.* Kuenenia stuttgartiensis, Water Research , 116188 (2020).

[98] H. Daims, S. Lücker, and M. Wagner, A new perspective on microbes formerly known as nitrite-oxidizing bacteria, Trends in Microbiology **24**, 699 (2016).

[99] M. Meincke, E. Bock, D. Kastrau, and P. M. Kroneck, Nitrite oxidoreductase from *Nitrobacter hamburgensis*: redox centers and their catalytic role, Archives of Microbiology **158**, 127 (1992).

[100] D. Castignetti and H. B. Gunner, Differential tolerance of hydroxylamine by an *Alcaligenes sp.*, a heterotrophic nitrifier, and by *Nitrobacter agilis*, Canadian Journal of Microbiology **28**, 148 (1982).

[101] O. J. Hao and J. M. Chen, Factors affecting nitrite buildup in submerged filter system, Journal of Environmental Engineering **120**, 1298 (1994).

[102] R. Blackburne, G. Carvalho, Z. Yuan, and J. Keller, Selective production of nitrite using hydroxylamine as inhibitor of nitrite oxidation, Water Environmental Management , 189 (2004).

[103] X. Zhang, Y. Xia, C. Wang, J. Li, P. Wu, L. Ma, Y. Wang, Y. Wang, F. Da, and W. Liu, Enhancement of nitrite production via addition of hydroxylamine to partial denitrification (PD) biomass: Functional genes dynamics and enzymatic activities, Bioresource Technology , 124274 (2020).

[104] D. Haase, B. Hermann, O. Einsle, and J. Simon, Epsilonproteobacterial hydroxylamine oxidoreductase (εHAO): characterization of a 'missing link'in the multihaem cytochrome c family, Molecular Microbiology **105**, 127 (2017).

[105] J. Simon and M. G. Klotz, Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations, Biochimica et Biophysica Acta (BBA)-Bioenergetics **1827**, 114 (2013).

[106] T. E. Hanson, B. J. Campbell, K. M. Kalis, M. A. Campbell, and M. G. Klotz, Nitrate ammonification by *Nautilia profundicola* AmH: experimental evidence consistent with a free hydroxylamine intermediate, Frontiers in Microbiology **4**, 180 (2013).

[107] Y. Liu, G.-M. Ai, M.-R. Wu, S.-S. Li, L.-L. Miao, and Z.-P. Liu, *Photobacterium sp.* NNA4, an efficient hydroxylamine-transforming heterotrophic nitrifier/aerobic denitrifier, Journal of Bioscience and Bioengineering (2019), https://doi.org/10.1016/j.jbiosc.2018.12.014.

[108] H.-S. Joo, M. Hirai, and M. Shoda, Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* No. 4, Journal of Bioscience and Bioengineering **100**, 184 (2005).

[109] D. Sorokin, Heterotrophic nitrification by bacteria belonging to the genus *Alcaligenes*, Microbiology **58**, 4 (1989).

[110] D. Y. Sorokin and G. A. Dubinina, Oxidation of hydroxylamine by heterotrophic bacteria, Microbiology **55**, 230 (1986).

[111] M. S. Jetten, P. De Bruijn, and J. G. Kuenen, Hydroxylamine metabolism in *Pseudomonas* PB16: involvement of a novel hydroxylamine oxidoreductase, Antonie Van Leeuwenhoek **71**, 69 (1997).

[112] S. K. Padhi, S. Tripathy, S. Mohanty, and N. K. Maiti, Aerobic and heterotrophic nitrogen removal by *Enterobacter cloacae* CF-S27 with efficient utilization of hydroxylamine, Bioresource Technology **232**, 285 (2017).

[113] M. A. Campbell, G. Nyerges, J. A. Kozlowski, A. T. Poret-Peterson, L. Y. Stein, and M. G. Klotz, Model of the molecular basis for hydroxylamine oxidation and nitrous oxide production in methanotrophic bacteria, FEMS Microbiology Letters **322**, 82 (2011).

[114] W. Versantvoort, A. Pol, M. S. Jetten, L. van Niftrik, J. Reimann, B. Kartal, and H. J. O. den Camp, Multiheme hydroxylamine oxidoreductases produce NO during ammonia oxidation in methanotrophs, Proceedings of the National Academy of Sciences **117**, 24459 (2020).

[115] F. Sabba, C. Picioreanu, J. Pérez, and R. Nerenberg, Hydroxylamine diffusion can enhance N_2O emissions in nitrifying biofilms: a modeling study, Environmental Science & Technology **49**, 1486 (2015).

[116] T. Kolasa and W. Wardencki, Quantitative determination of hydroxylamine, Talanta **21**, 845 (1974).

[117] D. Frear and R. Burrell, Spectrophotometric method for determining hydroxylamine reductase activity in higher plants, Analytical Chemistry **27**, 1664 (1955).

[118] J. H. Butler and L. I. Gordon, An improved gas chromatographic method for the measurement of hydroxylamine in marine and fresh waters, Marine Chemistry **19**, 229 (1986).

[119] S. Liu, H. Vereecken, and N. Brüggemann, A highly sensitive method for the determination of hydroxylamine in soils, Geoderma **232**, 117 (2014).

[120] M. M. Foroughi, H. Beitollahi, S. Tajik, M. Hamzavi, and H. Parvan, Hydroxylamine electrochemical sensor based on a modified carbon nanotube paste electrode: application to determination of hydroxylamine in water samples, International Journal Electrochemistry Science **9**, 2955 (2014).

[121] C. Zhang, G. Wang, M. Liu, Y. Feng, Z. Zhang, and B. Fang, A hydroxylamine electrochemical sensor based on electrodeposition of porous ZnO nanofilms onto carbon nanotubes films modified electrode, Electrochimica Acta **55**, 2835 (2010).

[122] J. Bremner, A. Blackmer, and S. Waring, Formation of nitrous oxide and dinitrogen by chemical decomposition of hydroxylamine in soils, Soil Biology Biochemistry **12**, 263 (1980).

[123] D. Jiang, W. Khunjar, B. Wett, S. Murthy, and K. Chandran, Characterizing the metabolic trade-off in *Nitrosomonas europaea* in response to changes in inorganic carbon supply, Environmental Science & Technology **49**, 2523 (2015).

[124] Y. Ma, S. Sundar, H. Park, and K. Chandran, The effect of inorganic carbon on microbial interactions in a biofilm nitritation–anammox process, Water Research **70**, 246 (2015).

[125] R. Yu, M. J. Kampschreur, M. C. v. Loosdrecht, and K. Chandran, Mechanisms and specific directionality of autotrophic nitrous oxide and nitric oxide generation during transient anoxia, Environmental Science Technology **44**, 1313 (2010).

[126] M. Pijuan, J. Tora, A. Rodríguez-Caballero, E. César, J. Carrera, and J. Pérez, Effect of process parameters and operational mode on nitrous oxide emissions from a nitritation reactor treating reject wastewater, Water Research **49**, 23 (2014).

[127] P. Wunderlin, J. Mohn, A. Joss, L. Emmenegger, and H. Siegrist, Mechanisms of N_2O production in biological wastewater treatment under nitrifying and denitrifying conditions, Water Research **46**, 1027 (2012).

[128] Y. Law, P. Lant, and Z. Yuan, The confounding effect of nitrite on N_2O production by an enriched ammonia-oxidizing culture, Environmental Science & Technology **47**, 7186 (2013).

[129] B.-J. Ni, L. Peng, Y. Law, J. Guo, and Z. Yuan, Modeling of nitrous oxide production by autotrophic ammonia-oxidizing bacteria with multiple production pathways, Environmental Science & Technology **48**, 3916 (2014).

[130] O. Van Cleemput and L. Baert, Nitrite: a key compound in N loss processes under acid conditions?, Plant and Soil **76**, 233 (1984).

[131] D. J. Arp and L. Y. Stein, Metabolism of inorganic N compounds by ammonia-oxidizing bacteria, Critical Review Biochemistry Molecular Biology **38**, 471 (2003).

[132] M. J. Kampschreur, R. Kleerebezem, W. W. de Vet, and M. C. van Loosdrecht, Reduced iron induced nitric oxide and nitrous oxide emission, Water Research **45**, 5945 (2011).

[133] V. A. Samarkin, M. T. Madigan, M. W. Bowles, K. L. Casciotti, J. C. Priscu, C. P. McKay, and S. B. Joye, Abiotic nitrous oxide emission from the hypersaline Don Juan Pond in Antarctica, Nature Geoscience **3**, 341 (2010).

[134] J. Heil, B. Wolf, N. Brüggemann, L. Emmenegger, B. Tuzson, H. Vereecken, and J. Mohn, Site-specific ^{15}N isotopic signatures of abiotically produced N_2O, Geochimica et Cosmochimica Acta **139**, 72 (2014).

[135] C. Döring and H. Gehlen, Über die Kinetik der Reaktion zwischen Hydroxylamin und salpetriger Säure, Z. Anorg. Allg.Chem. **312**, 32 (1961).

[136] L. B. Clapp, Sulfamic acid and its uses, Journal of Chemical Education **20**, 189 (1943).

[137] A. Anthonisen, R. Loehr, T. Prakasam, and E. Srinath, Inhibition of nitrification by ammonia and nitrous acid, Journal (Water Pollution Control Federation) , 835 (1976).

[138] M. Hughes and H. Nicklin, Autoxidation of hydroxylamine in alkaline solutions, Journal of the Chemical Society A: Inorganic, Physical, Theoretical , 164 (1971).

[139] A. Kock and H. W. Bange, Nitrite removal improves hydroxylamine analysis in aqueous solution by conversion with iron (III), Environmental Chemistry **10**, 64 (2013).

[140] F. T. Bonner, L. S. Dzelzkalns, and J. A. Bonucci, Properties of nitroxyl as intermediate in the nitric oxide-hydroxylamine reaction and in trioxodinitrate decomposition, Inorganic Chemistry **17**, 2487 (1978).

[141] K. E. Mampaey, M. K. De Kreuk, U. G. van Dongen, M. C. van Loosdrecht, and E. I. Volcke, Identifying N_2O formation and emissions from a full-scale partial nitritation reactor, Water Research **88**, 575 (2016).

[142] K. Chandran, L. Y. Stein, M. G. Klotz, and M. C. van Loosdrecht, Nitrous oxide production by lithotrophic ammonia-oxidizing bacteria and implications for engineered nitrogen-removal systems, Biochemical Society Transactions **39**, 1832 (2011).

[143] C. H. Frame, E. Lau, E. J. Nolan IV, T. J. Goepfert, and M. F. Lehmann, Acidification enhances hybrid N_2O production associated with aquatic ammonia-oxidizing microorganisms, Frontiers in Microbiology **7** (2016), https://doi.org/10.3389/fmicb.2016.02104.

[144] B.-J. Ni and Z. Yuan, Recent advances in mathematical modeling of nitrous oxides emissions from wastewater treatment processes, Water Research **87**, 336 (2015).

[145] J. A. Kozlowski, M. Stieglmeier, C. Schleper, M. G. Klotz, and L. Y. Stein, Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and *Thaumarchaeota*, The ISME Journal (2016), 10.1038/ismej.2016.2.

[146] L. Y. Stein, Surveying N_2O-Producing Pathways in Bacteria, Methods Enzymology **486**, 131 (2011).

[147] X. Zhu-Barker, A. R. Cavazos, N. E. Ostrom, W. R. Horwath, and J. B. Glass, The importance of abiotic reactions for nitrous oxide production, Biogeochemistry **126**, 251 (2015).

[148] H. J. Beaumont, N. G. Hommes, L. A. Sayavedra-Soto, D. J. Arp, D. M. Arciero, A. B. Hooper, H. V. Westerhoff, and R. J. van Spanning, Nitrite reductase of Nitrosomonas europaea is not essential for production of gaseous nitrogen oxides and confers tolerance to nitrite, Journal Bacteriology **184**, 2557 (2002).

[149] R. Yu, O. Perez-Garcia, H. Lu, and K. Chandran, *Nitrosomonas europaea* adaptation to anoxic-oxic cycling: insight from transcription analysis, proteomics and metabolic network modeling, Sci.ence of Total Environment **(accepted)** (2018), 10.1016/j.scitotenv.2017.09.142.

[150] T. J. Goreau, W. A. Kaplan, S. C. Wofsy, M. B. McElroy, F. W. Valois, and S. W. Watson, Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen, Applied Environmental Microbiology **40**, 526 (1980).

[151] M. Poth and D. D. Focht, ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification, Applied Environmental Microbiology **49**, 1134 (1985).

[152] R. A. Kester, W. De Boer, and H. J. Laanbroek, Production of NO and N_2O by pure cultures of nitrifying and denitrifying bacteria during changes in aeration, Applied Environmental Microbiology **63**, 3872 (1997).

[153] L. J. Shaw, G. W. Nicol, Z. Smith, J. Fear, J. I. Prosser, and E. M. Baggs, *Nitrosospira spp.* can produce nitrous oxide via a nitrifier denitrification pathway, Environmental Microbiology **8**, 214 (2006).

[154] I. C. Anderson, M. Poth, J. Homstead, and D. Burdige, A comparison of NO and N_2O production by the autotrophic nitrifier Nitrosomonas europaea and the heterotrophic nitrifier Alcaligenes faecalis, Applied Environmental Microbiology **59**, 3525 (1993).

[155] B. Kartal, L. van Niftrik, J. T. Keltjens, H. J. Op den Camp, and M. S. Jetten, Anammox—Growth physiology, cell biology, and metabolism, Advances in Microbial Physiology **60**, 212 (2012).

[156] G. W. Watt and J. D. Chrisp, Spectrophotometric method for determination of hydrazine, Analytical Chemistry **24**, 2006 (1952).

[157] R. Kleerebezem and M. C. Van Loosdrecht, A generalized method for thermodynamic state analysis of environmental systems, Critical Reviews in Environmental Science and Technology **40**, 1 (2010).

[158] T. Lotti, R. Kleerebezem, C. Lubello, and M. Van Loosdrecht, Physiological and kinetic characterization of a suspended cell anammox culture, Water Research **60**, 1 (2014).

[159] W. M. Haynes, *CRC handbook of chemistry and physics* (CRC press, 2014).

[160] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, Molecular biology of the cell. Fifth Edition (New York, 2007) Book section Chapter 11. Membrane transport of small molecules and the electrical properties of membranes., pp. 651–694.

[161] W. J. Maalcke, J. Reimann, S. de Vries, J. N. Butt, A. Dietl, N. Kip, U. Mersdorf, T. R. Barends, M. S. Jetten, and J. T. Keltjens, Characterization of anammox hydrazine dehydrogenase, a key N_2-producing enzyme in the global nitrogen cycle, Journal of Biological Chemistry **291**, 17077 (2016).

[162] Z.-B. Yao, Q. Cai, D.-J. Zhang, P.-Y. Xiao, and P.-L. Lu, The enhancement of completely autotrophic nitrogen removal over nitrite (CANON) by N_2H_4 addition, Bioresource tTchnology **146**, 591 (2013).

[163] W. R. Van Der Star, C. Dijkema, P. de Waard, C. Picioreanu, M. Strous, and M. C. van Loosdrecht, An intracellular pH gradient in the anammox bacterium Kuenenia stuttgartiensis as evaluated by ^{31}P NMR, Applied Microbiology and Biotechnology **86**, 311 (2010).

[164] L. A. van Niftrik, J. A. Fuerst, J. S. S. Damsté, J. G. Kuenen, M. S. Jetten, and M. Strous, The anammoxosome: an intracytoplasmic compartment in anammox bacteria, FEMS Microbiology Letters **233**, 7 (2004).

[165] J. van de Vossenberg, D. Woebken, W. J. Maalcke, H. J. Wessels, B. E. Dutilh, B. Kartal, E. M. Janssen-Megens, G. Roeselers, J. Yan, and D. Speth, The metagenome of the marine anammox bacterium '*Candidatus* Scalindua profunda' illustrates the versatility of this globally important nitrogen cycle bacterium, Environmental Microbiology **15**, 1275 (2013).

[166] J. Kostera, M. D. Youngblut, J. M. Slosarczyk, and A. A. Pacheco, Kinetic and product distribution analysis of NO reductase activity in *Nitrosomonas europaea* hydroxylamine oxidoreductase, JBIC Journal of Biological Inorganic Chemistry **13**, 1073 (2008).

[167] M. Henze, W. Gujer, T. Mino, and M. Van Loosdrecht, *Activated sludge models ASM1, ASM2, ASM2d and ASM3* (IWA publishing, 2000).

[168] M. C. van Loosdrecht, P. H. Nielsen, C. M. Lopez-Vazquez, and D. Brdjanovic, *Experimental methods in wastewater treatment* (IWA publishing, 2016).

[169] R. Blackburne, Z. Yuan, and J. Keller, Partial nitrification to nitrite using low dissolved oxygen concentration as the main selection factor, Biodegradation **19**, 303 (2008).

[170] P. L. Noophan, L. Figueroa, and J. Munakata-Marr, Nitrite oxidation inhibition by hydroxylamine: experimental and model evaluation, Water Science and Technology **50**, 295 (2004).

[171] A. Apha, Standard methods for the examination of water and wastewater, WEF Federation **22** (2012).

[172] L. Poughon, C. Dussap, and J. Gros, Energy model and metabolic flux analysis for autotrophic nitrifiers, Biotechnology and Bioengineering **72**, 416 (2001).

[173] K. Hanselmann, Microbial energetics applied to waste repositories, Experientia **47**, 645 (1991).

[174] J. Pérez, T. Lotti, R. Kleerebezem, C. Picioreanu, and M. C. van Loosdrecht, Outcompeting nitrite-oxidizing bacteria in single-stage nitrogen removal in sewage treatment plants: a model-based study, Water Research **66**, 208 (2014).

[175] Y. Law, B.-J. Ni, P. Lant, and Z. Yuan, N_2O production rate of an enriched ammonia-oxidising bacteria culture exponentially correlates to its ammonia oxidation rate, Water Research **46**, 3409 (2012).

[176] B.-J. Ni, M. Ruscalleda, C. Pellicer-Nacher, and B. F. Smets, Modeling nitrous oxide production during biological nitrogen removal via nitrification and denitrification: extensions to the general ASM models, Environmental Science & Technology **45**, 7768 (2011).

[177] C. Domingo-Félez, M. Calderó-Pascual, G. Sin, B. G. Plósz, and B. F. Smets, Calibration of the comprehensive NDHA-N_2O dynamics model for nitrifier-enriched biomass using targeted respirometric assays, Water Research **126**, 29 (2017).

[178] A. Guisasola, K. Chandran, B. F. Smets, J. Baeza, J. Carrera, and J. Lafuente, Observation and mathematical description of the acceleration phenomenon in batch respirograms associated with ammonium oxidation, Water Science and Technology **54**, 181 (2006).

[179] I. Jubany, J. Carrera, J. Lafuente, and J. A. Baeza, Start-up of a nitrification system with automatic control to treat highly concentrated ammonium wastewater: experimental results and modeling, Chemical Engineering Journal **144**, 407 (2008).

[180] B. Petersen, K. Gernaey, M. Henze, and P. Vanrolleghem, Calibration of activated sludge models: A critical review of experimental designs, Biotechnology for the environment: Wastewater treatment and modeling, waste gas handling , 101 (2003).

[181] G. Sin, S. W. Van Hulle, D. J. De Pauw, A. Van Griensven, and P. A. Vanrolleghem, A critical comparison of systematic calibration protocols for activated sludge models: A SWOT analysis, Water Research **39**, 2459 (2005).

[182] X.-D. Hao and M. van Loosdrecht, Model-based evaluation of COD influence on a partial nitrification-Anammox biofilm (CANON) process, Water Science and Technology **49**, 83 (2004).

[183] L. Y. Stein, D. J. Arp, and M. R. Hyman, Regulation of the synthesis and activity of ammonia monooxygenase in *Nitrosomonas europaea* by altering pH to affect NH_3 availability, Applied and Environmental Microbiology **63**, 4588 (1997).

[184] M. J. Kampschreur, W. R. van der Star, H. A. Wielders, J. W. Mulder, M. S. Jetten, and M. C. van Loosdrecht, Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment, Water Research **42**, 812 (2008).

[185] T. Machida, T. Nakazawa, Y. Fujii, S. Aoki, and O. Watanabe, Increase in the atmospheric nitrous oxide concentration during the last 250 years, Geophysical Research Letters **22**, 2921 (1995).

[186] R. T. Ferrell and D. M. Himmelblau, Diffusion coefficients of nitrogen and oxygen in water, Journal of Chemical and Engineering Data **12**, 111 (1967).

[187] A. Tamimi, E. B. Rinker, and O. C. Sandall, Diffusion coefficients for hydrogen sulfide, carbon dioxide, and nitrous oxide in water over the temperature range 293-368 K, Journal of Chemical and Engineering data **39**, 330 (1994).

[188] B. K. Mobarry, M. Wagner, V. Urbain, B. E. Rittmann, and D. A. Stahl, Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria, Applied and Environmental Microbiology **62**, 2156 (1996).

[189] R. I. Amann, B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl, Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations, Applied and Environmental Microbiology **56**, 1919 (1990).

[190] H. Daims, A. Brühl, R. Amann, K.-H. Schleifer, and M. Wagner, The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set, Systematic and Applied Microbiology **22**, 434 (1999).

[191] M. Wagner, G. Rath, H.-P. Koops, J. Flood, and R. Amann, In situ analysis of nitrifying bacteria in sewage treatment plants, Water Science and Technology **34**, 237 (1996).

[192] H. Daims, J. L. Nielsen, P. H. Nielsen, K.-H. Schleifer, and M. Wagner, In situ characterization of *Nitrospira*-like nitrite-oxidizing bacteria active in wastewater treatment plants, Applied and Environmental Microbiology **67**, 5273 (2001).

[193] S. Lücker, J. Schwarz, C. Gruber-Dorninger, E. Spieck, M. Wagner, and H. Daims, Nitrotoga-like bacteria are previously unrecognized key nitrite oxidizers in full-scale wastewater treatment plants, The ISME journal **9**, 708 (2015).

[194] Q. Su, A.-R. Schittich, M. M. Jensen, H. Ng, and B. F. Smets, Role of Ammonia Oxidation in Organic Micropollutant Transformation during Wastewater Treatment: Insights from Molecular, Cellular, and Community Level Observations, Environmental Science & Technology (2021), DOI: 10.1021/acs.est.0c06466.

[195] L. Y. Stein and M. G. Klotz, Nitrifying and denitrifying pathways of methanotrophic bacteria, Biochemical Society Transactions **39**, 1826 (2011).

[196] P. Wilfert, P. S. Kumar, L. Korving, G.-J. Witkamp, and M. C. van Loosdrecht, The relevance of phosphorus and iron chemistry to the recovery of phosphorus from wastewater: a review, Environmental Science & Technology **49**, 9400 (2015).

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LIST OF PUBLICATIONS

JOURNAL ARTICLES

6. **A. Soler-Jofra**, L. Schmidtchen, Ll. Olmo, M.C. M. van Loosdrecht, J. Pérez, *Short and long term impact of continuous hydroxylamine feeding in a granular sludge partial nitritation reactor*, (submitted).

5. **A. Soler-Jofra**, J. Pérez, M.C. M. van Loosdrecht, *Hydroxylamine and the nitrogen cycle: a review.*, Water Research **116723**, (2020).

4. **A. Soler-Jofra**, M.Laureni, M. Warmerdam, J. Pérez, M.C. M. van Loosdrecht, *Hydroxylamine metabolism of Ca. Keunenia stuttgartiensis*, Water Research **184**, 166-188, (2020).

3. **A. Soler-Jofra**, R.Wang, R. Kleerebezem, M.C. M. van Loosdrecht, J. Pérez, *Stratification of nitrifier guilds in granular sludge in relation to nitritation*, Water Research **148**, 479-491 (2019).

2. **A. Soler-Jofra**, C. Picioreanu, R. Yu, K. Chandran, M. C. M. van Loosdrecht, J. Pérez, *Importance of hydroxylamine in abiotic N_2O production during transient anoxia in planktonic axenic Nitrosomonas cultures*, Chemical Engineering Journal **335**, 756-762 (2018).

1. **A. Soler-Jofra**, B.Stevens, M. Hoekstra, C. Picioreanu, D. Sorokin, M.C.M. van Loosdrecht, J. Pérez, *Importance of abiotic hydroxylamine conversion on nitrous oxide during nitritation of reject water*, Chemical engineering Journal **287**, 720-726 (2016).

CONFERENCE CONTRIBUTIONS

Presenting author is underlined.

5. **A. Soler-Jofra**, J. Pérez, M.C.M. van Loosdrecht, *Influence of hydroxylamine on aerobic and anaerobic ammonium oxidation.*, IWA Nutrient Removal and Recovery Conference, **Helsinki, Finland (online)**, (September 2020).

4. M. Laureni, N. de Jonge, **A. Soler-Jofra**, G. Stouten, C. Lawson, R. Broekman, D. Weissbrodt, R. Kleerebezem, C. Picioreanu, D. McMillan, M. Pabst, J.L. Nielsen, M.C.M. van Loosdrecht, *Proteome-level response of Ca. Kuenenia to different organic electron donors and implications for microbial competition*, IWA Nutrient Removal and Recovery Conference, **Brisbane, Australia**, (November 2018).

3. **A. Soler-Jofra**, B. Stevens, M. Hoekstra, D. Sorokin, R. Yu, K. Chandran, C. Picioreanu, M.C.M. van Loosdrecht, J. Pérez, *It is not all about biology: environmental niche, kinetics and mitigation of abiotic N_2O production from hydroxylamine and free nitrous acid*, 23rd European nirogen cycle Meeting, **Alicante, Spain**, (September 2018).

2. **A. Soler-Jofra**, R. Wang, R. Kleerebezem, M.C.M. van Loosdrecht, J. Pérez, *Which factors are impacting NOB repression in partial nitritation granular sludge? Applicability of in-situ batch tests*, IWA Biofilm Granular sludge conference, **Delft,The Netherlands** , (March 2018).

1. **A. Soler-Jofra**, C. Picioreanu, M.C.M van Loosdrecht, J. Pérez, *Importance of hydroxylamine in abiotic N_2O production: reaction kinetics and mitigation strategies*, Fifth International Conference on Nitrification and Related Processes, **Vienna, Austria**, (July 2017).

CURRICULUM VITÆ

Aina Soler-Jofra was born in Vilassar de Mar, Barcelona, Spain on September 17th 1992. On 2010 she started her studies on Biotechnology in Universitat Autònoma de Barcelona. As part of her Biotechnology studies she performed a 6 months Erasmus+ internship in the Environmental Biotechnology Department in Delft University of Technology, where she studied the abiotic N_2O emissions from the nitrogen cycle. She stayed in the Netherlands for quite some more time, as she started a Master of Science in Life Science and Technology in Delft University of Technology (2015-2017). As part of the master program, she briefly returned to Spain for 3 months to perform and internship in IRTA (Institute of Agrifood Research and Technology).

From March 2017, she continued her master thesis project about abiotic N_2O emissions and hydroxylamine as a PhD candidate in the Environmental Biotechnology Department under the guidance and supervision of Julio Pérez and Mark M.C.M. van Loosdrecht. She also did part of her research in collaboration with GENOCOV group from Universitat Autònoma de Barcelona. After her PhD, she is continuing her research career in wastewater treatment and circular economy in Eurecat, Spain.

Besides science, Aina loves travelling, sun and spending time with her friends and family.