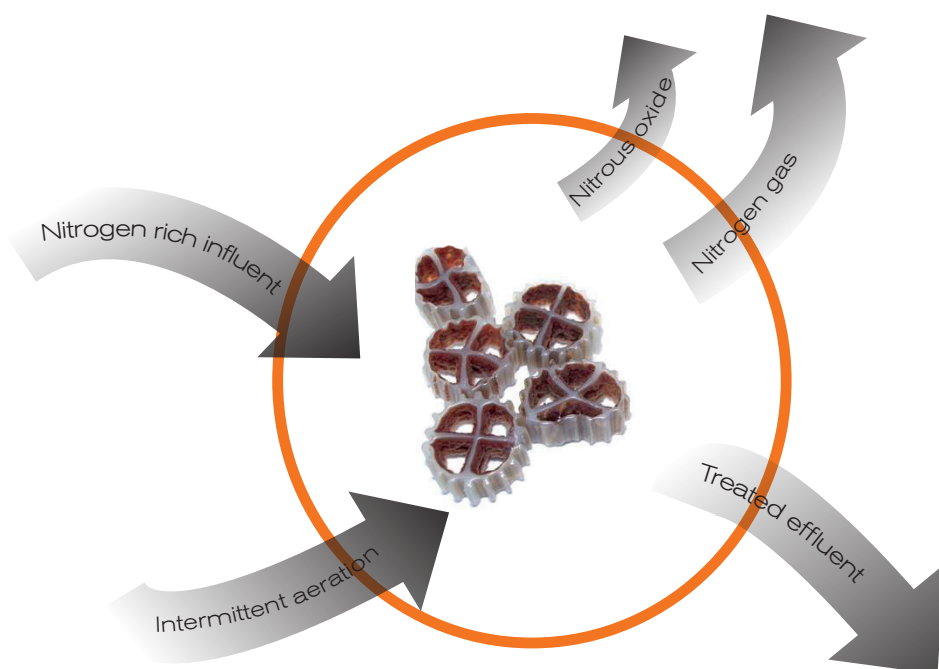


Start-up and operational strategies for deammonification plants

- a study with one-stage moving bed biofilm reactors treating reject water

Linda Kanders



Mälardalen University Press Dissertations
No. 290

START-UP AND OPERATIONAL STRATEGIES FOR DEAMMONIFICATION PLANTS

**- A STUDY WITH ONE-STAGE MOVING BED
BIOFILM REACTORS TREATING REJECT WATER**

Linda Kanders

2019



School of Business, Society and Engineering

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Akademin för ekonomi, samhälle och teknik

Abstract

To limit eutrophication, wastewater treatment plants use biological methods to convert degraded nitrogen to nitrogen gas. Deammonification, or partial nitrification in combination with anammox, has been shown to be an energy efficient process. This process is currently implemented in approximately 150 full-scale plants, and mainly on reject waters, the liquid fraction after dewatering of anaerobic digestion at municipal wastewater treatment plants. Implementation has been impeded by the slow growth of anammox bacteria, and 99% of the full-scale plants using the process have been using different methods to inoculate the process with anammox bacteria from elsewhere. Separate reject water installations, however, have shown high nitrous oxide emissions, which could increase the total carbon footprint.

The objective of this thesis was to develop and validate a start-up concept using the moving bed biofilm reactor (MBBR) technique applied to reject water, and to investigate how the operational strategies could be optimized to limit potential nitrous oxide emissions. The results show that a one-stage deammonification process based on the MBBR technology with indigenous anammox bacteria originating from the reject water can be set up within an applicable time frame (<100 days). This was validated in two laboratory reactors and in two full-scale studies. Reject water originating from both mesophilic and thermophilic digested sludge was used. Anammox growth and nitrogen reduction were detected with fluorescence in situ hybridization (FISH) and chemical analysis, respectively. The start-up time was 72 days in the laboratory and 120 days in full-scale. In laboratory scale, there was no improvement in start-up time when adding external anammox inoculum. Results from a screening study of seven reject waters and their content of anammox bacteria using qPCR indicated the presence of 10^4 – 10^5 genome units anammox per mL in reject water, which could be sufficient for starting up deammonification plants within an applicable time frame.

A final case study shows the potential of decreasing nitrous oxide emissions when a full-scale plant treating reject water was modified from nitrification/denitrification using a Sequencing Batch Reactor (SBR) to a deammonification process using the MBBR technique. The nitrous oxide emissions decreased from 10% to 0.1–0.7% of total nitrogen load with the change of operation mode. Further optimization by pH set point led to lower emission values. This effect is thought to be linked to the lower aeration ratio and increase in complete denitrification of dissolved nitrous oxide at higher pH.

To my family
Till min familj

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Lund, Sweden, in April 2019

Summary

The available nitrogen load in the environment is increasing due to anthropogenic activity. To limit the flow of nitrogen to the oceans and seas, which causes eutrophication and oxygen deficiency, wastewater treatment plants (WWTPs) are converting dissolved nitrogen back to dinitrogen gas. To meet increasing nitrogen loads and increasingly strict effluent demands, wastewater treatment utilities must consider new effective process solutions. The wastewater utilities must operate within the frame of regulations, total environmental impact and economic restrictions.

The deammonification process — that is, nitrification in combination with anammox — has proven to be an energy efficient alternative for biological nitrogen removal, especially in treatment of reject water and has been implemented in around 150 plants to date. However, if external seeding of anammox bacteria is not used, many view the start-up time as a bottleneck for implementation. The potential for high emissions of nitrous oxide, a potent greenhouse gas, has also put the implementation of separate reject water treatment into question.

The objective of this thesis was to develop and validate a concept for starting up a one-stage deammonification plant for mesophilic and for thermophilic digested reject water, without the use of an external anammox inoculum. Further, the quantities of anammox bacteria were evaluated in using qPCR (quantitative polymerase chain reaction) of reject water streams before and after the digester, to determine the existence of indigenous anammox bacteria in reject water. Finally, a case study was performed on a reject water treatment, comparing the nitrous oxide emissions when operating in nitrification/denitrification mode (using sequencing batch reactor technique) to those when operating in deammonification mode (using the moving bed biofilm reactor technique).

The first study describes a full-scale start-up of a one-stage deammonification plant that was completed within 10 months. Technical

issues, possibly inhibition and shortage of dilution water as well as lack of a heating source were the most likely reasons for the relatively long start-up time. The study shows that nitrification is the limiting step and that low oxygen concentrations markedly limit the process performance.

The second study evaluates whether an external seeding source of anammox bacteria would decrease the start-up time. The two parallel laboratory reactors showed similar development of anammox in the biofilm, detected by fluorescence in situ hybridization (FISH), and nitrogen reduction, detected using chemical analyses. Results from the third study confirmed that non-inoculated start-ups can also be performed on thermophilic reject water, using the same methodology, within a reasonable time scale (~100 days). This was demonstrated both in laboratory and in full-scale. A fourth study concludes that anammox bacteria is present in reject water (10^4 – 10^5 GU/mL), independent of the substrate going into the digester, pre-treatment of the sludge or digestion temperature. Reject water from mesophilic digestion and sludge with high sludge age could be used as a safe source of anammox bacteria. Hygienization and dewatering act as a sink of anammox bacteria in the WWTP.

To recommend the implementation of a one-stage deammonification plant as a side-stream treatment of reject water, potential emission of nitrous oxide was also considered. In a fifth study, emissions from a full-scale reject water treatment operating as deammonification using a moving bed biofilm reactor was much lower (0.1–0.7% compared to 10% of total nitrogen load) when operating in nitrification/denitrification mode using sequencing batch reactor mode. A complementary carbon footprint calculation in the thesis supports the recommendation of a side-stream reject water treatment, as long as the emissions of nitrous oxide from the side-stream treatment can be kept below 1.2%. The driver for this is high carbon equivalents for usage of external carbon for denitrification, which is needed for alternative treatment in the main-line as well as in the side-stream treatment.

In conclusion the performed studies show a valid methodology for rapid start-up of one stage deammonification plants, using mesophilic as well as thermophilic reject water. The studies further show the importance of measuring and take action against potential high emissions of nitrous oxide and the thesis is suggesting ways to minimize these emissions.

Sammanfattning

Den totala mängden tillgängligt kväve ökar i miljön på grund av mänsklig aktivitet. För att begränsa kväveflödet till sjöar och hav, vilket orsakar övergödning och syrebrist, omvandlar avloppsreningsverk det lösta kvävet tillbaka till kvävgas. För att möta den ökade kvävebelastningen och strängare utsläppskrav måste avloppsreningsverk överväga nya effektiva processlösningar. Reningsverken måste agera inom ramarna för lagar och regler, total miljöpåverkan samt ekonomiska möjligheter.

Deammonifikations-processen, nitrifikation i kombination med anammox (anaerob ammoniumoxidering) har visat sig vara ett energieffektivt alternativ för biologisk kväverening, speciellt för det s.k. rejektvattnet, och har idag implementerats i cirka 150 anläggningar. I de fall inte en extern tillförsel (ymp) av anammox-bakterier används, ser dock många uppstartstiden som en flaskhals för genomförandet. Risker för höga lustgasutsläpp, en stark växthusgas, har dock gjort implementeringen av separat rejektvattenrening tvivelaktig.

Syftet med denna avhandling var att utveckla och validera ett koncept för att starta en enstegsanläggning för deammonifikation, från mesofilt samt termofilt rötat rejektvatten, utan tillförsel av extern anammox-ymp. Vidare har mängderna av anammoxbakterier i rejektvattenflöden utvärderats både före och efter rökammaren för att bestämma mängden av anammoxbakterier i avloppsvatten genom användning av qPCR (quantitative Polymerase Chain Reaction). Slutligen utfördes en fallstudie vid fullskalig rejektvattenbehandling, varvid lustgasutsläppen jämfördes vid drift i nitrifikations/denitrifikations-läge (då Sekvensiell Batchreaktorteknik – SBR användes) med deammonifikation (där rörligt bärrmaterial, så kallad Moving Bed Biofilm Reactor – MBBR, användes).

Den första studien visar en fullskalig uppstart av en enstegsanläggning för deammonifikation, som genomfördes inom 10 månader. Tekniska problem, eventuell toxicitet samt begränsning av mängden utspädningsvatten och brist

på värmekälla, var troligtvis orsaken till den relativt långa uppstartstiden. Studien visar att under drift så är nitrifikation det begränsande steget, och låga syrekoncentrationer begränsar processens prestanda.

Den andra studien utvärderar om en extern ymp av anammoxbakterier minskar starttiden. De två parallella laboratoriereaktorerna visade liknande utveckling av anammox i biofilmen, vilket påvisades via fluorescens in situ-hybridisering (FISH), samt kvävereduktion, detekterad genom kemiska analyser. Resultaten från den tredje studien bekräftade att uppstarter utan ymp också kan utföras på rejektvatten från termofil rötning, med samma metod inom rimlig tid (~ 100 dagar). Detta bevisades både i laboratoriet och i fullskalan. En fjärde studie visar att anammoxbakterier är närvarande i rejektvatten (10^4 – 10^5 GU / ml), oberoende av substratet till röttkammaren, förbehandling på slammet eller rötningstemperaturen. Både rejektvatten från mesofil rötning, samt aktivt slam från avloppsreningsverk med hög slamålder, verkar som en säker källa till anammoxbakterier. Hygienisering och avvattnings fungerar som en sänka av anammoxbakterier i avloppsreningsverk.

För att kunna rekommendera genomförandet av en enstegs deammonifikationsanläggning för separat behandling av rejektvatten, granskades också potentiella utsläpp av lustgas. I en femte studie var utsläppen från en fullskalig anläggning med deammonifikation med bärarteknik (MBBR) mycket lägre, än vid drift i nitrifikations/denitrifikationsläge med användning av SBR-teknik (0,1–0,7 procent jämfört med 10 procent av inkommande kvävebelastning). En kompletterande beräkning i avhandlingen, av koldioxidavtrycket, ger stöd för att separat avloppsvattenbehandling av avloppsvatten är att rekommendera, så länge som utsläppen av lustgas från den separata behandlingen kan hållas under 1,2 procent av inkommande kväve. Motivet till denna siffra är att kompensera för användning av externt kol för denitrifikation, som behövs för alternativ behandling såväl i huvudlinjen som vid separat behandling.

Sammanfattningsvis visar avhandlingen en validerad metod för snabb uppstart av en enstegs anläggning för deammonifikation med användning av mesofilt samt termofilt rötat rejektvatten. Studierna visar vidare vikten av att mäta och vidta åtgärder mot potentiellt höga utsläpp av lustgas vid separat rening av rejektvatten. Avhandlingen föreslår sätt att minimera dessa utsläpp.

List of papers

Publications included in the thesis

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

- I. Plaza, E., Stridh, S., Örnmark, J., **Kanders, L.**, and Trela, J. (2011) Swedish Experience of the Deammonification Process in a Biofilm System. Proceedings of the IWA/Water Environment Federation, Nutrient and Recovery Conference 2011(1), 1067–1079
- II. **Kanders, L.**, Areskoug, T., Schneider, Y., Ling, D., Punzi, M., Beier, M. (2014) Impact of seeding on the start-up of one-stage deammonification MBBRs. *Environ. Technol.*, 35:2767–2773 <https://doi.org/10.1080/09593330.2014.920421>
- III. **Kanders, L.**, Ling, D., Nehrenheim, E. (2016) Rapid start-up of one-stage deammonification MBBR without addition of external inoculum. *Water Sci. Technol.*, 74:2541–2550 <https://doi.org/10.2166/wst.2016.406>
- IV. **Kanders, L.**, Beier, M., Nogueira, R., Nehrenheim, E. (2018) Sinks and sources of anammox bacteria in a wastewater treatment plant – screening with qPCR. *Water Sci. Technol.*, 78:441–451 <https://doi.org/10.2166/wst.2018.318>
- V. **Kanders, L.**, Yang, J-J., Baresel, C., Zambrano, J. (2019) Full-scale comparison of N₂O emissions from SBR N/DN operation versus one-stage deammonification MBBR treating reject water – and optimization with pH set-point. *Water Sci. Technol.*, (*In press*) <https://doi.org/10.2166/wst.2019.163>.

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- I. Operational planning and process evaluation together with colleagues during the start-up and writing the part of manuscript regarding start-up.
- II. Design and planning of the study, data collection with co-authors, and analysis of results and writing together with co-authors.
- III. Design and planning of the study, most of the data collection, analysis of results and writing the manuscript.
- IV. Design and planning of the study, data collection, analysis of results and writing the manuscript.
- V. Managing the research project, including design and planning of the study, analysis of results and writing the manuscript together with co-authors.

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Nomenclature

Abbreviations

AOB	Ammonia oxidizing bacteria
COD	Chemical Oxygen Demand
FISH	Fluorescence <i>In Situ</i> Hybridization
GHG	Greenhouse gas
HRT	Hydraulic Retention time
MBBR	Moving Bed Biofilm Reactor
NOB	Nitrite oxidizing bacteria
N/DN	Nitrification/Denitrification
PNA	Partial nitrification and Anammox
qPCR	Quantitative Polymerase Chain Reaction
SBR	Sequencing Batch Reactor
SRT	Sludge Retention Time
DS	Dry Solids
TSS	Total Suspended Solids
WWTP	Wastewater Treatment Plant

Symbols

CO _{2e}	Carbon dioxide equivalents
H ⁺	Hydrogen ion
HNO ₂	Free nitrous acid, FNA
N ₂	Dinitrogen gas
N ₂ O	Nitrous oxide, laughing gas
NH ₃	Ammonia or free ammonia, FA
NH ₄ ⁺	Ammonium
NO	Nitric oxide
NO ₂ ⁻	Nitrite
NO ₃ ⁻	Nitrate

Processes and terms

anammox	oxidation of ammonia with nitrite into dinitrogen gas and nitrate
denitrification	reduction of an oxidized form of nitrogen towards dinitrogen gas
deammonification	the combination of nitrification and anammox process also denoted as PNA
nitrification	oxidation of ammonia to nitrate
nitritation or partial nitrification	oxidation of ammonia to nitrite
reject water	liquid from dewatered digestate

1 Introduction

Wastewater treatment plants (WWTPs) are an essential part of the urban infrastructure. Nitrogen is one of the nutrients that need to be removed during wastewater treatment to limit eutrophication. This is achieved by biological nitrogen removal, which converts soluble ammonia into dinitrogen gas. As nitrogen loads on WWTPs increase due to increased protein-rich food consumption and co-digestion with food waste (Banks et al., 2018; Tumlin and Mattsson, 2013), and the increasing population and effluent treatment demands are getting stricter, existing utilities must find new or more effective processes to remove the nitrogen from the wastewater. The operational space for the utilities encompasses environmental concerns, regulations and economical aspects.

During the past decade, deammonification, i.e. nitrification in combination with anaerobic ammonia oxidation — also known as anammox — has been the preferred process for treatment of nitrogen-rich wastewaters because of its energy efficiency (Fux and Siegrist, 2004) and its lower carbon footprint in comparison to other biological nitrogen-removal processes, taking only the operational costs into account (Joss et al., 2009). However, due to higher substrate concentrations and higher temperatures in separate side-stream treatments in comparison to treatment in the main-line, high emissions of nitrous oxide (N_2O) may also occur, contributing to greenhouse gas (GHG) emissions and, consequently, global warming. Emissions have been shown to vary widely in magnitude (Kampschreur et al., 2009b), and may contribute to up to 80% of the total carbon footprint of the plant (Daelman et al., 2013). Therefore, new implementations of nitrogen removal processes should consider atmospheric emissions and not only water effluent quality. To be able to contribute to fulfil the Paris agreement (UNFCCC, 2015), anthropogenic GHG emissions must according to Rockström et al. (2017) peak by 2020 and halve by 2030.

The nitrification process has been known since the beginning of the last century, while anammox bacteria was first discovered in the late 1990’s (Beier et al., 1998; Hippen et al., 1997; Mulder et al., 1995). In the past twenty years, the number of full-scale plants and scientific publications using the anammox process have increased rapidly, together with knowledge and practical experience in this area (Figure 1). Early research carried out in the laboratory showed that anammox bacteria were slow-growing organisms, even slower than ammonia oxidizing bacteria (AOB). Moreover, start-up times in full-scale were reported to range from one to two-and-a-half years (Rosenwinkel and Cornelius, 2005; van der Star et al., 2007; Wett, 2006). The reasons for the long start-up time were often related to technical problems (Plaza et al., 2011; van der Star et al., 2007) and can today also be linked to lack of process knowledge. As a result, several actors on the market started to offer seeding material to reduce the start-up time for new plants, and the predominant practice today is to inoculate new plants with anammox sludge from anammox reactors already in operation. This market has grown, and to the author’s knowledge, of the 150 deammonification plants currently in operation or during commissioning, 148 have been seeded with inoculum from other anammox plants. The other two start-ups in full scale are in focus for the present thesis. Despite new findings regarding the doubling time of anammox bacteria, many researchers still refer to the bacteria as “extremely slow-growing” and formulate new research questions based on this assumption.

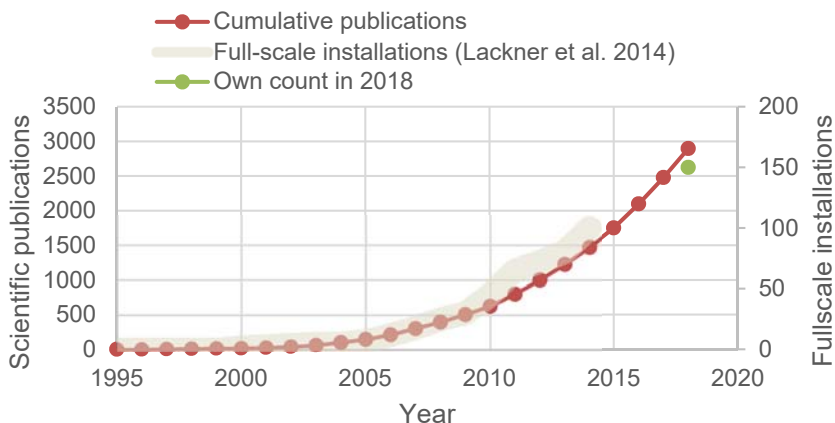


Figure 1. Number of full-scale deammonification installations worldwide (data adapted from Lackner et al. 2014) and authors own count in 2018 together with cumulative number of scientific publications from the Web of Science database with the topic ‘anammox’ or ‘deammonification’ between 1995 and 2018.

The aspect of start-up times and methodologies becomes even more relevant when starting to implement deammonification in the main-stream treatment of the wastewater line. There is a large potential for many utilities in energy savings and increasing biogas production instead of using the carbon source for denitrification of nitrate (NO_3^-). Many researchers have investigated this topic (for example, Gilbert et al., 2015; Hoekstra et al., 2018; Laurenzi, 2017; Malovanyy et al., 2015; Persson et al., 2017) with positive results but there are still no full-scale installations relying in the deammonification process in the main-stream.

This thesis aims to add a puzzle piece to the cumulative knowledge of the deammonification process, and more specifically, the start-up of the anammox process for full-scale plant operation with the Moving Bed Biofilm Reactor (MBBR) technique on reject water. Previous reported studies started up without anammox seeding all make use of the MBBR technique (Mehrddad et al., 2014; Rosenwinkel and Cornelius, 2005; Schneider et al., 2009; Zekker et al., 2012a) and it is therefore the technique of choice.

The thesis is located in the interphase of the theoretical knowledge of these types of processes and the full-scale operational aspect. The transfer from level of knowledge to operation in full scale is often difficult. In this case, the use of a biofilm application includes mass-transfer limitations, including the stratification of biofilm and interactions of several microorganisms. Full-scale operation also includes variations in essential boundary conditions such as flow, temperature and the mass transfer of oxygen.

On the other hand, these studies in full scale have shown how direct implementation of new-found knowledge can be put into practice. Although the study is limited to a biofilm application treating reject water, its conclusions are, in several ways, applicable to similar wastewater streams such as leachate and industrial nitrogen rich streams, or even main-stream treatment, as well as to similar techniques such as granules and suspended flocs.

1.1 Objective and research questions

This thesis discusses the implementation of a one-stage deammonification plant for reject water treatment. The scientific questions address typical problems for a process designer, water utility or plant operator. The research questions thus arise from the main evaluation factors: important operational parameters, time and method for starting up a stable process as well as operating costs and environmental considerations such as carbon footprint.

The objective of the research was to develop and validate a start-up strategy for a MBBR treating reject water and to investigate the potential

advantages of using external anammox seeding during start-up. In connection with these objectives, knowledge about anammox quantity in different types of reject waters was regarded as essential. Further, in order to optimize the operation of the process, N₂O emissions have been evaluated and automatic control has been used as a mitigation tool to limit emissions. Consequently, the work has focused on answering the following research questions:

- What operational parameters are the most important to minimize start-up time and operation of a one-stage deammonification process? (Papers I and III)
- How does external anammox inoculum influence the start-up time of a one-stage deammonification process? (Paper II)
- How does substrate to the digester, hygienization as pre-treatment of substrate and the digestion conditions influence concentration of anammox bacteria in reject water? (Paper IV)
- How can automatic control during operation of a one-stage deammonification process influence the N₂O emissions? (Paper V)

1.2 Outline of the thesis

The thesis includes five papers, denoted with roman numerals — Papers I, II, III, IV and V — and the outline of the thesis work is summarized in Figure 2. Paper I summarizes the start-up and operation of one of the first deammonification plants in full scale, at the Himmerfjärden WWTP in Stockholm in 2007. This start-up was carried out without any addition of external anammox seeding and took 10 months in total to grow a sufficient biofilm.

Experiences from this start-up were evaluated, and in Paper II these experiences were taken into the laboratory, where two reactors were run and external anammox seeding was applied for one of the reactors. As substrate, reject water from a nearby *mesophilic* anaerobic digester (Sjölunda WWTP, Malmö) was used.

Paper III covers two laboratory reactors treating reject water from *thermophilic* anaerobic digestion, and represents one of the first studies on the topic. Earlier experiences from a full-scale start-up test at Bekkelaget in Oslo indicated an uncertainty regarding the combination of thermophilic reject water and anammox bacteria, since anammox is mesophilic and

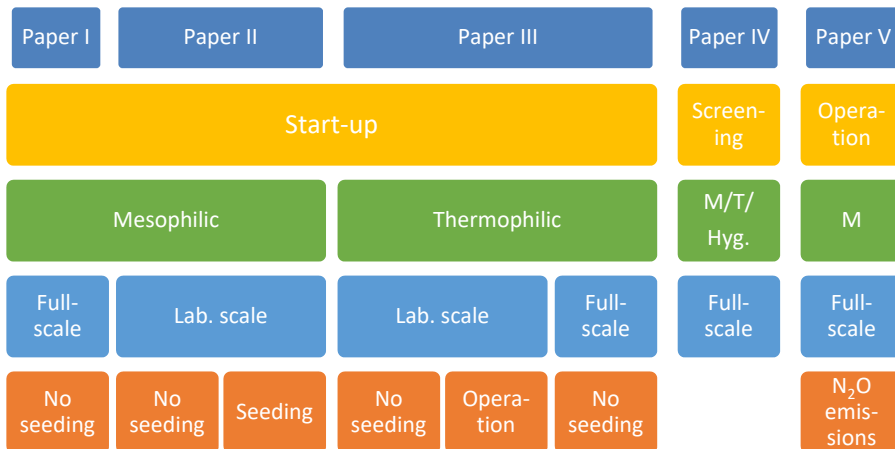


Figure 2. Outline of the thesis work. Numbered papers (blue) in relation to start-up versus operational strategies (yellow), use of reject water from mesophilic or thermophilic digestion (green), use of full-scale or laboratory reactors (blue) and whether external anammox seeding was used in the reactors or not (orange). M/T/Hyg is an acronym for mesophilic or thermophilic digestion conditions and hygienization, which is a pre-treatment method used for sludge.

treatment at higher-than-mesophilic temperatures could irreversibly inhibit anammox activity (Dosta et al., 2008) or produce inhibiting compounds for both AOB and anammox bacteria (Figdore et al., 2011). Again, laboratory reactors were used, but this time with thermophilic reject water. One reactor was fully seeded with deammonification carriers and only operated on this substrate while the other was performing a start-up of the process. The paper also describes the subsequent start-up in a full-scale plant.

Paper IV considers the microbiological community, focusing on the quantification of anammox bacteria in the reject water, the substrate for the deammonification process. This study was needed to quantify the abundance of bacteria in different types of reject water, and to confirm the hypothesis regarding the potential for a non-seeded start-up. If reject water from mesophilic and thermophilic digesters operating on sludge from municipal WWTPs contained sufficient anammox bacteria to perform a successful start-up within a reasonable timeframe (considered to be below six months), the next question was: how does the hygienization or thermal hydrolysis (high temperature treatment) upstream of the digesters influence the quantities of anammox bacteria and how does the substrate entering the digesters influence these quantities? To experimentally investigate this, samples from seven Nordic digesters, with substrate and reject water characteristics covering the

aspects above, were analysed using qPCR to quantify viable anammox bacteria.

After having analysed the results in Papers I–IV, the indicative study was further operationalized by investigating a sustainable operation of these plants. There had been a discussion in the research community about the potential GHG emissions, especially those of N₂O, from this type of reject water process. Therefore, it was deemed necessary to focus on this aspect to understand the full potential of the process. Therefore, Paper V addressed the N₂O emissions when a full-scale reject water treatment changed operation from nitrification/denitrification operation in a Sequencing Batch Reactor (SBR) to a one-stage deammonification MBBR. The paper also reveals the potential of minimizing the N₂O emissions by using a different automatic control strategy. In this study, different pH set points are in focus.

Chapter 2 provides an introduction and theoretical background to the subject, Chapter 3 introduces the methodologies used, Chapter 4 summarizes the results from Papers I–III and discusses research questions 2 and 3 and partly question 1, addressing start-up questions. Chapter 5 answers research question 4 as well as question 1, in part, which refers to operational strategies. Chapter 6 presents conclusions of the work, while Chapter 7 defines further research to be done.

2 Biological nitrogen removal in wastewater treatment

2.1 The nitrogen web

The conversion from dinitrogen gas, via plants and animals, back to dinitrogen gas in the biosphere has been referred to as “the nitrogen cycle”, but more recent research suggests that the process is better described as a nitrogen web (Kuypers et al., 2018). There are six main conversion processes for nitrogen: fixation, nitrification, denitrification, anammox, assimilation and ammonification. The conversion of nitrogen into different states is described in Figure 3.

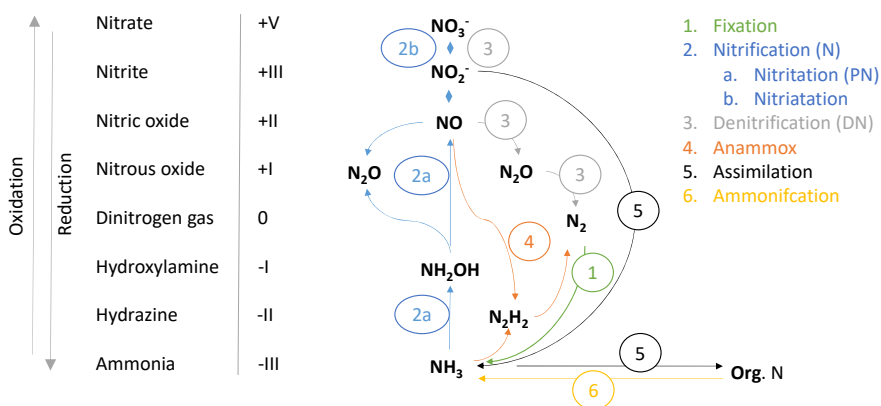


Figure 3. The nitrogen web (modified from Kuypers et al. 2018).

Anthropogenic nitrogen fixation has increased rapidly since the 1960s, mainly through the Haber–Bosch technique and fossil fuel generated deposits, resulting in increased available nitrogen in the biosphere. Annual anthropogenic fixation of nitrogen is in the same order of magnitude as bacterial fixation in terrestrial and marine ecosystems (Kuypers et al., 2018). This positive net flux of nitrogen into the biosphere, causing not only eutrophication (by emissions of NH_4^+ and NO_3^-) in water bodies, but also contributing to formation of potent GHGs, such as N_2O .

N_2O is an intermediate or side-product to nitrification and denitrification, and there is a risk that it may be formed anywhere nitrogen conversion processes occur, whether in the environment or in engineered systems. The gas is a potent GHG (298 CO_2e), based on a 100-year horizon, since it is stable once it reaches the stratosphere and it is also ozone depleting (Ravishankara et al., 2009). Nitric oxide (NO) is also an intermediate for many of the processes (Figure 3), and indirectly contributes to global warming, since it contributes to the formation of O_3 . However, NO gas is not considered further in this thesis. The Intergovernmental Panel on Climate Change (IPCC) have estimated nitrous oxide emissions from WWTPs to be 0.5% of the incoming nitrogen load (IPCC, 2006). However, measurements in full-scale plants have shown both much higher and lower emissions (Kampschreur et al., 2009b).

2.2 Wastewater treatment and nitrogen removal

In order to protect water bodies from eutrophication, toxic nitrogen compounds (NH_3 and NO_2^-) or oxygen depletion by unlimited access of nitrogen, WWTPs in the European union are strictly regulated (European Economic Community, 1990). To date there are no regulations for GHG emissions from WWTPs, but several countries have implemented rules stating that WWTPs are obliged to estimate, calculate and report their emissions (e.g. Australia, Germany and Sweden). These demands are closely linked to the UN Sustainable Development Goal (SDG) no. 13: to take climate action. Other SDGs which direct or indirectly relate to the treatment of wastewater and sludge handling are: focusing on clean water and sanitation (goal 6), affordable and clean energy (goal 7) and taking concerns of life below water (goal 14). A third concern for wastewater utilities is the economic aspect in relation to the continuing search for more effective treatment methods.

Nitrogen removal in wastewater treatment is preferably done with biological methods. Alternative methods such as chemical stripping or evaporation are more expensive and only applicable at very high nitrogen concentrations, and since inorganic fertilizers are cheap, the economic drivers for reusing the nitrogen in wastewater is low (Siegrist, 1996). Therefore, the goal of nitrogen removal is to transform the sewage nitrogen to dinitrogen gas (N_2), see Figure 3. The most commonly used processes for nitrogen removal in the main-line are nitrification, denitrification and assimilation, whereas deammonification can be applied as reject water treatment in warmer side-stream treatments.

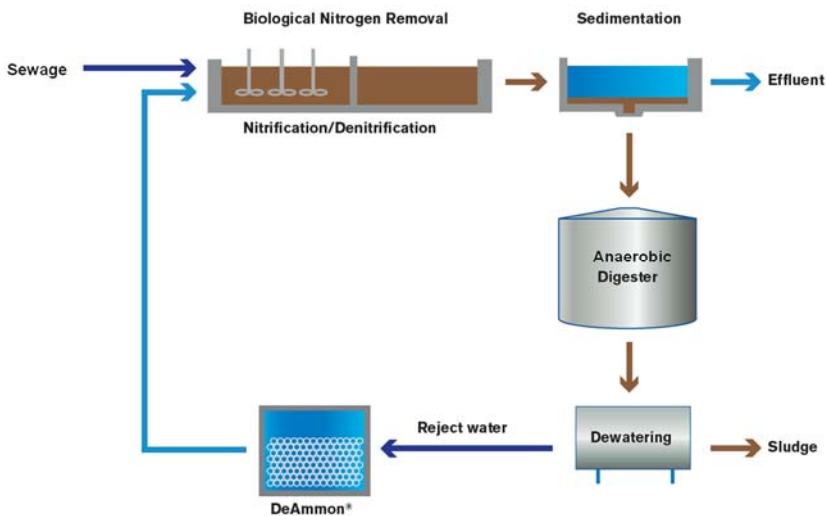


Figure 4. Conceptual WWTP for municipal sewage with side-stream reject water treatment. With courtesy from Purac AB.

Sewage reaching the municipal WWTPs contains nitrogen in the form of dissolved ammonium (NH_4^+) and particulate, organic nitrogen. A simplified, conceptual WWTP is shown in Figure 4. Approximately 40% of the influent nitrogen is assimilated in the biological sludge or included in particulate material, whereas 40% is converted into dinitrogen gas via nitrification/denitrification in the main treatment line, resulting in approximately 20% of the influent nitrogen leaving the plant via the effluent. The sludge consisting of organic, particulate material, as well as excess sludge from the biological treatment, are treated by anaerobic digestion in many WWTPs, whereby approximately 50% of the organic matter is converted into methane-rich gas, while the nitrogen in the sludge is released as dissolved ammonium, again via ammonification. In this way, the

supernatant from dewatered, anaerobic, digested sludge contains high concentrations of ammonium. This reject water, also referred to as the side-stream, liquid digestate or digestate supernatant, is in many cases returned untreated to the main process, creating an internal load. Without separate treatment, the reject water contributes 10–20% of the total nitrogen load of a WWTP (Siegrist, 1996).

2.2.1 Reject water and its characteristics

Table 1 shows characteristics of municipal wastewater and reject water (la Cour Jansen et al., 2019a). In comparison to municipal wastewater, reject water has much higher concentrations of nitrogen, higher temperatures, lower COD/N ratio and a lower alkalinity-to-nitrogen ratio. Composition and temperature of municipal wastewater may vary depending on where in the world it comes from, but reject water is relatively stable in composition both over time and region because of its origin. The flow of the reject water is only a few percent of the influent, which makes it suitable for a separate side-stream treatment.

A driver for many WWTPs in Scandinavia today is to increase biogas production in order to become energy neutral. By applying co-digestion, in which external organic and nitrogen-rich material is added to digesters (for example household waste), the proportion of nitrogen in reject water has increased over time. In addition, increased hygienization demands on sludge as well as pre-treatment of sludge by high temperature treatment (causing disintegration of the cells and improved digestibility) is becoming more common. The average ammonia concentrations in the reject water are therefore expected to increase even further.

Table 1. Characteristics of influent wastewater (sewage) in comparison to reject water after anaerobic digestion (la Cour Jansen et al., 2019a)

	Flow	Temp	TN	NH ₄ -N	COD	sCOD	sCOD/ NH ₄ -N	Alk/ NH ₄ -N
		C°	mg/L	mg/L	mg/L	mg/L		mol:mol
Municipal wastewater	100 %	6–19	20– 80	12–50	210– 740	80– 300	6–7	~0.8– 8.1 ¹
Reject water from AD of municipal WW sludge	~1%	25– 35	100– 1100	95– 1000	800– 4000	600– 3000	3–6	~0.14– 1.4

AD = anaerobic digestion

¹ Highly dependent on raw water composition

2.2.2 Reasons for implementing a side-stream reject water treatment

Wastewater utilities must operate within the boundaries of the requirements stipulated by law, environmental aspects and economic aspects. As the nitrogen load in the influent is increasing and stricter restrictions are being implemented on the effluent content of nitrogen, the capacity for nitrogen removal in the main-line may become limited. The limitations in the main treatment can be i) lack of nitrification capacity, ii) lack of denitrification capacity, or iii) lack of alkalinity. Each of these limitation could be solved with additional investment or operational costs. Side-stream reject water treatment could therefore be an alternative for handling this additional load, as well as to avoid disturbances in the main-line caused by intermittent reject water loads. Even without nitrogen conversion limitations in the main-line, a side-stream treatment of reject water with deammonification is beneficial from an operational costs view (Fux and Siegrist, 2004). However, in Sweden, the majority of WWTPs still treat their reject water in the main-line (Stenström et al., 2017).

2.3 Nitrogen processes used in wastewater treatment and their microorganisms

In this thesis, nitrogen removal through partial nitrification and anammox is in focus; in some studies denoted as PNA. Other processes, such as oxidation of nitrite (NO_2^-) and denitrification also occur simultaneously in the process and therefore influence the performance of the deammonification process. The following chapter presents these processes, and a summary of kinetic parameters for the microorganisms is presented in Table 2, p. 14. For comparison, *acetoclastic methanogens*, which mainly produce methane from acetate and are used in biogas production, are also included in this table. A separate chapter focuses on N_2O production and reduction. There is still much to discover regarding the biodiversity of nitrogen conversion; for a comprehensive summary including enzymatic conversions or different anammox species, the reader is referred to reviews by Kuypers et al. (2018) and Oshiki et al. (2015).

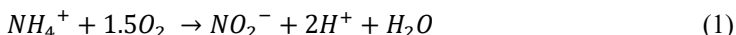
2.3.1 Nitrification

Nitrification is the biological transformation of ammonia to nitrate in aerobic conditions. The nitrification can occur in one, two or three steps, by the same or different bacterial groups, which all are chemoautotrophic, i.e. they obtain energy by the oxidation of ammonia and use carbon dioxide as the carbon source (Kuypers et al., 2018).

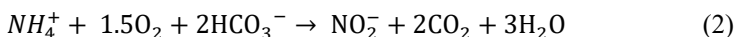
Nitritation or partial nitrification

First, ammonia is oxidized to hydroxylamine (NH_2OH), but since this is an endergonic reaction, all AOBs save energy by further oxidizing hydroxylamine to nitric oxide (NO) and nitrite. This step is called nitritation or partial nitrification (PN). Examples of microorganisms performing these processes include *Nitrosomonas*, *Nitrospira* and *Nitrosococcus*, all of which belong to the group of Alphaproteobacteria and Betaproteobacteria (Kuypers et al., 2018). There are also Ammonium Oxidizing Archea (AOA) that perform these reactions, which are commonly found in sea, but are also found in WWTPs (Könneke et al., 2005).

The nitritation process can be written chemically as



or including the metabolism of AOBs,



Two moles of hydrogen ions are produced for each mole of oxidized ammonia, contributing to a decreasing pH during the reaction. Since HCO_3^- is also consumed as a carbon source, alkalinity can limit how much ammonia can be oxidized. AOBs are inhibited by their substrate free ammonia (FA or NH_3) and by free nitric oxide (FNA or HNO_2) (Anthonisen et al., 1976). AOBs are also known for producing N_2O , a strong GHG, by performing nitrifier-denitrification or reducing NO by oxidizing hydroxylamine.

Nitratation

The oxidation of nitrite to nitrate, nitratation, can be done by nitrite-oxidizing bacteria, NOB, to gain energy. Examples of such bacteria are *Nitrobacter* and *Nitrospira*, which belong phylogenetically to Betaproteobacteria and Gammaproteobacteria.

The nitrification process can be described chemically as

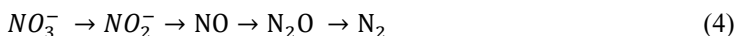


The differences in kinetics of AOB and NOBs can be used to achieve a partial nitrification system only producing NO_2^- . Firstly, NOBs are more sensitive to lower oxygen concentrations, see K_{s,O_2} in Table 2. Secondly, at higher temperatures (>20 °C), NOBs has a lower doubling time compared to AOB (Hellinga et al., 1998) and they are more sensitive to concentrations of both FA and FNA (Anthonisen et al., 1976). In addition, NOBs also have a longer lag phase following anaerobic conditions.

A group of bacteria belonging to the *Nitrospira* have recently been found to have the ability to oxidize ammonia to nitrate; this group is denoted comammox, for complete ammonium oxidation (Daims et al., 2015; Van Kessel et al., 2015).

2.3.2 Denitrification

Denitrification (DN) is reduction of nitrate to dinitrogen gas in several steps. Nitrate is reduced to nitrite, nitric oxide, nitrous oxide and further to dinitrogen gas, as shown in Equation 4. Each step is regulated by separate enzymes, and microorganisms may have the ability to perform one or more steps in the chain. The process can be performed by many diverse heterotrophic microorganisms that can use nitrate or nitrite as electron acceptor and gain energy in combination with an organic or inorganic source (such as sulphide). The bacteria would gain more energy by using pure oxygen as electron acceptor, and therefore denitrification only takes place under anoxic conditions.



Nitrous oxide (N_2O), can accumulate and be emitted to the atmosphere when there is an imbalance between production and reduction of nitrous oxide in the denitrification chain. On the other hand, denitrification is the only known sink for N_2O in wastewater treatment. This process is regulated by the enzyme nitrous oxide reductase (N_2O reductase) and is known to be sensitive to pH (Pauleta et al., 2013).

In specific conditions, such as with a high COD/N ratio, denitrification can be replaced by dissimilatory nitrate reduction to ammonia (DNRA), which retains the nitrogen in the system as ammonium (Kraft et al., 2011; van den Berg et al., 2016). Anammox bacteria have also been found to be able to perform denitrification (Güven et al., 2005; Kartal et al., 2007).

Table 2. Kinetic parameters for commonly used microorganisms in WWTPs.

Parameter	Units	Ammonia oxidation	Nitrite oxidation	Anammox	Denitri-fication	Acetocl. methano-gens
Electron donor		NH ₃	NO ₂ ⁻	NH ₄ ⁺	Org. matter	Acetate
Electron acceptor		O ₂	O ₂	NO ₂ ⁻	NO ₃	CO ₂ (1)
Product		NO ₂ ⁻	NO ₃ ⁻	N ₂	N ₂	CH ₄
Carbon source		CO ₂	CO ₂	CO ₂	Org. comp.	Org. comp.
Oxygen demand	gO ₂ /gN	3.43	1.14	–	–	–
COD dem incl. ass.	gO ₂ /gCOD	–	–	–	4.0	–
Alkalinity change	eqv/mol N	-2	–	+0.066	+1	–
Yield, Y ²	gVSS/gCOD or gNH ₄ -N	0.10–0.12 (20 °C)	0.05–0.07 (20 °C)	0.15 (32–33 °C)	0.5–0.55 (20 °C)	0.03–0.04 (35 °C)
Maximum growth rate, μ _{max} ²	day ⁻¹	0.6–0.8 (20 °C)	0.6–1.0 (20 °C)	0.1–0.35 (30–38 °C, Fig.5)	3–6 raw ww 5–10 meOH (20 °C)	0.3–0.5 (35 °C)
Recalculated maximum growth rate, μ _{max} (30 °C) ³	day ⁻¹	–	–	0.1–0.2 (30 °C)	6–12	0.2–0.35
Theoretical doubling time, t ⁴	day	0.9–1.2 (20 °C)	0.7–1.2 (20 °C)	2.0–6.9 (30–38 °C)	0.1–0.2 (20 °C)	1.4–2.3 (35 °C)
Theoretical doubling time, t (30 °C)	day	~0.4 ⁵	~0.6 ⁵	3–9 ⁴	0.06–0.12 ⁴	2.0–3.0 ⁴
O ₂ half-saturation time, K _s ²	mg/L	0.5–1.0	0.5–1.5	–	–	–

1. No external electron acceptor occurs during fermentation
2. la Cour Jansen et al., 2019b
3. Recalculating using the assumption that the reaction rate doubles with every 10 °C up to the optimum temperature according to van't Hoff-Arrhenius

relationship $\mu(T_1) = \mu(T_2)\theta^{(T_1-T_2)}$, where θ is set to 1.07 (Rittmann and McCarty, 2001)

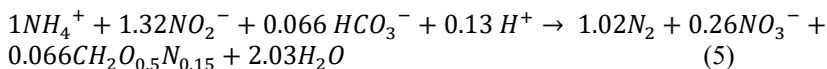
4. Doubling time, t , calculated from μ_{max} above with

$$t = \frac{\ln 2}{\mu_{max}}$$

5. Van Hulle et al., 2010

2.3.3 Anammox

Anammox is the abbreviation of ANaerobic AMMonium Oxidation and means that ammonium is oxidized with nitrite as electron acceptor. The anammox bacteria are autotrophic and uses carbon dioxide as carbon source and are sometimes denoted AnAOB. During anoxic conditions, they can transform nitrite and ammonia to dinitrogen gas with NO_3^- as a by-product. This is done through reduction of NO_2^- to NO , which together with NH_4^+ forms hydrazine (N_2H_2). The hydrazine is then finally oxidized to dinitrogen gas (N_2) (Kartal et al., 2011). The anammox process can be described chemically as (Strous et al., 1998):



However, Lotti et al. (2014a) presented an alternative stoichiometry with a $\text{NO}_2^-/\text{NH}_4^+$ ratio of 1.146 instead of 1.32. Equation 5 indicates that ammonia and nitrite are consumed at the ratio 1:1.32. The process therefore needs >50% nitrite content to work optimally. Besides dinitrogen gas, nitrate is also formed at a rate of 11% of ammonium reduced, which means that not all ammonia can be transformed to dinitrogen gas through anammox reactions.

Anammox bacteria readily form biofilms or granules, especially in the presence of oxygen (Lotti et al., 2014a), and have a characteristic reddish colour due to the high content of cytochrome *c*. The bacteria have an intracellular compartment, the anammoxosome, where the reaction with N_2H_2 can take place (Fuerst, 2005). They belong to the phylum Planctomycetes, and so far, five genera belonging to *Candidatus Scalindua*, *Kuenenia*, *Anammoxoglobus*, *Jettenia* and *Brocardia* have been identified in the anammox phylogenetic tree, comprising ten known species (Kartal et al., 2013; Ma et al., 2016). Since anammox are autotrophic bacteria, the bacterial yield is low.

The anammox process was first predicted via thermodynamic calculations (Broda, 1977) before it was detected in a pilot-scale wastewater plant (Mulder et al., 1995), and later cultivated at laboratory scale (Strous et al., 1998; Van

de Graaf et al., 1996). In early work, it was also found in a rotating biological contactor treating landfill leachate (Helmer and Kunst, 1998). The bacteria have been difficult to cultivate in pure culture and their kinetic parameters have been updated in several studies, see Table 2 and Figure 5.

2.3.3.1 Anammox and doubling time

Since they were discovered, anammox bacteria have been classed as “extremely slow-growing bacteria”, although in the past ten years, the new findings for growth rate has stabilized at around 2–4 days doubling time in different laboratory reactors operating on synthetic medium and inoculated with anammox bacteria from other reactors, see Figure 5. The reasons for the differences in doubling time may be the use of different cultivation methods: sequencing batch reactor, biofilm, membrane bioreactor or gel beads (Zhang et al., 2017). There are also differences in reactor temperature (Lotti et al., 2014a) and differences in the type of bacteria in the population (Oshiki et al., 2015; Tsushima et al., 2007). Another reason for the variation could be the base for calculating μ_{max} , from which the doubling time is calculated, which may be determined from the counts of anammox bacteria or from the nitrogen removal rate and biomass yield (Van Hulle et al., 2010). The doubling time for anammox bacteria at 30–38 °C of 2–7 days (Figure 5; Lotti et al., 2015; Strous et al., 1999, 1998; Tang et al., 2011; Tsushima et al., 2007; Van de Graaf et al., 1996; Van Der Star et al., 2008; Zhang et al., 2017) can be compared with other microorganisms used in the treatment plant, such as AOBs (0.4 days at 30 °C) or acetoclastic methanogens (2–3 days at 30 °C). Anammox bacteria are still among the slowest-growing microorganisms in

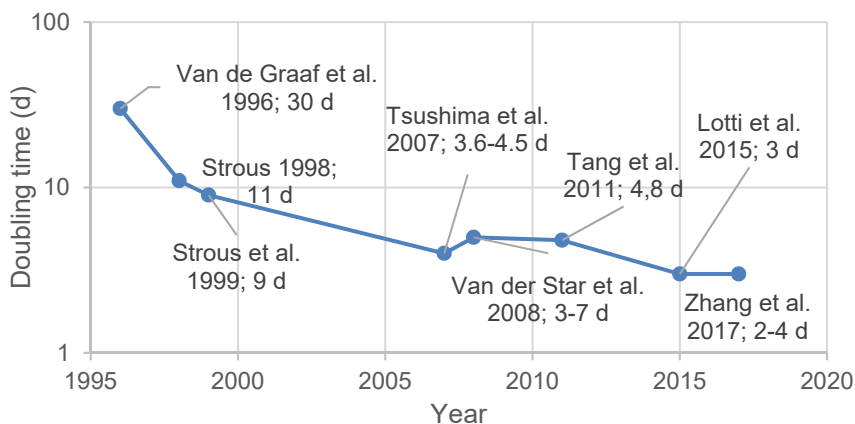


Figure 5. Anammox doubling time according to the literature operated in laboratory on synthetic water. Note that the y-axis is logarithmic.

WWTPs but the differences are not as large as previously believed (see Table 2).

Few publications refer to μ_{\max} in full-scale reactors operating with real wastewater. Van der Star et al., (2007) reported doubling times of 2–19 days from a high-loaded granular reactor, as calculated from the increase in reaction rates. They obtained increased doubling times when calculating growth using gene copy number (9–17 days). Another full-scale reactor operating in SBR mode had an estimated doubling time of 15–25 days, calculated over four months by comparing conversion rates (Joss et al., 2009). Park et al. (2010) operated a laboratory SBR on real reject water and inoculated it with anammox bacteria in parallel with a MBBR inoculated with activated sludge, achieving doubling times of 5.3 and 8.9 days during the growth phase. These reactors had an operating temperature of 35 °C. Doubling time in another MBBR in pilot scale, also inoculated with activated sludge and operating at a temperature of 32–34 °C, was calculated to be 6.3 days using qPCR (Mehrdad et al., 2014). In general, doubling times for anammox calculated on real wastewater are higher than corresponding data in purified cultures in laboratory scale.

2.3.3.2 Anammox in natural habitats

Anammox bacteria were first discovered in engineered systems like WWTPs before they were found in the environment (Kuypers et al., 2003; Mulder et al., 1995). Later they were detected in marine sediments, thereafter in the water column and in freshwater sediments (Penton et al., 2006; Rysgaard et al., 2004; Schmid et al., 2007; Schubert et al., 2006). Within a few years it was concluded that these bacteria are responsible for 30–50% of nitrogen conversion to dinitrogen gas in the world's oceans (Kuypers et al., 2005). This means that anammox bacteria are widespread in the environment. In WWTPs, they are also found in parts of the process that have not been inoculated (Chamchoi and Nitisravut, 2007; Toh et al., 2002; Wang et al., 2015). In a digester, typically, a few percent of the microbiota are related to the phylum Planctomycetes, to which anammox bacteria belong (Schnürer, 2016).

2.3.4 Deammonification in comparison to nitrification/denitrification processes

Deammonification has several advantages over the conventional nitrification/denitrification system, although it requires particular boundary conditions. Combining nitritation and anammox results in a complete autotrophic system, with no external organic carbon source requirement,

which saves 60% of the aeration demand in comparison to the conventional nitrification/denitrification process, since only 50% of the ammonia is oxidized to nitrite (Joss et al., 2009). It also generates less sludge due to the low microbial yield (see Table 2). If the main-line is limited by alkalinity, deammonification will also reduce the need to add alkalinity since the reject water only contains 1 mol HCO_3^- per mol $\text{NH}_4\text{-N}$ (see Table 1), and a complete nitrification would require 2 mol. So far, most implementations with deammonification have been under mesophilic conditions, such as in reject water treatment (Lackner et al., 2014). In reject water treatment, the suppression of NOBs is seldom a problem due to higher temperatures and higher substrate concentrations of FA for the reasons mentioned earlier.

By treating reject water with deammonification instead of conventional N/DN processes, less nitrogen is converted by AOB and no or minor activity by denitrifying bacteria occurs. This means that less ammonia is oxidized, less aeration or stripping and minor incomplete denitrification. These are all factors that decrease the formation and stripping of N_2O .

2.3.5 Nitrous oxide formation and reduction

Nitrous oxide can be formed in three microbial-driven reactions: autotrophic denitrification or oxidation of hydroxylamine performed by AOBs, or by incomplete denitrification performed by many heterotrophic bacteria. The different production pathways are illustrated in Figure 6. Denitrification is the only metabolic pathway to reduce nitrous oxide during formation of dinitrogen gas (Desloover et al., 2012; Massara et al., 2017).

1. Autotrophic denitrification
2. Oxidation of hydroxylamine (NH_2OH)
3. Incomplete heterotrophic denitrification
4. Complete denitrification, reduction by N_2O reductase

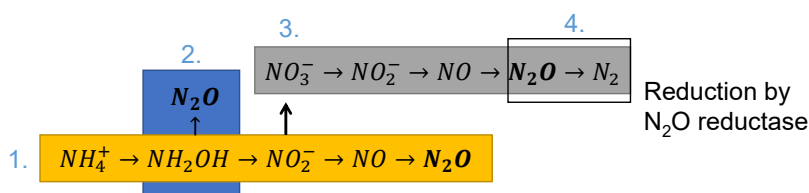


Figure 6. Main pathways for production of nitrous oxide in biological nitrogen conversion.

Depending on environmental conditions, nitrous oxide can be produced via the three different routes presented above. In biological nitrogen removal in WWTPs, some conditions can trigger nitrous oxide production and emission. Nitrous oxide production is well correlated to ammonium oxidizing rates, and may occur at high ammonia concentrations, peak ammonia loads, limited oxygen and high temperatures. It is also known to be positively correlated with nitrite concentrations (Law et al., 2012; Tallec et al., 2006). Referring to the denitrification route, nitrous oxide accumulates in the water phase when there is a higher production than reduction of the gas. This can depend on acceleration of the preceding step, increased nitrate or nitrite concentrations, peak loads or transition from aerobic to anoxic conditions (Firestone et al., 1980; Schneider et al., 2012). Further, it may take place with limitation or inhibition of N_2O reductase, the enzyme responsible for reducing the gas further to dinitrogen gas. This can occur in the presence of oxygen, low pH or with a lack of biodegradable substances (Hynes and Knowles, 1984). However, the anammox bacteria does not have N_2O as an intermediate substance and is not expected to produce N_2O (Kartal et al., 2007).

2.4 Factors influencing the deammonification process

There are many environmental factors influencing the deammonification process when implemented at a WWTP. These are crucial at the sensitive initial phase of start-up and then later for increasing the volumetric load or mitigating N_2O emissions. Some of the parameters are set at the design stage, such as hydraulic retention time (HRT) and sludge retention time (SRT), whereas others may be adjusted more easily during operation of the plant, such as dissolved oxygen concentration in water phase (DO), nitrite and nitrate concentrations and to some extent temperature, alkalinity and pH. Some parameters are dependent on substrate — in this case, composition of reject water, e.g. dissolved or particulate nitrogen and organic compounds as well as macro- and micronutrients or toxins. The addition of polymer to improve dewatering characteristics and anti-foam agents are both known to decrease oxygen transfer to the water (Phillips et al., 1960; Zhang et al., 2018) and thereby affect the deammonification process. Independently of process choice, Desloover et al., (2012) concluded that mitigation of N_2O formed during biological nitrogen removal processes is feasible: by minimizing the production, minimizing the emissions and maximizing the reduction of nitrous oxide.

2.4.1 Temperature

The process temperature has a large effect on the kinetic reactions as well as other process parameters. The temperature dependence of the growth rate can be estimated with the Arrhenius relationship. The microorganisms used in reject water treatment are mostly mesophilic bacteria, with temperature optima around 35–38 °C and a rapid decrease in activity above 40 °C (Lotti et al., 2014b; Van Hulle et al., 2007). Toh et al., (2002) had no success with cultivating anammox in thermophilic conditions. The temperature of the reject water depends on the digestion temperature and temperature losses or temperature recovery from the anaerobic digestion to the reactor tank.

2.4.2 Hydraulic retention time and sludge retention time

HRT is the average time for the water to pass through the system, and is calculated using Equation 10, p.35. By retaining the sludge in the process for longer than the HRT, the SRT can be separated, and is potentially longer than the HRT. The SRT in the system needs to be longer than the doubling time for the bacteria at the process temperature to keep the bacteria in the system. How much longer depends on competing microorganisms and inhibiting conditions. Different technical solutions use different means of retaining or washing out the desired bacteria in the system. During mesophilic conditions, in this case at 30 °C, the minimum SRT for AOB is 0.4 d (Table 2). Only the aerated SRT are relevant when considering the AOBs. Anammox bacteria need a longer SRT than other bacteria, a minimum of four days at 30 °C (Table 2). SRT is not an applicable term in a biofilm system, and the sludge age cannot be controlled. See Chapter 2.5.1 for more regarding biofilms.

2.4.3 Oxygen concentration

Oxygen is the limiting substrate in the nitrification process, and the process is therefore very sensitive to oxygen concentrations. The rate dependence for AOBs can be described using a Monod function with 50, 60 and 80% of μ_{\max} approximately for 1, 2 and 3 mg DO/L respectively (Van Hulle et al., 2007). Oxygen concentrations can be used as a selective tool to favour AOB growth above NOB growth as mentioned in Chapter 2.3.1.

On the other hand, oxygen is inhibiting for anammox and denitrifying bacteria. For anammox bacteria this inhibition is temporary and reversible (Strous et al., 1997), whereas denitrifying bacteria are facultative anaerobes. Oxygen is measured and can be controlled in the bulk liquid, but is lower in floc, granules or biofilm, due to mass transfer limitations, see Chapter 2.5.1.

Since oxygen concentrations influence both the nitrification and denitrification process, oxygen control is important in order to control N_2O production and reduction.

2.4.4 pH and alkalinity

Like temperature, pH influences many microbial and chemical nitrogen transformations. pH directly affects enzymatic reaction kinetics or affects reactions indirectly by poisoning the equilibrium of their substrates: NH_4^+/NH_3 with $pK_a = 9.25$ or NO_2^-/HNO_2 with $pK_a = 3.3$ (Tomaszewski et al., 2017). AOBs and NOBs have a pH optima between 7 and 8 (Van Hulle et al., 2010), and the nitrification process itself influences the pH and alkalinity due to the production of protons and consumption of inorganic carbon (Equation 1 and 2). Anammox is sensitive to pH fluctuations and has reported optima between 6.5 and 8.3 (Tomaszewski et al., 2017). The last step of denitrification from N_2O to N_2 has been shown to be more sensitive to change in pH than preceding steps (Pauleta et al., 2013).

Both nitrification and anammox are dependent on inorganic carbon (measured as alkalinity) due to their autotrophic nature (Wett and Rauch, 2003; Zekker et al., 2012b). On the other hand, denitrification produces alkalinity, which is desirable when using reject water as substrate, where alkalinity could be limiting the process.

2.4.5 Nitrogen as substrate and inhibitor

Free ammonia, NH_3 , the substrate for AOB, is also inhibiting. NH_3 is also inhibiting for the NOBs (Aktan et al., 2012; Anthonisen et al., 1976; Jaroszynski et al., 2012). Tang et al. (2010) showed that anammox were inhibited in a range of 57–178 mg/L NH_3 , whereas Jaroszynski et al., (2012) showed inhibitory effects at 2 mg/L NH_3 . Anammox is also sensitive to high NO_2^- concentrations; activity is reduced by 50% at 100 mg/L (Strous et al., 1999) to 400 mg/L (Lotti et al., 2012). NOBs have shown to be more sensitive to the pH equilibrium ion, FNA, than AOBs (Anthonisen et al., 1976). This means that during start-up of the processes, these nitrogen concentrations must be low to avoid inhibition during the sensitive growth phase.

2.4.6 Organic compounds and other nutrients

If anaerobic digestion, the preceding biological step of reject water treatment, is operating well, the degradable organic content in the reject water will be low. Methanol, which can be added as electron donor in DN processes, has been shown to be toxic to anammox (Güven et al., 2005). Other, non-toxic organic compounds enhance heterotrophic activity and therefore compete with anammox for NO_2^- as substrate during anoxic periods, and aerobic heterotrophs consume oxygen during aerobic conditions. Slow degradable or non-degradable COD does not influence the deammonification substantially. Particulate COD in the form of colloids may lower oxygen transfer in the process and in this way limit the rate of ammonium oxidation (Zhang et al., 2018). Lack of micro- or macro nutrients or presence of toxins can limit the growth of AOB and anammox bacteria.

2.4.7 Seeding source

To start the process, suitable bacteria must be available. AOBs are assumed to be dormant in the activated sludge before the digestion or to come from elsewhere. The anammox bacteria are either added as external seeding/inoculum at the start of the process or supplied continuously together with the substrate. If added only once, in the form of sludge, they are washed out within a few HRTs. However, when inoculum is added as biofilm on carriers, they are retained in the system. How the anammox inoculum develops in the reactor will depend on bulk concentrations in combination with kinetic selection.

2.4.8 Load

The nitrogen load on the system can be calculated according to biofilm area ($\text{gN}/\text{m}^2\text{d}$) or as volumetric load ($\text{gN}/\text{m}^3\text{d}$), as shown in Equations 7 and 8, page 34–35. The load must coincide with the reduction rates to avoid inhibiting concentrations of substrate. The load for anammox bacteria specifically, is only calculated by area, since they are only established on the carrier material, whereas the load for AOBs can be related to both biofilm area and the volumetric load. This load for nitrification can be compensated to relate only to the aerated time of the system. The load is linked to the concentration of total nitrogen (TN) or $\text{NH}_4^+\text{-N}$ and inflow.

2.5 The moving bed biofilm reactor (MBBR)

The deammonification process comprises aerobic nitrification and anaerobic anammox processes. Since these processes require different oxygen conditions, they can either be separated spatially (in two different tanks), temporally (in time), or they can take place simultaneously (as in a biofilm process). In a one-stage process, stratification of biofilms in combination with intermittent aeration or low oxygen concentrations form aerobic and anaerobic zones in the reactor, suitable for both processes. This configuration is often very stable and easy to operate (Jaroszynski and Oleszkiewicz, 2011). With respect to N_2O emissions, a one-step process is preferred over a two-stage process, since the nitrite concentrations are always low (<10 mg NO_2 -N/L) and nitrite is a trigger for N_2O production (Massara et al., 2017). This thesis only considers the one-stage process.

The MBBR was first developed for the Nordic countries in order to retain sufficient biomass for nitrogen removal at lower temperatures (Hem et al., 1994; Ødegaard et al., 1994). Since a high SRT is crucial for retaining anammox bacteria in the system and this bacteria are known to stick to surfaces or grow in clusters (Kuenen, 2008), the MBBR is very suitable for the deammonification process. With this technique, the anammox bacteria are retained in the system, while the HRT can be shorter than needed for anammox retention (>4 d, see Table 2). This enables decoupling of biomass retention and HRT. The anammox is designated as the carrier material, but the AOB can grow both in the outer layer of the biofilm on the carrier material and as suspended flocs in the water phase if the HRT allows it.

Reactors can be fed by a continuous or intermittent flow; the different flows influence the load over time. Continuous feeding requires a buffer tank in front of the reactor to buffer the reject water produced when dewatering is in operation.

2.5.1 Biofilm

The biofilm consists of microbes, extracellular polymeric substance (EPS) and inorganic particles. A cryosection of a deammonification (or PNA) biofilm is shown in Figure 7 (Suarez et al., 2015). The anammox bacteria (green) are located close to the carrier material, whereas the AOBs (red and purple) grow at the surface, closest to the water phase. The excess sludge produced in the biofilm detaches because of the shear forces and leaves the reactor with the liquid effluent.

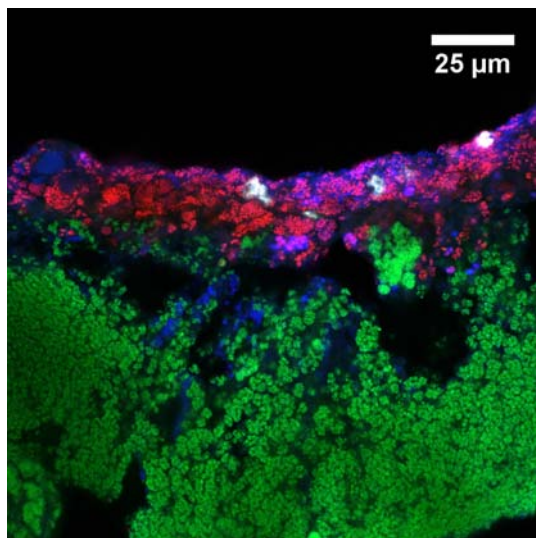


Figure 7. Biofilm with deammonification. Microscopy of a cryosection, using FISH-analysis. Anammox bacteria (green), AOB (red and purple) and white (protozoa). Image courtesy of Carolina Suarez and Malte Hermansson at University of Gothenburg and Frank Persson at Chalmers University of Technology.

On top of the biofilm there is a stagnant boundary layer of water, which limits substrate transport from the water phase to the biofilm, especially in low-turbulence flows (Lotti et al., 2014b; Mašić et al., 2010). A biofilm process is usually not limited by the kinetics of its microbiology, but rather by the mass-diffusion transfer of substrate from the bulk medium to the microorganisms in the biofilm (Ødegaard et al., 1994). This means that there are always different concentrations of substances in the water phase and in the layers of the biofilm (Kindaichi et al., 2007; Okabe et al., 2002). These different concentrations drive a constant transport of substrates and products to and from the biofilm. Proper mixing ensures a completely stirred tank

reactor (CSTR), but also limits the laminar layer at the biofilm and enhances substrate and product exchange between the bulk and the biofilm, as well as between the water and the gas phase. However, excessive mixing may increase the shear stress on the biofilm and limit its thickness.

In the studied process, the biofilm grows on a carrier material (see Chapter 3.2) that moves freely in the reactor, with aeration and/or mixing as energy input for the movement. Carriers have a protected surface, in which the biofilm is usually not scoured off due to contact with other carriers or walls. The thickness of the biofilm influences the community in the biofilm. A thick biofilm (>400 μm) may limit substrate transfer but, on the other hand, hosts different types of communities. A thin biofilm ($\sim 50 \mu\text{m}$) may select for certain types of bacteria (Piculell et al., 2016).

2.5.2 Chemostat

When the SRT is equal to the HRT, the system is operating as a chemostat. Up to moderate loads, in the systems studied (0.6–0.8 $\text{kgN}/\text{m}^3\text{d}$) the HRT is sufficient to also retain AOB in the suspended system. To increase the load, the SRT can be decoupled from the HRT as in a hybrid solution, with a sludge retaining system, such as sedimentation or lamellae (Veuillet et al., 2014). However, for a larger increase in the deammonification rates in such a system, the nitrite concentrations need to increase (Zhao et al., 2013); this may risk higher N_2O emissions, as discussed in Chapter 2.3.5.

2.5.3 Aeration

Oxygen is supplied through an aeration system consisting of blower(s) and diffusion system. Aeration may be continuous or intermittent, with switching for the blower. The oxygen concentration in the water phase can be controlled to a set point, regulated via a PI regulator¹, blower(s) and/or air valve(s). The transfer of gaseous oxygen to the water (oxygen transfer), depends on the oxygen concentration in the water phase, the penetration depth in the biofilm or floc, and the volumetric mass transfer coefficient, $K_{\text{L}}a$. In designing aeration systems for wastewater treatment and calculating the energy demand for aeration, the α -factor (ratio of $K_{\text{L}}a$ in wastewater to $K_{\text{L}}a$ in clean water) in the water is of high importance. Carrier material enhances the oxygen transfer rate, especially with a medium-to-coarse aeration system (Daigger and Boltz, 2017). Both mixing and aeration improve stripping of metabolites produced in the liquid phase, including CO_2 , NO , N_2O and N_2 .

¹ PI-regulator (proportional–integral regulator)

3 Methodology

The methodology used to answer the research questions of this thesis took advantage of the different full-scale plants as well as laboratory reactors with continuous operation, all using the MBBR technique. Reject water from different locations was used as substrate, and biofilms grown on different types of carrier material were used. The water phase, as well as sludge samples described in Paper IV, were characterized using chemical and physical methods. Anammox bacteria were analysed using two different molecular methods. Nitrous oxide (N₂O) was measured in both water phase and gas phase with two different methods as described below.

3.1 Types of reactors and instrumentation

The first study was performed in full scale; however, to answer specific questions such as the influence of an external anammox inoculum and the influence of reject water from thermophilic digestion, laboratory reactors were used. Table 3 presents the different reactors relating to the different papers, the substrates and the type of carriers used.

Table 3. Overview of the different reactors and their on-line instrumentation used in the study

	Himmerfjärden WWTP	Lund Univ. Biotech.	Bekkelaget WWTP	Slottshagen WWTP	8 selected WWTP
Type	Full-scale	Lab reactors	Full-scale	Full-scale	Full-scale
Place	Södertälje Sweden	Lund Sweden	Oslo Norway	Norrköping Sweden	Sweden/ Norway
Year of evaluation	2007 – 2008	2013 – 2016	2014	2018	2017
Volume	2x700 m ³	3 L	305 m ³	1000 m ³	N/A
Reject water	From plant	Sjölunda, Malmö/ Bekkelaget, Oslo	From plant	From plant	–
Instrumentation	pH, DO, T, Conductivity	pH, DO, T	pH, DO, T, NH ₄ ⁺	pH, DO, T, NH ₄ ⁺ and temporarily N ₂ O(liq/g)	–
Carriers used	K1H	YL-1/ K1H/ HXF12KLL	HXF12KLL	K1H	–
Paper	I	II, III	III	V	IV

3.1.1 Laboratory reactors

Laboratory reactors were set up at Lund University, Division of Biotechnology, as illustrated in Figure 8. For details, see the Methods sections in Papers II and III.

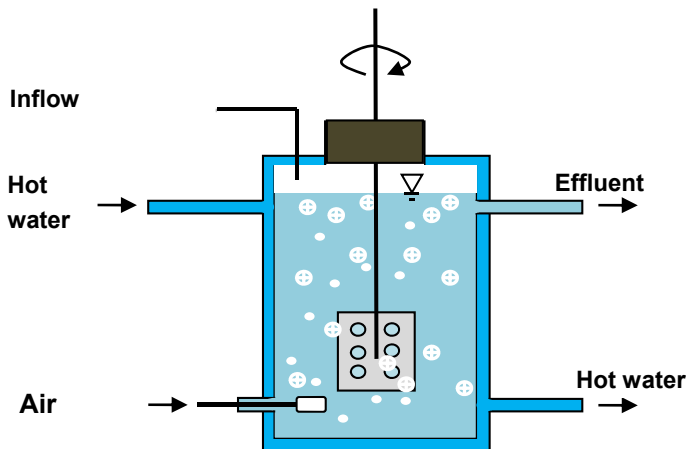


Figure 8. Schematic of laboratory reactors used at Lund University. Illustration courtesy of Therese Areskoug (2014).

3.1.2 Reject water treatment at Himmerfjärden WWTP, Södertälje

One of the first full-scale one-stage deammonification plants in the world is located in Södertälje, Sweden, at the Himmerfjärden WWTP. Applying experience from another full-scale research project in Hattingen, Germany (2000–2003) (Rosenwinkel and Cornelius, 2005) and results of on-site pilot tests (see, for example, Cema et al., 2006), the plant was designed by Purac AB in collaboration with Institut für Siedlungswasserwirtschaft und Abfalltechnik (ISAH) at Leibniz University in Hannover, Germany. The plant was built in existing pre-sedimentation basins, and to get an even distribution of the carrier material, each line was divided into four different zones, of which the last three were filled with carrier material of type K1H (AnoxKaldnes, Veolia Water Technologies). To limit plug-flow in the reactors, a recirculation was used from the effluent zone to zone 1, for the majority of the time (see Figure 9). The start-up phase and five months of operation are described in Paper I to evaluate the most important parameters to consider during start-up and operation.

There are two digesters at Himmerfjärden WWTP, which receives wastewater sludge from the WWTP as well as household food waste (30% of the organic load). At the time of starting up the reject water plant, the reject water load was approximately 10% of the total load into the main plant (350 kgN/d compared to 3 500 kg N/d) but an increase was expected due to co-digestion of sludge with nitrogen rich organic substrate. This WWTP is represented in Paper IV as Plant no. 2. The plant was operated between 2007–2017 and after closing, the carriers were transported to Slottshagen WWTP, see Chapter 3.1.4.

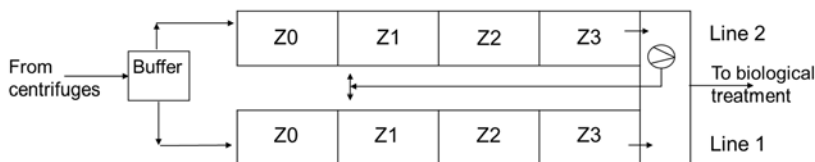


Figure 9. Overview of the two different lines with the three different zones at the plant in Himmerfjärden. Only zones 1–3 contain carrier material, zone 0 is filled with water but does not contain any carriers.

3.1.3 Reject water treatment at Bekkelaget WWTP, Oslo

The first configuration of the deammonification plant at Bekkelaget was also set up in existing basins. There was an existing configuration with two lines, with two basins in a row, all of the same size (305 m³). The start-up of deammonification at Bekkelaget reported in this thesis was preceded by an attempt to start-up in two-stage mode with separate nitrification and anammox stages. However, after successful laboratory tests of a one-step process, both in operation and start-up modes, the plant was started up in one of the four basins. After the start-up the carriers were moved to a bespoke new tank of 550 m³.

The two digesters at Bekkelaget WWTP are operated in thermophilic mode, and receive primary and excess sludge from the wastewater treatment. The reject water load in relation to the total load was approximately 13% (550 kgN/d compared to 4 400 kg N/d). The start-up at this plant is described in Paper III; this is different from the previous start-up in that the plant uses thermophilic reject water as substrate. The plant is represented in Paper IV as Plant No 6.

3.1.4 Reject water treatment at Slottshagen WWTP, Norrköping

The reject water treatment plant at Slottshagen WWTP was originally built as a sequencing batch reactor (SBR) with a nitrification/denitrification process, to which methanol was added as an external energy source. In 2018, the plant received carrier material from Himmerfjärdsverket WWTP in Södertälje, and the plant was rebuilt as a one-stage deammonification plant. Figure 10 shows the process tank.

The two digesters at Slottshagen WWTP are operated in mesophilic mode. The plant receives primary and excess sludge from the wastewater treatment. The reject water load in relation to the total load was approximately 15% (250 kgN/d compared to 1650 kg N/d). In this plant, nitrous oxide emissions were measured in nitrification/denitrification mode (Stenström et al., 2014) and have been repeatedly measured and are described in Paper V in deammonification mode. The purpose of the latter measurements was to evaluate the possibility of minimizing N_2O emissions with automatic control.



Figure 10. The reject water tank for deammonification at Slottshagens WWTP in Norrköping. The buffer tank can be seen on the right. Insert: Carrier material taken from the tank with biofilm attachment.

3.2 Carrier material for biofilm attachment

Different types of carrier material were used in the different studies. In full scale, the choice of carriers was based on technical and economic considerations. All carriers used are made of high-density polyethylene (HDP).



Figure 11. Different kinds of carrier material used in the study; K1H (AnoxKaldnes, Veolia Water Technologies), YL-1 (Jiangsu Yulong, China) and HXF12KLL (Stöhr, Germany). A staple (12 x 6 mm) is used as a size reference.

The carriers K1H (AnoxKaldnes, Veolia Water Technologies), YL-1 (Jiangsu Yulong, China) and HXF12KLL (Stöhr GmbH, Germany) have protected surface areas of 500, 385 and 700 m²/m³, respectively (Areskoug, 2013; Rusten et al., 2006; Stöhr GmbH, 2019) and are shown in Figure 11. The volumetric filling degree of carrier material was between 32 and 60% in the studies. For details see Papers I, II and III.

3.3 Reject water used as substrate

Reject water originates from dewatering of anaerobic-digested sludge, which is an outcome from a range of biological, chemical and mechanical processes, each of which may influence the quality and composition of the reject water. In this study, all reject water is produced by decanter centrifuges. The study mainly focuses on the use of reject water from municipal digestion of wastewater sludge. However, in Paper IV, reject waters from digestion of food and industrial wastes are included, to broaden the conclusions about anammox quantities in reject water from biogas plants in general. A characterization of the different reject waters used in the study is presented in Table 4.

Table 4. Reject water of different origins used in the study. Data are mean \pm standard deviation (mg/L) and number of analyses are shown in brackets

Parameter	Himmerfjärden, Södertälje	Sjölunda, Malmö	Bekkelaget, Oslo	Slottshagen, Norrköping
COD	–	690 \pm 290 (9)	–	–
sCOD	590 \pm 330 (96)	660 \pm 140 (9)	970 \pm 160 (58)	–
TOC	–	–	–	590 \pm 800 (10)
PO ₄ -P	–	23 \pm 4.0 (5)	–	–
Total alkalinity (mg HCO ₃ /L)	–	3900 \pm 410 (17)	4 000 \pm 850 (58)	5 700 \pm 200 (12)
NH ₄ -N	890 \pm 110 (100)	990 \pm 150 (25)	780 \pm 150 (58)	1220 \pm 130 (14)
TSS	–	510 \pm 140 (8)	1 220 \pm 450 (58)	2220 \pm 2640(12)
VSS	–	390 \pm 120 (5)	–	–

The ammonium concentration in the reject water is very similar to the ammonium concentration in the digester; therefore, the concentration of ammonium in the reject water depends on the substrate feed to the digestion and the degree of organic matter degradation. Nitrite was rarely detected in the reject water, but a few mg of nitrate was often detected. It should be noted that these analyses were all done with the same analysis technique (spectrophotometry with HL cuvettes) so analytical bias cannot be excluded. The pH of reject water is alkaline, often around 7.8–8.2, due to the high concentrations of NH₃ or NH₄⁺. The temperature is in the range of 28–37 °C (Table S1, Paper IV). If thermophilic digestion is applied, there is often a recovery of heat after the digestion and before the centrifuges, thus reject water from mesophilic digestion is in the temperature range for mesophilic processes when it reaches the deammonification process. The sCOD/NH₄-N ratio is in the range of 0.7–0.8 and the alkalinity in the range of 0.9–1.2 on a molar basis, which is typical for reject water. Since the European ban of mercury use in analytical chemistry for COD analyses (discussed in for example, Kolb et al., 2017), increasing numbers of plants are using the TOC parameter instead. The relation between COD and TOC depends on the type of carbon in the sample, and varies between 3.5–6.0 in wastewater streams (Balmér, 2015). In reject water, this parameter is estimated at 3.5 according to preliminary data from Himmerfjärden WWTP during this research. Thus, in the final study in this thesis (Slottshagen), only TOC, and not COD, is presented.

3.4 Chemical analysis and calculations

To adjust and calculate the critical key parameters in the process, several chemical parameters are measured in the water phase in addition to the physical parameters of suspended solids and biomass.

3.4.1 Chemical analysis of water and sludge samples

Inorganic nitrogen compounds ($\text{NH}_4\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$) and soluble COD (sCOD) were analysed using Hach-Lange (later Hach) test kits (Hach, Germany) and a spectrophotometer according to the manufacturer's instructions. For details about pre-treatment of the samples, the reader is referred to Papers I–V. Total suspended solids (TSS) and alkalinity were measured according to standard methods 2320 and 2540D (APHA, 2005) or Swedish standard methods SS-EN 872:2005 and SS 028139-1. Chemical analyses were used to calculate ratios, key parameters, load, reductions and conversion rates.

3.4.2 Calculation of free ammonia

Concentration of FA or NH_3 is positively correlated with the ammonium concentration, temperature and pH, and can be calculated according to Anthonisen et al. (1976):

$$\text{NH}_3 = \frac{17}{14} \cdot \frac{\text{NH}_4\text{-N} \cdot 10^{\text{pH}}}{e^{6.344/(273+T)} + 10^{\text{pH}}} \text{ [mg/L]} \quad (6)$$

where T is expressed in degrees Celsius ($^{\circ}\text{C}$) and $\text{NH}_4\text{-N}$ is in mg/L.

3.4.3 Determination of biomass quantity on carriers

Biomass quantity on the carrier material was approximated with dry solids. Dry solids on the carriers, $\text{DS}_{\text{carrier}}$ was determined by drying, weighing and washing with 37% HCl with repeated drying and weighing. For details of number of carriers chosen, the reader is referred to Papers II and III.

3.4.4 Calculation of load, reduction and HRT

Nitrogen loading rate (NLR) was calculated using the formula

$$NLR = \frac{NH_4-N_{in} * Flow_{in,d}}{Area_{carrier\ material}} [gN/m^2d] \quad (7)$$

where NH_4-N_{in} is the concentration in the influent to the reactor (mg/L), $Flow_{in,d}$ is the daily flow to the reactor (m^3/d) and $Area_{carrier\ material}$ is the total protected area of the carrier material (m^2).

The volumetric nitrogen load NLR_{volume} is determined according to

$$NLR_{volume} = \frac{NH_4-N_{in} * Flow_{in,d}}{V_{reactor}} [kgN/m^3d] \quad (8)$$

where $V_{reactor}$ is the reactor volume (m^3).

The reduction rate of inorganic nitrogen, R_{inorgN} , was determined with the formula

$$R_{inorgN} = \frac{100 * (NH_4-N_{in} - NH_4-N_{reactor} - NO_2-N_{reactor} - NO_3-N_{reactor})}{NH_4-N_{in}} [\%] \quad (9)$$

where $NH_4-N_{reactor}$, $NO_2-N_{reactor}$, $NO_3-N_{reactor}$ are the inorganic nitrogen concentrations in the reactor (mg/L).

The HRT is defined as

$$HRT = \frac{V_{reactor}}{Flow_{in,h}} [h] \quad (10)$$

where $Flow_{in,h}$ is the hourly influent flow (m^3/h).

3.4.5 Calculation of doubling time

The maximum growth rate, μ^*_{max} , was estimated during exponential growth of anammox bacteria during start-up with the formula adapted from van der Star et al. (2007):

$$\mu^*_{max} = \frac{\ln \frac{r_2}{r_1}}{t_2 - t_1} [d^{-1}] \quad (11)$$

where r_2 is the conversion rate at time t_2 and r_1 the conversion rate at t_1 . This is a simplification of the actual growth rate μ_{\max} , calculated by looking at increases in conversion rates instead of the actual increase of biomass. By applying the least-squares method to the logarithm of the conversion rate with respect to time, an estimate can be calculated. The estimation of μ_{\max}^* is based on constant yield during this time.

The doubling time t is then estimated with the following formula:

$$t = \frac{\ln 2}{\mu_{\max}^*} [\text{d}] \quad (12)$$

3.5 Quantification of anammox bacteria

To detect and then quantify anammox bacteria, which is central to three of the research questions in this thesis, FISH (Fluorescence *In Situ* Hybridization) was used in Papers I, II and III, whereas qPCR (Quantitative Polymerase Chain Reaction) was used in Paper IV. Bacterial analysis of biomass is more expensive than chemical analyses in the water phase, and needs more extensive sample preparation. In addition, biomass analyses are destructive, which may be a bottleneck when running laboratory reactors with a limited amount of biomass. Therefore, such analyses are performed less frequently than chemical analyses.

3.5.1 FISH analyses

FISH analyses involve labelling specific bacterial DNA with fluorescent probes and imaging them using a microscope fitted with a filter of a specific wavelength. By hybridizing a fluorescently labelled DNA probe at a specific site in the bacterial ribosomal RNA, the desired bacteria can be detected (van Loosdrecht et al., 2016). In the study in Paper I, samples were sent to an external laboratory (Vermicon, Germany) for analysis, whereas in Papers II and III, the analyses were done at the division of Biotechnology at Lund University with help of a quick test kit (VIT-anammox; Vermicon, Germany). These tests indicate the presence of the anammox genera and species *Candidatus Brocadia*, *Kuenenia*, *Scalindula*, *Anammoxoglobus propionicus* or *Jettenia asiatica*, i.e. they are aimed at all known anammox bacteria (Kartal et al., 2013). The detection limit for this method is 1000 cells/mL. More information about the sample preparation can be found in Paper II. No statistical analysis was performed to evaluate the uncertainty of these analyses, but the results were more of a guide towards the first detection of

anammox, and subsequently used to indicate anammox growth, rather than for quantitative analysis.

An advantage of the FISH analyses is that only active bacteria in the sample are fluorescent, since the probe targets rRNA; however, a disadvantage is that the analysis must be performed on fresh, and not frozen samples. This makes the work time consuming, since time series of samples cannot be saved and analysed simultaneously. In total, a minimum of ten images (in pairs) was taken, and an average percentage of anammox bacteria out of total viable bacteria was estimated. An example with two pairs of images is presented in Figure 12. A next step for analysis with the FISH kit would be to use an image analysis tool to digitalize the results.

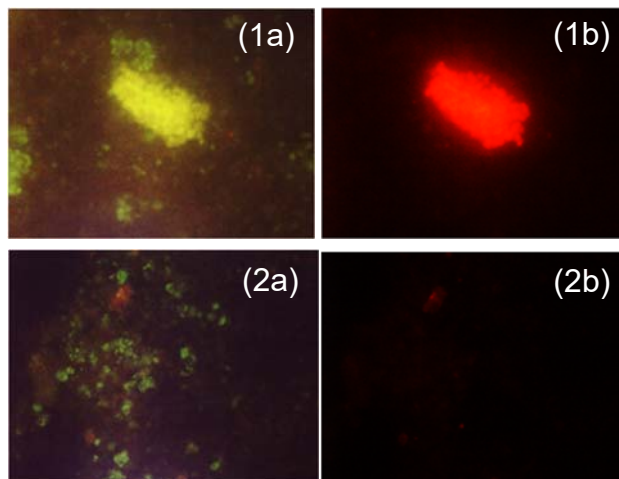


Figure 12. Examples of images from FISH analysis of two samples (1 and 2). (a) All viable cells show green fluorescence, and (b) all anammox cells show red fluorescence. Sample 1 indicates the presence of anammox, whereas sample 2 indicates viable cells, but no anammox bacteria. These images were taken from the laboratory-scale reactor at day 96 without seeding in Paper II.

3.5.2 qPCR

qPCR is a molecular method in which a specific DNA sequence belonging to certain bacteria is amplified for detection. The identification is usually based on the 16S rRNA gene (van Loosdrecht et al., 2016). The method involves several steps with varying temperature sequences to double the quantity of the DNA sequence at each step. By combining this with a fluorescent probe, the original sample can be quantified with a plate reader. The qPCR analyses

were used in Paper IV to screen and quantify different samples from WWTPs and digesters for anammox bacteria to evaluate the impact of digestion mode and pre-treatment of sludge on anammox quantities in reject water. In a second round of analyses, dead cells (cells with damaged cell walls) were excluded by pre-treatment of the samples with propidium monoazide bromide (PMA). More information about the primers sets and probes used can be found in Paper IV.

An advantage of using qPCR is that the samples can be frozen and stored over time and then be analysed together. The reason for using qPCR and not FISH is that microscopic analysis with FISH is not sensitive enough to detect the low concentrations of anammox bacteria expected in non-enriched cultures such as activated sludge or reject water from dewatering. The detection limit of this analysis is 500 copies/mL.

3.6 Measurements of N₂O

The last research question, no 4, is related to the risk of N₂O emissions from biological nitrogen removal processes. Paper V presents measurements of N₂O from the treatment plant in Slottshagen. Neither CH₄ nor CO₂ emissions were measured, since CH₄ preliminary data show that it is present at relatively low concentrations and emissions of CO₂ from reject water treatment is considered to be of biogenic origin, and is therefore not taken into account (IPCC, 2006). In order to analyse the N₂O produced in the water phase and the gas actually emitted to the atmosphere, two measurement methods were used simultaneously. Each method logged data continuously once per minute. The experimental set-up can be seen in Figure 13.

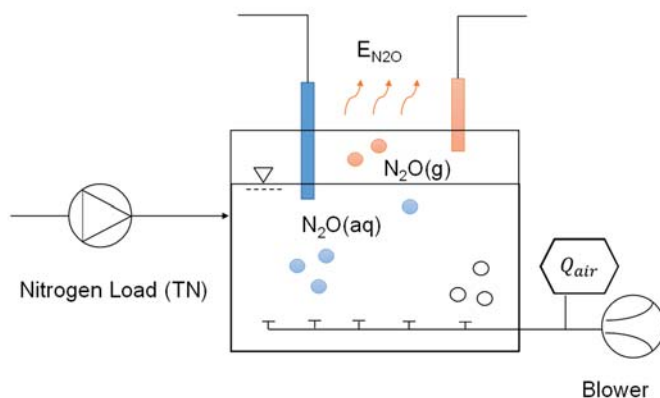


Figure 13. Method for measuring N₂O in water phase and in gas phase.

3.6.1 In water phase

A microsensor of the Clark type (Unisense, Aarhus, Denmark) was used to measure N₂O in the water phase. The sensor was calibrated according to the manufacturer's instructions. The sensor was connected to a transmitter, which was connected to the programmable logic controller (PLC) system for logging. The measurements are reported in mg N₂O-N/L.

3.6.2 In gas phase

The actual emissions from the plant were calculated by multiplying the N₂O concentration in the off-gas with the airflow from the reactor. The reject water tank was covered with a concrete lid and it was assumed that the concentration of N₂O in the gas space above the water surface and under the lid was equal to the concentration of gas leaving the plant. A spectrophotometer (Fresenius, GA2020) was used to measure the N₂O concentrations in the gas phase. It was further assumed that the plant only emitted N₂O during aeration, when there was an actual gas flow through the gas space, since preliminary data from earlier measurements done at the plant showed that this was the case (Baresel et al., 2016). The emission was calculated using the formula

$$E_{N_{2}O-N} = \sum_{i=1}^{1440} (c_{N_{2}O-N(g)} * Q_{air} * \Delta t_i) \quad (13)$$

where $E_{N_{2}O-N}$ (kg/d) is the emission rate per day, $c_{N_{2}O-N(g)}$ is the N₂O concentration in the off-gas (recalculated from ppmv N₂O to kg N₂O-N/m³ using molar volume at the current temperature), Q_{air} is the airflow from the blower (m³/min) and the time interval is Δt (min). The airflow from the blower was calculated from the energy requirement (kW) at that time, which is a linear relationship for this blower.

$$F_{TN} = \frac{E_{N_{2}O-N}}{TN} \quad (14)$$

where TN is the total nitrogen load into the plant during this time. The emissions, $E_{N_{2}O-N}$ were then related to the total nitrogen load into the reactor to obtain the formation or emission factor, F_{TN} .

3.7 Controlling the deammonification process with intermittent aeration

Intermittent aeration has been used throughout the studies in this thesis. This allows alternating aerobic conditions, not only in the biofilm but also in the suspended biomass. Different methods of controlling the intermittent aeration have been used in the different studies (see Chapter 5.1.2), but the oxygen concentration set point for the water phase has, however, been either 2.0 or 3.0 mg/L. Alternative ways of controlling one-step systems are, for example, via pH control (Wett et al., 1998) or aeration based on the ratio $\text{NO}_{3,\text{prod}}/\text{NH}_4\text{-N}_{\text{rem}}$ (Christensson et al., 2013), by fixed time (Yang et al., 2015), or as in a modelling study performed by Sander, (2014), by calculating microbiological activity by ΔNH_4 and ΔNO_2 including deactivation and reactivation of NOB by intermittent aeration. The reason for choosing intermittent aeration is the belief in effective out-selection of NOBs, due to their longer lag phase after an anoxic period in comparison to AOBs, as well as the deactivation of the NOBs during the anoxic periods (Beier et al., 2016; Park et al., 2004). Higher oxygen concentrations (2.0–3.0 rather than 0.3–1.3 mg DO/L) may be needed in the bulk to compensate for the non-aerated periods, and in modelling studies, have also enhanced the activity differences between AOBs and NOBs, resulting in a more stable nitrification process (Beier et al., 2016).

Figure 14 shows a *conceptual pattern* for the nitrogen compounds and pH in water phase. This helps to interpret the results presented in Chapters 4 and 5. Data from grab samples during the aeration cycle have been approximated with a sinusoidal pattern in the figure, since this form has been observed with on-line NO_2^- meters (unpublished results). Samples were taken in studies done in laboratory scale in Papers II and III and have been verified with data obtained in full scale in Paper V. The figure shows, that ammonium is oxidized to nitrite during aerobic conditions. The mass balances in water phase seldom close since the consumption of ammonium and produced nitrite occur simultaneously in the biofilm. During anoxic conditions, the anammox continues to reduce ammonium together with nitrite, but at the same time, ammonium increases due to the influent load. The pattern for NO_3 concentrations is different in different studies, and depends on the NOB activity. In some systems, a clear increase of NO_3 can be seen during aerobic conditions, which indicates NOB activity, whereas in other systems, a minor increase can be observed during non-aerated times — probably due to anammox activity. In addition, if detectable concentrations of NO_3 are produced aerobically, these can also be seen to be reduced by denitrification and/or anammox bacteria during non-aeration times.

pH may also exhibit an ambivalent pattern in the water phase. pH often decreases during aeration due to the proton-producing nitrification (Equation 1). However, in some reactors, the stripping of CO_2 actually increases the bulk pH, especially in the first part of the aeration period. This case is not illustrated in Figure 14 but is shown in Figure 7 of Paper I.

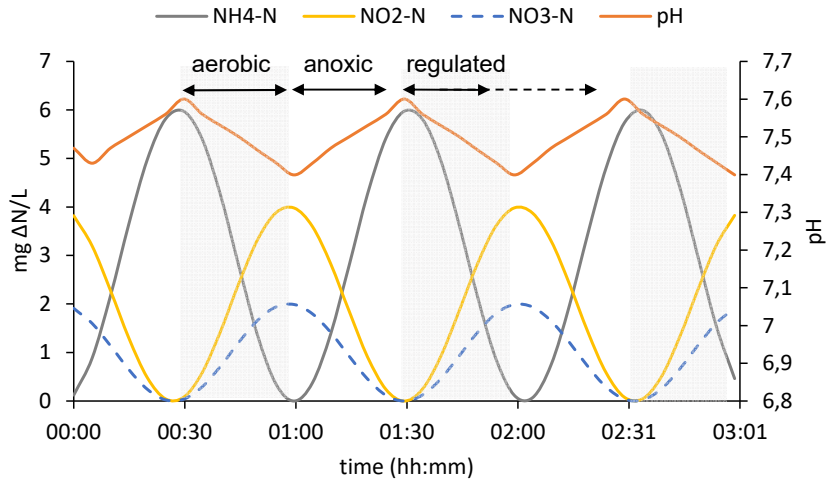


Figure 14. Conceptual pattern in changes of nitrogen concentrations and pH in the water phase during intermittent aeration. The shaded areas represent an oxygen concentration of 2 mg/L. NH_4 and NO_3 is often in the range of 100 mg N/L but here only the delta values are shown for simplicity. Similar data was collected during studies done in laboratory scale from Paper II and III and have been verified from data obtained in full scale in Paper V.

4 Results and discussion part I: Start-up of one-stage deammonification plants

This chapter presents the developed concept used for start-up in five different studies, as presented in Papers I–III. The methodology is described and the time for start-up is discussed. From Papers II and IV, the use of external anammox bacteria versus the use of indigenous bacteria are also addressed. These topics are related to research questions 1, 2 and 3 stated in Chapter 1.1. The first step to establish a biofilm with deammonification is to grow AOBs and thereafter optimize for growth of the anammox bacteria in the biofilm.

4.1 Start-up of nitrification biofilm

To favour growth of the bacteria, a cultivation temperature of 30 °C was chosen. This is within the range the process is expected to run at during operation. To achieve a nitrification biofilm, the retention time must be short enough to select only for microorganisms that attach to surfaces, in this case the carrier material. Suspended microorganisms will then be washed out. At a cultivation temperature of 30 °C, Table 2 show that a $HRT=SRT < 0.4$ d would sustain neither AOB nor NOBs in suspension, however biofilm-forming bacteria can be sustained in the system. Substrate concentrations, i.e. ammonia, oxygen and alkalinity, should be adjusted to an optimal range as discussed in Chapter 2.4. Since higher concentrations of ammonia are inhibiting, the influent reject water should be diluted until the biomass established can maintain reduction in the applied load. The alternative to maintaining low concentrations is to start with the reactor full of water low in ammonia and then increase the load in accordance with the reduction; however, this would result in very low loading over long time (i.e. low

inflows, long SRT in suspension) and would not favour biofilm growth, rather it would favour growth in suspension, and the long SRT may introduce NOBs. The dilution rate is 2–8 times, depending on the reject water concentrations and resources available (Papers I–III). This could be done with water low in ammonium, like effluent water from the main-line or tap water. In laboratory scale, the temperature requirement can be fulfilled by using a water bath (Papers II and III). In full scale, dilution water heated by heat exchangers is a way to maintain the process water temperature to support the mesophilic anammox bacteria (see Paper III). The mesophilic conditions will not only enhance the growth rate of the AOB, but also limit the growth of NOB, as mentioned in Chapter 2.3.1. In the primary study presented in Paper I, warm water from a gas scrubber was used, since this was both warm and low in ammonia. In hindsight, this water was thought to be inhibiting to the anammox process, and was later stopped, although no inhibition tests were performed. This resulted in a lower average temperature during start-up than desired (see Table 5, p.46).

Oxygen is the limiting substrate for ammonia oxidation in a deammonification biofilm (Szatkowska et al., 2007). To enhance the growth rate of nitrifying biofilm, oxygen as substrate should be present in excess to also reach inside the biofilm, and a set point of 3.0 mg DO/L was chosen during start-up. However, the supply should not be continuous or too prolonged, since experience has shown that this will also select for NOB growth in the biofilm. The aeration time depends on the load, oxygen concentrations and biofilm thickness at the time. In the studies described in Papers I–III, the aeration time was adjusted manually, in order to meet the requirement to convert available ammonia and alkalinity to nitrite. Once the nitrification starts, this results in only 50–60% of ammonium conversion because of the limited amounts of alkalinity (see Paper II, Figure 2 and Paper III, Figures 1a and 2a, during the first 50 days of operation). In Paper II, decreasing the nitrification below 50% by limiting the time of the aeration was tested, but this did not succeed with manual control. However, this might have been possible with automatic control, with feedback of ammonium concentration.

Difficulties with manual control of the aeration time resulted in occasional pH drops caused by consumption of alkalinity. This resulted in large pH variations, which could create unfavourable and stressful conditions for growing microorganisms. To avoid this, the aeration time was subsequently controlled with a PI regulator. The PI regulator had an average moving pH as input and the time of the intermittent aeration as output. This has been used for operation in both laboratory and full-scale plants outside this study and was further developed in Paper V. The pH control also ensures that the autotrophic bacteria have access to alkalinity as a carbon source.

Once a stable aerobic autotrophic biofilm is established on the carriers, with a critical amount of biomass, it is time to shift the conditions and optimize for the anammox bacteria. Results from Papers I and II show that the start-up time for an aerobic autotrophic biofilm with stable production of nitrite is approximately two weeks; however, in Paper I, the aerobic conditions were maintained until day 56 in an attempt to control the NO_2^- concentration. In Paper III, an already established aerobic autotrophic biofilm was used as the starting point for cultivation of anammox bacteria, and the above steps were therefore not needed.

In the start-up described in Paper I, methanol and later, pre-settled water in combination with aeration, were used to enhance the establishment of a biomass matrix on the carrier material. This protocol had been used in earlier studies with success (Rosenwinkel and Cornelius, 2005) but was not applied in later studies. This earlier study also used pH adjustment with sodium hydroxide to increase the NH_3 concentration, thereby limiting growth of NOB, but this was not necessary in the present studies. An alternative solution to dilution for keeping ammonium concentrations low during start-up is to combine nitrification or nitrification with denitrification by supplying an external energy source. This was used in a two-step start-up by van der Star et al. (2007), but since the ethanol used can cause 30% inhibition of anammox bacteria (Güven et al., 2005), this method is not recommended for start-up of a deammonification system.

4.2 Start-up of anammox biofilm

4.2.1 Suitable concentrations of essential parameters for cultivation of anammox

For further cultivation of the biofilm with anammox bacteria, the conditions should be modified. At the same time, it is important to maintain AOB activity to produce sufficient nitrite so that it does not become limiting for the anammox bacteria. Similar to the establishment of autotrophic AOBs in the biofilm, this means optimal substrate and carbon source concentrations (NH_3 , NO_2^- , HCO_3^-), modest-to-alkaline pH (7–8) and high temperatures (30 °C). With intermittent aeration and a critical biofilm thickness, both aerobic and anoxic conditions can be met. Successful ranges for the critical parameters from the four different start-ups are presented in Table 5: two in laboratory scale and two in full scale without addition of external inoculum. The results comprise data from 100 days of anammox enrichment in the biofilm.

Table 5. Conditions obtained in the reactor during the period for cultivation of anammox without external inoculum. Minimum, maximum, mean and standard deviation together are presented with the number of samples in brackets.

Paper	I	II	III	III
	Full-scale	Laboratory	Laboratory	Full-scale
Reject water from (Table 4)	Himmerfjärden Line 1, zone 2	Sjölunda	Bekkelaget	Bekkelaget
Evaluation days	d 100–d 200	d 1–d 100	d 1–d 100	d 1–d 100
Dilution water	Condensate water from gas stripper	Tap water	Tap water	Effluent
External heat		Water bath	Water bath	Excess heat from blower
Temperature (°C)	20–28, 23±2, (25)	23–30, 30±1, (43)	28–31, 30±0.5, (31)	24–33, 29±2, (34)
HRT (h)	5–24, 11±23, (25)	7–150, 16±20, (47)	13–67, 20±10, (29)	13–200, 22±20, (99)
Dissolved oxygen (mg/L)	0, (25)	1.0–5.7, 2.8±0.8, (29)	1.5–4.5, 2.9±0.8, (35)	1.8–3–3, 2.9±0.3, (34)
Aeration (min of total 60 min)	No aeration	11–50, 27±15, (47)	13–40, 20±9, (34)	0–20, 13±4, (100)
pH (–)	7.7–8.2, 7.9±0.14, (26)	6.3–8.9, 7.4±0.5, (55)	6.0–8.2, 7.3±0.4, (38)	6.7–7.8, 7.2±0.3, (35)
Alkalinity (mg HCO ₃ /L)	350–800, 520±120, (14)	24–1000, 290±200, (44)	100–1200, 300±220, (25)	120–1400, 420±250, (34)
NH ₄ -N (mg/L)	56–410, 170±100, (27)	51–350, 110±50, (42)	17–240, 83±45, (25)	4.5–230, 100±37, (35)
NH ₃ (mg/L)	2–32, 10±8, (24)	0.2–98, 7±16, (42)	0–36, 3.4±6.8, (26)	0–11, 2±2, (34)
NO ₂ -N (mg/L)	0.8–84, 31±23, (27)	0–230, 88±58, (42)	0.5–75, 32±27, (25)	10–94, 53±23, (35)
NO ₃ -N (mg/L)	0.3–29, 11±7, (27)	0.6–22, 5±4, (33)	1–71, 14±19, (25)	1–14, 2.5±2.1, (35)
sCOD (mg/L)	65–240, 140±56, (16)	99–250, 160±55, (4)	150–640, 290±150, (9)	170–430, 280±58, (19)
TSS (mg/L)	50–130, 80±23, (10)	25–340, 100±110, (6)	–	220–380, 320±55, (11)
Biofilm DS per litre carrier (mg/L)	3–5, 4±1, (13)	1.3–2.6 (at day 104), (2)	4.6–5.2, 5.0±0.2, (4)	8.5–14, 11±2, (3)
Start-up time (R _{inorg, N} >80%)	200–300 days	134 days	72 days	120 days

Continue Table 5.

Paper	I	II	III	III
Load (gN/m²d)	0.5–4.4, 2±1.2, (19)	0–4.0, 1.8±0.8, (43)	0.7–2.9, 1.3±0.6, (26)	0.1–1.0, 0.7±0.2, (35)
Estimated μ^*_{\max} (d⁻¹)	0.05 (8) R ² =0.58 Day 288–312 Total Line 1	0.11 (9) R ² =0.98 Day 103–121	0.20 (4) R ² =0.99 Day 43–53	0.11 (5) R ² =0.76 Day 110–120
Approximate doubling time(d) (calculated from μ_{\max} with Eq. 12)	14	6.1	3.5	6.0

Starting with the temperature, the relatively low average temperature during start-up in Paper I (23±2 °C) is probably one of the reasons why this start-up took longer in comparison to the later start-ups with higher average temperatures (29–30 °C). Another parameter from Paper I, (in line 1, zone 2, from which the data in Table 5 was collected) which differs from the other studies is the anaerobic conditions. The NO₂⁻ was mostly produced in the preceding zone 1. Without nitrifying capacity in zone 2, this resulted in a higher pH and hence higher FA concentrations, which were later shown to have a negative effect on the anammox bacteria (Jaroszynski et al., 2012). From this point of view, nitrification with intermittent aeration could be beneficial for cultivation of anammox, even though they are reversibly inhibited by oxygen. Another potential positive reason for aeration when growing anammox bacteria is that oxygen seems to stimulate the aggregation of the anammox bacteria (Lotti et al., 2014a), which is positive for biofilm formation. By contrast, anaerobic conditions seem to lead to a suspension of the anammox bacteria, which can then be washed out at the applied HRTs. A third reason for combining growth of anammox with aeration is that the anammox bacteria attach where the concentration of NO₂⁻ (the limiting substrate) is highest, i.e. at the biofilm surface. By later growing in the depth of the biofilm, the anammox bacteria are protected from oxygen by the AOBs and extracellular polysaccharides. Therefore, anammox growth should be combined with nitrification. The anammox and AOBs could be considered to have a ‘syntrophic relationship’ — the term used more broadly for a relationship that benefits both partners (Morris et al., 2013) — in a micro-aerated environment with modest NH₃/NH₄⁺ concentrations and available inorganic carbon, such as a nitrification biofilm. AOBs make use of the available oxygen, supplying anammox bacteria with NO₂ (both aspects are positive for anammox bacteria), and anammox bacteria maintain low nitrite

concentrations (preventing substrate inhibition) by converting this to dinitrogen gas.

Alkalinity is an essential substrate for the autotrophic bacteria and can become limiting with excessive aeration. In the study in Paper II, the alkalinity was periodically very low, due to difficulties with manual adjustment of the aeration times, as discussed in Chapter 4.2.1, which could have limited the processes. Simultaneous denitrification can be beneficial for the process, either in the biofilm or in the bulk, since it produces some alkalinity. Denitrification also reduces nitrate concentrations, which is positive from a total nitrogen reduction perspective. However, the sCOD/NH₄-N for the four start-ups were 0.8, 1.4, 3.5 and 2.8, respectively, showing a higher ratio for the thermophilic reject water; and dissolved organic material in this range is not believed to contribute substantially to denitrification or to interfere with the growth of anammox.

The four studies presented here had average concentrations of NH₄-N, NO₂-N and NH₃ in the range of 83–170, 31–88 and 2–10 mg/L, respectively, which are shown here as suitable for anammox growth. By applying dilution to obtain approximately 180 mg NH₄-N/L in the influent, and oxidizing 50% of ammonium to nitrite, this provides appropriate concentrations for anammox growth. These low NH₄-N concentrations also allow for a slightly alkaline pH set point (pH >7.0), without compromising with a higher NH₃ concentration. All of these conditions favour the growth of anammox bacteria.

In order to create a spatial separation of the two autotrophic bacterial groups with different oxygen concentrations, a critical biomass thickness is needed. In these studies, the mass, but not the thickness of the biofilm was measured, and a positive correlation was assumed between mass and thickness. In these studies, 1–11 g DS/L_{carrier} was reached during the cultivation period. The lower value was obtained when starting with virgin carrier material and the higher values were obtained from an already operating nitrification biofilm. Since oxygen only reversibly inhibits anammox activity, excess oxygen above the amount consumed by AOBs can be applied, but to reduce the start-up time this balance should be optimized. Unfortunately, the results presented in this thesis does not allow for any estimates regarding the concentration gradients of, for example, oxygen in the biofilm. Further research would be helpful at this point, to increase the possibilities of obtaining optimum conditions for growth of slower-growing anammox bacteria.

The total load on the biofilm is not crucial during the second part, as long as the influent is supplying a critical mass of indigenous anammox bacteria. However, the relation of the load and the HRT to the aeration is more critical. The aerated load in the first part should correspond to the maximum

conversion rate of the AOBs. Either the load or the aeration time can be adjusted to meet this criterion. Since the purpose of the second part is to grow *anaerobic* ammonium oxidation bacteria, the aeration time should be kept to a minimum, as discussed earlier.

For each start-up study the exponential growth phase for anammox, detected by the nitrogen conversion rate was identified. At this time the maximum growth rate is estimated and, from this, the doubling time is calculated using equations 11 and 12. The results show relatively short doubling time (3.5, 6.0, 6.1, 14 d) and no compensation of the data was done in relation to the intermittent aeration. However, there is some uncertainty in the data, such as the small number of data points in Paper III and low coefficient of determination in Paper I. The results achieved can be compared to doubling times shown in Table 5, achieved in highly enriched anammox cultures with synthetic medium, strict anoxic conditions and temperatures between 30–38 °C. In conclusion, it can be summarized that the parameters achieved in the last three studies (Paper II and III) are suitable conditions for growing anammox bacteria in full-scale reactors.

Another important parameter when cultivating anammox bacteria, which is not included in Table 5, is the SRT. Paper IV showed that the SRT is a significant factor and is positively correlated with the concentration of anammox bacteria (copies/g VS). In addition, previously reported studies started up without inoculum all use a biofilm technique, as mentioned in the introduction.

Once the parameters have been set for optimal conditions for anammox growth, it is time for what in the studies has been informally denoted as the “wait-and-see-phase”. Anammox growth can be detected by several methods. The results from Paper III, in Figures 1c and 2b, show that anammox growth can be detected in the nitrogen conversion data obtained using chemical or on-line measurements, with molecular methods (as shown in Paper II, Figure 3 and Paper III, Table 3) or by a rapid biofilm growth detected by DS analyses as in Paper I, Figure 2. These results indicate that the actual nitrogen conversion is the last sign of anammox activity and that molecular methods may be more informative at an earlier stage to show that the development is on the right track. Guided by molecular analysis, it may be possible to prepare an increase of load in time to increase nitrogen reduction rates and, in this way, reduce the start-up time.

4.2.2 The role of external anammox inoculum

In Paper I, a start-up was performed without external anammox seeding, resulting in a start-up in the range of 200–300 days. This is in the same range of previously published studies in full scale (Lackner and Horn, 2013; Mehrdad et al., 2014; Rosenwinkel and Cornelius, 2005; Zekker et al., 2012a). With these results in mind, research question 2 in this thesis relates to whether using external inoculum positively influences the start-up time of the process.

In Paper II, one reactor was run with external anammox inoculum and one was run without inoculum. When the carriers, with established deammonification biofilm, were added as inoculum to the first reactor with established nitrification biofilm, there was an immediate reduction of nitrogen. The reduction of $1.3 \text{ g N / m}^2_{\text{tot}} \text{ d}$ corresponds to a rate of $11.8 \text{ g N / m}^2_{\text{seed}} \text{ d}$. The immediate and high rate of response implies that the nitrifying biofilm (89% of the carrier area) was supplying the seeded carrier (11% of carrier area), so that nitrite was not limiting the anammox conversion rate. This also indicates that the microorganisms were adapted to the substrate and there was no real lag phase (the seeded carriers came from the same plant as the substrate, Sjölanda WWTP). Because the load of nitrogen at that time was higher than the reduction capacity of the anammox bacteria, the exponential phase of anammox growth could not be detected until later, when the inorganic nitrogen reduction ratio increased to 80% and FISH analyses detected 14% anammox in the virgin biofilm (at day 96). In parallel with this reactor, the reactor without inoculum was run on the same substrate. Growth of anammox bacteria was detected in similar quantities in the biofilm from this reactor at the same time, and both reactors needed the same start-up time (see also Figure 16).

The result from Paper II shows that external inoculum is not necessary for more efficient start-up, and in this study it did not shorten the start-up time. Thus, it was concluded that the quantity of indigenous anammox bacteria in reject water from the mesophilic digester was sufficient to seed a new deammonification reactor.

If external seeding is used (like one of the reactors in Paper II) in operational strategies, the origin and the size of the seeding should be considered. When using biofilm as an external seeding source for anammox, there is an initial imbalance between the established biofilm and the new growing biofilm. Since nitrite production is not limited at this stage, the seeded carriers can grow at their maximum growth rate. To ensure unlimited nitrite (indicated by NO_2^- concentrations in the bulk) for the new growing biofilm, the aeration time was increased (Paper II). However, this led to

production of nitrate. Thus, start-up would be simpler and lead to a more stable system if only indigenous anammox bacteria is used.

There are different theories of how and whether the origin of the inoculum sludge (type or genera) influence the anammox community later on (Date et al., 2009). Hu et al. (2010) conclude that one seeding source can be used to treat different types of wastewater, whereas another study showed a changed community of anammox with alternating substrate (high or low in COD) (Park et al., 2010).

One should also consider the size of the inoculum. By adding more than 11% (as in Paper II), the full capacity of the plant could potentially be reached within a shorter time. Reactors can also be fully-inoculated. This was done in Norrköping WWTP (the plant in Paper V) with carriers from Himmerfjärden WWTP (the plant in Paper I) (unpublished data). However, maintaining these amounts of carrier material (600 m³) for future plants does not seem to be a reasonable undertaking for any company, taking the uncertainty of this market into account.

4.2.3 Reject water from mesophilic versus thermophilic digestion as an anammox seeding source

Reject water used in Papers I and II originated from mesophilic digestion, whereas the reject water at Bekkelaget WWTP was from a thermophilic digester. In Paper III, two laboratory reactors were set up in parallel, one for continuous operation fed with reject water from Bekkelaget (LO) and one for start-up, using carrier material from Bekkelaget with established nitrification biofilm (LS). In reactor LO, the source of 100% inoculum with deammonification biofilm was from a previous laboratory trial (Paper II), operated on mesophilic water, i.e. the biofilm was not accustomed to the new substrate. Stable conditions with >80% of inorganic nitrogen removal were reached after 11 days, which, in this case is likely to correspond to a lag phase for the established bacteria. The final load in this reactor (3.3 ± 1.6 gN/m²d) was not as high as in previous laboratory trials (5.6 gN/m²d, Paper II), in which the bacteria had become acclimatized to mesophilic digested reject water. In the parallel reactor, the start-up of the deammonification biofilm preceded as planned, keeping the parameters given in Table 5. This resulted in a start-up time of 72 days.

By aiming for the same boundary conditions in a full-scale start-up at Bekkelaget WWTP as in the laboratory trials, the start-up succeeded in 120 days. All four start-ups, demonstrate that enough indigenous anammox bacteria is present in the reject water, whether it originates from mesophilic or from thermophilic digestate.

4.2.4 Where do anammox bacteria thrive?

The results from Paper IV, in which seven digesters and their reject water were analysed for quantity of anammox bacteria using qPCR, show 10^4 – 10^5 copies/mL in the reject water (Figure 15 and Paper IV). As discussed in this paper, this amount of anammox bacteria could be sufficient to cultivate a critical amount of anammox bacteria within a reasonable time frame (<100 d).

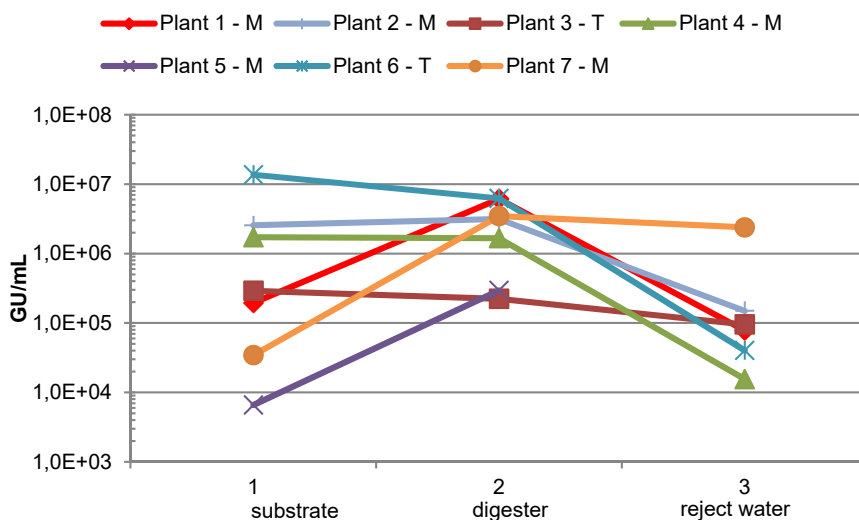


Figure 15. Quantification of anammox bacteria by qPCR analyses from substrate (to digester), in digester and in reject water after dewatering. The reject water sample from Plant 7 is considered as an outlier. Data collected from Paper IV. M=mesophilic digestion, T=termophilic digestion.

Since anammox bacteria have previously been enriched from activated sludge, they were believed to be present at some concentration in the digester substrate containing activated sludge (Chamchoi and Nitorisavut, 2007; Toh et al., 2002). However, it was not known how different digestion substrate (activated sludge or other organic material), pre-treatment of substrate with high-temperature (hygienization) or digestion temperature (mesophilic or thermophilic) influence the quantities of anammox bacteria in the reject water (for a schematic flow scheme, see Figure 1 in Paper IV).

In Paper IV, samples were taken before and after high-temperature treatment before the digester. qPCR analysis showed that substrate from two plants treated with thermal hydrolysis process (THP) decreased the anammox

quantities, whereas the other two substrates with 70 °C temperature treatment did not influence the quantities of anammox bacteria. High-temperature treatment, as hygienization, would by definition be expected to decrease the concentrations of pathogenic as well as useful microorganisms.

Referring to Figure 15, it is interesting that in five out of seven samples, the quantity of anammox bacteria increases from the substrate to the digester. For three plants, the increase is by two orders of magnitude, which suggests a growth of anammox bacteria in the digester. In two plants, the increase is less than this, but these substrates already contained high quantities of bacteria ($>10^6$ GU/mL). The two plants which showed a minor decrease of bacteria quantities through the reactor are both thermophilic digesters (Plants 3 and 6). This indicates that mesophilic digesters can be considered a safe source of anammox, independent of the pre-treatment or substrate, whereas thermophilic digestion can retain, but not necessarily grow, anammox bacteria. This could indicate that thermophilic digesters are more dependent on the anammox content in the substrate to the digester. However, it should be noted that the thermophilic digesters had the shortest SRT of the digesters investigated, 12 and 14 days, respectively (Table 1, Paper IV) which could also be a limiting factor for enrichment of anammox bacteria in the digester. Referring to Table 2, anammox bacteria need a longer SRT than other bacteria, a minimum of 4 days at 30 °C, which is in the same range as acetoclastic methanogens (2–3 days). This means that the SRT in a digester may be long enough to also sustain anammox bacteria.

The decrease of anammox bacteria from the digester to the reject water, seems evident and self-explaining when measuring in terms of GU/mL sample, since the dewatering process separates biomass from reject water. All plants in the study use the same decanter centrifuge dewatering technique. All seven digesters in the study thus contain anammox bacteria quantities determined by qPCR above 10^5 – 10^6 copies/mL. This implies that digested sludge, whether dewatered or not, is a crucial source of anammox bacteria. From the anammox seeding perspective, it may be an advantage to maintain a high TSS in the reject water during start-up. This is another advantage of applying the MBBR technique, which has proven to be not as sensitive to high TSS concentrations as sludge or granule techniques, where TSS from reject water treatment would be retained in the system. However, this should be done with care, since adding anaerobic sludge into aerated zones might cause other complications.

4.2.5 Start-up time for anammox growth

The reduction of inorganic nitrogen over time is shown in Figure 16, calculated using Equation 9. In Paper I, the full-scale start-up was achieved within 10 months but only 6–7 months of this was considered to be related to the actual start-up process. The difference in time was due to purely technical issues, such as lack of access to dilution water and potential inhibition and therefore lack of heating source for the process. In Paper II, the start-up was considered equal between the seeded reactor and the non-seeded reactor. Both reactors were considered to be in full operation at day 134 (4.4 months), but then 56 days were spent on only operating in nitritation mode. In Paper III, the biomass in laboratory scale already contained active AOB in a total biomass of approximately 5 g DS/L_{carrier} and it took 72 days to reach 80 % of design load. In full scale the biomass was 8.5 g DS/L_{carrier} at the start, and it took 120 days to reach full conversion (>80% TN_{red}) from this point. The difference in start-up time between the first (Paper I) and the second full-scale (Paper III) plants could first of all be acknowledged as due to experience and knowledge. Since 2007, many scientific papers have been published on the topic of how to achieve optimum conditions for anammox bacteria to grow, theoretically or in the laboratory.

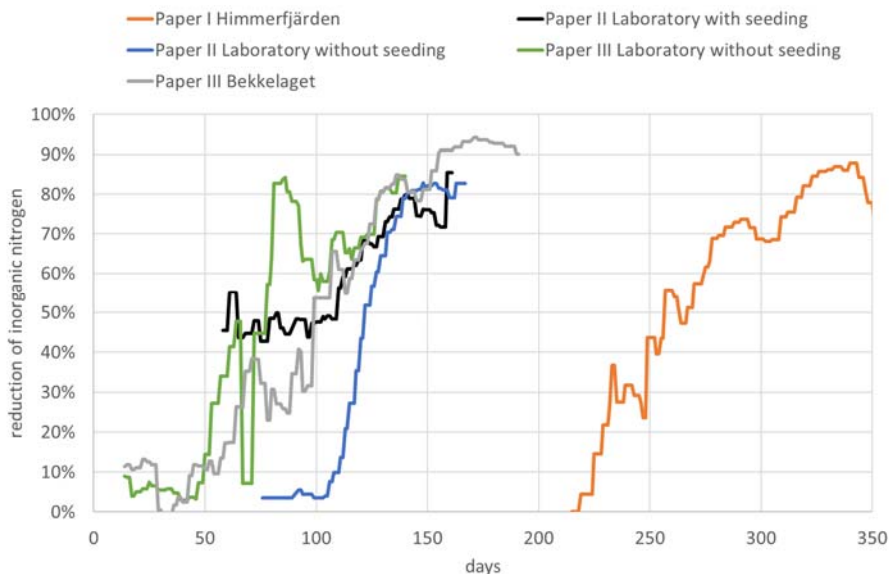


Figure 16. Reduction of inorganic nitrogen versus time for five different start-up reactors. The line is representing a moving average of 14 days in order to better clarify the exponential trend of reduction. During the time of exponential growth, the load has been kept stable. Data collected from Paper I, II and III.

The start-up times mentioned above are in the same range as start-up times reported by Lackner et al. (2015) and Christensson et al. (2013) using seeding material in MBBRs. However, Christensson et al. (2013) reported an 80% reduction in all times and considered the start-up time to be two months in the WWTP in Växjö. This is also an option, to only load a fraction of the design load corresponding to the seeding capacity, until the virgin carriers grow biofilm for deammonification. However, this strategy could risk formation of NOBs as seen in Paper II, Figure 2.

Many scientific papers state in their introduction that the long start-up time limits the implementation of the deammonification processes. Therefore, many research questions take this stand point; for example, Ali et al., 2015; Ali and Okabe, 2015; Jin et al., 2012; Massara et al., 2017. The results from the studies included in this thesis instead show that i) the start-up time related to process in relation to the total construction time and technical commission of a plant is not critical for either the total project time or budget, and ii) by cultivating the anammox bacteria using the suggested conditions, they do not need to be referred to as an “extremely slow-growing bacteria”. The growth rate is in a similar range to acetoclastic methanogens, which grow in all anaerobic digesters at WWTPs and are not referred to as a huge limitation for implementation of the anaerobic digestion process.

In full-scale implementations, the start-up time for a one-stage deammonification plant is not only limited by the growth rate of anammox bacteria. Many other factors, such as competing organisms and partly inhibiting, non-optimal conditions, influence the time taken for full nitrogen reduction capacity to be reached. This is influenced by the project schedule, selected start-up strategy, specific process knowledge and other external and often unexpected technical factors, which often occur during process commissioning, which are not unique for the start-up of the deammonification process.

5 Results and discussion part II: Operation of one-stage deammonification plants

This chapter discusses the operation of a one-stage deammonification plant and the parameters that should be in focus in order to increase its capacity, to have stable process conditions and limit nitrous oxide emissions. Based on the results presented in Paper V, nitrous oxide emissions are discussed in the context of how they influence the total carbon footprint of a reject water treatment.

5.1 Process limitation by AOBs

Chapter 4 mainly presents results and discussion of the growth of anammox bacteria. Once a one-stage deammonification process with MBBR is started up and sufficient anammox biomass is enriched in the carriers, the performance is limited by the rate of AOBs and their supply of nitrite to anammox bacteria. This is the conclusion in Paper I (see Figure 8) and in Paper III, Figure 1b and Paper V, Table 1, in which the observed nitrite concentrations are always low in these type of systems when in operational mode.

Therefore, to increase the capacity of the system, first of all, parameters related to the amount of AOB biomass and activity should be taken into special account. The AOBs are located in both the biofilm and in the suspended biomass. To what extent they exist in the suspended biomass depends on the HRT in the system and hence the load. In comparison with the start-up phase, both load and temperature vary in the system during operation, due to intermittent operation of dewatering units and seasonal changes.

The nitrifying rate in the biofilm is limited by ammonia and oxygen concentration as well as by organic loading rates (Ødegaard, 2006). The ammonium concentration in a reject water process is considered to be non-limited. In a pure nitrification system with suspended biomass, the oxygen uptake rate increases according to Monod kinetics (See Chapter 2.4.3). The plant in Bekkelaget operates at 3.0 mg DO/L during aeration, whereas the Slottshagen plant operates at 2.0 mg/L due to a lower volumetric load (0.25 kgN/m³d, compared to 1.0 kgN/m³d at Bekkelaget). Aerobic degradation of organic compounds competes with AOB activity for oxygen and may, in particulate form, limit the oxygen transfer. Expressed as sCOD, soluble organic compounds have not been shown to compete or inhibit the processes at the levels presented in the different studies (sCOD/NH₄-N = 1.1–2.2 in Paper III). High TSS has also not been shown to disturb the process (at least up to TSS < 1600 mg/L, Paper III).

Another way to compensate the process for being limited by AOB capacity in relation to anammox capacity is to design the process with lower filling degree of carrier material. In this way the HRT (which is equal to the SRT in a chemostat) at a specific load will be the same but with a smaller amount of anammox biomass due to the lower biofilm area.

5.1.1 The importance of automatic control and accurate measurements

In order to calculate key-parameters such as NLR, HRT and NH₃ (see Chapter 3.4) to control the process with dynamic response from the control system, common on-line measurements such as flow, ammonium, temperature, oxygen and pH are important parameters to log. These parameters and the sensors used to measure them are reliable and have been used in wastewater treatment for many years. Nitrite, nitrate, TSS and organic compounds can also be measured on-line, but have so far not been shown to have the same importance for process performance control. However, to be able to minimize N₂O emissions, as further discussed in Chapter 5.2, monitoring of parameters such as NO₂⁻, N₂O (aq) and N₂O (g) with on-line sensors is valuable.

In Paper I, conductivity was used as an indirect measurement of the process performance. A reduction in conductivity represents a reduction of ammonium ions. The results in Paper I shows that these parameters exhibit a good correlation (Figure 9, Paper I), but as the price of NH₄⁺-sensors decreases, direct measurement of ammonium becomes the better option. The salt concentration may vary in digested substrates, and therefore there is a risk of bias in conductivity measurements over time.

The results in Paper I show that NH_3 concentration is one of the most important parameters to control, and that it should be maintained below 15 mg NH_3/L . However, operational experience has shown that NH_3 concentration below 3-5 mg/L is preferable (see Table 5 and Paper V, Table 1). By controlling the pH, the NH_3 is controlled indirectly, as long as the reduction of nitrogen is stable in the process (see Equation 6). An issue with substrate-inhibited systems is that once a disturbance decreases the reduction rate and the turnover, the substrate concentration, in this case NH_3 , starts to increase, further inhibiting the process. To stop this negative spiral, a control system can respond and stop this evolving. If nothing is done, this can accelerate and result in permanent inhibition and process collapse, which can only be recovered with the help of warm dilution water or by stopping loading and allowing the process to recover over time with the help of low reduction rates.

5.1.2 Intermittent aeration

Intermittent aeration has been used throughout these studies. In Papers I–III, the aeration period was adjusted manually in the range of 10–50 minutes, out of a fixed 60-minute cycle. Fixed aeration times are acceptable in a system with a stable load, such as in laboratory reactors, but result in large variations in process performance and pH in a dynamic full-scale reactor. Automatic adjustment of the aeration times, in relation to load, can be used to minimize aeration, thereby saving energy and avoiding NO_3^- accumulation.

In Paper V, the aeration time was instead related to process pH. The time for aeration was changed with a PI regulator, with a total fixed cycle of 60 minutes. The input to the regulator was pH and the output was aeration time. Due to the system properties, this sometimes resulted in oscillation, but this improved after adjustment of the regulator set points (data not shown). The value of controlling the aeration with a pH set point is discussed in Paper V and further discussed in Chapter 5.2. It should be emphasized that no chemicals were added to change pH, only aeration time was used to influence the bulk pH.

5.2 N₂O production, reduction and emissions during intermittent aeration

In Paper V, net production and reduction of nitrous oxide in the water phase and the actual emissions were measured from a full-scale plant. Only N₂O was measured, since this gas is assumed to be the dominating gas in relation to GHG emissions for these processes.

It is important to distinguish between the production and the emission of nitrous oxide in a system with alternating oxygen conditions. Since N₂O shows good solubility in water¹, it can, at low concentrations, remain dissolved during non-aerated times. The gas is stripped off during aerated times, and therefore the N₂O in the water phase is low during aeration.

The results in Paper V show that nitrous oxide gas is produced during non-aerated periods and could potentially be reduced during this time, especially at higher pH set points. Figure 17 shows the net production and net reduction of N₂O during anoxic periods in the water phase, and the actual emissions during the aerobic period. The actual emissions during the aerobic period are substantially lower at a higher pH set point. The reason is most probably because emissions at the higher set point correspond only to N₂O production either via hydroxylamine oxidation or autotrophic denitrification during aerobic conditions whereas N₂O production during anoxic conditions via incomplete DN are also reduced by complete denitrification. It should be noted that anoxic condition allowing for complete denitrification may occur in the biofilm also during aerated periods.

Comparing Figure 14 with Figure 17, it can be concluded that the net production of N₂O is highest when the NO₂⁻ concentrations are at their highest levels. This is in line with other studies (Kampschreur et al., 2009b). Continuous feeding to keep ammonia oxidation rates low (Law et al., 2011) has also been shown to minimize N₂O emissions, and introduction of biofilm has also shown positive effects (Park et al., 2000). Results from Paper V indicate extremely high emissions when the plant was operating in SBR mode with nitrification followed by denitrification with ethanol as carbon source; the formation factor was up to 10% of total nitrogen load in (Stenström et al., 2014). A large part of these emissions are thought to be linked to inefficient dosage of ethanol and aeration capacity, leading to incomplete denitrification as a result of the first technical limitation as well as autotrophic denitrification at low oxygen concentrations due to the second limitation.

¹ The mole fraction solubility of N₂O in water at 30 °C is 3.805×10^{-4} at 1 atm (compared with 2.122×10^{-5} for oxygen, 5.41×10^{-4} for carbon dioxide and 1.108×10^{-5} for nitrogen, (Gevantman, 1994)).

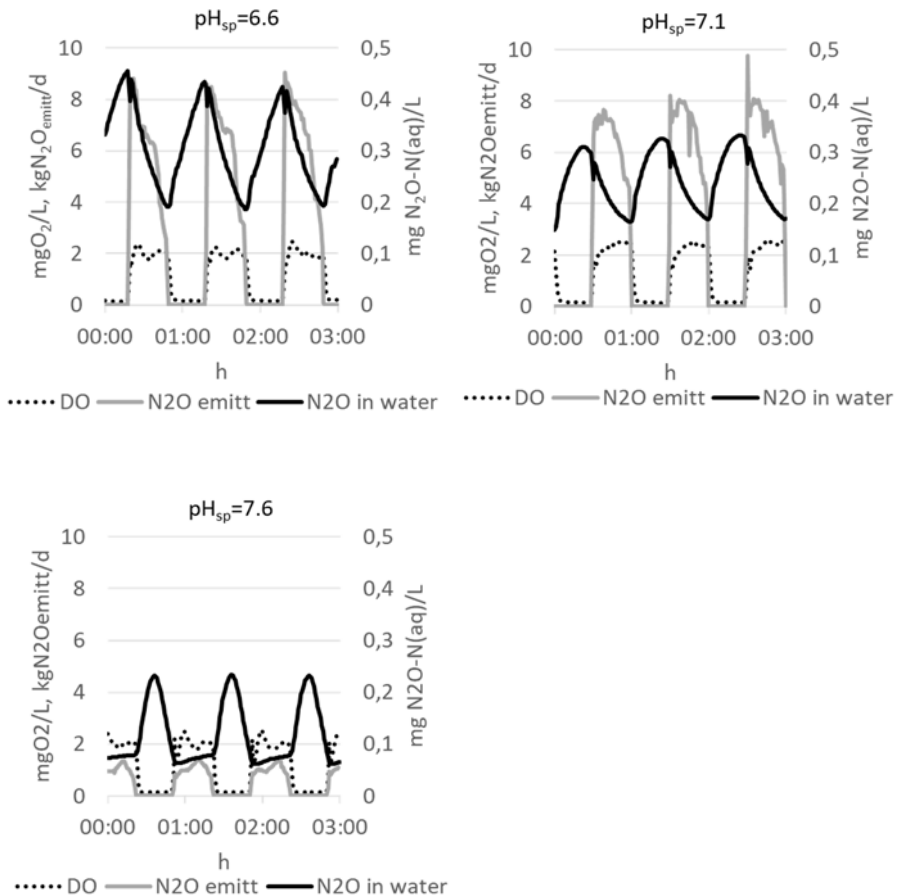


Figure 17. Detailed study of dissolved oxygen (DO), nitrous oxide in water (N_2O in water) and emitted nitrous oxide (N_2O emitt) during three aeration cycles (each cycle is 1 hour) in deammonification mode. Three representative hours are shown for one representative day (day 17, 30 and 46) from each phase. Adapted from Paper V.

5.3 Operational costs and carbon footprint

The major driver for implementing a side-stream reject water treatment is savings in operational costs and/or limitations in the treatment of the nitrogen load in the main-line (Chapter 2.2.2). But what if the installed treatment is emitting additional nitrous oxide, and in this way increases the carbon footprint per kilogram nitrogen treated, instead of decreasing it as intended? This chapter calculates actual operational costs and carbon footprint related to the case study in Paper V. The two operational modes compared for side-stream reject water treatment are N/DN and deammonification. Thereafter, the total carbon footprint is compared between treatment of reject water nitrogen load in the main-line or in a side-stream deammonification treatment in relation to direct emissions of N_2O .

5.3.1 Implementation of deammonification decreases operational costs and carbon footprint

At Slottshagen WWTP, the reject water was initially treated with N/DN as described in Paper V. Table 6 shows the results from a comparison of operational costs associated with reject water treatment done in N/DN mode and deammonification mode. The energy use in deammonification mode of 1.5 kWh/kg N_{in} is similar to results achieved in a system operating at lower oxygen concentrations (1.45–1.75 kWh/kg NH_4-N at 1.3 mg DO/L) with continuous aeration (Christensson et al., 2013). However, comparing data between full-scale plants is difficult, since technical aspects (such as water depth, aeration system) as well as chemical aspects of the substrate (influencing the α -factor), organic loading and temperature during the measurement period have a large impact on the energy use. From Table 6, it can be concluded that the newly installed deammonification plant at Slottshagen WWTP, has lowered the operational cost (associated with nitrogen removal from reject water) by >90%. These figures should be compared with treatment of the nitrogen in the main-line at Slottshagen WWTP with nitrification/denitrification at 0.47 €/kg N based on electricity use and periodic addition of ethanol (M. Eliasson, personal communication, 25th of February 2019). The theoretical data calculated by Gustavsson et al., (2008) resulted in a cost of 0.79 €/kg $N_{removal}$, considering chemicals only (ethanol), for a nitritation/*denitritation* process, in another Swedish reject water treatment plant. These calculations clearly show the lower operational costs associated with the deammonification in comparison to the N/DN or the nitritation/*denitritation* process. Table 6 also shows the carbon footprint linked to operation in kilogram carbon dioxide equivalents (kg CO_{2e}). A

larger CO_{2e} footprint is associated with the N/DN mode and is dominated by the need for an external organic carbon source, which here is assumed to be of biogenic origin. If a non-fossil-based product could be used, the differences would be linked only to the extra use of electricity. These carbon footprints are similar to calculations made by Joss et al. (2009), of 4.5 kg CO₂/kg N_{eliminated} for N/DN (treated in main-line) and 0.6 kg CO₂/kg N_{eliminated} for side-stream treatment in the deammonification mode.

A fourth parameter which is not taken into consideration in relation to this case study is the differences in sludge production between completely autotrophic process and a partly heterotrophic process with higher biomass yield. Sludge production is higher in the N/DN process, which is associated with higher costs as well as a carbon footprint for disposal, although it might generate a positive addition for biogas production. In 2004, Fux and Siegrist calculated an extra cost for sludge disposal of 0.3–0.75 €/kg N_{removal} in N/DN relative to the deammonification mode.

Table 6. Costs and carbon footprint associated with operation for side-stream treatment of reject water nitrogen load when operating in N/DN and deammonification modes. Based on data provided by the operational staff at Slottshagens WWTP. Data from March 2017 in N/DN mode and March 2018 in deammonification mode, and based on a load of 232 kgN/d

		N/DN	Deammonification
External carbon	COD/kgN _{in}	3.3	0
	kg EtOH/kgN _{in}	2.2	0
Energy use for aeration	kWh/kg N _{in}	2.8	1.5
Operating cost ^{1,2}	€/kg N _{in}	1.5	0.14
Carbon footprint (excluding direct emissions) ^{3,4}	kg CO _{2e} /kg N _{in}	4.4 – 5.4	0.1 – 0.6

1. 0.09 €/kWh (M. Eliasson, personal communication)
2. 0.56 €/kg ethanol (M. Eliasson, personal communication)
3. 0.415 kg CO_{2e}/kWh (EU average electricity mix) and 0.058 kg CO_{2e}/kWh (Nordic electricity mix), (Elforsk, 2008)
4. 1.913 kg CO_{2e}/kg ethanol, calculated from stoichiometry for denitrification. The external carbon source used here is not considered to be of biogenic origin and hence its degradation is considered to contribute to the anthropogenic emission of carbon dioxide.

5.3.2 Carbon footprint including N₂O emissions to the atmosphere

As long as the investment costs for a side-stream treatment can be on a sustainable level in comparison to the savings in operational cost, the choice of side-stream reject treatment in deammonification mode seems obvious. However, many studies have shown large nitrous oxide emissions from all biological nitrogen removal processes and side-stream reject water treatment plants in particular (Kampschreur et al., 2009a). Therefore, emissions to the atmosphere should be added when comparing carbon footprints for different treatment alternatives for reject water loads. Paper V in this thesis shows direct emissions of up to 10% N₂O-N of total nitrogen load in N/DN mode whereas 0.1–0.7% was measured in deammonification with MBBR which corresponds to an additional 30 kg CO_{2e}/kgN_{in} and 0.4–2.1 kg CO_{2e}/kgN_{in}, respectively. This can be compared with direct emissions of 1.9 kg CO₂/kg N_{eliminated} for side-stream treatment with deammonification from Joss et al. (2009). This means that direct emissions of N₂O dominates the total carbon footprint of the process. The evaluation of the data in Paper V shows that N₂O emissions are associated with, for example, high substrate concentrations and temperatures generating high ammonia oxidation rates. These conditions may not have occurred and triggered N₂O production if the nitrogen was treated in the main-line.

5.3.3 Implementing side-stream reject water treatment based on carbon footprint

Figure 18 shows the parameters and data used for the calculation of the total carbon footprint, used in the coming section, linked to the case study in Paper V. The total carbon footprint of the process is calculated using the energy use for aeration (based on Nordic electricity mix), external organic carbon source (ethanol) and direct emissions of N₂O. Potential differences in removal capacity are not taken into consideration.

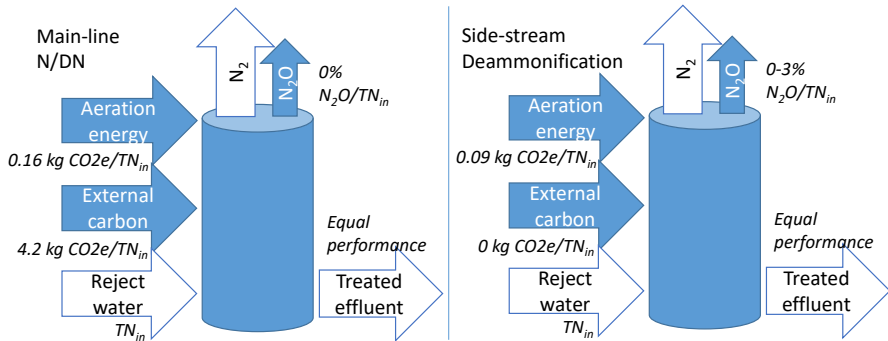


Figure 18. System for calculation of total carbon footprint for reject water treatment in main-line or in side-stream. Dark arrows mark included parameters for the calculations: energy use for aeration, external carbon source (ethanol) and direct emissions of N₂O.

Here treatment of reject water nitrogen is compared between main-line and side-stream. In the main-line the N/DN process is used while deammonification is used as side-stream treatment. The direct emissions of N₂O in main-line are assumed to be zero, because these additional emissions linked to reject water load specifically are difficult to quantify; however, this is a very optimistic approach. Further, the calculation in the main-line is done with and without addition of external organic carbon source (in this case, ethanol). The comparison is expressed in kg CO₂e/kgN_{in}.

Figure 19 shows the results from a comparison of total carbon footprint related to nitrogen load in the main-line or in a side-stream treatment. The lowest CO_{2e} occurs in the main-line treatment with no addition of external carbon. Since the impact of the carbon source highly influences the CO_{2e} emissions, the choice of electricity does not influence the carbon footprint significantly; nevertheless, this calculation with side-stream treatment is done with two different electrical mixes. The figure shows that if side-stream treatment of reject water emits less than 1.2–1.4 % N₂O/TN_{in}, it is beneficial from a carbon footprint point of view, to install a side-stream reject water treatment. However, if the emissions are higher than 1.2–1.4 % N₂O/TN_{in} the side-stream treatment cannot compensate, due to less dosage of an external organic energy source (or extra energy used for aeration). Since the emissions from the one-stage deammonification plant in Slottshagen, Norrköping emits 0.1–0.8 %, which is well below 1.2 %, the side-stream treatment of the reject water can be considered favourable from economic and environmental aspects and no trade-offs between the two needs to be made.

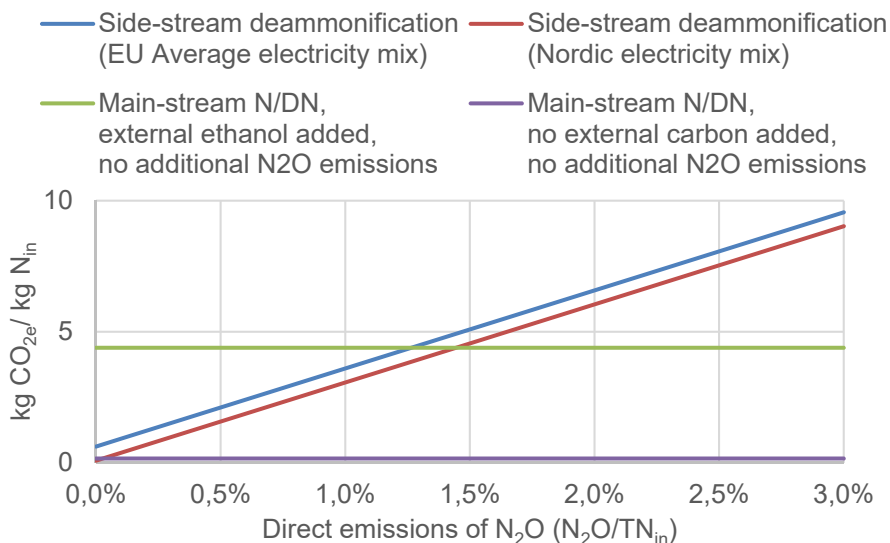


Figure 19. Comparison of different treatment methods for reject water load; deammonification with two different electricity mixes and additional nitrous oxide emissions or treatment in main-line, with or without external carbon. It is assumed that reject water treatment in the main-line does not contribute to additional emissions of nitrous oxide.

With this knowledge, a decision of how to weigh operational costs, GHG emissions and total carbon footprint can be made. A study by Flores-Alsina et al. (2014) showed a simulation tool which considers the different aspects of investment and operational costs, effluent water quality and GHG emissions in a plant-wide operational strategy. In this thesis, no investment costs have been included, but this would be of interest for further studies.

Minimizing the direct emissions of nitrous oxide is essential for the WWTPs in the effort to mitigate global warming. Determining how to minimize the emissions of direct GHGs such as nitrous oxide, and specifically from these types of nitrogen-rich processes is a demanding task. However, one option, as suggested in Paper V, is to operate the unit with intermittent aeration in combination with biofilm to enable complete denitrification at anoxic conditions. In the range of 1.0–3.0 mg DO/L most of the gas is believed to be stripped during aeration, while the reaction rates for AOBs increase by 60% (Van Hulle et al., 2007). This can shorten the aeration periods correspondingly and therefore lengthen the anoxic periods in favour of denitrification, thus enhancing the possibilities to reduce N₂O production. However, since the ammonia oxidizing rate is process-limiting, larger process volumes are then required. To avoid direct emissions of nitrous oxide it is therefore recommended to design the plant with sufficiently large volume, allowing for periods with denitrification rates. If this is not done, a separate treatment of the off-gas is recommended to limit direct N₂O emissions.

In conclusion, when treatment of nitrogen load from reject water is performed in a side-stream treatment with deammonification, given that there is limited capacity to treat this load in the main-line, this can be recommended as long as the N₂O emissions do not exceed 1.2% of TN_{in}. Above this emission factor, emissions from the treatment in the main-line should be evaluated and compared to find the best, most sustainable solution.

6 Conclusions

Deammonification as a side-stream treatment is now an established process. To achieve a successful start-up and a stable operation with low carbon footprint, a validated operational methodology based on the level of knowledge is needed. To ensure this, an adaptive control strategy, preferably integrated in the control system of the plant, is of importance. The studies described in this doctoral thesis show that deammonification plants based on MBBR technology can be started up in full-scale within 100 days without using external inoculum. This is true for reject waters originating from either mesophilic or thermophilic digestion. This research also shows that by measuring and controlling the process with relatively simple instrumentation, the emissions of the potent GHG nitrous oxide can be limited.

The most important operational parameters during start-up of a one-stage deammonification process were defined and validated to be:

- Temperature: operation at mesophilic conditions in order to shorten the start-up time
- Avoiding inhibiting conditions by using dilution water: moderate levels of NH_4 , NO_2 and NH_3 favour growth. Load should be adjusted in relation to ramping of the reduction to avoid substrate inhibition of the system.
- Adaptive aeration control: in order to increase AOB growth and limit NOB growth. Maintain stable oxygen concentration during aeration and adjust aeration time (amount of air supplied) in relation to load. Intermittent aeration is the only tool used to limit NOB growth in these studies, which have not been a substantial problem in any of the start-ups. However, this most probably relates to the relatively high temperatures applied (25–30 °C).

- Control pH: which will react on changes in microbiological activity, preferably with automatic regulation via aeration, in order to avoid pH variations that could hamper the growth of the biofilm, and ensure access to carbon source in the form of alkalinity at all times, as well as controlled concentrations of NH_3 .
- Fixation of anammox bacteria on established nitrifying biofilm: this lowers the pH in the reactor and therefore also the FA, it creates an attractive environment for the anammox to attach on the biofilm and may aggregate the anammox bacteria rather than keep them suspended, reducing the risk of wash out.

The use of a molecular method to detect the growth of anammox bacteria at an early stage of the start-up period may be beneficial but will not contribute to the change of the operation strategy. Positive signs of anammox bacteria will provide the start-up team with confidence that the conditions chosen are beneficial during the 'wait-and-see' phase. This might save some time in preparing an increase of nitrogen load.

The use of external anammox inoculum is not necessary for starting up the process, and the results show that it does not reduce the start-up time.

The studies also indicate that neither substrate type nor pre-treatment of digestion substrate influence the quantities of anammox bacteria in the reject water, since growth of anammox was indicated in mesophilic digesters. In the two thermophilic digesters investigated, there was no increase of anammox bacteria. In an overall balance of a WWTP, anammox bacteria seem to be enriched in activated sludge with nitrification (long sludge ages) as well as in the digester, enabling high enough SRT for enrichment. Decrease in anammox quantities was only seen with hygienization of the sludge when THP was applied. The dewatering process separates biomass, and hence the anammox, when applying a sludge separation process such as centrifugation.

In the operational period, the AOB rate is limiting for the deammonification process. Therefore, aeration strategies, oxygen concentrations and oxygen transfer are important parameters to consider in order to improve the process. However, it should be noted that by increasing the AOB rate, increases in nitrous oxide production may also occur.

When N₂O emissions were compared from a full-scale plant first operated in nitrification/denitrification mode with SBR and later in deammonification mode with MBBR, a decrease in N₂O emissions from 10 % to 0.1–0.7 % of total nitrogen load was observed. *The lower emissions during deammonification were reached during operation of the process at a higher pH set point.* The reasons for the lower emissions are believed to be an increase of the denitrification rate of dissolved N₂O in the water phase during anoxic conditions at higher pH as well as a shorter average aeration time. By comparing the implementation of a separate reject water treatment, considering the carbon footprint of treating the reject water load in the main-line and assuming no additional nitrous oxide emission, it can be concluded that a separate reject water treatment is favourable if the N₂O emissions are lower than 1.2%, which prevailed in this case study.

7 Future directions

Further potential improvements to the start-up strategy for deammonification processes treating reject water remain to be investigated. To optimize the environment for the growing anammox bacteria in the biofilm, the relationship between oxygen concentrations and aeration time could be further explored, possibly by using microsensors as a suitable measurement technique. Increasing the set-point temperature and pH in relation to NH_3 concentrations should be looked into further as well as external addition of alkalinity or even micronutrients to optimize the conditions, particularly for anammox growth.

In Paper IV, the possibility of anammox enrichment in digesters is discussed based on the results obtained, and this topic is ripe for further investigation. In biogas production systems, in which methane production is limited by high FA concentrations, a recirculation system with deammonification as a side-stream treatment could enhance the methane production, which could improve biogas production. Further, the differences in anammox species should be explored in order to differentiate the conditions for anammox bacteria in the main-stream, in the side-stream and potentially in the digester.

The limitation of the nitrite production rate during operation is potentially a problem that needs to be solved. On one hand, there is a consensus that nitrous oxide production is positively correlated with the ammonium oxidation rate, and therefore the rates should not be pushed further. At the design stage, compact solutions with high oxidation rate might not be positive from a carbon footprint perspective. On the other hand, there are solutions that should be investigated, into how to denitrify the still soluble nitrous oxide that is produced; within the one-stage deammonification process, as shown in Paper V, or in treatment of the off-gas from these systems. The implementation of membrane bioreactors with low aeration flux are also of

interest in relation to nitrous oxide production, since stripping during aeration increases emissions but not necessarily the production, of the strong GHG.

The future potential for the deammonification process lies in the implementation of main-stream in the effort for WWTPs to become energy neutral. One challenge for this implementation is not the growth of anammox, but rather the supply of nitrite and specific limitation of the growth of NOBs. Interestingly, anammox grown in biofilm seems to be less sensitive to low temperatures than anammox in suspension (Lotti et al., 2014b), which suggests that the MBBR technique should also be used for implementation in the main-line. In addition, there are many countries where main-line wastewater treatment is carried out at moderate temperatures (20–25 °C) all year round, where the implementation could start to prove the concept in full scale.

Lastly, a suggestion of a more explorative nature is the interesting combination of nitrification, anammox and algae. The algae could produce oxygen for the AOBs, which produce nitrite. The nitrite, in combination with ammonia supply anammox with the substrate to reduce nitrogen to dinitrogen gas. However, some critical obstacles remain, e.g. the critical supply or sensitivity to light.

In conclusion, the implementation of anammox in the WWTP is here to stay, and it will be interesting to follow, or take part in, the further development of future process combinations in order to protect our lakes and oceans from excessive nitrogen loads.

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