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Mainstream Anammox

potential & feasibility of autotrophic nitrogen removal

Maaike HOEKSTRA

Mainstream Anammox

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Proefschrift

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Door

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Summary

Currently wastewater treatment plants (WWTP) consume a lot of energy and surface area. While the incoming water contains chemical energy (BOD) and reusable resources which are not effectively utilized. The ideal is to develop a treatment scheme which allows for the efficient removal of pollutants while minimizing the energy input and maximizing the recovery of energy and resources present in the wastewater. This thesis describes the potential and feasibility of implementing of the partial nitritation/anammox (PN/A) process in the mainstream of a municipal WWTP. Implementation of this technology will allow a complete re-design of the conventional wastewater treatment scheme from an energy consuming into an energy producing system.

In wastewater treatment plants nitrogen is currently removed in two sequential microbial conversions: nitrification and denitrification. For the nitrification step oxygen is needed and for the denitrification step anoxic conditions and BOD are required. The PN/A technology can be used to optimize the municipal mainstream wastewater treatment technology. In the PN/A process the incomplete oxidation of ammonium to nitrite (by aerobic ammonium oxidising bacteria, AOB) is combined with the anaerobic ammonium oxidation (by anammox bacteria). The first advantage is; due to the autotrophic nature of the pathways used, there is no longer a need for carbon to remove nitrogen through denitrification. The carbon in the wastewater can therefore be used for different means for instance for the production of biogas. A second advantage of the PN/A technology is the use of biofilms for (part of) the biomass. Biofilms/granules can lead to higher biomass concentrations in the reactor and therefore higher volumetric loading rates can be applied. Biofilms are easier to separate from water compared to sludge flocs, so a more compact sludge retention system can be built (compared to current secondary clarifiers). Thirdly all nitrogen conversions can take place in the same reactor, omitting the two different zones/tanks for nitrification/denitrification.

In this thesis the potential and feasibility of implementation of the PN/A process in the mainstream of a municipal WWTP is explored. The introduction, the **first chapter**, giving an overview of the nitrogen cycle, the current pathways for nitrogen removal from wastewater. Followed by the general concept of PN/A technology.

In **chapter 2** the pilot-scale research at Dokhaven, Rotterdam is described. This pilot-scale study was a demonstration installation funded by the EU as part of the LIFE+ CENIRELTA project. From this study multiple research questions were formulated, related to: biomass retention, temperature effect on anammox bacteria, the suppression of nitrite oxidising bacteria (NOB), influence of incoming BOD and long-term stability of the nitrogen conversions. A selection of topics will be addressed in the successive chapters, other topics will be studied in follow-up pilot-scale work (which is not included in this thesis).

A mathematical model describing heterotrophic growth, nitritation and anaerobic ammonium oxidation (anammox) combined in a CSTR was developed. To delineate the boundaries of operational conditions (i.e. SRT, influent COD) for the implementation of the PN/A technology at different temperatures. These sets of engineering parameters and influent wastewater characteristics represent the “operational window” in which the PN/A process is likely to be successfully applied under mainstream conditions. The results are described in **chapter 3**.

In **chapter 4** the temperature effects on anammox bacteria in laboratory experiments are described. Laboratory experiments were carried out, since it is hard to study purely the temperature effect in

pilot-scale research. Short- and long-term temperature effects in a temperature range from 20°C-30°C were studied.

In **chapter 5** the competition between AOB and NOB is described, since NOB will compete with AOB and anammox bacteria for substrate in the PN/A biofilm. NOB will produce the undesired nitrate. In our laboratory scale research the effects were studied in a characterized biofilm grown under substrate limiting conditions. The results indicate that the substrate limited growth resulted in a biofilm without stratification. In these biofilms it was impossible to supress NOB based on temperature or dissolved oxygen concentration, in batch tests. To supress NOB in a biofilm growth conditions with a surplus of substrate or a nitrite sink (anammox or denitrifying bacteria) will be needed.

In the **last chapter,** the outlook, I reflect on the PN/A technology and related research. Before the first full-scale installation can be built, several topics should be studied. Firstly, the long-term effects of fluctuating temperatures. Secondly, the modelling and pilot-scale research have shown that a good and stable carbon removing step, prior to the PN/A reactor is essential. Removing the BOD in the A-stage (or A-stage-like step) is the most efficient way as to use it for biogas production through digestion. The suppression of NOB in the biofilm is the third crucial factor in operating the PN/A reactor. This also relates to the fourth point: proven long-term stability. The fifth point is related to the reactor design, for the pilot reactor the design of a CSTR (completely mixed tank) was chosen, but it is impossible to simultaneously reach good effluent quality and high conversion rates. For a full-scale design a plug flow should be chosen; suppression of NOB, oxidising BOD and effluent polishing will be easier in such a system.

Samenvatting

Het zuiveren van afvalwater kost veel energie en oppervlakte. Het afvalwater bevat energie, gebonden in biologische verbindingen (BZV) en herbruikbare grondstoffen, deze worden op dit moment nauwelijks gebruikt. Een ideale zuivering, zuivert het afvalwater op een energy efficiënte manier, waarbij zoveel mogelijk energie en grondstoffen worden teruggewonnen. In dit proefschrift is de potentie en haalbaarheid van de implementatie van de partiële nitritatie/anammox (PN/A) technologie beschreven. De implementatie van deze technologie in communale afvalwater zuivering, biedt de mogelijkheid om de zuivering te veranderen van een energie consumerende installatie in een energie producerende installatie.

In rioolwaterzuivering installaties (rwzi) wordt stikstof op dit moment verwijdert via twee microbiële processen in serie: nitrificatie en denitrificatie. Voor de nitrificatie stap is zuurstof nodig en voor de denitrificatie stap anoxische condities en BZV. Met de PN/A technologie wordt gebruik gemaakt van de gedeeltelijke oxidatie van ammonium naar nitriet (door AOB, ammonium oxiderende bacteriën) en anaerobe oxidatie van ammonium door anammox bacteriën. Het eerste voordeel is dat, door het autotrofe karakter van de omzetting, CO_2 een geschikte koolstofbron is. De koolstof in het afvalwater kan daarom gebruikt worden voor andere dolen, bijvoorbeeld de productie van biogas of bio plastics. Een tweede voordeel is het gebruik van biolfims, dit zorgt ervoor dat er hogere biomassa concentraties en hogere volumetrische belastingen bereikt kunnen worden. Daarnaast kunnen biofilms makkelijker van water gescheiden worden dan vlokken zodoende kan het slibretentie systeem compacter gebouwd worden (in vergelijking met de nabezinkers in het actief slib systeem). Ten derde vinden alle conversies plaats in dezelfde reactor, er is niet langer behoefte aan verschillende compartimenten en zones.

In dit promotie onderzoek is de potentie en de haalbaarheid van de implementatie van de PN/A technologie onderzocht. De inleiding, het **eerste hoofdstuk,** geeft een overzicht van de stikstof cyclus, de huidige stand van zaken op het gebied van stikstof verwijdering en een introductie van de PN/A technologie.

In het **tweede hoofdstuk** wordt het pilot-schaal onderzoek in Dokhaven, Rotterdam beschreven. Deze studie was een demonstratie project gefinancierd door de EU binnen het LIFE+ CENIRELTA project. Dit demonstratie project resulteerde in meerdere onderzoeksvragen gerelateerd aan, biomassa retentie, het temperatuur effect op anammox bacteriën, het onderdrukken van nitriet oxiderende bacteriën, de invloed van inkomend BZV en de lange termijn stabiliteit van het proces. Een aantal van deze onderzoeksvragen worden behandeld in de opvolgende hoofstukken, andere zullen bestudeerd worden in vervolg onderzoeken die geen onderdeel zijn van dit proefschrift.

Er is een mathematisch model, gebaseerd op heterotrofe groei, nitritatie en anammox in een volledig gemengd systeem ontwikkeld. Dit model kan helpen om de condities te definiëren die nodig zijn voor de implementatie van de technologie in afvalwater van verschillende temperaturen (zoals de biomassa retentie tijd en de inkomende BZV concentratie). Het model en de resultaten zijn beschreven in het **derde hoofdstuk**.

In het **vierde hoofdstuk** wordt het effect van temperatuur op anammox bacteriën beschreven. Dit onderzoek is uitgevoerd in het laboratorium, omdat het moeilijk is om specifiek het temperatuur effect te onderzoeken op pilot-schaal. In afvalwater gaat de verandering van tempartuur vaak gepaard met andere veranderingen in afvalwater samenstelling. Lange en korte termijn effecten in de temperatuur range van 20°C-30°C zijn bestudeerd.

In het **vijfde hoofdstuk** wordt de competitie tussen AOB en NOB onderzocht. In de PN/A biofilm zullen de NOB in competitie zijn met de AOB en anammox bacteriën, de NOB produceren het ongewenste nitraat. In onze laboratorium testen is de competitie tussen AOB en NOB onderzocht in een biofilm die gekweekt is onder substraat limitatie. De resultaten laten zien dat de substraat limitatie leidt tot een biofilm zonder stratificatie. In deze biofilms konden wij de NOB niet onderdrukken (met behulp van zuurstof concentratie of temperatuur) zonder de AOB ook te limiteren. Om de NOB te kunnen onderdrukken, moet de biofilm gegroeid worden onder condities met een overmaat aan substraat of een nitriet consumerende populatie.

Het **laatste hoofdstuk** is de outlook, een reflectie van mij op de PN/A technologie en het gerelateerde onderzoek. Voordat de eerste volle schaal hoofdstroom anammox installatie gebouwd kan worden, moeten er nog een aantal onderwerpen onderzocht worden. Ten eerste, het lange termijn effect van de fluctuerende temperaturen. Ten tweede tonen het model en het pilot-schaal onderzoeken aan dat een goede en stabiele BZV verwijderingsstap voor de PN/A reactor essentieel is. Het verwijderen van BZV in een A-trap (of een installatie zoals de A-trap in het AB-systeem) is de meest efficiënte manier om BZV te verwijderen en bied de mogelijkheid voor hergebruik. Het onderdrukken van NOB is de derde belangrijke factor, vooral de lange termijn onderdrukking is nog omringd met vragen. Het laatste punt van aandacht is het reactor ontwerp, in het pilot-schaal onderzoek is gekozen voor een compleet gemixte tank, daardoor was het onmogelijk om tegelijkertijd hoge belasting en goede effluent kwaliteit te halen. Voor een volle schaal ontwerp zal gekozen worden voor een propstroom-reactor, in een dergelijk systeem is de onderdrukking van NOB, het oxideren van BZV en effluent optimalisatie makkelijker.

1

Introduction to this thesis

Overall objective

Wastewater is treated in wastewater treatment plants (WWTP) in most parts of the developed world and for public health reasons it should be treated like this everywhere. The currently used treatment plants consume a lot of energy and require a large surface area. The incoming water contains chemical energy (expressed in Chemical/Biological Oxygen Demand (COD and BOD)) and reusable resources, which are currently not effectively used. The ideal is to develop a treatment scheme which allows for the efficient removal of pollutants while minimizing the energy input and maximizing the recovery of energy and resources present in the wastewater. In the Netherlands there is a strong interest to improve the WWTP, for instance through the formation of "Energy and Resource factories" of the Dutch water associations (Energie en Grondstoffenfabriek).

This thesis describes the potential and feasibility for implementation of the partial nitritation/anammox (PN/A) process in the mainstream of a municipal WWTP (in short: Mainstream Anammox). Implementation of this technology will allow a complete re-design of the conventional wastewater treatment scheme from an energy consuming into an energy producing system.

Nitrogen cycle

Nitrogen is an important element in all living systems and 78% of the air we breathe is nitrogen gas. There are a lot of different organisms responsible for the conversions of the different nitrogen species (N-species can be found in Table 1-1, conversions in Table 1-2). Together these conversions form the nitrogen cycle, a simplified version was already proposed in 1890 (Winogradsky, 1890). Discoveries in the last decades have changed the simple nitrogen cycle in a web-like structure as shown in Figure 1-1.

Table 1-1: Chemical formula of the nitrogen species used in this thesis and their names.

Chemical formula	Name
N_2	(di) Nitrogen (gas)
NH_4	Ammonium
NH_2OH	Hydroxylamine
NO_2	Nitrite
NO_3	Nitrate
NO	Nitric oxide
N_2O	Nitrous oxide / laughing gas
N_2H_4	Hydrazine

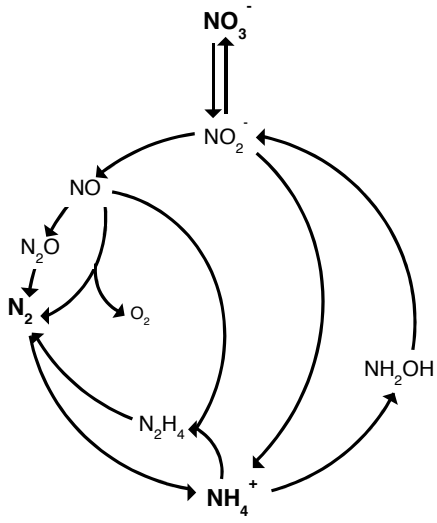

Figure 1-1: Graphical representation of the microbial conversions in the nitrogen cycle.

Table 1-2: Microbial conversions in the nitrogen cycle.

Pathway		Need of:		Abbreviation organisms (if used)
		Oxygen	External BOD	
Nitrogen fixation	$N_2 \rightarrow NH_4$		x	
Nitrification	$NH_4 \rightarrow NO_2 \rightarrow NO_3$	x		AOB & AOA (first step) NOB (second step) Comammox (complete conversion)
Denitrification	$NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$		(x)	
Dissimilatory nitrate reduction to ammonia	$NO_3 \rightarrow NO_2 \rightarrow NH_4$		x	DNRA
Anaerobic ammonium oxidation	$NH_4+NO_2 \rightarrow N_2+NO_3$			Anammox

Ammonium or nitrate are an important component in fertilizers. To fixate nitrogen for the use as fertilizer the Haber-Bosch process is used. This process was developed in the first half of the 20th century. The Haber-Bosch process requires high pressures (around 200 atm) and high temperatures (at least 400 °C). The process uses natural gas as a hydrogen source and air as a nitrogen source. The replacement of this energy consuming chemical process by a biological process was not economically feasible until now (Eekert van et al., 2012).

Nitrogen in the WWTP, current situation

Wastewater treatment uses a combination of different physical, chemical and biological processes aimed at removing soluble and particulate compounds. If these compounds would be discharged in the receiving water bodies, they would be a risk for public health and the environment. An unbalance in the eco-system could result, for instance in eutrophication (uncontrolled algal blooms). Therefore in a WWTP oxygen consuming compounds, pathogens and nutrients are removed.

This thesis focusses on the removal of nitrogen from the wastewater, mainly originating from urine. Removal of nitrogen from wastewater is currently done through biological processes. The natural conversion capacities of certain bacteria are enhanced to convert the polluting (soluble) compound (ammonium, nitrite, nitrate) in a harmless component (nitrogen gas). The natural processed are enhanced in a technical system were mass transfer and mixing are optimised compared to a natural system.

The most commonly used wastewater treatment system is the activated sludge system, first described by Arden and Lockett in 1914 and schematically represented in Figure 1-2.

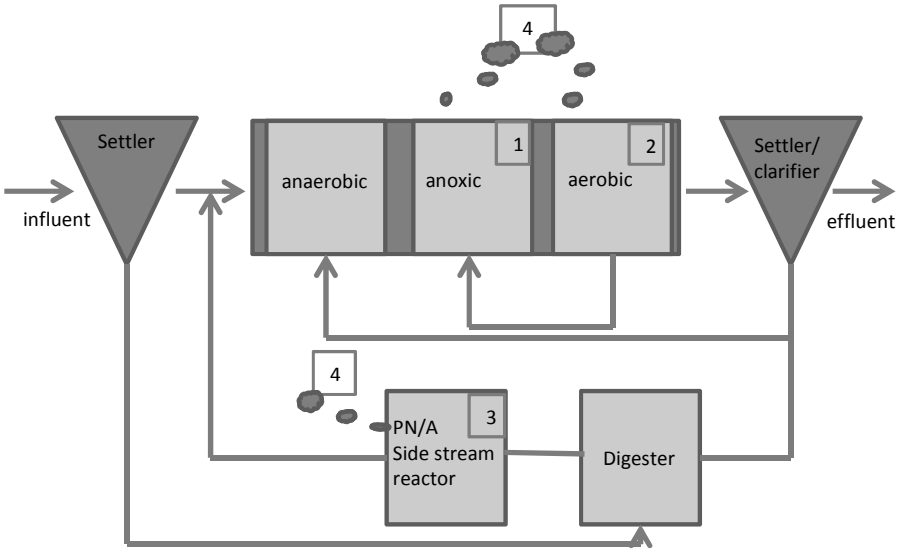

Figure 1-2: Schematic representation of a wastewater treatment plant.

A WWTP using the activated sludge system consist of several stages with different environmental characteristics in separate tanks, zones in a tank or stages of a cycle; the different conditions are separated in space or time. The main regulators are the time a microorganism is allowed to reside in the system (solid retention time, SRT) and the availability of an electron acceptor such as oxygen or nitrate.

After entering the treatment plant the large solid fractions are separated from the wastewater by sieving, flotation or sedimentation (or a combination of these). The fractions that are sieved out of the sewage are incinerated and the biological fraction is digested. The water enters the first tank, in a biological phosphate removal process this tank is anaerobic and the activated sludge is mixed with

the water. In the next tank/stage (1) a recycle flow is added, this flow contains nitrate which is denitrified with the carbon present in the wastewater. In the last aerobic tank/stage the ammonium is nitrified to nitrate (2) and is recycled to the denitrifying stage. In order to oxidise ammonium and nitrite, oxygen needs to be externally added to the wastewater. The energy used for the oxygen addition (mainly compressors for aeration) are one of the largest costs related to wastewater treatment. Phosphate can be removed chemically by precipitation or biological in the same series of stages/tanks. The sludge is separated from the water by sedimentation and recycled in settlers.

The bacteria will use the chemical energy (named: BOD), present in the polluting compounds, for growth and therefore for increasing their concentration in the wastewater treatment system. These excess bacteria need do be dealt with, the first step is often concentration followed by digestion and/or incineration. Sludge handling is a large fraction of the operation costs of a WWTP. Part of the chemical energy present in the wastewater can be reclaimed by digesting the produced biomass and producing methane (biogas). Methane can be converted into electrical energy, lowering the net energy consumption of the WWTP.

The water leaving the digester contains high concentrations of ammonium and this can be directly recycled to the beginning of the WWTP or autotropically removed in a reactor by the partial nitritation anammox (PN/A) technology (3). The PN/A technology uses two different bacteria (AOB and anammox bacteria) to convert ammonium into nitrogen gas. In an engineered system these bacteria can live together in one biofilm and will use CO_2 as carbon source. This means they are autotrophs, these organism produce complex organic compounds from CO_2, in this case using energy from ammonium oxidation. The advantage of an autotrophic system is that there is no need for BOD to denitrify (most denitrifies are not autotrophic, they are heterotrophic). Since there is hardly any BOD left in the digested water, it is a good implementation for autotrophic nitrogen removal. These flows usually have a temperature of above 20°C and ammonium concentrations over 0.5 $gNH_4\text{-}N\ L^{-1}$. Under these conditions the energy savings potential and stable operation of the PN/A technology have been well proven (Lackner et al., 2014; Morales et al., 2015; van der Star et al., 2007).

WWTPs are known to emit gasses, in the form of CO_2, methane, NO and N_2O. These gassed can contribute to the global warming, therefore emissions should be minimised if possible. From the nitrification, denitrification and PN/A (4) step emissions in the form of NO and N_2O have been measured (Kampschreur et al., 2009). It would be more sustainable to limit the greenhouse gas emissions from the WWTP.

Nitrogen in the WWTP, potential

Currently the ammonium present in the wastewater is converted into nitrogen gas and emitted into the atmosphere. Simultaneously nitrogen is fixed into the form of ammonium in the Haber-Bosch process. Theoretically the reuse of the nitrogen from the wastewater in the form of fertilizer is a good idea. A challenge is the low concentration of ammonium in the wastewater. Increasing the concentration of ammonium can for example be done by source separation in the toilets, and separation of rainwater from sewage water (Larsen and Gujer, 1996; Otterpohl, 2002). It is currently not economically feasible to reuse ammonium from the wastewater as compared to fixation in the Haber-Bosch process (Eekert van et al., 2012).

Application of the circular economy is based on the reuse of ammonium, but as long this is not economically feasible the WWTP could be improved, especially minimising energy consumption. The

currently used treatment plants consume high amounts of energy and surface area. Both can be reduced by using the aerobic granular sludge technology (Nereda) (Pronk et al., 2015). This technology is based on the same biological processes as a common activated sludge system, but the bacteria are grown in dense granules instead of flocs. Due to these granules higher biomass concentrations can be reached in the reactor, resulting in a smaller footprint of the plant also the overall energy consumption will be lower.

In the mainstream of a municipal wastewater treatment plants nitrogen is currently removed in two sequential microbial conversions: nitrification and denitrification. For the nitrification step oxygen is needed and for the denitrification step anoxic conditions and BOD are required. The PN/A technology can be used to optimize the municipal wastewater treatment technology (Bozkurt et al., 2016). In the PN/A process the incomplete oxidation of ammonium to nitrite (by aerobic ammonium oxidising bacteria, AOB), is combined with the anaerobic ammonium oxidation (by anammox bacteria).

Due to the autotrophic nature of the pathways used, there is no need for carbon to remove nitrogen through denitrification. The carbon in the wastewater can therefore be used for different means for instance for the production of biogas (Jetten et al., 1997). Electricity produced from the biogas can make the treatment plant energy neutral. This is especially of interest for countries with an unsecure electricity supply or to avoid the costs of connection to the electricity grid. Reusing carbon in different ways is also a possibility, for instance by producing biopolymers (Valentino et al., 2017). A second advantage of the PN/A technology is the use of biofilms for (part of) the biomass. As discussed before biofilms/granules can lead to higher biomass concentrations in the reactor and therefore higher volumetric loading rates can be applied. Granular sludge is easier to separate from water compared to sludge flocs, so a more compact sludge retention system could be built (compared to current secondary clarifiers). Thirdly all nitrogen conversions can take place in the same reactor, omitting the two different zones/tanks for nitrification/denitrification and the recirculation pumps to pump water around over these tanks.

Challenges for the implementation of the PN/A technology will be the long-term stability (partly related to the BOD removing step, prior to the PN/A reactor)(De Clippeleir et al., 2013; Lotti et al., 2014a), NOB suppression (Gilbert et al., 2014; Lotti et al., 2014a) and conversion rates at low temperature (Dosta et al., 2008; Hendrickx et al., 2014).

Different approaches PN/A

A possible approach to combining the partial nitritation and anammox processes is through application of a biofilm process, with the bacteria growing as granular sludge or on a carrier. In the outer layer of the biofilm, aerobic AOB are oxidising ammonium to nitrite, and in the anoxic deeper layers, anammox bacteria convert ammonium with nitrite to dinitrogen gas (Hao et al., 2001; Kartal et al., 2010; Rosenwinkel and Cornelius, 2005; van der Star et al., 2007). The use of a biofilm system has the main advantage that high biomass concentrations can be reached. Biofilms can be grown on carriers or without. The use of carriers will lead to a known surface area and easy biofilm retention, drawbacks are the need of plastic carriers and the low oxygen mass transfer (high mixing or aeration velocities will be needed) (Nogueira et al., 2015). Other proposed possibilities to implement this technology are: a hybrid system with anammox bacteria in a biofilm and AOB in flocs (Gilbert et al., 2015; Laureni et al., 2016) in suspension or a two stage system with the nitritation and anammox

conversions occurring in different biomasses in separate reactors (Cao et al., 2017; Pérez et al., 2015).

The PN/A sludge can be grown under mainstream conditions or in side-stream water (after the digestion) and added to the mainstream water (bio-augmentation). The difficulty of bio-augmentation is that it is still hard to prove that the conversion is occurring through the anammox pathway. When BOD is present it is also possible that nitrite is denitrified.

As delineated above, there are multiple ways to implement the PN/A technology in the mainstream of a wastewater treatment plant, different companies and research groups use different approaches. For instance the use of bio augmentation in the EssDe process (Wett et al., 2013). The use of biofilms in a sequential batch reactor (SBR) (Lotti et al., 2014a; Seuntjens et al., 2016). Or the use of biofilms in a continuous system (Cho et al., 2011; Lotti et al., 2015b; Persson et al., 2014). Hybrid systems continuous or in SBR mode (Laureni et al., 2016; Malovanyy et al., 2015). The use of intermitted aeration (Gilbert et al., 2014; Ma et al., 2015). Or a two-step system (Pérez et al., 2015; Reino et al., 2016).

A large part of my PhD project, was related to the PN/A demonstration installation at the WWTP Dokhaven in Rotterdam. This work was partly funded by the European Union in the form of a LIFE^{+} project: CENIRELTA (Cost Effective NItrogen REmoval from wastewater with Low Temperature Anammox) and was a collaboration between Waterschap Hollandse Delta, Paques and STOWA. The results of 3.5 years of running this installation can be found in chapter 2 and in the STOWA report (in Dutch) (CENIRELTA: demonstratieproject Anammox in de hoofdstroom op rwzi Dokhaven) (Hendrickx et al., 2017).

Outline of the thesis

This thesis describes the potential and feasibility of implementation of the PN/A process in the mainstream of a municipal WWTP. This introduction is the first chapter followed by four chapters, these chapters form the core of the thesis and describe part of the scientific work done in the last 5 years of research.

In **chapter 2** the pilot-scale research done at Dokhaven, Rotterdam is described. Before converting a whole treatment plant to this new PN/A technology a proof of principle is needed, therefore a pilot-scale reactor was in operation for 3.5 years. The aim of this research was to evaluate the biological feasibility of the technology to remove nitrogen from municipal mainstream wastewaters. Therefore different topics were studied. Firstly, the effect of decreasing temperatures on total nitrogen removal capacity. Secondly, the suppression of NOB and overcapacity in activity of AOB and anammox bacteria. The set-up was designed to evaluate the potential volumetric conversion capacity on effluent of the BOD removing step at fluctuating temperatures, with an effluent demand of total soluble nitrogen below 10 mgN L^{-1} (current effluent requirement for most WWTPs in the Netherlands). A point of interest during this reactor run was the stability of the system. When applying a technology on a full-scale in a WWTP the system needs to be resilient, not treating the wastewater will not be acceptable.

From this pilot-scale study multiple research questions were formulated, which are addressed in the following chapters. A mathematical model describing heterotrophic growth, nitritation and anaerobic ammonium oxidation (anammox) combined in a CSTR was developed, described in **chapter 3**. The

aim of this study was to delineate the boundaries of operational conditions (i.e. SRT, influent COD) for the implementation of the PN/A technology at different temperatures. These sets of engineering parameters and influent wastewater characteristics represent the “operational window” in which the PN/A process is likely to be successfully applied at mainstream conditions. The SRT could be controlled by biomass retention and the amount of incoming COD could be influenced by optimizing the COD removing step. The temperature of the wastewater was a parameter which cannot be regulated, the wastewater flow is too big to be heated up. The nitrifying stage of the WWTP of Rotterdam-Dokhaven, the Netherlands was used as case study.

In **chapter 4** the temperature effect on anammox bacteria was studied in laboratory experiments, since pure temperature effects were hard to study in pilot-scale research. A highly enriched and fast growing anammox community at 30°C was used to study the effect on the anammox bacteria by decreasing the temperature to 25°C and 20°C. To measure the long-term temperature dependency of the conversions the bacteria were at 30°C, 25°C, and 20°C. Short-term temperature effects were measured using short dynamic temperature experiments. The activity of the anammox bacteria was deteriorating during long-term cultivations at 25°C and 20°C.

In **chapter 5** the competition between AOB and NOB was studied, since NOB will compete with AOB and anammox bacteria in the PN/A biofilm. NOB will produce the undesired nitrate. This competition is studied before, but in literature different effects of lowering the DO and temperature are described. The observations are based on different species and biofilm structures. In our laboratory scale research the effects were studied in a characterized biofilm grown under substrate limiting conditions.

The **last chapter** is the outlook, my reflections on the PN/A technology and related research. The outlook contains a new treatment scheme for the WWTP, based on my experience with the PN/A technology. Before the first full-scale installation can be build, some final topics should be studied, these are identified as well and some recommendations for future work are included.

2

Towards mainstream Anammox; lessons learned from pilot-scale research at WWTP Dokhaven

Abstract

The aim of this research was to study the biological feasibility of the Partial Nitritation/Anammox (PN/A) technology to remove nitrogen from municipal mainstream wastewaters. During stable process operations at summer temperatures (23.2±1.3°C) the total nitrogen removal rate was 0.223±0.029 kgN $(m^3\ d)^{-1}$ while at winter temperatures (13.4±1.1°C) the total nitrogen removal rate was 0.097±0.016 kgN $(m^3\ d)^{-1}$. NOB suppression was successfully achieved at the complete temperature range of municipal mainstream wastewater, although an increase in nitrate production was observed after prolonged cultivation at low temperature. Despite the presence of NOB as observed in activity tests, their activity could be successfully suppressed by controlling the dissolved oxygen (DO) concentration. An overcapacity of AOB and anammox activity was always present. Long term stability is a focus point for future research, especially in relation to the stability of the BOD (biological oxygen demand) removing step, preceding the PN/A reactor.

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Introduction

One of the available technologies to treat municipal wastewater is the AB-system. Wastewater treatment plants (WWTPs) designed according to this technology are set-up as a two stage system with a high loaded A-stage (Adsorption) and a lower loaded B-stage (Belebung) (Böhnke, 1977). In the A-stage the biological oxygen demand (BOD) is removed and in the B-stage the ammonium is nitrified. To remove nitrate from the effluent of the nitrification process, wastewater is recycled to the A-stage where the BOD in the influent can serve as electron donor for the denitrification process. The main advantage of this process is the relative low external energy requirement and compact construction, with full nitrification. An example of an AB-process upgraded to partial denitrification, is the Dokhaven treatment plant in Rotterdam, the Netherlands (De Graaff et al., 2016).

In the two-sludge type AB-system it was not simple to implement the pre-denitrification process for nitrogen elimination. Due to the large recycle flow required for returning nitrate rich effluent from the B-stage to a partially anoxic A-stage, the hydraulic load on the settlers of both stages was strongly increased. The total nitrogen removal capacity of the treatment plant is still limited, for a further improvement would result in extreme large recycle flows. In AB-system based treatment plants it would therefore be favourable when complete nitrogen removal could be introduced in the B-stage of the process. This can be achieved by implementing the Partial Nitritation/Anammox (PN/A) process in the current B-stage. In the PN/A process the first step of nitrification, the oxidation of ammonium to nitrite (nitritation), is combined with the anaerobic ammonium oxidising (anammox) process. Both nitritation and anammox are autotrophic processes that do not require BOD, but the anammox bacteria do require a high sludge age for their implementation. Removal of BOD in the A-stage of the AB-process facilitates high sludge ages and high percentages of autotrophic biomass in the B-stage, potentially enabling the PN/A process.

The PN/A technology, can optimize the current municipal mainstream wastewater treatment technology (Bozkurt et al., 2016). There are multiple advantages to the process. Firstly there is no longer a need for carbon in order to remove nitrogen through denitrification. The carbon in the wastewater can therefore be used for different means, for instance for the production of biogas (Jetten et al., 1997). Electricity produced from the biogas could make the treatment plant autarkic with respect to energy. This is especially of interest to countries with an unsecure electricity supply or to avoid the costs of connection to the electricity grid. The carbon in the wastewater could also be reused in different ways, for instance for producing biopolymers (Valentino et al., 2017). A second advantage of the PN/A technology is the use of biofilms for (part of) the biomass. Biofilms can lead to higher biomass concentrations in the reactor and therefore higher volumetric loading rates can be applied. Biofilms are easier to separate from water compared to sludge flocs, so a more compact sludge retention system could be built (compared to current secondary clarifiers). And lastly, due to the exclusion of heterotrophic denitrification and therefore the need for BOD, this technology could be implemented in streams with BOD:N ratios, too low for heterotrophic denitrification. Specifically for AB-systems that need upgrading for full denitrification, effluent recycle (pump energy) will no longer be required.

There are different ways of implementing the PN/A process in the B-stage of an AB-process (Cao et al., 2017). Two types of organisms can be combined in a biofilm, in the form of granules or carrier material (Kartal et al., 2010; Rosenwinkel and Cornelius, 2005). In this biofilm the Ammonia Oxidising Bacteria (AOB) are oxidising part of the ammonium to nitrite and the anammox bacteria convert

ammonium together with the produced nitrite, to dinitrogen gas. Since the organisms grow as a biofilm it is possible to combine the aerobic zone (for AOB) and the anoxic zone (for anammox bacteria) in a single reactor at the same time. Another possibility would be to cultivate the AOB as flocs in suspension and the anammox bacteria as a biofilm, the so called hybrid system (Wett et al., 2013). It is also possible to separate the AOB from the anammox bacteria in a two-stage system. With AOB in the first, aerated, reactor and anammox bacteria in the second reactor (Pérez et al., 2015).

The PN/A technology is currently implemented in side stream flows of wastewater treatment plants and in industrial wastewater streams, characterized by high temperatures and high ammonium concentrations (Lackner et al., 2014). Laboratory and pilot-scale research on the mainstream application is currently pursued worldwide. But a limited amount of work is done on pilot-scale installations fed with real wastewater (Lotti et al., 2015b; Malovanyy et al., 2015; Seuntjens et al., 2016; Wett et al., 2013). The laboratory and pilot-scale results indicate that the main challenge of implementing the technology are the low water temperatures during winter. At low temperatures, the total conversion rates decrease and suppression of Nitrite Oxidising Bacteria (NOB) proved difficult (Cao et al., 2017; Laureni et al., 2016; Lotti et al., 2014a).

Before converting treatment plants to this new technology a proof of principle is needed, therefore a pilot-scale reactor was in operation for over 3.5 years. The aim of this research was to evaluate the biological feasibility of the technology to remove nitrogen from municipal mainstream wastewaters. Therefore different topics were studied. Firstly, the effect of decreasing temperatures on total nitrogen removal capacity. Secondly, the suppression of NOB needed for stability and finally the need for an overcapacity in activity of AOB and anammox bacteria. The set-up was designed to evaluate the potential volumetric conversion capacity on effluent of the A-stage at fluctuating temperatures, with an effluent demand of total soluble nitrogen below 10 mgN L^{-1}(current effluent requirement for most WWTPs in the Netherlands). A point of interest during this reactor run was the stability of the system. When applying a technology on a full scale in a WWTP the system needs to be resilient, not treating the wastewater will not be acceptable. Here we describe the results and discuss the lessons learned.

Materials and Methods

Dokhaven-Sluisjesdijk WWTP

The municipal wastewater treatment plant at Dokhaven, Rotterdam, the Netherlands, has a treatment capacity of about 560,000 p.e.. The treatment plant was designed as an AB-system and was built underground. In the A-stage biochemical oxygen demand (BOD) is removed in a high-loaded reactor by adsorption (HRT of 1 h in dry weather conditions, SRT 0.3 d) with the aim of maximizing biogas production by anaerobic digestion. In the B-stage the remaining BOD is oxidized and ammonium is nitrified to nitrate (HRT 3 h; SRT 7 d). The treatment plant was not originally designed for denitrification. To remove the nitrogen from the water, the effluent of the B-stage is recycled to the beginning of the A-stage, where the nitrate can be denitrified together with the incoming BOD. Phosphorus is chemically removed by $Fe^{3+}Cl_3$ dosage in the A-stage.

Reactor set-up and operating conditions

The reactor has been operated from 2013 to 2016 and is comprised of a well-mixed reactor of four cubic meter and a separator for granular biomass retention. A schematic representation of the installation can be seen in Figure 2-1. The complete reactor run was divided into different periods, a

description of the different periods can be found in Table 2-1. The influent of the reactor was the effluent of the intermediate sedimentation tank after the A-stage, before the influent entered the reactor it was buffered in the buffer tank. In this tank NH_4Cl could be dosed and the temperature of the influent was controlled. In period 1-3 ammonium chloride was dosed to the reactor influent, to decrease the BOD/N ratio and to correct for the decrease in ammonium in the influent, due to the recycling of the effluent for denitrification, in period 4 the dosing was stopped. By changing the hydraulic retention time (HRT), the volumetric nitrogen load was kept constant over all four periods. Oxygen transfer and mixing were done by fine-bubble aerators. The dissolved oxygen concentration was controlled by a gas recycling system with a constant gas flow to which fresh air was introduced. pH was controlled at 7.2 by the addition of 33 w/v% NaOH. The reactor was inoculated with granular biomass from a full-scale one-stage PN/A side stream reactor operated at 30°C-35°C.

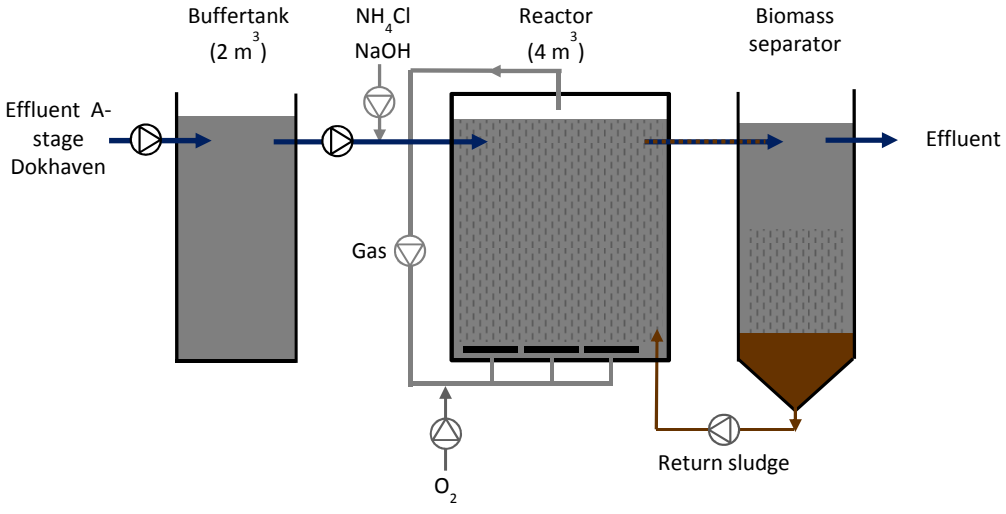


Figure 2-1: Lay-out of the PN/A reactor.

Table 2-1: Overview of the different periods, described in this publication.

Period	Inoculation date	# days	HRT [d]	NH_4Cl dosing	Temperature range [°C]	Inf NH_4 [mgNH_4-N L^{-1}]	Inf BOD/N
1	Feb 2014	91	0.12±0.02	+	16.1-21.1	33.0±7.5	0.6±0.2
2	May 2014	153	0.11±0.01	+	18.8-25.5	32.6±5.4	0.4±0.1
3	Dec 2014	83	0.10±0.00	+	10.4-17.5	30.3±4.9	1.2±0.4
4	July 2015	180	0.05±0.01	-	10.5-24.7	14.2±3.8	2.5±1.8

Calculating conversion rates

Based on the influent and effluent concentrations of the N-species, the conversion rates of the reactor were calculated. Based on the assumption that the only conversions taking place in the reactor were autotrophic nitrogen conversions, specific rates for anammox bacteria, AOB and NOB

were derived. Due to the presence of oxygen in the reactor it was assumed that the incoming BOD was converted aerobically and not through denitrification. The calculations are based on the stoichiometry in equations 1-3, for the AOB, NOB and anammox conversions (Lotti et al., 2014b). The ratio between nitrate production and ammonium consumption was used as an indicator of the significance of NOB activity. When there is only AOB and anammox activity, 7.5% of the consumed NH_4-N will be converted to NO_3-N.

$$NH_4 + 0.09HCO_3 + 1.38O_2 \rightarrow 0.09CH_{1.4}O_{0.4}N_{0.2} + 1.04H_2O + 0.98\,NO_2 + H^+ \quad (1)$$

$$NO_2 + 0.0025NH_4 + HCO_3 + 0.49O_2 \rightarrow 0.0125CH_{1.4}O_{0.4}N_{0.2} + 0.0075H_2O + NO_3 + 0.0025H^+ \quad (2)$$

$$1.146NO_2 + 1NH_4 + 0.071HCO_3 + 0.057H^+ \rightarrow 0.986N_2 + CH_{1.74}O_{0.31}N_{0.2} + 2.002H_2O + 0.161NO_3 \quad (3)$$

Ex situ batch test

Ex situ batch tests were conducted regularly, to identify the maximum activity of AOB, NOB and anammox bacteria. To measure the maximum anammox specific activity (SA_{AMX}^{max} ($mgNO_2$-N (gVSS d)$^{-1}$)), anoxic monomeric batch test were used, these test are described elsewhere (Lotti et al., 2012). In short, the experiments were performed in bottles with OxiTop heads. The granular biomass was washed (0.2 mm sieve) and suspended in filtered supernatant (filtered through filter paper). The pH of the medium was maintained at 7 using 25 mM HEPES (N-2-hydroxylehyl-piperazine-N0-2-ethane sulphonic acid) buffer, the pH was set using NaOH. Nitrate was added to the solution to avoid low redox values. The bottles were made anoxic by sparging with N_2 gas and incubated in a temperature controlled shaker. After stabilisation of the headspace pressure a substrate solution was injected, leading to concentrations of 25 $mgNH_4$-N L^{-1} , 25 $mgNO_2$-N L^{-1} and 21.8 $mgHCO_3$ L^{-1}. By measuring the increase of the headspace pressure (due to N_2 production), the anammox activity could be calculated. The substrate solution was injected three times and reported values are averages of the last two injections in four different bottles. Experiments were executed at 20°C and at the reactor temperature.

To measure the maximum AOB and NOB activity (SA_{AOB}^{max} ($mgNO_2$-N (gVSS d)$^{-1}$); SA_{NOB}^{max} ($mgNO_3$-N (gVSS d)$^{-1}$)), aerobic batch tests were used. In these tests mixed liquid of the reactor was used, the pH was stabilized at 7 using 25 mM HEPES buffer, the pH was set by using NaOH and temperature was controlled by placing the bottles in a temperature controlled water bath. A flow of compressed air was used to mix and aerate the sample through fine bubble aeration. At the start of the experiment, concentrated ammonium and nitrite solutions were fed to the bottles to obtain a concentration of 40 mg NH_4-N L^{-1} and 20 mg NO_2-N L^{-1}. Conversion rates were measured by measuring N-species concentrations at different time points. Ammonium, nitrite and oxygen were not limited during these experiments. Experiments were done in duplicates.

Analytical methods

Analytical measurements for inorganic N-compounds were conducted with an online grab sample of the influent and effluent, filtered samples were measured online every half an hour with a

spectrophotometric method (Applikon Biotechnology, Delft). In addition to the online measurements, 24 h collected samples (time & flow proportional) were measured with Dr. Lange test kits for N-species. Measurements for BOD and biomass concentration (gTSS L^{-1} and gVSS L^{-1}) were carried out according to standard methods (APHA, 2005).

The morphology and size distribution of the granules were monitored by image analysis, with a Lexmark Optra image analysis system.

FISH was used to analyse the distribution of the different microorganisms in the granule. The collected granules were washed in phosphate buffer and fixed using paraformaldehyde. Slicing was accomplished by embedding the granules in a tissue freezing medium (Leica Microsystems) and cut in the frozen state with a microtome-cryostat (Leica CM1900-Cryostat) into 20 µm slices. Dried slices were stored on a microscopic glass slide, and FISH was performed. Hybridisation with fluorescent labelled oligonucleotide probes and analysis of the samples were performed as described in Lotti *et al.* 2014b, a formamide concentration of 35% was used for hybridisation with the probes specific for Kuenenia-like and Brocadia-like anammox bacteria (AMX-820) (Schmid et al., 2000), AOB (mix of NEU-653, NSO-190 and NSO-1225) (Mobarry et al., 1996; Wagner et al., 1995), NOB (mix of NTSPA-0712 and NIT-1035) (Daims et al., 2000; Juretschko et al., 1998) and eubacteria (EUB-338) (Daims et al., 1999).

Genomic DNA was extracted using the Ultraclean Microbial DNA extraction kit supplied by MOBIO laboratories Inc. (CA, USA) according to the manufacturers protocol, with the exception that the bead-beating was substituted by a combination of 5 minutes heating at 65°C and 5 minutes beat-beating, to ensure maximum yields. The extracted genomic DNA was subsequently used for a two-step PCR reaction targeting the 16Sr-RNA gene of most bacteria and archaea. For this we used the primers, U515F (5' – GTGYCAGCMGCCGCGGTA - 3') and U1071R (5'- GARCTGRCGRCRRCCATGCA- 3') as used by Wang et al. (Wang and Qian, 2009). The first amplification was performed to enrich for 16s-rRNA genes. The following chemicals were used: 2x iQ™ SYBR® Green Supermix (Bio-rad, CA, USA), 500nM primers each and finally 1-50ng genomic DNA added per well to a final volume of 20µl. The protocol was: denaturation at 95°C for 5min and 20 cycles at 95°C for 30 seconds, 50°C for 40 seconds, 72°C for 40 seconds and a final extension at 72°C for 7 minutes. During the second step, 454-adapters (Roche) and MID tags at the U515F primer, were added to the products of step one. The second step was similar to step one, except that Taq PCR Master Mix (Qiagen Inc, CA, USA) was used, the program was run for 15 cycles and the template was diluted 10 times. After the second amplification, 12 PCR products were pooled equimolar and purified over an agarose gel using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, The Netherlands). The resulting library was sent for 454 sequencing and run in 1/8 lane with titanium chemistry by Macrogen Inc. (Seoul, Korea).

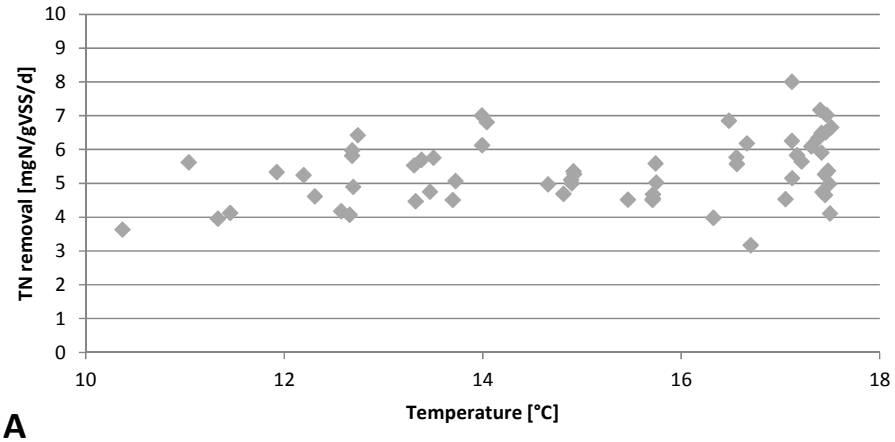
After analysis the library was imported into CLC genomics workbench v7.5.1 (CLC Bio, Aarhus, DK) and (quality, limit=0.05) trimmed to a minimum of 200bp and an average of 284bp. After trimming the sample was de-multiplexed resulting in twelve samples with an average of 7800 reads per sample. A build-it SILVA 123.1 SSURef Nr99 taxonomic database was used for BLASTn analysis on the reads under default conditions. The top result was used to determine taxonomic affiliation and species abundance.

Results

The PN/A pilot-scale reactor was operated for 3.5 years, under different influent conditions and temperatures. The operational period of the reactor is described in four periods, in which a stable process was obtained Table 2-1. During stable process operations, at summer temperatures (23.2±1.3°C) a total nitrogen removal rate of 0.223±0.029 kgN $(m^3\ d)^{-1}$ was achieved and at winter temperatures (13.4±1.1°C) the total nitrogen removal rate was 0.097±0.016 kgN $(m^3\ d)^{-1}$. The average granular size over the complete period of operation was 1.2±0.4 mm. The average nitrite concentration in the reactor was 1.1±1.0 $mgNO_2$-N L^{-1}. Graphs of loading rates, removal rates and temperature can be found in the supplemental material (supplemented material).

Effect of temperature on conversions

During periods 3 and 4 the temperature decreased to temperatures below 15°C, either due to the natural decent of the wastewater temperature (period 3) or due to forced cooling of the influent (period 4). The temperature effect on the conversion rates observed is presented in Figure 2-2. In period 3 (winter 2014-2015) the decrease in temperature had no negative effect on the conversions. In period 4 (winter 2015-2016) there was a small negative trend in conversions (due to a lower ammonium removal rate) and an increase in nitrate production at decreasing temperatures (for NO_3 concentrations, see supplemented material). It is important to note that the reactor under the influence of a protozoa bloom at this time. In period 4 the BOD/N ratio in the influent of the pilot reactor was higher compared to period 3. The fluctuations in BOD/N could not directly be related to the changes in temperature.



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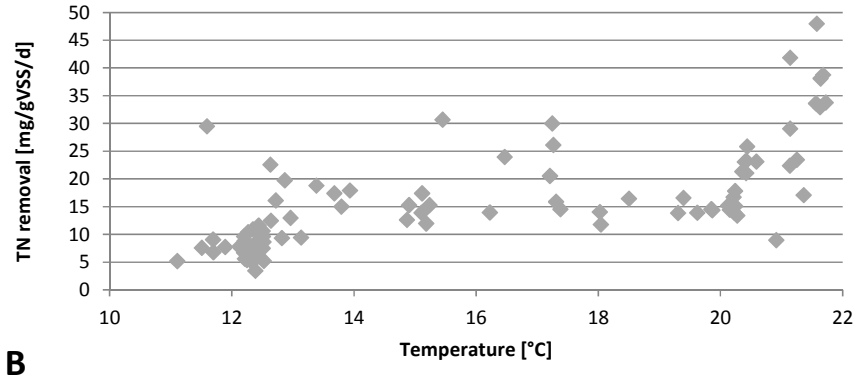

Figure 2-2: Effect of temperature on the total nitrogen removal, in A: period 3 and B: period 4. Average BON/N ratio of period 3 was 1.2±0.4 and 2.5±1.8 for period 4.

Maximal biomass capacity vs actual activity

The reactor was started up several times with sludge originating from full-scale side stream PN/A reactors containing granular biomass. The sludge was immediately active under municipal mainstream conditions and no adaptation period was needed. If the inoculation sludge did contain a significant fraction of floccular biomass, this fraction was washed out of the reactor in the first week of operation. The overcapacities of the AOB, NOB and anammox activity (Table 2-2) were estimated based on the ratio of the SA^{max} (maximal specific activity in tests outside of the reactor) and the SA (actual specific activity in the reactor). Rates can be found in the overview table in the supplemented materials. During the entire run there was an overcapacity of the anammox activity. A smaller AOB overcapacity was observed and NOB were present in the biomass, but barely active during continuous operation of the reactor. This also led to large NOB overcapacity values. The granules can be seen in Figure 2-3 and Figure 2-4.

Table 2-2: Ratio of maximum activity (in ex situ tests) and activity in reactor, values are based on biomass specific rates.

period	# days after inoculation	Anammox	AOB	NOB
1	1	4,06	6,06	89,30
2	22	2,76	1,64	21,99
3	6	16,47	3,43	$SA_{NOB}^{max} = 0$
4	3	12,52	2,39	8,49

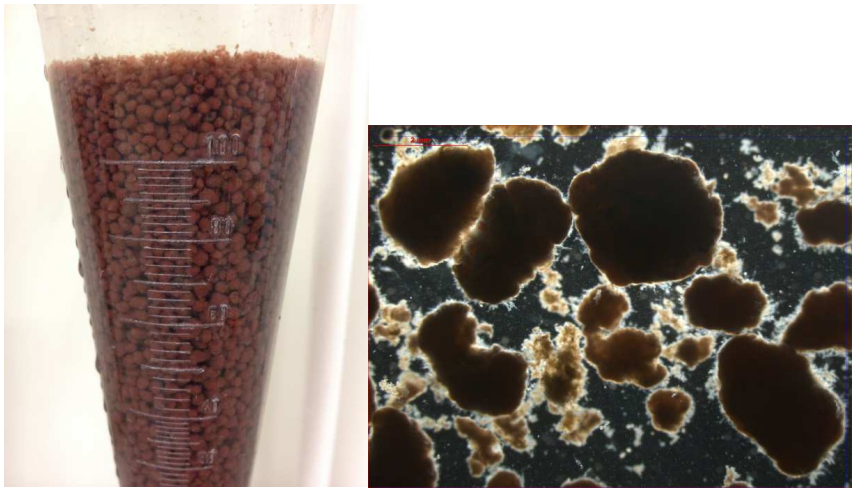

Figure 2-3: Granules in the inoculum of the PN/A reactor. Scale bar is 2 mm.

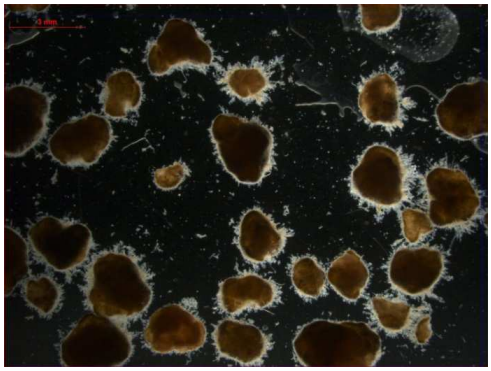

Figure 2-4: Granules after 120 days in the reactor in period 4. Scale bar is 3 mm.

For municipal mainstream application of an anammox based technology it is important that anammox bacteria are retained and remain active within the system. If the actual activity (SA_{amx} in the reactor during normal operation) was compared to the maximal activity in batch tests in the laboratory (SA_{amx}^{max}), the anammox overcapacity was fluctuating, but always present. Directly after inoculation the anammox overcapacity was highest, after prolonged cultivation overcapacity decreased, due to a decrease in SA_{amx}^{max} in time . The nitrogen removal rates of the biomass in the reactor were not limited by the anammox capacity, but limited by nitrite production since only low concentrations of nitrite were detected. In nitrification batch tests, at high oxygen concentrations (> 5mg L^{-1}), the nitrogen mass-balance closed based on ammonium, nitrite and nitrate, so there was no anammox activity at these oxygen concentrations. Based on the ex situ batch tests at different temperatures an anammox temperature dependency was estimated: an increase of 5°C (15°C - 20°C) increased the SA_{AMX}^{max} with a factor 2 and an activation energy of 96 kJ mol^{-1} (12°C – 24.5°C).

There was a large difference between the SA_{NOB} and the SA_{NOB}^{max}, this indicates that NOB were successfully supressed in the reactor (Table 2-3). In batch tests, with a surplus of oxygen, ammonium

and nitrite present, the sludge could be fully nitrifying. But in the pilot reactor, with low dissolved oxygen concentrations and anammox bacteria competing for nitrite, aerobic nitrite oxidation was minimal. The effect of DO on the suppression NOB activity in the pilot reactor is illustrated in Figure 2-5. High DO concentrations were present during a short period of time. At these high oxygen concentrations the reactor was fully nitrifying, but after lowering the DO the anammox pathway became active again.

As in the case of the anammox bacteria activity, there was always an overcapacity of AOB, although this overcapacity was fluctuating. The AOB activity was the limiting process step in the reactor, not due to maximum AOB capacity, but due to oxygen limitation.

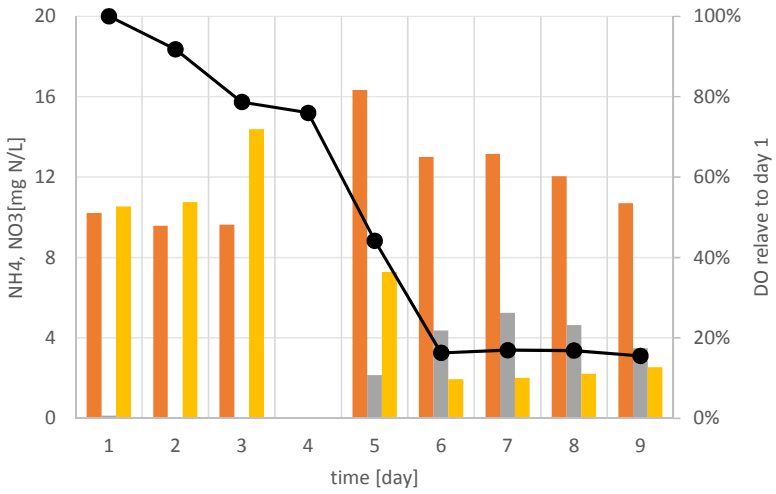

Figure 2-5 Influent and effluent of the reactor with different oxygen concentrations (data are 24h averaged values), temperature is constant at 24°C. Influent ammonium in orange bars, effluent ammonium in grey bars, effluent nitrate in yellow bar and relative DO in blue line.

Table 2-3: Average specific NOB activity for all 4 periods in the reactor and in ex situ batch test.

period	SA_{NOB} mgN (gVSS d)$^{-1}$	SA_{NOB}^{max} mgN (gVSS d)$^{-1}$
1	3.6±3.0	35.8±12.1
2	3.2±2.8	41,5±6.1
3	0.2±01	10,4±6.0
4	0.4±0.4	33,6±27.6

Long-term stability

During different reactor runs, the long-term stability was a point of concern. The longest period of stable performance that was achieved was six months. Some perturbations were caused by clear technical problems in the pilot-plant or the full-scale A-stage reactor, but there have also been periods without evident technical problems in which the nitrogen removal rate would eventually decrease. Decrease in nitrogen removal rates was in some cases due to an increase of NOB or a decrease in AOB activity. It was observed that during periods with instable A-stage performance the conversions decreased, probably due to high BOD in the incoming water leading to growth of heterotrophic bacteria and protozoa. An incidental peak load of BOD and particulates from the A-stage caused no problem for the conversions in the reactor, but long-term exposure to these conditions led to a decrease in nitrogen conversions.

Biomass composition

The microbial community within the biomass was analysed by sequencing samples at the beginning and end of period 2, the end of period 3 and the beginning and end of period 4. The observed species related to autotrophic nitrogen removal are shown in Table 2-4. The dominant anammox species identified in the reactor was always *candidatus Brocadia,* in period 3 an uncultured *Brocadia* species and in the other periods *candidatus Brocadia sinica.* In periods 2 and 4 a small fraction of uncultured *candidatus Jettenia* was present in the reactor. The inoculation sludge of periods 2 and 4 originated from the same full scale PN/A reactor, this might explain the presence of *Jettenia* and the different types of *Brocadia.* In general no change in types of anammox species was observed after inoculation. In period 3 the relative abundance of anammox bacteria was higher compared to the other two sequenced periods, but the SAA_{amx}^{max} of this sludge was relatively low, which could be due to the presence of a large fraction of inactive (anammox) biomass. The AOB and NOB species present in the sludge were uncultured *nitrosomonas* and *nitrospira* species. An overview of the other species identified in the biofilm is presented in the online supplemented material. There was no indication of the presence of a complete nitrifier (comammox bacterium) based on QPCR results (Abbas et al., in prep).

Table 2-4: Sequencing results with the relative abundance on genus level of the organisms involved in nitrogen removal.

Period	# days after inoculation	AMX_broc	AMX_jett	AOB	NOB_nitrosp
2	22	3,91%	0,70%	1,46%	0,84%
	146	1,22%	1,13%	0,49%	0,35%
3	201	8,90%	#N/A	2,10%	1,36%
4	2	3,99%	0,70%	4,02%	#N/A
	202	1,27%	#N/A	1,98%	2,70%

FISH analysis was performed on sliced granules. These results, for the sludge at the end of period 4, can be found in Figure 2-6. In figure A it can be seen that the core of the granule (left bottom) consisted of anammox bacteria, further away from the core was a layer of AOB and on the outside (right top) was a layer of bacteria that did not hybridise with the probe for AOB or anammox bacteria, these are probably heterotrophs. In a second FISH experiment (B) it became clear that the NOB were present in a deeper layer of the biofilm, compared to the AOB (core is in the left top).

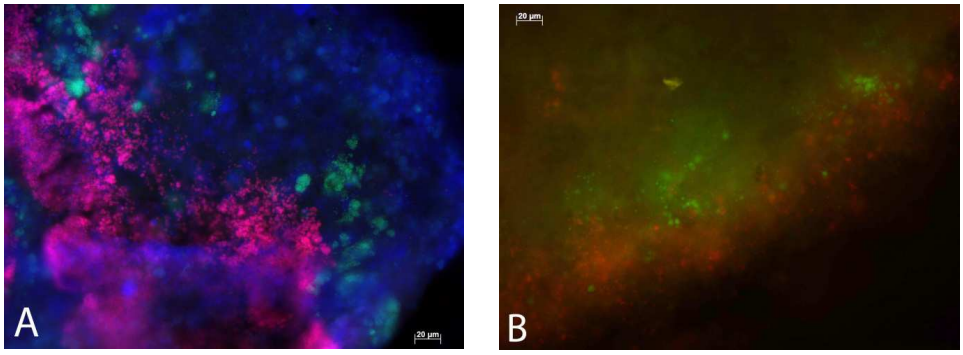

Figure 2-6: FISH analysis performed on sliced granules at the end of period 4, hybridisation was accomplished with A: CY3-red (anammox), Cy5-blue (Eubacteria) and Fluos-green (AOB) labelled probes. The abruption on the right side of the picture is the outside of the granule. B: CY3-red (AOB) and Fluos-green (NOB) labelled probes. Core of the granule is in the left top.

An important question on biomass composition is what happens to the heterotrophic biomass, did the autotrophic and heterotrophic biomass experience the same SRT? Or is there an uncoupling of the two SRTs, for instance due to the growth of heterotrophs in suspension and autotrophs in a biofilm. Based on the removal of BOD and ammonium in the reactor an estimation of the community composition of the grown biomass was made. The average BOD load over the periods described in this paper was 0.349±0.338 kgBOD $(m^3\ d)^{-1}$ and the average ammonium removal rate was 0.151±0.070 kgNH_4-N $(m^3\ d)^{-1}$. If all species in the biomass experienced the same SRT, the system would be strongly dominated by heterotrophs (>90%), as shown in Table 2-5. From the qualitative evaluation of the FISH analysis (Figure2-6) it was estimated that the fraction of autotropic biomass was around 80%, significantly higher than the theoretical 10% if the SRT of heterotrophs and autotrophs would be the same. Another indication of the uncoupling of the SRTs was the amount of biomass in the system. The calculated amount of grown heterotrophic bacteria would be 157 mgVSS $(L\ d)^{-1}$, if this was retained in the system the amount of biomass would have grown with 4.7 gVSS L^{-1} in a month, this is higher than the measured increase in the system. Therefore it can be concluded that the SRTs of the heterotrophic and autotrophic biomass was uncoupled.

Table 2-5: Biomass yields for anammox bacteria, AOB and heterotrophic bacteria and the corresponding biomass production rate, according to average substrate consumption rates (349 mgBOD $(m^3\ d)^{-1}$ and 138 mgNH_4-N $(m^3\ d)^{-1}$), the conversion factor from BOD to VSS of was 1.4 used. 55% of the converted ammonium was consumed by AOB.

	$Y_{XH/BOD}$ gBOD gBOD^{-1}	$Y_{XA/NH4}$ gBOD gNH_4-N^{-1}	$Rate_X$ mgVSS $(L\ d)^{-1}$	Community composition %	Reference
AOB		0.15	8.1	4.7	(Wiesmann, 1994)
Anammox bacteria		0.16	7.1	4.1	(Strous et al., 1998)
Heterotrophs (O_2)	0.63		157.1	91.2	(Henze et al., 2000)

Discussion

In this study a reactor design for a PN/A system with a purely granular biomass was used. During stable process operations, at summer temperatures (23.2±1.3°C) the total nitrogen removal rate was 0.223±0.029 kgN $(m^3\ d)^{-1}$ and at winter temperatures (13.4±1.1°C) the total nitrogen removal rate was 0.097±0.016 kgN $(m^3\ d)^{-1}$. Effluent polishing was not a goal of this research, therefore small amounts of ammonium (5 $mgNH_4$-N L^{-1}) were present in the effluent. The removal capacities obtained in this study are higher than conversions reported in literature (at 20°C) for a B-stage (0.210 kgN $(m^3\ d)^{-1}$, (Lotti et al., 2015b)), an aerobic granular sludge system (0.17 kgN $(m^3\ d)^{-1}$,(Pronk et al., 2015)) and a conventional activated sludge system (0.1 kgN $(m^3\ d)^{-1}$ (Metcalf and Eddy, 2003)). Indicating that a full granular PN/A system could achieve at least comparable volumetric loads to conventional systems.

The different periods with decreasing temperatures show different effects on the nitrogen removal. In period 3 the decrease in temperature, and therefore decrease in biomass specific activity, was compensated by an overcapacity of the sludge and no change in nitrate production per ammonium consumed was observed. In period 4 an increase in nitrate production was observed at decreasing temperatures.

NOB suppression

Different operational strategies to supress NOB are suggested in literature: Intermitted aeration, (Gilbert et al., 2015; Regmi et al., 2015; Wett, 2007), sequential batch operation (Laureni et al., 2016) and NH_4:DO ratio (Hao et al., 2001; Pérez et al., 2014). In the completely mixed tank reactor used in this study, the NH_4:DO was successfully used to supress the NOB activity, even if the NOB where present in the granular sludge. Biofilm simulations in general suggest that AOB tend to grow more on the outside and NOB deeper in the biofilm (Hao et al., 2002; Vannecke, 2015). This was also confirmed by experimental observations and by FISH images of granules from the pilot reactor (Matsumoto et al., 2010; Poot et al., 2016; Vlaeminck et al., 2010). This means that NOB will indeed suffer more from oxygen limitation then AOB and can therefore be effectively suppressed by oxygen limitation. Suppression of NOB activity is essential for proper process control, strategies relying solely on population changes will have a delay in control actions of several days/weeks making them less practical.

Ammonium conversion

The nitrogen removal rates presented in this study are limited by the AOB activity, due to the limits of oxygen mass transfer. Under stable conditions the AOB conversions in the reactor are lower than the maximal AOB capacity, due to oxygen limitation. In biofilm systems the concentration of the different substrates needs to be balanced (Hao et al., 2001). Increasing the DO could lead to less anoxic volume in the granules and consequently partial inhibition of anammox bacteria. For a good conversions the nitrite produced in the outer layers of biofilms/granules needs to be converted in the anammox core. The oxygen concentration determines the depth of the aerobic AOB activity layer and the remainder of the biofilm/granule volume is available for anammox conversion. This ensures that dissolved oxygen concentration, biofilm/granule thickness and ammonium loading rates are strongly coupled. An increase in biomass (more granules or a thicker layer of AOB) could increase the AOB conversions.

The average SA_{amx}^{max} (20°C) during the described reactor run was 70 mgN $(gVSS*d)^{-1}$. Other values reported in literature are diverse: SA_{amx}^{max} 68.8 mgN $(gVSS*d)^{-1}$ (20°C enriched anammox reactor) (Hoekstra et al., 2017), SA_{amx} 600 mgN $(gVSS*d)^{-1}$ (20°C enriched anammox reactor) (Hendrickx et al., 2012), SA_{amx}^{max} 50 mgN $(gVSS*d)^{-1}$ (15°C PN/A reactor) (Laureni et al., 2016) and SA_{amx}^{max} 13.5 mgN $(gTSS*d)^{-1}$ (20°C PN/A reactor) (Gilbert et al., 2014). The general conclusion is, that the order of magnitude of the anammox activity that can be maintained in this system is in a similar range as the reported values in literature.

Reactor design

There are different methods for implementing PN/A technology in the mainstream of a municipal wastewater treatment plant (Cao et al., 2017; Fernández et al., 2016). The reactor described in this paper was operated as a completely mixed tank under continuous gas mixing. This design was selected for its simplicity, but there is a limitation for optimization of the effluent quality. In a full-scale implementation a plug flow set-up or SBR (sequential batch reactor) would be chosen, in which optimization for effluent quality is possible while achieving adequate repression of NOB activity by inclusion of a gradient in DO in the plug flow bioreactor.

Due to the low substrate concentration in the influent of the mainstream PN/A reactor, and potential high conversion rates, the HRT of this (kind of) system will always be short (few hours). Under the low temperatures that mainstream WWTPs operate under during winter, the SRT needed to retain the autotrophic biomass in the reactor will be long. For a reactor experiencing yearly temperature fluctuations like the Dokhaven plant, a SRT of 100 days is needed to obtain an average of 80% nitrogen removal (chapter 3, this thesis). Combining these two restrictions will lead to a small HRT:SRT ratio, which will result in the need of an efficient autotrophic biomass retention, while obtaining preferential wash-out of the inevitably formed heterotrophic biomass.

One of the main challenges will always be separating granules from the effluent. Several options have been proposed in literature, like cyclones or tilted plate clarifiers. Their practical operation aspects at a large scale are still to be evaluated. This system for biomass (as biofilm) retention will be integrated in the PN/A reactor. The existing/secondary clarifiers will be used for the removal of heterotrophic floccular biomass.

On of the risks using granular sludge is trapping of the granules in sludge flocs, that leave these high-rate separation systems together with the effluent. Especially when a preceding A-stage gets disturbed, a significant amount of sludge can wash out of the intermediate clarifier in the PN/A stage, leading to floc/granule separation problems. Inadequate retention of granular biomass results in a decrease of biofilm surface area, limiting the conversions required. This problem can be avoided when mobile or fixed carriers are used instead of granular biomass, since they are easier to retain in the reactor. The disadvantage of carrier material is the limitation of surface, therefore higher DO concentrations (energy use) or bigger reactors, compared to the granular system, will be needed.

Impact of BOD

Independent of the reactor design chosen and the optimized process control, the PN/A system will always be dependent on the treatment performance of the BOD removing step. In combination with a good BOD removing step before the PN/A process, this technology can significantly improve the wastewater treatment plant (Bozkurt et al., 2016; Jetten et al., 1997; Verstraete and Vlaeminck, 2011).

During the operation of the reactor at the Dokhaven treatment plant, it was observed that the conversion rates of the PN/A reactor decreased when the A-stage was instable. The incoming BOD was most likely consumed by heterotrophs. This layer of heterotrophs could have multiple negative effects. Firstly, the heterotrophic layer could act as an additional diffusion layer that increases the external mass transfer (Nogueira et al., 2015). Secondly, the heterotrophic growth could consume so much oxygen that the AOB becomes oxygen limited. And finally, it is possible that the heterotrophic layer changes the stratification in the biofilm and will therefore make the suppression of NOB more difficult. Particulates in the influent of the PN/A reactor caused protozoa blooms in the sludge. The heterotrophic biomass and protozoa were observed as a fluffy growth on the outside of the granules.

A-stage instability was often correlated with rain weather conditions leading to large hydraulic loading of the A-stage. Uncoupling rainwater from sewage water within the sewage system, will therefore simplify the implementation of the PN/A technology for domestic wastewater treatment. In a plug flow reactor design, the negative impact of BOD in the influent on the PN/A process can be minimized. When the BOD to ammonium ratio is elevated, high aeration levels in the first part of the reactor could be introduced, oxidizing all the BOD quickly, to avoid oxygen competition between heterotrophs and AOB.

Based on the possible energy gain as the main driver for implementing PN/A technology, we strongly suggest that implementation of the PN/A technology to a wastewater treatment plant is combined with the optimization of the BOD removing step, prior to the PN/A reactor. The vulnerable floc structure in the A-stage might lead to limitations in A-stage optimization (De Graaff et al., 2016). And in addition, the decrease in temperature of the sewage might lead to a decrease in activity of the A-stage, which will lead to an increase of incoming BOD for the PN/A system during winter (Smitshuijzen et al., 2016). Possibilities are the use of membranes, sieves or sand filters. The use of a membrane for biomass separation in the A-stage might lead to problems with clogging and fouling at municipal mainstream wastewater temperatures (Laurinonyte et al., 2017). A combination of two techniques might be a solution, for instance an A-stage with a settler and subsequently a sand filter.

The FISH and sequencing data show different results in the abundance of autotrophic nitrogen converting organisms. Estimations based on FISH results indicate that 80% of the biomass was composed of AOB, NOB or anammox bacteria. In the sequencing results less than 15% of the counts is related to nitrogen conversions. This difference is probably caused by limitations in DNA extraction from the biofilm.

Outlook

We propose that the next step in the development of a municipal mainstream PN/A process focusses on the long-term stability. In the research described in this publication, the stability in the long-term is insufficient. The cause of the decrease of the nitrogen removal rate cannot always be identified. It is possible that it is related to the competition between AOB, NOB and anammox bacteria, but also the bloom of heterotrophic bacteria and protozoa, may have played an important role.

The PN/A technology will not be implemented in the wastewater treatment plant of Dokhaven immediately, the current B-stage with the recycling for denitrification is sufficient to achieve effluent standards of 20 mg N L^{-1}. Future research will focus on the resilience of the system and a proof of principle regarding the effluent quality, based on a different reactor design. If the Dokhaven

treatment plant will be required to generate a better effluent quality in the future, the (partial) adaptation of the B-stage to PN/A reactor is a viable and good option.

Conclusion

During stable process operations, high nitrogen removal rates were obtained. Suppression of NOB activity was successfully achieved at the complete temperature range of municipal mainstream wastewater, based on DO concentration. Overcapacity of AMX was always present. The AOB activity was the limiting process step. Long term stability will be a point for future focus, especially the stability of the BOD removing step preceding the PN/A reactor. The incoming BOD in the reactor will lead to growth of heterotrophic bacteria and protozoa.

Supplemented material

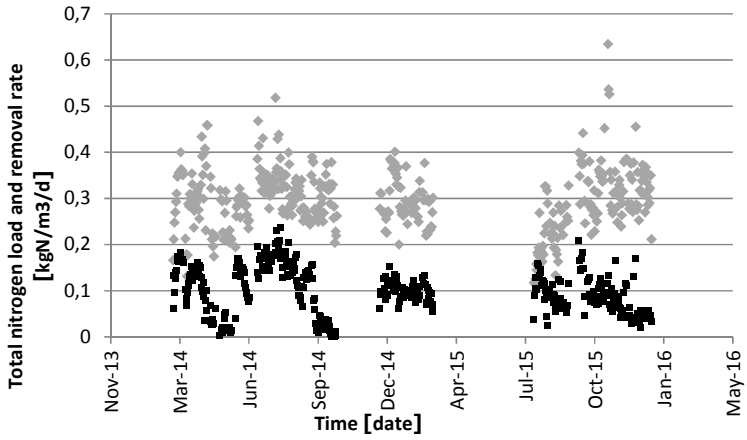

Figure 2-7: Total nitrogen loading and removal rates in the complete period described in this paper. In grey the loading rate and in black the removal rate.

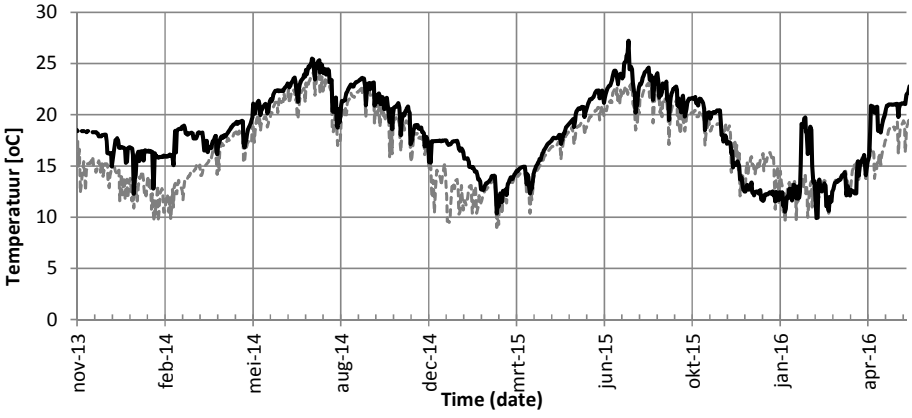

Figure 2-8: Temperature profile of the influent of the reactor (black) and the B-stage in Dokhaven (gray).

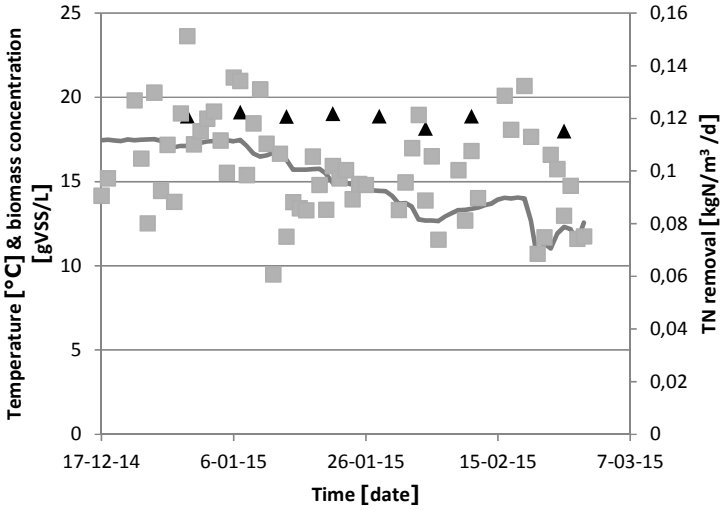

Figure 2-9: Period 3 (10/12/14) temperature (line), biomass concentration (black triangles) and the total nitrogen removal grey squares).

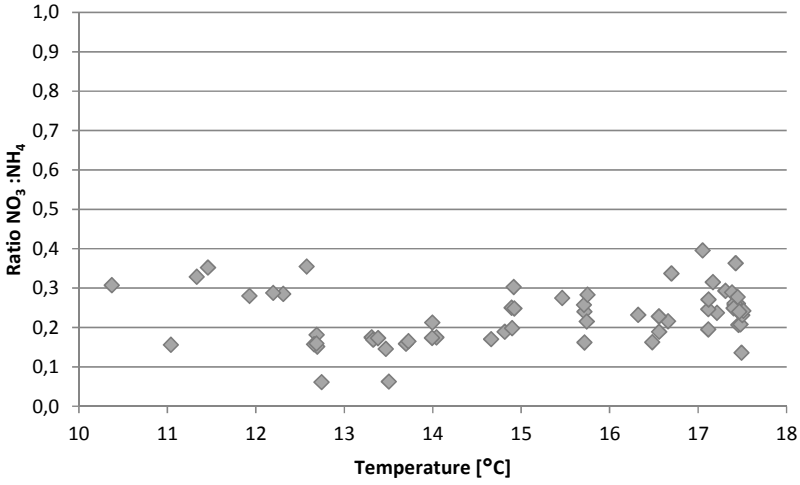

Figure 2-10: Period 3 (10/12/14), ratio of NO_3-N production over the NH_4-N consumption at different temperatures.

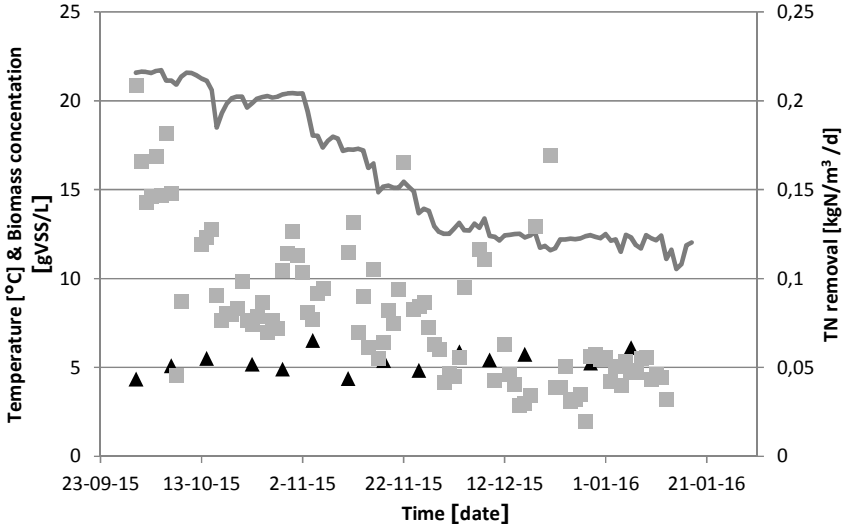

Figure 2-11: Period 4 (21/7/15) temperature (line), biomass concentration (black triangles) and the total nitrogen removal (grey squares).

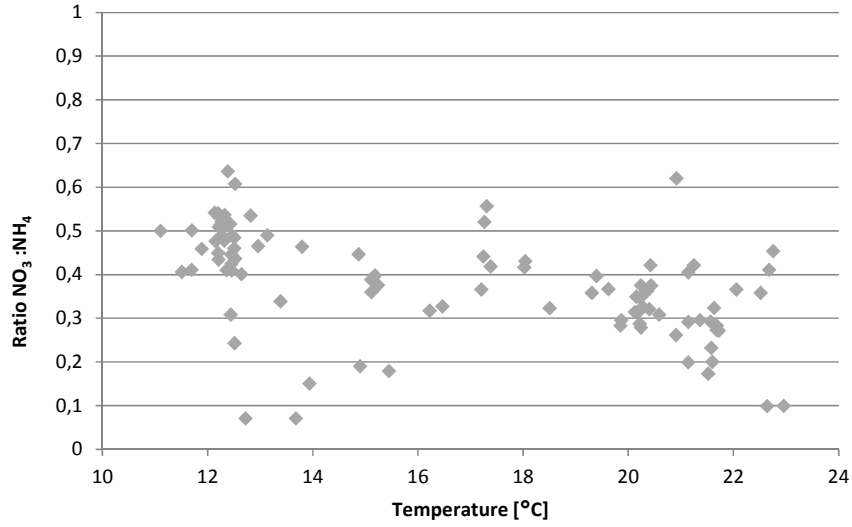

Figure 2-12 Period 4 (21/7/15), ratio of NO_3-N production over the NH_4-N consumption at different temperatures.

Table 2.6: overview table SA values are in mgN/gVSS/d.

Datum	Temp [°C]	SA_AOB	SA_AMX	SA_NOB	SA_AOB_max	SA_AMX_max_pi	SA_NOB_max	SA_AMX_max_2
19-2-2014	Period 1							
20-2-2014	18	4.59	6.93	1.12	55.22±12.85	36.71±5.41	24.27±11.35	83.71±4.55
10-3-2014	18	16.57	21.33	8.32	38.32±5.55	80.19±1.33	34.56±1.80	102.05±1.84
26-3-2014	16.5	48.27	59.94	14.90	83.83±3.82		56.00±8.22	
8-4-2014	16	27.69	39.51	4.93	57.25±0.16	60.47±5.74	34.32±4.76	123.13±2.90
6-5-2014	19	24.41	25.62	9.33	-4.48±12.07	40.61±1.49	29.73±15.45	53.74±1.05
20-5-2014	Period 2							
11-6-2014	20	31.98	43.65	1.91	52.33±6.54	120.51±3.69	42.01±8.05	120.51±3.69
7-7-2014	23	56.93	78.07	3.79	66.72±19.42	187.83±4.02	35.60±6.53	148.83±0.00
12-8-2014	24.5	85.54	127.16	6.76	71.81±0.65	246.64±7.46	49.83±26.36	139.68±3.92
13-10-2014	21.4	11.49	3.70	0.17	39.79±34.71	105.79±31.97	38.60±1.54	105.79±31.97
10-12-2014	Period 3							
16-12-2014	17	2.07	3.19	0.18	7.11±1.17	52.51±1.63		89.92±1.54
12-1-2015	17	2.81	3.44	0.13	22.41±8.81	53.42±3.11	9.64±1.72	58.27±4.50
2-2-2015	13.5	3.59	6.54	0.43	20.09±0.69	10.84±0.13	10.65±0.39	30.99±0.32
12-2-2015	13.5	3.24	5.27	0.31		8.86±0.18		
2-3-2015	12	4.02	4.92	0.18	11.06±1.99	10.71±2.65	7.49±0.74	25.35±0.22
21-7-2015	Period 4							
23-7-2015	23	13.37	13.72	0.79	31.93±2.04	171.77±8.51	6.71±0.31	144.72±5.43
31-8-2015	22.5	10.53	0.00	0.03	16.64±4.37	56.14±0.96	4.30±1.36	47.46±1.01
24-9-2015	20	13.76	5.55	0.11	41.66±9.63	45.00±0.54	45.09±0.83	45.00±0.54
22-10-2015	20	13.92	14.98	0.91	22.38±7.70	33.40±0.69	34.03±5.10	33,40±0.69
3-12-2015	12.5	10.39	9.50	0.51	33.23±14.03	10.63±0.24	79.28±12.93	55.49±4.79

Table 2-7: Results of the sequence data on the genus level. The first 20 results are presented, with genus counts and genus name.

period 2 day 22		period 2 day 146		period 3 day 201	
438	undetermined Genus from Anaerolineaceae	316	Thermomonas	608	undetermined Genus from Anaerolineaceae
425	undetermined Genus from SJA-28	293	Flavobacterium	470	Candidatus Brocadia
219	Candidatus Brocadia	279	Acidovorax	438	PHOS-HE36
213	Flavobacterium	250	undetermined Genus from SJA-28	262	Flavobacterium
173	Novosphingobium	214	undetermined Genus from Comamonadaceae	172	undetermined Genus from Ardenticatenia
153	undetermined Genus from Saprospiraceae	179	Cloacibacterium	139	undetermined Genus from Saprospiraceae
141	Denitratisoma	172	Brevundimonas	128	Limnobacter
137	Limnobacter	159	undetermined Genus from Sphingomonadaceae	111	Nitrosomonas
137	undetermined Genus from SC-I-84	159	Dechloromonas	97	undetermined Genus from PHOS-HE51
122	undetermined Genus from Mitochondria	133	Denitratisoma	90	Parafilimonas
117	Ferruginibacter	131	undetermined Genus from ST-12K33	84	Denitratisoma
112	undetermined Genus from Comamonadaceae	128	undetermined Genus from Anaerolineaceae	82	Pseudoxanthomonas
105	undetermined Genus from Sphingomonadaceae	113	Pseudomonas	80	undetermined Genus from Neisseriaceae
100	undetermined Genus from Ardenticatenia	111	Phenylobacterium	72	Nitrospira
98	Arenimonas	105	Thauera	72	undetermined Genus from Sphingomonadaceae
95	Dokdonella	93	undetermined Genus from SC-I-84	71	Novosphingobium
94	Terrimonas	80	Candidatus Brocadia	63	Taibaiella
83	undetermined Genus from Fimbriimonadales	79	undetermined Genus from Rhodocyclaceae	61	undetermined Genus from Comamonadaceae
82	Nitrosomonas	78	Rhodobacter	58	undetermined Genus from Fimbriimonadaceae
73	Taibaiella	74	Candidatus Jettenia	58	Rhodobacter
71	Pseudoxanthomonas	66	Simplicispira	58	Hydrogenophaga

period 4 day 2		period 4 day 201	
1425	undetermined Genus from Saprospiraceae	469	Flavobacterium
689	undetermined Genus from SJA-28	324	undetermined Genus from Saprospiraceae
340	undetermined Genus from Anaerolineaceae	199	undetermined Genus from Anaerolineaceae
248	Nitrosomonas	157	Nitrospira
246	Candidatus Brocadia	157	undetermined Genus from Comamonadaceae
155	Denitratisoma	124	Acidovorax
132	undetermined Genus from Comamonadaceae	123	SJA-28
113	Acidovorax	121	Aquimonas
112	Nannocystis	115	Nitrosomonas
108	undetermined Genus from NS9 marine group	111	Terrimonas
98	undetermined Genus from OPB56	98	Novosphingobium
94	Dokdonella	93	Aquabacterium
88	undetermined Genus from BRC1	92	Hydrogenophaga
86	undetermined Genus from Sphingobacteriales	90	undetermined Genus from Sphingomonadaceae
76	undetermined Genus from Blastocatellaceae (Subgroup 4)	77	undetermined Genus from Mitochondria
75	undetermined Genus from Nitrosomonadaceae	75	undetermined Genus from Subgroup 6
73	undetermined Genus from Xanthomonadaceae	75	Variovorax
57	Limnobacter	74	Candidatus Brocadia
55	undetermined Genus from Cytophagaceae	72	Rhodobacter
55	undetermined Genus from Ardenticatenia	69	undetermined Genus from SC-I-84
54	undetermined Genus from Xanthomonadales	69	Dokdonella

3

Defining the operational window for mainstream wastewater treatment using anammox, a modelling study

Abstract

The aim of this study was to define the operational window for mainstream application of the Partial Nitritation/Anammox technology (PN/A technology), for sewage treatment using computational modelling. Two main factors of the influent water were identified, that determine the effectiveness of this technology; the temperature of the wastewater and the concentration of incoming COD (chemical oxygen demand). The temperature in mainstream wastewater will be fluctuating and cannot be altered, therefore it is good to know what limitations in conversions the fluctuating temperatures yield. The concentration of incoming COD could be influenced, by optimizing the COD removing step prior to the PN/A reactor.

The results in this theoretical study highlight that long SRT's will be needed to cultivate autotrophic biomass under municipal mainstream wastewater conditions. Due to the long SRT (50-130d) requirement, even low COD concentrations in the influent of the PN/A reactor, would lead to a large fraction of heterotrophic biomass and inert solids in the total particulate concentration. In turn, extremely high biomass concentrations (>20 gVSS L^{-1}) would be required. Only when effective uncoupling of the SRT of autotrophs and heterotrophs could be achieved, moderate biomass concentrations are adequate to achieve a good treatment performance.

In preparation for publication

Hoekstra M., T. Lotti, R. Kleerebezem, J. Pérez, M.C.M. van Loosdrecht; Defining the operational window for mainstream wastewater treatment using anammox, a modelling study

Introduction

In wastewater treatment plants (WWTP's) nitrogen is currently removed in two sequential microbial conversions: nitrification and denitrification. For the nitrification step oxygen is needed and for the denitrification step anoxic conditions and COD are required. An alternative process for nitrogen removal is the Partial Nitritation/Anammox technology (PN/A) which exclusively depends on autotrophic microorganisms and is therefore also referred as autotrophic nitrogen removal (Kartal et al., 2010). In the PN/A process the incomplete oxidation of ammonium to nitrite (by aerobic ammonium oxidising bacteria), is combined with the anaerobic ammonium oxidation (by anammox bacteria).

The PN/A technology is currently implemented in side stream process flows at municipal wastewater treatment plants and in industrial wastewater treatment. These flows have usually temperatures above 20°C and ammonium concentrations over 0.5 gNH_4-N L^{-1} (Lackner et al., 2014; Morales et al., 2015; van der Star et al., 2007). Under these conditions the energy savings potential and stable operation have been well proven. The PN/A technology can also be used to optimize the municipal mainstream wastewater treatment technology, eliminating the need for COD to remove nitrogen through denitrification and the complete nitrogen removal can take place in one reactor (Bozkurt et al., 2016).

Considering that the PN/A process in the mainstream of a wastewater treatment plant would take place after a COD removing step, the COD removal efficiency attained in the first step will surely influence the performance and growth of heterotrophic bacteria of the following PN/A reactor. Heterotrophic growth could easily overgrow biofilm systems and minimises the fraction of autotrophic biomass in reactor systems, leading to very low volumetric conversion rates (Hao and van Loosdrecht, 2004). The organic carbon removed in the first process step could be used to generate biogas. Either the COD is concentrated and obtained sludge is digested to biogas or direct anaerobic wastewater treatment could be applied (De Graaff et al., 2016). The application of PN/A in the mainstream of a municipal wastewater treatment plant will be a step forward in obtaining an energy neutral wastewater treatment plant (Kartal et al., 2010).

An approach to combine the partial nitritation and anammox processes is through application of a biofilm process, with the bacteria growing as granular sludge or on a carrier. In the outer layer of the biofilm, aerobic ammonia oxidizing bacteria (AOB) are oxidising ammonium to nitrite, and in the anoxic deeper layers, anammox bacteria convert ammonium with nitrite to dinitrogen gas (Hao et al., 2001; Kartal et al., 2010; Rosenwinkel and Cornelius, 2005; van der Star et al., 2007). The use of a biofilm system has the main advantage, that high biomass concentrations can be reached. Other proposed possibilities to implement this technology are: a hybrid system with anammox bacteria in a biofilm and AOB in flocs in suspension or a two stage system with the nitritation and anammox conversions occurring in different biomasses in separate reactors (Cao et al., 2017).

Empirical studies on the applicability of anammox technology in mainstream wastewater treatment were performed in laboratory as well as in pilot scale studies (De Clippeleir et al., 2013; Laureni et al., 2016; Lotti et al., 2014a; Malovanyy et al., 2015; Xu et al., 2016). The results indicated that one of the main challenges of implementing the technology will be the low water temperatures during winter and the COD concentrations in the influent (Cao et al., 2017; Fernández et al., 2016). Due to the low temperatures the growth rate of the biomass will be low and good biomass retention will be needed.

Recent studies indicate that anammox bacteria can have a higher growth rate than previously assumed (Lotti et al., 2015a; Zhang et al., 2017), certainly at higher temperatures, which will lead to the possibility to implement the technology in non-biofilm systems (Cao et al., 2015). To the extent of our knowledge, there are no reports that this can be extrapolated to low temperature regimes (Hoekstra et al., 2017).

Identification of the boundary conditions, that need to be fulfilled for effective implementation of the PN/A process in the mainstream of a wastewater treatment plant is needed, in order to determine the minimum COD removal efficiencies and SRT that should be obtained. To define this operational window for an anammox based process receiving influent with different amounts of COD, a model was created and applied in this study.

A mathematical model describing heterotrophic growth, nitritation and anaerobic ammonium oxidation (anammox) combined in a CSTR was developed. The biological processes considered were described based on the activated sludge model n.1 platform (ASM1) (Henze et al., 2000). Transport limitations due to biofilm formation were not considered in this model, as reflected in the apparent affinity constant values used in the simulation (Characklis and Marshal, 1990; Picioreanu et al., 2016). Biomass retention by e.g. settling was not mechanistically described, but imposed on the system as a specific SRT-value. In the biofilm nitrite oxidising bacteria (NOB) might compete with the AOB for oxygen and with the anammox bacteria for nitrite. The objective of the model was not to investigate competition between AOB, NOB and anammox bacteria, it was to define the engineering limits of the process, assuming that this competition could be adequately controlled. In multiple publications the successful suppression of NOB was described (Gilbert et al., 2015; Laureni et al., 2016; Wett et al., 2013).

The aim of the present study was to delineate the boundaries of operational conditions (i.e. SRT, influent COD) for the implementation of the PN/A technology at different temperatures. These sets of engineering parameters and influent wastewater characteristics represent the “operational window” in which the PN/A process is likely to be successfully applied at mainstream conditions. The SRT could be controlled by biomass retention and the amount of incoming COD could be influenced by optimizing the A-stage. The temperature of the wastewater was a parameter that cannot be regulated. The B-stage of the WWTP of Rotterdam-Dokhaven, the Netherlands was used as case study. The reactor flow, flow characteristics and wastewater compositions were modelled according to Dokhaven plant.

Material and Methods

Model concept

The mathematical model used in this study describes heterotrophic growth, nitritation and anaerobic ammonium oxidation (anammox) combined in a CSTR. The biological processes considered were described based on the activated sludge model n.1 platform (ASM1,(Henze et al., 2000)) with two main modifications: (i) The nitrification process was replaced by nitritation (by AOB), full suppression of NOB was assumed; (ii) inclusion of the anammox process. In order to obtain the desired goal, the nitratation process was considered as outcompeted and therefore NOB activity was not included. Mass transfer limitations were not considered in the model resulting in a fully aerobic reactor and thus the anammox process was simulated without considering oxygen inhibition.

It was assumed that the biomass retention system used (e.g. common settler), was acting the same way on every particulate compound. In other words, every particulate compounds had the same SRT. In the second stage of the research, two different SRTs were defined for different groups of particulates.

The kinetic parameters used were taken from the ASM1 (Henze et al., 2000) or literature as stated in Table 3-1. The temperature dependency of the growth rates of the microorganisms involved is described by an Arrhenius-type equation. The slow growth rate of microorganisms at low temperatures makes experimentation very time consuming, this is why a modelling study could be of interest. Model simulations were conducted in AQUASIM (Reichert, 1994).

Model input

The Dokhaven treatment plant (Rotterdam, The Netherlands) was used as a case study, since implementing mainstream anammox in this plant is a plausible option (Lotti et al., 2015b). In the scenario of retrofitting the plant with the implementation of the PN/A process for nitrogen removal in place of the actual B-stage. The volume of the reactor used in the simulations was the same of the actual B-stage (11290 m^3), while the yearly average flow entering the B-stage (reduced of the flow actually recycled to the pre-denitrification unit ahead the A-stage) was considered as the wastewater inflow (200000 m^3/d). Assuming that in this scenario all soluble nitrogen present in the municipal wastewater would pass unaffected the A-stage (i.e. nitrogen assimilation for biomass growth in A-stage is neglected) entering the PN/A reactor, the influent ammonium concentration used for simulations (17 mg N L^{-1}) was calculated as the yearly average nitrogen load (3400 kg N d^{-1}) divided by the influent flow. The COD characteristics in terms of *readily biodegradable COD* (*rbCOD*, S_S), *slowly biodegradable COD* (*sbCOD*, X_S) and *inert COD* (*inertCOD*, X_I) fractions were input according to Dokhaven's data and model according to ASM1. All input variables are presented in Table 3-1.

The different scenarios simulated at constant temperature were characterized by different SRTs and different total COD concentrations in the influent. Every scenario was evaluated at different operational temperatures: 10, 15 and 20°C. Initially the SRT was considered equal for each type of particulate component in the system such as the different bacterial populations (X_{AOB} , X_{AMX} , X_{het}) and particulate COD fractions (X_S , X_I).

When granular or hybrid technology is applied, heterotrophic bacteria are expected to grow mainly in the bulk suspension as flocculent sludge due to their high specific growth rate and substrate limitation in the biofilm and their presence in the influent wastewater (suspended solids load coming from A-stage effluent) (Laureni et al., 2016; Lotti et al., 2015b). This results in uncoupling of the SRT for heterotrophic biomass and for the other particulate components (SRT_H) from the autotrophic biomass (SRT_{AUT}). In the simulations with different SRT-values for heterotrophic and autotrophic biomass, SRT_H was imposed as 50 times the HRT. This value is based on the average SRT of the current flocculent sludge in the B-stage in Dokhaven.

To mimic the conditions in a real WWTP, the effect of temperature fluctuations was studied in addition to the constant temperature model. The yearly fluctuations were based on an average temperature pattern of the influent wastewater of Dokhaven 2008-2012. The starting point of the fluctuation model was the biomass composition at 10°C with an SRT of 130 d. Five years of

fluctuations were run, to obtain a stable response. In this study the last year of these five years is presented.

Table 3-1: Parameters used for the simulations (*, different defined values were used).

Parameter (units)	Value
Volume B-stage (m^3)	11290
Solids Retention Time for X_{HET}, X_S and X_I (SRT_H, d)	Variable*
Solids Retention Time for X_{AOB}, X_{AMX} (SRT_{AUT}, d)	Variable*
Hydraulic retention time (h)	1.35
Dissolved oxygen (gO_2/m^3)	1
Temperature (T, °C)	10, 15, 20, 30 and a yearly profile
Influent ($Q_{in,}$ m^3/d)	200000
Ammonium concentration inflow (gN/m^3)	17
readily biodegradable COD concentration (rbCOD, S_S) inflow ($gCOD/m^3$)	10*
readily hydrolysable COD concentration (or slowly biodegradable COD, sbCOD, X_S) inflow ($gCOD/m^3$)	30*
inert COD concentration (inertCOD, X_I) inflow ($gCOD/m^3$)	3*

In the Table 3-2, Table 3-3 and Table 3-5 the stoichiometry of the used reactions is described together with the kinetic and stoichiometric parameters. The stoichiometric matrix was based on the matrix used by Hao *et al.* 2001. The nitrate formation from nitrite in the anammox reaction resulted in a theoretical yield of 14 gNO_3-N per 16 $gCOD_{biomass}$ and the use of ammonium as N-source for biomass (Hao et al., 2001).

Table 3-2. Stoichiometric Matrix i_{NBM}: N content in X_{AOB}, X_{HET} and X_{AMX}, i_{NXI}: N content in X_I, f_{XI}: Fraction of biomass leading to X_I

Process	O_2	NH_4^+	NO_2^-	NO_3^-	S_S	AOB	HET	AMX	X_I	X_S
Growth of X_{AOB}	$1-3.43/Y_{AOB}$	$-1/Y_{AOB}-i_{NBM}$	$1/Y_{AOB}$			1				
Decay of X_{AOB}		$i_{NBM}-i_{NXI}\cdot f_{XI}$				-1			f_{XI}	$1-f_{XI}$
Growth of X_{HET}	$1-1/Y_{HET}$	$-i_{NBM}$			$-1/Y_{HET}$		1			
Decay of X_{HET}		$i_{NBM}-i_{NXI}\cdot f_{XI}$					-1		f_{XI}	$1-f_{XI}$
Growth of X_{AMX}		$-1/Y_{AMX}$	$-1.225/Y_{AMX}$	$0.21/Y_{AMX}$				1		
Decay of X_{AMX}		$i_{NBM}-i_{NXI}\cdot f_{XI}$			$1-f_{XI}$			-1	f_{XI}	
Hydrolysis					1					-1
Units	g O_2 m^{-3}	g N m^{-3}	g N m^{-3}	g N m^{-3}	g COD m^{-3}	g COD m^{-3}	g COD m^{-3}	g COD m^{-3}	g COD m^{-3}	g COD m^{-3}

Table 3-3: Kinetic rate expressions, based on the matrix used by Hao *et al.* 2001.

Process	Process rate (d^{-1})
Growth of X_{AOB}	$\mu_{\max,AOB} \cdot \frac{S_{O_2}}{K_{O_2,AOB} + S_{O_2}} \cdot \frac{S_{NH_4^+}}{K_{S,NH_4^+,AOB} + S_{NH_4^+}} \cdot X_{AOB}$
Decay of X_{AOB}	$b_{AOB} \cdot X_{AOB}$
Growth of X_{HET}	$\mu_{\max,HET} \cdot \frac{S_{O_2}}{K_{O_2,HET} + S_{O_2}} \cdot \frac{S_S}{K_{S,S,HET} + S_S} \cdot X_{HET}$
Decay of X_{HET}	$b_{HET} \cdot X_{HET}$
Growth of X_{AMX}	$\mu_{\max,AMX} \cdot \frac{S_{NH_4^+}}{K_{S,NH_4^+,AMX} + S_{NH_4^+}} \cdot \frac{S_{NO_2^-}}{K_{S,NO_2^-,AMX} + S_{NO_2^-}} \cdot X_{AMX}$
Decay of X_{AMX}	$b_{AMX} \cdot X_{AMX}$
Hydrolysis	$k_h \cdot \frac{X_S / X_{HET}}{K_X + X_S / X_{HET}} \cdot \left(\frac{S_{O_2}}{K_{O_2,HET} + S_{O_2}} + \eta_H \frac{K_{O_2,HET}}{K_{O_2,HET} + S_{O_2}} \right) \cdot X_{HET}$

Table 3-4: Kinetic parameters (20 ºC, if not stated otherwise).

Symbol	Definition	Value	Unit	References
$\mu_{max,AOB}$	Maximum specific growth rate	0.8	d^{-1}	(Hao et al., 2001)
b_{AOB}	Decay rate	0.05	d^{-1}	(Hao et al., 2001)
Y_{AOB}	Growth yield	0.15	g COD g^{-1} N	(Wiesmann, 1994)
$K_{O2,AOB}$	Half-saturation coefficient for oxygen	0.3	mg O_2 L^{-1}	(Wiesmann, 1994)
$K_{S,NH4+}$	Half-saturation coefficient for ammonium	2.4	mg N L^{-1}	(Wiesmann, 1994)
$\mu_{max,HET}$	Maximum specific growth rate	6.0	d^{-1}	(Henze et al., 2000)
b_{HET}	Decay rate	0.62	d^{-1}	(Henze et al., 2000)
Y_{HET}	Growth yield	0.67	g COD g^{-1} N	(Henze et al., 2000)
$K_{O2,HET}$	Half-saturation coefficient for oxygen	0.2	mg O_2 L^{-1}	(Henze et al., 2000)
$K_{S,S}$	Half-saturation coefficient for COD	20	mg N L^{-1}	(Henze et al., 2000)
$\mu_{max,AMX}$	Maximum specific growth rate 20°C	0.028	d^{-1}	(Hao et al., 2002)
$\mu_{max,AMX}$	Maximum specific growth rate 30°C	0.33	d^{-1}	(Lotti et al., 2015a)
b_{AMX}	Decay rate	0.004	d^{-1}	(Hao et al., 2002)
Y_{AMX}	Growth yield	0.17	g COD g^{-1} N	(Lotti et al., 2014b)
$K_{S,NH4+}$	Half-saturation coefficient for ammonium	0.07	mg N L^{-1}	(Strous et al., 1998)
$K_{S,NO2-}$	Half-saturation coefficient for nitrite	0.5	mg N L^{-1}	assumed
k_{Hyd}	Maximum specific hydrolysis rate	3	d^{-1}	(Henze et al., 2000)
K_X	Half-saturation coefficient for hydrolysis	0.03	gCOD g^{-1}	(Henze et al., 2000)
η_H	Correction factor for hydrolysis under	0.4	dimensionless	(Henze et al., 2000)
i_{NBM}	N content in X_{AOB}, X_{HET} and X_{AMX}	0.07	g N g^{-1} COD	(Henze et al., 2000; Strous et
i_{NXI}	N content in X_I	0.02	g N g^{-1} COD	(Henze et al., 2000)
f_{XI}	Fraction of inert COD, X_I, generated in	0.07	gCOD g^{-1}COD	Adjusted from (Henze et al.,

Table 3-5: Temperature dependence for growth, decay and hydrolysis, temperature in °C, based on equations used by Hao *et al.* 2001.

Definition	Equation
Maximum specific growth rate	$\mu_{\max,AOB}(T) = 0.8 \cdot e^{0.094 \cdot (T-20)}$
Maximum specific growth rate	$\mu_{\max,HET}(T) = 6 \cdot e^{0.069 \cdot (T-20)}$
Maximum specific growth rate	$\mu_{\max,AMX}(T) = 0.0536 \cdot e^{0.096 \cdot (T-20)}$
Decay rate	$b_{AOB} = 0.05 \cdot e^{0.094 \cdot (T-20)}$
Decay rate	$b_{HET} = 0.62 \cdot e^{0.069(T-20)}$
Decay rate	$b_{AMX} = 0.004 e^{0.096(T-20)}$
Maximum specific hydrolysis rate	$k_{Hyd} = 3 \cdot e^{0.11 \cdot (T-20)}$
Half-saturation coefficient for hydrolysis	$K_X = 0.03 \cdot e^{0.11(T-20)}$

Results

SRT effect on Nitrogen Removal Efficiency

First the influence of the SRT on the nitrogen removal efficiency was evaluated for different long term stable temperatures (Figure 3-1) The influent COD was constant and equal to 10 mgCOD L^{-1} of rbCOD, 30 mg L^{-1} of sbCOD and 3 mg L^{-1} of inert COD, which represent the yearly average concentrations in the effluent of the A-stage settler in Dokhaven. The ammonium concentration in the influent was 17 mgNH_4-N L^{-1} and the HRT was fixed at 1.35 h.

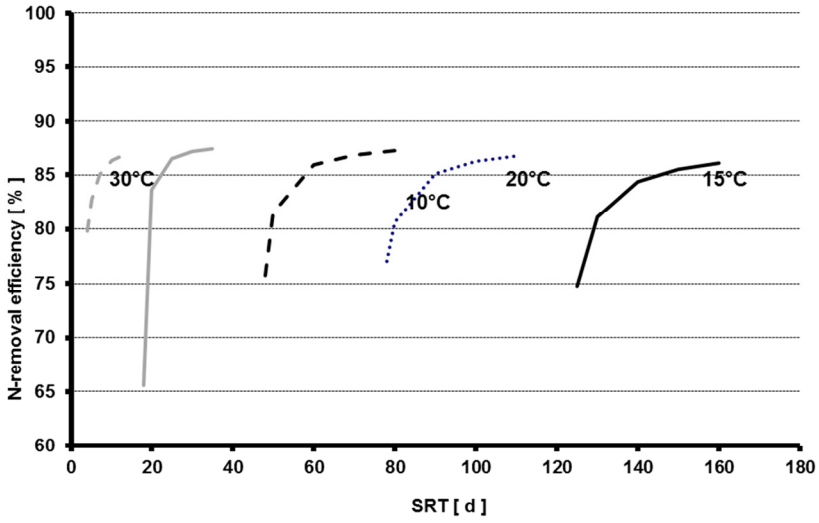

Figure 3-1: Influence of the solids retention time (*SRT*) on the nitrogen removal efficiency at 30°C (in grey) for two different maximal growth rates for the anammox bacteria (dashed line μ_{max_AMX} 0.33 d^{-1} and solid line μ_{max_AMX} 0.14 d^{1}), 20°C (black dashed line), 15°C (black dotted line) and 10°C (black solid line).

For each temperature the minimum SRT value shown in Figure 3-1 represented the shortest SRT applicable to maintain anammox bacteria (related to maximal growth rate of the anammox bacteria) in the system. As for all biological processes, the N-removal efficiency improved with increasing SRT-values. The particular SRT at each temperature will be named SRTmin and defined as the shortest SRT with more than 80% nitrogen removal. In Table the SRTmin for each temperature is reported.

Based on the required SRT, the maximal biomass concentration in the effluent could be calculated and it became clear that only a small amount of biomass in the effluent could be tolerated. At 20°C the required SRT is 50 days, which leads to the particulate concentration of 6.0 gVSS L^{-1} in the reactor and the effluent cannot contain more than a maximum of 6.7 mgVSS L^{-1}. A higher suspended solids wash-out would mean a too low SRT is obtained. (The biomass concentration multiplied by the reactor volume: 6.0 gVSS L^{-1}*11290 $m^3_{reactor}$ =67740 kgVSS total biomass; total biomass in reactor divided by SRT: 67740 kgVSS/50 d = 1354.8 kgVSS d^{-1} biomass in effluent; dividing the total amount of biomass in the effluent by the flow: 1354.8 kgVSS d^{-1} /200000 m^3_{flow}=6.7 mgVSS L^{-1}).

Table 3-6: SRTmin for each simulated temperature, SRT with more than 80% N-removal; COD in influent was equal to yearly average at Dokhaven's WWTP: *rbCOD*=10 mg/L; *sbCOD*=30 mg/L; *inertCOD*=3 mg/L.

Temperature [°C]	**SRTmin [d]**
30 (high AMX growth rate)	5
30 (normal AMX growth rate)	20
20	50
15	80
10	130

With a nitrogen removal efficiency of 80%, the simulations indicate that the effluent of the reactor would contain 3.4 mgTN-N L^{-1}. Based on the stoichiometry used in this model, 9% of the consumed ammonium would be converted to nitrate (anammox bacteria will produce nitrate during growth). Therefore the major part of the nitrogen in the effluent would be nitrate (1.34 mgNO_3-N L^{-1}).

SRT effect on solids concentration and composition in the reactor

In Figure 3-2 the effect of the different SRTs on the accumulation of particulate compounds (sludge) is reported for 10, 15 and 20°C, the values of growth at 30°C were omitted since most municipal wastewaters will be below that temperature. The heterotrophic growth in this model system was aerobic not anoxic (denitrification) since no anoxic zone is present in the model description. Denitrification could be an optimisation aspect in the application of the technology, to further reduce the nitrate in the effluent. The inert particulates originated partly from inert particulate COD entering the reactor (3 mgCOD L^{-1} in these simulations) and partly, roughly the other half was from decayed biomass. In this model the assumption was made that all the inert material entering the reactor would be incorporated in the sludge and therewith only washed out from the system by the excess sludge. It was clear that relative high sludge concentrations would need to be maintained in order to have an effective anammox process at lower temperatures.

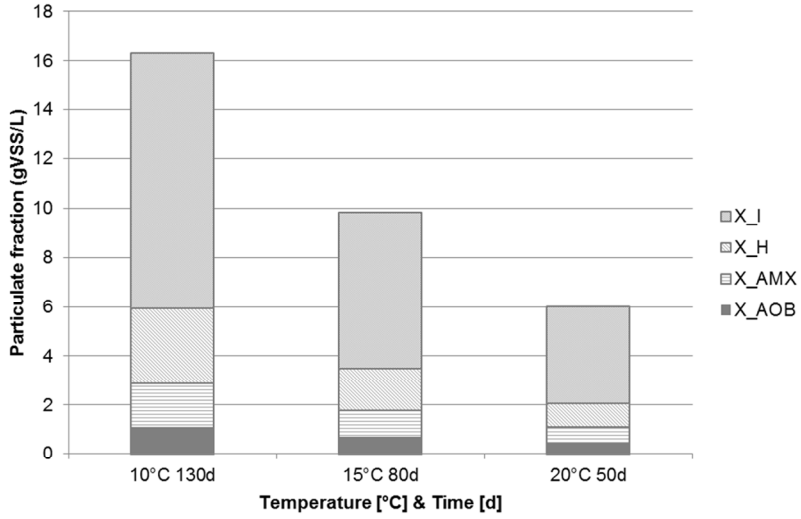

Figure 3-2*:* Particulate fractions as function of the SRT at 20°C, 15°C and 10°C; COD in influent: rbCOD=10 mg/L; sbCOD=30 mg/L; inertCOD=3 mg/L; in legend: X_I=inertCOD; X_H=heterotrophic biomass; X_AMX=anammox biomass; X_AOB=AOB biomass.

Effect of influent COD on solids concentration in the reactor

The influx of COD had a significant effect on the sludge accumulation and growth in the PN/A tank. Therefore the sensitivity of the model results to the influent COD content was evaluated (Figure 3-3). Four cases were chosen, the effluent produced by the A-stage in Dokhaven (which is the influent of the PN/A reactor) (reference: rbCOD 10 mg L^{-1}; sbCOD 30 mg L^{-1}), an extremely efficient A-stage (rbCOD 1 mg L^{-1}; sbCOD 1 mg L^{-1}), an A-stage which efficiently removed readily biodegradable COD but left unconverted some slowly biodegradable COD (rbCOD 1 mg L^{-1}; sbCOD 30 mg L^{-1}) and an A-stage which presented low COD removal efficiency (rbCOD 30 mg L^{-1}; sbCOD 50 mg L^{-1}). The SRT used in the simulations corresponds to the SRTmin (Table 3-6) for each temperature investigated and the inert COD in the influent was 10% of the rbCOD. The results show that the total biomass concentration increased with increasing influent COD concentrations (Figure 3-3A). Since for the nitrogen removal just the autotrophic fraction of the biomass (accounting for AOB and anammox bacteria) was relevant, the autotrophic fraction is presented in Figure 3-3. These data show that the percentage of autotrophic biomass decreased with the increase in COD in the influent. In order to achieve a high percentage of autotrophic biomass, the COD removal in the first stage need to be exceptionally high.

From Figure 3-3 it was clear that autotrophic biomass counted for less than 25% of the total particulate concentration in the reactor as soon as some COD (more than 2 mgCOD L^{-1}) was present in the influent. A large fraction of the particulates in the reactor were inert particulates and heterotrophic biomass, which did not contribute to the total nitrogen removal. To obtain satisfactory nitrogen removal, high biomass concentrations would be needed and biomass concentrations above 10 gVSS L^{-1}, might prove difficult to establish in the reactor.

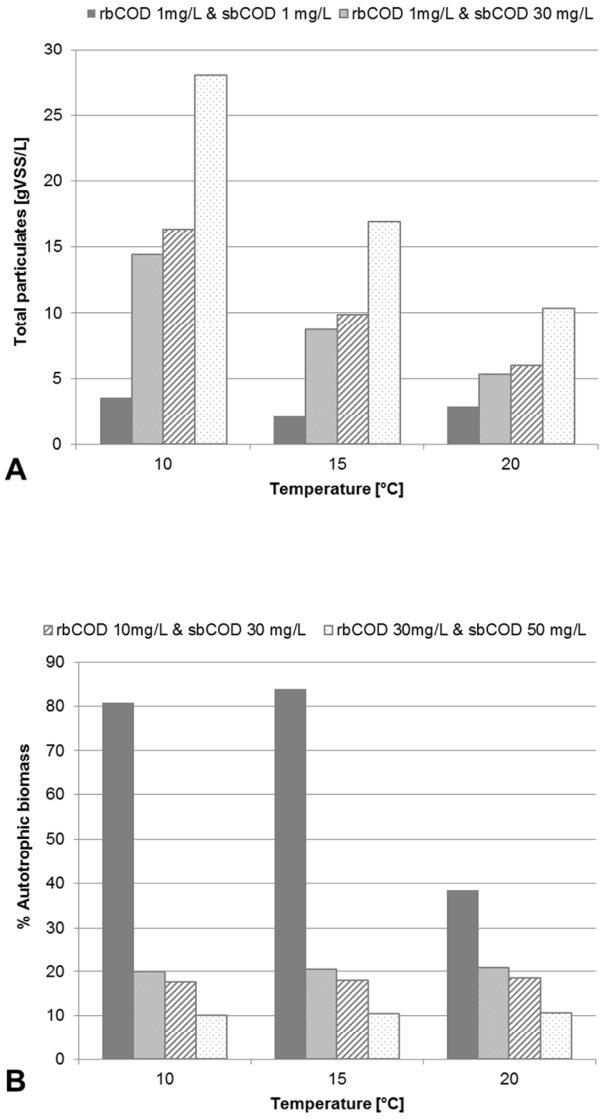

Figure 3-3*:* Total solids concentration in the PN/A reactor as function of the influent COD at 20, 15 and 10°C and SRT=SRTmin. Legend refers to the incoming COD rbCOD : sbCOD mgCOD/L, inertCOD in the influent was 10% if the rbCOD. A: total biomass B: % autotrophic biomass.

Effect of selective washout of heterotrophic biomass

It was clear that, certainly at lower temperatures the faction of autotrophic biomass in the sludge was very limited and required high total particulate concentrations. The model simulations dictated that a separated retention of autotrophic biomass from the heterotrophic was a requisite for successful application of PN/A technology in the mainstream. In the simulation the SRT of the

heterotrophs and other solid components (SRT_H) was uncoupled from the SRT of the autotropic biomass (SRT_{AUT}). This would lead to an increase in the percentage of autotrophs in the biomass due to the decrease in heterotrophic and inert particulates. In the following simulations the SRT_H was considered equal to 50 times the HRT of the system, while the SRT_{AUT} was set as the SRTmin previously obtained. The factor 50 is based on the ratio of HRT and SRT in the B-stage currently used in Dokhaven. In Figure 3-4 the results from simulations similar to those depicted in Figure 3-3 are depicted, but with the decoupled SRT. The total particulate concentrations were influenced by the different COD-load scenarios.

The autotrophic fraction of the particulates clearly beneficiated from the decoupled SRT(Figure 3-4). The percentages of autotrophic biomass of the total particulates increased and the increase of COD in the influent had a smaller effect on the ratio's between the different solid fractions.

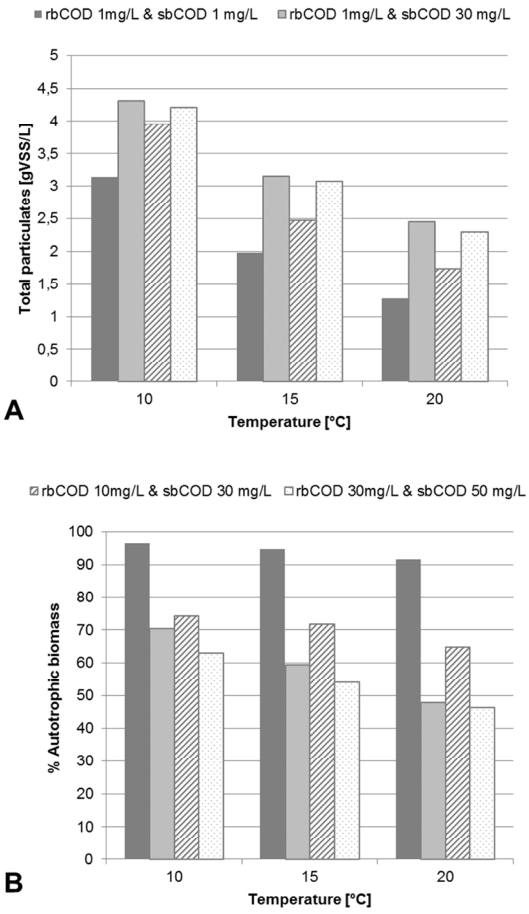

Figure 3-4: Total solids concentration in the PN/A reactor as function of the influent COD at 20, 15 and 10°C, the SRT of the heterotrophs and other solid components (SRT_H= 50*HRT) was uncoupled from the SRT of the autotropic biomass (SRT_{AUT} = SRTmin). Legend refers to the incoming COD rbCOD : sbCOD mgCOD/L, inertCOD in the influent is 10% if the rbCOD. A: total biomass B: % autotrophic biomass.

Effect of yearly fluctuations

A yearly temperature fluctuation profile based on an average temperature pattern of the influent wastewater of Dokhaven 2008-2012 (supplemented material) was used to model the impact of variable temperatures on the PN/A treatment performance. Five years of fluctuations were modelled, to obtain a stable response, the last year is presented in Figure 3-5. The SRT was fixed at one value during the complete simulation. The influent contained the 10 mgCOD L^{-1} of *rbCOD*, 30 mgCOD L^{-1} of *sbCOD* and 3 mgCOD L^{-1} of *inert COD*, our standard case. No uncoupling of the SRTs was introduced, since only the total nitrogen removal was studied (biomass concentrations in coupled and uncoupled systems can be found in the online supplemented material).

With a fixed SRT, the particulate concentration in summer would be higher than needed, creating an overcapacity. This capacity could be used to remove nitrogen during winter. The yearly average total nitrogen removal can be found in Table 3-7. Depending on the local legislation on the effluent characteristics, it would be possible to discharge higher effluent concentrations during winter compared to the summer, as long as the yearly average concentrations remained below the limits. In this model the NOB activity was omitted and therefore nitrite accumulated in the effluent during winter, to a maximum of 13 mgNO_2-N L^{-1} in March with an SRT of 60d (data in online supplemented material).

Table 3-7: Yearly average total nitrogen removal at different SRTs.

SRT (d)	Yearly average nitrogen removal (%)
60	50.2
80	75.4
100	83.7
120	86.4

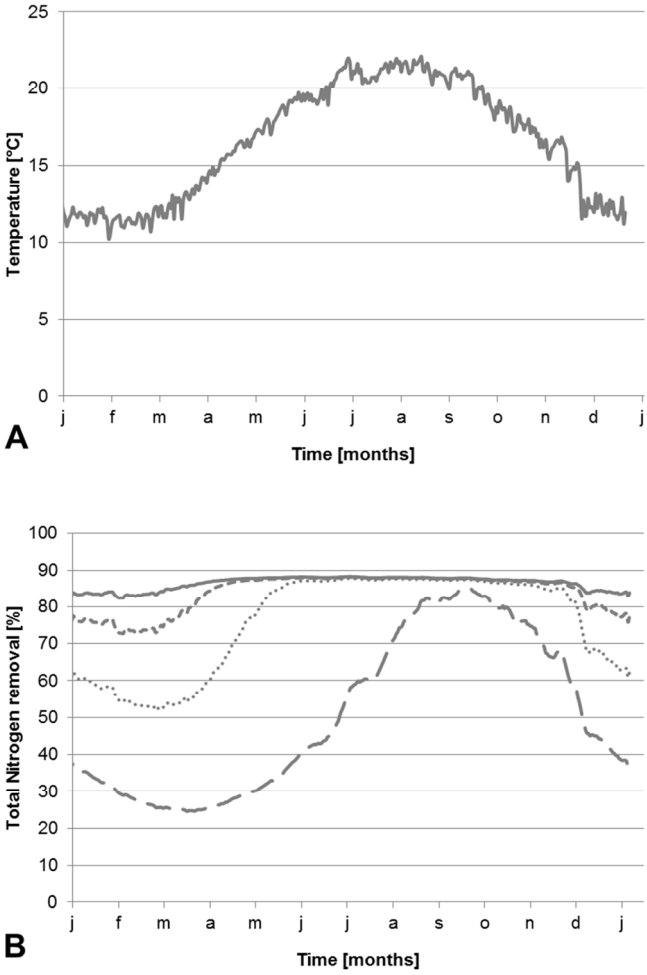

Figure 3-5: A: Yearly temperature fluctuations, averages of the temperature of Dokhaven influent 2008-2012 B: Total nitrogen removal during a year, after 5 years computational modelling. With different fixed SRTs; 60d (long-dashed line), 80d (dotted line), 100d (short-dashed line) and 120d (solid line).

Discussion

Modelling aspects

The model used in this study was a strong simplified version of a typical activated sludge model. Simplifications were included in order to focus on specific design aspects of the PN/A technology. Different assumptions/simplifications were made during model development: regarding the NOB suppression, growth rate of anammox bacteria, the temperature dependence of the anammox bacteria, the fate of the inert particulates in the biofilm and the SRT of the heterotrophic biomass.

An effective nitrogen conversion via the nitritation-anammox route was assumed to occur, i.e. it was assumed that effective suppression of NOB could be achieved. Extensive research has been oriented towards the suppression of NOB in PN/A processes and promising results have been reported (Gilbert et al., 2015; Laureni et al., 2016; Wett et al., 2013). NOB were omitted from this model, but long term NOB suppression under mainstream conditions might yield further challenges (Cao et al., 2017).

There were two different values, for the maximum specific growth rate for anammox bacteria, used in this study, a 'conservative' low growth rate (Oshiki et al., 2011; Strous et al., 1998; Van Der Star et al., 2008) and a high growth rate, recently described in literature for cultures grown above 30°C (Lotti et al., 2015a; Zhang et al., 2017). It remains unclear which environmental factors enabled these recently described high growth rates, at higher temperatures and not at lower temperatures. But when these rates can be achieved at temperatures below 30°C in a full scale process, the process design will be affected significantly.

The temperature dependency of the maximum growth rate values of the microorganisms involved in this model were described by an Arrhenius-type equation, described in the materials and methods section. The temperature effect on anammox was studied in multiple studies and the long term temperature effect on anammox was always bigger than expected, based on normal Arrhenius-type equations (Dosta et al., 2008; Hendrickx et al., 2014; Hendrickx et al., 2012; Hoekstra et al., 2017; Lotti et al., 2014c). To the extent of our knowledge it is not clear weather this temperature effect is due to a flaw of our cultivation techniques or an intrinsic problem of lower temperatures for the anammox bacteria, therefore the standard temperature dependence was used in this model.

The output of the model depended strongly on the assumptions around the fate of heterotrophic biomass and inert solids. In this model, it was first assumed that all particulates have the same SRT. Therefore the inert solids from the influent accumulated in the sludge and diluted the autotrophic biomass. The fate of inert particulates is very important and needs further research. Inert solids were also produced by decay/mineralisation of the produced biomass. The decay rate is an uncertain parameter but the general conclusions drawn here, are not influenced by moderate changes of the adopted default values.

When uncoupling the SRT of the autotrophic biomass from the SRT of the heterotrophic biomass, an SRT for heterotrophic biomass of 50*HRT was assumed. The value of 50 is based on the fate of the floccular biomass in the current B-stage of Dokhaven. In literature comparable values can be found for flocs in hybrid systems (Laureni et al., 2016).

Impact of model results for full scale application

The results of the model at 30°C, showed that implementation in tropical conditions may occur at SRT-values of 20 days or shorter. This suggests that PN/A can be achieved in traditional activated sludge process that are operated at comparable SRT-values in subtropical conditions, as was recently proposed for a treatment plant in Singapore (Cao et al., 2015).

Soluble COD in the influent has no direct negative impact on the anammox process (Kartal et al., 2007), but the degradable COD would be converted into biomass. Due to the long SRTs in a PN/A system even a low COD concentration in the influent would lead to a sharp increase in the total particulate concentration. The A-stage used as a case study in this paper was performing well on the level of COD removal, with an effluent of 10 mg soluble COD L^{-1}, 30 mg particulate COD L^{-1} and 3 mg inert COD L^{-1} (De Graaff et al., 2016). These values lead to a COD:N ratio of 2.8, which is on the high end, but considered suitable for PN/A (Jenni et al., 2014). The PN/A technology is currently implemented in side stream process flows at municipal wastewater treatment plants or in industrial wastewater treatment, these streams generally have a COD:N ratio below 2 (Lackner et al., 2014).

To avoid the presence of as much particulate concentrations in the influent of the PN/A process as possible, we would like to stress that implementation of the PN/A systems would be combined with an optimization of the carbon removing step. This might be achievable with a membrane filtration or sieving. However filtration is unfeasible when large amounts of biodegradable COD are present in the water at relative low temperatures, due to too large complications due to membrane fouling and filter clogging (Laurinonyte et al., 2017).

In most cases sewage pre-treatment in an A-stage process will not be able to achieve adequately low COD-concentration (< 2 mg/L) for the implementation of the PN/A technology with one pooled SRT. In this case uncoupling of the SRTs of the heterotrophic and autotropic biomass is a prerequisite to achieve good N-removal at moderate particulate concentrations (< 10 g/L) in the system. With the uncoupling of the SRTs of the two groups, a particulate concentration around 4 gVSS L^{-1} can be established, this value can be achieved in mainstream systems with biomass retention (Metcalf and Eddy, 2003). Uncoupling the SRT of heterotrophic and autotrophic biomass will be possible to a profound extent, since heterotrophs preferentially grow in bulk suspension or flocs due to their high specific growth rate, the oxygen limited condition applied in the reactor and their presence in the influent wastewater (Lotti et al., 2015b). Reactor configurations in which this uncoupling will occur; will be a biofilm reactor (granular or moving bed biofilm) or a hybrid system (for instance IFAS) (Christensson and Welander, 2004; Laureni et al., 2016). In a pure granule system there will be a natural uncoupling of SRTs due to the shear stress, removing the outside of the granule quicker, compared to the core of the biofilm (Lotti et al., 2015b).

The SRTs needed for a PN/A system will be high, due to the low growth rate of the autotropic biomass, therefore good biomass retention is needed, this leads to a relatively low values of the HRT:SRT ratio. In side stream systems, compared to mainstream conditions, the temperature is higher, which leads to higher growth rates and the concentration of substrate in the wastewater is higher. Since it is difficult to accurately measure biomass concentration in the effluent of a full scale system, not much side stream treatment plants report their values of SRT. For the anammox reactor at Sluisjesdijk, Rotterdam, the Netherlands an SRT of 45-160 d was reported, with an HRT of 19.4 h (van der Star et al., 2007). This yields an approximate HRT:SRT ratio of 0.018 (0.8d/45d). Under

mainstream conditions, the HRT will be shorter, there is less substrate in the water and the SRT needs to be long, due to low temperatures. This leads to a relative low HRT:SRT ratio, compared to side stream systems. For the system proposed in this paper the HRT:SRT ratio will be: 0.0005 (0.05d/100d) for 80% removal with yearly fluctuations. The big difference in HRT and SRT will lead to the need of excellent granulation and small amounts of autotrophic cells in the effluent. Separating flocs and granules might be a challenge, especially small granules might be trapped in flocs and are therefore washed out. Mobile or fixed carriers may have an advantage in selective retention of anammox bacteria in the system, compared to granules.

In the Netherlands, the wastewater treatment plant is due to treat its wastewater to a yearly average effluent quality. It could be possible to aim for good effluent quality during summer (< 2mgN L^{-1}) and accept higher effluent values during the coldest months of the year (for instance 15 mgN L^{-1}, in the form of NO_3^-). It is worth to note that in these modelling results the effluent did contain nitrite during winter (see online supplemented material). In this model the NOB are assumed to be effectively suppressed, so no nitrate is produced. But in reality NOB might grow in winter on the nitrite, that is not consumed by anammox bacteria. Whether it is possible to introduce NOB suppression during the subsequent summer months would need to be investigated.

Conclusions

The model described in this study can be considered as a helpful tool for evaluating the potential for application of the partial nitritation/anammox (PN/A) process in mainstream municipal wastewater treatment. It defined an operational window of the PN/A technology for the treatment of municipal wastewater and it is pinpointing some of the challenges of the PN/A technology. Anammox bacteria are known to be slow growing, keeping these bacteria in the PN/A reactor is essential. The COD load to the PN/A process has large influence on the required particulate concentrations in the PN/A reactor. Therefore the COD removal step, prior to the PN/A reactor, has to perform optimal. In practise, some COD will enter the PN/A reactor. This COD will lead to heterotrophic growth and an increase in total particulate concentration. Uncoupling of the SRTs of autotrophic biomass and heterotrophic biomass is therefore a prerequisite for effective implementation of PN/A process, without the need to have particulate concentrations above 20 gVSS L^{-1}.

Supplemented material

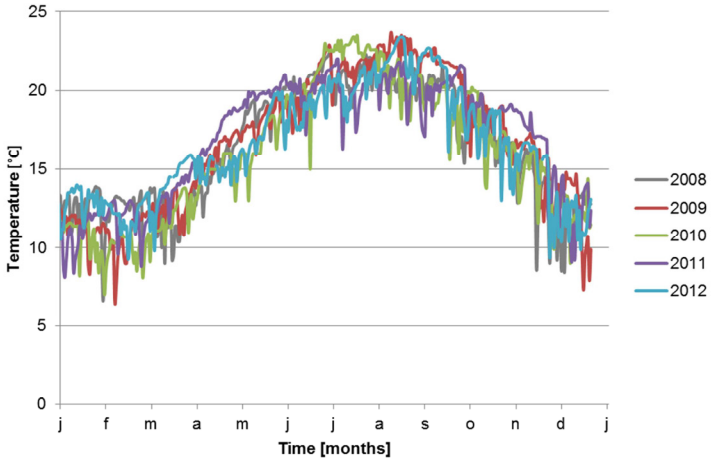

Figure 3-6: Yearly temperature fluctuations, the temperature of Dokhaven influent 2008-2012.

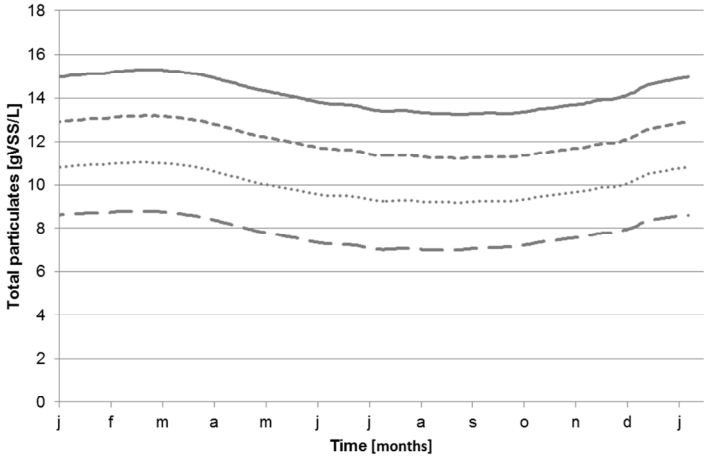

Figure 3-7: Total particulates during a year, after 5 years computational modelling, the SRT was identical for all particulates. With different fixed SRTs; 60d (long-dashed line), 80d (dotted line), 100d (short-dashed line) and 120d (solid line).

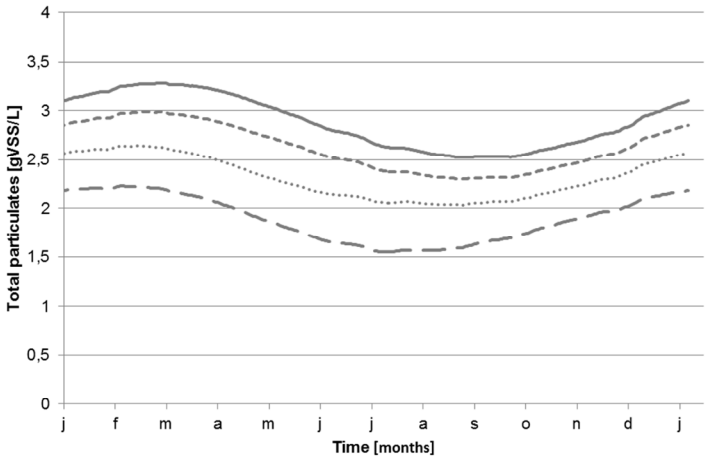

Figure 3-8: Total particulates during a year, after 5 years computational modelling, with uncoupled SRT for the different particulates. With different fixed SRTs for the autotrophic biomass; 60d (long-dashed line), 80d (dotted line), 100d (short-dashed line) and 120d (solid line).

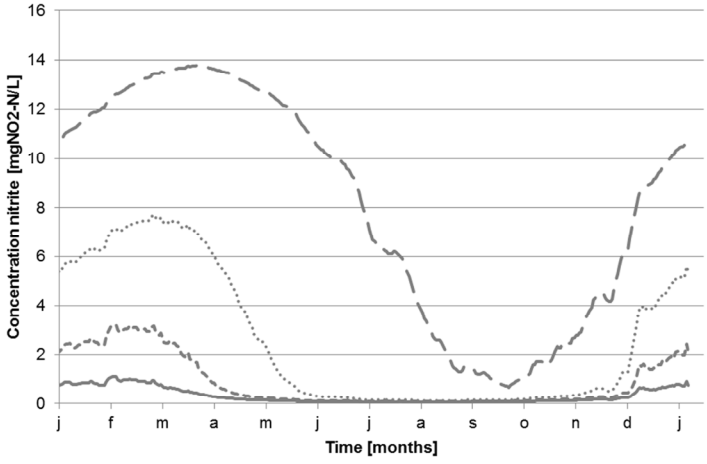

Figure 3-9: Nitrite concentration in the effluent during a year, after 5 years computational modelling. With different fixed SRTs; 60d (long-dashed line), 80d (dotted line), 100d (short-dashed line) and 120d (solid line).

4

Deterioration of the anammox process at decreasing temperatures and long SRTs

Abstract

The implementation of autotrophic nitrogen removal in the mainstream of a municipal wastewater treatment plant is currently pursued. Among the crucial unknown factors are the kinetic properties of anaerobic ammonium oxidising (anammox) bacteria at low temperatures. In this study we investigated the adaptation of a fast-growing anammox culture to a lower temperature. In a membrane bioreactor a highly enriched anammox community was obtained at 30°C, 25°C and 20°C. This culture was exposed to long and short term temperature changes. In short term experiments the decrease in biomass specific activity due to decrease in temperature can be described by an activation energy of 64±28 kJ mol^{-1}. Prolonged cultivation (months) implies that cultivation at low temperatures resulted in deterioration of biomass-specific activity (Ea^{LT} equal to 239 kJ mol^{-1}). The growth rate and specific anammox activity in the system decreased from 0.33 d^{-1} and 4.47 gNO_2-N $gVSS^{-1}$ d^{-1} at 30°C to 0.0011 d^{-1} and 0.037 gNO_2-N $gVSS^{-1}$ d^{-1} at 20°C. The reason for the deterioration of the system was related to the required long SRT in the system. The long SRT leads to an increase of non-active and non anammox cells in the reactor, thereby decreasing the biomass specific activity.

Published as

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Introduction

Anammox bacteria are interesting organisms from both a microbiological and an engineering point of view. Anammox stands for ANaerobic AMMonium OXidation and refers to the anaerobic conversion of ammonium with nitrite to nitrogen gas (the conversion was first called anoxic ammonium removal by Mulder,1989). The bacteria catalysing this conversion were discovered in a sulphide dependent denitrifying stage of a wastewater treatment pilot plant in Delft, the Netherlands, and named after its conversion: the anammox bacteria (Mulder, 1989). These autotrophic organisms fix CO_2 by obtaining reduction equivalents from oxidation of nitrite to nitrate the anammox bacteria therefore produce nitrate proportional to their growth rate (Van De Graaf et al., 1997).

From an engineering point of view the anammox bacteria are interesting for applications in wastewater treatment systems (Jetten et al., 1998). The anammox conversion makes a full autotrophic process for ammonium removal into nitrogen gas possible. This allows the conversion of the organic carbon into methane which can be used as energy supply for the wastewater treatment process. There is a whole set of partial nitritation/anammox (PN/A) processes developed (Lackner et al., 2014; Morales et al., 2015; van der Star et al., 2007). Implementation of the anammox process is mostly based on biofilm or granular sludge processes because through this method a high biomass concentration and therewith a high volumetric treatment capacity can be achieved.

The PN/A technology is currently applied in streams with high ammonium concentrations and mesophilic temperatures (Abma et al., 2010; Lackner et al., 2014). The application of PN/A in the mainstream of a wastewater treatment plant will be a major step forward in obtaining an energy neutral wastewater treatment plant (Kartal et al., 2010). One of the biggest challenges for implementation of this technology in the waterline of a sewage treatment plant is the temperature of the wastewater; in moderate climates the wastewater temperature reaches 10°C during winter. Lab and pilot scale research on the mainstream application of anammox is currently pursued worldwide (De Clippeleir et al., 2013; Malovanyy et al., 2015; Wett et al., 2013; Xu et al., 2016). These studies usually focus on in the full PN/A processes or actual wastewater, making it difficult to evaluate the exact contribution of the anammox conversion as well as the intrinsic impact of temperature on the anammox population.

Since anammox bacteria have a low growth rate, their kinetic characteristics are important in the process design. Verified kinetic information is however very sparsely available in the literature (Isaka et al., 2006; Lotti et al., 2014b; Strous et al., 1999). The cause of the limited availability of kinetic data can be found due to the method of cultivation of anammox bacteria. Anammox bacteria have a tendency to grow in biofilms and due to their low growth rate, the cultivation in biofilms is convenient. Biofilms can be developed in a granular sludge process or on carrier material, leading to high biomass concentrations in a reactor. Unfortunately identification of kinetic properties of bacteria in a biofilm process is complicated by the combined effect of microbial kinetics and mass transfer processes (Characklis and Marshal, 1990; Chu et al., 2003; Harremoes, 1982). To solve this bottleneck, a membrane bioreactor (MBR) has been used to cultivate the anammox bacteria as planktonic cells or in small flocs (Lotti et al., 2014b; Oshiki et al., 2013; Van Der Star et al., 2008). The advantages of this system are subsequently the absence of mass transfer limitations. In biofilm systems not all cells will be exposed to the same conditions due to the substrate and product concentration gradients. Secondly, the homogeneous distribution of cells and of the growth rate in the system. In a biofilm the actual growth rate of cells may be highly variable as a function of their

location. And finally, an accurate establishment of a biomass dilution rate (i.e. growth rate) by controlled removal of biomass results in a well-defined growth rate in the steady state. In systems with biomass retention such as biofilms or flocs, a small fraction of the biomass washes out of the system with the effluent. This fraction is hard to identify and is often neglected, leading to an underestimation of the growth rate.

In previous research, using different biofilm based reactor configurations, doubling times in the order of magnitude of 7 – 11 days were reported for cultures grown at 30°C (Oshiki et al., 2011; Strous et al., 1998; Van Der Star et al., 2008). In an MBR with a suspended cell culture, anammox doubling times down to 3 days were obtained at 30°C and accurate kinetic parameters could be measured (Lotti et al., 2015a). The doubling times that are until now reported for anammox cultures at lower temperatures are much higher, 77 d at 10°C (Lotti et al., 2014c); 17 d at 20°C (Hendrickx et al., 2012); 63 d at 10°C (Hendrickx et al., 2014); 80 d at 12.5°C (Laureni et al., 2015). For marine anammox species it is known that they can grow at temperatures below 10°C (Dalsgaard and Thamdrup, 2002; Rysgaard et al., 2004) however kinetic characterisation of these anammox communities has not been reported. These data seem to indicate that the growth rate of anammox gets strongly reduced at a lower temperature. Therefore we aimed to study the potential growth rate under optimal conditions at lower temperatures.

In this study a highly enriched fast-growing anammox community at 30°C was used to study the effect of decreasing the temperature to 25°C and 20°C on the anammox bacteria. The short-term temperature dependency of the activity of the anammox-dominated community was measured in stable enrichments grown at 30°C, 25°C, and 20°C, using dynamic temperature experiments. Short-term temperature effects were compared to data of long-term-cultivated enrichments.

Material and Methods

Reactor operations

The experiments described in this paper were conducted in an MBR with a working volume of 10L. The reactor set-up and general operational properties have been described elsewhere (Lotti et al., 2015a; Lotti et al., 2014b; Van Der Star et al., 2008). The biomass in the reactor originates from the full-scale anammox internal circulation reactor of Dokhaven WWTP in Rotterdam (van der Star et al., 2007). Prior to this experiment the biomass was used in other studies (Lotti et al., 2015a; Lotti et al., 2014b) and the reactor had been in operation for more than 6 years. When the temperature of the reactor was shifted, 15 mL of sludge from the low temperature mainstream anammox pilot reactor in Dokhaven WWTP in Rotterdam was added (Lotti et al., 2015b) in order to increase the microbial diversity in the system.

The synthetic medium utilised was composed of 30 mM ammonium sulphate (840 mgNH_4-N L^{-1}); 60 mM sodium nitrite (840 mgNO_2^--N L^{-1}); 0.51 mM of Ca^{2+} (added as $Ca_2Cl_2 \cdot 2H_2O$); 0.41 mM of Mg^{2+} (added as $MgSO_4 \cdot 7H_2O$); 5 mM of phosphate (added as pH-buffer by dissolving KH_2PO_4 and K_2HPO_4 salts); 0.08 mM of Fe^{2+} (added as $FeSO_4 \cdot 7H_2O$) and 1.25 mL L^{-1} of trace element solution (Van Der Star et al., 2008). During the 20°C run the EDTA concentration was elevated from 0.08 to 0.11 mM, because precipitations were observed in the medium.

The system was first operated at 30 °C for 12 months, followed by a 10 month period of 25 °C and finally a 12 month period of 20 °C. The changes in temperature were made in steps of 1 °C per day. The hydraulic and solid retention time (HRT and SRT) are depicted in Table 4.1. The HRT was set with the aim to operate under nitrite limiting conditions (below 0.5 $mgNO_2^-$ L^{-1}). The feeding flow and sludge bleed were monitored by weighting the sludge collection vessel and medium vessel, calculating the flow by measuring the weight difference over a period of time. The solids retention time (SRT) was controlled by purging mixed liquor (5 min every 30 min) at different flow rates and sampling. The dilution rate was controlled close to the maximal growth rate of the bacteria, based on measurements of the residual nitrite concentrations. Before feeding to the reactor, the medium was flushed with nitrogen gas in order to remove traces of oxygen. To keep anoxic conditions the reactor was continuously sparged at 80 mL min^{-1} with 95%N_2 – 5%CO_2 gas. The pH was controlled at a value of 7 using a 0.1 M bicarbonate buffer using an applikon ADI 1030 Bio controller.

Table 4-1: Reactor configurations of the enrichment reactor used in this study.

	30°C	25°C	20°C
Hydraulic retention time (HRT) [d]	1.65	2.5	2.45 - 11.83
Solid retention time (SRT) [d]	3 - 4.20	10	50 - 950
Loading rate [$mgNO_2$-N L^{-1} d^{-1}]	510	336	71-350

Analytical methods

Daily assessments of nitrite and nitrate concentrations in the reactor were executed with Merckoquant® test strips. Weekly measurements of ammonium, nitrite and nitrate were conducted using Hach Lange cuvette tests and analysed with a LASA 20 spectrophotometer.

The concentrations of solids, determined as total suspended solids and the fraction corresponding as volatile suspended solids (VSS), were determined according to Standard Methods (APHA, 2005). The yield was based on measurements of the biomass production and nitrite consumption.

Specific anammox activity

The maximum specific anammox activity (SAA^{max}) $\left(q_{NO_2^-}^{max},\ mg\ NO_2 - N\ gVSS^{-1}\ d^{-1}\right)$ was obtained via batch testing procedure. The medium was fed to the reactor at an elevated rate for a period of 15-20 to minutes to obtain a nitrite concentration between 20 and 30 $mgNO_2$-N L^{-1}. At this point the substrate feed was stopped and the nitrite uptake rate was determined by measuring nitrite depletion. Ammonium was not limited during stable reactor operation; therefore a surplus of ammonium (around 150 $mgNH_4$-N L^{-1}) was present in the solution during these tests. The sludge and effluent control were switched off during this period of time. The biomass concentration (g VSS L^{-1}) was used to convert the volumetric conversion to biomass specific anammox activity.

Dynamic temperature response

The short-term temperature response was analysed via dynamic temperature experiments. The temperature profile was adjusted from the initial temperature to 15-30°C and back to the initial temperature in the course of 8 h. The feeding flow was adjusted and monitored, such that nitrite would not become limiting or exceed 25 mg NO_2 -N L^{-1}. The dynamic temperature response was fitted with the Arrhenius equation (Equation (1)) using nonlinear regression for determination of the apparent activation energy (Ea, kJ mol^{-1}). The data were fitted with a singular Arrhenius coefficient

for the entire temperature range (15-30 °C), using the measured SAA ($mgNO_2$-N $gVSS^{-1}$ d^{-1}) to fit SAA^{max} ($mgNO_2$-N $gVSS^{-1}$ d^{-1}), and optimal temperature in T_{ref} (C) and error (r).

$$SAA(T) = SAA^{max} * \left(\frac{\exp\left(-\frac{Ea}{r * T}\right)}{\exp\left(-\frac{Ea}{r * T_{ref}}\right)} \right) \quad (1)$$

Microbial community analysis

An extensive analysis of the microbial community was performed using denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization (FISH). Samples were taken every 1-2 months to evaluate any community changes during different temperature periods. To estimate the average floc size, the size of 50 flocs was measured, with an image analyser.

Fluorescence in situ hybridisation

The collected samples were washed in phosphate buffer and fixed using paraformaldehyde. Hybridisation with fluorescent-labelled oligonucleotide probes and analysis of the samples were performed as described in Lotti *et al.* 2014b, a formamide concentration of 30% was used for hybridisation of the probes used in this study (Table 4-2). The rough estimation of the size of the side population was based on the analysis of 20 microscopic fields.

Denaturing gradient gel electrophoresis

The collected samples were washed in a phosphate buffer and centrifuged. The pellet was stored at -80 °C. DNA extraction and DGGE were carried out as described by Lotti *et al*. 2015a. Extracted DNA was used to amplify the 16S rRNA gene using a primer with near perfect matches to most anammox species: AN314 and a bacterial specific primer: Bac907rM (Table 4-2). After purification of the PCR products out of the agarose gel and sequencing, the obtained sequences were compared to sequences stored in GenBank using Blastn.

Table 4-2: Oligonucleotides probes used in this study for FISH analysis and primers used for DGGE.

Oligonucleotide	Target organism	Sequence (5'-3')	Reference
Bfu-613 FISH	*C.* Brocadia fulgida	GGA TGC CGT TCT TCC GTT AAG CGG	(Kartal et al., 2008)
Amx1015 FISH	*C.* Brocadia anammoxidans	GAT ACC GTT CGT CGC CCT	(Schmid et al., 2000)
Kst1273 FISH	*C.* Kuenenia stuttgartiensis	TCG GCT TTA TAG GTT TCG CA	(Schmid et al., 2000)
EUB 338 FISH	Bacteria	GCT GCC TCC CGT AGG AGT	(Daims et al., 1999)
AN314 DGGE	Anammox bacteria	CCT ACG GGA GGC AGC AG	(Muyzer et al., 1993)
Bac907rM DGGE	Bacteria	CCG TCA ATT CMT TTG AGT TT	(Muyzer and Smalla, 1998)

4

Results

Long-term operation at three different temperatures

The MBR was operated for 12 months at 30°C, for 10 months at 25°C and for 12 months at 20°C. Stable conversions and biomass concentrations were achieved at each temperature. Several studies have found that ammonium and nitrate do not inhibit the anammox process up to concentrations of 1 g N L^{-1}, but nitrite has been reported as an inhibitive compound. Nitrite levels above 350-400 mg NO_2-N L^{-1} completely inhibit the process (Dosta et al., 2008; Lotti et al., 2012). Therefore, nitrite concentration was kept below 30 mg NO_2-N L^{-1} , during batch tests. Since cells were grown almost suspended, oxygen inhibition can occur at very low values. Special care was taken to prevent trace amounts of oxygen entering the system. To avoid accumulation of nitrite during reactor operations the volumetric loading rate was decreased stepwise at the different temperatures by increasing the HRT. At each temperature the SRT was adjusted to the maximal achievable growth rate of the biomass, based on the residual nitrite concentration in the reactor. A small but measureable nitrite concentration ensures the anammox cells operate close to their maximal growth rate.

During the stabilisation at the different temperatures, the biomass concentration and the maximum specific anammox activity (SAA^{max}) were measured. The results of the operation at 20°C are depicted in Figure 4-1. At day 100 the sludge removal was stopped and the only biomass removed from the system was during sampling. Therefore the SRT was increased from 50 to 950 days, leading to an increase in the biomass concentration. This change was introduced, because the SAA decreased and nitrite started to accumulate in the system. From day 70 to 190 the activity decreased from 250 to 30 $mgNO_2$–N $(gVSS\,d)^{-1}$.

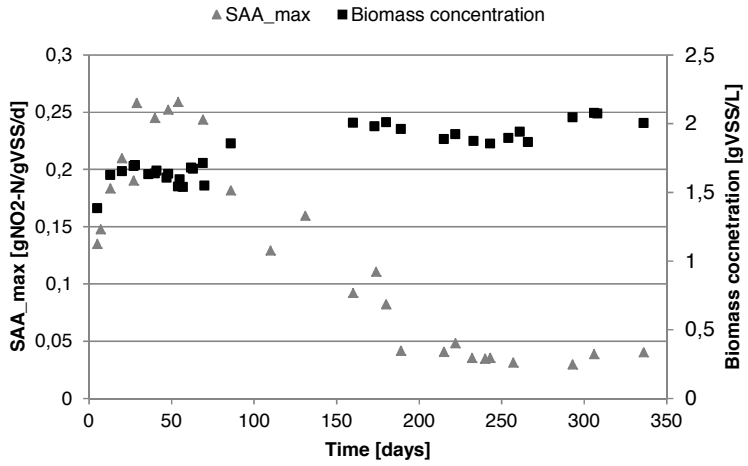

Figure 4-1: Maximum specific anammox activity ($SAA^{max\ LT}$) during long-term cultivation of anammox bacteria in an MBR at 20°C. Biomass concentration is shown in black squares and $SAA^{max\ LT}$ in grey triangles.

During stable reactor performance the different process parameters were measured. Based on these values different kinetic parameters were calculated, which are reported in Table 4-3. The biomass yield and the nitrite to ammonium ratio remained constant at 30°C and 25°C. Since the biomass yield remained constant, the $SAA^{max\ LT}$ and the maximum growth rate, decreased with a similar percentage from 30°C to 25°C. In this case the changes in decay and maintenance rate are not incorporated, since both values will be small and no significant changes were expected. The trend of the affinity constant for nitrite during long term operations cannot be accurately defined because too strongly fluctuating residual nitrite concentrations were measured (between 0.015 and 0.8 mg NO_2-N L^{-1}), compared to the fluctuations in $SAA^{max\ LT}$.

Table 4-3: Kinetic parameters for the anammox-enriched community obtained at three different temperatures during long-term cultivation.

Parameters	30 °C	25 °C	20 °C
$Y_{X/NO2\text{-}N}$ [gVSS g NO_2 -N^{-1}]	0.098±0.01	0.111 ± 0.017	0.028 ± 0.001
$SAA^{max\ LT}$[g NO_2-N $gVSS^{-1}$ d^{-1}]	4.19 ± 0,001	0.904 ± 0.174	0.0368 ± 0.0058
NO_2/NH_4	1.219 ± 0.08	1.00 ± 0.01	1.16 ± 0.03
NO_3/NH_4	0.211 ± 0.07	0.14 ± 0.11	0.10 ± 0.01

The values for growth at 20°C are different from the values at higher temperatures. The amount of nitrate produced per ammonium consumed decreased significantly with decreasing temperatures. The long term $SAA^{max\ LT}$ trend with temperature was fitted with an Arrhenius equation (see Figure 4-2). A significant decrease in $SAA^{max\ LT}$ is seen with a high apparent activation energy of 239 kJ mol^{-1}.

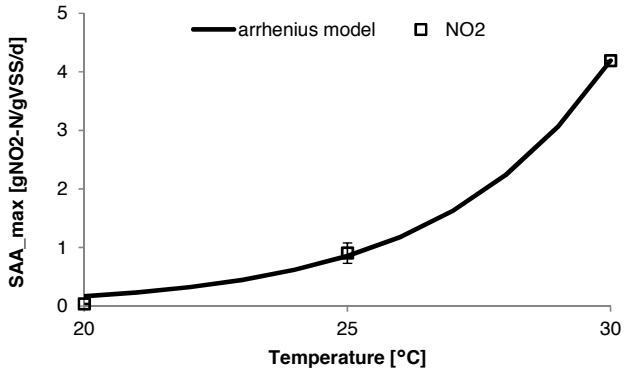

Figure 4-2: Maximum specific anammox activity as a function of temperature after long term cultivation at 30°C, 25°C and 20°C. Arrhenius equation is fitted (solid line) through $SAA^{max\ LT}$ data points (open squares), the data points are averages of long-term stable reactor performance and corresponding standard deviations. The error bars of the data points at 20°C and 30°C are too small to be seen.

Microbial community analysis

In the interest of accurate estimations of the kinetic parameters it is preferred to have a free suspended (planktonic) cell culture or small flocs without significant mass transfer limitations. In previous experiments conducted at 30°C cells could be grown in single cells. When the operational temperature of the system was decreased to 25°C, the biomass flocculated with floc sizes of approximately 50 – 150 µm. Microscopic images showed that the small flocs were composed of filamentous bacteria and anammox bacteria (Figure 4-3). In the 20°C period of operations the amount of non-anammox bacteria increased significantly there was a fraction of biomass present in the reactor which did not hybridise with the anammox and Eubacterial FISH probe (Figure 4-4). These organisms are probably gram-positive bacteria, since no hybridisation took place in the FISH experiment for gram-negative bacteria. Higher organisms, protozoa, were occasionally observed in the sludge as well (picture in supplemented material Figure). Due to the growth in the form of flocs, it was impossible to obtain free cells in a fish protocol and counting individual cells was therefore impossible. A rough estimation of the side population could be made; at 30°C there were hardly any non anammox cells present; at 20°C there were 20-40% of non anammox cells present. The increase in side population was related to the increased SRT. The increased SRT will lead to the accumulation of more heterotrophs, grown on the minimal organic carbon present in the influent as well on exudates or decay products of anammox bacteria.

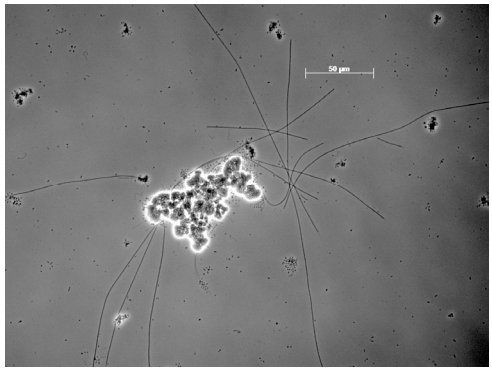

Figure 4-3: Microscopic phase contrast picture of floc and filamentous bacteria, during 25°C cultivation.

Throughout this study the anammox bacteria population was shown to be a member of the genus *Candidatus Brocadia,* based on FISH. Hybridisation took place with the anammox probe for*C. Brocadia fulgida* (BFU-613). No hybridisation took place with the ***C****. Brocadia anammoxidans* – specific probe (AMX-1015) or the ***C****. Kuenenia stuttgartiensis* specific probe (KST-1273) (data not shown).

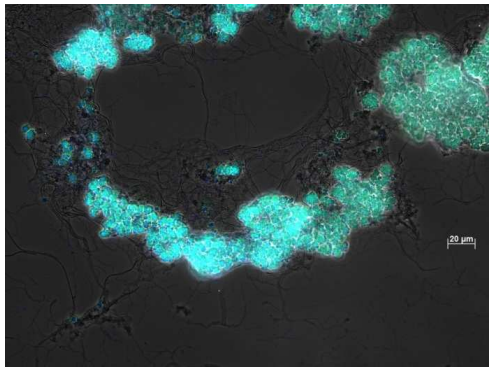

Figure 4-4 FISH picture of the biomass enriched at 20°C, hybridised with Eubacteria (blue) and AMX (BFU 613, green). Unstained side population can be clearly seen. There are a limited number of cells that are stained with the eubacteria probe and not with the anammox bacteria probe

Microbial community analysis by sequencing of the dominating DGGE bands confirmed 99% identity with *C. Brocadia TKU 1 & 2,* at all temperatures (supplemented material). FISH analysis revealed that anammox was the dominating species in bio volume in the reactor. However, a side population can clearly be seen in microscopic images. The bacterial DGGE results show three groups of bands with the highest intensity present throughout all temperature operations. Those sequences where blasted, corresponding with denitrifying bacterium enrichments and a chloroflexi bacterium. Due to the simple composition of the culture, no metagenomics sequencing was executed.

Short-term temperature response

The short-term temperature response was analysed via dynamic temperature experiments; the feed flow was adjusted and monitored, such that nitrite would not become limiting (0.5 mgNO_2 L^{-1}) or

exceeding 30 mg NO_2 -N L^{-1}. An example of an experiment is depicted in Figure 4-5. The temperature profile was adjusted from 25°C to 15°C, to 30°C and back to the initial temperature in the course of 8 h. Every half hour nitrite concentrations were measured. The dynamic temperature response in activity was fitted with the Arrhenius equation using nonlinear regression for determination of the apparent activation energy (Ea, kJ mol^{-1}).

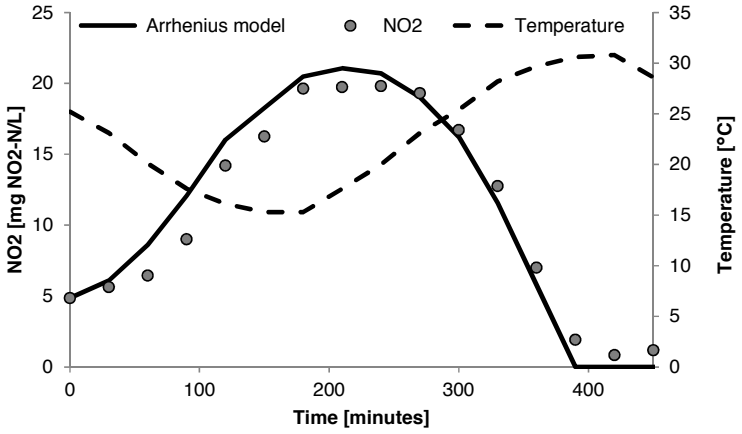

Figure 4-5: Dynamic temperature experiment biomass was cultivated at 25°C. Temperature (dashed line), nitrite samples (filled circles) and fitted nitrite concentrations based on the best estimate for the activation energy in the reactor model (solid line)

Dynamic temperature experiments were performed with the stable biomass for 30°C, 25°C and 20°C. The results of the experiments are shown in Figure 4-6 and Table 4-4. In Figure 4-6 it can be seen that the SAA decreased with decreasing temperatures in a short-term test and the $SAA^{max\ ST}$ was lower for the biomass cultivated at lower temperatures.

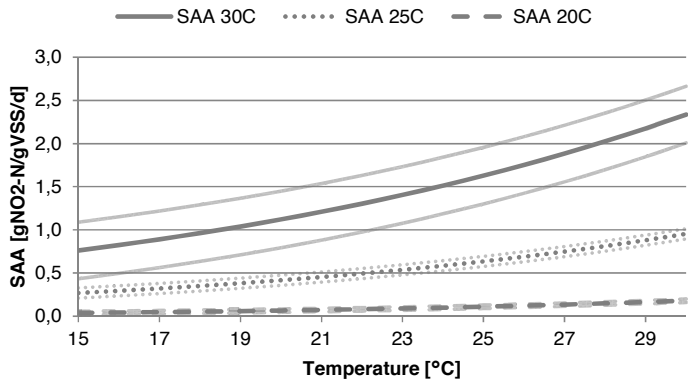

Figure 4-6 Temperature dependency of the specific anammox activity (SAA) as estimated from dynamic temperature experiments with biomass cultivated at 30°C (solid line), 25°C (dotted line) and 20°C (dashed line). Results are averages of multiple experiments (2 or 3) at 30°C, 25°C and 20°, with the combined standard deviations of these experiments as slightly transparent lines.

Table 4-4 presents the data of the eight different dynamic temperature experiments, the differences in the activation energies fall within the standard deviations of the different measurements. The r^2 of the fit is rather low, indicating a suboptimal fit when using one activation energy for the entire temperature range.

Table 4-4: Arrhenius model parameters obtained from short-term temperature experiments for anammox biomass cultivated at different temperatures. Data are averages for two dynamic temperature experiments performed during 30 °C cultivation period and three dynamic temperature experiments from the 25°C and 20°C cultivation periods.

Parameters	30 °C	25 °C	20 °C
Ea [kJ mol^{-1}]	54.1±20.1	61.0±12.8	73.6±46.6
$SAA^{max\ ST}$ [g NO_2-N $gVSS^{-1}$ d^{-1}]	2.39±0.82	0.95±0.47	0.19±0.18
r^2	0.73±0.20	0.94±0.03	0.84±0.087

Discussion

Combination of long- and short-term effect of temperature

If the results of the long term cultivation and the short-term dynamic temperature experiments are combined, it is evident that the long-term temperature impact is much stronger. The maximum biomass-specific activity, after prolonged cultivation at decreasing temperatures, decreases dramatically (Ea^{LT}, 239 kJ mol^{-1}) compared to the short term temperature responses (Ea^{ST}, 64±28 kJ mol^{-1}). As shown in Figure 4-1, the biomass specific activity ($SAA^{max\ LT}$) slowly decreases during long term cultivation at low temperatures. In Figure 4-6 can be seen that the maximum activity at 30°C is lower for the biomass grown at 20°C, compared to the biomass grown at 30°C. But the short term temperature effect is comparable for all cultivation temperatures; a short-term temperature dependency independent of the cultivation temperature is observed. The deterioration of the long term biomass specific activity is not reversible in a short term experiment.

The short term temperature dependency obtained for anammox bacteria in this study is similar with the results obtained by other authors with similar cultivation temperatures Ea: 70 kJ mol^{-1} for 20-43°C (Strous et al., 1999) 63 kJ mol^{-1} for 10-40°C (Dosta et al., 2008) and 83.1 kJ mol^{-1} for 15-30°C (Lotti et al., 2015c). Activation energies of 61 kJ mol^{-1} for 6.5-37°C and 51 kJ mol^{-1} for -2-13°C have been reported for anammox cultures from marine sediments that were adapted to colder climates (≤10°C)(Dalsgaard and Thamdrup, 2002; Rysgaard et al., 2004).

During all the short-term dynamic temperature experiments, the SAA always increased with temperature. This indicates that the optimum temperature, for anammox biomass cultivated at 30°C, 25°C and 20°C, is equal to or higher than the maximum temperature (= 30°C) tested in these experiments. A change in temperature optimum for anammox bacteria, cultivated at different temperatures, has been reported in literature for a C. *Brocadia fulgida*-like strain and a marine anammox strain (Hu et al., 2013; Rysgaard et al., 2004). Nonetheless, this observation was not confirmed in other studies (Lotti et al., 2015c). Also in this study no strong adaptation of the optimum temperature was observed.

The long term temperature effect on the SAA of this system was bigger compared to data reported in literature. After decreasing the temperature, the maximum biomass specific activity slowly decreased, in terms of weeks. This observation is inconsistent with results obtained in other studies where prolonged cultivation of anammox did not result in a slow decrease in time of the SAA as observed in this work (Dosta et al., 2008; Hu et al., 2013; Lotti et al., 2015c). The point of discussion in these studies is the question whether the cultivation times are long enough to properly study the long term temperature effects, because the long term temperature effects occurred over a period of moths in this study. In literature higher growth rates are reported for anammox cultures at lower temperatures, 0.009 d^{-1} at 10°C (Lotti et al., 2014c); 0.04 d^{-1} at 20°C (Hendrickx et al., 2012); 0.011 d^{-1} at 10°C (Hendrickx et al., 2014); 0.0087 d^{-1} at 12.5°C (Laureni et al., 2015), the main difference between these studies and the work described in this paper is the way of cultivation, the absence of a biofilm in our study.

Side population

Anammox bacteria have to date never been isolated in pure culture. A side population is always present in anammox cultivations; therefore all the anammox strains are still referred to as *candidatus*. The increasing amount of side population biomass in this study can be linked to the increasing SRT. At the high dilution rates imposed at 30°C, prokaryotic and eukaryotic grazers may have been washed out from the system. At the increasing SRT values required to avoid wash-out of anammox at lower temperatures, the undesired side population maintains itself in the system. The side population can grow directly on anammox bacteria through grazing, grow on decaying biomass or grow on microbial products excreted by anammox. Fourthly, it is possible that organisms grow on the chemical oxygen demand present in the demineralised water, used to prepare the medium (personal communication, E.I. Prest, 2015;(Moussa et al., 2005)). Some of the organisms of the side population are denitrifiers and can consume part of the nitrate produced by anammox bacteria. This is the likely cause of the changed ammonium to nitrate ratio at different cultivation temperatures; the consumption of nitrite by denitrifying bacteria or the change in stoichiometry of the anammox

bacteria cannot be ruled out. The type of organisms in the side population is observed in similar systems (Gonzalez-Martinez et al., 2016; Speth et al., 2016).

In a biofilm system anammox cells potentially are better protected against these direct or indirect grazing communities (Namkung and Rittmann, 1986). Unfortunately there are no inhibitors known that will only inhibit the side population and not the anammox bacteria; therefore it was not possible to repress selectively the non-anammox biomass.

Possible explanation for the observed strong long-term temperature effect

The results of this study would imply that long-term cultivation at low temperatures results in deterioration in terms of biomass specific conversion rates; the negative effect on the growth yield is less severe. This kinetic deterioration does not occur during short term temperature fluctuations.

The decrease in activity could be explained by the fact that a part of the biomass dies, becomes inactive or is not anammox biomass. At both downward steps in the temperature the SRT is increased substantially; therefore non anammox cells can accumulate in the system and thereby give a decreased value of the measured SAA (Moussa et al., 2005). In our reactor we observed a side population, which could lead to a decrease in specific activity. At the long SRT applied at 20°C protozoa were observed in the sludge; these protozoa might graze on the anammox biomass. The fact that the SAA is decreasing much more compared to the yield could be explained by the presence of dormant or dead anammox cells. The cells are formed, but do not retain their activity. These cells might be identified by FISH, but do not contribute to conversions in the reactor (Kaprelyants et al., 1993; Konopka, 2000; Monballiu et al., 2013). Unfortunately the standard life/dead staining procedure cannot be used on anammox cells due their different membrane composition with ladderane lipids. All these factors will have a small impact on the relative change in activity during short term temperature fluctuations as compared to long term temperature cultivation.

The above explanations can explain the strong long term temperature effects on primarily the SAA and secondary the growth yield observed in this study, which have not been reported in literature before. This is most likely an indirect effect: The increased SRT required for maintaining anammox biomass at lower temperatures facilitated a side population that grows on anammox biomass. Kinetically this suggests an increase in the decay coefficient, resulting in a decrease of the net-growth rate of the anammox bacteria, requiring an increase in SRT. This can be a self-amplifying effect, where an increase in SRT imposes an increase in decay/grazing, and thereby with a decrease in the net-growth rate of anammox, requiring an increase in SRT again.

The major difference between this study and the studies described in literature is the cultivation in biofilms in all studies in literature. There were good reasons to try to omit a biofilm in this study; in a free cell and fast growing culture, the kinetic parameters of anammox bacteria could have been accurately measured. But due to the formation of flocs from 25°C, the possible dormancy of a part of the anammox population and the increased side population, the measurements of the kinetic parameters might be concealed. The next step in this topic of research will be finding out whether the decrease in growth rate described in this study and in literature is intrinsic for anammox bacteria or whether it is a flaw of the cultivation technologies. The answer to this question will also be

important for the application of the PN/A technology in the mainstream of the wastewater treatment plant .

Conclusion

In a MBR a fast-growing highly enriched anammox culture was obtained at 30°C. This culture was exposed to long- and short-term temperature fluctuations. Long-term cultivation at low temperatures resulted in a very strong decrease in growth rate, yield and biomass specific activity. While short-term experiments showed a regular temperature response (activation energy (Ea) of 64±28 kJ mol^{-1}) the long-term temperature effect was very strong (Ea was 239 kJ mol^{-1}). To maintain an anammox culture in the membrane reactor, the SRT was increased at decreasing temperatures. This increase in SRT induced an increase in non-active anammox biomass and an increase in the side population suggesting an indirect temperature effect of long term cultivation at temperatures lower than 30°C.

Supplemented material

Table 4-5: Results of the DGGE gel, depicted in figure 4-8. The results which do not give information of phyla level were selected out.

Band nr.	Description	Max. identity
1	Bacterium enrichment culture clone SRAO_63 16S ribosomal RNA gene, partial sequence	99%
	Uncultured planctomycete clone 5GA_Pla_HKP_17 16S ribosomal RNA gene, partial sequence	99%
	Uncultured Chlorobi bacterium clone R2-77 16S ribosomal RNA gene, partial sequence	88%
2	Uncultured Sphingobacteriales bacterium isolate DGGE gel band G3-12 16S ribosomal RNA gene, partial sequence	88%
3	Uncultured planctomycete clone Pla_seed-6 16S ribosomal RNA gene, partial sequence	91%
	Uncultured Planctomycetales bacterium partial 16S rRNA gene, clone F16	91%
	Uncultured Firmicutes bacterium clone GASP-WB2W1_G12 16S ribosomal RNA gene, partial sequence	89%
4	Uncultured Chloroflexi bacterium clone Paddy_500_3702 16S ribosomal RNA gene, partial sequence	82%
5	Uncultured planctomycete clone Pla_PO55-1 16S ribosomal RNA gene, partial sequence	95%
	Uncultured Chloroflexi bacterium gene for 16S rRNA, partial sequence, clone: HUY-B04	94%
6	Uncultured Chitinophagaceae bacterium clone MH-223 16S ribosomal RNA gene, partial sequence	96%
8	Uncultured Flavobacteria bacterium clone GASP-KC3S1_C02 16S ribosomal RNA gene, partial sequence	93%
10	Uncultured Sphingobacterium sp. clone MCW2A1 16S ribosomal RNA gene, partial sequence	97%
13	Uncultured Owenweeksia sp. clone K1DN8 16S ribosomal RNA gene, partial sequence	95%
	Uncultured Owenweeksia sp. clone K1DN10 16S ribosomal RNA gene, partial sequence	90%
14	Denitrifying bacterium enrichment culture clone NOB_3_F2 16S ribosomal RNA gene, partial sequence	94%
	Denitrifying bacterium enrichment culture clone NOB_2_D10 16S ribosomal RNA gene, partial sequence	94%
16	Uncultured Chloroflexi bacterium clone C2_338 16S ribosomal RNA gene, partial sequence	96%
17	Denitratisoma sp. enrichment culture clone KU a 16S ribosomal RNA gene, partial sequence	82%
19	Uncultured Lewinella sp. clone G3B 16S ribosomal RNA gene, partial sequence	78%
20	Uncultured planctomycete clone Pla_PO55-1 16S ribosomal RNA gene, partial sequence	92%
21	Comamonas sp. RPWA5.3 16S ribosomal RNA gene, partial sequence	88%
	Comamonas nitrativorans strain AN13 16S ribosomal RNA gene, partial sequence	88%
22	Uncultured Lautropia sp. clone De25 16S ribosomal RNA gene, partial sequence	83%
23	Uncultured planctomycete clone 5GA_Pla_HKP_17 16S ribosomal RNA gene, partial sequence	99%
24	Ignavibacterium album strain 14rA 16S ribosomal RNA gene, partial sequence	86%
25	Ignavibacterium album strain 14rA 16S ribosomal RNA gene, partial sequence	99%
26	Denitrifying bacterium enrichment culture clone NOB_3_F2 16S ribosomal RNA gene, partial sequence	92%
	Denitrifying bacterium enrichment culture clone NOB_2_D10 16S ribosomal RNA gene, partial sequence	92%
27	Uncultured Firmicutes bacterium partial 16S rRNA gene, clone EJIR08_37	90%
28	Uncultured Chloroflexi bacterium clone C2_338 16S ribosomal RNA gene, partial sequence	96%
30	Denitratisoma sp. enrichment culture clone KU a 16S ribosomal RNA gene, partial sequence	85%
	Uncultured beta proteobacterium clone Zac715 16S ribosomal RNA gene, partial sequence	85%
31	Uncultured Chlorobi bacterium clone R2-77 16S ribosomal RNA gene, partial sequence	88%

32	Uncultured Bacteroidetes bacterium clone R1-2 16S ribosomal RNA gene, partial sequence	96%
	Uncultured Bacteroidetes bacterium clone R2-37 16S ribosomal RNA gene, partial sequence	95%
	Uncultured Flavobacteria bacterium clone AUVE_15E04 16S ribosomal RNA gene, partial sequence	95%
33	Uncultured Verrucomicrobia bacterium clone F1bUF12 16S ribosomal RNA gene, partial sequence	92%
	Uncultured Verrucomicrobia bacterium clone JAB SMS 108 16S ribosomal RNA gene, partial sequence	79%
34	Uncultured Chloroflexi bacterium clone C2_338 16S ribosomal RNA gene, partial sequence	94%
	Uncultured Chloroflexi bacterium clone C2_314 16S ribosomal RNA gene, partial sequence	94%
36	Ignavibacterium album strain 14rA 16S ribosomal RNA gene, partial sequence	97%
	Uncultured Chlorobi bacterium clone T12 16S ribosomal RNA gene, partial sequence	93%
37	Uncultured Chloroflexi bacterium clone C2_338 16S ribosomal RNA gene, partial sequence	89%
38	Uncultured Chloroflexi bacterium clone C2_338 16S ribosomal RNA gene, partial sequence	97%
39	Uncultured Planctomycetales bacterium clone Dok1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured Planctomycetales bacterium clone DTU1-9 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-2 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured planctomycete clone 5GA_Pla_HKP_47 16S ribosomal RNA gene, partial sequence	99%
40	Uncultured Planctomycetales bacterium clone Dok1 16S ribosomal RNA gene, partial sequence	100%
	Uncultured Planctomycetales bacterium clone DTU1-9 16S ribosomal RNA gene, partial sequence	100%
	Candidatus Brocadia sp. TKU-2 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured planctomycete clone 5GA_Pla_HKP_47 16S ribosomal RNA gene, partial sequence	99%
41	Uncultured Planctomycetales bacterium clone Dok1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured Planctomycetales bacterium clone DTU1-9 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-2 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured planctomycete clone 5GA_Pla_HKP_47 16S ribosomal RNA gene, partial sequence	99%
42	Uncultured Planctomycetales bacterium clone Dok1 16S ribosomal RNA gene, partial sequence	100%
	Uncultured Planctomycetales bacterium clone DTU1-9 16S ribosomal RNA gene, partial sequence	100%
	Candidatus Brocadia sp. TKU-2 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured planctomycete clone 5GA_Pla_HKP_47 16S ribosomal RNA gene, partial sequence	99%
43	Uncultured Planctomycetales bacterium clone Dok1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured Planctomycetales bacterium clone DTU1-9 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-2 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured planctomycete clone 5GA_Pla_HKP_47 16S ribosomal RNA gene, partial sequence	99%
44	Uncultured Planctomycetales bacterium clone Dok1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured Planctomycetales bacterium clone DTU1-9 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-2 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured planctomycete clone 5GA_Pla_HKP_47 16S ribosomal RNA gene, partial sequence	99%
45	Uncultured Planctomycetales bacterium clone Dok1 16S ribosomal RNA gene, partial sequence	96%
	Uncultured Planctomycetales bacterium clone DTU1-9 16S ribosomal RNA gene, partial sequence	96%
	Candidatus Brocadia sp. TKU-2 16S ribosomal RNA gene, partial sequence	95%

	Candidatus Brocadia sp. TKU-1 16S ribosomal RNA gene, partial sequence	95%
	Uncultured planctomycete clone 5GA_Pla_HKP_47 16S ribosomal RNA gene, partial sequence	95%
46	Uncultured Planctomycetales bacterium clone Dok1 16S ribosomal RNA gene, partial sequence	100%
	Uncultured Planctomycetales bacterium clone DTU1-9 16S ribosomal RNA gene, partial sequence	100%
	Candidatus Brocadia sp. TKU-2 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured planctomycete clone 5GA_Pla_HKP_47 16S ribosomal RNA gene, partial sequence	99%
47	Uncultured Planctomycetales bacterium clone Dok1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured Planctomycetales bacterium clone DTU1-9 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-2 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured planctomycete clone 5GA_Pla_HKP_47 16S ribosomal RNA gene, partial sequence	99%
48	Ignavibacterium album strain 14rA 16S ribosomal RNA gene, partial sequence	98%
49	Uncultured Chloroflexi bacterium clone C2_338 16S ribosomal RNA gene, partial sequence	95%
50	Uncultured Planctomycetales bacterium clone Dok1 16S ribosomal RNA gene, partial sequence	100%
	Uncultured Planctomycetales bacterium clone DTU1-9 16S ribosomal RNA gene, partial sequence	100%
	Candidatus Brocadia sp. TKU-2 16S ribosomal RNA gene, partial sequence	99%
	Candidatus Brocadia sp. TKU-1 16S ribosomal RNA gene, partial sequence	99%
	Uncultured planctomycete clone 5GA_Pla_HKP_47 16S ribosomal RNA gene, partial sequence	99%

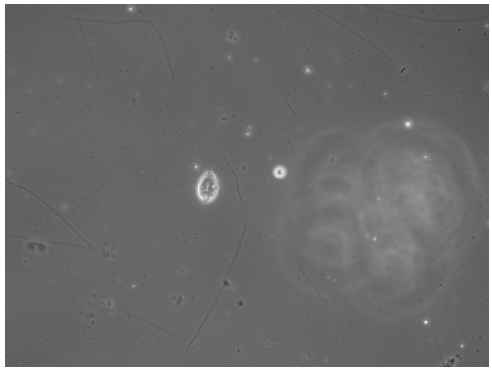

Figure 4-7: Microscopic picture of a protozoa in the reactor, during cultivation at 20°C. Size of the protozoa is 7,5 µm.

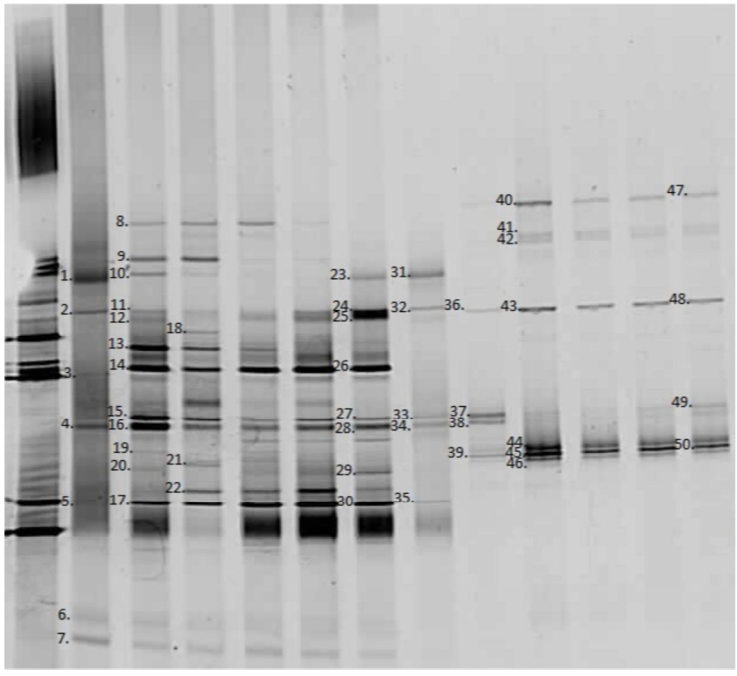

Figure 4-8: DGGE gel, successive lanes are:1: ladder, 2&8: 30 degrees average growth rate,3&9: 30 degrees high growth rate, 4&10:25 degrees just after decrease in temperature,5&11: 25 degrees after 10 months of cultivation, 6&12: 20 degrees after 1 month of cultivation, 7&14:20 degrees after a year of cultivation. In the 2-7th lane the general bacteria primer (Bac907rM) is used in the last 6 lanes the anammox bacteria primer (AN314) is used . Details about the species represented by each band is given in Table 4-5.

5

Competition between AOB and NOB, the influence of DO and temperature

Abstract

The aim of this research was to study the possibility of suppression of Nitrite Oxidising bacteria (NOB) in a nitrifying granular sludge cultivated under ammonium limiting conditions. The effect of dissolved oxygen (DO) concentration and temperature on NOB repression was evaluated. Nitrification was stably achieved in a sequential fed batch reactor (2L) at 20°C, treating low-strength synthetic wastewater. To simulate the low ammonium/nitrite concentrations occurring in completely mixed systems the reactor was fed during a prolonged period, instead of being operated in a dump fill mode. In the reactor a granular type of sludge occurred. Using the obtained enrichment culture the effect of DO concentration and temperature on the conversions by ammonium oxidising bacteria (AOB) and nitrite oxidising bacteria was evaluated. Batch test results at different temperatures (10°C - 20°C) and DO concentrations (0.1-4 g/L) showed a consistently higher maximum NOB nitrogen conversion activity compared to the AOB activity. At 20°C, without substrate limitation (DO, ammonium and nitrite), the specific activity for AOB was 0.27±0.08 gN (gTSS d)$^{-1}$ and for NOB 0.75±0.10 gN (gTSS d)$^{-1}$. *Nitrospira* spp. were found to dominate the NOB population during the entire reactor operation. Even though clear clustering of AOB and NOB was observed, no stratification of AOB and NOB was observed in the biomass aggregates. The biofilm structure obtained when cultivated at rate limiting ammonium concentration did not allow to supress nitrite oxidation by adjusting the DO level. To supress NOB effectively in biofilm or granular systems, cultivation at non-limiting ammonium concentrations or the presence of anammox bacteria is necessary.

In preparation for publication

Hoekstra M., R. Kleerebezem, M.C.M. van Loosdrecht; Competition between AOB and NOB, the influence of DO and temperature

Introduction

Research on nitrogen removal from mainstream municipal wastewater is recently focused on the implementation of the Partial Nitritation/Anammox (PN/A) technology. The use of the autotrophic PN/A technology has been associated with lower energy requirements and smaller reactors compared to conventional activated sludge systems (Cao et al., 2017; Kartal et al., 2010). PN/A technology is currently used for treating wastewater with high temperatures (> 30°C) and high ammonium concentrations, (0.5-1.5 gNH_4-N L^{-1})(Lackner et al., 2014) and shows promising results for implementation in mainstream municipal wastewater treatment plants (WWTP) (Bozkurt et al., 2016).

The main challenges for implementation of the PN/A technology in the mainstream of a municipal wastewater treatment system is controlling the competition between Ammonium Oxidising Bacteria (AOB), anammox bacteria (AMX) and Nitrite Oxidising Bacteria (NOB), and the decreasing conversion rates at low temperatures (Cao et al., 2017; Fernández et al., 2016; Lotti et al., 2014a). The decrease in anammox activity at decreasing temperatures has been studied and found to be higher than expected based on conventional temperature models (Dosta et al., 2008; Hendrickx et al., 2014; Hoekstra et al., 2017). The temperature effects on nitrifier species, which are relevant to wastewater treatment systems are less well described (Blackburne et al., 2007; Guo et al., 2010). In biofilms, the structure of the biofilm has a significant influence on conversion rates and competition (Picioreanu et al., 2016; Poot et al., 2016). The PN/A technology can be implemented in different ways, biofilms (granules or carriers)(Kartal et al., 2010; Rosenwinkel and Cornelius, 2005), hybrid systems (biofilms and flocs) (Wett et al., 2013) or two-stage systems (Pérez et al., 2015).

To successfully implement the PN/A technology in the mainstream of a municipal WWTP the NOB activity in the system needs to be suppressed. Different approaches to supress the NOB have been proposed. We have focussed on the studies done in mainstream conditions (ammonium concentrations < 100mgNH_4-N L^{-1}, neutral pH and temperatures ≤ 20°C). A first method is based on intermitted aeration and relies on anoxic nitrite uptake by anammox or heterotrophic denitrifying bacteria, alternating with aerobic ammonium oxidation by AOB (Chandran and Smets, 2000; Knowles et al., 1965). AOB are known to recover quicker from oxygen limitation than NOB, enabling the suppression of NOB activity (Kornaros et al., 2010). A second approach is the use of the DO:NH_4 ratio to suppress the NOB, this has been shown during long term cultivation on laboratory-scale (Isanta et al., 2015; Poot et al., 2016; Reino et al., 2016).

When reactors are operated under conditions with excess ammonia (or in SBR mode), stratification of the biofilm generally occurs (Hao et al., 2001; Matsumoto et al., 2010; Vlaeminck et al., 2010; Winkler et al., 2012b). In this layered structure, with AOB on the outside and NOB behind the AOB layer the NOB will be outcompeted by AOB when the biofilm is diffusion limited in oxygen (NOB might have grown in in a period of reactor instability) (Pérez et al., 2014; Poot et al., 2016). At lower ammonium loading rates, ammonium rate limiting conditions, NOB are more difficult to outcompete (Hao et al., 2001) likely due to absence of a strong stratification of microbial populations in such biofilm systems. Based on the competitive advantage of AOB at elevated ammonium concentrations (Picioreanu et al., 2016), NOB can effectively be outcompeted in a sequential batch reactor (SBR) or a plug flow reactor, because the biomass is exposed most of the time to elevated ammonium concentrations.

The aim of this study was to investigate the potential for NOB suppression based on DO and temperature in biomass grown at limiting nitrogen concentrations. Therefore we set-up an aerated sequential fed batch reactor (prolonged feed phase with a settling period for biomass retention) and fed with ammonium as sole substrate. When the enrichment culture was obtained we evaluated the effect of oxygen concentrations and temperature on the kinetics of ammonium and nitrite oxidation by AOB and NOB.

Materials and Methods

Reactor set-up and operating conditions

An Applikon 3L jacketed glass bioreactor containing an open nitrifying culture with a working volume of 2 litres was operated at 20^{o}C. The reactor was inoculated with sludge from the nitrifying stage of the wastewater treatment plant in Dokhaven, Rotterdam, the Netherlands and has been running for a year when the experiments started. The SBR system was operated in cycles of 6 hours: 10 minutes water fill phase, 180 minutes aerated feed phase, 120 minutes aerated reaction phase, 30 minutes of settling and 20 minutes of effluent discharge. During the fill phase, 900 mL of tap water was fed into the reactor. During the feed phase, 100 ml of concentrated medium was added to the reactor. One litre of effluent was removed during the effluent discharge, resulting in a hydraulic retention time (HRT) of 0.5 days. The SRT in the reactor was controlled by removal of sludge from the reactor during the reaction phase. During fill, feed and reaction phase, aeration, stirring and pH control were provided, with a gas flowrate of 1.5 L/min of compressed air and a stirring speed of 250 rpm. Weekly mechanical cleaning of the reactor was performed to prevent biofilm formation on the walls. The reactor was covered to avoid algae growth. The nitrogen load of the reactor was 0.4 gNH_4-N (L d^{-1}). The composition of the concentrated medium can be found in Table 5-1 and was based on the medium described in previous research (Kampschreur et al., 2008). The pH was kept at 7.0 using Na_2CO_3 (0.25M) and H_2SO_4 (0.5M).

Online measurement of DO, pH, temperature, Na_2CO_3 and H_2SO_4 dosage enabled continuous monitoring of the system. During several cycles the NO, N_2O, CO_2 and O_2 concentrations in the dewatered off-gas were measured online using a Rosemont Analytical multicomponent gas analyser. Off-gas dewatering was performed with a reflux condenser operated at 4^{O}C. The SRT was calculated based on the time when substrate was present and effluent suspended solids were taken into account.

Table 5-1: Composition of the feeding medium.

Concentrated feed	**(g/l)**	**trace solution**	**(g/l)**
NH_4Cl	4.008	EDTA	10.00
NaCl	1.841	$FeCl_3 \cdot 6H_2O$	1.50
$NaH_2PO_4 \cdot 2H_2O$	0.819	H_3BO_3	0.15
$MgSO_4 \cdot 7H_2O$	0.345	$CuSO_4 \cdot 5H_2O$	0.03
$CaCl_2 \cdot 2H_2O$	0.081	KI	0.18
KCl	0.292	$MnCl_2 \cdot 2H_2O$	0.10
yeast extract	0.0035	$Na_2MoO_4 \cdot 2H_2O$	0.06
	(ml/l)	$ZnSO_4 \cdot 7H_2O$	0.12
trace solution	11	$CoCl \cdot 6H_2O$	0.15

Dynamic batch tests

To measure the conversion rates of AOB and NOB at different temperatures and dissolved oxygen concentrations, dynamic batch tests were designed. The cycle of the SBR was stopped after the water fill phase and if needed the temperature of the water jacket was changed. A concentrated solution of ammonia and nitrite was added to the reactor (final concentration of 25-35 mgN L^{-1} for both ammonium and nitrite). After stabilisation of the DO concentration the conversion rates were measured by measuring the nitrogen species in the solution. After taking 5 measurements (with a 5 min interval) at one DO, the DO was changed and after stabilisation another 5 samples were taken. The DO concentrations were varied by mixing N_2/CO_2 into the airflow. An example of the results of a dynamic batch test can be found in Figure 5-5. Among the different batch tests there was a significant difference between the maximum activities, therefore the data was normalized. The results from the tests were analysed and fitted to a modelled Monod curve to estimate the apparent half saturation coefficients for oxygen.

$$SA_{norm} = \left(\frac{SA_{measured}}{SA^{max}_{this\ test}}\right) * SA^{max}_{this\ temperature}$$

Analytical methods

Daily assessment of nitrite and nitrate concentrations in the reactor were done with Merckoquant® test strips. Weekly measurements of ammonium, nitrite and nitrate were conducted using Hach Lange cuvette tests and analysed with a LASA 20 spectrophotometer. Hydroxylamine was measured according to the protocol described by Soler-Jofra *et al*. (Soler-Jofra et al., 2016).

The concentrations of solids, determined as total suspended solids (TSS) were measured according to Standard Methods (APHA, 2005). The morphology and size distribution of the biomass aggregates was monitored by image analysis, with a Lexmark Optra image analysis system.

FISH was used to analyse the distribution of the different microorganisms in the biomass. The collected small granules were washed in phosphate buffer and fixed using paraformaldehyde. Slicing was accomplished by embedding the granule in a tissue freezing medium (Leica Microsystems) and cut in frozen state with a microtome-cryostat into 20 µm slices (Leica CM1900-Cryostat). Dried slices were stored on a microscopic glass slide. Hybridization with fluorescent labelled oligonucleotide probes and analysis of the samples was performed as described by Lotti *et al.* 2014b. A formamide concentration of 35% was used for hybridization with the probes specific for, AOB (mix of NEU-653, NSO-190 and NSO-1225) (Mobarry et al., 1996; Wagner et al., 1995), NOB (mix of NTSPA-0712 and NIT-1035) (Daims et al., 2000; Juretschko et al., 1998) and eubacteria (EUB-338) (Daims et al., 1999).

Genomic DNA was extracted using the Ultraclean Microbial DNA extraction kit supplied by MOBIO laboratories Inc. (CA, USA) according to the manufacturers protocol, with the exception that the bead-beating was substituted by a combination of 5 minutes heating at 65°C and 5 minutes beat-beating, to ensure maximum yields. The extracted genomic DNA was subsequently used for a two-step PCR reaction targeting the 16Sr-RNA gene of most bacteria and archaea. For this we used the primers, U515F (5' – GTGYCAGCMGCCGCGGTA - 3') and U1071R (5'- GARCTGRCGRCRRCCATGCA- 3') as used by Wang et al. (Wang and Qian, 2009). The first amplification was performed to enrich for 16s-rRNA genes. The following chemicals were used: 2x iQ™ SYBR® Green Supermix (Bio-rad, CA, USA), 500nM primers each and finally 1-50ng genomic DNA added per well to a final volume of 20µl. The protocol was: denaturation at 95°C for 5min and 20 cycles at 95°C for 30 seconds, 50°C for 40

seconds, 72°C for 40 seconds and a final extension at 72°C for 7 minutes. During the second step, 454-adapters (Roche) and MID tags at the U515F primer, were added to the products of step one. The second step was similar to step one, except that Taq PCR Master Mix (Qiagen Inc, CA, USA) was used, the program was run for 15 cycles and the template was diluted 10 times. After the second amplification, 12 PCR products were pooled equimolar and purified over an agarose gel using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, The Netherlands). The resulting library was sent for 454 sequencing and run in 1/8 lane with titanium chemistry by Macrogen Inc. (Seoul, Korea).

After analysis the library was imported into CLC genomics workbench v7.5.1 (CLC Bio, Aarhus, DK) and (quality, limit=0.05) trimmed to a minimum of 200bp and an average of 284bp. After trimming the sample was de-multiplexed resulting in twelve samples with an average of 7800 reads per sample. A build-it SILVA 123.1 SSURef Nr99 taxonomic database was used for BLASTn analysis on the reads under default conditions. The top result was used to determine taxonomic affiliation and species abundance.

Results

Long term reactor operations

The reactor with the nitrifying culture was operated for more than two years. Obtaining a stable culture (based on conversions) proved difficult. An increase in the trace element concentration and elongation of the SRT were used to stabilise the conversions. Higher organisms (worms and mites) were observed in the sludge during periods of instability. During the 130 days described in this paper, the reactor was operating with stable conversions. Ten days prior to the period described in this paper 50 ml of fresh nitrifying sludge from the B-stage of the wastewater treatment plant of Dokhaven, Rotterdam was added to the system. The SRT was increased from 10.79±3.54 d to 19.46±7.40 d to improve the robustness of the system.

The conversion rates of the system were dictated by the feeding rate, only small amounts of ammonia and nitrite accumulated during the feeding period of three hours. In the first 10 days of the period described, more nitrite (1.5 mgNO2-N/L) was leftover at the end of the feed period. After increasing the SRT, more ammonium was left (<2mgNH_4-N L^{-1}). The substrate concentrations during a cycle are shown in Figure 5-1. Volumetric conversion rates for ammonium and nitrite were 0.40 gN (L $d)^{-1}$, resulting in an overall biomass specific activity (SA) of 0.37±0.04 gN (gTSS $d)^{-1}$ (in the period with the high SRT). The biomass concentration averaged over the same period was 1.09±0.11 gTSS L^{-1}, corresponding to an average yield of 0.18 gTSS gNH_4-N^{-1}. The long term biomass concentration and SRT are shown in Figure 5-2.

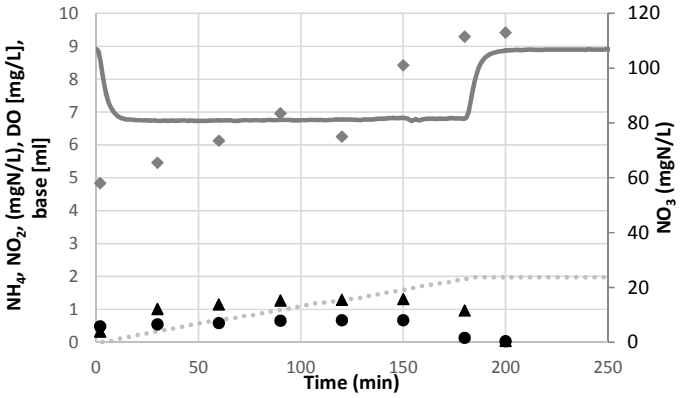

Figure 5-1: Cycle measurement of an average cycle around day 10, long term nitrification cultivation at 20°C, the feed of medium starts at 0 min and ends at 180 min. Concentrations of ammonium (dots), nitrite (triangles), nitrate (diamantes), amount of added base (dashed line) and DO concentration (solid line).

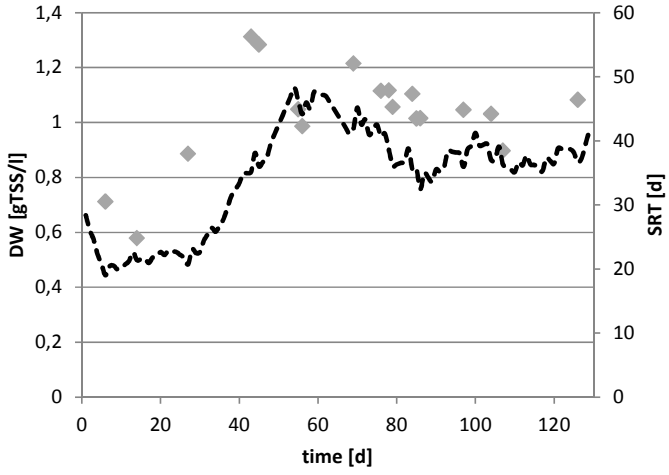

Figure 5-2: Long term data of nitrifying SBR at 20 degrees, yield and SRT are moving averages over 2 weeks . Aerobic SRT (dashed line) and biomass concentration (diamantes).

Microbiological analysis

The biomass in the reactor grew in the form of dense, small granules (or flocs, depending on the definition used) as shown in Figure 5-3. During the reactor run, the average granule diameter was 181±172 µm. When the biomass was crushed strong micro colonies were observed, these colonies probably contain AOB.

In nitrifying mixed cultures like the one described in this paper, some growth of heterotrophic bacteria will occur. Heterotrophic biomass can grow on the yeast extract present in the medium, traces of organic carbon in tap water and aeration air or on decaying biomass. Assuming a typical biomass yield of aerobic heterotrophs of 0.4 gBiomass per gram organic carbon expressed as COD, a

concentration of heterotrophs grown on yeast extract of 0.013 gTSS L^{-1} could be expected (approximately 1% of the total biomass).

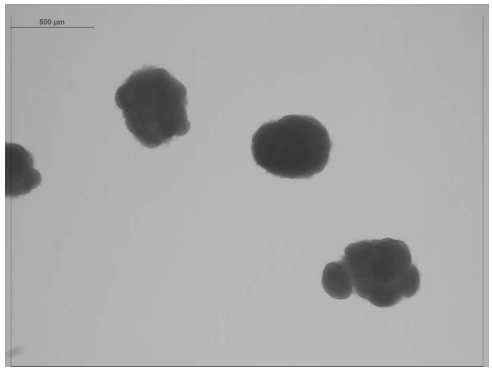

Figure 5-3: Microscopic picture of the biomass in the nitrifying SBR at 20 degrees.

Fish analysis was done on sliced granules. Upon hybridisation with AOB, NOB and eubacterial probes it was observed that active biomass was predominantly present in the outer layer of the granule (Figure 5-4). In the outer layer, different colonies of AOB and NOB were found, without a clear stratification in depth. In the core of the granule a structure was seen by phase contrast imaging, but limited hybridisation was observed. This biomass potentially is made up of dead cells or extracellular polymeric substances (EPS).

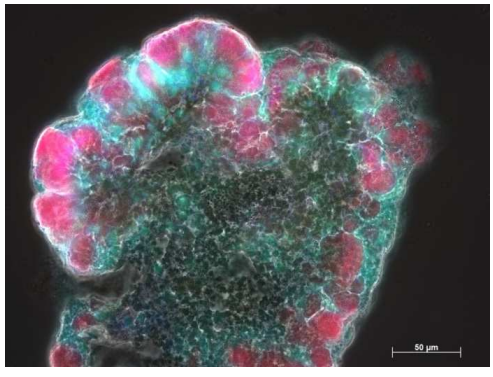

Figure 5-4: Picture of a sliced granule after hybridisation with FISH probes, AOB-Cy3 (red), NOB-fluos (green), Eub-Cy5 (blue) and phase contrast. No cells that were hybridised with the eubacterial probe and not with the AOB or NOB probe were observed.

To identify the type of AOB and NOB in the biomass, DNA was extracted, PCR-amplified and sequenced. The results of this analysis indicated that 50% of the OTU's is closely related (99%) to *candidatus Nitrospira defluvii* and *Nitrospira moscoviensis*. In the sequencing data no NOB from the nitrobacter or nitrotoga genus were detected. The AOB belong to the species *Nitrosomonas sp. Is79A* (97% related), corresponding to 6% of the OTU's.

A QPCR analysis indicated that comammox bacteria were present at 6.9±1.3% of the total bacterial population and the complete NOB population was measured to be 20.1±3.2% of the total bacterial population (Abbas et al., in prep).

Effect of temperature and DO

To measure the ammonium and nitrite conversion rates at low temperatures and low dissolved oxygen concentrations, dynamic batch tests were designed. The batch tests were performed in the reactor, with the biomass cultivated at 20°C, with an SRT of 19.46±7.40 d (second phase of cultivation in Figure 5-2). During the tests the DO concentration was varied by supplying air and dinitrogen gas (with supplemented carbon dioxide) in different ratio's and the tests were performed at different temperatures (10°C , 15°C and 20°C). A concentrated solution of ammonium and nitrite was supplied to the reactor (starting concentration of 25-35 mgN L^{-1} for both ammonium and nitrite) to obtain non-limiting substrate concentrations. After stabilisation of the DO (i.e. when stable conversion occurred) the conversion rates were measured by measuring the nitrogen species in the solution. After identifying the conversion rates, the DO was changed to a new value. Herewith conversion rates at different DO could be measured within one batch test. The results of an example of a dynamic batch test can be found in Figure 5-5.

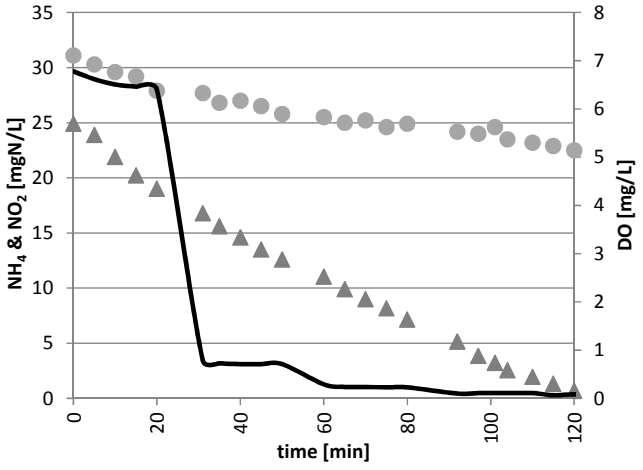

Figure 5-5: Nitrification by the enriched nitrifying sludge in a batch test at 20°C. DO concentration were varied (solid line) by varying the oxygen concentration in the gas flow, ammonium (dots) and nitrite (triangles) concentrations.

Multiple dynamic batch tests were done at three different temperatures (10°C, 15°C and 20°C). The data from these batch tests can be looked upon from different angles, the first is the difference in AOB and NOB activity. The maximum specific NOB activity (SA_{NOB}^{max} = 0.75±0.10 gN (gTSS d)$^{-1}$) was high compared to the maximum ammonium conversion rate (SA_{AOB}^{max} = 0.27±0.08 gN (gTSS d)$^{-1}$). During a normal cycle, the conversions of AOB and NOB were the same and defined by the supply rate of ammonium. The SA in the regular SBR cycle regime was 0.37±0.04 gN (gTSS d)$^{-1}$ for both organisms. The data suggest that for AOB no (or only a small overcapacity) was present, the NOB seemed to have a twice higher conversion capacity.

The maximum biomass specific activities of AOB and NOB as measured at high DO concentrations at different temperatures are presented in Figure 5-6. The conversion rates for both organisms decreased with decreasing temperatures, but the activity of NOB decreased by a factor of 2 (activation energy 44 kJ mol^{-1}) and the rate of AOB by a factor of 5 (activation energy 118 kJ mol^{-1}) (values for SA can be found in supplemented material).

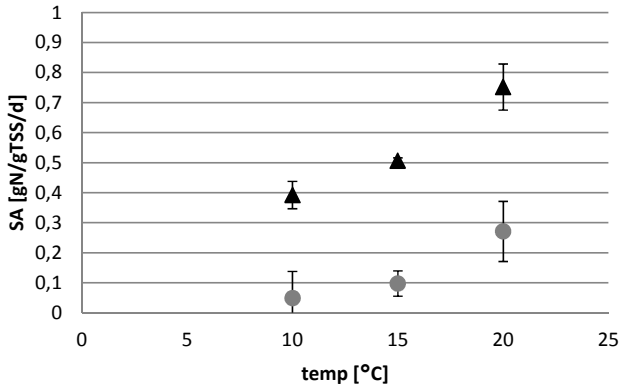

Figure 5-6: Results of multiple batch tests at different temperatures, with nitrifying sludge enriched at 20°C. At high DO concentration, in the presence of excess ammonium and nitrite. Biomass specific activity of AOB dots, biomass specific activity NOB in triangles.

A third point of interest is the effect of the dissolved oxygen concentration. To evaluate the effect of the oxygen concentration, the results of multiple batch tests were combined in 3 different figures (Figure 5-7, Figure 5-8 and Figure 5-9) for the 3 different temperatures (10°C, 15°C and 20°C). For the different tests, done at different days, there was a significant difference in maximum activity at high DO. The data presented is normalized (not normalized data can be found in supplemented material). The Monod equation was used to estimate the apparent half saturation coefficients for oxygen, the range for K_0 for AOB was 0.43-7.60 and for NOB 0.25-0.46, due to the poor fit of the Monod equation these values have big standard deviations. But it was clear from the conversion rates and the K_O values that the NOB in this culture had higher affinities for oxygen.

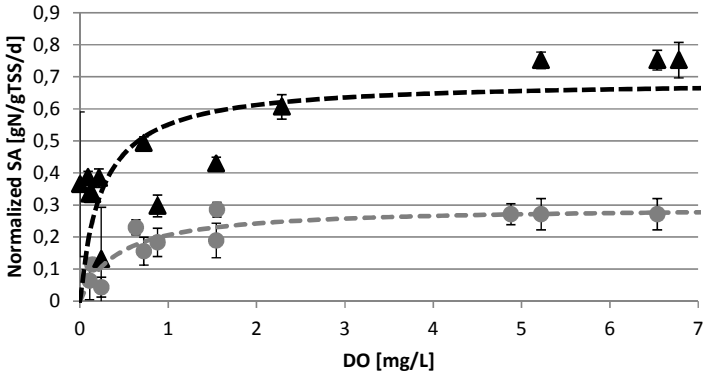

Figure 5-7: Results of 4 batch tests at 20°C, with nitrifying sludge enriched at 20°C. Normalized to be able to compare the different batch tests. Biomass specific activity of AOB dots, biomass specific activity NOB in triangles. Monod model in dashed line.

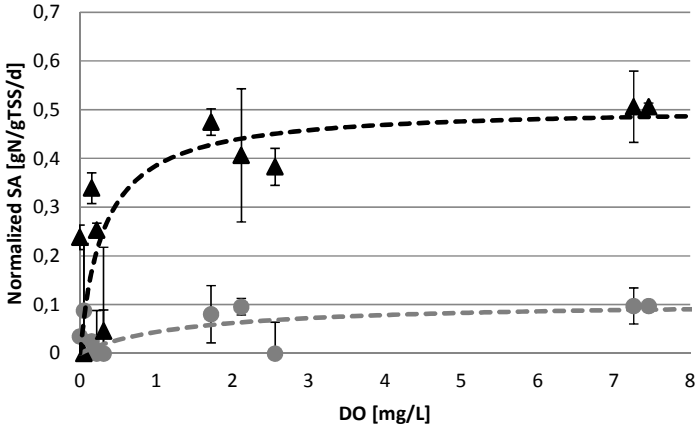

Figure 5-8: Results of 2 batch tests at 15°C, with nitrifying sludge enriched at 20°C. Normalized to be able to compare the different batch tests. Biomass specific activity of AOB dots, biomass specific activity NOB in triangles. Monod model in dashed line.

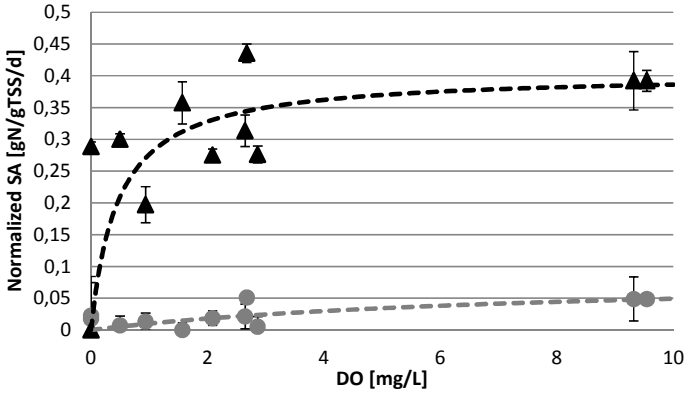

Figure 5-9: Results of 2 batch tests at 10°C, with nitrifying sludge enriched at 20°C. Normalized to be able to compare the different batch tests. Biomass specific activity of AOB dots, biomass specific activity NOB in triangles. Monod model in dashed line.

N_2O data

During stable reactor operations, no NO and very low concentrations of N_2O were measured in the off-gas. N_2O in the off-gas was only measurable during the feed period. After all ammonium and nitrite was consumed, no N_2O was detected in the off gas. The fraction of N_2O-N produced per ammonium converted was 0.05% (N_2O-N per NH_4-N). In the dynamic batch tests, the emissions were higher, 1.97±1.79% and 1.34±1.42% of the nitrogen load, for N_2O and NO respectively. The emission percentages at 20°C were similar to the emission of the 10°C tests. No correlation between the emissions and the nitrite or dissolved oxygen concentrations could be found. These observations were similar to those described in other nitrifying systems (Chandran et al., 2011). When ammonium and nitrite were present in the reactor, hydroxylamine concentrations were 0.05 mgN L^{-1}, when no substrate accumulated hydroxylamine was below the detection limit.

Discussion

Conversion rates

A nitrifying enrichment culture was established in an SBR with a long feeding period. Low (rate limiting) nitrite and ammonium concentrations during conversion were observed during feeding. The volumetric conversion rate 20°C was 0.40 gN (L d)$^{-1}$ during a normal cycle, with a biomass specific activity (SA) of 0.37±0.04 gN (gTSS d)$^{-1}$. In batch tests with no substrate limitation (DO, ammonium and nitrite), the SA^{max} for AOB was 0.27±0.08 gN (gTSS d)$^{-1}$and for NOB 0.75±0.10 gN (gTSS d)$^{-1}$. At 20°C the conversion rates reported in literature are often higher, for instance for the laboratory enrichment culture grown by Kampscheur *et al.* (2008) in a sequencing batch reactor with a dump feed: SA_{AOB}^{max} of 1.96 gN (gTSS d)$^{-1}$ and SA_{NOB}^{max} 1.3 gN (gTSS d)$^{-1}$ (Kampschreur et al., 2008). Moussa *et al* (2005) reported an SA_{AOB}^{max} of 1.2 gN (gVSS d)$^{-1}$ and SA_{NOB}^{max} 0.9 gN (gVSS d)$^{-1}$ in an SBR with a dump feed, operated at 30°C, SRT 100d (Moussa et al., 2005). The difference in biomass specific activity might be explained by the presence of inactive/inert biomass in the core of the small granules in our reactor (as was observed by FISH). The inactivity in the core might be caused by the elongated SRT.

In batch tests, the maximum AOB activity was comparable to the actual activity during normal operation, whereas the NOB activity was doubled. The maximum conversion rates of NOB were more than twice as fast as the AOB. In highly enriched nitrifying cultures it was observed that NOB from the *Nitrobacter* species have higher (approximately 3 times, both at 30°C) maximum rates as compared to AOB (Hunik et al., 1994; Wiesmann, 1994). But specific activities of *Nitrospira* type NOB were proposed to be considerably lower compared to N*itrobacter* (approximately 9 times, both at 30°C), and also lower compared to AOB (Blackburne et al., 2007). The dominance of *Nitrospira* NOB in this study does therefore not explain the observed relatively high nitrite oxidation rates in batch experiments.

In literature multiple studies described the adaptation/change in community of AOB cultures cultivated under different conditions (Dytczak et al., 2008). AOB cultivated under non ammonium rate limiting conditions (in a SBR) showed higher rates compared to AOB cultivated under ammonium rate limiting conditions (in a CSTR) (Terada et al., 2013). Cultivation at high substrate concentrations and low SRT selected for an AOB culture with high rates (Wu et al., 2016). These effects can be explained by a theory based on K-strategist and r-strategist, similar to the difference in *Nitrobacter* and *Nitrospira.* The composition of the AOB community can explain the relatively slow AOB in our study, but it does not explain the absence of the AOB overcapacity.

The biomass cultivated in this study was grown under substrate limitation (nitrite and ammonium), the enzyme complex responsible for ammonium conversion (AMO and HAO) is a complex system requiring a lot of proteins, and therefore significant energy to produce it. It is possible that the amount of enzymes is balanced by the required activity in the cell when the substrate is available in limiting amounts. A similar response is documented for *E. coli* (Ihssen and Egli, 2004). The central enzyme of NOB (NXR) is more simple and less energy intensive to produce and might not be balanced by the substrate limitation. Other possible explanations for the lack of AOB overcapacity, could be the selective washout or a higher decay rate of AOB.

Effect of oxygen concentration

The apparent half saturation coefficients for oxygen, for AOB were on the high end of the reported spectrum in literature. Contrary to that, the values for NOB were on the low end of the spectrum (Blackburne et al., 2007; Laanbroek and Gerards, 1993; Wiesmann, 1994). Overall the K_O of NOB was lower compared to the K_O of AOB. The difference in biofilm structure in the different cited studies has a significant effect on apparent half saturation constants, for instance the different sizes of the micro colonies (Picioreanu et al., 2016). In a stratified biofilm where NOB have been observed to be located in deeper biofilm layers, NOB would have more DO limitation compared to AOB on the biofilm surface (Hao et al., 2001). Based on this effect, the DO:NH_4 ratio can be used to selectively suppress the NOB (Lotti et al., 2014a; Pérez et al., 2014; Winkler et al., 2012b). Since the biofilms cultivated at low substrate concentration did not develop this stratification, the DO could not be used to selectively suppress the NOB.

Effect of temperature

The effect of decreasing temperature in a short term experiment (decrease from 20°C to 10°C, biomass cultivated at 20°C) was bigger for AOB (activation energy 118 kJ mol^{-1}) compared to NOB (activation energy 44 kJ mol^{-1}). A large range of activation energies has been described in literature (24.6-317 kJ mol^{-1})(Guo et al., 2010; Randall et al., 1982; Weon et al., 2004). The difference in

temperature dependencies between AOB and NOB is well known, and suggests that outcompeting of NOB on growth rate is only possible at higher temperatures (> 25°C) (Hellinga et al., 1998; Weon et al., 2004). Our data confirm this general trend and suggest that outcompeting NOB at lower temperatures is more complicated to achieve. This observation seems to hold true independent of the dominant microorganism responsible for nitrite oxidation, *Nitrobacter* or *Nitrospira*.

Microbial community

The results of the sequencing data seem to underestimate the AOB abundance, compared to the FISH data. The ratio observed by FISH imaging is probably the more accurate ratio, since sequencing results are semi-quantitative on the ratio between organisms (Kleiner et al., 2017; Props et al., 2017)

Comammox bacteria were detected in the nitrifying sludge which is an environment with low ammonium and nitrite concentrations, high DO and long SRT. Therefore the proposed high yield of comammox bacteria compared to AOB and NOB might have been an advantage in the biomass cultivated in this study (Costa et al., 2006; Daims et al., 2016; Van Kessel et al., 2015). But the ecological niche of comammox bacteria is not completely defined yet (Pjevac et al., 2016). Evaluation of the obtained *Nitrospira* sequences from our system indicated that potentially a third of the NOB population might have the comammox metabolism (based on qPCR). This seems too low to explain the relative high fraction of NOB observed in this system based on sequencing data.

Suppression of NOB

From literature it is known that competition between AOB and NOB can be influenced by the growth conditions, high substrate concentrations will lead to a segregated biofilm in which NOB can be suppressed based on oxygen limitation (Hao et al., 2001; Poot et al., 2016). A complementary way to outcompete NOB is the presence of a nitrite sink in the form of anammox or denitrifying bacteria. Anammox will consume the nitrite produced by AOB and multiple laboratory and pilot scale studies have shown this effect (Laureni et al., 2016; Lotti et al., 2015b; Lotti et al., 2014a). The combination of a nitrite sink with intermitted aeration is also possible (Gilbert et al., 2015; Ma et al., 2015; Wett et al., 2013).

Conclusion

The aim of this research was to study the possibility of NOB suppression based on DO and temperature, in a biofilm cultivated at low substrate concentration at 20°C (substrate concentrations similar to a CSTR). When nitrifying bacteria are cultivated in a SBR reactor with slow ammonium addition, (ammonium rate limiting conversions), a granular type of sludge occurs without stratification of ammonium and nitrite oxidising bacteria. Under these conditions it is not feasible to effectively suppress the nitrite oxidising bacteria. For granular sludge and biofilm systems effective presence of anammox (or denitrifiers) in the deeper layers is required to effectively outcompete nitrite oxidising bacteria in systems operated under ammonium rate limiting concentrations.

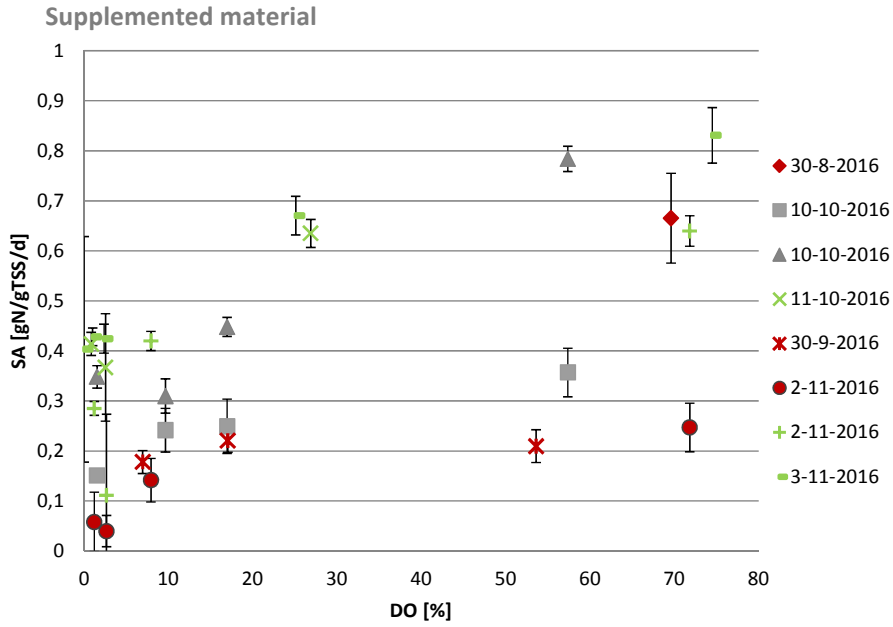

Figure 5-10: Data of 4 batch tests at 20°C,. SA_{AOB} in red, SA_{NOB} in green.

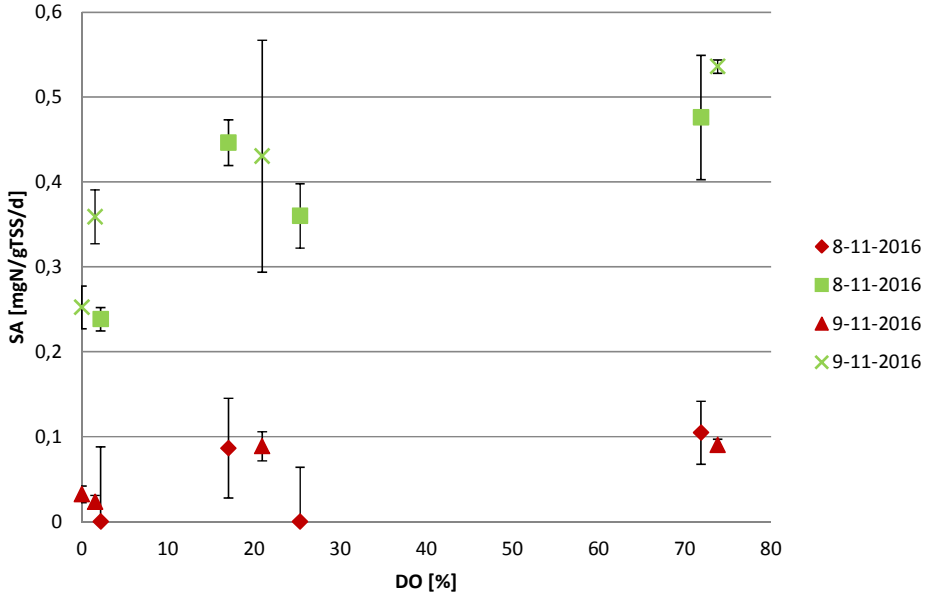

Figure 5-11: Data of 2 batch tests at 15°C,. SA_{AOB} in red, SA_{NOB} in green.

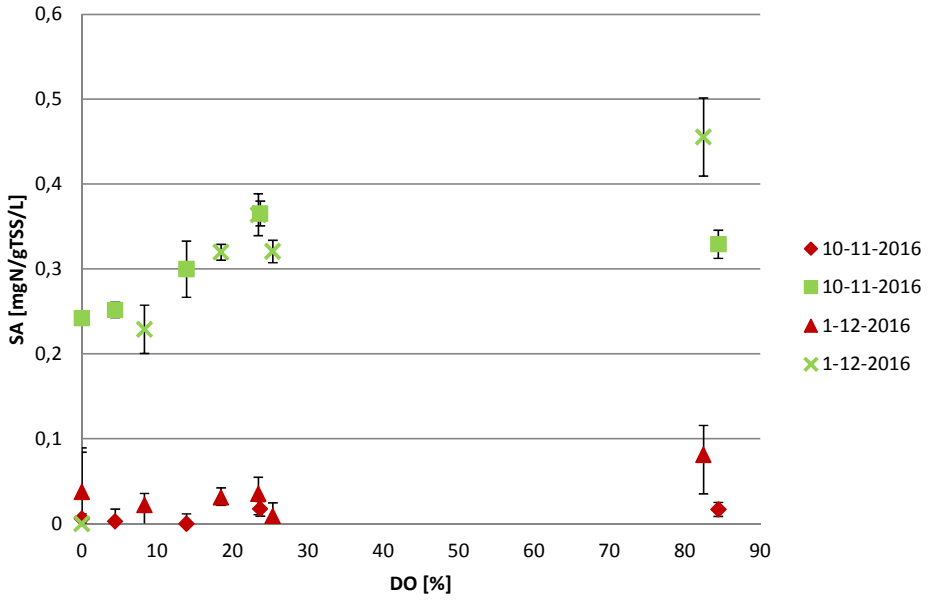

Figure 5-12: Data of 2 batch tests at 10°C,. SA_{AOB} in red, SA_{NOB} in green.

Table 5-2: high DO, excess ammonium and nitrite, cultivated at 20 degrees, substrate limited.

Temp [°C]	SA AOB [gN/gTSS/d]	SA NOB [gN/gTSS/d]
20	0.27±0.08	0.75±0.10
15	0.10±0.01	0.51±0.04
10	0.05±0.05	0.39±0.09

Table 5-3: Results of the sequence data on the genus level, the first 30 hits. AOB are binned in the 'others' fraction.

Taxonomy	day 73	day 128
Nitrospira	0,5798	0,5821
Others	0,2254	0,2416
Rhodobacter	0,1082	0,1012
Geobacter	0,0221	0,0105
Kaistobacter	0,0108	0,0003
Niabella	0,0102	0,0112
Serratia	0,0055	0,0010
Rhodocyclus	0,0038	0,0065
Sediminibacterium	0,0036	0,0130
Methylibium	0,0026	0,0097
Sphingopyxis	0,0022	0,0003
Halomonas	0,0022	0,0000
Gemmatimonas	0,0021	0,0013
Desulfosporosinus	0,0017	0,0000
Legionella	0,0016	0,0021
Flavobacterium	0,0015	0,0002
Citrobacter	0,0011	0,0010
Desulfovibrio	0,0011	0,0002
Rhodococcus	0,0009	0,0000
Microbacterium	0,0008	0,0002
Rhodanobacter	0,0007	0,0008
Rhodoferax	0,0007	0,0005
Dechloromonas	0,0007	0,0013
Hyphomicrobium	0,0007	0,0006
Dokdonella	0,0007	0,0003
Bacillus	0,0004	0,0000
Clostridium	0,0004	0,0001
Uliginosibacterium	0,0004	0,0002
Delftia	0,0004	0,0001
Dok59	0,0003	0,0005

6

Outlook

The implementation of the Partial Nitritation/Anammox (PN/A) process in the mainstream of a municipal wastewater treatment plant has been a hot topic within the biofilm/environmental technology arena in recent years. The work presented in this thesis contributes to the general effort to scale up this technology to the mainstream of municipal wastewater treatment plants. Before the implementation of the technology, the wastewater treatment plant of Dokhaven was used as a case-study. In the following pages I will highlight the main challenges I still see for the PN/A process. The first paragraph is a proposal for a new treatment scheme based on the PN/A technology, the second paragraph describes the essential steps that need to be taken before full-scale implementation can occur and the last paragraph contains suggestions for future research.

Treatment scheme

The proposed treatment scheme for municipal wastewater treatment plants (WWTPs) with PN/A technology in the mainstream is shown in Figure 6-1. The first step consists of the removal of solids and particulates (everything people throw in the toilet that is not biologically degradable) from the wastewater, due to sieving (not shown). Followed by a BOD (biological oxygen demand) removing step in the form of a high loaded A-stage (Adsorption) as in the AB-system (Böhnke, 1977). In this A-stage the water is mixed with sludge and aerated, the sludge has a short retention time (0.3d) and most BOD is removed together with the surplus sludge. The sludge/water separation can be improved by iron dosing which is also needed for phosphate removal. The removed sludge can be digested, to produce energy in the form of biogas. For separation of the produced sludge, membranes instead of clarifiers have been suggested (Laurinonyte et al., 2017). In warmer climates direct anaerobic treatment (UASB) can be a good alternative for the A-stage (Molinuevo et al., 2009). After the first BOD removal and collection stage mainly ammonium and potentially a small amount of BOD remains in the wastewater. The ammonium can be effectively removed in a PN/A stage, by the action of AOB (ammonium oxidising bacteria) and anammox bacteria.

For a full-scale PN/A reactor a plug flow design is chosen for effective suppression of NOB (nitrite oxidising bacteria). In a plug flow reactor ammonium will not be the limiting factor for the most time, a good condition for effective NOB repression. Using biofilms will offer a compact reactor/settling design and the potential to reach long solid retention times. The biofilm can be grown in the form of granular sludge or on carriers in a moving bed reactor. A compact biofilm separation unit (inside or outside the reactor) can be used to retain the biofilm in the reactor. For final effluent polishing like for suspended solids and post denitrification, a rapid filtration system can also be used, depending on the effluent quality demand. Secondary clarifiers can also be used for suspended solid removal.

In the proposed configuration a side stream reactor will not be needed, since the ammonium load can be effectively handled in the mainstream. However, having a side stream PN/A reactor might be useful to rapidly deliver biomass when bio-augmentation is needed.

This proposed treatment scheme might increase the energy generating capacity and could decrease the footprint (less surface and material costs) of the WWTP, compared to a conventional activated sludge system. Since most BOD will be removed from the sludge in the A-stage and can therefore be digested. The PN/A technology will be a good upgrade of the current AB-systems and will enable us to treat wastewaters in which the COD/N ratio is too low for denitrification. In an economical comparison with conventional treatment systems, the PN/A technology is the most energy efficient technology. The new treatment technology, based on aerobic granular sludge (Nereda) was not

taken into account. In this technology all conversions take place in one tank (which is operated as a sequential batch reactor), therefore it is likely even lower in in investment costs.

For the WWTP Dokhaven in Rotterdam there is an additional reason to look into the PN/A technology, the plant is built on the site of an old harbour. The installation is underground; it covers about four hectares and is in part two floors high. The installation is designed as an AB-system and, in principle, not suitable for far-reaching nitrogen removal. Despite various optimisations in recent decades, the total nitrogen removal remains limited to 60% and the annual average nitrogen concentration in the effluent is between 15-20 mg N L^{-1}. With conventional technologies, further removal of nitrogen is impossible due to the limited underground space. The application of mainstream PN/A technology can be a sustainable and cost-effective method of improving effluent quality at the WWTP Dokhaven and similar WWTPs.

Full-scale application

In the work described in this thesis, steps were taken in the direction of full-scale implementation of the PN/A technology in the mainstream of the municipal wastewater treatment plant. But before the first full-scale will be built, there are some final steps to be taken or topics to be studied, these are numbered in Figure 6-1. Interesting, but not essential (for full-scale implementation) research topics are discussed in the next paragraph

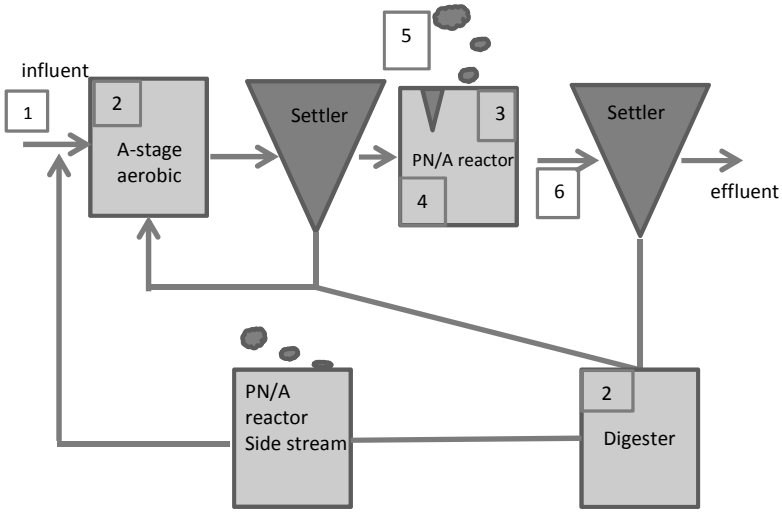

Figure 6-1: Proposed treatment scheme of a municipal wastewater treatment plant with implementation of the PN/A technology in the mainstream water line.

The temperature fluctuations of the influent (1) are an important parameter, this parameter cannot be influenced, but will have a significant effect on the design parameters of the plant (mainly on the required SRT of the autotrophic biomass (chapter 3)). The long-term effect of fluctuating temperatures needs to studied further, in the pilot work described in chapter 2, there is no clear long-term temperature effect observed. But in a laboratory scale anammox reactor, negative effects were observed (work described in chapter 4 and in literature (Laureni et al., 2016; Lotti et al.,

2015c)). If for implementation of the first full-scale a location with higher influent temperatures could be chosen, the implementation would be less delicate.

The modelling and pilot-scale research have shown that a good and stable carbon removing step (2), prior to the PN/A reactor is essential. A stable A-stage will help to increase the autotrophic fraction of the biomass and will lower the overall required biomass concentration. In an A-stage, the floc structure is vulnerable, a potentially improved flocculation process would decrease the BOD entering the PN/A reactor (De Graaff et al., 2016). And removing the BOD in the A-stage (or A-stage-like step) is the most efficient way to use the BOD for biogas production through digestion.

In the PN/a reactor the suppression of NOB in the biofilm (3) is another crucial factor. In the pilot study there were some indications that NOB started to proliferate after long-term operation. Whether this was due to the decreasing temperatures, change in biofilm structure or a long-term effect, remains unclear. This also relates to point 4, to prove the long-term stability of the system. Before (re)designing a full-scale installation, a demonstration is required that shows the conversions can be stable for a long period.

As described above, for the reactor design a plug-flow reactor can be chosen (5). To the extent of our knowledge a plug-flow design for mainstream anammox is not tested on a pilot-scale, so far.

One of the most essential objectives of the PN/A reactor is retaining the autotrophic biomass (6). Due to the low biomass production rates of AOB and anammox bacteria, a washout of sludge easily leads to disturbance in the operation. In the design proposed by Paques, there is a system for granule retention inside of the PN/A reactor. Separating granules from flocs has proven difficult in some reactors, when carriers are used the biofilms are easier retained within the system. But the use of carriers will lead to low oxygen transfer rates to the biofilm (Nogueira et al., 2015).

In my opinion, these six research topics described above, need to be addressed before a full-scale reactor can be operated.

Future research

This thesis took shape as an engineering thesis, mainly due to the work related to the pilot-scale research. Some of the proposed research topics are applied research, but there are also more fundamental topics which can be of interest for future research.

In relation to the greenhouse gas emissions, the role of hydroxylamine in the Ncycle, might be important. Hydroxylamine is an intermediate of the AOB pathway and a precursor for NO formation (and NO can react to N_2O). The presence of hydroxylamine is known to supress NOB (which is desired in the PN/A technology) and its role in the anammox pathway is currently under discussion. Knowledge of the role and triggers of hydroxylamine production could help to decrease the greenhouse gas emissions from a WWTP. Some preliminary measurements of N_2O in off gas are done, indicating that the emissions strongly relate to the mechanism of operation and reactor design.

Secondly, the removal rate of pharmaceuticals/micro pollutants in the redesigned wastewater treatment plant will be interesting, preliminary results from the pilot in Dokhaven have not shown much difference between the current B-stage and the PN/A pilot.

Another topic of interest might be the role of protozoa on the biofilm. The incoming BOD particulates will be consumed by protozoa. This layer on the biofilm could have multiple negative effects. Firstly, the layer acts as an additional diffusion layer, Secondly, the heterotrophic growth can consume so much oxygen that the AOB become oxygen limited. And finally, it is possible that the heterotrophic layer changes the stratification in the biofilm (a less dense biofilm can be formed between the stalks of the protozoa) and will therefore make the suppression of NOB more difficult. The protozoa were temporarily observed as a fluffy growth on the outside of the granules (Figure 6-2).

Figure 6-2: Granule from the pilot-scale reactor in Dokhaven, suffering from a protozoa bloom. Scale bar is 1 mm.

The SRT is an important factor in the PN/A technology. The SRT of the heterotrophic and autotrophic fractions should be uncoupled, to avoid high biomass concentrations and low percentages of autotrophic biomass. Recently it is proposed that the Extracellular Polymeric Substances (EPS) of the different autotrophic species (AOB, NOB and anammox bacteria) is not identical. In full-scale side stream PN/A reactors it was incidentally observed that the AOB activity is washed out of the system within a short time period. This could be explained by the presence of two different types of EPS, if they do not bind well to each other. The AOB layer could be a loosely bound shell around the anammox core and due to increased shear the complete AOB shell might disconnect from the granule. The EPS of the different species is not yet well characterized, this is the current focus of multiple people in our research group.

In literature growth rates of anammox bacteria at 30°C are described (Lotti et al., 2015a; Zhang et al., 2017). At lower temperatures the deterioration of anammox activity was observed (chapter 4). The mechanistic basis for these observations remains unclear. It could be related to viruses, behaviour of the ladderane lipids (Rattray et al., 2010) or availability of trace elements (Liu and Ni, 2015). The limited knowledge of the anammox biology (pathway and enzymatic conversions) is limiting the understanding of the temperature effect on anammox conversions.

In enrichment reactors, as used in this research, multiple bacteria species will be present in the reactor. This has a direct effect on activity measurements, measuring species specific rates will be impossible, since all measured rates are values for the 'complete mixed biomass'. To be able to compare the rates in enrichments studies among each other and to pure culture studies, we need to know what the amount of (for instance anammox) bacteria is in the biofilm. Secondly, it needs to be

known whether the bacteria are actively participating in conversions. Currently it is not possible to accurately measure these two. Thereby limiting the possibility to compare different measurements.

An interesting new organisms was discovered recently, the comammox bacteria (a *Nitrospira* type) (Daims et al., 2016; Van Kessel et al., 2015). These bacteria can execute the complete nitrification pathway, converting ammonium to nitrate. Comammox bacteria were identified in the nitrifying sludge, described in chapter 5, and are absent in the sludge from the pilot scale reactor. The presence of this organisms might explain strange results observed in previous research, for instance cases in which the amount of *Nitrospira* was much higher than what was proposed based on conversions (Winkler et al., 2012a). The ecological niche of the organism is not well defined, more knowledge of this organism will help in understanding all the conversions in the nitrogen removing biofilm.

More on the engineering side, a combination of anammox with DNRA (dissimilatory nitrate reduction to ammonium) could be interesting. The DNRA bacteria will convert the nitrate, inevitably produced by anammox, back to ammonium, which can be removed by anammox bacteria. This would increase the nitrogen removing capacity of the treatment plant. Anammox bacteria are also capable of executing the DNRA pathway themselves (in the presence of BOD), a pathway which is not yet studied in great detail (Kartal et al., 2007).

Considering the results of this thesis and the large amount of research currently being done worldwide, I believe that in the coming decade the PN/A technology will find its implication on a full-scale. For instance in emerging countries in which it will lead to a substantial improvement of water quality. Or as an upgrade for plants with limiting nitrogen removal capacity, but with a BOD removing step (A-stage, UASB). I hope that the technology and this thesis will contribute to the optimization of wastewater treatment. Hopefully the WWTP will evolve from a treatment plant to an energy and resource producing plant in the future.

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Curriculum vitae

Maaike Hoekstra was born on May 17th 1987 in Gouda and lived in Waddinxveen for 18 years. In 2005 she started her bachelor study Life Science and Technology at the Leiden University and Delft University of Technology. She interrupted her bachelor in 2007-2008 to be a board member of the study association, LIFE. In 2009 she started the master program Cell Factory at the TU Delft, including an internship at Bioclear, Groningen and a masters end project in the Environmental Biotechnology group under the supervision of Mario Pronk, Yuemei Lin and Mark van Loosdrecht. After graduating she worked on the aerobic granular sludge treatment technology Nereda at DHV, for a year. Coming back to Environmental Biotechnology at the TU Delft in 2013 for a PhD project on mainstream anammox under supervision of Robbert Kleerebezem and Mark van Loosdrecht. Her interest in wastewater brought her to Hoogheemraadschap Hollands Noorderkwartier, where she started in September 2017.

List of publications

Journal Articles

Hoekstra M, F.A. de Weerd, R. Kleerebezem, M.C.M. van Loosdrecht; Deterioration of the anammox process at decreasing temperatures and long SRT's; (2017); Environmental Technology

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

Soler-Jofra, A., B. Stevens**, M. Hoekstra M.,** C. Pichioreanu, D. Sorokin, M.C.M. van Loosdrecht, J. Pérez; (2015); Importance of abiotic hydroxylamine conversion on nitrous oxide emissions during nitritation of reject water; Chemical Engineering Journal

Lotti, T., R. Kleerebezem, C.S. van Erp Taalman Kip, T.L.G. Hendrickx, J. Kruit, **M. Hoekstra**, M.C.M. van Loosdrecht.; (2014); Anammox growth on pretreated municipal wastewater; Environmental Science & Technology

Other publications

Hendrickx, T.L.G., J. Vogelaar, S. Geilvoet, O. Duin, C.S. van Erp Taalman Kip, **M. Hoekstra;** (2017); CENIRELTA: demonstratieproject Anammox in de hoofdstroom op rwzi Dokhaven, STOWA rapport 2017-27

Geilvoet, S., C.S. van Erp Taalman Kip, O. Duin, T. Hendrickx, **M. Hoekstra**; (2016); CENIRELTA: hoofdstroom-Anammox op RWZI Rotterdam-Dokhaven; H2O

Conference contributions

Hoekstra, M., S.P. Geilvoet, C.S. van Erp Taalman Kip, T.L.G. Hendrickx, R. Kleerebezem, M.C.M. van Loosdrecht; (2017); Autotrophic nitrogen removal from mainstream wastewater, during winter; poster presentation; IWA international conference on biofilm reactors, Dublin

Hoekstra, M., S.P. Geilvoet, C.S. van Erp Taalman Kip, T.L.G. Hendrickx, R. Kleerebezem, M.C.M. van Loosdrecht; (2017); Autotrophic nitrogen removal from mainstream wastewater, during winter; poster presentation; Frontiers International Conference on Wastewater Treatment, Palermo

Hoekstra, M., R. Kleerebezem, M.C.M. van Loosdrecht; (2016); Kinetics of anammox bacteria in a membrane bioreactor, the effect of temperature; poster presentation; MEWE Copenhagen 2016

Mishima, I., **M. Hoekstra**, J. Pérez, M.C.M. van Loosdrecht; (2016); Characteristics of Nitrogen Removal with Partial Nitrification and Anammox in a Fixed-film Reactor; Annual Conference of Japan Society on Water Environment, Japan

Hoekstra, M., J. Pérez, A. Soler-Jofra, M.C.M. van Loosdrecht; (2016); The contribution of hydroxylamine in a partial nitritation/anammox biofilm; poster presentation; MEWE Copenhagen 2016

Hoekstra, M., S.P. Geilvoet, C.S. van Erp Taalman Kip, T.L.G. Hendrickx, R. Kleerebezem, M.C.M van Loosdrecht; (2016); Autotrophic nitrogen removal from mainstream wastewater, during winter; oral presentation; ecoSPT16 Cambridge

Hoekstra, M., R. Kleerebezem, S.P. Geilvoet, C.S. van Erp Taalman Kip, T.L.G. Hendrickx, M.C.M van Loosdrecht; (2015) Behaviour of anammox bacteria at temperatures below 20 °C; Poster presentation; Nutrient removal and recovery IWA, Gdansk 2015

Geilvoet, S., C.S. van Erp Taalman Kip, T.L.G. Hendrickx, **M. Hoekstra;** (2015); Anammox technology in the main stream of wastewater treatment plant Rotterdam-Dokhaven; Oral presentation; Nutrient removal and recovery IWA, Gdansk 2015

Hoekstra, M., S. Geilvoet, C.S. van Erp Taalman Kip, T.L.G. Hendrickx, R. Kleerebezem, M.C.M. van Loosdrecht; (2015); Autotrophic nitrogen removal from mainstream wastewater in the winter; Oral presentation; Young water professionals Benelux, Leeuwarden 2015

Hoekstra, M., T. Lotti, R. Kleerebezem, C.S. van Erp Taalman Kip, T.L.G. Hendrickx, M.C.M. van Loosdrecht; (2014); Towards energy neutral wastewater treatment by implementing Anammox in the mainstream, a pilot scale evaluation and implementation at Dokhaven WWTP; Oral presentation, Leading Edge Technology conference IWA, Abu Dhabi 2014

Hoekstra, M., T. Lotti, R. Kleerebezem, M. van Loosdrecht; (2014); The effect of temperature on maximal growth rate of anammox bacteria; Poster presentation; Ncycle meeting; Ghent 2014

Workshops & invited talks

Hoekstra, M., S. Geilvoet, C.S. van Erp Taalman Kip, T.L.G. Hendrickx; CENIRELTA: Nitrogen removal from municipal wastewater based on the Anammox process; Workshop: “Anammox based systems: Current Status, Challenges and Opportunities”, 6th IWA International Conference on Microbial Ecology and Water Engineering MEWE (2016), Technical University of Denmark - DTU, Copenhagen; oral presentation

Hoekstra, M.; A cold and granular CANON; Workshop: “Mainstream NOB Out-Selection and Anammox Retention: Where Do We Stand After 5 Years of R&D?”, WEF/IWA Chicago, (2016)

Lotti, T., R. Kleerebezem, C.S. van Erp Taalman Kip, T.L.G. Hendrickx, J. Kruit, **M. Hoekstra**, M.C.M. van Loosdrecht; Anammox growth on pretreated municipal wastewater; Workshop: “Anammox goes mainstream”, Amsterdam International Water Week (2015), Amsterdam; organisation and oral presentation

Hoekstra, M., S. Geilvoet, C.S. van Erp Taalman Kip, T.L.G. Hendrickx; CENIRELTA: Nitrogen removal from municipal wastewater based on the Anammox process; Workshop: “Anammox goes mainstream”, Amsterdam International Water Week (2015), Amsterdam; organisation and oral presentation

Hoekstra, M.; In zomer en winter stikstof verwijderen uit huishoudelijk avalwater; Vakantie cursus, TU Delft, (2014)

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Proposition 11: Your thesis is never finished, you just run out of time.
And if you run out of time, you still have to write the acknowledgements! Writing a PhD thesis might be a personal quest and the topic might be some sort of a island within the research group, but I have not done it alone.

Mario and Yuemei, thank you for offering me a masters end project in 2011. *EPS production by aerobic granular sludge at high salt concentrations* was the start of my time at Environmental BioTechnology (EBT). I learned a lot from both of you (in this first year, but also in the following years) and you showed me the fun of granular sludge and wastewater research. And you showed me the possibility of a career in wastewater. Of which I will always be grateful. Our collaboration became less during my PhD research, but I'm convinced that our ways will never completely separate. The (waste) water world is small.

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Wat betreft het CENIRELTA team (Tim, Stefan, Charlotte, René, Olaf, Jaap): jullie hebben mij kennis laten maken met de echte wereld van afvalwater. Elke donderdag in Rotterdam probeerde we de activiteiten van de pilot (sorry, demonstratie installatie) te doorgronden of dat echt gelukt is, zal de toekomst ons leren! Door jullie realiseerde ik me dat er meerdere manieren zijn om met afvalwater bezig te zijn: er is meer dan de universiteit. Dat ik nu bij een waterschap werk (ook al is het niet Hollandse Delta) heb ik helemaal aan jullie te danken.

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At my first conference in Abu Dhabi, I found some fellow mainstream anammox people and the team kept growing. Michele, Jan-Michael, Eva, Dries, Haydée, Jan, Siegfried, Vojtěch and all other mainstream anammox researchers, we have met at many conferences and workshops and thanks to all of you the topic felt more alive.

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In short, only my name is on the cover of this thesis, but luckily did not do it alone.

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