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Developing Anammox for mainstream municipal wastewater treatment

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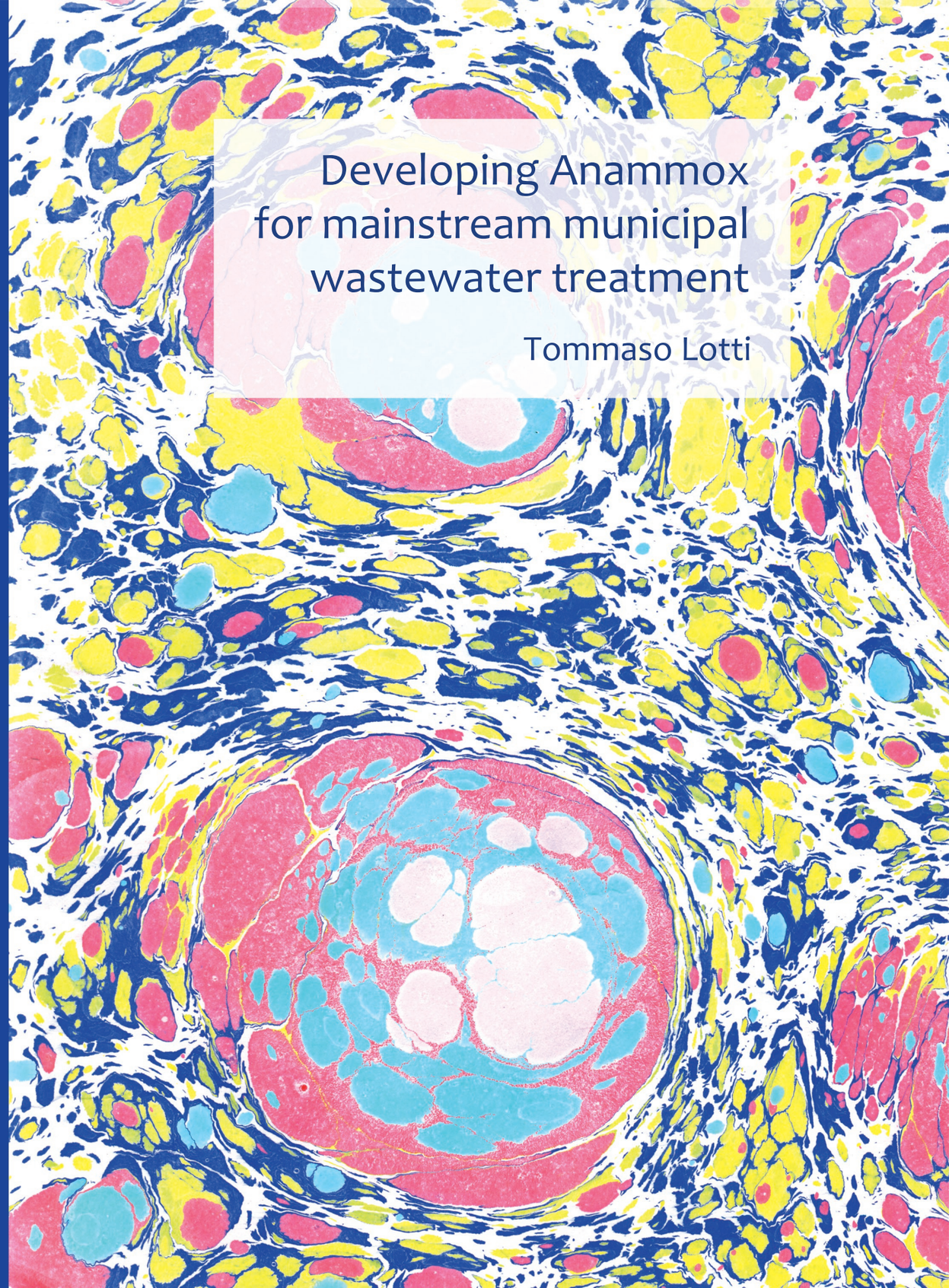

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Developing Anammox for mainstream municipal wastewater treatment

Proefschrift

ter verkrijging van de graad van doctor

aan de Technische Universiteit Delft,

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voorzitter van het College voor Promoties,

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alla mia famiglia

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Summary

Conventional wastewater treatment plants (WWTPs), like activated sludge systems, are energy demanding requiring a large electrical energy supply (e.g. 25 kWh PE^{-1} $year^{-1}$) which, especially during peak-load periods, may account for an important quote of the grid installed power of the surrounding area. Only across the EU, there are 16000 WWTPs that consume around 10000 GWh $year^{-1}$ of electricity. Furthermore, the volume of wastewater treated in WWTPs in the EU is increasing with a rate of around 7% each year. Besides the related financial costs, this energy consumption creates an additional environmental burden.

Considering that energy in Europe is mainly produced from the burning of fossil fuels, it has been calculated that energy consumption from WWTPs creates emissions of more than 27 Mtonnes $year^{-1}$ of CO_2 in the EU. Concerns about greenhouse gas emissions on a global level and cost issues on a microeconomic level have become major driving forces towards a more efficient usage of energy in wastewater treatment. In conventional wastewater treatment about 50% of the energy input is consumed in the aeration systems in order to remove organic matter (Chemical Oxygen Demand, COD) while about 25% is consumed in the nitrogen removal process (nitrification/denitrification) (Siegrist et al., 2008). Autotrophic nitrogen removal by anammox bacteria is to date the most efficient and environmentally friendly process for the treatment of ammonium in wastewaters and its application can save up to 60% of the energy input needed for nitrification. Application of anammox to municipal sewage treatment appears as a prerequisite to allow treatment scenarios for wastewater treatment plants with a net energy production (Kartal et al., 2010a). In a treatment scheme where nitrogen is removed via an autotrophic metabolic pathway such as partial nitritation/anammox (PN/anammox),the COD load, which is

conventionally oxidized to Co_2 partly with oxygen and partly with nitrate (No_3^-) in the denitrification process, can be used to generate energy in the form of methane-rich biogas via the anaerobic digestion process.

Whilst the application of anammox related technologies in the side-stream is at present state of the art, the feasibility of this energy-efficient process in main-stream conditions is still under investigation. Lower and variable operating temperatures and ammonium concentrations, together with a demand for high and stable nitrogen removal efficiency, represent the main challenges to overcome for this appealing new frontier of the waste water treatment field.

The research described in this thesis aimed at investigating the physiology and kinetic properties of anammox bacteria and their interaction with other microbial communities under municipal wastewater conditions with the ultimate scope of elucidating the boundary conditions for the application of the anammox-based process (PN/anammox) in the treatment of municipal sewage. This fundamental knowledge allow to design and successfully implement at lab- and pilot-scale the completely autotrophic nitrogen removal process for the treatment of municipal sewage. This thesis comprises therefore both fundamental and applied research which main results and achievements are briefly illustrated in this *summary*.

Although anammox related technologies are currently widely applied for nitrogen removal from sewage sludge digester rejection water, many aspects of the anammox process like the kinetic characteristics and the reaction stoichiometry are still under investigation. Parameter values reported in literature are often influenced by mass transfer limitation or by the presence of inactive cells and a significant side population. In **Chapter 2** a membrane bioreactor (MBR) based method for growing a highly enriched anammox microbial community is described. The almost pure free-cell suspension of highly active anammox bacteria was used for detailed kinetic and stoichiometric analysis of the anammox process. The yield of biomass production on ammonium uptake was calculated to be 0.071 C-mol N-mol^{-1}, value that was then experimentally confirmed in **Chapter 3**. The elemental biomass composition was measured as $CH_{1.74}O_{0.31}N_{0.20}S_{0.01}P_{0.01}$ (22.1 g C-mol^{-1}). From the yield and the elemental biomass composition the macro-chemical

reaction equation was identified and validated by long-term reactor operations. The anammox culture described in **Chapter 2** exhibited an unreported high biomass specific maximum growth rate of 0.21 d^{-1} corresponding to a doubling time of 3.3 days at 30°C. Using an experimental methodology based on imposing dynamic process conditions combined with process modeling and parameter estimation, the intrinsic nitrite half saturation constant was identified to be as low as 35 µg-N L^{-1}. This was confirmed to be a stable value in the tested pH range of 6.8-7.5.

Using the same system, in **Chapter 3** the stoichiometric and kinetic properties of a suspended anammox enrichment culture were investigated at decreasing solid retention times. This procedure enabled the maximum growth rate (μ^{max}) of the anammox enrichment culture to increase to 0.334 d^{-1}, which is four times higher than previously reported in literature and almost 60% higher than observed in **Chapter 2**. Even though researchers have speculated about the possibility of higher rates before, these speculations were always based on indirect measurements of the kinetic properties. Herewith **Chapter 3** reports the first direct experimental evidence for a significant increase in growth rate of an anammox enrichment culture. Since the biomass yield of the enrichment culture established is largely comparable to previous studies, it can be concluded that the increased growth rate results from an equivalent increase in biomass specific electron transfer capacity. Detailed molecular analysis did not reveal either a shift in dominant anammox strain nor major mutations in the dominant strain, suggesting that the actual reasons for the increase in electron transfer capacity is due to small changes in the metabolic machinery. The dominant strain throughout this experiment was closely related to *Candidatus* Brocadia Sp.40 (99% similarity). In this study anammox bacteria were cultivated applying a novel selection strategy based on the maximization of the electron transfer capacity demonstrating that maximum growth rate is not an intrinsic process property but that it can be increased significantly when the adequate cultivation conditions are imposed. The anammox enrichment became faster through training showing kinetics comparable with other chemolithoautotrophs and it is thereby concluded that anammox can no longer be regarded as intrinsically slow growing microorganism.

Nitrite is one of the main substrates of the anammox metabolism, but it is also an inhibitor. Its negative effect on anammox activity has been reported

widely during the past decade. Although the adverse effect is clear, conflicting reports exist on the level at which it occurs and its reversible/irreversible nature. In order to elucidate this important aspect, an in-depth study on nitrite inhibition was performed in which the influence of environmental factors was evaluated (**Chapter 4**). Anammox activity was measured in anammox granules by continuously monitored standardized manometric batch tests extending the interpretation by evaluation of lag times, maximum conversion rates during the tests and substrates/product conversion ratios. The granules, dominated by anammox organisms belonging to the *Brocadia* type, where sampled from a single-stage anammox full-scale reactor. The observed 50% activity inhibition for nitrite (IC_{50}) was 0.4 g-N L^{-1}. It was shown that biomass relatively quickly (and totally) recovers from high nitrite concentrations. The recovery after exposure indicates that the adverse effect of nitrite is reversible and thus inhibitory rather than toxic in nature. The effect of the presence of ammonium and oxygen during nitrite exposure has also been evaluated. Similarities between exposures at three different pH values suggest that nitrite rather than nitrous acid is the actual inhibiting compound. Overall the results reported in **Chapter 4** further underline that the anammox process can be a stable process not prone to temporarily adverse effects of oxygen and nitrite in the reactors. From our experience and previous observations we speculate that cultivation conditions and status of aggregation influence the inhibitory effect of nitrite and that in several cases where high nitrite is reported as a cause of activity loss, it might well be that activity loss has *resulted* in the accumulation of high nitrite concentrations rather than *causing* them.

The temperature effect on anammox activity is a crucial aspect that needs to be clarified for the successful implementation of anammox related processes at mainstream conditions. Lower operating temperatures in fact, together with lower ammonium concentrations and the demand for high and stable nitrogen removal efficiency, represent the main challenges to overcome for this appealing new frontier of the waste water treatment field. In **Chapter 5** is reported the short-term effect of temperature on the maximum biomass specific activity of anaerobic ammonium oxidizing bacteria as evaluated by means of batch tests. The experiments were performed on anammox biomass sampled from two full-scale reactors and two lab-scale reactors, all characterized by different reactor configurations and operating conditions. The

results indicate that in the temperature range of 10-30°C the temperature dependency for the anammox conversion cannot be accurately modeled by one single Arrhenius coefficient (i.e. θ) as typically applied for other biological processes. The temperature effect is increasing at lower temperatures, complicating the implementation of a stable mainstream process in winter conditions. Nevertheless, we observed adaptation of anammox bacteria after long term cultivation at 20 and 10°C indicating that also the history of the sludge impacts the temperature effect. Anammox sludge cultivated in an aerated partial nitritation/anammox process and/or in biofilm seemed to be less influenced by a decrease in temperature then anammox sludge grown under non aerated conditions and/or in suspension. The results reported in **Chapter 5** indicate that the temperature effect is stronger for anammox than for ammonium oxidizing bacteria (AOB), suggesting that, in order to maintain overall a good nitrogen removal along daily and seasonal temperature fluctuations, process control to balance the activity of both microbial groups needs to be adaptive to changes in relative rates of the two processes. Implications for modeling and process design are finally discussed.

In **Chapter 6** the application of the single-stage PN/anammox process at conditions relevant for sewage treatment was investigated in a lab-scale gas-lift sequencing batch reactor with granular sludge operated for more than 500 days. The reactor was operated at temperatures between 20 and 10°C and fed with synthetic autotrophic medium with ammonium (60 and 160 mg-N L^{-1} as only nitrogen compound at an HRT of 0.23-0.3 d. In the presence of ammonium dissolved oxygen was shown to be an effective control parameter, even at higher level than previously assumed (up to 2.5 mg-O_2 L^{-1}, for the suppression of the undesired nitratation process catalyzed by nitrite oxidizing bacteria (NOB). This control strategy guaranteed the effective suppression of the nitratation process both at 20 and 15°C, allowing nitrogen removal rates of 0.44 and 0.40 g-N_{Tot} L^{-1} d^{-1}. Unlike previously reported, these high removal rates were obtained together with optimal nitrogen removal efficiencies of 86 and 73%, respectively, fulfilling a decisive prerequisite for the implementation of the PN/anammox process in the main-stream of WWTPs. Anammox bacteria were shown to grow in the system, with estimated growth rate of 0.017 d^{-1} at 15°C. Operating conditions influencing N_20 emissions were also investigated and resulted in the observation of a positive correlation with the nitrite concentration in the bulk

whilst no clear correlation could be noticed between N_20 emissions and DO concentration or temperature. Unfortunately prolonged operation at 10°C caused a slow but unrestrainable decrease in anammox activity and process efficiency. Nevertheless, since in general these temperatures (winter conditions) do not extend over long time in moderate climates this is not seen as a limitation for the applications of anammox-based technologies in the mainstream of wastewater treatment plants. **Chapter 6** represents therefore a proof of concept for the application of the autotrophic nitrogen removal in a single reactor with granular sludge at mainstream conditions.

The next logical step in this challenging exploration of anammox bacteria capabilities was to investigate their behavior in the real mainstream of a sewage treatment plant. In **Chapter 7** we report the evaluation of the anammox process in a granular sludge fluidized bed lab-scale reactor continuously fed with the actual effluent of the A-stage of the WWTP of Dokhaven, Rotterdam (The Netherlands). In order to exclude the influence of oxygen and the competition for nitrite on anammox growth, the reactor was anoxic and nitrite was dosed continuously to support anammox activity only. The effect of influent COD and related heterotrophic growth by denitrification was instead included in the evaluation. The exclusion of oxygen was also intended in order to better evaluate the effect, if any, of potential toxic compounds in wastewater (e.g. from the influent or the addition of polyelectrolyte and technical grade iron salts in the A-stage). The system was operated for more than ten months at temperatures between 20 and 10°C. Volumetric N-removal rates obtained were comparable or higher than those of conventional N-removal systems, with values higher than 0.4 g-N L^{-1} d^{-1} when operated at 10°C. The biomass specific N-removal rate at 10°C was on average 50±7 mg-N g-vss^{-1} d^{-1} during last month of operations, almost two times higher than previously reported activities at this temperature. FISH analysis revealed that the dominant anammox species was *Candidatus* Brocadia Fulgida throughout the experimentation. Evidence for growth of anammox bacteria at main-stream conditions (i.e. anammox biomass increase and nitrate production in absence of oxygen) was demonstrated for the entire temperature range tested (10-20°C). Capability of granulation in the mainstream matrix under operative conditions was also proved since new granules were shown to be actively formed and efficiently retained in the proposed system. COD was also

consumed during the process, but heterotrophs could not outcompete anammox bacteria. In **Chapter 7** the capability of anammox bacteria to thrive under municipal wastewater conditions (low temperature, low ammonium concentration and presence of COD) was demonstrated for the first time, opening new perspective for the implementation of a more efficient (municipal) wastewater treatment chain.

For the application of the autotrophic nitrogen removal process, the first step of partial nitritation performed by ammonium oxidizing bacteria (AOB) has to be also accomplished in order to produce the nitrite used in the anammox process. During partial nitritation the nitratation process performed by nitrite oxidizing bacteria (NOB) has to be suppressed. Even though in **Chapter 7** it was demonstrated that anammox itself does not represent a problem, the managing of AOB and NOB activities in order to meet effluent standards might prove more complex for the direct application of the partial nitritation-anammox process on municipal wastewater. With the aim of evaluating the coupling of the anammox and partial nitritation processes at municipal wastewater conditions and the simultaneous suppression of the nitratation process, a pilot-scale experimentation was performed. In **Chapter 8** we report the evaluation of the process in a plug-flow granular sludge based pilot-scale reactor (4 m^3) continuously fed with the actual effluent of the A-stage of the WWTP of Dokhaven, Rotterdam. The one-stage partial nitritation-anammox system was operated for more than ten months at 19±1 °C. Observed average N-removal and ammonium conversion rates were comparable or higher than those of conventional N-removal systems, with 182±46 and 315±33 mg-N L^{-1} d^{-1} respectively. Furthermore, considering the higher biomass concentration obtainable in granular systems and the possibility for further anammox enrichment in the biomass, kinetics much higher than conventional systems appear to be feasible. BOD was also oxidized in the system with average removal efficiency of 90%. The system was shown to efficiently retain granules enriched in anammox bacteria with a small fraction of nitrifiers and heterotrophs located in the outer rim. At the same time, suspended flocs enriched in heterotrophs, and a small fraction of nitrifiers, were preferentially washed-out, allowing the system to withstand occasional COD and solids shock loads. The results reported in **Chapter 8** show that the proposed reactor configuration with granular sludge has the potential to be successfully applied

for the completely autotrophic nitrogen removal from the mainstream of WWTPs.

In summary, the research described in this thesis showed for the first time the feasibility of an innovative technology for the removal of nitrogen from wastewater and posed a solid background to open *de facto* a new era in which the treatment of wastewater will move from the actual energy depleting to an energy generating process.

Tommaso Lotti, september 2015

Samenvatting

Conventionele rioolwaterzuiveringsinstallaties (RWZI's), zoals actief slib systemen, hebben hoge eisen qua elektrische energievoorziening (typisch 25 kWh PE^{-1} $jaar^{-1}$) die, in het bijzonder tijdens piekbelasting, een grote belasting vormen voor het electriciteitsnetwerk. Alleen al in de EU zijn er 16.000 RWZI's die gezamenlijk ongeveer 10.000 GWh $jaar^{-1}$ aan elektriciteit verbruiken. Verder neemt de hoeveelheid afvalwater die behandeld wordt in RWZI's in de EU toe met ongeveer 7% per jaar. Naast de gerelateerde financiële kosten, zorgt dit energieverbruik voor een extra belasting van het milieu. Gezien het feit dat energie in Europa voornamelijk wordt geproduceerd uit de verbranding van fossiele brandstoffen, is berekend dat het gezamenlijke energieverbruik van alle RWZI's in de EU verantwoordelijk is voor meer dan 27 Mton/jaar aan CO_2 emissie. De bezorgdheid over de uitstoot van broeikasgassen op mondiaal niveau en de kosten op een micro-economisch niveau zijn belangrijke drijvende krachten om te komen tot een efficiënter gebruik van energie in de behandeling van afvalwater. Bij gebruikelijke afvalwaterbehandeling wordt ongeveer 50% van de energie verbruikt in het beluchtingssysteem om organisch materiaal (ook wel uitgedrukt als Chemisch Zuurstof Verbruik; CZV) te verwijderen terwijl ongeveer 25% wordt verbruikt voor de stikstofverwijdering door middel van nitrificatie en denitrificatie (Siegrist et al ., 2008).

Autotrofe stikstofverwijdering door Anammoxbacteriën is de meest efficiënte en milieuvriendelijke procesvorm voor de behandeling van ammonium uit afvalwater. Door gebruik te maken van het Anammoxproces kan tot wel 60% bespaard worden op het energieverbruik ten opzichte van conventionele stikstofverwijdering. Toepassing van Anammox op huishoudelijk afvalwater wordt als een interessante optie gezien voor toekomstige rioolwaterzuiveringsinstallaties met een netto energieproductie in plaats van -

gebruik (Kartal *et al.*, 2010a). In een toekomstig afvalwaterbehandelingsproces waarin stikstof wordt verwijderd via een microbiële autotrofe conversie zoals partiële nitritatie / Anammox (PN/A). Het CZV, dat gewoonlijk wordt geoxideerd tot CO_2 met zuurstof of nitraat (tijdens denitrificatie), wordt omgezet naar methaanrijk biogas via anaerobe vergisting. Op dit moment worden alleen nog maar deelstroombehandelingen met behulp van het Anammoxproces toegepast, maar de mogelijkheden om in de toekomst de hoofdstroom ermee te behandelen wordt op diverse plaatsen onderzocht. De voornaamste te overwinnen uitdagingen om tot een succesvolle, stabiele en efficiënte stikstofverwijdering in de hoofdstroom met behulp van Anammox te komen zijn de variabele en lage temperaturen en concentraties ammonium.

Het onderzoek dat in dit proefschrift is beschreven was erop gericht inzicht te krijgen in de fysiologie en de kinetische eigenschappen van Anammox-bacteriën en hun interactie met andere microbiële gemeenschappen onder huishoudelijk afvalwater-condities, zodat uiteindelijk de randvoorwaarden voor de toepassing van een Anammox gebaseerd proces beschreven kunnen worden voor de behandeling van huishoudelijk afvalwater. De vergaarde fundamentele kennis kan worden benut om een volledig autotroof stikstofverwijderingsproces te ontwerpen voor de behandeling van stedelijk afvalwater. Dit proefschrift omvat derhalve zowel fundamenteel als toegepast onderzoek en de belangrijkste resultaten en prestaties worden kort toegelicht in deze samenvatting.

Hoewel Anammox-gerelateerde technologieën op dit moment op grote schaal toegepast worden voor de verwijdering van stikstof uit slibvergistingswater, zijn vele aspecten van het Anammoxproces, zoals de kinetische eigenschappen en de stoichiometrie, nog zwak gekarakteriseerd. De parameterwaarden uit de literatuur zijn, bijvoorbeeld, vaak beïnvloed door stofoverdrachtslimitatie of door de aanwezigheid van inactieve cellen en een significante zijpopulatie. In **Hoofdstuk 2** wordt een membraanbioreactor (MBR) proces voor het kweken van een hoogverrijkte Anammox culture beschreven. De bijna pure, vrije celsuspensie van zeer actieve Anammox-bacteriën werd gebruikt voor een gedetailleerde kinetische en stoichiometrische analyse van de Anammoxproces. De biomassaopbrengst op basis van ammoniumopname werd berekend 0.071 mol C-N-mol^{-1}, terwijl de elementaire samenstelling van de biomassa werd gemeten als $CH_{1.74}O_{0.31}N_{0.20}S_{0.01}P_{0.01}$ (22.1 g C-mol^{-1}).

Middels de groeiopbrengst en de elementaire samenstelling van de biomassa werd de macro-chemische reactie vergelijking vastgesteld en gevalideerd door langdurige reactorbedrijfsvoering. De beschreven Anammoxcultuur in **Hoofdstuk 2** vertoonde een niet eerder gemelde hoge maximale specifieke groeisnelheid van 0.21 d^{-1} wat overeenkomt met een verdubbelingstijd van 3.3 dagen bij 30 °C. Met behulp van een experimentele methode die gebaseerd is op het opleggen van dynamische procesomstandigheden, gecombineerd met procesmodellering en parameterschatting, werd de intrinsieke affiniteitsconstante voor nitriet vastgesteld op 35 pg-N L^{-1}. Deze waarde was constant in het geteste pH-bereik van 6.8-7.5.

Met behulp van hetzelfde systeem, werden in **Hoofdstuk 3** de stoichiometrische en kinetische eigenschappen van een gesuspendeerde Anammox-verrijkingscultuur onderzocht bij steeds lager wordende slibleeftijden. Tijdens deze proeven kon de maximale groeisnelheid (μ^{max}) van de Anammox verrijkingscultuur toenemen tot 0.334 d^{-1}, welke vier maal hoger is dan eerder gerapporteerd werd in de literatuur en bijna 60% hoger dan de waargenomen groeisnelheid uit **Hoofdstuk 2**. Onderzoeken gaven eerder aanwijzingen voor hogere groeisnelheden voor Anammox bacteriën, maar deze vermoedens waren tot dusver altijd gebaseerd op indirecte metingen van kinetische eigenschappen. **Hoofdstuk 3** van dit proefschrift rapporteert het eerste directe experimentele bewijs voor een significante toename van de groeisnelheid van een Anammox verrijkingscultuur. Aangezien de biomassaopbrengst van de gebruikte verrijkingscultuur grotendeels vergelijkbaar is met eerdere studies, kan worden geconcludeerd dat de toegenomen groei een gevolg is van de equivalente toename van de biomassa specifieke elektronenoverdrachtscapaciteit. Gedetailleerde moleculaire analyse leverde geen verschuiving van de dominante Anammox species of belangrijke mutaties in de dominante soort op, wat suggereert dat de werkelijke redenen voor de verhoging van de elektronoverdrachtcapaciteit kunnen worden toegewezen aan kleine veranderingen in het metabolisme. De dominante stam in dit experiment was nauw verwant aan *Candidatus Brocadia* Sp.40 (99% overeenkomst). In deze studie werden Anammoxbacteriën gekweekt door toepassing een nieuwe selectiestrategie die gebaseerd is op het maximaliseren van de elektronenoverdrachtcapaciteit. Hieruit bleek dat de maximale groeisnelheid geen intrinsieke proceseigenschap is, maar dat deze aanzienlijk

kan worden verhoogd wanneer de geschikte kweekomstandigheden worden opgelegd. De Anammox cultuur is sneller geworden door training. Dit onderzoek laat een vergelijkbare snelheid met andere chemolithoautotrofe kinetiek zien. Het is daardoor te concluderen dat Anammox niet meer als intrinsiek langzaam groeiend micro-organisme kan worden beschouwd.

Nitriet is een van de belangrijkste substraten van het Anammox metabolisme, maar kan ook remmend werken. Het afgelopen decennium is het negatieve effect van nitriet op de Anammoxactiviteit op grote schaal onderzocht. Maar de bevindingen zijn vaak tegenstrijdig over de mate het remmende effect en of dat het omkeerbaar is of niet. Om dit belangrijke aspect op te helderen, werd een verdergaand onderzoek naar nitrietremming uitgevoerd waarbij de invloed van omgevingsfactoren werd geëvalueerd (**Hoofdstuk 4**). Anammoxactiviteit werd gemeten in Anammoxkorrels in continu gemonitorde standaard barometrische batchtesten, welke waren uitgebreid met een interpretatie en evaluatie van de vertragingstijden, maximale conversie en substraat- / productconversie-ratio's. De onderzochte Anammoxkorrels waren afkomstig uit een een-traps Anammoxreactor, waarvan is vastgesteld dat de dominante Anammoxsoort tot de Brocadia behoort. 50% inhibitie activiteit als gevolg van nitriet (IC_{50}) werd vastgesteld bij 0.4 $mg{\cdot}L^{-1}$. De gebruikte biomassa herstelde relatief snel (en totaal) van hoge concentraties nitriet. Het herstel na blootstelling geeft aan dat het remmende effect van nitriet omkeerbaar is. Tevens is het effect van de aanwezigheid van ammonium en nitriet onder aerobe condities geëvalueerd. De respons bij blootstellingen op drie verschillende pH-waarden suggereerd dat nitriet in plaats van salpeterigzuur de werkelijke remmende verbinding is. De resultaten beschreven in **Hoofdstuk 4** onderstrepen dat het Anammoxproces een stabiel proces is dat ongevoelig blijkt voor de tijdelijke nadelige effecten van zuurstof en nitriet. Uit ervaring en eerdere observaties bestaan vermoedens dat kweekomstandigheden en de aggregatietoestand invloed hebben op de mate van remming door nitriet. In een aantal gevallen waarin hoge nitrietconcentraties werden gerapporteerd als een oorzaak van activiteitsverlies zou het goed kunnen dat dit activiteitsverlies juist de oorzaak is van deze hoge concentraties aan nitriet.

Het temperatuureffect op de Anammox-activiteit is een cruciaal aspect dat moet worden opgehelderd voor de succesvolle implementatie van

Anammoxgerelateerde processen op huishoudelijk afvalwater. Lagere temperaturen vormen, samen met lage concentraties ammonium en de eis van efficiënte en stabiele verwijdering van stikstof, de belangrijkste te overwinnen uitdagingen bij de ontwikkelingen van deze grensverleggende afvalwatertechnologie. **Hoofdstuk 5** handelt over het korte-termijn-effect van temperatuur op de specifieke maximale activiteit van anaërobe ammonium-oxiderende bacteriën. De experimenten werden uitgevoerd met behulp van batchtesten op Anammox biomassa welke afkomstig was uit twee full-scale reactoren en twee laboratoriumschaal reactoren, met elk zijn eigen specifieke reactorkenmerken en bijbehorende condities. De resultaten laten zien dat in het temperatuurbereik van 10-30 °C de temperatuursafhankelijkheid van de Anammoxconversie niet nauwkeurig gemodelleerd kan worden door middel van één typische Arrhenius vergelijking gebaseerde coëfficiënt zoals bij de meeste andere biologische processen. Het temperatuureffect is groter bij lagere omgevingstemperaturen wat een stabiele implementatie voor een hoofdstroomtoepassing tijdens winterse condities bemoeilijkt. Desalniettemin werd een adaptatie van de Anammoxbacteriën waargenomen wanneer deze langdurig bij een temperatuur van tussen de 10 en 20 °C werden gekweekt wat aangeeft dat ook de voorgeschiedenis van de biomassa invloed heeft op de prestaties van biomassa. Anammox slib dat is gekweekt in een gecombineerd nitritatie / Anammox-proces (biofilm) leek minder gevoelig te zijn voor temperatuurdalingen dan strikt anaeroob gekweekte Anammoxbiomassa (zowel suspensie als biofilm). De in **Hoofdstuk 5** beschreven resultaten geven aan dat het temperatuureffect sterker is bij Anammox dan ammonium-oxiderende bacteriën (AOB). Dit impliceert dat om een dagelijkse goede stikstofverwijdering te houden, ondanks temperatuurschommelingen en andere seizoensgebonden procescontroles, een evenwicht gevonden moet worden waaronder zowel de activiteit behouden blijft als microbiële populatie in stand gehouden wordt die voldoende adaptief is om met wisselende condities om te gaan. Implicaties voor het modelleren en procesontwerp worden aan het eind van dit hoofdstuk besproken.

In **Hoofdstuk 6** is de toepassing van een een-traps PN/Anammox-proces onder omstandigheden die relevant zijn voor de behandeling van huishoudelijk afvalwater onderzocht in een laboratoriumschaal gas-lift sequencing batch reactor met korrelslib. De reactor werd voor meer dan 500 dagen bedreven. De

reactor opereerde bij temperaturen tussen 10 en 20 °C en is gevoed met een synthetisch autotroof medium dat 60 en 160 mg NH4-N L^{-1} als enige stikstofverbinding bevatte. De reactor draaide met een hydraulische verblijftijd 0.23-0.3 dagen. Opgeloste zuurstof bleek in aanwezigheid van ammonium een effectieve controleparameter, zelfs wanneer zuurstofspanning hoger was dan tot nu toe werd aangenomen. Bij zuurstofconcentraties tot 2.5 mg O_2 L^{-1} kon het ongewenste nitratatieproces onderdrukt worden. Nitratatie is het biokatalytisch proces waarbij nitriet wordt geoxideerd tot nitraat door nitriet oxiderende bacteriën (NOB). De vastgestelde regelstrategie garandeerde een effectieve onderdrukking van de nitratatie, zowel bij 20 en 15 °C. Stikstofverwijderingssnelheden van 0,44 en 0,40 g Ntot L^{-1} d^{-1} werden gemeten. Deze hoge omzettingssnelheden werden verkregen bij een optimale stikstofverwijdering-efficiëntie van respectievelijk 86 en 73%. Deze waarden zijn vereist voor een succesvolle implementatie van een PN/Anammox proces in de hoofdstroom van een RWZI. Bij 15 °C is aangetoond dat Anammoxbacteriën groeien met een geschatte snelheid van 0.017 d^{-1}. Ook is onderzocht hoe bedrijfsvoeringscondities van invloed zijn op N_20-emissies. Hierbij werd een positieve correlatie met de nitrietconcentratie waargenomen, terwijl er geen duidelijk verband was met de zuurstofconcentratie of temperatuur. Wanneer een Anammoxreactor langdurig werd bedreven bij 10 °C werd er een afname waargenomen in zowel activiteit als procesefficiëntie. Aangezien deze temperaturen niet voor een lange aaneengesloten periode voorkomen in een gematigd klimaat, hoeft dit niet als een cruciale beperking te worden gezien voor Anammox-gebaseerde technologieën in de hoofdstroom van een RWZI. **Hoofdstuk 6** beschrijft een beproeving van het concept voor de toepassing van autotrofe stikstofverwijdering in de hoofdstroom van een rioolwaterzuiveringsinstallatie met behulp van een enkele reactor met korrelslib.

De volgende logische stap bij de verkenning naar de mogelijkheden van Anammoxbacteriën is het onderzoek naar hun gedrag in de hoofdstroom van de afvalwaterzuivering. **Hoofdstuk 7** beschrijft de proef waarin een fluïde bed reactor met Anammoxkorrels continue werd gevoed met effluent van de A-trap van de RWZI Dokhaven te Rotterdam (Nederland). Om te voorkomen dat in de reactor met behulp van zuurstof nitriet zou worden geoxideerd tot nitraat in plaats van gereduceerd door Anammox werd deze reactor anoxisch met

nitriet bedreven. Omdat het effluent van de A-trap mogelijk nog residueel CZV bevat bestaat onder deze condities nog wel de mogelijkheid dat nitriet heterotroof wordt gedenitrificeerd. Ook kon het effect van eventuele toxische componenten (zoals polyelectrolyt of ijzerzouten welke worden toegepast in de A-trap) op de anammox bacteriën worden onderzocht. Gedurende meer dan 10 maanden werd het systeem bij temperaturen tussen de 10 en 20 °C bedreven. De vastgestelde volumieke N-conversies van dit systeem waren meer dan 0.4 gN L^{-1} d^{-1} en zijn vergelijkbaar of hoger dan bij conventionele stikstofverwijdering. De specifieke stikstofverwijderingssnelheid bij 10 °C was tijdens de laatste maand 50±7 mg N-g-vs^{-1} d^{-1}, wat bijna 2 maal zo hoog is als eerder gerapporteerde waarden bij deze temperatuur. Door middel van FISH analyse is vastgesteld dat de dominante Anammoxsoort *Canditatus Brocadia fulgida* was. Bewijs voor de groei van de Anammoxbacterie onder hoofdstroomcondities (dat wil zeggen toename van Anammoxbiomassa en nitraatproductie in afwezigheid van zuurstof) werd aangetoond voor het gehele temperatuurbereik (10-20 °C). Tijdens de bedrijfsvoering zijn ook nieuwe, actieve Anammoxkorrels gevormd die niet uitspoelden uit het gebruikte systeem. Residueel CZV werd heterotroof omgezet, maar deze heterotrofe bacteriën bleken niet in staat de Anammoxpopulatie te verdringen. In **Hoofdstuk 7** wordt voor het eerst het vermogen van Anammoxbacteriën om onder huishoudelijk afvalwatercondities (lage temperatuur, lage ammoniumconcentraties en de aanwezigheid van CZV) te groeien aangetoond, wat een nieuw perspectief opent voor de ontwikkeling van een efficiëntere behandeling van huishoudelijk afvalwater.

Bij de toepassing van autotrofe stikstofverwijdering zal de eerste stap van dit proces moeten bestaan uit een partiele nitritatie teneinde nitriet beschikbaar te hebben voor het Anammoxproces. Deze stap zal worden uitgevoerd door ammonium-oxiderende bacteriën (AOB). Het nitratatieproces (welke uitgevoerd wordt door nitriet-oxiderende bacteriën; NOB) zal zoveel mogelijk moeten worden onderdrukt. Hoewel in **Hoofdstuk 7** is aangetoond dat Anammox onder de gegeven condities prima werkt, blijkt dat het controleren van ammonium- en nitrietoxidatie in huishoudelijk afvalwater complexer in elkaar steekt dan werd verwacht. Hiertoe is een experiment op pilot schaal opgezet. Bij dit experiment is getracht een systeem weg te zetten waarin simultane partiële nitrificatie en anaerobe ammonium oxidatie

plaatsvond en waarin de aerobe oxidatie van nitriet naar nitraat werd voorkomen. **Hoofdstuk 8** handelt over deze propstroom korrelreactor op pilot schaal (4 m^3) welke continue gevoed werd met het effluent van de A-trap van de RWZI Dokhaven te Rotterdam. Dit was een één-traps Nitritatie-/Anammoxsysteem dat voor meer dan 10 maanden gedraaid heeft bij een temperatuur van 19±1 °C. De waargenomen gemiddelde stikstofverwijderings- en ammoniumomzettingssnelheden waren respectievelijk 182±46 en 315±33 mg-N L^{-1} d^{-1}. Deze waarden zijn vergelijkbaar of hoger dan die van conventionele stiktofverwijderingssystemen. Daarnaast zijn in dit soort korrelreactorsystemen veel hogere biomassaconcentraties haalbaar waardoor een verdere verrijking van Anammoxcellen mogelijk is. Hiermee zijn nog veel hogere omzettingssnelheden haalbaar. In het toegepaste korrelsysteem werd ook nog BZV geoxideerd met een gemiddeld verwijderingsrendement van 90%. Het systeem bleek efficiënt bij de retentie van Anammoxkorrels welke in de buitenste schil een fractie van nitrificerende en heterotrofe bacteriën bleek te bevatten. Tegelijkertijd werden vlokken, welke grotendeels bestaan uit heterotrofe organismen en een klein deel nitrificeerders, selectief uitgespoeld, waardoor het systeem incidentele CZV- en vaste stofbelastingen kan weerstaan. De resultaten welke beschreven worden in Hoofdstuk 8 laten zien dat de voorgestelde reactorconfiguratie potentieel succesvol kan worden toegepast bij de volledige autrotrofe verwijdering van stikstof in de hoofdstroom van een rioolwaterzuiveringsinstallatie.

Samengevat, het onderzoek dat beschreven is in dit proefschrift laat voor de eerste maal zien dat deze innovatieve technologie voor de verwijdering van stikstof uit afvalwater toepasbaar is. Deze technologie leidt een tijdperk in waarin we *de facto* zullen overgaan van een energieverbruikende afvalwaterzuivering naar een zuivering die energie oplevert.

Tommaso Lotti, september 2015

Chapter 1

General introduction

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Wastewater treatment (WWT) is a combination of many different physical, chemical and biological processes aimed at removing soluble and particulate compounds which when uncontrolled discharge would represent a risk for the public health and for the environment. In brief, the aim of wastewater treatment is to remove pathogens, reduced compounds which would cause oxygen depletion in the receiving water bodies and nutrients which emission in the environment would cause an unbalance in the eco-systems self-regulation mechanisms resulting in uncontrolled algal blooms (i.e. eutrophication). While for the removal of particulate compounds, simple physical-chemical processes are sufficient (e.g. coagulation, flocculation, sedimentation), the removal of soluble compounds requires more complex chemical-biological processes, with the biological ones preferred cause of their economical and environmental advantages. In the biological processes the ecophysiology of certain bacteria is exploited to convert the polluting soluble compounds in a form which is either harmless or easier to be disposed. In order to sustain their metabolism and to oxidize the reduced soluble pollutants, an oxidant (usually oxygen) needs to be externally added to the wastewater. The energy used for the oxygen addition (i.e. compressors for the aeration) constitutes one of the largest costs related to wastewater treatment. The bacteria would then use the chemical energy present in the polluting compounds for growth, increasing their concentration in the wastewater treatment systems. The removal (e.g. solid/liquid separation in a clariflocculator) and handling (e.g. by landfill disposal or incineration) of such "excess" of bacteria (the so called *excess sludge*) constitutes another large aliquot of the operational costs related to WWT. Part of the chemical energy originally present in the wastewater mainly in the form of particulate and soluble organic compounds is comprised in the excess sludge. This can be recovered in the form of methane via the anaerobic sludge digestion process. Such methane is then converted into electrical energy lowering thereby the net energy consumption of the wastewater treatment plant (WWTP). Obviously a treatment scheme which allows the efficient removal of pollutants while minimizing the energy input for aeration and maximizing the recovery of the chemical energy present in the wastewater would be most advantageous.

The research presented in this thesis aimed at implementing to the treatment of municipal wastewater a novel biological process, the partial

nitritation/anammox process, which allows a complete re-design of the conventional wastewater treatment scheme from an energy depleting into an energy producing system.

Before dealing with the potential attractive future of WWT though, it is useful to start with a brief introduction to its past and present followed by a more thorough introduction to the actual protagonists of this work: the anammox bacteria.

Origins of wastewater treatment

During the Neolithic period (ca. 10,000 B.C.) movement by nomadic tribes addressed the waste created by human activities. This nomadic movement allowed the earth or the soils treat the waste. In the ancient waste handling technologies developed as societies started living in more permanent settlements. These varied by the skills the various cultures developed. The City of Ur, by 3500 B.C., had an average population of 25,000 people per km^2 (a high population density which produced considerable waste). The populace of the city dealt with their waste problem by simply sweeping their wastes into the streets. The first proves of wastewater management can be found back in the 3rd millennium B.C. in the sewer systems found in Babylonia (Mesopotamian Empire) (Angelakis et al., 2005). One of the most ancient systems of wastewater management was constructed in Mohenjo-Daro near the river Indus (present Pakistan) at about 2500-1500 B.C. Private and public houses were equipped with toilets. Water used for washing and bathing as well as rain water flowed through special grooves into canals which were built with the necessary slope to transport the water into the river Indus. These installations demonstrate a high hygienic standard of an early culture. Moving back to the Mediterranean cultures, we see developments in waste treatment technologies. In the Egyptian city of Herakopolis (2100 B.C.), the average person treated their wastes much like those in Ur, they threw the wastes into the streets. However, in the elite and religious quarters, there was a deliberate effort made to remove all wastes, organic and inorganic to locations outside the living and/or communal areas, which usually meant the rivers. There is also religious teachings that dealt with waste. As example of religious teaching dealing with waste Mosaic laws (1300 B.C.) told "to remove his own refuse and

bury it in the earth" while the Talmud called for the streets of Jerusalem to be "washed daily". The Minoan Culture on the Island of Crete between 1500 and 1700 B.C. had a highly developed waste management system. They had very advanced plumbing and designed places to dispose of organic wastes. Knossos, the capital city, had a central courtyard with baths that were filled and emptied using terra-cotta pipes. This piping system is similar to techniques used today. They had flushing toilets, with wooden seats and an overhead reservoir. Wastewater was then collected and drained into large sewers built of stone. The Minoan royals were the last group to use flushing toilets until the re-development of that technology in 1596.

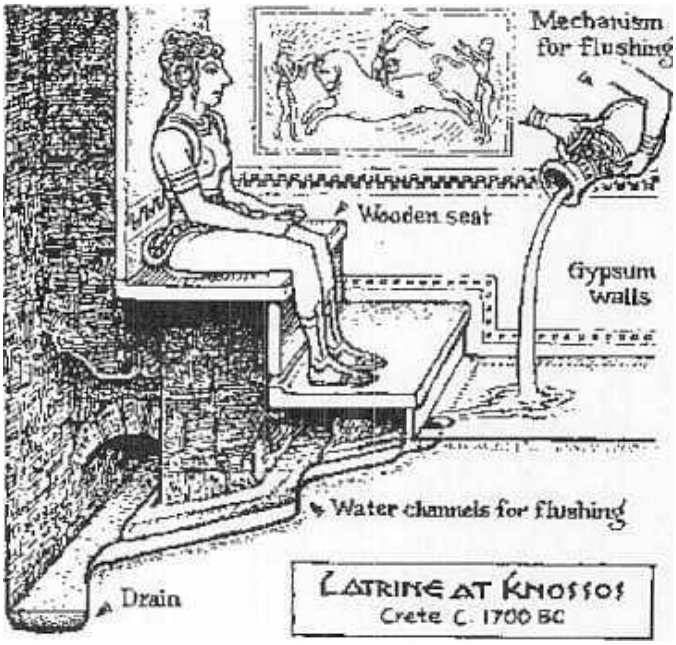

Figure 1. Latrine facilities in Knossos, Crete (present Greek)

The first dumps were developed by the Greeks (Athens) circa 500 B.C. In the development of waste management, Athens, in 320 B.C., passed the first known edict banning the disposal of refuse in the streets. In the continued development of waste management, by 300 B.C., one of the responsibilities of the Greek city-state was the removal of waste. "The expenses [for waste removal were] covered by levees on landowners". This system was sufficiently viable to last for eight hundred years, until the general breakdown of civic order. In the use of water the early Greeks understood the relationship

between water quality and general public health. This concern was passed onto the Romans. During the Roman Empire, besides the famous aqueducts for drinking water delivery, all the big cities where furnished with very complex sewer systems. In the capital, the city of Rome, the sewer system carrying the wastes to the river Tiber was designed and built so well that nowadays is still perfectly working, more than 20 centuries after its construction. The awareness about the relationship between water quality and public health and their sanitation systems for public health was so developed that health concerns caused by wastes and sewage disposal were regularly reported in the roman chronicles (Burian et al., 1999). A drastic loss of knowledge, also about hygienic practice, followed the fall of the Roman Empire. Waste and sewage was again discharged onto the streets and in the canals at all levels of society. As a direct consequence, during the Middle-Ages, epidemics raged in most of the European cities. The relationship between the dumping of excrements onto the streets, polluted drinking wells and diseases was not fully recognized anymore. A curious unware side effect was that beer became a popular drink in the north of Europe because of its wholesome effect (which was caused by the boiling step in the production process) and probably in a similar way part of the benefic effects attributed to wine in the south of Europe were related to the disinfecting potential of alcohol. Although some steps forward were made in the sanitation issues between the 12th to the 15th century, mainly by spotted proclamations of parliaments and religious orders, the collection and disposal of wastewater was neither adequate nor widely spread among cities population. A detrimental common behavior was to use the city canals and rivers both as sewer and drinking water supply which led to the rapid spread of epidemics. Another peculiar side effect of this terrible sanitary situation was the born of summer holidays: the unbearable stench in the cities caused by the high temperature in fact, made persons that could afford it to move away from the cities in this season. During the Renaissance, concern about waste and wastewater management grew again together with the increasing population living in towns and cities. For example in England Henry the VII outlawed slaughterhouses in cities and towns because of the risk for public health. In the hub of Renaissance, my sweet home town Florence, better houses had had latrines since the 14th century. Waste was conducted down and out of the houses in underground depositaries, and communal statutes required that these be emptied outside the city walls or into the river Arno (in which people

bathed and fished) but not into the streets. Here in the Netherlands, during the time of the Republic, "Hoogheemraden" (Waterboards) were in charge of making laws to prohibit certain type of draining to the surface waters and of flushing periodically the canals of the cities with fresh water. Despite increasing regulations and awareness among the ruling class, the inadequacy of sanitation became more and more urgent issue with increasing city populations during the industrial revolution. The world-wide epidemic of cholera in the 19th century and the discovery of the correlation between contaminated wells and cholera by the English physician John Snow in 1854, led to the awareness of the importance of safe drinking water and wastewater management. The Great Plague in London in 1857 forced the British government to widely introduce water closets and sewers ('seawards') in cities with the aim of using water to transport pathogenic wastes to the sea. During the following decades many other western cities imitate the example of London constructing sewer systems. The increasing population density and efficiency in wastewater collection led to an increase pollution load carried by the sewers to the surface water around cities, resulting in a drastic decline of the water quality. The irrigation fields introduced near London in 1860 represent the first attempt of the implementation of a sewage treatment system. Solid debris was efficiently removed from the wastewater thanks to another invention of this period, the septic tank, invented by Louis Moureas in 1860. However its introduction did not solve the surface water pollution issue since the effluent of the septic tank was still largely untreated. The quest for an efficient system for the treatment of both particulate and soluble polluting compounds from wastewater had begun.

Experiments to increase the specific wastewater load [m^3 (ha day)$^{-1}$] compared with that of irrigation fields resulted in the development of intermittent soil filtration ("Wastewater Farming"). Edward Frankland developed trickling sand filter technology in 1868. In the same period experiments of this kind were performed in USA, Germany and London (Dumbar 1899; Roeckling 1899). All these experiments and attempts, even if not completely understood in their biological mechanisms, led finally to the development of continuously operated trickling filters (Table 1). One of the first large-scale plants was built in the form of contact beds using large pieces of

coke and operated with intermittent filtration, in Stahnsdorf near Berlin (Müller 1907).

Table 1. Development of irrigation fields and trickling filters - increase in specific load (Wiesmann 2007).

Year	Process	Specific load [m^3 (ha·h)$^{-1}$]
1860	Irrigation fields prepared on suitable soil and level area	0.24-0.36
1878	Irrigation fields with drain trenches and soil fields	4-8
1884	Irrigation fields and preliminary sedimentation	8-10
1886-1900	Intermittent soil filtration	30-40
1890	Intermittent filtration with contact beds	120
1903	Trickling filter	500-2000
1960	High-load trickling filter	8000

In the meantime that sanitary engineering was making progress, milestone discoveries in the basic understanding of microbiology posed the basis for a better use of biological processes. After the foundation of the field of bacteriology by Ferdinand Cohn in the 19th century, other major contributions were given by the studies of Louis Pasteur and Robert Koch. However, it was not until the late 19th century and the work of Martinus Beijerinck and Sergei Winogradsky, the founders of general microbiology, that the true breadth of microbiology was revealed.

Finally, thanks to the study on suspended growth treatment and the discovery of activated sludge by Ardern and Lockett in 1914, the first generation of activated sludge systems was built in the 1920's. At the present time activated sludge systems are still the most frequently applied technology for biological wastewater treatment.

Conventional wastewater treatment

The activated sludge systems consist of different stages where circumstances are designed in order to create eco-systems favoring the development of microbial communities capable of removing organic carbon and nutrients (i.e. nitrogen and phosphate). The microbial community present

in these systems is very large and divers, including viruses, protozoa, metazoa, fungi, algae and bacteria, with the latter being the most abundant group. Selection mechanisms are used in order to regulate the microbial community composition and the biological process they catalyze. The main regulators in this respect are the time that a microorganism is allowed to reside in the system (named as *solid retention time*, SRT) and the availability of an electron acceptor such as oxygen (O_2) and nitrate (NO_3^-). An activated sludge plant consist of several stages with different biochemical characteristics which can be either compartments physically separated (e.g. different tanks), different zones of the same compartment (e.g. where some parts are aerated and some others are not) or the same compartment in which the different operations are separated in time within a cycle (e.g. *sequencing batch reactor*, SBR). In brief, the biochemical stages of an activated sludge plant can be described as follow:

- aerobic: where organic carbon is oxidized to carbon dioxide (CO_2) thus liberated in the atmosphere and ammonia (NH_3/NH_4^+, the main form of nitrogen in wastewater) is oxidized to nitrate (*nitrification* process).
- anoxic: where nitrogen is removed via the reduction of nitrate to dinitrogen gas (N_2) and its subsequent emission to the atmosphere (*denitrification* process).
- anaerobic: where external electron acceptors are absent and bacteria capable of removing phosphate (i.e. phosphate accumulating organisms, PAO) are selected.

The way these different steps are joined together in a treatment chain and the configuration and management of the single stages highly depend on local conditions and effluent requirements. As highlighted in the first paragraph microorganisms use the chemical energy in the wastewater for growth resulting in the production of *excess sludge*. The chemical energy is mainly present in the form of organic compounds which total concentration is measured by standard methods as *chemical oxygen demand* (COD). Heterotrophic bacteria harvest the energy released from the oxidation of COD to CO_2 in the catabolic phase and invest part of this energy for growth in the anabolic phase of their metabolism. In simple words then, part of the influent COD is oxidized to CO_2 and part is incorporated into new biomass. The production of new biomass requires also the use of some nutrients (mainly nitrogen and phosphorus) which are again harvested, and therefore removed, from the wastewater. The portion of influent COD that is converted to *excess sludge* can be manipulated till a certain extent by process operations. Finally,

the energy associated with the organic carbon contained in the newly formed biomass together with the organic solids naturally present in the sewage, can be converted into methane (CH_4) -rich biogas via an anaerobic process performed at higher temperature (*anaerobic digestion*, AD). The AD process is therefore a technology which allows us to harvest part of the chemical energy present in the wastewater and fix it in a form, methane, which can be easily used (e.g. for the production of electrical energy) and transported.

Simplifying, the different biological processes performed in an activated sludge plant can be summarized with the following (*not balanced*) biochemical reactions:

- Organic carbon oxidation

$$COD + O_2 \rightarrow CO_2 + biomass \quad (1)$$

- Nitrogen removal by coupling of the *nitrification* (eq.2) and *denitrification* process (eq.3)

$$NH_4^+ + 1.5 \cdot O_2 \rightarrow NO_3^- + biomass \quad (2)$$

$$NO_3^- + COD \rightarrow N_2 + CO_2 + biomass \quad (3)$$

- Biological (eq.4) and chemical (eq.5) removal of phosphorus by formation and removal of either P-rich biomass or precipitates

$$COD + PO_4^{3-} \rightarrow CO_2 + P_{rich} biomass \quad (4)$$

$$Metal\ ions + PO_4^{3-} \rightarrow Insoluble\ salts \downarrow\ (precipitates) \quad (5)$$

- Anaerobic digestion of excess sludge and influent organic solids

$$biomass \rightarrow CH_4 + CO_2 \quad (6)$$

Summarizing, the most important pollutants which have to be removed from wastewater are COD, ammonium and phosphate. COD and ammonium require a large input of oxygen (air) and therefore a large input of electrical energy for the air-pumps (aeration). During the oxidation of COD (eq.1) its chemical energy (± 14 kJ/g-COD) is mainly dispersed as metabolic heat while a smaller fraction is converted into new biomass. Some nitrogen and phosphorus

are also removed this way by assimilation in the new biomass. COD is also required for nitrogen removal via the denitrification process (eq.3). When the COD content of wastewater is insufficient for complete nitrogen removal, the necessary COD (e.g. in the form of methanol, acetate, etc.) is purchased and externally dosed. Phosphorus is mainly removed via either accumulation in the biomass of some specialized microorganisms (eq.4) or chemical precipitation (eq.5) followed by separation from the clean effluent.

Overall, the conventional wastewater treatment described above is an energetically very inefficient system where the energy present in the form of chemical bonds is mostly lost whilst a large amount of electrical energy is used.

A different approach

A better approach would be to think at the pollutants present in the wastewater as a potential source of energy and design the treatment process aiming at maximizing harvesting energy (Jetten et al., 1997; Kartal et al., 2010a). Currently, wastewater treatment plants with a primary settler collect only about 30% of these organics in the first stage. Approximately 60% of the energy usage of conventional activated sludge systems is due to aeration. By retaining as much organic matter as possible from the wastewater influent (both soluble and particulate COD), the aeration energy required to fully oxidize these organics can be drastically lowered while the collected organic substances can be used to produce biogas rich in methane and thus energy. This COD concentration step is already feasible with the present state of the art thanks to a wastewater treatment technology developed in the nineteen-seventies at the University of Aachen (Germany) during the last severe energy crisis, the Adsorption-Belebung (AB)-process (Böhnke, 1978). In the A-stage of an A-B process (Böhnke 1978; Versprille et al., 1984) in fact sewage is led to an activated sludge system characterized by a very low retention time (i.e. very-high-load; maximum 30 minutes hydraulic residence time, biomass specific load of 2-5 kgBOD kgMLSS^{-1} d^{-1} and solid residence time of less than one day), where the selection of fast growing microorganisms allow most of the soluble COD to be converted into biomass that can then be flocculated and separated from the effluent in conventional settlers together with the non-degraded suspended and colloidal influent organic material. In such a way more than 70% of the

organic matter present in the influent can be trapped in the first adsorption step (A-stage). Phosphate will be partly removed by assimilation in the newly formed biomass together with part of the influent nitrogen (57±10% and 28±7% , respectively, according to Roest et al., 2012). Depending on the wastewater composition and local regulations a more efficient phosphorus removal might be required which can be accomplished by metal ions and/or flocculant addition in the same A-stage in order to increase the chemical precipitation of phosphate containing minerals. The organic matter concentrated in the settler can be efficiently converted into methane-rich biogas in a conventional anaerobic digester. This approach would indeed maximize the recovery of the chemical energy present in the wastewater but without leaving enough COD for the denitrification process. Luckily, the recently discovered biochemical process *anaerobic ammonium oxidation* (anammox) does not require COD for nitrogen removal and represent therefore the missing piece of the long desired puzzle depicting an energy efficient wastewater treatment plant. Depending on the wastewater characteristics, application of anammox for nitrogen removal from municipal sewage allows treatment scenarios for wastewater treatment plants (WWTP) with a net energy production (Kartal et al., 2010a). The resulting treatment chain (not including the pretreatment for grit, fat and grease removal) is schematized in Figure 2.

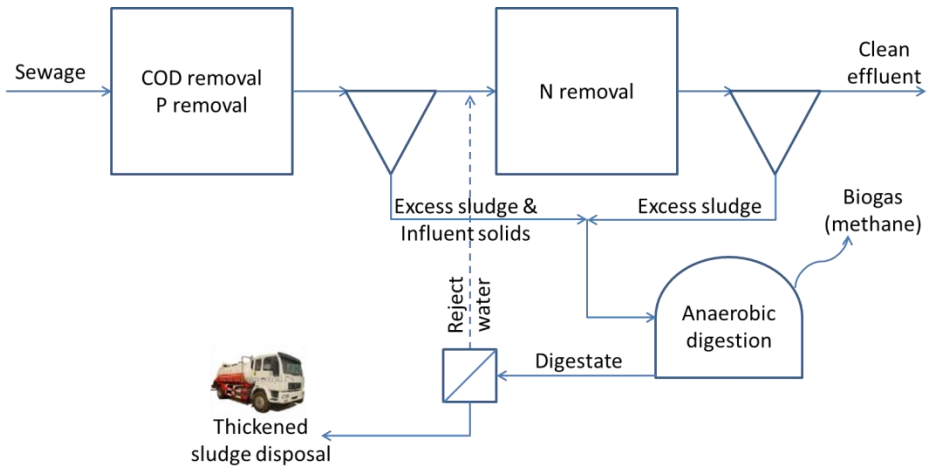

Figure2. Flow diagram of the proposed treatment scheme for a net energy producing sewage treatment plant

Due to the low growth rate of the anammox bacteria application is currently limited to higher temperatures (25-40 °C). With respect to the state of the art applications of the anammox process (Hu et al., 2013a [**Ch.1**]; Lackner et al., 2014), the characteristics of the municipal sewage are very different mainly in terms of temperature and nitrogen concentration. As will be exhaustively discussed later in this thesis, the drastic difference in wastewater characteristics poses several problems of both biological and technological nature.

The ultimate scope of this thesis was to investigate the boundary conditions for application of the anammox process in the treatment of municipal sewage.

Before entering in the details of the four years of research that are condensed in this thesis, I will first provide a general introduction to anammox bacteria and their current and potential future application in the field of environmental biotechnology.

The anaerobic oxidation of ammonium

Introduction

The anaerobic ammonium oxidation (Anammox) process is the oxidization of ammonium to N_2 in the absence of oxygen with nitrite as electron acceptor. Unlike other microbial processes involved in the nitrogen cycle, such as nitrification and denitrification, research on the anammox process was directed to improve nitrogen removal from wastewater. This occurred very shortly after it was first discovered in a denitrifying pilot plant in the late 1980s (Mulder et al., 1995) because of the perceived potential of this process for the wastewater treatment industry (Jetten et al., 1997; Strous et al., 1999a; van Dongen et al., 2001).

Nevertheless, the application of the process for nitrogen removal was not straightforward. The first obstacle encountered for the lab-scale research and industrial application of this process was the slow growth rate of the responsible microbial community (Jetten et al., 2009; Strous et al., 1998). In the laboratory, this hurdle was overcome by using systems with very efficient biomass retention such as sequencing batch reactors (SBR, Strous et al., 1998). At industrial scale, the first full-scale anammox reactor was started for the

treatment of rejection water at the sludge treatment plant in Sluisjesdijk, Rotterdam, the Netherlands in 2002 (van der Star et al., 2007). This up-flow anammox reactor was coupled in cascade with a SHARON (Single reactor system for High-rate Ammonium Removal Over Nitrite) reactor which produces a ~50:50 mixture of ammonium and nitrite (van der Star et al., 2007). Later on, the first full-scale CANON (Completely Autotrophic Nitrogen removal Over Nitrite) system, also known as one-stage anammox, was started at the wastewater treatment plant (WWTP) in Strass, Austria (Wett, 2007). In this system, partial nitritation and anammox processes happen in the same reactor under oxygen limitation (Third et al., 2001).

Autotrophic nitrogen removal is currently applied in about 100 full-scale installations for the treatment of a variety of ammonium rich municipal and industrial wastewaters: tannery, food-processing, semiconductor, fermentation, yeast, distillery, winery industries (for a review see Lackner et al., 2014; Vlaeminck et al 2012). The nitrogen load treated by these systems strongly varies with a maximum of 11 tons-N d^{-1} (Tongliao Meihua industry, China; unpublished data). Furthermore, encouraging results were reported for technical-scale installations treating black water digestate (de Graaff et al., 2011), digested manure (Villegas et al., 2011), urine (Udert et al., 2008) and pharmaceutical wastewaters (Tang et al., 2011).

The industrial application of the anammox process requires case-specific design, determined by the complexity of different types of wastewater. Many characteristics and contents of wastewater, such as pH, salinity, temperature, COD, nitrite and ammonium, heavy metals and antibiotics could affect anammox process stability significantly (Fernandez et al., 2009; Kartal et al., 2006; Lotti et al., 2012a; Strous et al., 1999a).

In this review different wastewater treatment processes using anammox bacteria and their advantages are summarized. Subsequently, process stability, N_2O emissions from anammox-based processes and perspectives for the future application of the anammox process are discussed.

Physiology of the anammox bacteria

Since their discovery, the research on the application of the anammox bacteria and the quest for understanding their physiology and ecological importance have gone hand in hand both from applied and fundamental points of view. The availability of anammox enrichment cultures not only helped to improve the application of this process, but also provided the possibility to study the physiology of the anammox bacteria in detail and develop tools for their detection in the environment. To date, anammox bacteria belonging to five genera have been identified: *Candidati* 'Brocadia', 'Kuenenia', 'Scalindua', 'Anammoxoglobus' and 'Jettenia'. These five genera together form a deeply branching, monophyletic order *Brocadiales* in the phylum *Plactomycetes* (Jetten et al., 2010a). All described species possess an organelle-like cell compartment bound by a single curved membrane, the so-called anammoxosome (van Niftrik et al. 2008). Even though anammox bacteria are slow-growing microorganisms that divide approximately once every week, recent studies revealed that these microorganisms contribute significantly to the release of fixed nitrogen from the oceans (Arrigo, 2005; Devol, 2003; Kuypers et al., 2005, Lam and Kuypers, 2011; Devol, 2015). This is most likely due to the fact that they are geared towards converting their substrates at very low concentrations; in other words, they have a very high affinity to their substrates ammonium and nitrite (sub-micromolar range, Strous et al. 1999), which is also of utmost importance from an applied perspective. In the absence of molecular oxygen, anammox bacteria activate ammonium through the oxidizing power of nitric oxide (NO). In short, anaerobic ammonium oxidation is a three-step process with NO and hydrazine as intermediates: First, nitrite is reduced to NO, then the produced NO reacts with ammonium to form hydrazine, catalyzed by the unique hydrazine synthase enzyme, and finally hydrazine is oxidized to N_2 (Kartal et al., 2011). The anammox cells are packed with cytochrome c type proteins (~30% of the protein complement) including the enzymes that perform the aforementioned key catabolic reactions. Cytochrome peroxidase stain revealed that these cytochrome c proteins were located at or in close proximity to the inside of the anammoxosome membrane suggesting that catabolic reactions take place in the anammoxosome (van Niftrik et al. 2008).

Many different aspects of the anammox bacteria and process have been reviewed thoroughly. The following reviews are recommended for further information and overview of the breakthroughs achieved in the last decade:

the description of the order Brocadiales, enrichment and taxonomy (Jetten et al., 2010a), cell biology (van Niftrik and Jetten, 2012), genome analyses, physiology and biochemistry (Kartal et al., 2013) and the presence and activity of anammox bacteria in natural ecosystems (Lam and Kuypers, 2011).

Application of the anammox process: state of the art

Over the last decade the cumulative nitrogen load increased almost 60 fold indicating that the anammox-based processes are becoming increasingly widespread (Figure 1). Currently, the anammox process is successfully implemented for full-scale wastewater treatment to treat ammonium rich wastewaters at mesophilic temperatures (Abma et al., 2010; van der Star et al., 2007; Wett, 2007). In order to supply anammox with nitrite (the electron acceptor for the oxidation of ammonium), two different strategies are used. Nitrite can be either produced in a separate aerated reactor and subsequently be fed into an anoxic anammox reactor (e.g. SHARON-Anammox, van Dongen et al., 2001) or produced in an oxygen limited single stage system (e.g. CANON, Third et al., 2001, Sliekers et al. 2003). In the literature, many different names are used to refer to these two different configurations (Table 1), but hereafter we will refer to them as two-stage or one-stage system, respectively. Besides the name, the type of sludge applied varies considerably in the anammox field, seemingly depending on where the technology was developed: flocculent (Switzerland, Austria), granular (Netherlands), biofilm on support (Sweden, Belgium), hybrid (Austria) (Table 1).

One-stage processes have generally lower capital costs than two-stage ones since no additional tank for nitritation is required. On the other hand, two-stage systems might allow more flexibility and a more stable process since nitritation and anammox processes can be controlled and optimized separately. The choice between the two different configurations depends of many local factors (e.g. previous presence of a nitritation unit, interest rate, space availability, wastewater characteristics) and is therefore highly case specific. Nevertheless, to the best of our knowledge, among approximately 40 autotrophic N-removal systems operating at full scale, only four are implemented with the two-stage scheme (Desloover et al., 2011; van der Star et al., 2007).

Table 2. Process options and names for nitrogen removal systems involving the anammox process

Biomass Type	Number of Reactors	Names	Nitrogen Removal Rate [kg-N m^{-3} d^{-1}][11]	Reference
Suspended	1	Single suspended-growth SBR[1]	0.5*	Joss et al., 2009
	2	NAS[2]	0.26	Desloover et al., 2011
Granular	1	CANON[3]	2*	Third et al., 2001; Abma et al., 2010
	2	SHARON[4,5]-anammox	0.6[12]*	Van Dongen et al., 2001
Hybrid	1	DEMON[6]	0.6*	Wett 2007
Biofilm	1	ANITA-Mox[7]	1.1	Christensson et al., 2011
	1	DeAmmon[8]	0.3-0.4*	Rosenwinkel et al., 2005
	1	OLAND[9]	0.05	Kuai and Verstraete 1998
	1	aerobic deammonification	1.23[13]	Hippen et al., 1997
	1	SNAP[10]	0.31-0.45	Furukawa et al., 2006

Notes:

[1] No acronym or commercial name is reported, [2] New Activated Sludge, [3] Completely Autotrophic Nitrogen Removal Over Nitrite, [4] Stable High rate Ammonium Removal Over Nitrite; the name only refers to nitritation where nitrite oxidation is avoided by choice of residence time and operation at elevated temperature, [5] Sometimes the nitrification–denitrification over nitrite is addressed by this term, [6] Name only refers to the process in an SBR under pH-control, [7] Commercial name used by Veolia for 1-stage nitritation-anammox in moving bed biofilm reactor (MBBR), [8] Commercial name used by Purac for 1-stage nitritation-anammox in moving bed biofilm reactor (MBBR), [9] Oxygen-Limited Autotrophic Nitrification Denitrification, [10] Single-stage Nitrogen removal using the Anammox and Partial nitritation; name only refers to the process on a biofilm surface layer, [11] Data is for full-scale application [when available and denoted by (*)] or lab-scale installations. [12] Only the aerobic HRT is included in the rate calculations (van der Star et al., 2007; Kampschreur et al., 2008a), [13] Nitrogen removal rate expressed as g N m^{-2} d^{-1} (calculated from reported data).

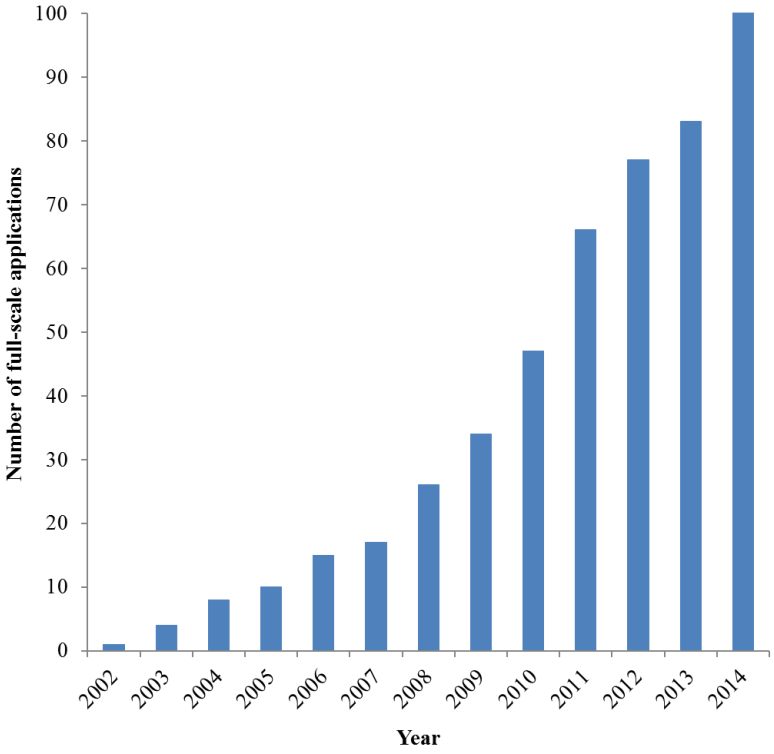

Figure 1. Number of full-scale nitritation-anammox systems implemented worldwide in the last decade

For the one-stage processes, different reactors such as SBR, gas-lift, rotating biological contactor (RBC) and moving bed biofilm reactors (MBBR) were used to establish the microaerobic conditions where aerobic ammonia oxidizing bacteria (AOB) and anammox bacteria coexist (Egli et al., 2001; Jaroszynski et al., 2011; Joss et al., 2009; Sliekers et al., 2003). When biomass is structured in biofilm or granule (self-aggregated biofilm), AOB are active in the aerobic outer layer due to limited oxygen diffusion, while anammox bacteria are active in the internal anoxic core (Figure 2). Aerobic ammonium oxidation produces a suitable amount of nitrite for anammox metabolism and prevents oxygen, which would inhibit the anammox bacteria, from fully penetrating the biofilm (Sliekers et al., 2003; Strous et al., 1997; Third et al., 2001). For suspended growth systems a very low oxygen concentration is applied combined with

on/off aeration. Model based investigations suggested that given a certain ammonium surface load on the biofilm, an optimal dissolved oxygen concentration level and an optimal biofilm thickness exist at which the maximum nitrogen removal occur (Hao et al., 2002a).

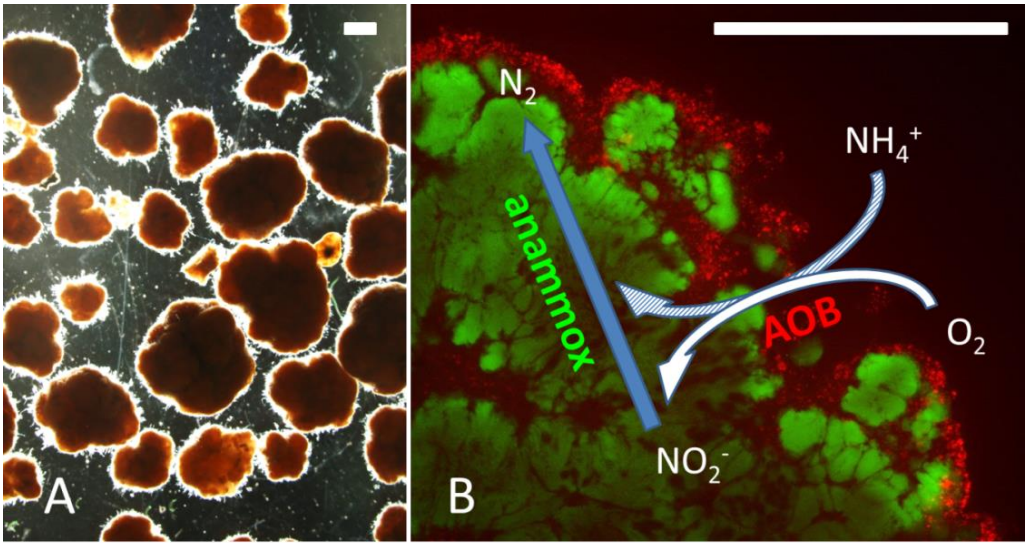

Figure 2. A: granular sludge originating from a single-stage PN/anammox system; B: A fluorescence *in situ* hybridization (FISH) micrograph of a cross-section (20 µm thick) of a nitritation-anammox granule superimposed with the reactions that show the conversion of ammonium to N_2. Anaerobic and aerobic ammonium oxidizing bacteria are depicted in green and red, respectively. (Pictures by Tommaso Lotti)

In one-stage systems, nitrate production by nitrite oxidizing bacteria (NOB) is prevented due to their lower affinity to oxygen compared to AOB and for nitrite compared to anammox bacteria (Blackburne et al., 2008a). It was proposed by several authors that in case of high strength wastewaters, inhibition of nitrite oxidizers by free ammonia (FA) might serve as an additional tool to maximize nitrogen removal efficiency. However, the significant disagreement on the effect of FA on NOB in literature (Anthonisen et al., 1976; Fux et al., 2003; Vadivelu et al., 2007) would advise against relying on ammonia levels for nitrate oxidation suppression.

Nitrate produced either by anammox or nitrite-oxidizing bacteria could be removed by nitrate reduction processes such as heterotrophic denitrification. However in order to implement the anammox process, pre-removal of COD is

currently (see perspectives section) considered as a requirement. Given the low yield and growth rate of anammox bacteria, heterotrophic growth should be minimized to maintain a high fraction of anammox bacteria in the sludge. Other electron donors than COD, such as methane or sulfide could also be used for nitrate reduction by denitrifying microorganisms. Furthermore, recently anammox bacteria were shown to successfully compete with heterotrophs for COD at COD/N ratio of 0.5 g-COD g-N^{-1} (Güven et al., 2005; Kartal et al., 2007a; Winkler et al., 2012a) opening new possibilities for process optimization.

Advantages of anammox process

Conventional nitrogen removal from ammonium-rich wastewater is accomplished in two separated steps: nitrification, which is mediated by aerobic ammonia- and nitrite-oxidizing bacteria and denitrification carried out by denitrifiers, which reduce nitrate to dinitrogen gas with the input of suitable electron donors such as COD. Aeration and input of organic substrates (usually methanol) determine that these two processes are: (1) remarkably energy consuming, (2) associated with the production of excess sludge and (3) produce significant amounts of greenhouse gases such as CO_2 and N_2O and ozone-depleting NO. Anammox bacteria convert ammonium and nitrite directly to N_2 in the absence of O_2; therefore, this process does not require aeration and other electron donors. Nevertheless, oxygen is still required for the production of nitrite by AOB. However, in partial nitritation/anammox systems, oxygen demand is strongly reduced because only half of the ammonium needs to be oxidized to nitrite instead of full conversion to nitrate. The autotrophic nature of anammox bacteria and AOB guarantee a low yield and thus a reduced sludge production. Furthermore, anammox bacteria easily form stable self-aggregated biofilm (granules) allowing reliable operation of compact systems characterized by high biomass concentration and conversion rate up to 5-10 kg N m^{-3} d^{-1} (van Loosdrecht, 2008). Overall, efficient application of the anammox process in wastewater treatment results in a significant cost reduction (60%, Siegrist et al., 2008; van Dongen et al., 2001) and lower CO_2 emissions.

Nitrous oxide emissions from one and two-stage systems

Nitrous oxide (N_2O) is an intermediate of denitrification, a side product of aerobic ammonium oxidizing bacteria under certain conditions and it is a major greenhouse gas with 300 times more global warming potential than carbon dioxide. In WWTPs, there are several nitrogen conversion processes that produce significant amount of N_2O, including nitrification, denitrification and chemical reactions between nitrite and hydroxylamine (Kampschreur et al., 2009a). Nitrous oxide is neither an intermediate nor a side product of the anammox metabolism (Kartal et al., 2011). Furthermore, a recent study showed that when an anammox enrichment culture (± 80 % anammox bacteria) was subjected to a high NO load (300 mg-N d^{-1}) there was no significant production of N_2O (Kartal et al., 2010b). In one-stage systems, however, oxygen is supplied to allow the growth of AOB, which could contribute to N_2O production via two pathways: (1) AOB can reduce nitrite to N_2O using ammonium or hydrogen as electron donor (Bock et al., 1995), (2) chemical reactions of the unstable intermediates hydroxylamine produced by AOB (Colliver and Stephenson, 2000). The emission of N_2O in a full-scale one-stage nitritation-anammox process was reported to be 1.3% of the nitrogen load (Weissenbacher et al., 2010) or 0.6 and 0.4% of the removed nitrogen load during intermittent and continuous aeration, respectively (Joss et al., 2009). Recently the emission of N_2O in a pilot-scale one-stage nitritation-anammox process was measured both with intermittent and continuous aeration and reported to account for 0.4-2% of the nitrogen load (Yang et al., 2013). In full-scale two-stage nitritation/anammox process on the WWTP Dokhaven-Sluisjesdijk (Rotterdam, Netherlands) was reported that 1.7% of the removed nitrogen load was emitted as N_2O from the nitritation reactor and 0.6% from the anammox reactor (Kampschreur et al., 2008a). In another two-stage full-scale system N_2O emissions accounted for the 6.6% (Desloover et al., 2011). De Graaff and coauthors (2011) registered an N_2O emission of maximum 1.0% of the total nitrogen load in an SBR anammox reactor treating the partially-nitritated effluent of black water anaerobic digestion. Recently 2.4% of the nitrogen removed was reported to be emitted as N_2O in a one-stage lab-scale reactor operating at 12 °C (Hu et al., 2013b). These findings suggest that N_2O is emitted from both one-stage and two-stage systems. In order to fully comprehend the scale of N_2O emissions from these two types of reactor systems and to be able

to compare them with N_2O emissions from conventional nitrification-denitrification reactors systems, further studies are necessary.

Process stability

The long doubling time (11-20 days) of anammox bacteria (Jetten et al., 2009) makes biomass retention of utmost importance for stable autotrophic nitrogen removal. In most cases, biomass retention is achieved by growing fast-settling biomass and is therefore a more delicate aspect for suspended growth systems than for biofilm systems. However, when sludge settling is implemented in a separate step such as in the settler of a CSTR or during the settling phase of an SBR, optimization is possible. In case of flocculent biomass in a SBR, occasional sludge-flotation problems have been reported (Joss et al., 2009). Larger aggregates are in general considered more suitable for single-stage systems (Hao et al., 2002a). Furthermore, they were reported to show higher anammox and lower nitritation/nitratation activity (Vlaeminck et al., 2010; Winkler et al., 2012b). In order to improve the retention of large aggregates, hydro-cyclones were installed on an SBR with flocculent biomass (Wett et al., 2010). However, also SBRs without such selective biomass retention showed long term process stability (Joss et al., 2009). Larger aggregates (granules) are characteristic of systems applying gas-lift and up-flow reactors since they are based on the continuous washout of poorly settling biomass (Abma et al., 2010). Regarding solids and COD load fluctuations, biofilm and granular systems appear intrinsically more robust than flocculent biomass based systems. In fact, in biofilm and granular sludge systems, small particles and the flocculent material resulting from heterotrophic growth are easily separated from the biofilm and therefore more efficiently removed, without compromising autotrophic biomass retention.

Industrial effluents and sewage composition often have a high complexity, which makes the successful implementation and stable operation of the anammox process a challenging issue (van der Star et al., 2007). It has been reported that chemical composition of applied wastewater would affect anammox activity significantly (van Dongen et al., 2001). The effect of several of factors, namely dissolved oxygen (DO) and substrate concentration on the stability of the anammox process are discussed below.

Anammox bacteria are active in the absence of O_2, which makes dissolved oxygen (DO) a critical operational parameter for anammox-based processes. It was initially reported that anammox bacteria are reversibly inhibited at very low DO concentrations (0.5% air saturation, Strous et al., 1997). In lab-scale bioreactors it has been repeatedly observed that anammox activity recovered even after prolonged exposition to fully aerobic conditions (>8 mg-O_2 L^{-1}, unpublished data based on a decade of reactor operation experience). Nevertheless, excessive DO would also lead to the growth of NOB that could compete for nitrite and oxygen with anammox and AOB and eventually lead to a collapse of the whole system. Based on the intricate interplay between aerobic and anaerobic microorganisms, DO was suggested as a control parameter combined with the nitrogen concentration in the bulk liquid. Joss and coauthors (2009) suggest that DO should be maintained at a certain set-point (<1 mg-O_2 L^{-1}) and choose to regulate the operations of the SBR cycle via direct nitrogen measurement with an ion-selective ammonium probe. On the other hand, in continuous systems DO level could be controlled via frequent automated colorimetric ammonium measurements (Abma et al., 2010).

Ammonium is not inhibitory to anammox bacteria, but its other substrate, nitrite, is a potent inhibitor (Strous et al., 1999a). It was reported that long-term exposure (longer than 1 week) to 980 mg-N L^{-1} to ammonium did not have negative effects on the activity of the anammox bacteria (Strous et al., 1999a), whereas batch incubations with free ammonia concentrations as low as 20-25 mg-N L^{-1} could be inhibitory for the anammox bacteria (Fernandez et al., 2012).

It was reported that nitrite concentrations higher than 0.1 g-N L^{-1} inhibits the anammox activity completely, but this inhibition was reversible and addition of a trace amount of hydrazine or hydroxylamine would restore the activity rapidly (Strous et al., 1999a). The earlier studies on the inhibitory effect of nitrite on the anammox bacteria suggest that this effect is based on exposure time as well as nitrite concentration (Dapena-Mora et al., 2007; Egli et al., 2001; Strous et al., 1999a). A recent comprehensive study on nitrite inhibition suggested that exposure to nitrite concentrations as high as 1 g-N L^{-1} for as long as 24 hours could still be reversible (Lotti et al., 2012b [**Ch.4**]). This study is in perfect agreement with many unpublished and unplanned experiments on nitrite exposure conducted in our laboratories over the last decade, which suggest that anammox bacteria do survive high concentrations of nitrite when

a pump breaks in the weekend. In addition, anammox biomass cultivated at low nitrite concentrations (<1 mg-N L^{-1}, Wett, 2007) seems to be more sensitive to transient nitrite accumulations, while cultivation at higher nitrite concentrations allow stable long term operations at concentrations considered otherwise toxic (Lotti et al., 2012b [**Ch.4**]).

Finally, we refer the reader to specific studies present in literature (a good overview on inhibiting compounds is provided by Jin et al., 2012) where the effect of other types of inhibitors on anammox activity are described in detail whilst in this thesis they are only listed for sake of completeness: phosphates, sulfides, salinity, heavy metals, suspended solids and organic substances such as alcohols, aldehydes, phenols, and antibiotics.

Perspectives for the future applications of the anammox process

Several prospective applications of anammox process have emerged based on recent research efforts on the physiology of the anammox bacteria: (1) The application of the anammox process for municipal wastewater treatment (2) combined ammonium and methane removal coupled to nitrite reduction (3) nitrate removal coupled to organic acid oxidation and (4) NOx removal coupled to ammonium oxidation.

(1) To date, most of the autotrophic nitrogen removal systems reported in literature were operated above 25 °C and influent nitrogen concentrations over 0.1 g-N L^{-1} (Van Hulle et al., 2010). The next immediate frontier for anammox-related processes is therefore the application of the anammox process at lower temperatures and lower nitrogen concentrations. This would allow extending its application to municipal sewage treatment opening new possible scenarios in the energy balance of wastewater treatment plants (Kartal et al., 2010a). In order to maximize the energy recovery from the municipal wastewater and avoid excessive organic load to the autotrophic nitrogen removal step, pre-removal of organic carbon can be established after the A-stage in an A/B process, after anaerobic digestion or physiochemical pretreatment (Alvarez et al., 2008; Jetten et al., 1997; Joss et al., 2009; Kartal et al., 2010a; Wett, 2007).

The main challenge for applying anammox in the mainline of municipal wastewater treatment is to achieve a high-rate process with good biomass

retention and a low effluent nitrogen concentration at low water temperatures (8-15 °C). Recently, a breakthrough was made in the application of the anammox process in this temperature range (Hu et al., 2013b). It was reported that a laboratory scale one-stage system running at 25 °C could be adapted to operate at 12 °C very rapidly (10 days). Moreover, this system was operated for over 300 days without nitrite accumulation or detectable NOB activity and was able to remove over 90% of the supplied nitrogen at 12 °C (Hu et al 2013b). Recently, an increasing numbers of other studies focused on the application of anammox-based technologies for the treatment fo municipal wastewater aiming at improving the understanding of microbial community interactions, system feasibility and capability, suitable process configuration, long term effect of temperature fluctuations and process optimization (*this thesis*; Dosta et al., 2008; Isaka et al., 2008; Vàzquez-Padìn et al., 2011; Winkler et al., 2011; Hendrickx et al., 2012; Cao et al., 2013; De Clippeleir et al., 2013a; Hu et al., 2013b; Wett et al., 2013; Gilbert et al., 2014; Lotti et al., 2014a,b [**Ch.6,7**]; Regmi et al., 2014; Gilbert et al., 2015; Isanta et al., 2015; Laureni et al., 2015; Lotti et al., 2015a,b [**Ch.5,8**]; Morales et al., 2015 *among others*). The need for further understanding was the driving force for the research described in this thesis.

As mentioned above, one-stage anammox reactors are successfully applied in full-scale sewage treatment plants to treat high ammonium loaded wastewaters such as anaerobic digester effluents (Van der Star et al., 2007; Abma et al., 2010). Along with ammonium, methane is also a major end product of anaerobic digestion and it is often collected from the off-gas and used as fuel after purification. On the other hand, it is unfeasible and difficult to recover dissolved methane, which leaves the system with the effluent wastewater and is eventually released to the atmosphere and contributes to the greenhouse effect. A wastewater stream containing methane and ammonium as electron donors create a perfect niche for employing the anammox process together with the recently discovered bacteria that are able to couple methane oxidation to nitrite reduction (n-damo) (Ettwig et al., 2008). Recently, two enrichment cultures containing n-damo and anammox bacteria were reported to be able to remove ammonium and methane simultaneously with externally supplied nitrite as the electron acceptor (Luesken et al., 2011; Zhu et al., 2011). In the future, a combination of these processes would be able to remove

ammonium, dissolved methane and nitrite simultaneously without the need of extra aeration and the addition of electron donors.

Currently, the anammox process is only used for the removal of ammonium. Growth of anammox bacteria on ammonium and nitrite results in nitrate production because these microorganisms oxidize a part of nitrite to nitrate to deliver the electrons necessary for carbon fixation. Consequently, nitrate is always present in the effluent of wastewater treatment systems that employ anammox bacteria. It was recently shown that anammox bacteria are also able to oxidize a multitude of organic compounds (formate, acetate, propionate, methylamines) coupled to the reduction of nitrate and/or nitrite (Güven et al., 2005; Kartal et al., 2007a,b; Kartal et al., 2008; Winkler et al., 2012). The co-oxidation of organic acids could increase the potential for the anammox process in wastewater treatment. In lab-scale reactors, anammox bacteria could outcompete heterotrophic denitrifiers at C:N ratios of below 2:1, which would imply that the anammox process could be applied to wastewaters with both organic compounds and ammonium (Jenni et al., 2015; Li et al., 2015). Moreover, this phenomenon could be exploited to remove the residual nitrate present in the effluent of anammox bioreactors. It is encouraging that both anammox species could be enriched from the same seed sludge and that all know anammox species could convert organic acids without the need of induction (Kartal et al., 2007a; Kartal et al., 2008; Winkler et al., 2012a).

Finally, it was recently shown that anammox bacteria were also able to convert NO (Kartal et al., 2010b). Surprisingly, instead of converting it to N_2O like denitrifiers, they coupled its reduction to ammonium oxidation, directly forming N_2. In a lab-scale reactor, when anammox bacteria were exposed to 300 mg-N d^{-1} NO load, 20% of the supplied NO was removed stoichiometrically coupled to ammonium oxidation. In total, 4.5 % of the influent concentration of ammonium was removed by anammox bacteria via this pathway with no appreciable N_2O production (Kartal et al., 2010b). Such a reaction could also be implemented in full-scale anammox bioreactors, which would then be designed to remove NO from flue gases.

Thesis scope and outline

The main goal of this study was to investigate the application of anammox based nitrogen removal process to the treatment of municipal wastewater. The feasibility of this biological process would allow a complete redesign of the conventional municipal wastewater treatment plants (WWTP) opening treatment scenarios with a net energy production, and therefore greatly contributing to increasing societal sustainability. Autotrophic nitrogen removal by anammox bacteria is to date the most energy efficient and environmentally friendly process for the treatment of ammonium in wastewaters. Due to the low growth rate of the anammox bacteria, application is currently limited to higher temperatures (25-40 °C). Application of anammox for the nitrogen removal from municipal sewage pose the challenges of achieving a high-rate process with good biomass retention and a low effluent nitrogen concentration at low water temperatures (8-25 °C).

Anammox related technologies are currently widely applied for nitrogen removal from sewage sludge digester rejection water with a total of about 100 full-scale installations. Nevertheless, many aspects of the anammox process like the kinetic characteristics and the reaction stoichiometry are not yet properly established. Parameter values reported in literature are often hampered by mass transfer limitation or by the presence of a significant side population. In **Chapter 2** a membrane bioreactor (MBR) based method for growing a highly enriched anammox microbial community is described. The almost pure free-cells suspension of highly active anammox bacteria was used for detailed kinetic and stoichiometric analysis of the anammox process.

Extension of the operational window of the anammox process is restricted by the "supposed" low growth rate of the responsible microorganisms. In **Chapter 3** the development of the maximum biomass specific growth rate of a highly enriched suspended culture of anammox bacteria was explored in the same system used in **Chapter 2**. By step-wise increasing the biomass dilution rate (decreasing the imposed solids retention time, SRT) a value of 0.33 d^{-1} could be achieved. This value is almost five times higher than the highest previously reported value. The main reason for the higher growth rate was identified as a higher biomass specific substrate uptake rate. Phylogenetic analysis showed a stable microbial community structure independent of the

dilution rate applied. The results described in **Chapter 3** demonstrate the capacity of an anammox community to modify its functional properties in response to a change in the cultivation conditions imposed without a change in the dominant anammox strain. This research demonstrates that the low growth rate can no longer be regarded as a bottleneck in anammox conversion process, opening, inter alia, new perspectives for the development of energy generating municipal wastewater treatment schemes.

The anaerobic ammonium oxidation performed by anammox bacteria uses nitrite as electron acceptor instead of oxygen. Part of the ammonium present in the wastewater needs therefore to be oxidized to nitrite (partial nitritation, PN, performed by ammonium oxidizing bacteria, AOB) in order to sustain anammox metabolism and allow nitrogen removal through dinitrogen gas production. Nitrite, besides a substrate, is also an inhibitor of the anammox metabolism. Although the adverse effect is clear, conflicting reports exist on the level at which it occurs and its reversible/irreversible nature. In **Chapter 4** an in depth study on nitrite inhibition was performed in which the influence of environmental factors was evaluated. Anammox activity was measured in anammox granular sludge by continuously monitored standardized manometric batch tests extending the interpretation by evaluation of lag times, maximum conversion rates during the tests and substrates/product conversion ratios. The recovery after exposure indicates that the adverse effect of nitrite is reversible and thus inhibitory rather than toxic in nature. Similarities between exposures at three different pH-values indicate that nitrite rather than nitrous acid is the actual inhibiting compound.

The pronounced fluctuation in time of the municipal wastewater temperature poses the challenge of maintaining an efficient nitrogen removal process both in summer and winter conditions. Whilst the effect of temperature on the activity of nitrifying bacteria is widely reported in literature, the effect of temperature variability in the range characteristic of municipal wastewater in the case of anammox bacteria was still lacking. In **Chapter 5**, we evaluated the effect of temperature change on anammox activity and the role which long term adaptation might play. Anammox biomass tested was obtained from two full-scale reactors and two lab-scale reactors, all characterized by different reactor configurations and operating conditions. The results obtained are compared to literature data and the practical implications

for modeling and for application of anammox for sewage treatment are discussed.

In order to maintain a stable an efficient nitrogen removal process, the activity of AOB and anammox bacteria needs to be balanced along the temperature fluctuation typical of municipal wastewater. Furthermore the undesired production of nitrate (nitratation) catalyzed by nitrite oxidizing bacteria (NOB) has to be suppressed in order to maximize the nitrogen removal efficiency. **Chapter 6** aimed at demonstrating the feasibility of granular sludge single stage autotrophic nitrogen removal at low temperature. A closely monitored air-lift sequencing batch reactor was fed with synthetic medium at conditions relevant for main-stream applications and operated at decreasing temperatures of 20, 15 and 10 °C. The granular sludge reactor configuration was chosen with the objective to achieve adequate biomass retention and high volumetric N-conversion rates as well as to investigate the interactions among different cohabiting bacterial populations. A control strategy for successful suppression of the undesired nitratation process is demonstrated and discussed. The basics engineering information for a good design of an anammox based municipal wastewater treatment system are provided.

In natural ecosystems such as Northern European soils and marine sediments, anammox bacteria thrive at low temperatures (<10 °C) and very low ammonium concentrations (μM range), indicating that there is no fundamental limitation for the anammox process to develop under municipal wastewater conditions. Nevertheless no report is yet available describing the application of anammox based process treating actual municipal wastewater. The study reported in **Chapter 7** aimed at demonstrating the feasibility of the anammox process from pretreated sewage. A granular sludge based fluidized lab-scale reactor was fed with ammonium containing effluent of the A-stage of the WWTP of Dokhaven (Rotterdam). The capability of anammox bacteria to grow at mainstream condition in the temperature range of 10-20 °C was demonstrated as well as the stability of the formed granules. The effect of the heterotrophic growth caused by the COD present in the influent was discussed and the volumetric and specific nitrogen removal rates observed were compared with previous reports and conventional systems.

When dealing with the development of new technologies, encouraging results obtained at lab-scale and especially in reactors fed with synthetic wastewater may lead to very different outcome when tested at larger scale and with actual wastewater. The basic knowledge and experience gained with the studies reported in the previous chapters were used to design and operate a PN/anammox pilot-scale system fed with the effluent of the A-stage of the WWTP of Dokhaven, Rotterdam. The results obtained during 10 months of operation at 19±1°C are presented in **Chapter 8**. A granular sludge plug-flow reactor with integrated tilted plate settler was chosen with the objective to achieve adequate biomass retention and high volumetric N-conversion rates while maximizing the nitrogen removal efficiency. Average N-removal and ammonium conversion rates observed were comparable or higher than those of conventional N-removal systems. The kinetic limiting-step and the effect of heterotrophic growth due to the oxidation of dissolved organic compounds were identified and discussed.

Chapter 2

Physiological and kinetic characterization of a suspended cell anammox culture

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Abstract

Anammox related technologies are currently widely applied for nitrogen removal from sewage sludge digester rejection water. Nevertheless, many aspects of the anammox process like the kinetic characteristics and the reaction stoichiometry are still subject of debate. Parameter values reported in literature are often hampered by mass transfer limitation or by the presence of a significant side population. In this study a membrane bioreactor (MBR) based method for growing a highly enriched anammox microbial community is described. The almost pure free-cells suspension of highly active anammox bacteria was used for detailed kinetic and stoichiometric analysis of the anammox process. The anammox culture enriched during this study had a biomass specific maximum growth rate of 0.21 d^{-1} which is higher than ever reported before in literature. Using an experimental methodology based on imposing dynamic process conditions combined with process modeling and parameter estimation, the intrinsic nitrite half saturation constant was identified to be as low as 35 µg-N L^{-1}. This was confirmed to be an accurate estimation in the pH range of 6.8-7.5.

Introduction

The anammox process is the anaerobic conversion of ammonium and nitrite to dinitrogen gas (Van de Graaf et al., 1996) catalyzed by autotrophic deep-branching Planctomycetes (Strous et al., 1999a). Anammox bacteria are usually considered slow-growing microorganisms with reported minimal doubling times of 4-15 days (Strous et al., 1998; van der Star et al., 2008). The high abundance of free anammox bacteria in marine environments (Schmid et al., 2007) show that there is no fundamental limitation for these bacteria to grow as free cells. Nevertheless, the enrichment cultures used to study these microorganisms typically consist of agglomerates or biofilms. Full-scale anammox reactor technology is heavily exploiting granular sludge based

systems (van der Star et al., 2007; Wett, 2007). Granular sludge-based reactor design (Nicolella et al., 2000) leads to compact reactors, which combine a short hydraulic retention time (HRT) with a long and stable solid retention time (SRT). However, biofilm or granular sludge reactor systems are not the most suitable systems for investigating the intrinsic properties of the microorganisms (van der Star et al., 2008). The correct assessment of bio-kinetic parameters such as substrate affinities, maximum growth rate or maintenance need is hindered by the mass transfer limitations within the floc or granule (Harremoës, 1977; Chu et al., 2003). Transport limitations potentially lead to underestimation of the observed maximum specific growth rate (μ^{max}) (Characklis, 1990) and overestimation of the intrinsic affinity constant (K_S). Moreover the presence of an undefined side population and the occurrence of inactive or dormant cells at longer SRT complicate the derivation of intrinsic reaction kinetics of the organisms.

The full stoichiometry of the anammox metabolism currently used for bioprocess design and modeling purposes is based on Strous et al. (1998). In that study stable conditions (comparable to a steady state in a chemostat) were achieved in a SBR, enabling mass-balancing under defined conditions. However the reported degree of enrichment for anammox cells was only 74% and the carbon balance relied on a set of data (collected over a period of about 200 days) of dry weight measurements affected by high standard deviation (up to ±50%). The electron balance of the measured conversion rates used to calculate the stoichiometric equation had an error of 15 %. Until now this stoichiometry has not been checked independently. Kinetic parameters of interest such as the affinity constant for nitrite have not been properly reported in literature (Strous et al., 1998; van der Star et al., 2008). The parameter values currently known are probably adequate for design purposes but they may lead to systematic inaccuracy in competition studies (e.g. biofilm modeling studies). For the correct evaluation of such physiological and kinetic parameters the availability of a highly enriched (negligible amount of non-anammox bacteria present) suspended culture (no mass transfer limitation) of anammox bacteria is essential. The membrane bioreactor (MBR) enables cultivation of slow-growing microorganisms with full biomass retention but without a selection on settling ability and thereby floc or granular sludge formation. Moreover it is possible to operate an MBR at dilution rates close to

the maximum growth rate of the concerned organisms, minimizing the effect of maintenance, decay and cryptic growth of a side population. Such a reactor system therefore is a valuable tool for identification of intrinsic stoichiometric and kinetic parameter values of slow growing microorganisms like anammox (van der Star et al., 2008). Van de Star et al. (2008) was the first to report on the suspended growth of anammox bacteria in a MBR system. The suggested requirements for obtaining suspended cell growth were low levels of bivalent ions in the medium (i.e., calcium and/or magnesium) and the addition of yeast extract to the standard medium from van de Graaf et al. (1996). The study could not differentiate between the effects of these two factors since they were applied at the same time. In this study we used the MBR system from van der Star et al. (2008) to characterize in detail a suspension culture of anammox bacteria for the stoichiometric and kinetic characteristics. We used a method for kinetic characterization that is based on the combination of dynamic modeling and a continuous process exposed to dynamic conditions e.g. short time changes in nitrogen load. Provided that the experiment is started in a stirred tank reactor in steady state, the methodology described here allows for efficient kinetic characterization of a slow growing culture in a few days.

Materials and methods

Inoculum

The reactor was inoculated with granular sludge from the upper part of the lower compartment of the full-scale anammox reactor of Dokhaven-Sluisjesdijk wastewater treatment plant in Rotterdam, the Netherlands (van der Star et al., 2007). The reactor contains granular anammox sludge and treats reject water after partial nitritation in a SHARON reactor. The inoculum, was confirmed to consist of a "*Brocadia*" enrichment by *fluorescence in situ hybridization* (FISH), the sludge hybridized with AMX-820 and not with KST-157 oligonucleotide probes (Schmid et al., 2001). The reactor was inoculated with 1.8 L of settled granular biomass.

Reactor operation

The reactor was operated as reported by van der Star et al. (2008) with the following changes:

a) The liquid volume was 10 L (V_L) and the reactor was fed continuously with 6 L d^{-1} medium with different compositions, resulting in a HRT of 1.67 days.
b) The headspace volume was 5 L (V_H).
c) To maintain anoxic conditions and to provide buffering capacity, the reactor was sparged continuously at 50 mL min^{-1} with an industrially prepared mixture of Argon and CO_2 (95 and 5%, respectively). The gas entering the reactor was supplied from pressurized bottles with Brooks mass flow controller (MFC; Brooks Instrument, Hatfield, PA, USA).
d) The pressure inside the reactor was maintained constant at 12 hPa by connecting the outflow gas-tube to the bottom of a water-filled vessel to act as a water-lock. Since day 333 on a dithionite solution (200 mM) in which the gas (Argon/CO_2, 95/5%) was bubbling (through a "fine-bubbles" porous sparger) before entering the reactor was also implemented in the set-up.
e) During normal operation the pH was not controlled, but was always between 6.8 and 7.5. Since day 451 the addition of bicarbonate was discontinued and a phosphate pH-buffer (tot-P 15 mM) was added to the medium in order to set the pH at 7.0 (Table 1).
f) Temperature was controlled at 30°C, and the stirring speed was 200 rpm.
g) The reactor was fed with a concentrated medium according to van der Star et al. (2007) with the changes reported in Table 1. The vitamin solution mentioned in Table 1 contained (mg L^{-1}): Folic acid (2.0), Riboflavin (5.0), Biotin (2.0), Thiamine (5.0), Nicotinic acid (5.0), Calcium Pantothenate (5.0), Vitamin B12 (0.1), p-Arninobenzoic acid (5.0), Thioctic acid (5.0), Monopotassium phosphate (900.0). Every time a new vessel of feeding-medium (20-50 L) was prepared, it was intensively sparged with nitrogen gas for 2-4 hours before connecting it to the reactor.

The start-up period, from the day of inoculation (the designated experimental day 1) until the design volumetric nitrogen loading rate (1 g-N L^{-1} d^{-1}) was achieved, lasted one month. During the start-up period the nitrite-loading rate was increased step-wise. The solid retention time (SRT) was controlled during the entire experimental period by means of a remote-

controlled (peristaltic) pump removing part of the mixed liquor. The excess sludge pump was operated 5 minutes every two hours. The reactor was operated for more than 600 days.

Table 1. Different medium compositions used in this study.

Nutrient	Dimension	Van der Star et al., 2008	Days 0-63	Days 64-88	Days 89-124	Days 125-231	Days 232-450	Days 451-612
Ammonium	mM	120	60	60	60	60	60	60
Nitrite	mM	120	60	60	60	60	60	60
Calcium	mM	1.0	1.0	1.0	0.5	0.5	0.5	0.5
Magnesium	mM	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Bicarbonate	mM	15	15	15	15	15	15	0
Phosphate	mM	0.18	0.18	0.18	0.18	0.18	0.18	15
Vitamin solution	mL L^{-1}	0	0	0	0	10	0	0
Yeast extract	mg L^{-1}	1	0	0	0	0	0	0
SRT	d	12	15	12	12	12	12	12

Measurements

Size distribution of MBR aggregates

The size distribution of the aggregates from the MBR was determined with the aid of image analysis (Tijhuis et al., 1994).

Biological Oxygen Monitor (BOM)

Biological oxygen monitor (BOM) measurements were conducted to determine the maximum volumetric oxygen uptake rate (OUR^{max}) and the maximum biomass specific oxygen uptake rate ($q_{O_2}^{max}$). BOM-measurements

are small-scale (≈43 mL) batch experiments conducted in closed reactors equipped with a DO electrode. The reactor was incubated with an oxygen-saturated sample from the reactor and after substrate dosage the oxygen uptake rate was measured. The OUR^{max} value was subsequently determined by estimating the slope of the oxygen depletion curve. By relating the OUR^{max} to the actual biomass concentration, $q_{O_2}^{max}$ can be calculated.

Maximum nitrite removal rate

For the evaluation of the maximum volumetric nitrite removal rate ($Rate_{NO_2^-}^{max}$, g-NO_2^--N L^{-1} d^{-1}) batch tests were conducted in the MBR reactor. A solution containing ammonium 7.1 M (as $(NH_4)_2SO_4$) and nitrite 8.6 M (as $NaNO_2$) was injected (4 mL) into the reactor immediately after the influent flow was stopped and the concentrations were monitored in time (starting ammonium and nitrite concentrations 12.9 mM and 3.4 mM, respectively). Starting nitrite concentration was chosen in order to allow substrate non-limiting conditions and avoid the inhibitory effect of nitrite (Lotti et al., 2012b [**Ch.4**]). The amount of ammonium dosed with the injection (28.4 mM) was calculated based on the complete consumption of the dosed nitrite (34.4 mM) considering a nitrite on ammonium consumption ratio of 1.21. The duration of the batch test was 0.5-2 hours. Maximum biomass specific nitrite removal rate ($q_{NO_2^-}^{max}$) was calculated by relating $Rate_{NO_2^-}^{max}$ to the biomass concentration in the reactor.

CO_2, N_2O, NO, N_2, O_2 in the gas-phase

CO_2, N_2O, NO and O_2 concentrations in the dewatered gas leaving the reactor were measured on-line using a Rosemont Analytical multicomponent gas analyzer. Off-gas dewatering was achieved in a reflux condenser operated at 4°C with a Permapure filter. Measured partial pressures were converted to molar fluxes by correcting for the air pressure and the gas flow rate applied. N_2 was measured off-line on an Agilent 6890 gas chromatograph. Mass transfer limitations could be neglected due to the high stirrer speed (200 rpm) and the maximum aggregates diameter (≈30 μm).

Mass flow

The gas flow rate of the dewatered outflowing gas was measured with a Bronkhorst MFC Calibrator, Bronkhorst Hi-Tec, Veenendaal, Netherlands.

Biomass concentration

Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to the Standard Methods (APHA 2005). Total organic carbon (TOC) was measured in a SHIMADZU 5050A as the difference between total carbon (TC) and inorganic carbon (IC). TC present in the sample was combusted or decomposed to CO_2 in a combustion tube filled with oxidation catalyst, heated to 680°C and supplied by high purity oxygen. Resulting CO_2 was then detected in a non-dispersive infrared gas analyser (NDIR). For IC measurement the sample was acidified and resulting CO_2 was detected in a NDIR gas analyser. During the experimentation on kinetic characterization biomass concentrations were estimated from CO_2 uptake in the bioreactor and checked by TOC measurement.

Elemental composition of biomass

The carbon, hydrogen, nitrogen and sulfur content of the enriched sludge was measured using a PerkinElmer CHNS ANALYZER 2400 (Waltham, Massachusetts, USA) after washing the sludge with a physiological salt solution and drying under vacuum at -50°C. Phosphorus content of the biomass was measured after digesting (according to standard methods, APHA 2005) one gram of freeze-dried sample using commercial test kits according to the protocol of the manufacturer (brand: Dr.Lange test kits, Hach-Lange GmbH, Düsseldorf, DE, kits LCK348) and determined on a designated spectrophotometer (DR 2800). Oxygen content was calculated as the remainder after subtracting the ash and CHNSP content of the samples.

Soluble nitrogen compounds

Ammonium, nitrite and nitrate were measured via spectrophotometric flow injection analysis (QuickChem 8500 series 2 FIA System, Lachat Instruments, Loveland, Colorado, USA). The methods applied were QuikChem®Methods 10-107-06-5-E for ammonium (range 0.1 to 10.0 mg-N L^{-1}, measurement of NH_3 after increasing pH and volatilization) and 10-107-04-1-C for nitrate/nitrite (range 0.01 to 2.0 mg-N L^{-1}, direct measurement of nitrite, or measurement proceeded by reduction of NO_3^- to NO_2^- to yield the concentration of "NO_3^-+NO_2^-") according to the protocol of the manufacturer. The length of the sample loop of the nitrate/nitrite detection was increased in order to obtain a measurement range from 0.005±0.001 to 10±0.01 mg-N L^{-1}.

Sampling Procedure

Samples were taken directly from the reactor by means of a 30 mL syringe filled with deep-frozen (-26°C) steel spherical beads (0.5 mm diameter, specific heat 0.107 cal g^{-1} K^{-1}). The amount (grams) of beads contained in the syringe was adapted to the sample volume in order to achieve the necessary temperature decrease (from 30°C to 2°C) and therefore to stop the microbial conversion. All samples were filtered at 0.45 µm before analysis.

Molecular Methods

Fluorescence in situ hybridization

Samples were fixed for fluorescence in situ hybridization (FISH) as described by Pernthaler et al. (2001). Briefly, cells were washed in phosphate buffer, fixed in paraformaldehyde and spotted onto Teflon-coated multi-well slides. After dehydration by immersion into ethanol solutions (50, 80, 98%), the cells were hybridized with the following fluorescently-labeled oligonucleotide probes: EUB-mix (mix of oligonucleotides EUB-338, EUB-338 II and EUB-338 III), Nso-190, Neu-653, AMX-820, KST-157 or Bfu-613. Details on the target organisms and the sequences can be found in Table 2. Probe and hybridization details are available at Probebase (Loy et al., 2003). Microscopic observations were performed with a Zeiss Axioplan epifluorescence microscope (Zeiss, Stuttgart, Germany). Fixation took place every two-three weeks. On day 472, the enrichment level was estimated by counting those cells which were visible under the microscope, but which did not hybridize with the AMX-820 probe (the number of non-anammox cells). This number was compared to the total number of visible (anammox and non-anammox) cells (circa 10,000).

DNA Extraction, PCR Amplification, and Phylogenetic Analysis

On day 451 a sample (5 mL cell suspension) was taken from the reactor and directly centrifuged for 5 min at 13,000 rpm at 4°C. The cell pellet was stored at -20°C. Genomic DNA was extracted from the cells using the UltraClean Soil DNA Extraction Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's protocol. The quality of the extracted DNA was analyzed by agarose gel electrophoresis. Subsequently, the extracted DNA was used to amplify the nearly complete 16S rRNA gene using primers Pla-46f and Univ-

1392r (see Table 2 for details). The PCR products were analyzed with agarose gel electrophoresis, purified using the Qiaquick PCR purification kit (Qiagen, Düsseldorf, Germany) and sequenced by a commercial company (BaseClear, Leiden, The Netherlands). The sequences were first compared to sequences stored in GenBank using blastn. Thereafter, they were imported into the SILVA database (Pruesse et al., 2007) with the ARB software program (Ludwig et al., 2004). The sequences were automatically aligned and alignments were corrected by hand after which a tree was created using the neighbor-joining algorithm with Felsenstein correction.

Table 2. Oligonucleotides probes implied for FISH analysis.

Probe	Target organisms	Sequence (5'-3')	Reference
Eub-338	Bacteria	GCT GCC TCC CGT AGG AGT	Amann et al. (1990a)
Eub-338 II	Bacteria	GCA GCC ACC CGT AGG TGT	Daims et al. (1999)
Eub-338 III	Bacteria	GCT GCC ACC CGT AGG TGT	Daims et al. (1999)
Nso-190	AOB of the beta-Proteobacteria	CGA TCC CCT GCT TTT CTC C	Mobarry et la. (1996)
Neu-653	Halophilic and halotolerant *Nitrosomonas* sp.	CCC CTC TGC TGC ACT CTA	Wagner et al. (1995)
Amx-368	Anammox bacteria	CCT TTC GGG CAT TGC GAA	Schmid et al. (2003)
Amx-820	"Kuenenia"/"Brocadia"	AAA ACC CCT CTA CTT AGT GCC C	Schmid et al. (2000)
Kst-157	"Kuenenia"	GTT CCG ATT GCT CGA AAC	Schmid et al. (2001)
Bfu-613	"Brocadia Fugida"	GGA TGC CGT TCT TCC GTT AAG CGG	Kartal et al. (2008)
Pla-46f	Planctomycetes	GAC TTG CAT GCC TAA TCC	Neef et al. (1998)
Univ-1392r	Bacteria	ACG GGC GGT GTG T	Ferris et al. (1996)

Kinetic Characterization Experiment

On day 587 the 48 hours-long experiment for the kinetic characterization was started (hereafter referred to as *kinetic characterization experiment*). The experiment consisted of a change in the operation of the bioreactor (i.e., ammonium and nitrite loading rates) and on-line registration/off-line measurement of the response of the key variables in the system (*on-line*: N_2O, NO, CO_2, pH; *off-line*: NH_4^+, NO_2^-, NO_3^-, N_2, TOC). Ammonium and nitrite loading rates were linearly increased to 1.058 g-N L^{-1} d^{-1} during the first 24h of the experiment and then linearly decreased during the subsequent 24h until the usual ammonium and nitrite load were established again (for both 0.504 g-N L^{-1} d^{-1}). Details on the experimental set-up and procedure are reported below. The measured response of the system variables allowed for calculation of important state variables such as the biomass yield, the biomass specific substrates uptake rate and the biomass growth rate as a function of the actual process conditions as described in the *Calculations* section.

Experimental Set-Up

On day 587 the 48 hours-long experiment for the kinetic characterization was started. Two additional feed vessels (*vessel-a* and *vessel-b*) were connected to the feeding-medium vessel (*vessel-c*) by two distinct tubing lines equipped with two (peristaltic) pumps as indicated in Figure 1 (before entering in *vessel-c* the two tubing lines were connected for practical reasons). The medium contained in *vessel*-c entered the reactor by means of a (peristaltic) pump at a flow rate of 6 L d^{-1} throughout the experimentation. *Vessel-a* contained a concentrated medium (20 L) prepared according to Table 1 with 300 mM ammonium and 300 mM nitrite (*medium-a*). *Vessel-b* contained a concentrated medium (20 L) prepared according to Table 1 without substrates (*medium-b*). *Medium-a* entered *vessel-c* (starting volume of 20 L) by means of a (peristaltic) pump at a flow rate of 6 L d^{-1} during the first 24 hours of the experimentation. At hour 24 (corresponding to the maximum imposed load) the composition of the medium contained in *vessel*-c (20 L) was according to Table 1 with 120 mM ammonium and 120 mM nitrite. The pump operating *medium-a* (final volume 14 L) was then stopped and the pump operating *medium-b* was started. *Medium-b* entered *vessel-c* by means of a (peristaltic) pump at a flow rate of 18 L d^{-1} during the second 24 hours of the experimentation (hours 24-48). At hour 48 the

composition of the medium contained in *vessel*-c was back to steady state condition (Table 1) and the dynamic part of the experiment was therefore concluded. The pump operating *medium-b* (final volume 2 L) was then stopped and steady state operations were applied. pH was registered as constant at 7.0 throughout the experimentation due to phosphate buffer (tot-P 15 mM).

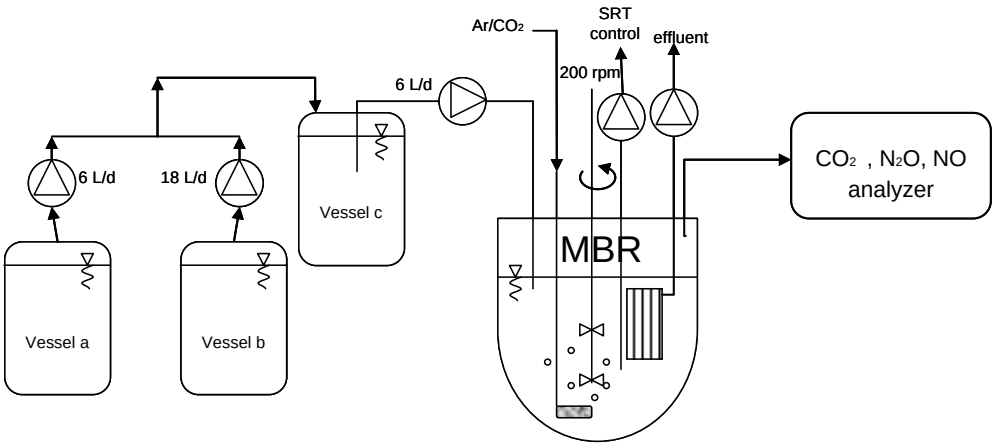

Figure 1. Experimental set-up for the kinetic characterization experiment.

Experimental Procedures

Prior to conducting an experiment, the bioreactor was operated at constant loading rate, HRT and SRT until steady state (at least five volume changes). The experiment subsequently consisted of a change in the operation of the bioreactor (i.e., ammonium and nitrite loading rates) and on-line registration/off-line measurement of the response of the key variables in the system (*on-line*: N_2O, NO, CO_2, pH; *off-line*: NH_4^+, NO_2^-, NO_3^-, N_2, TOC). The off-line measurements were conducted on samples taken directly from the reactor at regular time intervals (5 to 10 mL sample volume). Samples were taken by means of a 30 mL syringe filled with deep-frozen (-26°C) steel spherical beads (0.5 mm diameter, specific heat 0.107 cal g^{-1} K^{-1}). The amount (grams) of beads was adapted to the sample volume in order to achieve the necessary temperature decrease (from 30°C to 2°C) and therefore to stop the microbial conversion. All samples were filtered at 0.45 µm before analysis. The measured

response of the system variables allowed for calculation of important state variables such as the biomass yield, the biomass specific substrates uptake rate and the biomass growth rate as a function of the actual process conditions as described below in the *Calculations* section.

Calculations

Mass Balances

Since the carbon dioxide flux entering the bioreactor was known, on-line measurement of the CO_2 concentration in the dewatered gas leaving the bioreactor allows for direct estimation of the biomass production rate ($R_X = -R_{CO2}$, *C*-mol h^{-1}). Measured CO_2 uptake rates (R_{CO2}) were corrected for the dissolved carbon dioxide and bicarbonate in the reactor effluent: on-line measurement of the operational pH allows for the estimation of the CO_2 and bicarbonate concentration in the bulk. Measurements of the soluble nitrogen compounds (NH_4^+, NO_2^-, NO_3^-) together with the measurement of the volatile nitrogen compounds allows for estimation of the actual substrates uptake rate (R_{NH4}, R_{NO2}) and the actual products production rate (R_{NO3}, R_{N2}). R_{N2O} and R_{NO} were also estimated even if their contribution to the total nitrogen balance was smaller than 0.1%. Using all these measurements a full mass balance can be established over the reactor. A metabolic system that catalyzes one dominant catabolic and anabolic reaction has two degrees of freedom and requires measurement of two rates for system identification. Since more measurements are available with the system described here, the individual measurements can be checked.

State Variables

Based on the observed carbon dioxide uptake rate (R_{CO2}, *C*-mol h^{-1}) and ammonium uptake rate (R_{NH4}, N-mol h^{-1}), the biomass yield ($Y_{X/NH4}$) can be calculated according to:

$$Y_{X/Nh_4} = \frac{R_X}{R_{NH_4}} = \frac{R_{CO_2}}{R_{NH_4}} \qquad (1)$$

It is assumed that all CO_2 taken up is converted into biomass. The experiments described in this Chapter were conducted starting from steady state. The steady state biomass concentration (X^{SS}) can be calculated from R_{CO2} and the reactor-suspension outflow rate (Q_W, L h^{-1}; $Q_W = V_L\ SRT^{-1}$):

$$X^{SS} = \frac{-R_{CO_2}}{Q_w} \quad (2)$$

During the dynamic (non-steady state) experiment the biomass concentration (X) is calculated by integration with respect to time of the biomass mass balance:

$$X_{t+\Delta t} = X_t - \frac{(Q_w \cdot X_t + R_{CO_2}) \cdot \Delta t}{V_L} \quad (3)$$

During the experiments biomass concentrations calculated based on R_{CO_2} were verified by TOC and gravimetric (TSS and VSS) measurements at regular intervals. Knowing the actual biomass concentration allows for calculation of the actual specific ammonium uptake rate (q_{NH_4}, N-mol C-mol^{-1} h^{-1}) and the actual specific growth rate (μ, h^{-1}):

$$q_{NH_4} = \frac{R_{NH_4}}{X \cdot V_L} \quad (4)$$

$$\mu = \frac{R_X}{X \cdot V_L} = \frac{-R_{CO_2}}{X \cdot V_L} \quad (5)$$

Actual specific nitrate, dinitrogen gas, nitrous oxide and nitric oxide production rate (q_{NO_3}, q_{N_2}, q_{N_2O}, q_{NO}, N-mol C-mol^{-1} h^{-1}) can be calculated with Equation (4) considering the respective non-specific production rate (R_{NO_3}, R_{N_2}, R_{N_2O}, R_{NO}, N-mol h^{-1}). Actual specific nitrite uptake rate (q_{NO_2}, N-mol C-mol^{-1} h^{-1}) can be equally calculated with Equation (4) considering the respective non-specific uptake rate (R_{NO_2}, N-mol h^{-1}).

Statistical Methods

A detailed explanation of the statistical methods used in this study is reported by Verheijen et al. (2009).

Modeling

A model representing the MBR operations was implemented in AQUASIM (Reichert 1998). Anammox was the only microbial activity considered and,

except when explicitly stated, it was modeled according to Hao et al. (2002b). In the model the maximum specific growth rate was equal to 0.21 d^{-1} and the biomass yield was equal to 0.104 g-VSS $g\text{-}N^{-1}$. Other parameters like the Henry constants or acid dissociation constant were taken from literature. The soluble compounds considered were ammonium, nitrite, nitrate, dinitrogen gas, carbon dioxide, bicarbonate, carbonate, and protons. The only particulate compound considered was anammox biomass. The compounds considered in the gas phase were Argon, dinitrogen gas, and carbon dioxide.

Nitrite Half-Saturation Constant

The nitrite half-saturation constant (or nitrite affinity constant, $K_{NO_2^-}$, mg-N L^{-1}) was estimated from the reactor data during steady state operation (i) and from the data of the *kinetic characterization experiment* (ii):

(i) Before the evaluation of the maximum volumetric nitrite uptake rate ($Rate_{NO_2^-}^{max}$) by means of batch tests, the nitrite level at steady state operation was measured in three replicates by sampling as described above in a 2 hours period. The traditional Monod-based Equation (6) represents the dependency of the biomass specific substrate uptake rate (q_S) on the substrate concentration (C_S) and on the substrate half-saturation constant (K_S).

$$q_S = q_S^{max} \frac{C_S}{C_S + K_S} \qquad (6)$$

$K_{NO_2^-}$ can then be calculated with Equation (7) considering (i) the definition of the biomass specific substrate uptake rate ($q_S = \frac{Rate_S}{X}$), (ii) assuming the biomass concentration (X) as constant throughout the batch test and (iii) considering that the reactor was previously operated under nitrite limitation at steady state:

$$K_{NO_2^-} = C_{NO_2^-} \frac{Rate_{NO_2^-}^{max} - Rate_{NO_2^-}}{Rate_{NO_2^-}} \qquad (7)$$

where the actual nitrite uptake rate ($Rate_{NO_2^-}$) was equal to the nitrite loading rate (nitrite removal efficiency >>99%).

(ii) The results from the *kinetic characterization experiment* were fitted by the model output calibrating the nitrite half saturation constant. The best data

fitting was evaluated by minimizing the sum of squared residuals according to the method of least squares. The nitrite half saturation constant was therefore identified.

Results

Reactor Operation

The membrane bioreactor was inoculated with granular sludge enriched in anammox bacteria originating from the full-scale installation of the Dokhaven WWTP in Rotterdam, The Netherlands (van der Star et al., 2007) and operated for more than 600 days. The nitrite-loading rate was increased step-wise by increasing the influent flow rate (medium composition remained constant) each time the reactor nitrite concentration was observed to be stable and below 10 mg-N L^{-1} for few days. Within one month the reactor could be operated at the design conversion rate of 0.5 g-NO_2-N L^{-1} d^{-1} (total nitrogen-loading rate of 1 g-N L^{-1} d^{-1}) at 30°C and at sludge retention time (SRT) of 15 days. On day 63 the SRT was decreased to 12 days and maintained constant throughout the experimentation. A few days after the design nitrite-loading rate was reached, the reactor was operated under nitrite limitation as visible from bulk nitrite concentration reported in Figure 2. The results of in-situ batch tests in time confirmed that anammox biomass always had a significant overcapacity: the maximum nitrite removal rate (MNRR) was 2-2.5 times higher than the applied nitrite load (n = 47). From day 100 onwards the reactor demonstrated a steady nitrogen removal with a total nitrogen removal efficiency of 80-85%. Ammonium and nitrate concentration in the effluent were on average both equal to 140 mg-N L^{-1}. During the whole period at steady state (days 100-612), the molar ratio between nitrite removed and ammonium removed (NO_2/NH_4 ratio) and the molar ratio between nitrate produced and ammonium removed (NO_3/NH_4 ratio) were 1.219±0.08 and 0.211±0.07, respectively. The average NO_2/NH_4 ratio and NO_3/NH_4 ratio values during the different experimental periods correspond to different average bacterial aggregation status (granules, flocs, micro-flocs/free cells) as reported in Table 3. Oxygen concentration in the outflowing gas was measured occasionally on-line for 2-15 consecutive days and it was always below the analyzer detection limit (0.001% of oxygen saturation at 30°C). Maximum volumetric oxygen

uptake rate (OUR^{max}) was also measured occasionally by means of BOM-measurement since day 121 and no oxygen removal capacity could be detected (12-24 hours tests), indicating the absence of any aerobic microorganism.

Table 2. Molar ratio between nitrite removed and ammonium removed (NO_2/NH_4 ratio) and molar ratio between nitrate produced and ammonium removed (NO_3/NH_4 ratio) during different experimental periods corresponding to different average bacterial aggregation status (granules, flocs, micro-flocs/free cells).

Dominant bacterial aggregation status	Period [days]	NO_2/NH_4 ratio*	NO_3/NH_4 ratio*
Granules	0-100	1.22±0.07	0.20±0.04
Flocs	100-300	1.25±0.07	0.23±0.05
Suspended	300-612	1.21±0.05	0.20±0.04

***** mean ± standard deviation

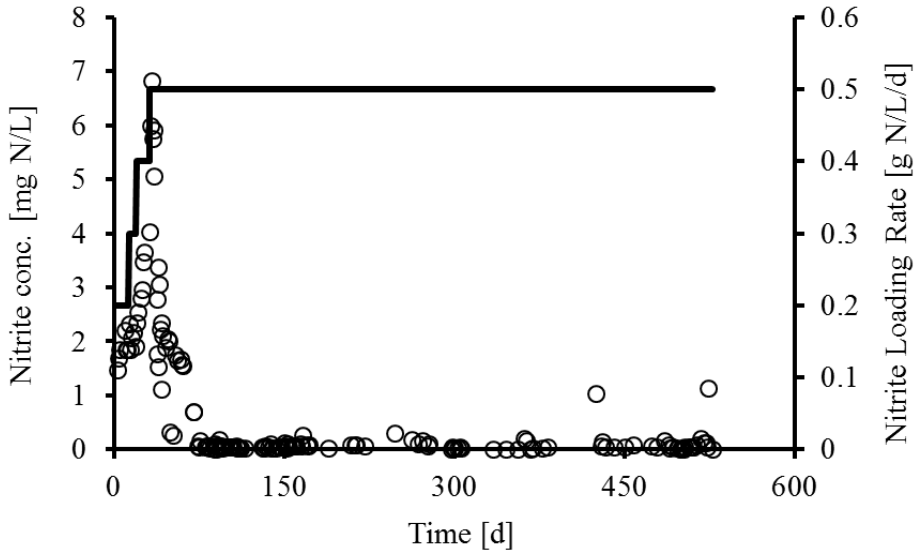

Figure 2. Nitrite concentration in the reactor (circles, left vertical axis) and nitrite-loading rate applied (continuous line, right vertical axis).

The particle size distribution was frequently measured throughout the reactor operations. In Figure 3 the results of three measurement campaigns conducted on day 0, 98 are 164 are reported to show the progressive decrease of the aggregates' size. The granules of the inoculum (diameter distribution mode equal to 1 mm) progressively changed to smaller granules/flocs during the first 100 days of operation. After the first 140-150 days the floc-dimension distribution stabilized at floc-diameter size smaller than 90-100 µm (95^{th} percentile equal to 50 µm, Fig. 3).

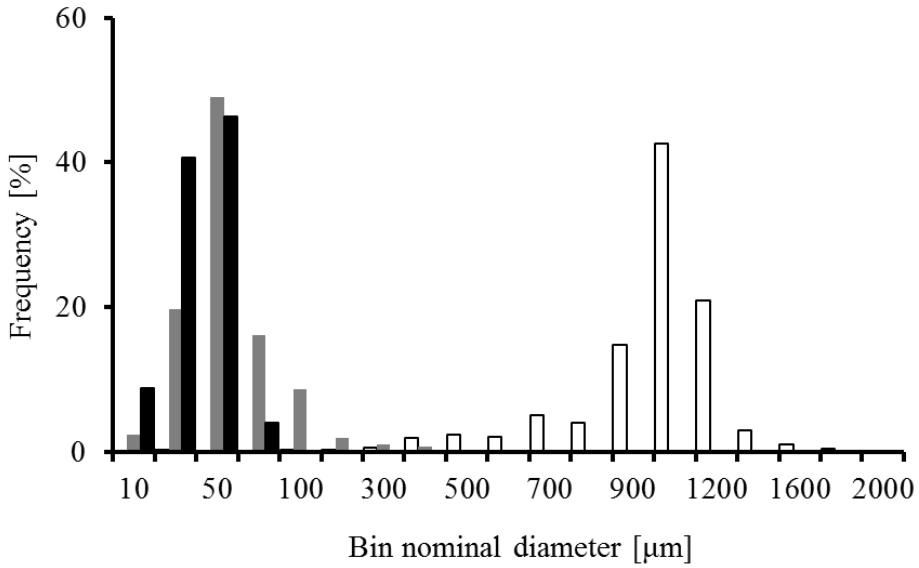

Figure 3. Aggregate size distribution as frequencies ([%], number of aggregates on total number of aggregates investigated) in discrete intervals ([µm], in figure the middle point of each bin is reported). Vertical bars describe the aggregate size distribution on day 0 (inoculum, white), on day 98 (gray) and on day 164 (black).

After the first 300 days of operations biomass was completely suspended and in fact unable to settle within 24 hours tests. Weekly conducted microscope investigations revealed that the biomass was mainly present as free cells with few small flocs with diameter comprised between 10 and 50 µm. The biomass concentration inside the reactor continuously decreased during the first three months of operation. During the same period the ash-content decreased resulting in an increasing VSS on TSS ratio. After approximately day

100 the biomass concentration was stable and maintained throughout the experimentation at about 0.52 g-TSS L^{-1} and 0.47 g-VSS L^{-1} (g-VSS g-TSS^{-1} ~ 0.90, Fig. 4).

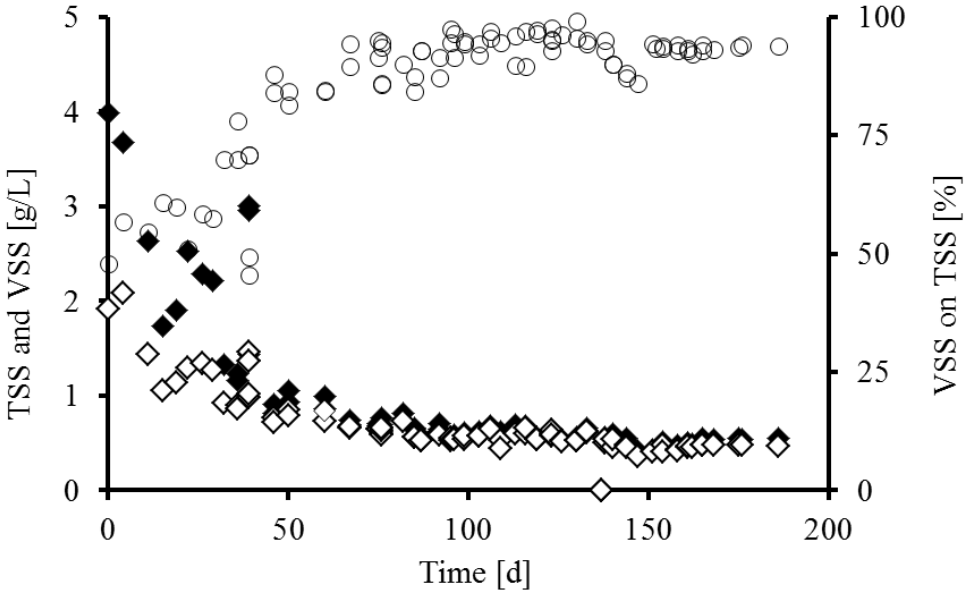

Figure 4. Biomass concentration inside the reactor as TSS ([g L^{-1}], closed diamonds, left vertical axis), VSS ([g L^{-1}], opened diamonds, left vertical axis) and VSS/TSS ratio ([%], open circles). Only dynamic part of the data set (first 200 days) is shown.

Microbial Community and Enrichment Level

In 2007 the anammox bacteria of the full-scale anammox reactor of Dokhaven wastewater treatment plant, was shown to consist of Candidatus "Brocadia" (Van der Star et al., 2007). When the MBR was inoculated (May 2010) this was still the main group since hybridization took place with the "Kuenenia"/"Brocadia"-specific probe (AMX-820), but not with the "Kuenenia"-specific probe (KST-157) (Fig.5, top). Throughout the whole experimental period all cells hybridizing with the "Kuenenia"/"Brocadia"-specific probe (AMX-820) were also found to hybridize (>99.5%) with the *Candidatus* Brocadia Fulgida-specifc probe (Bfu-613, Kartal et al., 2008, Fig.5, bottom). Since free cells were obtained, quantification using FISH was possible

by counting each individual cell hybridized with the Brocadia Fulgida-specific probe (Bfu-613) against the total number of bacteria present in the sample (hybridized with EUB-mix). The enrichment level at day 483 was 98±1% (ca. 10,000 cells counted).

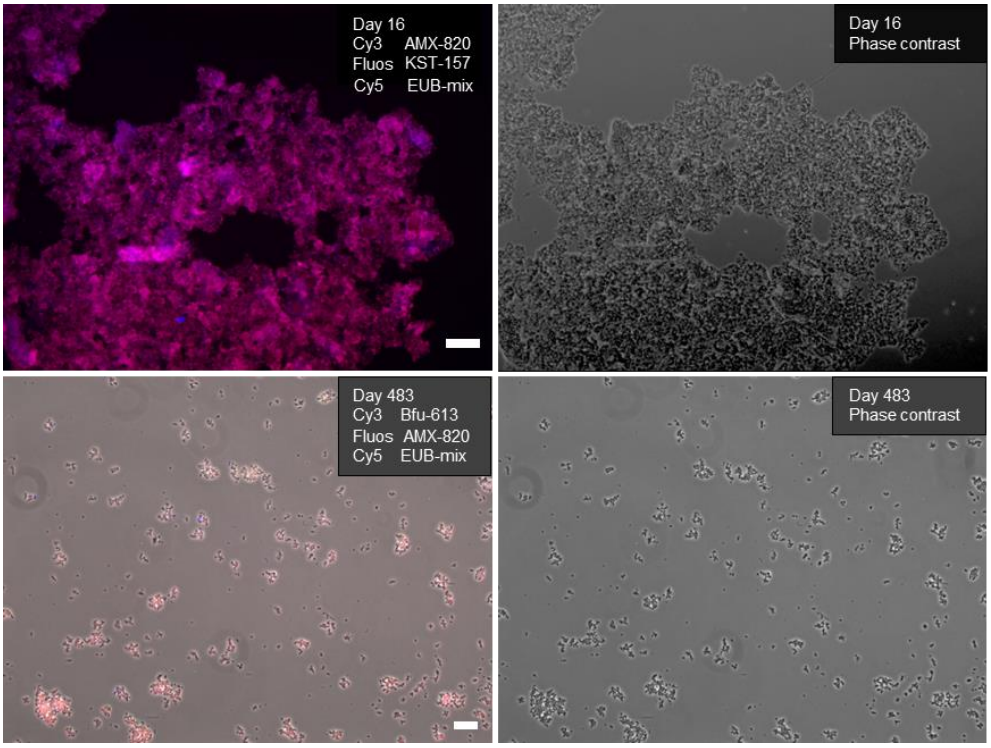

Figure 5. The bacterial population in the membrane bioreactor as shown by FISH (left) and phase contrast microscopy (right). Fixation took place on day 16 (top), 483 (bottom). Scale bar is 50 µm (top) and 10 µm (bottom). The oligonucleotide probes used are specified on the figure. Cy3 is depicted in red, Fluos in green and Cy5 in blue.

The bacterial community structure was confirmed by 16S rRNA sequence analysis. The sequences of the sample on day 451 showed the strongest similarity (99%) similarity with *Candidatus* Brocadia sp. 40 (Kieling et al., 2007), 97.5% similarity with *Candidatus* Brocadia Caroliniensis (Vanotti et al., 2011) and 97% with *Candidatus* Brocadia Fulgida (Kartal et al., 2008). Figure 6 shows a phylogenetic tree based on the 16S rRNA sequences of the strains. The sequence of the probe Bfu-613 has only one mismatch with the 16S rRNA sequences of the clones described in this study.

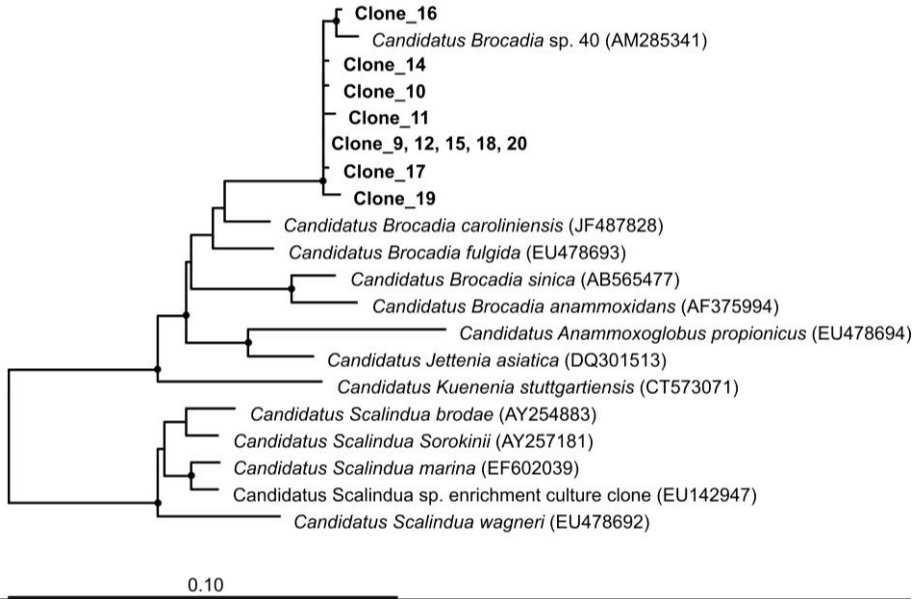

Figure 6. Phylogenetic tree of 16s rDNA gene sequences showing the affiliation of anammox bacteria. The tree has been calculated using a maximal likelihood method, RAxML which is implemented in the ARB v5.2 software package. Bootstrap was performed for 250 round and values of >90% are indicated by a solid black dot. For calculations the bac_var_ssuref:bacteria filter (SSU_ref_108_silva database) has been applied to the data set to filter out noise. In total 1271 positions (ecoli pos. 594-1528) were used for calculation. Clones were calculated in the tree using 'quick add sequences using parsimony' tool. In total 711 positions (ecoli pos. 811-1528) were used. All Clones are highlighted in bold. The scale-bar represents 10% sequence divergence.

Elemental Biomass Composition

The elemental biomass composition evaluation was measured on a dried sample taken on day 485, when the anammox enrichment purity was shown to be 98±1%. The C, H, N, S and P content were measured in two replicates and the elemental composition of the biomass was determined to be $CH_{1.74}O_{0.31}N_{0.20}S_{0.01}P_{0.01}$. The biomass molecular weight results therefore equal to 22.1 g-VS C-mol^{-1}.

Affinity for Nitrite

Kinetic Characterization by Changing the Loading Rate in Time

In order to accurately determine the nitrite affinity constant for anammox bacteria, the nitrogen loading rate (NLR, sum of ammonium and nitrite loading rate) supplied to the reactor was linearly increased for 24 hours starting from the steady state-nitrogen loading rate (NLR^{SS}, 1 g-N L^{-1} d^{-1}). The experiment started on day 587, after more than two weeks of stable operation in which bacteria grew as free cells. Mass transfer limitation could therefore be considered negligible. After 24 hours from the beginning of the experiment the nitrogen loading rate reached 210% of the NLR^{SS} (2.1 g-N L^{-1} d^{-1}). NLR was then continuously decreased for 24 hours at the end of which the steady state-nitrogen loading rate was achieved again. Results are shown in Figure 7 where on the *x-axis* time zero corresponds to the experimental day 587. The dynamics in the imposed NLR on the system (Fig. 7, A) resulted in corresponding dynamics in the ammonium, nitrite and nitrate concentration inside the reactor (Fig. 7, B). From the bulk concentrations and the applied load, the NH_4^+ and NO_2^- uptake rate and the NO_3^- production rate were calculated (Fig. 7, C). The ratio between nitrite and ammonium uptake (NO_2/NH_4 ratio) and the ratio between nitrate production and ammonium uptake (NO_3/NH_4 ratio), were calculated from off-line concentration measurements and from the known ammonium and nitrite loading rate. Throughout the experiment NO_2/NH_4 ratio and NO_3/NH_4 ratio were equal to 1.21±0.01 (n = 34) and 0.20±0.01 (n = 34), respectively (Fig. 7, D). From the online signal of CO_2 concentration in the off-gas and pH, a mass balance of inorganic carbon was established and the carbon fixation rate (equal to biomass growth rate) was calculated (Fig. 7, E). Throughout the experiments no limitations in ammonium or carbon dioxide were observed while nitrite remained the limiting substrate. From the actual CO_2-uptake rate the biomass concentration as a function of time was estimated using Equation 3 as shown in Figure 7, F. The biomass concentrations calculated from CO_2-uptake correspond adequately to the biomass concentration measured as TOC indicating that the CO_2-data are representative for the biomass production. From the actual ammonium and carbon dioxide uptake rate the actual yield can be calculated according to Equation 1.

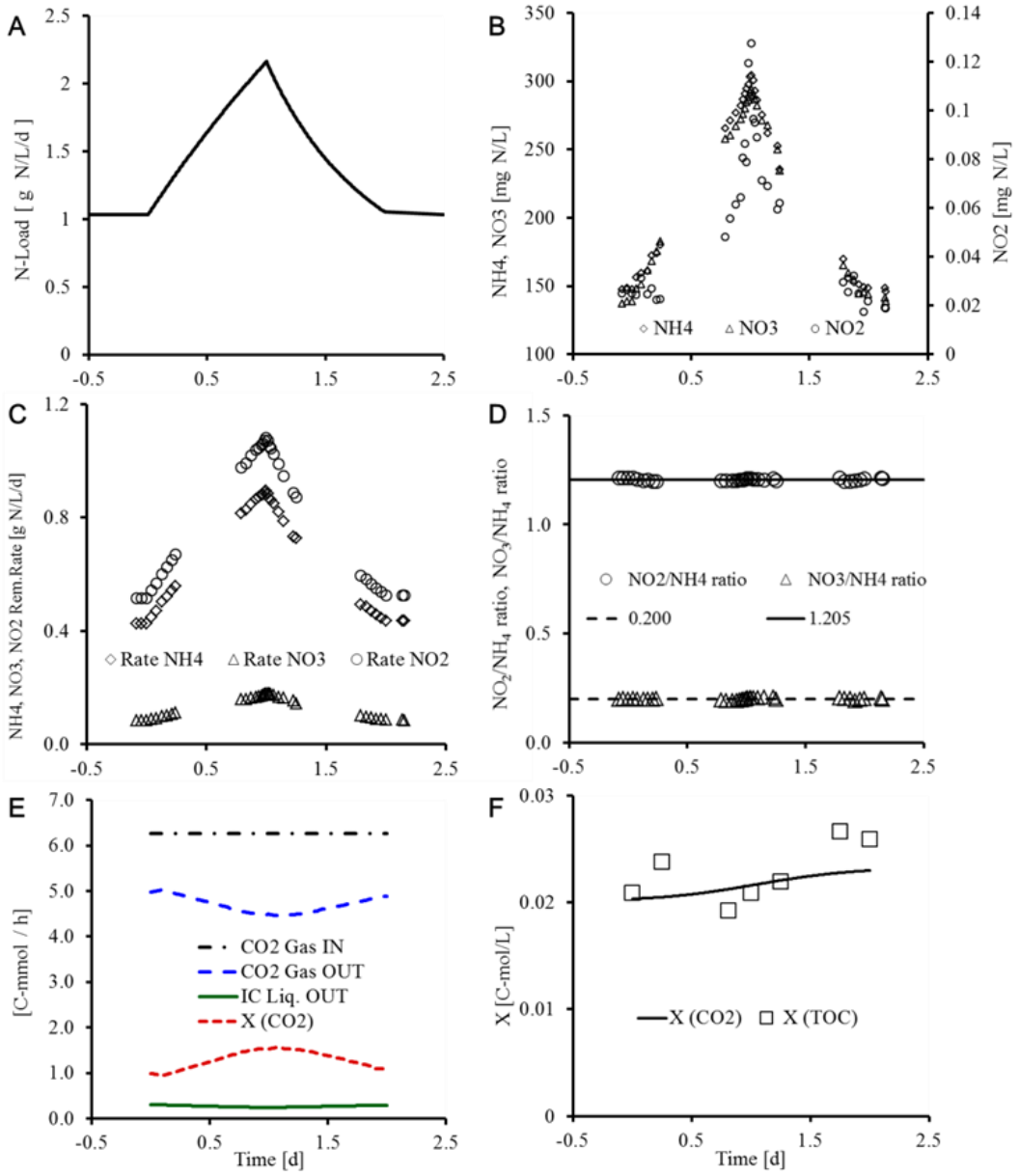

Figure 7. Experiment for kinetic parameter determination (i.e., affinity constant for nitrite). In alphabetical order the graphs show as a function of time the imposed total nitrogen loading rate (**A**); the ammonium [NH_4], nitrite [NO_2] and nitrate [NO_3] concentrations in the reactor as measured off-line (**B**); the ammonium [Rate NH_4], nitrite [Rate NO_2] uptake rate and the nitrate production rate [Rate NO_3] calculated from off-line concentration measurements and the ammonium and nitrite loading rate (**C**); the ratio between nitrite and ammonium uptake (NO_2/NH_4 ratio, circles) and the

ratio between nitrate production and ammonium uptake (NO_3/NH_4 ratio, triangles) calculated from off-line concentration measurements and the ammonium and nitrite loading rate (**D**); the carbon dioxide entering [CO_2 Gas IN] and leaving [CO_2 Gas OUT] the system in the gas phase, the inorganic carbon leaving the system in the liquid phase [IC liq. OUT] and the carbon fixation rate (biomass growth rate) as calculated from R_{CO2} [X (CO_2)] (**E**); the biomass concentration as calculated from R_{CO2} [X (CO_2)] and measured as total organic carbon [X (TOC)] (**F**).

The actual yield during the NLR dynamic change was 0.071±0.002 C-mol NH_4-mol^{-1}. It is worth to note that both the NO_3/NH_4 ratio and the biomass yield (Y_{X/NH_4^+}) were rather constant. This suggests the correctness of the hypothesis formulated by Van de Graaf et al. (1996) according to which nitrate is produced from nitrite to generate reducing equivalents for CO_2 fixation.

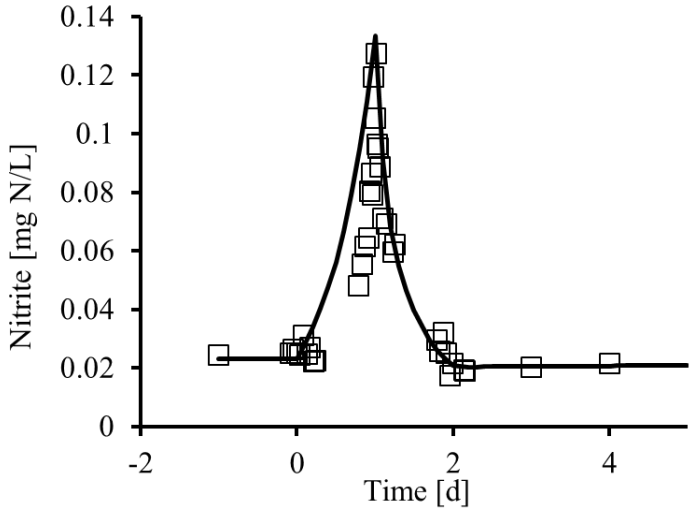

Figure 8. Nitrite concentration as measured during the experiment of the nitrogen loading rate dynamic change (squares) and as simulated by the model (solid line). The model was run 100 days before imposing the NLR dynamic change in order to reach the steady state: day zero in the figure represents the experiment starting time on day 587.

A model describing the anammox process and the MBR operations during the experiment was implemented. Using all the off-line and on-line measurements and considering the elemental composition of the biomass, a full mass balance could be established over the reactor. The carbon and nitrogen balance were closed at 88% and 92%, respectively. The maximum specific nitrite uptake rate (q_s^{max}, mg-N C-mol^{-1} d^{-1}) used in the model was calculated by the result of a batch test conducted 24 hours before the experiment and the biomass

concentration present in the reactor. The nitrite affinity constant for nitrite ($K_{NO_2^-}$) of anammox bacteria was calibrated in order to fit the model output. The best data fitting was evaluated by minimizing the sum of squared residuals according to the method of least squares (Figure 8). The nitrite half saturation constant was identified as equal to 0.035 mg-N L^{-1} (2.5 µM). Since this nitrite half saturation constant was identified in a suspended culture where mass transfer limitation does not act, it can be considered as the intrinsic half saturation constant for anammox bacteria.

From Reactor Operation and Maximum Rate

Throughout the entire experimental period the maximum nitrite removal rate (g-NO_2^--N L^{-1} d^{-1}) was measured using short-term batch experiments. From the nitrite concentration measured in the bulk during normal operation and the maximum rate measured the same day during the batch test (Eq. 7), the nitrite half-saturation constant ($K_{NO_2^-}$) was calculated (Figure 9). The apparent nitrite half-saturation constant was evaluated to be between 0.5 and 2.5 mg-N L^{-1} the first 70 days, 0.047±0.04 mg-N L^{-1} on days 70-200 and 0.038±0.011 on days 200-504.

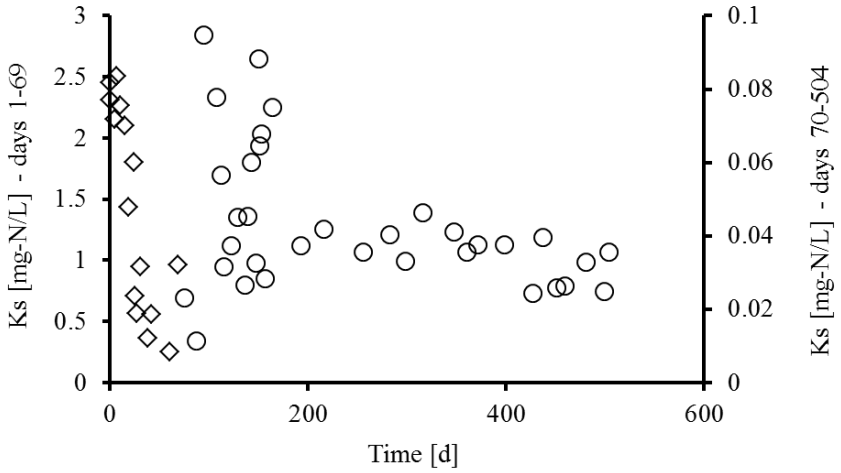

Figure 9. Nitrite half-saturation constant calculated according to Eq.7 from in reactor maximum nitrite removal rate ($Rate_{NO_2^-}^{max}$) measurements conducted throughout the eperimentation. Results on days 1-69 (diamonds) are reported on the left vertical axis, results on days 70-504 (circles) are reported on the right vertical axis.

The values of the (apparent) nitrite half-saturation constant (mg-N L^{-1}) estimated by batch test were related to the mean diameter (µm) of the aggregates measured by means of image analysis (Fig. 3) and are shown in Figure 10. A direct link between higher mass transfer limitation associated to bigger aggregate size and higher half saturation constant was observed. Already at average aggregate size larger than 100 µm mass transfer limitation gets important resulting in an apparent nitrite half saturation constant of about 0.1 mg-N L^{-1}. The half saturation constant seems to increase as exponential function of the average aggregate size up till aggregates as large as 700-800 µm, while for bigger aggregates the $K_{NO_2^-}$ seems to stabilize at values of about 2.3±0.2 mg-N L^{-1}. The $K_{NO_2^-}$-data for mean diameters smaller than 700 µm were interpolated with an exponential function in which the constant was set equal to the identified intrinsic half-saturation constant expressed in mg-N L^{-1} (0.035) (Figure 8). The so obtained exponential equation (Eq. 8) can be used to make a rough estimation of the apparent nitrite half-saturation constant ($K_{NO_2^-}$, mg-N L^{-1}) relative to a bacterial aggregate of a certain diameter (D, µm; valid for D<700 µm).

$$K_{NO_2^-} = 0.035 \cdot \exp(0.0062 \cdot D) \qquad (8)$$

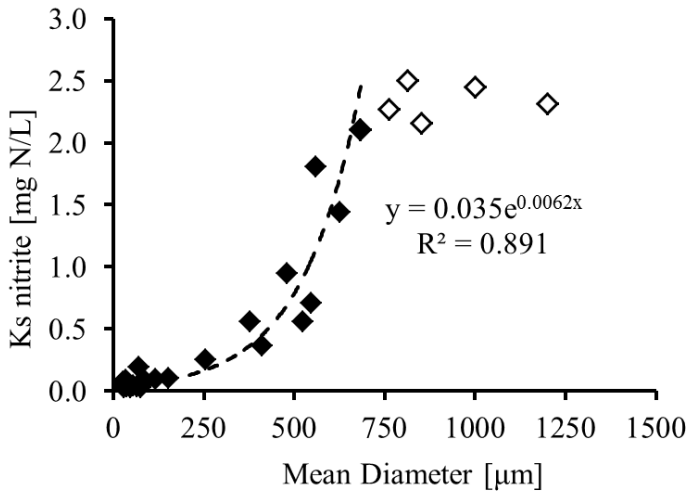

Figure 10. Nitrite half saturation constant [$K_{NO_2^-}$, mg-N L^{-1}] and relative aggregate size [µm]. Data relative to aggregate size smaller than 700 µm (closed diamonds) were interpolated with an exponential curve in which the pre-exponential factor was set equal to the intrinsic $K_{NO_2^-}$ estimated in this study (0.035 mg-N L^{-1}).

Biomass Yield and μ^{max}

The biomass yield (Y_{X/NH_4^+}) was shown to be constant (0.071±0.002 C-mol NH_4^+-mol^{-1}) during the *kinetic characterization experiment* when NLR increased up to 210% of the nitrogen loading rate at steady state (NLR^{SS}, 1 g-N L^{-1} d^{-1}). Taking into account the constant biomass yield and therefore neglecting maintenance, the ratio between biomass specific growth rate and substrate uptake rate is constant ($^{\mu}/_{q_S} = constant$). Furthermore, (i) by realizing that at steady state the actual biomass specific growth rate (μ) is equal to the inverse of the SRT (reliably controlled at 12 days), and (ii) assuming that the biomass concentration in the reactor (X) was constant throughout the batch-evaluation of the maximum nitrite uptake rate, the μ^{max} can be calculated as:

$$\mu^{\max} = \frac{q_S^{\max}}{q_S SRT} = \frac{Rate_S^{\max}}{Rate_S SRT} \qquad (9)$$

Given (i) the maximum nitrite uptake rate of 1.25 g N L^{-1} d^{-1} measured during the in-reactor batch test conducted the day before the NLR started to increase, (ii) the actual nitrite uptake rate (equal to the imposed nitrite load at steady state) of 0.50 g-N L^{-1} d^{-1} and (iii) the SRT of 12 days, a maximum biomass specific growth rate of anammox bacteria of 0.21 d^{-1} was calculated. The corresponding doubling time would then be 3.3 days. Even taking into account the decay rate at 35°C reported by Scaglione et al. (2009, 0.0048 d^{-1}), the μ^{max} would be equal to 0.206 d^{-1}, corresponding to a doubling time of 3.37 days (one hour longer than without considering maintenance).

Stoichiometry

The biomass yield was calculated during the *kinetic characterization experiment*. From the obtained yield (0.071 C-mol NH_4^+-mol^{-1}) and the elemental composition of the biomass, the stoichiometry of the Anammox process was calculated considering ammonium as the N-source and nitrite/nitrate as the electron-donor couple for inorganic carbon reduction during anabolism:

$$1NH_4^+ + 1{,}146NO_2^- + 0{,}071HCO_3^- + 0{,}057H^+ \rightarrow$$
$$0{,}986N_2 + 0{,}161NO_3^- + 0{,}071CH_{1,74}O_{0,31}N_{0,20} + 2{,}002H_2O \qquad (10)$$

From the measurements during the operational period between days 300 and 612 and the elemental composition of the biomass the stoichiometry of the anammox process was calculated using data reconciliation (Verheijen et al., 2009). The data-set used is reported in Table 4.

Table 4. Data-set used for the calculation of the stoichiometry using data reconciliation (Verheijen et al., 2009).

Entity	Mean	Error	Unit
Gas inflow	3.00	0.01	nL h^{-1}
Argon	95	0.02	%
CO_2	5	0.02	%
Headspace Pressure	1.039	$1.0 \cdot 10^{-4}$	Atm
Influent discharge	0.25	0.0021	L h^{-1}
SRT-control outflow	0.0347	0.0021	L h^{-1}
pH	7.0	0.1	-
Influent			
NH_4^+	60.0	0.60	N-mmol L^{-1}
NO_2^-	60.0	0.60	N-mmol L^{-1}
Effluent			
NH_4^+	10.56	0.12	N-mmol L^{-1}
NO_2^-	0.0079	0.0028	N-mmol L^{-1}
NO_3^-	10.34	0.12	N-mmol L^{-1}
Biomass	21.10	5.00	C-mmol L^{-1}

NO, N_2O emissions

During the steady-state operations of the MBR nitric oxide (NO) and nitrous oxide (N_2O) were produced at rate of 0.14±0.03 and 2.4±1.5 µmol-N L^{-1} h^{-1}, respectively (21 measurements). These NO and N_2O production rates accounted for the 0.003±0.002 and 0.056±0.02% of the imposed nitrogen load and for the 0.001±0.0006 and 0.20±0.01% of the total nitrogen removal rate, respectively.

During the experiment of the dynamic change of the N-load, NO and N_2O concentration in the off gas were monitored on-line. The resultant profile of NO and N_2O emission rates are reported in Figure 11. N_2O production was affected by the varying operational conditions inside the reactor, reaching a maximum production rate of about 4 μmol-N L^{-1} h^{-1} in correspondence of the peak of the applied nitrogen load (Fig. 11 and Fig. 7, A). NO, instead, was constantly emitted at the same rate as during steady state operations.

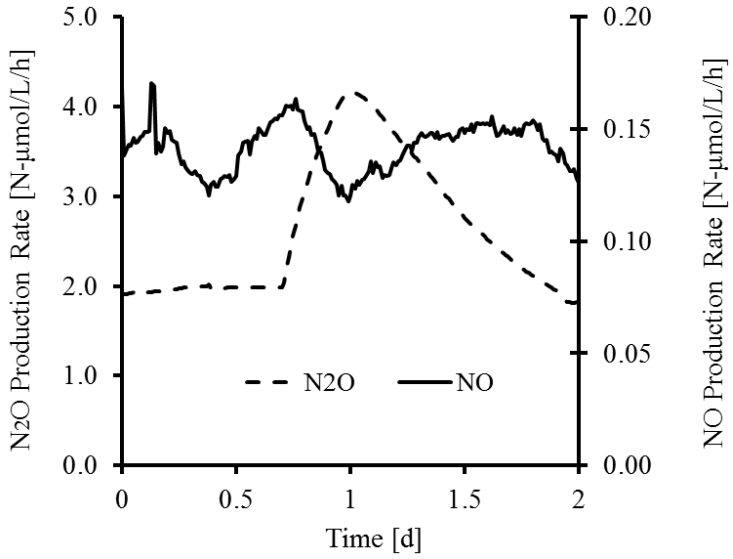

Figure 11. Nitrous oxide (N_2O, dashed line, left axis) and nitric oxide (NO, solid line, right axis) production during the experiment of the dynamic change of the N-load as calculated from on-line measurements (see for other measurements figure 7 and 8).

Oxygen Effect on the Aggregation Status of Anammox Biomass

In several occasions during the reactor run, the link between uncontrolled oxygen leak in the reactor and the aggregation of anammox free-cells into small flocs was noticed. On day 291, when biomass was present in the reactor in the form of small flocs (<100 μm), an attempt in which the influent medium was intensively sparged with helium (certified pure gas for analytical purposes) for 24 hours before being fed to the system was conducted and bacterial suspension was achieved in 10-15 hour time-span. 20 mL of air were then

injected in the feeding medium vessel (50 L) to verify the influence of oxygen and free-cells aggregated into small flocs in few hours. On day 300 measures were taken in order to ensure complete anaerobic conditions inside the reactor. These changes in medium preparation and gas-circuit were effective to obtain an almost stable suspension for more than 300 days: in several occasions the suspension turned temporarily back to the previous small flocs-status without apparent reason probably due to uncontrolled oxygen leakage into the reactor or oxygen content in the feeding medium; furthermore, each time the membrane was replaced (every 2-3 weeks) and therefore the reactor vessel was opened letting air enter the headspace, small flocs appeared within few hours. Growth in suspension could be restored within few hours by increasing temporarily the flow rate of the inflowing gas.

Figure 12. Syringe connected to the reactor mixed liquor by means of which the biomass settling properties were monitored.

As oxygen concentration in the reactor headspace was constantly below the analyzer detection limit, the oxygen-loading rate (μmol O_2 L^{-1} d^{-1}) causing free-cells to aggregate into small flocs was investigated. On day 450, after biomass was in suspension for ten consecutive days, a gas-bag (10 L) filled with air and N_2 at room temperature (23±2°C) was connected to the vessel containing degassed feeding medium. Oxygen loading rate supplied to the reactor was increased stepwise rising the air content of the gas-bag. Biomass aggregation status was monitored by the settling velocity in a 50 mL syringe connected by norprene tube (tube total internal volume of 4 mL) to the reactor mixed liquor (Fig. 12). As settling properties due the presence of micro-flocs were noticed, the biomass aggregation status was identified through microscope analysis on mixed liquor samples taken directly from the reactor with air-tight syringes previously flushed with nitrogen gas. Great care was taken when the sample was poured on the microscope slide in order to minimize exposure to oxygen; the whole microscope analysis procedure took no more than 3 minutes. An oxygen loading rate of 0.68±0.06 μmol O_2 L^{-1} d^{-1} (oxygen in the medium fed to the reactor equal to 1.1 μmol-O_2 L^{-1}) was found to be sufficient to make the free-cells aggregate into small flocs (<100 μm) within one hour. In two distinct occasions, on day 481 and on day 502, the experiment was repeated and the result was confirmed. In figure 13 the phase-contrast pictures relative to the aforementioned oxygen loading rate experiments are reported. At room temperature, a degassing method with 99.6% oxygen removal efficiency was therefore required to allow bacteria as free-cells.

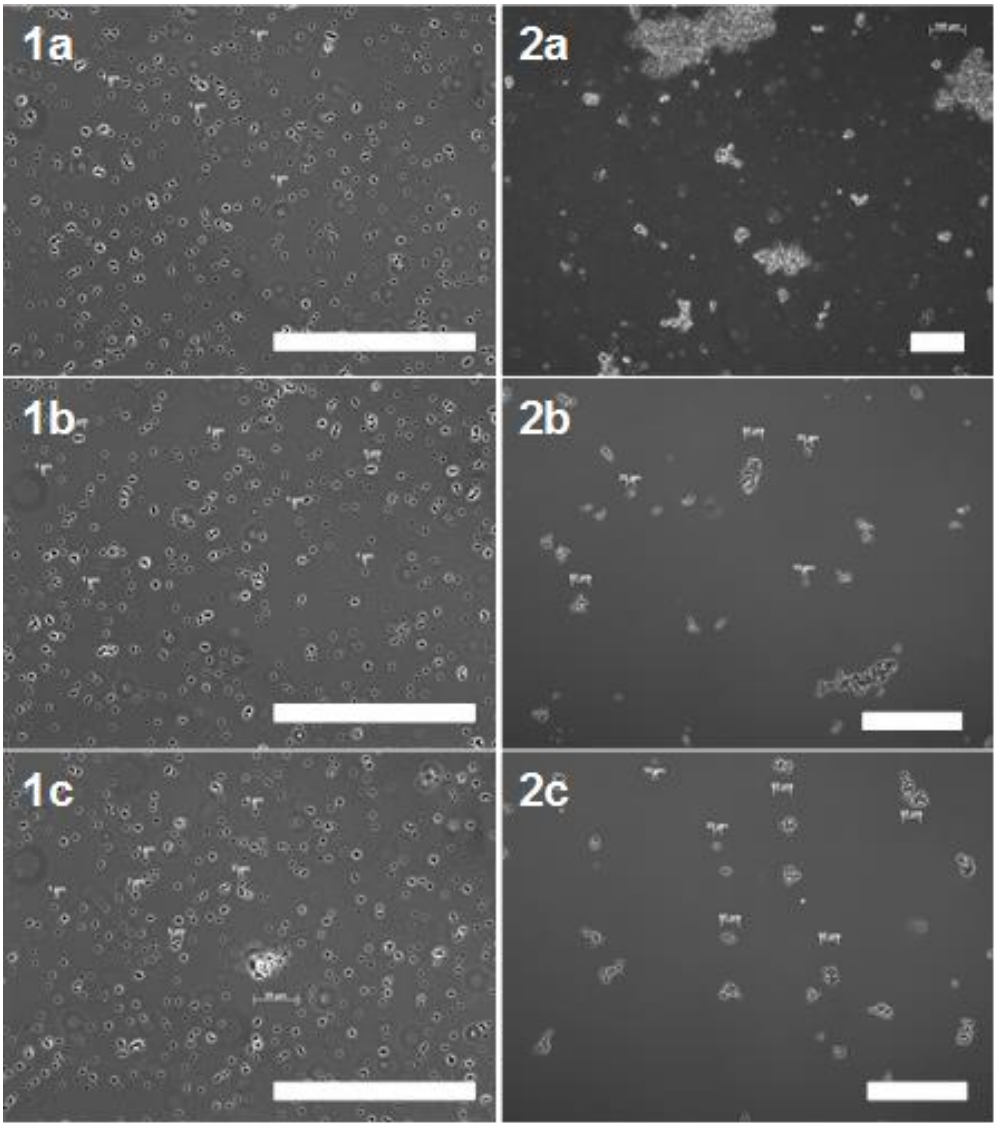

Figure 13. Phase-contrast pictures taken under the microscope representing the biomass aggregation status before (1) and after (2) the critical oxygen loading rate was supplied to the reactor. The experiment was conducted on day 450 (a), 481 (b) and 502 (c). Bar=100 µm.

Discussion

Anammox growth in suspension culture in a MBR

Van der Star et al. (2008) was successful in obtaining a free cell enrichment of anammox bacteria for the first time and reported that the trigger was the reduction of calcium and magnesium levels and the addition of small amounts of yeast extract together with other factors like (a) the absence of selective pressure for settling, (b) a high growth rate (short SRT) and (c) low shear stress. The MBR presented in this study met the indications (a), (b) and (c) reported by van der Star and coworkers (2008) throughout the entire experimental period. This study differentiated between the effect of lowering of calcium and magnesium concentration and the addition of yeast extract, whereas the two changes in operation were performed at the same time by van der Star et al. (2008). Yeast extract was not used in this study. However, the effect of the stress caused by the potential lack of micronutrients contained in the yeast extract, was tested by vitamin addition to the feeding medium. The use of vitamins instead of the yeast extract was chosen in order to avoid the addition of the COD equivalent of the yeast extract to the medium. Calcium concentration was also halved in the present study. None of these medium composition changes imposed single cell growth. Instead, it was found that the absence of trace amounts of oxygen in the feeding medium was the key factor for growth of anammox bacteria as free cells. An oxygen loading rate of no more than 0.68±0.06 μmol-O_2 L^{-1} d^{-1} (oxygen in the medium fed to the reactor equal to 1.1 μmol-O_2 L^{-1}) was found to be sufficient to destabilize the free-cell suspension and stimulate biomass aggregation into small flocs (<100 μm) within a few hours (please see the corresponding section in Results). A degassing method with 99.6% oxygen removal efficiency was therefore required in this study to enable growth as free-cells. The mechanism of oxygen induced biomass aggregation was not investigated. The COD equivalent of the yeast extract used in the experiment of van der Star et al. (2008) and the resultant heterotrophic side population present in their system luckily ensured enough oxygen consumption to obtain the virtually complete anaerobiosis needed to obtain a free cell anammox culture. The same oxygen scavenging feature could be obviously fulfilled by any aerobic chemolithoautotrophs such as, for instance, aerobic ammonium oxidizing bacteria (AOB). As opposed to

the aerobic heterotrophic bacteria, the autotrophic aerobes were potentially not capable of maintaining an adequately low dissolved oxygen concentration to avoid floc formation. A recent publication also reported planktonic cell suspension of another fresh-water anammox organism: *Candidatus* Brocadia sinica (Oshiki et al., 2013). The system presented in that study indeed respected the preconditions indicated in this paper (no O_2 and low Ca^{++}). Summarizing, in order to obtain an anammox suspension culture, the following criteria have to be met: (a) the absence of selective pressure for settling, (b) a high growth rate (short SRT), (c) low levels of calcium and magnesium in the feeding medium and (d) virtually complete anaerobiosis. Fulfilling these criteria allowed us to establish a highly enriched (98±1%) suspended culture of anammox bacteria enabling accurate kinetic characterization. The expected link between biomass aggregate size and apparent nitrite half-saturation constant ($K_{NO_2^-}$) was confirmed during the experimental period prior to the obtainment of the free-cells suspension. An exponential equation to estimate the apparent $K_{NO_2^-}$ as function of the aggregate diameter was proposed.

Nitrite half-saturation constant

The half-saturation constant (or affinity constant, K_S) is an important parameter for the engineering of biological processes as well as for the understanding of bacterial ecology. In literature a wide range of values have been reported for the K_S for nitrite of anammox bacteria (0.003 to 13.7 mM, Puyol et al., 2013). Parameter identification experiments have been conducted with either continuous reactors (Ni et al., 2012; Chen et al., 2011; van der Star et al., 2008) or using batch experiments (Puyol et al., 2013; Oshiki et al., 2013; Strous et al., 1999a). This high variability is not surprising when considering the difference in type of anammox used, differences in methods of cultivation and investigation and, most of all, the aggregation status of the biomass. Biomass cultivated in aggregates in fact (i.e. granules or flocs), leads to the estimation of the *apparent* affinity only, while a free cell suspension is necessary for the estimation of the *intrinsic* one. In this study we evaluated the half saturation constant for nitrite ($K_{NO_2^-}$) by conventional methods (results from batch tests and steady state operations) and a dedicated novel method for the accurate determination of this important kinetic parameter. The intrinsic K_S for nitrite for

anammox bacteria was estimated to be 2.5 µM (0.035 mg-N L^{-1}). We did not observe a different K_S for pH between 6.8 and 7.5 which supports previous observations (Puyol et al., 2014; Lotti et al., 2012b [**Ch.4**]; Strous et al., 1999a) that nitrite and not nitric acid is the growth substrate. We would like to stress that for accurate estimation of Ks values a sampling method as applied in this study (immediate stop of biological conversion by sudden temperature decrease) is essential. In presence of highly active biomass a small (few seconds) delay between the sampling and the stop of the nitrite uptake (i.e., by physical solid/liquid separation by filtration) would lead to a serious underestimation of $K_{NO_2^-}$; in our case a delay of 10 sec would decrease the measured nitrite concentration by 50 %. In (aerated) nitrite-limited systems (such as CANON, OLAND, deammonification), nitrite oxidizing bacteria compete with anammox bacteria for nitrite. The estimated K_{NO_2} of NOBs vary considerably both between different studies and between different species (12-955 µM, Both et al., 1992; Hunik et al., 1993; Schramm et al., 1999). In (aerated) nitrite-limited systems NOBs reside in the outer aerobic layer of the biofilm while anammox bacteria occupy the anoxic core. Nitrite is produced in the outer aerobic layer by ammonium oxidizing bacteria (AOB). The reported value for $K_{NO_2^-}$ by anammox is lower than those reported for single cell NOB which gives them the opportunity to effectively compete even if they are a bit further away from the source (AOB) then NOB's. The measured $K_{NO_2^-}$ of anammox bacteria was also lower than the one reported for denitrifiers (4-25 µM, Almeida et al., 1995; Betlach and Tiedje, 1981). A higher affinity for nitrite is a major competitive advantage for anammox bacteria over competitors (NOB and denitrifiers). In natural ecosystems the low $K_{NO_2^-}$-value may play an important role in the relative abundance of anammox bacteria in marine environments (Schmid et al., 2007) and in the capacity of organotrophic anammox bacteria to outcompete denitrifying bacteria (Winkler et al., 2012a).

Biomass specific growth rate

Under continuous cultivation at an SRT of 12 days, anammox bacteria were shown to grow at an actual growth rate of 0.083 d^{-1} for more than 580 days (doubling time, $t_d = \ln(2) \cdot SRT = t_d$ = 8.3 days). Typical doubling times reported until now were 15-30 days (Strous et al., 1998; Fux et al., 2004). Taking

into account the maximum conversion capacity of anammox bacteria determined in this study, the maximum specific growth rate was estimated to be as high as 0.21 d^{-1} (t_d = 3.3 days). There have been some earlier reports on fast growing anammox bacteria. Tsushima et al. (2007), during the exponential growth phase in shake flasks at 37°C, estimated an anammox bacteria doubling time of 3.6-5.4 days by quantitative polymerase chain reaction (qPCR). From the maximum conversion capacity of a continuous reactor (at 38°C), van der Star et al. (2008) estimated a t_d of 5.5-7.5 days. An even lower t_d of 3 days was suggested based on microscopic observations and qualitative community structure estimations (van der Star et al., 2008). Recently, the presence of anammox bacteria and activity in a full-scale WWTP in Singapore operated at an SRT of about 5 days and 28-30°C has been reported (Cao et al ., 2013). The fastest doubling time (1.8 days) was reported by Isaka et al. (2006), based on the comparison of the number of anammox cells of two different reactors inoculated at the same time under similar conditions and operated at 37°C. From the comparison of the cell numbers at two different points in time (each measurement in one of those two different reactors) the growth rate was calculated. The validity of the methods used by Isaka et al. (2006) is highly questionable and the obtained results were therefore generally considered doubtful (van der Star et al., 2008). It should be noted that the maximum specific growth rate reported in this study was observed at lower temperature (30°C) than those comparable maximum specific growth rates reported in literature (37-38°C). For normal biological process a change in growth rate by a factor 2 is expected with a temperature rise of 8-10°C. Our observations indicate that anammox bacteria have a higher growth rate then hitherto generally assumed. This might be due to the following:

- in a suspension system operated at higher growth rates, contrary to a biofilm systems, all cells are active;
- in many past experiments with biomass retention systems, cells removed with the effluent were generally neglected during calculations resulting in an overestimation of the SRT and therefore in an underestimation of the corresponding growth rate.

Stoichiometry of the anammox process

Since Strous and coworkers (1998) reported for the first time the stoichiometry of the process carried out by anammox bacteria, every scientific study used that equation. As stated by the authors themselves, the stoichiometry was obtained by mass balancing on about 200 days of experimental data and the an estimated 90% retention of growing biomass in the reactor; the evaluation of the stoichiometry was furthermore affected by a 50% uncertainty in the volatile solids measurements. The electron balance of the conversion rates used by Strous for data reconciliation had also a significant error (15% more electrons in product then in substrates). For this reason the estimated ammonium uptake rate and nitrate production rate were 5.4% and 4.3% lower compared to the measured rates, respectively. For balancing reasons the nitrite uptake rate needed to be increased by 3.1%. The correct knowledge of the macro-chemical reaction equation of a biological process is crucial both for scientific research and for process design and control.

In this study the availability of a high purity anammox culture actively growing in a controlled system (MBR) enabled accurate identification of the anammox macro-chemical reaction equation. The biomass yield observed was found to be constant during an experiment in which the biomass specific nitrite uptake rate (nitrite as the limiting substrate) increased from 39 to 83% of the maximum biomass specific nitrite uptake rate (*kinetic characterization experiment*), indicating that the yield value measured approximated the maximum biomass yield and maintenance processes played only a minor role at the relatively high dilution rates applied. The elemental biomass composition of the enriched high purity anammox culture was $CH_{1.74}O_{0.31}N_{0.20}S_{0.01}P_{0.01}$ (biomass molecular weight of 22.1 g C-mol^{-1}). Whereas the strictly autotrophic nature of anammox bacteria (inorganic carbon, IC, as C-source) has been specifically investigated, (Strous et al., 1998, Güven et al. 2005, Kartal et al., 2007a,b), the nitrogen-source for biomass production utilized is to date unclear. Data reconciliation (Verheijen et al., 2009) has been applied to the measurements obtained during long-term steady state reactor operations (Table 4) using the elemental biomass composition measured in this paper. Calculated conversions were balanced for element and charge conversion and the resulting stoichiometry was calculated together with the propagated errors. Data

reconciliation was applied both by assuming different N-sources in the anabolic reaction (ammonium, nitrite or nitrate were considered) and without any constrains on the N-source used (Table 5). Since the results considering either nitrite or nitrate as the N-source were identical, these two cases are reported together.

Table 5. Measurements from long-term reactor operations at steady state (Table 2A, supporting online material) were used for data reconciliation (Verheijen et al., 2009) together with the elemental composition of the biomass. Calculated conversions were balanced for element and charge conservation and the resulting stoichiometry was calculated. Statistics from data-reconciliations were also calculated. (I.C., inorganic carbon)

	No constraints		**NH_4^+ as N-source**		**NO_2^-/NO_3^- as N-source**	
Compounds	**Stoich.Coef.**	**+/- Error**	**Stoich.Coef.**	**+/- Error**	**Stoich.Coef.**	**+/- Error**
NH_4^+	-1	0	-1	0	-1	0
NO_2^-	-1.225	0.016	-1.189	0.0029	-1.225	0.0035
I.C.	-0.073	0.0081	-0.091	0.0014	-0.073	0.0011
H^+	-0.024	0.019	-0.020	0.018	-0.024	0.019
N_2	1.000	0.0081	0.982	0.00028	1.000	0
NO_3^-	0.210	0.0035	0.207	0.0032	0.210	0.0033
$CH_{1.74}O_{0.31}N_{0.20}$	0.073	0.0081	0.091	0.0014	0.073	0.0011
H_2O	1.950	0.014	1.937	0.012	1.950	0.012

Statistics	**No constraints**	**NH_4^+ as N-source**	**NO_2^-/NO_3^- as N-source**
p-value [%]	74.83	15.89	94.28
Weighted Sum-of-Squares	1.2198	6.5945	1.2214

Ammonium as N-source for the growth of a microorganism which already uses ammonium in the catabolic reaction seems the most logic N-source. In the case

of anammox therefore, it is assumed that ammonium is the preferred N-source. In this study the experimental data were well described by a model based on this assumption. However it was observed that when assuming nitrite/nitrate as N-source the system description was even more consistent as visible from the statistical analysis performed (Table 5). Furthermore, the stoichiometric coefficients obtained assuming nitrite/nitrate as N-source and without any constraints were almost identical, except for the different errors. However, the hypothesis of ammonium as N-source could not be rejected (p-value > 5%).

Table 6. The conversion rates and the elemental composition of the biomass reported by Strous et al. (1998) were used for data reconciliation (Verheijen et al., 2009). The conversion rates were balanced for element and charge conservation and the resulting stoichiometry was calculated. Statistics from data-reconciliations were also calculated. (I.C., inorganic carbon)

	No constraints		**NH_4^+ as N-source**		**NO_2^-/NO_3^- as N-source**	
Compounds	**Stoich.Coef.**	**+/- Error**	**Stoich.Coef.**	**+/- Error**	**Stoich.Coef.**	**+/- Error**
NH_4^+	-1	0	-1	0	-1	0
NO_2^-	-1.320	0.033	-1.184	0.012	-1.234	0.015
I.C.	-0.066	0.007	-0.087	0.006	-0.081	0.005
H^+	-0.128	0.013	-0.074	0.005	-0.093	0.006
N_2	1.026	0.009	0.987	0.001	1.000	0.000
NO_3^-	0.258	0.020	0.197	0.013	0.222	0.015
$CH_2O_{0.5}N_{0.15}$	0.066	0.007	0.087	0.006	0.081	0.005
H_2O	2.031	0.008	1.994	0.000	2.006	0.000

Statistics	**No constraints**	**NH_4^+ as N-source**	**NO_2^-/NO_3^- as N-source**
p-value [%]	46.19	0.00	1.49
Weighted Sum-of-Squares	2.5800	25.8100	12.3500

The value of the biomass yield calculated during the *kinetic characterization experiment* (0.071 C-mol NH_4^+-mol^{-1}) falls outside the confidence interval (stoichiometric coefficient ± 2·error) of the yield calculated during data-reconciliation only in the case of ammonium as the N-source while for the other two cases considered resulted within. Data reconciliation was applied the same way to the conversion rates reported by Strous et al. (1998) (Table 6). The system of Strous et al. (1998) could be described only without considering any constrain on the N-source used, while when either ammonium or nitrite and nitrate were considered as N-source there was no adequate system description based on the measured data (p-value < 5%) this was probably related to the aforementioned significant error in the electron balance. From the present data set it was not possible to reach a conclusion, but in future experiments it would be worthwhile to evaluate the exact N-source for anammox metabolism.

Nitric oxide and nitrous oxide emission

In this study a not previously reported high purity anammox culture was shown to emit nitric and nitrous oxide at steady state at a production rate accounting for the 0.003±0.002 and 0.056±0.02% of the imposed nitrogen load and for the 0.001±0.0006 and 0.20±0.01% of the total nitrogen removal rate, respectively. NO and N_2O emissions were evaluated also during the experiment of the dynamic change of the N-load (Fig. 11). While N_2O production was affected by the varying operational conditions inside the reactor, NO was constantly emitted at the same rate as during steady state operations. From the data reported it is difficult to identify the cause of the dynamics of N_2O production: e.g. nitrite accumulation, varying respiration or carbon fixation rate (Fig. 8). NO is part of the regular anammox metabolism and as such some NO emissions are expected (Kartal et al., 2010b). N_2O instead is not part of the anammox metabolism (pure cell study by Kartal et al. (2007)) and production seems to be caused by a side reaction. This might be a biological reaction catalyzed by other community members (e.g. Kampschreur et al., 2008) or a chemical reaction biologically enhanced as suggested by Kampschreur et al. (2011).

Conclusions

A method for growing anammox bacteria as free-cells in high purity was here shown univocally for the first time. Oxygen was found to be the key factor for inducing anammox bacteria to aggregate at an oxygen loading rate as low as 0.7 µmol-O_2 L^{-1} d^{-1}. Growing an almost pure and highly active suspended anammox culture enabled accurate estimation of a set of stoichiometric and kinetic parameters for anammox bacteria. The yield of biomass production on ammonium uptake was calculated to be 0.071 C-mol N-mol^{-1}, whereas the elemental biomass composition was measured as $CH_{1.74}O_{0.31}N_{0.20}S_{0.01}P_{0.01}$ (22.1 g C-mol^{-1}). From the yield and the elemental biomass composition the macro-chemical reaction equation was identified and validated by long-term reactor operations. The data-set used in this study to calculate the anammox stoichiometry by means of data reconciliation was more consistent than the one used by Strous et al. (1998) due to the smaller error in the electron balance and the higher accuracy in the carbon balance. Furthermore, instead of using independent averaged rates, this data-set consisted of the original measurements obtained during long-term steady state operations which were even cross correlated during error propagation. The anammox culture enriched during this study exhibited an unreported high biomass specific maximum growth rate of 0.21 d^{-1} corresponding to a doubling time of 3.3 days at 30°C. The nitrite half saturation constant for anammox bacteria as free-cells (intrinsic $K_{NO_2^-}$) was identified to be equal to 0.035 mg-N L^{-1} at pH = 7.0. This value was confirmed in a broader pH range of 6.8-7.5.

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Chapter 3

Faster through training: the anammox case

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Abstract

Anaerobic ammonium oxidizing (anammox) bacteria based technologies are widely applied for nitrogen removal from warm (25-40 ˚C) wastewater with high ammonium concentrations (~ 1 gNH_4-N L^{-1}). Extension of the operational window of this energy and resource efficient process is restricted by the "supposed" low growth rate of the responsible microorganisms. Here we demonstrate that the maximum specific growth rate (μ^{max}) of anammox bacteria can be increased to a μ^{max} value of 0.33 d^{-1} by applying a novel selection strategy based on the maximization of the electron transfer capacity in a membrane bioreactor. This value is four times higher than the highest previously reported value. The microbial community was strongly dominated by anammox bacteria closely related (99%) to *Candidatus* Brocadia sp.40 throughout the experiment. The results described here demonstrate the remarkable capacity of a phylogenetically stable anammox community to adjust its growth rate in response to a change in the cultivation conditions imposed.

Introduction

The anaerobic ammonium oxidation (anammox) process is the oxidization of ammonium to molecular nitrogen (N_2) in the absence of oxygen with nitrite as the electron acceptor. Due to the perceived potential of the anammox process for the wastewater treatment industry (Jetten et al., 1997; Siegrist et al., 2008; Kartal et al., 2010a), research on the anammox process was directed to improve nitrogen removal from wastewater very shortly after it was first discovered in a denitrifying pilot plant in the late 1980s (Mulder et al., 1995). Autotrophic nitrogen removal by anammox bacteria is currently applied in almost 100 full-scale installations for the treatment of a variety of ammonium rich municipal and industrial wastewaters: anaerobic digestion reject water, tannery, food processing, semiconductor, fermentation, yeast, distillery, winery

industries (van Hulle et al., 2010; Vlaeminck et al., 2012; Lackner et al., 2014). Nevertheless, the introduction of this process in wastewater treatment practice was not straightforward. The major obstacle encountered for the application of the anammox process was the very low growth rate of the responsible microbial community (Strous et al., 1999b; Strous et al., 2006; Kuenen et al., 2008; Jetten et al., 2009; Joss et al., 2009; Wett et al., 2010). Low biomass specific growth rates have important implications for bioprocess design, like either a very large bioreactor volume or the necessity for highly effective biomass retention. These restrictions limit the application of the anammox process to warm wastewaters (25-40 ˚C). Typical doubling times (T_d) considered during anammox based process design are 15-30 days (Strous et al., 1998; Wett, 2007). Application of anammox for the nitrogen removal from municipal sewage (diluted water and 10-25 °C) allows treatment scenarios for wastewater treatment plants (WWTP) with a net energy production (Kartal et al., 2010a). Energy-positive wastewater treatment would increase societal sustainability, and anammox is a key process that facilitates this goal. Recent studies reported encouraging results for the application of anammox at low temperatures, but the low growth rate in this condition still pose hard technological challenges (Lotti et al., 2014a,b [**Ch.6,7**]; Gilbert et al., 2014; Lotti et al., 2015a [**Ch.5**]). There are indications in the literature that anammox bacteria may have a higher growth rate, but no clear evidence for this has been described. Several authors (Isaka et al., 2006; Tsushima et al., 2007; van der Star et al., 2007) have suggested higher growth rates based on quantitative polymerase chain reaction (qPCR) data for the increase of anammox specific 16S-DNA in lab cultures. van der Star *et al.* (2008) and Lotti *et al.* (2014c) [**Ch.2**] suggested based on the maximum conversion capacity observed in their reactors compared to the applied load that the growth rate of anammox bacteria might be higher than previously reported. The aim of this study was therefore to evaluate the maximum growth rate for anammox bacteria under controlled conditions. We operated a membrane bioreactor (MBR) to a step-wise decrease in the solid retention time (SRT), and therewith, an increase in the actual biomass growth rate required to achieve stable operation. The results obtained are discussed in the light of previous maximal growth rate estimates as well as the putative cellular modifications allowing the improvement in kinetic properties observed.

Materals and methods

Reactor operations

The experiment was conducted in a membrane bioreactor (MBR) with a working volume of 10 L. The reactor set-up and operations have been described elsewhere (van der Star et al., 2008 and Lotti et al., 2014c [**Ch.2**]). The operational run of the reactor described in this paper was inoculated with an anammox enrichment established in a previous study (Lotti et al., 2014c [**Ch.2**]). Prior to inoculation the biomass was stored at 4°C in the presence of nitrate (0.1÷0.5 g-N L^{-1}) for about 1.5 year. The reactor used by Lotti *et al.* (2014c) [**Ch.2**], was originally inoculated with granular anammox sludge originating from a full-scale anammox reactor (Sluisjesdijk, Rotterdam, the Netherlands (van der Star et al., 2007)).

The synthetic medium utilized was composed of: 30 mM ammonium sulphate (60 mM of ammonium), 60 mM sodium nitrite; 0.51 mM of Ca^{2+} (added as $CaCl_2 \cdot 2H_2O$); 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 $FeSO_4 \cdot 7H_2O$); 1.25 mL L^{-1} of trace element solution (van der Star et al., 2008). The medium contained equal concentrations of ammonium and nitrite (60 mM), resulting in an equal ammonium and nitrite volumetric load of 504 mg-N L^{-1} d^{-1}, respectively (total nitrogen load of 1.0 g-N L^{-1} d^{-1}) and in nitrite limiting conditions. The temperature and hydraulic retention time (HRT) were maintained constant throughout the experimentation at 30°C and 1.67 d, respectively. Solids retention time (SRT), controlled by purging mixed liquor (5 min every 30 min) at different flow rates, varied decreasing from the initial value of 12 d to the 3 d applied during the last experimental period. Before feeding to the reactor, the medium was intensively sparged with nitrogen gas in order to remove traces of oxygen.

Kinetic and stoichiometric parameters evaluation

Throughout the experimentation the maximum volumetric nitrite and ammonium removal rate ($Rate_{NO_2^-}^{max}$ and $Rate_{NH_4^+}^{max}$, [mg-N L^{-1} d^{-1}]) were measured

in the reactor in non-limiting conditions. To determine the *in situ* maximum volumetric rates, the influent flow rate was temporarily increased to obtain a bulk liquid nitrite concentration of 20-30 mg-N L^{-1} (corresponding ammonium concentration of 210-220 mg-N L^{-1}). Feeding was subsequently stopped and nitrite and ammonium depletion were measured in time. The $Rate_{NO_2^-}^{max}$ and $Rate_{NH_4^+}^{max}$ values were identified through linearization of the nitrite and ammonium depletion curves. The maximum biomass specific substrate uptake rates ($q_{NO_2^-}^{max}$ and $q_{NH_4^+}^{max}$, [mg-N g-VSS^{-1} d^{-1}]) were calculated through dividing the $Rate_{NO_2^-}^{max}$ and $Rate_{NH_4^+}^{max}$ values by the biomass concentration in the reactor ([g-VSS^{-1} L^{-1}]). From the $Rate_{NO_2^-}^{max}$ and, the nitrite concentration measured in the bulk in steady state and the applied nitrite load, the nitrite half-saturation constant ($K_{NO_2^-}$) was calculated as $K_{NO_2^-} = C_{NO_2^-} \frac{Rate_{NO_2^-}^{\max} - Rate_{NO_2^-}}{Rate_{NO_2^-}}$ according to Lotti *et al.* (2014c) [**Ch.2**]. The analytical methods used to measure nitrogen compounds concentrations have been described elsewhere (Lotti et al., 2014c [**Ch.2**]). Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to the Standard Methods (APHA, 2005). The actual biomass yield was calculated as the ratio between the inorganic carbon uptake rate and the ammonium removal rate obtained by means of mass balance through the gas and liquid phase as previously described (Lotti et al., 2014c [**Ch.2**]).

Microbial community characterization

Fluorescence *in situ* hybridization (FISH) and the evaluation of the anammox enrichment level was conducted monthly as described elsewhere (Lotti et al., 2014c [**Ch.2**]). Briefly, cells were washed in phosphate buffer, fixed in paraformaldehyde and spotted onto Teflon-coated multi-well slides. After dehydration by immersion into ethanol solutions (50, 80, 98%), the cells were hybridized with the following fluorescently-labeled oligonucleotide probes: EUB-mix (mix of oligonucleotides EUB-338, EUB-338 II and EUB-338 III), AMX-820 and Bfu-613. Details on the target organisms and the sequences can be found elsewhere (Lotti et al., 2014c [**Ch.2**]).

Total genomic DNA was extracted from the sludge samples collected from the reactor. Extraction was performed using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), following the manufacturer's recommendations. The extracted DNA was evaluated both qualitatively and quantitatively on 1% (w/v) agarose. After DNA extraction different methods were applied for identifying anammox (i) and non-anammox bacteria (ii).

(i) The extracted DNA was used to amplify the nearly complete 16S rRNA gene of anammox bacteria using An7f and An1388r (Penton et al., 2006). The PCR products were analysed with agarose gel electrophoresis, purified using the QIAquick PCR Purification Kit (QIAGEN, Düsseldorf, Germany) and sequenced (Macrogen Europe, Amsterdam, The Netherlands). The sequences were first compared to sequences stored in GenBank using blastn. Thereafter, they were imported into the SILVA database (Pruesse et al., 2007) using the ARB software program (Ludwig et al., 2004).

(ii) Total genomic DNA was extracted from the sludge samples collected from the reactor. A set of universal primers for the domain bacteria, BAC341F (containing a 40-bp GC-clamp) and BAC907RM (M=A/C) (Schaefer and Muyzer, 2001), was used to target the 16S rRNA gene fragment. The PCR protocol was as followed, an initial denaturation at 95 °C for 5 min, followed by 32 cycles of denaturing at 95 °C for 30 s, primer annealing at 55 °C for 40 s and extension at 72 °C for 40 s. After the last cycle, a final extension at 72 °C for 30 min was applied. Before loading the PCR product onto the DGGE gel they were quantified on an agarose gel and 250 ng was used for analysis.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was carried out in the Bio-Rad DCode System (Bio-Rad, Hercules, CA, USA). Electrophoresis was run in 1 mm thick gels, containing 6% acrylamide-bis-acrylamide mix content (w/v). The denaturing gradient of the gel started from 20 to 70% UF (100% denaturants is defined as 7 M urea and 40% (v/v) deionized formamide). Gels were submerged in 1× TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.4). Electrophoresis of 16S rRNA PCR products was run for 16 h at a constant voltage of 100 V and a temperature of 60°C. After electrophoresis, the gel was stained for 30 min with a 5 mL 1× TAE solution

containing a 10x concentrated SYBR gold nucleic acid stain (Molecular Probes, Eugene, OR, USA) in the dark, and visualized in a Safe Imager Blue-Light Transilluminator (Invitrogen, Carlsbad, CA, USA). The gel images were captured by a digital camera placed in the GeneSnap system (Syngene, Cambridge, UK).

Internal Transcribed Spacer (ITS)

Six samples were chosen from the MBR reactor population to check for minor diversity difference within the anammox community during the experimental run. The ITS region was chosen as the resolution of diversity at the genus level is much higher (Boyer et al., 2001) than the commonly used 16S rRNA gene. For this reason a new assay was setup to detect the ITS of anammox using a combination of primers An1388F_16S ('5-CACACCGCCCGTCAAGC-3'), the reverse complement of the An1388R primer used by Penton *et al.* (Penton et al., 2006) and the newly developed An85R_23S ('5-CTCCCCGAAGCTTATCGCAG -3') (this study). After optimization, the following protocol was used for amplification, an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 30 s, primer annealing at 62 °C for 45 s and extension at 72 °C for 45 s. After the last cycle, a final extension at 72 °C for 10 min was applied. For amplification we used the following chemicals; Taq PCR Master Mix Kit (QIAGEN, Düsseldorf, Germany; order no. 201445), 0.5 uM of each primer and about 10ng of extracted genomic DNA. The resulting PCR products were purified over an agarose gel and cleaned using a QIAquick Gel Extraction Kit (QIAGEN, Düsseldorf, Germany). Subsequently the purified material was cloned using a TOPO® TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). About twenty clones per sample were picked for sequencing and assembled using Codoncode Aligner v3.7 software (CodonCode Corp., Centerville, MA, USA). The corrected sequences were imported into ARB software (Ludwig et al., 2004) and aligned against known ITS sequenced in the database using newest rRNA SILVA databases (both 16 and 23S). A tree was created using the Neighbor-Hood Joining algorithm on the basis of 381 common base-pairs.

Results

Reactor operations

Suspended anammox enriched biomass from a previous study (Lotti et al., 2014c [**Ch.2**]) was inoculated in a membrane bioreactor (MBR). The initial biomass concentration in the reactor was about 2 g-VSS L^{-1}. The reactor was fed with synthetic medium and initially operated at 12 days of solids retention time and 1.67 days of hydraulic retention time (HRT). The equal ammonium and nitrite concentrations in the feeding medium resulted in nitrite limiting conditions due to a larger nitrite removal compared to ammonium removal according to anammox stoichiometry. Ammonium effluent concentration was on average 189±12 mg-N L^{-1} resulting in a constant nitrite to ammonium consumption ratio of 1.29±0.04. The same average consumption ratio (same stoichiometric coefficient) was observed during maximum activity measurements. Full nitrite conversion was reached after 18 days of operation and after two months the experimental period described in this paper was started (we defined this as experimental day one). During steady state operations with SRT equal to 12 days (days 1-67), the biomass concentration in the reactor was on average 0.50±0.06 g-VSS L^{-1}. On day 68 the SRT was decreased from 12 to 4.1 days by increasing sludge withdrawal from the system. The resulting minimum generation time (T_d = ln(2)·SRT) required for the bacteria to prevent wash-out from the system decreased from 8.3 to 2.8 days. The change in kinetic properties induced by the SRT decrease was monitored by measuring the maximum volumetric specific nitrite removal rate ($Rate_{NO_2^-}^{max}$), biomass and nitrite concentrations (FIG. 1, Table 1).

The lower SRT imposed resulted in a gradual increase of the nitrite concentration and decrease of biomass concentration (days 68-124) (FIG. 1, *bottom*). In continuously operated bioreactors, higher concentration of the limiting substrate (nitrite in this case) results in a microbial activity closer to its maximum according to the well-known Monod equation. Within one month the nitrite concentration stabilized at an average level of 0.4±0.1 mg-N L^{-1}, whilst the $Rate_{NO_2^-}^{max}$ fluctuated between 522 and 683 mg-NO_2-N L^{-1} d^{-1} (days 95-187) (FIG. 1, *top*). Biomass concentration decreased due to the lower SRT imposed and stabilized only in the period 125-187 days (constant SRT of 4.1 d) at the level of 0.29±0.02 g-VSS L^{-1} (FIG. 1, *bottom*).

Table 1. Average results obtained for each operational period.

Reference period	SRT	$Rate_{NO_2^-}^{max}$	NO_2^-	VSS
	[d]	[mg-N L^{-1} d^{-1}]	[mg-N L^{-1}]	[g L^{-1}]
0-67	12	1293±15	0.05±0.01	0.50±0.06
68-124	12 → 4.1	628±128	0.28±0.18	0.42±0.03
125-187	4.1	586±46	0.46±0.11	0.29±0.02
188-270	4.1 → 3.0	598±49	0.23±0.36	0.24±0.08
271-296	3.0	503±8	2.15±1.12	0.12±0.03

After long term steady state conditions were established in the reactor operated at 4.1 days SRT (days 125-187), the nitrite concentration decreased to levels below 0.1 mg-N L^{-1} suggesting that the biomass specific maximum nitrite removal rate of the reactor had increased (if it is assumed the affinity for nitrite was unaffected). This hypothesis was confirmed by *in situ* batch tests and $Rate_{NO_2^-}^{max}$ was measured to increase from an average of 586 mg-NO_2-N L^{-1} d^{-1} to values above 650 mg-NO_2-N L^{-1} d^{-1} (FIG. 1).

In the next experimental period the imposed SRT was decreased stepwise until gradual nitrite accumulation occurred. Nitrite started to accumulate on day 271 when the reactor was operated at an SRT of 3.0 days, corresponding to minimum generation time of 2.1 d. When the nitrite concentration in the bulk stabilized (2.8±0.7 mg-N L^{-1}), the reactor was operated for ten days longer (equal to more than three times the operational SRT) in order to reach steady state conditions (days 286-296). During this last period the biomass concentration in the reactor was on average 0.12±0.03 g-VSS L^{-1}.

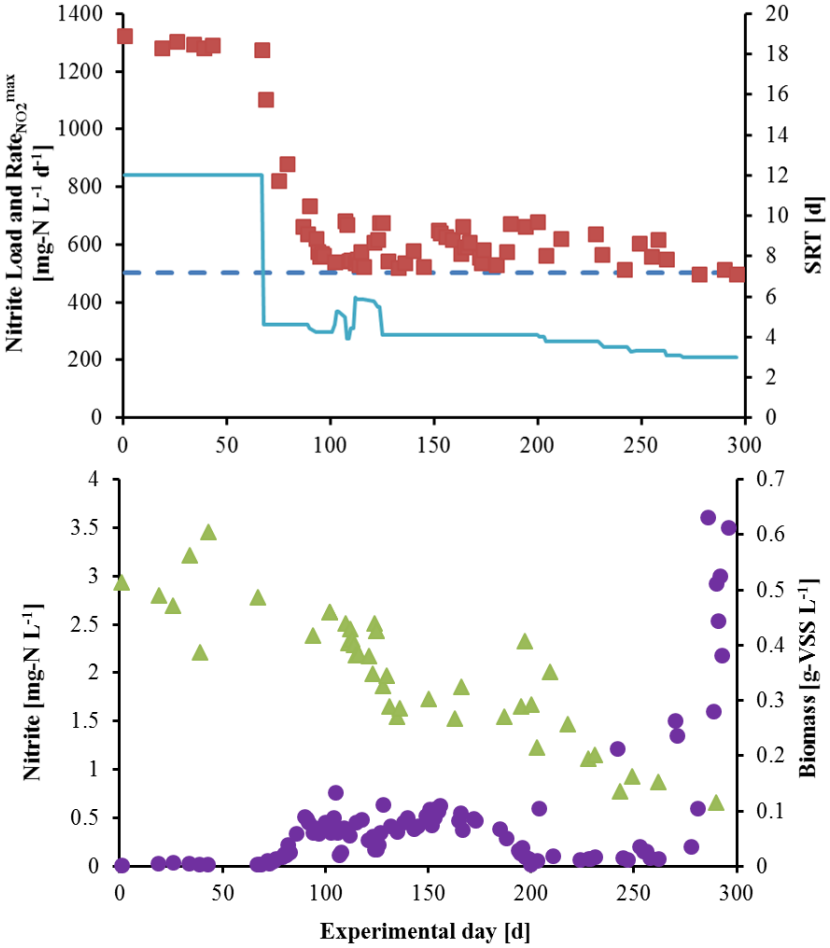

Figure 1. Reactor operation. ***Top*:** *left axis*: volumetric nitrite loading rate (- - -); maximum volumetric nitrite removal rate as determined in batch experiments ($Rate_{NO_2^-}^{max}$, ■). *Right axis*: solids retention time (SRT, ——). ***Bottom*:** *left axis*: nitrite concentration (●). *Right axis*: biomass concentration (▲).

Kinetic and stoichiometric parameters identification

The nitrite half-saturation constant ($K_{NO_2^-}$) was regularly estimated throughout the experimentation. The average $K_{NO_2^-}$ value obtained was 0.036±0.021 mg-N L^{-1} and no trend in time could be observed. This value is close to the previously more accurately established $K_{NO_2^-}$ of 0.038±0.011 mg-N L^{-1} (Lotti et al., 2014c [**Ch.2**]) suggesting that the affinity constant was largely

unaffected by the actual growth rate imposed. Considering the measured $K_{NO_2^-}$ and the average nitrite concentration during the period of non-limiting conditions described above (0.4 mg-N L^{-1}), it can be assessed that anammox activity was on average 91% of its maximum during days 95-187. Similar considerations suggest that during days 286-296 the anammox activity was on average 99% of its maximum.

Table 2. Dataset for the calculation of the yield coefficient. Operational SRT is reported for completeness.

SRT	pH	Off-gas CO_2	Q_{IN}=Q_{OUT}	Gas-Flow IN	Gas-Flow OUT	Yield
d	-	%	L d^{-1}	mL min^{-1}	mL min^{-1}	C-mol N-mol^{-1}
12.0	6.95	3.597	6.0	100	104.4	0.068
12.0	6.78	3.778	6.0	100	104.4	0.057
4.1	6.95	3.541	6.0	100	104.4	0.082
3.8	6.71	3.736	6.0	100	104.4	0.079
3.5	6.79	4.159	6.0	200	204.4	0.075
3.5	6.93	4.304	6.0	300	304.4	0.059
3.4	6.78	3.671	6.0	100	104.4	0.084
3.0	7.05	3.496	6.0	100	104.4	0.067

Considered for calculations: Temperature of 303.15 K, reactor headspace pressure of 1.02 atm, CO_2 concentration in the influent gas equal to 4.7%, Henry's law constant for CO_2 solubility equal to 29.76 L·atm mol^{-1}, ammonium removal rate of 391 mg-N L^{-1} d^{-1} (27.9 mM d^{-1}), common acid dissociation constants for the carbonate equilibrium.

The actual yield was monthly (n=8) estimated throughout the experimentation by performing inorganic carbon and ammonium mass balances over the reactor gas and liquid phases. The average yield was 0.071±0.010 C-mol NH_4^+-mol^{-1} (Table 2), similar to what has been reported in literature (0.066 and 0.071 C-mol NH_4^+-mol^{-1} according to Strous et al., 1998 and Lotti et al., 2014c [**Ch.2**], respectively).

Microbial community composition

The enrichment level of the anammox community was evaluated by counting individual cells hybridized with the anammox-specific probe (Amx-820) against the total number of bacteria present in the sample (hybridized with EUB-mix). The enrichment level at day 172 was 97±2% (ca. 10,000 cells counted). Fluorescence *in situ* hybridization (FISH) analysis was regularly conducted as a simple method to evaluate the occurrence of change in the anammox population. Throughout the experimental period complete overlapping between the signal of the probe Amx-820 and the more specific probe Bfu-613 (Kartal et al., 2008) was always observed suggesting that no population shift occurred. Furthermore all FISH analysis conducted (n = 10) showed an almost complete overlapping between Amx-820 (Bfu-613) and EUB-mix probe indicating that non-anammox populations accounted for a minor fraction of the microbial community throughout the experimentation.

In order to analyse more in detail the anammox community, the strain dominant in the reactor on day 5 (S1) and 248 (S2) was evaluated by phylogenetic analysis based on the anammox specific 16S rRNA sequences. These sequences were related to the clone library results relative to the inoculum previously obtained (clones 1 to 12) (Lotti et al., 2014c [**Ch.2**]). The phylogenetic tree is shown in Figure 2. Among the different samples there was > 99.9% similarity of the 16S rRNA sequences. The samples sequences had 97% resemblance with *Candidatus* Brocadia Fulgida (Kartal et al., 2008) and 99% with *Candidatus* Brocadia sp. 40 (Kieling et al., 2007).

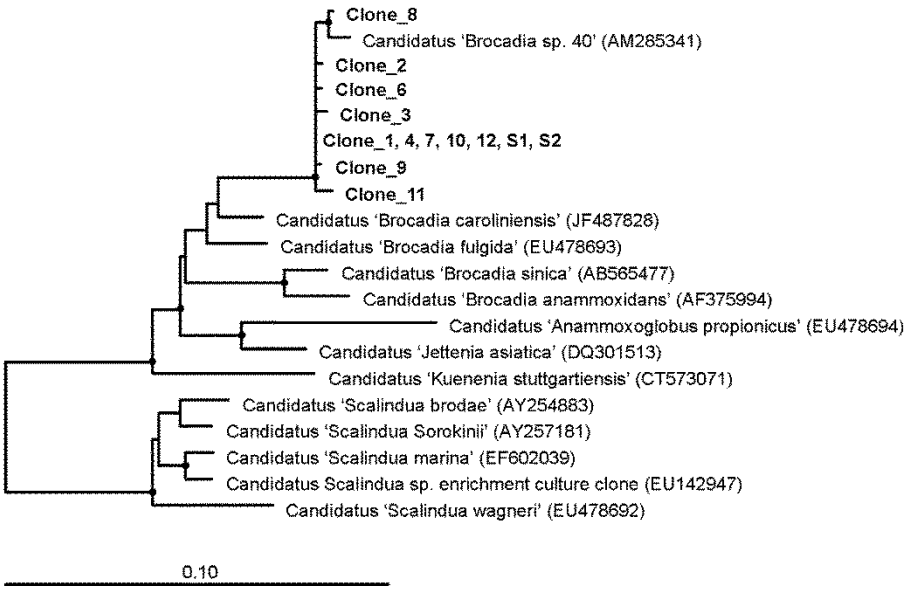

Figure 2. Phylogenetic tree of full 16S rRNA gene sequences. All samples are highlighted in bold. The scale-bar represents 10% sequence divergence. Samples refer to the clones from the inoculum (1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12) and to the biomass sampled from the reactor on day 5 (S1) and 248 (S2). The phylogenetic tree has been calculated using a maximal likelihood algorithm RAxML which is implemented in the ARB v5.2 software package. Bootstrap was performed for 250 round and values of >90% are indicated by a solid black dot. For calculations the bac_var_ssuref:bacteria filter (SSU_ref_108_silva database) has been applied to the data set to filter out noise. In total 1271 positions (ecoli pos. 594-1528) were used for calculation. Samples were calculated in the tree using 'quick add sequences using parsimony' tool. In total 711 positions (ecoli pos. 811-1528) were used.

The biodiversity of the satellite microbial population was analysed by denaturing gradient gel electrophoresis (DGGE) performed on amplified DNA extracted from the anammox biomass from the full-scale reactor of Sluisjesdijk (van der Star et al., 2007), from the inoculum used in this study and from the biomass sampled from the reactor on days 123, 133 and 161 (FIG.3). Minor differences were noticed in both the number and thickness of the bands for the different microbial populations and no trend in time was visible. Interestingly the biodiversity of the satellite microbial population seemed stable in time.

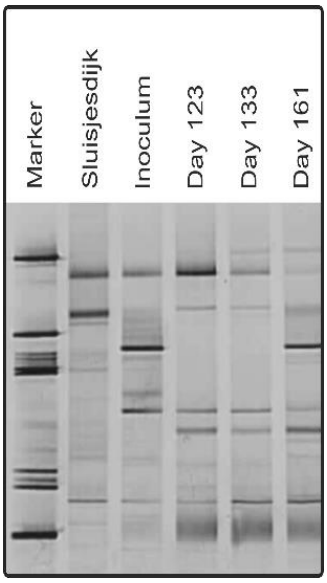

Figure 3. Denaturing Gradient Gel Electrophoresis (DGGE). DGGE was performed on amplified DNA extracted from the anammox biomass from the full-scale reactor of Sluisjesdijk (Rotterdam, The Netherlands), from the inoculum used in this study and from the biomass sampled from the reactor on days 123, 133 and 161.

Sequence comparison of the ITS region was performed on the DNA extracted from the anammox biomass from the full-scale reactor of Sluisjesdijk (van der Star et al., 2007) (clones 1÷19), from the inoculum used in this study (clones 20÷38) and from the biomass sampled from the reactor on days 123, 133, 161 and 284 (clones 39÷120) (FIG. 4). The diversity of the anammox population was found higher in the biomass from the full-scale reactor of Sluisjesdijk (clones 1÷19 in Figure 4) than both in the inoculum used in this study and in the biomass sampled from the MBR (clones 20÷120 depicted as "group of 101 clones" in Figure 4).

In the ITS region none to four base-pair variations (group of 101 clones) where found among the samples originating both from the different operational periods and from the inoculum resulting in 99.8% (STD=0.81) average similarity. Furthermore, mutations were found randomly distributed suggesting no evolution. Together with the narrow diversity observed, these results indicated that during the time course of the experiment with increasing

growth rate no change in anammox bacterial strain had occurred. It is important to note that the biomass used here as inoculum was previously cultivated for more than 600 days in the same MBR used in this study and was therefore the result of a long selection process (Lotti et al., 2014c [**Ch.2**]).

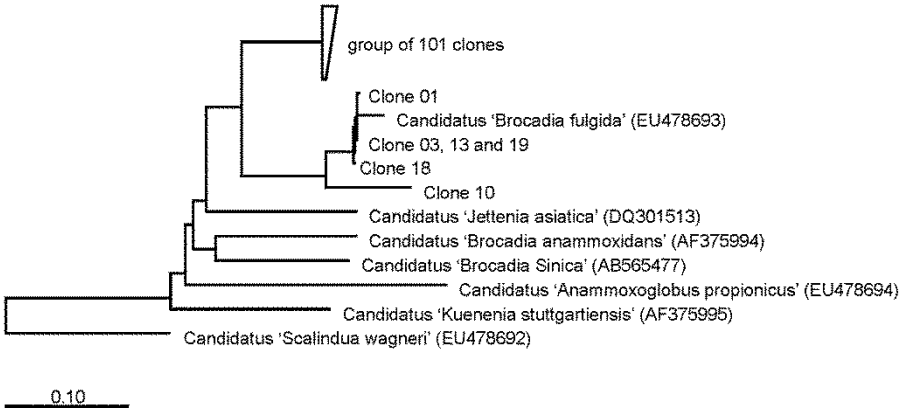

Figure 4. Internal transcribed spacer (ITS). The scale-bar represents 10% sequence divergence. Clones refer to the anammox biomass from the full-scale reactor of Sluisjesdijk, Rotterdam (clones 1÷19), to the inoculum used in this study (clones 20÷38) and to the biomass sampled from the reactor on day 123, 133, 161 and 284 (clones 39÷120). Given the high similarity (AVG 99.8%) of their sequences, clones 20÷120 were clustered together and depicted as "group of 101 clones" in the figure.

Discussion

15 years after the first report on the kinetic properties of anammox bacteria by Strous *et al.*(1998), anammox bacteria are still considered to be very slow growing microorganisms (e.g. Strous et al., 1999a; Strous et al., 2006; Kuenen et al., 2008; Kartal et al., 2012; Shi et al., 2013; Ali et al., 2014). In this work we have shown, by step-wise decreasing the SRT (increasing the flow rate of mixed liquor discharge) in a MBR, that the maximum specific growth rate of an anammox enrichment culture can be increased to values that are much higher than reported before. While in fact in this study a growth rate as high as 0.334 d^{-1} was observed at 30°C, highest values previously reported in literature, after correction for the temperature of cultivation using an activation energy of 65.7 kJ mol^{-1} (Lotti et al., 2015a [**Ch.5**]), vary from 0.11 d^{-1} (Liu & Ni, 2015) to 0.21 d^{-1} (Lotti et al., 2014c [**Ch.2**] and Isaka et al., 2006). Furthermore, these highest

literature values were indirectly estimated (e.g. estimated by model fitting procedures or from maximum nitrite removal rate compared to design load) and not directly estimated as the inverse of the SRT as in the present study. The highest directly estimated growth rate value reported in literature is 0.083 d^{-1} (Lotti et al., 2014c [**Ch.2**]), thus four times lower than the growth rate observed in the present study. It should be also noted that in their recent publication Liu & Ni (2015) reported a positive effect of iron with specific growth rate increase (after temperature correction) from 0.08 to 0.11 d^{-1} for an increase in iron concentration in the feeding from 0.03 to 0.09 mM. The iron concentration used in this study instead, was maintained at a lower level of 0.08 mM throughout the experimentation, while higher growth rates were observed. The higher biomass specific growth rate reported in this paper leads to the question why in this case anammox bacteria could grow so much faster than previously observed?

First of all it should be noted that cultivation under non-limiting conditions (e.g. in batch) is a prerequisite for identification of the maximum biomass specific growth rate of a microorganism. In most of the studies reported in literature instead, the anammox process was operated under nitrite limiting conditions in view of the inhibition potential of this substrate (Strous et al., 1999a; Lotti et al., 2012b [**Ch.4**]). Secondly, biomass retention systems like those usually applied for cultivation of anammox bacteria, result in the formation of flocs or biofilms further complicating the determination of the maximum growth rate. The experimental setup used in this study enabled anammox growth in free-cell suspension at well-defined SRT for several generations. Herewith for the first time precise growth rate values are obtained.

The maximum biomass specific growth rate (μ^{max}) can be regarded as the resultant from the maximum biomass specific substrate uptake rate ($q_{NH_4^+}^{max}$, [g-NH_4-N g-VSS^{-1} d^{-1}]), the yield of biomass produced per unit of substrate removed ($Y_{X/NH_4^+}^{max}$, [g-VSS g-NH_4-N^{-1}]) and the decay rate (b, [d^{-1}]). A change in one or more of these three terms must have occurred in order to allow the higher μ^{max} observed in the experiment described in this paper.

The biomass yield value determined during this work was fairly constant throughout the experimentation (0.071±0.010 C-mol NH_4^+-mol^{-1}) and similar to literature values (Strous et al., 1998; Jetten et al., 2009; Lotti et al., 2014c

[**Ch.2**]) and does therefore not explain the higher growth rates established in this study as such.

The fraction of active cells in the biomass may have been higher than estimated in previous studies due to a lower synthesis of extracellular polymeric substances (EPS) by the suspended cells. However, considering that EPS normally accounts for no more than 40% of the total volatile solids present in autotrophic flocs/biofilms (Pellicer-Nácher et al., 2013), a shift in the carbon fixation route from EPS to active biomass at increasing growth rate cannot account for the four times increase in μ^{max} observed. Furthermore, the anammox free-cells cultivated in this study were shown to be able to aggregate (i.e. by means of EPS interactions) as soon as transiently exposed to oxygen (Lotti et al., 2014c [**Ch.2**]), indicating that EPS were still present.

The fraction of active cells may also be reduced by the accumulation of a significant fraction of inactive cells at higher SRT due to decay. The anammox decay rate has been reported as 0.0048 d^{-1} (Scaglione et al., 2009). This is significantly lower (1-6%) than the actual inverse solids removal rate (SRT^{-1}) rates applied in our work, suggesting that decay was irrelevant in this study. A significant fraction of inactive cells cannot be excluded for the high SRTs studies in the literature (e.g. operating at SRT of 100 days, about 50% of the anammox bacteria could have been inactive). Again this cannot explain the difference with the high observed growth rates in the current study.

Herewith the most plausible explanation for the increase in the maximum biomass specific growth observed is a major increase in the maximum biomass specific substrate uptake rate as observed at higher growth rates. Table 3 summarizes the main kinetic characteristics of the different steady states described in this paper, in comparison with values reported in literature. During the reactor run reported in this study, $q_{NH_4^+}^{max}$-values increased with a factor three, resulting in the highest $q_{NH_4^+}^{max}$ for anammox ever reported. The direct correlation between the SRT during cultivation and the actual value for $q_{NH_4^+}^{max}$ is evident.

Table 3. Summary of the kinetics measured in this study and comparison with literature values. The kinetics are reported together with the SRT maintained during cultivation.

Reactor	SRT of cultivation	μ^{max}	$q_{NH_4^+}^{max}$	q_e^{max} [a]	Reference period of this study	Reference
	[d]	[d^{-1}]	[g-NH_4-N g-VS^{-1} d^{-1}]	[e-mol C-mol^{-1} h^{-1}]	[d]	
MBR after 1.5 year storage at 4°C	n.a.	0.135 [b1]	1.11	0.22	Start Up	This study
MBR	4.1	0.244 [c]	1.88	0.37	125-187	This study
MBR	3.8	0.263 [c]	2.48	0.49	188-248	This study
MBR	3.3	0.303 [c]	2.82	0.56	249-270	This study
MBR	3.0	0.334 [c]	3.38	0.67	286-296	This study
Full-scale anammox (long-term run) [d]	45-160	0.030 [b1] (0.060 [b2])	0.25	0.05	-	Lotti et al., 2012b [**Ch.4**]
Full-scale anammox (start up in 2013) [d]	Start Up	0.049 [b1]	0.40	0.08	-	This study
SBR	10-100	0.065 [e]	0.66 [f]	0.13	-	Strous et al., 1998
MBR	12	0.083 [c] (0.210 [g])	2.01	0.40	-	Lotti et al., 2014c [**Ch.2**]

[a] Maximum biomass specific electron transfer rate calculated considering biomass molar weight of 22.1 g-VS C-mol^{-1} and assuming that 3 e-mol NH_4^+-mol^{-1} are transferred in the anammox catabolism

[b1] Calculated from q_{NH4}^{max}, considering yield of 0.122 g-VS g-NH_4-N^{-1}; [b2] Calculated from qPCR data during start up in 2006 (van der Star et al., 2007)

[c] Calculated as equal to SRT^{-1}

[d] Full-scale anammox reactor at Sluisjesdijk-Dokhaven WWTP, Rotterdam (van der Star et al., 2007)

[e] Calculated from mass balance

[f] Adapted from (Strous et al., 1999a)

[g] Calculated from the maximum conversion capacity of the reactor compared to steady state operations

n.a.: not appliable

The anammox biomass yield values measured in this and other work do not deviate significantly from thermodynamic biomass yield predictions for other autotrophic nitrogen conversions such as aerobic ammonium oxidation, aerobic nitrite oxidation, or autotrophic denitrification with reduced sulphur compounds. The comparable thermodynamic growth efficiency implies that the low growth rates reported previously for anammox were the result from a significantly lower biomass specific electron flux through the catabolic and anabolic pathway compared to other chemolitoautotrophs. Previously we have proposed that, as rule of thumb, biomass specific electron transfer rates (q_e^{max}) are restricted by a maximum value of 3 e-mol C-mol^{-1} h^{-1} at 25 °C (4.7 at 30 °C) (Heijnen and Kleerebezem, 2010). In Table 3 the measured q_e^{max}-values for anammox are reported. The highest q_e^{max}-value for anammox identified in this work (0.67 e-mol C-mol^{-1} h^{-1}) is in the same order of magnitude as the maximum value proposed by Heijnen and Kleerebezem (2010), and comparable to other chemolithoautotrophs such as aerobic ammonium oxidizing bacteria (0.33 e-mol C-mol^{-1} h^{-1} (Wiesmann, 1994)), nitrite oxidizing bacteria (0.29 e-mol C-mol^{-1} h^{-1} (Wiesmann, 1994)), sulfur oxidizing bacteria (3.0 e-mol C-mol^{-1} h^{-1} (Banciu et al., 2004)) and autotrophic denitrifiers (2.5 e-mol C-mol^{-1} h^{-1} (Oh et al., 2000)). The q_e^{max}-values shown were recalculated at 30 °C using an Arrhenius type correlation with an activation energy of 69 kJ mol^{-1} (Roels, 1983). The growth rates and electron transfer rates identified in this work demonstrate that anammox cannot be regarded anymore as an unusually slow growing microorganism.

From the previous section we can conclude that the increase in maximum growth rate observed most likely originates primarily from a more than three times increase in the q_e^{max}-value at decreasing SRT. Herewith the question remains *why* the q_e^{max} increased at decreasing SRT. We have identified three potential reasons for the increase observed: (i) the selection of a different anammox strain (competition), (ii) directed evolution of the dominant anammox strain, or (iii) metabolic adaptation by e.g. different expression levels for some genes.

(i) Results of the phylogenetic analysis revealed that the dominant anammox strain present in the reactor belonged to the same species throughout the experimental period (99% related to *Candidatus* Brocadia sp.

40) (FIG. 2). Overall, we found no indication for a shift in population towards a different (faster) anammox strain growing into the system and explaining the increase in growth rate.

(ii) Previous research has demonstrated repeatedly that directed evolution allows for selection of mutant strains that have functional characteristics that provide a competitive advantage over other cells. Contrary to what was observed in other chemostat studies performed at increasing dilution rates (e.g. Wick et al., 2001; Wick et al., 2002; Kuyper et al., 2005), we did not observe a decrease in affinity constant for the limiting substrate in time. Even though only full metagenomic analysis can confirm the absence of mutations, the minor and randomly distributed changes encountered in the ITS region (FIG. 4) suggest that no major mutations have occurred in the dominant anammox strain. Premising that solid conclusions cannot be formulated due to the limitations related to ITS rRNA sequencing and that minor mutations may have occurred, our results suggest that directed evolution, whilst not rejectable, is unlikely to explain the increase in growth rate observed.

(iii) Strong metabolic adaptation appears unlikely due to the time scale relative to the observation of the improved kinetics. While in fact up-regulation is reported to take place within few generations after the imposition of the selective pressure (Wick et al., 2001), in our case more than 40 generations were needed to observe an improvement in kinetic properties after the imposition of the shorter SRT (TAB.3).

None of the previous explanations provides a convincing clarification of the change in kinetic properties observed. Herewith the question remains unanswered which cellular modification of anammox enabled the strong increase in electron transfer rate observed. In principle, minor modifications at the protein level may already induce a major change in kinetic properties, but only detailed whole-genome analysis may be capable of identifying such a modification (de Kock, 2012). It should be noted that maximization of the electron transfer capacity was not a selection factor in the cultivation systems used for anammox bacteria in previous studies. Traditional anammox cultivation systems are characterized by long or very long SRT-values (low

growth rates), such as biofilm based bioreactor systems where competition is based on biofilm forming capacity as well as substrate affinity.

It is concluded that anammox can no longer be regarded as a microorganism characterized by peculiarly low kinetics since the activity observed in this study is comparable to other chemolithoautotrophs. The higher kinetic properties observed open new field of application of the anaerobic ammonium oxidation process. This raises the question if adequate cultivation conditions may also result in significantly higher growth rates for other intriguing microbial processes, such as the recently discovered nitrite-dependent anaerobic oxidation of methane (Raghoebarsing et al., 2006).

Conclusions

The stoichiometric and kinetic properties of an anammox enrichment culture were investigated at decreasing solid retention times in a membrane bioreactor. It was shown that this procedure enabled the maximum growth rate of the anammox enrichment culture to increase to 0.334 d^{-1}, which is four times higher than previously reported. Even though researchers have speculated about the possibility of higher rates before, these speculations were always based on indirect measurements of the kinetic properties. Herewith this is the first direct experimental evidence for a spectacular increase in growth rate of an anammox enrichment culture. Since the biomass yield of the enrichment culture established is largely comparable to previous studies, it can be concluded that the increased growth rate results from an equivalent increase in biomass specific electron transfer capacity. Detailed molecular analysis did not reveal either a shift in dominant anammox strain nor major mutations in the dominant strain, suggesting that the actual reasons for the increase in electron transfer capacity is due to small changes in the metabolic machinery. The dominant strain throughout this experiment was closely related to *Candidatus* Brocardia Sp.40 (99% similarity). In this study we cultivated anammox bacteria applying a novel selection strategy based on the maximization of the electron transfer capacity demonstrating that maximum growth rate is not an intrinsic process property but that it can be increased significantly when the adequate cultivation conditions are imposed. The anammox enrichment became faster through training and anammox can

therefore not be regarded anymore as an intrinsically slow growing microorganism.

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Chapter 4

The effect of nitrite inhibition on the anammox process

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Lotti T., Van der Star W.R., Kleerebezem R., Lubello C., van Loosdrecht M.C.M. (2012) The effect of nitrite inhibition on the anammox process. Water Research 46 (8), pp. 2559-69.

Abstract

The negative effect of nitrite on anammox activity has been reported widely during the past decade. Although the adverse effect is clear, conflicting reports exist on the level at which it occurs and its reversible/irreversible nature. An in-depth study on nitrite inhibition therefore was performed in which the influence of environmental factors was evaluated. Anammox activity was measured in anammox granules by continuously monitored standardized manometric batch tests extending the interpretation by evaluation of lag times, maximum conversion rates during the tests and substrates/product conversion ratios. The granules where obtained from a one-stage anammox reactor, the dominant anammox organisms belonged to the *Brocadia* type. The observed 50% activity inhibition for nitrite (IC_{50}) was 0.4 g N L^{-1}. The activity recovered fully after removal of the nitrite. Conversion in fresh medium after exposure to up to 6 g NO_2^--N L^{-1} for 24 hours showed less then 60 % loss of activity. Presence of ammonium during nitrite (2 g N L^{-1}) exposure resulted in a stronger loss of activity after nitrite exposure (50% and 30% in presence and absence of ammonium respectively). Presence of oxygen during nitrite incubation led to a maximum activity reduction of 32%. The recovery after exposure indicates that the adverse effect of nitrite is reversible and thus inhibitory rather than toxic in nature. Similarities between exposure at three different pH values indicate that nitrite rather than nitrous acid is the actual inhibiting compound.

Introduction

The anaerobic ammonium oxidation (anammox) process represents a cost-effective nitrogen removal process for treatment of ammonium-rich wastewater (Fux and Siegrist, 2004; Van Dongen et al., 2001), getting rapidly introduced in practice worldwide (van der Star et al., 2007). The responsible microorganisms grow on ammonium with nitrite as electron acceptor resulting

in production of dinitrogen gas. The anaerobic and autotrophic nature of these organisms permits significant savings in aeration energy, no need for organic carbon and a lower sludge production. The bacteria performing the anammox process form the order "Brocadiales" within the phylum *Planctomycetales* (Jetten et al., 2010b) of which the two "candidatus" genera *Brocadia* and *Kuenenia* are the most relevant for wastewater treatment.

One of the most critical aspects in the anammox process stability is nitrite, since it is the electron acceptor in the process and converted by anammox bacteria, but also a potential inhibiting compound. Nitrite concentrations as low as 5 and 40 mg N L^{-1} have been reported as strongly inhibitive (Wett (2007) and Fux (2003), respectively). Strous et al. (1999), who first reported the adverse effect of nitrite, found a complete but reversible, inhibition of the process at 100 mg N L^{-1}. Other authors reported similar concentrations as detrimental for the anammox process, but indicating the nitrite inhibition either as reversible or irreversible (e.g. Fux et al., 2004; Jetten et al., 2005; López et al., 2008; Van Dongen et al., 2001). A few reports indicate even higher nitrite tolerance (Cho et al., 2010; Dapena-Mora et al., 2007; Egli et al., 2001; Fernández et al., 2012) with the highest reported non-inhibitory value reported by Kimura et al. (2010) (toxicity threshold higher than 300 mg N L^{-1}).

The wide range of observations regarding nitrite which was observed makes it difficult to predict, model or design anammox-based technologies. Therefore an in-depth study of nitrite inhibition to anammox bacteria was made. Particular emphasis was given to the recovery of the anammox bacteria after exposure to nitrite. We differentiate between inhibition, defined as a phenomenon which is reversible and depending on the time of exposure and the concentration of inhibiting compound, and toxicity, that is defined as the irreversible process of activity loss depending on the time of exposure and the concentration of the toxicant.

The use of standardized manometric batch tests was first proposed by Dapena-Mora et al. (2007). This method was modified to increase accuracy and reliability, and used it as a reproducible methodology for our research. The standard evaluation of maximum conversion rates was extended by evaluating also the duration of lag times and substrates/products conversion ratios.

Materials and methods

Manometric test equipment

The assays were performed in closed bottles equipped with manometric sensors including a data storage system for 360 datapoints (OxiTop Control AN6 (WTW, Weilheim, Germany)). The system was used previously for evaluation of anammox activity (Scaglione et al., 2009). The manometric devices consisted of 340 mL vials provided with a measuring head with a pressure transducer (sensitivity level 1 hPa). Each vial had two lateral holes closed with a puncturable rubber septum for substrate injections and sampling.

Origin of the biomass

The biomass used originates from the full-scale anammox reactor of Dokhaven-Sluisjesdijk wastewater treatment plant. The reactor contains granular anammox sludge and treats reject water after partial nitritation in a SHARON reactor. The size distribution of the granules was determined with the aid of image analysis (Thijus et al., 1994). 94% of the granules analyzed presented a diameter of 1.1±0.2 mm. During the experimental period, the anammox reactor was operated at the design volumetric load of 7.1 kg N m^{-3} d^{-1} (van der Star et al., 2007). During 2010 the average reactor conditions where: temperature 34±2.5 °C, pH 7.2±0.4 and concentrations of nitrogen in effluent were 50±20 mg NH_4^+-N L^{-1}, 15±15 mg NO_2^--N L^{-1}, 95±20 mg NO_3^--N L^{-1} (de Kreuk, 2011). The biomass was confirmed to consist of a "*Brocadia*" enrichment during the period of the tests by *fluorescence in situ hybridization* (FISH), the sludge hybridized with AMX820 and not with KST157 probes (Schmid et al., 2001).

General procedure for manometric tests

After sampling the granular sludge was brought under non-aerated conditions to the laboratory (30 minutes travel time) and directly used for the tests. The biomass was washed and re-suspended in a *washing medium*: a medium containing the microelements needed to avoid nutrient limitation (Van De Graaf et al., 1996) as well as 25 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) buffer. The pH value of the medium was set to 7.5 with

0.1 M NaOH or H_2SO_4. After this, the headspace and liquid phase (200 mL) were sparged with nitrogen gas to obtain anoxic conditions. The bottles were placed in a thermostatic shaker, at 170 rpm and 30 °C until the headspace pressure (rising as a result of the temperature change) had stabilized. Then overpressure was released (by inserting a needle connected to a water-filled vessel to act as a water-lock) and substrates were injected. The *injected solutions* contained $NaNO_2$, $(NH_4)_2SO_4$ and NH_4HCO_3 dissolved in high purity water obtained through a milli-Q™ system. The initial concentration of ammonium and nitrite was 50 mg N L^{-1} unless mentioned otherwise. To avoid inorganic carbon limitation the initial bicarbonate concentration was set to 32.7 mg L^{-1} while taking into account that part of the inorganic carbon partitioned to the headspace during equilibration. The pressure increase caused by the nitrogen gas production and accumulation in the headspace was automatically measured and recorded during the entire test for subsequent processing. Once the pressure reached a constant value (and all nitrite was assumed to be converted), a liquid sample was taken for chemical analysis (pH, ammonium, nitrite and nitrate).

Preliminary tests

To assess the accuracy and reliability of the method for measurement of the maximum specific anammox activity (MSAA) a set of preliminary assays was performed in duplicate according to *par.2.3*. The same test runs were performed with different batches of biomass during the entire experimental period to exclude any effect on changes in biomass composition in the full scale reactor.

- Ammonium and nitrite level: Starting concentrations of 40, 50, 60, 70 and 80 mg N L^{-1} of ammonium and nitrite were tested. The initial biomass concentration was 1.0 g VSS L^{-1}. The test was also used to evaluate the nitrogen balance for each experiment.

- Biomass level: Varying levels of biomass (0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 g VSS L^{-1}) were used for tests with 40 mg NH_4^--N L^{-1} and 40 mg NO_2^--N L^{-1} as well as 60 mg NH_4^--N L^{-1} and 60 mg NO_2^--N L^{-1}.

- Effect of buffer solution: Biomass was suspended in 5.3 mM phosphate buffer (Dapena-Mora et al., 2007), HEPES (25 mM) buffer or non-buffered medium (the rest of the medium composition was standard as described above). The experiments were performed in triplicate.

Activity after nitrite exposure tests

When exposure to a (potentially) inhibitory compound was tested, the procedure described in *par.2.3* was preceded by exposure to nitrite only (as $NaNO_2$) or to ammonium (as $(NH_4)_2SO_4$) and nitrite for 1 or 24 hours and subsequent washing (Figure 1, *protocol A*). When toxicity to ammonium and nitrite was tested, ammonium and nitrite had equimolar nitrogen concentrations in the exposure medium. Sodium nitrate was added to the exposure medium of a control assay (unexposed biomass) at a concentration of 70 mg NO_3^--N L^{-1} to avoid sulphate reduction.

The protocol used in these tests (*protocol A*) is illustrated in Figure 1 and consisted of the following stages:

i) Anoxic exposure to various concentrations toxicant for 1, 2 or 24 hours

ii) sampling for determination of pH and measurement of ammonium, nitrite and nitrate concentrations, followed by

iii) washing and resuspension of the biomass in *washing medium*;

iv) sparging to obtain anoxic conditions and transferring the vessels to a thermostatic shaker (170 rpm and 30 °C);

v) waiting for ca 60 min to allow for pressure stabilization followed by removal of overpressure by insertion of a needle connected to a neoprene tube immersed in water;

vi) dosage by injection of a concentrated solution of ammonium, nitrite and inorganic carbon (procedure: see previous paragraph);

vii) second dosage of substrates (only performed when explicitly stated; procedure: see previous paragraph);

viii) sampling for *chemical analysis*.

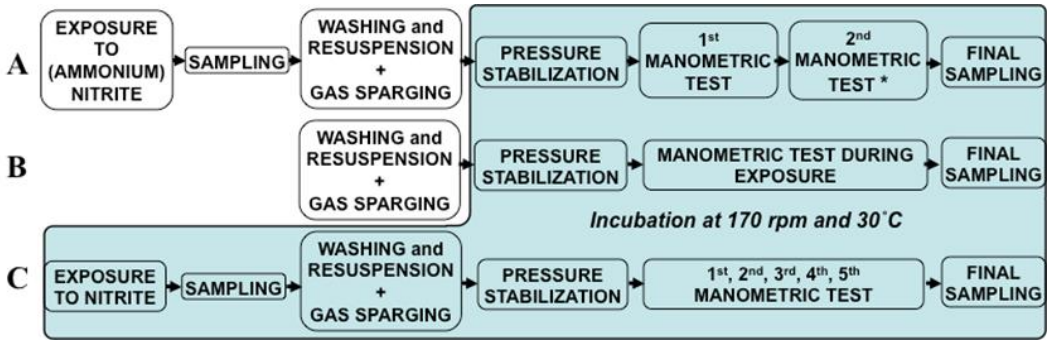

Figure 1. Adopted experimental procedures: *protocol A*: activity after exposure tests; *protocol B*: nitrite inhibition test; *protocol C*: long term activity after exposure.

***** Only when explicitly stated, the second manometric test was performed.

Nitrite Inhibition tests

The inhibition of nitrite on anammox activity was tested by *protocol B* (protocol adapted from *protocol A*, Figure 1).

Biomass was prepared in a *washing medium* (see previous paragraph), which also contained 85 mg N L^{-1} ammonium (as NH_4HCO_3). After pressure equilibration, pressure was reduced, 0.5-4 mL nitrite solution was added to achieve the desired starting concentration (Table 1) and the manometric test was started. The control assays consisted of manometric tests with 50 mg N L^{-1} ammonium and 50 mg N L^{-1} nitrite.

Nitrite inhibition test conditions

Inhibition-related tests were either focused on the immediate effect of exposure to nitrite only (as $NaNO_2$), or the effect after exposure to ammonium (as $(NH_4)_2SO_4$) and nitrite for a designated time as described above. An overview of the test conditions is shown in Table 1.

Table 1. Test conditions [a]

Test type	Protocol	Exposed chemical	Exposure concent. [mg N L^{-1}]	Exposure time [h]	Biomass concent. [g VSS L^{-1}]	Number of standard manometric tests after exposure	Other conditions
Inhibition	B	NO_2^-	0, 100, 150, 200, 250, 300, 400, 500, 1000, 3000[b]	-	1.5 ÷ 2	0	presence of 85 mg NH_4^+-N L^{-1}
Activity after exposure	A	NO_2^-	0, 500, 1000, 2000, 6000	1 , 24	1.5 ÷ 2	2	
Activity after exposure	C	NO_2^-	1000	24	1.5 ÷ 2	5[c]	presence of 100 mg NH_4^+-N L^{-1}
Activity after exposure	A	NO_2^- + NH_4^+	0, 250, 500, 1000, 2000	1 , 24	1.5 ÷ 2	2	
Activity after exposure Aerobic conditions	A	NO_2^- + NH_4^+	0, 250, 500, 1000, 2000	1 , 24	1.5 ÷ 2	2	During toxicity DO (mg L^{-1}): 5.0[d]
pH effect	A	NO_2^-	0, 500, 1000	2	1.5 ÷ 2	1	pH = 6.8, 7.8[e]

[a] All assays were conducted as two replicates according to *protocol A* except when explicitly stated.

[b] For the manometric tests with nitrite starting concentrations of 1 and 3 g N L^{-1}, the activity decreased in time and no maximum could be found; MSAA was considered as the average activity 60 to 80 minutes after the injection.

[c] Assay conducted with four replicates

[d] During the exposure, the biomass was continuously sparged with a mixture of air and nitrogen gas in order to keep a dissolved oxygen concentration (DO) in the bulk of 5 mg O_2 L^{-1}. The air and the nitrogen gas flows were regulated by mass flow controllers at a total rate of ca 50 mL min^{-1}.

[e] The *exposure medium* pH was set to 6.8 and 7.8 respectively with 0.1 M $NaOH/H_2SO_4$, while pH during the manometric tests after exposure was 7.5 as usual. After the exposure and after the manometric test pH was measured to verify it had remained constant.

Long term effect after exposure

To evaluate the effect of successive injections of non-toxic levels of ammonium and nitrite after nitrite exposure, tests were performed using an extension of *protocol A* (*protocol C*) at 24 h of exposure to 1000 mg N L^{-1} nitrite. The control assay was performed by two standard manometric tests without toxicity exposure. The assay was conducted in four replicates. In addition, manometric measurements took place also during the exposure phase (24-hour long manometric test; called *Exposure phase* in *par 3.4*). The *suspension medium* also contained 0.1 g N L^{-1} $(NH_4)HCO_3$. A concentrated nitrite solution (prepared dissolving $NaNO_2$ in milli-Q water) was injected in the vessels in order to obtain 1 g N L^{-1} as initial nitrite concentration. After washing, the biomass was suspended in the *suspension medium*, degassed in order to remove oxygen and five successive feedings (called batch *1-5* in *par 3.4*) were injected (initial ammonium and nitrite concentrations of up to 50 mg N L^{-1}). The first manometric test after washing was conducted 2 hours after the toxicity exposure. Subsequent manometric tests were performed after 8, 23, 27 and 94 hours respectively after the 24 hours toxicity exposure.

Calculations

Accuracy of the method

The relative error of the method was calculated based on the total nitrogen mass balance considering both liquid and gas phase. The total amount of dinitrogen gas produced during the test was calculated from the overpressure recorded by the transducer in the gas phase at the end of the assay by using the ideal gas low equation. The absolute change in CO_2 partial pressure due to carbon fixation is less than 4% of the change in N_2 partial pressure. It was therefore neglected in the evaluation. The amount of substrate consumed and product produced was calculated by measuring the concentrations of ammonium, nitrite and nitrate at the beginning and at the end of each essay.

Maximum specific anammox activity and activity percentage

From the recorded data of pressure increase in time, the N_2 produced was calculated: with a headspace volume of 140 mL and assuming ideal gas conditions, 1 hPa equals $5.55 \cdot 10^{-3}$ mmol N_2 at 30°C. Figure 2 shows a typical response of a manometric test, where data taken from one of the preliminary tests are reported. The nitrogen gas production rate was calculated through linear regression of a set of 20 datapoints (N_2 [mmol] in headspace versus time [min]) corresponding to a time interval of at least 10 minutes. The curve describing the N_2 production rate in time (obtained by numerical differentiation of the elaborated experimental data) typically presented an initial positive slope (reactivation phase, corresponding to phase I in Figure 2), followed by a plateau corresponding to the highest values (corresponding to phase II in Figure 2) and a final negative slope ending on the abscissa (substrate limiting phase, corresponding to phase III in Figure 2). The average of the values on the plateau (identified by $d^2N_2/dt^2=0$) was considered as the maximum anammox activity and expressed as mmol N_2 min^{-1}. Dividing this value by the known amount of biomass present in the bottle at the beginning of the test, the maximum specific anammox activity was calculated and expressed as g N_2-N (g VSS)$^{-1}$ d^{-1}. The amount of biomass grown during each manometric test can be calculated by considering the complete consumption of the limiting substrate during the test (50 mg NO_2^--N L^{-1} for all tests except 85 mg NH_4^+-N L^{-1} for the *immediate inhibition to nitrite* test), the average starting biomass concentration (1.5 ÷ 2 g VSS L^{-1}) and the yield of biomass on the limiting substrate (0.09 g VSS g NO_2^--N^{-1} and 0.12 g VSS g NH_4^+-N^{-1} according to Strous et al., 1998). This value corresponds to less than 0.31-0.69% of the initial biomass concentration for *nitrite-limited* and *ammonium-limited* test respectively, indicating that the biomass concentration can be considered constant throughout the test.

Figure 2 shows a typical response of a manometric test, where data taken from one of the preliminary tests are reported. The percentage of activity that was maintained after the exposure to, or in presence of, inhibitory compounds was calculated with respect to the average of the activities of the control assays (unexposed biomass).

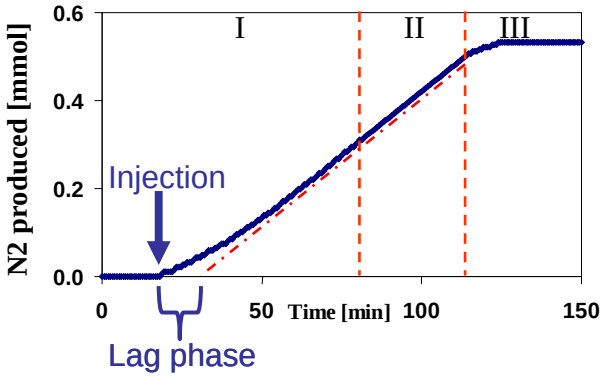

Figure 2. Typical response of a manometric test –nitrogen gas produced [mmol] was calculated through ideal gas law from recorded data [hPa]–. The data shown are derived from a *preliminary test*. The dash dot line is a graphical representation of the calculation to determine the *lag phase*.

Substrates/products molar ratio and lag phase

The molar ratios of substrates (nitrite on ammonium converted, *R_NiAm*) and of product (nitrate produced on ammonium converted, *R_NaAm*) was calculated from N-compound concentrations in the liquid samples before and at the end of the test, according to the following relation:

$$R\_NiAm = \frac{\left[NO_2^-\right]_{START} - \left[NO_2^-\right]_{END}}{\left[NH_4^+\right]_{START} - \left[NH_4^+\right]_{END}} \quad (1)$$

$$R\_NaAm = -\frac{\left[NO_3^-\right]_{START} - \left[NO_3^-\right]_{END}}{\left[NH_4^+\right]_{START} - \left[NH_4^+\right]_{END}} \quad (2)$$

Lag phase

In order to quantify the delay between the substrates injection and the occurrence of the maximum specific anammox activity a parameter called *lag*

phase was defined. The part of the curve of the nitrogen gas development in time at the moment the maximum specific anammox activity occurred was extrapolated till the abscissa and the *lag phase* was defined as the difference between the time relative to this intercept and the injection time (see Figure 2 for a graphical representation of the calculation).

Analytical procedures

Soluble nitrogen compounds were measured via spectrophotometric flow injection analysis (QuickChem 8500 series 2 FIA System, Lachat Instruments, Loveland, Colorado, USA). The methods applied were QuikChem®Methods 10-107-06-5-E for ammonium (range 0.1 to 10.0 mg N L^{-1}, measurement of NH_3 after increasing pH and volatilization) and 10-107-04-1-C for nitrate/nitrite (range 0.01 to 2.0 mg N L^{-1}, direct measurement of nitrite, or measurement proceeded by reduction of NO_3^- to NO_2^- to yield the concentration of "NO_3^-+NO_2^-") according to the protocol of the manufacturer. The length of the sample loop of the nitrate/nitrite detection was increased in order to obtain a measurement range from 0.05±0.01 to 10±0.01 mg N L^{-1}. TSS and VSS were determined according to the Standard Methods (APHA, 2005).

Results

Accuracy of the method

A set of preliminary assays was performed in duplicate to assess the accuracy and reliability of the method to estimate the maximum specific anammox activity (MSAA). The tested initial concentrations of ammonium and nitrite of 40 to 80 mg N L^{-1} (total nitrogen concentration of 80 to 160 mg N L^{-1}) were chosen in order to be below the inhibition threshold indicated in literature (Dapena-Mora et al., 2007; Egli et al., 2001; Strous et al., 1999a), but high enough to allow for complete reactivation of the biomass before the limited substrate (nitrite) gets depleted. 50 mg NH_4^+-N L^{-1} and 50 mg NO_2^--N L^{-1} (total nitrogen concentration of 100 mg N L^{-1}) were chosen as standard initial concentration for the manometric tests. The variations in the MSAA-values identified in ten replicates proved to be less then 2%. The MSAA values were

furthermore shown to be independent of the biomass concentration (0.2-4 g VSS L^{-1}) and showed a low (5 %) variability. 2 g VSS L^{-1} was chosen as appropriate biomass concentration for further testing. Without a pH-buffer solution the pH increased during the tests from 7.5 to 7.9. Both the HEPES and phosphate buffer enabled a constant pH (7.5 except when explicitly stated otherwise) throughout the experiment. HEPES buffer (25 mM) was selected as buffer solution in subsequent experiments. There was no indication that these buffers negatively influenced the anammox activity.

The accuracy of the measurements was checked by evaluating the nitrogen mass balance. The nitrogen balance had always less then 5% inaccuracy indicating that the pressure measurement was accurate. The observed *R_NiAm* ratio was 1.35 (±0.07) and the *R_NaAm* ratio was 0.28 (±0.06), indicating a normal growth of the anammox bacteria in the test. Repeated testing with the same biomass was performed. The experiments showed an increase from the first to the second test of 1.4 to 4% and an increase of 2-8% (compared to the first test) in the 3rd and the 4th tests. The second, third and fourth test gave almost the same results as the first test indicating there was no strong positive or negative effect of the test system.

Inhibition of anammox conversion due to nitrite

Experiments in which MSAA was measured with varying nitrite concentrations (in the presence of 85 mg NH_4^+-N L^{-1}) were performed according to *protocol* B to evaluate the inhibitive effect of nitrite on the conversion process. MSAA decreased with increasing initial (50 and 500 mg NO_2^--N L^{-1}) concentrations (Figure 3). The residual activity in the presence of 1000 and 3000 mg NO_2^--N L^{-1} was 7 (Figure 3) and 3% respectively. For inhibition of nitrite a half maximal inhibitory concentration (IC_{50}) of 400 mg N L^{-1} was determined.

At initial nitrite concentrations up to 500 mg N L^{-1} the length of the *lag phase* increased, with a maximum of 70 minutes. For initial nitrite concentrations until 500 mg NO_2^--N L^{-1} the *R_NiAm* and *R_NaAm* values (Figure 4) were close to the standard stoichiometric ratios (Strous et al., 1998). At higher nitrite levels the *R_NiAm* ratio increased just as the *R_NaAm* levels 2.97 and 0.75 respectively at 3000 mg NO_2^--N L^{-1}.

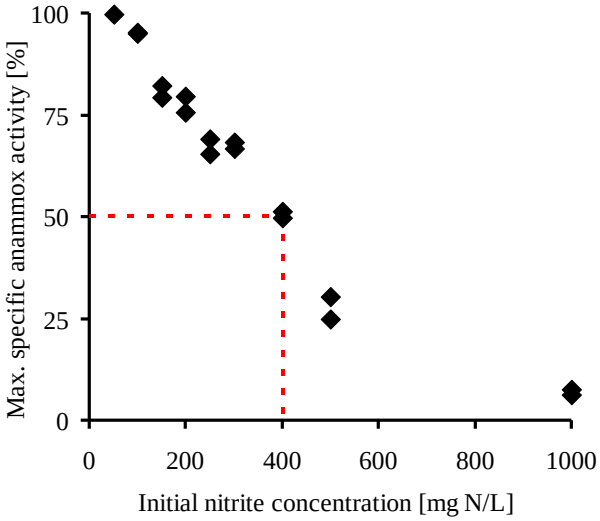

Figure 3. Maximum specific anammox conversion rate (MSAA, as a % of conversion of non-exposed biomass).

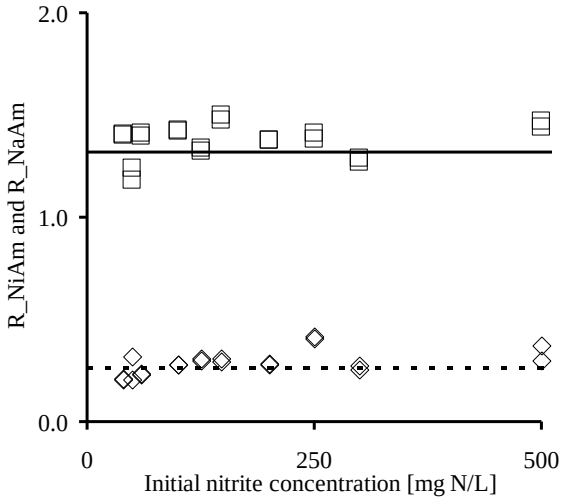

Figure 4. *R_NiAm* (squares) and *R_NaAm* (diamonds) during manometric activity tests at different initial nitrite concentrations; the continuous and dashed lines represent literature values for *R_NiAm* and *R_NaAm* for anammox bacteria (Strous et al., 1998).

Exposure tests

Exposure tests were performed according to *protocol* A and consisted of activity tests under standard (non-toxic) conditions after pre-incubation at potentially toxic concentrations of nitrite or ammonium and nitrite. After incubation for 1, 2 or 24 hours with nitrite or ammonium and nitrite, biomass was washed with washing medium to remove the (possibly) nitrogen compounds. Thereafter activity tests where performed.

Exposure to ammonium and nitrite

Exposure to concentrations of 250 to 2000 mg N L^{-1} of ammonium and nitrite (total nitrogen concentration of 500 to 4000 mg N L^{-1}) did lead to partial degradation of ammonium and nitrite during the exposure phase. During 24 hours exposure to an initial concentration of 250 mg N L^{-1} ammonium and nitrite, nitrite was fully removed. During the exposure to higher concentrations, the exposure time was not sufficient to convert all the nitrite present (less than 40%). Exposure for 1 hour did lead 15% ammonium and nitrite conversion at the lowest concentration of 250 mg N L^{-1}. The percentage of anammox activity that remained after 1 and 24 hours exposure to the tested substrate concentrations is shown in Figure 5, 1A. Exposure to higher concentrations resulted in lower activity values. Concentrations up to 500 mg N L^{-1} ammonium and nitrite had no significant effect on the activity for both exposure times. After 1 hour exposure at concentrations of 2 g N L^{-1}, the MSAA was reduced by 32%. Exposure for 24 hours to 1 and 2 g N L^{-1} ammonium and nitrite resulted in a reduction of MSAA by 50%. The length of the *lag phase* after the exposure to ammonium and nitrite (Figure 5, 2A) ranged from 4 to 12 minutes for concentrations up to 500 mg N L^{-1} for both the exposure durations. For higher exposure concentrations, the effect of the exposure duration and concentration increased strongly to 77 minutes after exposure of 24 hours at 2 g N L^{-1}. Lag times after exposure of 1 hour never exceeded 24 minutes.

R_NiAm and *R_NaAm* increased after exposure to higher ammonium and nitrite concentrations and also increased upon longer exposure (Figure 5, 3A). Although the nitrate production levels were stable, ammonium removal levels decreased when the biomass had been exposed to higher ammonium and nitrite levels (Figure 5, 4A).

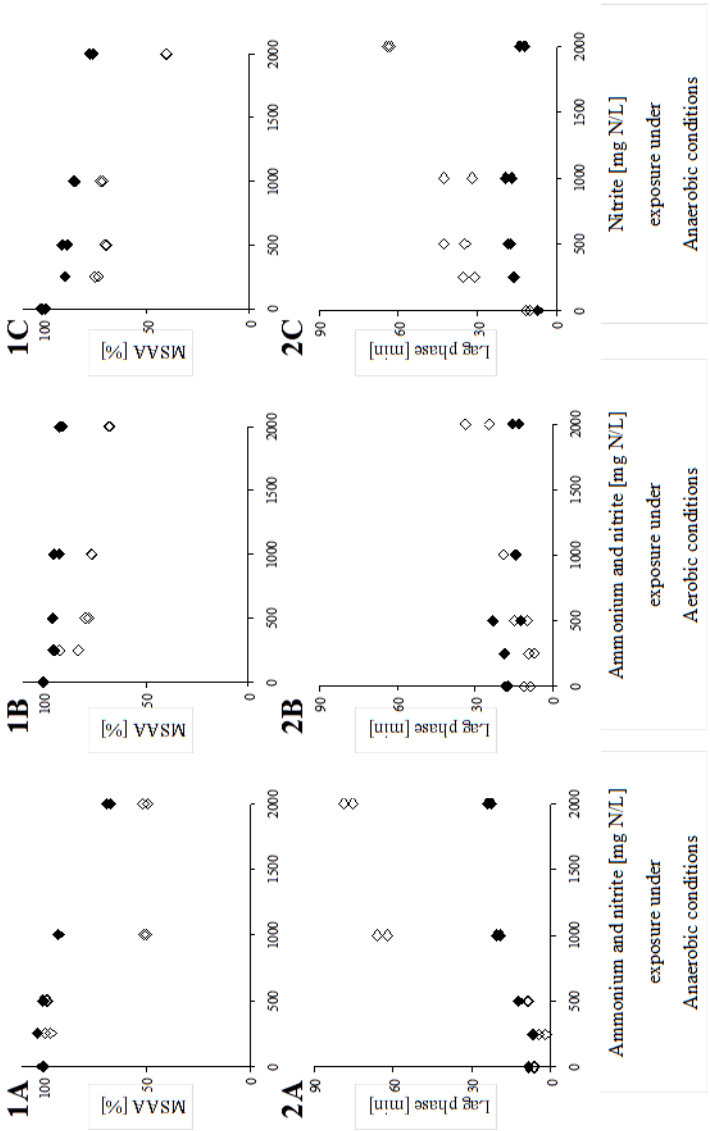

Figure 5. Anammox activity assays after exposure for 1h (closed symbols) and 24h (open symbols) to different concentrations of ammonium and nitrite in anoxic conditions (column A), ammonium and nitrite in aerobic conditions (column B), nitrite in anoxic conditions (column C). The concentration on the x-axis corresponds to the nitrite concentration during exposure (mg N L^{-1}). Row 1 indicates the maximum specific anammox conversion rate (MSAA, as a % of conversion of non-exposed biomass).

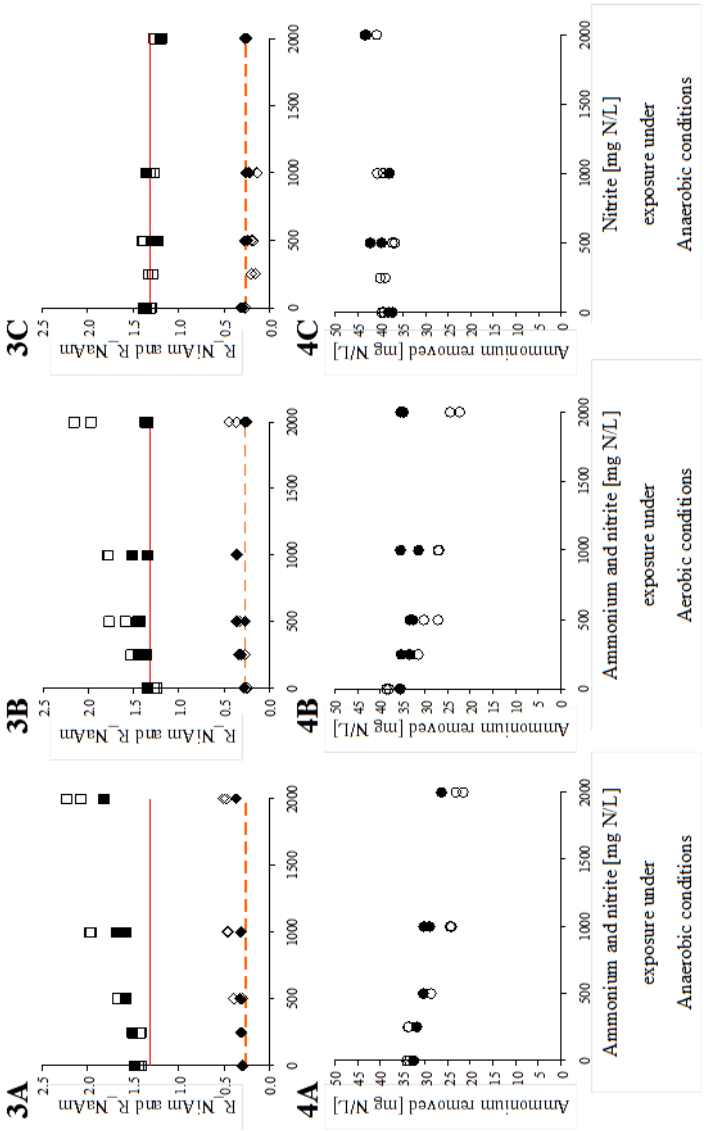

Figure 5. (continuation) Row 2 indicates the length of the *lag phase* (minutes) after exposure. Row 3 indicates the conversion ratios *R_NiAm* (equation 1, ■,□) and *R_NaAm* (equation 2, ♦,◊); the continuous and dashed lines represent standard values for *R_NiAm* (1.32) and *R_NaAm* (0.26) (Strous et al., 1998). Row 4 indicates ammonium removed during standard manometric batch test after exposure (mg N L^{-1}, *right y-axis*, ●,○).

Exposure to ammonium and nitrite in an aerobic environment

To assess whether the presence of oxygen has an extra effect on the exposure to ammonium and nitrite, activity tests (under anoxic conditions) were conducted after exposure in the presence of oxygen. Preliminary tests in which biomass was exposed to 50 mg NH_4^+-N L^{-1}, 50 mg NO_2^--N L^{-1} and 8 mg O_2 L^{-1} for 24 hours showed no activity loss and *lag phase* values less than 30 minutes during the standard anoxic activity test conducted afterwards (data not shown). Exposure to oxygen inhibited conversion of ammonium nitrite during the exposure phase completely, as the difference between initial and final values was less than or equal to the experimental error. Subsequent standard anoxic activity tests resulted in a reduction of less than 10% in MSAA after 1 hour exposure (Figure 5, 1B). Exposure for 24 hours resulted in higher losses in MSAA after exposure at 1 g N L^{-1} (24%) and 2 g N L^{-1} (32%) ammonium and nitrite. The *lag phase* increased with increasing exposure time but less compared to exposure under anoxic conditions with a maximum lag time of 33 minutes after exposure for 24 hours at 2 g N L^{-1} ammonium and nitrite (Figure 5, 2B). Also after exposure in the presence of oxygen, nitrate production was about 9.9 mg N L^{-1} (as expected after the conversion of 50 mg N L^{-1} nitrite), but ammonium conversion levels were less than expected (83% after exposure for 1 hour to 2 g N L^{-1} and 60% after exposure for 24 hour to 2 g N L^{-1}) causing increasing discrepancies with the standard stoichiometric ratios (Figure 5, 3B, 4B). During a second test conducted after exposure (both for 1 and 24 hours) the ammonium conversion levels markedly increased (at least 88% of the expected conversion) coming closer to the values predicted by the theoretical biochemical reaction.

Exposure to nitrite only

The activity after exposure for 1 or 24 hours with nitrite (in the absence of ammonium) is shown in Figure 5, 1C. No significant nitrite conversion took place during exposure. At 1 g NO_2^--N L^{-1} the concentration nitrate produced was less than 4 mg N L^{-1}, which was similar to a biomass-free control (results not shown). After 1 hour exposure the negative effect of nitrite was limited with a maximum activity reduction of 22% after exposure at 6 g N L^{-1}. Exposure for 24 hours resulted in higher losses in MSAA. The effect of 24 hours exposure to 2 g

N L^{-1} was similar to exposure to 1 g N L^{-1}. The activity reduction caused by exposure to 6 g N L^{-1} was 60%. A second injection of nitrite and ammonium after exposure resulted in an increase in activity of 6 to 23% (Figure 6) compared to the previous test. The increase was higher at increasing exposure concentration and exposure time for exposure concentrations until 2 g N L^{-1}.

The *lag phase* in general did not increase due to nitrite exposure, except for the long term high nitrite concentrations. A second test with this sludge showed a normal lag time again, indicating recovery of the biomass (Figure 5, 2C).

Nitrate production and ammonium consumption in activity tests after exposure to nitrite were 5 to 11 mg N L^{-1} and 37 to 43 mg N L^{-1} respectively, giving a *R_NaAm* ratio between 0.14 and 0.31 (Figure 5, 3C). A decrease in removed ammonium at increasing exposure concentrations was not observed (Figure 5, 4C).

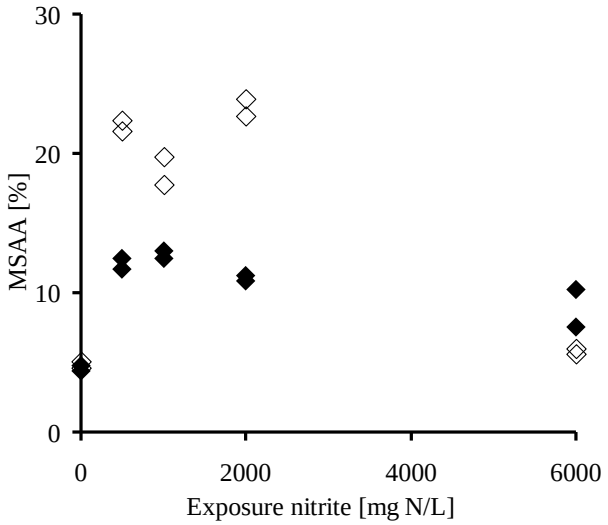

Figure 6. Activity increase in the second manometric test with respect to the first manometric test after exposure for 1h (♦) and 24h (♦) to different concentrations of nitrite.

Effect of pH on exposure to nitrite only

The effect of pH on incubation with nitrite was evaluated by tests at pH 6.8 and 7.8 during two hours nitrite exposure. Under these conditions the MSAA was reduced by 18 and 19% respectively (Figure 7) comparable to the incubation at pH 7.5 (Figure 5, 1C). No significant difference in MSAA during standard tests after exposure for two hour at pH 6.8 and pH 7.8 was observed. As with tests at pH 7.5 (see *par.3.3.2*), no nitrite was removed in absence of ammonium during the exposure. Often nitrous acid is indicated as the compound leading to toxicity (Anthonisen et al., 1976). The activity tests at different pH did not indicate that is the case for inhibition of anammox bacteria by nitrite; i.e. a 10 times increased nitrous acid concentration at pH 6.8 compared to 7.8 at equal total nitrite concentration did not affect the inhibition observed. It therefore seems that the total nitrite concentration determines the extent of inhibition.

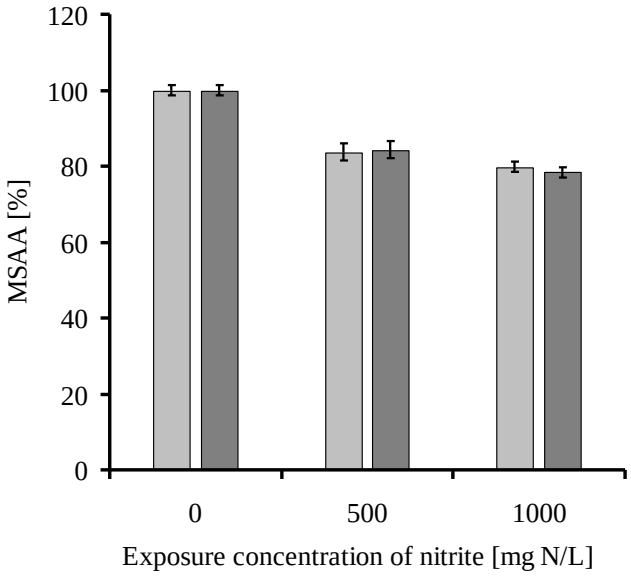

Figure 7. Maximum specific anammox activity (as a % of conversion of non-exposed biomass) after two hours exposure to nitrite at pH 6.8 (light grey) and 7.8 (dark grey). Error bars represent standard deviation.

Long term recovery of anammox activity after nitrite exposure

The reversibility of nitrite inhibition was evaluated after exposure of anammox biomass to 1000 mg NO_2^--N L^{-1} for 24 hours. The anammox activity was followed during this incubation. Biomass was then washed and five successive manometric tests (Batch *1-5* tests) were conducted with initial ammonium and nitrite concentration of 50 mg N L^{-1} during the following 98 hours.

Incubation at 1000 mg NO_2^--N L^{-1} resulted in a progressive loss of activity. After 2 hours the activity loss was 93-94% with respect to the control assay. After washing the sludge to remove nitrite activity was regained with a *lag phase* of ca 20 minutes, while in the successive tests with the same sludge the *lag phase* was less than 8 minutes. The maximum specific activity increased during the first three consecutive tests conducted after washing of the biomass, while there was negligible difference between the 3rd, 4th and 5th test (Figure 8 and Tab. 2). The nitrate to ammonium ratio during these tests remained in the normal range.

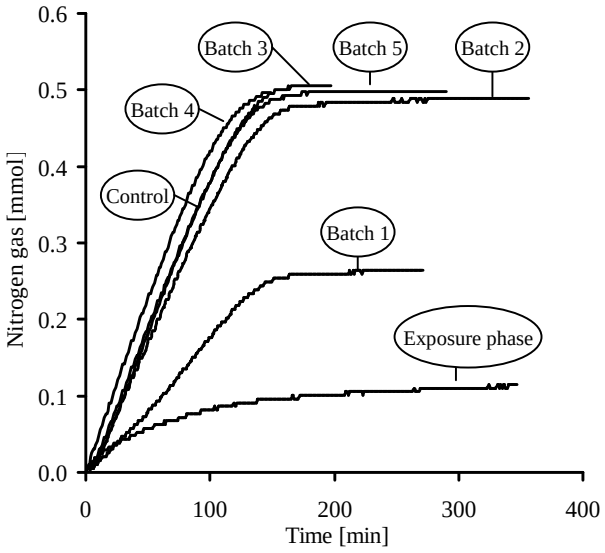

Figure 8. Nitrogen gas production in time during the whole manometric tests. Labels indicate the abbreviation of the corresponding manometric test. Only the first 350 minutes of the 24 hours *Exposure phase* test are shown.

Table 2. 24-Hour long exposure to 1 g N L^{-1} nitrite, followed by five successive manometric tests conducted after biomass washing and relative to an unexposed control assay: maximum specific anammox activity (MSAA) and duration of each test.

Manometric test	Manometric test duration [h]	MSAA [g N_2-N (g VSS)$^{-1}$ d^{-1}]
Control assay	2	0.458
Exposure phase	24	0.061[a]
Batch 1	7	0.209
Batch 2	11.5	0.390
Batch 3	4	0.430
Batch 4	4	0.454
Batch 5	4	0.439

[a] This is not the MSAA but the SAA (specific anammox activity) calculated 1h after the injection

Discussion

1) Suitability and accuracy of the tests

The manometric batch tests used in this work are adaptions of manometric batch tests first developed as standardized tests for biological oxygen demand (BOD) determination and were already successfully adapted to nitrogen production for evaluation of the anammox process by Dapena-Mora et al. (2007) and Caffaz et al. (2008). The approach used in the present work furthermore prevented the possible effect of inorganic carbon limitation (Kimura et al., 2011; Lotti et al., 2009) and pH fluctuation was avoided with a 25 mM HEPES buffer. Furthermore the method applied avoided any disturbances of the process during the batch measurement of the activity. Disturbances that may occur include volume and/or temperature fluctuations due to manual sampling in the case of tracking the substrates concentration or the nitrogen gas production. Together with the high measurement frequency (one measurement per minute), the experimental setup allows for detailed analysis of the development of the activity in time. In a too short test the *lag phase* may

not have ended before (near) depletion of ammonium and nitrite and thus lead to underestimation of the maximal activity. The presence of a plateau in the N_2 production rate is a confirmation that the maximum activity was reached during the test. The results showed a high accuracy of the manometric test for the measurement of the maximum specific anammox activity. The tests were very reproducible (<5% variation) and when evaluated with liquid samples the nitrogen balances closed. Clearly the manometric test can be reliably used for anammox activity evaluation when used in a careful way.

2) Inhibition of the anammox process by nitrite

A wide range of data on nitrite inhibition has been reported (Table 3) using different determination systems and biomass in different aggregation states. Unlike results obtained from measurements directly within the (laboratory scale) reactor, the results of batch tests outside the reactor environment are relatively consistent. Inhibition appears to be more severe in case of suspended and flocculent biomass (Table 3), suggesting that the outer layer of the biofilm (biofilm on support/carriers or granular biomass) can act as a protecting shell for the inner core (due to diffusion and, in case of non-complete inhibition, residual nitrite removal rate). This is supported by the studies of Fernández et al. (2012) and Cho et al. (2010) who investigated nitrite inhibition using biomass in different aggregation states (Table 3). Despite differences in (I) dominating anammox taxon, (II) average MSAA and (III) -slight- different experimental conditions, the IC_{50} reported for biofilm systems (400 and 350 mg N L^{-1} for Cho et al. (2010) and Fernández et al. (2012) respectively) is similar to the value found in this work (400 mg N L^{-1}, Figure 3). 350-400 mg N L^{-1} can thus be regarded as an accurate and relatively situation independent value for the IC_{50} in case of biofilm or granular sludge. From nitrite concentration ranges experienced by anammox microorganisms during normal reactor operation (Table 3), no indications for inhibition-adaption could be inferred.

Table 3. Reported values for nitrite inhibition on the anammox process: the table includes studies where specific tests on nitrite effect have been conducted.

Reference	Nitrite level [mg N L^{-1}]	Determination method	Batch (B) or in react. (R)	Accuracy	Test length
Strous et al., 1999a	100 ("complete inhib.")	NO_2^- conv. rate	B	n.r.	n.r.
Egli et al., 2001	180 ("complete inhib.")	NO_2^- conv. rate	B	n.r.	70 h[c]
Fux et al., 2002	60 ("strong inhib.")	NO_2^- conv. rate	R	n.r.	2 days
Li et al., 2004	70 ("serious inhib.")	nitrite accumulation	R	n.r.	12 h
Fux et al., 2004	80 (80% loss)	NO_2^- conv. rate	R	n.r.	n.r.
Jung et al., 2007	70 (inhibition)	nitrite accumulation	R	n.r.	n.r.
Dapena-Mora et al., 2007	350 (IC_{50})	manometric	B	7±4%	n.r.
López et al., 2008	100 (inhibition)	nitrite accumulation	R	n.r.	n.r.
Bettazzi et al., 2010	>60 (28% reduction at 75)	manometric	B	4.5±3.3%	6-16 h
Fernández et al., 2012	≈350 (IC_{50}[c])	manometric	B	n.r.	n.r.
Fernández et al., 2012	≈120 (IC_{50}[c])	manometric	B	n.r.	n.r.
Cho et al., 2010	≈400 (IC_{50}[c])	NO_2^- conv. rate	B	n.r.	40 h
Cho et al., 2010	≈230 (IC_{50}[c])	NO_2^- conv. rate	B	n.r.	40 h
Kimura et al., 2010	>300 (37% loss at 430)	NO_2^- conv. rate	B	n.r.	1 h
Oshiki et al., 2011	224 (IC_{50})	NO_2^- conv. rate	B	n.r.	n.r.
present work	400 (IC_{50})	manometric (batch)	B	4.2±0.7	4-8h

Table 3 (continuation). Reported values for nitrite inhibition on the anammox process: the table includes studies where specific tests on nitrite effect have been conducted.

Reference	Anammox taxon	Aggregation status	Process config.[a]	Reactor volume (L)[b]	pH	Normal operation nitrite level [mg N L^{-1}]
Strous et al., 1999a	*Candidatus* Brocadia anammoxidans	homogenized aggregates	2	10	7, 7.4, 7.8	0÷1.4
Egli et al., 2001	Kölliken microrganisms[d]	suspended	2	2	7	84
Fux et al., 2002	Kölliken microrganisms[d]	suspended	2	2500	7.5	n.r.
Li et al., 2004	n.r.	n.r.	1	3	7.5-8	0÷20[c]
Fux et al., 2004	Kölliken microrganisms[d]	biofilm on support[e*]	2	3.5	8.0	18±17
Jung et al., 2007	n.r.	granular	2	3.35	7.5-8	0÷35
Dapena-Mora et al., 2007	C. Kuenenia Stuttgartiensis	flocculent	2	1	7.8	0÷15[c]
López et al., 2008	C.Brocadia anammoxidans	granular	2	15	7.5-8.2	"close to zero"
Bettazzi et al., 2010	C. Brocadia anammoxidans	flocculent	2	40	n.d.	"close to zero"
Fernández et al., 2012	C. Kuenenia Stuttgartiensis	biofilm on support[e**]	2	5	7.8	0÷25[c]
Fernández et al., 2012	C. Kuenenia Stuttgartiensis	flocculent	2	1	7.8	0÷15[c]
Cho et al., 2010	C. Brocadia anammoxidans	granular	2	1.25	n.d.	n.r.
Cho et al., 2010	C. Brocadia anammoxidans	homogenized granules	2	1.25	n.d.	n.r.
Kimura et al., 2010	C. Brocadia anammoxidans	gel carriers	2	0.5	n.d.	0÷60[c]
Oshiki et al., 2011	C. Brocadia sinica	flocculent[f]	2	0.8	7.0-7.5	n.r.
present work	C. Brocadia anammoxidans	granular	2	70000	7.5	0÷85[g]

[a] Nitritation and anammox processes in a single reactor (1) or in separate reactors (2)
[b] When experiment are conducted in batch this column reports information on the origin of the biomass used
[c] Implied from Figure
[d] The nucleotide sequence of the 16S rRNA gene of the Kölliken anammox organism was deposited in the GenBank database under accession no. AJ250882 (Egli et al., 2001)
[e] PVC as carrier material ([e*]); Zeolite as carrier material ([e**])
[f] Biofilm samples were dispersed by magnetic stirring for 2h (aggregates diameter <100 μm)
[g] Reported by van der Star et al. (2007)
n.r. not reported; n.d. not determined

3) Mode of action of nitrite

In literature the adverse effect of nitrite is referred to as reversible inhibition (Strous et al., 1999a), irreversible inhibition (Jetten et al., 2005; Van Dongen et al., 2001) and toxicity (e.g. Wett, 2007). Reversible inhibition refers to a reversible decrease of the catabolic activity during exposure, while toxicity or "irreversible inhibition" refers to damages to the microorganisms associated with an irreversible decrease of the microbial activity. In order to clarify whether nitrite exposure resulted in reversible inhibition or toxicity, the reversibility of the exposure should be assessed. In the present work it is shown that after nitrite exposure to 1 g N L^{-1} for 24 hours the anammox activity can be fully recovered by decreasing nitrite concentration through biomass washing within two days (Figure 8 and Table 2), confirming the results obtained by Kimura et al. (2010) with anammox in gel-carriers.

The recovery of anammox bacteria from nitrite inhibition during an exposure of up to 24 hours, contradicts the attribution of reactor failures to nitrite exposure in many reactor studies (e.g. Egli et al. (2001), Fux et al. (2004), López et al. (2008), Table 3). Furthermore inhibition was reported in an SBR system at concentrations higher than 100 mg NO_2^--N L^{-1} (Strous et al., 1999a) and in a long-term experiment irreversible inhibition was reported when nitrite concentration was maintained at 40 mg N L^{-1} over several days (Fux et al., 2004). On the other hand, a full-scale reactor (origin of biomass in this work) was shown to operate at a total nitrogen conversion rate of 7.1 kg N m^{-3} d^{-1} for months at a nitrite concentration of 40 to 80 mg N L^{-1} (van der Star et al., 2007) and was capable of full recovery within a few days after exposure to 350 mg NO_2^--N L^{-1} for more than one day (de Kreuk, 2011). Thus, while it seems logic to associate a non-working anammox reactor with high nitrite levels, such a level

is –at least after short exposure– probably an effect rather than a cause of damaged biomass.

4) Role of nitrous acid

For nitrifiers and denitrifiers, nitrous acid –rather than the dissociated form nitrite– is toxic (Anthonisen et al., 1976; Zhou et al., 2011). The mode of action is related to the ease of diffusion of nitrous acid over the cell wall, where it dissociates resulting in a drop in pH and loss of the proton motive force (pmf). Also for the anammox process, nitrite toxicity is generally regarded to be HNO_2 related (e.g. Fernández et al., 2012), although there are no anammox related measurements supporting this assumption. Direct toxicity tests at pH 7, 7.4, and 7.8 however indicated that the ion itself (NO_2^-) is the actual inhibitor (Strous et al., 1999a). In our exposure tests at 500/1000 mg N L^{-1} nitrite at different pH-values, the remaining activity decreased by only 1.8 to 2.7% (at 500 and 1000 mg N L^{-1} respectively) although the difference in HNO_2 concentration varied by factor 10. Comparable changes in HNO_2 at constant pH a much larger decrease in activity was found. Combining the results of both tests a clear correlation between the decrease in activity and the total nitrite concentration was found. This is a strong indication that the actual inhibitor is nitrite rather than nitrous acid for anammox bacteria and that the toxicity is not due to dissipation of the pmf by HNO_2. It could well be that indeed the ladderane membrane lipids in the anammox cell membrane form such a tight membrane (Sinninghe Damsté et al., 2005) that HNO_2 diffusion is effectively prevented. However the specific mechanism for nitrite inhibition remains unclear.

5) Effect of exposure time and exposure conditions

The results presented in this work show that increased concentrations and exposure time increase the loss of activity. The joint effect of concentration and exposure time could not be correlated straightforwardly. Inhibition by nitrite also took place in the absence of ammonium and when cells were not catabolically active (Figure 5, 1C). For exposure to concentrations of up to 1 g NO_2^--N L^{-1}, the absence of ammonium during the exposure seemed to have a more severe effect, while the effect was reversed at higher values (Figure 5, 1B,

1C). However even after 24 hours exposure to 6 g NO_2^--N L^{-1} the activity maintained in the first manometric test after biomass washing was still 40% of the control activity and a second feeding leads to an additional increase of about 7% (Figure 6), showing that even such an extreme nitrite level did not seem to completely destroy the anammox metabolism.

Anammox bacteria are strictly anoxic and their inhibition by oxygen has been shown (Strous et al., 1997; Van Dongen et al., 2001). This inhibition was demonstrated to be reversible even at concentrations as high as 9 mg L^{-1} (calculated based on "37°C, 1.5 bar and 18% oxygen concentration in the headspace", Egli et al., 2001) and 3.7 mg L^{-1} (based on "32°C, 1 bar of absolute pressure and 50% air saturation", Strous et al., 1997).

From the results presented in this work, the effect on activity in tests *after* one hour exposure in the presence of 5 mg L^{-1} O_2 was negligible for concentrations up to 2 g N L^{-1} of ammonium and nitrite (Figure 5, 1B). Oxygen even seemed to decrease the inhibiting effect of substrates resulting in 30% loss of activity in batch tests (compared to a 50% loss after exposure in the absence of oxygen) after exposure for 24 hours to 2 g N L^{-1} ammonium and nitrite (Figure 5, 1A, 1B) and in an overall shorter *lag phase* (Figure 5, 2A, 2B). The results show that when the nitrite exposure concentration is sufficiently low to have actively metabolizing cells (<1 g N L^{-1} of ammonium and nitrite in anoxic condition), activity during exposure resulted in higher activity loss than when exposure took place under non metabolizing conditions (presence of oxygen, or absence of ammonium).

Wett (2007) reported that when the anammox process is carried out in presence of oxygen, nitrite is inhibiting anammox activity already at low concentrations (irreversible toxicity at 50 mg N L^{-1} and detrimental effect on the process already at 5 mg N L^{-1}). This is not in line with our results. We could however not find an explanation for this difference, except that maybe different types of anammox bacteria where present in the studies or that the suspended growth process described by Wett (2007) missed a protective effect of biofilm growth.

6) Ammonium sorption and conversion ratios

The inhibition effect on general metabolism and biomass synthesis was evaluated based on the decrease of the specific nitrogen gas production rate (which corresponds to catabolic activity) and on the *R_NiAm* and *R_NaAm* ratios, which are indicators for the anabolic activity (nitrite oxidation to nitrate functions as the electron-donating reaction for CO_2 fixation, Strous et al., 1998). After exposure to high concentration of ammonium however these ratios varied strongly (Figure 5, 3A, 3B, ascending trend in *R_NiAm* and *R_NaAm* ratios). The results showed that the higher the ammonium exposure (both in aerobic and anoxic conditions) the smaller the amount of ammonium converted (Figure 5, 4A, 4B) while nitrite and nitrate conversions were constant. The increased *R_NiAm* and *R_NaAm* ratios could be taken as an indication of a disturbed metabolism (and thus of concern), but could also be an effect of release of ammonium adsorbed or precipitated as e.g. struvite on the granules during the exposure. Since after the exposure to nitrite (in the absence of ammonium) the ratios (Figure 5, 3C) as well as the amount of ammonium converted (Figure 5, 4C) are in agreement with theoretical values disturbance of the metabolism is unlikely.

An assay (in triplicate) conducted to test the extent of ammonium sorption, confirmed that during 24 hours anoxic exposure in presence of 1 g N L^{-1} ammonium the granules had an ammonium sorption capacity of 14 to 16 mg N g VSS^{-1} (data not shown). Also Bassin et al. (2011) reported ammonium sorption capability of anammox granular sludge.

Due to ammonium sorption (either inside the cells, adsorbed to the EPS matrix or trapped in minerals such as struvite), the use of conversion ratios based on ammonium to characterise anammox activity might be biased. The conversion ratio of nitrate produced on nitrite consumed (*R_NaNi*) thus serves as a better indicator for the active growth of anammox bacteria (see Figure 9). Using the stoichiometry from Strous et al. (1998) *R_NaNi* would amount to 0.20. Note that in complex systems some of the produced nitrate can be converted by denitrification to nitrite or ammonium by regular heterotrophic bacteria or even certain type of anammox bacteria (Kartal et al., 2007a).

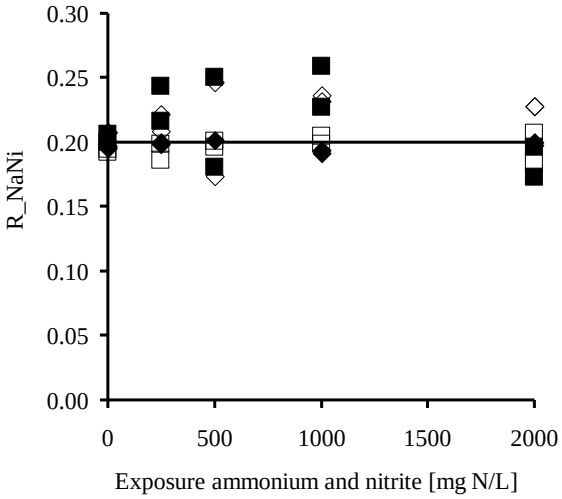

Figure 9. Conversion ratio of nitrate produced on nitrite consumed (R_NaNi) during manometric test after exposure for 1h (■ and ♦) and 24h (□ and ◊) to different conc. of ammonium and nitrite in anoxic (♦ and ◊) and aerobic (■ and □) conditions; the continuous line represents R_NaNi for anammox bacteria (Strous et al., 1998).

7) Implications for reactor operation

The results reported in this work show the robustness of the anammox process to inhibition by nitrite as well as to oxygen and that sufficient operational flexibility exists to prevent long term detrimental effects of short-term exposure. High nitrite concentrations may accumulate in anammox bioreactors due to inorganic carbon limitation or failure of the previous process in a treatment scheme, or an unbalance between ammonium oxidation and anammox activity. To minimize the loss in anammox activity operational measures should aim for lowering the reactor nitrite concentration as fast as possible (exposure time has a greater impact than concentration levels). This can be established by diluting the reactor supernatant with nitrite-free medium (usually influent) and this is therefore likely to be an effective strategy as already described by Wilsenach (2006).

From this study as well as from literature data (Table 3) biofilm and granular sludge systems seem to be more protected from inhibition by nitrite. The

tolerance to nitrite enables stable reactor operations at relatively high nitrite concentrations (85 mg N L^{-1}, van der Star et al., 2007) in biofilm systems.

Conclusions

Despite earlier reports on potential nitrite toxicity for the anammox process this study shows that inhibition levels of nitrite are rather high (IC_{50} of 0.4 g N L^{-1}), and that biomass relatively quickly (and totally) recovers from high nitrite concentrations. All our experiments showed that increasing exposure times to high nitrite concentration increased the inhibition. In several cases where high nitrite is reported as a cause of activity loss, it might well be that activity loss has *resulted* in the high nitrite concentrations rather than *causing* them. It was also again confirmed that anammox bacteria can well resist aerated periods. Nitrite rather then nitrous acid was observed as the inhibiting compound. Overall our results further underline that the anammox process can be a stable process not prone to temporarily adverse effects of oxygen and nitrite in the reactors.

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Chapter 5

Effect of temperature change on anammox activity

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Abstract

Autotrophic nitrogen removal appears as a prerequisite for the implementation of energy autarchic municipal wastewater treatment plants. Whilst the application of anammox related technologies in the side-stream is at present state of the art, the feasibility of this energy-efficient process in main-stream conditions is still under investigation. Lower operating temperatures and ammonium concentrations, together with a demand for high and stable nitrogen removal efficiency, represent the main challenges to overcome for this appealing new frontier of the waste water treatment field. In this study we report the short-term effect of temperature on the maximum biomass specific activity of anaerobic ammonium oxidizing (anammox) bacteria as evaluated by means of batch tests. The experiments were performed on anammox biomass sampled from two full-scale reactors and two lab-scale reactors, all characterized by different reactor configurations and operating conditions. The results indicate that for the anammox conversion the temperature dependency cannot be accurately modeled by one single Arrhenius coefficient (i.e. Θ) as typically applied for other biological processes. The temperature effect is increasing at lower temperatures. Adaptation of anammox bacteria after long term cultivation at 20 and 10°C was observed. Implications for modeling and process design are finally discussed.

Introduction

To date, most of the autotrophic nitrogen removal systems reported in literature were operated at temperatures exceeding 25 °C and influent nitrogen concentrations over 0.1 g-N L^{-1} (van Hulle et al., 2010). The application at lower temperatures and lower nitrogen concentrations would allow to extend the application potential of anaerobic ammonium oxidizing (anammox) processes to municipal sewage treatment opening new possible scenarios in designing energy producing wastewater treatment plants (Jetten et al., 1997; Kartal et al.,

2010a; Siegrist et al., 2008). In order to maximize energy recovery from municipal wastewater removal of the organic carbon as a first step would be most advantageous. This first step could then either be a UASB reactor at tropical climates (Aiyuk et al., 2006) or a concentration step at lower temperature regions. This concentration can be performed by physical (e.g. sieving), chemical (e.g. precipitation) or biological (Versprille et al., 1984) methods, or combinations thereof. The concentrated sludge can then subsequently be digested anaerobically to methane containing biogas. The remaining liquid contains ammonium that can be removed in an autotrophic process based on anammox (Abma et al., 2010, Jetten et al., 1997; Joss et al., 2009; Kartal et al., 2010a; van der Star et al., 2007, Wett, 2007). To facilitate autotrophic nitrogen removal on pretreated sewage a process based on prevention of growth of nitrite oxidizing bacteria (NOB) and retaining anaerobic ammonium oxidation bacteria in the system in symbiosis with ammonium oxidizing bacteria (AOB) has to be developed. The main challenge for applying anammox in the main-stream of a wastewater treatment plant (WWTP) is to achieve a high rate process with good biomass retention and low effluent nitrogen concentration at low water temperatures. A very large decrease in specific anammox activity has been reported after lowering the temperature of reactors operated at warm temperatures (>25 °C) (Dosta et al., 2008; Hu et al., 2013b; Isaka et al., 2008; Lotti et al., 2014a,b [**Ch.6,7**]; Vázquez-Padín et al., 2011). Despite this decrease in activity several laboratory studies have reported 1-stage partial nitritation/anammox at lower temperatures (≤25 °C) (de Clippeleir et al., 2011; de Graaf et al., 2011; Dosta et al., 2008; Hendrickx et al., 2012; Hu et al., 2013b; Isaka et al., 2008; Lotti et al., 2014a,b [**Ch.6,7**]; Vázquez-Padín et al., 2011; Winkler et al., 2011). The effect of temperature on the anammox activity is therefore a topic of interest, as well as their response to temperature fluctuations after long-term cultivation in colder conditions (i.e. <20°C). In view of the use of anammox biomass from side-stream processes (i.e. >25°C) to bio-augment reactors at lower operational temperatures, it is also important to evaluate the temperature effect on biomass grown under different operating conditions.

In this work the short-term effect of temperature on the maximum biomass specific activity of anammox bacteria was evaluated by means of batch tests. Anammox biomass tested was obtained from two full-scale reactors and two

lab-scale reactors, all characterized by different reactor configurations and operating conditions. The results obtained are compared to literature data and the practical implications for modeling and for application of anammox for sewage treatment are discussed.

Materials and Methods

Batch test

The maximum specific anammox activity (MSAA, expressed as gN_2-N $gVSS^{-1}$ d^{-1}) measuring procedure was applied according to Lotti et al. (2012b [**Ch.4**]). All experiments were conducted in three replicates. The batch tests conducted at different temperature of incubation were started in parallel the same day the biomass was sampled from the original reactor. The anammox activity for each condition tested was evaluated during successive feedings (injection of substrates) until a stable MSAA was measured in two consecutive tests (less than 5% difference). Two to three consecutive batch tests, conducted in a time frame of one to two days, were needed before stabilization. Before the experiment was conducted, biomass was sampled from the original reactor operating in steady state conditions and maintained at the tested temperature in presence of substrates (i.e. ammonium and nitrite) for 1 day. The amount of substrates converted during this phase resulted in a negligible (<1%) increase in the biomass concentration. The effect of temperature was evaluated by measuring the maximum specific anammox activity at different temperature. From the analysis of these data the apparent activation energy (E_a, kJ mol^{-1}) was calculated as the slope of the regression line of the Arrhenius plot. In engineering practice however, the use of the temperature coefficient Θ (theta, K^{-1}) is usually preferred. For sake of clarity, in equation 1 we report the relation between Θ and E_a.

$$\theta = \frac{E_a}{R \cdot T_1 \cdot T_2} \qquad (1)$$

Where R is the ideal gas constant and T_1 and T_2 are the temperatures (expressed in Kelvin) at which the kinetic is measured.

The bioreactor configuration, temperature of operation and volume of the four reactors from where anammox biomass was sampled are reported in Table I together with the biomass aggregation status and the type of process performed. Worth to mention that the full-scale airlift reactor in Olburgen (Abma et al., 2010) and the lab-scale sequencing batch airlift reactor (SBAR) are 1-stage partial nitritation/anammox systems: the process is operated aerobically (0.5-1.5 mg-O_2 L^{-1}) and ammonium oxidizing bacteria (AOB) are therefore abundant in the system. The full-scale side-stream anammox internal circulation (IC) reactor of Dokhaven WWTP in Rotterdam (van der Star et al., 2007) and the lab-scale membrane bioreactor (MBR, Lotti et al., 2014c [**Ch.2**]) are operated anaerobically with nitrite and ammonium containing influent. The SBAR was inoculated with biomass from the airlift reactor in Olburgen and was operated at 20°C for 8 months and at 10°C for 6 months before the tests were conducted (see Lotti et al., 2014b [**Ch.6**]). The MBR was inoculated with biomass from the full-scale anammox IC reactor in Rotterdam. The biomass samples from the full-scale reactors of Rotterdam and Olburgen, suspended in the original supernatant with the addition of 250 mg-N L^{-1} of $NaNO_3$, were transported to our laboratories within one and two hours, respectively.

Table 1. Process conditions of the reactors from which the biomass used during the experimentation was originating.

Reactor	Volume	Temperature	Biomass aggregation status	Process type
	m^3	°C		
Airlift reactor	600	30-35	Granular	Nitritation/Anammox
IC reactor	70	30-35	Granular	Anammox
MBR	0.01	30	Free-cell suspension	Anammox
SBAR@20	0.003	20	Granular	Nitritation/Anammox
SBAR@10	0.003	10	Granular	Nitritation/Anammox

Biomass characterization

Fluorescence in situ hybridization (FISH) analysis was conducted as described by Hu et al. (2013b). From each microbial community cultivated in the different reactors, the dominant anammox strain was identified by FISH: stain with strain-specific oligonucleotide probes. The degree of enrichment of the anammox population was evaluated by microscopy as described elsewhere (Lotti et al., 2014c [**Ch.2**]).

Results

Effect of temperature

The biomass specific anammox maximum activity (MSAA, g-N_2-N g-VSS^{-1} d^{-1}) was measured by means of manometric batch tests. The tests were conducted on anammox biomass sampled from four distinct reactors operated at different temperature and in different conditions (Table I). The results of the batch tests are reported in Figure 1 in terms of MSAA-values (*left*) and after normalization at 30°C (*right*). Each data point represents the average of the three replicates. The standard deviation was below 10% of the average value. The MSAA values reported in this study were the one observed after recovery from the temperature shock effect (Hwang and Oleszkievicz, 2007) that was observed during the first 24-48 hours after the biomass was sampled from the original reactor. The difference in specific activity observed for the different biomass samples (Fig.1, *left*) can be attributed to difference in anammox enrichment level and operative conditions. For instance the highest MSAA at 30°C was measured for the biomass originating from the MBR where indeed the anammox enrichment level was higher than 95% (Lotti et al., 2014c [**Ch.2**]). Sludge samples originating from reactors with a combined nitritation anammox process are characterized by lower MSAA values at all temperatures due to the large fraction of ammonium oxidizing biomass in the sludge, resulting in a decreased fraction of anammox bacteria.

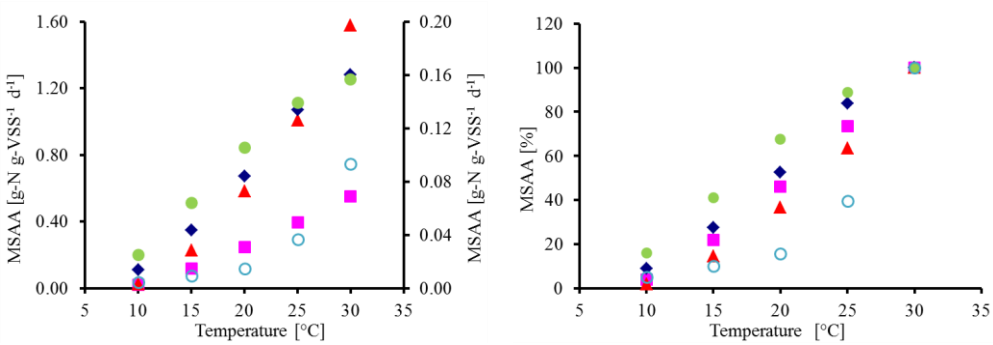

Figure 1. ***Left:*** Biomass specific anammox activity (MSAA) measured at different temperatures for the biomass originating from the IC reactor in Dokhaven, from the MBR (left axis), from the Airlift reactor in Olburgen and from the SBAR operating at 20 (SBAR@20) and 10°C (SBAR@10) (right axis). ***Right:*** MSAA-values normalized at 30°C. IC reactor in Dokhaven (squares), MBR (triangles), Airlift reactor in Olburgen (diamonds), SBAR@20 (closed circles), SBAR@10 (open circles).

The temperature effect on the anammox activity was firstly evaluated by plotting MSAA results in a conventional Arrhenius plot. The coefficient of determination (R^2) of the linear regression of the data set for each biomass tested was below 0.9 suggesting that the effect of temperature was not satisfactory described by a singular E_a (root mean squared error, RMSE, higher than 0.22). As example, in Figure 2 (*left*) the Arrhenius plot relative to the biomass from the full-scale 1-stage partial nitritation/anammox system in Olburgen shows different slope of the linear regression (thus different E_a which is equal to the additive inverse of the slope times the ideal gas constant) for different temperature intervals (30-20, 20-10°C) and each of these slopes falls outside the confidence interval (slope±2·standard error) of the slope of the linear regression of the whole temperature interval 30-10°C. The activation energy data are therefore presented for several temperature intervals as shown in Figure 2 (*right*). The E_a-values and relative standard errors depicted in the bar chart of Figure 2 can be found in Table 2.

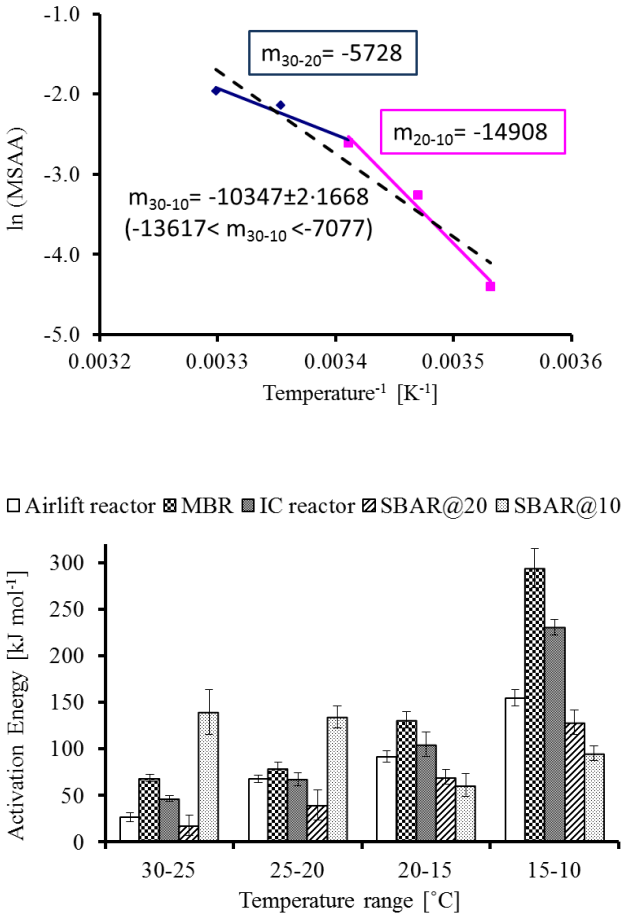

Figure 2. ***Top:*** Arrhenius plot for the anammox conversion by sludge from the full-scale 1-stage partial nitritation/anammox airlift reactor in Olburgen. ***Bottom:*** activation energy (Ea) relative to the different biomass studied and the different temperature intervals; the error bars represent the standard errors.

For all the biomass tested the effect of temperature change increases (higher E_a-values) with decreasing temperatures (Fig.2, *right*). In other words the anammox activity seems to decrease more upon a five degrees temperature decrease at lower temperatures, resulting in a sharper increase of E_a. The only exception is represented by the anammox biomass cultivated at 10°C that is characterized by a lower E_a values at temperatures below 20°C. Overall the free-cell suspension from the MBR exhibited stronger effect of temperature than granular biomass.

Table 2. Activation energies (E_a, kJ mol^{-1}) and relative standard errors for different temperature ranges and for biomass samples originating from different reactors as calculated from maximum biomass specific anammox activity (MSAA) measured during batch tests.

Temperature range (°C)	Origin of the biomass				
	Airlift reactor	MBR	IC reactor	SBAR@20	SBAR@10
30-25	26.6±5.1	68.6±4.2	46.6±2.7	17.7±11.0	139.4±24.2
25-20	67.7±4.0	79.8±5.8	67.6±7.1	40.0±16.0	134.3±12.1
20-15	91.8±6.1	131.3±9.3	104.9±13.3	69.9±8.2	61.1±12.3
15-10	154.7±8.9	293.7±20.8	230.5±8.3	128.8±13.4	95.4±7.9

Biomass characterization

The anammox bacteria investigated in this study all bound to the FISH probe specific for *Candidatus* Brocadia. In particular, based on FISH probes, the dominant anammox strain was *Candidatus* Brocadia Fulgida (Kartal et al., 2008) for the full-scale IC reactor of the Dokhaven WWTP, the MBR and the SBAR, while it was *Candidatus* Brocadia Sinica (Hu et al., 2010) for the full-scale airlift reactor in Olburgen.

Discussion

E_a values and impact on modeling

In this study, for the biomass cultivated at temperature ≥20°C (Table I), the temperature dependency of the MSAA as expressed in the E_a increased at lower temperatures (10-20°C) compared to higher temperatures (20-30°C) (Figure 2 and Table AI).

Also Isaka and coauthors (2008) found similar behavior with estimated E_a-values of 93-94 and 33 kJ mol^{-1} for temperature ranges of 6-28 and 28-37°C, respectively. For anammox bacteria growing at low temperature Dalsgaard and Thamdrup (2002) and Rysgaard et al. (2004) reported values of 61 (in the range 6.5-37°C) and 51 kJ mol^{-1} (in the range -2-13°C), respectively, for anammox bacteria from marine sediments. From literature results appears a clear difference in terms of optimal temperature range for marine (Dalsgaard & Thamdrup, 2002; Rysgaard et al., 2004) and fresh water anammox bacteria (Dosta et al., 2008; Hendrickx et al., 2012; Isaka et al., 2008; Oshiki et al., 2011; Strous et al., 1999a). The effect of temperature was studied in literature for different fresh water anammox organisms and for different temperature ranges: E_a of 70 kJ mol^{-1} (in the range 20-43°C) for *Candidatus* Brocadia anammoxidans (Strous et al., 1999a), 63 kJ mol^{-1} (in the range 10-40°C) for *Candidatus* Kuenenia Stuttgartiensis (Dosta et al., 2008). A decrease in temperature optimum for anammox bacteria from 35 to 25°C was reported by Hu et al. (2013b) after long-term cultivation at 12°C. In our study instead, even for the biomass cultivated at 10°C, an increase in incubation temperature always resulted in an increase in MSAA, suggesting that the optimum temperature was equal or higher than the maximum temperature tested (i.e. ≥30°C, Figure 1).

Excluding the case of anammox bacteria cultivated at 10°C, the reported results suggest that cultivation in partial nitritation/anammox systems (airlift reactor and SBAR@20) provide more resilience to temperature changes than cultivation in absence of oxygen (IC reactor and MBR), with average E_a-values in the temperature range 15-30°C of 52.3 kJ mol^{-1} for the formers and of 83.1 kJ mol^{-1} for the latters (Figure 2 and Table AI). Anammox biomass cultivated in suspension exhibited the strongest effect of temperature (highest E_a) suggesting that growth in biofilm mitigate somehow the effect of changes in the incubation conditions (temperature in this case) similarly to what previously reported in the case of nitrite inhibition (Lotti et al., 2012b [**Ch.4**]).

Modelling studies on anammox-based processes reported in literature made use of the Arrhenius equation to take into account the temperature dependency of the specific growth rate (e.g. Hao et al., 2002b; Koch et al., 2000; Volcke et al., 2010). To the best of our knowledge every study used the activation energy of 70 kJ mol^{-1} reported by Strous et al. (1999) and 30°C as

reference temperature. Made 100 the activity at 30°C, this means an activity of 39 at 20°C and of 14 at 10°C. Following this approach would therefore lead to a systematic overestimation of the anammox activity at 10°C (see Figure 1, *right* for comparison). On the other hand for biomass cultivated at temperatures around 30°C and for the temperature range between 20 and 30°C, the Arrhenius equation with the E_a from Strous et al. (1999) was able to estimate the specific anammox activity observed in this study with an inaccuracy smaller than 30%. In fact, when estimating the E_a in the range 15-30°C for the biomass cultivated at temperatures around 30°C a value of 65.7 kJ mol^{-1} was obtained (30°C as reference temperature; mean square error estimation method used) (Figure 3). It seems therefore that temperatures close to 10°C represent a sort of turning point for the metabolism of anammox bacteria cultivated at mesophilic temperatures. Overall these observations suggest that the conventional method to consider the effect of dynamic variations of the operative temperature on anammox activity cannot be reliably used when exploring the low temperature range (<15°C).

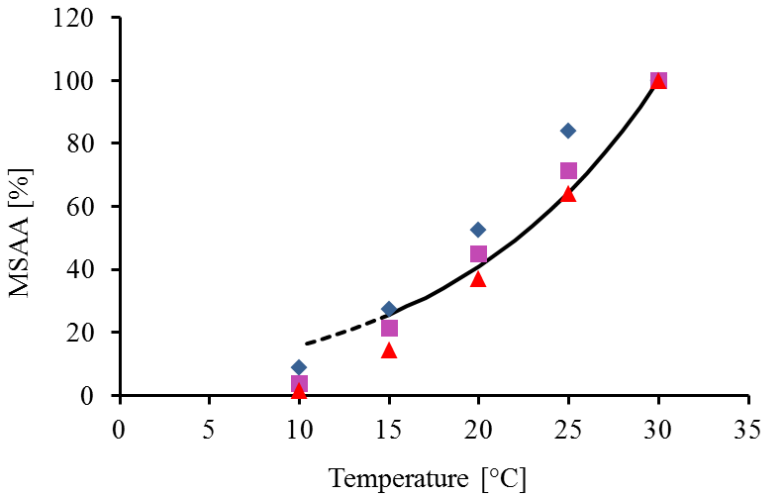

Figure 3. Biomass specific anammox activity (MSAA) measured at different temperatures for the biomass originating from the IC reactor in Dokhaven (squares), from the airlift reactor in Olburgen (diamonds) and from the MBR (triangles) after normalization at 30°C. The solid line (continuous for the temperature range 15-30°C and dashed for 10-15_C) represents the Arrhenius equation using 30°C as reference temperature and an activation energy (Ea) equal to 65.7 kJ mol^{-1}.

Adaptation to low temperatures

The least pronounced effect of temperature change below 20°C was registered for the biomass cultivated in the SBAR at 20 and 10°C. Since the SBAR was inoculated with granular biomass from the 1-stage partial nitritation-anammox airlift reactor in Olburgen and in both case the dominant organism belonged to the "Brocadia" type of anammox bacteria, adaptation might have played a role similarly to the suggestion by Dosta et al. (2008) and Hu et al. (2013b). The adaptation to low temperature of the anammox bacteria cultivated in the SBAR is shown by the E_a in the interval 10-20°C, which is lower than for the other biomass tested (Fig.2). Adaptation to lower temperatures then, is an additional factor to consider when modeling the anammox process at low temperatures, the extent of which is difficult to assess a priori. Furthermore, adaptation at lower temperatures likely depends on the duration of operations at a certain temperature, making the accurate modeling of a dynamic system even more challenging. Nevertheless, considering the long-term operations at 10 and 20°C of the SBAR where the biomass samples used in this study were originating from, the E_a-values in the low temperature range reported in Figure 2 and in Table AI might be considered representative for anammox bacteria adapted to low temperatures.

Impact on reactor operation

Considering the activation energy of the nitritation process reported in literature (e.g. 70 kJ mol^{-1}, Wiesmann, 1994), the results presented here highlight a different temperature dependency between ammonium oxidizing bacteria (AOB) and anammox in the low temperature range (especially below 15°C). As an example in Figure 4 the temperature dependency of the anammox biomass from airlift reactor in Olburgen as observed in this study, is compared to the temperature dependency of the AOB population in the same reactor as calculated with the Arrhenius law.

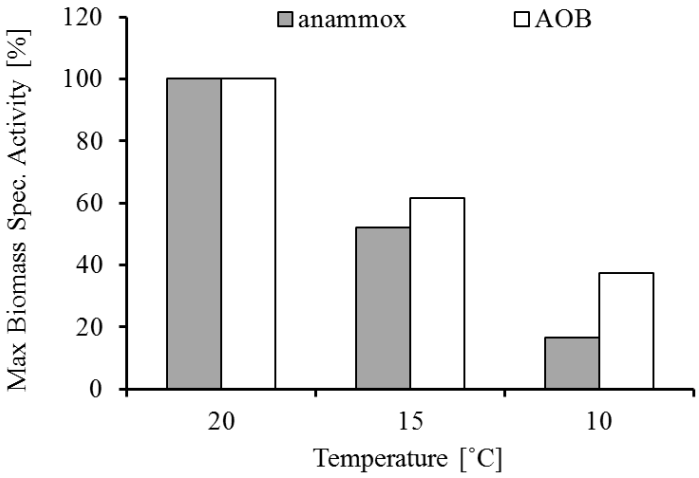

Figure 4. Maximum biomass specific activity (mg-NH_4-N g-VSS^{-1} d^{-1}) as measured for anammox biomass originating from the full-scale installation in Olburgen (grey bars) and as calculated using the Arrhenius equation for AOB (Ea=70 kJ mol^{-1}, Wiesmann, 1994) (white bars). Data are normalized for the activity at 20°C.

The different response of anammox and AOB to temperature variations becomes important for implementing a single stage partial nitritation/anammox system in the main-stream of the WWTP. In these systems the coupling of AOB and anammox conversion rates is considered a prerequisite in order to successfully implement the completely autotrophic nitrogen removal process. The AOB and anammox biomass concentration ratio in the sludge can be assumed to be constant, but with decreasing temperature the AOB become relatively more active. This suggests that the DO has to be adjusted to maintain a proper balance between nitrite production by AOB and nitrite uptake by anammox bacteria. If that balance is not maintained nitrite might easily accumulate hindering the suppression of the undesired production of nitrate by nitrite oxidizing bacteria (NOB) which could therefore result in the reduction of the nitrogen removal efficiency of the process. The acclimatization of the anammox bacteria to low temperatures would plead for a process choice that is not depending on the cultivation of anammox sludge in a side stream reactor at higher temperatures. This would potentially lead to a lower specific anammox activity at low temperatures and thereby more difficulty to balance AOB and anammox activities properly.

Conclusions

The effect of temperature on anammox activity cannot be described with one temperature coefficient for the range of 10-30°C. Also the history of the sludge impacts the observed temperature effect. Adapted anammox sludge has a higher specific rate at lower temperatures. The temperature effect is stronger for anammox then for AOB, this makes that process control to balance the activity of both microbial groups needs to be adaptive to changes in relative rates of the two processes in order to maintain overall a good nitrogen removal. Anammox sludge cultivated in an aerated partial nitritation/anammox process and/or in biofilm seemed to be less influenced by a decrease in temperature then anammox sludge grown under non aerated conditions and/or in suspension.

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Chapter 6

Simultaneous partial nitritation and anammox at low temperature with granular sludge

This chapter has been published as:

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Abstract

Autotrophic nitrogen removal in the main stream appears as a prerequisite for the implementation of energy autarchic wastewater treatment plants. To investigate autotrophic nitrogen removal a lab-scale gas-lift sequencing batch reactor with granular sludge was operated for more than 500 days. The reactor was operated at temperatures between 20 and 10°C on autotrophic medium with ammonium (60 and 160 mg-N L^{-1}) as only nitrogen compound at an HRT of 0.23-0.3d. The dissolved oxygen (DO) concentration was shown to be an effective control parameter for the suppression of the undesired nitratation process. DO control guaranteed the effective suppression of the nitratation both at 20 and 15°C, allowing nitrogen removal rates of 0.4 g-N_{Tot} L^{-1} d^{-1} at nitrogen removal efficiencies of 85-75 %. Prolonged operation at 10°C caused a slow but unrestrainable decrease in anammox activity and process efficiency. This study represents a proof of concept for the application of the autotrophic nitrogen removal in a single reactor with granular sludge at main stream conditions.

Introduction

The application of the anammox process for the treatment of concentrated and warm wastewaters characterized by temperatures exceeding 25 °C and influent nitrogen concentrations over 0.1 g-N L^{-1} (mainly the liquid fraction of the digestate) is nowadays part of the state of the art (van Hulle et al., 2010). The application at lower temperatures and lower nitrogen concentrations would allow to extend the application potential of anammox-related processes to municipal sewage treatment opening new possible scenarios in designing energy producing wastewater treatment plants (Jetten et al., 1997; Kartal et al., 2010a; Siegrist et al., 2008). In order to maximize energy recovery from municipal wastewater removal of the organic carbon as a first step would be most advantageous. This first step could either be a UASB reactor at tropical

climates (Aiyuk et al., 2006) or a concentration step at lower temperature regions. This concentration can be performed by physical (e.g. sieving), chemical (e.g. precipitation) or biological (Versprille et al., 1984) methods, or combinations thereof. The concentrated sludge can then subsequently be digested anaerobically resulting in methane containing biogas. The remaining liquid contains ammonium that can be removed in an autotrophic process based on anammox (Abma et al., 2010, Jetten et al., 1997; Joss et al., 2009; Kartal et al., 2010a; van der Star et al., 2007, Wett, 2007). To facilitate autotrophic nitrogen removal on pretreated sewage a process based on prevention of growth of nitrite oxidizing bacteria (NOB) and retaining anaerobic ammonium oxidation (anammox) bacteria in the system in symbiosis with ammonium oxidizing bacteria (AOB) has to be developed. The main challenge for applying anammox in the water line of a WWTP is to achieve a high rate process with good biomass retention and low effluent nitrogen concentration at low water temperatures. A very large decrease in specific anammox activity was reported after lowering the temperature of reactors operated at 30-32 °C (Dosta et al., 2008; Isaka et al., 2008; Vazquez-Padin et al., 2011). Despite this decrease in activity several laboratory studies have reported 1-stage partial nitritation/anammox at lower temperatures (≤25 °C) and reasonable conversion rates (de Clippeleir et al., 2013a; Szatkowska et al., 2007; Vazquez-Padin et al., 2011; Winkler et al., 2011; Hu et al., 2013b; Lotti et al., 2014a [**Ch.7**]). In natural ecosystems such as Northern European soils and marine sediments, anammox bacteria thrive at low temperatures (<10 °C) and very low ammonium concentrations (μM range) (Hu et al., 2011; Lam and Kuypers 2011; van de Vossenberg et al., 2008), indicating that there is no fundamental limitation for the anammox process to develop under municipal wastewater conditions. This putative capability was recently confirmed by the study of Lotti et al. (2014a [**Ch.7**]) where anammox bacteria were shown to grow under anoxic conditions in a lab-scale fluidized bed reactor fed with pretreated municipal wastewater.

This study aimed at demonstrating the feasibility of granular sludge-anammox based single stage (aerated system where both AOB and anammox bacteria coexist) autotrophic nitrogen removal on lab-scale at low temperature. A sequencing batch granular sludge based lab-scale reactor (2.7 L) was fed with synthetic medium at conditions relevant for main-stream

applications. The ammonium concentration in the reactor was during regular operation always below 70 mg-N L^{-1} and nitrite below 30 mg-N L^{-1}. A closely monitored granular sludge process operated in an air-lift sequencing batch reactor (SBAR) was chosen with the objective to achieve adequate biomass retention and high volumetric N-conversion rates as well as to investigate the interactions among different cohabiting bacterial populations. The reactor was operated at decreasing temperatures of 20, 15 and 10°C. The results obtained during 18 months of operation of the lab-scale reactor are presented.

Material and methods

Long term reactor operation

Experiments were conducted in a lab-scale air-lift reactor (working volume, 2.7 L) with granular biomass operated in a sequencing fed-batch mode (SBAR, sequencing batch air-lift reactor). The reactor was inoculated with granular biomass capable of autotrophic nitrogen removal enriched at room temperature during a previous study (Winkler et al., 2012b). The experimental period described in this study was preceded by a long start-up in which the reactor was operated at room temperature (22±3°C) and fed with ammonium as the only nitrogen source. After eight months of operation at room temperature, the reactor was temperature controlled using a thermostatic bath connected to the double wall of the reactor (referred to as experimental day one). The reactor was operated in five phases as reported in Table 1.

The exchange ratio ($V_{Effluent}/V_{Reactor}$) was fixed at 0.56. The hydraulic retention time (HRT) was fixed at 7.2h during phase I-IV and it was decreased to 5.4h in phase V decreasing the cycle length from 4 to 3 hours. The total cycle time was 4 hours during the first 483 days and 3 hours for the last two months of the experimental period. The 4-hours cycle consisted of one hour of *aerobic feeding phase*, about two hours of *aerobic reaction phase*, one hour of *anoxic reaction phase* (DO setpoint equal to zero), 4min of settling phase and 5min of decant phase. In the 3-hours cycle the *aerobic* and *anoxic reaction phases* were shortened to one hour and 51 minutes, respectively. In the 4-hours (3-hours) cycle the ratio between *aerobic* and *anoxic* time was 2.85:1 (2.4:1), resulting in the cycle scheme reported in Figure 1. The settling time of four minutes was

chosen such that only particles with a settling velocity larger than 12 m h^{-1} were effectively retained in the reactor.

Table 1. Description of the different operational phases throughout the experimentation.

Phase	Period	Temperature	$NH_{4\,Influent}^{+}$	Cycle length	Volumetric N-Load
	day	°C	mg-N L^{-1}	hour	g-NH_4^+-N L^{-1} d^{-1}
I	1-110	20	160	4	0.53
II	111-425	15	160	4	0.53
III	426-440	15 ÷ 10	160	4	0.53
IV	440-483	10	130-60	4	0.43-0.27
V	484-541	10	60	3	0.27

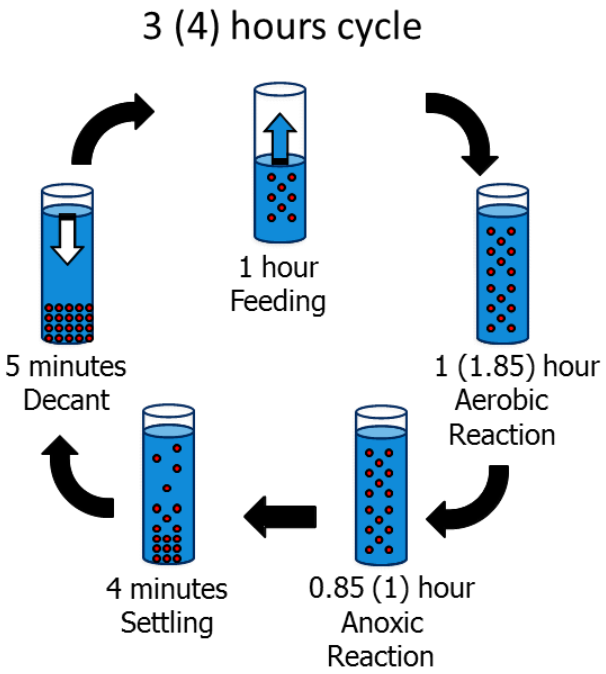

Figure 1. Schematic view of the operations and their duration for the 3-hours and 4-hours cycle.

Analytical procedures

The CO_2 concentration in the off-gas was measured online with an infrared CO_2 analyser. Dry weight (TSS), ash content and volatile suspended solids (VSS, dry weight minus ash content) were determined according to standard methods (APHA 2005). Bed volume of the settled bed was determined by reading the height of the biomass bed directly from the scale on the reactor at the end of a settling and effluent withdrawal period (in total 4 or 5 minutes settling time). Solid retention time (SRT) was determined performing a mass balance over the solid phase: the biomass in the reactor and the effluent biomass, which consists of granules and eroded material from the granule surface. It should be noted that a solid mass balance results in determination of an average SRT, and therefore underestimates the sludge age in the inner part of the granule. Concentrations of ammonium, nitrite and nitrate were measured regularly with Dr.Lange test kits in filtered influent/effluent samples and in samples directly taken every 10 or 20 minutes from the reactor during one cycle (cycle measurement). From cycle measurement data, the actual volumetric activity of AOB, NOB and anammox bacteria was calculated as described elsewhere (Vázquez-Padín et al., 2011) and expressed as ammonium converted, nitrate produced and total nitrogen removed, respectively, per unit of reactor volume and time (*Rate*, mg-N L^{-1} d^{-1}). In the calculation of the volumetric activity of each microbial group, only the appropriate part of the cycle was considered: the aerobic phase for AOB and NOB and the anoxic phase for anammox volumetric activities, respectively. Nitrite and nitrate concentrations were regularly checked with Merck indicator papers. Aerobic and anoxic batch tests were conducted inside the reactor in order to evaluate the maximum volumetric conversion potential of AOB/NOB and anammox, respectively ($Rate^{max}$, mg-N L^{-1} d^{-1}). The biomass specific maximum conversion capacity was calculated dividing $Rate^{max}$ by the total biomass concentration present in the reactor at the time the batch test was conducted (q^{max}, mg-N g-VSS^{-1} d^{-1}). When the batch tests were conducted the reactor volume was 2.7 L and DO was controlled either at zero (anoxic test) or at 70-90% of the oxygen solubilisation level (aerobic) by temporarily imposing the appropriate DO set-point. Excess of substrate was ensured by manually injecting concentrated solutions of ammonium sulphate and sodium nitrite. Changes in morphology of the granules were followed by image analyses (IA), with a Lexmark Optra

image analysis system. Also pictures were taken with this system to follow the development of the granules. When the reactor was operated at 15°C, N_2O was measured on-line during several cycles by an on-line Servomex 4900 infrared gas analyzer. Together with the online data of gas flow (air and N_2) entering and leaving the system (by MFCs), a mass balance could be established and the N_2O emission rate was calculated in terms of percentage of the N-load and of the total nitrogen removal rate.

Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) was performed on the nitritation-anammox SBR as described previously (Schmid et al., 2000). Oligonucleotide probes used in this study are listed in Table 2. DAPI (4',6-diamidino-2-phenylindole) was used to stain the whole community DNA.

Table 2. Oligonucleotide probes and target microorganisms as well as references used in this study. Probe sequence are reported in Loy et al. (2003).

Probe	Specificity	Reference
Amx 820	Kuenenia and Brocadia-like anammox bacteria	Schmid et al., 2001
Bfu 613	*Candidatus* Brocadia Fulgida	Kartal et al., 2008
NEU 653	AOB	Wagner et al., 1995
NTSPA 712	NOB	Daims et al., 2000
NIT 1035	NOB	Juretschko et al., 1998
EUB 338 I	Most bacteria	Amann et al., 1990b
EUB 338 II	Most bacteria	Amann et al., 1990b
EUB 338 III	Verrucomicrobiales	Daims et al. 1999

Results

Long term operation

The reactor was operated in five phases over a time period of 540 days. Each phase is characterized by the temperature of operation and the ammonium load applied (Table 1). For a comprehensive view of the operational performance throughout the experimental period the reader is referred to Figure 2. This graph shows the operational parameters such as N-Load, DO and temperature as well as effluent concentrations of ammonium, nitrite and nitrate. In the same figure the development in time of the calculated volumetric conversion rates as well as the resultant ammonium and total nitrogen removal efficiencies are reported. The solids retention time (SRT) was on average 150 days throughout the experimentation. The biomass concentration was 3.6±0.3 g-VSS L^{-1} whilst the mass of VSS on TSS ratio (g-VSS g-TSS^{-1}) was on average 0.9. AOB and NOB inhibition by free ammonia (NH_3) and free nitrous acid (HNO_2) (Anthonisen et al., 1976) could be excluded throughout the whole experimental period, since their concentrations were calculated to be below 0.5 and 0.02 mg-N L^{-1}, respectively. The volumetric conversion rates registered during periods of steady state operations (in terms of soluble compounds) are displayed in Table 3 and are based on the difference between the total soluble nitrogen compounds in the influent and effluent of the reactor. Important operational parameters are also reported in the same table together with the ratio between ammonium converted and nitrate produced during one cycle (NO_3^-/NH_4^+ ratio). Considering a fixed anammox stoichiometry (i.e. nitrate produced per ammonium removed), the NO_3^-/NH_4^+ ratio can be directly related with NOB activity and can be used to monitor the relevance of nitratation in the autotrophic nitrogen removal process (Lotti et al., 2014c [**Ch.2**]). Note that in a completely autotrophic system, as the one described in this work, complete coupling of AOB and anammox activity together with successful suppression of nitratation by NOB, would result in a NO_3^-/NH_4^+ ratio equal to 0.11.

Table 3. Ammonium conversion ($Rate_{NH_4^+}$) and total nitrogen removal rate ($Rate_{Tot-N}$) and corresponding efficiencies as well as operational parameters during the different phases of the reactor run.

Phase	Period	Temp.	$NH_{4\,Inf.}^+$	$NH_{4\,Eff.}^+$	DO	NO_3^-/NH_4^+ ratio
	Days	°C	mg-N L^{-1}	mg-N L^{-1}	mg-O_2 L^{-1}	-
I	83-107	20	160	5.0	1.0	0.11
II	380-411	15	162	18.1	2.1	0.12
IV	441-450	10	130	23.8	1.9	0.20
IV	455-460	10	130	41.2	1.2	0.11
IV	464-476	10	102	29.3	0.9	0.11
V	492-498	10	61	27.1	0.7	0.17
V	522-534	10	61	18.0	1.2	0.43

Phase	Period	NH_4^+ conversion rate	Tot-N removal rate	NH_4^+ removal efficiency	Tot-N removal efficiency
	Days	g-N L^{-1} d^{-1}	g- N_{Tot} L^{-1} d^{-1}	%	%
I	83-107	0.50	0.44	97	86
II	380-411	0.48	0.40	89	73
IV	441-450	0.35	0.21	82	48
IV	455-460	0.30	0.19	68	45
IV	464-476	0.24	0.15	71	43
V	492-498	0.15	0.10	56	39
V	522-534	0.19	0.09	70	36

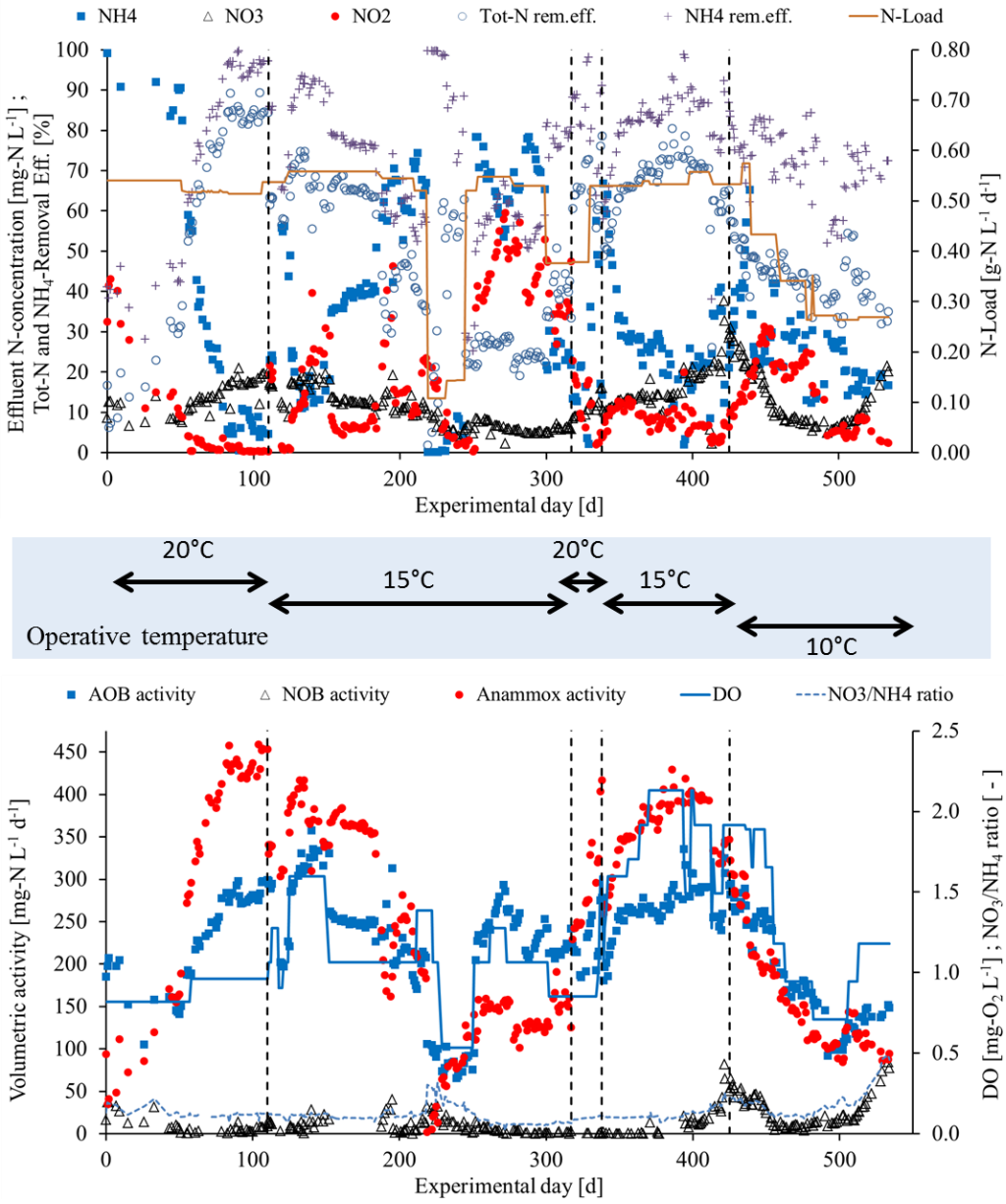
NH4
NO3
NO2
Tot-N rem.eff.
NH4 rem.eff.
N-Load
Effluent N-concentration [mg-N L-1] ; Tot-N and NH4-Removal Eff. [%]
N-Load [g-N L-1 d-1]
Experimental day [d]
20°C
15°C
20°C
15°C
10°C
Operative temperature
AOB activity
NOB activity
Anammox activity
DO
NO3/NH4 ratio
Volumetric activity [mg-N L-1 d-1]
DO [mg-O2 L-1] ; NO3/NH4 ratio [-]
Experimental day [d]

Figure 2. ***Top Graph*****:** Effluent soluble N-compounds concentrations as well as total nitrogen and ammonium removal efficiencies (*left axis*); volumetric ammonium nitrogen load (*right axis*); ***Bottom Graph***: Calculated bacterial activities: expressed as ammonium removal rate (AOBs), nitrate production rate (NOBs), ammonium and nitrite removal rate (anammox) (*left axis*); dissolved oxygen (DO) maintained during the aerobic phases of the SBR-cycle, ratio between nitrate produced and ammonium consumed (NO_3^-/NH_4^+ ratio) (*right axis*). The vertical dashed lines indicate the days in which reactor temperature was changed; the temperature of operation is indicated in the blue box.

During the first 80 days of Phase I the volumetric nitrogen removal rate (NRR) increased exponentially and stabilized at 0.44 g-N_{Tot} L^{-1} d^{-1} during the last month of operation (83-107), before the reactor temperature was decreased from 20 to 15 °C. The short term effect of the decrease in temperature was a sharp decrease in anammox activity (NRR from 0.45 to 0.34 g-N_{Tot} L^{-1} d^{-1}), while AOB activity was hardly affected. This differential change in bacterial activities resulted in accumulation of ammonium and nitrite in the effluent up to about 20 mg-N L^{-1}. One week after decreasing the temperature the process stabilized. The reactor was then successfully operated for two months (days 120-183) in which nitrogen was removed at an average rate of 0.37 g-N_{Tot} L^{-1} d^{-1} with an efficiency of 66%. During days 188-195 excessive condensate in the gas recirculation circuit caused an undesired increase of the mixing gas flow rate: from about one to over two normal-L min^{-1}. The consequent excessive shear stress partly damaged the granular structure (granules disruption), resulting in destabilization of the process and lower removal rates. The sludge bed volume in this period decreased due to higher washout and higher compaction of the settled bed. During the period between days 180 and 340 experiments were conducted in the reactor aiming to investigate mutual competition among bacterial populations involved in the process (AOB, NOB and anammox) and nitrous oxide emissions during different operative conditions. During the same period operational and technical problems occurred which caused fluctuations in the reactor performance: undesired ammonium limitation (days 219-231); loss of 8% of the biomass due to uncontrolled effluent withdrawal (day 275).

When the reactor was operated at 15 °C with the aim of maximizing the process performance (days 340-411), an exponential increase in nitrogen removal rate was registered (days 340-380). The process was then stable for one month performing an average nitrogen removal rate of 0.40 g-N_{Tot} L^{-1} d^{-1}

(Table 3). On day 411 the reactor experienced another unintentional sharp increase of the mixing gas flow rate and part of the biomass (~5%) was removed with the effluent.

The reactor temperature was decreased stepwise since day 426 and was maintained stable at 10 °C since day 436 (Table 1). After initial stabilization (days 436-454, NRR ~ 0.20±0.01 g-N_{Tot} L^{-1} d^{-1}), the performance of the reactor worsened in time. Anammox activity, and consequently NRR, continuously decreased in time while nitrate production by NOB increased as shown by the NO_3^-/NH_4^+ ratio in Table 3. A test was conducted in which the reactor temperature was temporarily increased to 15°C (days 503-513). After the temperature was decreased again to 10°C, NOB activity rapidly increased with a constant trend that lasted until the end of the experimentation (Table 3 and Fig. 2).

In phases I, II and III the nitrogen load was maintained at 0.53 and then decreased in phases IV and V down to 0.27 g-NH_4^+-N L^{-1} d^{-1}, as described in Table 1. In two occasions the load was decreased for a short time by decreasing the ammonium concentration in the feeding medium in order to evaluate the impact of the medium nitrogen concentration on bacterial activities. Ammonium concentration in the feeding medium was reduced by 80% during days 219-244 and by 30% during days 300-329. The first load reduction (days 219-244) resulted in complete ammonium depletion during the first 90 minutes of the cycle operation. The absence of ammonium during the remaining two and a half hours of the cycle, caused starvation for AOB and anammox bacteria. This resulted in a sharp increase of NOB activity since the competition for oxygen (with AOB) and for nitrite (with anammox) was no longer present (Fig. 5). The second time the load was decreased (days 300-329), instead, ammonium was still present in the effluent as reported in Table 4. The lower ammonium concentration in the influent resulted in lower ammonium concentration during the whole cycle, but did not cause any appreciable alteration of the conversions catalyzed by the different microbial community members (Table 4).

Table 4. Effect of the decrease of the N-load on the overall conversion rates performed by the microbial community members. The test was conducted during operational Phase II (15°C).

Exp. Day	Volumetric N-load	$NH_{4\,Inf.}^{+}$	$NH_{4\,Eff.}^{+}$	AOB	NOB	anammox [a]
d	g-NH_4^+ L^{-1} d^{-1}	g-N L^{-1} d^{-1}	g-N L^{-1} d^{-1}	g-N L^{-1} d^{-1}	g-N L^{-1} d^{-1}	g-N L^{-1} d^{-1}
299	0.53	159	65.4	255.8	6.3	114.7
300	0.38	112	21.6	244.1	6.8	122.2

[a] Anammox activity expressed as the consumption of NH_4^+-N and NO_2^--N (mg N_2-N·L^{-1}·d^{-1}).

Cycle measurements and maximum conversion rates

Cycle measurements were conducted throughout the experimentation in order to closely evaluate production and consumption of nitrogen compounds throughout the different cycle phases (Fig. 1). Changes in nitrogen concentrations during one cycle of operation in phase I (20°C) and phase II (15°C) are depicted in Fig. 3 together with the dissolved oxygen concentration measured online. During the aerobic *feeding phase* nitrate concentration decreased due to a dilution effect, whilst nitrite dilution was compensated by AOB activity. During the aerobic reaction phase ammonium depletion is due to both AOB and anammox activity, whilst nitrate production is due to anammox and NOB activity. Finally in the anoxic reaction phase AOB and NOB were limited by oxygen availability and nitrite and ammonium depletion are therefore mainly due to anammox activity.

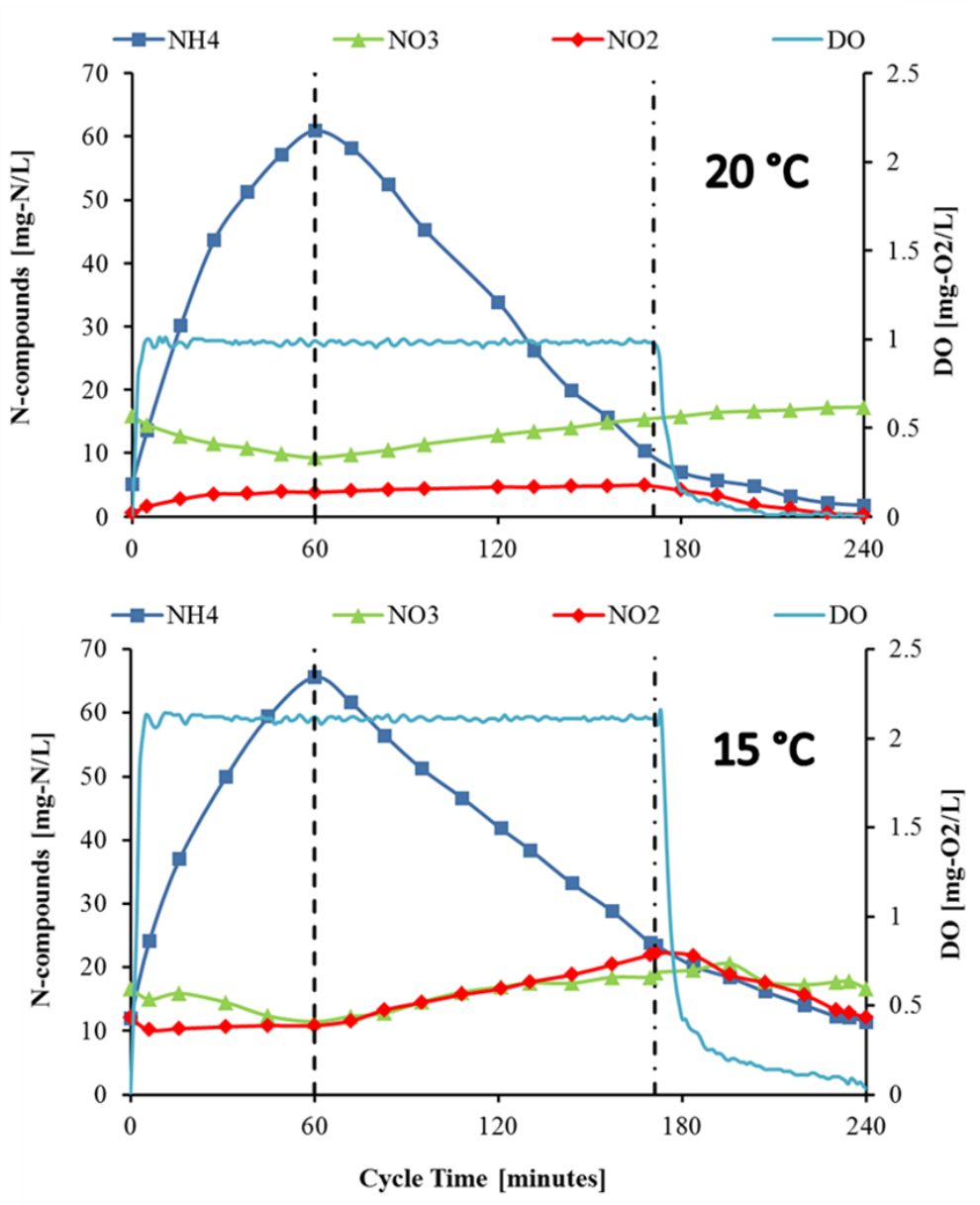

Figure 3. Cycle measurement conducted on day 100 (Phase I, *above*) and 385 (Phase II, below) when reactor was operating at 20 and 15°C, respectively. Vertical lines represent the end of feeding phase/beginning of aerobic reaction phase (dash) and the end of aerobic reaction phase/beginning of anoxic reaction phase (dash dot). During this test corresponding ammonium and total nitrogen removal efficiencies were respectively 98 and 87% (*above*) and 93 and 75% (*below*). The NO_3^-/NH_4^+ ratio was 0.11 in both tests.

Maximum activity tests were also conducted the same day or the day after cycle measurements, enabling comparison with the actual activity and therewith the extent the system was biomass limited. The development in time of the maximum conversion capacity of AOB, NOB and anammox, also provides insights in the development of the different microbial community members in time. Maximum activity tests were performed directly in the reactor adjusting the dissolved oxygen concentration: fully aerobic conditions (DO over 6 mg-O_2 L^{-1}) for AOBs and NOBs and anoxic conditions for anammox bacteria. Substrate concentrations were not limiting throughout the test: ammonium, nitrite and nitrate concentrations were higher than 20 mg-N L^{-1}. In Table 5 the maximum conversion rate catalyzed by AOB, NOB and anammox during batch tests (*Maximum volumetric conversion rate*) are compared to the activity measured during normal reactor operations (*Actual volumetric conversion rate*). Note that different units are used for different microbial processes: ammonium conversion rate for nitritation (mg-NH_4^+-N L^{-1} d^{-1}), nitrate production rate for nitratation (mg-NO_3^--N L^{-1} d^{-1}) and total nitrogen removal for the anammox process (mg-N_{Tot} L^{-1} d^{-1}).

In Phase I anammox operated under substrate limitation as shown in Table 5 by the difference between actual and maximum activity. When temperature was decreased to 15°C in phase II, the decrease in maximum activity was sharper for anammox than for AOB. This resulted in nitrite accumulation in the bulk and in the complete exploitation of anammox capacity (actual/maximum ratio close to 100%). When the reactor was operated stably with the aim of maximizing nitrogen removal, anammox grew in the system and their maximum volumetric activity increased from 127 on day 282 to 541 mg-N_{Tot} L^{-1} d^{-1} on day 418. Anammox was then operating under substrate limitation and the same held true for the successive experimental periods (phase III, IV and V). AOB and NOB activity was limited by oxygen throughout the experimental period. The limitation was stronger for NOB than for AOB during the whole reactor run except in Phase V.

Table 5. Comparison between Maximum (batch test) and actual volumetric conversion rate (reactor operations) catalyzed by AOB, NOB and anammox (*AMX*) and expressed as ammonium conversion, nitrate production and total nitrogen removal rate, respectively. (*n.d.* not determined)

			Maximum Volumetric Rate			Actual Volumetric Rate			Actual / Maximum		
Phase	**Exp. Day**	**Temp.**	**AOB**	**NOB**	**AMX**	**AOB**	**NOB**	**AMX**	**AOB**	**NOB**	**AMX**
	d	°C	mg-N L^{-1} d^{-1}	mg-N L^{-1} d^{-1}	mg-N_{Tot} L^{-1} d^{-1}	mg-N L^{-1} d^{-1}	mg-N L^{-1} d^{-1}	mg-N_{Tot} L^{-1} d^{-1}	%	%	%
I	56	20	1033	203	725	193	1	281	19	0	39
I	86	20	1207	120	n.d.	284	3	n.d.	24	2	n.d.
I	96	20	n.d.	n.d.	869	n.d.	n.d.	419	n.d.	n.d.	48
II	118	15	n.d.	n.d.	341	n.d.	n.d.	321	n.d.	n.d.	94
II	180	15	1330	11	n.d.	252	0	357	19	0	n.d.
II	182	15	1313	21	362	247	0	354	19	1	98
II	282	15	1161	41	127	260	5	123	22	12	97
II	316	15	n.d.	n.d.	164	n.d.	n.d.	160	n.d.	n.d.	98
II	418	15	956	206	541	244	33	319	26	16	59
III	433	12	799	439	311	274	54	269	34	12	86
IV	447	10	616	335	245	260	40	195	42	12	80
IV	471	10	590	221	223	173	10	141	29	5	63
V	510	15	n.d.	n.d.	215	n.d.	n.d.	142	n.d.	n.d.	66
V	516	10	n.d.	n.d.	170	n.d.	n.d.	104	n.d.	n.d.	61
V	547	10	800	511	99	136	99	43	17	19	44

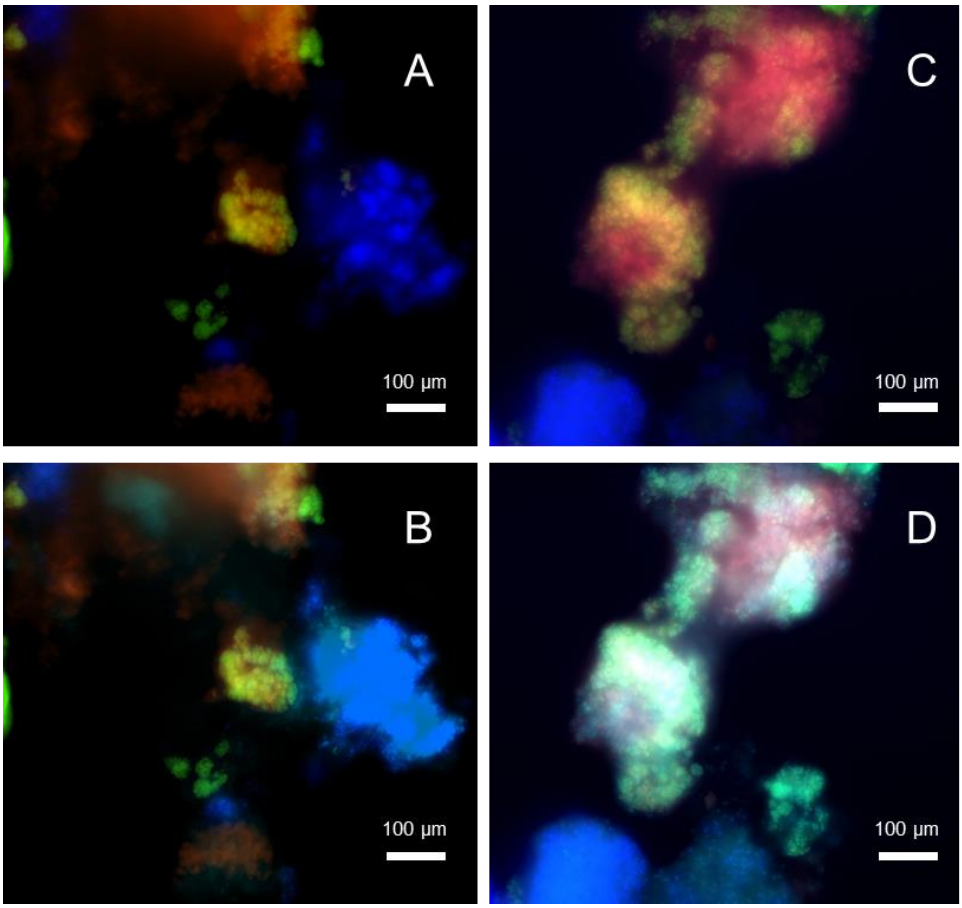

Figure 4. FISH analysis performed on pottered biomass samples. Probes used for pictures A and C: Blue: Anammox (Amx 820); Red: NOB (mix of NTSPA 712 and NIT 1035); Green: AOB (NEU 653). In pics B and D also DAPI staining was applied (Light blue). Pics A and B refer to biomass sampled on day 191, while C and D refer to day 265.

During normal reactor operation NOB activity was successfully suppressed by oxygen limitation during the first 400 days of operations (Table 5 and Fig. 2) even if they were always present in the sludge as shown by the positive maximum conversion rates in Table 5 and by FISH analysis in Figure 4. The maximum nitratation capacity decreased during Phase I indicating that NOB were washed out of the system. NOB population increased during the period of operational problems (188-340) and were abundant during the rest of the reactor run (Table 5). Nevertheless the operative conditions maintained in the reactor were able to suppress most of the nitratation capacity until the end of

Phase IV. In Phase V the nitratation capacity more than doubled and it was not possible to suppress NOB activity during normal operations. The loss of system stability in Phase V is also indicated by the similar limitation experienced by AOB and NOB on day 800 (Table 5).

Microbial community characterization

Fluorescence in situ hybridization was performed in order to identify the bacterial population distribution. Samples from reactor mixed liquor and from effluent samples were fixed and analyzed occasionally. Tests were conducted in order to verify which strain of AOB, NOB and anammox were present in the sludge in order to identify the best set of probes to use. The probes used were specific for Anammox bacteria (Amx 820, depicted in dark blue), AOBs (Neu 653, depicted in green), NOBs (Ntspa 712, depicted in red) and all organisms containing DNA (DAPI, depicted in light blue). In Figure 4 an example of the obtained results relative to biomass sampled on days191 and 265 are reported. NOB were detected throughout the full experimental period *Candidatus* Brocadia Fulgida (Kartal et al., 2008) was identified to be the dominant anammox strain during the whole reactor run. Satellite heterotrophic bacterial population growing on the products of biomass decay is expected to be present in the system due to the extremely long SRT maintained. However the non-autotrophic bacterial population was very small as indicated by the almost complete overlapping between probes specific for autotrophic bacteria and DAPI (Fig. 4).

N_2O emissions

When detailed cycles measurements were done also the concentration of nitrous oxide (N_2O) in gas phase was analysed online. Together with the online data of gas flow entering and leaving the system (by mass-flow controllers, MFCs), the N_2O emission rate was calculated as percentage of the total nitrogen load (N-load) and as percentage of the total nitrogen removal rate (TN-removal rate). The N_2O emission rate varied quite strongly but correlated with the nitrite concentration in the bulk. No clear correlation could be noticed between N_2O emissions and DO concentration or temperature. For nitrite

concentrations between 30 and 50 mg-N L^{-1}, the N_2O emission relative to N-load and TN-removal rate was 2.3±0.9% and 11.5±3.5%, respectively (13 cycles measured). For nitrite concentrations lower than 15 mg-N L^{-1}, the N_2O emission relative to N-load and TN-removal rate was 2.4±0.7% and 4.0±1.6%, respectively (4 measurements). The period when nitrite concentration was below 15 mg-N L^{-1} corresponded also to higher total nitrogen removal rates registered in the reactor, that is why the difference between N_2O emission relative to N-load and TN-removal rate is smaller.

Discussion

Autotrophic nitrogen removal was investigated in a granular lab-scale sequencing batch air-lift reactor for 541 days. The influent medium contained only ammonium as nitrogenous compound at concentrations ranging from 60 to 160 mg-N L^{-1} (Table 1). During the reactor operations at 20 and 15°C high nitrogen removal rates and efficiencies were registered, both in terms of ammonium and total nitrogen, prerequisite for the scale up of the process toward main stream applications. Once stable operation was established, the temperature was stepwise decreased from 20 to 15, to 10°C. In order to facilitate the comparison between literature and the present study, in Table 6 a summary of the biomass and reactor performance of systems operated in conditions relevant for main-stream application: operative temperature ≤20°C and reactor ammonium concentration <50 mg-N L^{-1}.

At 20°C the reactor showed ammonium and total nitrogen removal efficiencies above 95% and about 85%, respectively (Table 3). The corresponding nitrogen removal rates were 0.51 and 0.45 g-N L^{-1} d^{-1}, respectively, corresponding to biomass specific removal rates of 0.15 and 0.13 g-N $gVSS^{-1}$ d^{-1}. During this optimal performance period, the nitrite concentrations in the effluent were below 1 mg-N L^{-1}, demonstrating that by imposing an anaerobic period in the end of the operational cycle could adequately lower the nitrite concentrations that had built up in the aerobic period.

Table 6. Lab-scale anammox reactors operated at influent nitrogen concentration <200 mg-N L^{-1} and temperature ≤20°C. SBAR: sequencing batch airlift reactor; UASB: anaerobic up-flow sludge blanket; SBR: sequencing batch reactor; RBC: rotating biological contactor; MBR: membrane bioreactor.

Reference	Temp.	Reactor	Including nitritation	Influent	NH_4^+ (NO_2^-) reactor conc.
	°C			mg-N L^{-1}	mg-N L^{-1}
Hendrickx et al., 2012	20	gaslift	no	69	<3 (<3 [a])
Osaka et al., 2012	18	Upflow column with non-woven	no	20-50	n.r.
Ma et al., 2013	16	UASB-like	no	51	<10 [a] (<5 [a])
Vázquez-Padín et al., 2011	15 (20)	SBR	yes	175	n.r.
De Clippeleir et al., 2013a	14	RBC	yes	50	<5 [a] (<15 [a])
Hu et al., 2013b	12	SBR	yes	70	<20 [a] (<0.1)
Hendrickx et al., 2014	10	SBR/MBR	no	61	n.r.
Lotti et al., 2014a [**Ch.7**]	10	Fluidized bed	no	59	14 (13)
this study	20	SBAR	yes	160	<65 [f] (<5 [f])
this study	15	SBAR	yes	160	<70 [f] (<25 [f])
this study	10	SBAR	yes	130	<70 [f] (<30 [f])

Reference	Tot-N rem. rate	Tot-N rem. efficiency	Specific activity	Dominant anammox species	Note
	$g\text{-}N_{Tot}\ L^{-1}\ d^{-1}$	%	$g\text{-}N\ g\text{-}VSS^{-1}\ d^{-1}$		
Hendrickx et al., 2012	0.26	84 [b]	n.r.	n.r.	
Osaka et al., 2012	0.07-0.14	<30 [a]	<0.001 [a]	mix of different anammox species	Biomass enriched from fresh-water sediments
Ma et al., 2013	2.28	~50 [a]	0.47	n.r.	Start-up at 30°C, days 137-200 at 16°C
Vázquez-Padín et al., 2011	0.2 (0.5)	29 [b] (55 [b])	0.13 [c] (0.33 [c])	n.r.	Operations for more than 1000 days at 20°C and last 100 days at 15°C
De Clippeleir et al., 2013a	0.53	42	n.r.	n.r.	31 days operation at this Temp.
Hu et al., 2013b	0.025 [d]	92	0.036 [e]	*Candidatus* Brocadia Fulgida	Stoichiometric oxygen load
Hendrickx et al., 2014	0.027	81 [b]	0.039	*Candidatus* Brocadia Fulgida	Biomass enriched from activated sludge
Lotti et al., 2014a [**Ch.7**]	0.34	46	0.06	*Candidatus* Brocadia Fulgida	Pretreated municipal wastewater with the addition of nitrite as influent
this study	0.44	86	0.24 [g]	*Candidatus* Brocadia Fulgida	days 83-107
this study	0.4	73	0.15 [g]	*Candidatus* Brocadia Fulgida	days 380-411
this study	0.2	47	0.07 [g]	*Candidatus* Brocadia Fulgida	days 436-460

n.r.: not reported
[a] Depicted from graph
[b] Calculated from reported values of total nitrogen removal and loading rate
[c] Calculated from reported values of total nitrogen removal rate and reactor biomass concentration
[d] Calculated from the reported nitrogen (ammonium) load, ammonium removal efficiency and the ratio of nitrate production to ammonium consumption. Nitrite was reported as below detection limit in both the influent and the effluent
[e] Specific activity at 10°C calculated considering 0.6 g-protein g-VSS^{-1}
[f] As observed during cycle measurements
[g] Calculated from Table 4 considering a biomass concentration of 3.6 g-VSS L^{-1}

Nitrite is also reported to enhance undesired N2O emissions during ammonium oxidation by AOBs (Kampschreur et al., 2008b) and it's therefore crucial from an environmental point of view to be able to operate the partial nitritation/anammox process at low nitrite concentrations. Also in this study a positive correlation between the nitrite concentration and N2O emission rate was observed.

When the reactor was operated at 15°C, ammonium and total nitrogen removal efficiencies of about 89% and 73% were obtained, corresponding to volumetric removal rates of 0.48 and 0.41 g-N L^{-1} d^{-1}, respectively. In terms of biomass specific rates, they correspond to 0.13 and 0.11 g-N gVSS^{-1} d^{-1}. Dosta and co-authors (2008), reported a specific total nitrogen removal rate of 0.02 g-N_{Tot} gVSS^{-1} d^{-1} 15°C, while Vázquez-Padín (2009) reported a maximum of 0.12 g-N_{Tot} gVSS^{-1} d^{-1} at 20°C, but both with much lower removal efficiencies and higher influent nitrogen concentration than those reported in this study. Also Winkler and co-authors (2011) operated a partial nitritation/anammox reactor at ambient temperature reaching 0.16 g-N_{Tot} gVSS^{-1} d^{-1}. In that study, however, the influent contained 40 mg-N L^{-1} of nitrite besides ammonium and was dosed anaerobically, therefore notably facilitating anammox reaction during operations. The same process was studied by Vázquez-Padín et al. (2011) and by De Clippeleir et al. (2013a) reaching total nitrogen volumetric removal rates of 0.20 and 0.53 g-N_{Tot} L^{-1} d^{-1} operating their system at 15 and 14°C, respectively, but in both cases with very low removal efficiencies (Table 6). Furthermore, the total nitrogen volumetric removal rate at 10°C obtained in this study was eight times higher than values recently reported by Hu et al. (2013b) and Hendrickx et al. (2014) at similar temperature (Table 6). Also when comparing biomass specific anammox activity at 10°C, a factor two can be noticed between this

study and the activities reported by the aforementioned authors (0.036 g-N_{Tot} $gVSS^{-1}$ d^{-1} by Hu et al., 2013b and 0.039 g-N_{Tot} $gVSS^{-1}$ d^{-1} by Hendrickx et al., 2014).

Interestingly, also Hu et al. (2013b), Hendrickx et al. (2014) and Lotti et al. (2014a [**Ch.7**]) found that *Candidatus* Brocadia Fulgida was the dominant anammox species in their systems (Table 6). The fact that the same anammox microorganism dominated the only three systems operated at temperature close to 10°C might suggest that *Candidatus* Brocadia fulgida may have a competitive advantage at low temperatures. This can only be confirmed when temperature effects on various individual anammox species have been performed.

The exponential increase in NRR during the initial period of Phase II can be used to estimate the growth rate of anammox bacteria at 15°C. In this period the nitrite concentration was on average 8.6 mg-N L^{-1}. The specific anammox activity in the aerated reactor equalled the conversion under anoxic conditions and with excess nitrite during batch tests (Table 5) indicating anammox biomass (and not substrate) limited conversion. Obviously all the oxygen diffusing into the granules was consumed by AOB, preventing oxygen inhibition of the anammox bacteria. Through linear interpolation of the natural logarithm of the NRR in time (days 123-132) an anammox growth rate of 0.017 d^{-1} was estimated.

During the operations at 15°C the ammonium concentration in the influent was decreased by 30% keeping all other influent concentrations constant. The lower ammonium load during the cycles did not cause any appreciable alteration of the conversions catalyzed by the different microbial community members (Table 4). These results indicate that, as far as ammonium does not become limiting during the cycle (as during days 219-244), wastewater with low concentrations of ammonium can efficiently be treated provided that a plug-flow or batch process is applied, ensuring that biomass is exposed to relatively high NH_4^+-concentrations.

The factor that was limiting nitrogen removal during the reactor run depended on the temperature of operation (Fig. 2; Table 5). At 20°C the nitrite production by nitritation process limited anammox activity, whereas the DO concentration limited the nitrite production rate by AOB. During operation at 15°C the anammox activity became the limiting step for the conversion. When

the reactor was operated at 10°C the anammox conversion capacity decreased further potentially due to oxygen presence in the core of the granule. The decreased AOB activity at this low temperature leads to more oxygen penetration into the interior of the granular sludge. Likely a proper DO control would be needed for a good process operation and balance between AOB activity and Anammox activity at these low temperatures.

These results confirm the importance of the granular structure in terms of the correct balance between the spatial distribution of AOB and anammox bacteria and the aerobic and anoxic volume within the granules (Winkler et al., 2011; Volcke et al., 2010). Similar observations of properly running systems which destabilize after a temperature decrease were reported in literature: the anammox-SBR operated by Dosta et al. (2008) lost its stability when the temperature was lowered from 18 to 15°C; nitrate formation by nitratation continuously increased in time in the system of De Clippeleir et al. (2013a) when the temperature was gradually decreased from 29 to 14°C; a strong decrease in anammox bacteria abundance (100-fold decrease in the copy numbers) was observed by Hu et al. (2013b) after temperature decreased from 25 to 12°C.

The comparison between maximum and actual activities shows that AOB and NOB were strongly limited by oxygen throughout the experimentation (Table 5). NOB presence was detected throughout the entire operational period (Table 5; Figure 4). During operational Phases I to IV NOB experienced stronger limitation than AOB and nitratation was successfully suppressed (Fig. 2). When the influent ammonium concentration was temporarily decreased by 80% (Phase II, days 219-244), ammonium became limiting for most of the cycle duration resulting in limiting conditions for both AOB and anammox. As a result, NOB activity rapidly increased as shown in Figure 5 by the NO_3^-/NH_4^+ ratio.

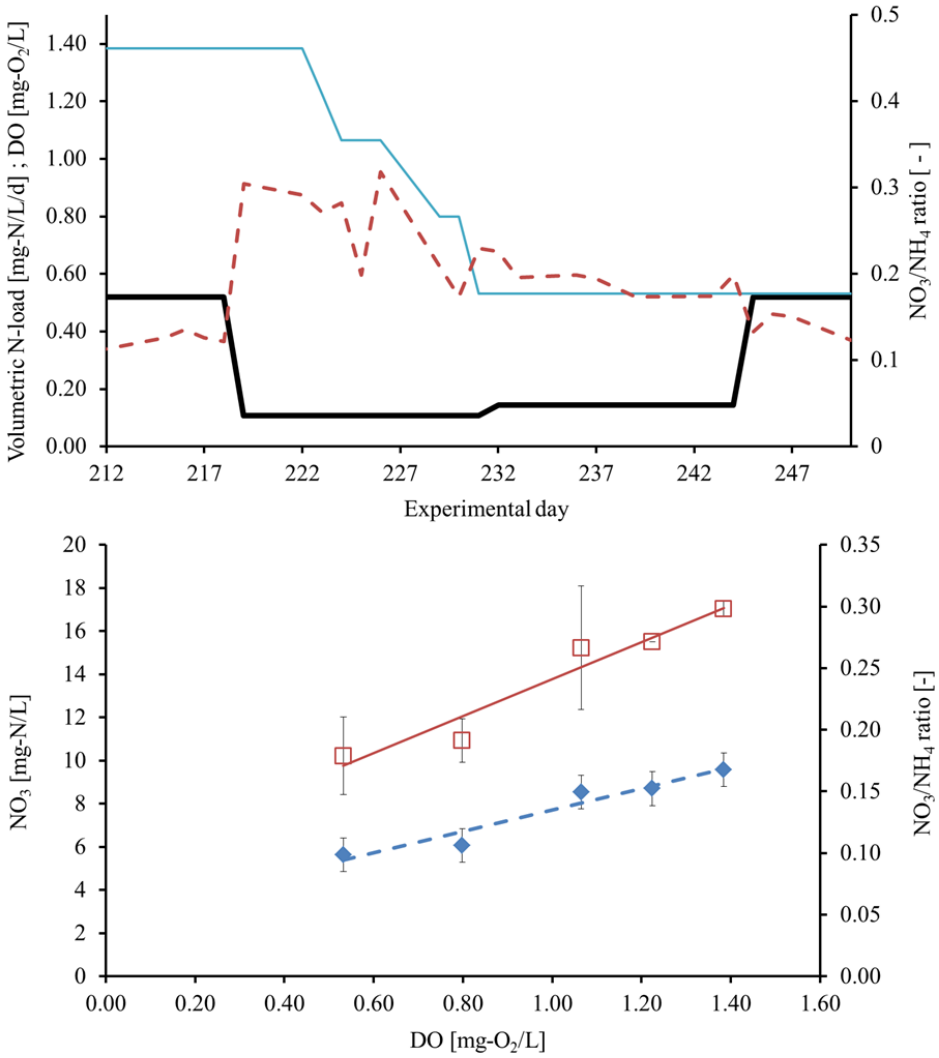

Figure 5. Effect of the N-load and the oxygen concentration on the suppression of NOB conversion. ***Top:*** N-load (solid thick line), dissolved oxygen (*DO*, solid thin line), NO_3^-/NH_4^+ ratio (dashed line); ***Bottom:*** NO_3^-/DO ratio (closed diamonds), NO_3^-/NH_4^+ ratio (open squares). Error bars represent the standard deviation.

Nevertheless lowering the controlled DO concentration resulted in an increase of ammonium concentration and a reduction of the NOB activity within few days. Nitratation could then be efficiently suppressed during the

following ten days of operations (six times the HRT) at lower influent ammonium concentration. The fact that during this period the nitrite concentration in the bulk was non-limiting (average effluent nitrite concentration equal to 4 mg-N L^{-1}) suggests the potential of suppressing nitritation based on the competition for oxygen against AOB rather than on the competition for nitrite against anammox. This suggestion is supported by the study of Jemaat at el. (2013) who reported the successful suppression of nitratation at 20°C in a system with excess of ammonium and nitrite by a proper oxygen control. Picioreanu et al. (2004) showed that in a biofilm segregation of the microbial populations takes place based on growth rate. It's therefore expected that in biofilm systems AOB will reside mainly in the external part of the aerobic layer, relegating NOB more internally. Previous studies reported that NOB reside more internally than AOB (Vlaemminck et al., 2010; Lotti et al., 2015b [**Ch.8**]) supporting that in biofilm NOB are more prone to oxygen limitation. Furthermore, larger aggregates have been shown to enhance nitratation suppression also by modeling (Volcke et al., 2010) and experimentally (Winkler et al., 2012b).The results reported in this study are not sufficient to exhaustively explain the uncontrolled increase of the NOB activity during Phase V. Nevertheless a hypothesis can be formulated in which a combination of factors had contributed. Lower ammonium load together with higher oxygen concentrations could have resulted in higher NOB activity due to decreased competition for oxygen with AOB (Fig. 2; Table 5; Jemaat et al., 2013). The reduced competition for nitrite due to lower anammox activity instead, is unlikely to have influenced NOB activity given the non-limiting nitrite concentration during Phase V (14.6 mg-N L^{-1}). In the current study prolonged operation for more than 100 days at 10°C caused a slow but unrestrainable decrease in anammox activity and process efficiency. Since in general these conditions do not extend over long time in moderate climates this is not seen as a limitation for the applications of anammox-based technologies in the main-stream of wastewater treatment plants, but in climates with long cold winters the long-term process effectiveness remains a point of attention.

Overall the results reported here indicate that for the successful implementation of completely autotrophic nitrogen removal at main-stream conditions the oxygen supply rate has to be accurately regulated on the ammonium load. This tight control of the aeration is necessary to supply the

AOB population the amount of oxygen merely sufficient to produce enough nitrite for the anammox metabolism and to avoid ammonium limitation due to excessive nitritation. The importance of an efficient DO-control was also highlighted in modelling studies (Hao et al., 2002b; Volcke et al., 2010; Joss et al., 2011). Furthermore, in order to maximize nitritation rate whilst ensuring an effective suppression of nitratation at low temperatures, the aeration control should probably account also for the influent ammonium concentration and flow rate as recently suggested by experimental results (Bartrolí et al., 2010; Jemaat et al., 2013; Regmi et al., 2014) and supported by mathematical modeling of nitrifying biofilm reactors (Pérez et al., 2009; Brockmann and Morgenroth, 2010; Jemaat et al., 2013). Whilst in our lab-reactor, with constant (or stepwise changed) influent concentrations and flow rate, the aeration control could rely on online DO-measurements only, in practise additional measurements and a more complicated control scheme might be necessary. The currently used control scheme in side stream applications for granular sludge anammox might be a better start for a control scheme. In these reactors the aeration flow rate is controlled by a feed-back control loop from an ammonium effluent measurement. This proved in practise a more stable and simple control procedure then a direct DO control scheme.

The pronounced decrease in anammox biomass specific activity reported in literature at decreasing temperatures (Dosta et al., 2008; Hu et al., 2013b) might limit anammox applications at these temperatures. In moderate climates as in the Netherlands very low temperatures generally occur during 2 months a year. A reasonable strategy for full-scale application has to be developed to 'survive' this period. Build-up of sufficient anammox overcapacity during warm seasons can be exploited during cold seasons. The nitritation capacity change due to the yearly temperature fluctuation appears as less problematic because of the oxygen limiting conditions at which AOB are grown in the system as also shown in this paper (Table 5). Another important aspect in a partial nitritation/anammox system with granular sludge is the ability of the aerobic organisms residing in the outer layer to prevent oxygen from penetrating more internally and therefore reducing the anoxic volumetric fraction of the granules and inhibiting part of the anammox population. The temporary increase of shear stress in such a system should be therefore avoided in order to prevent granules disruption causing anammox inhibition by oxygen.

The N_2O emissions reported in this study are higher than the observations of Hu et al. (2013b) (~2.4% of the nitrogen removed) as well as the emissions measured in full-scale partial nitritation/anammox systems by Kampschreur et al. (2009b) (1.2% of the nitrogen load). De Clippeleir et al. (2013a) instead, observed comparable N_2O emission of 5% of the nitrogen load. This should not surprise considering the higher and fluctuating nitrite concentrations in the systems described here and by De Clippeleir et al. (2013a), compared to the system of Hu et al. (2013b) and the effect of nitrite on the production of N_2O (Kampschreur et al., 2008a). Optimization of the operational conditions, especially regarding nitrite accumulation, would be therefore fundamental in real application in order to minimize N_2O emissions.

Conclusions

Autotrophic nitrogen removal was demonstrated at conditions relevant for sewage treatment in a lab-scale gas-lift sequencing batch reactor with granular sludge. In the presence of ammonium dissolved oxygen was shown to be an effective control parameter for the suppression of the undesired NOB activity even at higher level than previously assumed (up to 2.5 mg-O_2 L^{-1}). This control strategy guaranteed the effective suppression of the nitratation both at 20 and 15°C, allowing nitrogen removal rates of 0.44 and 0.40 g-N_{Tot} L^{-1} d^{-1}. Unlike previously reported, these high removal rates were obtained together with optimal nitrogen removal efficiencies of 86 and 73%, respectively, fulfilling a decisive prerequisite for the implementation of the process in the main-stream of WWTPs. Anammox bacteria were shown to grow in the system, with estimated growth rate of 0.017 d^{-1} at 15°C. A positive correlation between nitrite concentration in the bulk and N_2O emissions was observed whilst no clear correlation could be noticed between N_2O emissions and DO concentration or temperature. In the current study anammox was unstable at prolonged winter temperatures (10°C). Since in general these conditions do not extend over long time in moderate climates this is not seen as a limitation for the applications of anammox-based technologies in the main-stream of wastewater treatment plants.

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Chapter 7

Anammox growth on pretreated municipal wastewater

This chapter has been published as:

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Abstract

Autotrophic nitrogen removal from municipal wastewater enables development of energy autarkic wastewater treatment plants. In this study we report the evaluation of the anammox process in a granular sludge fluidized bed lab-scale reactor continuously fed with the actual effluent of the A-stage of the WWTP of Dokhaven, Rotterdam. The reactor was anoxic and nitrite was dosed continuously to support anammox activity only. The system was operated for more than ten months at temperatures between 20 and 10°C. COD was also consumed during the process, but heterotrophs could not outcompete anammox bacteria. Volumetric N-removal rates obtained were comparable or higher than those of conventional N-removal systems, with values higher than 0.4 g-N L^{-1} d^{-1} when operated at 10°C. The biomass specific N-removal rate at 10°C was on average 50±7 mg-N g-VSS^{-1} d^{-1} during last month of operations, almost two times higher than previously reported activities at this temperature. FISH analysis revealed that the dominant anammox species was *Candidatus* Brocadia Fulgida throughout the experimentation. Evidence for growth of anammox bacteria at main-stream conditions was demonstrated for the entire temperature range tested (10-20°C) and new granules were shown to be actively formed and efficiently retained in the system.

Introduction

To date, most of the autotrophic nitrogen removal systems reported in literature were operated at temperatures exceeding 25 °C and influent nitrogen concentrations over 0.1 g-N L^{-1} (van Hulle et al., 2010; van der Star et al., 2007). The application at lower temperatures and lower nitrogen concentrations would allow to extend the application potential of processes involving anammox to municipal sewage treatment opening new possible scenarios in designing energy producing wastewater treatment plants (Jetten et al., 1997; Siegrist et al., 2008; Kartal et al., 2010a). In order to maximize energy recovery

from municipal wastewater energetic valorization of organic carbon in a first step would be most advantageous. This first step could either be a UASB reactor at tropical climates (Aiyuk et al., 2006) or a COD-concentration step at lower temperature regions. This concentration can be performed by physical (e.g. sieving), chemical (e.g. precipitation) or biological (Versprille et al., 1984) methods, or combinations thereof. The concentrated sludge can subsequently be digested anaerobically to methane containing biogas. The remaining liquid contains ammonium that can be removed in an autotrophic process based on anammox (van der Star et al., 2007; Jetten et al., 1997; Wett et al., 2007; Joss et al., 2009; Abma et al., 2010). To facilitate autotrophic nitrogen removal on pretreated sewage a process based on prevention of growth of nitrite oxidizing bacteria (NOB) and retaining anaerobic ammonium oxidation (anammox) bacteria in the system in symbiosis with ammonium oxidizing bacteria (AOB) has to be developed. The main challenge for applying anammox in the water line of WWTP is to achieve a high rate process with good biomass retention and low effluent nitrogen concentrations at low water temperatures. A very strong decrease in specific anammox activity was reported after lowering the temperature of warm reactors (Dosta et al., 2008; Isaka et al., 2008; Vázquez-Padìn et al., 2011). Despite this decrease in anammox activity, several laboratory studies have reported 1-stage partial nitritation/anammox at lower temperatures (≤25 °C) and reasonable conversion rates higher than 0.2 g-N L^{-1} d^{-1} at 15°C (Vázquez-Padìn et al., 2011; Winkler et al., 2011; De Clippeleir et al., 2013a; Hu et al., 2013b). Hendrickx et al. (2012) and Isaka et al. (2008) studied the anammox process at low temperature in anoxic reactors (2^{nd} step of the 2-stages partial nitritation/anammox process) fed with synthetic feeding-medium and volumetric N-removal rates of 0.26 and 0.36 g-N L^{-1} d^{-1} were reported at 10 and 6.3°C, respectively. Until now no report is available describing the application of anammox based process treating actual municipal wastewater. In natural ecosystems such as Northern European soils and marine sediments, anammox bacteria thrive at low temperatures (<10 °C) and very low ammonium concentrations (µM range) (Hu et al., 2011; Lam and Kuypers, 2011; van de Vossenberg et al., 2008), indicating that there is no fundamental limitation for the anammox process to develop under municipal wastewater conditions. This study aimed at demonstrating the feasibility of the anammox process from pretreated sewage on lab-scale, and evaluate the specific activities of anammox granular sludge under these conditions. A granular sludge based

fluidized lab-scale reactor (1.8 L) was fed with ammonium containing effluent of the A-stage of the WWTP of Dokhaven (Rotterdam). In order to have only anammox conversion nitrite was added to the pretreated wastewater. Using this approach we could exclude the potential effect of oxygen required to grow ammonium oxidizing bacteria and the competition for nitrite by nitrite oxidizing bacteria and focus on the anammox conversion.

The main objective of this work was to investigate the capacity of anammox bacteria to grow at main-stream conditions as well as to achieve adequate biomass retention and high volumetric N-conversion rates. Furthermore this work also aimed at proving granules stability at temperatures as low as 10°C as granules disintegration may occur as a result of lowering the temperature as it was previously observed for anaerobic granules (McKeown et al., 2009). The reactor was operated at temperatures between 20 and 10°C. The results obtained during 10 months of operation of the lab-scale reactor are presented.

Materials and methods

Reactor operation

The experimentation was conducted in a lab-scale upflow fluidized granular sludge reactor (working volume, 1.8 L) with granular biomass operated in continuous. The reactor constituted of two joined cylindrical parts with different diameters: most of the biomass resided in the lower part (D=6 cm) where was fluidized, while the upper part (D=9.3 cm) was used as settler. In Figure 1 the reactor is depicted together with digital and microscopic pictures of the reactor biomass. The reactor was inoculated with about 100 mL (6.6 g-VSS) of settled granular biomass from the full-scale ANAMMOX® reactor of Dokhaven WWTP.[2] The wastewater applied for conducting this study originates from the main line of the municipal wastewater treatment plant of Dokhaven (560,000 p.e., Rotterdam, The Netherlands) and was continuously fed to the reactor at a flow of 12÷36 L d^{-1} (hydraulic retention time, HRT, of 1.2÷3.6 h). The influent was the effluent of the A-stage (HRT 1h (dry weather conditions, DWC), solids retention time, SRT, 0.3 days) after settling. For a description of the WWTP we refer to literature (Kampschreur et al., 2008). Prior to enter in the reactor the influent passed through an additional buffer tank (50 L); the HRT of

the buffer tank was 1.1÷3.3 d. Nitrite was dosed to the actual wastewater to obtain an influent concentration of 40 mg-N L^{-1} by means of a peristaltic pump (0.3 L d^{-1}). Anoxic effluent from the reactor was recirculated at a flow rate of 1.4 m^3 d^{-1} by means of a peristaltic pump to increase the upflow velocity to approximately 20 m h^{-1} in order to keep the bed fluidized. The reactor temperature was controlled by means of a cryostat connected to the double wall of the reactor. The reactor operations were divided in three different phases, each one characterized by different operative temperatures: phase I, 20 °C (days 1-52); phase II, 15 °C (days 60-127); phase III, 10 °C (days 134-385). During the transition between two consecutive phases the temperature was stepwise decreased. pH was not controlled in the reactor, but the pH of the influent was set at 7.0-7.5 by addition of NaOH (0.1 M). All tubing and connectors were of butyl rubber, norprene or polyvinylchloride to limit oxygen intrusion. The reactor headspace was connected by means of a norprene tube to a cylinder filled with water to act as a water-lock and avoid air entrance. The water column in the water-lock was 10 cm, resulting in an overpressure inside the reactor of about 10 hPa. The nitrogen gas produced during anammox reaction could be observed by gas bubble formation in the water-lock.

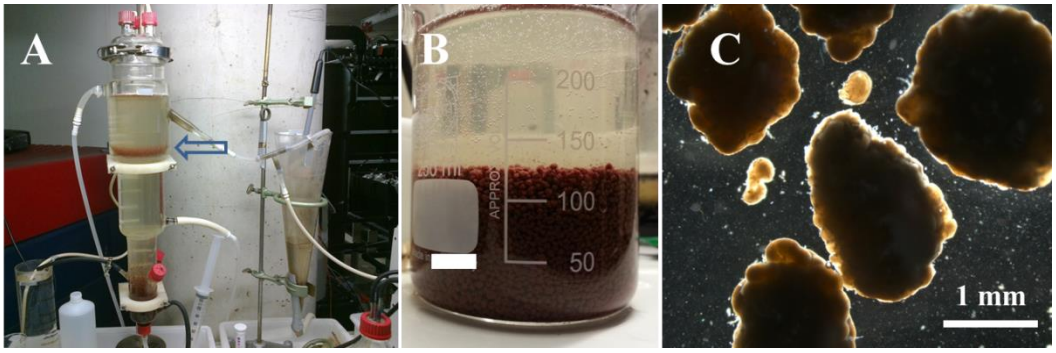

Figure 1. Images of the fluidized bed reactor (A), of the granular biomass (B) and a microscopic picture of the granules (C). The arrow indicates the settled biomass accumulated on the horizontal annulus at the bottom of the top part of the reactor.

Analytical procedures

Dry weight (TSS), ash content and volatile suspended solids (VSS, dry weight minus ash content) were determined according to standard methods (APHA,

1998). Bed volume of the settled bed was determined by reading the height of the biomass bed directly from the scale on the reactor after allowing the biomass to settle for 5 minutes after the feeding and recirculation pumps were temporarily stopped. Concentrations of ammonium, nitrite and nitrate were measured regularly with Dr.Lange test kits in filtered influent/effluent samples. From influent/effluent data, the actual volumetric conversion rate of ammonium, nitrite and nitrate were calculated. The ratio between nitrate produced and ammonium consumed (NO_3/NH_4 ratio) was calculated in order to monitor anammox anabolism since in the anammox metabolism nitrate production is coupled with carbon fixation (Lotti et al., 2014c [**Ch.2**]). Nitrite and nitrate concentrations were regularly checked with Merck indicator papers. Anoxic batch tests were conducted inside the reactor in order to evaluate the maximum volumetric nitrogen removal potential of anammox ($Rate_{Tot\text{-}N}^{max}$, g-N L^{-1} d^{-1}). During the batch test (4-6 hours long) the concentration of ammonium, nitrite and nitrate in the bulk liquid was measured at regular interval (20-30 min). When the batch tests were conducted the recirculation flow was manually increased to such an extent that granules were fluidized in the whole reactor volume and mass transfer limiting conditions were no longer present: recirculation flow of 2.2 m^3 d^{-1} corresponding to a superficial liquid velocity of approximately 32 m h^{-1}. Excess of substrate was ensured by manually injecting concentrated solutions of ammonium sulfate and sodium nitrite to obtain a final concentration over 50 mg-N L^{-1}. Changes in morphology of the granules were followed by image analyses (IA) (Tijhuis et al., 1994), with a Lexmark Optra image analysis system. Also pictures were taken with this system to follow the development of the granules.

Calculations

The average solid retention time (SRT) was determined through performing a solids mass balance: the biomass in the reactor and the effluent biomass, which consists of granules and eroded material from the granule surface. The biomass specific maximum conversion capacity (q^{max}, g-N_2 g-VSS^{-1} d^{-1}) was calculated by dividing the maximum volumetric N-removal rate with the total biomass concentration present in the reactor at the time the batch test was

conducted. Net growth rate was calculated from the increase of the ammonium removal rate in time as $\mu = \ln(Rate_{t2}/Rate_{t1})/(t2 - t1)$.

Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) was performed on the biomass from the reactor as described previously (Schmid et al., 2000) with probes specific for Kuenenia and Brocadia-like anammox bacteria (AMX-820; Schmid et al., 2001), Kuenenia stuttgartiensis (KST1273; Schmid et al., 2000), Brocadia Fulgida (BFU613; Kartal et al., 2008), AOB (mix of NEU653, NSO190 and NSO1225; Wagner et al., 1995; Mobarry et al., 1996), NOB (mix of NTSPA0712 and NIT1035; Daims et al., 2000; Juretschko et al., 1998). Prior biomass sampling, the recirculation flow was increased to 2.2 m^3 d^{-1} in order to fluidized the granules in the whole reactor. In total 2-5 mL of settled granules were homogenized by pottering prior FISH was conducted. The qualitative or quantitative presence of a target was evaluated by combining a specific probe with an equimolar mixture of EUB338I, EUB338II, and EUB338III targeting all bacteria (Loy et al., 2003). Ten pictures were considered for each analysis.

Results

Reactor operation

The lab-scale upflow fluidized granular sludge reactor was inoculated in July 2012 (experimental day zero). The granules of the inoculum had an average ferret diameter of 1.5 mm as measured by IA. The lab-scale reactor was operated with temperature control for ten months. The different phases of operations corresponded to different temperatures as described in Table 1.

The influent ammonium concentration fluctuated between 8 and 65 mg-N L^{-1} as usual in municipal wastewater. The influent flow was manually changed in order to maintain non limiting ammonium and nitrite concentrations in the reactor effluent. In Table 1 the average influent concentrations and standard deviations of ammonium and nitrite for the different phases of operations are shown, together with the nitrogen load applied and the average hydraulic retention time. The influent contained on average 57±7 mg-COD L^{-1}, resulting in

a COD/NH_4^+ ratio of 1.9. The biomass concentration in the reactor increased from the initial 3.6 to 6.7 g-VSS L^{-1} during last period of operations, whilst settled sludge volume increased from 100 to 310 mL.

Table 1. Operational phases and corresponding influent characteristics and operational parameters.

			Influent characterization					
Phase	**Period**	**Temp.**	**NH_4^+**	**NO_2^-**	**COD**	**Solids**	**HRT**	**[a] N-Load**
	Exp. Days	°C	mg-N L^{-1}	mg-N L^{-1}	mg-COD L^{-1}	mg-VSS L^{-1}	h	g-N L^{-1} d^{-1}
I	1-52	20	31 ± 4	66 ± 25	61 ± 5	4 ± 6	1.3	1.80 ± 0.53
II	60-127	15	27 ± 6	36 ± 15	55 ± 12	5 ± 5	1.2	1.25 ± 0.31
III	134-285	10	30 ± 9	29 ± 11	58 ± 8	5 ± 5	2.8	0.60 ± 0.31

[a] Sum of ammonium and nitrite load

Table 2. Volume specific ($Rate_{Tot\text{-}N}^{max}$) and biomass specific ($q^{max}$) total N removal rate during in-situ anoxic batch tests; ammonium ($Rate_{NH4\text{-}N}$) and total-N ($Rate_{Tot\text{-}N}$) removal rate during normal operations as well as the ratios between nitrite and ammonium conversion rates (NO_2/NH_4 ratio) and nitrate production and ammonium conversion rates (NO_3/NH_4 ratio).

Exp. Day	Temp.	$Rate_{NH4\text{-}N}$	$Rate_{Tot\text{-}N}$	$Rate_{Tot\text{-}N}^{max}$	$Rate_{Tot\text{-}N}^{max}/Rate_{Tot\text{-}N}$	q^{max}	NO_2/NH_4 ratio	NO_3/NH_4 ratio
d	°C	g-N L^{-1} d^{-1}	g-N L^{-1} d^{-1}	g-N L^{-1} d^{-1}	%	g-N_2 g-VSS^{-1} d^{-1}	-	-
11	20	0.41	1.40	1.61	116	0.43	1.26	0.21
39	20	0.72	1.85	2.38	129	0.60	1.25	0.24
62	15	0.29	0.69	1.46	213	0.29	1.47	0.21
69	15	0.34	0.76	1.52	201	0.30	1.32	0.21
90	15	0.49	1.19	1.58	133	0.30	1.43	0.18
129	13	0.19	0.51	0.69	135	0.12	1.41	0.14
144	10	0.12	0.34	0.34	103	0.06	1.70	0.09

The sludge volume index (SVI) increased from 17 to 28 mL g-VSS^{-1} during the ten months of operations and was on average 23±5 mL g-VSS^{-1}. The solids in the influent and effluent on average accounted for less than 5 and about 10 mg-VSS L^{-1}, respectively, resulting in a solid retention time between 60 and 145 d. The efficiency of solids retention was estimated as 99%. The maximum nitrogen removal capacity was measured during in situ batch tests. The results from batch tests are reported in Table 2.

Throughout the experimental period Anammox was cultivated in excess of substrate, with effluent ammonium and nitrite concentration equal to 14±9 and 13±11 mg-N L^{-1}, respectively. Nevertheless the microbial conversion was limited by mass transfer as shown by the difference between maximum nitrogen removal rate ($Rate_{Tot\text{-}N}^{max}$) as measured during batch tests and the actual nitrogen removal rate ($Rate_{Tot\text{-}N}$) measured during normal operations (Table 2). During normal operations the system was likely mass transfer limited due to insufficient mixing; the recirculation flow was merely sufficient to fluidize the granular sludge bed and preferential flows occurred. In Figure 2 an overview of the total nitrogen load applied as well as the total nitrogen removal rate with relative removal efficiency and the ammonium removal rate observed are reported together with the temperature of operations. The influent for the reactor came from a buffer vessel and the net oxygen input into the reactor is considered negligible. A small fraction of the COD in the influent was converted in the reactor, resulting in an effluent concentration of 50±5 mg-COD L^{-1} and therefore in an average COD removal rate of 51±11 mg-COD L^{-1} d^{-1}. Assuming that COD was removed through heterotrophic denitrification using nitrate (or nitrite) as electron acceptor and neglecting growth, it would have corresponded to a nitrogen removal rate of 18±4 (30±6) mg-N L^{-1} d^{-1}. Heterotrophic biomass was present in the granules, but also as a small layer of settled biomass accumulated on the horizontal annulus of the top part of the reactor (Fig.1, A). The biomass of this accumulated layer was composed by loose flocs and it was removed from the system every two-three weeks. The day after the accumulated layer was removed a temporary decrease in total nitrogen removal rate ($Rate_{Tot\text{-}N}$) was always registered.

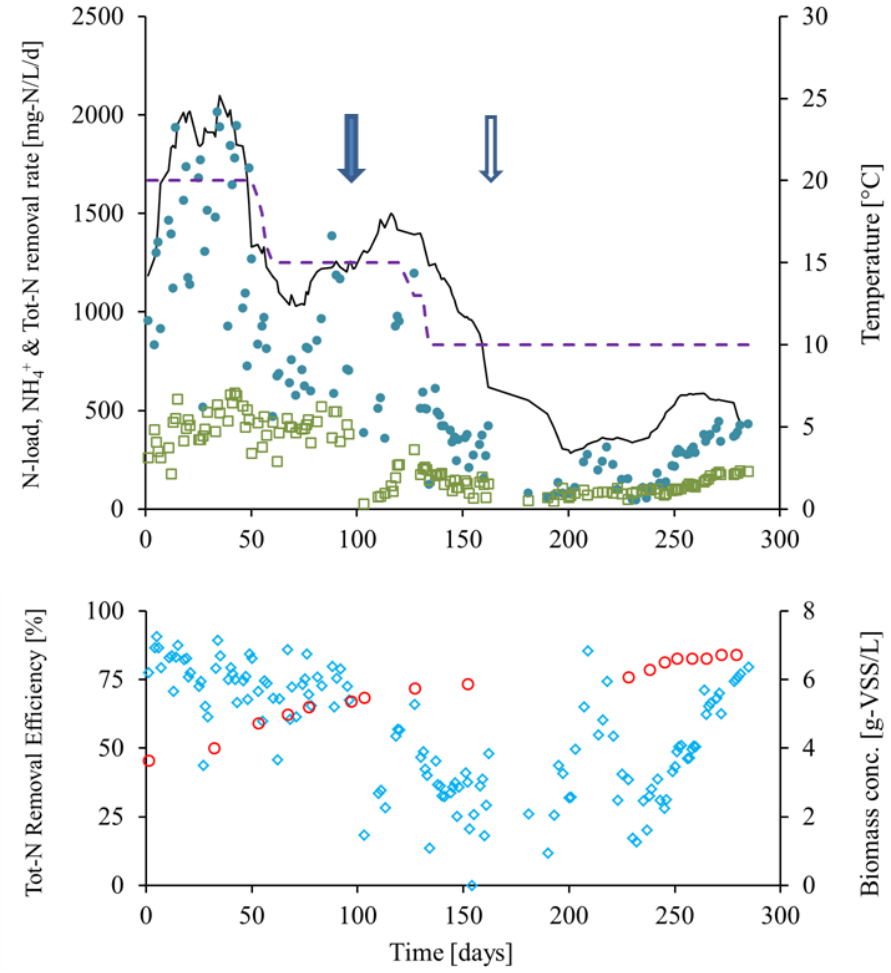

Figure 2. Time course of reactor operation. *Top*: Total nitrogen load and nitrogen removal rate (solid line and closed circles, mg-N L^{-1} d^{-1}); ammonium removal rate (open squares, mg-N L^{-1} d^{-1}); temperature (dashed line, °C). Closed arrow indicates an unwanted oxygen leak into the reactor experienced during days 95-100. Open arrow indicates the failure of the influent pump on day 163. *Bottom*: Total nitrogen removal efficiency (open diamonds, %) and biomass concentration (open circles, g-VSS L^{-1}).

In Phase I the volumetric total N conversion increased from approximately 1 to 2 g-N L^{-1} d^{-1}, corresponding to biomass specific rate (q) of 0.3 to 0.5 g-N g-VSS^{-1} d^{-1}. In the same period ammonium removal rate increased from 0.26 to 0.59 g-

N L^{-1} d^{-1} whilst the biomass concentration in the reactor increased from 3.6 to 4.7 g-VSS L^{-1} (Figure 2). From the increase of the ammonium removal rate over time the anammox growth rate was estimated to be equal to 0.020 d^{-1}, corresponding to a generation time (T_d) of 35 d. At the beginning of Phase II, when the temperature was lowered from 20 to 15°C, a sharp decrease in conversion rate was observed. After a few days of stabilization the conversion rate increased from 0.65 to about 1.2 g-N L^{-1} d^{-1} (Fig.2, *Top*), corresponding to a biomass specific rate of 0.14 to 0.24 g-N g-VSS^{-1} d^{-1}, respectively. In the same period ammonium removal rate increased from 0.39 to 0.50 g-N L^{-1} d^{-1} whilst the biomass concentration e increased from 4.7 to 5.4 g-VSS L^{-1} (Figure 2). From the increase of the ammonium removal rate over time the anammox growth rate was estimated to be equal to 0.009 d^{-1} (T_d=77 d). Note that likely the actual rate was higher since we could not accurately estimate anammox removal as suspended solids in the effluent. An oxygen leakage into the reactor during days 95-100 (see closed arrow in Fig. 2) caused a temporarily deterioration of the reactor performance. After the problem was solved the conversion rate increased again up to about 0.9 g-N L^{-1} d^{-1} (Fig. 2, *Top*). After the reactor performance was stable for a few days, the reactor temperature was stepwise decreased to 10°C in two weeks. In parallel with the decrease in temperature at the beginning of Phase III, the conversion rate decreased and stabilized at about 0.35 g-N L^{-1} d^{-1}. After two weeks of stable operations the influent pump broke down (day 163, see open arrow in Figure 2). This pump failure resulted in the accumulation of nitrite in the reactor up to a concentration of more than 200 mg-N L^{-1}. After this technical failure of two weeks, the $Rate_{Tot\text{-}N}$ decreased to 0.08 g-N L^{-1} d^{-1}. After about 50 days of fluctuating performance, the $Rate_{Tot\text{-}N}$ constantly increased during the last two months of Phase III, from about 0.1 up to 0.43 g-N L^{-1} d^{-1} (Fig.2, *Top*), corresponding to biomass specific $Rate_{Tot\text{-}N}$ ranging from 0.02 to 0.08 g-N g-VSS^{-1} d^{-1}, respectively. During the same period ammonium removal rate increased from 0.09 to 0.19 g-N L^{-1} d^{-1} whilst the biomass concentration increased from 6.1 to 6.7 g-VSS L^{-1} (Figure 2). From the increase of the ammonium removal rate over time the anammox growth rate was estimated to be equal to 0.005 d^{-1} (T_d=132 d). Nitrate production, which in absence of oxygen is considered as proof of anammox growth (Lotti et al., 2012a), was observed throughout the experimental period resulting in an average effluent concentration of 3±2 mg-N L^{-1}. In Figure 3 the ratio between nitrate production and ammonium consumption rate (NO_3/NH_4 ratio) is

depicted in time together with the ratio based on reported anammox stoichiometry (0.26; Strous et al., 1998) and the temperature of operation.

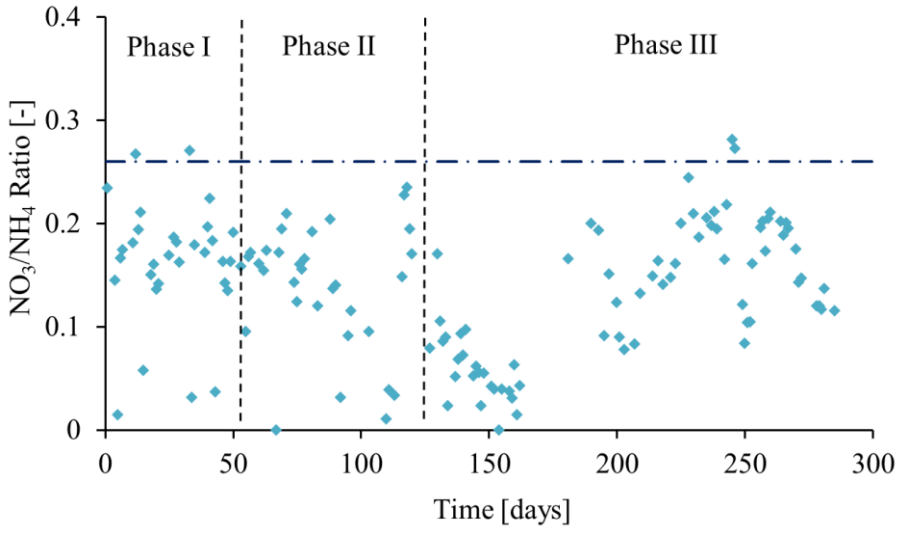

Figure 3. Time development of the ratio between the nitrate production and the ammonium consumption rate (NO_3/NH_4 ratio, *diamonds*) together with the theor. ratio (*dash-dot line*) based on reported anammox stoichiometry (0.26; Strous et al., 1998).

Microbial community characterization

Stable granules enriched in anammox bacteria were obtained throughout the entire experimental period, irrespective of the lower nitrogen concentrations, lower operational temperatures, solids in the influent and heterotrophic growth on COD. Most of the biomass in the reactor was in the form of granules with very little flocculent material. The granular sludge occupied the lower part of the reactor whereas a thin and loose layer of flocs accumulated on the flat annulus at the bottom of the settling part as aforementioned (Figure 1, A). When the dry mass of this accumulated layer was measured in Phase III to be compared with the total amount of biomass present in the system a value of 0.35 g-TSS was determined, showing that about 97% of the biomass was always present in the form of granules (~12 g-TSS). The anammox bacteria dominating the microbial community belonged to

the strain *Candidatus* Brocadia Fulgida (Kartal et al., 2008) throughout the experimental period (complete overlapping between AMX-820 and BFU-613 oligonucleotide probes). Anammox enrichment level increased during the period of operations as visually shown in Figure 4. Autotrophic nitrifiers could not be detected. The granular size increased in time from 1.5 mm of average ferret diameter to 2.1 mm as measured by means of image analysis (data not shown).

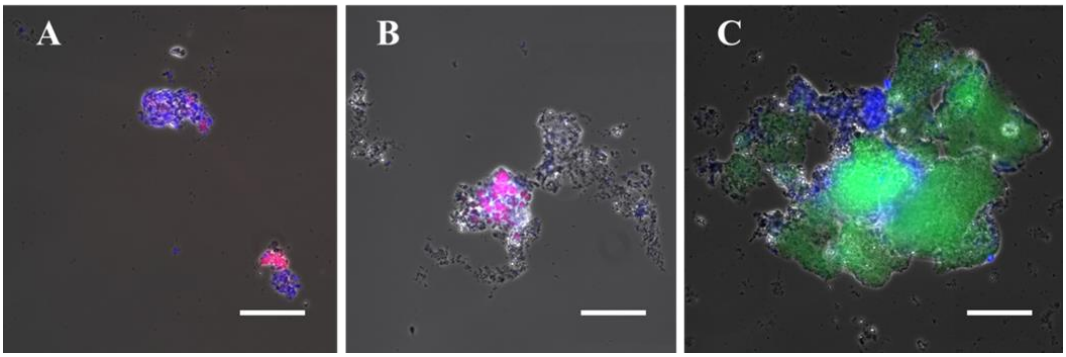

Figure 4. FISH analysis performed on granules depicting anammox (red) and eubacteria (blue) (A and B) or anammox (green) and eubacteria (blue). The FISH analysis were conducted on the inoculum (A) and on reactor mixed liquor samples fixed on day 105 (B) and on day 284 (C). Scale bars are 50 µm.

Discussion

Reactor performance

The anammox process was operated in an upflow fluidized granular sludge reactor for ten months. The reactor was fed with ammonium containing effluent of the highly loaded A-stage of the Dokhaven WWTP after settling. The reactor was operated in different phases at temperatures between 20 and 10°C. Volumetric and biomass specific total nitrogen removal rates were higher than in conventional nitrogen removal systems (for a comparison see Metcalf & Eddy, 1991). A volumetric and biomass specific nitrogen removal rate of 0.43 g-N L^{-1} d^{-1} and 0.08 mg-N g-VSS^{-1} d^{-1} were observed during operations at 10°C, respectively. This specific removal rate was higher than what reported for nitrifiers at similar temperatures (Guo et al., 2010). The volumetric nitrogen removal rates observed were comparable with conventional denitrification

systems. Considering the COD and nitrogen removal rates in the reactor the total nitrogen removal rate observed can only in minor part be justified by heterotrophic denitrification and has to a large extent to be attributed to anammox metabolism. With decreasing temperature the maximal anammox activity decreased (maximal biomass specific activity at 10 °C was 20% of the activity at 20 °C). This decrease is stronger than generally observed for nitrifying systems. Temperature coefficients were calculated from the biomass specific activity reported in Table 2; values of activation energy (Ea) of 100 and 160 kJ mol^{-1} were calculated for the temperature decrease from 20 to 15 and to 10°C, respectively. These Ea-values are higher than previously reported in similar temperature ranges (Dosta et al., 2008; Hu et al., 2013b; Hendrickx et al., 2014). Recently, Hendrickx et al. (2014) reported a biomass specific activity at 10°C of 0.044 g-N g-VSS^{-1} d^{-1} for an anammox culture cultivated at the same temperature, while Hu et al. (2013b) observed an anammox biomass specific activity of 0.036 g-N g-$VSS_{anammox}^{-1}$ d^{-1} in batch tests performed at 10°C using biomass cultivated at 12°C in a partial nitritation-anammox system. In our study the biomass specific anammox activity was not only higher than previously reported, but was also obtained in a bioreactor fed with actual wastewater from the main-stream of a sewage treatment plant (STP). Furthermore, the anammox process was operated at nitrite concentrations of 13±11 mg-N L^{-1} with peaks due to experimental errors over 60 mg-N L^{-1}, showing that high level of nitrite can be tolerated at low temperatures.

Observed volumetric and biomass specific nitrogen removal rates ranged from 0.4 g-N L^{-1} d^{-1} and 0.08 g-N g-VSS^{-1} d^{-1} to 2 g-N L^{-1} d^{-1} and 0.50 g-N g-VS^{-1} d^{-1} during operations at 10 and 20 °C, respectively. These rates were higher than those of conventional systems showing the feasibility of the implementation of a more compact and energy-efficient system for the nitrogen removal from sewage. Nevertheless it should be noted that when effluent standards are met, lower substrate concentrations in the reactor bulk might result in lower rates than observed in this study. Considering that the anammox fraction in the biomass could be roughly estimated as 40% at 20°C and 80% at 10°C, indication of anammox specific conversions rates are 1.25 and 0.10 g-N g-$VSS_{anammox}^{-1}$ d^{-1} at 20 and 10°C, respectively.

Anammox growth

The biomass concentration in the reactor increased from the initial 3.6 to 6.7 g-VSS L^{-1} at the end of the experimental period. Assuming a yield of anammox biomass growth on ammonium uptake of 0.11 g-VSS g-N^{-1} (Strous et al., 1998), an SRT of 100 days and considering the cumulative ammonium uptake, 11.1 g-VSS of anammox biomass could potentially have grown in the system during the reactor run. Granules remained dense and well-shaped throughout the experimentation despite the low temperature and heterotrophic growth. Granulation at these low temperature has been previously reported for both aerobic (e.g. Bao et al., 2009) and anaerobic biomass (e.g. Rebak et al., 1999), whilst in this study, the capability of granulation of anammox bacteria at temperatures as low as 10°C was reported for the first time. The increase of the sludge volume index of 65% registered during the reactor run, cannot justify the three-fold increase in settled granular sludge bed volume (Fig. 3), indicating that new granules were actively formed and maintained in the system throughout the reactor run. The size of the granules in the system increased in time up till an average ferret diameter of about 2 mm. This increase in granules size and the observed increase in SVI were likely a consequence of the weaker shear stress conditions experienced in the presented system compared to the full-scale internal circulation (IC) reactor where the inoculum was originating from. Throughout the experimentation nitrate was produced in the system (Fig. 3). Since in anammox metabolism nitrate production is coupled with inorganic carbon fixation,[24] this results suggested that anammox bacteria were growing in the system. Summarizing, the increase in ammonium conversion rate, nitrogen removal rate and biomass concentration (Fig. 2), the nitrate production (Fig. 3) and the increase in anammox enrichment level in time (Fig. 4) provided, according to the authors, sufficient indication that anammox bacteria had actively grown in the system at any temperature tested between 20 and 10°C (Table 1).

In this study the capability of anammox bacteria to thrive under municipal wastewater conditions was demonstrated for the first time, opening new perspective for the implementation of a more efficient (municipal) wastewater treatment chain (Kartal et al., 2010a). Nevertheless, for the application of the autotrophic nitrogen removal process, the first step of partial nitritation performed by ammonium oxidizing bacteria (AOB) has to be also accomplished

in order to produce the nitrite used in the anammox process. During partial nitritation the nitratation process performed by nitrite oxidizing bacteria (NOB) has to be suppressed. Even though anammox itself likely does not represent a problem, the managing of AOB and NOB activities in order to meet effluent standards might prove more complex for the direct application of the partial nitritation-anammox process on municipal wastewater (De Clippeleir et al., 2013a). Further studies should focus therefore on the coupling of the anammox and partial nitritation processes at municipal wastewater conditions and the simultaneous suppression of the nitratation process.

Conclusions

- Stable anammox process between 20 and 10°C in a granular sludge fluidized bed lab-scale reactor continuously fed with real A-stage effluent with the addition of nitrite
- Observed volumetric N-removal rates comparable or higher than conventional activated sludge systems, with values higher than 0.4 g-N L^{-1} d^{-1} when operated at 10°C.
- *Candidatus* Brocadia Fulgida as dominant microorganism throughout the experimentation as evaluated by FISH
- Anammox bacteria growth at main-stream conditions was shown as well as the active formation of new granules

Aknowledgements

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Chapter 8

Pilot-scale evaluation of anammox based mainstream nitrogen removal from municipal wastewater

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Abstract

Autotrophic nitrogen removal in the main stream wastewater treatment process is suggested to be a prerequisite of energy autarkic wastewater treatment plants (WWTP). Whilst the application of anammox related technologies in the side-stream is at present state of the art, the feasibility of this energy-efficient process at main-stream conditions is still under development. Lower operating temperature and ammonium concentration, together with required high nitrogen removal efficiency, represent the main challenges to face in order to reach this appealing new frontier of the waste water treatment field. In this study we report the evaluation of the process in a plug-flow granular sludge based pilot-scale reactor (4 m^3) continuously fed with the actual effluent of the A-stage of the WWTP of Dokhaven, Rotterdam. The one-stage partial nitritation-anammox system was operated for more than ten months at 19±1 °C. Observed average N-removal and ammonium conversion rates were comparable or higher than those of conventional N-removal systems, with 182±46 and 315±33 mg-N L^{-1} d^{-1}, respectively. BOD was also oxidized in the system with average removal efficiency of 90%. Heterotrophic biomass was growing preferentially in flocs and was efficiently washed out of the system. Throughout the experimentation the main bottleneck was the nitritation process that resulted in nitrite limiting conditions for the anammox conversion. Anammox bacteria were able to grow under main-stream WWTP conditions and new granules were formed and efficiently retained in the system.

Introduction

To date, most of the autotrophic nitrogen removal systems (including anaerobic ammonium oxidation (anammox)-related processes) reported in literature was operated at temperatures higher than 25 °C and influent nitrogen concentrations above 0.1 g-N L^{-1} (van Hulle et al., 2010). The application at lower

temperatures and lower nitrogen concentrations would allow extending the application of anammox-related processes to municipal sewage treatment opening new possible scenarios in designing energy producing wastewater treatment plants (Jetten et al., 1997; Siegrist et al., 2008; Kartal et al., 2010a). In order to maximize energy recovery from municipal wastewater, removal of the organic carbon as a first step would be most advantageous. This first step could either be an upflow anaerobic sludge blanket (UASB) reactor under tropical climates (Aiyuk et al., 2006) or a chemical oxygen demand (COD)-concentration step, which could be achieved by physical (e.g. sieving), chemical (e.g. precipitation) or biological (e.g. A-stage; Versprille et al., 1984) methods, or combinations thereof. The concentrated sludge can subsequently be digested anaerobically resulting in the production of biogas containing methane. The remaining liquid contains ammonium that can be removed in an autotrophic process based on anammox (Jetten et al., 1997; Kartal et al., 2010a; van der Star et al., 2007; Wett et al., 2007; Joss et al., 2009; Abma et al., 2010; Jaroszynski and Oleszkiewicz, 2011). To facilitate autotrophic nitrogen removal on pretreated sewage a process based on prevention of growth of nitrite oxidizing bacteria (NOB) and retaining aerobic ammonium oxidizing (AOB) and anammox bacteria in the system has to be developed. The main challenge for applying anammox in the main stream of a WWTP is to achieve a high-rate process with good biomass retention and low effluent nitrogen concentration at low water temperatures. A very large decrease in specific anammox activity was reported after lowering the temperature of warm reactors (Dosta et al., 2008; Isaka et al., 2008; Vázquez-Padìn et al., 2011). Despite this decrease in activity several laboratory studies have reported 1-stage partial nitritation/anammox at lower temperatures (≤25 °C) and reasonable conversion rates (Dosta et al., 2008; Isaka et al., 2008; Vázquez-Padìn et al., 2011; Winkler et al., 2011; de Clippeleir et al., 2013a; Hu et al., 2013b; Lotti et al., 2014b [**Ch.6**]; Gilbert et al., 2014; Persson et al., 2014). Recently, few studies have also reported the implementation of the anammox process (under anoxic conditions) treating low strength wastewaters at low temperature (Hendrickx et al., 2014; Lotti et al., 2014a [**Ch.7**]; Ma et al., 2013). Among these studies, Lotti et al. (2014a [**Ch.7**]) and Ma et al. (2013) used sewage samples as influent, under well controlled laboratory scale conditions. To the best of our knowledge there are no studies describing the application of an anammox-based process treating actual main-stream wastewater at pilot-scale. In natural ecosystems

such as Northern European soils and marine sediments, anammox bacteria thrive at low temperatures (<10 °C) and very low ammonium concentrations (µmol L^{-1} range) (van de Vossenberg et al., 2008; Hu et al., 2011; Lam and Kuypers, 2011), indicating that there is no fundamental limitation for the anammox process to develop under municipal wastewater conditions. This study aimed at demonstrating the feasibility of anammox based autotrophic nitrogen removal from pretreated sewage in pilot-scale. A plug-flow granular sludge based pilot-scale reactor (4 m^3) was continuously fed with the effluent of the A-stage of the WWTP of Dokhaven, Rotterdam (Kampschreur et al., 2008a). A granular sludge process with integrated tilted plate settler (TPS) was chosen with the objective to achieve adequate biomass retention and high volumetric N-conversion rates. A plug flow configuration was chosen to maximize the removal efficiency. The results obtained during 10 months of operation of the pilot plant at 19±1 °C are presented.

Material and methods

Dokhaven-Sluisjesdijk WWTP

The municipal wastewater treatment plant of Dokhaven (Rotterdam, The Netherlands) has a treatment capacity of about 560,000 p.e.. The plant is composed of two distinct parts: the main water line and the sludge treatment facilities. The wastewater applied for conducting this study originates from the main line of the treatment scheme. For a description of the sludge-line as well as the entire WWTP please see (van der Star et al., 2007) and Kampschreur et al. (2008a). The installation of Dokhaven was built entirely underground in the late 1970s and designed as an A-B system (Kampschreur et al., 2008a). In the A-stage (hydraulic retention time, HRT, 1h in dry weather conditions, DWC; solids retention time, SRT, 0.3 days) biochemical oxygen demand (BOD) is removed in a high-loaded reactor and mostly converted into sludge with the aim of maximizing biogas production by anaerobic digestion. In the B-stage (HRT 3h in DWC; SRT 7 days), the remaining BOD is oxidized and ammonium nitrified to nitrate. The treatment scheme was originally not designed for N-removal. Depending on the inflow of the treatment plant (dry or rain weather condition) part of the nitrate-rich effluent of the B-stage is recirculated to the head of the plant for partial denitrification. The total nitrogen removal of the plant is

currently limited to a yearly average of 60%, with an effluent concentration of 15-20 mg-N L^{-1} mainly in the form of nitrate. Under current legislation this effluent concentration needs to be decreased; the application of anammox could be a compact solution, avoiding a conventional and, because of the underground situation, highly expensive retrofit of the treatment plant. Phosphorus is chemically removed by ferric chloride ($FeCl_3$) dosage in the A-stage, reaching an effluent concentration of about 1 mg-P L^{-1}. In Figure 1 the current treatment scheme of the Dokhaven WWTP is shown as well as how it would be after replacement of B-stage with an anammox based N-removal process.

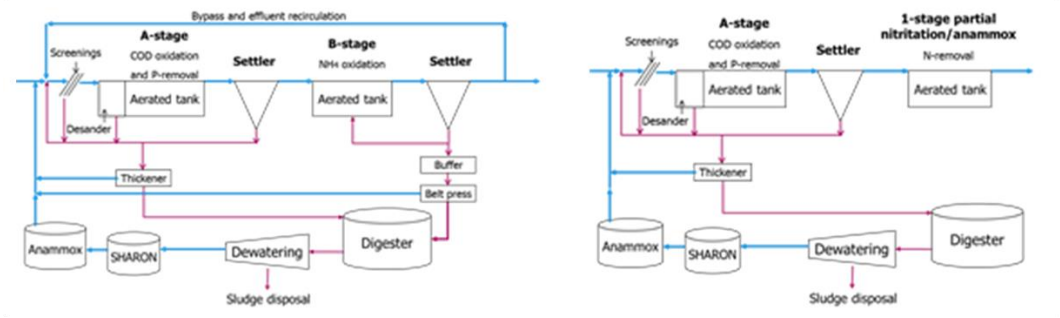

Figure 1. Actual (left) and proposed (right) treatment scheme of Dokhaven WWTP.

Pilot set-up and operating conditions

Experiments were conducted in a plug-flow granular pilot-scale reactor (working volume of 4 m^3, Figure 2) consisting of four identical compartments and operated from July 2012 to April 2013. The reactor was equipped with an external TPS for granular biomass retention operating at a surface loading rate of 5-15 m h^{-1}. Oxygen transfer and mixing were provided by fine-bubbles aerators. The dissolved oxygen (DO) concentration was controlled at 0-2 mg-O_2 L^{-1} by a gas recycle system, while maintaining a constant gas velocity and thereby mixing intensity (Mosquera-Corral et al., 2005). pH was controlled at 7.0-7.5 by addition of NaOH (0.1 mol L^{-1}). The influent of the pilot reactor was the effluent of the A-stage after settling, characterized by an average BOD/N ratio of 0.67 g-O_2 g-N^{-1}. Before dosage to the reactor the influent was buffered

in a 2 m^3 tank (HRT of 0.75-1 h) that was maintained at 19±1°C by a thermostat. The HRT of the pilot reactor varied between 1.5 and 2 h. An NH_4Cl solution (20%) was transiently dosed to the pilot influent to compensate for the lower ammonium concentration caused by the recirculation in the main line for denitrification as described above. When operated in full anammox mode such recirculation would not occur. The reactor was inoculated with granular biomass (~800 L settled sludge) from the full-scale 1-stage partial nitritation/ANAMMOX® reactor of Olburgen (The Netherlands; Abma et al., 2010). Bio-augmentation was not performed during the experimental period.

Figure 2. Setup of the one-stage partial nitritation/anammox pilot reactor used for the experimentation.

SRT determination

The solids retention time (SRT) was calculated through mass balance over the sludge. In this study distinction was made between the SRT of granules and the SRT of suspended/flocculent biomass and only the former was estimated (Winkler et al., 2012c). The granular part was separated from the flocculent part by means of a sieve with 200 µm mesh size before measuring the solid concentration in the reactor and in the effluent. The influent contained no granules.

Biomass yield

Estimation of the community composition was based on literature values of the COD-based biomass yield for heterotrophs ($Y_{XH/COD}$) and of the ammonium-based biomass yield for autotrophs ($Y_{XA/NH4}$), respectively. It was assumed that all COD consumed would be metabolized by heterotrophic bacteria. Both aerobic (Heterotrophs (O_2)) and anoxic (Heterotrophs (NO_3^-)) heterotrophic consumption of COD were considered by means of different yields. Two different scenarios were considered for the nitrogen cycle:

i) considering the stoichiometric coupling of AOB and anammox metabolisms (Rate-$NH_{4,AOB}$=1.32·Rate-$NH_{4,anammox}$), the ammonium consumption rate was assumed to be divided between AOB (57%) and anammox bacteria (43%).;

ii) half of the ammonium converted was assumed to be oxidized to nitrate by NOB, while the other half was assumed to be divided between AOB and anammox again considering the stoichiometric coupling described above.

For the conversion from COD to VSS a factor of 1.4 was used (Scherer et al., 1983).

Ex-situ batch test

Anoxic ex-situ manometric batch tests were regularly conducted at 20 °C in order to measure the biomass specific maximum anammox activity (MSAA, mg-N_2 g-VSS^{-1} d^{-1}) as described elsewhere (Lotti et al., 2012b [**Ch.4**]). Aerobic ex-situ batch test were regularly conducted at 20 °C in order to measure the biomass specific maximum AOB and NOB activity (mg-NH_4-N g-VSS^{-1} d^{-1} and mg-NO_3-N g-VSS^{-1} d^{-1}, respectively). During the aerobic ex-situ batch test DO was controlled at 80% of the saturation and starting ammonium and nitrite concentration were both set at 40 mg-N L^{-1} by the addition of concentrated solutions prepared with $(NH_4)_2SO_4$ and $NaNO_2$, respectively. Conversion rates were measured by measuring N-species concentration in time (one sample every 15 min for a total of about ten samples). Ex-situ aerobic batch tests were performed with either granules or a mix of granules and flocs. The pH was maintained at 7.5 using 25 mM HEPES (N-2-hydroxyethyl-piperazine-N0-2-ethane sulfonic acid) buffer. The pH value of the medium was set to 7.5 with 0.1M NaOH or H_2SO_4.

Analytical methods

Analytical measurements for inorganic N-compounds were conducted with Dr. Lange test kits, while measurements for COD, BOD and biomass concentration (as g-VSS L^{-1}) according to standard methods (APHA, 1998). The ratio between nitrate production and ammonium conversion rate (NO_3/NH_4-conversion ratio hereafter) was calculated as indicator of the significance of NOB activity (nitratation) in the overall autotrophic nitrogen removal process: a minimum theoretical NO_3/NH_4-conversion ratio of 0.11 represents a process where nitratation is completely suppressed and AOB and anammox activities are balanced to maximize nitrogen elimination (i.e. aerobic ammonium conversion rate by AOB is 1.32 times higher than the anaerobic ammonium oxidation rate by anammox bacteria; the anammox stoichiometry is according to Strous et al., 1998). The maximum theoretical value of one represents full nitrification to nitrate (ammonium removal rate equal to nitrate production rate). Fluorescence in situ hybridization (FISH) was conducted as described by Hu et al. (2013b) with probes specific for Kuenenia and Brocadia-like anammox bacteria (AMX-820), Kuenenia stuttgartiensis (KST1273), Brocadia Fulgida (BFU613), AOB (mix of NEU653, NSO190 and NSO1225), NOB (mix of NTSPA0712 and NIT1035). The references for these probes can be found in Lotti et al. (2014a [**Ch.7**]). Microscopic images from sliced and entire granules were taken with a light microscope (Axioplan 2, Zeiss). Slicing was accomplished after fixation in 4% paraformaldehyde. Granules were embedded in a tissue freezing medium (Leica Microsystems) hardened by freezing (-20 °C) and cut in the frozen state with a microtome-cryostat (Leica CM1900-Cryostat) into 20 µm thin slices. Dried slices were kept on a microscopic glass slide, and FISH was performed. The morphology and size distribution of the granules were monitored by image analyses (IA), with a Lexmark Optra image analysis system (Tijhuis et al., 1994). The volume assessment of granular and non-granular sludge was performed in regular Imhoff cones (1 L) after settling time of 5 and 30 minutes. The distinction between granular and non-granular biomass, clearly visible by eyes, was confirmed by the fact that the volume of the former stayed

about constant, while the volume of the latter sharply decreased between 5 and 30 minutes due to compaction.

Results

Reactor performance

Throughout the whole experimental period (including disturbance periods) nitrogen removal rates fluctuated between 30 and 240 mg-N L^{-1} d^{-1} and amounted on average to 182±47 mg-N L^{-1} d^{-1} during periods without technical problems. The HRT of the system was 1.5-2.0 h while the SRT for granular sludge was estimated as 120±30 d. The biomass concentration in the reactor was 4±0.5 g-VSS L^{-1}, corresponding to an average biomass specific N-removal rate of 46 mg-N g-VSS^{-1} d^{-1} during periods without technical problems. During the operational period described in this study, the process experienced a number of technical problems: i) failure of the influent pump resulting in prolonged starvation and repeated exposure of the biomass to anaerobic conditions (end December 2012- half January 2013); ii) failure of the return-sludge pump from the TPS compartment resulting in uneven distribution of biomass in the system and exposure to anaerobic conditions (first week of March 2013); iii) operational problems with the A-stage settler in the main-line of the Dokhaven WWTP resulting in high solid content in the influent to the pilot (third week of March 2013). Ex-situ batch test results showed that the anammox biomass specific activity was maintained despite these temporary failures/disturbances throughout the experimentation (Figure 3). Three periods of operation of 2-3 weeks in which no technical failures/disturbances occurred were analyzed and closely evaluated (Dec. 2012, Feb. and Apr. 2013).

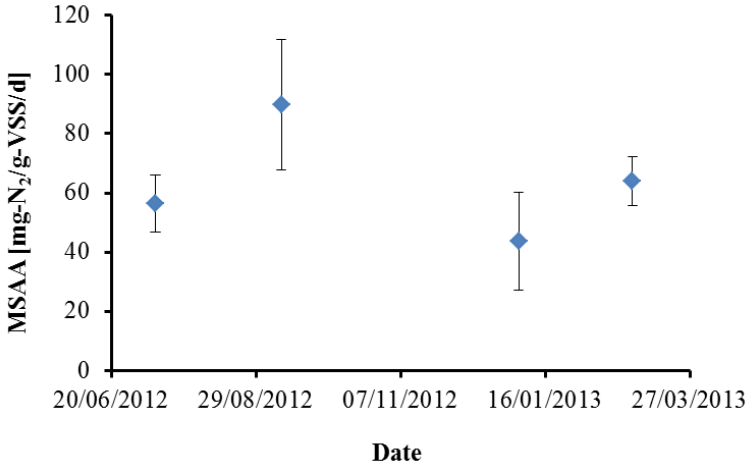

Figure 3. Biomass specific maximum anammox activity (MSAA, mg-N_2 g-VSS^{-1} d^{-1}) as measured during anoxic ex-situ manometric batch tests conducted in triplicates.

The average influent and effluent composition during periods of stable (undisturbed) operations are shown in Table 1; whereas the conversions in the same period are given in Table 2.

Table 1. Influent and effluent average composition during periods without technical problems.

		NH_4^+	NO_2^-	NO_3^-	P-tot	BOD	COD	TSS
		mg-N L^{-1}	mg-N L^{-1}	mg-N L^{-1}	mg-P L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}
Influent	Average	26.8	1.0	1.7	1.3	17.4	62.1	30
	Std.Dev.	5.3	0.7	1.8	0.6	7.3	17.0	10
Effluent	Average	6.8	2.3	9.1	1.2	1.7	25.3	30
	Std.Dev.	5.4	0.4	3.1	0.5	0.8	5.9	30

Table 2. Average reactor performance during periods without technical problems.

	NH4 Conv. Rate	**NH4 Conv. Efficiency**	**Tot-N Removal Rate**	**Tot-N Removal Efficiency**	**NO_3/NH_4 conversion ratio**
	[mg-N L^{-1} d^{-1}]	[%]	[mg-N L^{-1} d^{-1}]	[%]	[-]
Dec. 2012	297±26	75±12	158±17	37±5	0.41±0.06
Feb. 2013	319±40	86±11	189±59	46±13	0.35±0.16
Apr. 2013	324±15	64±7	191±27	33±4	0.36±0.04

In order to compare the performance of the pilot installation in the periods reported in Table 1 and Table 2 with the B-stage of the Dokhaven WWTP in periods with similar conditions, only the measurement of the latter relative to the period between May and September 2012 were considered (Figure 4). In fact, during that period, the wastewater temperature (20.0±1.7 °C) was comparable to the operative temperature in the pilot installation (19±1°C). The effluent concentrations for this pilot plant testing period show that the conversion rates and removal efficiencies for ammonia and total N are similar or better than in the full-scale system at similar HRT in both installations (Figure 4).

Nitrite formation was found to be the rate limiting process during the whole experimental period. When nitrite was temporarily added to the influent wastewater (10÷44 mg-NO_2-N L^{-1} added), higher N-removal rates up to 550 mg-N L^{-1} d^{-1} were observed. The DO was not changed in this period showing that anammox bacteria were limited by nitrite and not inhibited by oxygen.

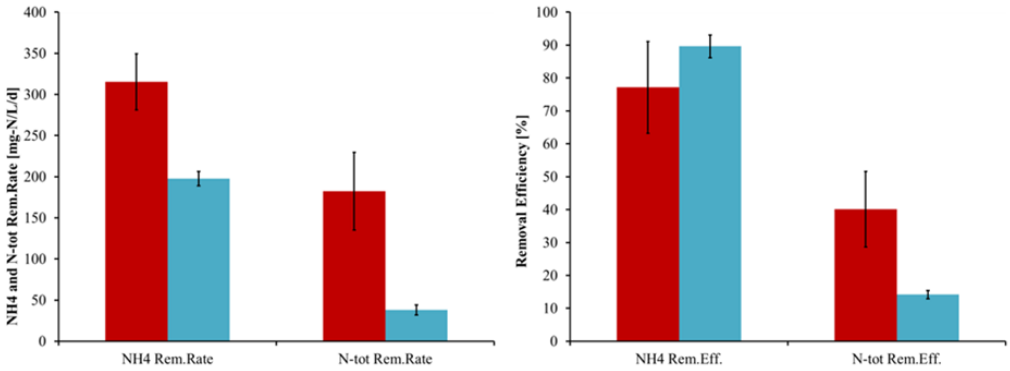

Figure 4. Comparison of the performance between the pilot installation (*red bars*) and the B-stage of the Dokhaven WWTP (*blue bars*). For the latter only the period May÷September 2012 was considered (wastewater temperature of 20.0±1.7°C). *Left*: ammonium and total inorganic nitrogen removal rate [mg-N L^{-1} d^{-1}]; *Right*: ammonium and total inorganic nitrogen removal efficiency [%].

Results from ex-situ anoxic batch tests conducted at 20°C showed that the maximum nitrogen removal capacity was always higher than the N-removal rate observed in the pilot (data not shown) indicating that anammox biomass was always under substrate (nitrite) limiting conditions. The development of ammonium conversion and nitrogen removal rates during periods of improving performances (e.g. positive trend of the N-removal rates) are reported in Figure 5 together with the NO_3/NH_4- conversion ratio and the nitrite concentration in the effluent. It should be noted that the concomitant presence of significant (compared to the autotrophic nitrogen removal process) heterotrophic denitrification and fluctuation in the influent BOD/N ratio would make the correct interpretation of the NO_3/NH_4-conversion ratio more difficult. Nevertheless in the system described here the nitrogen removal was mainly autotrophic (and not heterotrophic) as discussed later in a dedicated section.

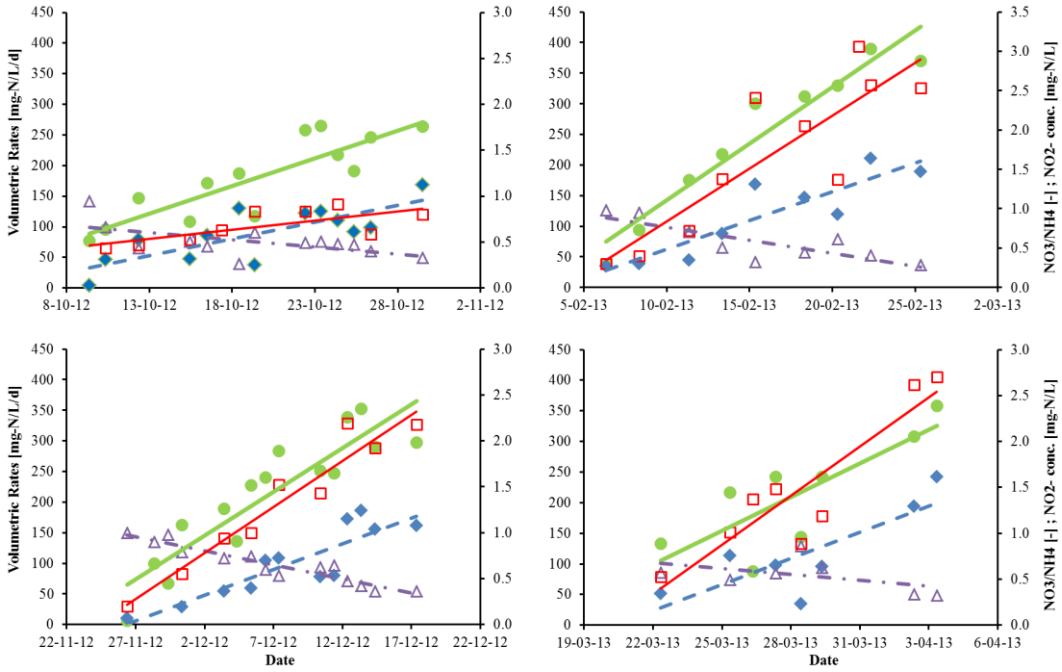

Figure 5. Change in volumetric ammonium conversion rate (green circles) and total nitrogen removal rate (blue diamonds) [*left y-axis*], NO3/NH4-conversion ratio (purple triangles) and nitrite effluent concentration (red squares) [*right y-axis*] during four distinct periods of increasing performances (i.e. positive trend of total nitrogen removal rate). The DO was constant (1.5±0.2 mgO_2 L^{-1}) during these four periods.

An increase in total nitrogen removal rate was always associated with an increase in ammonium oxidation rate and the accumulation of nitrite in the effluent. The NO_3/NH_4-conversion ratio also decreases indicating repression of NOB and more conversion by anammox bacteria. The increasing ammonium conversion at a similar dissolved oxygen concentration in the bulk liquid leads to more oxygen limitation in the granular sludge, which leads to NOB activity decrease. The maximum biomass specific anammox activity (MSAA) was regularly measured under anoxic conditions and values of 63±17 mg-N_2 g-VSS^{-1} d^{-1} were measured. In a time period of 61 days (July-September 2012) MSAA increased from 57 to 90 mg-N_2 g-VSS^{-1} d^{-1} (Figure 3). Considering that for bacteria cultivated under certain conditions the biomass specific maximum activity is constant, the observed increase of MSAA in time indicated that anammox bacteria grew and were retained in the system.

Biomass Production and Composition

To facilitate autotrophic nitrogen removal the prevention of the accumulation of heterotrophic biomass in the system would be desirable. The maximum biomass concentration in a system is technically limited. The accumulation of heterotrophic biomass would therefore reduce the amount of the AOB and anammox population present, resulting in lower conversion rates.

Table 3. Biomass yields for anammox, AOB, NOB and heterotrophic bacteria and the corresponding biomass production rate and relative community composition according to average substrate consumption rates (453 mg-COD L^{-1} d^{-1} and 216 mg-NH_4-N L^{-1} d^{-1}). In parenthesis are the results of the calculations according to the scenario (ii) in which NOB activity was considered (see Materials and Methods section). Both oxygen (*column* O_2) and nitrate (*column* NO_3^-) have been considered as electron acceptor in the COD oxidation process.

	$Y_{XH/COD}$	$Y_{XA/NH4}$	$Rate_X$	Community Composition		Reference
				O_2	NO_3^-	
	g-COD g-COD^{-1}	g-COD g-NH_4-N^{-1}	mg-VSS L^{-1} d^{-1}	%	%	
anammox		0.16	11 (5)	4.7 (2.3)	5.4 (2.6)	[Strous et al., 1998]
AOB		0.15	13 (18)	5.8 (7.9)	6.6 (9.0)	[Wiesmann, 1994]
NOB		0.041	(3)	(1.4)	(1.6)	[Wiesmann, 1994]
Heterotrophs (O_2)	0.63		204	89.5 (88.4)		[Henze et al., 2000]
Heterotrophs (NO_3^-)	0.54		175		88.0 (86.8)	[Henze et al., 2000]

In order to investigate if selective wash out of heterotrophic biomass growing in flocs occurred, we evaluated the biomass production and microbial

community composition based on average substrate consumption rates observed during the whole experimental period (453 mg-COD L^{-1} d^{-1}; 216 mg-NH_4-N L^{-1} d^{-1}). If autotrophic and heterotrophic biomass would experience the same SRT (i.e. both growing in granules) a sludge productivity of about 200 mg-VSS L^{-1} d^{-1} would be expected and the system would be strongly dominated by heterotrophs (over 86%, Table 3). The difference between oxygen and nitrate as electron acceptor of the COD oxidation was negligible for the resultant community composition as in either case the heterotrophic biomass would dominate the population. The enrichment level of AOB and anammox would be lower than 10% either considering or neglecting NOB activity (Table 3).

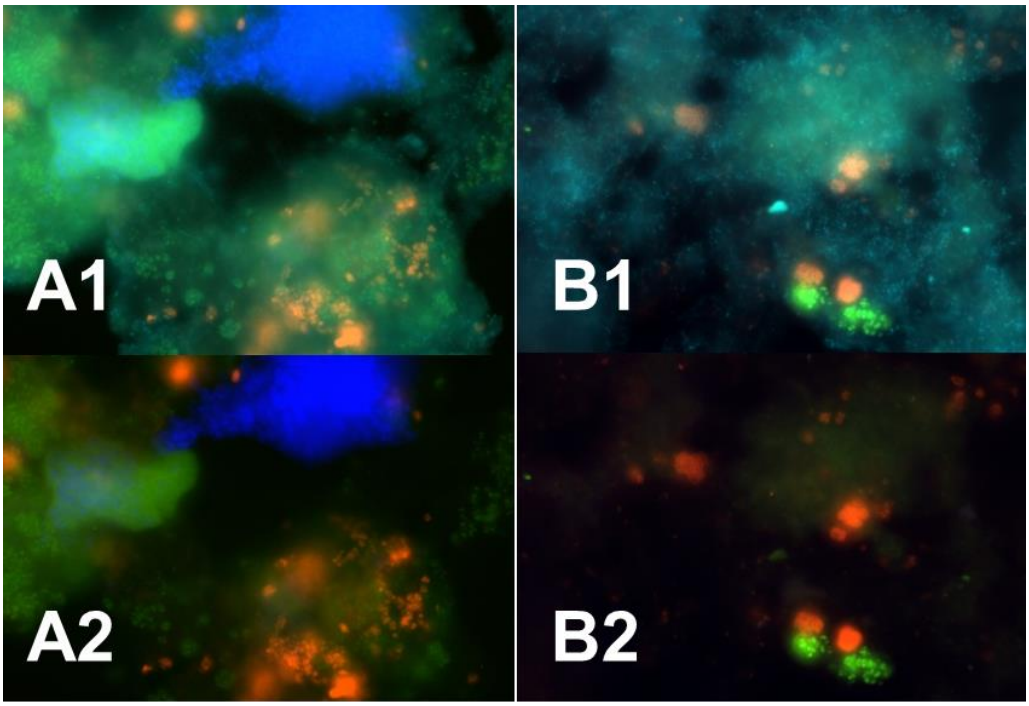

Figure 6. A: FISH analysis performed on granules depicting anammox (dark blue), AOBs (green) and NOBs (red) and with (A1) or without (A2) DNA staining with DAPI (light blue); **B:** FISH analysis performed on suspended flocs depicting anammox (dark blue), AOBs (green), NOBs (red) and with (B1) or without (B2) DNA staining with DAPI (light blue).

From the qualitative evaluation of the results of FISH analysis conducted throughout the experimentation we could estimate that autotrophic bacteria

(i.e. anammox, AOB and NOB) microorganisms always accounted for more than 50% of the total biomass present in the system (e.g. as shown in Figure 6).

Granular sludge characteristics

Autotrophic nitrogen removal was performed in a granular sludge based system. Granules enriched in aerobic ammonium-oxidizing and anammox bacteria were stable in the system despite 30±10 mg-TSS L^{-1} in the influent and heterotrophic growth on the BOD load. Anammox bacteria were mainly present in the granular sludge together with nitrifiers (AOBs and NOBs) and a relatively minor fraction (<50%) of other microorganisms (i.e. heterotrophs) (Figure 6, A1-A2 and Figure 7, B). Nitrifiers were present also in the flocculent biomass, but in smaller relative abundance with respect to the granular biomass (Figure 6, B2). The flocculent biomass was dominated by other microorganisms (i.e. heterotrophs) and the presence of anammox bacteria was negligible (Figure 6, B1).

FISH analysis was performed also on sliced granules. The results from the analysis conducted on a reactor sample collected in April 2013 are depicted in Figure 7.

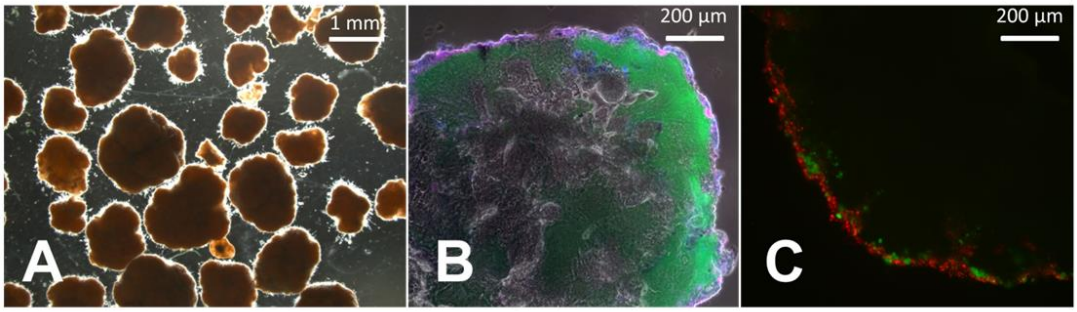

Figure 7. Microscopic images of granules (**A**) as well as FISH image of sliced granules (**B** and **C**). FISH was conducted on sliced granules and hybridization was accomplished with Cy3-red (AOB+NOB), Cy5-blue (Eubacteria), and Fluos-green (anammox)-labeled probes (**B**) and with Cy3-red (AOB) and Fluos-green (NOB)-labeled probes (**C**).

According to measurements in Imhoff cones, the settled sludge volume occupied 220±30 mL L^{-1} on average (corresponding to an average SVI of 50±15 mL g-SS^{-1}), of which 200±30 and 20±30 mL L^{-1} were granules and flocs,

respectively. The granular sludge volume increased during the operational period from 200±20 to 230±30 mL L^{-1}, while the volume of flocs stayed about constant. Ex-situ aerobic batch tests performed with either granules or a mix of granules and flocs, showed a similar biomass specific aerobic ammonium conversion rate (data not shown). Given the contribution of granules and flocs to the overall sludge volume (~10:1), it was clear that the aerobic ammonium conversion capacity of the system mainly resided in the granules. The size distribution of the granules was measured twice on December 2012 and on March 2013 indicating an increase in granule size during this period (Figure 8).

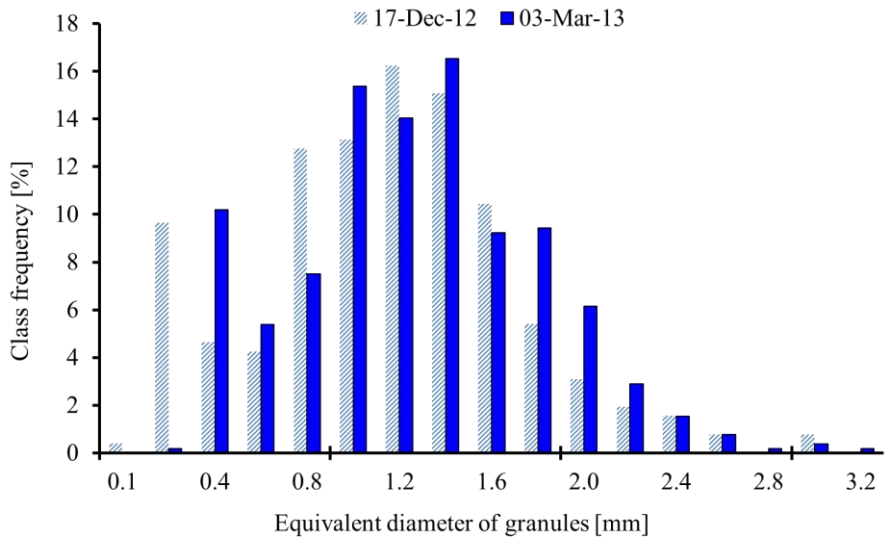

Figure 8. Size distribution of granular biomass. More than 1000 particles analyzed. Analysis was conducted on December the 17th 2012 (*light blue bars*) and on March the 13th 2013 (*dark blue bars*).

Discussion

Potential of anammox based system

The system described here was inoculated only once with granular sludge enriched in AOB and anammox bacteria. The anammox population was dominated by *Candidatus* Brocadia Fulgida (Kartal et al., 2008) throughout the

experimentation based on FISH analysis (complete overlapping between Bfu-613 and Amx-820 probes). The volumetric and biomass specific ammonium conversion rates reported in this study (Table 1, 2) were in the same order of magnitude as a B-stage system and superior to a conventional activated sludge process (CAS, Figure 9). Please note that the ammonium conversion rates reported in literature for CAS systems are based on yearly average data and therefore comprise also data relative to the winter periods. Considering the biomass concentration in our system (4±0.5 g-VSS L^{-1}, corresponding to ~220 mL L^{-1}) and the much higher potential biomass concentration in granular systems (e.g. 6-12 g-VSS L^{-1} according to Liu and Tay [2004] and ~600 mL L^{-1} according to Abma et al. [2010], respectively), the reported results appear even more promising for full scale application.

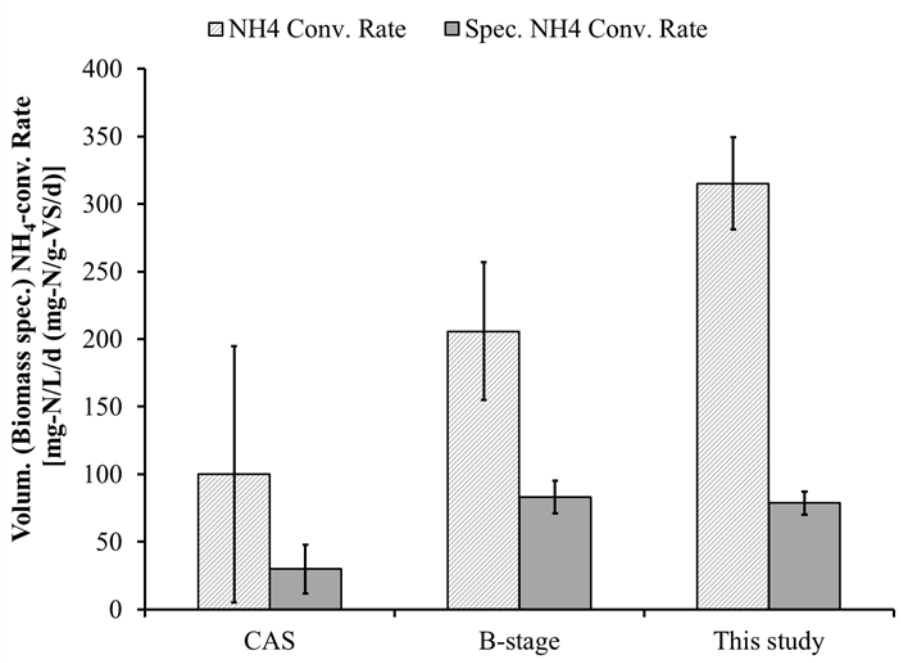

Figure 9. Volumetric (light gray) and biomass specific (dark gray) ammonium conversion rates from CAS systems (yearly average) (Lotti et al., 2012b [**Ch.4**]), B-stage (average data for the months May-September for the period 2010-2012 from the Dokhaven WWTP) and observed in our pilot installation.

Heterotrophic or autotrophic N-removal ?

The total nitrogen removal rate increased during several periods of stable operations, reaching average levels of 180 mg-N L^{-1} d^{-1} (Table 2), which were comparable with conventional denitrification systems (Metcalf and Eddy, 1991). Anammox bacteria were responsible for most of the nitrogen removal, since in periods with high nitrogen removal rates (160-240 mg-N L^{-1} d^{-1}), BOD conversion rate was 198±87 mg-BOD L^{-1} d^{-1}, corresponding to a potential denitrification (nitrate to nitrogen gas) of 69±30 mg-N L^{-1} d^{-1}. However, considering the oxygen level in the reactor during those periods (>1 mg-O_2 L^{-1}), the presence of heterotrophic biomass in flocs and the continuous mixing, oxygen appeared as the most likely electron acceptor used for BOD oxidation instead of oxidized nitrogen compounds such as NO_2^- and NO_3^-. Considering the granular biomass the heterotrophic bacteria were mainly located in the external rim of the granules (Figure 7, B) where the presence of the anoxic conditions necessary for denitrification were unlikely. Furthermore, we could not find a positive correlation between total nitrogen and BOD removal rates. Also in literature it was reported that fast growing aerobic heterotrophs preferentially grow where the availability of substrates (O_2 and BOD) is higher due to the less pronounced gradient of substrate concentrations (i.e. in flocs or in the external rim of granules; Picioreanu et al., 2004). Overall, the results presented here showed that the measured increase in total nitrogen removal rate could not be explained by heterotrophic denitrification and was mainly due to anammox activity.

Potential of granular sludge based system

Granules were successfully maintained throughout the experimental period under main-stream conditions. Despite the presence of COD and solids in the influent and their fluctuations in time (Table 1), granules maintained their shape (Figure 8), consistence and excellent settling properties. The granular sludge based system described here was resistant to process failures/disturbances and technical issues. The system was able to recover the capability of nitrogen removal and COD conversion even after extended periods of anaerobiosis and starvation. The wash-out of flocculent material seemed to be essential and on average the effluent contained 30 mg-TSS L^{-1}. This can be improved by

optimizing the intermediate settler after the A-stage. If this is not possible an effluent polishing (e.g. by sand filtration) might be needed depending on prevailing effluent discharge criteria. The operational flexibility of the TPS with respect to hydraulic variations, allowed an efficient solid liquid separation in which granules are retained and most of the flocs washed out, making the system capable of dealing with sudden and sharp increase in solid load as indicated when the A-stage settler had a failure. The increased solid load was maintained for about one week resulting in a protozoa bloom. These protozoa entirely covered the surface of the granules, anchoring the stalks inside the biomass and feeding on the suspended particles in the bulk liquid. The increased amount of particulate COD and the products of predation resulted in a sudden increase of the flocculent biomass in the system resulting in fluctuating DO concentrations and in a decrease of the ammonium conversion rate. Nevertheless the temporary increase of shear stress due to higher mixing power and the increase in superficial loading rate in the TPS, caused the washout of most of the protozoa population, restoring the usual features of the granules and the ammonium conversion capacity of the system (Figure 10). At the same time, the total volume of granular biomass in the system remained constant, suggesting that no granules were washed out due to increased superficial loading rate and there were no structural changes.

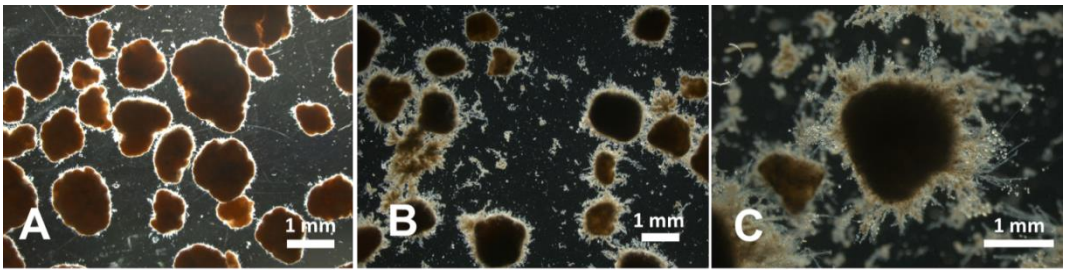

Figure 10. Black background picture of granules during normal operations (**A**) and during the episode of vorticella-like protozoa blooming (**B** and **C**).

FISH images (Figure 6, A-B) showed a higher fraction of autotrophic bacteria as expected from calculations assuming that all bacteria of the microbial community experienced the same SRT (Table 3). According to this calculation the microbial population would consist of over 88% heterotrophs and only a minor fraction of autotrophic bacteria (slightly higher than 10%), with a small prevalence of AOB abundance with respect to anammox (Table 3). The analysis

of effluent and reactor solids (Figure 6, A-B) together with the above considerations on expected microbial community composition (Table 3) suggested that heterotrophs mainly grew in suspended flocs and were efficiently washed out of the system. The estimated SRT for granules (120±30 d), the absence of bioaugmentation and the length of the reactor run, demonstrated that new granules were formed in the pilot system. Together with the qualitative evaluation of the microbial composition of the granules performed by FISH (Figure 6, A1-A2; Figure 7, B) and the stability of the MSAA in time (Figure 3), the results reported indicate that anammox bacteria actively grew in the system. Visual observation of the microbial community composition within a granule confirmed that anammox bacteria were located in the middle of the granule and a smaller fraction of AOB, NOB and heterotrophs were located on the outer shell of the granule (Figure 7, B), whereas flocs were strongly dominated by heterotrophs with a smaller fraction of AOB and NOB (Figure 6, B1-B2). Therefore, due to preferential wash out of flocs and erosion of the surface of the granules, AOB/NOB and, most of all, heterotrophs would experience a shorter retention time. This would lead to a higher SRT for granules (especially the inner part where anammox reside) and hence to an enrichment of anammox bacteria in the biomass (Winkler et al., 2012c).

Potential of operations at higher DO

The decrease of the NO_3/NH_4-ratio during period of nitrite accumulation shows that the increase of NOB activity was smaller than the increase of AOB activity during periods of stable operations (Figure 5). These results indicate that NOB suppression was mainly based on oxygen concentration rather than nitrite concentration. In literature the debate is open on the actual values of the oxygen half-saturation constants for AOB and NOB involved in wastewater treatment systems (Sin et al., 2008). The oxygen level adopted in the system described here (>1 mg-O_2 L^{-1}) was normally considered too high for the suppression on nitrite oxidation (Bernet et al., 2001; Blackburne et al., 2008b; Joss et al., 2009) in conventional activated sludge systems. The biomass grown in the system described here consisted of a similar proportion of AOB and NOB, suggesting the potential for complete nitrification (Figure 6, A-B). Nevertheless the nitratation process was partially suppressed during pilot operations and

nitrite accumulated in the effluent. Biomass that is organized in biofilm therefore, seems to represents an additional advantage in NOB suppression even when high oxygen levels are maintained (Vázquez-Padìn et al., 2010). Considering that segregation of the microbial populations takes place in a biofilm based on growth rate (Picioreanu et al., 2004), it was expected that AOB would reside mainly in the external part of the aerobic layer, relegating NOB to the more internal parts of the aerobic outer rim of the granules. This positioning of AOB and NOB in a biofilm, which represents a clear advantage for the former in the competition for oxygen, was reported before (Vlaeminck et al., 2010) and was occasionally visible in our granules as well (Figure 7, B). Larger aggregates were shown to enhance nitratation suppression also by modeling (Volcke et al., 2010) and experimentally (Winkler et al., 2012b). Engineering wise, operations at moderate DO levels (around 1-2 mg L^{-1}) would be beneficial for the accuracy of the control and would allow higher specific ammonium conversion rates. Due to their higher biomass concentration and the possibility to successfully operate at higher DO levels, granular systems appear as intrinsically more compact than flocculent or hybrid (mixture of flocs and granules) systems.

Practical application

The current results demonstrate the feasibility of anammox based process (completely autotrophic nitrogen removal process) at 19±1°C. The temperature at the Dokhaven WWTP is at, or above, this temperature during 5-6 months per year. In winter, temperature can drop to as low as 9°C for short periods of time. Further work is required to demonstrate the feasibility of the proposed anammox based process throughout the year. Nitrogen removal at Dokhaven WWTP is currently achieved by applying an effluent recirculation from the B to the A stage for pre-denitrification. With the anammox based process this recycle is no longer required, as denitrification by anammox takes place in the same reactor as partial nitritation. The theoretical potential of the completely autotrophic nitrogen removal process is to reach up to 89% N removal (11% nitrate production according to anammox stoichiometry; Strous et al., 1998), assuming no residual ammonium and nitrite. Current results showed an achieved removal efficiency of up to 46%, showing that progress still needs to

be/ made in lowering residual ammonium and nitrite concentrations. Furthermore, the NO_3/NH_4-conversion ratio decreased to a minimum of 0.35, which is still well above the theoretical potential of 0.11. To further decrease this ratio, more efficient suppression of the NOB activity is required.

Conclusions

The reported results show that the proposed reactor configuration with granular sludge has the potential to be successfully applied for the completely autotrophic nitrogen removal from the mainstream of WWTPs. The system was shown to efficiently retain granules enriched in anammox bacteria with a small fraction of nitrifiers and heterotrophs located in the outer rim. At the same time, suspended flocs enriched in heterotrophs and a small fraction of nitrifiers were preferentially washed-out. New granules were formed, while also the granule size effectively increased, and efficiently retained in the system. Throughout the experimentation the main bottleneck was represented by the nitritation process since anammox activity was always present in overcapacity. Observed N-removal and especially ammonium conversion rates were higher than those of conventional N-remo val systems. Furthermore, considering the higher biomass concentration obtainable in granular systems and the possibility for further anammox enrichment in the biomass, kinetics much higher than conventional systems appear to be feasible. However a better and stable effluent quality and attention for suspended solids handling would be needed for widespread introduction of an energy neutral WWTP based on A-stage combined with partial nitritation/anammox technology.

Aknowledgements

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Chapter 9

Outlook and Perspectives

The application of the combined partial nitritation/anammox process for nitrogen removal from municipal wastewater (MWW) is gaining more and more attention in the past years. After the first publications indicating the substantial benefits of its implementation in the mainstream of sewage treatment plants (Jetten et al., 1997; Mulder, 2003; van Loosdrecht et al., 2004; Kartal et al., 2010a) many research groups around the world are currently engaged with this challenging quest (*this thesis*; Dosta et al., 2008; Isaka et al., 2008; Vàzquez-Padìn et al., 2011; Winkler et al., 2011; Hendrickx et al., 2012; Cao et al., 2013; De Clippeleir et al., 2013a; Hu et al., 2013b; Wett et al., 2013; Gilbert et al., 2014; Regmi et al., 2014; Isanta et al., 2015; Laureni et al., 2015; Morales et al., 2015 *among others*). Due to all these research efforts many steps forward have been made, nevertheless some important issues need still to be solved.

Without pretending to be exhaustive in this section an attempt is made for highlighting those aspects which still need to be understood properly and therefore deserve further studies.

Suppression of the nitratation process

Maintaining sufficiently high nitrogen removal rates (e.g. above 50 gN m^{-3} d^{-1}; Metcalf & Eddy, 2013) whilst reliably achieving the required effluent quality at temperatures of 10-25°C and with low influent nitrogen concentrations (lower than 100 gN m^{-3}) represent one of the main challenges towards PN/anammox application to sewage treatment.

Recently, nitrogen removal from synthetic wastewater without COD at nitrogen concentrations representative for MWW and low temperature has been reported.

Combined partial nitritation/anammox reactors can be established at both 12°C (Hu et al. 2013b) and 10°C (Gilbert et al. 2014). Volumetric nitrogen removal rates reported by Gilbert et al. (2014) and by Hu et al. (2013b) were however low (15 and 25 gN m^{-3} d^{-1}, respectively) mainly due to very low biomass specific conversion rates (36 gN $kgVSS^{-1}$ d^{-1} and 1.7 gN $gTSS^{-1}$ d^{-1}, respectively). In Chapters 6 of this thesis, combined PN/anammox process in a granular system

at 10°C could be established at higher nitrogen removal rates of almost 200 gN m^{-3} d^{-1} corresponding to biomass specific anammox activity of 54 gN $kgVSS^{-1}$ d^{-1}.

Although the significant negative impact of low temperature on anammox activity (Ch. 5) a high nitrogen removal rate of 430 gN m^{-3} d^{-1} has been demonstrated using an upflow fluidized anammox granular sludge reactor treating pretreated municipal wastewater with the addition of nitrite (Ch.7). In this case biomass specific anammox activity was up to 80 gN $kgVSS^{-1}$ d^{-1}.

At low temperature then, volumetric and biomass specific nitrogen removal rates observed in anoxic anammox systems supplied with nitrite were much higher than those observed in combined PN/anammox systems. This was confirmed also in Chapter 8 where higher nitrogen removal rates were observed when nitrite was externally dosed compared to regular operations where nitrite was produced by AOB. These results indicate that the main bottleneck for achieving stable nitrogen removal at the desired rate might be represented by the nitrite production process by AOB. In combined PN/anammox systems, ammonium oxidation to nitrite (nitritation) is mainly limited by oxygen availability which is controlled at low level with the aim of suppressing the undesired nitratation process performed by NOB. Efficiently suppress nitrite oxidation to nitrate while maintaining the nitritation process at the desired rate in order not to limit anammox conversion, seems therefore one of the main challenges to face towards the application of combined PN/anammox process for the treatment of MWW.

NOB suppression

Control of the dissolved oxygen (DO) has been proven as an effective strategy for the suppression of the nitratation process at temperatures as low as 15°C, but after prolonged operations at 10°C nitrite oxidation by NOB had a negative impact on the effluent quality (Ch.6). Oxygen-based suppression of the nitratation process rely on the assumption that the affinity constant for oxygen for AOB is lower than for NOB ($K_{O2,AOB}<K_{O2,NOB}$). Although it is commonly considered that NOB have a higher oxygen affinity constant than AOB (e.g. Sin et al., 2008), recent observations by Liu and Wang (2013) showed that NOB can become better oxygen competitor than AOB upon long term

cultivation at low DO concentrations resulting in nitrate production. Accordingly, a recent study conducted by Regmi et al. (2014) observed that the NOB population in their nitrifying reactor had a lower apparent DO half-saturation constant than AOB and was able to compete with AOB for oxygen uptake at low DO concentrations. Also Gilbert et al., (2015) in each of their four distinct systems with biofilm attached on inert carriers, granular and suspended sludge, could not efficiently suppress nitrite oxidation to nitrate in spite of a low operational DO (0.3 mg L^{-1}). Remarkably, in all these studies the dominant NOB genus in their systems was Nitrospira instead of Nitrobacter. On the other hand operations at higher DO (1-3 mg L^{-1}) in a system dominated by Nitrobacter allowed a stable partial nitritation at prolonged low temperature conditions (12.5°C, Isanta et al., 2015). Isanta and co-authors (2015) suggest that operations at high nitrite concentrations favoured Nitrobacter (r-strategist) against Nitrospira (K-strategist) which resulted in a dominant NOB population with a higher affinity constant for oxygen compared with AOB (Blackburne et al., 2007; Downing and Nerenberg, 2008). Mechanistic understanding on the effect of cultivation conditions in the competition between Nitrospira and Nitrobacter and between each of these two different genus and AOB is still lacking in literature and it would be of upmost importance for the implementation of an efficient and robust (partial) nitritation process.

A recent modelling study for PN/anammox in granular sludge suggests the ratio between DO and ammonium concentration as the most important parameter affecting the competition among AOB and NOB (Pérez et al., 2014). The mechanistic hypothesis behind it considers AOB activity in PN systems as limited by two substrates at the same time: ammonium and oxygen (e.g. according to Monod equation). Since the affinity for oxygen is more properly defined as the ratio between the growth rate and the oxygen half saturation constant (μ / K_{O_2}), higher ammonium concentrations (i.e. higher growth rate), could result in higher AOB's oxygen affinity, thus favouring AOB against NOB in the competition for oxygen. According to this hypothesis, a plug-flow reactor configuration (or SBR) would be more advantageous than a CSTR. While in fact the concentration in a CSTR would be the one required in the effluent, in a plug-flow (SBR) a concentration gradient along the reactor length (in time during operational cycle) will be established. Zones (time periods) with higher ammonium concentrations would allow operations at a higher DO resulting in

higher nitritation rates while guaranteeing an effective suppression of the nitratation process. This concept has been proven also experimentally by obtaining a stable partial nitritation at low temperatures (down to 12.5°C) in a granular airlift reactor fed with synthetic wastewater with low ammonium concentration (70 gN m^{-3}) with ammonium conversion rates in the order of 700 gN m^{-3} d^{-1} (Isanta et al., 2015). This study, together with the results obtained in Ch. 7, demonstrate the technical feasibility of efficiently controlling the two processes separately (the first aerobic, the second fully anoxic) which may result easier than controlling two balanced processes in one single system and indicates that these two processes could be easily integrated in series, in a two-stage autotrophic N-removal system with granular sludge. Nevertheless, the impact of the presence of COD in the influent to the PN system potentially altering the oxygen flux to the nitrifying granular biomass needs still to be investigated as well as the feasibility of coupling the two processes to obtain a stable effluent quality considering the fluctuation in wastewater characteristics typical for municipal wastewater. In any case a single stage nitrogen removal process would be economically more appealing compared with a two stage system because of the supposed reduced footprint and capital costs together with the need of controlling two separate processes instead of only one.

Overall then, competition between AOB and NOB seems still poorly understood. Elucidation of the role of simultaneous multiple-substrates limitation should be the objective of further studies. While for the basic understanding, the use of suspended cultures might be easier in order to avoid complications related to mass transfer limitation associated with biofilm structures (apparent affinity constant instead of intrinsic one, Chapter 2), the role of substrate concentration gradients along the biofilm thickness should be also thoroughly investigated by means of both experimental and modelling studies.

Optimization of the COD removal process

Municipal wastewaters with typical organic matter concentrations of 400-500mg COD/L contain a potential chemical energy of 1.5-1.9 kWh per m^3 of wastewater, which is more than twice the energy demand of a conventional activated sludge system (CAS) (McCarty et al., 2011). In a CAS system this

energy is largely destroyed by aerobic mineralization of the sewage organic matter to CO_2. Currently, CAS systems with a primary settler collect only about 30% of these organics in primary sludge, while more than 70% can be trapped in the first adsorption step of the A-B process (Böhnke 1978) (Ch.1). Increasing the efficiency of COD sequestration process in order to maximize the production of methane-rich biogas via anaerobic sludge digestion would therefore be beneficial from an energetic point of view. Furthermore the optimization of the COD sequestration step would be beneficial for the implementation of PN/anammox since it would lower the COD/N ratio of the wastewater to be treated by this autotrophic nitrogen removal process. Influent with lower COD/N ratios would have the double advantage of making easier the operations of the PN/anammox process (see following section) and would maximize the economic benefits of its implementation compared with conventional systems. The biodegradable organic carbon in fact fosters the growth of heterotrophic bacteria (HET), which are characterized by higher growth rates and yields compared with AOB and anammox bacteria, leading to higher sludge production and therefore decreasing the solid retention time (SRT). Additionally, HET compete with ammonia oxidising bacteria (AOB) for oxygen and with anammox bacteria for nitrite and therefore COD fluctuations in the influent might hamper process control. Given the slow growth rate of anammox bacteria observed at low temperatures (Ch. 6 and 7 *among others*), a long SRT is needed in order to avoid washout. Since the long retention time is necessary mainly for anammox bacteria (slowest growth rate) a sludge retention strategy aimed at selectively retaining anammox and washout other bacteria could potentially solve the issue. In Ch.8 it has been demonstrated how the use of granular biomass allow the selective removal of heterotrophic biomass and an efficient retention of granules enriched in anammox bacteria, but this needs still to be confirmed in the long term and at temperatures below 20°C. The control of different SRTs in a single bioreactor has been previously demonstrated for biofilm systems growing on inert carrier materials (e.g. Di Trapani et al., 2011). In systems where anammox is mainly growing in small granules, extensive efforts have been made to selectively anammox biomass by means of a hydrocyclone (Wett et al., 2013) and screening or sieving (De Clippeleir et al. 2013b), but long term feasibility at mainstream conditions is still lacking. Other potential solution could be offered by the bioaugmentation of mainstream reactor by the anammox biomass grown in the sidestream (Wett

et al., 2013). The last option has the disadvantage that it would require the implementation of a sidestream treatment which is not strictly needed in the proposed configuration with PN/anammox in the mainstream. Apart from the additional reactor required, it could in principle have good potential but given the limited N-load going to the side stream (15-20% in A-B process configuration) and the large and sudden temperature decrease which anammox bacteria grown in the sidestream would experience in winter (Ch.5), its effectiveness still needs to be proven. A mechanistic based model to elucidate the impact of COD oxidation (with oxygen and nitrite/nitrate as electron acceptor) on the SRT along with the daily and seasonal fluctuations, would be needed to support evaluation of the potential of the PN/anammox process in the mainstream. Modelling results could help the design of experimental studies aimed at investigating the impact of COD and suitable technologies/practices to guarantee the maintenance of the SRT needed for anammox and AOB bacteria.

Temperature effect

While the general effect of temperature on bacterial activity is well known, its quantification for different groups of bacteria cultivated under different operative conditions is still poorly studied (Ch.5). Adaption phenomena after prolonged cultivation at low temperature such as decrease of the temperature coefficient (θ) and decrease of optimum temperature have been recently observed for anammox bacteria (Ch.5-6; Dosta et al., 2008; Hu et al., 2013b; Gilbert et al., 2015). Furthermore, indications that thick biofilms provide more favourable conditions for withstanding decreasing temperature than thinner biofilms or suspended biomass has been recently reported (Ch.5; Gilbert et al., 2015). Additionally, the temperature dependency of anammox bacteria seems to be more pronounced at lower temperature ranges not allowing to model temperature dependency with a single θ as common practice (Ch.5; Isaka et al., 2008). Unfortunately though, mechanistic reasons explaining these observations have not been elucidated yet. Overall the impact of temperature and how it can be influenced by the cultivation history of the biomass and its aggregation status, still lacks a proper mechanistic understanding which

disclosure might help in mitigating the negative effect of seasonal temperature fluctuations.

Strong activity reduction when decreasing the operative temperature has been repeatedly observed in this thesis as well as previous reports. This activity reduction would pose operational problems during winter periods in moderate climates. Whether bioaugmentation (Wett et al., 2013) would not be proven to be feasible, accumulation of overcapacity in summer to be exploited in winter, thus counterbalancing the effect of temperature decrease, might potentially be a solution. Anammox decay rate has been reported to be rather low by means of repeated fed/batch experiments (Scaglione et al., 2009), but more studies performed with different anammox types and under different and continuous cultivating conditions would be desirable. Accumulation of large amounts of anammox biomass (i.e. conversion overcapacity) would result in a lower biomass specific load which may lead to incomplete substrate penetration in the biofilm resulting in prolonged famine conditions during the warmer periods of the year when specific activity is higher (i.e. lower substrate penetration depth). Operations in a plug-flow reactor (or more CSTR in series) with periodic redistribution of the granular sludge along the substrate concentration gradient could potentially solve this issue (*this thesis*). Again, in order to verify the latter hypothesis and to explore the potential of large nitrogen removal overcapacity accumulation and its effect on granular/biofilm stability more fundamental as well as applied research should be performed.

COD consumption by anammox

The ability of certain anammox species to couple fatty acids oxidation to nitrate reduction to ammonium has been previously reported (Güven et al., 2005; Kartal et al., 2007a). If part of the COD could be consumed by anammox bacteria this would mitigate the negative effect of influent organics described above (SRT decrease). Previous studies have shown that *Candidatus* Brocadia fulgida is able to oxidise acetate and out-competes other anammox types in the presence of acetate (Kartal et al., 2008; Winkler et al., 2012a, 2012b) and recently Jenni et al. (2015) found the same genus as dominant anammox microorganism in their sequencing batch reactor both when feeding acetate and glucose. Concerning the experiments described in this thesis, *Candidatus*

Brocadia fulgida was found as dominant anammox organisms in all systems despite different cultivation conditions and the use of organic-free synthetic medium as well as pretreated MWW with presence of COD. Even if apparently organic carbon is oxidized to CO_2 and not assimilated into biomass, the oxidation of fatty acids might occur for the conservation of energy, which could in turn lead to a higher biomass yield (Kartal et al., 2008). Unfortunately the latter hypothesis has not yet been proven. Elucidating the effect of COD on the competition among different anammox types and the extent and effect of COD oxidation by both physiological and kinetic perspectives bacteria may contribute to effective integration of autotrophic N-removal in mainstream sewage treatment. It may furthermore facilitate implementation of the autotrophic N-removal process for treatment of industrial WW with higher COD/N ratio's.

Growth rate increase

In Chapter 3 a substantial increase of anammox growth rate has been observed confirming and extending some previous reports (Ch.2; Isaka et al., 2006; Tsushima et al., 2007; van der Star et al., 2007; Lotti et al., 2015c [**Ch.3**]), but the mechanistic basis for this observation is still unclear. Considering that in conventional circumstances anammox bacteria are the slowest growing *players* in the investigated autotrophic nitrogen removal process and how beneficial would be to make use of a *faster* anammox microorganism (this thesis; Pérez et al., 2014), the observations made in Ch.3 surely need further investigation.

Considering the results reported in this thesis and the enthusiastic and fruitful research effort currently made worldwide by both academy and public stakeholders, I convincingly believe that in the coming decade this technology will be implemented and can result in a change from energy consuming to energy producing wastewater treatment plants. My hope is that the improvement of the economical profitability of the wastewater purification sector will contribute to make wastewater treatment a consolidated practice also in the emerging countries with a substantial improvement of water quality and thus of the state of health of the environment and human communities.

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Patents

Patent WO/2014/171819 (Publication Date: 23-10-2014; International Application Number: PCT/NL2014/050204) Process for biological removal of nitrogen from wastewater. Inventors: **T. Lotti**, T.L.G. Hendrickx, M.C.M. van Loosdrecht, J. Kruit

Patent NL PD 015626 (Pending; International Application Number: PCT/SE01/01699). Extracellular polymers from granular sludge as sizing agents. Inventors: Y. Lin, **T. Lotti**, M.C.M. van Loosdrecht

Awards

Jaap van der Graaf Award 2014. Witteveen+Bos Raadgevende ingenieurs B.V., Deventer, the Netherlands

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