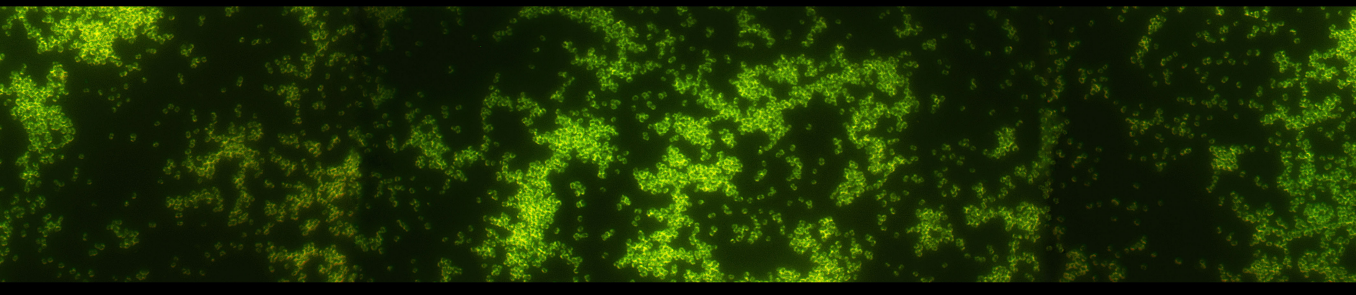
Growth and Metabolism of Anammox Bacteria



Wouter van der Star

Propositions accompanying the thesis
"Growth and metabolism of anammox bacteria"
Wouter van der Star

1. The one-reactor nitritation-anammox process is preferable over the two-reactor configuration for nitrogen removal.

2. In the decision when to build a demonstration reactor or a first full-scale reactor PR considerations should also be taken into account.

3. Microorganisms employing anoxic nitrite fermentation (with the catabolism: $5NO_2^- + 2H^+ \rightarrow 3NO_3^- + N_2 + H_2O$) do exist and are likely to be present in anammox enrichments.

4. The minimum doubling time of anammox bacteria is probably less than three days. However, such a fast growth rate has never been convincingly observed in the laboratory.
Isaka et al. 2006. Appl Microbiol Biotechnol 70(1) p47,
Tijhuis et al. 1993. Biotechnol Bioeng 42(4), p509-519.

5. The physiological difference of organisms in oxic (presence of oxygen) and anoxic (absence of oxygen) environments is much smaller than the difference between anoxic and anaerobic (absence of oxygen and oxidised nitrogen compounds). It is therefore useful that also in microbiology the concepts anaerobic and anoxic are employed.

6. Pure culture studies are useful to generate hypotheses and develop experimental systems for study of the behavior of natural systems, but they should not be used to *predict* the behavior of natural systems.

7. The "great plate count anomaly" has been succeeded by the "great general primer anomaly".

8. The application of a membrane bioreactor for the anammox process on full-scale is not economically feasible.
Chapter 3 of this thesis

9. The proposal that "detoxification" is the physiological function of a metabolic capability in bacteria is often only an indication that all other explanations for the observed phenomenon have failed.

10. Those who choose to work exclusively with one microorganism for their entire career will obtain a tunnel-vision.

11. The most important dish from the kitchen of (bio)nanotechnology is baked air. Presently, research funding agencies are more than satisfied with this product.

12. It is already hard enough for foreign researchers to adapt to the local culture and it would therefore be too much to stimulate them to also learn the local language.

13. Coincidence does not exist (however, it often seems to manifest itself as a result of a lack of knowledge).

These propositions are considered opposable and defendable and as such have been approved by the supervisors, prof.dr.ir. M.C.M. van Loosdrecht and prof.dr.ir. M.S.M. Jetten.

Stellingen behorende bij het proefschrift
"groei en metabolisme van anammox bacteriën"
Wouter van der Star

1. Het één-reactor nitritatie-anammox-proces is voor stikstofverwijdering te verkiezen boven de twee-reactor configuratie.

2. Bij het nemen van de beslissing om een eerste praktijkschaalreactor te bouwen, dienen ook PR argumenten in overweging genomen te worden.

3. Microorganismen die anoxische nitriet-fermentatie toepassen (met als katabolisme: $5NO_2^- + 2H^+ \rightarrow 3NO_3^- + N_2 + H_2O$) bestaan en zijn waarschijnlijk aanwezig in anammox ophopingsculturen.

4. De minimale verdubbelingstijd voor anammoxbacteriën is waarschijnlijk minder dan drie dagen. Een dergelijk hoge groeisnelheid is echter nog nooit overtuigend waargenomen onder laboratoriumomstandigheden.
Isaka et al. 2006. Appl Microbiol Biotechnol 70(1) p47.
Tijhuis et al. 1993. Biotechnol Bioeng 42(4), p509-519.

5. Het fysiologische verschil tussen organismen in oxische (aanwezigheid van zuurstof) en anoxische (afwezigheid van zuurstof) milieus is veel kleiner dan het verschil tussen organismen onder anoxische en anaerobe (geen zuurstof of geoxideerde bron van stikstof) omstandigheden. Het is daarom nuttig dat ook in de microbiologie de concepten anaeroob en anoxisch worden toegepast.

6. Reinculture-studies zijn nuttig om hypotheses te genereren en om experimentele systemen te ontwikkelen voor het gedrag binnen natuurlijke systemen, maar ze behoren niet gebruikt te worden om het gedrag van natuurlijke systemen te *voorspellen*.

7. De "grote anomalie in plaattellingen" is opgevolgd door de "grote anomalie in algemene primers".

8. De toepassing van de membraan bioreactor voor het anammox proces op praktijkschaal is economisch niet haalbaar.
Hoofdstuk 3 van dit proefschrift

9. Het voorstel dat detoxificatie de fysiologische functie is van een bepaalde metabole route in een bacterie, is vaak uitsluitend een aanwijzing dat geen overtuigende verklaring voor het waargenomen fenomeen kon worden gevonden.

10. Zij, die ervoor kiezen om hun hele carrière uitsluitend met één microorganisme te werken, zullen een tunnel-visie krijgen.

11. Het belangrijkste gerecht uit de keuken van de (bio)nanotechnologie is gebakken lucht. Op dit moment zijn onderzoeksfinanciers zeer tevreden met dit product.

12 Het is absoluut noodzakelijk voor de (wetenschappelijke) ontwikkeling van buitenlandse onderzoekers dat zij de locale taal spreken.

13 Toeval bestaat niet (hoewel het zich vaak lijkt te manifesteren door een gebrek aan voorkennis).

Deze stellingen worden opponeerbaar en verdedigbaar geacht en zijn als zodanig goedgekeurd door de promotoren, prof.dr.ir. M.C.M. van Loosdrecht en prof.dr.ir. M.S.M. Jetten.

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FISH image of planktonic cells of the anammox bacterium "Kuenenia stuttgartiensis"

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PROEFSCHRIFT

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J.T. Fokkema, voorzitter van het College voor Promoties,
in het openbaar te verdedigen op dinsdag 15 april 2008 om 15:00 uur

door

Wouter Roelof Lambertus VAN DER STAR

scheikundig ingenieur
geboren te Deurne

Dit proefschrift is goedgekeurd door de promotoren

Prof.dr.ir. M.C.M. van Loosdrecht

Prof.dr.ir. M.S.M. Jetten

samenstelling promotiecommissie:

Rector Magnificus	Technische Universiteit Delft, *voorzitter*
Prof.dr.ir. M.C.M. van Loosdrecht	Technische Universiteit Delft, *promotor*
Prof.dr.ir. M.S.M. Jetten	Technische Universiteit Delft en Radboud Universiteit Nijmegen, *promotor*
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Chapter 1

Introduction

Outline

The anoxic ammonium oxidation (anammox) process is one of the main biological nitrogen conversion processes and is characterized by the reaction of nitrite with ammonium -in the absence of oxygen- to form dinitrogen gas. The knowledge on the anammox process started only at the end of the twentieth century and the existence of the process itself came to many as a surprise: more than hundred years after the other basic players in the nitrogen cycle -nitrification, denitrification and nitrogen fixation- had been identified. During those 100 years, good reasons seemed to exist why the anammox process could not take place: the biological oxidation of ammonium under oxic conditions was already cumbersome (performed by dedicated slow-growing organisms), and the harsh conditions required for chemical oxidation of ammonium were well known from industrial processes.

In this Chapter, the history of research on the anammox process is placed in the context of the start of research on other nitrogen converting processes. After evaluating the (harsh) conditions for chemical ammonium oxidation and explaining the biological mode of action of aerobic ammonium oxidation, the characteristics of the anammox process, the bacteria responsible for it, and the application in wastewater treatment are introduced. An outline of the research described in this thesis concludes this Chapter.

Discovery of the anoxic ammonium oxidation (anammox) process

In 1977, Engelbert Broda predicted on thermodynamic and evolutionary grounds, that ammonium oxidation under anoxic conditions with nitrate or nitrite as electron acceptor should "exist or have existed" (1977). Oxidation of ammonium with an electron acceptor other than oxygen was also predicted already by researchers of the marine environment based on mass balance studies (RICHARDS 1965), later even in combination with a thermodynamic justification (CLINE and RICHARDS 1972). These predictions were put forward however in an era when the general belief was that biological ammonium oxidation under anoxic conditions was simply impossible.

In 1985, indeed ammonium removal was observed for the first time under anoxic conditions in a pilot scale denitrifying reactor at the baker's yeast factory Gist-Brocades (now part of DSM) in Delft, The Netherlands (HEIJNEN 1988; MULDER 1989; VAN DE GRAAF et al. 1990; MULDER et al. 1995). In this reactor, nitrate was added to achieve sulfide oxidation coupled to nitrate reduction, but surprisingly, ammonium (thought to be not reactive under anoxic conditions) was removed as well (MULDER et al. 1995). The new biological process was named

the "anammox" process, acronym for an*ox*ic ammonium oxidation (MULDER 1989; VAN DE GRAAF et al. 1990) but also an*aerob*ic ammonium oxidation[1] (MULDER et al. 1995; VAN DE GRAAF et al. 1995). The pilot plant in Delft where the anammox process took place was scaled down to lab-scale and successful enrichment of the organism responsible for the reaction was only achieved, after nitrite (rather than nitrate) was identified as the electron acceptor (VAN DE GRAAF et al. 1996)

About ten years after the initial observations in Delft, in Germany (HIPPEN et al. 1996; HIPPEN et al. 1997) and Switzerland (BINSWANGER et al. 1997; SIEGRIST et al. 1998) the production of dinitrogen gas instead of nitrate ("nitrogen losses") were reported in full-scale rotating disc contactors treating (ammonium-rich) wastewater originating from landfill leachates. Since ammonium was the only electron donor that was present in significant amounts in these wastewaters, all converted ammonium was expected to end up in the aqueous effluent as nitrate, instead of being "lost" to the atmosphere (as dinitrogen gas). The conversion in these reactors was initially related to nitrifier denitrification, reported earlier by POTH and FOCHT (1985), but eventually attributed to a combination of nitrifiers and anammox bacteria (see Box 1 in Chapter 2 for details).

Enrichment cultures (50-90% pure, e.g. SCHMID et al. 2000; EGLI et al. 2001; STROUS et al. 2006; TSUSHIMA et al. 2007b; KARTAL et al. 2008; LÓPEZ et al. 2008) were the only sources of information on the anammox process, as numerous attempts to isolate the organism responsible for the process failed (STROUS et al. 1999a). To prove that the enriched organisms were indeed responsible for the anammox process, cells were physically purified up to 99.6%, using density gradient centrifugation (the "Percoll" method, STROUS et al. 1999a). Since the purified cells performed the characteristic anammox conversion (conversion of ammonium and nitrite) at high rate, the conversion in the reactor could be ascribed to the main population of the enrichment. The concentrated cell-solution also allowed for sequencing of the 16S rRNA-gene, which provided evidence that the organism was a deep-branching Planctomycete (STROUS et al. 1999a). The organism was named *Candidatus* "Brocadia anammoxidans".

[1] In the literature on wastewater treatment, a clear distinction is made between anoxic (the absence of oxygen) and anaerobic (absence of any external electron acceptors like oxygen and nitrate/nitrite). Contradictory to this convention, within (micro)biology the absence of oxygen is indicated as anaerobic. To avoid confusion, the wastewater terminology will be used in this thesis and therefore the anammox process will be referred to as anoxic ammonium oxidation.

Discovery of the classical players of the nitrogen cycle

The biological conversions of nitrogen have had scientific interest since the second half of the 19th century. The conversion of dinitrogen gas (N_2) to ammonium (nitrogen fixation), oxidation of ammonium to nitrate (nitrification), and reduction of nitrate to dinitrogen gas (denitrification) were discovered in these 50 years. The combination of these reactions made it possible to interpret nitrogen conversions as a biogeochemical cycle (Figure 1.1) and the thus formed cycle expressed the general view on nitrogen conversions in most of the 20th century. In this paragraph, a brief history of the different processes in the classical nitrogen cycle is given.

Remarkable similarities exist between the discovery of the "classical" nitrogen conversion processes and the discovery of the anammox process. Despite the fact that research on the classical nitrogen removal processes started more than 100 years before the postulation of the existence of the anammox process, the knowledge in these processes developed in the same sequence:

- Postulation of the process
- Proof that it was microbiological in nature
- Identification of the main microorganisms

As an introduction to the processes of nitrogen fixation, nitrification and denitrification, the first years of research on these processes will be described.

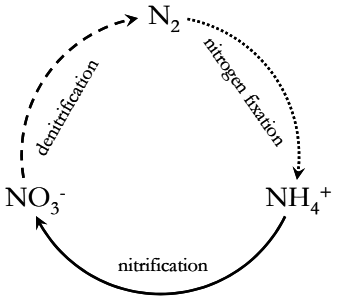

FIGURE 1.1 *The classical nitrogen cycle with nitrification, denitrification, and nitrogen fixation.*

Denitrification

Pasteur was wrong when he ascribed the reduction of nitrate to "lactic yeast" (PASTEUR 1859), but with his research, he was the first to show a nitrogen converting process to be of biological nature. This observation marked in the fifties of the 19th century, together with the observation of Reiset (1856), that nitrogen was released into the atmosphere during decay of plant and animal material, the start of research on the biology of the nitrogen cycle (PAYNE 1986). The process was called denitrification by GAYON and DUPETIT (1883) [according to Payne (1986)]. The requirement of organic matter for denitrification -following the notion by

WARINGTON (1884) that nitrate was removed after fresh soil was added to experimental systems- was proven by MUNRO (1886). The isolation of two nitrate reducing organisms twenty years after the proof of their activity by MEUSEL (1875) offered the possibility to establish unequivocally its intermediates nitrite, nitric oxide and nitrous oxide.

Nitrification

The biological nature of the *production* of nitrate was stated only 20 years after the discovery of denitrification (SCHLŒSING and MÜNTZ 1877) and this process was called nitrification. After Warington (1878) found proof of the microbiological nature of the process, Winogradsky showed (after a non reproducible cultivation-claim by HERAEUS 1886) that specific groups of bacteria were responsible for nitrification, by isolating for the first time an ammonium oxidizing bacterium (WINOGRADSKY 1890). WINOGRADSKY's isolations (1892) of *Nitrosomonas europaea* and *Nitrobacter* (the latter was later named *Nitrobacter winogradskyi* in his honor, BUCHANAN 1918) also showed that labor in nitrification was divided between ammonium oxidation to nitrite by ammonium oxidizing bacteria (AOB) and nitrite oxidation to nitrate by nitrite oxidizing bacteria (NOB) respectively. Hydroxylamine was identified as the main intermediate (MUMFORD 1914) of AOB, who also reported the emission of nitrous oxide (N_2O) and nitric oxide (NO) by nitrifiers.

Nitrogen fixation

The fixation of dinitrogen gas was hypothesized to be performed by leguminous plants in the 1850 by BOUSSIGNAULT. Lachmann specified this by suggesting that nitrogen fixation took place within the nodules of the roots of these plants. Definite proof however came by HELLRIEGEL and WILFARTH (1888), who showed that nitrogen-addition was vital for non-leguminous plants, and that nitrogen fixation took only place in leguminous plants where root nodules were present. The bacteria which were present in root nodules (named *Rhizobium leguminosum*) were cultivated shortly after by FRANK (1889), after having established their origin as an "infection[2]" from the soil (FRANK 1879).

Free-living nitrogen-fixing bacteria were discovered only six years later using ammonium and nitrate-free enrichment cultures by WINOGRADSKY (1895). The isolated organism was named "Clostridium pastorianum*"* (now *Clostridium pasteurianum*) and only grew under anoxic conditions.

[2] Frank used the word "infection" to indicate that the organism originated from ground and not from the plant itself. He did not mean the negative connotation of the word.

Using similar enrichment cultures, Beijerinck (1901) isolated two aerobic microorganisms, which he named *Azotobacter chroococcum* and "Azotobacter agilis" (now known as *Azomonas agilis*).

Although the organisms were enriched on nitrogen-free medium or cultivated directly from the root nodules, the actual capability of nitrogen fixation (i.e., the measurement of the conversion of dinitrogen gas to ammonium) was shown only in 1902 (BEIJERINCK and VAN DELDEN 1902).

The expansion of research on the nitrogen cycle

From the introduction of the concepts of nitrification, denitrification and nitrogen-fixation in the 19th century, together with an increased amount of research worldwide, also research in the nitrogen cycle (measured as the number of papers with one of these key words in title or abstract) increased in an exponential manner (Figure 1.2) until about 1910. From the 1960ies on, again a continuous growth of publications took place, which coincided with the increased interest in the application of nitrogen as fertilizer as part of the "green revolution": the aspiration to ban hunger from the earth by providing food for everyone (GAUD 1968).

The number of papers on the anammox process over the years followed the same trend as the previously identified nitrogen converting processes: circa 10 years after a hesitant start in the 1980ies, the number of articles on the anammox process has increased exponentially from 2000 on. Extrapolation of the present increase in publications would indicate that within six years from now, the number of anammox publications will be similar to the number of publications, which are dedicated to the other major conversion reactions in the nitrogen cycle. It can also be noted from Figure 1.2, that cycles of about 20 years of increasing interest on a specific topic took place after which a stabilization of the number of papers occurred.

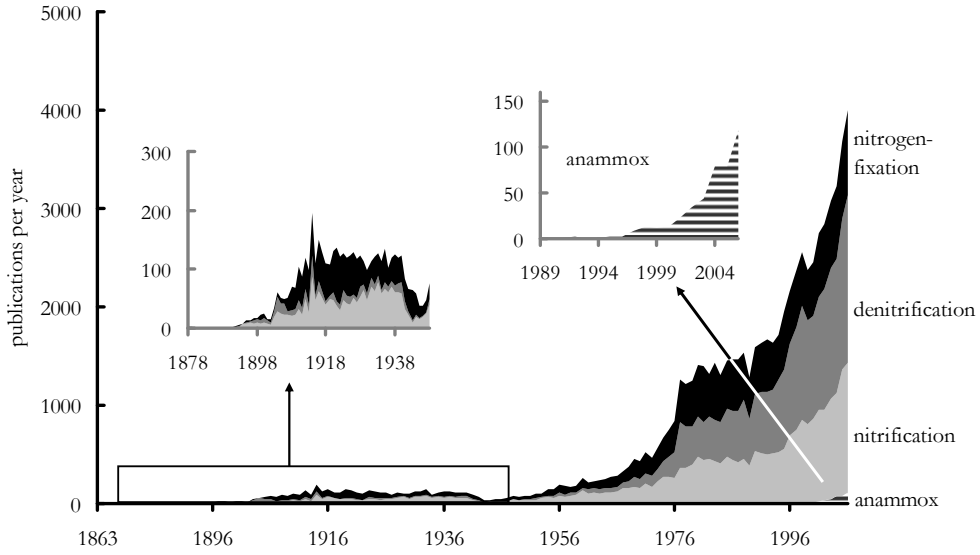

FIGURE 1.2 *Research in the biological conversions of nitrogen from its cradle in the end of the 19th century until 2006. Articles with keywords "denitrification", "nitrification", "nitrogen fixation" and "anammox OR anaerobic ammonium oxidation" indexed by Chemical Abstracts were taken into account.*

Chemical conversion of ammonium or ammonia

Despite the energy that can be gained from oxidation of ammonium, the reactivity at room temperature is low. The only known chemical conversion process at low temperature (5°C) is oxidation with the highly reactive hypochlorite (ClO^-) to chloramine (NH_2Cl). The almost purely covalent character of the N-H bond and the small (NH_3) or non-existent (NH_4^+) dipole are explanations for the remarkable stability of ammonium/ammonia (see e.g. ZUMDAHL and ZUMDAHL 2007). This makes it hard for nucleophiles as well as for electrophylic compounds to attack the molecule. Therefore, only strong oxidants like hypochlorite can convert ammonium in solution at room temperature. For other chemical oxidation reactions of ammonia or ammonium always high temperatures are required. The temperature for the Ostwald process (production of nitric oxide and then nitric acid) is 900°C. Also in the Haber-Bosch process of ammonia production, temperatures of 400°C are required to obtain a reasonable reaction rate and overcome the thermodynamic equilibrium restriction. An overview of operating temperatures in the main industrial ammonia or ammonium conversions is given in Table 1.1.

TABLE 1.1 *Reactions for the most important commercial processes of production of nitrogen containing inorganic chemicals from ammonia or ammonium (Bohnet et al. 2007).*

reaction/ process name	reaction equation	temperature [°C]	pressure [bar]	catalyst	use
Haber-Bosch[1]	$N_2+3H_2 \rightarrow 2NH_3$	400	>100	magnetite (Fe_3O_4) (or ruthenium)	fertilizer (intermediate)
Ostwald	$4NH_3+5O_2 \rightarrow 4NO+6H_2O$	500-900	2-11	platinum/ rhodium	fertilizer (intermediate)
Raschig (second step)	$NH_2Cl+NH_3+NaOH \rightarrow N_2H_4+NaCl+H_2O$	130-150	30		rocket fuel
Raschig (first step)	$NH_3+NaClO \rightarrow NH_2Cl+NaOH$	5	1	-	hydrazine production (intermediate)

[1] Ammonia production is given here instead of ammonia conversion, since this is the overall reaction. However, because the reaction rate in this equilibrium process is also relevant for the reversed -ammonia consuming- reaction, the reaction is included in this table.

Biological ammonium oxidation

Nitrifying bacteria (and nitrifying crenarchaea, KÖNNEKE et al. 2005) are the only microorganisms growing on the oxidation of ammonium besides anammox bacteria. To this purpose, all nitrifiers carry an ammonium mono-oxygenase (AMO) enzyme, in which ammonium and oxygen directly react to hydroxylamine. The role of O_2 can only be taken over by nitrogen dioxide (NO_2), which is subsequently converted to NO (SCHMIDT and BOCK 1997). Growth however on ammonium and NO_2 has not been shown. Nitrate and nitrite are known to be capable of replacing oxygen during denitrification in many organisms by accepting electrons directly from the respiratory chain. However, the specific requirement of molecular oxygen in the AMO enzyme of nitrifiers renders regular denitrification (via NO and N_2O) based on ammonium impossible for these organisms.

The requirement of molecular oxygen as an oxidant is characteristic for mono-oxygenases. The biological conversion of stable molecules like ammonium, methane and other alkanes is (almost exclusively) performed by oxygenase-containing microorganisms. In these oxygenases, oxygen (normally present in the unreactive triplet state) is either first activated to its singlet state (which is 87 kJ/mol higher); or the oxygen molecule is partly reduced to (enzyme bound) highly reactive superoxide ($-O_2^{2-}$) or peroxide ($-O_2^{-}$). Despite the clear advantages oxygen, it is not the only possible biological oxidant for these molecules. In the past decade, indications of anoxic biological conversion of alkanes (AITKEN et al. 2004) methane (BOETIUS et al. 2000; RAGHOEBARSING et al. 2006) and ammonium (the anammox process) have

appeared with sulfate, nitrate or nitrite as electron acceptors. How these unreactive compounds are activated is still largely unknown.

The anammox process

As the anammox process is object of study in this thesis, the nature of the conversion and the physiology of the organisms is introduced briefly here. Extensive reviews (JETTEN et al. 2002; JETTEN et al. 2005a; OP DEN CAMP et al. 2006; FRANCIS et al. 2007) give a more in-depth introduction to the process and its characteristic organisms.

Stoichiometry

Ammonium, nitrite and bicarbonate are the main substrates in the anammox process (VAN DE GRAAF et al. 1996). The coupling of the nitrogen atom from ammonium and the nitrogen atom from nitrite to form dinitrogen gas (N_2) comprises the catabolic reaction (1.1):

$$NH_4^+ + NO_2^- \longrightarrow N_2 + 2H_2O \qquad (1.1)$$

The anammox process is autotrophic, that is, HCO_3^- is the carbon source for the produced biomass in the anabolism (reaction 1.2). Nitrite oxidation to nitrate generates the electrons, which are required for this HCO_3^- reduction process (VAN DE GRAAF et al. 1996):

$$HCO_3^- + 2.1NO_2^- + 0.2NH_4^+ + 0.8H^+ \longrightarrow CH_{1.8}O_{0.5}N_{0.2} + 2.1NO_3^- + 0.4H_2O \qquad (1.2)$$

If catabolism (1.1) and anabolism (1.2) are combined using the experimentally obtained yield of carbonate on ammonium (0.066 mol C/mol NH_4^+, by STROUS et al. 1998), the following overall reaction equation (1.3) can be derived: (1.3)

$$1NH_4^+ + 1.13NO_2^- + 0.066HCO_3^- + 0.053H^+ \longrightarrow 0.99N_2 + 0.066CH_{1.8}O_{0.5}N_{0.2} + 0.14NO_3^- + 2.00H_2O$$

This stoichiometry is in quite close accordance with the experimentally found stoichiometry (STROUS et al. 1998): (1.4)

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \longrightarrow 1.02N_2 + 0.066CH_{1.8}O_{0.5}N_{0.2} + 0.26NO_3^- + 2.03H_2O$$

Growth rate

Anammox organisms grow notoriously slow. The doubling time is several days under the known optimal conditions (STROUS et al. 1998; TSUSHIMA et al. 2007a). Autotrophic growth is an energy expensive process and therefore always leads to low growth rates when compared with heterotrophic growth. However, the extremely slow growth of anammox bacteria can not be explained by autotrophy alone. The energy gained in the catabolism (calculated per mol of electrons) is comparable to the -also autotrophic- nitrification process, but the growth rate is

much lower. Other potential explanations for the slow growth might be that anammox bacteria have an intrinsically low conversion rate (of ammonium and nitrite) or that the enrichment takes place under suboptimal growth conditions in the current cultivation systems.

Intermediates in the catabolism

Substantial uncertainty exists on the intermediates in the catabolism. There is general agreement however that hydrazine (N_2H_4) is an intermediate. The oxidation of this compound (of which the anhydrous form has been in use as a rocket fuel) to N_2 is the actual energy generating step. Nitrite is not directly converted to hydrazine but via hydroxylamine and/or nitric oxide (NO) (VAN DE GRAAF et al. 1997; STROUS et al. 2006). A schematic representation of three possible metabolic schemes is given in Figure 1.3.

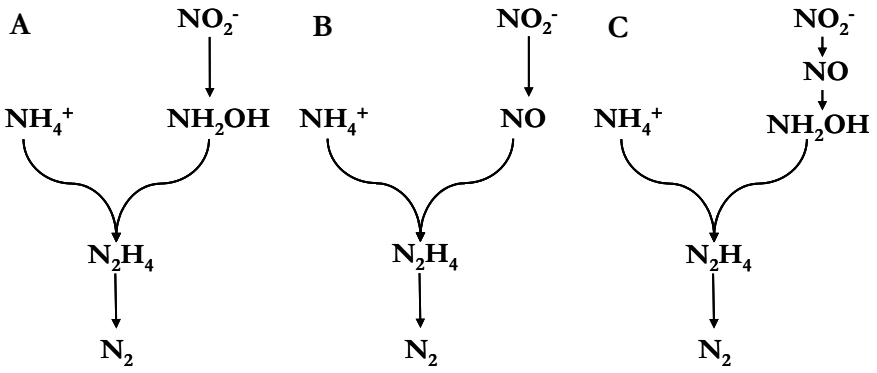

Figure 1.3 *Catabolic schemes for the anammox process with hydrazine as the main intermediate. Additional potential intermediates are hydroxylamine (A), nitric oxide (B), or hydroxylamine and nitric oxide (C).*

Important enzymes are hydroxylamine oxidoreductase (HAO, purified by SCHALK et al. 2000), hydrazine oxidase (HZO, purified by SHIMAMURA et al. 2007) and the nitrite reductases ccNir (partially purified by SCHALK 2000) as well as cd_1Nir (found in the "Kuenenia" genome, STROUS et al. 2006). Since these enzymes are all capable of performing a multitude of nitrogen-converting reactions, it is -as with the intermediates- at this moment unclear which enzyme is responsible for which reaction.

Cell plan, phylogeny and niche differentiation

All identified anammox bacteria form a monophyletic group within the Planctomycetes (STROUS et al. 1999a). A characteristic of Planctomycetes is their (for bacteria) unusually high level of cell-organisation, as each cell consists of one or more internal membrane-surrounded compartments with a varying (and largely unknown) function (LINDSAY et al. 2001; FUERST

2005). The main internal compartment in anammox bacteria is the anammoxosome and this compartment is hypothesized to be the locus of the catabolism (VAN NIFTRIK et al. 2004). An implication of this hypothesis is, that a proton motive force is generated over the anammoxosome membrane for the coupling of energy generation and anabolic activities. The anammoxosome is surrounded by the riboplasm (location of ribosomes and chromosome), which on its turn is surrounded by the paryphoplasm (LINDSAY et al. 2001; VAN NIFTRIK et al. 2008, Figure 6.1). As there is no consensus on the characteristics (in particular: the permeability) of the membrane between paryphoplasm and riboplasm, the status of the paryphoplasm (as a true internal compartment, or as a region which is similar to the periplasm in Gram-negative bacteria) is under debate.

Seven anammox species have been identified: "Brocadia anammoxidans" (STROUS et al. 1999a), "Brocadia fulgida" (KARTAL et al. 2004), "Kuenenia stuttgartiensis" (SCHMID et al. 2000), "Anammoxoglobus propionicus" (KARTAL et al. 2007b), "Scalindua brodae" (SCHMID et al. 2003), "Scalindua wageneri" (SCHMID et al. 2003) and "Scalindua sorokinii" (SCHMID et al. 2003). Since no anammox bacterium has been obtained in pure culture, all species have the *Candidatus* status. "Brocadia" and "Kuenenia" were generally enriched in lab-scale enrichments with a medium similar to that of VAN DE GRAAF et al. (1996) irrespective of the used inoculum (SCHMID et al. 2000; VAN DONGEN et al. 2001b; CHAMCHOI and NITISORAVUT 2007). The addition of fatty acids has led to enrichment of "Anammoxoglobus" (KARTAL et al. 2007b) and "Brocadia fulgida" (KARTAL et al. 2004, 2008). Despite the considerable diversity in different enrichment systems, in marine systems always "Scalindua sorokinii" is the dominant anammox species (SCHMID et al. 2007).

Metabolic versatility

Besides the conversion of ammonium and nitrite, the anammox genera "Brocadia", "Anammoxoglobus" and "Kuenenia" were also shown to be capable of cometabolizing the fatty acids propionate, acetate and formate (GÜVEN et al. 2005; KARTAL et al. 2007b, 2008). The oxidation of these fatty acids (to CO_2) is coupled to the reduction of nitrate -via nitrite- to ammonium (KARTAL et al. 2007a). In this way, anammox bacteria are capable of producing their own ammonium (and nitrite) to perform their "standard" catabolism (Figure 1.4). Whether the energy from the conversion of nitrate to ammonium can also be harvested and thus forms an additional catabolism is not known. Surprisingly, the fatty acids seem not to be incorporated into biomass, but completely converted to CO_2 (KARTAL et al. 2007b, 2008). Since anammox bacteria perform the energy-expensive CO_2-fixation also via acetate (which is the reverse of the described

acetate oxidation), this is a surprising and presently ununderstood characteristic of anammox bacteria.

Besides conversion of fatty acids, "Kuenenia stuttgartiensis" was shown to be capable of oxidation of Fe^{2+} (to Fe^{3+}) with nitrate as electron acceptor, as well as the reduction of Fe^{3+} (to Fe^{2+}) and Mn^{4+} to Mn^{2+} with formate as electron donor (STROUS et al. 2006). As with the conversion of fatty acids, the metabolic function -and whether actual growth on these substrates is possible- is not known.

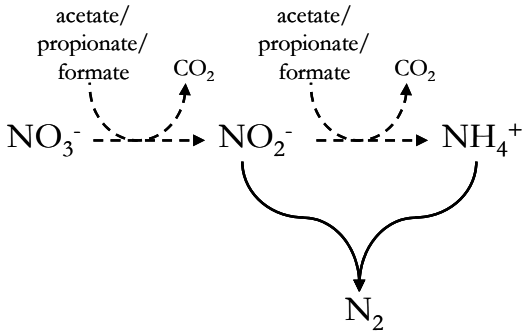

Figure 1.4 *Metabolic versatility of anammox bacteria: besides the conversion of nitrite and ammonium (solid lines), anammox bacteria are also capable of using short chain fatty acids as electron donor for ammonification (dashed lines). Reduction of Fe^{3+} and Mn^{4+}, as well as the oxidation of Fe^{2+} is also possible, but the stoichiometry of these reactions is not known.*

Application of the anammox process

The anammox process offers many advantages for the removal of ammonium from wastewater. Conventionally, ammonium is removed via nitrification (to nitrate), followed by denitrification. In anammox-based processes, only about half of the ammonium is oxidized to nitrite (and *not* to nitrate), after which the produced nitrite reacts with the remaining ammonium to form dinitrogen gas. The main advantages are:

- No requirement for an external oxidant.
- Lower energy requirements due to lower aeration costs.
- No CO_2 emission (due to the autotrophic nature of the nitritation and the anammox process, CO_2 is consumed instead of produced).

Especially in waste streams containing high ammonium levels, but low amounts of organics, the anammox process can be successfully applied. Two main reactor configurations are possible for anammox-based ammonium removal (Figure 1.5):

(i) A two-reactor configuration in which part of the ammonium is first oxidized to nitrite (partial nitritation) in an aerated reactor, after which the anammox process takes place in a second (anoxic) reactor (VAN DONGEN et al. 2001b).

(ii) A one-reactor configuration in which the partial nitritation and the anammox process both take place in the same (aerated) reactor. In this configuration, nitrification takes place on the (aerobic) outer layer of a floc or granule within the reactor, whereas the anammox process takes place in the anoxic zone deeper in the biofilm (HIPPEN et al. 1997; KUAI and VERSTRAETE 1998; THIRD et al. 2001). Also the alternation of oxic and anoxic periods by switching the aeration flow on and off is a method to obtain nitritation and the anammox process in one reactor (WETT 2006).

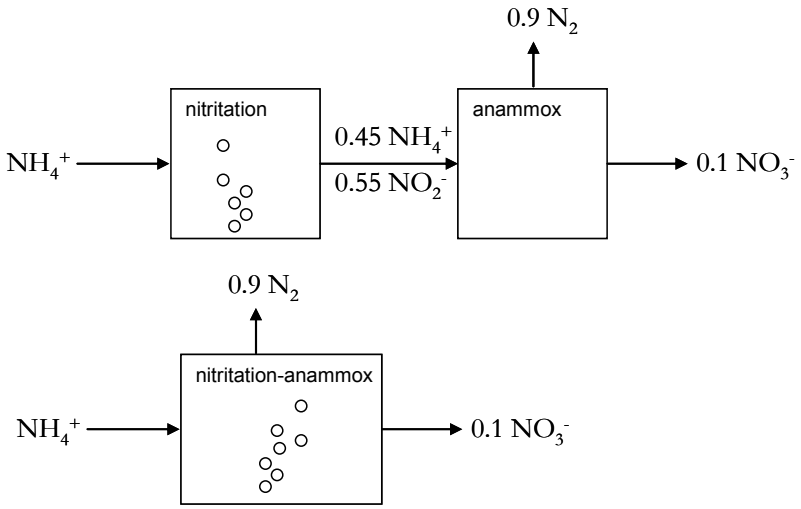

Figure 1.5 *Removal of ammonium with the anammox process can be applied in two configurations. In a two-reactor configuration, nitritation takes place in the first (aerated) reactor and the anammox process in the second (anoxic) reactor (top figure), whereas in the one-reactor configuration, both processes take place in the same (aerated) reactor (bottom figure).*

Presently, the anammox process is successfully employed -in both configurations- in the removal of wastewater from sludge digestates, landfill leachates and several industrial wastewaters (see table 2.4). Besides the potential application of the anammox process for the removal of ammonium, also removal processes for ammonium nitrate with the anammox process have been employed on lab-scale (MULDER et al. 1995; KALYUZHNYI et al. 2006; PATHAK et al. 2007; CHAMCHOI et al. 2008). In this process, partial denitrification (nitrate reduction to nitrite) is

coupled to the anammox process. If fatty acids are the electron donor for this denitrification, also anammox bacteria themselves are capable of this reduction (GÜVEN et al. 2005; KARTAL et al. 2007b).

Outline of this thesis

The research described in this thesis is part of the research program "Improved applicability of the anammox process". The project is funded by the Dutch Technology Foundation STW (Stichting Toegepaste Wetenschappen) under project number NPC.5987 and is a collaboration between the Department of Biotechnology of Delft University of Technology and the Department of Microbiology of Radboud University Nijmegen. The research performed in Nijmegen has focused on the ecophysiology of anammox bacteria (KARTAL 2008).

This thesis describes the research executed in Delft which was performed partly on lab-scale and partly on full-scale. The work on lab-scale offers new insights in the growth and metabolism of the anammox process, whereas the research on full-scale evaluates whether certain lab-scale observations -known already at the beginning of this research in 2003- can be translated directly to full-scale. The thesis is divided into two parts: "Growth" and "Metabolism". A schematic representation of the links between the different parts of this thesis is outlined in Figure 1.6.

Part I: Growth of anammox bacteria

Chapter 2 describes the startup of the anammox process on full-scale and evaluates its performance. Important parameters during the startup and operation of the full-scale reactor are compared to the values obtained on lab-scale. To evaluate the applicability of different reactor types, limiting factors for operation of the anammox process in several bioreactors have been assessed. *Chapter 3* evaluates the possibility to grow anammox bacteria as single cells to determine whether biofilm growth is essential to anammox bacteria. Dedicated operation of a membrane bioreactor resulted indeed in cultivation of anammox bacteria as free cells with an unparalleled production rate and an extremely high level of enrichment.

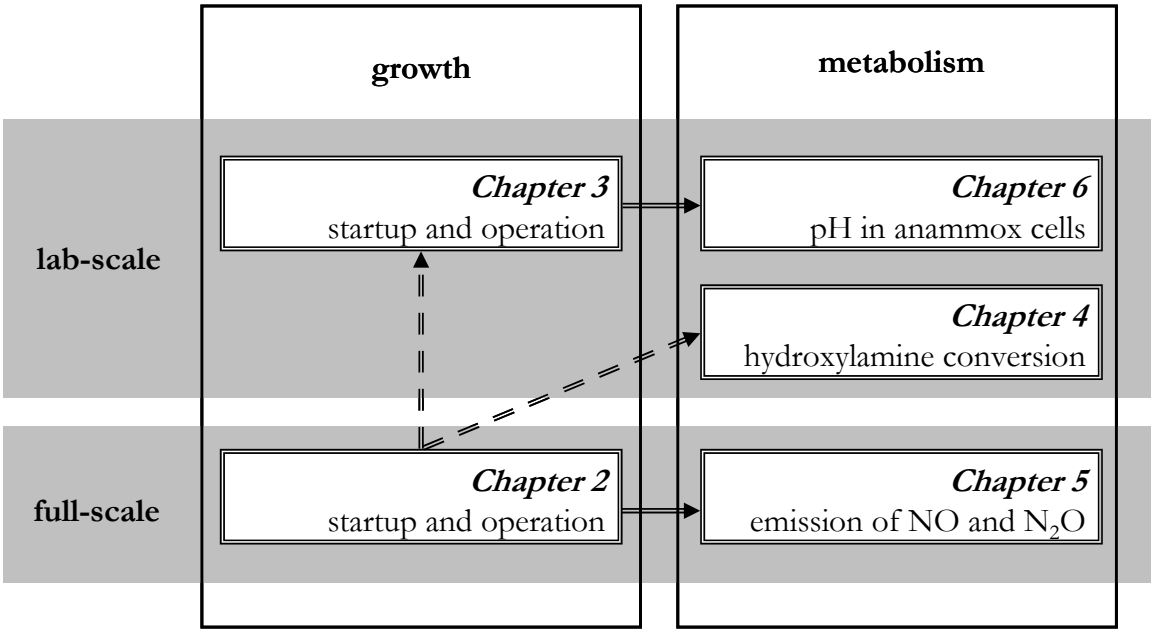

Figure 1.6 *Outline of the thesis. Dashed arrows indicate origin of the inoculum for the cultivation reactor. Solid arrows indicate the use of (biomass from) this reactor directly for experiments.*

Part II: Metabolism of the anammox process

To evaluate the different reactions in the catabolism (Figure 1.3), the response of anammox bacteria to hydroxylamine is described on lab-scale in *Chapter 4*. Addition of hydroxylamine (possibly an intermediate itself in the anammox process, proposal A and C in Figure 1.3) is known to result in the transient accumulation of hydrazine. This transient hydrazine accumulation is studied dynamically -both experimentally and with the aid of a mathematical model- and a mechanism for the process is suggested. In *Chapter 5*, the emission of nitric oxide (NO, intermediate in the anammox process according to proposals B and C in Figure 1.3) and nitrous oxide (N_2O) (which directly or indirectly contribute to the greenhouse effect) is evaluated from the full-scale nitritation-anammox process (in a two-reactor configuration) described in Chapter 2. Time-dependent measurements allowed the correlation of the emission with the operational parameters. The emission levels were (especially for the anammox reactor) markedly different from lab-scale observations. Finally, in *Chapter 6*, the location of the catabolism within the anammox cell is investigated. If the anammoxosome is the locus of the catabolism -as it is hypothesized-, also the proton motive force must be generated over the anammoxosome membrane. This hypothesis is tested by determining the pH difference between the region inside the anammoxosome and the rest of the cytoplasm using ^{31}P NMR.

2

Chapter 2

Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam

This Chapter has been published as

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Abstract

The first full-scale anammox reactor in the world was started at Dokhaven-Sluisjesdijk WWTP in Rotterdam (NL). The reactor was scaled-up directly from laboratory-scale to full-scale and treats up to 750 kg-N/d. In the initial phase of the startup, anammox conversions could not be identified by traditional methods, but quantitative PCR proved to be a reliable indicator for growth of the anammox population, indicating an anammox doubling time of 10-12 days. The experience gained during this first startup in combination with the availability of seed sludge from this reactor, will lead to a faster start-up of anammox reactors in the future. The anammox reactor type employed at Dokhaven-Sluisjesdijk WWTP was compared to other reactor types for the anammox process. Reactors with a high specific surface area like the granular sludge reactor employed at Dokhaven-Sluisjesdijk WWTP provide the highest volumetric loading rates. Mass transfer of nitrite into the biofilm is limiting the conversion of those reactor types that have a lower specific surface area. Now the first full-scale commercial anammox reactor is in operation, a consistent and descriptive nomenclature is suggested for reactors in which the anammox process is employed.

Introduction

The anammox process is the anoxic oxidation of ammonium with nitrite as electron acceptor (VAN DE GRAAF et al. 1996). The process is performed by bacteria in the order of the *Plantomycetales* (STROUS et al. 1999a) and is -with an estimated doubling time of 11 days (STROUS et al. 1998)- characterized by an extremely slow growth rate. The autotrophic growth mode (in combination with the high maintenance requirement due to the slow growth rate) leads to an overall stoichiometry showing a relatively low biomass yield (STROUS et al. 1998): (1.4)

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \longrightarrow 1.02N_2 + 0.066CH_{1.8}O_{0.5}N_{0.2} + 0.26NO_3^- + 2.03H_2O$$

The substrate nitrite is toxic to anammox organisms at levels above 50-150 mg-N/L, and stops the process completely (STROUS et al. 1999b). The anammox conversion was first observed in an autotrophic denitrification reactor with a sulfide-limiting loading for the denitrification process, in the presence of ammonium (MULDER et al. 1995). Since this first description, anammox-based processes for wastewater treatment have been developed and in recent years the first applications have occurred. In 2002 the first full-scale anammox reactor has been taken into operation at the sludge treatment plant of Dokhaven-Sluisjesdijk WWTP, Rotterdam, NL. The

reactor was fed with partially nitritated sludge liquor from an adjusted SHARON process (MULDER et al. 2001; VAN DONGEN et al. 2001b). The reactor is now fully operational (ABMA et al. 2007).

In this paper the course of the reactor start up followed by its stable operation is described. The operation is compared with anammox-related parameters previously reported in studies on laboratory scale. Furthermore, the various anammox-based processes now reaching full-scale are compared and an evaluation of possible limiting factors in anammox reactor-configurations is discussed.

Experimental

The anammox reactor

The full-scale anammox reactor is a 70 m^3 reactor designed by Paques BV (Figure 2.1). The reactor combines a high loading rate with efficient biomass retention, characteristics which the anammox process has in common with anaerobic wastewater treatment. The lower compartment (ca. 40 m^3) is mixed by influent and downcomer flow as well as by gas recycled from the top of the reactor. On top of the lower compartment, gas is collected for the riser of the gas lift. The liquid moves from the lower compartment to the less mixed and thus stratified upper compartment, serving mainly for biomass retention and effluent polishing. The feed is introduced from the bottom of the reactor and is (during loads lower than ca. 8 m^3/h or 150 kg-N/d) mixed with an additional recirculation flow from the effluent of the reactor to maintain adequate upflow velocity and shear stress to favor granule formation. The design load was 500 kg-N/d (7.1 kg-N/m^3/d) but the practical maximum loading is determined by the amount of nitrogen in the sludge digestate (on average ca. 700 kg-N/d).

Description of the sludge treatment facility

The anammox reactor is located at the sludge treatment site of Dokhaven-Sluisjesdijk WWTP (Rotterdam, NL, 51°53'48.8"N, 4°27'13.8"E). This site treats the sludge of the municipal wastewater treatment plant Dokhaven-Sluisjesdijk (620,400 population equivalents (p.e.), defined as 136 g COD/per person). At the sludge treatment site, sludge is thickened and digested (residence time ca. 30 days, temperature 32-33ºC). The centrifuged digestate (containing ca. 1200 mg/l NH_4^+-N) can be heated or cooled and is fed to a SHARON-type nitritation reactor (MULDER et al. 2001). The temperature of the nitritation reactor is kept at 33°C and the reactor can be operated with nitritation alone, or with nitritation-denitrification (see: start-up strategy).

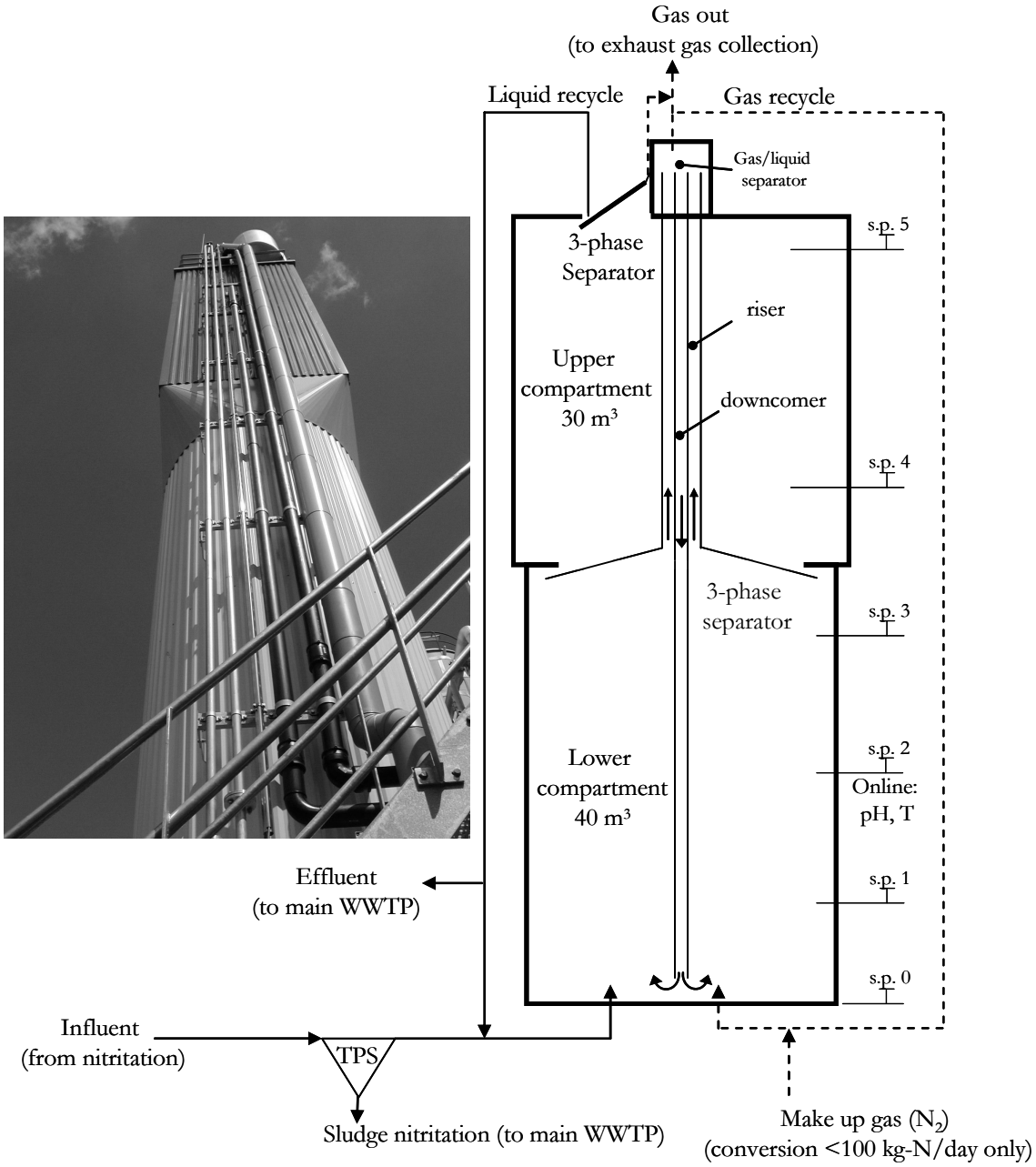

Figure 2.1 *The full (70 m³) scale anammox reactor at Dokhaven-Sluisjesdijk WWTP in Rotterdam, designed to treat 500 kg-N/m³/d. The photograph shows the upper compartment (dark grey), as well as the lower compartment (light grey). The schematic picture shows the internal airlift, gas recycle, six sample points (s.p.), as well as the tilted plate settler (TPS) before the anammox reactor.*

Nitrite oxidation to nitrate is avoided by controlling aerobic residence time: by controlling the aeration, the "aerated hydraulic retention time" is set long enough to enable growth of ammonium oxidizers, but short enough to lead to wash-out of (undesired) nitrite oxidizers (HELLINGA et al. 1998; MULDER et al. 2001). The effluent of the nitritation reactor (largely) serves -after passing a tilted plate settler- as influent for the anammox reactor. The effluent of the anammox reactor is returned to the influent of the main WWTP.

Startup strategy

Scale-up strategy

Based on 10 L lab-scale experiments with the nitritation-anammox process on centrifuged sludge digestate from Dokhaven-Sluisjesdijk WWTP (VAN DONGEN et al. 2001b), the reactor was directly scaled up 7000 fold to full-scale without building a pilot plant first. However, it was anticipated that some of the teething problems associated with new technologies -which normally would be identified and solved on pilot scale- would now show up during the startup of this first full-scale installation. To detect these expected problems, and to follow the startup well, several extra sample points and a flexible measurement loop were installed in addition to an online monitoring system.

Inoculation

The reactor was initially inoculated with nitrifying sludge (originating from the B-stage, see Figure 5.1) from the Dokhaven-Sluisjesdijk wastewater treatment plant. The inoculum had a sludge age of 7 days. After the startup, from day 622 to 1033, on 29 occasions with a total amount of 9.6 m^3, settled biomass from an anammox enrichment reactor was added in portions between 24 and 500 L. The enrichment reactor was a 5 m^3 well-mixed reactor connected to a settler located in Balk (NL), running at 35ºC with a conversion of 5 kg-N/m^3/d. The enrichment reactor had a hydraulic retention time of 0.7 days and was fed with synthetic medium containing -besides nutrients similar the medium of VAN DE GRAAF et al. (1996)- 1.8 kg-N/m^3 NH_4Cl and 1.6 kg-N/m^3 $NaNO_2$.

Control of nitrite concentration and loading

The concentration of nitrite during the startup is of crucial importance for growth: a too low level will result in substrate limitation and thus slower growth, while concentrations above 50-150 g-N/L can already lead to inhibition (STROUS et al. 1999b; EGLI et al. 2001; DAPENA-

MORA et al. 2007). These inhibition values are especially low compared with the nitrite concentration in a nitritation reactor running on sludge digestate (ca. 600 mg-N/L).

The start-up consisted of two phases. During the first 900 days, the startup regime was characterized by a relatively high influent flow rate (on average 3.6 m^3/h, HRT=19.4 h) with a low concentration of nitrite (on average 120 mg-N/L). The low nitrite level was achieved by operating the nitritation reactor as a nitritation-denitrification reactor by alternating periods of aeration (for nitritation) with non-aerated periods with methanol dosing (for denitrification). This mode of operation was similar to that of the nitritation reactor before the anammox reactor was installed. During the startup of the anammox reactor, the aim was to produce an effluent containing nitrite at non-toxic levels. An additional economical advantage of this mode of operation was that the nitrogen removal of the sludge treatment as a whole remained high during this phase in the startup of the anammox reactor.

In the second part of the startup, after about 900 days, methanol dosing to the nitritation reactor was completely stopped and the reactor was running as a nitritation reactor with nitrite effluent concentrations close to 600 mg-N/L. In this phase, the loading rate of the anammox reactor was carefully balanced with the available conversion capacity by varying the influent flow rate. The nitrite:ammonium ratio of circa 1:1 -which is required for the anammox process- was obtained automatically because the nitrification was limited by the amount of alkalinity. Therefore, the acidifying effect of nitritation could be balanced for circa 50% by the carbonate present in the sludge digestate (VAN DONGEN et al. 2001b).

Ammonium, nitrite and nitrate measurement

Daily measurements of ammonium, nitrite and nitrate served as basis for calculation of nitrogen load and conversion rate. Influent concentrations in the anammox reactor were based on the daily averaged effluent concentration of the nitritation reactor. An automated sampling device was employed for daily flow-rate proportional sampling. The sample was stored at 4°C. For anammox effluent samples, a daily grab-sample from the effluent of the anammox reactor was taken and analyzed immediately. Ammonium (47-130 mg-N/L), nitrite (0.6-6 mg-N/L) and nitrate (0.23-13.5 mg-N/L) were detected using commercial test kits (brand: Dr. Lange test kits, Hach Lange GmbH, Düsseldorf, DE, kits LCK304, LCK 341 and LCK339 respectively). The analysis was performed on a designated spectrophotometer (LASA 20).

Quantitative polymerase chain reaction (Q-PCR)

Q-PCR of partial 16S rRNA genes was performed on samples taken every 2-5 days during the first 1000 days of the startup. Samples were taken from sample point two of the reactor (Figure 2.1).

DNA extraction

DNA extraction was performed as described by ZHOU et al. (1996). However, after initial centrifugation and before the final centrifugation a bead beating step was introduced using 1 ml of Zirconia/Silica 100 μm beads and 1 ml of extraction buffer (pH 8.0, containing 100mM Tris-HCl, 100mM EDTA, 100mM phosphate and 1.5M NaCl).

Q-PCR

Extracted DNA was diluted 10 times Tris-EDTA buffer. 1 μl of the diluted DNA extract was mixed with 19 μl of hybridization essay (LightCycler DNA master hybridization probe, from Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, CH) for Q-PCR. Q-PCR was performed using a LightCycler 2.0 (Roche Diagnostics). PLA-46F (NEEF et al. 1998) was used as forward primer. A reverse primer (AMX-667R) was designed to amplify a part of 16S rDNA genes of the anammox cluster with containing 621 base pairs. Three types of hybridization probes were designed and mixed in a 1:2:1 molar ratio: (i) AMX-361 (hybridizing with all known anammox bacteria), (ii) AMX-381 (hybridizing with all known anammox bacteria) and (iii) AMX-382 (hybridizing with "Kuenenia" and "Brocadia"). Primer and probe details and sequences can be found in Table 2.1. The Q-PCR method was validated and calibrated by using serial dilution of a lab-scale anammox enrichment from a of toilet-water treatment. Detection in the enrichment was linear with the concentration in the range from 10^1 to 10^7 cells/ml. In view of the large amount of non-anammox DNA in the sample 10^4 cells/ml was taken as the detection limit.

The program for the Q-PCR assay consisted of 2 minutes denaturation at 95^0C, followed by 50 cycles of denaturation (heating to 95^0C) immediately followed by annealing combined with real time-detection (15 sec at 55^0C) and elongation (27 sec at 72^0C). The procedure was concluded with a melt-curve analysis between 40 and 85°C.

TABLE 2.1 *Oligonucleotides used in Q-PCR.*

name	Probe sequence[1] 5' -- 3'	hybridization	reference
PLA-46F	GGA TTA GGC ATG CAA GTC	Planctomycetes	NEEF et al. (1998)
AMX-667R	ACC AGA AGT TCC ACT CTC	anammox bacteria	this Chapter
AMX-361	AGA ATC TTT CGC AAT GCC CG-F	anammox bacteria[2]	this Chapter
AMX-381	L-AAG GGT GAC GAA GCG ACG CC	most anammox bacteria	this Chapter
AMX-382	L-AAG GTG ACG AAG CGA CGC C	"Kuenenia" and "Brocadia"	this Chapter

[1] Fluorophores mentioned: L=LightCycler Red 640, F=fluorescein isothiocyanate

[2] This probe partly overlaps with FISH probe AMX-368 (CCTTT**CGGGCATTGCGAA**).

FISH

Fluorescence In Situ Hybridization (FISH) analysis of the anammox population was performed regularly in the first 400 days of the startup to detect anammox growth. The *Plantomycetales*-specific probe PLA-46, as well as the probe AMX-820 (hybridizing with anammox bacteria "Brocadia" and "Kuenenia") were employed. On day 1330, FISH was performed to determine the genus of the anammox population of the reactor at Dokhaven-Sluisjesdijk WWTP in Rotterdam and of the inoculation reactor in Balk. The probes used were AMX-368 (hybridizing with all known anammox bacteria), AMX-820 (hybridizing with "Kuenenia" and "Brocadia") and KST-157 (hybridizing with "Kuenenia"). Probe and hybridization details (SCHMID et al. 2000; SCHMID et al. 2003; SCHMID et al. 2005) are available at Probebase (LOY et al. 2003).

Online measurements

Temperature, conductivity and pH sensors (Endress+Hauser, Reinach CH) were located in a measurement loop in the lower compartment of the reactor. The pH sensor was calibrated once every two months. Values were averaged over 24 hours for further evaluation. Nitrite concentrations at sample point 2 and in the effluent were determined spectrophotometrically every 20 minutes using a fully automated analyzer following the manufacturer's protocol (ADI 2019, Applikon, Schiedam NL).

Granule size

1000 ml of reactor volume was sieved on sieves with a 0.25 and 0.45 mm mesh. The sieved granules were collected, resuspended in water, and the volume was estimated using an Imhoff funnel after settling.

Control of granulation

In view of the low growth rate of anammox bacteria, and the required high loading rate, efficient biomass retention is required. A high upflow velocity (BEUN et al. 2000) is a crucial factor in the formation of well settling biomass that can remain in the reactor. The hydraulic retention time varies with varying nitrogen load during the startup. Therefore, the hydraulic retention time cannot be used efficiently to maintain a high upflow velocity. Thus, from day 1130 on, a flow-adjustable recycle stream from the top of the reactor was mixed with the influent to maintain a sufficiently high upflow rate (2-3 m/h) during the phases in the startup when the influent rate was not high enough to ensure proper granulation.

Sludge withdrawal

After the reactor was converting at its design capacity of 500 kg-N/d, sludge was removed periodically from the bottom of the reactor. A total amount of 36 m^3 of sludge was removed on 26 occasions from day 1341 to day 1432 in amounts varying from 0.5 to 2 m^3. The sludge was used to inoculate an anammox reactor in Lichtenvoorde (NL) and a one-reactor nitritation-anammox process in Olburgen (NL).

Results

Description of the startup

Based on the behavior of biomass growth and anammox activity (Figures 2.2 and 2.3), the startup period of the anammox reactor can be divided into two main phases.

First 800 days: periods of growth, but no detectable anammox activity

The reactor was inoculated with activated sludge from the main treatment plant, since the effect of inoculation with excess anammox sludge from available enrichments (on 1-25 L scale) would not be significant on a 70 m^3 scale. The presence of (low amounts) of anammox bacteria was shown already on lab-scale by the successful anammox enrichment from activated sludge of the Dokhaven-Sluisjesdijk wastewater treatment plant (VAN DONGEN et al. 2001b). The predominant activity in the first period was a rather low nitrite conversion (ca. 0.025 kg-N/m^3/d, Figure 2.2), probably due to denitrification which consumed nitrite with some excess COD in the anammox reactor. The nitrite removal was not coupled to ammonium removal, nor could nitrate production be detected (as would be expected if the anammox process were the dominant process). Since the anoxic oxidation of ammonium is (by definition) a clear indicator of

anammox activity, the *absence* of ammonium removal indicated that anammox activity was not visible from the conversion rate. In order to smoothen the rather fluctuating measurements and have a more robust indication of when ammonium removal occurred during a sufficiently long time interval, a 30-day moving average of the daily measured conversion rates was applied. The time-averaged ammonium conversion reached a value higher than 0.1 kg NH_4^+-N/m^3/d only from day 530. Significant nitrate production was not detected at all during this period. Q-PCR however provided an indication that anammox bacteria grew in the reactor (Figure 2.2). From day 73 on, a first period of growth could be detected by molecular methods.

Day 800-1250: periods of increase in conversion

From day 800 on, the 30-day moving average ammonium conversion was always significant and thus anammox activity could be detected by traditional measurements (Figure 2.3). Nitrate production, also a clear indicator of anammox activity, was only significant from day 925 on, while granule formation was significant from day 848 (Figure 2.3).

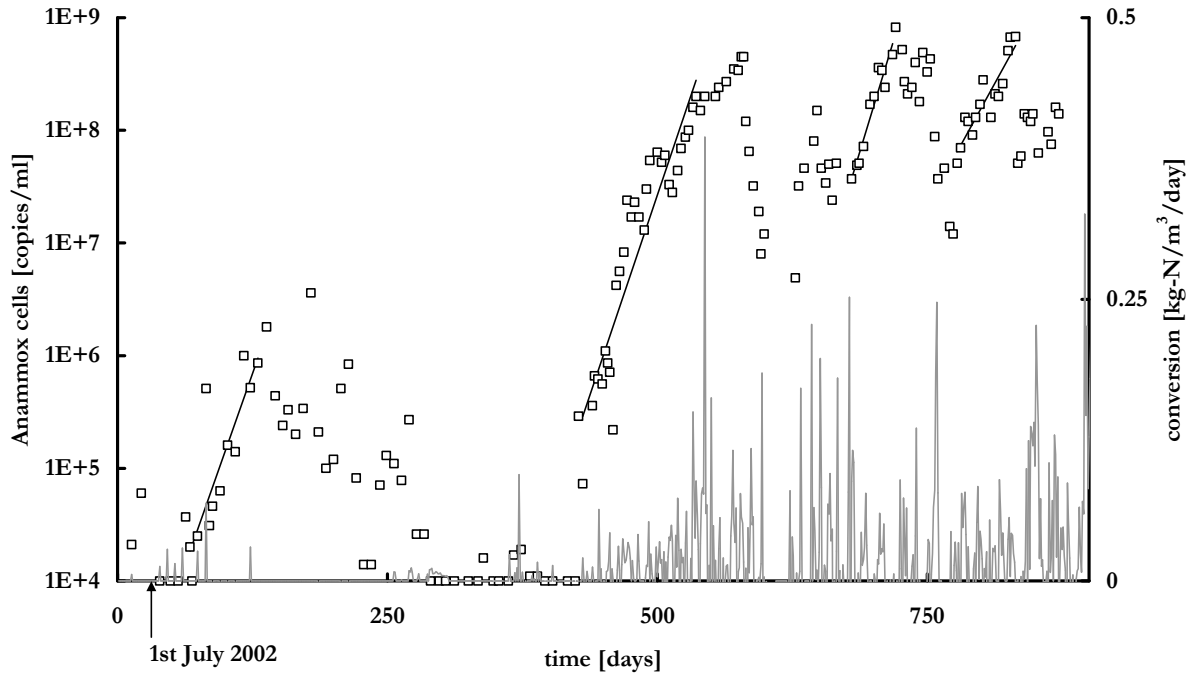

Figure 2.2 *Startup of the anammox reactor. The anammox cell concentration as measured by Q-PCR (□); the black lines represent the trend of the cell concentration during periods of exponential growth which were used for estimation of growth rates* μ *(Table 2.2); ammonium conversion is shown in grey.*

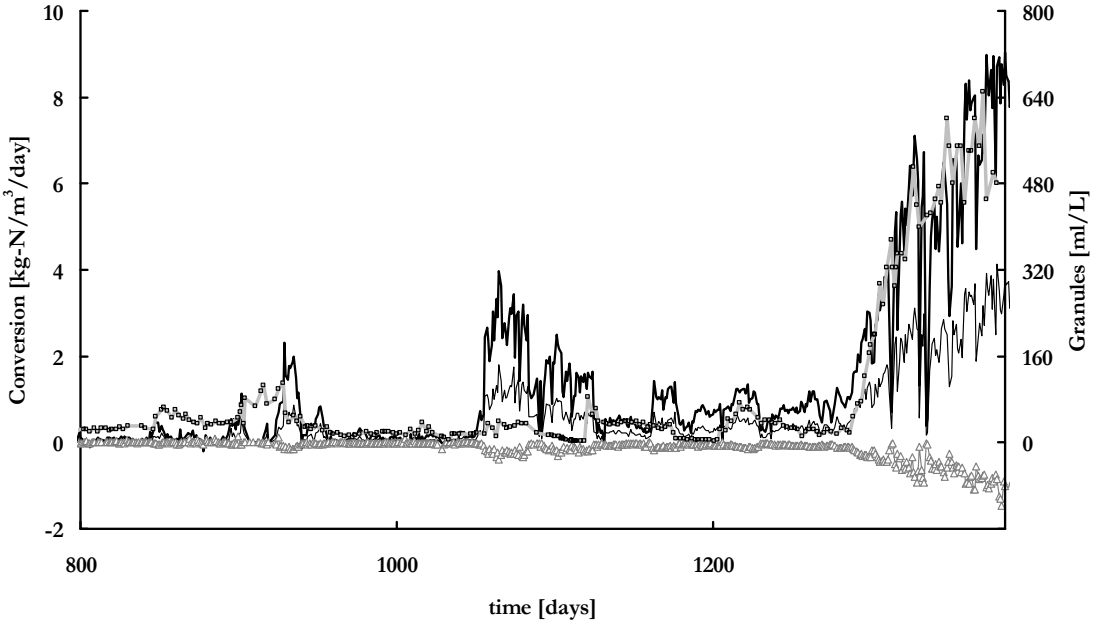

Figure 2.3 *Nitrogen conversion rate in the anammox reactor between days 800 and 1400 (before the design conversion of 7.1 kg-N/m³/d was achieved). Conversion of total nitrogen (thick black line), ammonium (thin black line), and nitrate (△, negative, because it is production) are shown on the left axis. The relative volume of granules (thick grey line with black squares ■, Imhoff value) is shown on the right axis.*

On day 900, the operation of the nitritation reactor was changed from nitritation-denitrification to nitritation only, which led to a rise in nitrite level of the anammox influent (Figure 2.4). The nitrite level rose from 130±268 mg-N/L to 575±175 mg-N/L. Several periods of growth could be distinguished, but for various reasons (see also "Problems during startup"), the enhanced conversion levels could not be maintained. In all those periods along with the conversion, the amount of granules increased.

Stable operation

From day 1235 on a stable conversion took place which could be gradually increased until the design load of 500 kg-N /d (7.1 kg-N/m^3/d) was reached on day 1359. From then on, sludge was removed to serve as an inoculum for new reactors (Lichtenvoorde, Olburgen) and if possible, the load of the anammox reactor was increased. The maximum attained conversion of 9.5 kg-N/m^3/d was limited by the available influent load and is not a maximum volumetric conversion of the anammox reactor (Figure 2.5).

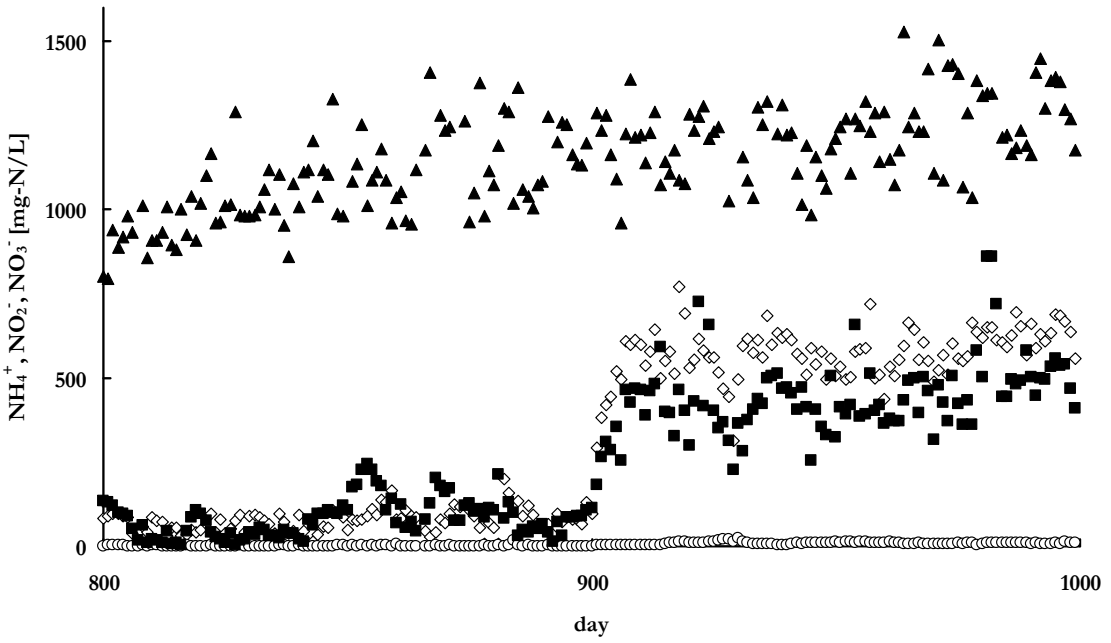

Figure 2.4 *Evolution of concentration of nitrogen compounds during the transition from operation of SHARON reactor as nitritation-denitrification reactor with methanol addition (until day 900) to operation with nitritation only. Influent ammonium (▲), effluent ammonium (■), nitrite (◊) and nitrate (○).*

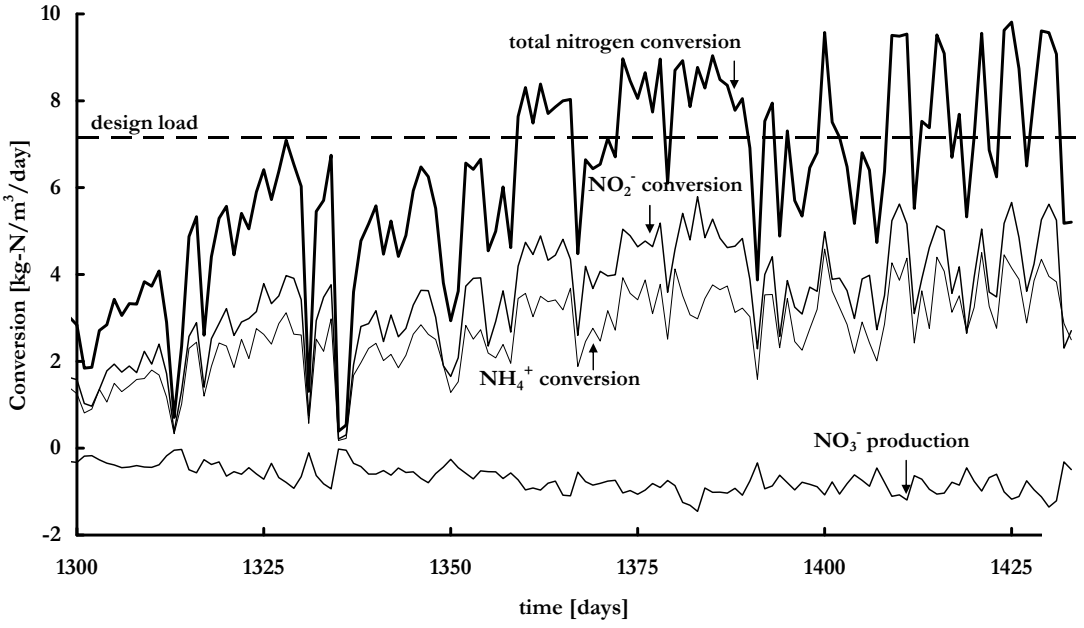

Figure 2.5 *Conversion during the days that the final the design load was achieved. Total nitrogen conversion (thick black line), nitrite conversion (thick grey line), ammonium conversion (thin black line) and nitrate production (indicated as "negative conversion"; thin grey line).*

Problems during start-up

Often during the startup (day 0-1250) the increasing amount of anammox biomass could not be maintained due to various accidents. A list of problems is given below:

- *Fall in conversion due to incidental nitrite toxicity.* This was caused by a too high loading rate and occurred mainly when nitrite analyzers were defect, or not properly calibrated.
- *Wash-out of biomass due to sudden changes in the hydraulic regime.* When during once-through operation (before day 1134, when no liquid recycle flow existed), the loading rate was increased, the upstream velocity was increased accordingly. This incidentally led to biomass washout because of a too sudden increase in upstream velocity.
- *Breakthrough of toxic methanol from nitritation.* Methanol is extremely toxic to anammox bacteria (GÜVEN et al. 2005), and in the period that methanol was dosed on the nitritation reactor (until day 900) this issue was of continuous concern. Although mixing-time calculations and incidental measurements indicated that methanol was converted before reaching the anammox reactor, and although breakthrough of methanol into the anammox reactor was never detected, it was incidentally suspected to have caused problems.
- *Freezing of pipes leading to a stop in the influent.* During the winter of 2002 (around day 150), during periods of low influent flow, freezing of the anammox influent lead to a stop in the feed.
- *Incidental failing of pumps and compressor; which hampered mixing or loading.* Mechanical problems occurred once in a while, mainly in the first 500 days of the startup.
- *Discharge of wastewater from mobile chemical toilets directly in the sludge line.* This wastewater (From Dixi, Papendrecht NL) contains amongst others nitrification inhibitors. These chemicals (or their degradation products during the sludge digestion or nitritation) had a strong adverse effect on the anammox process. The conversions in the nitritation process were however not visibly influenced. If this wastewater had been supplied to the main treatment plant (as is normal routine), then this would not have been a problem in view of the large dilution in the main plant and the associated longer residence time.

Exponential increase in biomass and conversion

Under laboratory conditions the maximum attainable doubling time in biofilm reactors is 11 days (STROUS et al. 1998). In view of this low growth rate it is interesting to see if this value was also attained in the full-scale reactor. For this purpose, the growth rate and doubling time of the organisms were determined from four periods of exponential increase in anammox cell

copies during the preliminary phase of the startup (Figure 2.2 and Table 2.2), and for six periods of exponential increase in conversion (Figure 2.6 and Table 2.3). Only those periods of exponential increase were taken into account, where no inoculation had taken place during the weeks before the calculation. Growth rates from the Q-PCR data were determined with the least squares method from the logarithm of the number of gene copies/ml with respect to time:

$$\ln C = \ln C_0 + \mu \cdot t \tag{2.1}$$

where C [gene copies/ml] is the gene copy concentrations at time, t [days], C_0 [gene copies/ml] is the initial gene copy concentration and μ [day^{-1}] is the specific biomass growth rate.

The loading rate of the anammox reactor was always adjusted to fit the maximum possible conversion rate, and thus to avoid limitation. This results in a balance between loading rate and conversion

$$R = \left[\frac{\phi_V}{V}(C_{in} - C)\right] = \frac{\mu}{Y_{sx}} C_x \tag{2.2}$$

where R [kg-N/m^3/d] is the conversion rate, ϕ_V [m^3/d] is the influent flow rate, V [m^3] is the reactor volume, Y_{sx} [kg-C/kg-N] is the yield of biomass on nitrite and C_x [kg-C/m^3] is the biomass concentration. During exponential growth this becomes:

$$\ln R = \ln\left(\frac{\mu}{Y_{sx}} C_{x,0} e^{\mu t}\right) = \ln\left(\frac{\mu}{Y_{sx}} C_{x,0}\right) + \mu t = \ln R_0 + \mu t \tag{2.3}$$

where R_0 [kg-N/m^3/d] is the initial conversion. This is similar to the equation for increase in the biomass concentration. For the periods of exponential increase in anammox cell copies, doubling times of 9 to 17 days were estimated. The doubling times based on conversion ranged from 2 to 18 days.

TABLE 2.2 *Exponential increase in anammox cells during the initial phase of the startup, as determined by Q-PCR.*

Start day	Duration [days]	μ [day^{-1}]	Doubling time[1] [days]	RR[2] -
67	63	0.063	11.1	0.70
431	105	0.065	10.6	0.84
680	38	0.073	9.5	0.92
778	54	0.040	17.3	0.83

[1] The biomass doubling time is $\frac{\ln 2}{\mu}$

TABLE 2.3 *Exponential increase in nitrogen conversion, as determined from the mass balance.*

Start day	Duration [days]	μ [day^{-1}]	Doubling time[1] [days]	RR[2] -
887	13	0.28	2.8	0.81
912	9	0.21	3.3	0.91
967	9	0.34	2.1	0.91
1284	44	0.037	18.5	0.67

1 The doubling time calculated from the conversion is $\frac{\ln 2}{\mu}$.

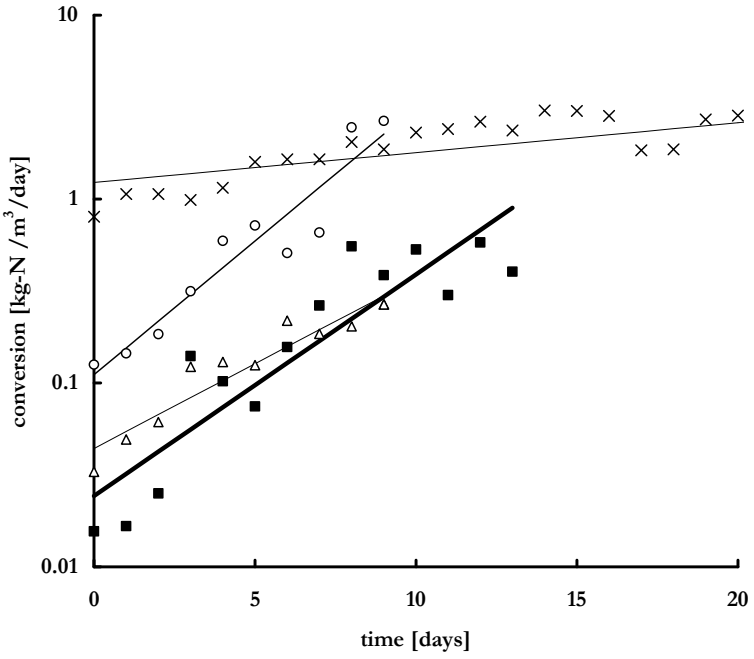

Figure 2.6 *Determination of growth rates from periods of exponential increase in conversion from days 887* (■)*, 912* (Δ), *967* (●) *and 1284* (x) *(see Table 2.3).*

FISH

From the weekly FISH analysis in the first 400 days, only an *estimate* of the relative population could be given. At the low levels of anammox organisms present, this resulted in low but fluctuating amounts. Anammox organisms could be detected nearly always however.

The population (as determined on day 1330) consisted of "Brocadia anammoxidans" with a relative abundance of 50-60%. The inoculation reactor in Balk contained "Kuenenia stuttgartiensis" with a similar relative abundance. This means that in the inoculation reactor and the full-scale reactor different anammox bacteria were present.

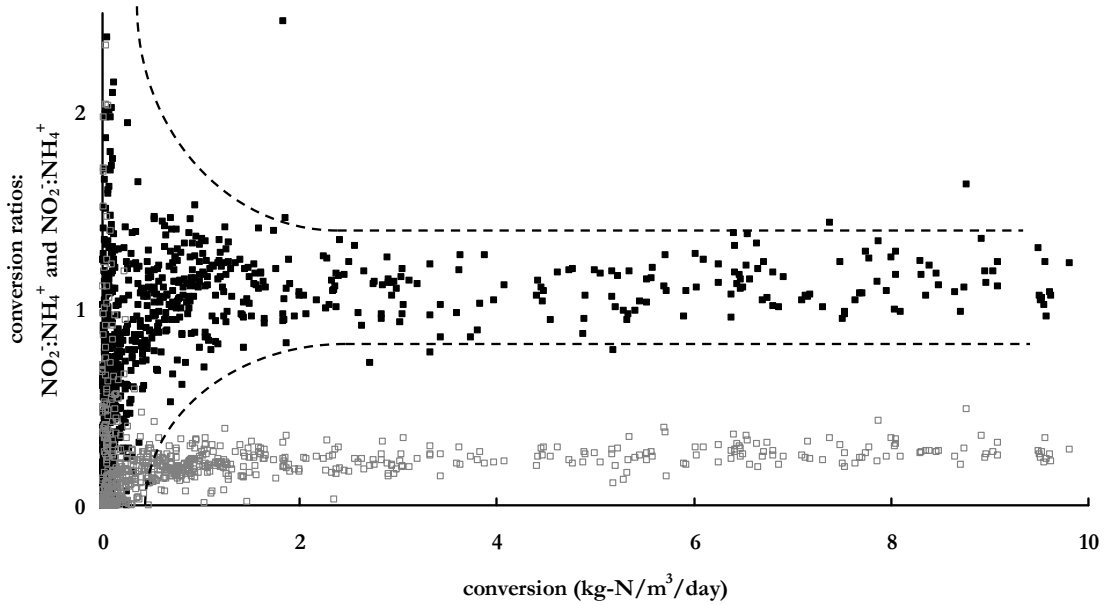

Figure 2.7 *Conversion ratio of nitrite and ammonium (*$Y_{NO_2^-/NH_4^+}$ *, ■); ratio between nitrate production and ammonium conversion (*$Y_{NO_3^-/NH_4^+}$ *, □).*

Stoichiometry

The ratio between nitrate production and ammonium consumption ($Y_{NO_3^-/NH_4^+}$), as well as the ratio between nitrite conversion and ammonium conversion ($Y_{NO_2^-/NH_4^+}$) are given as a function of the total nitrogen conversion rate in figure 2.7. Both ratios vary considerably on days where the total nitrogen conversion is below 1-1.5 kg-N/m^3/d, but become stable at conversions above 1.5 kg-N/m^3/d. The average value for $Y_{NO_2^-/NH_4^+}$ at a conversion above 1.5 kg-N/m^3/d is 1.31 (average over 193 days, standard deviation (st.dev.) of the mean 0.032, st.dev. 0.46) and $Y_{NO_3^-/NH_4^+}$ is 0.25 (193 days, st.dev. of the mean 0.006, st.dev 0.09). These values are well in line with values found in lab-scale reactors (STROUS et al. 1998). The pH and temperature in the reactor at different levels of conversion are presented in Figure 2.8. It is apparent from these data that the anammox process in this reactor configuration can run stably at a 6-10 kg-N/m^3/d level, in a pH range between 7 and 8 and in a temperature range of 30 to 40°C.

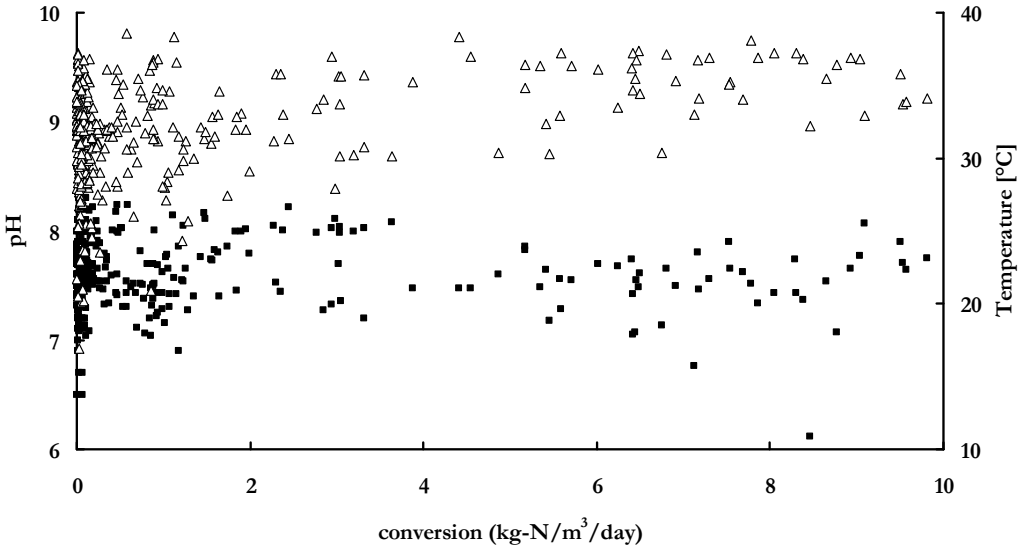

Figure 2.8 *pH* (■) *and temperature* (Δ) *at different conversion ratios.*

Discussion

Direct startup

To save time and resources, the anammox technology was scaled up directly from lab-scale to full-scale. This approach has proven to be effective in the introduction of other new nitrogen removing technologies (e.g. the BABE process (SALEM et al. 2004) and the SHARON process (VAN KEMPEN et al. 2001)), mostly because the operational costs (mainly personnel) of a pilot plant are approximately the same as when running a full-scale plant. At low conversion rates, the slow growth rate of the anammox bacteria made the assessment of different changes in the process operation relatively time-consuming, which would not have been different at pilot-scale.

The determination of growth of anammox bacteria by amplification with Q-PCR was one of the solutions to overcome this problem. However, to be fully effective, the Q-PCR should be used on daily rather than on weekly basis. Furthermore, also the time-delay between sample-taking and the communication of the results of the Q-PCR during this startup hampered the direct assessment of the status of the reactor.

The inoculation with seed sludge from an enrichment reactor in Balk proved to be very helpful to see the effects of changes in wastewater composition or process conditions. The reactor in Balk was running on synthetic feed under well known lab-conditions, and thus the

startup was relatively easy. The size of the reactor (4 m^3) was large enough to produce enough sludge to actually see the effects after inoculation. In the same period as the anammox reactor startup took place, a one reactor anammox process was scaled up in Strass (AT) in a step-wise way, from a 5 L inoculum (from a pilot scale anammox reactor in Zürich, FUX et al. 2002) to 300L, to 2.4 m^3, and finally to 500 m^3 (WETT 2006). The whole scale-up took also more than 3 years. This is another indication that, at least for the anammox process, directly building a full-scale plant did not cost extra time and saved the cost of running a pilot plant on-site.

Growth rate

The detection of anammox bacteria by Q-PCR has been the only parameter from which the success of the enrichment during the first 900 days could be determined. Only recently the use of Q-PCR for quantification of anammox bacteria has been reported (TSUSHIMA et al. 2007a), but detection was normally mainly based on conversions or population estimation from FISH. Q-PCR has proven to be very valuable during the initial phase of the startup, when anammox conversions were too low to reflect the biomass growth. To my knowledge, this is the first successful industrial application of Quantitative PCR as a biomass growth monitor. During the four identified phases of exponential biomass growth (Figure 2.2, Table 2.2), doubling times of 9-17 days could be estimated, which slightly higher than achieved doubling time in biofilm reactors (17.4 days, STROUS et al. 1998), but longer than recently reported minimum doubling times (of 3.5-8 days Chapter 2; TSUSHIMA et al. 2007a). It is possible however that (as was estimated in the lab-scale SBR) the actual growth rate was slightly higher and that part of the produced biomass was removed with the effluent. Suspended solid concentrations in influent and effluent were only monitored incidentally, and a possibly higher growth rate due this washout could not be calculated. If one tries to use the increase in nitrogen conversions to indirectly characterize the biomass growth, much quicker "doubling times" (2-3 days) are obtained. This unusually high increase in activity seemed to occur only for short periods (ca. one week), suggesting that the boost of nitrogen removal –at least here- does not correspond to growth, but rather to biomass reactivation after certain process disturbances. However, a reliable biomass estimate could not be given because of the large differences in conditions within the reactor volume during this phase of the startup. The results of FISH-analysis during the first 400 days could not be quantified, and also a reliable trend could not be identified from those images. This shows once again that (contrary to Q-PCR) when the population is not strongly enriched, FISH cannot be used to follow the growth.

Anammox species

The enriched species ("Brocadia anammoxidans") is different from the inoculation reactor ("Kuenenia stuttgartiensis"). Interestingly, it is also different from the dominant species in two other enrichments from Dokhaven-Sluisjesdijk WWTP B-stage sludge or the anammox reactor, where the enriched organism was "Kuenenia stuttgartiensis" (VAN DONGEN et al. 2001b) or "Brocadia fulgida" (KARTAL et al. 2004). Apparently the anammox-diversity of the nitrifying sludge is (at least at Dokhaven-Sluisjesdijk WWTP) large, and which organism is enriched is determined not by inoculation but by (for "Brocadia" and "Kuenenia" largely unknown) niche differentiation or other minor aspects. The kinetics and stoichiometry of all these anammox bacteria seem to be similar, so for the overall conversion it seems not to matter which organism is enriched. The anammox bacteria in the inoculum from Balk were clearly active when introduced in the full-scale reactor as their introduction into the reactor led to an increase in conversion. However, finally they seemed to be replaced by "Brocadia anammoxidans".

Granule formation

Increases in volumetric nitrogen conversion were always coupled to an increase in the volume of the biomass granules in the reactor (Figure 2.3). During the final phase of the start-up (after ~1300 days), the nitrogen conversions seem linearly dependent on the total volume of biomass granules. This strongly indicates that in the period of the sharpest improvement in conversion most of the active biomass was present in the granules. In other periods of growth the total volume of granules also increased but to lesser extent than in the final period. Probably in those periods a significant part of the activity was performed by the suspended biomass, as this type of biomass is more easily removed from the reactor upon a (slight) change in process conditions or upon incidental occurrence of higher nitrite levels (DAPENA-MORA et al. 2004).

Volumetric conversion

The maximum attained volumetric conversion rates in the reactor (more than 9 kg-N/m^3/d) were limited by the availability of nitritated sludge digestate and thus it is not clear if this is a maximum capacity related to the reactor-type. The full-scale nitrogen conversions are high compared to the usual conversion reported at lab-scale, but the gas production is comparable to that of anaerobic methanogenic reactors (ca. 10 m^3 gas/$m^3_{reactor}$/d). For 0.5-10L lab reactors, 0.2-2 kg-N/m^3/d are normally obtained (STROUS et al. 1998; FURUKAWA et al. 2003; WYFFELS et al. 2004), although a maximum of 8.9 kg-N/m^3/d (SLIEKERS et al. 2003) and even

26 kg-N/m^3/d (TSUSHIMA et al. 2007b) have also been reported. The footprint of the reactor (only 10 m^2) gives rise to a high surface specific conversion (75 kg-N/$m^2_{reactor}$/d).

Because all substrates (ammonium, nitrite and (bi)carbonate) are available in the liquid phase, and because the liquid concentration of the final product (N_2) is low enough not to influence the process, gas-liquid transfer is not a process rate limiting step in granular sludge anammox reactors. The lab-scale maximum conversions are therefore generally limited by the biomass retention capacity. In the full-scale reactor however, biomass retention during stable operation has proven to be more effective. The low biomass concentrations in the top part of the reactor, where the anammox biomass is actually separated from the effluent, show that biomass washout is very small (SRT is 45-160 days based on effluent measurements). The maximum attainable conversion is therefore probably only limited by the amount of biomass which can be present in the lower compartment.

Choice of reactor type

Anammox reactors

Several types of reactors have –under various names (Box 1, Table 2.4)- been employed for pilot scale and full-scale installations (Table 2.5) where anammox is the dominant process. The volumetric conversion in these reactors is markedly lower than that in the reactor at Dokhaven-Sluisjesdijk WWTP, and is generally limited by availability of substrate. All substrate-limited reactors are -by definition- over-designed, and it is not possible to compare reactor types based on these conversions. Therefore, maximum conversions for different anammox reactors were estimated by calculating the rates of possible process steps which are limiting the conversion (Table 2.6). Apart from the hydraulic limitation in the granular sludge reactor from Dokhaven-Sluisjesdijk WWTP, nitrite flux into the biofilm is another potential limitation. The maximum nitrite flux to an anammox-containing biofilm was calculated using the half-order substrate flux derived from penetration theory assuming zero-th order reaction kinetics (HARREMOËS 1977).

By assuming a concentration of nitrite of 15 mg-N/L in the bulk, a conversion flux of 17 g-NO_2^--N/m^2/d (equivalent to 30 g-N/m^2/d) can be estimated. Due to lower biofilm specific areas, rotating disk contactors and moving bed reactors attain lower maximum conversions of 7 kg-N/m^3/d respectively, based on this limitation. The small diameter of the granular sludge (ca. 1 mm) yields –based on nitrite limitation- a conversion capacity which is so high (90 kg-N/m^3/d), that nitrite limitation cannot be the main limitation in this reactor. The efficiency of biomass retention or eventually the gas-liquid separation unit determines in this case the maximum conversion.

TABLE 2.4 *Process options and names for nitrogen removal involving the anammox process*

process name proposed in this Chapter	number of reactors	source of nitrite	alternative process names	first reference
two-reactor nitritation-anammox process (FUX et al. 2001)	2		SHARON[1,2]-anammox	VAN DONGEN et al. (2001b)
			two stage OLAND[3]	WYFFELS et al. (2004)
			two stage deammonification	TRELA et al. (2004)
one-reactor nitritation-anammox process	1	NH_4^+ nitritation	aerobic deammonification	HIPPEN et al. (1997)
			OLAND[3]	KUAI and VERSTRAETE (1998)
			CANON[4]	THIRD et al. (2001)
			aerobic/anoxic deammonification	HIPPEN et al. (2001)
			deammonification	SEYFRIED et al. (2001)
			SNAP[5]	LIEU et al. (2005)
			DEMON[6]	WETT (2006)
			DIB[6,7]	LADIGES et al. (2006)
one-reactor denitrification-anammox process		NO_3^- denitrification	anammox[8]	MULDER et al. (1995)
			DEAMOX[9]	KALYUZHNYI et al. (2006)
			denammox[10]	PATHAK and KAZAMA (2007)

1 Acronym of Sustainable 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.
2 Sometimes the nitrification-denitrification over nitrite is addressed by with this term.
3 Acronym of Oxygen-Limited Autotrophic Nitrification-Denitrification.
4 Acronym of Completely Autotrophic Nitrogen removal Over Nitrite.
5 Acronym of Single-stage Nitrogen removal using the Anammox process and Partial nitritation; Name only refers to the process on a biofilm surface layer.
6 Name only refers to the process in an SBR under pH-control.
7 Acronym of Deammonification in Interval-aerated Biofilm systems.
8 System where anammox was found originally. Whole process was originally designated as "anammox".
9 Acronym of DEnitrifying AMmonium OXidation; This name only refers to denitrification with sulfide as electron donor.
10 Acronym of DENitrification-anAMMOX process; this name only refers to denitrication with organic matter as electron donor.

One-reactor nitritation-anammox processes

The combined nitritation-anammox process is –also under various names (Box 1, Table 2.4)- applied on full-scale, and also found incidentally in existing nitrifying rotating disk contactors (Table 2.5). For nitritation-anammox reactors, besides the nitrite limitation mentioned above, oxygen consumption by aerobic nitrifiers plays a role in process design. Oxygen transfer was indeed indicated as the limiting process for a lab-scale air-lift (SLIEKERS et al. 2003) and for a

pilot scale moving bed reactor (SZATKOWSKA et al. 2007). The oxygen limitation can stem from the slow diffusion into the biofilm or from the gas-liquid transfer. For the oxygen gas-liquid transfer rate a simplified correlation was used assuming a transfer of 0.55v% air per meter of reactor height (HEIJNEN and VAN 'T RIET 1984), whereas the oxygen penetration in the biofilm was calculated with the same approach as for nitrite. Estimations show that oxygen penetration is limiting the rotating disk contactor and the moving bed reactor with conversions of 2.5 and 1.2 kg-N/m^3/d, respectively. For the other reactors, gas-liquid oxygen transfer is potentially limiting as well. With a superficial gas velocity 0.025 m/s the oxygen transfer is ca 15 kg O_2/m^3/d (equivalent to a conversion of 8 kg-N/m^3/d).

Recommendations for future start ups

The low maximum specific growth rate of anammox bacteria will lead to a longer startup period for any anammox process compared to other nitrogen removal technologies. However, the startup period can be considerably shortened if the following two factors are taken into account:

- The biomass produced by the existing anammox reactor can be used for the inoculation of other reactors. Therefore, only 3-5 successful anammox doubling times are required to achieve full operation.
- Inoculation with large amounts of active biomass enables a fast check if the installation (including safety and automation systems) is functioning well. For example, the effect of unexpected toxicities can be seen immediately.

The startup of a full-scale one-reactor nitritation-anammox process treating a digested potato-wastewater in Olburgen (NL) (startup to design capacity in 6 months) inoculated with biomass from the reactor in Rotterdam indeed confirm that the faster startup of an anammox-based nitrogen removal process is possible with the larger biomass amounts now available and applying the lessons learned during the start-up of the process. Also the reactor in Glarnerland (CH) could be started up in a short time period with the inoculum–as well as with the experience gained- from the reactor in Strass (AT) (WETT 2007).

Box 1 Terminology

In the 1970ies, the common knowledge that ammonium could be oxidized under oxic conditions only, and even so not directly to N_2, was challenged by Broda's (1977) prediction of anoxic ammonium oxidation with nitrite or nitrate -instead of oxygen- as electron acceptor. Despite his postulation of these "lithotrophs missing from nature", it took years to find them:

- In an anoxic reactor *in Delft (NL)* which was fed with nitrate and ammonium, unexpectedly ammonium was removed (MULDER 1989; VAN DE GRAAF et al. 1990; MULDER et al. 1995). The responsible organism for the oxidation of ammonium was enriched with nitrite as electron acceptor (VAN DE GRAAF et al. 1996) and the process was named the **anammox process**. In 1999 it was found that the anammox process was performed by deep branching Planctomycetes. (STROUS et al. 1999a).
- The high removal of ammonium from a nitrifying rotating disk contactor *in Mechernich (DE)* in the absence of COD was noticed and thought to be linked to the aerobic oxidation of ammonium to dinitrogen gas (HIPPEN et al. 1997). The process was named **aerobic deammonification**.
- Also in a rotating biological contactor *in Kölliken (CH)*, high nitrogen losses occurred (SIEGRIST et al. 1998). They were attributed to either a combination between nitritation and the anammox process, or to the activity of nitrifiers alone.
- In lab-scale nitrifying rotating disk contactors *in Gent (BE)* nitrogen losses occurred, which were attributed to the activity of nitrifiers in the oxic and anoxic layers of the biofilm (KUAI and VERSTRAETE 1998). The process was named Oxygen Limited Autotrophic Nitrification-Denitrification **(OLAND)**.

During the following years, it was discovered that the nitrogen losses in the nitrifying systems in Switzerland (EGLI et al. 2001), Germany (HELMER et al. 2001) and Belgium (PYNAERT et al. 2003) were all caused by partial nitritation by aerobic ammonium oxidizers, followed by anoxic ammonium oxidation by anammox organisms in the deeper layers of the biofilm. The finding of unexplainable nitrogen losses at different locations -in combination with lacking consensus on both the responsible mechanism as well as the type of organisms performing the reaction- has led to several names for processes in which anammox organisms play a major role. These names, combined with others (often based on the names mentioned above) for certain configurations of these processes have lead to an unclear terminology in the (scientific) literature (Table 2.4).

Because the first full-scale anammox installations are now running, it would be helpful to clarify this situation by using the following *descriptive* terms, which are unambiguous for the scientific community:

- The *anammox process* for the anoxic combination of ammonium and nitrite to form dinitrogen gas.
- *One-reactor nitritation-anammox process* as the occurrence of the nitrite production and the anammox process in one reactor.
- *Two-reactor nitritation-anammox process* for the partial oxidation of ammonium to nitrite in an aerated reactor, followed by an anoxic reactor, where only the anammox process takes place.
- *One-reactor denitrification-anammox process* for the anoxic processes of denitrification from nitrate to nitrite, combined with the anammox process. This was the original process configuration in which anammox process was discovered (MULDER et al. 1995).
- The *anammox reactor* for the reactor in which only the anammox process takes place.
- *Anammox organisms*: the dedicated organisms (until now found to be a monophyletic group within the Planctomycetes) capable of performing the anammox process.

TABLE 2.5 *Conversions in full-scale and pilot scale (>1m³) reactors for the anammox process and the one-stage nitritation-anammox process in comparison with the corresponding lab scale reactors showing the highest conversions.*

process	location	reactor type	volume [m³]	area specific Conversion[1] [g-N/m²/d]	maximum conversion [kg-N/m³/d]	limitation	organism	reference
anammox process	Rotterdam NL	granular sludge	70	n.d.	10 (20)[2]	feed (NO_2^-)	"Brocadia"	this chapter
	Lichtenvoorde NL	granular sludge	100	n.d.	1	feed (NO_2^-)	"Kuenenia"	ABMA AND HAARHUIS, pers. comm.
	Hattingen[3] DE	moving bed	67	5	1	na[4]	n.d.	THÖLE et al. (2005)
	Mie prefecture JP	granular sludge	58	n.d.	3	feed (NH_4^+)	n.d.	TOKUTOMI et al. (2007)
	Datianshang CN	anaerobic biofilm	245	n.d.	0.6	not reported	"Brocadia"/ "Kuenenia"	XU et al. (2007 and pers. comm.)
	Balk NL	granular sludge	5	n.d.	4	feed (NO_2^-)	"Kuenenia"	This chapter
	Stockholm[5] SE	moving bed	2	0.5	0.1	feed (NO_2^-)	"Brocadia"[6]	GUT et al. (2006)
	Zürich CH	SBR	2.5	n.d.	2	feed (NO_2^-)	n.d.	FUX et al. (2002)
one-reactor nitritation-anammox process	Olburgen NL	airlift	600	n.d.	1.2	feed	"Brocadia"	ABMA AND HAARHUIS, pers. comm.
	Strass AT	SBR	500	n.d.	0.7	feed	"Brocadia"[7]	WETT (2007)
	Ruhrgebiet DE	MBR	660	n.d.	0.4	feed	"Brocadia"/ "Kuenenia"	DENECKE et al. (2007)
	Glarnerland CH	SBR	400	n.d.	0.6	feed	n.d.	WETT (2007)
	Pitsea GB	RDC[8]	240	7	1.7	feed	"Scalindua"	SCHMID (2003)
	Hattingen[9] DE	moving bed	102	6	1	not reported	n.d.	THÖLE et al. (2005)
	Mechernich DE	RDC	80[10]	2	0.6[4]	aeration	n.d.	HIPPEN (1997)
	Kölliken CH	RDC	33	2	0.4	feed	n.d.	SIEGRIST (1998)
	Stockholm[5] SE	moving bed	4	2	0.5	aeration	"Brocadia"	SZATKOWSKA (2007)

1 Conversion per area of biofilm.
2 Estimated conversion in the lower compartment.
3 Reactor 2, when no aeration was employed there, and reactor 1 was operated as a nitritation reactor.
4 Setup changed before maximum conversion was reached.
5 Reactor was first operated as a two reactor nitritation-anammox process, followed by one reactor nitritation-anammox operation.
6 Reported by GUT (2006).
7 Reported by INNEREBNER et al. (2007)
8 Plant consists of 4 lines of 3 RDC's in series. The values for the first cylinders are presented.
9 Reactor 1.
10 Not reported, value based on a biofilm surface of 250 m^2/m^3.

Table 2.6 *Estimated volumetric conversion limitations and fluxes (shown in brackets) in different types of reactors for the anammox process and the one-reactor nitritation-anammox process. The strongest limitation for each reactor is shown in* **grey-bold**.

	reactor type	particle diameter [m]	surface area[1] [m^2/m^3]	maximum volumetric conversion rate[2] [kg-N/$\mathbf{m^3}$/d] (conversion flux [g-N/$\mathbf{m^2}$/d]) limiting process: nitrite penetration[3,5,6]	oxygen penetration[3,4,7]	oxygen transfer[8]	hydro-dynamics
anammox process	granular sludge	0.001	3000	90 (30)	-	-	**12**[9]
	biofilm moving bed	0.01	250	**7** (30)	-	-	n.d.
	biofilm packed bed[10]		250	**7** (30)	-	-	n.d.
	biofilm sheets reactor		250	**7** (30)	-	-	n.d.
one-reactor nitritation-anammox process	airlift/bubble column	0.001	3000	89 (30)	15 (5)	**8**	n.d.
	rotating disk contactor		250	7 (30)	**2.5** (10)[11]	n.d.	n.d.
	moving bed	0.01	250	7 (30)	**1.2** (5)	8	n.d.
	sequencing batch reactor	0.001	3000	89 (30)	15 (5)	**8**	n.d.

1 Specific surface areas are estimated based on (NICOLELLA et al. 2000b) and the references cited in table 2.4 .

2 In one-reactor nitritation-anammox reactors the ammonium conversion is the amount of ammonium that is removed (not only the amount of NH_4^+ oxidized by aerobic ammonium oxidizing bacteria to NO_2^-).

3 Based on penetration into the biofilm (assuming zero-th order conversion kinetics) $\Phi_i = \sqrt{2 q_{iX} C_x D_i C_{b,i}}$ (HARREMOËS 1977) where subscript i is nitrite or oxygen, biofilm concentration C_x=80 kg DW/m^3.

4 Assumed oxygen bulk concentration = 1 mg/L; Diffusion coefficient D_{O_2} = $2.4 \cdot 10^{-9}$ m^2/s, specific oxygen conversion rate (nitrifiers) $q_{oxygenX}$ = $3.2 \cdot 10^{-5}$ kg O_2/kg DW/s (WIESMANN 1994).

5 Nitrite was assumed to be the limiting substrate in the anammox reaction for the anammox reactor (to avoid nitrite toxicity, STROUS et al. 1998) as well as for the two-reactor nitritation-anammox process (to prevent in-growth of nitrite oxidizers, THIRD et al. 2001).

6 Assumed NO_2^- bulk concentration = 15 mg-N/L; Diffusion coefficient $D_{NO_2^-}$ = $1.9 \cdot 10^{-9}$ m^2/s, specific NO_2^- conversion rate (anammox) $q_{nitriteX}$ = $8.3 \cdot 10^{-6}$ kg NO_2^--N/kg DW/s (STROUS et al. 1998).

7 Oxygen consumption is assumed to be caused by aerobic ammonium oxidizers as nitrite oxidizers are not supposed to be significantly present in the one-reactor nitritation-anammox process.

8 Oxygen transfer of 0.55% per meter height (HEIJNEN and VAN 'T RIET 1984) and superficial gas velocity taken at 0.025 m/s (flooding limit).

9 Gas production of more than 10 $m^3_{gas}/m^3_{reactor}$/d will lead to failing biomass retention in this type of reactors (NICOLELLA et al. 2000b).

10 The low shear stress in this type of reactor could lead to less dense biofilms, leading to a lower penetration depth for nitrite.

11 An oxygen bulk concentration of 4 mg/L was assumed as an average between concentrations in and outside the liquid.

Conclusion

The anammox process is the first wastewater treatment process based on notoriously slow-growing microorganisms that has reached the commercial and full-scale level. For the first time, a full-scale startup could be successfully monitored with Q-PCR during the periods when conversion did not give a clear proof of anammox activity yet. The biomass growth rate during periods of exponential growth could be estimated from Q-PCR to be 9-17 days, which is in correspondence with literature values. However, periods of faster (2-3 days) exponential increase in nitrogen conversion seemed to be correlated more to an increase in the biomass activity, rather than to actual biomass production.

Future startup of anammox reactors can take advantage of the experiences gained at Dokhaven-Sluisjesdijk WWTP, and can use sludge from existing reactors as an inoculum. The large inoculum will enable the fast check of the reactor performance, and decrease the number of multiplications of anammox organisms required before the design conversion is reached; thus decreasing the startup time of future anammox reactors.

3

Chapter 3

The membrane bioreactor: a novel tool to grow anammox bacteria as free cells

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Abstract

In a membrane bioreactor, fast growth of anammox bacteria was achieved with a sludge residence time (SRT) of 12 days. This relatively short SRT resulted in a -for anammox bacteria- unprecedented purity of the enrichment of 97.6%. The absence of a selective pressure for settling, and dedicated cultivation conditions led to growth in suspension as free cells and the complete absence of flocs or granules. Fast growth, low levels of calcium and magnesium, and possibly the presence of yeast extract and a low shear stress are critical for the obtainment of a completely suspended culture consisting of free anammox cells. During cultivation, a population shift was observed from *Candidatus* "Brocadia" to *Candidatus* "Kuenenia stuttgartiensis". It is hypothesized that the reason for this shift is the higher affinity for nitrite of "Kuenenia". The production of anammox bacteria in suspension with high purity and productivity makes the membrane bioreactor a promising tool for the cultivation and study of anammox bacteria.

Introduction

The cultivation of slow-growing microorganisms requires efficient retention of biomass and relies mostly on the ability of microorganisms to form biofilms or aggregates such as flocs or granules. Startup of these reactors can be impeded by insufficient biomass build-up: a continuous loss of "small" amounts of biomass via the effluent might lead to significantly longer observed biomass doubling times in the case of slow-growing microorganisms (STROUS et al. 1998). Granular sludge-based reactor design (NICOLELLA et al. 2000a) leads to compact reactors, which combine a short hydraulic retention time (HRT) with a long and stable solid retention time (SRT). Processes employing slow-growing organisms like nitrification (TANAKA and DUNN 1982), anaerobic digestion (LETTINGA et al. 1980), ferrous ion oxidation (EBRAHIMI et al. 2005), and phosphate removal (DE KREUK and VAN LOOSDRECHT 2004) all can be implemented successfully, on lab-scale as well as on full-scale, in retention-based reactors with a high volumetric loading rate. Typical examples of such reactors are airlifts (HEIJNEN et al. 1990), sequencing batch reactors (SBR, IRVINE et al. 1977; WILDERER and MCSWAIN 2004), internal circulation reactors (PEREBOOM and VEREIJKEN 1994) and upflow anaerobic sludge bed reactors (UASB, LETTINGA et al. 1980; MCHUGH et al. 2004).

Although particle-based bioreactors are advantageous for the cultivation of slow-growing microorganisms, and thus valuable from a technological point of view, the produced granules are

not the most suitable forms for the study of these microorganisms. Biokinetic parameters such as substrate affinities, maximum growth rate or maintenance need cannot be well assessed due to diffusion limitations within the floc or granule itself (HARREMOËS 1977; CHU et al. 2003). Disruption procedures performed in order to obtain single cells for several microbial tests -such as the most probable numbers (MPN) method and fluorescence in situ hybridization (FISH)- require large amounts of biomass and often introduce biases in the obtained results. Finally, also the energy required for bacteria to agglomerate (e.g., additional production of extracellular polymers) might reduce the observed maximum specific growth rate (μ_m) and agglomeration therefore also potentially leads to underestimation of μ_m (CHARACKLIS 1990).

In the membrane bioreactor (MBR), biomass retention is not based on settling of biomass. The effluent is withdrawn via a membrane which is impermeable for microbial cells. Unlike reactors with granular biomass, the MBR enables cultivation of slow-growing microorganisms with full biomass retention but without a selection on settling ability. This reactor type is currently employed for the growth of sensitive cells like plant/animal cells as well as for cell tissue production (DRIOLI and DE BARTOLO 2006). The MBR is also employed in full-scale wastewater treatment (SUTTON 2006; YANG et al. 2006), where the membrane separation reduces the surface area which is normally required for settling of flocculated sludge.

In this study, the possibility of cultivating slow-growing anammox cells in an MBR -with high purity and productivity- is demonstrated. The anammox process is the biological conversion of ammonium and nitrite to dinitrogen gas (VAN DE GRAAF et al. 1996). The process is performed by slow-growing deep-branching Planctomycetes (STROUS et al. 1999a). Anammox bacteria are autotrophic and have a notoriously low growth rate with minimum doubling times of several days (STROUS et al. 1998; TSUSHIMA et al. 2007a). Despite considerable interest in their cultivation, only enrichments (which typically contain 60 to 80% anammox bacteria) are available consisting of agglomerates or biofilms. This might even lead to the perception that anammox bacteria are preferentially growing in biofilms or granules. This perception is strengthened by the -valid- observation that selection for biofilm growth *in granular sludge or biofilm reactors* often improves with better biomass retention (STROUS et al. 1998). However, the high abundance of suspended anammox bacteria at the oxic-anoxic interface in several marine systems (SCHMID et al. 2007) indicates that growth as free cells is (also) a natural mode of growth.

The anammox process is applied on full-scale in biofilm-based bioreactors (ROSENWINKEL and CORNELIUS, 2004; CHAPTER 2). Contrary to problems associated with these reactors in the study of anammox organisms, for the application of the anammox process in nitrogen removal, these types of reactors are preferable over membrane bioreactors, since

anammox bacteria easily form sludge granules or biofilms which form a simple and economic way to obtain high biomass concentrations in the reactor. Moreover, wastewater always contains a certain amount of suspended solids. Since these solids are also (unintentionally but efficiently) retained by membrane filtration -and because the biomass production of anammox bacteria is relatively low due to their autotrophic nature- the sludge activity is also expected to decrease rapidly in a full-scale MBR-based anammox process.

In this Chapter, the successful enrichment of the slow-growing anammox bacteria in a lab-scale membrane bioreactor to high purity as single cells is reported. The resulting high production of completely suspended anammox bacteria during more than nine months makes the reactor a promising tool for study of the anammox process.

Experimental

Inoculation of the MBR

The reactor was inoculated with granular anammox sludge from the bottom of the lower compartment of the full-scale anammox reactor (Chapter 2) at the Dokhaven-Sluisjesdijk wastewater treatment plant in Rotterdam (NL). After removal of the 20% heaviest (i.e., quickest settling) fraction of the solids (with a high precipitates content), the reactor was inoculated with 1.5 liters of the remaining granular biomass.

Reactor operation

A 15 L reactor was used for the cultivation (see Figure 3.1). The liquid volume was 8 L and the reactor was fed continuously with 3.9-4.1 L/day medium with different compositions, resulting in a hydraulic retention time of 2 days. The liquid level was maintained via a liquid level-controlled (peristaltic) effluent pump connected to a membrane microfiltration module type Zeeweed, (Zenon Environmental, Ontario, CA.) which was placed within the reactor vessel. The membrane fiber (absolute pore size: 0.1 μm) was designed for operation in membrane bioreactors for wastewater treatment and is impermeable for microbial cells. The lab-scale module that was used (ZW1) consists of about 100 tubes (diameter ca. 1 mm, length ca. 300 mm). The module was replaced every 10 days to avoid biofilm growth on the membrane surface, and was subsequently cleaned (outside the reactor) with a protease-containing detergent (Tergazyme, Alconox, NY US). Great care was taken to remove all detergent after cleaning by prolonged and intensive rinsing with water. Replacement of the membrane took only 1-2 minutes and mixing

was stopped during the replacement to avoid the entrance of large amounts of air into the reactor.

To maintain anoxic conditions and to provide buffering capacity, the reactor was sparged continuously at 25 ml/min with 95% Ar-5% CO_2. pH was not controlled, but was always between 7.1 and 7.5. The temperature was controlled at 38°C, and the stirring speed was 160 rpm. To avoid growth of phototrophic organisms (and the related oxygen production, which would enable growth of other non-anammox microorganisms like nitrifying bacteria), the reactor was covered completely by a PVC cover (1 mm thickness) to prevent penetration of light. The reactor was fed with a concentrated medium according to VAN DE GRAAF et al. (1996) containing 120 mM ammonium and 120 mM nitrite (Table 3.1).

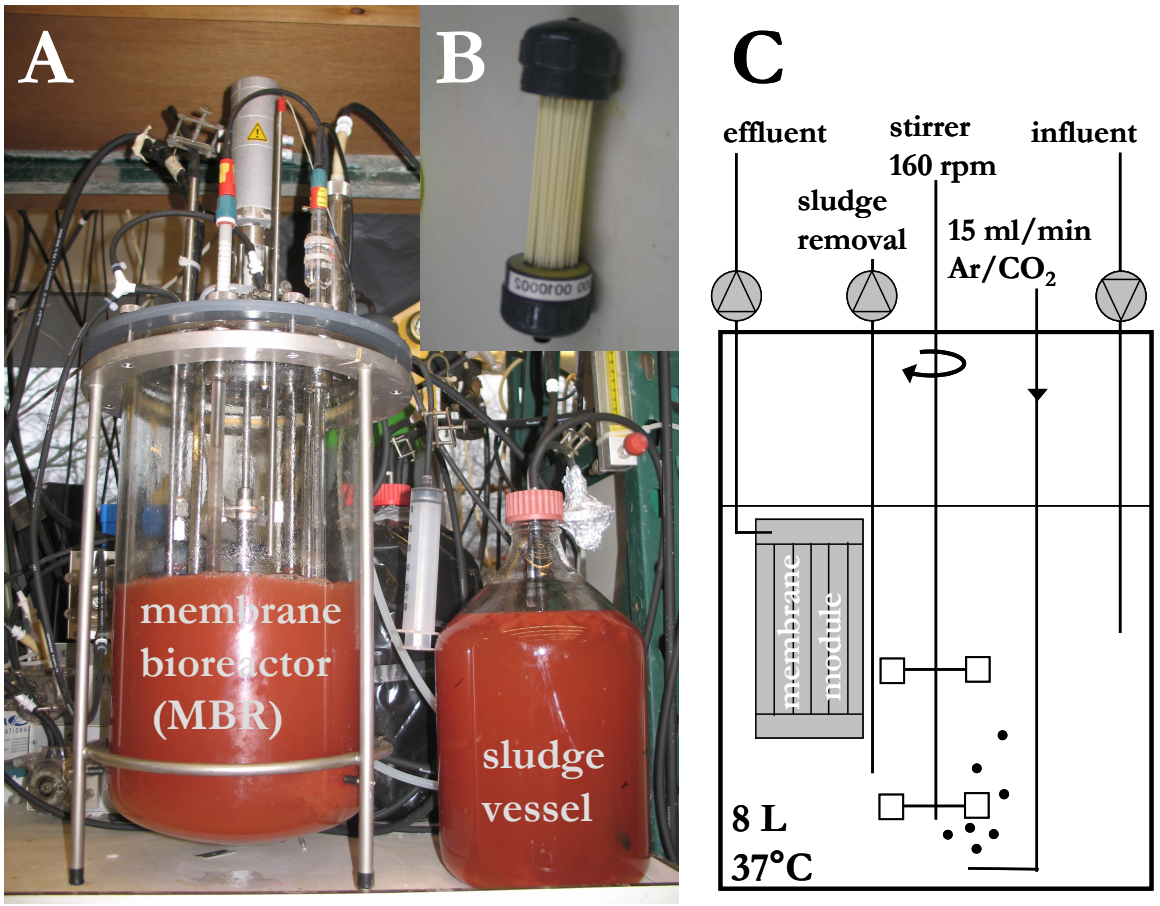


Figure 3.1 *(A) Photograph of the membrane bioreactor (MBR) for enrichment of anammox bacteria as free cells and the sludge withdrawal vessel; (B) photograph of the membrane module used; (C) scheme of the MBR showing the position of the membrane, gas sparger, influent line and sludge withdrawal pump. The MBR contains the completely suspended red anammox cells, which stay in suspension also in the (non-mixed!) sludge withdrawal vessel. The membrane (B) is completely immersed in the reactor.*

Table 3.1 *Different medium compositions for enrichment of anammox organisms.*

nutrient	dimension		VAN DE GRAAF et al. (1996)		initial period (this Chapter)		final period (this Chapter)		TRIGO et al. (2006)	
ammonium	mg-N/L	(mM)	420	(30)	1680	(120)	1680 [a]	(120)[a]	366	(26)
nitrite	mg-N/L	(mM)	420	(30)	1680	(120)	1680	(120)	370	(26)
calcium	mg/L	(mM)	49	(1.2)	164	(4.1)	41	(1.0)	1.5	(0.038)
bicarbonate	mg-C/L	(mM)	60	(5.0)	179	(15)	179	(15)	60	(5.0)
magnesium	mg/L	(mM)	30	(1.2)	39	(1.6)	9.9	(0.41)	5.8	(0.24)
EDTA	mg-C/L	(mM)	6.5	(0.054)	6.0	(0.050)	6.0	(0.050)	2.0	(0.017)
phosphate	mg-P/L	(mM)	6.2	(0.20)	5.7	(0.18)	5.7	(0.18)	2.3	(0.073)
iron	mg/L	(mM)	1.0	(0.018)	2.5	(0.045)	2.5	(0.045)	2.3	(0.041)
yeast extract	mg/L		-		-		1.0		-	

[a] This value was reduced from day 70 on to 1400 mg-N/L (100 mM).

The start-up period began with a first week in which the feed contained 100 mM sodium nitrate to avoid potential sulfate reduction. In this week, the influent ammonium nitrite levels were increased gradually in three steps from 20 mM to 120 mM. Then, after 30 days of full biomass retention (practically infinite SRT), the solid retention time (SRT) was controlled to ca. 16 days by removing once per day (during 30 minutes) 0.5 L from the reactor using an excess-sludge pump which was not connected to the membrane, but which pumped out the reactor-suspension directly. Operation of this excess-sludge pump was computer-controlled. This day, when the SRT started to be controlled to 16 days, is from here on designated as day 1 of the experiment.

On day 85, after a moment of incomplete conversion of nitrite, the medium composition was adjusted. Calcium and magnesium levels in the medium were lowered by 75%, and addition of 1 mg/L yeast extract was started (adjusted medium composition in Table 3.1). On day 127, the SRT was reduced to 12 days. This SRT was maintained for another 100 days. From day 150 on, ammonium was lowered from 120 mM to 100 mM to reduce the excess of ammonium in the reactor. The reactor was operated for more than 250 days.

Determination of nitrite, nitrate, ammonium and nitric oxide

Ammonium (2-47 mg-N/L, 0.14-3.4 mM), nitrite (0.015-0.6 mg-N/L, 1.1-43 μM), nitrate (0.23-13.5 mg-N/L, 16-964 μM) were analyzed using commercial Dr. Lange test kits (Hach Lange GmbH, Düsseldorf DE) and determined on a designated spectrophotometer (CADAS 50S). The off-gas was collected every 20 minutes in a 1 L sample bag and nitric oxide (NO) was

determined from this bag by a chemiluminescence analyzer (CLD700e, Ecophysics Dürnten, CH). N_2O was determined occasionally by gas chromatography: 100 µl off-gas samples were directly injected on a Hayesep Q 80/100 Ultimetal micropacked column (0.25 m x 1/16" x 1 mm) in a Varian 3800 gas chromatograph (Varian, Palo Alto, CA US). N_2O was determined with an electron capture detector with a lower limit of ca. 2 ppm.

Community analysis using molecular methods

Fluorescence in situ hybridization

Samples were fixed for fluorescence in situ hybridization (FISH) as described by PERNTALER et al. (2001). Briefly, cells were washed, fixed in paraformaldehyde and spotted onto Teflon-coated multi-well slides. After dehydration, the cells were hybridized with the following fluorescently-labeled oligonucleotide probes: EUB-338, PLA-46, AMX-368, AMX-820 or KST-157. Details on the target organisms and the sequences can be found in Table 3.2. Microscopic observations were performed with a Zeiss Axioplan epifluorescence microscope (Zeiss, Stuttgart DE). Fixation took place on day 35, 64, 77, 149, 196 and 267. On day 267, 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 (ca 10,000).

DNA extraction, PCR amplification and phylogenetic analysis

Samples (5 ml cell suspension) were taken from the reactor (on day 17 and 118) and directly centrifuged for 5 minutes at 13,000 g. The cell pellets were 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.

TABLE 3.2 *Oligonucleotides used in this Chapter*

oligo-nucleotide	target organisms	sequence (5'-3')	reference
EUB I		GCT GCC TCC CGT AGG AGT	
EUB II	bacteria	GCA GCC ACC CGT AGG TGT	DAIMS et al. (1999)
EUB III		GCT GCC ACC CGT AGG TGT	
PLA-46	Planctomycetes	GAC TTG CAT GCC TAA TCC	NEEF et al. (1998)
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. (2001b)
PLA-46F	Planctomycetes	GGA TTA GGC ATG CAA GTC	NEEF et al. (1998)
907RM	bacteria	CCG TCA ATT CMT TTG AGT TT[1]	MUYZER et al. (1997)
1392R	universal	ACG GGC GGT GTG TAC	SCHÄFER and MUYZER (2001)

[1] M denotes A or C.

Subsequently, the extracted DNA was used to amplify the nearly complete 16S rRNA gene using primers PLA-46F, 907RM and 1392R (see Table 3.2 for details). The PCR products were analyzed with agarose gel electrophoresis, purified using the Qiaquick PCR purification kit (Qiagen, Düsseldorf, DE) and sequenced by a commercial company (BaseClear, Leiden, NL). 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. The nearly complete 16S rRNA sequences were deposited in the Genbank database under accession numbers EU361730 (day 17, TUD-1) and EU361731 (day 118, TUD-2).

Results

General reactor operation

Within four weeks after inoculation (the designated day 1), the reactor could be operated at a conversion rate of 0.7 kg-NO_2^--N/m^3/d. The reactor was operated under nitrite limitation and nitrite consumption was generally complete (>>99%) throughout the study. The inoculum consisted of granules, and this changed to small flocs in the first 60-65 days. Lowering of calcium and magnesium ion levels in the medium, in combination with the addition of yeast extract to the reactor medium resulted in disappearance of the flocs and the culture changed fully to suspended free cells with the red color which is characteristic for anammox cells (Figure 3.1). Removed sludge did not settle at all. In the biomass effluent vessel (which was not stirred and had a

residence time of 2.5 days) most of the biomass remained in suspension and only a small fraction of the biomass accumulated as a floatation layer after several weeks of operation.

The membrane bioreactor could be operated stably for more than 250 days with very little maintenance at solid retention times of 16 and 12 days. The correspondingly achieved effective growth rates (where μ_m=1/SRT) of 0.0026 and 0.0035 h^{-1} represent doubling times of 11 and 8.3 days respectively. Occasional in-reactor determination of the maximum conversion capacity during operation at a solid retention time of 12 days was performed by increasing the influent flow for 1-2 hours to such an extent, that not all nitrite was converted and thus a slow nitrite accumulation could be observed. By subtraction of this nitrite accumulation from the imposed nitrite loading rate, the nitrite conversion rate under these non-nitrite limiting conditions could be estimated and related to the conversion rate under normal operating conditions. These short experiments indicated that the reactor was operated at 70-90% of the maximum conversion rate. A clear relation between operating conditions and the differences between individual measurements could not be observed. The nitrite:ammonium conversion ratio ($Y_{NO_2^-/NH_4^+}$) was 1.1-1.3 and the nitrate:ammonium ($Y_{NO_3^-/NH_4^+}$) ratio 0.10-0.25. These numbers are in accordance with values obtained in other reactors (STROUS et al. 1998; SCHMID et al. 2000; LÓPEZ et al. 2008). Nitric oxide was on-line measured in the off-gas of the MBR, and the levels were generally well-below 1 ppm. The (off-line) N_2O level was below 2 ppm. The fraction of the nitrogen converted to NO and N_2O therefore was below 0.01%.

Membrane fouling was heavier in the first 60 days of operation, but never led to clogging of the membrane before its scheduled replacement (every 10 days). Membrane replacement did not lead to disturbances in conversion during the first 120 days, but after that, the conversion started again only after a delay of about one hour. Probably, the absence of heterotrophic organisms and nitrifiers during the 120 days of stable operation (see next paragraph), increased the time which was required to remove the oxygen leaking into the reactor during the procedure (STROUS et al. 1997a), thus inhibiting the anammox process temporarily. The inability of the culture to remove oxygen also became apparent from batch tests in the presence of ammonium under oxic conditions. In these batch tests, the oxygen concentration dropped less than 0.2 mg/L/h per hour, so oxygen consumption could not be detected.

Microbial community analysis

The anammox bacteria in the inoculum consisted of *Candidatus* "Brocadia" (Chapter 2), and on day 35 this was still the main population since hybridization took place with the "Kuenenia"/"Brocadia"-specific probe (AMX-820), but not with the "Kuenenia"-specific probe

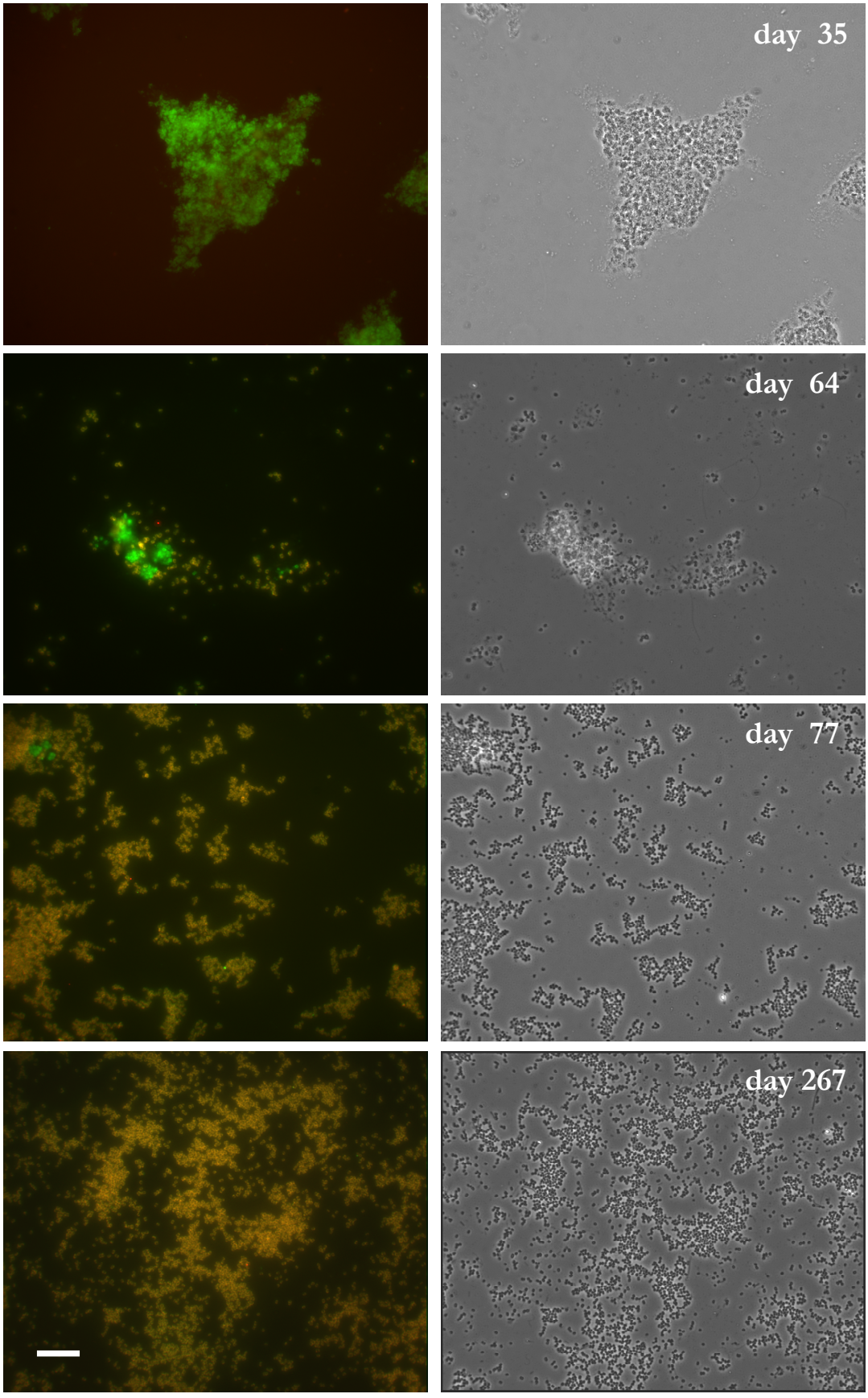
(Figure 3.2). However, the sample on day 64 shows also a significant number of "Kuenenia" cells, which became the main population after day 149. After this date no change in the main population or level could be seen and "Brocadia" could not be detected anymore (<0.5%). The level of enrichment increased from 60-80% in the first 100 days to more than 90% after day 100. No cells could be found at any time that did not hybridize with the "Kuenenia"/"Brocadia" specific probe (AMX-820), but which did hybridize with the Planctomycetes-specific PLA-46 probe or with the all-anammox specific AMX-368 probe in any of the samples. This indicates that other described anammox bacteria were not present (or only present in very low amounts). Since free cells were obtained, quantification using FISH was possible by viewing each individual cell and determining whether this cell had hybridized with the "Kuenenia"/"Brocadia" specific probe (AMX-820). The enrichment level at day 267 was 97.6±0.2% (ca. 10,000 cells counted).

The population change from *Candidatus* "Brocadia" to *Candidatus* "Kuenenia" was confirmed by 16S rRNA sequence analysis. The sequences of the sample on day 17 showed the strongest similarity (98.9%) with *Candidatus* "Brocadia" sp. 40 (KIELING et al. 2007). The sample on day 118 showed a sequence similarity of 100% with a *Candidatus* "Kuenenia stuttgartiensis" strain (Kölliken enrichment, EGLI et al. 2001), and 99.7% with the original *Candidatus* "Kuenenia stuttgartiensis" (Stuttgart enrichment, SCHMID et al. 2000). Figure 3.3 shows a phylogenetic tree based on the 16S rRNA sequences of the strains.

Affinity for nitrite

The nitrite level in the reactor fluctuated considerably but was generally between 2 and 6 μM. Since the reactor was operated at 70% to 90% of the maximum specific conversion rate, the half saturation constant (K_S) must be 10-50% of this range (as $\frac{\mu}{\mu_m} = \frac{C_{NO_2^-}}{C_{NO_2^-} + K_S} \rightarrow K_S = C_{NO_2^-} \frac{\mu_m - \mu}{\mu}$, where $C_{NO_2^-}$ is the nitrite concentration in the reactor) and is thus estimated to be between 0.2 and 3 μM. The low nitrite level is another indication that the bacteria are really present as free cells, since any significant agglomeration would have led to a lower (apparent) substrate affinity due to diffusion limitation (i.e., a higher K_S).

Figure 3.2 *(next page) The reactor population in the reactor in time as shown by FISH (left) and phase contrast microscopy (right). The anammox population shifts from "Brocadia" (green, hybridization with the "Brocadia"/"Kuenenia"-specific probe AMX 820 in cy3) to "Kuenenia" (orange, hybridization also with "Kuenenia"-specific probe KST-157 in cy5). The purity in "Kuenenia" increases to 96.7% on day 267. Scale bar is 10 μm.*


day 35
day 64
day 77
day 267

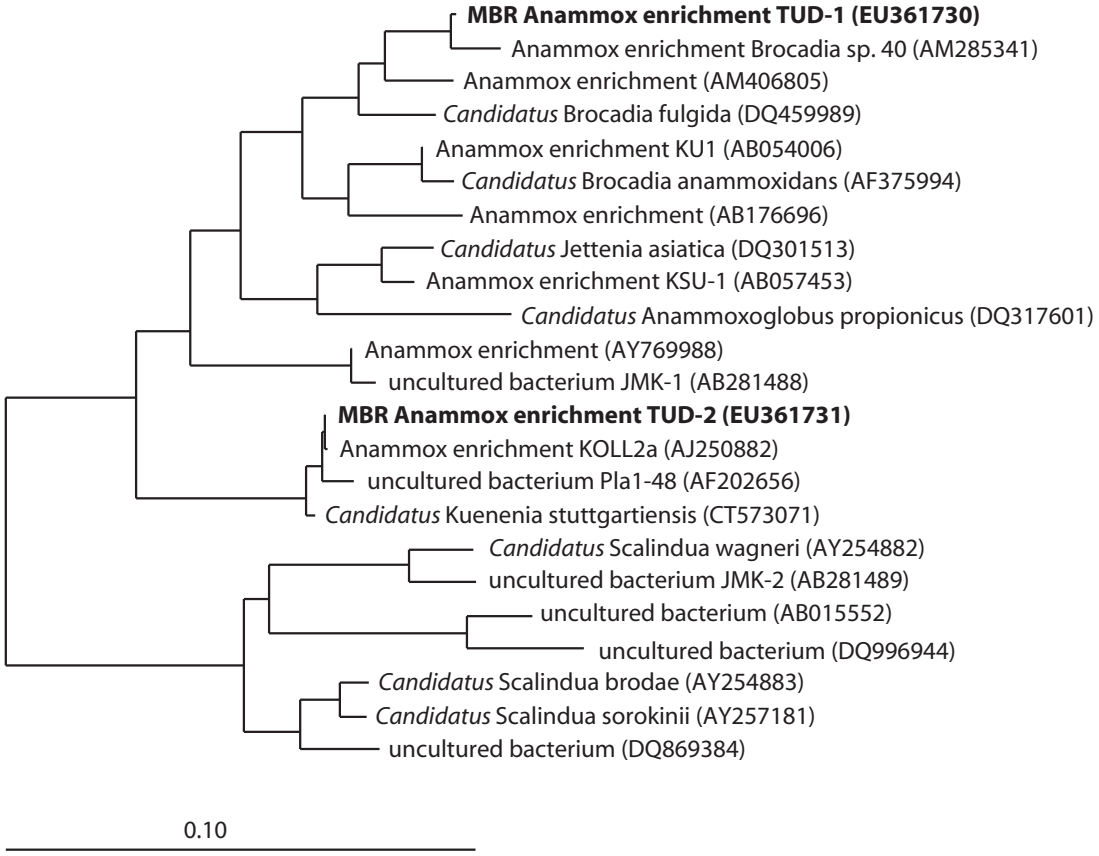


Figure 3.3 *Phylogenetic tree based on nearly complete 16S rRNA sequences obtained from the dominant organisms of the membrane bioreactor (MBR). The sequence obtained from enrichment TUD-1 was related to members around Candidatus "Brocadia fulgida". The sequence obtained from enrichment TUD-2 was related to members around Candidatus "Kuenenia stuttgartiensis". The sequence of Thermotoga maritime was used as an outgroup, but pruned from the tree. Bar indicates 10% sequence difference.*

Discussion

Why was a suspension culture achieved?

Although the obtainment of aggregates is a logic consequence of enrichment reactors which select on settling ability (like sequencing batch reactors, gaslift reactors, etc.), the reversed statement is not necessarily true. Thus, an enrichment system where no selection on settling ability is present (like the MBR) might still lead to the formation of aggregates. Aggregates were indeed present in the first 70 days of this enrichment, and also in other membrane bioreactors

where the anammox process (WYFFELS et al. 2004; TRIGO et al. 2006) or nitrification (WYFFELS et al. 2003) were employed.

What was then the trigger for growth as free cells? It appears that the direct cause was the reduction of calcium and magnesium levels, which took place in combination with the addition of small amounts of yeast extract. Bivalent ions (like calcium and magnesium) are known flocculation enhancers (MAHONEY et al. 1987; SOBECK and HIGGINS 2002; PEVERE et al. 2007). Moreover, small calcium or magnesium precipitates in the reactor (e.g. hydroxyapatite, calcite) might act as nucleation seeds for the growth of small granules. If the presence of flocs were a result of extracellular polymeric substances (EPS) -produced as a result of stress because of the absence of a micronutrient- addition of yeast extract potentially has led to a lower EPS production and may thus have aided suspended growth by reducing the related stress of the bacteria. This study unfortunately can not differentiate between the effect of addition of yeast extract on one hand, and lowering of calcium and magnesium levels on the other, since addition of yeast extract coincided with lowering of calcium and magnesium levels. Because the effect of bivalent ions on flocculation is a much more established effect, it is assumed here that this has been the determining parameter in the production of suspended anammox cells. However, a low level of bivalent ions can not be the only determining factor, since TRIGO and coworkers (2006) obtained granules even after calcium and magnesium levels were lowered (see Table 3.1 for an overview of feed conditions).

An important other difference in reactor operation is the applied solid retention time: in the MBR reactor of this study, the SRT was carefully controlled at 12 or 16 days whereas the retention time was "nearly infinite" in the MBRs of WYFFELS et al. (2004) and TRIGO et al. (2006) since no biomass (and hence also no precipitate) was actively removed. Besides the fact that competition in systems with an extremely high SRT is much lower (because the growth rate is lower and this enhances the required time for a change in community composition), a low growth rate is a known enhancer of granule formation (DE KREUK and VAN LOOSDRECHT 2004). A (relatively) short solid retention time therefore seems to be also important for obtaining suspended cells.

A low shear stress is another parameter which is disadvantageous for aggregation and can therefore aid suspended growth (BEUN et al. 2000; LIU and TAY 2002). With the absence of baffles in the reactor and the low stirring speed (160 rpm) this condition is also met in this reactor.

In conclusion, it seems that the cultivation of anammox bacteria as suspended cells is only possible when several requirements are satisfied: (i) the absence of selective pressure for

settling (MBR or chemostat cultivation), (ii) a high growth rate (obtained by short solid retention time), (iii) low levels of bivalent ions (i.e., calcium and/or magnesium). Furthermore, addition of yeast extract and low shear stress might play a role in the obtaining of a suspended culture of anammox bacteria.

Growth rate of the anammox bacteria

With the continuous cultivation at an SRT of 12 days, this is the first report of a reactor study on anammox bacteria with a doubling time (t_d) of less than 10 days (as $t_d = \ln(2) \cdot SRT$). Typical doubling times in anammox reactors are 15-30 days (STROUS et al. 1998; FUX et al. 2004). Moreover, the produced biomass could be efficiently harvested because it was obtained separate from the effluent (which was removed via a membrane). In view of the maximum conversion capacity in the reactor, the minimum doubling time for anammox bacteria was estimated to be 5.5-7.5 days. Also from the rate at which the population shift from "Kuenenia" to "Brocadia" took place, a doubling time could be estimated. Assuming from microscopic observations that "Kuenenia" constituted 10% of the population on day 64, and 90% on day 77 (and assuming that the growth of "Kuenenia" was not substrate-limited during this period, and the total amount of anammox ("Brocadia" and "Kuenenia") biomass was constant), the "Kuenenia" biomass had increased by a factor 9 in 13 days at an SRT of 16 days. The growth rate required for this is $\mu = \frac{1}{SRT} + \frac{\ln\left(\frac{C_{"Kuenenia",day\,77}}{C_{"Kuenenia",day\,64}}\right)}{t_{day\,77} - t_{day\,64}} = 0.062 + 0.17 = 0.23\ day^{-1}$ (where $C_{"Kuenenia",\ day\ i}$ = concentration of "Kuenenia" bacteria on day i), which corresponds to a doubling time of only 3 days. Although this is an indication for a relatively fast growth of anammox bacteria, it should be stressed that it only constitutes a one-time observation based on qualitative population estimations and can therefore only be regarded as an *indication* for this fast growth.

From these observations it can be concluded that the doubling time of anammox bacteria is at most 5.5-7.5 days, but possibly as low as three days. Also the growth rate of anammox bacteria during the exponential growth phase (in shake flasks, TSUSHIMA et al. 2007a) was estimated to be within this range. By quantitative PCR, the doubling time in those systems was estimated to be 3.6-5.4 days. ISAKA et al. (2006) have reported a doubling time of 1.8 days, which is faster than the fastest estimates of the doubling time; not only in the present study but also in the study of TSUSHIMA et al. However, the growth rate estimation by ISAKA et al. (2006) was based on the comparison of the number of anammox cells of two different reactors inoculated at the same time under similar conditions. 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 method used by ISAKA et al. (2006) is highly questionable and the obtained results are therefore doubtful.

Affinity constants for nitrite

The K_S of anammox bacteria for nitrite was estimated to be 0.2-3 μM in this study (with "Kuenenia") and below <5 μM by Strous et al. (1999b) (with "Brocadia"). The estimated K_S values for nitrite for (aerobic) nitrite oxidizers vary considerably (12-955 μM, BOTH et al. 1992; HUNIK et al. 1993; SCHRAMM et al. 1999) both between different studies and between different species, but the values obtained for anammox bacteria are definitely lower than the lowest reported affinity for nitrite oxidizers.

In (aerated) nitrite-limited systems, nitrite oxidizing bacteria compete with anammox bacteria for nitrite. Nitrite oxidizers maintain themselves in the aerobic part of the biofilm, while anammox bacteria reside in the anoxic regions. Due to the required diffusive transport of nitrite from the aerobic to the anoxic region, nitrite oxidizers will observe somewhat higher nitrite concentrations than anammox cells. In the competition this can be compensated by a lower value for the affinity constant for anammox bacteria. It has been predicted by mathematical modeling that an affinity constant ratio between anammox and nitrite oxidizing bacteria of $\frac{K_{S,NO_2^-}^{nitrite\ oxidizers}}{K_{S,NO_2^-}^{anammox}} > 3$ is required for anammox bacteria in order to maintain themselves in a nitritation-anammox biofilm processes (HAO et al. 2002). The estimated lower affinity constants of anammox bacteria for nitrite in this experimental study indeed suggest that competition in these systems can be solely based on nitrite, and thus the one-reactor nitritation-anammox process does not have to take place under strict oxygen limitation. Operation without oxygen limitation could be advantageous for process control, since a strictly oxygen-limited system is harder to operate because the oxygen load has to be carefully balanced with influent loading rate.

Also in natural systems, a higher affinity for nitrite is a major competitive advantage that might be responsible for the relatively large abundance of anammox bacteria in marine systems (SCHMID et al. 2007). However, in natural systems also competition for nitrite with denitrifying microorganisms takes place. Still, the affinity for nitrite of anammox bacteria could be in the same range as the affinity of denitrifiers (4 μM - 25 μM, BETLACH and TIEDJE 1981; ALMEIDA et al. 1995) and therefore, the outcome of competition between those two groups can not be assessed.

Niche differentiation of different anammox bacteria

In lab-scale enrichments with ammonium and nitrite, always either "Brocadia" or "Kuenenia" cells become dominant. Therefore, a clear niche difference exists between the "Brocadia"/"Kuenenia" group, and other genera, like *Candidatus* "Scalindua" (detected under marine conditions) or *Candidatus* "Anammoxoglobus" (enriched under propionate addition). The niche differentiation between *Candidatus* "Brocadia" and *Candidatus* "Kuenenia" themselves however is still unresolved. It has been suggested that "Brocadia" cells are more susceptible to nitrite inhibition, and therefore are not enriched in reactor systems at high nitrite levels (GAUL et al. 2005). The recent finding of several "Brocadia" enrichments in full-scale reactors -which are operated at higher nitrite levels- [Olburgen NL, Rotterdam NL, (Chapter 2)] disproves this hypothesis. Also aeration does not seem to play a determining role, as no clear difference could be found between the population of (aerated) one-reactor nitritation-anammox processes ["Brocadia" in Olburgen NL (Chapter 2) and Strass CH (INNEREBNER et al. 2007); but "Kuenenia" in Gent BE (PYNAERT et al. 2003)] and (non-aerated) anammox reactors ["Brocadia" in Rotterdam NL; "Kuenenia" in Lichtenvoorde (NL) (Chapter 2)].

In the MBR described in this Chapter, the switch from a "Brocadia"-dominated culture to a "Kuenenia"-dominated culture took place during cultivation at an SRT of 16 days. The minimum specific growth rate of 0.0026 h^{-1} which was required for cultivation at this SRT is around or below the maximum specific growth rate of both "Kuenenia" and "Brocadia" organisms. Therefore -and because the switch took place in such a short period of time- the competition has probably not taken place based on growth rate. Selection in MBRs is (like in chemostats, HARDER and KUENEN 1977) generally based on (apparent) affinity for the limiting substrate, in this case nitrite. In lab-scale enrichments (without additions of acids, or extra salts, etc.) in sequencing fed-batch reactors in time the culture always seems to become dominated by "Kuenenia" (several unpublished occasions in Nijmegen (NL) and Delft (NL)). Also in these cases, affinity for nitrite might be the determining factor.

Based on these observations it is hypothesized that "Kuenenia" is an affinity (K) strategist, and "Brocadia" is a growth rate (r) strategist. The affinities of both "Brocadia" (STROUS et al. 1999b) and "Kuenenia" (this Chapter) could not be assessed with enough accuracy to prove this hypothesis. Therefore, more reports of population switches -preferably in studies where replicate reactors can be operated- as well as determination of K_S and μ_m are necessary to confirm this hypothesis. The hypothesis also entails that operation of the anammox process at a shorter SRT will lead to enrichment of "Brocadia".

Function and necessity of aerobic ammonium oxidizing bacteria in the anammox process

Based on side-by-side spatial organization of aerobic ammonium oxidizing bacteria (AOB) and anammox bacteria in the anoxic layers of in (aerated) one-reactor nitritation-anammox systems (PYNAERT et al. 2003; 2004) a "significant role" was postulated for AOB in the "autotrophic nitrogen removal" process (PYNAERT et al. 2003). Although nitrifying bacteria were present in the inoculum reactor (Chapter 5), no nitrifying bacteria could be detected in the anammox reactor (<0.1%) after 100 days of cultivation, and therefore the presence of AOB does not seem to be necessity to the anammox process. The absence of AOB is inconsistent with the data from WYFFELS et al. (2004), where a consortium of AOB and anammox bacteria was present. The main difference between the two reactors was the biomass retention, which was (nearly) complete in WYFFELS' setup (since no biomass was actively removed in their MBR), but short and defined (12-16 days) in this study. It is therefore likely that nitrifiers could not maintain themselves in the reactor described in this Chapter due to the lower retention time. An advantage of the presence of nitrifiers (and other aerobic heterotrophs) in anammox reactors is potentially a higher stability of the system after process disturbances. Due to their O_2 scavenging capacity, anoxic conditions can be easily maintained, whereas the absence of this capacity in this highly enriched MBR might have caused the delayed anammox activity after air intrusion in the reactor.

Conclusion

Ten years ago, the sequencing batch reactor was presented as the "powerful tool" to obtain a stable enrichment of anammox bacteria (STROUS et al. 1998). The SBR is now widely used for the enrichment of anammox bacteria. With the successful enrichment of anammox bacteria in a suspended culture, the membrane bioreactor promises to be an even more powerful tool: the availability of ample amounts of almost pure suspended anammox cells offers great opportunities for the research on anammox physiology.

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4

Chapter 4

Response of anammox bacteria to hydroxylamine

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Abstract

Anoxic ammonium oxidation is a recent addition to the microbial nitrogen cycle, and its metabolic pathway, including the production and conversion of its intermediate hydrazine, is not well understood. Therefore, the effect of hydroxylamine addition on the hydrazine metabolism of anammox bacteria was studied both experimentally and by mathematical modeling. It was observed, that hydroxylamine was disproportionated biologically in the absence of nitrite into dinitrogen gas and ammonium. Little hydrazine accumulated during this process, however a rapid hydrazine production was observed when nearly all hydroxylamine was consumed. A mechanistic model is proposed in which hydrazine is suggested to be continuously produced from ammonium and hydroxylamine (possibly via nitric oxide), and subsequently oxidized to N_2. The electron acceptor for the hydrazine oxidation is hydroxylamine, which is reduced to ammonium. A decrease in the hydroxylamine reduction rate therefore leads a decrease in the hydrazine oxidation rate, resulting in the observed hydrazine accumulation. The proposed mechanism was verified by a mathematical model which could explain and predict most of the experimental data.

Introduction

The anammox process is the oxidation of ammonium with nitrite as electron acceptor -in the absence of oxygen- resulting in the formation of dinitrogen gas (N_2) (VAN DE GRAAF et al. 1996). This process has so far only been shown to be performed by certain organisms, which are deep-branching Planctomycetes (STROUS et al. 1998). Because of its unique ability to combine two different nitrogen compounds to form dinitrogen gas (VAN DE GRAAF et al. 1997), and its relevance to the global nitrogen cycle (DALSGAARD et al. 2003; KUYPERS et al. 2003; RYSGAARD et al. 2004; KUYPERS et al. 2005; SCHUBERT et al. 2006; SCHMID et al. 2007), the anammox process has gained much attention lately. Furthermore, the process was successfully applied in wastewater treatment (Chapter 2; WETT 2006; ROSENWINKEL and CORNELIUS 2004)

Although several catabolic pathways of the anammox bacteria have been proposed (VAN DE GRAAF et al. 1997; STROUS et al. 2006), no definite catabolic scheme is available since none of the intermediates has been detected under physiological conditions. Hydrazine is suggested to be an intermediate, because of its production upon addition of hydroxylamine (VAN DE GRAAF et al. 1997) or nitric oxide (NO) (KARTAL 2008) in enrichment cultures. Whether hydroxylamine and/or nitric oxide are intermediates in the anammox process as well is unclear. The presence of

the nirS enzyme (which converts nitrite exclusively to nitric oxide) in the genome of *Candidatus* "Kuenenia stuttgartiensis" however implies that NO is an intermediate (STROUS et al. 2006).

The production of hydrazine upon addition of hydroxylamine in microorganisms is unique and thus hydrazine measurements following hydroxylamine addition can be regarded as a "benchmark" for anammox bacteria (JETTEN et al. 2005b). Therefore, hydroxylamine addition experiments were used during several enrichment studies (ZHENG et al. 1997; EGLI et al. 2001; VAN DONGEN et al. 2001a; SCHMID et al. 2003; KARTAL et al. 2007b, 2008), and the subsequent production of hydrazine was regarded as an indicator of successful enrichment.

The unavailability of pure anammox cultures makes the research into the metabolism particularly challenging. Several complementary approaches have been used over the years to elucidate the metabolism. First, enzymes were purified from anammox enrichment cultures (SCHALK et al. 2000; CIRPUS et al. 2005; SHIMAMURA et al. 2007); second, anammox proteins were expressed in *E. coli* (HUSTON et al. 2007); third, the genetic blue-print of an anammox bacterium was unraveled in an environmental genome project (STROUS et al. 2006); and finally, batch experiments were performed with physically purified cells (STROUS et al. 1999b; KARTAL et al. 2007a).

Additionally, valuable information can also be obtained by disturbance of the metabolism of anammox bacteria by sudden addition of relevant chemicals. Perturbations lead to an imbalance in the anammox metabolism and -therefore- to measurable changes in the concentration of intermediates. In this Chapter, this perturbation approach has been used by adding hydroxylamine, and monitoring the concentration of ammonium and hydrazine in time. This dynamic response under different conditions was used to evaluate the possible interaction between different metabolic reactions in anammox cells and to evaluate the role of hydroxylamine in anammox enrichments. Based on the data, a metabolic model was constructed that was able to predict most of the observed phenomena.

Methods

Biomass origin

Two different anammox species were used in this study. Most experiments used biomass collected from a lab-scale anammox reactor, containing granular biomass from an earlier enrichment (VAN DONGEN et al. 2001b). The reactor volume was 15 L with a liquid volume of 8 L. The pH was not controlled, but stable at 7.5±0.4. The temperature was controlled at 38 °C and the hydraulic retention time was 2.5 days. The reactor was fed with a medium which was similar to the one described by VAN DE GRAAF et al. (1996), but with increased ammonium and

nitrite levels to obtain a total nitrogen load of 100 mM/day. The reactor contained granules with an average diameter of 0.3 mm and a total biomass concentration of 5 g-C/L. To maintain anoxic conditions and to provide a pH buffer, the reactor was flushed at 25 ml/min with 95%Ar-5% CO_2 (v/v). FISH analysis (hybridization with probes AMX-820 and KST-1273) showed that the enriched organism was *Candidatus* "Kuenenia stuttgartiensis" in a proportion of about 70% of the total biomass. To assess whether the findings were strain-specific, a second set of experiments was performed with a *Candidatus* "Brocadia fulgida" enrichment from a sequencing batch reactor under similar conditions (KARTAL et al. 2004).

Biomass preparation

30 ml samples from the enrichment reactor were taken and washed with "nitrogen free medium": a medium solution similar to VAN DE GRAAF et al. (1996), but containing no ammonium, nitrate or nitrite. Granules were washed until the nitrate concentration was below 0.1 mM. The biomass was transferred to 100 ml bottles and the liquid volume was set to 50 ml with the nitrogen free medium solution. The bottles contained septa for sample taking, and were made anaerobic by sparging with 95%Ar-5% CO_2 (v/v). The bottles were placed in a thermostated shaker (37 °C, 150 rpm), and 1 ml 100 mM ammonium (as $(NH_4)_2SO_4$) was added to achieve a starting concentration of about 2 mM. After acclimatizing for two hours, experiments were started with addition of NH_2OH.

Hydroxylamine addition tests

0.25 to 5 ml of 100 mM freshly prepared $NH_2OH.HCl$ solutions were added to the test bottles to achieve initial hydroxylamine concentrations of 0.5 to 10 mM. Samples were taken from the bottles -after short (5 seconds) settling of the granular biomass- and immediately filter-sterilized (0.2 μm filters). The filtrate was analyzed for hydroxylamine, hydrazine, ammonium, and -for selected samples- for nitrate and nitrite. Concentrations were corrected for the volume removed by sampling. The biomass content was determined after the experiment by measurement of dry weight (D.W.). As a negative control, the same experiment (initial hydroxylamine concentration: 4 mM) was performed with the nitrogen free medium in the absence of biomass.

Influence of ammonium, short chain fatty acids and oxygen

Freshly prepared hydroxylamine (2 ml, 100 mM) was added to the test bottles to achieve an initial concentration of 4 mM. Ammonium, formate, acetate or propionate was added immediately to achieve a concentration of 2 mM in the test bottles. In the aerobic experiment, the sample bottles were flushed with air (rather than with Ar-CO_2 gas) during preparation.

Effect of hydroxylamine on NO, N_2O and NO_2

Because of the small volume of the sample bottles, NO, N_2O and NO_2 could not be measured in the batch tests, and therefore hydroxylamine addition experiments were also conducted directly in the 15 L enrichment reactor which contained the *Candidatus* "Kuenenia stuttgartiensis" biomass. The experiment was started by stopping influent and effluent pumps for three hours, followed by addition of hydroxylamine (50 ml of 1 M NH_2OH giving a final concentration of 6 mM). Samples were taken regularly for hydroxylamine, ammonium, nitrite, nitrate and N_2O. pH was measured on line and off-gas was collected continuously in sample bags. From these sample bags, NO and NO_2 were determined automatically every 20 minutes.

Analytical procedures

Hydroxylamine (30-100 µM) was measured spectrophotometrically using the method of FREAR and BURRELL (1955). Briefly, 100 µl of 0.5 M phosphate buffer (pH 7), 200 µl of 12 m% trichloroacetic acid (TCA) and 200 µl of 1 m% quinolinol in absolute ethanol were added to 2.7 ml of (diluted) sample. After shaking, 1 ml of 10.6 m% (1 M) Na_2CO_3 solution was added. The mixture was heated for one minute in a water bath at 100 °C. After cooling (10 minutes), the hydroxylamine was determined spectrophotometrically at 705 nm (Novaspec 4049, Biochrom, Cambridge UK). Hydrazine detection (3-30 µM) was performed using a modification of the method of WATT and CHRISP (1952). 100 µl of hydrazine reagent (4 g para-dimethyl-aminobenzaldehyde dissolved in 20 ml ethanol and 2 ml concentrated HCl) was added to 900 µl sample. After 20 minutes, the sample was measured spectrophotometrically at 458 nm (Novaspec 4049, Biochrom, Cambridge UK).

Ammonium (1-143 µM), nitrite (43-429 µM) and nitrate (16-964 µM) were detected using commercial test kits (Dr. Lange, Hach Lange GmbH, Düsseldorf DE, kits LCK304, LCK 341 and LCK339 respectively) and determined on a designated spectrophotometer (CADAS 50S). The standard deviation of the measurements was estimated to be 5% of the measured value.

Acetate and propionate were determined by gas chromatography (Chrompack CP 9001 equipped with a Hewlett Packard HP INNOWAX column). pH was measured at the beginning and end of each experiment and was always in the optimum pH range of anammox activity (STROUS et al. 1997b).

NO and NO_2 were measured in the gas phase using a chemiluminescence analyzer (CLD 700EL, Ecophisics, Ann Arbor MI US). N_2O in the liquid was measured off-line with a reversed Clark-type sensor (Unisense, Århus, DK).

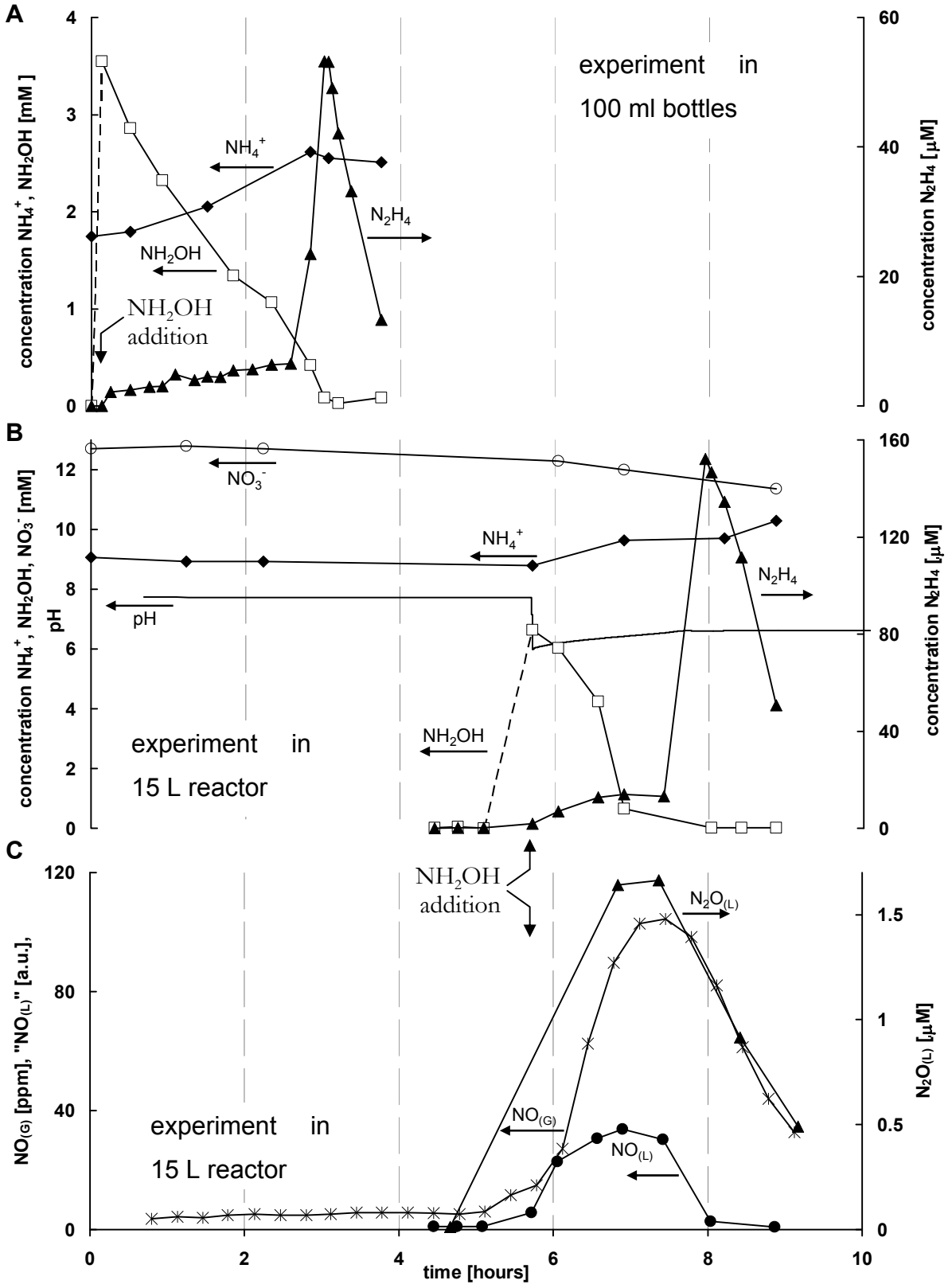
Mathematical modeling

Simulations with the proposed kinetic models were performed in the AQUASIM software (REICHERT 1998). The reaction volume was modeled as one homogeneously mixed compartment. Rate equations were of Monod-type saturation for all relevant substrates. Reaction stoichiometry and rate equations that were used are presented in the discussion section. Initial solute and biomass concentrations in the simulated batch tests were set to the measured values.

Thermodynamic calculations

The Gibbs energy of the reaction ($\Delta G_R^{0'}$) was always calculated under standard conditions in the biological frame of reference (temperature: 298 K, pressure: 1 bar, concentration of reactants and products: 1 M, pH: 7). Values for the Gibbs energy of formation (ΔG_f^0) of H_2O, N_2, NH_4^+ and NO were taken from the Handbook of Chemistry and Physics (*Thermodynamic properties of aqueous systems* 2006). For hydroxylamine and hydrazine, ΔG_f^0-values of -23.4 and 127.8 kJ/mol were used, respectively. These values were calculated from standard reduction potentials (LATIMER 1952).

Figure 4.1 *(see next page) Measured evolution of different nitrogen compounds after hydroxylamine addition to anammox enrichments in: (A) small (100 ml) bottles, (B, C) large (15 L) reactor. Hydroxylamine addition leads first to ammonium production and a slight hydrazine accumulation. A transient hydrazine peak appears upon near-completion of hydroxylamine. Experimental conditions: (A) 100 ml bottle, biomass 3.5 g DW/L, initial hydroxylamine concentration 4 mM, added at t=0 h; (B, C) 15 L cultivation reactor after a stop in the feed (t=0); hydroxylamine was added after 5.7 hours. Symbols on the charts: (A, B) on left axis NH_4^+ ♦, NH_2OH □, NO_3^- ○, pH —, on right axis N_2H_4 ▲; (C) on left axis $NO_{(L)}$ ●, $NO_{(G)}$ ✳, on right axis N_2O ▲. a.u. = arbitrary units, subscripts L and G denote liquid phase and gas phase, respectively.*


A
experiment in
100 ml bottles
NH_4^+
N_2H_4
NH_2OH
NH_2OH addition
concentration NH_4^+, NH_2OH [mM]
concentration N_2H_4 [µM]
B
NO_3^-
NH_4^+
N_2H_4
pH
NH_2OH
experiment in
15 L reactor
concentration NH_4^+, NH_2OH, NO_3^- [mM]
pH
concentration N_2H_4 [µM]
C
NH_2OH addition
$N_2O_{(L)}$
$NO_{(G)}$
$NO_{(L)}$
experiment in
15 L reactor
$NO_{(G)}$ [ppm], "$NO_{(L)}$" [a.u.],
$N_2O_{(L)}$ [µM]
time [hours]

Results

Hydrazine and hydroxylamine evolution in time

Results of batch experiments in which hydroxylamine was added to the anammox cells -in the absence of nitrate and nitrite- are shown in Figure 4.1A. In all hydroxylamine addition tests, little hydrazine accumulated (maximum concentration ~5-10 μM) as long as hydroxylamine was still present. When hydroxylamine was nearly completely converted, the hydrazine concentration rose within five minutes (up to 10 times higher than the starting concentration), followed by a gradual decrease. Ammonium production was significant in all experiments until the appearance of the hydrazine peak. Thereafter, no significant ammonium production could be detected. In the negative control (in which no biomass was present) a hydroxylamine conversion rate of only 0.1 mM/h was estimated, and no hydrazine could be detected at all. Thus, the rate of "chemical" hydroxylamine conversion is too low to explain the observed behavior, which indicates that the process is biological in nature.

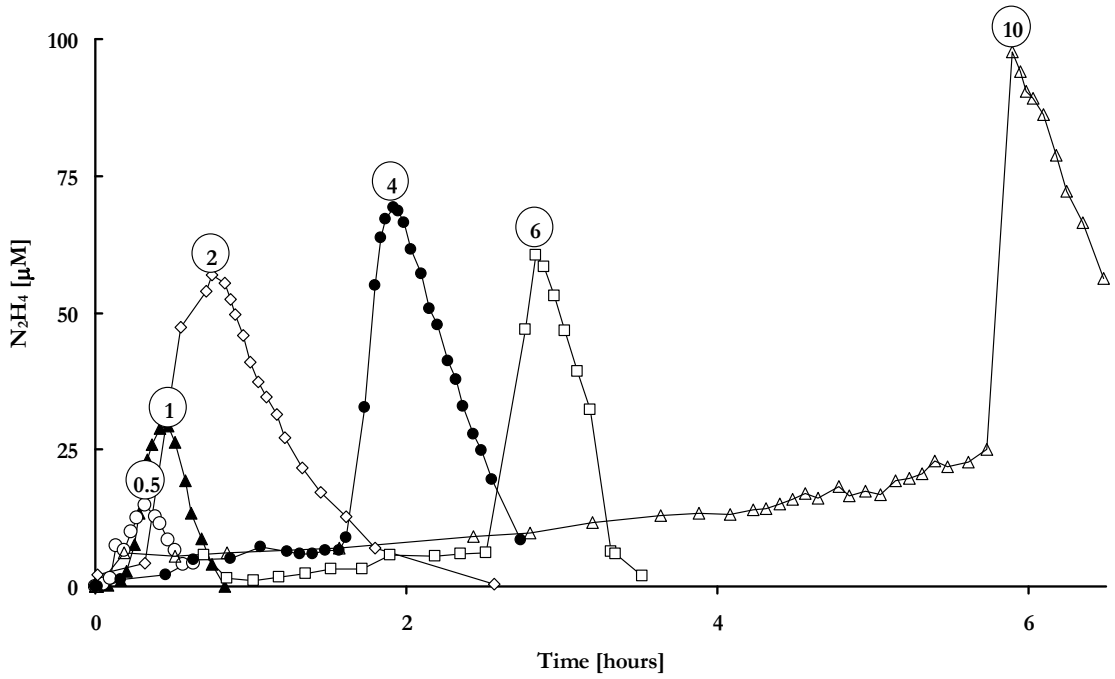


Figure 4.2 *Measured evolution of the hydrazine concentration after the addition of varying amounts of hydroxylamine. Initial hydroxylamine concentrations (mM) are denoted in circles above the hydrazine maximum. Higher initial hydroxylamine levels led to a delayed apparition of the hydrazine peak.*

TABLE 4.1 *Experimental conditions and main results of the hydroxylamine addition batch tests.*

experiment	initial NH_2OH concen-tration	time to N_2H_4 peak	height of N_2H_4 peak	NH_4^+ produced at N_2H_4 peak	$\frac{-\Delta NH_4^+}{\Delta NH_2OH}$	dry weight	NH_2OH conversion rate	N_2H_4 disappea-rance rate *after* N_2H_4 peak
[unit]	$[mM]$	$[h]$	$[\mu M]$	$[mM]$	$\left[\frac{mol}{mol}\right]$	$\left[\frac{g}{L}\right]$	$\left[\frac{mmol}{gDW \cdot h}\right]$	$\left[\frac{mmol}{gDW \cdot h}\right]$
different initial NH_2OH concentrations	0.5	0.32	15	n.d.	n.d.	5[1]	0.31	0.0086
	1	0.47	26	n.d.	n.d.	5[1]	0.42	0.0070
	2	0.43	62	0.26	0.23	5.1	0.91	0.010
	2	0.75	57	0.39	0.20	4.8	0.71	0.017
	4	1.5	53	1.3	0.31	5.2	0.51	0.010
	4	1.9	69	1.9	0.48	3.7	0.43	0.017
	6	2.8	61	1.1	0.19	4.5	0.47	0.024
	10	5.9	98	2.8	0.28	3.0	0.57	0.024
different biomass concentrations	4	0.8	82	0.9	0.22	7.8	0.66	0.012
	4	4.3	59	1.0	0.27	2.1	0.41	0.006
acetate addition (2 mM)	4	3.5	62	1.0	0.25	n.d.	-	-
presence of oxygen	4	(no peak)		-	-	n.d.	-	-
different NH_4^+ (6 mM)	4	3.1	96	n.d.	n.d.	n.d.	-	-
concentrations (9 mM)[2]	6	2.2	152	2.5	0.41	5.1	0.52	0.021
"B. fulgida" enrichment	4	4.0	28	0.5	0.25	n.d.	-	-
Control (no biomass)	4	-	no N_2H_4	-	n.a.	n.a.	n.a.[3]	-

[1] Estimate based on the amount of biomass in the reactor and the sample size.

[2] This experiment was performed with 8 liters of biomass; within the biomass cultivation reactor.

[3] The conversion rate was estimated at 0.1 mM/h (at 4 mM NH_2OH).

From the experiments with varying biomass and hydroxylamine concentration can be seen, that higher amounts of biomass in the experiment resulted in a shorter time before the hydrazine peak appeared (Table 4.1) and in a more rapid conversion of hydroxylamine. Higher initial concentrations of hydroxylamine resulted in a later appearance of the hydrazine peak with the same amount of biomass (Table 4.1, Figure 4.2).

N_2O and NO production

Experiments in the 15 L reactor (Figures 4.1B and 4.1C) showed similar trends with the experiments in small (100 ml) bottles for the hydroxylamine, ammonium and hydrazine evolution (Figure 4.1A). Furthermore, the N_2O level in the liquid rose to 2 μM after hydroxylamine addition, and remained constant until all hydroxylamine was converted. After the conversion of hydroxylamine, the N_2O level dropped again. NO in the gas phase initially increased to 100 ppm (4 μM; this value corresponds to about 2 μM in the liquid phase at equilibrium) and decreased again after the hydrazine peak had reached its maximum. The slow logarithmic decrease of NO in the gas phase after the hydrazine peak can be explained by the slow gas dilution rate (the headspace had a volume of 7 L, which is large compared to the gas flow rate of 10 ml/min).

Therefore, NO production can be assumed to have stopped already when all hydroxylamine had been converted. NO_2 in the gas phase was always below the detection level (<1 ppm or < 5% of the measured NO level).

Measurements of nitrite seemed to show an accumulation (up to 34 μM) upon hydroxylamine addition. This level decreased again after conversion of hydroxylamine. However, a critical evaluation of the analytical procedure showed that the measured "nitrite" value can be fully attributed to the interference of nitric oxide on the analytical determination. Control measurements with water -containing no nitrite- saturated with 100 ppm NO (therefore containing 2 μM NO in the liquid) showed already a value of 10 μM "nitrite". Although clearly not a measure for nitrite, these measurements are nevertheless useful as a qualitative indication of the NO concentration in the liquid and are therefore shown in Figure 4.1C (in arbitrary units, a.u.). Corroborated with the NO measurements in the gas phase, these measurements confirm the observation that NO accumulated slightly upon hydroxylamine addition, but disappeared again directly after full conversion of the hydroxylamine.

Influence of the presence of acetate, formate and propionate

It has been shown that anammox bacteria can convert short chain fatty acids to CO_2 as an additional energy source while nitrite is reduced to N_2 via ammonium. The produced ammonium is immediately turned over to combine with nitrite to form dinitrogen gas (KARTAL et al. 2007a). Formate, acetate or propionate were added together with hydroxylamine in a series of batch tests. However, none of these acids were found to affect the conversion of hydroxylamine (Table 4.1), indicating that the conversion of NH_2OH could neither be further stimulated nor inhibited by these organic acids. The other general trends -such as the ammonium production and the sharp hydrazine peak- were also observed when the organic acids were added.

Influence of ammonium, nitrate and oxygen

Varying the ammonium concentration in the batch tests did not affect the overall behavior. Initial values of 2, 6 and 9 mM (the latter not in the 100 ml vessels but directly in the 15 L reactor) all resulted in hydroxylamine conversion, ammonium production and sudden hydrazine accumulation (Table 4.1). As the experiment in the 15 L reactor -which gave similar results to the 100 ml batch tests- was performed in the presence of nitrate, nitrate (at least up to 12 mM) did not influence the general behavior. Also in the presence of oxygen, hydroxylamine conversion had a very similar rate to the conversion in the absence of oxygen. Hydrazine production however was not observed at all under these conditions (Table 4.1).

Influence of anammox species

To determine whether the sudden appearance of hydrazine was a specific effect for the employed enrichment (consisting of *Candidatus* "Kuenenia stuttgartiensis"), a batch experiment was also performed with a culture containing *Candidatus* "Brocadia fulgida". A qualitatively similar behavior was observed (sudden appearance of hydrazine and about 25% ammonium production), but the hydroxylamine conversion was slower, and thus the hydrazine peak appeared later than in similar experiments with *Candidatus "*Kuenenia stuttgartiensis". Also the height of the hydrazine peak was lower (Table 4.1).

Discussion

The general trends

In the experiments described in this Chapter, hydroxylamine was converted in the absence of added electron acceptors by anammox bacteria. Control experiments in the absence of biomass (both in this study as –in the presence of nitrite- by VAN DE GRAAF et al. 1997) did show a very low conversion of hydroxylamine, indicating that the observed conversion was not chemical in nature. When hydroxylamine is converted by anammox bacteria, the main products are ammonium (about 25% of the converted hydroxylamine) and N_2. The nitrogen gas was not measured, but it is most likely the main end product because no other gaseous nitrogen compounds (such as N_2O, NO and NO_2) were detected in appreciable amounts. The conversion into ammonium and dinitrogen gas indicates that the overall hydroxylamine conversion is a disproportionation: (4.1)

$$3NH_2OH + H^+ \rightarrow N_2 + NH_4^+ + 3H_2O \qquad \Delta G_R^{0'} = \text{-228 kJ/mol } NH_2OH$$

According to this disproportionation, the yield of ammonium from hydroxylamine is 0.33 mol/mol. This value is close to the experimentally determined one of about 0.25 mol/mol.

Although hydrazine accumulation upon hydroxylamine conversion was also detected by others (VAN DE GRAAF et al. 1997; SCHALK et al. 1998; EGLI et al. 2001; SCHMID et al. 2003), the hydrazine peak never appeared as suddenly as in this study. The number of hydrazine samples taken in each experiment in this study is substantially larger compared to those in hydroxylamine addition experiments by most others. It is therefore possible that the hydrazine accumulation has also occurred in the previously reported experiments just as suddenly as in this study, but that the sampling frequency there was too low to notice this behavior. The maximum concentration of hydrazine reported in other studies is markedly higher than in this study (up to 2.8 mM in (EGLI et al. 2001), compared with 100 μM in this study). It was not possible however to correlate these

higher values in hydrazine level to differences in experimental conditions or to a different type of anammox bacteria that was used in these experiments.

The observation that the rate of hydroxylamine conversion in the presence of oxygen was similar to the rate in the absence of oxygen was quite unexpected, since oxygen is inhibiting the conversion of ammonium and nitrite by anammox bacteria. Hydrazine accumulation could not be detected however upon (near) hydroxylamine depletion (Table 4.1). This indicated that hydrazine was not formed during this experiment, or that the formed hydrazine was immediately oxidized.

Influence of initial biomass and initial hydroxylamine concentration

The relationship between the (initial) hydroxylamine/biomass ratio and the time until the hydroxylamine peak appeared is linear. Therefore, the kinetics of hydroxylamine consumption fit well a zero order reaction rate (Figure 4.3). This indicates that in the chosen range of initial hydroxylamine/biomass ratio neither hydroxylamine limitation nor inhibition took place. Also the time to the hydrazine peak is proportional to the initial hydroxylamine/biomass ratio (Figure 4.3), which reflects a first order dependency of the hydroxylamine conversion rate on the biomass concentration. The initial hydroxylamine conversion rate was 0.6 (±0.2) mM NH_2OH/g-DW/h on average. From the linear part of the decreasing slope of the hydrazine peak, a net hydrazine conversion rate of 0.012 (±0.006) mM N_2H_4/g DW/h was estimated (see Table 4.1). This rate is 20 to 80 times lower than the initial hydroxylamine conversion rate. The hydrazine peak height reached a maximum of about 70-100 μM (Figure 4.2) for an initial hydroxylamine/biomass ratio of 0.4 mmol NH_2OH/g DW. Essentially, the behavior of the experiments at higher initial hydroxylamine/biomass ratios were -after lower hydroxylamine biomass ratios were reached during the experiment due to the conversion of hydroxylamine- similar to the experiments started at those lower ratios. Only for those experiments which were started at an initial hydroxylamine/biomass ratio lower than 0.4 mmol NH_2OH/g DW, the hydrazine peak was significantly lower.

Influence of the anammox species

The behavior of the *Candidatus* "Kuenenia stuttgartiensis" enrichment which was employed in this study was qualitatively similar to the behavior of the *Candidatus* "Brocadia fulgida" enrichment. It cannot be excluded that the quantitative differences between the two enrichments (in conversion rate and peak height) could possibly be species-specific. It is however also possible that the mode of enrichment, the level of enrichment, differences in sample

preparation, or the time delay between sample taking (in Nijmegen) and performance of the test (in Delft) 16 hours later have led to the measured differences in peak height and hydroxylamine conversion rate.

Influence of other community members

Because no pure anammox culture is available for experiments at this moment, information regarding the anammox process is generally obtained using enrichment cultures. The possible contribution of other members of the anammox community to the hydroxylamine conversion was not determined in this study. Other possible community members are denitrifiers, nitrifiers and sulfate reducing bacteria. However, no hydrazine accumulation upon hydroxylamine addition by bacteria other than anammox bacteria has ever been reported. Furthermore, since hydroxylamine conversion and hydrazine production were always observed in anammox cultures (at several levels of enrichment, and probably with different community compositions), it is unlikely that the other community members played a significant role in the hydroxylamine conversion.

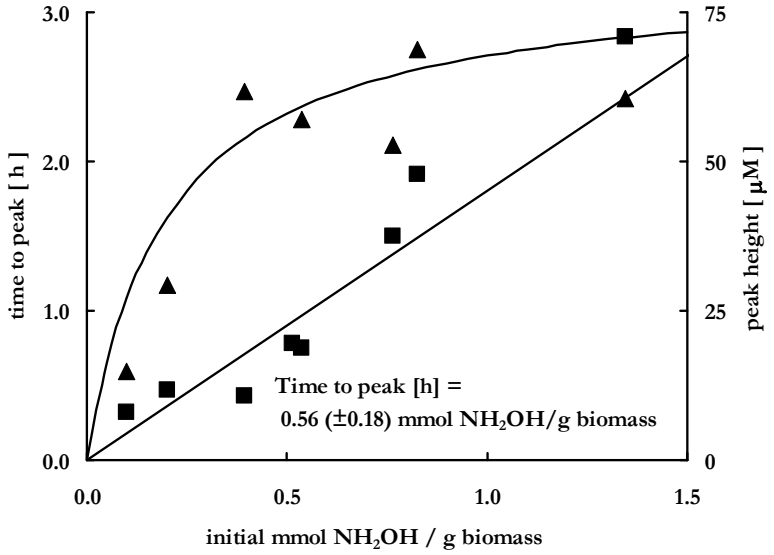


Figure 4.3 *Results from batch tests in 100 ml bottles at varying initial hydroxylamine and biomass concentrations show that the time from hydroxylamine addition until the time of appearance of the hydrazine peak is proportional to the biomass-specific hydroxylamine concentration* (■). *In contrast, the maximum hydrazine concentration in each experiment* (▲) *is stable at a hydroxylamine/biomass ratio above 0.4 mmol NH_2OH / g dry weight (DW).*

Possible mechanism of hydrazine accumulation

The sudden accumulation of hydrazine upon full conversion of hydroxylamine in the batch tests could be caused by: (i) a quick increase in *production* of hydrazine, and/or (ii) a sharp decrease in its *conversion*. The measured concentration changes presented in Figures 4.1 and 4.2 show, that hydrazine concentrations increased gradually before they abruptly increased. Possibly, at a hydroxylamine concentration >0.2 mM, hydrazine is continuously turned over -that is, produced and consumed at the same rate in different reactions- with only a very small excess in its production rate. This would also be consistent with the production of $^{15}N\equiv N^{14}$ when ^{15}N labeled hydroxylamine or ammonium are employed in hydroxylamine addition experiments (VAN DE GRAAF et al. 1997).

A sudden rise in the net hydrazine *production* upon near completion of hydroxylamine could only be explained by assuming a toxic or inhibitive effect of hydroxylamine on the hydrazine production rate, as postulated by SHIMAMURA et al. (2007). This mechanism could potentially explain the hydrazine production upon hydroxylamine addition in the *presence* of nitrite and ammonium, as inhibition of the hydrazine oxidation reaction would immediately lead to hydrazine accumulation when hydrazine is already being produced from ammonium and nitrite. This work (in the *absence* of nitrite) does not suggest any inhibition by hydroxylamine, because if hydroxylamine were to inhibit hydrazine oxidation, a stronger inhibition at higher concentrations is likely. Therefore, addition of hydroxylamine would have led to accumulation of hydrazine, *immediately* after hydroxylamine addition (when the concentration was the highest). The *delayed* hydrazine production observed in this study -taking place only upon near complete consumption of the hydroxylamine - could therefore not be explained by hydroxylamine inhibition.

A sudden stop in hydrazine *conversion* could however also be explained by a limiting amount of electron acceptor in the later stage of the experiments. A possible reduction reaction in the system is the hydroxylamine conversion to ammonium. When coupled to hydrazine oxidation, the $\Delta G_R^{0'}$ of this reaction is -318 kJ/mol NH_2OH and thus the reaction is thermodynamically favorable. If this reaction would cease or significantly slow down (e.g., because of depletion of one of the electron carriers, or because of the low affinity for its substrate) the oxidation of hydrazine would suddenly stop as well.

Two possible schemes -based on such a sudden stop in hydrazine conversion upon near complete conversion of hydroxylamine- that could explain the observed disproportionation and sudden accumulation of hydrazine are shown in Figures 4.4A and 4.4B. The hydrazine conversion after the hydrazine peak, could run via a generalized (possibly multiple-enzyme) hydrazine disproportionation reaction (scheme A) or could involve the reverse

of the hydrazine formation step, thus producing hydroxylamine and ammonium (scheme B). In reaction mechanism B (Figure 4.4B), reaction 3B is the formation of hydroxylamine and ammonium from hydrazine, which is the reverse of reaction 1. The -small- amounts of hydroxylamine produced in this way can be converted again to ammonium with the remaining hydrazine (which is converted to N_2). Both mechanisms thus result in the same overall reaction (eq 4.1). However, the actual reversibility of the hydrazine producing reaction is not known, since the enzyme capable of this conversion has not been purified and the plausibility of the reversibility assumption thus cannot be tested.

In both schemes in Figure 4.4, the following three metabolic regimes can be distinguished from the evolution of concentrations of nitrogen compounds in the hydroxylamine addition batch experiments:

Regime I — Hydroxylamine combination with ammonium to form hydrazine is the rate limiting step. Hydrazine oxidation to N_2 is coupled to hydroxylamine reduction to ammonium. In this regime, the hydroxylamine concentration falls and ammonium increases, while the hydrazine concentration remains at very low levels.

Regime II — The ammonium production is strongly slowed down when hydroxylamine is nearly depleted. Therefore, hydrazine oxidation (which is coupled to hydroxylamine reduction) is stopped, and as a result, hydrazine accumulates.

Regime III — The hydrazine concentration falls due to its removal via two possible mechanisms: (A) direct disproportionation into ammonium and N_2 (Figure 4.4A), or (B) disproportionation into ammonium and hydroxylamine, the latter further reacting to ammonium while the remaining hydrazine is converted to N_2 (Figure 4.4B).

These reaction schemes involving NH_2OH do not exclude NO participation in the hydroxylamine disproportionation or in the anammox metabolism, as both alternatives (A and B) could possibly be extended to have NO as an additional intermediate (as is shown in Figure 4.4, in grey). The hydroxylamine combination with ammonium to form hydrazine is still the overall reaction in these schemes, but hydroxylamine is first converted to nitric oxide. Nitric oxide (instead of hydroxylamine) subsequently combines with ammonium to form hydrazine. Since the overall reaction is the same, also the electron balance remains unaffected: the three-electron oxidation of hydroxylamine to NO is balanced by the three-electron reduction from the combination of NO and ammonium to form hydrazine.

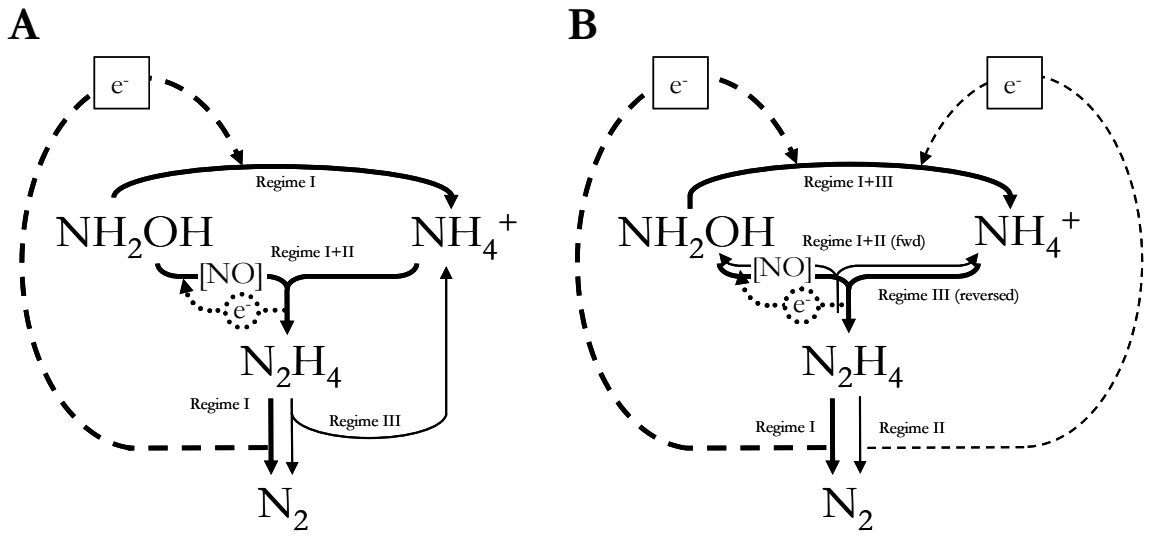


Figure 4.4 *Two reaction mechanisms (A and B) can explain the response of ammonium and hydrazine towards hydroxylamine additions in anammox enrichments. Three regimes can be distinguished in each mechanism. Regime I (at high hydroxylamine levels) involves a continuous turnover of hydrazine with ammonium and N_2 production. Regime II (at low hydroxylamine levels) is a stop in the hydroxylamine reduction, which ceases the hydrazine oxidation, leading to sudden hydrazine accumulation. Regime III (at relatively high hydrazine levels, and low hydroxylamine levels) is in mechanism A: hydrazine disproportionation into ammonium and dinitrogen gas; or in mechanism B: the slow hydrazine removal via oxidation to N_2 and reversing of the hydrazine combination reaction. The possible place of NO as an additional intermediate in the hydrazine formation and the resulting electron flows are shown in dots.*

Mathematical model

A mathematical model was constructed to test the hypothetical metabolic schemes proposed for the hydroxylamine degradation. The model could describe the sudden hydrazine accumulation and its subsequent consumption. The reaction equations were based on alternatives A and B and were constructed in such a way that in all reactions, the electron and element balances were satisfied. Ammonium (NH_4^+) is considered in all reactions instead of ammonia (NH_3) because at the working pH (7.5) this is the dominant species, at least in the extracellular solution. pH effects were not taken into account in this model. The notations and parameters used in the model equations are presented in Table 4.2.

Table 4.2 *Parameters for the kinetic model of the hydroxylamine disproportionation in the experiment shown in Figure 4.5. Model A involved a separate hydrazine disproportionation reaction, whereas in model B the hydrazine formation reaction was considered to be reversible. The two reaction schemes are presented in Figure 4.4.*

parameter	name	unit	value	
			model A	model B
k_1	rate constant of hydrazine formation[1]	$\frac{mmol}{gDW \cdot h}$	0.25	0.29
k_2	rate constant of ammonification[1]	$\frac{mmol}{gDW \cdot h}$	0.30	0.31
k_{3A}	rate constant for hydrazine disproportionation[1]	$\frac{mmol}{gDW \cdot h}$	0.024	-
k_{3B}	rate constant for reversed hydrazine formation[1]	$\frac{mmol}{gDW \cdot h}$	-	0.080
K_{1,NH_2OH}	half saturation constant for hydroxylamine conversion with ammonium into hydrazine[1]	mM	0.0011	0.010
K_{2,N_2H_4}	half saturation constant for hydrazine oxidation to N_2 [1]	mM	0.00063	0.0005
K_{2,NH_2OH}	half saturation constant for hydroxylamine reduction to ammonium[1]	mM	0.14	0.20
K_{3A,N_2H_4}	half saturation constant for hydrazine[1]	mM	0.044	-
K_{3B,N_2H_4}	half saturation constant for hydrazine[1]	mM	-	0.0061
C_x	biomass concentration[2]	$g\ DW/L$	7.8	
C_{0,N_2H_4}	initial hydrazine concentration[2]	mM	0	
C_{0,NH_4^+}	initial ammonium concentration[2]	mM	1.8	
C_{0,NH_2OH}	initial hydroxylamine concentration[2]	mM	4.2	

1 Parameters were fitted to the measured nitrogen species concentrations.
2 Initial conditions taken from measured values.

The first reaction is the hydrazine forming step from ammonium and hydroxylamine:

Reaction 1: $NH_4^+ + NH_2OH \rightarrow N_2H_4 + H_2O + H^+$ (4.2)

with rate $r_1 = k_1 \cdot \frac{C_{NH_2OH}}{C_{NH_2OH} + K_{1,NH_2OH}} \cdot C_x$ $\Delta G_R^{0'}$ = -47 kJ/mol NH_2OH

The rate dependency of the ammonium concentration was neglected because (i) the measured ammonium levels (2-3 mM) were much higher than physiological half-saturation coefficients (K_M) for ammonium (which are generally in the 10-100 μM range) and (ii) batch experiments did not indicate sensitivity of the reaction rate to the ammonium concentration (Table 4.1).

In a second reaction, the hydrazine oxidation to N_2 is coupled to hydroxylamine reduction to NH_4^+:

Reaction 2: $2\ NH_2OH + N_2H_4 + 2\ H^+ \rightarrow N_2 + 2\ NH_4^+ + 2\ H_2O$ (4.3)

with rate $r_2 = k_2 \cdot \frac{C_{N_2H_4}}{C_{N_2H_4} + K_{2,N_2H_4}} \cdot \frac{C_{NH_2OH}}{C_{NH_2OH} + K_{2,NH_2OH}} \cdot C_x$ $\Delta G_R^{0'}$ = -318 kJ/mol NH_2OH

Application of the combination of these first two reactions in a mathematical model is capable of reproducing the hydroxylamine consumption and the swift hydrazine accumulation (results not shown). However, for the hydrazine consumption when all hydroxylamine is depleted (regime III), a separate reaction has to be introduced.

Hydrazine disappears in this third reaction, which can be either the hydrazine disproportionation (case A, Figure 4.4A) or the reversed reaction 1 with production of ammonium and hydroxylamine (case B, Figure 4.4B). In the case of direct disproportionation of hydrazine, reaction 3A is employed:

Reaction 3A: $N_2H_4 + 4/3H^+ \rightarrow 1/3\ N_2 + 4/3\ NH_4^+$ (4.4)

with rate $r_{3A} = q_{3A} \cdot \frac{C_{N_2H_4}}{C_{N_2H_4} + K_{3A,N_2H_4}} \cdot C_x$ $\Delta G_R^{0'}$ = -180 kJ/mol N_2H_4

In the second alternative (B), the reaction is the reverse of reaction 1:

Reaction 3B: $N_2H_4 + H^+ + H_2O \rightarrow NH_4^+ + NH_2OH$ (4.5)

with rate $r_{3B} = q_{3B} \cdot \frac{C_{N_2H_4}}{C_{N_2H_4} + K_{3B,N_2H_4}} \cdot C_x$ $\Delta G_R^{0'}$ =+47 kJ/mol N_2H_4

The $\Delta G_R^{0'}$ for reaction 3B is close enough to zero to make the assumption of reversibility plausible, since at the actual concentrations -after the hydrazine peak- of ammonium (~10^{-3} M), hydroxylamine (~10^{-5} M) and hydrazine (~10^{-4} M), the $\Delta G_R'$ is much closer to zero (~15 kJ/mol).

The kinetic model described above could in case A as well as in case B explain the experimentally determined evolution of hydrazine concentration over time satisfactory

(Figure 4.5). The model parameters (i.e., maximum rate constants and half-saturation coefficients) were experimentally determined from the batch experiment with 7.8 g DW/L biomass and 4.2 mM hydroxylamine. All the parameters for this kinetic model are shown in Table 4.2.

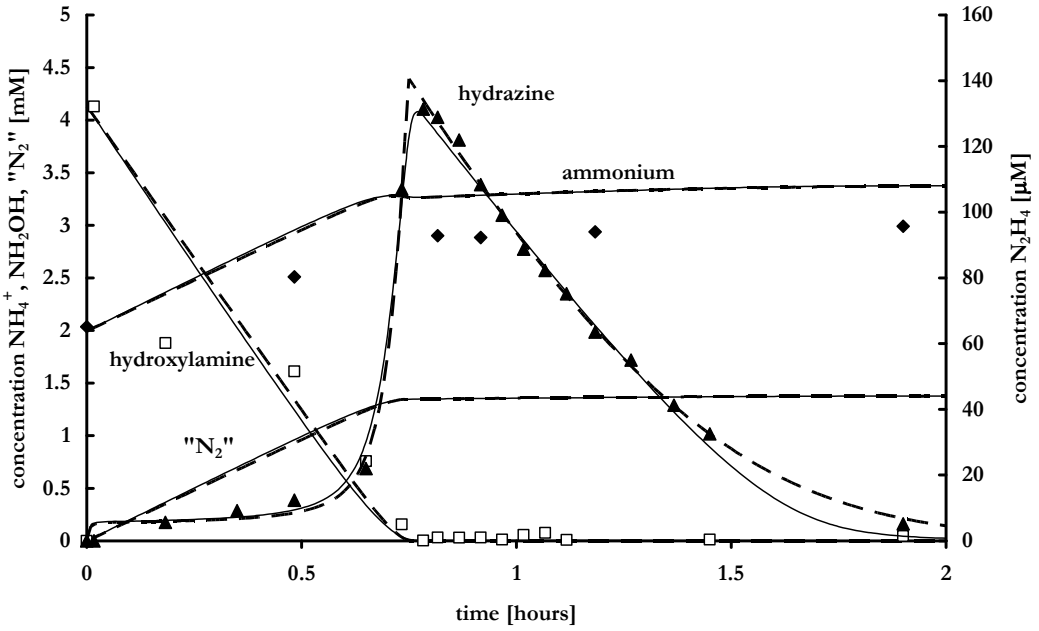


Figure 4.5 *The experimental results of a batch experiment with hydroxylamine addition (hydroxylamine: □; hydrazine: ▲; ammonium: ♦) are fitted with the kinetic model A (dashed lines) and B (solid lines). The models are both capable of describing the observed phenomena quantitatively. Experimental conditions: Initial NH_2OH concentration: 4.2 mM, biomass concentration: 7.8 g DW/L, and all other parameters were taken from Table 4.2. N_2 is represented as cumulative production of mmol N_2 per mol liquid volume.*

The kinetic model with parameters thus obtained in one batch experiment, can be used to predict the effect of varying biomass and initial hydroxylamine levels in the other experiments. However, the model is not capable of predicting exactly the time at which the hydrazine peak occurs. The differences can be fully attributed to variances in the measured biomass-specific hydroxylamine reduction rate. Because the overall reaction of the model is the disproportionation of hydroxylamine in ammonium and N_2, the final ammonium production is fixed at 1/3 of the converted hydroxylamine. Since the measured production is actually about 25% lower, this shows a systematic error in the prediction of the ammonium level.

In the described mathematical model, the affinity for hydroxylamine is much lower than the affinity for hydrazine (that is, $K_{2,NH_2OH} >> K_{2,N_2H_4}$). A lower difference in affinities in the model will result in a more gradual accumulation of hydrazine (because the hydroxylamine

reduction, and thus the hydrazine oxidation are slowed down earlier), which would not be in agreement with the observed behavior. In anammox bacteria, the enzyme responsible for ammonification is unknown, but based on genomic (STROUS et al. 2006) and enzymological (SCHALK et al. 2000; KARTAL et al. 2007a) information, penta heme nitrite reductase (nrfA), hybrid cluster protein (HCP), and the cd1 nitrite reductase (nirS) are candidates for this conversion. The reported half-saturation constants of these enzymes -purified from other bacteria than anammox bacteria- with hydroxylamine as a substrate are high, ranging from 0.6 mM to 30 mM (SINGH 1974; BAMFORD et al. 2002; CABELLO et al. 2004). The values reported in these studies are more than two orders of magnitude higher than the K_M value of these enzymes for nitrite (which is in the μM range). The model requirement of a low affinity for hydroxylamine in the ammonification reaction therefore seems to be also plausible based on the nature of the enzyme(s) performing this conversion.

Source of NO and N_2O

N_2 gas was the main oxidation product of hydroxylamine conversion. From the nitrogen mass balance during the experiment in the 15 L reactor, the amount of hydroxylamine which was converted into nitrous and nitric oxide was estimated to be at maximum 0.01%. However, small amounts of N_2O and NO did accumulate during hydroxylamine conversion (Figure 4.1C). This could indicate (i) that nitrous oxide and/or nitric oxide were intermediates in the hydroxylamine disproportionation, (ii) that they were the end products of side-reactions, or (iii) that they were produced by other -non anammox- community members. The different options are discussed below.

(i) If nitric oxide or nitrous oxide were intermediates in the hydrazine formation reaction (reaction 1), this implies oxidation of hydroxylamine to NO/N_2O, followed by reduction to form hydrazine as proposed on the basis of genome data (STROUS et al. 2006). Since NO is probably an intermediate in the anammox process -more specifically: a substrate in the hydrazine formation- as is suggested based on genome information (STROUS et al. 2006), this is indeed a possible mechanism. As no indications exist, that N_2O can be converted by anammox bacteria, it is at the moment unlikely that it is an intermediate.

(ii) Hydroxylamine can also be converted to NO and N_2O by the hydroxylamine oxidoreductase (HAO) enzyme, present in substantial amounts in anammox bacteria (SCHALK et al. 2000), and this side-reaction could be the source of the NO and/or N_2O. Furthermore, nitrous oxide production via one of several potential nitric oxide detoxification enzymes

identified from the anammox genome (KARTAL et al. 2007a) is another possible anammox-related source of nitrous oxide.

(iii) Given the low amounts of N_2O and NO produced during the experiment, the possibility that other community members (see previous paragraph) are producing the nitrogen oxides cannot be fully excluded. These other bacteria would then start to produce NO/N_2O upon hydroxylamine addition. Denitrification by heterotrophs is improbable, as there is no known reaction of denitrification on hydroxylamine and nitrite/nitrate was absent in the experiments. Aerobic ammonium oxidizing bacteria, present in small quantities in anammox enrichments, are known to produce NO and N_2O, especially under anoxic conditions (SLIEKERS et al. 2002), and this could be a source of NO and N_2O.

Implications of the mechanism for the "normal" anammox metabolism

The production of hydrazine in the presence of excess hydroxylamine was originally considered as a strong indication that hydrazine and hydroxylamine were both intermediates in the anammox process (VAN DE GRAAF et al. 1997). However, recent evidence points towards reduction of nitrite to nitric oxide (not hydroxylamine) as the fist step in the anammox metabolism, suggesting that nitric oxide and hydrazine are the intermediates in the anammox process (SCHMIDT et al. 2002; STROUS et al. 2006). The subsequent conversion of nitric oxide and ammonium to hydrazine comprises the coupling of two nitrogen atoms, in combination with a three-electron reduction reaction. *If* hydroxylamine were an *additional* intermediate, there would be a separation of the three-electron reduction (NO to hydroxylamine) from the formation of the N-N bond (combination of hydroxylamine and ammonium to hydrazine). If the combination of hydroxylamine and ammonium via the mechanism that was proposed in this study takes place directly (and not via NO, see Figure 4.4), the possibility exists that also under physiological conditions hydroxylamine is an additional -possibly enzyme bound- intermediate in the anammox process.

Conclusions

Hydroxylamine was disproportionated by anammox enrichments into ammonium and N_2. Hydrazine accumulated slightly during the conversion of hydroxylamine, but rose sharply when nearly all hydroxylamine had been consumed. NO and N_2O were also produced in low amounts. It was suggested that hydrazine was an intermediate in the hydroxylamine disproportionation. In this hypothesis, the sudden accumulation of hydrazine was caused by the sudden stop in the conversion, while the production still continued. Although the experiments were mostly

performed with one anammox species (*Candidatus* "Kuenenia stuttgartiensis"), comparable results in a test with *Candidatus* "Brocadia fulgida" indicated the described phenomenon is a general anammox characteristic. Two simple kinetic models were capable of explaining the observed behavior. Both models predict a very low hydroxylamine affinity for the hydroxylamine reduction to ammonium compared to the reaction of hydroxylamine with ammonium to form hydrazine.

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5

Chapter 5

Dynamics of nitric and nitrous oxide emission during full-scale reject water treatment

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* both authors contributed equally to this paper

ABSTRACT

The emission of NO and N_2O from a full-scale two-reactor nitritation-anammox process was determined during a measurement campaign at the Dokhaven-Sluisjesdijk municipal WWTP (Rotterdam, NL). The NO and N_2O level in the off-gas responded to the aeration cycles and the aeration rate of the nitritation reactor and to the nitrite and dissolved oxygen concentration. Due to the strong fluctuations in the NO and N_2O level in the nitritation as well as in the anammox reactor, only time-dependent measurements could yield a reliable estimate of the overall NO and N_2O emissions. The NO emission from the nitritation reactor was 0.2% of the nitrogen load and the N_2O emission was 1.7%. The NO emission from the anammox reactor was determined to be 0.003% of the nitrogen load and the N_2O emission was 0.6%. Emission of NO_2 could not be detected from the nitritation-anammox system. Denitrification by ammonium oxidizing bacteria was considered to be the most probable cause of NO and N_2O emission from the nitritation reactor. Since anammox bacteria have not been shown to produce N_2O under physiological conditions, it is also suspected that ammonium oxidizing bacteria contribute most to N_2O production in the anammox reactor. The source of NO production in the anammox reactor can be either anammox bacteria or denitrification by heterotrophs or ammonium oxidizing bacteria. The emission was compared to measurements at other reject water technologies and to the main line of the Dokhaven-Sluisjesdijk WWTP. The N_2O emission levels in the reject water treatment seem to be in the same range as for the main stream of activated sludge processes. Preliminary measurements of the N_2O emission from a one-reactor nitritation-anammox system indicate that the conversion is lower than in two-reactor systems. Based on the results and previous work it seems that a low dissolved oxygen or a high nitrite concentration are the most likely cause of NO and N_2O emission by ammonium oxidizing bacteria.

INTRODUCTION

With the introduction of more stringent standards for nutrient removal, nitrogen removal from reject water in side stream processes has become common practice in many communal wastewater treatment plants (WWTPs). Since reject water flows originating from sludge treatment, are relatively small (compared to the main line) and have a high ammonium content (typically 500-1500 mg-N/L), cost-effective nitrogen removal in small reactors can be achieved

(VAN LOOSDRECHT and SALEM 2006). Amongst the possible treatment options are the classical nitrification-denitrification, nitrification-denitrification over nitrite (SHARON, HELLINGA et al. 1998; MULDER et al. 2001) and nitrification combined with augmentation of the main treatment line (SALEM et al. 2004). Recently, the two-reactor nitritation-anammox process has been applied in at the Dokhaven-Sluisjesdijk WWTP in Rotterdam (NL) (Chapter 2). After the sludge digestion, ammonium is first oxidized to nitrite in a SHARON-type partial nitritation process. Because alkalinity is limiting the reaction, only half of the ammonium is converted into nitrite (VAN DONGEN et al. 2001b). The thus produced 1:1 mixture of ammonium and nitrite constitutes the feed for the anammox reactor, where it is converted into dinitrogen gas (N_2).

The production of nitric oxide (NO) and nitrous oxide (N_2O) can be detected during nitrogen removal at WWTPs (SOMMER et al. 1998; FUERHACKER et al. 2001). This is not surprising as NO and N_2O are known to be produced by ammonium oxidizing bacteria (AOB) (LIPSCHULTZ et al. 1981), nitrite oxidizing bacteria (NOB) (FREITAG and BOCK 1990) and denitrifying micro-organisms (FIRESTONE et al. 1979). In most bacteria, NO and N_2O are produced as a side-product, and are not present as an intermediate in the main energy-generating pathways. However, in denitrifying micro-organisms NO and N_2O are intermediates of the catabolic respiratory pathway, in which nitrate or nitrite is reduced to N_2. AOB can produce NO and N_2O either as a side-product in the catabolic pathway (oxidizing ammonium to nitrite), or, alternatively, by denitrification of nitrite with ammonium, hydrogen or pyruvate as electron-donor (COLLIVER and STEPHENSON 2000; SCHMIDT et al. 2004). The potential for emission of N_2O and NO by anammox organisms is unknown. Although low levels of NO and N_2O were detected in the off-gas of anammox enrichments (STROUS et al. 1999b), it is unclear whether this production is caused by the anammox bacteria themselves, or by other community members. The recently hypothesized role of NO in the anammox catabolism (SCHMIDT et al. 2002; STROUS et al. 2006) could point towards NO emission by anammox bacteria themselves. N_2O does not seem to play a role in the anammox metabolism, since in batch tests with physically purified anammox cells (purity higher than 99%, STROUS et al. 1999a) N_2O was not turned over (KARTAL et al. 2007a). NO_2 emission from WWTPs is rarely detected, but was observed in reject water treatment (STÜVEN and BOCK 2001). NO_2 can originate from chemical oxidation of NO with oxygen (UDERT et al. 2005) and was also observed to serve as electron acceptor for AOB (SCHMIDT and BOCK 1997). NO_2 is not known to play a role in the pathways of NOB, denitrifying micro-organisms or anammox bacteria.

NO and NO_2 are important atmospheric trace gasses, with a direct effect on the ozone chemistry of the atmosphere (CRUTZEN 1979). N_2O is a significant greenhouse gas, having an

approximately 300-fold stronger effect than carbon dioxide (IPCC 2001). In addition to the effects on the atmosphere, NO and N_2O have a large biological impact. NO is a free radical and is toxic to a wide range of organisms. Due to its hydrophobicity, NO can easily diffuse over cell membranes. In addition to the reactivity of NO with proteins containing transition metals and oxygen, it has a mutagenic effect in bacteria (ZUMFT 1993). Inactivation of Vitamin B_{12} (cobalamin) is the main toxic action of N_2O (WEIMANN 2003). Besides the environmental effect of nitrogen oxides, NO, NO_2 and N_2O gases are all toxic to humans and therefore relatively low maximum 8-hour exposure limits are defined by the Dutch government (MAC-values, as of 1/1/2007) of 0.2, 0.2 and 83 ppm respectively.

The production of NO and N_2O in full-scale reject water treatment is largely unknown. Due to the high volumetric nitrogen conversion rates, significant emissions can however be expected. Objective of this study was to characterize the emission of nitrogen oxides from a full-scale reject water facility where the two-reactor nitritation-anammox process was applied. Especially the impact of dynamic process conditions on the emission of NO and N_2O was studied. The overall emission levels were related to treatment options for similar (high nitrogen containing) wastewaters and to the mass balance of the total municipal WWTP at which the investigated reject water treatment was located.

METHODOLOGY

The emission of NO, NO_2 and N_2O from the full-scale nitritation and anammox reactors was measured intensively during a measurement campaign at the Dokhaven-Sluisjesdijk municipal WWTP. Additionally, grab samples were collected from the off-gas of the main stream treatment processes at this WWTP. Furthermore, NO and N_2O were determined in the off-gas of lab-scale nitritation and anammox reactors, and of two industrial treatment plants where the nitritation-anammox process (in a one-reactor and in a two-reactor configuration) was employed.

Design and dimensions of full-scale reactors

Main treatment plant and sludge treatment site

The Dokhaven-Sluisjesdijk WWTP mainly treats municipal wastewater (620,400 population equivalents, defined as 136 g COD/per person, nitrogen load of 4200 kg-N_{kj}/day) from the city of Rotterdam (NL). The main line consists of a high-loaded first stage for COD removal (A-stage, HRT 1 hour, dry weather conditions (DWC), SRT 0.3 days) and chemical phosphate removal, followed by a low-loaded second stage (B-stage, HRT 3 hours, DWC, SRT

7 days) where nitrogen removal takes place (Figure 5.1). The produced A- and B-stage sludges are treated at the Dokhaven-Sluisjesdijk sludge treatment site, where -after thickening- the sludges are combined and digested (SRT ca. 30 days, temperature 32-33ºC). The digested sludge is centrifuged and the centrate (reject water) is fed to the nitritation reactor for partial nitritation. The ammonium and nitrite containing effluent of the nitritation reactor serves as the influent for the anammox reactor. The treated reject water is returned to the A-stage of the WWTP.

Nitritation reactor

The nitritation reactor (1500 m^3) is a closed reactor without any biomass retention (SHARON-type partial nitritation reactor). The reactor was originally operated for nitritation-denitrification over nitrite (the original SHARON configuration, MULDER et al. 2001), but methanol addition for denitrification has been discontinued during the start-up of the anammox process (Chapter 2). The reactor is fed continuously with reject water. During aeration, the dissolved oxygen concentration is controlled at 2.5 mg/L by aeration flow rate. The temperature in the reactor is controlled at 32-33°C by additional heating of the influent when needed. pH is recorded but not controlled. The aeration of the nitritation reactor is discontinuous, because it is used to maintain a constant aerobic (solid) retention time of 1.4 days (the hydraulic retention time is dependent on the reject water flow and is 2-3 days). Aeration takes place in aeration cycles of two hours where the length of the aerated time during the cycle is dependent on the hydraulic load of the system (on average, aeration takes place during 80 to 90 minutes of this 2-hour period). When the reactor is not aerated, gas is removed from the headspace of the reactor to maintain a pressure of -0.1 mbar(g).

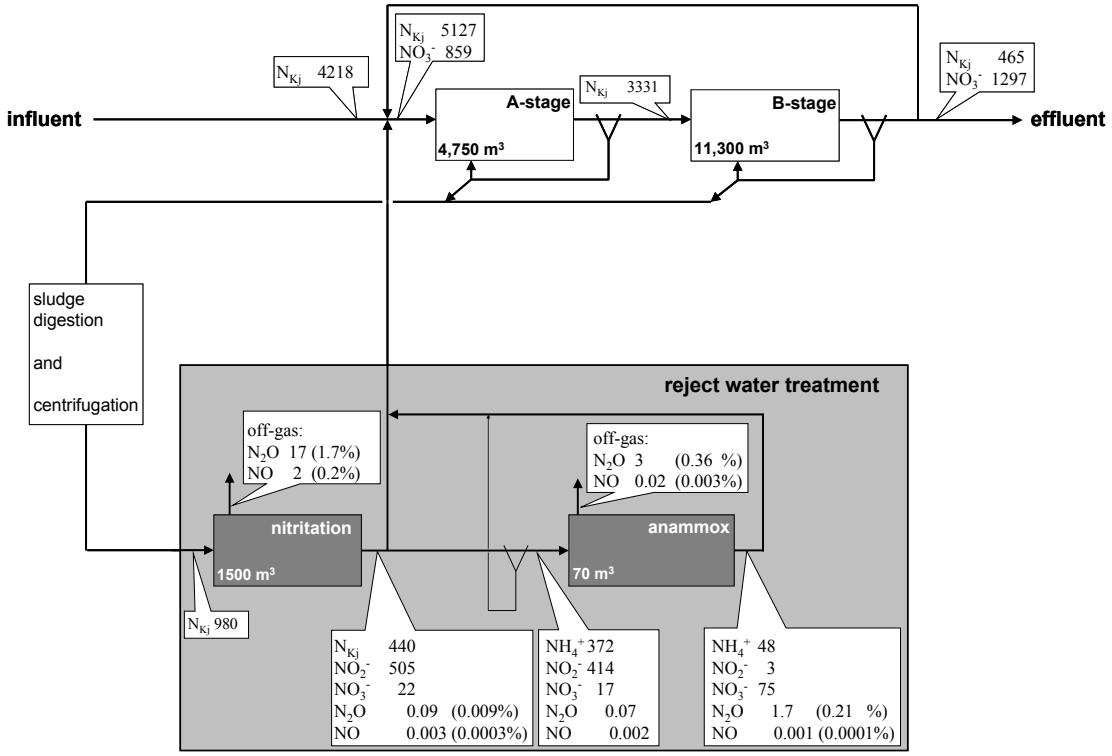

Figure 5.1 *Average nitrogen loads (in kg-N/day) of influent and effluent of the nitritation and anammox reactor (in grey), as well as from the main line of the WWTP during the measurement period. Emissions of NO and N_2O are shown in the liquid as well as the off-gas of the reject water treatment. Percentages are relative to the nitrogen load of the reactor.*

Anammox reactor

The anammox reactor (70 m^3) is fed with the effluent of the nitritation reactor. The reactor consists of two compartments (Figure 2.1), of which the upper compartment is employed as a settling system. The lower compartment is well-mixed by a gas lift driven by the gas production. In addition to gas mixing by the gas-lift, part of the off-gas is recycled to the bottom of the reactor. The remaining off-gas is sent to the plant-wide off-gas collection system. The chosen influent flow of the anammox reactor is dependent on the biological conversion capacity of the reactor and was designed for conversion of 500 kg-N/day. The remaining effluent of the nitritation reactor is returned to the main wastewater treatment plant. To decrease the risk of periods with no feed to the anammox reactor (after a prolonged stop of the centrifuges who produce the reject water), the influent flow of the anammox reactor was controlled by the water level in the nitritation reactor: at lower liquid levels in the nitritation reactor (possibly as a result of a lower influent flow of the nitritation), the influent flow of the anammox reactor was reduced. As an –unintentional– result, the water flow to the anammox reactor increased during aeration of

the nitritation reactor, as gas hold-up during the aeration phase increased the water level. The reactor temperature was 35-36°C.

Measurement campaign

From 10th to 13th of April 2006 (day 1383-1386 of operation of the anammox reactor, see Chapter 2), during 75 hours, on-line NO and NO_2 measurements and off-line N_2O measurements were performed on the off-gas of the full-scale nitritation and anammox reactors treating the reject water at Dokhaven-Sluisjesdijk WWTP. During this period, the reactors were operating normally. Reject water is, unlike municipal wastewater, relatively constant in temperature, weekly flow and composition over the year, which leads to stable reactor performance. Therefore, the 75-hour measurement period was considered representative.

To test the effect of process conditions on the emissions, short-term experiments were performed in the full-scale reactors:

- The influent flow to the nitritation reactor was paused for six hours. During this period the reactor was not aerated for four hours.
- During normal operation, the influent flow rate of the anammox reactor was based on the liquid level in the nitritation reactor (see paragraph "Design and dimensions of full-scale reactors"). This was changed to a fixed flow rate for 25 hours. During this period, the flow rate was reduced by 30% during one hour.

The recorded dissolved oxygen concentration during the first 26 hours of the measurement campaign was lower than the actual concentration due to probe fouling. This resulted in operation at an oxygen level, which was higher than during normal operation, resulting in a higher aeration flow.

Nitrogen mass balance

The acquired on-line data for NO and off-line data for N_2O during the measurement week, in combination with the average of routine lab analyses of the nitrogen species in the water phase performed by the Waterboard, served as the basis for a nitrogen balance of the nitritation and anammox reactor. For NO and N_2O, both the concentration in the off-gas and the dissolved concentration in the effluent were taken into account.

Nitrogen balance of the nitritation reactor

The nitritation reactor has shown stable conversion for 2006 (Figure 5.2). Therefore, the average levels over 2006 of Kjeldahl-Nitrogen (IN 1265±41 mg-N/L, OUT 574±17 mg-N/L), nitrite (OUT 654±16 mg-N/L) and nitrate (OUT 28±6 mg-N/L) were taken as the most reliable steady state values for the mass balance. The 71 daily-averaged determinations were evenly distributed over the year and were performed spectrophotometrically according to standards of the Dutch standardization institute (Kjeldahl-N, NEN6646, ammonium, NEN11732, nitrite/nitrate NEN13395). Nitrite (<0.6 mg-N/L) and nitrate (<1 mg-N/L) in the influent were not determined regularly, but could not be detected during incidental measurements during the measurement campaign. The influent flow (773 m^3/day), aeration flow ($2.2 \cdot 10^4$ Nm^3/h) and NO level (51 ppm) in the gas phase were the gas-flow weighed average over a representative period of 10 hours during the measurement period (35.12-45.10h). The gas phase N_2O level (135 ppm) was the average of 13 samples during one cycle of the nitritation reactor (25.10-27.10h, aeration flow $5.0 \cdot 10^4$ Nm^3/h).

The k_La for oxygen (17.8 h^{-1}) of the nitritation reactor was calculated based on oxygen uptake rate (OUR) and DO (2 g/m^3) during aerated periods. The OUR (69 g O_2/$m^3_{reactor}$/hour) was estimated based on the stoichiometric oxygen requirement from the steady state nitrite and nitrate production. The salt-corrected maximum solubility of oxygen at 35°C (C^*_{ox}) was estimated as 6.48 g/m^3. The corresponding k_La for NO (16.6 h^{-1}), N_2O (16.9 h^{-1}) and NH_3 (14.1 h^{-1}) were obtained after correction for differences in diffusion coefficients (WISE and HOUGHTON 1968; JANSSEN and WARMOESKERKEN 1997).

Levels of NO (0.0040 mg-N/L) and N_2O (0.11 mg-N/L) in the liquid were calculated based on k_La and maximum solubility for NO (27.2 mg-N/L, ZACHARIA and DEEN 2005) and N_2O (640 mg-N/L, JANSSEN and WARMOESKERKEN 1997). The NH_3 level (12 ppm) in the gas phase was calculated based on the k_La, the NH_3/NH_4^+ ratio (pKa= 9.25, THERMODYNAMIC PROPERTIES OF AQUEOUS SYSTEMS 2006) and solubility of NH_3 (257g-N/L, JANSSEN and WARMOESKERKEN 1997).

Nitrogen balance of the anammox reactor

The load of the anammox reactor varied considerably during 2006 and thus yearly averaged concentration values were not indicative for the situation during the measurement period. Therefore, the average of the two lab-measurements within the measurement week were taken for ammonium (IN 620 mg-N/L, OUT 80 mg-N/L), nitrite (IN 690 mg-N/L, OUT

4.5 mg-N/L) and nitrate (IN 30 mg-N/L, OUT 126 mg-N/L). For NO (70 ppm) and the influent flow (25 m^3/h), the average was taken from 10 hours during the measurement period (35.12-45.10h). For N_2O in the gas phase (4335±43 ppm), the average of 12 gas samples (between 25.6 and 27.5h) was taken. Production of dinitrogen gas was not measured, but calculated from the mass balance. Since no external gas was introduced into the reactor, the outgoing gas flow could be estimated from the produced amount of gas. Because the k_La of the anammox reactor was not known, NO (0.0019 mg-N/L) and N_2O (2.8 mg-N/L) levels in the liquid could only be calculated based on equilibrium and are thus underestimated. The calculation of the NH_3 (1.4 ppm) in the gas phase was also calculated based on equilibrium and is thus an overestimation.

Nitrogen balance of the complete WWTP

For calculation of the emissions from the Dokhaven-Sluisjesdijk site as a whole, the NO and N_2O emissions (in kg-N/h) were calculated relative to the nitrogen conversion of the specific reactors during the measurement campaign. For the A- and B-stage of the main treatment plant, the nitrogen load was relatively stable, and yearly averaged nitrogen conversion rates were employed to calculate the relative emissions.

Lab reactors

A 2 L lab-scale nitritation reactor was used for testing the reproducibility of the dynamics of nitrogen oxides emission under controlled conditions. The reactor was inoculated with sludge from the Dokhaven-Sluisjesdijk nitritation reactor. It was fed with synthetic influent (containing 1200 mg-N/L ammonium sulphate, 1200 mg-C/L $NaHCO_3$ and trace elements according to KAMPSCHREUR et al. (2007). Other process conditions: HRT 1.5 days, temperature 35°C, aeration flow 800 ml/min, DO= 3-4 mg/L, no pH control (6.8-7.2). During determination of the behavior of the reactor under anoxic conditions, the aeration flow was replaced by addition of dinitrogen gas at the same rate. Online NO (in the gas phase, as described by KAMPSCHREUR et al. 2007) and N_2O (in the liquid phase) analysis were performed during the experiments.

Online NO and NO_2 analysis, combined with incidental N_2O analyses from a continuously operated 8 L lab-scale anammox membrane bioreactor (see Chapter 3 for details), fed with a synthetic influent based on VAN DE GRAAF et al. (1996) (HRT 2 days, SRT 12 days, temperature 37°C, conversion 1.5 kg-N/m^3/day, fraction anammox bacteria >90%) served as a comparison for the full-scale anammox reactor.

NO and N_2O determination in other full-scale reactors

In order to correlate the emissions of the nitritation and the anammox reactor with emissions from other reactors, NO and N_2O emissions from four full-scale reactors were determined by grab sampling. The observed emissions were related to the nitrogen conversion.

- The A-stage (4750 m^3, mainly COD removal) of the main line of Dokhaven-Sluisjesdijk WWTP (Rotterdam, NL) (3 samples in three hours) during dry-weather conditions. The aeration rate was 30,000 Nm^3/h and dissolved oxygen was 0.5-1.0 mg/L.
- The B-stage (11300 m^3, nitrogen removal) of the main line of Dokhaven-Sluisjesdijk WWTP (Rotterdam, NL) (three samples in three hours) during dry-weather conditions. The B-stage consisted of three separate treatment lines, in which the dissolved oxygen concentration varied from 0.3 to 2.0 mg/L. The aeration rate of the three lines was 17,000 Nm^3/h and the off-gas of the three lines was merged.
- A 100 m^3 anammox reactor (Lichtenvoorde, NL) treating (on measurement days) 1.7-1.9 kg-N/m^3/day from a tannery wastewater (FRIJTERS et al. 2007) after nitritation (two samples over two months). The off-gas consisted only of the gas, which was produced within the reactor itself.
- A 600 m^3 one-reactor nitritation-anammox process (Olburgen, NL) treating 1.2 kg-N/m^3/day ammonium from a potato company (three samples over three months), operated at aeration rates of 1700-2300 Nm^3/h and at a dissolved oxygen concentration of circa 2 mg/L.

Measurement of NO, NO_2, N_2O, nitrate, nitrite, ammonium

From the headspace of the nitritation reactor and from the gas recycle loop of the anammox reactor, off-gas was collected continuously (at 1 L/min) during the measurement campaign. NO and NO_2 measurements of the anammox and the nitritation reactor were alternated every three minutes by a computer-controlled valve system. NO and NO_2 analysis was performed by chemiluminescence (CLD700e, Ecophysics Dürnten, CH).

N_2O was measured off-line on an Agilent 6890 gas chromatograph (HP Porapak Q Column 1 m x 2 mm i.d., nitrogen gas as carrier gas at 25 ml/min, electron capture detector, temperature of the injector, column and detector were 125, 60, and 300°C respectively). N_2O in the liquid phase was detected using a modified Clark electrode (Unisense, Århus, DK).

Process performance data were obtained from on-line monitoring systems for pH (nitritation and anammox reactor), dissolved oxygen (nitritation reactor) and nitrite (effluent of the anammox reactor). Also influent flow (nitritation and anammox reactor) and aeration flow (nitritation) were taken from the on-site available data. Twice weekly spectrophotometric analyses for ammonium, nitrite and nitrate according to standards of the Dutch standardization institute (NEN) were obtained from the Waterboard. To validate samples of the Waterboard and determine short-term dynamics during the measurement campaign, grab samples were regularly taken from the water phase of both reactors and analyzed for ammonium, nitrite, nitrate and phosphate using commercial spectrophotometric test kits (Dr.Lange, Hach-Lange GmbH, Düsseldorf, DE) and for total suspended solids (standard filter-based method).

RESULTS

Nitrogen balance of the nitritation and anammox reactor

Evaluation of the regular analyses by the Waterboard showed that the influent and effluent concentrations in the nitritation and the anammox reactor were stable over the first semester of 2006 (Figure 5.2), even although the daily averaged influent flow in both reactors fluctuated strongly. The anammox reactor was in the last phase of its start-up (Chapter 2) and therefore showed a steady increase in load in the first three months. The nitrogen balance based on measurements in the liquid phase for the main line of Dokhaven-Sluisjesdijk WWTP and for the nitritation and anammox reactor is shown in figure 5.1. In the nitritation reactor, on average 52% of the incoming nitrogen load was converted to nitrite. During the measurement period, the anammox reactor handled 82% of the effluent flow of the nitritation reactor. The remaining part was directed without further treatment to the main line of the WWTP.

The yearly averaged performance data of the nitritation reactor, in combination with the performance data of the anammox reactor during the measurement campaign, were combined with the measurements of NO, NO_2 and N_2O in the off-gas of the nitritation and anammox reactors to make an overall mass balance (Figure 5.1, shaded area). The N_2O and NO levels in the liquid phase as well as in the gas phase were taken into account for this balance. The emission of NO_2 of both reactors was negligible (<2.5% of the NO level). The nitritation reactor emitted 1.7% of its nitrogen load as N_2O, and 0.2% as NO. The mass balance of the nitritation reactor fitted well with an error of less than 2% (more nitrogen in the effluent than in the influent). N_2 production therefore did not play a significant role in the reactor.

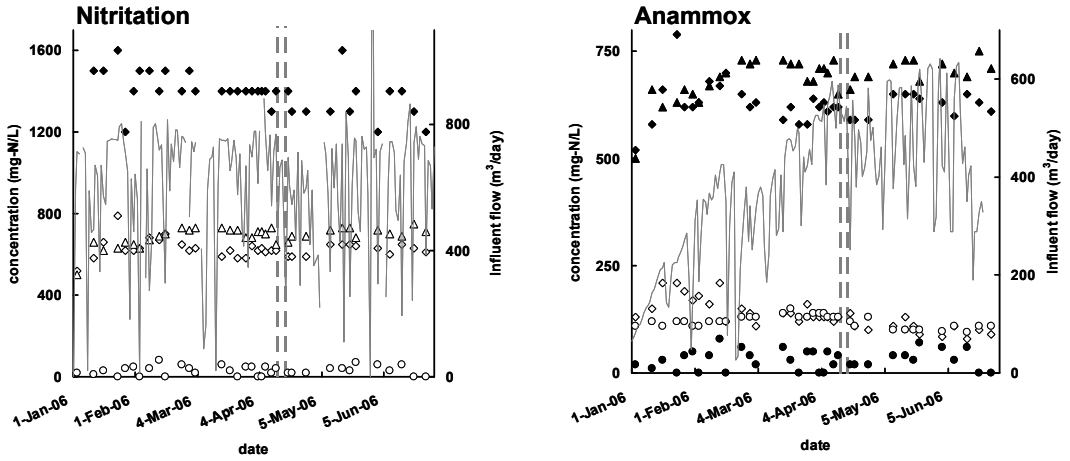

Figure 5.2 *Operation of the nitritation and the anammox reactor during the first 6 months of 2006, indicating influent flow (solid line), levels of ammonium (♦,◊), nitrite (▲,Δ) and nitrate (●,○) in the influent (closed symbols) as well as in the effluent (open symbols). Nitrite and nitrate levels in the influent of the nitritation reactor (below 1 mg-N/L during incidental measurements) were not determined regularly and are therefore not reported. The period in which the off-gas measurements were performed is indicated with dashed lines.*

The emission of N_2O and NO from the anammox reactor was 0.6% and 0.003% of the nitrogen load respectively. More than 90% of the nitrogen load was removed from the water phase; the remaining 10% was present in the liquid as nitrate (9%) and -to a small extent- as nitrite (0.3%). The off-gas flow was calculated by a conversion-based estimation of the N_2 production.

Dynamic behavior of NO and N_2O emission from the nitritation reactor

During aeration of the nitritation reactor, an approximately stable NO concentration in the off-gas was detected of about 50 ppm (±10 ppm) (Figure 5.3). During the non-aerated period -in which the gas flow was only caused by the generated underpressure of the headspace of the nitritation reactor- the NO concentration decreased to about 15 ppm. Increased NO levels at the start and at the end of the aeration period were observed in several aeration cycles. After 26 hours in the measurement campaign, the oxygen sensor of the nitritation reactor was cleaned, leading to a more accurate and higher oxygen measurement and –since the aeration level was controlled by the dissolved oxygen concentration- to a lower aeration rate. Despite the lower aeration rate, the NO concentration in the off-gas remained constant, leading to a decreased NO

emission. However, the concentration peak of NO during the start of the aeration period seemed to be slightly higher.

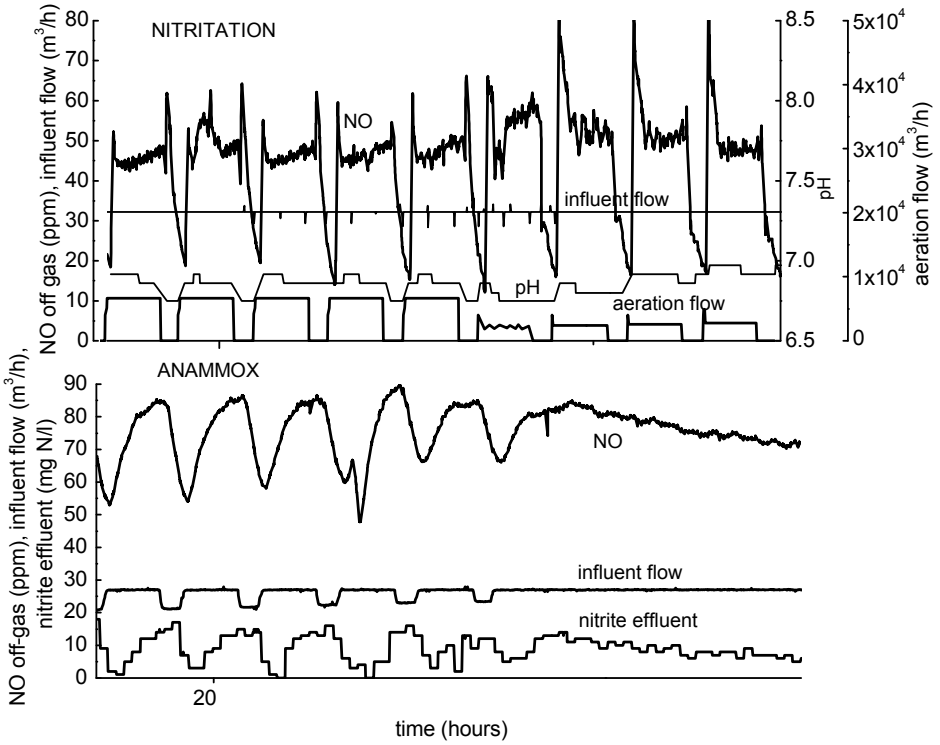

Figure 5.3 *Dynamic profiles of NO in off-gas of the nitritation and anammox reactor. Relevant operational data are also depicted (pH, aeration and influent flow of the nitritation reactor; influent flow and effluent nitrite concentration in the anammox reactor).*

The average N_2O level in the off-gas during normal operation of the nitritation reactor was 135 ppm and varied strongly within the two-hour aerated-non-aerated period (Figure 5.4, detailed measurement is performed during one cycle only). The N_2O level decreased more than 10-fold between the start and the end of the aerated period in the liquid phase as well as in the off-gas.

An experiment with a prolonged non-aerated phase of 5.5 hours and no influent flow during the first 4.5 hours (Figure 5.5) showed that N_2O accumulated in the liquid phase during the non-aerated period. In this period, the dissolved oxygen concentration decreased and no gas stripping occurred. Start of the influent addition did not influence the N_2O accumulation. This indicated, that N_2O accumulation did not depend on availability of organic carbon (which was present in relatively low concentrations in the influent). The interpretation of the profile of the

NO emission during the anoxic period is less straight-forward: NO accumulates during the first 2.5 hours of the anoxic period, but decreases during the next 2.5 hours.

Dynamic behavior of NO and N_2O emission from the anammox reactor

In the anammox reactor, influent flow, nitrite level and the NO concentration in the off-gas were fluctuating with a two-hour periodicity (Figure 5.3, 17 to 27 hours). The NO level in the off-gas of the anammox reactor was between 50 and 90 ppm, having the highest values simultaneously at the end of the aerated period in the nitritation reactor. Nearly all of the incoming nitrite (690 mg-N/L) was converted, but the low levels of nitrite in the effluent (0-18 mg-N/L) fluctuated with the periodicity of the influent flow. The nitrite level in the effluent fluctuated with the fluctuating load, and grab samples indicated that these fluctuations in the nitrite level were even stronger in the lower compartment where typical levels of 40 mg-N/L were detected.

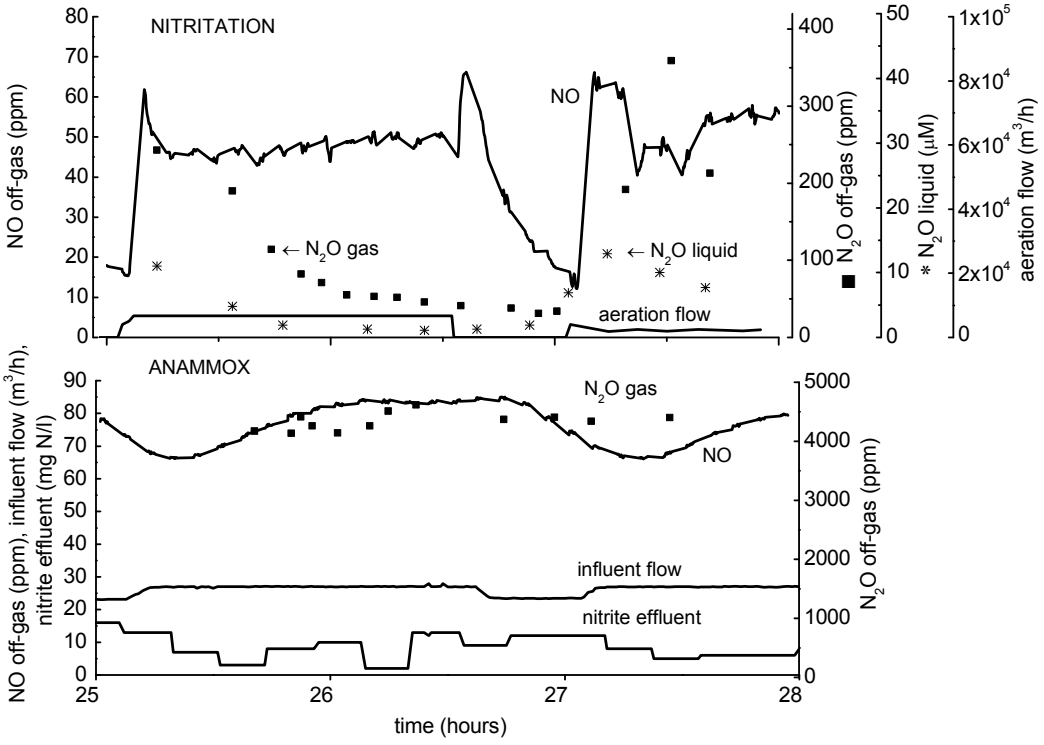

Figure 5.4 *Dynamic profiles of NO and N_2O concentration ■ in the off-gas of the nitritation reactor and the anammox reactor and N_2O in the liquid of the nitritation reactor *. Relevant operational data are also depicted (aeration of the nitritation reactor, influent flow and effluent nitrite concentration in the anammox reactor).*

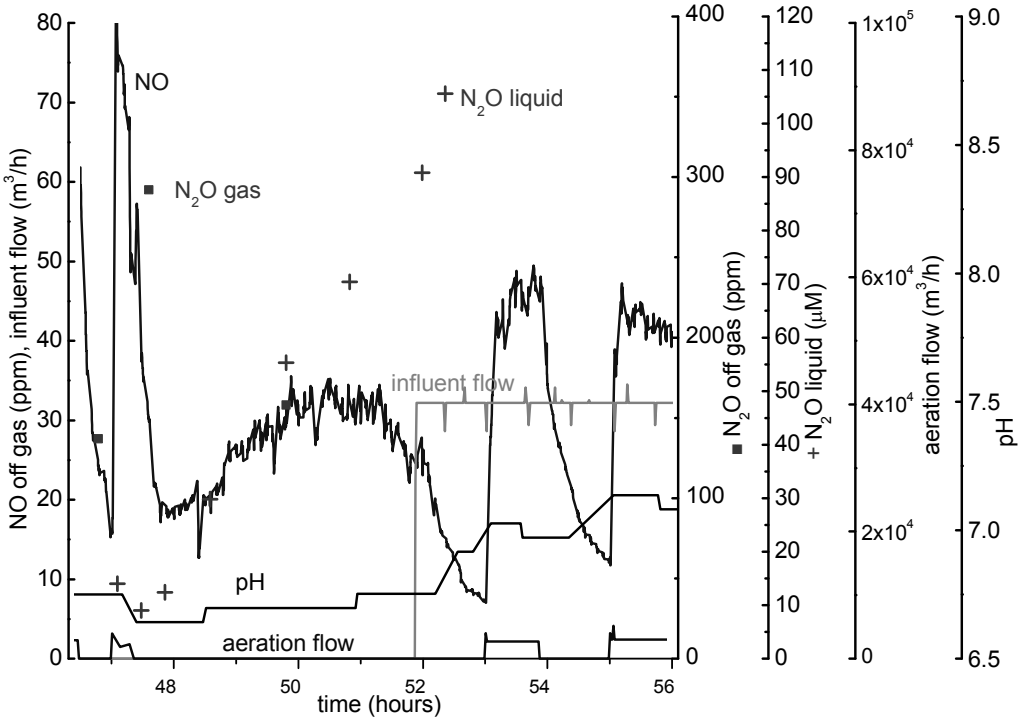

Figure 5.5 *Dynamic profiles of NO and N_2O concentration ■ in off-gas and N_2O in liquid + of the nitritation reactor during temporary influent shutdown. Relevant operational data are also depicted (pH, aeration and influent flow).*

When the influent flow was set at a constant level, the periodicity of the nitrite and NO levels disappeared (Figure 5.3, from t=27 hours), indicating a relation between influent flow, NO and nitrite level. At the constant influent flow rate of 27 m^3/h, the NO level in the off-gas was 70 ppm and the nitrite level in the effluent was 7 mg-N/L. After a sudden reduction of the influent flow (at t=52.1 hours, with 30%), the NO level decreased by about 40% and the nitrite level in the effluent was also reduced significantly (Figure 5.6). A similar trend was observed later during the measurement campaign (t=71.8 hours).

The N_2O level in the off-gas was on average 4335 ± 150 ppm (*n=12*) and showed no relation with the periodicity of influent flow (Figure 5.4).

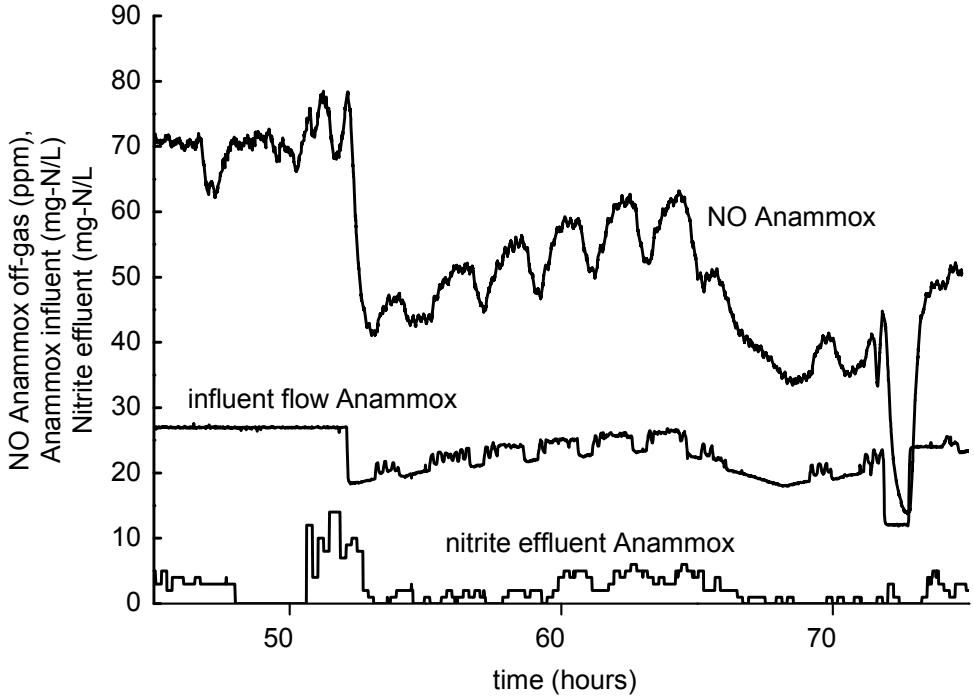

Figure 5.6 *Dynamic profiles of NO in the off-gas and nitrite in the effluent of the anammox reactor upon changes in the influent flow.*

NO and N_2O emission in the lab-scale nitritation and anammox reactors

The NO emission from the lab-scale nitritation reactor -converting synthetic influent- confirmed the phenomena observed on full-scale. The NO emission was constant during aeration (approximately 4.6 ppm at 0.8 L/min aeration) and decreased in the non-aerated phase. The NO emission was 0.15 % of the nitrogen load (average of four cycles of two hours, the non-aerated period was 0.5 hours). The increased NO emission at the start and end of an aeration period was not observed. However, the NO production did increase during the non-aerated phase: when N_2 gas was used for stripping, the NO level in the off-gas increased up to four-fold. N_2O was produced during both the aerated and non-aerated periods. However, the N_2O concentration in the non-aeration period was more than three times as high as in the aerated period. Since the N_2O level was measured in the liquid phase, measurements of N_2O did not allow quantitative interpretation due to measurement inaccuracies.

The lab-scale anammox reactor showed no detectable N_2O production (< 2 ppm) and NO levels were about 1 ppm (0.004% of the load).

NO and N_2O emission levels and loads in evaluated full-scale reactors

The levels of NO and N_2O in the off-gas of the other reactors that were analyzed are depicted in Figure 5.7 in absolute values and as a function of conversion. The highest concentrations in the off-gas can be found in anammox reactors (Figure 5.7A), but the absolute emissions (Figure 5.7B) from anammox reactors are relatively low. The nitrifying B-stage of the Dokhaven-Sluisjesdijk WWTP emits the largest amount of N_2O; both in absolute values (Figure 5.7A) as relative to the nitrogen converted (Figure 5.7B). The nitritation reactor emits the most NO of the reactors that are considered here, both related to the nitrogen load and to the amount of nitrogen that was converted. However, the B-stage of the Dokhaven-Sluisjesdijk WWTP emits a larger amount of NO than the nitritation reactor (Figure 5.8), because the nitrogen load of the main line is higher than the load of the nitritation reactor.

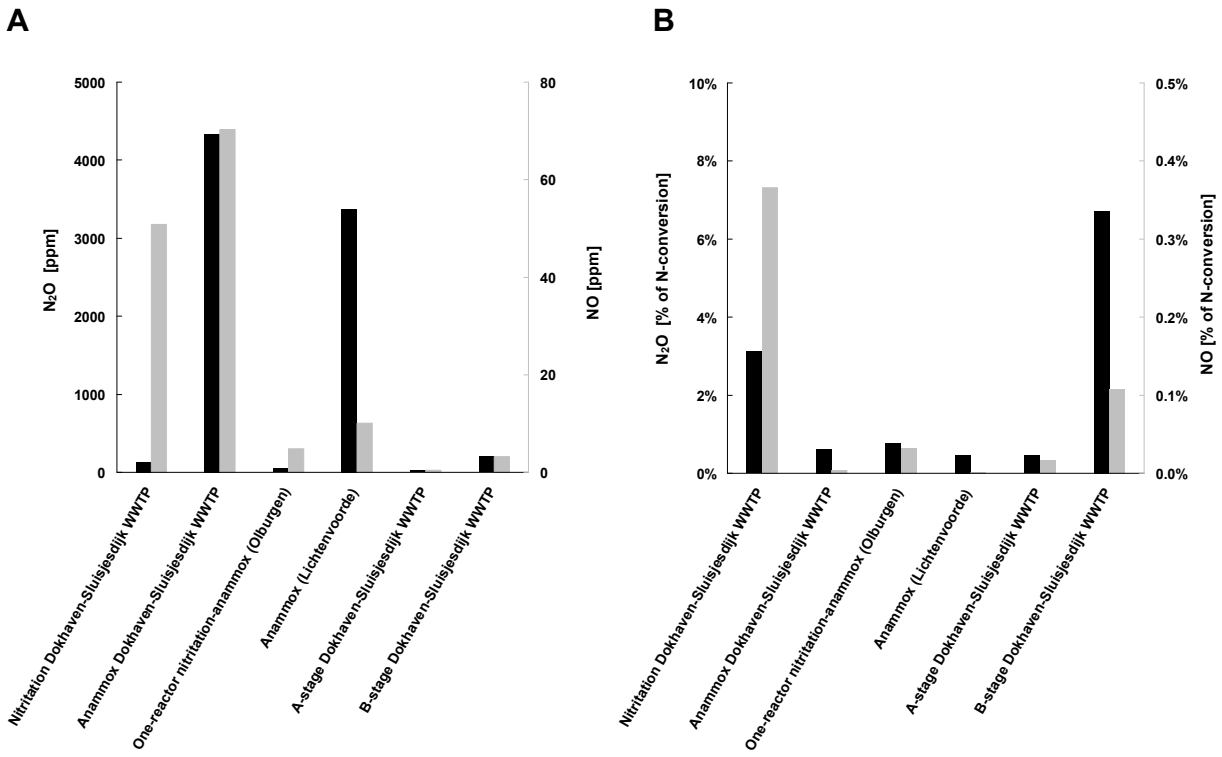

Figure 5.7 *Emission of N_2O (black) and NO (grey) in the evaluated treatment plants.* ***A****. Concentration of NO and N_2O in the off-gas.* ***B****. Emission of NO and N_2O as percentage of the nitrogen converted in the reactor.*

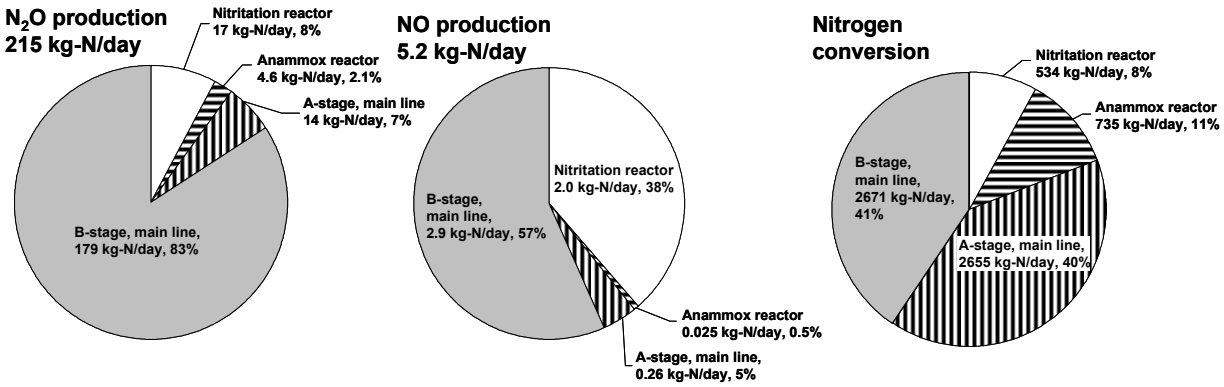

Figure 5.8 *Estimation of NO and N_2O emission as well as the nitrogen conversion rates (kg-N/day) by the different treatment units in the Dokhaven-Sluisjesdijk treatment plant, based on the operational conditions during the measurement campaign.*

DISCUSSION

Impact of application of full-scale reject water treatment systems on emission of NO and N_2O

In this study, the NO emission from the nitritation reactor was determined to be 0.2% of the nitrogen load and the N_2O emission 1.7%. The NO emission from the anammox reactor was determined to be 0.003% of the nitrogen load and the N_2O emission 0.6% (Figure 5.1, emission into the gas and the liquid phase have been taken into account). At this moment, no data are available in literature for NO and N_2O emission from other full-scale nitritation and anammox systems.

Emission compared to main line of WWTPs

To estimate the impact of reject water treatment using the two-reactor nitritation-anammox system on emission of NO and N_2O from the total WWTP, a comparison was made to the emission from the main line of the Dokhaven-Sluisjesdijk WWTP. NO and N_2O emission data have been reported in literature for both full-scale and lab-scale activated sludge systems, but it is not possible to use these data to estimate a standard emission level. Often the presented percentages for full-scale systems are extrapolated based on a few grab samples. Such a procedure has a large bias, as both influent characteristics and operational variations introduce fluctuations in the emission values. Furthermore, the varying sampling and measurement techniques and the unique set-up of every treatment plant, creates even larger fluctuations. The NO emission values in pilot WWTPs range from 0.02% of the nitrogen load (SCHMID et al. 2001a) to 0.07% of the ammonium converted in the nitrification stage of a reject water treatment

pilot (STÜVEN and BOCK 2001). The N_2O emission in literature varies widely from 0.02% of the nitrogen load (SOMMER et al. 1998) in a full-scale activated sludge system to above 20% in a lab-scale nitrifying-denitrifying reactor at low COD/N ratios (ITOKAWA et al. 2001). A study in 25 full-scale WWTPs also showed a large variation with an average N_2O emission of 0.6% of the nitrogen load and a maximum emission of 14.6% (WICHT and BEIER 1995).

The grab samples that were taken for estimation of the contribution of the main line at Dokhaven-Sluisjesdijk WWTP indicated that the emission of the B-stage is relatively large; 5% of the nitrogen load to this stage is converted into N_2O and 0.12% into NO. The N_2O emission from the main line of Dokhaven-Sluisjesdijk WWTP is relatively high compared to most other values reported in literature, whereas the NO emission lies within the range of most reported values. The applied AB-system at Dokhaven-Sluisjesdijk WWTP is special, as it includes a two-sludge system, in which the nitrifying B-stage contains a much higher concentration of nitrifiers and the hydraulic retention time in the B-stage is approximately five times lower than the aerobic retention time in conventional activated sludge systems. Additionally, the dissolved oxygen concentration in the reactor was low (<1 mg O_2/L) compared to the level which is generally applied in WWTPs.

Due to the higher nitrogen load of the B-stage compared to the reject water treatment system, the largest NO and N_2O emission is caused by the B-stage (83% of the total N_2O emission and 57% of the total NO emission, figure 5.8). Extrapolation of the few grab sample measurements at the Dokhaven-Sluisjesdijk WWTP (as indicated in Figure 5.7B) shows that the application of reject water treatment seems to decrease the N_2O emission and slightly increases the overall NO emission. However, care should be taken not to over-interpret a few grab samples. Continuous monitoring of the NO and N_2O emission in the main line over longer periods are necessary to verify these extrapolations and assumptions.

Environmental effect

The absolute N_2O-emission of the reject water treatment of 9,000 kg-N_2O/year (6,000 kg-N/year) [based on full operation of the nitritation and anammox reactor system, N_2O production of 2.3% of the nitrogen load, and the actual (2006) ammonium load of 668 kg-N/day] is close to the European Environmental Agency plant-wide threshold level (of 10,000 kg-N_2O/year) above which reporting to the European Pollutant Release and Transfer Register (E-PRTR) is required.

The Intergovernmental Panel on Climate Change (IPCC) applies a value of 0.5% of the nitrogen load as an estimate of the N_2O emission from wastewater, regardless whether the

nitrogen is converted in WWTPs or in effluent-receiving rivers and estuaries (IPCC 2006). The emission of the reject water treatment at Dokhaven-Sluisjesdijk WWTP is higher than this value. The preliminary point measurements of the main treatment plant indicated that also here the emissions were higher than the IPCC estimate. If this incidental observation is indicative for more treatment plants in the Netherlands, the contribution of wastewater treatment to the total anthropogenic N_2O emission in the Netherlands is higher than the estimated 2.3% for 2005 (BRANDES et al. 2007). This contribution is significant but small compared to the contribution of agriculture (53% in 2005, BRANDES et al. 2007).

The estimated anthropogenic emissions of NO and NO_2 in the Netherlands (345 kton $NO+NO_2$/year in 2005, BRANDES et al. 2007) were circa six times higher than the emission for N_2O (57 kton/year N_2O, in 2005, BRANDES et al. 2007). Since the NO emissions from the Dokhaven-Sluisjesdijk reject water treatment system were about ten times lower than the N_2O emissions, the contribution of this type of reject water treatment (and probably municipal wastewater treatment as a whole) could be considered a negligible source of NO.

Health effect

The levels of NO and N_2O were above the MAC-values, not only in the nitritation and the anammox reactor, but also in the A-stage (for N_2O) and B-stage of the main Dokhaven-Sluisjesdijk WWTP (Figure 5.7). The N_2O in the anammox reactor, and NO concentration in all other reactors, were about 100 times higher than the MAC value, which introduces the risk that plant personnel is subjected to too high levels. However, in the anammox reactor at Dokhaven-Sluisjesdijk WWTP, a proper off-gas collection system is already in place. The potential health risk of NO and N_2O has to be taken into account in the operation and periodic maintenance of wastewater treatment plants.

Measurement strategies

Due to the strong fluctuations in NO and N_2O level (Figures 5.3-5.6) in both the nitritation as well as in the anammox reactor, only time-dependent monitoring yielded a reliable estimate of the NO and N_2O emissions. Moreover, the relationship between the emissions and different operational conditions (loading rate, oxygen concentration etc.) could only be determined due to the registration of their dynamics. This may be valid for any full-scale nitrogen-converting reactor where -even if operational conditions are stable- the concentration and flow of influent will fluctuate, presumably leading to fluctuations in the N_2O and NO emissions.

Correlation of the presented emission data with lab-scale observations

Nitritation reactor

No NO and N_2O emission data are available for lab-scale nitritation systems that use retention time to achieve partial nitritation. In lab-scale reactors which use oxygen limitation to achieve partial nitritation, significantly higher NO and N_2O emissions are measured than in the full-scale and lab-scale retention-based reactors in this study. In a lab-scale nitritation system, 5.4% of the converted nitrogen was emitted as N_2O at a dissolved oxygen level of 0.1 mg O_2/L (ZHENG et al. 1994). Furthermore, in a nitrifying lab-scale airlift reactor, operated at a dissolved oxygen concentration below 0.032 mg O_2/L, 3.3% of the consumed ammonium was emitted as NO and 5.5% as N_2O (SLIEKERS et al. 2005).

Anammox reactor

The N_2O emission from the full-scale anammox reactor of 0.6% of the converted nitrogen was much higher than the emission of lab-scale anammox enrichment reactors, where values of 0.03-0.06% (STROUS et al. 1998), <0.1% (VAN DE GRAAF et al. 1997; WYFFELS et al. 2004), and <0.01% (this Chapter) were found. Batch experiments with physically purified anammox cells did not show any N_2O turn over (KARTAL et al. 2007a), indicating that the low amount of N_2O formed in the enrichments may have been produced by other community members. In general, nitrifying bacteria are detected in these systems (WYFFELS et al. 2004). Experiments with biomass from a one-reactor nitritation-anammox system yielded under anoxic conditions a production of 0.8% N_2O of the converted nitrogen (WYFFELS et al. 2003).

For NO emission in lab-scale anammox reactors, values varying up to three orders of magnitude (0.00025-0.0005% (STROUS et al. 1998) to 0.1% of the converted nitrogen (VAN DE GRAAF et al. 1997)) are reported. It is therefore not surprising that the full-scale value of 0.003% is within this range.

Which pathways may cause the NO and N_2O emission?

Nitritation reactor

Three processes can be distinguished for the production of NO and N_2O in the nitritation reactor: (i) production by AOB, (ii) production by denitrifying micro-organisms or (iii) by chemical production. In AOB, two pathways can be distinguished for NO and N_2O production: escape of unstable intermediates of the hydroxylamine oxidation to nitrite and emissions during denitrification.

The chemical pathways leading to NO emission are dependent on the presence of nitrous acid (HNO_2) (UDERT et al. 2005). As a result, chemical NO production increases upon a decrease in pH. Such a pH dependence of NO production did not occur in the nitritation reactor. Therefore, chemical NO production is not considered to be the main cause for the NO production. The known mechanisms for chemical N_2O emission all require hydroxylamine as reactant (UDERT et al. 2005). Hydroxylamine is produced biologically and intracellularly during ammonium oxidation (for which oxygen as electron acceptor is required), so it is expected to be turned over during the aerobic phase, but not during the anoxic phase. As N_2O accumulates during the anoxic phase, chemical formation of N_2O from hydroxylamine can be excluded as the main pathway leading to N_2O production.

The oxygen dependence of the NO and N_2O accumulation indicates that denitrification causes the main NO and N_2O production. Whether heterotrophic denitrification or denitrification by AOB causes the emission cannot be unambiguously determined based on these data. It is most likely that denitrification by AOB causes the emission, because the presence of organic carbon had no clear effect on the NO and N_2O emission. The availability of readily degradable organic matter in the influent of the nitritation reactor is low and influent addition to the nitritation reactor did not affect the NO and N_2O emission.

Anammox reactor

Since there are no indications of N_2O turnover by anammox bacteria, and since N_2O production was never reported to such a high extent in lab-scale reactors, it seems that anammox bacteria could not be responsible for N_2O formation. From the nitrogen balance over the first three years of operation of the full-scale anammox reactor at the Dokhaven-Sluisjesdijk WWTP, no indications for significant N_2 production through denitrification could be found (Chapter 2), which indicates that N_2O formation was not a side-product from regular denitrification. Incomplete denitrification –possibly yielding N_2O only- however could be a possible source. Another possible pathway leading to N_2O formation is denitrification by AOB. AOB originating from the nitritation reactor can be transported to the anammox reactor, as the settler between the nitritation and the anammox reactor only removes about 50% (J.W. Mulder and W.R.L. van der Star (2006), unpublished observations) of the solids from the nitritation reactor effluent (particularly since nitritation reactor sludge has a low settleability due to the absence of sedimentation-based biomass retention). The denitrifying pathway of AOB would yield N_2O only, and this route is often linked with low oxygen levels (POTH and FOCHT 1985) and is also occurring in anoxic conditions (KAMPSCHREUR et al. 2008). Therefore, from the available

information at this moment, denitrification by AOB is the most probable cause of N_2O production.

The NO emission was much lower than the N_2O emission and could be caused by the anammox bacteria, as NO is a potential intermediate of the anammox catabolism (Figure 1.3, schemes B and C). The emission of NO during production of N_2O in heterotrophic denitrifiers or in AOB however cannot be excluded.

Factors influencing the emissions

The use of full-scale reactors for research purposes includes the drawback that determination of factors influencing the emissions is impeded due to variability of influent conditions and the impossibility of separately changing parameters as would be possible on lab-scale. Nevertheless, the most important factors that are expected to determine the emission of NO and N_2O from reject water treatment systems are discussed here in light of the results obtained in this study.

Nitrite

Increased nitrite concentrations are known to increase NO and N_2O emission from nitrifying cultures (COLLIVER and STEPHENSON 2000; KAMPSCHREUR et al. 2008). The high nitrite level in the nitritation reactor (more than 100 times higher than in complete nitrification) probably increased the overall emission of nitrogen oxides from the reactor compared to reactors that fully nitrify to nitrate. Due to the large reactor volume and relatively long overall retention times, nitrite variation over the aeration cycles is not significant, which is confirmed by the grab samples during the measurement campaign (data not shown). For this reason, a possible effect of nitrite level on the emission of nitrogen oxides could not be confirmed in this study.

In the anammox reactor, the nitrite concentration in the effluent indicated that the nitrite in the sludge bed of the reactor fluctuated, corresponding with the influent flow. The level of NO production in the anammox reactor was strongly dependent on the influent flow and -as a possible consequence- on the nitrogen load and nitrite accumulation in the system (Figure 5.3). The NO emission profile indicates that the production of NO in the anammox reactor could have been directly dependent on the nitrite level, as was observed in nitrifying reactors (KAMPSCHREUR et al. 2008). The N_2O emission did not fluctuate significantly with influent flow or with nitrite levels. However, interpretation is impeded due to the high solubility of N_2O in water, which makes it harder to observe changes in N_2O via sampling the off-gas.

Dissolved oxygen

At Dokhaven-Sluisjesdijk WWTP, the largest NO and N_2O emissions originate from nitrifying systems: the B-stage of the main line and the nitritation reactor. The higher contribution of the nitrifying compartment to NO emissions was also found by SOMMER et al. (1998), where in a full-scale WWTP the NO emission was 0.02% of the nitrogen load in the nitrification stage and 0.0004% in the denitrification stage.

Dissolved oxygen concentration largely impacts the level of NO and N_2O emission. An increase in NO and N_2O production upon a decrease in dissolved oxygen concentration, is reported in different nitrogen converting reactors (e.g. in nitrifying lab-scale reactors (SLIEKERS et al. 2005; KAMPSCHREUR et al. 2008) and in a denitrifying pilot scale reactor (TALLEC et al. 2006)). Also in the full-scale nitritation reactor which is described in this Chapter, N_2O accumulated strongly during the anoxic phase (Figure 5.5). This probably caused the increased emission at the start of the aeration phase. During the aeration phase, the N_2O emission -and thus the N_2O production- decreased.

NO also accumulated during the anoxic phase in the nitritation reactor, but when the anoxic period was prolonged the NO production decreased. Interpretation of the NO accumulation is impeded, as NO was only measured in the off-gas and mass transfer was low during anoxic periods. Accumulated NO during the anoxic phase is expected to be stripped from the liquid phase at the beginning of the aeration phase, leading to a peak in the NO emission. The increased NO production under anoxic conditions in the lab-scale nitritation reactor -where NO was stripped by dinitrogen gas addition during the anoxic phase- supports the assumption that NO did accumulate during the anoxic phase in the full-scale system.

The nitritation reactor contained suspended biomass, in which no large oxygen gradients are expected to be present. Reactors containing flocculated or granulated biomass are expected to encounter more NO and N_2O emission caused by oxygen limitation, since due to the larger oxygen gradients, part of the nitrifying biomass will always experience low (or zero) oxygen levels. The relatively high emission of NO and N_2O from the B-stage of the Dokhaven-Sluisjesdijk site compared to other WWTPs, is also likely to be caused by the low oxygen levels that were present in the B-stage (ranging from 0.3 to 2 mg O_2/L).

The absence of oxygen in the anammox system, could stimulate the occurrence of denitrification (probably by AOB, see section "Which pathways may cause the NO and N_2O emission?"), which was hypothesized to cause the majority of the N_2O emission from the anammox reactor.

Aeration rate

The two-fold higher aeration flows (expected to result in about two-fold higher dissolved oxygen concentration[3]) in the first 26 hours, surprisingly did not influence the NO level in the off-gas. The NO level at both aeration flows was similar (with a difference of less than 10%). The resulting NO emission, and thus the NO production, was higher at higher aeration flow and -as a consequence- also at higher dissolved oxygen concentration. This is an interesting response. It indicates, that the NO emission was not dependent on biological conversion rates of ammonium and oxygen, as the rates are not expected to change due to the increase of aeration flow or dissolved oxygen concentration. It is not likely that oxygen limitation was the cause of NO formation, since with increasing dissolved oxygen concentration the NO emission did not decrease (oxygen limitation was not expected to play a role anyway under the operating conditions as the dissolved oxygen concentration was above 2.5 mg O_2/L and no serious mass transfer limitation was expected due to the small size of the sludge flocs).

However, since the NO concentration in the off-gas did not change significantly upon this change in airflow rate, the NO concentration in the liquid must have remained stable[4] (at the oxygen levels at which the phenomenon took place). A possible explanation for the constant concentration of NO in the liquid phase of the reactor, is that the NO-intermediate level within the nitrifying cells is thus maintained at a stable level. If this were general behavior of ammonium oxidizing bacteria, it implies that that above a certain dissolved oxygen concentration, the emission is independent of the dissolved oxygen concentration and solely dictated by NO-stripping through aeration.

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[3] Assuming that the oxygen uptake rate (OUR) remains the same (since conversion is limited by availability of alkalinity, and not by DO level), the product $k_La\cdot(DO^*-DO)$ should be constant (where DO* is the equilibrium DO under atmospheric conditions). Assuming that the k_La is linearly dependent on airflow (valid at the applied low superficial gas velocities, HEIJNEN et al. 1992), the driving force (DO*-DO) should be twofold lower at a two-fold higher aeration flow. This driving force in the final situation is 8-2.5=5.5 mg/L. At the two-fold higher aeration flow before calibration of the DO probe, the driving force should be 5.5/2=2.75 mg/L. This driving force corresponds to a calculated DO of (8-2.75)=5.25 mg/L, which is about twice the value at a normal aeration rate.

[4] Assuming that the NO production rate and the k_La_{NO} both are linearly dependent on the airflow, the driving force (C_{NO}-C^*_{NO}) (where C^*_{NO} is the solubility of NO at the NO concentration in the off-gas) should remain the same. As the off-gas NO level did not change, also C^*_{NO}, did not change. Therefore, the NO level in the liquid must have been (approximately) stable as well.

pH

The pH varied in the nitritation reactor: during the aerated phase it decreased as a result of ammonium oxidation; during the anoxic period it increased (both by approximately 0.2 units). Surprisingly, this had no effect on the NO and N_2O emission dynamics. If HNO_2 were the substrate of the chemical or biological reactions involving the formation of NO and N_2O, a decrease in pH -and thus an increase of HNO_2 can be expected to lead to increased NO and N_2O levels. During the aeration phase however (when the pH decreased, Figure 5.3), the off-gas NO level was constant and the N_2O level increased. During a prolonged non-aerated phase (Figure 5.5), NO and N_2O both increased when the pH increased. Other factors, like oxygen level, aeration rates and conversion rates, influence the NO and N_2O emission of the nitritation reactor more than the pH in the pH-range of 6.6 to 7.2.

Measures to prevent NO and N_2O emission from reject water treatment

Nitritation reactor

The dissolved oxygen level is a process parameter which strongly affects the NO and N_2O emission (see section "Factors influencing the emissions"). The N_2O and to a lesser extent NO in the nitritation reactor accumulated during the non-aerated phase, which led to N_2O and NO emission due to stripping in the aerated phase. Production of NO and N_2O during the anoxic phase could probably be largely prevented by avoiding anoxic periods. This can be easily applied by building a smaller reactor where a suitable HRT can be achieved without having anoxic phases. During the aerated phase, efficient oxygen control is an important factor that can limit NO and N_2O emission. Since the NO level in the off-gas is constant above a certain oxygen level, increased aeration will lead to more NO stripping and thus to a higher emission (as is the case during the present operation). If the oxygen set-point however is chosen too low, increased NO production due to oxygen-limitation is expected to increase the off-gas NO level.

Anammox reactor

The emission prevention measures which can be introduced in the anammox reactor, depend on the source of NO and N_2O production in the anammox reactor. If AOB -originating from the nitritation reactor- are the main source, a more effective removal of the nitrifying sludge from the effluent of the nitritation reactor can be applied before introduction into the anammox reactor. If denitrifiers (or anammox bacteria) are the main cause of the NO and N_2O production, reactor operation should be aimed at controlling factors that lead to the production, as presence of these micro-organisms in the reactor cannot be prevented. Reactor control could therefore

potentially be aimed at a lower nitrite level, as an increase in nitrite level in the anammox reactor increases the NO emission. A too low nitrite level however will lead to a lower conversion capacity, since the penetration of nitrite into the anammox granules becomes too low (Chapter 2).

Alternative configurations

High nitrite levels are known to increase the emission of NO and N_2O from nitrifying systems. A two-reactor nitritation-anammox reactor configuration means a high nitrite level in the nitritation reactor to generate a 50/50 ammonium/nitrite load for the anammox reactor. In one-reactor nitritation-anammox reactors (HIPPEN et al. 1997; KUAI and VERSTRAETE 1998; THIRD et al. 2001), nitrite is immediately turned over, resulting in a 10-50 times lower nitrite level which possibly leads to lower emissions of NO and N_2O. Incidental off-gas analysis of such a reactor on full-scale indeed indicated lower emissions, but the limited amount of grab samples did not justify strong conclusions. Alternatively, a one-reactor nitritation-denitrification system (the original SHARON configuration, HELLINGA et al. 1998) could be applied, where nitrite levels are also lower, which potentially leads to lower emissions. Application of classical denitrification instead of an anammox-based process however obviously leads to a need for electron donor and as a consequence to higher costs and substantial CO_2 emissions (JETTEN et al. 2002). Furthermore, there is the risk that the denitrification step also will lead to substantial NO and N_2O emissions.

CONCLUSIONS

During reject water treatment by the two-reactor nitritation-anammox process, NO and N_2O are emitted. Due to the strong fluctuations in NO and N_2O level in both the nitritation as well as in the anammox reactor, only time-dependent measurements yielded a reliable estimate of the NO and N_2O emissions. The emission of the total full-scale nitritation-anammox system (based on such time-dependent measurements) consisted mainly of N_2O (2.3% of the nitrogen load). The larger part of the N_2O (75%) and NO (99%) was produced in the nitritation reactor. NO_2-emission from the nitritation-anammox system was not observed.

Denitrification by ammonium oxidizing bacteria was considered the main source of NO and N_2O emission from the nitritation reactor. Since anammox bacteria have not been shown to produce N_2O on lab-scale, and since no activity by denitrifying microorganisms could be detected, ammonium oxidizing bacteria were also suspected to contribute most to the N_2O

production in the anammox reactor. The main process conditions that influence the NO and N_2O emission are dissolved oxygen level, nitrite concentration and aeration rate.

The levels of NO and N_2O emission from reject water treatment seem to be in the range of the absolute (nitrogen load based) emissions from main line of WWTPs. Time-dependent measurement of the dynamics of NO and N_2O emissions in full-scale WWTPs and investigation of several different types of reactors are required to generate a reliable picture of the overall impact of WWTPs and application of reject water treatment on the total anthropogenic NO and N_2O emission.

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6

Chapter 6

An intracellular pH gradient in the anammox bacterium "Kuenenia stuttgartiensis" as evaluated by ^{31}P NMR.

This Chapter has been submitted for publication as:

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Abstract

The cytoplasm of anoxic ammonium oxidizing (anammox) bacteria consists of three compartments separated by membranes. It has been suggested that a proton motive force may be generated over the membrane of the innermost compartment, the "anammoxosome". ^{31}P Nuclear Magnetic Resonance (NMR) spectroscopy was employed to investigate intracellular pH differences in the anammox bacterium *Candidatus* "Kuenenia stuttgartiensis". With *in vivo* NMR, spectra were recorded of active, highly concentrated suspensions of "K. stuttgartiensis" in a wide-bore NMR tube. At different external pH values and metabolic rates, two stable and distinct phosphate peaks were apparent in the recorded spectra. These peaks were equivalent with pH values of 7.4 and 6.3 and suggested the presence of a proton motive force over an intracytoplasmic membrane in "K. stuttgartiensis". This study provides for the second time -after discovery of acidocalcisome-like compartments in *Agrobacterium tumefaciens*- evidence for an intracytoplasmic pH gradient in a prokaryotic cell.

Introduction

Anammox bacteria constitute a deeply branching monophyletic group inside the phylum Planctomycetes (STROUS et al. 1999a). They grow by the anoxic oxidation of ammonium with nitrite as the electron acceptor, leading to the direct production of dinitrogen gas, N_2 (VAN DE GRAAF et al. 1996). Although overlooked for almost a century of research on the nitrogen cycle, the anammox process is now known to play a major role in the production of N_2 in a wide variety of (marine) ecosystems (SCHMID et al. 2007) and is currently applied in wastewater treatment (Chapter 2).

All Planctomycetes have a differentiated cytoplasm with different intracytoplasmic compartments separated by membranes (FUERST 2005). Anammox bacteria have three compartments (Figure 6.1, STROUS et al. 1999a; LINDSAY et al. 2001; VAN NIFTRIK et al. 2008): (i) the "riboplasm" which contains the chromosome and ribosomes; (ii) the "anammoxosome" located inside the riboplasm; (iii) the "paryphoplasm" that surrounds the riboplasm much like the periplasm surrounds the cytoplasm in Gram-negative bacteria. However, the Gram-negative periplasm is in open contact with the cell's surroundings via porins in the outer membrane, whereas both genomic (STROUS et al. 2006) and experimental (VAN NIFTRIK et al. 2008) evidence suggest that the paryphoplasm is not open to the environment and should be considered a cytoplasmic compartment. Whether the paryphoplasm is a true intracytoplasmic compartment as

in other Planctomycetes (LINDSAY et al. 2001) or whether it should be regarded a "special" periplasm as is suggested by genome analysis is presently not clear.

For anammox bacteria, it has previously been suggested that the anammoxosome contains the enzymes involved in the anammox catabolism and that a proton motive force is generated over its membrane (VAN NIFTRIK et al. 2004). The observation that all or most cytochrome c proteins of anammox bacteria reside inside the anammoxosome (VAN NIFTRIK et al. 2008) and that the anammoxosome membrane is highly curved (VAN NIFTRIK et al. 2007) is consistent with this idea.

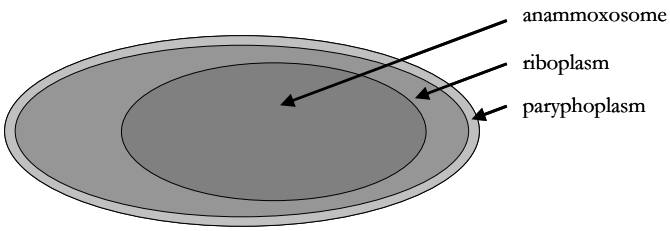

Figure 6.1 *Schematic representation of the different compartments in anammox bacteria (LINDSAY et al. 2001; FUERST 2005). The cytoplasm is divided into the paryphoplasm (the outer-most compartment), the riboplasm (where the ribosomes and chromosome are located) and anammoxosome (where most or all of the cytochromes c are present and where the catabolism has been hypothesized to take place).*

Ladderane lipids are the most abundant membrane lipids of anammox bacteria. These lipids contain concatenated cyclobutane rings and may therefore make the membranes less permeable to solutes and protons (SINNINGHE DAMSTÉ et al. 2002). The slow growth rate of anammox bacteria (doubling time of several days, see Chapter 3; STROUS et al. 1998; TSUSHIMA et al. 2007a) continues to be a major challenge in anammox research. The inability to grow anammox bacteria (possibly as a result of the slow growth) in pure culture only adds to this challenge. Enrichment cultures (with enrichment levels of 50-90% of the population (e.g. SCHMID et al. 2000; EGLI et al. 2001; STROUS et al. 2006) are the only source of anammox bacteria for experimental investigation. Because of the relatively large non-anammox population (10-50%) in such cultures, additional physical purification of anammox cells (i.e. with Percoll density gradient centrifugation, STROUS et al. 1999a) is necessary for unambiguous results. However, the recent enrichment of the anammox bacterium "Kuenenia stuttgartiensis" (Chapter 3) makes it possible to obtain large amounts of suspended cells with a high degree of enrichment (up to 97.6%), which has enabled the experiments of the present study.

In this Chapter, the nature of the intracytoplasmic compartmentalization of cells of "K. stuttgartiensis" was addressed with *in vivo* ^{31}P-NMR. In this approach, the shift of the $HPO_4^{2-}/H_2PO_4^-$ peak can be directly related to the $HPO_4^{2-}/H_2PO_4^-$ ratio and can thus be used to detect intracytoplasmic pH differences. The method has previously been successfully applied to estimate the (relatively acidic) vacuolar pH in several plants (ROBERTS et al. 1980), yeasts (NICOLAY et al. 1982) and fungi (HESSE et al. 2000), as well as in mitochondria (OGAWA and LEE 1984). In the latter case, the pH difference between the mitochondrial matrix and the cytoplasm constitutes the pH component of the proton motive force, and has been shown to be a good indicator of cell activity. If the anammoxosome is indeed the locus of anammox catabolism, generation of a proton motive force over its membrane would be expected. The present study provides further evidence for this concept, by showing that two distinct and stable $HPO_4^{2-}/H_2PO_4^-$ ratios are present in active cells of "K. stuttgartiensis".

Materials and Methods

Origin and preparation of the biomass

Biomass for all experiments was harvested from an 8 liter anammox membrane bioreactor (MBR), fed with synthetic medium (see Chapter 3 for details). The biomass retention time was 12 days, corresponding to a doubling time of 8.3 days. At the time of harvesting, the biomass concentration in the MBR was ca. 2 g-C/L, and the conversion was 50 mM NH_4^+/day. "Kuenenia stuttgartiensis" (100% sequence similarity of the 16S rRNA with the "Kuenenia" from the Kölliken enrichment, Chapter 3) was the only anammox species that could be detected (with fluorescence in situ hybridization) and constituted 97.6% of the population. Biomass collected during 3-4 days was concentrated 100 times in a single centrifugation step and used for *in vivo* NMR experiments within 24 hours after concentration. The biomass produced by this procedure was dark red and highly viscous, but still liquid. Further concentration was not possible and led to degradation of the biomass, resulting in extensive foaming. Cells remained in suspension up to five days after preparation.

NMR

^{31}P NMR spectra were recorded at 121.5 MHz on a Bruker AV-300 (Bruker Rheinstetten, DE) wide bore spectrometer equipped with a ^{31}P/^{13}C probe (diameter 20 mm), and collected in 60 minute blocks of 6700 scans. Temperature was controlled at 298 K. Spectra were calibrated by the NADH/NADPH peak at 10.8 ppm. pH values were determined by comparing the

pH-sensitive shifts of inorganic phosphate in the cytoplasm with a calibration curve for inorganic phosphate (DEN HOLLANDER et al. 1981).

In vivo fed-batch NMR

For the experiment, the 20 mm NMR tube was employed as a miniature fed-batch reactor (Figure 6.2). Ten to fifteen ml of concentrated biomass was added to the NMR-tube. Mixing and anoxic conditions were maintained by sparging continuously with N_2. The pH was buffered with carbonate. Ammonium nitrite (200 mM) was added as the substrate via a syringe pump at 1 ml/h for one to twenty hours. The first spectra were recorded without substrate supply or N_2 flushing. Subsequently, spectra were recorded for 1-2 h while the tube was flushed with N_2 without substrate supply. Finally, spectra were recorded during 1-4 h with substrate supply and flushing with N_2. For experiments at different external pH values, the concentrated cell solution was buffered with 200-400 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer at pH 6.5, 6.9, 7.4 and 7.8. After one hour of anoxic conditions without substrate supply, 200 mM ammonium nitrite solution was added for two hours.

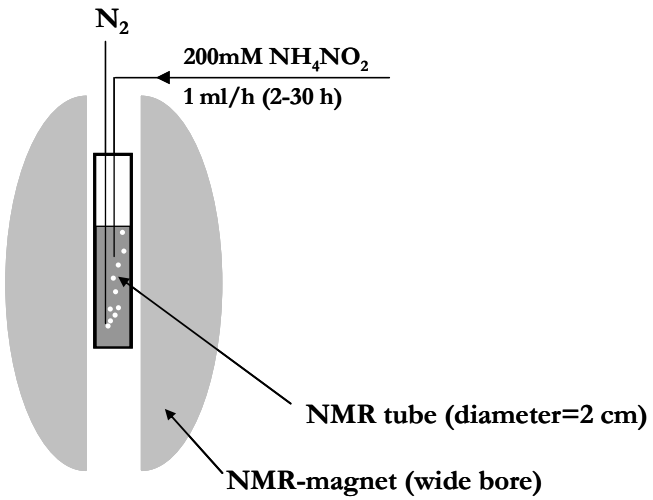

Figure 6.2 *The experimental setup for in vivo ^{31}P NMR employing a high purity, well-mixed anammox culture consisting of fully suspended "Kuenenia stuttgartiensis" cells.*

Results

Response to anoxic conditions and feed of ammonium nitrite

The cell suspension was harvested from the membrane bioreactor, concentrated to 15 ml, transferred to a 20 mm (i.d.) wide bore NMR tube and mounted into the 300 MHz NMR machine which was tuned for ^{31}P at 121.5 MHz (see Materials and Methods). "Kuenenia

stuttgartiensis" was shown to constitute 97.6% of the cells in the suspension. The ^{31}P NMR-spectra for this experiment are shown in Figure 6.3. Initially, the suspension was supposedly oxic, since no efforts were made to maintain anoxic conditions and the oxygen consumption rate of the suspension was below the detection limit (Chapter 3). The initial spectra displayed two distinct peaks, the first at 2.52 ppm (corresponding to pH 7.1, which was the medium pH) and a peak of lower intensity at 1.55 ppm (corresponding to pH 6.4, see Figure 6.3-I). After the removal of any remaining oxygen by N_2 sparging, the peak at 2.52 ppm (pH 7.1) shifted to 3.1 ppm (pH 7.8) but retained its shape (Figure 6.3-II).

Because of this peak shift, a shoulder in the recorded spectrum became apparent (2.84 ppm, pH 7.4, Figure 6.3-II/III). pH measurement at the end of the experiment confirmed that the pH of the solution had shifted to 7.8, and that the 3.1 ppm peak could thus be assigned to the extracellular environment. During N_2 sparging, the smaller peak at 1.55 ppm shifted to a slightly more acid value (1.43 ppm, pH 6.3, Figure 6.3-II/III). After the induction of anammox activity by substrate supply, the peaks remained at these values (Figure 6.3-IV/V).

To investigate the stability of the observed peaks, "Kuenenia stuttgartiensis" cell suspensions were harvested, concentrated and resuspended in HEPES buffers with different pH values. Figure 6.4 shows the in- and extracellular pH values derived from the recorded spectra and confirms that the position of the main peak corresponded to the external pH. Furthermore, the peak which had previously been present as a shoulder at pH 7.4 as well as the smaller peak at pH 6.4 always remained at the same positions. When the medium pH was 6.9 or 7.4, only two peaks were present (at the medium pH and at pH 6.3) as the compartment responsible for the peak around 7.3-7.4 was too close -or equal- to the medium pH to a give a clearly independent peak/shoulder.

To show that the "Kuenenia stuttgartiensis" cells had been active in the previous incubations, spectra were also recorded during a longer time (4 hours, 26,800 spectra) to increase the sensitivity in order to enable the detection of phosphate-containing organic metabolites. Besides the above-mentioned peaks of the inorganic phosphate species, these spectra showed peaks at -18.6, -10.8, -5.2, -0.8, 4.1 and 4.5 ppm (Figure 6.5). The peaks at -18.6 and -5.2 could be ascribed to γ- and β-ATP respectively, whereas the peak at -10.8 ppm was NAD(P)H. The peak at -0.8 ppm is consistent with phosphoenolpyruvate (PEP). The peaks above 4 ppm may indicate the presence of phosphate esters (a monoester at 4.5 ppm and a diester at 4.1). No signs of poly-phosphate were found.

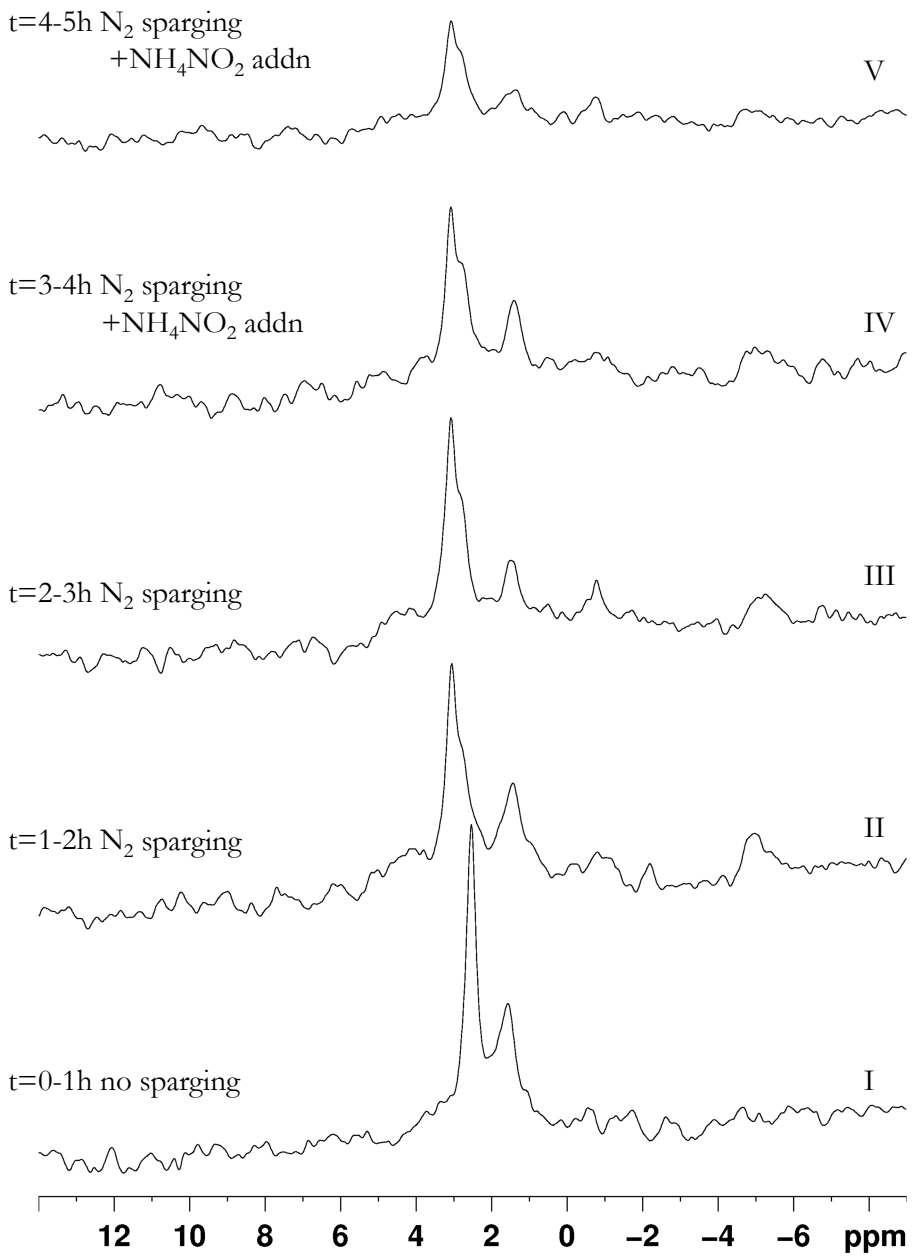

Figure 6.3 *In vivo ^{31}P spectra of resting anammox bacteria under non-gassed conditions (I) and under dinitrogen gas sparging conditions (II/III). There is a shift in the main phosphate peak from 2.52 ppm (pH 7.1 in I) to 3.1 ppm (pH 7.8 in II), while a shoulder appears at 2.84 ppm (pH 7.4 in II). A peak at 1.55 ppm (pH 6.4) is present throughout the experiment. Start of the active conversion of catabolic substrates ammonium and nitrite (IV/V) does not significantly change the pattern. Acquisition time = 1 hour (6700 spectra).*

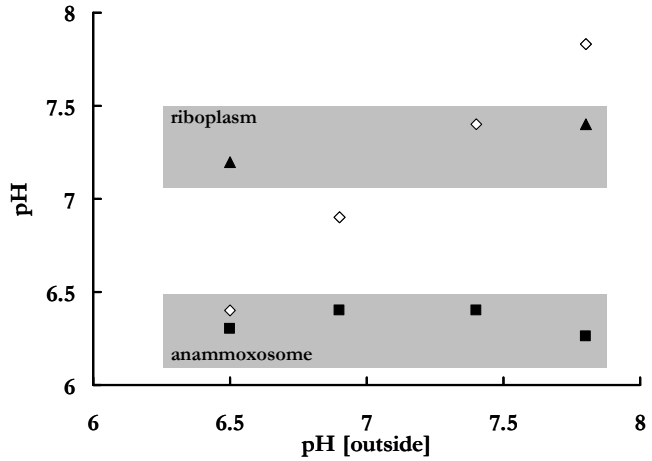

Figure 6.4 *pH values in the intracytoplasmic compartments of cells of "K. stuttgartiensis" as revealed by ^{31}P NMR. Symbols represent the assigned pH for: ◇ extracellular medium; ■ anammoxosome compartment, and ▲ riboplasm compartment. At pH 6.9 and 7.4, the pH of the riboplasmic compartment was too close to the medium pH to a give a clearly independent peak/shoulder.*

Discussion

Allocation of the peaks

The experiments clearly showed that apart from the external medium, two intracytoplasmic compartments were present with pH values of 7.4 and 6.3. These pH values were maintained over an external pH range from 6.5 to 7.8 (Figure 6.4). This observation can be explained in several ways. Firstly, it may be possible that the two pH values resulted from two different populations. However, since the cell suspension that was used contained only very few cells that were not "Kuenenia stuttgartiensis" (Chapter 3), this is unlikely. Even if other bacteria contributed to the NMR signal, a minor contribution to the pH 7.4 peak would be expected rather than the production of a second acidic peak at pH 6.3, since the cytoplasm of these -presumably uncompartmentalized- contaminants would be expected to have neutral or slightly alkaline pH relative to the external medium.

Second, it may be possible that the "Kuenenia stuttgartiensis" population was in two different physiological states, resulting in two distinct pH values of the cytoplasm. This was also unlikely, because vigorous mixing of the NMR tube by sparging of N_2 and addition of substrates did not cause the disappearance of either of the two peaks. Thus, the population must have been physiologically homogeneous.

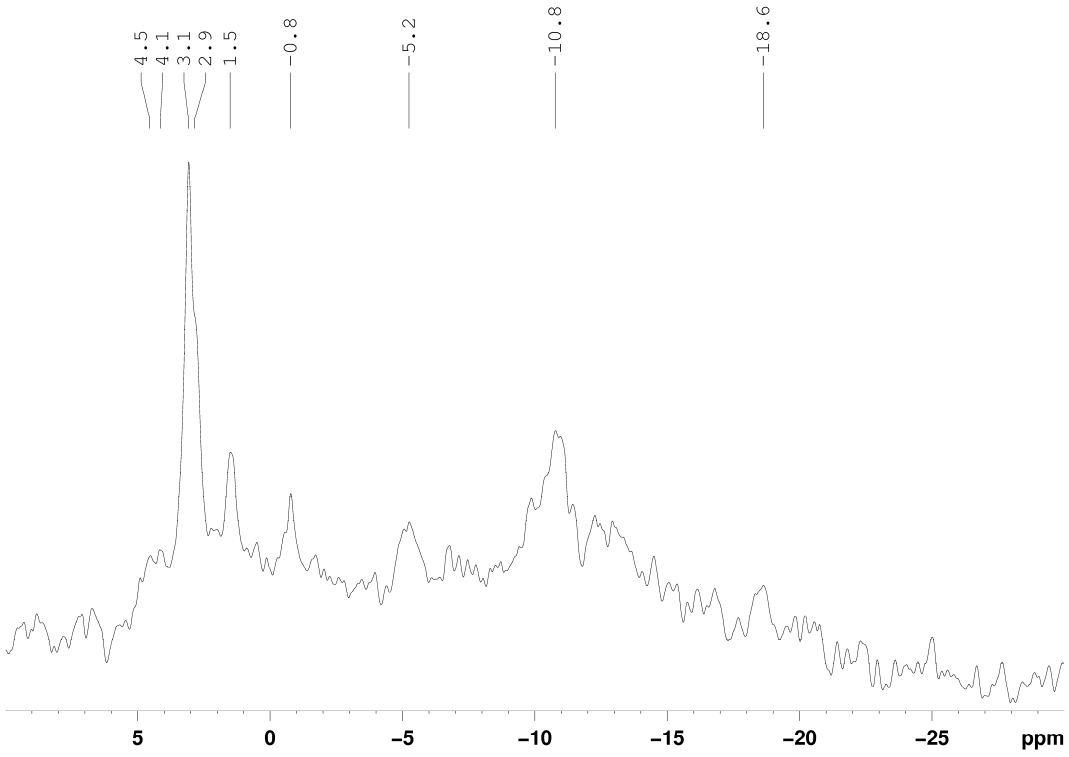

Figure 6.5 *Full in vivo ^{31}P spectra of anammox bacteria converting ammonium nitrite. Acquisition time = 4 hours (26,800 spectra), line width = 25 Hz. The three pH related peaks are: 1.5 ppm (pH 6.3, assigned to the anammoxosome), 2.9 ppm (shoulder, pH 7.4, assigned to the riboplasm) and 3.1 ppm (pH 7.8, the extracellular pH). Four non-pH peaks are visible at -18.6, -10.8, -5.2, -0.8, 4.1 and 4.5 ppm, which can probably be assigned to β-ATP (-18.6), NADH/NADPH (-10.8 ppm), γ-ATP (-5.2) phosphoenolpyruvate (PEP, -0.8 ppm) and NDP-glucose (-0.8 ppm). The peaks at 4.1 and 4.5 ppm are probably a phosphate diester and a phosphate monoester respectively.*

Third –and most plausibly- the two peaks may have resulted from the presence of two intracytoplasmic compartments with different pH values. The allocation of the peaks to different compartments (anammoxosome, riboplasm and paryphoplasm) cannot be assessed directly by ^{31}P NMR. Although the allocation of peaks to specific compartments is usually verified with pH-sensitive fluorophores, this is impossible for "K. stuttgartiensis", since these molecules cannot pass the ladderane membranes of living anammox bacteria.

If the anammoxosome indeed plays a role in energy generation (see below), then a pH gradient would be expected over the anammoxosome membrane. All presently available experimental evidence indicates that the final metabolic step of the anammox catabolism (hydrazine oxidation to dinitrogen gas, VAN DE GRAAF et al. 1997; STROUS et al. 2006) takes place inside the anammoxosome. This step feeds four electrons into the respiratory chain and simultaneously releases four protons. Thus, this step has the potential to acidify the

anammoxosome and build up a proton motive force over its membrane. The attribution of the lower pH value of the present study to the anammoxosome is therefore the only one consistent with previous experimental evidence.

The detection of two compartments at different pH is not necessarily inconsistent with an anammox cell consisting of three separate compartments. In the first place, it is possible that no pH gradient exists between two of the three compartments (for example the riboplasm and the paryphoplasm). Second, it is possible that no pH gradient exists between the paryphoplasm and the external medium. That would be expected if the anammox paryphoplasm is actually more similar to the Gram-negative periplasm (STROUS et al. 2006). Finally, the third compartment may have been too small (and/or may not contain enough phosphate) to be visible with ^{31}P NMR.

The anammoxosome as the location for energy generation

Since the proton motive force in general is constant for a given organism, also a more or less constant pH difference is expected between the two sides of the membrane over which the proton motive force is created. In bacteria, this generally results in a constant pH difference between the outside pH and the cytoplasm. In the investigated anammox bacterium, this constant difference was not found. But since two internal compartments exerted a pH which was independent of the outside pH, it seems logic that the proton motive force was generated over this membrane. The only other reported compartment in a prokaryote in which the proton motive force was shown to take place is the acidic acidocalcisome-like compartment in *Agrobacterium tumefaciens* (SEUFFERHELD et al. 2003).

The proton motive force in bacteria generally constitutes a more or less constant potential of circa 150-200 mV. If the proton motive force is generated over the anammoxosome membrane -with a pH difference of about 1 unit-, then the pH component of the proton motive force is circa 60 mV for "K. stuttgartiensis".

If this interpretation is correct, the anammoxosome is functionally similar to mitochondria in eukaryotic cells as both these compartments (organelles) would provide the cell with energy in the form of ATP. Also the pH difference between mitochondria and the cytosol in eukaryotes -e.g. 35-53 mV for rat liver (OGAWA and LEE 1984) and 60 mV in human hepatocytes (liver cells, HOEK et al. 1980; STRZELECKI et al. 1984)- is similar to the estimated pH difference found between the anammoxosome and the cytoplasm. In contrast to the anammoxosome, the pH in mitochondria is -instead of being more acidic- about one unit more alkaline. Therefore, the orientation of the membrane-bound ATP synthases must be reversed compared to mitochondria: whereas in mitochondria the ATP synthesizing domain of the ATP synthase is directed towards

the mitochondrial matrix, it can be expected to be directed towards riboplasm in anammox bacteria (VAN NIFTRIK et al. 2004).

Function of PEP and NDP-glucose

The production of PEP immediately upon energizing the system is consistent with the acetyl CoA route, which is the most probable CO_2 fixation pathway of anammox bacteria (SCHOUTEN et al. 2004; STROUS et al. 2006). The production of this metabolite upon addition of the substrates ammonium and nitrite is another indicator that the cells were actively metabolizing during the experiment. The same can probably be concluded from production of the (unidentified) phosphate mono ester.

The production and consumption of glycogen always proceeds via the coupling of glucose units coming from ADP- or UDP-glucose. Finding NDP-glucose is therefore an indication that anammox bacteria actively store glycogen. This is not surprising, as glycogen indeed has been observed in the riboplasm of all known anammox genera (VAN NIFTRIK et al. 2008). Furthermore, some kind of reduced storage material is required to restore the anammox catabolism after a period of inactivity. The indications of presence (VAN NIFTRIK et al. 2008) and production (this Chapter) of glycogen suggest that glycogen may serve this role in anammox bacteria.

Conclusion and outlook

The presence of two intracytoplasmic compartments in cells of "Kuenenia stuttgartiensis" was shown by ^{31}P NMR. The two different and stable pH values observed in these cells provide the first direct evidence of an intracytoplasmic pH gradient in an anammox bacterium. Together with the previously detected exclusive presence of cytochrome c in the anammoxosome and the curvature of the anammoxosomal membrane, this study provides strong support for the notion that anammox catabolism occurs inside the anammoxosome, and that a proton motive force is created over its membrane.

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Summary

Growth and metabolism of anammox bacteria

The anoxic ammonium oxidation (anammox) process is the conversion of nitrite and ammonium -under anoxic conditions- to form dinitrogen gas. The process is performed by a clade of bacteria, which are deep-branching Planctomycetes. The bacteria are characterized by a slow growth rate (with a doubling time of several days) and are inhibited by their own substrate nitrite, as well as by oxygen. Possibly because of their slow growth rate, no pure culture has ever been obtained, and all information regarding the process -as well as the organisms responsible for it- has to be derived from the study of enrichments (50-90% pure) or from physically purified cells.

The anammox process is a promising technique for the treatment of ammonium-rich wastewaters. After the biological oxidation of half of the ammonium to nitrite (partial nitritation), the produced ammonium nitrite is converted in the anammox process. The partial nitritation and the anammox process can be both performed in separate reactors or in one (aerated) reactor.

In this thesis, growth (Chapters 2 and 3) and metabolism (Chapters 4, 5 and 6) of the anammox bacteria are treated on full-scale (Chapters 2 and 5) as well as on laboratory scale (Chapters 3, 4 and 6).

Growth

On lab-scale, anammox bacteria are enriched most successfully in systems with efficient biomass retention, like sequencing batch reactors, granular sludge reactors and biofilm reactors. The startup of the first full-scale anammox reactor (in a configuration where partial nitritation and the anammox process take place in separate reactors) is treated in *Chapter 2*. The described 70 m^3 full-scale reactor was a granular sludge reactor which was optimized for biomass retention. The reactor was scaled up directly from lab-scale to full-scale -without the intermediate step of a pilot plant- and the step from lab-scale to full-scale took three years. In the first phase of the startup, quantification of the number of anammox bacteria, which were present in the reactor by quantitative polymerase chain reaction (Q-PCR) was a reliable indicator of growth of the anammox bacteria. This was particularly useful, since at that stage, the growth of anammox bacteria could not be observed from conversion rates yet. Also on full-scale, biomass retention and granule formation proved to be the most crucial steps during the startup. The volumetric conversion of 10 kg-N/m^3/day is high compared to lab-scale systems, but is probably only ca.

60% of the maximum attainable conversion of this type of reactor. For those types of anammox reactors for nitrogen removal which do not consist of granular sludge, the amount of biofilm area is the determining factor for the maximum conversion rate and generally much lower conversions can be reached.

To evaluate whether an actual necessity existed for anammox bacteria to grow in biofilms and granules, anammox bacteria were enriched in a membrane bioreactor in *Chapter 3*. In this type of reactor, no cells leave the reactor via the effluent and thus no active selection for granular biomass takes place. When the reactor was operated at a solid residence time (SRT) of 16 days, at reduced levels of calcium and magnesium (and in the presence of yeast extract), suspended non-settling cells appeared and granules were completely absent. Even at a reduced SRT of 12 days (doubling time 8.3 days) stable operation was possible. The purity of the biomass was estimated to be 97.6%, which was the highest level of enrichment ever achieved for anammox reactors. The population changed from "Brocadia" (present in the inoculum) to "Kuenenia stuttgartiensis", which could be an indication that "Kuenenia" is an affinity (K) strategist, whereas "Brocadia" is a growth rate (r) strategist.

Metabolism

The catabolic intermediates of the anammox process are hydrazine, in combination with nitric oxide (NO) and/or hydroxylamine. The addition of hydroxylamine and the subsequent transient production of hydrazine can therefore be regarded as a benchmark for the anammox process. In *Chapter 4*, the kinetics of this conversion were studied in detail for "Kuenenia stuttgartiensis". Hydrazine accumulated slightly after addition of hydroxylamine and remained low until near completion of the hydroxylamine. At that moment, the hydrazine level suddenly rose to ca. 100 μM, after which it gradually disappeared. The overall reaction was a disproportionation of hydroxylamine into ammonium and dinitrogen gas. The observed sudden accumulation of hydrazine could only be explained by assuming that hydrazine was an intermediate in this process. The sudden accumulation was therefore not a sudden increase in production of hydrazine, but an imbalance in the metabolism at low levels of hydroxylamine which resulted in a sudden stop in the conversion of the hydrazine. Two simple mathematical models, based on the continuous turn over of hydrazine during hydroxylamine conversion, were capable of quantitatively explaining the observed phenomena. Similar behavior of cells of "Brocadia fulgida" suggested that the behavior was general for anammox bacteria.

The production of nitric oxide, another potential intermediate in the anammox process, was studied in combination with the emission of greenhouse gas nitrous oxide (N_2O) in a

full-scale two-reactor nitritation-anammox process in *Chapter 5*. The NO and N_2O emissions in the nitritation reactor were 0.2% and 1.7% of the nitrogen load respectively and 0.003% and 0.6% for the anammox reactor. The NO emission in the nitritation reactor was higher at higher aeration flows and NO seemed to be produced mainly in the period when the nitritation reactor was aerated. The N_2O emission on the other hand seemed to be mainly produced during anoxic periods. The NO emission in the anammox reactor varied with varying influent flow, possibly as a result of varying nitrite levels. The emission level of N_2O of the anammox reactor was surprisingly high (ca 4000 ppm), but since this reactor was not aerated, the emission load was relatively low.

Anammox bacteria have a unique cell plan consisting of several membrane-surrounded compartments. In the main compartment, the anammoxosome, the anammox catabolism is hypothesized to take place. Therefore, also the proton motive force is probably generated between the anammoxosome and the riboplasm by which the anammoxosome is surrounded. ^{31}P nuclear magnetic resonance (NMR) spectroscopy was used to evaluate the pH difference over the anammoxosome membrane of "Kuenenia stuttgartiensis" *in vivo*. Two compartments with stable pH values of 6.3 and 7.4 respectively were found in actively converting cells. The pH values were independent of the external pH and were visible already upon exposing the cells to anoxic conditions. The lower pH value was assigned to the anammoxosome, whereas the pH of 7.4 was assigned to the riboplasm. The stability of the pH in both compartments is a strong indication that the anammoxosome is the locus of the catabolism and thus functionally resembles the eukaryotic mitochondrion. After *Agrobacterium tumefaciens*, this is the second bacterium for which a pH gradient within the cell has been shown.

The research described in this thesis showed that valuable information on a slow-growing organism could be obtained, although the organism was not available in pure culture. The applied mixed culture approach allowed successful scale-up and estimation of greenhouse gas emissions, as well as the obtainment of information regarding growth mode, turnover of intermediates and the location of the catabolic processes.

Wouter van der Star, February 2008

Samenvatting

Groei en metabolisme van anammoxbacteriën

Het anoxische ammoniumoxidatieproces (anammox) is de omzetting van nitriet en ammonium tot stikstofgas onder anoxische omstandigheden. Het proces wordt uitgevoerd door een groep bacteriën, die een vroege afsplitsing vormen binnen de Planctomyceten. Anammox bacteriën worden gekarakteriseerd door een lage groeisnelheid (met een verdubbelingstijd van enkele dagen), inibitie door zuurstof en hun eigen substraat: nitriet. Mogelijkerwijze vanwege hun lage groeisnelheid zijn ze nog nooit in reinculture verkregen. Daarom komt alle informatie over het anammoxproces -en over de organismen die ervoor verantwoordelijk zijn- uit ophopingsculturen of van fysiek gezuiverde cellen.

Het anammoxproces is de basis voor veelbelovende technologieën voor de behandeling van ammonium-rijk afvalwater. Na biologische oxidatie van de helft van het aanwezige ammonium tot nitriet (partiële nitritatie), wordt het geproduceerde ammoniumnitriet omgezet in het anammox proces tot (voornamelijk) stikstofgas. Het partiële nitritatieproces en het anammoxproces kunnen worden uitgevoerd in afzonderlijke reactoren of in één (beluchte) reactor.

In dit proefschrift worden groei (hoofdstuk 2 en 3) en metabolisme (hoofdstuk 4, 5 en 6) van anammoxbacteriën beschreven op praktijkschaal (hoofdstuk 2 en 5) en op laboratorium-schaal (hoofdstuk 3, 4 en 6).

Groei

Op laboratoriumschaal kunnen anammoxbacteriën het meest succesvol worden opgehoopt in systemen met efficiënte biomassaretentie, zoals in "sequencing batch reactoren", korrelslib- en biofilmreactoren. De opstart van de eerste anammoxreactor op praktijkschaal wordt behandeld in *hoofdstuk 2*. De 70 m^3 praktijkschaal korrelslibreactor werd direct opgeschaald van laboratoriumschaal (8 liter) zonder de tussenstap van een pilot-reactor. De stap van laboratoriumschaal naar praktijkschaal duurde drie jaar. In de eerste fase van de opstart, was kwantitatieve polymerase-kettingreactie (Q-PCR) een betrouwbare indicator voor groei van de anammoxbacteriën. Deze techniek was vooral nuttig omdat in deze fase de groei van de anammoxbacteriën nog niet kon worden bepaald aan de hand van omzettingssnelheden. Biomassaretentie en korrelvorming bleken van cruciaal belang tijdens de opstart. De

volumetrische omzettingssnelheid van 10 kg-N/m^3/dag was hoog in vergelijking met reactoren op laboratoriumschaal. Deze conversie is echter naar schatting slechts 60% van de maximale conversie voor dit reactortype. Voor andere typen reactoren -die geen korrelslib bevatten- is de hoeveelheid biofilmoppervlak de bepalende factor en kunnen in het algemeen veel lagere maximale omzettingssnelheden worden bereikt.

Om te onderzoeken of anammoxbacteriën zouden kunnen worden gecultiveerd als vrije cellen in suspensie (en niet noodzakelijkerwijze in biofilms, zoals algemeen werd aangenomen), werden anammoxbacteriën opgehoopt in een membraanbioreactor (MBR). In *hoofdstuk 3* wordt de MBR-opstelling en manier van bedrijven beschreven. Omdat cellen de reactor niet kunnen verlaten via de effluent, vindt er geen actieve selectie plaats voor korrelslib of geaggregeerd slib. Wanneer de reactor werd bedreven met een slibleeftijd (SRT) van 16 dagen, met een verlaagd calcium en magnesium niveau, groeiden de bacteriën als volledig gesuspendeerde cellen, die niet uitvlokten wanneer gestopt werd met roeren. Zelfs bij een verkorte slibleeftijd van 12 dagen (verdubbelingstijd 8,3 dagen) was het mogelijk de reactor stabiel te bedrijven. De zuiverheid van de biomassa werd geschat op 97,6%, wat de hoogste mate van ophoping in anammoxreactoren tot nu toe was. Na enkele maanden van continu-bedrijf veranderde de populatie van "Brocadia" (aanwezig in het inoculum) naar "Kuenenia stuttgartiensis". Dit zou een indicatie kunnen zijn, dat "Kuenenia" een affiniteits-(K)-strateeg is, terwijl "Brocadia" een groeisnelheids-(r)-strateeg is.

Metabolisme

Behalve hydrazine zijn stikstofmonoxide (NO) en/of hydroxylamine (NH_2OH) katabole intermediairen van het anammoxproces. De tijdelijke ophoping van hydrazine na toevoeging van hydroxylamine kan daarom worden beschouwd als een anammox-karakteristiek. In *hoofdstuk 4* werd deze omzetting bestudeerd voor "Kuenenia stuttgartiensis". Een aantal experimenten bij verschillende initiële hydroxylamineconcentraties toonde aan, dat de toevoeging van hydroxylamine leidde tot een lichte ophoping van hydrazine, die aanwezig bleef tot de hydroxylamine bijna helemaal was omgezet. Op dat moment steeg de hydrazineconcentratie plotseling tot ongeveer 100 µM, waarna het weer geleidelijk verdween. De totaalreactie was de disproportionering van hydrolamine tot ammonium en stikstofgas. De plotselinge ophoping van hydrazine die werd waargenomen kon slechts worden verklaard door aan te nemen dat hydrazine een intermediair was in dit proces. De accumulatie was daarom niet een snelle toename in de productie van hydrazine, maar een onbalans in het metabolisme bij lage hydroxylamine-concentraties, die resulteerde in het plotselinge stoppen van de productie van hydrazine. Twee eenvoudige kinetische modellen, gebaseerd op de voortdurende productie en consumptie van

hydrazine tijdens de omzetting van hydroxylamine waren in staat om de waarnemingen kwantitatief te beschrijven. Vergelijkbaar gedrag van "Brocadia fulgida"-cellen gaf aan dat dit gedrag een algemene karakteristiek van anammox bacteriën zou kunnen zijn.

De productie van stikstofmonoxide, een potentieel intermediair in het anammoxproces, werd bestudeerd in combinatie met de uitstoot van lachgas (N_2O) -beiden zijn (in)directe broeikasgassen- in het twee-reactor nitritatie-anammoxproces op praktijkschaal in *hoofdstuk 5*. De NO- en N_2O-emissies in de nitratiereactor waren respectievelijk 0,2% en 1,7% van de stikstofbelasting en 0,003% en 0,6% in de anammox reactor. De NO-emissie in de nitritatiereactor was hoger bij hogere beluchtingsdebieten en NO leek voornamelijk geproduceerd te worden wanneer de reactor werd belucht. N_2O leek daarentegen vooral geproduceerd te worden tijdens de niet-beluchte periodes. De NO-emissie uit de anammoxreactor varieerde met een variërend influentdebiet, mogelijk als gevolg van variërende nitrietconcentraties. De N_2O-concentratie in het afgas was verbazingwekkend hoog (ongeveer 4000 ppm), maar omdat deze reactor niet werd belucht was de daadwerkelijke emissie (in kg-N/m^3/dag) relatief laag.

Anammoxbacteriën hebben een unieke cel die bestaat uit verschillende door membranen omgeven compartimenten. Volgens de meest gangbare hypothese vindt het anammox katabolisme plaats in het binnenste (en grootste) compartiment: het anammoxozoom. Het is daarom mogelijk dat een protonengradient bestaat tussen anammoxozoom en het riboplasma dat eromheen ligt. Dit impliceert het bestaan van verschillende pH waarden in de anammox compartimenten. ^{31}P Kernspinresonantiespectroscopie (NMR) is gebruikt om *in vivo* het pH verschil te meten dat aanwezig is over het anammoxozoommembraan van "Kuenenia stuttgartiensis". Twee compartimenten met stabiele pH waarden van respectievelijk 6,3 en 7,4 werden gevonden in actieve cellen. De pH waarden waren onafhankelijk van de externe pH en waren al detecteerbaar -in afwezigheid van substraat- bij het blootstellen van de cellen aan anoxische omstandigheden. De laagste pH waarde werd toegewezen aan het anammoxozoom, terwijl de pH van 7,4 werd toegewezen aan het riboplasma. De stabiliteit van de pH in beide compartimenten is een sterke indicatie dat het anammoxozoom de locus is van het katabolisme en daarom functioneel gezien lijkt op het mitochondrion in Eukaryote cellen. Na *Agrobacterium tumefaciens* is dit de tweede bacterie waarin een pH gradient binnen de cel is aangetoond.

Het onderzoek dat beschreven staat in dit proefschrift laat zien dat waardevolle informatie over een langzaam-groeiend organisme kan worden verkregen, hoewel het organisme niet in reinculture beschikbaar is. De gebruikte mengculture-benadering maakte een succesvolle opschaling mogelijk, maar ook een schatting van broeikasgasemissies en gaf informatie over de

manier van groeien, omzetting danwel productie van intermediairen en de locatie van de katabole processen.

Wouter van der Star, februari 2008

Nawoord: Roeren in tomatensoep

Dit proefschrift is het tastbare resultaat van een promotieonderzoek op het gebied van anammoxbacteriën. De interessante karakteristieken van deze beestjes hebben vier jaar lang mijn werk en mijn humeur bepaald. De mooie toepassingsmogelijkheden in combinatie met de beperkte kennis die we over hen hebben heeft me altijd aangetrokken (en dat is ook niet van de ene op de andere dag opgehouden). Eén aspect maakte dat onderzoek bijzonder weerbarstig: de enorm trage groei zorgde ervoor, dat het verlies van mijn cultuur me zó een halfjaar achterstand opleverde. Voor iemand die al moeite heeft met het in leven houden van zijn eigen planten thuis, is dat op zijn minst een uitdaging te noemen. Het zorgde er wel voor dat mijn gedachten dus ook vier jaar lang gericht waren op de nukken van een rode brei met anammoxbacteriën, die nog het meest weghad van licht gebonden tomatensoep.

Vrienden (en andere slachtoffers) hebben dat aan den lijve ondervonden. Bij de minste aanmoediging (en vaak ook zonder dat) heb ik er vaak en graag over verteld. En collega's hebben me ontelbare malen uit de brand geholpen door "nog even een monstertje te nemen en te bellen als het nitriet te hoog was". Hoewel misschien niet altijd uitgesproken: jullie aandacht en hulp werd zeer gewaardeerd!

Mark van Loosdrecht was als promotor en aanvankelijk als directe begeleider mijn eerste aanspreekpunt. Mark, de discussies die we hebben gehad over het onderzoek en de implicaties ervan waren altijd interessant en diepgaand. Je helikopter-view maakte het voor mij vaak mogelijk dingen te zien waar ik nog niet aan gedacht had, en je enorm snelle begrip maakte discussies altijd scherp en betekenisvol. In het algemeen kwam ik terug met meer ideeën dan er tijd beschikbaar was, maar dat mocht de pret niet drukken. Het was een voorrecht bij je te mogen promoveren. De broodnodige focus in het onderzoek kwam van Cristian Picioreanu. Cristi, jij vormde een belangrijke stimulans om doelen te specificeren, om dingen echt af te maken en tot een succesvol einde te brengen. Zonder die inbreng weet ik niet waar ik nu had gestaan. Jammer was dat slechts één hoofdstuk zich leende voor meer modelleerwerk, want de discussies daarover blijven me toch met meeste bij. Mike Jetten volgde als projectleider het onderzoek van een afstandje en is mijn tweede promotor. Mike, de vrijheid die je het Delftse onderzoek gaf en je altijd snelle en gedetailleerde commentaar op manuscripten was bijzonder prettig in onze samenwerking.

Binnen het Kluyverlab heb ik al die tijd op dezelfde kamer gezeten. De goede sfeer en fijne (wetenschappelijke en niet-wetenschappelijke) gesprekken zijn er eigenlijk altijd aanwezig geweest. Dat kwam voor een niet onbelangrijk gedeelte, omdat Merle er ook al die jaren heeft gezeten en altijd aandacht had voor ieders wel en wee. Bovendien kon ik bij haar terecht voor alle

vragen over afvalwaterzuivering "in het echt" en korrelgroeimechanismen. With the other PhD students and post-docs Sirous, António and Joao research seemed to be a 24/7 activity, but always in a very relaxed atmosphere. Met de komst van Marlies kwam er iemand naast me zitten die ook aan stikstof werkte en mijn passie voor metabolisme en dynamiek deelde. Dat leidde tot prettige discussies en -last but not least- tot een succesvolle "veldtest" die is beschreven in hoofdstuk 5. Toen in de laatste 2 jaar ook Weren en Raymon als promovendi begonnen, leek het bijna wel een echte "stikstofkamer" (met heuse "N-Cycle meetings") te worden! Met Leon -die me steeds heeft geleerd dat wat logisch is vanuit het referentiekader van een chemisch technoloog, niet de enige (maar wat mij betreft wel de meest handige) waarheid hoeft te zijn- ben ik nog niet helemaal uitgewerkt: door mijn huidige werk aan biogrout, werken we meer samen dan ooit!

Udo introduceerde me met een geweldig Fingerspitzengefühl in de kunst van de anammoxophoping. Door zijn bijdrage in het laatste jaar aan het draaien van de MBR is de cirkel nu helemaal rond. Rondom het onderzoek was verder de hulp van Rob (gesterkt door zijn AIO hamer), Dirk, Max, Stef, Johan, Sjaak en alle mannen van de werkplaats onontbeerlijk.

Als alles waar ik aan begonnen was gelukt was, dan was dit boekje nog lang niet uit geweest. De hulp van Simon de Vries en Marc Strampraad (NO metingen), Chrétien Simons en Ulf Hanefeld (lage concentatie hydrazine metingen) en tenslotte Arthur Kroon en Bert Wielders (anammox lab-on-a-chip) tijdens de projecten die niet tot een hoofdstuk leidden wordt echter zeer gewaardeerd.

Ook de andere leden van de vakgroep, Margarida (veel succes volgende week!), Katja, Guus, Jasper, Shabir, Raji, Gijs (altijd inspirerend voor anammox-onderzoekers!), Robbert, Gerard (je was bovendien uitgebreid betrokken bij het MBR-verhaal), Yang, Cao, Dimitri, Mario, Rolf, Ann-Charlotte, Mirjam, Kees, Sander, Claudia, Geert, Krisztina, Esengül wil ik bedanken voor de prettige sfeer. Voor een biertje met aansluitend eten op de vrijdagmiddag waren (vooral in het begin) Loesje, Peter, Emile en later Nicolette altijd wel te porren. Het is maar goed dat de op dat soort avonden gedane uitspraken niet altijd in de openbaarheid zijn doorgedrongen ;-).

Many students helped with the research. I want to thank Bruno, Alessandro, Estefanía, Maarten, Bert and Caspar for their contribution and critical questions!

Het onderzoek was een door STW gefinancierd samenwerkingsproject met de groep van Mike Jetten in Nijmegen. We were indeed having a successful project in the end Boran! Marc wil ik bedanken voor zijn uitgebreide bijdrage aan het de beschrijving van het P-NMR werk en voor de altijd bemoedigende woorden. Bij de praktijkschaal-anammoxreactor "Op Sluisjesdijk" kon ik altijd een emmertje slib halen (en niet alleen van de anammoxreactor) en kon de veldproef zonder

problemen uitgevoerd worden: Jan-Willem, Dennis, Floor, en de anderen: bedankt! Ook met Paques was door Wiebe's inbreng de samenwerking altijd goed en constructief. Mijn vrijdagse tochten naar Wageningen waren door de rustige benadering van Cor Dijkema en Pieter de Waard -ook toen een dikke anammoxsmurrie jullie dure probe overspoelde- altijd bijzonder prettig (en het resultaat mag er wezen!).

Oud-huisgenoten (Froukje, Ronald, Rogier, Robert, Rick, Jorien, Edwin, Bram, Rutger, Philip, Ilse en natuurlijk Jeroen), oud-studiegenoten (Aimée, Jacqueline, Mark, Annemieke, Ricardo, Esther en Jeanet), mijn ouders, Joke en Geert waren die in het begin genoemde al dan niet gewillige slachtoffers voor het aanhoren van mijn hoogte- en dieptepunten. Dank!

Door vergelijkbare promotie-pieken en -dalen ging ik al die jaren met Emile. Onze avondlijke discussies bij een -altijd welverdiend- glas wijn (of wisky) en onze hikes zijn onvergetelijk. Hopelijk houden we het nog even vol zo!

Misschien wel het belangrijkste moment gedurende mijn promotie was de Canada-reis in 2003, want hoewel we in het zelfde lab rond bleken te lopen, hadden we elkaar nog nooit leren kennen: Lieve Beatrix, jij hebt ook alle baaldagen van dichtbij meegemaakt en dat zal niet altijd een pretje zijn geweest... Tijdens alle drukte en tijd die ik 's avonds meestal met deze "laatste loodjes" bezig was dwong je me om me ook op andere dingen te richten: gelukkig maar!

List of Publications

Thesis chapters

Van der Star WRL, Abma WR, Blommers D, Mulder JW, Tokutomi T, Strous M, Picioreanu C and Van Loosdrecht MCM. **2007**. *Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam*. **Chapter 2**. Water Res **41**(18) 4149-4163.

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Kampschreur MJ, Van der Star WRL, Wielders HA, Mulder JW, Jetten MSM and Van Loosdrecht MCM. **2008**. *Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment*. **Chapter 5.** Water Res **42**(3) 812-826.

Other papers

Abma W, Mulder JW, van der Star WRL and Strous M. **2006**. *Anammox® in Rotterdam overtreft verwachtingen*. H_2O **39**(10) 47-49.

Abma W, Schultz CE, Mulder JW, van Loosdrecht MCM, van der Star WRL, Strous M and Tokutomi T. **2007**. *The advance of Anammox*. Water21 **36** (February) 36-37.

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Curriculum vitae

Wouter van der Star was born in Deurne in 1978. After attending the Sint-Willibrord Gymnasium, he obtained his VWO-diploma in 1996. At Delft University of Technology, he studied chemical and biochemical engineering and finished his studies with a Master's thesis on the mathematical modeling of biocorrosion. In 2003, he joined the environmental biotechnology group of this university for a study on growth and metabolism of anammox bacteria, which has resulted in this thesis. Since 2007, Wouter is employed as a researcher at the Dutch Delta-institute Deltares (Delft NL), where he works on the microbially-enhanced conversion of sand to sandstone.

Growth and Metabolism of Anammox Bacteria

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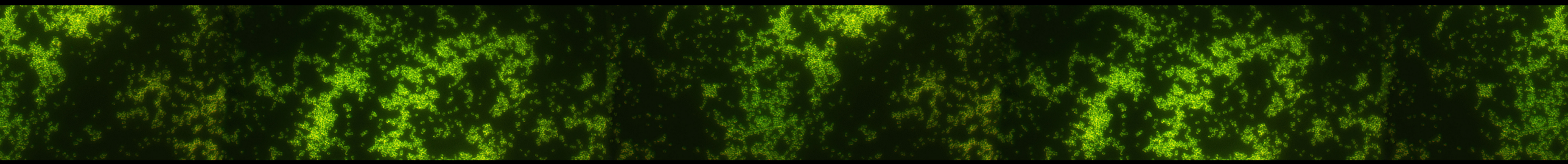

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