Ammonium oxidation at the oxic/anoxic interface

Olav Sliekers

Stellingen behorende bij het proefschrift

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1. CANON is gezien de robuustheid en de omzettingsnelheden een uitstekend proces om ammoniumrijk afvalwater te behandelen.

   Dit proefschrift

2. Het zal blijken dat het Anammox proces op zeer veel plaatsen op de wereld, zowel in de bodem als ook in mariene en zoetwatermilieus een belangrijke rol in de stikstofcyclus speelt.

   Dit proefschrift

3. Het 16 S rRNA is een onbetrouwbare moleculaire klok

   McGenity, T.J., Gemmell R.T., Grant, W.D. and Stan-Lotter H. (2000). Origins of halophilic microorganisms in ancient salt deposits. Env. Microbiol. 2(3), 243-250

4. Detectie van 16 S rRNA moleculen met DNA microarrays kan nu al gebruikt worden voor relatief eenvoudige ecosystemen zoals aanwezig in sommige voedselproducten geproduceerd met behulp van micro-organismen.

   Peplies, J., Glöckner, F.O. and Amann, R.(2003) Optimization Strategies for DNA Microarray-Based Detection of Bacteria with 16 S rRNA-Targeting Oligonucleotide Probes. Appl. Environ. Microbiol. 69:1397-1407.

5. Het is nog maar de vraag of signaalmoleculen van bacteriën in de natuur een grote rol spelen.

   Uroz, S., D'Angelo-Picard, C., Carlier, A., Elrasi, M., Sicot, C., Petit, A., Oger, P., Faure, D. and Dessaux, Y. (2003). Novel bacteria degrading N-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. Microbiology UK 149:1981-1989

6. Het onderscheid tussen anaeroob en aeroob is onvoldoende scherp.

   Boga H.I. and Brune A. (2003). Hydrogen-dependent oxygen reduction by homoacetogenic bacteria isolated from termite guts. Appl. Environ. Microbiol. 69: 779-786

7. Directed evolution met behulp van lange termijn selectie is een onderschatte methode voor de verandering van eigenschappen van micro-organismen.

Arensdorf, J.J. Loomis, A.K., DiGrazia, P.M., Monticello, D.J. and Pienkos, P.T. (2002) Chemostat approach for the directed evolution of biodesulfurization gain-of-function mutants. Appl.Environ.Microbiol. 68:691-698

8. De verlaging van de K_La, de zuurstof overdrachtscoëfficient, door *Pseudomonas aeruginosa in vitro*, speelt *in vivo* geen rol.

Sabra, W., Kim, E-J and Zeng, A-P (2002). Physiological responses of Pseudomonas aeruginosa PAO1 to oxidative stress in controlled microaerobic and aerobic cultures. Microbiology 148:3195-3202.

9. Gezien het feit dat grote producenten van software XML niet gaan gebruiken om hun producten gemakkelijk te laten aansluiten op software van de concurrenten, zal de mogelijkheid voor de klant om een gezonde keuze te maken op basis van kwaliteit en prijs, nog lang op zich laten wachten.

Kendall Grant Clark, April 2003, XML column: "At Microsoft's mercy.", www.xml.com

10. De afschaffing van de vrijstelling voor ecotax op groene stroom, met de reden dat het geld anders toch maar naar het buitenland vloeit, is een bewijs dat het innovatiebeleid in het binnenland op het gebied van milieuvriendelijke energie niet doeltreffend genoeg is geweest.

11. Ter vermindering van de bureaucratie zou het kabinet niet zo zeer alle regels goed tegen het licht moeten houden, maar vooral de procedures en de efficiëntie van de administratie.

12. Anaerobe methaan oxidatie is een hot topic.

Schouten, S., Wakeham, S.G., Hopmans E.C. and Sinninghe Damsté, J.S. (2003) Biogeochemical evidence that thermophilic archaea mediate the anaerobic oxidation of methane. Appl. Environ. Microbiol. 69: 1680-1686

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Proefschrift

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft
op gezag van de Rector Magnificus prof. dr. ir. J.T. Fokkema
voorzitter van het College van Promoties
in het openbaar te verdedigen op dinsdag 16 december 2003 om 13.00 uur

door Arne Olav SLIEKERS
doctorandus in de biologie
geboren te Hoogeveen

Dit proefschrift is goedgekeurd door de promotoren:

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

Prof. dr. J.G. Kuenen

Samenstelling promotiecommissie

Rector Magnificus	Voorzitter
Prof. dr. ir. M.S.M. Jetten	Technische Universiteit Delft en Katholieke Universiteit Nijmegen, promotor
Prof. dr. J.G. Kuenen	Technische Universiteit Delft, promotor
Prof. dr. J.T. Pronk	Technische Universiteit Delft
Prof. dr. N.P. Revsbech	Aarhus Universitet, Denemarken
Prof. dr. H.J. Laanbroek	Katholieke Universiteit Nijmegen
Prof. dr. ir. A.J.M. Stams	Wageningen Universiteit
Dr. R.J.M. van Spanning	Vrije Universiteit Amsterdam

Dr. ir. M. Strous heeft als begeleider in belangrijke mate aan de totstandkoming van het proefschrift bijgedragen

Het in dit proefschrift beschreven onderzoek werd uitgevoerd in het Kluyverlaboratorium voor Biotechnologie van de Technische Universiteit Delft en bij de vakgroep microbiologie van de Katholieke Universiteit Nijmegen, en werd mede mogelijk gemaakt door een financiele bijdrage van de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO) uit de stichting Aard en Levens Wetenschappen (ALW), Nummer: 80533422/P, Programmasubsidie.

Published and distributed by: DUP science
DUP science is an imprint of
Delft University Press
PO Box 98
2600 MG Delft
The Netherlands
Telephone: +31 15 27 85 678
Telefax: +31 15 27 85 706
E-mail: info@library.tudelft.nl

ISBN 90-407-2459-8

Printed in The Netherlands

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Chapter 1

General Introduction

The nitrogen cycle

In the cycling of elements on earth, the nitrogen cycle has an important place. In the nitrogen cycle, nitrogen compounds in different oxidation states (-3 to +5) are transferred between organisms and their environment. In Figure 1.1, the various microbial nitrogen transformations are depicted.

All living organisms require nitrogen for biomass production. Nitrogen is an important atom in proteins and in RNA, which make up the cellular machinery and regulate all life functions, as well as in DNA, the blueprint. In general, living organisms contain between 10-15% of their biomass as nitrogen. For example, algae have a relative stable content of nitrogen in their biomass, as expressed in the Redfield Ratio, 106 C : 16 N : 1 P (Redfield et al., 1963).

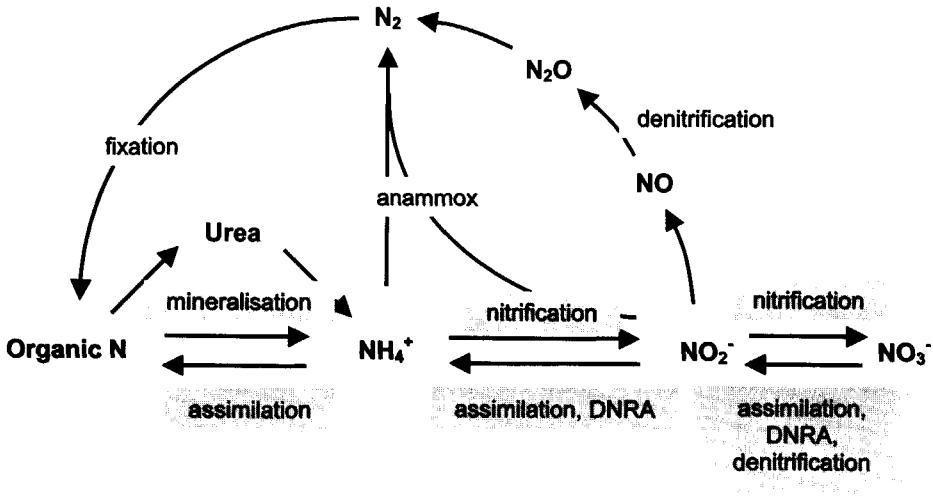

Figure 1.1: Microbial transformations in the N-cycle

Plants, fungi and bacteria, can take up inorganic nitrogen from their surroundings in the form of ammonia or nitrate. When nitrate serves as a N-source it is converted to nitrite, which is subsequently reduced to ammonia in a process called assimilatory nitrate reduction. Organisms feeding on organic material can take up nitrogen in the form of amino acids already present in the diet. Depending on the C/N ratio of the food, the C/N of the organism and the amount of carbon used for energy generation, net mineralization of nitrogen will take place and the nitrogen diffuses into the environment or is actively excreted in the form of urea or uric acid. Urea can be hydrolysed to ammonia by a variety of microorganisms.

$$NH_2CONH_2 + H_2O \rightarrow 2\ NH_3 + CO_2$$

If nitrogen is limiting in an environment, dinitrogen gas can be fixed to ammonia by autotrophic organisms like cyanobacteria, but also by heterotrophic bacteria like Azotobacter (Bock and Wagner, 2001)(Oelze, 2000). In turn, these organisms can be a food source for other organisms, resulting in mineralization of the fixed nitrogen. Mineralized nitrogen like urea or ammonia can be oxidised by heterotrophic and autotrophic nitrifiers, which oxidize ammonia with oxygen to nitrite (Bock and Wagner, 2001).

$NH_3 + 1.5\ O_2 \rightarrow NO_2^- + H_2O + H^+$ ($\Delta G^{o'} = -273$ kJ/mol)

Aerobic nitrite oxidizers can oxidize nitrite with oxygen, yielding nitrate (Bock and Wagner, 2001). Nitrite oxidation is regarded as the second step in nitrification.

$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$ ($\Delta G^{o'} = -74$ kJ/mol)

Nitrite as well as nitrate can be subsequently converted by various processes. Under anaerobic or oxygen limited circumstances, the produced nitrite from ammonia oxidation can be converted with ammonia to dinitrogen gas by a process called anammox, an acronym for anaerobic ammonia oxidation (Strous, 1999a).

$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$ ($\Delta G^{o'} = -358$ kJ/mol)

Depending on the availability of electron donor, nitrate as well as nitrite, produced by nitrification can be used as electron acceptor in the process called denitrification. During denitrification, several more or less oxidized nitrogen compounds can be reduced to dinitrogen gas (Zumft, 1997).

$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

As already mentioned, nitrate can also be incorporated into biomass during assimilatory nitrate reduction via nitrite, but can also be reduced to ammonia via nitrite in a process called DNRA (dissimilatory nitrate reduction to ammonia).

Nitrification, denitrification, anammox

Knowledge of the microbial processes in the nitrogen cycle is needed to understand the impact of human nitrogen input in natural ecosystems. Application of this knowledge about microbial processes will result in more efficient ways to treat nitrogen rich wastewaters. Below we discuss the most relevant processes of the microbial nitrogen cycle for this purpose.

Nitrification

Nitrification is the oxidation of ammonia to nitrate. This is a two-step process. Aerobic ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrite, and aerobic nitrite oxidizing bacteria (NOB) oxidize nitrite to nitrate. The bacteria involved are autotrophic, although some species are known to have a mixotrophic metabolism (Bock and Wagner, 2001).

Autotrophic AOB belong mainly to the β-subclass of the proteobacteria (Figure 1.2), divided in the genera *Nitrosomonas* and *Nitrosospira*. Some species belong to the genus *Nitrosococcus*, which is assigned to the γ-subclass. Nitrite oxidisers can be found in the δ, γ and α subclasses of the proteobacteria, as well as in a distinct *Phylum*, known as *Nitrospira* (Bock and Wagner, 2001). In their habitat, ammonia and nitrite oxidisers are frequently found in clusters (Bock and Wagner, 2001). Attached nitrifiers are assumed to be more active and better survivors than free-living cells (Stehr et al., 1995a)(Stehr et al., 1995b).

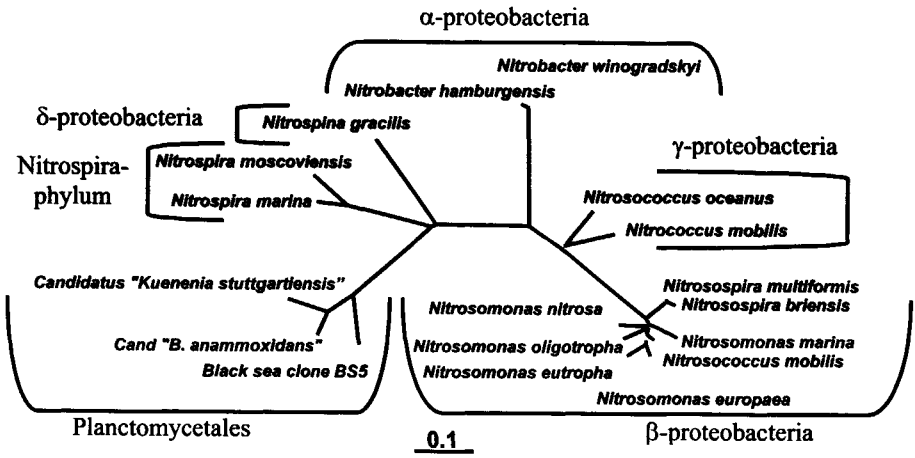

Figure 1.2: Phylogenetic tree showing the relation ship between nitrifying bacteria and anaerobic ammonia oxidisers

The ammonia is oxidized to nitrite in a two-step reaction, involving two different enzymes. Ammonium monooxygenase (AMO) catalyses the conversion of ammonia to hydroxylamine, which is subsequently converted to nitrite by the enzyme hydroxylamine oxidoreductase (HAO) (Bock and Wagner, 2001). Nitrite can be converted into nitrate by nitrite oxidizers without intermediates, catalysed by the enzyme nitrite oxidoreductase (NO_2OR) (Bock and Wagner, 2001). The genes of

AMO, HAO and NO_2OR have been identified in some representatives of the nitrifying bacteria. In addition, NO_2OR and HAO have been purified and characterized (Bock and Wagner, 2001)(Hooper et al. 1997), whereas purification of AMO from autotrophic AOB was unsuccessful so far.

Although some heterotrophic bacteria also convert ammonia to nitrite, the rate per amount of biomass at which these bacteria convert ammonia, is low (Kuenen and Robertson, 1994). The reaction in these bacteria is not coupled to growth and it is proposed to be, amongst others, a sink for reducing equivalents (Richardson et al, 1998).

During the last decades, it became clear that AOB have a more versatile metabolism as generally assumed. It was already known for some time that AOB produce nitric and nitrous oxide, especially at reduced oxygen levels (Goreau et al., 1980)(Jørgensen et al. 1984). Several ammonia oxidizers can use hydroxylamine, organic substances and hydrogen as electron donor (Bock et al., 1995). AOB can oxidize ammonia under anoxic conditions, albeit at a very low rate, using nitrite as an alternative electron acceptor (Jetten et al., 1998). This might contribute to the ability of these bacteria to survive under prolonged anaerobic conditions (Abelovich and Vonshak, 1992). Homologues of denitrification enzymes have been found in AOB (Beaumont et al., 2002). Moreover, AOB have the ability to use NO_2 gas instead of oxygen, and the internal NO_2 cycle, proposed to be responsible for this, might even be part of the main metabolic route for ammonia oxidation (Schmidt et al., 2002). The availability of the genome of *Nitrosomonas*, by the DOE Joint Genome Institute (Chain et al., 2003) and the possibility of genetic engineering of *Nitrosomonas*, will give more insight into the versatile metabolism and its significance for functioning of the cells. It's already clear that NO and N_2O production, generally referred to as 'nitrifiers denitrification' (Wrage et al, 2001) does not depend on the homologues of denitrification-enzymes (Beaumont et al., 2002), making the term 'nitrifiers denitrification' obsolete. The exact source of N_2O and NO production on the enzyme level is still unknown. The enzyme HAO and the NO_2-cycle might be responsible for the N_2O and NO emission by nitrification as well (Hooper, 1968)(Hooper et al., 1977). It might be that the mechanism of N_2O and NO production is different for anaerobic, microaerobic and aerobic metabolism.

Nitrite oxidisers of the genus *Nitrobacter* have a more versatile metabolism as well. Several *Nitrobacter* species are able to grow on pyruvate and acetate. The growth rate is slower than lithotrophic growth with nitrite (Bock and Wagner, 2001). Although isolated bacteria of the genus *Nitrospira* are strictly lithotrophic (Koops and Pommerening-Röser, 2001), it has been shown that a *Nitrospira* related nitrite oxidiser could fix CO_2 and simultaneously take up pyruvate (Daims et al, 1999). The capability to take up low quantities of organic compounds is known for all other obligate chemolithotrophs, studied so far. Mixotrophic properties of NOB can play an important role in competition for oxygen with AOB under oxygen limited conditions. (Laanbroek et al., 1994)

Denitrification

During denitrification, nitrogen oxides serve as electron acceptor. In this respiratory process, nitrate is sequntially reduced via nitrite, nitric oxide and nitrous oxide to dinitrogen gas. The denitrifying bacteria cover a large diversity of genera of bacteria and archaea, with a spectrum of heterotrophic and autotrophic metabolism, and can be present in a variety of environments, including high temperature and high alkalinity. Both organic and inorganic compounds, like sulfur, hydrogen and iron (II), can serve as an electron donor (Zumft, 1997). Bacteria capable of denitrification are almost all facultative anaerobes. It seems that only a very few obligate anaerobic bacteria are able to denitrify (Zumft, 1997).

Two enzymes can catalyse nitrate reduction to nitrite. One is membrane bound (NarG) and closely related to the NO_2-OR of *Nitrobacter* (Zumft, 1997)(Ferguson, 1994). The other is located in the periplasm and referred to as NapA. Also, nitrite reduction to nitric oxide (NO) can be catalysed by two different enzymes, a cytochrome cd_1 (NirS) and a copper containing one (Cu-Nir) (Zumft, 1997). A Cu-Nir homologue is also found in *Nitrosomonas europaea* (Beaumont et al., 2002). The nitric (Nor) and nitrous oxide (Nos) reductases catalyse further reduction of nitric oxide via nitrous oxide to dinitrogen gas.

Accumulation of intermediates of denitrification is possible since not all denitrifiers posses all the enzymes needed for complete reduction of nitrate to dinitrogen gas (Ferguson, 1994, Otte et al. 1996). In addition, the extent of each reduction step during denitrification depends on the pH and the type of carbon source (Ferguson, 1994). This is reflected by the fact that denitrifiers contribute to nitric and nitrous oxide emissions to the atmosphere in soils (Kester et al.1997).

Denitrification generally takes place under anoxic conditions. However, some denitrifying bacteria use nitrite or nitrate when oxygen is present in amounts as high as 90% of air saturation. This process is called aerobic denitrification (Kuenen and Robertson, 1994).

DNRA

Nitrate can also be reduced to ammonia by a variety of microorganisms, in a process called DRNA (Dissimilatory Reduction of Nitrate to Ammonia). The ammonia produced is not for assimilatory purposes and thus ammonia is released into the environment. Apart from a nitrate reductase, a nitrite reductase, NrfA, which reduces nitrite to ammonia, is involved in this process (Cole, 1996)(Simon, 2002).

DNRA occurs in soils, freshwater and marine environments. The importance and role of DNRA in natural environments has recently started to be acknowledged.

Denitrification and DNRA can occur simultaneously and DNRA can be of quantitative importance in environments with a high carbon/nitrate ratio or high sulfide concentrations (Fazzolari et al., 1998)(Brunet and García-Gil, 1996). Moreover, plants like *Glyceria maxima* have a positive

effect on the amount of bacteria able to reduce nitrate to ammonia (Nijburg et al. 1996). DNRA can lead to high nitrite concentrations in freshwater environments (Kelsko et al, 1997).

The relatively huge marine microorganism *Thioploca* is able to store high amounts of sulfur and nitrate within their cells. The nitrate is reduced to ammonia during sulfur oxidation (Otte et al, 1999). *Thioploca* filaments are able to shuttle nitrate to the deeper parts of sediments (Zopfi et al, 2001).

Anammox

The denitrifying bacteria described above are not the only bacteria producing dinitrogen gas, and ammonia can be oxidized under anaerobic conditions as well. Recently, it became clear that slow growing autotrophic bacteria belonging to the order of the Planctomycetales are able to do both. Nitrite and ammonia are converted to dinitrogen gas by these bacteria and this proces is called anammox, an acronym for ANaerobic AMMonia OXidation (Mulder et al., 1995)(Van de Graaf et al. 1996). Hydrazine and hydroxylamine are intermediates in this process (Jetten et al., 1998).

The growth rate of Anammox bacteria is very low. The doubling time is 11 days at best. The cells are sensitive to oxygen, but the inhibition by oxygen is fully reversible (Strous et al, 1997). This suggests that the cells are frequently exposed to oxygen in their natural niche, as can be the case at the oxic/anoxic interface. The affinity constant for nitrite and ammonia is below 5 µM (Strous et al,

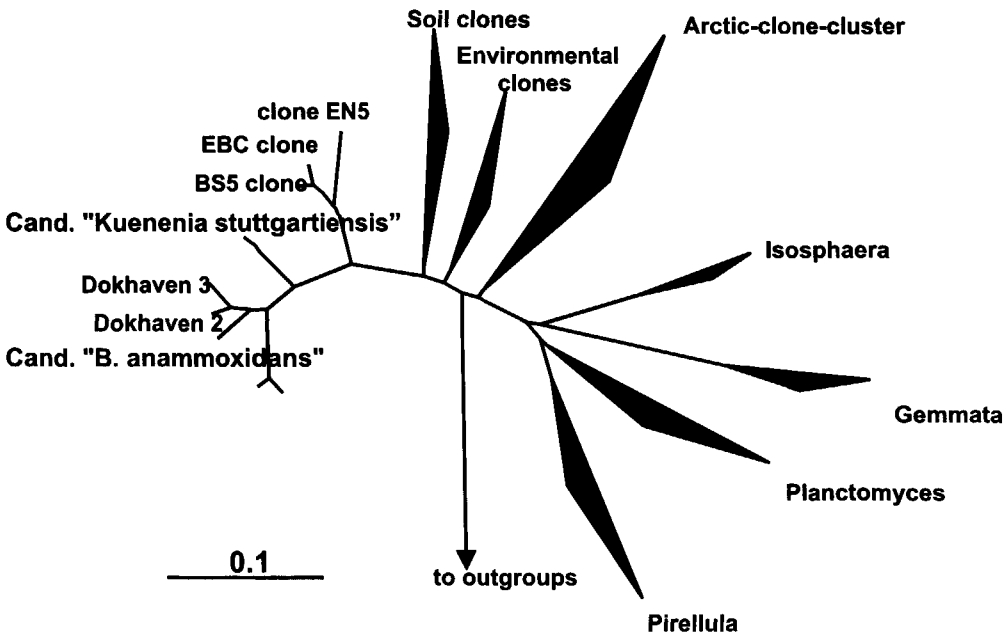

Fig. 1.3: Phylogenetic tree showing the relationship between bacteria of the order Planctomycetales

1999b). Like other bacteria of the order *Planctomycetales*, anammox bacteria have intracytoplasmic membrane formed compartments. One of these is called the anammoxosome and the oxidation of ammonia takes place in this compartment. Unique lipids, called ladderanes, surround the anammoxosome. These lipids can provide a rigid membrane structure. It is proposed that this rigid structure is needed to avoid hydrazine leakage through the anammoxosome. 10 % leakage of the intermediate hydrazine could cause 50 % loss of yield (Sinnighe Damste et al. 2002). In the cell, the enzyme HAO is only found in the anammoxosome (Lindsay et al, 2001). This enzyme can catalyse the oxidation of hydrazine to dinitrogen gas.

Thus far, Anammox is mainly studied in labscale reactors and pilot or full-scale plants for wastewater treatment. Although the diversity amongst the anaerobic ammonia oxidizers is far from clear yet, several genera of the order of planctomycetales are able to convert ammonia under anaerobic conditions. The families Brocadia and Kuenenia are already described (Kuenen and Jetten, 2001), but phylogenetic trees suggest that there are more genera to be discovered (Figure 1.2, Figure 1.3).

The search for anaerobic ammonia oxidizers in natural environments, and their importance in these environments has just begun (Thamdrup and Dalsgaard, 2002). The exact ecological niche is yet unknown. Although ammonia, needed for anammox, can be derived from mineralization or excretion, other bacteria must produce the required nitrite from ammonia or nitrate. The oxic/anoxic interface, where other groups of either aerobic or anaerobic bacteria are able to provide the anammox cells with nitrite, would be an excellent habitat.

Oxygen availability and interactions between nitrogen converting bacteria

Although the atmosphere on earth contains 21 % of oxygen, this does not mean that oxygen is available everywhere in ecosystems. In sediments, flocs, soils and particles, oxygen availability can be severely limited. The diffusion rate of oxygen into the deeper layers of the sediment is lower than the oxygen consumption rate. This oxygen diffusion limitation leads to typical oxygen profiles as shown in Figure 1.4. At the oxic/anoxic interface, where oxygen is still present in low amounts and anaerobiosis is spatially very close, bacterial activity is usually high (Ward, 1997). Oxic/anoxic interfaces can have a span of tens of meters in the water column of aquatic environments, or less than ten micrometer in sediments (Lorenzen et al., 1998).

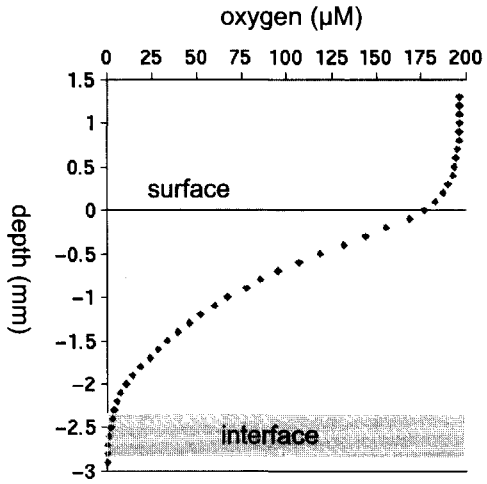

Figure 1.4: Oxygen profile of a sediment, showing the oxic/anoxic interface

At the oxic/anoxic interface, an ideal situation exists where anaerobic and aerobic bacteria can have extensive interactions. Possible interactions in the nitrogen cycle are the interactions between nitrifying and denitrifying bacteria (Ward, 1997). In this case nitrifying bacteria produce nitrite or nitrate needed for denitrification (figure 1.5a). Also aerobic and anaerobic ammonia oxidation can interact (figure 1.5b). In this case aerobic ammonia oxidizers partly oxidize ammonia to nitrite due to oxygen limitation and anaerobic ammonia oxidizers combine nitrite and ammonia to dinitrogen gas.

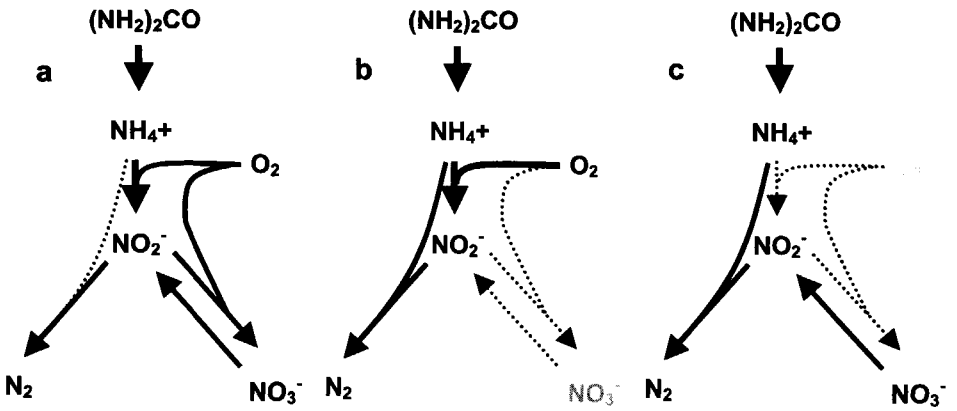

Figure 1.5: Possible interactions between nitrogen converting bacteria at the oxic/anoxic interface: nitrification/denitrification(a), partial nitrification/anammox (b) and denitrification/anammox (c). Solid arrows represent the transformations actually taking place during certain interactions and dashed arrows are transformations, which are possible but are taking place during other interactions.

Such a combination could explain the N-losses in several wastewater treatment systems as well as in sediments. Also possible is a combination of denitrification of nitrate to nitrite and anaerobic ammonia oxidation (Figure 1.5c). Such an interaction was probably the case in the first pilot reactor in which anammox was discovered. This reactor received an effluent from a methanogenic reactor and this contained amongst others ammonia, sulfide and was amended with nitrate (Mulder et al, 1995)(Van der Graaf 1996).

Human impact on the nitrogen cycle

Humans can have a high impact on the input of several nitrogen compounds in the environment. Urea, ammonia and nitrate are frequently used as fertilizer. Moreover, waste from manure also can contribute to large nitrogen deposition in agricultural soil. Due to nitrification and denitrification, N_2O or NO is produced and released into the atmosphere (Kester et al. 1997). N_2O is a persistent greenhouse gas, with an atmospheric half-life time of more than 100 years. Ammonia release in aquatic natural environments can cause eutrophication and oxygen depletion. Ammonia release in the atmosphere can cause acidification of soils due to nitrification as well as biodeterioration of masonry. Apart from eutrophication, an elevated nitrate concentration in drinking water can cause toxic effects in the gastrointestinal tract of infants. It is evident from this that the release of nitrogen compounds in the environments, other than dinitrogen gas, from human activity must be kept at a minimum.

Nitrogen removal from wastewater

To reduce the impact of humans on the nitrogen cycle and the consequences thereof, actions were taken to reduce the output of several nitrogen compounds. Amongst these actions, wastewater treatment is one of the most direct and used options. Wastewater treatment systems can have various waste streams with different ammonia strengths. Municipal wastewater often contains relatively low amounts of ammonia (10-60 mg N/l). When surplus of sludge is treated by anaerobic digestion, a waste stream is produced with high concentrations of ammonia (500-2000 mg N/l). Ammonia is usually removed by nitrification to nitrate and subsequent denitrification, to yield the environmental harmless dinitrogen gas. Sometimes, wastewaters do not contain enough organic compounds to support complete denitrification and methanol must be added. Recently, several new options for nitrogen removal for high strength wastewater were developed. One of these processes is the so-called SHARON process (Hellinga et al. 1998). SHARON is an acronym for Single reactor High activity Ammonium Removal Over Nitrite. The microbial principle behind this process is to circumvent nitrite oxidation to nitrate, by applying a low hydraulic retention time (HRT) and a high temperature (above 30 °C). At this temperature, the growth rate of the AOB is higher than the growth rate of NOB. The HRT is too short to maintain NOB in the reactor, but long enough to prevent wash out of the AOB. The absence of nitrite oxidation reduces aeration costs, but also reduces methanol costs if the produced

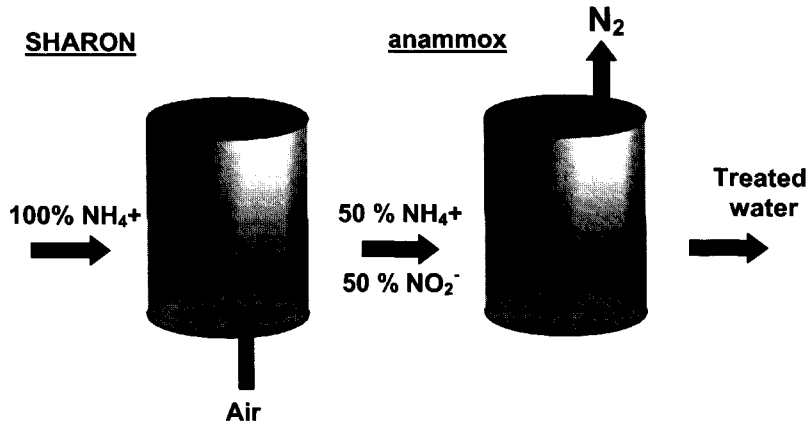

Figure 1.6: Schematic overview of the SHARON-anammox process

nitrite is subsequently removed by denitrification. A combination of SHARON and anammox, also aims at reduced aeration costs by circumventing nitrite oxidation (Figure 1.6). However, no methanol at all is needed and SHARON-anammox is for example suited to treat sludge digestion effluent (Jetten et al. 1997). For this purpose, two reactors are needed. Reduction of investment costs and also operational costs can be achieved when aerobic and anaerobic ammonia oxidation would take place in one reactor.

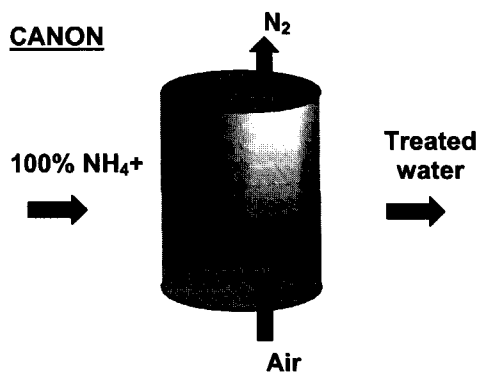

Figure 1.7: Schematic view of the CANON process

A combination of anaerobic and anaerobic ammonia oxidation is realised in the OLAND process. OLAND is an acronym for Oxygen-Limited Autotrophic Nitrification-Denitrification. In this (patented) process the anaerobic metabolism of aerobic ammonia oxidisers is held responsible for the observed anaerobic ammonium removal (Kuai & Verstreate, 1998). However, aerobic ammonia oxidisers metabolize at a slow rate under anaerobic conditions (Jetten et al., 1998). Therefore, a combination with Planctomycetes capable of anammox would be much more desirable. These bacteria

oxidize ammonium under anaerobic conditions at a rate of at least twenty times higher. A combination of partial nitrification and anammox in one reactor (figure 1.7) is patented (Dijkman & Strous, 1999) and has been modelled (Hao et al., 2002). This combined aerobic and anaerobic ammonia oxidation, referred to as CANON (Completely Autotrophic Nitrogen removal Over Nitrite), has been suggested for a new generation of sustainable wastewater treatment systems (Jetten et al, 1997), where sludge digestion and subsequent N-removal is taking an important place in the overall process for municipal wastewater treatment, reducing CO_2-output and energy input.

Outline of this thesis

The aim of this thesis was to study the interactions between autotrophic nitrogen converting bacteria at oxygen-limited conditions. Furthermore, applications and consequences of these interactions in wastewater treatment technology were investigated. First, the interactions between aerobic and anaerobic ammonia oxidisers were studied in one reactor CANON system (Chapter 2). Then, in chapter 3, the interactions between anaerobic ammonia oxidisers, aerobic ammonia oxidisers and aerobic nitrite oxidisers are described. In Chapter 4, it is shown that high N-removal rates can be achieved using interactions between aerobic and anaerobic ammonia oxidisers in a gas lift reactor. In chapter 5 and 6, the interactions between aerobic ammonia and nitrite oxidisers are addressed. Chapter 7 shows that interactions between aerobic and anaerobic ammonia oxidizers are also possible using urea as the energy source. Chapter 8, finally shows that planctomycete-like anaerobic ammonia oxidizers are also present in natural environments and that extensive interactions between anammox bacteria and other bacteria are also possible outside man-made environments.

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Chapter 2

Completely Autotrophic Nitrogen removal Over Nitrite in one single reactor

Previously published as:
A.O. Sliekers, N. Derwort, J.G. Kuenen, M. Strous, M.S.M Jetten. (1998) Completely Autotrophic Ammonia Removal Over Nitrite in One Single Reactor. Water Research **14**: 23-45

Abstract

The microbiology and the feasibility of a new, single-stage, reactor for completely autotrophic ammonia removal were investigated. The reactor was started anoxically after inoculation with biomass from a reactor performing anaerobic ammonia oxidation (Anammox). Subsequently, oxygen was supplied to the reactor and a nitrifying population developed. Oxygen was kept as the limiting factor. The development of a nitrifying population was monitored by Fluorescent In Situ Hybridisation and off-line activity measurements. These methods also showed that during steady state, anaerobic ammonium-oxidizing bacteria remained present and active. In the reactor, no aerobic nitrite-oxidizers were detected. The denitrifying potential of the biomass was below the detection limit. Ammonia was mainly converted to N_2 (85%) and the remainder (15%) was recovered as NO_3^-. N_2O production was negligible (less than 0.1%). Addition of an external carbon source was not needed to realize the autotrophic denitrification to N_2.

Introduction

Biological nitrogen removal is generally used for elimination of nitrogen from wastewater. Ammonia is especially abundant in many wastewater streams, although nitrate or nitrite can also be present. Ammonia removal is often achieved using nitrification/denitrification systems. In such systems, nitrifying bacteria oxidize ammonia to nitrate under oxic conditions, and nitrate is subsequently or simultaneously reduced to dinitrogen gas, under anoxic conditions (Jetten et al. 1997). Nitrifying bacteria are mainly autotrophic and derive energy from the oxidation of ammonia or nitrite. The oxidized nitrogen compounds can be used as alternative electron-acceptor by denitrifying bacteria. The denitrifiers are mostly heterotrophic and need reduced organic compounds (COD) for their energy and carbon supply. Many wastewaters do not contain enough COD, and addition of an exogenous carbon-source, such as methanol, is often necessary to achieve complete denitrification (Jetten et al. 1997, van Loosdrecht and Jetten 1998).

Recently a range of new microbial processes has been described and investigated in the laboratory: aerobic denitrification and heterotrophic nitrification (SND, Simultaneous Nitrification/Denitrification), anaerobic ammonium oxidation and denitrification by autotrophic nitrifying bacteria. These processes might explain some ununderstood N-losses in wastewater treatment practice (van Loosdrecht and Jetten 1998). Autotrophic denitrification is also possible with elemental sulfur as an electron donor, but sulfur has to be added (Kuai and Verstraete, 1999).

Although the denitrifying bacteria cover a large diversity of genera, with a spectrum of heterotrophic and autotrophic metabolism, the aerobic ammonia and nitrite oxidizers belong to a very restricted group of autotrophs. *Nitrosomonas* and *Nitrosospira* are the best-known aerobic ammonia oxidizers and *Nitrobacter* and *Nitrospira* are well known nitrite oxidizers.

The nitrifyers are typically aerobic bacteria although research has shown that they can have an anaerobic metabolism (Bock et al. 1995, Bock et al. 1988, Schmidt et al. 2001).
During the last decade, it has been discovered that certain bacteria, belonging to the Planctomycetales, are capable of simultaneous ammonia and nitrite removal under anoxic conditions. In this process, which has become known as Anammox (acronym for: ANaerobic AMMonia OXidation), ammonia and nitrite are converted to dinitrogen gas, with the concurrent production of biomass from carbon dioxide (Strous et al. 1999, Jetten et.al. 1999) .

So far, Planctomycete-like bacteria, capable of Anammox, have been clearly shown to be present in a number of reactors (Strous et al. 1999, Schmid et al. 2000, Egli at al. 2001, Jetten et al.1999, Twachtmann 1998).

Recent reports have shown that it is possible to have substantial nitrogen losses in reactors with a low dissolved oxygen concentration and with low amounts of COD present in the wastewater. The nitrogen loss could not be accounted for by the amount of COD present in the wastewater (Helmer and Kunst 1998, Kuai and Verstraete 1998, Siegrist et al. 1998, Helmer et al. 2001). It is very

likely that, in these systems, an autotrophic denitrification process is taking place; the bacteria capable of Anammox might be involved in these processes.

In such cases, ammonia would be converted partly to nitrite (equation 1) by oxygen-limited aerobic ammonia oxidisers and subsequently, anaerobic ammonium oxidizers would convert ammonia with nitrite to dinitrogen gas (equation 2). Combined, these oxygen-limited systems could be described by equation 3.

$$1\ NH_3 + 1.5\ O_2 \rightarrow 1\ NO_2^- + H_2O + H^+ \qquad \text{equation 1}$$

$$1\ NH_3 + 1.32\ NO_2^- + H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2\ H_2O \qquad \text{equation 2}$$

$$1\ NH_3 + 0.85\ O_2 \rightarrow 0.11\ NO_3^- + 0.44\ N_2 + 0.14\ H^+ + 1.43\ H_2O \qquad \text{equation 3}$$

Because Anammox-bacteria are reversibly inhibited by low (0.5% air saturation) concentrations of oxygen, (Strous et al. 1997), the process described by equation 3 must occur under oxygen limiting conditions, i.e. the aerobic ammonia oxidizers will have to remove virtually all of the oxygen from the liquid. However, aerobic ammonia oxidizers are known to produce N_2O and NO at low oxygen conditions (Goreau et al. 1980, Kester et al. 1997). The combined Anammox/Nitrification process, in one reactor system, may thus lead to a variety of nitrogen based compounds.

In this paper, a completely autotrophic process is described, in which aerobic ammonia-oxidizers and anaerobic ammonia oxidizers simultaneously oxidize ammonia to dinitrogen gas and a small amount of nitrate. This is achieved in one single reactor, at oxygen-limited conditions, without the production of N_2O or NO. This process has been called CANON, which is an acronym for Completely Autotrophic Nitrogen-removal Over Nitrite.

Material and methods

Reactor system and control-equipment

A sequencing batch reactor (SBR) with a working volume of 2 L was used. Two cycles were performed each day. One complete cycle consisted of a 11.5 hour filling period, with 1 L of synthetic wastewater at a flow rate of 1.45 ml/min, a 0.25 hour settling period of the biomass, and a 0.25 hour drawing period of 1 L liquid. At the beginning of each cycle, 1L of liquid was present in the reactor and, at the end of the filling period, 2 L was present. The hydraulic retention time was thus 1 day. The gas flowrate was kept constant at 7.9 ml/min controlled by a mass-flow controller (Brooks Instrument, Veenendaal, The Netherlands). The reactor was stirred at 100 rpm and the temparature was 30°C. Dissolved Oxygen was measured using a Clark-type oxygen electrode with a detection limit of 0.3% air-saturation (equivalent to 0.02 mg/l) (Ingold, Urdorf, Switzerland). The oxygen electrode was calibrated every two weeks. The pH was kept at 7.8 with 0.5M Na_2CO_3 and 1M HCl with an ADI 1030 Biocontroller (Applicon, Schiedam, The Netherlands). Biofilms were removed from the fermentor parts and the wall every week.

Origin of Biomass

The biomass used for inoculation originated from an Anammox-SBR-reactor in which 80% of the biomass consisted of Anammox bacteria (Strous et al. 1998).

Media

Synthetic wastewater was composed as described in Table 2.1.

Table 2.1: Composition of the synthetic wastewater used in this study. Values are in g/l.

	Synthetic wastewater A	Synthetic wastewater B
$KHCO_3^-$	1.25	1.25
$KH_2PO_4^-$	0.025	0.025
$CaCl_2.2H_2O$	0.3	0.3
$MgSO_4$	0.2	0.2
$FeSO_4$	0.00625	0.00625
Trace elements solution [a]	1.25 ml/l	1.25 ml/l
$(NH_4)_2SO_4$	1.2	0.62
$NaNO_2$	1	-
EDTA	0.00625	0.00625

[a] Trace element solution according to Schmidt (Belser and Schmidt, 1978).

Synthetic wastewater A contained, mainly nitrite and ammonia to support Anammox activity (equation 2) and this wastewater was used for experiments under anoxic conditions. Synthetic wastewater B contained no nitrite but did contain ammonia to establish aerobic ammonia oxidation and, due to oxygen limitation, only part of the ammonia was converted to nitrite. The resulting mixture of ammonia and nitrite supports subsequently anaerobic ammonia oxidation (equation 3). Wastewater B was fed into the reactor from day 80 onwards.

Experimental set-up

In order to start up the Anammox process, the reactor was filled with one liter of synthetic wastewater containing 14 mg NH_4^+-N and 14 mg NO_2^--N (wastewater A, Table 2.1, with altered ammonia and nitrite concentrations). Anammox biomass was added to a final concentration of 1g/L protein (2.4g dry weight). After a two hours, when the nitrite was consumed, the sequencing batch mode was started, with wastewater A (Table 2.1). The reactor was kept anoxic by sparging with helium. The concentration of nitrogen compounds in the reactor were determined twice a week and the concentration of the biomass in the reactor was determined every week. The reactor was considered to be in "steady state" when concentrations of N-compounds, biomass and the N-conversion-rates in the reactor were constant. When a "steady state" was achieved, the composition and activity of the biomass were determined every week and this was done by taking biomass from the reactor for activity measurements and Fluorescence In Situ Hybridization (FISH). During "steady state" the effluent gas was analyzed for NO, NO_2, N_2O and N_2. After a "steady state" was achieved and analysed, the helium was replaced with air. The transition-state that followed and the subsequent "steady state" were also analysed, as described for the first "steady state", with the exception that, during the transition state, no anaerobic ammonia oxidation activity was measured and no FISH was conducted.

Throughout the whole experiment, the concentration of nitrogen compounds of every new batch of synthetic wastewater was determined. Ammonia was always present in excess.

Activity measurements

The maximum aerobic ammonia oxidation activity and the maximum aerobic nitrite oxidation activity of the biomass were measured under fully aerobic conditions. Biomass was taken from the reactor and incubated in an Erlenmeyer flask at 30 °C in a rotary shaker. Nitrite and nitrate were measured over time, during 1 to 2 hours. Maximum anaerobic ammonia oxidation activity was measured as follows: biomass from the reactor was transferred into serum bottles with gas tight rubber stoppers. The flasks were made anaerobic using 95% Ar/ 5% CO_2. Nitrite was added to a maximum of 100mg/l NO_2^-. Ammonia was already present in the biomass suspension, due to the ammonia surplus in the reactor. Nitrite and ammonia consumption and nitrate production were tracked during a 2 hour period.

Maximum denitrification activity of the biomass was measured by incubating biomass with 1 g/l yeast extract, under anaerobic conditions, using bottles with rubber stoppers and a headspace of 95% Argon/ 5% CO_2.

Fluorescent In Situ Hybridisation (FISH)

Biomass samples were fixed immediately for 2 to 3 hours with 4% (w/v) paraformaldehyde. (Amann, 1995) FISH-analysis was carried out as described by Juretschko (Juretschko et al. 1998). The 16 S rRNA gene probes used were Amx820 (Strous et al. 1999, Schmid et al. 2000), Nit1035 (Juretschko et al., 1998), Neu 653 (Juretschko et al. 1998) , Nsp436 (Stephen et al. 1998), and Ntspa1026 (Juretschko et al. 1998).

All gene probes were labeled with either of the fluorophores Cy3 and Fluorescine and were purchased from Interactiva (Ulm, Germany).

Chemical analysis

The concentrations of ammonia, nitrite and nitrate were measured colorimetrically. (Cataldo et al. 1975, Weatherburn 1967, Griess-Romein van Eck 1966). Dry weight was measured after drying the filtered biomass in a microwave for 10 min at 300 W. Protein was measured according to the method of Lowry. (Lowry et al. 1951) Nitrous oxide, oxygen and dinitrogen gas were measured with a Gas Chromatograph (Fisons Instruments, Italy) equipped with a Molsieve 5A, a Hayesep column, an Electron Capture Detector and a Hot Wire Detector (Intersience, Breda, The Netherlands). NO and NO_2 gas were measured using chemiluminescense (Kester et al. 1994).

Results and discussion.

Ammonia removal under anoxic conditions.

Biomass was added to the reactor and checked for activity. After starting the sequencing batch-mode, the total biomass content in the reactor decreased to 630 mg within two weeks, and stayed constant. Two weeks after the start-up of the Anammox-reactor, the concentrations of NH_4^+-N and NO_3^--N showed no further change and the reactor was therefore considered to be at "steady state". The characteristics of the process at this time are listed in Table 2.2. The main product of NO_2^- and NH_4^+ conversion was N_2, as confirmed by gas chromatography. The molar ratio of NO_2^-/ NH_3 consumption was 1.27 and the ratio of NO_3^- production/NH_3 consumption was 0.28. This was as expected for the Anammox process (Strous et al.1998). Nitrite could not be detected in this period.The maximum aerobic ammonia or nitrite oxidation activity tests showed that the biomass did not produce nitrite or nitrate at oxic conditions, respectively (Table 2.2).

Table 2.2. Characteristics of the process during steady state under anoxic conditions and during steady state under oxygen limiting conditions. The values between brackets are standard deviations

Parameter	anoxic conditions	oxygen-limitation
Test period (day no.)	14-36	50-70
Nitrite consumption[b]	0.20 (0.012)	-[a]
Ammonia consumption[b]	0.16 (0.01)	0.075 (0.003)
Nitrate production[b]	0.045 (0.005)	0.011 (0.0011)
Total dry weight (g)	1.5	1.5
Total protein (mg)	630 (25)	624 (21)
Ammonia out[c]	103	56
Ammonia in[c]	257	131
Nitrogen removal[b]	0.315	0.064
Activity in reactor[d]	0.24	0.1
Maximum Anammox activity[d]	0.25	0.15
Max. aerobic NH_4^+ ox. activity[d]	0	0.655
Max. aerobic NO_2^- ox. activity[d]	0	0
Max. denitrification act.[d]	0	0

[a] Due to simultaneous production and consumption of the nitrite, this figure could not be calculated.

[b] (kg NO_2^--N/$m^3_{reactor}$/day)

[c] (mg NH_4^+-N/l)

[d] (kg NH_4^+-N/kg dw/day)

Apparently, little, if any nitrifiers were present in the biomass during this period. This was also confirmed by FISH-analysis. No nitrite oxidizers and aerobic ammonia oxidizers were present (detection limit < 1%). When cells were hybridized with probes specific for the planctomycete-like Anammox bacteria, a large percentage of the cells reacted with the probes (Fig. 2.3). Taken together, these observations showed that the Anammox-process was fully operative in the SBR.

Transition phase.

After 5 weeks, the influent gas of the same reactor was changed from Helium to air, and at the same time, synthetic wastewater containing only NH_4^+-N was supplied to the reactor (Synthetic wastewater B, Table 2.1). The oxygen concentration increased immediately from 0 to 0.5 mg/l but dropped gradually to values below detection limit (<0.1% air-saturation/ < 0.07 mg/l) within 6 days. Also, after one day of air supply, the nitrite concentration increased to 31 mg NO_2^--N/l, but it dropped to less than 0.6 mg NO_2^--N/l within 6 days (Fig 2.1).

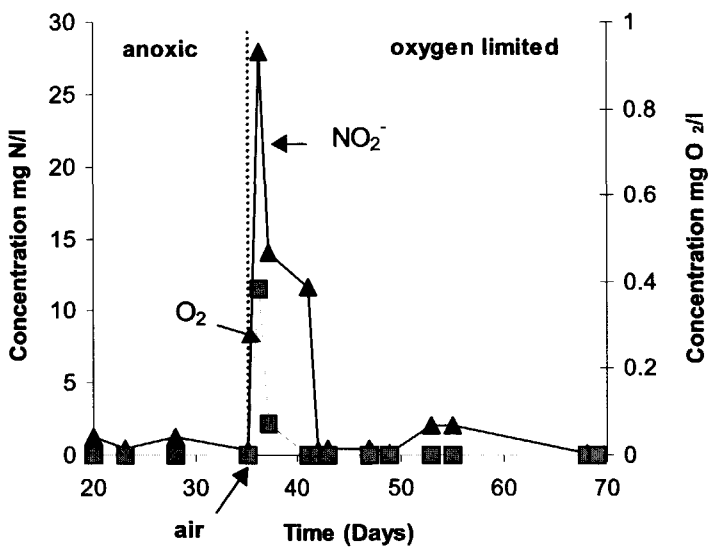

Figure 2.1: Oxygen concentration (Squares), Nitrite concentration (Triangles) under anoxic conditions, during the transition period and under oxygen limited conditions. At day 36, air was supplied to the reactor, and a transient peak of nitrite and oxygen could be observed.

During two weeks the maximum oxic ammonia oxidation activity increased dramatically (Fig 2.2). Also, the NH_4^+-conversion-rate in the reactor increased compared to the conversion rate immediately after the start of the air addition (Fig 2.2). During the two week transition phase, an aerobic nitrifying population developed, whereas this population was originally not detectable during the preceding Anammox "steady state". It should be noted that the number of nitrifiers in permanent

anoxic Anammox-reactors was found to be very low (10^3-10^4 cells/mg VS) in results presented previously (Van de Graaf et al. 1996). So, it was expected that a small population of aerobic ammonia oxidizing bacteria would be present in the biomass during the anoxic period. However, to explain the build-up of nitrite in the first few oxic days of the experiment, it was calculated that a much larger part, at least 0.1% (which corresponds to 10^6 cells/mg prot, according to Van de Graaf et al., 1996) of the population must have been aerobic ammonia oxidizers in this experiment. This difference might have resulted from the strict anaerobe techniques applied by Van der Graaf et al. (1996).

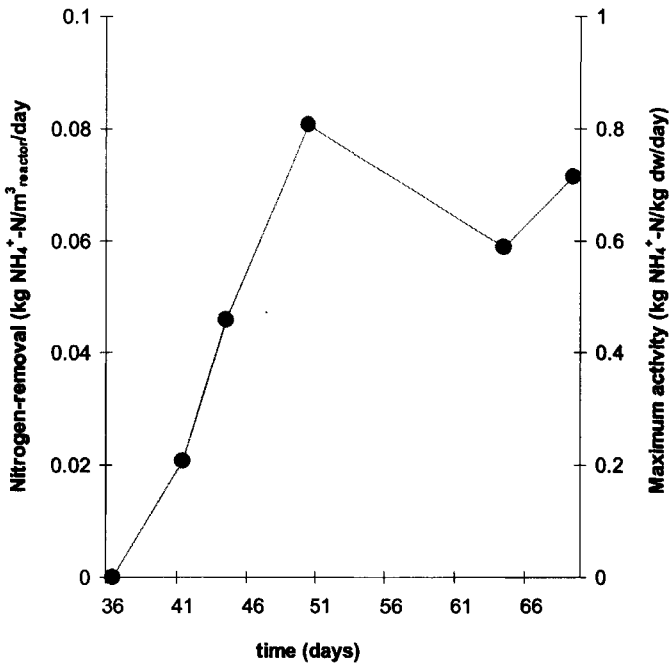

Figure 2.2. Ammonia removal (squares) and the maximum aerobic ammonia oxidation activity (circles) from the day that air was supplied to the reactor. Note that at day 36 the composition of the synthetic wastewater was changed from $NH_4^+ + NO_2^-$ to NH_4^+ only (table 1). Therefore, the Nitrogen-removal could not be calculated.

Ammonia removal under oxygen-limited conditions

From week 7 on, the reactor was considered to be in "steady state" mode, because from this point onward, the ammonia conversion rate in the reactor was constant. The characteristics of this "steady state" are listed in Table 2.2. The total amount of biomass in the reactor was fairly constant. The dry weight/protein ratio was calculated to be 2.4. The conversion rate in the reactor was 0.15 g

NH_4^+-N/day. From this, the activity of the biomass in the reactor was calculated to be 0.1 kg NH_4^+-N/kg dw/day. The maximum anaerobic ammonium oxidation activity was 0.15 kg NH_4^+-N/kg dw/day (Table 2). Per mole ammonia consumed 0.146 mole nitrate was formed (Table 2.2).

The ammonia/nitrate ratio in the reactor was almost as expected for aerobic/anaerobic ammonia oxidation (equation 3). Only 0.25 mg NO_2^--N/l was present in the reactor, and the production of NO and N_2O were negligible (about 0.05 %). NO_2 gas was not detected. The main product was identified as dinitrogen-gas.

The absence of N_2O and NO production is surprising since different studies have shown the production of these gases by ammonia-oxidizing bacteria at low oxygen conditions. However, Kester et al. (1997) showed that a pure culture of *Nitrosomonas* was producing N_2O and NO, but not in presence of a nitrite consumer, in this case a pure culture of *Nitrobacter*.

During the anaerobic ammonium oxidation activity measurement, nitrate was formed, but nitrate was not formed during the aerobic nitrite oxidation activity measurements. No nitrite or nitrate removal was observed in the activity test for heterotrophic denitrification.

The maximum aerobic nitrite oxidation activity of the sludge was zero, and the absence of aerobic nitrite oxidizers was confirmed by FISH, using probes for known *Nitrospira* and *Nitrobacter*. From FISH analysis, it became clear that a large part of the biomass consisted of bacteria belonging to the *Nitrosomonas* and the planctomycete-like anaerobic ammonia oxidizers (Figure 2.3). Estimates from FISH-analysis of the fractions of aerobic ammonia oxidizers and anaerobic ammonia oxidizers were 45% (±15%) and 40% (±15%) respectively. This means that the aerobic ammonia oxidizers fraction increased from at least 0.1% (estimated from activity) to 45% (±15%) after the start of aeration. The fraction of anaerobic ammonia oxidizers decreased from 80% (±15%) to 40% (±15%), and the decrease in maximal Anammox activity (Table 2.2) was consistent with this estimation.

No external carbon source was present in the medium and the observed ammonia removal to dinitrogen gas was a completely autotrophic process. Heterotrophic denitrifiers did not seem to be active in this process.

Under oxygen limiting conditions, bacteria capable of the Anammox process consumed the nitrite, produced by aerobic ammonia oxidizers, while the aerobic nitrite oxidizers were apparently not involved. It seems that nitrite-oxidizing bacteria can be only present when oxygen is not limiting. In the oxygen-limiting situation, presented in this paper, nitrite oxidizers had to compete for oxygen with the aerobic ammonia oxidizers and had to compete for nitrite with anaerobic ammonia oxidizers. Another possibility is that nitrite-oxidizers were inhibited by free ammonia (Abeling 1992), and during the experiments presented in this paper, ammonia was present in excess in the reactor, thus ammonia could have inhibited the nitrite oxidizers. In that case, the high ammonia concentration in the reactor was needed to establish the combined aerobic and anaerobic ammonia oxidation.Although the N-removal was quite low, it must be emphasized that the aim of this experiment was to study the microbiology of the process and that the reactor set-up was not yet optimized, with respect to

gas/liquid oxygen-transfer. Future studies will be done using a reactor with a better O_2 transfer rate such as an airlift reactor.

The process described in this paper has been named the CANON process, an acronym for Completely Autotrophic Nitrogen Removal Over Nitrite.

Conclusions

1. Under oxygen limiting conditions, completely autotrophic ammonia removal to dinitrogen gas was achieved in one sequencing batch reactor. This process was very stable and easy to maintain during the period tested (three months). *Nitrosomonas*-like bacteria converted partially ammonia to nitrite and Anammox bacteria converted the mixture of ammonia and nitrite mainly to dinitrogen gas. The sludge showed no heterotrophic denitrification potential during activity tests, and the dinitrogen gas was therefore produced by Anammox bacteria.
2. Under oxygen limiting conditions, 15% of the consumed ammonia was converted to nitrate. This is what was expected in a combined Anammox/partial nitrification process. This nitrate was produced by Anammox bacteria, because the activity experiments showed that the biomass from the reactor produced nitrate under anoxic conditions, but not under oxic conditions. In addition, aerobic nitrite-oxidizers were not detected.
3. The process has a high potential for application, when the maximum oxygen transfer rate is high.

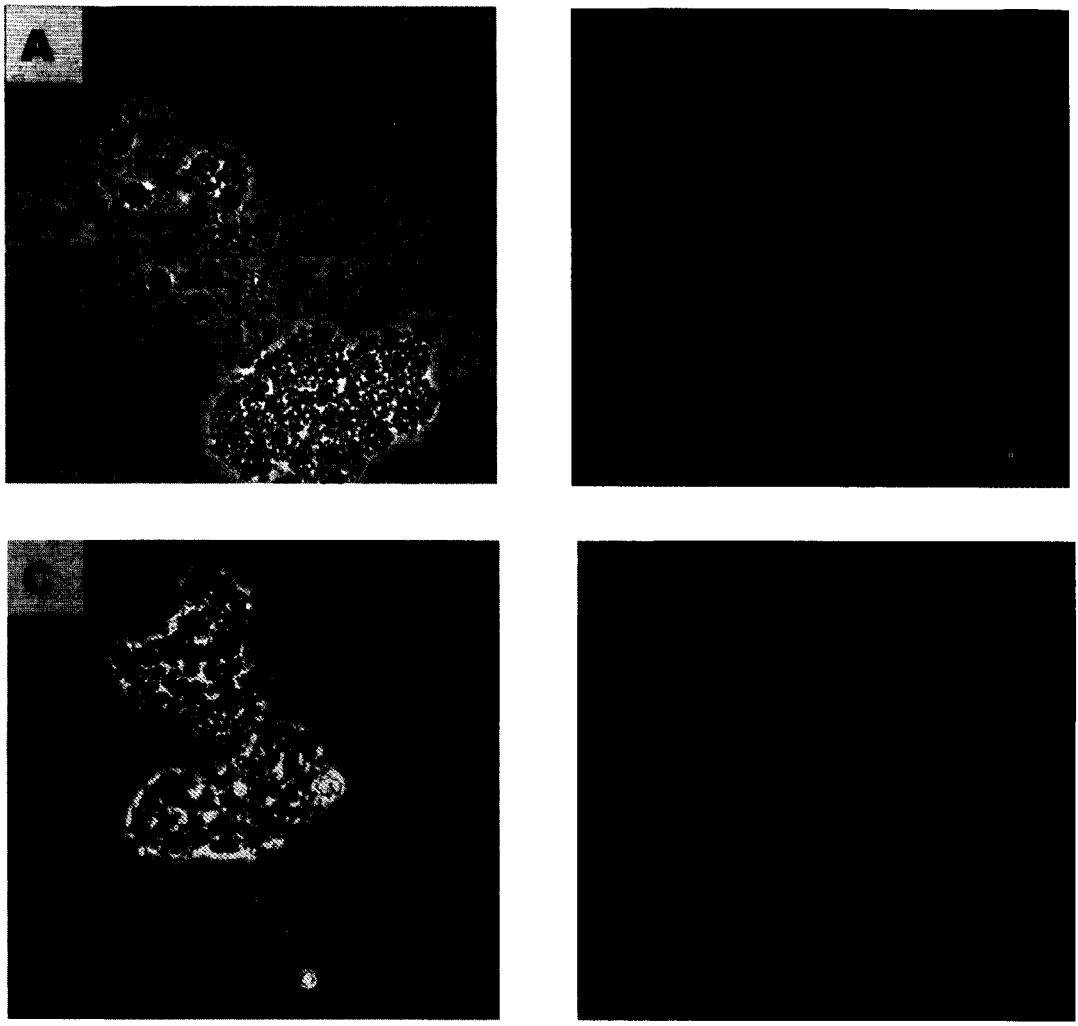

Figure 2.3: Micrographs of flocs at 400 x magnification. A: phasecontrast-micrograph of a floc from the period without air-supply. B: fluorescense micrograph of the same floc hybridized with Amx 820-Cy3 (red color) and NEU fluos (green color). Only Anammox bacteria are present. C: phasecontrast-micrograph of a floc from the period with air-supply; D: fluorescense-micrograph of the same floc hybridized with Amx 820 (red) and NEU (green). Anammox bacteria and a *Nitrosomonas* species are both present.

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Chapter 3

The CANON System (Completely Autotrophic Nitrogen-removal Over Nitrite) under ammonium limitation: Interaction and competition between three groups of bacteria.

Previously published as:

Third, K.A, Sliekers, A.O., Kuenen, J.G. & Jetten, M.S.M. (2001) The CANON System (Completely Autotrophic Nitrogen-removal Over Nitrite) under Ammonium Limitation: Interaction and Competition between Three Groups of Bacteria. System.Appl.Microbiol. **24**: 588-596.

Abstract

The CANON system (Completely Autotrophic Nitrogen Removal Over Nitrite) can potentially remove ammonium from wastewater in a single, oxygen-limited treatment step. The usefulness of CANON as an industrial process will be determined by the ability of the system to recover from major disturbances in feed composition. The CANON process relies on the stable interaction between only two bacterial populations: *Nitrosomonas*-like aerobic and *Planctomycete*-like anaerobic ammonium oxidising bacteria. The effect of extended periods of ammonium limitation was investigated at the laboratory scale in two different reactor types (sequencing batch reactor and chemostat). The lower limit of effective and stable nitrogen removal to dinitrogen gas in the CANON system was 0.1 kg N m^{-3} day^{-1}. At this loading rate, 92% of the total nitrogen was removed. After prolonged exposure (> 1 month) to influxes lower than this critical NH_4^+-influx, a third population of bacteria developed in the system and affected the CANON reaction stoichiometry, resulting in a temporary decrease in nitrogen removal from 92% to 57%. The third group of bacteria were identified by activity tests and qualititative FISH (Fluorescence In Situ Hybridisation) analysis to be nitrite-oxidising *Nitrobacter* and *Nitrospira* species. The changes caused by the NH_4^+-limitation were completely reversible, and the system re-established itself as soon as the ammonium limitation was removed. This study showed that CANON is a robust system for ammonium removal, enduring periods of up to one month of ammonium limitation without irreversible damage.

Introduction

A co-operation between aerobic and anaerobic ammonium oxidising bacteria under oxygen limitation has been observed previously (Strous 2000, Chapter 2). This is a promising new principle for wastewater treatment, as only a single oxygen-limited step might be needed to remove ammonium from wastewater. The process relies on the interaction of two groups of autotrophic bacteria under oxygen-limiting conditions that perform two sequential reactions, simultaneously. Under oxygen limitation, ammonium is oxidised to nitrite by aerobic ammonium oxidisers, such as *Nitrosomonas* and *Nitrosospira* (eqn. 1).

$$1\ NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O \qquad \text{Eqn. 1}$$

The nitrite produced in this reaction can be used by planctomycete-like anammox bacteria, which anaerobically oxidise ammonium using nitrite as electron acceptor (Strous, 2000) (eqn. 2).

$$1\ NH_4^+ + 1.3\ NO_2^- \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2\ H_2O \quad \text{Eqn. 2}$$

As the nitrite also serves as an electron donor for the formation of biomass from carbon dioxide, the formation of nitrate in the reaction is stoichiometrically coupled to growth. The combination of the above two reactions results in nitrogen removal according to (eqn 3.):

$$1\ NH_4^+ + 0.85\ O_2 \rightarrow 0.435\ N_2 + 0.13\ NO_3^- + 1.3\ H_2O + 1.4\ H^+ \quad \text{Eqn. 3}$$

The interaction of aerobic and anaerobic ammonium oxidising bacteria under oxygen-limitation results in an almost complete conversion of ammonium to dinitrogen gas, along with small amounts of nitrate. A high loss of nitrogen has been reported in several systems with high ammonium loading and low organic carbon content of the wastewater (Helmer *et al.* 2001, Helmer and Kunst, 1998, Helmer *et al.* 1999, Hippen *et al.*, 1997, Koch *et al.* 2000, Kuai and Verstraete, 1998, Siegrist *et al.*, 1998*)*. The autotrophic conversion of ammonium into dinitrogen gas was defined microbiologically (Strous *et al.*, 1997) and the process has been named CANON, an acronym for Completely Autotrophic Nitrogen-removal Over Nitrite (Dijkman and Strous, 1999). If ammonium removal can be achieved in a single reactor, it would represent a very economical and efficient option for water treatment, especially for wastewater rich in ammonium but devoid of organic carbon (COD). Ammonium removal from wastewater is traditionally performed using oxic nitrification to nitrate, involving high aeration demands, followed by anoxic denitrification of the nitrate to nitrogen gas, in a separate tank. The

CANON process is completely autotrophic, therefore avoiding COD addition, which is often required for the heterotrophic denitrification step in traditional systems. In addition, the entire nitrogen removal can be achieved in a single reactor with very low aeration, greatly reducing space and energy requirements. The autotrophic process consumes 63% less oxygen and 100% less reducing agent than traditional nitrogen removal systems. Unlike other autotrophic nitrogen removal systems, such as the SHARON-anammox process (Jetten *et al.*, 1997) where the nitrite is generated in a separate reactor, there is no requirement for nitrite addition in the CANON system. Thus, an ammonium-rich wastewater can be fed directly to a single oxygen-limited reactor at a suitable loading rate. Nitrogen removal rates of up to 0.3 kg N_{total} m^{-3} day^{-1} have been reported for the CANON process (Chapter 2).

If the CANON process is to be applied on a large scale, it is important to know the limitations of the system, and whether or not severe disturbances to the system are reversible. The CANON process relies on the harmonious and balanced interaction between only two groups of bacteria, the aerobic and the anaerobic ammonium oxidising bacteria. If under certain conditions the balance is disturbed, for example if other groups of bacteria, such as aerobic nitrite oxidisers develop, they may interfere with nitrogen removal. The following study investigated the effects of severe ammonium limitation, as may occur during periods of low nitrogen loading in wastewater treatment. The effect of ammonium limitation on the CANON system was studied in two different reactor types. The bacterial populations were monitored by Fluorescence In Situ Hybridisation (FISH) to investigate changes in the composition of the biomass under ammonium limitation. Together with activity measurements and mass balances, changes in the bacterial populations and hence in the nitrogen removal characteristics were monitored. The reversibility of the changes imposed by the limitation was investigated.

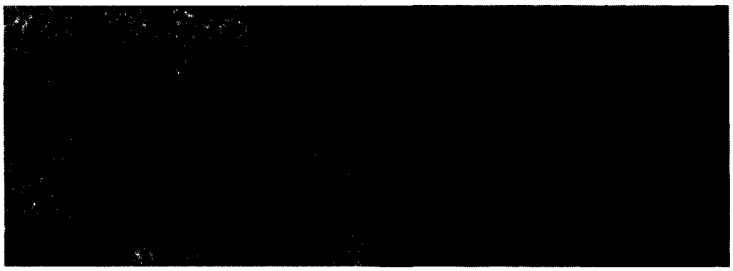

Figure 3.4a: FISH analysis of CANON SBR biomass under NH_4^+-limitation at day 14 (A: Phase contrast x 630 magnification, and B: The same section of biomass showing *Nitrosomonas* like aerobic ammonium-oxidising cells stained with probe FLUOS-Neu 653 (green) and anammox cells stained with probe CY3-Amx820 (red).

Figure 3.4b: FISH analysis of biomass in the CANON-SBR under NH4+-limitation at day 14; A: Phase contrast at x 630 magnification, and B: Nitrospira-like nitrite-oxidising cells stained with CY3-NITSP (red), Nitrosomonas-like cells stained with probe Fluos-NIT (green) along with anammox cells stained with probe CY5-Amx820 (blue).

Materials and Methods

Reactor Systems and Control Equipment

The Sequencing Batch Reactor (SBR)

A glass vessel (height 0.21 m, diameter 0.14 m) without baffles, with a 2 L working volume and equipped with a water jacket (maintained at 30° C) was used as the SBR. The glass vessel was fitted with a fermentor lid containing a turbine propellor stirrer, acid/base inflow tubes, feed inflow tube, dissolved oxygen probe, pH probe, outflow tube, level controller and a sampling port (Fig. 3.1). The vessel was stirred at 100±10 rpm. Dissolved oxygen was measured using a Clark-type oxygen electrode (Ingold, Switzerland), which was calibrated every two weeks. The dissolved oxygen concentration was monitored using an Applikon 1030 biocontroller (Applikon, The Netherlands), which also controlled the pH at 7.8 using 0.5 M Na_2CO_3 and 1 M HCl. The gas-flow was maintained constant using a mass-flow controller (Brooks Instruments, The Netherlands). During anaerobic

periods the reactor was flushed with Helium, and during aerobic periods air was used. The pumps and stirrer were controlled by timers.

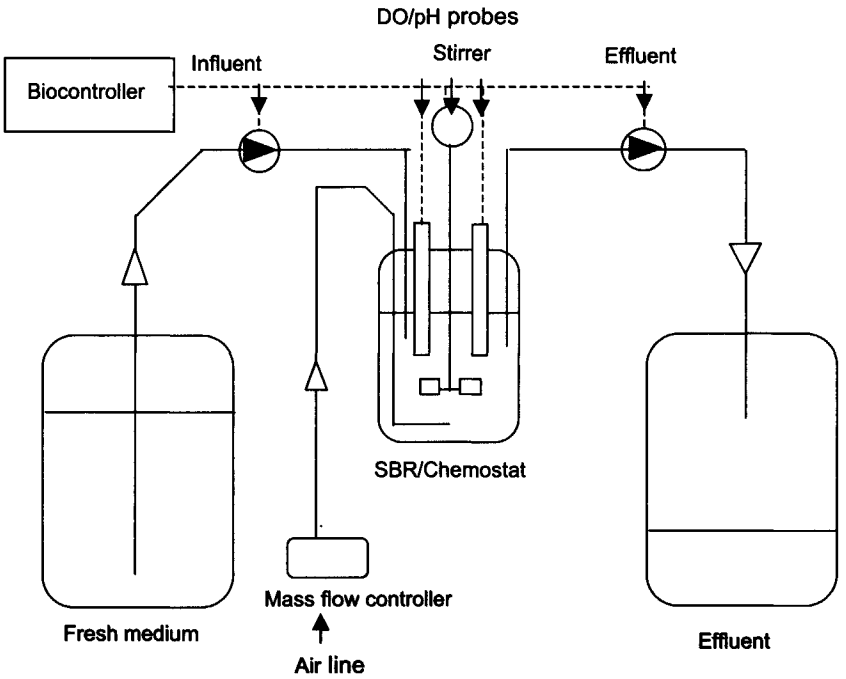

Figure 3.1: Experimental set-up of the reactor system. The SBR was converted to a chemostat by adjusting the effluent flow rate equal to the influent flow rate to maintain a constant volume, and the cyclic settling and decant phases were removed.

The SBR was run as a continuously-fed sequence batch reactor with a 12 hour cycle. It was filled continuously with fresh medium over 11.5 hours at a flow rate of 1.45 mL min^{-1}. The minimum volume was 1 L, and at the end of the cycle the final volume was 2 L. After the filling period, the stirrer and influent pump were stopped and the biomass aggregates were allowed to settle for 15 minutes. In the remaining 15 minutes of the cycle, 1 L of supernatant was removed by an effluent pump.

The Chemostat

The same 2 L vessel used for the SBR was converted to a chemostat and maintained at a constant volume (1.5 L) by a level controller. The feed flow rate started initially at 2.6 L day^{-1} (dilution rate 0.072 h^{-1}). To enable biomass retention the reactor was fitted with a settling device in the outflow tube, in which the upflow rate of the outflowing liquid was slower than the settling rate of the biomass. Hence, biomass settled in the tube (diameter around 5 cm) and was periodically returned to the reactor (every 2-3 days). The stirrer and inflow pump were turned off for half an hour each cycle, to mimic the SBR and to make a comparison possible.

Origin of Biomass

Anammox

The biomass was taken from the outflow tank of a 15 L Anammox-SBR-reactor in which 80% of the biomass consisted of planctomycete-like Anammox bacteria (Strous *et al.*, 1999b). The biomass in the outflow tank was 2-3 months old. The biomass was never separated from its supernatant liquid before inoculation, as it has been shown to significantly decrease its initial activity.

Nitrifying biomass

The nitrifying biomass was taken from a refrigerated sample from a previously oxygen-limited nitrifying chemostat, known to actively oxidize ammonium at a rate of 106 nmoles NH_4^+ mg protein^{-1} min^{-1}.

Synthetic wastewater

Ammonium and nitrite were added to a mineral medium in the required amounts (see Results section) in the form of $NaNO_2$ and $(NH_4)_2SO_4$. The composition of the mineral medium was (g L^{-1}): $KHCO_3$ 1.25, KH_2PO_4 0.025, $CaCl_2.2H_2O$ 0.3, $MgSO_4.7H_2O$ 0.2, $FeSO_4$ 0.00625, EDTA 0.00625 and 1.25 mL L^{-1} of trace elements solution. The trace element solution contained (g L^{-1}): EDTA 15, $ZnSO_4.7H_2O$ 0.43, $CoCl_2.6H_2O$ 0.24, $MnCl_2.4H_2O$ 0.99, $CuSO_4.5H_2O$ 0.25, $NaMoO_4.2H_2O$ 0.22, $NiCl_2.6H_2O$ 0.19, $NaSeO_4.10H_2O$ 0.21, H_3BO_4 0.014, $NaWO_4.2H_2O$ 0.05. The separate components of the medium were autoclaved at 120° C to avoid bacterial growth in the feed vessels, although the reactors were not run aseptically.

Activity measurements

The maximum *aerobic* NH_4^+ and NO_2^--oxidizing activity of the biomass were measured under oxygen saturation conditions. A 10 mL sample of biomass suspension was taken from the reactor and put into a 250 mL Erlenmeyer flask. Nitrite and ammonium were added (if not already in the medium) to final concentrations of about 2 mM. The flask was then incubated at 30° C on a rotary shaker. Ammonium, nitrate and nitrite were measured every half an hour for up to two hours. To measure the maximum *anaerobic* ammonium oxidation activity, biomass was taken out of the reactor and put into a serum bottle with a gas-tight rubber stopper. The flask was made anaerobic by flushing with Argon. Nitrite and ammonium were added to the flasks to final concentrations of about 2 mM. Nitrite and ammonium consumption and NO_3^- production were measured over time during 4 hours. All activities were calculated from the rate of substrate disappearance or product appearance per concentration of biomass present (i.e. nmoles mg protein^{-1} min^{-1}).

Dry weight determinations

A sample of biomass suspension (2 or 5 mL) was taken from the reactor and filtered through 0.2 μm filter paper. The filter paper was then dried in a microwave for 25 minutes at a low temperature (180° C), and the weight of the dried biomass was determined. Protein measurements of the biomass were performed according to the method of Lowry (Lowry *et al.*, 1951) and showed that the protein concentration was consistently approximately half of the measured dry weight. Protein concentrations were thus calculated as half of the measured dry weight.

Figure 3.8a: FISH analysis of CANON chemostat biomass under NH4+-limitation (day 65); A: Phase contrast x 630 magnification, B: Nitrosomonas-like aerobic ammonium-oxidising cells stained with probe FLUOS-Neu 653 (green), Nitrospira-like nitrite-oxidising cells stained with CY3-NITSP (red) and anammox cells stained with CY5-Amx820 (blue). Samples were prepared with 40% formamide concentration.

Figure 3.8b: FISH analysis of chemostat biomass (day 65); A: Phase contrast x 630 magnification, and B: *Nitrospira*-like nitrite-oxidising cells stained with CY3-NITSP (red), *Nitrobacter*-like nitrite-oxidising cells stained with FLUOS-NIT (green) and anammox cells stained with CY5-Amx820 (blue). Samples were prepared with 40% formamide concentration.

Fluorescence in situ hybridization (FISH)

Fixation of cells for in situ hybridisation

The method was adapted from Amann (1995). Cells were washed in 10 mM phosphate buffer (pH 7.2, containing 130 mM NaCl) and resuspended in the same buffer. One volume of the cell suspension was mixed with 3 volumes of the same buffer (freshly prepared and containing 4% paraformaldehyde) and kept on ice for 1-3 hours. The fixative was then removed by centrifugation and the cells were resuspended in 0.5 mL phosphate buffer. Finally, 0.5 mL of absolute ice-cold ethanol was added and the cells stored at -20° C until used.

Immobilisation of fixed cells on microscope slides

The immobilisation step was adapted from Juretschko *et al.* (1998). 5-10 µL aliquots of cell suspension were loaded in each gelatine-coated well of a teflon-printed slide (Nutacon, the Netherlands). After loading, the slide was dried for 10 min at 46° C. The cells were then dehydrated by successive passage through 50, 80 and 90% ethanol (3 minutes each) and air dried.

Hybridization of immobilised cells

This step was also adapted from Juretschko *et al.* (1998). To each well on the teflon-printed slide 10 µL of freshly prepared hybridisation buffer (pH 8, 20 mM Tris/HCl, 180 mM NaCl, formamide 35%), and 1 µL probe working solution containing 5 ng of each of the respective dye-labelled probes was added. The 16S rRNA gene probes used were Amx820 for planctomycete-like anammox cells (Strous *et al.*, 1999a), Neu 653 for *Nitrosomonas*-like aerobic ammonium-oxidizng bacteria and NIT and Nspa for *Nitrobacter* or *Nitrospira*-like aerobic nitrite-oxidizing bacteria (Juretschko *et al.*, 1998). Probes were synthesized and directly labelled with the hydrophilic sulphoindocyanine flourescent dye Cy3 or the flourochrome 5(6)-carboxy-fluorescin-N-hydroxysuccinimide ester (FLUOS) (Interactiva, Germany).

The slides were incubated at 46° C for 90 min in 50 mL Falcon tubes, containing a tissue paper moisturised with 2 mL of hybridisation buffer. After hybridisation, the slides were washed rapidly with 20 mM Tris/HCl (pH 8, 48° C and containing 5 mM EDTA and NaCl concentrations depending on the applied formamide concentration in the buffer. For 35% formamide, 80 mM NaCl was added. The slides were rapidly immersed in the same buffer and incubated at 48° C. After 20 min the slides were rinsed with cold MilliQ water, air dried rapidly and stored at -20° C until studied under the microscope.

Epifluorescence microscopy and documentation

The slides were embedded in vectashield (Vector laboratories, USA) and analysed with a Zeiss Axioplan 2 imaging fluorescent microscope (Zeiss, Weesp, the Netherlands). Photomicrographs were taken using LeicaQFluoro imaging software (Leica, the Hague).

Chemical Analyses

Nitrate, nitrite and ammonium were measured colorimetrically as described previously (Strous *et al.* 1998, Chapter 2). Gas measurements were performed by gas chromatography (Interscience, Breda, Netherlands) equipped with two columns (Molsieve 5A and a Hayesep column), an Electron Capture Detector and a Hot Wire Detector (Interscience, Netherlands).

Results

The Sequencing Batch Reactor (SBR) for investigation of CANON under NH_4^+-limitation

Start-up of the CANON system (non-limiting NH_4^+ supply)

To start the CANON process, nitrifying biomass (0.01 g L^{-1}) and oxygen (7.9 mL min^{-1} air) were introduced into an anammox culture, which had been anaerobically oxidising ammonium with nitrite as electron acceptor (feed concentrations 18 mM NO_2^- and 25 mM NH_4^+). The feed was changed to contain NH_4^+-N only (12 mM) and was fed at a rate of 1.0 mmole NH_4^+ L^{-1} h^{-1}. The change in gas flow from helium, during anammox operation, to air in the CANON system resulted in a rapid increase of the dissolved oxygen to around 3% of air saturation (0.24 mg L^{-1}). Within a few hours the oxygen concentration was reduced to below the detection limit (<0.1% sat.), indicating immediate activity of the added nitrifying biomass.

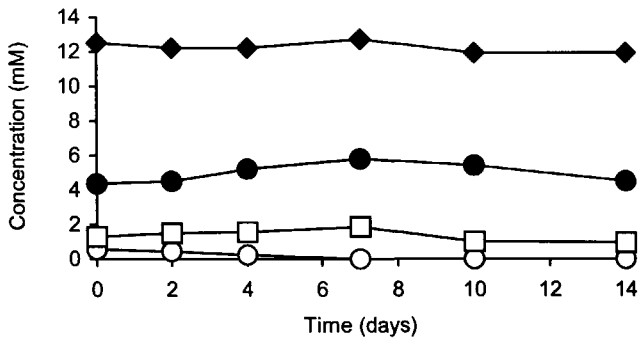

Figure 3.2: Concentrations of ammonium, nitrate and nitrite over time in the CANON reactor with non-limiting ammonium (12 mM) in the feed supply (◆ feed NH4+, ○ NO2-, □ NO3- and ● NH4+).

The concentrations of ammonium, nitrite and nitrate were followed over time for 2 weeks. Ammonium was always present in excess, while nitrite levels gradually reached zero (Fig. 3.2). This indicated that the anammox bacteria were consuming all the available nitrite produced by the aerobic ammonium oxidisers and that a cooperation had been established between the aerobic and anaerobic ammonium oxidizers. The molar ratio of $NO_{3\,produced}^-/NH_{4\,consumed}^+$ was about 0.13, corresponding to the overall CANON stoichiometry (Eqn 3). The nitrogen removal characteristics during the period where ammonium was in excess are shown in Table 3.1. Nitrogen was removed in the SBR at a rate of 0.11 kg N m^{-3} day^{-1}. The nitrogen removal characteristics of the SBR were similar to the removal rates in the chemostat investigated later (± 10%), hence the values given in Table 3.1 are an average of the

rates observed in both the SBR and the chemostat. No nitrite oxidising activity could be detected in the aerobic activity tests in the SBR or chemostat when ammonium was in excess (Row 8, Table 3.1).

CANON under NH_4^+-limitation in the SBR

The ammonium influx was decreased stepwise to investigate the effect of ammonium limitation on the stability of the CANON system. At an ammonium influx of 0.36 mmoles NH_4^+ L^{-1} h^{-1} (feed conc. 5.2 mM NH_4^+), true ammonium limitation was achieved. At the beginning of the SBR cycle the dissolved oxygen increased to values greater than 25% air saturation and then fell slowly, reaching 0 - 3% half way through the cycle (Fig. 3.3). This was due to the changing oxygen transfer capacity (k_La (h^{-1})) of the reactor, caused by the changing volume over the 12-hour cycle (i.e. filling from 1 to 2 L). The presence of high oxygen concentrations during the initial part of the cycle caused a reversible inactivation of anaerobic ammonium oxidation during this period, resulting in accumulation of nitrite to around 1.2 mM (Fig. 3.3). The recovery of anammox activity towards the end of the cycle resulted in removal/reduction of all of the nitrite that had accumulated.

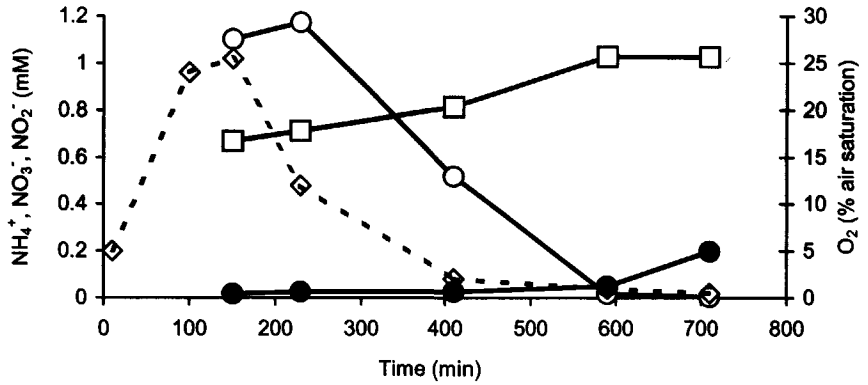

Figure 3.3: **Profile of nitrogen compounds during one SBR cycle with ammonium limitation, showing large fluctuations during one cycle (● NH_4^+, ○ NO_2^-, □ NO_3^- and -◇- DO concentration, as % air saturation).**

Biomass samples were taken 14 days after the onset of NH_4^+-limitation to examine the composition of the bacterial community. Qualitative FISH analysis showed that planctomycete-like anammox bacteria and *Nitrosomonas*-like aerobic ammonium oxidisers dominated the biomass (Fig. 3.4a). The analysis also showed the presence of very small amounts of *Nitrospira* sp. (Fig. 3.4b). The presence of these nitrite-oxidising bacteria was surprising, as aerobic activity tests failed to detect any nitrite oxidising activity (Row 8, Table 3.1).

Table 3.1: Characteristics of CANON nitrogen removal in both the chemostat and SBR (N removal characteristics for the two reactor types were similar (± 10%), hence an average is given).

Reactor characteristic	**NH_4^+-excess** (1 mmole $NH_4^+ L^{-1} h^{-1}$)	**NH_4^+-saturation*** (0.40 mmoles $NH_4^+ L^{-1} h^{-1}$)	**NH_4^+-Limitation** ($\leq$0.36 mmoles $NH_4^+ L^{-1} h^{-1}$)
1. Nitrogen load (kg N m^{-3} day^{-1})	0.22	0.12	0.07
2. Nitrogen consumption (kg N m^{-3} day^{-1})	0.11	0.12	0.07
3. Nitrogen removal (kg N m^{-3} day^{-1})	0.08	0.11	0.04
4. % Nitrogen removal	36	92	57
5. Dry weight conc. (g/L)	1.0	0.92	0.75
Activity Tests			
6. Max. aerobic NH_4^+-oxidising activity**	72	74	33
7. Max. anaerobic NH_4^+-oxidising activity	38	27	7
8. Max. aerobic NO_2^- oxidising activity**	0	0	15

* "NH_4^+-saturation" refers to the situation where ammonium was neither limiting nor in excess.

** Maximum aerobic activity tests were performed in separate shake flask tests with optimum aeration. All activity measurements are given in nmoles mg $protein^{-1}$ min^{-1}.

CANON with prolonged NH_4^+-limitation

The SBR was maintained for a period of two months with a constant NH_4^+-influx of 0.36 mmoles NH_4^+ L^{-1} h^{-1} (NH_4^+-limitation). The concentrations measured daily in the reactor over time (Fig. 3.5) showed there were particularly high concentrations of nitrate present in the reactor. The molar ratio of $NO_3^-{}_{produced}/NH_4^+{}_{consumed}$ in the CANON system with NH_4^+ in excess had been around 0.13, as expected for the CANON system (Eqn. 3). However under prolonged NH_4^+-limitation this molar ratio had increased to around 0.40, indicating increased nitrate production by aerobic nitrite-oxidising bacteria.

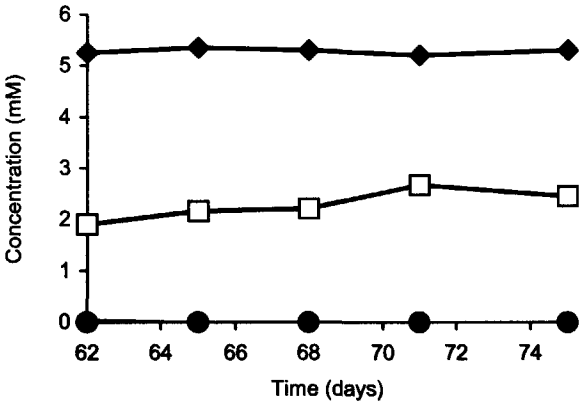

Figure 3.5: Concentrations of ammonium, nitrate and nitrite during operation of the CANON sequence batch reactor under prolonged NH_4^+-limitation (◆ feed NH_4^+, ○ NO_2^-, □ NO_3^- and ● NH_4^+).

Activity tests conducted during the period of NH_4^+-limitation indeed indicated a gradual increase in the aerobic nitrite oxidising activity of the biomass. When ammonium had been in excess, up to 100% of the nitrite produced by aerobic NH_4^+-oxidation was reduced to dinitrogen gas by anammox-like bacteria. After two months of NH_4^+-limitation, the percentage of nitrite reduced anaerobically had decreased to only 31% of the total nitrite produced, and the remaining 69% NO_2^- was oxidised aerobically to nitrate (activities are given in Table 3.1, rows 6-8). The growth of aerobic nitrite-oxidising bacteria in the system due to the ammonium limitation resulted in a reduction of nitrogen removal capacity from 92% to 57% N-removal (row 4, Table 3.1).

After the 3 months of prolonged ammonium limitation, the feed was changed back to an excess of ammonium (12 mM feed, influx 1 mmole L^{-1} h^{-1}). Within one week, the molar ratio of NO_3^-/NH_4^+ gradually decreased from a ratio of 0.4 to its original ratio of 0.1, indicating that aerobic and anaerobic

ammonium oxidising bacteria had re-established their co-operation, leaving no oxygen or nitrite for the aerobic nitrite oxidising bacteria.

The chemostat for investigation of CANON under NH_4^+-limitation

The oxygen transfer rate in the CANON-sequencing batch reactor fluctuated during each cycle due to the changing volume over the 12 hour cycle (continuous filling from 1 to 2 L). The changing oxygen transfer rate resulted in large changes in the dissolved oxygen concentration during one cycle (Fig. 3.3), which made it difficult to determine the changes in bacterial activity and similarly the mass balances over time. To eliminate these "non steady-state" fluctuations, a chemostat reactor system with biomass retention was run in parallel to investigate effects of ammonium limitation on the CANON system. After 30 days, a steady-state with ammonium in excess (0.57 mmoles L^{-1} h^{-1}) at a dilution rate of 0.072 h^{-1} was established. A stable interaction between the aerobic and anaerobic ammonium oxidising bacteria had been reached, as indicated by the molar ratio of $NO_3^-{}_{produced}/NH_4^+{}_{consumed}$ of 0.1 (Eqn. 3). The ammonium consumption rate at steady-state was 0.4 mmoles NH_4^+ L^{-1} h^{-1} (or 0.13 kg N m^{-3} day^{-1}). Interestingly, the ammonium conversion rate was very similar to that observed in the SBR with NH_4^+ in excess (0.11 kg N m^{-3} day^{-1}). From GC measurements it was calculated that around 0.82 moles of O_2 were being used for every mole of NH_4^+, as expected according to CANON nitrogen removal (Eqn. 3).

From day 47 onwards, the NH_4^+-influx was decreased gradually to impose NH_4^+-limitation on the system (Fig. 3.6). The NH_4^+-influx was decreased from 0.40 to 0.21 mmoles L^{-1} h^{-1} on day 50 by increasing the hydraulic retention time from 13.8 hours to 22.8 hours. The decrease in the NH_4^+-influx caused an immediate increase in the oxygen concentration (up to 3.5%), in turn causing an increase in NO_2^- concentration from 0 to 1.6 mM, due to partial inactivation of the anammox bacteria. The biomass was exposed to nitrite and oxygen for 5 days. The nitrite gradually disappeared after 5 days in the presence of oxygen, together with an increase in NO_3^- levels, indicating growth of aerobic nitrite-oxidising bacteria. The HRT of 23 days was maintained for two weeks. At day 70, the HRT was increased further to 30 days, lowering the NH_4^+-influx even further to 0.16 mmoles L^{-1} h^{-1}.

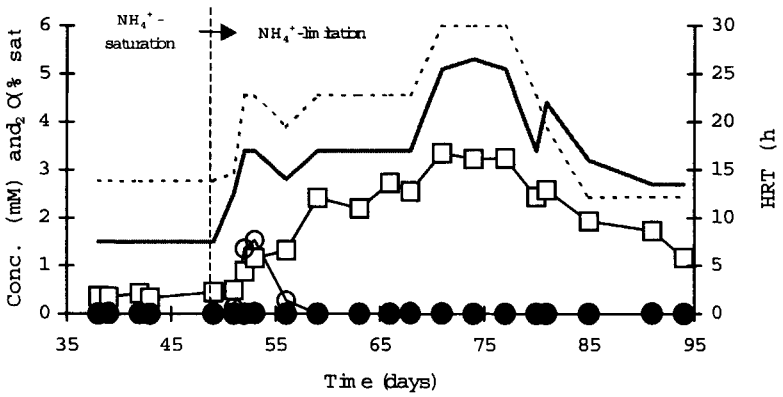

Figure 3.6: Change in nitrogen compounds (● NH_4^+, ○ NO_2^- and □ NO_3^-), O_2 concentration (grey line) and hydraulic retention time (HRT, dotted line) during operation of the CANON chemostat before and during NH_4^+-limitation.

It was interesting to note that the further lowering of the NH_4^+-influx directly increased the oxygen concentration, and in turn increased the nitrate production due to increased aerobic nitrite oxidation (Fig. 3.6). However, when the ammonium limitation was removed (day 85), the nitrate concentration in the reactor decreased accordingly and within one week the cooperation between the aerobic and anaerobic ammonium oxidisers had been re-established, leaving no nitrite or oxygen for the nitrite oxidisers. The shift in bacterial populations caused by the ammonium limitation was completely reversible.

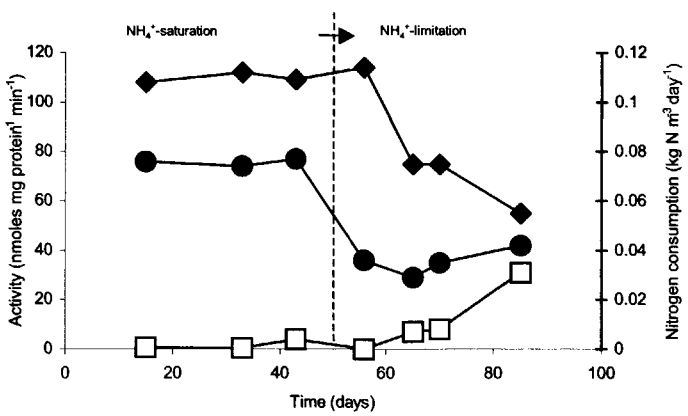

Figure 3.7: Changes in nitrification activity over time, measured in shake flask tests with optimum aeration (● NH_4^+ consumption rate, □ NO_3^- production rate) and total nitrogen consumption (◆).

Aerobic activity tests with optimum aeration were conducted regularly during operation of the chemostat before and after the onset of ammonium limitation (Fig. 3.7). When ammonium was in excess, no aerobic nitrite-oxidising activity was detected in the chemostat, as indicated by the negligible nitrate-production rate. After the onset of NH_4^+-limitation, the aerobic nitrate-production rate increased steadily over time to 25 nmoles NO_3^- mg protein^{-1} min^{-1}. At day 85 in the chemostat, both the aerobic ammonium- and nitrite-oxidising activities were high (Fig. 3.7), and it was suspected that the anaerobic ammonium-oxidising bacteria had been outcompeted from the system. The anaerobic ammonium oxidising activity of anammox had decreased from 27 nmoles NH_4^+ mg protein^{-1} min^{-1} (ammonium not limiting) to 7 nmoles NH_4^+ mg protein^{-1} min^{-1} (row 7, Table 3.1). The anammox bacteria were still present and active, albeit at a low rate. FISH analysis of biomass from the chemostat at day 65 showed the presence of all three bacterial populations (Figs 3.8a, b).

Discussion

The CANON system could effectively remove nitrogen in a single oxygen-limited treatment step. The lower limit for stable nitrogen removal to dinitrogen gas was 0.4 mmoles L^{-1} h^{-1} (0.12 kg N m^{-3} day^{-1}). At and above this ammonium influx, a stable interaction existed between aerobic and anaerobic ammonium oxidising bacteria under oxygen limitation. The system was easy to maintain during the test period (3 months) both in a Sequencing Batch Reactor (SBR)-type as in a chemostat with biomass retention. Retention of the biomass, in the form of flocculated material, is required in the first place by the unusually low growth rate of the anaerobic ammonium oxidisers. At the same time the presence of dense biomass-flocs creates (a)biotic physical and chemical gradients allowing for coexistence and (co)performance of microbial processes and populations with complementary and/or opposed environmental requirements.

During the normal ammonium saturation operation of the CANON system, the aerobic nitrite oxidising bacteria in the flocculated material experience a double limitation of nitrite and oxygen. They compete with the *Nitrosomonas*-like bacteria for oxygen, and with the anammox-like bacteria for nitrite. The aerobic and anaerobic ammonium oxidisers, on the other hand, experience only a single limitation. The *Nitrosomonas*-like bacteria are limited by their oxygen supply, and the anammox-like bacteria are dependent on the aerobic ammonium oxidisers for their nitrite supply (nitrite-limited). As long as the single limitation for each of these two groups of bacteria can be maintained, the CANON system can run effectively, and the nitrite oxidisers will be outcompeted. However, during ammonium limitation both aerobic and anaerobic ammonium oxidisers are limited by their respective electron donor (NH_4^+) *and* their acceptors (O_2 and NO_2^-), due to competition with the nitrite oxidisers.

During ammonium limiting operation, an initial increase in the oxygen and nitrite concentrations occurred in both the chemostat and the SBR. The ammonium influx was not enough for the aerobic ammonium oxidisers to consume all the available oxygen. The remaining oxygen inhibited the anammox bacteria, and nitrite accumulated temporarily (Figs. 3.3, 3.6). The simultaneous presence of excess nitrite and oxygen were perfect for the survival and growth of the nitrite oxidising bacteria. Their competition for nitrite with anammox and for oxygen with ammonium-oxidising bacteria was relieved, and the cells could start to produce nitrate. The interaction between aerobic and anaerobic ammonium oxidising bacteria was interrupted, and nitrogen removal efficiency was reduced from 92% to 57% (Row 4,Table 3.1).

The opportunity for nitrite-oxidisers to develop in the system occurred when the NH_4^+-limitation caused an increase in the dissolved oxygen concentration. This could be prevented in a plant situation by the inclusion of dissolved oxygen-controllers, which control the airflow and prevent the increase in dissolved oxygen if the incoming ammonium concentration decreases. If the dissolved oxygen concentration does not increase, then there is no room for the nitrite oxidisers to proliferate.

Similarly, if the oxygen could be limited until all the nitrite has been anaerobically reduced by anammox-bacteria, the nitrogen removal efficiency could be maintained during low-loading periods. Control of aeration would therefore play an important role at full scale, and is currently being investigated.

Both the sequencing batch reactor and the chemostat achieved very similar nitrogen removal rates. In the "non-steady state" conditions in the SBR during NH_4^+-limitation, the bacteria were exposed to fluctuating availabilities of electron donors and acceptors (Fig. 3.3), however the overall efficiency compared to the chemostat was not affected. Anammox-bacteria were reversibly inhibited by the fluctuating oxygen conditions, yet their activity resumed as soon as the oxygen was no longer inhibiting. Prolonged exposure to oxygen in such systems might lead to the loss of anammox bacteria from the reactor. However, anaerobic activity tests showed that there was still anaerobic NH_4^+-oxidation activity after one month of ammonium limitation and oxygen excess, albeit at a low rate (7 nmoles/mg protein/min; Table 3.1). FISH analysis confirmed that anammox cells were still present in significant numbers (Fig 3.7). Furthermore, the interactive operation between the aerobic and anaerobic ammonium oxidising bacteria could be re-established within one week as soon as the ammonium limitation had been relieved.

Other autotrophic systems for removing ammonium from wastewater include the SHARON-Anammox process (Van Dongen *et al.* 2001) and the OLAND (oxygen limited autotrophic nitrification-denitrification) process (Kuai & Verstraete, 1998). The nitrogen-removal rate observed in this study (0.12 kg N m^{-3} day^{-1}) compares well with the rates reported in the other autotrophic systems (0.8 and 0.05 kg N m^{-3} day^{-1}, respectively). Under ammonium-saturated operation of CANON in the SBR and chemostat, the process was limited principally by the oxygen transfer rate to the aerobic ammonium-oxidisers. A recent investigation looked at increasing the oxygen transfer rate by using a gas-lift reactor for the CANON system. Nitrogen removal rates of up to 1.5 kg N m^{-3} day^{-1} have been reported when the gas-transfer rate is optimised (Chapter 4), showing that the CANON could be a very useful nitrogen removal process for very high strength ammonium wastewaters.

The ability of the CANON system to withstand ammonium limitation for up to one month without irreversible damage shows that the CANON system could be a robust and effective industrial system to remove ammonium from wastewater with a very low organic load.

Acknowledgements

The authors thank Murdoch University for their fellowship during this study, Katinka van de Pas-Schoonen and Markus Schmid (TU Munich) for help with FISH analysis and Marc Strous for critical discussion.

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CANON and Anammox in a gaslift reactor

Previously published as:
Sliekers A.O., Third K.A., Abma W., Kuenen J.G. and Jetten M.S.M. (2003) CANON and Anammox in a gaslift reactor. FEMS letters **218**: 339-344

Abstract

Anammox (Anoxic Ammonium Oxidation) and CANON (Completely Autotrophic Nitrogen Removal over Nitrite) are new and promising microbial processes to remove ammonia from wastewaters characterized by a low content of organic materials (COD).

These two processes were investigated on their feasibility and performance in a gaslift reactor. The Anammox as well as the CANON process could be maintained easily in a gas lift reactor, and very high N-conversion rates were achieved. An N-removal rate of 8.9 kg N $(m^3$ reactor$)^{-1}$ day^{-1} was achieved for the Anammox process in a gas-lift reactor. N-removal rates of up to 1.5 kg N $(m^3$ reactor$)^{-1}$ day^{-1} were achieved when the CANON process was operated. This removal rate was 20 times higher compared to the removal rates achieved in the laboratory previously. Fluorescence In Situ Hybridisation (FISH) showed that the biomass consisted of bacteria reacting to NEU, a 16S rRNA targeted probe specific for halotolerant and halophilic Nitrosomonads and of bacteria reacting to Amx820, specific for planctomycetes capable of Anammox.

Introduction

Anammox is an anoxic microbiological process in which ammonia, together with nitrite, is converted to dinitrogen gas according to reaction 1 (Strous et al, 1998). Also, some nitrate is formed from nitrite. This reaction is thought to be needed for autotrophic CO_2-fixation. Also, it has been suggested that CO_2-fixation can be uncoupled from the catabolic reaction, i.e. the stoichiometric conversion of nitrite and ammonia to dinitrogen gas can proceed without production of cell material and nitrate (Strous et al., 1999a).

$1\ NH_3 + 1.32\ NO_2^- + H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2\ H_2O$ (reaction 1)

The bacteria, shown to be responsible for the Anammox process belong to the order of Planctomycetales. The bacteria are autotrophic and do not need organic carbon to support growth (Kuenen and Jetten, 2001). Although the bacteria are anaerobic, their activity is only reversibly inhibited by oxygen. Furthermore, Anammox bacteria are inhibited by high nitrite concentrations (Van de Graaf et al, 1996)(Strous et al., 1999b).

Anammox-bacteria have been enriched from inocula from different waste water treatment plants and are characterized by a low maximum growth rate, and thus have to be grown in a reactor with sufficient biomass retention (Van Dongen et al. 2001)(Egli et al., 2001). Anammox bacteria have also been detected in several (pilot) wastewater treatment systems with high nitrogen-losses and low COD input (Schmid et al. 2000)(Helmer et al, 2000).

To remove ammonia from wastewater using Anammox bacteria, these bacteria must be provided with sufficient nitrite. Nitrite can be produced from ammonia by aerobic autotrophic ammonia oxidizing bacteria, according to reaction 2 .

$1\ NH_3 + 1.5\ O_2 \rightarrow 1\ NO_2^- + H_2O + H^+$ (reaction 2)

However, bacteria oxidizing ammonia to nitrite need oxygen, whereas bacteria converting ammonia and nitrite to dinitrogen gas are anaerobic. It was recently shown that both types of bacteria can co-exist in one reactor, provided that the system was kept oxygen limited. The process is called CANON, which stands for Completely Autotrophic Nitrogen removal Over Nitrite (Chapter 2)(Chapter 3). This process appeared to be particularly suitable for the removal of ammonia from wastewater that does not contain enough organic material to support the conventional Nitrification/Denitrification process (Chapter 2). Ammonia is partly oxidized to nitrite by oxygen limited aerobic ammonia oxidizers, according to reaction 2. The nitrite produced, together with a part of the remaining ammonia, is converted to dinitrogen gas by Anammox bacteria according to reaction 1, leading to the overall reaction 3 (reaction 3 and figure 1).

$1\ NH_3 + 0.85\ O_2 \rightarrow 0.11\ NO_3^- + 0.44\ N_2 + 0.14\ H^+ + 1.43\ H_2O$ (reaction 3)

In order to maintain the oxygen-limitation in practice, the ammonia influx to such reactors is maintained higher than the oxygen influx (Chapter 2). In laboratory-scale CANON Sequencing Batch Reactors, relatively low N-conversion rates have been reached until now. It was evident that the gas-liquid mass transfer of oxygen was the rate-limiting step in these reactors (Chapter 2). Gas-Lift reactors are reported to have a relatively high gas-liquid mass transfer of oxygen (Garrido et al, 1997). Therefore, the current study was performed to evaluate the performance of a gaslift reactor carrying out the Anammox and the CANON process.

Material and Methods

Gaslift reactor and set-up.

A 1.8 L gas lift reactor was used (Tijhuis et al., 1994). No special device for biomass retention was mounted or present. Synthetic wastewater (described below) was added at the top of the reactor. Gas was sparged from the bottom of the reactor with at a maximum gas flow of 200 ml/min for fluidization of the biomass. 95 % Ar /5% CO_2 (Hoekloos, Schiedam, the Netherlands) and compressed air were used as gases (Table 1). When the reactor was kept anoxic, only Ar/CO_2 was used. When oxygen limited conditions were needed, Ar/CO_2 mixed with air, or solely air was used. Oxygen was measured using a Clark-type electrode (Ingold, Zurich, Switzerland). The oxygen concentration was controlled by manual variation of the air supply.

When only CO_2/Ar was used, the pH was measured but not controlled. When the gas contained air, pH was adjusted with 0.5 M Na_2CO_3 and 1 M HCl, using a ADI 1020 bio-controller (Applicon, Schiedam, The Netherlands).

Synthetic wastewater

Synthetic wastewater was prepared by adding ammonia and nitrite to a mineral medium in the form of $NaNO_2$ and $(NH_4)_2SO_4$. The composition of the mineral medium was (gL^{-1}): $KHCO_3$ 1.25, KH_2PO_4 0.025, $CaCl_2.2H_2O$ 0.3, $MgSO_4.7H_2O$ 0.2, $FeSO_4$ 0.00625, EDTA 0.00625 and 1.25 mL L^{-1} of trace elements solution(Chapter 2). Synthetic wastewater A was used for studying the Anammox process. It contained therefore $(NH_4)_2SO_4$ (6.4 g L^{-1}) as well as $NaNO_2$ (6.75 g L^{-1}). To study the CANON process under oxygen-limited conditions, wastewater B was used, containing only $(NH_4)_2SO_4$ (7.3 g L^{-1}) , but no nitrite.

Origin of Biomass

The biomass used for inoculation with anaerobic ammonia oxidizing bacteria originated from an Anammox Sequencing Batch Reactor in which 80% of the biomass consisted of planctomycete-like Anammox bacteria (Strous et al. 1998). Biomass with aerobic ammonia oxidizing bacteria was obtained from an oxygen-limited ammonia oxidizing sequencing batch reactor (Chapter 3).

Experimental setup

The evaluation of the gaslift reactor experiments consisted of two parts. During the first period, the gaslift reactor was kept anoxic and during the second period, the reactor was kept oxygen-limited.

In the first three months, the anoxic gaslift reactor was used in order to grow and maintain a stable consortium of bacteria capable of Anammox. During this period biomass retained in the 20 L effluent flask was returned manually to the reactor. After the initial period, limited amounts of air were

carefully introduced to support activity and growth of aerobic ammonia oxidizers. During this period, the goal was to achieve simultaneous aerobic/anaerobic ammonia oxidation. The performance of the reactor was evaluated at least every week and this was done by monitoring the ammonia, nitrate and nitrite concentrations in the influent and effluent and by subsequent calculation of the N-removal rate. Biomass was not returned to the reactor in this period.

Chemical analysis

The concentration of ammonia, nitrite and nitrate was determined colorimetrically using standard procedures (Chapter 2). Dry weight was measured after drying filtered biomass in a microwave for 10 min at 300 W.

Fluorescence In Situ Hybridisation (FISH)

Biomass samples were fixed immediately for 2 to 3 hours with 4% (w/v) paraformaldehyde. FISH-analysis and DAPI staining was carried out as described by Juretschko et al.(1998). The 16S rRNA gene probes used were Amx820 (Schmid et al. 2000), NIT3 (Juretschko et al. 1998), NEU (Juretschko et al. 1998), Nsp436 (Juretschko et al. 1998) (Stephen at al. 1998) and Ntspa1026 (Juretschko et al. 1998). All gene probes were labelled with either of the fluorophores Cy3 and Fluorescine and were purchased from Interactiva (Ulm, Germany). To estimate the relative amount of some bacterial groups (e.g. nitrite oxidizers), the DAPI stained cells of two flocs per fixed sample were counted, as well as the fluorescently stained cells of interest.

Results

Ammonia removal under anoxic conditions.

To investigate the possibility to grow and stably maintain Anammox-biomass in a gaslift reactor, the reactor was filled with synthetic wastewater without nitrogen compounds and was inoculated with Anammox biomass. The reactor was kept anoxic and at a pH of 7.5 by sparging with a mixture of 95% Argon and 5% CO_2. Accordingly, the oxygen-concentration in the reactor remained always below the detection limit. After inoculation, the Anammox activity of the biomass was checked by adding ammonia and nitrite to the reactor at 14 mg-N l^{-1} each. After one hour, when all of the added nitrite and most of the ammonia had been converted, synthetic wastewater A with nitrite and ammonia was pumped continuously into the reactor.

At the beginning of the experiment, the superficial gas flow was relatively low, 0.00324 m h^{-1} (40 ml min^{-1}), to ensure that the biomass aggregates were not disrupted by the relatively high gas flows (and thus high shear-forces) used. At this gas flow, the aggregates were fully suspended. The gas flow was then manually increased during the first days to a maximum of 0.015 m h^{-1} (200 ml min^{-1}). It appeared that this had no significant effect on the activity of the Anammox-biomass. Higher gas-flows were not tested.

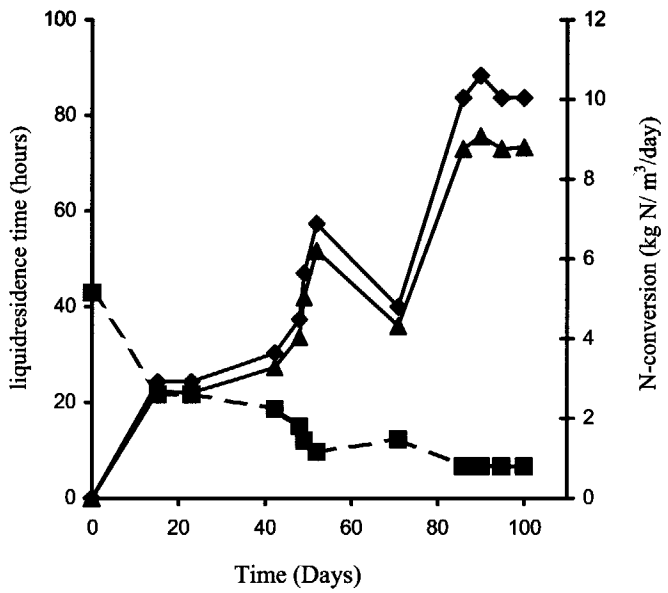

Figure 4.1: N-load (♦), Liquid residence time(■) and N-conversion(▲) during the anoxic period in the reactor (first part of the experiment).

The liquid residence time at the beginning of the experiment was 43 hours. During the experiment the nitrogen-loading rate was gradually increased. To increase the loading-rate, the liquid residence time was gradually decreased, while the ammonia and nitrite concentration in the inflowing synthetic wastewater were kept constant. During the first part of the experiment the N-loading rate could be increased to 10.7 (±0.3) kg N $(m^3$ reactor$)^{-1}$ day^{-1} (Fig. 4.1), and at that time (day 86-100) the ammonia consumption rate was 3.8 kg NH_3-N $(m^3$ reactor$)^{-1}$ day^{-1}, and the nitrite consumption rate was 5.1 kg NO_2^--N $(m^3$ reactor$)^{-1}$ day^{-1}. The total nitrogen removal rate was 8.9 (±0.2) kg N $(m^3$ reactor$)^{-1}$ day^{-1}. The liquid residence time at that moment was 6.7 hours. The ratio between ammonia consumption and nitrate production was 0.2. Nitrite was present at a very low concentration of 4 (±1.5) mg l^{-1}. The N-loss was therefore as high as 80% during this part of the experiment. No aerobic ammonium and aerobic nitrite oxidisers were detectable by Fluorescence In Situ Hybridization. The biomass consisted mainly (>80%) of Anammox-bacteria, which reacted with the Amx820 fluorescent probe.

Ammonia removal under oxygen limited conditions.

After 100 days of succesful operation of the anaerobic gaslift reactor, it was decided to investigate ammonia removal under oxygen-limited conditions and therefore the composition of the synthetic wastewater was changed, so that it only contained ammonia (synthetic wastewater B). The gas was then changed to a mixture of Ar/CO_2 and air (Table 4.1), and a pH-control device was installed.

Table 4.1: Gas-composition, oxygen concentrations in the bulk liquid and N-removal rate in the gaslift reactor. The first 100 days, the gaslift was operated in Anammox mode. At day 100, nitrifying biomass was added, and the mode was switched to CANON.

Day no.	Gas composition and flow (ml min^{-1})		N-loss (%)	N-removal rate (kg N $(m^3_{reactor})^{-1}day^{-1}$)
	air	Ar/CO_2		
0-100	0	200	93[1]	8.9[2]
101	10	200	90	0.3
103	30	170	ND[3]	ND[3]
104	75	100	95	0.8
107	200	0	93	1.5
111	200	0	92	1.5
111-161	200	0	92[1]	1.5

[1] avarage value

[2] maximal removal

[3] not determined

In order to establish a mixed culture of bacteria capable of both aerobic and anaerobic ammonia oxidation, nitrifying biomass (0.25 g dw l^{-1}) was added to the Anammox reactor. Shortly after the addition of air to the influent gas, the oxygen concentration in the bulk liquid increased to 4.4 mg l^{-1}.

The amount of air in the gas was gradually increased (Table 4.1). This was done to prevent too high oxygen concentrations and to prevent too abrupt high nitrite production rates, which could lead to high nitrite concentrations in the reactor. Too high nitrite and oxygen concentrations would inhibit bacteria capable of anaerobic ammonia oxidation (Strous et al. 1997)(Strous et al 1999b). After three days, when the bulk oxygen concentration dropped from 4.4 mg l^{-1} to 3.7 mg l^{-1}, indicating that the specific oxygen consumption rate of the biomass had increased, the Ar/CO_2 was removed from the influent gas and the gas consisted of only air from this moment onwards. The oxygen concentration in the reactor decreased further and after one week the oxygen concentration in the reactor stayed constant at a value of about 0.5 mg l^{-1}, indicating that the specific oxygen consumption rate of the biomass had now dramatically increased. The combined aerobic/anaerobic process was stable within 7 days and the N-conversion stayed at 1.5 kg $(m^3 \text{ reactor})^{-1}$ day^{-1} during a period of 60 days and longer periods were not tested (Table 4.2).

Table 4. 2: Values of different parameters during steady-state of the oxygen-limited period (day 111-161)

Parameter	Value
NH_4^+-concentration in feed (mg N l^{-1})	1545 ± 62
Oxygen-concentration (mg l^{-1})	0.5 ± 0.07
NH_4^+-concentration (mg.N l^{-1})	899 ± 61
NO_2^--concentration (mg.N l^{-1})	6 ± 2
NO_3^--concentration (mg. N l^{-1})	45 ± 7
NH_4^+/NO_3^--ratio	0.07 ± 0.01
HRT (hours)	10
Dillution-rate (per hour)	0,1
N-load (kg-N day^{-1})	3.7 ± 0.15
N-conversion (kg N $(m^3 \text{ reactor}^{-1})$ day^{-1})	1.5 ± 0.2
N-removal efficiency (%)	42 ± 4.7

During this period of 60 days the process-parameters were as listed in Table 4.2. A high excess of ammonia was observed (899 mg NH_3-N l^{-1}) and a relatively low conversion efficiency (42%). This high excess had been maintained intentionally to ensure oxygen-limitation. At the start of oxygen-limited operation of the reactor, it was not known how high the maximum N-removal rate would be at a gas flow of 0.015 m h^{-1} (200 ml $min.^{-1}$). The ratio between ammonia consumption and nitrate production was 0.07. The N-loss was, as expected for CANON, high and was 39% of the N-load, and 93% of the N-conversion.

FISH showed that the biomass consisted of mainly anaerobic Anammox bacteria reacting with probe Amx820 and aerobic ammonia oxidizers reacting to probe NEU, but a very small population of aerobic nitrite oxidizers reacting with Ntspa1026, specific for some *Nitrospira*-species, was also present (<2%). No reaction was observed with the probes Nsp436 and NIT3 indicating the absence of *Nitrosospira* and *Nitrobacter* like bacteria.

Discussion

During the first part of the experiment, it was tried to maintain Anammox in a gaslift reactor and to maintain a as high as possible N-conversion rate. It became clear that a relatively high ammonia removal rate (8.9 kg N $m^3_{reactor}{}^{-1}$ day^{-1}) can be achieved, and to our knowledge, such a high volumetric conversion rate for Anaerobic Ammonia Oxidation has never been reported before. Compared to other reactor setups and other processes the volumetric N-removal rate is very high as well (Table 4.3).

Table 4.3: Overview of the N-conversion in kg N $(m^3$ reactor$)^{-1}$ day^{-1} in different reactor set-ups.

Process	Reactor	N-conversion	Reference
Single autotrophic processes			
Anammox	FBR[1]	4.8	Van de Graaf et al (1996)
Anammox	SBR[1]	7	Strous (pers.comm)
Anammox	Gaslift	8.9	This paper
Nitrification	BAS[1]	5[2]	Tijhuis et al. (1994)
Combined autotrophic processes			
CANON	SBR[1]	0.07	Chapter 2
CANON	Gaslift	1.5	This chapter
SHARON	CSTR[1] + SBR[1]	1	Van Dongen (2001)
OLAND	SBR[1]	0.05	Kuai & Verstreate (1998)
Deammonification	RBC[1]	0.3	Seyfried et al. (2001)
Combined autotrophic/heterotrophic processes			
N/D[1]	BAS[1]	3.75	Van Benthum et al. (1998)

[1] FBR = Fluidised Bed Reactor, SBR = Sequencing Batch Reactor, BAS = Biofilm Airlift System, CSTR = Continuous Stirred Tank Reactor, N/D = Nitrification / Denitrification, RBC= Rotating Biological Contactor.

[2] This is the ammonium removal rate. In all other cases, the total nitrogen removal is presented.

The nitrate production/ammonium consumption ratio was somewhat lower than expected. A ratio of 0.2 was observed, whereas a ratio of 0.3 was expected based on the stoichiometry for Anammox (reaction 1), as calculated from experiments in a Sequencing Batch Reactor (Strous et al. 1998). As nitrate production is thought to be coupled to biomass production, this might be an indication that the conditions in a gas lift reactor are not as optimal for supporting growth of anaerobic ammonia oxidizers as were the conditions in an SBR. However, on the basis of the high N-conversion rates achieved, it is clear that a gas-lift reactor is suited to maintain and grow bacteria capable of

Anammox. Probably, an even higher N-conversion rate could be achieved when a better biomass retention is applied.

During the second part of the experiment it became clear that a gaslift reactor is also very well suited for the CANON process. The nitrate production/ammonia removal ratio was again somewhat lower than can be expected from the predicted CANON-stoichiometry (reaction 3), which might be due to reduced growth of anaerobic ammonia oxidisers. Apparently, this has no effect on the stability of the process, since the process could be maintained easily for two months, and probably much longer. A very small population of aerobic nitrite oxidizers was present, but their activity must have been very low. The presence of this small population can be caused by the higher bulk oxygen concentration compared to previous studies with the CANON system with excess of ammonia (Chapter 2, Chapter 3). The absence of a large and active population of nitrite oxidizers at ammonia excess is in agreement with the predictions of the model of the CANON system (Hao et al. 2002).

The major rate-limiting step was probably still the oxygen-transfer from the gas to the liquid. This can be concluded from the fact that there was a large excess of ammonia. Higher N-removal rates might be achieved when the gas-liquid oxygen transfer coefficient could be increased further. Another possibility is that the specific area of the flocs is too small to achieve a good liquid-floc mass transfer of oxygen. However, no quantification of the flocs and specific area were conducted during this experiment to adress this question.

The amount of nitrifying biomass may be also a rate limiting factor, because the oxygen concentration was low (below 0.5 mg l^{-1}) but not zero. The amount of biomass can be increased by applying a better biomass-retention. Previous experiments showed that when an SBR, with good biomass retention, is used to perform the CANON process, the oxygen concentration can fall below the detection limit, i.e. below 0.04 mg l^{-1} (Chapter 2). Nevertheless, it is confirmed that, when a gaslift reactor with very good gas-liquid transfer capabilities was used, like in this study, N-removal rates can be increased.

Compared to other set ups (Table 4.3), a good N-conversion rate was achieved. The N-conversion was 20 times higher as compared to CANON in an SBR, which is probably due to lower oxygen mass transfer rates in the SBR. Compared to SHARON-Anammox, CANON in a gaslift is slightly better for removal of ammonia from high strength wastewater streams. Moreover, CANON uses one reactor, whereas for SHARON-Anammox, two reactors are needed. In addition, the N-conversion of SHARON-Anammox is limited by the maximal strength of the wastewater being treated. Compared to Nitrification/Denitrification, the N-removal rate of CANON is lower. However, to support denitrification, COD is needed, which is not always present in sufficient amounts in the wastewater, and addition of costly exogenous carbon sources, such as methanol, is needed.

A new ammonia removal process has been applied in this study, with less oxygen demand and without organic carbon demand (Chapter 2) in one single reactor. In this chapter, it was shown that this new process is suited for treatment of high strength wastewater. Moreover, the high nitrogen

removal capacity of this process enables compact reactor design, resulting in lower investment costs. Still, factors like maximum oxygen transfer rates and biomass retention are good candidates for optimisation.

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Chapter 5

Nitrifying bacteria at the oxic/anoxic interface

Submitted as:

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Nitrifying bacteria at the oxic/anoxic interface.

Abstract

Nitrification, i.e. the oxidation of ammonia to nitrate via nitrite was studied under very low or limiting oxygen conditions, using mixed cultures and different reactor set-ups. The community composition was analyzed using Fluorescent In Situ Hybridisation (FISH). At low oxygen concentrations (2-8 μM O_2), *Nitrosomonas* and *Nitrospira* cells converted ammonia completely to nitrate. Under these conditions, hardly any N_2O or NO was produced. The nitrite oxidizing bacteria, but not the ammonia oxidizing bacteria, were characterized by a very low K_s (1.3 μM) for oxygen. Under oxygen limitation, with ammonia in excess, ammonia was converted to mainly nitrite by *Nitrosomonas* like cells, which had now a very low K_s (2.3 μM) for oxygen as well. NO and N_2O were emitted; up to 3% and 10% of the NH_4^+-N converted, respectively. It became clear that these two situations, i.e. low oxygen conditions and oxygen limitation, were very distinct but fully reversible.

Introduction:

Nitrification is known as an aerobic process, during which ammonia is converted to nitrate by chemolithotrophic bacteria. Aerobic ammonia oxidizing bacteria (AOB) like *Nitrosospira* and *Nitrosomonas* oxidize ammonia to nitrite, and aerobic nitrite oxidizing bacteria (NOB) like *Nitrospira* and *Nitrobacter* oxidize nitrite to nitrate.

The natural habitat of the nitrifiers is on one hand any aerobic environment where sufficient ammonia is becoming available (i.e. from mineralization processes) to support their growth. On the other hand, nitrifiers can be found at the oxic/anoxic interface where the ammonia, mineralized from anaerobic degradation meets the oxygen. At such interfaces, the volumetric activity of nitrifiers is relatively high (Lorenzen et al., 1998). Usually, the nitrifying bacteria are cultivated under fully oxygenated conditions, but it is likely that most of the nitrifying bacteria have adapted to the low and limiting amounts of oxygen, present at the oxic/anoxic interface. In addition, it has been observed that aerobic ammonia and nitrite oxidizers keep some of their activity in anoxic environments (Bock et al. 1995)(Bock et al. 1988)(Schmidt et al., 2001). *Nitrosomonas* cells, for example can use nitrite as electron acceptor when ammonium or hydrogen are provided as electron donor (Bock et al., 1995). Nitrifiers have been detected in small numbers in systems, which were anoxic for several years (Abellovich 1985, 1987). Both AOB and NOB could also be readily enriched from anoxic sludge, which showed anaerobic ammonium oxidation activity (Van der Graaf et al. 1996, Chapter 2) It appears that nitrifying bacteria are well suited to be active at very low oxygen concentrations and that they can survive anoxic conditions for a very long time.

When oxygen is limiting, both AOB and NOB have to compete for oxygen. At low oxygen concentrations, pure cultures of the nitrite-oxidizer *Nitrobacter winogradsky* appeared to be a poor competitor for oxygen compared to aerobic ammonia oxidizers (Laanbroek and Gerards, 1993). The mixotrophic *Nitrobacter hamburgiensis*, however, was a better competitor for oxygen. In the presence of organic matter, it was able to maintain itself in high cell numbers in a mixed culture. However, the affinity (the ratio V_{max}/K_s) of *Nitrobacter hamburgiensis* was lower than the affinity of *Nitrosomonas* (Laanbroek et al., 1994). Only at the highest dilution-rate the affinity of *Nitrobacter hamburgiensis* was almost as high as the affinity of *Nitrosomonas* (Laanbroek et al., 1994).

During the last decades, it became clear that the metabolism of aerobic ammonia oxidizers and aerobic nitrite oxidizers is more complex than just oxidizing ammonia to nitrite and nitrite to nitrate, respectively. Aerobic ammonia oxidizers are known to produce N_2O and NO at reduced concentrations of oxygen (Jørgensen et al., 1984)(Goureau et al., 1980). N_2O is a persistent greenhouse gas and aerobic ammonia oxidizers can be responsible for the largest part of the N_2O production in some ecosystems (Davidson 1986, Downes, 1988). However, from experiments presented in literature, it is difficult to predict the production of NO and N_2O at a certain oxygen concentration, due to the fact that only the oxygen concentrations of the headspace were measured

(Jørgensen et al., 1984), or due to the usage of pure cultures which were mostly isolated from fully oxygenated systems (Goureau et al., 1980).

Knowledge about nitrification at low or limiting oxygen concentrations is important for both natural and man-made ecosystems such as wastewater treatment systems. In conventional wastewater treatment systems ammonia is oxidized to nitrate and nitrate is reduced again to nitrite and further reduced to dinitrogen gas (Van Loosdrecht and Jetten, 1998). In this way, large amounts of oxygen and COD, needed for the oxidation from nitrite to nitrate and the subsequent reduction of nitrate again to nitrite, are sometimes required. Application of a relatively high dilution rate (1 d^{-1}) in reactor systems appeared to be a good way to prevent the oxidation of nitrite to nitrate. This concept was tested on pilot and full scale systems in a process called SHARON (Van Dongen et al., 2001, Mulder 2001). It was shown that lowering the oxygen concentration can also lead to formation of nitrite instead to nitrate during oxidation of ammonia (Bernet et al., 2001). Nitrite can be subsequently reduced to dinitrogen gas. The reduction of nitrite can be achieved by adding COD to support denitrification. Relatively recently, it has been discovered that nitrite can also be converted together with ammonia to dinitrogen gas by chemolithotrophic bacteria capable of anaerobic ammonia oxidation (Mulder et al., 1995)(Van de Graaf et al., 1996)(Strous et al., 1999). In the latter case, no additional COD is needed and a two step process of SHARON and anammox has been proposed for ammonia removal from waste water (Van Dongen et al. 2001). The cooperation of aerobic and anaerobic ammonia oxidation can also be achieved in the CANON process, which is performed in a single reactor system, under oxygen limitation (Chapter 2, Chapter 3, Chapter 4, Schmidt et al. 2002a, Schmidt et al 2002b, Hao et al., 2002). Under these conditions, the culture is dominated by oxygen limited AOB and planctomycetes, which are responsible for the oxidation of ammonia with nitrite to dinitrogen gas (Chapter 2).

The objective of this study was to get more knowledge about the ecophysiology of nitrifying bacteria under very low oxygen conditions and under oxygen limiting conditions, with special attention to N_2O and NO production during nitrification.

Material and methods

Reactor systems and Control equipment

All reactors:

The temperature was kept at 30 °C. Water evaporation was prevented by cooling the outlet gas via a condensator connected to a cryostat operating at 10 °C. Dissolved Oxygen was measured using a Clark-type oxygen electrode (Ingold, Urdorf, Switzerland). The oxygen probe was calibrated every two weeks. The pH was kept at 7.8 and controlled using 0.5M Na_2CO_3 and 1M HCl with an ADI 1030 Biocontroller (Applicon, Schiedam, The Netherlands).

retentostat

For the retentostats a fermenter of 1.5 L was used. Biomass retention was achieved by placing a pipe with a 2 cm diameter for liquid withdrawal, to achieve liquid/biomass separation in the outlet. Medium was continuously fed into the reactor at a dilution rate of 0.01 h^{-1}.

A mixture of air and N_2 was sparged through the reactor at a gas-flow of 200 ml min^{-1}. Calibrated mass-flow controllers regulated this gas flow. The reactor was stirred at 100 rpm. Biofilms were removed from the fermentor parts and the wall every week.

chemostat

A reactor of 1.5 L was used for the chemostats. A long needle with a diameter of 0.8 mm was used for liquid withdrawal, to prevent liquid/biomass separation in the outlet (see results). Fermentor parts and the fermentor wall were cleaned from biofilms twice a week. The cleaning was done within 15 minutes. The reactor was stirred at 300 rpm. A mixture of air and N_2 was sparged through the reactor at a gas-flow of 100 ml min^{-1}. Under oxygen-limited conditions, the dilution rate (D) was 0.025 h^{-1}. Under low oxygen conditions the dilution rate (D) was 0.005 h^{-1}.

SBR (Sequencing Batch Reactor)

For the SBR, a glass vessel (height 0.21 m, diameter 0.14 m) without baffles, with a 2 L working volume was used. The SBR was run as a continuously fed sequence batch reactor with a 12-hour cycle and retention of biomass. It was filled continuously with fresh medium over 11.5 hours at a flow rate of 1.45 mL min^{-1}. The minimum volume was 1 L, and at the end of the cycle the final volume was 2 L. After the filling period, the stirrer and influent pump were stopped and the biomass aggregates were allowed to settle for 15 minutes. In the remaining 15 minutes of the cycle, an effluent pump removed 1 L of supernatant. The reactor was stirred at 300 rpm. A mixture of air and N_2 was sparged through the reactor at a gas-flow of 20 ml min^{-1}.

Medium

Ammonia was added to a mineral medium in the required amounts (see Results section) in the form of $(NH_4)_2SO_4$. The composition of the mineral medium was (g L^{-1}): $KHCO_3$ 1.25, KH_2PO_4

0.025, $CaCl_2.2H_2O$ 0.3, $MgSO_4.7H_2O$ 0.2, $FeSO_4$ 0.00625, EDTA 0.00625 and 1.25 mL/L of trace elements solution. The trace element solution contained (g L^{-1}): EDTA 15, $ZnSO_4.7H_2O$ 0.43, $CoCl_2.6H_2O$ 0.24, $MnCl_2.4H_2O$ 0.99, $CuSO_4.5H_2O$ 0.25, $NaMoO_4.2H_2O$ 0.22, $NiCl_2.6H_2O$ 0.19, $NaSeO_4.10H_2O$ 0.21, H_3BO_4 0.014, $NaWO_4.2H_2O$ 0.05. The separate components of the medium were autoclaved at 120° C to avoid bacterial growth in the feed vessels, although the reactors were not run aseptically.

Origin of Biomass

Biomass for inoculation was taken from CANON reactors, running at oxygen limitation (Sliekers et al. 2002, Third et al. 2001)

Chemical analysis

The concentration of ammonia, nitrite and nitrate was measured colorimetrically (Chapter 2). Protein was measured according to the method of Lowry. (Lowry et al. 1951) Nitrous oxide, oxygen and dinitrogen gas were measured with a Gas Chromatograph (Fisons Instruments) equipped with a Molsieve 5A, a Hayesep column, an Electron Capture Detector and a Hot Wire Detector (Intersience, Breda, The Netherlands). NO_X gasses were measured using chemiluminescense. (Kester et al., 1994)

Kinetic measurements

Oxygen consumption rates and the corresponding oxygen and ammonia concentrations were measured in a 5 ml biological oxygen monitor equipped with a Clark type oxygen electrode. Cell suspensions were prepared from 10 times concentrated culture suspensions by washing with medium without ammonia and passing several times through a 0.4 mm diameter needle to disrupt the small flocs. Ammonia and nitrite consumption rates and their corresponding concentrations were also measured in a 50 ml Erlenmeyer flask with culture suspension, using colorimetric ammonia, nitrite and nitrate determinations. Kinetic parameters were calculated using the computer program Grafit 3.0 (Erithacus Software Ltd, Horley, UK)

Fluorescence In Situ Hybridisation (FISH)

Biomass samples were fixed immediately for 3 hours with 4% (w/v) paraformaldehyde (Amann, 1995). FISH-analysis and DAPI staining were carried out as described by Juretschko (Juretschko et al. 1998). The fluorescent 16 S rRNA gene probes (Interactiva, Ulm, Germany) used were Pla46 (Schmid et al. 2000), specific for planctomycetes; Nit1035 (Juretschko et al.1998), specific for *Nitrobacter* cells; Neu 653 (Juretschko et al. 1998), specific for Salt-tolerant *Nitrosomonas* cells; Nsp436 (Stephen et al. 1998), specific for Nitrosospira cells; and Ntspa1026 (Juretschko et al. 1998); specific for *Nitrospira* cells. Percentages of fractions, reacting with a specific probe, were calculated as described previously (Schmid et al., 2000)

Results

Nitrification at the oxic/anoxic interface in a retentostat

The retentostat was inoculated with bacteria from a CANON reactor. In the first few weeks an increasing activity of ammonia oxidation to nitrite was observed. The mixture of air and nitrogen was adjusted in such a way that the oxygen concentration was always 2.3 μM (± 1 μM), and in such a way that the superficial gas-flow was kept constant. After 18 days, also nitrite oxidation to nitrate could be observed. Finally, all the ammonia from the medium was converted to nitrate at a constant oxygen concentration of 2.3 μM (Figure 5.1).

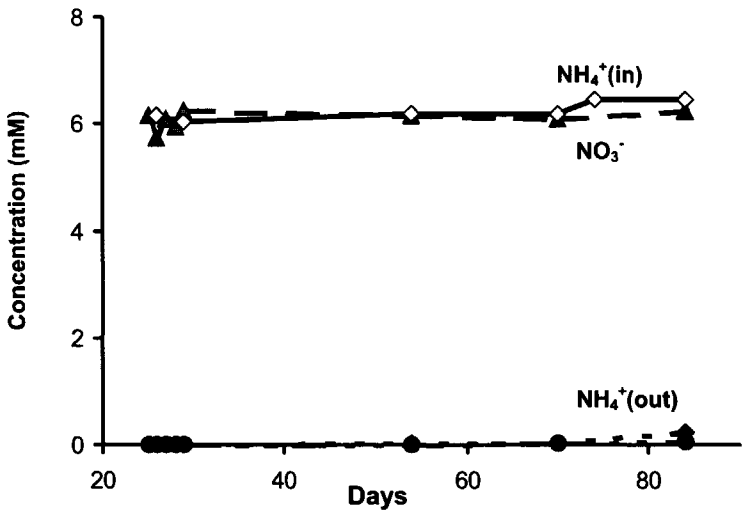

Figure 5.1: Steady state of nitrification at 2.3 μM O_2 in a retentostat. In this situation, the bacteria experienced ammonia limitation and low oxygen concentrations. All of the ammonia consumed was converted to nitrate, and hardly any N_2O and NO was emitted. At day 70, the inflowing NH_4^+ concentration was slightly increased to show that the culture was close to oxygen limitation. Exact values are presented in Table 5.1 situation A of the retentostat.

At steady state conditions, hardly any N_2O or NO was produced (Table 5.1, situation A). At steady state conditions the K_s and V_{max} of the biomass were determined (Table 5.2). The V_{max} was also corrected for the fraction size of the AOB and NOB respectively (Table 5.2). The fraction size was determined using FISH and DAPI staining. FISH and DAPI staining showed that the AOB, made up about 65% of the cells, and they belonged to the salt tolerant *Nitrosomonas* group, reacting with the NEU probe. The NOB were mainly *Nitrospira* (Ntspa1026; 15% of the population), and *Nitrobacter* (NIT3; 3% of the population).

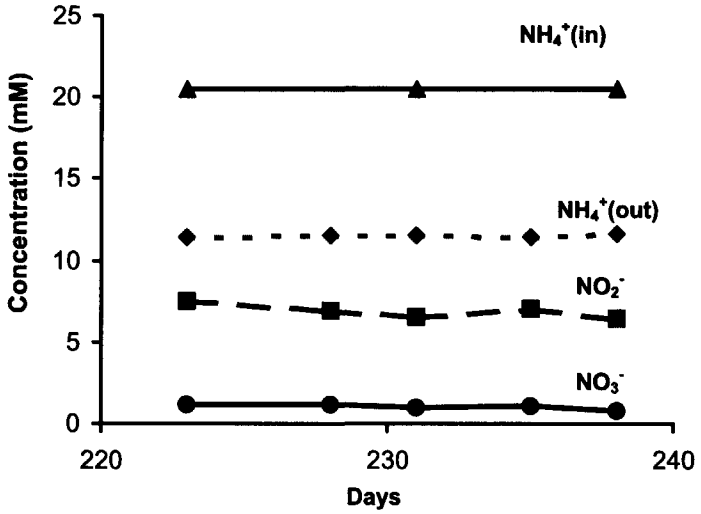

Figure 5.2: Steady state of nitrification in a retentostat at ammonia in excess and oxygen limitation. Almost all of the ammonia consumed was converted to nitrite, 3% of the consumed N was converted to NO and 10% to N_2O. Exact values are presented in Table 5.1 situation B of the retentostat.

In order to create oxygen limiting conditions, the ammonia concentration in the medium was increased to 20 mM, while the gas composition as well as the gas-flow was kept constant. After a few weeks, ammonia was converted mainly to nitrite (Figure 5.2). A relatively small amount of nitrate was still present, but no nitrite-oxidation activity could be measured anymore. Also, a reasonable part of the ammonia was not oxidized, indicating an oxygen limitation. Indeed, oxygen was now below detection-level (<1 μM).

Table 5.1: steady state values of N-compounds (mM) in the reactor. Values within brackets are standard deviations.

situation	NH_4^+ in	NH_4^+ out	NO_2^- out	NO_3^- out	NO[a] out	N_2O[a] out
Retentostat						
A (low oxygen[c])	6.25(0.07)	0.01(0.01)	0 (0.01)	6.21 (0.1)	0.01	0.015
B (oxygen limitation[d])	20.5	11.5(0.08)	6.9(0.44)	1(0.18)	0.3	0.5
Chemostat						
A (low oxygen[c])	5	0.01(0.005)	0.18(0.15)	4.6(0.3)	ND[b]	ND[b]
B (oxygen limitation[d])	18.1(0.7)	16(0.9)	1.65(0.4)	0.3(0.2)	0.03	0.1

[a] calculated by dividing the amount produced per hour by the fluid flow ($L\ h^{-1}$)

[b] not determined

[c] O_2-concentration = 2,3 μM

[d] O_2-concentration < 1μM

A significant production of nitrous oxide (10% of the NH_4^+-N consumed) and nitric oxide (3% of the NH_4^+-N consumed) was now observed (Table 5.1).

FISH analysis showed that the AOB were *Nitrosomonas* cells, like in the first situation. NOB were not detected. Under steady state conditions the ammonia-oxidizing bacteria had a $K_s(O_2)$ of 2.3 μM. As can be seen in Table 5.2 the affinity for oxygen (V_{max}/K_s) increased two times, but the affinity for the substrate ammonia decreased about 5 times. The V_{max} stayed constant. Surprisingly, the affinity (V_{max}/K_s) for oxygen of the ammonia oxidizers was still lower than that of the nitrite oxidizers under ammonia and nitrite limited conditions.

Table 5.2: Kinetic parameters of the biomass during the different pseudo steady states in the retentostat. Values within Brackets are standard deviations. Situation A and B of the retentostat are the same situations as presented in Table 5.1

parameter	Situation A		Situation B	
	AOB	NOB	AOB	NOB
$K_s\{O_2\}$ [a]	6.1 (0.3)	1.3 (0.8)	2.3 (0.6)	-[f]
$K_s\{S\}$ [b]	113	15	620	-[f]
$V_{max}\{O_2\}$ [c]	146	34	146	0
$V_{max}\{S\}$ [b]	97	67	106	0
$V_{max}\{O_2\}$ [d]	225 (25)	189 (25)	170 (15)	0
Affinity O_2 [e]	37	145	74	-[f]
Affinity NH_4^+	0.86	-	0.17	-[f]

[a] in μM

[b] substrate

[c] in nmol (mg $prot^{-1}$) min^{-1}

[d] V_{max} corrected for the fraction size of AOB or NOB

[e] V_{max}/K_s

[f] NOB cells not present.

Nitrification in a chemostat.

Real competition can only be studied in a chemostat in which the hydraulic retention time equals the solids retention time, although this situation probably does not occur in nature or man made environments very often. Nitrifying bacteria tend to form aggregates and easily stick to the wall and fermentor parts, making a chemostat with suspended cells difficult to achieve. A needle of 0.8 mm diameter was placed into the reactor as a medium outlet to ensure high liquid flow in the outlet, even at low dilution rates. This prevented liquid/biomass separation in the outlet. Secondly the fermentor parts were cleaned twice a week to prevent biofilm accumulation.

The results from the chemostats were very similar to the results obtained in the retentostats (Table 5.1). During oxygen limitation ammonia was mainly converted to nitrite whereas during

ammonia limitation (O_2 = 2.3 ± 1 μM), ammonia was converted to nitrate. N_2O and NO were only produced during oxygen limitation (< 1 μM O_2). FISH analysis showed that during low oxygen but ammonia limiting conditions, the ammonia oxidizers were *Nitrosomonas* cells and that the nitrite oxidizers were *Nitrospira* cells.

Reversibility and NO-toxicity.

In nature, oxygen concentrations and fluxes can change, and a SBR was started to check the effect of changing conditions and to check the reversibility of the process. First, oxygen limitation was achieved (O_2 < 1 μM) and alternating conditions with low oxygen (O_2 = 8 μM) or oxygen limitation (Figure 5.3).

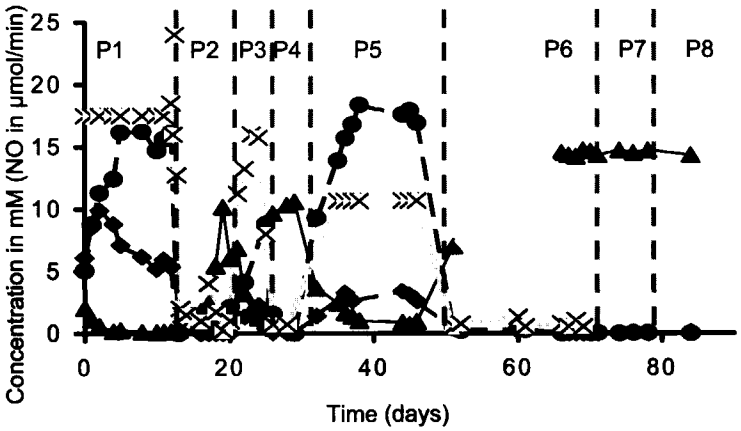

Figure 5.3: Fluctuation in NH_4^+ (diamonds), NO_2^-(circles), NO_3^- (triangles) and NO (crosses) in the SBR during several pseudo steady states. P1, P3, P5 are periods with oxygen limitation and ammonia excess. Ammonia is then converted to mainly nitrite but also NO is emitted (N_2O results not shown). P2, P4 and P6 are periods with low oxygen conditions and ammonia limitation. No NO is produced and all ammonia is converted to nitrate. P7 and P8 are the same as P2, P4 and P6, but in these periods NO is added at a concentration of 20 and 40 ppm, respectively. This had no effect on the formation of nitrate.

Under oxygen limitation, ammonia was mainly converted to nitrite and some N_2O and NO were produced. Ammonia was in excess. Under low oxygen conditions and ammonia limitation at the same time, ammonia was converted to nitrate and hardly any NO and N_2O production was observed, like in the previous experiments. When the system was again under oxygen limitation, nitrite was produced, instead of nitrate and again there was N_2O and NO production albeit in lower amounts than in the first steady state. Placing the system back to low oxygen conditions resulted in nitrate production, but hardly any NO production.

To check the effect of nitric oxide on the biomass, NO was continuously added to a concentration of 20 ppm, and later 40 ppm, in the ingoing gas stream at the end of the experiment. NO addition did have no effect on nitrite-oxidizers. Ammonia was still completely converted to nitrate.
At day 13 and 28, cells were fixed for FISH analysis. From FISH analysis, it could be concluded that cells reacting with the NEU probe were the bacteria responsible for ammonia oxidation, while *Nitrospira* cells were responsible for nitrite oxidation. A small fraction of *Nitrobacter* cells were also detected, but *Nitrospira* cells were far more dominant. The nitrite oxidizing population was not present at day 13 but was present at the 28th day, after a period of ammonia-limitation. A few cells reacting to the probe specific for planctomycetes, pla46, were also present, but in very low amounts.

Discussion:

The nitrite oxidizers were in most cases *Nitrospira*-like cells. The ecological niches of *Nitrospira* and *Nitrobacter* are not yet very well defined. *Nitrobacter* cells could be favored under mixotrophic conditions (Laanbroek et al., 1994) or high nitrite and oxygen conditions, like in batch enrichments (Juretschko et al., 1998). The results in our study suggest that one of the ecological niches for *Nitrospira* is low oxygen and nitrite limitation.

To our knowledge, this is the first study, which clearly showed that nitrite-oxidizers can be cultivated successfully at very low oxygen concentrations. However, the activity of nitrite-oxidizing bacteria becomes very low when they have to compete for oxygen with aerobic ammonia oxidizers. Due to the fact that biomass retention was applied, the effect of washout could be prevented, resulting in a situation, more realistic to natural environments. Nitrifying bacteria easily attach to many different materials, probably to prevent washout, and to compensate for relatively low maximum growth rates. Biomass retention, however, did not influence the composition of the N-products going out of the reactor. In the chemostat, the retentostat and in the SBR, the formation of nitrite instead of nitrate was observed under oxygen limiting conditions. Also, in all systems, N_2O and NO production were only observed under oxygen limiting conditions.

At steady state conditions during oxygen limitation, the ammonia-oxidizing bacteria had a $K_s(O_2)$ of 2.3 μM. The affinity (V_{max}/K_s) for oxygen increased two times, compared to the low oxygen situation, but the affinity for the substrate ammonia decreased about 5 times. This is in accordance with the fact that the bacteria were now in an oxygen-limited situation with excess of ammonium. The V_{max} stayed constant. Apparently the bacteria are not capable of altering the V_{max}, by producing more enzymes, but can change the K_s by producing enzymes, including oxidases and mono-oxygenases, with a lower K_m. Another possibility is that a closely related but different population of ammonia oxidizers was now present. The (eco)physiological background of this phenomenon was not further investigated. The V_{max} is quite low as compared to some V_{max}-values reported in literature. In fact, it is five times lower than the highest maximum rates reported (Jetten et al. 1997). However the K_s for oxygen is also much lower than the values normally reported. This means that the bacteria present in the experiment convert ammonia faster at low oxygen concentrations. Surprisingly, the affinity (V_{max}/K_s) of the AOB for oxygen was still lower than the affinity for oxygen of the NOB. Although the NOB have a much lower yield compared to the yield of AOB (Hao et al., 2002), preliminary calculations show that this difference in yield cannot explain the fact that AOB win the competition in our experiments. Since the results indicate that NO is not toxic for nitrite oxidizers, something else might be toxic for nitrite oxidizers such as the excess of ammonia during oxygen limitation (Anthonisen et al., 1976). Another reason might be that the K_s-values are too low to allow an exact determination, due to the detection limit of a Clark-type oxygen electrode. In fact, Table 2 shows that at very low K_s values, large standard deviations are present. Also, deviations in the fraction size of fluorescently stained cells cannot be circumvented. These deviations do not allow an exact calculation

of the competitiveness of the different groups of bacteria. Modeling should give insight in the possible situations obtainable within the range of the standard deviations. Moreover, the effect of ammonia on nitrite oxidation deserves further investigation. It is reasonable though, that when a third group of bacteria, the anaerobic ammonia oxidizers (anammox), is present, which competes for nitrite with the nitrite oxidizers, that nitrite oxidizers cannot win the competition anymore. This is proven to be the case in models and experiments with CANON systems at oxygen limitation (Hao et al., 2002)(Chapter 2, Chapter 3).

The two situations, i.e. formation of mainly nitrite under oxygen limited conditions and the formation of mainly nitrate under low oxygen conditions, were completely reversible. This is an advantage in wastewater treatment practice. A change in the ammonia or oxygen flux has no irreversible consequences. By adjusting the oxygen or ammonia influx, the system can be directed to the desirable situation again, irrespective of the reactor configuration.

Complete nitrification of ammonia to nitrate can be achieved at very low oxygen concentrations. Thus, the concentration difference between the ingoing gas and the liquid can be kept high, leading to a higher oxygen transfer rate.

Under oxygen limited conditions, the formation of the environmentally hazardous gasses, NO and N_2O, was observed. This seems to be a disadvantage to apply oxygen limitation in wastewater treatment practice. However, in CANON systems, where a mixture of oxygen limited AOB and anammox bacteria were active at very low intermediate NO_2^- concentrations, no N_2O and NO were produced (Chapter 2). Apparently, the presence of Planctomycetes capable of anaerobic ammonia oxidation seem to influence the NO and N_2O production of systems in which aerobic ammonia oxidation is taking place under oxygen limitation by preventing nitrite accumulation (Schmidt et al, 2002a). When nitrite oxidizers were present, which oxidize the nitrite further to nitrate, N_2O and NO were not produced. The same observation was done in previous experiments with pure cultures of nitrite and ammonia oxidizers (Ketser et al., 1997). The exact mechanism, regulation and the role of N_2O and NO production by aerobic ammonia oxidizers are still unknown (Schmidt et al., 2002b).

Acknowledgements

The authors would like to thank Markus Schmid and Katinka van de Pas for help with the FISH analysis and Rob van Spanning, Bertus Beaumont, Hendrikus Laanbroek, Anette Bollman and Marc Strous for the valuable discussion. We also would like to thank the ALW for the financial support (project number:80533422/P).

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Chapter 6

Nitrite oxidizing bacteria at limiting oxygen concentrations: inhibition and modelling

Submitted as:
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Abstract

During nitrification in wastewater treatment plants, periodic build up of nitrite can cause severe inhibition of the activated sludge biomass. The mechanism behind nitrite accumulation in oxygen-limited systems fed with ammonia was investigated, using two chemostats with nitrite oxidizing bacteria. One reactor was oxygen-limited and the other was nitrite-limited. Inhibition of nitrite oxidation was studied by addition of ammonia, NO and N_2O to these cultures. No inhibition of free ammonia up to 20 mM of total ammonia and ammonium was observed. Also, no inhibitory effect of N_2O was observed, with the concentrations tested (0.01% v/v). Inhibition by NO was not observed due to consumption by chemical or biological reactions. The possible role of variation in kinetic parameters on nitrite build up was studied by mathematical modeling. This was done with a suspended cell model using previously determined kinetic parameters (Chapter 5). The modeling showed that, within deviation of the measured parameters, two situations were possible. One in which all nitrogen was converted to nitrate and another situation in which nitrite oxidizing bacteria were inactive resulting in nitrite accumulation. Application of a biofilm model gave essentially the same results. Presumably, the affinity (V_{max}/K_s) for oxygen of nitrite oxidizing bacteria was previously overestimated.

Introduction:

Insight into the process of nitrification at limiting oxygen concentrations is important to understand natural as well as man-made ecosystems such as wastewater treatment systems. In natural systems, oxygen-limiting conditions are frequently observed (Thamdrup & Dalsgaard, 2002). During nitrification in wastewater treatment plants, periodic build up of nitrite can cause severe inhibition of the activated sludge biomass. On the other hand, in wastewater treatment practice, permanent oxygen limitation can be used to intentionally limit or even circumvent nitrite oxidation (Garrido et al., 1997). This is practiced in processes like the CANON (Completely Autotrophic Nitrogen removal Over Nitrite) and OLAND processes (Chapter 2)(Kuai and Verstraete, 1998).

Nitrification is known as a two-step oxic process, during which ammonia is converted to nitrate by two groups of chemolithotrophic bacteria. Aerobic ammonia oxidizing bacteria (AOB) like *Nitrosospira* and *Nitrosomonas* oxidize ammonia to nitrite, and aerobic nitrite oxidizing bacteria (NOB) like *Nitrospira* and *Nitrobacter* oxidize the nitrite further to nitrate (Bock and Wagner, 2001) (Koops and Pommerening-Röser, 2001) (Watson et al. 1989) Under anoxic conditions, Planctomycete-like bacteria can convert nitrite and ammonia to dinitrogen gas. This process is known as anammox, an acronym for anaerobic ammonia oxidation (Strous et al. 1999)(Kuenen and Jetten, 2001)(Jetten et al, 1997).

Both AOB and NOB have to compete for oxygen, when it is present in limiting amounts. It was shown that lowering of the oxygen influx leads to formation of nitrite instead of nitrate during oxidation of ammonia, in mixed microbial communities (Bernet et al., 2001)(Chapter 5). The nitrite-oxidizer *Nitrobacter winogradskyi* appeared to be a poor competitor for oxygen compared to pure cultures of aerobic ammonia oxidizers (Laanbroek and Gerards, 1993). During oxygen limitation, the ammonia supplied in these experiments was partially oxidized to nitrite, but not to nitrate. The mixotrophic *Nitrobacter hamburgensis* however, was a better competitor for oxygen (Laanbroek et al. 1994). In presence of organic matter at high cell numbers it was able to maintain itself in mixed cultures during oxygen limitation, and part of the ammonium was oxidized completely to nitrate. However, the affinity (the ratio V_{max}/K_s) of *Nitrobacter hamburgiensis* was lower than the affinity of *Nitrosomonas* (Laanbroek et al., 1994). In mixed microbial communities of ammonia and nitrite oxidizing bacteria, oxygen limitation resulted in the formation of nitrite, instead of nitrate (chapter 5). The hypothesis that this was due to a higher affinity for oxygen of the AOB could not be substantiated due to experimental shortcomings. Although the half saturation constants for oxygen of the NOB were lower ($K_s(O_2)$ =1.8 μM) compared to the constants of the AOB ($K_s(O_2)$ =2.3 μM), the values measured had large standard deviations (± 0.6 μM) due to the limited sensitivity of the oxygen sensors used, and the possible errors in quantification of bacterial cells using FISH (chapter 5).

Experiments as well as mathematical modeling showed that nitrite oxidizers are also poor competitors in co-cultures of anaerobic and aerobic ammonia oxidizers (Hao et al. 2002)(Chapter

2)(Chapter 3) In this case, nitrite oxidizers have two competitors. They have to compete for oxygen with the aerobic ammonia oxidizers and for nitrite with the anaerobic ammonia oxidizers. In the situation that ammonia also becomes limited, and thus aerobic and anaerobic ammonia oxidizers have to compete for ammonia, nitrite oxidizers will reappear and become active as well (Chapter 3).

When oxygen is the only limiting compound in a system, which is fed with ammonia as the energy source, ammonia will automatically be in excess. This is not depending on whether the ammonia will be oxidized to nitrite (Laanbroek and Gerards, 1993) or to nitrate (Laanbroek et al. 1994). The inhibitory effect of free ammonia on the nitrifying microorganisms was postulated a long time ago (Lees, 1963). It is assumed that the NOB are more sensitive than the AOB to free ammonia inhibition (Anthonisen *et al.*, 1976). High concentrations of ammonium are believed to be the main responsible factor causing the nitrite accumulation in the nitrification/denitrification process carried out via nitrite (Fdz-Polanco et al, 1996).

It has been documented that aerobic ammonia oxidizers produce N_2O and NO under oxygen limitation (Goureau et al, 1980) (Chapter 5). These gasses might also be inhibiting for nitrite oxidizing activity. However, the amounts of these gasses present in cultures can become very low when NOB are also present (Kester et al. 1997). Moreover, NO did not inhibit nitrite oxidizers in other investigations (Chapter 5, Stüven *et al.* 1992).

The objective of this study was to investigate the effect of ammonia, NO and N_2O on NOB and also to model the interactions between the ammonia and nitrite oxidisers, using the kinetic parameters previously determined (Chapter 5).

Material and methods

Continuous culture

Two reactors of 1.5 L were used as chemostats for cultivation of a mixed nitrite oxidizing culture. A long needle with a diameter of 0.8 mm was used for liquid withdrawal. Fermentor parts and the fermentor wall were cleaned to remove biofilms twice a week. The cleaning was done within 15 minutes. The reactors were stirred at 300 rpm. The temperature was kept at 30 °C. Water evaporation was prevented by cooling the outlet gas via condensators connected to a cryostat operating at 10 °C. Dissolved Oxygen was measured using a Clark-type oxygen electrode (Ingold, Urdorf, Switzerland). The oxygen probes were calibrated every two weeks. The pH was kept at 7.8 and controlled using 0.5M Na_2CO_3 and 1M HCl with an ADI 1030 Biocontroller (Applicon, Schiedam, The Netherlands). Air was fed into the reactor at a rate of 20 ml/min in one reactor, and a combination of N_2 (40%) and air (60%) at a rate of 20 ml/min in the other reactor. The gas flow rate was kept constant using mass-flow controllers (Brooks Instrument BV, Veenendaal, The Netherlands).

Media

Nitrite was added to a mineral medium in the required amounts (see Results section) in the form of $NaNO_2$. The composition of the mineral medium was (g L^{-1}): $KHCO_3$ 1.25, KH_2PO_4 0.025, $CaCl_2.2H_2O$ 0.3, $MgSO_4.7H_2O$ 0.2, $FeSO_4$ 0.00625, EDTA 0.00625 and 1.25 mL/L of trace elements solution. The trace element solution contained (g L^{-1}): EDTA 15, $ZnSO_4.7H_2O$ 0.43, $CoCl_2.6H_2O$ 0.24, $MnCl_2.4H_2O$ 0.99, $CuSO_4.5H_2O$ 0.25, $NaMoO_4.2H_2O$ 0.22, $NiCl_2.6H_2O$ 0.19, $NaSeO_4.10H_2O$ 0.21, H_3BO_4 0.014, $NaWO_4.2H_2O$ 0.05.

Origin of Biomass

Biomass for inoculation of the chemostat culture was obtained from the nitrifying second stage of the wastewater treatment plant "Dokhaven" of the city of Rotterdam, the Netherlands. The average concentrations of nitrite and nitrate in this stage are 0 and 30 mg $N.l^{-1}$, respectively.

Batch experiments

Samples were taken from the reactors and centrifuged at 16,000 rpm for 40 minutes. The cells were then washed with synthetic wastewater and re-suspended in half of the volume to concentrate the samples. Hepes-buffer (pH=7.8) was added to an end concentration of 30 mM. The batch experiments were performed in 58 ml serum bottles. The suspended cells (10 ml) with nitrite (2 mM) were added to the bottles. Different possible inhibitors were added to different bottles. The bottles were closed with rubber stoppers. 50 µl of gaseous NO or N_2O (100%) were added through the rubber stop with a

syringe (to a concentration of 0.1%). In case ammonia inhibition was studied, ammonia was added to an end concentration of 8 mM. The bottles incubated at 30 °C and samples were collected every 30 minutes. Gas samples were collected from the head-space of the bottles and analysed by gas chromatography for NO and N_2O. Control experiments, without any NO, N_2O or ammonia, were performed simultaneously.

Chemical analysis

The concentration of ammonia, nitrite and nitrate was measured colorimetrically (Chapter 2). Protein was measured according to the method of Lowry. (Lowry et al. 1951) The OD was measured at 420 nm.

Kinetic measurements

Oxygen consumption rates and the corresponding oxygen and ammonia concentrations were measured in a 5 ml biological oxygen monitor equipped with a Clarck type oxygen electrode. Cell suspensions were prepared from 10 times concentrated culture suspensions by washing with synthetic wastewater without ammonium and passing several times through a 0.4 mm diameter needle to disrupt the small flocs. Ammonia and nitrite consumption rates at different concentrations were also measured in a 50 ml Erlenmeyer flask, using colorimetric ammonia, nitrite and nitrate determinations. Kinetic parameters were calculated using the computer program Grafit 3.0 (Erithacus Software Ltd, Horley, UK)

Fluorescence In Situ Hybridisation (FISH)

Biomass samples were fixed immediately for 3 hours with 4% (w/v) paraformaldehyde (Schmid et al. 2000). FISH-analysis and DAPI staining were carried out as described previously (Schmid et al, 2000). The fluorescent 16 S rRNA gene probes (Interactiva, Ulm, Germany) used were Nit1035 (Juretschko et al.1998), specific for *Nitrobacter* cells and Ntspa1026 (Juretschko et al. 1998); specific for some of the *Nitrospira* cells. Percentages of fractions, reacting with a specific probe, were calculated as described previously (Schmid et al., 2000)

Modeling and simulations.

Two models were used. One model simulated a retentostat with suspended single cells. The model was implemented in the computer language C. The expressions used are listed in Table 6.1. The expressions were continuously evaluated using a step size of 1 minute, according to figure 6.1. Smaller steps did not result in different results, whereas larger steps resulted in deviations and the accuracy was lower. Nitrogen incorporation into the biomass was neglected as well as maintenance, endogenous respiration and anaerobic metabolism. The kinetic parameters used were taken from chapter 5.

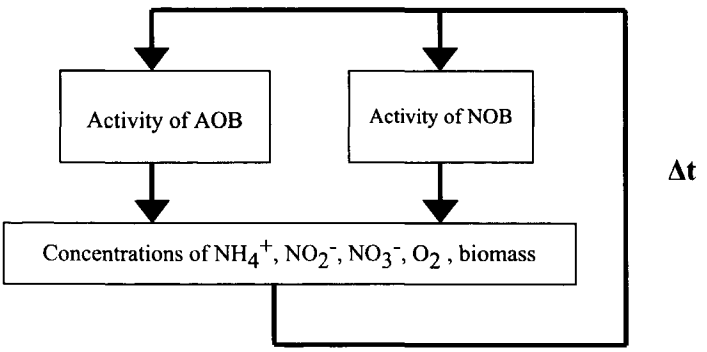

Figure 6.1: Schematic overview of the single cell model.

Table 6.1a: Expressions of the processes used in the simplified single cell model.

Process	Process rate
Activity of aerobic ammonia oxidisers (Vaob)	$V_{max}^{AOB}\{O_2\} \cdot \frac{S(O_2)}{K_{s\{O_2\}}^{AOB} + S(O_2)} \cdot \frac{S(NH_4^+)}{K_{s\{NH_4^+\}}^{AOB} + S(NH_4^+)} \cdot X_{AOB}$
Activity of aerobic nitrite oxidisers (VNOB)	$V_{max}^{NOB}\{O_2\} \cdot \frac{S(O_2)}{K_{s\{O_2\}}^{NOB} + S(O_2)} \cdot \frac{S(NO_2^-)}{K_{s\{NO_2\}}^{NOB} + S(NO_2^-)} \cdot X_{NOB}$
Change in oxygen concentration	$-V_{AOB} - V_{NOB} + K_L a \cdot (S(O_{2\{in\}}) - S(O_2)) - S(O_2) \cdot F$
Change in ammonia concentration	$-\frac{V_{AOB}}{1.5} + S(NH_4^+\{in\}) \cdot F - S(NH_4^+) \cdot F$
Change in nitrite concentration	$-\frac{V_{NOB}}{0.5} + \frac{V_{AOB}}{1.5} - S(NO_2^-) \cdot F$
Change in nitrate concentration	$-\frac{V_{NOB}}{1.5} + S(NO_3^-) \cdot F$
Change in biomass concentration (NOB)	$\frac{V_{NOB}}{0.5} \cdot Y_{NOB} - (1-R) \cdot X_{NOB} \cdot F$
Change in biomass concentration (AOB)	$\frac{V_{AOB}}{1.5} \cdot Y_{AOB} - (1-R) \cdot X_{AOB} \cdot F$

A second model was used, describing the processes in a biofilm with nitrite, ammonia and anaerobic ammonia oxidizers (Hao et al., 2002). This model was implemented in AQUASIM (Reichert et al., 1998). The oxygen concentration was 0.6 mg/l. The processes for anaerobic ammonia oxidation were inactivated when we used this model. Also, the kinetic parameters for oxygen were changed according

to previous studies (Chapter 5). The μ_{max} was estimated from the V_{max} and the yield (chapter 5). All other kinetic parameters, biofilm and mass transfer parameters were the same as described previously (Hao et al., 2002).

Table 6.1b: The values of the parameters used in the simplified single cell model.

Parameter	Value
Reactor volume	1 liter
Influent flow (F)	0.0002 l/min
Effluent flow (F)	0.0002 l/min
Influent NH_4^+ concentration	10 mM
Biomass retention (R)	0-100 %
Initial AOB concentration (X_{AOB})	5 mg
Initial NOB concentration (X_{NOB})	5 mg
Oxygen transfer coefficient (K_La)	/min
$S(O_2)max$	230 μM
V_{max} (O_2) AOB	see results section
Ks {O_2} AOB	see results section
Ks {NH_4^+} AOB	100 μM
Ks (NO_2-) NOB	50 μM
Ks {O_2} NOB	see results section
V_{max} (O_2) NOB	see results section
Yield NOB (Ynob)	0.0003 mg protein/μmol NO_2^-
Yield AOB (Yaob)	0.0012 mg protein/μmol NH_4^+

Results

Two chemostats were filled with medium and inoculated with nitrifying activated sludge from the WWTP "Dokhaven". Nitrite (10 mM) was added to both reactors. When nitrate production was observed, the reactors were run at a dilution rate of 0.015 h^{-1}. After two weeks the chemostats were in steady state as judged from stable concentrations of N-compounds in the reactor. One of the two reactors was kept nitrite limited and fed with an influent of 30 mM nitrite. The bacteria in this reactor converted all of the nitrite to nitrate at an oxygen concentration of 140 µM. The other reactor was kept oxygen limited and fed with an influent of 25 mM nitrite, and 5 mM of nitrite was not consumed (Table 6.2). The oxygen concentration in this reactor was 4 µM. Samples were taken for FISH analysis and were analyzed with the fluorescently labeled 16 S rRNA gene probes Nitspa1026 and NIT1035. In both reactors, the culture appeared to contain a mixture of *Nitrospira* and *Nitrobacter* species. However, *Nitrobacter* dominated the nitrite limited reactor, whereas *Nitrospira* was the dominant nitrite oxidizer in the oxygen limited reactor (Table 6.2). The V_{max} and K_s of the biomass was determined and as expected, the mixed culture from the oxygen limited reactor had a lower apparent K_s for oxygen (Table 6.2). Moreover, the oxygen limited culture also had a higher V_{max} (Table 6.2).

Table 6.2: Different parameters of the two chemostats and the biomass present in these chemostats.

Parameter	Nitrite limited reactor	Oxygen limited reactor
NO_2^- in influent (mM)	30	25
NO_2^- in effluent (mM)	0	5
V_{max} (nmol NO_2^- /mg prot/min)	90	140
$K_s(O_2)$ (µM)	62.5	6.1
Nitrobacter %[a]	69	32
Nitrospira %[a]	31	68

[a] percentage of the nitrite oxidizers

To determine the effect of NO and N_2O on the rate of nitrite oxidation, biomass was taken from both reactors and put into gas tight bottles. N_2O or NO was added to the headspace. There was no influence of these gasses on the nitrite oxidation rate, compared to the controls in which only air was present. At the end of the experiment the headspaces were analyzed for NO and N_2O. In the bottles, to which N_2O was added, N_2O was still present in the headspace at the initial concentration of 0.01 % v/v. In the bottles amended with NO, this compound was no longer present at the end of the experiments, after one hour. A chemical or biological process had consumed the NO gas. Similar inhibition experiments were conducted to check the influence of ammonia (8mM) on the rate of nitrite oxidation. Again, no inhibition was observed. To determine the influence of ammonia on the growth of nitrite oxidizers,

ammonia was added to both chemostat cultures after three months of continuous operation. The total ammonia concentration applied was 20 mM. No change in the nitrite or nitrate concentrations was observed within 48 hours. In addition, the nitrite oxidizing bacteria bacteria were not washed out of the reactors, and the OD did not change, indicating that the cultures remained in steady state and hence that growth was still occurring at the same rate. During the time these experiments were conducted, no ammonia oxidation activity was observed.

The inhibition experiments did not give conclusive answers about the mechanism responsible for the absence of nitrite oxidizers under oxygen limiting conditions in previous studies (Chapter 5). The most obvious explanation for this absence is a better affinity for oxygen of the ammonia oxidizers. However, previous measurements indicated that this is not necessarily the case. Therefore, these results were validated using mathematical modeling. A simple model was constructed, describing a nitrifying retentostat, to check what kind of results could be expected within the standard deviations, previously reported. With a biomass concentration of 5 mg/l and a biomass retention of 90% (0.9), the K_s and the V_{max} of both organisms were changed in each run of the model. Within the standard deviations of previous reported kinetic parameters, different situations could be obtained (Table 6.3).

Table 6.3: Kinetic parameters used and results of the modelling with the simplified single cell model (influent NH_4^+ = 10 mM).

Situation	Parameter				Results			
	Vmax AOB	**Vmax NOB**	**K_s AOB**	**K_s NOB**	**NH_4^+**	**NO_2^-**	**NO_3^-**	**O_2**
	nmol/mg/min	nmol/mg/min	µM	µM	mM	mM	mM	µM
1	170	190	2.3	1.3	6.9	0.1	3.0	0.37
2	170	190	1.7	1.3	6.9	0.2	2.9	0.28
3	170	190	1.7	2.1	5.9	3.9	0.2	0.28
4	190	165	1.7	2.1	5.9	4.1	0	0.24

In all runs, ammonia was in excess. The V_{max} was previously determined as 170 (±20) nmol O_2/mg/min for AOB and 190 (±25) nmol O_2/mg/min for NOB, and the K_s for oxygen was determined previously as 2.3 (±0.6) µM for AOB and 1.3 (±0.8) for NOB (Chapter 5). In the first run of the model, the V_{max} and the K_s was set at the mean values for each group of organisms. The ammonia, that was converted, was oxidized completely to nitrate (Table 6.3, situation 1). In the second run, the $K_s(O_2)$ of the ammonia oxidizers was set to the lower limit, but the ammonia oxidizers could still not outcompete the nitrite oxidizers. In the third run the $K_s(O_2)$ of the NOB was set to the higher limit and the K_s of the ammonia oxidizers was set to the lower limit. Now, the ammonia oxidizers could almost completely outcompete the nitrite oxidizers, as observed in previous experiments (Chapter 5). In the fourth run, the $K_s(O_2)$ of both groups of organisms was the same as in the third run, but the V_{max} of the

ammonia oxidizers was set to the higher limit and the V_{max} of the NOB was set to the lower limit. The ammonia was converted to nitrite. Thus, within the standard deviations, nitrite build up as well as ammonia oxidation completely to nitrate is possible. Using the biofilm model (Hao et al, 2002) did not give essentially different results, compared to the simplified model in which single planktonic cells were present.

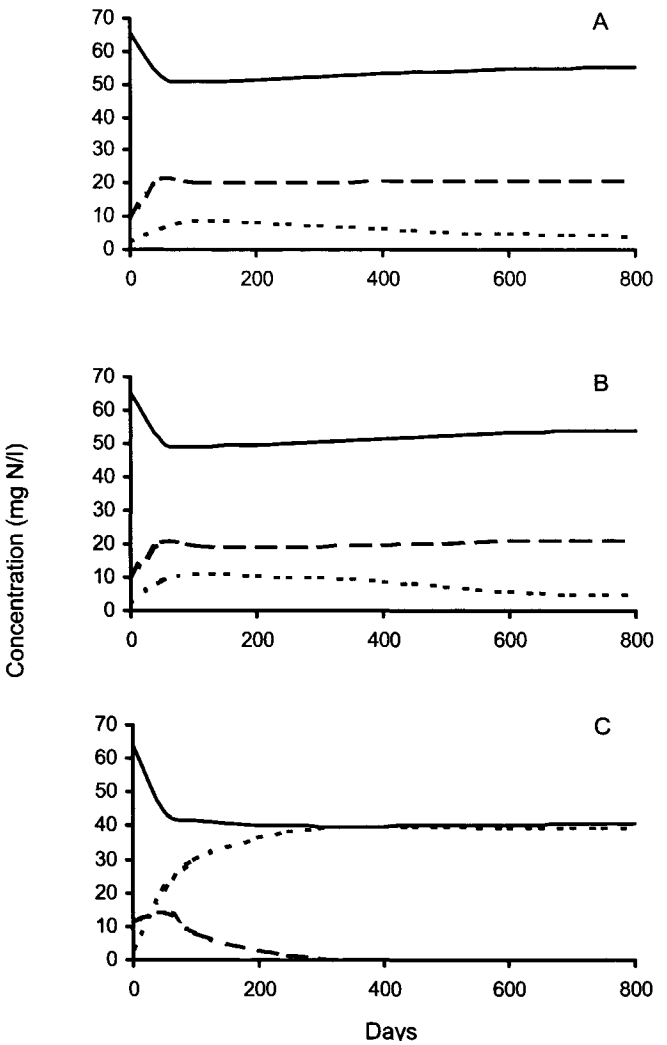

Figure 6.2: Ammonium (solid line), nitrite (stripes) and nitrate (dotted line) concentrations in a modeled biofilm reactor. Situation A,B and C corresponds to situation 1, 3 and 4 from table 6.3, respectively.

Also, nitrite build up as well as conversion to nitrate was observed when the kinetic parameters were changed within the standard deviations (Figure 6.2.). It should be noted that in order to operate this model for our simulations, the anammox reaction was inactivated.

Discussion

The inhibition experiments with ammonia indicated that an ammonium concentration up to 20 mM under the conditions tested, was not inhibiting oxidation of nitrite. The ammonium concentration in the reactors was fairly stable during the time of the experiments. Therefore, growth and activity of ammonia oxidisers during the experiments could be neglected. There is a large controversy on the effect of ammonia. Some reports conclude that ammonia is inhibiting, whereas other investigations did not observe this effect (Table 6.4). In our experiments, ammonia was directly added to a nitrite oxidizing culture whereas the reports, which claim that free ammonia is inhibiting, used mixed cultures of ammonia and nitrite oxidizers. Therefore, the possible occurrence and effects of oxygen limitation, i.e. possible competition for oxygen, in the latter reports cannot be ruled out. Therefore, we assume that ammonia is not inhibiting, and that NOB will likely adapt to high NH_3 concentrations in wastewater treatment practice.

Table 6.4: Reported values and results of free ammonia ($N\text{-}NH_3$) concentrations tested. Values are referred to free ammonia concentration when nitrite accumulation starts (inhibition) or the highest value tested (not inhibiting).

Tested or inhibitory $N\text{-}NH_3$ values ($mg \cdot l^{-1}$)	Remarks	Reference
0.1-1	Inhibiting, mixed cultures of *Nitrobacter* and *Nitrosomonas*	Anthonisen *et al* (1976), Abeling and Seyfried (1992)
0.5	Inhibiting, mixed cultures of *Nitrobacter* and *Nitrosomonas*	Fdz-Polanco et al (1994)
6.1	Not inhibiting, *Nitrobacter vulgaris* in pure cluture	Stüven et al (1992)
14	Not inhibiting, mixed nitrite oxidizing culture	This Paper

N_2O gas was not toxic to the NOB (up to 0.1%). During the experiments with addition of NO gas, NO was not detectable anymore at the end of the experiments. NO could have reacted with oxygen to NO_2 gas, which in turn reacts with water to form NO_2^- and NO_3^-. During the batch experiment the added NO gas was therefore probably converted to nitrite and nitrate, or consumed by the NOB. Without continuous supply of NO gas the effect of NO gas on the nitrite oxidisers could not be measured. To test the effect of NO gas one has to perform experiments with a constant gas flow of NO. This has been done recently with mixed cultures, and no inhibition was observed (Chapter 5). Also in pure cultures of *Nitrobacter vulgaris* , 5 ppmv NO was not inhibitory. Hydroxylamine has been postulated to be inhibitory to nitrite oxidizers at a concentration of 30 μM. However, such high concentrations have not been detected in an ammonium oxidizing culture yet (Stüven et al, 1992)(Bock et al. 1995)(Sliekers, unpublished results). Taken together, it is doubtful whether there is

any inhibitory component present under oxygen limitation in systems with a coculture of AOB and NOB.

Another explanation of the observed absence of nitrite oxidation at oxygen limiting conditions in a system fed with ammonia, is that AOB have better affinity (V_{max}/K_s) for oxygen than NOB. However, previous measurements (Chapter 5) suggested that this was not the case. Interestingly, the $K_s(O_2)$ of oxygen limited NOB, measured in the present study, is higher than the $K_s(O_2)$ of NOB previously measured (Chapter 5). This difference in half saturation constants could be due to different inocula. Therefore, during this study, modelling was performed with the kinetic parameters taken from Chapter 5. During the modelling the kinetic parameters were varied within the standard deviations of the previous measurements. It turned out that two situations were possible: one, in which ammonia was converted to nitrate and another in which ammonia is converted to nitrite. In the first situation NOB have a better affinity for oxygen than AOB and therefore all the nitrite produced by the AOB is further oxidized to nitrate. In the second situation, the affinity for oxygen of the AOB is better than the affinity of NOB, and therefore all of the oxygen is used for ammonia oxidation to nitrite. The latter situation, i.e. oxidation of ammonia to nitrite, resembles the observations in the oxygen limited reactor systems in chapter 5. As inhibitory components could not be found in the present study, it is likely that the affinity of nitrite oxidisers, previously measured (Chapter 5) is overestimated, due to the insensitivity of the oxygen sensor at very low oxygen concentrations. An oxygen microsensor with a guard cathode could have been useful in such experiments, as these electrodes can be very well calibrated and are sensitive to low concentrations of oxygen (Revsbech 1989). The absence of nitrite oxidation activity at oxygen limited conditions in systems fed with ammonia is probably due to a better affinity for oxygen of the aerobic ammonia oxidisers (Laanbroek et al., 1994). A more realistic model which describes the nitrification in a retentostat at low or limiting oxygen concentrations with suspended aggregates could also give more insight, instead of using two models, as done in this chapter, which both do not exactly describe the real situation. A possible alternative explanation is that compounds produced by ammonia oxidising bacteria, known as homeserinelactones (Batchelor et al., 1997), could influence activity of NOB.

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Chapter 7

Nitrification and Anammox with urea as the energy source

Accepted for publication in Systematic and Applied Microbiology as:
A. Olav Sliekers, Suzanne Haaijer, Markus Schmid, Harry Harangi, Karin Verwegen, J. Gijs Kuenen and Mike S.M. Jetten (2003) Nitrification and Anammox with Urea as the Energy source

Abstract

Urea is present in many ecosystems and can be used as an energy source by chemolithotrophic aerobic ammonia oxidizing bacteria (AOB). Thus the utilization of urea by AOB as well as anaerobic ammonia oxidizing (Anammox) bacteria was investigated, using enrichments cultures, inoculated with activated sludge, and molecular ecological methods. In enrichment cultures grown with ammonia a population established in 2 weeks, which was dominated by halophilic and halotolerant AOB as determined by fluorescence in situ hybridization (FISH) experiments, with the 16S rRNA targeting oligonucleotide probe NEU. In other batch enrichment cultures using urea, the AOB population was assessed by PCR amplification, cloning and phylogenetic analysis of amoA and ribosomal 16S genes. While only one of the 48 16S rRNA gene clones could be identified as AOB (*Nitrosomonas oligotropha*), the amoA approach revealed two more AOB, *Nitrosomonas europaea* and *Nitrosomonas nitrosa* to be present in the enrichment. FISH analysis of the enrichment with probe NEU and newly designed probes for a specific detection of *N. oligotropha* and *N. nitrosa* related organisms, respectively, showed that *N. oligotropha* like AOB formed about 50% of the total bacterial population. Also *N. nitrosa* (about 15% of the total population) and *N. europaea* (about 5% of the total population) were relatively abundant. Additionally, continuous enrichments were performed under oxygen limitation. When ammonia was the energy source, the community in this reactor consisted of Anammox bacteria and AOB hybridizing with probe NEU. As the substrate was changed to urea, AOB related to *N. oligotropha* became the dominant AOB in this oxygen limited consortium. This resulted in a direct conversion of urea to dinitrogen gas, without the addition of organic carbon.

Introduction

Urea is an important source of nitrogen in many ecosystems. A variety of vertebrates excrete urea causing large nitrogen inputs (Shand et al., 2002). In marine environments, urea can be the predominant nitrogen source for primary production, and the pool of dissolved urea can be one or two orders of magnitude higher than the ammonia pool (Rees et al., 2002)(Sambrutto, 2000). Urea is also used in agriculture for fertilization. The enzyme urease, which hydrolyses urea to ammonia and carbondioxide, is very stable and many microorganisms produce the enzyme. (Mobely, 1989). Among them is a wide variety of aerobic ammonia oxidizing bacteria (AOB) (Koops and Pommerening-Röser, 2001). The fact that several AOB do posses the capability of hydrolysing urea, suggests that urea can be an important energy source.

Interestingly, autotrophic ammonia oxidizers have been detected in, and isolated from, several acid soils (De Boer and Laanbroek, 1989)(Klemedtsson et al., 1999) (Martikainen et al.,1993) (Stephen et al, 1998). The usage of urea as an energy source might explain why autotrophic nitrification still proceeds in these low pH environments (De Boer & Laanbroek, 1989)(Burton and Prosser, 2001). This theory is consolidated by the fact that isolates from acid soils fail to grow with ammonia at low pH (De Boer et al., 1989)(Burton and Prosser, 2001) whereas growth on urea was observed at pH values ranging from 4 to 7.5 (De Boer and Laanbroek, 1989)(Burton and Prosser, 2001). It was postulated that urea might diffuse over the membrane at a wide range of pH values, whereas free diffusible ammonia is only present in sufficient amounts at pH values above 7 (Burton and Prosser, 2001). How the capability to hydrolyse urea directs the niche differentiation of aerobic ammonia oxidizing bacteria is not really known. Experiments showed that the extinction dilution method on urea yielded mostly *Nitrosospira* spp., while dilution series with ammonia as the energy source, resulted in isolation of *Nitrosomonas* spp. (Aakra et al, 1999). This might explain why *Nitrosospira* is a common ammonia oxidizer in acid soils (Martikainen et al, 1993). Nevertheless, several *Nitrosomonas* like species (e.g. *N. nitrosa*, *N. ureae* and *N. oligotropha*) do posses ureolytic activity (Koops and Pommerening-Röser, 2001).

Recently, bacteria capable of anaerobic ammonia oxidation (Anammox) have been detected in natural environments (Dalsgaard et al. 2003)(Chapter 8)(Thamdrup and Dalsgard, 2002). These bacteria belong to the order of the Planctomycetales (Strous et al, 1999)(Kuenen and Jetten, 2001), and were previously discovered in wastewater treatment systems. They convert ammonia and nitrite to dinitrogen gas, according to Equation 1(Strous et al, 1998). However, Anammox bacteria have to be provided with nitrite. In nitrate-rich ecosystems, this can be done by partial denitrification of nitrate to nitrite. In oxygen limited systems aerobic ammonia oxidizers might be the dominant source of nitrite. Based on the latter, an ammonia removal process was developed, called CANON, in which aerobic and anaerobic ammonia oxidizers coexist at very low oxygen concentrations, according to Equation 2 (Chapter 2, Chapter 3)(Schmidt et al. 2002)(Pynaert, 2003).

$1\ NH_3 + 0.066\ HCO_3^- + 1.32\ NO_2^- + H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2\ H_2O$ (Equation 1)

$1\ NH_3 + 0.85\ O_2 \rightarrow 0.11\ NO_3^- + 0.44\ N_2 + 0.14\ H^+ + 1.43\ H_2O$ (Equation 2)

Anaerobic ammonia oxidizers, in combination with aerobic ammonia oxidizers, might play an important role in nitrogen conversion in soils as well. Urea might serve as an alternative energy source for a consortium of anaerobic and aerobic ammonia oxidizers.

Urea can also be found in some wastewater streams, often in combination with organic pollutants. During the elimination of these organic pollutants, most of the urea is already hydrolysed to ammonia before it enters the nitrification step (Garrido et al. 2001). However, it has been proposed to add a carbon source to achieve complete urea hydrolysis by heterotrophic bacteria in urea containing wastewater without organic carbon (Rittstieg et al. 2001). Since this is costly and has also ecological consequences, urea conversion by CANON or Anammox biomass would be more favourable.

The present study aimed at enrichment of ammonia oxidising bacteria from wastewater treatment sludge using urea or ammonia as the energy source. The bacteria present in the enrichment cultures were identified by using molecular methods. The ability of Anammox bacteria to utilise urea was studied and finally the coexistence between aerobic and anaerobic ammonia oxidizers growing with urea as the energy source was investigated.

Material and methods

Reactor systems and Control equipment

All reactors:

Stirred glass fermentor vessels (height 0.21 m, diameter 0.14 m, working volume 2 L) were operated at a temperature of 30 °C. Water evaporation was prevented by a condensator connected to a cryostat operating at 10 °C. Dissolved oxygen was measured by a Clark-type oxygen electrode (Ingold, Urdorf, Switzerland). The pH was kept at 7.8 and controlled using 0.5M Na_2CO_3 and 1M HCl with an ADI 1030 Biocontroller (Applicon, Schiedam, The Netherlands).

Batch reactor enrichments

The batch reactors were filled with 2 liters of medium and ammonia or urea was added. The inoculum consisted of an activated sludge sample retrieved from the aeration step of the municipal wastewater treatment plant in Weurt, The Netherlands. One ml of inoculum was added to the reactor and the concentration of nitrogen compounds was determined at least every two days. The stirring rate was 300 rpm.

SBR (Sequencing Batch Reactor)

The reactor was run as a continuously fed sequence batch reactor with a 12 hour cycle. It was filled continuously with fresh medium over 11.5 hours at a flow rate of 1.45 mL min^{-1}. The minimum volume was 1 L, and at the end of the cycle, the final volume was 2 L. After the filling period, the stirrer and influent pump were stopped and the biomass aggregates were allowed to settle for 15 minutes. In the remaining 15 minutes of the cycle, an effluent pump removed 1 L of supernatant. In case urea was added continuously to the reactor, cleaning of the tubing was performed every week, although some hydrolysis of urea in the tubing could not be prevented. The stirring rate was 100 rpm.

Chemical analysis

The concentration of ammonia, nitrite and nitrate was measured colorimetrically (Chapter 2). Urea was determined by hydrolysis to ammonia with Soya bean urease (Merck). The ammonia present before and after the hydrolysis was determined and from these results, the urea concentration was calculated.

Medium

Urea was added to a mineral medium in the required amounts (see Results section) and ammonia was added likewise in the form of $(NH_4)_2SO_4$. The composition of the mineral medium was (g L^{-1}): $KHCO_3$ 1.25, KH_2PO_4 0.025, $CaCl_2.2H_2O$ 0.3, $MgSO_4.7H_2O$ 0.2, $FeSO_4$ 0.00625, EDTA 0.00625 and 1.25 mL/L of trace elements solution. The trace element solution contained (g L^{-1}): EDTA 15, $ZnSO_4.7H_2O$ 0.43, $CoCl_2.6H_2O$ 0.24, $MnCl_2.4H_2O$ 0.99, $CuSO_4.5H_2O$ 0.25, $NaMoO_4.2H_2O$ 0.22, $NiCl_2.6H_2O$ 0.19, $NaSeO_4.10H_2O$ 0.21, H_3BO_4 0.014, $NaWO_4.2H_2O$ 0.05.

DNA isolation, amplification of amoA and 16S rRNA genes and phylogeny inference

High molecular weight DNA was isolated from reactor samples according to the protocol published by Juretschko et al. (1998). 16S rRNA gene and amoA gene amplification, cloning, sequencing and phylogenetic analysis were performed according to Purkhold et al. (2000).

Probe design, fluorescence in situ hybridization, microscopy.

For the probes used in this study, sequences, target sites and optimal formamide concentrations in the hybridization buffers are displayed in Table 7.1. Probes Nolig630, Nolig1023 specific for *Nitrosomonas oligotropha* and closely related sequences, as well as Nnitro1253 specific for *Nitrosomonas nitrosa* and related sequences were designed using the probe design tool of the ARB package. Probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Thermohybaid (Ulm, Germany). Hybridizations were performed as described by Amann (1995). Simultaneous hybridization with probes requiring different stringency was realized by a successive-hybridization procedure (Wagner et al., 1994). Optimal hybridization conditions for all probe were determined by using the hybridization and wash buffers described by Manz et al. (1992). The in situ probe dissociation for every probe was measured by assessing the relative fluorescence intensity of biofilm bacteria after hybridization at different stringencies as described by Daims et al. (1999b). For image acquisitions a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Jena, Germany) was used together with the standard software package delivered with the instrument (version 3.1). Percentages of fractions, reacting with a specific probe, were calculated as described previously (Schmid et al., 2000)

Nucleotide sequence accession numbers.

The sequences obtained in this study are available in GenBank under accession no. AY343318 (16S rRNA) and AY343319, AY343320, AY343321 (*amoA*).

TABLE 7.1. Probe sequences, target sites, formamide concentrations in the hybridization buffer required for specific *in situ* hybridization

Trivial name (reference)	OPD[a] designation	Specificity	Sequence 5'-3'	Target site[b]	% Formamide/ mM [NaCl]
AMX820 (Schmid *et al.*, 2000)	S-*-Amx-0820-a-A-22	*Candidatus* "K. stuttgartiensis", *Candidatus* "B. anammoxidans"	AAAACCCCTCTACTTAGTGCCC	820-841	40/56
Nolig630 (this study)	S-S-Nolig-630-a-A-22	*Nitrosomonas oligotropha*	GCTCTGCAGTTTCAAACGCAGT	630-651	30/112
Nolig630c (this study)	S-S-Nolig-630-b-A-22	used as unlabeled competitor to S-S-Nolig-630-a-A-22	GCCCTGCAGTTTCAAACGCAAT	630-651	30/112
Nolig1023 (this study)	S-S-Nolig-1023-a-A-21	*Nitrosomonas oligotropha*	TCAGATTCCCTTTCAGGCACA	1023-1043	40/56
Nnitro1253 (this study)	S-S-Nnit-1253-a-A-20	*Nitrosomonas nitrosa*	CCCCCTCGCAGGTTGGCAAC	1253-1272	40/56
Nnitro1253c (this study)	S-S-Nnit-1253-b-A-20	used as unlabeled competitor to S-S-Nnitro-1253-a-A-20	CCCCCTCGCGGGTTGGCAAC	1253-1272	40/56
Neu (Wagner *et al.*, 1995)	S-*-Neu-0653-a-A-18	Halophilic and halotolerant *Nitrosomonas* sp.	CCCCTCTGCTGCACTCTA	653-670	40/56
Nsv 443 (Mobarry *et al.*, 1996)	S-F-Nsp-0444-a-A-19	*Nitrosospira*-cluster	CCGTGACCGTTTCGTTCCG	444-462	30/112
Eub 338 (Daims *et al.*, 1999a)	S-D-Bact-0338-a-A-18	Eubacteria	GCTGCCTCCCGTAGGAGT	338-355	0/900
Eub 338 II (Daims *et al.*, 1999a)	S-D-Bact-0338-b-A-18	Refer to Daims *et al.*, 1999a	GCAGCCACCCGTAGGTGT	338-355	0/900
Eub 338 III (Daims *et al.*, 1999a)	S-D-Bact-0338-c-A-18	Refer to Daims *et al.*, 1999a	GCTGCCACCCGTAGGTGT	338-355	0/900

[a] Oligonucleotide probe database (Alm *et al.*, 1996), [b] 16S rRNA position, [c] Formamid in the hybridization buffer and [NaCl] in the washing buffer, respectively.

Results

Enrichment of nitrifying bacteria.

Two sets of enrichment experiments were conducted in pH and temperature controlled batch reactors. They were inoculated withl ml of activated sludge. Either urea (5 mM) or ammonium (8 mM) served as the energy source.

In the enrichments on ammonia, a nitrifying population readily developed in less than 48 hours in all batch experiments, indicating that the inoculum contained enough bacteria able to grow on ammonia. In all cases, a transient build up of nitrite was observed. FISH was performed with the 16S rRNA targeting oligonucleotide probes NEU and NSV. NEU targets halophilic and halotolerant β-proteobacterial ammonia oxidizing bacteria, whereas NSV targets the *Nitrosospira* cluster. In the batch experiments on ammonia, a dominant population developed which hybridized with probe NEU probe. NSV positive bacteria could not be detected.

Also in the batch experiments with urea a nitrifying population established in less than 48 hours. During growth on urea, build up of ammonia was never observed, indicating that the ammonia oxidation rate was at least as twice high as the hydrolysis rate of urea to ammonia. To assess the composition of the ammonia oxidizing bacteria population in the urea enrichment samples FISH was again conducted with probes NEU and NSV. In accordance with the ammonia enrichment samples no NSV positive bacteria could be detected. However, only a minor amount of bacteria hybridized with probe NEU. To determine the identity of the ammonia oxidizers present in the enrichments on urea, DNA was extracted from the biomass present in the reactor. After amplification of stretches of the 16S rRNA gene as well as the amoA gene clone libraries were constructed and 48 16S rRNA gene clones were sequenced and phylogenetically analysed. Only one of these clones was actually related to known ammonia oxidizers. Surprisingly, the highest similarity of this sequence was observed to *Nitrosomonas oligotropha* (98% figure 7.1a). The sequences obtained from the amoA library expressed a higher diversity of ammonia-oxidizing bacteria. Although most of the clones (60%) were related to *Nitrosomonas oligotropha*, clones related to *Nitrosomonas europaea* (20% of the clones) and *Nitrosomonas nitrosa* (20% of the clones) were also found (figure 7.1b).

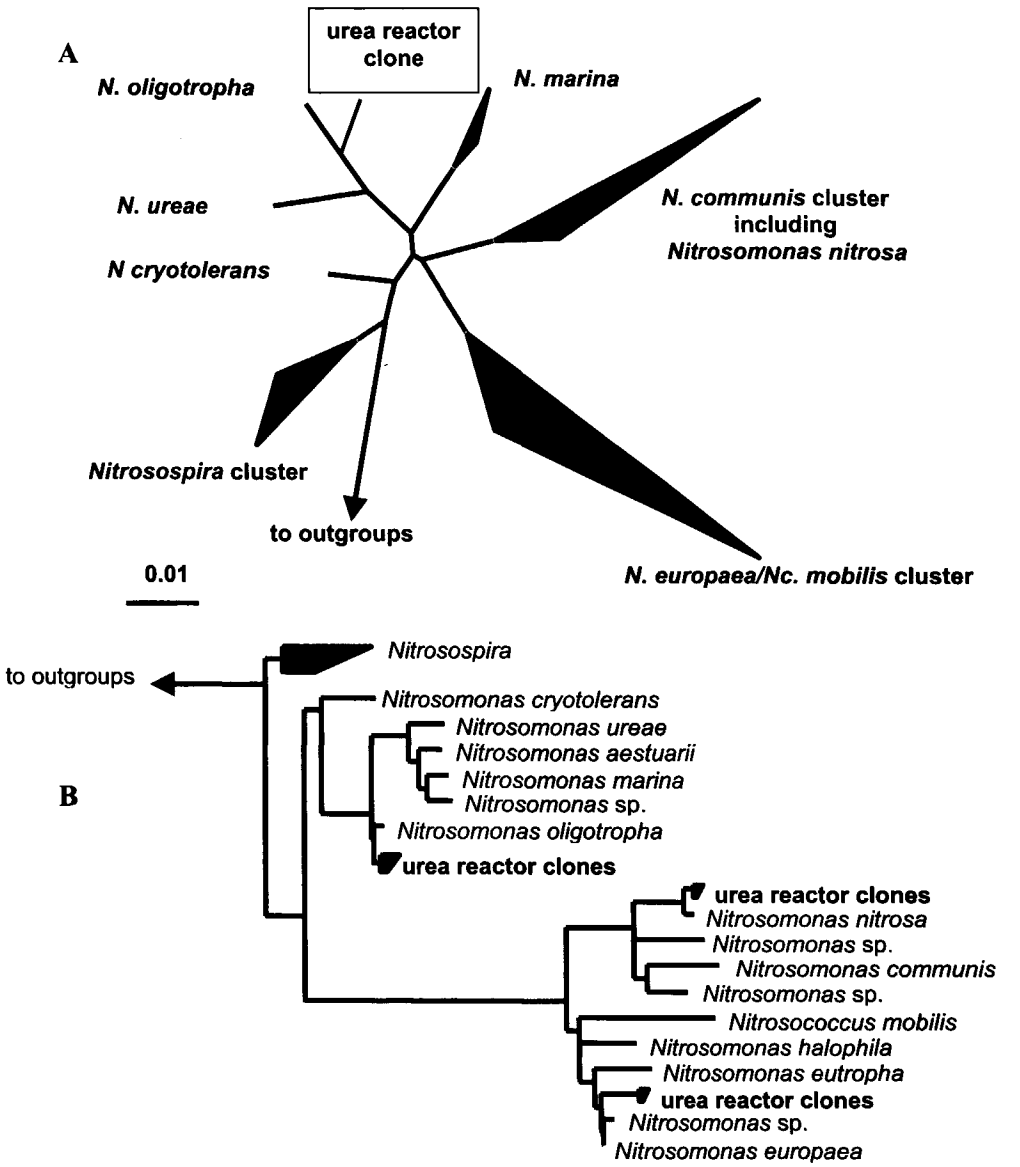

Figure 7.1: 16S rRNA (a) based and AmoA deduced amino acid based (b) phylogenetic tree reflecting the relation of clones derived from the urea fed reactor and other betaproteobacterial ammonia oxidizing bacteria. Maximum likelihood was used as treeing method. The scale bar indicates 1% sequence divergence

It was not possible to determine correctly the ammonia oxidising species of the bacterial population in the ureum reactor with the previously published probes NEU and NSV. Thus on the basis of results from the 16S rRNA and amoA gene clone library three new oligonucleotide probes Nolig630, Nolig1023, Nnitro1253 were constructed to detect *N. oligotropha* and *N. nitrosa* and related organisms (Table 7.1). Since probes Nolig630 and Nnitro1253 had only one weak mismatch to the target sites of other ammonia oxidizing bacteria, competitor oligonucleotides were applied to ensure a specific binding of both probes (see Table 7.1). The newly designed probes for *N. oligotropha* bound to 60-70% of all aerobic ammonia oxidizers in the sample, whereas 25-30% of all aerobic ammonia oxidizers could be identified as *N. nitrosa* (Figure 7.2). The rest (about 5%) of the aerobic ammonia oxidizing bacteria were hybridizing with probe NEU and were most likely *N. europaea* type organisms (Figure 7.2). Together the AOB made up 60-70% of the EUB cell counts.

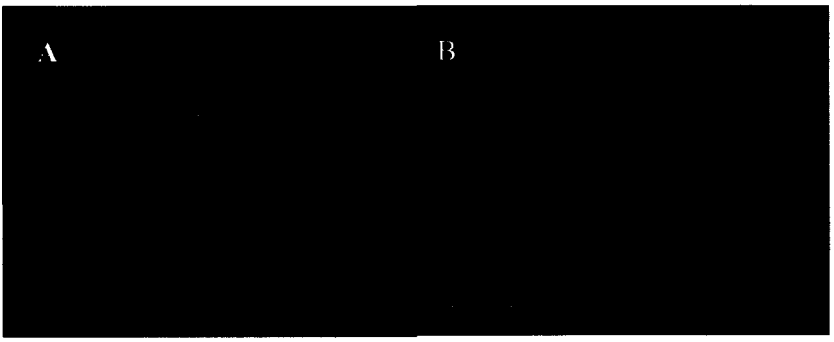

Figure 7.2: *In situ* identification of aerobic ammonia oxidizing bacteria in aggregates from a lab scale reactor fed with ureum. Hybridization was performed simultaneously with Cy3-labeled probe Nolig1023 (red), Fluos-labeled probe Nnitro1253 (with competitor, green) and the Cy5-labeled Eubmix (blue) (A). *N. oligotropha* cells appear purple, because overlapping Cy3 and Cy5 labeling. *N. nitrosa* cells appear turquois, because overlapping Fluos and Cy5 labeling. Bacteria other then aerobic ammonia oxidizers appear blue. Hybridization was also performed simultaneously with Cy3-labeled probe Nolig630 (with competitor, red), Fluos-labeled probe Nnitro1253 (with competitor, green) and the Cy5-labeled Neu (blue)(B). *N. oligotropha* cells appear red, *N. nitrosa* cells appear green and halophilic and halotolerant ammonia oxidizers appear blue.

Anammox and urea utilization.

Additional experiments were performed to check whether anaerobic ammonia oxidizers or a consortium of aerobic and anaerobic ammonia oxidizers could also utilize urea. A sequencing batch reactor was filled with medium and 1.5 mM of nitrite and 1 mM of ammonia was added. Initially, the reactor was run in batch mode, i.e. the pumps were not started yet. Anammox biomass was added and checked for activity by following the ammonia and nitrite disappearance. Within one hour most of the nitrite and all of the ammonia were utilized. Then, 5 mM of urea was added, together with 2 mM of

nitrite. No change in the urea concentration was observed within one week, indicating that this Anammox biomass was not able to hydrolyse urea.

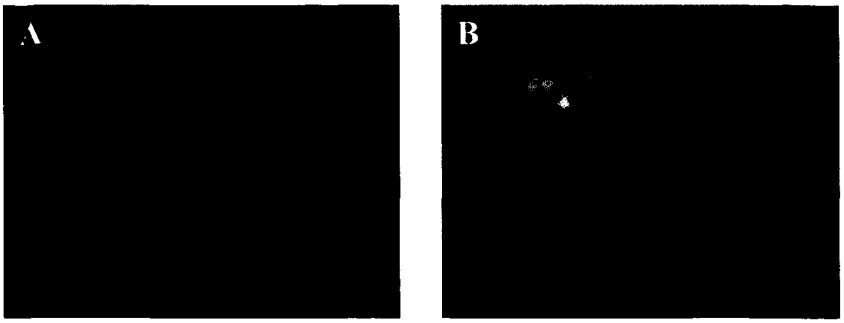

Figure 7.3: FISH analysis of the population in CANON reactor with ammonia as the energy source (A) and in the CANON reactor with urea as the energy source (B). Bacteria reacting with NEU are stained green, anaerobic ammonia oxidizers reacting to Amx820 are stained blue, and bacteria reacting to the probe specific for *N. oligotropha* are stained red.

CANON with ammonia as the energy source

Since anammox bacteria alone were not able utilize urea, a consortium of anaerobic and aerobic ammonia oxidizers was tested. In the same reactor, with the same anammox biomass, the pumps were turned on to initiate the sequencing batch mode, and the reactor was fed with ammonia and nitrite. From ammonia, nitrite and nitrate measurements, it was clear that the Anammox process proceeded in the reactor as expected (Table 7.2). After a week, the gas was changed from dinitrogen gas to air and the medium contained now only ammonia, but no nitrite. Within two weeks the cooperation of aerobic and anaerobic ammonia oxidizers was established in accordance to previous experiments (Sliekers et al. 2002, Third et al. 2001). The conversion of ammonia into N_2 and NO_3^- proceeded according to equation 2 (Table 7.2). Samples were taken for FISH. The biomass consisted mainly of bacteria hybridizing with probes AMX820 and NEU (Figure 7.3a).

Table 7.2. Concentrations of nitrogen compounds in the influent and effluent in the CANON reactor.

Mode	Influent (mM)			Effluent (mM)			
	NH_4^+	Urea	NO_2^-	NH_4^+	Urea	NO_2^-	NO_3^-
Anammox*	10.1	0	10	3.2	0	0	2.1
CANON (period 1)*	10.2	0	0	5.3	0	0	0.8
CANON (period 2)*	10	0.9	0	5.1	0	0	0.8
CANON (period 3)*	0.5	16.8	0	2.2	13.0	0	0.7

* The period with the anammox process took one week. The three CANON periods were each two weeks long.

CANON with urea as the energy source

The medium was changed into a medium, which contained 1 mM of urea and 10 mM of ammonia, and after two weeks, the medium was changed and contained now only urea as the energy source (Table 7.2). Urea was converted to dinitrogen gas, following the expected stoichiometry of the CANON process (Sliekers et al 2002). Again samples were taken for FISH analysis The biomass not only consisted of bacteria reacting to AMX820 and NEU, but also a substantial part of the bacteria hybridised to the new probe targeting bacteria related to *Nitrosomonas oligotropha* (Figure 7.3b).

Discussion

In this study we describe enrichment experiments where activated sludge samples were incubated with either ammonia or ureum. FISH performed with probe NEU on enrichments grown with ammonia revealed halophilic and halotolerant β-proteobacterial ammonia oxidizing bacteria as the dominant species in this sample. In contrast to this finding, probe NEU did only hybridise to a minor amount of bacteria in the enrichment fed with urea. This had been expected since it was reported previously, that *Nitrosospira*-like bacteria were dominant in the batch enrichments on urea (Aakra et al., 1999). However, probe NSV specific for *Nitrosospira* spp. did not hybridize with any of the cells in the reactor. Therefore, we amplified the 16S rRNA genes from the ureum enriched biomass, but only one of the 48 sequenced clones could be identified as the 16S rRNA gene of a known ammonia oxidising bacterium, i.e. *N. oligotropha*. No *N. europaea* sequence was found in this clone library, which is in contrast to observations with FISH. This phenomenon of under representation of 16S rRNA gene sequences was described and discussed before (Juretschko et al. 1998, Purkhold et al., 2000, Mendum et al., 2001). The amoA approach was more sensitive in accordance with previous reports (Rotthauwe et al., 1997, Purkhold et al., 2000). This approach showed that the sample from the ureum reactor harbored at least three different Nitrosomonads (*N. oligotropha*, *Nitrosomonas nitrosa* and *N. europaea*) rather than the expected *Nitrosospira* species.

Interestingly, the diversity of the ammonia oxidizing population was higher in enrichments grown with urea compared to enrichments grown with ammonia. Recently, it was shown that pH influences the diversity in ammonia-oxidizing communities as well (Egli et al. 2003). A higher diversity may influence the stability of a nitrogen removing system in a positive way (Egli et al. 2003).

In contrast to *N. europaea*, *N. oligotropha* and *N. nitrosa* are urease positive (Koops and Pommerening-Röser, 2001). FISH performed with the newly constructed probes and probe NEU showed that the urea enrichment was dominated by *N. oligotropha*. The formation of this *N. oligotropha* dominated consortium is in contrast to findings published by Aakra et al. (1999). This could be explained by the use of a different inocculum or of applied growth conditions, which favoured *N. oligotropha*. However, the stability of the formation of a *N. oligotropha* dominated population was confirmed by studies in a urea fed CANON set up. Here growth conditions differed very much from the batch experiments. Also in this case *N. oligotropha* was an abundant ammonia oxidising bacterium in the system.

Another surprising fact is also that *N. oligotropha* could not be detected in the original sample. Apparently, the diversity of AOB in activated sludge samples is quite high even if just a few species occur in detectable abundances. This is supported by the fact ammonia oxidising bacteria are able to maintain themselves in a system even during a very long period of starvation (Abelovich et al., 1992).

If a tight coupling between urea-hydrolysis and ammonia oxidation were present in the cell, it would be expected that *Nitrosomonas europeae* related bacteria would not be present in the enrichments on

urea, because they are not known to be able to hydrolyse urea. Probably, some of the ammonia from urea hydrolysis diffused into the medium.

Anammox bacteria did not hydrolyse urea in our test. Normally, urea is readily hydrolysed and only stable for a few days when bacterial hydrolysing activity is present. The duration of the experiment with the anammox culture was one week, which should have been long enough to test the potential of the culture. The absence of this ability of urea hydrolysis is not surprising. The reactor was filled with biomass, which had never seen any urea. Since the affinity of anaerobic ammonia oxidizers for ammonia is very high (Strous et al, 1999b) it is not surprising that anammox bacteria were able to retrieve some of the produced ammonia from the aerobic ammonia oxidizers. The question remains whether or not anammox bacteria exist, which are able to hydrolyse urea and whether coexistence between aerobic and anaerobic bacteria, with urea as the energy source would exist in nature. That will probably depend on the availability of urea compared to the availability of ammonia. The more ammonia is present the smaller the necessity for urea hydrolysis. Cooperation between aerobic and anaerobic ammonia oxidizers is likely to occur only at oxic/anoxic interfaces (Chapter 8). Often ammonia diffuses from the anoxic environments to the oxic environments due to mineralisation in the anoxic part. This ammonia is readily oxidized at the oxic/anoxic interface and the ratio between urea and ammonia probably gets higher upwards the oxic/anoxic interface. Urea, likely to diffuse from the upper layers, can be a good alternative energy source there. However, no data about urea availability in soils are available, but in marine environments, urea is especially more available in the lower euphotic zone (Sambrotto 2000). Thus, oxic/anoxic interfaces in the lower euphotic zone might be a place where coexistence between aerobic and anaerobic ammonia oxidizers with urea as the predominant energy source exists.

Acknowledgements

The authors would like to thank Katinka van de Pas for help with the FISH analysis, and Huub Op den Camp, Hendrikus Laanbroek, Annette Bollman, Rob van Spanning, Bertus Beaumont, and Marc Strous for the valuable discussion. We also would like to thank the ALW (Dutch Science Foundation for Earth and Life Sciences) for the financial support (project number:80533422/P).

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Chapter 8

Anaerobic ammonium oxidation by Anammox bacteria in the Black Sea

Previously published as:
Kuypers M., Sliekers A.O., Lavik G., Schmid M., Jørgensen B.B., Kuenen J.G., Sinninghe Damsté J.S., Strous. M. and Jetten M.S.M. (2003) Anaerobic Ammonium oxidation by anammox bacteria in the Black Sea. Nature 422, 608 - 611

The availability of fixed inorganic nitrogen (nitrate, nitrite and ammonium) limits primary productivity in many oceanic regions (Falkowski, 1997). The conversion of nitrate to N_2 by heterotrophic bacteria (denitrification; Fig. 8.1a) is believed to be the only important sink for fixed inorganic nitrogen in the ocean (Codispoti et al. 1985). Here we provide evidence for bacteria that anaerobically oxidize ammonium with nitrite to N_2 in the world's largest anoxic basin, the Black Sea. Phylogenetic analysis of 16S ribosomal RNA gene sequences shows that these bacteria are related to members of the order *Planctomycetales* performing the Anammox (anaerobic ammonium oxidation) process in ammonium-removing bioreactors (Strous et al., 1999). Nutrient profiles, fluorescently labelled RNA probes, ^{15}N tracer experiments, and the distribution of specific ladderane membrane lipids (Sinninghe Damsté et al., 2002) indicate that ammonium diffusing upwards from the anoxic deep water is consumed below the oxic zone by anammox bacteria. This is the first time anammox bacteria have been identified and directly linked to the removal of fixed inorganic nitrogen in the environment. The widespread occurrence of ammonium consumption in suboxic marine settings indicates that anammox may play an important role in the oceanic nitrogen cycle (Bender et al., 1989)(Codispoti et al., 1991)(Dalsgaard & Thamdrup, 2002).

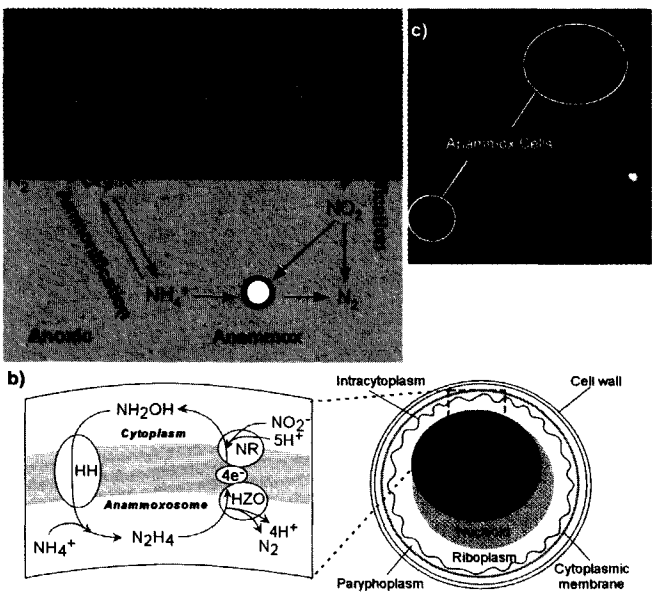

Figure 8.1. **Morphology and physiology of anammox bacteria and their role in the marine nitrogen cycle. (a) Simplified marine nitrogen cycle including the anammox 'sink'; Org. N: organic nitrogen. (b) Morphology of the anammox cell and proposed model for the anammox process; HH: hydrazine (N_2H_4) hydrolase; HZO: hydrazine oxidizing enzyme; NR: nitrite reducing enzyme. (c) Fluorescence *in situ* hybridization of filter material from station 7617 (142 m water depth). Green cells: total Eubacteria stained with EU338 probe; Red cells (encircled): anammox bacteria stained with a new specific probe (AmxBS820).**

The Black Sea is the world's largest anoxic basin and is a model for both modern and ancient anoxic environments. It is characterized by a high ammonium concentration in the deep waters (up to 100 μM), while only trace amounts of fixed inorganic nitrogen are present in the 'suboxic' zone (Codispoti et al, 1991)(Sorokin et al., 2002) where nitrate, manganese oxide or iron oxide reduction occurs (Froelich et al., 1979). This apparent ammonium sink in the suboxic zone strongly suggests that ammonium is oxidized anaerobically to N_2 (Thamdrup & Dalsgaard, 2002)(Richards, 1965)(Murray et al., 1995). Indeed, bacteria able to oxidize ammonia anaerobically have recently been discovered in laboratory bioreactors and wastewater treatment systems (Strous et al, 1999)(Kuenen & Jetten, 2001). These so-called 'anammox' bacteria belonging to the Order *Planctomycetales* directly oxidize ammonia to N_2 with nitrite as the electron acceptor (Fig 8.1b):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad (1)$$

During an R/V Meteor cruise in December 2001 we investigated the role of anammox in the Black Sea water column using microbiological and biogeochemical techniques. Consistent with earlier studies (Murray et al., 1995)(Codispoti et al, 1991)(Sorokin et al., 2002) we observed a nitrate maximum at the bottom of the oxic zone in the western basin (site 7605; 42°30.71'N 30°14.69'E; Fig. 8.2a). This maximum is caused by the mineralization of phytoplankton-derived organic nitrogen coupled to aerobic nitrification.

Ammonium concentrations are high in deep waters but drop to background values above 97 m water depth (Fig. 8.2a). Aerobic nitrification cannot account for the consumption of ammonium since O_2 is absent below 80 m (Fig. 8.2b). Nitrate, however, penetrates 15 m deeper in the water-column indicating that nitrate could be the oxidizer of ammonium (Murray et al., 1995). Alternatively, anammox bacteria could be using nitrite instead of nitrate to oxidize ammonium. Nitrite is an intermediate of denitrification and a nitrite peak is present at the base of the nitrate peak (Fig. 8.2a). Anammox in the suboxic zone could be coupled to nitrate reduction to nitrite by denitrifiers (Murray et al. 1995), similar to the process in anammox bioreactors (Mulder et al., 1995). To check for anammox activity in the suboxic zone we anaerobically incubated water samples from various depths after addition of ^{14}N-nitrite and ^{15}N-ammonium. Since the anammox process combines 1 mol ^{15}N-ammonium and 1 mol ^{14}N-nitrite to 1 mol $^{29}N_2$ (equation (1)), the depth distribution of $\delta^{29}N_2$ (Fig 8.2c) expresses the potential anammox activity. The $\delta^{29}N_2$ record shows a clear peak in the zone of nitrite and ammonium disappearance, whereas no significant anammox activity is observed outside the suboxic zone. Specific biomarkers, so-called 'ladderane' lipids (Sinnighe Damsté et al., 2002), were used to trace anammox bacteria in particulate organic matter collected from various depths across the suboxic zone. Ladderane lipids are the main building blocks of a unique bacterial membrane that surrounds the 'anammoxosome', a special compartment of the anammox cell, where the anaerobic oxidation of

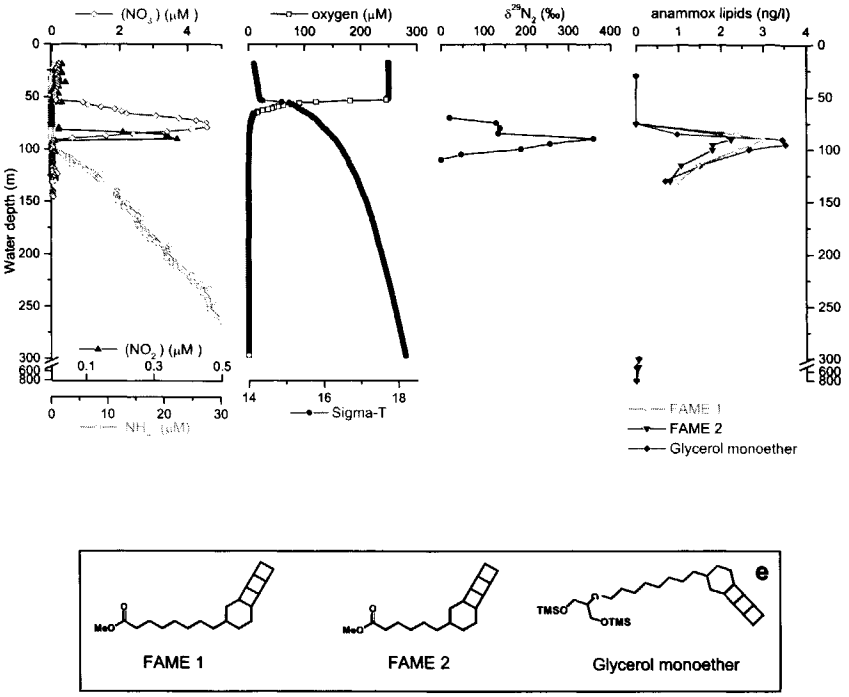

Figure 8.2. **Chemical zonation and distribution of anammox indicators across the Black Sea chemocline. (a) Fixed inorganic nitrogen species, (b) water density and oxygen concentrations, (c), interface peak of potential anammox activity expressed as anaerobic $^{15}NH_4^+$ oxidation by $^{14}NO_2^-$ to $^{29}N_2$, (d) peak of three ladderane membrane lipids specific for anammox bacteria, and (e) molecular structures of the three ladderane membrane lipids specific for anammox bacteria presented in (d). The suboxic zone is indicated by gray shading. Density (sigma-T), nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), and oxygen profiles from Station 7605 (42°30.71'N 30°14.69'E). Ladderane lipid data from Stations 7605 and 7620 (42°55.56'N 30°03.65'E) was used to create a composite plot for the ladderane glycerol monoether and for the fatty acid methyl esters, FAME 1 and 2.**

ammonium to N_2 takes place (Fig. 8.1b). Three different ladderane lipids were detected in the saponified total lipid extracts with a depth distribution (Fig. 8.2d, e) similar to that of the potential anammox activity (Fig. 8.2c), indicating that anammox bacteria could indeed be responsible for the anaerobic oxidation of ammonium. A clone library was generated from DNA extracted from Black Sea water at the depth of maximum ladderane abundance (90 m), after amplification of the 16S ribosomal RNA gene with primers specific for Planctomycetes (Schmid et al. 2000). Phylogenetic analysis of the 16S rRNA sequences confirms that the Planctomycetes, tentatively named *Candidatus* Scalindua sorokinii, from the suboxic zone of the Black Sea are related to bacteria known to be capable of anammox (87.9 % sequence similarity to *Kuenenia*, 87.6% to *Brocadia*, Fig. 8.3). In fact, the sequence obtained from the Black Sea is nearly identical (98.1%) to a sequence recently obtained from a bioreactor shown to have anammox activity (Schmid et al., unpublished data). Based on the

sequence obtained from the Black Sea, we designed an oligonucleotide probe, labeled with Cy3 fluorochrome, for fluorescence *in situ* hybridisation (FISH). This probe gave a bright and specific

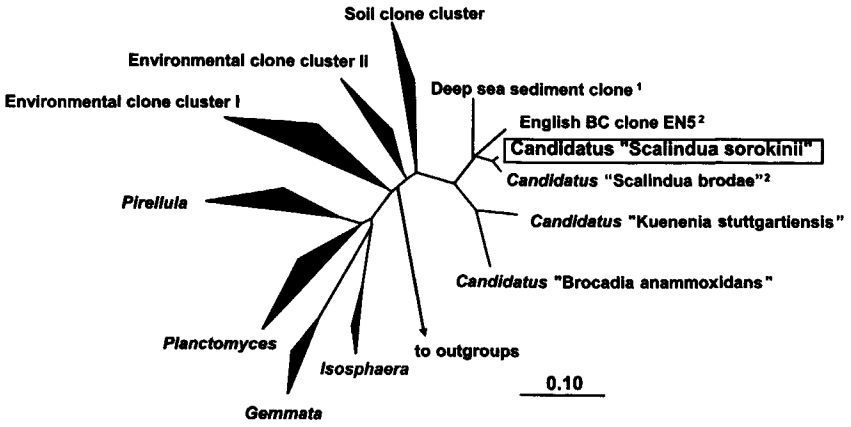

Figure 8.3. Phylogenetic tree of 16S rRNA gene sequences showing the order *Planctomycetales* and the position of the anammox affiliated organisms from the Black Sea (indicated by a rectangle). The black triangles indicate phylogenetic groups. The bar represents 10% estimated sequence divergence. (1) Li et al, 1999); (2) M. Schmid *et al.*, unpublished data

signal with cells that have the unusual doughnut shape characteristic for anammox bacteria in bioreactors. Ladderane biomarkers and cells hybridizing with the new FISH probe (Fig. 8.1c) were also found in the suboxic zone at the shelf break (Station 7617, 43 38,04'N 30 02,54'E), indicating that anammox bacteria are not restricted to the strongly stratified central basin but are also present in the more dynamic peripheral current (Sorokin, 2002). The combined results clearly indicate that anammox bacteria are abundant and active in the Black Sea.

Could these anammox bacteria be responsible for the observed ammonium sink in the suboxic zone of the Black Sea?

Assuming that the concentration profile of ammonium represents a steady state, an anaerobic ammonium oxidation rate of ~0.007 μM day^{-1} was calculated for the suboxic zone of the central basin using a reaction diffusion model. This rate is comparable to aerobic ammonium oxidation rates (0.005-0.05 μM day^{-1}) determined for the nitrate maximum of the western central basin of the Black Sea (Ward and Kilpatrick, 1991). An anammox rate of 2-20 fmol ammonium $cell^{-1}$ day^{-1} (1 fmol = 10^{-15} mol) was found in laboratory bioreactors (Strous et al, 1999). Assuming a similar range of cell specific activity for the Black Sea, 300-3000 anammox cells ml^{-1} would be needed to account for the observed ammonium oxidation rates in the suboxic zone. Counts of cells stained with the new FISH probe gave an anammox cell density of ~1900 (±800) cells ml^{-1} (0.75% of all cells counted by DAPI) at the nitrite peak.

While we acknowledge the uncertainty involved in the extrapolation of laboratory derived anammox activities to the natural environment, the rates of net ammonium and nitrate consumption calculated as a function of depth indicate that nitrate reduction by denitrifiers coupled to anammox accounts for a substantial loss of fixed inorganic nitrogen. In fact, the downward flux of nitrate (~7 μmol m^{-2} h^{-1}) is sufficient to oxidize all the ammonium (~5 μmol m^{-2} h^{-1}) diffusing up into the suboxic zone. If we assume that the area (3.10^5 km^2) below the shelf break (< 200 m) (Sorokin, 2002) represents the total surface area of the suboxic zone, 0.3 Tg fixed inorganic nitrogen per year may be lost through nitrate reduction coupled to anammox. For comparison, the annual primary production of phytoplankton in the whole basin is about 80 Tg carbon (Sorokin, 2002), which is equivalent to 14 Tg fixed organic N if we assume an atomic C/N ratio of 6.6 for phytoplankton (Redfield et al., 1963). Since more than 95% of this phytoplanktonic organic nitrogen is recycled in the upper 80 m (Karl and Knauer, 1991), anammox might consume more than 40% of the fixed nitrogen that sinks down into the anoxic Black Sea water.

Moreover, these results demonstrate that anammox bacteria are abundant and play an important role in the nitrogen cycle of the Black Sea. In fact, the widespread occurrence of ammonium consumption in suboxic marine waters as well as in sediments (Thamdrup and Dalsgaard, 2002) suggests that anammox bacteria could play an important but as yet neglected role in the oceanic loss of fixed nitrogen.

Methods:

Nutrient analyses.

Water samples for nutrient analyses were obtained by a pumpcast-CTD-system. Prior to analyses, $ZnCl_2$ was added to the samples from the anoxic part of the water column to precipitate sulfide. Nitrate, nitrite and ammonium concentrations (detection limits; 0.1, 0.01, and 0.5 μM, respectively) were determined on board with an autoanalyzer, immediately after sampling.

^{15}N incubations and analysis:

Black Sea water collected from specific water depths was flushed with argon for 1 hour and, after addition of 500 μM $^{15}NH_4Cl$ and 100 μM $Na^{14}NO_2^-$, incubated for 4 days at *in situ* temperatures. Subsequently, the samples were stored at 4 °C until analysis. $^{29}N_2$:$^{28}N_2$ ratios were determined by gas chromatography isotope ratio mass spectrometry.

Lipid analysis. Particulate organic matter for lipid analyses was collected from specific water depths by *in situ* filtration of large volumes (~1000 l) of water through 292 mm diameter, precombusted (at 450 °C) glass fibre filters (GFF; nominal pore size, 0.7 μm) using *in situ* pumps. Since filtration through 0.7 μm pore size filters may lead to an undersampling of anammox cells, the calculated ladderane lipid concentrations represent minimum values. The GFF filters were Soxhlet extracted for 24 h to obtain the total lipid extracts. Aliquots of the total extracts were saponified after addition of an internal standard and separated into fatty acid and neutral lipid fractions. The fatty acid fractions were

methylated and the neutral fractions silylated and analysed by gas chromatography-mass spectrometry (GC-MS) for identification and quantification of ladderane lipids. Repeated concentration measurements were within ±10%.

Molecular Cloning and Phylogeny: DNA extraction, isolation, and cloning were done as described previously (Schmid et al., 2001). Phylogenetic analysis was done using the ARB software package (Schmid et al., 2000). The phylogenetic tree is based on maximum likelihood analysis of different data sets.

Fluorescence *in situ* hybridisation and microscopy: Filter material was stained with an oligonucleotide probe specific for Planctomycetes (Pla46, S-P-Planc-0046-a-A-18) (Neef et al., 1998), a newly designed Anammox probe (AmxBS820, S-*-BS-820-a-A-22 (5'-TAATTCCCTCTACTTAGTGCCC-3')), an eubacterial probe (EUB338, S-D-Bact-0338-a-A-18) (Amann, 1990), as well as 4,6-diamidino-2-phenylindole (DAPI) to determine the abundance of anammox and total bacteria. FISH and DAPI staining was done as described (Pernthaler et al., 2001) and the average number of anammox bacteria was determined by analyzing 20 different slides.

Flux calculations: Nitrate, and ammonium fluxes, and ammonium oxidation rates were calculated from the concentration profiles and a vertical diffusion coefficient (K_z) using the program 'Profile' (Berg et al., 1998). Published estimates of the vertical diffusion coefficient for the suboxic zone vary over an order of magnitude (0.02 $cm^2.s^{-1}$ to 0.7 $cm^2.s^{-1}$) (Sorokin, 2002)(Oguz et al, 2001)(Lewis, 1991). Most calculations of chemical fluxes (Oguz et al, 2001)(Lewis, 1991) have, however, used a K_z value close to the lower end of the range. Accordingly, a K_z of 0.04 $cm^2.s^{-1}$ was used here. The model predicted zones of net ammonium and nitrate consumption at 106-93 m and 88-94 m, respectively.

Acknowledgements. We thank L. Neretin for discussions; the Romanian and Turkish authorities for access to their national waters; the crew of the R/V *Meteor* for excellent collaboration; S. Krüger, F. Pollehne, T. Leipe (IOW, Warnemünde) for operating the pumpcast and generously providing the CTD data; J. Eygensteyn, A. Pol, H. op den Camp; K. van de Pas and G. Klockgether for analytical assistance. C. Hanfland and the AWI, Bremerhaven, generously provided the *in situ* pumps. The investigations were supported by the MPG, the University of Nijmegen, the TU Delft and the DFG. M. Kuypers was financially supported by the EC Human Potential Programme Research Training Networks Activity (CT-net); O. Sliekers was supported by a grant of the ALW, 80533422/P; M. Schmid was supported by the EU grant evk1- 2000-00054.

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Chapter 9

Integration and Outlook

The CANON process

In the previous chapters the microbiology, feasibility and stability of the CANON process have been documented. However before the CANON process can be applied in pilot and full scale treatment plants several issues have to be addressed.

Spontaneous establishment of mixed microbial communities containing aerobic ammonium oxidizing bacteria (AOB) and anaerobic ammonium oxidizing bacteria (AAOB) in some full scale plants, might give the impression that the start up the CANON process should be quite easy, but in the following paragraphs we will point out a number of possible bottlenecks.

Starting Procedure

In our laboratory experiments, CANON reactors were started by addition of anammox biomass. On full scale, such a procedure would require a large amount of anammox biomass (about 10 m^3). At the moment such amounts are not yet available, although the first full scale anammox reactor has recently been started at the Dokhaven waste water treatment plant in Rotterdam, The Netherlands. An alternative possibility for start up of a CANON reactor would be the enrichment of anammox bacteria in an oxygen limited nitrifying reactor. Although it has not been tested yet, this procedure will probably take at least six months, the period needed to establish a fully operational anammox reactor inoculated with activated anaerobic sludge. Augmentation of an oxygen limited nitrifying reactor with anammox biomass could greatly enhance the start up procedure.

Biomass

Availability of anammox biomass is thus essential for both start-up procedures. Since it has become clear that different treatment plants and ecosystems contain different anaerobic ammonia oxidizing bacteria (Brocadia, Kuenenia and Scalindua) it is difficult to predict which anammox bacteria would be most suitable for a certain type of wastewater. This could be dependent on other components than ammonia and nitrite present in the wastewater (e.g. trace elements, carbonate, organic acids). Also the hydrodynamics, biofilm structure, oxygen penetration depth and oxygen inhibition kinetics of different anammox bacteria might be important for the niche differentiation. Obtaining such knowledge will require considerable effort. A defined mixture of different anammox bacteria will have to be exposed to various types of waste waters under different environmental conditions for extended periods of time. This would provide information on selection and competition among AAOB and their potential habitats. To this goal, environmental genomics will be of tremendous help in linking genetic potential and biochemistry to the different ecological niches of the various AAOB

To unravel the effects of floc size and oxygen penetration depth, the use of new microelectrodes for oxygen and nitrite, and confocal scanning laser microscopy with newly developed 16S rRNA and ISR gene probes are essential.

High strength waste water vs. low strength wastewater.

In this thesis (Chapter 2 & 4), it was demonstrated that the process could be used for treatment of wastewater with both low and high ammonia concentrations. In general, treatment of high strength wastewater will result in high conversion rates at a relatively high liquid residence time and hence biomass retention should be less critical. However, the N-conversion rate in synthetic wastewater with low concentrations of ammonia (chapter 2) was relatively low. A lower liquid retention time in combination with a higher oxygen transfer coefficient might overcome the low conversion rate but the biomass retention in such system should be efficient. Additional experiments would be needed to document the operational boundaries with respect to the liquid retention time and the solid retention time possible. A biofilm gas lift suspension reactor would be the recommended tool for such a study. Introduction of support media, which would enable high solid retention times in combination with low hydraulic retention times, could enhance the performance of such a reactor even further.

Interactions at the oxic/anoxic interface.

In chapter 5 and 6, the presence and absence of nitrite oxidizing bacteria in oxygen limited nitrifying cultures was studied. We did not obtain any evidence for a nitrogen compound being responsible for the inhibition of NOB. It is feasible that the inhibition of NOB is related to the presence of homoserine lactone like signal molecules produced by AOB. Indication of such an inhibition might be derived from experiments in which nitrite oxidisers are supplemented with spent media from AOB cultures. This remains to be tested. The original hypothesis that the NOB would not be able to maintain themselves due to poorer kinetic properties (K_s, V_{max} and μ_{max}) could not be substantiated or ruled out as the measurements of the parameters did not yield sufficient accurate data. In the following paragraph, we give suggestions to a more definite answer.

Kinetic parameters

First of all, more exact determination of the kinetic parameters is needed. This can be achieved in two ways. Pure cultures could be isolated from low oxygen and oxygen limited nitrifying reactors. The isolation in pure culture of such nitrifying bacteria will be quite difficult, due to very slow growth rates and long incubation times. However, with the use of these pure cultures, maintained under oxygen limited conditions, the Ks and Vmax could be determined more accurately. Also the negative effects of aggregate disruption could be circumvented by, for example, high density cell suspensions.

(Micro) electrodes with a guard electrode will be necessary for oxygen measurement in the nanomolar range, since exact values at very low oxygen concentrations are needed. Another method for a more exact determination of the Ks and V_{max} values is the use of microsensors in combination with FISH and image analysis on whole aggregates. In this way the bacteria are studied without disruption, isolation or other effects, which could cause deviations between measured and in situ kinetic parameters. However, many bacterial flocs must be examined to get reliable values.

Fluctuating environmental conditions

In chapter 5, one experiment was performed in which the environmental conditions were fluctuated. It would be interesting to see what would happen if the intervals of fluctuation would be adjusted to more natural day/night rhythms and examined for longer periods of time (months). What kind of bacteria would develop? What would happen with the concentrations of the energy source? Modelling may help us to give some more insight into the heterogeneity of the population, and will give insight into what values for K_s and V_{max} will be favourable for NOB and AOB. New data from reactors with different ammonia and nitrite oxidizing populations under fluctuating environmental conditions will be necessary to allow further predictive modelling experiments.

Anammox and interactions in natural environments

In chapter 8, it was shown that anaerobic ammonia oxidation does occur in natural environments and that anammox bacteria in the Black sea are related to the previous AAOB. The anammox process is dependent on the continuous supply of both nitrite and ammonia. Ammonia is liberated in various decomposition and mineralisation processes. Nitrite can be provided either by aerobic ammonia oxidising bacteria under oxygen limitation or by denitrifying bacteria under carbon limitation and nitrate surplus.

In marine sediments, where micro-oxic and anoxic environments are spatially very close, denitrification as well as nitrification could provide the nitrite for anammox bacteria.

In water columns where the oxic/anoxic interface spans ten to twenty meters, like the Black Sea, nitrite production for anaerobic ammonia oxidation is most likely to occur by only one of the processes (denitrification or nitrification). To establish which process provides the nitrite for anammox several different ^{15}N experiments will have to be performed. Oxic incubation with only ^{15}N-ammonia is needed to establish the potential of the AOB. Anoxic incubation with only ^{15}N-nitrate enables determination of the potential of denitrification. Addition of a combination of ^{15}N-ammonia with ^{14}N-nitrate to water or sediment samples might be sufficient to look at coupling between denitrification and anammox, by comparing the amount of $^{29}N_2$ and $^{28}N_2$ formed. FISH analysis and PCR based methods could provide additional information on the bacteria which supply nitrite to anammox. For example, if there is no nitrifying potential close to the depth where anammox takes place, and no aerobic ammonia oxidisers are detected, it is not likely that aerobic ammonia oxidizers provide the

nitrite for anammox. Careful measurement of the oxygen concentration and calculation of the oxygen fluxes will also give information.

Summary

Ammonium oxidation at the oxic/anoxic interface

Olav Sliekers

Humans can have a high impact on the input of several nitrogen compounds in the environment, resulting in for example eutrophication and acid rain. The release of nitrogen compounds in the environments, other than dinitrogen gas (N_2), from human activity must be kept at a minimum. Several microbial processes can transform nitrogen compounds. Knowledge of the microbial processes in the nitrogen cycle is needed to understand this cycle and to understand the impact of human nitrogen input in natural ecosystems. Application of this knowledge will result in more efficient ways to treat nitrogen rich wastewaters.

The research, described in this thesis, was aimed at a better understanding of nitrification and anaerobic ammonia oxidation (anammox) at the oxic/anoxic interface. Nitrification is the aerobic oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) via nitrite (NO_2^-). Anammox is the anaerobic oxidation of ammonia with nitrite to dinitrogen gas (N_2). Both processes are mediated by autotrophic bacteria. Nitrification is carried out by two types of bacteria, i.e. aerobic ammonia oxidizers (AOB) and aerobic nitrite oxidisers (NOB). Anammox is mediated by one group of planctomycetes. Bacteria capable of anammox were only recently discovered in man-made ecosystems and can be used as an alternative for denitrification to achieve complete conversion to dinitrogen gas. A combination of aerobic ammonia oxidation and anaerobic ammonia oxidation can treat ammonia rich wastewater streams without the need of a costly carbon source, like methanol. Such a combination is achieved in the patented CANON (completely autotrophic ammonia removal over nitrite) process.

First, the microbiology and the feasibility of the CANON process were investigated (Chapter 2). A reactor was started anoxically after inoculation with biomass from a reactor performing anaerobic ammonia oxidation (anammox). Subsequently, air was supplied to the reactor and a nitrifying population developed within 14 days while anaerobic ammonium-oxidizing bacteria remained present and active. Oxygen was kept as the limiting factor in the reactor and the cooperation, between aerobic and anaerobic ammonia oxidizers, remained stable throughout the experiment. In the reactor, no aerobic nitrite-oxidizers were detected using Fluorescence In Situ Hybridization (FISH). Ammonia was mainly converted to N_2 (85%) and the remainder (15%) was recovered as NO_3^-. Production of N_2O, a greenhouse gas, was negligible (less than 0.1%). Addition of a carbon source was not needed to realize the autotrophic denitrification to N_2.

To maintain a stable cooperation between aerobic and anaerobic ammonia oxidizers, oxygen must be limiting and ammonia must be in excess. In the experiments described in Chapter 2, ammonia was always in excess. If oxygen is in excess, NOB might proliferate and oxidize nitrite to nitrate.

Anammox bacteria might deteriorate due to oxygen inhibition or shortage of nitrite. Since the ammonia concentration in wastewater is not always constant, the application of CANON will depend on the ability of the system to recover from major disturbances in feed composition. Therefore, the effect of extended periods of ammonium limitation was investigated in Chapter 3. A CANON reactor was started, with ammonia in excess, and 92% of the total nitrogen was removed. After prolonged exposure (> 1 month) to ammonium limitation, a population of NOB, identified using FISH, developed in the system and the NOB affected the CANON reaction stoichiometry, resulting in a temporary decrease in nitrogen removal from 92% to 57%. However, the changes caused by the ammonium-limitation were completely reversible, and the system re-established itself as soon as the ammonium limitation was removed. This study showed that CANON is a robust system for ammonium removal, enduring periods of up to one month of ammonium limitation without irreversible damage.

The volumetric ammonium removal in the experiments, described in Chapter 2 and 3, was quite low. This was due to limited mass transfer rates of oxygen. Therefore, the anammox process as well as the CANON process was investigated on their feasibility and performance in a gaslift reactor. The Anammox as well as the CANON process could be maintained easily in a gas lift reactor, and very high N-conversion rates were achieved. An N-removal rate of 8.7 kg N/ m^3 reactor /day was achieved for the Anammox process in a gas-lift reactor. N-removal rates of up to 1.5 kg N/m^3 reactor/day were achieved when the CANON process was operated. This removal rate was 20 times higher compared to the removal rates achieved in the laboratory previously and very promising when compared to alternative processes for N-removal.

Partial nitrification is the basis of the CANON system. Moreover, during nitrification in wastewater treatment plants, periodic build up of nitrite, due to partial nitrification and absence of anammox bacteria, can cause severe inhibition of the activated sludge biomass. Therefore, the interactions between AOB and NOB at low and limiting oxygen concentrations were investigated in Chapter 5 and 6. At low oxygen conditions (2-8 μM O_2), ammonium was converted completely to nitrate. Under these conditions, hardly any N_2O or NO was produced. The NOB, but not the AOB, were characterized by a very low K_s (1.3 μM) for oxygen. Under oxygen limitation, with ammonia in excess, ammonia was converted to mainly nitrite by AOB, which had now a very low K_s (2.3 μM) for oxygen as well. NO and N_2O were emitted; up to 3% and 10% of the NH_4^+-N converted, respectively. It became clear that these two situations, i.e. low oxygen conditions and oxygen limitation, were very distinct but fully reversible. Surprisingly, the affinity (the V_{max}/K_s ratio) for oxygen of the NOB was better than the affinity of AOB, although the kinetic parameters were characterized by large standard deviations. The mechanism behind nitrite accumulation in oxygen-limited systems fed with ammonium was further investigated. Inhibition of nitrite oxidation was studied by addition of ammonia, NO and N_2O to cultures in which NOB were present and active. The tested compounds, which are typically present under oxygen limited conditions in systems fed with ammonia had no

inhibitory effect on nitrite oxidation. The possible role of the large standard deviations in kinetic parameters on nitrite build up was studied by mathematical modeling. This was done with a suspended cell model using previously determined kinetic parameters. The modeling showed that, within the limits of the deviations in the measured parameters, two situations were possible. One in which all nitrogen was converted to nitrate and another situation in which nitrite oxidizing bacteria were inactive resulting in nitrite accumulation. Application of a biofilm model gave essentially the same results. The affinity (V_{max}/K_s) for oxygen of nitrite oxidizing bacteria could have been previously overestimated. Alternatively, the nitrite build-up in oxygen limited systems are the result of overlooked mechanisms, in particular microbial interactions mediated by signal molecules, which are known to be produced by nitrifiers.

Urea is present in many ecosystems and sometimes in industrial wastewaters. Urea can be used as an energy source by AOB. Thus, the utilization of urea by AOB as well as anammox bacteria was investigated in Chapter 7. In enrichment cultures grown with ammonia, a population established, which was dominated by halophilic and halotolerant AOB. In other batch enrichment cultures using urea, *N. oligotropha* like AOB formed about 50% of the total bacterial population. Also *N. nitrosa* (about 15% of the total population) and *N. europaea* (about 5% of the total population) were relatively abundant. This was shown by analysis of a 16S rRNA and an AmoA clone library as well as FISH. Additionally, continuous enrichments were performed under oxygen limitation. When ammonia was the energy source, the community in this reactor consisted of anammox bacteria and halophilic and halotolerant AOB. As the substrate was changed to urea, AOB related to *N. oligotropha* became the dominant AOB in this oxygen limited consortium. This resulted in a direct conversion of urea to dinitrogen gas, without the addition of organic carbon.

In Chapter 8, evidence is provided for the presence of bacteria that anaerobically oxidize ammonium with nitrite to N_2 in the world's largest anoxic basin, the Black Sea. Phylogenetic analysis of 16S ribosomal RNA gene sequences showed that these bacteria are related to anammox bacteria present in the reactors of Chapters 2,3,4 and 7 and closely related to anammox bacteria in other bioreactors. Nutrient profiles, FISH, ^{15}N tracer experiments, and the distribution of specific ladderane membrane lipids indicated that ammonium diffusing upwards from the anoxic deep water is consumed below the oxic zone by anammox bacteria. This was the first time anammox bacteria have been identified and directly linked to the removal of fixed inorganic nitrogen in the environment. The widespread occurrence of ammonium consumption in suboxic marine settings indicates that anammox may play an important role in the oceanic nitrogen cycle. Until now, the conversion of nitrate to N_2 by heterotrophic bacteria (denitrification) was believed to be the only important sink for fixed inorganic nitrogen (nitrate, nitrite and ammonium) in the ocean.

Finally, aspects for further research are given in chapter 9. For purposeful and widespread application of the CANON process, the starting procedure of such systems should be investigated further. Anammox can be enriched in oxygen limited nitrifying reactors, but this takes at least six

months. If a quick start-up procedure is used like described in this thesis, i.e. introducing oxygen in limited amounts in an anammox reactor, large amounts of anammox biomass are needed. Although anammox is starting to be applied in full scale reactors, more knowledge is needed about the diversity and niche differentiation between anammox bacteria to successfully kick start full scale reactors by inoculation or augmentation with anammox biomass, transferred from other reactors. To apply CANON for the removal of ammonium from low strength wastewaters at a sufficient high volumetric rate, the boundaries with respect to the liquid retention time and the solids retention time should be investigated, because anammox bacteria are slow growing bacteria. The mechanisms behind the absence of nitrite oxidation in oxygen limited systems remains puzzling. On one hand, more sensitive equipment like microelectrodes should be used to determine kinetic parameters of nitrifying bacteria grown at low or limiting oxygen conditions. On the other hand, the role of signal molecules must be studied. To investigate the interactions between bacteria involved in nitrogen transformations in natural environments, ^{15}N tracer studies are proposed. These studies include, of course, a possible role for anammox.

Samenvatting

Ammonium oxidatie op het oxisch/anoxisch grensvlak

Olav Sliekers

Mensen kunnen een grote invloed hebben op de input van stikstofverbindingen in de natuur en dat leidt tot bijvoorbeeld eutrofiering en zure regen. De uitstoot van stikstofverbindingen in de natuur door mensen, anders dan stikstof gas, moet dan ook tot een minimum beperkt worden. Tijdens verscheidende microbiële processen kunnen stikstofverbindingen omgezet worden. Kennis van omzettingen door micro-organismen in de stikstof cyclus is nodig om de stikstof cyclus te kunnen begrijpen en om het effect van menselijk handelen op de natuur te begrijpen. Toepassing van kennis omtrent de stikstof cyclus leidt tot efficiëntere manieren om stikstofhoudend afvalwater te behandelen.

Het onderzoek dat beschreven is in dit proefschrift doelde op een beter begrip van nitrificatie en anaërobe ammonium oxidatie (anammox) op het oxisch/anoxisch grensvlak. Nitrificatie is de aërobe oxidatie van ammonium (NH_4^+) via nitriet (NO_2^-) naar uiteindelijk nitraat (NO_3^-). Anammox is de anaërobe oxidatie van ammonium met nitriet naar stikstof gas (N_2). Beide processen worden door autotrofe bacteriën uitgevoerd. Nitrificatie wordt door twee groepen bacteriën uitgevoerd, te weten aerobe ammonium oxideerders en aerobe nitriet oxideerders. Anammox wordt uitgevoerd door bacteriën behorende tot de orde van de planctomyceten. Bacteriën die het Anammox proces kunnen uitvoeren werden recentelijk ontdekt in laboratoriumreactoren en kunnen worden gebruikt als een alternatief voor denitrificatie om een complete omzetting te krijgen van ammonium naar stikstofgas. Een combinatie van aerobe en anaerobe ammonium oxideerders kan ammoniumrijk afvalwater behandelen zonder de noodzaak van een dure koolstofbron zoals methanol. Een dergelijke combinatie wordt gebruikt in het CANON-proces (completely autotrophic Nitrogen removal Over Nitrite).

Eerst werd de microbiologie en de haalbaarheid van het CANON-proces onderzocht (hoofdstuk 2). Een reactor werd geënt met biomassa uit een andere reactor waar anaerobe ammonium oxidatie plaatsvond. Daarna werd de reactor belucht en een nitrificerende populatie ontwikkelde zich in de reactor, terwijl de anaerobe ammonium oxiderende bacteriën nog steeds actief bleven. Zuurstof werd in limiterende hoeveelheden toegevoegd en de samenwerking tussen aerobe en anaerobe ammonium oxideerders was stabiel gedurende het hele experiment. Aerobe nitriet oxideerders waren afwezig in de reactor, hetgeen werd aangetoond met Flourescence In Situ Hybridization (FISH). Ammonium werd voornamelijk omgezet naar stikstofgas (85%) en de rest werd naar nitraat omgezet (15%). Productie van N_2O, een broeikasgas, was verwaarloosbaar (minder dan 0.1 %). Er hoefde geen koolstofbron worden toegevoegd om de autotrofe omzetting van ammonium naar stikstof gas te verkrijgen.

Om een stabiele samenwerking tussen aerobe en anaerobe ammonium oxideerders te houden, moet zuurstof de limiterende factor zijn, terwijl ammonium in overvloed aanwezig moet zijn. Tijdens de experimenten, beschreven in hoofdstuk 2, was ammonium altijd in overvloed. Als zuurstof in overvloed aanwezig is en ammonium limiterend, kunnen aerobe nitriet oxideerders zich gaan ontwikkelen en nitriet in nitraat omzetten. Anammoxbacteriën kunnen dan afsterven wegens inhibitie door zuurstof of een tekort aan nitriet. Aangezien de concentratie van ammonium niet altijd constant is in afvalwater, zal de toepassing van het CANON-proces afhangen van de mogelijkheid om zich te herstellen van grote veranderingen in de ammoniumconcentratie. Daarom werd het effect van lange perioden van ammoniumlimitatie onderzocht (hoofdstuk 3). Een CANON-reactor werd opgestart met een overvloed aan ammonium en 92% van de totale stikstof werd verwijderd. Vervolgens werd gedurende meer dan een maand, ammoniumlimitatie toegepast en een nitriet oxiderende bacteriepopulatie ontwikkelde zich in de reactor, wat resulteerde in een verstoring van de CANON-stochiometie en een tijdelijke afname in de stikstofverwijdering van 92 % naar 57 %. Echter, de veranderingen veroorzaakt door de ammoniumlimitatie waren volledig terug te draaien en het CANON-systeem herstelde zichzelf zodra de ammoniumlimitatie werd opgeheven. Deze studie toonde aan dat CANON een robuust systeem is voor ammoniumverwijdering en een periode van een maand van ammoniumlimitatie kan doorstaan zonder blijvende schade.

De volumetrische ammonium omzetting was behoorlijk laag gedurende de experimenten beschreven in hoofdstuk 2 en 3. Dit werd veroorzaakt door lage zuurstof overdracht snelheden. Daarom werden het Anammox- en het CANON-proces onderzocht in een gaslift reactor. Beide processen deden het goed in een gaslift reactor en hoge stikstof omzettingssnelheden konden worden bereikt. Een stikstofomzettingsnelheid van 8.7 kg N/ m^3 reactor /dag kon worden gehaald met het Anammox-proces en 1.5 kg N/ m^3 reactor/ dag kon worden bereikt met het CANON-proces. Deze laatste waarde was 20 keer hoger dan de snelheid die werd gehaald gedurende de vorige experimenten met het CANON-proces en veelbelovend indien deze waarde wordt vergeleken met andere processen voor stikstofverwijdering.

Partitiële nitrificatie is de basis van het CANON-proces. Tevens kan periodieke ophoping van nitriet in waterzuiveringssystemen, wegens partitiële nitrificatie en de afwezigheid van Anammoxbacteriën, de biomassa ernstig inhiberen. Om deze redenen werden de interacties tussen aerobe ammonium oxideerders en aerobe nitriet oxideerders onderzocht bij lage en limiterende zuurstof concentraties (hoofdstuk 5 en 6). Bij lage zuurstofconcentraties (2-8 μM O_2) werd ammonium vo9lledig omgezet naar nitraat. Onder deze condities werd er nauwelijks N2O en NO geproduceerd. De nitriet oxideerders, in tegenstelling tot de ammonium oxideerders, hadden een erg lage K_s (1.3 μM). Gedurende zuurstoflimitatie en ammonium in overvloed werd ammonium omgezet naar nitriet door aerobe ammonium oxideerders, die nu ook een lage K_s waarde hadden. Zowel NO als N2O werden geproduceerd tot respectievelijk 3 % en 10 % van de omgezette NH_4^+-N. Het werd duidelijk dat deze twee situaties, te weten: lage zuurstofcondities en zuurstoflimitatie, volledig verschillend

maar omkeerbaar waren. Verassend was dat de affiniteit (de Vmax/Ks ratio) voor zuurstof van de nitriet-oxideerders beter was dan de affiniteit van de ammoniumoxideerders, hoewel de kinetische parameters grote standaarddeviaties hadden. Het mechanisme achter de nitrietophoping in zuurstofgelimiteerde systemen werd verder bestudeerd. Inhibitie van nitrietoxidatie werd bestudeerd door toevoeging van ammoniak, NO en N2O aan cultures waarin nitriet-oxeerders aanwezig en actief waren. De geteste stoffen, die typisch aanwezig zijn in zuurstofgelimiteerde systemen gevoed met ammoniak had geen inhiberende effect op nitriet oxidatie. Vervolgens werd gekeken met mathematische modellering naar de grote standaard deviaties van de kinetische parameters. Modellering toonde aan dat binnen de grenzen van de standaarddeviaties twee compleet verschillende situaties mogelijk waren. Een situatie waarin alle stikstof werd omgezet naar nitraat en een situatie waarin nitriet-oxideerders inactief waren, hetgeen resulteerde in nitriet ophoping. Het is mogelijk dat de affiniteit van de nitriet-oxideerders voorheen overschat zijn. Ook is het mogelijk dat de nitrietophoping veroorzaakt wordt door mechanismen waar over heen is gekeken, zoals het effect van signaal moleculen, waarvan het bekend is dat deze door nitrificeerders worden gemaakt.

Ureum is aanwezig in verscheidende ecossytemen en soms ook in industrieel afvalwater. Ureum kan worden gebruikt als energiebron door aerobe ammonium oxideerders. Daarom werd het gebruik van ureum door zowel aerobe als anaerobe ammoniumoxideerders bestudeerd (hoofdstuk 7). In batch ophopingscultures met ammonium ontwikkelde zich een populatie die werd gedomineerd door halofiele en halotolerante ammonium oxideerders. In batch ophopingscultures met ureum domineerden bacteriën gerelateerd aan *Nitrosomonas oligotropha*. Ook *Nitrosomonas nitrosa* (15% van de populatie) en *Nitrosomonas europaea* (5% van de populatie) waren aanwezig. Dit werd aangetoond door zowel analyse van een 16 S rRNA en een AmoA klonenbank als ook door het gebruik van FISH. Tevens werden ophopingen in continu-systemen onder zuurstoflimitatie uitgevoerd. Wanneer ammonium de energiebron was, bestond de populatie in de reactor uit Anammoxbacteriën en halofiele en halotolerante ammoniumoxiderende bacterien. Wanneer ureum als energiebron werd toegediend, werden ammonium oxideerders gerelateerd aan *Nitrosomonas oligotropha* de dominante aerobe ammonium-oxideerders in het zuurstofgelimiteerde consortium. Dit resulteerde in een directe omzetting van ureum naar stikstofgas.

In hoofdstuk 8 worden bewijzen geleverd voor de aanwezigheid van bacteriën, die anaeroob ammonium kunnen oxideren met nitriet naar stikstofgas in het grootste anoxische basin ter wereld, de Zwarte Zee. Fylogenetische analyse van 16 S rRNA gen sequenties toonde aan dat deze bacteriën nauw verwant zijn aan de Anammoxbacteriën in de reactoren beschreven in hoofdstukken 2, 3, 4 en 7. Nutriënt-profielen, FISH, ^{15}N-tracer experimenten en de distributie van specifieke ladderaan membraanlipiden toonden aan dat de ammonium, dat opwaarts diffundeert vanuit anoxisch diepere wateren, beneden de oxische zone wordt geconsumeerd. Dit was de eerste keer dat Anammoxbacteriën werden aangetoond in natuurlijke ecosystemen. Het wereldwijd voorkomen van ammonium oxidatie in suboxische mariene milieus doet vermoeden dat anammox een belangrijke rol

speelt in de mariene stikstofcyclus. Tot dusver werd gedacht dat de omzetting van nitraat naar stikstofgas door heterotrofe bacteriën (denitrificatie) de enige belangrijke afvoer van gefixeerde stikstof (nitraat, nitriet en ammonium) in de oceaan was.

Tenslotte worden in hoofdstuk 9 ideeën gegeven voor verder onderzoek. Voor doeltreffend en wijdverspreide applicatie van het CANON proces, is verder onderzoek naar de opstart procedure van het proces noodzakelijk. Anammoxbacteriën kunnen worden opgehoopt in zuurstof gelimiteerde systemen, maar dat zal enige maanden kosten. Een snelle opstart zoals gebruikt in de proeven beschreven in dit proefschrift, te weten: gelimiteerd zuurstof toevoegen aan draaiende anammoxreactoren, zal grote hoeveelheden van Anammox-biomassa vergen. Hoewel Anammox reeds toegepast begint te worden in grootschalige reactoren, is meer kennis nodig omtrent de diversiteit en nichedifferentiatie van Anammoxbacteriën, teneinde de kickstart van grootschalige reactoren door beënting met Anammox-biomassa uit andere reactoren succesvol te laten verlopen. Om CANON toe te passen voor ammoniumverwijdering uit afvalwater met lage ammoniumgehaltes, zullen de grenzen van de hydrolic retention time (HRT) en de solids retention time (SRT) onderzocht moeten worden, aangezien Anammoxbacteriën langzaam groeien. Het mechanisme verantwoordelijk voor de afwezigheid van nitriet-oxideerders in zuurstofgelimiteerde reactors blijft onduidelijk. Enerzijds zullen veel gevoeligere instrumenten zoals micro-elektrodes gebruikt moeten worden om kinetische parameters te bepalen van bacteriën die onder zuurstofgelimiteerde omstandigheden worden gekweekt. Anderzijds moet de eventuele rol van signaalmoleculen worden onderzocht. Om een beter beeld te krijgen van interacties tussen bacteriën die stikstof omzetten in natuurlijke omgevingen zijn ^{15}N-tracer studies voorgesteld met uiteraard een mogelijke rol voor Anammoxbacteriën.

Dankwoord

Het proefschrift is bijna af, maar dat zeg ik al een paar maanden. Toch is het nu echt gebeurd want de afspraak met de drukker is al gemaakt en over niet al te lange tijd zal de verdediging plaatsvinden. Het waren al met al een paar hele leuke jaren, waarin ik veel geleerd heb. Ook is het eindresultaat, "het boekje", redelijk dik geworden. Maar dat heb ik niet alleen gedaan. Gelukkig ben ik in de gelegenheid om alle medeplichtigen hier met naam en toenaam te noemen en te bedanken.

Als eerste wil ik mijn promotoren Mike Jetten en Gijs Kuenen bedanken, die altijd veel interesse in mijn werk hadden, veel voor mij geregeld hebben, en ook alle vertrouwen hadden in mijn eigen ideeën maar die mij ook, waar het nodig was, bijstuurden. Marc Strous heeft mij niet alleen in het begin ingewerkt, maar is ook geestelijk vader van de CANON en heeft regelmatig kritisch naar mijn resultaten gekeken. Uit Australië kwam er goede versterking, namelijk Katie Third, waarmee ik een half jaar bijzonder effectief mee heb kunnen samenwerken zoals duidelijk te zien is in hoofdstuk 3 en 4. Goede studenten waren er ook! Ik hoop dat Niels Derwort, Marit Stafsnes en Suzanne Haaijer net zoveel van mij hebben kunnen leren als dat ze resultaten geleverd hebben. Waarvoor dank!! Ook wil ik Marcel Kuypers bedanken voor de samenwerking en niet te vergeten Markus Schmid maar ook Harry Haranghi, Karin Verwegen, Wiebe Abma, Luis Campos Gomez en "De Denen": Niels Peter Revsbech, Lars Peter Nielsen, Michael Nielsen en Anette Bollmann. De "nitrificatie-etentjes" met Anette Bollmann, Ingo Schmidt en Bertus Beaumont wil ik ook zeker even noemen.

I will never forget the expedition on the Black Sea. I'm just thankful for the possibility given to me to join this expedition. I would especially like to thank all the people from the "water-column"-group for the work we did together and I would like to thank all participants for making this trip really nice. Christmas on a ship in the middle of the Black Sea is something special.

Fantastic were also the Dutch-UK N-cycle meetings, which were always a perfect combination of nice people, nice pubs, tasty beer, interesting science and good food and some more tasty beer.

Dankbaar ben ook ik nog steeds Janneke Krooneman, Robert van der Geize en Malgosia Swiatek voor de begeleiding tijdens mijn studie en het geven van een basis voor het uitvoeren van onderzoek.

Dan zijn er nog zoveel mensen die het werk gemakkelijker en vooral leuk hebben gemaakt. De koffieclub in Delft was altijd weer gezellig. Personen als Pascal, Astrid, Jos, Sjaak en Theo hebben het werk in Delft een stuk gemakkelijker gemaakt. In Nijmegen heb ik het ook prima naar mijn zin gehad. Alle collega's: bedankt!

Ook wil ik Danny bedanken voor het voorrecht om een jongen op te zien groeien. Toen ik begon aan promotieonderzoek was Danny nog maar een baby van drie maanden oud. Nu is het al een kleuter die naar groep twee gaat. Hij zorgde er voor dat ik compleet vergat wat ik die dag gedaan had zodra ik thuis kwam. Ania, jou wil ik bedanken voor jouw geduld, steun en die heerlijke momenten buiten mijn

werk. De snelheid waarmee jij je leven op hebt gepakt in Nederland is een geweldige prestatie. Ook Ilse en Geert, Roos, Fiore en Nikée, bedankt voor de gezelligheid, steun en de interesse in mijn werk. Vrienden en kennissen: bedankt voor de gezelligheid, interesse en steun!

Mijn ouders verdienen een speciale plek in dit proefschrift. Niet alleen hebben jullie meegeholpen (bijvoorbeeld spullen naar Bremen brengen voor de expeditie naar de Zwarte Zee) maar als ik terug denk aan hoe ik vroeger van jullie altijd mocht kliederen met potjes en oplossingen dan is dit proefschrift eigenlijk een logisch vervolg.

Olav Sliekers

Curriculum Vitae

The author of this thesis was born on the 5th of May 1973 in Hoogeveen, the Netherlands. In 1992, he went to Groningen to study biology. He attended a broad range of courses about ecology, microbiology, biochemistry and molecular biology. At the laboratory of Prof. dr. R.A. Prins, he specialized in microbial ecology under supervision of Janneke Krooneman. During this period, he investigated the aerobic and micro-aerobic degradation of halo-aromatic compounds. Subsequently, he specialized in microbial physiology at the laboratory of Prof. dr. L. Dijkhuizen under supervision of Robert van der Geize and studied the genes encoding enzymes involved in sterol degradation. He went to the University of Gdańsk, Poland, for four months to specialize in biochemistry at the laboratory of molecular and cellular biology of Prof. dr. hab. M. Zylicz under supervision of M. Swiątek. During his stay in Poland, he purified truncated mutants of λO and looked at the degradation of these mutants by the ClpX/P proteolytic system. After receiving his Master of Science degree, he started his PhD study in 1998 at the TUDelft under supervision of Prof. dr. J.G. Kuenen and Prof. dr. M.S.M. Jetten. The results are written in this thesis. Now he's working as a scientist at Purac BV in Gorinchem, The Netherlands. Purac produces lactates and gluconates via fermentation.

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Abbreviations and acronyms

Anammox	ANaerobic AMMonia Oxidation
AOB	Ammonia Oxidizing Bacteria
BAS	Biofilm Airlift System
CANON	Completely Autotrophic Nitrogen-removal Over Nitrite
COD	Chemical Oxidation Demand
CSTR	Continuous Stirred Tank Reactor
CTD	Conductivity Temperature Depth
FBR	Fluidized Bed Reactor
FISH	Fluorenscence In Situ Hybridization
OLAND	Oxygen-Limited Autotrophic Nitrification-Denitrification
RBC	Rotating Biological Contactor
SBR	Sequencing Batch Reactor
SHARON	Single reactor High activity Ammonium Removal Over Nitrite
NOB	Nitrite Oxidizing Bacteria

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