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ecophysiology of the anammox bacteria

ECOPHYSIOLOGY OF THE ANAMMOX BACTERIA

Een wetenschappelijke proeve op het gebied van de Natuurwetenschappen, Wiskunde en Informatica

Proefschrift

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door

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geboren op 01 April 1979 te İstanbul

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Ecem'e

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chapter 1
general introduction

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In nature inorganic nitrogen atoms can exist in different oxidation states from -3 (NH_4^+) to +5 (NO_3^-). Most of the nitrogen compounds representing these oxidation states can be converted to one and other through microbial activity. Microbiologists link these conversions with the so-called Nitrogen cycle (N-Cycle, Figure 1). In the beginning of the 20th century most of the reactions depicted in the N-Cycle, nitrogen fixation, nitrification, denitrification, were already described for a long time (Winogradsky, 1949), and the N-Cycle was assumed to be complete. In this "complete" N-Cycle, there was no reaction accounting for the possibility of the anaerobic oxidation of ammonium even though this reaction, with nitrite as electron acceptor, is associated with a substantial release of Gibbs free energy, -358 kJ/mol (Broda, 1977). Moreover, oceanographers had already noticed that ammonium accumulating in an anoxic fjord was far less than that would be expected if ammonium was to be inert under anoxic conditions (Richards, 1965).

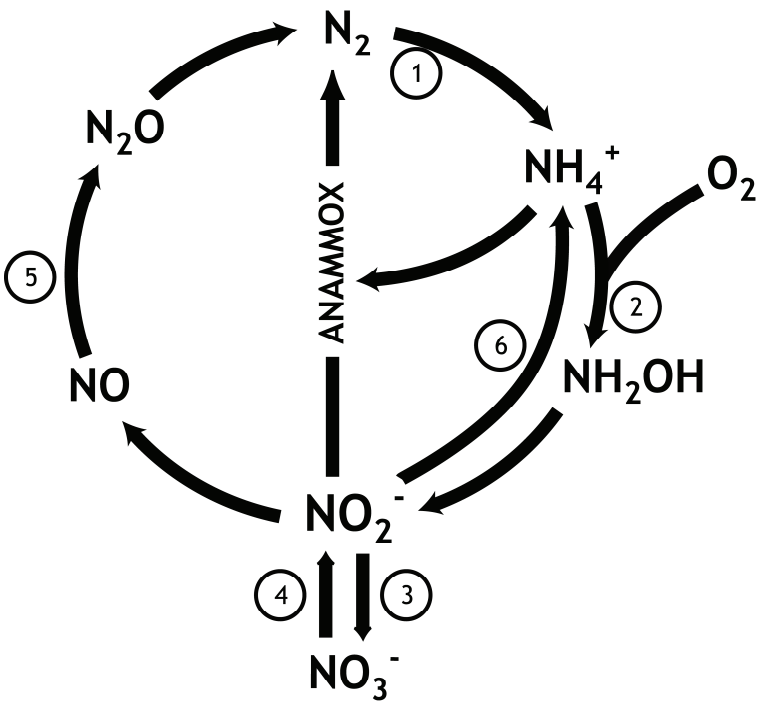

Figure 1| The Nitrogen Cycle

1. Nitrogen Fixation
2. Aerobic ammonium oxidation
3. Nitrite oxidation
4. Nitrate reduction
5. Denitrification
6. Nitrite reduction to ammonium

The first evidence for the existence of anaerobic ammonium oxidation (anammox) was from a pilot wastewater treatment plant (wwtp) (Mulder, et al. 1995). This was followed by the molecular identification of the bacteria responsible for the anammox reaction (Strous et al. 1999a). Physically separated cells of a representative of the phylum Planctomycetes, Candidatus "*Brocadia anammoxidans*" were shown to oxidize ammonium with nitrite under strictly anoxic conditions to dinitrogen gas (Strous, et al. 1999a).

Physiology, biochemistry and diversity of the anammox bacteria

Surveys of different wastewater treatment plants using anammox specific 16S rRNA gene primers and anammox specific oligonucleotide probes have revealed the presence of at least three other anammox bacteria, which have been named *Kuenenia stuttgartiensis*, *Candidatus* "Scalindua wagneri" and *Candidatus* "Scalindua brodae" (Figure 2). Close relatives of the *Candidatus* "Scalindua spp" were found to have significant contributions to the loss of fixed nitrogen in the marine environments (Kuypers, et al. 2003, Kuypers, et al. 2005, Francis, et al. 2007, Schmid, et al. 2007). *Candidatus* "Anammoxoglobus propionicus", *Candidatus* "Brocadia fulgida", anammox bacteria that could co-oxidize organic acids, were enriched from sludge from a wastewater treatment plant using a medium containing ammonium, nitrite and propionate (*A. propionicus*) or acetate (*B. fulgida*) (Kartal, et al. 2007a,

Kartal et al., in press). Interestingly three of the four described anammox genera could be enriched from the same activated sludge inoculum (Strous, et al. 2006, Kartal, et al. 2007a, Kartal, et al. in press). The genus *Candidatus* "Scalindua" is so far the only anammox species detected in the natural environment (Penton, et al. 2006, Schubert, et al. 2006, Schmid, et al. 2007). These findings raise a question about the niche differentiation of the anammox bacteria: which compounds or conditions determine which anammox species occupy a given ecosystem? Chapters two, three and four (in part) of this thesis address this question.

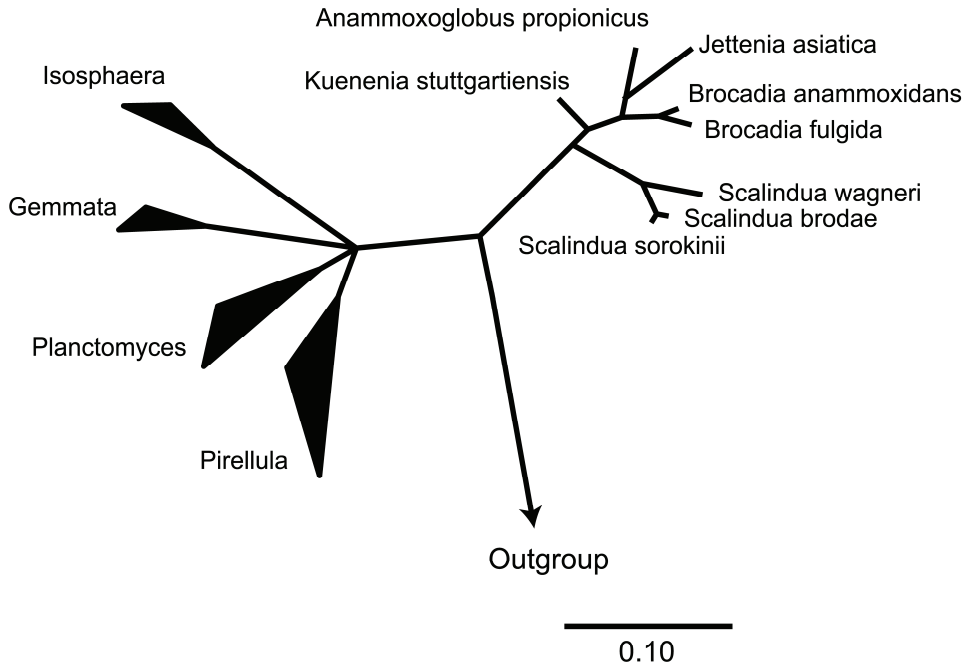

Figure 2 | Phylogenetic tree showing the relationships of known anammox species to each other, to other Planctomycetes and other reference organisms.

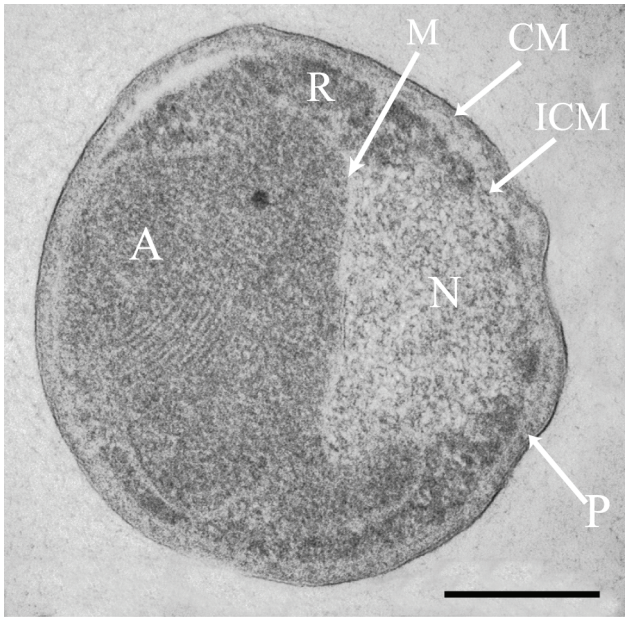

Figure 3 | Transmission electron micrograph of *Candidatus* "Anammoxoglobus Propionicus"

An anammoxosome (A) containing tubule-like structures, riboplasm (R) containing the nucleoid (N) apposed to the anammoxosome membrane (M), paryphoplasm (P) separated from riboplasm by an intracytoplasmic membrane (ICM) and the cytoplasmic membrane (CM). Bar = 200 nm

Examination of the anammox bacteria with transmission electron microscopy revealed that anammox bacteria shared the same compartmentalized cell plan of other Planctomycetes (Lindsay, et al. 2001, Schmid, et al. 2003, Kartal, et al. 2007a, Figure 3). The anammox

bacteria contain a single membrane bound organelle-like compartment tentatively named the "anammoxosome". This unique organelle-like compartment is surrounded by a membrane mainly consisting of ladderane lipids containing 3 to 5 concatenated cyclobutane rings (Sinninghe Damsté, et al. 2002). The anammoxosome represents a large part of the anammox cell volume (Lindsay, et al. 2001). It is hypothesized that the anammox catabolism takes place in the anammoxosome and is associated with its membrane (van Niftrik, et al. 2004).

Anammox bacteria are slow growers, they double every 11-20 days, but they are very well suited to convert their substrates. They have K_s values for ammonium and nitrite below 5 µM (Strous, et al. 1999b). They are reversibly inhibited by very low levels (< 1 µM) of oxygen and (ir)reversibly inhibited by high nitrite (>10mM) concentrations. The biochemical conversions leading to the formation of dinitrogen gas from nitrite and ammonium were the topic of a number of studies. The first biochemical pathway hypothesis for anammox process was based on ^{15}N-labeling experiments (Van de Graaf, et al. 1997), however the findings were far from conclusive. According to this tentative model hydrazine and hydroxylamine were the intermediates of the anammox reaction (Figure 4A). In this first scheme nitrite was reduced to hydroxylamine by a "nitrite reducing enzyme" (Jetten, et al. 2001). Then the hydroxylamine and ammonium were condensed to hydrazine by the so-called hydrazine hydrolase. Hydrazine was then oxidized to dinitrogen gas by a hydroxylamine oxidoreductase (HAO) like enzyme. Such a protein with both hydroxylamine and hydrazine oxidizing capacity was purified from *Candidatus* "Brocadia anammoxidans" (Schalk, et al. 2000). This protein was present exclusively in the anammoxosome as shown by immunogold labeling (Lindsay, et al. 2001). Recently, the genome of the anammox bacterium *Kuenenia stuttgartiensis* was reconstructed from a bioreactor metagenome. Annotation of the *Kuenenia stuttgartiensis* genome revealed a gene that encoded for a "nitrite reducing enzyme", but, unexpectedly, this was a cytochrome cd1 type nitrite reductase (Strous, et al. 2006). This type of nitrite reductase reduces nitrite to nitric oxide, and not to hydroxylamine. Based on the genomic analysis a modified anammox pathway was proposed. According to this pathway, nitrite was first reduced to nitric oxide; ammonium was then combined with NO to form hydrazine which was later oxidized to dinitrogen gas (Figure 4B). Recently a second protein that oxidized only hydrazine at a higher rate than the protein purified by Schalk et al. (2000) was purified from the anammox bacterium KSU-1 (Shimamura, et al. 2007). This HAO-like enzyme did not convert and was inhibited by hydroxylamine (Shimamura, et al. 2007). This suggests that the oxidation of hydrazine and hydroxylamine are mediated by two distinct enzymes, and that it is unlikely that the protein isolated by Schalk et al. is the one that oxidizes hydrazine in the anammox process. The intermediates of anammox biochemistry are investigated in chapter five of this thesis.

In the anammox process nitrite is not only reduced to nitric oxide (or hydroxylamine); a part of it is oxidized to nitrate to release electrons for CO_2 fixation. The chemolithoautotrophic life style of the anammox bacteria has been established by $^{14}CO_2$ incorporation into the cells and confirmed by mass balances (Strous, et al. 1999a). Based on the isotopic signatures of anammox specific biomarkers (extremely ^{13}C-depleted, -47‰ compared to CO_2) it was proposed that the acetyl-CoA pathway was used (Schouten, et al. 2004). Indeed, all genes necessary for an acetyl-CoA pathway were detected in the genome of *Kuenenia stuttgartiensis*, whereas all other carbon fixation pathways were missing or incomplete (Strous, et al. 2006).

Anammox bacteria do not only convert nitrite and ammonium, they are generalists. They also use Fe^{3+}, Mn^{4+}, nitrite and nitrate as electron acceptors, and Fe^{2+}, formate, acetate, propionate and methylamines as electron donors (Güven, et al. 2005, Strous, et al. 2006, Kartal, et al. 2007a, Kartal, et al. in press). The nitrate reduction pathway by anammox bacteria is specially interesting because during this metabolism anammox bacteria use of their end products (nitrate) to produce their substrates (nitrite and ammonium) with an

organic acid as the electron donor. This metabolism is addressed in chapters two and three; its mechanism is described in chapter six.

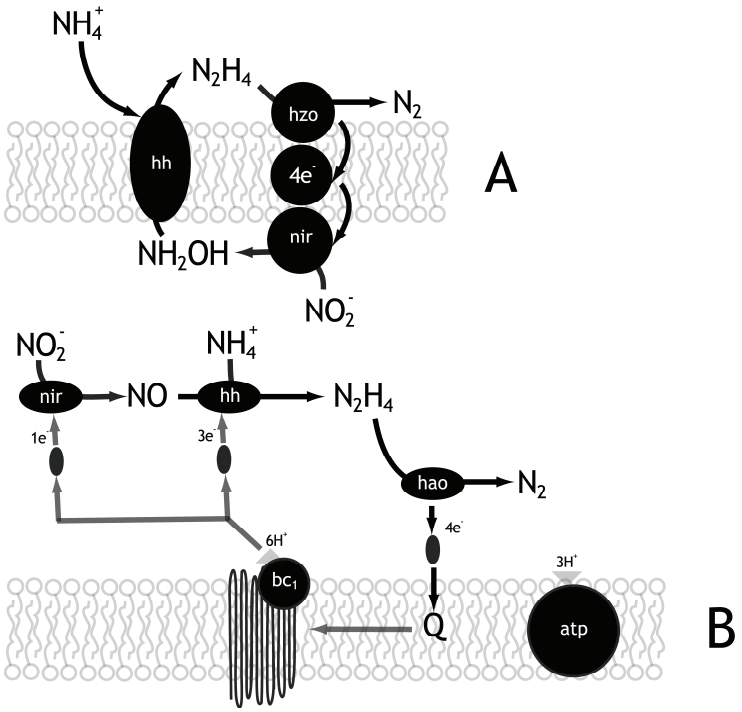

Figure 4 I Different hypotheses on the anammox catabolic pathway. 1A Proposed in Jetten et al. 2003 1B Proposed in Strous et al. 2006

Hunting for the Anammox Bacteria

In the past decade of anammox research three main state of the art approaches were used to trace anammox bacteria in wastewater treatment plants and in the environment: molecular techniques, detection of anammox lipids and labeling experiments (Kuypers, et al. 2003, Risgaard-Petersen, et al. 2003, Risgaard-Petersen, et al. 2004, Kuypers, et al. 2005, Schmid, et al. 2005, Kartal, et al. 2007).

Molecular Techniques

For the analysis of both environmental and wastewater treatment plant samples polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH) based techniques have been the methods of choice for the detection and quantification of anammox bacteria.

The detection of anammox bacteria depends strongly on the proper choice of PCR primers. Anammox bacteria are underrepresented in 16S rRNA clone libraries using so-called universal primers because of the mismatches in the primers with the anammox 16S rDNA template. When Planctomycetes or anammox specific primers (such as Pla46F, Amx368F, Amx820R) are used the relative amount of retrieved anammox sequences from a given sample increases (Schmid, et al. 2005, Penton, et al. 2006). Recently, anammox specific primer sets were developed and tested in quantitative PCR assays to quantify anammox cells in biomass from different wastewater treatment plants (Tsushima, et al. 2007a). With further optimizations in the future this technique could be applied to detect and quantify anammox bacteria in nature and waste water treatment plants.

Fluorescence in situ hybridization (FISH) is a tool to both detect and quantify anammox bacteria in both environmental samples and waste water treatment plants. Anammox bacteria can successfully be detected with domain, phylum, genus and species specific probes. Probes EUB338I, II and III which target the 16S rRNA of most bacteria have mismatches with members of the Planctomycetes phylum (Amann, et al. 1990, Daims, et al. 1999). A substitute probe with two nucleotide difference with the original probe (EUB338IV) should be used as a general probe to detect anammox microorganisms (Schmid, et al. 2005). Anammox bacteria belong to the phylum Planctomycetes; probe Pla46 that targets

Planctomycetes hybridizes with the 16S rRNA of anammox bacteria and is good for initial experiments (Neef, et al. 1998).
For specific detection of anammox microorganisms in nature and in wastewater treatment plants there are several oligonucleotide probes available. Probe Amx368 was designed to detect all anammox microorganisms (Schmid, et al. 2003). Probe Amx820 was designed to detect *Kuenenia spp*. and *Candidatus* "Brocadia spp." (Schmid, et al. 2001). In order to detect these genera separately, probe Kst1275 and Ban162 are designed to hybridize with *Kuenenia spp.* and *Candidatus* "Brocadia spp." respectively (Schmid, et al. 2000, Schmid, et al. 2001). The probe Apr820 is designed to specifically detect *Candidatus* "Anammoxoglobus spp." (Kartal, et al. 2007). Recent discovery of *Candidatus* "Scalindua spp." in natural ecosystems and wastewater treatment plants showed that the previously constructed probes were not sufficient (Schmid, et al. 2003). The probes BS820, Sca1309 and Scabr1114 are designed to detect *Candidatus* "Scalindua spp." exclusively (Kuypers, et al. 2003, Schmid, et al. 2003). If the probes Amx820, BS820 and Apr820 are used together as competitors, better results can be achieved for the detection of different types of anammox bacteria simultaneously (Schmid, et al. 2003, Kartal, et al. 2007). Today there are specific oligonucleotide probes for all known anammox species. The increasing amount of available validated anammox sequences will lead to the design of improved probes for detection and *in situ* identification of the anammox bacteria.

Anammox Lipids

Anammox bacteria have unique membrane lipids (ladderanes) that make excellent biomarkers for detection (Kuypers, et al. 2003, Schmid, et al. 2003, Kartal, et al. 2007). Anammox bacteria also produce hopanoids and branched fatty acids that can be detected, but these are not specific for anammox bacteria (Sinninghe Damsté, et al. 2002, Sinninghe Damsté, et al. 2004, Sinninghe Damsté, et al. 2005). The anammox lipids are characterized by substantial ^{13}C depletion relative to their carbon source (CO_2). This feature of the anammox lipids can also be used to confirm their "anammox" origin (Schouten, et al. 2004).

Labeling Experiments

Since the discovery of the anammox process, the anammox bacteria have been detected in many natural ecosystems and are currently believed to contribute significantly to the loss of inorganic nitrogen in the oceans – next to denitrification (Trimmer, et al. 2003, Risgaard-Petersen, et al. 2004, Rysgaard, et al. 2004, Arrigo, 2005, Kuypers, et al. 2005, Meyer, et al. 2005). The relative contributions of anammox and denitrification to marine N-losses were estimated via the incubation of sediments or water in sealed containers (Thamdrup & Dalsgaard, 2002, Trimmer, et al. 2003, Kuypers, et al. 2005). These containers were amended with excess ^{15}N-labelled nitrogen species (such as ^{15}N-ammonium and ^{15}N-nitrate). Based on the stoichiometry of anammox and denitrification, the contributions of both processes could be derived from the recovery of the label in the produced N_2; where denitrification converts ^{15}N-nitrate or ^{15}N-nitrite to $^{15}N^{15}N$, anammox converts it exclusively to $^{14}N^{15}N$ (in the presence of unlabeled ammonium). The ratio of $^{15}N^{15}N$ to $^{14}N^{15}N$ can be measured by isotope-ratio mass spectrometry (IR-MS). Complementary labeling experiments with ^{15}N-labeled NH_4^+ and NO_2^- are sufficient to quantify anammox activity in environmental samples. Under anoxic conditions only anammox species can form the unique $^{14-15}N_2$ from $^{15}NH_4^+$ and $^{14}NO_2^-$, and from the rate of evolution of $^{14-15}N_2$ *in situ* anammox rates can be calculated and compared to the rate of denitrification.

Anammox Bacteria in Nature

As mentioned above, the existence of the anammox process was completely overlooked for a long time. In nature, under anoxic conditions, denitrification was regarded as the only pathway for the loss of fixed nitrogen in the natural environments. If this was true, ammonium would accumulate to a large extent in nature. However as observed by Richards,

in certain marine ecosystems, there was an unaccountable ammonium deficiency (Richards, 1965). When the anaerobic ammonium oxidizing bacteria were first discovered, it was believed that the anammox process would not be significant in nature due to the long doubling times of these bacteria (Zehr & Ward, 2002). However, the activity and the presence of the anammox bacteria in nature were shown in the Black Sea, the World's largest anoxic basin (Kuypers, et al. 2003). Later it was also shown in the oxygen minimum zones of the Benguela and Peru upwelling systems, important sites for primary production in the Atlantic and Pacific Oceans, that the anammox bacteria contribute significantly to the loss of fixed nitrogen (Kuypers, et al. 2005, Thamdrup, et al. 2006, Hamersley, et al. 2007). In other studies anammox bacteria and their activity have been detected in the anoxic or sub-oxic zones of many marine and freshwater ecosystems (Trimmer, et al. 2005, Penton, et al. 2006, Schubert, et al. 2006, Rich, et al. 2007, Schmid, et al. 2007). These examples do not only illustrate the importance of the anammox process for the marine ecosystems, but also have clear implications towards the ubiquity of the anammox bacteria. In the last five years, anammox bacteria have been recognized as important actors in the global biogeochemical nitrogen cycle (Devol, 2003, Ward, 2003, Arrigo, 2005, Brandes, et al. 2007, Francis, et al. 2007). In some of the studies mentioned above the types of anammox species that were mediating the anammox process were also investigated. In all of these studies exclusively *Candidatus* "Scalindua spp" were detected (Kuypers, et al. 2003, Kuypers, et al. 2005, Penton, et al. 2006, Rich, et al. 2007, Schmid, et al. 2007, Woebken, et al. 2007). A comprehensive list of the manmade and natural eceosystems where anammox bacteria were detected is given in Table 1.

Application of the Anammox Bacteria

Conventional nitrogen removal processes depend on a combination of nitrification and denitrification. This increases the cost per removed nitrogen due to energy requirement for the aeration in the nitrification step and the need for electron donor in the denitrification step. The anammox reaction is emerging as an attractive alternative to replace the nitrification and denitrification processes (Ahn, 2006, Abma, et al. 2007, van der Star, et al. 2007).

In the last 3 years, the first anammox wastewater treatment plants have been implemented as a cost effective (60% less costs) and environment friendly (90% less CO_2 emissions) nitrogen removal system compared to conventional ammonium removal technology (Abma, et al. 2007). As already mentioned anammox bacteria have a low growth rate, and a form of sludge retention is required. Such systems like Sequencing Batch Reactor, SBR; Membrane BioReactor, MBR; Rotating Biological Contactor, RBC; etc. could be used to cultivate these organisms (Strous, et al. 1998, Pynaert, et al. 2003, Wyffels, et al. 2003, Kartal, et al. 2006, Trigo, et al. 2006). In addition solid support materials like glass beads (Strous, et al. 1997), Kaldness rings (Helmer-Madhok, et al. 2002) or non-woven biomass carriers (Fujii, et al. 2002, Furukawa, et al. 2003, Imajo, et al. 2004) can be used. A comparison of the various reactor systems was described in detail in Pynaert, et al. (2004) and van der Star, et al. (2007). In two recent studies it was shown that anammox process with two different reactor setups (RBC and SBR) could be applied to high-salinity wastewaters (Windey, et al. 2005, Kartal, et al. 2006). Chapter 4 describes the adaptation of a freshwater anammox community to high salinity.

Anammox bacteria require nitrite as electron acceptor for the anaerobic oxidation of ammonium, and for the application of anammox process in wastewater treatment different setups are used to provide nitrite: one-reactor and two-reactor systems. The common purpose in the application these systems is providing anammox bacteria with nitrite, a compound rarely found in wastewater at high concentrations. In both systems part of available ammonium is converted to nitrite by aerobic ammonium oxidizers, and the remaining ammonium and the formed nitrite is converted to dinitrogen gas by anammox bacteria.

One Reactor Systems

The cooperation between the aerobic and anaerobic ammonium oxidizing bacteria is the microbial basis of CANON (Completely Autotrophic Nitrogen removal Over Nitrite), OLAND (Oxygen-Limited Autotrophic Nitrification-Denitrification) and the so-called deammonification reactor systems. In these systems, aerobic ammonium-oxidizing bacteria (AOB) and the planctomycete-like anammox bacteria perform two sequential reactions simultaneously (Kuai & Verstraete, 1998, Third, et al. 2001, Helmer-Madhok, et al. 2002, Pynaert, et al. 2002, Sliekers, et al. 2002, Toh & Ashbolt, 2002, Schmidt, et al. 2003, Sliekers, et al. 2003)

In the CANON reactor system, under oxygen limitation, the supplied ammonium is partly oxidized to nitrite by aerobic ammonium oxidizing bacteria (AOB). The produced nitrite is utilized with the remainder of the ammonium by anammox bacteria and converted into dinitrogen gas. The feasibility of the CANON concept has been established by carefully introducing limited amounts of oxygen to anammox SBR systems. Within 2 weeks a new stable consortium of AOB and anammox becomes operative (Sliekers, et al. 2002). FISH analysis of the CANON biomass has shown that about 40% of the community consisted of AOB, also the anammox cells constituted about 40% of the community. No aerobic nitrite oxidizing bacteria (NOB, *Nitrospira* or *Nitrobacter* species) have been detected in these systems with FISH or activity tests. The NOB are only able to develop in the CANON reactor after prolonged exposure (>1 month) to ammonium limitation (Third, et al. 2001). In RBC based systems where the supply of ammonia and oxygen are difficult to control it is possible for the NOB to coexist with AOB and anammox bacteria (Egli, et al. 2001, Egli, et al. 2003, Pynaert, et al. 2004); however it is also possible to have biofilms without NOB (Helmer-Madhok, et al. 2002).

The upper limits of nitrogen loading of both anammox and CANON processes were explored in lab scale gas lift and sequencing batch reactors (Dapena-Mora, et al. 2004a, Sliekers, et al. 2005). In such reactors anammox Planctomycetes were able to remove 8.9 kg N m^{-3} reactor day^{-1}. In the same set-up the combined action of AOB and anammox Planctomycetes achieved 1.5 kg N removal m^{-3} reactor day^{-1}, which is more than sufficient to start application trials. The 1.5 kg N m^{-3} reactor day^{-1} removal capacity was also reached in a RBC system which contained both *Kuenenia*-like anammox bacteria and *Nitrosomonas* related aerobic ammonium-oxidizing bacteria (Pynaert, et al. 2003, Pynaert, et al. 2004). In a recent study the highest total nitrogen removal rate, 26 kg N m^{-3}reactor day^{-1}, was reported by Tsushima, et al (2007b).

The distribution of AOB and anammox bacteria in a CANON system was investigated using ^{15}N-labelled substrate and nitrite microsensors (Nielsen, et al. 2004). Under oxygen-limited conditions (<5 µM O_2), AOB were restricted to the outer shell (<100 µm) of the CANON aggregates, while anammox bacteria were found in the central anoxic parts. The larger type aggregates (>500 µm) accounted for about 68% of the anammox potential whereas 65% of the nitrification potential was found in the smaller aggregates (<500 µm). Analysis with O_2 and nitrite microsensors showed that the thickness of the activity zones varied as a function of the bulk oxygen and nitrite concentrations and the flow rate. This is in good agreement with the biofilm models developed by Hao et al (2002ab, 2004). In a separate study, urea was tested as an alternative energy source for the microbial consortium in the CANON reactor system (Sliekers, et al. 2004). Urea is a major source of nitrogen input to both wastewater streams and natural ecosystems. Human excretion and leachate from agriculture fields are the two main sources of urea. Urea conversion by CANON biomass is much more cost-effective (compared to conventional ammonium removal) since it is evident that the system is completely autotrophic and does not require additional organic carbon (Sliekers, et al. 2004). Tests with lab-scale reactors demonstrated that when urea is supplied to the CANON reactor it is immediately converted to dinitrogen gas and full capacity is reached within two weeks of adaptation (Sliekers, et al. 2004). In these two weeks, the urease-positive *Nitrosomonas oligotropha* and *Nitrosomonas nitrosa* become the dominant AOB in the urea-converting CANON systems. Anammox bacteria do not hydrolyse urea

themselves, but rely on the urea degradation of *N. oligotropha* and *N. nitrosa* to be supplied with sufficient amounts of ammonium and nitrite (Sliekers, et al. 2004). Further tests have clearly demonstrated that CANON systems are well suited to treat separately collected urine waste (Udert, et al. 2003, Wilsenach & van Loosdrecht, 2003).

Two Reactor Systems

The possibility to use the SHARON (Single reactor system for High rate Ammonium Removal Over Nitrite) process in combination with anammox has also been investigated. SHARON process was developed for the removal of ammonium via the so-called nitrite route (Jetten, et al. 1997, Hellinga, et al. 1998, Mulder, et al. 2001). It has been tested for 2 years in the laboratory and successfully scaled-up from two litres to an 1800 m^3 full- scale plant (Hellinga, et al. 1998, Hellinga, et al. 1999, Mulder, et al. 2001, Jetten, et al. 2002). In the SHARON process, 53% ammonium is oxidized for to nitrite at 1.2 kg N m^{-3} day^{-1}, without pH control. If necessary, the ammonium/nitrite ratio in the effluent of the SHARON process can be fine-tuned by adjusting the pH between 6.5 and 7.5 (van Dongen, et al. 2001). The effluent of this SHARON reactor is fed to an anammox reactor that removes all nitrite and the remaining ammonium. The specific activity of the anammox biomass is relatively high: 0.8 kg N (kg dry weight)$^{-1}$ day^{-1} and the load can be increased to more than 2 kg N m^{-3} day^{-1} (van Dongen, et al. 2001, Fux, et al. 2002).

Table 1 I Distribution of anammox bacteria in natural and man-made ecosystems around the world

Place	Method of detection	Reference
Man-made systems		
Delft, The Netherlands	^{15}N, Enrichment, FISH, lipids, clone library	Strous et al. 1999a
Nijmegen, The Netherlands	^{15}N, Enrichment, FISH, lipids, clone library	Kartal et. al 2004, 2006, 2007a, 2007b
Ghent, Belgium	Enrichment, FISH, clone library	Pynaert et al. 2002, Wyffels, et al. 2003, Windey et al. 2005
Rotterdam, The Netherlands	Enrichment, FISH, Real Time PCR	Van der Star et al 2007
Stuttgart, Germany	Enrichment, FISH, clone library	Schmid et al. 2000
Dubendorf, Switzerland	Enrichment, FISH, clone library	Egli et al. 2001
Hannover, Germany	Enrichment, FISH	Helmer-Madhok et al. 2002
Kumamoto, Japan	Enrichment, FISH	Fuji et al. 2002
Sydney, Australia	Enrichment	Toh et al 2002
Athens, Georgia, USA	Enrichment	Dong et al. 2003
Kirinya, Jinja, Uganda	FISH	Jetten et al. 2003
Pitsea, United Kingdom	Enrichment, FISH, lipids, clone library	Schmid et al. 2003
Hangzhou, China	Enrichment	Zheng et al. 2004
Kyungsan, Korea	Enrichment	Ahn et al. 2004
Kanagawa, Japan	Enrichment, FISH	Imajo et al. 2004
Lyngby, Denmark	Enrichment	Schmidt et al 2004
Mechernich, Germany	Enrichment	Li et al. 2004
Santiago de Compostela, Spain	Enrichment, FISH	Dapena-Mora et al. 2004b
Yongin, Korea	Enrichment, FISH	Liu et al. 2005
Bejing, China	Enrichment	Wang et al. 2005
Chiba, Japan	Enrichment, FISH	Isaka et al. 2006
Perth, Australia	Enrichment, FISH	Third et al. 2005
Sapporo, Japan	Enrichment, FISH	Tsushima et al. 2007
Katmandu, Nepal	Enrichment, FISH	Pathak et al. 2007
Strass, Austria	Enrichment, clone library	Innerebner et al. 2007
Moscow, Russia	Enrichment	Kalyuzhnyi et al. 2006
Pereira, Colombia	Enrichment	Paredes et al. 2007
Baltimore, USA	Enrichment, FISH, PCR	Tal et al. 2006

Place	Method of detection	Reference
Natural systems		
Skagerak (North Sea)	^{15}N, nutrient profiles	Thamdrup and Dalsgaard 2002
Black Sea	^{15}N, nutrient profiles, FISH, lipids, clone library	Kuypers et al, 2003
Golfo Dulce, Costa Rica	^{15}N, nutrient profiles	Dalsgaard et al. 2003
Thames estuary, United Kingdom	^{15}N	Trimmer et al. 2003
Arctic Sea (East Greenland)	^{15}N, nutrient profiles	Rysgaard et al. 2004
Arctic Sea (NW Greenland)	^{15}N, nutrient profiles	Rysgaard et al. 2004
Mertz Sea, Antartica	Clone library	Bowman et al. 2003
Randers Fjord, Denmark	^{15}N, nutrient profiles, FISH, clone library	Risgaard-Petersen et al. 2004
Benguela OMZ, Namibia	^{15}N, nutrient profiles, FISH, lipids, clone library	Kuypers et al. 2005
Chesapeake Bay, U.S.A.	^{15}N, FISH, clone library	Tal et al. 2005
Baltimore Inner Harbor, USA	Enrichment, FISH, PCR	Tal et al. 2005
Gullmarsfjorden, Sweden	^{15}N, nutrient profiles	Engström et al. 2005
Long Island, U.S.A.	^{15}N, nutrient profiles	Engström et al. 2005
Barents Sea, Russia	FISH, nutrient profiles	Schmid et al. 2007
North Sea, North of the Friesian Front	FISH, nutrient profiles	Schmid et al. 2007
Lake Tanganyika, Tanzania	^{15}N, FISH, clone library	Schubert et al. 2006
Tidal river system, Australia	^{15}N, nutrient profile	Meyer et al. 2005
East Hanna Shoal, North of Alaska	clone library	Penton et al. 2006
Juan de Fuca Ridge, Pacific Ocean	clone library	Penton et al. 2006
Turning Basin, USA	clone library	Penton et al. 2006
Washington Margin Sediment, USA	clone library	Penton et al. 2006
Florida Everglades, USA	clone library	Penton et al. 2006
Cape Svyatoi Nos, Russia	clone library	Penton et al. 2006
Sheriff's Marsh, USA	clone library	Penton et al. 2006
Shallow Bud Inlet, USA	clone library	Penton et al. 2006
Peruvian OMZ, Peru	^{15}N, nutrient profile, FISH, lipids, clone library	Hamersley et al. 2007
Arabian Sea, Oman	Lipids, nutrient profile	Jaeschke et al. 2007
Xinyi River, China	Enrichment, clone library	Zhang et al. 2007

chapter 2

candidatus "anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria

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Abstract
The bacteria that mediate the anaerobic oxidation of ammonium (anammox) are detected worldwide in natural and man made ecosystems, and contribute up to 50% to the loss of inorganic nitrogen in the oceans. Two different anammox species rarely live in a single habitat, suggesting that each species has a defined but yet unknown niche. Here we describe a new anaerobic ammonium oxidizing bacterium with a defined niche: the co-oxidation of propionate and ammonium. The new anammox species was enriched in a lab scale bioreactor in the presence of ammonium and propionate. Interestingly, this particular anammox species could out-compete other anammox bacteria and heterotrophic denitrifiers for the oxidation of propionate in the presence of ammonium, nitrite and nitrate. We provisionally named the new species *Candidatus* "Anammoxoglobus propionicus".

Introduction
In the anammox process ammonium and nitrite are directly converted to dinitrogen gas under anoxic conditions. In recent years the anammox process is emerging as an efficient and cost-effective alternative to conventional nitrogen removal from wastewater (Grommen & Verstraete, 2002, Kartal, et al. 2004). Moreover, anammox bacteria are recognized as important actors in the global biogeochemical nitrogen cycle (Strous & Jetten, 2004, Arrigo, 2005).

Anammox bacteria have been detected in many ecosystems ranging from wastewater treatment plants to arctic ice (Rysgaard & Glud, 2004, Schmid, et al. 2005). Anammox bacteria have been shown to contribute to the loss of fixed nitrogen in the Black Sea, the World's largest anoxic basin, and in the Benguela upwelling system, an important site for primary production in the Atlantic Ocean (Kuypers, et al. 2003, Kuypers, et al. 2005). Anammox activity has also been detected in sediments off the coast of Greenland, in estuaries in Denmark, the United Kingdom and Australia (Risgaard-Petersen, et al. 2004, Rysgaard, et al. 2004, Meyer, et al. 2005, Trimmer, et al. 2005). These examples do not only illustrate the importance of the anammox process for the marine ecosystems, but also have clear implications towards the ubiquity of anammox bacteria.

So far three genera capable of anaerobic ammonium oxidation have been identified and named provisionally *Candidatus* "Brocadia", *Candidatus* "Kuenenia" and *Candidatus* "Scalindua" (Schmid, et al. 2005). All the described genera have an anammoxosome and unique ladderane lipids in their membranes (Sinninghe Damsté, et al. 2002, Sinninghe Damsté, et al. 2005). It is a challenge to cultivate the bacteria mediating this reaction due to their long doubling times (10-20 days) and low biomass yields.

Anammox bacteria were assumed to have a completely chemolithoautotrophic lifestyle. However, Güven et al. showed that anammox bacteria are also capable of oxidizing propionate to CO_2 (Güven, et al. 2005), and thus they have a more versatile metabolism than previously assumed.

Different anammox species are rarely found in the same ecosystem, and there are large phylogenetic distances between different species. This indicates that each species occupies a distinct, but yet unknown ecological niche. However, until now there is no clear niche definition for the anammox genera. In the present paper we describe a new anammox genus and species with a clear niche: this species oxidizes organic acids at high rate and so out-competes the other anammox species for nitrite in the presence of propionate. The new species is particularly interesting for wastewater treatment practice because it could be applied in the treatment of nitrate and/or COD containing waters. The new anammox species also out-competed all other bacteria (e.g. heterotrophic denitrifiers) in competition studies, demonstrating the first clear-cut niche for an anammox bacterium.

Materials and Methods

Enrichment and cultivation of Anammox bacteria

Two sequencing batch reactors (SBR, working volume 4 l) were used for enrichment and cultivation of anammox bacteria (Strous, et al. 1998). Each SBR cycle consisted of 11 h and 45 min of filling, 5 min of settling of the biomass and 10 min of drawing of the liquid. During each filling period, 1 l of mineral medium (containing nitrite, ammonium and nitrate) described by Van de Graaf et al. (1996) and 250 ml of a propionate solution (concentrations are specified at the results section) were added continuously to the reactor at flow rates of 1.4 $ml \cdot min^{-1}$ and 0.35 $ml \cdot min^{-1}$ respectively. To maintain anoxic conditions, the reactors and the medium vessels were flushed continuously with Ar/CO_2 (95/5%, 10 $ml \cdot min^{-1}$). The SBRs were stirred at 200 rpm with a turbine stirrer. The temperatures of the SBRs were kept constant at 33°C with water jackets. CO_2 present in the supplied gas was sufficient to buffer the solution and to keep the pH in the SBR between 7.0 and 7.3.

Anammox Activity Assays

Biomass (5 ml with ~5 $mg \cdot ml^{-1}$ protein) was harvested from the SBR described above. The biomass sample was washed 3-5 times with mineral medium until the nitrite, nitrate and ammonium in the sample were less than 20 µM. The biomass was then transferred to 30 ml serum bottles. The bottles were sealed with 5 mm butyl rubber stoppers and were made anoxic by alternately applying under-pressure and Argon gas. An overpressure of 1 bar was maintained in the bottles. The soluble substrates were added to the bottles from 100 mM anoxic stock solutions. To measure anaerobic ammonium oxidation activity, final concentrations of 3 mM NO_2^- and NH_4^+ were used. In order to measure nitrate reduction activity, final concentrations of 5 mM NO_3^- and 12.5 mM formate, 3 mM acetate or 2 mM propionate were used. To measure the intermediate accumulation of hydrazine, final concentrations 5 mM NH_2OH and 5 mM NH_4^+ were used. The bottles were incubated at 33°C and were shaken continuously at 300 rpm for 6 h.

Analytical Methods

Nitrate, nitrite, propionate, acetate and formate were measured with high performance liquid chromatography (HPLC). Water samples from the batch incubations and the SBR were centrifuged and 10 µl from the supernatant was injected with a Hewlett Packard 1050 series autosampler. A sodium hydroxide solution was used as the liquid phase at a flow rate of 1.5 $ml \cdot min^{-1}$. The anions were separated using a hydroxide gradient elution from 1 mM to 15 mM in 9 min. Separations were performed on a 4x250mm Ionpac AS11-HC (Dionex, UK) column at 30°C. Anions were detected using a CD25 conductivity detector (Dionex, UK). Ammonium was measured colorimetrically at 420 nm after a 30 min reaction of 40 µl sample containing 0.5 – 5 mM ammonium with 760 µl 0.54 % ortho-phthalaldehyde, 0.05 % β-mercaptanol and 10% ethanol in 400 mM potassium phosphate buffer (pH 7.3) (modified from Taylor et al. (1974). Hydroxylamine, hydrazine and protein were determined as described before (Strous, et al. 1998).

DNA Extraction and Retrieval of 16S rRNA Sequences

Biomass was harvested from the SBR by centrifugation of a 1.5 ml sample. DNA was extracted as described by Juretschko et al. (Juretschko, et al. 1998). DNA was then suspended in 50 µl ultrapure water, and kept at 4°C for 24 h until further analysis.A primer combination of Pla46F (E. *coli* positions 46-63) forward and universal reverse (630R) (E. *coli* positions 1529-1545) primers were used for the preferential amplification of 16SrRNA genes of the members of the Planctomycetes (Schmid, et al. 2003).PCR amplificates were cloned directly using the TOPO TA cloning kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. Plasmid-DNA was isolated with the Flex prep kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ). Plasmids were digested with 5U EcoRI enzyme in EcoRI buffer for 3 h at 37°C. The digestion products were examined for an insert with the expected size by agarose (1%) gel electrophoresis. Sequencing was done with BigDye Terminator Cycle Sequencing v2.0 kit (Applied Biosystems, Foster City, CA). The reaction mixtures were analyzed with the 3700 DNA analyzer (Applied Biosystems, Foster City, CA). The complete sequences of the 16S rRNA gene fragments were determined by using M13 forward and reverse primers targeting vector sequences adjacent to the multiple cloning site.

Phylogenetic Analysis and Probe Design

Phylogenetic analysis of the sequences and design of oligonucleotide FISH probes were conducted with ARB software as previously described (Schmid, et al. 2003). Phylogenetic analyses were performed with maximum likelihood, neighbor joining and maximum parsimony methods with 50% sequence conservation filters for Planctomycetes. Since no differences between all calculated trees in terms of branching order could be observed for the anammox bacteria, the tree based on Treepuzzle (Strimmer & von Haeseler, 1996) analysis with the 50% conservation filter for Planctomycetes is presented here. Bootstrap values were 100% for each branching in the anammox bacteria cluster.

Fluorescence *in situ* Hybridization (FISH)

Biomass (1 ml) was harvested from the enrichment cultures, and hybridizations with fluorescent probes were performed as described previously (Schmid, et al. 2000). All probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Thermo Electron Corporation (Ulm, Germany). The following probes were used to monitor the enrichment of the anammox population as described in Schmid et al. 2003: EUB 338 (S-D-Bact-0338-a-A-18, specific for most but not all bacteria, Amann et al. 1990), EUB 338 II (S-D-Bact-0338-b-A-18, specific for Planctomycetales, Daims, et al. 1999), EUBIII 338 (S-D-Bact-0338-c-A-18, specific for Verrucomicrobiales, Daims et al. 1999), Pla46 (S-P-

Planc-0046-a-A-18, specific for Planctomycetales) Amx 368 (S-*-Amx-0368-a-A-18, specific for all known anammox genera, Schmid et al., 2003), Amx 820 (S-*-Amx-0820-a-A-22, specific for *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis", Schmid et al. 2000). Probe Apr 820 (S-*-Apr-0820-a-A-21) was designed in the present study for specific hybridizations with *Candidatus* "Anammoxoglobus propionicus".

Electron Microscopy

Biomass (1 ml) was harvested from the enrichment culture. The sample was frozen in a BAL-TEC HPM010 (Liechtenstein) high pressure freezer, and it was cryosubstituted using 2% osmium tetroxide and 0.5% uranyl acetate in a Leica (Germany) automatic freeze substitution unit and embedded in epon. Later the sample was prepared and inspected as previously described (Lindsay, et al. 2001).

Lipid Analysis

Biomass (20 ml) from the enrichment culture was freeze dried and ultrasonically extracted using methanol (MeOH) ×3, MeOH/dichloromethane (DCM) mixture (1:1, v/v) ×3 and DCM ×3. The combined extracts were dried using rotary evaporation and methylated using BF_3/MeOH with a known stable carbon isotopic composition [-43 ‰ vs Vienna Pee-Dee Belemnite (VPDB)]. Very polar material was removed by eluting the methylated extract over a small silica column with ethyl acetate as eluent. Alcohols in the resulting fraction were transformed into trimethylsilyl (TMS) ethers by heating with bis-(trimethyl-silyl)-trifluoroacetamide with a known isotopic composition, -40 ‰ vs VPDB) in pyridine at 60 °C for 20 min. The sample was analysed using gas chromatography (GC) and lipids were identified using gas chromatography mass spectrometry (GC/MS). Stable carbon isotopes of the lipids were measured using a ThermoFinnigan Delta^{PLUS} XL isotope ratio monitoring irms-GC/MS system as described previously (Schouten, et al. 2004). The isotopic compositions of fatty acids and alcohols were corrected for additional carbon from sample derivatization.

$\delta^{13}C$ values of the total dissolved inorganic carbon (DIC) content of the reactor supernatant was analysed in duplicate using a ThermoFinnigan Gas Bench II coupled to a DELTA^{PLUS} irmMS. Water samples (1 ml) were taken from the SBR, stored with saturated $HgCl_2$ solution, and biomass was removed by centrifugation. Headspace analysis was performed on the supernatant after reaction with H_3PO_4 (1 h, room temperature). The analysis was repeated 10 times and gave a standard deviation of 0.2 ‰. Stable isotope ratios were determined relative to a laboratory standard ($NaCO_3$, -0.57 ‰ vs VPDB) which was calibrated against NBS-18 carbonate (International Atomic Energy Agency, IAEA).

$\delta^{13}C$ values of the propionate solution were determined using Elemental analysis–irmMS. The Carlo Erba Flash elemental analyzer, coupled to a ThermoFinnigan Delta^{PLUS} irmMS system, gave a standard deviation of 0.02 ‰. Laboratory standards, calibrated using NBS-22 oil (IAEA) were used to determine the stable carbon isotope ratios.

Separation of Suspended Cells from Biofilm Aggregates by Percoll Centrifugation

Percoll (6 ml, Pharmacia) and 3 ml Tris-HCl (20 mM, pH 8) was centrifuged in 10 ml glass tubes (15°C, 10.000 x g, 30 min) to pre-form a density gradient. Biomass was harvested from the enrichment culture, and aggregates were disrupted by mild sonication (30 s, 150 W, tip diameter 9.5 mm, 20-33°C). After sonication, the cell suspension was centrifuged. The pellet was suspended in 1 ml Tris-HCl (20 mM, pH 8), and the mix was gently pipetted onto the Percoll gradient. The tubes were centrifuged for another 30 min at 15°C (10.000 x g). The suspended cells were recovered as a light-brown band from the Percoll gradient. The suspended cells were washed in Tris-HCl (20 mM, pH 8) to remove the Percoll.

Results

A robust procedure for enrichment of anammox bacteria is well established. The sequencing batch reactor (SBR), the mineral medium and the activated sludge inoculum have been used successfully in previous studies to enrich *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis" (Strous, et al. 1998, van Dongen, et al. 2001). In the present study we used the same experimental procedure and the same type of inoculum. The anammox enrichment culture was started with an activated sludge sample originating from secondary stage of Dokhaven municipal wastewater treatment plant (Rotterdam, The Netherlands, van Dongen, et al. 2001). The major difference in the present study was the addition of propionate to the enrichment culture as an alternative electron donor for anammox bacteria.

In the first four months after the inoculation of the SBR the concentrations of nitrite and ammonium were gradually increased from 2.5 mM to 45 mM. The influent concentration of nitrate was maintained at 6 mM at all times. Parallel to the increase of nitrite and ammonium, the concentration of propionate was increased from 0.8 mM to 15 mM. The effluent concentration of nitrite and propionate was always below detection limits (10 μM). The population dynamics of the enrichment culture were monitored by fluorescence *in situ* hybridisation (FISH) probes throughout the enrichment (see below).

After four months, the anammox activity of the enrichment culture was 15 NH_4^+ μmol·g protein^{-1}·min^{-1}. In the absence of propionate for each mol of ammonium 1.7 mol of NO_2^- was converted, similar to the previously reported values (Strous, et al. 1998). When the biomass was incubated with hydroxylamine, there was a transient accumulation of 0.3 mM hydrazine, a phenomenon unique to anammox bacteria (Figure 1). The nitrate-reducing activity of the enrichment culture with small organic acids was 2.8 NO_3^- μmol·g protein^{-1}·min^{-1} for formate, 0.7 for acetate and 1 for propionate. The acetate and propionate oxidation rates with the current biomass were notably higher than rates observed with *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis" (Table 1). Consistently, in these control incubations of *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis" with nitrate and organic acids (acetate and propionate) the nitrate reduction rates were significantly lower (data not shown). These controls showed a transient accumulation of up to 1 mM NO_2^-. In the test with the biomass from the enrichment culture this transient nitrite accumulation was not observed.

Table 1 | Organic acid oxidation rates (μmol·g prot^{-1}·min^{-1}) of different anammox species.

	Candidatus "Brocadia anammoxidans"	*Candidatus* "Kuenenia stuttgartiensis"	*Candidatus* "Anammoxoglobus propionicus"
Formate	6.5 ± 0.6	5.8 ± 0.6	6.7 ± 0.6
Acetate	0.57 ± 0.05	0.31 ± 0.03	0.79 ± 0.07
Propionate	0.12 ± 0.01	0.12 ± 0.01	0.64 ± 0.05

To identify the bacteria responsible for propionate oxidation, suspended cells were separated from aggregated cells with Percoll centrifugation. Anammox cells were not detected in suspended cells, and no nitrate-reducing activity was observed when these bacteria were incubated with propionate and nitrate. Only the aggregated cells oxidized propionate. Anammox cells made up 80% of the population in these aggregates as estimated with FISH.

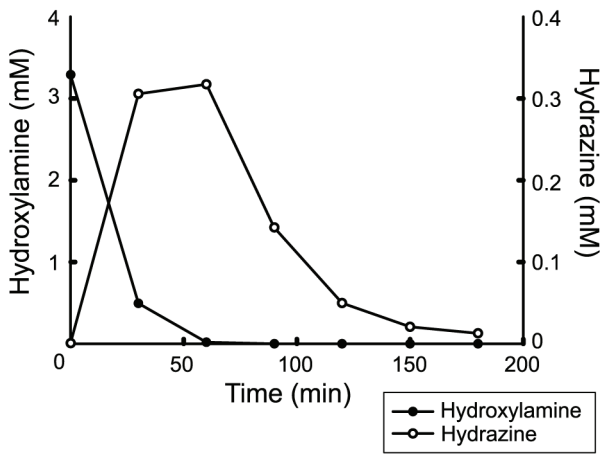

Figure 1 | Production of hydrazine (empty circles) from hydroxylamine (circles) under anoxic conditions.

When the anammox enrichment culture was examined with fluorescence *in situ* hybridization (FISH), it was observed after four months that 80% of the bacterial population hybridized with the Planctomycetes specific probe S-P-Planc-0046-a-A-18, and 99% of the Planctomycetes hybridized with the general anammox probe S-*-AMX-0368-a-A-18 (Schmid, et al. 2005). Surprisingly, there was no hybridization with species-specific probes for either *Candidatus* "Brocadia anammoxidans" or *Candidatus* "Kuenenia stuttgartiensis" or *Candidatus* "Scalindua *sp.*" (Schmid, et al. 2005), indicating that a new anammox species had indeed been enriched.

In order to determine the affiliation of the new anammox species present in the enrichment culture, we applied a Planctomycetes specific full cycle rRNA approach. DNA was extracted from the biomass from the enrichment culture. 16S rRNA gene sequences of Planctomycetes were amplified using PCR with the primers Pla46F and 630R, and cloned. Twenty clones were randomly selected for sequencing. Near full-length 16S rRNA sequences were obtained. All of the 16S rRNA genes had the same sequence and subsequent phylogenetic analysis showed that this sequence formed a new branch outside the known anammox genera. This new anammox phylotype had at most 91 % sequence similarity to the known anammox species indicating that this new organism represented by 16S rRNA gene sequence "BK7" indeed constituted a new genus in anammox line of descent in the phylum Planctomycetes (Figure 2).

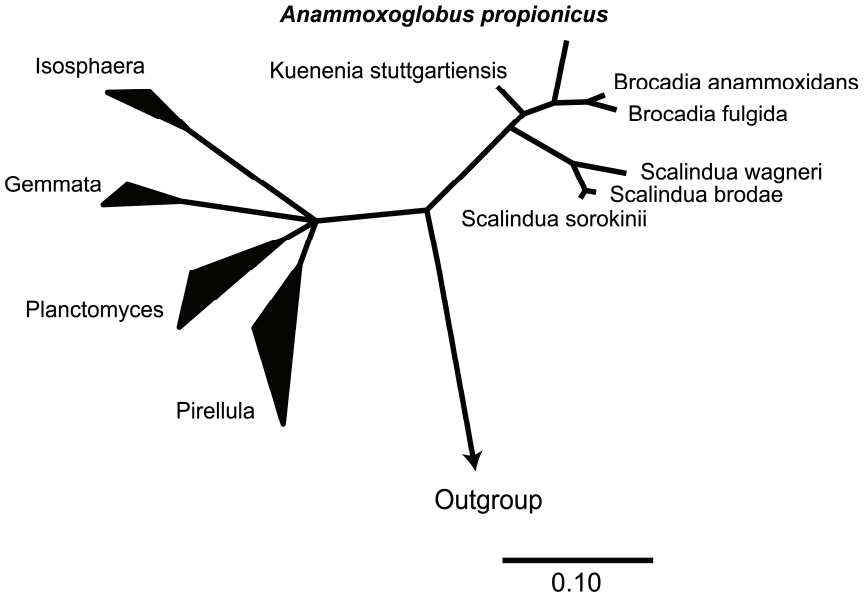

Figure 2 | Phylogenetic tree showing the relationships of the propionate enrichment culture 16S rRNA gene BK7 (*Candidatus* "A. Propionicus"), to other Planctomycetes and other reference organisms.

A new oligonucleotide probe S-*-Apr-820-a-A-21 was constructed to match specifically with this new 16S rRNA gene sequence. At the time of the inoculation no anammox cells were detected. During the enrichment there was a gradual increase in the population of the new anammox species. After four months the new species made up 80% of the aggregated population.
One characteristic of the previously described anammox organisms is the presence of a membrane-bound intracytoplasmic compartment known as the anammoxosome (Sinninghe Damsté, et al. 2005). Transmission electron microscopy (TEM) was performed on thin sections prepared from the biomass containing the new anammox organism. The new anammox species displayed typical EM features of anammox bacteria: a single membrane bound anammoxosome containing tubule like structures, an anammoxosome-membrane-attached nucleoid and riboplasm with ribosome-like particles separated from paryphoplasm at the cell rim by an intracytoplasmic membrane (Figure 3).

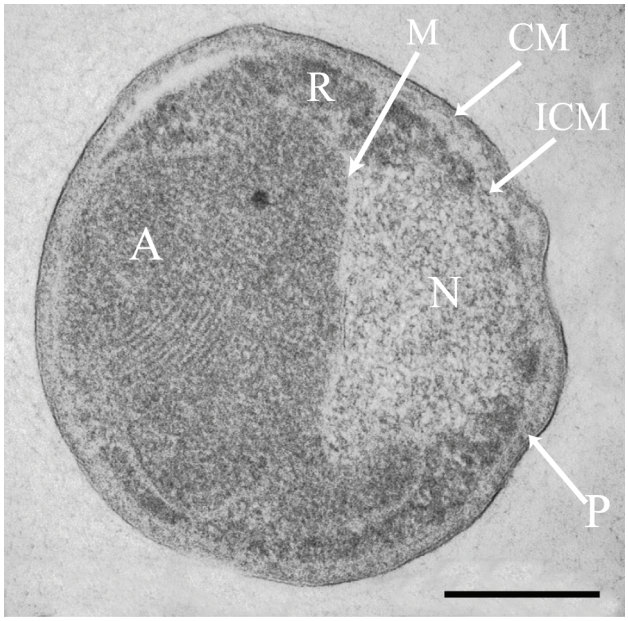

Figure 3 | Transmission electron micrograph of thin-sectioned *Candidatus* "Anammoxoglobus propionicus" Cells have the conventional anammox cell components:

An anammoxosome (A) containing tubule-like structures, riboplasm (R) containing the nucleoid (N) apposed to the anammoxosome membrane (M), paryphoplasm (P) separated from riboplasm by an intracytoplasmic membrane (ICM) and the cytoplasmic membrane (CM). Bar in micrograph = 200nm

The most abundant lipids in the enrichment culture were the ladderane fatty acids b and c (Figure 5), specific for anammox bacteria (Sinninghe Damsté, et al. 2002). Another lipid that is most likely to originate from the new anammox species is the 14-methylpentadecanoic acid (i-C16) which is rarely found in bacteria but has been reported in anammox before (Schmid, et al. 2003, Sinninghe Damsté, et al. 2005). The sample also contained the more unusual lipids 10-methylhexadecanoic acid and 9,14-dimethylpentadecanoic acid which appear to be characteristic for Planctomycetes (Sittig & Schlesner, 1993, Sinninghe Damsté, et al. 2005). Other compounds known to occur in anammox bacteria, i.e. bacteriohopane-tetrol, ladderane mono-ethers, ladderane dialkyl glycerol diethers or ladderane alkyl glycerol ether/esters, were not observed in this enrichment culture.
The stable carbon isotope composition of the lipids of the enrichment culture was used to elucidate the carbon source of the anammox bacteria: did they use CO_2 or propionate? Both carbon sources had similar $\delta^{13}C$ values (Table 2), but only for CO_2 a significant fractionation would be expected (Schmid, et al. 2003, Schouten, et al. 2004). The $\delta^{13}C$ values of the lipids were variable and ranged from -25.4 ‰ (non-specific C17:0 fatty acid) to -76.7 ‰ (ladderane fatty acid, Table 2). Table 2 also shows that based on the $\delta^{13}C$ values the less specific lipids, C27 hopanoid ketone (37 ‰), squalene (41 ‰) and 9,14-dimethylpentadecanoic acid (30 ‰), must have also been biosynthesized by the new anammox species.

Table 2 | δ ^{13}C isotopic depletion of the identified lipids and substrates (^{13}C ‰ vs. VPDB (Vienna Pee-Dee Belemnite)

Lipid	^{13}C ‰ vs. VPDB
i-C16	-61.7
16:0	-35.9
9,14-di Me-$C_{15:0}$	-58.6
17:0	-25.4
18:0	-29.8
Ladderane **b**	-70.1
Ladderane **c**	-76.6
Squalene	-66.8
C_{27} hopanoid ketone	-62.5
DIC	-25.6
Propionic acid	-23.5

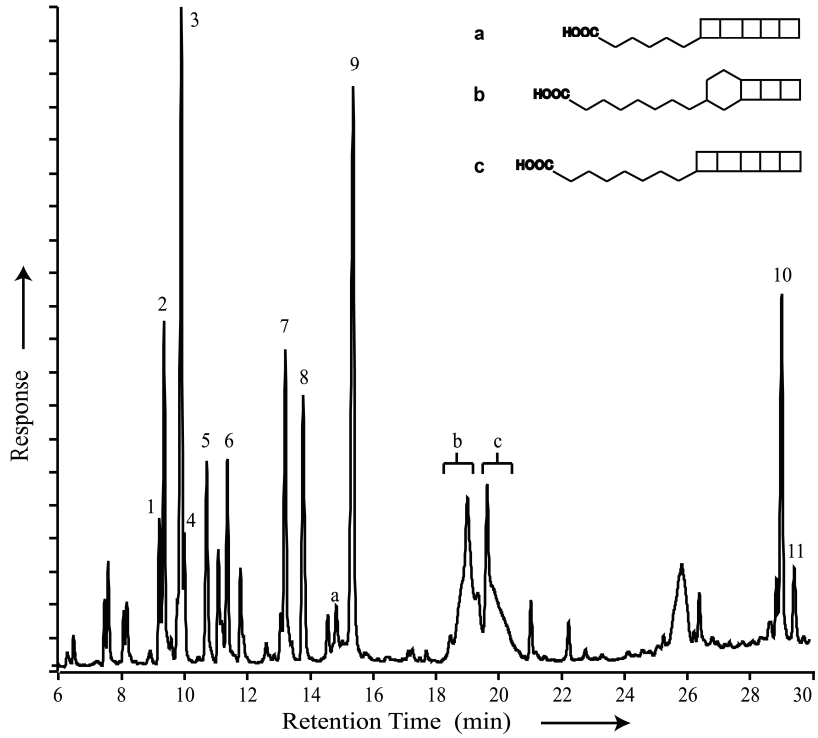

Figure 4 | Gas chromatogram of the total lipid fraction of the new anammox enrichment culture. Key: 1 = *i*-C_{16} fatty acid, 2 = $C_{16:1}$ fatty acid, 3 = $C_{16:0}$ fatty acid, 4 = 9,14-dimethylpentadecanoic acid, 5 = 10-methylhexadecanoic acid and 9-methylhexadecanoic acid, 6 = $C_{17:0}$ fatty acid, 7 = $C_{18:1}$ fatty acid, 8 = $C_{18:0}$ fatty acid, 9 = $C_{19:1}$ fatty acid, 10 = squalene, 11 = C_{27} hopanoid ketone. Ladderane lipids are; **a** = C_{20} fatty acid with 5 cyclobutane rings, **b** = C_{20} fatty acid with 3 cyclobutane rings and one cyclohexane ring, **c** = C_{22} fatty acid with 5 cyclobutane rings.

The presence of only one type of anammox bacteria in the above described enrichment culture, and the high nitrate and organic acid conversion rates of this anammox bacterium suggested that the presence of propionate defined its niche relative to the other anammox bacteria. In order to verify this hypothesis we conducted a competition experiment between *Candidatus* "Brocadia anammoxidans" and the new anammox species as follows: an SBR consisting of 60% *Candidatus* "Brocadia anammoxidans" and 40% of the new species was started. Nitrite, ammonium and nitrate loads on the reactor were 90, 90 and 6 mmol·day^{-1} respectively at all times. Propionate load was increased from 2.5 to 15 mmol·day^{-1}. The effluent concentrations of nitrite and propionate were below detection limits (10 μM) at all times.

Over four months after the introduction of the propionate, the new anammox species gradually grew to be the dominant anammox species, and at the end of the four month period 99% of the Planctomycetes consisted of this species as determined with FISH (Figure 5). Apparently *Candidatus* "Brocadia anammoxidans" was outcompeted.

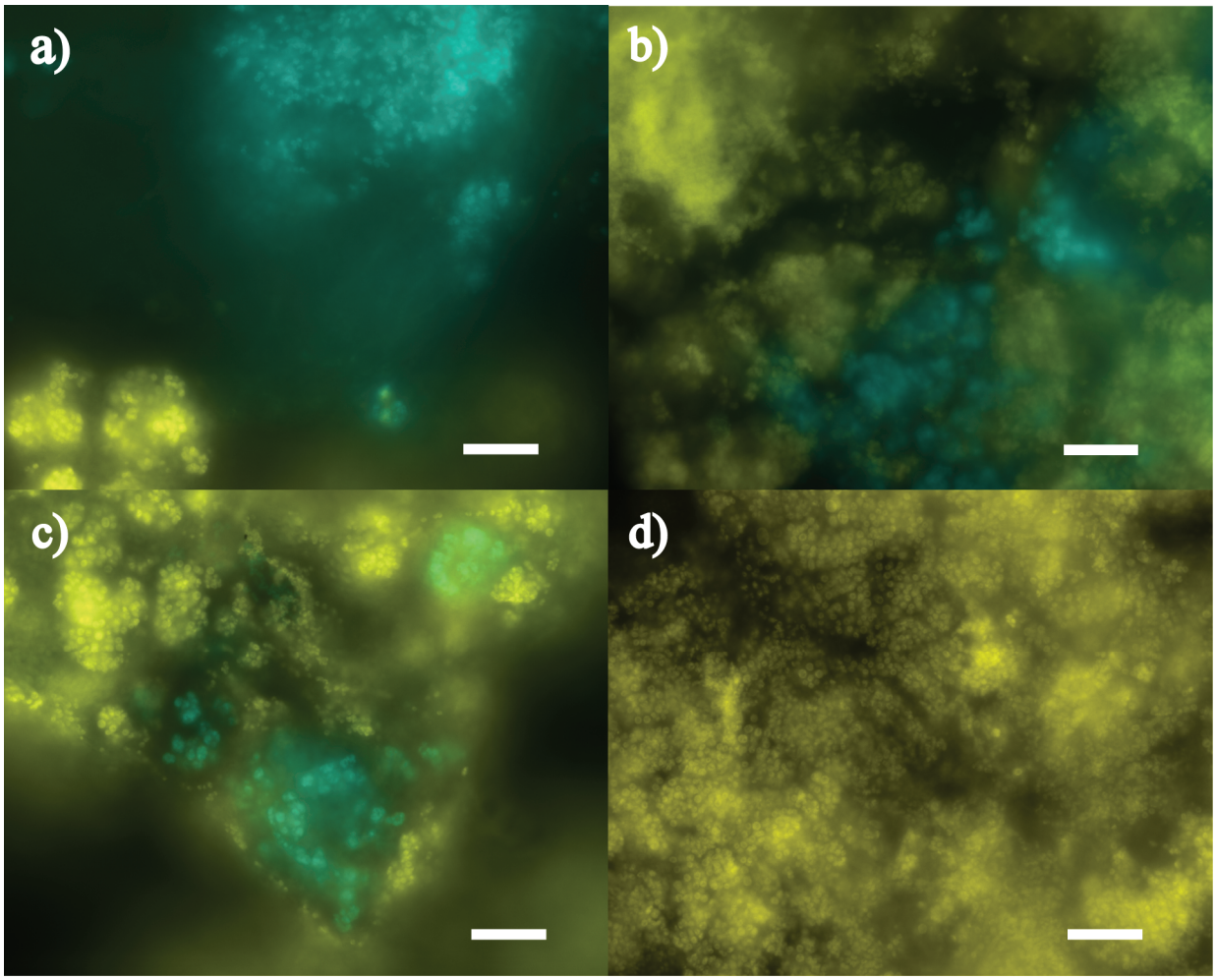

Figure 5 | Monitoring of the disappearance of *Candidatus* "Brocadia anammoxidans" from the propionate enrichment culture over 4 months with fluorescence *in situ* hybridization. *Candidatus* "Anammoxoglobus propionicus" is hybridized with Cy3-labeled probe S-*-Apr-0820-a-A-21 and depicted in yellow. *Candidatus* "Brocadia anammoxidans" is hybridized with cy5-labeled S-*-Amx-0820-a-A-22 probe and depicted in cyan. (a 0, b 1 month, c 2 months, d 4 months,Bar=20 µm)

Discussion

Niche differentiation is a poorly understood phenomenon in general. For example, the aerobic ammonia-oxidizing bacteria are a relatively well-described group of bacteria; however, the niches of *Nitrosomonas* and *Nitrosospira* are still not well known (Bollmann, et al. 2002). For the anammox case it has been previously observed that the different genera are seldom found in the same habitat or enrichment culture. This indicated that each anammox species has a well-defined (but as yet unknown) niche. The cell structure and key physiology of anammox bacteria have been well documented (Strous, et al. 1999a, Lindsay, et al. 2001), and so far are the same for all anammox genera. There is no evidence on factors determining the niche of *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis". The present study is the first clear-cut description of a specific niche for an anammox bacterium. Here we show that the availability of propionate is the factor that determines the niche for the new anammox bacterium described in this paper.

According to the yield calculations using Gibbs free energy dissipated by the oxidation of propionate and/or ammonium, two possible outcomes of enrichment could be predicted as an effect of propionate addition in the presence of ammonium (Heinen, et al. 1992): depending on their affinity for propionate either heterotrophic denitrifying bacteria or anammox bacteria would become the dominant species in the enrichment culture. When anammox bacteria are grown on nitrite and ammonium as the substrates, an 80% enriched anammox culture is achieved. If the heterotrophic denitrifying bacteria would have used all

the available propionate, anammox bacteria would only make 30 to 45 % of the total population. Despite the fact that sufficient quantity of organic material (propionate) was supplied, heterotrophic bacteria never became the dominant organisms in the enrichment culture. Furthermore, when propionate was incubated with the Percoll separated bacteria that co-exist with anammox bacteria, organic acid oxidation was not observed.

Based on batch activity measurements, 16S rRNA gene based phylogenetic analysis, fluorescence *in situ* hybridization, electron microscopy and lipid analysis we propose that the anammox bacteria enriched in this study define a new genus affiliated with the anammox bacteria in the order Planctomycetes. We propose to provisionally name the new species *Candidatus* "Anammoxoglobus propionicus". The production of hydrazine from hydroxylamine, the presence of ladderane lipids, the presence of an anammoxosome and other ultrastructural features and substrate conversion rates were all comparable to the known anammox bacteria. The one important difference of *Candidatus* "Anammoxoglobus propionicus" is its capability to oxidize small organic acids (acetic and propionic) at a higher rate and without a transient nitrite accumulation.

Candidatus "Anammoxoglobus propionicus" was enriched from the same inoculum previously used to cultivate other anammox species. When propionate was introduced to a co-culture of *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Anammoxoglobus propionicus", the latter became the dominant species in four months. This indicated that *Candidatus* "Anammoxoglobus propionicus" had a better affinity for nitrite in the presence of propionate. Further, it demonstrated the ability of *Candidatus* "Anammoxoglobus propionicus" to compete for organic electron donors better than both heterotrophic denitrifiers and other anammox bacteria. The complete disappearance of *Candidatus* "Brocadia anammoxidans" from the culture in a four month period could only explained if the effective growth rate of this species was 0. This indicated that the higher affinity of *Candidatus* "Anammoxoglobus propionicus" for nitrite (the limiting substrate) completely prevented the growth of *Candidatus* "Brocadia anammoxidans" in the presence of propionate. Furthermore, during the batch activity tests with *Candidatus* "Anammoxoglobus propionicus", transient nitrite accumulation was not observed in contrast to activity tests with the other anammox species. This suggests that the *Candidatus* "Anammoxoglobus propionicus" species are more efficient in using the nitrite produced from the nitrate giving them advantage over the other anammox species.

Up to 51 ‰ relative depletion of the ladderane lipids compared to the dissolved inorganic carbon (DIC) suggests that the carbon acquisition pathway used by the *Candidatus* "Anammoxoglobus propionicus" species was similar to that of other anammox species (i.e. *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Scalindua sorokinii") because they also biosynthesize lipids which are depleted by 47-49 ‰ relative to DIC (Schouten, et al. 2004). This strong depletion is characteristic for anammox bacteria and thought to be caused during carbon fixation via the acetyl-CoA pathway (Schouten, et al. 2004). The $\delta^{13}C$ of the propionate medium was -23.5 ‰. Most of the carbon fractionation occurs during carbon fixation. Thus, if *Candidatus* "Anammoxoglobus propionicus" incorporated propionate directly into its biomass, much less carbon fractionation would have been expected in the lipids. Apparently, *Candidatus* "Anammoxoglobus propionicus" does not incorporate propionate into biomass. This is consistent with previous results (Güven, et al. 2005), but still surprising, because the known pathways for propionate conversion proceed via acetyl-CoA (Plugge, et al. 1993), presumably the end product of the carbon fixation pathway of anammox bacteria. Possibly propionate oxidation occurs only for generation of energy. The mechanism of propionate oxidation by *Candidatus* "Anammoxoglobus propionicus" will be further investigated in the future.

Due to the long doubling time of anammox bacteria it is difficult to measure the effect of organic acids on the yield or growth rate of the anammox bacteria. Previously it has been reported that three to four months are necessary to enrich anammox bacteria in a lab scale reactor (van Dongen, et al. 2001). This start-up period is likely to be determined by factors

such as the viability and initial cell count of anammox bacteria present in the activated sludge inoculum and physical factors such as diffusion into the aggregates. Hence, it is difficult to draw conclusions about the growth rate of anammox bacteria from the start-up times of different anammox enrichment cultures. The introduction of propionate during the start-up of the anammox enrichment culture did not have an apparent effect on the length of the start-up period. Still, on thermodynamic grounds it was expected that start-up time would be lower; in the view of the above it cannot yet be ruled out that anammox bacteria grow faster with propionate and consequently anammox reactors would have a shorter start-up time.
Currently, the anammox process is designed and applied for treating ammonium rich wastewater containing no or little organic material (Kartal, et al. 2004). The fact that anammox bacteria can oxidize, and compete successfully for organic acids in a lab-scale reactor is a significant discovery. As a result, in the future, the anammox process could be designed and used for the treatment of nitrate rich wastewaters using limited amounts of organic material.
Anammox bacteria seem to have a more versatile metabolism than before observed. At present the contribution of anammox to the marine nitrogen cycle is calculated based solely on the anaerobic oxidation of ammonium (Dalsgaard, et al. 2003, Kuypers, et al. 2003, Kuypers et al. 2005). However, it is possible that anammox bacteria also compete for organic compounds such as organic acids and nitrate in the environment. Thus, the total contribution of anammox bacteria to the nitrogen losses from oceans might still be underestimated.

Description of *Candidatus* "Anammoxoglobus propionicus"

Anammoxoglobus propionicus **(glo.bus L. mas. n. sphere; referring to the spherical shape; pro.pi.o.ni.cus L. mas. adj. of propionic; referring to the compound defining the niche of the species)**

Anaerobic chemolithoautotrophic coccoid cells with diameters ranging between 0.7 to 2 µm. Cells oxidize ammonium with nitrite as the electron acceptor and with CO_2 as the main carbon source. Cells oxidize propionate, acetate and formate with nitrate as the electron acceptor. Cells convert hydroxylamine to hydrazine. Cells possess a membrane bound intracytoplasmic compartment containing tubule like structures, known as the anammoxosome. Cells contain ladderane lipids, more specifically 14-methylpentadecanoic acid, 10-methylhexadecanoic acid, 9,14-dimethylpentadecanoic acid and ladderane fatty acids b and c. 16S rRNA sequence similarity to the closest relative (*Candidatus* "Brocadia fulgida") is 91%.

chapter 3

candidatus "brocadia fulgida": an autofluorescent anaerobic ammonium oxidizing bacterium

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Abstract
The anaerobic ammonium oxidizing (anammox) bacteria are detected in many natural ecosystems and wastewater treatment plants worldwide. The present study describes the enrichment of the anammox bacteria in the presence of acetate. Our results extend the concept that the anammox bacteria can be enriched to high densities in the presence of substrates for heterotrophic growth. Batch experiments showed that among the tested biomass the biomass from the *Candidatus* "Brocadia fulgida" enrichment culture oxidizes acetate at the highest rate. Continuous cultivation experiments showed that in the presence of acetate, ammonium, nitrite and nitrate, *Candidatus* "Brocadia fulgida" out-competed other anammox bacteria. Our results indicated that *Candidatus* "Brocadia fulgida" did not incorporate acetate directly to their biomass. *Candidatus* "Brocadia fulgida" exhibited the common characteristics of anammox bacteria: the presence of an anammoxosome and ladderane lipids and the production of hydrazine in the presence of hydroxylamine. Interestingly, the biofilm aggregates of this species showed a strong autofluorescence. It is the only known anammox species exhibiting this feature. The autofluorescent extracellular polymeric substance had two excitation (352 and 442 nm) and two emission (464 and 521) maxima.

Introduction
Anaerobic ammonium oxidizing (anammox) bacteria conserve energy via oxidation of ammonium with nitrite in the absence of oxygen. The discovery of the anammox process in the second half of the nineties was the start of a series of fundamental changes in our understanding of the biological nitrogen cycle (Strous & Jetten, 2004). Many studies in the last decade showed that the anammox bacteria are ubiquitous in the natural ecosystems and contribute significantly to the global loss of fixed nitrogen (Kuypers, et al. 2003, Kuypers, et al. 2005, Hamersley, et al. 2007, Jaeschke, et al. 2007, Schmid, et al. 2007). Furthermore, the anammox process has been applied successfully for ammonium removal from wastewater on both full (van der Star, et al. 2007), and lab scale (Pynaert, et al. 2003, Kartal, et al. 2004, Jetten, et al. 2005).
In nature anammox bacteria were first detected in the Black Sea, the world's largest anoxic basin (Kuypers, et al. 2003). Since then they have been shown to contribute significantly to nitrogen gas production in two important sites for primary production: the Benguela and Peru upwelling systems (Kuypers, et al. 2005, Hamersley, et al. 2007). Besides, anammox bacteria and their activity have also been detected in the Arabian Sea (Jaeschke, et al. 2007), which contains the largest oxygen minimum zone in the world, and in many natural systems (Risgaard-Petersen, et al. 2004, Rysgaard & Glud, 2004, Meyer, et al. 2005, Trimmer, et al. 2005, Penton, et al. 2006, Schubert, et al. 2006). Still, it is a challenge to cultivate anammox bacteria because of their long doubling times (10-20 days) and low biomass yields (Strous, et al. 1998, Kartal, et al. 2007a).
The anammox bacteria form a distinct, deep branching phylogenetic group in the Order *Planctomycetales*. So far four genera capable of anaerobic ammonium oxidation have been described and named provisionally *Candidatus* "Brocadia", *Kuenenia*, *Candidatus* "Scalindua" and *Candidatus* "Anammoxoglobus" (Strous, et al. 1999a, Schmid, et al. 2000, Schmid, et al. 2003, Kartal, et al. 2007a). All characterized anammox bacteria have a membrane-bound intracytoplasmic compartment, the anammoxosome, and unique ladderane lipids in their cellular membranes (Sinninghe Damsté, et al. 2002, Schmid, et al. 2003, Sinninghe Damsté, et al. 2005, Kartal, et al. 2007a).
Anammox bacteria were assumed to have a completely chemolithoautotrophic lifestyle. However recent studies have shown that they have a more versatile metabolism. In addition to the anaerobic oxidation of ammonium they can also use organic acids and iron as electron donor, and iron and manganese as electron acceptor (Güven, et al. 2005, Strous, et al. 2006, Kartal, et al. 2007ab). Two anammox genera (*Kuenenia* and *Candidatus* "Anammoxoglobus") were shown to be able to reduce nitrate and/or nitrite to ammonium using organic acids as

electron donor (Kartal, et al. 2007b). This trait could give anammox bacteria a competitive advantage in the usually ammonium-limited natural ecosystems (Kartal, et al. 2007b).
One particular species, provisonally named *Candidatus* "Anammoxoglobus propionicus" was shown to out-compete other anammox bacteria as well as heterotrophic denitrifers for propionate as supplementary electron donor in the presence of ammonium (Kartal, et al. 2007a). Interestingly, while oxidizing propionate this anammox species persisted in its autotrophic lifestyle: it did not assimilate propionate directly but apparently still used CO_2 as its only carbon source.
In the present paper we show that anammox bacteria can also be enriched in the presence of acetate, a more environmentally relevant intermediate in the anaerobic degradation of organic matter than propionate. Feeding of ammonium, nitrate, nitrite and acetate led to the enrichment of *Candidatus* "Brocadia fulgida". The biomass from the enrichment culture was capable of oxidizing other organic compounds such as formate, propionate, monomethylamine and dimethylamine. One other interesting feature of biofilm aggregates of this anammox bacterium was the presence of autofluorescent extracellular substances, never observed before in anammox biofilms.

Materials and Methods

Enrichment and Cultivation of Anammox Bacteria

A sequencing batch reactor (SBR, working volume 4 l) was used for enrichment and cultivation of anammox bacteria (Strous, et al. 1998). Each SBR cycle consisted of 11 h and 45 min of filling, 5 min of biomass settling and 10 min of drawing of the liquid. During each filling period, 1 l of mineral medium containing nitrite, ammonium and nitrate (Van de Graaf, et al. 1996) and 500 ml of a sodium acetate solution (concentration specified in the results section) were added continuously to the reactor at flow rates of 1.4 $ml \cdot min^{-1}$ and 0.35 $ml \cdot min^{-1}$ respectively. To maintain anoxic conditions, the reactors and the medium vessels were flushed continuously with Ar/CO_2 (95/5%, 10 $ml \cdot min^{-1}$). The SBR was stirred at 200 rpm with a six-bladed turbine stirrer. The temperature of the SBR was maintained at 33°C with a water jacket. CO_2 present in the supplied gas was sufficient to buffer the solution and to keep the pH in the SBR between 7.0 and 7.3. The SBR was inoculated with an activated sludge sample originating from the secondary stage of Dokhaven municipal wastewater treatment plant (Rotterdam, NL).

Anammox Activity Assays

Biomass (5 ml with ~5 $mg \cdot ml^{-1}$ protein) was harvested from the SBR described above. The biomass sample was washed 3-5 times with mineral medium (see above) without substrates until the nitrite, nitrate and ammonium in the sample were less than 20 µM. The biomass was then transferred to 30 ml serum bottles. The bottles were sealed with 5 mm butyl rubber stoppers and were made anoxic by alternately applying under-pressure and Argon seven times. An overpressure of 1 bar was maintained in the bottles. The soluble substrates were added to the bottles from 100 mM anoxic stock solutions. To measure anaerobic ammonium oxidation activity, final concentrations of 3 mM NO_2^- and NH_4^+ were used. To measure nitrate reduction activity, final concentrations of 5 mM NO_3^- and 12.5 mM formate, 3 mM acetate, 2 mM propionate, 6 mM monomethylamine or 6 mM dimethylamine were used. To measure the transient accumulation of hydrazine, final concentrations 4 mM NH_2OH and 5 mM NH_4^+ were used. The bottles were incubated at 33°C and were shaken continuously at 300 rpm for 2 to 5 h.

Analytical Methods

Nitrate, nitrite, propionate, acetate and formate were measured with high performance liquid chromatography (HPLC) as described previously (Kartal, et al. 2007a). Ammonium was measured colorimetrically after the reaction with ortho-phthalaldehyde as described previously (Kartal, et al. 2006). Hydroxylamine, hydrazine and protein were determined as described before (Strous, et al. 1998).

DNA Extraction, Retrieval of 16S rRNA Sequences and Phylogenetic Analysis

Biomass was harvested from the SBR by centrifugation of a 1.5 ml sample. DNA was extracted as described in Juretschko, et al. (1998). DNA was then suspended in 50 µl ultrapure water, and kept at 4°C for 24 h until further analysis. A primer combination of Pla46F (*Escherichia coli* positions 46-63) forward and universal reverse (630R) (*E. coli* positions 1529-1545) primers was used for the preferential amplification of 16S rRNA genes of the members of the Planctomycetes (Schmid, et al. 2003). PCR amplificates were cloned directly using the TOPO TA Cloning kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. Plasmid-DNA was isolated with the Flex prep kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ). Sequencing and retrieval of the cloned 16S rRNA genes and phylogenetic analyses were performed as described (Kartal, et al. 2007a).

Fluorescence *in situ* Hybridization (FISH)

Phylogenetic analysis of the sequences and design of oligonucleotide FISH probes were done with ARB software as previously described (Schmid, et al. 2003). Biomass (1 ml) was harvested from the enrichment culture fixed in paraformaldehyde, and hybridizations with fluorescent probes were performed as described previously (Schmid, et al.

2000). All probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Thermo Electron Corporation (Ulm, Germany). The following probes were used to monitor the enrichment of the anammox population as described in Schmid et al. 2003: Amx 368 (S-*-Amx-0368-a-A-18, specific for all known anammox genera, Schmid, et al., 2003), EUB 338 (S-D-Bact-0338-a-A-18, Amann, et al. 1990), EUB 338 II (S-D-Bact-0338-b-A-18), EUB 338 III (S-D-Bact-0338-c-A-18, together with EUB and EUBII specific for most bacteria, Daims, et al., 1999), Pla46 (S-P-Planc-0046-a-A-18, specific for Planctomycetales). Probe Bfu 613 (S-*-Bfu-0613-a-A-24) was designed for specific hybridizations with *Candidatus* "Brocadia fulgida".

Electron Microscopy

Small aggregates of biomass from the SBR were cryofixed, freeze-substituted, embedded in Epon, and analyzed with transmission electron microscopy as described previously (van Niftrik, et al. 2007).

Lipid Analysis

The biomass from the SBR and reactor supernatant were separated using centrifugation and subsequently the biomass was frozen and freeze dried. Total lipids were extracted from the biomass and were analyzed using gas chromatography (GC) and gas chromatography mass spectrometry (GC/MS) as described previously (Kartal, et al. 2007a). Ladderane and other lipids were identified on basis of published mass spectral data (Sinninghe Damsté, et al. 2005). The stable carbon isotopes of lipids were measured using a ThermoFinnigan Delta C isotope ratio monitoring MS GCirm/MS which has been described previously (Schouten, et al. 2004). $\delta^{13}C$ values were corrected for the additional carbon obtained during derivitization.

Analysis of the $\delta^{13}C$ for Mineral and Acetate media

The reactor supernatant was analyzed for $\delta^{13}C$ values of total dissolved inorganic carbon (DIC) as described previously (Kartal, et al. 2007a). $\delta^{13}C$ values of the sodium acetate crystals used for reactor medium preparation were determined using Elemental Analysis irmMS as described before (Kartal, et al. 2007a).

Extraction and Analysis of Fluorescent Extracellular Polymeric Substances

Biomass (20 ml) was harvested from the SBR and the extracellular polymeric substances (EPS) were extracted by sequential incubation with formaldehyde (40% v/v, 4°C, 1 h) and NaOH (200 mM, 4°C, 4 h) (Liu & Fang 2002). Subsequently the biomass was separated from the extract with centrifugation (20000 g, 4°C, 20 min). The optimal excitation and emission wave lengths for the fluorescent EPS were determined with a fluorescence spectrophotometer (Varian, CA, USA). To determine the approximate molecular weight of the fluorescent compound the extract was passed through ultrafiltration membranes with different molecular mass cut-offs (300000, 100000, 50000, 10000 and 3000 Da) and after each filtration step the fluorescence was monitored.

Results

In this study we used the same experimental setup (a sequencing batch reactor, SBR), the same medium and the same inoculum used previously to enrich the anammox bacteria *Candidatus* "Brocadia anammoxidans", *Kuenenia stuttgartiensis* and *Candidatus* "Anammoxoglobus propionicus". The only difference was the addition of acetate to the reactor as an extra electron donor. The acetate-carbon:ammonium ratio in the medium was ultimately 1:6.

In the first four months of the enrichment culture the concentrations of nitrite and ammonium in the influent medium was increased from 3 mM to 45 mM, while the nitrate concentration was maintained at 6 mM. Simultaneously, the acetate concentration was increased from 1 mM to 30 mM. The concentrations of acetate and nitrite in the effluent were almost always below the detection limit (10 μM). The microbial population of the enrichment culture was monitored with fluorescence *in situ* hybridization (FISH) analysis.

In the first two months, the anammox population increased to approximately 50% and in the following two months to 80% of the enrichment culture as determined with FISH analysis. When genera-specific FISH probes were used it appeared that the only detectable anammox bacteria in the enrichment culture belonged to *Candidatus* "Brocadia" genus. After the first four months of the enrichment culture, the anammox biomass gradually became autofluorescent. The autofluorescence was prevalent in the same wavelength as fluorescein iso-thiocyanate (FITC, excitation/emission 490/520) and cy3 (excitation/emission 550/570); thus, FISH analysis with these dyes became impossible. The fluorescence was observed only in the aggregated anammox cells, not the single cells, indicating that the source of the fluorescence was the extracellular matrix. The extracted extracellular polymeric substances (EPS) were fluorescent between 390-630 nm. There were two excitation maxima, 352 and 442 nm, and two corresponding emission maxima, 464 and 521 nm respectively (Figure 1).

This indicated two different fluorescence sources. Membrane ultrafiltration showed that the mass of the fluorescent polymers were between 3-10 kDa.

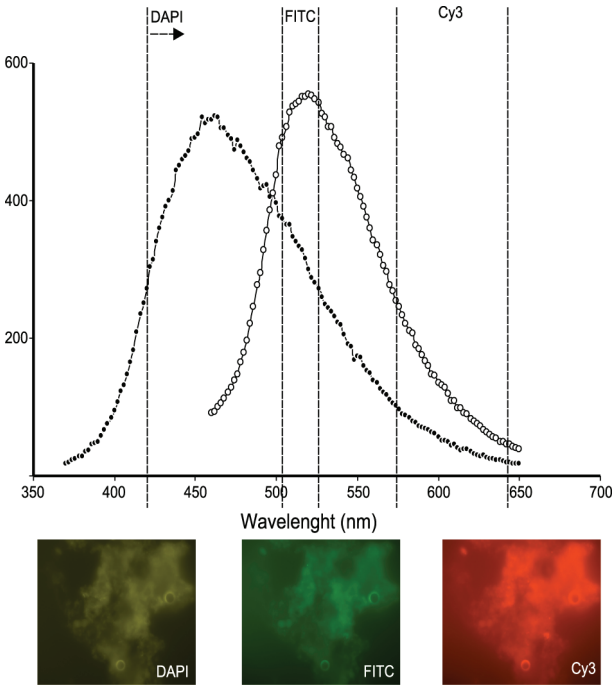

Figure 1 | Fluorescence spectrum of the extracellular polymeric substances extracted from the *Candidatus* "Brocadia fulgida" enrichment culture.Dashed lines indicate the boundaries of our emission filters (DAPI, FITC, Cy3). Excitation wavelengths: filled circles 352 nm, empty circles 442 nm. Y axis is in arbitrary intensity units. The micrographs show the autofluorescence in different channels.

To determine the precise affiliation of the anammox species present in the enrichment culture we applied a Planctomycetes specific full cycle rRNA approach. DNA was extracted from the the enrichment culture biomass. 16S rRNA gene sequences of Planctomycetes were amplified using PCR with the primers Pla46F and 630R, and cloned. Twenty clones were randomly picked for sequencing. Near full-length 16S rRNA sequences were obtained. All of the 16S rRNA genes had the same sequence and branched within the *Candidatus* "Brocadia" lineage (Figure 2).

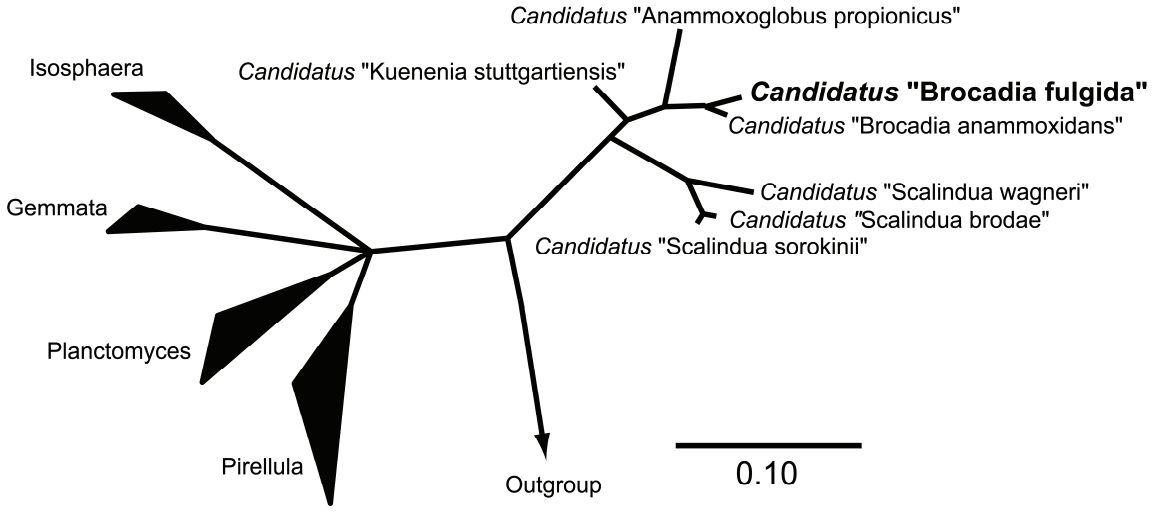

Figure 2 | Phylogenetic tree showing the relationships of the acetate enrichment culture 16S rRNA gene clone (*Candidatus* "Brocadia fulgida"), other anammox bacteria, Planctomycetes and other reference organisms.

A new oligonucleotide probe S-*-Bfu-613-a-A-24 was constructed to match specifically with the dominant 16S rRNA gene sequence from the enrichment culture. Retrospective analysis

of biomass samples showed that upon inoculation no anammox cells were detected. During the enrichment there was a gradual increase in the population of the anammox species binding with the new probe (Figure 3). This species became dominant in two months and after four months it out-competed all other bacteria and made up 80% of the population.

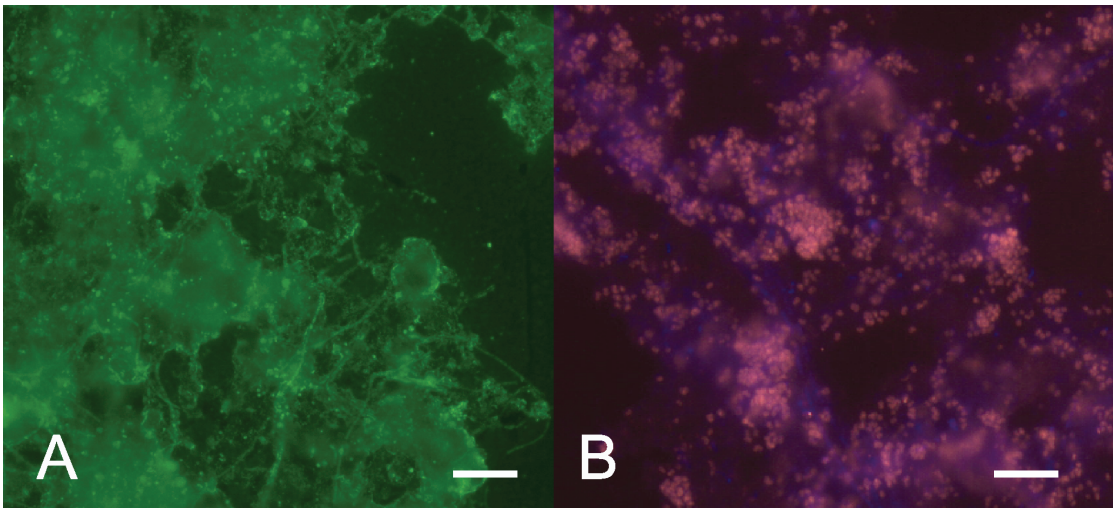

Figure 3 | Monitoring of the enrichment of *Candidatus* "Brocadia fulgida" from inoculum over 2 months with fluorescent in situ hybridization. *Candidatus* "Brocadia fulgida" is double hybridized with pla 46 (cy5, blue) and Bfu613 (cy3, red) and depicted in pink. Other bacteria are hybridized with FLUOS-labeled EUB mix probes and are depicted in green. *Candidatus* "Brocadia fulgida" was enriched from <1% (A) to 80% in 60 days (B). Bar = 20 µm

This 80% enriched anammox biomass had a specific activity of 15 µmol NH_4^+·g protein^{-1}·min^{-1}. When the biomass from the enrichment culture was incubated in the absence of acetate, per mol of ammonium 1.5 mol of NO_2^- was converted, similar to the previously reported values (Strous, et al. 1998). When the biomass was incubated with hydroxylamine, there was a transient accumulation of 0.19 mM hydrazine, a phenomenon unique to anammox bacteria (Figure 4).

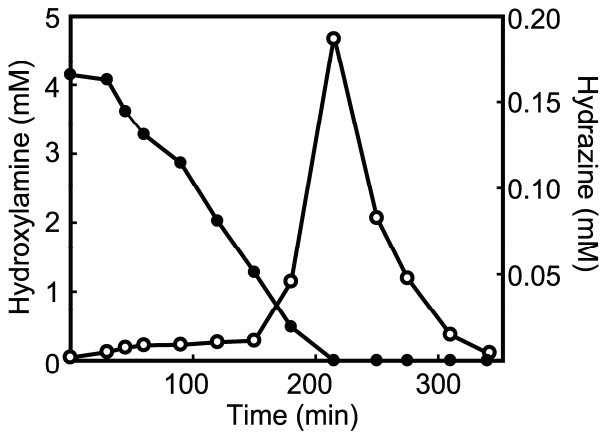

Figure 4 | Production of hydrazine (empty circles) in the presence of hydroxylamine (circles) under anoxic conditions

The nitrate-reducing activity of the enrichment culture with small organic compounds was 3±0.22 NO_3^- µmol·g protein^{-1}·min^{-1} for formate, 1.5±0.06 for acetate, 0.88±0.02 for propionate, 1±0.09 for monomethylamine and dimethylamine. In the incubations with acetate and propionate there was a transient nitrite accumulation of 0.8±0.06 and 0.3±0.02 mM respectively. There was no detectable transient nitrite accumulation in the incubations with monomethylamine, dimethylamine and formate. The formate and acetate oxidation rates with the current biomass were notably higher than rates observed with *Candidatus* "Brocadia anammoxidans", *Kuenenia stuttgartiensis* and *Candidatus* "Anammoxoglobus

propionicus" (Table 1). The propionate oxidation rate of the biomass was 3 times higher than *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis", but half the rate of *Candidatus* "Anammoxoglobus propionicus".

Table 1 | Organic acid oxidation rates (μmol·g prot^{-1}·min^{-1}) of different anammox species

	Candidatus "Brocadia anammoxidans" [a]	*Kuenenia stuttgartiensis*[a]	*Candidatus* "Anammoxoglobus propionicus" [a]	*Candidatus* "Brocadia fulgida"
Formate	6.5 ± 0.6	5.8 ± 0.6	6.7 ± 0.6	7.6±0.6
Acetate	0.57 ± 0.05	0.31 ± 0.03	0.79 ± 0.07	0.95±0.04
Propionate	0.12 ± 0.01	0.12 ± 0.01	0.64 ± 0.05	0.31±0.007

a These rates are taken from Kartal *et al* (2007a)

The presence of ladderane lipids, another unique characteristic of all the anammox bacteria, was also investigated in the enrichment culture. The most abundant ladderane lipids in the total lipid extract from the SBR were the anammox specific ladderane fatty acids **b** and **c** (Figure 5). Large amounts of C16 fatty acids were also present. Although these straight chain fatty acids are present in many other organisms they were also shown to be incorporated into intact membrane ladderane lipids, thus confirming their presence in anammox bacteria (Boumann, et al. 2006).

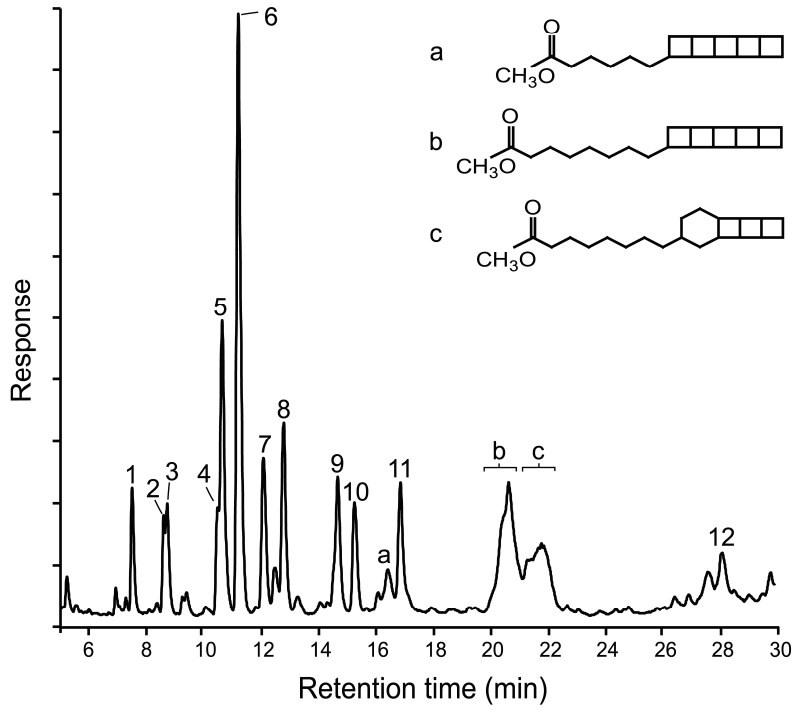

Figure 5 | Gas chromatogram of the total lipid fraction from the acetate enrichment culture. Key: 1= C14:0 fatty acid, 2 = i-C15 fatty acid, 3 = C15:0 fatty acid, 4 = i-C16 fatty acid, 5 =C16:1 fatty acid, 6 = C16:0 fatty acid, 7 = 10-methylhexadecanoic acid, 8 = C17:1 fatty acid, 9 = C18:1 fatty acid, 10 = C18:0 fatty acid, 11 = C19:1 fatty acid, 12 = squalene. (a) = C18 ladderane fatty acid with 5 cyclobutane rings, (b) = C20 ladderane fatty acid with 5 cyclobutane rings, (c) = C20 ladderane fatty acid with 3 cyclobutane rings and one cyclohexane ring.

To determine if the anammox bacteria in the enrichment culture used CO_2 or acetate for the synthesis of the ladderane lipids (i.e. as C source) their stable carbon isotope composition were measured. Both potential carbon sources had similar $\delta^{13}C$ values (Table 3), but only for CO_2 a significant isotopic fractionation would be expected (Schouten, et al. 2004, Londry and Des Marais 2003). The $\delta^{13}C$ values were variable and ranged from -32.8 ‰ (non-specific $C_{16:1}$ fatty acid) to -84.2 ‰ (ladderane lipid c), indicating that CO_2 was the most likely main carbon source for the lipids.

Table 2 | $\delta^{13}C$ isotopic depletion of selected lipids and substrates [(^{13}C ‰ vs. VPDB (Vienna Pee-Dee Belemnite)]

Lipid / Medium	$\delta^{13}C$ ‰ vs VPDB
i16:0	-67.0
16:1	-32.8
16:0	-42.1
10MeC15	-60.8
Ladderane **b**	-72.5
Ladderane **c**	-84.2
Sodium acetate	-28.4
Medium DIC	-27.6

A typical characteristic of the previously described anammox organisms is the presence of a membrane-bound intracytoplasmic compartment known as the anammoxosome (Lindsay, et al. 2001). Transmission electron microscopy (TEM) was performed on thin sections prepared from the enriched biomass containing the new anammox organism. The new anammox species displayed typical ultrastructural features of anammox bacteria: a single membrane bound anammoxosome containing tubule like structures, and riboplasm with ribosome-like particles separated from paryphoplasm at the cell rim by an intracytoplasmic membrane (Figure 6).

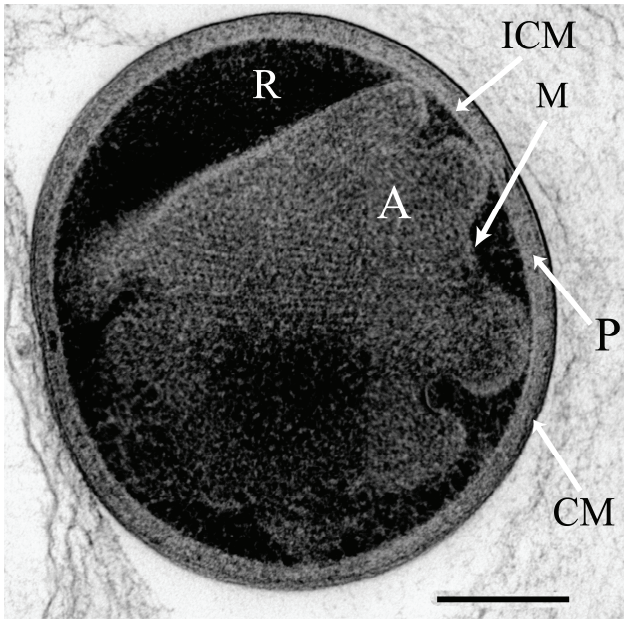

Figure 6 | Transmission electron micrograph of thin-sectioned *Candidatus* "Brocadia fulgida".

Cells have the conventional anammox cell components: the anammoxosome (A), the anammoxosome membrane (M), riboplasm (R), paryphoplasm (P) separated from riboplasm by an intracytoplasmic membrane (ICM) and the cytoplasmic membrane (CM). Bar = 200 nm

Discussion

Compared to aerobic ammonium oxidizers, anaerobic ammonium oxidizing (anammox) bacteria have a more divergent 16S rRNA gene phylogeny. Such a considerable degree of evolution could indicate that anammox species are quite different from each other and each might occupy a distinct ecological niche (while belonging to the same ecological guild). The characterization of *Candidatus* "Brocadia fulgida", the fifth species of anammox bacteria currently described, indicates that the anammoxosome, the ladderane lipids and the capability to produce molecular hydrazine are shared among all anammox bacteria and define the guild of anaerobic ammonium oxidizers as a whole. On the other hand, the presence of organic acids in the medium seems to define some degree of niche differentiation. Previously, *Candidatus* "Anammoxoglobus propionicus" was shown to out-compete other anammox bacteria for propionate (Kartal, et al. 2007a). In the present study, in the presence of acetate *Candidatus* "Brocadia fulgida" was shown to be more successful competitor than

other anammox bacteria. It should be noted that both these species were previously also transiently observed in enrichment cultures without supplemented organic acids. These previous studies showed that the inoculum used in the present study potentially contained all known wastewater anammox species.
Altogether these anammox species constitute an almost undetectable minority of the complete population of the inoculum. It is interesting that none of the dominant bacteria could compete successfully for nitrite with a chemolithoautotrophic anammox bacterium under denitrifying conditions. Thermodynamic maximal biomass yield calculations based on the Gibbs free energy changes associated with the oxidation of acetate and ammonium showed that if the denitrifiers would have used all the available acetate, a coculture of a heterotrophic acetate-oxidizing denitrifier with *Candidatus* "B. fulgida" would be expected. According to these calculations the anammox population would only make up 40% of the total biomass. But apparently this did not happen: despite the fact that a sufficient quantity of organic material (acetate) was supplied, heterotrophic bacteria never became the dominant organisms in the enrichment culture. Instead, *Candidatus* "B. fulgida" was shown to constitute 70-80% of the total population by FISH. Based on the same yield calculations, if all of the remaining 20-30% were denitrifiers, they would be responsible for 20-40% of the acetate consumption. Thus it is possible that the side population could be contributing to the acetate oxidation in the continuous culture and the batch incubations.
Previously, the organic acid dependent nitrate reduction pathway of the anammox bacterium *Kuenenia stuttgartiensis* was elucidated, and it was shown that this anammox bacterium was capable of dissimilatory nitrate reduction to ammonium (Kartal, et al. 2007b). Previous studies and the results of the present study indicate that the organic acid oxidation could be a common trait among anammox species. It is conceivable that *Candidatus* "B. fulgida" performs nitrate reduction through the same pathway. This hypothesis will be investigated in the future with ^{15}N labeled nitrogen tracer studies. In the present study, *Candidatus* "B. fulgida" was characterized as a typical anammox bacterium with one exception: the presence of an autofluorescent extracellular substance around its cells.
In sequencing batch reactors (SBR) anammox bacteria grow in biofilm aggregates because this type of bioreactor selects for the well settling biomass. In this case, these biofilm aggregates became fluorescent after several months of enrichment. The fluorescence was prevalent in a wide wavelength range (390 - 630 nm) making fluorescence *in situ* hybridization (FISH) studies with FLUOS and Cy3 labeled probes impossible after four months of enrichment. Interestingly, the single cells of anammox bacteria in the enrichment culture were not fluorescent, indicating that the source of the fluorescence was the extracellular polymeric substances (EPS) that form the matrix of the aggregates. When these polymeric substances were extracted from the biomass it was clear that they were indeed autofluorescent. When analyzed with fluorescence spectrophotometer it was determined that there were two distinct excitation and emission maxima. This suggested that there were at least two different compounds or moieties contributing to the autofluorescence of the biofilm aggregates. Via ultrafiltration we also determined that the fluorescent substance had a mass between 3-10 kDa. All described anammox enrichment cultures form EPS as a matrix to build biofilm aggregates, but none are autofluorescent. Hence it was highly likely that the enriched anammox bacterium was the source of the autofluorescence. Based on this feature we propose to provisionally name this anammox species *Candidatus* "Brocadia fulgida". The molecular structure and detailed properties of this compound remains unknown, but will be investigated in the future with mass spectrometry.
The ladderane lipids unique to anammox bacteria were the most abundant lipid type in the *Candidatus* "Brocadia fulgida" enrichment culture. These lipids were depleted in ^{13}C up to 56.6‰ relative to the medium dissolved inorganic carbon (DIC). This strong depletion is characteristic for anammox bacteria and is consistent with carbon fixation via the acetyl-CoA pathway (Schouten, et al. 2004). The ^{13}C depletion in the ladderane lipids of *Candidatus* "Brocadia fulgida" was similar to other described anammox species (depleted 47-51 ‰

relative to DIC) suggesting they use a similar carbon acquisition pathway. The other possible carbon source, sodium acetate, had a $\delta^{13}C$ of -28.4 ‰ ± 0.2 ‰ (relative to VPDB), suggesting this was not the direct source of carbon for the ladderane lipids. Most of the carbon fractionation occurs during carboxylation; thus, if *Candidatus* "Brocadia fulgida" incorporated acetate directly into its biomass, much less carbon fractionation would have been expected in the lipids (Londry and Des Marais, 2003). This is striking because instead of directly converting acetate into acetyl-CoA, *Candidatus* "Brocadia fulgida" apparently chooses the hard way; it degrades acetate to CO_2 and then "re-fixes" this carbon via acetyl-CoA. The genome analysis of the anammox bacterium *Kuenenia stuttgartiensis* revealed at least 3 different acetate-CoA ligases. One might speculate that anammox bacteria oxidize acetate in the periplasm via an unknown mechanism, for example via oxalate. It is also possible that the heterotrophic bacteria dissimilate some of the acetate to CO_2 and the produced CO_2 is then used as carbon source for the anammox bacteria. The mechanism of acetate oxidation by *Candidatus* "Brocadia fulgida" enrichment culture will be a topic for future research.

It is possible that acetate oxidation occurs only for the conservation of energy. This would lead to a higher biomass yield for the anammox bacteria per mole of oxidized ammonium. Theoretical yield calculations showed that a 55% increase in the biomass yield would be expected compared to anammox bacteria grown in the absence of acetate. This increase would in theory lead to shorter start-up periods for anammox bioreactors. In the case of the *Candidatus* "Brocadia fulgida" enrichment culture there was no significant difference in the startup time compared to the previous enrichment cultures. However, it is very difficult to follow such an increase precisely during an anammox enrichment because knowledge on the viability and initial cell count of anammox bacteria present in the inoculum is lacking and physical factors such as diffusion limitation into the aggregates could also be important. An alternative explanation for the lack of an increase in the yield of the anammox bacteria could be the possibility that the most of the acetate is oxidized by the 20-30% side population, common in all anammox enrichment cultures (Strous, et al.1999a, Strous, et al. 2006, Kartal, et al. 2006, Kartal, et al. 2007a). However as mentioned before, such a high acetate consumption would not have resulted in a highly (70-80%) enriched anammox culture.

Biomass from the *Candidatus* "Brocadia fulgida" enrichment culture was also able to oxidize methylamines with nitrate and/or nitrite as electron acceptors. The end product of the oxidation of methylamine with nitrate or nitrite is ammonium, and *Kuenenia stuttgartiensis* reduces nitrate and nitrite to ammonium. In many natural ecosystems, such as the water column of the oceans, anammox bacteria are usually limited by ammonium, and methylamines are available in freshwater and marine ecosystems (Wang & Lee, 1990, Fitzsimons, et al. 2006). Hence, the oxidation of methylamines to ammonium could be a valuable survival strategy for anammox bacteria in nature in the absence of ammonium because the end product for the oxidation of methylamines is ammonium.

In wastewater treatment the anammox process is used for the removal of ammonium. Application of anammox is dependent on a source of nitrite. The co-oxidation of organic acids could increase the potential for the anammox process in wastewater treatment as follows: (1) anammox bacteria are still enriched to a very high density in the presence of organic acids and ammonium so the process could also be applied to wastewater containing both organic compounds and ammonium; (2) sometimes nitrate is available as an electron acceptor, and nitrate could be used directly by anammox bacteria (or other heterotrophs present in the reactor biomass) with organic acids as a co-substrate; (3) currently the theoretical maximum ammonia removal efficiency with anammox is 90%. By supplying organic acids as co-substrate, ammonia removal can become close to 100% efficient.

Description of *Candidatus* "Brocadia fulgida"

Brocadia fulgida (Brocad.ia L.fem.n referring to the place of discovery of the first anammox species; fulgid.a L. fem. adj. of shining, referring to the strong autofluorescence of the biofilm aggregates)

Anaerobic chemolithoautotrophic coccoid cells with diameters ranging between 0.7 to 1 µm. Cells oxidize ammonium with nitrite as the electron acceptor and with CO_2 as the main carbon source. Cells oxidize propionate, acetate, formate, monomethylamine and dimethylamine with nitrate and/or nitrite as the electron acceptor. Cells produce hydrazine transiently in the presence of hydroxylamine. Cells possess a membrane bound intracytoplasmic compartment containing tubule like structures, known as the anammoxosome. Cells contain ladderane lipids. 16S rRNA sequence similarity to the closest relative (*Candidatus* "Brocadia anammoxidans") is 94%. Accession number of the 16S rRNA gene sequence is DQ459989.

chapter 4

adaptation of a freshwater anammox population to high salinity wastewater

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Abstract
For the successful application of anaerobic ammonium oxidation (anammox) in wastewater practice it is important to know how to seed new anammox reactors with biomass from existing reactors. In this study, a new high salinity anammox reactor was inoculated with biomass from a freshwater system. The changes in activity and population shifts were monitored. It was shown that freshwater anammox bacteria could adapt to salt concentrations as high as 30 $g \cdot l^{-1}$, provided the salt concentration was gradually increased. Higher salt concentrations reversibly inhibited anammox bacteria. The nitrogen removal efficiency and maximum anammox activity of the salt adapted sludge was very similar to the reference freshwater sludge. Fluorescence *in situ* hybridization analysis revealed that the freshwater anammox species *Kuenenia stuttgartiensis* was dominant in both salt adapted sludge and freshwater sludge. These results show that gradual adaptation may be the key to successful seeding of anammox bioreactors.

Introduction
In the last 3 years, the anaerobic ammonium oxidation (anammox) process has been successfully implemented as a cost-effective and environment friendly N-removal system in the Netherlands (Kartal, et al. 2004, Jetten, et al. 2005, Op den Camp, et al. 2006). Anammox bacteria oxidize ammonium under anoxic conditions with nitrite as the electron acceptor, and conserve energy for CO_2 fixation. Compared to conventional nitrification-denitrification dependent nitrogen removal systems, the anammox process requires less oxygen and no organic material (e.g. methanol) supply. Hence the costs and CO_2 emissions of nitrogen removal are reduced by up to 60% and 90% respectively (van Dongen, et al. 2001).
Unfortunately, the slow growth rate (doubling time about 15 d) of anammox bacteria leads to long start-up and recovery time after disturbances. A similar problem of long start-up time was also encountered in 1980s when the upflow anaerobic sludge blanket (UASB) technology was first introduced to wastewater treatment (Lettinga, et al.1980, Lettinga, et al.1987). Due to the difficulty of granule formation from flocculant sludge, the first UASB reactors had a long start-up time. By the beginning of the 1990s this problem had already been solved by seeding new reactors with already formed granules (Lettinga & Pol, 1991). Currently, UASB reactors are seeded with already formed granules, reducing the start-up time from months to a matter of weeks. Seeding might also be the solution to long start-up time in the anammox case.
When a new reactor is seeded the biomass has to adapt to the new conditions. Adaptation could either lead to the acclimation of the existing bacterial population to the new conditions or a significant shift in the microbial community. Important conditions like ion concentrations, pH and temperature could be the potential sources of such effects.
Salinity is an important parameter for wastewater treatment because many industrial wastewaters rich in ammonium also contain high salt concentrations (landfill leachate, leather industry, etc.). Until now in natural saline ecosystems only anammox species belonging to the "Scalindua" genus have been detected (Thamdrup & Dalsgaard, 2002, Kuypers, et al. 2003, Schmid, et al. 2003, Kuypers, et al. 2005). The other three genera are known to inhabit freshwater ecosystems (Strous, et al. 1999a, Schmid, et al. 2000, Kartal, et al. 2007a). Two anammox genera are rarely found in the same ecosystem. This indicates that each anammox genus has a distinct, but yet unknown niche. Thus, it would be expected that a freshwater anammox population would change dramatically during exposure to salinity. In the oxygen-limited autotrophic nitrification denitrification (OLAND) process aerobic and anaerobic ammonium oxidizers coexist (Kuai & Verstraete, 1998). Recently the OLAND process was demonstrated to be active under saline conditions (Windey, et al. 2005). It was shown that the anammox bacteria in this OLAND reactor could adapt to salt concentrations up to 30 $g \cdot l^{-1}$ provided that the increase was gradual (Windey, et al. 2005). The population dynamics of the OLAND system were not studied.

In the current paper we present a pilot study for the seeding of anammox reactors. We used anammox sludge adapted to freshwater to seed a reactor that was fed with high salt concentration influent. The effect of salt concentration on the anammox activity and anammox population dynamics was monitored.

Materials and Methods

Operation of Laboratory Scale Bioreactors

Two sequencing batch reactors (SBRfresh and SBRsalt working volumes 2.5 l) were used for enrichment and cultivation of anammox bacteria (Strous, et al. 1998). During the first 11.5 h of the cycle the SBRs were filled continuously with synthetic wastewater (van de Graaf, et al. 1996) containing ammonium and nitrite (concentrations are specified at the results section). After the filling period, the stirrer, influent liquid and gas supplies were stopped and the aggregates were allowed to settle for 15 min. In the remaining 15 min of the total cycle, part of the liquid (depending on the desired hydraulic retention time) was purged by an effluent pump. A mixture of 90% NaCl and 10% KCl was added to the mineral medium for the SBR running under salt stress (SBRsalt, concentrations are specified at the results section). To maintain anoxic conditions, the reactors and the medium vessels were flushed continuously with Ar/CO_2 (95/5 %, 10 ml·min^{-1}). The SBRs were stirred at 200 rpm with a turbine stirrer. The temperature of the SBRs were kept constant at 25°C with a heating finger and counter cooling with water (6°C). CO_2 present in the supplied gas was sufficient to buffer the solution and to keep the pH in the SBRs between 7.0-7.3.

Batch Anammox Activity Tests

Biomass (5 ml with ~5 mg·ml^{-1} protein) was harvested from the SBRs described above. The biomass sample was washed 3-5 times with mineral medium until the nitrite, nitrate and ammonium in the sample were less than 20 µM. For the incubations with salt the biomass was suspended in mineral medium solution containing 90% NaCl and 10% KCl (5, 10, 30, 45, 60, 75, 90 g salt/l), for the incubations without salt, the biomass was suspended in mineral medium without salt. In all cases the biomass suspension was then transferred to 30 ml serum bottles. The bottles were sealed with 5 mm butyl rubber stoppers and were made anoxic by alternately applying under-pressure and argon. An overpressure of 1 bar was maintained in the bottles. The soluble substrates were added to the bottles from 10 mM stock solutions. To measure anaerobic ammonium oxidation activity, final concentrations of 3 mM NO_2^- and NH_4^+ were used. The bottles were incubated at 30 °C and were shaken continuously at 300 rpm for 1 to 6 hour(s) depending on their ammonium oxidation rates.

Analytical Methods

Nitrate was measured colorimetrically at 420 nm after a 30 min reaction of 40 µl sample containing 0.5 – 5 mM nitrate with 10 µl saturated sulfamic acid solution (to remove nitrite), 0.2 ml 5% salicylic acid in 98% sulfuric acid and 2 ml cold (4°C) 4 M NaOH (Cataldo, et al. 1975). Nitrite was measured colorimetrically at 540 nm after a 20 min reaction of 1 ml sample containing 0.01 – 0.05 mM nitrite with 1 ml 1% sulfanilic acid in 1M HCl and 1 ml 0.1% N-naphtylethylenediamine (van Eck, 1966). Ammonium was measured colorimetrically at 420 nm after a 30 min reaction of 40 µl sample containing 0.5 – 5 mM ammonium with 760 µl 0.54 % ortho-phthalaldehyde, 0.05 % β-mercaptanol and 10% ethanol in 400 mM potassium phosphate buffer (pH 7.3) (modified from Taylor, et al. 1974).

Fluorescence *in situ* Hybridization (FISH)

Biomass (1 ml) from the enrichment culture was harvested, and hybridizations with fluorescent probes were performed as described previously (Schmid, et al. 2000). All probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Thermo Electron Corporation (Ulm, Germany). The following probes were used to monitor the enrichment of the anammox population as described in Schmid et al. 2003: EUB 338 (S-D-Bact-0338-a-A-18, specific for most but not all bacteria, Amann, et al. 1990), EUB 338 II (S-D-Bact-0338-b-A-18, specific for Planctomycetales, Daims, et al. 1999), EUBIII 338 (S-D-Bact-0338-c-A-18, specific for Verrucomicrobiales, Daims et al. 1999), Pla46 (S-P-Planc-0046-a-A-18, specific for Planctomycetales), BS 820 (S-*-BS-820-a-A-22, specific for *Candidatus* "Scalindua wagneri", Schmid, et al. 2003) Amx 820 (S-*-Amx-0820-a-A-22, specific for *Candidatus* "Brocadia anammoxidans" and *Kuenenia stuttgartiensis*, Schmid, et al. 2000), and Kst 1273 (S-*-Kst-1273-a-A-20, specific for *Kuenenia stuttgartiensis*, Schmid, et al. 2000) .

Results

Sludge (500 ml) from the rotating biological contactor (RBC) from Pitsea wwtp, UK was used to inoculate a sequencing batch reactor (SBR, 2.5 l). Initially, 2 – 5% of the inoculum consisted of "Scalindua" species. During four months after inoculation, the sludge became 60 – 70% enriched with anammox bacteria (as determined with fluorescence *in situ* hybridization, FISH). Approximately equal amounts of *Kuenenia stuttgartiensis* and *Candidatus* "Scalindua wagneri" made up the anammox population.

At this point (t = 0) half of the sludge from the anammox bioreactor (SBRfresh) was used to seed another reactor (SBRsalt). Initially, the same medium (15 mM NO_2^- and 15 mM NH_4^+) was supplied to both reactors with the same hydraulic retention time (HRT) of 3 days. In the next 180 days the nitrogen load on both of the SBRs was increased gradually by increasing the NO_2^- and NH_4^+ concentrations to 45 mM each and decreasing the HRT to 1.5 days. At the same time the influent salt concentration of SBRsalt was gradually increased to 30 g·l^{-1} (Fig 1). Between t = 180 and t = 360 days both reactors were run at steady state conditions without any change in the nitrogen load and the salt concentrations. Nitrate was always produced in both cases, indicating a good anammox growth (van de Graaf, et al. 1996). During this 180-day period the N-removal performance of SBRsalt and SBRfresh did not change (Figure 1). Evidently the freshwater anammox bacteria had fully adapted to seawater conditions, indicated by growth and stable reactor performance.

At t = 360 the influent salt concentration of SBRsalt was further increased to 45 g·l^{-1}. Five days after this increase, the anammox activity of the reactor was completely lost. At t = 365 the influent salt concentration for SBRsalt was decreased back to 30 g·l^{-1}, and medium (30 mM NO_2^- and 30 mM NH_4^+) was supplied with an HRT of 3 days. Nitrite in the influent was now completely removed and the anammox activity had obviously recovered (Figure 1). Apparently a salt concentration of 45 g·l^{-1} reversibly inhibited the anammox sludge.

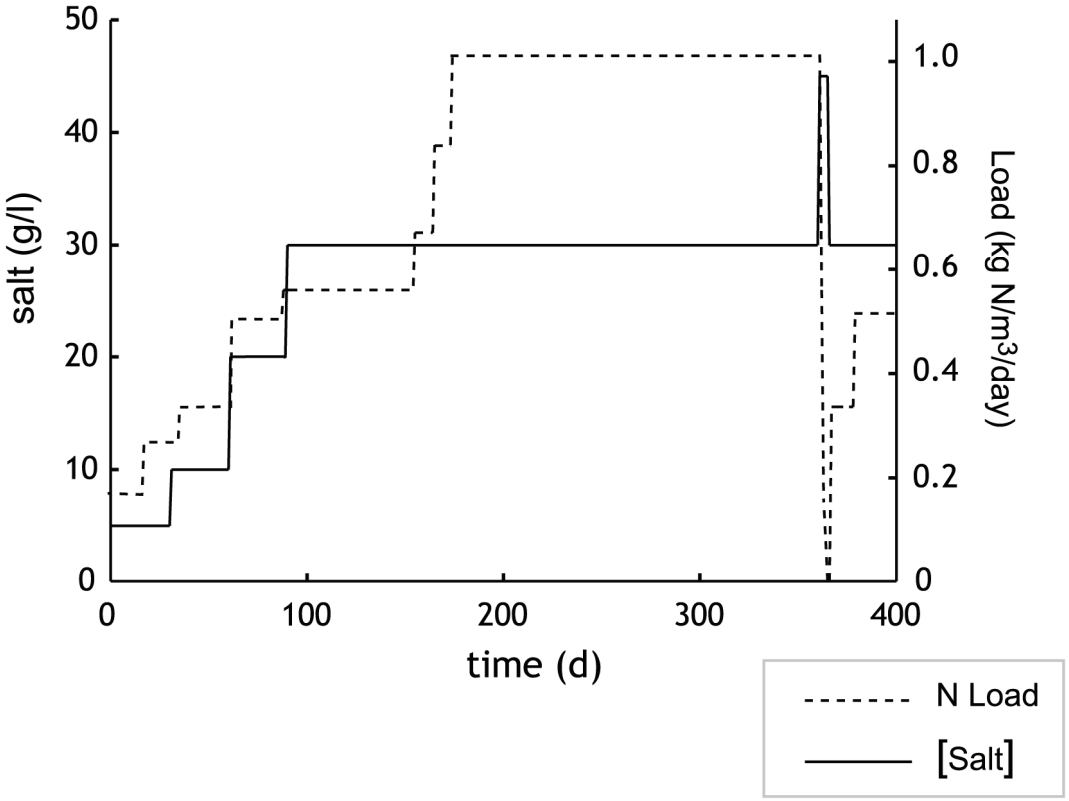

Figure 1 | Adaptation of anammox bacteria to salinity.

Biomass harvested from the two enrichment cultures (SBRfresh and SBRsalt) during the steady state period was incubated in batch reactors and the maximum anaerobic ammonium oxidation rate was determined as a function of salt concentrations (Figure 2). Biomass from SBRfresh had a maximum anaerobic ammonium oxidation rate of 25 µmol·g protein^{-1}·min^{-1} at 10 g·l^{-1} salt. At 30 g·l^{-1} salt concentration biomass from SBRfresh had significantly reduced anammox activity (Fig. 2). At a salt concentration of 45 g·l^{-1} there was a lag phase of 20 min before the reaction started, and activity was 15% of the maximum. The activity was lost at 60 g·l^{-1} salt. At salt concentrations between 5 – 45 g·l^{-1}, the usual anammox stoichiometry (NO_2^- : NH_4^+ = 1.3) was observed, whereas at 0 g·l^{-1} and above 60 g·l^{-1} the stoichiometry changed to 2 and 1.6 respectively.
Biomass from SBRsalt had a maximum anaerobic ammonium oxidation rate of 21 µmol·g protein^{-1}·min^{-1} at 30 g·l^{-1} salt. At lower and higher salt concentrations, anaerobic ammonium oxidation rate of the biomass dropped, but the biomass was still active at 75 g·l^{-1} salt. There was no detectable lag phase for biomass from SBRsalt up to 75 g·l^{-1}. The activity was lost at 90 g·l^{-1} salt.
Biomass from SBRfresh lost activity above 10 g/l salt, whereas the biomass from SBRsalt had a maximum activity at 30 g/l (Figure 2). This indicated that the anammox bacteria do not have an intrinsic resistance to high salt concentration, but they adapted to higher salt concentrations due to the stepwise increments in the salt concentration.

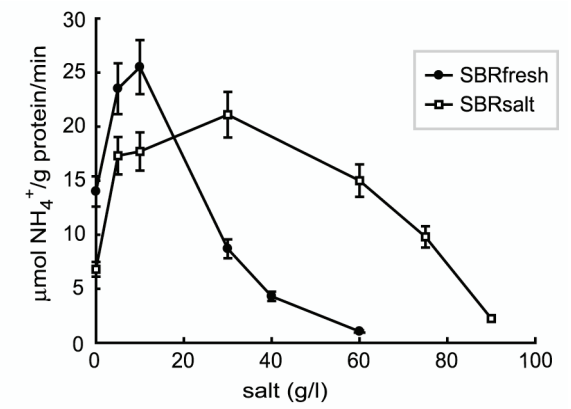

Figure 2 | Short batch experiments with salt and freshwater adapted anammox biomass under different salt concentrations

SBRfresh and SBRsalt were monitored with FISH throughout the cultivation of the anammox bacteria. Probes specific for Planctomycetes (S-P-Planc-0046-a-A-18) (Neef, et al.1998), *Kuenenia stuttgartiensis* (S-*-Kst-1273-a-A-20) (Schmid, et al. 2000), *Candidatus* "Scalindua wagneri" (S-*-BS-820-a-A-22) (Kuypers, et al. 2003), and most bacteria (EUB, EUB II and EUB III mix) (Amann, et al. 1990, Daims, et al. 1999) were used for the analysis.
At t = 0 the anammox bacteria comprised of 70-80% of the total bacteria and 99% of the Planctomycetes in both of the enrichment cultures. As mentioned above, 50% of the anammox population was *Kuenenia stuttgartiensis* and the remaining 50% was *Candidatus* "Scalindua wagneri". At t = 360 both reactors still consisted of 70-80% anammox bacteria, however the abundance of the two species had changed. The anammox population in SBRfresh consisted of 99 % *Candidatus* "K. stuttgartiensis" and 1 % *Candidatus* "S. wagneri" as detected with FISH microscopy. In SBRsalt 70 % of the anammox population consisted of *Candidatus* "K. stuttgartiensis" and 30 % was still *Candidatus* "S. wagneri". Even though the dominant species was *Candidatus* "K. stuttgartiensis", apparently the salinity helped sustain a viable amount (30% of the total anammox population) of *Candidatus* "S. wagneri" species in the system.

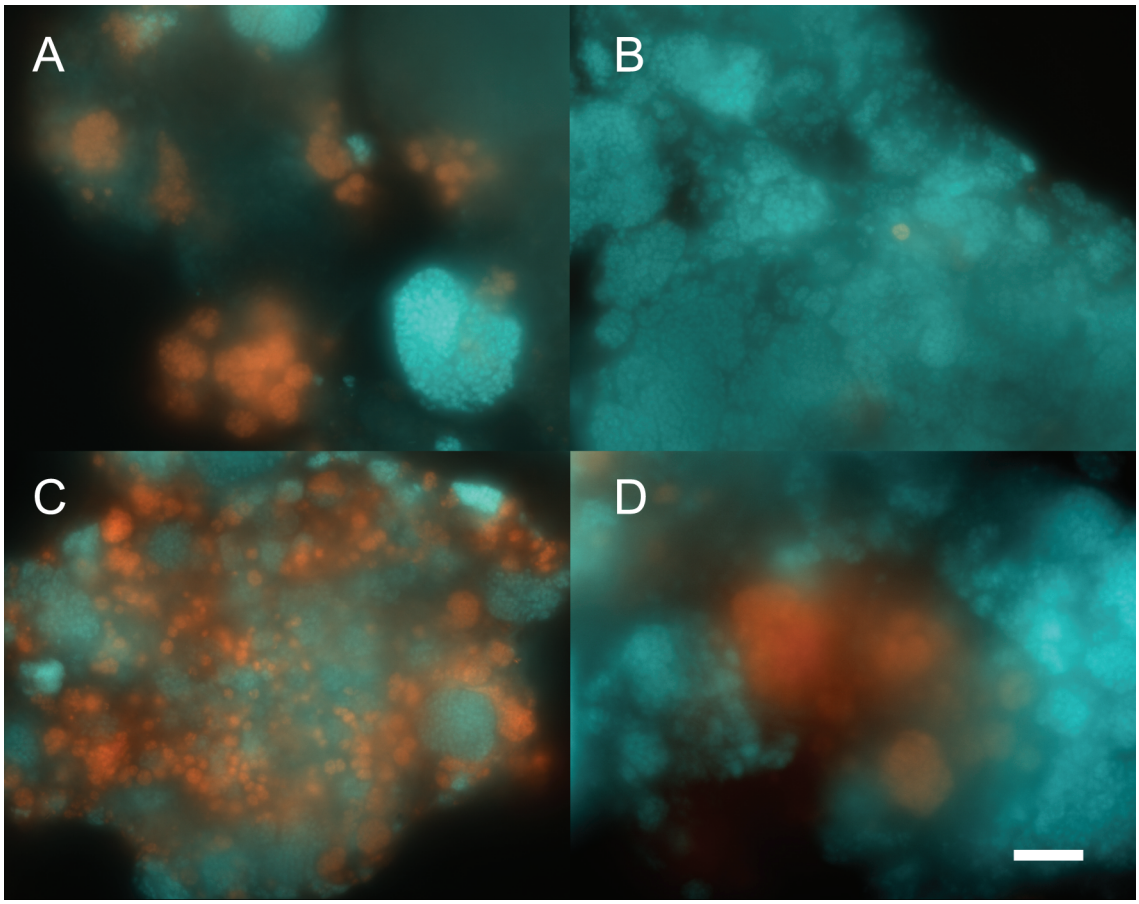

Figure 3 | Changes in anammox populations in SBRsalt and SBRfresh with fluorescent *in situ* hybridization. *Kuenenia stuttgartiensis* is hybridized with Cy3-labelled probe S-*-Kst-1273-a-A-20 and depicted in cyan. *Candidatus* "Scalindua wagneri" is hybridized with cy5-labelled S-*-BS-820-a-A-22 probe and depicted in orange. (A) SBRfresh T = 0 , B) SBRfresh T = 300 days, C) SBRsalt T = 0, D) SBRfresh T = 300) Bar = 20 µm

Discussion

The anammox process is a cost-effective and environmentally friendly N-removal system. The main challenge for the application of the anammox process is the slow growth of the anammox bacteria. An optimal seeding strategy might be the solution to the slow start-up problem of the anammox process.

Here we used biomass adapted to low salinity wastewater to seed another anammox reactor supplied with high salinity wastewater. The results showed that when salt was gradually introduced to the anammox reactor over a ninety-day period by increasing the salt concentration every 30 days (approx. two anammox generations), anammox bacteria retained activity at salt concentrations up to 30 $g \cdot l^{-1}$ (0.5 M). Recently, Windey et al. (2005) demonstrated that the OLAND process was active under saline conditions up to 30 $g \cdot l^{-1}$, confirming that the anammox bacteria in this reactor also could adapt to high salt concentrations. In the present study, anammox activity in the SBRsalt was lost almost immediately when the salt concentration in the influent was increased to 45 $g \cdot l^{-1}$. In other words 30 $g \cdot l^{-1}$ was the limit to the anammox adaptation to salt also in this case.

The adaptation to salt did not have an adverse effect on maximum anaerobic ammonium oxidation activity. However, the rate maxima were achieved at higher salt concentrations, whereas biomass from SBRfresh had significantly reduced activity at 30 $g \cdot l^{-1}$. This illustrates that anammox biomass did not have an intrinsic resistance to salt, but rather adapted to the presence of salt.

As mentioned above 30 $g \cdot l^{-1}$ was the limit of anammox adaptation to salt. Yet, in batch incubations at 45 $g \cdot l^{-1}$ salt concentration, the salt adapted biomass was still active (90 % of

maximum activity). Thus, it is surprising that when 45 $g \cdot l^{-1}$ was introduced to SBRsalt; the anammox activity disappeared. Apparently the duration of the exposure to 45 $g \cdot l^{-1}$ was also important. The short (1-6 h) batch experiments did not reveal the toxic effect of 45 $g \cdot l^{-1}$ salt. Thus, short batch incubations are very useful to measure sludge activity under different conditions, but they provide only limited information about toxicity.

Two possible outcomes of the adaptation of a freshwater biomass to salinity would be (1) the acclimation of the existing population or (2) a population shift. The anammox seed sludge used in this study consisted of approximately equal amounts of marine (Scalindua) and fresh water (Kuenenia) anammox species. In SBRfresh, in the absence of salinity, 99% of the anammox population was *Candidatus* "K. stuttgartiensis" species. A probable outcome of an increase of salt concentration to 30 $g \cdot l^{-1}$ (marine salinity) in SBRsalt could have been a population shift towards the marine anammox species if salinity of the marine ecosystem was a major factor defining the niche of the Scalindua genus. However, FISH analysis showed that the presence of sea-level salinity (30 $g \cdot l^{-1}$) in SBRsalt did not give *Candidatus* "S. wagneri" a competitive advantage over *Candidatus* "K. stuttgartiensis". At the end of one year of reactor operation the marine anammox bacteria only constituted 30 % of the total anammox population. The similar maximum ammonium oxidation activity of both reactors showed that the freshwater species *Candidatus* "K. stuttgartiensis" contributed to the activity of the biomass and could adapt to salt concentrations as high as 30 $g \cdot l^{-1}$, and sustain anammox activity. Apparently the salt concentration was not the sole factor defining the niche of the Scalindua species. Nonetheless the presence of 30 % Scalindua species in SBRsalt indicated that salinity could be one of the factors contributing to the exclusive presence of this species in the marine environments.

In the light of the previous results and the present study it would be safe to conclude that the anammox process can be applied to high salinity wastewaters. More importantly, it is not necessary to seed such an anammox reactor with salt water anammox species. Sludge from a reactor working at low salinity could be used to seed another reactor designed to operate at high salt concentrations as long as salinity is introduced during an adaptation period.

chapter 5

intermediates of the anaerobic oxidation of ammonium

Abstract

There are several hypotheses on the metabolic pathway of the anammox bacteria, but unambiguous experimental validation of these proposed pathways is lacking. In this study, the intermediates of the anammox process have been determined with a complimentary array of different methods. Involvement of nitric oxide and hydrazine as intermediates in the anammox process has been established. *Kuenenia stuttgartiensis* cells stopped converting nitrite and ammonium upon reaction with a powerful nitric oxide scavenger (2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide, PTIO). Production of nitric oxide by active *Kuenenia stuttgartiensis* cells was visualized by diaminofluorescein-2-diacetate that becomes fluorescent upon reaction with nitric oxide. Tracer experiments with $^{15}NO_2^-$ and $^{15}NH_4^+$ showed that hydrazine is turned over by the anammox bacteria. Moreover, there was a transient hydrazine accumulation when *Kuenenia stuttgartiensis* cells were incubated with nitric oxide and ammonium. Involvement of a cytochrome bc1 complex was shown by the inhibition of *Kuenenia stuttgartiensis* cells with pentachlorophenol, a specific inhibitor of the bc1 complex. At this point, hydroxylamine could not been ruled out as the third possible anammox intermediate.

Introduction

In 1977, based on thermodynamic calculations, Broda predicted that anaerobic oxidation of ammonium could occur with nitrite or nitrate as the electron acceptor (Broda, 1977). The bacteria capable of anaerobic ammonium oxidation (anammox) at the expense of nitrite were first identified in 1999 following their discovery in a pilot wastewater treatment plant (Mulder, et al. 1995, Strous, et al. 1999a). Subsequent studies showed that the anammox reaction is unique to a monophyletic group of bacteria within the phylum Planctomycetes (Schmid, et al. 2000, Schmid, et al. 2003, Kartal, et al. 2007a). Screening of many different natural ecosystems revealed the ubiquitous presence of anammox bacteria in sub-oxic and anoxic environments, where the anammox bacteria were shown to be key players in the global biogeochemical nitrogen cycle (Kuypers, et al. 2003, Kuypers, et al. 2005, Hamersley, et al. 2007, Schmid, et al. 2007). According to the current estimates, anammox bacteria may account for at least 50% of the loss of fixed nitrogen globally (Brandes, et al 2007), thus representing a major source of atmospheric N_2.

The anammox bacteria are also present in man-made ecosystems such as wastewater treatment plants (Mulder, et al. 1995, van der Star, et al. 2007). The application of these bacteria to wastewater treatment is of great interest because the anammox process reduces CO_2 emissions compared to conventional ammonium removing wastewater treatment plants by 90% and is more cost-effective than conventional ammonium removal technology (van Dongen, et al. 2001).

Anammox bacteria contain a bacterial organelle, the anammoxosome, accounting for a major portion of the cell volume (Lindsay, et al. 2001). Analysis of the anammoxosome membranes showed that they contain unique ladderane lipids. These rigid molecules form a dense, relatively impermeable membrane (Sinninghe Damsté, et al. 2002). Immunogold labeling demonstrated that hydroxylamine oxidoreductase (HAO, Schalk, et al. 2000), one of the most highly expressed proteins of the anammox bacteria, was present exclusively inside the anammoxosome (Lindsay, et al. 2001). Based on these findings it was postulated that the anammoxosome was the locus of anammox catabolism and that a proton motive force was generated over its membrane (van Niftrik, et al. 2004).

The biochemical mechanism of anaerobic ammonium oxidation is still largely unknown. Based on labeling experiments, it was previously postulated that hydrazine (N_2H_4) and hydroxylamine (NH_2OH) were the intermediates of anaerobic ammonium oxidation; see Figure 1A (Van de Graaf, et al. 1997). Later, analysis of the genome of the anammox bacterium *Kuenenia stuttgartiensis* indicated that nitric oxide (NO) could also be an intermediate (Figure 1B, Strous, et al. 2006). The assembly of the genome enabled the postulation of a pathway in molecular detail. However, unambiguous experimental evidence

for this pathway or any of its possible intermediates was still missing. For example, the role of hydrazine in anaerobic ammonium oxidation was based on the observation that hydrazine was produced in the presence of hydroxylamine (Van de Graaf, et al. 1997). Direct evidence that hydrazine was actually turned over with the physiological substrates nitrite and ammonium was still lacking. In the present study we used a complementary array of methods with specific reagents, heavy isotopes, fluorescent labeling, and inhibitors to demonstrate that nitric oxide and hydrazine were indeed intermediates. So far no compelling evidence could be found to include or exclude hydroxylamine as another intermediate.

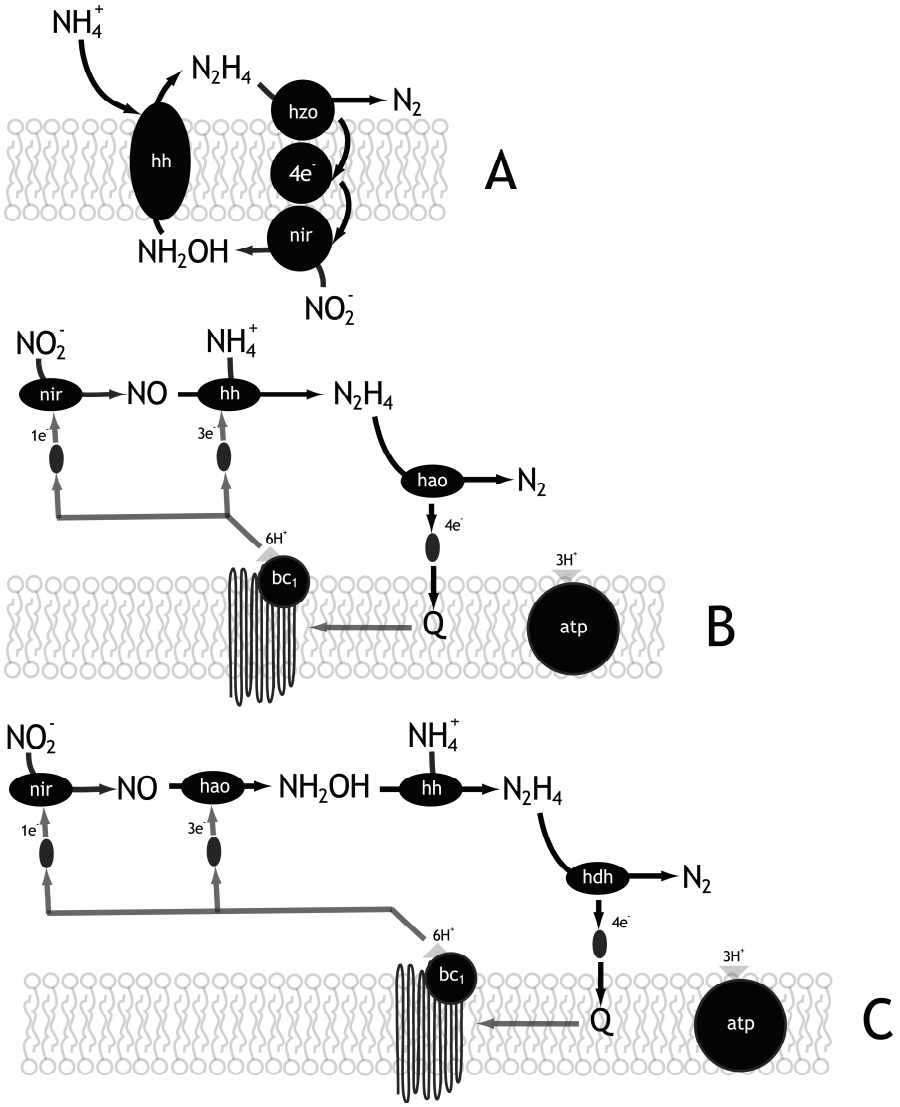

Figure 1 | Different hypotheses on the anammox catabolic pathway. 1A proposed in Jetten et al. 2003. 1B proposed in Strous et al. 2006. 1C proposed in this study.

Materials and Methods

Kuenenia stuttgartiensis cells were harvested from the effluent of a laboratory scale anammox enrichment culture, and were concentrated via centrifugation. The cells were resuspended to a protein concentration higher than 1 $mg \cdot l^{-1}$. A part of the cell suspension was diluted 100 times, chemically fixed, and hybridizations with fluorescently labeled oligonucleotide probes were performed as described previously (Schmid, et al. 2005).

The suspensions were transferred to 8 ml serum bottles. The vials were made anoxic by alternately applying under-pressure and helium or argon gas seven times. The vials were transferred to an anaerobic chamber with a 95/5 Ar/H_2 atmosphere. The argon in the anaerobic chamber was made oxygen-free by passing Ar over a Pd catalyst. In the anaerobic chamber, the cell suspensions were diluted 5 times with anaerobic mineral medium (Van de Graaf, et al. 1996) to a final volume of 40 or 8 ml and transferred to glass vials. All preparations (e.g. addition of

substrates, inhibitors) for different incubations were handled in the anaerobic chamber. All experiments were run in duplicate. All non labeled salts were purchased as molecular grade (more than 99.95% pure, Merck, Germany) unless stated otherwise. All labeled compounds were 99% pure and purchased as sodium or chloride salts (Cambridge Isotope Laboratories Inc., USA). All gaseous compounds were of the highest purity available.

For the detection of N_2H_4 turnover by anammox cells during anaerobic ammonium oxidation, 2 mM $^{15}NO_2^-$ and 2 mM NH_4^+ (or NO_2^- and $^{15}NH_4^+$) and 1 mM unlabelled N_2H_4 were added to the *Kuenenia stuttgartiensis* cell suspensions. The vials were kept dark and incubated in the dark for 60 min at 33°C and were shaken continuously at 300 rpm. Liquid samples were taken at 0, 30 and 60 min and centrifuged. The supernatant of each sample was transferred to a clean eppendorf cup and kept at 4°C until the end of the experiment. NO_2^- and NH_4^+ were determined as described previously (Kartal, et al. 2006). N_2H_4 was determined colorimetrically at 420 nm after reaction of 100 µl sample with 900µl 2% (w/v) paradimethylaminobenzaldehyde (PDB), 10% (v/v) ethanol in water. The isotopic composition of N_2H_4 was determined by matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectroscopy (MS) after reaction with PDB. For MALDI-TOF analysis 0.3 µl PDB-reacted N_2H_4 samples were directly mixed on a MALDI-TOF plate with an equal volume of sample buffer containing 20 mg/ml α-cyano-4-hydroxycinnamic acid in 0.05% (v/v) trifluoroacetic acid (TFA), 50% (v/v) acetonitrile. MALDI-TOF MS measurements were performed in the mass range of 100–1000 Da on a Bruker III mass spectrometer, set to reflectron mode.

For the detection of nitric oxide (NO) turnover by anammox during anaerobic ammonium oxidation, 2 mM NO_2^- and NH_4^+ were added to the *Kuenenia stuttgartiensis* cell suspensions in amber vials. In parallel, nitrite-depleted cell suspensions were incubated in the presence of NH_4^+. After a 5 min pre-incubation, diaminofluorescein-2-diacetate (DAF2-DA, Calbiochem) was added to a final concentration of 10 µM. The vials were incubated in the dark for 30 min at 33°C and were shaken continuously at 300 rpm. The cells were then harvested by centrifugation, and washed in mineral medium (see above) three times to remove the excess chromophore. The cells were resuspended in mineral medium. A liquid sample (5 µl) of the cell suspensions was pipetted on a microscope slide. The samples were dried in the dark. The preparations were examined with a Zeiss Axioplan2 epifluorescence microscope (Zeiss, Jena, Germany).

For the determination of the activity of *Kuenenia stuttgartiensis* with NO and NH_4^+, single cell suspensions were incubated with NO (1% of the headspace) and 2mM NH_4^+. Gas samples were analyzed in a chemoiluminescence NO_x analyzer. Liquid samples were taken every 30 min and analyzed for NH_4^+ and N_2H_4.

To determine the effect of a powerful NO scavenger (2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide, PTIO, Akaike & Maeda, 1996), on the anammox bacteria three incubations were run in parallel. To all the incubations 2 mM NO_2^- and NH_4^+ were added. In one of the incubations PTIO (100 µM) was added at 0 min, and to the other it was added at 45 min, no PTIO was added to the third incubation. Liquid samples were taken from the incubations at 0, 20, 45 and 60 min, and centrifuged. The supernatant of each sample was transferred to a clean eppendorf cup and kept at 4°C until the end of the experiment, and analyzed. NO_2^- and NH_4^+ were determined as described previously (Kartal, et al. 2006).

The effect of acetylene on the anammox bacteria was determined as follows: *Kuenenia stuttgartiensis* single cell suspensions were transferred to 40 ml glass vials. NO_2^- and NH_4^+ were added to the incubations to a final concentration of 2 mM. The vials were incubated at 33°C and were mixed with a magnetic stirrer at 500 rpm. The vials were continuously gassed with Ar/CO_2 (95/5) with a flow of 10 ml·min^{-1}. The gas effluent from the vials was connected to a chemoiluminescence NO_x analyzer for online measurement of NO. At 15 min, 100 µl acetylene was added to the vials. As a negative control 100 µl air and 100 µl nitrogen were added to separate incubations. Liquid samples were taken from the incubations at 0, 15, 30 and 60 min, and centrifuged. The supernatant of each sample was transferred to a clean eppendorf cup and kept at 4°C until the end of the experiment, and analyzed. NO_2^- and NH_4^+ were determined as described previously (Kartal, et al. 2006).

PTIO and acetylene were used to determine the role of NH_2OH in the anammox catabolism as follows: to *Kuenenia stuttgartiensis* single cell suspensions 1 mM NH_2OH and NH_4^+ were added. PTIO (1 mM) or acetylene (1 ml) was added in different experiments. Samples were taken every 30 min for 330 minutes, centrifuged and the supernatants were analyzed for NH_4^+, NH_2OH and N_2H_4.

To determine the role of cytochrome bc1 complex in the central anammox catabolism, *Kuenenia stuttgartiensis* cell suspensions were incubated with specific inhibitors of the bc1 complex. NO_2^- and NH_4^+ were added to the incubations to a final concentration of 2 mM. Pentachlorophenol was added to a final concentration of 10 µM. Liquid samples were taken, and NO_2^- and NH_4^+ were determined as described previously (Kartal, et al. 2006).

Results

The aim of this study was to determine the actual intermediates of the anammox catabolism *in situ*. We used *Kuenenia stuttgartiensis* single cells originating from a continuous laboratory enrichment culture. Fluorescence *in situ* hybridization analysis (FISH) showed that cells of *Kuenenia stuttgartiensis* made up >95% of the population in the cell suspensions prepared from this effluent.

Comparative genomics indicated that anammox catabolism starts with the reduction of nitrite to nitric oxide (Figure 1B). To address the possible role of nitric oxide in anaerobic ammonium oxidation, the following experiments were performed. First, the cells were incubated with ammonium and nitrite in the presence of diaminofluorescein-2-diacetate (DAF2-DA). The latter molecule reacts with nitric oxide to form a fluorescent product (Nagano, 1999, Guo, et al. 2003). When the anammox cells were subsequently inspected under an epifluorescence microscope they displayed the characteristic green fluorescence, indicative of the production of NO (Figure 2). When nitrite-depleted cells were incubated in the presence of ammonium with DAF-2DA, there was no detectable fluorescence, suggesting that nitrite is the precursor for NO production. When cells were incubated with ammonium, nitrite and the nitric oxide scavenger PTIO (2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide) they did not consume nitrite and ammonium (Figure 3). When PTIO was added to actively metabolizing cells incubated with nitrite and ammonium, anammox catabolism was immediately and completely inhibited (Figure 3). These experiments suggested that nitric oxide is an essential intermediate of anaerobic ammonium oxidation.

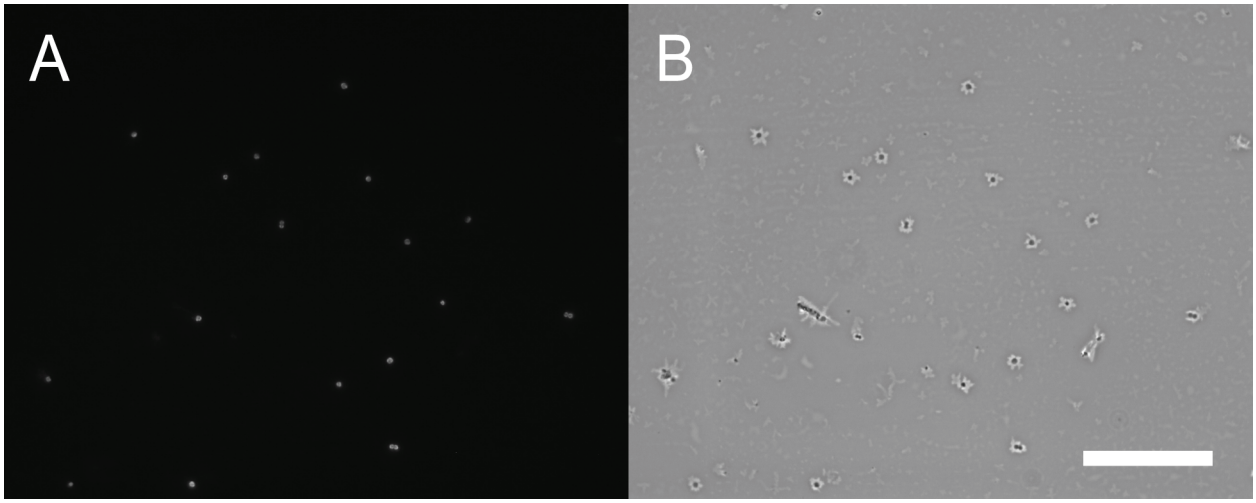

Figure 2 | Epifluorescence (A) and phase-contrast (B) micrographs of anammox cells after reaction with DAF2-DA.

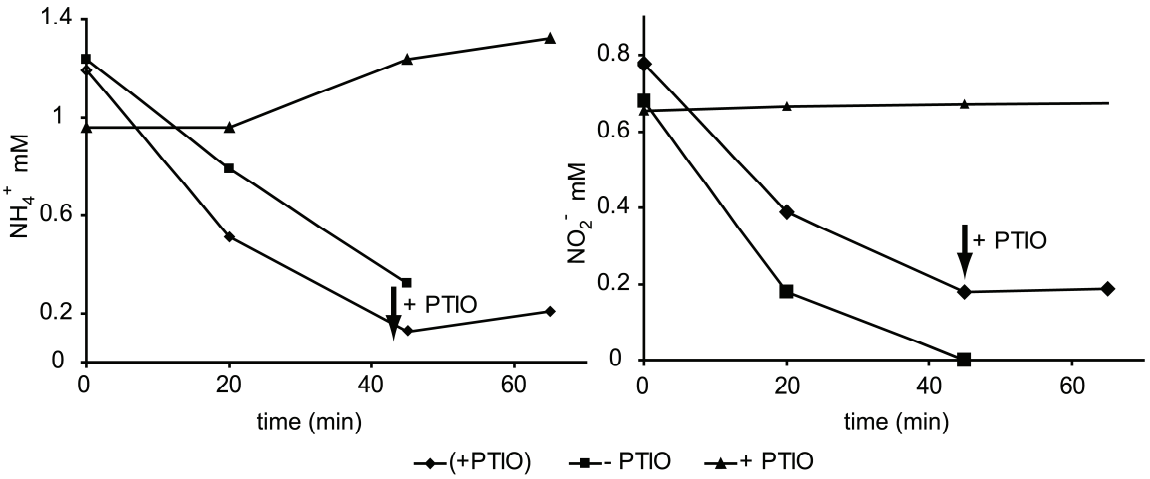

Figure 3 | Inhibition of the anammox bacteria by PTIO

The turnover of hydrazine, the postulated second intermediate in the anammox pathway was demonstrated by incubating *Kuenenia stuttgartiensis* cell suspensions with $^{15}NO_2^-$ and

$^{14}NH_4^+$ (or $^{14}NO_2^-$ and $^{15}NH_4^+$) in the presence of $^{14\text{-}14}N_2H_4$. If the cells would convert the substrates via hydrazine, $^{15\text{-}14}N_2H_4$ would be expected to appear. This non-labeled N_2H_4 was used as a "pool" to trap possible formed $^{15}N_2H_4$. The incorporation of the ^{15}N isotope of nitrite or ammonium into the hydrazine pool was indeed detected (Figure 4) after reaction of hydrazine with paradimethylaminobenzaldehyde followed by matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectroscopy.

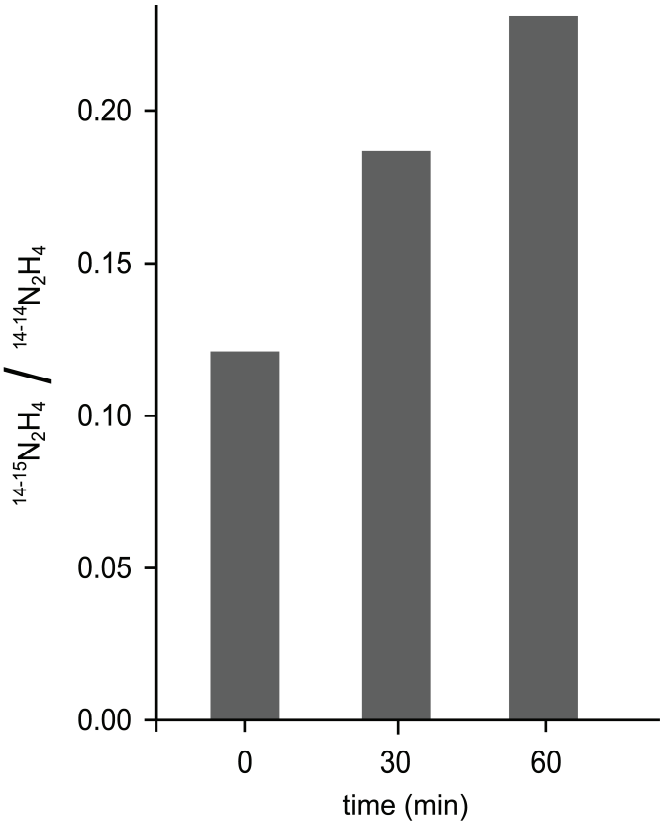

Figure 4 | Incorporation of the label from $^{15}NO_2^-$ to $^{15\text{-}14}N_2H_4$

According to figure 1B, nitric oxide reacts with ammonium, leading to the production of hydrazine. To confirm this hypothesis experimentally, *Kuenenia stuttgartiensis* cell suspensions were incubated with nitric oxide and ammonium: a transient accumulation of hydrazine was observed (Figure 5). Such hydrazine accumulation could be prevented by addition of acetylene, a known inhibitor of anaerobic ammonium oxidation (MSM Jetten, personal communication). When acetylene was added to actively metabolizing cells incubated with nitrite and ammonium, consumption of both substrates halted and nitric oxide accumulated. These experiments indicated that nitric oxide is a precursor of molecular hydrazine and that the pathway from nitric oxide to hydrazine in the anammox catabolism is inhibited by acetylene.

In order to determine the involvement of NH_2OH in the anammox catabolism, cells were incubated with NH_2OH and NH_4^+ in the presence of PTIO. When cell suspensions were incubated with or without PTIO there was always a transient N_2H_4 accumulation indicating that NO was not an intermediate in the conversion of NH_2OH and NH_4^+ to hydrazine. When the cells were incubated with NH_2OH and NH_4^+, in a separate experiment, in the presence of acetylene, NH_2OH was consumed, but there was no detectable N_2H_4 formed. This indicated that acetylene inhibited the N_2H_4 forming enzyme, the so called hydrazine hydrolase.

According to the current hypothesis cytochrome bc1 complex plays an integral role in the anammox catabolism. When *Kuenenia stuttgartiensis* cell suspensions spiked with 10 µM pentachlorophenol, a structural analogue of quinol, cells stopped converting NO_2^- and NH_4^+ immediately.

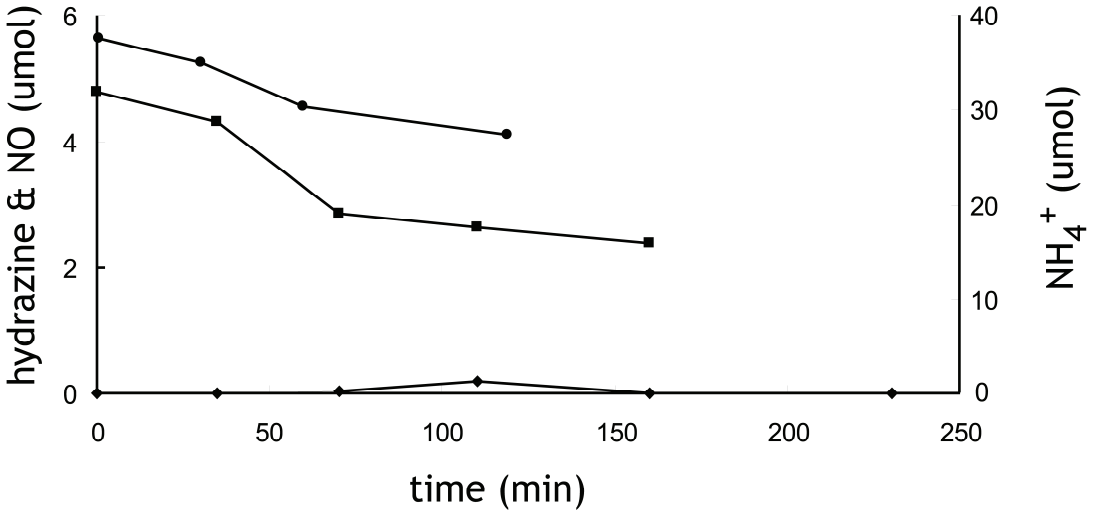

Figure 5 | Transient accumulation of N_2H_4 in the presence of NO and NH_4^+

Discussion

The experiments of this study showed that nitric oxide (NO) and hydrazine (N_2H_4) were intermediates of anaerobic ammonium oxidation (anammox) and that at present, a role for hydroxylamine cannot be excluded.

When cells were incubated with or spiked with 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO, a potent NO scavenger) all cell activity ceased. This showed that when NO was removed from the cells, the catabolic anammox cycle was interrupted. In the same experiment there was a slight increase in the ammonium concentration after the addition of PTIO. This might be explained by the dissociation of the intracellular N_2H_4 (and potentially NH_2OH) pools to ammonium. NO formation in the anammox cells was visualized by fluorescence microscopy. Actively metabolizing *Kuenenia stuttgartiensis* became fluorescent in the presence of diaminofluorescein-2-diacetate (DAF2-DA). The inhibition of anammox cells by an NO-scavenger and the detection of *in situ* detection of NO with a fluorescent dye suggested that NO is an intermediate of the anammox reaction. Experiments with acetylene addition to active *Kuenenia stuttgartiensis* cells revealed that there was an immediate but transient accumulation of NO. This suggested that the reduction of NO_2^- to NO, catalyzed by a cd1 nitrite reductase (NirS) was the first step of the anammox process.

Transient accumulation of N_2H_4 in the presence of NH_4^+ and NH_2OH was the first experimental indication that NH_2OH and N_2H_4 could be the intermediates of the anammox reaction (Van de Graaf, et al. 1997). However, these experiments could not exclude the possibility that NH_2OH was first converted to NO and then NO reacted with NH_4^+ to form N_2H_4 (Strous, et al. 2006). Indeed when *Kuenenia stuttgartiensis* cells were incubated with NO and NH_4^+ there was a transient accumulation of N_2H_4 (Figure 5). This indicated that NO could be a precursor of hydrazine. Because 1 µM NH_2OH has a severe inhibiting effect on the N_2H_4 oxidizing enzyme (Shimamura, et al. 2007), N_2H_4 accumulates up to 1 mM in NH_2OH and NH_4^+ incubations. In the NO and NH_4^+ incubations this NH_2OH inhibition most likely does not occur; thus the levels of hydrazine accumulation remain low (µM range). Moreover, reaction of NO with NH_4^+ needs 3 electrons, whereas the reaction of NH_2OH with NH_4^+ is independent of electrons. This means that with NO, 75% of the N_2H_4 can still be oxidized (to N_2 with 4 electrons) coupled to NO reduction, whereas with NH_2OH there are no electron acceptors available for N_2H_4 oxidation (apart from NH_2OH itself that can be reduced to ammonium, Schalk et al. 2000).

It is still possible that both NO and NH_2OH are intermediates of the anammox reaction. There are two plausible pathways for the evolution of N_2H_4 in the presence of NO: either NH_4^+ directly reacts with NO, or first NO is reduced to NH_2OH and then NH_2OH reacts with

NH_4^+. When *Kuenenia stuttgartiensis* cells were incubated with NH_2OH and NH_4^+ both in the presence and absence of PTIO N_2H_4 turnover was observed. This experiment clearly showed that the conversion of NH_2OH did not proceed via NO. At present it can not be ruled out that the conversion of NH_2OH with NH_4^+ to N_2H_4 is a side or detoxification reaction. If the reduction of NO to NH_2OH would occur under physiological conditions, presumably this reaction would be performed by the highly abundant HAO-like protein purified by Schalk et al. (2000) operating in reverse compared to the ammonium oxidizing bacteria (Hooper et al. 1979). In the anammox reaction, the HAO-like enzyme would catalyze the reduction of NO with three electrons to NH_2OH, while in aerobic ammonium oxidation HAO oxidizes NH_2OH to nitrite accepting four electrons. The recent discovery that anammox bacterium KSU-1 does contain at least 2 separate NH_2OH and N_2H_4 oxidizing proteins, makes it less likely that HAO is involved in hydrazine oxidation (Shimaura et al. 2007; Schalk et al. 2000). The future experiments will focus on the possibility of *in situ* detection of NH_2OH with ^{15}N labeled tracers.

All previous experiments on the detection of N_2H_4 were performed with NH_2OH and NH_4^+ as substrates instead of NH_4^+ and NO_2^-. It was likely that the production of N_2H_4 from NH_2OH was merely a side reaction. These experiments did not present any evidence for the turnover of N_2H_4 in the anammox catabolism. To determine the physiological turnover of N_2H_4 from NH_4^+ and NO_2^- we incubated with $^{15}NO_2^-$ or $^{15}NH_4^+$ in the presence of non-labeled N_2H_4. Part of the ^{15}N-label was recovered as $^{15\text{-}14}N_2H_4$, clearly showing that the anammox reaction proceeds through N_2H_4. This experiment showed that the previously observed molecular N_2H_4 was not a byproduct, but indeed was a real intermediate of anaerobic ammonium oxidation.

According to the current working hypothesis the 4 electrons released in the last step of the anammox reaction, the oxidation of N_2H_4 to N_2, are transferred via c type cytochromes and through low potential quinol to the cytochrome bc1 complex. These electrons in turn are used to initiate the next anammox cycle. In our experiments when we spiked *Kuenenia stuttgartiensis* cell suspensions with 10µM pentachlorophenol (PCP, a structural quinol analogue) all cell activity ceased. This strong inhibition of the anammox reaction by a specific bc1 complex inhibitor suggested that this part of the respiratory chain is involved in energy conservation from anammox and that its role in electron transport from hydrazine oxidation to nitrite reduction is not backed up by other systems.

The experiments of this study have falsified the postulated pathway of figure 1A and are consistent with both pathways figures 1B/C. Anammox catabolism starts with the one electron reduction of NO_2^- to NO by a cd1 type nitrite reductase; this is potentially followed by a three electron reduction of NO to NH_2OH, presumably by the HAO-like enzyme similar to the one described by Schalk et al, that was found to constitute over 10% of total the anammox proteins (Schalk, et al. 2000). This step could be followed by the condensation of NH_2OH and NH_4^+ to form hydrazine. Alternatively, NO and ammonia could be condensed directly to hydrazine by hydrazine hydrolase with three electrons as suggested by Strous et al. (2006). The electrons from the oxidation of hydrazine (presumably catalyzed by the recently purified hydrazine oxidizing enzyme, (Shimamura, et al. 2007) are shuttled to nitrite reductase and NO:NH_2OH oxidoreductase (or to HH that combines NO and NH_4^+) through Q cycle that involves a bc1 type cytochrome complex.

The unraveling of the intermediates of the anammox reaction most certainly will give more incentive to study the proteome and biochemistry of the anammox bacteria. Future research will concentrate on studying protein abundance and using protein purification to elucidate the enzymes that are responsible for the production of N_2H_4, the conversion of nitrite and nitrate and the transfer of electrons to the bc1 complex.

chapter 6

anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium

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Abstract

Anaerobic ammonium oxidizing (anammox) bacteria oxidize ammonium with nitrite and produce N_2. They reside in many natural ecosystems and contribute significantly to the cycling of marine nitrogen. Anammox bacteria generally live under ammonium limitation, and it was assumed that in nature anammox bacteria depend on other biochemical processes for ammonium. In this study we investigated the possibility of dissimilatory nitrate reduction to ammonium by anammox bacteria. Physically purified *Kuenenia stuttgartiensis* cells reduced $^{15}NO_3^-$ to $^{15}NH_4^+$ via $^{15}NO_2^-$ as the intermediate. This was followed by the anaerobic oxidation of the produced ammonium with nitrite. The overall end-product of this metabolism of the anammox bacteria was $^{15}N^{15}N$ dinitrogen gas. The nitrate reduction to nitrite proceeds at a rate of 0.3 ± 0.02 $f$mol·cell^{-1}·day^{-1} (10% of the "normal" anammox rate). A calcium-dependent cytochrome c protein with a high (305 µmol·min^{-1}·mg protein^{-1}) rate of nitrite reduction to ammonium was partially purified. We present evidence that dissimilatory nitrate reduction to ammonium occurs in Benguela upwelling system at the same site where anammox bacteria were previously detected. This indicates that anammox bacteria could be mediating dissimilatory nitrate reduction to ammonium in natural ecosystems.

Introduction

Anaerobic ammonium oxidizing (anammox) bacteria oxidize ammonium with nitrite under anoxic conditions. In the 1960s, ammonium deficits were already observed in anoxic basins, but this observation did not change the general belief that ammonium was essentially inert under anoxic conditions (Richards, 1965). Heterotrophic denitrification was thus considered to be the only anoxic sink for inorganic nitrogen. Soon after the discovery of the anammox process in a denitrifying pilot plant (Mulder, et al. 1995) and the identification of the responsible bacteria (Strous, et al. 1999a), the first direct evidence for the activity and presence of anammox bacteria in the marine environment was provided (Thamdrup & Dalsgaard, 2002, Kuypers, et al. 2003). Since then, the anammox bacteria have been detected in many natural ecosystems and are currently believed to contribute significantly to the loss of inorganic nitrogen in the oceans – next to denitrification (Trimmer, et al. 2003, Risgaard-Petersen, et al. 2004, Rysgaard, et al. 2004, Arrigo, 2005, Kuypers, et al. 2005, Meyer, et al. 2005).

The relative contributions of anammox and denitrification to marine N-losses were estimated via the incubation of sediments or water in sealed containers (Thamdrup & Dalsgaard, 2002, Trimmer, et al. 2003, Kuypers, et al. 2005). These containers were amended with excess ^{15}N-labelled nitrogen species (such as $^{15}NH_4^+$ and $^{15}NO_3^-$). Based on the stoichiometry of anammox and denitrification, the contributions of both processes could be derived from the recovery of the label in the produced N_2; where denitrification converts $^{15}NO_3^-$ or $^{15}NO_2^-$ to $^{15}N^{15}N$, anammox converts it exclusively to $^{14}N^{15}N$ (in the presence of unlabeled ammonium). In such experiments, the contribution of a third process, dissimilatory nitrate reduction to ammonium (DNRA) was assumed to be insignificant compared to denitrification and anammox (Risgaard-Petersen, et al. 2003). However, if DNRA would convert a significant amount of $^{15}NO_3^-$ to $^{15}NH_4^+$, this would lead to the production of $^{15}N^{15}N$ by anammox bacteria (Trimmer, et al. 2003). Thus the contribution of anammox bacteria to N-losses would be underestimated.

Recently, it was shown that anammox bacteria have a more versatile metabolism than previously assumed. They were shown to use organic acids as electron donors to reduce nitrate and nitrite and to out-compete heterotrophic denitrifiers for these compounds (Güven, et al. 2005, Strous, et al., 2006, Kartal, et al. 2007a). The end product of nitrate reduction by anammox bacteria is dinitrogen gas. Apparently, under certain conditions, anammox bacteria can also produce $^{15}N^{15}N$ from $^{15}NO_3^-$, even in the absence of dissimilatory nitrate reducers.

The biochemical pathway of this metabolism is yet unknown. In theory there are two possible pathways for the conversion of nitrate into N_2 by anammox bacteria. N_2 could be

produced either by 'classical' denitrification via nitrite, nitric oxide and nitrous oxide (Figure 1). Alternatively in dissimilatory nitrate reduction, nitrate could first be reduced to nitrite and subsequently to ammonium via a multiheme nitrite reductase as described for many enteric bacteria (Cole, 1996, Simon, 2002). The ammonium could then be combined with nitrite to form N_2 via the usual anammox pathway (Figure 1).

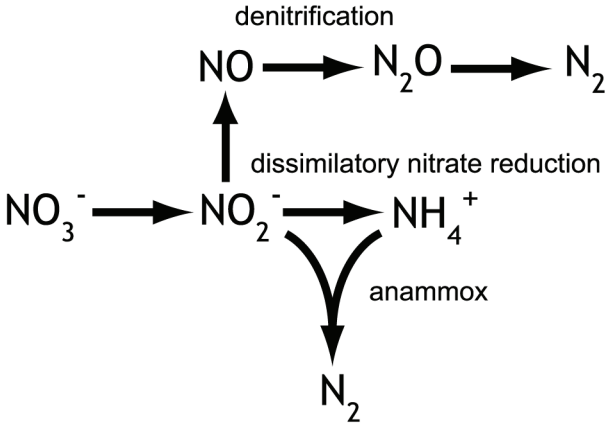

Figure 1 | Two possible routes of nitrate reduction by anammox bacteria

In the present study we show that $^{15}N^{15}N$ production by anammox bacteria is relevant under field conditions. The pathway of $^{15}N^{15}N$ production was resolved by isotope ratio mass spectrometry after trapping ^{15}N-labeled intermediates in pools of unlabeled intermediates. The key enzyme (a calcium-dependent multiheme nitrite reductase) was partially purified and candidate genes were identified in the genome of the anammox bacterium *Kuenenia stuttgartiensis* (Strous, et al. 2006). The environmental implications of the observed activity are discussed.

Materials and Methods

Physical Separation of *Kuenenia stuttgartiensis* Cells on Percoll Density Gradient

Percoll (6 ml, Pharmacia) and mineral medium (3 ml, van de Graaf, et al. 1996) were centrifuged in 10 ml glass tubes (15°C, 10.000 · g, 30 min) to pre-form a density gradient. *Kuenenia stuttgartiensis* cells were harvested from the effluent of a laboratory scale anammox enrichment culture, and were concentrated via centrifugation. The pellet was re-suspended in 1 ml mineral medium, and the mix was applied onto the Percoll gradient. The tube was centrifuged for another 30 min at 15°C (6000 · g). The target cells were recovered as a bright red band halfway the Percoll gradient. They were washed in mineral medium (see above) to remove the Percoll.

Fluorescence *in situ* Hybridization (FISH)

After the experiments, physically purified anammox cells were chemically fixed, and hybridizations with fluorescently labeled oligonucleotide probes were performed as described previously (Schmid, et al. 2005). All probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Thermo Electron Corporation (Ulm, Germany). The following probes were used to determine the purity of the Percoll separated *Kuenenia stuttgartiensis* cells as described in Schmid et al. 2005: Amx 368 (S-*-Amx-0368-a-A-18, specific for all known anammox genera, Schmid, et al. 2003), Kst 1273 (S-*-Kst-1273-a-A-20, specific for K. stuttgartiensis, three mismatches with the closest known relative, (Schmid, et al. , 2000)), EUB 338 (S-D-Bact-0338-a-A-18, (Amann, et al. 1990)), EUB 338 II (S-D-Bact-0338-b-A-18, (Daims, et al. 1999)), EUB 338 III (S-D-Bact-0338-c-A-18, together with EUB and EUBII specific for most bacteria, (Daims, et al. 1999)) (Schmid, et al. 2005). Cell suspensions were diluted 20 times, placed in a counting chamber (Bürker and Türk, 2.5×10^{-5} mm^3) and 20 fields were counted under the microscope per sample.

Activity Assays with Purified Cells

The purified cells ($6.6\pm0.4\times10^9$ cells/ml) were suspended in 2 ml of incubation buffer containing substrates ($^{15}NO_3^-$, ^{13}C-formate, and the corresponding pool). ^{15}N-nitrate (98%) and ^{13}C-formate (99%) were purchased as sodium salts (Cambridge Isotope Laboratories Inc., USA). Non labeled pools were used to trap possible intermediates formed during nitrate reduction. Pools of NH_4Cl (10mM and 2mM), $NaNO_2$ (2mM) and N_2O (2.5mM 99.998% pure) were added to separate incubations. All non labeled salts were purchased as molecular grade (more than 99.95% pure, Merck). Cell suspensions without electron donors were incubated as negative controls. All incubations were conducted in duplicate. The suspensions were transferred to 3 ml (Exetainer, Labco) vials or to 8 ml serum bottles. The vials were made anoxic by alternately applying under-pressure and helium or argon gas seven times. The vials were incubated at 33°C and were shaken continuously at

300 rpm for 6 or 9 h. The cell activity was stopped by adding 100 µl of saturated mercuric chloride solution to the vials. The incubations were followed in a time course; two vials were harvested at 0, 120, 240 and 360 minutes. $^{15}N^{14}N$:$^{14}N^{14}N$ and $^{15}N^{15}N$:$^{14}N^{14}N$ ratios were determined by gas chromatography isotope ratio mass spectrometry (VG Optima, Micromass, UK) (Kuypers, et al. , 2003, Kuypers, et al. , 2005). The VG Optima is designed for measurements of natural abundance isotopes at 0.2 ‰ ^{15}N changes. $^{15}NH_4^+$ was analyzed after chemical conversion of ammonium into N_2 with sodium hypobromite (NaOBr) (Risgaard-Petersen, et al. 1995).

^{15}N Incubations with the Benguela Upwelling System Water and Analysis

For the ^{15}N incubations we slightly modified the method previously described by Dalsgaard, et al. (2003). Briefly, 250 ml of Namibian shelf water (site M202) collected from 68m depth with the pump-CTD were flushed with helium for 15 minutes after addition of either 5 µmol $Na^{15}NO_3$, 2.5 µmol $^{15}NH_4Cl$, or 5 µmol $Na^{15}NO_3$ + 2.5 µmol $^{14}NH_4Cl$. The in situ concentrations of NO_3^- and NH_4^+ were 6 µM and about 1 µM respectively (Kuypers, et al. 2005). The ^{15}N incubations were started immediately after sampling. The water was transferred into 12-ml vials (Exetainer, Labco) and incubated for up to 48 hours at *in situ* temperatures. Samples were taken after 0, 12, 24 and 48 h by removing 1 ml water while replacing it with helium. Mercuric chloride was added to the samples to stop biological activity and the samples were stored at 4ºC until analysis. The ratios of different N_2 species were determined as described in the previous section.

Enzyme Assays and Partial Purification of a Calcium Dependent Nitrite Reductase

Kuenenia stuttgartiensis and *Candidatus* "Brocadia anammoxidans" cells were washed twice under anaerobic conditions with 50 mM anoxic Tris-HCl pH 7.8, 1 mM DTT and 1 mM $CaCl_2$ and suspended anoxically inside a glove box (95% N_2, 5% H_2) in 4 ml buffer and passed through a French pressure cell at 4°C. Unbroken cells and debris were removed by centrifugation in gas tight tubes at 40.000·g. The supernatant was used as cell extract and contained about 12 mg protein per ml. Nitrite and nitrate reductase assays were carried out in anaerobic quartz cuvettes as described elsewhere (Schouten, et al. 2004, Strous, et al. 2006). Including calcium in the extraction and assay buffer resulted in very high nitrite reductase activity (Einsle, et al. 1999). Calcium dependent nitrite reductase was partially purified from the membrane fraction of *B. anammoxidans*. Cells (100g) were washed in 20 mM HEPES pH 7.0 containing 2 mM $NaNO_2$ and 1 mM $CaCl_2$ (bufferA) and subsequently disrupted by 5 passes through a French pressure cell. Unbroken cells and debris were removed by centrifugation for 15 min at 12,000·g. The membrane fraction was obtained after centrifugation for 1.5h at 140,000·g followed by washing in buffer A. Proteins were solubilized from the membrane by 1% D-lauryl maltoside (1 h at 4°C) and applied to anion exchange chromatography (macro Q). Proteins were eluted using a NaCl gradient in buffer A containing 0.05% D-lauryl maltoside. Fractions showing high calcium dependent nitrite reductase activity were pooled, concentrated and applied onto a monoQ (HR 10/10) column. Proteins were again eluted using the same NaCl gradient, and concentrated using centriprep 10KDa filters. Pooled fractions were loaded on a superdex 200 HR10/30 column and eluted in buffer A containing 0.05% D-lauryl maltoside and 0.2 M KCl. Active fractions were stored at -80°C.

Substrate specificity of the fractions showing the highest calcium dependent nitrite reductase activity were tested with NO_2^-, NO_3^-, NO, NH_2OH, N_2O and N_2H_4. Inhibitor tests were performed with sodium azide (1mM), KCN (0.1-1.0 mM), and diethyldithiocarbamate (DDC, 1 mM). Product analysis was performed with reaction mixtures containing 10 mM Ti(III) citrate, 1.0 mM Methylviologen, 50 mM sodium phosphate buffer pH 7.0, and 1 mM substrates (NO_2^-, NH_2OH or NO). BLASTP and FASTA searches with various NrfA and NrfB proteins were conducted against the genome assembly of *Kuenenia stuttgartiensis* (Strous, et al. 2006).

Results

Single cell suspensions consisting of 80-90% of *Kuenenia stuttgartiensis* anammox bacteria were harvested from an anammox enrichment culture (Kartal, et al. 2006). The *K. stuttgartiensis* cells were physically separated from the other community members by percoll density gradient centrifugation. Fluorescence *in situ* hybridization (FISH) analysis of cell suspensions after percoll treatment showed that there were more than 500 cells of *K. stuttgartiensis* for each remaining contaminating cell (Figure 2).

These cell suspensions ($6.6\pm0.4\times10^9$ cells·ml^{-1}) were incubated with $^{15}NO_3^-$ as the electron acceptor and ^{13}C-formate as the electron donor. The main products of nitrate reduction with formate by anammox bacteria were $^{15}N^{15}N$ and $^{13}CO_2$ (Figure 3, leftmost bar). Interestingly, $^{15}N^{15}N$ only evolved after a lag phase of two hours (Figure 4). Initially, nitrite accumulated transiently and was the initial product of nitrate reduction (Figure 4). The rate of nitrate reduction to nitrite by the purified *K. stuttgartiensis* was 0.3 ± 0.02 *f*mol·cell^{-1}·day^{-1} (approximately 10% of the "normal" anaerobic ammonium oxidation activity of these cells).

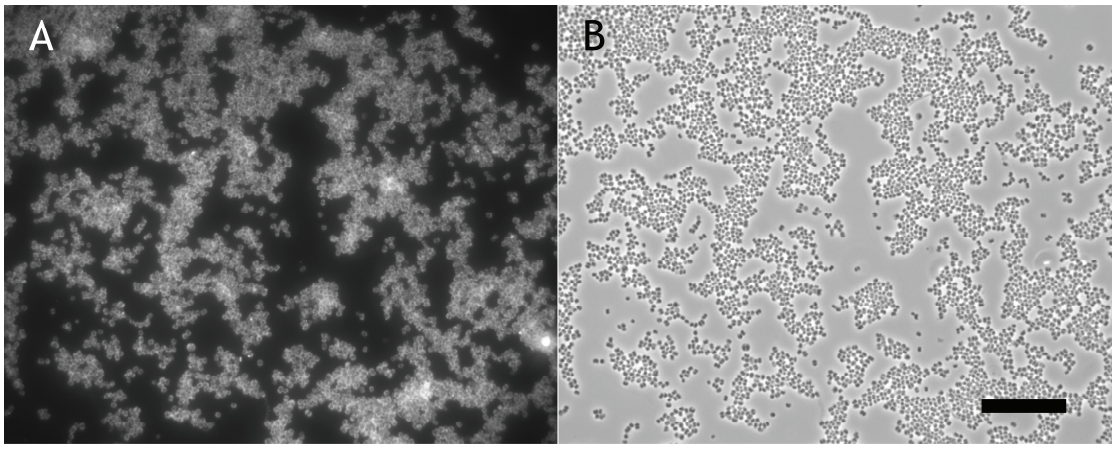

Figure 2 | Fluorescence *in situ* hybridization (A) and phase contrast micrographs (B) of the percoll purified *K. Stuttgartiensis* cells after incubations. Bar 20µm.

In separate incubations in the presence of one of the non-labeled compounds NO_2^-, NH_4^+ and N_2O the mechanism of nitrate reduction by *Kuenenia stuttgartiensis* was investigated. Cell suspensions of *K. stuttgartiensis* obtained by gradient centrifugation were incubated with formate and $^{15}NO_3^-$ (the only labeled compound) in the presence of one of the non-labeled compounds NO_2^-, NH_4^+ and N_2O (2 mM, 10 or 2 mM and 2.5 mM respectively). These non-labeled compounds were used as "pools" to trap possible ^{15}N-labeled intermediates. Figure 3 shows the recovery of the ^{15}N-label from the different pools in these experiments. In the presence of unlabeled nitrite, most of the nitrate label was recovered in the nitrite pool. In the presence of unlabeled ammonium, the label was partially (2-10 %) recovered as $^{15}NH_4^+$. Most of the label in this case was recovered as $^{14}N^{15}N$. In the presence of unlabeled nitrous oxide (N_2O) 0.26 % of ^{15}N was recovered in N_2O, and most of the ^{15}N-label accumulated as $^{15}NO_2^-$.

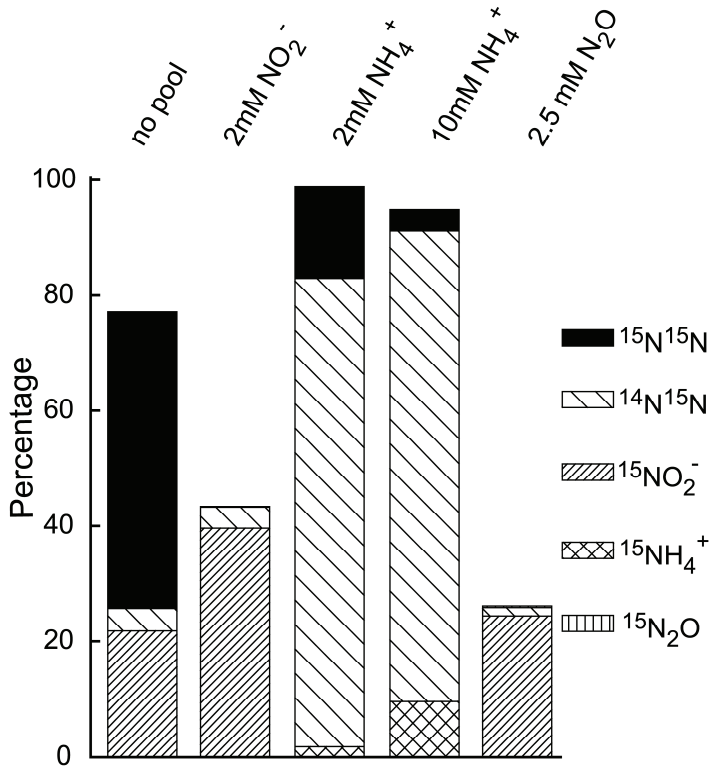

Figure 3 | Relative amounts of ^{15}N label recovered in different compounds after incubation. All incubations initially contained 2 mM (=100% on the y axis) $^{15}NO_3^-$ and 10 mM ^{13}C-Formate. The heights of the bars indicate the percentage of $^{15}NO_3^-$ converted in the incubations. Heights of the different parts of the bars represent percentage of concentrations of the specific compounds relative to the amount of converted $^{15}NO_3^-$.

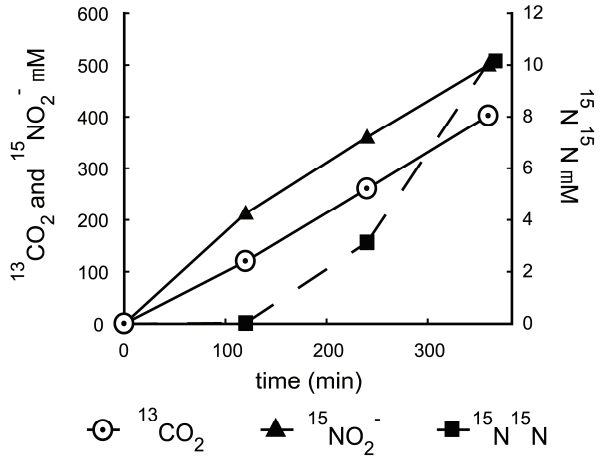

Figure 4 I Transient accumulation of $^{15}NO_2^-$ during incubation with $^{15}NO_3^-$ and ^{13}C-Formate. The end products $^{15}N^{15}N$ and $^{13}CO_2$ are also shown. The standard deviations of the data points are within the data labels, and therefore not shown.

Different $^{14}NH_4^+$ concentrations were used to investigate whether it was possible to prevent $^{15}N^{15}N$ production by anammox bacteria by supplying a high background of $^{14}NH_4^+$. Even in the presence of 10 mM $^{14}NH_4^+$ (a fivefold excess compared to $^{15}NO_3^-$), $^{15}N^{15}N$ production by anammox bacteria was detectable (2%). The amount of detectable $^{15}N^{15}N$ was four times higher (8%) in the presence of 2 mM $^{14}NH_4^+$ and $^{15}NO_3^-$ (equal amounts of ammonium and nitrate).

Strictly anaerobically prepared cell extracts of *Kuenenia stuttgartiensis* and *Candidatus* "Brocadia anammoxidans" were used to determine nitrate and nitrite reductase activities. The cell free extracts of both *Kuenenia stuttgartiensis* and *Candidatus* "Brocadia anammoxidans" contained nitrate reductase activities between 30-40 nmol·min^{-1}·mg protein^{-1}. When $CaCl_2$ and $NaNO_2$ (1 mM) was included in the extraction and assay buffers high (400-500 nmol·min^{-1}·mg protein^{-1}) nitrite reductase activity was observed. This calcium-dependent nitrite reductase could be partially purified from the membrane fraction. A 25 kDa multi heme protein with a very high (305 µmol·min^{-1}·mg protein^{-1}) rate of nitrite reduction to ammonium was recovered. The native molecular mass of the *K. stuttgartiensis* protein on superdex 200 was 42 kDa. This suggested a homodimeric enzyme. Sodium azide and the copper chelating agent diethyldithiocarbamate (DDC), known inhibitors for cd1 and Cu nitrite reductases respectively, did not inhibit the enzyme at 1 mM, while 1 mM KCN inhibited the enzyme by more than 95%. This indicated that the enzyme at hand was most likely a cc type nitrite reductase. The most active fractions converted NO_2^-, NO and NH_2OH at 305; 800 and 500 µmol·min^{-1}·mg protein^{-1} respectively and the end-product of the reaction was ammonium. Ca^{2+} could not be replaced by other divalent ions. EDTA also acted as an inhibitor, probably by chelating the Ca^{2+} ions that apparently stabilized the enzyme.

When the genome assembly of Kuenenia was analyzed for multiheme proteins, the most likely candidate gene which could code for this calcium dependent nitrite reductase was identified as CAJ71137, an unusual 25.2 kDa multiheme protein located in gene clusters of multiheme (CAJ71138; CAJ71139) and cytochrome b (CAJ71140) proteins (Figure 5).

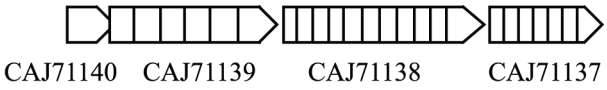

Figure 5 I Layout of a gene cluster in present *K. Stuttgartiensis* genome that may encode a cytochrome c nitrite reductase. Orf CAJ71140 encodes a hypothetical cytochrome b. The other orfs encode hypothetical multiheme cytochrome c proteins. Vertical bars indicate putative heme c binding motifs.

The possibility of nitrate reduction to ammonium in the environment was also investigated. The incubation of oxygen minimum zone water from the Benguela System (site M202; 68 m) after the addition of $^{15}NO_3^-$ and $^{14}NH_4^+$ resulted in the production of $^{14}N^{15}N$ as well as $^{15}NH_4^+$ (Figure 6).

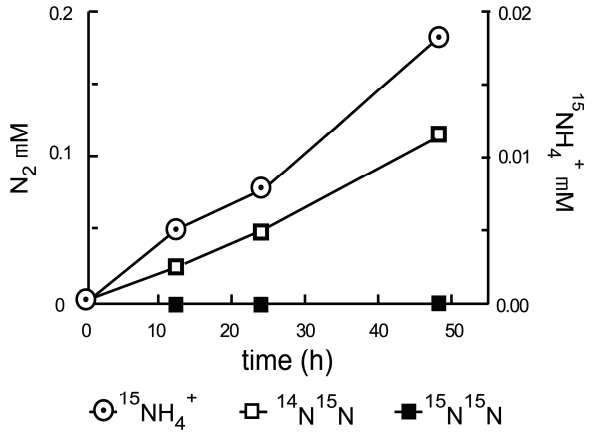

Figure 6 | Production of $^{15}NH_4^+$ from $^{15}NO_3^-$ in the sample from Benguela upwelling system at 68m at site M202. The standard deviations of the data points are within the data labels, and therefore not shown.

Discussion

Until recently the anammox bacteria were thought to be specialists, capable of only the anaerobic oxidation of ammonium. However, the unraveling of the *Kuenenia stuttgartiensis* genome combined with the latest experimental studies showed that anammox bacteria have a more versatile metabolism than assumed (Güven, et al. 2005, Strous, et al. 2006, Kartal, et al. 2007a).

In the present study it was shown that the anammox bacterium *Kuenenia stuttgartiensis* produced $^{15}N^{15}N$ from $^{15}NO_3^-$ while oxidizing an organic energy source - a metabolism at first sight strikingly similar to denitrification. Apart from $^{15}N^{15}N$ production, two other phenomena currently also considered indications of denitrification were also mimicked by *K. stuttgartiensis*, namely transient nitrite accumulation and nitrous oxide production (Codispoti, et al. 2001).

When an unlabeled pool of nitrite was applied, a substantial part of the converted $^{15}NO_3^-$ was recovered as $^{15}NO_2^-$, indicating that the metabolism proceeded via nitrite. When an unlabeled pool of ammonium was applied, most of the $^{15}NO_3^-$ was converted to $^{14}N^{15}N$. Moreover a significant part of the label was recovered as $^{15}NH_4^+$. Interestingly, anammox bacteria still converted nitrate all the way to ammonium even in the presence of external ammonium. Apparently, the anammox bacteria reduced nitrate to ammonium via dissimilatory nitrate and nitrite reduction, and subsequently converted nitrite and ammonium into dinitrogen gas by the usual anammox metabolism. Thus, although the final product is the same as for denitrification, $^{15}N^{15}N$, the pathway proceeded in a completely different way.

These results indicated that anammox bacteria do not reduce nitrate through conventional denitrification via N_2O. Only 0.26 % of ^{15}N label from nitrate was detected in the N_2O pool (Figure 3). A possible explanation for the production of N_2O could be NO detoxification. NO is one of the proposed intermediates of the anammox reaction (Strous, et al. 2006), and three genes possibly involved in NO detoxification were identified in the *K. stuttgartiensis* genome: flavorubredoxin *norVW* (CAJ73918; CAJ73688) and bacterial hemoglobin (CAJ72702). Homologues of these genes were shown to be involved in NO detoxification in other bacteria and the end product was also N_2O (Wu, et al. 2003). Furthermore, hydroxylamine oxidoreductase (of which more than eight paralogues are encoded in the *K. stuttgartiensis* genome) is another potential source of N_2O, well known for aerobic nitrifiers (Hooper & Terry, 1979). Aerobic ammonium oxidizing bacteria and denitrifiers are also known to convert NO to N_2O with norBC nitric oxide reductases (Zumft, 2005). Currently, in the oxic environments nitrification and in anoxic environments denitrification are assumed as the only sources of N_2O production. However, NO detoxification by anammox or other bacteria in the anoxic zones could be another potential source for N_2O production.

Enzyme assays, partial purification, inhibition studies and in silico analysis of the K. stuttgartiensis genome together suggested that anammox bacteria could employ a calcium-dependent multiheme nitrite reductase for dissimilatory nitrite reduction to ammonium

similar to many enteric bacteria (Cole, 1996, Simon, 2002). A candidate gene (CAJ71137) encoding for this multiheme nitrite reductase was located in a gene cluster containing three multiheme proteins (penta- deca-, hexa-) preceded by a membrane-anchored cytochrome b. A similar gene organization is known for enteric bacteria which employ a decaheme NrfB protein to transfer electrons to homodimeric pentaheme NrfA protein. In future studies, further purification and N-terminal sequencing of the 25 kDa protein will be used to finally link the nitrite reduction activity of anammox bacteria to the candidate gene (CAJ71137).

Nitrogen gas production by anammox bacteria through nitrate reduction proceeds via two reactions: dissimilatory nitrate reduction to ammonium followed by anaerobic oxidation of ammonium. The dissimilatory nitrate reduction to ammonium also proceeds in two steps; first nitrate is reduced to nitrite, than nitrite is reduced to ammonium. In our experiments nitrate reduction to nitrite started immediately, however, nitrogen gas evolved only after a lag phase of 120 minutes. Also the accumulation of nitrite only occurred in the absence of ammonium. Evidently, nitrite reduction to ammonia was the rate-limiting step in the $^{15}N^{15}N$ production by anammox bacteria. Apparently, this does not compromise the fitness of these relatively slow growing bacteria because the habitat as a whole is limited by the supply of organic electron donors and not by the capacity for nitrate reduction. Therefore, the oxidation of organic compounds coupled to nitrate reduction may contribute significantly to the success of the anammox bacteria in the marine environment.

Recently anammox bacteria were shown to be present and active in the oxygen minimum zone of the Benguela upwelling system (Kuypers et al. 2005). There, at site M202 (68 m) the highest number of anammox cells (22000 cells·ml^{-1}) and the highest concentration of anammox specific ladderane lipids were detected (Kuypers et al. 2005). In the present study when we incubated samples from the same site and same depth in the presence of non-labeled ammonium and $^{15}NO_3^-$, most of the converted $^{15}NO_3^-$ ended up in $^{14}N^{15}N$ and 10% of the amended $^{15}NO_3^-$ ended up in $^{15}NH_4^+$. The production of $^{14}N^{15}N$ and the apparent lack of $^{15}N^{15}N$ production showed that the anammox bacteria were the sole contributors to nitrogen gas production in this sample. Moreover the conversion of $^{15}NO_3^-$ to $^{15}NH_4^+$ indicates that dissimilatory nitrate reduction to ammonium also occurred in the same sample. Apparently dissimilatory nitrate reduction to ammonium was a significant supply of ammonium for the anammox bacteria at this site; thus, they did not depend only on ammonium produced via mineralization and/or upward diffusion from the sulfidic zone. Finally, our results indicated that dissimilatory nitrate reduction to ammonium (DNRA) occurs in the oxygen minimum zones (OMZs) of the oceans; still more research is needed to identify the bacteria that mediate this process in nature.

Anammox bacteria have been shown to contribute to the loss of fixed nitrogen in many other natural suboxic and anoxic environments (Kuypers et al. 2003; Risgaard-Petersen et al. 2004; Rysgaard et al. 2004; Kuypers et al., 2005; Meyer et al., 2005). Previously, it was assumed that in such environments anammox bacteria were dependent on other bacteria for the production of nitrite and ammonium (Kuypers, et al. 2006) and they did not join the competition for nitrate. Recent results show that anammox bacteria do use nitrate directly and even out-compete heterotrophs for organic acids in the presence of ammonium (Kartal et al., 2007a). Generally, nitrate concentrations are substantially higher than nitrite concentrations in marine settings (Jaffe, 2000). Apparently the reduction of nitrate to nitrite is the limiting step in the overall reduction of nitrate to N_2. This limitation is not caused by the bacteria themselves, because in marine sediments there is a high capacity for nitrate reduction to nitrite by many bacteria (Knowles, 1982). Apparently, all those microbes compete for limiting amounts of electron donor. Our results showed that anammox bacteria were capable of converting nitrate via nitrite to ammonium. Hence, under ammonium-limited conditions anammox bacteria would be capable of producing the ammonium they require from nitrate. Consequently organic acid oxidation coupled to nitrate reduction by anammox bacteria could be a valuable survival strategy for anammox bacteria because they would no

longer be dependent on other microorganisms for the production of the nitrite and ammonium.

chapter 7

integration and outlook

The experiments described in this thesis addressed several aspects of the niche differentiation and eco-physiology of the anaerobic ammonium oxidizing (anammox) bacteria. In chapters 2-4 some conditions or compounds that favor one anammox species over others were investigated. In chapters 5 and 6 the intermediates and possible pathways in anammox bacteria were studied. On the basis of the results of these chapters several general conclusions and research directions with regard to inoculum, organic acid additions, salt tolerance, and application of anammox bacteria can be made and discussed.

Source of Anammox Bacteria

It is intriguing that the activated sludge inoculum (secondary stage sludge of the Rotterdam WWTP) used for the enrichment cultures described in the second and third chapters of this thesis, and several previous studies contained all three described freshwater anammox species (van Dongen, et al. 2001, Schmidt, et al. 2002; Güven, et al. 2004, Strous, et al. 2006). The amount of anammox cells in the secondary stage sludge is generally below the FISH detection limit of 0.1% of visible cells. Nevertheless, when the proper conditions were applied (anoxia, presence of ammonium, nitrite and bicarbonate, biomass retention, and suitable organic carbon source) anammox enrichment cultures developed within 3-6 months and one anammox species became dominant. Factors that might influence the selection for a certain species are discussed below.

Oxidation of Organic Acids

When no organic acid is provided in anammox enrichments on synthetic medium described by van de Graaf et al. (1996), generally either a *Brocadia* or *Kuenenia* dominated anammox culture is obtained. Results of previous research (Güven, et al. 2005) and the results of this thesis pointed out that the ability to oxidize organic acids is a common trait of all anammox bacteria. However, as chapters two and three indicated, the rates for the oxidation of organic acids were different for the different anammox species. It was shown in these chapters, that organic acid oxidation is most likely the key for understanding the niche of at least two different anammox species. *Candidatus* "Anammoxoglobus propionicus" seems well equipped to oxidize propionate effectively and out-compete all other bacteria for propionate in the presence of ammonium, while *Candidatus* "Brocadia fulgida"became the dominant species if acetate was included in the medium. It may be argued that the *Brocadia* and *Anammoxoglobus* lineages form one "guild" in the anammox tree of life, whereas *Kuenenia* and *Scalindua* lineages may each represent separate guilds. It is possible that the organisms in the *Brocadia/Anammoxoglobus* branch are better equipped for oxidizing small organic compounds. At present, the affinities of these species to small organic acids are unknown. Results of chapter two showed that *Candidatus* "Anammoxoglobus propionicus" completely outcompeted *Candidatus* "Brocadia anammoxidans" in only four months (10-20 generations). The most likely explanation for such a rapid take over suggests that *Candidatus* "Anammoxoglobus propionicus" apparently has a better affinity to nitrite, the limiting substrate, than *Candidatus* "Brocadia anammoxidans" in the presence of propionate. A comparative analysis of the affinity of different anammox species to different small organic compounds could also reveal why one species has competitive advantage over the other.

One question arising from the results of chapters two and three do not cover is the determination of actual increases of yield of the anammox bacteria due to the oxidation of organic acids. Calculations, based on the Gibbs free energy release of the oxidation of acetate and propionate, suggest that there should be a 50% increase in the yield. However, it was not possible to observe such an increase in yield of anammox aggregates. This point remains to be resolved in future studies on the anammox process by using continuous reactor systems containing suspended biomass. A probable approach would be a continuous culture experiment with ^{13}C-bicarbonate in the presence and absence of organic acids. Based on the increase in the incorporation of ^{13}C-bicarbonate to the anammox biomass in the

presence of an organic acid, the increase in the yield of the anammox bacteria due to organic acid oxidation could be calculated.
Unexpectedly, analysis of the anammox lipids revealed that the anammox bacteria incorporated neither propionate nor acetate directly to their biomass. The degree of natural fractionation in the anammox lipids suggested that the anammox bacteria degraded these compounds to the level of CO_2, and then fixed carbon through the acetyl CoA-pathway. It would be an interesting topic of future research to investigate why the anammox bacteria do not incorporate organic acids directly to their biomass, but rather degrade them to CO_2. Unconditional burning of the organic compounds to conserve energy might make ecological sense in the electron donor limited natural ecosystems where the anammox bacteria usually reside.

High Salinity

In a previous study on the activated sludge inoculum (Rotating Biological Contactor (RBC), Pitsea UK) of the anammox enrichment culture described in chapter four, it was reported that 20% consisted of *Candidatus* "Scalindua sp." and that there were no other detectable anammox species present (Schmid, et al. 2003). However, after several months of further enrichment using the medium described by van de Graaf (1996), *Kuenenia stuttgartiensis* accounted for 50% of the anammox population; the remaining 50% was *Candidatus* "Scalindua wagneri". This provided the perfect mixture to study the effect of salinity on anammox bacteria, because the genus *Scalindua* is regarded as a "marine" species, while the genus *Kuenenia* represents a "freshwater" anammox species. However, after 10 months of cultivation at 3% salt (90% NaCl/10% KCl, 25°C), *Kuenenia stuttgartiensis* became the dominant species in the enrichment culture. The results of this study showed that salinity was not the key factor for defining the niche of *Candidatus* "Scalindua wagneri", and that a "freshwater" anammox species could adapt to and survive at high salinity. In many studies, in different marine settings, *Candidatus* "Scalindua sp." are the only anammox bacteria detected (Schmid, et al. 2007), indicating that the marine environment (3% salt, low temperatures, low substrate concentrations) might determine the niche of the *Scalindua* lineage. However, it is still not clear which compound or conditions or combination might be the key factor in selecting for the *Scalindua* genus in the marine environment. Of course, at this point it cannot yet be ruled out that there are other anammox species waiting to be discovered in the marine ecosystems. But the cell numbers determined by FISH and rate measurements with ^{15}N isotopes do not leave much room for undetected anammox diversity (Schmid, et al. 2007).
One final interesting point of the salt study was the difference of the effect of salinity in batch and continuous cultures. In batch cultures the anammox bacteria retain their activity until 90 g/l salt, but in the continuous culture experiments the anammox activity was lost at 45 g/l. This suggested that the time of exposure to the stress factor is quite important and the results from batch incunbations should be interpreted carefully.

Implications for the Application of the Anammox Process

At present, the anammox process is designed for removal of only ammonium and nitrite from wastewaters (van der Star, et al. 2007). Chapters two and three of this thesis point out that the anammox bacteria are not specialists; they can use nitrate (an end product of anammox metabolism) as an electron acceptor and small organic compounds as electron donors. This concept could be used as a polishing step to decrease the nitrate concentrations in the anammox effluent. Clearly, the C/N ratio is a key issue in the successful application of the anammox bacteria to nitrogen and carbon containing wastewaters. It was shown previously that the anammox bacteria cannot compete with denitrifiers when the C/N ratio is higher than 1 (Güven, et al. 2004). It is probable that by adjusting the C/N ratio the anammox process could also be applied to wastewaters containing nitrate and organic acids.

In a recent study, it was also shown that the biomass of a RBC comprised of aerobic and anaerobic ammonium oxidizing bacteria could also adapt to 3% salt (Windey, et al. 2005). Another study on an aerobic ammonium oxidizing reactor showed that aerobic ammonium oxidizing bacteria were not affected by salt concentrations up to 4% (Moussa, et al. 2006). Along with chapter four these two studies show that the anammox process or OLAND/CANON type processes could be applied to high salinity wastewaters.
The study described in chapter four indicated that different types of wastewater may not need different types of anammox species. In that study, a freshwater anammox species could grow under high salinity conditions. If this is the case for other factors, it might be possible that anammox reactors treating different wastewaters could be started with the same seed biomass. The anammox species that favors the new settings might dominate the reactor eventually.
Recent studies showed that the aerobic ammonium oxidation was not unique to bacteria (Könneke, et al. 2005). Crenarchaea present in wastewater treatment plants (Park, et al. 2006) or aquacultures could also oxidize ammonium with oxygen (Könneke, et al. 2005). The aerobic ammonium oxidizing bacteria are the partners of ammonium oxidizers in the Completely Autotrophic Nitrogen removal Over Nitrite (CANON) systems (Sliekers, et al. 2002). It is already known that the ammonium oxidizing crenarchaea supply nitrite for the anammox bacteria in the natural environments (Lam, et al. 2007), and anammox bacteria and ammonium oxidizing archaea (AOA) live in close association in marine snow (Woebken, et al. 2007). It can be hypothesized that such cooperation could also be implemented to a new CANON type system based on the co-operation of the AOA and anammox bacteria. This of course depends on a better understanding of the niche differentiation of nitrifying crenarchaea relative to nitrifying bacteria.

Intermediates

In chapters five and six intermediates of the two catabolic reactions mediated by the anammox bacteria are described.
In chapter five data that support two alternative explanations for the catabolic pathway of the anammox bacteria are presented. The major difference between the two possibilities is that hydroxylamine is regarded as an intermediate in one and not in the other. In other words either hydroxylamine (eq. 1) or nitric oxide (eq. 2) is the direct precursor of hydrazine. When *Kuenenia stuttgartiensis* cells were incubated with nitric oxide and ammonium or hydroxylamine and ammonium, there was a transient hydrazine accumulation. This accumulation was much higher in the incubations with hydroxylamine.
As it can be seen in equation 1, the reaction of hydroxylamine with ammonium to hydrazine is electron neutral, whereas the same reaction with nitric oxide (eq. 2) requires three electrons.

$$NH_4^+ + NH_2OH \xleftrightarrow{HH} N_2H_4 + H_2O + H^+ \qquad 0\ V \qquad \text{equation 1}$$

$$NH_4^+ + NO + 2H^+ + 3e^- \xleftrightarrow{HH} N_2H_4 + H_2O \qquad +\ 0.06\ V \qquad \text{equation 2}$$

This means the 75% of the hydrazine has to be oxidized to N_2 (eq. 3) coupled to the reduction of nitric oxide (eq. 2). This could explain why less hydrazine accumulates in the incubations with nitric oxide.

$$N_2H_4 \xleftrightarrow{HDH} N_2 + 4H^+ + 4e^- \qquad -\ 0.75\ V \qquad \text{equation 3}$$

The pathway with only nitric oxide as the only intermediate does not explain the abundant presence of hydroxylamineoxidoreductase-like (HAO) protein purified by Schalk et al. (2000). If the scenario with both nitric oxide and hydroxylamine as intermediates was to be true, the role of the HAO could be the conversion nitric oxide to hydroxylamine (eq. 4).

$$NO + 3H^+ + 3e^- \xleftrightarrow{HAO} NH_2OH \qquad +0.03V \qquad \text{equation 4}$$

This matter may be resolved by *in situ* detection of hydroxylamine during anaerobic ammonium oxidation, following the approach that was successful for the *in situ* detection of hydrazine turnover described in chapter five. Ultimately purification of the protein responsible for the production of hydrazine is necessary to resolve the kinetics of this reaction.

An interesting point of the anammox reaction is the involvement of a cytochrome bc1 complex. If the proposed anammox pathway is correct, the cytochrome bc1 complex would be connected to hydrazine:ubiquinone oxidoreductase, an enzyme unique in nature. It would be very interesting to characterize this enzyme in the future, since the *Kuenenia stuttgartiensis* genome contains at least 3 copies for bc1 complex in close association to octaheme HAO-like proteins (Strous, et al. 2006).

In chapter six, intermediates of nitrate reduction by anammox bacteria are described. This metabolism of the anammox bacteria proceeds via the production of the anammox substrates, nitrite and ammonium, from the reduction of nitrate with an organic electron donor. Nitrate reduction to ammonium by anammox bacteria has very interesting implications for the survival of anammox bacteria in nature. They could utilize nitrate and organic acids under ammonium limitations, which is the usual case in sub-oxic zones of the marine ecosystems. Although the experiments in chapter 6 were performed with the anammox bacterium *Kuenenia stuttgartiensis*, one may generalize the outcome to other anammox bacteria. Based on the fact that organic acid oxidation coupled to nitrate (nitrite) reduction is a common trait among the described anammox species (see chapters 2 and 3), we may expect that the marine species *Candidatus* "Scalindua sp." can also reduce nitrate all the way to ammonium. A recent study showed that the crenarchaeal and bacterial ammonium oxidizing bacteria supplied nitrite to the anammox bacteria in the Black Sea in about equal amounts (Lam, et al. 2007). It would be interesting to investigate if this is the case for all natural anoxic systems or whether different environmental conditions require different solutions to the problem of nitrite supply for anammox bacteria.

In isotope tracer studies for the estimation of the relative contributions of denitrification and anammox to the total N_2 production, amounts of $^{15}N^{15}N$ and $^{45}N^{15}N$ were directly linked to the contributions of denitrification and anammox, respectively. However, in chapter 6, it is shown that anammox bacteria also produce $^{15}N^{15}N$ from $^{15}NO_3^-$. In future quantification experiments with isotope tracers, $^{15}N^{15}N$ can no longer be directly linked to denitrification only. In previous ^{15}N tracer experiments, the contributions of anammox to global loss of fixed nitrogen could have been underestimated.

Another point of interest that arises from chapters five and six is the regulatory mechanisms of the anammox bacteria. In chapter five it is stated that nitrite is used both as an electron acceptor in the anammox catabolism and as an electron donor for the carbon fixation pathway. There must be a regulatory system in the anammox bacteria to resolve this dilemma, and divert some of the nitrite for oxidation to nitrate and the remaining part to reduction to nitric oxide.

In chapter six, the results clearly show that the anammox bacteria keep on producing ammonium when there is an excess of ammonium in the system. Common sense would suggest that the anammox bacteria should somehow shut down the ammonium producing nitrite reductase (NrfA type), and divert nitrite to nitric oxide reductase (NirS type) and first use the available ammonium. However, it seems that such regulatory system does not operate in anammox bacteria. Still, one could argue that evolution would not force such a

regulatory system because anammox bacteria never see these compounds (nitrate, ammonium, organic acids) in such abundance in their natural ecosystems. In the fifth chapter it is shown that the anammox bacteria still synthesize hydrazine when there is more then enough supplied in the medium. Future studies on anammox biochemistry are needed to understand the the regulatory systems of the anammox bacteria.

One other interesting aspect of the anammox metabolism is the assumed cell density dependency of anammox bacteria. The idea was that anammox cells would stop activity below a certain cell density. This was explained by the assumption that the anammox cells would leak hydrazine, and this would disable anammox bacteria to spark the next catabolic cycle (Strous, 2000). Recent studies showed that anammox bacteria reside in natural ecosystems as single cells and as small clusters and that they are at least as active as anammox bacteria in laboratory scale bioreactors (Schmid, et al. 2007). This suggests that anammox bacteria most likely do not leak hydrazine to the environment at such levels to completely halt their metabolism. It is possible that the anammox bacteria form micro niches in the natural environment, maybe even with 3-5 cells, and this is enough to maintain a local hydrazine concentration. Of course it is also possible that the anammox bacteria are dependent on another molecule, maybe one that they excrete, as a quorum sensing agent.

A topic for future research could be the study of protein translocation in an anammox cell. There are two membranes in the anammox cell where a protein could be sent to: the anammoxosome membrane, and the intracytoplasmic membrane. It is intriguing how the anammox bacteria could regulate the correct protein targeting. It is likely that in the future, this mechanism could be resolved by proteomics and bioinformatics.

It has been more than a decade since the discovery of the anammox bacteria, and it is now clear that they were not the last mystery of the nitrogen cycle. Recently, nitrifying crenarchaea, nitrite dependent methane oxidation, denitrifying foraminifera and nitrite oxidizing phototrophs were identified (Könneke, et al. 2005, Raghoebarsing, et al. 2006, Risgaard-Petersen, et al. 2006, Griffin, et al. 2007). Still, the anammox process holds many secrets yet to be resolved. It seems that the topic most open to discoveries is the fundamentals of the anammox biochemistry. Many surprises are bound to come out of studies aiming to unravel the mysteries of the anammox metabolic pathways.

samenvatting

özet

summary

Lange tijd werd gedacht, dat anaërobe ammonium oxidatie (anammox) door levende organismen niet mogelijk was. Ruim een decennium geleden bleek dat het wel mogelijk was, waarmee het proces één van de meest recente ontdekkingen is in de stikstofcyclus. In de anammox reactie wordt ammonium (NH_4^+) door bacteriën geoxideerd naar stikstofgas (N_2), waarbij nitriet (NO_2^-) wordt gebruikt als elektronenacceptor. Binnen acht jaar na hun ontdekking bleek dat anammox bacteriën zelfs een sleutelrol vervullen binnen de stikstofcyclus op aarde. Bovendien bleek anammox een relatief goedkoop en milieuvriendelijk alternatief voor de traditionele methode om ammonium uit afvalwater te verwijderen.
Het was al snel bekend dat de anammox reactie door meerdere soorten bacteriën gedaan kon worden, en dat sommige soorten wel met elkaar in hetzelfde milieu voorkwamen, maar dat in sommige omgevingen toch vooral één soort domineerde. Een van de vragen voor dit proefschrift was: welke omstandigheden of stoffen (zoals organische zuren) bepalen welke soort anammox bacteriën daar groeit? Bovendien waren de tussenproducten van het anammox metabolisme nog niet bekend, en die kennis is noodzakelijk om het anammox proces beter toepasbaar te maken voor afvalwaterzuivering. Ook wilden we een beter begrip krijgen van de microbiële ecologie van deze bacteriën.

Havasız amonyum yükseltgenmesi (HAY) doğadaki biyolojik azot döngüsünün en son keşfedilen proseslerinden biridir. Havasız amonyum oksidasyonunu yapan bakteriler amonyumu nitrit ile yükseltgeyerek azot gazi açığa çıkarırlar. Bu tür bakterilerin ilk kez bulunmasından sonraki sekiz yıl içinde yapılan araştırmalar ile bu bakteri grubunun azot döngüsünün en önemli üyelerinden olduğu ortaya çıkarılmıştır. Ayrıca bu bakterilerin atık su arıtımında kullanılması konvansiyonel amonyum giderimine göre daha ucuz ve daha çevre dostu bir sistemin geliştirilmesini sağlamıştır.
Bu tezin amacı havasız amonyum yükseltgenmesi yapan bakterilerin organik asitler bazındaki niş farklılıklarının saptanması ve HAY prosesinin ara maddelerinin belirlenerek bu prosesin atık su arıtımında daha başarılı bir şekilde kullanılmasını sağlamak ve bu bakterilerin doğal ortamdaki ekolojisini daha iyi anlamaktır.
Bu doktora tezinin ilk bölümü HAY bakterilerinin fizyolojisi, biyokimyası, çeşitliliği konusunda yapılan çalışmaları aktarmaktadır. Bu bölümde ayrıca HAY bakterilerinin biyolojik azot döngüsündeki önemi ve HAY teknolojisinin atık su arıtımında kullanılması da anlatılmıştır.
Tezin ikinci bölümü amonyumun yanı sıra propiyonik asidi de yükseltgeyen yeni bir HAY bakterisinin çoğaltılmasını anlatmaktadır. Bu tür HAY bakterilerinin çoğaltılabilmesi için daha önce *Candidatus* "Brocadia anammoxidans" ve

Anaerobic ammonium oxidation (anammox) is one of the latest additions to the nitrogen cycle. In the anammox reaction ammonium is oxidized to dinitrogen gas with nitrite as the electron acceptor. Within eight years following their discovery, the anammox bacteria have been identified as the key players in the global nitrogen cycle. Moreover, the anammox process has emerged as a cost-effective and environment-friendly alternative to conventional ammonium removal from wastewater streams.
The aim of this thesis was to get a better understanding of the niche differentiation of the anammox bacteria with respect to organic acids, and to identify the intermediates of the anammox metabolism to facilitate a better application of the anammox process to wastewater treatment and to improve the understanding of the microbial ecology of these bacteria.
The first chapter of this thesis is an overview of the physiology, biochemistry, diversity and the methods of detection of the anammox bacteria, the relevance of the anammox process to the biological nitrogen cycle, and the application of this process as a wastewater treatment technology.
The second chapter describes the enrichment of a propionate oxidizing anammox bacterium. For the enrichment of these anammox bacteria, the same medium composition and the same reactor setup used for the enrichment of *Candidatus* "Brocadia

Het eerste hoofdstuk beschrijft de fysiologie, biochemie en diversiteit van anammox bacteriën. Ook worden detectiemethoden voor deze bacteriën behandeld, de betekenis van anammox voor de biologische stikstofcyclus en de toepassing van het anammox proces in technologie voor afvalwaterzuivering. Het tweede hoofdstuk beschrijft de ophoping van een propaanzuur oxiderende anammox bacterie. De bacteriën konden worden opgehoopt uit B-trap biologisch actief slib, in medium met dezelfde samenstelling en in een zelfde type reactor als eerder gebruikt voor ophopingen van zowel *Candidatus* "Brocadia anammoxidans" als *Kuenenia stuttgartiensis*. Het enige verschil was toevoeging van propaanzuur bij een koolstof/stikstof verhouding lager dan 1. Na een aantal maanden bestond de ophopingscultuur voor 80% uit *Candidatus* "Anammoxoglobus propionicus". Deze bacteriesoort oxideert propaanzuur sneller dan andere anammox bacteriën. In aanwezigheid van nitriet, nitraat, ammonium en propaanzuur kon *Candidatus* "Anammoxoglobus propionicus" andere anammox bacteriën en heterotrofe denitrificerende bacteriën wegconcurreren, wat wijst op een hogere affiniteit voor de limiterende substraten nitriet en propaanzuur dan bij andere bacteriën. *Candidatus* "Anammoxoglobus propionicus" toplam bakteri kültürünün %80'ini meydana getirmiştir. *Candidatus* "Anammoxoglobus propionicus" türü, propiyonik asidi diğer HAY bakterisi türlerinden daha hızlı oksitlemektedir. Bu bakteri nitrit, nitrat, amonyum ve propiyonik asit kullanıldığında aktif çamurda bulunan diğer bakteri türlerinden daha iyi rekabet ederek reaktörün egemen türü haline gelmiştir. Bu sonuç *Candidatus* "Anammoxoglobus propionicus"un sınırlayıcı besiler olan nitrit ve propiyonik aside yüksek bir afinitesi olduğunu göstermektedir. Yeni HAY bakterisi daha önce çoğaltılan HAY bakterilerin olduğu tüm ortak özelliklere sahiptir. Bunlar Merdivensi lipitler, anamoksozom olarak adlandırılan bakteriyel organel, yavaş çoğalma, hidroksilaminden hidrazin üretilmesidir. *Candidatus* "Anammoxoglobus propionicus" ve *Candidatus* "Brocadia anammoxidans" içeren sürekli kültürle yapılan rekabet deneylerinde *Candidatus* "Anammoxoglobus propionicus"un sınırlayıcı besi olan nitrite *Candidatus* anammoxidans" and *Kuenenia stuttgartiensis* were used. The only difference was the addition of propionate in a C/N ratio below 1. After several months of enrichment using secondary stage activated sludge, *Candidatus* "Anammoxoglobus propionicus" constituted 80% of the enrichment culture. *Candidatus* "Anammoxoglobus propionicus" oxidizes propionate faster than other anammox bacteria. This bacterium also out-competed other anammox bacteria and heterotrophic denitrifiers in the presence of nitrite, nitrate, ammonium and propionate, indicating a higher affinity for the limiting substrates nitrite and propionate. It had all the previously observed features of the anammox bacteria: Ladderane lipids, an anammoxosome, slow growth, production of hydrazine in the presence of hydroxylamine. The Competition experiments in continuous cultures with *Candidatus* "Anammoxoglobus propionicus" and *Candidatus* "Brocadia anammoxidans" showed that *Candidatus* "Anammoxoglobus propionicus" had a better affinity to nitrite, the limiting substrate, than *Candidatus* "Brocadia anammoxidans".
The third chapter describes the enrichment of *Candidatus* "Brocadia fulgida", the autofluorescent and acetate oxidizing anammox bacterium. Besides the usual mineral medium used for the enrichment of the anammox bacteria, acetate

propionicus" had voor het overige alle eigenschappen van anammox bacteriën, zoals die eerder waren beschreven: ladderaan lipiden (speciale membraanlipiden, die tot nu toe alleen nog in anammox bacteriën zijn gevonden), een anammoxosoom (een uniek intern organel-achtig compartiment, waarvan wordt aangenomen, dat de anammox reactie er plaatsvindt), lage groeisnelheid, en vorming van hydrazine bij toevoeging van hydroxylamine. Competitie experimenten in continu culturen met *Candidatus* "Anammoxoglobus propionicus" en *Candidatus* "Brocadia anammoxidans" lieten zien, dat *Candidatus* "Anammoxoglobus propionicus" een hogere affiniteit had voor het limiterende substraat nitriet, dan *Candidatus* "Brocadia anammoxidans". In het derde hoofdstuk wordt de ophoping van *Candidatus* "Brocadia fulgida" beschreven. De ophopingscultuur kreeg azijnzuur toegevoegd aan het normale anammox medium. Na twee maanden was *Candidatus* "Brocadia fulgida" de dominante bacteriesoort in deze ophopingscultuur. De soort kon azijnzuur sneller oxideren dan de anammox bacteriesoorten, die tot dan toe waren onderzocht. Biofilm vlokjes van deze anammox soort werden autofluorescent na een paar maanden ophoping. Het was de eerste keer, dat dit verschijnsel werd waargenomen bij anammox bacteriën. Behalve ammonium en azijnzuur,

"Brocadia anammoxidans"tan daha kuvvetli bir afinite gösterdiği ortaya çıkmıştır. Üçüncü bolum kendiliğinden florasanlı olan ve asetat yükseltgeyen HAY bakterisi *Candidatus* "Brocadia fulgida"nın çoğaltılmasını anlatmaktadır. Bu bakteri türü de daha önce çoğaltılan tüm HAY bakteri türlerini içeren aktif çamur kullanılarak çoğaltılmıştır. Bu kültüre daha öncekilerden farklı olarak normal HAY besileri olan amonyum ve nitritin yanı sıra asetat da beslenmiştir. Reaktörün aktif çamurla aşılanmasından iki ay sonra *Candidatus* "Brocadia fulgida" reaktördeki egemen bakteri türü haline gelmiştir. Bu HAY bakterisi daha önce çoğaltılan HAY bakterilerinden farklı olarak kendiliğinden florasanlı olan bir madde üretmektedir. Bu bakteri, amonyumun yanı sıra format, asetat, propiyonat, mono ve di metilamin de yükseltgeyebilmektedir. Bu HAY bakterisi de diğer hay bakterilerinin taşıdığı tüm özellikleri göstermektedir: Yavaş çoğalma, anamoksozom, merdivensi lipitler ve hidrazin üretimi. Dördüncü bölüm tuzluluk içermeyen atık su ile çoğaltılan bir HAY bakteri kültürünün yüksek tuzluluğa alıştırılmasını anlatmaktadır. Yarı yarıya *Kuenenia stuttgartiensis* ve *Candidatus* "Scalindua wagneri" türlerini içeren iki özdeş bakteri kültürü daha önce kullanılan 33–37 °C deki sıcaklıklar yerine 25 °C de çoğaltılmıştır. Bu kültürlerden biri tuzluluk içermeyen diğeri ise yüksek tuzluluğu olan yapay atık su ile beslenmiştir. HAY bakterilerinin etkinliği

was fed to this enrichment culture. In two months *Candidatus* "Brocadia fulgida" became the dominant anammox bacterium in the enrichment culture. The biofilm aggregates of this anammox species became autofluorescent after several months of enrichment. This was observed for the first time for an anammox species. Besides ammonium, this bacterium was capable to oxidize compounds such as formate, acetate, propionate, monomethylamine and dimethylamine. Further, it had all the described anammox features (slow growth, anammoxosome, ladderane lipids, formation of hydrazine)
The fourth chapter describes the adaptation of a freshwater anammox population to high salinity. Two enrichment cultures were grown at a moderate 25 °C rather than elevated 33-37 °C like previous enrichment cultures. Initially the reactors contained a co-culture of *Kuenenia stuttgartiensis* and *Candidatus* "Scalindua wagneri". One of the cultures was subjected to gradually increasing salinity, and the other one was run with "freshwater" anammox medium. The anammox activity was sustained up to 3% salinity which corresponds to the salinity of seawater. Ultimately, the freshwater reactor was completely dominated by *Kuenenia stuttgartiensis*. In the 3% salinity reactor *Kuenenia stuttgartiensis* also dominated, but 30% of the community was still consisted of *Candidatus*

kon deze soort ook mierenzuur, propaanzuur, monomethylamine en dimethylamine oxideren. Ook *Candidatus* "Brocadia fulgida" had alle eerder beschreven anammox eigenschappen (trage groei, anammoxosoom, ladderaan lipiden, hydrazine vorming).
Hoofdstuk vier beschrijft de aanpassing van een anammox-populatie uit zoetwater aan verhoogde zoutconcentraties. De temperatuur in twee ophopingsculturen werd ingesteld op 25°C in plaats van 33-37°C, zoals bij eerdere ophopingen. In het begin van dit experiment bevatten de bioreactoren een co-cultuur van *Kuenenia stuttgartiensis* en *Candidatus* "Scalindua wagneri".Bij één van de twee culturen werd de zoutconcentratie gestaag opgevoerd, terwijl de andere cultuur gevoed bleef worden met normaal 'zoetwater' anammox medium. Anammox activiteit bleef behouden tot een zoutgehalte van 3 %, wat overeenkomt met het zoutgehalte van zeewater. Uiteindelijk werd de 'zoetwater' cultuur volledig gedomineerd door *Kuenenia stuttgartiensis*. *K. stuttgartiensis* kwam ook het meeste voor in de cultuur met 3% zout, maar hier bleef 30% van de anammox bacteriepopulatie bestaan uit *Candidatus* "Scalindua wagneri". Activiteitstests gaven aan, dat beide reactoren zeer vergelijkbare anaërobe ammonium oxidatiesnelheden hadden. Hiermee was aangetoond, dat het anammox proces toegepast kon worden op afvalwater met een hoog %3'lük tuzluluğa (deniz suyu tuzluluğu) kadar sürmüştür. Bunun üstündeki tuzluluğa (%4,5) kesikli kültür deneylerindeki bakterilerin dayanmasına rağmen sürekli kültürdeki bakteriler inhibisyona uğramıştır. Tuzluluk içermeyen atık suyla beslenen reaktördeki egemen HAY türü *Kuenenia stuttgartiensis* olmuştur. Yüksek tuzlulukla beslen reaktörde ise *Kuenenia stuttgartiensis* bakteri topluluğunun %70'ini *Candidatus* "Scalindua wagneri" ise %30'unu oluşturmuştur. Kesikli reaktörlerde yapılan aktivite testleri iki kültürün de amonyum giderme kapasitelerinin eşit olduğunu göstermiştir. Bu çalışma HAY prosesinin yüksek tuzluluk içeren atık sulara da uygulanabileceğini göstermiştir.
Beşinci bölümde HAY bakterilerinin katabolizması incelenmiştir. HAY prosesinin ara maddelerinin fizyolojik koşullar altında belirlenmesi için birbirini tamamlayan çeşitli yöntemler kullanılmıştır. Bu çalışmada *Kuenenia stuttgartiensis* hücre süspansiyonları kullanılmıştır. ^{15}N içeren ağır amonyum ve nitrit ile yapılan deneyler hidrazinin bir ara madde olduğunu ortaya çıkarmıştır. Azot oksit gideren bir kimyasal ile yapılan deneyler, HAY prosesinden azot oksit uzaklaştırıldığında prosesin durduğunu göstermiştir. Bunun yanı sıra azot oksidin Kuenenia stuttgartiensis hücrelerindeki varlığı azot oksitle tepkimeye girdikten sonra florasanlı hale gelen bir kimyasal ile "Scalindua wagneri". Activity tests showed that the two reactors had very similar anaerobic ammonium oxidation rates. It was shown that the anammox process could be applied to high salinity wastewater.
In the fifth chapter the pathway of the anammox process was investigated. An array of complementary methods was used to identify the anammox intermediates *in situ*. Single cell suspensions of *Kuenenia stuttgartiensis* were used in this study. Labeling experiments showed that in the anammox process hydrazine was turned over. Inhibition with a nitric oxide scavenger showed that nitric oxide was an intermediate of the anammox process. Nitric oxide was visualized in active *Kuenenia stuttgartiensis* cells with a nitric oxide reactive compound that becomes fluorescent upon reaction with nitric oxide. Incubations with nitric oxide and ammonium showed that these compounds were the precursors of hydrazine in the anammox reaction. The results of this study showed that nitric oxide and hydrazine were intermediates of the anammox reaction; hydroxylamine has not been ruled out as an intermediate. Inhibition of the anammox process with pentachlorophenol, a specific inhibitor of the cytochrome bc1complex, showed the involvement of the bc1 complex in the anammox catabolism. The nitrate reduction metabolism of the

zoutgehalte, zoals percolatiewater van stortplaatsen.
In de loop der tijd is in verscheidene hypotheses gespeculeerd over de metabole route voor het anammox proces, maar ondubbelzinnige experimentele onderbouwing ontbrak. Het vijfde hoofdstuk beschrijft onderzoek naar de biochemische route van het anammox proces in de cel. In deze studie werden losse cellen van *Kuenenia stuttgartiensis* gebruikt. Een aantal complementaire methodes werd gebruikt om de tussenproducten van het anammox proces *in situ* aan te tonen. Labelingsproeven met de stabiele stikstofisotoop ^{15}N bevestigden, dat hydrazine werd omgezet in het anammox proces. Het anammox proces kon worden geremd met PTIO, dat stikstofmonoxide (NO) wegvangt, waarmee duidelijk werd gemaakt, dat stikstofmonoxide een tussenproduct is. Stikstofmonoxide kon zichtbaar gemaakt worden in actieve *Kuenenia stuttgartiensis* cellen met diaminofluorescein-2-diacetaat, een indicator die fluorescent wordt zodra deze in de cel in contact komt met stikstofmonoxide. Door middel van incubatie experimenten met ammonium en stikstofmonoxide kon worden aangetoond, dat beide stoffen tussenproduct waren in de anammox reactie. Pentachloorfenol, een specifieke remmer van het cytochroom bc1 complex, kon de anammox reactie remmen, wat duidt op de betrokkenheid van het bc1 complex bij het

gözlemlenmiştir. Azot oksit ve amonyum ile inkübe edilen Kuenenia stuttgartiensis hücrelerinin hidrazin ürettiği açığa çıkarılmıştır. Bu çalışmanın sonucunda azot oksidin ve hidrazinin HAY prosesinin ara maddeleri olduğu ortaya çıkmıştır. Bununla birlikte hidroksilaminin bu prosesteki rolü tam olarak aydınlatılamamıştır. Sitokrom bc1 kompleksini (Kompleks 3) durduran pentaklorofenol, *Kuenenia stuttgartiensis* süspansiyonlarına eklendiğinde bakterilerin etkinliklerini yitirdikleri ortaya çıkmıştır. Bu sonuç kompleks 3' ün HAY prosesinde önemli bir yerinin olduğunu göstermiştir.
HAY bakterilerinin nitrat indirgeme metabolizması altıncı bolümde incelenmiştir. Bu çalışmada fiziksel olarak ayrıştırılmış *Kuenenia stuttgartiensis* hücre süspansiyonları kullanılmıştır. Bu süspansiyonlar ^{15}N-nitrat ve ^{13}C-format ile inkübe edilmiştir. Bu reaksiyonun son ürünün $^{15-15}N_2$ olduğu anlaşılmıştır. Reaksiyonun ara ürünlerinin ortaya çıkarılması için süspansiyonlar yine ^{15}N-nitrat ve ^{13}C-format ile fakat farklı deneylerde NO_2^-, NH_4^+, ve N_2O ile inkübe edilmişlerdir. Bu deneylerin sonucunda HAY bakterilerinin konvansiyonel denitrifkasyon bakterilerinin tersine nitratı, nitrit yolu ile amonyuma indirgedikleri ve daha sonra oluşan amonyum ve nitriti birleştirip azot gazi ürettikleri ortaya çıkmıştır. Nitriti amonyuma indirgeyebilen bir enzim kısmen ayrıştırılmış ve bu enzimi kodlaması olası

anammox bacteria is described in the sixth chapter. Also in this chapter single cell suspensions of *Kuenenia stuttgartiensis* were used. These cells were incubated with ^{15}N-nitrate and ^{13}C-formate. The end product of the nitrate reduction was determined to be $^{15-15}N_2$. Different non-labeled pools (NO_2^-, NH_4^+, and N_2O) were used in different incubations to determine the intermediates of this apparent denitrification process by anammox bacteria. This way the ^{15}N-labeled intermediates would be "trapped" in non-labeled pools for later analysis with mass spectroscopy. It was shown that unlike conventional denitrifiers, the anammox bacteria perform dissimilatory nitrate reduction to ammonium via nitrite, and subsequently combine the formed nitrite and ammonium through the usual anammox pathway. An enzyme capable of nitrite reduction to ammonium was partially purified, and candidate genes were identified in the *Kuenenia stuttgartiensis* genome. This newly discovered metabolism of the anammox reaction could be important for the survival of the anammox bacteria in the generally ammonium limited natural environments.
Taken together these results showed that the anammox bacteria are versatile, and could be applied in different nitrogen removal systems. The elucidation of the anammox metabolism remains a challenge for future research.

anammox katabolisme. Van hydroxylamine werd eerst gedacht, dat het een rol speelt in het natuurlijke anammox proces. Vervolgens werd aangenomen dat dit niet het geval was, maar het is mogelijk toch een tussenproduct.
In het zesde hoofdstuk wordt het nitraatreductie metabolisme van anammox bacteriën beschreven. Voor dit onderzoek werden ook losse cellen van *Kuenenia stuttgartiensis* gebruikt. Deze cellen werden geïncubeerd met ^{15}N gelabeld nitraat en ^{13}C gelabeld mierenzuur. Het eindproduct van de nitraatreductie bleek $^{15-15}N_2$ te zijn. Om de tussenproducten van dit op denitrificatie lijkende proces te bepalen werden niet-gelabelde pools gebruikt (NO_2^-, NH_4^+, N_2O) in verschillende experimenten om gelabelde tussenproducten in te vangen. Op deze manier konden gelabelde tussenproducten worden gedetecteerd vóórdat ze verder werden omgezet in de volgende stap in het metabolisme. In tegenstelling tot conventionele denitrificerende bacteriën reduceren anammox bacteriën nitraat via nitriet naar ammonium. Vervolgens combineren ze het ontstane ammonium en nitriet via de normale anammox route. Het enzym dat verantwoordelijk zou zijn voor de reductie van nitriet naar ammonium, werd gedeeltelijk gezuiverd, en kandidaat genen voor andere enzymen voor dit proces werden gevonden in het genoom van *Kuenenia*

genler *Kuenenia stuttgartiensis* bakterisinin genomunda belirlenmiştir. HAY bakterilerinin bu yeni keşfedilen metabolizması sınırlayıcı miktarda amonyum içeren doğal ortamlarda da HAY bakterilerinin diğer bakterilerle başarılı olarak rekabet etmelerini sağlayabileceği tahmin edilmektedir.
Bu tezdeki bütün sonuçlar bir arada ele alındığında HAY baterilerinin birçok redoks tepkimesinden enerji dönüşümü yapabildiği ve farklı atık sulardan azot giderme sistemlerinde kullanılabileceği ortaya çıkmaktadır. HAY bakterilerinin metabolizmasının tam olarak anlaşılması gelecekte yapılacak çalışmaların başlıca konusudur.

stuttgartiensis. Dit nieuw ontdekte onderdeel van het anammox metabolisme zou voor anammox bacteriën belangrijk kunnen zijn om te overleven in een omgeving met weinig ammonium.
De in dit proefschrift beschreven resultaten laten zien, dat anammox bacteriën veelzijdige organismen zijn, en dat ze toegepast zouden kunnen worden in meerdere systemen om stikstofverbindingen te verwijderen uit afvalwater. Meer onderzoek blijft nodig om het anammox metabolisme verder te ontrafelen.

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curriculum vitae

The author of this thesis was born in İstanbul on 1st April 1979. He graduated from Üsküdar American Academy in 1997. In 2001 he received the Bachelor of Science degree in environmental engineering from İstanbul Technical University. The same year he won the British Chevening Scholarship to study in Newcastle University, UK. Here, he worked on aerobic ammonium oxidizing bacteria under the supervision of Prof. dr. Tom Curtis. In 2002 he received the Master of Science degree in environmental engineering from Newcastle University. He spent the following year in Boğaziçi University Molecular Ecology lab. In 2003 he started his PhD in University of Nijmegen under the supervision of Prof. dr. ir. Mike Jetten. This work on the anaerobic ammonium oxidation resulted in the present thesis. Currently he is employed as a post-doc at the same department to investigate the biochemistry of anaerobic oxidation of ammonium.

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