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On the use of anammox in treating ammonium-rich wastewater

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Table of contents

Summary

This PhD thesis focused on the enrichment, characterization and possible use of anaerobic ammonium oxidizing bacteria. Anaerobic ammonium oxidation (also called the *anammox* process) involves the oxidation of ammonium with nitrite to N_2 coupled to autotrophic growth. The anammox process is well-defined, yet only one bacterium performing the anammox reaction was known at the start of this PhD work. Since the anammox process is thought of as an interesting alternative to denitrification in removing ammonium from wastewaters, the goals of the work were to enrich anammox bacteria from other sources, to understand the possible differences of those anammox bacteria with the one described previously, and to study the microbiological requirements for operating a two stage aerobic-anaerobic ammonium removal process involving nitritation and anammox.

Chapter 2 describes the enrichment of anammox bacteria from biofilm material from a rotating disk contactor, which was operated at the Kölliken landfill area to treat ammonium-rich leachate from which almost all carbon had been removed. Less than 1 g of Kölliken biofilm material was used to set up a series of sequential enrichments which were all incubated with ammonium and nitrite as energy source and bicarbonate as carbon source under strictly anoxic conditions. Stable anaerobic ammonium oxidation could relatively easy be obtained, however, due to the low growth rate of the bacteria involved it took several months and transfers to obtain a biomass of $OD_{600} \approx 0.1$ with a relative population size of 88 ± 5 % of anaerobic ammonium oxidizers. This enrichment culture was used to identify the responsible anammox bacterium and study its physiology. PCR amplification was used to amplify 16S rDNA fragments from the organisms of the enrichment culture, which were cloned and subjected to DNA sequencing. Based on 16S rDNA sequence analysis, one clone contained a 16S rDNA sequence 91% similar to that of the known anammox organism *Brocadia anammoxidans* but mostly related (98-99%) to other cloned sequences from a reactor system in Stuttgart. This sequence had been sufficiently divergent to propose another genus and species name Candidatus *Kuenenia stuttgartiensis*, suggesting that the anammox organism from the Kölliken biofilm belonged to the genus *Kuenenia* as well. Physiological experiments performed with the enrichment culture showed that N_2 was only produced when both ammonium and nitrite were present. The amount of N_2 produced was proportional to the ammonium concentration in the presence of constant amounts of nitrite provided that nitrite was always present in excess to the amount of ammonium. Anammox activity was optimal at 37 °C and pH 8 with a specific activity of 26.5 nmol N_2 min^{-1}(mg protein)$^{-1}$. Anammox activity was reversibly inhibited by low amounts of oxygen. 15 % of the converted nitrite was oxidized to nitrate probably to generate reduction equivalents for CO_2 assimilation. Both hydrazine and hydroxylamine were converted by the enrichment culture, suggesting they are intermediates in anaerobic ammonium oxidation, like was shown for *B. anammoxidans*. In contrast to Cand. *B. anammoxidans* Cand. *Kuenenia* sp. 'Kölliken' showed a higher tolerance to nitrite and phosphate and was actively oxidizing ammonium with nitrite irrespective of the cell density in the incubation. Anammox bacteria formed tight clusters of up to several hundred individual cells, embedded in an extracellular matrix. Electron microscopy (EM) and fluorescence *in situ* hybridization (FISH) revealed an inner protein rich area with a low content of DNA and rRNA, giving the cells a "doughnut" like appearance.

Chapter 3 was focused on unraveling the structure of the initial Kölliken biofilm and understanding how biofilm structure and community composition enabled the overall ammonium removal in the rotating biological contactor. Aliquots from the biofilm were investigated with different methods such as FISH (fluorescence *in situ* hybridization), rRNA dotblot hybridizations, 16S rDNA clone library construction, electron microscopy and thin sliced FISH-stained biofilm. It was discovered that the biofilm consisted mainly of four different groups of bacteria: aerobic and anaerobic ammonium oxidizing bacteria, nitrite oxidizing bacteria and filamentous bacteria from the phylum *Cytophaga-Flavobacterium-Bacteroides* (CFB). Nitrite oxidizing bacteria were present only in low amounts, whereas the other three groups each formed approximately one quarter of the total biomass. Population quantifications from FISH and from 16S rRNA dot blot hybridizations showed considerable differences, suggesting that both methods still suffer from over- and underdeterminations of certain bacterial groups. With FISH on thin biofilm transections (10 µm), high amounts of clusters of aerobic ammonium oxidizing bacteria, surrounded by fewer groups of nitrite oxidizing bacteria from the genus *Nitrospira*, were clearly seen at the top of the biofilm (oxic part). Only ammonium oxidizing bacteria from the *Nitrosomonas eutropha/europaea* group were found in the biofilm. The anammox bacteria were only detectable in the lower (presumably anoxic) part of the biofilm with a clear upper boundary, probably defined by the penetration depth of oxygen into the biofilm. Bacteria from the phylum *Cytophaga-Flavobacterium-Bacteroides* were present throughout the entire biofilm. The structure of the biofilm could explain the observed nitrogen loss and the occurrence of nitrate as sole inorganic nitrogen ion in the outlet. The current model of the biofilm assumes that ammonium is only partly converted to nitrite, and that the remaining ammonium and nitrite diffuse in the lower (anoxic) part of the biofilm were they are converted by the anammox bacteria to N_2. Interestingly, nitrite oxidizers were apparently not able to colonize the biofilm sufficiently to outcompete the anaerobic ammonium oxidizers for nitrite. Strangely enough, no anammox organisms or nitrite oxidizing bacteria were represented in the 16S rDNA clone library, although these two bacterial groups contributed to a significant proportion of bacteria present in this biofilm. Furthermore, several other very unusual sequences were detected. These bacteria were not present in high amounts and their physiology and presence in the biofilm remains unclear.

Chapter 4 was committed to studying the community development of aerobic ammonium oxidizers under different start-up conditions. In order to apply the anammox process effectively, it has to be ensured that ammonium and nitrite are presented to the bacteria in almost equimolar amounts. The stratified biofilms of the rotating biological contactor, described in Chapter 3, had just the right structure to fulfill this prerequisite Another possibility would be to separate both aerobic and anaerobic processes in a two reactor system. One reactor would contain aerobic ammonium oxidizing bacteria, converting ammonium to nitrite, and a second reactor with the anammox bacteria would use both ammonium and nitrite to produce dinitrogen gas (and some nitrate). Critical in the first reactor is to prevent nitrite oxidizing bacteria from establishing themselves. In this chapter we investigated which parameters would determine a successful development of a population of mainly anaerobic ammonium oxidizers starting from a regular aerobic activated sludge. The effect of different pHs, temperatures and dilution rates on the formation of nitrite and nitrate from ammonium and on the community composition of ammonium and nitrite oxidizers was determined with a high time resolution. All reactors were pH and temperature controlled continuously stirred tank systems and were inoculated with

activated sludge from the Werdhölzli wastewater treatment plant in Zürich. This sludge contained nitrifiers of four different lineages: *Nitrosomonas communis*, *Nitrosomonas urea/oligotropha*, *Nitrosomonas europaea/eutropha* as ammonium oxidizing and *Nitrospira* sp. as nitrite oxidizing bacteria. The reactors were fed with either a synthetic medium or with supernatant of the Werdhölzli sludge digester, containing both around 50 mM ammonium (as ammonium bicarbonate). Under all conditions, nitrate was produced only during the first three to four days after inoculation, after which the concentrations decreased to below 0.05 mM (detection limit). The highest nitrate concentrations (25 mM) were temporarily produced in the reactor which was fed with supernatant from the sludge digester. After 5 to 10 days, the nitrite concentration started to increase from less than 1 mM to nearly 50 mM. For most conditions, the residual ammonium concentration after 10 days was less than 5 mM. After 10 days, the nitrite production and ammonium removal rates remained more or less stable. Highest nitrite production was achieved in reactors operated at a pH between 7 and 7.5, and at 30°C.

Quantitative community analysis by fluorescence *in situ* hybridization with group-specific probes for ammonium and nitrite oxidizers led to the following results. Nitrite oxidizing bacteria from the genus *Nitrospira* were only detectable in the reactors up to a period of about two weeks. Parallel to the increase of nitrite in the reactor effluent, the relative population size of the ammonium oxidizing bacteria from the *N. europaea/eutropha* group increased from less than 1% directly after inoculation to a maximal relative amount of 60 to 70 % of the FISH stainable bacteria after four weeks of operation. In some cases, but not consistently, a second type of ammonium oxidizing bacteria occurred in the reactors, belonging to the *N. communis* lineage. Ammonium oxidizing bacteria from the *N. urea/oligotropha* lineage were only detectable in the first week after inoculation but did not develop to a larger sized population under the operating conditions of the reactors. Interestingly, reactors fed with synthetic medium could not be operated at higher dilution rates than 0.2 day^{-1} or else washout of the microbial community occurred, whereas those fed with supernatant from the sludge digester could be operated at 0.75 day^{-1} without loss of the ammonium oxidizing activity. It was assumed that use of sludge digester supernatant led to continuous reinoculation of ammonium oxidizers into the reactor, a hypothesis which was supported by the detection by PCR (polymerase chain reaction) of the ammonium monooxygenase gene (*amoA*) in DNA isolated from supernatant digester biomass.

In order to refine the community results for ammonium oxidizers with FISH, the diversity of the *amoA* gene was analyzed on PCR amplified *amoA* gene fragments from isolated community DNA. The amplified DNA was subsequently digested with different restriction enzymes and separated on high resolution gels (restriction fragment length polymorphisms or RFLP). Samples taken at different time points during operation of the reactors clearly showed a change in RFLP patterns during the first two weeks after reactor inoculation, where FISH had shown a homogenous composition. Apparently, more than one species of ammonium oxidizer could be present although with FISH only one group was detected. This refined analysis also demonstrated that repetition of the inoculation experiments under identical conditions with respect to temperature, pH and dilution rate did not lead to identical RFLP patterns of the *amoA* gene in the reactor community. The pattern of the reactors fed with supernatant of a sludge digester was finally dominated by the ammonium oxidizing bacteria from the supernatant although initially other ammonium oxidizing bacteria were present as well.

Chapter 5 describes attempts which were made to detect the presence of anammox bacteria in different wastewater treatments plants around Zürich and in the groundwater infiltrate at the Kölliken landfill. For detection, we focused on a region of the 16S rDNA amplified with PCR with primers targeting one region specific for *Planctomycetes* and one for all known anammox bacteria. All groundwater samples taken at the landfill site were positive for amplification of the anammox 16S rDNA fragment. This suggests that the anammox bacteria present in the rotating disk contactor were inoculated from the subsurface of the landfill. In DNA isolated from most wastewater treatment plants (aeration basin and sludge digester), no anammox 16S rDNA fragment could be amplified. However, in two activated sludges and in one sequencing batch reactor, positive amplification products were obtained, suggesting that anammox bacteria may occasionally also inhabit regular wastewater treatment systems.

Zusammenfassung

Ab Mitte der neunziger Jahre wurde in einer Anlage in Kölliken (AG), welche ammoniumreiches Sickerwasser einer Sondermüldeponie behandelte, ein unüblicher Stickstoffverlust beobachtet. Die Anlage besteht aus zwei Tauchtropfkörpern (TTK) welche als Unterlage für das Wachstum eines Biofilms dienen. Der erste TTK enfernt nur den organischen Kohlenstoff. Beim zweiten TTK, welcher das immer noch ammoniumreiche Abwasser (bis 30 mM) behandelt, wurde der Stickstoffverlust beobachtet. Dieser Verlust konnte nicht auf eine Kombinaton von Nitrifikation (Oxidation von Ammonium zu Nitrat via Nitrit) und Denitrifikation (Reduktion von Nitrat über Nitrit zu N_2) zurückgeführt werden. Dieser Stickstoffverlust wurde schon kurz nach Inbetriebnahme beobachtet und äusserte sich darin, dass die Ammoniumkonzentration im Einlauf viel höher war als die Nitratkonzentration im Ablauf. Ammonium und Nitrat waren die beiden häufigsten Stickstoffverbindungen im Zu- respektiv Ablauf. Da kurz vorher die Anaerobe Ammonium Oxidation (Anammox) an der Universität Delft (Niederlanden) entdeckt wurde, ist vermutet worden, dass dieser Stickstoffverlust auf einem mindestens ähnlichen Prozess beruht. Beim Anammox-Prozess wird Ammonium mit Nitrit unter anoxischen Bedingungen zu N_2 oxidiert. Diese Beobachtungen wurden die Basis für folgende Themen, die während der Dissertation behandelt wurden:

a) Anreicherung und Chrakterisierung eines Anammox Bakteriums von einem Tauchtropfkörper welcher ammoniumreiches Abwasser behandelt (Kapitel 2).
b) Zusammensetzung und Struktur des Anammox Biofilmes aus Kölliken (Kapitel 3).
c) Populationsanalysen von Nitrifizierenden Reaktoren während der Einlaufphase (Kapitel 4).
d) Detektion von Anammoxbakterien in verschiedenen Umweltproben mittels PCR (Kapitel 5).

Kapitel 2. Ein kleiner Teil des Biofilmes (weniger als 1g) wurde dazu verwendet, um die dort vorkommenden Anammoxbakterien anzureichern. Um dies zu erreichen, wurde der Biofilm unter anoxischen Bedingungen inkubiert. Ammonium und Nitrit wurden regelmässig zur Anreicherungskultur dazugegeben und dienten als einzige Energiequelle. Bikarbonat war die Kohlenstoffquelle. Nach ein paar Monaten wurde eine relative Populationsgrösse von $88 \pm 5\%$ Anammoxbakterien erreicht. Diese Anreicherungskultur wurde dazu verwendet, um das Bakterium aufgrund dessen 16S rDNA Sequenz zu identifizieren. Da die Homologie der 16S rDNA zwischen dem Anammox Bakterium aus Kölliken und dem aus Delft (Candidatus *Brocadia anammoxidans*) weniger als 91 % betrug, wurden physiologische Versuche durchgeführt. Aufgrund der kleinen Homologie der 16S rDNA gehören die beiden Anammoxbakterien zu zwei verschiedenen Gattungen. Wegen der unterschiedlichen Sequenzen in der rDNA können die beiden Anammoxorganismen mit diversen FISH-Sonden gut unterschieden werden. FISH steht für fluoreszierende *in situ* Hybridisierung. Das Kölliken Anammox Bakterium wies jedoch eine hohe Homologie (98 – 99%) auf zu einem mutmasslichen Anammox Bakterium aus Stuttgart (Candidatus *Kuenenia stuttgartiensis*), welches jedoch nur mit molekularbiologischen Methoden identifiziert wurde. Zuerst wurde gezeigt, dass nur N_2 produziert wurde, wenn sowohl Ammonium als auch Nitrit vorhanden war. Die N_2 Produktion nahm zu bei steigender Ammoniumkonzentration (0-6 mM), wenn genügend Nitrit vorhanden war (6 mM). Die höchste Aktivität wurde bei pH 8 und 37 °C beobachtet. Die maximale spezifische Aktivität bei optimalen Bedingungen (Temperatur, pH, Nitritkonzentration, anoxische Verhältnisse) war 26.5 nmol N_2 min^{-1}(mg Protein)$^{-1}$. Reduzierte Sauerstoffkonzentrationen hemmten die Aktivität reversibel. Da diese Anammoxbakterien sowohl in der Deponie als auch im Biofilm detektiert werden konnten, also Orten bei denen die Bakterien teilweise mit Sauerstoff in Kontakt kamen, ist eine gewisse Sauerstofftoleranz nicht weiter erstaunlich. Etwa 15 % des umgesetzten Nitrits wurde zu Nitrat oxidiert. Es wird angenommen, dass die bei dieser Oxidation frei werdenden Elektronen zur CO_2 Reduktion (und daher fürs Wachstum) gebraucht werden. Hydroxylamine (NH_2OH) und Hydrazine (N_2H_4) wurden von der Anreicherungskultur umgewandelt. Nitrifikanten konnten mit FISH nicht nachgewiesen werden, obwohl im Köllikenbiofilm einige aerobe Ammoniumoxidierer detektiert wurden. Demnach spielten diese Organismen keine wichtige physiologische Rolle in dieser Anreicherungskultur. Das angereicherte Bakterium der Gattung *Kueninia* wies eine höhere Nitrit- und Phosphattoleranz auf als *B. anammoxidans*. Zudem wurde keine Abahängigkeit der Aktivität von der Zelldichte beobachtet. Sonst sind die getesteten Eigenschaften zwischen beiden Anammox Bakterien sehr ähnlich. Zur Zeit wird das Genom von Cand. *K. stuttgartiensis* an der TU München sequenziert. Es ist daher nur eine Frage der Zeit, dass noch weitere Unterschiede zwischen beiden Organismen endtdeckt werden. Elektronenmikroskopie zeigt eine innere, proteinreiche aber Nukleinsäure arme Region. Dies äussert sich darin, dass Zellen, die mit FISH angefärbt wurden, eine nur schwach fluoreszierende innere Region besitzen. Die Bakterien wachsen in Aggregaten bis zu mehreren hundert Zellen, welche in einem extrazellulären Polymer eingebettet sind.

Kapitel 3. Der Biofilm, welcher zur Anreicherung der Anammox Bakterien verwendet wurde, wurde mit verschiedenen molekularbiologischen Methoden bezüglich Zusammensetzung und Struktur untersucht und mit der beobachteten Aktivität verglichen. Dazu wurde FISH (fluoreszierende *in situ* Hybridisierung mit

Epifluoreszenzmikroskopie und Konfokale Laser Scanning Mikroskopie: KLSM), rRNA dotblots, eine 16S rDNA Klonbibliothek, PCR (polymerase chain reaction), dünn geschnittener und mit FISH angefärbter Biofilm und Elektronenmikroskopie verwendet. Mit FISH konnten 4 wichtige Bakteriengruppen identifiziert werden: Aerobe und anaerobe Ammoniumoxidierer (Kandidatus *Kueninia sp.* und von der *Nitrosomonas europaea/eutropha* Gruppe), nitritoxidierende Bakterien der Gattung *Nitrospira* und fädige Bakterien der Ordnung *Cytophaga-Flavobacteria-Bacteroides* (CFB). Nitritoxidierer waren nur in sehr kleinen Menge vorhanden (KLSM: weniger als 5 %). Dies ermöglichte, dass ein Teil des Ammonium zu Nitrit und nicht weiter zu Nitrat oxidiert wurde. Das Ammonium/Nitrit Gemisch diffundierte in den unteren (anoxischen) Teil des Biofilms wo die beiden Substanzen von den Anammox Bakterien hauptsächlich zu N_2 umgewandelt werden konnte. Die Nitrifizierer im oberen Teil des Biofilmes sind im oberen (oxischen) Bereich des Biofilmes und nahe beieinander anzutreffen. Die Anammoxbakterien sind im unteren Bereich des Biofilmes zu finden und bilden eine deutliche obere Grenze, wahrscheinlich als Resultat der Gegenwart von Sauerstoff im oberen Biofilmteil. Nitrat, welches die grösste Menge an inorganischem Stickstoff im Ablauf darstellte, wurde von den Nitritoxidierer und Anammox Bakterien gebildet. Die fädigen Bakterien der Ordnung CFB konnten im ganzen Biofilm beobachtet werden, das heisst sowohl im oxischen als auch im anoxischen Teil. Die 16S rDNA Klonbibliothek enthielt eine Sequenz von einer *Nitrosomonas sp.*, welche die gleichen Targetsequenzen für FISH-Sonden aufwiesen wie die mit FISH angefärbten Ammoniumoxidierer. Interessanterweise wurden Sequenzen mit unbekannter Homologie (weniger als 85 % zu einer bekannten Art) gefunden. Es bleibt unklar, welche Funktion diese Bakterien inne haben, obwohl sie mengenmässig keine grosse Rolle spielten. Eine Sequenz wies 87 % Homologie zu einer *Cytophaga sp.* auf und enthielt auch die Targetsequenz der CF319a Sonde. Möglicherweise wurde mit dieser Sequenz das dominierende fädige Bakterium im Biofilm identifiziert. Jedoch ist es auch denkbar, dass aufgrund des Sauerstoffprofiles verschiedene fädige Bakterien im Biofilm vorhanden waren. Es wurde jedoch keine Sequenz von *Nitrospira sp.* oder Anammox Bakterien gefunden obwohl diese beiden Organismen einen grösseren Anteil an den vorhandenen Bakterien ausmachten.

Elektronenmikroskopie zeigt deutlich Aggregate der Nitrifizierenden und Anammox Bakterien. Auch hier zeigt es ich, dass die fädigen Bakterien der Ordnung CFB eine Art Netzwerk über die gesammte Tiefe des Biofilmes bildeten. Die Struktur und Zusammensetzung des Biofilmes stimmte sehr gut mit den beobachteten Umwandlungsprozessen überein.

Kapitel 4. Traditionell wird Stickstoff mit einer Kombination von Nitrifikation und Denitrifikation aus dem Abwasser entfernt. Diese Kombination eignet sich auch für tiefe Konzentrationen (im mM-Bereich und kleiner) hat jedoch den Nachteil, dass nicht die ganze Stickstoffracht eliminiert werden kann (etwa 40-70 %). Des weiteren müsste eine organische Kohlenstoffquelle dazugegeben werden, falls das C/N Verhältnis sehr klein ist (z.B. bei Ueberstand vom Faulturm). Eine Kombination von 2 Reaktoren, mit partieller Nitrifikation im ersten Reaktor und Anammox (anaerobe Ammonium Oxidation) im zweiten Reaktor, eignet sich deshalb ausgezeichnet für die Behandlung solch spezieller Abwässer. Bei der partiellen Nitrifikation muss darauf geachtet werden, dass keine Nitritoxidierer wachsen. Daher wurden Reaktoren unter verschiedenen Bedingungen (pH 7 und 7.5, Temperatur von 25° und 30 °C, Verdünnungsrate von 0.15 bis 0.7 pro Tag) mit Belebtschlamm vom Werdhölzli

gestartet, der einzigen Kläranlage von Zürich (Reinigungsleistung etwa 200´000m^3 pro Tag). Die meisten Experimente wurden einmal wiederholt, um die Reproduzierbarkeit zu testen. Es wurde die nitrifizierende Aktivität und sowohl quantitative als auch qualitative Veränderungen in der nitrifizierenden Population während drei bis fünf Wochen verfolgt. Das Medium war entweder synthetisch oder Faulwasser. (Temperatur im Faulturm: 35 - 40 °C). Die Ammoniumkonzentration betrug in allen Experimenten etwa 50 mM. Quantitative Populationsanalysen wurden mit FISH (fluoreszierende *in situ* Hybridisierung), konfokaler Laser scanning Mikroskopie und digitaler Bildauswertung durchgeführt. FISH hat, wie andere Methoden, auch gewisse Nachteile. So zum Beispiel braucht es eine gewisse Anzahl Ribosomen pro Zelle und je kleiner die Population (vor allem bei weniger als 5 % relatives Biovolumen) desto grösser der relative Fehler beim Quantifizieren. Je höher die Homologie in der 16S rDNA von zwei Arten, desto schweriger wird es, zwei Arten voneinander zu unterscheiden. Daher war es sinnvoll, als Ergänzung zu den FISH-Daten eine weitere Methode anzuwenden. Als Ergänzung wurde die „restriction fragment length polymorphism" (RFLP) Methode angewandt. Ein Teil der Ammoniummonooxygenase (*amoA*), welche auch als phylogenetischer Marker verwendet wird, wurde mit PCR amplifiziert, mit 2 Enzymen verdaut und auf einem hochauflösenden Gel aufgetragen. Zu Beginn waren drei Gruppen von Ammoniumoxidierern im Belebtschlamm vorhanden, welche mit FISH deutlich unterscheidbar sind, (nämlich *Nitrosomonas oligotropha/urea*, *Nitrosomonas communis* und *Nitrosomonas europaea/eutropha* vorhanden). Die nitritoxidierenden Bakterien gehörten ausschliesslich zur Gattung *Nitrospira*. Bei allen getesteten Bedingungen wurden die Nitritoxidierer und die Ammoniumoxidierer der *N. oligotropha/urea* Gruppe ausgewaschen. *N. communis* konnten sich nur bei synthetischem Abwasser bei pH 7 und 30 °C in grossen Mengen (>40 % relatives Biovolumen nach 4 Wochen) durchsetzen. Ammoniumoxidierer der *Nitrosomonas europaea/eutropha* Gruppe waren in allen Reaktoren zu jeder Zeit auffindbar. Die RFLP Analyse zeigte eine höhere Biodiversität als FISH. Diese Biodiversität war jedoch nicht reproduzierbar. Dies im Gegensatz zu FISH. Die Nitritoxidierer der Gattung *Nitrospira* wurden wahrscheinlich wegen der hohen Nitrit- und Ammoniumkonzentration kombiniert mit hoher Temperatur (und daher Verdünnungsrate) ausgeschwemmt. Eine Nitratproduktion wurde nur innerhalb der ersten 4 Tage beobachtet. Leider sind noch keine physiologischen Daten von *Nitrospiren* vorhanden, welche aus Abwasseranlagen isoliert wurden. Mit Faulwasser als Medium konnte eine höhere Verdünnungsrate (0.7 im Vergleich zu 0.2 pro Tag) gewählt werden. Dies ist wahrscheinlich auf einen steten Reinokulationseffekt von Ammoniumoxidierern zurückzuführen. Interessanterweise überleben Ammoniumoxidierer den Aufenthalt im Faulturm (2-3 Wochen bei 30-40 °C). Dies im Gegensatz den den Nitritoxidierern.

Kapitel 5. Versuche zur Auffindung von Anammoxbakterien in verschiedenen Umweltproben (Abwasserreinigungsanlagen aus der Region Zürich, Grundwasserproben der Deponie in Kölliken (AG)) wurden ebenfalls durchgeführt. Zur Detektion von Anammoxbakterien wurde ein PCR mit spezifischen Primern (Oligonukleotiden) durchgeführt (Amx820 und Pla46rc). Dieses PCR amplifizierte einen Teil der 16S rDNA aller bisher bekannten Anammoxbakterien. Der eine Primer (dessen umgekehrt komplementäre Sequenz nur im PCR verwendet wurde) detektiert in FISH Planktomyceten und der andere Primer detektiert alle bisher bekannten Anammoxbakterien (Amx820). In allen Grundwasserproben der Kölliken-Deponie

konnten Anammoxbakterien nachgewiesen werden. Dies bedeutet, dass die im Tauchtropfkörper gefundenen Anammoxbakterien von der lokalen Umgebung stammen. Kein PCR Produkt wurde von den meisten untersuchten Proben aus der Abwasserreinigung (Belüftungsbecken und dazugehörender Faulturm) erhalten. Jedoch wurden in 2 Belüftungsbecken und einem sequencing batch reactor (SBR) der Abwasserreinigungsanlage Meilen Anammoxbakterien detektiert. Dies bedeutet, dass Anammoxbakterien auch in üblichen Abwasserreingungsystemen mit hohem Schlammalter zum Teil vorkommen können.

Chapter 1: Introduction

Nitrogen (N) is an element with oxidation state from –3 to +5. In each oxidation state the nitrogen atom combines with atoms of hydrogen, oxygen and nitrogen. In this way at least one unique inorganic molecule exists per oxidation state (Fig. 1). Although some of these molecules are thermodynamically more stable than others, all oxidation states are possible in aqueous systems, because the oxidation state of N in a given environment is controlled by kinetics and not by thermodynamic equilibrium (Williams and Frausto da Silva, 1996). Usually, the activation energy of N-compounds is high (e.g. for nitritification, nitratification and anammox between 44 and 70 kJ/mol substrate). Although a large part of nitrogen on this globe is present in the solid state as part of minerals, its concentration there does not exceed the ppm range (per kg solid material, Strous, 2000). Nitrogen gas is the most abundant gas in the atmosphere (79% vol N_2) but as such not directly of use for organisms (Williams and Frausto da Silva, 1996; Brock *et al.*, 1997).
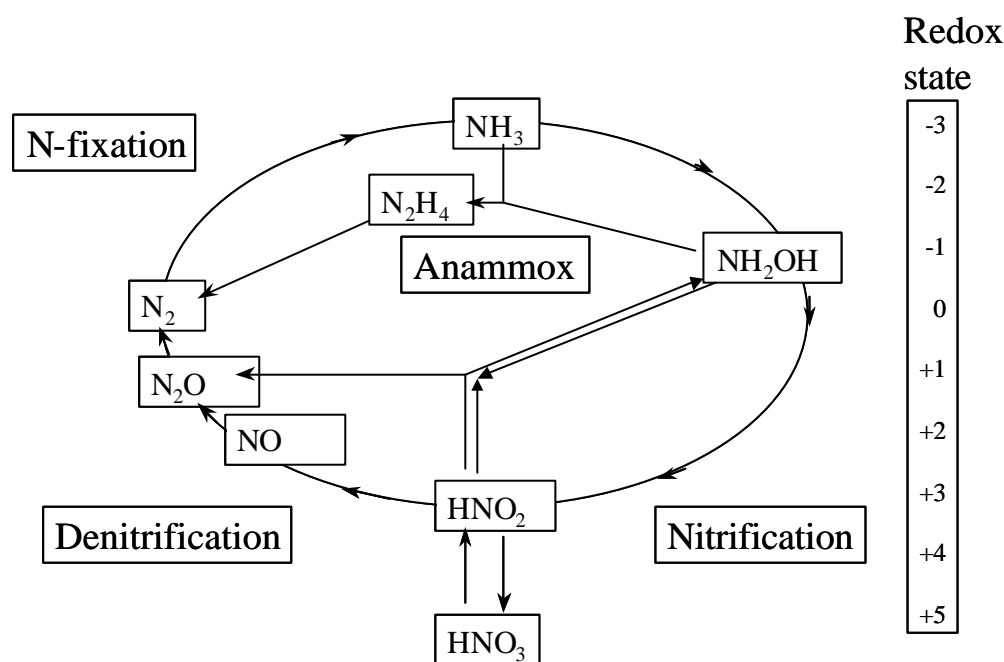

Fig. 1. N-cycle showing the educts, intermediates and products of the important processes of N-fixation, nitrification, denitrification and anammox (Figure taken from M. Strous).

Life depends on nitrogen. About 10 to 15% of the organic content of living organisms is formed by nitrogen. The turnover of nitrogen compounds in the biosphere is known as the nitrogen cycle (Fig. 1). From a microbiological point of view, the nitrogen cycle consists of five catabolic reactions (nitritification, nitratification, denitrification, dissimilatory nitrate reduction and anaerobic ammonium oxidation -*anammox*), three anabolic reactions (ammonium uptake, assimilatory nitrate reduction and nitrogen fixation), and ammonification (Brock *et al.*, 1997). Except for the anammox reaction, the full biological nitrogen cycle became evident years ago. In the early days, the main application of the N-cycle microbiology was to understand and improve

fertilizer efficiency in agriculture. Much later, the potential of nitrifiers and denitrifiers for nutrient removal from wastewater became generally recognized and research was directed to improve nitrogen removal from wastewater. Several years later, the contribution of nitrogen oxides in the atmosphere to ozone destruction and global warming was reassessed (von Schulthess *et al.*, 1994; Kuenen and Robertson 1994; Robertson, 1999), and the role of nitrification and denitrification in the formation of nitrogen oxides was again the focus of ´environment-driven´ N-cycle research.

Nitrogen, an environmental issue

It is evident that the protection of our water resources is of major importance on a global scale. An increasing population and industrialization will increase our water demand, placing even more pressure on water resources. Conventional wastewater treatment plants have not been designed for nitrogen removal, and many plants do not meet the current discharge standards of 10 mg N per liter (6 mg NO_3^-N/L in natural waters as limit in Switzerland). Plants with less input of energy and chemicals are needed to improve the current quality of the effluents. In this way, the quality of water being returned to rivers and other water sources will be significantly improved (Jetten *et al.*, 2002).

As a result of different anthropogenic activities, the loading of nitrogen compounds into the environment is increasing dramatically. In Switzerland, this increase causes problems in water, soil and air and, consequently, for humans, animals and plants, despite the fact that international communities have agreed to reduce nitrogen loadings. In our country, 120 000 tons of nitrogen compounds (as N) are released to water bodies annually. Of these 120 000 tons, 38 % are from agricultural sources, 35 % are from wastewater, 8 % are caused by emissions from transportation and incineration processes, and 17 % are from natural sources (decay of biomass, excrements of wild-life animals). To achieve effective reduction in nitrogen loading to water, it is necessary to take action in several areas (BUWAL, 1993). Swiss sewage works discharge approximately 28700 tonnes of nitrogen annually into receiving waters. If all the wastewater treatment plants of more than 10000 people-euqivalents would be expanded by the addition of denitrification zones, the nitrogen output would drop to approx. 13000 tonnes of nitrogen per year (BUWAL, 1996). Nitrification/denitrification as nitrogen elimination process from wastewater is sometimes far from optimal (40-70 % removing efficiency). Furthermore, 20 % of the annual nitrogen amount entering the wastewater treatment plant is derived from the sludge digester (Siegrist, 1996). Application of the recently developed CANON process (complete autotrophic nitrogen removal over nitrite) with a very high removal efficiency (> 90 %, van Dongen *et al.*, 2001) would therefore be beneficial.

Nitrogen converting reactions in wastewater treatment technologies

The two most important nitrogen converting processes in traditional nitrogen removal in wastewater treatment plants are nitrification (Table1, eq. 1, 2, 3, 4) and denitrification. These two processes and anammox (Table1, eq. 5) will be discussed in more detail. At present, the contribution of other nitrogen conversion processes (e.g., aerobic denitrification or anaerobic nitrification) to the nitrogen balance in wastewater

treatment plants is not quantitatively established (Schmidt *et al.*, 2002) and will not not be further treated here.

Table 1. Main stoichiometric reactions in nitrification, denitrification and anaerobic ammonium oxidation.

	Reaction	Energy yield (kJ/mol)[a]	Biomass g/mol[b]
Eq.1	$NH_3 + 1.5\ O_2 \rightarrow NO_2^- + H_2O + H^+$	-275	1.3-2.6
Eq.2	$NH_3 + O_2 + 2\ H^+ + 2\ e^- \rightarrow NH_2OH + H_2O$	-140.6	
Eq.3	$NH_2OH + H_2O \rightarrow NO_2^- + 4e^- + 5H^+$	22.5	
Eq.4	$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$	-74	0.9-1.8
Eq.5	$4\ NO_3^- + 5[CH_2O] + 4H^+ \rightarrow 2N_2 + 5CO_2 + 7H_2O$	-1180[c]	27[d]
Eq.6	$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$	-357	1.5

a) Gibbs free energy change (pH 7, 25 °C):
b) Per mol of oxidized ammonium or nitrite (Chapter 2, Egli *et al.*, 2001; Egli, 2000; Jetten *et al.*, 2002).
c) With acetate as electron donor
d) For growth of *Paracoccus denitrificans* with glutamate as carbon substrate.

The nitrification-denitrification scheme. Wastewater treatment plants in Switzerland either operate with nitrification alone, or with nitrification plus denitrification. In this process scheme ammonium is first oxidized with oxygen to nitrate, followed by denitrification. In this type of configuration organic carbon may become limited in the denitrifying zone, since most organic compounds were already oxidized in the aerobic nitrifying compartment. In the absence of sufficient organic carbon the denitrification process is not complete. To avoid carbon limitation in the denitrifying zone, most treatment plants first lead the wastewater to a stirred denitrifying basin with no air supply and afterwards to a basin with aeration in which nitrification occurs. Nitrate containing wastewater from the aeration basin is recirculated to and mixed with the organic carbon rich wastewater entering the denitrifying zone. Still, with such a combination only 40 to 70 % of the nitrogen reaching the wastewater treatment plant can be eliminated. The efficiency depends on the amount and presence of suitable organic substrates in the wastewater. In case when the denitrifying zone follows the nitrification basin external carbon sources like methanol are added to improve denitrification efficiency.

The SHARON process. Some wastewaters have a relatively high ammonium concentration (in the range of 50 mM) but a low organic carbon content, making it not easy to remove the ammonium by nitrification/denitrification without adding any organic carbon source (Hellinga *et al.*, 1998). In order to treat these wastewaters a

process was developed, in which ammonium is first oxidized to nitrite rather than nitrate and then subsequently denitrified (named the SHARON process, the acronym for **s**ingle reactor **h**igh **a**ctivity ammonia **r**emoval **o**ver **n**itrite). The main advantage of this process is a reduced air/oxygen input (Hellinga *et al.*, 1998; van Kempen *et al.*, 2001). Pilot plants using the SHARON process have been successfully operated for several years (van Kempen *et al.*, 2001; Fux *et al.*, 2002) without the need for sludge retention. The process usually runs at temperatures between 30 and 40°C and can reach up to 90% nitrogen removal, which is higher than can be achieved with usual nitrification and denitrification. Essential for the success of the SHARON process is the elimination of nitrite oxidizing bacteria from the reactor system. This is usually achieved by taking advantage of higher growth rates of ammonium oxidizers at higher temperatures and pH (Hellinga *et al.*, 1998), although it is still not known if this is generally true for all species of ammonium and nitrite oxidizers found in wastewater treatment systems (Chapter 4).

The CANON process. A newer process configuration for ammonium removal consists of a combination of nitritation and anaerobic ammonium oxidation. Since these two reactions occur fully autotrophically, the process is called **c**ompletely **a**utotrophic **n**itrogen removal **o**ver **n**itrite (or CANON). This process configuration eliminates the need for organic carbon addition to achieve denitrification (as in SHARON), since all involved bacteria are autotrophs (Strous, 2000; Egli *et al.*, 2001; Koops and Pommerening-Röser, 2001; Third *et al.*, 2001). In a first step of the CANON process, (part of the) ammonium is converted to nitrite, whereas in the second step ammonium is oxidized anaerobically with nitrite to form dinitrogen. For optimal efficiency, the anaerobic phase must be fed with slightly more nitrite than ammonium (Strous 2000; Egli *et al.*, 2001). The CANON process is already operational at technical scale in the Netherlands (Fig. 2 and 3. van Dongen *et al.*, 2001).
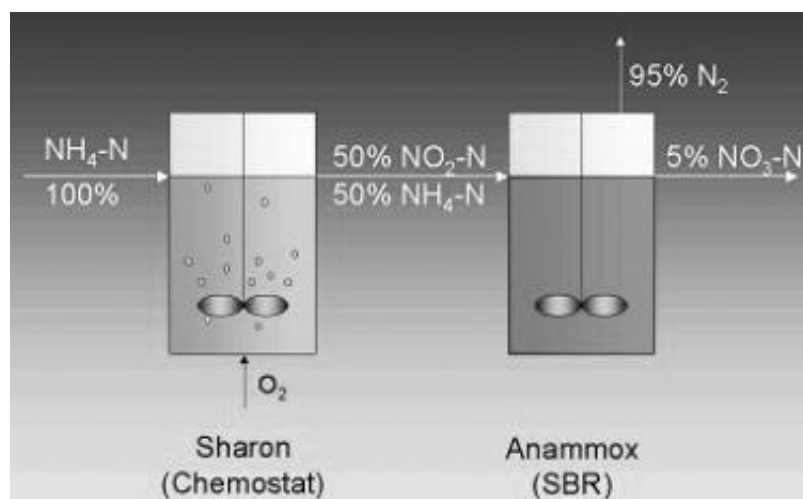

Fig.2. Schematic representation of the planned implementation of a combined SHARON-anammox process for the removal of ammonium from sludge digestion effluent (picture taken from Jetten *et al.*, 2002).
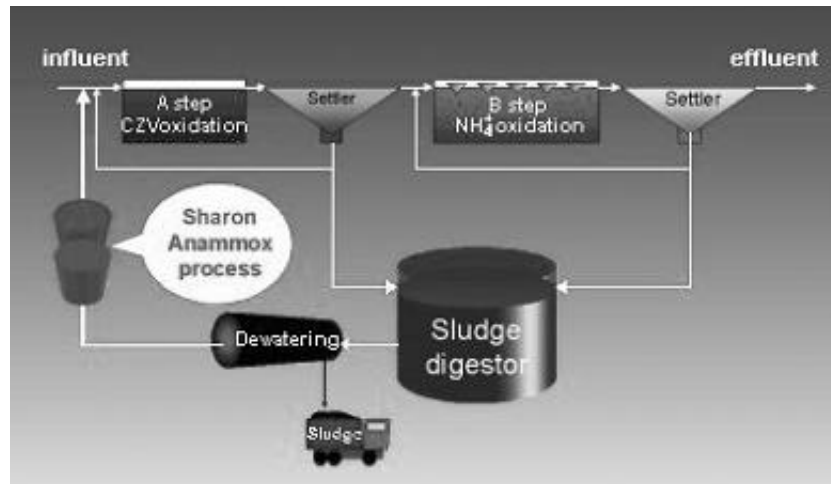
Fig. 3. Schematic representation of the combined SHARON-anammox process for the removal of ammonium from sludge digestion effluents (picture taken from Jetten *et al.*, 2002). Due to different growth rates of ammonium oxidizer and anammox bacteria, different reactors have to be used.

Biofilm configurations. In contrast to completely stirred-tank reactors a few biofilm systems treating ammonium-rich wastewater have also been investigated (Siegrist *et al.*, 1998; Helmer *et al.*, 2002; this PhD thesis). These biofilms were growing on rotating disk contactors, which were operated in such a way as to create oxygen gradients across the biofilm. The formation of a biofilm on the surface of the carrier material enables the growth and maintainance of slow growing microorganisms. On the other hand, physical parameters such as pH or oxygen supply are more difficult to control in a biofilm system compared to stirred reactors (Chapter 3, Helmer *et al.*, 2002). Two of these biofilm systems spontaneously developed aerobic and anaerobic ammonium oxidation. They were effectively removing ammonium from wastewaters without organic carbon and releasing mainly dinitrogen gas with small amounts of nitrate (Siegrist *et al.*, 1998; Hippen *et al.*, 2001; Helmer *et al.*, 2002). The formation of small amounts of nitrate was also observed in the CANON reactors. It is a consequence of the physiology of anammox bacteria (Third *et al.*, 2001). The biofilm systems are intriguing, since apparently their conditions are optimal for long term establishment of both aerobic and anaerobic ammonium oxidizers, whereas nitrite oxidizing bacteria seemed to remain only in relatively low numbers. Investigations by fluorescence *in situ* hybridisation with 16S rRNA targeted oligonucleotide probes demonstrated that classical ammonia-oxidizers of the β-subclass of *Proteobacteria* thrived within the surface region of the biofilms while the anaerobic ammonium oxidizers dominated the deeper presumably anaerobic biofilm layers (Chapter 3, Helmer *et al.*, 2002). The anammox organisms in both biofilms were very similar (Candidatus *Kuenenia stuttgartiensis*, see below).

Microbiology of nitrification, denitrification and anaerobic ammonium oxidation

Denitrification

This process makes use of N oxides (e.g. nitrate or nitrite) as terminal electron acceptors under anaerobic, microaerophilic, and occasionally aerobic conditions. The

process is an essential branch of the global N cycle, reversing dinitrogen fixation, and is associated with chemolithotrophic, phototrophic, diazotrophic, or organotrophic metabolism. Discovered more than a century ago and believed to be exclusively a bacterial trait, denitrification has now been found in halophilic and hyperthermophilic archaea and in the mitochondria of fungi, raising evolutionarily intriguing vistas (Zumft, 1997).

Important advances in the biochemical characterization of denitrification and the underlying genetics have been achieved with various organisms (like species from the genera *Pseudomonas*, *Paracoccus*, *Ralstonia*, and *Rhodobacter*). Pseudomonads (Baumann, 1997) represent one of the largest assemblies of the denitrifying bacteria within a single genus, favouring their use as model organisms. Around 50 genes are required within a single bacterium for the synthesis of the biochemical denitrification apparatus (Zumft, 1997). Numerous environmental factors can influence denitrification activity, such as nitrate and nitrite concentrations, pH, aeration, temperature, carbon availability, and relative activities of NO - and N_2O reductases (Bergsma *et al.*, 2002).

Aerobic ammonia oxidation

Nitrification is generally performed by aerobic autotrophic or mixotrophic microorganisms (Laanbroek *et al.*, 1994). Ammonium is oxidized first to nitrite by ammonia oxidizing microorganisms and then nitrite is converted to nitrate by nitrite oxidizers (see below). Both oxidation processes generate energy for the respective organisms (Table 1). Two enzymes (ammonium monooxygenase and hydroxylamine oxidoreductase) catalyze the oxidation of ammonia to nitrite (Table1, eq. 2 and 3). Hydroxylamine is the first oxidation product of aerobic ammonium oxidation (Table 1, eq. 2) but also the reduction product of nitrite in the anammox process (van de Graaf *et al.*, 1996).

Although the basic metabolism is more or less uniform for all ammonia-oxidizing bacteria, different physiological requirements exist among the different strains (Wagner *et al.*, 1995; Pommerening-Röser *et al.*, 1996; Suwa *et al.*, 1997; Koops and Pommerening-Röser 2001). For example, the substrate affinity (K_s value) for ammonium differs significantly among ammonia oxidizers (1.9-61 µM, Fig. 4). Many strains are able to use urea as ammonium source and the ability to hydrolyze urea is used as additional classification characteristic (Pommerening-Röser *et al.*, 1996). Ammonia oxidizers also differ considerably in salt requirement and salt tolerance. For example, the two species of *Nitrosococcus* from the γ *Proteobacteria* are obligately halophilic. Among ammonia oxidizers of the β subclass of the *Proteobacteria* both obligately halophilic species, moderately halophilic or halotolerant species and salt sensitive species are found.

species | ecophysiological parameters | | | preferred habitat
| | salt requirement | urease activity | substrate (NH₃) affinity (K_s) | |

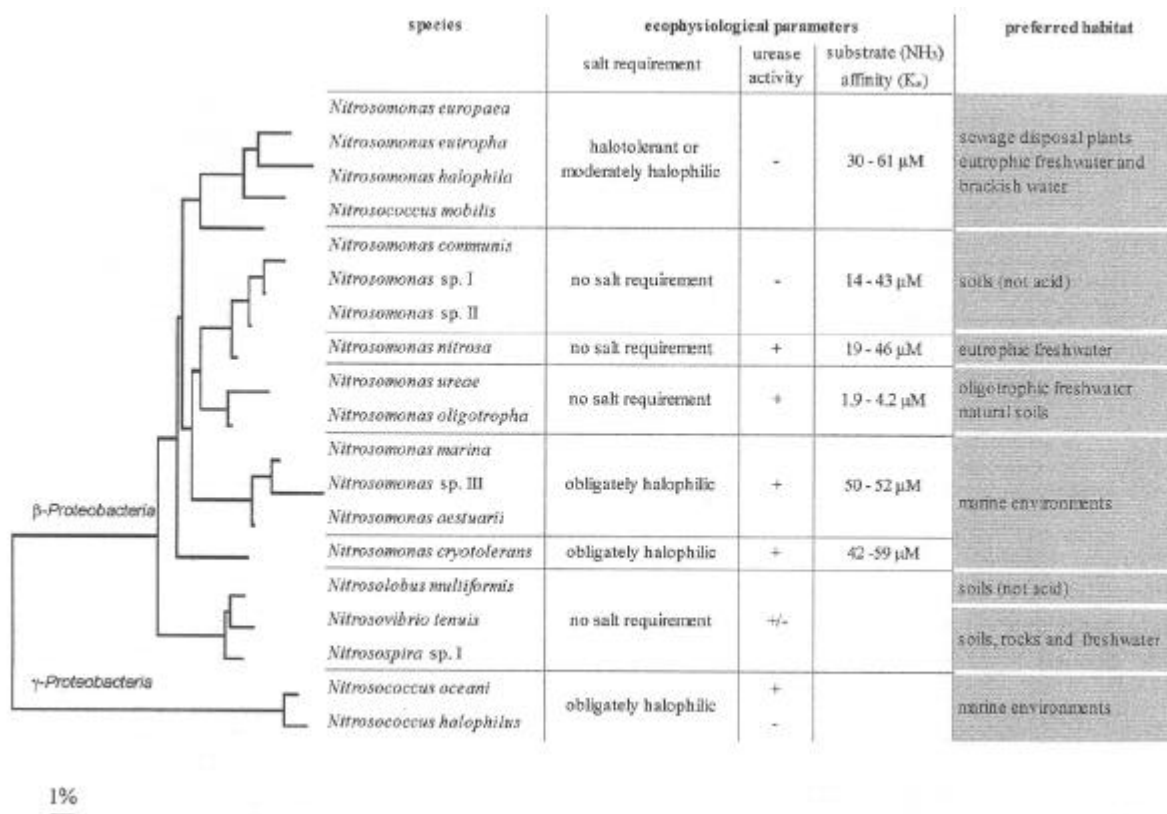| species	salt requirement	urease activity	substrate (NH$_3$) affinity (K$_s$)	preferred habitat
Nitrosomonas europaea *Nitrosomonas eutropha* *Nitrosomonas halophila* *Nitrosococcus mobilis*	halotolerant or moderately halophilic	-	30 - 61 µM	sewage disposal plants eutrophic freshwater and brackish water
Nitrosomonas communis *Nitrosomonas* sp. I *Nitrosomonas* sp. II	no salt requirement	-	14 - 43 µM	soils (not acid)
Nitrosomonas nitrosa	no salt requirement	+	19 - 46 µM	eutrophic freshwater
Nitrosomonas ureae *Nitrosomonas oligotropha*	no salt requirement	+	1.9 - 4.2 µM	oligotrophic freshwater natural soils
Nitrosomonas marina *Nitrosomonas* sp. III *Nitrosomonas aestuarii*	obligately halophilic	+	50 - 52 µM	marine environments
Nitrosomonas cryotolerans	obligately halophilic	+	42 - 59 µM	
Nitrosolobus multiformis	no salt requirement	+/-		soils (not acid)
Nitrosovibrio tenuis *Nitrosospira* sp. I				soils, rocks and freshwater
Nitrosococcus oceani *Nitrosococcus halophilus*	obligately halophilic	+ / -		marine environments

β-Proteobacteria

γ-Proteobacteria

1%

Fig. 4. Phylogenetic and physiological requirements of cultured ammonia oxidizing bacteria (picture taken from Koops and Pommerening-Röser, 2001).

Originally, all lithoautotrophic nitrifying bacteria were comprised within one family, named *Nitrobacteraceae*. At the time being, a total of 25 species of ammonia-oxidizers and eight species of nitrite-oxidizers are in pure culture (Koops and Pommerening-Röser, 2001). However, molecular methods have identified many more species which have not been cultured to purity yet (Gieseke *et al.*, 2001; Koops and Pommerening-Röser, 2001). Further phylogenetic investigations made it evident that various distinct groups of nitrifying organisms exist (Figs. 4 and 5) (Ward *et al.*, 1997; Gieseke *et al.*, 2001). At present ammonia oxidizing bacteria are classified in two phylogenetic groups (Fig. 4). One group is related to the γ subclass of the *Proteobacteria*. Its only genus, *Nitrosococcus*, is represented by two described marine species (Koops and Pommerening-Röser, 2001). The second group belongs to the β subclass of the *Proteobacteria*. Two clusters exist within this assemblage, the *Nitrosospira* cluster (with three genera) and the *Nitrosomonas* cluster. All members of the three genera of the *Nitrosospira* cluster are very closely related to each other, whereas the *Nitrosomonas* cluster reveals at least five distinct lineages of descent (Koops and Pommerening-Röser. 2001).
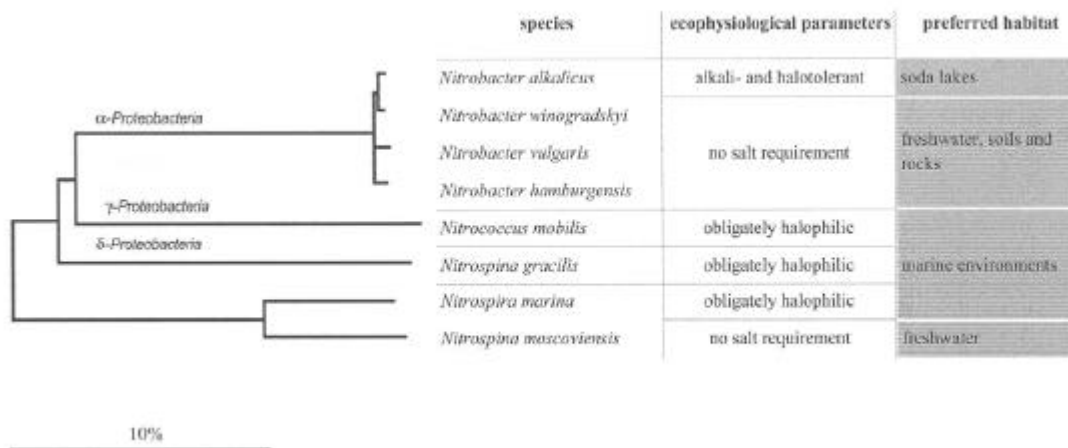
species	ecophysiological parameters	preferred habitat
Nitrobacter alkalicus	alkali- and halotolerant	soda lakes
Nitrobacter winogradskyi		
Nitrobacter vulgaris	no salt requirement	freshwater, soils and rocks
Nitrobacter hamburgensis		
Nitrococcus mobilis	obligately halophilic	
Nitrospina gracilis	obligately halophilic	marine environments
Nitrospira marina	obligately halophilic	
Nitrospina moscoviensis	no salt requirement	freshwater

10%

Fig. 5. Phylogenetic and physiological requirements of cultured nitrite oxidizing bacteria (picture taken from Koops and Pommerening-Röser, 2001).

The current perception of phylogenetic relationships and the diversity of ammonia-oxidizing bacteria is mainly based on comparative sequence analyses of their genes encoding the 16S rRNA and the ammonium monooxygenase (*amoA*). Phylogenetic classification of ammonia oxidizing bacteria by using 16S rRNA almost gives the same divisions as by using *amoA* sequence information (Purkhold *et al.*, 2000), which means that - in the case of ammonia oxidizing bacteria - community data obtained on the basis of 16S rDNA/rRNA analysis can be used to infer functionality. For example, cultivation-independent methods based on 16S rRNA sequence information (e.g. fluorescence *in situ* hybridization: FISH) was used to investigate the diversity and community composition of ammonia oxidizers in diverse environments (Gieseke *et al.*, 2001). PCR-mediated preferential amplification of 16S rDNA and *amoA* from ammonia oxidizing bacteria and subsequent cloning and sequencing have been extensively applied (Rotthauwe *et al.*, 1997; Purkhold *et al.*, 2000; Horz *et al.*, 2000; Gieseke *et al.*, 2001). Polymorphism methods, like RFLP, have been used to study the diversity of communities of ammonia oxidizers (Horz *et al.*, 2000). Potential biases introduced by DNA extraction, PCR amplification, and cloning methods make it very difficult to obtain quantitative information on the community composition by PCR-based methods alone. It is for this reason that *in situ* hybridization techniques in combination with different specific fluorescently labeled oligonucleotide probes targeting the ribosomal RNAs have been successfully used to unravel communities of ammonia oxidizers (Purkhold *et al.*, 2000; Gieseke *et al.*, 2001). The FISH-approach allows to compare community structure with the activity and spatial distribution of the detected organisms (Schramm *et al.*, 1996).

Community studies on wastewater treatment systems have indicated that *Nitrosomonas* strains form one of the most important populations. Three lineages (*Nitrosomonas europaea, Nitrosomonas oligotropha/urea,* and *Nitrosomonas communis* lineages) are routinely observed as dominant organisms in wastewater treatment plants but also in biofilm systems (Gieseke *et al.*, 2001). Very little is known about how and which nitrifying bacteria compete with other related nitrifiers and with heterotrophs in complex systems (Gieseke *et al.*, 2001). Specialized process configurations, like SHARON or CANON, often lead to the development of less diverse populations, probably as a result of adaptation to the process regimes (Logemann *et al.*, 1998; Third *et al.*, 2001). For example, *Nitrosomonas eutropha* was found as the dominant ammonia oxidizer in the SHARON process (Logemann *et al.*,

1998), whereas *Nitrosomonas*-like organisms were also mostly abundant in the CANON reactors (Third *et al.*, 2001).

Nitrite oxidation

Autotrophic oxidation of nitrite to nitrate (Table 1, eq. 4) is a usual subsequent step to ammonium oxidation, although the reaction is carried out by different microorganisms. The key enzyme for nitrite oxidation is the nitrite oxidoreductase.

Nitrite oxidizing bacteria also differ in their physiological requirements and capabilities. Most nitrite oxidizers, except those of the *Nitrobacter* group, are able to use only nitrite to generate biological useful energy. Some *Nitrobacter* strains can use organic compounds for energy metabolism (Bock and Koops, 1992). Nitrite oxidizers are abundant in marine systems, such as *Nitrococcus mobilis*, *Nitrospira marina* and *Nitrospina gracilis*, and therefore are thought to be obligately halophilic (Watson *et al.*, 1971). Others, like *Nitrospira moscoviensis*, are halotolerant (Ehrich *et al.*, 1995). Some other *Nitrobacter* isolates were isolated from marine environments and even from soda lakes (Sorokin *et al.*, 1998).

Nitrite-oxidizing bacteria have been classified into four groups (Fig. 5) (Watson *et al.*, 1971 and 1986; Bock and Koops, 1992; Ehrich *et al.*, 1995; Sorokin *et al.*, 1998). The major group, which belongs to the α subclass of the *Proteobacteria*, is represented by a single genus, *Nitrobacter*, with four described species (Bock and Koops 1992; Sorokin *et al.*, 1998). Two marine species, *N. mobilis* and *N. gracilis* (Waston *et al.*, 1971), were assigned to the γ and the δ subclass of the *Proteobacteria*, respectively (Koops and Pommerening-Röser, 2001). The two species of the genus *Nitrospira*, *Nitrospira marina* and *Nitrospira moscoviensis* (Ehrich *et al.*, 1995), are members of a distinct phylum close to the δ subclass of the *Proteobacteria*. Nitrite-oxidizing bacteria have been detected on the genus level by using monoclonal antibodies targeting different subunits of the nitrite oxidoreductase, the key enzyme of all known nitrite oxidizing bacteria, either directly via immunofluorescence microscopy or indirectly via immunoblotting (Aamand *et al.*, 1996; Bartosch *et al.*, 1999; Spieck *et al.*, 1996; Spieck *et al.*, 1998; Bartosch *et al.*, 2002). Phylogenetic studies suggest that *Nitrospira* bacteria may be widespread in terrestrial habitats (Bartosch *et al.*, 2002).

The question of the diversity of nitrite oxidizing bacteria in wastewater treatment plants has been intensively studied (Daims *et al.*, 2000 and 2001). On the basis of all available 16S rDNA sequence information the genus *Nitrospira* was divided into four distinct sublineages. Numerous bacteria related to the two isolated species from the genus *Nitrospira* have been detected by sequencing 16S rDNA fragments amplified from different environmental samples (not only wastewater treatment plants). These findings seem to suggest that *Nitrospira* are widely distributed in nature and probably contribute significantly to global nitrite oxidation. However, the lack of further pure cultures of *Nitrospira*-related bacteria restricts the knowledge on their physiology and genetics. In contrast to textbook knowledge, *Nitrospira*-like bacteria and not *Nitrobacter* spp., are the dominant nitrite oxidizers both in most full-scale wastewater treatment plants and in laboratory scale reactors (Daims *et al.*, 2000). Based on FISH data combined with microelectrode measurements, it was suggested that *Nitrospira*-like nitrite oxidizers represent K strategists adapted to low nitrite and oxygen concentrations, while *Nitrobacter* sp., as an R strategist, thrives if nitrite and oxygen are present in higher concentrations (Daims *et al.*, 2001).

Anaerobic ammonium oxidation

Ammonia can be used as an inorganic electron donor, both aerobically and anaerobically. The anaerobic reaction proceeds with nitrite as electron acceptor and is called the anammox process. The free energy for this reaction (–357 kJ/mol, Table 1, eq. 6 and Fig. 1) is even more favorable as aerobic ammonium oxidation. Evidence for the occurrence of anaerobic ammonium oxidation was obtained seven years ago in a pilot plant in Delft in the Netherlands, where it was noticed that ammonium disappeared from the reactor effluent together with nitrite with a concomittant production of dinitrogen gas (Mulder *et al.*, 1995). A novel metabolic pathway was postulated for ammonium oxidation with nitrite (van de Graaf *et al.*, 1996). In follow-up studies, the biological nature of the anammox process and the responsible microorganisms were investigated in great detail (Strous *et al.*, 1999a). Although the anammox bacterium could not be obtained in pure culture until now, it could be enriched and further purified on selective gradients to sufficient purity (99%) to characterize it. The group in Delft discovered that the anammox bacterium in their reactors was an unknown organism and proposed a new genus and species name for it (Candidatus *Brocadia anammoxidans*) (Strous *et al.*, 1999b). The physiology of the organism was mainly investigated on a highly enriched culture (80% anammox microorganisms) grown in a sequencing batch reactor (Strous *et al.*, 1998). The anammox pathway proceeds via hydroxylamine and hydrazine as intermediates (Fig. 1). From cell extracts of Candidatus *Brocadia anammoxidans*, a new type of hydroxylamine oxidoreductase (HAO) was purified to homogeneity (Schalk *et al.*, 2000). The enzyme was able to oxidize hydroxylamine and hydrazine. Inhibition experiments with H_2O_2 showed that hydroxylamine binds to a P-468 cytochrome, which is assumed to be the putative substrate binding site. The amino acid sequences of several peptide fragments of HAO from *B. anammoxidans* showed clear differences with the deduced amino acid sequence of HAO from the aerobic ammonia-oxidizing bacterium *Nitrosomonas europaea*.

Since the discovery of the anammox process in Delft, evidence for anammox activity has been obtained in a variety of engineered systems, such as a rotating biological contactor (Siegrist *et al.*, 1998; Helmer and Kunst, 1998; Helmer *et al.*, 2002; Schmid *et al.*, 2000; Pynaert *et al.*, 2002), sequencing batch reactors (Strous *et al.*, 1998), enrichments (Strous *et al.*, 1999a; Egli *et al.*, 2001; Toh *et al.*, 2002), pilot plants for ammonium removal (van Dongen *et al.*, 2001; Fux, 2003), and even marine sediments (Thamdrup and Dalsgaard, 2002). In some cases (Thamdrup and Dalsgaard, 2002) ^{15}N measurements suggested that nitrate rather than nitrite was reduced to dinitrogen (albeit with a transient accumulation of nitrite) with a concomittant removal of ammonium. Only in one other case have bacteria carrying out the anammox reaction been enriched to sufficiently high purity to characterize their physiology (Chapter 2; Fig. 1 and 6). The physiology of these two anammox bacteria is now better understood (Jetten *et al.*, 1999; Strous *et al.*, 1999a; Schalk *et al.*, 2000; Egli *et al.*, 2001; Schmid et al., 2001a). Anammox is a newly and not yet fully explored part of the biological nitrogen cycle, and the process offers new opportunities for wastewater engineers and microbiologists.
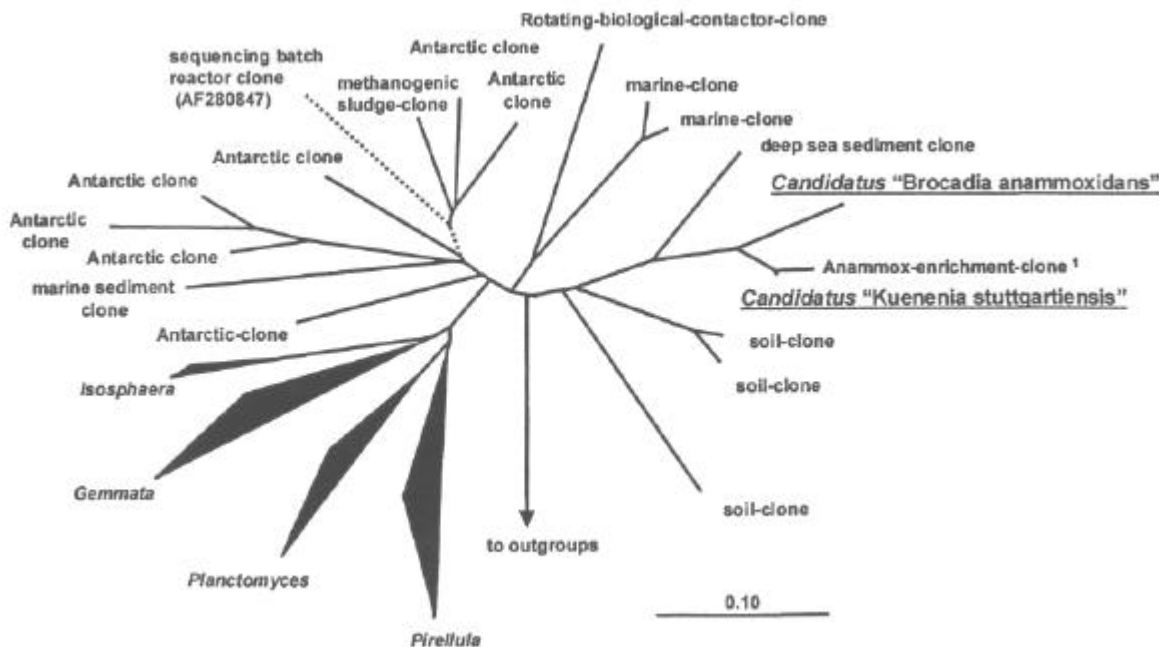
Fig. 6. 16S rDNA based phylogenetic tree reflecting the relationships of Candidatus *Kuenenia stuttgartiensis* and Candidatus *Brocadia anammoxidans*, cloned 16S rDNA sequences of environmental organisms with unknown physiology affiliated to the order Planctomycetales, the "classical" planctomycetes (genera *Pirellula*, *Planctomyces*, *Gemmata* and *Isophaera*), and other reference organisms. The triangles indicate phylogenetic groups. The bar represents 10 % estimated sequence divergence. [1]Sequence published by Egli (Egli *et al.*, 2001). Figure is from Schmid (Schmid *et al.*, 2001b). Rotating biological contactor clone is from the 16S rDNA clone library made from DNA extracted from the Kölliken biofilm (Chapter 3).

From the original enrichment culture in Delft, one microorganism (named Candidatus *B. anammoxidans*) was identified as catalyst carrying out the anammox reaction. Candidatus *B. anammoxidans* belongs to the *Planctomycetes* (Strous *et al.*, 1999b), a morphologically and phylogenetically distinct group of organisms. They form one of the major divisions of the domain Bacteria but the understanding of their ecological functions is limited (Fuerst, 1995). Species within the order *Planctomycetales* lack peptidoglycan, an almost universal polymer found within the domain Bacteria. Instead, protein is the major constituent of their cell walls. Among the domain Bacteria, this lack of peptidoglycan is a characteristic shared only with the *Chlamydiae* and the cell-wall-free *Mycoplasms* (Lindsay *et al.*, 2001).

The characterization and identification of *B. anammoxidans* has also led to the development of suitable oligonucleotide probes targeting the 16S rRNA thereby enabling the possibility for detection of anammox bacteria in various wastewater engineering systems (Siegrist *et al.*, 1998; Schmid *et al.*, 2000; Hippen *et al.*, 1901). By applying this set of oligonucleotides, two different anammox bacteria could be clearly separated. The second anammox bacterium, now named Candidatus *Kuenenia stuttgartiensis* (Schmid *et al.*, 2000), has been found in a variety of wastewater treatment plants (Chapter 3, Schmid *et al.*, 2000; Hippen *et al.*, 2001). It originates from a slightly different phylogenetic position as *Brocadia*.

Outline of this thesis

The topic of this PhD thesis was initiated with an unusual observation on nitrogen removal in the middle of the nineteen nineties at the landfill in Kölliken (AG,

Switzerland). A biofilm system, grown on a rotating biological contactor (RBC), was used to treat the landfill leachate for ammonium after almost all organic carbon was removed in a previous stage reactor. Soon it became evident that the inlet concentration of ammonium was much higher than the outlet concentration of nitrate, whereas both compounds were the main inorganic nitrogen compounds in the inlet and outlet (Siegrist *et al.*, 1998). Since no organic carbon was present any longer in the leachate at this point, this seemed to exclude denitrification as process for nitrogen removal. Instead, it was assumed that a similar type of anaerobic ammonium oxidation, as had been described earlier in the Netherlands (Mulder *et al.*, 1995), was occurring in the Kölliken reactor system. Since the anammox process has potential economical advantages for reactor systems designed for nitrogen removal and nothing much was known about the process in Kölliken, it was decided to focus on the following four major topics during the PhD work.

a) Enrichment and characterization of an anammox bacterium from the rotating biological contactor treating ammonium-rich leachate (Chapter 2).

b) Composition and structure of the ammonium removing biofilm grown of the rotating disk contactor treating an ammonium-rich wastewater (Chapter 3)

c) Population analysis during start-up of nitritation-reactors (Chapter 4).

d) Finally, some work was started to trace anammox bacteria in different environments by using the polymerase chain reaction (Chapter 5).

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Chapter 2: Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate

Abstract

Anaerobic ammonium oxidation with nitrite to N_2 (anammox) is a recently discovered microbial reaction with interesting potential for nitrogen removal from wastewater. We enriched an anammox culture from a rotating disk contactor (in Kölliken, Switzerland) that was used to treat ammonium-rich leachate with low organic carbon content. This enrichment led to a relative population size of 88 ± 5 % anammox bacteria. The microorganism carrying out the anammox reaction was identified by analysis of the 16S rDNA sequence and by fluorescence *in situ* hybridization (FISH) with 16S rRNA targeting probes. The percentage sequence identity between the 16S rDNA sequences of the Kölliken anammox organism and the archetype anammox strain Candidatus *Brocadia anammoxidans* was 90.9%, but between 98.5 and 98.9% with Candidatus *Kuenenia stuttgartiensi*s, an organism identified in a biofilm by molecular methods. The Kölliken culture catalyzed the anaerobic oxidation of ammonium with nitrite in a manner seemingly identical to that of Candidatus *B. anammoxidan*s, but exhibited higher tolerance to phosphate (up to 20 mM) and to nitrite (up to 13 mM) and was active at lower cell densities. Anammox activity was observed only between pH 7 and 9, with an optimum at pH 8 and a temperature optimum at 37 °C. Hydroxylamine and hydrazine, which are intermediates of the anammox reaction of Candidatus *B. anammoxidan*s, were utilized by the Kölliken organisms, and approximately 15% of the nitrite utilized during autotrophic growth was converted to nitrate. Electron microscopy showed a protein-rich region in the center of the cells surrounded by a doughnut-shaped region containing ribosomes and DNA. The cells did not contain an unusual high amounts of nitrogen. The doughnut-shape region was observed with FISH as having a higher fluorescence intensity. Similar to Candidatus *B. anammoxidans,* the Kölliken anammox organism typically formed homogenous clusters containing up to several hundred cells within an extracellular matrix.

Introduction

Nitrogen removal is an important aspect of wastewater treatment often accomplished by microbial processes such as nitrification and denitrification. These reactions have been known for a long time (Winogradsky, 1890; Kluyver and Donker 1926; Beijerinck and Minkman 1910) and have been successfully applied in most modern wastewater treatment plants. Recently, a new microbial process for nitrogen removal was observed in a fluidized bed-reactor in Delft (The Netherlands) (Mulder *et al.*, 1995). The process, called anaerobic ammonium oxidation (anammox), involves oxidation of ammonium to N_2 with nitrite as electron acceptor under strictly anoxic

conditions (van de Graaf *et al.*, 1995 and 1996; Strous *et al.*, 1997). ^{15}N-studies have shown that one N-atom of the produced N_2 originates from nitrite, whereas the other originates from ammonium. Hydroxylamine (NH_2OH) and hydrazine (N_2H_4) were identified as intermediates of the anammox process (van de Graaf *et al.*, 1996; Fig. 1).
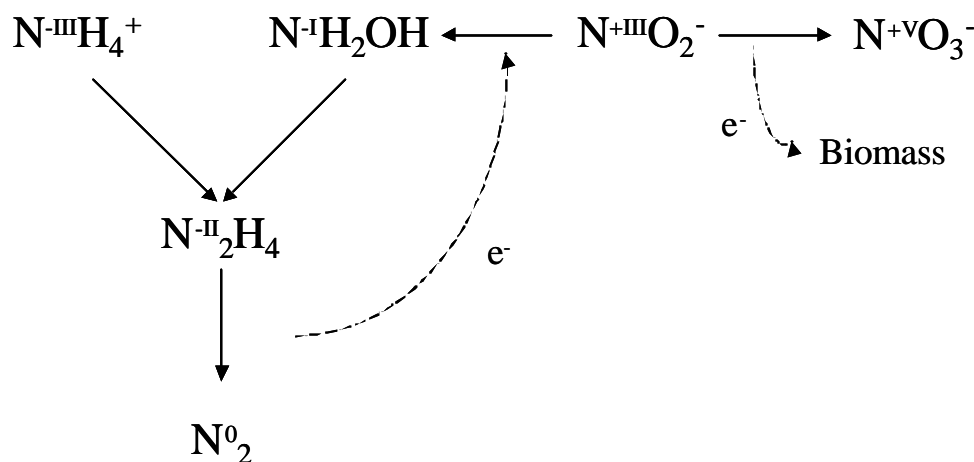
$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad\qquad (Eq.\ 1)$$

Fig. 1. Anammox reaction as suggested (van de Graaf *et al.*, 1996).

The existence of bacteria capable of catalyzing the anammox reaction (Eq. 1) had already been predicted in 1977 (Broda, 1977) based on thermodynamic calculations. Further studies have shown that the anammox process is carried out by an autotrophic bacterium (van de Graaf *et al.*, 1996), which has recently been shown to belong to the order *Planctomycetales* (Fuerst, 1995) and has been named Candidatus *Brocadia anammoxidans* (Strous *et al.*, 1999a). This organism was also postulated to couple the oxidation of nitrite to nitrate for generating reducing equivalents for CO_2 fixation (van de Graaf *et al.*, 1997). Candidatus *B. anammoxidans* grows slowly and has been reported to have a doubling time of 11 days at pH 8 and 40 °C (Jetten *et al.*, 1999).
The advantages of the anammox process over the traditional combination of nitrification and denitrification for wastewater treatment are lower oxygen demand, which is needed by nitrifiers for partial oxidation of ammonium to nitrite, and no requirement for external carbon sources because the process is autotrophic. However, process startup could be hindered by the relatively low growth rate of anammox bacteria. Implementing the anammox process as a manageable wastewater treatment technology will also require a better understanding of the range of permissible nitrite, ammonium, and organic carbon loads, and oxygen and pH regimes. The basic physiological aspects of the anammox process have been studied in depth mostly by a single research group in Delft (Jetten *et al.*, 1999). However, anammox activity has been suspected for other wastewater treatment plants with uncharacterized high ammonium losses (Helmer and Kunst 1998; Siegrist *et al.*, 1998; Schmid *et al.*, 2000; Pynaert *et al.*, 2002). To understand the anammox process and its importance in natural or engineered systems, it is desirable to identify and understand other anammox bacteria besides the archetype strain Candidatus *B. anammoxidan*s. Unfortunately, anammox organisms have been extremely difficult to cultivate in pure culture. Even Candidatus *B. anammoxidans* has only been purified to apparent homogeneity by Percoll density centrifugation (Strous *et al.*, 1999a). Here, we

describe the enrichment of anammox activity from biofilm material from a rotating biological contactor (RBC). Emphasis was placed on characterizing the anammox organism in this enrichment and on comparing its physiological characteristics to those of Candidatus B. *anammoxidan*s.

Material and Methods

Biomass and mineral medium

The culture was enriched from biofilm material from an anammox/nitrifying RBC in Kölliken near Aarau, Switzerland. This reactor exhibited 40–70% removal of inorganic nitrogen at very low organic carbon concentrations (Siegrist *et al.*, 1998). Anammox bacteria were enriched in 2.25-l Schott flasks containing approximately 2 l mineral medium with ammonium as electron donor, nitrite as electron acceptor and bicarbonate as carbon source. The bottles were closed with gastight butyl septa and incubated in an anaerobic chamber filled with nitrogen gas and traces of H_2. The initial pH was 7 and was adjusted regularly with HCl. The medium contained initially in demineralized water: 3 mM $(NH_4)_2SO_4$, 6 mM $NaNO_2$, 25 mM $KHCO_3$, 1 mM K_2HPO_4, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2 ml of trace element solution 1 and 1 ml trace element solution 2 per liter. Trace element solution 1 contained (per liter demineralized water) 10 g $Na_2EDTA·2H_2O$ and 5 g $FeSO_4$. Trace element solution 2 contained (per liter demineralized water) 15 g $Na_2EDTA·2H_2O$, 0.43 g $ZnSO_4·7H_2O$, 0.24 g $CoCl_2·6H_2O$, 0.99 g $MnCl_2·4H_2O$, 0.25 g $CuSO_4·5H_2O$, 0.22 g $NaMoO_4·2H_2O$, 0.19 g $NiCl_2·6H2O$, 0.08 g Na_2SeO_3, 0.014 g H_3BO_4.

Physiological experiments

All experiments were performed in 60-ml serum bottles filled with 43 ml medium and inoculated with 2 ml of an anammox bacteria suspension from the enrichment culture. The bottles contained a 15-ml headspace and were closed with butyl rubber stoppers (Maagtechnic, Dübendorf, Switzerland) and aluminum crimp seals. After inoculating the anammox culture and before starting incubations, the headspace was exchanged by applying several cycles of vacuum and overpressure (1.5 bar) or by flushing with a gas mixture containing 20% (mol% or mol-ppm) CO_2 (99.998% purity) and 80% He (99.9990%). The initial gas pressure of the headspace was 1.5 bar. All experiments were performed in triplicate at 37 °C and pH 7, unless stated otherwise. To study the effect of pH, a 20 mM phosphate buffer was used for experiments between pH 6 and 8, and a 50 mM Tris/HCl-buffer was used for the range between pH 8 and 9. The initial pH of the medium was adjusted by mixing different amounts of KH_2PO_4 and K_2HPO_4 and remained constant during the experiment. In the pH experiments, the headspace was flushed only with He (99.996%), and only 1 mM instead of 25 mM $KHCO_3$ was added. In control experiments without ammonium or nitrite, $(NH_4)_2SO_4$ or $NaNO_2$ were replaced with 2 mM Na_2SO_4. In experiments testing oxygen sensitivity, initial oxygen concentrations were set at 0.25, 0.5, 1 and 18%. Different amounts of oxygen gas were added to reach the different concentrations. The bottles containing 18% O_2 were flushed after 64 h with aligal62 [=20% CO2 (99.5%) and 80% Ar (99.996%)]. All gases were purchased from Carbagas (Rümlang, Switzerland).

The C and N content of the cells were determined with a CHNS-O analyzer (Carlo Erba Instruments, Milano, Italy). Cells used for C and N analysis were washed three times with distilled water and lyophilized prior to analysis.

Nucleic acid extraction
Genomic nucleic acids were isolated from a 15-ml sample from the enrichment culture after four rounds of subculturing. The cells were pelleted and resuspended in a screw-capped vial in 1 ml of homogenization buffer (200 mM Tris-HCl pH 8.5, 1.5% SDS, 10 mM EDTA, 1% sodium desoxycholate, 1% nonidet-P40, 5 mM urea and 10 mM dithiothreitol). Glass beads (0.5 g, 0.1 mm in diameter) were added and the mixture was shaken twice for 1 min at 4,000 rpm in a Braun Cell Homogenizer (Inotech, Dottikon, Switzerland) with a 1-min interval on ice. The glass beads were allowed to settle without centrifugation, and the supernatant was transferred to a fresh Eppendorf tube. This sample was mixed 1:1 with acidic phenol (pH 5.5) and incubated in a water bath at 65 °C for 5 min (Aiba *et al.*, 1981). After vortexing and centrifugation for 5 min at 13000 rpm (13K) and 4 °C, the water phase was transferred to a new Eppendorf tube and mixed 1:1 (v/v) with Tris/HCl buffered phenol (pH 8.0). After vortexing, chloroform/isoamylalcohol (24:1 v/v) was added, vortexed and centrifuged for 5 min as before. The water phase was again recovered and extracted with 1 ml of chloroform/ isoamylalcohol as before. The supernatant was transferred into a new Eppendorf tube, and DNA and RNA were pelleted with 0.7 volumes isopropanol and 0.1 volumes 3 M sodium acetate (pH 5.5) for 1 h at –80 °C. The tube was subsequently centrifuged for 15 min at 13 K to recover nucleic acids. The pelleted nucleic acids were washed with a solution of 70% (vol/vol) ethanol and 30% of a solution of 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) dissolved in RNase-free water.

Amplification of 16S rDNA
16S rDNA fragments from isolated total DNA of the enrichment culture were amplified with Taq DNA polymerase (GIBCO BRL, Life Technologies, Gaithersburg, Md.). The following eubacterial primers were used: 16S 6F (5-ggagagttagatcttggctcag-3) and 16S 1510R (5-gtgctgcagggttaccttgttacgact-3). As anammox-specific primers we used Pla46rc (5-ggattaggcatgcaagtc-3) and Amx820 (5-aaaacccctctacttagtgccc-3). Dimethylsulfoxide was added at 5%. Conditions for PCR were as suggested by the supplier, but with a cycling regime of: 4 min at 95 °C, then 35 cycles each of 45 s at 95 °C, 45 s at 56 °C, and 1 min at 72 °C. Final extension was carried out for 3 min at 72 °C. The 16S rDNA PCR products were cloned in *Escherichia coli* DH5α in vector pGEM-T-easy (Promega, Wallisellen, Switzerland) by established procedures (Sambrook *et al.*, 1989). Plasmid DNAs for sequencing were isolated by boiling preparation (Sambrook *et al.*, 1989). Plasmid inserts were sequenced on both strands by using a Thermosequenase Kit (Amersham, Little Chalfont, UK) with IRD-800 and IRD-700 labeled primers (MWG Biotech, Ebersberg, Germany). Universal vector-located primer sequences were used and, in addition, two primers which targeted a conserved region around position 785 of the cloned 16S rDNA fragment: EUB785R (5-ggattagatacccctg-gtag-3) and EUB785F (5-ctaccagggtatctaatc-3). Sequence transcripts were separated and analyzed on a LiCOR 4200L IR^2 automated DNA sequencer (LiCOR, Lincoln, Neb.). The nucleotide sequence of the 16S rRNA gene of the Kölliken anammox organism was deposited in the GenBank database under accession no. AJ250882. Sequence comparisons were calculated by direct FastA alignments (Pearson and Lipman, 1988).

Analytical measurements

Concentrations of O_2 and N_2 were measured with a gas chromatograph (GC) (type GC-8A; Shimadzu, Tokyo, Japan). A gas volume of 1 ml (at the pressure existing in the sample flask) was injected with a gas-tight syringe (GC syringe series A2; Supelco, Switzerland). The carrier gas was helium (99.999%). The GC was equipped with a thermal conductivity detector and two parallel packed columns. One column was packed with molecular sieve 5A 80/100 (Brechbühler, Schlieren, Switzerland), the other with Porapack Q 80/100 (Brechbühler). The concentration of nitrate (NO_3^-) was quantified by ion exchange chromatography using a DX500-apparatus with IONPAC-ATC1 (anion trap column), IONPAC-AG11 guard column, an analytical IONPAC-AS11–4-mm column, ASRSII-4-mm suppressor, and CD20 conductivity detector (Dionex, Olten, Switzerland).

The concentration of nitrite (NO_2^-) was determined either with ion chromatography as described above or colorimetrically. For the colorimetric quantification, cells were removed from the sample by centrifugation. Fifty µl of the sample was mixed with 950 µl of the reagent, which contained 1% sulfanilic acid, 0.05% N-(1-naphthyl)-ethylene diammoniumdichloride (Riedel-deHaën, purchased from Fluka, Switzerland) and 1 M of H_3PO_4. After 5 min of incubation at room temperature, the color development was complete and the absorbance at 540 nm was determined. Ammonium was analyzed spectrophotometrically as described elsewhere (Scheiner, 1976). Phenol reagent (0.4 ml) and hypochlorite reagent (0.6 ml) were added to a 1 ml sample, followed by incubation at room temperature in the dark for at least 2 h. The absorbance was measured at 635 nm. The concentration of hydroxylamine (NH_2OH) was determined according to the method of Frear and Burrel (Frear and Burrel, 1955). The following reagents were used: 1 g 8-quinolinol dissolved in 100 ml absolute ethanol, 1 M sodium carbonate, 12% (w/v) trichloroacetic acid and 0.05 M sodium phosphate buffer pH 6.8. Sodium phosphate buffer pH 6.8 (0.3 ml) and trichloroacetic acid solution (60 µl) were added to the sample (50 µl), which was followed by the addition of 8-quinolinol solution (0.3 ml). The solution was mixed, and the tube was closed and placed in a boiling water bath for 1 min. After cooling for 15 min at room temperature, the absorbance was measured at 705 nm.

The concentration of hydrazine (N_2H_4) was measured according to Watt (Watt, 1952). A 50-µl sample was incubated for 10 min at room temperature with 0.95 ml of a reagent containing 0.4 g *p*-dimethylaminobenzaldehyde, 20 ml ethanol and 2 ml of 37% HCl. The absorbance was measured at 458 nm. The absorbance increased linearly with hydrazine up to concentrations of 0.4 mM. All spectrophotometric measurements were performed with a Uvikon 860 spectrophotometer (Kontron, Switzerland).

In situ **hybridization (FISH)**

Cells were recovered by centrifugation and resuspended in phosphate-buffered saline (PBS), pH 7.4, consisting of 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per liter distilled water. The samples were fixed by adding three volumes of 4% paraformaldehyde (in PBS), mixing and incubating for 3 h on ice. Afterwards, the cells were washed twice with PBS. The fixed cells were suspended in a solution of 50% PBS and 50% ethanol, and the mixture was stored at –20 °C. Glass slides (Huber & Co., Reinach, Switzerland) were coated with gelatin (Amann *et al.*, 1990). Between 2 and 10 µl of a fixed sample was applied on a well of the coated slide, dried for 3 h at 45 °C and sequentially dehydrated in solutions of 50%, 80% and 100% ethanol

(vol/vol) in PBS for 3 min each. To start hybridization, 9 µl of hybridization buffer (containing the appropriate NaCl and formamide concentration for the used probe, Table 1) and 1 µl of a fluorescently labeled probe (at a concentration of 50 ng/µl) were added to a well. The hybridization was conducted for 90 min at 45 °C in a humidified chamber. Following hybridization, a stringent washing step was performed for 15 min at 48 °C in a buffer with the appropriate NaCl concentration (Table 1), 20 mM Tris-HCl, pH 7, 5 mM EDTA and 0.01% SDS. The samples were counterstained with DAPI (4,6-diamidino-2-phenylindole, Hicks *et al.*, 1992) at a final concentration of 10 mg/l for 5 min at room temperature and mounted in Citifluor (Citifluor, London, UK).

Table 1. Probes used for fluorescence *in situ* hybridization

Probe-name	Sequence (5´-3´)	Reference	% Formamide
EUB338	gctgcctcccgtaggagt	Amann *et al.*, 1990	0 %
EUB338II	gcagccacccgtaggtgt	Daims *et al.*, 1999	or in combination
EUB338III	gctgccacccgtaggtgt		with other probes
Alf1b	cgttcgytctgagccag	Manz *et al.*, 1992	20 %
Bet42a	gccttcccacttcgttt		35 %
Amx820	aaaacccctctacttagtgccc	Schmid *et al.*, 2000	40 %
Amx1240	tttagcatccctttgtaccaacc		60%
Amx432	gttaactcccgacagtgg		40 %
Amx997	tttcaggtttctacttctacc		20 %
Kst1275	tcggctttataggtttcgca		25 %
Nit3	cctgtgctccatgctccg	Wagner *et al.*, 1996	40 %
cNit3	cctgtgctccaggctccg		
Ntspa662	ggaattccgcgctcctct	Daims *et al.*, 2000	30 %
cNtspa662	ggaattccgctctcctct		
Pla46	gacttgcatgcctaatcc	Neef *et al.*, 1998	30 %
Pla886	gccttgc gaccatactccc		35 %
Pla886comp	gccttgcgaccatactccc		

All oligonucleotide probes were obtained from Microsynth (Balgach, Switzerland). Microscopy was performed on an Olympus BX50 microscope, equipped with filters HQ-CY3, HQ-FITC and HQ-DAPI (all from AF Analysentechnik). Digital images were taken with a CCD camera (type Sensys, Photometrics, Tuscon, Ariz., USA) and acquired in the program METAVIEW (Visitron, Puchheim, Germany). The relative population size of the anammox bacteria in the enrichment was determined by confocal laser scanning microscopy by M. Schmid at the Technical University of Munich on paraformaldehyde-fixed samples, stained with probe Amx820 and counterstained with the EUB mixture (Daims *et al.*, 1999), according to Schmid (Schmid *et al.*, 2000).

Electron microscopy
Freeze-fraction and transmission electron microscopy were carried out by E. Wehrli from the Laboratory for Electron Microscopy I, the central electron microscopy facility of the Biology Department of the Swiss Federal Institute of Technology (ETH-Zürich). Samples were prepared as described elsewhere (Wehrli and Egli, 1988).

Protein measurements
Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Cell suspensions (2 ml) were pelleted, resuspended in the same amount of 0.1 M NaOH and incubated for 1.5 h in a boiling water bath. An 0.8 ml-aliquot of this suspension was incubated with 0.2 ml of Bio-Rad dye-reagent for 15 min. The absorbance was measured at 595 nm. A standard curve was prepared from known concentrations bovine serum albumin (Fraction V, Sigma).

Results

Enrichment of anammox bacteria
Anammox activity was previously observed in an RBC operated at the landfill site in Kölliken (near Aarau, Switzerland) (Siegrist *et al.*, 1998; Koch *et al.*, 2000). The RBC was used to treat ammonium-rich leachate with low organic carbon content. Cultures incubated under anoxic conditions with approximately 0.5 g of biofilm material scratched from the RBC in 1 l of mineral medium immediately converted nitrite (6 mM) and ammonium (5.2 mM). After several additions of nitrite and ammonium to the same culture flask (in each case to a final concentration of 6 and 5.2 mM, respectively), stable production rates of dinitrogen and nitrate were obtained (data not shown) and the culture density increased slowly. The culture had a brownish-red color, probably due to a high cytochrome content (Jetten *et al.*, 1999). After reaching an OD600nm of 0.08, the enrichment culture was diluted tenfold and used to inoculate a new flask. This dilution and enrichment procedure was repeated three more times within 6 months and led to an increase of the relative population size of anammox bacteria in the culture. The enrichment culture was subsequently analyzed for the presence of anammox bacteria by cloning amplifiable 16S rDNA fragments, and by fluorescence *in situ* hybridization (FISH). Total DNA was isolated from the enrichment culture and 16S rDNA fragments were amplified by PCR using conserved eubacterial primers for almost the entire 16S rDNA length or using the primers Pla46rc and Amx820 (Table 1). The fragments were cloned, sequenced and compared to entries in the GenBank database. Among eleven plasmid inserts in the full-length 16S rDNA library, six were identical and revealed 90.9% sequence identity to the 16S rDNA sequence of Candidatus *B. anammoxidans* (Strous *et al.*, 1999a, GenBank accession number AJ131819). On the basis of such a high percentage of sequence identity with the archetype anammox bacterium, we suspected this 16S rDNA fragment to be representative of possible anammox bacteria in the Kölliken enrichment culture. Furthermore, the percentages of sequence identity of this 16S rDNA fragment of the Kölliken enrichment culture with 16S rDNA fragments derived from biofilm material of a trickling filter operating in Stuttgart were between 98.5% and 98.9% (from a 1407-nucleotide overlap), as determined by direct FastA alignments (GenBank accession numbers AF202655-AF202663, Schmid *et al.*, 2000). The 16S rDNA fragments analyzed from the reactors in Stuttgart have been proposed to originate from anammox microorganisms that were classified as Candidatus *Kuenenia stuttgartiensis* (Schmid *et al.*, 2000). Therefore, the organisms enriched from the biofilm material from Kölliken seem more similar to Candidatus *K. stuttgartiensis* and less similar to Candidatus *B. anammoxidan*s. We verified whether the near-full length 16S rDNA sequence of the Kölliken enrichment was representative for anammox bacteria in the culture by reamplifying a specific part of

the 16S rDNA using one primer targeting a region conserved for *Planctomycetales* (Pla46rc) and one for all known anammox bacteria (Amx820). Eleven cloned 16S rDNA fragments were sequenced, which were all identical to the previously determined, near-full length 16S rDNA sequence (AJ250882). Based on the cloned 16S rDNA sequence from Kölliken, we verified the presence of anammox bacteria in the enrichment culture by FISH. Several oligonucleotide probes (Amx432, Amx820, Amx997, Amx1240), which previously were found to specifically detect Candidatus *B. anammoxidans* in Delft enrichments (Schmid *et al.*, 2000), reacted positively with most organisms in our enrichment culture (Fig. 2).
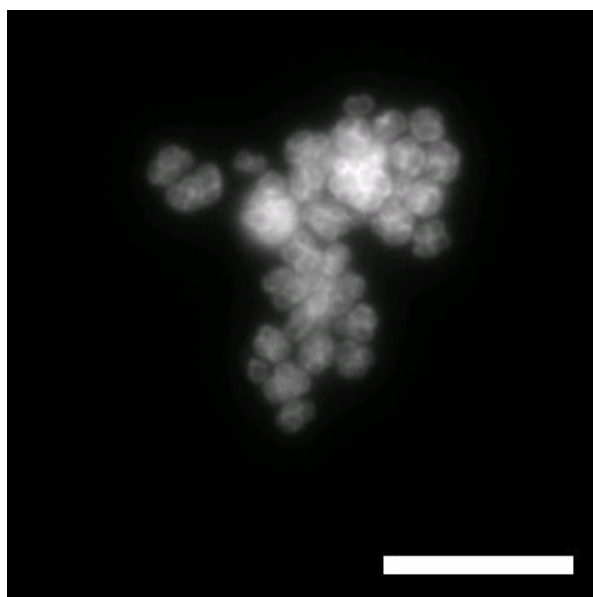
Fig. 2. Sample from the Kölliken anammox enrichment culture, stained with the probe Amx820. Note the optically lighter inside of the cells which results in their "doughnut" appearance. *Bar* 10 μm.

The probe Kst1275, which was developed for the anammox bacteria in Stuttgart (Schmid *et al.*, 2000), reacted positively as well. Several other oligonucleotide probes specific for Candidatus *B. anammoxidans* (i.e., Amx156, Amx223, Amx613 and Amx1154, Schmid *et al.*, 2000) did not react with the Kölliken organism, and the target sequences for these probes were not conserved in the 16S rDNA sequence derived from the Kölliken organism (not shown). Other general probes that reacted positively with most cells in the Kölliken biofilm enrichment were the ALF1b probe originally developed to detect α-Proteobacteria, the EUB338 general eubacterial probe with 0 % formamide, and the Pla46 probe for the order *Planctomycetales* (Neef *et al.*, 1998). Interestingly, the Pla886 probe (Neef *et al.*, 1998) did not hybridize due to two mismatches to the Kölliken anammox sequence (the competitor has one mismatch). The newly developed EUB338II and EUB338III probes (Daims *et al.*, 1999) had at least two mismatches to the Kölliken anammox sequence, although they were designed to detect members of the phyla *Verromicrobia* and *Planctomycetales*. However, the mixture of all three probes reacted positively. Similar to Candidatus *B. anammoxidans* (Strous, 2000*),* FISH-stained cells typically had an inner area with very low fluorescence intensity (Fig. 2). By counting FISH-stained cells with different specific probes, we estimated that the enrichment culture contained approximately 90% anammox organisms (of all DAPI-stained cells). Very precise determination,

however, was difficult since the anammox organisms tended to form stable and relatively large clusters (Figs. 2 and 3). By using confocal laser scanning microscopy on FISH-stained samples, a relative biovolume for the anammox population of 88% ± 5% could be determined (M. Schmid, personal communication). The relative population size of *B. anammoxidans*, used for the physiological experiments, was 80 ± 5 % (Strous, 2000). Since the probes Amx820, Kst1275, Amx1240 and Pla46 all stained the majority of the cells in the enrichment culture, and since the positively reacting cells were all of the same type, we concluded that the derived 16S rDNA sequence originated from the majority of cells in the enrichment. These cells most likely represent anammox bacteria; however, they differed from the Delft archetype strain *B. anammoxidan*s. Furthermore, no positively stained cells were observed with the probes Bet42a (Manz *et al.*, 1992) for the β-Proteobacteria, indicative of most known ammonium oxidizing bacteria, Ntspa662 for *Nitrospira* (Daims *et al.*, 2000) and Nit3 (Wagner *et al.*, 1996) for the genus *Nitrobacte*r. Thin sections of fixed enrichment samples were also analyzed by transmission and freeze-fraction electron microscopy (Fig. 3). Typically, clusters of different size were observed (up to approximately 17 µm in diameter, judged from electron micrographs, Fig. 3). Cells within the clusters seemed embedded in an extracellular matrix, apparently holding the cells tightly together. The clusters in the electron micrographs consisted only of one type of bacterium, similar to the observations with fluorescence microscopy. Cells imaged in electron microscopy on freeze fractions and in thin sections displayed both an inner and an outer membrane, and a putative S-layer (Fig. 3).
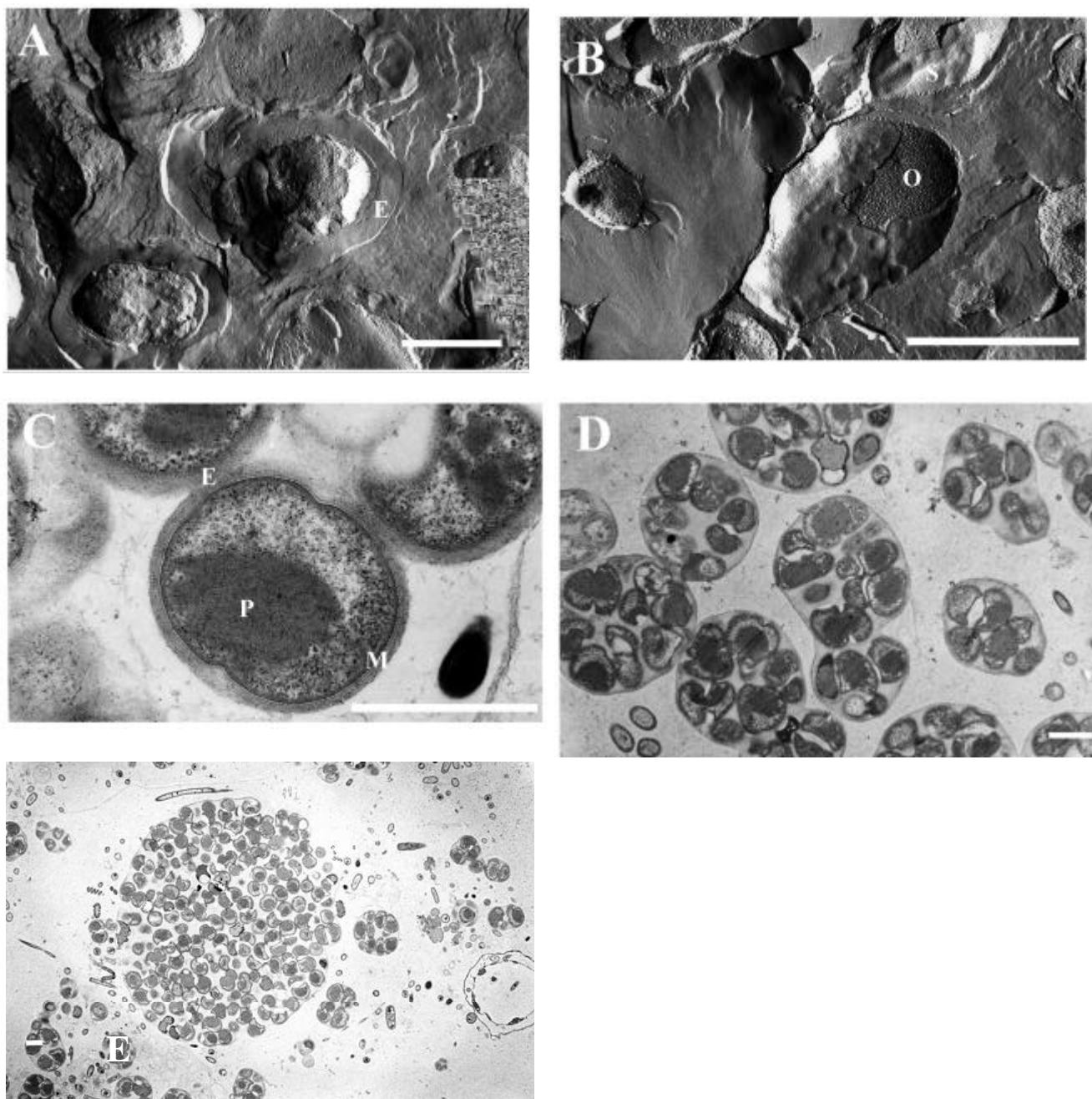
Fig. 3 A-E Electron micrographs of Kölliken anammox cells. Freeze-fractions show cells with a large extracellular polymer matrix (E, panel A) and the S-layer (S) and the outer membrane (O, panel B). With transmission electron microscopy a protein-rich area (P) within the cells (*dark region*, panel C), the (now reduced) extracellular polymer layer (E, panel C) and the membranes (M) are to be seen, panel D). Compact clusters of anammox cells of different sizes were present (panel D and E). *Bar* 1 μm.

It was not possible to elucidate from freeze-fraction electron microscopy whether there were crateriform structures as described for Candidatus *B. anammoxidans* (Strous *et al.*, 1999a). A high density, presumably protein-rich compartment was observed in the center of the cells (Fig. 3). This structure might be similar to the „anammoxosome" described for Candidatus *B. anammoxidans* (Fuerst, 1995; Strous, 2000; Lindsay *et al.*, 2001). Some cells displayed larger white areas that looked like membranes which had collapsed during fixation. Although there was a protein rich area, the nitrogen content of dry mass of the anammox enrichment was only about

10%. Approximately 46% of the dry weight was carbon, indicating no unusual carbon or nitrogen content (Egli, 2000).

Physiological experiments

To determine whether the enrichment culture from Kölliken was indeed performing the anammox reaction, several parameters in anoxic batch cultures were tested and changes in ammonium, nitrite, nitrate and N_2-gas concentrations were measured. A decrease of ammonium and nitrite concentrations and a concomittant production of nitrate and N_2 were observed only when both ammonium and nitrite were present (Fig. 4A).
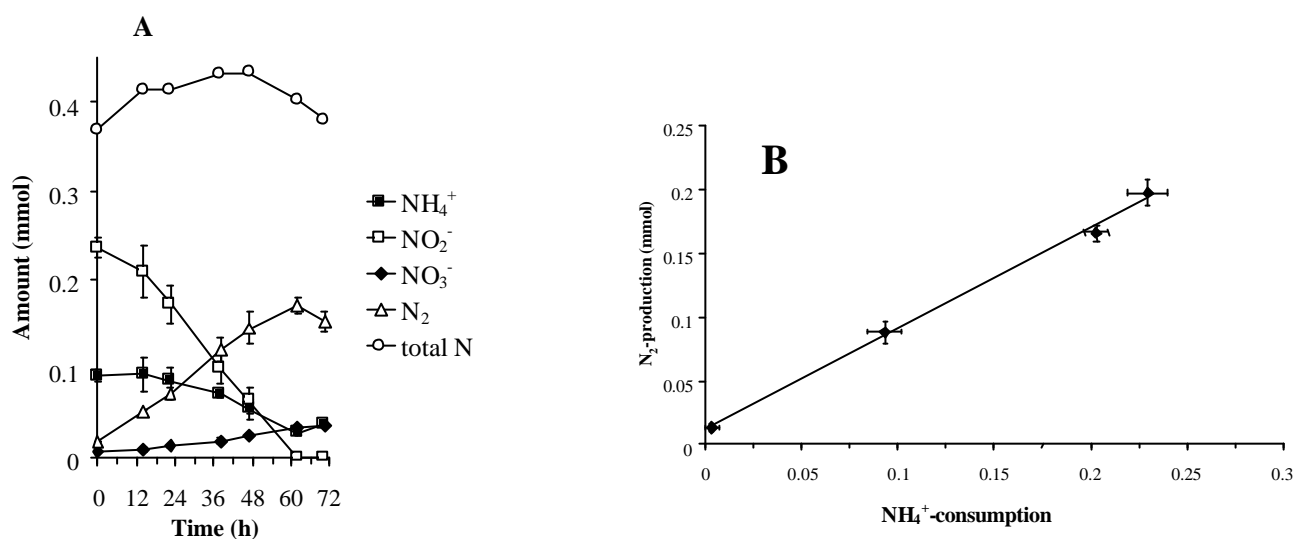
Fig. 4 A, B Anammox activity of the enrichment culture at pH 7.5 and 37 °C. A Disappearance of NH_4^+ and NO_2^- and concomitant formation of N_2 and NO_3^-. Note that the *y-axis* shows the total amount of NH_4^+, NO_2^-, NO_3^- and N_2 in mmol per incubation flask (= 45 ml liquid volume, 15 ml headspace). B Correlation of N_2-production with NH_4^+-consumption in flasks with constant NO_2^- concentrations but with increasing amounts of NH_4^+. N_2-production and NH_4^+-consumption were measured after 4 days. Error bars represent ± one standard deviation from the mean of triplicate incubations.

The rate of N_2 production was approximately 24 nmol N_2 min^{-1} (mg protein)$^{-1}$. When only nitrite or ammonium were added to the enrichment culture, no N_2 was produced and there were no changes in nitrite, nitrate or ammonium concentrations. This indicates that classical nitrification and denitrification reactions did not play a significant role during anoxic incubations. When the anammox culture was incubated with different ammonium concentrations (0, 2, 4 and 6 mM), but with the same nitrite concentration (5 mM), the production of nitrate and N_2 increased linearly with ammonium utilization (Fig. 4B). The overall stoichiometry of mol N_2-N production per mol of ammonium utilized was not significantly different from 1. In addition, nitrate production increased with nitrite utilization at an overall stoichiometry of 0.15 mol nitrate per mol nitrite, which is in agreement with the stoichiometries predicted

40

for the anammox process by Candidatus *B. anammoxidans* (Strous *et al.*, 1998). To determine the optimal temperature for the Kölliken anammox organisms, we carried out incubations at 11, 20, 25, 30, 37 and 45 °C. The highest anammox activity (as rate of N_2 production) was observed at 37 °C. No anammox activity was observed at 45 °C and the activity could not be restored by reducing the temperature to 37 °C. Anammox activity at 11 °C was approximately 24% of that at 37 °C which is slightly higher than expected from the Arrhenius equation.

To investigate any inhibitory effects of nitrite, batch cultures with different nitrite concentrations were incubated, keeping the initial ammonium concentration constant. At 5.3 mM nitrite, anammox activity started immediately (Fig. 5), and, as expected, nitrogen and nitrate production stopped when all nitrite was consumed. At 13.2 mM nitrite, anammox activity did not start until after 3 days (Fig. 5). Until then, no changes in the nitrite, ammonium and nitrate concentrations took place, and no N_2 was produced. Anammox activity stopped when all ammonium was depleted. At higher initial nitrite concentrations, no anammox activity was observed within the tested period. The specific N_2 production at 5.3 and 13.2 mM nitrite and pH 7 was similar at around 13 nmol min^{-1} (mg protein)$^{-1}$. The mean biomass concentration in all incubations was 65 μg protein per ml culture and did not change significantly during the experiments. Therefore, practically no growth occurred during this 1-week experiment, which reflects the slow growth of anammox bacteria.
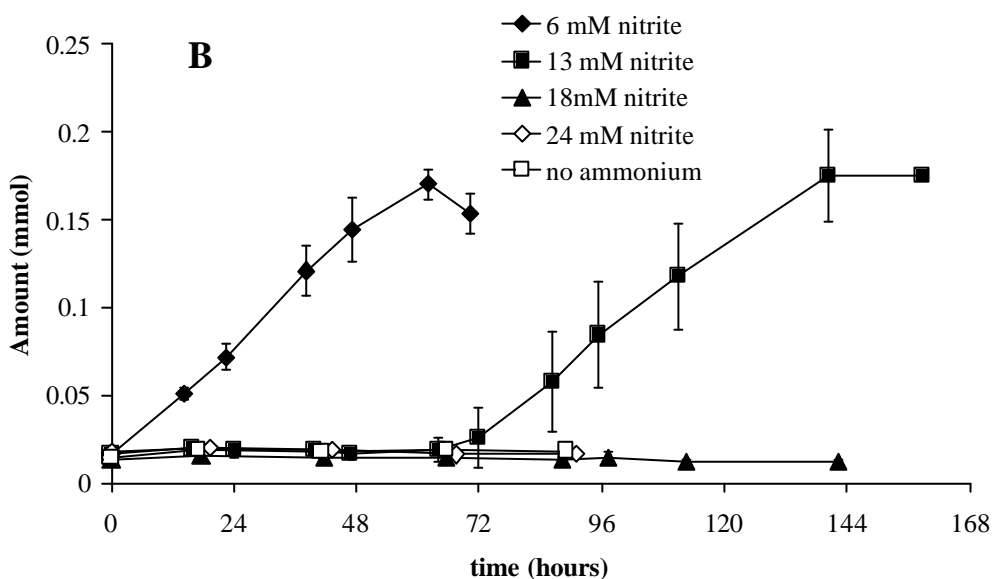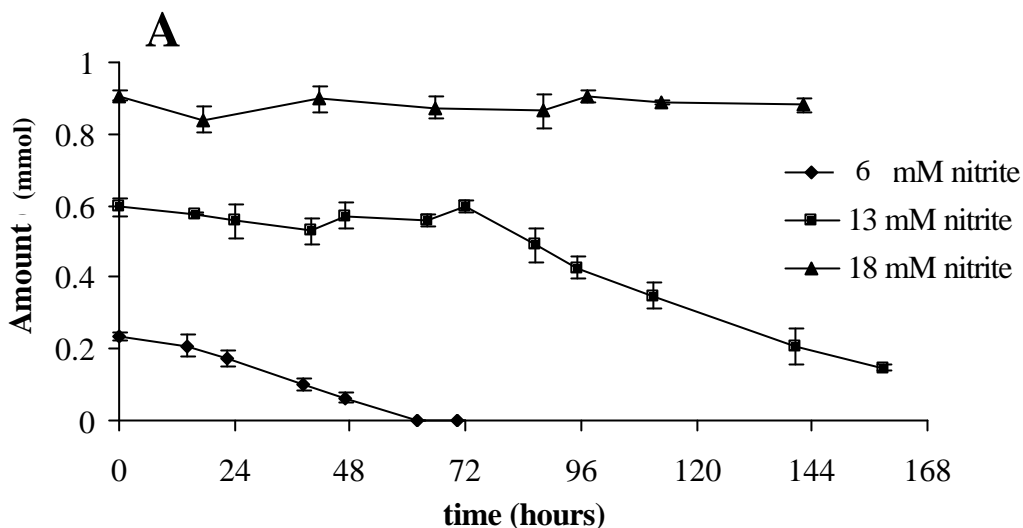
Fig. 5 A, B Effect of increasing nitrite concentrations on the anammox activity of the enrichment culture incubated at the same ammonium concentration (3 mM). A Nitrite concentrations and B N_2 production during incubation. 6 mM Nitrite (corresponding to 0.25 mmol per flask), 12 mM nitrite, 18 mM nitrite, 24 mM nitrite. Error bars represent $\pm$ one standard deviation from the mean of triplicate incubations.

To test the effects of pH of the medium on anammox activity, batch cultures at 37 °C were incubated with nitrite and ammonium at the following pH values: 6, 6.5, 7, 7.5, 8, 8.5 and 9. No anammox activity was observed at pH 6 and 6.5; ammonium and nitrite concentrations remained constant and neither N_2 nor nitrate was produced (Fig. 6). The highest activity was observed at pH 7.5 and 8 [24 and 26.5 nmol N_2 min^{-1}(mg protein)$^{-1}$, respectively].
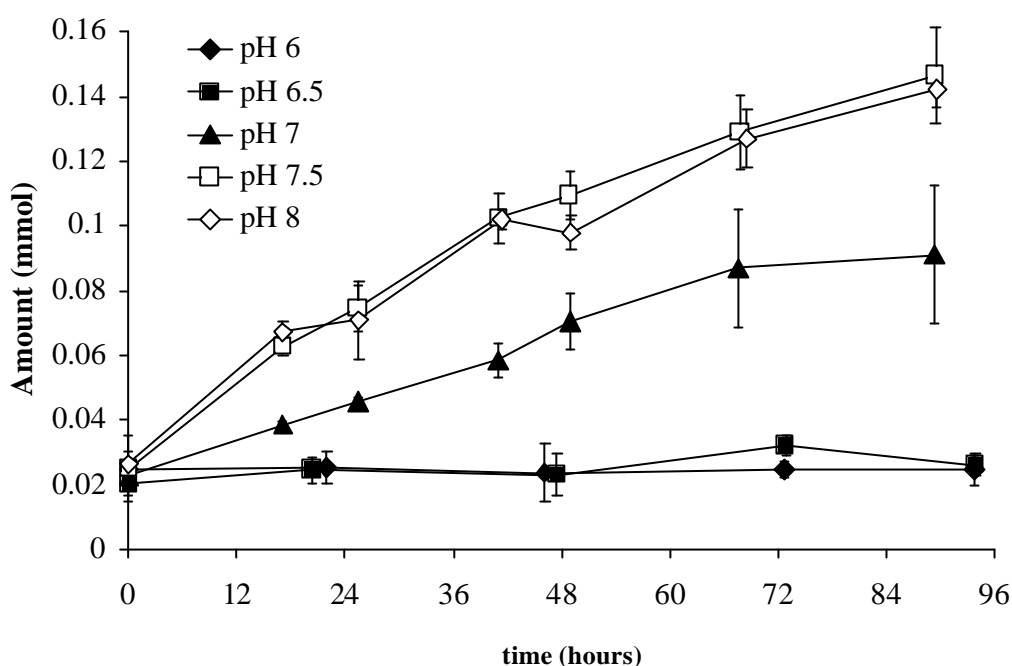
Fig. 6. Effect of pH on the activity of the enrichment culture. Formation of N_2 at pH of 6.5, pH 7, pH 7.5 and pH 8 are shown. Note that y-axis show absolute amounts in mmol per incubation flask (= 45 ml liquid volume and 15 ml headspace). Error bars represent ± one standard deviation from the mean of triplicate incubations.

The N_2-production rate at pH 7 was 56% of the maximum. Anammox activity was also observed at pH 8.5 and 9. There was no significant difference in protein levels of cultures incubated at pH 7, 7.5 and 8 compared to those at pH 6 and 6.5 (mean level: 20.9 µg protein per ml culture), again indicating that practically no growth occurred during the batch incubations. The effect of pH was tested in buffer containing 20 mM sodium phosphate. The N_2 -production rates with 20 mM phosphate (at pH 7) were essentially the same as with bicarbonate buffer (at pH 7), therefore, the anammox reaction was not inhibited by phosphate (Strous, 2000).

The sensitivity to oxygen was tested by adding different amounts of oxygen to the anoxic headspace in batch incubations. Initially, 0.25, 0.5, 1 or 18% of oxygen saturation was present in the headspace. Within 16 h, oxygen at the lowest concentrations (0.25 and 0.5%) had disappeared, probably due to any remaining aerobic microorganisms, and anammox activity started subsequently. Anammox activity was also observed in bottles with 1% initial oxygen concentration, but again only after oxygen had disappeared. No anammox activity occurred within 64 h in bottles with 18% initial oxygen, and anammox activity could not be restored after oxygen was removed. This indicates a reversible inhibition of the anammox process at low oxygen concentrations, but irreversible inhibition at higher oxygen concentrations.

It was tested whether the anammox enrichment culture could be stored at –80 °C with glycerol (15 %) and DMSO (10 %). After freezing at –80 °C during two days and thawing on ice, the bacteria were centrifuged and washed twice with anoxic medium to remove traces of glycerol and DMSO. The biomass was incubated in anammox medium at 37 °C. Since it was thought that hydrazine has an activation effect (Strous,

2000), hydrazine was also added to some of the incubations. Activity was only found without hydrazine present after freezing, but both in cultures frozen with glycerol and with DMSO. In both cases, nitrate was built beside N_2 and a decrease of ammonium and nitrite was observed, which is specific for anammox activity.

During cultivation of the anammox bacteria in Schott flasks as batch culture, ammonium sulfate $(NH_4)_2SO_4$ and sodium nitrite $(NaNO_2)$ were regularly added. Since only ammonium and nitrite was removed, the concentration of the counter ions increased. Upon transfer to a new flask, the biomass was centrifuged and resuspended in a new medium (as described in Material and Methods). Maximum salt concentrations of up to 20 g/L were therefore reached during batch incubations (Table 2). Even at such high salt concentrations anammox activity was still observed.

Table 2. Maximum ion concentration reached in the anammox batch cultures (in mM).

Na^+	K^+	SO_4^{2-}	NO_3^-	Cl^-
250	50	100	40	75

Metabolic process for the anammox pathway

To confirm that the anammox organisms from the RBC in Kölliken were carrying out a reaction similar to that of Candidatus *B. anammoxidans* (van de Graaf *et al.*, 1997, Fig. 1), we tested whether hydroxylamine and hydrazine were possible intermediates in anaerobic ammonium oxidation. In regular incubations with ammonium and nitrite (both at 5 mM), no hydroxylamine or hydrazine were detected at a concentration above the detection limit (i.e. 25 µM and 10 µM). In incubations with 2.8 mM hydroxylamine and 0.8 mM ammonium, hydroxylamine was indeed disappearing (Fig. 7A), and hydrazine was transiently produced (up to 0.31 mM), which is consistent with the hypothesis that ammonium is oxidized with hydroxylamine to hydrazine (Schalk *et al.*, 1998 and 2000). The concentrations of ammonium and N_2 also increased during this experiment, possibly due to hydrazine disproportionation. When hydrazine and nitrite were added to the enrichment culture, both compounds simultaneously disappeared at similar rates (Fig. 7B), which is consistent with the proposed reduction reaction of hydrazine and nitrite to form hydroxylamine (Fig. 1). Ammonium and molecular nitrogen were also produced. In sterile tubes the hydrazine concentration remained constant, indicating that hydrazine disproportionation is a biologically mediated process. When the enrichment culture was incubated with hydrazine and nitrate, ammonium and N_2 also increased whereas hydrazine decreased. The nitrate concentration remained at a constant level (Fig. 8).
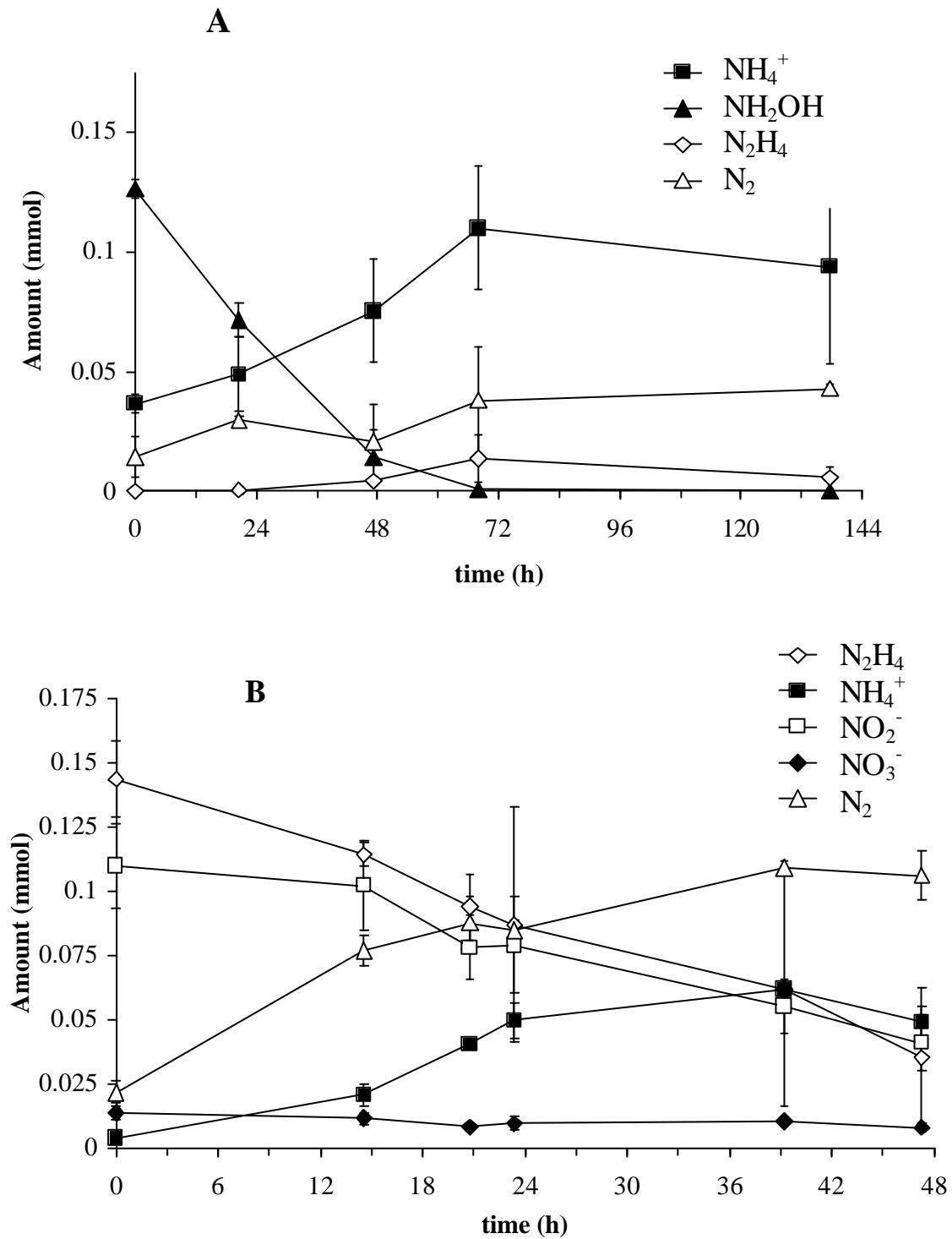
Fig. 7. A, B Addition of possible intermediates to the enrichment culture. A Conversion of hydroxylamine (NH_2OH) with ammonium. B The parallel decrease of hydrazine (N_2H_4) and nitrite. Error bars represent ± one standard deviation from the mean of triplicate incubations.
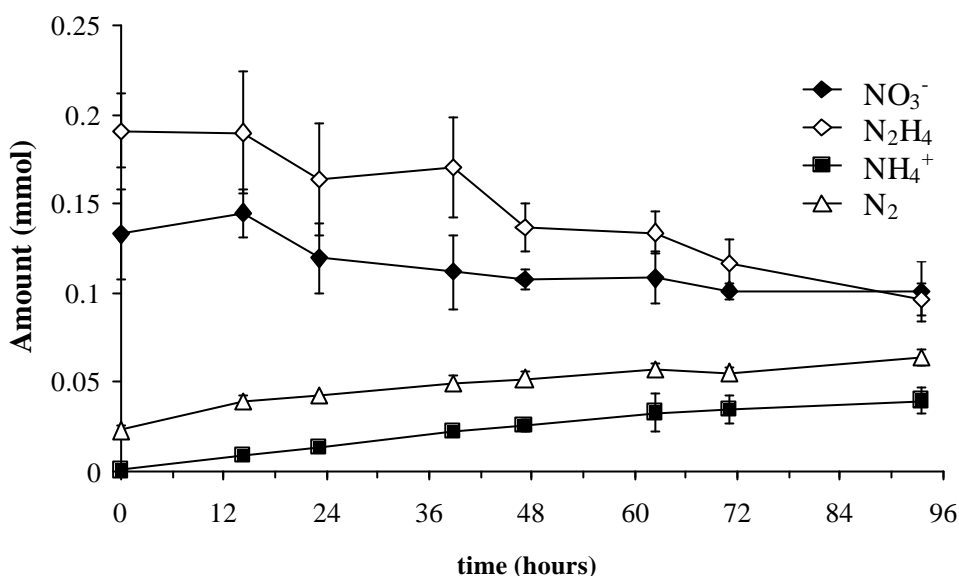
Fig. 8 Addition of nitrate and hydrazine to the enrichment culture. Error bars represent ± one standard deviation from the mean of triplicate incubations.

Discussion

The results presented in this work showed that microorganisms that carry out the anammox process could be enriched from biofilm material of the RBC in Kölliken. Although the experiments were not performed with a pure culture, we established that about 90% of the population was an anammox organism functionally similar to but taxonomically different than Candidatus *B. anammoxidan*s. The 16S rDNA sequence obtained for the Kölliken anammox organism showed highest percentage of identity (between 98.5% and 98.9%) to the 16S rRNA gene sequences of Candidatus *K. stuttgartiensi*s, a putative anammox organism in biofilm reactors in Stuttgart (Schmid *et al.*, 2000). Other sequences from GenBank showed much lower percentages of identity (less than 86%) to the sequence of the Kölliken organism. Based on several specific probe sequences developed for Candidatus *B. anammoxidans* and Candidatus *K. stuttgartiensi*s, and based on other group-specific probes, we showed that 90% of the organisms in the Kölliken enrichment culture possessed the 16S rRNA gene identified in this work. Despite the significant difference in 16S rDNA sequence of *B. anammoxidans* and the Kölliken organism (9.1%), their principal morphological properties were similar. Within the cells, a protein-rich region with lower content of ribosomes than the surrounding area was detected with electron microscopy and by FISH. In addition, the anammox organisms clumped in aggregates of up to 350 cells (judged from the electron micrographs and FISH pictures, Figs. 2 and 3). Since the 16S rDNA sequence of the anammox organisms from the Kölliken biofilm was most similar to Candidatus *K. stuttgartiensis* (Schmid *et al.*, 2000) it seems appropriate to address the Kölliken organisms with the same Candidatus *Kuenenia* genus name. Presently, it is not clear whether the few sequence differences found between the 16S rDNAs of Candidatus *K. stuttgartiensis* and the Kölliken organism represent true phylogenetic and physiological differences. However, since Candidatus *K. stuttgartiensis* was not further enriched to high relative population sizes (their relative abundance in the biofilms was 49%), any physiological differences between the Kölliken and the Stuttgart anammox bacteria still remain to be determined. The physiological experiments confirmed that the Kölliken culture indeed performed the anammox process. N_2 production took place when both ammonium and nitrite were

present and oxygen was absent, but not when ammonium or nitrite was added alone. Still, the anoxic oxidation of ammonium with nitrite could also have been catalyzed by „aerobic" ammonium oxidizers of the genus *Nitrosomonas* (Bock *et al.*, 1995), although the catalytic activity of these organisms is about 20-fold lower than that of Candidatus *B. anammoxidans* (Jetten *et al.*, 1999). However, since no cells in the Kölliken enrichment stained positively in FISH with probes for Bet42a (which would detect most ammonium oxidizers), Ntspa662 or Nit3 (Table 1), and a highly enriched culture (88% anammox organisms) was investigated, this strongly suggests that no classical ammonium- and nitrite-oxidizing bacteria were present at significant population sizes in the enrichment culture. Furthermore, when the nitrite concentration was kept constant, N_2 production increased linearly with increasing ammonium consumption. Therefore, nitrite removal and N_2 production could not be ascribed to denitrification, and ammonium removal could not be ascribed to nitrification at low oxygen levels. The concurrent removal of ammonium and nitrite with N_2 production can only be reasonably explained by the anammox reaction, catalyzed by anammox bacteria. Approximately 15% of the nitrite removed was oxidized to nitrate, which is similar to observations for Candidatus *B. anammoxidans* and seems to be due to the need for electrons to reduce CO_2 for autotrophic growth (van de Graaf *et al.*, 1996; Fig. 1). In addition, both hydroxylamine and hydrazine were converted by the enrichment culture, and a transient accumulation of hydrazine was observed when hydroxylamine was added, possibly due to condensation with ammonium. An increase in N_2 and ammonium concentrations was also observed when hydroxylamine was added, possibly due to its disproportionation. These results suggest that the anammox reaction mediated by the Kölliken organism proceeds as proposed for Candidatus *B. anammoxidans* (van de Graaf *et al.*, 1996). However, since no nitrate was consumed during incubation with hydrazine and ammonium, and nitrate was produced during autotrophic growth, the loss of nitrate observed previously at other sites was not only due to anammox activity (Thamdrup and Daalsgard 2002; Mulder *et al.*, 1995).

Based on the relatively low percentage of identity of the 16S rDNA sequences of Candidatus *B. anammoxidans* and the Kölliken anammox organism, one might expect that the organisms would have physiological differences. However, our data showed only a few physiological differences with respect to the anammox reaction. For example, the optimum pH and temperature of the two organisms were very similar, although the Kölliken organism still showed activity at pH 9, whereas Candidatus *B. anammoxidans* has an activity range between pH 6.7 and 8.3 (Strous 1999b). The Kölliken organisms exhibited higher tolerance to nitrite, up to 13 mM, compared to the 7 mM reported for Candidatus *B. anammoxidans* (Strous *et al.*, 1999b).

The highest observed anammox activity of the Kölliken organism (at pH 8 and 37 °C) was 26.5 nmol N_2 min^{-1}(mg protein)$^{-1}$, which is lower than that reported for Candidatus *B. anammoxidans* [55 nmol N_2 min^{-1}(mg protein)$^{-1}$ at pH 8 and 40 °C, Jetten *et al.*, 1999]. However, the Kölliken anammox organisms were active at low cell densities ($2*10^8$ cells per ml or 20 µg protein per ml), whereas the optimal activity of Candidatus *B. anammoxidans* was reached only in suspensions with a density higher than 10^{10} –10^{11} cells per ml (Strous *et al.*, 1999a). Furthermore the color of the enrichment culture was different which might be due to differences of the present cytochromes. Finally, there was no inhibitory effect of phosphate (up to 20 mM tested) on the activity of the Kölliken culture compared to a loss of activity of Candidatus *B. anammoxidans* when incubated with more than 2 mM phosphate (van de Graaf *et al.*, 1996). Perhaps this is due to a higher affinity of the Kölliken organism

for hydrazine, which may be lost from the metabolic pathway by autooxidation. Autooxidation rates of hydrazine were reported to increase with increasing phosphate buffer concentrations (Moliner and Street, 1989). In conclusion, converging lines of evidence show mainly phylogenetic and slight physiological differences between Candidatus *B. anammoxidans* and the Kölliken anammox organism, although the main nitrogen transformation reactions appear to be identical.

Acknowledgments

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Chapter 3: Composition and structure of an ammonium removing biofilm on a rotating disk contactor treating ammonium-rich wastewater without organic carbon

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Abstract

High nitrogen losses were observed in a rotating biological contactor (RBC) treating ammonium-rich (up to 500 mg NH_4^+-N/l) but organic carbon poor leachate of a landfill in Kölliken, Switzerland. The composition and spatial structure of the microbial community in the biofilm on the RBC was analyzed with specific attention for the presence of aerobic ammonia and nitrite oxdizing bacteria and anaerobic ammonium oxidizers. Anaerobic ammonium oxidation (*anammox*) involves the oxidation of ammonium with nitrite to N_2. First the composition and diversity of the biofilm community was determined from sequencing cloned PCR amplified 16S rDNA fragments. This revealed the presence of a number of very unusual 16S rDNA sequences, but very few sequences related to ammonia or nitrite oxidizing bacteria. From analysis of biofilm samples by fluorescence *in situ* hybridization with known phylogenetic probes and by dot blot hybridization of the same probes to total RNA purified from biofilm samples, the main groups of microorganisms constituting the biofilm were found to be ammonia oxidizing bacteria from the *Nitrosomonas europaea/eutropha* group, anaerobic ammonium oxidizing bacteria of the Candidatus *Kuenenia stuttgartiensis* type, filamentous bacteria from the phylum *Cytophaga-Flavobacterium-Bacteroides* (CFB) and nitrite oxidizing bacteria from the genus *Nitrospira*. Aerobic and anaerobic ammonium oxidizing bacteria were present in similar amounts of around 20 to 30% of the biomass, whereas members of the CFB phylum were present at around 7%. Nitrite oxidizing bacteria were only present in relatively low amounts (less than 5 % determined with fluorescence *in situ* hybridization). Data from 16S rRNA dot blot and *in situ* hybridization were not in all cases congruent. FISH analysis of thin-sliced and fixed biofilm samples clearly showed that the aerobic nitrifiers were located at the top of the biofilm in an extremely high density and in alternating clusters. Anammox bacteria were exclusively present in the lower half of the biofilm, whereas CFB-type filamentous bacteria were present throughout the biofilm. The structure and composition of these biofilms correlated very nicely with the proposed physiological functional separations in ammonium conversion.

Introduction

The landfill in Kölliken (near Aargau, Switzerland) was constructed during the nineteen seventies and filled until 1985 with solid waste of different origin. About 250,000 m^3 of waste material was stored in this landfill, resulting in several thousand m^3 of landfill percolate per year, containing a large variety of soluble organic and inorganic compounds. In the early ninetees a wastewater treatment plant was built on site to treat the percolated wastewater from the landfill. Among many other compounds, the landfill leachate flowing into the treatment plant contained a total of

100-400 mg/l dissolved organic carbon, which is quite high for an environmental sample (Münster, 1993) and 100-500 mg NH_4^+-N per liter. The treatment plant consists of two separate rotating disk contactors (RDC) (Fig. 1) with an extremely high surface area (5728 m^2 for contactor 1 and 7238 m^2 for contactor 2) enabling slowly growing and sticking bacteria to be maintained in the treatment system. The first compartment eliminates 88 % of the organic compounds (TOC) in the landfill leachate and a further 6 % of the organic carbon is allowed to absorb to activated carbon. This ensured an almost total removal of all organic compounds which are typical for the leachate of the chemical landfill (e.g., chlorinated hydrocarbons, phenols and anilines; P. Lais personal communication). The effluent wastewater from the first compartment therefore mainly contained high amounts of ammonium which was subsequently treated in a second rotating disk contactor.
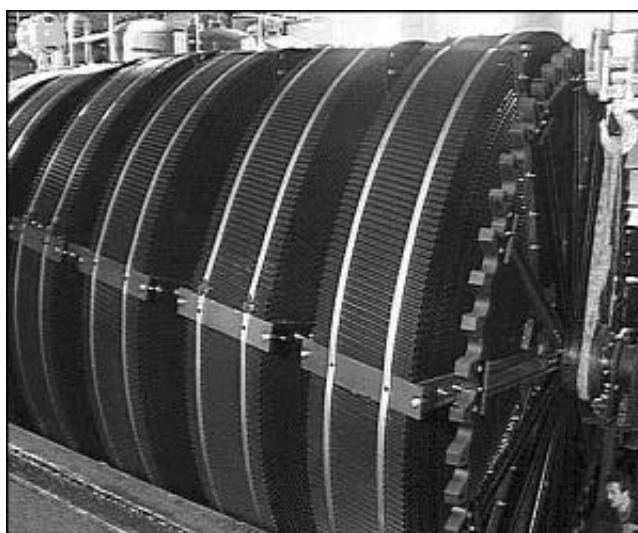

Fig. 1: Rotating disk contactor used in Kölliken to treat the leachate of the landfill.

Soon after the treatment plant was running properly (1994), it was found that the total load of inorganic nitrogen (mainly nitrate) coming out of the second compartment was much lower than the load of inorganic nitrogen (mainly ammonium) in the inlet (Fig. 2) (Siegrist et al., 1998). This was surprising since nitrogen elimination in municipal wastewater treatment plants was always thought to be due to a combination of nitrification and denitrification, which needs an organic carbon source. However, since practically no organic carbon was present in the influent to the second compartment reactor, the observed nitrogen removal could not be satisfactorily explained from nitrification and denitrification.

The phenomena of similar nitrogen 'loss' were also observed in other locations (van de Graaf et al., 1995; Helmer and Kunst 1998; Schmid et al., 2000; Thamdrup and Dalsgaard 2002; Toh et al., 2002). Further investigations then showed that autotrophic bacteria existed oxidizing ammonium with nitrite to N_2 under strictly anoxic conditions (Strous et al., 1997). The physiology and biochemistry of such anaerobic ammonium oxidizing (or *anammox*) bacteria is now better understood (Jetten et al., 1999; Strous et al., 1999b; Schalk et al., 2000; Egli et al., 2001; Schmid et al., 2001) and highly enriched cultures of different anammox bacteria exist (Strous et al., 1999a; Schmid et al., 2000; Egli et al., 2001). Their characterization has also led to the

development of suitable oligonucleotide probes targeting the 16S rRNA thereby enabling the possibility for detection of anammox bacteria in various wastewater engineering systems (Siegrist *et al.*, 1998; Schmid *et al.*, 2000; Hippen *et al.*, 2001).
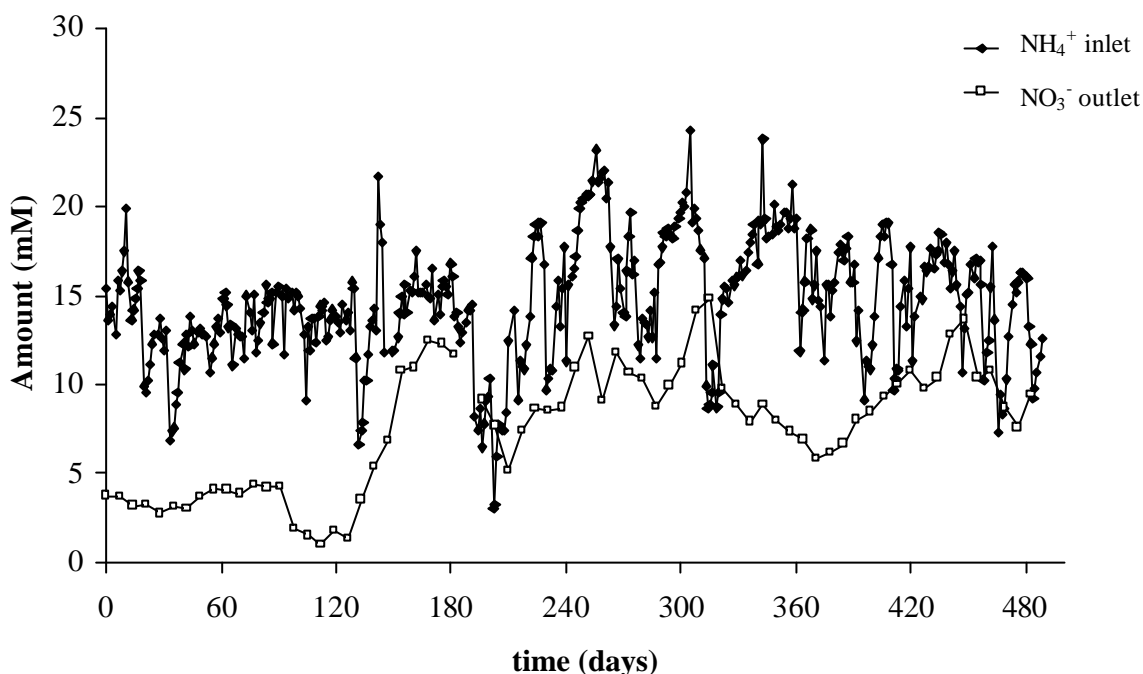

Fig. 2: Influent ammonium and effluent nitrate concentrations from the secondary rotating disk contactor. Nitrate was not present in the inlet and ammonium was absent from the outlet. Nitrite in the outlet was between 0 and 3 mM (mainly 0 mM). Note the consistent difference of approximately 30% between nitrogen in inlet and outlet. Data were provided by P. Lais (Landfill Kölliken).

In this work we focused on the characterization of the microbial community in the biofilms of the rotating biological contactor in the second compartment of the treatment plant in Kölliken, trying to link the community structure and composition with the nitrogen removal process in this unique reactor system. It has become clear from many studies that biofilms (Characklis and Wilderer 1989) provide many special characteristics for slow-growing bacteria, such as a substratum for attachment, stratified microenvironments due to gradients of oxygen (Gieseke *et al.*, 2001) and ammonium, nitrite or nitrate (Schramm *et al.*, 1996; Gieseke *et al.*, 2001), and protection in deeper layers against predation. We were wondering if the specific conditions of the Kölliken biofilms would be sufficient for a natural enrichment of anammox bacteria, perhaps also giving clues to their origin. For this purpose, we combined several molecular tools like fluorescence *in situ* hybridization (FISH), dot blot rRNA hybridization, PCR and 16S rDNA clone library construction, to unravel composition and spatial structure of the biofilms.

Materials and Methods

Fluorescence *in situ* hybridization and microscopy

Fixation of samples was performed by resuspending the cells in 4 % (w/v) paraformaldehyde in PBS (consisting of 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per litre distilled water at a pH of 7.4) and incubating during 1 h at room temperature. Afterwards, the cells were washed twice with PBS and finally resuspended in a 1:1 (v/v) mixture of PBS and ethanol and stored at –20 °C. Before pipetting on the wells, the samples were dissolved in a solution of 2.8 g sodium pyrophosphate (NaP_2O_4) per liter PBS to prevent aggregation. Glass slides (Huber & Co., Reinach, Switzerland) were coated with gelatine (Amann *et al.*, 1990). Between 2 µl and 10 µl of a fixed sample was applied on a well of the coated slide, dried for approximately 1 h at 46 °C and sequentially dehydrated in solutions of 50 %, 80 % and 100 % ethanol for 3 min each. To start hybridization, 9 µl of hybridization buffer was mixed with 1 µl of fluorescently labelled probe (at a concentration of 50 ng/µl) and added to the sample on the glass slide. The composition of the hybridization buffer was dependent on the melting characteristics of the oligonucleotide probe and consisted of buffer solution, containing 0.9 M NaCl, 20 mM Tris-HCl (pH 8), and 0.01 % SDS, mixed with formamide (Table 1). When necessary for increased selectivity, 1 µl of unlabelled competitor probe (at 50 ng/µl) was added to the hybridization mixture. Hybridization was conducted for 90 min at 46 °C in a moistured chamber. Following hybridization, a stringent washing step was performed for 10 min at 48 °C in a buffer with the appropriate NaCl concentration (Table 1), 20 mM Tris-HCl (pH 8) and 5 mM EDTA. The samples were counterstained with 4,6-diamidino-2-phenylindole (DAPI) at a final concentration of 10 mg/l for 5 min at room temperature (Hicks *et al.*, 1992) and mounted in Citifluor (Citifluor Ltd. London, U.K.). For quantification with confocal microscopy the EUB-Mix (Daims *et al.*, 1999) was labelled with Fluoros prime (MWG Biotech, Ebersberg, Germany) and the group-specific probe with the carboxycyanin dye Cy3. In case simultaneous hybridizations with two labelled probes were carried out (e.g., Ntspa662 and NEU for the structure of the biofilm) the probes were labelled with dyes fluorescein isothiocyanate (FITC) and Rhodamin red (both from MWG Biotech).

All oligonucleotide probes were obtained from Microsynth (Balgach, Switzerland) or MWG Biotech. Microscopy was performed on an Olympus BX50 microscope (Olympus Optical AG, Volketswil, Switzerland) equipped with filters HQ-CY3 for Cy3 and Rhodamin red labelled probes, HQ-FITC for FITC labeled probes and a HQ-filter for DAPI (all filters from AF Analysentechnik, Tübingen, Germany). Digital images were taken with a cooled CCD-camera (type Sensys, Photometrics Ltd., Tuscon, Arizona, USA) and acquired in the program METAVIEW (Version 4.1, Universal Imaging Corporation, Visitron Systems, Germany).

For image acquisitions with confocal laser scanning microscope, a Zeiss LSM 510 (Zeiss, Jena, Germany) equipped with an Ar ion laser (458 and 488 nm) and a HeNe laser (543 nm) was used together with the standard software package delivered with the instrument. All confocal laser microscope imaging was carried out at the facility of the Technical University of Münich at Freising, and the authors greatly acknowledge the help of S. Schmitz-Esser, K. Stöcker and M. Wagner.

Electron microscopy was performed with a Philips XL-30 scanning electron microscope (Philips, Eindhoven, The Netherlands) as described elsewhere (Volkland *et al.*, 2000).

Table 1: Probes used for FISH and dot blots

Probe	Sequence (5'-3')	competitor	Forma-mide	[NaCl] in wash buffer (M)	Target position[1]	Reference
EUB338 I EUB338 II EUB338III	gctgcctcccgtaggagt gcagccacccgtaggtgt gctgccacccgtaggtgt	-	0 % [2]	0.9	338-355	Amann *et al.*, 1990 Daims *et al.*, 1999
Amx820	aaaacccctctacttagtg	-	40 %	0.056	820-839	Schmid *et al.*, 2000
Amx1240	tttagcatccctttgtaccaacc	-	60 %	0.014	1240-1263	
Kst1275	tcggctttataggtttcgca		25 %	0.159	1275-1295	
NSO1225	cgccattgtattacgtgtg	-	35 %	0.08	1225–1244	Mobarry *et al.*, 1996
NEU	cccctctgctgcactcta	CTE	40 %	0.056	653–670	Wagner *et al.*, 1995
Ntspa662	ggaattccgcgctcctct	Comp-Ntspa662	35 %	0.08	662–679	Daims *et al.*, 2000
Nit3	cctgtgctccatgctccg	CNit3	40 %	0.056	1035–1048	Wagner *et al.*, 1995
CF319a	tggtccgtgtctcagtac	-	35 %	0.08	319-356	Manz, 1996
Nmo218	cggccgctccaaaagcat	-	35 %	0.08	218–235	Gieseke *et al.*, 2001
Nsv443	ccgtgaccgtttcgttccg	-	30 %	0.112	444–462	Mobarry *et al.*, 1996
Nse1472	accccagtcatgacccccc	-	50 %	0.028	1472–1489	Juretschko *et al.*, 1998
NmII	ttaagacacgttccgatgta	-	25 %	0.159	120–139	Pommerening-Röser *et al.*, 1996
NmV	tcctcagagactacgcgg		35 %	0.08	174–191	
Pla46	gacttgcatgcctaatcc	-	30 %	0.112	46-64	Neef *et al.*, 1998
CTE	ttccatccccctctgccg		40 %	0.056		Wagner *et al.*, 1995
Comp-Ntspa662	ggaattccgctctcctct		40 %	0.056		Daims *et al.*, 2000
cNit3	cctgtgctccaggctccg		40 %	0.056		Wagner *et al.*, 1996

1) Numbers refer to the *Escherichia coli* 16S nucleotide numbering.
2) In case of simultaneous hybridization of the EUB mix with other probes, the formamide concentration of the specific probe was used.

Biofilm slices

Biofilm samples were taken from the secondary stage RDC treating the infiltrate wastewater from the chemical landfill in Kölliken. Biofilms and support were cut with a sharp knife. An area of 3×5 cm^2 of biofilm was removed from the outer layer of the RDC including the support material. Care was taken to maintain the structure of the biofilm. After fixation with paraformaldehyde (procedure see above; samples were rinsed with PBS instead of centrifuged), a part of the biofilm was embedded in Cryoembedding Compound for low and medium temperatures (Microm Labor AG, Walldorf Germany) and frozen at -35°C. A small representative piece was selected and cut in slices with a thickness of approximately 10 μm with a MICROM HM 500

OM microtome (Carl Zeiss AG) while remaining at –35 °C. Slices were placed flat on gelatine coated microscope slides, dehydrated and hybridized as described above.

Nucleic acid extraction

For RNA extraction maximally 0.5 g of homogenized biofilm was suspended in 0.9 ml homogenisation buffer in a screw-capped vial (homogenisation buffer is 200 mM Tris-HCl pH 8.5, 1.5 % SDS, 10 mM EDTA, 1 % sodiumdesoxycholate, 1 % Nonidet-P40). Glass beads (0.4 g, 0.1 mm in diameter) were added and the mixture was shaken two times for 1 min at 4000 rpm in a Braun Cell Homogenizer (Inotech AG, Dottikon, Switzerland) with a 1-min interval on ice. The glass beads were allowed to settle without centrifugation, and the supernatant was transferred to a fresh eppendorf tube. This sample was mixed 1:1 with sodium acetate-buffered acidic phenol (pH 5.5) and incubated in a water bath at 65 °C for 5 min (Aiba *et al.*, 1981). After vortexing and centrifugation for 5 min at 15,000 × g and 4 °C, the water phase was transferred to a new eppendorf tube and mixed 1:1 (v/v) with Tris-HCl buffered phenol (pH 8.0). After vortexing, an equal volume of chloroform/ isoamylalcohol (24:1) was added, the mixture vortexed and centrifuged at 15,000 × g for 3 min. The water phase was recovered and reextracted with chloroform/isoamylalcohol, after which the nucleic acids in the water phase were precipitated. Nucleic acids were recovered by centrifugation, washed with 70 % ethanol and 30 % TE (10 mM Tris/HCl, 1 mM EDTA and pH 8) and dissolved in RNase free water. Digestion of DNA was performed for 30 min at 37 °C with RNase-free DNase I (10 U/µl, Amersham Biosciences Europe, Dübendorf, Switzerland). RNA was visualised on a 0.8 % agarose gel, and the RNA concentration and quality were measured with a spectrophotometer at 260 and 280 nm (using the conversion factor of $OD_{1\ 280nm} = 40$ µg RNA/ml). RNA independently extracted from three different biofilm pieces were finally pooled.

For DNA extraction the cells were collected from the biofilm sample as described above for RNA isolation and resuspended in 5 ml of TENP solution (is 50 mM Tris-HCl, 20 mM Na_2EDTA, 100 mM NaCl, 1 % [w/v] polyvinylpolypyrrolidone and pH 8). 1.5 ml of this suspension was removed to a screw capped eppendorf tube, containing 0.2 g glass beads. The mixture was shaken three times for 1 min at 4000 rpm in a Braun Cell Homogenizer with 1-min intervals on ice. The sample was heated to 100 °C in a boiling water bath for 2 min and subsequently cooled in liquid nitrogen, twice. After centrifugation for 5 min at 15,000 × g the water phase was recovered and the DNA was precipitated with sodium acetate (pH 5.5) and isopropanol. DNA was recovered by centrifuging for 15 min at 15,000 × g and 4°C, washed once and dissolved in 100 µl of 10 mM Tris-HCl (pH 7.5). The samples were further purified with a BioSpin® 30 chromatography column (Bio-Rad Laboratories, Hercules, CA) and subsequently with a MicroSpin™ S-400 HR column (Amersham Biosciences Europe). Nucleotide concentration and quality were determined from measuring the optical density at 260 and 280 nm.

Dot blot analysis

RNA (1 µg/µl) was serially diluted as follows: 1/3, 1/10, 1/30, 1/60, 1/100, 1/300, 1/1000. RNA dilutions were denatured at 94 °C for 2 min and immediately placed on ice until spotting on the membrane. As quantitative hybridization control oligonucleotides with a sequence reverse complementary to the hybridization probes

(Table 1) were used. A solution of 2.5 ng/µl of each oligonucleotide was hereto similarly as for the RNA samples serially diluted.

Nylon hybridization membrane (Hybond N$^+$, Amersham Biosciences) was moistened with 1 × SSC solution (SSC solution is 150 mM NaCl, 15 mM sodium citrate and pH 7), mounted in a Convertible™ Filtration Manifold System (Gibco BRL Life Technologies Inc., Gaithersburg, USA) and 20 µl of the diluted RNA and oligonucleotide solutions were pipetted in alternating columns, immediately followed after every pipetting step by vacuum suction. Every well was subsequently washed with 20 µl of 1 × SSC solution. After blotting, the membrane was cut into slices, each containing a dilution series of RNA and of the respective oligonucleotide. The RNA and oligos were crosslinked to the membrane using a Stratalinker$^®$ 1800 (Stratagene, La Jolla, USA) and dried to ambient air.

For hybridization, the membrane slices were each preincubated in a 10 ml polypropylene tube in a water bath at the respective hybridization temperature (Table 2) for 15 min with 2 ml of prehybridization buffer (containing 0.5 M sodium phosphate, 1 mM EDTA, 1 % *w/v* BSA (Fraction V Grade) and 7 % *w/v* SDS and with pH 7.2). Oligonucleotide probes were labelled with biotin 16-ddTUP (Enzo, Roche, Switzerland) by treatment with terminal deoxynucleotidyl transferase for 15 min at 37°C according to specifications of the supplier (Amersham Biosciences). To start hybridization 10 µl of the biotin labelled probe was added to the prehybridization mixture and the mixture was incubated in a water bath for 90 min at the appropriate hybridization temperature of the probe (Table 2).

After hybridization, the membranes were washed twice in 5 ml of a solution of 2 × SSC plus 0.1 % *w/v* SDS for 5 min at room temperature, twice in a preheated solution of 1 × SSC plus 0.1 % SDS for 5 min at hybridization temperature and twice in 1 × SSC solution for 5 min at room temperature, while rotating on a tube roller.

For detection of the biotin label, membranes were washed twice in blocking buffer (0.2 % I-Block Reagent, 1 × PBS, 0.5 % SDS) (Tropix) followed by an incubation for 30 min in blocking buffer. 2 µl of Avidix-alkaline phosphatase (Tropix) in 10 ml blocking buffer were used to conjugate with biotin during incubation for 20 min. Membranes were washed once in blocking buffer for 5 min, three times in wash buffer (1 × PBS, 0.5 % SDS) for 5 min and twice in assay buffer (0.1 M diethanolamine, 1 mM MgCl$_2$ and pH 10) for 5 min. Chemiluminescent substrate solution (10 µl CSPD (Concentrated Chemiluminescent Substrate for Alkaline Phosphatase) /ml assay buffer) was pipetted in drops on the membrane which was sealed in a polyethylene bag. Where nothing else was stated, all procedures were carried out at room temperature. Membranes were exposed to X-ray film (Hyperfilm™ MP, Amersham Biosciences Europe) for 1 to 30 minutes and developed with a Kodak X-omat 1000 processor (Eastman Kodak Company, Rochester, New York).

The film was scanned and the darkness of each spot quantified by a computing densitometer (Molecular Dynamics) with the program Image Quant (Version 3). Calculation of 'copy numbers' of target was done by comparing signal intensities of each RNA dilution to the oligonucleotide standard row. Comparison was made by hyperbolic curve-fitting with the programs KaleidaGraph and Excel.

Table 2: Hybridization temperatures

Probe	Melting temperature[1]	Used hybridization temperature
EUB I,II,III	59.5 °C	54.5 °C
AMX820	57.7 °C	52 °C
Nso1225	53.4 °C	48 °C
NEU	57 °C	52 °C
Ntspa662	57.2 °C	52 °C
CF319a	54.9 °C	50 °C

1) according to specifications of the manufacturer.

Protein determination

An amount of 2 ml of the anammox enrichment culture (Egli *et al.*, 2001) was centrifuged. Cells were resuspended in 2 ml 0.1 M NaOH and lysed during 90 min in a boiling water bath. Dilutions (1/50, 1/100, 1/200, 1/400, 1/800) were made. 0.8 ml of the (diluted) solution was mixed with 0.2 ml Bradford reagent (Bio-Rad Laboratories GmbH, München) and incubated for 15 min in the dark. After incubation, the absorption (at 595 nm) was measured, and protein content was calculated with a BSA standard curve.

PCR and construction of the clone library

16S rDNA fragments from isolated total DNA of the enrichment culture were amplified in the PCR with *Taq* DNA polymerase (Life Technologies, Gaithersburg, Md.). The following eubacterial primers were used: 16S 6F (5'-ggagagttagatcttggctcag-3') and 16S 1510R (5'-gtgctgcagggttaccttgttacgact-3'). Conditions for PCR were as suggested by the supplier, but with a cycling regime of: 4 min at 95 °C, then 35 cycles each of 45 s at 95 °C, 60 s at 56 °C, and 1 min at 72 °C. Final extension was carried out for 3 min at 72 °C. The 16S rDNA PCR products were cloned in *Escherichia coli* DH5α in vector pGEM-T-easy (Promega, Wallisellen, Switzerland) by established procedures (Sambrook *et al.,* 1989). Plasmid DNAs for sequencing were isolated by boiling preparation (Sambrook *et al.,* 1989). Plasmid inserts were sequenced on both strands by using a Thermosequenase Kit (Amersham, Little Chalfont, UK) with IRD-800 and IRD-700 labeled primers (MWG Biotech, Ebersberg, Germany). Universal vector-located primer sequences were used and, in addition, two primers which targeted a conserved region around position 785 of the cloned 16S rDNA fragment (EUB785F, 5'-ggattagatccctggtag-3' and EUB785R, 5'-ctaccagggtatctaatc-3'). Sequence transcripts were separated and analyzed on a LiCOR 4200L IR2 automated DNA sequencer (LiCOR, Lincoln, Neb.). Only those clones were fully sequenced which had a different G and T track. The nucleotide sequences of the 16S rRNA gene of the unknown organisms were deposited in the GenBank database under accession numbers AJ250882, AJ224539-224541 and AJ224938-224943. Sequence comparisons were calculated by direct FastA alignments (Pearson and Lipman, 1988).

Results
Composition of the Kölliken trickling filter biofilm

The composition of the microbial community in the Kölliken biofilms was determined by using different independent approaches. In first instance, a 16S rDNA clone library was constructed from 16S rDNA amplified from total biofilm DNA. The insert DNAs

of 26 clones were sequenced of which 9 were different (Table 3). Two different 16S rDNA sequences (AJ224938 and AJ224940) had 93% identical nucleotide sequence to each other but both at least 97 % sequence identity to 16S of *Sphingomonas* sp. Another sequence belonged to an organism related to the genus *Staphylococcus* (AJ224939). Furthermore, 16S rDNA of a putative ammonium oxidizer related to the genus *Nitrosomonas* was cloned (AJ224941), which also contained the target sequence for the Nse1472 probe (Table 1). However, this was the only sequence among the clone library related to the group of ammonia oxidizing bacteria. Five sequences showed very low percentages of sequence identity (85 % or less) to known species (AJ224539 through AJ224943). Of these, AJ224942 had around 87 % identical nucleotide sequence to a 16S rDNA sequence from the phylum *Cytophaga-Flavobacterium-Bacteroides* (CFB), but the target sequence for the Cf319a-FISH-probe was present in this sequence. AJ224539 grouped within the rather exotic bacterial division OP11, AJ224540 within OP 3 and AJ224541 within the *Bacillus/Clostridium* group (Hugenholtz *et al.*, 1998). AJ224943 grouped within the order of Planctomycetes. The phylogenetic position of this 16S rDNA sequence is not very closely related to the anammox bacteria as described elsewhere (Schmid *et al.*, 2001) and the sequence did not match to the general Amx820 probe. The 16S rDNA sequences of the anammox bacteria *Candidatus* Kuenenia stuttgartiensis or of any putative nitrite oxidizing bacteria were not detected in the analyzed representation of the biofilm clone library.

Table 3: Composition of the 16S rDNA clone library

Accession number	Putative microorganism (species/group)	Percentage identity to other GenBank-entry	Accession number of closest relative	Name of closest related species
AJ224938	*Sphingomonas* sp.	97.2 %	D84518	*Sphingomonas* sp. MK329
AJ224940		99.4 %	X85023	*Sphingomonas yanoikuyae*
AJ224939	*Staphylococcus*	99.5 %	D83362	*Staphylococcus epidermidis*
AJ224941	*Nitrosomonas*	99.7 % 99 %	AB079053 AF353160	*Nitrosomonas* sp. ENI-11 *N. europaea*
AJ224942	CFB-phylum	86.7 %	AB015254	*Cytophaga* sp. strain JTB250
AJ224539	OP11 division	82%[1]	AF424439	Uncultured candidate division OP11 bacterium MERTZ_2CM_219 16S
AJ224540	OP 3 division	93%[1]	AF424408	Uncultured candidate division OP3 bacterium MERTZ_2CM_315 16S
AJ224943	Planctomycetes	97%	AF314425	Uncultured bacterium PHOS-HE93

1) not along the complete 16S rDNA sequence

With FISH four different bacterial groups comprised the largest populations in the biofilm samples. Three of those were involved in nitrogen conversion based on their phylogenetic classification. These three were ammonia oxidizing bacteria from the

Nitrosomonas europaea/eutropha-group (FISH-probes NEU and Nse1472, Table 1), annamox bacteria (probe AMX820), and nitrite oxidizers from the genus *Nitrospira* (probe Ntspa662). The fourth main group consisted of filamentous bacteria detected with the probe Cf319a, suggesting they belong to the phylum *Cytophaga-Flavobacterium-Bacteroides* (CFB). Ammonia oxidizers from the *Nitrosococcus mobilis* lineage (NmV), *Nitrosospira spp.* (Nsv443), *N. communis* (NmII) and *N. oligotropha/urea* lineage (Nmo218) were not detectable. Around 70 % of all cells were stained with the EUB338 probe mixture using epifluorescence microscopy. Ammonia oxidizing bacteria were present between 10 % (compared to DAPI-staining) and 28 % (in CLSM-analysis compared to EUB stained biomass) of all cells. The quantity of Cf319a-stained bacteria was similar (7.6 % of DAPI-stained cells). Nitrite oxidizing bacteria were present in lower numbers (less than 5 %), which was confirmed with CLSM (2.6 %). When trying to quantify the population of anammox bacteria with CLSM it was found that the fluorescent signal of the EUB-hybridization on the same aggregates was too weak compared to the Amx820 and Pla46 signals. Therefore, the relative amount of Amx820 stainable biomass compared to EUB could not be determined. Judged from the Amx820- and NEU-stained biomass only, aerobic and anaerobic ammonium oxidizers were present in a similar amounts. Interestingly, the EUB338II and EUB338III probes (Daims *et al.*, 1999) which had both two mismatches to EUB338 (Amann *et al.*, 1990) stained a few aggregates with epifluorescence microscopy in the area where anammox bacteria prevailed (Fig. 3). Both newly designed probes (Daims *et al.*, 1999) were designed to detect members of the phyla *Verromicrobia* and *Planctomycetales*.
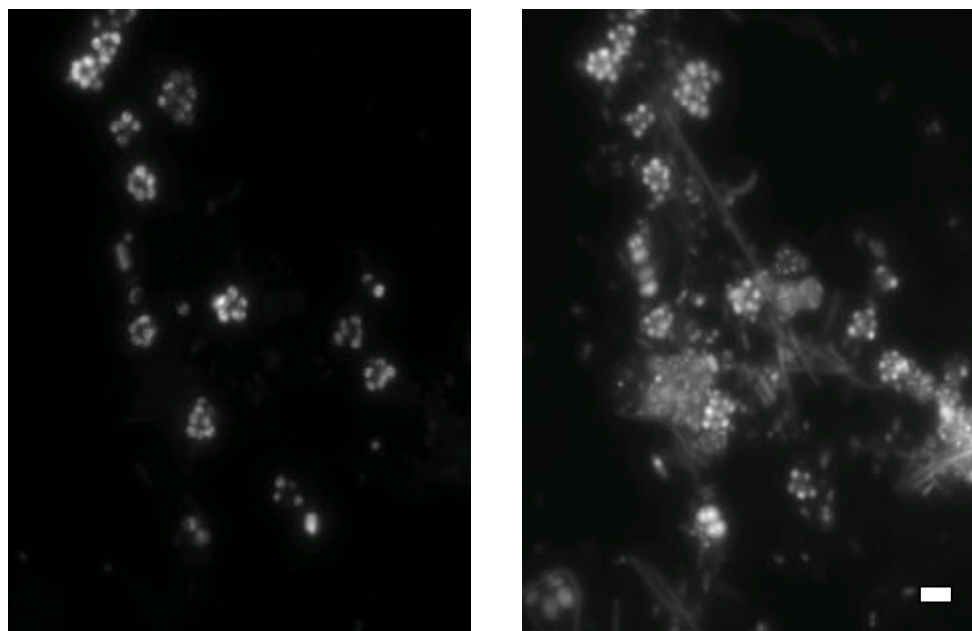

Fig. 3: EUB338II- and EUB338III- stained aggregates in the biofilm (left: EUB 338II and right: DAPI). Both EUB probes were designed to detect members of the phyla *Verromicrobia* and *Planctomycetales*. Bar corresponds 10 μm.

The relative abundance of the same phylogenetic groups as in FISH were investigated with RNA dot blot hybridisation (Fig. 4). Compared to the signal intensity of the hybridization of the EUB mixture (set as 100 %) the amount of rRNA stained with NEU was around 23 % (Table 4). The same relative amount was hybridizable with the more general probe for ammonia oxidizing bacteria Nso1225. The highest amount of

rRNA hybridized with the probe Cf319a detecting the filamentous bacteria from the CFB phylum (around 40 %). The quantity of nitrite oxidizing bacteria (detectable by hybridization with the probe Ntspa662) was 14 %. The amount of rRNA hybridizing to the Amx820 probe (representative for anammox bacteria) was extremely low (around 1 %) although FISH hybridizations had suggested a relative high number of anaerobic ammonium oxidizers in the biofilm.
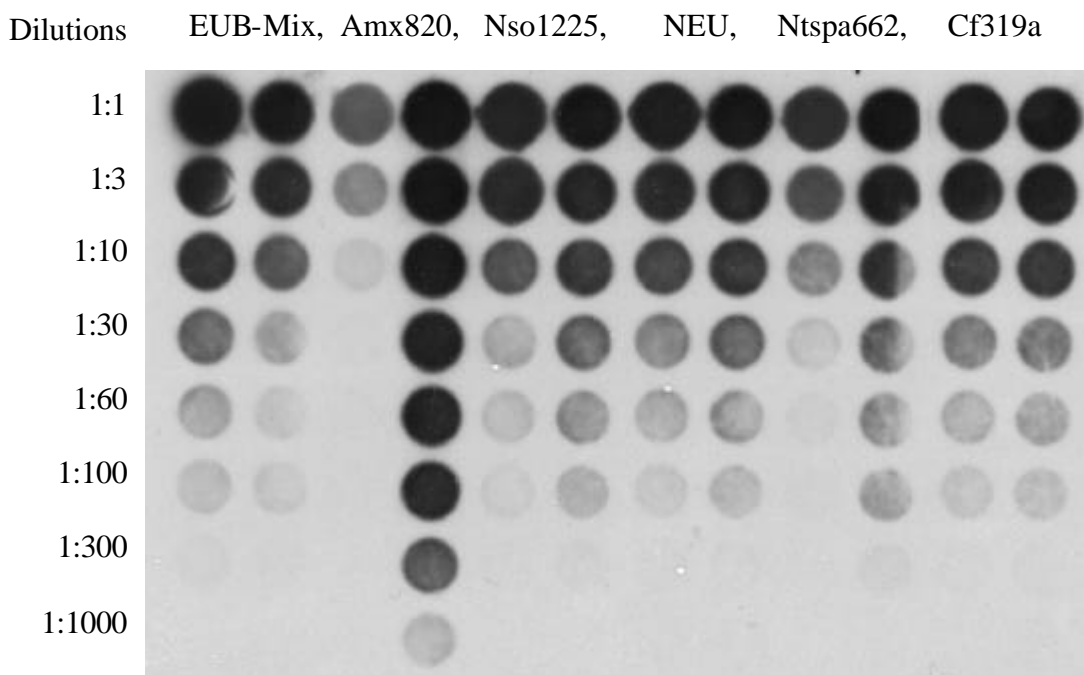

Fig. 4. rRNA dotblot hybridization with probes against the main organisms present in the biofilm. The first row of each hybridization corresponds to extracted and diluted rRNA and the second row to the oligonucleotide dilution series. Probes: EUB-mixture, Amx820, Nso1225, NEU, Ntspa662 and CF319a.

To explain this difference between FISH and rRNA dot blot hybridizations, hybridizations with Amx820 were carried out at lower temperatures (48°C and 50°C instead of 52°C) to rule out the possibility that the low signal was due to a too high hybridization temperature. However, this did not change the relative hybridization signal intensity. Hybridizations were then performed with extracted rRNA from the anammox enrichment culture which had previously been shown to contain 88 ± 5 % anammox bacteria (Egli *et al.*, 2001). This resulted in a relative signal intensity of 25.5 ± 3.8 % compared to the EUB signal intensity. Assuming that all cells in the enrichment culture contained comparable ribosome numbers, the extraction efficiency of RNA from anammox organisms was calculated to be only about 4 % (due to the 10 % of other bacteria with high extraction efficiencies). This would indicate that the Amx820-specific hybridizable population determined by dot blot hybridisation (1 % of EUB) should be corrected with a factor of 25.

The three sequences (i.e., AJ224539, AJ224540 and AJ224943) with very low sequence homologies to known species were present in very low numbers among total isolated RNA (less than 1 % as determined with rRNA dot blots). With specific FISH

probes targeting these sequences no significant signal was observed at various formamide concentrations, indicating that the abundance of microorganisms carrying those 16S rDNA sequences in the biofilm was most likely very low. Not a complete balance of all bacterial groups in the biofilm could be made according to our microscopic and dot blot analyses. With normal epifluorescence microscopy counting around 71 % of all DAPI-stained cells were detected with the EUB probe mixture and 56 % could be accounted for by all other used probes. With rRNA dotblot analysis, 78 % (102 % when correcting for the extraction efficiency of anammox RNA) of all bacteria were quantified compared to the EUB mixture. This suggests that some other populations were missed, perhaps those which had been picked up by cloning the 16S rDNA fragments (e.g., α-Proteobacteria and high GC Grampositive organisms). However, the contribution of those populations for the total ammonium conversion by the biofilm is likely to be of minor importance.

Table 4: Relative abundance of the major populations of the microbial composition of the Kölliken biofilm as determined with fluorescence microscopy and RNA dot blot hybridizations.

Probe	Representative for group	Epifluorescence[1] (DAPI =100 %)	CLSM[2]: (EUB-Mix = 100 %)	rRNA dotblots: (EUB: 100 %)
EUB-Mix	Most Bacteria	71.4 ± 19.2 (100)[3]	100	100
NEU	*Nitrosomonas europea/eutropha*	10.3 ± 3.8 (14)	28 ± 8.8	23 ± 4.1
Nso1225		24.7 ± 15.7 %	n.a.	23.3 ± 5.5
Amx820	*Candidatus* Kuenenia stuttgartiensis and Brocadia anammoxidans	33.4 ± 12.8 (46.8)	n.q.[4]	0.96 ± 0.86 (25)[5]
Cf319a	*Cytophaga Flavobacterium Bacteroides*	7.6 ± 10 (11.2)	n.q.	39.8 ± 0.8
Ntspa662	*Nitrospira*	< 5 % (7)	2.6 ± 2	13.7 ± 8.6
Total (NEU, Amx, Cf and Ntspa)		56.3 ± 26.6	n.q.	78 (102)[5] ± 25

1) Object counting on dispersed biofilm samples.
2) Biovolume determination on dispersed biofilm samples
3) Values within brackets indicate percentages compared to the EUB signal
4) n.a., not analyzed; n.q. not quantifiable
5) after correcting for RNA extraction efficiency

Structure of the biofilm

To elucidate the structural organization of the major populations in the ammonium removing biofilm in Kölliken, thin cross-section biofilm slices (10 μm) were again hybridized with the probes which covered most of the community (aerobic and anaerobic ammonium oxidizing bacteria, nitrite oxidizing bacteria and bacteria from the phylum CFB). Typically, the thickness of the biofilm samples was up to 750 μm (± 120 μm), varying strongly in the reactors depending on manual cleaning or spontaneous sloughing. Hybridization with probes NEU and Ntspa662 clearly showed

a very high abundance of nitrifying bacteria in the top layer of the biofilm (Figs. 5 and 6). Both ammonia oxidizing (NEU-stained) and nitrite oxidizing bacteria (Ntspa662-stained) typically formed dense clusters of around 2 µm to 15 µm in diameter, containing probably up to several hundred cells. Simultaneous hybridization of NEU and Ntspa662 with different fluorescent labels showed that ammonia and nitrite oxidizing bacteria were in close neighbourhood to each other (Fig. 6).
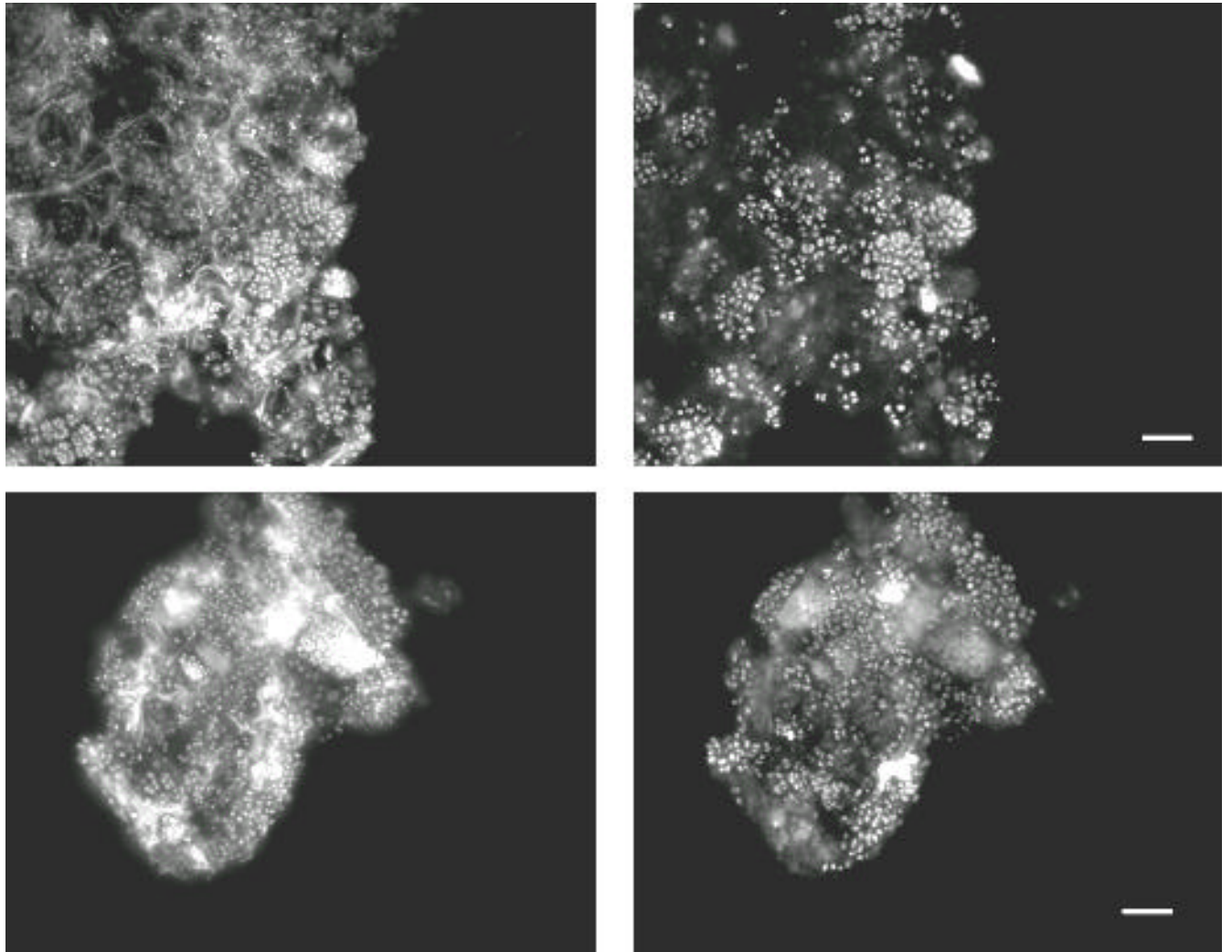

Fig. 5: Upper and lower row: upper (oxic) part of the biofilm stained with DAPI (left row) and with NEU (right). Note the high frequency and close neighborhood of the nitrifying aggregates.
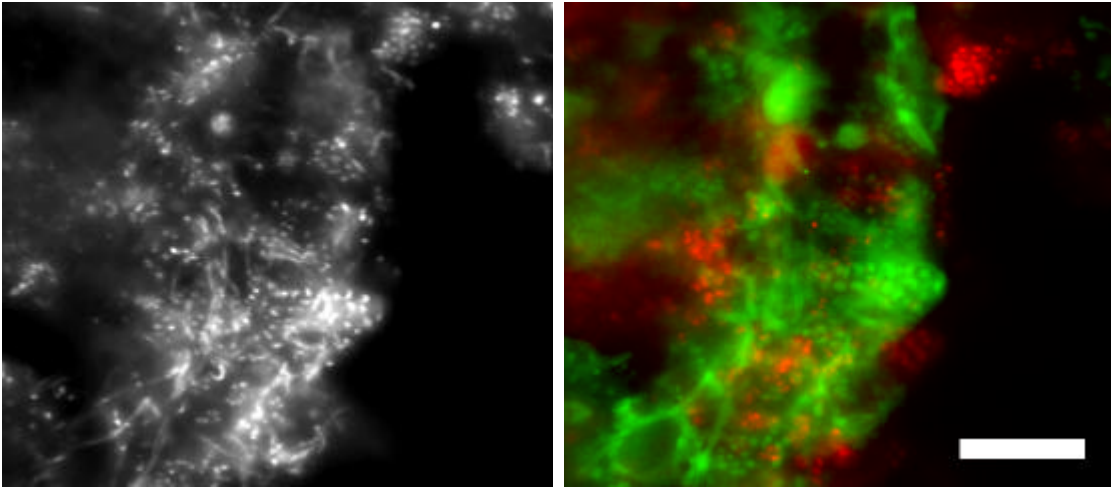
Fig 6: Upper part of the biofilm stained with DAPI (left) and simultaneously with Ntspa662 (FITC: green) and NEU (Rhodamin red). The bar has a size of 10 µm. Note the alternating occurrence of ammonia and nitrite oxidizing bacterial clusters, and the species homogeneity of the clusters.

In the lower part of the biofilm no hybridization occurred with the probes NEU and Ntspa662, but very strong hybridization with the Amx820 (Fig. 7) and Pla46 probes. A clear upper boundary of Amx820 stainable cells were clearly detectable (Fig. 8) and some aggregates were still visible with DAPI near this boundary. On the contrary, no Amx820 hybridizable signal was obtained in middle and top layers of the biofilm. This suggested the anaerobic ammonium oxidizing bacteria to be condensed to a clear dense layer at the bottom of the biofilm, above which no anammox bacteria sustained (Figs. 7, 8, 11). Anammox bacteria like ammonia and nitrite oxidizing bacteria formed dense large essentially homogeneous aggregates. In contrast to these three types, bacteria staining with CFB-probe were present throughout the biofilm (Figs. 9, 10 A and D, 11).
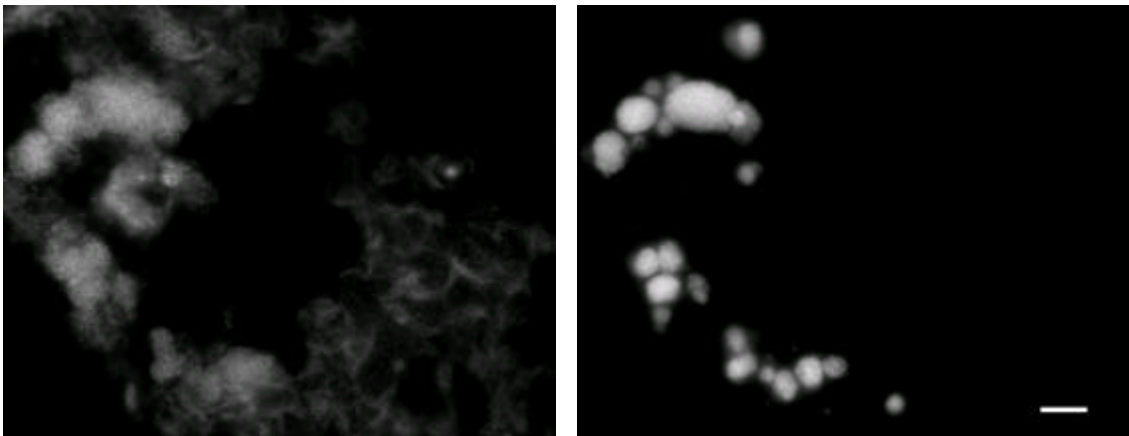

Fig. 7: Lower part of the biofilm stained with DAPI (left) and Amx820 (Cy3, right). Note again the dense clusters of anammox bacteria. Bar measures 10 µm.
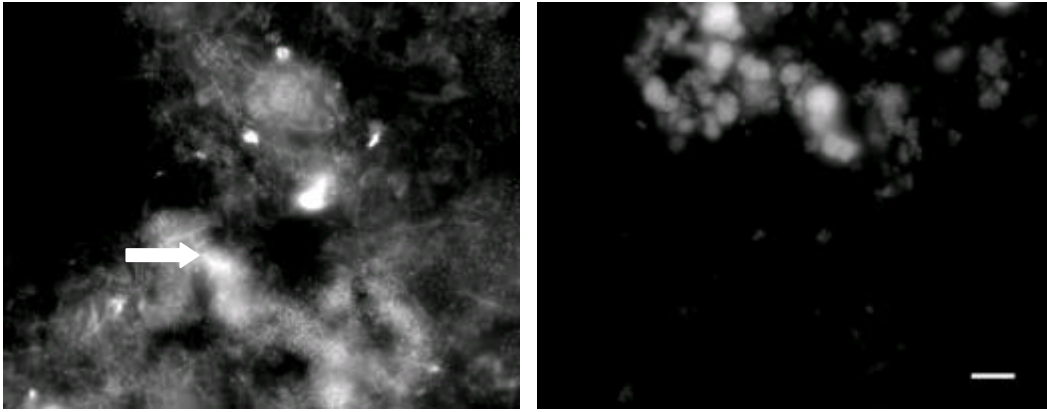
Fig 8: Part of the biofilm stained with DAPI (left) and Amx820 (right). Note the aggregates at the bottom in the DAPI-picture which were not stained with the Amx820-probe (arrow), whereas aggregates at the top of the picture stain for anammox bacterial clusters. This part may indicate the transition between anoxic and oxic conditions. Bar measures 10 µm.
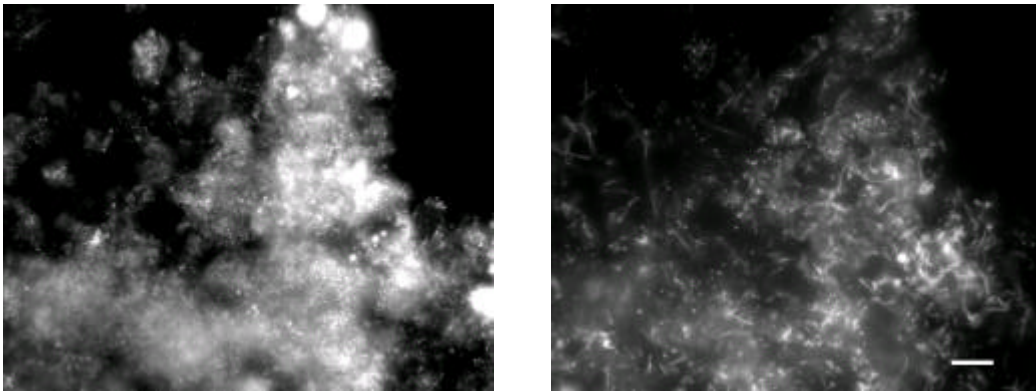

Fig. 9: Part of the biofilm stained with DAPI (left) and Cf319a (right). Only the filamentous bacteria were detected, whereas the large amount of aggregates (left picture) were only visible with DAPI. Bar measures 10 µm.
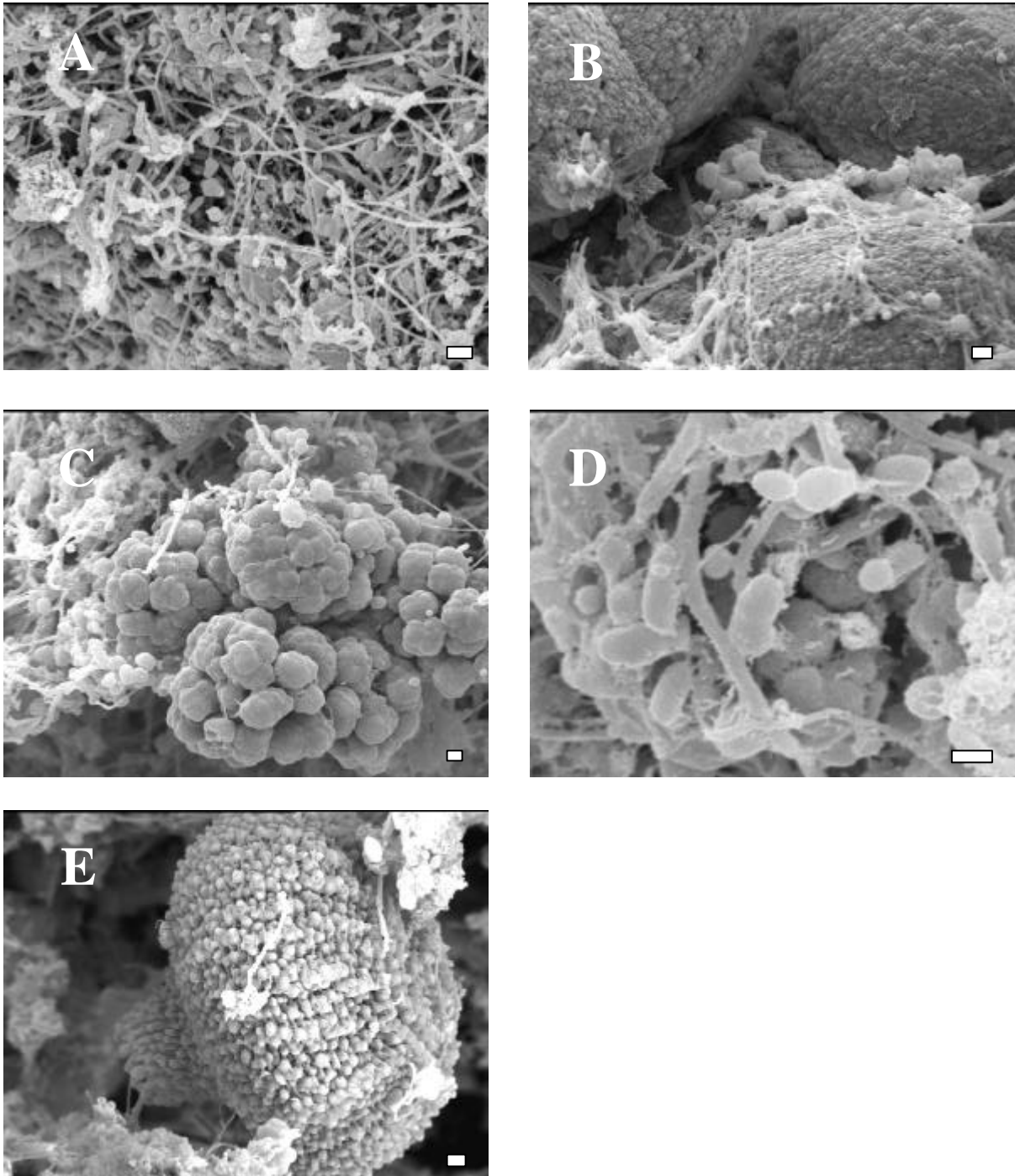
Fig. 10. SEM pictures from the Kölliken anammox biofilm. Note the cauliflower like aggregates nitrifying or anammox bacteria (C, E). Also here it is clearly recognizable that single cells as well as the aggregates are somehow embedded in a net of filamentous bacteria (A, B, C, D). Bar represents 1μm. A to E were pictures from different locations of the biofilm.

With electron microscopy the typical cauliflower aggregates were also seen on the surface of the biofilm, which based on FISH results, consisted of homogeneous clusters of ammonia and nitrite oxidizing bacteria (Fig. 10 C and E). Surface regions with more network like structures, consisting of (perhaps) elongated extracellular polymers and individual cells were also seen with SEM (Fig. 10 A and D). EM

observations of cracks in the surface allowed visualization of the filamentous bacteria at the deeper layers. Strange objects were also detected (Fig. 10 E), of which it is presently unclear whether they are consisting of bacterial cells. The bottom part of the biofilm consisted of mineral surface (Fig. 10 B) and the clusters of anammox bacteria could not be discerned by SEM viewing from the outside.
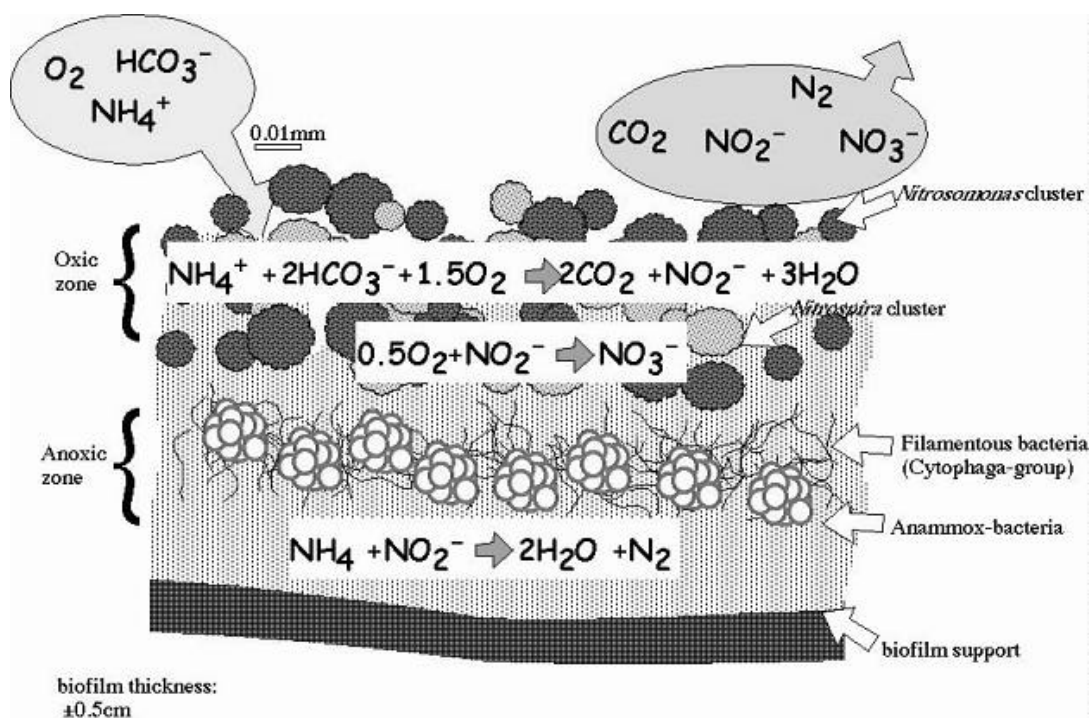

Fig. 11: Schematic structure of the anammox biofilm in Kölliken. The nitrifying bacteria were found in the upper (oxic) zone where part of the ammonium is oxidized to nitrite. Not all nitrite is further oxidized by *Nitrospira* to nitrate (also in the upper oxic zone). In the lower (anoxic) part, anammox bacteria convert the residual ammonium and nitrite to N_2 and nitrate. The filamentous bacteria were found throughout the biofilm.

Discussion

During the last few years several publications appeared showing the presence and activity of anammox bacteria in engineered systems or enrichment cultures (Siegrist *et al.*, 1998; Helmer and Kunst 1998; Jetten *et al.*, 1999; Schmid *et al.*, 2000; Egli *et al.*, 2001; Thamdrup and Dalsgaard 2002). Here we report on the structure and function of a biofilm spontaneously formed on the surface of a rotating disk contactor treating an ammonium-rich but organic carbon poor landfill exfiltrate of leachate. A major part (appr. 35 % as counted by fluorescence microscopy) of the biofilm community appeared to consist of anammox bacteria from the type *Candidatus* Kuenenia stuttgartiensis, suggesting that both the operating conditions of the treatment system and the type of infiltrate were creating optimal conditions for spontaneous inoculation and enrichment of anammox bacteria. Although the natural occurrence of anammox bacteria is still relatively obscure, they have been detected at quite different locations (Strous *et al.*, 1999a; Helmer and Kunst 1998; Schmid *et al.*, 2000; Toh *et al.*, 2002). Some evidence could also be obtained by PCR that in the case of the Kölliken biofilm the anammox bacteria were indeed entering the reactors from the leachate of the

landfill (Chapter 5), suggesting that in one case the subsurface is an environment where they originate.

The biofilm was formed mainly by four different bacterial groups. In the upper part of the biofilm mainly aerobic ammonia oxidizers of the *N. europaea/eutropha* group (which were stained with NEU, Nso1225 and Nse1472) could be seen. Data from all three methods (FISH on dispersed biofilm samples, confocal laser scanning with FISH and quantitative rRNA dot blotting) agreed more or less on the relative size of the aerobic ammonia oxidizing population (approximately 20 %). No ammonia oxidizing bacteria from other groups were present since the FISH probes NmII, Nsv443, NmV and Nmo218 (Gieseke *et al.*, 2001) did not give any signal at all. It might be that the high ammonium (up to 35 mM) and salt (10 g/l) concentrations in the influent of the treatment system were selecting for ammonia oxidizing bacteria from the *N. europaea/eutropha*-group, since these are known to resist such conditions better than other ammonia oxidizers, like those from the *N. oligotropha/urea* lineage (Gieseke *et al.*, 2001; Koops and Pommerening-Röser 2001). Nitrite oxidizing bacteria (*Nitrospira*) were clearly detectable although in a much lower quantity than the aerobic ammonia oxidizers. Their population size estimated by the three methods ranged between 3 and 14 %. The population of anaerobic ammonium oxidizers (mainly bacteria related to Candidatus *K. stuttgartiensis* since they stained with Amx820 and Kst1275) as determined by FISH sized around 33 %. A much lower relative size (1 %) of anammox bacteria was determined by rRNA dot blot hybridization. This is probably an underestimation of the true relative population size and due to a low extraction efficiency of total RNA from the anammox bacteria. It was possible to determine the extraction efficiency for the anammox bacteria by isolating and hybridizing total RNA from an anammox enrichment culture which was characterized previously (Egli *et al.*, 2001). When corrected for the extraction efficiency (4 %), the relative size of the anammox population determined with FISH (46 % of EUB-stained cells) and the corrected value of the rRNA dotblots (around 25 % of the EUB signal) were much better in agreement. This discrepancy indicated that care has to be taken with total RNA based quantification methods as long as the extraction efficiencies are unknown. Population size determinations of the filamentous shaped organisms from the phylum CFB by rRNA hybridization and FISH were also not in good agreement (40 % and 8 %, respectively). Unfortunately, we could not determine RNA extraction efficiencies for the CFB-stained bacteria, since we did not possess pure cultures of those. It might be, however, that due to their long size (10 µm in length was not unusual), the cells contained higher amounts of rRNA per cell than coccoid or rod-shaped bacteria.

The poor staining of the EUB mixture to the putative anammox aggregates in the CLSM analysis might be due to the fact that all three EUB-probe sequences have at least one mismatch to the anammox sequence (AJ250882), although the fluorescent signal was not obviously different at 0 or 40 % formamide with the anrichment culture (Egli *et al.*, 2001) with the EUB mixture. The fact that the anammox bacteria were not stained with EUB in CLSM could lead to a slight overestimation of the other populations with CLSM analysis. The different quantity of the Nso1225 stained cells in normal epifluroescence microscopy compared to the amount of NEU stained cells could be due to a very week fluorescent signal of the Nso1225 probe which therefore could lead to a wrong counting.

The clone library did not give a good representation of the abundance of the different strains in the biofilm. Although not a very high number of clones (i.e., 26) with 16S rDNA fragments were analyzed, no sequence for the anammox or nitrite oxidizing

bacteria was found, despite the large relative sizes of those populations. In contrast, some sequences representative for (probably) minor sized populations, such as *Sphingomonas sp.* were cloned. This clone library showed once more the limitation of this technique (Amann *et al.*, 2000). Interestingly, however, four sequences with percentages identity below 85 % were detected, which is at the division level for taxonomic positioning. Some of these proposed bacterial strains (such as within the OP3 and OP8 divisions) have never been detected by other means than cloned 16S rDNA sequences (Hugenholtz *et al.*, 1998). Their detection in the Kölliken biofilm suggested that the organisms belonging to these 16S rDNA sequences might naturally occur in the subsurface groundwater (GenBank entry AF424408, Table 3). However, despite extensive FISH probing, nothing more than very few single cells could be observed with probes for these sequences, which is not conclusive evidence for their significance or physiological role in the biofilm. It could not be ruled out, however, that these FISH-probes had a target position on the 16S rRNA with a low fluorescence signal (Fuchs *et al.*, 2001).

Structural analysis demonstrated that the aerobic nitrifiers were at the top and the anammox bacteria in the lower layer of the biofilm. Although we did not probe the oxygen profile in this biofilm, there is good reason to assume that oxygen did not penetrate much further than 100-200 µm below the surface (Koch *et al.*, 2000) and that the layer where oxygen became absent formed the boundary for the subsistence of the anammox bacteria. Anammox bacteria have been shown to lose their ribosomes (Schmid *et al.*, 2001) and their activity (Strous *et al.*, 1997; Egli *et al.*, 2001) when exposed to oxygen for prolonged times, which also may be one reason why no anammox clusters were detected in the middle of the biofilm although some clusters of bacteria were seen with DAPI. Very strikingly, the nitrifying bacteria formed alternating close homogeneous clusters at the top (oxic) layer of the biofilm. This suggests that not only a vertical decreasing gradient of oxygen, bicarbonate and ammonium existed in the biofilm, but also smaller horizontal gradients of nitrite, since the nitrite oxidizer population has to rely on nitrite produced by the aerobic ammonium oxidizers. At present there is also no proven explanation as to the reason why all three nitrogen converting populations form such strong clusters of individual cells, although such clustering has been observed before (Koops and Pommerening-Röser, 2001; Strous, 2000). The filamentous bacteria stained with Cf319 were present throughout the biofilm, meaning that no specific preference for oxygen existed, although we cannot exclude that actually different strains were targeted by FISH in the different zones of the biofilm. The function of the CFB bacteria might lie in providing a structural support for the biofilm (also seen in SEM pictures) as a whole and for other bacteria within the biofilm. Members of the CFB-phylum have been found in high abundance in various habitats, such as freshwater habitats, soil but also extreme habitats like Antarctic marine waters (Manz, 1996; Weller *et al.*, 2000). Accordingly broad is their physiological repertoire. In the biofilm they might profit from degrading traces of remaining carbon or of carbon released from dead bacteria. The main CFB bacterium in the biofilm might be represented by the sequence AJ224942, which, however, showed only low homology to species from the genus *Cytophaga* although still contained the FISH-target sequence of the probe CF319a.

The structural composition of the biofilm of the rotating disk reactors correlated very nicely to the chemical model of nitrogen conversion in this and other related biofilm systems (Fig. 11) (Siegrist *et al.*, 1998; Koch *et al.*, 2000; Hao *et al.*, 2002). The high amount of aerobic and anaerobic ammonium oxidizing bacteria in the biofilm, which are both autotrophic bacteria, explains the previously observed nitrogen loss from the

influent into the secondary treatment system. The occurrence of nitrate in the effluent from the secondary rotating disk contactor is the result from nitrate production of anammox and aerobic nitrite oxidizing bacteria in the biofilms. Anammox bacteria oxidize around 15 % of the total amount of consumed nitrite to nitrate to have electrons for CO_2 reduction and therefore biomass production (Egli *et al.*, 2001; Jetten *et al.*, 1999). Since there were more ammonia than nitrite oxidizing bacteria in the biofilm, only a part of the produced nitrite was further oxidized to nitrate by nitrite oxidizing bacteria. Therefore both ammonium and nitrite could diffuse to the lower (anoxic) part of the biofilm where the conversion to N_2 took place. From making some rough assumptions, it can be calculated that the potential activity of the anammox biomass is much higher than taking place in the RDC. At a measured biofilm thickness of 0.5 mm, a total surface area of the RDC of 7238 m^2 and an average biofilm density of 50,000 gCOD $*m^{-3}$ (Koch *et al.*, 2000) with 0.9 g dry mass/g COD, the total biomass equals 181 kg. Assuming that half of the wet biomass is protein, and anammox bacteria make up a fraction of 0.35, this would mean that 32 kg anammox protein is present. At a maximal specific activity of the anammox bacteria of 25 nmol N_2 $*min^{-1}$ $*mg$ protein^{-1} (Egli *et al.*, 2001) this would indicate that the anammox bacteria in the biofilm are potentially capable of producing 1140 mol per day. During 1.1.97 to 31.12.98, an average of 85 m^3 water containing as average 15 mM of ammonium was flowing into the second RBC per day. This equals an ammonium load of 1275 mol/day, of which 1/3 (425 mol) is apparently used by the anammox bacteria for dinitrogen production (Fig. 2). Therefore, it appears that the activity of the anammox bacteria in the biofilm is slightly limited, either by temperature, pH, ammonium, nitrite, or by bicarbonate. Taking into account that the pH in the rotating disk contactor is not optimal (pH 7.2-7.3) and the temperature is far from optimal (17 °C in the winter, 22-24 °C in summer; Egli *et al.*, 2001), the observed nitrogen elimination rate corresponds very well with the calculated rate. This might be reason (besides possible high fluxes), that the the nitrite oxidizers did not completely take over the biofilm from the anammox bacteria. Apparently, either the operating conditions (e.g. pH, oxygen regime, hydraulic retention time and temperature, or rotating speed) of the rotating disk contactor or other biological factors (e.g., the position in the biofilm), ensure that the anammox population have sufficient competitive advantage to sustain in the biofilm.

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Chapter 4: Population analysis during start-up of nitritation reactors

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Abstract

A combination of partial nitrification of ammonium to nitrite under oxic conditions (nitritation) and a subsequent anaerobic oxidation of ammonium with nitrite to N_2 (the anammox reaction) could be an alternative process for ammonium removal in wastewaters. This could be accomplished in a two reactor system with ammonia oxidizing bacteria in a first and anammox bacteria in the second reactor. However, the achievement of nitritation but not nitrate formation is critical for the effectivity of the two stage process. Here the conditions were investigated which promote establishment of a suitable microbial community performing nitritation only, starting from regular sewage sludge. Reactors were operated in duplicate at different conditions (pH, temperature, dilution rate) and were fed with 50 mM ammonium either as synthetic medium or as sludge digester supernatant. In all cases, stable nitritation could be achieved within 10 to 20 days after inoculation. Quantitative *in situ* hybridization analysis with group-specific fluorescent oligonucleotides (FISH) and restriction fragment polymorphisms of the *amoA* gene for ammonia monooxygenase from the microbial communities in the different reactors showed that nitrite oxidizing bacteria of the genus *Nitrospira* were only active directly after inoculation with sewage sludge (up to 4 days and detectable up to 10 days). The community of ammonia oxidizing bacteria changed within the first 15 to 20 days from a more diverse set of populations consisting of *Nitrosomonas communis*, *Nitrosomonas oligotropha/urea* and the *Nitrosomonas europaea/eutropha* group in the inoculated sludge to one dominated by one or two FISH-groups. Reactors operated at 30°C and pH 7.5 contained reproducibly homogeneous communities dominated by one RFLP-type from the *N. europea/eutropha* group. Duplicate reactors at pH 7.0 developed into diverse communities and showed transient population changes even within the ammonia oxidizer community. Reactors at pH 7.5 and 25°C formed communities undistinguishable by FISH, but different in *amoA* RFLP-types. No large differences in rates of ammonia oxidation and nitrite formation were seen in reactors operated with synthetic wastewater even though the individual community compositions were not identical. Communities in reactors fed with sludge digester supernatant contained a higher diversity and were constantly reinoculated with ammonium oxidizers from the supernatant. Therefore, such systems could be maintained at a higher dilution rate (0.75 d^1 compared to 0.2 d^1 for the synthetic wastewater reactors). We conclude from these results that it is relatively easy to achieve nitritation only and that there is a high diversity of ammonia oxidizing bacteria in activated sludge able to perform ammonia oxidation at high ammonium concentrations. Despite similar reactor performance with respect to chemical parameters, the underlying community structures were different, which may have an influence on stability during perturbations.

Introduction

Strong regulations exist nowadays for the removal of ammonium, which can lead to eutrophication of freshwater bodies and is toxic to the aquatic life (Hagiopan and Riley, 1998), from wastewater discharge. The current removal of ammonium in wastewater treatment plants consists of a combination of aerobic nitrification catalyzed by autotrophic organisms and anaerobic denitrification catalyzed by heterotrophic organisms. In a first step, ammonium is oxidized to nitrate via nitrite and in a second step, nitrate is reduced with organic carbon via nitrite to N_2. This combination was long considered as the only way to remove ammonium from wastewater. However, more recent economical considerations and discoveries urge to use either shortcuts in the nitrification-denitrification process, by going via nitrite instead of nitrate, or to apply the anammox process. In the anammox process ammonium is oxidized anaerobically with nitrite to N_2 by autotrophic bacteria (Jetten *et al*., 1999).

Although not directly applicable for the main aerobic treatment tanks, the combination of nitritation and denitrification or anammox (Hellinga *et al*., 1998; van Kempen *et al*., 2001) has interesting economical advantages for the separated treatment of ammonium-rich wastewater, like supernatant from sludge digester tanks. In fact, the amount of ammonium derived from the sludge digester can represent more than 20 % of the daily nitrogen load of the inlet of a wastewater treatment plant (Siegrist, 1996). In the first place, a combination of partial nitrification and anammox would require less oxygen input for the nitrification part (since only a part of ammonium is oxidized to nitrite and not all ammonium to nitrate). Secondly, no addition of external organic carbon is necessary, which is often practised in the nitrification-denitrification scheme by the addition of methanol (Hellinga *et al*., 1998), since the anammox process is carried out by autotrophic bacteria. One possible configuration for a combination of nitritation and anammox would be a two reactor system (Fig. 1), since the physiological requirements for both types of microorganisms are so different (e.g., oxygen vs. no oxygen). For a proper functioning of such a two reactor system, the optimal conditions for both aerobic and anaerobic ammonium oxidizing bacteria have to be generated and maintained (Fux, 2003).
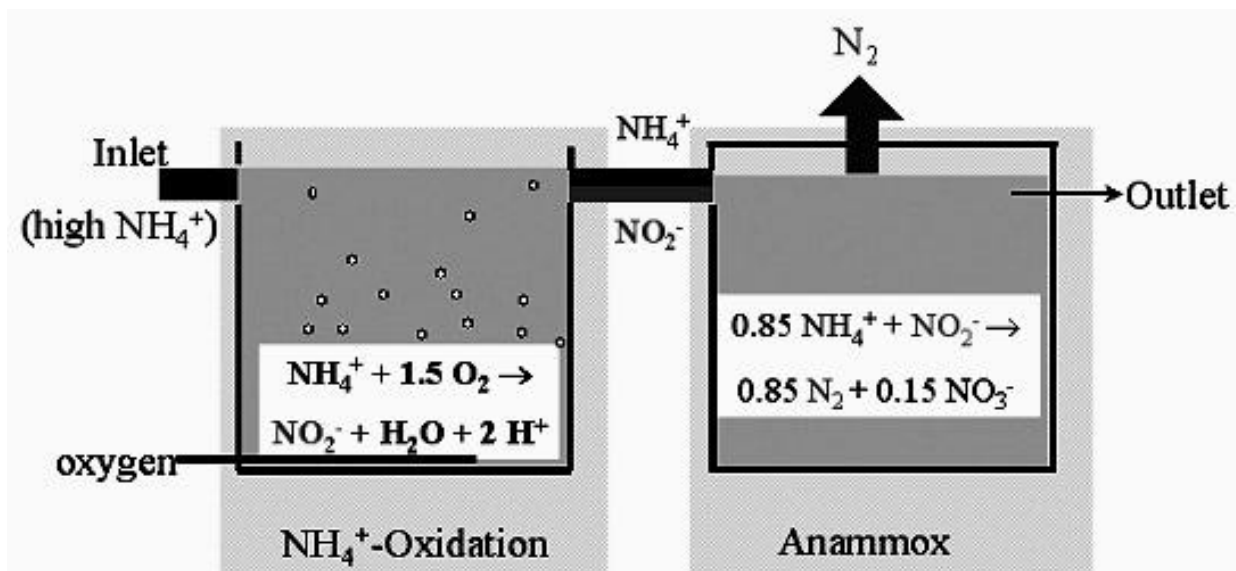
Fig. 1. Schematic overview of the two reactor setup for nitritation and subsequent anaerobic ammonium oxidation (anammox).

From recent work on the physiology of different anammox bacteria in enriched culture (Jetten *et al.* 1999; Strous, 2000; Schmid *et al.* 2000 and 2001; Egli *et al.* 2001) and in technical systems (Siegrist *et al.* 1998; Hippen *et al.* 1901; van Dongen *et al.* 2001), it is now known that stable anammox activities can actually be maintained during several years. The main requirements for the anammox process are equimolar (or slightly lower) nitrite and ammonium loads (Strous, 2000; Schmid *et al.* 2000; Egli *et al.* 2001; Hippen *et al.* 2001; van Dongen *et al.* 2001), mesophilic temperatures (28°C-37°C) (Hippen *et al.*, 2001; van Dongen *et al.*, 2001), absence of oxygen (Strous *et al.* 1997; Egli *et al.* 2001; van Dongen *et al.* 2001; Koch *et al.* 2000; Hao *et al.* 2002) or at least anoxic niches, like in biofilms or flocs (Hippen *et al.* 2001; Siegrist *et al.* 1998; Schmid *et al.* 2000). On the other hand, generation and maintenance of a nitritation reactor requires that nitrite oxidizing bacteria are washed out from the biomass and can no longer find suitable conditions to reestablish themselves. Unfortunately, ammonium and nitrite oxidizing bacteria can be found almost everywhere and, therefore, it might be difficult to find conditions favoring one over the other. Up to now, there exist 25 cultured species of ammonia oxidizing bacteria and eight cultured species of nitrite oxidizing bacteria (Koops and Pommerening-Röser, 2001). The different ammonia oxidizers cover the complete range of salt requirements (from no salt requirement to obligately halophilic) and of substrate affinities for ammonia and/or urea (Koops and Pommerening-Röser, 2001). Also nitrite oxidizing bacteria differ in ecophysiological requirements. *Nitrospira* generally is regarded as obligately chemolithotrophic, whereas *Nitrobacter* can thrive on organic compounds for energy generation (Bock, 1976). Members of *Nitrospira* prefer relatively low nitrite concentrations and is therefore found as most abundant nitrite oxidizer in wastewater treatment systems (Koops and Pommerening-Röser, 2001; Daims *et al.*, 2001). For example, *Nitrospira moscoviensis* was found to be inhibited at nitrite concentrations of 15 mM (Ehrich *et al.*, 1995). Mathematical modeling with physiological parameters of *Nitrosomonas* and *Nitrobacter* was used to predict the succes of nitritation (Hellinga *et al.*, 1998 and 1999), suggesting that mesophilic temperatures (between 35 and 40°C) and pHs between 7 and 8 are required. Normal temperatures in wastewater treatment plants (up to 20 °C) favor growth of nitrite oxidizers, with the result that ammonium is completely oxidized to nitrate. Since *Nitrobacter* is not commonly found in many wastewater treatment

plants (Daims *et al.,* 2001), it was interesting to study if nitritation could also be established from activated sludge under conditions of mesophilic temperature (25 and 30°C) and at near neutral pHs (7 and 7.5).

Here we focus on the effects of small temperature, pH, and dilution rate differences on the formation, composition and activity of the ammonia oxidizing biomass in bioreactors, inoculated from sewage sludge. The ammonia oxidizing community in the biomass of the nitritation reactors was quantitatively followed in time by using quantitative fluorescence *in situ* hybridization (qFISH). Changes in the diversity of the ammonia oxidizers were further studied by determining restriction fragment length polymorphisms of amplified *amoA* gene fragments (for the ammonia monooxygenase) from community DNA.

Material and Methods

Nitritation reactor setup and growth conditions

Nitritation reactors were inoculated from regular activated sludge and operated for periods of up to 50 days. The influences of pH (7 or 7.5), dilution rate (0.2 d^{-1} up to 1 d^{-1}), temperature (25°C or 30°C) and medium composition (synthetic -see below- or sludge digester supernatant) on the community development, nitrite and nitrate production and ammonium removal were tested (for a detailed operation plan, see Table 1). All sludge experiments were performed in pH and temperature controlled bioreactors (Bioengineering, Wald, Switzerland) in a total volume of 2.5 - 3 l. Each reactor was inoculated with activated sludge from the aeration basin from Werdhölzli, the wastewater treatment plant of Zürich, treating approximately 200,000 m^3 of wastewater each day. For inoculation, sludge biomass (same volume as used for the reactors) was immediately transported from the treatment plant to the laboratory, centrifuged for 10 min at 8,000 rpm (Centrikon H403, Kontron Instruments, Volketswil, Switzerland) and resuspended in 2.5-3 l of synthetic mineral medium (depending on the reactor volume) or in the same amount of sludge digester supernatant. Synthetic mineral medium contained 50 mM NH_4HCO_3, 50 mM phosphate buffer (61.5% KH_2PO_4 and 38.5 % K_2HPO_4 (M/M) for pH 7), 10 mM $KHCO_3$, 3 mM Na_2SO_4, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM EDTA in demineralized water. Furthermore, 2 ml of trace element solution 1 (containing 10 g $Na_2EDTA•2H_2O$ and 5 g $FeSO_4$ per liter demineralized water) and 1 ml of trace element solution 2 (containing per liter of demineralized water, 15 g $Na_2EDTA•2H_2O$, 0.43 g $ZnSO_4•7 H_2O$, 0.24 g $CoCl_2•6 H_2O$, 0.99 g $MnCl_2•4 H_2O$, 0.25 g $CuSO_4•5 H_2O$, 0.22 g $NaMoO_4•2 H_2O$, 0.19 g $NiCl_2•6 H_2O$, 0.08 g Na_2SeO_3, 0.014 g H_3BO_4) were added per liter mineral medium. Sludge digester supernatant was obtained from the sludge digesting tanks at Werdhölzli and an was used without any treatment. The approximate salt concentrations of the sludge supernatant were: chloride, 1.9 mM; sulfate, 0.08 mM; phosphate, <50 µM; Na^+, 3.9 mM; K^+, 3.1 mM; Mg^{2+}, 0.9 mM; Ca^{2+}, 2.6 mM; NO_3^-, <50 µM; NH_4^+, 56 mM.

Reactor cultures were flushed constantly with a mixture of oxygen and CO_2 to prevent an increase of the pH. For the experiments at pH 7.5, the cultures were flushed with Aligal28 (20% CO_2 and 80% O_2 with at least 99.5 % purity). Reactors operated at pH 7 were flushed with a gaseous mixture of 30% CO_2 (99.99% purity) and 70% O_2 (99.95%). All gases were purchased from Carbagas (Rümlang, Switzerland). The impeller speed of the reactor was 500 rpm and the pH was further maintained by automatic addition of 2 M KOH when necessary.

Table 1: Overview of the different experimental conditions tested for achieving nitritation

Reactor	Condition	Figure	Starting biomass	Medium	Inoculation time	Remarks
1 a	pH 7.5, 30 °C	2A, 3A, 4A	Activated sludge	Synthetic	Single reactor	Dilution rate from 0.2 to 0.3 d^1 at day 25. Half amount of starting sludge.
1 b	Repetition of 1 a	2B, 3B, 4B			1b and 2b together	pH increase at day 19 from 7.5 to 7.9
2 a	pH 7, 30 °C	2C, 3C, 4C			2a and 3a together	
2 b	Repetition of 2 a	2D, 3D, 4D			2b and 1b together	pH decrease at day 30 from 7 to 6.6
3 a	pH 7.5, 25 °C	2E, 3E, 4E			3a and 2a together	
3 b	Repetition of 3 a	2F, 3F, 4F			3b and 5 together	
4	pH 7, 30 °C	2G, 3G, 4G	Activated sludge and supernatant of sludge digester	supernatant of sludge digester	Single reactor	Dilution rates: 0.2 d^1 (day 0-15), 0.3 (15-17), 0.5 (17-20), 0.75 (20-22), 1.0 d^{-1} (22-27)
5	pH 7, 25 °C	2-4H			5 and 3b together	

Nucleic acid extractions

Total genomic DNA was isolated from sludge samples from the different reactors at regular time intervals. Cells (from 0.5 to 50 ml) were pelleted by centrifugation and resuspended in 0.9 ml of TEN homogenization buffer (TEN is a solution of 0.1 M NaCl, 10 mM Tris/HCl and 1 mM EDTA, pH 8.0) in a screw-capped vial. Glass beads (0.2 g, 0.1 mm in diameter) were added and the mixture was shaken twice for 1 min at 4,000 rpm in a Braun Cell Homogenizer (Inotech AG, Dottikon, Switzerland) with a 1-min interval on ice. The glass beads were allowed to settle without centrifugation and the supernatant was transferred to a fresh Eppendorf tube. This sample was mixed 1:1 with Tris-buffered phenol (pH 8). After vortexing, one volume of chloroform/isoamylalcohol (24:1 v/v) was added. After vortexing and centrifugation for 10 min at 15,000 × g and 4 °C, the water phase was transferred to a new Eppendorf tube and mixed with the same volume of chloroform/isoamylalcohol (24:1 v/v), vortexed and centrifuged for 10 min as before. DNA was precipitated with 2 volumes of ethanol and 0.1 volume of 3 M sodium-acetate (pH 5.5) for 1 hour at –80 °C or over night at –20 °C and recovered by centrifugation. The pelleted nucleic acids

were washed with a solution of 70% (v/v) ethanol plus 30% TE (TE is 10 mM Tris/HCl, 1 mM EDTA, pH 8) and dissolved in 10 mM Tris/HCl pH 7.5.

Amplification of *amoA*

Fragments of 491 basepairs of the *amoA* gene for ammonia monooxygenase were amplified from isolated sludge community DNA by using the polymerase chain reaction (PCR). Primers for *amoA* amplification were those published previously: amoA-1F (5'- ggggtttctactggtggt-3') and amoA-2R (5'-cccctc(GT)g(GC)aaagccttcttc-3') (Rotthauwe *et al.*, 1997). PCR reagents were used according to the supplier (Sigma, Buchs, Siwtzerland). PCR with *amoA* primers was carried out at a cycling regime of: 4 min at 95 °C, then 35 cycles of each 1 min 95 °C, 45 sec 60 °C, and 1 min 72 °C. Final extension was carried out for 3 min at 72 °C. The *amoA* PCR-products were either used directly for restriction fragment length polymorphism (RFLP) studies (see below) or cloned in vector pGEM-T-Easy (Promega, Wallisellen, Switzerland) and transformed into *Escherichia coli* DH5α by established procedures (Sambrook *et al.*, 1992). Selected *amoA* inserts were sequenced on GenElute™ - isolated plasmid DNA (Sigma) on both strands by using the Thermosequenase Kit (Amersham Biosciences Europe, Dübendorf, Switzerland) with IRD-800 and IRD-700 labelled universal vector-located primers (MWG Biotech, Ebersberg, Germany). Sequence transcripts were separated and analyzed on a LiCOR 4200L IR^2 automated DNA sequencer (LiCOR, Lincoln, Ne, USA).

RFLP

Amplified *amoA* fragments from community DNA of the reactors at different time points were digested simultaneously with *Rsa*I and *Hae*III (Amersham Biosciences Europe). Digested products were separated on SPREADEX EL 400 precast gels (Elchrom Scientific AG, Cham, Switzerland) together with a DNA size marker (M3, Elchrom), with a separation range from approximately 20 to 400 basepairs (bp). Gel electrophoresis was performed on a SEA 2000$^{®}$ apparatus (Elchrom) during 81 minutes (with buffer circulation and constant temperature of 55 °C) to ensure optimal and reproducible separation of the restriction fragments. After electrophoresis, the DNA was stained with SYBR gold (0.05 µl of concentrated stock/ml, Molecular Probes Europe BV, Leiden, Netherlands) and visualized under UV light. Digital images were recorded with the Q-EL™ 330 digital gel recording and analysis system (Elchrom) and further analyzed with the program RFLP-SCAN (CSP, Billerica, MA, USA). Similarity matrices were obtained by scoring the presence (= 1) and absence (= 0) for all bands in the different reactors, and producing a two-dimensional rectangular data matrix of binary codes, which was used for the clustering analysis. Bootstrapping using the program SEQBOOT from PHYLIP (Version 3.5, department of genomic sciences (www.gs.washington.edu) at the University of Washington (www.washington.edu)) (Filsenstein, 1993) was performed to generate 100 data sets. Clustering analysis was performed on the multiple data set by using the subroutine DOLLOP of PHYLIP with randomized input order. A consensus tree was produced with the subroutine CONSENSUS, and drawn with the program TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Fluorescence *in situ* hybridization (FISH)

Cells from up to 50 ml sludge culture were recovered by centrifugation and resuspended in phosphate-buffered saline (PBS), pH 7.4, consisting of 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per liter destilled water. The samples were

fixed with 4 % paraformaldehyde (in PBS) for 1 hour at room temperature. Afterwards, the cells were washed twice with PBS. The fixed cells were suspended in a solution of 50% PBS and 50% ethanol and stored at –20 °C. For FISH, 10 µl of the fixed sample (or multiple times 10 µl for qFISH) was applied on a well of a gelatine-coated (Amann *et al.* 1990) glass slide (Huber & Co., Reinach, Switzerland), dried for 1 h at 46°C and subsequently dehydrated in solutions of 50%, 80% (vol/vol, in 10 mM Tris/HCl of pH 7.5) and 100% ethanol for 3 min each. To start hybridization, 9 µl of hybridization buffer (with a composition dependent on the used probe, Table 2) and 1 µl of fluorescently labeled probe (at a concentration of 50 ng/µl) were added to a well. If necessary for increased selectivity, an unlabeled competitor (at 50 ng/µl) was added to the mixture. The hybridization was conducted for 1 h at 46°C in a humidified chamber.

Following hybridization, a stringent washing step was performed for 10 min at 48°C in a buffer with the appropriate NaCl concentration (Table 3). Samples were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Hicks *et al.*, 1992) at a final concentration of 10 mg/l for 5 min at room temperature and mounted in Citifluor (Citifluor, London, UK). All oligonucleotide probes were obtained from Microsynth (Balgach, Switzerland). Microscopy was performed on an Olympus BX50 microscope, equipped with filters HQ-CY3 and HQ-DAPI (both from Analysentechnik AG, Tübingen, Germany). Digital images were taken with a Sensys cooled CCD camera (Photometrics, Tuscon, Ariz., USA) and acquired in the program METAVIEW (Visitron, Puchheim, Germany).

Quantative FISH was performed with a Zeiss CSLM 510 confocal laser scanning microscope (Zeiss, Jena, Germany) equipped with a HeNe laser at 543 nm and an Ar-ion laser at 488 nm. Population sizes were determined as the relative biovolume taken up by cells stained with the Cy3 labeled specific probe compared to the biovolume of cells stained with all three Fluos labeled EUB-probes (Schmid *et al.*, 2000). Image analysis was performed with the delivered software package from Zeiss.

Table 2: Probes used for FISH and the corresponding hybridization and washing conditions.

Probe	Sequence (5'→ 3')	Target organisms	Formamide concentration (%)	Competitor	Reference
EUB338	gctgcctcccgtaggagt	Eubacteria	0 or together with specific probes		Amann *et al.* 1990
EUB338II	gcagccacccgtaggtgt				Daims *et al.* 1999
EUB338III	gctgccacccgtaggtgt				
NEU	cccctctgctgcactcta	Halophilic and halotolerant members of the genus *Nitrosomonas*	40	CTE	Wagner *et al.* 1995
Ntspa662	ggaattccgcgctcctct	*Nitrospira* genus	35	Comp Ntspa 662	Daims *et al.* 2000
NmII	ttaagacacgttccgatgta	*Nitrosomonas communis* lineage	25		Pommerening *et al.* 1996
Nsv443	ccgtgaccgtttcgttccg	*Nitrosospira* spp.	30		Schramm *et al.* 1998
Nso1225	cgccattgtattacgtgtga	Ammonia-oxidizing β-proteobacteria	35		Juretschko *et al.* 1998
Nse1472	accccagtcatgacccccc	*N. europaea*	50		
Nsm156	tattagcacatctttcgat	Various *Nitrosomonas* spp.	5		Mobarry *et al.* 1996
Nso190	cgatcccctgcttttctcc	Ammonia-oxidizing β-proteobacteria	50		
Nmo218	cggccgctccaaaagcat	*Nitrosomonas oligotropha* lineage	35		Gieseke *et al.* 2001
NmV	tcctcagagactacgcgg	*Nitrosococcus mobilis* lineage	35		Mobarry *et al.* 1996
CTE	ttccatcccctctgccg				
CompNtspa662	ggaattccgctctcctct				

Table 3: NaCl - concentrations for the stringent washing step.

Formamide	NaCl concentration (M)
0 %[1]	0.9
25 %	0.159
30%	0.112
35%	0.08
40%	0.056
50 %	0.028
60 %	0.014

1) No 5 mM EDTA at 0 % formamide.

Analytical measurements

Concentrations of nitrite, nitrate, chloride, sulfate and phosphate were determined by ion-exchange chromatography using a DX500-apparatus with an IONPAC-ATC1 anion trap column, a IONPAC-AG11 guard column, an analytical IONPAC-AS11-4 mm column, ASRSII-4mm suppressor and CD20 conductivity detector (from Dionex, Olten, Switzerland). The concentrations of ammonium, sodium, potassium, magnesium and calcium were quantified by ion-exchange chromatography using the same DX500-apparatus but with an IONPAC-CTC1 cation trap column, a IONPAC-CG12 guard column, an analytical IONPAC-CS12-4mm column, CSRS Ultra 4mm suppressor and CD20 conductivity detector (Dionex, Olten, Switzerland).

Protein concentrations in sludge samples were measured using the Bio-rad protein assay (BIO-RAD Laboratories, GmbH, München Germany). Cells were pelleted, resuspended in 0.1 M NaOH and incubated for 1.5 h in a boiling water bath. An aliquot (0.8 ml) of the diluted suspension was mixed with 0.2 ml Bio-rad dye-reagent and incubated for 15 min in the dark. The absorbance was measured at 595 nm. A standard-curve was created using known BSA-concentrations (BSA is bovine serum albumin, Fraction V, Sigma, Buchs, Switzerland).

Results

Nitritation activity under synthetic medium conditions

The effects of pH and temperature on the formation of active ammonia, but not nitrite oxidizing cultures were tested in continuously stirred tank reactors first with sterile synthetic medium containing 50 mM ammonium and HCO_3^- as sole carbon source. In all cases nitrite production started five to ten days after inoculating the reactors with fresh activated sludge, increasing afterwards to reaching an effluent concentration of between 40 and 50 mM (Fig. 2). Under all conditions, except in reactor 3A, the ammonium concentration dropped from an influent concentration of 50 mM to levels around 10 mM or below. This indicated that ammonium oxidation to nitrite only was effectively taking place. Nitrite oxidizing activity was small in most reactors and limited to the first five days after inoculation. Afterwards, a slow decline was observed. Nitrate was never produced in amounts higher than 7 mM. Protein concentrations –an indication for the total biomass in the reactors- decreased constantly from between 200 to 300 μg protein/ml directly after inoculation to more or less constant levels after two weeks of between 20 and 30 μg protein/ml (not shown). This was expected, since the community was forced to change from mixed heterotrophic (in activated sludge) to one dominating by autotrophic organisms (due to carbonate as only carbon source in the medium). The protein concentration corresponds approximately to the expected yield of ammonium oxidation (Egli, 2000).
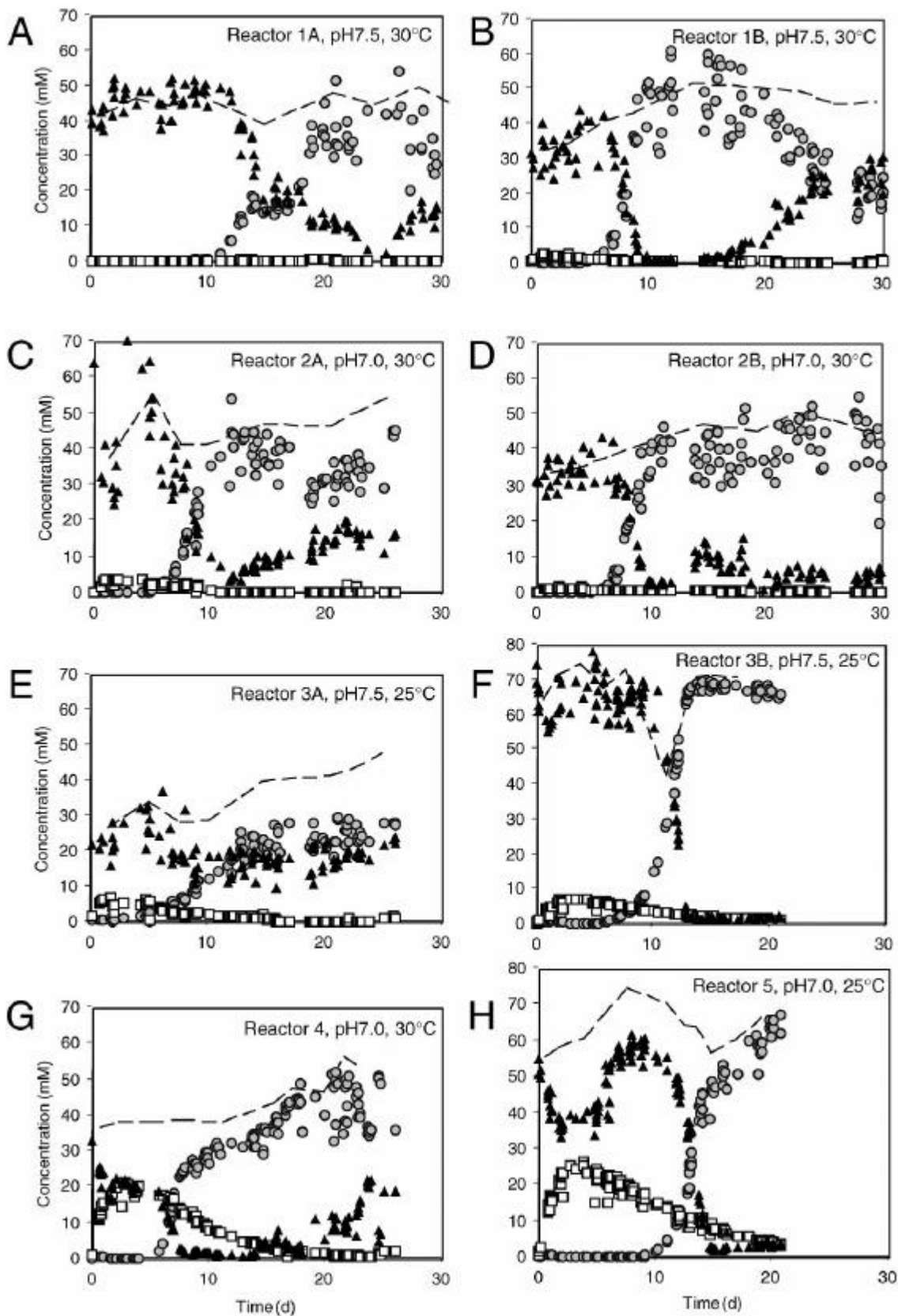
Fig. 2. Nitrogen dynamics in the different reactors during incubation time. Letters of panels correspond to Table 1 and to those in Figures 3 and 4. Shown are ammonium concentrations (black triangles), nitrate (open squares), nitrite (grey circles) and total nitrogen (dotted line), as sum of ammonium, nitrate and nitrite concentrations (all in mM) all in the reactors. Experiments in reactors 1A, 1B and 2B were operated for longer periods but not shown for clarity of presentation.

Contrary to our expectations, no large differences occurred in soluble nitrogen parameters (ammonium, nitrite and nitrate) during time at different pH and temperature. The only consistent observation was a stronger nitrate production at lower incubation temperatures, and a slightly longer lag in the occurrence of nitrite production (Figs. 2E and F). Due to technical reasons, not all reactors could be operated at the same time, but repetitions of the nitritation reactors at different starting points (and therefore slightly different composition of inoculated sludge) resulted in mostly similar dynamics of the nitrogen compounds (Figs. 2C and D).

Nitritation activity with sludge digester supernatant
Two reactors were operated with sludge digester supernatant instead of synthetic medium (reactors 4 and 5). In both cases, nitrite production also started after about 10 days, but relatively high amounts of nitrate (20-25 mM) were produced during the first two weeks of operating time of both reactors (Figs. 2G and H). After five days, however, no new net nitrate production occurred, since nitrate concentrations in the effluent from both reactors followed the washout expected at this dilution rate (not shown). The peak and subsequent decline in nitrate production occurred before the onset of measurable nitrite concentrations in the effluent. Nitrite production started earlier in the reactor operated at 30°C than the one at 25°C, which is consistent with the observations for the reactors running with synthetic medium. However operating conditions, both reactors finally established effective ammonium oxidation to high nitrite but low nitrate concentration.

Effects of dilution rates and pH stability
In two cases, the effect of increasing the dilution rate on nitrite production was tested. In reactor 1A (operated with synthetic medium at pH 7.5 and 30°C) an increase of dilution rate from 0.2 to 0.3 d^{-1} from day 25 to day 52 resulted in a complete washout of ammonium oxidizing activity from the reactor (onset shown in Fig. 2A). Ammonium concentrations after day 45 reached 50 mM and nitrite concentrations in the effluent decreased to below 5 mM (not shown). Reactor 4, which was fed with sludge digester supernatant, showed a completely different behavior (Fig. 2G). In this case, the dilution rate could be increased to about 0.75 d^{-1} without dramatic increase of ammonium levels in the effluent. Only at a dilution rate of 1.0 d^{-1} (after day 22) ammonium concentrations started to increase above 10 mM in the effluent. This indicated that reactors fed with sludge digester supernatant can be operated at higher dilution rates. This difference seemed to be the result of a reinoculation of ammonia oxidizers from the non-sterile sludge digester supernatant (see below).

The effects of pH changes on the activity of a stable nitritation system were investigated in two reactors. A pH change from 7.5 to 7.9 in reactor 1B at day 19 (operated with synthetic medium at 30°C) resulted in an immediate loss of ammonia oxidizing activity. Ammonium concentrations in the effluent increased and nitrite concentrations decreased to about 20 mM (Fig. 2B). A pH decrease from pH 7 to pH 6.6 in reactor 2B at day 30 and beyond also resulted in partial loss of ammonia oxidizing activity. Both nitrite and ammonium concentrations in the effluent changed to between 20 and 25 mM (not shown). These results indicated that the nitritation systems were relatively sensitive to pH changes outside the optimal range of 7 to 7.5.

Population analysis of the communities in different reactor configurations

We were interested to determine whether specific operating conditions would lead to very defined communities of ammonia oxidizers in the reactors. Hereto, sludge samples were analyzed at regular time intervals with qFISH and *amoA* RFLP. According to our focus we only applied FISH-16S rRNA probes targeting the ammonia and nitrite oxidizing community (Table 2). At time of inoculation the activated sludge contained relatively low numbers of ammonia oxidizing bacteria from three different lineages (*Nitrosomonas oligotropha/urea*, *N. communis*, *N. europaea/eutropha* (Koops and Pommerening-Röser, 2001)) and nitrite oxidizing bacteria from the genus *Nitrospira* (Daims *et al.,* 2000). The relative volume of Nmo218 stained compared to all EUB stained cells determined with confocal laser scanning 4.5 % ± 3.4 of the biovolume. Not quantifiable but judged from visual observations the biovolume of NmII stained cells was very similar to Nmo218 and there were even lower numbers of NEU stained cells (< 1 %). The relative level of Ntspa662 stained cells (indicative for *Nitrospira*) in the inoculated sludge was 3.25 % ± 3 (Fig. 3C).

In general, the population analysis of all reactors confirmed the trends observed in nitrogen parameters (Fig. 2). The community of ammonia oxidizing bacteria increased under all conditions, parallel to the observed increase of nitrite concentrations in the effluent. On the other hand, the nitrite oxidizer community never established itself, but decreased and was no longer quantifiable after ten days (Figs. 3A, D and G). Interestingly, similar nitritation activities (Fig. 2) could be the result of different ammonia oxidizing communities. Under conditions of pH 7.5 and 30°C and with synthetic medium, the community of ammonia oxidizers developed to an almost exclusive population of NEU-stainable cells (Figs. 3A and B). In the biomass of these two reactors, the relative amount of cells stained with the more general probe Nso190 was the same as that stained with NEU. Both reactors operated at pH 7 and 30°C established two different FISH-groups of ammoniua oxidizing bacteria. In both reactors, there was an initial increase of a population stainable with the NEU-probe (Figs. 3C and D), which decreased after approximately 10 days of incubation at the expense of an increase of a second group of ammonia oxidizers, stainable with the more general probe Nso190. Under conditions of pH 7.5 and at 25°C the increase in the population of ammonia oxidizers was less pronounced than at 30°C (Figs. 3E and F). FISH community quantification with the probes NEU and the more general probe Nso190 gave the same values, suggesting that the ammonia oxidizing community under these conditions again developed reproducibly to one dominated by NEU-stainable cells. This suggests that pH rather than temperature is the factor for selection of the NEU-stainable population.

Both reactors operated with sludge digester supernatant also developed to a community dominated by NEU-stainable cells. Despite a pH of 7.0, no transition to other Nso190-stainable organisms took place in these reactors, since relative levels of the Nso190-stainable population were the same as those of the NEU-stainable population and the probe NEU stained all nitrifying aggregates (not shown). It might be that in the case of these two reactors the increased dilution rate or the salt composition of the sludge digester supernatant (Table 4) were selecting for the NEU-population.
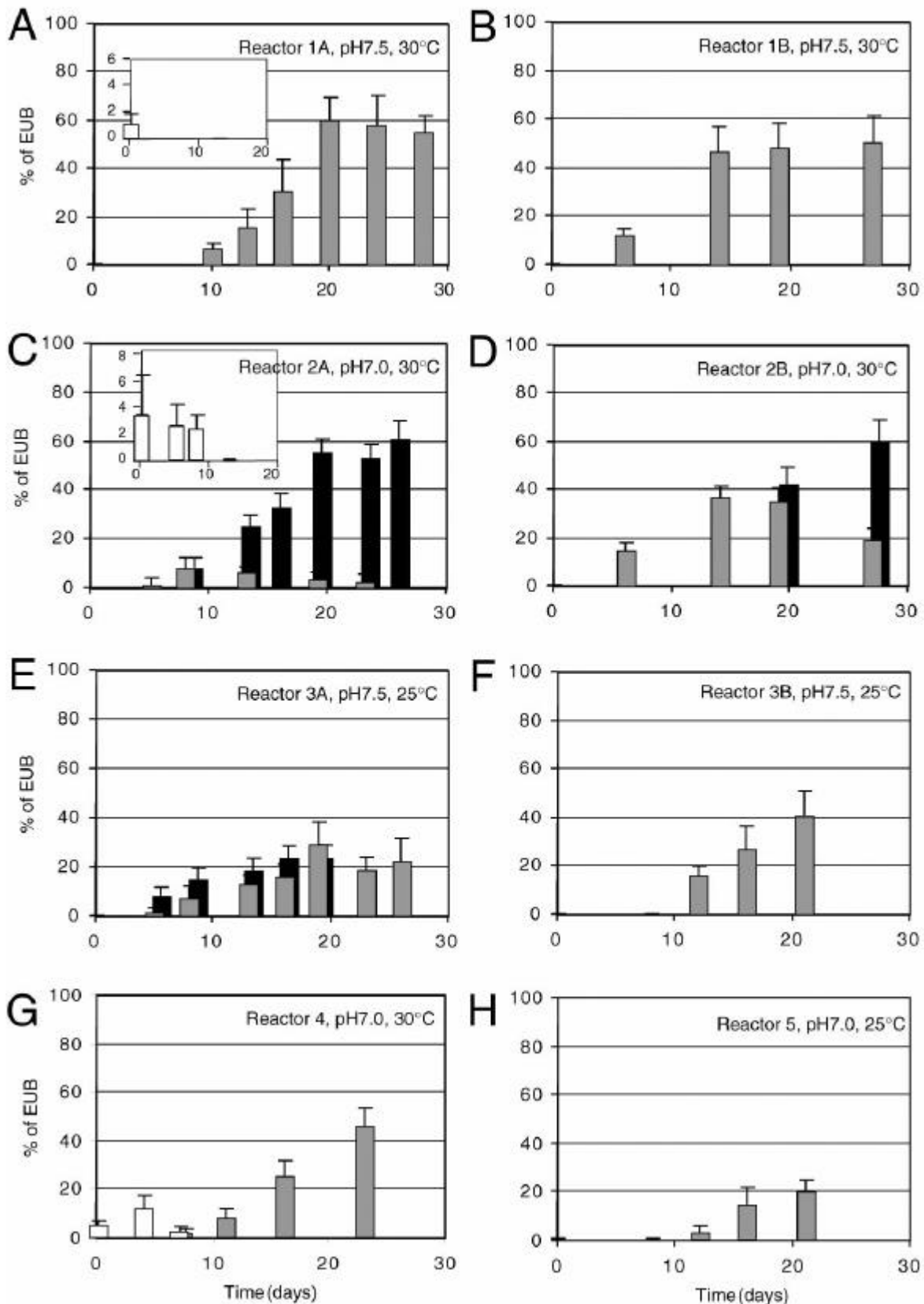
Fig. 3. Community composition development of ammonia and nitrite oxidizers in the different reactors during operation time. Panel numbers correspond to those in figures 2 and 4. Shown are relative biovolumes of FISH probe-stainable groups compared to the biovolume taken up by the biomass stainable with the EUB probe mixture (% of EUB). Probes used for quantitation were NEU (grey bars), representative for the *N. eutropha/europea* group, Nso190 (black bars), representative for nearly all ammonium oxidizing bacteria from the β-subgroup of *Proteobacteria* and Ntspa662 (white bars), for *Nitrospira*. Other probes were also used (see

Table 2), but populations detectable by those probes were either too small to be quantifiable (less than 1% of EUB) or absent. Note that in some instances the population sizes were detectable but appear as very small bars on the scale (e.g., day 0, 6 and 8 in some reactors). The inset in panels A and C shows the *Nitrospira* populations on an enlarged scale.

Diversity of the *amoA* gene in the different communities
Since most FISH probes were not species specific we used RFLP-analysis of the *amoA* gene to investigate the strain-level diversity of the involved ammonia oxidizing bacteria under the different reactor conditions. For this purpose, sludge DNA was extracted, a part of the *amoA* gene amplified by PCR, digested with two enzymes (*Rsa*I and *Hae*III) and the products separated on high resolution acrylamide gel (Fig. 4). Both reactors operated at pH 7.5 and 30°C with synthetic medium developed into a homogeneous identical population (Figs. 4A and B, Fig. 5), except for some minor banding differences. The RFLP of this population was mostly represented by the banding pattern derived from one of the cloned sequences (see below, Fig. 4F, lane 'NITa'). The sampling point at day 8 for reactor 1B (Fig. 4B) illustrated a sort of transition state, during which the NEU-stainable population was not dominating the reactor yet. The banding intensity of the main bands in reactor 1B also reflected the relative increase of the NEU-stainable population between day 6 and 14 in FISH analysis (Figs. 3B and 4B), indicating that there was no major bias during PCR amplification. As expected from qFISH analyses, the RFLP-patterns in reactors running under conditions of pH 7.0 and 30°C differed considerably from those at pH 7.5 and 30°C, and at pH 7.5 and 25°C. Moreover, both reactors operated under the same conditions developed into a different community (Figs. 4C and 4D). Reactor 2A (Fig. 4C) showed a transient occurrence of the same NITa-RFLP-group as observed in reactors 1A and 1B between days 0 and 13, which was then taken over by a different RFLP-group. Representative bands for the NITa-group of reactor 1 (183, 97, 71, 52, 41 and 40 bp) were also visible in reactor 2B (Fig. 4D) and more intense as in reactor 2A. This is consistent with the changes in relative amounts of NEU-stainable cells (Fig. 3D). However, from day 16 onwards a different RFLP-pattern started to appear (represented by a double band at 185, and bands at 140, 79 and 66 bp). These RFLP patterns may originate from different *amoA* sequences. At pH 7.5 and 25°C again a different type of community developed, although this had not been observed with qFISH analysis (Figs. 4E and F). In addition, the communities in both these reactors were not identical. In reactor 3A a dominant community developed which was represented by bands at 140, 50, 45 and 42 bp (Fig. 4E). This was most similar to the RFLP-type of cloned sequence NITc (see Fig. 4H, lane 'NITc'). The community in reactor 3B was dominated by an RFLP-type similar to that observed in reactors 1A and 1B, although consistently the 79-bp band was lacking (Fig. 4F). Both communities were relatively homogenous at all time points, indicating that no major transitions in the community structure took place. Patterns in the communities of reactors 4 and 5, which were fed with supernatant of sludge digester, were very similar to one another but very different from those of the reactors fed with synthetic medium (Figs. 4G and H). Interestingly, the RFLP-patterns derived from the community of the reactors were also very similar to that of DNA from the biomass of the sludge digester supernatant itself (Figs. 4G and H, lanes 'SL'). This was a confirmation for the hypothesis that those reactors were continuously inoculated from the non-sterile sludge digester feed. We suspect that different populations make up the community of ammonium oxidizers in reactors 4 and 5. For example, the pattern of cloned sequence NITb (Fig. 4H, lane ''NITb') could be found among the complex patterns in reactors 4 and 5. The effect of increasing dilution rate in reactor 4 was also

immediately visible as a change in community structure (Fig. 4G, lanes 18 and 26), which had not become evident from FISH analysis.

From each of the reactors 1A, 2A and 3A, approximately ten cloned *amoA* gene fragments were sequenced. From reactor 1A, one clone were from DNA isolated at day 28 and 9 clones of DNA retrieved after 31 days of operation, from reactor 2A from day 8 (one clone) and day 19 (9 clones), and from reactor 3A from day 13 (one clone) and 19 (19 clones). One type of sequence was detected among the reactor 1A-clones, (named NITa) which had the highest homology to GenBank entry AF272483 (98%). This sequence groups in the *N. europaea/eutropha* group. The RFLP pattern of this cloned sequence (Fig. 4F, lane NITa) was indeed representative for the patterns in reactors 1A and B. One sequence of the reactors 2A and 3A clones (NITc) displayed 99% nucleotide identity to GenBank entry AF276485. This sequence groups in the *Nitrosomonas* cluster 6a containing the cultured species *N. urea* and *N. oligotropha* (Purkhold *et al.,* 2000). The RFLP-type of NITc was found in reactors 2A and 3A (Figs. 4C and E). One additional sequence cloned from reactor 3A-DNA (NITb) displayed 99.8% identity to the sequence in AF276476. This sequence grouped near cluster 6 (Purkhold *et al.,* 2000), however, its RFLP-type was not clearly visible in reactor 3A, but only in the sludge digester supernatant for reactor 4 (Fig. 4G).

The clustering of RFLP patterns from the different reactors was analyzed by DOLLOP (Fig. 5). In most cases, the banding pattern of all samples of one reactor cluster together although the pattern changed during time. The banding pattern of rector 4 and 5 group quite close together because of very similar banding patterns of the two reactors (Fig. 4). An exception is reactor 3A with no obvious trend in the banding pattern during time and therefore no similar clustering.
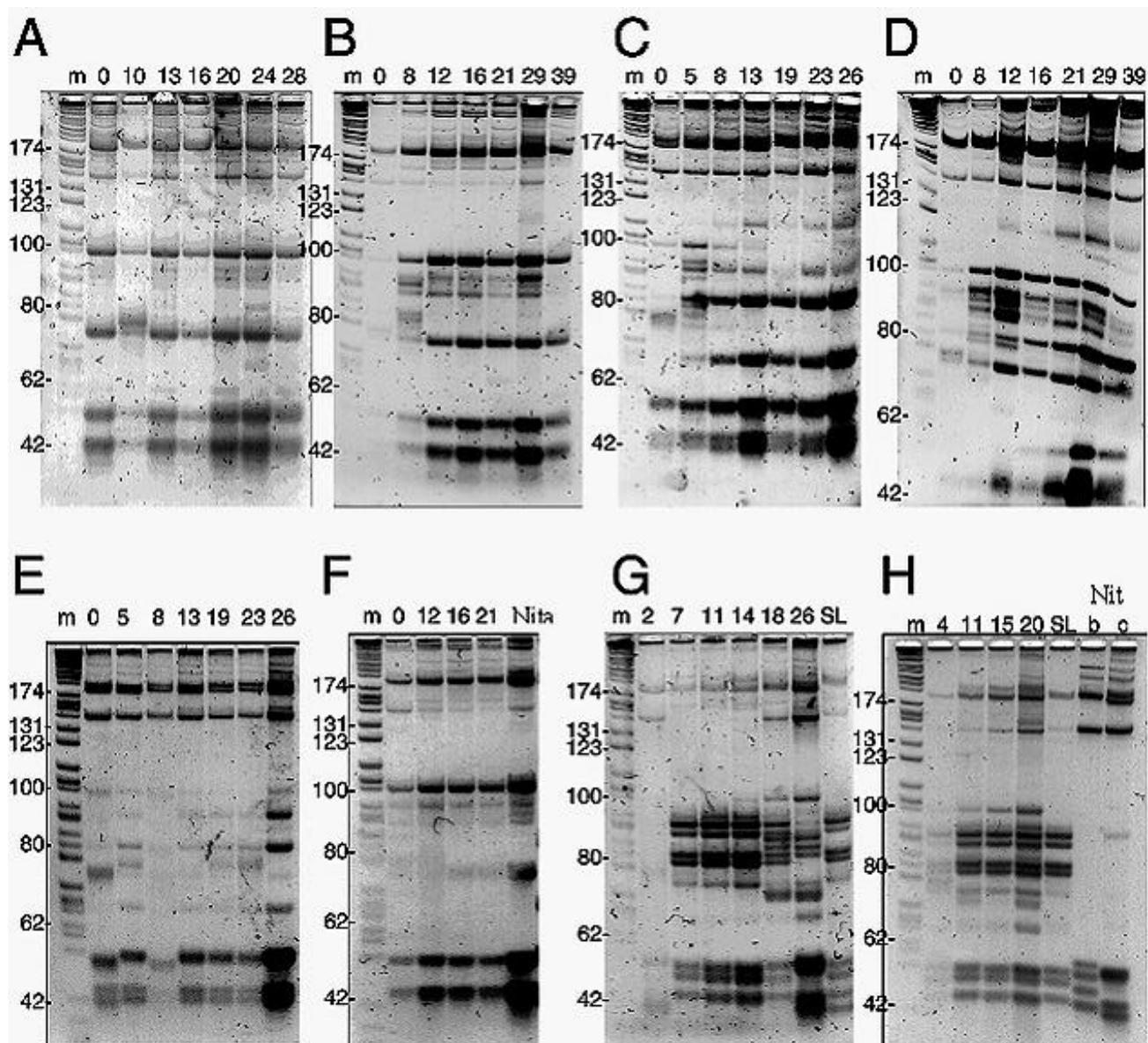
Fig. 4. RFLP patterns of the *amoA* amplifiable DNA in the communities from the different reactors. Panels correspond to the assignments given in figures 2 and 3. Sizes of some of the bands in the M3 marker are indicated in bp on the left of each panel. Lanes are marked according to the sampling day, or with 'm', M3 marker; NITa, NITb and NITc, cloned and sequenced *amoA* fragments; SL, incoming biomass from the sludge digester.
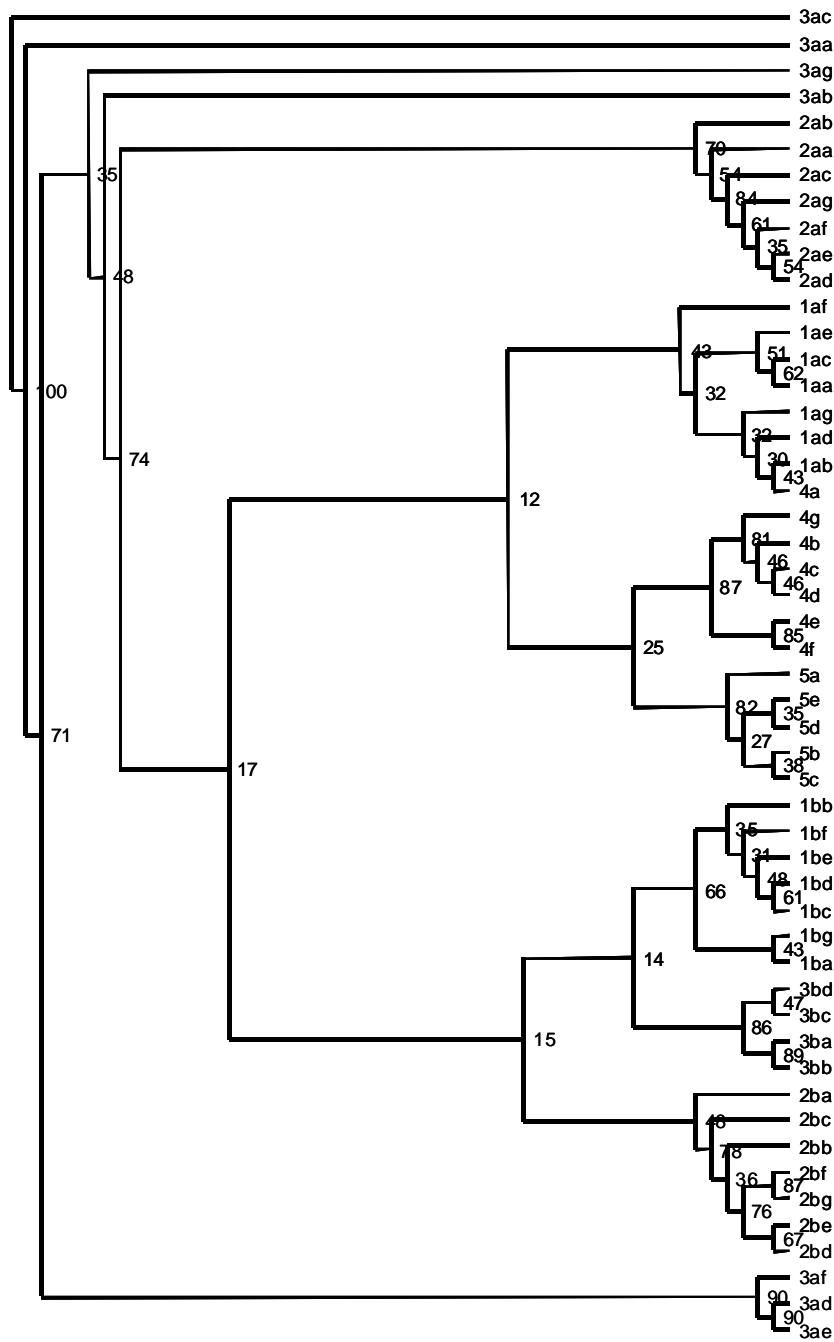
Fig. 5. Clustering of RFLP patterns from the different reactors as analyzed by DOLLOP on 100 replicated bootstrapped data sets of the banding patterns of Fig.4. Numbers at the nodes indicate the number of times that particular node appeared among all 100 datasets.

Discussion

Despite only small differences in operating parameters (pH 7.0 or 7.5; temperature of 25°C or 30°C), the communities of ammonia oxidizers which developed in the nitritation reactors were remarkably different. Only at pH 7.5 and 30°C the sludges developed into almost identical communities of ammonia oxidizers belonging to the *N. eutropha/europea* group (stainable with NEU and represented by *amoA* sequence NITa), even though the reactors were started at different time points with different sludges. All other conditions led to communities either from the same FISH- group but different in *amoA* RFLP (at 25°C and pH 7.5), or even different FISH groups (at pH 7.0 and 30°C). This suggests that pH is the parameter determining the selection of *N. eutropha/europea* ammonia oxidizers, more than temperature. The two reactors operated with sludge digester (reactors 4 and 5) supernatant established a community with very similar RFLP patterns, at least until the time point of changing dilution rates (reactor 4, day 18) even though the reactor temperatures were different. The composition of this community was clearly determined by continuous reinoculation from the sludge digester supernatant, which may therefore be an important factor in technical scale applications. Interestingly, pure cultures of *N.communis* do not show a salt requirement (Koops and Pommerening- Röser, 2001), however the synthetic medium contained quite high phosphate and potassium concentrations. The ammonia oxidizing bacteria stained with Nmo218 (containing the pure cultures *N. urea* and *oligotropha*) were washed out in all reactors. These bacteria have oligotrophic freshwater as preferred habitat and a very low Ks for NH_3 (less than 5 µM) which makes these organisms good competitors in a multispecies wastewater treatment system (Koops and Pommerening- Röser, 2001), however, at high salt and ammonia concentrations in our reactors, it is no surprise, that Nmo218 stained cells washed out of the system.

One important conclusion from our work is that communities of ammonia oxidizers with different composition and complexity may form in reactor systems without obvious differences in performance of chemical parameters (such as nitrite formation or ammonium oxidation). From a perspective of performance stability, it might be wiser to chose for conditions favouring a more complex communty of ammonia oxidizers. However, this was not tested systematically in our experiments. Both the relatively homogeneous sparse communities at pH 7.5 and 30°C (reactors 1A and B) were easily disturbed by a slight increase in dilution rate and pH increase. On the other hand, also the more diverse community at pH 7.0 and 30°C (reactor 2B), lost nitritation activity soon after a pH decrease. The only system resisting a strong dilution rate increase was the complex community established with the sludge digester supernatant at pH 7.0 and 30°C (reactor 4), although with an equally strong shift in community composition.

Surprisingly, all conditions tested led to a development of sludges performing nitritation without nitrate formation. Nitrite oxidizing bacteria from the genus *Nitrospira* were present in the beginning of all reactors incubations, but gradually lost activity and were washed out almost completely from the reactors. This could be demonstrated by qFISH. Nitrite oxidizers definitively remained more and longer active at lower temperatures (25°C) and in the reactors operated with sludge digester supernatant. The better survival of nitrite oxidizers at lower temperatures is also known from other systems (Hellinga *et al.*, 1998; Fux *et al.*, 2002). On the other hand, the temperature difference alone cannot explain the better survival of nitrite oxidizers in the sludge digester operated reactors. One of the factors may be the higher salt

concentration in the synthetic medium, primarily consisting of tenfold higher potassium ion, phosphate and sulphate concentrations (K^+: 60 mM compared to 3, sulphate: 3 mM opposed to 0.08), and twofold higher concentrations of sodium and chloride. The other factor may lie in a reinoculation of nitrite oxidizers from the sludge digester, similar to ammonia oxidizers. It is known from literature that freshwater *Nitrospira* are salt sensitive (Koops and Pommerening-Röser, 2001), however information is based on a few pure cultures of *Nitrospira* and the term salt sensitivity was not further specified. Nitrate production stopped very soon after inoculation in all reactor systems (after approximately 5 days), already before nitrite started to accumulate in the reactor. This suggests that not so much nitrite itself, but the high ammonium concentrations prevailing at that time in the reactor (around 50 mM) were responsible for the decline of nitrite oxidizing activity. Since most *Nitrospira* species are adapted to low nitrite concentrations (Daims *et al.*, 2001) they may have further become inhibited by the increasing nitrite concentrations.

Our results show that it should be possible to obtain stable nitritation reactors starting from activated sludge under a variety of conditions (at least within the boundaries of pH 7 to 7.5 and temperatures of 25°C and 30°C). On a technical scale, engineers have for several years applied the CANON process for the treatment of ammonium-rich wastewater (Completely Autotrophic Nitrogen-removal Over Nitrite) (Hippen *et al.*, 2001; Third *et al.*, 2001; Fux, 2003). If the nitritation process were to be combined with anaerobic ammonium oxidation, it would at least be very important to maintain temperature and pH at the higher end (i.e., pH 7.5 and temperatures 30°C or higher), since this would lead to optimal activity of anaerobic ammonium oxidizers (Egli *et al.*, 2001; Jetten *et al.*, 2000). There is no particular need for complete oxidation of ammonium to nitrite in the nitritation reactors, since anammox bacteria need almost equimolar amounts of nitrite and ammonium for their energy generation (van de Graaf 1996). From the prospective of dimensioning of the aerobic ammonium oxidation reactor, it seems rather necessary to increase the dilution rates in the nitritation reactor to around 0.8 to 1 d^{-1} (Fux, 2003). Otherwise, larger reactor volumes would be needed, which causes higher costs and need more space. This would indicate that operation of the system with sludge digester supernatant is favourable, since through reinoculation of ammonium oxidizing bacteria such a type of reactor is less susceptible to high dilution rates.

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Chapter 5: Molecular detection of anammox bacteria in different environments

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Abstract

Anaerobic ammonium oxidation (anammox) is a relatively recent discovery but has now been observed in different (mostly technical) systems around the world. The source for anammox bacteria, however, remains obscure. In this work, the presence of putative anammox bacteria in different environmental samples was investigated using PCR with specific primers for the 16S rDNA of this type of bacteria. Samples were taken from six different wastewater treatment plants (including an SBR with a much higher sludge age fed with municipal activated sludge) around Zürich and from six monitoring wells at the chemical landfill in Kölliken (CH), which had previously been recognized as a site where anammox activity occurred. Anammox specific DNA was amplified from DNA of two wastewater treatment plants and from the SBR, all with relatively high sludge ages. However, anammox DNA was only amplifiable from the activated sludge but not from sludge digester samples. Anammox specific DNA was also amplified from biomass recovered from leachate of all monitoring wells at Kölliken. This suggests that anammox bacteria originate in the underground but can also be minor inhabitants of wastewater treatment plants.

Introduction

The anammox process involves the oxidation of ammonium with nitrite to N_2 under strictly anoxic conditions (Strous *et al.*, 1997; Egli *et al.*, 2001). Only in a few cases have bacteria carrying out this reaction been enriched to sufficiently high purity to characterize their physiology (Strous *et al.*, 1999a; Egli *et al.*, 2001). Evidence for anammox activity, however, has been obtained in a larger variety of engineered systems, such as a rotating biological contactor (Siegrist *et al.*, 1998; Helmer and Kunst, 1998; Schmid *et al.*, 2000; Pynaert *et al.*, 2002), sequencing batch reactors (Strous *et al.*, 1998), enrichments (Strous *et al.*, 1999a; Egli *et al.*, 2001; Toh *et al.*, 2002) and other pilot plants (van Dongen *et al.*, 2001; Fux *et al.*, 2002). This suggested that anammox bacteria can build reasonably sized populations provided that the reactor conditions are suitable for the anammox process. Still, the source of the inoculum and the natural environment of occurrence of anammox bacteria are not well known although the anammox process was predicted since a long time (Broda, 1977). In one case, anammox activity was reported in marine sediments (Thamdrup and Dalsgaard, 2002). These authors showed by ^{15}N activity measurements that nitrate rather than nitrite was reduced to dinitrogen (albeit with a transient accumulation of nitrite) with a concomittant removal of ammonium. However, since the two highly enriched anammox cultures *Candidatus* Brocadia anammoxidans and Kuenenia stuttgartiensis (Strous, 2000; Egli *et al.*, 2001) only convert ammonium to N_2 with nitrite but not with nitrate as electron acceptor, the observed nitrate reduction itself is not a strong proof for anammox activity in natural environments even though a transient accumulation of nitrite was observed. By comparative sequence analysis of the 16S rDNA of enriched anammox bacteria and of environmental clones (Strous, 2000; Schmid *et al.*, 2000; Egli *et al.*, 2001) it was found that all known anammox bacteria contain conserved sequence motifs distinguishing them from other related bacteria. On the basis of these motifs, it was possible to derive oligonucleotides for

use in fluorescence *in situ* hybridization (FISH) targeting anammox bacteria in microbial communities (Neef *et al.*, 1998, Schmid *et al.*, 2000). In this way, anammox bacteria were detected in rotating disk contactors, pilot plants and marine sediments (van de Graaf et al., 1995; Siegrist *et al.*, 1998; Strous *et al.*, 1999b; Schmid et al., 2000; Hippen *et al.*, 2001; Thamdrup and Dalsgaard, 2002). Some studies suggested that the natural origin of anammox bacteria was the deeper saturated zone of soils (Chapter 3).

Here we used the polymerase chain reaction (PCR) to detect the possible presence of anammox bacteria in natural and engineered systems. Sources of material were the leachate at different monitoring wells in a chemical landfill in Kölliken (Siegrist *et al.*, 1998; Egli *et al.*, 2001; Table 1), activated sludge, and sludge digester material from different wastewater treatment plants in the region of Zürich.

Materials and Methods

Fluorescence *in situ* hybridization (FISH) and microscopy

Fixation of the samples and FISH was carried out as described previously (Chapter 4). Samples were dispersed by ultrasonic treatment and counterstained with DAPI (4,6-diamidino-2-phenylindole; Hicks *et al.*, 1992) and mounted in Citifluor (Citifluor Ltd., London, U.K.). All oligonucleotide probes were obtained from Microsynth (Balgach, Switzerland). The probe Amx820 (5´ aaaacccctctacttagtg3´; Schmid *et al.*, 2000) was used to detect putative anammox bacteria. Microscopy was performed on an Olympus BX50 microscope as described elsewhere (Chapters 3 and 4).

Nucleic acid extraction

Activated sludge samples and samples form sludge digesters were taken from wastewater treatment plants in Regensdorf, Bassersdorf, Opfikon, Meilen, Dübendorf and Uster (all in the Zürich area, Table 2) and from a sequencing batch reactor (SBR) in Meilen, which was fed with wastewater from the aeration basin of the main treatment process. An amount of 4.5 ml of sludge, containing between 2.5 and 3.5 g total suspended solids (TSS) per liter, was centrifuged and resuspended in 0.9 ml extraction buffer and was used for DNA extraction by glass bead disruption and column purification as described previously (Chapter 4). The purified DNA from this volume of sludge was finally dissolved in 200 µl of 10 mM Tris/HCl (pH 7.5).

Leachate was collected from five different monitoring wells at the landfill in Kölliken (near Aarau, Switzerland) (Siegrist *et al.*, 1998; Egli *et al.*, 2001) and from the final wastewater infiltrate before entering the first trickling filter. Hereto four litres of leachate were collected by pumping the leachate to the surface. The DNA from leachate samples was extracted with the following slightly different procedure. Cells from a 2 liter sample of leachate were pelleted by centrifugation and resuspended in a screw-capped vial in 0.9 ml of TEN homogenization buffer (0.1 M NaCl, 10 mM Tris/HCl pH 8.0 and 1 mM EDTA pH 8.0). Glass beads (0.2 g, 0.1 mm in diameter) were added and the mixture was shaken twice for 1 min at 4,000 rpm in a Braun Cell Homogenizer (Inotech AG, Dottikon, Switzerland) with a 1-min interval on ice. The glass beads were allowed to settle without centrifugation, and the supernatant was transferred to a fresh Eppendorf tube. This sample was extracted once with Tris-buffered phenol (pH 8) and twice with chloroform/isoamylalcohol (24:1, v/v). DNA was precipitated from the water phase by mixing with 2 volumes of ethanol and 0.1

volumes of 3 M sodium acetate (pH 5.5) at –80 °C for one hour and by centrifuging, then washed once with ethanol/TE (70:30 (v/v), TE is 10 mM Tris and 1 mM EDTA pH 8), and finally dissolved in 10 mM Tris/HCl pH 7.5.

PCR

The reaction solution for the PCR contained 5 µl polymerase buffer (from a 10 x concentrated stock), 0.25 µl *Taq* DNA polymerase (5 U/µl), 0.4 µl of deoxynucleotide mixture (25 mM each) and 43.35 µl double destilled H_2O. Forward (Amx820, 5´ aaaaccctctacttagtg 3´) and reverse primer (Pla46rc, 5´ ggattaggcatccaagtc 3´) were added each at 10 ng per reaction. All reagents were provided from Sigma (Buchs, Switzerland) except for the primers which were from Microsynth (Balgach, Switzerland). The sequence of the Pla46rc primer is the reverse complement of its published version (Neef *et al.*, 1998). The primers for the eubacterial 16S rDNA amplification were 16S 6f (5´ ggagagttagatcttggctcag 3´) and 16S 926r (5´ ccgtcaattcctttragttt 3´) (Liu *et al.*, 1997). An amount of 1 µl of the purified (diluted) sample DNA was finally added to start the PCR. DNA from the enriched culture of *Candidatus* Kuenenia stuttgartiensis (Egli *et al.*, 2001) was used as positive control for the PCR. Amplification was performed in a thermocycler (Eppendorf Mastercycler gradient, Eppendorf-Netheler-Hinz GmbH, Hamburg) at the following conditions: initial denaturing step for 4 min at 95 °C, 35 cycles of 1 min at 95°C, annealing at 50 °C for 1 min and extension for 1.5 min at 72°C and a final elongation step of 4 min at 72 °C. Optimal annealing temperature for the Amx820/Pla46rc primer set was determined by running a gradient program from 41°C until 54°C. PCR products (10 µl of the reaction) were separated on a 0.8% agarose gel and visualized by staining with ethidium bromide.

Analytical measurements

Ammonium was quantified in Kölliken using a photometric test (test number 303-305 and CADAS 30, which were both from Dr. Lange AG, Düsseldorf, Germany). TOC was quantified with a TOC-Analyser 5000A (Shimadzu, Tokyo, Japan). These data were kindly provided by P. Lais.

Results and discussion

Anammox amplifiable 16S rDNA at the Kölliken landfill area

In order to find a possible source of natural anammox bacteria, we focused on the region around the landfill in Kölliken (near Aarau, Switzerland), since the trickling filter systems treating the landfill's leachate had been shown to contain a substantial enrichment of anammox bacteria of the type *Candidatus* K. stuttgartiensis (Chapter 3). DNA was extracted from biomass collected from leachate from monitoring wells with the highest ammonium concentrations at the landfill area and from the wastewater finally flowing into the treatment system (Table 1). With the conserved primers Pla46rc and Amx820, which until now target all known anammox bacteria (Strous, 2000; Schmid *et al.*, 2000; Egli *et al.*, 2001) products of the appropriate size of 0.8 kb were obtained for DNA of all sampling sites (Fig. 1).

Fig. 1: Detection of anammox amplifiable DNA in the leachate of Kölliken by using the primer set Amx820/Pla46rc. Lanes: 1, DNA size marker; 2 and 3, anammox enrichment culture (37 and 4 ng DNA, respectively); 4 and 5, well P0 (48 and 5 ng DNA); 6 and 7, well P1 (65 and 7 ng); 8 and 9, well P2 (420 and 42 ng); 10 and 11, influent into the reactors (335 and 34 ng); 12 and 13, well P3 (175 and 18 ng); 14, 15 and 16, well P8 (20, 2 and 0.2 ng); 17, empty, 18, anammox enrichment; 19, negative control (water); 20, size marker.

The maximal amount of PCR product was found between an annealing temperature of 50 and 54 °C (Fig. 2). Since DNA from the anammox enrichment culture gave a product of the same size and the negative control with only water gave no product, these results suggest that anammox bacteria were present in the leachate before entering into the trickling filter system and therefore must be native to the groundwater site or to the landfill material. From looking at the amount of product in DNA dilutions of the different samples, it appeared that well P8 contained the highest amount of anammox amplifiable material (Fig. 1). No clear correlation could be observed between ammonium and TOC concentration and the abundance of anammox amplifiable material. The TOC content is quite high for an anvironmental sample (Münster, 1993).
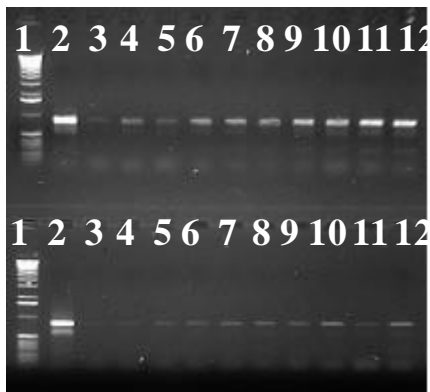
Fig. 2: Effect of different annealing temperatures on the amount of PCR product obtained with the Amx820/Pla46rc anammox primer set, using 0.2 ng (upper panel) and 0.02 ng (lower panel) of DNA isolated from the anammox enrichment culture. Lanes (left to right): 1kb standard (lane 1), positive control with 2 ng DNA from the enrichment culture (Egli *et al.*, 2001; lane 2), 40.85 (lane 3), 41.8 (lane 4), 43.25 (lane 5), 45.05 (lane 6), 47.0 (lane 7), 49.0 (lane 8), 50.9 (lane 9), 52.5 (lane 10), 53.6 (lane 11), 54.1°C (lane 12), respectively.

Table 1. TOC and NH_4^+ concentrations of the leachate in different monitoring wells.

Monitoring well[a]	Ammonium concentration (mM)[b]	TOC (mg C/l)[b]
P0	28.6	504.6
P1	32.5	107
P2	34	102.6
Influent to the reactors	26.2	289.5
P3	4.1	85.5
P8	8.1	21.7

a) The temperature of the leachate at the sampling time ranged from 14.6 to 16.3 °C
b) Temperature, ammonium and TOC concentrations were determined three days before sampling for DNA extraction.

Anammox-specific amplifiable DNA in wastewater treatment plants
To test for the presence of anammox bacteria in wastewater treatment plants, we isolated DNA from samples of the aerated stage of the activated sludge and of the sludge digester, where anoxic conditions prevail. PCR products with the anammox specific primers were found for activated sludge from Bassersdorf, Opfikon and the sequencing batch reactor in Meilen (Figs. 3 and 4). No anammox organisms on the other hand were detected in activated sludge from Regensdorf, Meilen, Dübendorf and Uster, nor in any of the sludge digesters (Fig. 3 and 4). Since in all cases DNA could be amplified with the 16S rDNA primer set, we conclude that no inhibiting factors were present in the samples which could have been responsible for the absence of Amx/Pla amplifiable DNA.
With FISH and probe Amx820 on fixed sludge samples, however, no anammox bacteria were detected in any of the wastewater treatment plants.
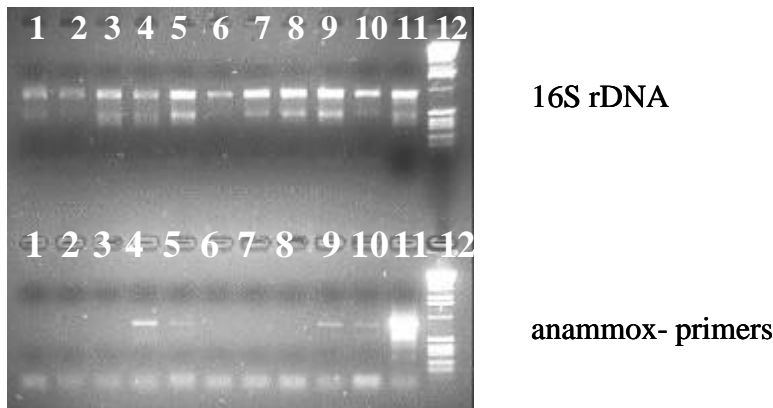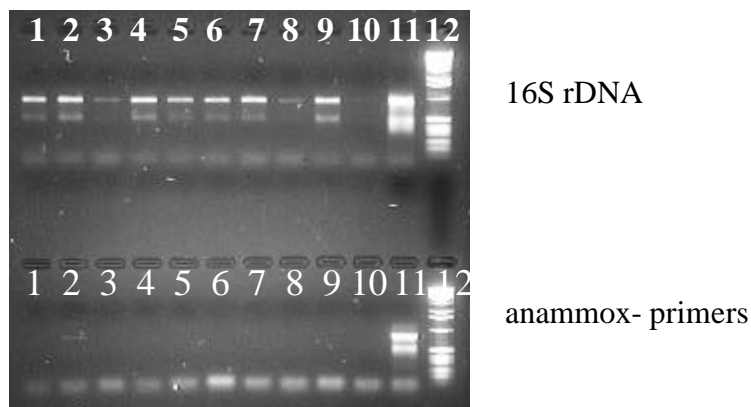
Fig. 3: PCR products of different sludge DNA samples. Upper panel: 16S rDNA eubacterial primers, bottom panel: anammox primers. Lanes: (1 through 5, approximately 0.05-0.1 ng DNA) 1, Regensdorf sludge digester; 2, Regensdorf, activated sludge; 3, Bassersdorf sludge digester; 4, Bassersdorf activated sludge; 5, SBR Meilen; 6 through 10 (approximately 0.5 – 1 ng DNA), same order as 1 through 5; 11, anammox enrichment culture; 12, DNA size marker. The lane number is the same for 16S rDNA and anammox-PCR.

Fig. 4: PCR products of different sludge DNA samples. Upper panel: 16S rDNA eubacterial primers, bottom panel: anammox specific primers. Lanes: 1, Opfikon sludge digester; 2, activated sludge; 3, Meilen sludge digester; 4, activated sludge; 5, Dübendorf sludge digester; 6, activated sludge; 7, Uster sludge digester; 8, activated sludge; 9, Bassersdorf sludge digester; 10, negative control (water); 11, anammox enrichment culture; 12, DNA size marker. All DNAs in 10^{-5} dilution (approx. 0.5-1 ng DNA). The lane number is the same for 16S rDNA and anammox-PCR.

The detectable presence of anammox specific DNA in the tested treatment plants correlated more or less with the sludge age (Table 2), with Bassersdorf and SBR Meilen having the longest sludge age. The plant in Regensdorf, however, has a longer sludge age than Opfikon and it might have been expected that anammox specific DNA would also have been detected in the Regensdorf plant. In a way, it was unexpected not to find anammox DNA in samples from the sludge digesters. First of all, the conditions in the sludge digesters were anoxic and the temperatures were between 35 °C and 40 °C, which both favour the proliferation of anammox bacteria. The reason for their absence in the sludge digesters might lay in the absence of sufficient nitrite for their growth. In the activated sludge basins sufficient ammonium and nitrite are formed and temporarily and locally anoxic conditions may even exist, forming niches for the anammox bacteria. Provided that the sludge age is higher than

the (slow) doubling rate (appr. 10 days) of the anammox bacteria (Jetten *et al.*, 1999), anammox bacteria may become an established member of the wastewater treatment sludge community. The detectable presence of anammox bacteria in a few wastewater treatment systems is in agreement with the results of others (Toh *et al.*, 2002).

Table 2. Sludge age for the different wastewater treatment plants. Tempartures in the sampling basin were nearly 20 °C.

Treatment plant	Sludge age (days)
Bassersdorf	20
Opfikon	10
Regensdorf	13.3
Uster	7.5
Dübendorf	10
Meilen	2.5
SBR Meilen	60

Conclusions

The results of this work demonstrate that anammox bacteria occur in some wastewater treatment plants at low numbers (favouring those with high sludge ages, although not necessarily all wastewater treatment systems with a high sludge age have anammox bacteria.) Since we could also detect anammox DNA in groundwater leachate samples at the Kölliken landfill, this suggests that the anammox organisms originate from an underground source. The Kölliken rotating disk reactors basically formed a unique spontaneous inoculation (by material from the probably anoxic underground containing high amounts of ammonium) and enrichment environment (since ammonium was abundant, aerobic ammonium oxidizers present and organic carbon absent). The origin of anammox bacteria in the wastewater treatment plants remains unclear. However, they generally tend not to proliferate in wastewater treatment plants, which mostly carry out a regime favouring nitrification and denitrification in the presence of naturally provided or by addition of extra organic carbon.

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Final Discussion

This dissertation was focused on investigating physiological aspects, structures and development of ammonium oxidizing bacterial communities in wastewater treatment systems. Special emphasis was given to systems treating wastewaters with relatively high ammonium loads but little organic carbon. Under these conditions it was expected that the process of anaerobic ammonium oxidation would occur. It was reasoned that alternative process technologies (i.e. combination of partial nitrification and anammox instead of full nitrification and denitrification) for ammonium removal from (special) wastewater streams (e.g. supernatant of sludge digester) could be further developed on the basis of this microbiological dissertation and its engineering counterpart.

The current practise for nitrogen transformation in Swiss wastewater treatment plants is to either apply a combination of full nitrification and denitrification or to oxidize ammonium to nitrate only. Since no regulation exists on the release of nitrate, the practise of nitrification only is still in use. The nitrification-denitrification cycle in wastewater treatment plants is relatively robust against perturbations although its efficiency in nitrogen removal is far from optimal (usually between 40 and 70%) and some concern exists that potential greenhouse gases (NO and N_2O) are released from the denitrification process (von Schulthess *et al.*, 1994; Bergsma *et al.*, 2002). If denitrification in wastewater treatment plants is carried out after the nitrification stage, a lack of organic compounds for the reduction of nitrate may occur, which is the reason for the common practise of adding methanol at the stage of denitrification.

More recently, other process configurations have been developed with the purpose of diminishing the oxygen input for full nitrification and reducing carbon input for denitrification. In one of those, the SHARON (**s**ingle reactor **h**igh **a**ctivity ammonia **r**emoval **o**ver **n**itrite) process, ammonium is oxidized to nitrite only and then nitrite is reduced to dinitrogen with organic compounds (Hellinga *et al.*, 1998; van Kempen *et al.*, 2001). The overall costs for the SHARON process are estimated at about $ 1.7 per kg removed NH_4^+, of which power input (over 90 % for aeration) accounts for 35 % and methanol for 25 % (Hellinga *et al.*, 1998). In another configuration, it was recognized that a full autotrophic ammonium removal is possible when aerobic ammonium oxidation to nitrite and anaerobic oxidation of ammonium with nitrite to dinitrogen gas (the *anammox* reaction) are combined (the process was given the acronym CANON: Completely Autotrophic Nitrogen Removal Over Nitrite). Both SHARON and CANON have indeed been shown to be practicable, although the final process configurations and reactor technology still leave room for creativity and optimisation (van Dongen *et al.*, 2001). When compared to SHARON the costs of extra investments with CANON for a second anammox reactor balance those of methanol and aeration in the nitritation/ denitrification set-up (van Dongen *et al.*, 2001). It was with the possible combination of aerobic and anaerobic ammonium oxidation in mind, that this dissertation was started.

In the beginning the anammox process seemed to be the most difficult to control. Anammox activity had first and only been observed in Delft (Netherlands) in the nineteen nineties (Mulder *et al.*, 1995). The responsible microorganism has later been identified as *Brocadia anammoxidans* and is now relatively well characterized both on the physiological and biochemical level (Strous *et al.*, 1999; Schalk *et al.*, 2000). In our own case, hints for possible anammox activity had been obtained from nitrogen balance calculations on a rotating disk contactor treating an ammonium-rich leachate from the landfill in Kölliken (Siegrist *et al.*, 1998; Koch *et al.*, 2000). During this

dissertation, the anammox activity from biofilm material from the contactor in Kölliken could successfully be enriched to sufficiently high purity (about 90%) that the responsible microorganism could be identified and its physiology studied. Interestingly, the enriched anammox bacterium was different from *Brocadia*. Parallel to our work, Schmid et al. (Schmid *et al.*, 2000) identified a new Candidatus genus *Kuenenia* from a biofilm treatment system and called their strain Candidatus *Kuenenia stuttgartiensis*. This strain was closely related to ours (Egli *et al.*, 2001) based on the 16S rDNA sequence. Despite the distinct phylogenetic difference between *Brocadia* and *Kuenenia*, they differed little in their physiological characteristics (Chapter 2). We obtained clear hints that the *Kuenenia* organism may be more robust against nitrite, oxygen and phosphate exposure, and it seems to retain its per cell activity more easily than *Brocadia* (Chapter 2). Currently, the *Kuenenia* genome is being sequenced at the TU München, which will likely reveal more interesting aspects about anaerobic ammonium oxidation and the general characteristics of these types of microorganisms. The pathway for anaerobic ammonium oxidation produces a few interesting intermediates, such as hydrazine (N_2H_4) and hydroxylamine. Anammox organisms seem to be the only organisms able to convert hydrazine (Schalk *et al.*, 1998; Jetten *et al.*, 1999; Jetten *et al.*, 2002). The oxidation of hydrazine to dinitrogen gas is mediated by a hydroxylamine oxidoreductase (HAO), which –interestingly- is also present in *Nitrosomonas europaea* (Jetten *et al.*, 2002). Therefore, biochemical evolutionary relationships may actually exist between aerobic and anaerobic ammonium oxidizers although the sequence homology based on amino acid level is rather low (Schalk *et al.*, 2000). Anammox organisms are planctomycetes. They have a very unusual cell compartmentalization (Lindsay *et al.*, 2001) with a protein rich area within the cell which actually may contain (some of) the enzymes for anaerobic ammonium oxidation (Strous *et al.*, 2000; Lindsay *et al.*, 2001). The complete genome sequence may also shed more light on this compartmentalization and its possible functions.

In order to obtain a feasible engineering solution for the combination of anammox and nitrification, it was important to study the physiological properties of the different nitrogen converting bacteria involved. More information was also needed on the community structures formed in the reactor systems. One of these, which actually was the source for the anammox bacteria studied here, was a biofilm reactor. The rotating disk reactors operated in Kölliken for the treatment of secondary leachate (after passage through a system removing most of the organic carbon but not the ammonium) pertained the conditions necessary to maintain both anammox bacteria and aerobic ammonium oxidizers (Chapter 3). The most likely explanation for the simultaneous occurrence of both aerobic and anaerobic ammonium oxidizers is that an oxygen gradient was formed across the 500 μm thick biofilm due to the activity of the aerobic ammonium oxidizers at the top creating anaerobic conditions at the bottom. Apparently, also ammonium and nitrite gradients formed across the biofilm. The reason for these gradients might be that the ammonium load was larger than the necessary oxygen supply. By this way, part of the ammonium diffused to the deeper layers, since it was not converted by the aerobic ammonia oxidizers. The aerobic ammonium and nitrite oxidizers competed for oxygen in the top biofilm layers, with the ammonium oxidizers being seemingly more effective. The result of this would be that the nitrite formed by the aerobic ammonia oxidizers was also partly transported to deeper layers. At these layers the anaerobic ammonium oxidizers found a perfect niche, since they did not have to compete with the aerobic ammonium and nitrite

oxidizers. Nitrite oxidizers were present in low numbers at the biofilm surface only and therefore they removed some of the nitrite before it reached the lower biofilm layers. In previous studies (Koch *et al.*, 2000), a mathematical model which describes the kinetics of the aerobic ammonia-oxidizing, aerobic nitrite-oxidizing and anaerobic ammonium-oxidizing micro-organisms was formulated. This model allowed a good prediction of the distribution of the populations within the biofilm as well as of rates of ammonium, nitrite and nitrate degradation along the rotating disk contactor in a steady state and during short-term experiments (Koch *et al.*, 2000). The immobilization in a biofilm probably was another factor for the successful enrichment of anammox bacteria, which are very slow growers and would easily have been washed out from suspended mixed systems. To illustrate this last point, several research groups have unsuccessfully tried to enrich anammox bacteria from wastewater treatment plant primary sludge, though the organisms can be detected by FISH or PCR in very low numbers (Blackall, pers. Communication, Chapter 5). Another yet unresolved aspect of the structure and physiology of both aerobic and anaerobic ammonium oxidizers is their strong tendency to form globular cauliflower like clusters, which might actually be very important for their physiology. The source or 'natural' environment for anammox bacteria is also somewhat mysterious. Anammox bacteria have been detected now in different wastewater treatment plants, in the Netherlands (Jetten *et al.*, 2002), Germany (Schmid *et al.*, 2000), Switzerland (Siegrist *et al.*, 1998; Egli *et al.*, 2001), UK, Australia (Toh and Ashbolt, 2002) and Japan (Jetten, 2001). Recently anammox cells were detected in a fresh water swamp in Uganda (Jetten, 2001). Evidence for anammox activity was also found in marine sediments (Dalsgaard and Thamdrup, 2002) and thus the organisms may prevail in environments with strong gradients of nitrogen compounds and oxic/anoxic interfaces. It was interesting that we could detect the anammox bacteria found in the rotating disk reactors in the incoming landfill leachate and in several landfill monitoring wells (Chapter 5). This suggests that the rotating disk reactor was inoculated from landfill material and that the underground of the landfill was a source and perhaps enrichment for anammox bacteria (landfills often result in anoxic environments and quite high ammonium concentrations).

For technical reasons, the rotating disk reactor cannot reach a very high capacity in ammonium removal due to its long hydraulic retention times. For this reason, a two-reactor system was proposed, consisting of a first aerated reactor, in which nitritation would take place, and a second in which anoxic conditions would prevail, allowing the anammox process to proceed. Due to the slow growth rate of both aerobic and anaerobic ammonium oxidizers, both reactors could be operated as sequencing batch reactors in order to reduce their volumetric size but maintaining high activity (Strous *et al.*, 1998). Quite essential for the process is to avoid growth and establishment of nitrite oxidizing bacteria in the aerated reactor. In fact, such a configuration was successfully operated on pilot scale at the wastewater treatment plant Werdhölzli (Fux, 2003). With FISH analysis it became obvious, that an active community of aerobic ammonia oxidizing bacteria was present, even at the high ammonium concentrations (15 mM average and 220 mM maximum). This community also tolerated high nitrite concentrations (20 mM on average and around 220 mM at maximum; Fux, 2003). At the temperature of around 30 to 40 °C, a stable long term nitrite production could be maintained. By testing different pH values and temperatures (pH 7 and 7.5; 25° and 30 °C) we wanted to investigate under which conditions nitrite oxidizing bacteria would not be able to establish themselves in the reactor. To our surprise, under all these conditions nitrite oxidizing bacteria were

washed out, although at 25°C significantly more and longer nitrite oxidation was observed. From our own data and from literature, one therefore has to assume that a combination of temperature (Hellinga *et al.*, 1998), high nitrite (Daims *et al.*, 2001) and free ammonia concentrations, and short hydraulic retention times are successful to eliminate nitrite oxidizers. Based on fluorescence *in situ* hybridization (FISH) combined with microelectrode measurements, it has been suggested that *Nitrospira*-like nitrite oxidizers, which form the most abundant nitrite oxidizer population in regular activated sludge, are adapted to low nitrite and oxygen concentrations (Daims *et al.*, 2001). Therefore, *Nitrospira* strains present in the inoculum to the reactors could probably not grow under the conditions of the high ammonium concentrations and higher temperatures typical for sludge digester supernatant. Long term stability of an active nitrite-producing community might also be effected by fluctuations in ammonium load. To test this, extended periods of ammonium limitation were applied in a laboratory scale CANON system. In these experiments it was found that *Nitrospira* and *Nitrobacter* populations increased at lower ammonium loads (Third 01).

Interestingly, almost none of the conditions and experimental repetitions resulted in the same community structures of ammonia oxidizers when starting from regular sludge as inoculum. Repetitions are –unfortunately- a neglected aspect in many studies of engineered systems. Almost none of the experimental data described in the literature on community structures of ammonia oxidizers show independent repetitions and, therefore, the value of those analyses is rather limited. For example, it would be valuable to know if a complex community behaves the same irrespective of the composition of its individual 'components'. On the other hand, it could also be that certain communities are more resistant to stresses and perturbations, for which we obtained some hints (Chapter 4). But if community structures are not reproducibly measured no general conclusions can be drawn from fluctuations of individual populations. We also observed that important differences occurred when nitritation reactors were fed with sterile 'synthetic medium' or with supernatant of a sludge digester. Although in both cases effective nitritation developed in the reactors, operation with real sludge supernatant allowed a much shorter hydraulic retention time, because of constant reinoculation of ammonia oxidizers which were present in the supernatant. This reinoculation effect is due to the survival of ammonia oxidizing bacteria in the anaerobic sludge digester during two to three weeks at high temperatures (30-40 °C), although these bacteria were only detectable with PCR but not with FISH. The survival of ammonium but not nitrite oxidizers in sludge digesters was also demonstrated by measuring production of nitrite but not nitrate upon aerating sludge digester supernatant (C. Fux, unpublished). The communities in those reactors operated with sludge supernatant were different than those in reactors operated with synthetic medium. This might be due to a competitive advantage of some strains of ammonia oxidizing bacteria present in the inoculum when cultivated in a closed system, which disappears when constant reinoculation of other strains from the sludge digester takes place.

A successful operation of partial nitrification and anammox during prolonged times (up to years) has also been shown by others (Hippen *et al.*, 2001; van Dongen *et al.*, 2001). This indicates a high interest and need for energy (and therefore cost) savings treating an ammonium-rich wastewater and the reduction of the amount of nitrogen released into the environment. During the past years, clear and convincing data about the combination of partial nitrification and anammox were published. Since the CANON-process (van Dongen *et al.*, 2001) has very promising economical potential

and worldwide application possibilities, it is no surprise that this process has been patented. These patents on the other hand may limit the speed of introducing the anammox process to treat wastewater. Currently, three patents have been registered (Mulder A. Anoxic ammonium oxidation (1992). van Loosdrecht MCM and Jetten MSM. Method for treating ammonia-comprising wastewater (1997). Dijkman H and Strous M. Process for ammonia removal from wastewater. Patent PCT/NL99/00446 (1999)).

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Epilogue

Acknowledgments

Curriculum Vitae

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