Re/Views in Environmental Science & Bio/Technology **1:** 51–63, 2002.

Review

Improved nitrogen removal by application of new nitrogen-cycle bacteria

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Key words: ammonium, anammox, denitrification, nitrification, nitrite, planctomycetales

Abstract

In order to meet increasingly stringent European discharge standards, new applications and control strategies for the sustainable removal of ammonia from wastewater have to be implemented. In this paper we discuss a nitrogen removal system based on the processes of partial nitrification and anoxic ammonia oxidation (anammox). The anammox process offers great opportunities to remove ammonia in fully autotrophic systems with biomass retention. No organic carbon is needed in such nitrogen removal system, since ammonia is used as electron donor for nitrite reduction. The nitrite can be produced from ammonia in oxygen-limited biofilm systems or in continuous processes without biomass retention. For successful implementation of the combined processes, accurate biosensors for measuring ammonia and nitrite concentrations, insight in the complex microbial communities involved, and new control strategies have to be developed and evaluated.

Abbreviations: anammox – anaerobic ammonium oxidation; CANON – completely autotrophic nitrogen removal over nitrite; FISH – fluorescence in situ hybridization; ISR – intergenic spacer region; Sharon – Single reactor system for high rate ammonia removal over nitrite

1. Introduction

It is evident that the protection of our water resources is of major importance on a global scale. This is for example reflected in directive 91/271 of the European Community which "aims to protect the environment from any adverse effects due to discharge of (untreated) urban and industrial waters".

Table 1. Qualitative comparison of several components of the anammox technology with conventional nitrogen removal systems

System	Sharon	Anammox	CANON	Conventional nitrification denitrification
Number of reactor	1	1	1	2
Feed	wastewater	ammonium nitrite mixture	wastewater	wastewater
Discharge	NH_4^+, NO_2^-	N_2, NO_3^-	N_2, NO_3^-	NO_3^-; N_2O, N_2
Conditions	oxic	anoxic	oxygen limited	oxic; anoxic
Oxygen requirement	low	none	low	high
pH control	none	none	none	yes
Biomass retention	none	yes	yes	none
COD requirement	none	none	none	yes
Sludge production	low	low	low	high
Reactor capacity (kg N/ m^3 day)	1	6–12	1–3	0.05–4
Bacteria	Aerobic NH_4^+ oxidizers	Planctomycetes	Aerobic NH_4^+ oxidizers + planctomycetes	Nitrifiers + various heterotrophs

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.

Thus many cost-effective wastewater treatment 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. This paper reviews a new combined system for nitrogen removal based on partial nitrification of ammonia to nitrite, together with anaerobic ammonium oxidation. Such system has no need for external carbon addition, has hardly any sludge production, and uses less energy and oxygen than conventional systems. In Table 1, three process options of the new system are presented and compared to a conventional nitrogen removal system based on autotrophic nitrification and heterotrophic denitrification. The options are: a Sharon reactor system for the production of ammonium and nitrite mixtures, an anammox system, and an oxygen-limited one reactor nitrogen removal system (CANON).

The review first summarizes the microbial aspects of the anammox process, followed by an overview of the applied aspects of the anammox process, and finally addresses the implementation of the combined partial nitrification-anammox process in wastewater treatment.

2. Microbiology of anaerobic ammonia oxidation

2.1. *History*

The first studies on the aerobic oxidation of ammonia by nitrifying bacteria go back more than one hundred years (Winogradsky, 1890) and the biochemical principles of the process were already described in 1926 (Kluyver & Donker 1926). Therefore, it is not so surprising that the microbiology of anaerobic ammonia oxidation (equation 1) was 'overlooked' for a long time (Jetten et al. 1999, 2001).

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

The first experimental confirmation of anaerobic ammonia oxidation (anammox) was described and patented by Mulder et al. (1995, 1992). The microbial nature of the process was verified and nitrite was shown to be the preferred electron acceptor. Hydroxylamine and hydrazine were identified as important intermediates.

Table 2. Properties of *Candidatus* Brocadia anammoxidans

Phylogenetic position:	Planctomycetales
Morphological features:	Coccoid, Proteinaceous S layer, crateriform structures, no peptidoglycan, internal compartment 'anammoxosome'
Reaction:	$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$
Intermediates:	Hydazine, hydroxylamine
Enzymes:	Hydroxylamine oxidoreductase
Aerobic rate:	0 nmol (mg protein) $^{-1}$ min^{-1}
Anaerobic rate:	60 nmol (mg protein) $^{-1}$ min^{-1}
Growth rate:	0.003 h^{-1}
Doubling time:	10.6 days
K_s (ammonium):	5 μM
K_s (nitrite):	< 5 μM
Oxygen:	Reversible inhibition

Initial experiments, albeit at suboptimal conditions, indicated that the microbial community responsible for the process had an extremely low growth rate (doubling time more than 3 weeks). Therefore, reactor systems with very efficient biomass retention had to be applied to obtain sufficient biomass. The sequencing batch reactor (SBR) was chosen for this purpose, and further optimized for the quantitative study of the anammox community (Strous et al. 1998). Biomass cultivated in this system was used to determine (Table 2) several important physiological parameters (Strous et al. 1999a). The use of a medium for strict autotrophic growth in the SBR system gave rise to persisting stable and strongly selective conditions. The biomass in the community was dominated for more than 70% by a morphologically conspicuous bacterium. This unusual bacterium was physically separated from the other community members by an optimized percoll density gradient centrifugation (Strous et al. 1999b). This method produced very pure cell suspensions that contained less than one contaminating bacterium per 200 target bacteria.

2.2. *Molecular identification, diversity and in situ detection*

These purified suspensions had high anammox activity and the cells incorporated radioactive bicarbonate. DNA extracted from the purified cells was used as a template for PCR amplification with an universal 16S rDNA primer-set. The dominant 16S rDNA sequence obtained was planctomycete-like, and branching very deep within the planctomycete lineage of descent (Figure 1). Based on this finding, the anaerobic ammonium oxidizing planctomycete-like bacterium was named '*Candidatus* Brocadia anammoxidans'. 'Brocadia' refers to the place of discovery at the pilot plant of Gist-brocades by Mulder (Mulder et al. 1995), and 'anammoxidans' reflects the unique metabolic properties of this bacterium (Kuenen & Jetten 2001). The sequence of the 16S rDNA gene was used to design very specific oligonucleotide probes for B. anammoxidans to be applied for fluorescence in situ hybridization (FISH). All these probes hybridized very specifically with the target organism both in purified cell suspensions, as well as in complex mixed microbial communities. In addition, the oligonucleotide probes were used to survey (Table 3) the presence of planctomycete-like anammox bacteria in wastewater treatment systems with a limited supply of oxygen and a very high nitrogen load (Van Dongen et al. 2001; Schmid et al. 2000). The observed probe binding patterns indicated that in addition to B. anammoxidans other planctomycete-like bacteria occurred in these treatment systems. The diversity of planctomycete-like bacteria in those waste water systems was investigated further (Schmid et al. 2000). Phylogenetic analysis of the retrieved 16S rDNA sequences from these treatment systems showed a previously not recognized genus level diversity within the *Planctomycetales* (Figure 1). The retrieved sequences formed a clearly separated monophyletic cluster with less than 90% 16S rDNA sequence similarity to B. anammoxidans. Therefore, one of these new anammox bacteria was provisionally named '*Candidatus* Kuenenia stuttgartiensis'. FISH with specific probes for K. stuttgartiensis demonstrated that these anammox bacteria dominated the microbial biofilm communities of the investigated plants (Schmid et al. 2000). Recent studies have indicated that K. stuttgartiensis is in many ways very similar to B. anammoxidans (Egli et al. 2001). K. stuttgartiensis cells have the same overall cell structure and do also produce hydrazine from exogeneously supplied hydroxylamine. Future research will be necessary to evaluate the differences in specific activity and nitrite tolerance of the two organisms.

Previous investigations pointed out that it is possible to deduce the growth rate of certain organisms in an environmental system from the FISH signal intensities, which are directly proportional to the content of ribosomal RNA in the cell (Poulsen et al. 1993). However, this approach is not applicable to

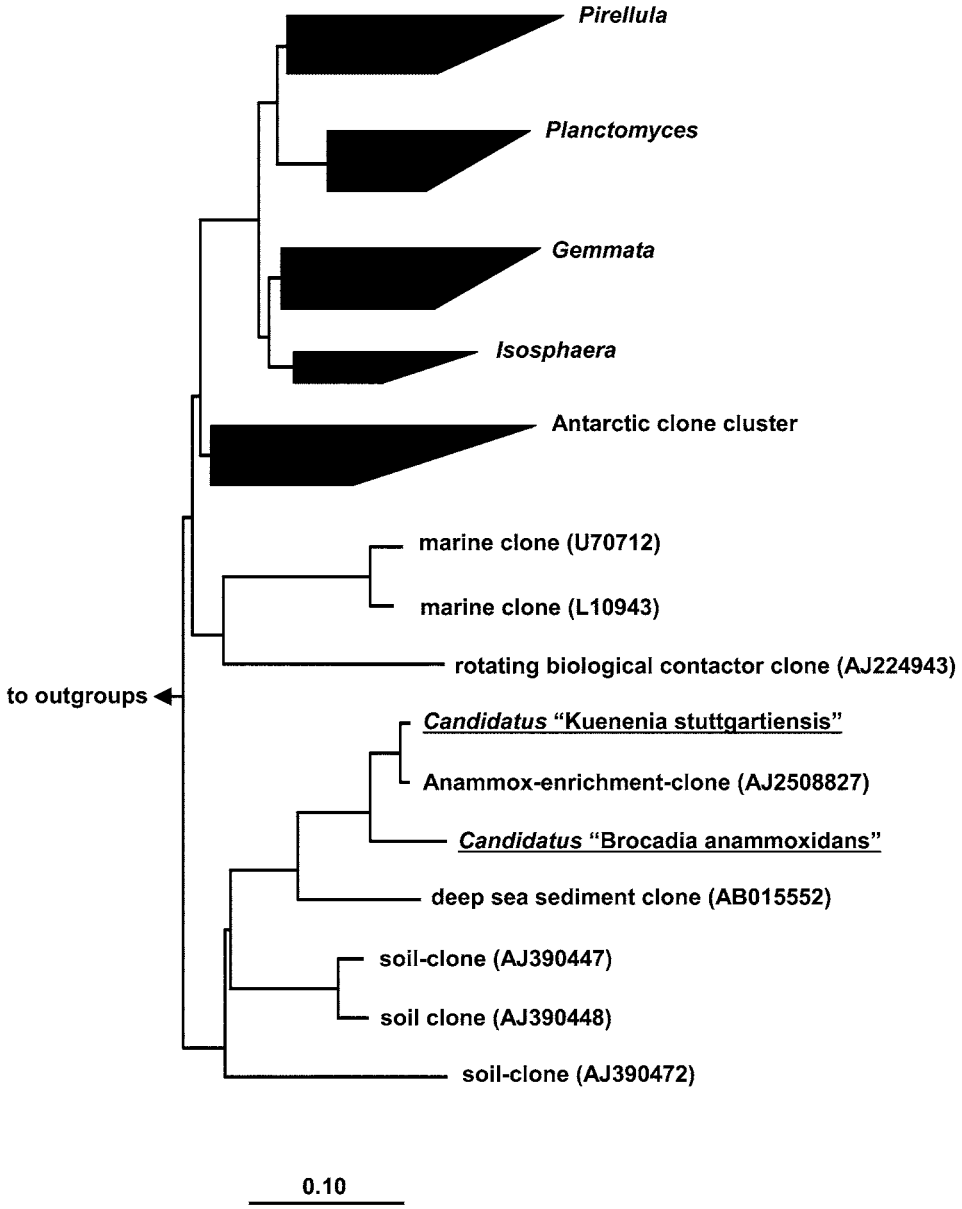

Figure 1. 16S rDNA based phylogenetic dendrogram reflecting the relationships of *Candidatus* "Kuenenia stuttgartiensis" and *Candidatus* "Brocadia anammoxidans" to organisms affiliated to the order *Planctomycetales*, and other reference organisms. The trapezoids indicate phylogenetic groups. For a better view environmental derived sequences mainly originating from the Antarctic were pooled in the Antarctic clone cluster. Genbank accession numbers of organisms other than anaerobic ammonium oxidizing bacteria are given in brackets. The tree is based on results of maximum likelihood analyses on different data sets. The bar represents 10% estimated sequence divergence.

nitrifying and anammox systems, since at least the ammonia oxidizing bacteria maintain a stable ribosome content even when they are starved (Morita, 1993) or inhibited (Wagner et al. 1995). Cangelosi and Brabant (1997) showed that the physiological activity is better reflected by the cellular concentration of precursor rRNA. This could be detected *in situ* by fluorescently labeled oligonucleotide probes targeting the intergenic spacer region (ISR) between the 16S rRNA and the 23S rRNA (Oerther et al. 2000). In a recent study the ISRs of B. anammoxidans and K. stuttgartiensis were sequenced and, subsequently, probes for the *in situ* detection of these ISRs were constructed (Schmid et al. 2001). The combined application of

Table 3. Survey of various nitrogen removal system for the presence of planctomycete like anammox bacteria

System	Source of ammonia	Mode of detections	Reference
Rotating Biological Contractor (Kollikon, CH)	Leachate	Anammox activity FISH	Egli et al. 2001
Rotating Biological Contractor (Mechernich, BRD)	Leachate	Anammox activity FISH	Helmer et al. 2001 Schmid & Wagner
Trickling filter (Stuttgart, BRD)	Wastewater	N-loss Anammox activity FISH	Schmid et al. 2000
Biofilm reactor (Sydney, Australia)	Coke oven water	Anammox activity FISH	Jetten 2001
Freshwater wetland (Uganda)	Water	Nitrogen loss FISH	Strous & van Kuijck pers. comm.
Fluidized bed reactor	Mineral medium	Anammox activity	van de Graaf et al. 1996
Sequencing batch reactor	Mineral medium	Anammox activity FISH	Strous et al. 1998
Sequencing batch reactor	Waste water	Anammox activity FISH	van Dongen et al. 2001

these probes and rRNA targeted probes in an experiment with oxygen inhibited B. anammoxidans cells showed that during inhibition the anammox-organisms kept a ribosome content, which was clearly detectable with rRNA targeted FISH. A suitable *in situ* detection of activity was only possible by ISR targeted probes. These experiments proved the ISR targeting FISH as a powerful method for the detection of activity changes in cultures of anammox organisms during deliberate variation of environmental conditions. Further research in terms of enlarging the ISR sequence data sets of anammox-organisms and a subsequent design of specific ISR targeting probes could lead to a system, which allows monitoring of the active anaerobic ammonium oxidizing cells during startup and maintenance of full scale anammox-plants, without the need for elaborate laboratory activity tests.

2.3. *Biochemistry of anaerobic ammonium oxidation in Candidatus* B. anammoxidans

The biochemistry of the aerobic ammonium-oxidizing bacterium *Nitrosomonas europaea* has been studied quite well (Hooper et al. 1997) with main focus on the enzymes ammonia monooxygenase and hydroxylamine oxidoreductase (HAO). Knowledge gained from these studies was the basis for unraveling the metabolic pathway for anaerobic ammonium oxidation in B. anammoxidans. A series of ^{15}N-labeling experiments showed that ammonium and nitrite are combined to yield dinitrogen gas (Van de Graaf et al. 1997). In batch experiments with excess hydroxylamine and ammonium, a transient accumulation of hydrazine was observed, indicating that hydrazine is an important intermediate of the anammox process. In our working hypothesis (Figure 2), the oxidation of hydrazine to dinitrogen gas is supposed to generate four electrons for the initial reduction of nitrite to hydroxylamine . As far as we know, the occurrence of free hydrazine in microbial nitrogen metabolism is rare, if not unique (Jetten et al. 1999). The oxidation of hydrazine to dinitrogen gas is also mediated by the HAO enzyme of *N. europaea*. High HAO activity in cell extracts of B. anammoxidans indicated that a similar enzyme might be operative in the anammox mechanism (Schalk et al. 2000). From these extracts, a new type of HAO enzyme was purified to homogeneity. Many of the amino acid sequences of the B. anammoxidans HAO peptide fragments were unique. The B. anammoxidans enzyme contained several c-type cytochromes similar to the enzyme of *N. europaea*. The B. anammoxidans enzyme was also able to catalyze the oxidation of both hydroxylamine and hydrazine. The purified HAO enzyme was used to raise polyclonal antibodies for localization studies with immunogold labelling (Lindsay et al. 2001). The B. anammoxidans enzyme was found to be only present inside a membrane

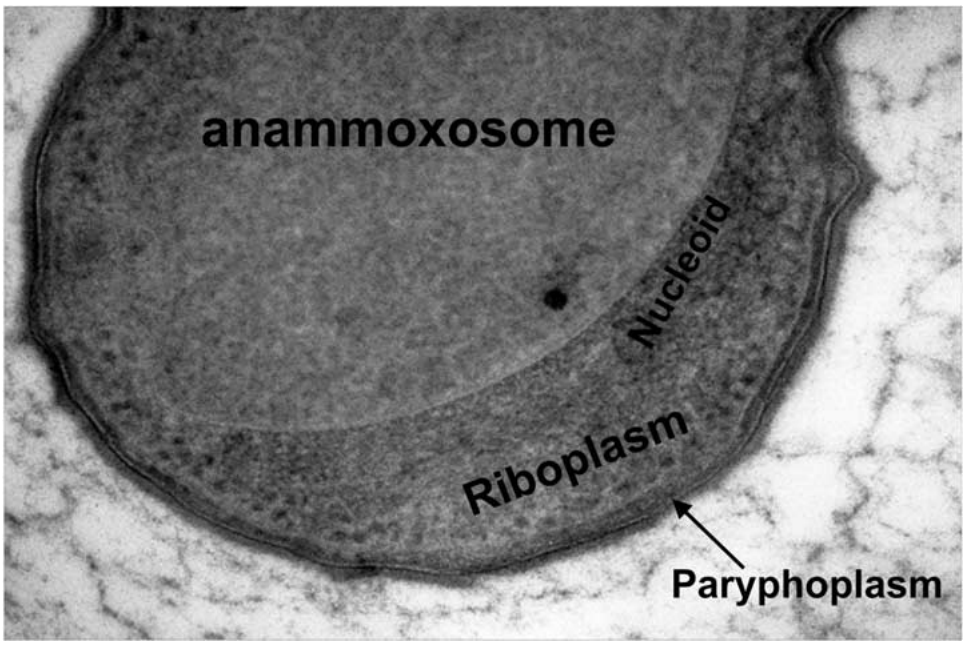

Figure 2. Electron micrograph of the planctomycete like anammox bacterium *Candidatus* Brocadia anammoxidans, showing typical compartimentalization. Internal compartment containing the enzyme hydroxylamine oxidoreductase, is named anammoxosome. The middle compartment containing the nucleoid and ribosomes is shown as riboplasm. The most outer compartment is named paryphoplasm (Lindsay et al. 2001).

bounded region of the cytoplasm, that made up more than 30% of the cell volume (Figure 3). It appears that this "organelle" may play an important role in the catabolism of B. anammoxidans, and was therefore named the 'anammoxosome'. Whether the anammoxosome does contain more enzymes involved in the conversion of nitrite and hydrazine is topic of future investigations.

3. Application of the anammox process

3.1. *Combined anoxic and oxic ammonium oxidation*

From an applied point of view, it is important to know how B. anammoxidans and related planctomycetes cope with the presence of oxygen. Experiments with B. anammoxidans cells showed that oxygen as low as 2 μm completely, but reversibly inhibited anammox activity (Jetten et al. 1999; Strous et al. 1997b). Once the oxygen was removed from these incubations, the B. anammoxidans cells resumed their metabolism. The obligate anaerobic nature of B. anammoxidans is in sharp contrast to the more versatile metabolism of aerobic ammonium-oxidizing bacteria (Schmidt et al. 2001). Most of these aerobic bacteria are known to be facultative anaerobes. Under anoxic condition they can use a variety of electron donors (hydrogen, pyruvate, and ammonia) for the reduction of nitrite (Bock et al. 1995; Schmidt & Bock 1997; Schmidt & Bock 1998). The enzyme responsible for nitrite reduction is a copper-type nitrite reductase, which is present in the periplasm of *Nitrosomonas*. The electron transfer pathway from the electron donors to nitrite may serve as an alternative to oxygen dependent ammonium oxidation. The product of nitrite reduction is nitric oxide, which is extremely cytotoxic. In order to prevent the accumulation of the gas to toxic levels, *Nitrosomonas* has the genetic potential to synthesize a membrane bound nitric oxide reductase, which catalyzes the reduction of nitric oxide to nitrous oxide (Beaumont et al. 2001).

In addition, the molecule nitrogen dioxide seems to play an important role in both the aerobic and anaerobic conversion of ammonia in the organisms, and a new complex NO cycle has been proposed (Figure 4). In this model, N_2O_4 (dimeric form of NO_2) is assumed to be the oxidizing agent for ammonia oxidation (Schmidt et al. 2001). Beside hydroxylamine, NO is a product of ammonia oxidation. Under oxic conditions (Figure 4) NO is re-oxidized to NO_2 (N_2O_4), again providing the ammonia monooxygenase with

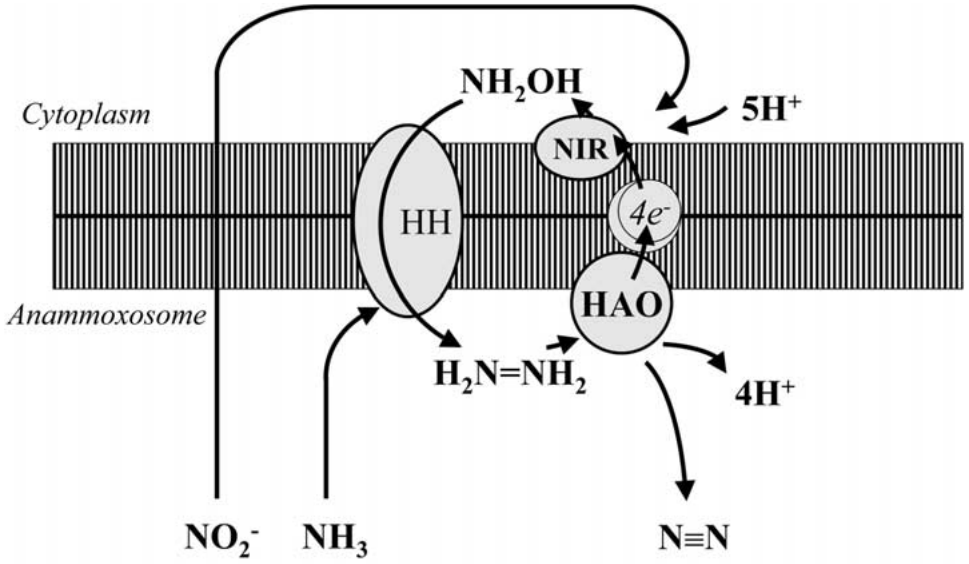

Figure 3. Proposed mechanism of anaerobic ammoium oxidation in Brocadia anammoxidans. HAO, hydroxylamine oxidoreductase, oxidizes hydrazine to dinitrogen gas and 4 electrons.; NIR, the nitrite reductase uses the 4 electrons to reduce nitrite to the level of hydroxylamine. Hydroxylamine and ammonium are assumed to be converted to hydrazine by a putative hydrazine hydrolase (HH).

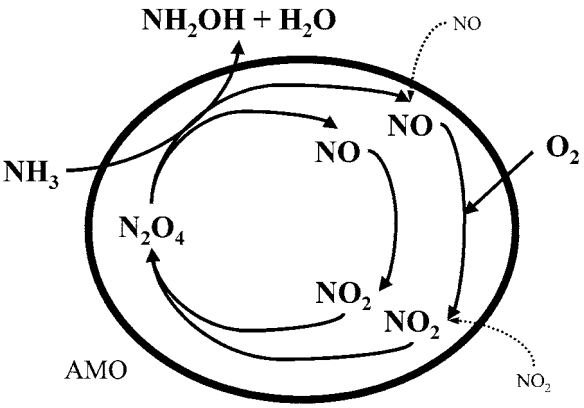

Figure 4. NO_x cycle during aerobic and anaerobic ammonium oxidation by *Nitrosomonas eutropha* N_2O_4 is used as oxidant for ammonia conversion to hydroxylamine and nitric oxide (Schmidt et al. 2001). Nitric oxide is oxidized to nitrogen dioxide.

the oxidizing agent (NO_x-cycle). Since detectable NO_x concentrations were small, nitrogen oxides seem to cycle in the cell (possibly enzyme-bound) and therefore, the total amount of NO_x per cells is expected to be low. This hypothetical model is in good accordance with the described mechanisms of the aerobic ammonia oxidation (Dua et al. 1979). According to the new NO_x cycle model, O_2 is used to oxidize NO. The product NO_2 is then consumed during aerobic and anaerobic ammonia oxidation. The oxygen of hydroxylamine still originates from molecular oxygen, but is incorporated via a "NO-NO_2-shuttle" (Schmidt et al. 2001). Due to the limited amount of nitrogen dioxide that can be applied, the maximum anaerobic ammonia conversion activity *Nitrosomonas* can achieve, is about 2 nmol NH_4^+ min^{-1} (mg protein)$^{-1}$. This is much lower than the activity of B. anammoxidans (60 nmol NH_4^+ min^{-1} (mg protein)$^{-1}$), but could be high enough to survive in or adapt to prolonged periods of oxygen limitation. On the basis of this anaerobic metabolism, *Nitrosomonas* and B. anammoxidans might even be able to coexist under oxic limiting conditions. In such a situation, *Nitrosomonas* will oxidize the ammonium to nitrite and keep the oxygen concentration low, while B. anammoxidans can convert the toxic nitrite with the remaining ammonium to dinitrogen gas. Experimentally, it was possible to establish such oxygen-limited system by carefully and gradually supplying air into an existing anammox reactor system (Sliekers et al. 2001). The *Nitrosomonas*-like bacteria consumed the supplied oxygen very effectively, keeping the actual oxygen levels below the detection limit of 2 μm. The nitrite concentration in the system never exceeded 1 mm, showing that the B. anammoxidans cells remained active despite the introduction of air into the system. After several months of stable operation, nearly all of the ammonium (30 mm) supplied was converted into dinitrogen gas and some nitrate, according to equation 2.

$$2.5NH_4^+ + 2.1O_2 \rightarrow 0.2NO_3^- + 1.15N_2 + 3.6H_2O + 2.8H^+ \quad (2)$$

Biomass from this reactor converted ammonia and nitrite simultaneously at a rate of 35 nmol NH_4^+ min^{-1} (mg protein)$^{-1}$ in anaerobic incubations. During aerobic experiments, ammonium was converted to nitrite only at a rate of 30 nmol NH_4^+ min^{-1} (mg protein)$^{-1}$. In oxic incubations, nitrate was never observed. The microbial composition of the biomass was analyzed with FISH with probes specific for B. anammoxidans and *Nitrosomonas europaea*. Initially B. anammoxidans dominated (70%) the community, but gradually more and more *Nitrosomonas*-like bacteria were detected. Aerobic nitrite oxidizing bacteria (either *Nitrobacter* or *Nitrospira*) were never detected, consistent with the absence of nitrite-oxidizing activity in oxic batch tests.

This co-operation of aerobic and anaerobic ammonium oxidizing bacteria is relevant for wastewater treatment. Ammonium can now be removed in a simple, single oxygen-limited step (Table 1) that was patented (Van Loosdrecht & Jetten 1997; Dijkman and Strous 1999) and named CANON (Completely Autotrophic N-removal Over Nitrite). CANON also refers to the way the two groups of bacteria interact: performing two sequential reactions simultaneously. Several other oxygen-limited processes for one-step ammonium removal have been described (Helmer et al. 2001; Kuai & Verstraete 1998). Microscopic analysis of the biomass present in these system has to reveal which groups of bacteria are actively involved in the nitrogen conversion in those processes. Finally, stable conversion of ammonium into dinitrogen gas has also been achieved in alginate co-immobilized anammox and nitrifying cells in air loop reactors (Martins dos Santos et al. 1998).

3.2. *Combined Sharon and anammox processes*

The introduction of anammox to N-removal would lead to a substantial reduction of operational costs. Wastewater that contains high amounts of ammonium and little organic COD, such as sludge liquor or landfill leachate are prime targets for an anammox application (Jetten et al. 1997; Strous et al. 1997a). The anammox process would replace the conventional denitrification step completely and would also save half of the nitrification aeration costs. Before anammox can be applied, several important questions need to be answered. Firstly, can the anammox community cope with the variable and harsh conditions of wastewater treatment, compared to the optimal laboratory conditions it has been studied in?

Secondly how can the process be introduced most efficiently? Is the CANON process the favorite option, or should the nitrification and anammox steps be engineered separately?

Thirdly, what parameters should be monitored during start-up and steady state operation of such systems?

Some of these questions have been addressed in feasibility studies with sludge liquor on laboratory scale. Others are subject of a large multi-disciplinary project sponsored by the European Community.

In general, the composition of sludge liquor did not negatively affect the activity of anammox biomass cultivated on synthetic wastewater in laboratory scale reactor systems. The pH (7.0–8.5) and temperature (30–37 °C) range for the anammox process were well within the values observed for digester effluents.

The possibility to combine (Figure 5) the anammox process with a preceding nitritification system such a Sharon (Single reactor system for high ammonia removal over nitrite) was tested recently (Van Dongen et al. 2001). The Sharon process was originally developed for the removal of ammonium via the so called nitrite route (Hellinga et al. 1997). It was tested for 2 years in the laboratory and successfully (!) scaled-up from two liters to full scale (1800 m^3) (Mulder et al. 2000). Sharon is essentially a chemostat without biomass retention, in which the dilution rate is higher than the maximum growth rate of nitrite-oxidizing bacteria, but lower than the growth rate of ammonium-oxidizing bacteria. Under these conditions, nitrite is the stable end product of nitrification. The ratio of ammonium and nitrite needed for the anammox process is about one. For sludge liquor, this ratio can be achieved without any pH control, because these effluents contain bicarbonate as the counter-ion for ammonium. Thus, when half of the ammonium in the liquor is converted, the alkalinity of the water is nearly depleted, leading to a pH drop and preventing further nitrification (equation 3):

$$NH_4^+ + HCO_3^- + 0.75O_2 \rightarrow 0.5NH_4^+ + 0.5NO_2^- + CO_2 + 1.5H_2O \quad (3)$$

The feasibility of Sharon process for the production of equal amounts of ammonium and nitrite was demonstrated in a 20 liter laboratory system with sludge digester effluent from the wastewater treatment

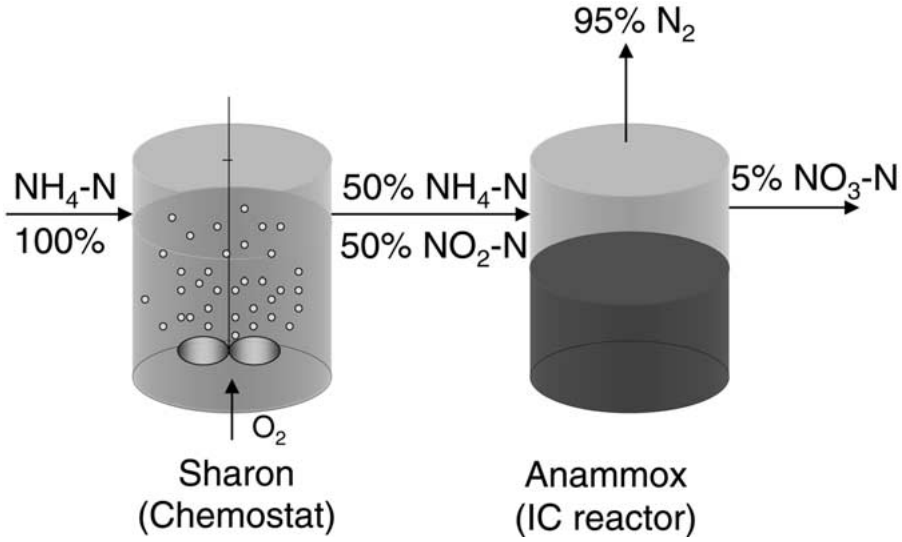

Figure 5. Schematic representation of the combined sharon-anammox process for the removal of ammonium from sludge digeation effluents.

plant, Dokhaven-Sluisjesdijk, in Rotterdam, the Netherlands. The ammonium in the liquor was oxidized for 53% to nitrite at nitrogen load of 1.2 kg N m^{-3} day^{-1}. It was not necessary to apply a pH control in the Sharon system. The ammonium and nitrite ratio in the effluent of the Sharon process could be fine-tuned by adjusting the pH between 6.5 and 7.5. The effluent of this Sharon reactor was used to start up an anammox SBR system. The reactor was inoculated with nitrifying sludge from the B-step of the Dokhaven treatment plant. Initially the biomass consisted of about 5–10% of aerobic ammonium oxidizing bacteria reacting with probes Neu653. After 60 days of operation, the first micro-colonies of planctomycete-like anammox bacteria could be made visible using probe Amx820, long before any measurable anammox activity could be observed. After 120 days, the system was fully operational, removing all nitrite at a nitrogen load of 0.75 kg N m^{-3} day^{-1}. Some surplus ammonia remained in the system. The specific activity of the anammox biomass in this system was very high: 0.8 kg N (kg dry weight)$^{-1}$ day^{-1}. The characteristics of the biomass were consistent with those of B. anammoxidans. The cells produced hydrazine from hydroxylamine, converted ammonia and nitrite in the expected 1 to 1.3 stiochiometry, produced some nitrate, and reacted with 16S rDNA probes specific for anammox bacteria (Van Dongen et al. 2001).

4. Implementation of Sharon-anammox and CANON technology

Feasiblity studies have shown that the Sharon-anammox technology is ready for large scale implementation (Van Dongen et al. 2001). Important for such an implementation of the technology are the following issues: effective on line monitoring of nitrogen compounds, very efficient biomass retention, a good balance between aerobic and anaerobic ammonium oxidation, and long term stability of the process.

4.1. *Biosensors for nitrate and nitrite measurements*

The monitoring of the nitrogen compounds in anammox systems (Table 1) will be most conveniently supplied by fast-responding sensors placed directly in the liquor of the reactors (Lynggaard et al. l996). Recently long-term stable biosensors for NO_x^- and NO_2^- based on bacterial reduction of the ionic species to N_2O gas have been developed, and they have been shown to work satisfactorily for periods of several months in wastewater treatment systems (Larsen et al. 1997; Revsbech et al. 2000; Nielsen et al., in press). The overall chemistry and high ionic strength of the liquor, which causes difficulties in conventional ion-selective electrodes, does not affect the biosensors. A factor that may cause problems in the biosensors, however, is the over-saturation with N_2 gas in the anammox reactor of the combined Sharon-anammox process. This could lead to bubble formation within

the sensors. Biosensors may still be used in the effluent if this is purged with air to remove excess N_2 gas. With respect to the CANON process, the formation of N_2 is more than balanced by the reduction of O_2, and the N_2 produced by the anammox process is stripped away with the aeration gas. An alternative to the biosensors is the use of UV absorption for NO_x^- determination, but then no separation between NO_x^- and NO_2^- can be obtained, and the accuracy is also considerably lower than the one offered by the biosensors. Oxygen sensors with electrical zero calibration may be used for on-line control of the very low oxygen concentrations that should be maintained in the CANON process.

4.2. *Balancing aerobic and anoxic ammonium oxidation*

About 50% of the ammonium needs to be oxidized to nitrite in the proposed system configurations (Table 1). If the wastewater originates from an anaerobic sludge digestion process ammonium and bicarbonate are present in a one-to-one ratio. This gives an opportunity for a self-evident, natural control. If the aerobic ammonium oxidation has proceeded for 50% all alkalinity is consumed and the conversion will stop due to a drop in pH. Van Dongen et al. (2001) showed that indeed a stable and good N-removal is possible by this natural process control. For very strict effluent standards it is possible to adjust the pH in a minor way in order to adjust the ammonium/nitrite effluent of the Sharon process such that it matches exactly the requirements of the anammox process. Since in practice the HRT in the Sharon process is relatively long and in the anammox process short (van Dongen et al. 2001) it is most feasible to apply a feed back control to the fast anammox part of the integrated process. This could be based on ammonium and nitrite concentration measurements in the anammox effluent. If the influent doesn't contain the required alkalinity as for some industrial wastewaters, pH correction will be needed. In this case too pH control could be used most favorably to regulate the ammonia:nitrite ratio, this again based on, for instance, anammox effluent N-species concentration measurements.

An alternative option would be to use measurements in the Sharon reactor. However, since the time constants of this process are considerably longer than the ones of the anammox process, a lower control performance can be expected. Of course feedforward control could anticipate the effect of disturbances, but this would require a thorough mathematical model and the measurement of these disturbances. Future research will focus on the feasibility of this alternative. An important aspect is of course that if the anammox process is supplied with the correct feed no direct process control is needed, since the reaction will then run to completion. In case a reactor system is devised where the N-removal can occur in a single reactor process, the controllers main objective will have to be the balancing of the oxygen and ammonium flux into the biofilm in the proper stoichiometry. If the requirements on the ammonium effluent are not too strict, this can probably be done by controlling the dissolved oxygen setpoint in a cascade way from a controller of the reactor ammonium concentration that uses ammonium concentration measurements in the reactor. Setting the ammonium set point to a value of about 0.5 mg N/l will yield good N-removal. From the modeling work (Van Loosdrecht pers. comm.) it is clear that the DO set-point and the ammonium surface load are thigthly coupled. From a controllability point of view it is then most beneficial to select an ammonium loading rate such that the DO can be controlled at around 1 mg/l. If a very low N-effluent is required ($<$ 1 mgN/l) correct control of the aeration becomes very crittical and gradients in the reactor will result in many local variations that may negatively affect performance. Indeed it might be that the system is part of the time ammonium rather than oxygen limited. In such situation mixing properties of the reactor will need adequate attention.

The process control system must also make sure that nitrite oxidizing bacteria are kept out of the system. In a suspended sludge system this can be done by setting the SRT such that nitrite oxidizers wash out (Hellinga et al. 1998). However, this only works for systems run at higher temperatures where the nitrite oxidizers grow slower than the ammonium oxidizers. In oxygen limited biofilm systems on the other hand, ammonium can be partially oxidized to nitrite (Bernet et al. 2001; Garrido et al. 1997; Picioreanu et al. 1997) since in this system gradients occur due to the diffusion processes. Hence, anammox processes are also feasible at the lower bulk oxygen concentrations. In these cases, however, steady state analysis showed that a mixture of nitrite and nitrate is produced. Only when the nitrite is continuously removed from the system by denitrification, nitrite oxidizers can be competed out of the process. This can be done by conventional denitrification or by using anammox. Anammox has the advantage that these organisms can be positioned in the same bioflm as the aerobic nitrifiers. For hetero-

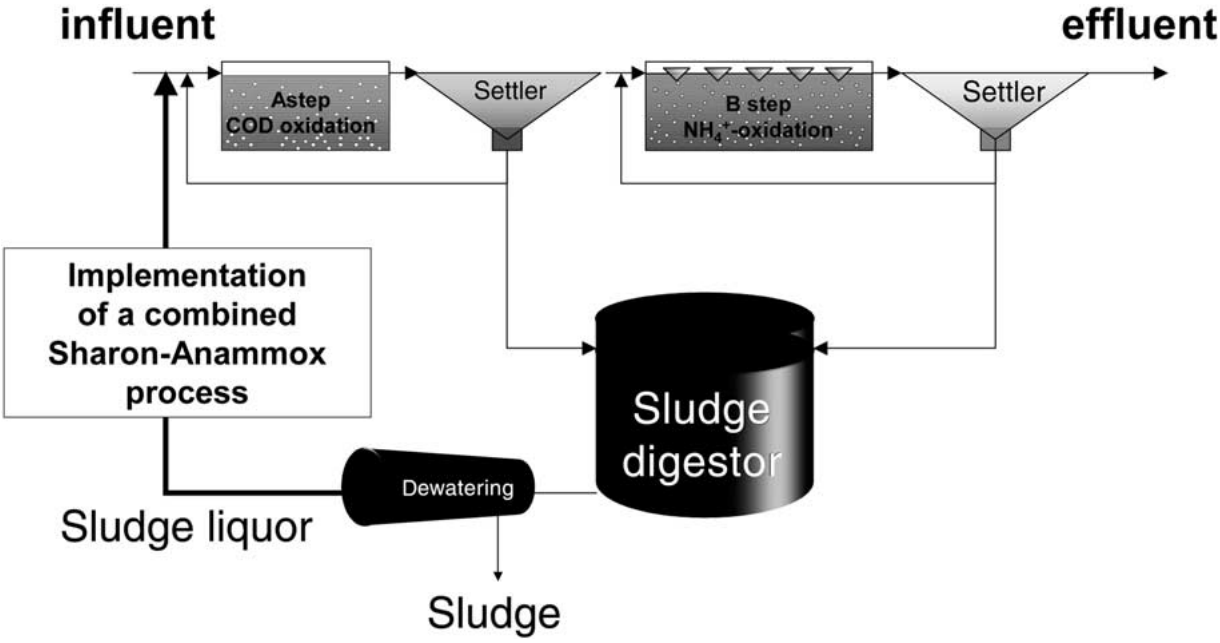

Figure 6. Schematic representation of the planned implementation of a combined sharon-anammox process for the removal of ammonium from sludge digestion effluent.

trophic denitrifiers this can be done by alternatingly recycling the reactor content over an aerated and a non aerated biofilm compartment. Very recent experiments (Third et al. 2002) and simulations (Van Loosdrecht pers comm) showed that the combined competition for the nitrite oxidizers (for oxygen with ammonium oxidizers and for nitrite with anammox) can lead to a stable process. Again in this case cascade DO control based on effluent ammonium measurements seems to be the most appropriate.

4.3. *Long term stability*

A second purpose of process control is of course the need to keep the process stable. Due to the slow response of the biomass, it is important to prevent any significant deterioration of anammox population or ingrowth of nitrifiers. This is not only due to the slow growth rate of anammox bacteria but also due to the slow wash-out of nitrifiers once they have accumulated in the system. In the Sharon process this has been shown to be the reciprocal of the difference between the maximal specific growth rate and the wash out rate, whereas in biofilms this is mainly the biofilm turnover rate. To achieve biomass stability, first , the anammox biomass has to be protected against high nitrite and oxygen concentrations. This can simply be achieved by redox or oxygen measurements and appropriate control actions. Secondly, one has to consider that most wastewater treatment plants are highly dynamic. It is essential that ingrowth of nitrite oxidizers is prevented under all circumstances, since it will take a very long time before they are washed out again (days to weeks, see above). Small daily variations do not seem to be a problem due to the slow response of the biomass, but a period of a few days in which no or little influent is supplied, requires special measures to be taken. Although it may be stated that these control strategies could be regarded as 'trouble-shooting', they are certainly not trivial for an anammox process in practice.

4.4. *Design and scale up*

The Sharon-anammox process was patented (Van Loosdrecht & Jetten 1997) and is presently evaluated for full-scale implementation (Figure 6) by Paques, a company specialized in anaerobic waste treatment. Based on the design of the combined system a cost estimate of 0.75 Euro per kg N removed was made. This very low compared to other processes that have been tested on pilot plant scale for N removal from sludge liquors. Paques has taken a worldwide license on the anammox technology, and will execute

the scale up of the anammox process. In order to make the anammox technology suitable for large-scale applications, a thorough pilot plant program will be performed. Main objectives of the pilot plant program are to establish the design criteria for full-scale implementation of the technology and to determine the performance of the technology under real wastewater conditions. The design of the pilot plant, based on the Paques' IC® reactor, has already started at the Rotterdam wastewater treatment plant.

5. Conclusions

Brocadia anammoxidans and Kuenenia stuttgartiensis are planctomycete-like bacteria responsible for the anammox process. These anaerobic ammonium-oxidizing bacteria are natural partners of aerobic ammonium oxidizers in oxygen-limited systems, and together they convert ammonium directly into dinitrogen gas.

Feasibility studies have shown that the combination of partial nitrification and anammox is ready for full scale implementation for the treatment of waste streams with a high ammonium and low BOD content. Full scale introduction of the anammox technology will lead to substantial savings in energy and resources.

Acknowledgement

The research on anaerobic ammonium oxidation was financially supported by the European Community EESD EVK1-CT-2000-00054, the Foundation for Applied Sciences (STW), the Foundation of Applied Water Research (STOWA), the Netherlands Foundation for Life Sciences (NWO-ALW), the Royal Netherlands Academy of Arts and Sciences (KNAW), DSM-Gist, and Grontmij consultants. We gratefully acknowledge the contributions of the many co-workers and students over the years.

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