ELSEVIER

FEMS Microbiology Reviews 27 (2003) 481–492

FEMS MICROBIOLOGY Reviews

www.fems-microbiology.org

New concepts of microbial treatment processes for the nitrogen removal in wastewater

Ingo Schmidt [a,*], Olav Sliekers [b], Markus Schmid [b], Eberhard Bock [c], John Fuerst [d], J. Gijs Kuenen [b], Mike S.M. Jetten [a], Marc Strous [a]

[a] *University of Nijmegen, Department of Microbiology, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands*

[b] *Delft University of Technology, Kluyver Laboratory for Biotechnology, Department of Microbiology and Enzymology, Julianalaan 67, 2628 BC Delft, The Netherlands*

[c] *University of Hamburg, Institute for Botany, Department of Microbiology, Ohnhorststraße 18, 22609 Hamburg, Germany*

[d] *University of Queensland, Department of Microbiology, Brisbane, Qld. 4072, Australia*

Received 3 March 2002; received in revised form 21 February 2003; accepted 27 February 2003

First published online 16 April 2003

Abstract

Many countries strive to reduce the emissions of nitrogen compounds (ammonia, nitrate, NO_x) to the surface waters and the atmosphere. Since mainstream domestic wastewater treatment systems are usually already overloaded with ammonia, a dedicated nitrogen removal from concentrated secondary or industrial wastewaters is often more cost-effective than the disposal of such wastes to domestic wastewater treatment. The cost-effectiveness of separate treatment has increased dramatically in the past few years, since several processes for the biological removal of ammonia from concentrated waste streams have become available. Here, we review those processes that make use of new concepts in microbiology: partial nitrification, nitrifier denitrification and anaerobic ammonia oxidation (the anammox process). These processes target the removal of ammonia from gases, and ammonium-bicarbonate from concentrated wastewaters (i.e. sludge liquor and landfill leachate). The review addresses the microbiology, its consequences for their application, the current status regarding application, and the future developments.

Keywords: Nitrification; Anammox; Canon; SHARON; NO_x cycle; Aerobic and anaerobic ammonia oxidation; Nitrogen removal; Wastewater treatment

Contents

* Corresponding author. Tel.: +31 (024) 3652568; Fax: +31 (024) 3652830. *E-mail address:* i.schmidt@sci.kun.nl (I. Schmidt).

0168-6445/03/$22.00
doi:10.1016/S0168-6445(03)00039-1

1. Introduction

The three ammonium removal processes that are the focus of this review make use of (combinations of) three groups of chemolithoautotrophic bacteria: the well known 'aerobic' ammonia and nitrite oxidizers (Eqs. 1–3) and anaerobic ammonia oxidizers (Eq. 4). They all derive energy for microbial growth (CO_2 fixation) from the oxidation of inorganic nitrogen compounds:

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O\ [\Delta G^{\circ\prime} -275\ \text{kJ mol}^{-1}] \quad (1)$$

$$NH_4^+ + N_2O_4 \rightarrow 0.33NO_2^- + 1.33H^+ + 0.33N_2 + 2NO + 1.33H_2O\ [\Delta G^{\circ\prime} -295\ \text{kJ mol}^{-1}] \quad (2)$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^-\ [\Delta G^{\circ\prime} -74\ \text{kJ mol}^{-1}] \quad (3)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O\ [\Delta G^{\circ\prime} -357\ \text{kJ mol}^{-1}] \quad (4)$$

For each of these three processes, microbiological aspects important for wastewater treatment are reviewed in the next sections.

2. Phylogeny and ecological niche

The evolutionary relationships (based on 16S rDNA phylogeny) of the chemolithoautotrophs of the nitrogen cycle are relevant to wastewater treatment, because 16S rDNA-based probing of populations of these organisms has been remarkably successful. Such 16S rDNA probes have been used to quantify the amounts of nitrifiers or anaerobic ammonia oxidizers in wastewater treatment plants [1–4]. In several cases probing resolved the mechanism of not-understood nitrogen conversions [5–7]. The probes were used to measure the in situ growth rates of relevant organisms in the actual plant [8,9]. The characterization of populations of nitrifiers using probes does not yet enable solid conclusions to improve plant management, but in view of the rapidly accumulating genomic data (http://www.arb.de), phylogenetic (16S) gene-probing may become part of the standard tools for daily plant management and optimization in the coming years.

2.1. Proteobacterial ammonia oxidizers

These ammonia oxidizing bacteria form two monophyletic groups, one within the beta- and one within the gamma-proteobacteria [3]. They are generally considered as aerobic chemolithoautotrophs, but recently organic compounds have been described that can serve them as carbon and energy source (see below). The beta-ammonia oxidizers comprise the well known genera *Nitrosomonas* and *Nitrosospira* [10], *Nitrosococcus* is the gamma-proteobacterial genus [11], but does not include *Nitrosococcus mobilis*, that is related to *Nitrosomonas*. Different members of these genera have been found to dominate different wastewater treatment plants or natural ecosystems [2,4,8,12–17], but general relationships between the ecological niche and evolutionary position are often still obscure. The SHARON process (single reactor system for high-rate ammonium removal over nitrite; discussed below) is carried out largely by *Nitrosomonas eutropha* [18]. The same bacterium is also one of the most capable denitrifiers (among nitrifiers) and was found to dominate the nitrifier denitrification (NO_x process, see below). Salty wastewaters were found to be dominated by *N. mobilis* [2]. The genome project of *Nitrosomonas europaea* nears completion. Although the relevance of this organism for wastewater treatment is disputable, it will still provide an invaluable source of information.

2.2. Aerobic nitrite oxidizers

The second step of nitrification, the oxidation of nitrite to nitrate, is performed by nitrite oxidizing bacteria, e.g. members of the genera *Nitrobacter*, *Nitrococcus* and *Nitrospira* [19]. The first two genera are part of the alpha-proteobacteria, while *Nitrospira* is phylogenetically unrelated to any other cultivated species and forms a separate division [20].

Several strains of *Nitrobacter* and one strain of *Nitrospira* are the only nitrite oxidizers that are not restricted to marine environments [21,22]. There is some evidence that *Nitrospira* is the more specialized nitrite oxidizer. The other genera are more versatile, being facultative autotrophs and anaerobes, able to grow on heterotrophic substrates such as pyruvate and also capable of the first step of denitrification (the reduction of nitrate to nitrite) [21]. It appears that the genomes of nitrite oxidizers will not become available in the near future.

2.3. Anaerobic ammonia oxidizers

Anaerobic ammonia oxidation (anammox) is mediated

by a group of planctomycete bacteria [23], two of which have been named provisionally ('*Candidatus* Brocadia anammoxidans' [24] and '*Candidatus* Kuenenia stuttgartiensis' [6]). Retrieval of 16S rDNA sequences from anammox wastewater treatment has revealed several relatives of both species [7,25] and at least one other distinct group (Schmid et al., unpublished results). The molecular biodiversity of anammox bacteria is much larger than the diversity of their proteobacterial counterparts [26]. It is not yet known if or how the niche differentiation correlates with the phylogeny. This issue is important for application because the long start-up times of anammox reactors could be reduced significantly if it could be predicted how to seed a new reactor. The lack of pure cultures of anammox bacteria makes a genomic approach less straightforward in the near future.

3. Physiology

3.1. Proteobacterial ammonia oxidizers

The physiology of conventional, 'aerobic' ammonia oxidizers is not completely understood. Only recently, it was discovered that these organisms also have an anaerobic metabolism (see below).

The proteobacterial ammonia oxidizers can obtain their energy for growth from both aerobic or anaerobic ammonia oxidation. Most likely ammonia (NH_3) and not ammonium (NH_4^+) is the substrate for the oxidation process [21]. The main products are nitrite under oxic conditions and dinitrogen, nitrite and nitric oxide under anoxic conditions [27,28]. Aerobic (Eq. 1) and anaerobic ammonia oxidation (Eq. 2) is initiated by the enzyme ammonia monooxygenase (AMO), that oxidizes ammonia to hydroxylamine. Oxygen and dinitrogen tetroxide (dimer of NO_2) are the most likely electron acceptors for this enzyme [27–33] (Eqs. 4 and 5).

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad [\Delta G^{\circ\prime} -120 \text{ kJ mol}^{-1}] \tag{5}$$

$$NH_3 + N_2O_4 + 2H^+ + 2e^- \rightarrow NH_2OH + 2NO + H_2O \quad [\Delta G^{\circ\prime} -140 \text{ kJ mol}^{-1}] \tag{6}$$

The hydroxylamine resulting from ammonia oxidation is further oxidized to nitrite (Eq. 7) by the hydroxylamine oxidoreductase (HAO) [34–36].

$$NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^- \quad [\Delta G^{\circ\prime} -289 \text{ kJ mol}^{-1}] \tag{7}$$

The four reducing equivalents derived from this reaction enter the AMO reaction (Eqs. 5 and 6), the CO_2 assimilation, and the respiratory chain [37]. The reducing equivalents are transferred to the terminal electron acceptors O_2 (oxic conditions) or nitrite (anoxic conditions) [33,37]. The reduction of nitrite under anoxic conditions leads to the formation of N_2 resulting in a N-loss of $45 \pm 15\%$. Under anoxic conditions the ammonia oxidation activity is relatively low (2.5 nmol NH_3 (g protein)$^{-1}$ min^{-1}). The doubling time is about 30 days at best and the biomass yield is 0.13 ± 0.019 g dry weight (g NH_3-N)$^{-1}$. The K_s value for the substrate ammonia is about 20 μM at pH values between 6.7 and 8.3. These organisms are reversibly or irreversibly inhibited by various carbon compounds [38,39]. In contrast to aerobic ammonia oxidation [40], ammonia oxidation under anoxic conditions is not inhibited by acetylene [41].

In the presence of oxygen, the produced NO can be oxidized to NO_2. Therefore, only small amounts of NO are detectable in the gas phase of *N. eutropha* cell suspensions [42]. According to Eq. 2 N_2O_4 is the oxidizing agent also under oxic conditions [41]. Hydroxylamine and NO are produced as intermediates. While hydroxylamine is further oxidized to nitrite (Eq. 7), NO is (re)oxidized to NO_2 (N_2O_4) (Eq. 8).

$$2NO + O_2 \rightarrow 2NO_2\ (N_2O_4) \tag{8}$$

Recently, a model was developed to explain the role of NO_x in the metabolism of the ammonia oxidizers [41].

Under oxic conditions (> 0.8 mg O_2 l^{-1}) aerobic nitrifiers convert ammonia to nitrite (see above). At an oxygen concentration below 0.8 mg O_2 l^{-1} they use small amounts of the produced nitrite as terminal electron acceptors producing NO, N_2O, and N_2 [43]. In the absence of nitrogen oxides, up to 15% of the converted ammonia can be denitrified [44]. *N. eutropha* was shown to nitrify and simultaneously denitrify under fully oxic conditions in the presence of NO_2 or NO. Interestingly, there is no fixed stoichiometry measurable between ammonia and NO_2 (NO) consumption under oxic conditions. The ratio of ammonia to NO_x consumption range between 1000:1 and 5000:1. Obviously, nitrogen oxides have a regulatory function in the metabolism of nitrifiers under oxic conditions, stimulating the denitrification activity [45]. Influenced by nitrogen oxides, ammonia oxidizers convert ammonia to gaseous dinitrogen (about 60% of the converted ammonia) and nitrite (just about 40% of the converted ammonia) [42]. The specific aerobic ammonia oxidation activity is stimulated by NO_2, with values increasing from 33 μmol NH_3 (g protein)$^{-1}$ min^{-1} without NO_x addition to 280 μmol NH_3 (g protein)$^{-1}$ min^{-1} and a denitrification activity of 150 μmol NO_2^- (g protein)$^{-1}$ min^{-1} in the presence of 50 ppm NO_2 [42]. The biomass yield and the affinity for ammonia remain unchanged. Control experiments with *N. europaea* and *Nitrosolobus multiformis* have yielded similar results. The reaction mechanism is the same, but the activities vary. Nitrogen oxides are toxic for many other microorganisms (nitrite oxidizers, heterotrophic bacteria) [46]. Reducing the cell number and the ac-

tivity of the nitrite oxidizers by adding NO_x [42] can be desirable in wastewater treatment, because the nitrite formed by the ammonia oxidizers is not further oxidized to nitrate (i.e. nitrite oxidizers). This is important since the nitrite is needed for the denitrification by the ammonia oxidizers.

3.2. Aerobic nitrite oxidizers

As mentioned above, nitrite oxidizers are often more versatile than ammonia oxidizers. When growing autotrophically with nitrite, the biomass yield is 0.036 g dry weight (g nitrite-N)$^{-1}$, at a maximum growth rate of 0.04 h^{-1} [47]. The apparent activation energy of nitrite oxidation is 44 kJ mol^{-1} [48]. Like the ammonia oxidizers, these bacteria can have high substrate affinities (around <70 μM for nitrite and <25 μM for oxygen). It has been reported that hydroxylamine, ammonia and NO can inhibit nitrite oxidizers [49], but a mechanism for such inhibitions has not yet been proposed.

The key enzyme of nitrite oxidizing bacteria is the membrane-bound nitrite oxidoreductase [50] which oxidizes nitrite with water as the source of oxygen to form nitrate [51]. The electrons released from this reaction are transferred via a- and c-type cytochromes to a cytochrome oxidase of the aa_3-type. However, the mechanism of energy conservation in nitrite oxidizers is still unclear. Neither Hollocher et al. [52] nor Sone et al. [53] was able to find any electron transport chain-linked proton translocation, which is necessary to maintain a proton motive force for ATP regeneration. Thus, NADH is thought to be produced as the first step of energy conservation [49]. Nitrite oxidizers are generally lithoautotrophic organisms [54]. Higher growth rates are obtained when the cells are growing mixotrophically [55,56]. Several strains of *Nitrobacter* are capable of heterotrophic growth under oxic as well as anoxic conditions [57,58]. Heterotrophic growth is significantly slower than lithoautotrophic growth [57], although 10–50-fold higher cell densities are obtained [21]. Some strains of *Nitrobacter* were shown to be denitrifying organisms as well. Under anoxic conditions, nitrate can be used as an acceptor for electrons derived from organic compounds to promote anoxic growth [59]. Since the oxidation of nitrite is a reversible process, the nitrite oxidoreductase can reduce nitrate to nitrite in the absence of oxygen [60]. Nitrite oxidation occurs obligatory under oxic conditions. The involved organisms are much more sensitive to oxygen limitation than ammonia oxidizers are. Already at dissolved oxygen concentrations of about 0.5 mg l^{-1} nitrite oxidation is completely inhibited [61]. Additionally, *Nitrobacter* is inhibited at high oxygen concentrations [62]. Thus, the oxygen content of a nitrite oxidizing nitrification vessel has to be maintained carefully to avoid accumulation of nitrite. With sufficient oxygen supply nitrite oxidation proceeds at a faster rate than conversion of ammonia to nitrite. Therefore, high nitrite concentrations are rarely found neither in natural environments nor in wastewater treatment plants [21].

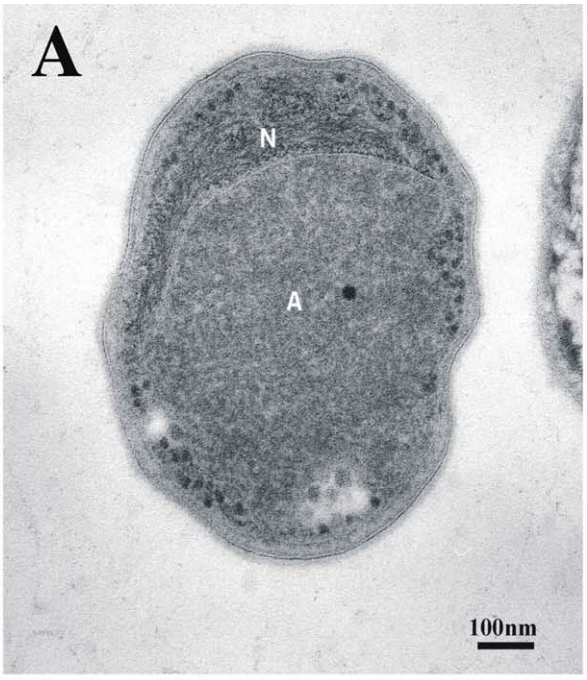
3.3. Anammox

The physiology of the anaerobic ammonia oxidizer '*Candidatus* Brocadia anammoxidans' has been studied in detail. The bacterium is a chemolithoautotroph, has a doubling time of 11 days and the biomass yield is 0.13 g dry weight (g NH_3-N)$^{-1}$ [63]. It has a very high affinity for the substrates ammonia and nitrite [64]. It is reversibly inhibited by oxygen and irreversibly by nitrite (at concentrations in excess of 70 mg N l^{-1} for several days) and phosphate (>60 mg P l^{-1} for several days) [64–66]. The apparent activation energy is approximately the same as for aerobic ammonia oxidation: 70 kJ (mol NH_3)$^{-1}$ [64]. '*Candidatus* Kuenenia stuttgartiensis' has a higher, but still low, tolerance to nitrite (180 mg N l^{-1}) and phosphate (600 mg P l^{-1}) [7]. Both bacteria have a similar temperature (37°C) and pH (8) optima.

Anammox bacteria do not consume ammonia and nitrite in a ratio of 1:1, as might be expected from their catabolism (Eq. 4), but in a ratio of 1:1.3. The excess nitrite (0.3 mol of nitrite per mol of ammonia) is oxidized anaerobically to nitrate. The electrons derived from this oxidation are probably used for the fixation of CO_2 [66].

The biochemistry of the anammox bacteria is not yet completely resolved. It is known that the anaerobic oxidation of ammonia proceeds via hydrazine (N_2H_4), a volatile and toxic intermediate [67,68]. An enzyme that resembles HAO from aerobic ammonia oxidizers is responsible for the oxidation of hydrazine to dinitrogen gas ($\Delta G^{\circ\prime} = -288$ kJ mol^{-1}) [69]. It has been postulated that the electrons from this oxidation are channelled to nitrite leading to the production of hydroxylamine ($\Delta G^{\circ\prime} = -22.5$ kJ mol^{-1}). Hydroxylamine and ammonia could yield hydrazine in a condensation reaction ($\Delta G^{\circ\prime} = -46$ kJ mol^{-1}), which completes the catalytic cycle.

The ultrastructure of *B. anammoxidans* has many features in common with previously described planctomycetes (Fig. 1). These microorganisms have a proteinaceous cell wall lacking peptidoglycan and are thus insensitive to ampicillin. Anammox catabolism is at least partly located in a membrane-bound intracytoplasmic compartment, known as the anammoxosome [69]. All anammox cells have exactly one anammoxosome [69]. Anammoxosomes can be isolated intact from anammox cells [70]. They contain little or no RNA or DNA [69] and are surrounded by a dedicated membrane that is very impermeable because it consists of ladderane lipids [70]. The bacterial nucleoid is located on the outside of the anammoxosome membrane; it is extremely condensed as is the case for the other planctomycetes. Fig. 1 shows the ultrastructure of the anammox bacterium *Candidatus* 'Brocadia anammoxidans'. Interestingly, *B. anammoxidans* [70] as well as aerobic ammonia



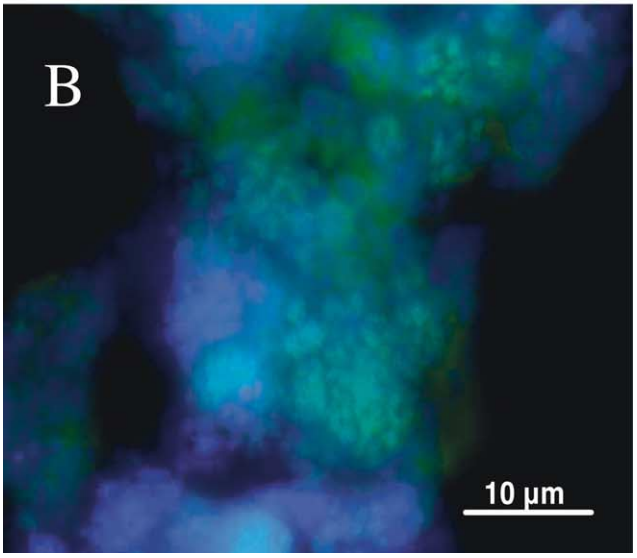


Fig. 1. A: Ultrastructure of the anammox bacterium *Candidatus* 'Brocadia anammoxidans'. A, anammoxosome; N, bacterial nucleoid. Bar: 100 nm. B: Two different anammox microorganisms in a mixed population. Green: *Candidatus* 'Kuenenia stuttgartiensis' and, Blue: *Candidatus* 'Brocadia anammoxidans Dokhaven 1'.

oxidizers like *Nitrosomonas* develop internal membrane systems.

3.4. Heterotrophic nitrification

The oxidation of ammonia [71], hydroxylamine [72] or organic nitrogen compounds, e.g. oximes [73], to nitrite and nitrate by various chemoorganotrophic microorganisms is called heterotrophic nitrification. The latter is a co-metabolism which is not coupled to energy conservation [74]. Heterotrophic nitrifiers are found among algae [75], fungi [76] and bacteria [77]. Compared to those of autotrophic nitrifiers nitrification rates of heterotrophic nitrifiers are low [78]. Therefore, heterotrophic nitrification was thought to occur preferentially under conditions which are not favorable for autotrophic nitrification, e.g. acidic environments [79].

3.5. Denitrification

Denitrification is the reduction of oxidized nitrogen compounds like nitrite or nitrate to gaseous nitrogen compounds. This process is performed by various chemoorganotrophic, lithoautotrophic, and phototrophic bacteria and some fungi [80,81], especially under oxygen-reduced or anoxic conditions [82]. Denitrification can be described as a kind of anoxic respiration. Electrons originated from e.g. organic matter, reduced sulfur compounds, or molecular hydrogen are transferred to oxidized nitrogen compounds instead of oxygen in order to build up a proton motive force usable for ATP regeneration. Enzymes involved are the nitrate reductase, the nitrite reductase, the nitric oxide reductase, and finally the nitrous oxide reductase [83,84]. Dinitrogen is the main end product of denitrification while the nitrogenous gases (nitric oxide and nitrous oxide) are occurring as intermediates in low concentrations [85]. However, these gases are also released as end products when denitrification enzymes are not completely expressed, e.g. when the concentration of dissolved oxygen is too high [86]. Denitrification also occurs in the presence of oxygen. The range of oxygen concentrations permitting aerobic denitrification is broad and differs from one organism to another [77]. The onset of aerobic denitrification is not depending on oxygen sensitivity of the corresponding enzymes, but rather on regulation of oxygen- or redox-sensing factors involved in the regulation on a transcriptional level.

4. Processes for N-removal

The newly discovered anaerobic ammonia oxidizing planctomycetes and the anaerobic metabolism of proteobacterial ammonia oxidizers open up new possibilities for nitrogen removal from wastewater. More specifically, the paradigm that the only way to biologically convert wastewater ammonium to dinitrogen gas necessitates the complete oxidation to nitrate followed by heterotrophic denitrification, has become obsolete. In this section the application of the new microbial possibilities is discussed.

4.1. Partial nitrification

Partial nitrification is the oxidation of wastewater ammonium to nitrite (Eq. 1, Fig. 2), but not to nitrate. To achieve partial nitrification, the subsequent oxidation of nitrite to nitrate must be prevented. Partial nitrification

can be combined with the anammox process (see below), but even if it is combined with conventional denitrification (the so called 'nitrite route'), already a significant benefit is achieved in terms of use of resources [48]. The process needs less aeration, the subsequent denitrification consumes less COD (chemical oxygen demand), since only nitrite and not nitrate has to be reduced to molecular nitrogen (N_2). This is cost-effective if the low C/N ratio of the wastewater necessitates the addition of a synthetic electron donor, such as methanol. In that case the process also emits less CO_2 to the atmosphere.

The oxidation of nitrite to nitrate can be prevented in at least two ways. First, by making use of the difference in activation energy between ammonia and nitrite oxidation (68 kJ mol^{-1} and 44 kJ mol^{-1}, respectively). The high activation energy of ammonia oxidation makes the rate of this process more dependent on temperature. The SHARON process (Fig. 2) makes use of the different growth rates of ammonia and nitrite oxidizers at sufficiently high temperatures (more than 26°C) [48,87]. It works at a hydraulic retention time higher than the growth rate of nitrite oxidizers but lower than ammonia oxidizers (about 1 day). Because this process has no sludge retention nitrite oxidizers are not able to remain in the SHARON reactor and they are washed out. Because SHARON depends on high temperature, it is not suitable for all wastewaters (but many wastewaters high in ammonium also have a high temperature, such as sludge liquor). Furthermore, because there is no sludge retention and the hydraulic retention time is fixed, the volumetric ammonium reactor loading depends on the ammonium concentration. Thus, the process costs also depend on the ammonium concentration (rising costs with decreasing ammonium concentration). Aeration is not only necessary for oxygen supply, but also to strip CO_2 from the reactor to control the pH. SHARON still makes use of denitrification (with added methanol) to reduce the nitrite to dinitrogen gas. Methanol is supplied periodically while the aeration is switched off. The stripping of CO_2 combined with the addition of methanol neutralizes all the protons formed in Eq. 1 – if bicarbonate is the counter-ion for the wastewater ammonium. SHARON has been scaled-up and applied successfully at the Rotterdam wastewater treatment plant, for the treatment of sludge liquor. The 1500-m^3 reactor is in operation for 2 years and treats 1000 kg N day^{-1} [88].

A variation on the SHARON process does make use of sludge retention. Instead of the hydraulic retention time, here the sludge age is controlled (in SHARON, the hydraulic retention time equals the sludge age) [89]. This allows higher ammonium loading rates and more efficient aeration. The process also makes use of a second principle to prevent nitrite oxidation; at low oxygen concentrations (less than 0.4 mg l^{-1} or 5% air saturation) and with surplus ammonium, nitrite oxidizers are unable to grow, and nitrite becomes the stable end product of nitrification. It is unclear why nitrite oxidizers are inhibited; inhibition of

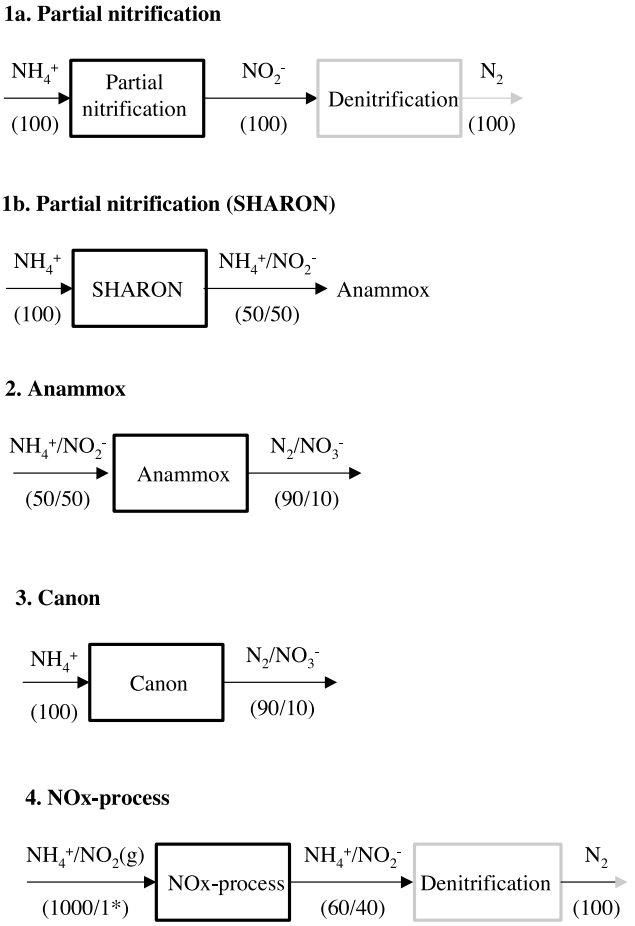


Fig. 2. Flux diagrams of the partial nitrification (1a.), SHARON (1b.), anammox (2.), Canon (3.), and NO_x process (4.). (<number>) N-compound in % (values idealized; they may vary depending on process parameter), (g) gaseous NO_2 (nitrogen dioxide). *In the presence of oxygen the supplemented NO_2 acts as regulatory signal (not as a substrate), inducing the denitrification activity of the aerobic ammonia oxidizers.

nitrite oxidizers by ammonia and a lower affinity for oxygen and/or nitrite have been suggested as possible explanations, but we still lack mechanistic evidence. This process has not yet been applied at full scale.

4.2. Anammox

The anammox process (Fig. 2) is the denitrification of nitrite with ammonia as the electron donor [90,91]. Anammox needs a preceding partial nitrification step, that converts half of the wastewater ammonium to nitrite. A modified SHARON process has been applied successfully in the laboratory to generate such ammonium/nitrite mixtures [91,92]. By simply not supplying any methanol and removing the anoxic periods, a SHARON reactor yields the desired ammonium/nitrite mixture, without the need for feedback control. This is possible because after 50% of the ammonium is oxidized, the decrease in pH (to 6.7) prevents the oxidation of the remaining ammonium. By limiting the oxygen supply to a nitrification reactor with

sludge retention, the same result can be obtained, although feedback control might be necessary [89].

The first full-scale anammox reactor is currently being built in Rotterdam, The Netherlands, as an addition to the SHARON reactor that is already in place. The reactor is estimated to have a return on investment of less than 7 years, because addition of methanol (currently used to sustain the denitrification) will no longer be required.

Laboratory experiments and design calculations have shown that anammox reactors will be extremely compact with volumetric ammonium loading rates of more than 15 kg N m^{-3} day^{-1}. Depending on the ammonium concentration of the wastewater and the reactor design, the dinitrogen gas produced by the process could at least partially mix the reactor (analogous to upflow anaerobic sludge blanket process (UASB) reactors), leading to very low power consumption. Additional mixing could be provided by recycling part of the produced dinitrogen gas. Due to the low growth rate of the responsible bacteria, sludge retention is extremely important. The reactor should be well mixed (to keep the redox potential in the 'denitrification zone' and prevent formation of toxic sulfide) and should not be overloaded, because high nitrite concentrations (more than 70 mg N l^{-1} NO_2^- '*Candidatus* Brocadia anammoxidans', more than 180 mg N l^{-1} NO_2^- '*Candidatus* Kuenenia stuttgartiensis') for extended periods are also detrimental to the process [64,66].

On laboratory scale, anammox has been tested in different reactors: fixed bed [89], fluidized bed [66], sequencing batch [63], and gas-lift reactors (unpublished results) all appeared to be suitable, although the economics of the process differ for the different reactor configurations (depending on existing reactors already in place that could be adapted to the process). One of the main challenges of the anammox process is the long start-up time. Because the anammox planctomycetes grow so slowly (see above) it takes between 100 and 150 days before an anammox reactor inoculated with activated sludge reaches full capacity [92]. Experience in anaerobic wastewater treatment (with UASB reactors) has shown that this problem may be overcome once the first full-scale anammox plants are in operation and seeding will become possible.

4.3. Canon

Canon is an acronym for 'completely autotrophic nitrogen removal over nitrite'. This concept (Fig. 2) is the combination of partial nitrification and anammox in a single, aerated reactor [89,93,94]. The name 'Canon' also refers to the way the two groups of bacteria cooperate: they perform two sequential reactions (Eqs. 1 and 4) simultaneously. The nitrifiers oxidize ammonia to nitrite, consume oxygen and so create anoxic conditions the anammox process needs. Canon has been tested extensively on laboratory scale. The volumetric loading rate (1.5 kg N m^{-3} day^{-1} in a gas-lift reactor) [94] is lower than for anammox and also somewhat lower than has been achieved with high-end dedicated nitrification reactors. However, because only one reactor is required, the economics might still be advantageous when the daily ammonium load is low. Canon would need process control, to prevent nitrite build-up by oxygen excess.

The Canon concept has not been purposefully tested on pilot or full scale, but is known to occur accidentally in sub-optimally functioning full-scale nitrification systems [25,95–97]. Such systems combine three processes (Eqs. 1, 3 and 4), and convert ammonium to mixtures of nitrate and dinitrogen gas. The stoichiometry of the released products is influenced by e.g. the bacterial population and the physical parameters.

4.4. NO_x process

Controlling and stimulating the denitrification activity of *Nitrosomonas*-like microorganisms by adding nitrogen oxides offers new possibilities in wastewater treatment [98]. In the presence of NO_x *Nitrosomonas*-like microorganisms nitrify and denitrify simultaneously even under fully oxic conditions with N_2 as main product (Fig. 2). Just about 40% of the ammonia load is converted to nitrite. Besides a 50% lower oxygen demand in the nitrification step (since nitrite is used as terminal electron acceptor), the subsequent denitrification step consumes less COD. The N-conversion in a combined nitrification/denitrification without NO_x supply is shown in Eqs. 9–11 and N-conversions influenced by nitrogen oxides in Eqs. 12–14. The [H] represents the reducing equivalents (e.g. supplied by an external C-source). These results might vary depending on the composition of the wastewater.

Conventional plant

Nitrification

$$3NH_4^+ + 6O_2 \rightarrow 3NO_3^- + 6H^+ + 3H_2O \quad (9)$$

Denitrification

$$3NO_3^- + 3H^+ + 15[H] \rightarrow 1.5N_2 + 9H_2O \quad (10)$$

Sum

$$3NH_4^+ + 6O_2 + 15[H] \rightarrow 1.5N_2 + 3H^+ + 12H_2O \quad (11)$$

Plant with NO_x supply

Nitrification

$$3NH_4^+ + 3O_2 \rightarrow N_2 + 4H_2O + NO_2^- + 4H^+ \quad (12)$$

Denitrification

$$NO_2^- + H^+ + 3[H] \rightarrow 0.5N_2 + 2H_2O \quad (13)$$

Sum

$$3NH_4^+ + 3O_2 + 3[H] \rightarrow 1.5N_2 + 3H^+ + 6H_2O \quad (14)$$

NO_x (NO/NO_2) is the regulatory signal inducing the denitrification activity of the ammonia oxidizers and it is only added in trace amounts (NH_4^+/NO_2 ratio about 1000/1 to 5000/1) [45]. As a consequence about 50% of the reducing equivalents [H] are now transferred to nitrite as terminal electron acceptor (Eq. 12) instead of oxygen. Therefore the oxygen consumption of the process is reduced (Eqs. 12 and 14).

The new method for N-removal was developed in a laboratory scale reactor system allowing a performance increase as well as a decrease of the operating costs. The new process offers the possibility to be integrated into existing wastewater treatment plants with minimal financial and technical efforts.

One 2-m^3 pilot plant for the treatment of wastewater from intensive fish farming and a pilot plant of the company Nitra GmbH (Germany) at a municipal wastewater treatment plant (sludge liquor) were tested [99]. We will present data of a 3.5-m^3 plant. The installation is working for 22 months, treating highly loaded wastewater. Exhaust fume containing ammonia is processed via a washer and the effluent is treated in a nitrification/denitrification system equipped with the NO_x method. The highly loaded wastewater (about 2 kg NH_4^+-N m^{-3}) is fed into the nitrification reactor (3 m^3), which is connected to a stirred but not aerated denitrification tank (0.5 m^3). The contact between the sewage and biomass is mediated via membrane surfaces (cross-flow filtration). The nitrification step is aerated with about 65 l air min^{-1} supplied with 200 ppm NO_2.

The performance data of the nitrification step (without denitrification step) are presented in Fig. 3. The volume load of the plant was increased from about 2 kg NH_4^+-N m^{-3} day^{-1} to about 4.7 kg NH_4^+-N m^{-3} day^{-1}. The denitrification activity of the nitrifying biomass was very sensitive towards the NO_2 supply. When the NO_2 supply in the nitrification step was turned off between days 100 and 112, this led on short-term to an increased ammonia concentration caused by a reduced ammonia oxidation activity. The long-term effect was more interesting: a significantly increased nitrite concentration was detectable between days 112 and 150. Obviously, the denitrification activity of the ammonia oxidizers decreased when NO_2 was not present (days 100–112). Under the influence of NO_2, the ammonia oxidizers again increased their denitrification activity (days 112–150).

During the 22-month operation time, the ammonia consumption in the nitrification step was about 3.5 times higher than the nitrite production. Since hardly any nitrate (<1 g NO_3^--N m^{-3}) was formed and NO and N_2O were only detectable in small amounts (in waste gas <40 ppm), strong evidence is given that the N_2 production by ammonia oxidizers is mainly responsible for the average N-loss of about 67% (Fig. 4). Control tests under laboratory conditions confirmed these results [28,42]. The remaining N-load (nitrite) was removed in the small denitrification step with methanol as carbon source. Including the denitrification step into the analysis of the nitrogen balance of the treatment plant, the N-loss was about 97% (Fig. 4). The costs to equip an existing sewage plant with a system for the NO_x supply were calculated with EUR 10 000–55 000 depending on the dimension of the plant. The method causes additional operating costs of EUR 0.05–0.08 per kilogram ammonia-N (NO_2 supply). The following savings have to been taken into account: the external C-source can be reduced up to 80%, and the supply of oxygen can be reduced up to 50%.

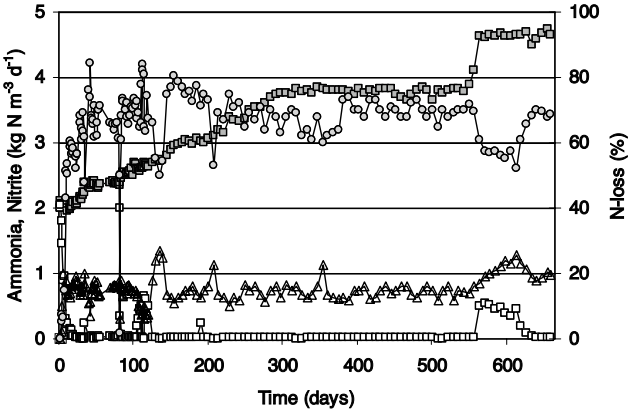


Fig. 3. Nitrogen balance of the NO_x process. Ammonia in (■), ammonia out (□), nitrite out (△), N-loss in the nitrification step (●). The N-loss is defined as the rate of soluble N-compounds converted into gaseous N-compounds (mainly N_2).

4.5. Further application

The OLAND process (oxygen-limited nitrification and denitrification) is described as a new process for one-step ammonium removal without addition of COD [100]. Apart from the basic fact that nitrifiers are involved, the mechanism is not yet understood and the ammonium loading rates are low. It seems possible that OLAND will be based on either the Canon concept (a combination

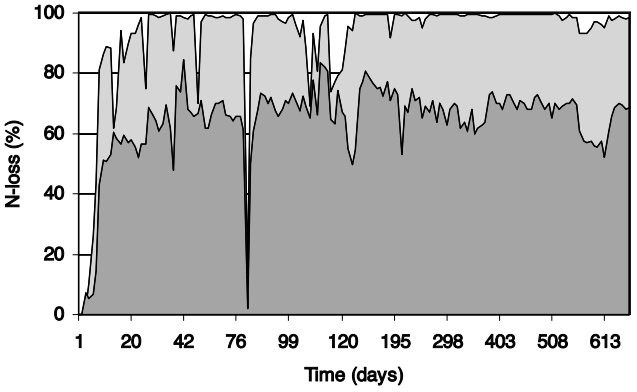


Fig. 4. N-losses in the nitrification step (NO_x process) and denitrification step; dark gray: nitrification step, gray: denitrification step.

Table 1
Operational characteristics of wastewater treatment processes for nitrogen removal

	Conventional nitrification denitrification	NO_x	OLAND	SHARON	Anammox	Canon	Aerobic deammonification
Aerobic ammonia oxidizers	many	*N. eutropha*	unknown	*N. eutropha*	absent	*N. eutropha*	unknown salt tolerant ammonia oxidizer
Aerobic nitrite oxidizers	many	absent	unknown	absent	absent	absent	*Nitrobacter*
Anaerobic ammonia oxidizers	absent	absent	unknown	absent	*B. anammoxidans*, *K. stuttgartiensis*	*B. anammoxidans*, *K. stuttgartiensis*	*K. stuttgartiensis*
Biofilms or suspension	biofilms/suspension	suspension	biofilms	suspension	biofilms	biofilms	biofilms
NH_4^+ loading (kg N m^{-3} reactor day^{-1})	2–8	5	0.1	0.5–1.5	10–20	2–3	1–2
N-removal efficiency	95%	95%	85%	90%	90%	90%	60%
Process complexity	separate oxic and anoxic compartments or periods, methanol dosing	separated oxic and anoxic compartments, methanol dosing, membrane for sludge retention	aeration needs to be tuned to ammonia loading	separate oxic and anoxic compartments or periods, methanol dosing	preceding partial nitrification needed	aeration needs to be tuned to ammonia loading	aeration needs to be tuned to ammonia loading
Application status	established	pilot plant	laboratory	two full-scale plants	full scale initiated	laboratory	two full-scale plants
Investment costs	medium	medium	medium	medium	low	medium	medium
Operational costs	high	low	unknown	low	very low	low	low

of aerobic and anaerobic ammonia oxidizers) or the NO_x process (nitrifier denitrification in the presence of NO_x).

The 'aerobic deammonification' process is another one-step ammonium removal process that does not depend on COD [25]. It has been tested on pilot plant and full scale, and converts part of the ammonium to dinitrogen gas and part to nitrate. Recently, it was discovered that this process is based on the Canon concept, with nitrifiers and anaerobic ammonia oxidizers cooperating under oxygen limitation. Because the 'aerobic deammonification' process evolved in reactors designed for conventional nitrification, the process design (a rotating biological contactor) is not optimal and the nitrogen loading rates and removal efficiency are low (Table 1).

5. Conclusion

Over the past 25 years, a significant amount of resources have been invested to construct wastewater treatment plants. Unfortunately, the performance of many of these facilities has not fulfilled the requirements of the discharge permits. In many cases, newly constructed plants have had to be retrofitted or modified at considerable expense to meet the discharge requirements and to provide more reliable performance. The need to conserve energy and resources is well documented, and therefore more attention is being paid to the selection of processes that conserve energy and resources. Operation and maintenance costs plus reliable process control are extremely important to operating agencies. Thus, the operability of treatment plants is receiving more attention. To design and operate a wastewater treatment system (activated sludge system) efficiently, it is necessary to understand the biochemistry of the involved microorganisms and basic research is the keystone to optimize established processes and to invent new and innovative systems. Discovering the group of anammox microorganisms opened new ways in nitrogen removal. Processes like SHARON and Canon have been developed, meeting the needs of treatment plants to handle e.g. high nitrogen-loaded wastewater. Also the discovery of the versatility of aerobic ammonia oxidizers led to the development of new treatment processes (e.g. NO_x process). In the future the combination of the different groups of nitrogen converting microorganisms and the optimization of the process management (adaptation according to the wastewater composition; design of the treatment plants, temperature, oxygen and NO_x supply) will improve the nitrogen removal. One of these options is a complete nitrogen removal (ammonia to N_2) by a mixed population of 'aerobic' ammonia oxidizers and anammox bacteria under anoxic conditions in the presents of NO_2 [101].

The interest in small treatment systems has often been overshadowed by concern over design, construction, and operation of large regional systems. Small systems were often designed as small-scale models of large plants. As

a consequence, many are operationally energy and resource intensive. New and innovative techniques like those described in the review might offer a solution for many treatment plants. The activated sludge process has been used extensively in its original form as well as in many modified forms. In the method used and the design of the process, consideration must be given to selection of the reactor type, loading criteria, sludge production, oxygen requirements and transfer, nutrient requirements, control of filamentous organisms, and effluent characteristics. More specific characteristics for the biological part are the operation factors like reaction kinetics, oxygen transfer, nature of the wastewater, local environmental conditions, construction, operation mode, and maintenance costs.

In view of these considerations, we believe there is no single best process for ammonium removal from wastewater. In each case it has to be evaluated which process is most suitable. Table 1 compares the different characteristics of new and established processes to allow a proper evaluation which method is best for a specific application.

Acknowledgements

The authors gratefully acknowledge the support of the EU in the 5th framework project ICON (EVK1-CT2000-00054). This is CWE (Center for Wetland Ecology) publication 2002-271.

References

[1] Daims, H., Nielsen, P.H., Nielsen, J.L., Juretschko, S. and Wagner, M. (2000) Novel Nitrospira-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: diversity and in situ physiology. Water Sci. Technol. 41, 85–90.

[2] Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Roser, A., Koops, H.P. and Wagner, M. (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge – *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. 64, 3042–3051.

[3] Purkhold, U., Pommerening-Roser, A., Juretschko, S., Schmid, M.C., Koops, H.P. and Wagner, M. (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. 66, 5368–5382.

[4] Schramm, A., De Beer, D., Wagner, M. and Amann, R.I. (1998) Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl. Environ. Microbiol. 64, 3480–3485.

[5] Helmer, C., Kunst, S., Juretschko, S., Schmid, M.C., Schleifer, K.H. and Wagner, M. (1999) Nitrogen loss in a nitrifying biofilm system. Water Sci. Technol. 39, 13–21.

[6] Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., Metzger, J.W., Schleifer, K.H. and Wagner, M. (2000) Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst. Appl. Microbiol. 23, 93–106.

[7] Egli, K., Fanger, U., Alvarez, P.J.J., Siegrist, H., Van der Meer, J.R. and Zehnder, A.J.B. (2001) Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Arch. Microbiol. 175, 198–207.

[8] Gieseke, A., Purkhold, U., Wagner, M., Amann, R. and Schramm, A. (2001) Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Appl. Environ. Microbiol. 67, 1351–1362.

[9] Schmid, M., Schmitz-Esser, S., Jetten, M. and Wagner, M. (2001) 16S–23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. Environ. Microbiol. 3, 450–459.

[10] Bateman, A. (1997) The structure of a domain common to Archaebacteria and the homocystinuria disease protein. Trends Biochem. Sci. 22, 12–13.

[11] Steinert, K., Wagner, V., Kroth-Pancic, P.G. and Bickel-Sandkotter, S. (1997) Characterization and subunit structure of the ATP synthase of the halophilic archaeon *Haloferax volcanii* and organization of the ATP synthase genes. J. Biol. Chem. 272, 6261–6269.

[12] De Bie, M.J.M., Speksnijder, A.G.C.L., Kowalchuk, G.A., Schuurman, T., Zwart, G., Stephen, J.R., Diekmann, O.E. and Laanbroek, H.J. (2001) Shifts in the dominant populations of ammonia-oxidizing β-subclass Proteobacteria along the eutrophic Schelde estuary. Aquat. Microb. Ecol. 23, 225–236.

[13] Kowalchuk, G.A., Bodelier, P.L.E., Heilig, G.H.J., Stephen, J.R. and Laanbroek, H.J. (1998) Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. FEMS Microbiol. Ecol. 27, 339–350.

[14] Speksnijder, A.G.C.L., Kowalchuk, G.A., Roest, K. and Laanbroek, H.J. (1998) Recovery of a *Nitrosomonas*-like 16S rDNA sequence group from freshwater habitats. Syst. Appl. Microbiol. 21, 321–330.

[15] Kowalchuk, G.A., Stienstra, A.W., Heilig, G.H.J., Stephen, J.R. and Woldendorp, J.W. (2000) Changes in the community structure of ammonia-oxidizing bacteria during secondary succession of calcareous grasslands. Environ. Microbiol. 2, 99–110.

[16] Nold, S.C., Zhou, J.Z., Devol, A.H. and Tiedje, J.M. (2000) Pacific northwest marine sediments contain ammonia-oxidizing bacteria in the beta subdivision of the Proteobacteria. Appl. Environ. Microbiol. 66, 4532–4535.

[17] Phillips, C.J., Harris, D., Dollhopf, S.L., Gross, K.L., Prosser, J.I. and Paul, E.A. (2000) Effects of agronomic treatments on structure and function of ammonia-oxidizing communities. Appl. Environ. Microbiol. 66, 5410–5418.

[18] Logemann, S., Schantl, J., Bijvank, S., Van Loosdrecht, M.C.M., Kuenen, J.G. and Jetten, M. (1998) Molecular microbial diversity in a nitrifying reactor system without sludge retention. FEMS Microbiol. Ecol. 27, 239–249.

[19] Prosser, J.I. (1989) Autotrophic nitrification in bacteria. In: Advances in Microbial Physiology, Vol. 30 (Rose, A.H. and Tempest, D.W., Eds.), pp. 125–181. Academic Press, London.

[20] Ehrich, S., Behrens, D., Lebedeva, E., Ludwig, W. and Bock, E. (1995) A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, *Nitrospira moscoviensis* sp nov and its phylogenetic relationship. Arch. Microbiol. 164, 16–23.

[21] Bock, E., Koops, H.-P., Harms, H. and Ahlers, B. (1991) The biochemistry of nitrifying organisms. In: Variations of Autotrophic Life (Shively, J.M., Ed.), pp. 171–200. Academic Press, London.

[22] Watson, S.W., Bock, E., Harms, H., Koops, H.-P. and Hooper, A.B. (1989) Nitrifying bacteria. In: Bergey's Manual of Systematic Bacteriology (Stanley, J.T., Bryant, M.P., Pfennig, N. and Holt, J.G., Eds.), pp. 1808–1834. Williams and Wilkens, Baltimore, MD.

[23] Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., Van de Pas-Schoonen, K.T., Webb, R., Kuenen, J.G. and Jetten, M.S.M. (1999) Missing lithotroph identified as new planctomycete. Nature 400, 446–449.

[24] Jetten, M.S.M., Wagner, M., Fuerst, J., Van Loosdrecht, M.C.M., Kuenen, G. and Strous, M. (2001) Microbiology and application of

the anaerobic ammonium oxidation ('anammox') process. Curr. Opin. Biotechnol. 12, 283–288.
[25] Hippen, A., Helmer, C., Kunst, S., Rosenwinkel, K.H. and Seyfried, C.F. (2001) Six years' practical experience with aerobic/anoxic deammonification in biofilm systems. Water Sci. Technol. 44, 39–48.
[26] Koops, H.P. and Pommerening-Roser, A. (2001) Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiol. Ecol. 37, 1–9.
[27] Rees, M. and Nason, A. (1966) Incorporation of atmospheric oxygen into nitrite formed during ammonia oxidation by *Nitrosomonas europaea*. Biochim. Biophys. Acta 113, 398–401.
[28] Schmidt, I. and Bock, E. (1997) Anaerobic ammonia oxidation with nitrogen dioxide by *Nitrosomonas eutropha*. Arch. Microbiol. 167, 106–111.
[29] Andersson, K.K. and Hooper, A.B. (1983) O_2 and H_2O are each the source of one O in HNO_2 produced from NH_3 by *Nitrosomonas*: ^{15}N-NMR evidence. FEBS Lett. 164, 236–240.
[30] Dua, R.D., Bhandari, B. and Nicholas, D.J.D. (1979) Stable isotope studies on the oxidation of ammonia to hydroxylamine by *Nitrosomonas europaea*. FEBS Lett. 106, 401–404.
[31] Hyman, M.R. and Wood, P.M. (1985) Suicidal inactivation and labeling of ammonia monooxygenase by acetylene. Biochem. J. 227, 719–725.
[32] Hyman, M.R. and Arp, D.J. (1993) An electrophoretic study of the thermal-dependent and reductant-dependent aggregation of the 28 kDa component of ammonia monooxygenase from *Nitrosomonas europaea*. Electrophoresis 14, 619–627.
[33] Schmidt, I. and Bock, E. (1998) Anaerobic ammonia oxidation by cell free extracts of *Nitrosomonas eutropha*. Antonie van Leeuwenhoek 73, 271–278.
[34] Arciero, D.M. and Hooper, A.B. (1993) Hydroxylamine oxidoreductase from *Nitrosomonas europaea* is a multimer of an octa-heme subunit. J. Biol. Chem. 268, 14645–14654.
[35] Bergmann, D.J., Arciero, D.A. and Hooper, A.B. (1994) Organization of the hao gene cluster of *Nitrosomonas europaea*: genes for two tetraheme c cytochromes. J. Bacteriol. 176, 3148–3153.
[36] Sayavedra-Soto, L.A., Hommes, N.G. and Arp, D.J. (1994) Characterization of the gene encoding hydroxylamine oxidoreductase in *Nitrosomonas europaea*. J. Bacteriol. 176, 504–510.
[37] Wood, P.M. (1986) Nitrification as a bacterial energy source. In: Nitrification (Prosser, J.L., Ed.), pp. 63–78. IRL Press, Oxford.
[38] Hyman, M.R. and Wood, P.M. (1983) Methane oxidation by *Nitrosomonas europaea*. Biochem. J. 212, 31–37.
[39] Hyman, M.R., Sansome-Smith, A.W., Shears, J.H. and Wood, R.M. (1985) A kinetic study of benzene oxidation to phenol by whole cells of *Nitrosomonas europaea* and evidence for further oxidation to hydroquinone. Arch. Microbiol. 43, 302–306.
[40] Hynes, R.K. and Knowles, R. (1978) Inhibition by acetylene of ammonia oxidation in *Nitrosomonas europaea*. FEMS Microbiol. Lett. 4, 319–321.
[41] Schmidt, I., Bock, E. and Jetten, M.S.M. (2001) Ammonia oxidation by *Nitrosomonas eutropha* with NO_2 as oxidant is not inhibited by acetylene. Microbiology 147, 2247–2253.
[42] Zart, D. and Bock, E. (1998) High rate of aerobic nitrification and denitrification by *Nitrosomonas eutropha* grown in a fermentor with complete biomass retention in the presence of gaseous NO_2 or NO. Arch. Microbiol. 169, 282–286.
[43] Poth, M. and Focht, D.D. (1985) ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. Appl. Environ. Microbiol. 49, 1134–1141.
[44] Poth, M. (1986) Dinitrogen production from nitrite by a *Nitrosomonas* isolate. Appl. Environ. Microbiol. 52, 957–959.
[45] Schmidt, I., Zart, D. and Bock, E. (2001) Gaseous NO_2 as a regulator for ammonia oxidation of *Nitrosomonas eutropha*. Antonie van Leeuwenhoek 79, 311–318.
[46] Mancinelli, R.L. and McKay, C.P. (1983) Effects of nitric oxide and nitrogen dioxide on bacterial growth. Appl. Environ. Microbiol. 46, 198–202.
[47] Both, G.J., Gerards, S. and Laanbroek, H.J. (1992) Kinetics of nitrite oxidation in two *Nitrobacter* species grown in nitrite-limited chemostats. Arch. Microbiol. 157, 436–441.
[48] Hellinga, C., Schellen, A.A.J.C., Mulder, J.W., Van Loosdrecht, M.C.M. and Heijnen, J.J. (1998) The SHARON process: an innovative method for nitrogen removal from ammonium-rich waste water. Water Sci. Technol. 37, 135–142.
[49] Sundermeyer, H. and Bock, E. (1981) Energy metabolism of autotrophically and heterotrophically grown cells of *Nitrobacter winogradskyi*. Arch. Microbiol. 130, 250–254.
[50] Tanaka, Y., Fukumori, Y. and Yamanaka, T. (1983) Purification of cytochrome a_1c_1 from *Nitrobacter agilis* and characterization of nitrite oxidation system of the bacterium. Arch. Microbiol. 135, 265–271.
[51] Aleem, M.I.H., Hoch, G.E. and Varner, J.E. (1965) Water as the source of oxidant and reductant in bacterial chemosynthesis. Proc. Natl. Acad. Sci. USA 54, 869–873.
[52] Hollocher, T.C., Kumar, S. and Nicholas, D.J.D. (1982) Respiration-dependent proton translocation in *Nitrosomonas europaea* and its apparent absence in *Nitrobacter agilis* during inorganic oxidations. J. Bacteriol. 149, 1013–1020.
[53] Sone, N., Yanagita, Y., Hon-Nami, K., Fukumori, Y. and Yamanaka, T. (1983) Proton-pump activity of *Nitrobacter agilis* and *Thermus thermophilus* cytochrome c oxidases. FEBS Lett. 155, 150–153.
[54] Bock, E., Düvel, D. and Peters, K.-R. (1974) Characterization of a phage-like particle from cells of *Nitrobacter*. I. Host-particle correlation and particle isolation. Arch. Microbiol. 97, 115–127.
[55] Steinmüller, W. and Bock, E. (1976) Growth of *Nitrobacter* in the presence of organic matter. I. Mixotrophic growth. Arch. Microbiol. 108, 299–304.
[56] Watson, S.W., Bock, E., Valois, F.W., Waterbury, J.B. and Schlosser, U. (1986) *Nitrospira marina* gen. nov. sp. nov. a chemolithotrophic nitrite oxidizing bacterium. Arch. Microbiol. 144, 1–7.
[57] Bock, E. (1976) Growth of *Nitrobacter* in the presence of organic matter. II. Chemoorganic growth of *Nitrobacter agilis*. Arch. Microbiol. 108, 305–312.
[58] Steinmüller, W. and Bock, E. (1977) Enzymatic studies on autotrophically, mixotrophically and heterotrophically grown *Nitrobacter agilis* with special references to nitrite oxidase. Arch. Microbiol. 115, 51–54.
[59] Bock, E., Wilderer, P.A. and Freitag, A. (1988) Growth of *Nitrobacter* in the absence of dissolved oxygen. Water Res. 22, 245–250.
[60] Sundermeyer-Klinger, H., Meyer, W., Warninghoff, B. and Bock, E. (1984) Membrane-bound nitrite oxidoreductase of *Nitrobacter*: evidence for a nitrate reductase system. Arch. Microbiol. 140, 153–158.
[61] Hanaki, K., Wantawin, C. and Ogaki, S. (1990) Nitrification at low levels of dissolved oxygen with and without organic loading in a suspended-growth reactor. Water Res. 24, 297–302.
[62] Eigner, U. and Bock, E. (1972) Synthesis and breakdown of polyphosphate fraction in cells of *Nitrobacter winogradskyi*. Arch. Mikrobiol. 81, 367–378.
[63] Strous, M., Heijnen, J.J., Kuenen, J.G. and Jetten, M.S.M. (1998) The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. Appl. Microbiol. Biotechnol. 50, 589–596.
[64] Strous, M., Kuenen, J.G. and Jetten, M.S.M. (1999) Key physiology of anaerobic ammonium oxidation. Appl. Environ. Microbiol. 65, 3248–3250.
[65] Strous, M., Van Gerven, E., Kuenen, J.G. and Jetten, M. (1997) Effects of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (Anammox) sludge. Appl. Environ. Microbiol. 63, 2446–2448.
[66] Van de Graaf, A.A., De Bruijn, P., Robertson, L.A., Jetten, M.S.M. and Kuenen, J.G. (1996) Autotrophic growth of anaerobic ammo-

nium-oxidizing micro-organisms in a fluidized bed reactor. Microbiology UK 142, 2187–2196.
[67] Van de Graaf, A.A., De Bruijn, P., Robertson, L.A., Jetten, M.S.M. and Kuenen, J.G. (1997) Metabolic pathway of anaerobic ammonium oxidation on the basis of N-15 studies in a fluidized bed reactor. Microbiology UK 143, 2415–2421.
[68] Schalk, J., De Vries, S., Kuenen, J.G. and Jetten, M.S.M. (2000) Involvement of a novel hydroxylamine oxidoreductase in anaerobic ammonium oxidation. Biochemistry 39, 5405–5412.
[69] Lindsay, M.R., Webb, R.I., Strous, M., Jetten, M.S., Butler, M.K., Forde, R.J. and Fuerst, J.A. (2001) Cell compartmentalisation in planctomycetes: novel types of structural organisation for the bacterial cell. Arch. Microbiol. 175, 413–429.
[70] Sinninghe Damsté, J.S., Strous, M., Rijpstra, W.I.C., Hopmans, E.C., Geenevasen, J.A.J., Van Duin, A.C.T., Van Niftrik, L.A. and Jetten, M.S.M. (2002) Linearly concatenated cyclobutane lipids form a dense bacterial membrane. Nature 419, 708–712.
[71] Van Niel, E.W.J., Arts, P.A.M., Wesselink, B.J., Robertson, L.A. and Kuenen, J.G. (1993) Competition between heterotrophic and autotrophic nitrifiers for ammonia in chemostat cultures. FEMS Microbiol. Ecol. 102, 109–118.
[72] Ralt, D., Gomez, R.F. and Tannerbaum, S.R. (1981) Conversion of acetohydroxamate and hydroxylamine to nitrite by intestinal microorganisms. Eur. J. Appl. Microbiol. Biotechnol. 12, 226–230.
[73] Castignetti, D. and Hollocher, T.C. (1984) Heterotrophic nitrification among denitrifiers. Appl. Environ. Microbiol. 47, 620–623.
[74] Wood, P.M. (1988) Monooxygenase and free radical mechanism for biological ammonia oxidation. In: The Nitrogen and Sulfur Cycles (Cole, J.A., Ed.), pp. 217–243. Cambridge University Press, Cambridge.
[75] Spiller, H., Dietsch, E. and Kessler, E. (1976) Intracellular appearance of nitrite and nitrate in nitrogen-starved cells of *Ankistrodesmus braunii*. Planta 129, 175–181.
[76] Stams, A.J.M., Flameling, E.M. and Marnette, E.C.L. (1990) The importance of autotrophic versus heterotrophic oxidation of atmospheric ammonium in forest ecosystems with acid soil. FEMS Microbiol. Ecol. 74, 337–344.
[77] Robertson, L.A. and Kuenen, J.G. (1990) Combined heterotrophic nitrification and aerobic denitrification in *Thiosphaera pantotropha* and other bacteria. Antonie van Leeuwenhoek 57, 139–152.
[78] Robertson, L.A. and Kuenen, J.G. (1988) Heterotrophic nitrification in *Thiosphaera pantotropha*: oxygen uptake and enzyme studies. J. Gen. Microbiol. 134, 857–863.
[79] Killham, K. (1986) Heterotrophic nitrification. In: Nitrification (Prosser, J.I., Ed.), pp. 117–126. IRL Press, Oxford.
[80] Shoun, H. and Tanimoto, T. (1991) Denitrification by the fungus *Fusarium oxysporum* and involvement of cytochrome P-450 in the respiratory nitrite reduction. J. Biol. Chem. 25, 1527–1536.
[81] Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61, 533–616.
[82] Focht, D.D. and Chang, A.C. (1975) Nitrification and denitrification process related to wastewater treatment. Adv. Appl. Microbiol. 19, 153–186.
[83] Zumft, W.G., Viebrock, A. and Körner, H. (1988) Biochemical and physiological aspects of denitrification. In: The Nitrogen and Sulfur Cycles (Cole, J.A. and Ferguson, S.J., Eds.), pp. 245–279. Cambridge University Press, Cambridge.
[84] Zumft, W.G. and Körner, H. (1997) Enzyme diversity and mosaic gene organization in denitrification. Antonie van Leeuwenhoek 71, 43–58.
[85] Zumft, W.G. (1992) The denitrifying prokaryotes. In: The Prokaryotes, 2nd edn. (Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-H., Eds.), pp. 554–582. Springer, New York.
[86] Körner, H. and Zumft, W.G. (1989) Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stuzeri*. Appl. Environ. Microbiol. 55, 1670–1676.
[87] Hellinga, C., Van Loosdrecht, M.C.M. and Heijnen, J.J. (1999) Model based design of a novel process for nitrogen removal from concentrated flows. Math. Comp. Model Dyn. 5, 351–371.
[88] Mulder, J.W., Van Loosdrecht, M.C.M., Hellinga, C. and Van Kempen, R. (2001) Full-scale application of the SHARON process for treatment of rejection water of digested sludge dewatering. Water Sci. Technol. 43, 127–134.
[89] Strous, M., Van Gerven, E., Ping, Z., Kuenen, J.G. and Jetten, M.S.M. (1997) Ammonium removal from concentrated waste streams with the Anaerobic Ammonium Oxidation (Anammox) process in different reactor configurations. Water Res. 31, 1955–1962.
[90] Mulder, A. (1992) Anoxic ammonia oxidation. U.S. Patent documents 427849.
[91] Van Loosdrecht, M.C.M. and Jetten, M.S.M. (1997) Method for treating ammonia-comprising wastewater. PCT/NL97/00482.
[92] Van Dongen, U., Jetten, M.S.M. and Van Loosdrecht, M.C.M. (2001) The SHARON((R))-Anammox((R)) process for treatment of ammonium rich wastewater. Water Sci. Technol. 44, 153–160.
[93] Koch, G., Egli, K., Van der Meer, J.R. and Siegrist, H. (2000) Mathematical modeling of autotrophic denitrification in a nitrifying biofilm of a rotating biological contactor. Water Sci. Technol. 41, 191–198.
[94] Third, K.A., Sliekers, A.O., Kuenen, J.G. and Jetten, M.M. (2002) The CANON System (Completely Autotrophic Nitrogen-removal Over Nitrite) under ammonium limitation: Interaction and competition between three groups of bacteria. Syst. Appl. Microbiol. 24, 588–596.
[95] Siegrist, H., Reithaar, S., Koch, G. and Lais, P. (1998) Nitrogen loss in a nitrifying rotating contactor treating ammonium-rich wastewater without organic carbon. Water Sci. Technol. 38, 241–248.
[96] Helmer, C., Tromm, C., Hippen, A., Rosenwinkel, K.H., Seyfried, C.F. and Kunst, S. (2001) Single stage biological nitrogen removal by nitritation and anaerobic ammonium oxidation in biofilm systems. Water Sci. Technol. 43, 311–320.
[97] Hippen, A., Rosenwinkel, K.H., Baumgarten, G. and Seyfried, C.F. (1997) Aerobic deammonification – a new experience in the treatment of wastewaters. Water Sci. Technol. 35, 111–120.
[98] Bock, E., Schmidt, I., Stüven, R. and Zart, D. (1996) Verfahren zur biologischen Umsetzung von in Wasser gelöstem Ammonium unter Verwendung ammoniak-oxidierender Bakterien. Az.: DE 196 17 331.0-41.
[99] Schmidt, I., Zart, D., Stüven, R. and Bock, E. (2001) Ein neues Verfahren zur Entfernung von Ammonium-Stickstoff aus Abwasser. Chem. Ing. Tech. 73, 879–882.
[100] Kuai, L. and Verstraete, W. (1998) Ammonium removal by the oxygen-limited autotrophic nitrification–denitrification system. Appl. Environ. Microbiol. 64, 4500–4506.
[101] Schmidt, I., Hermelink, C., Van de Pas-Schoonen, K., Strous, M., op den Camp, H.J., Kuenen, J.G. and Jetten, M.S.M. (2002) Anaerobic ammonia oxidation in the presence of nitrogen oxides (NOx) by two different lithotrophs. Appl. Environ. Microbiol. 68, 5351–5357.