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REVIEW ARTICLE

Biochemistry and molecular biology of anammox bacteria

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Abstract

Anaerobic ammonium-oxidizing (anammox) bacteria are one of the latest additions to the biogeochemical nitrogen cycle. These bacteria derive their energy for growth from the conversion of ammonium and nitrite into dinitrogen gas in the complete absence of oxygen. These slowly growing microorganisms belong to the order Brocadiales and are affiliated to the Planctomycetes. Anammox bacteria are characterized by a compartmentalized cell architecture featuring a central cell compartment, the "anammoxosome". Thus far unique "ladderane" lipid molecules have been identified as part of their membrane systems surrounding the different cellular compartments. Nitrogen formation seems to involve the intermediary formation of hydrazine, a very reactive and toxic compound. The genome of the anammox bacterium *Kuenenia stuttgartiensis* was assembled from a complex microbial community grown in a sequencing batch reactor (74% enriched in this bacterium) using a metagenomics approach. The assembled genome allowed the *in silico* reconstruction of the anammox metabolism and identification of genes most likely involved in the process. The present anammox pathway is the only one consistent with the available experimental data, thermodynamically and biochemically feasible, and consistent with Ockham's razor: it invokes minimum biochemical novelty and requires the fewest number of biochemical reactions. The worldwide presence of anammox bacteria has now been established in many oxygen-limited marine and freshwater systems, including oceans, seas, estuaries, marshes, rivers and large lakes. In the marine environment over 50% of the N_2 gas released may be produced by anammox bacteria. Application of the anammox process offers an attractive alternative to current wastewater treatment systems for the removal of ammonia-nitrogen. Currently, at least five full scale reactor systems are operational.

Keywords: *Nitrogen cycle; anaerobic ammonium oxidation; hydrazine; metagenome; anammoxosome; Brocadiales*

Physical properties of inorganic nitrogen compounds

Nitrogen (N) is a group 5B element with oxidation states from –3 to +5 (Table 1). In each oxidation state the nitrogen atom combines with atoms of hydrogen, oxygen or other nitrogen atoms. In this way at least one unique inorganic molecule exists per oxidation state. Although some of these molecules are thermodynamically more stable than others, all oxidation states are possible in aqueous systems, because the oxidation state of N in a given environment is controlled by kinetics (the activation energy of the N-compounds is high), and not by thermodynamic equilibrium. The bulk of nitrogen on this globe is present in solid state (rocks). However, the dinitrogen gas in the atmosphere (79% vol.) is the most important nitrogen source available to biology.

The nitrogen cycle

Life depends on nitrogen. A gross chemical formula for a living organism is $CH_2O_{0.5}N_{0.15}$. From a microbiological point of view, the turnover of nitrogen compounds

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Table 1. Physical properties of inorganic nitrogen compounds. Thermodynamic values: ° refers to standard conditions (pH 0, 25°C) and °′ to physiological conditions (pH 7, 25°C).

Compound	Formula	Oxidation state nitrogen	$\Delta H_f°$ (kJ mol^{-1})	$\Delta G_f°'$ (kJ mol^{-1})	S° (J mol^{-1} K)	pK
Ammonium	NH_4^+	−3	133.1	−79.4	713	9.2
Hydrazine	N_2H_4 (aq)	−2	34.4	128.5	−316	6.1
Hydroxylamine	NH_2OH (aq)	−1	−98.7	−22.9	−254	6.0
Dinitrogen gas	N_2 (g)	0	0	0	0	—
Nitrous oxide	N_2O (g)	+1	82.4	104.6	−74	—
Nitric oxide	NO (g)	+2	90.6	86.9	12	—
Nitrite	NO_2^-	+3	−105.0	−37.4	−227	3.3
Nitrogen dioxide	NO_2 (g)	+4	33.3	51.5	−61	—*
Nitrate	NO_3^-	+5	−208.2	−111.7	−324	−1.5

*Nitric dioxide reacts in water to nitrite and nitrate.

Table 2. The enzymes of the nitrogen cycle and the reactions they catalyze. Reactions are shown as redox half reactions where the enzyme itself acts as the primary electron acceptor or donor.

Process/enzyme	Reaction			Equation number	$E^{0'}$ (V/e$^-$)	Location
Nitritification						
Ammonia monooxygenase	$NH_4^+ + O_2 + H^+ + 2e^-$	→	$NH_2OH + H_2O$	1	0.73	Transmembrane
Hydroxylamine oxidoreductase	$NH_2OH + H_2O$	→	$NO_2^- + 5H^+ + 4e-$	2	−0.06	Periplasm
Nitratification/anammox						
Nitrite oxidoreductase	$NO_2^- + H_2O$	→	$NO_3^- + 2H^+ + 2e-$	3	−0.43	Membrane associated
Hydrazine hydrolase	$NH_4^+ + NO + 2H^+ + 3e^-$	→	$N_2H_4 + H_2O$	4	0.34	Anammoxosome
Hydrazine oxidoreductase	N_2H_4	→	$N_2 + 4H^+ + 4e-$	5	−0.75	Anammoxosome
Denitrification & dissimilatory nitrate reduction						
Nitrate reductase	$NO_3^- + 2H^+ + 2e^-$	→	$NO_2^- + H_2O$	6	0.43	Membrane associated, periplasm or cytoplasm
Nitrite reductase	$NO_2^- + 2H^+ + e^-$	→	$NO + H_2O$	7	0.34	Periplasm
Nitric oxide reductase	$2NO + 2H^+ + 2e^-$	→	$N_2O + H_2O$	8	1.17	Transmembrane
Nitrous oxide reductase	$N_2O + 2H^+ + 2e^-$	→	$N_2 + H_2O$	9	1.36	Periplasm
Dissimilatory nitrite reductase	$NO_2^- + 8H^+ + 6e^-$	→	$NH_4^+ + 2H_2O$	10	0.75	*

*Reported with the reducing activity both at the outside and inside of the cytoplasmic membrane.

in the biosphere (the nitrogen cycle) is made up of five catabolic processes (nitritification, nitratification, denitrification, dissimilatory nitrate reduction and anammox), three anabolic processes (ammonium uptake, assimilatory nitrate reduction and nitrogen fixation), and ammonification (a necessary result of the biological food chain). The most important enzymes of the nitrogen cycle and the reactions that they catalyze are summarized in Table 2.

In the early days, the main application of N-cycle microbiology was to understand and improve fertilizer efficiency in agriculture. Not until the 1960s did the potential of nitrifiers and denitrifiers for nutrient removal from wastewater become generally recognized, and research directed to improve nitrogen removal from wastewater. In the 1980s the contribution of nitrogen oxides in the atmosphere to ozone destruction and global warming was reassessed, and the role of nitrification and denitrification in the production of these compounds was again the focus of "environment-driven" N-cycle research.

However, the last 10 years has showed us that our knowledge of the microbial nitrogen cycle and its major players is far from complete (Jetten, 2008). Spectacular discoveries such as anaerobic ammonium oxidation (anammox) (Jetten *et al.*, 1998; Strous *et al.*, 1999), ammonium oxidation by Crenarchaea (AOA) (Koennecke *et al.*, 2005; Francis *et al.*, 2007), the interaction between

these two groups (Lam *et al.*, 2007), nitrate reduction to dinitrogen gas by foraminifera (Risgaard-Petersen *et al.*, 2006), nitrite-oxidizing phototrophs (Griffin *et al.*, 2007), nitrite-dependent anaerobic methane oxidation (N-DAMO) (Raghoebarsing *et al.*, 2006; Ettwig *et al.*, 2008), hyperthermophilic N_2-fixing methane-producing archaea (Mehta and Baross, 2006), and genome sequencing of several N-cycle organisms (Chain *et al.*, 2003; Starkenburg *et al.*, 2006; Strous *et al.*, 2006; Arp *et al.*, 2007; Stein *et al.*, 2007) provide examples that there is an enormous biodiversity and metabolic capability of nitrogen conversions hidden in the microbial world of which we know only very little to date (Jetten, 2008). Further, new sequence technologies and the refinement of molecular methods indeed showed how many secrets the vast majority of functional microbial diversity in the environment still hides (Yooseph *et al.*, 2007).

On the other hand increased combustion of fossil fuels and high demand for nitrogen in agriculture and industry indicate that mankind continues to transform the global N-cycle at a high rate (Galloway *et al.*, 2008). Vast amounts of anthropogenic nitrogen are lost to the environment and cause a cascade of problems, e.g. increased fresh water nitrate levels and increased nitrous oxide production, that may increase global climate change (Duce *et al.*, 2008). Improved knowledge of the microbes that are involved in nitrogen transformations is necessary to understand and eventually counteract the negative effects of nitrogen pollutions. This review focuses on one of the above-mentioned recent additions to the nitrogen cycle: the anaerobic ammonium oxidation (anammox). Since its discovery in 1995 the anammox process evolved from a largely unexplored part of the biological nitrogen cycle to general text book knowledge. In the last years it has become clear that anammox bacteria may be a major player in the global nitrogen cycle.

History of anammox research

Until the end of the 20th century the general opinion was that ammonium is an inert molecule under anoxic conditions: activation by oxygen was assumed to be required for its metabolism as known for nitrifying bacteria. Based on thermodynamical calculations, the Austrian biochemist Broda (1977) predicted the existence of microorganisms capable of ammonium oxidation with nitrite or nitrate as the electron acceptor. Unexplainable loss of ammonium under anoxic conditions was already reported a decade earlier (Richards, 1965) in studies of the nitrogen balance in anoxic fjords. Thirty years later, a similar observation in a denitrifying bioreactor in Delft, The Netherlands (Mulder *et al.*, 1995) initiated an active search for the microorganisms involved. The first description of an anammox bacterium dates from 1999 when Strous *et al.* (1999) were able to physically purify anammox cells from a laboratory enrichment culture. The purified anammox cells converted ammonium and nitrite into dinitrogen gas in the absence of oxygen and fixed cellular carbon from CO_2 only. The first anammox bacterium was named "Brocadia anammoxidans" and since it was not pure by classical microbiological standards, it was given the status of *Candidatus*. Anammox cells display a complex cell architecture with a central compartment, reminiscent to that of other members of the Planctomycetes, to which anammox bacteria are phylogenetically related (see below). Although the enrichment and/or detection of microorganisms capable of anaerobic growth on ammonium were unsuccessful for a long time, presently many research groups are studying various aspects of the anammox process (Pilcher, 2005; Kuenen, 2008). So far, five anammox genera have been described, with 16S rRNA gene sequence identities of the species ranging between 87 and 99% (Jetten *et al.*, 2009). Despite this relatively large phylogenetic distance, all anammox organisms belong to the same monophyletic cluster (order) named the Brocadiales and are related to the order Planctomycetales. The Brocadiales branch deeply inside the phylum Planctomycetes (Strous *et al.*, 1999; Schmid *et al.*, 2005; 2007). Four "*Candidatus*" anammox genera have been enriched from activated sludge: "Kuenenia" (Schmid *et al.*, 2000; Strous *et al.*, 2006), "Brocadia" (Strous *et al.*, 1999; Kuenen and Jetten, 2001; Kartal *et al.*, 2008), "Anammoxoglobus" (Kartal *et al.*, 2007b) and "Jettenia" (Quan *et al.*, 2008). The fifth anammox genus, "*Candidatus* Scalindua" (Kuypers *et al.*, 2003; Schmid *et al.*, 2003; van de Vossenberg *et al.*, 2008), has often been detected in natural habitats, especially in marine sediments and oxygen minimum zones (OMZ) (Dalsgaard *et al.*, 2005; Penton *et al.*, 2006; Schmid *et al.*, 2007; Woebken *et al.*, 2008).

Growth and metabolism of anammox bacteria

Anammox bacteria are slow growers; cells double only once per 11–20 days. In nature, these microorganisms thrive at very low substrate concentrations, probably reducing their doubling time *in situ* even further. Furthermore, anammox bacteria are obligate anaerobes and their metabolism is reversibly inhibited above 2 μM oxygen (Strous *et al.*, 1997). Current microbiological isolation techniques are not designed to deal with slow growing microorganisms. The sequencing batch reactor (SBR) was applied and optimized for the enrichment and quantitative study of anammox bacteria (Strous *et al.*, 1998). The SBR technique ensured long term reliable

operation (>1 year) under stable conditions and substrate-limiting conditions with efficient biomass retention (less than 10% of the growing biomass was washed out) and homogeneous distribution of substrates, products and biomass aggregates. Stable enrichments were obtained after a period of 90–200 days from SBRs sparged with oxygen-free gas and inoculated with a suitable environmental sample (wastewater sludge, river or marine sediment) (Strous *et al.*, 1999; Kartal *et al.* 2007b; 2008; van de Vossenberg *et al.*, 2008). The reactors were fed with ammonium, nitrite, bicarbonate and nitrate, the latter to avoid low redox potentials. Nitrite and ammonium inlet concentrations were gradually increased, such that the *in situ* reactor levels were maintained in the micromolar range by microbial activity. During the 90–200 days enrichment the SBR content slowly turns red (heme proteins constitute approximately 20% of the cellular protein mass) and the anammox bacteria increase to at least 70% of the population. The SBR enrichment cultures result in the growth of anammox biomass as biofilm aggregates. Very recently a membrane reactor was applied successfully for cultivation of anammox bacteria as single cell suspensions (van der Star *et al.*, 2008b). Under the microscope, the bacteria are observed as small coccoid cells with a diameter of approximately 800 nm. In laboratory-scale reactors operating under steady state conditions, ammonium, nitrite and bicarbonate are converted according to the overall equation (Strous *et al.*, 1998):

$$1\,NH4^+ + 1.32\,NO_2^- + 0.066\,HCO_3^- + 0.13\,H^+ \rightarrow 1.02\,N_2 + 0.26\,NO_3^- + 0.066\,CH_2O_{0.5}N_{0.15} + 2.03\,H_2O. \quad (1)$$

Anammox bacteria derive their energy for growth from the 1:1 chemolithotrophic conversion of ammonium and nitrite into N_2 ($\Delta G^{o\prime} = -275$ kJ mol^{-1} NH_4^+). Bicarbonate serves as the sole carbon source for the synthesis of cell biomass ($CH_2O_{0.5}N_{0.15}$), making the organisms autotrophs. Cell carbon fixation involves the acetyl-CoA pathway (Strous *et al.*, 2006). Based on the formation of biomass and nitrate in an SBR reactor performing the anammox process and on stoichiometric calculations it was hypothized that the reducing equivalents for the reduction of CO_2 are derived from the oxidation of nitrite to nitrate (Strous *et al.*, 1998). Anammox bacteria are characterized by a high affinity towards their substrates. Ammonium and nitrite are utilized down to very low concentrations (K_s < 5 μM). However, the metabolic activity is relatively low (15–80 μmol of N_2 formed per g dry weight of cells per min), which may explain the low growth rates to some extent.

Recent research revealed that anammox bacteria may not be strict chemolithoautrophic specialists, but could favor a more versatile lifestyle. Next to ammonium, the microorganisms are capable of using ferrous iron (Fe^{2+}) and a variety of organic compounds, including carboxylic acids (formate, acetate, propionate, methylamines), as electron donors (Strous *et al.*, 2006; Kartal *et al.*, 2007b; 2008). The enrichment of "*Candidatus* Anammoxoglobus propionicus" (Kartal *et al.*, 2007b) and "*Candidatus* Brocadia fulgida" (Kartal *et al.*, 2008) raises interesting questions as to the niche differentiation and species-specific adaptive mechanisms. Both species were enriched from the same wastewater treatment sludge by operating two SBR in parallel under identical conditions except for the addition of an extra carbon compound. "*Candidatus* B. fulgida" specifically became the dominant anammox species when the reactor was amended with acetate, whereas "*Candidatus* A. propionicus" was dominant in the SBR fed with propionate. Although both species are able to oxidize acetate and propionate, a comparison shows that the specific rate of acetate oxidation is somewhat higher in "*Candidatus* B. fulgida", while "*Candidatus* A. propionicus" somewhat favors propionate. These small differences apparently provide the organisms their competitive advantage. Remarkably, all other nitrate or nitrite-reducing microorganisms in the inoculum were outcompeted indicating that the presence of ammonium yielded the anammox cells with a strong selective advantage.

Apart from nitrite, anammox bacteria also employ Fe^{3+}, manganese oxides and nitrate as electron acceptors in their metabolism (Strous *et al.*, 2006). The use of nitrate is especially interesting. Like in classical denitrification the compound is converted into dinitrogen gas but via quite a different route. Nitrate is reduced to nitrite and ammonium, which combine to form N_2 by the anammox mechanism (Kartal *et al.*, 2007a) and as such the anammox bacteria are able to disguise themselves as denitrifiers.

Cell biology of anammox bacteria

Most prokaryotic cells have a similar structure, consisting of cell wall, cytoplasmic membrane and cytoplasm. For taxonomic purposes, bacterial cells can be subdivided into two classes based upon differences in cell envelope structure: Gram-negative and Gram-positive. The cell envelope of Gram-positive bacteria consists of the cytoplasmic membrane and a thick, highly cross-linked layer of peptidoglycan. The Gram-negative cell envelope consists of the cytoplasmic membrane, the periplasmic space, which is filled by a less cross-linked peptidoglycan gel, and the outer membrane. The outer membrane is a bilayer membrane composed of phospholipids (on the periplasmic side) and lipopolysaccharides (on the outside). Although intracellular membrane-bounded organelles are one of the defining features of eukaryotic cells, there are also prokaryotes that contain intracellular membrane systems.

Planctomycetes available in pure culture were shown to have more or less complex compartmentalization, involving a single intracytoplasmic membrane defining a major cell compartment (Figure 1; Fuerst, 2005). As concluded from electron microscopy observations, chemical analysis, genome sequencing and resistance to beta-lactam antibiotics and other cell wall-targeting antibiotics, Planctomycetes lack the otherwise universal bacterial cell wall polymer peptidoglycan (König *et al.*, 1984; Liesack *et al.*, 1986; Stackebrandt *et al.*, 1986; Fuerst, 1995). Further, their cell wall is not surrounded by one membrane on the outer and one membrane on the inner side of the cell wall as is the case for other Gram-negative bacteria. Instead there are two membranes on the inner side and no membrane on the outer side of the cell wall, which consists mainly of proteins. The outermost of these two membranes is closely positioned to the cell wall. This membrane has been defined as the cytoplasmic membrane based on the detection of RNA directly on its inner side by immunogold labeling. The other, innermost, membrane has been defined as an intracytoplasmic membrane as it is on the inside of the cytoplasmic membrane. The outermost cytoplasmic compartment of the cell (between these two membranes) has been named "paryphoplasm". The location of the paryphoplasm is the same as the Gram-negative periplasm, but where the former is inside the essential cell boundary, the latter is not. The organization of the cell envelope of Planctomycetes is therefore fundamentally different from the other Gram-negative bacteria (Lindsay *et al.*, 2001).

In the Planctomycetes *Pirellula* and *Isosphaera*, the intracytoplasmic membrane surrounds a single interior cell compartment, the "riboplasm", which holds the DNA as well as the ribosomes (Figure 1; Lindsay *et al.*, 2001). In *Isosphaera* the intracytoplasmic membrane exhibits a large invagination into the riboplasm. In the Planctomycete *Gemmata* and anammox bacteria the riboplasm itself contains a second membrane-bounded compartment (Figure 1; Strous *et al.*, 1999; Lindsay *et al.*, 2001). In *Gemmata* this compartment contains the cell DNA and is surrounded by a double membrane. In anammox bacteria the compartment is bounded by a single bilayer membrane and has been named "anammoxosome". The cytoplasm in anammox bacteria is thus divided into three cytoplasmic compartments separated by single bilayer membranes (van Niftrik *et al.*, 2008a; 2008b): (1) the outer region, i.e. the paryphoplasm, occurs as an outer rim defined on its outer side by the cytoplasmic membrane and cell wall and on the inner side by the intracytoplasmic membrane; (2) the riboplasm, containing DNA, ribosomes and storage

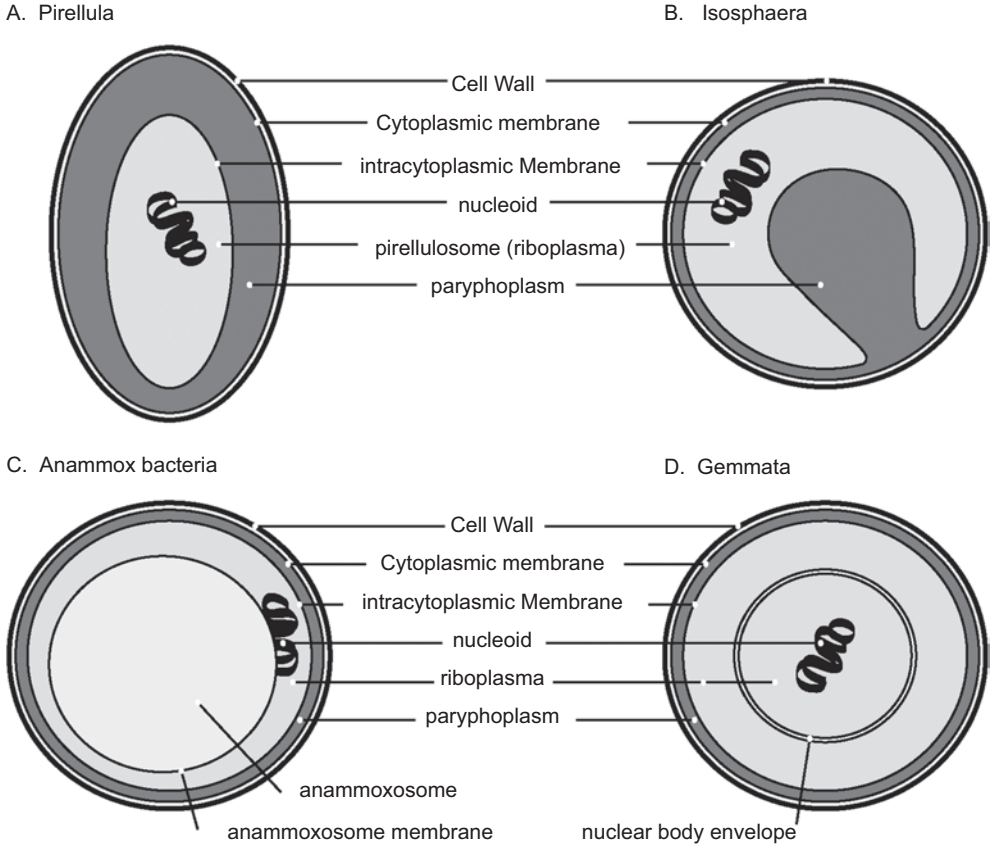

Figure 1. Cell plan of Planctomycetes including anammox bacteria. Adapted from Lindsay *et al.* (2001).

materials (glycogen granules); and (3) the inner ribosome-free compartment, the anammoxosome, bounded by the anammoxosome membrane and comprising 50–70% of the total cell volume. The membrane bounding this compartment is often highly curved, possibly to increase the surface to volume ratio.

Apart from whether the anammoxosome compartment has a specific cellular function, it can be questioned whether this compartment is a true separate compartment, i.e. that there are no membrane-links between the anammoxosome membrane and the intracytoplasmic membrane. Detailed studies using transmission electron microscopy and electron tomography (van Niftrik *et al.*, 2008a; 2008b) never revealed clear membrane-links between the anammoxosome and intracytoplasmic membrane. Also, the anammoxosome compartment was vertically inherited to the daughter cells upon cell division. Cytochrome peroxidase staining showed that cytochrome *c* proteins were located inside the anammoxosome. In conclusion, these results are supportive of the anammoxosome being a separate membrane-bounded compartment.

Unique ladderane lipids

Like in all other living organisms, the membranes of anammox bacteria are composed of glycerolipid bilayers. Glycerol moieties are linked to fatty acid residues both via ester bindings (typical of the bacteria and eukarya) and via ether bindings (typical of the archaea). The membrane lipid composition of anammox bacteria has been studied using gas chromatography mass spectrometry (GC-MS) and high field nuclear magnetic resonance (NMR) spectrometry (Sinninghe Damsté *et al.*, 2002; 2004). Extraordinary lipid structures were discovered which comprised hydrocarbon chains with 3 or 5 linearly concatenated cyclobutane rings, designated [3]- and [5]-ladderanes respectively, with the [3]-ladderanes condensed to a cyclohexane ring (Figure 2). These ladderane structures are either esterified or etherified to a glycerol backbone via an alkyl chain. The concatenated cyclobutane ring systems are unique in nature. To elucidate the full structure of the intact ladderane lipid species, the intact phospholipids were analyzed by high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Boumann *et al.*, 2006). It was demonstrated that phosphocholine (PC) and phosphoethanolamine (PE) are the major headgroup moieties of ladderane lipids and that the molecular diversity of the anammox lipid species is predominantly accomplished by the wide variety of hydrocarbon tails at the *sn*–1 position of the glycerol backbone (Figure 2). Cell fractionation shows that the anammoxosome membrane is particularly enriched with ladderanes (Sinninghe Damsté *et al.*, 2002).

Molecular modeling indicated that the ladderane lipids surrounding the anammoxosome are tightly packed (Sinninghe Damsté *et al.*, 2002). The unusual density makes them impermeable for apolar compounds, like fluorophores, that readily pass through common membranes. Since anammox metabolism involves formation of gaseous intermediates, notably nitric oxide (NO) and hydrazine (see below), proton leakage and the loss of intermediates could easily become detrimental. By their tightly packed nature, the ladderane lipids might minimize the losses.

Recently, intact ladderane phospholipids and core lipids were studied in different species of anammox bacteria, representing four of the five known genera

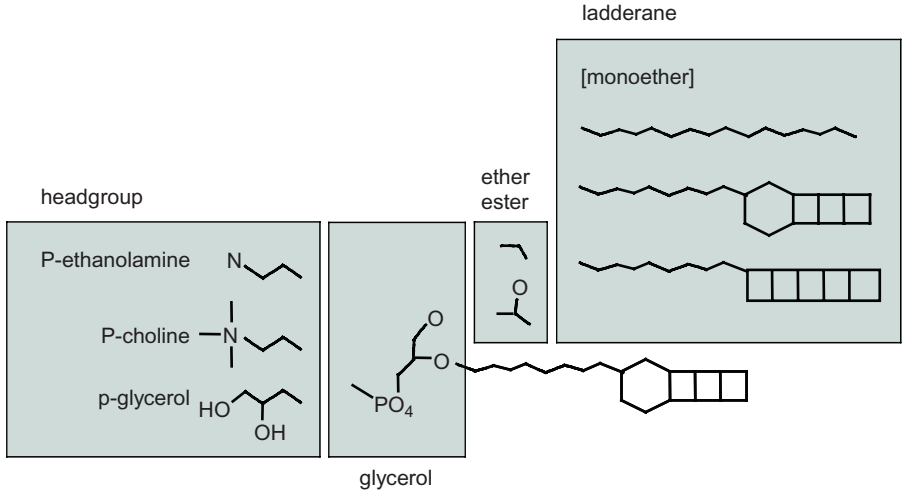

Figure 2. Composition of the unique ladderane lipids from anammox bacteria based on literature (Sinninghe Damsté *et al.*, 2002; Boumann *et al.*, 2006; Rattray *et al.*, 2008).

(Rattray *et al.*, 2008). Each species was shown to contain C18 and C20 ladderane fatty acids with either three or five linearly condensed cyclobutane rings and a ladderane monoether containing a C20 alkyl moiety with three cyclobutane rings. The broad distribution of ladderane lipids among anammox species is consistent with their putative physiological role to provide a dense membrane around the anammoxosome. In contrast to the core lipids, large variations were observed in the distribution of ladderane phospholipids, i.e. different combinations of hydrophobic tail types attached to the glycerol backbone sn-1 position, in combination with different types of polar attached to the sn-3 position. The fact that intact ladderane lipids made up a high percentage of the lipid content of *Kuenenia stuttgartiensis*, suggests that ladderane lipids are also present in membranes other than the anammoxosome. Finally, all four investigated species contained a C27 hopanoid ketone and bacteriohopanetetrol. This supports the finding that hopanoids are anaerobically synthesized by anammox bacteria (Damsté *et al.*, 2004). Their stable isotope composition and uniqueness makes ladderanes perfect proxies for past anammox activity (see below).

The anammoxosome and energy metabolism

The hypothesis that the anammox reaction takes place inside the anammoxosome compartment (with concomitant build up of a proton motive force) was based on the immunogold localization of hydrazine/hydroxylamine oxidoreductase (HAO), one of the key enzymes of this process (Figure 3; van Niftrik, 2008). The HAO antiserum used was raised against a purified HAO-like enzyme from "*Candidatus* Brocadia anammoxidans" (Schalk *et al.*, 2000). The results obtained in two independent studies left no doubt that the anammoxosome contains HAO (Lindsay *et al.*, 2001; van Niftrik *et al.*, 2008b). However, the question remains whether this protein acts together with proteins of the electron transport chain to actively translocate protons across the anammoxosome membrane to the anammoxosome. Protons could then flow back to the riboplasm along the proton gradient through ATPases, which convert the energy stored in this gradient to ATP. To address this point, immunolocalization of the anammox ATPases was initiated. The genome was analyzed for ATPase gene clusters, four putative clusters were found, and parts of the catalytic subunits were expressed in *Escherichia coli*, in order to be used in antibody production. The resulting four antisera were used in Western blot analysis, immunofluorescence and immunogold localization pilot experiments but methodological caveats were encountered that still need to be overcome. As a consequence, the location of the anammox ATPases still remains to be determined. However, other aspects of this research presented additional evidence for the postulated hypothesis. The cytochrome peroxidase staining (see above, van Niftrik *et al.*, 2008a) could only detect cytochrome *c* proteins associated with the anammoxosome, especially along a 150-nm rim on the inside of the anammoxosome membrane. This indicates that anammox cytochromes *c* proteins are predominantly located in this area, which is in good accordance with the hypothesis that the cytochromes involved in the electron transport chain are located on the inside of the anammoxosome membrane. Another indication that the anammoxosome is indeed used for energy metabolism is the highly folded anammoxosome membrane. This curvature may be used to increase the

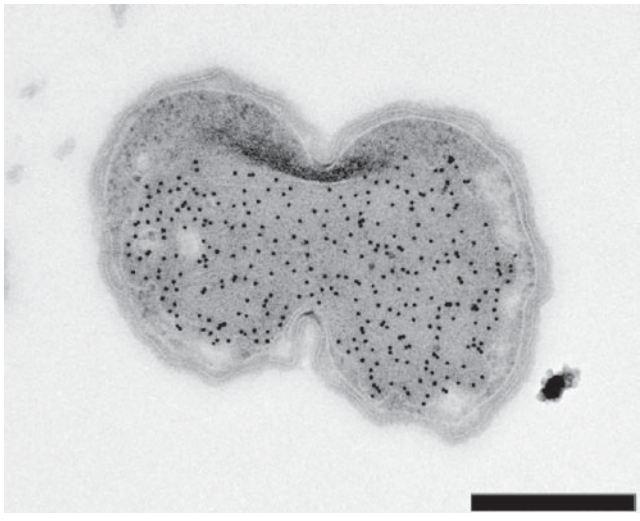

Figure 3. Electron micrograph showing the immunogold localization of hydrazine/hydroxylamine oxidoreductase (black dots) to the anammoxosome compartment in the anammox bacterium "*Candidatus* Kuenenia stuttgartiensis". Scale bar, 500 nm.

membrane surface available for the enzymes involved in catabolism, as holds for the mitochondrial inner membrane (cristae). In conclusion, the results obtained thus far support the hypothesis that the anammoxosome compartment is used for energy generation.

The anammoxosome and growth

The combination of slow growth and respiration makes an interesting case. Mitchell's chemiosmotic theory predicts a lower limit for the respiratory rate: as soon as the rate of respiratory proton extrusion drops below the rate of uncoupled proton influx (due to membrane leakiness), energy can no longer be conserved via respiration. Bacteria can limit proton leakiness to a certain extent, by adjusting membrane composition and width. Still, calculations using the data from these papers show that in some natural ecosystems the activity of anammox bacteria is impossible to explain with standard chemiosmotic theory. These bacteria must have a strategy to overcome this problem, and this strategy might depend on the presence of intracytoplasmic compartments.

In case of the nitrifiers, it is assumed that the large membrane surface area serves to accommodate more respiratory proteins (the specific activity of the proteins involved might be low). More membrane surface area could then lead to a higher maximum growth rate (as has been argued for methanotrophs). However, this hypothesis alone cannot explain the internal membrane systems of the nitrifiers. For if a large surface area was all that was needed, then why are there no filamentous nitrifiers? One might argue that the costs of cell wall biosynthesis would be prohibitive for these autotrophs. On the other hand, a filamentous morphology would lead to a higher substrate affinity, compared to the internal membranes where substrates need to travel longer by diffusion before they are converted. The internal organization of the membrane surface area might also be explained as follows: A large continuous, external membrane surface would lead to a large uncoupled influx of protons (due to membrane leakiness). This is no problem as long as enough substrates are available to make good use of all that membrane surface area. But once the substrates become limiting, the large surface area would make energy conservation impossible; extruded protons would diffuse back into the cells through the membrane and energy would no longer be conserved.

The nitrifier internal membrane systems could overcome this problem. The component lamellae appear to be organized in distinct vesicles – that is, the different lamellae are completely separate systems. Thus, *Nitrosococcus oceanus* might adjust its activated membrane surface area to the availability of substrate. When plenty of substrate is available all membrane surface area would be activated. When substrate limitation sets in, only one of the internal membranes would be activated and the bacterium would still be capable of energy conservation. Thus, the organization of the internal membranes could enable these bacteria to cope with a large range of growth rates – from very slow in times of need to moderately fast in times of plenty.

Because anammox bacteria have only a limited internal membrane surface area, one would not expect this kind of versatility in this case – and neither was it found: the maximum activity of anammox bacteria is low compared to nitrifiers. In the anammox case, there is no large internal membrane surface area and neither is the internal volume very large (as has been found for *Thioploca* or *Thiomargarita*). Thus, the anammox compartment does not seem to be designed to boost substrate turnover nor to store large amounts of substrate. Still, since the compartment is the location of the key catabolic enzymes, it most likely has a role in catabolism.

One might argue that the function of the anammoxosome would be to internalize the hydrazine pool and so limit hydrazine losses. However, comparison of time constants for diffusion and conversion inside the anammoxosome shows that the turnover of hydrazine is too slow to prevent disappearance of hydrazine out of the compartment by diffusion. Thus, this strategy would not be advantageous to the cells.

So what could be the advantage of the internal membrane/compartment in the anammox case? One feature of respiration over internal membranes has not been considered yet: to start up respiration (to activate the membrane) fewer protons need to be translocated, because: (1) although curved, the internal membrane surface area is smaller; and (2) the internal volume is much smaller than the external medium. Together this would lead to a closer contact of opposing charges inside the anammoxosome and a larger potential energy per proton translocated.

The following speculation is meant to stimulate the discussion on how this effect could be used to make respiration possible at extremely low growth rates – for example a doubling time of half a year. For most organisms such growth rates would only occur under extreme substrate limitation. But for anammox this is only 10 times slower than the maximum growth rate. We envisage a respiration process that is characterized by a sequence of two alternating phases. In the first phase of such a respiratory cycle, all substrates present in the cells are consumed quickly, at a rate that exceeds the diffusion of fresh substrates from the bulk liquid (or immediate surroundings) back into the cells. Protons are translocated, the proton motive force is generated, the internal membrane is activated and some ATP is generated. After all substrates are depleted, the proton motive force dissipates. In the second phase, the respiratory

enzymes are silent and fresh substrates diffuse back into the cells without being converted. When the substrate levels inside the compartment are restored, the next cycle starts. The advantage of the anammoxosome in this scheme is the following: in each cycle, some energy is wasted in the activation of the membrane. By making use of the internal compartment, this loss is minimized and this would increase the energy efficiency of the bacteria in this dynamic scheme. Thus, internal compartments might make extremely slow growth possible.

Genomics of anammox bacteria

In 2006 the genome sequence of *Kuenenia stuttgartiensis* was published, being the first one from an anammox bacterium (Strous *et al.*, 2006). The assembly was performed from a metagenome obtained from a complex microbial community grown in an SBR in which *K. stuttgartiensis* made up 74% of the microbial population. Ultimately, five supercontigs (4.2 Mb total) could be assembled. The five remaining gaps could not be closed and the size of these gaps remains unknown. However, the near completeness and correct assembly of the *K. stuttgartiensis* genome was confirmed by the lack of any suspicious redundancy or missing essential genes in major biosynthetic pathways, DNA replication, transcription, translation and protein translocation. Except from leucyl-tRNA synthetase, the 64 clusters of orthologous groups of proteins (COGs) present in all currently sequenced bacterial genomes represented in the STRING database appeared to be present and from this the genome was estimated to be more than 98% complete.

Carbon dioxide fixation and respiration

Among planctomycetes, *K. stuttgartiensis* is the only known chemolithoautotroph; therefore it cannot be predicted which pathway for carbon fixation it would use, although some indications were obtained from ^{13}C-carbon analyses (Schouten *et al.*, 2004). The genome of *K. stuttgartiensis* codes for a complete acetyl-CoA (Wood-Ljungdahl) pathway, while all other known carbon fixation pathways are either missing or incomplete (Strous *et al.*, 2006). In the Wood-Ljundahl route two molecules of CO_2 are reduced and bound to coenzyme A (HS-CoA) to form acetyl-CoA. Use of the acetyl-CoA pathway is indeed consistent with the strongly depleted carbon found experimentally in ladderane lipids of anammox bacteria (Schouten *et al.*, 2004), with the activity of two key enzymes (formate dehydrogenase and carbon monoxide dehydrogenase) in cell-free extracts (Strous *et al.*, 2006) and with the one-carbon metabolism described in related Planctomycetes (Bauer *et al.*, 2004; Chistoserdova *et al.*, 2004). However, like in acetogens, one-carbon metabolism in *K. stuttgartiensis* appears to involve folate, not methanopterin; thus, the genes are completely different from those encoding methanopterin-dependent one-carbon metabolizing proteins found in related Planctomycetes (Chistoserdova *et al.*, 2004). Acetyl-CoA is the substrate for all cell constituents starting with the gluconeogenesis/glycolysis route and the tricarboxylic acid cycle as intermediary pathways. All genes of the pathways are found in the genome, except for a gene coding ATP citrate lyase. The apparent absence of this enzyme might indicate that the citric acid cycle was preferably used for amino acid metabolism (Huynen *et al.*, 1999).

The reducing equivalents required for CO_2 reduction are derived from the oxidation of reduced quinone or NADH. Clusters coding for NADH:ubiquinone oxidoreductase (complex I), a sodium-translocating NADH:quinone oxidoreductase and a formate:quinone oxidoreductase complex were identified in the genome and this complies with such electron transfer.

Versatile lifestyle

The genome data from *K. stuttgartiensis* are also supportive of the recently discovered versatile lifestyle of anammox bacteria (see above; Kartal *et al.*, 2007b; 2008). The central carbon metabolism was completely reversible, and transporters for organic acids and amino acids were identified. Furthermore, respiration is highly redundant in the *K. stuttgartiensis* genome: at least 200 genes were predicted to be directly involved in respiration (Strous *et al.*, 2006). So far, a comparable level of redundancy has only been observed for versatile heterotrophic bacteria such as *Geobacter sulfurreducens* and *Shewanella oneidensis* (Heidelberg *et al.*, 2002; Methe *et al.*, 2003), whereas the aerobic ammonia oxidizer *Nitrosomonas europaea* has only 50 such genes (Chain *et al.*, 2003). The finding of three gene clusters encoding complex III (cytochrome bc_1) and four gene clusters coding for ATP synthase complexes provides strong evidence that ATP is synthesized by a chemiosmotic mechanism.

The unique anammox metabolism

Anammox itself is the one-to-one combination of ammonium and nitrite into dinitrogen gas and the oxidation of part of the nitrite to nitrate to generate reducing equivalents for carbon dioxide fixation (Figure 4). For this metabolism, the following genes were identified in the *K. stuttgartiensis* genome: a nitrate::nitrite oxidoreductase (*nar*GH), a nitric oxide::nitrite oxidoreductase of the cd_1 type (*nir*S, Baker *et al.*, 1997) and nine divergent paralogues of hydroxylamine/hydrazine oxidoreductase (HAO/HZO, Hooper *et al.*, 1997). Recently, two articles described the evolution of N-cycle enzymes

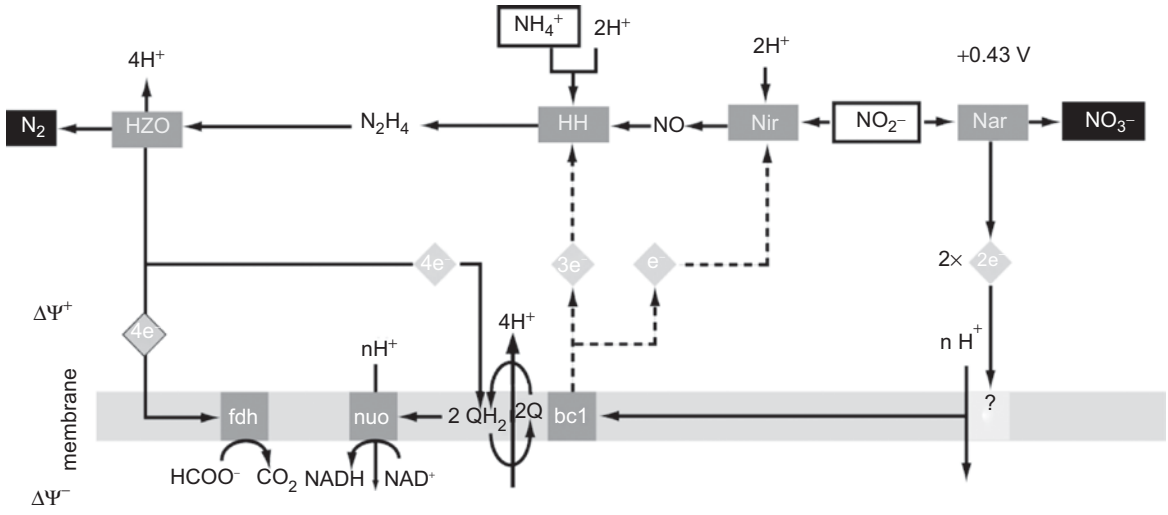

Figure 4. Hypothetical scheme showing a combination of the central catabolism of *K. stuttgartiensis* together with the nitrate reductase to generate low-redox-potential electrons for the acetyl-CoA pathway. Abbreviations: Nir, nitrite reductase; HH, hydrazine hydrolase; HZO, hydrazine dehydrogenase; Nar, nitrate reductase; Q, ubiquinone; fdh, formate dehydrogenase, nuo, NADH:ubiquinone oxidoreductase, Q(H_2), (reduced) ubiquinone; bc1, bc1-complex;. Symbols: light diamonds, cytochromes; dark diamond with a black rim, ferredoxin; solid arrows, reductions; dashed arrows, oxidations. $\Delta\Psi^+$ and $\Delta\Psi^-$ are thought to represent the anammoxosome and riboplasmic compartments, respectively.

in general and for HAO/HZO in more detail (Klotz and Stein, 2008; Klotz *et al.* 2008). Comparison of the cyclic electron flow in aerobic and anaerobic ammonium oxidizers (Strous *et al.*, 2006) revealed a striking similarity and a central positioning of HAO/HZO proteins. These HAO/HZO proteins are assumed to be functional analogs as both oxidize hydroxylamine and hydrazine, and deliver the released electrons to ubiquinol via cytochrome *c* proteins. In such a case, HAO and HZO may be interchangeable, and define the hydroxylamine/hydrazine-ubiquinol redox module (HURM) of both aerobic and anaerobic ammonium oxidizers. According to this hypothesis, anammox bacteria most likely made possible the first complete recycling of fixed nitrogen to the dinitrogen pool and fulfilled this role until the emergence of copper enzymes (Klotz and Stein, 2008).

The capability for the oxidation of nitrite to nitrate (and vice versa) mediated by NarGH is consistent with previous experimental evidence (van de Graaf *et al.*, 1997; Schalk *et al.*, 2000; Güven *et al.*, 2005; Kartal *et al.*, 2007a). The presence of NirS is surprising because nitric oxide was previously not recognized to be an intermediate of anammox nitrogen metabolism. The nitrate (NarGH) and nitrite (NirS) reductases are also known as the first steps of conventional denitrification. However, the genes coding for nitric oxide reductase and nitrous oxide reductase are absent in the anammox genome assembly, making it very unlikely that *K. stuttgartiensis* is capable of complete conventional denitrification; it is more likely that nitric oxide is an intermediate of the anammox pathway itself.

So far two reaction steps were still missing. First, the critical step in which ammonium is combined with nitric oxide and a nitrogen–nitrogen bond is forged to yield hydrazine ("hydrazine hydrolase") and second, the reduction of nitrite to ammonium. The latter reaction is performed by anammox bacteria under stress conditions (Strous and Jetten, 2004) and is also necessary to explain the experimentally verified capability to produce dinitrogen gas from nitrate in the absence of ammonium and with organic acids as the sole source of electrons (Güven *et al.*, 2005; Kartal *et al.*, 2007a). Without denitrification, anammox bacteria could still produce dinitrogen gas from nitrate by first reducing half of the nitrate all the way to ammonium and then proceed with the anammox pathway as usual. This six electron reduction of nitrite to ammonium is well known and normally catalyzed by dissimilatory nitrite reductase (NrfA), a pentaheme cytochrome *c*, which forms a dimer (Simon, 2002). However, no obvious orthologue for NrfA, containing the CxxCK motif, is present in the genome. Candidate genes for these two missing steps were identified by focusing on domains of interest rather than on genes without orthologues in the databases (Strous *et al.*, 2006). Special emphasis was given to heme and iron sulfur cluster binding sites because the enzyme complexes mediating these steps should at least have the potential to accept and donate electrons (three electrons for hydrazine hydrolase and six for dissimilatory nitrite reductase). Most of the genes which contained such domains coded for small proteins presumably involved in electron transfer only and not in catalysis. Interestingly, the main genetic novelty, genes which contained new combinations of domains involved in both electron transfer and catalysis, could be identified in just three operons. One of these operons

codes for a candidate complex consisting of two pentaheme and one decaheme cytochrome *c* proteins. Although these genes are not orthologous to a dissimilatory nitrite reductase (NrfA), the presence of five or 10 heme-c binding sites still makes this complex the most likely candidate to code for a functional homologue of NrfA in *K. stuttgartiensis*.

The other two complexes are the most likely candidates for the exciting role of hydrazine hydrolase (HH). The first candidate operon suggests that the biological formation of hydrazine from ammonium and nitric oxide is catalyzed by a beta-propellor protein (such as nitrous oxide reductase) with the aid of a quino-cofactor (which is also used in methylamine oxidation by methylotrophic bacteria). The second candidate operon identified suggested a role for a new multicopper oxidase (such as the nitrite reductase, NirK), a flavin containing amine oxidase and several integral membrane proteins.

Summarizing, the *K. stuttgartiensis* genome assembled form the SBR metagenome allowed an *in silico* reconstruction of the anammox metabolism and identification of the genes likely to be involved. Although biochemical alternatives may be possible, the depicted pathway is the only one consistent with the available experimental data, thermodynamically and biochemically feasible, and consistent with Ockham's razor: it invokes minimum biochemical novelty and requires the fewest number of biochemical reactions. Currently, the founded and detailed hypothesis is being experimentally validated (see also below).

Ladderane lipid biosynthesis

Ladderanes and their derivatives are of fundamental interest to physical organic chemists due to their ring strain and electronic properties (Nouri and Tantillo, 2006). Chemical synthesis studies underscore the extraordinary nature of the biosynthesis (Mascitti and Corey, 2004; 2006a; 2006b). The mode of ladderane biosynthesis is totally unknown and certain to be unprecedented because of the structural novelty and high ring strain (Macitti and Corey, 2004). The genome of *K. stuttgartiensis* was shown to code the putative pathways for the biosynthesis of fatty acids, squalene, hopanoids and ubiquinones (Strous *et al.*, 2006), consistent with the existing experimental evidence (Damsté *et al.*, 2004). Fatty acid biosynthesis is represented by four operons, one of which consists of a conspicuous combination of homologues of known fatty acid biosynthesis genes, S-adenosyl methionine (SAM) methylases, putative radical SAM enzymes and a gene similar to phenylacetate-coenzyme A ligase. Radical SAM enzymes seem to be frequently reserved for the most difficult chemical reactions (Sofia *et al.*, 2001; Layer *et al.*, 2004). Interestingly, the genome of *K. stuttgartiensis* encodes 62 putative radical SAM enzymes, far more than in any genome sequenced so far. Apparently this anammox bacterium has extensive capabilities for the biosynthesis of presently unknown organic molecules. Although the exact pathway could not be immediately inferred, it seems to involve methylation, cyclization via oxidative radical chemistry, and addition of an aromatic residue, combined with regular fatty acid elongation.

Cell biology and protein sorting

The biogenesis of the membrane-bound anammoxosome compartment would require the bacteria to selectively translocate some proteins into the anammoxosome and others across the cytoplasmic membrane. The same would apply to the correct insertion of membrane proteins. The redundancy in respiratory functions (see above), as well as the presence of multiple paralogues for putative transporters of ammonia, nitrite and nitrate would make it possible to selectively translocate proteins in different directions. Multiple paralogues could have different signal peptides used for protein sorting. An attempt was made to discover a pattern in the N-terminal and C-terminal amino acid sequences of these paralogues, but no significant pattern could be found (Strous *et al.*, 2006). This was caused by (1) the large redundancy (i.e. nine different copies of hydroxylamine oxidoreductase, at least one of which was known to be present inside the anammoxosome (Figure 3); (2) the general lack of conservation in protein sorting signals; and (3) the difficulty of predicting the correct start codon.

For protein translocation, the *K. stuttgartiensis* genome encodes both the complete, non-redundant general apparatus (Sec, including the signal recognition particle and receptor) and the twin arginine pathway (TAT) for the translocation of folded proteins. *K. stuttgartiensis* would need the latter for the translocation of iron-sulfur and molybdopterin enzymes such as nitrate reductase, which contain conventional TAT signals (Berks *et al.*, 2003). Bacterial protein secretion systems I to V were shown to be absent. In eukaryotes, the subcellullar targeting of proteins is mediated by specific N-terminal and C-terminal signal sequences recognized by tetratricopeptide repeat (TPR) proteins (D'Andrea and Regan, 2003). Interestingly, TPR proteins are conspicuously frequent in the *K. stuttgartiensis* genome. While TPR domains are ubiquitous among higher eukaryotes, bacterial genomes generally only have few (less than five) genes which encode these domains. In contrast, 90 genes with multiple (1–12) TPR domains were identified in the *K. stuttgartiensis* genome, far more than in any bacterial genome sequenced so far. Eight of these genes comprise a family of integral membrane proteins with seven TPR domains. In the absence of clear homologues

to complete targeting systems, the mechanism of anammoxosomal protein targeting remains unresolved. However, based on the present data, it is most likely to involve TPR proteins, which could function in signal recognition and as membrane receptors.

Biochemistry and bio-energetics of the anammox process

From the biochemical point of view the mechanism of the anammox process, in particular the way the inert ammonium molecule is handled and the way ATP is conserved, is very intriguing. The hypothesized anammox pathway (Strous *et al.*, 2006) comprises a minimum set of three redox reactions: (1) the one-electron reduction of nitrite to NO (Table 2, Equation (3)); (2) the condensation of NO and ammonia with the input of three electrons yielding hydrazine (Table 2, Equation (4)); and (3) the four-electron oxidation of hydrazine to produce dinitrogen gas (Table 2, Equation (5)).

Preliminary biochemical support came from experiments with ^{15}N-labeled substrates (ammonium, nitrite and nitrate) (van de Graaf *et al.*, 1997). When incubations with anammox bacteria were amended with hydroxylamine (NH_2OH), a transient accumulation of a nitrogenous compound identified as hydrazine (N_2H_4) was observed. Hydrazine is one of the most powerful reductants in nature and its synthesis is so far unique to anammox bacteria. At first, both hydroxylamine and hydrazine were postulated to be intermediates in the anammox process (van de Graaf *et al.*, 1997). The effect of hydroxylamine addition on the hydrazine metabolism of anammox bacteria was studied both experimentally and by mathematical modeling (van der Star *et al.*, 2008a). It was observed that hydroxylamine was disproportionated biologically in the absence of nitrite into dinitrogen gas and ammonium. Little hydrazine accumulated during this process. However, rapid hydrazine production was observed when nearly all hydroxylamine was consumed. A mechanistic model was proposed in which hydrazine was suggested to be continuously produced from ammonium and hydroxylamine (possibly via nitric oxide) and subsequently oxidized to N_2. The electron acceptor for hydrazine oxidation was hydroxylamine, which is reduced to ammonium. A decrease in the hydroxylamine reduction rate, therefore, led to a decrease in the hydrazine oxidation rate, resulting in the observed hydrazine accumulation.

From the *K. stuttgartiensis* genome data, however, an intermediary role of nitric oxide (NO) rather than hydroxylamine seems more likely (Strous *et al.*, 2006). The genome apparently lacks a nitrite:hydroxlamine reductase, instead cd_1 nitrite:nitric oxide oxidoreductase (NirS) is present. However, recently it was shown that the HAO of aerobic ammonium-oxidizing bacteria could convert NO via hydroxylamine into ammonium using reduced methylviologen as electron donor (Kostera *et al.*, 2008). It remains to be established if this is a physiological relevant reaction.

A hydrazine dehydrogenase/oxidase (HZO), which catalyzes the oxidation of hydrazine with cytochrome *c*, has been purified from the anammox strain KSU-1 (Shimamura *et al.*, 2007). This enzyme has oxidizing activity toward hydrazine but not toward hydroxylamine. The dimeric octaheme protein with high specific activity and high substrate affinity is abundantly present in the cells. Two copies of homologous protein are encoded on the genome of *K. stuttgartiensis*. It is not yet verified whether the enzyme reaction proceeds according to Equation (5) (Table 2). Also from this anammox bacterium a hydroxylamine oxidoreductase (HAO) was purified which showed opposite characteristics: high oxidizing activity towards hydroxylamine and low activity towards hydrazine (Shimamura *et al.*, 2008). These enzymatic properties were similar to those of the HAO purified from "*Candidatus* Brocadia anammoxidans" (Schalk *et al.*, 2000). The strain KSU-1 *hao* gene exists upstream of the *hzo*B gene, which codes for the HZO. The *hao* gene sequence showed 87% identity with a polypeptide encoded by an open reading frame (kustc1061) in the genome of *K. stuttgartiensis*. These findings suggest that both HZO and HAO are indispensable enzymes and well conserved in anammox bacteria.

The presence of a dissimilatory nitrite reductase (NrfA) was experimentally validated by partially purifying a calcium-dependent nitrite reductase from cell-free extracts of "*Candidatus* Brocadia anammoxidans" that contained high (400–500 nmol min^{-1} mg $protein^{-1}$) nitrite reductase activity (Kartal *et al.*, 2007a). Ultimately a 25 kDa multiheme protein with a very high rate of nitrite reduction to ammonium (305 μmol min^{-1} mg $protein^{-1}$) was recovered. The native enzyme seems to occur as a homodimer. Sodium azide and the copper chelating agent diethyldithiocarbamate (DDC) did not inhibit the enzyme at 1 mM, while 1 mM KCN inhibited the enzyme by more than 95%. The most active fractions converted NO_2^-, NO and NH_2OH at high rates, and in all cases the end-product of the reaction was ammonium. Ca^{2+} could not be replaced by other divalent ions. As stated above when the genome assembly of *K. stuttgartiensis* was analyzed for multiheme proteins, the most likely candidate gene which could code for this calcium-dependent nitrite reductase was identified as kustc0392, encoding an unusual 25.2 kDa multiheme protein located in a gene cluster with other multiheme protein encoding genes (kustc0393, kustc0394) and a cytochrome *b* gene (kustc0395).

Finally, the purification of the "hydrazine hydrolase" (HH), that mediates hydrazine synthesis (Table 2, Equation (4)) will make it possible to prove that the postulated gene cluster (see above; Strous *et al.*, 2006) is really encoding a HH.

Anammox bacteria are believed to conserve the energy derived from the conversion of ammonium and nitrite by a chemiosmotic mechanism. This means that the electrons derived from hydrazine oxidation are transferred via ubiquinone to the cytochrome bc1 complex (complex III). The bc1 complex shuttles the electrons towards nitrite reduction and hydrazine synthesis. Coupled to this electron transfer, protons are translocated across a membrane system, thus creating a proton motive force. Intermediary electron transfer would be accomplished by a set of cytochrome *c*-type proteins (Cirpus *et al.*, 2005; Huston *et al.*, 2007).

During growth, part of the nitrite is oxidized to nitrate which is hypothesized to generate the electrons for CO_2 fixation. The acetyl-CoA pathway depends on electrons at very low redox potential for NAD^+ reduction (–0.32 V), CO_2 reduction to formate (–0.44 V) and acetyl-CoA synthesis (–0.5 V). In most cases these electrons are derived from the oxidation of molecular hydrogen (Drake and Daniel, 2004). However, anammox bacteria derive their electrons from the anaerobic oxidation of nitrite to nitrate (+0.43 V), making use of the acetyl-CoA pathway a challenge. The genomic data allowed the deduction of a biochemical pathway that explains how the acetyl-CoA pathway can be reconciled with nitrite as electron donor for carbon fixation (Figure 4; Strous *et al.*, 2006). In this model, part of the exceptional high reducing power electrons derived from hydrazine oxidation are channeled towards NAD^+ and CO_2 reduction to sustain carbon fixation. The replenishment of the hydrazine pool to compensate for the hydrazine invested in carbon fixation requires no additional enzymes except reverse electron transport. The nitrite oxidation is likely to be catalyzed by a nitrate oxidoreductase NarGH which is present in the *K. stuttgartiensis* genome (Strous *et al.*, 2006). Remarkably, the *nar* gene cluster seems to lack the gene (*narI*) coding for a ubiquinone-binding subunit. Instead six genes are present encoding cytochrome *c*-type proteins, which might facilitate the electron transport.

Many details with respect to anammox biochemistry and bioenergetics remain to be verified experimentally, including hard evidence for the postulated intermediates, the purification and characterization of the key metabolic enzymes and respiratory complexes involved in the electron transfer processes. In addition, the cellular localization (anammoxosome) and membrane orientation of the proton-motive processes have to be resolved.

Ecology and environmental importance of anammox bacteria

Detection of anammox bacteria in the environment

A range of suitable methods is available for the detection of anammox bacteria and their activity in natural and man-made ecosystems (Risgaard-Petersen *et al.*, 2003; Schmid *et al.*, 2005). In environmental samples, PCR amplification with general 16S rRNA gene-targeted primers and subsequent phylogenetic analysis of the product is commonly used to detect previously undescribed organisms. However, anammox bacteria may be underrepresented in general 16S rRNA gene clone libraries since the widely used "universal" primer set for 16S rRNA gene amplification has several mismatches. The use of a more specific primer, i.e. Pla46F (a Planctomycete-specific forward primer) or amx386F (an anammox specific primer) together with a general eubacterial reverse primer or a specific anammox reverse primer (i.e. amx820R) may increase relative amounts of Planctomycete or anammox 16S rRNA gene sequences (Schmid *et al.*, 2000; 2007; Penton *et al.*, 2006). Recently, a more functional PCR approach using primers amplifying anammox genes encoding hydroxylamine/hydrazine oxidoreductase (HAO/HZO) proteins showed that these genes are suitable targets for molecular ecological studies on both aerobic and anaerobic ammonium-oxidizing bacteria (Quan *et al.*, 2008; Schmid *et al.*, 2008). For a proper evaluation of the contribution of the anammox process to nitrogen cycling in a particular habitat, the combination of different (rRNA and non-rRNA) methods is necessary. Fluorescence *in situ* hybridization (FISH) is an excellent tool to collect both qualitative and quantitative data of anammox bacteria in environmental samples. It can also be used to validate the findings of clone libraries. Probe design will improve as more validated anammox sequences become available, also including data from metagenome projects. In addition to FISH, a 16S rRNA gene-based real time PCR method was developed for quantification of anammox bacteria (Tsushima *et al.*, 2007a). FISH-MAR and ISR probing are advanced techniques that allow the measurement of activity and growth at the single-cell level (Schmid *et al.*, 2001). Raman-FISH combines stable-isotope Raman spectroscopy and FISH for the single cell analysis of identity and function (Huang *et al.*, 2007). Confocal Raman microscopy (CRM) was introduced as a new non-invasive technique to determine the distribution of different microorganisms and other substances inside physiological intact microbial communities (Pätzold *et al.*, 2006). Anammox bacteria were identified without pretreating the samples just by its Raman vibrational signature. Using the resonance Raman effect of cytochrome *c* the microbial distribution of nitrifiers and

anammox bacteria in microbial aggregates obtained from biological wastewater treatment was recorded (Pätzold *et al.*, 2008). Based on a reference database of bacteria assumed to be found, the grouping of bacteria down to strain level was possible.

Tracer experiments with ^{15}N-labeled ammonium and nitrite are commonly used for the detection of anammox activity (Risgaard-Petersen *et al.*, 2003). Under anoxic conditions, ^{15}N-labeled ammonium reacts uniquely, in a 1:1 ratio with unlabeled ^{14}N-nitrite to $^{29}N_2$ ($^{14}N^{15}N$) via the anammox reaction. A lot of effort was put into development and use of this method by several research groups (e.g. see Dalsgaard *et al.*, 2005). The ^{15}N isotope technique can also be combined with the addition of inhibitors. Differential effects of acetylene and methanol on anammox and denitrification helped to elucidate the contribution of these main pathways of N_2 production in marine sediments (Jensen *et al.*, 2007). However, it should be kept in mind that inhibition studies on complex ecosystems such as marine sediments have to be interpreted with care. Compounds that inhibit one group of microorganisms under the given conditions could enhance the activity of other microorganisms.

In addition, very sensitive biosensors for online nitrite monitoring have become available for the sensitive detection of anammox activity in reactor systems or sediments (Nielsen *et al.*, 2005; Kindaichi *et al.*, 2007). Although the conversion of hydroxylamine to hydrazine is a unique feature of anammox bacteria, this assay requires rather high anammox cell numbers.

The unique ladderane lipids of anammox bacteria (see above) can also be used as biomarkers. Lipids from anammox bacteria are characterized by substantially lower ^{13}C content than their carbon source (Schouten *et al.*, 2004). Because the ^{13}C content of anammox ladderanes is approximately 45‰ depleted compared to their carbon source, the isotopic composition of anammox lipids in environmental samples can thus be an additional confirmation of their origin. Lipids from other autotrophic organisms generally are 20 to 30‰ depleted (Rattray *et al.*, 2008). The ladderane lipids are also applied as proxies for past anammox activity (Jaeschke *et al.*, 2008). Analyses of the distribution of fossil ladderane lipids in a sediment core from the northern Arabian Sea revealed concentrations of ladderane lipids between 0.3 and 5.3 ng g sediment^{-1} during the past 140 kyr, suggesting that the anammox process constituted an important sink for fixed inorganic nitrogen in the Arabian Sea over the last glacial cycle.

Anammox in the marine ecosystem

Inorganic nitrogen is one of the key nutrients in marine waters that may limit primary productivity. Ammonium can be assimilated, but may also be used as an energy source when oxidized first to nitrite and then to nitrate in the process of nitrification. The nitrite and nitrate can subsequently be reduced to nitrogen gas in the suboxic zone by anaerobic ammonium oxidation (anammox) or denitrification (Kuypers *et al.*, 2003; 2005). At the moment we know very little of how and to what extent the different groups of nitrogen cycle bacteria contribute to the biogeochemical cycling of (marine) nitrogen. In 2003, the first direct evidence was provided for the presence of anammox bacteria in the world's largest anoxic basin, the Black Sea, supported by nutrient profiles, 16S rRNA gene clone libraries, fluorescence *in situ* hybridization, ^{15}N activity tests, and ladderane lipid analysis (Kuypers *et al.*, 2003). Since then follow-up studies have shown that anammox bacteria play a dominant role in the removal of fixed nitrogen in the Benguela and Peru upwelling systems, two of the world's most important primary production sites (Kuypers *et al.*, 2005; Thamdrup *et al.*, 2006; Hamersley *et al.*, 2007). The analysis of the vertical distribution of anammox activity (^{15}N-labeling) through the suboxic zone of the central Black Sea showed that anammox rates increased with depth through the upper suboxic zone and reached a maximum of similar at the sharp interface between nitrate and ammonium, below which rates decreased toward the depth of sulfide accumulation (Jensen *et al.*, 2008). Since heterotrophic denitrification was not detected, anammox was the prevailing sink for fixed nitrogen in this ecosystem. Also in the Benguela upwelling system anammox bacteria may even be the only sink for fixed nitrogen. Furthermore, the presence of anammox bacteria in marine and estuarine sediments was established (Risgaard-Petersen *et al.*, 2004; Rysgaard *et al.*, 2004; Meyer *et al.*, 2005; Tal *et al.*, 2005; Amano *et al.*, 2007; Hietanen, 2007; Schmid *et al.*, 2007; Rich *et al.*, 2008). Based on these studies, it is now estimated that anammox bacteria might contribute more than 50% to global, present day nitrogen losses from the oceans (Brandes *et al.*, 2007). Thus, anammox bacteria may represent a large but presently unexplored sink in the biogeochemical cycling of nitrogen in the ocean with large consequences for the past and present marine carbon cycle (Francis *et al.*, 2007; Brandes *et al.*, 2007). Recent findings indicate that both ammonium-oxidizing bacteria, such as *Nitrosococcus oceanii*, and ammonium-oxidizing Crenarchaea, such as *Nitrosopumilus maritimus*, contribute to marine ammonium oxidation (Lam *et al.*, 2007; Woebken *et al.*, 2008) in the Black Sea and Namibian OMZ and are likely coupled to anammox in indirect and direct manners, respectively. Based on ^{15}N incubations, expression studies and diffusion modeling, each process is assumed to supply about half of the nitrite required by anammox (Lam *et al.*, 2007). Because anammox bacteria contribute substantially to nitrogen loss in marine suboxic waters, such nitrification–anammox coupling would potentially also occur in oceanic oxygen minimum zones (OMZ)

and would act as a short circuit connecting regenerated ammonium to direct nitrogen loss. In this way canonical denitrification is bypassed (Capone and Knapp 2007), which until recently was believed to be the main sink for fixed inorganic nitrogen in the oceans. Hannig *et al.* (2007) demonstrated that dynamic changes in the N_2 producing processes may occur. After a massive inflow of oxygenated North Sea water, causing a complete ventilation of the Baltic Sea, and the reestablishment of the redoxcline, a shift from denitrification to anammox as the main N-loss process was observed.

So far the temperature range suitable for anammox bacteria has been estimated between –2°C (sea ice, Greenland) and 43°C (laboratory tests). A recent study investigated the role of anammox in deep sea hydrothermal vents (Byrne *et al.*, 2008). Samples were collected from five hydrothermal vent sites from the Mid-Atlantic Ridge at depths ranging from 750 to 3650 m. Evidence for the occurrence of anammox bacteria in these particular habitats was demonstrated by 16S rRNA gene analyses, ladderane lipid analysis and measurement of $^{29}N_2$ production in isotope-pairing experiments at 60 and 85°C.

Microbial interactions in the marine N-cycle

Presently it is calculated that anammox bacteria may contribute approximately 50% to the global loss of fixed nitrogen. This calculation is based on the assumption that the only source of ammonium for the anammox bacteria is the upward diffusion of ammonium derived from anaerobic mineralization in the sulfidic zone to the OMZ. Other possible sources of ammonium such as dissimilatory reduction of nitrate to ammonium (DNRA) are not well studied in the marine ecosystem. It was recently shown that DNRA could supply anammox bacteria with the necessary ammonium (Kartal *et al.*, 2007a). The source of sufficient nitrite and the competition for limiting amounts of nitrite is not clear. On one hand, in oxygenated parts of the ocean aerobic ammonium-oxidizing microbes may provide nitrite for anammox bacteria, while nitrite-oxidizing bacteria may compete for nitrite. Cooperation between anammox bacteria and aerobic ammonium- oxidizing bacteria has been confirmed in laboratory experiments (Third *et al.*, 2001; Schmidt *et al.*, 2002a; 2002b; Sliekers *et al.*, 2002; Vlaeminck *et al.*, 2007). In anoxic parts of the ocean, nitrate-reducing bacteria may produce nitrite for anammox bacteria under electron donor limitation, while denitrifying microbes will also compete for nitrite when sufficient electron donor is available.

Until recently, betaproteobacterial ammonium-oxidizing bacteria (*Nitrosomonas*, *Nitrosospira* or *Nitrosococcus*) were assumed to be responsible for marine nitrification, although their actual cell numbers are relatively low. However, some evidence has been provided that Crenarchaea, which can account for about 20% of all prokaryotic cells in the global ocean, may be involved in the process of nitrification and thus provide nitrite for anammox bacteria (Koenneke *et al.*, 2005; Wuchter *et al.*, 2006; Francis *et al.*, 2007; Prosser and Nicol, 2008). Two recent studies (Lam *et al.*, 2007; Woebken *et al.*, 2007) indicated that anammox bacteria and Crenarchaea may cooperate in oxygen-limited ecosystems. In the Black Sea anammox bacteria inhabit the aerobic–anaerobic interface, where both ammonium and nitrite can be found. Interestingly, nitrite is supplied by aerobic ammonium-oxidizing bacteria and Crenarchaea occupying different sub-oxic zones (Lam *et al.*, 2007). Half of the nitrite is estimated to be produced by gammaproteobacterial nitrifiers that occupy the zone where virtually no oxygen is measured, and the other half by Crenarchaea living in the zone with relatively higher oxygen concentrations.

The natural nitrite-converting microbial partners of the marine ammonium-oxidizing microbes have not yet been thoroughly established and may depend on local physiological conditions. For instance under low oxygen conditions anammox bacteria may be favored, while aerobic nitrite-oxidizing bacteria which convert nitrite to nitrate may be more important under higher oxygen conditions. The organisms which are responsible for the aerobic oxidation of nitrite in the process of marine nitrification remain largely unexplored. The existing knowledge is mostly based on molecular data and actively growing cultures of nitrite-oxidizing bacteria are scarce.

The response of anammox bacteria in the presence of denitrifying competitors to (surplus) carbon compounds in marine ecosystems is unexplored. Some fresh water anammox bacteria like *"Candidatus* Brocadia fulgida" or "*Candidatus* Anammoxoglobus propionicus" can effectively compete for acetate or propionate (see above), but it is not yet known how marine anammox bacteria respond to increased carbon availability. The enrichment cultures of "*Candidatus* Scalindua" species (van de Vossenberg *et al.*, 2008) are a very good starting point to investigate this competition by adding increasing amounts of acetate.

Anammox in freshwater and terrestrial ecosystems

The first direct evidence for the anammox process in a lacustrine system, Lake Tanganyika, the second largest lake in the world, was provided by Schubert *et al.* (2006). Incubations with ^{15}N-labeled nitrate showed that anammox occurred in the suboxic water layer at 100–110 m water depth. Laderane lipid biomarkers were found in filtered water from the same depths and FISH revealed up to 13,000 anammox bacteria per ml (1.4% of total counts). Phylogenetic analyses of 16S rRNA

genes indicated the presence of sequences most closely related to the known anammox bacterium "*Candidatus* Scalindua brodae". Anammox rates were comparable to those reported for the marine water column. Up to approximately 13% of the produced N_2 could be attributed to the anammox process whereas the remainder was related to denitrification.

In a survey of the biodiversity and abundance of aerobic and anaerobic ammonium-oxidizing bacteria in sediment samples from the Xinyi River (China) 16S rRNA genes and bacterial cells (FISH) closely related to the known anammox bacterium *Candidatus* "Brocadia anammoxidans" were found (Zhang *et al.*, 2007).

Also in soil ecosystems, habitats with high ammonium and low oxygen concentrations may prevail, but the presence and activity of anammox in soil ecosystems has never been investigated. However, very recently a molecular survey showed that anammox 16S rRNA sequences could be retrieved from several soils samples (Penton *et al.*, 2006) and ground water (Clark *et al.*, 2008).

Application of anammox bacteria

Conventional wastewater treatment technology for nitrogen removal makes use of nitrification followed by denitrification. Ammonium is oxidized to nitrate by ammonium and nitrite oxidizing bacteria and the resulting nitrate is reduced to dinitrogen gas with a suitable electron donor (usually methanol). Application of these processes is not very cost-effective (high oxygen demand, high sludge production and need of external electron donor supply) or environment-friendly (i.e. there is CO_2 and N_2O production, which contribute to global warming). The applications of anammox bacteria in combination with partial nitrification by aerobic ammonium-oxidizing bacteria offer a quite attractive alternative (Jetten *et al.*, 1997; 2001; 2002, Schmidt *et al.*, 2003; Ahn, 2006; Op den Camp *et al.*, 2006).

Anammox bacteria require nitrite as electron acceptor for the anaerobic oxidation of ammonium. The common purpose in the application of the one-reactor and two-reactor systems is providing anammox bacteria with nitrite, a compound rarely found in wastewater at high concentrations. This means that in both systems part of available ammonium has to be converted into nitrite by aerobic ammonium oxidizers. In turn, the remaining ammonium and the formed nitrite are converted to dinitrogen gas by anammox bacteria. Some reactor systems include the CANON ("completely autotrophic removal of nitrogen over nitrite"), the DEMON (pH-controlled "deammonification"), and OLAND ("oxygen-limited autotrophic nitrification-denitrification") processes (Kuai and Verstraete, 1998; Third *et al.*, 2001; 2005; Pynaert *et al.*, 2004; Wett, 2006; Vlaeminck *et al.*, 2007; 2008).

While several processes are performed in a single tank, the SHARON ("single reactor system for high-rate ammonium removal over nitrite") process takes advantage of the partial nitrification by aerobic ammonium oxidizing bacteria under oxygen limitation in a separate tank (Hellinga *et al.*, 1998). Nitrite oxidizing bacteria are washed out due to their long doubling times resulting in an effluent consisting of a 1:1 ammonium:nitrite ratio, which is the perfect influent for an anammox reactor (van Dongen *et al.*, 2001). Application of marine anammox bacteria to remove nitrogen from high strength and salty wastewaters and single-tank options exploiting the denitrification potential of anammox bacteria are currently studied (Windey *et al.*, 2005; Kartal *et al.*, 2006). It was shown that both highly enriched anammox biomass and an OLAND type mixed AOB–anammox culture could be adapted to high salt concentrations (up to 3% salt, seawater salinity) with gradual increase of salt content of the influent wastewater. In addition, up-flow fixed-bed biofilm column reactors with nonwoven fabric sheets as biomass carrier were used to develop high-rate anammox biofilm reactors (Tsushima *et al.*, 2007b). The first anammox reactor on an industrial scale (75 m^3) in the world was started in Rotterdam (NL, Abma *et al.*, 2007; van der Star *et al.*, 2007). The reactor was scaled up directly from laboratory-scale to full-scale and treats up to 750 kg-N d^{-1}. The reactor shows stable performance at this high loading rate which seems to be the result of the formation of anammox granules with high densities and high settling velocities. Using biomass from the reactor as an inoculum, two more large-scale reactors were started.

Fundamental knowledge of anammox metabolism and gene expression is highly relevant to optimize and extend the application of anammox bacteria in the future. Understanding the metabolic capabilities and competitive fitness of anammox bacteria will help to fine tune the operational conditions and thus stability of anammox reactors systems, and may be instrumental to shorten the relatively long start-up times. The discovery that anammox bacteria use nitric oxide as an intermediate will open new application possibilities in removal of NO_x from exhaust and flue gasses.

Conclusion

The combined research efforts outlined in this review provide the beginning of understanding that is necessary to assess the environmental importance and success of the anammox bacteria in the global nitrogen cycle. It also contributes directly to our environment and economy because anammox technology is a new opportunity

for nitrogen removal from wastewater: cheaper and with lower carbon dioxide emissions than existing technology. Last, the results contribute to the unraveling of the pathway of anaerobic ammonium oxidation, the understanding of the genomic blueprint and the biosynthesis of a prokaryotic organelle, which are so far all unique to the anammox bacteria.

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