

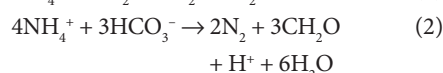
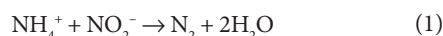
## TIMELINE

# Anammox bacteria: from discovery to application

J. Gijs Kuenen

**Abstract** | Anaerobic ammonium oxidation (anammox) bacteria, which were discovered in waste-water sludge in the early 1990s, have the unique metabolic ability to combine ammonium and nitrite or nitrate to form nitrogen gas. This discovery led to the realization that a substantial part of the enormous nitrogen losses that are observed in the marine environment — up to 50% of the total nitrogen turnover — were due to the activity of these bacteria. In this Timeline, Gijs Kuenen recalls the discovery of these unique microorganisms and describes the continuing elucidation of their roles in environmental and industrial microbiology.

Thermodynamic tables are mines of information. They allow scientists to speculate on combinations of suitable electron donors and acceptors, and propose unexpected ways that microorganisms might make a living. In 1977, I recall reading Broda's<sup>1</sup> description of “two kinds of lithotrophs missing in Nature”, and the subsequent discussion in my laboratory about microbial reactions that feature ammonium as an electron donor for denitrification with nitrite ( $\text{NO}_2^-$ ) (see Equation 1) and photosynthesis (see Equation 2).



Back then, we were unaware of whether these possibilities had been investigated by our scientific predecessors, but assumed that they had tried and failed to establish a biological basis for these reactions. In addition, the conventional wisdom at that time was that ammonium was chemically inert, and its oxidation required oxygen and a mixed-function oxygenase. Consequently, it was thought that anaerobic oxidation of ammonium would not be feasible.

Ten years after these discussions, Arnold Mulder from the Gist Brocades Fermentation Company introduced me to some fascinating observations from their denitrifying pilot plant — the disappearance of ammonium at the expense of nitrate ( $\text{NO}_3^-$ ) and a clear increase in nitrogen production (FIG. 1). The name he gave to this potential process was ‘anammox’, a term which was based on the metabolism that was thought to be involved: anaerobic

ammonium oxidation<sup>2</sup>. Arnold had tried, but was unable, to enrich, grow or identify the organism that was responsible and was unsure whether the anammox process was a spontaneous chemical reaction or a biologically mediated reaction. Recalling Broda's<sup>1</sup> prediction, I proposed to add  $^{15}\text{N}$ -labelled ammonium to the pilot plant (fed with  $^{14}\text{N}$ -nitrate) to establish whether ammonium was actually the source of the nitrogen gas. A student in my laboratory, Astrid van de Graaf, carried out the experiment in a laboratory-scale fluidized bed reactor, and was able to observe mixed-label  $^{14,15}\text{N}_2$  gas<sup>3,4</sup>. Despite the excitement of this discovery and its potential ramifications, we realized from the outset that extraordinary claims require extraordinary evidence. Astrid, with the help of Lesley Robertson, designed a range of experiments to prove the biological nature of the reaction and enrich for the responsible microorganism. In addition, she used  $^{15}\text{N}$ -labelled compounds, which included nitrite, nitrate and hydroxylamine, to identify the intermediates and products of the reaction. Heat treatment, gamma-ray treatment and an analysis of temperature optima finally convinced us that we were dealing with a true biological process. It also became clear that the anammox enrichment culture preferred nitrite over nitrate, as shown in Equation 1.

## Hydrazine in the anammox reaction

Around this time, Mike Jetten joined our group as a fellow of the Royal Academy of Arts and Sciences. In the laboratory, we were able to reproduce the anammox reaction using samples that were taken

from the pilot plant, but nitrogen production ceased within 24–48 hours. Batch enrichments of the process using a selection of different media failed to prolong the reaction. The eventual solution to this problem was to develop a flow-through system that comprised a fluidized bed reactor that was fed continuously with mineral medium, which contained ammonium and nitrite rather than nitrate. The bacteria that were responsible for the anammox process were able to grow when attached to sand particles, and, eventually, the biomass turned slightly pink owing to massive cytochrome synthesis. Under the electron microscope, we could observe enrichment of the biomass with clusters of coccus-like cells that constituted approximately 60% of the total biomass. The  $^{15}\text{N}$ -labelling experiments revealed that combining  $^{15}\text{N}$ -labelled ammonium with  $^{14}\text{N}$ -labelled nitrite or  $^{14}\text{N}$ -labelled nitrate (and vice versa) resulted in the expected mixed-labelled  $^{14,15}\text{N}_2$ . Surprisingly, however, combining  $^{14}\text{N}$ -ammonium with  $^{15}\text{N}$ -hydroxylamine also produced  $^{14,15}\text{N}_2$  gas<sup>5</sup>. This finding indicated that ammonium was not oxidized first to hydroxylamine, as in the aerobic oxidation of ammonium, but that hydroxylamine, or a related compound, reacted with ammonium to produce a doubly labelled intermediate. We postulated that this intermediate was hydrazine, which we speculated could be produced by Equation 3.

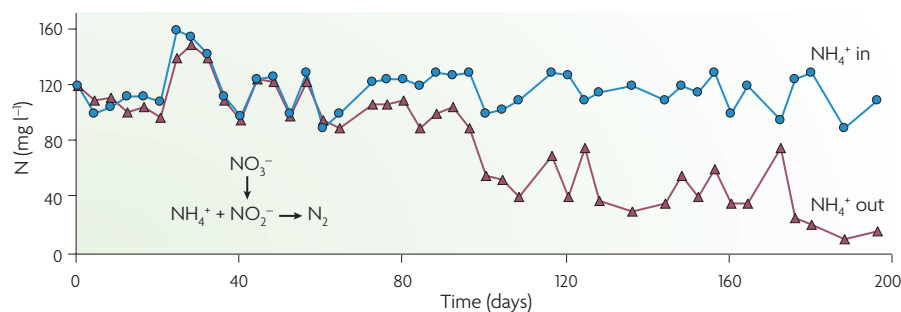


As predicted, we were able to detect hydrazine in the enrichment culture following the addition of a pulse of hydroxylamine<sup>6</sup>. Confirming that hydrazine was produced was exciting, as it is an energy-rich compound that can be used as a rocket fuel. Indeed, hydrazine is a powerful, yet potentially toxic, molecule that can be used as an energy source by bacteria.

When Astrid completed her thesis in 1997, we were convinced that an extremely unusual bacterium was present in our enrichment cultures; however, the identity of the anammox bacterium remained elusive because a pure culture could not be obtained and the enrichments were too crude to determine even simple bacterial growth rates and kinetic properties.

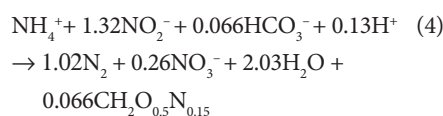
## Anammox bacterium is a Planctomycete

Marc Strous, a Ph.D. student, next took over the hunt, and we collaborated with Gerard Muyzer to help us characterize the 16S ribosomal RNA (rRNA) gene of the



**Figure 1 | Ammonium concentration of the influent and effluent from an anoxic denitrifying pilot reactor as a function of time.** The reactor received the effluent from a methanogenic pilot reactor, which contained ammonium, sulphide and some organic compounds. It was also supplemented with nitrate ( $\text{NO}_3^-$ ) from a nitrifying plant. The ammonium entering the reactor varied from 80 to 120 mg of nitrogen per litre (shown as circles). As expected from anaerobic treatment, the effluent ammonium concentration (shown as triangles) followed the influent concentration for the first 60–80 days, but then, unexpectedly, began to deviate downwards and, eventually, was almost depleted. This revealed that ammonium was removed in an oxygen-free (anoxic) reaction. The process was called 'anammox' (anoxic ammonium oxidation). In the mixed culture, other bacteria converted the nitrate into nitrite ( $\text{NO}_2^-$ ), which, as shown later, was the preferred electron acceptor for the anammox bacteria. The addition of  $^{15}\text{N}$ -labelled ammonium in the presence of normal  $^{14}\text{N}$ -nitrate led to the production of mixed-labelled  $^{14,15}\text{N}_2$  gas, which was shown to be the end product of the anammox reaction. Heat treatment, gamma-ray irradiation and temperature optima provided evidence for the true biological nature of the anammox process. The big challenge was to identify the slowly growing, novel anammox bacteria. Figure modified, with permission, from REF. 3 © (1995) Blackwell Publishing.

organism. After much effort and creativity, Marc finally managed to cultivate the organism reproducibly and with high biomass yield in a sequencing fed-batch reactor, and obtained an exponentially growing culture that was up to 70% enriched for anammox microorganisms<sup>7</sup>. Indeed, the main hurdle in purifying the bacterium was, and still is, their extremely low growth rates, with a doubling time of approximately 2 weeks. An analysis of mass balances showed that the organism uses carbon dioxide as its carbon source to produce biomass ( $\text{CH}_2\text{O}_{0.5}\text{N}_{0.15}$ ) and that nitrite not only functions as an electron acceptor for ammonium oxidation, but also as an electron donor for the reduction of carbon dioxide (see Equation 4).



Growth of the microorganism was reversibly inhibited even by oxygen concentrations that were below 0.5% air saturation<sup>8</sup>. Electron-microscopy (EM) analysis of the enrichment culture, which was done in collaboration with John Fuerst and colleagues, showed coccoid cells that shared similarities with bacteria that belong to the Planctomycetes. Given its unusual structure, we then tried to separate the organism from its contaminants by gradient Percoll centrifugation. This process yielded a 99.6% pure culture that was capable

of carrying out the anammox reaction and carbon dioxide fixation. PCR amplification of the DNA from this culture and subsequent sequence information revealed that the so-called universal primer we had used to amplify the 16S rRNA gene contained several mismatches to the anammox sequence. On the basis of its 16S rRNA gene, we could at last confirm that the organism was a member of the Planctomycetes. In 1999, a characterization of the bacterium that was responsible for the anammox reaction was published by this group<sup>9</sup>, and the causative organism was named *Candidatus Brocadia anammoxidans*. The development of 16S rRNA-based fluorescence *in situ* hybridization (FISH) probes also allowed specific identification of the organism in the enrichment culture.

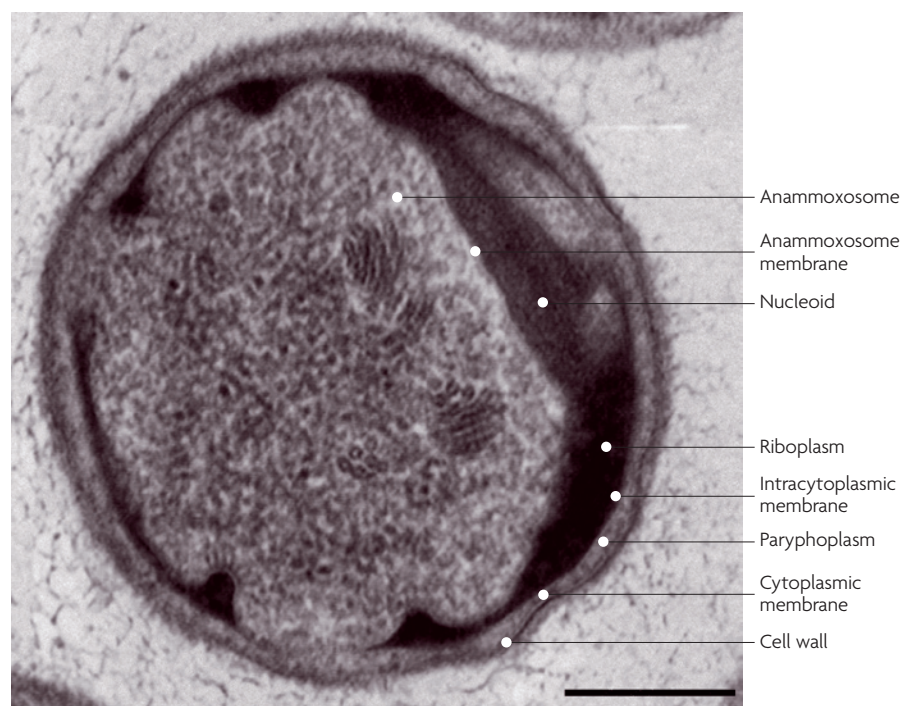
In contrast to all other known prokaryotes, Planctomycetes typically, and uniquely, possess membrane-bound sub-cellular compartments (FIG. 2), the function of which is still under investigation. EM studies indicated that anammox bacteria have a separate, anammox-specific membrane-bound compartment, which we termed the 'anammoxosome'. In collaboration with the Fuerst group<sup>10</sup>, Jos Schalk<sup>11</sup> discovered that this compartment contained large quantities (more than 10–15% of total cell protein levels) of a hydroxylamine oxidoreductase (HAO)-like enzyme, which we postulated was

responsible for the oxidation of hydrazine to  $\text{N}_2$  gas. HAO can oxidize both hydroxylamine and hydrazine, and contains 24 cytochrome *c* units per trimer that are involved in the electron-transfer process. Recent work by Shimamura *et al.*<sup>12</sup> has revealed that the organism not only expresses the HAO enzyme in high concentrations, but also encodes a true hydrazine-oxidizing enzyme; this protein has 16 cytochrome *c* units per dimer, has a significantly higher affinity for hydrazine than HAO and is strongly inhibited by hydroxylamine.

### Anammox research: the next phase

From this point onwards, research on anammox bacteria gained momentum. Using the previously developed 16S rRNA sequences and FISH probes, Jetten, Wagner and colleagues tested other nitrifying waste-water-treatment plants for the presence of anammox bacteria. These studies identified other species of anammox bacteria among the Planctomycetes, one of which was named *Candidatus Kuenenia stuttgartiensis*<sup>13</sup>. A collaboration with the group of Jaap Sinninghe Damsté<sup>14</sup> led to the discovery that anammox bacteria express various unusual lipids that contain ladderanes — lipids built from concatenated cyclobutane rings, which form a molecular ladder — and their structure was confirmed by chemical synthesis<sup>15</sup>. These molecules are thought to render the anammoxosome membrane less permeable to the toxic hydrazine, which is thought to be formed within the organelle. To date, ladderanes have only been found in association with anammox bacteria, and thus can be used to positively identify these organisms in their natural or man-made environment<sup>16,17</sup>.

In 2001, Mike Jetten established his own group in Nijmegen, The Netherlands, which resulted in a major step up in our joint research. One of the big goals was to obtain the genome sequences of anammox bacteria. As no pure cultures were available, DNA isolated from the Percoll purified culture was used for sequencing. In 2006, a major milestone in anammox bacteria research was achieved when the genome sequence of a representative anammox bacterium was published<sup>18</sup>. Because of the unavailability of a pure culture, this enterprise was in fact a progenitor metagenomics project and, in addition to the genome of *Candidatus K. stuttgartiensis*, many partial genomes, including the satellite populations of the anammox culture, were obtained simultaneously. Identification of



**Figure 2 | Transmission electron micrograph of a *Candidatus Kuenenia stuttgartiensis* cell.** As a member of the Planctomycetes, *Candidatus K. stuttgartiensis* contains subcellular compartments, including the anammoxosome, where energy conservation takes place (FIG. 3). The sample was high-pressure frozen, freeze substituted and Epon embedded. The riboplasm is the equivalent of the ribosome-containing cytoplasm in most other bacteria. The scale bar represents 200 nm. Photograph courtesy of L. van Niftrik, Radboud University, Nijmegen, The Netherlands.

these sequenced satellite bacteria is currently in progress; however, to date there is no evidence that any of these bacteria are specific for the anammox process.

#### ***Candidatus K. stuttgartiensis***

Analysis of the genome of *Candidatus K. stuttgartiensis* by Strous and colleagues<sup>18</sup> revealed a range of interesting physiological and biochemical properties that could, in part, be checked and confirmed by direct activity tests and biochemical enzyme assays.

For example, the biochemical route that is used for carbon dioxide fixation was revealed to be the acetyl-CoA pathway, as predicted by stable carbon isotopic fractionation of anammox bacteria<sup>19</sup>. In this pathway, one molecule of carbon dioxide is reduced to the level of a B12-bound methyl group and a second molecule of carbon dioxide is reduced to carbon monoxide. The methyl and carbon monoxide group combine to produce acetyl-CoA, which can be further metabolized by reduction to pyruvate. This pathway requires high energy, low-redox-potential electrons, which can be generated from the oxidation of hydrazine.

After genes were identified that encode putative enzymes for inorganic-nitrogen metabolism, the postulated pathway for energy metabolism and hydrazine formation was modified. In the original model, it was proposed that hydroxylamine is a true intermediate that would combine directly with ammonium to form hydrazine. In this case, hydroxylamine would have to be formed directly from nitrite or nitric oxide. An analysis of the genome revealed the presence of an analogue of a nitric oxide-producing nitrite reductase and several homologues of the gene that encodes HAO. One or more of these HAO enzymes could be responsible for the formation of hydroxylamine from either nitrite or nitric oxide. However, nitric oxide is a radical, and its direct attack of ammonium, with subsequent uptake of three more electrons to yield hydrazine, would also be a viable alternative for the reaction, as postulated in REF. 18 (FIG. 3).

Hydrazine, as an energy-rich compound, can donate its electrons to produce reduced ferredoxin. The four high-energy electrons from ferredoxin can then be used for energy conversion and generation of a proton motive force (PMF). After passage

through the electron-transport chain, the electrons have lost most of their energy and must then flow back for the next cycle of hydrazine formation (FIG. 3a). The PMF would be used to energize an ATPase that is localized in the anammoxosomal membrane to produce ATP in the riboplasm.

Reduced ferredoxin can also act as an electron donor for carbon dioxide fixation in the acetyl-CoA pathway. The electrons that are lost by carbon dioxide reduction are then replenished by electrons from the nitrite–nitrate oxidation reaction. However, the low-energy electrons from this oxidation need to be passed through the quinone pool by PMF-driven reversed electron transport, as postulated in FIG. 3b.

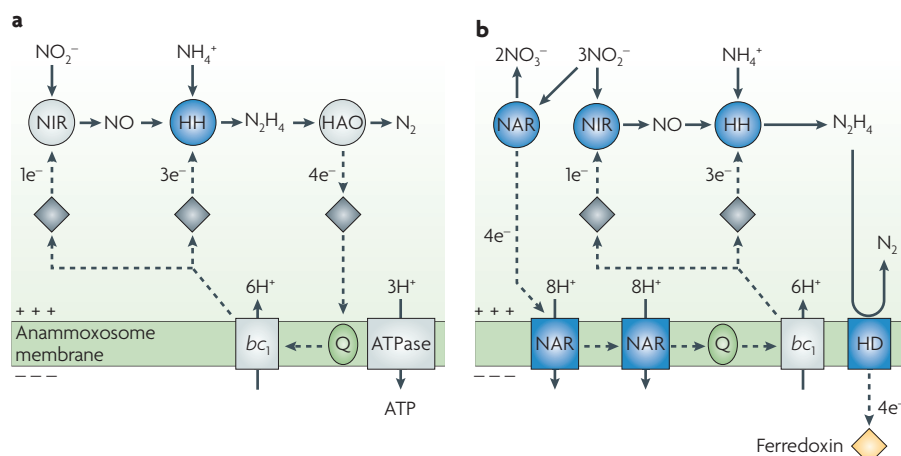
Unexpectedly, physiological and genomic analyses revealed that autotrophic anammox bacteria can oxidize formate, acetate and propionate to carbon dioxide<sup>20</sup>. The organic compounds were only dissimilated, not assimilated, as shown using <sup>14</sup>C-labelled substrates<sup>21</sup>. Interestingly, both nitrite and nitrate could serve as electron acceptors, with ammonium being the final product. As the organism can generate both nitrite and ammonium from nitrate, the surprising consequence is that this organism can produce its own inorganic electron acceptor and donor from nitrate using formate, acetate and propionate as electron donors. This pathway leads to the production of N<sub>2</sub> gas through a pathway that is different from the conventional denitrification pathway<sup>22</sup>.

#### **Taxonomy and ecological niches**

Elucidation of the genome sequence of *Candidatus K. stuttgartiensis* allowed further analysis of the taxonomic position of the anammox bacteria within the Planctomycetes. As a result of this analysis, it was argued that this group of bacteria should be placed in a super phylum that comprises Planctomycetes, Verrucomicrobia, Chlamydiae and less known phyla, rather than being considered an early branch in the evolutionary tree<sup>23</sup>.

The taxonomic position of *Candidatus K. stuttgartiensis* and other anammox species are shown in FIG. 4. Although the evolutionary distance between the different identified anammox families and species is substantial, there is no indication that their physiologies, metabolisms and ultrastructures are significantly different. Our current understanding is that they can be differentiated on the basis of their habitats and ecological niches. The *Candidatus Scalindua* family is found primarily, but not exclusively, in marine





**Figure 3 | Hypothetical catabolism and reversed electron transport in the anammoxosome.**

**a** | Pathway of ammonium oxidation that uses nitrite as the electron acceptor for the creation of a proton motive force (PMF) over the anammoxosomal membrane. Nitrite ( $\text{NO}_2^-$ ) is reduced to nitric oxide, which then combines with ammonium to produce hydrazine, with the uptake of one plus three low-energy electrons. The oxidation of hydrazine to nitrogen yields four high-energy electrons, which flow downhill through the quinone (Q) pool and the  $\text{H}^+$ -translocating cytochrome  $bc_1$  complex, thereby generating a PMF that is inside positive. The PMF energizes the proton-translocating ATPase for the production of ATP in the riboplasm. The electrons are recycled from quinone–cytochrome  $bc_1$  oxidoreductase the hydrazine-forming reactions. **b** | PMF-driven reversed electron transport combines central catabolism with nitrate ( $\text{NO}_3^-$ ) reductase to generate ferredoxin for carbon dioxide reduction in the acetyl-CoA pathway. Hydrazine can donate high-energy electrons to ferredoxin, but these electrons are not recycled. Nitrite oxidation to nitrate through nitrate reductase (NAR) compensates for this, but yields low-energy electrons that must be ‘energized’ by the PMF to be fed back into the anoxic ammonium oxidation (anammox) reaction. This ‘energization’ is accomplished by a nitrate reductase that operates at the expense of the PMF. HAO, hydrazine oxidoreductase; HD, hydrazine dehydrogenase; HH, hydrazine hydrolase; NIR, nitrite oxidoreductase. Figure modified, with permission, from *Nature* REF. 18 © (2006) Macmillan Publishers Ltd.

At the aerobic–anaerobic interface (for example, in a biofilm, in a sediment or in stratified water bodies), interesting interactions and competition can occur between the anaerobic anammox bacteria and the aerobic ammonium- and nitrite-oxidizing bacteria — the aerobic nitrite oxidizers compete with the aerobic ammonium oxidizers for oxygen, and the anammox bacteria compete with the ammonium oxidizers and nitrite oxidizers for ammonium and nitrite, respectively<sup>27</sup> (FIG. 5). Notably, the anammox bacteria and aerobic nitrite-oxidizing bacteria at this interface require aerobic ammonium-oxidizing bacteria to produce one of their substrates: nitrite. Experiments that used an oxygen-limited reactor, which was fed with ammonium only, revealed that at low oxygen concentrations (up to 2 mg per litre in the bulk phase) coexisting small clumps of *Candidatus B. anammoxidans* and separate clumps of a *Nitrosomonas eutropha* strain dominated the reactor biomass<sup>28,29</sup>. All of the removed ammonia was converted to  $\text{N}_2$  gas by a coupled nitrite-formation–anammox reaction, and only a few nitrite oxidizers could be detected. This oxygen-limited combined process for the removal of ammonium has been patented (the CANON (completely autotrophic nitrogen removal over nitrite) process)<sup>30,31</sup>, and is discussed below. When the oxygen concentration was gradually increased, coexisting populations of three organisms, including a nitrite oxidizer, were obtained.

### Environmental activity and global impact

In the early 1990s, the Gist Brocades Fermentation Company closed their pilot plant, leaving the reactor in our laboratory as the only confirmed source of anammox bacteria. Consequently, the Delft-Nijmegen team<sup>13</sup> and a Swiss team<sup>32</sup> began to look for evidence of the anammox reaction elsewhere, particularly in waste-water-treatment plants. The organisms were eventually detected and identified in many waste-water-treatment plants and a large range of marine and fresh-water habitats. Using  $^{15}\text{N}$ -nitrogen compounds, Thamdrup and Dalsgaard<sup>33</sup> looked for anammox activity in marine sediments from Danish coastal regions, using labelled ammonium or nitrate in an isotope-pairing technique<sup>34,35</sup>. This analysis provided the first clear evidence that anammox activity was detectable in different natural habitats; however, the convincing proof that the mixed-labelled  $\text{N}_2$  gas was due to anammox bacteria came from a cold Christmas expedition to the Black Sea,

environments, such as the Black Sea, the coasts of Namibia, Chile, Peru<sup>24,25</sup>, and, more recently, Lake Tanganyika, a fresh-water lake in western Tanzania<sup>26</sup>. There is also evidence of organisms that belong to the *Candidatus Scalindua* family in sediments off the coast of Gothenburg and the San Pedro basin (W. Berelson, unpublished communication). Recently, the Jetten team successfully enriched for these organisms from the Gullmarsfjord coastal sediment, Sweden, using a sequencing fed-batch reactor. *Brocadia* and *Kuenenia* species are the most commonly found organisms in the enrichments from waste-water-treatment plants and large-scale anammox reactors. Interestingly, a member of a new anammox family, *Candidatus Anammoxoglobus propionicus*, was discovered in fed-batch enrichments (in a minerals medium that contained ammonium and nitrite) that were supplemented with increasing concentrations of propionate<sup>21</sup>. Competition experiments between this bacterium and *Candidatus B. anammoxidans* demonstrated that the higher

capacity of *Candidatus A. propionicus* for propionate metabolism gave it a competitive advantage, and pointed to a clearly defined ecological niche for this organism.

As mentioned above, the habitat of anammox bacteria requires the simultaneous presence of ammonium and nitrite, which can be found at or near the aerobic–anaerobic interface of sediments and water bodies. Ammonium is produced by the anaerobic degradation of organic matter, both through ammonification and dissimilatory nitrate and/or nitrite reduction. The nitrite may originate from nitrate reduction, which, in turn, may be due either to common denitrifying organisms (lithoautotrophic or organo-heterotrophic) or nitrate reduction by the anammox bacteria in the presence of organic compounds such as formate, acetate or propionate. When ammonium diffuses upwards and meets the oxygen that is diffusing downwards, the nitrite could also be derived from nitrification by aerobic bacterial or crenarchaeal ammonium oxidizers.

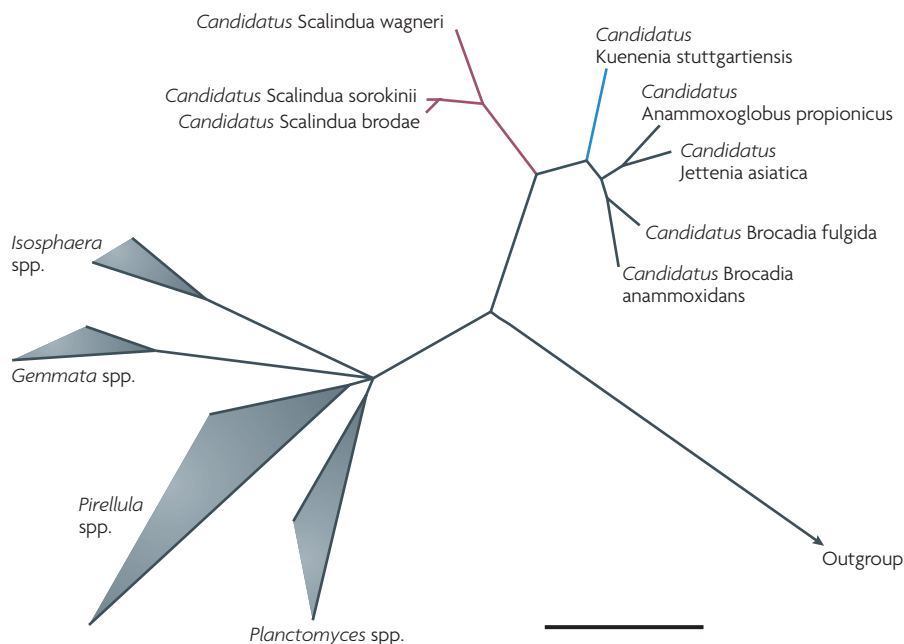


Figure 4 | **A 16s ribosomal RNA-gene-based phylogenetic tree of anammox bacteria.** Illustrates the relationships of the different families of anaerobic ammonium oxidation (anammox) bacteria among the Planctomycetes. The sequence divergence of the Planctomycetes from other Bacteria (indicated as outgroup) is high. The scale bar represents 10% sequence divergence. Figure courtesy of M. Jetten and colleagues, Radboud University, Nijmegen, The Netherlands.

which was undertaken in close collaboration with Marcel Kuypers and colleagues from the Max Planck Institute, Bremen<sup>36</sup>. At the aerobic–anaerobic interface, at a depth of 90 metres, it was possible to link the production of mixed-labelled nitrogen with coexisting nitrite and ammonium. Furthermore, molecular evidence, including 16S rRNA, FISH and the presence of ladderanes, indicated the involvement of anammox bacteria. These measurements also demonstrated the quantitative significance of anammox activity in the Black Sea. Indeed, it was estimated that this process contributes at least 30% of the total nitrogen turnover in this inland sea. Dalsgaard and colleagues<sup>37</sup> also detected high anammox activity in a coastal bay in Costa Rica, and they also observed that the contribution of the anammox process to the total turnover of nitrogen in the water column was as much as 35% of the total nitrogen turnover. They also pointed out that the chemistry of this body of water was similar to major upwelling areas around the world, and therefore predicted that anammox activity could be responsible for a major part of global nitrogen turnover. In major upwelling zones, such as the coasts of Peru, Chile and Namibia, cold, nutrient-rich water reaches the photic zone, which leads to high primary production

and, subsequently, high mineralization rates, including high nitrogen turnover. An expedition by Kuypers and colleagues<sup>24</sup> to the Benguela upwelling zone, Namibia, confirmed the massive contribution of the anammox process to marine nitrogen turnover in that upwelling zone. Considerable supporting evidence has since confirmed its global importance<sup>25,38,39</sup>. The evidence for anammox activity in waste-water-treatment systems of different configurations is overwhelming, and recent research now shows that the anammox process is almost ubiquitous in freshwater systems, including marshes, peat bogs and large lakes, such as Lake Tanganyika<sup>26,40,41</sup>.

#### The industrial application of anammox

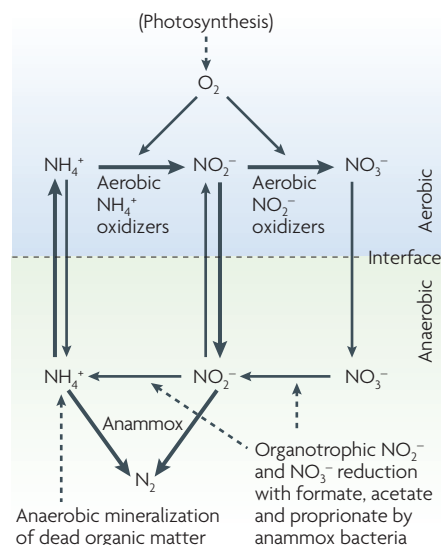
From the beginning it was realized that the anammox process had great potential for the removal of undesired ammonium from waste gas or waste water. For this reason, Arnold Mulder and colleagues<sup>2</sup> patented the process immediately, even without direct proof of its biological nature. Much later, when it was possible to grow cultures reproducibly, we revived the original ideas for use in ammonium removal, in collaboration with the Paques Company (Balk, The Netherlands). At that time, Mark van Loosdrecht and colleagues had devised a large-scale process — termed

the SHARON (single reactor system for high-rate ammonium removal over nitrite) process — to convert ammonium exclusively to nitrite, rather than nitrate (which is costly to remove)<sup>42</sup>. This process also turned out to be ideally suited to produce the 50:50 mixture of nitrite and ammonium that is required in the anammox process. This mixture was produced in the laboratory in an aerated, stirred tank reactor under controlled temperature, oxygen supply and dilution rate. Under these conditions, the nitrite oxidizers, such as *Nitrobacter* spp., were washed out. Subsequently, the effluent was fed to an anammox reactor, and the result was a complete conversion of the ammonium to  $N_2$  gas and additional nitrate, as predicted by Equation 3 (REF. 42).

Based on this successful laboratory-scale experiment and a large European Union-sponsored, multidisciplinary study<sup>43</sup>, a full-size plant (volume of 80 m<sup>3</sup>) was built in Rotterdam, The Netherlands, to treat high-ammonium effluent from the Dokhaven–Sluisjesdijk waste-water-treatment plant. Owing to the low growth rates of anammox microorganisms, and other technical issues, the start-up period was approximately 2 years. The reactor was inoculated with nitrifying sludge from the plant and its start-up phase was monitored using real-time PCR to follow the growth of the microorganisms. In September 2006, the reactor was in full operation and was converting between 8–10 kg of nitrogen per m<sup>3</sup> every day, a performance level that was twice its design capacity<sup>44</sup>. Two more anammox reactors, which are fed from a SHARON reactor, are now in operation. A third large-scale, oxygen-limited reactor that functions on the principle of the CANON process is also in operation.

#### Outlook for anammox research

All over the world, research groups are working on diverse aspects of the molecular biology, biochemistry, ultrastructure, physiology and metabolism, and ecology of anammox bacteria, as well as assessing the impact of their activities on the environment and their applications in waste-water and waste-gas treatment. Although most field-activity measurements are not hindered by the lack of pure cultures, other research can be delayed because: first, the organisms grow too slowly; second, the cultivation of these organisms requires considerable experience; and third, if large amounts of biomass are required, more elaborate cultivation



**Figure 5 | Interaction and competition among aerobic and anaerobic nitrifiers.** An aerobic-anaerobic interface that might exist at the surface of a sediment or biofilm, or at the interface of a stratified water body. The interactions between three nitrifiers only are considered. Ammonium is released by the anaerobic degradation of organic material and diffuses upwards to the aerobic interface. Oxygen is derived from above from photosynthesis. Both the anaerobic ammonium oxidation (anammox) bacteria and nitrite oxidizers are dependent on the nitrite ( $\text{NO}_2^-$ ) that is generated by the aerobic ammonia oxidizers. Anammox bacteria compete with the two aerobes for ammonia and nitrite, respectively, whereas the aerobic ammonium oxidizers compete with anammox for ammonium and with the nitrite oxidizer for oxygen. This simplified model includes the possibility of nitrite formation by anammox bacteria themselves, but not by heterotrophic bacteria. When an oxygen-limited, gently stirred reactor is fed with a minerals-ammonium medium, the three types of organisms grow in flocs or in suspended biofilms, and compete as described above. At low oxygen concentrations, a mixture of aerobic ammonium oxidizers and anammox bacteria will be selected, a principle on which the CANON (completely autotrophic nitrogen removal over nitrite) process for ammonium removal is based. The nitrate ( $\text{NO}_3^-$ ) that is produced in this case is primarily due to anammox bacteria.

equipment is needed. Fortunately, a new cultivation system for anammox bacteria has been developed in which the organisms grow in suspension at significantly higher enrichment and have a doubling time of approximately 8 days — this represents a 50% improvement over the earlier sequencing fed-batch techniques (W. van der Star, A. Miclea, U. van Dongen, G. Muyzer, C. Picioreanu and M. van Loosdrecht, personal communication).

To increase our knowledge of the biochemistry of these fascinating bacteria, research priorities will focus on developing a greater understanding of the mechanisms that underlie the build-up of electrochemical gradients — in particular, the PMF — that act across the anammoxosome and other cellular compartments. To this end, one of the big obstacles is the preparation of purified intact anammoxosomes<sup>45</sup>. Understanding other aspects of the ultrastructural development of anammox bacterial cells, including the mechanisms of cell and anammoxosome multiplication, is also important. Laura van Niftrik<sup>46</sup> recently found convincing EM evidence that the anammoxosome is a completely separate, highly folded organelle that is responsible for energy metabolism, and contains not only HAO but also high concentrations of cytochrome *c* along a 150-nm ring within the compartment<sup>46</sup>. It was also discovered that the cells undergo binary fission through a division ring, whereas the anammoxosome is vertically inherited from the mother to the daughter cells<sup>47</sup>. Equally challenging are the questions of how the autotrophic and organotrophic metabolism of this organism are regulated and how alternative electron acceptors are exploited. The biochemical pathway for ladderane synthesis<sup>17</sup>, and in particular how the organism synthesizes concatenated cyclobutane rings, is also an important and intriguing issue that requires further investigation. Research in this area may also help to identify whether or not these molecules or their products leave any traces for geological dating. The use of  $^{15}\text{N}$ -labelled nitrogen compounds for measuring anammox activity, including anammox activity in the presence of organic compounds in the field or in waste-water-treatment plants, presents another complex challenge for future research. In addition, it is important that field measurements can be used to positively identify the responsible organism in the light of the enormous diversity of anammox bacteria and their various ecological niches.

Several important questions remain to be answered on the global impact of the anammox process. How do anammox organisms interact with denitrifiers? What is the role of organic compounds in this process? And, how do anammox organisms interact with nitrifiers and dissimilatory nitrate reducers? Recently Lam *et al.*<sup>48</sup> have shown that in the Black Sea bacterial and crenarchaeal nitrification<sup>49</sup> contribute equally to the provision of nitrite for the anammox process, thereby underlining the importance of the Crenarchaeal nitrifiers in nitrogen

cycling in the marine environment. One could hope that the discovery of anammox Planctomycetes will inspire others to look for other Bacteria or even Archaea that can grow at the expense of anaerobic ammonium oxidation. Hopefully, further studies will determine how the anammox process interacts with the major global-element cycles, how seasonal and spatial parameters determine the fate of nitrogen compounds in the environment and which ecological niches are occupied by the huge diversity of anammox organisms.

In terms of industrial usefulness, the application of anammox bacteria in wastewater treatment — in particular for the removal of highly contaminated nitrogen waste water that has a low organic content — is now a proven technology. However, its application is far from trivial and, in many cases, will require tailor-made solutions that will be based on insight into the ecophysiology of anammox bacteria together with technological knowledge.

The discovery of the anammox process and the microorganisms that are responsible for its activity have been a prime example of the value of multidisciplinary collaboration. It has been an immensely rewarding and well-supported scientific interaction that has involved a wide range of experts from all over the world.

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#### DATABASES

Entrez Genome Project: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj>  
*Candidatus* Brocadia anammoxidans | *Candidatus* Kuenenia stuttgartiensis | *Nitrosomonas europaea*

#### FURTHER INFORMATION

J. Gijs Kuenen's homepage: <http://www.bt.tudelft.nl>

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