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Anammox and beyond

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How we did it!

Anammox and beyond

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Summary

When looking back and wonder how we did it, I became even more aware of how my wanderings in microbiology are all linked, from the start of my PhD with Hans Veldkamp on sulphur-oxidizing bacteria in chemostats. My interests broadened from obligate chemolithoautotrophic bacteria to facultative organisms and the question about the ecological niches of these different metabolic types. The sulphide oxidizing bacteria also may be used to produce elemental sulphur, which can easily be removed from wastewater. This fitted in a long-standing collaboration with Dmitry Sorokin on the ecophysiology and application of alkaliphilic sulphur bacteria. Then came the denitrifying sulphur-oxidizing bacteria and their application to remove sulphide from wastewater, which lead to our interest in nitrate, nitrite and ammonium removal in general. The big surprise was the serendipitous discovery of the ‘anammox’-process, whereby ammonium is anaerobically oxidized to dinitrogen gas with nitrite as electron acceptor. The early days of our anammox research are the main focus of this article, which describes the struggle of growing and identifying the most peculiar bacteria we ever came across. A specialized organelle, the anammoxosome was shown to be responsible for the key ammonium oxidation, whereby a rocket fuel, hydrazine, turned out to be an intermediate. Soon after we became aware that anammox is everywhere and in the marine environment makes up a major portion of the nitrogen cycle. The intense scientific collaboration with Mike Jetten and Mark van Loosdrecht and colleagues led to our further understanding and application of this fascinating process, which is briefly

summarized in this article. My broader interest in environmental microbiology and microbial ecology has been a regularly returning theme, taking me all over the world to great collaborations lasting to this very day.

It all began when I followed a full-time 6-week microbiology course with Hans Veldkamp at the Groningen University (NL), who taught the course in the style of the famous Pacific Grove class of Cees Van Niel, which Veldkamp also had attended in the 1950s (Veldkamp, 1987). We learned the essence of the Beijerinck enrichment cultivation of a great variety of microorganisms and metabolic types. I just fell in love with microbiology, did an MSc study with him on the selection of bacteria under nutrient limitation in home built chemostats and successfully reproduced the early work by Holger Jannasch, showing that one can easily isolate bacteria with crossing growth curves by running carbon- and energy-limited continuous cultures at low and high dilution rates. In this way, I became a member of the Delft School of Microbiology and from then on, I was lost in the wonders of microbiology and the use of continuous culture to study quantitative (eco)physiology (Kuenen, 2019). My PhD with Veldkamp was on the physiological properties of a newly isolated spirillum-like obligate chemolithoautotrophic sulphur oxidizer, *Thiomicrospira pelophila* (Kuenen and Veldkamp, 1972) for comparison I also studied a well-known specialist sulphur oxidizer *Thiobacillus neapolitanus* (today a *Halothiobacillus* species) (Kuenen and Veldkamp, 1973). The aim was to understand its lifestyle or ecological niche and the reasons why these organisms were **obligate** autotrophs. We were also wondering why organisms would ‘choose’ (i.e. what is the evolutionary advantage) to be obligate chemolithoautotrophic and this was the start of a long journey after my PhD thesis into the specialist versus generalist (facultative) style of metabolism. It led to a range of publications with my students (Gottschal and Kuenen, 1980; Beudeker *et al.*, 1982) about sulphur-oxidizing obligate-, and facultative chemolithoautotrophs and even the advantage of being a chemolithoheterotrophic sulphur oxidizer. We pinpointed the important metabolic capabilities of their ecological niches and were able to confirm this

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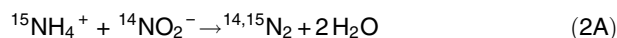
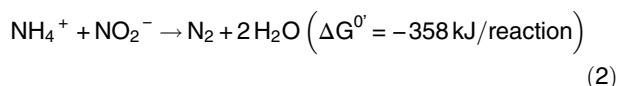
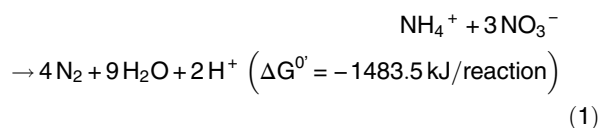
by successful chemostat enrichments of the different metabolic types.

When I moved to Delft in 1980, Piet Bos joined my team and together with our students, we extended our interests into all kinds of potential applications of sulphur oxidizers among which acidophilic sulphur oxidizers involved in leaching from ores and coal (Pronk *et al.*, 1990) and bacteria capable to oxidize organic sulphur compounds like dimethyl sulphide (Suylen *et al.*, 1986) (de Zwart and Kuenen, 1992). In collaboration with Gatzte Lettinga in Wageningen University (NL) and the Paques Company (Balk, NL), and our students we developed a successful large-scale process for sulphur removal, especially hydrogen sulphide, in the form of insoluble sulphur particles from (industrial) waste streams. The Delft scientific aim was to find out what is the basis for the successful enrichment of bacteria producing (insoluble) sulphur in competition with organisms converting the sulphide all the way to sulphate (Stefess *et al.*, 1996) (Visser *et al.*, 1997). The key was oxygen limitation, and this is the trick applied in several hundreds of large-scale operations on a global scale, today. In the early 1990s, Dmitry Sorokin (Moscow) joined our team and when looking for alkaliphilic sulphur oxidizers that would be the preferred organisms to do the sulphide to sulphur conversion, he discovered an overwhelming variety of (halo) alkaliphilic sulphur oxidizers living in soda lakes. The early focus was on the obligately autotrophic *Thioalkalivibrio* and *Thioalkalimicrobium* species with totally different survival strategies of the ecophysiological R and K-type, as demonstrated in a number of chemostat studies with pure and mixed cultures (Sorokin *et al.*, 2001; Sorokin and Kuenen, 2005). The understanding of their ecophysiology and geographical distribution was later extended in studies by two PhD students of Dmitry Sorokin, Gerard Muyzer and me (Foti *et al.*, 2006; Banciu *et al.*, 2008). Among the alkaliphiles are denitrifiers (Sorokin *et al.*, 2004) and *tandem* denitrifiers (Sorokin *et al.*, 2003), which only in combination perform the complete denitrification, the first organism producing nitrite and the second converting the nitrite into dinitrogen gas (Sorokin *et al.*, 2003). Dmitry Sorokin also studied a range of other peculiar sulphur oxidizers, for example, the strange *Catenococcus thiocyclus* (Sorokin *et al.*, 1996), which is capable of litho-heterotrophic growth exploiting the thiosulfate tetrathionate oxidation/reduction cycle to oxidize sulphide.

Hydrogen sulphide removal was also one of the prime goals of a totally novel wastewater treatment at the Gist Brocades biotechnological factory in Delft. The concept developed by Sef Heijnen (Heijnen *et al.*, 1991) was a series of fluidized bed reactors (vertical cylindrical columns of 18 m high and 5 m in diameter) with suspended flocs or granules of microorganisms initially attached to sand particles as support. The carbon and ammonium-rich effluent of

the yeast and penicillin production was first treated anaerobically to recover methane as fuel. As the factory-wastewater also contained considerable concentrations of sulphate, hydrogen sulphide from dissimilatory sulphate reduction was the unavoidable and undesired by-product. It was decided to kill two birds with one stone by oxidizing the ammonium in the effluent of the treatment process aerobically to nitrate and then feed-back the nitrate to the sulphide containing effluent of the methanogenic reactor. This worked out well and the sulphide was reoxidized to sulphate with the nitrate as electron acceptor converted to dinitrogen gas. In an ensuing collaboration, we discovered that the responsible dominant sulphide oxidizer was the facultatively chemolitho(auto)trophic, denitrifying *Thiosphaera pantotropha*, in other words, a typical versatile mixotroph consuming a mixture of the remaining acetate and sulphide while denitrifying (Robertson and Kuenen, 1983). Further physiological studies by Lesley Robertson showed that the organism also was capable of mixotrophy (i.e. simultaneous use) of oxygen and nitrate at significantly high oxygen concentrations (Robertson *et al.*, 1995).

In order to optimize this denitrifying step further, Arnold Mulder at Gist brocades built a separate denitrifying pilot fluidized bed reactor. To this reactor, he fed the sulphide-containing effluent of the methane plant with externally added nitrate under strictly anoxic conditions. The nitrate was consumed as expected, most likely by a *Thiosphaera pantotropha* type of organism, but after around 80 days, ammonium began to disappear at the expense of nitrate, which apparently was converted to dinitrogen gas (Mulder, 1989). Was this unexpected reaction the anaerobic ammonium oxidation with nitrate as theoretically predicted by Broda in a paper of 1977? I remembered being very excited by that publication (Broda, 1977), which based on thermodynamic data listed a number of those reactions that might be possible energy-yielding reactions for microbiological life:



We were not aware of any report in the literature but later we discovered some papers in which an unexplained disappearance of ammonium was mentioned (Chick, 1906; Buswell, 1923). This had never been followed up and given the solid fact that a fitting nitrogen balance could be obtained only by taking gaseous

nitrogen into account, Arnold and colleagues decided to patent the process (Mulder, 1989) (EP0327184, 1995) as a novel possibility to remove undesirable ammonium from waste or wastewater under the name 'anammox'. They approached us to find out what might be going on. I decided the priority was to prove that ammonium and nitrate were indeed being combined to give dinitrogen gas and that the ammonium disappearance was of biological nature. After a most cumbersome procedure to revive the anammox process in a pilot fluidized-bed reactor Astrid van de Graaf added (^{15}N)-labelled ammonium to the reactor which was fed unlabelled (^{14}N)-nitrate and to our immense joy the mass spectrometer showed that the majority of the label ended up in mixed labelled $^{14,15}\text{N-N}_2$ gas (reaction 2A). The reaction immediately stopped after pasteurization or X-ray irradiation (Van de Graaf *et al.*, 1990). We also excluded the possibility that oxygen was involved and in fact, oxygen severely inhibited the reaction. With this quantitative evidence, we were able to convince the national science foundation (STW/NWO) that something special was going on and they supported Astrid's PhD project (Van de Graaf *et al.*, 1990). Then came the real hard work. All attempts to enrich the anammox activity in a regular batch culture with a spectrum of substrates and mixtures failed. Samples taken from the fluidized bed reactor incubated under anoxic/anaerobic conditions would consume ammonium and nitrate or nitrite simultaneously for a short while, but the reaction always petered out showing that our incubation conditions were insufficient, even if the medium was based on the effluent of the original methane reactor. Hence, in an attempt to mimic the environment in the large fluidized bed, we decided to try cultivating the anammox bacteria on sand particles with recycling of the liquid to keep the particles suspended and a constant low

bleed of the spent medium. The culture was fed with nitrate and ammonium and under these conditions, we were able to keep the anammox reaction going, but it did not lead to a substantial enrichment. Organic compounds did not improve the turnover, but reduced sulphur compounds gave a slight improvement. However, when Astrid van de Graaf supplemented the standard nitrate with nitrite, the ammonium consumption increased significantly and one of the columns turned slightly pinkish, with small gas bubbles forming on the surface of the particles.

This was when we observed the nearly stoichiometric conversion of ammonium and nitrite into dinitrogen gas. That was the beginning of the successful enrichment of the microorganisms responsible for the anammox reaction. Gradually, i.e. over quite a number of years, the handling of the fluidized bed and recycling improved, and we could reproducibly cultivate the anammox culture on a minerals medium with a mixture of equal amounts of ammonium and nitrite as energy source and bicarbonate as the carbon source, i.e. a true inorganic minerals-medium plus vitamins. These experiments were not a matter of a few days but rather of weeks and months due to the apparent slow growth of the responsible organisms. The first estimates were that the organisms were growing a rate below 0.001 h^{-1} . In hindsight, the continuous presence of nitrate helped to keep sulphate-reducing bacteria and methanogens at an insignificant level (Mulder *et al.*, 1995; van de Graaf *et al.*, 1995, 1996).

Given the composition of the medium, the responsible anammox organism had to be an autotroph capable of deriving metabolically useful energy from the reaction (2). During this process in the order of 10% of the nitrite was converted to nitrate and this might account for the reduction-equivalents required for CO_2 fixation.

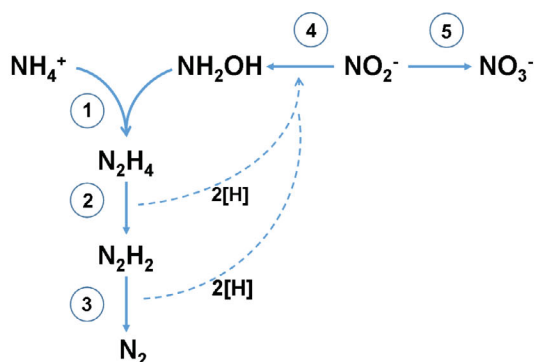
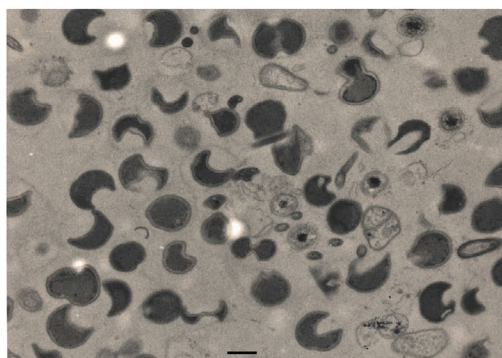


Fig. 1. (L). The dominant species in the enrichment culture in an early thin-section electron micrograph of resuspended Anammox biofilm grown on synthetic (autotrophic) medium in a fluidized bed reactor (FBR). Bar, 1 μm . (R) Initial proposal for the metabolic pathway for anaerobic ammonium oxidation. Consumption and production of H_2O or H^+ is not indicated. Ammonium combines with hydroxylamine to form hydrazine (step 1). Reducing equivalents derived from N_2H_4 then reduce nitrite to form even more hydroxylamine and the oxidation of N_2H_4 to N_2 provides more electrons to form more hydroxylamine (steps 2, 3 and 4). Nitrate formation from nitrite could generate reducing equivalents for biomass growth (step 5). (R) Reproduced with permission from Van de Graaf *et al.* (1997). [Color figure can be viewed at wileyonlinelibrary.com]

However, reproducibility was wavering, and a pure culture was still very far away. Even now (2019), pure cultures of anammox bacteria are not available. Under the phase-contrast microscope, we saw the 50%–70% enrichment of a somewhat coccoid irregularly shaped bacterium (Fig. 1). This culture allowed us to study the carbon and nitrogen balance (in and output) of the reactor, with the clear notion that ammonium, nitrite and bicarbonate were the only substrates provided. We wondered if hydroxylamine might be an intermediate as is the case for aerobic ammonium-oxidizing bacteria. We also guessed that the first compound containing the N–N bond might be hydrazine (NH₂–NH₂).

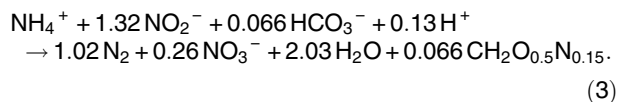
At that time, Mike Jetten joined us as a Royal Academy Fellow, which greatly helped enforcing our small team. From then on, he became an important (co)author of many of the ensuing anammox papers.

When we pulsed the culture with hydroxylamine, a peak of hydrazine appeared transiently. Experiments with ¹⁵N labelled ammonium, nitrate, nitrite and hydroxylamine supported this hypothesis. Different combinations of the ¹⁵N-compounds with unlabelled ammonium and/or the N-oxides including NH₂OH and NO were also performed. Unexpectedly hydroxylamine was not the product of ammonium oxidation but rather replaced nitrite to combine with ammonium to form mixed labelled nitrogen gas. Apparently, nitrate and/or nitrite was converted to hydroxylamine to react with the ammonium. We published the first rough metabolic pathway for anammox in 1997, as shown in Fig. 1 (Van de Graaf *et al.*, 1997). However, when considering the reactivity of hydroxylamine versus another possible candidate, nitric oxide (NO), we speculated that it might well turn out to be the latter. This was supported by the literature (Hooper *et al.*, 1997). Following this up, PhD student Jos Schalk isolated a major protein from these organisms, identified as a hydroxylamine oxidoreductase (HAO), supporting the early idea that an HAO type enzyme might be involved in the anammox metabolism (Schalk *et al.*, 2000). Indeed, it had been reported that an HAO-enzyme was capable of oxidizing hydrazine to dinitrogen gas (Hooper *et al.*, 1997).

Today, metagenomics, or even metaproteomics, would quickly help us identifying the dominant organism in the enrichment culture, but in the early 1990s, the most realistic option was cultivating, further enrichment and purification.

In 1995 Marc Strous had also joined our team as the third PhD student. He set out to cultivate the biomass in a sequencing batch reactor (SQBR) in order to allow the further enrichment of our slowly growing anammox bacteria. An SQBR is essentially a flow-through system with biomass retention: The culture is fed continuously with substrate for a couple of hours. Then the feed is stopped and particles with the (anammox) biofilm can settle. Then 60%–70% of

the overlying liquid is removed and the feed is started again. Under these conditions, the slowly growing organism can be enriched effectively (Strous *et al.*, 1998). Indeed, this turned out to be a breakthrough: in a little more than a year Mark obtained a dense dark red culture converting nitrite and ammonium in a reproducible way, which allowed us to obtain a fitting nitrogen- and carbon balance of the Anammox reaction including the CO₂ fixation, which on the basis of biochemical and enzymological tests could be identified as the acetyl-CoA-pathway:



It also permitted us to make a first estimate of the growth rate: in the order of 10 days doubling time (specific growth rate 0.0027 h⁻¹). The yield was 0.066 C-mol/mol of NH₄⁺ and a maximum ammonium consumption rate of 45 nmol/mg protein/min. Based on its peculiar morphology (Fig. 1), we estimated that about 70% of the culture was dominated by the enriched anammox bacterium. The SQBR-culture also allowed a further determination of key physiological parameters. One of the surprising properties was the extremely high affinity for ammonium (expressed as a very low-affinity constant of less than 1 micromolar).

Soon after we began to try and determine the 16 S RNA sequences in the mixture, using all the available know-how, but the sequences revealed by the so-called 'universal' probe did not show any dominant bacterium. Hence it was decided to artificially enrich the organism using a Percoll gradient. This delivered a clear red band in the centrifuge tube, which by microscopic inspection contained up to 95% of the desired morphology and carried out both the desired anammox reaction and carbon dioxide fixation when primed with a trace of N₂H₄ or NH₂OH, or ¹⁴CO₂. When we applied the universal probe on this concentrated sample a weak band appeared and upon sequencing of this PCR product it turned out to have a sequence only partially overlapping with the universal probe. On that basis, we could identify the organism as a member of the Planctomycetales, which had recently been described by John Fuerst and colleagues (Fuerst, 1995). Our bacterium still was not much related to any of the other members of this Eubacterial phylum but on electron microscopical inspection it turned out to have the peculiar intracellular structure observed for the other Planctomycetes (see Fig. 2A, EM and cartoon). However, unlike the latter, it had a very dominant intracellular membrane-bound 'organelle', which we later identified as the anammoxosome, where energy conversion takes place. Later it was shown conclusively that they are independent cell organelles (Neumann *et al.*, 2011). The first identified anammox-bacterium was named *Candidatus* Brocadia anammoxidans (Strous *et al.*, 1999). A totally unexpected

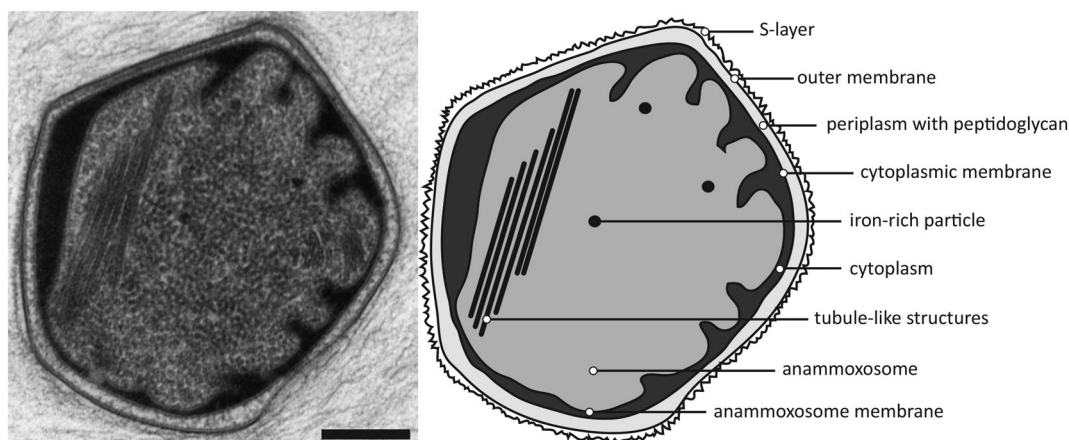


Fig. 2. Transmission electron micrograph (A) and cartoon of a section through an anammox cell. Scale bar, 0.2 μm . Reproduced with slight modifications with permission from Peeters and Van Niftrik (2019).

discovery was the presence of ladderanes, totally unknown lipids containing concatenated cyclobutene rings, which turned out to be unique for anammox bacteria (Sinninghe Damsté *et al.*, 2002).

In the meantime, two microbial ecologists from Denmark (Dalsgaard and Thamdrup, 2002) had picked up the message that the anammox reaction could be identified by exploiting the ^{15}N tracer technique and they convincingly demonstrated that the anammox reaction could also be observed in sediments of the Arhus Bay. Further careful field experiments showed that the anammox reaction could be demonstrated in a great variety of anoxic sediments where both N-oxides and ammonium were simultaneously present. One of our own PhD students, Olav Sliemers, joined an expedition organized by the Max Planck Marine Microbiology Institute to look for anammox at the aerobic/anaerobic interface of the Black Sea where both nitrite and ammonium had been detected. In the paper of Marcel Kuypers *et al.* (Kuypers *et al.*, 2003) it was established that the anammox reaction could be quantitatively identified at the interface. *In situ* filtered water contained bacteria that positively responded to the (specific) fluorescent ribosomal RNA probe of anammox bacteria and the collected biomass contained the peculiar ladderane lipids unique to them. In this way, the reaction and the presence of the responsible bacteria could be firmly established. Following publications by us and others using ^{15}N tracer technique (Dalsgaard *et al.*, 2005; Schmid *et al.*, 2007), proved that anammox bacteria can be found all over the world and have a wide geographical distribution with dominant hot spots in oceanic up-flow zones. Quantitative assays indicate that the anammox reaction is responsible for at least 40%–50% of the total global marine N-turnover. In marine environments, *Candidatus* Scalindua species were dominant. Screening of wastewater treatment plants usually also detects

anammox bacteria, often in biofilms of the nitrifying step. Presently, five different genera are known with nine different species, but today's databases show a large spectrum of other unidentified anammox species or subspecies (cited from Kartal *et al.*, 2012).

In 1999 Mike Jetten (Delft and Nijmegen(NL) and Michael Wagner (München(DE) and Vienna(AU)) convinced the Genoscope (Evry France) company to try and sequence the dominant DNA in one of our (70%) enriched fluidized-bed reactors containing a new anammox enrichment of *Candidatus* Kuenenia stuttgartiensis, which my colleagues had kindly named after me (Schmid *et al.*, 2000). In fact, this sequencing project became the first metagenomic analysis of its kind. It took several years to reassemble the DNA sequence but eventually, 98.5% of the DNA was covered (Strous *et al.*, 2006) The genome was rather large (4.27 Mb) and according to the further annotation contained a remarkably large number (>200) of genes of cytochrome sequences including HAO type of enzymes. In the order of 30% of the proteins of *Candidatus* K. stuttgartiensis is cytochrome-linked. This clearly explained the red colour and spectrum of the anammox enrichments.

Much later, with all the new developments in DNA sequencing, the genome of Kuenenia was further confirmed and finished (Speth *et al.*, 2012) by the group of Mike Jetten who had moved to Nijmegen in 2000 and teamed up with Huub op den Camp. Using this knowledge in combination with extensive biochemical and enzymological research his team, including a few of our joint PhD students, made major advances in the further analysis of the pathways, first published in 2006 (Strous *et al.*, 2006) and further extended in a number of publications reviewed in two papers of 2012 and 2016 (Kartal and Keltjens, 2016). Structural analysis by Laura van Niftrik and her students in the Nijmegen-group has shown

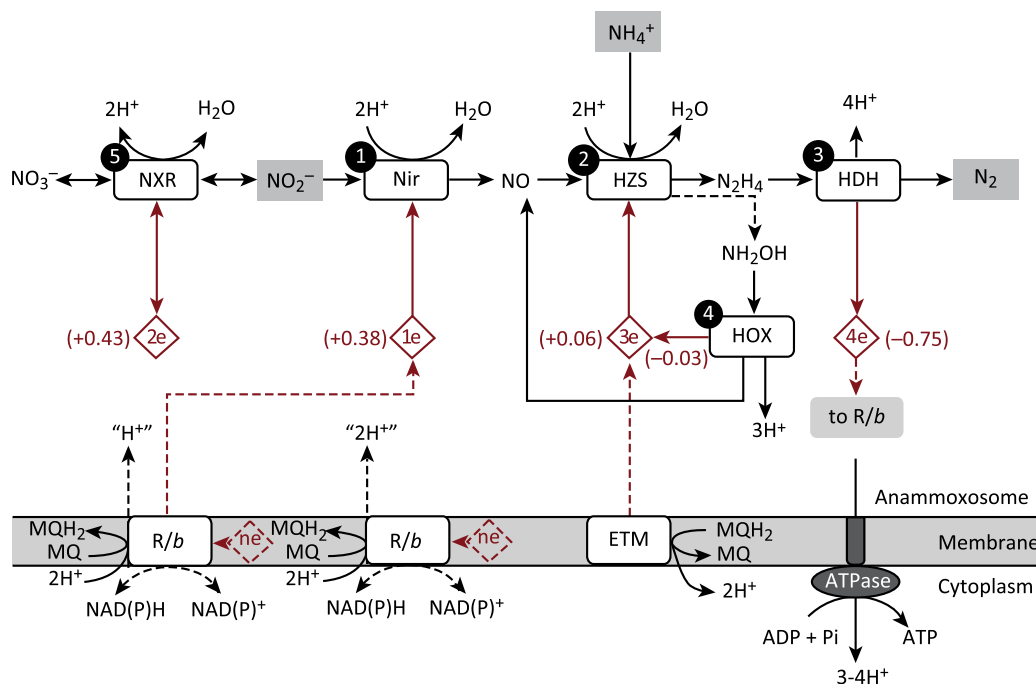


Fig. 3. Schematic overview of the anammox energy metabolism in the anammoxosome. The main reactions are indicated by numbers: 1. nitrite reduction to nitric oxide by nitrite reductase (Nir), 2. combining nitric oxide with ammonium by hydrazine synthase (HZS) 3. the oxidation of hydrazine by hydrazine dehydrogenase (HDH), 5. NXR nitrite oxidoreductase, 4. HOX hydroxylamine oxidase, R/b Rieske-heme complexes (bc1 complexes), ETM electron transfer module, MQ Menaquinone. The dotted lines indicate the assumed flow of electrons between the pathway and the electron transport chain in the anammoxosomal membrane. Reproduced with slight modifications with permission from Peeters and Van Niftrik (2019).

that the membrane-bound anammoxosome is the site for energy conversion (Neumann *et al.*, 2011; Neumann *et al.*, 2014) and research summarized by Boran Kartal, Jan Keltjens and colleagues (2012 and 2016) show that NO rather than hydroxylamine is the intermediate in the pathway in many anammox bacteria, but it cannot be excluded that in some other anammox bacteria hydroxylamine reacts with ammonium (Oshiki *et al.*, 2016). Particularly interesting is the observation that anammox bacteria can be grown on ammonium and NO instead of nitrite (Hu *et al.*, 2019).

2019's state of the art is shown in a summary of the structure of anammox bacteria and the pathways in Figs 2 and 3, in which the research in Nijmegen played a major part, supplemented with research from all over the world, and reviewed in three publications (Kartal *et al.*, 2012; Kartal and Keltjens, 2016; Peeters and van Niftrik, 2019).

In Fig. 2 the detailed ultrastructure of the anammox cell is shown in the EM photo and the cartoon. The S-layer and outer membrane surrounds the periplasmic space, containing peptidoglycan. Further inside the cytoplasmic membrane borders the cytoplasm (originally called the riboplasm since it contains the ribosomes) and then, inside, the separate, membrane-bound anammoxosome, with tubule like structures the role of which is unknown.

The anammoxosomal membrane and its metabolic role in energy conversion are shown schematically in Fig. 3. Many enzymes have now been identified by a combination of genomic and sophisticated biochemical work including enzyme purification and crystallography, but others still remain to be identified: The synthesis and oxidation of hydrazine is proceeding via enzyme systems 1, 2 and 3: 1. reduction of nitrite to NO by Nir (requiring 1 electron from R/b), 2. combination of NO with ammonium to hydrazine with HZS, requiring three electrons provided by electrons from menaquinone via postulated electron transport module located in the membrane, and 3. finally oxidation of hydrazine to nitrogen gas by hydrazine dehydrogenase. The high energy-electrons from the hydrazine oxidation are fed into the Rieske heme (R/b) complexes, where, most likely, a bifurcation process directs electrons to NADPH required for CO₂ fixation in the reductive acetyl-CoA pathway. The role of the NXR-complex (5) is the supplementation of electrons from the nitrite oxidation to nitrate to replenish the electrons withdrawn from the cyclic electron flow in order to balance the overall electron budget. The role of HOX (4) may be to recycle hydroxylamine formed as a by-product. The location of H⁺-ATPase is firmly established as facing toward the cytoplasm to allow ATP synthesis at the expense of the proton motive force over the membrane.

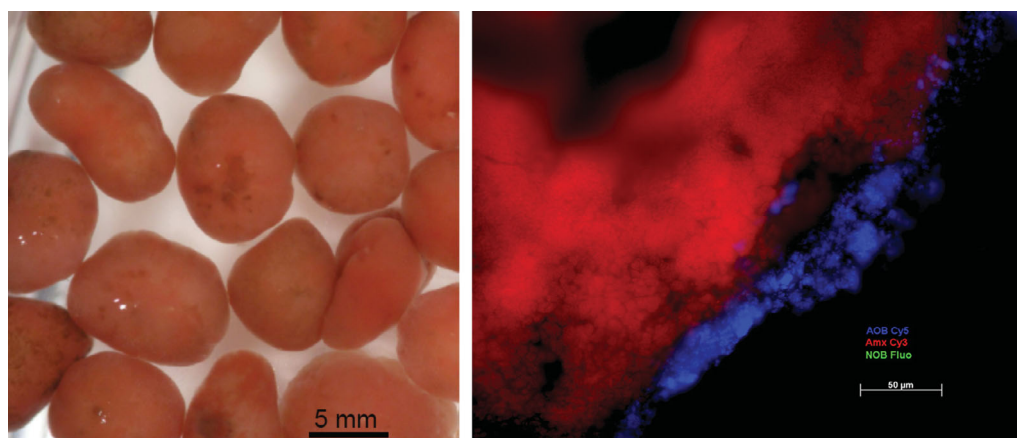


Fig. 4. Combined granules of aerobic nitrifying with anammox bacteria, with the ammonium-oxidizing bacteria in a thin layer outside and the anammox bacteria on the outside as shown in the right panel with specific fluorescent probes for the two physiological types of Bacteria. Reproduced with permission from Winkler *et al.* (2011).

One puzzling aspect discovered by Boran Kartal and Mark Strous (Kartal *et al.*, 2007) is that anammox bacteria are capable to anaerobically oxidize a few organic compounds, formate, acetate and propionate but appear not to be able to assimilate these organic compounds. The oxidation process not only can exploit nitrite but also nitrate as the electron acceptor in a dissimilatory nitrate reduction process leading to ammonium. The genome contains a nitrite reductase leading to ammonium formation, but it remains a mystery why acetate and propionate should not be able to serve as a carbon source. It should be noted that in this process the anammox organism produces its own substrates nitrite and ammonium which, when used as an energy source, will lead to the formation of nitrogen derived from nitrate only and the derived dinitrogen will carry the exclusive label of the nitrate. It would explain the presence of traces of $^{15,15}\text{N}_2$ in our early labelling experiments to prove the presence of anammox in the revived anammox reactor at Gist brocades in 1990 (Van de Graaf *et al.*, 1990).

Since the turn of the 20th-century anammox research has become widespread if not ubiquitous in all its aspects, ranging from genomics, proteomics, structural and biochemical to molecular and environmental and ecological microbiology. Given the early patent deposited by Arnold Mulder one might wonder what happened to that part of the story and its possible application. In the 1990s great scepticism existed as to the identity and feasibility of exploitation of this inherently 'slow' growing microbial culture. No company was interested but in our institute Mark van Loosdrecht asked Udo van Dongen to try and set up a duo reactor system, where ammonium was first aerobically oxidized to nitrite, and then feed the resulting effluent to a second anammox fluidized bed reactor (van Dongen *et al.*, 2001). A principal trick was to

limit the oxygen supply to the first reactor, by which a 50:50% nitrite/ammonium effluent could be created. Under these conditions, the ammonium oxidizing bacteria largely outcompeted the nitrite oxidizers. When this mixture was fed to the second reactor all ammonium was removed and the predicted output was some remaining nitrite and nitrate due to CO_2 fixation as shown in reaction (3).

This result was enough to convince the Wastewater treatment authorities, Dokhaven, in Rotterdam in combination with Paques Company (Balk, NL) to collaborate with us in the start-up of a pilot plant to remove excess ammonium from an anaerobic sludge digester. With support from the EU, an 18 m-tall fluidized bed reactor was built and put in operation (van der Star *et al.*, 2007). Although the start-up from scratch was cumbersome and the system took more than a year to function, the most difficult part turned out to trim the nitrite producing reactor. If too much nitrate was produced by nitrite oxidizers in the oxygen-limited reactor the anammox reactor would not properly function, due to too high redox potential. Fortunately, the plant performed well after 2 years. The investment paid itself back rather soon because in the previous operation of the plant all ammonium had to be oxidized to nitrate which under the specific Dokhaven conditions had to be denitrified by adding expensive methanol.

Soon after we established (Third *et al.*, 2001; Winkler *et al.*, 2011), that the aerobic and anaerobic reaction could be combined in one reactor under oxygen limitation and the newest developments in the application are now large-scale up-flow-reactors with granular flocs, which are operated under oxygen limitation (Abma *et al.*, 2007). The granules contain a thin (40 μm) layer of nitrite forming (nitrifying) bacteria and a core of anammox

bacteria as shown in Fig. 4 (Winkler *et al.*, 2011). This is a nice example of applied microbial ecology or ecotechnology. Different types of anammox reactor systems are now operating in wastewater treatment plants all over the world. The application of anammox also allows a novel approach to make conventional anaerobic wastewater treatment more sustainable (Kartal *et al.*, 2010) and even energy-producing.

The ecology of anammox is a fascinating aspect of its existence. Anammox bacteria must live where ammonium and nitrite are simultaneously present or produced as is the case in the granules shown. Generally, this occurs at the interface of aerobic or anaerobic conditions as can be found in sediments and biofilms or wherever turbulent waters mix nitrite-containing waters with anaerobic water with ammonium. The nitrite may originate from ammonium oxidizing bacteria or Archaea and from denitrifying or ammonifying microbes. This inevitably leads to competitions and cross-feeding patterns depicted in Fig. 5.

In view of all these interactions and our early puzzle and questions about which organism or organisms in the raw enrichment were responsible for the anammox reaction, it was logical to also look for (i) heterotrophic nitrification by a range of bacteria and (ii) since the early enrichments of anammox contained substantial quantities of autotrophic nitrifiers, the capability of autotrophic bacteria like *Nitrosomonas* to oxidize NH_2OH (van Niel *et al.*,

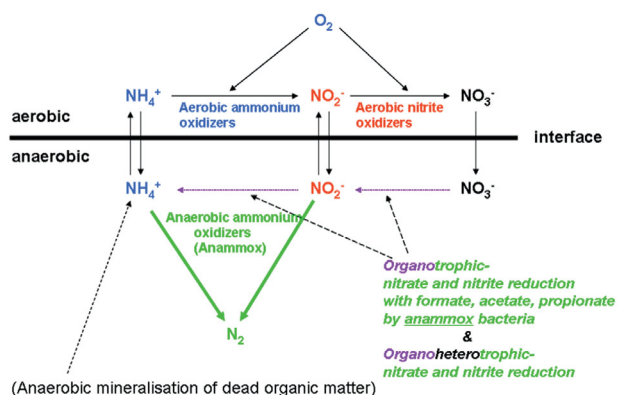


Fig. 5. Ecology of anammox: Under anoxic conditions, ammonium can be provided by mineralization of organic matter. Ammonium and nitrite may also be produced by the organo-heterotrophic metabolism of bacteria using nitrate or nitrite as electron acceptor. Anammox bacteria may also produce their own substrate ammonium and nitrite by dissimilatory nitrate reduction to ammonium (DNRA) with organic compounds as electron donors. Under aerobic conditions, nitrite may originate from autotrophic ammonium oxidizers as well as from heterotrophic ammonium oxidizers. NH_2OH , NO and even N_2H_4 may also be intermediates. At the interface, where all kinds of limitation will occur, including oxygen limitation, competition takes place 1) for ammonium between anammox bacteria and nitrifiers and bacteria assimilating ammonium as nitrogen-source, 2) for nitrite between denitrifiers, dissimilatory nitrite reducers and nitrite oxidizers, 3) for ammonium, oxygen, nitrate, nitrite, N_2O between the nitrifiers and heterotrophic bacteria.

1993; de Bruijn *et al.*, 1995; Otte *et al.*, 1999b). However, based on the extensive metagenomic analysis, we now are pretty sure that apart from the proper minerals medium no specific microorganisms are required to maintain any known anammox culture. Why, however, anammox bacteria can only be cultivated in some kind of flow-through system in which part of the spent medium is removed is still a big question.

Our research on nitrate-reducing bacteria got another exotic turn when Bo Jørgensen invited us to join an MPI expedition to Chile to try and find out more about the physiology of colourless sulphur bacterium (*Mari*)*Thioploca araucae*, living in centimetres long filaments in the marine mud off the continental shelf off Chile. It was observed that this giant bacterium stored massive quantities of nitrate intracellularly, up to 0.5 M concentrations. It turns out that this organism makes a living by autotrophic and mixotrophic metabolism, using the sulphide produced several centimetres down in the mud with nitrate as an electron acceptor for dissimilatory nitrate reduction to ammonium. Nitrite is excreted as intermediate. The giant filament can move actively up and down in the mud. At the surface, it takes up the nitrate from the nitrate-containing water coming in from the upwelling zone and by moving down it can reach the very active sulphate reduction zone. By its size and motility, it can bridge the (cm's wide) gap between the electron-donor and electron-acceptor and thus outcompete much smaller sulphide oxidizing bacteria (Otte *et al.*, 1999a). In 2006, we re-visited the place and were able to rule out that denitrification was an alternative in this *Thioploca* species (Høgslund *et al.*, 2009), though recently it has been shown that some of these large colourless sulphur bacteria (Beggiatoaceae) do have the genetic potential to perform both denitrification and dissimilatory nitrate reduction (Schutte *et al.*, 2018). Since *Thioploca* excretes nitrite in the presence of plenty of ammonium in the anaerobic sediment we also looked for anammox bacteria attached to *Thioploca* filaments with a specific fluorescent probe for anammox bacteria. We did observe a few cells (J. van de Vosseberg and J.G. Kuenen unpublished results) and later the presence of anammox in those sediments was supported by the vertical isotopic distribution of N-compounds in the environment (Prokopenko *et al.*, 2013).

What else to expect for future anammox research? This is carefully analysed in the recent review by Peeters and Van Niftrik (2019) and I just give a few examples: how is the proton motive force built up over the anammoxosomal membrane, what is the role and identity of the many HAOs and other key enzymes of the ammonium oxidation and production of reducing power for CO_2 assimilation via the acetyl CoA pathway. How are the ladderanes synthesized and is their role to contain intermediates in the anammoxosome? What is

the niche differentiation of the big spectrum of the anammox bacteria and what is the role of organic compounds and alternative (in)organic electron donors and alternative extracellular polysaccharides, glycolipids or proteins in this diversity? Finally, how to obtain a truly pure culture so that the organisms can finally lose their *Candidatus*-prefix.

The question where did it begin inevitably leads to the question where did it end? Microbiology remained my hobby and in fact, after my formal retirement in 2005 more time became available for research.

Ken Nealson at USC did not need much time to lure me into spending 2–3 months each year at USC in Los Angeles, which lasted another 12 years. There I fell in love with hyper-alkaliphiles, which can grow up to pH 12.3 or 12.5. They live in alkaline, ‘serpentine’ springs North of San Francisco in ‘The Cedars’, high up in the mountains near the Russian River (Morrill *et al.*, 2013). A wealth of anaerobes have been identified (Suzuki *et al.*, 2017). The only pure cultures obtained are microaerophilic bacteria, which have been named *Serpentinomonas* spp. (Suzuki *et al.*, 2014). Their genomes have been sequenced and *in vivo* and *in situ* transcriptomics have indicated that they live primarily as autotrophs on hydrogen gas that originates from the serpentinization process where water reacts with reduced iron at greater depth and temperature in the mountains. They grow slowly and at very low density, but we have managed to cultivate them in chemostats growing at pH values between 10 and 12.3 in order to reveal the secrets of being a hyper-alkaliphile. The results are still in the works and it is interesting to mention that some of the isolates can denitrify, which closes the circle of my interests in nitrogen cycling.

In the context of this article I will just mention my short but intense exploration in the physiology of the predatory *Bdellovibrio* in the lab of Syd Rittenberg (Kuenen and Rittenberg, 1975), the honour of meeting Cees van Niel in person and sabbaticals with Yehuda Cohen (Jørgensen *et al.*, 1979) in Eilat Israel, Bo Jørgensen (Kuenen *et al.*, 1986) in Arhus, Denmark and Dave Ward (Ruff-Roberts *et al.*, 1994) in Bozeman Montana (US). Last but not least I have a lifelong interest and involvement in the (eco)physiological research of yeasts directed by my colleagues in the lab, Lex Scheffers (W.A.), Hans van Dijken (J.P.) and my successor Jack T. Pronk at TUDelft.

In Delft, I still have the privilege of remaining the bouncing board for some anammox research. I am now part of Mark van Loosdrecht’s group who has played a major role in the application side of anammox and also involved in ecophysiological research concerning denitrifying bacteria and organisms performing dissimilatory nitrate reduction and bacteria, which prefer N₂O as terminal electron acceptor (van den Berg *et al.*, 2016).

Physiological and competition experiments are carried out to study the selection and performance of the different physiological types (Conthe *et al.* 2018). Such cultures are grown next to anammox bacteria, which are studied in modern recycling reactors with membrane modules. In these reactors, anammox bacteria are grown as single suspended cells and under these conditions, the cultures reach a ‘purity’ of almost 98% of one species. The method, first developed by van der Star *et al.* (2008)), is now the routine method of growing these organisms in Delft and Nijmegen for any study concerning the ‘omics’, physiology and structure studies.

This is my story about ‘how we did it’ and how it began. Here it ends for the author but is comforting to realize that the wonders of microbiology will continue forever.

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