Reviews in Environmental Science & Bio/Technology (2004) 3: 255–264

Application, eco-physiology and biodiversity of anaerobic ammonium-oxidizing bacteria

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Received 4 June 2004; accepted 6 December 2004

Key words: Anammox, Brocadia, denitrification, nitrification, nitrite, nitrogen removal, planctomycetes, 16S rRNA gene, urea

Abstract

The demand for new and sustainable systems for nitrogen removal has increased dramatically in the last decade. It is clear that the conventional systems cannot deal with the increasing nitrogen loads in a cost effective way. As an alternative, the implementation of the anammox (*an*aerobic *amm*onium *ox*idation) process in the treatment of wastewater with high ammonium concentrations has been started. The compact anammox reactors can sustain high nitrogen loads without any problems. The highest observed anammox capacity is 8.9 kg N removed m^{-3} reactor day^{-1}. The first 75 m^3 anammox reactor is operating in Rotterdam, the Netherlands, combined with the partial nitrification process Single reaction system for High Ammonium Removal Over Nitrite (SHARON). Partial nitrification and anammox can also be combined in one reactor systems like Completely Autotrophic Nitrogen removal Over Nitrite (CANON) or Oxygen Limited Ammonium removal via Nitrification Denitrification (OLAND) where aerobic ammonium-oxidizing bacteria (AOB) and anammox bacteria cooperate under oxygen-limitation. These systems remove about 1.5 kg N m^{-3} reactor day^{-1}. In addition to ammonium, urea can also be converted in the CANON system after a two-week adaptation period. The ecophysiological properties of the anammox bacteria make them very well suited to convert ammonium and nitrite. The K_s values for ammonium and nitrite are below 5 μM. However, nitrite above 10 mM is detrimental for the anammox process, and oxygen reversibly inhibits the process at concentrations as low as 1 μM. Acetate and propionate can be used by the anammox bacteria to convert nitrite and nitrate, whereas methanol and ethanol severely inhibit the anammox reaction. The enzyme hydroxylamine/hydrazine oxidoreductase (HAO), one of the key enzymes, is located in the anammoxosome, which is a membrane bound organelle. The membranes of the anammox bacteria contain unique ladderane lipids and hopanoids. The bacteria responsible for the anammox reaction are related to the Planctomycetes. The first anammox bacteria were isolated via Percoll centrifugation and characterized as *Candidatus* ''Brocadia anammoxidans''. Survey of different wastewater treatment plants using anammox specific 16S rRNA gene primers and anammox specific oligonucleotide probes has revealed the presence of at least three other anammox bacteria, which have been tentatively named *Candidatus*

''Kuenenia stuttgartiensis'', *Candidatus* ''Scalindua wagneri'' and *Candidatus* ''Scalindua brodae''. A close relative of the latter, *Candidatus* ''Scalindua sorokinii'' was found to be responsible for about 50% of the nitrogen conversion in the anoxic zone of the Black Sea, making the anammox bacteria an important player in the oceanic nitrogen cycle.

Abbreviations: ANAMMOX – anaerobic ammonium oxidation; AOB – aerobic ammonium-oxidizing bacteria; CANON – completely autotrophic nitrogen removal over nitrite; NOB – nitrite-oxidizing bacteria; OLAND – oxygen limited ammonium removal via nitrification denitrification; RBC – rotating biological contactor; SHARON – Single reactor system for high ammonium removal over nitrite; SBR – sequencing batch reactor

1. Introduction

Over the last decade, growing populations and industrialization increased the drinking and potable water requirement and wastewater discharge. Clearly the increase in the water demand will put more pressure on the global water resources. In order to protect the potable water reservoirs from the discharge of the untreated domestic and industrial wastewaters, the European Union has been implementing more and more stringent directives (Directive 91/271/EEC).

Typical wastewater treatment plants are principally designed to remove the carbonaceous material from the waste streams and do not reach the 10 mg l^{-1} discharge standard for total nitrogen. New and sustainable technologies are needed to comply with the stringent discharge standards (van Loosdrecht et al. 2004).

The anammox reaction, a microbial process long overlooked, is emerging as an attractive alternative to replace the nitrification and denitrification processes that are primarily used to remove nitrogen from wastewater (Jetten et al. 2001, 2002; Strous & Jetten 2004).

In the anammox process, chemolithoautotrophic bacteria convert 1 mol of nitrite and 1 mol of ammonium directly to dinitrogen gas with hydrazine as an intermediate (Jetten et al. 1998). Due to extremely low growth rate, anammox bacteria can only be cultivated with very efficient biomass retention (Strous et al. 2002). The bacteria responsible for the process have been physically purified from highly enriched cultures by Percoll density gradient centrifugation (Strous et al. 1999b). The first purified anammox bacterium is named *Candidatus* ''Brocadia anammoxidans'' (Kuenen & Jetten 2001).

2. Properties of the anammox bacteria

The anammox bacteria are very well suited to convert their substrates. They have K_s values for ammonium and nitrite below 5 μM (Strous et al. 1999a). However, they are reversibly inhibited by very low levels (< 1 μM) of oxygen and irreversibly inhibited by high nitrite (> 10 mM) concentrations (Strous et al. 1997, 1999a, 2002). The anammox pathway has been elucidated using ^{15}N-labelling experiments, which showed that hydrazine is an important intermediate. As far as we know, the occurrence of free hydrazine in microbial nitrogen metabolism is rare, if not unique (Jetten et al. 1998). The chemolithoautotrophic life style of the anammox bacteria has been established by $^{14}CO_2$ incorporation into the cells and confirmed by mass balances (Strous et al. 1999b). In order to elucidate the pathway for carbon dioxide fixation of the anammox bacteria, $^{13}CO_2$ was supplied to an anammox Sequencing Batch Reactor (SBR) culture. The labelling pattern of the extremely ^{13}C-depleted (−47%) lipids showed that either the reductive pentose phosphate or the acetyl-CoA pathway was used (Schouten et al. 2004). In recent studies it has been shown that anammox bacteria are severely inhibited by methanol and ethanol at concentrations below 1 mM (Güven et al. 2004, 2005). However the anammox bacteria are able to use acetate and propionate as energy source for the reduction of nitrite and nitrate (Hao et al. 2004; Güven et al. 2004; 2005). At C/N ratios above 1 the anammox bacteria are no longer able to compete with heterotrophic denitrifying bacteria (Güven et al. 2005).

The HAO enzyme hydroxylamine/hydrazine oxidoreductase was purified from *Candidatus*

''Brocadia anammoxidans'' (Schalk et al. 2000). Unique peptide sequences of tryptic fragments have been used to locate the *hao* gene in the *Candidatus* ''Kuenenia stuttgartiensis'' genome. The purified enzyme has also been used to raise polyclonal antibodies for localization studies (Lindsay et al. 2001). Using immunogold electron microscopic analysis, the enzyme has been found to be present exclusively inside one of the membrane bounded organelles (the anammoxosome), that made up more than 30% of the cell volume (van Niftrik et al. 2004) (Figure 1). This dedicated intracytoplasmic compartment has been found to be surrounded by a membrane nearly exclusively composed of unique ladderane lipids (Sinninghe Damste et al. 2002, 2004a, b; Schmid et al. 2003). These lipids are composed of pentacycloanammoxic acids, which contain five linearly concatenated cyclobutane rings (Mascitti & Corey 2004). The ladderane lipids occur both as ether and ester lipids in all three groups of anammox bacteria (Jetten et al. 2003). Due to the very slow metabolism of the anammox bacteria a very dense and impermeable membrane is required to maintain concentration gradients during the anammox reaction. Such a membrane also protects the cell from the toxic intermediates. The anammoxosome membrane, having a lower degree of rotational freedom and being significantly denser (1.5 kg dm^{-3}) and impermeable than a conventional membrane (1.0 kg dm^{-3}), is perfectly suited for both of the above-mentioned tasks (Sinninghe Damste et al. 2002). The recent finding of hopanoids, which act as rigidifiers in the membranes of the anaerobic anammox bacteria, gives further support to the necessity of a very dense membrane to limit diffusion of protons and intermediates (Sinninghe Damste et al. 2004a, b; van Niftrik et al. 2004).

3. Application of anammox bacteria

Both in natural and man-made ecosystems anammox bacteria have to be provided with their substrates ammonium and nitrite. Natural anoxic ecosystems such as marine sediments can contain substantial amounts (mM range) of ammonium due to the degradation of organic matter (Kuypers et al. 2003; Trimmer et al. 2003). In the case of water columns and marine ecosystems, nitrate-reducing bacteria are the most likely source of nitrite (Dalsgaard et al. 2003; Kuypers et al. 2003). Nitrite can also be produced by aerobic ammonium oxidizing bacteria operating at the oxic-anoxic interface of many ecosystems (Schmidt et al. 2002)

The cooperation between the aerobic and anaerobic ammonium oxidizing bacteria is the microbial basis of the integrated reactor system *C*ompletely *A*utotrophic *N*itrogen removal *O*ver *N*itrite (CANON) and *O*xygen-*L*imited *A*utotrophic *N*itrification-*D*enitrification (OLAND) process. In these systems, aerobic ammonium-oxidizing bacteria (AOB) and the planctomycete-like anammox bacteria perform two sequential reactions simultaneously (Kuai & Verstraete 1998; Philips et al. 2002; Pynaert et al. 2002a, b, 2003, 2004; Schmidt et al. 2003; Sliekers et al. 2002, 2003; Third et al. 2001; 2005; Wyffels et al. 2003a, b).

In the CANON reactor system, under oxygen limitation, the supplied ammonium is partly oxidized to nitrite by AOB. The produced nitrite is utilized with the remainder of the ammonium by

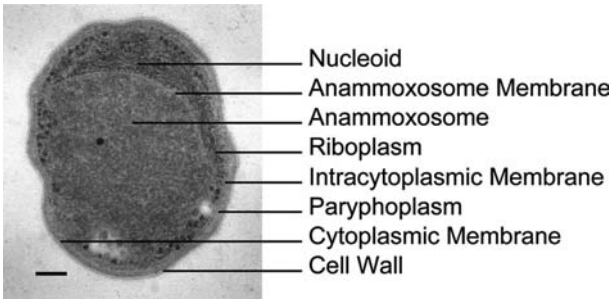

Figure 1. Electron micrograph of the bacterium *Candidatus* ''Brocadia anammoxidans'' showing typical compartmentalization. The bar is 100 nm.

anammox bacteria and converted into dinitrogen gas. The feasibility of the CANON concept has been established by carefully introducing limited amounts of oxygen into anammox SBR systems. Within 2 weeks a new stable consortium of AOB and anammox becomes operative (Sliekers et al. 2002). Fluorescence *in situ* hybridisation (FISH) analysis of the CANON biomass shows that about 40% of the community consisted of AOB, also the anammox cells constitute about 40% of the community. No aerobic nitrite oxidizing bacteria (NOB, *Nitrospira* or *Nitrobacter* species) have ever been detected. Activity tests confirm the absence of NOB. The NOB are only able to develop in the CANON reactor after prolonged exposure (>1 month) to ammonium limitation (Third et al. 2001). The situation is quite different in Rotating Biological Contactor (RBC) based systems where the supply of ammonium and oxygen are difficult to control. In these reactors all three groups of bacteria, AOB, NOB and anammox can coexist simultaneously (Egli et al. 2002; 2004; Pynaert et al. 2003).

The upper limits of nitrogen loading of both anammox and CANON processes were explored in gas lift reactors (Sliekers et al. 2003; Dapena-Mora 2004a–c) (Figure 2). In these reactors anammox planctomycetes were able to remove 8.9 kg N m^{-3}reactor day^{-1}. In the same setup the combined action of AOB and anammox planctomycetes achieved 1.5 kg N removal m^{-3} reactor day^{-1}, which is more than sufficient to start application trials. The 1.5 kg N m^{-3} reactor day^{-1} removal capacity was also reached recently in a RBC system which contained both the "*Candidatus* Kuenenia" anammox bacteria and *Nitrosomonas* related AOB (Pynaert et al. 2003, 2004).

The distribution of AOB and anammox bacteria in a CANON system was investigated using ^{15}N-labelled substrate and novel nitrite microsensors (Nielsen et al. 2004; 2005). Under oxygen-limited conditions (<5 μM O_2), AOB were restricted to the outer shell (<100 μm) of the CANON aggregates, while anammox bacteria were found in the central anoxic parts. The larger type aggregates (>500 μm) accounted for about 68% of the anammox potential whereas 65% of the nitrification potential was found in the smaller aggregates (<500 μm). Analysis with O_2 and nitrite microsensors showed that the thickness of the activity zones varied as a function of bulk oxygen and nitrite concentrations and flow rate. This is in good agreement with the biofilm models developed by Hao et al. (2002a, b; 2004).

Figure 2. CANON oxygen-limited gas lift reactor in which aerobic ammonium-oxidizing bacteria and anammox bacteria cooperate to remove ammonium directly into dinitrogen gas (Sliekers et al. 2003).

In a separate study, urea was tested as an alternative energy source for the microbial consortium in the CANON reactor system (Sliekers et al. 2004). Urea is a major source of nitrogen input to both wastewater streams and natural ecosystems. Human excretion and leachate from agriculture fields are the two main sources of urea. It has been proposed that a carbon source should be supplied for complete urea hydrolysis by heterotrophic bacteria in wastewaters without organic carbon (Rittstieg et al. 2001). Urea conversion by CANON biomass is much more cost-effective since it is evident that the system is completely autotrophic and does not require additional organic carbon (Sliekers et al. 2004). Tests with

lab-scale reactors demonstrated that when urea is supplied to the CANON reactor it is immediately converted to dinitrogen gas and full capacity is reached within two weeks of adaptation (Sliekers et al. 2004). In these two weeks, the urease-positive *Nitrosomonas oligotropha* and *Nitrosomonas nitrosa* become the dominant AOB in the urea-converting CANON systems. Anammox bacteria do not hydrolyse urea themselves, but rely on the *N. oligotropha* and *N. nitrosa* urease activity to be supplied with sufficient amounts of ammonium and nitrite (Sliekers et al. 2004). Further tests have clearly demonstrated that CANON or anammox systems are well suited to treat separately collected urine waste (Wilsenach & van Loosdrecht 2003; Udert et al. 2003).

The possibility to use the SHARON (*S*ingle reactor system for *H*igh rate *A*mmonium *R*emoval *O*ver *N*itrite) process in combination with anammox has also been investigated. SHARON process was developed for the removal of ammonium via the so-called nitrite route (Jetten et al. 1997; Van Hulle et al. 2004). It has been tested for 2 years in the laboratory and successfully scaled-up from 2 l to an 1800 m^3 full- scale plant (Jetten et al. 1997; 2002; Mulder et al. 2001; van Dongen et al. 2001). In the SHARON process, the ammonium is oxidized for 53% to nitrite at 1.2 kg N m^{-3} day^{-1}, without pH control. The ammonium/nitrite ratio in the effluent of the SHARON process can be fine-tuned by adjusting the pH between 6.5 and 7.5. The effluent of this SHARON reactor is fed to an anammox SBR that removes all nitrite. The specific activity of the anammox biomass is relatively high: 0.8 kg N (kg dry weight)$^{-1}$ day^{-1} and the load can be increased to more than 2 kg N m^{-3} day^{-1} (van Dongen et al. 2001; Fux et al. 2002).

The SHARON-anammox process has been patented and implemented in the wastewater treatment plant in Rotterdam (Figure 3). Based on the design of the combined SHARON-anammox process a cost estimate of 0.75 € kg^{-1} N is made (van Dongen et al. 2001; van Loosdrecht et al. 2004). This is very low compared to the 2–5 € kg^{-1} N that is calculated for other processes that have been tested on pilot plant scale for N-removal from sludge digestion liquors (van Dongen et al. 2001; Jetten et al. 2002).

As already mentioned anammox bacteria have an extremely low growth rate, and thus only systems with very efficient biomass retention like the SBR or RBC can be used to cultivate these organisms (Strous et al. 1998; Dapena-Mora 2004a; Fux et al. 2002, 2004; Pynaert et al. 2003; Wyffels 2003a, Schmidt et al. 2004). In addition solid support materials like glass beads (Strous et al. 1997), Kaldnes rings (Helmer et al. 2002) or non-woven biomass carriers (Fujii et al. 2002; Furukawa et al. 2003; Imajo et al. 2004) are good alternatives to ensure that anammox biomass is retained in the reactor systems. A comparison of the various reactor systems was recently described in detail (Schmidt et al. 2003; Sliekers et al. 2004; Pynaert et al. 2004).

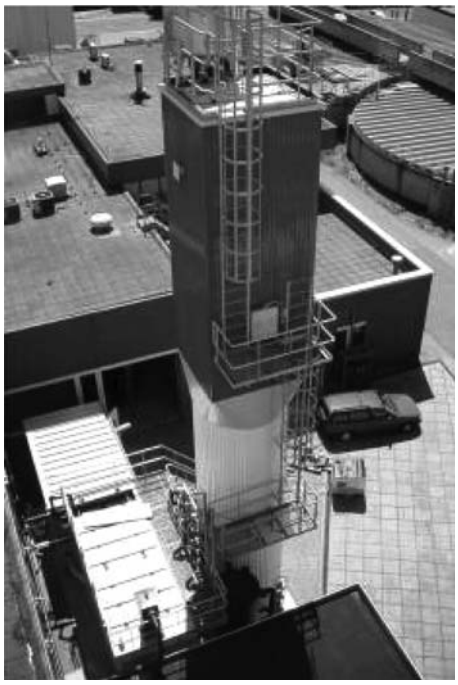

Figure 3. Anammox reactor at Rotterdam WWTP (Courtesy Paques and ZHEW) in EU-icoN project.

During the start up period of the 75 m^3 anammox wastewater treatment plant in Rotterdam, biomass from the anammox reactor was investigated for its potential nitrogen removal capacity in a laboratory scale SBR. A 2 l SBR reactor was inoculated with 800 ml sludge from the 75 m^3 anammox reactor in Rotterdam. FISH studies showed that the anammox bacteria constituted 0–1% of the initial biomass. The nitrogen load was increased gradually from 0.14 kg m^{-3}

reactor day^{-1} to 3 kg m^{-3} reactor day^{-1} in 96 days (Figure 4). At the end of this 96-day period, 85% of the biomass consisted of anammox bacteria (Figure 5), and the specific activity of the biomass was 1.02 kg N (kg dry weight)$^{-1}$ day^{-1}. The 16S rRNA gene was amplified from DNA extracted from the reactor, and the dominant 16S rRNA gene had a 96% sequence identity to *Candidatus* ''Brocadia anammoxidans'' (Figure 6).

A striking feature of this anammox bacterium is its very bright autofluorescence. This seems to be unique for this anammox species and may occur due to the polysaccharides excreted by these anammox bacteria for floc and aggregate formation. This phenomenon was the inspiration to name this anammox bacterium *Candidatus* ''Brocadia fulgida'' (Figure 6).

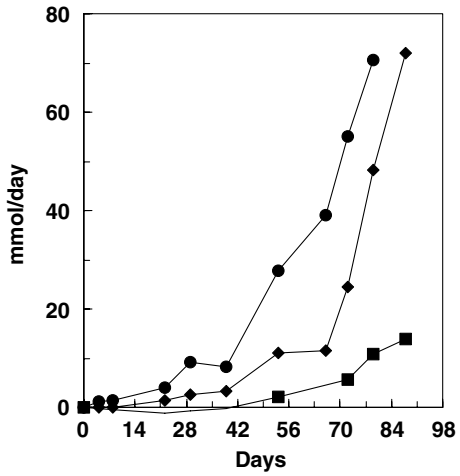

Figure 4. Consumption of nitrite (•) and ammonium (♦), production of nitrate (■) in anammox SBR reactor inoculated with biomass from the Rotterdam plant.

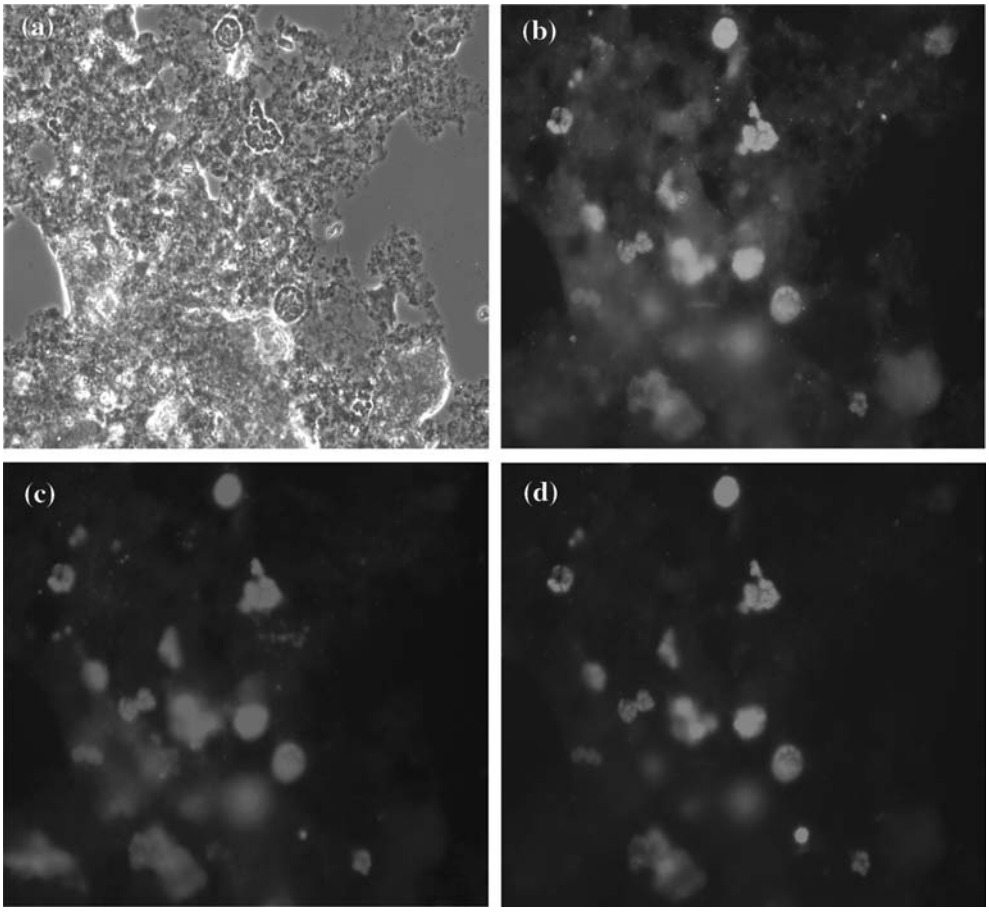

Figure 5. FISH micrographs of the anammox biomass in lab-scale SBR after 80 days of enrichment. (a) phase contrast picture; (b) Green FLUOS-AMX368 showing all anammox cells; (c) Purple Cy5-EUB338 showing nearly all bacterial cells; (d) Red Cy3-AMX820, showing all Brocadia cells.

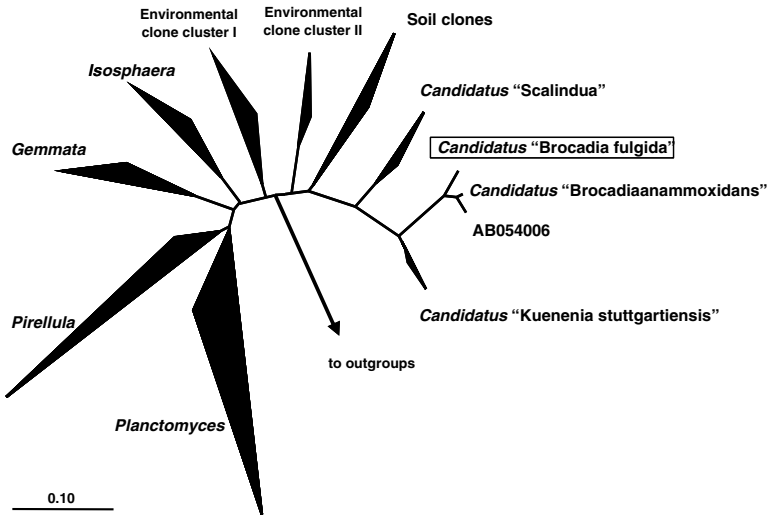

Figure 6. 16S rRNA gene based phylogenetic tree reflecting the relationship of Candidatus "Brocadia fulgida" to other anammox organisms, other *Planctomycetes* and other reference organisms. Tree reconstruction was performed as published by Schmid et al., 2003. The triangles indicate phylogenetic groups. The bar represents 10% sequence divergence.

4. Biodiversity of anammox bacteria

Many water treatment systems and fresh water ecosystems also appeared to contain significant populations of anammox bacteria, some of those were only distantly related (Figure 6; less than 90% similarity on 16S rRNA gene) to the *Candidatus* "Brocadia" branch (Fujii et al. 2002; Helmer et al. 2002; Toh & Asbolt 2002; Toh et al. 2002; Dong & Tollner 2003; Egli et al. 2003; Jetten et al. 2003; Pynaert et al. 2003). These anammox bacteria have been named *Candidatus* "Kuenenia stuttgartiensis" (Egli et al. 2001; Schmid et al. 2000, 2005). Furthermore a new group of 'Scalindua' anammox bacteria have been discovered in the Black Sea (Kuypers et al. 2003) and in a UK wastewater treatment plant (Schmid et al. 2003). In this treatment plant about 20% of the population consists of two new anammox species, named *Candidatus* "Scalindua wagneri" and *Candidatus* "Scalindua brodae". *Candidatus* "Scalindua sorokinii" in the Black Sea is the first anammox bacterium directly linked to the removal of fixed inorganic nitrogen from a natural ecosystem. Recent field experiments have revealed that anammox can contribute as much as 70% to dinitrogen gas production in marine ecosystems (Kuypers et al. 2003; Thamdrup & Dalsgaard 2002). Other studies also indicate that marine anammox bacteria play a very important role in the oceanic nitrogen cycle (Dalsgaard & Thamdrup 2002; Dalsgaard et al. 2003; Trimmer et al. 2003; Ward 2003; Risgaard-Petersen et al. 2004; Rysgaard & Glud 2004).

5. Conclusions

As a sustainable and low cost alternative to the presently used nitrification-denitrification processes, the combination of partial nitrification and anammox is ready for full scale implementation in nitrogen removal which will lead to substantial savings in energy and resources. Both in natural and man-made ecosystems anammox bacteria contribute significantly to dinitrogen gas formation. Since anammox bacteria have many unique properties, current and future research on the biodiversity, physiology and the application of the anammox bacteria are bound to reveal new and interesting information that can be utilized for the practical use of the process.

Acknowledgements

The research on anaerobic ammonium oxidation is supported by the Foundation for Applied Sciences (STW grant NPC 5987), the European Union (grant EESD EVK1-CT-2000-00054), the Foun-

dation of Applied Water Research (STOWA), the Netherlands Foundation for Earth & Life Sciences (NWO-ALW, Biogeosphere grants 853.00.012 and 853.00.031), and Paques BV.

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