APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2005, p. 1677–1684 Vol. 71, No. 4
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.4.1677–1684.2005

MINIREVIEW

Biomarkers for In Situ Detection of Anaerobic Ammonium-Oxidizing (Anammox) Bacteria

Markus C. Schmid,[1] Bart Maas,[2] Ana Dapena,[3] Katinka van de Pas-Schoonen,[2] Jack van de Vossenberg,[2] Boran Kartal,[2] Laura van Niftrik,[1] Ingo Schmidt,[2] Irina Cirpus,[1] J. Gijs Kuenen,[1] Michael Wagner,[4] Jaap S. Sinninghe Damsté,[5] Marcel Kuypers,[6] Niels Peter Revsbech,[7] Ramon Mendez,[3] Mike S. M. Jetten,[1,2] and Marc Strous[2]*

Kluyver Laboratory for Biotechnology, Delft University of Technology, Delft,[1] Department of Microbiology, Faculty of Science, Radboud University Nijmegen, Nijmegen,[2] and Department of Biogeochemistry, Royal Netherlands Institute for Sea Research (NIOZ), Den Burg, Texel,[5] The Netherlands; Department of Chemical Engineering, University of Santiago de Compostela, Santiago de Compostela, Spain[3]; Department of Microbial Ecology, University of Vienna, Vienna, Austria[4]; Max Planck Institute for Marine Microbiology, Bremen, Germany[6]; and Department of Microbial Ecology, University of Aarhus, Aarhus, Denmark[7]

The existence of anaerobic ammonium oxidation (anammox) was hypothesized based on nutrient profiles and thermodynamic calculations (5, 31, 44). It was first discovered about 1 decade ago (25) in a pilot plant treating wastewater from a yeast-producing company in Delft, The Netherlands. The anammox reaction is the oxidation of ammonium under anoxic conditions with nitrite as the electron acceptor and dinitrogen gas as the product. Hydroxylamine and hydrazine were identified as important intermediates (51). Due to their very low growth rates (doubling time in enrichments is at best 11 days) the cultivation of the anammox bacteria proved to be tedious and required very efficient biomass retention (41, 43). A physical purification of anammox organisms from enrichment cultures was achieved with percoll density centrifugation (42). The purified cells performed the anammox reaction after activation by hydrazine. Based on phylogenetic analysis, the discovered anammox organism branched deep in the *Planctomycetes* phylum (Fig. 1A and B, [42]) and was named "*Candidatus* Brocadia anammoxidans" (19).

After the first discovery, nitrogen losses, which could only be explained by the anammox reaction, were reported in other wastewater treatment facilities including landfill leachate treatment plants in Germany, Switzerland, and England (11, 14, 15, 36), as well as in semitechnical wastewater treatment plants in Germany (34), Belgium (30), Japan (12), Australia (48), and the United States (10, 45). Molecular techniques showed the presence of organisms affiliated with the anammox branch within the *Planctomycetes* in all these wastewater treatment plants.

Nutrient profiles and ^{15}N tracer studies in suboxic marine and estuarine environments indicated that anammox is also a key player in the marine nitrogen cycle (8, 46, 49). In addition, 16S rRNA gene analysis, fluorescence in situ hybridization (FISH), the distribution of specific anammox membrane lipids, nutrient profiles, and tracer experiments with [^{15}N]ammonia showed the link between the anammox reaction and the occurrence of the anammox bacterium "*Candidatus* Scalindua sorokinii" in the suboxic zone of the Black Sea (20).

The anammox reaction has also been tested for implementation for full-scale removal of ammonia in wastewater treatment (13, 52, 53). The detection and identification of active anammox organisms in environmental samples combined with information on environmental conditions can facilitate the search for possible biomass sources to be used as an inoculum for laboratory, semitechnical, or full-scale anammox reactors. Additionally, such information could provide insights into the niche differentiation of anammox organisms. This review summarizes the recent advances made in the 16S rRNA gene-based techniques for the detection of anammox bacteria. A convenient PCR detection method for anammox organisms is presented in which anammox-specific FISH probes were used as primers. Furthermore, methods which link activity and the detection of anammox bacteria, such as the combination of FISH and microautoradiography (FISH-MAR) (22) as well as FISH targeting the intergenic spacer region (ISR) between the 16S and 23S rRNA are discussed and compared to conventional methods to detect anammox activity.

Each of these approaches by itself only addresses limited aspects, such as abundance, activity, or physiology. Thus, a combination of rRNA-based and non-rRNA-based methods is necessary to allow a comprehensive study of anammox bacteria in their ecosystems.

NON-rRNA-BASED INDICATORS FOR THE PRESENCE AND ACTIVITY OF ANAMMOX BACTERIA

Tracer experiments with ^{15}N-labeled ammonium and nitrite are the method of choice for the detection of anammox activity. Under anoxic conditions, labeled [^{15}N]ammonium reacts

* Corresponding author. Mailing address: Department of Microbiology, Radboud University Nijmegen, Toernooiveld 1, NL 6525 ED Nijmegen, The Netherlands. Phone: 31 24 36 52657. Fax: 31 24 36 52830. E-mail: m.strous@science.ru.nl.

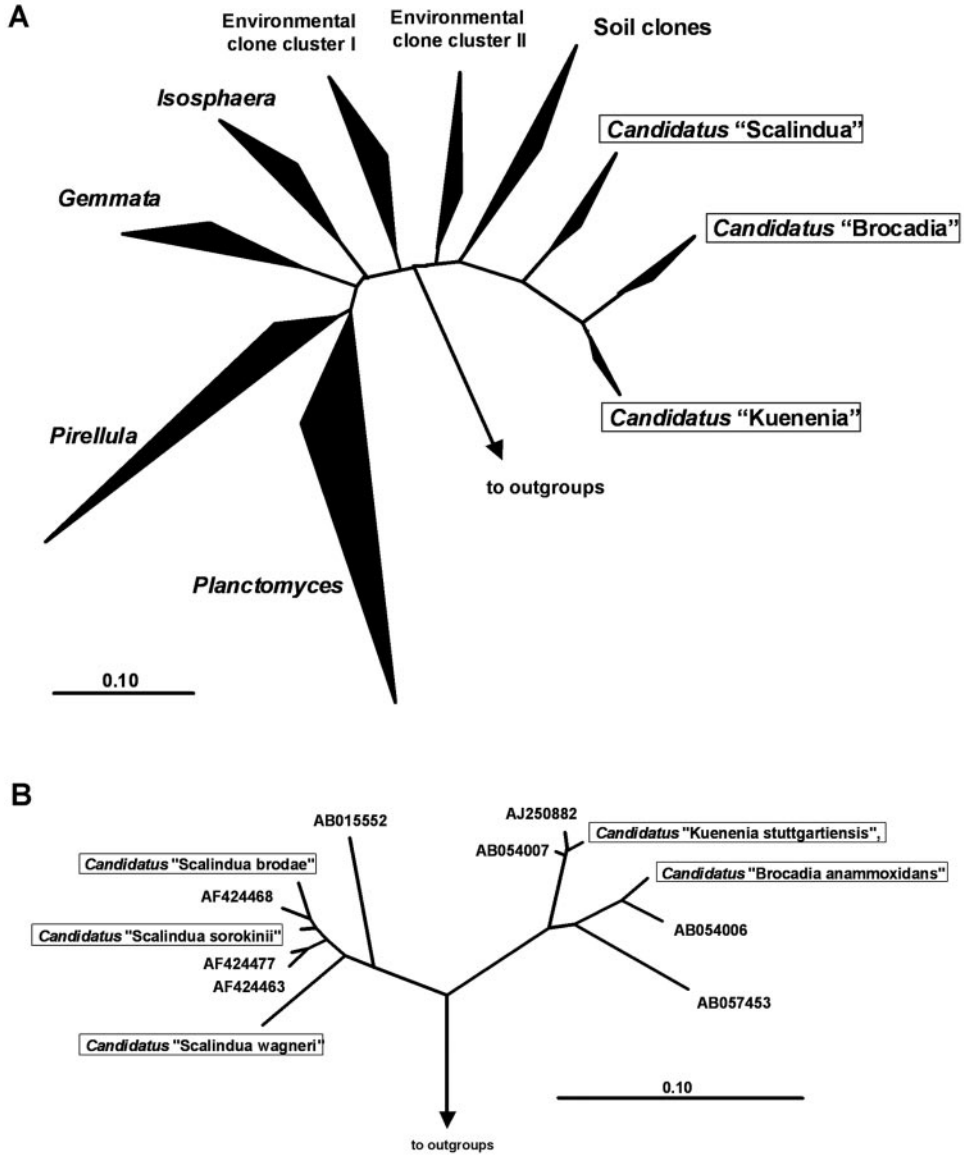

FIG. 1. (A) 16S rRNA gene-based phylogenetic tree reflecting the relationship of "*Ca.* Scalindua," "*Ca.* Brocadia," and "*Ca.* Kuenenia" to other *Planctomycetes* and other reference organisms. Tree reconstruction was performed by maximum likelihood analysis with a 50% conservation filter for *Bacteria*. Neighbor-joining and maximum parsimony analysis with different conservation filters verified the tree topology. The triangles indicate phylogenetic groups. The bar indicates 10% sequence divergence. (B) 16S rRNA gene-based phylogenetic tree reflecting the relationship of "*Ca.* Scalindua brodae," "*Ca.* Scalindua sorokinii," "*Ca.* Scalindua wagneri," "*Ca.* Brocadia anammoxidans," and "*Ca.* Kuenenia stuttgartiensis" to other sequences (given as GenBank accession numbers) derived from environments and bioreactors performing the anammox reaction. Tree reconstruction was performed by maximum likelihood analysis with a 50% conservation filter for *Planctomycetes*. Neighbor-joining and maximum parsimony analysis with 50% conservation filters for *Bacteria* and *Planctomycetes* verified the tree topology. The bar indicates 10% sequence divergence.

uniquely, in a 1:1 ratio with unlabeled [^{14}N]nitrite, to $^{29}N_2$ ($^{14}N^{15}N$) via the anammox reaction. This method has been used successfully to assess the contribution of anammox to nitrogen conversions in marine and estuarine environments such as the oxygen minimum zones in the Black Sea and Golfo Dulce, where the numbers of anammox cells are low (8, 20, 46, 49).

Anammox bacteria have unique lipids (38, 54) that can be used as biomarkers for the presence of anammox cells in the environment (20, 36). The ladderane lipids are especially suitable, but anammox bacteria also produce characteristic, branched fatty acids (38). Recently, it has been found that anammox bacteria also produce a variety of hopanoids (39). Anammox bacteria are the first strict anaerobes that have been shown to biosynthesize these bacterial membrane rigidifiers. Lipids from anammox bacteria are characterized by substantially lower ^{13}C content than their carbon source (36, 37). The ^{13}C content of ladderane and other lipids is approximately 45‰ depleted compared to their carbon source, whereas lipids from other autotrophic organisms generally are 20 to 30‰ depleted. The isotopic composition of anammox lipids in en-

vironmental samples can thus be an additional confirmation of their origin.

The conversion of hydroxylamine to hydrazine is a unique reaction catalyzed by anammox bacteria (51), which can also be used specifically to detect anammox activity in environmental samples (36). Because this assay requires rather high anammox cell numbers, it can only be used in samples where anammox bacteria forms a substantial part (10 to 20%) of the microbial population (36). If anammox bacteria are present in such high numbers, and this is really the case in some habitats (32), anammox activity can also be directly assessed by measuring the simultaneous consumption of ammonium and nitrite under anoxic conditions (25, 50) or the pressure exerted by the nitrogen gas produced in the anammox reaction (9).

In the future, anammox bacteria might also be detected in environmental samples via immunofluorescence with antibodies raised against anammox-specific proteins, e.g., hydroxylamine oxidoreductase (33). A genomics project on "*Candidatus* Kuenenia stuttgartiensis" is under way and will reveal more anammox-specific genes and proteins, which could be exploited as specific targets for antibodies in future experiments.

Recently very sensitive biosensors for online nitrite monitoring have become available (16, 27), making the sensitive detection of anammox activity in reactor systems or sediments possible (32).

16S rRNA-BASED PHYLOGENY OF ANAMMOX BACTERIA

In 1999 the first 16S rRNA sequence of an anammox organism, "*Ca*. Brocadia anammoxidans," was placed within the phylum *Planctomycetes* (42). In the past three years, many surveys of wastewater treatment systems as well as marine samples have been completed. Thereby, the anammox organisms "*Ca*. Kuenenia stuttgartiensis," "*Ca*. Scalindua sorokinii," "*Candidatus* Scalindua brodae," and "*Candidatus* Scalindua wagneri" have been discovered, and their 16S rRNA sequences are known (Fig. 1A and B) (20, 34, 36). Phylogenetic analysis of these new anammox 16S rRNA sequences has shown that these bacteria form a monophyletic branch within the phylum *Planctomycetes* (Fig. 1). This branch consists of three distinct genera with about 90% 16S rRNA sequence similarity to each other (Fig. 1A) (34, 36). The low 16S rRNA sequence similarities of anammox organisms to other genera of the *Planctomycetes* such as *Gemmata*, *Isosphaera*, *Planctomyces*, or *Pirellula* (below 80%) suggest that the anammox branch might be a second order within the *Planctomycetes*. However, the peculiarity of long phylogenetic distances between individual members of the *Planctomycetes* remains to be solved. There is no indication of a single dramatic evolutionary event, because both the length of the stem and the length of the branches of the planctomycete phylogenetic tree are within the range found in other bacterial groups. The theory that *Planctomycetes* are an old lineage (4) may be a good alternative explanation, because the organisms would have had more time to evolve into well-separated individual genera. The architecture of the planctomycete ribosome could also play a role. It might be less sensitive to changes, which would allow the 16S rRNA genes of individual species and genera to drift further apart. Hopefully, genome comparisons of several planctomycetes (*Rhodopirellula baltica*, *Gemmata obscuriglobus*, and "*Ca*. Kuenenia stuttgartiensis") will help to solve these phylogenetic questions. Doubtless, *Planctomycetes* and especially anammox organisms are able to deal with changes in the rRNA operon structure as well as insertion and deletion in their rRNA genes. Most importantly, while members of the genera *Planctomyces*, *Pirellula*, and *Gemmata* have genomically separated rRNA genes (56), anammox organisms do possess linked 16S and 23S rRNA genes (35). Thus, the 16S rRNA and the 23S rRNA are transcribed together with their ISR, which can serve as a target for fluorescence in situ hybridizations (see below). Furthermore, the 16S rRNA gene of members of the genera "*Ca*. Kuenenia" and "*Ca*. Brocadia" contain an insertion of 20 nucleotides located within helix 9 (beginning at *Escherichia coli* position 158). A secondary structural analysis led to the prediction of the two new subhelices 9a and 9b (35). The existence of the 9a and 9b helices in the mature 16S rRNA was shown by FISH analysis with the probe S-S-Kst-0157-a-A-18 and probe S-S-Ban-0162-a-A-18, respectively. Interestingly, this insertion is absent from all other 16S rRNA gene sequences in the ARB database (about 28,000 entries as of March 2004; www.arb-home.de). The only exception is a 14-bp insertion in one 16S rRNA gene sequence derived from an industrial wastewater treatment plant and affiliated with a different branch of *Planctomycetes* (21).

DETECTION OF ANAMMOX ORGANISMS BY PCR

In environmental samples, PCR amplification with general 16S rRNA gene-targeted primers and subsequent phylogenetic analysis of the product is the method of choice to detect previously undescribed organisms (3). In the anammox case this approach is useful if the presence of a new anammox organism is expected or if the cell counts are too low for FISH. However, planctomycetes such as anammox organisms are still underrepresented in general 16S rRNA gene clone libraries. One explanation could be a reduced recovery of anammox DNA by the applied DNA extraction method, as was observed earlier for aerobic ammonia oxidizing bacteria (18). Additionally, PCR amplification might also introduce biases in the anammox template-to-product ratio (see reference 28). A more directed PCR approach with the primer S-P-Planc-0046-a-a-18 (Pla46F, a planctomycete-specific forward primer) (Table 1) together with either the reverse primers 1390R (*E. coli* positions 1390 to 1407; 5′-GACGGGCGGTGTGTACAA-3′) (34, 57) or 630R (*E. coli* positions 1529 to 1545; 5′-CAKAAAGGAGGTGATCC-3′) (18, 36) increased relative amounts of planctomycete 16S rRNA gene sequences. Still, these primer pairs and the subsequent cloning procedure did not yet yield a quantitative representation of anammox bacteria in the various samples investigated (11, 20, 30, 34, 36). In a semitechnical plant in Stuttgart, Germany, in which the abundance of anammox organisms was 99% of the planctomycete population and over 40% of the bacterial population, even with the planctomycete-specific primer pair Pla46F/1390R, just 9 clones out of 25 carried anammox 16S rRNA genes (34). Therefore, primers specific for the amplification of the 16S rRNA genes of anammox organisms are required. For this purpose some anammox-specific FISH probes have been successfully applied as PCR primers (Table 1). Most effective are the combinations of

TABLE 1. Oligonucleotide probes used for the detection of anammox organisms and their suitability for use as PCR primers

OPD[a] designation trivial name [reference]	Specificity	Sequence 5′-3′	% Formamide/ mM [NaCl][b]	Tested as PCR primer[c]
S-P-Planc-0046-a-A-18 (Pla46; [26])	*Planctomycetales*	GACTTGCATGCCTAATCC	25/159	F (58°C)
S-P-Planc-0886-a-A-19 (Pla 886; [26])	*Isosphaera*, *Gemmata*, *Pirellula*, *Plantomyces*	GCCTTGCGACCATACTCCC	30/112	—
S-D-Bact-0338-b-A-18 (Eub338II; [7])	Bacterial lineages not covered by probes EUB338 and EUB338II	GCAGCCACCCGTAGGTGT	0/900	—
S-D-Bact-0338-d-A-18[d] (Eub338IV)	Bacterial lineages not covered by probes EUB338, EUB338II, and EUBIII	GCAGCCTCCCGTAGGAGT	0/900	
S-*-Amx-0368-a-A-18 (36)	All anammox organisms	CCTTTCGGGCATTGCGAA	15/338	F/R (56°C)
L-*-Amx-1900-a-A-21 (35)	Genera "*Ca.* Brocadia" and "*Ca.* Kuenenia"	CATCTCCGGCTTGAACAA	30/112	—
S-*-Amx-0820-a-A-22 (AMX 820, [34])	Genera "*Ca.* Brocadia" and "*Ca.* Kuenenia"	AAAACCCCTCTACTTAGTGCCC	40/56	F/R (56°C)
S-G-Sca-1309-a-A-21 (36)	Genus "*Ca.* Scalindua"	TGGAGGCGAATTTCAGCCTCC	5/675	R (56°C)
S-*-Scabr-1114-a-A-22 (36)	"*Ca.* Scalindua brodae"	CCCGCTGGTAACTAAAAACAAG	20/225	R (56°C)
S-*-BS-820-a-A-22 (20)	"*Ca.* Scalindua wagneri" "*Ca.* Scalindua sorokinii"	TAATTCCCTCTACTTAGTGCCC	40/56	R (56°C)
S-S-Kst-0157-a-A-18 (35)	"*Ca.* Kuenenia stuttgartiensis"	GTTCCGATTGCTCGAAAC	25/159	—
S-*-Kst-1275-a-A-20 (34)	"*Ca.* Kuenenia stuttgartiensis"	TCGGCTTTATAGGTTTCGCA	25/159	—
S-S-Ban-0162(B.anam.)-a-A-18 (34)	"*Ca.* Brocadia anammoxidans"	CGGTAGCCCCAATTGCTT	40/56	—
S-*-Amx-0156-a-A-18 (34)	"*Ca.* Brocadia anammoxidans"	CGGTAGCCCCAATTGCTT	40/56	—
S-*-Amx-0223-a-A-18 (34)	"*Ca.* Brocadia anammoxidans"	GACATTGACCCCTCTCTG	40/56	—
S-*-Amx-0432-a-A-18 (34)	"*Ca.* Brocadia anammoxidans"	CTTAACTCCCGACAGTGG	40/56	—
S-*-Amx-0613-a-A-22 (34)	"*Ca.* Brocadia anammoxidans"	CCGCCATTCTTCCGTTAAGCGG	40/56	—
S-*-Amx-0997-a-A-21 (34)	"*Ca.* Brocadia anammoxidans"	TTTCAGGTTTCTACTTCTACC	20/225	—
S-*-Amx-1015-a-A-18 (34)	"*Ca.* Brocadia anammoxidans"	GATACCGTTCGTCGCCCT	60/14	—
S-*-Amx-1154-a-A-18 (34)	"*Ca.* Brocadia anammoxidans"	TCTTGACGACAGCAGTCT	20/225	—
S-*-Amx-1240-a-A-23 (34)	"*Ca.* Brocadia anammoxidans"	TTTAGCATCCCTTTGTACCAACC	60/14	—
I-*-Ban-0071(B.anam.)-a-A-18 (35)	"*Ca.* Brocadia anammoxidans"	CCCTACCACAAACCTCGT	10/450	—
I-*-Ban-0108(B.anam.)-a-A-18 (35)	"*Ca.* Brocadia anammoxidans"	TTTGGGCCCGCAATCTCA	10/450	—
I-*-Ban-0222(B.anam.)-a-A-19 (35)	"*Ca.* Brocadia anammoxidans"	GCTTAGAATCTTCTGAGGG	10/450	—
I-*-Ban-0389(B.anam.)-a-A-18 (35)	"*Ca.* Brocadia anammoxidans"	GGATCAAATTGCTACCCG	10/450	—
I-*-Kst-0031(K.stutt.)-a-A-18 (35)	"*Ca.* Kuenenia stuttgartiensis"	ATAGAAGCCTTTTGCGCG	10/450	—
I-*-Kst-0077(K.stutt.)-a-A-18 (35)	"*Ca.* Kuenenia stuttgartiensis"	TTTGGGCCACACTCTGTT	10/450	—
I-*-Kst-0193(K.stutt.)-a-A-19 (35)	"*Ca.* Kuenenia stuttgartiensis"	CAGACCGGACGTATAAAAG	10/450	—
I-*-Kst-0288(K.stutt.)-a-A-20 (35)	"*Ca.* Kuenenia stuttgartiensis"	GCGCAAAGAAATCAAACTGG	10/450	—

[a] OPD, oligonucleotide probe database designation according to reference 1. Original reference is given in parentheses.
[b] Percent formamide in the hybridization buffer and concentration of NaCl in the washing buffer, respectively, required for specific in situ hybridization.
[c] F, forward primer; R, reverse primer. The commonly applied annealing temperature is given in parentheses. Note that probes used as forward primers are complementary and reverse to the given probe sequences. —, not determined.
[d] Target sequence suggested by reference 7.

the primer S-P-Planc-0046-a-a-18 (forward primer) with either the reverse primer S-*-Amx-0820-a-A-22 for Brocadia-like bacteria, reverse primer S-*-BS-820-a-A-22 and reverse primer S-*-Scabr-1114-a-A-22 for Scalindua-like bacteria, or reverse primer S-*-Amx-0368-a-A-18 for all known anammox bacteria (34–36). They can all be used at an annealing temperature range of 56 to 58°C (for a PCR program, see reference 11 or 34). The combination of the forward primer S-P-Planc-0046-a-a-18 and reverse primer Amx-0368-a-A-18 has been tested with 10 different wastewater treatment plant samples, 5 of which yielded an amplification product of anammox 16S rRNA genes. The specificity of the PCR with these primers is excellent. Subsequent phylogenetic analysis showed that only anammox 16S rRNA genes were amplified. By applying the different sets of primers described above, it is thus possible to distinguish between the different groups of anammox organisms. The application of these primer pairs could be extended to various kinds of PCR-based methods such as denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphisms, or quantitative PCR. However, since more and more genera and species responsible for anammox are being discovered, it is strongly recommended that PCR products be tested by either direct sequencing or dot blot analysis with 16S rRNA-specific probes to confirm their phylogenetic affiliations.

FISH AS THE GOLD STANDARD FOR THE DETECTION OF ANAMMOX ORGANISMS

In various studies probes for FISH have been used to collect both qualitative and quantitative data of anammox bacteria in environmental samples (15, 34, 36). Since anammox organisms are affiliated with *Planctomycetes*, the probe S-P-Planc-0046-a-A-18 (Table 1) (26), which also hybridizes with the 16S rRNA gene of anammox bacteria, is a good probe for initial experiments. However, it should be explicitly noted that the probe S-P-Planc-0886-a-A-19, specifically constructed to detect members of the genera *Pirellula*, *Gemmata*, *Planctomyces*, and

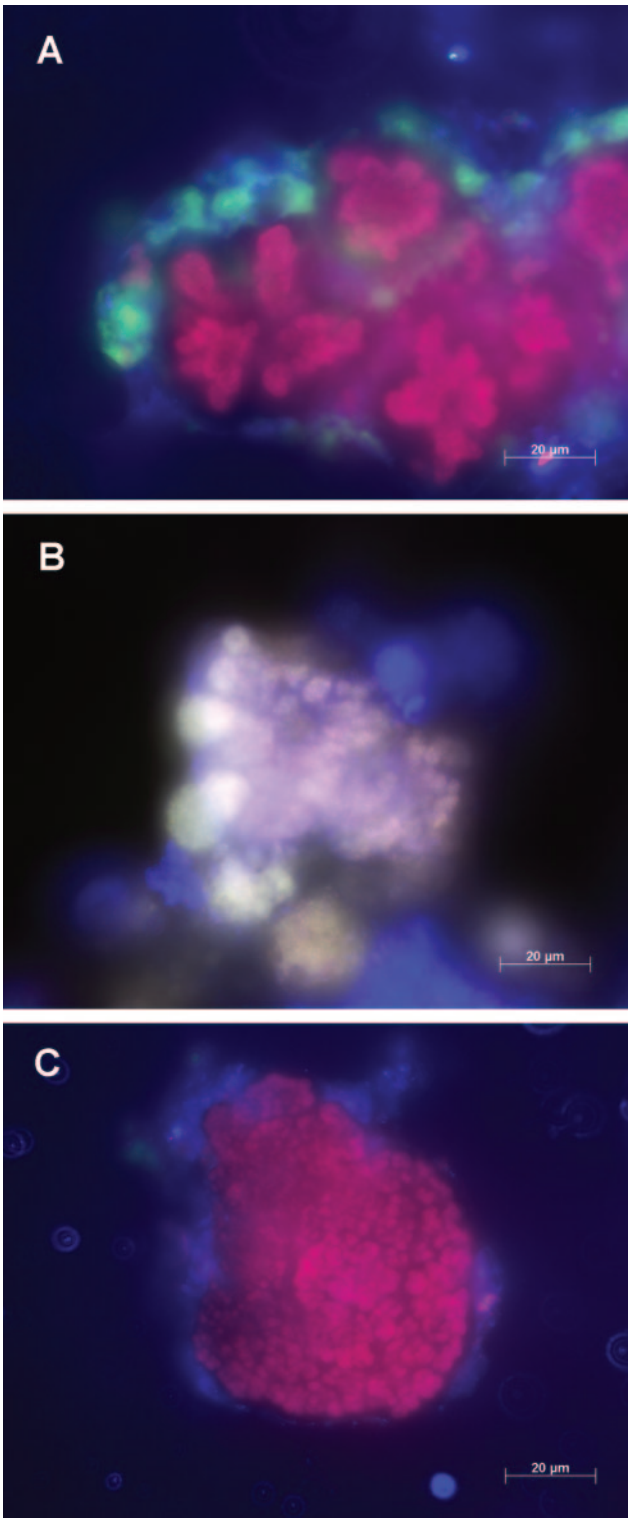

FIG. 2. (A) FISH of an anammox enrichment sample from a 2L laboratory reactor containing mostly "*Ca*. Kuenenia stuttgartiensis" with probe S-*-Kst-1275-a-A-20 (labeled with Cy3; red) and probe S-*-Amx-0820-a-A-22 (labeled with Cy5; blue). Overlapping red and blue labels result in purple "*Ca*. Kuenenia stuttgartiensis" cells. Remaining blue cells might be another type of anammox bacteria. Autofluorescence is depicted in green. (B) FISH of an anammox biomass sample containing "*Ca*. Scalindua" with probe S-*-Amx-0368-a-A-18 (labeled with Cy3; red), probe S-*-BS-820-a-A-22 (labeled with Fluos; green), and the Eub probe mix (7) (labeled with Cy5; blue). Overlapping red, green, and blue labels result in anammox organisms that appear yellow-white. (C) ISR-FISH of an anammox enrichment sample from a 2-liter laboratory reactor containing "*Ca*. Brocadia anammoxidans" with the probe mix targeting the ISR of "*Ca*. Brocadia anammoxidans" (labeled with Cy3; red) and probe S-*-Amx-0820-a-A-22 (labeled with Cy5; blue). Overlapping red and blue labels result in anammox organisms that appear purple.

Isosphaera, does not hybridize with the 16S rRNA of anammox organisms or any other environmental clone with a phylogenetic position between the described planctomycete genera and anammox. In addition probe S-D-Bact-0338-a-A-18 (2), which targets the 16S rRNA of almost all *Bacteria*, has mismatches to the respective target site of members of the phylum *Planctomycetes*. In 1999 the substitute probe S-D-Bact-0338-b-A-18 was constructed with two nucleotide substitutions in the sequence of the original probe S-D-Bact-0338-a-A-18 (7). However, the 16S rRNAs of *Isosphaera* and anammox organisms have only one mismatch to the probe S-D-Bact-0338-a-A-18, which results in a weak hybridization signal for *Isosphaera* and anammox organisms with this probe (34). We suggest that the probe S-D-Bact-0338-d-A-18 (Table 1) be used as a general probe, as it has no mismatch to the 16S rRNAs of *Isosphaera* and anammox organisms.

A specific detection of anammox bacteria in environmental samples was initially performed in bioreactors and wastewater treatment plants. Most of these samples contained large amounts of a single strain of anammox bacteria (to a maximum of about 50%) (15, 34), which were affiliated with "*Ca*. Kuenenia" and "*Ca*. Brocadia." Consequently, the majority of the initial probes designed for the detection of anammox organisms targeted either "*Ca.* Brocadia" or "*Ca*. Kuenenia" (Table 1) (34, 35, 42). Probe S-*-Amx-0820-a-A-22 as an exception hybridized with the 16S rRNA of both. To distinguish between the two genera, probes targeting helices 9a and 9b, which are unique for both "*Ca*. Kuenenia" and "*Ca*. Brocadia," proved to be very suitable (Table 1, probe S-S-Kst-0157-a-A-18 and probe S-S-Ban-0162-a-A-18) (35). Probe S-*-Kst-1275-a-A-20 (Table 1), which was specific for the 16S rRNA of "*Ca*. Kuenenia stuttgartiensis," was only slightly better than S-S-Kst-0157-a-A-18 (35) in terms of signal strength at its highest stringency of 25% formamide in the hybridization buffer (Fig. 2A). Finally, the 23S rRNA targeting probe L-*-Amx-1900-a-A-21 was constructed to specifically detect the 23S rRNA of "*Ca*. Brocadia" and "*Ca*. Kuenenia" (35).

The recent discovery of members of the anammox genus "*Ca*. Scalindua" in a landfill leachate treatment plant in Pitsea (England) (36) and in marine environments (20, 32) showed that the probes constructed for "*Ca*. Kuenenia" and "*Ca*. Brocadia" were not sufficient to detect all anammox bacteria. Consequently, probe S-G-Sca-1309-a-A-21, probe S-*-Scabr-1114-a-A-22, and probe S-*-BS-820-a-A-22 (Table 1) were constructed to specifically detect the different 16S rRNAs of "*Ca*. Scalindua species" (20, 36). Though probe S-*-Amx-0820-a-A-22 does not target the 16S rRNAs of "*Ca*. Scalindua" in general, it did hybridize in some cases with the 16S rRNAs of "*Ca*. Scalindua" cells (data not shown), if probe S-*-BS-820-a-A-22 was not used as a competitor. Therefore, it is recommended probe S-*-Amx-0820-a-A-22 and

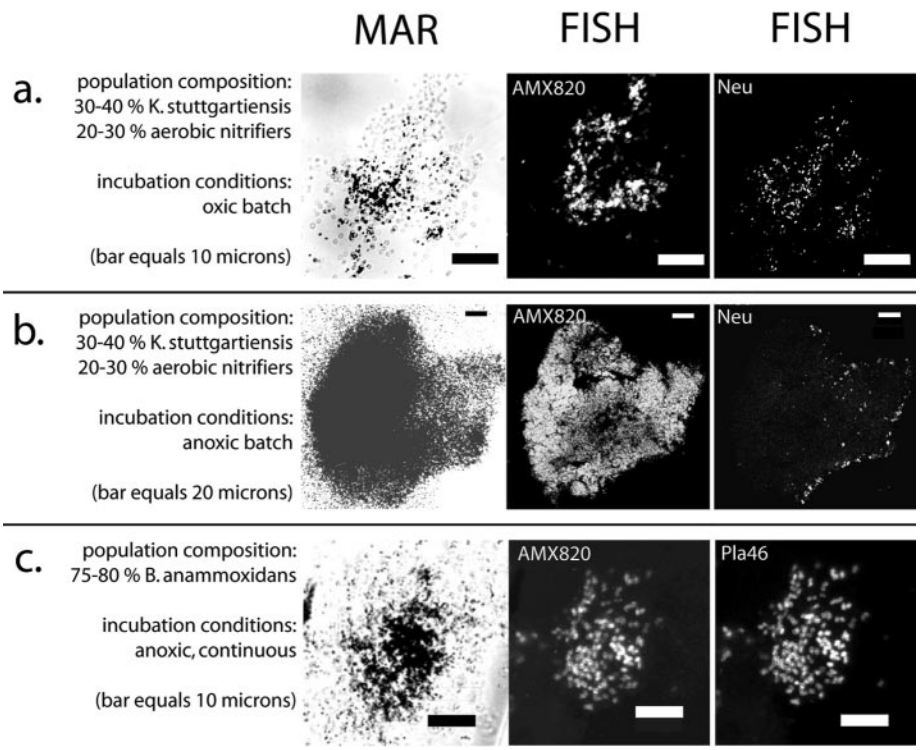

FIG. 3. MAR-FISH (22). Panels A to C present the results of one experiment. The MAR signal is shown in the first column; the other two columns give FISH images of the same area and indicate the probes that were used. Probe AMX820 (S-*-Amx-0820-a-A-22) and probe Pla46 (S-P-Planc-0046-a-A-18) are listed in Table 1. Details for probe Neu can be found at the ProbeBase website (23). Shown are the labeling patterns of a mixed culture containing "*Ca.* Kuenenia stuttgartiensis" (30 to 40%) and *Nitrosomonas eutropha* (30 to 40%) after aerobic incubation with ammonium (A), of the same culture after anaerobic incubation with ammonium and nitrite (B), and of an enrichment culture of "*Ca.* Kuenenia stuttgartiensis" (70 to 80%) after 24-h labeling in a continuous setup, under nitrite limitation (C).

probe S-*-BS-820-a-A-22 be applied together (differently labeled) with a minimal stringency of 30% formamide in the hybridization buffer. Additionally, probe S-*-Amx-0368-a-A-18 was designed to detect the 16S rRNAs of all anammox species (Fig. 2B) (36). Further probe details can be found at the probeBase (23) website (www.microbial-ecology.net/probebase).

The application of the probes specific for "*Ca*. Scalindua species," revealed that the plant in Pitsea is exceptional so far, since it is the only habitat that contains two different anammox species ("*Ca*. Scalindua wagneri" and "*Ca*. Scalindua brodae") (36) in almost equal amounts. Most importantly, the detection of "*Ca*. Scalindua species" in marine environments with FISH probes has shown the important role of anammox bacteria in the global nitrogen cycle (20, 32).

ASSESSMENT OF THE METABOLIC ACTIVITY OF ANAMMOX BACTERIA BY ADVANCED FISH APPROACHES

FISH as outlined above delivers qualitative and quantitative data about the bacterial population in a sample. To gain more insights into the metabolic activity of anammox organisms and still use the high potential of FISH, two advanced FISH approaches are available.

One of these approaches uses FISH targeting the ISR between the 16S and 23S rRNA (35). For many fast-growing organisms it has been shown that the FISH signal intensity is directly proportional to the concentration of ribosomes and precursor-rRNA molecules in the cells (see reference 29). However, for betaproteobacterial ammonia oxidizing bacteria and anammox organisms, it has been shown that ribosome content does not decrease significantly during periods of starvation (24) or inhibition (35, 55). This property is most likely linked to their rigid and specialistic obligate chemolithotrophic way of life, which includes extreme resistance to starvation. Thus, the cellular rRNA content does not reflect the physiological activity of these organisms. For these slowly growing bacteria, the precursor rRNA concentrations are a direct measure of the ribosome turnover rate (≈growth rate) in the cells (6). To learn more about the in situ activity of anammox organisms, the ISR between the 16S rRNA and 23S rRNA has been targeted with fluorescently labeled oligonucleotide probes. This sequence stretch is only present in precursor rRNA and not in mature ribosomes (35). However, a single oligonucleotide probe is not bright enough for the detection of the ISR in anammox organisms. Sufficient signal amplification can only be achieved by simultaneous application of four probes targeting the ISR (35). It has been shown with the ISR-targeted probes that anammox organisms indeed immediately arrest their metabolism when they are exposed to oxygen (17, 35). A variety of experiments with ISR-targeting probes have been performed with other samples (Fig. 2C), which showed that the ISR-targeted FISH (ISR-FISH) has great potential for monitoring activity changes in enrichment

cultures of anammox bacteria and ecosystems. Therefore, this method would also be very suitable for the effective monitoring of anammox activity during the startup of a reactor. However, a disadvantage of this approach is the lack of evolutionary pressure on the ISR sequences, which might cause even two strains of the same species to have sequence differences. Therefore, the two sets of ISR probes that are available for "*Ca*. Brocadia anammoxidans" and "*Ca*. Kuenenia stuttgartiensis" hardly allow a comprehensive application of ISR-FISH. This means that new probes have to be designed for each specific ecosystem.

The FISH-MAR combination (22) is a more general method for the determination of the metabolic activity of anammox organisms. FISH-MAR directly links the uptake of radiolabeled substrates with specific organisms in a complex environmental sample.

FISH-MAR experiments have been helpful to demonstrate that anammox bacteria are chemolithoautotrophs, which incorporate carbon dioxide as the main carbon source (17). Because anammox bacteria are not available in pure culture, FISH-MAR has been useful in excluding the possibility that other bacteria in the enrichment culture were responsible for the carbon dioxide uptake measured in mass balances over these cultures (41). For successful FISH-MAR with anammox bacteria, it is sometimes necessary to incubate the culture with the radiolabeled carbon dioxide in a continuous cultivation setup (Fig. 3C). In batch experiments the amount of incorporation has not always been sufficient for FISH-MAR.

Good uptake of radiolabel in batch experiments is possible with cocultures of anaerobic and aerobic ammonium oxidizers (40, 47). These cultures can be incubated both aerobically to measure carbon dioxide uptake by nitrifiers and anaerobically to measure carbon dioxide uptake by the anammox bacteria (Fig. 3A and B).

FISH-MAR can be applied successfully with the very slow growing anammox bacteria. However, due to the long incubation times necessary for sufficient incorporation of the label, the results may not always reflect the physiological state of the organisms at the time of sampling. The problem can be overcome with ISR-FISH.

CONCLUSION

A range of suitable methods is available for the detection and enumeration of anammox bacteria and their activity in natural and man-made ecosystems. For a proper evaluation of the contribution of the anammox process to nitrogen cycling in a particular habitat, the combination of different (rRNA and non-rRNA) methods is necessary. Primers for FISH have to be chosen wisely and in a nested approach to ensure the correct enumeration and identification of the anammox bacteria present. FISH-MAR and ISR probing are advanced techniques that make possible the measurement of activity and growth at the single-cell level.

ACKNOWLEDGMENTS

The research on anaerobic ammonium oxidation over the years was financially supported by the European Union (EESD EVK1-CT-2000-00054), the Foundation for Applied Sciences (STW), the Foundation of Applied Water Research (STOWA), The Netherlands Foundation for Earth and Life Sciences (NWO-ALW, Biogeosphere grants 853.00.012 and 853.00.031), the Royal Netherlands Academy of Arts and Sciences (KNAW), DSM-Gist, and Paques Natural Solutions.

We gratefully acknowledge the contributions of the many coworkers and students.

REFERENCES

1. **Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin.** 1996. The oligonucleotide probe database. Appl. Environ. Microbiol. **62:**3557–3559.
2. **Amann, R. I., B. J. Binder, R. J. Olsen, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
3. **Amann, R. I., W. Ludwig, and K-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
4. **Brochier, C., and H. Philippe.** 2002. Phylogeny: a non-thermophilic ancestor for bacteria. Nature **417:**244.
5. **Broda, E.** 1977. Two kinds of lithotrophs missing in nature. Z. Allg. Mikrobiol. **17:**491–493.
6. **Cangelosi, G. A., and W. H. Brabant.** 1997. Depletion of pre-16S rRNA in starved *Escherichia coli* cells. J. Bacteriol. **179:**4457–4463.
7. **Daims, H., A. Brühl, R. Amann, K.-H. Schleifer, and M. Wagner.** 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. **22:**434–444.
8. **Dalsgaard, T., D. E. Canfield, J. Petersen, B. Thamdrup, and J. Acuña-González.** 2003. Anammox is a significant pathway of N_2 production in the anoxic water column of Golfo Dulce, Costa Rica. Nature **422:**606–608.
9. **Dapena-Mora, A., J. L. Campos, A. Mosquera-Corral, and R. Méndez.** 2003. Studies of inhibition on the anammox process, abstr. 4.3-039. *In* Proceedings of the 4th European Congress of Chemical Engineering, 21 to 25 September 2003, Granada, Spain.
10. **Dong, X., and E. W. Tollner.** 2003. Evaluation of anammox and denitrification during anaerobic digestion of poultry manure. Biores. Technol. **86:**139–145.
11. **Egli, K., U. Franger, P. J. J. Alvarez, H. R. Siegrist, J. R. Van der Meer, and A. J. B. Zehnder.** 2001. Enrichment and characterization of an anmmox bacterium from a rotating biological contractor treating ammonium-rich leachate. Arch. Microbiol. **175:**198–207.
12. **Fujii, T., H. Sugino, J. D. Rouse, and K. Furukawa.** 2002. Characterization of the microbial community in an anaerobic ammonium-oxidising biofilm cultured on a non-woven biomass carrier. J. Biosci. Bioeng. **94:**412–418.
13. **Fux, C., M. Boehler, P. Huber, I. Brunner, and H. R. Siegrist.** 2002. Biological treatment of ammonium-rich wastewater by partial nitritation and subsequent anaerobic ammonium oxidation (anammox) in a pilot plant. J. Biotechnol. **99:**295–306.
14. **Helmer, C., S. Kunst, S. Juretschko, M. C. Schmid, K-H. Schleifer, and M. Wagner.** 1999. Nitrogen loss in a nitrifying biofilm system. Water Sci. Tech. **39:**13–21.
15. **Helmer-Madhok, C., M. C. Schmid, E. Filipov, T. Gaul, A. Hippen, K. H. Rosenwinkel, C. F. Seyfried, M. Wagner, and S. Kunst.** 2002. Deammonification in biofilm systems: population structure and function. Water Sci. Tech. **46:**223–231.
16. **Jetten, M. S. M., M. C. Schmid, I. Schmidt, M. Wubben, U. van Dongen, W. Abma, A. O. Sliekers, N. P. Revsbech, H. J. E. Beaumont, L. Ottosen, E. Volcke, H. J. Laanbroek, J. L. Campos Gomez, J. A. Cole, M. C. M. van Loosdrecht, J. W. Mulder, J. Fuerst, D. Richardson, K. T. van de Pas-Schoonen, R. Mendez Pampin, K. Third, I. Cirpus, R. van Spanning, A. Bollmann, L. P. Nielsen, H. J. M. Op den Camp, C. Schultz, J. Gundersen, P. Vanrolleghem, M. Strous, M. Wagner, and J. G. Kuenen.** 2002. Improved nitrogen removal by application of new nitrogen-cycle bacteria. Rev. Environ. Sci. Biotechnol. **1:**51–63.
17. **Jetten, M. S. M., M. Strous, K. T. Van de Pas-Schoonen, J. Schalk, U. Van Dongen, A. A. Van de Graaf, S. Logemann, G. Muyzer, M. C. M. Van Loosdrecht, and J. G. Kuenen.** 1998. The anaerobic oxidation of ammonium. FEMS Microbiol. Rev. **22:**421–437.
18. **Juretschko, S., G. Timmermann, M. C. Schmid, K-H. Schleifer, A. Pommerening-Röser, H-P. Koops, and M. Wagner.** 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. **64:**3042–3051.
19. **Kuenen, J. G., and M. S. M. Jetten.** 2001. Extraordinary anaerobic ammonium oxidising bacteria. ASM News **67:**456–463.
20. **Kuypers, M. M. M., A. O. Sliekers, G. Lavik, M. Schmid, B. B. Jøgensen, J. G. Kuenen, J. S. Sinninghe Damsté, M. Strous, and M. S. M. Jetten.** 2003. Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Nature **422:**608–611.
21. **LaPara, T. M., C. H. Nakatsu, L. Pantea, and J. E. Alleman.** 2000. Phylogenetic analysis of bacterial communities in mesophilic and thermophilic bioreactors treating pharmaceutical wastewater. Appl. Environ. Microbiol. **66:**3951–3959.

22. **Lee, N., P. H. Nielsen, K. H. Andreasen, S. Juretschko, J. L. Nielsen, K.-H. Schleifer, and M. Wagner.** 1999. Combination of fluorescent *in situ* hybridization and microautoradiography—a new tool for structure-function analyses in microbial ecology. Appl. Environ. Microbiol. **65:**1289–1297.
23. **Loy, L. A., M. Horn, and M. Wagner.** 2003. probeBase: an online resource for rRNA-targeted oligonucleotide probes. Nucleic Acids Res. **31:**514–516.
24. **Morgenroth, E., A. Obermayer, E. Arnold, A. Brühl, M. Wagner, and P. A. Wilderer.** 2000. Effect of long-term idle periods on the performance of sequencing batch reactors. Water Sci. Technol. **41:**105–113.
25. **Mulder, A., A. A. van de Graaf, L. A. Robertson, and J. G. Kuenen.** 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol Ecol. **16:**177–184.
26. **Neef, A., R. I. Amann, H. Schlesner, and K.-H. Schleifer.** 1998. Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. Microbiology **144:**3257–3266.
27. **Nielsen, M., N. P. Revsbech, L. H. Larsen, and A. Lynggaard-Jensen.** 2002. On-line determination of nitrite in wastewater treatment by use of a biosensor. Water Sci. Technol. **45:**69–76.
28. **Polz, M. F., and C. M. Cavanaugh.** 1998. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. **64:**3724–3730.
29. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354–1360.
30. **Pynaert, K., B. F. Smets, S. Wyffels, D. Beheydt, S. D. Siciliano, and W. Verstraete.** 2003. Characterization of an autotrophic nitrogen-removing biofilm from a highly loaded lab-scale rotating biological contactor. Appl. Environ. Microbiol. **69:**3626–3635.
31. **Richards, F. A.** 1965. Anoxic basins and fjords. *In* J. P. Ripley and G. Skirrow (ed.), Chemical oceanography, p. 611–645. Academic Press, London, United Kingdom.
32. **Risgaard-Petersen, N., R. L. Meyer, M. C. Schmid, M. S. M. Jetten, A. Enrich-Prast, S. Rysgaard, and N. P. Revsbech.** 2004. Anaerobic ammonia oxidation in an estuarine sediment. Aquatic Microbiol. Ecol. **36:**293–304.
33. **Schalk, J., S. DeVries, J. G. Kuenen, and M. S. M. Jetten.** 2000. Involvement of a novel hydroxylamine oxidoreductase in anaerobic ammonium oxidation. Biochemistry **39:**5405–5412.
34. **Schmid, M., U. Twachtmann, M. Klein, M. Strous, S. Juretschko, M. S. M. Jetten, J. Metzger, K.-H. Schleifer, and M. Wagner.** 2000. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst. Appl. Microbiol. **23:**93–106.
35. **Schmid, M., S. Schmitz-Esser, M. Jetten, and M. Wagner.** 2001. 16S–23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. Environ. Microbiol. **3:**450–459.
36. **Schmid, M., K. Walsh, R. Webb, W. I. C. Rijpstra, K. T. van de Pas-Schoonen, M. J. Verbruggen, T. Hill, B. Moffett, J. Fuerst, S. Schouten, J. S. Sinninge Damsté, J. Harris, P. Shaw, M. S. M. Jetten, and M. Strous.** 2003. *Candidatus*"Scalindua brodae," sp. nov., *Candidatus*"Scalindua wagneri," sp. nov.: two new species of anaerobic ammonium oxidizing bacteria. Syst. Appl. Microbiol. **26:**529–538.
37. **Schouten, S., M. Strous, M. M. M. Kuypers, W. I. C. Rijpstra, M. Baas, C. J. Schubert, M. S. M. Jetten, and J. S. Sinninghe Damsté.** 2004. Stable carbon isotopic fractionations associated with inorganic carbon fixation by anaerobic ammonium-oxidizing bacteria Appl. Environ. Microbiol. **70:**3785–3788.
38. **Sinninghe Damsté, J. S., M. Strous Rijpstra, W. I. C., Hopmans, E. C., Geenevasen, J. A. J., Van Duin, A. C. T., Van Niftrik, L. A., and M. S. M. Jetten.** 2002. Linearly concatenated cyclobutane lipids form a dense bacterial membrane. Nature **419:**708–712.
39. **Sinninghe Damsté, J. S., W. I. C. Rijpstra, S. Schouten, J. A. Fuerst, M. S. M. Jetten, and M. Strous.** 2004. The occurrence of hopanoids in planctomycetes: implications for the sedimentary biomarker record. Org. Geochem. **35:**561–566.
40. **Sliekers, A. O., N. Derwort, L. Campos, J. G. Kuenen, M. Strous, and M. S. M. Jetten.** 2002. Completely autotrophic ammonia removal over nitrite in a single reactor system. Water Res. **36:**2475–2482.
41. **Strous, M., J. J. Heijnen, J. G. Kuenen, and M. S. M. Jetten.** 1998. The sequencing batch reactor as a powerful tool to study very slowly growing microorganisms. Appl. Microbiol. Biotechnol. **50:**589–596.
42. **Strous, M., J. Fuerst, E. Kramer, S. Logemann, G. Muyzer, K. T. van de Pas-Schoonen, R. Webb, J. G. Kuenen, and M. S. M. Jetten.** 1999. Missing lithotroph identified as new planctomycete. Nature **400:**446–449.
43. **Strous, M., J. G. Kuenen, J. A. Fuerst, M. Wagner, and M. S. M. Jetten.** 2002. The anammox case: a new experimental manifesto for microbiological eco-physiology. Antonie Leeuwenhoek **81:**693–702.
44. **Strous, M., and M. S. M. Jetten.** 2004. Anaerobic oxidation of ammonium and methane. Ann. Rev. Microbiol. **53:**99–117.
45. **Tal, Y., J. E. M. Watts, S. B. Schreier, K. R. Sowers, and H. J. Schreier.** 2003. Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system. Aquaculture **215:**187–202.
46. **Thamdrup. B., and T. Dalsgaard.** 2002. Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Appl. Environ. Microbiol. **68:**1312–1318.
47. **Third, K., A. O. Sliekers, J. G. Kuenen, and M. S. M. Jetten.** 2001. The CANON system (completely autotrophic nitrogen-removal over nitrite) under ammonium limitation: interaction and competition between three groups of bacteria. Syst. Appl. Microbiol. **24:**588–596.
48. **Toh, S. K., and N. J. Ashbolt.** 2002. Adaptation of anaerobic ammonium-oxidising consortium to synthetic coke-ovens wastewater. Appl. Microbiol. Biotechnol. **59:**344–352.
49. **Trimmer, M., J. C. Nicholls, and B. Deflandre.** 2003. Anaerobic ammonium oxidation measured in sediments along the Thames estuary, United Kingdom. Appl. Environ. Microbiol. **69:**6447–6454.
50. **Van de Graaf, A. A., A. Mulder, P. de Bruijn, M. S. Jetten, L. A. Robertson, and J. G. Kuenen.** 1995. Anaerobic oxidation of ammonium is a biologically mediated process. Appl. Environ. Microbiol. **61:**1246–1251.
51. **Van de Graaf, A. A., P. De Bruijn, L. A. Robertson, M. S. M. Jetten, and J. G. Kuenen.** 1997. Metabolic pathway of anaerobic ammonium oxidation on the basis of N-15 studies in a fluidized bed reactor. Microbiology **143:**2415–2421.
52. **Van Dongen, U., M. S. M. Jetten, and M. C. M. van Loosdrecht.** 2001. The Sharon-anammox process for the treatment of ammonium rich wastewater. Water Sci. Technol. **44:**153–160.
53. **Van Loosdrecht, M. C. M., X. Hao, M. S. M. Jetten, and W. Abma.** 2004. Use of anammox in urban wastewater treatment. Water Supply **4:**87–94.
54. **Van Niftrik, L. A., J. A. Fuerst, J. S. Sinninghe Damsté, J. G. Kuenen, and M. S. Jetten, M. Strous.** 2004. The anammoxosome: an intracytoplasmic compartment in anammox bacteria. FEMS Microbiol. Lett. **233:**7–13.
55. **Wagner, M., G. Rath, R. Amann, H.-P. Koops, and K.-H. Schleifer.** 1995. *In situ* identification of ammonia oxidizing bacteria. Syst. Appl. Microbiol. **18:** 251–264.
56. **Ward, N. L., F. A. Rainey, B. P. Hedlund, J. T. Staley, W. Ludwig, and E. Stackebrandt.** 2000. Comparative phylogenetic analyses of members of the order *Planctomycetales* and the division *Verrucomicrobia*: 23S rRNA gene sequence analysis supports the 16S rRNA gene sequence-derived phylogeny. Int. J. Syst. Evol. Microbiol. **50:**1965–1972.
57. **Zheng, D., E. W. Alm, D. A. Stahl, and L. Raskin.** 1996. Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. **62:**4504–4513.