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Candidatus "Scalindua brodae", sp. nov., *Candidatus* "Scalindua wagneri", sp. nov., Two New Species of Anaerobic Ammonium Oxidizing Bacteria

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Abstract

Anaerobic ammonium oxidation (anammox) is both a promising process in wastewater treatment and a long overlooked microbial physiology that can contribute significantly to biological nitrogen cycling in the world's oceans. Anammox is mediated by a monophyletic group of bacteria that branches deeply in the *Planctomycetales*. Here we describe a new genus and species of anaerobic ammonium oxidizing planctomycetes, discovered in a wastewater treatment plant (wwtp) treating landfill leachate in Pitsea, UK. The biomass from this wwtp showed high anammox activity (5.0 ± 0.5 nmol/mg protein/min) and produced hydrazine from hydroxylamine, one of the unique features of anammox bacteria. Eight new planctomycete 16S rRNA gene sequences were present in the 16S rRNA gene clone library generated from the biomass. Four of these were affiliated to known anammox 16S rRNA gene sequences, but branched much closer to the root of the planctomycete line of descent. Fluorescence *in situ* hybridization (FISH) with oligonucleotide probes specific for these new sequences showed that two species (belonging to the same genus) together made up >99% of the planctomycete population which constituted 20% of the total microbial community. The identification of these organisms as typical anammox bacteria was confirmed with electron microscopy and lipid analysis. The new species, provisionally named *Candidatus* "Scalindua brodae" and "Scalindua wagneri" considerably extend the biodiversity of the anammox lineage on the 16S rRNA gene level, but otherwise resemble known anammox bacteria. Simultaneously, another new species of the same genus, *Candidatus* "Scalindua sorokinii", was detected in the water column of the Black Sea, making this genus the most widespread of all anammox bacteria described so far.

Key words: ammonium – nitrite – nitrate – 16S rRNA approach

Introduction

Anaerobic ammonium oxidation (anammox) is the oxidation of ammonium with nitrite as the electron acceptor and dinitrogen gas as the product [10]. Anammox catabolism proceeds via hydrazine (N_2H_4) and hydroxylamine (NH_2OH). The reaction is exergenic (deltaG = –358 kJ/mol) and provides the energy for anammox anabolism, e.g. the fixation of CO_2 with nitrite as the electron donor (leading to the anaerobic production of nitrate). The process is mediated by obligately anaerobic chemolithoautotrophic bacteria, two of which have been named provisionally: *Candidatus* "Brocadia anammoxidans" [25] and *Candidatus* "Kuenenia stuttgartiensis" [22]. These species (and genera) form a monophyletic cluster inside the *Planctomycetales*, one of the major divisions of the *Bacteria*. Recently it was shown that this order might be the closest to the root of the bacterial domain [3]. Planctomycetes have a proteinaceous cell wall without peptidoglycan and a differentiated cytoplasm

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with membrane-bounded intracytoplasmic compartments that are different and unique for each species [14]. Anammox bacteria have a compartment known as the anammoxosome, which is the locus of anammox catabolism. The lipid bilayer membrane surrounding the anammoxosome contains unique 'ladderane' lipids: concatenated cyclobutane moieties that are either ether and/or ester linked to the glycerol backbone, methylated or occur as free alcohols [23]. The other membranes of anammox bacteria contain lipids typical for planctomycetes in general: iso, normal and 10-methyl hexadecanoic acid.

Anammox bacteria grow very slowly with a doubling time of 3 weeks under laboratory conditions [26]. They are inhibited by low concentrations of the substrate nitrite, are only active in the lab at high cell densities (>10^9 ml^{-1}) and have a low biomass yield [18, 21]. For these reasons their isolation is almost impossible and the identification of anammox bacteria has depended on the use of molecular techniques combined with density gradient centrifugation and activity measurements [27]. In the present paper, the evidence for presence and identity of anammox organisms is based on a 16S rRNA gene library from a wastewater treatment plant (wwtp), fluorescence *in* situ hybridization (FISH), activity measurements, electron microscopy and lipid analysis.

The wwtp, located in Pitsea (UK), is a nitrifying rotating disk reactor operated under oxygen limitation and with a high (87%) nitrogen loss. In similar systems in Switzerland (Kollikon rotating biological contactor [7]), Germany (Mechernich biological contactor [8]), and Belgium (laboratory scale bioreactor [20]) uniform communities of *Candidatus* "Kuenenia stuttgartiensis" affiliated anammox organisms have consistently been detected in high numbers. Although the biomass from the UK had high anammox activity, no anammox bacteria could be detected with a general anammox probe (S-*-Amx-0820-a-A-22, Table 1), indicating that known anammox species were below the FISH detection limit. With a full-cycle rRNA approach we now show that a new genus of anammox bacteria is present in the UK wwtp. By coincendence, another species (*Candidatus* "Scalindua sorokinii") belonging to the same genus was detected in the water column of the Black Sea [12].

Table 1. Probe sequences, target sites, formamide concentrations in the hybridization buffer required for specific *in situ* hybridization

Trivial name (reference)	OPD[a] designation	Specificity	Sequence 5′-3′	Target site	% Formamide/ mM NaCl[c]
Pla 46 (Neef et al. 1998) [19]	S-P-Planc-0046-a-A-18	Planctomycetales	GACTTGCATGCCTAATCC	46–63[b]	25/159
Pla 886 (Neef et al. 1998) [19]	S-P-Planc-0886-a-A-19	*Isosphaera, Gemmata, Pirellula, Plantomyces*	GCCTTGCGACCATACTCCC	886–904[b]	35/80
– (this study)	S-G-Sca-1309-a-A-21	genus *Cand.* "Scalindua"	TGGAGGCGAATTTCAGCCTCC	1309–1329[b]	5/675
– (this study)	S-*-Scabr-1114-a-A-22	*Cand.* "*Scalidua brodae*"	CCCGCTGGTAACTAAAAACAAG	1114–1135[b]	20/225
– (this study)	S-*-Amx-0368-a-A-18	all Anammox organisms	CCTTTCGGGCATTGCGAA	368–385[b]	15/338
– Kuypers et al. 2003 [12]	S-*-BS-820-a-A-22	*Cand.* "*Scalidua wagneri*" *Cand.* "*Scalidua sorokinii*"	TAATTCCCTCTACTTAGTGCCC	820–841[b]	20/225
– Schmid et al. 2000 [22]	S-*-Amx-0820-a-A-22	*Cand.* "Brocadia anammoxidans" and *Cand.* "Kuenenia stuttgartiensis"	AAAACCCCTCTACTTAGTGCCC	820–841[b]	40/56
Eub 338 (Amann et al. 1990) [2]	S-D-Bact-0338-a-A-18	Many but not all *Bacteria*	GCTGCCTCCCGTAGGAGT	338–355[b]	0/900
Eub 338 II (Daims et al. 1999) [6]	S-D-Bact-0338-b-A-18	To be used in combination with probe EUB338	GCAGCCACCCGTAGGTGT	338–355[b]	0/900
Eub 338 III (Daims et al. 1999) [6]	S-D-Bact-0338-c-A-18	To be used in combination with probe EUB338	GCTGCCACCCGTAGGTGT	338–355[b]	0/900

[a]Oligonucleotide probe database [1], [b]16S rRNA position, *E.coli* numbering [5], [c]% Formamide in the hybridization buffer and mM NaCl in the washing buffer, respectively, required for specific *in situ* hybridization.

Materials and Methods

Operation of the wastewater treatment plant (wwtp)

The Pitsea Landfill Site wastewater treatment plant operates five Rotating Biological Contactors (RBCs). Three 40 m^3 units, each with a surface area of 10,000 m^2 were commissioned in 1985, while two larger units (60 m^3 volume and 15,000 m^2 area) were commissioned six years later. The system is designed to treat 300,000 m^3 y^{-1}. Operating at ≥20 °C the units take 3–5 months to reach 100% efficiency. The treated leachate is discharged into the Thames Estuary. Maximum relevant permitted discharge allowances are 1,095 m^3 d^{-1}, 20 mg l^{-1} ammonia and a BOD_5 of 40 mg l^{-1}.

Biomass sampling procedures

The sample was taken from RBC 1, one of the smaller units, which was treating 8,500 litres of leachate per hour on the day of sampling. Biofilm was taken from the outlet on 1st August 2001. Seven hundred millilitres was scraped off the vanes with a sterile spatula and placed in a sterile, 1 l bottle. Seventy millilitres of 0.5 M $NaNO_3$ was then added and gently mixed in. The sample was posted in an insulated container to the Netherlands, arriving the following morning.

Anammox activity

Anoxic batch incubations were carried out in 100 ml flasks containing 50 ml medium (5 mM $NaNO_2$ and 2.5 mM $(NH_4)_2SO_4$ or 2.5 mM $(NH_4)_2SO_4$ and 5 mM $NH_2OH \cdot H_2SO_4$) and 3 g/l protein of sampled biomass (see above), as described previously [28]. Ammonium, nitrite, nitrate, hydrazine and hydroxylamine concentrations were measured colorimetrically as described previously [28].

Retrieval of 16S rRNA gene sequences

Biofilm material was suspended in 10 ml of DNA extraction buffer (100 mM Tris/HCl [pH 8.0]; 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB) [32]. Total genomic DNA was extracted as described previously [11].

The preferential PCR amplification of 16S rRNA genes of members of the *Planctomycetales* was performed with Pla46F (*E. coli* positions 46–63; [22]) forward primer in combination with the universal reverse primer (*E. coli* positions 1529–1545) [11]. PCR was performed with the Tgradient cycler (Biometra, Goettingen, Germany) as described previously [22]. Negative controls (no DNA added) and positive controls (DNA from a *Candidatus* "Brocadia anammoxidans" enrichment culture) were included in all sets of amplifications. Optimum annealing temperature for the used primer set in combination with the DNA retrieved from the biofilm was determined by using the annealing temperature gradient function of the Tgradient cycler. Eight different annealing temperatures between 50 °C and 65 °C were tested. The optimal annealing temperature for the primer set Pla46F/630R regarding yield and specificity was 60 °C. The presence and size of amplification products were determined by agarose (1%) gel electrophoresis of 5 µl aliquots of the PCR products.

The biofilm-derived 16S rRNA gene amplificates were cloned directly by using the TOPO TA Cloning kit following the instructions of the manufacturer (Invitrogen, Groningen, The Netherlands). Plasmid-DNA was isolated with the FlexiPrep Kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ). Plasmids with an insert of the expected size were identified by agarose (1.0%) gel electrophoresis after EcoRI digestion (5 U, Eco RI-buffer for 3 h at 37 °C). Sequencing was done non radioactively by using the BigDye Terminator Cycle Sequencing v2.0 kit (Applied Biosystems, Foster City, CA). The reaction mixtures were analyzed with the 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). The complete sequences of the 16S rRNA gene fragments were determined by using M13 forward and reverse primers targeting vector sequences adjacent to the multiple cloning site as well as primers 609F (*E. coli* positions 785–806; 5′-TTAGATACCCC(A/G/T)GTAGT-3′) and 699R (*E. coli* positions 1099–1114; 5′-AGGGTTGCGCTCGTTGC-3′) targeting the 16S rRNA gene.

Phylogenetic analysis

The 16S rRNA gene sequences retrieved in this study were added to the 16S rRNA gene sequence databases of the Technical University of Munich (currently encompassing more than 25,000 small subunit rRNA sequences) by use of the ARB program package [16, 29]. 16S rRNA sequences were aligned automatically using the respective tool of the ARB package. Subsequently, the alignments were corrected by visual inspection. Phylogenetic analysis of 16S rRNA sequences were performed by applying neighbor-joining, ARB parsimony and maximum likelihood analysis (fast DNAml) [17] to different data sets. Bootstrapping was performed using the PHYLIP parsimony tool (100x resampling) (Phylogeny Inference Package Version 3.5c, University of Washington, Seattle). Checks for chimeric sequences were conducted by independently subjecting the first 5′ 454 base positions, the middle 455 base positions, or the last 454 3′ base positions for phylogenetic analysis.

Probe design, fluorescence in situ hybridization (FISH) and microscopy

For the probes used in this study, sequences, target sites and optimal formamide concentrations in the hybridization buffers are displayed in Table 1 (all probes can also be found on www.probeBase.net [15]. Probes S-G-Sca-1309-a-A-21, S-*-Scabr-1114-a-A-22, S-*-BS-820-a-A-22, S-*-AMX-0368-a-A-18 were designed using the probe design tool of the ARB package. All probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Interactiva (Ulm, Germany). Hybridizations were performed as described previously [22]. Simultaneous hybridization with probes requiring different stringency was realized by a successive-hybridization procedure [31]. Optimal hybridization conditions for probes S-G-Sca-1309-a-A-21, S-*-Scabr-1114-a-A-22, S-*-BS-820-a-A-22, S-*-AMX-0368-a-A-18 were determined by using the hybridization and wash buffers described previously [18]. I*n situ* probe dissociation curves were recorded by measuring the relative fluorescence intensity of biofilm bacteria after hybridization with probes S-G-Sca-1309-a-A-21, S-*-Scabr-1114-a-A-22, S-*-BS-820-a-A-22, S-*-AMX-0368-a-A-18 at different stringencies [6]. After hybridization and air-drying slides were embedded in Vectashield (Vector Laboratories Inc., Burlingame, CA). For image acquisitions a Zeiss axioplan 2 microscope (Zeiss, Jena, Germany) was used.

Electron microscopy

Samples were prepared and inspected as described previously [14].

Lipid analysis

Biomass from the WWTP was ultrasonically extracted with methanol (MeOH), MeOH/dichloromethane (DCM), and DCM (×3). The extracts were combined, the solvent was removed by a rotary evaporator, and an aliquot of the extract was methylated with BF3/methanol. Subsequently, this fraction was chromatographed over a small silica column using ethyl acetate as the eluent to remove very polar material. This fraction was silylated with BSTFA in pyridine at 60 °C for 30 min to convert al-

cohols in TMS ethers. This fraction was analysed with gas chromatography and gas chromatography-mass spectrometry (GC/MS) as described elsewhere.

Compound-specific stable carbon isotope ratios were determined using a Thermofinnigan Delta lus XL isotope ratio monitoring GC/MS system. The gas chromatograph was equipped with a fused silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 (0.12 mm film thickness) and used helium as carrier gas. Samples dissolved in ethyl acetate were injected at 70 °C and the oven was programmed to 130 °C at 20 °C/min and then to 320 °C at 4 °C/min, followed by an isothermal hold for 10 min. Most values reported were determined by duplicate analyses and were averaged. Isotopic compositions are reported in standard delta notation relative to the VPDB standard. δ^{13}C-values of fatty acids and alcohols were obtained by correcting their measured δ^{13}C-values for the isotopic composition of carbon added during the derivatization step.

Nucleotide sequence accession numbers

The sequences obtained in this study are available in GenBank under accession numbers AY254882 and AY254883.

Results

The Pitsea Landfill Site wastewater treatment plant was commissioned in 1985 using an activated sludge inoculum. Typical flow rate of leachate through the plant is about 30 m^3 h^{-1} with an influent ammonium concentration of 350–400 mg l^{-1} and an effluent concentration of about 10 mg l^{-1}. During the early years of operation, mass-balance between influent and effluent indicated a high percentage (> 90%) transformation of ammonium to nitrite/nitrate measured as total oxidized nitrogen (TON). However, presently the typical effluent TON is reduced to about 60 mg l^{-1}.

When sampled, RBC 1 was reducing the ammonia concentration in the leachate from 349 mg l^{-1} at the influent to 3.5 mg l^{-1} at the outlet, a conversion rate of 5.8 g N m^{-2} d^{-1}. Concurrently, respective nitrite and nitrate concentrations increased from 0.7 and < 0.1 mg l^{-1} at the inlet to 8.3 and 33 mg l^{-1} at the outlet. This lowered alkalinity from 2,817 to 1,560 mg $CaCO_3$ l^{-1}. The intense microbial activity raised the temperature of the water from 22 to 25 °C.

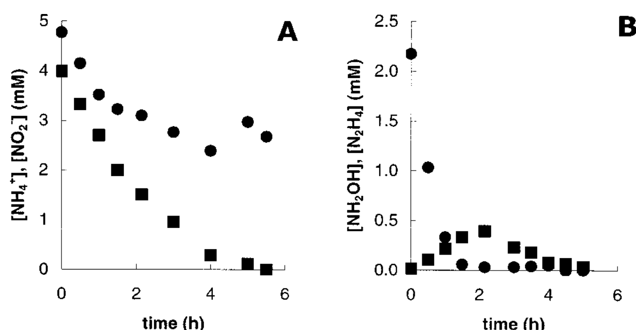

Fig. 1. Anammox activity of biomass from the wwtp (Pitsea, UK). A. Simultaneous consumption of ammonia (circles) and nitrite (squares) under anoxic conditions. B. Production of hydrazine (squares) from hydroxylamine (circles) under anoxic conditions.

Anammox activity of sampled biomass was measured in batch incubations (Fig. 1). The anammox activity was 5.0 ± 0.5 nmol/mg protein/min, 25% of the activity found previously for 80% enriched *Candidatus* "Brocadia anammoxidans" or *Candidatus* "Kuenenia stuttgartiensis" under similar conditions. The stoichiometry of the anammox reaction was 1:1.3 (mol NH_4^+/mol NO_2^-), the same as reported previously for anammox. Nitrate production was not detected; this could be explained by the simultaneous activity of other, non-anammox denitrifying bacteria, that consumed the nitrate produced by anammox [30]. When the biomass from the wwtp was incubated with ammonium and hydroxylamine (NH_2OH), a transient accumulation of hydrazine (N_2H_4) was found (Fig. 1). This is again consistent with results obtained with the known anammox bacteria *Candidatus* "Brocadia anammoxidans" or *Candidatus* "Kuenenia stuttgartiensis". Production of free hydrazine is one of the unique features of anammox bacteria and indicates that the anammox activity of the UK wwtp proceeds via the usual anammox biochemical pathway and is not a result of nitrifier denitrification.

To link the detected anammox activity to the presence of anammox bacteria, the sampled biomass was initially inspected using fluorescence *in situ* hybridization (FISH) with the as-yet general anammox probe S-*-Amx-0820-a-A-22 (see Table 1). Surprisingly, no bacteria could be detected with this probe. However, hybridization with the probe S-P-Planc-0046-a-A-18 specific for all *Planctomycetales* (Table 1) did show that a large planctomycete population constituted about 20% of the total microbial community.

Since a clear assignment of these planctomycetes to anammox organisms was not possible by FISH with existing probes we applied a *Planctomycetales* specific full cycle rRNA approach. Thus DNA was extracted from biofilm of the wwtp. 16S rRNA gene sequences of planctomycetes were amplified by PCR with the primers Pla46F and 630R and cloned. Ten clones of the resulting clone library were randomly sequenced and almost full-length 16S rRNA sequences (average length 1,490 bases without primer) were obtained. Subsequent phylogenetic analysis showed that four sequences branched relatively close to the known anammox genera and formed a sequence cluster around the anammox species *Candidatus* "Scalindua sorokinii", discovered recently in the Black Sea [12] and close to a clone derived from a deep sea sediment. The other 6 clones were affiliated to previously described members of the *Plantomycetales* other than anammox. The four new anammox affiliated clones clones split in two sequence types represented in Fig. 2 by clone EN8 (marked *Candidatus* "Scalindua brodae", see Discussion section, 3 clones, 98% sequence similarity to *Candidatus* "Scalindua sorokinii" and 99.9% similarity to each other) and clone EN5 (marked *Candidatus* "Scalindua wagneri" (see Discussion section, 1 clone). The latter had approximately 93% sequence similarity to both the EN8 and the *Candidatus* "Scalindua sorokinii"

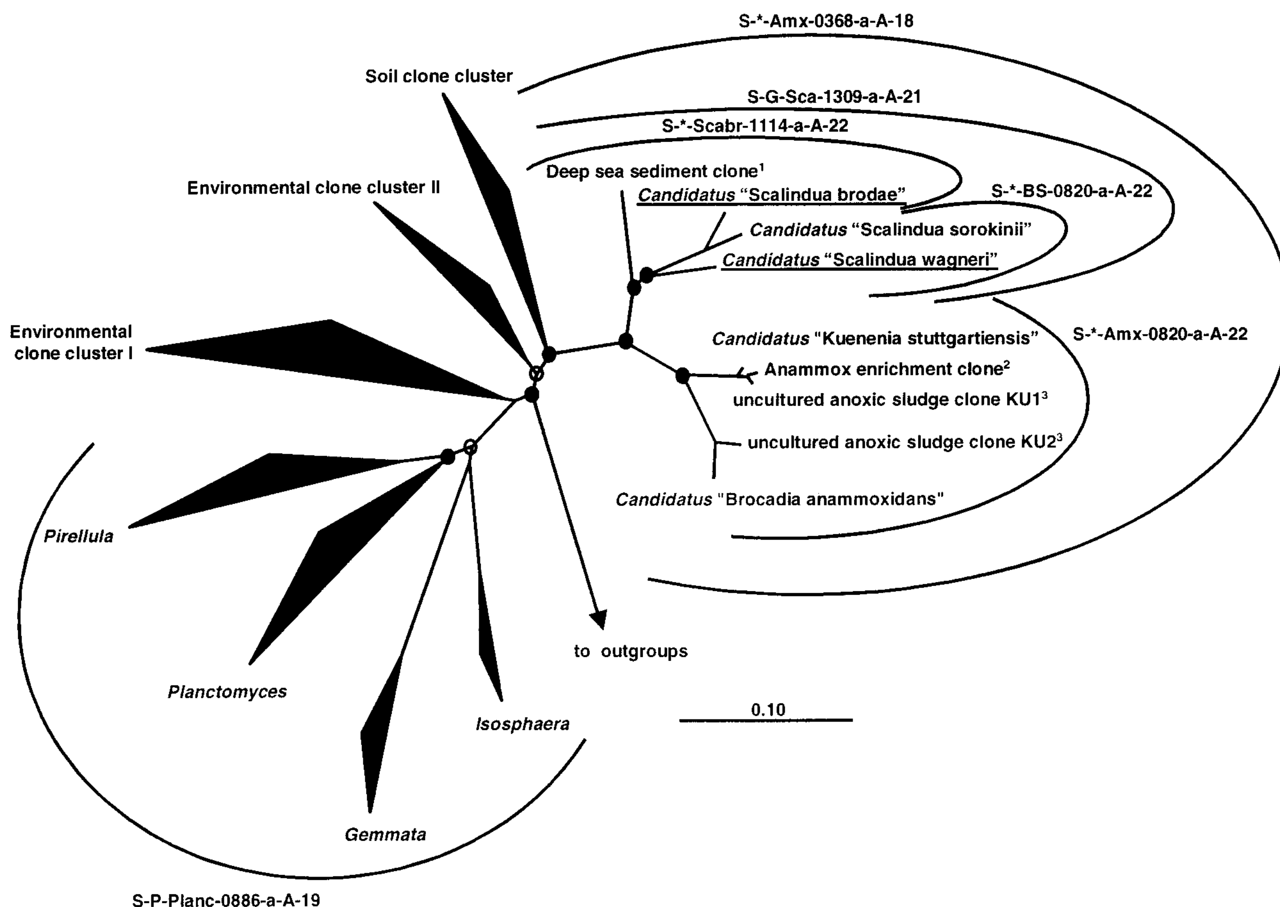

Fig. 2. Phylogenetic tree reflecting the relationships of the wwtp (Pitsea, UK)16S rRNA gene EN8 (*Candidatus* "Scalindua brodae"), gene EN5 (*Candidatus* "Scalindua wagneri"), *Candidatus* "Scalindua sorokinii", *Candidatus* "Kuenenia stuttgartiensis", *Candidatus* "Brocadia anammoxidans", other *Planctomycetales,* and other reference organisms. The triangles indicate phylogenetic groups. Phylogenetic analyses were performed with maximum likelihood, neighbor joining and maximum parsimony methods with 50% sequence conservation filters for *Bacteria* as well as *Plantomycetales.* Since no differences between all calculated trees in terms of branching order could be observed the tree based on maximum likelihood analysis with the 50% conservation filter for *Bacteria* is presented here. Filled circles indicate parsimony bootstrap values higher than 75%. Empty circles refer to values between 50 and 75%. The bar represents 10% estimated sequence divergence. Probe Pla 46 binds to all *Planctomycetales* in the tree and specificities of other probes used in this study as well as probe Pla 866 are indicated by brackets around the targeted species. For further information about the probes, refer to Table 1. References: [1]Li et al. 1999 [13]; [2]Egli et al. 2001 [7]; [3]Imajo et al. 2001 [9].

sequences. This indicates that the two organisms represented by 16S rRNA gene sequences EN5 and EN8 constitute new species of the genus "Scalindua", the third anammox lineage described so far. Three new oligonucleotide probes S-G-Sca-1309-a-A-21, S-*-Scabr-1114-a-A-22, S-*-BS-820-a-A-22 (the latter also described in [12]) were constructed to match specifically with one or more of these new sequences and additionally, probe S-*-AMX-0368-a-A-18 was designed to detect all recognized anammox organisms (for probe specifications see Table 1 and Fig. 2). Optimal hybridization stringencies for all four probes were determined by visual inspection of the hybridization intensity with fixed biofilm samples from the wwtp using increasingly stringent conditions. For each probe the highest formamide concentration, which still yielded a maximum probe intensity, was chosen as optimal stringency (see Table 1). The specificity of the new probes was tested by hybridization of enrichment cultures of *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis" at stringent conditions. No signal could be obtained with probes S-G-Sca-1309-a-A-21, S-*-Scabr-1114-a-A-22, S-*-BS-820-a-A-22. However probe S-*-AMX-0368-a-A-18 exhibited strong signals with both enrichment cultures. Furthermore both new anammox organisms in the wwtp Pitsea could be detected specifically with probe S-*-AMX-0368-a-A-18.

FISH with probes S-G-Sca-1309-a-A-21, S-*-Scabr-1114-a-A-22, S-*-BS-820-a-A-22, S-*-AMX-0368-a-A-18 showed that each of the two anammox species repre-

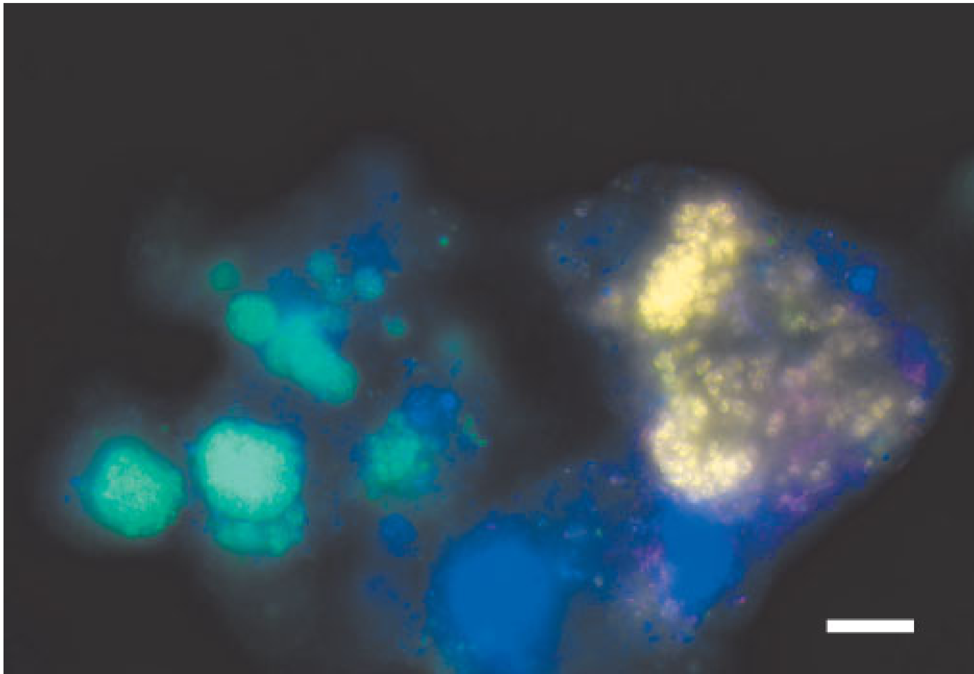

Fig. 3. *In situ* identification of *Candidatus* "Scalindua brodae" (clone EN8) and *Candidatus* "Scalindua wagneri" (clone EN5) like organisms in biofilm of the wwtp (Pitsea, UK) by simultaneous hybridization with Cy3-labeled probe S-*-Scabr-1114-a-A-22, Fluos-labeled probe Pla46 and the Cy5-labeled Eub probe mix (Table 1). *Candidatus* "Scalindua brodae" appears yellow-white, because all three labels overlap (yellow-white color is caused by a slight overexposure of the green and red channel compared to the blue). Other planctomycetes including *Candidatus* "Scalindua wagneri" are labeled turquoise, because of the overlapping Fluos and Cy5 labeling.

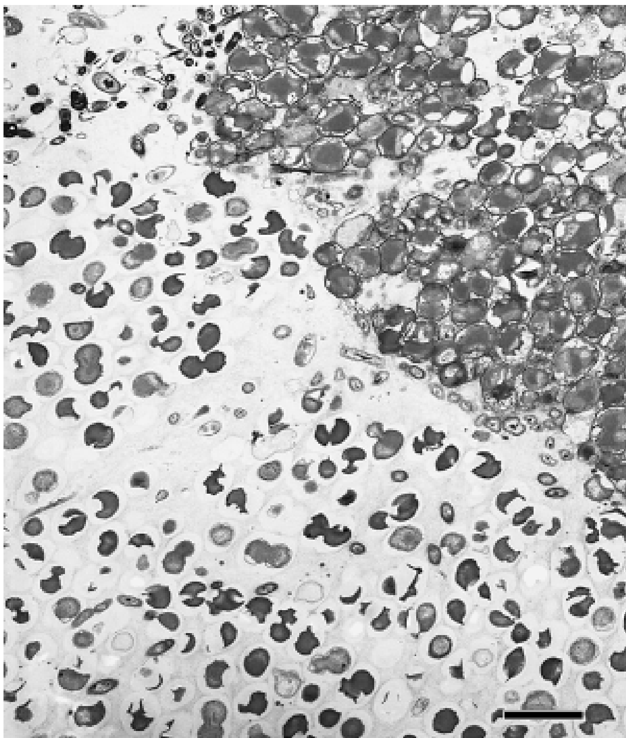

Fig. 4. Transmission electron micrograph of thin section of chemically fixed wwtp biofilm floc microcolonies showing groups of cells of two distinct morphotypes, both sharing anammox cell plan. Morphotype 1 forms a microcolonial group of closely packed cells in the top-right of the image, while morphotype 2 forms an adjacent group of well-spaced cells in the bottom left. Bar = 2 μm (plate 84908).

sented by the 16S rRNA gene sequences EN8 and EN5 (Fig. 2) contributed about 50% to the total planctomycete population (Fig. 3). They both formed clusters consisting of only one species; the clusters hybridizing with probe S-G-Scabr-1309-a-A-21 appeared to be less dense than the clusters hybridizing with S-*-BS-820-a-A-22 (Fig. 3). Since together the two species constituted more than 99% of all *Planctomycetales* (as determined by simultaneous hybridization of probes S-G-Sca-1309-a-A-21, S-*-Scabr-1114-a-A-22, S-*-BS-820-a-A-22, S-*-AMX-0368-a-A-18 with S-P-Planc-0046-a-A-18) in the UK wwtp, other planctomycete 16S rRNA gene sequences belonged to a negligible part of the bacterial community.

Anammox bacteria have a unique ultrastructure, with an intracytoplasmic membrane that surrounds an organelle-like intracytoplasmic compartment, the anammoxosome, which is also the locus of anammox catabolism. This compartment contains no ribosomes and leads to the visualization of ring-shaped cells with FISH. Such rings were also observed with all applied anammox bacteria specific probes (Fig. 3).

To confirm the presence of anammoxosomes in the new planctomycetes, the biomass was inspected with electron microscopy. Transmission electron microscopy of thin sections prepared from the UK wwtp biomass showed the presence of cells consistent with the anammox structural plan as previously described [14]. The cell plan was detected regardless of whether they were fixed chemically or via cryotechniques, though with superior preservation via the latter. Two distinct morphotypes of cells with such a cell plan were detected in the biofilm floc, each grouped in a distinct microcolony arrangement,

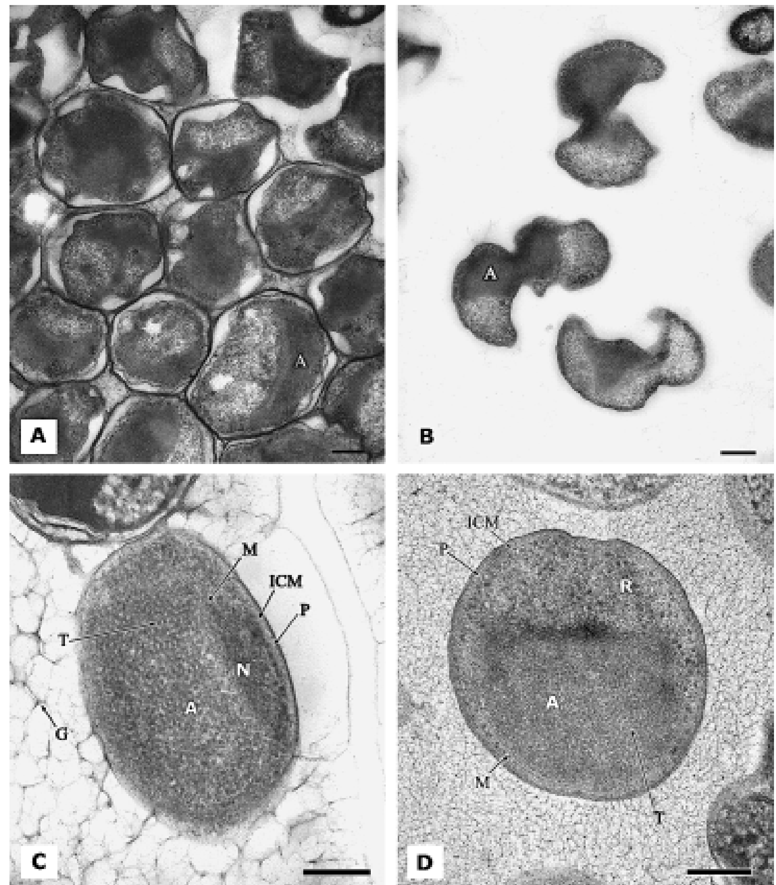

Fig. 5. A. Transmission electron micrograph of thin-sectioned chemically fixed anammox floc microcolony of morphotype 1 cells, showing close apposition of cells with each other and glycocalyx. Each cell displays an anammoxosome (A) and riboplasm, and is extensively plasmolysed with retraction of cytoplasmic membrane from wall in each case. Bar = 200 nm (plate 84903) **B.** Transmission electron micrograph of thin-sectioned chemically fixed microcolony of morphotype 2. Cells in this microcolony are only loosely bound to each other and no clear glycocalyx is visible. Each cell displays an anammoxosome (A) and riboplasm. Although cells are not plasmolyzed, they exhibit strong distortion of shape. Bar = 200 nm. (plate 84899). **C.** Transmission electron micrograph of thin-sectioned cryosubstituted cell of morphotype 1 from an anammox floc showing well-preserved structure of internal organization of the anammox cell plan including anammoxosome (A) containing many tubules (T), riboplasm containing the nucleoid (N) apposed to the anammoxosome membrane (M), and paryphoplasm (P) separated from ribosplasm by an intracytoplasmic membrane (ICM). The cell is embedded in an extensive glycocalyx (G). Bar = 200 nm (plate 86253). **D.** Transmission electron micrograph of thin-sectioned cryosubstituted cell of morphotype 2 from an anammox floc showing well-preserved structure of internal organization of the anammox cell plan including anammoxosome (A) containing many tubules (T), riboplasm containing the nucleoid (N) apposed to the anammoxosome membrane (M), and paryphoplasm (P) separated from a large riboplasm (R) by an intracytoplasmic membrane (ICM). The cell is embedded in an extensive glycocalyx. Bar = 200 nm (plate 71976).

either with cells closely packed (morphotype 1) or widely spaced (morphotype 2). See Fig. 4. Chemically fixed cells of morphotype 1 exhibited only some aspects of the anammox plan; these include the characteristic and diagnostic anammoxosome compartment and a nucleoid closely apposed to this structure (Fig. 5a). Extensive plasmolysis was seen in this morphotype when chemically fixed, and in contrast to what was observed in chemically fixed morphotype 2. Cryosubstituted morphotype 1 cells did not display any plasmolysis and all elements of the classical anammox cell plan were present including a single membrane-bounded anammoxosome containing tubules, nucleoid apposed to anammoxosome membrane, riboplasm with ribosome-like particles separated from the paryphoplasm at the cell rim by an intracytoplasmic membrane (Fig. 5c) . Also consistent with *Candidatus* “Brocadia anammoxidans“ but not with non-anammox planctomycete genera was the relatively electron-transparent paryphoplasm. Thus, the three characteristic compartments of the anammox cell plan, the anammoxosome, the nucleoid-containing riboplasm and the paryphoplasm were demonstrated in morphotype 1, if cells were prepared via cryosubstitution. Chemically fixed cells of morphotype 2 displayed no plasmolysis but did display a strong distortion of cell shape (Fig. 5b), clearly apparent when compared to cryosubstituted cells (Fig. 5d). The anammox plan including tubule-containing anammoxosome, nucleoid in riboplasm, and paryphoplasm were also clear in cryosubstituted morphotype 2 (Fig. 5d) but the anammoxosome appeared less extensive in area occupied within the cell than in the case of morphotype 1. Typical of bacterial biofilms, both morphotypes 1 and 2 were embedded in a glycocalyx well-preserved in the case of cryosubstituted floc but in chemically fixed material only partially preserved in the case of morphotype 1. The presence and abundance of two morphotypes with anammox cell plan was consistent with the discovery of two phylotypes from 16S rRNA sequence data and FISH. Since the two types of colony morphologies could be discriminated both with FISH and with electron microscopy, morphotypes and phylotypes could be paired, with morphotype 1 belonging to 16S rRNA sequence EN5 and morphotype 2 belonging to 16S rRNA sequence EN8.

The chemical structure of the membrane lipids is another unique feature of known anammox bacteria. Anammox bacteria contain lipids characterized by linearly concatenated cyclobutane (ladderane) moieties, which fulfil an essential physiological role [23]. They form a dense membrane around the anammoxosome, which

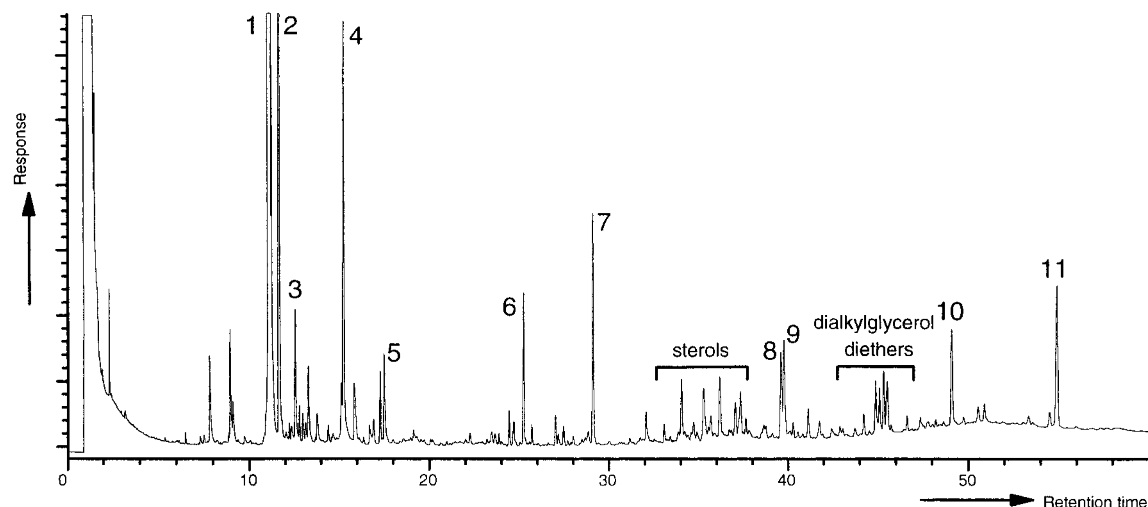

Fig. 6. Gas chromatogram of the total lipid fraction of the extract of the biomass of the wwtp. Key: 1 = $C_{16:1}$ fatty acid, 2 = $C_{16:0}$ fatty acid, 3 = 10-methylhexacosanoic acid, 4 = $C_{18:1}$ fatty acid, 5 = $C_{19:1}$ fatty acid, 6 = 1-hexadecenyl glycerol monoether, 7 = squalene, 8 = tetrahymanol, 9 = diplopterol, 10 = 1,2-tetradecyl, ladderane-glycerol diether, 11 = bacterio-hopanetetrol. Fatty acids and alcohols were analyzed as the methyl ester and trimethyl silyl ether derivatives, respectively.

might contain the intermediate products of the anammox reaction (e.g. hydrazine) within this cell compartment. Since these lipids play an essential role in the physiology of anammox bacteria the UK WWTP biomass was analysed by gas chromatography/mass spectrometry for their lipid content (Fig. 6). The most abundant lipids present were the $C_{16:1}$, $C_{16:0}$ and $C_{18:1}$ fatty acids. Other, slightly less abundant lipids include: bacteriohopanetetrol, dialkyl glycerol esters, diplopterol, tetrahymanol, hop-22(29)-ene, squalene, 1-glycerol-hexadecene and a suite of other fatty acids. Two lipids could be attributed as likely originating from anammox bacteria: 10-methyl-hexadecanoic acid and a ladderane glycerol diether (Fig. 6). 10-Methyl-hexadecanoic acid has been reported to occur in planktomycetes [24], in *Candidatus* "B. anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis" [23], where it is thought to be a membrane lipid of the cell membrane. The ladderane glycerol diether was identified on the basis of its mass spectrum. It was different from the ladderane membrane lipids reported in *Candidatus* "B. anammoxidans" [23] since it contained a ladderane alkyl moiety at position 2 and an, as yet uncharacterised, acyclic C_{14} alkyl moiety at position 1 of the glycerol unit.

To provide further evidence for the biological origin of the 10-methyl-hexadecanoic acid and the ladderane glycerol diether in the mixed microbial community of the UK WWTP, we applied compound-specific stable carbon isotope analysis. Unpublished work has shown that the lipids of anammox bacteria in culture are substantially depleted in ^{13}C (~38‰ for the branched fatty acid and ~43% for ladderane lipids) relative to its carbon source (i.e. dissolved inorganic carbon) compared to other autotrophic organisms (~20–30‰). Although the reasons for this depletion are presently unclear, it could be used to assign specific lipids to anammox bacteria. The stable carbon isotopic compositions of 10-methyl-hexadecanoic acid ($\delta^{13}C$ = ca. –38‰) and the ladderane glycerol diether ($\delta^{13}C$ = ca. –45‰) were, indeed, substantially depleted relative to the other bacterial lipids (–20‰ < $\delta^{13}C$ < –30‰), confirming their origin from anammox bacteria. In addition, bacteriohopanetetrol ($\delta^{13}C$ = ca. –34‰) and two acyclic dialkyl diethers ($\delta^{13}C$ = ca. –41‰) were also substantially depleted in ^{13}C, suggesting a (partial) origin from anammox bacteria. Bacteriohopanetetrol has indeed also been found as an abundant lipid in enrichments cultures of *Candidatus* "B. anammoxidans" (Sinninghe Damsté et al., unpublished results).

Discussion

Based on mass balances over the wastewater treatment plant (wwtp) in Pitsea, UK, batch activity assays of its biomass, 16S rRNA gene based phylogenetic analysis combined with fluorescence *in situ* hybridization, electron microscopy, and lipid analysis, we suggest that two new species of anammox bacteria, belonging to the same genus, are responsible for the nitrogen losses observed in this wwtp. All results are internally consistent and also consistent with results obtained previously with other anammox bacteria. For ease of future reference, we propose to provisonally name the new species *Candidatus* "Scalindua brodae" (corresponding to 16S rRNA sequence EN8, morphotype 2) in honour of the Austrian theoretical chemist Engelbert Broda, who was the first to recognize the possibility of anaerobic ammonium oxidation [3]; *Candidatus* "Scalindua wagneri" (corresponding to 16S rRNA sequence EN5, morphotype 1) in honour of

the German microbial ecologist Michael Wagner, who has contributed much to the field of microbial ecology and phylogeny of anammox in particular.

Interestingly, a third member of the genus "Scalindua" (*Candidatus* "Scalindua sorokinii") was simultaneously detected in the water column of the Black Sea [12]. This habitat was also characterized by significant anammox activity and the presence of ladderane lipids. With its detection in two very different habitats, "Scalindua" is possibly the most widespread anammox genus identified so far.

The new genus is very similar to known anammox bacteria, in all aspects investigated: production of hydrazine from hydroxylamine, presence of ladderane lipids, presence of an anammoxosome and other ultrastructural features, substrate conversion rate – approximately – 20 nmol NH_4^+/mg protein/min (corrected for the anammox part of the population, 20%). The only clear difference between *Candidatus* "Scalindua" and the other anammox genera is the absence of the recently described helices 9a and 9b in the 16S rRNA [21]. Thus this characteristic feature for *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis" can no longer be considered a common feature of all anammox bacteria.

On the 16S rRNA level, the new genus is quite distant from the other anammox genera. The average sequence similarity between members of *Candidatus* "Scalindua" and *Candidatus* "Brocadia" was only 85%. Indeed, the phylogenetic depth of the anammox lineage is remarkable; it is much deeper than the lineage of aerobic betaproteobacterial ammonia oxidizers (sequence similarity between its most distant members is about 90%). Yet, all anaerobic ammonium oxidizers have very similar physiology and morphology. Among the *Betaproteobacteria*, organisms with the same degree of relatedness on the 16S rRNA level as the anammox bacteria, have phenotypes that are completely unrelated: *Nitrosomonas europaea*, a chemolithoautotroph and *Neisseria gonnorhoea*, a parasitic chemoorganoheterotroph, also have 85% sequence similarity. It is clear that the long phylogenetic distances between individual members of the *Planctomycetales* remains unresolved. Planctomycetes grow slowly, and the anammox bacteria in particular (doubling time 3 weeks), so rapid evolution is not a logical explanation in this case. Neither can a single event of 'quantum evolution' explain the divergence of anammox bacteria, because their phylogentic tree is not characterized by a long stem and short branches. Three possible explanations remain: (1) the planctomycetes are indeed an old lineage and the individual genera had more time to evolve; (2) the architecture of the planctomycete ribosome leaves room for more degrees of freedom (e.g. caused by a less efficient repair system), and (3) individual species and genera have drifted further apart on a genetic level without compromising ribosome function; With the soon available full genome sequences of several planctomycetes (*Pirellula marina, Gemmata obscuriglobus* and *Candidatus* "Kuenenia stuttgartiensis"), this question might be resolved in the near future.

Since anammox bacteria of different genera rarely occur in the same wwtp or enrichment culture, it seems that they all occupy their own niche and environmental conditions select for only one of the different genera. At present we have no clue as to which properties are responsible for niche differentiation of anammox bacteria. From an applied point of view, it remains impossible to predict whether seeding of new anammox reactors with biomass from an existing reacter would be successful. This question is all the more urgent since the start up of anammox reactors takes a long time due to the slow growth of the bacteria. Availability of biomass from different sources with a well-characterized anammox population might help to eventually solve this problem. In this context, the new probes in this paper are an important contribution to the detection and characterization of anammox populations both in man-made and natural ecosystems.

Description of *Candidatus* "Scalindua brodae", *Candidatus* "Scalindua wagneri"

Scalindua brodae (sca.lin'.du.a L. fem. n. _scala_ ladder; L. v. _induo_ dressed out or fitted with; L. f. n. _scalindua_ dressed out with ladders, referring to the presence of unique ladderane lipids; _brodae_ in honour of Engelbert Broda; _wagneri_ in honour of Michael Wagner).

Obligately anaerobic chemolithoautotrophic coccoid cells with a diameter of approximately 1 µm. Cells oxidize ammonium with nitrite as the electron acceptor and with CO_2 as the main carbon source. Cells convert hydroxylamine to hydrazine. Cells have a proteinaceous cell wall and possess a membrane bound intracytoplasmic compartment known as the anammoxosome. Cells contain ladderane lipids, more specifically 1,2-tetradecyl, ladderane-glycerol diether. Cells of *S. wagneri* form tightly packed clusters and display plasma lysis after glutaraldehyde fixation whereas cells of *S. brodae* are more loosely packed and show no plasma lysis but cell distortion after glutaraldehyde fixation. 16S rRNA sequence similarity between the two species is 93%.

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