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PhD Thesis

-Summary-

**Identification by molecular methods of the microbial community
from an anammox bioreactor for treatment of residual fluids, rich
in toxic nitrogen compounds**

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Introduction

The evolution of man and of the society determined the accumulation of reactive forms of nitrogen both in the ecosystems and atmosphere, caused by the intensification of anthropogenic activities. Intensive agriculture, industrialization and urbanization has led to an increased impact on water, soil and air quality, aquatic ecosystem biodiversity and implicitly on water resources. It is estimated that human activities contribute, globally, to 30%-45% of the total fixed nitrogen, in terrestrial and aquatic ecosystems.

Pollution by toxic nitrogen compounds determines soil and water acidification, reduction of terrestrial and aquatic ecosystems, reduction of the ozone layer, climate change, directly affecting human health (Galloway et al., 2003).

The residues resulted as a consequence of human activities represent a considerable source of reactive forms of nitrogen and residual water fluxes will increase subsequently to population growth and intensive urbanization. Nitrogen removal from wastewater streams is conventionally done by microbial processes such as nitrification and denitrification. The efficiency of these two processes depends on spatial and temporal differentiation, because of the different conditions and microorganisms involved. Nitrification is based on high nitrogen demand and denitrification involves an external source of electron donors (methanol, acetate, ethanol, lactate, glucose) because organic carbon naturally present in residual waters is limited (van Dongen et al., 2001). The limitations of the conventional nitrification/denitrification system are represented by high oxygen requirements, pH control, no retention, total organic carbon (Jetten et al., 2002).

An alternative, efficient and less expensive method was identified by the discovery (Mulder et al., 1995) of the anammox process (anaerobic ammonium oxidation) and its microbiological nature (van de Graaf et al., 1996). The anammox process is in fact the anaerobic oxidation of ammonium to nitrogen gas, having nitrite as electron acceptor. The anammox process being autotrophic there is no need for external organic carbon addition for denitrification (van de Graaf et al., 1996), resulting in the reduction of costs for electron donor acquisition. If anammox is combined with an initial nitrification step, only a part of the ammonium needs to be nitrified to NO_2^- , while the anammox bacteria combines the ammonium left with nitrite to form

nitrogen gas, reducing the oxygen needed and so the aeration costs for nitrification (Jetten et al., 2002).

Although anammox bacteria have been enriched initially from wastewater treatment systems (van de Graaf et al., 1996; Strous et al., 1999), their presence and activity is not restricted to such ecosystems (Schmid et al., 2003; Thamdrup and Dalsgaard, 2002; Dalsgaard et al., 2003; Kuypers et al., 2003). Globally, it is assumed that the anammox activity is responsible for 30-70% of the total N_2 produced in the oceans (Devol, 2003).

Phylogenetically belonging to the phylum *Planctomycetes*, sharing common characteristics with cultivated genera like the lack of peptidoglycan in the cell wall and internal compartmentalization (Lindsay et al., 2001), the anammox bacteria identified so far are grouped in four genera, all having *Candidatus* names: *Brocadia* (Strous et al., 1999), *Kuenenia* (Schmid et al., 2000), *Anammoxoglobus* (Kartal et al., 2007) and *Scalindua* (Schmid et al., 2003). Because different anammox genera rarely are identified in the same wastewater treatment system, or enrichment culture they seem to occupy different micro-niches; also, the significant phylogenetic distances (>91%) between species indicate the existence of distinct but, yet, unknown ecological niches.

In this study, by using a membrane bioreactor, a highly enriched (97.6%) anammox culture was obtained, using a solids retention time of 12 days (van der Star et al., 2008). The goal of this experiment was to identify the microorganisms growing in the enrichment culture by using molecular techniques based on 16S rDNA like PCR-DGGE (Polymerase chain reaction, Denaturing Gradient Gel Electrophoresis) and FISH (Fluorescence In Situ Hybridization). It is also interesting to study the side population and its members in this reactor, and other reactors in which the anammox process is present or absent and also to try to determine if these bacteria originate in the inoculum or actively grow, under anammox conditions.

Objectives

General objective: identification, by molecular methods, of community members from the culture enriched in the membrane bioreactor.

Specific objectives:

1. Identification of the anammox members of the community enriched in the membrane bioreactor and establishment of the enrichment level of the culture.
2. Obtainment of a community profile of the non-anammox populations and establishing the phylogenetic affiliation of these microorganisms.
3. Screening of several reactors, positive or negative for the anammox process, for identification of non-anammox bacteria found in the membrane bioreactor.

Materials and methods

Biological material

The biological material used in this study was collected from several reactors, in which the anammox process did/did not take place. Details on the reactors are given in tab 1. From the membrane bioreactor (MBR) biomass was collected 2-3 times/week; for the full-scale anammox reactor samples were collected from the inferior compartment of the reactor (sample point 2, van der Star et al., 2007), once per week, for 10 weeks. Two additional samples came from the start-up period (2005). For all other reactors, samples were collected once, at a time when the reactors were functioning in optimal conditions.

Molecular methods

DNA extraction

Genomic DNA was isolated from the samples collected by using UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA).

Table 1. List of reactors from which biomass was collected for the experiments.

	Bioreactor/process	Reactor type	Dominant population
	Membrane bioreactor	MBR	Brocadia/Kuenenia
Positive control	Full-scale anammox reactor	UASB	Brocadia
	Full-scale one reactor nitrification-anammox (CANON/OLAND/deammonification)	BCB	Brocadia
	Lab-scale sequencing batch reactor	SBR	Brocadia
Negative control	Nitrification/denitrification and acetate/phosphate removal bioreactor	SBR	Nd
	SHARON (lab-scale)	Chemostate	AOB
	SHARON (full-scale)	-	AOB
	Nitrification reactor (lab-scale)	SBR	AOB NOB

PCR amplification

The extracted DNA was amplified by PCR using several primer pairs (tab. 2), *Planctomycetes* specific (for identification of anammox bacteria) or general/universal bacterial primers (for identification of the non-anammox members of the community. For identification of the main population, DNA isolated on days 1, 18, 52, 105, 262 and 331 was amplified using Pla-46F/907R(M) and Pla-46F/1392R primer pairs.

Table 2. Primers used for partial 16S DNA amplification.

Name	Specificity	Sequence (5'-3')	References
Pla-46F	<i>Planctomycetes</i>	GGA TTA GGC ATG CAA GTC	Neef et al. (1998)
907R(M)¹	<i>Bacteria</i>	CCG TCA ATT CMT TTG AGT TT1	Muyzer et al. (1993)
1392R	Universal	ACG GGC GGT GTG TAC	Schaefer and Muyzer (2001)
341F (GC)²	<i>Bacteria</i>	CCT ACG GGA GGC ACG AG	Muyzer et al. (1993)

¹M indicates the presence of adenine (A) or cytosine (C)

²GC-clamp attached at the 5' end, formed of 40 nucleotides (sequence: 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3')

DGGE (Denaturing Gradient Gel Electrophoresis)

This molecular method, based on the 16S rRNA gene was used to determine the structure of the non-anammox community from the membrane bioreactor and control reactors, as well as to establish the temporal dynamics of this MBR community during the experiment. For PCR product analysis we used 6% (w/v) polyacrylamide gels. Initially the denaturant gradient used was 20%-60% urea and formamide (100% denaturant solution consists of a mixture of 7M urea and 40% v/v formamide). For amplification of the distance between migrating fragments, we subsequently used a narrow gradient (35%-55% UF) which also favors band excision for sequencing purposes. Electrophoresis was performed in 1xTAE buffer, at 100V for 16 hours. After 16 h gels were incubated in etidium bromide solution for 30 min, than washed in MilliQ (30 min) and photographed using a BioRad GelDoc system.

The polyacrylamide gel was transferred on the trans-illuminator and bands were excised and incubated over night 4°C) in 50 µl ultrapure water. DNA was re-amplified using the same primer pair and PCR protocol, and re-DGGE was performed using identical electrophoresis conditions. Initial PCR products were migrated alongside with products from the re-amplification to check for band “purity” and if the band is identical to the original profile

After re-amplification, PCR products from the band of interest are purified by agarose gel electrophoresis for sequencing, using QIAquick Gel Extraction Kit (Qiagen, Germany).

Oligonucleotidic probes design

Based on the sequences retrieved after PCR-DGGE oligonucleotidic probes were designed, using PROBE DESIGN option of ARB software. The probes (18 nucleotides, tab. 3) were synthesized by a commercial company (Thermo Fisher Scientific, Ulm, Germany) and were fluorescently labeled, at 5' end using Cy3. Optimal hybridization conditions were determined by successively testing different formamide concentrations in the hybridization buffer. *In situ* hybridization was performed simultaneously, using 3 probes, labeled with different fluorochromes: CHL probes (Cy3), AMX-820 (Fluos) and a mixture o EUB probes (Cy5).

Fluorescence *in situ* hybridization (FISH)

FISH was used in this study mainly for the identification of the dominant members of the membrane bioreactor community and determination of the purity of the enrichment. For FISH,

samples collected from the MBR (15 ml cell suspension) were fixed in paraformaldehyde; cells were immobilized on teflon coated plates, hybridized with oligonucleotidic probes, washed for removal of un-hybridized probes and mounted for visualization, using a epifluorescence microscope (Zeiss Axioplan 2, Zeiss, Jena, Germany). Fixation was performed for samples collected on days 35, 64, 77, 149, 196 and 267. The probes used for hybridizations are described in tab 3.

On day 267, the enrichment level was estimated by counting visible cells (using the epifluorescence microscope) which did not hybridize with AMX-820 probe (non-anammox cells) and this number was compared to the number of total visible cells (anammox and non-anammox, ca. 10,000).

We also used FISH to determine if non-anammox members of the MBR community represent a satellite population for anammox bacteria, using the oligonucleotidic probes designed in this study to confirm/infirm if the non-anammox microorganisms are present in the control reactors.

Sequencing and comparative sequence analysis

PCR products were purified from the gels using QIAquick Gel Extraction Kit (Qiagen, Germany). Purified products were sequenced by a commercial company (BaseClear, Leiden, The Netherlands). The sequences retrieved were compared to sequences from public databases using NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), imported into ARB software and aligned using the alignment tool of the software (Ludwig et al., 2004). The alignment was manually corrected and a phylogenetic tree was constructed using Neighbor-Joining algorithm with Felsenstein correction (Saitou and Nei, 1987).

Table 3. Oligonucleotidic probes used for FISH.

Oligonucleotidic probes	Target organism	Sequence (5'-3')	References
EUB I EUB II EUB III	<i>Bacteria</i>	GCT GCC TCC CGT AGG AGT GCA GCC ACC CGT AGG TGT GCT GCC ACC CGT AGG TGT	Daims et al. (1999)
AMX-820	<i>Kuenenia/Brocadia</i>	AAA ACC CCT CTA CTT AGT GCC C	Schmid et al. (2000)
KST-157	<i>Kuenenia</i>	GTT CCG ATT GCT CGA AAC	Schmid et al. (2001)
PLA-46	<i>Planctomycetes</i>	GAC TTG CAT GCC TAA TCC	Neef et al. (1998)
AMX-368	Anammox bacteria	CCT TTC GGG CAT TGC GAA	Schmid et al. (2003)
CHL_408	Uncultured Chlorobi-like organisms	GCT AAA AGC CCT TCG TCC	Miclea et al., unpublished
CHL_485	Uncultured Chlorobi-like organisms	GGG CTT CCT CTG AGC ATA	Miclea et al., unpublished
CHL_730	Uncultured Chlorobi-like organisms	TAT GGC CCA GAT GAC TGC	Miclea et al., unpublished
CHL_834b	Uncultured Chlorobi-like organisms	ACT GAC CCC TAT GGG ACC	Miclea et al., unpublished

Results

Identification of anammox bacteria by partial amplification of 16S rRNA gene followed by sequencing

Partial amplification of 16S rRNA gene

For the first 120 days of constant operations of the MBR, from the biomass samples collected we isolated genomic DNA; to establish the identity and/or genetic diversity of the anammox species present in the bioreactor we partially amplified the 16S rRNA gene, using Pla 46F-907R(M) and Pla46F-1392R. Of all samples tested, bands corresponding to the PCR products from genomic DNA isolated on days 18 and 105 were excised from the 1% agarose gel, and the amplification products were purified for sequencing.

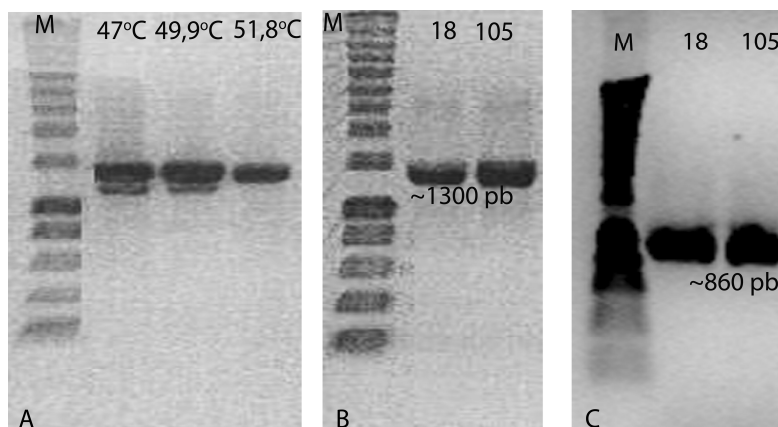


Figure 1. Partial amplification of 16S rRNA gene for identification of anammox bacteria from the membrane bioreactor. (A) Temperature gradient PCR, to determin the optimal primer annealing temperature. (B) PCR products obtained by amplification with primers pair Pla46F-1392R, separated by agarose gel electrophoresis. (C) Using the primer pair Pla46F-907(M) we amplified 860 bp fragments, based on genomic DNA isolated from the biomass collected on days 18 and 105.

Sequencing and comparative sequence analysis

The sequence retrieved for day 18 had 99% similarity with a strain of *Brocadia* genre, *Candidatus Brocadia* sp. 40 (Kieling et al., 2007), while the sequence retrieved for day 105 had 99% similarity with a strain of *Candidatus Kuenenia stuttgartiensis* (enriched in Kölleken, Switzerland by Egli et al., 2001) and with the original *Candidatus Kuenenia stuttgartiensis* (enriched in Stuttgart, Germany, by Schmid et al., 2001; Strous et al., 2001, Strous et al., 2006).

Identification of anammox bacteria by FISH

For the biomass fixed on day 35 (fig 2), a high number of cells, forming large aggregates, were specifically labeled after hybridization with probe AMX-820. No signal was detected after hybridization with probe KST-157. Simultaneous hybridization of fixed cells with probe AMX-820 (specific for anammox bacteria) and the EUB mixture was used to show that bacteria involved in anaerobic oxidation of ammonium form the dominant population in the MBR, the hybridization signals for the two probes overlapping.

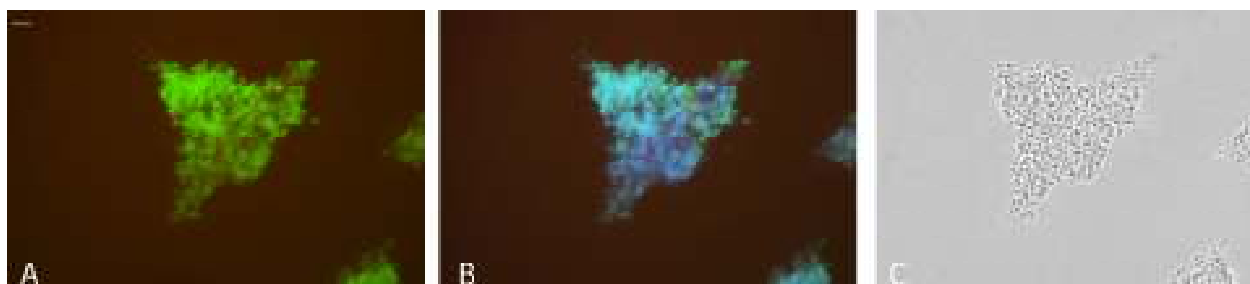


Figure 2. The dominant anammox population four weeks after MBR inoculation. (A) *In situ* hybridization is positive for AMX-820 (green) but not for KST-157 (red). (B) Overlapping image for probes KST-157, AMX-820 and EUB-338. (C) Phase contrast image of the anammox biomass from the MBR. Bar indicates 10 μ m. Objective: x100

FISH for the biomass fixed on day 64 (fig 4) confirmed the results obtained by amplification and DNA sequencing, the rRNA from the anammox cells specifically hybridizing with both AMX-820 and KST-157 probes. Comparing epifluorescence images from day 35 and 64 we could observe a difference in abundance, initially *Brocadia* was the dominant anammox bacteria, but *Kuenenia* is taking its place in the MBR. We could also observe the coccoid morphology (1.5 μ m in diameter) and a ring shaped hybridization signal, specific for Planctomycetes (Neef et al., 1998, Schmid et al., 2000).

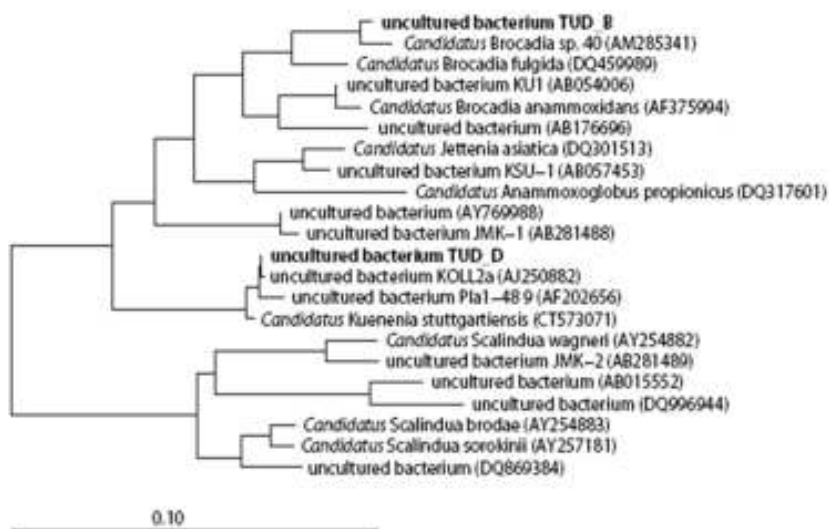


Figure 3. Phylogenetic tree based on nearly complete 16S rDNA sequences from the dominant bacteria in the MBR. TUD_B is the sequence retrieved from the biomass collected on day 18 and TUD_D from day 105. *Thermotoga maritima* was used as outgroup. Bar indicates 10% divergence.

We could also observe that the different anammox species in the membrane bioreactor are present as aggregates (cells hybridizing with AMX-820) and as free cells, those hybridizing with KST-157 (fig 4).

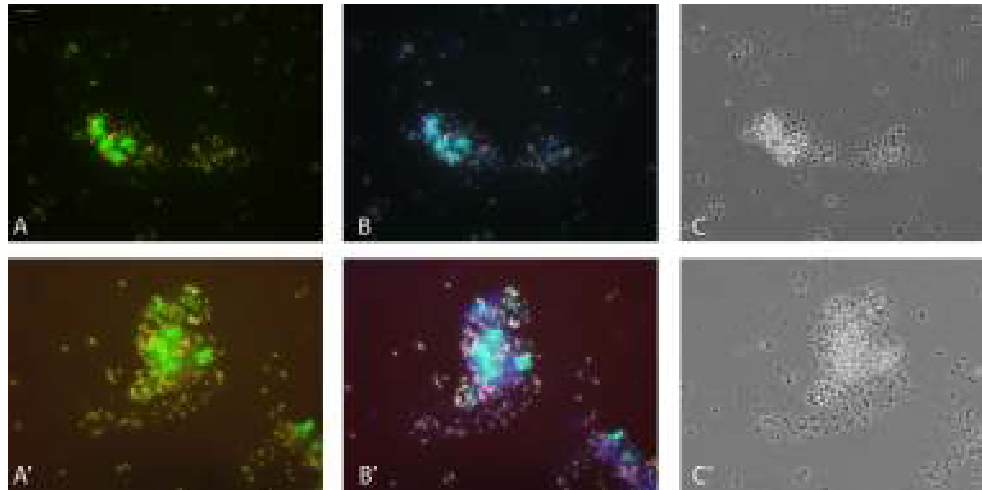


Figura 4. The anammox dominant population is changing, after day 64 (A, A'). Overlapping hybridization signal for KST 157, AMX-820 and EUB 338 mixture, labeled with Cy5(B, B'). The anammox community by phase contrast microscopy (C, C'). Bar indicates 10 μ m. Objective: x100

After 64 days of stable operations, at a SRT= 16 days, a population shift took place, from *Brocadia* to *Kuenenia*, FISH on day 77 (fig. 5) showing that *Kuenenia* became the dominant anammox population in the MBR, the hybridization signals being positive with both anammox specific probes (orange signal, obtained by the overlap of signals for both probes).

For the first 120 days of stable operation conditions, no cells hybridized with other anammox specific probes like Pla-46 and AMX-368, suggesting that, at least in this period of time, *Candidatus Brocadia* and *Candidatus Kuenenia* were the only anammox bacteria present in the MBR.

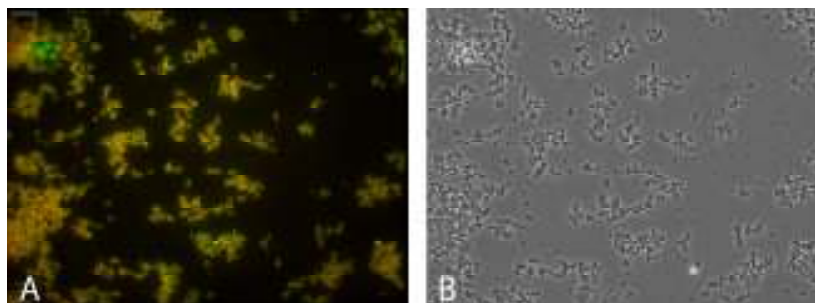


Figure 5. FISH on day 77 shows the considerable reduction of hybridization signal for probe AMX-820 (green) and the predominance of cells labeled in orange, by hybridization with probe KST-157 and AMX-820 (A). Phase contrast image of *Kuenenia* free cells (B). Bar indicates 10 μ m. Objective: x100

Because a suspension of free cells was obtained, it was possible to estimate the purity of the enrichment. On day 267, approximately 10,000 cells were counted and the enrichment level was estimated to be $97.6 \pm 0.2\%$ (van der Star et al., 2008).

Identification of non-anammox members of the microbial community in the MBR

The community profile obtained by DGGE and the phylogenetic analysis

The anammox bacteria were detected in the enrichment culture from the MBR only by amplification with *Planctomycetes*-specific primers; partial amplification of 16S rDNA using bacterial specific primers 341F-GC/907RM (amplifying a region of ca. 560 bp, from *E. coli* position 341 to 926) could allow the identification of non-anammox bacteria from the enrichment culture.

The evolution of the non-anammox community in the first 120 days was determined by amplification of genomic DNA from 10 samples collected from the bioreactor on days 1, 10, 18, 28, 35, 52, 74, 83, 105, 114. In the first 75 days, the solids retention time (SRT) was controlled at 16 days. The non-anammox community seems to contain three different species; a single band was excised from the gel (fig. 6, band #1). On day 85, the Ca and Mg ions concentration was lowered by 75% and the medium composition was adjusted by adding yeast extract (1mg/l, van der Star et al., 2008).

These adjustments modified the DGGE profile, an increase in the number of bands could be detected on the gel, at least 6 bands are visible (bands #2, #3 and #4, #5, #6 were excised and

purified for sequencing). Two other bands (#7, #8), from the control sample, were excised from the polyacrylamide gel, the electrophoretic migration pattern from this sample being similar to the one from day 1 of operations.

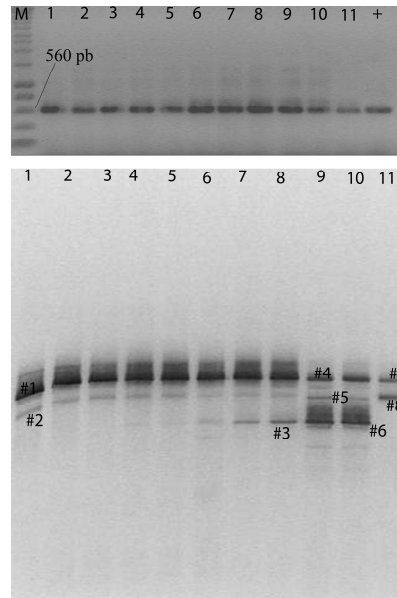


Figure 6. MBR's non-anammox community structure in the first 120 days of stable conditions. The positive amplification of genomic DNA with 341F-GC/907RM primer pair (up) and DGGE profile on a 6% polyacrilamide gel, using a gradient of 20%-60% urea and formamide (down). In the first 20 days (lanes 1-3), no modifications were noted in the structure of the community, but starting with day 28 (lane 4) the number of bands increased, on day 114 (lane 10), at least 6 bands were visible. Sample 11 was collected as positive control, from another anammox reactor, at present not functioning.

Phylogenetic analysis of sequences retrieved from the bands excised from DGGE gels in the first 120 days, groups the non-anammox bacteria with uncultured microorganisms belonging to the *Chlorobi* phylum (fig. 7) but sequence similarity is only 88%. Bands 1, 4, 7 (fig. 6) form a separate cluster from the one formed by bands 3, 6, 8. Bands corresponding to these sequences had the same electrophoretic mobility in the polyacrylamide gel, and the formation of two different clusters suggests the existence of two different species.

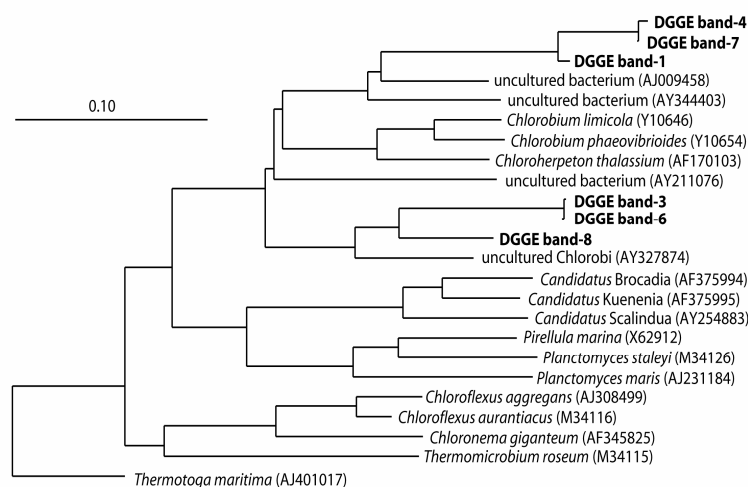


Figure 7. Phylogenetic position of non-anammox bacteria from the MBR, based on 16S rDNA analysis. The tree was constructed using the Neighbor-Joining method and *Thermotoga maritima* was used as outgroup. Bar indicates 10% divergence.

To check if these bacteria could come from the medium (not sterilized), a sample was collected on day 140; genomic DNA was isolated and amplified with the same primer pair as for samples from the MBR. No similarity could be observed by DGGE analysis between electrophoretic migration patterns of samples from the MBR and the medium; also, microscopically, no *Chlorobium* like bacteria could be observed, as no extracellular sulphur depositions, characteristic for these bacteria (Imhoff et al., 2003) could be visualized.

To determine the temporal dynamics and structural modifications of the non-anammox community from the MBR, all samples collected from the bioreactor, during stable functioning, were analyzed by PCR-DGGE. Till day 135, no modifications could be observed on the DGGE profile, 3 dominant fragments were observed; a total number of five bands with variable intensity appeared, but not all could be excised for sequencing. Comparing the migration pattern (fig. 8), a drastic modification of the composition could be observed at the end of the experiment, two of the dominant bands disappeared and a new band, #10 (species) appeared.

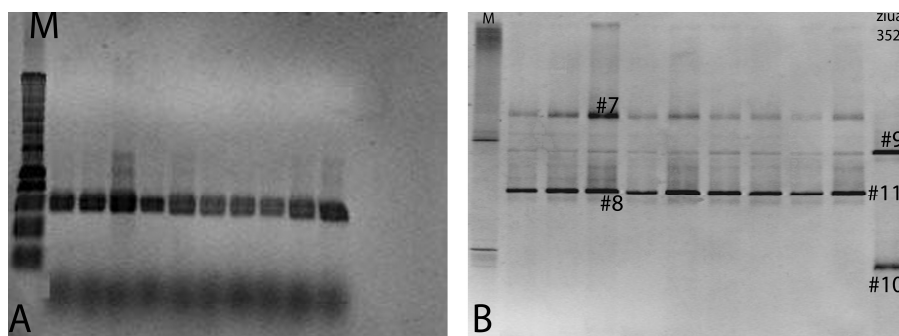


Figure 8. Partial amplification of 16S rRNA gene (A) and electrophoretic migration of PCR products on DGGE gel (B) from day 105 to day 135. M is molecular weight marker and the numbers correspond to the bands excised for sequencing.

On day 127, SRT was lowered from 16 to 12 days. DGGE profile, after this modification in cultivation conditions, showed, initially, a reduction in the number of dominant bands. After several days after functioning in these new conditions (30 days), the same three dominant bands appeared on the gel (fig. 9).

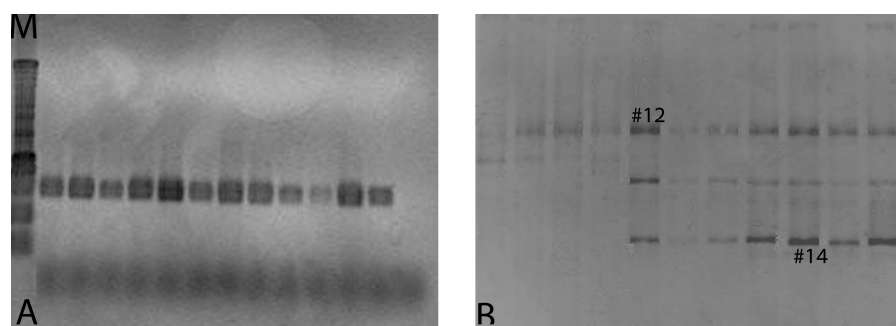


Figure 9. Amplification products (A) and the corresponding DGGE profile (B) from day 135 to day 165, indicating the evolution of the non-anammox community after reduction of SRT. #12, #14 represent the bands excised for sequencing.

On day 150, the concentration of ammonium from the influent was lowered from 120 mM to 100 mM. On the DGGE profile, some of the dominant bands disappeared and new bands, in the highly denaturant part of the gel appeared (fig. 10). After this day, the same cultivation conditions were maintained during the rest of the experiment, and after day 220 to the end of the experimental period, the composition/structure of the non-anammox community stabilizes.

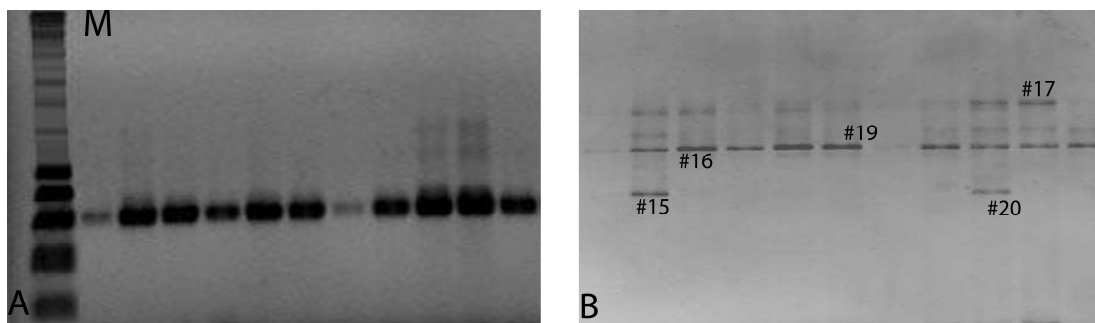


Figura 10. Evolution of the non-anammox MBR community after reduction of SRT and ammonium concentration, from day 165 to day 220. (A) Partial amplification of 16S rDNA (B) DGGE analysis of the PCR products, indicating the sequenced DNA fragments.

The phylogenetic analysis of the sequences indicates low diversity of the non-anammox population from the enrichment culture. All three clusters group together with uncultured bacteria, phylogenetically affiliated to the *Chlorobi* phylum. SB_MBR10 is related to these sequences, but not grouping with any of these clusters, the similarity with the rest of the sequences being around 87%.

The sequences: SB_MBR9, 15, 19, 22 and 24 (99% similarity to each other), also grouped with uncultured microorganisms, belonging to the *Bacteroidetes* phylum. SB_MBR 16 is grouping with uncultured bacteria belonging to this phylum, but the similarity with the sequences in this cluster is under 85% (fig. 11B).

Fluorescence in situ hybridization confirms the presence of non-anammox bacteria in the MBR community

For the biomass fixed for FISH, collected on day 63, initially, all four newly designed probes gave a positive hybridization signal.

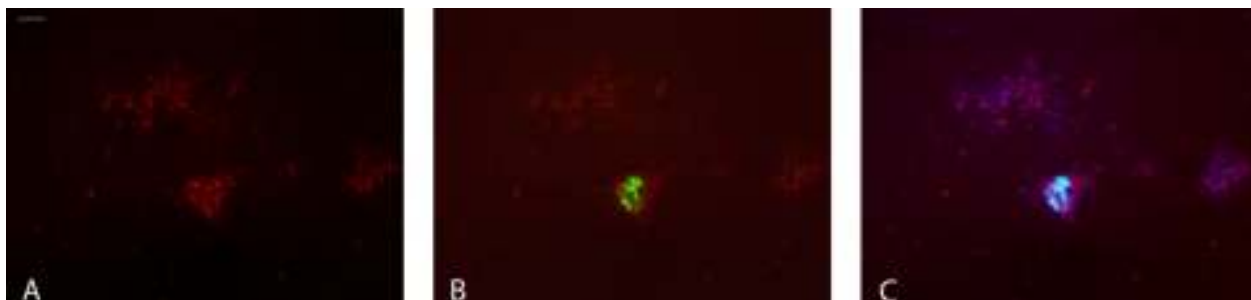


Figure 12. CHL_408 probe hybridizes with non-anammox bacterial cells from the MBR. (A) *In situ* identification of non-anammox bacteria, by hybridization with CHL 408, labeled with Cy3 (A). Simultaneous hybridization with CHL_408, labeled with Cy3 (red hybridization signal) and AMX-820 labeled with Fluor (green hybridization signal) (B). Overlapping image of hybridization with CHL_408, AMX-820, EUB-338 mix (C). Bar indicates 10 μm . Objective: x100.

For all oligonucleotidic probes, a maximum hybridization signal was obtained with 15% formamide in the hybridization buffer. The majority of cells, specifically labeled with the CHL probes are localized near the anammox aggregates, but dispersed cells were also visualized. Morphologically, the non-anammox bacteria are small rods (maximum 0.5 μm), comparatively to anammox cells (1.5 μm).

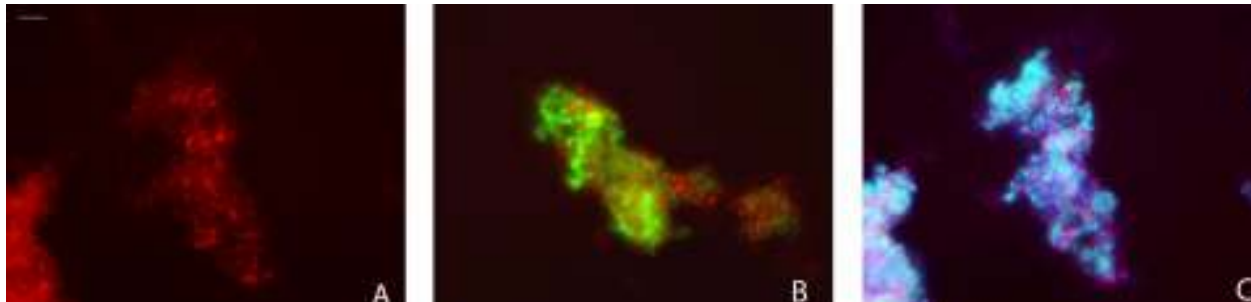


Figure 13. *In situ* identification of non-anammox bacteria by hybridization with CHL_485 probe (A). Simultaneous hybridization of the biomass from the MBR with CHL_485, labeled with Cy3 and AMX-820, labeled with Fluor (B). Signal overlap of probes CHL_485, AMX-820, EUB 338 (Cy5-blue) showing that most of the non-anammox cells were localized near the anammox aggregates (C). Bar indicates 10 μm . Objective: x100.

After day 150, the presence of these microorganisms was not detected by *in situ* hybridization. The results obtained by FISH confirm the DGGE analysis and show the structural modifications in the non-anammox community.

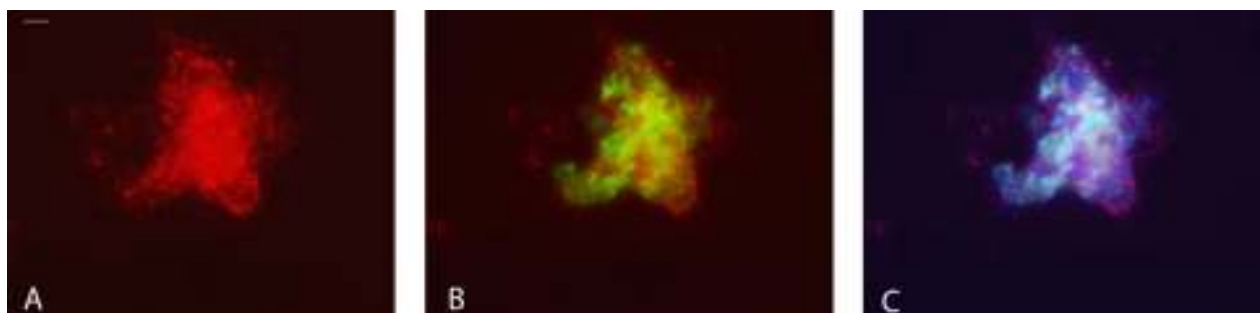


Figure 14. (A) The oligonucleotidic probe CHL_730 hybridizes with the biomass from the membrane bioreactor. (B) Simultaneous hybridization with CHL_730 (Cy3) and AMX-820 (Fluos) probes. (C) MBR's non-anammox community, identified by FISH with probes CHL_730, AMX-820, EUB 338 (Cy5-blue). Bar indicates 10 μ m. Objective: x100.

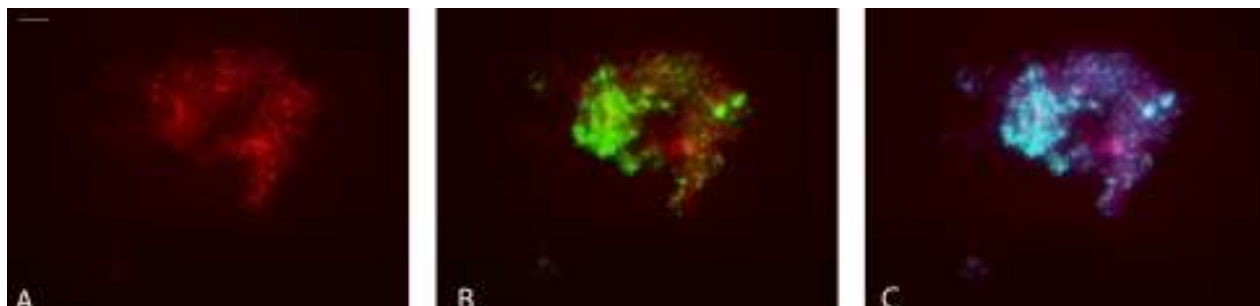


Figure 15. *In situ* hybridization of MBR biomass using CHL_834b probe, labeled with Cy3 (A), the same new probe and AMX-820 labeled with Fluos (B). Signal overlap in simultaneous hybridization with CHL probe (red signal), the anammox specific probe (green signal) and the EUB mixture (blue signal). suprapunerea semnalelor în hibridarea simultană cu sonda specifică pentru microorganismele non-anammox (semnal roșu), cu sonda specifică pentru bacteriile anammox (semnal verde) și amestecul de sonde EUB (semnal albastru) specifice pentru totalitatea bacteriilor (C). Bar indicates 10 μ m. Objective: x100.

Evaluation of non-anammox bacteria present in control reactors

The full-scale anammox reactor

The DGGE profile, based on 16S rRNA gene, showed a higher complexity of the non-anammox community in the full-scale reactor, the banding pattern contains at least ten bands; the non-anammox community from the full-scale reactor is characterized by similarity of the banding patterns, in the initial period (2005) as well as during the ten weeks in 2007. Comparing, by denaturing gradient gel electrophoresis, the profiles of the lab-scale and full-scale non-anammox community, at least two fragments with similar electrophoretic mobility were present (fig. 16, B). The sequences retrieved were included in the phylogenetic analysis (named

SB_RFS), together with the sequences from the MBR (fig. 11A și B). According to the phylogenetic analysis, 3 of the full-scale sequences grouped with MBR sequences, forming two different clusters, affiliated to uncultured *Chlorobium* like bacteria; 3 other sequences group with sequences from the MBR affiliated to the *Bacteroidetes* phylum, and other two sequences are affiliated to *Proteobacteria*.

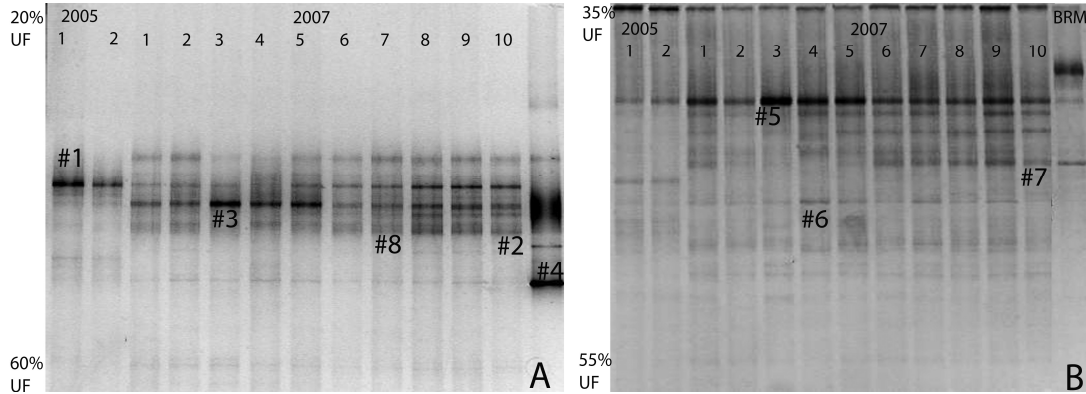


Figure 16. Non-anammox bacteria diversity in the full-scale reactor. (A) the non-anammox community profile during the start-up and after two years of functioning, using a 20%-60% urea and formamide gradient. (B) Comparison of non-anammox community structure from the full-scale and lab-scale anammox reactors (denaturant gradient of 35%-55%).

Based on the collected samples, the composition of the non-anammox microbial community from the full-scale anammox reactor was analyzed by FISH (fig 17). Of all four oligonucleotidic samples, only for CHL_485 a positive signal was not obtained.

The structure of the non-anammox community in the other control reactors

The DGGE profile showed the presence of common bands, with similar electrophoretic mobility (fig. 18). Based on this profile, no sequence could be retrieved.

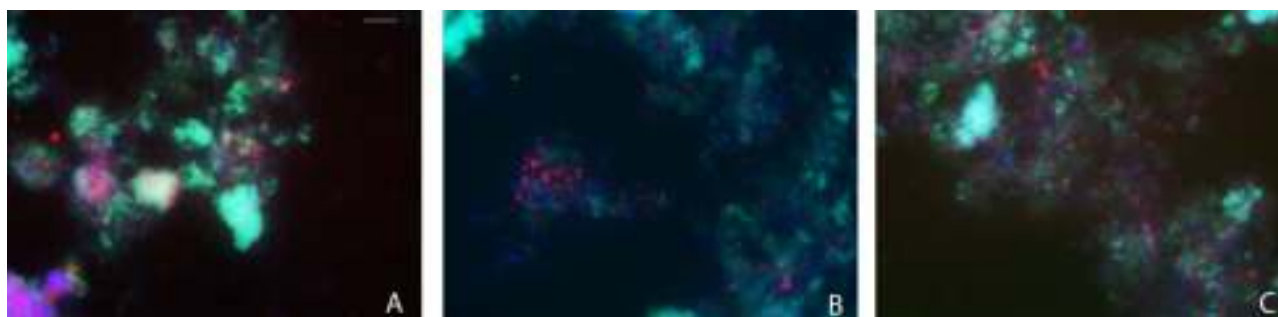


Figure 17. The non-anammox bacterial population from the membrane bioreactor was present also in the full-scale reactor. Probes CHL_408 (A), CHL_730 (B), CHL_834b (C) specifically hybridize with the microorganisms in the full-scale anammox reactor. FISH was performed by simultaneous hybridization with probes CHL, AMX-820 (Fluos) and EUB mix (Cy5). Bar indicates 10 μ m; objective: x100.

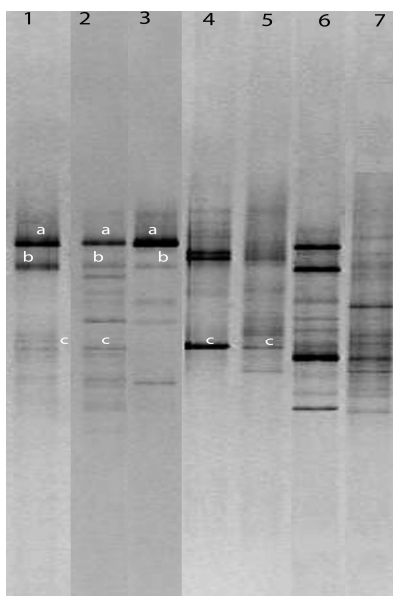


Figure 40. DGGE profiling of control reactors samples: full-scale anammox reactor (lane1), one reactor nitrification-anammox (lane2), lab-scale SBR (lane3), nitrification-denitrification and acetate/phosphate removal (lane4), nitrification reactor (lane5), full-scale SHARON reactor (lane6), lab-scale SHARON reactor (lane7). a, b, c, represent common migration patterns.

The presence of the non-anammox bacteria identified in the MBR was also detected, by FISH, in the control reactors (tab. 4).

Table 4. *In situ* hybridization with the CHL probes for samples from all the reactors

Bioreactor	Process	Dominant population	Hybridization result			
			CHL_408	CHL_730	CHL_834b	CHL_485
Membrane bioreactor (MBR)	Anammox	<i>Brocadia</i> , <i>Kuenenia</i>	+	+	+	+
Full-scale anammox reactor	Anammox	<i>Brocadia</i>	+	+	+	–
Full-scale one reactor nitrification-anammox	CANON/OLAND/deammonification	<i>Brocadia</i>	+	+	-	+
Lab-scale SBR	Anammox	<i>Brocadia</i>	+	+	+	+
Lab-scale SBR	Nitrification/denitrification and acetate/phosphate removal		-	+	+	+
Lab-scale SHARON	Nitrification	AOB	-	-	-	-
Full-scale SHARON	Nitrification	AOB	-	-	+	+
Lab-scale SBR	Nitrification	AOB	-	-	-	-

Discussions

Achievement of a suspension culture

Although the membrane bioreactor is not a settling based system (van der Star et al., 2008), anammox cell aggregates were present in the first 70 days of the experiment. Using an inoculum with a high sedimentation capacity, like granular sludge (from the full-scale anammox reactor) can improve biomass retention because granules offer a specific habitat for anammox bacteria, favoring their attachment, their resistance to the hydraulic pressure in the system and thus, to the growth of microorganisms (Gaul et al., 2006). The presence of bacterial aggregates was noticed in other MBR systems, in which the anammox (Wyffels et al., 2004; Trigo et al., 2006) or nitrification (Wyffels et al., 2003) took place.

Other experimental data show that in systems with submerged membranes, the biomass tends to grow in suspension, because the substrate is more available to the active biomass, and less diffusion problems (than for granules) appear (Cicek et al., 1999). Indeed, in the MBR used in this experiment, a suspension culture was achieved, but the disappearance of the granules

remains to be explained. It is possible that, the growth of anammox bacteria as free cells was caused by the reduction of the concentration of bivalent cations (Ca^{2+} and Mg^{2+}), a change in culture conditions done at the same time as the addition of yeast extract. Bivalent ions determine the formation of flocs (Mahoney et al., 1987; Sobeck and Higgins, 2002; Peveré et al., 2007). If the presence of flocs is determined by the extracellular polymeric substances (EPS), produced because of the lack of a micro-nutrient, than the addition of yeast extract reduced the production of EPS and favored suspended growth.

Nevertheless, lowering the concentration of bivalent ions cannot be the only reason for the formation of a suspended culture, because even at lower concentrations than those encountered in the MBR the biomass were granular (Trigo et al., 2006).

A major difference from the other membrane bioreactors mentioned was the SRT of 16 or 12 days, while in the other bioreactors, the SRT was practically infinite (no biomass was removed). The competition in systems with high SRT is much lower and the growth rate is also low, favoring granule formation (de Kreuk and van Loosdrecht, 2004). In this context, a relatively low SRT seems to be necessary to obtain a suspended culture.

Suspended growth can also be caused by low shear stress and speed of rotations (160 rpm) (van der Star et al., 2008).

The shift of anammox populations

Anammox biodiversity is proved by numerous experiments at lab-scale, in wastewater treatment systems and in aquatic ecosystems (van de Graaf et al., 1996; Strous et al., 1999; Kartal et al., 2007; Kuypers et al., 2003). Different genera have been identified in different environmental conditions. In lab-scale enrichment cultures, in which N-NH_4^- is an electron donor and NO_2^- acceptor, either *Brocadia*, or *Kuenenia* becomes dominant. There are clear differences between these two genera and other genera like *Scalindua* (identified in marine ecosystems, Kuypers et al., 2003), or *Anammoxoglobus propionicus* (enriched in a culture with propionate addition, Kartal et al., 2007).

Because different genera of anammox bacteria rarely are encountered in the same treatment system or enrichment culture, it seems that each genre occupies its own niche, and environmental conditions select for only one of these genera. Also, significant phylogenetic

distances between different anammox species indicate distinct ecological micro-niches, unknown so far (Kartal et al., 2007; Schmid et al., 2003). Between *Brocadia* and *Kuenenia* there is only 90.5% sequence similarity at 16S DNA level and 87.8% at 23S rDNA level, and between the *Brocadia-Kuenenia* group and the rest of *Planctomycetes*, 78% and respectively 72% (Schmid et al., 2001).

An important factor for the differentiation of the ecological strategies of *Kuenenia* and *Brocadia* species can be the substrate affinity and the sensibility for different inhibition factors (Gaul et al., 2006). *Kuenenia* seems to have a higher tolerance capacity for high nitrite and phosphate concentrations than *Brocadia* (Strous et al., 1999; Egli et al., 2001). Nevertheless, recent data mentioned that in full-scale reactors (operated at high nitrite levels) *Brocadia* was enriched (van der Star et al., 2007).

In the membrane bioreactor used in this study a population shift from *Brocadia* to *Kuenenia* took place. The inoculum for the MBR was collected from the full-scale anammox reactor in Rotterdam, which contains *Brocadia*. The inoculum for the full-scale anammox reactor came from a lab-scale enrichment culture, fed with synthetic wastewater which contains, besides nutrients similar to van de Graaf medium (1996), 1.8 kg-N/m³ NH₄Cl and 1.6 kg-N/m³ NaNO₂, and the enriched species in this bioreactor is *Kuenenia* (van der Star et al., 2007). After inoculation, the biomass extant in the inoculum, acclimatizes to the new conditions or the microbial community suffers a significant change, caused by ion concentration, pH, temperature (Kartal et al., 2006).

At a SRT of 16 days *Kuenenia* became dominant. The minimum specific growth rate at this SRT (0.0026/h) is approximately equal or under the value for the maximal specific growth rate for *Brocadia* and *Kuenenia*. It is way, because the population shift took place in a short period of time (max. 40 days from using the SRT of 16 days), the competition is probably not based on growth rate. The selection in the membrane bioreactor is based on apparent affinity for the limiting substrate (nitrite, in this case). Based on these observations we can assume that the strategy for *Brocadia* is based on growth rate and for *Kuenenia* on affinity (van der Star et al., 2008).

The diversity of non-anammox bacteria in the MBR

In this study, by using a membrane bioreactor, a highly enriched anammox culture was obtained (97.6%), using a relatively short SRT, of 16 and 12 days (van der Star et al., 2008). The strategy for the characterization of the total community in the enrichment culture was based on the use of molecular techniques based on the 16S rRNA gene, like PCR-DGGE and FISH. Because anammox 16S rDNA was amplified only by using specific *Planctomycetes* primer Pla-46 (Neef et al., 1998) we considered that a general primer pair would amplify the 16S rDNA from minority members of the community from the enrichment culture (341FGC/907RM, Muyzer et al., 1993). Truly, none of the sequences retrieved from the MBR, by amplification with general bacterial primers, had no similarity to anammox species, from the enrichment or other *Planctomycetes* member.

During the entire experimental period, the non-anammox community structure is relatively stable and formed by a low number of species. In the first 120 days of stable functioning of the MBR, the DGGE profile indicates the presence of three dominant species, which, based on the phylogenetic analysis of the sequences retrieved group into three different clusters, having 88% sequence similarity with uncultured bacteria from the *Chlorobi* phylum. By fluorescence *in situ* hybridization was observed that these bacteria are located nearby anammox aggregates and inside the aggregates. This specific localization was visible only when the aggregates were formed of *Brocadia* sp., initially dominant in the MBR. The medium composition modification, on day 85 (reduction by 75% of calcium and magnesium ions concentrations and the addition of 1mg/l yeast extract) does not influence the composition of the anammox population.

The operational change, on day 127, the reduction of SRT from 16 to 12 days, determines a clear oscillation in the non-anammox community structure, by the reduction of bands on DGGE profile; lowering the SRT implies higher competition for essential elements (de Kreuk and van Loosdrecht, 2004), explaining the lower diversity, but after 30 days the banding pattern indicates that the same three dominant species are back in the system. This modification did affect the structural dynamics of the non-anammox population but only during the adaptation period to the new conditions. Two species affiliated to the phylum *Chlorobi*, maintain themselves in the system till the end of the experiment (sequences SB_MBR20, 23). The

presence of these non-anammox species from the start to the end of the experiment, including after SRT lowered, suggests that these microorganisms grow and form a stable population under anammox conditions.

The phylum *Chlorobi* (green sulphur bacteria) is formed of a relatively small group of microorganisms, metabolically limited, physiologically well defined and closely related (genetically speaking), related to the phylum *Bacteroidetes*. All known strains have a photolithoautotrophic growth, using sulphur compounds, iron oxides or hydrogen as electron donors for photosynthesis; they have a highly specialized photosynthetic apparatus, one of the strains, *Chlorobium phaeobacteroides* MN1 has the most pronounced low light adaptation, being isolated from a depth of 100 m, in the Black Sea (Overmann et al., 1992). The light intensity, in this area of the Black Sea where this strain was discovered, corresponds to the light offered by a candle, at a distance of 50 m, in total darkness (Manske et al., 2005). As mentioned before, the MBR was covered with a PVC layer, so that the enrichment culture was not exposed to light, except during short periods of time, not enough for the growth and proliferation of phototrophic organisms. In the full-scale reactors, in which the anammox process takes place, the microbial community was never exposed to light.

In an enrichment culture used for the growth of *Kuenenia stuttgartiensis* and for the genome sequencing project of this species, sequences affiliated to the phylum *Chlorobi* were identified (Strous et al., 2006); these sequences group with the MBR non-anammox sequences. As for the MBR, no reduced sulphur was added to the medium. Another experiment, for the detection of the *dsrAB* gene, involved in dissimilatory sulphide reduction, present in many sulphur oxidizing bacterial genomes, did not identify the presence of this gene in the sequences from the sequencing genome project enrichment affiliated to *Chlorobi* (Loy et al., 2009). In another experiment by Li et al. (2009), for studying the diversity of the bacterial community from an anammox reactor, bacteria affiliated to *Chlorobi* represented 54% of the total number of sequenced clones (this sequences have less than 50% similarity with the sequences in the MBR), other community members being associated to the phylum *Bacteroidetes* (11%), *Proteobacteria*, *Chloroflexi*.

The other modification of conditions in the MBR, the reductions of ammonium concentration from 120 mM to 100mM, determined the apparition of new species in the

bioreactor, grouping separately from the already extant non-anammox bacteria, affiliated to the phylum *Bacteroidetes* (cluster formatted by the sequences SB_MBR9, 15, 19, 22, 24, sequence SB_MBR16). These microorganisms are present in numerous habitats like freshwater, soil, cold marine habitats, wastewater treatment systems (Manz et al., 1996). In a biofilm formed of nitrifying bacteria and anammox, the role of these microorganisms is to ensure structural support for the biofilm, or for other species in the biofilm. In this case, they can use low carbon amounts, left after nitrification or released by dead bacteria (Egli et al., 2003).

By fluorescent *in situ* hybridization, the non anammox bacteria specific for the MBR (present in the first 150) were also detected in the full-scale anammox reactor in which the main population is *Brocadia anammoxidans* (van der Star et al., 2007). Their presence at lab-scale and full-scale, in reactors in which *Brocadia anammoxidans* is the dominant anammox bacteria, could imply that these microorganisms form a “satellite” population for this anammox species. The fact that they were eliminated from the reactor, after the population shift, favors this hypothesis.

In the full-scale one reactor nitritation-anammox, the dominant anammox bacterium is also *Brocadia anammoxidans*; other bacteria present are ammonium oxidizers and nitrite oxidizers. The non-anammox bacteria detected in the MBR are also present in this reactor, their presence being detected by FISH with CHL probes. In the lab-scale anammox SBR, in which the dominant anammox bacteria is *Brocadia* sp., the CHL probes also hybridized with the biomass.

Although these non-anammox microorganisms were detected in lab-scale and full-scale systems, where *Brocadia* is dominant, suggesting the possibility of being “satellites” of this anammox bacteria, they are affiliated to sequences retrieved from the genome sequencing project, enrichment culture in which the main anammox organism was *Kuenenia* sp. (Strous et al., 2006).

The CHL probes also hybridized with biomass from the negative control reactors, the lab-scale nitrification-denitrification and acetate/phosphate removal reactor and the full-scale SHARON reactor. The influent for the first contains 50 mg/L ammonium (de Kreuk et al., unpublished).

Taking into account that the effluent of the full-scale SHARON reactor is the influent for the full-scale anammox reactor, and the MBR was inoculated with biomass from the full-scale

anammox reactor, it is possible that these non-anammox bacteria come from the SHARON reactor; still, biomass from the lab-scale SHARON did not hybridize with the CHL probes.

The molecular data gathered here allowed the detection of possible new taxa associated to wastewater treatment systems but are not enough to offer an explanation of the presence and role of these microorganisms in these systems. The fact that these *Chlorobium*-like microorganisms group mainly with uncultured members of the phylum *Chlorobi* and are distantly related to cultivated species may suggest that these microorganisms belong to an ancient line of green sulphur bacteria, with a different metabolism than these phototrophic anoxygenic bacteria.

General conclusions

Cultivation of anammox bacteria in suspension requires several conditions: the absence of a selective pressure for sedimentation, high growth rate obtained by using a short SRT, reduced concentrations of bivalent ions. Addition of yeast extract and low shear stress can contribute to obtaining a suspended culture.

By using a system like the MBR a highly pure enrichment culture can be achieved. (ca. 97.6%); the non-anammox members of the community are related to members of the *Bacteroidetes-Chlorobi* group.

The molecular data indicates active growth of the non-anammox bacteria, under anammox conditions.

The non-anammox microorganisms do not form a “satellite” community for any of the anammox species in the bioreactor, their presence being detected in the reactor where the anammox process does not take place.

The molecular data gathered in this study allowed the identification of possible new taxa associated to wastewater treatment systems but are not sufficient to explain the presence and role of the microorganisms in these systems.

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