Quantitative and qualitative effects of bioaugmentation on ammonia oxidisers at a two-step WWTP

S. M. Podmirseg, M. A. Schoen, S. Murthy, H. Insam and B. Wett

ABSTRACT

Large waste water treatment plants (WWTP) often operate nitrification in two different process environments: the cold-dilute sewage is treated in the mainstream nitrification/denitrification system, while the high strength ammonia liquors from sludge dewatering are treated in a separate high temperature reactor (SBR). This study investigates transfer from nitrifier biomass into a two-stage WWTP, commonly referred to as bioaugmentation. Besides the quantitation of ammonia oxidising bacteria (AOB), community differences were analysed with two techniques, denaturing gradient gel electrophoresis and real-time PCR melt curve analysis. It was shown that, without bioaugmentation, two distinct AOB communities establish in the mainstream and in the SBR, respectively. A gradual shift of the two AOB communities with increasing pump rates between the systems could be demonstrated. These molecular findings support process engineering experience, that cycling of waste activated sludge improves the ability of AOB to adapt to different process environments.

Key words | ammonia-oxidising bacteria, bioaugmentation, DGGE, habitat change, melt-curve analysis, qRT-PCR

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INTRODUCTION

Nitrification is a crucial step of biological nitrogen elimination in waste water treatment plants (WWTP), involving ammonia oxidising (AOB) and nitrite oxidising bacteria (NOB). Especially low temperatures during the cold season represent a challenge in maintaining nitrification. So, when it comes to meeting effluent criteria, plant operators may face capacity limits. Instead of increasing sludge retention time and activated sludge tank volumes, alternative strategies like bioaugmentation can be considered (Salem *et al.* 2003; Krhutková *et al.* 2006; Smith *et al.* 2008). The advantage of the bioaugmentation method, which is best applicable to slowly growing communities as AOB, is that it recycles active nitrifier biomass and is thus suited to increase the overall nitrogen removal rate (Rittmann 1996; Smith *et al.* 2008). The AOB examined in this study are classified into the β-Proteobacteria, including two genera (*Nitrosomonas* and *Nitrosospira)* and the γ-Proteobacteria with only one genus (*Nitrosococcus*) (Wagner *et al.* 1995). These organisms catalyse the oxidation of ammonia to nitrite with hydroxylamine as intermediate (Hooper *et al.* 1997). Regarding microbiological analyses of AOB, the quest for less time-consuming, culture-independent methods in this field has led to the establishment of various molecular techniques. Denaturing Gradient Gel Electrophoresis (DGGE) or Fluorescence *in situ* Hybridisation (FISH) are the most common methods (Amann *et al.* 1995; Nicolaisen & Ramsing 2002). In recent years, also quantitative real-time polymerase chain reaction (qRT-PCR) has been used for estimation of AOB abundance, allowing quantitation specificity up to the genus level (Zhang & Fang 2006). Moreover, the melt-curve analysis of real-time PCR experiments makes distinctions between different amplified sequences possible and has already been successfully applied to AOB populations (Mertoglu *et al.* 2008).

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In our study at the two-step WWTP in Salzburg (Austria), recycling of previously enriched sludge of a sidestream treatment system (SBR) into either the high-rate (A) or the low-rate stage (B) was tested. Microbiological aspects were examined by DGGE and qRT-PCR. The objective of this study was to demonstrate the effect of bioaugmentation on the AOB community in a full-scale WWTP. Additional insight into qualitative and quantitative changes and the adaptation capability of AOB to changing habitats should be gained. The findings could help to develop appropriate models and to improve and adjust bioaugmentation strategies.

METHODS

Sampling and extraction

At the WWTP in Salzburg a recycle pipe system, pumping waste activated sludge (WAS) from the low-rate stage (B) to the SBR tank (SBR) and from there either into the aeration tanks of the high-rate (A) or the low-rate stage (B) was installed. Samples were taken at the WWTP Salzburg from the A, B and SBR reactor. For a detailed set-up of the experiment see Figure 1. From each sample (2 L), a 0.5 L subsample was used for DNA extraction. DNA was extracted in three technical replicates (0.25 mL sample) using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA). Altogether four different bioaugmentation strategies were tested with increasing pumping rates to/from the SBR tank (0/0, 100/150, 200/300 and 300/400 $m^3 d^{-1}$). Sludge retention times (SRT) ranged from ~ 0.5 d (A), to 9–14 d (B) and 2–14 d (SBR). Sampling intervals were adjusted to periods equivalent to $2\frac{1}{2}$ sludge ages.

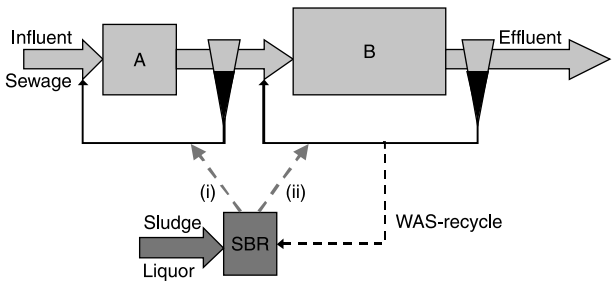

Figure 1 | Transfer scheme between the three different sludge systems, showing the two-step mainstream- (light grey) and sidestream treatment (dark grey). The standard sludge recycling systems of A and B are marked in black: A, high-rate biology for carbon removal; B, low-rate biology for nitrogen elimination; SBR, sidestream reactor for sludge liquor; WAS, waste activated sludge-recycle; (i) Bioaugmentation to the A-stage; (ii) Bioaugmentation to the B-stage.

The two operational strategies were (i) the Bioaugmentation to the A-stage and (ii) the Bioaugmentation to the B-stage, both in the warm and the cold season, respectively, with one sampling at each pump rate.

PCR amplification

For the amplification of AOB DNA a nested polymerase chain reaction (PCR) approach was performed. The first PCR run with the primer pair 63f/1378r (Heuer *et al.* 1997; Marchesi *et al.* 1998), amplicon length 1,358 bp, was followed by the amplification of the 16S rRNA of AOB with the primer pair 189f-GC/654r (Kowalchuk *et al.* 1997), amplicon length 465 bp. PCR was performed as described by Kowalchuk *et al.* (1997) for environmental samples but we reduced the number of cycles to 25.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed with the INGENYphorU® vertical electrophoresis system (Ingeny, Goes, The Netherlands). A 7%–8% acrylamide (w/v) and a 30%–70% denaturing chemical concentration (100% denaturant according to 7M urea plus 40% formamide in 1X TAE-buffer) were chosen. After PicoGreen DNA quantitation, 60 ng of DNA were loaded on the acrylamide gels. DGGE was run for 16 h at 60°C in 1X TAE buffer (pH 7.4) at 120 V. Gels were stained with silver nitrate (Sanguinetti *et al.* 1994) or with SYBR®Green I (Invitrogen™, Carlsbad, USA) if bands were excised.

Band excision and sequencing

Specific bands from the AOB gels were cut out after SYBR Green staining and reamplified through PCR. This step was repeated three times to obtain single band patterns. Cleaned-up PCR-amplified DNA was subjected to sequencing at the Eurofins MWG Operon company (Martinsried, Germany).

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Computer analysis

The GelCompar II software package (version 4.0, Applied Maths, Belgium) was used to analyse the DGGE gels. Banding patterns were normalised and cluster analysis was performed using the Dice correlation coefficient to obtain the pair-wise similarities and the Ward algorithm. The programme settings were at 1.0% Optimisation and 1.0% Position tolerance. The CLC DNA Workbench Software (Version 4.1.2) was chosen for the phylogenetic analysis of the cut-out bands and their closest relatives revealed through BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For the phylogenetic tree calculation the UPGMA algorithm and the Jukes Cantor substitution model were used.

Quantitative real-time PCR assay

For a quantitative estimation of the AOB abundance, real-time PCR analysis was performed with the Rotor-Gene 6,000 Real Time Thermal Cycler (Corbett Research, Sydney, Australia). Standard DNA was amplified from a pure culture of *Nitrosomonas europaea* (ATCC25978), with PCR conditions described by Hermansson & Lindgren (2001). The primer set 189f/RT1r, described by Kowalchuk *et al.* (1997) and Hermansson & Lindgren (2001) (amplicon length 116 bp) was chosen for the real-time PCR analysis. These primers were used in combination with the QuantiMix EASY SYG kit (Biotools, Madrid, Spain), which is based on the DNA-intercalating dye SYBR Green I. A final 20 μL reaction volume contained 1 X Quantimix reagent, 100 nM primer (189f and RT1, respectively), 0.4 μL 2% BSA, A.d. (GIBCO® ultra pure distilled water; DNAse, RNAse free, Invitrogen Corporation) and 2 μL 1:10 diluted template DNA. We used a three-step PCR approach and increased annealing temperatures (64°C) to avoid non-specific DNA-binding and primer-dimer formation. Furthermore, to exclude non-specific PCR amplification, a melt-curve analysis was performed with a transition rate of 1°C per step for 5 sec, starting from 60°C to 99°C. The five serial dilutions of Standard DNA product ranged from 10^6 to 10^2 gene copies μL^{-1}. For a higher accuracy of the results, all samples and standards were run in duplicate and standard dilutions were prepared fresh every time. The R^2 values for all standard curves were greater than 0.99. The calculation of gene copy number g^{-1} TS was performed with the Rotor-Gene 6000 Series Software 1.7.

RESULTS AND DISCUSSION

DGGE analysis

The DGGE technique was chosen to give a quick overview of the AOB diversity and detect changes in the community structure (Figure 2). The number of detectable bands ranged from only one to 15. The fingerprint pattern of the B samples remained similar throughout the whole experimental phase. Samples from the A tank and the SBR showed major shifts. SBR samples showed a clear numerical increase and a change of pattern with rising pump rates. Samples retrieved from the SBR tank during the stabilisation phase (0/0 m^3) had very few or only one band. This band, that was always present in SBR samples, could be related to our *Nitrosomonas europaea* pure culture. Having a closer look at the cluster analysis, the following trends can be stated. The effect of bioaugmentation was most obvious in the SBR samples in contrast to the B samples that seemed to be less influenced from the WAS recirculation system. During the bioaugmentation to the A-stage (Figure 2A), the SBR (0/0 m^3) samples clearly clustered apart from other samples and with increasing pump rates were more closely related to the other sample types, with the highest pump rate samples SBR (300/400 m^3) being closest to the B samples. The A samples showed a separate clustering. This trend was the same in both bioaugmentation campaigns to the A-stage (warm/cold season). Bioaugmentation to the B-stage (Figure 2B) rendered a completely different clustering than that to the A-stage. All samples but SBR (0/0 m^3) showed a similar banding pattern. Interestingly, A samples displayed the same fingerprint as B samples. The second bioaugmentation to the B-stage during the warm season was not as distinct, besides the same difference of SBR (0/0 m^3). No specific banding pattern for either the warm or the cold season could be detected throughout the four experimental phases.

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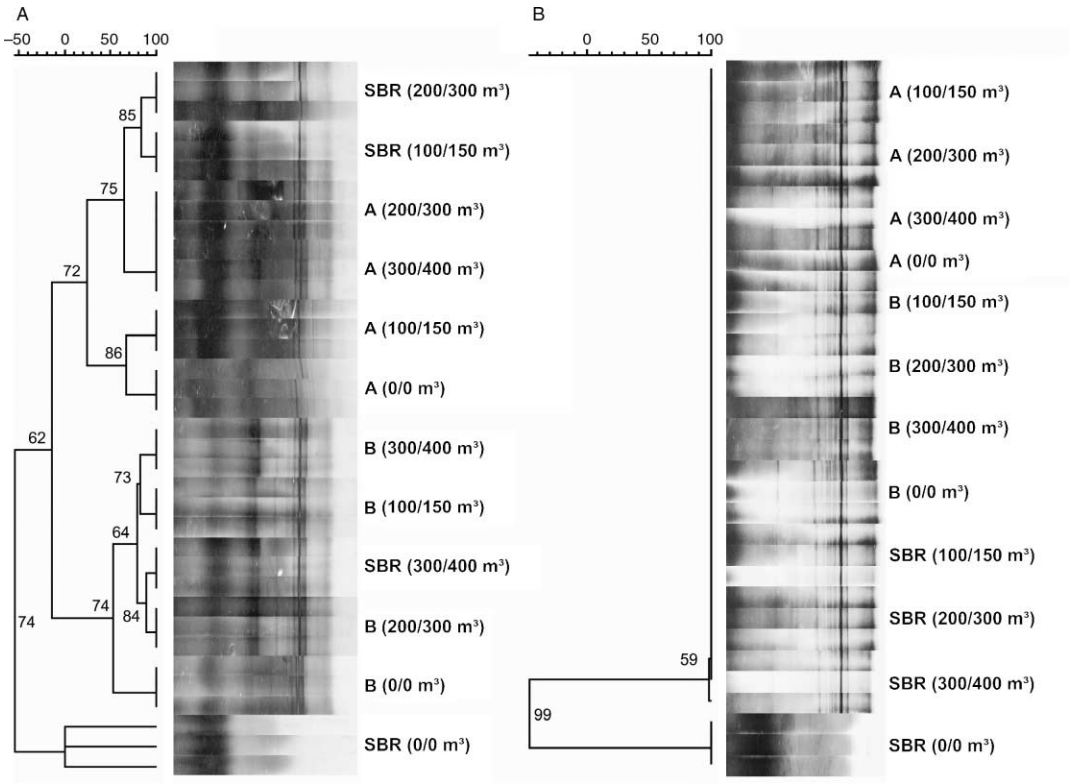

Figure 2 | AOB cluster analysis of DGGE fingerprints based on the 16S rRNA gene. A) Bioaugmentation to the A-stage (warm season), B) Bioaugmentation to the B-stage (cold season). Values at the branchings indicate the percentage of similarity; pump rates in brackets; samples not bioaugmented from the SBR are marked in italic.

Band excision and sequencing

Apart from the bands that could be assigned to the pure culture of *N. europaea*, two further bands were excised (Figure 3). Band 1 represents the most dominant one, found throughout all sampling types (except for SBR samples at 0/0 m^3). According to a BLAST query, this DNA sequence was most closely related to an uncultured bacterial clone (EU104159) that had been found in activated sludge and to an uncultured environmental clone found in the Evry WWTP (CU466881). The second band was never detectable in B tank samples and strongest in SBR sample of 0/0 m^3 or the lowest pump rate (100/150 m^3). With pump rates of 200/300 m^3 and 300/400 m^3 it completely disappeared in the SBR. A phylogenetic analysis showed Band 2 to be most closely affiliated with an uncultured bacterial clone (EU133841) and further to the uncultured *Nitrosospira* sequence (EF043007). Sequences were deposited at the GenBank database with the following accession numbers: Band 1 (GQ451711), Band 2 (GQ451712) and *N. europaea* 16S rRNA gene partial sequence (GQ451713).

The phylogenetic affiliation is shown in Figure 4. Both bands are related to yet uncultured groups. The amplicon with the primer pair 189f/654r renders only 465 bp long fragments which enables only reduced phylogenetic classification. Thus it is hard to determine sequence differences up to genus level. In the phylogenetic tree also *Nitrosococcus mobilis* (β-Proteobacteria) and *Dechloromonas sp.* (Rhodocyclales) sequences are added, that seem to be also amplified by the 189f/654r primer set. Also Boon *et al.* (2003) found *Nitrosococcus*-like sequences

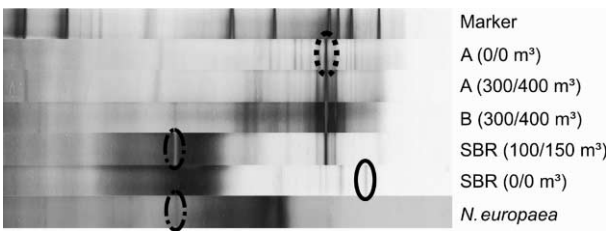

Figure 3 | Fingerprinting profiles of 16S rRNA gene sequences, obtained with the 189f-GC/654r primer pair. The circles indicate the excised bands used for phylogenetic analysis. Band 1 (dotted line), Band 2 (solid line). *N. europaea* (dash-dot line).

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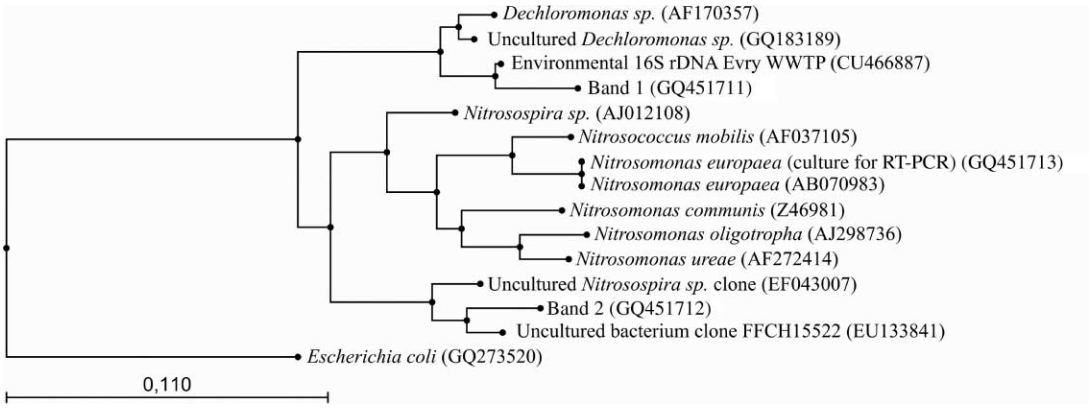

Figure 4 | Phylogenetic distance tree, based on the comparison of 16S rRNA gene fragments, that were amplified with the 189f/654r primer pair. *Escherichia coli* (γ-Proteobacteria) was used as an outgroup. Cut-out bands and the *Nitrosomonas europaea* pure culture are highlighted in bold letters. Accession numbers are given in brackets. The scale represents the sequence divergence.

with this primer pair. Therefore excision and sequencing of bands of interest is highly recommended.

Another fact to keep in mind is that even amplicons obtained from the pure culture of *N. europaea* rendered more than one band on the DGGE. For AOB, only one 16S rRNA gene copy per cell is assumed (Aakra *et al.* 1999), which allows the direct conversion of gene copy to cell numbers. Therefore, as stated by Kowalchuk *et al.* (1997), the multiple bands are rather due to degenerated primers, leading to slightly variable DNA fragments with different migration behaviours in the gels. Summarised, the AOB community might be less diverse than anticipated by the fingerprint patterns.

Quantitative real-time PCR analysis

To be able to better combine the DGGE and real-time PCR results, the CTO primer set 189f/654r for DGGE and 189f/RT1r for real-time PCR, with the same forward primers, was chosen (Kowalchuk *et al.* 1997; Hermansson & Lindgren 2001). These primers amplify AOB sequences of the whole β-Proteobacteria and *Nitrosococcus mobilis* and are therefore ideal for a general survey. The 16S rRNA gene based quantitative real-time PCR demonstrated alterations in AOB abundance throughout the experimental set-up and general differences among the three sample types (A, B, SBR). Our obtained values ranged from 2.76×10^9 gene copies g^{-1} TS (A-sample) to 2.38×10^{11} gene copies g^{-1} TS (SBR-sample). These minimum and maximum values were obtained during the bioaugmentation to the A-stage in the cold season and with 0/0 m^3 pump rate. Regarding each sampling type on its own (mean values ± standard errors (SE)), A-samples had the lowest gene copy numbers with $2.41 \times 10^{10} \pm 9.61 \times 10^9$ SE g^{-1} TS, followed by the B-samples $5.82 \times 10^{10} \pm 1.31 \times 10^{10}$ SE g^{-1} TS and the SBR-samples $7.91 \times 10^{10} \pm 1.84 \times 10^{10}$ SE g^{-1} TS, respectively. The general trends in AOB abundance, however, were not very consistent throughout the four bioaugmentation phases.

Comparing the results of the DGGE and the real-time PCR analysis, especially the high gene copy numbers in the bioaugmentation to the A-stage (cold season) was striking. With a closer look at the banding patterns, *N. europaea* seems to be the dominant AOB, making up for even all copy numbers in the SBR (0/0 m^3) sample. The rest of the AOB community in these samples, represented by other detected bands, is less influencing the total gene copy number. Applying this given fact on all sampling periods, the presence and intensity of the before mentioned *N. europaea* bands is clearly correlating with the real-time PCR results. Low values in most A-samples go along with the absence or very low intensity of this band. The bioaugmentation strategy seems to have a bigger effect on the community composition than on total abundance of AOB. The presence of 16S rDNA is not to be set equal with active biomass. As shown in the study by Schoen *et al.* (in preparation) that is dealing with the physical-chemical parameters of the here presented experiment, samples with low gene copy numbers of the 16S rRNA gene can still represent highly active AOB communities.

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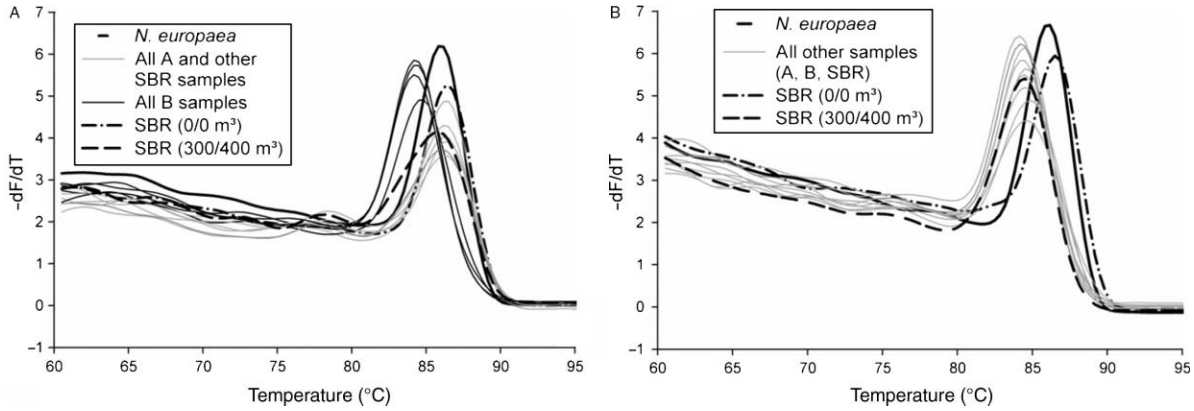

Figure 5 | Melt-curve analysis of the bioaugmentation strategies to the A-stage (A) (warm season) and the B-stage (B) (cold season). The negative first derivative of the fluorescence (F) plotted versus the temperature (°C).

Melt-curve analysis

The melt-curve analysis was performed mainly to detect any non-specific PCR amplification and primer-dimer formation. Furthermore, it also allowed clustering of the samples into different groups, with specific melting temperatures. The melting temperature (T_m) reflects the temperature at which 50% of a specific DNA fragment is dissociated and is besides the amplicon's length, dependent on the GC and AT concentration. Thus, it also enables discrimination between different DNA sequences. Bioaugmentation to the A- and the B-stage, respectively, revealed specific melt-curve results that were underlining the DGGE findings. Having a closer look at the bioaugmentation to the A-stage, Figure 5(A) shows, as in Figure 2(A), the initially closer related SBR and A-samples (close to T_m of *N. europaea*) and the B-samples with a lower T_m. The SBR samples at highest pump rate (300/400 m^3) were shifting towards the B-samples and therefore showing a broader peak in the melt-curve. Regarding the bioaugmentation to the B-stage (Figure 5(B)), A-samples, not affected by the bioaugmentation strategy, are similar to the B-samples and also the SBR samples adapt immediately to the B fingerprint pattern. Only at zero pumping rates two distinct sludge systems were detectable, the SBR (0/0 m^3) and mainstream (A and B) system.

The critical step in this whole experimental set-up was the temperature gradient among the three reactors (A, B and SBR), which led to a noticeable decrease in seeded biomass activity. Temperatures of the A-stage and the B-stage were on average 11.7°C during the cold and 16.2°C during the warm season periods. The temperature of the SBR ranged from 34.1°C down to 26.7°C at low and high pumping rates, respectively. The pump rate to the SBR itself is furthermore limited by the influence of colder WAS on the temperatures of the SBR, which is usually kept at 35°C. Despite the habitat changes for the AOB biomass, an overall positive effect on N-removal efficiency could be demonstrated (Schoen *et al.* in preparation). The molecular techniques used in this analysis indicated the gradual shift of the microbial community in the SBR and partly also in the A-stage, to the finally intended AOB fingerprint pattern of B-samples, from where WAS was recycled. This shift was most pronounced at highest pump rates, reflecting the accumulative effect of the stepwise, increasing pump rates on the microbial community composition.

CONCLUSION

The bioaugmentation strategy has a noticeable effect on the microbial community composition, especially in the SBR tank. With increasing pump rates, the two initially distinct sludge systems show a gradual shift of the fingerprint patterns. Molecular experiments revealed, however, that the effect was more accentuated on community composition than on AOB abundance. Sequenced bands were related to yet uncultured *Nitrosospira*-like and uncultured environmental sequences. Bands related to *Nitrosomonas europaea* could be detected in all SBR and B-samples and in some A-samples.

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