The inoculum effect on the ammonia-oxidizing bacterial communities in parallel sequential batch reactors

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ABSTRACT
Three identical sequential batch reactors (SBRs) were each inoculated with sludge from a full-scale wastewater treatment plant (WWTP) treating a waste stream of different origin, i.e. a hospital, a meat processing company, and a municipal WWTP. The SBRs were run in parallel for 84 consecutive days to investigate whether the reactors would become more phylogenetically similar or stay separated concerning their functionality and microbial communities. Overall, the nitrification functionality was high throughout the experiment, and the size and structure of the sludge flocs were very similar. The total bacterial and ammonia-oxidizing bacterial (AOB) communities were analyzed by PCR-DGGE. Cluster analysis demonstrated very distinct bacterial communities in the three SBRs, not showing any trend becoming more similar. The carrying capacity, dynamics and functional organization of the communities were assessed by DGGE analysis and based on these patterns the range-weighted richness, moving window analysis, and constructing Pareto–Lorenz evenness distribution curves were calculated. Between the SBRs, highly comparable internal structure and dynamics of the AOB communities were observed, although they had only one AOB DGGE band in common. These observations indicate that community characteristics such as the extent of biodiversity and dynamics are more important indicators of good microbial functionality than the presence of certain specific species.

1. Introduction
Nitrification is a two-step process defined as the biological transformation of reduced forms of nitrogen to nitrate (Koops and Pommerening-Röser, 2001). The first step is the conversion of ammonia to nitrite. The second step involves the further transformation of nitrite into nitrate. Nitrification is – together with denitrification – centrally positioned in the global nitrogen cycle, and as starting stage in the nitrogen removal, it fulfills a key role in wastewater treatment plants (WWTPs) (Kowalchuk and Stephen, 2001; Subbarao et al., 2006). An incomplete nitrification process can provoke serious ecological risks (e.g. eutrophication and foam formation) and health related problems (e.g. fish death, release of nitrite and/or nitrate in drinking water and methemoglobinemia) (Das et al., 2004; Jensen, 2003). Nevertheless, nitrification – and especially ammonia-oxidation – is a process sensitive to inhibition, because the number of different microorganisms that perform this function is limited, these species have low growth yields and are sensitive to changing environmental parameters such as temperature or pH (Anthonisen et al., 1976; Balmelle et al., 1992; Grunditz and Dalhammar, 2001; Prosser, 1989). Therefore, nitrification is considered an Achilles’ heel of wastewater treatment (Daims et al., 2006).

The biodiversity of the nitrifying communities is an important driver of nitrification functionality. Species
richness (number of different species) and species evenness (relative abundance among species) are two facets of biodiversity (Purvis and Hector, 2000). A positive relationship between species richness and ecosystem functioning has been observed (Bell et al., 2005). Although research investigating the relation between species evenness and productivity is scarce, it has been indicated that decreased evenness has an indirect lowering effect on the productivity and stability of the ecosystem (Balvanera et al., 2005; Wilsey and Potvin, 2000; Wittebolle et al., 2009a). It has already been suggested to engineer wastewater treatment plants to have higher diversity, because this could make processes such as nitrification more stable (Daims et al., 2001). However describing the community structure, i.e. evenness and richness, is still not straightforward. Recently, we suggested a pragmatic processing method for molecular fingerprinting techniques, such as DGGE, to evaluate the internal structure and dynamics of microbial communities with the aim to relate ecological data with specific functionality (Marzorati et al., 2008). Based on these fingerprints, the range-weighted richness (Rr), moving window analysis (Dy), and constructing Pareto–Lorenz evenness distribution curves (Fo) could be calculated (Marzorati et al., 2008) and linked the carrying capacity of an environment (number of individuals an environment with its resources can support) and the functional organization (result of the action of the microorganisms that are most fitting to the ongoing environmental–microbiological interactions).

In this research, three identical sequential batch reactors (SBRs) were each inoculated with sludge from a full-scale WWTPs of different type respectively from a hospital (SBR1), a meat processing company (SBR2), and a municipal WWTP (SBR3) at a final sludge concentration (VSS) of 4 g L$^{-1}$. The influent consisted of NH$_4$Cl (volumetric loading rate: between day 1 and day 17 gradually from 15 until 100 mg N L$^{-1}$ d$^{-1}$, and from day 17 until 84 fixed at 100 mg N L$^{-1}$ d$^{-1}$), supplemented with 13 mg L$^{-1}$ d$^{-1}$ Nutriflok as a source of macro- and micronutrients (Avecom, Beernem, Belgium) and 4.5 mg L$^{-1}$ d$^{-1}$ KH$_2$PO$_4$. Daily, two complete cycles (12 h in total) were performed, consisting of (i) a 10 h aeration phase (feeding during the first 0.5 h at 25 L h$^{-1}$), (ii) a 1 h 25 min settling phase, (iii) a 5 min decanting phase, (iv) a 15 min safety phase, and (v) a 15 min restarting phase of the aeration and pH control (prior to feeding). Each of the reactors had a separate acid–base pH controlling system (Consort R305, Turnhout, Belgium) to maintain the pH between 7.2 and 7.6. The hydraulic residence time (HRT) was 1.5 d. No sludge was removed, except for sampling (once a week, 50 mL).

### 2. Materials and methods

#### 2.1. Experimental set-up and sampling

Three simultaneously run 37.5 L sequential batch reactors were followed during 84 consecutive days. At day 0, the reactors were inoculated with sludge obtained from full-scale WWTP treating a waste stream of different origin, i.e. a hospital, a meat processing company, and a municipal WWTP. The SBRs were run in parallel during 84 consecutive days. At day 0, the reactors were inoculated with sludge obtained from full-scale WWTPs of different type respectively from a hospital (SBR1), a meat processing company (SBR2), and a municipal WWTP (SBR3) at a final sludge concentration (VSS) of 4 g L$^{-1}$. The influent consisted of NH$_4$Cl (volumetric loading rate: between day 1 and day 17 gradually from 15 until 100 mg N L$^{-1}$ d$^{-1}$, and from day 17 until 84 fixed at 100 mg N L$^{-1}$ d$^{-1}$), supplemented with 13 mg L$^{-1}$ d$^{-1}$ Nutriflok as a source of macro- and micronutrients (Avecom, Beernem, Belgium) and 4.5 mg L$^{-1}$ d$^{-1}$ KH$_2$PO$_4$. Daily, two complete cycles (12 h in total) were performed, consisting of (i) a 10 h aeration phase (feeding during the first 0.5 h at 25 L h$^{-1}$), (ii) a 1 h 25 min settling phase, (iii) a 5 min decanting phase, (iv) a 15 min safety phase, and (v) a 15 min restarting phase of the aeration and pH control (prior to feeding). Each of the reactors had a separate acid–base pH controlling system (Consort R305, Turnhout, Belgium) to maintain the pH between 7.2 and 7.6. The hydraulic residence time (HRT) was 1.5 d. No sludge was removed, except for sampling (once a week, 50 mL).

#### 2.2. Physico-chemical parameters

The nitrite and nitrate content of the effluent was analyzed by ion chromatography as previously described (Wittebolle et al., 2009). Ammonium (with Nessler), suspended solids (SS), and volatile suspended solids (VSS) were analyzed by standard methods (Greenberg et al., 1992). To evaluate the functional nitrification performance of the reactors, the nitrification efficiency was calculated by Eq. (1).

\[
\text{Nitrification efficiency}(\%) = 100 - \frac{\left(\text{NH}_4^+ - \text{N}\right)_{\text{effluent}} + \left(\text{NO}_2^- - \text{N}\right)_{\text{effluent}}}{\left(\text{NH}_4^+ - \text{N}\right)_{\text{influent}}} \times 100
\]

#### 2.3. Fluorescent in situ hybridization (FISH)

Activated-sludge samples were fixed according to the protocol of Amann et al. (1995). The method of Biesterfeld et al. (2001) was used to perform FISH assays with fluorescently labeled rRNA-targeting oligonucleotide probes. For the AOB species the Cy3 labeled NSO1225 probe (Mobarry et al., 1996), and for the Nitrospira sp. the Alexa488 labeled probe S-G-Ntspa-0662-a-A-18 (Daims et al., 2000) were applied. Probe mixtures and a hybridization buffer with a formamide concentration of 35% were used in hybridization reactions. Visualization of the samples was performed with a Nikon Eclipse TE300 epifluorescence microscope equipped with a Bio-Rad Radiance 2000 confocal system. ImageJ 1.36b software (freely available at http://rsb.info.nih.gov/ij) was applied for further image processing.

#### 2.4. Nucleic acid extraction

Mixed liquor samples of 50 mL were centrifuged at 3000 × g for 2 min and supernatants were discarded. Nucleic acids were extracted from 0.5 g of the centrifuged sludge samples by
using a low-pH hot-phenol extraction procedure as previously described (Stahl et al., 1988). The DNA concentration was measured spectrophotometrically at 260 nm (NanoDrop ND-1000 Spectrophotometer, Isogen Life Science, Sint-Pieters-Leeuw, Belgium) and set to 5 ng μL⁻¹ using DNase- and RNase-free filter-sterilized water (Sigma-Aldrich Chemie, Steinheim, Germany).

### 2.5. PCR amplification

PCR was performed with primers targeting all bacteria (P338f with GC-clamp and P518r) to obtain DNA amplicons for further analysis of the total bacterial community by DGGE (Øvreås et al., 1997). To study the AOB community, a nested PCR was performed within the first round primers CTO189AB, CTO189C and CTO653, which amplify specifically the beta-Proteobacterial ammonia-oxidizers (Kowalchuk et al., 1997). The second round was performed with total bacterial primers (P338f with GC-clamp and P518r) (Øvreås et al., 1997) that target all bacteria (to obtain DNA amplicons for further analysis by DGGE). PCRs were performed according to the protocol of Boon et al. (2002). Primer pairs NSR1113F–NSR 1264R (Dionisi et al., 2002) and FGPS872–FGPS1269 (Degrange and Bardin, 1995) were used to investigate the possible presence of respectively Nitrospira and Nitrobacter according to the protocols described by the authors mentioned. To amplify the AOA, PCR was performed with primers arch-amoAF and arch-amoAR according to the protocol of Francis et al. (2005).

### 2.6. DGGE analysis

DGGE gels (8% (w/v) polyacrylamide, and a denaturing gradient ranging from 45 to 60%) were run on a Bio-Rad DGene™ system (Hercules, CA, USA) as described previously (Boon et al., 2002). The obtained DGGE patterns were further processed using BioNumerics software version 2.0 (Applied Maths, Sint-Martens-Latem, Belgium). Range-weighted richness (Rr) values (Marzorati et al., 2008) were calculated based on the total number of bands (N₁), and the denaturing gradient comprised between the first and the last band of the pattern (D₀), according to Eq. (2).

\[
Rr = N^2 \times D_0
\]  

(2)

The matrix of similarities for the densitometric curves of the band patterns was calculated based on the Pearson product-moment correlation coefficients and was used to perform cluster analysis and moving window analysis (Wittebolle et al., 2005). Hereto, the correlation between day X and day X – 7 was plotted. This way, each data point in the graph is – on itself – a weekly based comparison. Eq. (3) recalculate these similarity percentage values to change percentage values.

\[
\text{change}\% = 100 - \text{similarity}\% \quad (3)
\]

The rate of change or \( \Delta\text{t}_{\text{week}} \) values (Wittebolle et al., 2008) were calculated as the average and standard deviation of the respective change% values. The higher the change between the DGGE profiles of day X and day X – 7, the higher the change per week data point, and the higher the \( \Delta\text{t}_{\text{week}} \) value will be.

In order to graphically represent the evenness of the bacterial communities, Pareto–Lorenz (PL) distribution curves (Lorenz, 1905) were set up, based on the DGGE profiles as previously described (Wittebolle et al., 2008). For each DGGE lane, the respective bands were ranked from high to low based on their intensities. Consecutively, the cumulative normalized number of bands was used as x-axis, and their respective cumulative normalized band intensities represented the y-axis. Finally, the curves were evaluated by a horizontal y-axis projection of their intercepts with the vertical 20% x-axis line. The latter enabled numerical interpretation of the PL curves. The more the Pareto–Lorenz curve deviated from the 45° diagonal (the theoretical perfect evenness line), the higher the y-value, thus the less evenness is observed in the structure of the studied community. The latter means that a smaller fraction of the different species was present in dominant numbers.

The desired DGGE bands were cut out, cloned with the pCR® 2.1-TOPO® cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manual instructions, and sequenced by IIT Biotech–Bioservice (Bielefeld, Germany). DNA sequence analysis was performed using the BLAST server of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) with the BLAST algorithm and specifically with the BLASTN program. Sequences were deposited in the GenBank database.

### 2.7. Reproducibility of the DGGE method

The low-pH hot-phenol nucleic acid extraction procedure was successfully applied before to detect different kinds of ammonium-oxidizers and both Nitrobacter as Nitrospira (nitrite-oxidizing) species (Mota et al., 2005). To test the reproducibility of the sampling, extraction, PCR and DGGE, triplicate sludge samples were analyzed. Similarity values above 97% (Wittebolle et al., 2009b) were noted between DGGE samples originating from the same reactor on the same day. This demonstrated the high reproducibility of the molecular methods.

### 3. Results

#### 3.1. Physico-chemical parameters

The dissolved oxygen (DO) and temperature were not significantly different between the three SBRs (DO: \( p = 0.22 \); temperature: \( p = 0.30 \)). The average value was 6.2 ± 0.4 mg O₂ L⁻¹ for DO, and 18.4 ± 1.9 °C for temperature, demonstrating that the latter two parameters did not limit nitrification. Although the VSS concentrations were significantly different between the three SBRs after stabilization ( \( p < 0.01 \)), a comparable VSS trend was visible (Fig. 1A). Starting at about 4 g VSS L⁻¹, the VSS concentrations gradually decreased during the first two months. Afterwards, from day 50 on, an approximately fixed VSS level (SBR1: 2.7 g VSS L⁻¹; SBR2: 2.0 g VSS L⁻¹; SBR3: 1.5 g VSS L⁻¹) was maintained. The functionality of the three nitrifying SBR reactors was assessed
study. The majority of the bands (designated 1 until 14 in Fig. 3C and D) were sequenced. According to the Ribosomal Database Project II (http://rdp.cme.msu.edu), DGGE bands 1 until 13 are related to the genus Nitrosoomonas. Table 2 lists per DGGE band the corresponding closest match from the NCBI database (http://www.ncbi.nlm.nih.gov) with indication of similarity percentage and source of origin. Sequence analysis revealed that band 14 gave 100% similarity with the uncultured Ferribacterium sp. partial 16S rRNA gene (AJ890204) found by DGGE in a lab-scale wastewater reactor. Therefore, band 14 can be considered as a result of primer non-specificity and was excluded from further data processing. By cluster analysis it was demonstrated that the AOB communities of the SBRs with different inoculum did not become more phylogenetically similar, but stayed apart throughout the experiment (Fig. 3C and D). Nevertheless, in each reactor some changes were visible over time. In SBR1 bands 5, 11, and 13 decreased in favour of bands 2, 4, 7, and 10. In SBR2 bands 10, 11, and 13 decreased in favour of bands 3 and 7. In SBR3 bands 5 and 8 decreased in favour of bands 6, 7, 10, and 12.

3.4. Pragmatic analysis of the AOB DGGE patterns

To further characterize the structure and dynamics of the ammonia-oxidizing community, a pragmatic analysis was applied to the AOB DGGE patterns. From week 1 until week 12, the number of DGGE bands was on average 18 ± 1, 13 ± 1, and 19 ± 1 for respectively SBR1, SBR2, and SBR3. These bands were spread over 13% of denaturing gradient both for SBR1 and SBR3, and 11% of denaturing gradient for SBR2. Based on Eq. (2), this corresponds with a range-weighted richness (Rr) between 38 and 47 for SBR1, between 16 and 22 for SBR2, and between 42 and 52 for SBR3. Thus, SBR2 had a medium, and SBR1 and SBR3 had a high Rr according to the classification of Marzorati et al. (2008).

To evaluate the dynamics (Dy) of the AOB communities, weekly based deviations were established with moving window analysis (Suppl. Fig. 1). Based on Eq. (3), Δdehy values were calculated. SBR1 had a Δdehy value of 8.8 ± 4.8%, SBR2 of 8.8 ± 4.5%, and SBR3 of 13.3 ± 6.0%. This indicated that comparable extents of dynamics were visible between the different reactors.

Pareto-Lorenz curve distribution patterns were plotted to assess the interspecies abundance ratios in the reactors. This way, the functional organization (Fo) of the bacterial communities was visualized. For the AOB community a medium Fo was detected for all SBRs as 20% of the bands (number based) corresponded with on average 56–57% of the cumulative band intensities (Fig. 4A–C). Thus, the functional organization was highly comparable between the different reactors.

Finally, the DGGE profiles of the three SBRs were classified in a combined plot according to their Rr, Dy and Fo values (Fig. 5). It was detected that the AOB communities of all SBRs were characterized by a medium Dy and Fo, and an Rr that was medium (SBR2) or high (SBR1 and SBR3).

4. Discussion

The central question of this research was whether microbial communities of different origin become more

![Fig. 1 – (A) Volatile suspended solids (VSS) concentration (g L⁻¹), and (B) nitrification efficiency (%) of SBR1 (×), SBR2 (□), and SBR3 (▲).](image-url)
It was observed that the three sludges of different origin and community composition, stayed apart over the course of the experiment, both for the total bacterial community as for the AOB subgroup. The microbial communities of the three reactors all had a similar high nitrification efficiency and highly comparable levels of internal structure (Rr and Fo) and dynamics (Dy) concerning their AOB communities. However, the phylogenetic composition of the communities was very different (based on DGGE and sequence analysis). For example, all the reactors had only one AOB DGGE band in common during the experiment. This might indicate that community characteristics, such as the extent of biodiversity and dynamics, are more important indicators for good process functionality than the presence of certain specific species.

Hence, the pragmatic processing based on the Rr, Dy and Fo does not take into account the phylogeny of the present species, only their internal structure and dynamics (Marzorati et al., 2008). This observation indicates the usefulness of such as the pragmatic processing methods, especially for engineered microbial systems.

At start-up, the inocula of the SBR reactors were very distinct both in terms of the total bacteria (less than 5% similarity) and AOB (20–45% similarity). Although internal community dynamics at a rate of about 10% of community change per week were noted during the course of the experiment, this did not lead to more similar bacterial communities. Even at the end of the run the respective SBR communities clustered separately. Among each other, the total bacterial communities of the SBRs were 5–20% similar, and the similarity between the AOB communities still ranged between 20 and 45% at the end of the test. Moreover, there was only one AOB band (n/C147) common in the three SBRs during the entire experiment. In a previous test using the same reactor set-up, replicate nitrifying reactors were run starting from the same inoculum. During the entire run, more than 70% of similarity between the respective AOB communities was observed, as in all replicates the same species were present and in very comparable amounts (Wittebolle et al., 2009b). Moreover, in the latter experiment, it was demonstrated that AOB communities of similar SBRs changed the most during start-up, when the VSS was decreasing. Once a stable VSS concentration was obtained, the AOB dynamics continued to

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Target</th>
<th>SBR1</th>
<th>SBR2</th>
<th>SBR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>P338f–P518r</td>
<td>Bacteria</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CTO189AB, CTO189C–CTO653</td>
<td>AOB</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arch-amoAF and arch-amoAR</td>
<td>AOA</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>NSR1113F–NSR 1264R</td>
<td>Nitrospira</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FGPS872–FGPS1269</td>
<td>Nitrobacter</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

+: Detection of specific amplification product, –: no detection.

Fig. 2 – Light microscopic images (400×) of (A) SBR1, (B) SBR2, and (C) SBR3 flocs on day 84, and confocal microscopic images (400×) of FISH analysis of (D) SBR1, (E) SBR2, and (F) SBR3 flocs focussed on the AOB (green) and Nitrospira (red) communities on day 84. Depicted scale bars measure 50 μm in length. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
be low during several months. In the current research, the experiment was run until the VSS concentration stayed stable for at least one month. During this timeframe, no convergence was noticed.

In wastewater treatment plants a lot of variation in the AOB species presence has been found (Rowan et al., 2003), ranging from the predominance of single AOB populations, e.g. Nitrosomonas europaea-like organisms (Okabe et al., 1999); Nitrosococcus mobilis-like bacteria (Juretschko et al., 1998); Nitrospira-like bacteria (Schramm et al., 1998), to the coexistence of several different AOB populations (Daims et al., 2001; Gieseke et al., 2001; Wittebolle et al., 2005, 2008).

Table 2 – 13 AOB DGGE bands with corresponding closest matches (BLASTN) via the NCBI database (http://www.ncbi.nlm.nih.gov) and indication of similarity percentages and sources of origin.

<table>
<thead>
<tr>
<th>Band</th>
<th>Closest match</th>
<th>% Similarity (no. of similar bp/total bp)</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uncultured Nitrosomonas sp. (AY543074)</td>
<td>99% (196/197)</td>
<td>Lab-scale biological aerated filter, Newcastle, UK</td>
</tr>
<tr>
<td>2</td>
<td>Uncultured beta-Proteobacterium (AY631514)</td>
<td>98% (193/197)</td>
<td>Tropical soil, Costa Rica</td>
</tr>
<tr>
<td>3</td>
<td>Uncultured bacterium (AB176884)</td>
<td>100% (197/197)</td>
<td>Sewage activated-sludge system, Tokyo, Japan</td>
</tr>
<tr>
<td>4</td>
<td>Uncultured beta-Proteobacterium (AY631514)</td>
<td>97% (191/197)</td>
<td>Tropical soil, Costa Rica</td>
</tr>
<tr>
<td>5</td>
<td>Uncultured Nitrosomonas sp. (AY583659)</td>
<td>100% (197/197)</td>
<td>Treated wastewater, lower Seine river, France</td>
</tr>
<tr>
<td>6</td>
<td>Uncultured Nitrosomonas sp. (EU734545)</td>
<td>100% (197/197)</td>
<td>Overland flow area treating wastewater, Sweden</td>
</tr>
<tr>
<td>7</td>
<td>Uncultured beta-Proteobacterium (AJ299048)</td>
<td>98% (195/197)</td>
<td>Ammonia-oxidizer enrichment from freshwater sediment, Lake Drontemeer, The Netherlands</td>
</tr>
<tr>
<td>8</td>
<td>Nitrosomonas sp. isolate (AJ621026)</td>
<td>100% (197/197)</td>
<td>Freshwater sediment, Lake Drontemeer, The Netherlands</td>
</tr>
<tr>
<td>9</td>
<td>Nitrosomonas ureae Nm10^7 (AF272414)</td>
<td>100% (197/197)</td>
<td>Soil, Sardinia, Italy</td>
</tr>
<tr>
<td>10</td>
<td>Uncultured bacterium (AB239537)</td>
<td>100% (197/197)</td>
<td>River sediment, Niida river, Japan</td>
</tr>
<tr>
<td>11</td>
<td>Uncultured Nitrosomonas sp. (DQ887682)</td>
<td>100% (197/197)</td>
<td>Overland flow area treating wastewater, Sweden</td>
</tr>
<tr>
<td>12</td>
<td>Uncultured bacterium (AB239537)</td>
<td>99% (196/197)</td>
<td>River sediment, Niida river, Japan</td>
</tr>
<tr>
<td>13</td>
<td>Uncultured beta-Proteobacterium (AJ299052)</td>
<td>99% (196/197)</td>
<td>Ammonia-oxidizer enrichment from freshwater sediment, Lake Drontemeer, The Netherlands</td>
</tr>
</tbody>
</table>

Fig. 3 – Cluster analysis based on DGGE profiles for the total bacteria at (A) the start and (B) the end of the experiment, and of the AOB subgroup at (C) the start and (D) the end of the experiment with indication of the 14 bands that were sequenced (dX = dayX). Note the clear-cut separation of the samples originating from different SBRs.
latter is also the case here. All analyzed samples rendered several DGGE bands, indicating the presence of different AOB. Nevertheless, all bands were found to be related to the genus *Nitrosomonas*, demonstrating the absence of *Nitrosospira*-like AOB in these sludges. This correlates with the finding that in traditional WWTP sludge *Nitrosomonas* lineages seem to prevail (Gorra et al., 2007; Harms et al., 2003; Layton et al., 2005; Limpiyakorn et al., 2005; Mota et al., 2005; Wittebolle et al., 2005). The fact that the lower AOB range-weighted richness of SBR2 correlated with the exclusive retrieval of AOA related genes in this research is interesting. As currently not much is known about the presence, stability and activity of AOA in WWTPs (Park et al., 2006), the reason behind this observation is speculative. Further investigation will have to unravel whether this finding is: (i) a result of the lower AOB diversity creating a niche for the growth of other ammonia-oxidizers, *in casu* AOA; (ii) because the AOA out-competed the AOB, or (iii) purely coincidentally. We believe that much about AOA can be learned both from their presence and absence in specific WWTPs, and therefore we would like to encourage the reporting of successful and unsuccessful detection attempts by other researchers. Further studies are warranted to elucidate the ecological role of AOA in wastewater treatment systems.

Concerning the nitrite-oxidizing bacteria, members of the genus *Nitrobacter* were not detected by PCR or FISH (<2 log units of cells per mg VSS). *Nitrospira* sp. on the other hand was detected in all sludge samples. Up to now, *Nitrobacter* (Coskuner and Curtis, 2002) or *Nitrospira* (Lydmark et al., 2007; Wittebolle et al., 2008) have been identified as the dominant nitrite-oxidizing bacteria in WWTPs. In the case both NOBs were detected, most frequently a higher amount of *Nitrospira*

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**Fig. 4** – Pareto-Lorenz distribution curves based on DGGE profiles of the AOB subgroup of (A) SBR1, (B) SBR2, and (C) SBR3 on days 1, 15, 28, 42, 56, 70 and 84. The dashed vertical line at the 0.2 x-axis level is plotted to evaluate the range of the Pareto values.

**Fig. 5** – Classification of the range-weighted richness (Rr), the dynamics (Dy), and the functional organization (Fo) of the AOB DGGE profiles of SBR1 (circles), SBR2 (squares), and SBR3 (triangles). According to Marzorati et al. (2008), samples with medium Rr (10 ≤ Rr ≤ 30) are represented by two shelled symbols, and samples with high Rr (Rr > 30) are represented by three shelled symbols. The barred areas represent the zone where – conceptually – the parameter ranges are conceived to offer chances for sustainability of the microbial communities (Marzorati et al., 2008).
sp. than Nitrotrichum sp. was observed (Burrell et al., 1998; Daims et al., 2001; Juretschko et al., 1998; Schramm et al., 1999, 1998). The latter researches and our findings correlate with the recent expectation that Nitrospira rather than Nitrotrichum would generally dominate typical WWTPs due to their better nitrite affinity (Blackburne et al., 2007; Daims et al., 2001).

All reactors maintained complete nitrification during the entire experimental run, apart from a start-up related temporarily drop in the nitrification efficiency of SBR2. Moreover, FISH analysis demonstrated the presence of small to large clusters of AOB and Nitrosospira sp. in the flocs. This is known to be important for enduring high nitrification efficiencies (Wittebolle et al., 2008). Nevertheless, the AOB communities were not static as indicated by moving window analysis, but experienced a change over time of on average 10% of the community per week.Comparable rates of change were observed for all SBRs. Also other researchers have observed that high and stable functionality can correlate with a changing microbiology in laboratory-scale activated-sludge reactors (Boon et al., 2000; Eichner et al., 1999; Forney et al., 2001; Kaewpipat and Grady, 2002; Saikaly et al., 2005; Wittebolle et al., 2008; Zumstein et al., 2000). This indicates that functional stability does not necessarily imply community stability.

Previously it was hypothesized that to achieve functional stability, a given arrangement among populations within a community is important (Briones and Raskin, 2003; Fernandez et al., 1999). Pareto–Lorenz evenness distribution curves were plotted to visualize the interspecies abundance ratios within the AOB community (Mertens et al., 2005; Rousseau et al., 1999). Over time, it was observed for the AOB community that 20% of the bands corresponded with an average 56–57% of the cumulative band intensities, indicating a medium functional organization according to Marzorati et al. (2008). This way it was concluded that at each moment only a small group of the present AOB was numerically dominant while the rest was only present in small amounts. Moreover, there was a change in the identity of the dominant DGGE bands per reactor over the course of the experiment. Therefore, the remaining less dominant species are thought to represent a pool of AOB which can proliferate to replace the current dominant AOBs according to the concept of functional redundancy.

5. Conclusion

In conclusion, a clear effect of the inoculum was observed in this experiment. Even during strictly parallel operation, the community did not become phylogenetically more similar on the total bacterial or the AOB level. Secondly, it was found that AOB diversity rather than the presence of a specific AOB species ensured nitrification functionality. Therefore, when bio-augmenting a WWTP to improve its nitrification functionality, the primary objective should be increasing the biodiversity. Finally, the usefulness of methods such as the pragmatic processing, especially for engineered microbial systems, was indicated. Both microbial ecologists and environmental engineers will benefit from the information gained by the further application of these techniques to full-scale WWTPs of different types.

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Appendix.
Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.watres.2009.06.034

References


