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Qualitative analysis of bacterial biocenoses in two sequencing batch reactors treating reject water under different technological conditions

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ABSTRACT

Complete nitrogen removal over nitrite (CANON) was used to treat reject water with ammonia concentrations ranging from 70 to 154mg·L^{-1}. Two experimental sequential batch reactors, SBR_A and SBR_B, differed in the time of the reject water inflow (6h40min vs 40min), process temperature (25 vs 29°C), and the number of aeration periods per day (3 vs 6, respectively). Nitrogen removal efficiency was higher in SBR_B (50-90%) than in SBR_A (40-80%). Analysis of total (PCR-DGGE) and active (RT-PCR-DGGE) bacteria revealed that the biodiversity of the bacterial biocenoses, expressed as the Shannon-Wiener Biodiversity Index, was higher in SBR_B (2.75-3.10) than in SBR_A (1.80-2.75).

INTRODUCTION

In wastewater treatment, reject water from sludge dewatering has a high ammonium-nitrogen concentration, which if recirculated to the main treatment stream, can cause problems with nitrification and denitrification, and affect the balance of the microbial community. Separate treatment of reject water is a solution to this problem that reduces the nitrogen load of the main stream and improves nitrogen removal (Fux et al. 2002).

For treating wastewater with a high concentration of ammonium nitrogen and a low C/N ratio, a combination of partial nitrification with the anammox (anaerobic ammonium oxidation) process is a promising method (Van Loosdrecht and Jetten 1998). In the first stage, about 50% of the influent ammonium can be oxidized to nitrite, reducing the amount of oxygen required by almost a half (Furukawa et al. 2009). This helps to make the combination of processes a cost-effective solution. Many researchers have studied the process from a technological point of view (Fux et al. 2002; Galí et al. 2007; Vega–De Lille et al. 2015; Zhang et al. 2010), but the impact of the high and unstable nitrogen concentrations in raw reject water on the biodiversity of anammox bacteria is not entirely clear.

Anammox bacteria need very specific environmental conditions to grow, and their growth is inhibited in nitrogen-rich wastewater, which has restricted the application and industrialization of the anammox process (Jin et al. 2012). In general, these bacteria grow very slowly (doubling time at 30-40°C is 10-14 days (Strous et al. 1998; van der Star et al. 2007)), their cell yield is low (Strous et al. 1998, 1999), and they are highly sensitive to changes in environmental conditions, which makes them very difficult to cultivate.

Here, we use the completely autotrophic nitrogen removal over nitrite process (CANON) to enable simultaneous partial nitrification and the anammox process in two sequencing batch reactors (SBRs) fed with diluted reject water from anaerobically digested sludge dewatering. We report that the biodiversity of the bacterial community was higher in the SBR with the higher temperature (29°C), shorter time of wastewater inflow (40 min), and six aeration periods per day than in the SBR with the lower temperature (25°C), longer periods of wastewater inflow (6 hours and 40 minutes), and 3 aeration periods per day.

MATERIALS AND METHODS

Experimental systems

Reject water was treated in two laboratory-scale SBRs, the volume of each SBR was 10L, the hydraulic retention time was around 1.6 days, and the sludge age was 50-100 days. In both SBRs, the CANON process was conducted, but the reactors differed in technological conditions. In SBR_A, the temperature was 25.0 with a range of ±0.5°C (25.0±0.5°C) and the feeding time was 6 hours and 40 minutes. In SBR_B, the temperature was 29.0±0.5°C, and feeding time was 40 minutes. In both reactors there were three 8-hour cycles per day (Figure 1).

For inoculation of both reactors, two-thirds of the final reactor volume was filled with regular activated sludge from municipal wastewater and one-third with digested sludge. Total suspended solids (TSS) content was 0.5g·L^{-1}.

The SBRs worked from July 2013 to January 2014 (181 days). The influent (1.5±0.2L·cycle^{-1}) contained NH_4^+-N between 70-154mg·L^{-1}, NO_2^--N between 0.4-24.0mg·L^{-1}, and NO_3^--N between 2.5-15.0mg·L^{-1}. The oxygen concentration was 0.25-0.30g·m^{-3} during the aeration phase and dropped to 0.00-0.05g·m^{-3} in the mixing phase of each cycle. Samples of sludge were collected from both SBRs at approximately two-week intervals. To prevent RNA degradation, RNA Later (Sigma-Aldrich) was added to each sample according to the manufacturer's instructions. Then samples were frozen and stored at -45°C until examination.

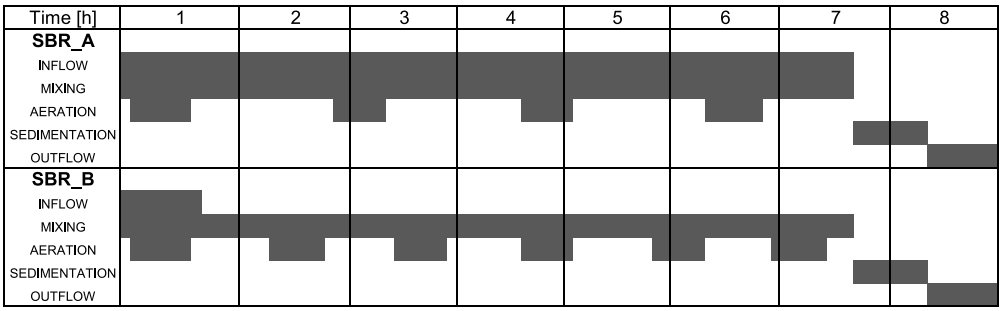

Figure 1. Different working conditions in the two experimental bioreactors: in SBR_A, the temperature was lower (25°C), the inflow of wastewater was longer (6h and 40min), and there were 3 aeration periods per day, whereas in SBR_B, the temperature was higher (29°C), the wastewater inflow was shorter (40min), and there were 6 aeration periods per day.

Concentration of nitrogen forms

To measure the concentrations of nitrogen forms (NH_4^+-N, NO_2^--N, NO_3^--N) in the influent (raw, diluted reject water) and in the effluent, colorimetric methods based on Merck Spectraquant® quick tests were used.

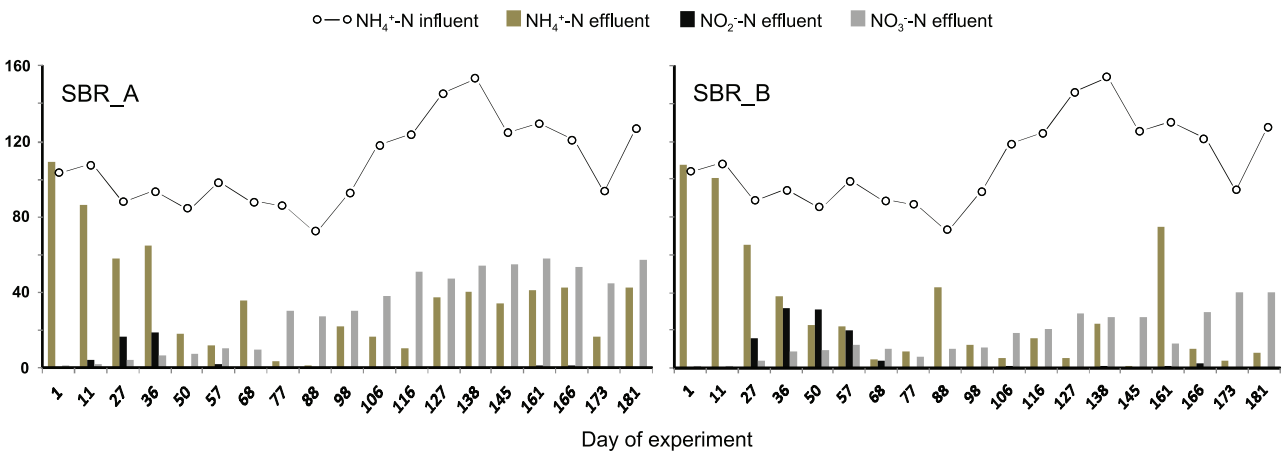

Figure 2. Concentrations of nitrogen forms in two experimental SBRs in which the CANON process was conducted. In the influent, NH_4^+-N concentration varied between 70-154mg·L^{-1}. The anammox process had started after day 65-70 of the experiment, resulting in a decrease in the concentration of nitrite and an increase in that of nitrate in the effluents from both SBRs.

Molecular analyses

Isolation of genetic material

Total genomic DNA was extracted from 0.2g of the sludge samples using a mechanical method described by Ziembińska et al. (2014). Total RNA was extracted from samples using a commercial Total RNA Mini Plus Kit (A&A Biotechnology) according to the manufacturer's instructions. The amount of DNA and RNA isolated from samples was measured spectrophotometrically (in triplicate) using Qubit (Invitrogen) and stored at -20°C until PCR was performed.

Reverse transcription reaction and PCR conditions

Total RNA isolated from the samples was used as a template for reverse transcription. In the first step, the inactivation of DNases was performed with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instructions. Next the RNA template was transcribed to cDNA with a TranScriba Kit (A&A Biotechnology) according to the manufacturer's instructions.

Amplification of the 16S rRNA gene of all bacteria was performed using the following primers: 338F-GC and 518R. The sequence of the forward primer was 5'-(CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC) TAC GGG AGG CAG CAG-3' and that of the reverse primer was 5'-ATT ACC GCG GCT GCT GG-3' (Muyzer et al. 1993). The amplification of PCR products was performed in a C1000^{TM} thermal cycler (BioRad) in a 30μL reaction containing 1.5 U GoTaq Flexi Polymerase (Promega), 1×buffer, 2mM $MgCl_2$, 5pmol·μL^{-1} of each primer, 20pmol·μL^{-1} of dNTPs and 0.5μL template DNA or cDNA. The temperature cycling conditions for the amplification of the 16S rRNA partial gene were previously described by Muyzer et al. (1993). PCR products were electrophoresed in 1% agarose gel with a 1kb DNA Ladder (Promega) and visualized under UV light.

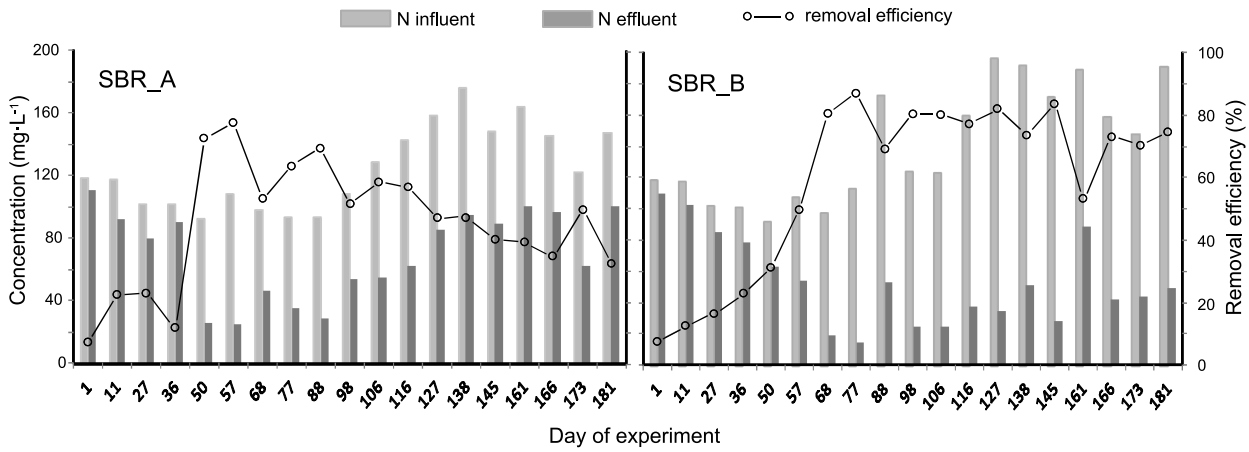

Figure 3. Total nitrogen concentrations and the efficiency of nitrogen removal in two experimental SBRs. SBR_A performed in an unstable manner after 50 days; its removal efficiency ranged between 40 and 80%. SBR_B performed in a stable manner. After 77 days of adaptation, its removal efficiency ranged from 52 to 90%.

Denaturing gradient gel electrophoresis (DGGE)

PCR products obtained with the 338F-GC and 518R primers were electrophoresed in the Dcode Universal Mutation Detection System (BioRad). Polyacrylamide gel (8%, 37.5:1 acrylamide-bisacrylamide, Fluka) with a gradient of 30-60% denaturant was prepared according to the manufacturer's instructions. Electrophoresis was performed in a 1×TAE buffer at a constant temperature of 60°C for 16 hours at 40V. Gels were stained with SYBR Gold (1:10 000, Invitrogen) in MiliQ water for 30min and destained in MiliQ water for the next 30min, then visualized under the UV light.

DGGE fingerprints were analyzed using Quantity One 1D software (BioRad). The Shannon-Wiener diversity index was calculated using the relative intensity of the bands in each sample, as previously described by Watanabe et al. (2004).

On the basis of the PCR-DGGE fingerprints, dendrograms were created using the Neighbor Joining with Dice coefficient method. The number of OTUs (operational taxonomic units) was defined as the number of lanes in the gel.

RESULTS AND DISCUSSION

Efficiency of nitrogen removal

During the whole experiment, the NH_4^+-N concentration fluctuated from 70 to 154mg·L^{-1}, according to the composition of the digested sludge and the fermentation efficiency. In the beginning of the experiment (adaptation phase), nitrogen removal was very low (5-23%).

After 30 days of the experiment, the concentration of nitrites peaked in the effluent, indicating that the balance had shifted towards nitrification. The anammox process, for

which nitrites are the substrate, had occurred after 65-70 days of the experiment (Figure 2). At this stage the concentration of nitrites had decreased and that of nitrates began to increase in the effluents from both SBRs.

SBR_A performed in an unstable manner (Figure 3). After 50 days, its nitrogen removal efficiency ranged between 40 and 80%, and tended to decrease after 90 days of the experiment. In SBR_B, adaptation lasted for 77 days, after which its nitrogen removal efficiency was 50-90%, without any clear trend (Figure 3).

Nitrogen removal was stable in SBR_B for the final 104 days of the experiment. Chu et al. (2015) showed that 20 days were needed to achieve 20-30% nitrogen removal efficiency in their lab-scale nitritation-anammox experiment, in which the SBR was fed with synthetic wastewater (containing 500mgN·L^{-1}). The performance of their SBR was unstable and nitrogen removal reached 90-95% only after 60 days of operation under stable conditions. After that, an increase in nitrogen load reduced nitrogen removal to 20-30%, and another 40 operational days were needed to achieve 80% nitrogen removal. Similarly, Gali et al. (2007) and Fux et al. (2002) have reported that conducting partial nitrification-anammox in a single SBR is very challenging.

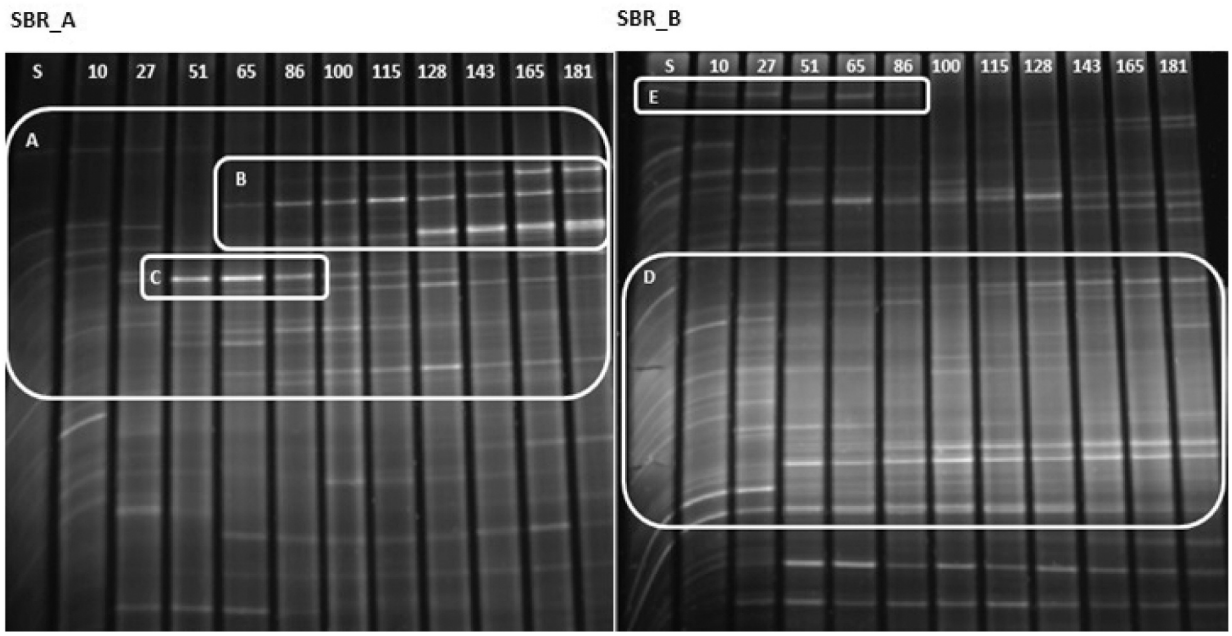

Figure 4. DGGE fingerprints of bacterial 16S rRNA gene fragments that were amplified from total DNA samples taken from the two experimental SBRs. S - inoculum; A, B, C, D, E - locations of dominant bands.

Changes in the biodiversity of the total bacterial community

To analyze the diversity of the bacterial communities in both SBRs throughout the experiment, PCR-DGGE with the 338F-GC and 518R primers was performed (Figure 4). In SBR_A, bands were located mainly in the upper part of the DGGE gel, which suggests that the bacterial community was rich in genotypes with high A and T content (frame A). As the experiment progressed and the system adapted to the experimental conditions, some bands appeared (frame B) and others faded out (frame C). In SBR_B, bands were located mainly in the middle and lower part of the gel, suggesting the presence of bacteria with genotypes with high G and C content (frame D), and some bands disappeared after the adaptation phase (frame E).

To compare the biodiversity of the systems, the Shannon-Wiener biodiversity index was calculated on the basis of densitometric analysis of the DGGE fingerprints

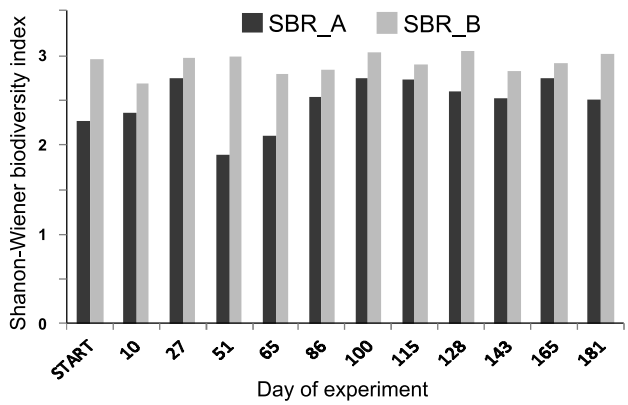

Figure 5. Shannon-Wiener bacterial biodiversity index for all bacteria in samples taken from SBR_A and SBR_B (based on the DGGE results presented in Figure 4). The structure of the bacterial community was more stable in SBR_B than in SBR_A.

(Figure 5). This index showed that the diversity of the bacterial community in SBR_B tended to be both higher and more stable.

The dendrograms created with Neighbor-Joining clustering of the DGGE fingerprints indicate that the examined bacterial communities differed (Figure 6). Samples taken from SBR_A from the time of inoculation (START) to day 86, which corresponds to the time that nitrogen removal efficiency was increasing and then high are clustered in one branch. Samples taken during the time that nitrogen removal efficiency

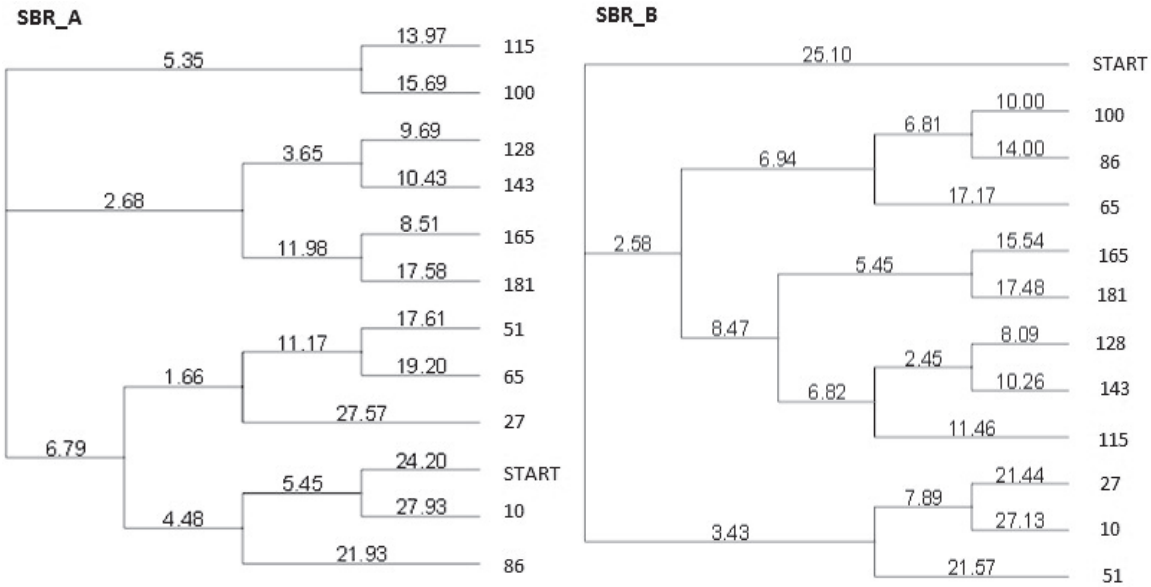

Figure 6. Dendrograms created with Neighbor-Joining clustering with Dice coefficient of the DGGE fingerprints from the total bacterial communities in the two experimental SBRs. The differencies in the clustering of the samples from the two SBRs reflect the differences in their bacterial biocenoses.

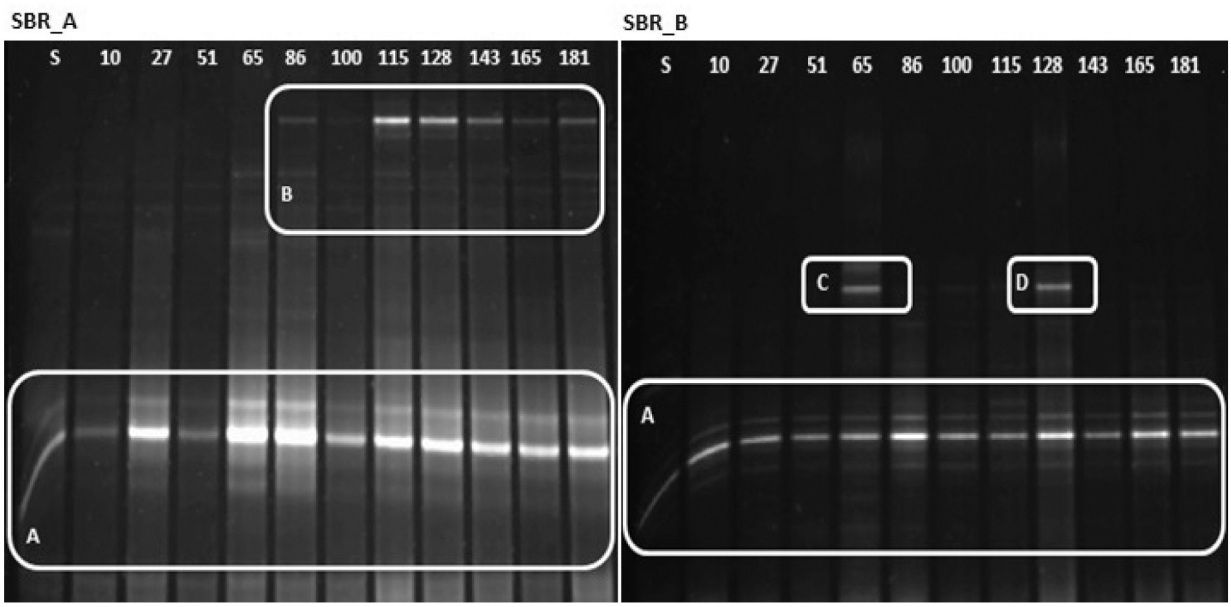

Figure 7. DGGE fingerprints of bacterial 16S rRNA 180 bp gene fragments amplified from cDNA templates after reverse transcription of the total RNA samples taken from the two experimental SBRs. S - inoculum; A, B, C, D - locations of dominant bands. In both SBRs, the most intense bands were present in all samples and located in the same part of the gel (frame A).

decreased grouped into a second branch (day 110 and 115) and a third one (days 128 to 181), indicating that the bacterial community changed during the decrease in nitrogen removal efficiency. In contrast, samples taken from SBR_B show a different pattern. The sample from the day of inoculation is in its own branch, and samples from days 10 to 51 (the adaptation phase, when removal efficiency was low) are in a second branch. Samples from day 65 to the end of the experiment, when the load of nitrogen was generally higher and removal efficiency was high, are in the third branch. This indicates that the bacterial community was relatively stable during the period of efficient nitrogen removal from SBR_B.

Changes in the biodiversity of the active bacterial community

To analyze the diversity of the active (living) bacteria in both SBRs throughout the experiment, reverse transcription (RT), cDNA amplification (PCR), and DGGE were performed (RT-PCR-DGGE). In gels obtained from both SBRs, the most intense bands are located in the same position (Figure 7, frame A). These bands suggest that anammox bacteria were present, which are normally found in activated sludge and can become active in low oxygen concentrations and other appropriate environmental conditions, such as those in this study (Liu et al. 2012a; Third et al. 2005). However, confirmation of this assumption needs further studies with the use of specific primers.

In the gel from SBR_A, some bands appeared after the adaptation phase of the treatment process, when the nitrogen removal efficiency was decreasing (frame B). In SBR_B the diversity of active bacteria was less than in SBR_A, as reflected by the lower number of bands in the gel. However, bacteria in SBR_B showed transient responses to the changes in physicochemical conditions, as indicated by the appearance and disappearance of bands during the experiment (frames C and D).

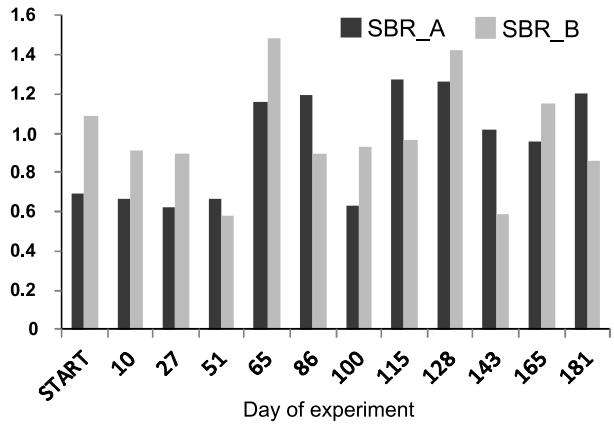

Figure 8. Shannon-Wiener bacterial biodiversity index for active bacteria in samples taken from SBR_A and SBR_B (based on the DGGE results presented in Figure 7). During the adaptation phase, the biodiversity of the bacterial community was higher in SBR_B than in SBR_A.

To compare the biodiversity of the two systems, the Shannon-Wiener biodiversity index was calculated on the basis of densitometric analysis of the DGGE fingerprints (Figure 8). This index shows that, during the adaptation phase, the biodiversity of bacteria was higher in SBR_B. Starting from day 80, however, and lasting until the end of the experiment, the biodiversity fluctuated in both SBRs.

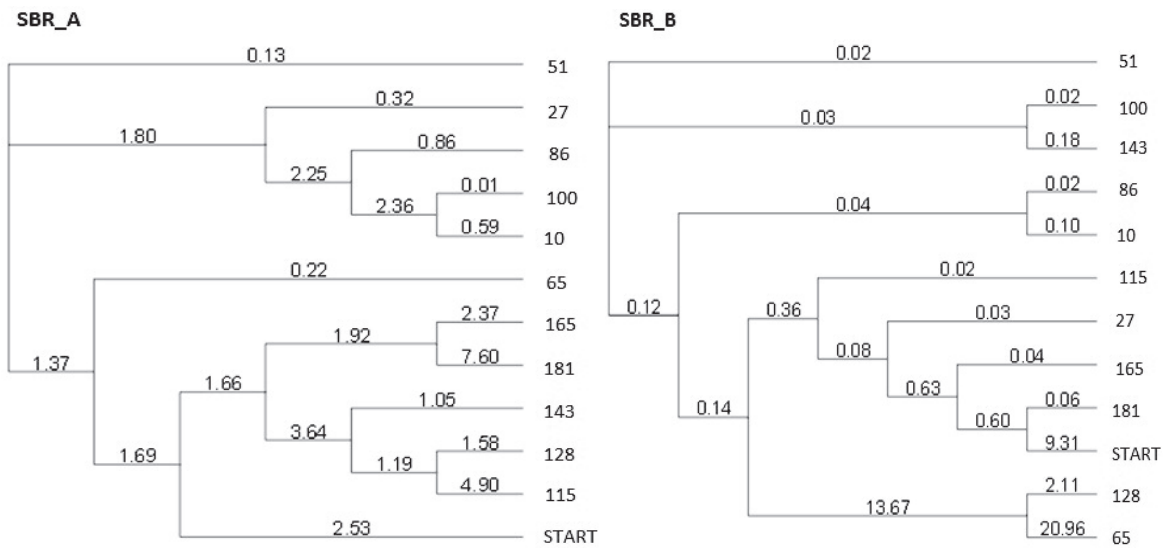

Figure 9. Dendrograms created with Neighbor-Joining clustering with Dice coefficient of the DGGE fingerprints from the active bacterial communities in the two experimental SBRs. The similarity in the clustering of the samples from both SBRs on the inoculation day and the final day of the experiment indicates similarities in the structure of the active bacterial communities in both reactors.

In the dendrograms created with Neighbor-Joining clustering of the DGGE fingerprints (Figure 9), the samples from both reactors taken on the inoculation day and during the final day of the experiment are clustered in one of the main branches. This indicates the genetic similarity of the active bacterial communities throughout the experiment.

Relationship between reactor performance and the characteristics of the microbial community

During the start-up of the CANON process, the different technological conditions in the two experimental SBRs influenced the physicochemical conditions for bacterial growth, which resulted in the development of different bacterial biocenoses. Liu et al. (2012a, b), Third et al. (2005), and van de Vossenberg et al. (2008) reported that the process temperature, characteristics of inoculation sludge, oxygen concentration, nitrogen concentration, and additional doses of nitrite can affect the microbial community and influence the efficiency of nitrogen removal.

Anaerobic ammonium oxidizing bacteria (anammox), because of their slow growth and long doubling time, are less sensitive to low concentrations and fluctuations of NH_4^+-N than aerobic ammonium oxidizing bacteria. According to Liu et al. (2012b) and Strous et al. (1998), at high concentrations of ammonia (from 480 to 1000mg·L^{-1}) the CANON system is dominated by bacteria related to *Nitrosomonas* and *Planctomycetes*. In the present experiment, the concentration of NH_4^+-N fluctuated from 70 to 154mg·L^{-1} (Figure 2). DGGE analysis of samples taken from both SBRs showed transient changes in bacterial biocenoses (Figure 4, 7), probably in response to the variable nitrogen load. Some genotypes, visible on the DGGE gels as intensely bright bands, appeared in the second half of the experiment (from day 65), but when NH_4^+-N increased, some other bands appeared and remained during the entire CANON start-up period. However, genetic identification of those would require another series of analyses with the use of primers specific to nitrifiers and anammox bacteria.

The biodiversity of the total bacterial community was higher and more stable in SBR_B (Figure 5), which had a higher temperature and a shorter time for wastewater inflow (Figure 1). In this reactor, the conditions were favorable for the CANON process, which was reflected by nitrogen removal efficiency (50-90%) that was higher than that observed in SBR_A (Figure 3).

Quantitative DGGE analysis of DNA and RNA diversity with the gels from SBR_B revealed that intense bands from different sampling times were present in the same parts of the gels (Figure 4, 7). With regard to SBR_A, however, intense bands were only observed in the analysis of active bacteria (Figure 7), but not in the analysis of the total bacterial community (Figure 4). The high tolerance of anammox bacteria to fluctuations in NH_4^+-N concentrations (Liu et al. 2012b; Strous et al. 1998) leads to the speculation that the intense bands present in the DGGE gels from both bioreactors (Figure 7, frame A) were derived from *Planctomycetes* bacteria.

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