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Biological and technical study of a partial-SHARON reactor at laboratory scale: effect of hydraulic retention time

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Abstract This study was on the technical and biological characteristics of a partial-SHARON submerged-filter bioreactor of 3 L. The main focus was the influence of the hydraulic retention time (HRT) on biofilms. For this purpose, we used molecular tools based on the partial 16S rRNA genes. The results showed that the HRT may affect the nitrification processes of a bioreactor using synthetic wastewater containing 600 mg/L of ammonia. It was found that an HRT of 0.5 day transformed 100 % of the ammonium into nitrite. However, when the HRT was decreased to 0.4 day, there was a significant reduction (35 %) in the quantity of ammonia transformed, which confirmed the complexity of the system operation. Moreover, a PCR-TGGE approach highlighted the differences observed. The results obtained showed that an HRT of 0.5 day reduced bacterial biodiversity in the biofilms, which were mainly formed by *Nitrosomonas* and *Diaphorobacter*. In contrast, an HRT of 0.4 day facilitated the formation of heterogeneous biofilms formed by nitrifying bacteria, such as *Nitrosomonas* sp., *Nitrosospira* sp., and *Nitrosovibrio* sp.).

Keywords SHARON process · Partial nitrification · Hydraulic retention time (HRT) · Wastewater treatment · Submerged biofilter · Nitrogen removal

Introduction

In the last 10 years, soaring population levels as well as a corresponding growth in industrial activity have led to increased amounts of wastewater in densely populated areas. This surfeit of waste is having an extremely negative impact on the environment. For example, high concentrations of nitrogen, one of the main compounds in wastewater, cause serious environmental problems such as oxygen depletion and eutrophication [1]. The EU Water Framework Directive 91/271/EEC clearly requires EU member states to protect the environment from any adverse effects due to the discharge of (untreated) urban and industrial waters. In this context, new technologies, such as the partial-SHARON/Anammox process, provide a cost-effective way to treat highly contaminated effluent [1, 2]. This combined process is an excellent alternative to conventional nitrification-denitrification processes since it reduces the organic matter (40 %) and oxygen (25 %) required for ammonia removal in comparison to more conventional technologies [3].

In order to fully understand the biodiversity of biological wastewater treatments, it is first necessary to identify the microbiota present and analyze their numerical significance. Culture-dependent methods have sometimes been regarded as inadequate for the analysis of microbial communities in natural environments because of the high numbers of unculturable bacteria. Furthermore, in recent years, molecular methods, based on the sequencing of PCR-amplified the partial 16S rRNA genes from DNA extracted from environmental samples, have been widely used to reveal intrinsic

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genetic biodiversity [4]. In particular, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) approaches yield large quantities of data regarding the diversity of microorganisms in their natural habitats. This has the advantage of permitting the taxonomic classification of community members [5].

The single reactor system for high-activity ammonia removal over nitrite (SHARON) process was described in detail by Hellinga et al. [6], who proposed the partial-SHARON technology. The partial-SHARON process is a modification of the traditional SHARON process, in which 100 % of the ammonium is converted into nitrite. In contrast, the partial-SHARON process, as its name implies, consists of a partial nitritation. More specifically, only 50 % of the ammonium is converted to nitrite. This process was developed for the elimination of ammonium by the "nitrite route" [7]. When the partial-SHARON process is used in combination with the Anammox process, nitrogen removal takes place in two steps. According to Van Dongen et al. [8], the Anammox process achieves an optimal performance with an ammonium–nitrite mixture of 50 % ammonium and 50 % nitrite. For this reason, the Anammox process has to be preceded by a partial-SHARON process involving a partial nitrification.

Molecular techniques have been used to provide a broader vision of the different biotechnological systems in wastewater treatment as shown in recent studies (e.g., [4]). These techniques have been used to obtain a wide range of data regarding microbiota in their habitats. In fact, they facilitate the study of non-cultivable bacteria by specifying the microbial populations that carry out these processes [9, 10]. For this reason, this research analyzed the following: (1) the hydraulic retention time (HRT) in a partial-SHARON reactor in which submerged filters were used to remove nitrogen; (2) the effect of the HRT on the structure of the bacterial community. In our study, molecular fingerprinting tools (PCR-TGGE) and scanning electron microscope (SEM) were used to evaluate the structure of the bacterial community.

Materials and methods

The SHARON bioreactor: bench-scale plant

The bench-scale plant used in our experiments consisted of a plastic SHARON bioreactor with a volume of 3 L. It was constructed as a submerged biofilter with PVC carriers (BioFlow 9). A schematic diagram of the experimental plant is shown in Fig. 1. The bioreactor received synthetic wastewater [2] from a peristaltic pump, and was operated in continuous flow.

The operating conditions in the bioreactor (i.e., HRT, pH, dissolved oxygen concentration, and temperature) were monitored every 24 h in order to verify that they remained stable. Four 15-cm air diffusers at the bottom of the vessel supplied oxygen from an air pump to ensure that the oxygen concentration in the bioreactor was maintained at 2 mg/L. All of the experimental work was performed at a pH of 7.5 and a temperature of 35 °C [11, 12], thanks to an adjustable thermostat.

Inoculation of the pilot plant

The partial-SHARON bioreactor was inoculated with mixed liquor from an aerobic reactor located in the Los Vados urban wastewater treatment plant (Granada, Spain). The mixed liquor was recirculated for 3 days until a biofilm formed on the surface of the plastic carriers used in the construction of the submerged biofilter. After inoculation, synthetic wastewater was fed into the bioreactor.

Synthetic wastewater

The synthetic wastewater [2] used in our study simulated the leachate from an anaerobic digester, since it contained a high concentration of ammonium and was low in organic matter (see Table 1).

To prepare the synthetic wastewater, 24 L of distilled water was poured inside the 60-L tank along with the exact quantity of the chemical compounds that made up the synthetic sewage medium. All components were then mixed and dissolved. The influent was continuously fed into the bioreactor by a peristaltic pump (Watson Marlow s-520) that pumped the synthetic wastewater at different flow rates.

Physico-chemical parameters

The physico-chemical parameters analyzed in our study were the following: pH, dissolved oxygen concentration, temperature, and nitrogen concentration in its various

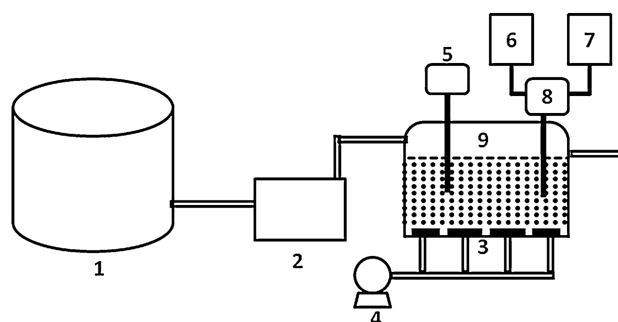

Fig. 1 Diagram of the pilot-scale partial-SHARON bioreactor used in the experiments. *1* Synthetic wastewater tank; *2* peristaltic pump; *3* oxygen diffusers (porous plates); *4* air pump; *5* thermostat; *6* tank of NaOH 0.1 M for pH control; *7* tank of H_2SO_4 0.1 M for pH control; *8* pH meter; *9* partial-SHARON bioreactor stuffed with carriers

Table 1 Composition of the synthetic wastewater in g/L used in the experiments

Chemical	g/L
$(NH_4)_2SO_4$	2.35
$NaHCO_3$	3.25
$CaCl_2$	0.30
KH_2PO_4	0.07
$MgSO_4$	0.02
$FeSO_4 \cdot 7H_2O$	0.009
H_2SO_4	0.005

Table 2 Conditions of the partial-SHARON bioreactor in experiments 1 and 2

Parameter	Experiment 1	Experiment 2	References
Oxygen demand (mg/L)	1.5	1.5	[13]
pH	7.5	7.5	[14]
Temperature (°C)	35	35	[3]
HRT (days)	0.5	0.4	

inorganic forms (ammonium, nitrite, and nitrate). Samples were taken every 24 h because of the slow growth of ammonia-oxidizing bacteria [8].

In constant pH, oxygen, and temperature conditions, two experiments were performed at different HRTs (0.4 and 0.5 day) with a view to analyzing the evolution of inorganic nitrogen concentration in the bioreactor and also the microbial diversity in the biofilm. Table 2 shows the conditions of both experiments.

pH

The pH was measured directly in the bioreactor at 8-h intervals, using a pH meter (Crison GLP 91) [15]. The equipment was adjusted daily with buffer solutions of pH 4.0 and 7.0.

Dissolved oxygen concentration

The dissolved oxygen concentration in the bioreactor was determined by means of a pulse oximeter (CRUCIBLE OXI320), which was calibrated according to the manufacturer's instructions.

Determination of ammonium, nitrite, and nitrate

Concentrations of the various inorganic forms of nitrogen (nitrite, nitrates and ammonium) were measured daily at the entry and exit points of the partial-SHARON bioreactor with an ionic chromatograph Metrohm. Nitrite and nitrate levels were measured with an anion column Metrosep A supp-4-250, and ammonium levels, with a cation column Metrosep C 2-150. A carbonate/bicarbonate solution was used as an eluent. Calibration curves of known concentrations of ammonium, nitrite, and nitrate (10, 500 and 1,000 mg/L) were also analyzed daily.

DNA extraction and PCR amplification of partial bacterial 16S rRNA genes

DNA was extracted from the biofilm that formed in the submerged biofilter. This was done by vortexing approximately 200 mL of plastic carriers from the biofilters with a saline solution, and then centrifuging them to obtain the biofilm fraction. Samples (approx. 200 mg) from the biofilm were collected with the FastDNA Kit and the FastPrep24 apparatus (MP-BIO, Germany).

Polymerase chain reaction (PCR) amplification was performed in two steps, following other research on TGGE and DGGE fingerprinting [4, 9]. One microliter (2–5 ng) of the DNA extracted was used as a template for all the PCRs. At the first PCR, the template was diluted 1:10. High-performance liquid chromatography (HPLC)-purified oligonucleotides were purchased from Sigma. AmpliTaq Gold polymerase (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) was used for all PCRs, which were performed in an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). Primers and conditions for each of the PCR reactions were those described in Molina-Muñoz et al. [9]. The final PCR products were cleaned and/or concentrated (when required) using Amicon Ultra-0.5 mL Centrifugal Filters (Eppendorf, Hamburg, Germany). Ten microliters (60–100 ng DNA) were loaded into each well for TGGE.

TGGE analysis

TGGE was performed using a TGGE Maxi system (Whatman-Biometra, Goettingen, Germany). The denaturing gels (6 % polyacrylamide [37.5:1 acrylamide:bis-acrylamide], 20 % deionized formamide, 2 % glycerol, and 8 M urea) were prepared and run with 2× Tris–acetate-EDTA buffer. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). The temperature gradient was optimized at 43–63 °C [9]. The bands were visualized by silver staining with the Gel Code Silver Staining kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Various PCR reactions were tested, and different TGGE gels were run to check the reproducibility of the results.

Analysis of TGGE fingerprints

The band patterns generated by TGGE were normalized, compared, and clustered by using the Gel Compar II v. 5.101 software (Applied Maths, Belgium). For cluster

analysis, the TGGE profile was compared by means of a band assignment independent method (Pearson product-moment correlation coefficient) as well as a method based on band presence/absence (Dice coefficient). In reference to band assignment, a 1 % band position tolerance (relative to the total length of the gel) was applied [4]. Dendrograms relating band pattern similarities were automatically calculated with unweighted pair group method with arithmetic mean (UPGMA) algorithms. The significance of UPGMA clustering was estimated by calculating the cophenetic correlation coefficients.

Range-weighted richness indices (R_r), which estimate the level of microbial diversity in environmental samples, were calculated, based on the total number of bands in each TGGE pattern (N) and the temperature gradient (°C) between the first and last band of each pattern (Tg), following Marzorati et al. [16]. The resulting values were divided by 100 [5] to keep an order of magnitude analogous to that of the R_r index, as originally described for DGGE in Marzorati et al. [16].

Pareto-Lorenz distribution curves rendered a graphical representation of the evenness of the bacterial communities in the different samples, based on the TGGE fingerprints [16]. The bands in each TGGE lane were ranked from highest to lowest based on intensity levels. The cumulative normalized band intensities for each TGGE lane were plotted against their respective cumulative normalized number of bands. The curves were numerically interpreted by the functional organization index (F_o), given by the horizontal *y*-axis projection on the intercept with the vertical 20 % *x*-axis line [16]. The calculation of the F_o indexes permitted the evaluation of the functional redundancy of the microbial communities analyzed by fingerprinting methods [16].

DNA reamplification and sequencing

Portions of individual bands on silver-stained TGGE gels were picked up with sterile pipette tips, placed in 10 μL of filtered autoclaved water, and 3 μL of the resulting DNA suspensions were used for reamplification with the appropriate primers. The PCR products were electrophoresed in agarose gels and purified with the Qiaex-II kit (Qiagen, Hamburg, Germany). The recovered DNA was directly used for automated sequencing in an ABI PRISM 3100 Avant Genetic Analyzer (Life Technologies, CA, USA).

Bacterial community analysis

The DNA sequences were analyzed and compared with the biocomputing tools provided online by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequence similarity analysis was performed with the BLASTn program [17]. ClustalX v. 2.0.3 software was used for the alignment of the DNA sequences. The graphical distribution of the main bacterial groups found is shown in this article.

Scanning electronic microscopy

The biofilm formed in the submerged biofilter was analyzed by scanning electron microscopy (SEM). Individual pieces of plastic carriers from the biofilter were fixed with glutaraldehyde (5 % v/v) in a 0.2 M sodium cacodylate buffer (pH 7.1), washed, and post-fixed in OsO_4, before being dehydrated with graded ethanol solutions (10, 30, 50, 70, 90, and 100 % ethanol). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). The samples were transferred to fresh 100 % ethanol and critical point-dried from liquid carbon dioxide at 36.1 °C and 7.37 Pa, using a Samdri 780B apparatus (Tousimis, Rockville, USA). Samples were coated with gold before being examined by variable pressure scanning electron microscopy (VP-SEM), model LEO 1430VP-SEM.

Results and discussion

Physico-chemical parameters at different HRT

Experiment 1: HRT of 0.5 day

The partial-SHARON bioreactor was fed with synthetic wastewater at a constant flow rate of 4.16 mL/min and an HRT of 0.5 day. The concentration of ammonium, nitrate, and nitrite was measured at the entry and exit points of the system. These results are shown in Fig. 2.

As can be observed in Fig. 2, after 5 days of operation, 100 % of the ammonium was converted to nitrite. After this period, the partial-SHARON bioreactor stabilized and maintained its high capacity for biotransformation. However, the higher nitrite concentration caused a sharp drop in the pH of the bioreactor. To correct this, it was necessary to add small amounts of NaOH 1 % (p/v), which kept the pH value at 7.5.

When the biotransformation capacity of ammonium into nitrite in submerged biofilters was compared with that of other systems such as conventional partial-SHARON bioreactors [8, 12], the results showed that submerged biofilters have higher levels (three times higher) of biotransformation. The high transformation capacity of submerged-biofilter systems should be regarded as an important operational factor for the development and future design of partial-SHARON/Anammox systems, which can be applied to the treatment of effluents with high nitrogen content such as landfill leachate [18].

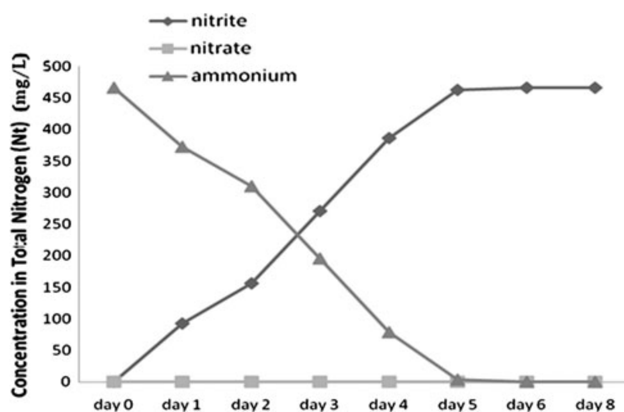

Fig. 2 Values of ammonium and nitrite expressed as total nitrogen detected in the effluent of a partial-SHARON bioreactor over time with an HRT of 0.5 day

Experiment 2: HRT of 0.4 day

Experiment 2 was performed at an HRT of 0.4 day and a constant flow rate of 5.20 mL/min of synthetic wastewater. In the same way as in experiment 1, the concentration of ammonium, nitrate, and nitrite was measured at the entry and exit point of the partial-SHARON bioreactor. The results are shown in Fig. 3.

As can be observed in Fig. 3, the transformation of ammonium into nitrite reached 60 % after 5 days of operation. After this period, the partial-SHARON bioreactor stabilized, and its capacity for the biotransformation of ammonia to nitrite remained constant. The increased nitrite concentration caused a sharp drop in the pH level of the bioreactor. To correct this, it was necessary to add small amounts of NaOH 1 % (p/v) to maintain the pH value at 7.5.

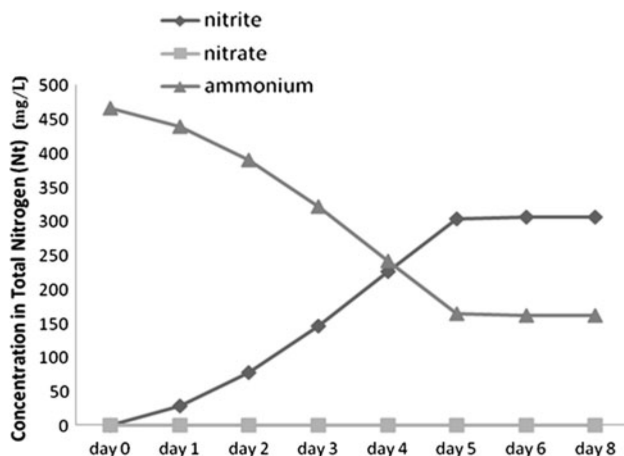

Fig. 3 Values of ammonium and nitrite expressed as total nitrogen detected in the effluent of a partial-SHARON bioreactor over time with an HRT of 0.4 day

The results obtained in the submerged-biofilter partial-SHARON system showed that working at experimental conditions of temperature (35 °C), oxygen concentration (1.5 mg/L), pH (7.5), and HRT from 0.5 to 0.4 days, an evident reduction in the biotransformation of ammonium to nitrite was observed when the HRT was decreased. When the bioreactor was operating at an HRT of 0.5 day, 100 % of the ammonium was converted to nitrites, whereas when the bioreactor was operating at an HRT of 0.4 day, only 60 % of the ammonium was converted to nitrites. However, undetectable amounts of nitrates were produced at the exit point of the partial-SHARON bioreactor. This low capacity of transformation of ammonium to nitrate in the bioreactor can be due to the operational conditions of the system that increase the biological activity of the ammonium-oxidizing bacteria and decrease the biological activity of the nitrite-oxidizing bacteria. In this sense, according to the bacterial community analysis obtained in our study (described below), the use of an HRT of 0.5 days, determined the production of highly specialized biofilms mainly integrated by *Nitrosomonas* sp., which are very effective in the oxidation of ammonium into nitrite.

According to Van Dongen et al. [8], the optimal ammonium and nitrite ratio in the effluents in partial-SHARON systems for their combination with Anammox bioreactors is 50 % ammonium and 50 % nitrite. In this context, our data suggest that in submerged-biofilter partial-SHARON systems, the ammonium–nitrite ratio can be modified by the HRT. Moreover, the results obtained in our experiments show that the submerged-filter technology applied to partial-SHARON processes increased the transformation of ammonium into nitrite and decreased the time required for the start-up of the bioreactors. This is evident when the data obtained in submerged-biofilter systems are compared with other technologies [12, 18, 19].

Study of the bacterial diversity in the partial-SHARON bioreactor

The structure of bacterial communities was analyzed by TGGE fingerprinting. The prevalent TGGE bands indicated the phylogenetic groups. The sequencing of the TGGE bands revealed that the prevalent bacteria populations were developmentally close to Proteobacteria and specifically to Alphaproteobacteria, Betaroteobacteria, Gammaproteobacteria, and Deltaproteobacteria. The bacteria populations in the partial-SHARON bioreactor varied, depending on operational conditions. Accordingly, the PCR-TGGE method showed significant differences in the structure of the bacteria community at HRTs of 0.5 and 0.4 day (see Fig. 4). The Pearson coefficient-based analysis permitted the identification of four clusters corresponding to the different treatments analyzed. On the other hand, the Dice

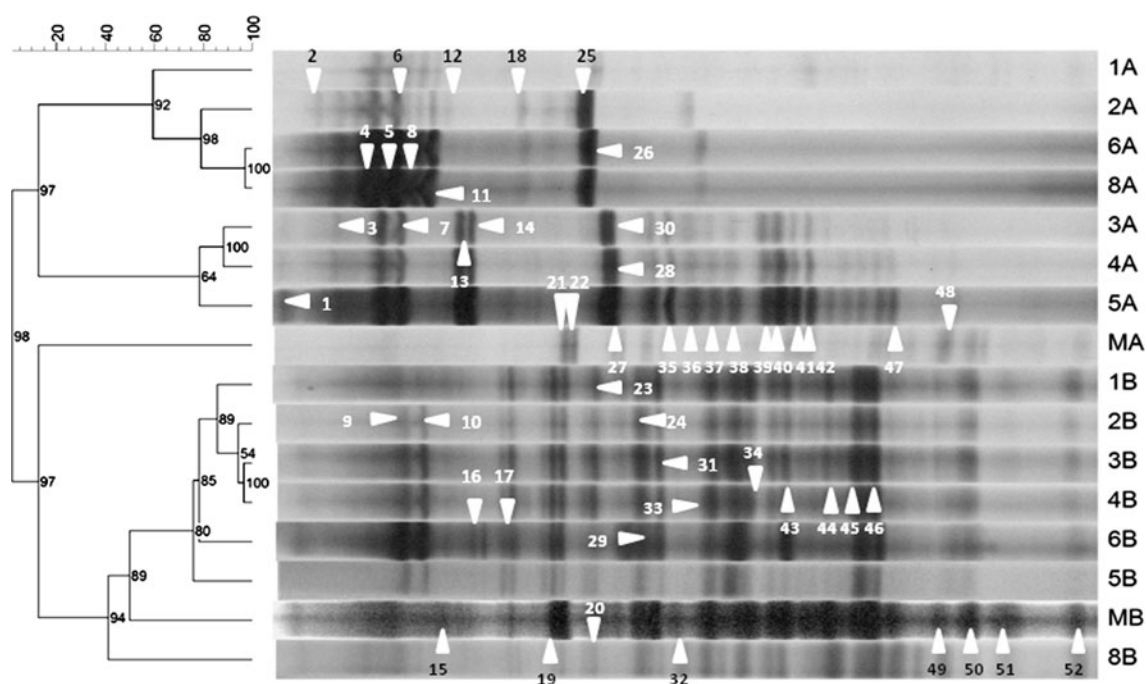

Fig. 4 Pearson coefficient-based analysis of the band patterns generated from 52 samples analyzed in the partial-SHARON bioreactor. Samples named with letter *A* corresponds to the first experiment and letter *B* corresponds to the second experiment. The *numbers* indicate the days on which samples were extracted from the bioreactor. *MA* and *MB* samples were taken at the beginning of the first and second experiment, respectively, related to zero time

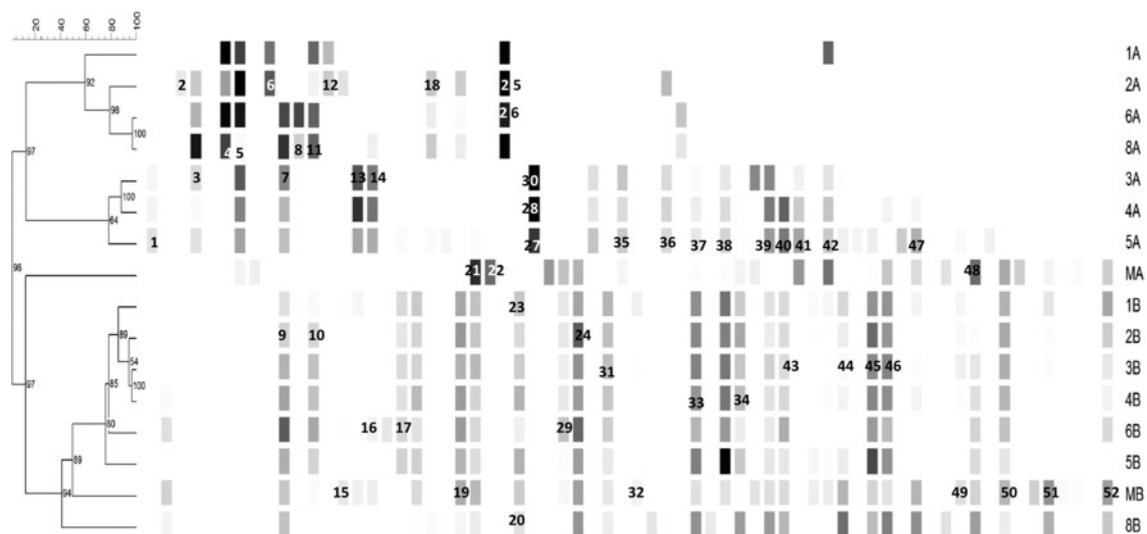

Fig. 5 Dice coefficient-based analysis of band patterns generated from all samples analyzed with a presence/absence matrix

coefficient was used to obtain 66 unique band classes in both experiments (see Fig. 5).

Study of biofilms formed at an HRT of 0.5 day

The TGGE profiles demonstrated that when the partial-SHARON system operated at an HRT of 0.5 day, a significant number of bands disappeared 48 h after its start-up (Fig. 4). For example, bands 21 and 22 related to *Pseudacidovorax* sp. and *Aquaspirillum* sp., vanished completely. However, after 2 days of operation, new bands were detected in the TGGE gels. Finally, the PCR-TGGE studies showed how the bacteria populations of the biofilms in the partial-SHARON system began to stabilize after 4 days of operation. Moreover, some bands gained in intensity over time, such as bands 4 (*Nitrosomonas*

eutropha), 14 (*Variovorax* sp.), and 27 (*Nitrosomonas europaea*).

After the stabilization of the partial-SHARON bioreactor (5 days after the pilot plant start-up), when 100 % of the ammonium was converted into nitrite, there was a significant decrease in the bacterial biodiversity of the biofilms in the submerged biofilter. Consequently, bands 38, 39, 40, 41, 42 and 47 were no longer detected in the TGGE gels (Fig. 4). In contrast, certain bands, such as 4 and 11 (*N. europaea* and *N. eutropha*), gained in intensity. In addition, a new band (band 8) appeared that was related to *Diaphorobacter* sp.

According to Khan and Hiraishi [20], and Anshuman et al. [21], *Diaphorobacter* sp. is an interesting bacteria in nitrogen removal processes. Nevertheless, to our knowledge, this is the first time that these microorganisms have been observed in a partial-SHARON bioreactor. Furthermore, our data showed that *N. europaea* and *N. eutropha*, which have a great affinity for ammonium, were prevalent over the rest of the ammonium-oxidizing bacteria [22, 23].

Samples 1A and 2A were collected from the partial-SHARON bioreactor when the concentration of ammonium was high and the concentration of nitrite very low. They showed a balanced community with low microbial diversity (Fig. 4). Similar results were obtained in samples 6A and 8A, which were taken from the bioreactor when the concentration of ammonium was very low and the concentration of nitrite was high (Fig. 4). This reflects that in extreme environments of high concentrations of ammonium or nitrite, microbial diversity decreases in the biofilms in order to preserve its functionality in changing environmental conditions.

The Pearson coefficient-based analysis (Fig. 4) allowed for the identification of two separate clusters of Bacteria, which corresponded to samples taken at two different sampling times: the start-up point and the stabilization point of the bioreactor. The sample taken at the start-up point clustered at 60 % and the one taken at the stabilization point clustered at 80 %, which indicated a good relationship between the compositions of the two bacteria cluster communities. Cluster analysis based on the Dice coefficient (Fig. 5) showed the same results as the Pearson-based clustering.

A total of 24 bands selected from the TGGE fingerprints targeting bacteria were successfully amplified and sequenced from TGGE gels (Table 3), corresponding to the dominant bacteria populations in biofilms formed in the partial-SHARON bioreactor. A prevalence of Proteobacteria in the set of sequences analyzed was found in the sampling periods. The main group of identifiable TGGE bands was related to Betaproteobacteria (59 %), whereas in order of abundance, the second group was Alphaproteobacteria (36 %) and the third group was Gammaproteobacteria (5 %) (Fig. 6). Ten TGGE bands were reamplified and sequenced from the TGGE gels corresponding to the dominant *Nitrosomonas* populations in the partial-SHARON bioreactor (Table 3). Five of these were closest to *N. eutropha* and five to *N. europaea*. Three sequences were related to *Diaphorobacter* in the TGGE gels.

In conclusion, the results of this experiment demonstrated that Proteobacteria and members of the genus *Nitrosomonas* dominated the composition of the bacteria communities of the submerged-biofilter partial-SHARON bioreactor at an HRT of 0.5 day. However, nitrite-oxidizing bacteria such as *Nitrobacter* were not detected in the TGGE gels.

Study of biofilms formed at an HRT of 0.4 day

In the second experiment, which was performed in the partial-SHARON bioreactor at an HRT of 0.4 day, the Pearson coefficient (Fig. 4) showed only one cluster of bacteria, which clustered at 80 % similarity. This result clearly indicates that there was less variation in the samples over time. Interestingly, the bands belonging to bacteria, such as *Roseobacter* sp. (Band 32) or *Burkholderia* sp. (band 50) disappeared, depending on the operating time. However, other microorganisms such as *Nitrosospira* sp. (band 10), *Nitrosomonas* sp. (band 10) and *Paracoccus* sp. (band 46) increased in abundance.

According to Hiroaki and Hiroshi [24], *Paracoccus* sp. is a common bacterium in wastewater treatment bioreactors with an important role in nitrogen removal. On the other hand, several bands had a high intensity level. This was the case of bands 31 (*Vibrio* sp.), 45 (*Rhodobacter* sp.), and 46 (*Catellibacterium* sp.), among others. The increasingly high intensity of these bands indicates that the development of these bacteria was favored by these conditions [25–27].

A total of 14 bands selected from the TGGE fingerprints targeting bacteria were successfully amplified and sequenced (Table 4). These bands corresponded to the dominant bacteria populations in the partial-SHARON bioreactor. The main group of identifiable TGGE bands was related to Proteobacteria and specifically to Alphaproteobacteria (56 %), Betaproteobacteria (40 %) and Delta-Protobacteria (4 %) (Fig. 6). Four TGGE bands were reamplified and sequenced from the TGGE gels corresponding to the dominant ammonium-oxidizing bacteria populations (*Nitrosospira*, *Nitrosomonas* and *Nitrosovibrio*) in a partial-SHARON bioreactor (Table 4). In these experiments, *Nitrosospira* sp. and *Nitrosovibrio* sp. were detected as a normal microbiota in the bioreactor working at an HRT of 0.4 day. However, these microorganisms were not identified in the TGGE gels when the bioreactor was operating at an HRT of 0.5 day. In all likelihood, when the HRT of the partial-SHARON bioreactor was reduced

Table 3 Bacteria obtained from the NCBI database from the sequencing of the bands extracted in experiment 1 (HRT 0.5 day)

No. band identification	Identities (bp)	% similarity	Experiment 1: name sequence reference	Phylogenetic class
1	97	96	HQ113216.1 *Hydrogenophaga* sp. CL-9.06	Betaproteobacteria
		96	GU300152.1 *Diaphorobacter oryzae* strain 3R2-14	Betaproteobacteria
3	85	100	GQ284427.1 *Acidovorax delafieldii* strain THWCSN39	Betaproteobacteria
		100	U51105.1 *Denitrifying* Fe < II > -oxidizing bacteria	Betaproteobacteria
4	83	100	M96402.1 *Nitrosomonas eutropha*	Betaproteobacteria
		100	HM446362.1 *Nitrosomonas europaea* strain PD60	Betaproteobacteria
5	109	100	M96402.1 *Nitrosomonas eutropha* 16S ribosomal RNA	Betaproteobacteria
		100	AY856378.1 *Nitrosomonas* sp. CNS332 16S ribosomal RNA	Betaproteobacteria
8	75	100	HQ183880.1 uncultured beta proteobacterium clone De385 16S	Betaproteobacteria
		98	GU300152.1 *Diaphorobacter* sp. 16 s ribosomal RNA	Betaproteobacteria
11	92	100	M96402.1 *Nitrosomonas eutropha* 16S ribosomal RNA	Betaproteobacteria
		100	HM446362.1 *Nitrosomonas europaea* strain PD60	Betaproteobacteria
12	83	100	HM921137.1 uncultured bacterium clone ar2e1016	Betaproteobacteria
		100	HM001269.1 *Methylophilus glucoseoxidans* strain B	Betaproteobacteria
13	91	100	HM124369.1 *Rhodobacter* sp. 16-62 16S ribosomal RNA	Alphaproteobacteria
		100	EU652478.1 *Catellibacterium* sp. JPB-2.07 16S ribosomal RNA	Alphaproteobacteria
14	107	100	HQ385754.1 *Variovorax* sp. 2C1-21 16S	Betaproteobacteria
		100	EF203908.1 *Variovorax paradoxus* isolate DB1	Betaproteobacteria
21	98	100	HQ259687.1 *Pseudacidovorax* sp. A14(2010)	Betaproteobacteria
		100	AF384190.1 *Aquaspirillum* sp. TG27	Betaproteobacteria
22	98	100	HQ259687.1 *Pseudacidovorax* sp. A14(2010)	Betaproteobacteria
		100	AF384190.1 *Aquaspirillum* sp. TG27	Betaproteobacteria
25	125	97	AJ245760 uncultured beta proteobacterium partial 16S rRNA	Betaproteobacteria
	124	97	JN217090 uncultured bacterium clone S252 16S ribosomal RNA	Betaproteobacteria
26	113	100	GU980069.1 uncultured bacterium clone HKTJ485	Betaproteobacteria
		100	EU542425.2 uncultured bacterium clone Er-MS-1	Betaproteobacteria
27	83	100	M96402.1 *Nitrosomonas eutropha* 16S ribosomal RNA	Betaproteobacteria
		100	HM446362.1 *Nitrosomonas europaea* strain PD60	Betaproteobacteria
28	97	100	HQ183880.1 uncultured beta proteobacterium clone De385	Betaproteobacteria
	96	98	GU300152.1 *Diaphorobacter oryzae* strain 3R2-14	Betaproteobacteria
30	96	100	M96402.1 *Nitrosomonas eutropha* 16S ribosomal RNA	Betaproteobacteria
		100	HM446362.1 *Nitrosomonas europaea* strain PD60	Betaproteobacteria
35	92	100	EU445263.1 *Agrobacterium tumefaciens* isolate EFLRI 121	Alphaproteobacteria
		100	AJ784210.1 *Rhizobium* sp. P033 partial 16S rRNA gene	Alphaproteobacteria
37	98	100	GU574708.1 *Parvibaculum* sp. EPR92	Alphaproteobacteria
		100	FJ528267.1 *Rhizobium* sp. Cs218	Alphaproteobacteria
38	95	95	GQ351376.1 uncultured bacterium isolate DGGE gel band	Alphaproteobacteria
39	75	100	X87274.1 *B.diminuta* 16S rRNA gene	Alphaproteobacteria
		100	U63935.1 *Caulobacter* sp. 16S ribosomal RNA gene	Alphaproteobacteria
40	76	100	GU949635.1 uncultured bacterium clone 4EU1038B12	Alphaproteobacteria
	73	100	EU256442.1 *Mesorhizobium mediterraneum* strain CCBAU	Alphaproteobacteria
41	84	100	GU420646.1 *Defluvibacter lusatiensis* clone AW171	Alphaproteobacteria
		100	GU415542.1 *Ochrobactrum anthropi* clone AW034	Alphaproteobacteria
42	99	100	EU635967.1 uncultured bacterium isolate DGGE band 13	Alphaproteobacteria
	98	98	FJ587218.1 *Pseudoxanthobacter* sp.	Alphaproteobacteria
47	90	100	HM629504.1 *Escherichia coli* strain BAB-286	Gammaproteobacteria
	89	98	HM629493.1 *Salmonella enterica* strain	Gammaproteobacteria

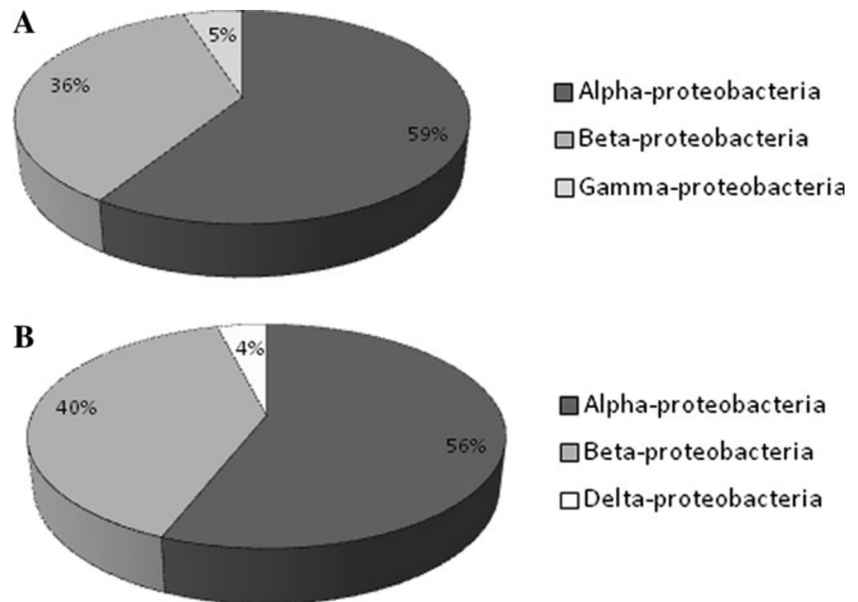

Fig. 6 Phylogenetic classes (%) detected in the partial-SHARON bioreactor at HRT of 0.5 day (**a**) and HRT of 0.4 day (**b**) analyzed by PCR-TGGE method

Table 4 Bacteria obtained from the NCBI database from the sequencing of the bands extracted in experiment 2 (HRT 0.4 day)

No. band identification	Identities (bp)	% similarity	Experiment 2: name sequence reference	Phylogenetic class
9	78	98	X84662.1 *Nitrosospira* sp. 16S rRNA gene	Betaproteobacteria
		98	M96405.1 *Nitrosovibrio tenuis* 16S ribosomal RNA	Betaproteobacteria
10	95	100	M96402.1 *Nitrosomonas eutropha* 16S ribosomal RNA	Betaproteobacteria
		100	HM446362.1 *Nitrosomonas europaea* strain PD60	Betaproteobacteria
15	99	100	FJ222605.1 *Albidovulum* sp. S1K1	Alphaproteobacteria
		100	HM705035.1 uncultured bacterium clone GB7N87002DSSDY	Alphaproteobacteria
16	65	100	GQ853528.1 uncultured *Ochrobactrum* sp.	Alphaproteobacteria
17	74	100	EF195167.1 *Alcaligenes* sp. RG-03/06 16S	Betaproteobacteria
24	70	100	HM001269.1 *Methylophilus glucoseoxidans* strain B	Betaproteobacteria
		100	GQ411499.1 *Methylophilus methylotrophus* strain NBCS15	Betaproteobacteria
31	98	100	EF079668.1 *Thiobacillus* sp. K6.2	Betaproteobacteria
	97	98	FM957479.1 *Vibrio* sp. MY-2008-U67	Betaproteobacteria
32	78	100	AM710422.1 uncultured bacterium partial 16S rRNA gene	Alphaproteobacteria
	79	98	AY576768.1 *Roseobacter* sp. 3X/A02/234	Alphaproteobacteria
33	87	95	AM922185.1 *Sphingopyxis* sp. Sulf-541	Alphaproteobacteria
34	94	98	HM687288.1 uncultured bacterium clone GB7N87001BDHL1	Alphaproteobacteria
	93	97	HQ596322.1 *Bradyrhizobium* sp. CNX333	Alphaproteobacteria
45	82	100	100 % HM124369.1 *Rhodobacter* sp. 16-62	Alphaproteobacteria
		100	100 % EU652478.1 *Catellibacterium* sp. JPB-2.07	Alphaproteobacteria
46	91	100	100 % HM124369.1 *Rhodobacter* sp. 16-62 16S ribosomal RNA gene	Alphaproteobacteria
52	52	100	100 % GQ183899.1 *Geothermobacter* sp.	Deltaproteobacteria

from 0.5 to 0.4 day, this caused the biodiversity of this specialized microbial group to increase.

In conclusion, the results of this experiment demonstrated that Proteobacteria and members of the genus *Nitrosomonas*, *Nitrosospira*, and *Nitrovibrio* dominated the composition of the bacterial community of the submerged-biofilter partial-SHARON bioreactor at an HRT of 0.4 day. However, as previously mentioned, nitrite-oxidizing bacteria such as *Nitrobacter* were not detected in the TGGE gels.

Comparison of the bacterial diversity obtained in experiment 1 (HRT of 0.5 day) and experiment 2 (HRT of 0.4 day)

A comparison of the results of the two experiments seems to indicate that the modification of the HRT affected the bacterial diversity of the biofilms formed in a partial-SHARON system with a submerged filter. In the experiment performed at an HRT of 0.5 day, bacterial diversity was significantly reduced when the bioreactor operated in stable conditions (after 5 days). In fact, fewer than 10 bands were observed, probably as a consequence of the high level of specific ammonium-oxidizing bacteria. In these conditions, 100 % ammonium was converted into nitrite, and thus all the microbial population was obliged to compete for ammonium. However, in experiment 2, in which the HRT of the bioreactor was adjusted to 0.4 day, the microbial biodiversity was extremely heterogeneous. This result could explain the evenness observed in the bacterial community. Since 65.5 % of the ammonium was converted to nitrite, this led to a less specialized bacterial community (see Figs. 4, 5). These data are in consonance with the results reported in Logemann et al. [28], and Marzorati et al. [16].

Table 5 Average range-weighted richness (R_r) and functional organization (F_o) indices of the bacterial communities in partial-SHARON bioreactor samples from the two experiments in this study

Experiment	No. of samples	R_r	F_o	Statistic program
1	8	2*	49	ANOVA analysis
2	8	5*	43	ANOVA analysis

* Statistically significant difference (Student's t test, $p < 0.05$)

The comparison of the results obtained in the two experiments highlighted the similarity of different bands. This indicated the presence of certain microorganisms, such as *Nitrosomonas* sp., which were constant in both experiments (see Tables 3, 4). This fact is hardly surprising since this microbial group can be regarded as predominant in an extreme environment with a high dilution rate, a temperature of 36 °C, and a high ammonia concentration [29]. These results suggest the significance of *Nitrosomonas* sp. in the biofilm formed in the submerged-filter partial-SHARON system and its important role in the biotransformation of ammonium into nitrite in this wastewater treatment biotechnology [22, 23].

Image analysis with Gel Compar II detected a total of 66 unique band classes in the TGGE fingerprints of bacteria among the 52 bands detected (Fig. 5). A total of 38 bands selected from the TGGE fingerprints targeting bacteria were successfully amplified and sequenced, representing the 73 % of the bands chosen for sequencing (Tables 3, 4).

The richness range-weighted (R_r) indices [14] showed significant differences with ANOVA analysis ($p < 0.05$) in both experiments (Table 5). The R_r of experiment 2 (HRT of 0.4 day) displayed higher average values than those of experiment 1 (HRT of 0.5 day). Construction of the Pareto-Lorenz curves of the bacterial community profiles in

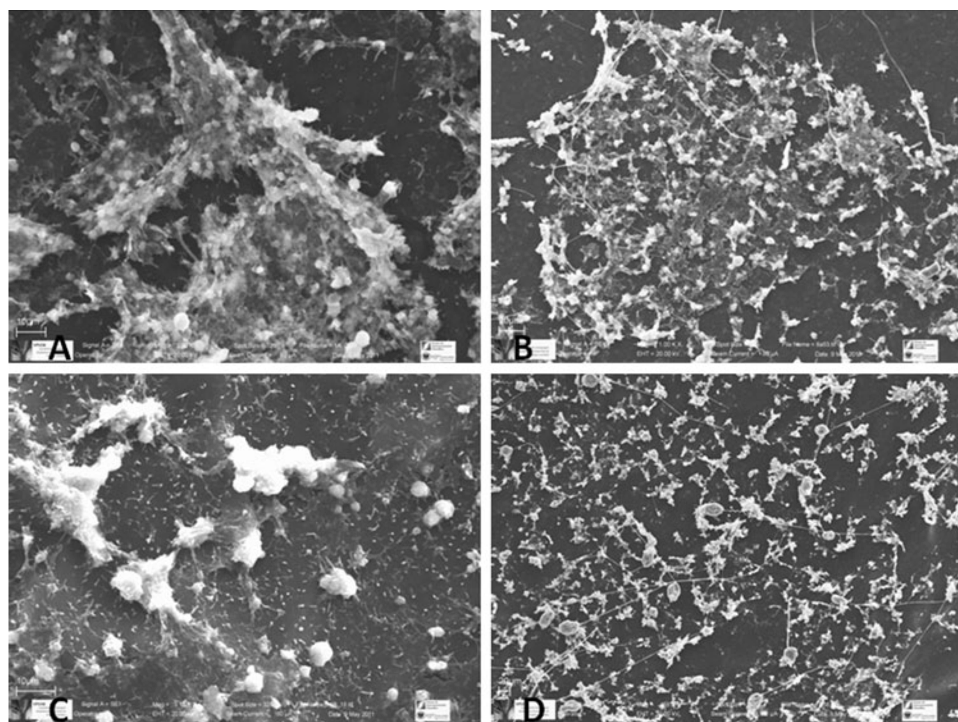

Fig. 7 SEM of the carriers used in a submerged-filter partial-SHARON bioreactor in different conditions. **a** Microscopic images of day 1 with an HRT of 0.5 (sample 1A); **b** microscopic images of day 8 with an HRT of 0.5 (sample 8A); **c** microscopic images of day 1 with an HRT of 0.4 (sample 1B); **d** microscopic images of day 8 with an HRT of 0.4 (sample 8B)

experiment 2 permitted the calculation of the functional organization (F_o) indices [14].

F_o indices of 49 and 43 % were obtained in the experiments 1 and 2, respectively. According to Marzorati et al. [16], F_o index values of around 45 % represent a balanced community, potentially able to preserve its functionality under changing environmental conditions. Obviously, these results reflect the higher specialization of the bacterial community in the submerged-filter partial-SHARON bioreactor and its capacity to adapt to different working conditions.

Scanning electronic microscopy

The colonization of the plastic carrier, used in the construction of the submerged-filter partial-SHARON bioreactor with an HRT of 0.5 and 0.4 day, was studied after 1 and 8 days (see Fig. 7). The results showed a rapid colonization of the carrier with the formation of a complex and heterogeneous biofilm. At the end of both experiments, the samples were found to contain a large number of different morphological types as well as filamentous bacteria. These results coincide with the data previously reported in this paper, which suggest the presence of complex microbiota in both experiments, independently of the HRT used in the wastewater system.

Conclusions

The results obtained in our study within the context of recent research in the field lead to the conclusion that the HRT affects the functioning of a partial-SHARON bioreactor built with submerged-filter technology. This is reflected in different levels of biotransformation of ammonium into nitrite. This signifies that the application of submerged-filter technology to the partial-SHARON system increases the biotransformation of ammonium into nitrite, in comparison to other technologies such as a fluidized bed. Moreover, our results show that the HRT affects the microbial diversity of biofilms formed in a partial-SHARON bioreactor, possibly as a result of the different nutritional conditions that arise when this variable is modified.

On the other hand, the use of an HRT of 0.5 day determines the formation of highly specialized biofilms (mainly by *Nitrosomonas* sp.), which are effective in the biotransformation of ammonium into nitrite. On the contrary, the use of an HRT of 0.4 day, determines the formation of more heterogeneous biofilms that allow a closer ammonium/nitrite ratio, which is more effective for the combined development of partial-SHARON/Anammox systems.

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