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PCR-DGGE Based Biodiveristy, Changeability and Genetic Similarity Analysis of Bacterial Community in Sequencing Batch Reactors Dealing with Reject Water

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Reject water, produced during stabilization and dewatering of activated sludge, contains a high load of biogenic compounds which are returned to bioreactors, so there is a necessity for its treatment within biological systems. The efficacy of such performance depends mainly on the activated sludge composition responsible for the biochemical processes. For biocenosis diversity and changeability, PCR-DGGE is the most commonly used tool. In this article we monitored the activated sludge bacterial communities of two sequencing batch reactors (SBRs) dealing with reject water but varying in their operational parameters. A physico-chemical analysis of the SBRs performance was also done. We used two different PCR primers sets to present a total overview of the changeability in complex microbial biocenosis during wastewater treatment. For biodiversity monitoring 16S rRNA gene fragments amplified with 338f-GC/518r primers appeared to be more suitable than the fragments amplified with 968f-GC/1401r – the index was higher though the changes were proportional for both cases. The results were confirmed with genetic similarity analysis presented as dendrograms.

Key words: PCR-DGGE, SBR, bacterial community monitoring, reject water.

1. Introduction

Biological wastewater treatment is one of the most important biotechnological processes in environmental protection. It is less harmful to the environment than chemical treatment because it is based on natural microbial activity in an intensified form, performed in activated sludge containing *Bacteria*, *Metazoa* and *Protozoa*. Nowadays, European Union Regulations require high quality effluent obtainment in wastewater treatment processes, underlining the need for efficient nitrogen removal. Several new biotechnologies were implemented to meet these regulations, but biological treatment also causes the increase of sludge volume and, as a consequence, the necessity for its stabilization and dewatering. Reject water, produced during the processes mentioned above, still possess a high load of biogenic compounds returned to bioreactors and they contain even 30%

of the total N and P load [12]. Such wastewater needs to be treated within biological systems, but the efficacy of such performance depends mainly on the activated sludge composition responsible for the biochemical processes. For a long time knowledge about microorganisms was based on research performed using culture-dependent methods. Recently, due to development in molecular tools, faster and more precise bacterial composition and changeability monitoring of the activated sludge has become possible. For biocenosis diversity and changeability analysis, PCR-DGGE (Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis) is the most commonly used tool. This method requires PCR amplification using a particular sort of primers with a GC clamp (a 30–40 GC nucleotides fragment with a high DNA melting temperature enabling the molecular resolution in the gel). In the case of environmental samples, the total bacterial DNA is used for DGGE purposes, so the PCR product contains DNA fragments of all bacteria, of the same in size, but different in sequence. For these analyses a universal molecular bacterial marker – 16S rRNA gene can be used. This gene possesses several domains of different properties, from which the V3 region is the most variable, thus the most often used for biodiversity analysis [1, 4, 7]. But this gene also possesses a less variable part of the gene (V6–V8) which is usually used for bacterial identification. The usage of both parts in the bacterial monitoring enables a precise and complete picture of the bacterial community in the system to be made.

In this article we monitor two activated sludge biocenoses from sequencing batch reactors (SBRs). The systems, both dealing with reject water, vary in their operational parameters. The monitoring was performed using two sets of PCR primers for the 16S rRNA gene, generating a product differing in size and from various gene regions. A physico-chemical analysis of the SBRs wastewater treatment efficacy was also performed. The main aim of the experiment was to present the differences in total bacterial community structure and the similarity at the genetic level. The biodiversity analysis was performed with two different PCR primer sets from the same material and molecular marker. This procedure was used to prove the necessity of such an experimental attitude to present the total complexity of microbial changeability in bacterial communities during wastewater treatment.

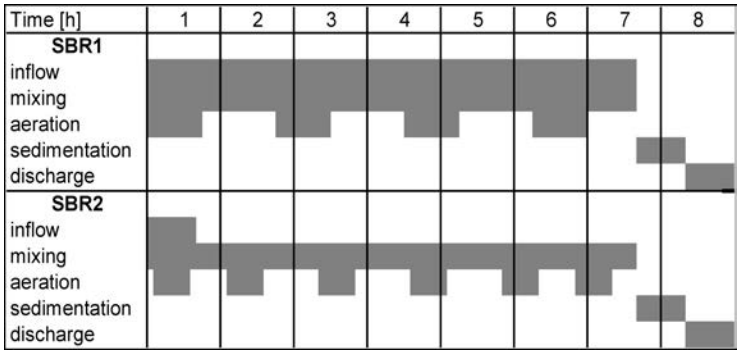
2. Materials and methods

2.1. Operational data and physico-chemical analysis

Two SBRs, each a volume of 10 L, were seeded with activated sludge from the municipal wastewater treatment plant in Gliwice, Poland. They were working in a configuration for 3 cycles/day under a temperature of $19.2 \pm 2^{\circ}$C. The feeding medium was reject water from the same Gliwice plant. SBR1 was fed

with medium constantly for 6 hours 40 minutes, SBR2 partially for 40 minutes. The operational scheme is presented on Table 1. The inflow to each reactor (2 ± 0.2 L/cycle) was provided by peristaltic pumps ISMATEC REGLODigitalMS (responsible for the inflow of wastewater and outflow of treated sewage). Additionally, the system was equipped with aeration and mixing devices and was controlled by electronic and analog timers.

Table 1. SBRs performance scheme.



The standard physico-chemical parameters such as: ammonia, nitrite, nitrate nitrogen concentration, total suspended solids (TSS), volatile suspended solids (VSS), alkalinity, phosphates, chemical oxygen demand (COD), temperature, pH were measured once a week. NH_4^+-N, NO_2^--N, NO_3^--N as well as COD, phosphates and alkalinity were measured according to MERCK methodology on spectrophotometers Spectroquant NOVA320 and Spectroquant NOVA620. For physical parameters (pH and temperature), the pH-meter WTW 340i with electrode was used. TSS and VSS were measured according to Standard Method APHA (1998).

2.2. Activated sludge sampling, DNA isolation and PCR conditions

Activated sludge samples (volume of 10 mL) were collected from both SBRs, pelleted by centrifugation (5 000 × g, 10 min, 4°C) and stored at −45°C until DNA isolation. Total genomic DNA was extracted from 0.2 g of the activated sludge samples using the mechanical method. The samples were washed three times with 1 × PBS buffer (Sigma) and disintegrated with bead beating (Roth, Germany) in lysis buffer (Tris-HCl 100 mM, EDTA 100 mM, NaCl 1,5 M; pH = 8.0). The samples were incubated 20 minutes with shaking at 1400 rpm, using a thermomixer (Eppendorf) and after this period of time 200 µL 10% SDS was added. After 30 minutes of incubation at 65°C, the samples were centrifuged twice at 13 000 rpm and placed on spin filters (A&A Biotechnology). DNA attached to the filter was washed twice with 70% ethanol solution (A&A

Biotechnology). The amount of DNA was measured spectrophotometrically using Qubit (Invitrogen) and stored at −20°C until PCR amplification.

Partial 16S rRNA gene amplification of all the bacteria was performed using primers: 338f-GC and 518r gene fragment [10]. The other part of the same gene for bacteria was amplified with primers: 968f-GC and 1401r [5, 11]. The primer sequences are presented in Table 2. The PCR reaction was performed in a 30 μL mixture with 1.5 U GoTAQ flexi Polymerase (Promega), 1× buffer, 2 mM $MgCl_2$, 5 pmol/μL of each primers and 20 pmol/μL of dNTPs. The total bacterial DNA from the activated sludge samples was used as DNA template in a concentration of 0.2 μg/μL. The amplification was performed in termocycler T-1000 (Bio-Rad) according to the scheme presented in Table 3. The PCR products were separated in 0.8% (w/v) agarose with ethidium bromide (10 μL/mL) in 1 × TBE buffer and visualized under UV light.

Table 2. PCR primers and PCR programs used in the study.

Primer	Sequence 5'-3'	Bibliography
968f-GC*	AACGCGAAGAACCTTAC	5, 11
1401r	CGGTGTGTACAAGGCCC	
338f-GC*	CCTACGGGAGGCAGCAG	10
518r	ATTACCGCGGCTGCTG	

*CG clamp: 5' CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCGCC 3'

Table 3. PCR programs used in the study.

Cycle steps	Primers			
	338f-GC/518r		968f-GC/ 1401r	
	Conditions			
	temp. [°C]	time [min]	temp. [°C]	time [min]
predenaturation	95	10	94	5
denaturation	95	1	9	1
annealing	53	1	53	1
elongation	72	2	72	1
final elongation	72	12	72	10
cycles	×30		×30	

2.3. Denaturing gradient gel electrophoresis: conditions and results analysis

The PCR products obtained in reactions with 338F-GC/518R and 968f-GC/1401r primers underwent electrophoretic separation in the Dcode Universal Mutation Detection System (BioRad). Polyacrylamide gel (8% for both 16S rRNA gene PCR products, 37:1 acrylamide-bisacrylamide, Fluka) with a gra-

dient of 30–60% denaturant was prepared according to the manufacturer's instruction. The gel was run for 10 h at 70 V and 15 h at 65 V for 180 bp and 465 bp fragments, respectively, in a 1× TAE buffer at a constant temperature of 60°C. The gel was stained with SYBR Gold (1:10 000, Invitrogen) in MiliQ water for 30 min and distained in MiliQ water for 40 min, then visualized under UV light and photographed using Quantity One 1D (BioRad).

The analysis of DGGE fingerprints was performed using Quantity One 1D software (BioRad). Bacterial biodiversity was estimated on the basis of densitometric measurements and the Shannon diversity index, as previously described [14].

3. Results

The two SBRs operated in parallel with reject water derived from sludge dewatering in the municipal wastewater treatment plant in Gliwice, Poland.

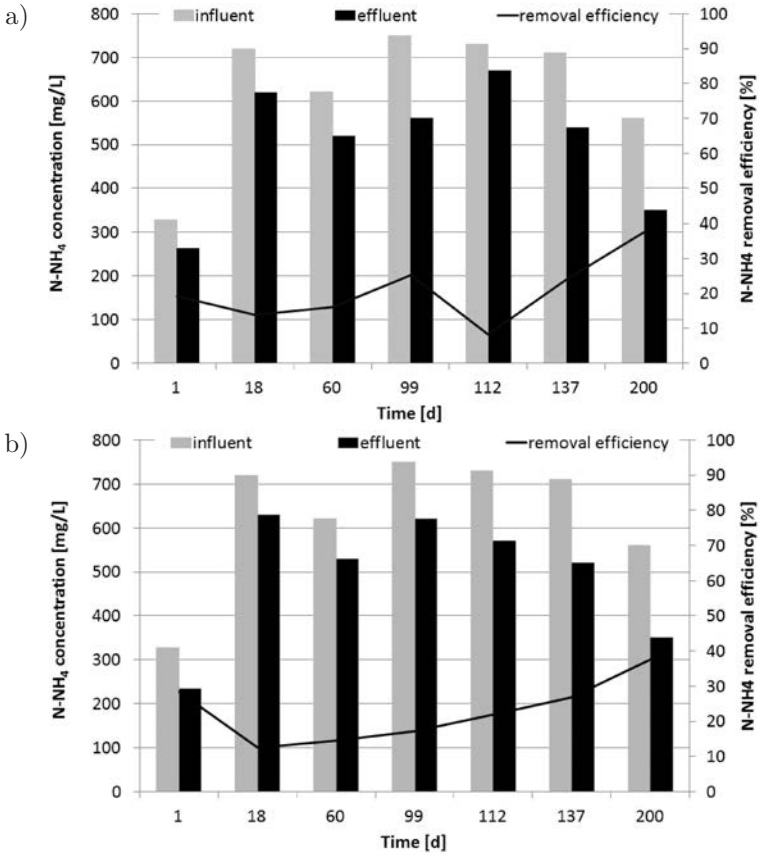


Fig. 1. The nitrogen concentration in the influent and its removal efficiency: a) SBR1, b) SBR2.

The analysis of nitrogen compound concentration (ammonia, nitrite and nitrate) was performed. The nitrogen concentration in the influent and its removal efficiency is presented in Fig. 1. Figure 2 presents the nitrogen compound changes during the experiment in both SBRs.

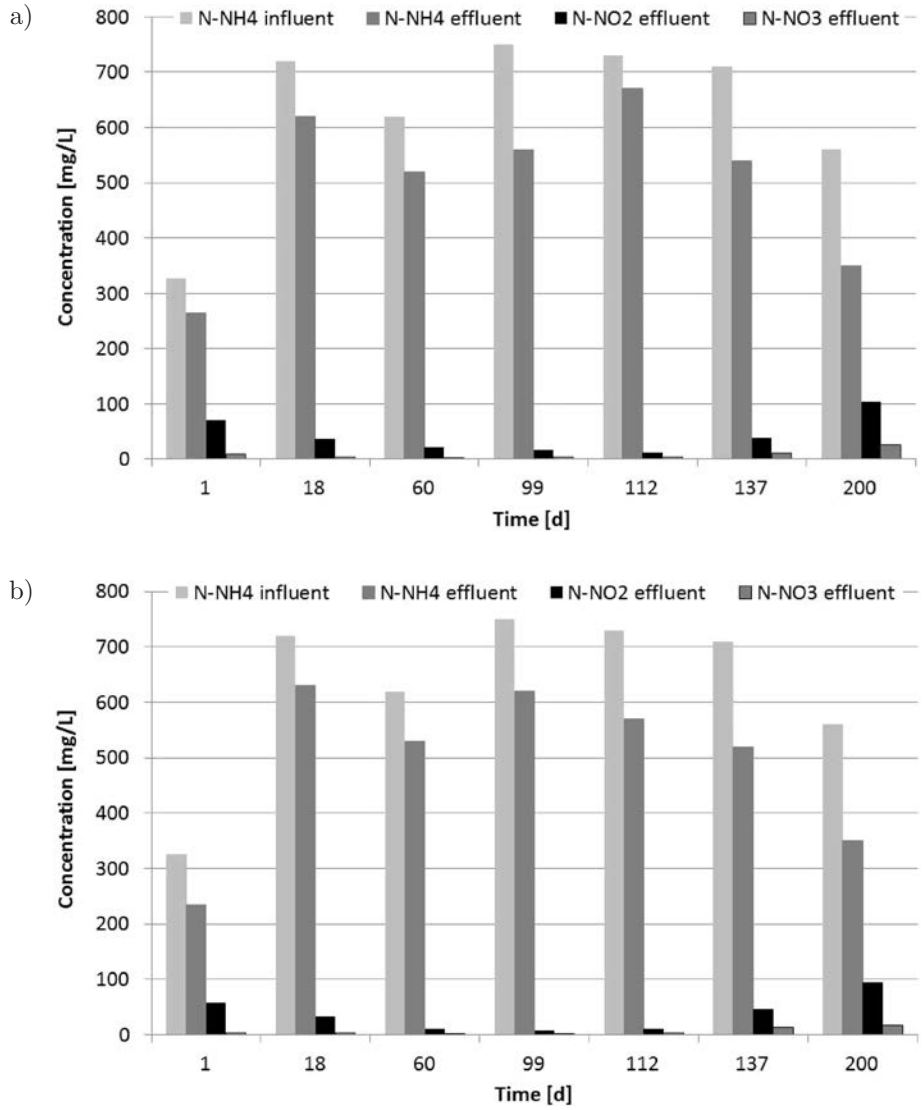


FIG. 2. Nitrogen compound changes during the experiment; a) in SBR1, b) in SBR2.

On the basis of total bacterial DNA obtained from the samples PCR reaction was performed. Two different PCR primers were used: 338f-GC and 518r and 968f-GC and 1401r generated products 180 bp and 465 bp in size, respectively.

DGGE separation of these products is presented in Fig. 3. On the basis of the DGGE fingerprints, densitometric analysis with Quantity One 1D Software (BioRad) was performed. These data were used for the Shannon biodiversity index calculation (Fig. 4) and dendrogram preparation using Neighbor Joining algorithm (Fig. 5).

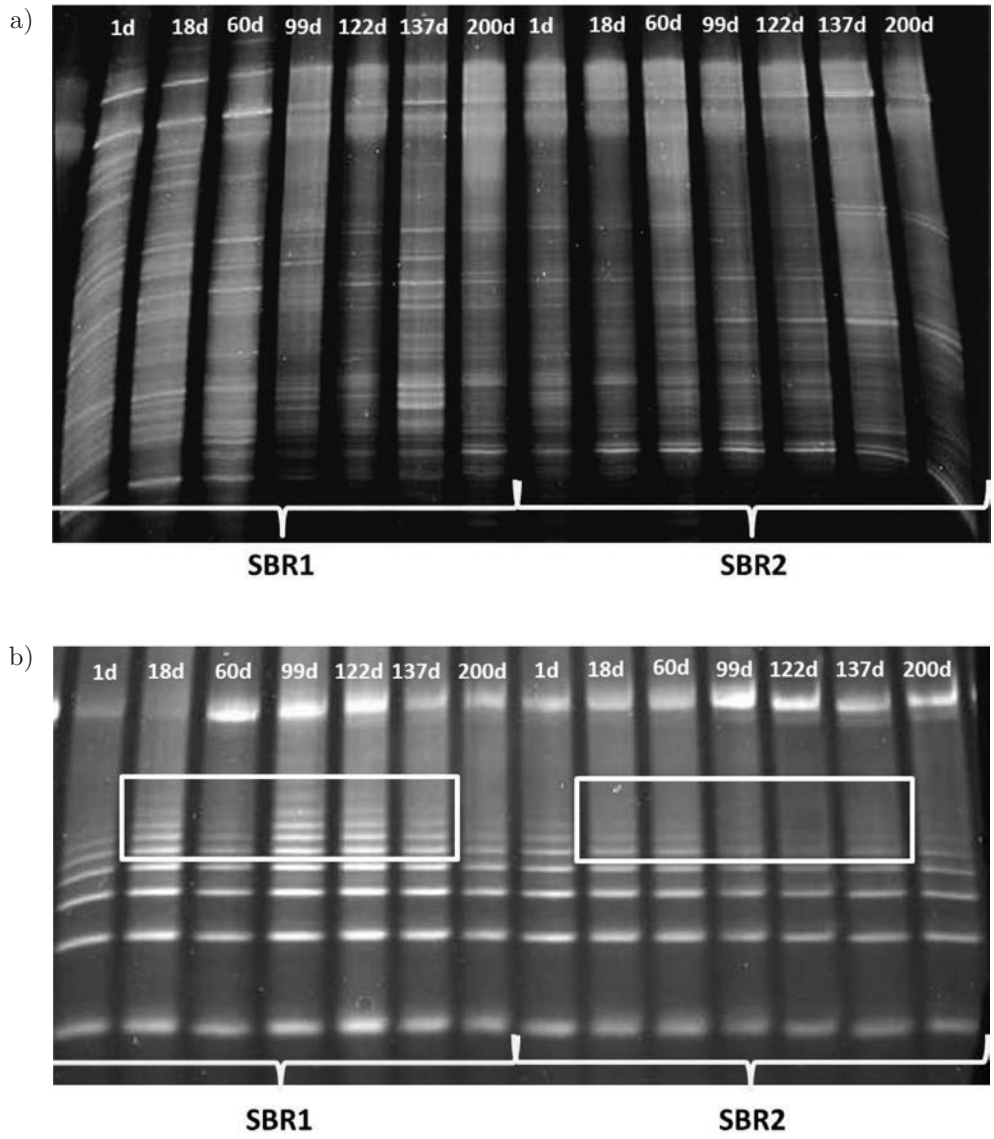
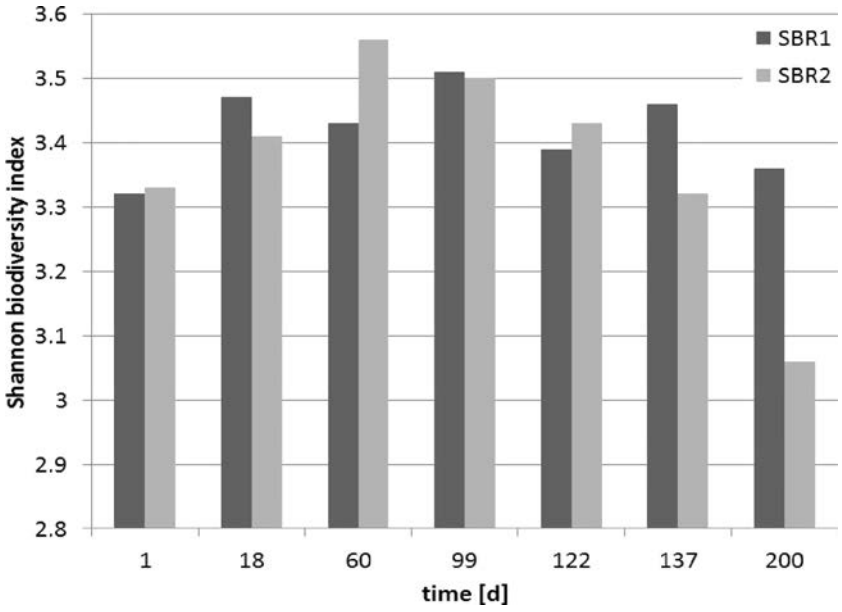


FIG. 3. DGGE fingerprint pattern obtained with 16S rRNA gene product; a) PCR product size of 180 bp, b) PCR product size of 465 bp product size (more diverse part of the fingerprints in frame).

a)



b)

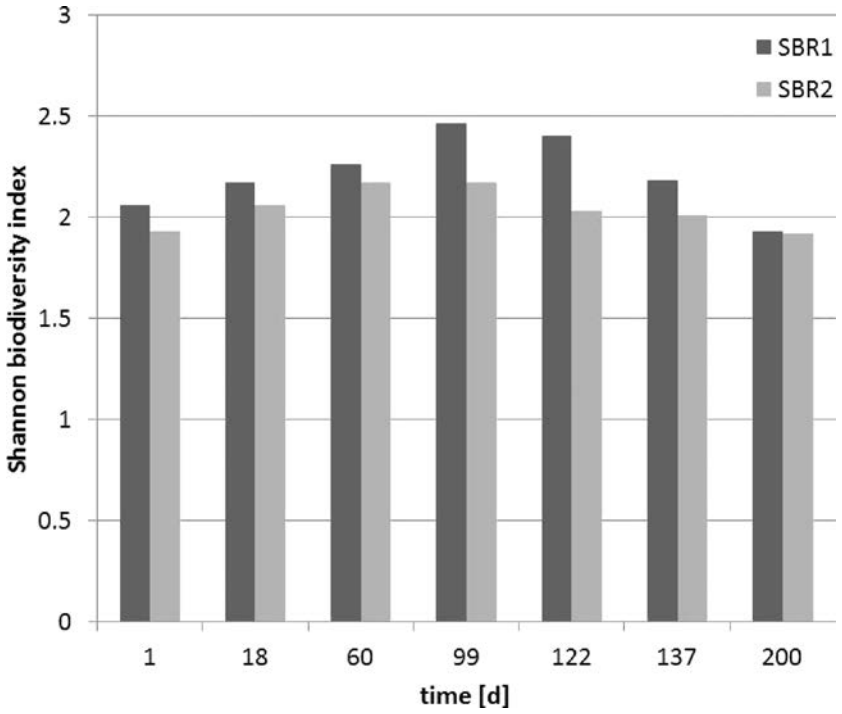


FIG. 4. Shannon biodiversity index calculated on the basis of DGGE fingerprints, a) for PCR product size of 180 bp, b) for PCR product size of 465 bp.

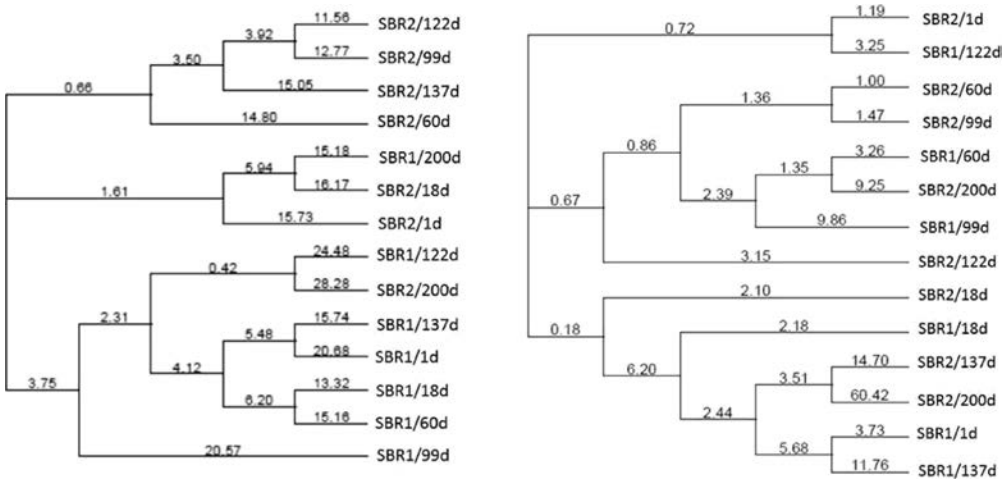


FIG. 5. Dendrograms presenting the genetic distance of the analyzed samples created on the basis of the Neighbor Joining algorithm, Dice coefficient above the branches; a) for PCR product size of 180 bp; b) for PCR product size of 465 bp.

4. Discussion

Two SBRs were treating reject water over a period of 200 days. Both bioreactors were monitored technologically with physico-chemical analysis and microbiologically using PCR-DGGE. Nitrogen concentration in the influent to the partial nitritation/Anammox reactor varied from 327 to 930 mg/L with the average value 666.4 ± 130.3 mg/L. Ammonia nitrogen removal efficacy during the total length of the experiment did not exceeded 45%. The maximum observed nitrogen removal efficiency was equal to 37.5% and 41.8% in SBR1 and SBR2, respectively (Fig. 1). In both reactors the effectiveness of the substance removal fluctuated over time. The highest ammonia removal efficiency was noted during the first 60 days of experiment and then there was a removal efficiency decrease from ca. 30% to 10% on average in both reactors. At the end of experiment a gradual increase in ammonia nitrogen removal efficiency was observed. Also inorganic nitrogen removal efficiency was low and not exceeding 30% and 35% in SBR1 and SBR2, respectively. As a consequence, the ammonia nitrogen was the main form of nitrogen in the effluent from the reactors. The other form was nitrite nitrogen. However, its concentration was very low and slightly exceeded 100 mg/L for SBR1 (Fig. 2). Generally, nitrite nitrogen is a potential inhibitor for the Anammox process and inhibition studies have shown that anammox bacteria are irreversibly inhibited by high nitrite concentrations (more than 100 g N mg/L) [13], thus its concentration in the reactor was kept at a low level, below 100 mg/L. Nevertheless, in the literature there is no uniformity about

the threshold values of nitrite inhibition. It has been reported [3] that nitrite concentrations as high as 350 g mg/L cause only a 50% inhibition. Also, the research of [9] showed a concentration of nitrite equal to 400 mg/L responsible for a 50% process inhibition. Moreover, they have demonstrated that nitrite inhibition can be reversible. It seems that the problem of process inhibition may be in free ammonia (FA) concentration rather than the nitrite concentration. Initially, it was reported that the Anammox process is not inhibited by ammonia nitrogen up to 1000 mg/L [13]. In [3] a 50% Anammox bacteria activity decrease at an ammonia concentration equal to 770 mg/L was observed. However, further studies have indicated that FA rather than ammonia nitrogen is the real inhibitor of the process [2, 8]. In short term tests, the IC_{50} of the free ammonia was 38 mg/L [6] and in a continuous operation, the Anammox process was unstable when the FA concentration exceeded 20–25 mg/L with even worse results, and the removal efficiency finally decreasing to zero when the FA concentration was more than 35–50 mg/L. In the presented study, the FA concentration was equal to 44.4±17.4 and 45.8±15.0 mg/L in SBR1 and SBR2, respectively. The idea was to maintain a high free ammonia concentration to inhibit aerobic nitrite oxidizers which compete with the Anammox bacteria for substrate (nitrite). However, it seems that at the same time such a high free ammonia concentration completely inhibits Anammox bacteria.

For the PCR-DGGE monitoring of bacterial biocenosis we used 16S rRNA gene amplicons differing in size and amplified from two distinctively variable molecular marker parts in order to present a total picture of the activated sludge bacterial community complexity during reject water treatment processes. As it has already been presented in the literature [1], PCR primers 338f/518r (180 bp fragment of the 16S rRNA gene of all bacteria) and 968f/1401r (465 bp fragment of the 16S rRNA gene of all bacteria), are successfully used for DNA amplification and DGGE resolution in order to present complete insight into bacterial community structure. The 180 bp fragment enables biodiversity monitoring due to the fact that this part of the gene is more variable and the fingerprints for such small amplicons are clearer and sharper. The larger fragment enables bacterial identification (separate DNA bands can be excised from the gel and undergo sequencing) but the biodiversity monitored with these primers is usually slightly lower [4, 15]. Out results are coherent with these studies [4, 15], the biodiversity index is higher for 180 bp PCR products (Fig. 4a) than for the 465 bp ones (Fig. 4b). DGGE resolution of 180 bp fragments gives more complex and variable fingerprints (Fig. 3a) while the 465 bp amplicons presents lower changeability and the fingerprints vary mainly in DNA bands brightness (Fig. 3b). However, for 465 bp fragment in case of samples from day 18 to 122 of the experiment, in both SBRs community seems to present very low changeability (Fig. 3b, frames). It has a reflection in the Shannon biodiversity index

(Fig. 4b) from day 18 to 122 of the experiment the biodiversity increased to the value of ca. 2.4 for SBR1 and to 2.2 for SBR2. Despite the biodiversity level difference for both sorts of primers, the changeability of the communities seems to be proportional in both cases - the higher biodiversity is observed between day 18 and 122 of the experiment. This period covers the time of the experiment when the highest ammonia concentration was present in the feeding medium (Figs. 1 and 2). It could be suspected that this parameter activated a large group of bacteria resistant and/or removing a high concentration of ammonia. It should be mentioned that SBR1, whose bacterial community was more variable and presented less stable effectiveness of ammonia removal than SBR2 (Fig. 1a). It could thus be suspected that in the case of this study, the higher biodiversity and changeability in the bacterial community could be one of the reasons for efficacy fluctuations. It seems probable that most of the bacteria in the community were not able to perform ammonia removal effectively, thus the bacterial changeability was higher with no visible dominant genotypes. It should be also underlined that the stable bacterial community in SBR2, whose composition is similar in the beginning and in the end of the experiment, covers the time with a comparable level of ammonia removal effectiveness (Fig. 1b).

Genetic distance analysis (Fig. 5) performed on both DGGE gels using a Neighbor Joining algorithm revealed that dendrogram constructed on the basis of 180 bp PBR fragments is more coherent (Fig. 5a) than those constructed on 465 bp PCR fragments (Fig. 5b). In the first case (Fig. 5a) SBR1 and SBR2 are clustered together and they seem to be more closely related than in the dendrogram for the larger fragments in which every clad consists of a SBR1 and SBR2 samples mixture with no clear grouping tendency.

5. Conclusion

This experiment revealed that for biodiversity monitoring with PCR-DGGE on 16S rRNA gene, fragments amplified with 338f-GC/518r primers are more suitable than these amplified with 968f-GC/1401r. The biodiversity index calculated for both DGGE gels differ and is higher for 180 bp PCR fragments. However, it should be underlined that the diversity changes are proportional for both gels. Higher concentration of ammonia in the feeding medium could be the reason for the biodiversity increase, but the more stable efficacy of ammonia removal was performed in SBR2, where this biodiversity was lower.

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