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Molecular Analysis of Temporal Changes of a Bacterial Community Structure in Activated Sludge Using Denaturing Gradient Gel Electrophoresis (DGGE) and Fluorescent *in situ* Hybridization (FISH)

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Abstract

Wastewater treatment based on activated sludge is known to be one of the most effective and popular wastewater purification methods. An estimation of microbial community variability in activated sludge allows us to observe the correlation between a particular bacterial group's appearance and the effectiveness of the removal of chemical substances. This research is focused on microbial community temporal changes in membrane bioreactors treating wastes containing a high level of ammonia nitrogen. Samples for this study were collected from two membrane bioreactors with an activated sludge age of 12 and 32 days, respectively. The activated sludge microbial community was adapted for the removal of ammonia nitrogen up to a level of 0.3 g NH_4^+–N g/VSS/d (VSS – volatile suspended solids). The methods – denaturing gradient gel electrophoresis (DGGE) based on 16S rRNA gene PCR products and fluorescent *in situ* hybridization (FISH) with 16S rRNA gene probes – revealed significant differences in the microbial community structure in the two bioreactors, caused mainly by a difference in sludge age. According to the results obtained in this study, a bioreactor with a sludge age of 12 days is characterized by a much higher microbial community diversity than a bioreactor with a sludge age of 32 days. Interestingly, the appearance of particular species of nitrifying bacteria was constant throughout the experiment in both bioreactors. Changes occured only in the case of the *Nitrosomonas oligotropha* lineage bacteria. This study demonstrates that the bacterial community of bioreactors operating with different sludge ages differs in total community structure. Nevertheless, the changeability of the bacterial community structure did not have any influence on the efficiency of nitrification.

Key words: activated sludge, ammonia nitrogen removal effectiveness, PCR-DGGE, FISH

Introduction

Activated sludge, as a mixture of microorganisms, is an excellent research material both for microbiology and technology, which allows researchers to find a method to effectively utilize different chemical substances. In the case of wastewater treatment, the removal of nitrogen compounds is one of the priorities. Despite the fact that studies in the field of deammonification as a process connected with nitrogen removal have recently expanded, nitrification is still the most commonly examined and used process.

Nitrification consists of two steps: nitritation (biological oxidation of ammonia) and nitratation (biological oxidation of nitrite). Ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) respectively carry out these steps. Many studies of nitrifying bacteria revealed that *Nitrosomonas* sp. and *Nitrobacter* sp. are the most common bacteria encountered in wastewater systems (Chain *et al*., 2003; Duddleston *et al*., 2000; Hommes *et al*., 2001; Kelly *et al*., 2005). Nowadays, due to the common use of molecular methods, it is known that the nitrifiers' group in activated sludge is much more diverse. Traditional cultivation techniques underestimate the number and the diversity of the nitrifiers groups, which are known to be fastidious and slow growing in the laboratory (Amman and Kuhl, 1998; Luxmy *et al*., 2000). Research based on cultivation-independent methods enabled researchers to extend the knowledge about ammonia and nitrite oxidizers.

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Descriptions and comparisons of activated sludge bacterial communities have been carried out since the early 90s. Molecular methods based on PCR reaction were introduced due to their sensitivity and the possibility of avoiding difficulties attributable to pure culture obtainment (Blackall *et al*., 1997; Snaidr *et al*., 1997; Juretschko *et al*., 1998). DNA isolation directly from bacterial cells in a bacterial community accelerates the identification procedures, assessment of composition and changeability of individual microbial groups without previous pure culture isolation (Holben *et al.*, 1998; Kowalchuk *et al.*, 1997). Currently, the basic tools used in a comparative analysis of bacterial communities without previous cultivation are DGGE (Denaturing Gradient Gel Electrophoresis) and FISH (Fluorescent *in situ* Hybridization). DGGE is based on the separation of DNA fragments of the same length, but with a different nucleotide sequence. The use of increasing gradient of denaturing factors (formamide and urea) makes the obtainment of detailed characteristics for the sample fingerprint possible (Curtis and Craine, 1998; van de Gast *et al.*, 2006). FISH uses fluorescently labeled oligonucleotides to study the presence and estimate the abundance of particular bacteria (Schuppler *et al.*, 1998).

In addition to the difficulties in the pure culture isolation of nitrifiers, the most problematic issues in studies of this group of bacteria in laboratory-scale experiments are a low bacterial growth rate and sensitivity to toxic shocks, such as pH and temperature swings (Luxmy *et al*., 2000; Rowan *et al*., 2003). Due to low bacterial growth (long doubling time), nitrifiers are more sensitive to washing out from the wastewater treatment plant than heterotrophic bacteria, while the system is operated on short sludge retention times. Sludge retention time (SRT), or sludge age, is a technological parameter determining the time during which the activated sludge stays in the reactor. SRT is calculated on the basis of the amount of suspended solids in the reactor and the amount of activated sludge removed from the reactor as excessive sludge and the amount of suspended solids in the effluent (Metcalf and Eddy, 1991).

Membrane bioreactors were used in this study. Such reactors have become very commonly used due to their advantages over settler-operated systems. Membrane reactors possess a filtration module (membrane) instead of a settler. The membranes are able to retain particles of different sizes, depending on the membrane type within the reactor (Bodzek *et al.*, 1997; Charcosset, 2006). Application of membranes in activated sludge systems enables a much more effective separation of solids from wastewater and can protect wastewater treatment plant from the problems connected with bulking. Membranes can be installed inside (submerged membrane reactors) or outside of the reactor. A membrane located inside the reactor reduces the space requirements and expenses connected with activated sludge pumping (Charcosset, 2006). Due to the lack of solids in the effluent in membrane reactors, sludge retention time would be the only crucial parameter responsible for bacterial withdrawal, because activated sludge flocs are removed from the system only during sludge age regulation. Thus, sludge age is an important parameter influencing the community structure because of the differences in the nitrifier species growth time.

The aim of the study was to compare activated sludge communities in two bioreactors containing activated sludge adapted to different sludge ages and to observe temporal changes in the bacterial communities. Such analysis was undertaken in order to check whether the communities performing a stable process of nitrification show any changeability on the bacterial level. An analysis performed to estimate the abundance of the main species and the structure of the bacterial communities pointed mainly to ammonia and nitrite-oxidizing bacteria.

Experimental

Materials and Methods

Reactor details and operational data. Bacteriological material from two completely mixed, laboratory-scale membrane bioreactors was used in this study. The membrane (pore size of 0.4 μm) was submerged in the reactor (Fig. 1). Activated sludge from a municipal wastewater treatment plant performing nutrient removal was used for seeding. The reactors were operated on with nitrification and fed with a synthetic medium containing high ammonia concentra-

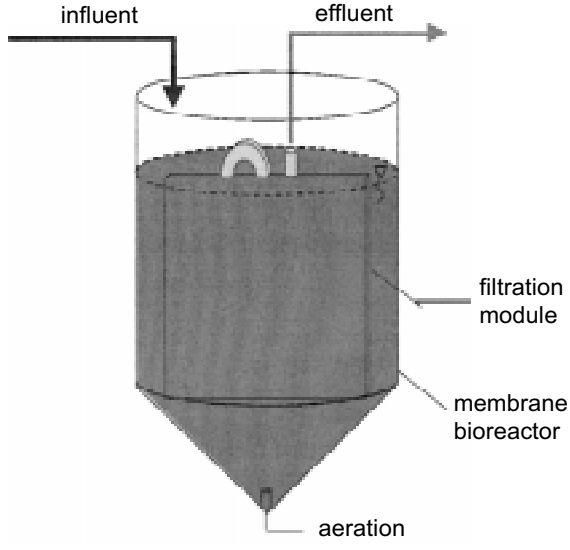

Fig. 1. The scheme of the membrane bioreactor.

tions. The wastewater was composed of 500–700 mg NH_4^+–N/l, 250 mg COD/l (COD – chemical oxygen demand) coming from CH_3COONa and broth extract and an amount of phosphorus (Na_2HPO_4) enabling 100:1 C:P ratio obtainment. The pH was maintained at a level of 7.5–8.0 using $NaHCO_3$.

Sludge age was calculated typically for activated sludge systems as follows:

$SRT = V_r \times X / Q_W \times X_W$

Since in the MBR reactor: $X_W = X$

than: $SRT = V_r / Q_W$

where: V_r – reactor volume, l; Q_W – amount of activated sludge removed daily from the reactor, l/d; X – biomass concentration within the reactor, g VSS/l; X_W – biomass contained in the waste sludge, g VSS/l. In order to obtain a particular sludge age, a constant and appropriate ($Q_N = V_r$ /SRT) volume of activated sludge was removed daily from each reactor (Sponza, 2002; Sponza, 2003).

The performance of the reactors was monitored by analysis of the influent and effluent. Nitrogen compound concentrations were determined colorimetrically – ammonia with Nessler reagent according to PN-C-04576-4:1994, nitrite with alfanaftyloamine reagent according to Hermanowicz and Dojlido (1999) and nitrate with dimethylphenol reagent according to ISO 7890-1. Due to daily sludge removal (in order to obtain a particular sludge age) and activated sludge suspended solids variations during the adaptation phase of the experiment, the substrate load was 0.3 g NH_4^+ – N g/VSS/d for reactor A and 0.24 g NH_4^+ – N g/VSS/d for reactor B (all the technological parameters of the investigated bioreactors are shown in Table I). The experiment was carried out for 8 months. Activated sludge samples were taken from the bioreactors within a period of 4 months each 2 weeks,

Table I
Technical parameters of two membrane bioreactors used in the study

Technical parameter	Bioreactor A	Bioreactor B
Bioreactor volume, l	25	36
Sludge age, days	12	32
Activated sludge removed daily for sludge age control, l	2.08	1.13
Substrate load, g NH_4^+ – N g/VSS/d	0.3	0.24
Volatile suspended solids, g VSS/l	0.6	1
Flow speed, l/d	9	9

after 4 months of adaptation to the high load of ammonia and upon achieving a particular sludge age.

Activated sludge samples for DGGE. Activated sludge samples 1A and 2A-6B (volume of 10 ml) were collected from both bioreactors at 2-week intervals (samples 6A and 6B were obtained at the 4 week mark), pelleted by centrifugation (10 000 × g, 10 min, 4°C) and stored at –20°C.

DNA extraction and PCR conditions. Total genomic DNA was extracted from 0.3 g of the activated sludge samples using an Ultra Clean Soil Isolation DNA Kit (MoBio Laboratories Inc., USA) according to the manufacturer's instructions and stored at –20°C until PCR amplification. Primers: 968F with a GC clamp (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC 3') and 1401R (5' CGG TGT GTA CAA GGC CC 3') were used for partial 16S rRNA bacterial gene PCR amplification (Felske *et al*., 1996). PCR was carried out in a 25 µl (total volume) reaction mixture containing 17 µl sterile MiliQ water, 2.5 µl 10 × PCR buffer – containing $(NH_4)_2SO_4$, 2.5 µl $MgCl_2$ (2 mM), 0.5 µl BSA (Bovine Serum Albumin, 3 mg/µl), 0.5 µl of both primers (20 pmol), 0.5 µl dNTPs (2.5 mM), 0.5 µl of genomic DNA and 0.5 µl *Taq* DNA polymerase (1U). All of the components were delivered by Fermentas, Lithuania.

PCR amplification was performed using an Eppendorf thermal cycler and the following steps: (1) the initial denaturation step (5 min at 94°C); (2) 30 cycles, each single cycle consisting of denaturation (1 min at 94°C), annealing (1 min at 53°C), and elongation (1 min at 72°C); and (3) the final extension step (10 min at 72°C). Products were evaluated in agarose gel (0.8% w/vol agarose, 1 × TAE buffer), stained with ethidium bromide (1% w/vol) in MiliQ water and photographed under UV light.

DGGE – denaturing gradient gel electrophoresis. The DGGE of PCR products obtained in reaction with 968F-GC and 1401R primers were performed using the Dcode Universal Mutation Detection System (BioRad). Polyacrylamide gel (6%, 37:1 acrylamide-bisacrylamide) with a gradient of 28–50% denaturant was prepared with a gradient former (Amersham Bioscience) according to the manufacturer's guidelines. The gel was run for 11 h at 80 V in a 1 × TAE buffer at a constant temperature of 60°C. The gel was stained with ethidium bromide (1% w/vol) in MiliQ water for 20 min and washed in MiliQ water twice for 15 min, then visualized under UV light and photographed.

Numerical analysis of the DGGE fingerprints. The DGGE banding patterns with 16S rDNA PCR products were analyzed using GelCompar II software (Applied Maths, Ghent, Belgium) in order to compare the fingerprint patterns obtained from the separation of the PCR products from samples 1A-6B. The clustering was done using the Pearson correlation coefficient and the UPGMA method (Unweighted Pair Group Method with Arithmetic mean). The principle of moving window analysis was used in order to evaluate the stability of the bacterial community (Possemiers *et al.,* 2004; Wittebolle *et al*., 2005).

Table II
rRNA-targeted oligonucleotide probes used in the study

Probe	Target organisms	Sequence (5' – 3')	Target site	% formamide	Reference
Nso 1225	B-proteobacterial ammonia-oxidizing bacteria	CGC CAT TGT ATT ACG TGT GA	1224 – 1243	35	Mobarry *et al*., 1996
NEU	most halophilic and halotolerant *Nitrosomonas* sp.	CCC CTC TGC TGC ACT CTA	653 – 670	40	Wagner *et al*., 1995
CTE	[a]	TTC CAT CCC CCT CTG CCG	653 – 670	–[a]	Wagner *et al*., 1995
Cluster 6a192	*Nitrosomonas oligotropha* lineage	CTT TCG ATC CCC TAC TTT CC	192 – 211	35	Adamczyk *et al*., 2003
Comp-Cluster 6a192	[b]	CTT TCG ATC CCC TGC TTC C	192 – 211	–[b]	Adamczyk *et al*., 2003
Ntspa-662	*Nitrospira* sp.	GGA ATT CCG CGC TCC TCT	662 – 679	35	Daims *et al*., 2001a
Comp-Ntspa662	[c]	GGA ATT CCG CTC TCC TCT	662–679	–[c]	Daims *et al*., 2001a
NIT3	*Nitrobacter* sp.	CCT GTG CTC CAT GCT CCG	1035 – 1052	40	Wagner *et al*., 1996
Comp NIT3	[d]	CCT GTG CTC CAG GCT CCG	1035 – 1052	–[d]	Wagner *et al.,* 1996
Nsv443	*Nitrosospira* sp.	CCG TGA CCG TTT CGT TCC G	444 – 462	30	Mobarry *et al*., 1996
EUB338	most bacteria	GCT GCC TCC CGT AGG AGT	338 – 355	35	Amann *et al*., 1990
EUB338 II	*Planctomycetales*	GCA GCC ACC CGT AGG TGT	338 – 355	35	Daims *et al*., 1999
EUB338 III	*Verrucomicrobiales*	GCT GCC ACC CGT AGG TGT	338 – 355	35	Daims *et al*., 1999

[a] used as unlabeled competitor together with probe S-*-Nsm-0651-a-A-18
[b] used as unlabeled competitor together with probe Cluster 6a192
[c] used as unlabeled competitor together with probe S-G-Ntspa-662-a-A-18
[d] used as unlabeled competitor together with probe S-G-Nbac-1035-a-A-18

The structural diversity of the bacterial community was estimated on the basis of the Shannon-Weaver diversity index, H (Eichner *et al*., 1999; Nübel *et al.,* 1999; Luxmy *et al*., 2000), estimated on the relative band intensities obtained from the DGGE fingerprints.

FISH – Fluorescent *in situ* hybridization: sample preparation, oligonucleotide probes, confocal microscopy and cell quantification. Activated sludge samples were fixed with a paraformaldehyde solution (4% paraformaldehyde in phosphate-buffered saline, PBS, pH 7.2) at 4°C for 3 hours and subsequently washed in PBS. Fixed samples were stored in PBS: ethanol (1:1) solution at –20°C. *In situ* hybridization was performed as described previously by Daims (Daims *et al*., 2005). 16S rRNA targeted fluorescently labeled oligonucleotide probes, the sequences and targeted sites are listed in Table II. The probes EUB338, EUB338 II and EUB338 III were mixed together (EUB338 mix) in the proportion 1:1:1 in order to detect all bacteria.

Details on the chosen oligonucleotide probes are available at probeBase (Loy *et al*., 2003). The probes were 5' labeled with the dye FLUOS (5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester), Cy3 or Cy5. Both the probes and unlabeled competitor oligonucleotides were obtained from Biomers, Ulm, Germany.

Prior to microscope observations, samples were embedded in Citifluor (Citifluor Ltd, UK) to reduce fluorochrome fading. A scanning confocal microscope (Zeiss LSM 510) equipped with an Ar-ion laser (488 nm) and two HeNeLasers (543 nm and 633 nm) was used to examine the microbial community. Image processing was performed using the standard software package delivered with the instrument (Zeiss LSM version 3.95). For cell quantification DAIME software was used.

Results and Discussion

Ammonia oxidation effectiveness in the experiment. The measurements of the ammonia, nitrite and nitrate in bioreactors A and B pointed to a high nitrification efficiency (Fig. 2). In both bioreactors the effectiveness of ammonia removal was maintained at a level of 99%. The effluent quality confirmed full nitrification obtainment. Such nitrification efficiency suggests that the bacterial community contains a considerable amount of ammonia and nitrite-oxidizing bacteria.

DGGE analysis of the bacterial community. The fingerprints obtained from DGGE separation of 16S rRNA gene fragments are shown in Figure 3. The DNA-based DGGE pattern had changed in both bioreactors during the experiment. The dendrogram (Fig. 4) obtained from DGGE fingerprint analysis presents the value of similarity among activated sludge samples. This could be calculated by summarizing the length of branches connecting particular samples of the dendro-

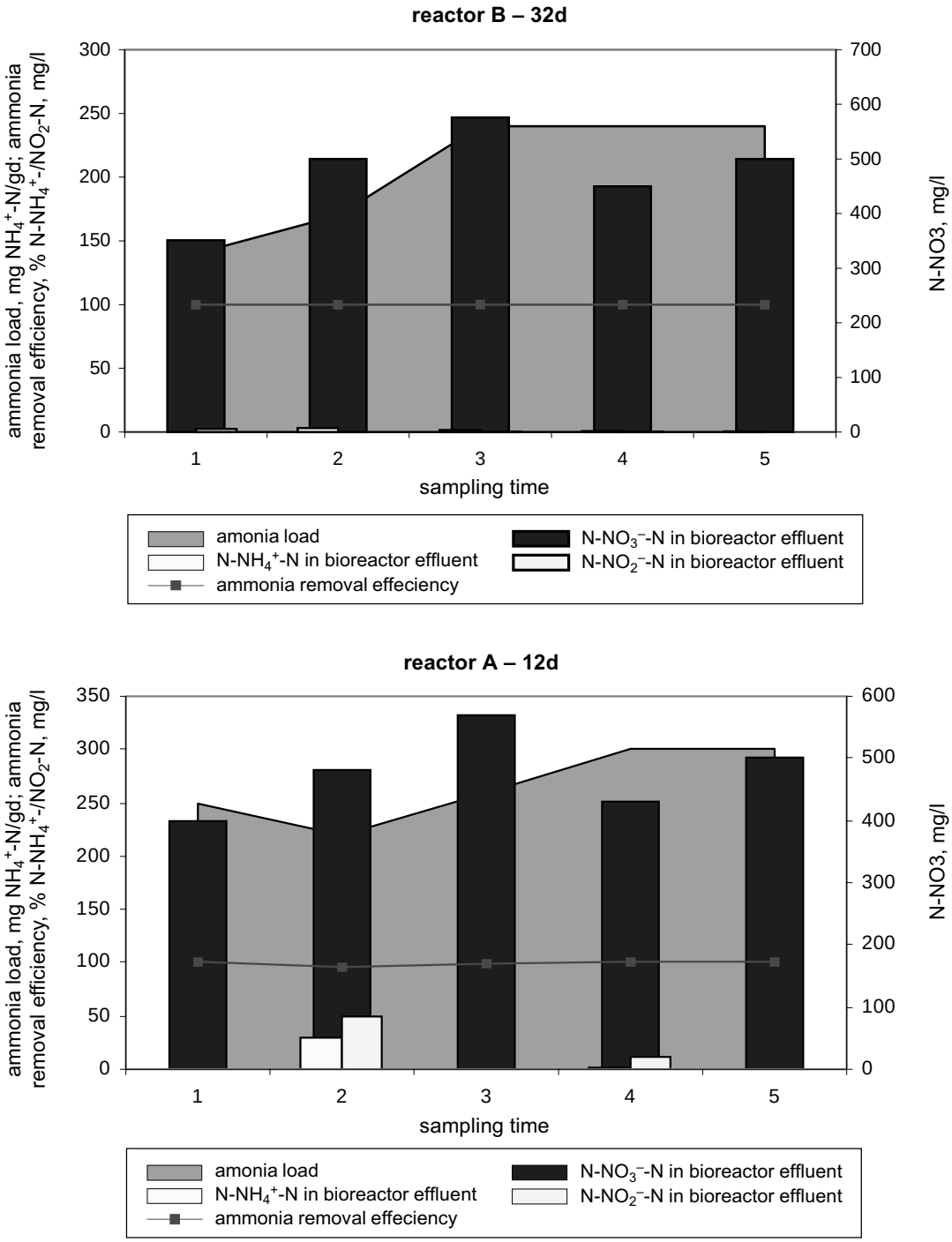

Fig. 2. The temporal dynamics of ammonia-nitrogen removal from bioreactors A and B.

gram. The lower this value is, the higher the similarity the samples present. This analysis revealed higher similarity values among samples collected from bioreactor B (shorter dendrogram branches connecting particular samples), which suggests a lower temporal variation in this community. Nevertheless, sample A6 seems to be more congruent to the bioreactor B cluster than to its own group. There is a possibility that at the end of the experiment, the bioreactor A community reached a level of homogeneity, which characterized the older sludge from bioreactor B.

The variability of the bacterial community according to the results obtained in Shannon-Weaver index estimations is higher in bioreactor A (Fig. 5). The diversity of the bacterial community changed slightly during the experiment. Interestingly, in the case of bioreactor A, the diversity increases (in the period of sampling time for samples 2A-3A) and then decreases after the third measurement (samples 3A-5A), while the diversity of bacteria decreases constantly at a similar rate in bioreactor B (between measurements for samples 2B-5B). The reason for such situation

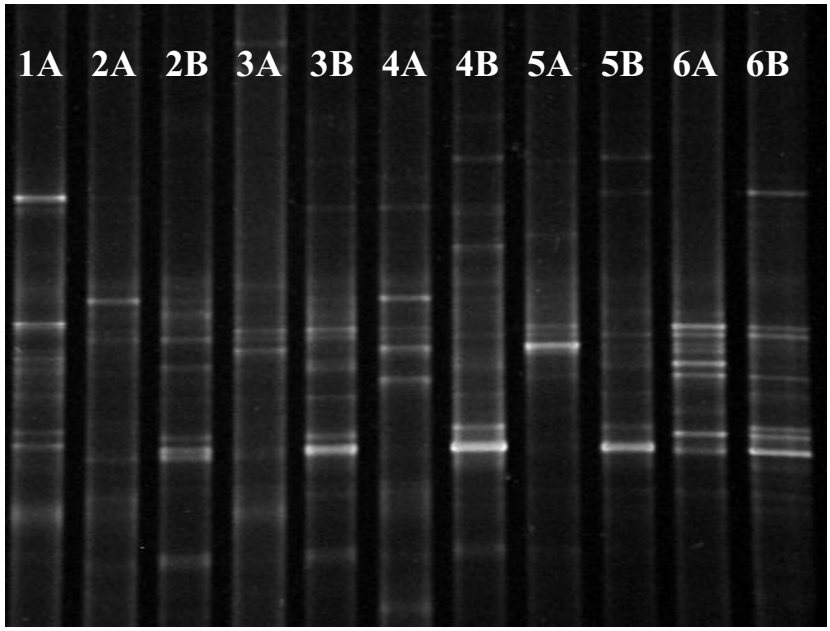

Fig. 3. DGGE pattern of 16S rRNA genes fragments with a size *ca.* 500 bp amplified using DNA obtained from activated sludge samples.

could be the difference in sludge age. In order to maintain the shorter age of the activated sludge, a larger volume of the sludge was removed from the bioreactor. In this situation, the ammonia and nitrite-oxidizers community is removed from the environment of bioreactor A faster, while the bacteria in bioreactor B have a longer time to multiply and change.

Results obtained from both bacterial communities underwent the moving window analysis (Fig. 6) in order to estimate bacterial community stability (Wittebolle *et al*., 2005). This analysis revealed that differences in sludge age could be the cause of the dissimilarities in the value of correlation coefficient. The higher the sludge age is, the higher the coefficient of correlation. The results obtained correlate with the performance of the bioreactors leading nitrification with high efficiency and stability. The analysis proved that bioreactors achieved stability by the first experimental sampling time, but in the case of bioreactor A, the microbial community steadily changed until the fourth measurement.

Diversity of nitrifying bacteria analyzed by fluorescent *in situ* hybridization (FISH). The appearance of the ammonia and nitrite oxidizers was identified

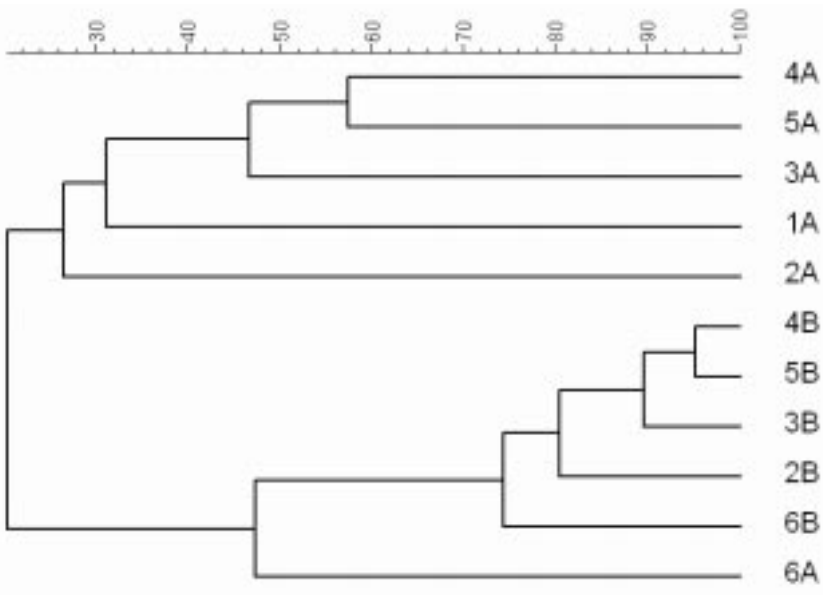

Fig. 4. DGGE tree based on Pearson correlation coefficient and UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering method

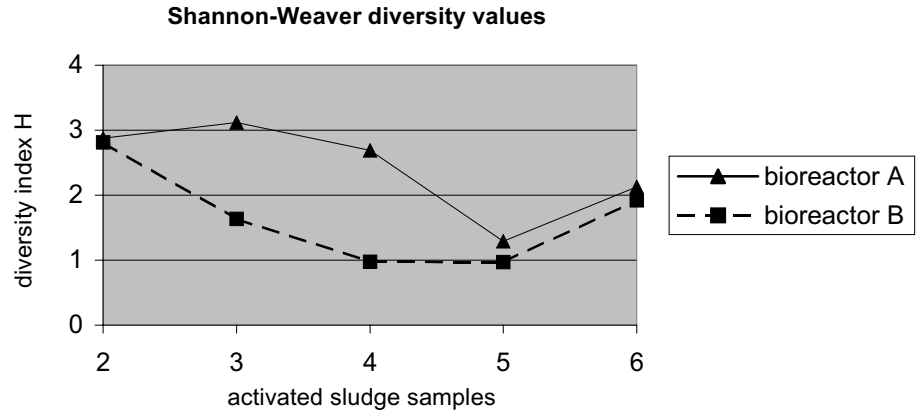

Fig. 5. Temporal dynamics of the Shannon-Weaver diversity values (H) for activated sludge samples (activated sludge samples from 2A to 6B)

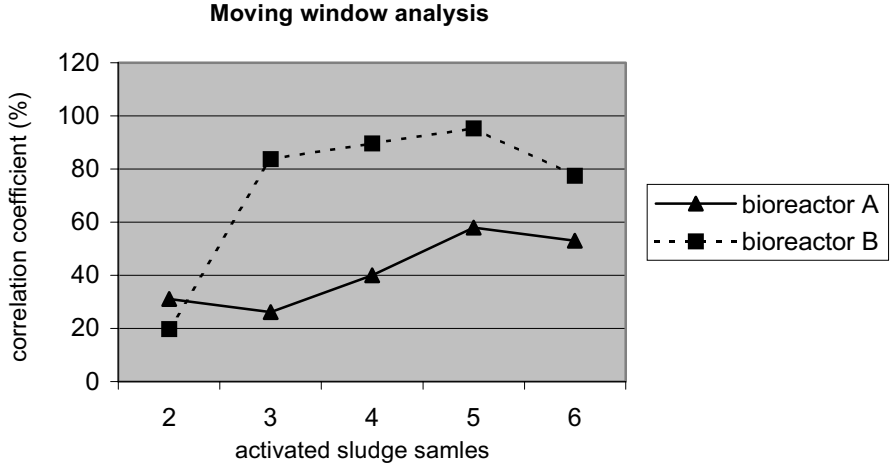

Fig. 6. Moving window analysis of DGGE fingerprints of sludge samples from bioreactors A and B (2A-6B) presents the difference of the correlation coefficient due to the differences in the sludge age

using molecular probes characteristic for α and β proteobacterial representatives of these functional groups as well as for the *Nitrospira* sp. group. The EUB mix probe detects most of the bacteria and was used as a control. Tables III and IV gather the results of the FISH investigation for the groups: *Nitrobacter* sp., *Nitrospira* sp., halophilic and halotolerant *Nitrosomonas* sp. Bacteria belonging to these groups appear in both bioreactors, while no representatives of *Nitrosospira* sp. group were found. The only difference is the presence of *Nitrosomonas oligotropha* lineage members in bioreactor A during the time of the experiment, while in bioreactor B such bacteria appear at the end of the experimental period. The obtained results confirm previous studies where *Nitrosomonas* sp. appearance was usually exhibited in the engineered high ammonia environments (Juretschko *et al*., 1998). Interestingly, these bacteria were absent in bioreactor B, which had activated sludge age of 32-days, during most of the experiment, which could mean that the

Table III
Structure of nitrifiers community in the activated sludge of reactor A (obtained by FISH analysis)

Activated sludge sample	*Nitrosomonas oligotropha* lineage (clad)	Most halophilic and halotolerant *Nitrosomonas* sp.	*Nitrosospira* sp.	*Nitrobacter* sp.	*Nitrospira* sp.	Amount of nitrifiers* (%)
2A	+	+	–	+	+	46.9
3A	+	+	–	+	+	37.9
4A	+	+	–	+	+	36.7

* amount of nitrifiers present in activated sludge as a percent of the total amount of bacteria

Table IV
Structure of nitrifiers community in the activated sludge of reactor B (obtained by FISH analysis)

Activated sludge sample	*Nitrosomonas oligotropha* lineage	Most halophilic and halotolerant *Nitrosomonas* sp.	*Nitrosospira* sp.	*Nitrobacter* sp.	*Nitrospira* sp.	Amount of nitrifiers* (%)
2B	–	+	–	+	+	64.6
3B	–	+	–	+	+	60.8
4B	+	+	–	+	+	48.6

* amount of nitrifiers present in activated sludge as a percent of the total amount of bacteria

presence of *Nitrosomonas* sp. in a well-working engineered system is not obligatory. The level of ammonia can be a relevant factor of selection in bacterial appearance in the environment (Pommerening-Röser *et al*., 1996). It is important to note that lab-scale artificial environments are known to abound in one group of ammonia oxidizers such as *Nitrosomonas*-like, *Nitrosospira*-like bacteria or a mixture of these groups (Schramm *et al*., 1998). In this case no representatives of *Nitrosospira* sp. were found in either bioreactor. This could suggest the existence of an unknown environmental factor that eliminates these bacteria from the environment and disturbs the disposition of the other bacterial groups. We might suspect that bioreactor conditions are not suitable for the desired bacteria's adaptation. Nevertheless, it was previously shown (Daims *et al.*, 2001b) that a large diversity of nitrifying bacteria appear in spite of a high ammonia and salt concentration known to be "extreme" conditions. It was also noted previously that different wastewater systems support different groups of bacteria as well as population richness (Rowan *et al*., 2003). The total amount of nitrifiers decreased during the experiment in both cases. However, the rate of the decline is slightly higher in the bioreactor with a shorter activated sludge age (bioreactor A) which could be caused by the larger volume of sludge removed from the bioreactor in order to maintain a particular sludge age. There is also a possibility that some groups of nitrifiers can adapt better to the environment than others. It could mean that the nitrifiers remaining in the bioreactor adapted to the environment gaining higher efficiency of the process. In such a case, the effectiveness of nitrification is maintained at a high level even in a situation where a high amount of bacteria is removed from the bioreactor.

Conclusions. This study found that two lab-scale environments of bioreactors dealing with the same wastewater vary in the homogeneity of the bacterial groups and that bioreactor A (sludge age of 12 days) is characterized by a much higher diversity of the genotypes than bioreactor B (sludge age of 32 days). Such differences probably occur due to the differences in sludge age. There were no disturbances of nitrification during the experiment and the efficiency of the process in both environments was very high. The total amount of nitrifiers decreased in both bioreactors during the experiment, but changes in the appearance of particular groups occurred only in the case of the *Nitrosomonas oligotropha* lineage bacteria. Bacteria belonging to this group appear in bioreactor B in the final stage of the experiment, while in bioreactor A they are constantly present. It can be concluded that the effectiveness of the bioreactors' performance does not depend on the level of the total bacterial diversity or the presence of a particular species.

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