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Molecular Analysis of Bacterial Community Diversity in Sequencing Batch Reactor (SBR) Operating in Autotrophic Conditions

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Abstract

In the present study the bacterial community structure in a sequencing batch reactor (SBR) operating in autotrophic conditions was determined in relation to temporal variations of NH_4-N, NO_2-N, NO_3-N levels in the effluent. Bacterial richness and composition were determined by PCR-DGGE (Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis) of 16S rRNA and *amoA* genes, and RISA (Ribosomal Intergenic Spacer Analysis) technique. The applied approaches revealed that the composition of bacterial population in the studied SBR varied in time. A positive correlation between Shannon index of ammonia-oxidizing bacteria (AOB) diversity and ammonia level in the effluent was observed. The variation of total bacterial diversity did not reflect the changes in nitrification efficiency.

Key words: AOB, bacterial community, DGGE, nitrification, RISA, SBR

Introduction

Ammonia is the major nitrogen compound in wastewater and it is removed by conversion to gaseous nitrogen forms *via* nitrification and denitrification. Nitrification, the aerobic oxidation of ammonia to nitrate *via* nitrite, is catalysed by two phylogenetically different groups of bacteria – ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) (Wagner and Loy, 2002). Activated sludge systems are widely used as a method of biological wastewater treatment, and ammonia oxidation is a key step in this process. Although nitrification has been intensively studied during the past several decades, it is still recognized as being consequently difficult to maintain in engineering systems. Therefore, in order to construct and maintain effective nitrogen removal process, it is necessary to know the biological diversity of bacteria participating in this process. Further understanding of AOB microbial ecology and other accompanying bacteria in activated sludge and monitoring their population changes are necessary in order to control wastewater treatment processes stability (Aoi *et al*., 2004). It has recently been suggested that the level of AOB diversity in activated sludge relates to the stability of the reactor (Daims *et al*., 2001), but information about the relation between total bacteria diversity and process efficiency is very limited.

Recently, several molecular techniques have been developed in order to study natural samples (Dahllöf, 2002). These molecular techniques identify microorganisms without isolation and reveal the enormous extent of microbial diversity. Specifically, denaturing gradient gel electrophoresis (DGGE) has emerged as a powerful tool. By DGGE of PCR-amplified fragments coding for 16S rRNA, DNA fragments of the same length but different base pair sequences can be separated. This method has recently been introduced into molecular microbial ecology to determine the genetic diversity of natural microbial communities and to identify the phylogenetic affiliation of community members (Muyzer *et al*., 1993). Ribosomal Intergenic Spacer (RIS) region located between the 16S and 23S rDNA genes in the ribosomal operon is extremely variable in size and sequence even within closely related taxonomic groups (Gürtler and Stanisich, 1996). Size patterns can be used to characterize different communities of Bacteria or Archea by PCR, oligo-probe or even long DNA probe (Jensen *et al*., 1993).

DGGE of 16S rRNA and *amoA* gene as well as RISA were used as culture independent techniques to

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estimate diversity and changes of bacteria in time. The combination of DGGE and RISA gives a chance for more precise information regarding the diversity of microbial communities during nitrification processes.

The purpose of this study was to examine the diversity of the total and the ammonia-oxidizing bacteria in activated sludge from a sequencing batch reactor, operating in autotrophic conditions, and to find a relation between nitrification efficiency and values of bacterial diversity.

Experimental

Materials and Methods

SBR operation. The experiment was carried out for 36 days. A 3.0 l bench scale reactor was used as a sequencing batch reactor system. Seed sludge was collected from SBR, treating artificial wastewater composed of NH_4Cl (0.250 g/l), $Na_2HPO_4 \times 12H_2O$ (0.046 g/l), NaCl (0.010 g/l), KCl (0.0047 g/l), $CaCl_2 \times 2H_2O$ (0.0047 g/l), $MgSO_4 \times 7H_2O$ (0.0167 g/l), $NaHCO_3$ (0.600 g/l), Na_2CO_3 (0.500 g/l), $FeCl_3 \times 6H_2O$, $MnSO_4 \times H_2O$, $ZnSO_4$, $CuSO_4$ (<0.0002 g/l) (Coelho *et al*., 2000; modified) and sodium acetate as the sole source of organic carbon (660 mg COD/d).

Each SBR operated in 24-hour cycle, with the following operating strategy: filling (0.25 h), aeration (23 h), settling (0.50 h) and decantation (0.25 h). The mean cell residence time was 18 ± 2.3 days and the total suspended solids averaged 2.2 g TSS/l. The reactor operated at about 2 mg/l of dissolved oxygen. The temperature was maintained at 20°C and the pH was kept between 7 and 8. During the filling period 1.0 l of synthetic wastewater was added to the reactor to make the final working volume of 2.5 l. The synthetic wastewater supplied to the reactor was the same as given above but without organic carbon.

Analytical measurements. Every day sampling was made at the influent and effluent of one cycle. All of the samples were filtered with a 0.2 mm micro-pore filter before being assayed. The following parameters were determined in wastewater: total suspended solids by drying at 103–105°C, ammonium by Nesslerization method or distillation method, nitrite and nitrate by colorimetric methods.

Total DNA extraction. Activated sludge samples were collected in 2–4-day intervals and kept in a refrigerator in –20°C before analysis. Genomic DNA was isolated as follows: 0.02 g as semi-dry weight of aggregated sludge sample was washed with sodium phosphate buffer (0.1 M; pH 8.0), pelleted by centrifugation, suspended in the proteinase K buffer (100 mM Tris-HCl; 10 mM EDTA; pH 8.0) and incubated at 55°C in the presence of sodium dodecyl sulphate, proteinase K and lysozyme. DNA was purified using phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v) solution, and precipitated with 2 volumes of ethanol (96%) and 0.1 volume of sodium acetate (pH 5.2). The pellet was washed with 70% ethanol, dried and re-suspended in 200 µl of TRIS/EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

Denaturant Gradient Gel Electrophoresis (DGGE). PCR amplification of bacterial 16S rRNA and *amoA* gene fragment was performed using primers given in Table I. A touchdown thermocycling program was used for PCR as described by Murray *et al*. (1996). PCR was performed in Eppendorf® Mastercycler Gradient (Eppendorf, Germany). The mixtures used for PCR amplification contained 50 ng of extracted total DNA, 0.5 µM of each primer, 100 µM of deoxynucleoside triphosphate mixture (Promega, Winsconsin, USA), 1 U of *Taq* DNA polymerase (Invitrogen, Life Technologies), 5 µl of reaction buffer (500 mM KCl, Triton X-100, pH 8.5), 1.5 mM $MgCl_2$ and sterile water to a final volume of 50 µl. The standard PCR amplification was carried out using the following program: 95°C

Table I
PCR primers used in this study

Primer	Sequence (5'-3')	Annealing temperature	Target gene	Source
357F *517R*	[a]CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	63°C TD	*V3 16S rRNA*	Muyzer *et al*., 1993
amoA2R *amoA1F*	CCC CTC TGC AAA GCC TTC TTC [b]GGG GTT TCT ACT GGT GGT	61°C	*amoA*	Nicolaisen and Ramsing, 2002
357F *EubR*	[a]CCT ACG GGA GGC AGC AG CAK AAA GGA GGT GAT CC	63°C TD	*V3-8 16S rRNA*	Muyzer *et al*., 1993 Juretschko *et al*., 1998
1 *2*	TTG TAC ACA CCG CCC GTC A GT ACT TAG ATG TTT CAG TTC	43°C	*ISR*	Dolzani *et al*., 1995

Clamps of PCR primers:
a – CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G
b – CGC CGC GCG GCG GGC GGG GCG GGG GCG GGG
TD – touchdown (Murray *et al*., 1996)

for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s, extension at 72°C for 1 min and single final elongation at 72°C for 5 min. Different annealing temperatures were used depending on the primers employed (Table I). The presence of PCR products was confirmed by analyzing 5 µl of the product on 1.2% agarose gel stained with ethidium bromide. A size of PCR products was estimated using molecular weight marker øX174 DNA/Hinf I (Promega, USA).

DGGE electrophoresis was performed with a D-CODE Universal Mutation System (Bio-Rad, Hercules, USA). The PCR samples with equal amounts of DNA were applied directly on 8% polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) in a 0.5×TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.5) with a denaturing gradient ranging from 30 to 70%. Denaturation of 100% corresponded to 7 M urea and 40% formamide. The gradient gel was cast with a gradient delivery system (Model 475, Bio-Rad, USA). Electrophoresis was run at a constant voltage of 60 V at 60°C. After appropriate time of electrophoresis, the gel was stained with SYBR gold (Molecular Probes, USA) at 10 000x dilution in 1×TAE buffer for 30 min. Stained gel was viewed with an ultraviolet transiluminator and recorded with CCD camera (Gel Logic 200, Eastman Kodak Company, USA). Bands were detected automatically from digital images of the gel using KODAK 1D 3.6 Image Analysis Software (Eastman Kodak Company, USA).

Ribosomal Intergenic Spacer Aanalysis (RISA). The bacterial RIS located between the small and large subunit of rDNA was amplified with primers *1* and *2* described previously by Dolzani *et al*. (1995) (Table I). Amplified fragments contained RIS plus approximately 380 bp corresponding to flanking regions of genes coding for 16S and 23S rRNA. PCR mixture components and conditions were the same as given above. After successful DNA amplification, the PCR products were separated in 5% polyacrylamide gel (29:1 acrylamide: bisacrylamide). Electrophoresis was carried out at 60V for 100 min in 1×TBE buffer (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA; pH 8.0). Sizes of PCR products were estimated using øX174 DNA/Hinf I (Promega, USA). The gel was stained, documented and digitalized as in case of DGGE.

Analysis of DGGE and RISA patterns. The structural diversity of the microbial community was examined by the Shannon index of general diversity *H* (Fromin *et al*., 2002). *H* was calculated on the basis of the bands on the gel tracks, using the densitometric curves. The intensity of the bands was reflected as peak heights in the densitometric curve. The equation for the Shannon index is:

$$H = -\sum(n_i/N)\log(n_i/N),$$

where n_i is the height of the peak and N the sum of all peaks heights of the densitometric curve. The relationships between the Shannon index and chemical parameters in the reactor effluent were analyzed by correlation analyses (van der Gucht *et al*., 2001). Since normal distribution could not be assumed, the correlation analyses were performed as Spearman's rank correlations using the program STATISTICA (StatSoft, USA).

Results

Nitrification in SBR. Over 90 % nitrification efficiency, measured as the amount of ammonia oxidized in SBR cycle, was observed during 36 days of the experiment. Fig. 1 shows the time courses of NH_4-N, NO_3-N, and NO_2-N concentrations in the effluent from the SBR reactor. The ammonia and nitrite concentrations in the effluent fluctuated slightly at the beginning of experiment but from 23rd day stabilized at about 0.008 and 0.18 g/l, respectively. Statistically significant (Student's t-test, $p<0.05$) increase in nitrate concentration in the effluent was observed from 20th day of the experiment. This result suggests that NO_2-N oxidation to NO_3-N improved with time.

DGGE. In order to monitor the changes within the microbial community of activated sludge, the diversity of the 16S rRNA and *amoA* genes were examined during 36 days of experiment. Samples were taken from the reactor in 2 to 4-day intervals. After DNA extraction and amplification, the PCR-amplified products were analyzed by DGGE. Figures 2 and 3A show the DGGE patterns of the V3 and V3-8 region of the 16S rRNA gene, whereas Fig. 3B shows the DGGE band pattern of the *amoA* gene.

The DGGE patterns obtained with primers targeting the *amoA* gene showed less variation than those obtained with primers for the 16S rRNA. Some differences were noted in position, intensity, and number of bands presented in the bacterial DGGE patterns throughout the collection period. Amplification of the V3 region of the 16S rRNA gene yielded the most intense patterns with the largest number of bands (maximum 14), followed by the *amoA* gen and the V3-V8 region of the 16S rRNA gene (minimum 2). Some bands were unique for a specific day, whereas other bands were obtained for each day of the experiment. A visible change in the DGGE patterns composition was noticed on about the 20th day of the experiment; some bands became dominant in the reactor (A bands, Fig. 3), while other bands vanished (A and B bands; Fig. 2).

The patterns of each PCR amplicon were compared with each other. To determine the information content of the banding patterns in terms of structural diversity they were analyzed by Shannon index (*H*) measurement. Thus, *H* was used as a parameter that reflects the diversity of bacterial community. The ammonia oxidizers community had the lowest average *H*

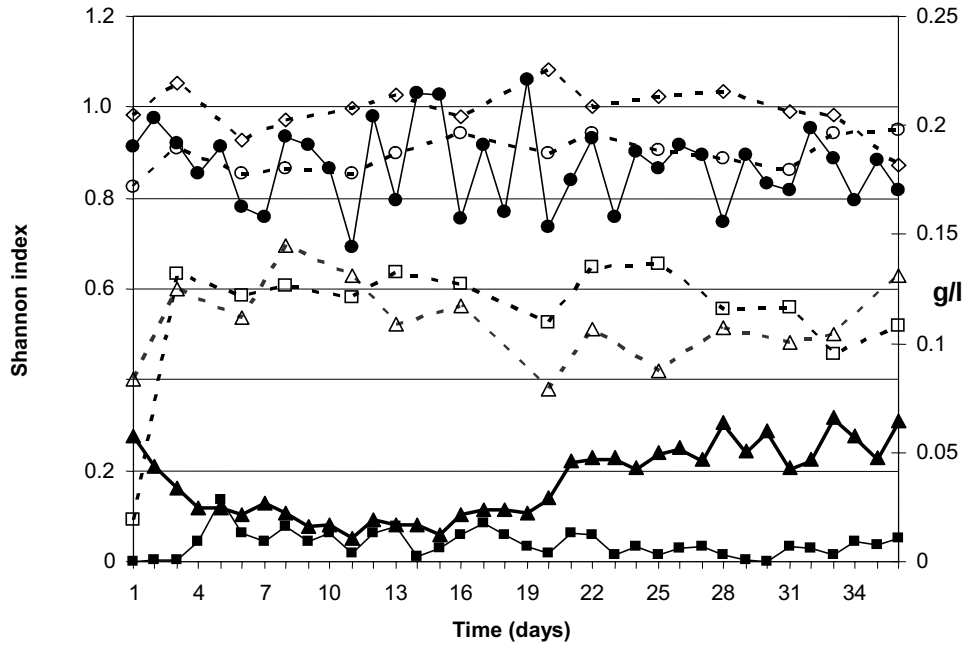

Fig. 1. Time courses of NH_4-N (■), NO_2-N (●), and NO_3-N (▲) concentrations in the effluent and values of Shannon indices for all analyzed markers.

V3 region of 16S rRNA (◊), V3-8 region of 16S rRNA (□), *amoA* (Δ), and RISA (○) during 36 days of the experiment.

(0.5279 ± 0.0914), similarly to bacterial community obtained by the V3-8 region of the 16S rRNA analysis (0.5475 ± 0.1421). In contrary, the average Shannon index for the total bacterial community analyzed by the V3 region of 16S rRNA was proportionally high (0.9946 ± 0.0524). Time-course values of Shannon indices for all analyzed markers are given in Fig. 1.

The relationships between the Shannon index and the chemical parameters in reactors effluent was analyzed by Spearman's rank correlation analyses (Table II). This analysis showed that the diversity of AOB was positively correlated with the concentration of ammonia in the effluent. The diversity of the total bacteria measured by the V3-8 region of 16S rDNA was negatively correlated with NO_3-N concentration in the effluent. No relationships were found between the diversity of total bacteria obtained by the V3 region of 16S rRNA and NH_4-N, NO_2-N, and NO_3-N levels in the effluent. Moreover, there was a lack of any statistically significant correlations between the sample richness obtained by the three analyzed primer sets.

RISA. Electrophoretic separation of PCR amplified ISR of the ribosomal operon from each time point resulted in distinct banding patterns (Fig. 4). These patterns comprised of 9 to 12 bands with one dominant band (about 720 bp) present in each sampling day. The bacterial diversity measured by Shannon index ranged from 0.8227 to 0.9512 (average value 0.8951 ± 0.0406). Most of the bands were obtained in each day of the experiment, whereas some were specific only for particular days. The most interesting band, with length of 160 bp, appeared about the 20th day, and

Table II
Spearman's rank correlation values between Shannon indices of bacterial diversity and NH_4-N, NO_2-N, and NO_3-N levels measured in the effluent of SBR

	V3-8 16S rDNA	V3 16S rDNA	*amoA*	RISA	NH_4-N	NO_2-N	NO_3-N
V3-8 16S rDNA	1.0000	0.2703	0.2000	0.2747	0.2967	0.0198	–0.5324[a]
V3 16S rDNA	–	1.0000	–0.4285	0.0505	–0.4857	0.2178	–0.0880
amoA	–	–	1.0000	0.1736	0.5384[a]	–0.2464	–0.3652
RISA 1	–	–	–	1.0000	0.0857	0.0704	0.2420
NH_4-N	–	–	–	–	1.0000	–0.3300	–0.6446[a]
NO_2-N	–	–	–	–	–	1.0000	–0.0176
NO_3-N	–	–	–	–	–	–	1.0000

a – statistically significant correlation ($p < 0.05$)

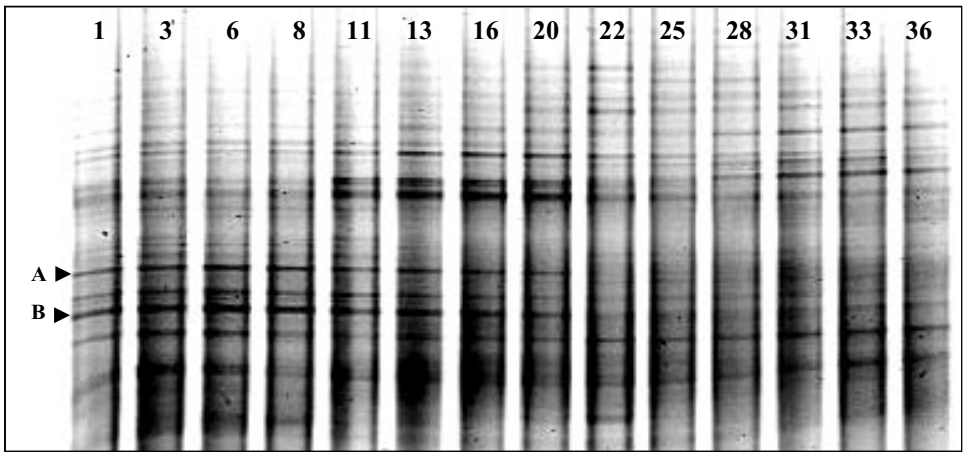

Fig. 2. PCR-DGGE analysis of V3 region of 16S rRNA in activated sludge samples.
Lane labels along the top show sampling days from startup of the process. A and B badges indicate the two bands that start to disappear about 20th day of the experiment. The gel was stained with SYBRgold (Molecular Probes, USA).

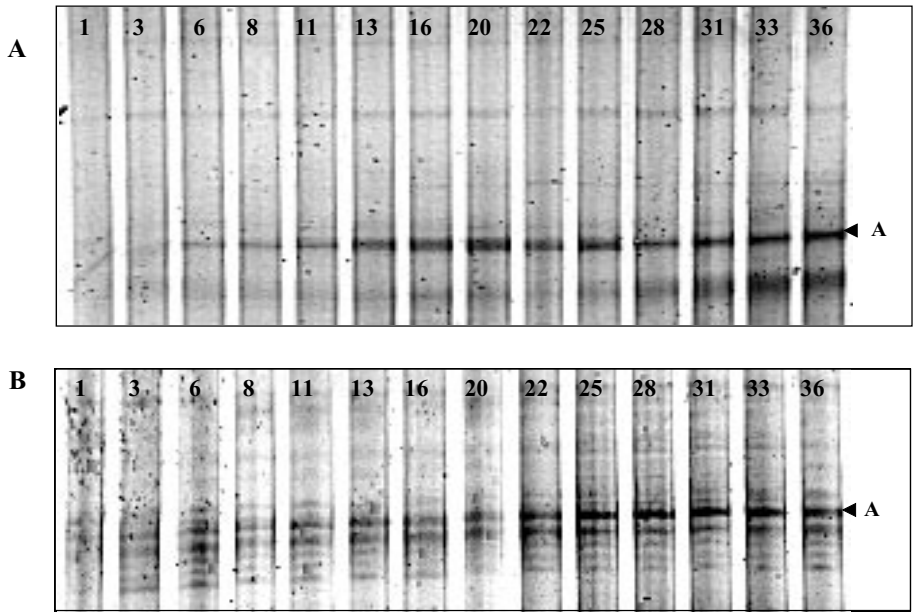

Fig. 3. PCR-DGGE analysis of V3-8 region of 16S rRNA (A) and *amoA* gene (B) in activated sludge samples.
Lane labels along the top show sampling days from startup of the process. A badges indicate the dominant bands. The gel was stained with SYBRgold (Molecular Probes, USA).

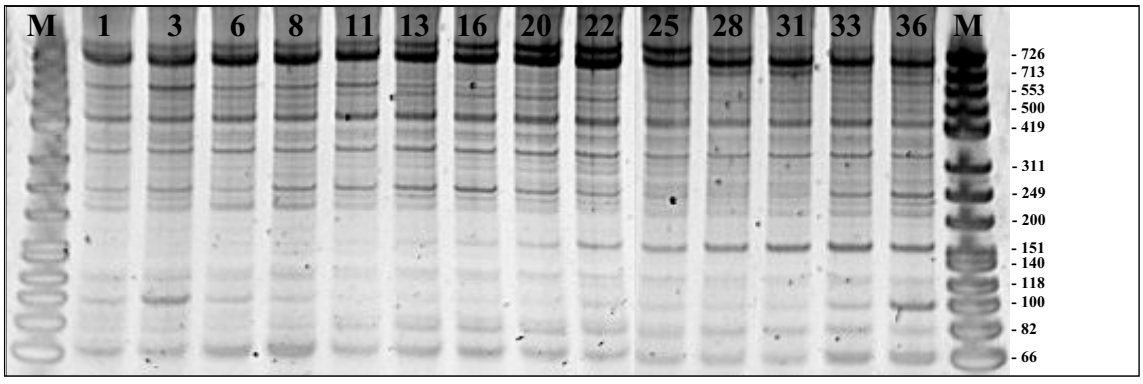

Fig. 4. Ribosomal Intergenic Spacer Analysis (RISA) of bacterial community from activated sludge.
Lane labels along the top show sampling days from startup of the process. Lane M: ΦX174 DNA/HinfI. The gel was stained with SYBRgold (Molecular Probes, USA).

became one of the dominant bands in the end of the experiment. There was no relation between Shannon indices values obtained on the base of RISA patterns and NH_4-N, NO_2-N, and NO_3-N levels in the effluent.

Discussion

DGGE patterns of samples obtained from complex ecosystem, such as activated sludge, are very difficult to interpret. Precise computer-based analyses are necessary to examine these patterns by means of densitometric curves. The number of obtained peaks and their intensity reflect the number and relative abundance of the dominant gene sequences (Boon *et al*., 2002).

In this work Shannon diversity index was applied to analyze the composition of the total and the AOB bacterial community in SBR in a 36-day long experiment. The synthetic wastewater did not contain organic carbon which promoted autotrophic conditions and through that the nitrification process. The samples were collected in 2–4-day intervals and analyzed by using two fingerprinting techniques: RISA and DGGE. The second technique was performed by applying three PCR primer pairs that amplify the V3 (Muyzer *et al*., 1993) and the V3-8 regions of the 16S rRNA gene and fragment of the *amoA* gene (Nicolaisen and Ramsing, 2002).

In order to compare the bacterial communities in the SBR, numerical indicators of microbial diversity were calculated from values of the DGGE and the RISA bands intensity in form of Shannon index (*H*). Diversity indices are a useful approach to estimate the diversity of microbial communities, *e.g.* the higher *H*, the greater microbial diversity (Hill *et al*., 2003).

In this study the lowest average diversity index (*H*) was obtained by applying *amoA* PCR-DGGE, whereas the highest was observed in the total bacterial diversity measured by the V3 region of the 16S rRNA gene analysis. In order to obtain more precise results regarding the microbial diversity the procedure was supplemented by RISA. The average *H* value was similar to that obtained by PCR-DGGE of the most variable region of 16S rDNA (V3), suggesting that this approach could effectively supplement and maybe even replace DGGE technique, when the necessary laboratory equipment is lacking.

The *amoA* primer set has previously been used to study AOB diversity in environmental samples and was acclaimed as a reliable tool to investigate ammonia-oxidizing bacteria diversity (Nicolaisen and Ramsing, 2002). Throughout 36 days of the experiment changes in band patterns, described by Shannon index, were observed. The positive correlation between the AOB diversity and the ammonia level in the effluent occurred, thus greater diversity was negatively correlated with nitrification efficiency. This fact may suggest that ammonia-oxidizing bacteria specialized with time and only species that are well adapted to high ammonia load remained in the activated sludge. This is the disadvantage in relation to the reactor performance as it has been suggested that greater diversity makes the nitrification process more stable (Rowan *et al*., 2003).

Similar results were obtained by the 16S rRNA V3-8 region analysis – increase in NO_3-N production during the experiment correlated with the decrease of microbial diversity. However, amplification of this region of 16S rRNA allowed to obtain only maximum 7 bands, thus it cannot reflect the whole bacterial population diversity. There is no statistically significant relation between the total bacterial richness (measured both by DGGE and RISA) and the nitrification efficiency, which suggests that even rich bacterial composition does not guarantee the efficiency of the process.

The performed time-course experiment allowed observing changes of the bacterial composition in the SBR during 36 days of operation. With time some bands appeared and some vanished, this being distinctly observed between 16^{th} and 22^{nd} day of operation. This shift can be observed in Fig. 1, where two bands (A and B) vanished about the 20^{th} day and in Fig. 4, where band A appeared at the same time. This change of bacterial composition coincides with a rise in nitrate production, that started to increase about 20^{th} day of process (Fig. 3). We infer from these results that the bacterial community, adapted previously to heterotrophic conditions, when subjected to autotrophic conditions shifted from more complicated and diverse to less complex but better adapted to particular substrate conditions. The above-mentioned observation suggests that biological processes, such as nitrification, could be more precisely described by the monitoring of single bands (species) responsible for a particular process than by analyzing whole populations.

In conclusion, PCR-DGGE as well as RISA were useful tools for studying bacterial community composition and changes in time during 36 days of a sequencing batch reactor operation. The results of this work suggest that there is no significant relation between total bacterial diversity and ammonia oxidation efficiency. Autotrophic conditions in the reactor favour only selected species causing a decrease in the diversity of microorganisms.

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Literature

Aoi S., Y. Shiramasa, Y. Masaki, S. Tsuneda, A. Hirata, A. Kitayama and T. Nagamune. 2004. Expression of *amoA* mRNA in wastewater treatment processes examined by competitive RT-PCR. *J. Biotechnol.* 111: 111–120.

Boon N., W. De Windt, W. Verstraete and W. Top. 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group specific 16S rDNA primers for the analysis of bacterial communities from different treatment plants. *FEMS Microbiol. Ecol.* 39: 101–112.

Coelho M.A.Z., C. Russo and O.Q.F. Araujo. 2000. Optimization of sequencing batch reactor for biological nitrogen removal. *Water Res.* 34: 2809–2817.

Dahllöf I. 2002. Molecular community analysis of microbial diversity. *Curr. Opin. Biotechnol.* 13: 213–217.

Daims H., U. Purkhold, L.E. Bjerrum, E. Arnold, P.A. Wilderer and M. Wagner. 2001. Nitrification in sequencing biofilm batch reactors: lessons from molecular approaches. *Water Sci. Technol.* 43: 9–18.

Dolzani L., E. Tonin, C. Lagatolla, L. Prandin and C. Monti-Bragadin. 1995. Identification of *Acinetobacter* isolates in the *A. calcoaceticus-A. baumannii* complex by restriction analysis of the 16S-23S rRNA intergenic-spacer sequences. *J. Clin. Microbiol.* 33: 1108–1113.

Fromin N., Hamelin J., Tarnawski S., Roesti D., Jourdain-Miserez K., N. Forestier N., S. Teyssier-Cuvelle S., Gillet F., Aragno M., and P. Rossi. 2002**.** Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environ. Microbiol*. 4: 634–643.

Gürtler V. and V.A. Stanisich. 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* 142: 3–16.

Hill T.C.J., K.A. Walsh, J.A. Harris and B.F. Moffett. 2003. Using ecological diversity measures with bacterial communities. *FEMS Microbiol. Ecol.* 43: 1–11.

Jensen A., J.A. Webster and N. Strauss. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphism. *Appl. Environ. Microbiol.* 59: 945–952.

Juretschko S., G. Timmermann, M. Schmid, K.H. Schleifer, A. Pommerening-Röser, H.P. Koops and M. Wagner. 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Appl. Environ. Microbiol.* 64: 3042–3051.

Murray A.E., J.T. Hollibaugh and G. Orrego. 1996. Phylogenetic composition of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Appl. Environ. Microbiol.* 62: 2676–2680.

Muyzer G., E.C. de Waal and A.G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rRNA. *Appl. Environ. Microbiol.* 59: 695–700.

Nicolaisen M.H. and N.B. Ramsing. 2002. Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. *J. Microbiol. Methods* 50: 189–203.

Rowan A.K., J.R. Snape, J.D. Fearnside, M.R. Barer, T.P. Curtis and I.M. Head. 2003. Composition and diversity of ammonia-oxidizing bacterial communities in wastewater treatment reactors of different design treating identical wastewater. *FEMS Microbiol. Ecol.* 43: 195–206.

Van der Gucht K., K. Sabbe, L. De Meester, N. Vloemans, G. Zwart, M. Gillis and W. Vyverman. 2001. Contrasting bacterioplankton community composition and seasonal dynamics in two neighboring hypertrophic freshwater lakes. *Environ. Microbiol.* 3: 680–690.

Wagner W. and A. Loy. 2002. Bacterial community composition and function in sewage treatment systems. *Curr. Opin. Biotechnol.* 13: 218–227.