ELSEVIER

FEMS Microbiology Ecology 39 (2002) 101–112

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Evaluation of nested PCR–DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants

Nico Boon, Wim De Windt, Willy Verstraete *, Eva M. Top [1]

Ghent University, Faculty of Agricultural and Applied Biological Sciences, Laboratory of Microbial Ecology and Technology, Coupure Links 653, B-9000 Ghent, Belgium

Received 4 July 2001; received in revised form 29 October 2001; accepted 30 October 2001

First published online 12 December 2001

Abstract

The diversity of bacterial groups of activated sludge samples that received wastewater from four different types of industry was investigated by a nested PCR–DGGE (denaturing gradient gel electrophoresis) approach. Specific 16S rRNA primers were chosen for large bacterial groups (Bacteria and α-Proteobacteria in particular), which dominate activated sludge communities, as well as for actinomycetes, ammonium oxidisers and methanotrophs (types I and II). In addition primers for the new Acidobacterium kingdom were used to observe their community structure in activated sludge. After this first PCR amplification, a second PCR with bacterial primers yielded 16S rRNA gene fragments that were subsequently separated by DGGE, thus generating 'group-specific DGGE patterns'. The community structure and diversity of the bacterial groups from the different samples was further analysed using different techniques, such as statistical analysis and Shannon diversity index evaluation of the band patterns. By combining the seven DGGE gels, cluster analysis, multidimensional scaling and principal component analysis clearly clustered two of the four activated sludge types separately. It was shown that the combination of molecular and statistical methods can be very useful to differentiate microbial communities. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Activated sludge; Community analysis; Nested polymerase chain reaction; Denaturing gradient gel electrophoresis

1. Introduction

Activated sludge of aerobic wastewater treatment plants (WWTP) consists of a complex mixture of microorganisms that are either generalists or specialists. For years, researchers have examined the microbial populations of these activated sludge communities in order to understand their specific biological processes [1]. The presence of certain microbial groups in wastewater treatment systems can cause problems such as poor solid separation, bulking and foaming [2] and needs a more thorough evaluation. When biodiversity is studied by conventional techniques, such as cultivation of bacteria on solid media, these results are quite biased, because a majority of microorganisms are not culturable using standard techniques [3]. For activated sludge the percentage of culturable bacteria in comparison with total cell counts is estimated to range between 1 and 15% [4,5].

During the last decade, methods based on direct PCR amplification and analysis of ribosomal RNA genes were developed and allowed a more comprehensive analysis of microbial communities in comparison with cultivation based techniques. The amplified fragments of 16S or 18S rRNA genes and especially the analysis of these genes by temperature or denaturing gradient gel electrophoresis (DGGE) have been frequently used to examine the microbial diversity of environmental samples and to monitor changes in microbial communities [6–9]. In a DGGE gel the number, precise position, and intensity of the bands in a gel track give an estimate of the number and relative

* Corresponding author. Tel.: +32 (9) 264 59 76; Fax: +32 (9) 264 62 48.
E-mail address: willy.verstraete@rug.ac.be (W. Verstraete).

[1] Present address: University of Idaho, Department of Biological Sciences, 353B Life Sciences Building, Moscow, ID 83844-3051, USA.

0168-6496/01/$22.00 © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0168-6496(01)00198-2

abundance of numerically dominant ribotypes in the sample. This approach allows a comparison of different microbial communities, but not without specific problems. The banding patterns of highly diverse microbial communities, present in soils, activated sludges and sediments, are usually very complex when Bacterial primers are used. Moreover, only the major populations of the analysed community are represented on these DGGE patterns and thus relatively less abundant but potentially very important species may not be detected by this molecular method [10].

A recent evolution in the direct amplification and analysis of ribosomal RNA genes is the use of specific primers, which allows to amplify and analyse the 16S rRNA genes of defined groups within a complex microbial community. The analysis of these group-specific PCR fragments on a DGGE gel provides a valuable tool for monitoring the structure and dynamics of microbial populations over time or under the influence of environmental changes. This approach has already been used in a few studies, which investigated specific microbial groups such as methanotrophic members of the α- and γ-Proteobacteria [11], actinomycetes [10], α- and β-Proteobacteria [12], ammonia-oxidising bacteria [13], Archaea [14] and fungi [15].

In this study, we aimed to standardise the use of DGGE for different group-specific PCR generated 16S rRNA gene fragments, and apply this method to analyse the structure of specific bacterial groups in activated sludge systems of different WWTP. This standardisation was made possible by performing a nested PCR approach. In a first PCR round, specific fragments were amplified by using group-specific primers for methanotrophic members of the α-and γ-Proteobacteria, actinomycetes, α-Proteobacteria, ammonia-oxidising bacteria and Acidobacterium, and in parallel a first set of Bacterial primers was used to amplify all members of the domain Bacteria. The second PCR round was performed with a second set of Bacterial primers and served to reduce and equalise the length of the specific fragments and to add a GC-clamp, necessary for DGGE analysis. Since all above mentioned groups belong to the domain of the Bacteria, the Bacterial primers used in the second PCR round should reamplify all fragments obtained after the first PCR round. The use of this group-specific approach can extend the possibilities of DGGE in microbial community analysis because it can better reveal subtle changes within or differences between microbial communities. To detect and quantify these small differences, the power of statistical tools, such as clustering analysis, multidimensional scaling (MDS), principal component analysis (PCA), diversity index evaluation and regression analysis were evaluated. We chose to limit the analysis to bacterial groups that are known to be present in activated sludge systems, and for which specific 16S rRNA primers have been designed and published previously.

2. Materials and methods

2.1. Activated sludge samples

Activated sludge samples were taken at 15 WWTP from different origins (Flanders, Belgium), i.e., domestic wastewater (samples A), carbohydrate rich wastewater from paper and starch related industries (samples B), protein and fat rich wastewater from food and meat related industries (samples C) and wastewater from textile industry (samples D). A list of the samples together with some operational parameters (SVI = sludge volume index; SRT = sludge retention time; Bx = sludge loading rate) is given in Table 1. The respective companies reported the operational parameters. All samples were collected from the aerated mixed liquid and 50 ml of sample was frozen at −20°C until use. Upon thawing, the total community DNA was extracted and purified as described previously [16].

2.2. PCR–DGGE analysis

All the used primers and PCR conditions are listed in Table 2, and for more information about these primers and PCR conditions, we refer to the original papers. In order to increase the sensitivity and to facilitate the DGGE by analysing fragments of the same length, a nested PCR technique was applied. In the first round different group-specific primers and one set of Bacterial primers were used, each with their own corresponding PCR protocol. During the second PCR round, the obtained fragments were reamplified by using the Bacterial primers P338F and P518r in one and the same PCR protocol (Table 2). Since all mentioned groups in Table 1 belong to the domain of the Bacteria, the Bacterial primers P338F and P518r used in the second PCR round should reamplify all fragments obtained after the first PCR round. After PCR, samples were stored at 4°C (few hours) or at −20°C (days).

The final concentrations of the different components in the mastermix were: 0.2 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1×Taq DNA Polymerase 10×Reaction Buffer ($MgCl_2$-free), 1.25 U/50 μl of Taq DNA Polymerase (Promega, Madison, WI, USA), 400 ng μl^{-1} of bovine serum albumin (Hoffmann-La Roche, Basel, Switzerland), and DNase and RNase free filter sterilised water (Sigma-Aldrich Chemie, Steinheim, Germany). During the first PCR round, 1 μl of extracted DNA was added to 24 μl of PCR mastermix and in the second PCR round, 1 μl of amplified product from the first round was added to 49 μl of PCR mixture. After each PCR amplification round, the size of the PCR product was verified on a 1% agarose gel.

DGGE, based on the protocol of Muyzer et al. [17], was performed using the Bio-Rad D Gene System (Bio-Rad,

Hercules, CA, USA). The PCR products of the second round were loaded onto 8% (w/v) polyacrylamide gels in 1×TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). The polyacrylamide gels were made with denaturing gradient ranging from 50 to 65% (actinomycetes, methanotrophs, Acidobacterium and α-Proteobacteria) or from 45 to 60% (Bacteria and ammonium oxidisers) (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run for 16 h at 60°C and 37 V (Bacteria, ammonium oxidisers and α-Proteobacteria) or 40 V (actinomycetes, methanotrophs, Acidobacterium). After the electrophoresis, the gels were soaked for 5 min in fixation buffer (10% ethanol, 0.5% acetic acid) (optional), and subsequently 10 min in SYBR Green I nucleic acid gel stain (1:10 000 dilution, FMC BioProducts, Rockland, ME, USA). The stained gel was immediately photographed on a UV transillumination table with a Video Camera Module (Vilbert Lourmat, Marne-la Vallé, France).

2.3. Cloning and sequencing analysis

The specific 16S rRNA gene fragments of the first PCR round were cloned by using the TOPO TA cloning kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. DNA sequencing was carried out by IIT Biotech-Bioservice (Bielefeld, Germany). Analysis of DNA sequences and homology searches were completed with the BLAST server of the National Centre for Biotechnology Information using the BLAST algorithm [18] for the comparison of a nucleotide query sequence against a nucleotide sequence database (blastn).

2.4. Analysis of DGGE patterns

The processing of the DGGE gels was done with the Bionumerics software 2.0 (Applied Maths, Kortrijk, Belgium). The calculation of the similarities is based on the Pearson (product–moment) correlation coefficient [19] and results in a distance matrix. The Pearson correlation is an objective coefficient that does not suffer from typical peak/shoulder mismatches as often found when band-matching coefficients are used. Clustering analysis, MDS and PCA, were performed with Bionumerics 2.0. The clustering algorithm of Ward [20] was used to calculate the dendrograms of each DGGE gel and a combination of all gels. By using MDS and PCA analysis, the different data of the complex DGGE patterns of one sample could be reduced to one point in a three dimensional space. MDS does not analyse the original data set, but the distance matrices of each DGGE using a similarity coefficient (Pearson correlation). A PCA analysis is different from MDS, because the data are directly analysed. However, for PCA, fingerprint types can only be processed by generating a band-matching table first. All bands are divided into classes of common bands and for each pattern, a particular band class can have two states: present or absent (binary matrix).

The structural diversity of the microbial community was examined by the Shannon index of general diversity H [21]. 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:

$$H = -\Sigma(n_i/N)\log(n_i/N)$$

where n_i is the height of the peak and N the sum of all peak heights of the densitometric curve. Regression analysis was performed with SPSS for Windows release 7.5.2. to investigate correlation between the Shannon index and operational parameters.

3. Results

The use of DGGE with 16S rRNA gene fragments, regenerated with group-specific primers, was evaluated by comparing the specific DGGE patterns of activated sludge samples from WWTP that receive different types of influents. To be able to correlate certain industrial activities with specific microbial populations, a rough classification was made into four influent types (Table 1).

3.1. Nested PCR

In the first PCR round, group-specific primers were used (Table 2). These specific forward and reverse primers were located before and after the 16S rRNA *Escherichia coli* positions 338 and 518 respectively. Therefore it was possible to use the Bacterial primer set P338F and P518r in a second PCR round. The result of this nested PCR approach was that a fragment of the same 16S rDNA region and the same length was obtained for all specific bacterial groups.

In our study, the size of the amplified fragments of first and second PCR rounds was evaluated on an agarose gel. All the PCR products were of the expected length. Although these primers were tested rigorously before (Table 2), we evaluated the specificity of the PCR amplification by cloning and sequencing three fragments for each specific primer set. The sequence data confirmed that no aspecific amplification had occurred (data not shown). After the first PCR round, the concentration of the PCR products sometimes differed, depending on the initial amount of specific template DNA in the sample. However, after the second PCR round, no differences in PCR product concentration were visible, based on band intensities. For the ammonium oxidiser-specific primers, we examined what the minimum ratio of amplified product over template DNA after the first PCR round should be in order to avoid amplification of non-group-specific template DNA, still present as background in the second PCR round. This

Table 1
Overview of the analysed activated sludges from the WWTP and their operational parameters

Sample	Source	SVI (ml g^{-1})	Bx (g COD g^{-1} d^{-1})	SRT (d)
Domestic wastewater influent				
A1	Municipal waste	107	0.1	10
A2	Hospital waste	120	0.2	17
A3	Municipal waste	160	0.3	4
A4	Municipal waste	220	0.4	5
Carbohydrate rich influent				
B1	Starch production	190	0.1	38
B2	Paper production	100	0.3	8
B3	Potato processing	100	0.1	15
B4	Paper production	30	0.3	6
Protein and fat rich influent				
C1	Food production	109	0.2	5
C2	Food production	160	0.05	50
C3	Slaughterhouse	110	0.2	20
C4	Meat production	70	0.1	21
Textile influent				
D1	Textile	48	0.2	31
D2	Textile	114	0.2	52
D3	Textile	140	0.1	31

COD = chemical oxygen demand; SVI = sludge volume index; Bx = sludge loading rate; SRT = sludge retention time.

was done by comparing the DGGE patterns after the second PCR reaction, in which the PCR product of the first round was either added as such, or serially diluted in a background of the first template DNA. The DGGE patterns showed that non-specific Bacterial fragments only appeared when the PCR product of the first round was diluted more than 10^3 times in the template background. This corresponded with a PCR product concentration of less than 0.3% of the total template DNA (or pg of amplified product per μl) (data not shown). These results strongly indicate that as long as the PCR product of the first round is visible on the gel, there should be no problem with amplification of aspecific ribosomal DNA in the second PCR round.

A potential effect of the second PCR round on the number and relative intensities of the different bands in the final DGGE profile was examined by comparing this profile with that obtained after only the first PCR with the specific primers. On the one hand, a nested PCR fragment, was generated by a first PCR with primer 31f (specific for the Acidobacterium group), without GC-clamp, and primer P518r, followed by a second PCR round with the Bacterial primers P63FGC (a GC-clamp was added to primer P63F), and 518r. On the other hand, another Acidobacterium specific PCR fragment was obtained in a single PCR run with primer 31f with GC-clamp (31fGC) in combination with primer P518r. These primer sets were chosen such that both fragments would only differ 40 bp in length

Table 2
PCR primers used in this study

Target	Primers	PCR conditions							References
		Number of cycles	Denaturation		Annealing		Elongation		
			°C	min	°C	min	°C	min	
First PCR round									
Bacteria	P63F, R1378r	30	95	1	53	1	72	2	[14]
Actinomycetes	F243, R1378r	35	95	1	63	1	72	2	[10]
Ammonium oxidisers	CTO189fAB, CTO189fC, CTO653r	35	94	1	57	1	72	2	[13]
Acidobacterium	31f, R1378r	30	95	1	53	1	72	2	[10,36]
Type I methanotrophs	MB10γ, R1378r	35	94	1	60	1	72	2	[10,36]
Type II methanotrophs	MB9α, R1378r	35	94	1	60	1	72	2	[10,11]
α-Proteobacteria	F203a, R1378r	35	94	1	56	1	72	2	[10,12]
Second PCR round									
Bacteria	P338F[a], P518r	30	95	1	53	1	72	2	[14]

Before each run of cycles, the temperature was held at 95°C for 10 min and after each run the temperature was kept 72°C for 12 min for final template elongation.
[a]A 5′ GC-clamp was added for DGGE analysis [17].

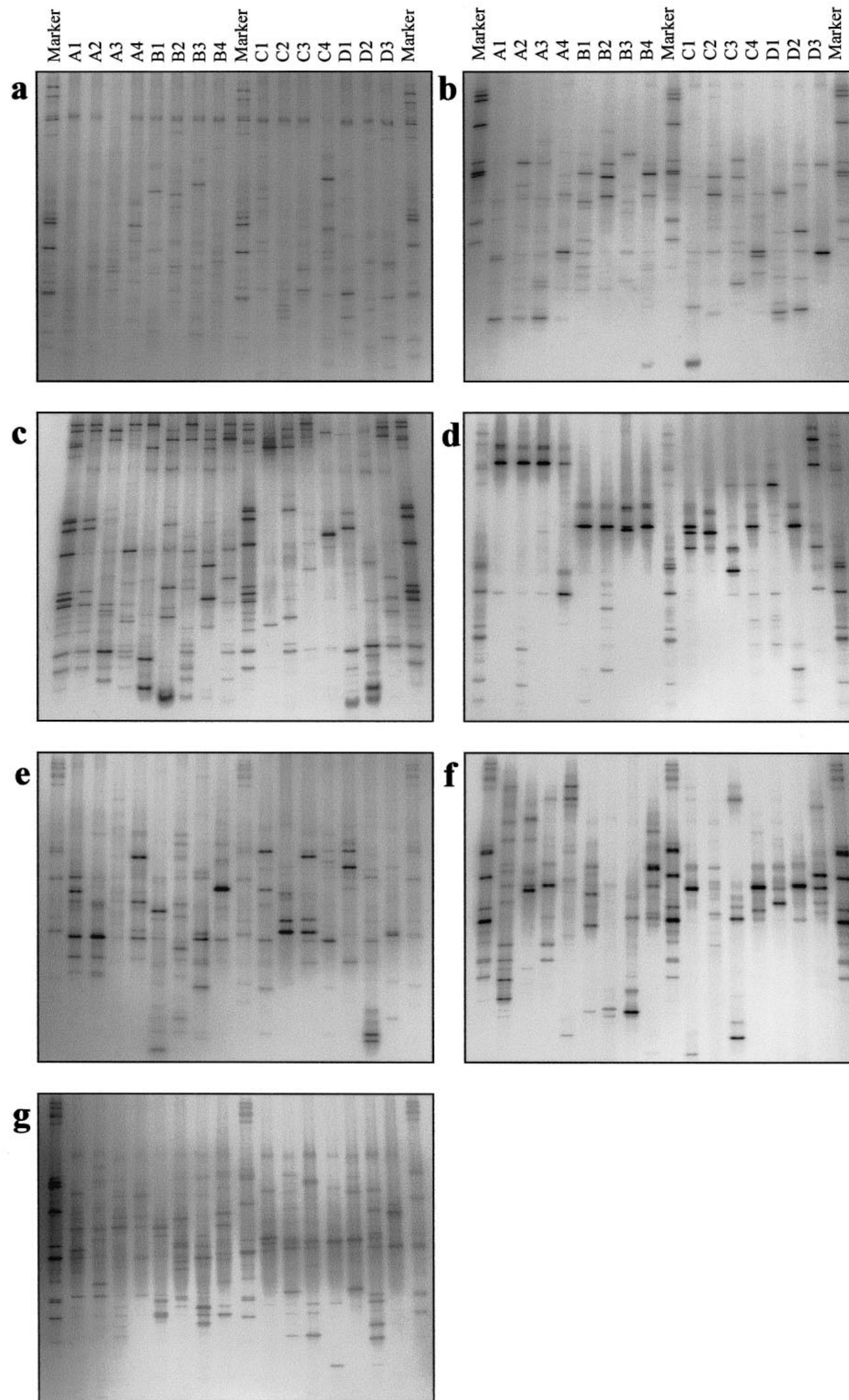

Fig. 1. DGGE analysis of the 15 activated sludge samples, listed in Table 1. Bacteria (a); actinomycetes (b); Acidobacterium (c); ammonium oxidisers (d); methanotrophs, type II (e) and type I (f); α-Proteobacteria (g).

and thus could both be easily analysed on the same DGGE gel. For four samples tested, both patterns were very similar both in position, number and intensities of the band, except that the positions of the P63FGC-518r PCR fragments were all shifted downwards in comparison to the 31fGC-518r fragments, due to the larger length of the latter fragment (data not shown). This suggests that the second PCR round did not drastically change the number nor the intensities of the DGGE bands, compared to a one-step PCR protocol with specific primers.

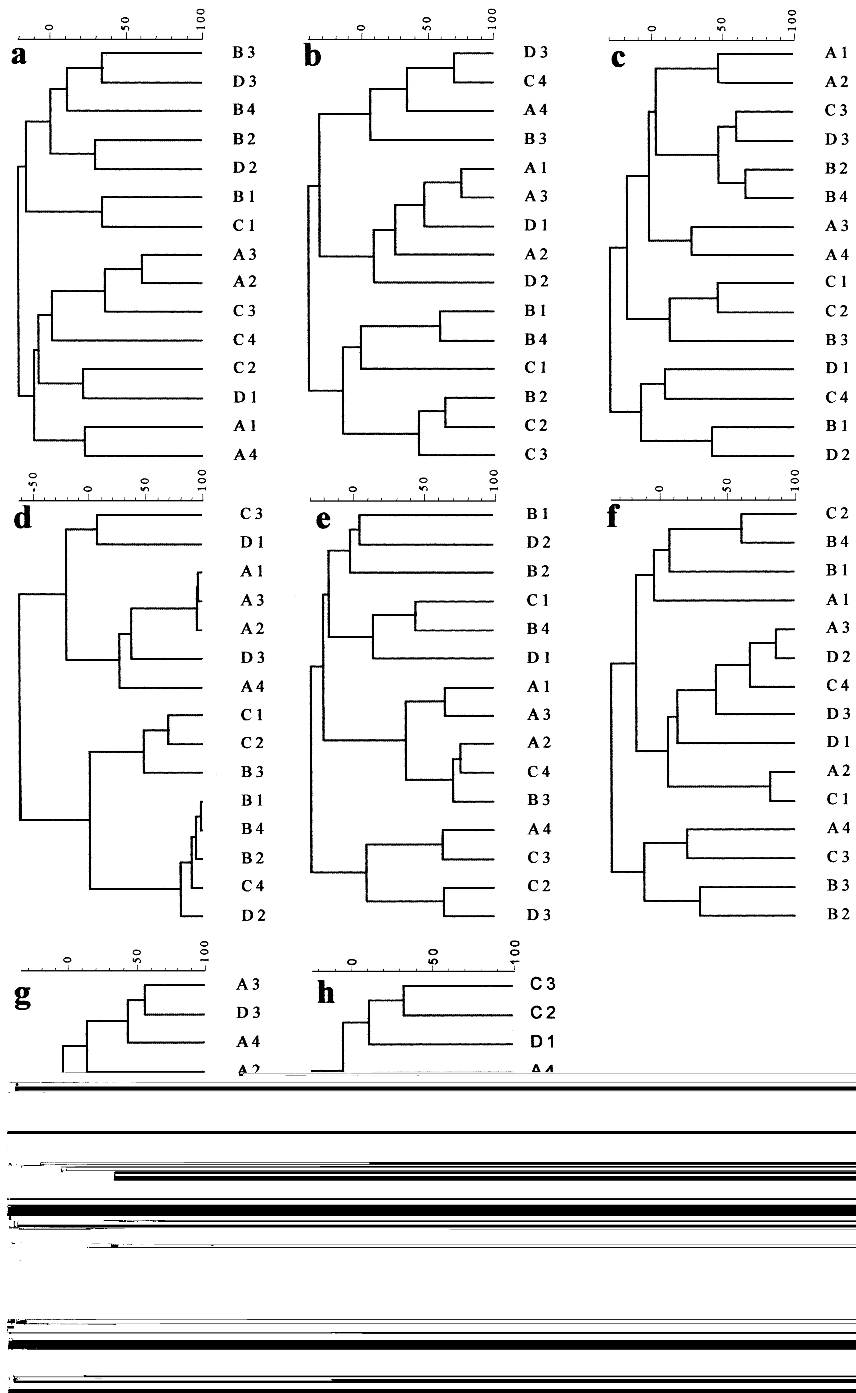

Fig. 2. Clustering analysis of the DGGE patterns of the 15 samples, listed in Table 1: Bacteria (a); actinomycetes (b); Acidobacterium (c); ammonium oxidisers (d); Methanotrophs, type II (e) type I (f); α-Proteobacteria (g); all different DGGE tracks combined (h).

3.2. *DGGE analysis of PCR-amplified 16S rRNA gene fragments*

Fig. 1 shows the seven DGGE gels of the different bacterial groups. The DGGE patterns obtained with the Bacterial primers did not show many intensive bands (Fig. 1a). A few dominant bacteria were present in some samples, but in all samples the high number of weak bands resulted in a smear. This is probably due to the high number of different Bacterial species present in the sludge. Also the DGGE patterns of the α-Proteobacteria were very complex (Fig. 1g). The DGGE pattern obtained with the Acidobacterium primers also contained a relatively high number of bands (Fig. 1c), which indicates that this group is highly diverse as well. Every activated sludge sample seemed to have a unique Acidobacterium population and common bands were exceptional. The actinomycete, methanotrophs and ammonium oxidising pop-

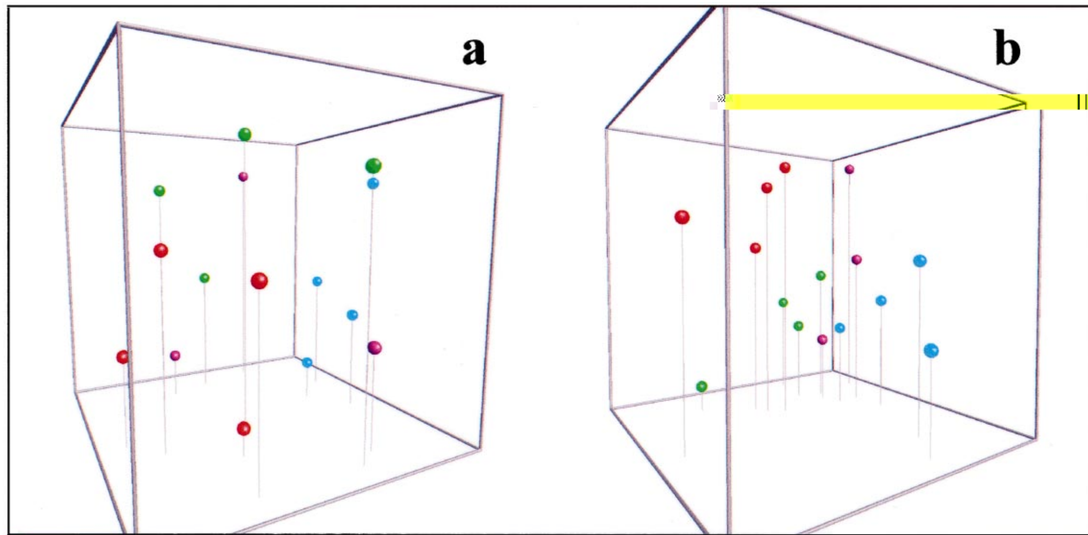

Fig. 3. Representation of two dimension techniques, calculated for each track of each DGGE gel with group-specific 16S rDNA fragments: MDS (a) and PCA (b). Samples A (blue dots); samples B (red dots); samples C (green dots); samples D (purple dots).

ulations in all sludge samples seemed to consist of only a limited number of dominant species, without a smear of weak bands (Fig. 1b,d–f). Striking were the patterns of the ammonium oxidising populations for the sludge samples of groups A and B. The four DGGE patterns within each of these two types of sludge showed great similarity among each other, although they came from four different WWTP. This was not the case for patterns from the sludge groups C and D.

3.3. Analysis of DGGE banding patterns

Highly diverse ecosystems, such as activated sludge, sediments and soils, have DGGE banding patterns that are very complex to interpret. Therefore, computer-aided analyses are necessary to examine these patterns by means of densitometric curves. In the densitometric curve of a gel track, the number of peaks and their intensity reflect the number and relative abundance of the dominant 16S rRNA gene sequences. Thus, a numerical comparison can be made based on these densitometric curves. To be able to perform such analyses on each gel, three standard patterns (markers) were included to allow a normalisation of the gels. The normalised gels were then used to perform the calculations for the different statistical analyses and for the calculation of the Shannon diversity index.

The different DGGE banding patterns were examined in three ways, i.e., clustering, analysis by dimensioning techniques (MDS and PCA) and Shannon diversity index analysis. First, the information of the different tracks was analysed for one type of group-specific fragments by calculating a distance matrix of all the possible gel tracks within the DGGE patterns by using the Pearson correlation. Based on the values of the resulting matrix, a cluster analysis was performed and the 15 different samples were visualised in dendrograms (Fig. 2). In the dendrograms, sometimes several sludge samples (three or four) of the same type of wastewater were located in one cluster, such as for samples A (Fig. 2a,b,d,e,g), samples B (Fig. 2a,b,d,g), samples C (Fig. 2a,b,d,g) and samples D (Fig. 2a,b,d,f,g). At first sight, no real conclusions or correlations could be made when Bacterial patterns were compared, while on the group-specific level, the clustering analysis in some cases led to interpretable results. Finally, a dendrogram was created based on the combination of all data from the seven different gels (Fig. 2h). Remarkably,

Table 3
Mean and standard deviation of the Shannon diversity index for the sludges of the different WWTP groups

	Samples A	Samples B	Samples C	Samples D	Average
Bacteria	1.08 ± 0.08	1.07 ± 0.06	1.06 ± 0.06	0.98 ± 0.09	1.05 ± 0.08
Actinomycetes	0.81 ± 0.14	0.70 ± 0.11	0.66 ± 0.12	0.59 ± 0.27	0.70 ± 0.16
Acidobacterium	1.04 ± 0.10	1.05 ± 0.07	0.91 ± 0.25	0.97 ± 0.09	1.00 ± 0.15
Ammonium oxidisers	0.45 ± 0.09^{D}	0.45 ± 0.16^{D}	0.58 ± 0.14	0.76 ± 0.09^{AB}	0.54 ± 0.17
Type I methanotrophs	0.91 ± 0.21	0.71 ± 0.14	0.78 ± 0.21	0.62 ± 0.19	0.86 ± 0.20
Type II methanotrophs	0.95 ± 0.06^{C}	0.88 ± 0.13	0.80 ± 0.05^{A}	0.83 ± 0.10	0.76 ± 0.10
α-Proteobacteria	1.08 ± 0.13	1.07 ± 0.13	0.97 ± 0.14	0.85 ± 0.20	1.00 ± 0.16

^{A,B,C,D}Samples which are significantly different (t-test), with $P < 0.05$.

this resulted in a dendrogram with two clusters, which clearly separated the patterns of the different activated sludge groups A and B. This was not the case for the activated sludge groups C and D, where more variation was present within each group of samples.

MDS and PCA are two alternative grouping techniques that can both be classified as dimensioning techniques. These techniques produce two or three-dimensional plots in which the entries are spread according to their relatedness. Unlike a dendrogram, a MDS or PCA plot does not provide clusters and thus the interpretation is more subjective. To perform the MDS and PCA analysis, all DGGE patterns available for the different activated sludge samples were included. MDS just replaces the clustering step and it is an alternative to the dendrogram methods, which often oversimplify the data available in a similarity matrix and tend to produce overestimated hierarchies. The MDS analysis shows that the different activated sludge groups in the three-dimensional plot are not grouped together (Fig. 3a). The samples of the fat and protein rich influents (C) and of the textile industry (D) are more distributed over the whole plot, while the other sample types are more or less grouped. This corresponds to the results of the overall cluster analysis of all DGGE patterns. A PCA analysis distinguishes itself from a MDS, because a PCA analyses the data directly by a binary band-matching table. Therefore, the same sets of data will result in a different PCA and MDS plot (Fig. 3b). The three principal components (PC) explained a low and almost equal percentage of the total variation (PC 1 = 12%; PC 2 = 10%; PC 3 = 9%). The PCA did not separate the different groups completely, but all the sludge samples of one wastewater type were localised together in the same area.

The third method that was used to compare the bacterial communities of the different samples, was the calculation of the Shannon diversity index H, based on the DGGE banding patterns of the specific groups. Diversity indices are useful as a first approach to estimate the diversity of microbial communities, i.e., the higher H, the greater the diversity of the microbial community. A diversity index consists of two components: (i) the total numbers of species present or species richness and (ii) the distribution of the number of individuals among those different species, called species evenness, or species equability [22]. The averages and the standard deviations of the Shannon index H values for each activated sludge group and for all samples are listed in Table 3. Also a two-tailed t-test was performed to investigate if the H values of different activated sludge groups were significantly different. The diversity index of ammonium oxidisers was significantly higher in textile wastewater activated sludge types (samples D) than in the activated sludge types A and B. The Shannon index for the methanotrophs type II population was significantly different between sludge groups A and C. The Bacteria and the α-Proteobacteria showed the highest average index H of the sludge samples. Remarkably, the standard deviation of the Bacterial indices was very low, in comparison with those of the other bacterial groups. The ammonium oxidiser community had the lowest average Shannon index H. This is a result of the very limited number of bands, visible in the gel (Fig. 1d).

The Shannon diversity indices of the different groups and the operational parameters were subjected to a correlation analysis. The diversity indices for the ammonium oxidisers correlated positively with the SRT ($P = 0.05$; $R^2 = 0.31$), while the index of the Bacteria ($P = 0.05$; $R^2 = 0.30$) had a negative correlation. The Shannon index of the actinomycetes was positively correlated to the SVI ($P = 0.10$; $R^2 = 0.22$).

4. Discussion

4.1. Nested PCR

In this study a nested PCR approach was chosen to facilitate the analysis of the 16S rRNA gene fragments of different bacterial subgroups by DGGE. A comparable approach has been used earlier by Heuer et al. [10] to monitor actinomycete community changes in potato rhizosphere and to investigate actinomycete diversity in different soils. They used the actinomycete-specific primer F243 (same as in this study), followed by the Bacterial primers F984GC and R1378. An advantage of the nested PCR approach in our study is that the final group-specific DGGE patterns can be directly compared (under the same DGGE conditions) to all the other group-specific and Bacterial patterns of the same sample, because the same 16S rRNA gene fragment was amplified in the second PCR. As a result, only small optimisation of the denaturing gradient and electrophoretic conditions is needed to obtain a good separation for all the fragments of the different bacterial groups. A second advantage of using nested PCR with specific primers is the increased sensitivity, which makes it possible to visualise also those species that are present in lower numbers. Phillips et al. [23] detected ammonium oxidisers by using a nested PCR approach with the specific primers, while the abundance of these ammonium oxidisers was maximum 0.01% of the total bacterial soil community.

The use of nested PCR should be evaluated with caution. A possible disadvantage of applying two successive PCR reactions is the introduction of an even greater bias due to preferential amplification [24]. However, according to previous studies this bias of preferential amplification may be overestimated, and the intensity of the 16S rDNA bands in a DGGE gel may correspond at least semiquantitatively with the abundance of the corresponding species [10,25].

The number and intensity of bands in a DGGE gel do not necessarily give an accurate picture of the number and abundance of the corresponding species within the micro-

bial community. One organism may produce more than one DGGE band because of multiple, heterogeneous rRNA operons [26–28]. On the other hand, partial 16S rDNA sequences do not always allow discrimination between species, such that one DGGE band may represent several species with identical partial 16S rDNA sequences [29]. In addition, in a mixture of target rDNAs present at very different concentrations, the less abundant sequences are not amplified sufficiently to be visualised as bands on a DGGE gel. Therefore, the banding pattern reflects only the most abundant rDNA types in the microbial community [17]. Because of these shortcomings inherent in 16S rDNA DGGE, the diversity index calculated from the DGGE banding patterns of amplified 16S rDNA sequences must be interpreted as only an indication and not an absolute measure of the degree of diversity in a bacterial community [9].

A problem with the application of group-specific primers for the analysis of natural microbial communities is that the prediction of their specificity relies on the available cultured isolates and on known sequences in the database, which may not adequately reflect the entire pool of 16S rDNA sequences in nature. The study of Purkhold et al. [30] clearly demonstrated that specific primers for ammonium oxidisers are, in many cases, not sufficiently specific. Also primer F243 might not be the ideal primer for all studies of actinomycetes because it does not match the 16S rDNA of all actinomycetes and it matches the 16S rDNA of a few non-actinomycetes [10]. Nevertheless, the primer is useful to enrich actinomycete 16S rDNA in order to improve the detection of this group from environmental samples [10]. Also in our study, there is no absolute certainty that all the amplified bands in the DGGE gels represent true members of the respective group. However, sequence analysis of three randomly chosen DGGE bands for each primer set confirmed that the sequences corresponded to bacteria belonging to the respective amplified group. In the future, thanks to the ever expanding sequence information, improved primer design will allow a more accurate development of group-specific PCR–DGGE approach.

4.2. DGGE analysis of different bacterial groups

To obtain an optimal separation of the PCR fragments on DGGE, slight modifications of the running voltage and the gradient had to be performed. After this optimisation it was possible to analyse the seven different groups of one sludge sample on one gel, using a voltage of 40 V and a gradient of 45–65%. A slight disadvantage of this approach is a less optimal separation of certain bands in some groups (data not shown).

For many sludge samples, several bands of some of the analysed specific groups were not visible in the corresponding Bacterial pattern. Heuer et al. [10] made a similar observation when they examined actinomycetes in soil samples with the same approach. This indicates that species, other than the specific groups, analysed in this study, were more abundant in these activated sludges, and therefore masked the presence of some of the members of these subgroups. The appearance of new bands when group-specific primers were used, confirms that diversity reflected by Bacterial DGGE patterns only relates to the numerically dominant species and not at all to the total number of different species in the environmental sample. Muyzer et al. [17] showed that the presence of a few dominant species leads to a simple pattern, and that species of less than 1% of the analysed community were not represented in the microbial community pattern. This will also be true for PCR–DGGE for certain very diverse bacterial groups: only species that are dominant within this specific group will be visible. For subgroups that still contain a large number of different 16S rRNA types, further subdivision using more specific primers could be useful if more detailed analysis is desired.

In this study, only one activated sludge sample of each WWTP was investigated. To monitor the reproducibility in time, two samples of the WWTP A1 were taken with an interval of 6 months, and their DGGE profiles of the Bacterial and Acidobacterium populations did not show much variation (data not shown). Other investigators also did not find evidence for variation in the WWTP they studied by PCR–DGGE and concluded that a single sample of an activated sludge plant was sufficient for a plant to plant comparison [8]. However, more elaborate studies to examine abiotic parameters, such as seasonal variation, rainfall, temperature, etc., are necessary to confirm the stability of microbial communities in activated sludge.

Fluorescent in situ hybridisation (FISH) analysis has shown before that on average 70–90% of the observable micro-organisms in activated sludge are binding with the Bacteria probe EUB [4]. In the same study the authors observed that the α-Proteobacteria accounted for 60–75% of the Bacteria. Whereas members of the β subclass were common in both high- and low-load aeration basins, members of the α subclass were more common in low-load basins [4]. Earlier work has also demonstrated that the α-Proteobacteria are numerically dominant in activated sludge [31,32] and that some species have been shown to form filaments in activated sludge systems [33,34]. Our DGGE patterns of the Bacteria and α-Proteobacteria are very complex, because these groups seem to have not only a high number of cells in the activated sludge microbial community, but also a high number of different species (ribotypes). This was also reflected in the Shannon diversity index. The Bacteria and the α-Proteobacteria have the highest average index H of the investigated samples. The high indices are the result of the high number of bands and the absence of a few very dominating bacteria. Within the profiles of the Bacteria, the variance of H was very

low. Therefore the comparison of different ecosystems by DGGE analysis would better be carried out by studying specific bacterial groups.

The kingdom Acidobacterium is a recently discovered bacterial lineage and at this time contains only a few cultured representatives [35,36]. The Acidobacterium group seems to be present in many ecosystems, particularly in soils, while only one study so far has reported its presence in wastewater [37]. The exact role and the ecological significance of these bacteria is still unknown, however recent developments indicate that members of the kingdom Acidobacterium are involved in methanol metabolism [38]. Barns et al. [36] have suggested that members of the Acidobacterium kingdom could be as genetically and metabolically diverse, as environmentally widespread and, perhaps, as ecologically important as the well-known Proteobacteria and Gram-positive bacterial kingdoms. To study the diversity of this kingdom, the latter authors designed a specific forward primer to clone Acidobacterium 16S rRNA gene sequences [36], but until now, DGGE analysis of this new bacterial kingdom has not yet been published. Remarkably, none of the DGGE patterns in the different samples were comparable. This was also observed by the absence of real groups in the clustering analysis. The Shannon diversity index of the Acidobacterium group was as high as the Bacteria and α-Proteobacteria, which could indicate that also in activated sludge a very diverse Acidobacterium community is present.

We included the analysis of actinomycetes in this study, because these bacteria can cause severe bulking and foaming in activated sludge plants [39–41]. Their hydrophobic cell surface is supposed to support adherence and stabilisation of interfaces and thus promote sludge flotation, leading to a higher SVI [42]. In our activated sludge samples only a few species were dominating the actinomycete community, based on the DGGE patterns, and the type of industrial influent seems to determine its species composition. A weak positive correlation was found between the Shannon index of diversity of the actinomycetes, and the SVI. Although the amount of samples investigated in this study was too low to draw firm conclusions from this observation, the correlation could be related to the role of these bacteria in sludge bulking.

Methanotrophs have already been isolated from different activated sludges [43–45] and they were also clearly present in the samples examined in this study. They are a phylogenetically heterogeneous group, belonging to the α- or γ-Proteobacteria. Seven genera of type I (γ-Proteobacteria) and type II (α-Proteobacteria) methanotrophs have been proposed [46]. These bacteria can utilise C-1 molecules, such as methane, methanol and formaldehyde. The samples analysed with group-specific primers for the type I and II methanotrophs, both with the same metabolic function, cluster differently. This phenomenon was already observed in groundwater, where both types react differently on changing conditions, such as biostimulation [47].

The 16S rRNA gene fragments related to the β-Proteobacteria subgroup of the ammonium oxidisers were examined because of their importance in the nitrogen removal process. The most prominent members of this β-subgroup in activated sludge are usually related to the genera *Nitrosomonas* or *Nitrosospira* [48]. Within this group of bacteria, clustering analysis of their DGGE patterns showed a clear similarity among the samples of domestic wastewater (group A) and also among those of the carbohydrate rich wastewater (group B). The ammonium oxidisers in the activated sludge samples of the textile industry (group D) were significantly more diverse than groups A and B, as observed by Shannon diversity index evaluation. In textile wastewaters, azo-dyes and their metabolites, i.e., chloroanilines, can be present and these compounds have a negative influence on the nitrification [49]. This could lead to a lower density of common ammonium oxidisers, which might result in a more diverse ammonium oxidising community. More work would have to be done however to confirm this hypothesis.

More and more often, the interpretation of complex DGGE patterns is carried out with techniques such as MDS [47,50] and PCA plots [51,52]. We also tried to analyse our data with both techniques, but no clearly defined groups could be observed. Activated sludge populations are very complex systems and the reduction into four groups based on influent type is too simplistic. The operational parameters and nutrient removal efficiencies were different for every plant and may have an important impact on the composition of the microbial communities. An indication for the latter interaction was given by the correlation analysis between the Shannon diversity indices of the different groups and the operational parameters. The regression model was significant, however the low R^2 values make it impossible to draw real conclusions. To know the effect of the parameters on the different bacterial subpopulations, experiments with one type of sludge and controlled changes of one parameter should be performed.

5. Conclusions

This study clearly shows that it is possible to obtain a view on activated sludge bacterial communities that was previously inaccessible. The integration of the fingerprinting data with statistical tools can be used to show the biological relationships between different activated sludges. A greater understanding of activated sludge microbiology is then expected to lead to improvements in analysis and control of activated sludge treatment processes. In the future, when specific primers will be designed for an increasing number of groups, a more complete picture of bacterial communities will be obtained. DGGE with group-specific 16S rRNA primers is not only useful to

compare different microbial communities, but also to monitor microbial communities in function of time. Combination of qualitative (such as DGGE with group-specific 16S rRNA primers) and quantitative techniques (such as FISH or real time PCR [53]) would be a next step in acquiring a good descriptive tool for microbial community analysis of activated sludges.

Acknowledgements

This work was supported by the project grant G.O.A. (1997–2002) of the 'Ministerie van de Vlaamse Gemeenschap, Bestuur Wetenschappelijk Onderzoek' (Belgium). We thank Steven Siciliano for the useful suggestions, Johan Goris for the help with the gel analysis, Siska Maertens and Katrien Crul for technical assistance and Winnie Dejonghe, Geert Rombaut, Vanessa Vermeirssen, Hanne Lievens, Dave Seghers and Elke Vincke for critically reading the manuscript. We also thank the different companies for their co-operation and sampling of the activated sludge and K. Smalla for providing us the sequence for the α-Proteobacteria-specific primer.

References

[1] Amann, R., Glockner, F.O. and Neef, A. (1997) Modern methods in subsurface microbiology: in situ identification of microorganisms with nucleic acid probes. FEMS Microbiol. Rev. 20, 191–200.

[2] Blackall, L.L., Burrell, P.C., Gwilliam, H., Bradford, D., Bond, P.L. and Hugenholtz, P. (1998) The use of 16S rDNA clone libraries to describe the microbial diversity of activated sludge communities. Water Sci. Technol. 37, 451–454.

[3] Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

[4] Wagner, M., Amann, R., Lemmer, H. and Schleifer, K.H. (1993) Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. 59, 1520–1525.

[5] Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D. and Schleifer, K.H. (1994) Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. Appl. Environ. Microbiol. 60, 792–800.

[6] Muyzer, G. and Smalla, K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol. 73, 127–141.

[7] van Elsas, J.D., Duarte, G.F., Rosado, A.S. and Smalla, K. (1998) Microbiological and molecular biological methods for monitoring microbial inoculants and their effects in the soil environment. J. Microbiol. Methods 32, 133–154.

[8] Curtis, T.P. and Craine, N.G. (1998) The comparison of the diversity of activated sludge plants. Water Sci. Technol. 37, 71–78.

[9] Eichner, C.A., Erb, R.W., Timmis, K.N. and Wagner-Döbler, I. (1999) Thermal gradient gel electrophoresis analysis of bioprotection from pollutant shocks in the activated sludge microbial community. Appl. Environ. Microbiol. 65, 102–109.

[10] Heuer, H., Krsek, M., Baker, P., Smalla, K. and Wellington, E.M.H. (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. 63, 3233–3241.

[11] Henckel, T., Friedrich, M. and Conrad, R. (1999) Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase. Appl. Environ. Microbiol. 65, 1980–1990.

[12] Gomes, N.C.M., Heuer, H., Schönfeld, J., Costa, R., Mendonça-Hagler, L. and Smalla, K. (2001) Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant Soil 232, 167–180.

[13] Kowalchuk, G.A., Bodelier, P.L.E., Heilig, G.H.J., Stephen, J.R. and Laanbroek, H.J. (1998) Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. FEMS Microbiol. Ecol. 27, 339–350.

[14] Øvreas, L., Forney, L., Daae, F.L. and Torsvik, V. (1997) Distribution of bacterioplankton in meromictic lake Saelevannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl. Environ. Microbiol. 63, 3367–3373.

[15] Smit, E., Leeflang, P., Glandorf, B., van Elsas, J.D. and Wernars, K. (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Appl. Environ. Microbiol. 65, 2614–2621.

[16] Boon, N., Goris, J., De Vos, P., Verstraete, W. and Top, E.M. (2000) Bioaugmentation of activated sludge by an indigenous 3-chloroaniline degrading *Comamonas testosteroni* strain, I2*gfp*. Appl. Environ. Microbiol. 66, 2906–2913.

[17] Muyzer, G., de Waal, E.C. and Uitterlinden, A. (1993) Profiling of complex microbial populations using denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

[18] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

[19] Pearson, K. (1926) On the coefficient of radical likeliness. Biometrika 18, 105–117.

[20] Ward, J.H. (1963) Hierarchical grouping to optimize an objective function. J. Am. Stat. Assoc. 58, 236–244.

[21] Shannon, C.E. and Weaver, W. (1963) University of Illinois Press, Urbana, IL.

[22] Kennedy, A.C. and Smith, K.L. (1995) Soil microbial diversity and the sustainability of agricultural soils. Plant Soil 170, 75–86.

[23] Phillips, C.J., Harris, D., Dollhopf, S.L., Gross, K.L., Prosser, J.I. and Paul, E.A. (2000) Effects of agronomic treatments on structure and function of ammonia-oxidizing communities. Appl. Environ. Microbiol. 66, 5410–5418.

[24] Suzuki, M. and Giovannoni, S. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62, 625–630.

[25] Heuer, H. and Smalla, K. (1997) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities. in: Modern Soil Mmicrobiology (van Elsas, J.D., Wellington, E.M.H. and Trevors, J.T., Eds.). Marcel Dekker, New York.

[26] Nubel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W. and Backhaus, H. (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. 178, 5636–5643.

[27] Rainey, F.A., Ward Rainey, N.L., Janssen, P.H., Hippe, H. and Stackebrandt, E. (1996) *Clostridium paradoxum* DSM 7308(T) contains multiple 16S rRNA genes with heterogeneous intervening sequences. Microbiology 142, 2087–2095.

[28] Cilia, V., Lafay, B. and Christen, R. (1996) Sequence heterogeneities among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. Mol. Biol. Evol. 13, 451–461.
[29] Vallaeys, T., Topp, E., Muyzer, G., Macheret, V., Laguerre, G., Rigaud, A. and Soulas, G. (1997) Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. FEMS Microbiol. Ecol. 24, 279–285.
[30] Purkhold, U., Pommerening-Roser, A., Juretschko, S., Schmid, M.C., Koops, H.P. and Wagner, M. (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: Implications for molecular diversity surveys. Appl. Environ. Microbiol. 66, 5368–5382.
[31] Mudaly, D.D., Atkinson, B.W. and Bux, F. (2001) 16S rRNA in situ probing for the determination of the family level community structure implicated in enhanced biological nutrient removal. Water Sci. Technol. 43, 91–98.
[32] LaPara, T.M., Nakatsu, C.H., Pantea, L. and Alleman, J.E. (2000) Phylogenetic analysis of bacterial communities in mesophilic and thermophilic bioreactors treating pharmaceutical wastewater. Appl. Environ. Microbiol. 66, 3951–3959.
[33] Neef, A., Witzenberger, R. and Kampfer, P. (1999) Detection of sphingomonads and in situ identification in activated sludge using 16S rRNA-targeted oligonucleotide probes. J. Ind. Microbiol. Biotechnol. 23, 261–267.
[34] Nielsen, J.L., Mikkelsen, L.H. and Nielsen, P.H. (2001) In situ detection of cell surface hydrophobicity of probe- defined bacteria in activated sludge. Water Sci. Technol. 43, 97–103.
[35] Sievert, S.M., Kuever, J. and Muyzer, G. (2000) Identification of 16S ribosomal DNA-defined bacterial populations at a shallow submarine hydrothermal vent near Milos Island (Greece). Appl. Environ. Microbiol. 66, 3102–3109.
[36] Barns, S.M., Takala, S.L. and Kuske, C.R. (1999) Wide distribution and diversity of members of the bacterial kingdom Acidobacterium in the environment. Appl. Environ. Microbiol. 65, 1731–1737.
[37] Ludwig, W. et al. (1997) Detection and in situ identification of representatives of a widely distributed new bacterial phylum. FEMS Microbiol. Lett. 153, 181–190.
[38] Radajewski, S., Ineson, P., Parekh, N.R. and Murrell, J.C. (2000) Stable-isotope probing as a tool in microbial ecology. Nature 403, 646–649.
[39] Davenport, R.J., Curtis, T.P., Goodfellow, M., Stainsby, F.M. and Bingley, M. (2000) Quantitative use of fluorescent in situ hybridization to examine relationships between mycolic acid-containing actinomycetes and foaming in activated sludge plants. Appl. Environ. Microbiol. 66, 1158–1166.
[40] Madoni, P., Davoli, D. and Gibin, G. (2000) Survey of filamentous microorganisms from bulking and foaming activated-sludge plants in Italy. Water Res. 34, 1767–1772.
[41] Seong, C.N., Kim, Y.S., Baik, K.S., Lee, S.D., Hah, Y.C., Kim, S.B. and Goodfellow, M. (1999) Mycolic acid-containing actinomycetes associated with activated sludge foam. J. Microbiol. 37, 66–72.
[42] Lemmer, H., Lind, G., Muller, E., Schade, M. and Ziegelmayer, B. (2000) Scum in activated sludge plants: Impact of non-filamentous and filamentous bacteria. Acta Hydrochim. Hydrobiol. 28, 34–40.
[43] Gisi, D., Willi, L., Traber, H., Leisinger, T. and Vuilleumier, S. (1998) Effects of bacterial host and dichloromethane dehalogenase on the competitiveness of methylotrophic bacteria growing with dichloromethane. Appl. Environ. Microbiol. 64, 1194–1202.
[44] Colquhoun, K.O. (1994) A proposed pathway for the biodegradation of hexamethylenetetramine. Water Sci. Technol. 30, 95–101.
[45] Aleshchenkova, Z.M., Samsonova, A.S. and Semochkina, N.F. (1999) Effects of degrading microorganisms on purification of wastewater from Lavsan production enterprises with the use of activated sludge. Appl. Biochem. Microbiol. 35, 404–406.
[46] Bowman, J., McCammon, S. and Skerratt, J. (1997) *Methylosphaera hansonii* gen. nov., sp. nov., a psychrophilic, group I methanotroph from Antarctic marine-salinity, meromictic lakes. Microbiology 143, 1451–1459.
[47] Iwamoto, T., Tani, K., Nakamura, K., Suzuki, Y., Kitagawa, M., Eguchi, M. and Nasu, M. (2000) Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. FEMS Microbiol. Ecol. 32, 129–141.
[48] Ballinger, S.J., Head, I.M., Curtis, T.P. and Godley, A.R. (1998) Molecular microbial ecology of nitrification in an activated sludge process treating refinery wastewater. Water Sci. Technol. 37, 105–108.
[49] Xiong, X.J., Hirata, M., Takanashi, H., Lee, M.G. and Hano, T. (1998) Analysis of acclimation behavior against nitrification inhibitors in activated sludge processes. J. Ferment. Bioeng. 86, 207–214.
[50] Schafer, H. etal. (2001) Microbial community dynamics in Mediterranean nutrient-enriched seawater mesocosms: changes in the genetic diversity of bacterial populations. FEMS Microbiol. Ecol. 34, 243–253.
[51] Rasmussen, L.D. and Sorensen, S.J. (2001) Effects of mercury contamination on the culturable heterotrophic, functional and genetic diversity of the bacterial community in soil. FEMS Microbiol. Ecol. 36, 1–9.
[52] Müller, A., Westergaard, K., Christensen, S. and Sørensen, S.J. (2001) The effect of long-term mercury pollution on the soil microbial community. FEMS Microbiol. Ecol. 36, 11–19.
[53] Amann, R. and Ludwig, W. (2000) Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. FEMS Microbiol. Rev. 24, 555–565.