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Evaluation and Analysis of Bacterial Communities from Different Waste Water Treatment Plants by Denaturing Gradient Gel Electrophoresis with Group Specific 16s rRNA

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Abstract The diversity of different 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 oxidizers and methanotrophs (Types I and II). In addition primers for the new *Acidobacterium* group 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 analyzed 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 (MDS) and Principal Component Analysis (PCA) 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 activated sludge microbial communities.

Keywords: *DGGE, 16s rRNA, Waste Water Treatment, PCR*

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1. Introduction

Activated sludge of aerobic wastewater treatment plants 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 denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) have been frequently used to examine the microbial diversity of environmental samples and to monitor changes in microbial communities [6,7,8,9]. In a DGGE gel the number and precise position of the bands in a gel track give an estimate of the number 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 constituents of the analyzed 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 amplifying and analyzing 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 the microbial subpopulations 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-oxidizing bacteria [13], Archaea [14] and fungi [15]. In this study, we aimed to simplify the use of DGGE for different group-specific PCR generated 16S rRNA gene fragments, and apply this method to analyze the structure of specific bacterial groups in activated sludge systems of different wastewater treatment plants. This standardization 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-oxidizing 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, which amplified an internal fragment, and served to reduce and equalize 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 those groups for which specific 16S rRNA primers have been designed and published previously, and which are known to be present and even important in activated sludge systems.

2. Materials and Methods

2.1. Activated Sludge Samples

Activated sludge samples were taken from ten different wastewater treatment plants (WWTP) from Ankleshwar, India. All samples were collected from the aerated mixed liquor 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. DNA Quantification

DNA was measured by staining it with SYBR Green I nucleic acid gel stain (1: 10,000 dilution; FMC BioProducts, Rockland, ME, USA) and the emission intensity was measured with a microplate spectrofluorometer (Perkin-Elmer,Shelton, USA).

Table 1. PCR primers used in this study

Target	Primers	Number of cycles	Denaturation		Annealing		Elongation		References
First PCR Round			°C	Min	°C	Min	°C	Min	
Bacteria	P63F, R1378r	30	95	1	53	1	72	2	221
Actinomycetes	F243, R1378r	35	95	1	63	1	72	2	126
Ammonium oxidisers	CTO189fABC, CTO653r	35	94	1	57	1	72	2	167
Acidobacterium	31f, R1378r	30	95	1	53	1	72	2	22,126
Type I Methanotrophs	MB10γ, R1378r	35	94	1	60	1	72	2	122,126
Type II Methanotrophs	MB9α, R1378r	35	94	1	60	1	72	2	122,126
α-Proteobacteria	F203a, R1378r	35	94	1	56	1	72	2	11,126
Second PCR Round									
Bacteria	P338F*, P518r	30	95	1	53		72	2	221

* A 5' GC-clamp was added for DGGE analysis [57]; Before each PCR run, the temperature was held at 95°C for 10 min and after each run the temperature was kept at 72°C for 12 min for final template elongation.

2.3. PCR-DGGE Analysis

All the used primers and PCR conditions are listed in Table 1, 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 analyzing 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 1). 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 MgCl2, 1X Taq DNA Polymerase 10X Reaction Buffer (MgCl2-free), 1.25u/50µl of *Taq* DNA Polymerase (Promega, Madison, WI, USA), 400 ng/µl of bovine serum albumin (Hoffmann-La Roche, Basel, Switzerland), and DNase and RNase free filter sterilized 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 (Denaturing Gradient Gel Electrophoresis), based on the protocol of Muyzer et al. [57], 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 1xTAE (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 45 to 60 % (*Bacteria* and ammonium oxidizers) (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run for 16 hours 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).

Table 2. Sequences of the primers used in this study

Primer	Target	Sequence (5'-3')	References
P63F	All *Bacteria*	CAGGCCTAACACATGCAAGTC	14
P338F	All *Bacteria*	ACTCCTACGGGAGGCAGCAG	14
P518r	Universal	ATTACCGCGGCTGCTGG	14
R1378r	All *Bacteria*	CGGTGTGTACAAGGCCCGGGAACG	10
F243	Actinomycetes	GGATGAGCCCGCGGCCTA	10
CTO189AB	Ammonium oxidizers	GGAGRAAAGCAGGGGATCG	13
CTO189C	Ammonium oxidizers	GGAGGAAAGTAGGGGATCG	13
CTO653r	Ammonium oxidizers	CTAGCYTTGTAGTTTCAAACG	13
31f	Acidobacterium	GATCCTGGCTCAGAATC	33
MB10©	Type I Methanotrophs	AAGCGGGGGATCTTCGGACC	11
MB9⟨	Type II Methanotrophs	GTTCGGAATAACTCAGGG	11
F203a	⟨-Proteobacteria	CCGCATACGCCCTACGGGGGAAA	12
		GATTTAT	
GC-clamp	-	CGCCGGGGGCGCGCCCCGGGCGGG	27
		GCGGGGGCACGGGGGG	

2.4. 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 (NCBI) using the BLAST algorithm [17] for the comparison of a nucleotide query sequence against a nucleotide sequence database (blastn).

2.5. 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 [18] 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, Multidimensional scaling (MDS) and Principal Component Analysis (PCA), were performed with Bionumerics 2.0. The clustering algorithm of Ward [19] 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 analyze 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 analyzed. For PCA analysis, 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 [20]. H was calculated on the basis of the bands on the gel tracks, using the densiometric curves. The intensity of the bands was reflected as peak heights in the densiometric 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 peak heights of the densiometric 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 wastewater treatment plants that receive different types of influents.

3.1. Validation of the Nested PCR Procedure

In the first PCR round, group specific primers were used (Table 1). These specific forward and reverse primers were located before and after the 16S rRNA *E. 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 rRNA gene 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 1), we evaluated the specificity of the PCR amplification by cloning and sequencing a few fragments after the first PCR run for each specific primer set. 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.

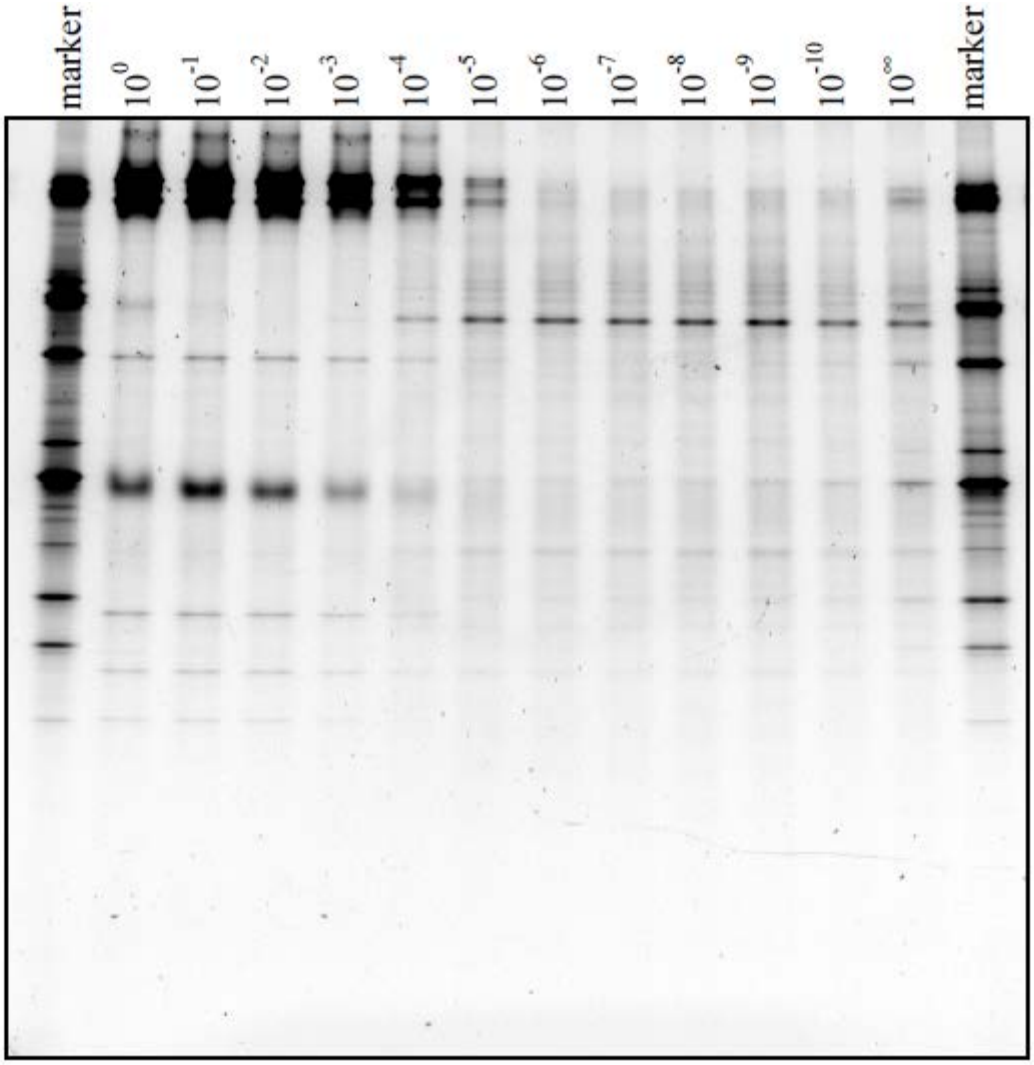

Figure 1. DGGE of the PCR fragments of the second PCR run with the general bacterial primers. At the top of the gel the dilution is mentioned of the first group specific PCR product CTO primers) with diluted DNA from sludge sample A1.

3.2. Determination of the Minimum Concentration of First PCR Product Required to Obtain a Specific Second PCR Product

In addition to sequencing amplified fragments, as described above, another way to insure that the DGGE patterns consisted of only group-specific bands, is the following. We examined what the minimum ratio of amplified product over template DNA should be after the first PCR round in order to avoid amplification of non-group-specific template DNA, still present as background in the second PCR round. This second template indeed consists not only of the specific first PCR fragment, but also still contains some extracted genomic DNA (25 times diluted). Thus if no or very little specific fragment would be amplified in the first PCR round, the genomic DNA could serve as template in the second PCR round, resulting in an aspecific second PCR fragment and thus an incorrect DGGE-pattern. Hence, the lowest concentration of first PCR fragment required to avoid aspecific bands in the final DGGE pattern has to be determined. The evaluation was based on the amplification of ammonium oxidizing bacteria with the CTO-primers. As template DNA, DNA extracted from wastewater treatment plant A1, which was characterized by good nitrification, was chosen. The first DNA template concentration was 0.12 µg/µl, and

since 1 µl was added to 25 µl of PCR mastermix, the final genomic DNA concentration in the mastermix was 4.8 ng DNA/µl. Using the CTO primers, a group specific PCR product of 15.75 ng DNA/µl was obtained. In order to examine the influence of the group specific PCR product, a tenfold dilution series of the first specific PCR product was made in water, containing 4.8 ng total genomic DNA/µl. A sample containing 4.8 ng DNA/µl was regarded as infinitely diluted. The concentration of total genomic DNA was thus constant while the concentration of specific PCR fragment varied. From this dilution series, 1 µl was added to 49 µl mastermix for the second PCR round, containing the general bacterial primers 338GC and 518r. A DGGE of these final PCR fragments was run for 17 hours at 38V on a gel with a denaturing gradient of 50-65%. The DGGE patterns showed that non-specific Bacterial fragments only appeared when the PCR product of the first round was diluted more than 103 times in the template background (Figure 1). This corresponded with a PCR product concentration of less than ca. 16 pg of amplified product per µl (or 0.3% of the total template DNA). These results strongly indicate that as long as the PCR product (a few µl) of the first round is visible on the gel, there should be no problem with amplification of aspecific ribosomal RNA genes in the second PCR round.

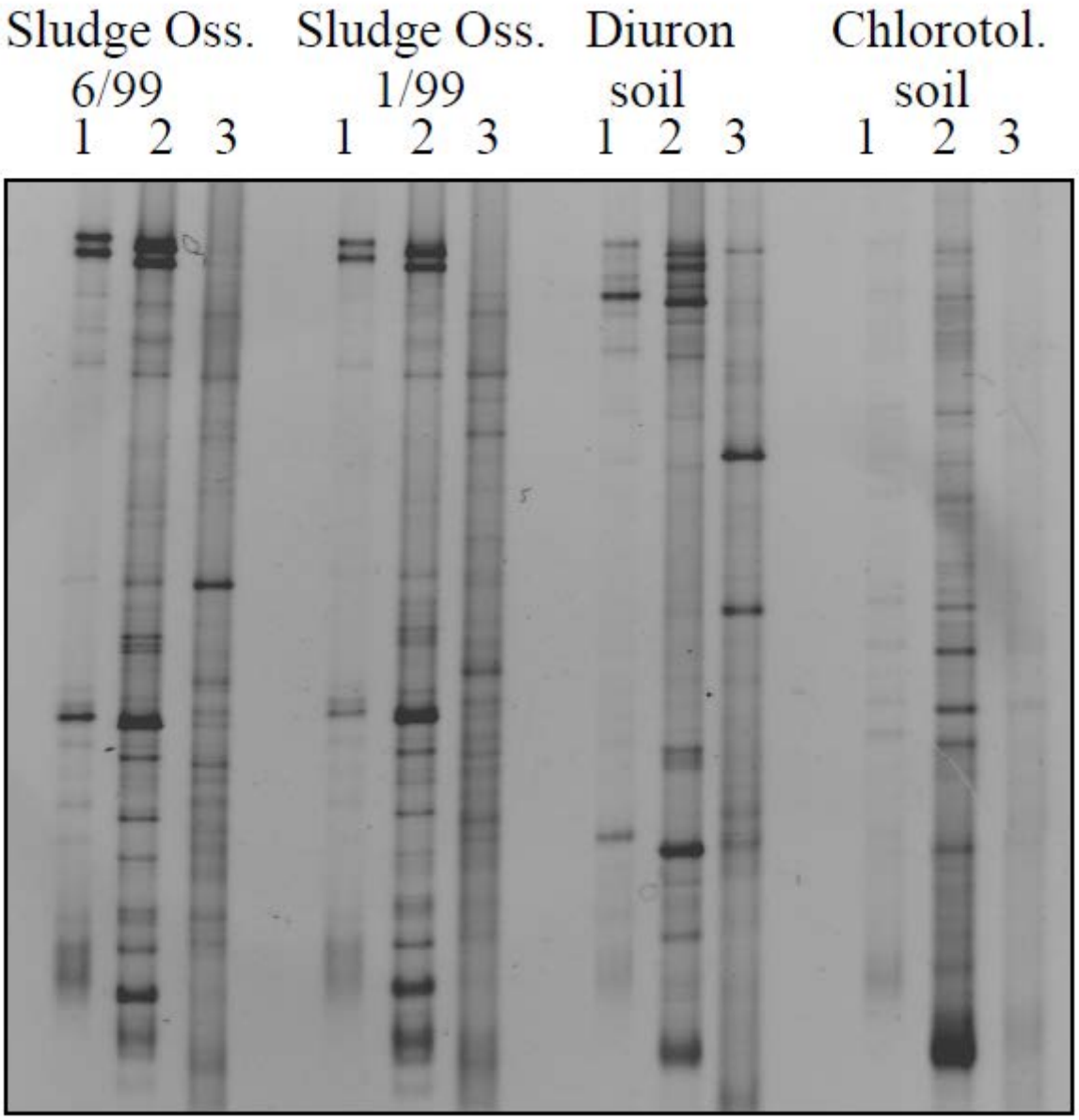

Figure 2. Analysis of the DGGE profiles of the different samples: (1) fragments of PCR with total DNA and *Acidobacterium*-primers; (2) fragments of a nested PCR on (1) with bacterial primers; (3) fragments of PCR with total DNA and bacterial-primers. The different DNA samples were from a wastewater treatment plant at two different dates (Sludge Oss.) and from two soils, treated with diuron and chlorotoluron

3.3. Validation of the Nested PCR Approach

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. In a first PCR reaction, a *Acidobacterium* specific PCR fragment was obtained in a single PCR run with primer 31f with GC-clamp (31fGC) in combination with primer P518r (lanes 1 of Figure 2). The 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 (lanes 1 of Figure 2). These primer sets were chosen such that both fragments would only differ 40 bp in length and thus could both be easily analyzed and compared on the same DGGE gel. In lanes 3 of Figure 2 PCR fragments obtained with primers P63FGC-518r are showed. For 4 samples tested, both patterns were very similar, except that the positions of the P63FGC-518r PCR fragments were all shifted downwards in comparison to the 31fGC-518r fragments, due to the slightly larger length of the latter fragment (Figure 2). These data suggest that the second PCR round did not drastically change the number nor the intensities of the DGGE-bands, compared to a 1-step PCR protocol with specific primers.

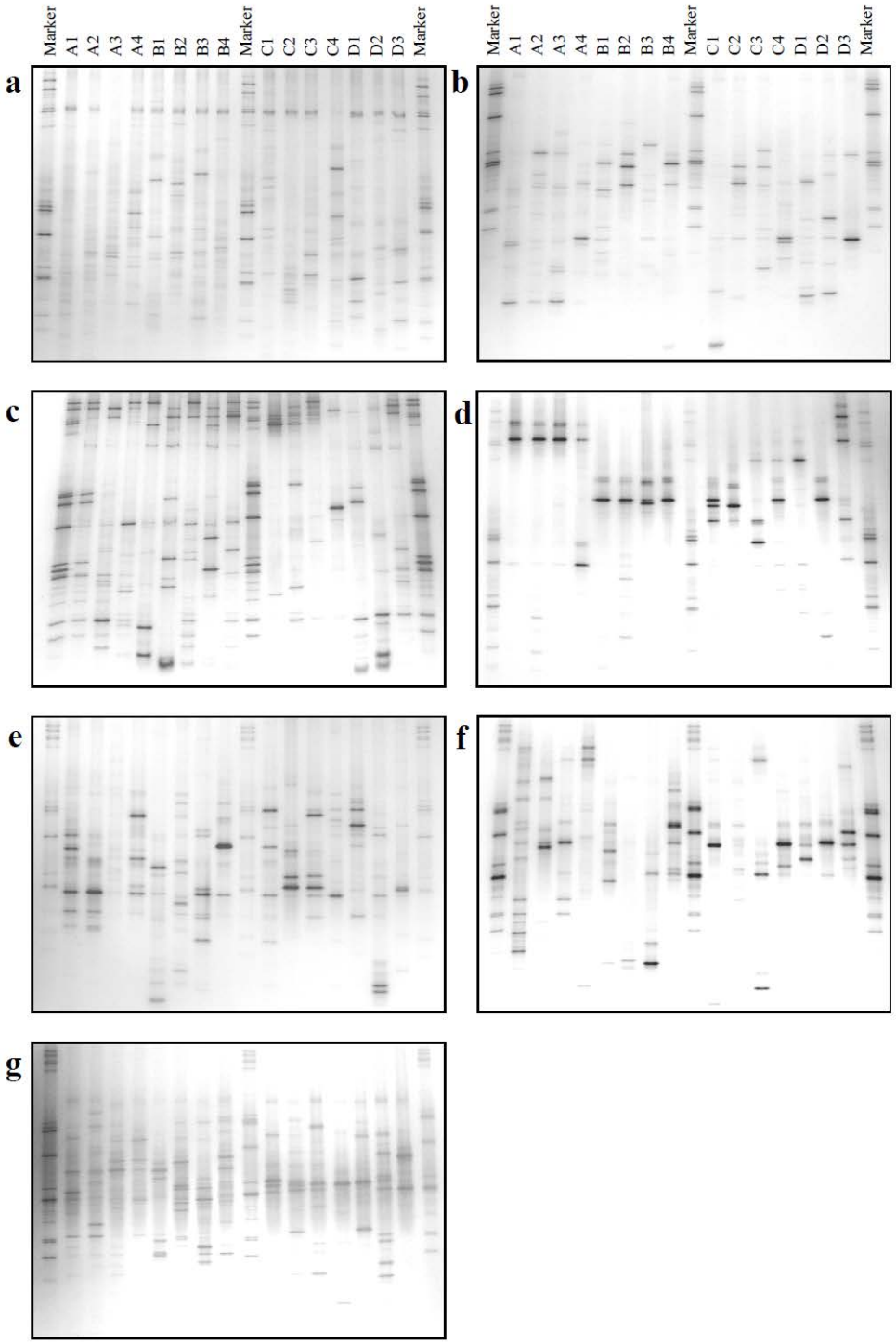

Figure 3. DGGE analysis of the 10 activated sludge samples: Bacteria (a); actinomycetes (b); *Acidobacterium* (c); Ammonium oxidizers (d); Methanotrophs - type II (e) and type I (f); α-Proteobacteria (g)

3.4. DGGE Analysis of PCR-amplified 16S rRNA Gene Fragments

Figure 3 shows the seven DGGE gels for all the activated sludge samples and for all the different bacterial groups. The DGGE patterns obtained with the Bacterial primers did not show many intensive bands (Figure 3a). 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 (Figure 3g). The DGGE pattern obtained with the *Acidobacterium* primers contained a relatively high number of bands as well (Figure 3c), which indicates that this group is also highly diverse. Every activated sludge sample seemed to have a unique *Acidobacterium* community and common bands were exceptional. The actinomycete, methanotrophs and ammonium oxidizing communities in all sludge samples seemed to consist of only a limited number of dominant species, without a smear of weak bands (Figure 3b, d, e and f). Striking were the patterns of the ammonium oxidizing 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 wastewater treatment plants. This was not the case for patterns from the sludge groups C and D.

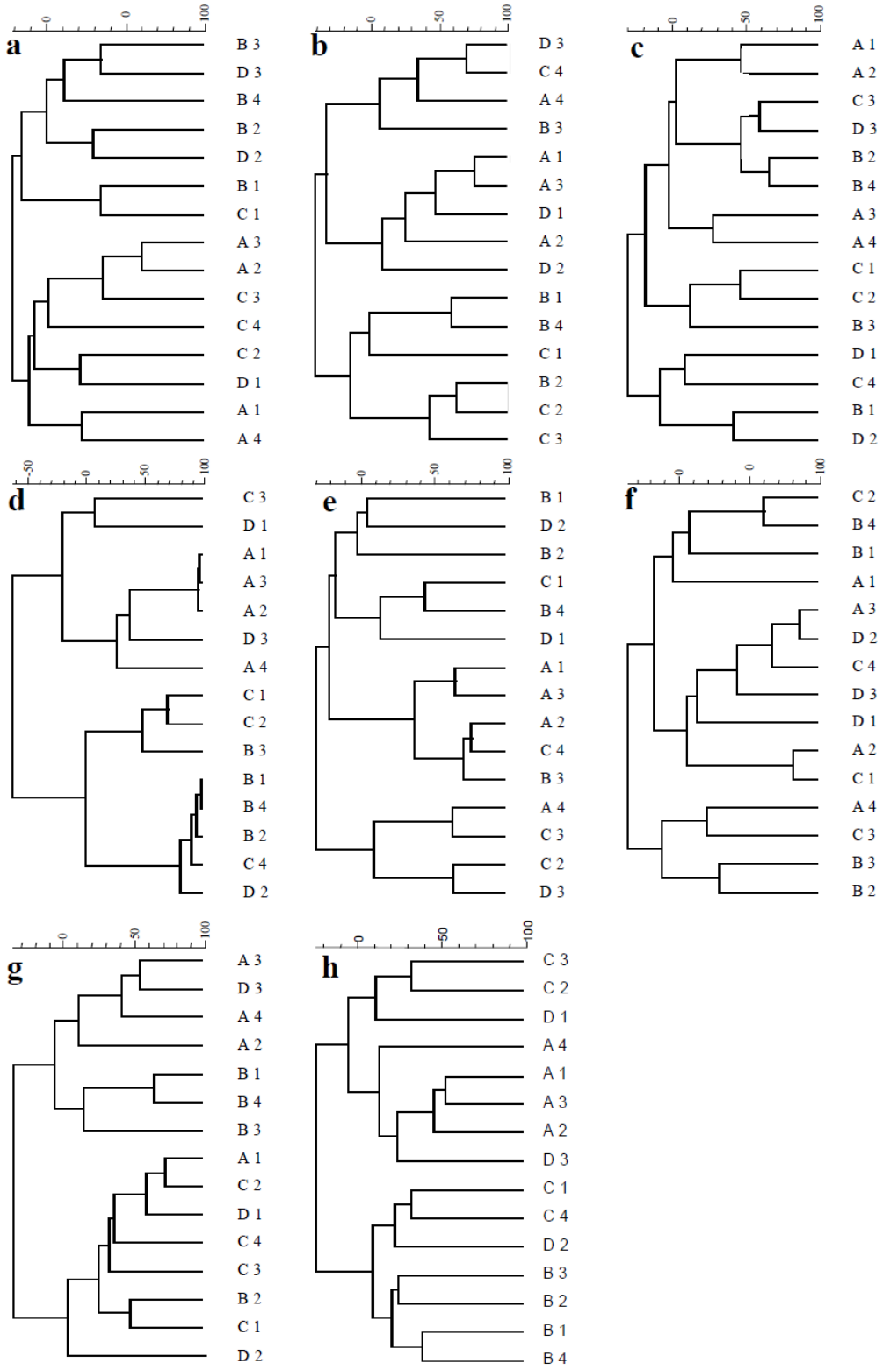

Figure 4. Clustering analysis of the DGGE patterns of the 10 samples: Bacteria (a); actinomycetes (b); *Acidobacterium* (c) ammonium oxidizers (d); methanotrophs - type II (e) type I (f); ⟨-Proteobacteria (g); all different DGGE-tracks combined (h)

3.5. 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. To be able to perform such analyses on each gel, three standard patterns (markers) were included to allow a normalization of the gels. The normalized 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 analyzed 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 visualized in dendrograms (Figure 4). In the dendrograms, sometimes several sludge samples (3 or 4) of the same type of wastewater were located in one cluster, such as for samples A (Figure 4a, b, d, e, g), samples B (Figure 4a, b, d, g), samples C (Figure 4a, b, d, g) and samples D (Figure 4a, 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 (Figure 4h). Remarkably, 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. Multidimensional Scaling (MDS) and Principal Component Analysis (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 oversimplifies the data available in a similarity matrix and tends to produce overestimated hierarchies. The MDS analysis shows that the different activated sludge groups in the three dimensional plot are not grouped together (Figure 5a). 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.

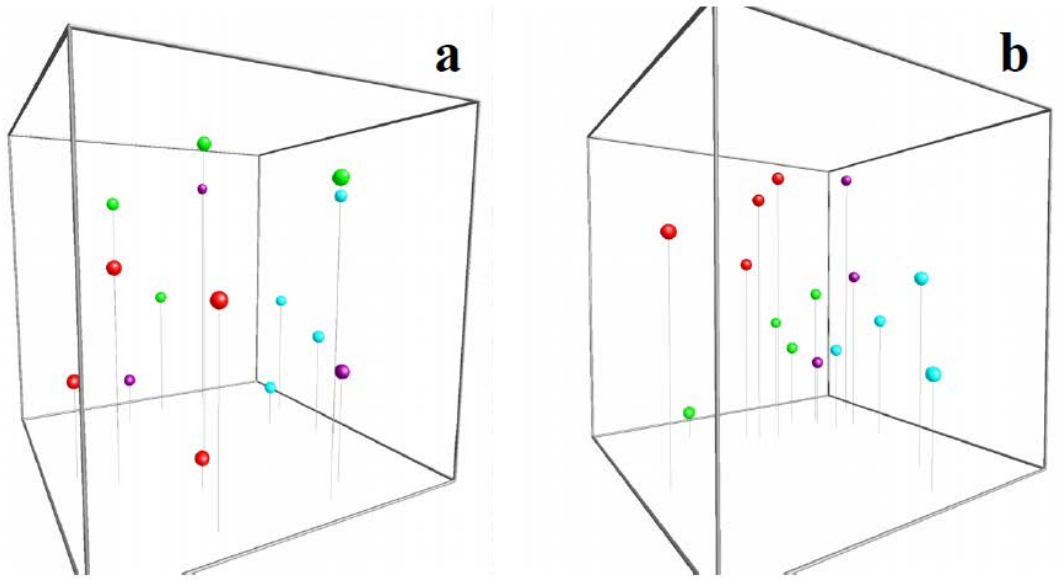

Figure 5. Reptrsentation of two dimension techniques, calculated for each track of each DGGE gel with group specific 16S rRNA gene fragments: Multi-Dimensional Scaling (a) and Principal Component Analysis (b): samples A (●); samples B (●);samples C (●); samples D (●)

Therefore, the same sets of data will result in a different PCA-and MDS-plot (Figure 5b). The three principal components (PC) explained a very 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 localized 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 number 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 [21]. 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. The Anderson-Darling test for normality showed that the Shannon Diversity Indices *H* for the different groups were normal distributed. 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 oxidizers 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 oxidizer community had the lowest average Shannon index *H*. This is a result of the very limited number of bands, visible in the gel (Figure 3d). The Shannon diversity indices of the different groups and the operational parameters were subjected to a correlation analysis. The diversity indices for the ammonium oxidizers correlated positively with the sludge retention time (SRT) (P=0.05; R2=0.31), while the index of the Bacteria (P=0.05; R2=0.30) had a negative correlation. The Shannon index of the actinomycetes was positively correlated to the sludge volume index (SVI) (P=0.10; R2=0.22).

Table 3. Mean and standard deviation of the Shannon Diverisity Indices for the sludges of the different wastewater treatment plant-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
Acidobacterium	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 ± 0.21	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

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 optimization 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 allows to visualize also those species that are present in lower numbers. Phillips et al. [22] detected ammonium oxidizers 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 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 microbial community. One organism may produce more than one DGGE band because of multiple, heterogeneous rRNA operons [23,24,25]. On the other hand, partial 16S rRNA gene sequences do not always allow discrimination between species, such that one DGGE band may represent several species with identical partial 16S rRNA gene sequences [26]. Some bacteria have more copies of the rRNA genes than others, and some lyse more easily than others. In addition, in a mixture of target rRNA genes present at very different concentrations, the less abundant sequences are not amplified sufficiently to be visualized as bands on a DGGE gel. Therefore, the banding pattern reflects only the most abundant ribosomal RNA types in the microbial community [27]. 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 rRNA gene sequences in nature. The study of Purkhold et al. [28] clearly demonstrated that specific primers for ammonium oxidizers 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 rRNA of all actinomycetes and it matches 16S rRNA of a few nonactinomycetes [10]. Nevertheless, the primer is useful to enrich actinomycete 16S rRNA gene fragments 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 optimization it

would be 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% (data not shown). A slight disadvantage of this approach is a less optimal separation of certain bands in some groups. For many sludge samples, several bands of some of the analyzed 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, analyzed 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. [27] showed that the presence of a few dominant species leads to a simple pattern, and that species of less than 1% of the analyzed community were not represented in the microbial community pattern. This will also be true for PCRDGGE 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 subdivison using more specific primers could be useful if more detailed analysis is desired. In this study, only one activated sludge sample of each wastewater treatment plant was investigated. To monitor the reproducibility in time, two samples of the wastewater treatment plant A1 were taken with an interval of 6 months, and their DGGE profiles of the Bacterial and *Acidobacterium* populations did not show much variation (Figure 2). Other investigators also did not find evidence for variation in the wastewater treatment plants they studied by PCR-DGGE and concluded that a single sample of an activated sludge plant was sufficient for a plant to plant comparison [6]. Fluorescent *in situ* hybridization (FISH) analysis has shown before that on average ca.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 to 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 lowload basins [4]. Earlier work has also demonstrated that the α-Proteobacteria are numerically dominant in activated sludge [29,30] and that some species have been shown to form filaments in activated sludge systems [31,32]. 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 DGGE analysis with a focus on specific rather than all bacterial groups would be better to compare different ecosystems. The kingdom *Acidobacterium* is a recently discovered bacterial lineage and at this time contains only a few cultured representatives [31,34]. 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 [35]. 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 [36]. Barns et al. [33] 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 [33], 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. The analysis of actinomycetes was included in this study, because these bacteria can cause severe bulking and foaming in activated sludge plants [37,38,39]. Their hydrophobic cell surface is supposed to support adherence and stabilisation of interfaces and thus promote sludge flotation, leading to a higher Sludge Volume Index (SVI) [40]. 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 Sludge Volume Index (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 [41,42,43] 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 [44]. These bacteria can utilize C-1 molecules, such as methane, methanol and formaldehyde. The DGGE patterns of the samples clustered differently when they were analyzed with the two different sets of group specific primers, i.e., for the Type I and II methanotrophs. This phenomenon has been observed before in groundwater, where both types of methanotrophs reacted differently on changing conditions, such as biostimulation [45]. Similarly, in a soil treated with herbicides, the diversity of type I methanotrophs decreased, while the diversity of type II increased [46]. The 16S rRNA gene fragments related to the β-Proteobacteria subgroup of the ammonium oxidizers 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* [47]. 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 oxidizers 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 [48]. This could lead to a lower density of common ammonium oxidizers, which might result in a more diverse ammonium oxidizing 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 [45,49] and PCA-plots [50,51]. We also tried to analyze our data with both techniques, but no clear 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 R2 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. 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 [52] would be a next step in acquiring a good descriptive tool for microbial community analysis of activated sludges.

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