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Analysis of bacterial diversity in river biofilms using 16 S rDNA PCR-DGGE: methodological settings and fingerprints interpretation

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Abstract

Reliability of bacterial diversity assessment using polymerase chain reaction (PCR) denaturing gradient gel electrophoresis (DGGE) analysis of 16S rDNA fragments was evaluated for a particular complex microbial assemblage: river epilithic biofilm. By comparing 3 routine protocols on replicates of one river biofilm sample, we found that common DNA extraction procedures gave comparable diversity (from 28.0 to 30.7 bands detected) and community composition (> 75% of homology) despite differences in the total amount of extracted DNA (from 0.9 to 4.2 µg). Therefore methodological improvements only concerned electrophoretic separation of DNA fragments (range of denaturing gradient from 35% to 70% and migration time = 18 h) and standardisation of DNA amounts used (PCR-template = 50 ng, gel loading = 700 ng). Using such a standardised methodology we found a good reproducibility of all steps of the procedure. When an *Escherichia coli* strain was introduced as a contaminant in a biofilm sample, we were able to recover ribotypes from the strain. As concerns fields sampling, a satisfactory repeatability of banding patterns from neighbouring pebbles (sampling point) allowed discriminating between the biofilm intrasite variability (various points from a cross-profile). These trials confirmed that PCR-DGGE is suitable to assess a reliable genetic fingerprint of epilithic biofilms in the river. Phylogenetic analysis of 40 partial sequences of 16S rDNA from DGGE gels of two sets of river biofilms samples proved evidences for the retrieval of DNA fragments related to phototroph Eukarya. However, in both cases plastidial 16S rDNA represented less than 25% of the analysed operational taxonomic units. Taking into account that Cyanobacteria, as members of the Bacteria, were also detected, sequence analysis of relevant bands from the pattern is required to target "bacteria", i.e. the functional group of prokaryotic microorganisms to which one commonly refers as a key component in sustaining the nutrient turnover.

Keywords: Bacterial diversity; Epilithic biofilm; 16 S rDNA; PCR-DGGE

1. Introduction

Diversity of natural microbial communities have become a task for understanding the dynamics of organisms particularly as concerns microbial consortia

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which are involved in biogeochemical cycling (Paerl and Pinckney (1996)).

Molecular tools to monitor the bacterial diversity of complex microbial assemblages have developed in the last decade using 16S rDNA based approaches recommended by Amann et al. (1995). Amongst these culture-independent approaches, genetic fingerprinting is considered as a suitable tool for rapid and comparative analysis of unknown natural communities (Ranjard et al. (2000)). One method, 16S rDNA based PCR-DGGE, proposed by Muyzer et al. (1993), has been widely used for studying bacterial communities of numerous environments: soils and sediments (Powell et al. (2003)), plankton of lake (Casamayor et al. (2000); Dumestre et al. (2001)) or sea (Schafer et al. (2001); Schauer et al. (2000)) and more recently epilithic biofilms (Jackson et al. (2001); Araya et al. (2003); Lyautey et al. (2003)).

Nevertheless, in the euphotic zone of aquatic ecosystems, the occurrence of phototroph micro-organisms may generate interference as plastids being related to bacteria by an endosymbiotic event (Cavalier-Smith (2000); Van den Hoek et al. (1995)). For planktonic communities, interference can be limited by decreasing the eukaryotic cells density by size-filtration (Schauer et al. (2000); Rappé et al. (1998)). Size filtration is not suitable for planktonic or benthic aggregated communities such as respectively marine snow (DeLong et al. (1993)) or epilithic biofilms (Lock (1993)) where all organisms are tightly clustered. Then it may be expected that part of the recorded operational taxonomic units (OTUs) could originate from phototroph eukarya that is to say undesirable bands which may overlap ribotypes of interest.

The present work aimed to examine the reliability of bacterial diversity assessment using PCR-DGGE analysis of 16S rDNA fragments for a particular complex microbial assemblage: epilithic biofilm. Two aspects were developed and discussed. On one hand, methodological aspects were investigated including optimisation of electrophoresis, comparison of three routine extraction methods, test of recovery of a contaminant culture of *E. coli* and sampling strategy. On the other hand, bacterial diversity results of a set of samples have been studied using a 16S rDNA based PCR-DGGE approach followed by DGGE bands phylogenetic sequence analysis.

2. Material and methods

2.1. Samples

Sampling was performed by the point contact method collecting for one site 1–3 pebbles in a sterile bag at 3 sampling points corresponding to 3 depths of a cross-profile (0.3, 0.5 and 0.7 m). In most cases, a mean sample was generated from mixing all the collected samples, excepted for repeatability experiments where they were treated separately.

Two sets of respectively 11 and 14 epilithic biofilm samples were collected from 2000 to 2003 by sampling river pebbles at 6 different sites of a large gravel bed river (river Garonne, SW France).

In some cases, natural biofilm samples were contaminated with aliquots of a pure culture of *E. coli* (strain K12 – MG1655) cultivated on a tryptone soy broth medium. Two contaminated samples were designed, with addition of cultured cells corresponding to 1% and 10% of the total biofilm bacterial density.

2.2. DNA extraction and purification

Prior to aliquoting and further analysis, the biofilms were recovered as a homogeneous suspension in particle free (0.22 μm) autoclaved water using a tissue homogeniser. After centrifugation (16 000 × *g*, 20 min, 4 °C) of an aliquot of 20–50 mg of biofilm dry matter, DNA extraction have been carried out on the biofilm pellet using three routine methods referenced A, B and C, described in Table 1. Method A is based on an enzymatic disruption of cells followed by a phenol-chloroform and chloroform extraction of DNA

Table 1
Reference and description of the main steps of the three routine extraction methods (A, B and C) used in this study

Methodology	Method A	Method B	Method C
Reference	Dumestre et al., 2001	Jackson et al., 2001	Qiagen DNeasy Plant Mini Kit
Cell lysis	Proteinase K SDS Lysozyme	Proteinase K SDS Liquid Nitrogen CTAB	RNase SDS
DNA extraction	Phenol-chloroform isoamyl alcohol, chloroform isoamyl alcohol	Chloroforme isoamyl alcohol	Guanidine hydrochloride
Purification			DNeasy column
Concentration	Centricon 100 (Millipore)	Isopropanol	

and a further concentration and washing of DNA using a microconcentrator (Amicon, Centricon 100, 100000 D cut-off) (Dumestre et al. (2001)), method B has already been used for epilithic biofilm DNA extraction and is based on an enzymatic and mechanical disruption of cells followed by a chloroform extraction and an isopropanol precipitation (Jackson et al. (2001)) and method C was designed for the purification of material extracted on plant samples and was carried out according to the manufacturer protocol (DNeasy Plant Mini Kit, Qiagen). After extraction, DNA concentration was determined by fluorimetry using the DNA Quantitation Kit Fluorescence Assay (Sigma, DNA-QF).

2.3. *Amplification*

The variable region V3 to V5 of the 16S rDNA was amplified using primers 341F-GC and 907R (Genset) designed to be specific to most bacteria (Muyzer et al. (1997)). The protocol of amplification used is described elsewhere (Lyautey et al. (2003)). Note that a defined amount of extracted DNA (50 ng) was always used as template for PCR amplification. Amplified DNA concentration was determined on 1.65% agarose gel using Precision Molecular Mass Ruler (BioRad).

2.4. *DGGE*

After optimisation experiments, perpendicular DGGE was performed using D-Code Universal Mutation Detection System (BioRad) as already described (Muyzer et al. (1997)). The gel contained a gradient of denaturant ranging from 35% to 70% (100% denaturant is 7 M urea and 40% deionised formamide). DGGE was run at 100 V for 18 h at 60°C.

After electrophoresis, the gel was stained with 2 × SYBR Green I (Sigma) and visualised by UV transillumination. The gel image was captured using a CCD camera and Biocapt software (Vilber Lourmat). Image analysis was done using Bio-1D + + software (Vilber Lourmat), which allows fragment detection and quantification. For each sample, a densitometric profile was generated to determine the relative contribution of each band to the total signal in the lane sample.

DGGE bands were scored as present or absent from DGGE gel analysis. Homology between samples banding patterns were calculated by means of the Jaccard similarity index: $J = 100(c/[a+b-c])$, where a is the number of bands of the sample A, b the number of bands of sample B and c the number of bands that are in common to samples A and B.

2.5. *DNA sequences and phylogenetic analysis*

For sequencing, selected bands were excised from DGGE gels and placed in a sterile Eppendorf containing 20 µL of sterile water and DNA was eluted using three cycles of freeze-thawing (−20°C/37°C). Five µL of the solution were used as template in PCR using the former protocol. A second DGGE was carried out to check the purity of excised bands that were excised twice, eluted and reamplified. The amplified products were sequenced (Genome Express, France). Sequence analysis and phylogenetic trees construction were done using the ARB software package (http://www.arb-home.de/, O. Strunk et al., ARB: a software environment for sequence data, Department of Microbiology, Technische Universität München, Munich, Germany, 1997). A total number of 40 partial 16S rDNA sequences have been deposited in the GenBank sequence database under accession numbers AY456641 to AY456680.

3. Results

3.1. *Protocol improvement*

3.1.1. *DGGE*

For the set of tested epilithic biofilm samples, the best resolving range of denaturant was from 35% to 70%. It was defined from an analysis of our samples on a DGGE gel with a 0–100% range of denaturant followed by a visual inspection of the migration area of the bands. Using this denaturant range, 7 different migration times (from 12 to 18 h) were tested and resolution of 2 co-migrating bands was achieved after a migration time of 18 h (Fig. 1). Using these parameters of migration, two amounts (400 and 700 ng) of a biofilm amplicon were loaded in the same gel providing different band patterns: a higher richness (29 vs.14 bands) was obtained with the higher load. Conversely less than 10% of the detected bands differed between triplicate amounts of DNA. However, the 14 bands detected for the 400 ng load corresponded to the 14 brightest bands of the 700 ng load suggesting that DNA amount may have a threshold effect on band detection sensitivity (Fig. 2). Therefore, standard conditions were loading of 700 ng, denaturant range of 35–70% urea-formamide, and migration time of 18 h under 100 V.

3.1.2. *PCR amplification*

The influence of the amount of DNA used as PCR template was investigated by considering the amplification yield (amplified DNA/template DNA) and the richness obtained from DGGE patterns (Fig. 3). The higher the amount of template was (from 10 to 100 ng, a range commonly found in the literature (Bosshard et al. (2000); Norris et al. (2002); van Hannen et al. (1999)), the lower the amplification yield was, decreasing from 207 to 40 ng of amplified DNA per ng of template DNA. The band richness varied with the amount of template

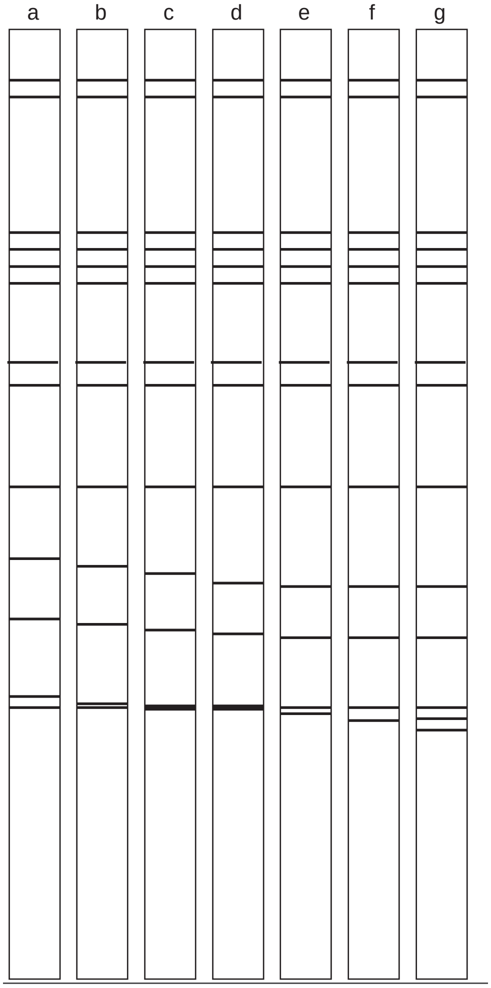

Fig. 1. Schematic representation of the DGGE gel obtained for replicate samples submitted to different migration times (a: 12 h; b: 13 h; c: 14 h; d: 15 h; e: 16 h; f: 17 h and g: 18 h).

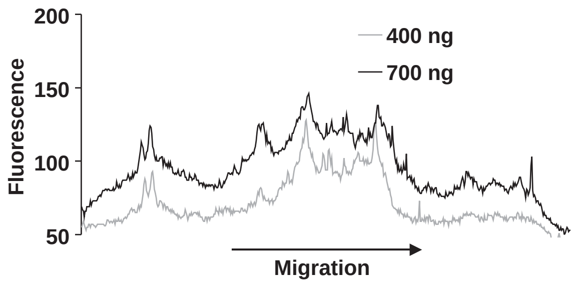

Fig. 2. Chromatograms of the fluorescence of two lanes of a DGGE gel loaded with respectively 400 and 700 ng of DNA obtained after amplification of a 500 bp fragment of 16SrDNA from river biofilms.

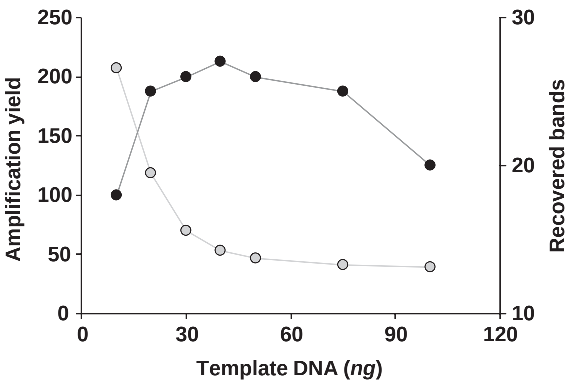

Fig. 3. Amplification yields (grey circles) and number of bands recovered in DGGE (black circles) for various quantities of epilithic biofilm DNA (ng) used as template of 16S rDNA PCR-DGGE analysis.

DNA (Fig. 3). Optimal richness values (more than 26 bands) were obtained between 30 and 50 ng of DNA template, which is the range of template used in most environmental studies. When the same DNA extract was amplified with 3 different PCR reactions, replicate amplicon being loaded in the same gel, dissimilarity between amplicon was 3% of the detected bands. When triplicate were loaded in three different DGGE gel, dissimilarity between amplicon was 10% of the detected bands. This confirmed that the amplification is not the step that introduces much variability in the whole analysis process.

3.1.3. Extraction methods comparison and influence on each step of the PCR-DGGE analysis

Extraction is known to be a critical step of this methodology since it determines the significance of the sample (target DNA) (Wintzingerode et al. (1997)). Two of the 3 routine extraction procedures tested (Methods A and B) gave comparable extraction yield (around 4 µg for 45 mg of biofilm dry matter) while 4 fold less DNA quantities (Table 2) were extracted by third method (method C). Differences between extraction techniques were significant according to Kruskal–Wallis test ($H = 6.49$; $n_1 = n_2 = n_3 = 3$; $p < 0.04$). However, amplification yields were not significantly different between amplicons A, B and C ($H = 3,2; n_1 = n_2 = n_3 = 3$; $p = 0202$) which averaged 22 ng of amplified DNA per ng of template DNA (Table 2). As concerns the number of detected bands (or richness), mean values were close to 29 bands analysing amplicons A, B or C (Table 2). Jaccard similarity index averaged 79.8% of the detected bands between methods A and B, 77.9% between methods A and C, whereas methods B and C shared 87.5% of the detected bands.

Table 2
Comparison of mean ± standard error of extracted DNA quantities (µg), amplification yield (ng of amplified DNA per ng of template DNA) and richness (number of detected bands) obtained with DGGE for biofilm triplicate samples (45 mg DM) using three different methods of extraction

Method	A	B	C
DNA quantity (µg)	4.2 ± 0.1	3.5 ± 0.5	0.9 ± 0.02
Amplification yield	22.6 ± 2.0	25.1 ± 1.9	19.8 ± 1.1
DGGE Richness	28.0 ± 1.0	29.3 ± 0.9	30.7 ± 0.7

3.2. Sampling strategy

3.2.1. Sample size

Three sets of triplicate biofilm samples (one replicate corresponding to the biofilm covering one pebble, average size around 12 cm) were compared using DGGE analysis to evaluate the community heterogeneity at local scale. All three sets gave similar results since the variation coefficient between the samples was lower than 5% in terms of specific richness, and 8–16% of the bands discriminated between the sample patterns. Then, one colonised pebble looked to be the satisfactory sample size to integrate the micro-heterogeneity at the point scale.

3.2.2. Site sampling

Comparison of sampling patterns for biofilms from different points of two distinct sites indicated the occurrence of an intra-site variability: differences in specific richness and in banding patterns were recorded between points from different depths (Table 3) as was reported for algae (Biggs (1996)). Comparison of the points and site DGGE fingerprints confirmed the relevance of an integrated sampling strategy: point samples *a* (1,2,3) and *b* (1,2,3,4) generated respectively 50 and 48 band positions; amongst them 10 and 14, respectively were not found in the pattern corresponding to the mean sample, whereas 21 for both cases were present in the mean sample but not in all point samples.

3.3. Quantitative and qualitative aspects of bacterial diversity

3.3.1. Bacterial contamination

The addition of an aliquot of known concentration of an *E. coli* pure culture to a natural biofilm sample generated two bands. Relative intensities of these bands were calculated versus the total intensities of the lane and showed values corresponding to the level of contamination, i.e. more intense for the 10% than for the 1% contamination. However recorded values differed from the expected one, especially for the 1% contamination (Table 4).

Table 3
Schematic representation of the banding patterns obtained for point samples collected at different depths (1, 2, 3, 4) for sampling site A and B (lower-case letters indicate point samples and upper case letters indicate mean site samples)

*a*1	*a*2	*a*3	A	*b*1	*b*2	*b*3	*b*4	B
X	X	X		X		X	X	X
X	X	X	X	X	X	X	X	X
X	X	X				X	X	X
X	X	X	X	X	X	X	X	
X	X	X	X	X	X	X	X	X
X	X	X	X	X	X		X	
X	X	X	X				X	X
		X				X		X
X	X	X	X			X		
X	X	X	X	X	X	X	X	X
X	X	X	X	X	X	X	X	X
X	X	X	X	X	X	X	X	X
X	X		X	X	X	X		X
X	X	X	X	X	X	X		X
	X	X	X			X		
X	X	X	X	X	X	X		X
X	X	X	X	X	X	X		X
X	X	X	X	X	X	X	X	X
	X	X	X			X	X	X
	X	X	X			X		
X	X	X	X	X	X	X		X
X	X	X	X			X		
	X			X	X	X	X	X
X		X	X	X		X	X	
		X	X	X	X	X	X	X
X			X	X	X	X		X
X	X	X	X	X	X	X	X	X
X	X	X	X		X		X	
X	X		X	X	X		X	X
X	X	X	X	X	X	X	X	X
X	X			X	X	X		X
X	X	X	X	X	X		X	X
X			X	X	X	X		
		X	X	X	X	X		
X			X	X	X	X	X	X
X			X	X	X	X	X	X
X			X	X		X	X	X
X	X	X	X	X	X	X	X	X
X			X			X	X	X
			X			X	X	
X						X	X	X
X			X		X	X		X
X						X		X
X						X		
X					X	X		
X			X	X	X	X		X
X						X	X	
X			X			X	X	X
X			X					
X			X					
X			X					
			X					

Table 4
Mean cumulated relative intensities ± standard error of the two bands corresponding to *E. coli* in the biofilm contaminated samples (corresponding to 1% and 10% of the total densities of bacteria) using the three extraction methods

Method	A	B	C
10% contamination	7.3%±0.7	8.1%±0.8	8.6%±1.1
1% contamination	6.9%±3.2	5.3%±0.2	4.9%±0.3

3.4. Sequencing and phylogenetic analysis

First, phylogenetic analysis was performed on 23 DNA sequences, corresponding to 20 OTUs from the 74 bands detected in the first set of 11 biofilm samples extracted with method A. Five OTUs were related to diatoms plastidial DNA and 16 to Bacteria among which 4 were related to Cyanobacteria (Fig. 4 (a)). Then, method C was applied on a set of 14 samples and 26 DNA sequences (corresponding to 20 OTUs excised from the 63 detected in the DGGE banding pattern) were analysed (Fig. 4 (b)): 4 were related to plastidial DNA and 16 to Bacteria of which 9 corresponded to Cyanobacteria.

Note that all co-migrating bands and that band located at the same positions in different patterns exhibited related sequences. Moreover, no chimeric sequences were retrieved.

Microscopic analysis of the set of samples showed 5 morphotypes of Cyanobacteria: *Anabaena sp., Chroococcus sp., Leptolyngbya sp., Oscillatoria sp. and Pseudanabaena sp.* and 70 taxa of algae: 57 species of Diatomophyceae, 12 Chlorophyceae and 1 Rhodophyceae.

4. Discussion

Applied to complex microbial communities, 16S rDNA based PCR-DGGE underwent a wide development as an useful and rapid routine analysis of bacterial diversity (Ranjard et al. (2000); Muyzer (1999)). Investigating methodological aspects of the procedure, we observed as for most techniques that the definition of a sampling strategy is a key point of the method reliability. Obviously, as reported for soils (Ellingsoe and Johnsen (2002); Ranjard et al. (2003)), the samples collected on the field must integrate the level of heterogeneity of the considered study scale: one sampling point of the river is satisfactorily described by one pebble (around 0.1 m^2 or from 0.5 to 2.5 g of biofilm dry matter) although one site requires to take into account various depths of the cross-profile. Due to the gel to gel variability (Moeseneder et al. (1999)), the number of samples that can be compared is limited to the number of wells of the used electrophoresis device (generally less than 20). An alternative way to overcome this limitation would be to use a reference patterns, included in all DGGE gel, that would allow comparing patterns coming from different gels. In the present work, to properly compare the maximum of cases, we chose to generate a mean sample for each site by mixing point samples from different depths. However, as mentioned in Chandler et al. (1997) and Schauer et al. (2000) we observed that the amounts of DNA, PCR template and loaded in DGGE, have also to be standardised since they proved to influence the recorded banding pattern. Following a standardised procedure for sample size and adapting DGGE parameters (range of denaturing gradient and migration time) as recommended by Diez et al. (2001) we observed that the three routine extraction procedures tested compared except for the amount of extracted DNA. It has been demonstrated that diversity depends on this parameter as a reflection of the sample metagenome (Wintzingerode et al. (1997); Lindström (1998); Martin-Laurent et al. (2001)). In the present work, no differences in the diversity was recorded although the different extraction procedures exhibited differences in DNA amounts extracted suggesting that the lowest amount of collected DNA (0.9 ng per 45 mg of biofilm dry mass) was sufficient to reliably describe the community diversity. Then, for routine analysis, the use of commercial ready-to-use extraction kits looked recommendable (cost, easy and safe to use: no phenol).

The recovery of *E. coli* ribotypes confirmed that PCR-DGGE is an adapted tool for the detection of bacterial populations in epilithic biofilms while it should be kept in mind that the method should not be appropriate for the most resilient autochthonous bacteria (Wintzingerode et al. (1997)). Nevertheless, as concern relative intensities, the bands corresponding to the 1% contamination accounted for less relative intensity than those of the 10% contamination, indicating that even if not exactly accurate, and mainly due to known amplification biases (Schauer et al. (2000); Nübel et al. (1997)), bands relative intensities might at least be used to infer the general changes in relative abundance of the dominant members of the community.

Phylogenetic analysis of DNA sequences retrieved from 16S rDNA PCR-DGGE analysis of 25 river biofilm samples showed that plastidial DNA of phototroph eukaryotes accounted in the recorded diversity of the microbial assemblage but for less than 25% of the sequences. The occurrence of plastidial related DNA fragments has already been reported for planktonic communities using the cloning approach (Rappé et al. (1998); Moreira and Lopez-Garcia (2002); Zwart et al. (2002)). Since previous studies on epilithic bacterial communities did not focused on sequence analysis, they did not report the presence of such interference (Jackson

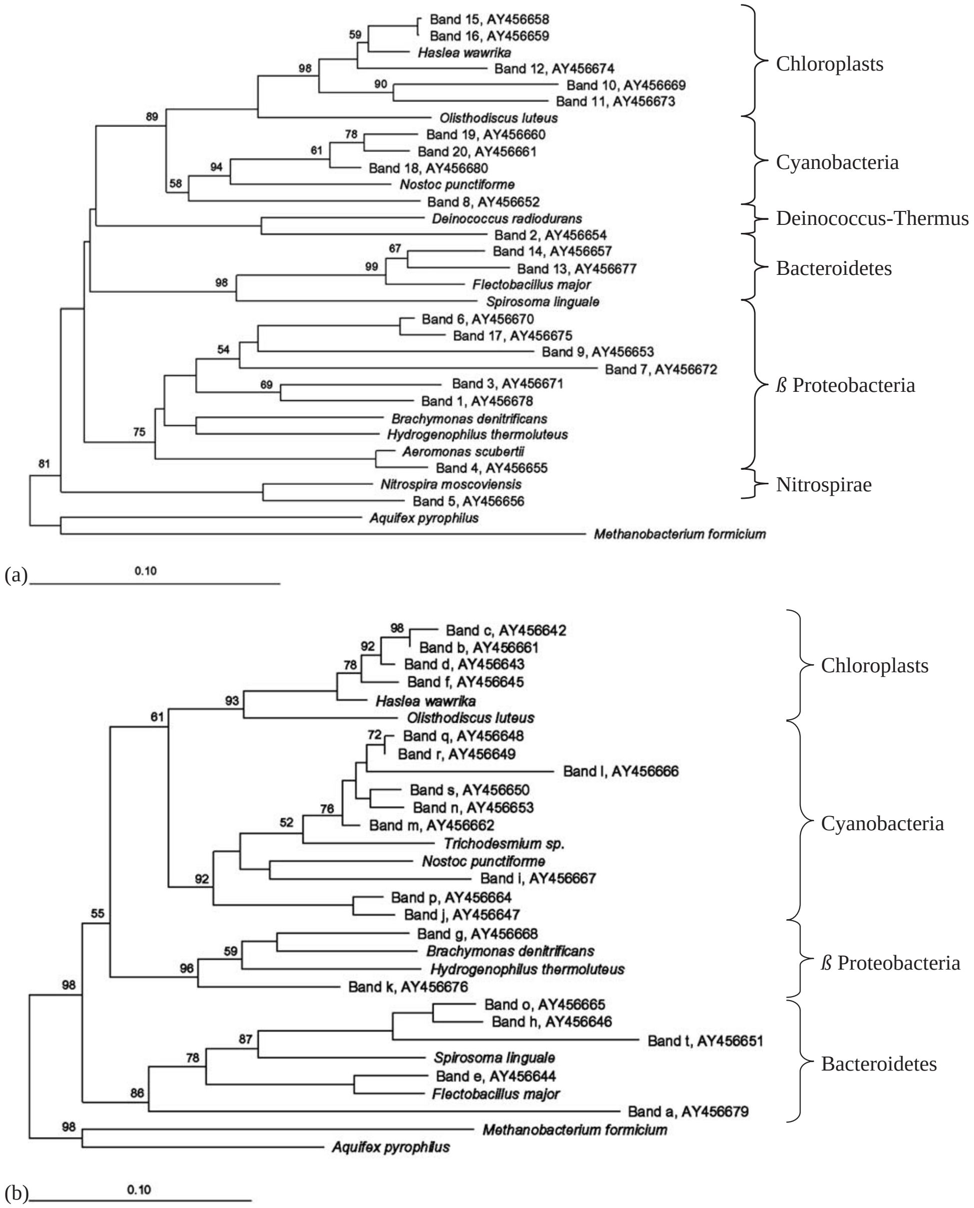

Fig. 4. Phylogenetic trees for the partial bacterial 16S rDNA sequences obtained using extraction method A (a) on a set of 11 samples and C (b) on a set of 14 samples. The tree was obtained using neighbour-joining with *Aquifex pyrophilus* and *Methanobacterium formicicum* as the outgroups. Bootstrap values are based on 1000 runs and are shown where >50. Similar trees were obtained using maximum-likelihood and maximum-parsimony analyses.

et al. (2001); Araya et al. (2003); Lyautey et al. (2003)). As members of the Bacteria Domain, Cyanobacteria were also detected in our samples. The number of OTUs compared to the morphotypes detected by microscopy.

The retrieval of DNA fragments related to phototroph organisms highlights a paradox in ecological studies of bacterial diversity analysis. From a phylogenetic point of view, plastids and Cyanobacteria are members of the domain Bacteria (Woese et al. (1990)), which explains their recovery using 16S rDNA as a phylogenetic marker. Ecologically, bacteria are considered in most studies as a functional group of decomposers, recycling organic matter into nutrients and CO_2. If this particular group is to be targeted, then care should be taken while analysing DGGE patterns (bacterial sample richness or community structure) from communities containing phototroph organisms (plankton, microbial mats or epilithic biofilms), not to overestimate the number of OTUs corresponding to bacteria in this ecological meaning.

As already mentioned, some attempts to avoid this interference have been described, such as size-filtration (Schauer et al. (2000); Rappé et al. (1998)), or combination of the most probable number method with DGGE for microbial mats where filtration is not reliable (Jonkers and Abed (2003); Jonkers et al. (2003)). However, for the first case, care should be taken to phototrophic picoeukaryotes that are not retained on commonly used filters (Rappé et al. (1998)); for the latter approach, its application in an exhaustive diversity study should introduce other biases commonly attributed to cultivation (Colwell (2000)). In theory, a very basic solution to avoid interference with Cyanobacteria and plastidial 16S rDNA should have been to limit the amplification of their corresponding DNA fragments. Basically, such aim should be achieved by cutting the target DNA fragment between the two PCR primers using a restriction enzyme. Unfortunately, an exhaustive analysis of restriction patterns using different enzymes on 16S rDNA database (ARB database) did not yield to any consistent results. After all, sequence analysis remains the most efficient way to select interesting OTUs among the DGGE fingerprint.

5. Conclusion

To conclude, assessment of bacterial diversity using fingerprinting techniques such as 16S rDNA based PCR-DGGE requires methodological settings and complement. In complex phototroph microbial assemblages such as river biofilms, such complements are bands sequencing followed by phylogenetic analysis which should allow to draw more substantial outcomes from the genetic fingerprints. Furthermore, the use of a standardised procedure (biofilm sample size, amount of template DNA, amount of loaded PCR product and DGGE parameters) provided repeatable and consistent results on a set of field samples. Thanks to this approach, the diversity of the biofilm bacteria could be assessed for a better understanding of the biological processes of the river.

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