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Differences in DNA Fingerprints of Bacterioplankton Community as Affected by Biomass Collection Approaches

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To find out different sampling approaches how to affect DNA fingerprinting, environmental genomic DNA was prepared with three sampling approaches. The DNA profiles of 5 bacterial subgroups (alpha-,beta-,gamma-*Proteobacteria*, *Bacteroidetes* and *Cyanobacteria*) and total bacteria were investigated by using denaturing gradient gel electrophoresis (DGGE) fingerprinting. The DGGE patterns showed alpha-*Proteobacteria* had more free-living OTUs, while gamma-*Proteobacteria* and *Bacteroidetes* had more particle-attached OTUs. *Cyanobacteria* and beta-*Proteobacteria* had approximately equal OTU number in the two fractions. Principle component analysis based on DNA banding patterns showed there were obvious differences among free-living, particle-attached fraction and whole bacterial community composition of 3 bacterial subgroups (alpha-, gamma-*Proteobacteria* and *Bacteroidetes*) and total bacteria, while the patterns of beta-*Proteobacteria* and *Cyanobacteria* were similar, suggesting the dynamics of bacterial subgroups can be better understand by combining different collection approaches.

Keywords: Free-living; particle-attached; DGGE pattern; 16S rDNA; bacterial groups.

For obtaining bacterial community information of aquatic environment, microbial cells collecting is the key step of DNA fingerprinting techniques. In eutrophic waters, 0.22 μm pore size filter membrane is used to collect free-living fraction of bacterioplankton, and a larger pore-size (>0.22 μm) filter membrane is used to collect particle-attached fraction[1-6]. However, in addition to researches focusing on particle-attached and free-living bacteria, sampling approach is usually employed with only the larger pore-size filter membrane, leaving free-living bacteria in water sample. Thus biodiversity information of free-living fraction is ignored.

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Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), one of the DNA fingerprinting techniques, have been widely used as sensitive and reproducible tool for analyzing microbial diversity of aquatic ecosystem[7-9]. Inherent shortcomings in carrying out PCR-DGGE, such as PCR bias[10], comigration of bands of different species[11], multiple bands from single species[12], multiple species from common band[13] have been discussed. Experimental improvements, including sampling size[4], DNA extracting method[14], electrophoresis time[15] have been studied. However, little is known about the impact of sampling approaches with different filters on DGGE pattern.

Previous work[5] showed a number of phylotypes targeted by bacterial primer sets were found solely on particles. Compositional and

functional differences observed between particle-attached and free-living communities may reflect different roles in the decomposition or biodegradation in aquifers[16]. Significant inter and intra-lake differences between free-living and particle-associated Actinobacteria were also observed [1]. These earlier studies on particle-attached and free-living microorganism suggested that the two fractions of bacterioplankton differed from each other and were not independent with each other. Based on the previous works, we proposed that different sampling approaches may have impact on bacterial DNA fingerprints, which perhaps result in the loss of bacterial biodiversity information of aquatic ecosystem. For verifying the hypothesis, we compared the impact of three sampling approaches on DGGE patterns in our work.

MATERIALS AND METHODS

Sampling stations

Lake Donghu (30°33'N, 114°23'E) is a eutrophic freshwater lake located in the Wuhan city with an average depth of 2.5 m and a total surface area of 32 km^2. Lake Donghu has been divided into five districts with different nutrient level by the artificial dikes[17]. Seven sampling stations were established according to the shape and trophic status of Lake Donghu. 2 L of water samples were collected from the seven stations of Lake Donghu.

DNA extraction and purification

For total environmental DNA extraction, 300 to 500 ml of water sample was filtered through 1.3 µm pore size GF/C filter membrane (Whatman, USA) to obtain particle-attached bacteria, then the filtrate was filtered through 0.22 µm pore size filter membrane (Millipore, USA) to obtain free-living bacteria. Equal volume water sample was filtered through 0.22 µm pore size filter membrane (Millipore, USA) to obtain total bacteria. filter membranes were transferred into 50 ml sterile polypropylene tubes containing 5 ml lysis buffer (0.5% SDS; 10 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; Protease K, 100 µg/ml) and incubated for more than 2 hours at 55°C. Subsequently, Extraction of genomic DNA was performed using a standard protocol with phenol/chloroform/isoamylalcohol. DNA in the supernatant was precipitated with 1D 3 volume 3 M sodium acetate and 3 volumes absolute alcohol for 2 hours at -20°C. After washing with 70% ethanol, The DNA pellet was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -70°C.

Nested PCR

A two- or three-step nested PCR approach was performed in this study to obtain sufficient PCR product for subsequent DGGE analysis. Firstly, nearly full-length 16S rRNA gene fragments were amplified with the universal primer pairs (9bfm/1512uR), and then the PCR product was used as template for the second PCRs with group-specific primers (Table 1). The group-specific PCR product was used as template in the final PCR with DGGE primers (Table 2). An additional 40 bp GC-rich nucleotide sequence (GC-clamp) was added to stabilize migration of the DNA fragment in the DGGE.

PCRs were performed on a Bio-Rad S1000 thermal cycler (Bio-rad Inc, USA). All PCRs used the similar basic thermal cycle protocol as described in previous work[18]. The annealing temperatures (ATs) were dependent on primer pairs used, and then optimized in a gradient PCR. A typical PCR reaction was carried out in 25-µl volumes and contained 1×Taq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 50 nM of each of the primers, 2.5 U of Taq DNA polymerase (Fermentas Inc. Hanover, USA) and 2 µl of template DNA. Briefly, an initial denaturation at 96°C for 4 min, 30 cycles were performed (96°C for 1 min, AT for 1 min, 74°C for 1 min) followed by a final extension at 74°C for 10 min. In addition, the extension time was prolonged to 90 s for the bacteria universal primer pair 9bfm/1512uR.

DGGE fingerprinting

DGGE was performed using the Bio-Rad DCode universal mutation detection system (Bio-Rad Inc, USA). Approximately equal amounts of PCR amplicons were loaded onto 1mm thick vertical gels containing 9 %(w/v) polyacrylamide and a linear gradient of denaturants (100% denaturant is defined as 7 M urea and 40% (v/v) formamide). Different denaturing gradients were applied to separate 16S rDNA fragments for obtaining a clear DNA banding pattern. The gels were prepared in 1×TAE buffer (40 mM Tris acetate, 40 mM acetic acid, 1 mM EDTA, pH 8.0), which was also used as the electrophoresis buffer. Electrophoresis was run

for 11 hours at a constant voltage of 140 V and 60°C. After electrophoresis, the gels were stained in 1×TAE buffer containing 1×SYBR Gold (Molecular Probes Europe BV, Leiden, the Netherlands). Images of DGGE patterns were snapped using a Gel Documentation system (Bio-Rad Inc., USA). Replication of the PCR and DGGE steps was performed to confirm the consistency of DGGE patterns.

Data processing and statistical analysis

Quantity One 4.6.2 (Bio-Rad Inc, USA) was used to analysis the gel images of DGGE patterns, and the banding patterns were labeled manually. Each DGGE pattern was turned into a matrix indicating the band present with band intensity and its absence with zero (if present in any other banding pattern). Each band type represented an operational taxonomic unit (OTU) and its intensity represented relative abundance in the community. Principle component analysis (PCA) on Canoco 4.51 was applied to compare the similarity of bacterial community composition among the sampling stations.

RESULTS

DGGE patterns

16S rRNA gene fragments of bacterial subgroups and total bacterial community from seven sampling stations were amplified by using a nested PCR protocol as previously described[18]. With different denaturing gradient, we had produced DNA banding patterns of these bacterial subgroups and total bacterial communities. Numbers of DGGE bands varied with the bacterial subgroups and total bacteria, some obvious changes of band intensity were detected in DGGE patterns. Most of the DGGE bands were common in attached, free-living fractions and whole community. Most particle-attached bacteria showed more complex community structure than the free-living fraction according to the OTU

Table 1. Group-specific PCR primers used in this study

Target group	Primer	Sequence (5' to 3')	Reference
α-*Proteobacteria*	Alf28f; Alf684r	ARCGAACGCTGGCGGCA; TACGAATTTYACCTCTACA	Ashelford *et al.,* 2002
β-*Proteobacteria*	Beta359f; Beta682r	GGGGAATTTTGGACAATGGG; ACGCATTTCACTGCTACACG	Ashelford *et al*., 2002
γ-*Proteobacteria*	Gamma395f; Gamma871r	CMATGCCGCGTGTGTGAA; ACTCCCCAGGCGGTCDACTTA	Mühling *et al*., 2009
Bacteroidetes	CFB555f; CFB968r	CCGGAWTYATTGGGTTTAAAGGG; GGTAAGGTTCCTCGCGTA	Mühling *et al*., 2009
Cyanobacteria, chloroplast	CYA361f; CYA785r	GGAATTTTCCGCAATGGG; GACTACWGGGGTATCTAATCC	Mühling *et al*., 2009
Universal (Bacteria and archaea)	9bfm; 1512uR	GAGTTTGATYHTGGCTCAG; ACGGHTACCTTGTTACGACTT	Mühling *et al*., 2009; Weisburg *et al*., 1991

Table 2. DGGE primers for nested PCR used in this study

Primers used for second-step PCR	Primers used for third-step PCR	Third-step PCR primer sequence (5' to 3')	Reference
Alf28f/Alf684r	341f-GC /518r	341f, CCTACGGGAGGCAGCAG	Muyzer *et al*., 1993
Beta359f/Beta682r	518f-GC/beta682r	518f, CCAGCAGCCGCGGTAAT	Muyzer *et al*., 1993
Gamma395f/Gamma871r	518f-GC/785r	518r, ATTACCGCGGCTGCTGG	Muyzer *et al*., 1993
CFB555f/CFB968r	CFB555r-GC/907r	785r, CTACCAGGGTATCTAATCC	Lee *et al*., 1993
CYA361f/CYA785r	518f-GC/CYA785r	907r, CCGTCAATTCMTTTGAGTTT	Muyzer *et al*., 1993
9bfm/1512uR	341f-GC/518r		

GC clamp, CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG (Muyzer *et al.,* 1993).

Table 3. Total OTU number of bacterial subgroups and bacteria in all seven sampling stations

	Particle-attached	Free-living	Whole community
alpha-*Proteobacteria*	100	137	86
beta-*Proteobacteria*	74	70	74
gamma-*Proteobacteria*	109	77	121
Bacteroidetes	163	149	167
Cyanobacteria	87	86	85
Bacteria	121	118	120

number (Table 3). Particle-attached bacteria, free-living bacteria and whole community OTU number of 3 groups (*Cyanobacteria,* beta-*Proteobacteria* and bacteria) were nearly same respectively.

Community structure analysis

Based on the matrixes of DNA band intensity, PCA was performed to analyze the bacterial community structure of particle-attached fraction, free-living fraction and whole community. The PCA results revealed that the bacterial community structure of free-living fraction was very different from the particle-attached fraction and whole community of alpha-, gamma-*Proteobacteria*, *Bacteroidetes* and bacteria along the first and second principal components (PCs), which together described 69.7%, 68.2%, 54.8% and 56.6% of the total variance respectively (Fig. 2). In addition, the particle-attached fraction and whole community did not separate from each other obviously in these DNA patterns, except bacteria. For β- *Proteobacteria* and *Cyanobacteria*, the two fractions and whole community did not group together clearly, which together described 69.4% and 57.8% of the total variance respectively (Fig.2)

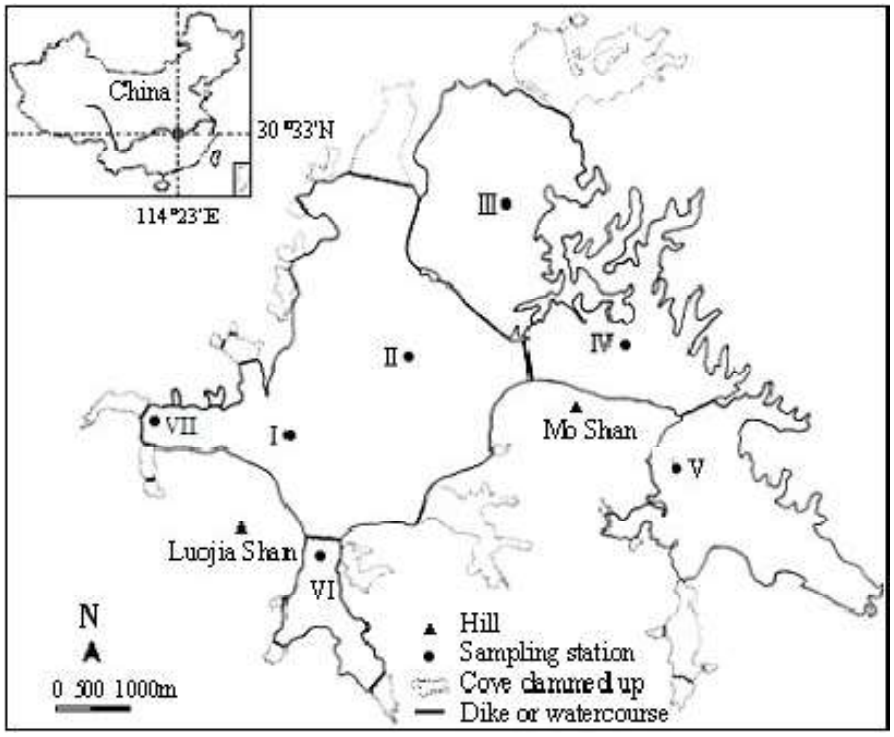

Fig. 1. Distribution of the sampling stations in Lake Donghu (Wuhan, China)

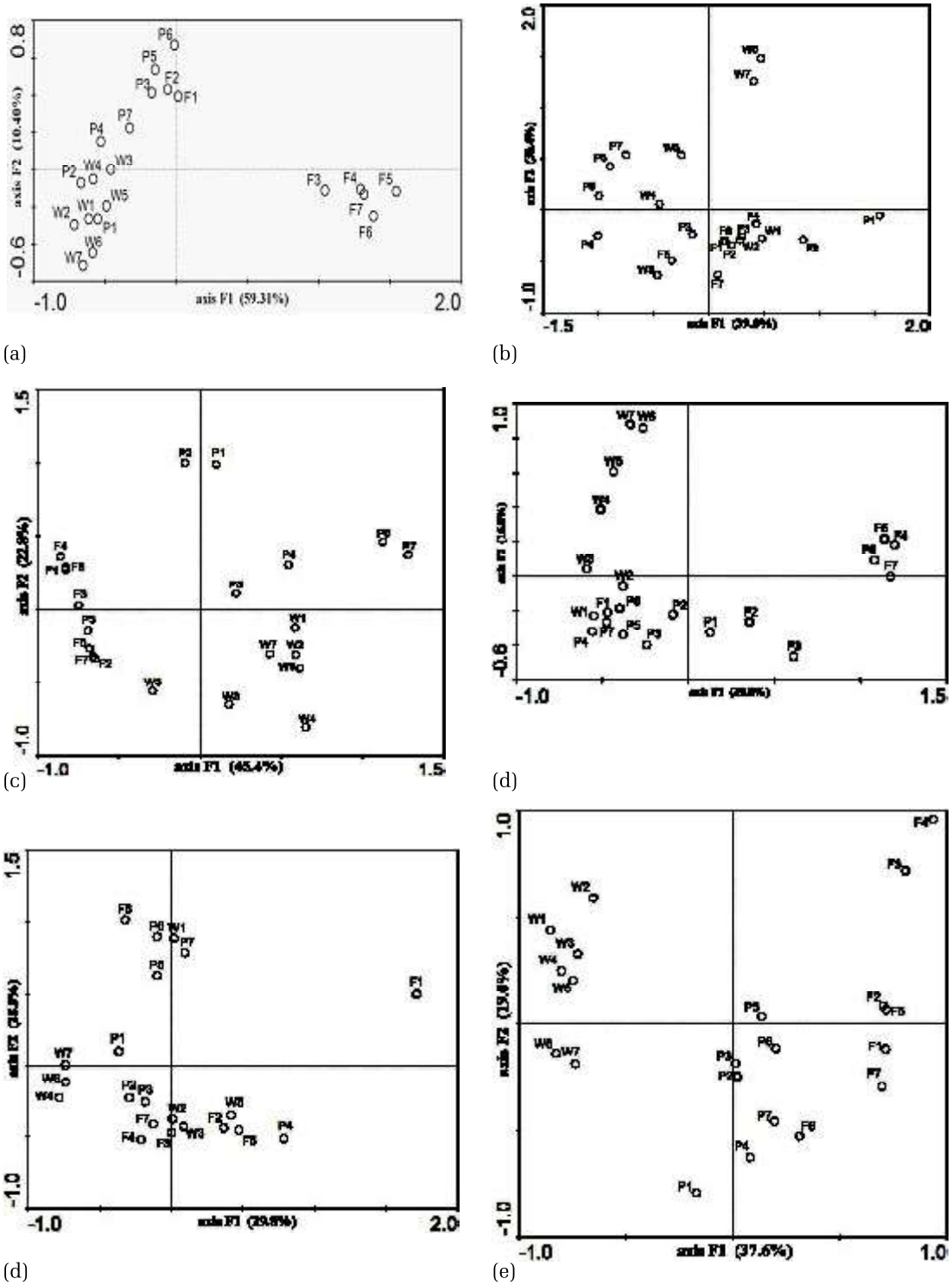

Fig. 2. PCA scatter plots of DNA banding patterns from alpha-*Proteobacteria* (A), beta-*Proteobacteria* (B), gamma-*Proteobacteria* (C), *Bacteroidetes* (D), *Cyanobacteria* (E) and bacteria (F). P1 to P7 represent the particle-attached bacteria, F1 to F7 represent the free-living bacteria, and W1 to W7 represent the whole bacteria communities, which were sampled from seven stations of Lake Donghu, China

DISCUSSION

In recent years, group specific PCR-DGGE has increasingly been used as a helpful tool in community structural analysis of bacterial subgroups[18, 19]. DNA templates for PCR derived from different sampling approaches perhaps change the DGGE pattern, resulting in misunderstanding on bacterial pattern succession. The growth abilities, population dominance and community structure of particle-attached and free-living bacterioplankton varied with trophic state, hydrological conditions and anthropogenic pollutions[2, 20]. Significant differences of community composition between the two fractions were found in freshwater, estuary and seawater[21-23]. Accordingly, environmental DNA was prepared with three sampling approaches in this study, which were used to find out whether these approaches have impact on the DGGE patterns.

Operational taxonomic unit succession pattern in the more typical marine station was clear, while in the station affected by riverine inputs was not clear[24]. The free-living bacteria presented a more complex community structure than the particle-attached fraction in seawater[4]. According to the results of our work, most of the particle-attached fractions of bacterioplankton community were more complex in freshwater ecosystem. It is possible that particle-attached bacteria are more sensitive to salinity. The highest number of bands among free-living bacteria was also observed in freshwater mesocosm during an induced diatom bloom[5]. However, free-living fraction of alpha-*Proteobacteria* was more complex contrary to the other subgroups. The results suggested that organic/inorganic particles may play a more important role, while the attachment to particle was not an advantage for alpha-*Proteobacteria* as reported by previous study[3]. In this work, the particle-attached bacteria exhibited a greater diversity of phylotypes than free-living fractions.

The study of attached bacteria in Mediterranean Sea suggested that particles were colonized by a relatively limited number of ubiquitous ribotypes, which were also detected frequently in seawater[25]. In accordance with the previous results, a great number of common DGGE bands were found in bacteria and all subgroups, suggesting particles were colonized by ubiquitous ribotypes in freshwater lake. To compare with diversity indices, DGGE pattern provided more information about the community structure, which can discriminate the bacterial community with similar diversity indices. Since alpha, beta-Proteobacteria and bacteria showed distinct differences in scatter plots of DNA banding patterns In contrast, the different fraction of Bacteroidetes distinguished from each other, which showed no significant differences in all diversity indices. That phenomenon suggested that there were similar species richness and abundance in the different fraction of Bacteroidetes, but with different population composition. In contrast to free-living fractions, community structure of particle-attached fractions were more similar to whole communities, suggesting the large amount of particle-attached bacterial DNA gained a competitive advantage over free-living fraction in PCR amplification. For research focus on bacterial community in freshwater ecosystem, particle-attached and free-living fractions should be analyzed separately by using different biomass collection approaches.

In summary, alpha-*Proteobacteria* had more free-living OTUs, while gamma-*Proteobacteria* and *Bacteroidetes* had more particle-attached OTUs. *Cyanobacteria* and beta-*Proteobacteria* had similar composition in the two fractions. Free-living fractions of alpha-, gamma-*Proteobacteria*, *Bacteroidetes* and bacteria were very different from the particle-attached fraction and whole community, suggesting that the dynamics of these bacterial subgroups can be better understand by combining different collection approaches.

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