

Cyanobacterial community diversity in the sediments of the Pearl River Estuary in China

Fu-Lin Sun^{1,2}, You-Shao Wang^{1,2}, Mei-Lin Wu¹, Cui-Ci Sun^{1,2}

¹ State Key Laboratory of Tropical Oceanography, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China.

² Daya Bay Marine Biology Research Station, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Shenzhen 518121, China.

(F-LS) E-mail: flsun@scsio.ac.cn. ORCID-iD: <http://orcid.org/0000-0003-3373-3000>

(Y-SW) (Corresponding author) E-mail: yswang@scsio.ac.cn. ORCID-iD: <http://orcid.org/0000-0001-9565-2666>

(M-LW) E-mail: mlwu@scsio.ac.cn. ORCID-iD: <http://orcid.org/0000-0002-3387-8122>

(C-CS) E-mail: scuici@scsio.ac.cn. ORCID-iD: <http://orcid.org/0000-0002-7332-5100>

Summary: Cyanobacterial community diversity in the sediment of the Pearl River Estuary in China was evaluated in this study by denaturing gradient gel electrophoresis (DGGE) during the wet and dry seasons. Nucleotide sequences obtained from DGGE bands were classified into five cyanobacterial clusters, including *Synechococcus*, *Cyanobium*, *Chroococcus*, *Prochlorales* and *Tolypothrix*. *Synechococcus* was identified as the dominant cyanobacterial group in the sediment samples; its distribution varied from the inner estuary to the outer estuary, with a wide range of salinity adaptation. Observed patterns of cyanobacterial communities changed markedly between sampling sites and seasons, suggesting that most cyanobacteria were not delivered via fresh water. Canonical correspondence analysis was conducted to determine the relationship between environmental variables and bacterial community structures during the dry season. The results suggested that the cyanobacterial community was significantly influenced by pH, salinity, PO₄-P and NO₃-N in sediments.

Keywords: estuary; sediment; cyanobacterial community diversity; DGGE.

Comunidad de cianobacterias en los sedimentos del estuario del río Perla en China

Resumen: La diversidad de la comunidad de cianobacterias en el sedimento del estuario del río Perla en China fue evaluada en este estudio por electroforesis en gel de gradiente desnaturalizante (DGGE) durante las estaciones húmeda y seca. Las secuencias de nucleótidos obtenidas de bandas DGGE se clasificaron en cinco grupos de cianobacterias, incluyendo *Synechococcus*, *Cyanobium*, *Chroococcus*, *Prochlorales* y *Tolypothrix*. *Synechococcus* fue identificado como el grupo dominante de cianobacterias en las muestras de sedimento, su distribución varió desde la parte interna del estuario hasta la externa, con un amplio rango de adaptación a la salinidad. Los patrones observados de las comunidades de cianobacterias cambiaron marcadamente entre diferentes sitios de muestreo en diferentes estaciones del año y sugirió que la mayoría de las cianobacterias no provenían a través del agua dulce. Se realizó un análisis de correspondencia canónica (CCA) para determinar la relación entre variables ambientales y estructuras de las comunidades bacterianas durante la estación seca. Los resultados sugirieron que las distintas comunidades de cianobacterias estaban significativamente influenciadas por el pH, salinidad, PO₄-P y NO₃-N en los sedimentos.

Palabras clave: estuario; sedimento; diversidad de la comunidad cianobacteriana; DGGE.

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INTRODUCTION

Cyanobacteria form part of numerous aquatic ecosystems and can be found in almost all types of habitat, including terrestrial, coastal and marine environments.

In these environments, cyanobacteria contribute massively to photosynthesis and nitrogen fixation (Whitton and Potts 2000). Cyanobacteria also affect the microbial food web and the primary production in the oceans (Campbell et al. 1994, Savage et al. 2010). The cyano-

bacterium *Synechococcus* is abundant in coastal waters, whereas the cyanobacterium *Prochlorococcus* only occurs at oceanic stations (Liu et al. 2004). Because of the high abundances and contributions to primary production of *Prochlorococcus* and *Synechococcus*, these changes may have large impacts on ocean ecosystems and biogeochemical cycles (Flombaum et al. 2013). Picoplankton and filamentous species may represent a small fraction of the cyanobacterial community in terms of biomass, but their importance is related to their contribution to phytoplankton diversity (Caroppo et al. 2006).

The above-mentioned species have been physiologically examined on strains in the laboratory; only a small number of naturally occurring microorganisms can be represented by cultivated isolates. Culture-independent molecular-based approaches such as 16S rRNA–DGGE were used to evaluate the distribution of cyanobacterial communities in complex systems (Boutte et al. 2006, Dadheech et al. 2009, Fewer et al. 2009). Polymerase chain reaction–DGGE (PCR–DGGE) is still a powerful technique and has been routinely employed for monitoring diversities of microbial communities under changing environments. Genetic diversity of cyanobacterial community in various environments has also been reported; environmental factors such as salinity and water temperature significantly affect cyanobacterial diversity (Al-Thukair et al. 2007, Boutte et al. 2008, Jing et al. 2009). The majority of these studies have focused on the cyanobacterial community in water. Chamberlain et al. (2014) reported that a cyanobacterial community structure can respond to biotic/abiotic variables and environmental conditions, and that it may also be influenced by runoff from terrestrial habitats in coastal water and sediment. The cyanobacterial community in sediment consists of benthic and planktonic representatives (Kormas et al. 2010). Correlation analysis has indicated that cyanobacterial abundance in water and sediment is affected more by water than by sediment properties (Quesada and Fernández-Valiente 1996). Several studies have focused on sediment cyanobacterial migration to water and demonstrated that the quantity of cyanobacteria recruitment is higher in shallow regions. Recruitment of benthic cyanobacteria can serve as a seed bank for the pelagic phase. Summer bloom development has also been linked to the emergence of cyanobacteria from sediment populations, and the absence of benthic recruitment reduces summer blooms (Tsuji-mura and Okubo 2003, Verspagen et al. 2005).

The Pearl River is the second largest river in China and is located in the subtropical zone of the country. A wet and warm southwest monsoon prevails during the wet season, while a dry and cold northeast monsoon predominates during the dry season. The water of the Pearl River plume exhibits great seasonal variation because the discharge is higher in the wet season than in the dry season. Salinity, pH and N and P nutrients showed a significant gradient along the estuary. As freshwater discharge was greatly reduced, salt water could intrude far into the river gates in the dry season (Dong et al. 2004). Salinity and pH in the dry season increased markedly in comparison with the wet season.

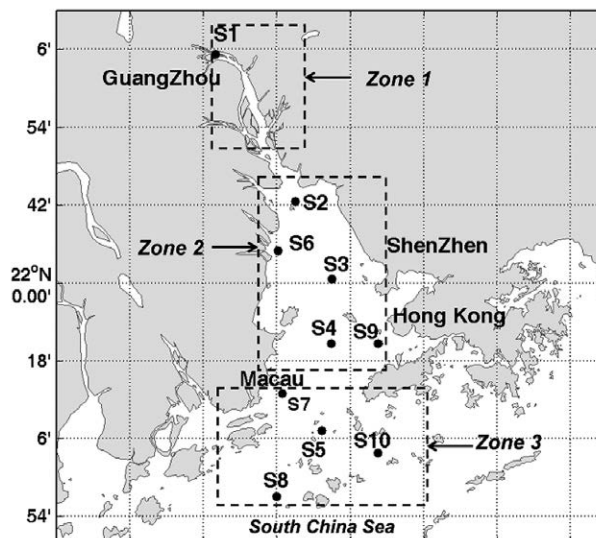


Fig. 1. – Sampling sites in the Pearl River Estuary.

In previous studies, cyanobacteria taxa have often been observed in the Pearl River Estuary (Sun et al. 2011, 2012, 2015) and these studies have indicated that cyanobacteria are the dominant group in this estuarine sediment. However, no studies on the ecological distribution of cyanobacteria in the Pearl River Estuary sediment have been reported. The present study aims first to understand the distribution pattern of the cyanobacterial community in the Pearl River Estuary sediment. Second, it aims to identify the major cyanobacterial phylogenetic groups and their diversity based on culture-independent techniques. Finally, it evaluates the impacts of environmental changes such as pH and salinity variations in the cyanobacterial community.

MATERIALS AND METHODS

Field sample collection

Two cruises were conducted in August 2009 during the wet season and in January 2010 during the dry season in the Pearl River Estuary. The geographic locations of the sampling sites are shown in Figure 1. Sediment samples were taken for three replicates at 0–2 cm depth with a core sampler and then stored in a refrigerator at -20°C . Bottom water samples for biochemical analysis were also collected from these ten sites. Water samples were filtered online through a 47-mm filter with $0.45\text{-}\mu\text{m}$ pore size filters (Millipore, USA), and then the filters were stored at -20°C .

Physicochemical parameters of bottom water

Salinity, temperature, and pH were determined *in situ* using the YSI 6600 V2 Sonde water quality monitoring system (YSI Incorporated, USA). Concentrations of ammonium ($\text{NH}_4\text{-N}$), nitrate ($\text{NO}_3\text{-N}$), nitrite ($\text{NO}_2\text{-N}$), silicate ($\text{SiO}_3\text{-Si}$) and phosphate ($\text{PO}_4\text{-P}$) were determined using standard analytical methods, as described in Sun et al. (2011). Results of physicochemical parameters are shown in Figure 2.

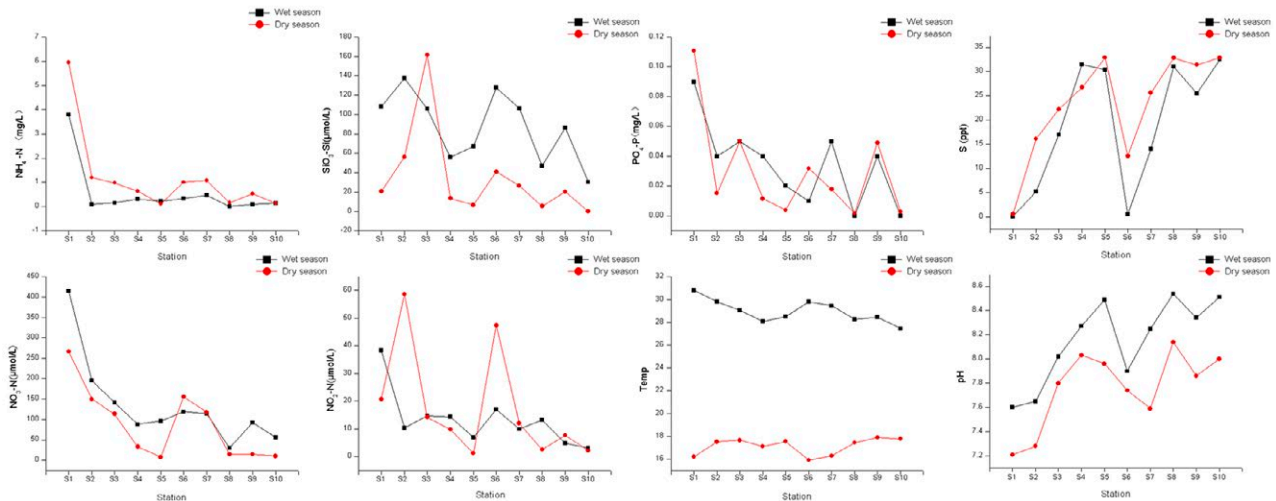


Fig. 2. – Water physicochemical parameters of the Pearl River Estuary.

Total community DNA extraction

Total community/genomic DNA were extracted from 1.0 g of wet sediment using the Soil DNA Kit (Biomiga Inc., USA). The quantity and quality of extracted DNA samples re-dissolved in TE buffer were estimated by electrophoresis of 2.5- μ L aliquots on 0.8% agarose gel and then compared against a molecular weight standard (Takara, Japan). These samples were stored at -20°C prior to analysis.

Amplification of 16S rRNA gene fragments

Primers CYA359-GC and CYA781R (equimolar mixture between primers CYA781Ra and CYA781Rb) were used for PCR amplification of the cyanobacterial 16S rRNA gene from environmental DNA (Nübel et al. 1997). PCR reactions were conducted in 50- μ L reaction volumes containing 2500 ng of bovine serum albumin, 15 pmol of each primer, 20 mmol dNTPs, 1.25 U of ExTaq DNA polymerase (Takara) and 2 μ L of diluted genomic DNAs (5–30 ng μL^{-1}). The thermal PCR protocol was as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 54°C for 1 min, chain extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were confirmed by analysing 2.5 μL of the PCR product on 1.5% (w/v) agarose gel, stained with ethidium bromide and visualized using AlphaImager (Alpha Innotech, Japan).

DGGE

An INGENYphorU-2 system (Ingeny International BV, Goes, NL) was used to perform DGGE analysis. Three replication products were mixed and loaded into 8% wt/vol acrylamide gels in 1 \times TAE buffer containing a linear chemical gradient of 40%–70% denaturant. Gels were run at 120 V for 18 h at 60°C , stained with an ethidium bromide solution for 30 min, and rinsed with distilled water. The gels were subsequently viewed under UV light on an AlphaImager imaging system (Alpha Innotech, Japan).

Analysis of DGGE patterns

DGGE bands that shared an identical migration position were considered to be of similar species. Bands were visually identified and distinguished by the distance migrated and intensity in the gel using BandScan 5.0 (PROZYME, USA). Each band was numbered and scored in each sediment sample. To assess the change in genetic diversity of cyanobacterial communities in different samples, DGGE banding patterns were analysed by multi-dimensional scaling (MDS) analysis with SPSS 18.0 for Windows (SPSS Inc., USA). For this purpose, dissimilarity indices were recorded in a binary matrix, which was then analysed by the Euclidean distance method. MDS is an ordination technique that represents samples as points in a multi-dimensional space. Sample communities with the highest similarity in the data set are shown as the closest plotted points, and communities with the lowest similarity are indicated by points that are farthest apart.

The relationship between DGGE profiles and the physicochemical properties of bottom water was investigated. To determine whether weighted-averaging techniques or linear methods were appropriate, detrended correspondence analysis (DCA) was performed using CANOCO for Windows 4.5 (Biometris, the Netherlands). The longest gradients derived from DCA were 9.677 for the analysis based on DGGE profiles. The results demonstrated that the data exhibited unimodal rather than linear responses to the environmental variables (Lepš and Šmilauer 2003). Thus, we performed canonical correspondence analysis (CCA) to explain our data. Manual selection of environmental variables, applying a Monte Carlo permutation test (499 permutations) with unrestricted permutation, was performed to evaluate the statistical significance.

Cloning of 16S rRNA gene fragments after DGGE analysis

DGGE bands were excised using a scalpel blade and then incubated at 4°C overnight in 20 μL of TE

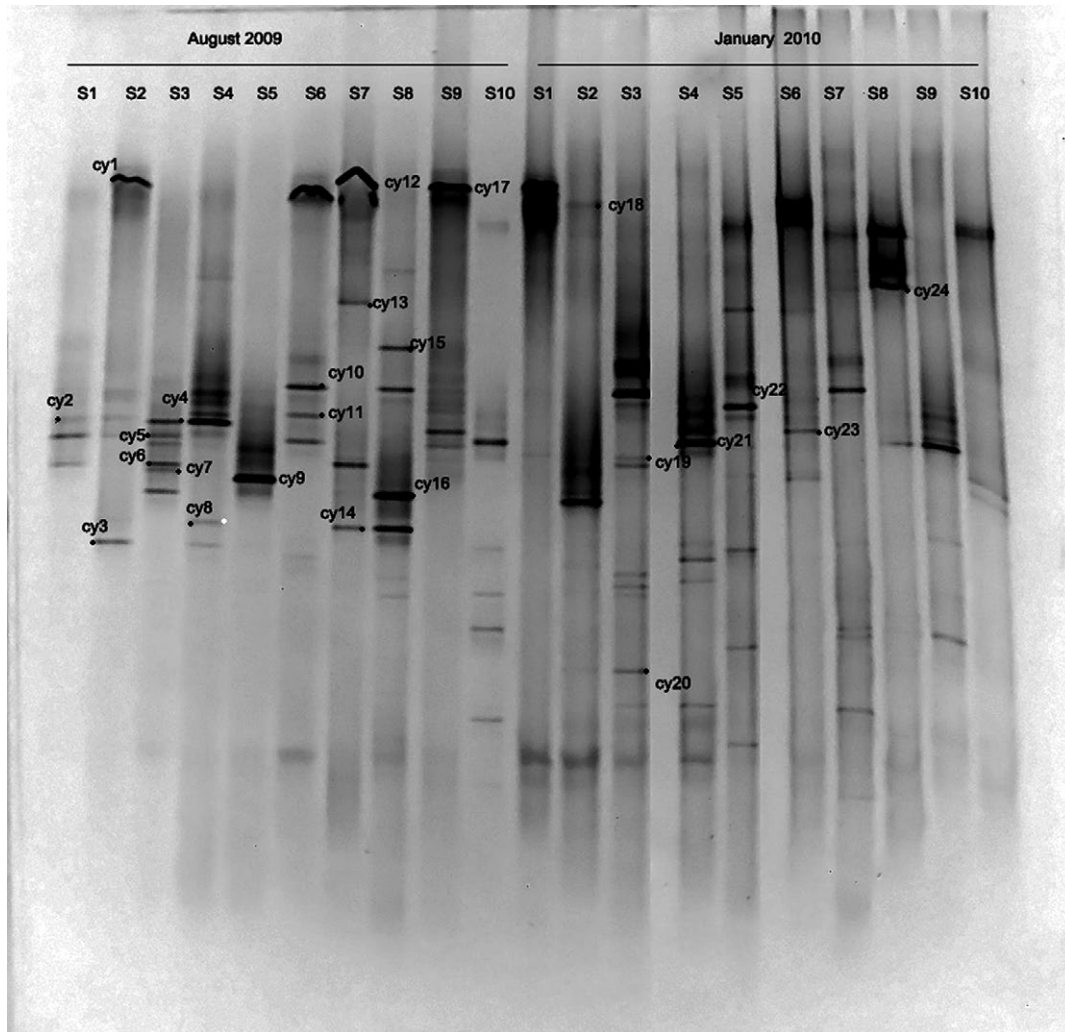


Fig. 3. – DGGE analysis of cyanobacteria in the Pearl River Estuary sediment. Labels of S1-S10 at the top of the lanes represent corresponding sampling sites. Sequencing bands have been indicated.

buffer before they were re-amplified. The positions of the excised bands in the DGGE gel were confirmed with repeated DGGE. Bands showing the expected melting position were amplified with the CYA359/CYA781R primer without GC-clamp. The PCR products were purified with the PCR Purification kit (Takara Co. Ltd) and subsequently cloned into *E. coli* DH5a cells using a pMD18-T cloning vector kit in accordance with the manufacturer's instructions (Takara Co. Ltd). Positive clones were identified by PCR amplification with the pMD18-T vector primer pairs T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and M13 (5'-CAG GAA ACA GCT ATG ACC-3'). The same program as for 16S rRNA gene amplification was used.

DNA sequencing and phylogenetic tree

Positive recombinants were submitted for sequencing using an ABI3730 DNA Sequencer (USA) with an M13 primer (Shanghai Major Biotech Co. Ltd). All sequences obtained in this study have been deposited to the GenBank nucleotide sequence database with accession numbers HQ849488-HQ849505, HQ849508,

HQ849509, HQ849513, HQ849515, HQ849517, HQ849520 and HQ849522. Nucleotide sequences were compared with those in the GenBank nucleotide database using the BLAST algorithm for identification. Phylogenetic trees of 16S rRNA gene sequences were generated using neighbour-joining algorithms in Mega 5.0. The support level for the phylogenies derived from neighbour-joining analysis was gauged by 1000 bootstrap replicates.

RESULTS

DGGE fingerprint profiles

Analyses of DGGE gel resulted in a total of 129 detectable bands in 50 different positions (Figs. 3, 4). The number of bands per sample varied between 2 and 11, indicating diverse cyanobacteria in estuary sediments. A total of 24 bands were excised and successfully sequenced. As indicated in Figure 4, the cyanobacterial community at sites S3, S4, S5, and S7 exhibited higher diversity and had more microbial genotypes in the dry season than in the wet season during our investigation. Meanwhile, the cyanobacte-

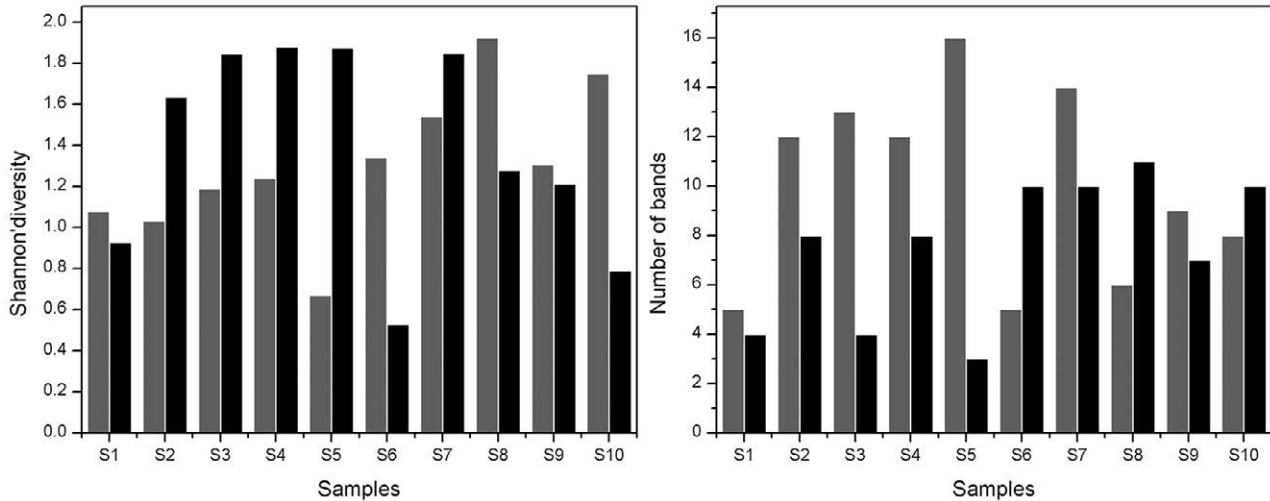


Fig. 4. – Comparison of the cyanobacterial diversity of sediment samples (H' was calculated on the basis of the number and relative intensities of bands on the gel track). Grey columns stand for samples in wet season; black columns stand for samples in dry season.

rial community at sites S6, S8 S9, and S10 exhibited higher diversity and had more microbial genotypes in the wet season than in the dry season.

MDS analysis of DGGE banding pattern

Two-dimensional plots of MDS scores for sediment samples showed spatial diversity in the sediment cyanobacterial community (Fig. 5). MDS analysis demonstrated that the cyanobacterial community in the wet season had a different structure from that in the dry season, with an MDS stress value of 0.18 (stress values below 0.2 indicate that an MDS ordination plot is a good spatial representation of the differences between data). In the dry season, the cyanobacterial community distinctly varies between the inner estuary (Zone 1), middle estuary (Zone 2), and outer estuary (Zone 3). In the wet season, no obvious difference was observed between the inner estuary, middle estuary and outer estuary.

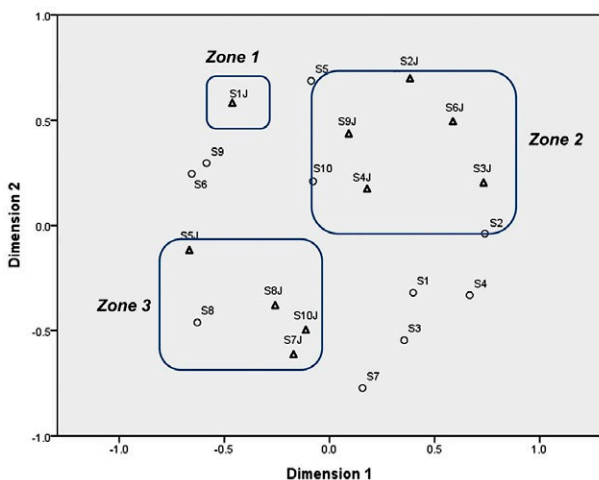


Fig. 5. – Two-dimensional plots of MDS analysis of DGGE patterns to compare broad-scale differences between cyanobacterial communities. S1-S10 represents samples in wet season; S1J-S10J represents samples in dry season.

Bottom water physicochemical parameters

Figure 2 shows the physicochemical properties of the bottom waters in the wet season and dry season. Large variations in these parameters at ten sampling sites were observed. Inorganic nutrients ($\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, and $\text{NH}_4\text{-N}$) showed a gradient of decrease from the inner to the outer estuary. The salinity and pH measured in this study showed a gradient of increase from the inner to the outer estuary and were higher in the dry season than in the wet season.

Microbial community composition in relation to environmental variables

On the basis of the CCA result, all environmental variables had high P-values ($P > 0.05$) in the wet season, indicating that water physicochemical parameters exhibited no significant correlation with the

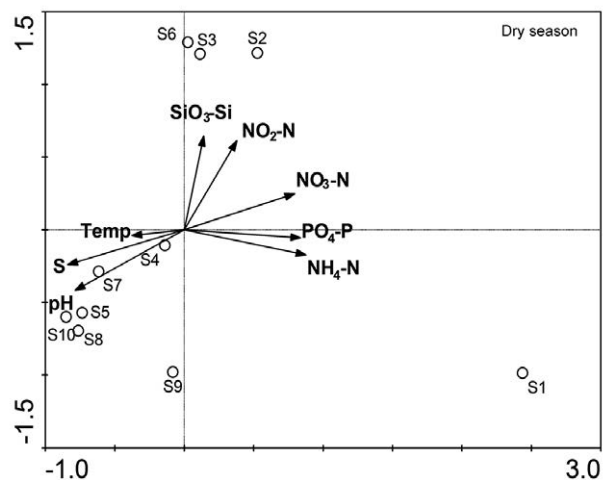


Fig. 6. – Canonical correspondence analysis ordination diagram of cyanobacterial communities in the dry season and wet season associated with environmental variables of silicate ($\text{SiO}_3\text{-Si}$), phosphate ($\text{PO}_4\text{-P}$), ammonium ($\text{NH}_4\text{-N}$), nitrate ($\text{NO}_3\text{-N}$), nitrite ($\text{NO}_2\text{-N}$); salinity (S), and temperature (Temp).

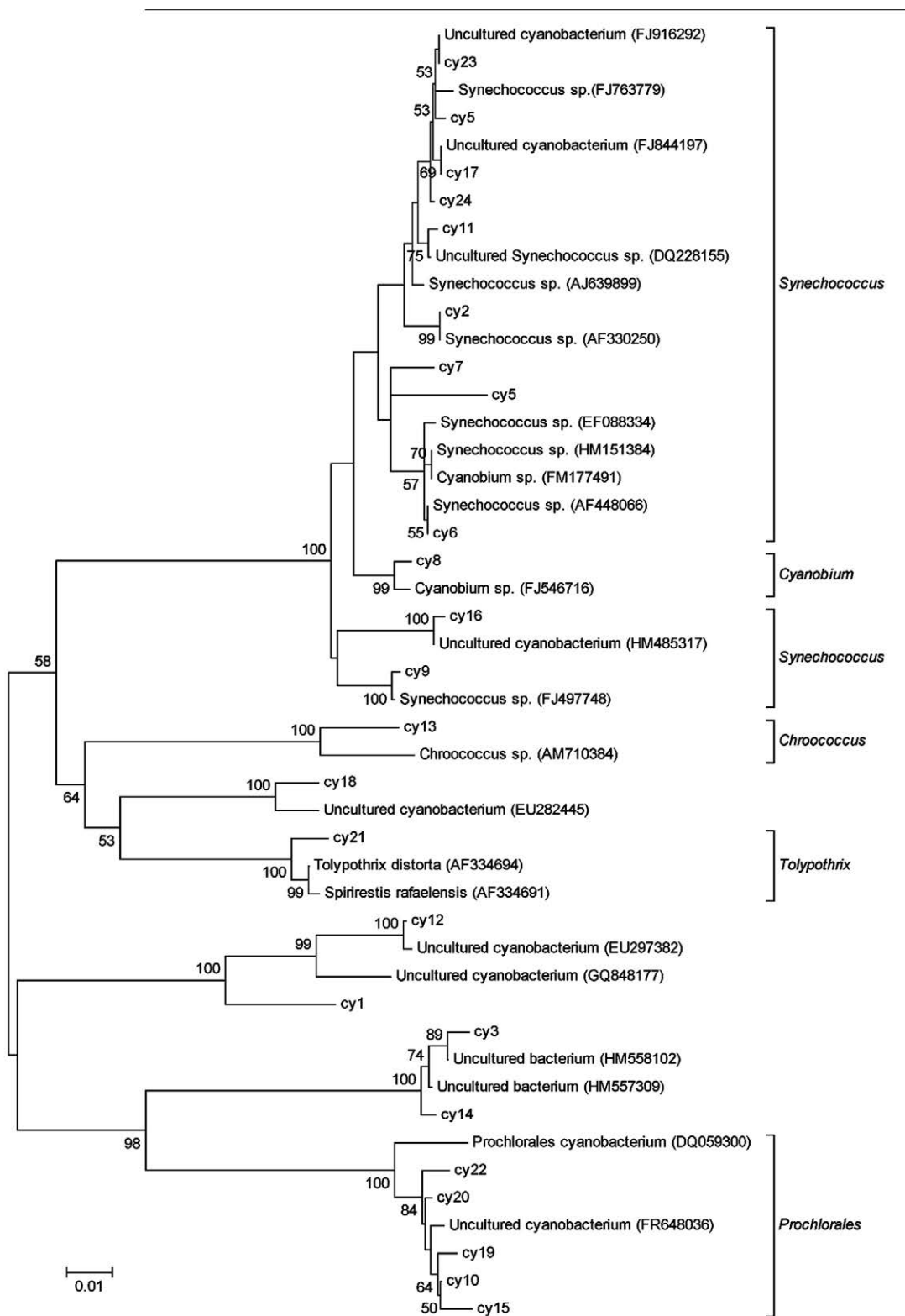


Fig. 7. – Phylogenetic tree based on 16S rRNA gene sequences representing the respective DGGE bands in Figure 3. Bootstrap analysis was based on 1000 replicates. Bootstrap values from distance analysis are depicted. Bootstrap values lower than 50% are not shown.

surface sediment cyanobacterial community. Biplots consisting of environmental variables and DGGE samples (lanes) were chosen to analyse the effects of the physicochemical properties of water in the dry season (Fig. 6).

The sum of all unconstrained eigenvalues indicated an overall variance of 5.145 in the dataset. The total variation that could be attributed to environmental variation was 2.593, as indicated by the sum of all canonical eigenvalues. The first and second axes ex-

plained 59.8% of the cumulative percentage variance in the data. Species–environment correlations were high, especially for axes 1 and 2 (0.996 and 0.988), indicating a strong relationship between samples and environmental variables. On the basis of the 5% level in a Monte Carlo permutation test, the sampling site emerged as a highly significant explanatory variable. CCA results in this study suggested that pH ($p=0.012$), $\text{PO}_4\text{-P}$ ($p=0.042$) and $\text{NO}_3\text{-N}$ ($p=0.042$) significantly influenced the cyanobacterial community composition in the sediments. Cyanobacterial communities of the sampling sites (S4, S5, S7, S8 and S10) at the outer estuary were positively affected by pH ($p=0.012$) and salinity ($P=0.040$).

Identification and phylogenetic analyses of predominant cyanobacterial phylotypes based on 16S rRNA gene sequences

For phylogenetic analyses, the 16S rRNA gene sequences of the 24 clones in this study were compared with those from the GenBank nucleotide database. Neighbour-joining analyses divided these sequences into five groups: *Synechococcus*, *Cyanobium*, *Chroococcus*, *Prochlorales* and *Tolypothrix* (Fig. 7). According to BLAST results, bands cy2, cy5, cy6, cy7 and cy9 sequences were identical to cultivable cyanobacteria. Band cy8 had 99% similarity with *Cyanobium* sp., and band cy13 had 100% similarity with *Chroococcus* sp. In the *Chroococcales* cluster, bands (cy2, cy5, cy6, cy7, cy9, cy11, cy17, cy23 and cy24) had 96–100% similarity to *Synechococcus* sp. In the *Prochlorales* cluster, band cy20 was 97% identical to 16S rRNA gene sequence of the *Prochlorales* cyanobacteria. Band cy21 was 98% similar to *Spirirestis rafaensis*, which belonged to the *Tolypothrix* cluster. However, four sequences (cy1, cy3, cy12, and cy14) were highly similar with uncultured cyanobacteria and could not be verified with a strong credible value to any defined genera in the phylogenetic analyses.

Combined DGGE bands with the phylogenetic group *Synechococcus* comprised the highest number of genotypes of the cyanobacterial community and reached 60% and 45.8% in the dry and wet seasons, respectively. *Prochlorales* and *Tolypothrix* had relatively high number of genotypes in January, exceeding 20%. Other taxa exhibited relatively low number of genotypes with less than 10%.

DISCUSSION

Main groups of cyanobacteria in sediments of the Pearl River Estuary

Twenty-four cyanobacterial phylotypes identified in this study are classified into five cyanobacterial genera (Figs 3 and 7). Most sequences exhibited 97–100% sequence homology to their representative taxa. Cyanobacterial phylotypes of the genera *Synechococcus*, *Cyanobium*, *Chroococcus*, *Prochlorales* and *Tolypothrix* have been reported for the first time in the sediments of the Pearl River Estuary. This study re-

vealed diverse predominant cyanobacteria in different estuarine zones. Many cyanobacterial species, which appeared in the inner estuary and the middle estuary, disappeared in the outer estuary, suggesting the estuary had a varied distribution of cyanobacterial community.

Synechococcus has a wide geographical distribution and is generally the dominant phytoplankton in nutrient-rich mixed waters (Partensky et al. 1999, Flombaum et al. 2013). The popularity of marine *Synechococcus* spp. in the aquatic system has been attributed to its ability to grow over a wide range of light intensities (Kana and Glibert 1987) and to utilize various nitrogen resources. *Synechococcus* was the species exhibiting the highest diversity and was detected at each sampling site of the Pearl River Estuary, which comprised about 50% of the total number of bands. Although some cyanobacteria can grow near optimal rates over a wide range of salinities, their occurrences in the field were restricted to the highest salinities (Nübel et al. 2000). Increasing salinities could result in aggregation of photosynthetically inhibited cells and, due to prolonged exposure, to increasing osmotic stress (Sellner et al. 1988). Some sequences of *Synechococcus* that existed in the middle estuary may not have been detected at the stations of the outer estuary.

Several sequences affiliated with *Prochlorales* were found to exhibit higher diversity in the dry season than in the wet season. *Prochlorales* can grow efficiently at an optimal growth temperature of 15 to 25°C and a pH of 8–10 (Burger-Wiersma et al. 1989). As a shallow water system, the temperature of the Pearl River Estuary sediment generally ranged from 16.19 to 17.90°C in the dry season (Fig. 2). This temperature was the optimal condition for *Prochlorales* growth. In the wet season, the temperature of the surface sediments exceeds the optimum temperature and can reach 27.45 to 30.82°C, which is not suitable for *Prochlorales* growth.

Distribution characteristics of cyanobacterial community

MDS results suggested divergence of cyanobacterial community among sampling sites and between seasons (Fig. 5). The cyanobacterial community in the wet-season samples differed from that in the dry-season samples, in accordance with its distribution in the MDS profile. In the dry season, the cyanobacterial community showed different geographical distributions between the inner estuary (S1), the middle estuary (S2, S3, S4, S6, and S9) and the outer estuary (S5, S7, S8 and S10). In the wet season, however, no obvious difference was observed between the inner estuary, the middle estuary and the outer estuary. In the wet season, the Pearl River Estuary was influenced by the large volume of runoff. In the dry season, the effect of runoff was largely decreased, and salt water intruded far into the estuary. The cyanobacterial community in the inner and middle estuaries was mainly affected by fresh water, but in the outer estuary it was regulated by seawater.

Salinity and pH in the estuary were higher in the dry season (salinity, 0.58–32.89; pH, 7.60–8.51) and lower in the wet season (salinity, 0.17–32.60; pH 7.21–8.14)

during the study period (Fig. 2). Significant changes in cyanobacteria at each station in different seasons indicated that cyanobacteria were subject to interference from environmental change. The notable seasonal variations of *Synechococcus* diversity in this region were probably caused by the changes in hydrographic conditions. Another study reported that salinity and water turbidity also significantly influenced marine *Synechococcus* diversity (Jing et al. 2009).

CCA results suggested that cyanobacterial community sampling from the outer estuary positively correlated with pH in the dry season (Fig. 6), suggesting that water environment was an important factor controlling the cyanobacterial community of the Pearl River Estuary in the dry season. As freshwater discharge was greatly reduced, salt water could intrude far into the river gates in the dry season (Dong et al. 2004). Salinity and pH in the dry season were markedly higher than in the wet season, thereby altering the cyanobacterial communities in the Pearl River Estuary. Thus, the cyanobacterial community in the dry season varied differently from the inner estuary to the outer estuary. Significant correlations between pH and cyanobacterial communities also supported the argument that fluctuation of pH could substantially change microbial communities directly through inhibitory physiological effects and indirectly through environmental effects (Day et al. 1989, Stepanauskas et al. 2003, Yannarell and Triplett 2005).

CONCLUSIONS

Cyanobacteria consist of oxygenic photosynthetic prokaryotes exhibiting diverse physiology and wide ecological tolerance that contribute to their competitive success over a broad spectrum of environments (Cohen and Gurevitz 2006). The roles of cyanobacteria in estuarine waters and sediments remain unknown because of inadequate information on the numbers and types of these organisms in the system. Benthic cyanobacteria could serve as seed banks for the pelagic phase in the Pearl River Estuary. Cyanobacterial communities exhibited interesting dynamics in the sediment samples in our study. Future studies should evaluate the physiological properties of different cyanobacteria using enrichment or pure culture techniques to elucidate the reason for cyanobacterial community shifts with changes in environmental conditions. In addition, monitoring more samples over a long period may be valuable to obtain more information regarding the dynamics of the cyanobacterial population in the Pearl River Estuary.

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