Response of bacterioplankton community structures to hydrological conditions and anthropogenic pollution in contrasting subtropical environments

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Abstract

Bacterioplankton community structures under contrasting subtropical marine environments (Hong Kong waters) were analyzed using 16S rRNA gene denaturing gradient gel electrophoresis (DGGE) and subsequent sequencing of predominant bands for samples collected bimonthly from 2004 to 2006 at five stations. Generally, bacterial abundance was significantly higher in the summer than in the winter. The general seasonal variations of the bacterial community structure, as indicated by cluster analysis of the DGGE pattern, were best correlated with temperature at most stations, except for the station close to a sewage discharge outfall, which was best explained by pollution-indicating parameters (e.g. biochemical oxygen demand). Anthropogenic pollutions appear to have affected the presence and the intensity of DGGE bands at the stations receiving discharge of primarily treated sewage. The relative abundance of major bacterial species, calculated by the relative intensity of DGGE bands after PCR amplification, also indicated the effects of hydrological or seasonal variations and sewage discharges. For the first time, a systematic molecular fingerprinting analysis of the bacterioplankton community composition was carried out along the environmental and pollution gradient in a subtropical marine environment, and it suggests that hydrological conditions and anthropogenic pollutions altered the total bacterial community as well as the dominant bacterial groups.

Introduction

In aquatic ecosystems, ubiquitous bacterioplankton is one of the major components of food webs and play key roles in biogeochemical cycles and energy flow. In the past two decades, various molecular techniques, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and automated ribosomal intergenic spacer analysis, have been used to quantify microbial biodiversity. Previous evidence indicated that bacterioplankton often show clear spatial patterns in terms of their distribution, abundance, and phylogenetic diversity in marine ecosystems, which are affected by both hydrodynamics and other anthropogenic factors. For example, Crump et al. (2004) showed a strong influence of residence time on microbial biogeography along an estuarine salinity gradient. Riemann & Middelboe (2002) showed pronounced differences in a bacterial community along a transect crossing the Skagerrak–Kattegat front. General ecological theories, for example taxa–area relationships, were also verified in microbial communities (Horner-Devine et al., 2004; Bell et al., 2005). It appears that bacteria in aquatic ecosystems also have definable biogeography similar to that of plants and animals (Martiny et al., 2006; Fuhrman et al., 2008).

In comparison with the work on spatial variations, there have been fewer studies on temporal variations of marine bacterioplankton that cover the whole seasonal cycle in coastal waters. For example, Pinhassi & Hagström (2000) examined the seasonal distribution of marine bacterioplankton in the northern Baltic Sea using whole-genome hybridization. Schauer et al. (2003) observed that the taxonomic composition of the bacterioplankton in an oligotrophic coastal system of NW Mediterranean Sea
changed gradually throughout the year. Morris et al. (2005) revealed temporal trends of bacterioplankton lineages in the North Atlantic Ocean using T-RFLP and quantitative rRNA hybridization. Kan et al. (2006a, b) observed variable and stable bacterial communities in the Chesapeake Bay in winter and summer, respectively. Fuhrman et al. (2006) provided a statistically robust demonstration of temporal patterns of bacterioplankton in the coast of southern California and indicated that the biogeography of bacterioplankton might modulate the function and response of the ecosystem. Notably, to the best of our knowledge, no seasonal study was performed in subtropical coastal environments and/or with complex natural and anthropogenic influence, for example sewage pollution.

Hong Kong (22°N, 113–114°E; Fig. 1), located at the southern coast of China, has typical subtropical coastal environments with complex and seasonally varying hydrography. A large amount of freshwater (an annual flow of 308 × 10^9 m^3) discharged from the Pearl River to the western waters of Victoria Harbor, Hong Kong, creates a sharp environmental gradient across the harbor: salinity increases, but nutrient loading decreases from the west to the east (Yung et al., 1999). The eastern areas of Hong Kong are predominantly affected by high salinity and nutrient-poor water from the South China Sea. In addition, in the middle part of Victoria Harbor, the water quality is severely affected as a consequence of rapid population growth and economic development, which has introduced sewage discharge in the last several decades (Yung et al., 1999). Hence, there are strong spatial and seasonal changes in the profiles of nutrient, salinity, and other environmental factors in Hong Kong waters (Connell et al., 1998; Yin, 2003). These make Hong Kong waters a good system to study the microbial biogeography of subtropical coastal environments. In this study, we used DGGE to study the changes in the bacterioplankton community structure and the relative abundance of major bacterioplankton species in Hong Kong waters over a period of 2 years (2004–2006). We aimed to illustrate the possible relationship between these changes and various environmental parameters under contrasting environmental conditions.

**Materials and methods**

**Station characterization and sampling**

We selected five sampling sites in Hong Kong waters based on their environmental characteristics, namely, Tung Lung Chau (TLC), Victoria Harbor East (VHE), Victoria Harbor (VH), Victoria Harbor West (VHW), and Peng Chau (PC) (Fig. 1). According to the results of a long-term monitoring by the Hong Kong Government (http://www.epd.gov.hk/), TLC is a mesotrophic environment; PC is a nutrient-rich estuarine environment; and VHE, VH, and VHW are anthropogenically nutrient-polluted stations. Detailed sampling station information (location, depth, etc.) was shown previously (Zhang et al., 2007). At each sampling station, 6 L (1 L for each replicate) of seawater from the surface (1 m below surface) and the bottom (1 m above bottom) of the sea were collected bimonthly from June 2004 to April 2006. The samples were filtered firstly through a 1.0-μm-pore-size polycarbonate membrane (47 mm diameter, Millipore), and subsequently through a 0.22-μm-pore-size membrane (47 mm diameter, Millipore) to collect particle-attached and free-living bacterioplankton, respectively. The membranes were immersed into 0.8 mL of extraction buffer (0.1 M of Tris-HCl, 0.1 M of Na_2-EDTA, 0.1 M of sodium...
phosphate, 1.5 M of NaCl, and 1% of CTAB) and stored on dry ice until DNA extraction.

**Determination of environmental parameters and bacterial abundance**

Temperature, salinity, pH, and dissolved oxygen content (DO) in the water column were measured *in situ* using an YSI 6600 Sonde. The concentration of nutrients including NH$_4^+$, NO$_2^-$, NO$_3^-$, total phosphate (TP), and silica (Si) was determined with a Skalar San autoanalyzer for both the surface and the bottom water samples after filtration through 0.7-μm GF/F (Whatman) filters (Knap et al., 1996). The concentration of total nitrogen (TN) and dissolved nitrogen (DN) was measured with a Shimadzu TOC analyzer, according to the protocols described by Knap et al. (1996). Suspended solid content, turbidity, chlorophyll $a$ (chl $a$) concentration, and 5-day biochemical oxygen demand (BOD$_5$) were obtained from the Environmental Protection Department of Hong Kong (http://www.epd.gov.hk/).

Fifty milliliters of each seawater sample were fixed with 4% of formaldehyde (final concentration) and stored on dry ice for the quantification of bacterial abundance. Bacterial abundances were determined using flow cytometry (Coulter Epics XL, Beckman) and SYBR Green I (Invitrogen) staining according to the methods described by Gasol & del Giorgio (2000).

**DNA extraction and PCR**

Total DNA of particle-attached and free-living bacteria on the filters was extracted and purified using proteinase K and sodium dodecyl sulfate concomitant with chloroform extraction and isopropanol precipitation, following the protocols for all samples also made it possible to use band supposed to occur homogeneously. The standardized protocols for all samples collected in 2 years, in which the biases introduced during DNA extraction and PCR amplification were supposed to occur homogeneously. The standardized protocols for all samples also made it possible to use band intensity as the relative abundance of OTUs for calculation. Previous studies suggest that the major bands on the DGGE gel represented dominant bacterial species *in situ* environments and the band intensity was directly related to the relative abundance of corresponding bacterial species within the sample (Murray et al., 1996; Fromin et al., 2002). In the current study, we used identical experimental protocols for all samples collected in 2 years, in which the biases introduced during DNA extraction and PCR amplification were supposed to occur homogeneously. The standardized protocols for all samples also made it possible to use band intensity as the relative abundance of OTUs for calculation of the diversity index and comparison among samples.

Fifty milliliters of each seawater sample were fixed with 4% of formaldehyde (final concentration) and stored on dry ice for the quantification of bacterial abundance. Bacterial abundances were determined using flow cytometry (Coulter Epics XL, Beckman) and SYBR Green I (Invitrogen) staining according to the methods described by Gasol & del Giorgio (2000).

**DNA extraction and PCR**

Total DNA of particle-attached and free-living bacteria on the filters was extracted and purified using proteinase K and sodium dodecyl sulfate concomitant with chloroform extraction and isopropanol precipitation, following the protocol described in detail in Zhang et al. (2008). Bacterial 16S rRNA genes for DGGE were amplified by a touch-down PCR program using the primer set 341F (5'-CCG TCA ATT CMT TTG AGT TT-3') and 907R (5'-CCG TCA ATT CMT TTG AGT TT-3') with a GC-clamp attached to the forward primer (Muyzer et al., 1993, 2004). The PCR reaction mixtures (50 μL) contained 2 μL of template DNA, 1 x Taq buffer (TaKaRa), 0.2 μM of each primer, 100 μM of each dNTP, and 2.5 U of Taq DNA polymerase (TaKaRa). The amplification protocol included a denaturing step at 95°C for 5 min, 10 touch-down cycles at 95°C for 30 s, 65–55°C for 30 s (~1°C per cycle), and 72°C for 30 s, 15 normal cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension step of 72°C for 10 min.

**DGGE and sequencing analysis**

Similar to our previous study (Zhang et al., 2007), random checking indicated that DGGE patterns of samples collected from surface and bottom seawater, as well as samples from six replicates, were highly similar (data not shown). Therefore, DGGE analyses for large amount of samples, and subsequent statistical analyses based on DGGE patterns, were performed using PCR products amplified from combined environmental DNA from six replicates of surface and bottom samples at each station. DGGE was carried out with a Bio-Rad Protean II system. PCR products were loaded onto an 8% polyacrylamide gel with a denaturing gradient of 45–75% [100% denaturant = 7 M urea, 40% (v/v) formamide] and electrophoresis was performed at 125 V for 18 h at 60°C in 1 x TAE buffer. After electrophoresis, the gel was stained for 20 min using SYBR Gold (1:1000 dilution; Invitrogen) and photographed with an Alpha Imager 2000 (Alpha Innotech Corporation). The middle portion of each selected DGGE band was excised, washed with Milli-Q water, and incubated in 50 μL of Milli-Q water at room temperature for 4 h. Two microliters of DNA from each excised band were used as the template for the same PCR-DGGE analysis to check for the band position and purity. PCR products were then purified and cloned into the vector with a TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. The insertion of DNA fragments was confirmed by the same PCR-DGGE procedure. The 16S rRNA genes were sequenced from both ends using the primers M-13F and M-13R with MegaBACE 500 (Amersham). The nucleotide sequences obtained with the two primers were assembled using the SEQUENCHER 4.2 (Gene Codes Corporation).

Phylogenetic affiliation of sequenced DGGE bands was determined by ARB software (http://www.arb-home.de/; Ludwig et al., 2004). Sequences from the DGGE gel, as well as their close relatives determined with BLASTN program on the NCBI homepage (http://www.ncbi.nlm.nih.gov/), were input into ARB to update the database (SLIVA Release 93). Sequence alignment was manually modified and the neighbor-joining phylogenetic tree was constructed with bootstrapping of 1000 replicates.

**Data analysis**

Previous studies suggest that the major bands on the DGGE gel represented dominant bacterial species *in situ* environments and the band intensity was directly related to the relative abundance of corresponding bacterial species within the sample (Murray et al., 1996; Fromin et al., 2002). In the current study, we used identical experimental protocols for all samples collected in 2 years, in which the biases introduced during DNA extraction and PCR amplification were supposed to occur homogeneously. The standardized protocols for all samples also made it possible to use band intensity as the relative abundance of OTUs for calculation of the diversity index and comparison among samples.
The DGGE band position and intensity were determined using a GELCOMPAR II software package (Applied Maths) and were modified manually. Band matching was performed with 1.00% position tolerance and 1.00% optimization.

Cluster analysis for comparison of bacterial community structures was performed based on the Pearson similarity correlation and the Ward dendrograming method in GELCOMPAR II software package. The relationship between the measured environmental parameters and the bacterial community structure revealed by DGGE was studied using the BIOENV analysis provided in PRIMER 5 software. BIOENV analysis selects the environmental parameters that may best explain the community pattern, maximizing the correlation between their respective similarity matrices with application of a weighted Spearman’s correlation coefficient.

### Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study were deposited in the GenBank under the following accession numbers: EF655903–EF655910.

### Results

#### Environmental characterization

As expected, Hong Kong waters showed clear seasonal patterns of temperature, salinity, DO, and Chl a (Supporting Information, Fig. S1). Water temperature at all five stations was usually higher in summer (26.5 ± 1.0 °C in June, August, and October) than in winter (19.9 ± 1.7 °C in December, February, and April). Salinity decreased from the eastern (34.1 ± 0.9 psu at TLC) to the western side (31.7 ± 2.5 psu at PC) and the variations between summer and winter were clearer at PC than at TLC. The DO concentration increased from 5.5 ± 0.8 mg L⁻¹ in summer to 7.5 ± 1.1 mg L⁻¹ in winter. TLC always showed lower concentrations of nutrients and BOD₅ than the other four stations (Fig. S1). Chl a was consistently low at TLC and higher in some summer months at the other four stations. Most environmental parameters (except salinity, DO, suspended solids, and turbidity) of surface and bottom seawaters did not differ largely and stratification was observed only in summer (Fig. S1).

Bacterial abundances, determined by flow cytometry, varied from 0.18 × 10⁶ to 2.56 × 10⁶ in the surface seawater and from 0.16 × 10⁶ to 2.34 × 10⁶ in the bottom seawater. Bacterial abundance also showed clear seasonal trends with higher abundances from April to October and lower in December and February (Fig. S2; one-way ANOVA, P < 0.05). However, the spatial difference of bacterial abundance in Hong Kong waters was not clear during the sampling period.

#### Seasonal pattern of bacterioplankton community

The number of DGGE bands detected ranged from 7 to 27 in all samples investigated (Fig. 2). Among the 10 temporal patterns investigated [5 stations × 2 populations (particle-attached and free-living bacteria)], seven showed the highest number of DGGE bands in summer and the lowest in winter from 2004 to 2006. Both particle-attached and free-living bacterial populations from TLC usually showed a higher band number in summer (e.g. June, August, and October of 2004 and 2005), resulting in a seasonal variation (one-way ANOVA, P < 0.05). However, only samples collected in April always showed a low band number in both particle-attached and free-living bacteria. Nevertheless, no clear temporal trend was found for samples from the three stations at Victoria Harbor (VHE, VH, and VHW) and PC. At the same time, particle-attached and free-living samples did not always show the same pattern at the same station. For example, particle-attached bacteria at TLC in June 2005 showed a relatively low apparent diversity (number of DGGE bands), while free-living bacteria at the same sampling time showed a relatively higher diversity. The inconsistency was also observed for samples collected in February 2006 (Fig. 2).

Generally, bacterial community structures, revealed by cluster analysis of the DGGE pattern, showed clear seasonal patterns except for the western part of Victoria Harbor (Fig. 3). Particle-attached (data not shown) and free-living bacterial (Fig. 3) community structures at TLC, VHE, VH, and PC were grouped into two large clusters mainly according to their sampling seasons. However, weak temporal trends were observed for samples from VHW, where the samples from summer and winter clustered together (Fig. 3).

![Temporal variations of particle-attached apparent bacterial diversity (number of DGGE bands) at TLC and VH of Hong Kong waters.](https://academic.oup.com/femsec/article-abstract/69/3/449/544790)
DO, TP, and BOD5 were observed as factors mostly correlated with bacterial communities. However, at VHW, temperature was the only parameter that best correlated with bacterial community structure in nine out of the 10 correlations (particle-attached bacteria from VH and PC, 5 stations). Turbidity or suspended solid concentrations were listed as significant environmental factors in six correlations. Nitrogen nutrient (NH4+, NO2−, TN, and DN) was another contributing parameter affecting bacterial community structures in Hong Kong waters (Table 1).

The bacterial community structure was more stable in summer than in winter. Among the 10 temporal dynamic patterns of bacterial community structure investigated (5 stations × 2 populations), samples collected in October and August clustered together in six and five patterns, respectively. Only two patterns showed that the samples collected at the same winter time (e.g., December, February, or April) formed the same cluster (Fig. 3).

BIOENV analysis was used to correlate multivariate DGGE profiles with environmental variables (Table 1). At each station, higher correlation values were obtained for free-living bacteria than for particle-attached bacteria. Temperature showed the highest correlation with the bacterial community structure in nine out of the 10 correlations (5 stations × 2 bacterial populations). Furthermore, in two correlations (particle-attached bacteria from VH and PC, Table 1), temperature was the only parameter that best correlated with bacterial communities. However, at VHW, DO, TP, and BOD5 were observed as factors mostly correlated with bacterial community structures, with correlation values of 0.727 and 0.802 for particle-attached and free-living bacteria, respectively. Turbidity or suspended solid concentrations were listed as significant environmental factors in six correlations. Nitrogen nutrient (NH4+, NO2−, TN, and DN) was another contributing parameter affecting bacterial community structures in Hong Kong waters (Table 1).

### Seasonal pattern of dominant bacterial species

In total, eight major DGGE bands were sequenced, based on their intensity and temporal variation, and their relative abundance, compared with the total PCR-amplified bacterial 16S rRNA gene, was calculated (Fig. 4). They accounted for an average of 47% of the total band intensity of DGGE gels. Six of them were affiliated to Proteobacteria, with three belonging to the γ subgroup, two to the α subgroup, and one to an uncultured δ subgroup. The other two DGGE bands were affiliated to Cyano bacteria (Synechococcus sp.) and Bacteroidetes (Cytophaga sp.). The relative density of six out of eight bands (except M-1 and M-2) showed a significant seasonal pattern (Fig. 5; one-way ANOVA, P < 0.05).

The sequence of the DGGE band M-1 showed 93% and 91% similarities to the 16S rRNA gene sequence of an uncultured and a cultured Legionella sp., respectively. Phylogenetic analysis based on the ARB database also indicated that it was closely related to a group of Legionella spp. (Fig. 4). M-1 showed a lower occurrence in particle-attached bacteria from three stations of Victoria Harbor than of other populations and stations (one-way ANOVA, P < 0.001). The highest percentage of M-1 reached 20.3% of the PCR-amplified 16S rRNA genes in the sample of free-living population at VHW in October 2005 (Fig. 5). Band M-2 showed a high sequence identity to, and was clustered with, uncultured Roseobacter spp. (Fig. 4). M-2 appeared as one of the major groups with an average of 15.3% of the total amplicon in most of both the particle-attached and the free-living bacterial communities in all the five stations (Fig. 5). Sequences of M-3 and M-4, which shared 97.9% sequence identity but were clearly separated on DGGE gel, were

### Table 1. BIOENV analysis showed the correlations (Corr.) between bacterial community structure and environmental (Env.) factors

<table>
<thead>
<tr>
<th>Corr.</th>
<th>Env. factors</th>
<th>Corr.</th>
<th>Env. factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC 0.551</td>
<td>T, Sal, NH4+</td>
<td>0.705 T</td>
<td>NO2, Turb</td>
</tr>
<tr>
<td>0.491 T</td>
<td>NH4, Turb</td>
<td>0.693 T</td>
<td>NH4, Si, Turb</td>
</tr>
<tr>
<td>0.491 T</td>
<td>NH4, NO2, Turb</td>
<td>0.670 NH4, Turb</td>
<td></td>
</tr>
<tr>
<td>VHE 0.701</td>
<td>T, SS</td>
<td>0.755 T</td>
<td>NO2, DN, SS</td>
</tr>
<tr>
<td>0.643 T</td>
<td>Si, SS</td>
<td>0.754 T</td>
<td>NO2, Si, TN, SS</td>
</tr>
<tr>
<td>0.630 T</td>
<td>NO2, DN, SS</td>
<td>0.751 T</td>
<td>Sal, NO2, DN, SS</td>
</tr>
<tr>
<td>VH 0.500</td>
<td>T</td>
<td>0.706 T</td>
<td>Sal, NO2, SS</td>
</tr>
<tr>
<td>0.467 T</td>
<td>Sal</td>
<td>0.703 T</td>
<td>NO2, TP, SS, Chl a</td>
</tr>
<tr>
<td>0.449 T</td>
<td>Sal, NO2</td>
<td>0.687 T</td>
<td>Sal, pH, NO2, SS</td>
</tr>
<tr>
<td>VHW 0.727</td>
<td>T, DO, NH4+, TP, Chl a</td>
<td>0.802 DO, NH4+, Turb, BOD5</td>
<td></td>
</tr>
<tr>
<td>0.727 T</td>
<td>TP</td>
<td>0.787 T</td>
<td>DO, NH4+, NO2, TP, BOD5</td>
</tr>
<tr>
<td>0.727 T</td>
<td>DO, TP</td>
<td>0.779 T</td>
<td>NH4+, NO2, TP, BOD5</td>
</tr>
<tr>
<td>PC 0.512</td>
<td>T</td>
<td>0.528 T</td>
<td>NO2</td>
</tr>
<tr>
<td>0.506 T</td>
<td>Si</td>
<td>0.502 T</td>
<td>Sal, NO2</td>
</tr>
<tr>
<td>0.467 T</td>
<td>Sal</td>
<td>0.484 T</td>
<td>Sal, NO2, Si</td>
</tr>
</tbody>
</table>

T, temperature; Sal, salinity; Turb, turbidity; SS, suspended solid.
grouped with *Glaciecola* spp. from cold environments (Fig. 4). M-3 showed a higher abundance in winter seasons, while M-4, in contrast, showed higher percentages in summer seasons (one-way *ANOVA*, \( P < 0.05 \)). Furthermore, the highest relative abundance of M-3 and M-4 appeared at VHW station. M-5 showed a high sequence identity and close phylogenetic relationship with uncultured *Synechococcus* spp. and *Bacteroidetes* spp. from hypersaline ecosystems (Fig. 4). The detectable signals of M-5 came from the samples collected at PC and VH (only free-living bacteria) in winter (Fig. 5). M-6 showed a 100% sequence identity to an uncultured *Synechococcus* sp. (AB294981) and was clearly grouped within cultured *Synechococcus* spp. Significantly, it bloomed in summer, especially for the particle-attached fractions of VHW (Fig. 5). Despite the low sequence identity (91%), M-8 was related to *Azospirillum* spp. based on the phylogenetic analysis (Fig. 4) and had higher abundances in summer at VHW (Fig. 5).

**Discussion**

*Effects of hydrological conditions on bacterial communities in Hong Kong waters*

The combined effects of annual river discharge, rainfall, and monsoon winds determined the seasonal profiles of temperature, salinity, and other environmental parameters in Hong Kong waters during our sampling period (Fig. S1), which was also recorded in previous studies (Yin, 2002, 2003). Overall, about 80% of the annual discharge from Pearl River and rainfall occurred in summer seasons with the maxima in June, July, and August (Yin, 2003). Although easterly winds occur throughout the year, north-easterly to easterly winds blow in winter and southerly to southwesterly winds in summer. As a result, during the period of December to April, the China Coastal Current that originates from the north dominates the Hong Kong
Fig. 5. Temporal patterns of relative abundance of sequenced DGGE bands at five stations in Hong Kong waters. The relative abundance was indicated with the percentage of intensity of each DGGE band to the intensity of all DGGE bands of each sample. The possible phylogenetic affiliations for sequences from DGGE gel are indicated. Refer to Fig. 1 for site abbreviations.
coastal water circulation. In summer, the southwest monsoon drives upwelling along the coast, together with the Pearl River discharge and maximal rainfall (Yin, 2003). Furthermore, data of long-term observation from the Environmental Protection Department of HKSAR and other studies indicated three contrasting environments, mesotrophic coast (TLC), anthropogenic polluted coast (VHE, VH, VHW), and eutrophic estuary (PC), were developed from the east to the west along Victoria Harbor in Hong Kong waters. Our previous study showed clear spatial variations of particle-attached and free-living bacterial communities using DNA fingerprinting and clone library analyses (Zhang et al., 2007). The present study indicated that the bacterioplankton in Hong Kong waters also showed clear seasonal patterns.

Generally, the effects of hydrological conditions on bacterial populations were observed in Hong Kong waters. Firstly, bacterial abundances differed between summer and winter seasons, which are mainly due to the influence of annual variations in temperature (Fig. S1). Clear seasonal patterns of community structures of particle-attached and free-living bacterial populations were observed at TLC, VHE, VH, and PC using cluster analysis of DGGE gel (Fig. 3). This indicated that substantially different bacterial populations existed in different seasons. Our finding at subtropical Hong Kong waters was consistent with those of global marine environments with totally different hydrological conditions, for example the Blanes Bay (Temperate Mediterranean Sea; Schauer et al., 2003; Pinhassi et al., 2006; Alonso-Sáez et al., 2007), the Gulf of Trieste (Temperate Adriatic Sea; Celussi & Cataletto, 2007), the Chesapeake Bay (Subtropical–temperate Atlantic; Kan et al., 2006a, b; 2007; Crump et al., 2007), the San Pedro Harbor (Subtropical Pacific; Fuhrman et al., 2006), the Banyuls-sur-mer Bay (Temperate Mediterranean Sea; Ghiglione et al., 2005), the English Channel (Mary et al., 2006), the Bermuda Sea (Morris et al., 2005), the Baltic Sea (Pinhassi & Hagstroem, 2000; Riemann et al., 2008), and the North Sea (Sapp et al., 2007). Most of the studies related seasonal bacterial community dynamics with environmental parameters (e.g. temperature, salinity, etc.). Indeed, in our study, BIOENV analysis showed that temperature was one of the driving forces for the variations detected by DGGE (Table 1). However, some investigations based on various lake systems showed less or no seasonal pattern of planktonic bacterial composition (Lindstroem, 1998; Yannarell et al., 2003; Kent et al., 2004; Yannarell & Triplett, 2005). One possible reason of this contrasting phenomena may be the closed vs. open nature of the systems.

Furthermore, only samples at TLC showed a clear seasonal pattern of DGGE band number (apparent diversity) of bacterial populations (Fig. 2). Because of the fact that TLC is the cleanest station, we supposed that the clear seasonal pattern of apparent bacterial diversity at TLC came from its pollution conditions and calculation of these ecological parameters from DGGE. The calculation of apparent diversity simply depends on the total number of DGGE bands, which include the weak bands (minor bacterial groups) as well. Bacterial community structure analysis, which was based on the cluster analysis of the similarity matrix from the DGGE gel pattern, considered the band intensity on DGGE gel (e.g. abundant bacterial groups might show a high band intensity; Muyzer & Smalla, 1998; Fromin et al., 2002). This indicated that the communities of major bacterial groups at TLC, VH, VHE, and PC followed a general seasonal pattern, while other factors (e.g. pollution, see discussion below) ‘stimulated or repressed’ minor bacterial groups, changing the species richness but not disturbing the seasonal pattern of general bacterial community structures (Fig. 3). Meanwhile, we cannot exclude the fact that the bacterial diversity displayed on DGGE gel was not representative of all bacterial communities due to the limitation of DGGE gel resolution.

**Effects of anthropogenic pollution on bacterial communities in Hong Kong waters**

Since the 1970s, the Hong Kong waters, especially in the Victoria Harbor area, have been severely polluted by domestic sewage and industrial effluents. In 1997, the estimated loading of total BOD, total suspended solids, and total toxic metals into the Harbor area was about 340 and 280 tons day\(^{-1}\), and 3000 kg day\(^{-1}\), respectively (Yung et al., 1999). There are 12 outfalls from 11 sewage-screening plants and one Stonecutters Island Sewage Treatment Works, which discharge about 1.7 million m\(^3\)\(\text{day}^{-1}\) primarily treated wastewater into the Harbor (near VHW, Fig. 1). A previous study on spatial diversity of bacterioplankton in the Hong Kong waters strongly indicated the influences of anthropogenic pollutions (Zhang et al., 2007). For example, the sequences of fecal indicators of *Bacteroides* and *Arcobacter* were only observed in the clone libraries from VH, but not from TLC and PC. Temporal patterns of bacterial communities, revealed in the present study, also showed possible effects of pollution. Bacterial community structure at VHW, the closest station to one of the largest sewage treatment works, was the only one that did not show clear seasonal patterns in bacterial community structures among the five stations. Samples, especially of free-living bacteria, from summer and winter mixed together in the cluster analysis (Fig. 3). Meanwhile, BIOENV analysis showed that VHW was the only station in which the bacterial community could be highly correlated with DO and BOD\(_5\) (Table 1). This suggested that the consistent and routine discharge of preliminarily treated sewage near VHW substantially affected the bacterial community and disturbed the natural
patterns of bacterial community structure produced by seasonal changes of the ecosystem. Furthermore, in the Hong Kong waters, a relatively small-scale area, apparent diversity (the number of detectable bands on DGGE gel) showed clear spatial variations for all sampling times (Fig. 2). A simple explanation of the variation was the influence of a consistent and large amount of pollution discharge in the Victoria Harbor area. TLC was the least affected by the pollution discharge and might be a reference site in comparison with other stations that have been receiving pollution discharge routinely. The nutrients, along with sewage discharge, might stimulate or repress bacterial growth and, consequently, affected specific groups of bacteria, which appeared as the presence or absence of weak bands on the DGGE gel. Although only eight major bands were excised and sequenced in the present study, a detailed previous study (Zhang et al., 2007) in which 28 bands (including weak bands) were sequenced supported the explanation.

To the best of our knowledge, the present study, for the first time, documented the long-term effects (disturbing seasonal pattern of bacterial community structure) of pollution on marine bacterioplankton. Furthermore, our study indicated that BOD$_5$ (combined with other nutrient parameters) may be an appropriate indicator when considering the anthropogenic effects on microbial biogeography.

**Dominant bacterial groups in subtropical Hong Kong waters**

Previous studies showed that *Roseobacter* spp. and its close relatives were one of the major marine bacterial lineages in coastal areas and played very important roles in the global carbon and sulfur cycle and climate (Selje et al., 2004; Buchan et al., 2005). Our results indicated that *Roseobacter* spp. was also abundant in the subtropical coastal Hong Kong area, with a high abundance of M-2, constituting 15.3% (range 4.8–27.9%) of the total band intensity in the DGGE profiles (Fig. 5). Furthermore, the present study showed that *Roseobacter* sp. was rather consistently distributed within two summer–winter cycles, with a clear temperature variation, which was different from previous studies (Buchan et al., 2005; Kan et al., 2007). Therefore, our study suggested that *Roseobacter* might play more important roles than what we previously thought in the global carbon and sulfur cycle because they might be more widely distributed and less sensitive to temperature changes. However, we (this study and Zhang et al., 2007) did not recover the other important marine bacterial group SAR 11 in Hong Kong waters, although it was observed frequently at coastal areas (Pommier et al., 2005).

Clear spatial and temporal patterns were observed for another abundant coastal species, *Synechococcus* sp. (M-6) (Fig. 5). The high percentages of *Synechococcus* in particle-attached populations (> 1.0 μm) and at TLC were in good agreement with their cell size and aggregation in *in situ* environments and the fact that TLC is the most oceanic environment with the least effect on fresh water discharge (Fig. 1). Furthermore, seasonal patterns showed that they always appeared in summer in Hong Kong waters (Fig. 5). Previous studies on the spatial diversity of total bacteria and temporal dynamics of cyanobacteria using clone library analysis verified the conclusion from the DGGE pattern (Zhang et al., 2007). A recent multiyear investigation in the Chesapeake Bay revealed a similar temporal distribution pattern of *Synechococcus*-type of cyanobacteria (Kan et al., 2007).

On DGGE gel, two major bands (M-3 and M-4) were clearly separated and showed different intensities in each sample, although their sequences had 98% similarity and both were closely related to *Glaciecola* sp. The different temporal patterns of M-3 and M-4 excluded the possibility that they were from the same bacterial strain. The two *Glaciecola* spp. accounted for an average of 21% of all bacterial signals on the DGGE gel, and in some samples (e.g. free-living bacteria at VHW), the percentages were > 40%. Phylogenetic analysis indicated that our *Glaciecola* spp. were similar to those isolated from cold environments. Previously, strains or environmental clones belonging to *Glaciecola* sp. were usually isolated from polar or subpolar seas (Bowman et al., 1998; Brown & Bowman, 2001; Van Trappen et al., 2004). The only exception was that Alonso-Sáez et al. (2007) found the blooming of *Glaciecola* from northwest Mediterranean coastal waters sampled in July, 2003. This suggested that some *Glaciecola* spp. might survive, adapt, and bloom in much warmer waters than previously thought. Our study also indicated that bacterial microdiversity might be a possible reason for the adaptation of *Glaciecola* spp. for < 2% sequence difference of their 16S rRNA genes. Similar to a previous study of *Prochlorococcus*, the diversification of different ‘ecotypes’ in the same ‘species’ of *Glaciecola* might help them confound viral attack and protistan grazing (Rocap et al., 2003).

The possible pollution-related bacteria were detected and showed clear spatial and temporal dynamics. *Cytophaga* sp. (M-5) was supposed to be an important user of organic matters in the ocean (in the Hong Kong waters, mainly originated from sewage and river discharge), and was critical in carbon budgets and cycles (Kirchman, 2002). Although several studies found that certain *Cytophaga* sp. showed seasonal patterns with maximum abundance in winter, very few studies investigated their seasonal distribution in marine ecosystems (Riemann & Middelboe, 2002). Our results indicated that *Cytophaga* sp. was abundant only at PC and VH in the winter season (Fig. 5), which was consistent with the observations in fresh water systems (Riemann & Middelboe, 2002). The other major bacterial group in Hong Kong waters was M-1. In spite of the relatively low similarity
among M-1 and known *Legionella* sequences in public database, our study was similar to previous studies (Atlas, 1999) of *Legionella* spp. that M-1 was more abundant at VHW and VH, which are close to the sewage outfall. The extremely high percentages (about 30%) of *Legionella*-like bacteria in certain areas (e.g. VHW) at certain times (e.g. October 2005) should be further investigated and evaluated carefully.

**Conclusion**

Being one of the few long-term spatio-temporal studies on marine bacterioplankton, the present study showed variations of particle-attached and free-living bacterial communities at different sites with contrasting environments in a subtropical coastal area. Possible combined effects of hydrological conditions and anthropogenic pollutions on bacterial communities were observed: hydrological effects determined the general bacterial community structure while anthropogenic pollutions affected nearby bacterioplankton in Hong Kong waters. Dominant bacterial species, determined by sequencing major DGGE bands and clone library (Zhang et al., 2007), in Hong Kong waters were *Proteobacteria*, *Cyanobacteria*, and *Bacteroidetes*. Temporal variation of eight dominant bacterial species indicated a controlling mechanism of natural and/or anthropogenic influence in coastal areas.

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**References**


Bacterioplankton community of contrasting coastal waters


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Dynamics of abiotic and biotic parameters in seawaters from different locations of Hong Kong waters from 2004 to 2006.

**Fig. S2.** Temporal variations of bacterial abundance, determined by flow cytometry, at five stations in Hong Kong waters.

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