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 summer than in winter. The general seasonal variations of bacterial community structure as indicated by cluster analysis of 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 presence and intensity of DGGE bands at the stations receiving discharge of primarily treated sewage. Relative abundance of major bacterial species, calculated by 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 bacterioplankton community composition was carried out along the environmental and pollution gradient in subtropical marine environment and suggest that hydrological conditions and anthropogenic pollutions altered total bacterial community as well as 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, 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 and Middelboe (2002) showed pronounced differences in bacterial community along a transect crossing the Skagerrak-Kattegat front. General ecological theories, e.g. taxa-area relationships, were also verified in microbial communities (Bell*, et al.*, 2005; Horner-Devine*, et al.*, 2004). 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 to 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 and 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 North Atlantic Ocean using T-RFLP and quantitative rRNA hybridization. Kan *et al*. (2006) observed variable and stable bacterial communities in the Chesapeake Bay in winter and summer, respectively. Fuhrman *et al*. (2006) provided statistically robust demonstration of temporal patterns of bacterioplankton in the coast of southern California and indicated the biogeography of bacterioplankton might modulate function and response of ecosystem. Notably, to our best knowledge, no seasonal study was performed in subtropical coastal environments and/or with complex natural and anthropogenic influence, e.g. 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 (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, the middle part of Victoria Harbor, the water quality is severely affected as a consequence of rapid population growth and economic development, which 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 factor in Hong Kong waters (Connell, *et al.*, 1998; Yin, 2003). These make Hong Kong waters to be a good system to study microbial biogeography of subtropical coastal environments. In this study, we used DGGE to study the changes in bacterioplankton community structure and relative abundance of major bacterioplankton species in Hong Kong waters over a period of two 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 5 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 meso-trophic 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 liters (1 L each for each replicate) of seawater from the surface (1 m below surface) and the bottom (1 m above bottom) of the sea were collected bi-monthly from June 2004 to April 2006. The samples were filtered through firstly a 1.0-μm-pore-size

polycarbonate membrane (47 mm diameter, Millipore) and subsequently 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, 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), 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, 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 biochemical oxygen demand (BOD_5) were obtained from the Environmental Protection Department of Hong Kong (http://www.epd.gov.hk/).

Fifty ml 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 and 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 details 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'-CCT ACG GGA GGC AGC AG-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; Muyzer*, et al.*, 2004). The PCR reaction mixtures (50 μl) contained 2 μl of template DNA, 1× r*Taq* buffer (TaKaRa), 0.2 μM of each primer, 100 μM of each deoxyribonucleoside triphosphate, 2.5 U of r*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 sec, 65-55°C for 30 sec (-1°C per cycle) and 72°C for 30 sec, 15 normal cycles at 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, 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 6 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 6 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 a 8% polyacrylamide gel with denaturing gradient of 45-75% (100% denaturant = 7 M urea, 40% (vol/vol) formamide) and electrophoresis was performed at 125 V for 18 h at 60°C in 1×TAE buffer. After electrophoresis, the gel was stained for 20 min using SYBR Gold (1:1,000 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 μl 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 DGGE gel, as well as their close relatives determined with BLASTN program on 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 1,000

replicates.

Data analysis

Previous studies suggest that major bands on DGGE gel represented dominant bacterial species in *in situ* environments and band intensity was directly related with 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 two 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 relatively abundance of OTUs for calculation of diversity index and comparison among samples (Fromin, *et al.*, 2002). DGGE band position and intensity were determined using a GelCompar II software package (Applied Maths) and were manually modified. 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 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* (Supplementary materials Fig. S1). Water temperature at all five stations was usually higher in summer (26.5 ± 1.0 °C in Jun, Aug, and Oct) than in winter (19.9 ± 1.7 °C in Dec, Feb, and Apr). 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. DO concentration increased from 5.5 ± 0.8 mg/L in summer to 7.5 ± 1.1 mg/L in winter. TLC always showed lower concentrations of nutrients and BOD_5 than other four stations did (Supplementary materials Fig. S1). Chl *a* was consistently low at TLC and higher in some summer months at 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 only observed in summer (Supplementary material Fig. S1).

Bacterial abundances, determined by flow cytometry, varied from 0.18×10^6 to

2.56×10^6 in the surface seawater and from 0.16×10^6 to 2.34×10^6 in the bottom seawater. Bacterial abundance also showed clear seasonal trends with higher abundances from Apr to Oct and lower in Dec and Feb (Supplementary material Fig. S2; One-way ANOVA, $p<0.05$). However, the spatial difference of bacterial abundance in Hong Kong waters was not clear in the sampling period.

Seasonal pattern of bacterioplankton community

The detected number of DGGE bands ranged from 7 to 27 in all samples investigated (Fig. 2). Among 10 temporal patterns investigated [5 stations × 2 populations (particle-attached and free-living bacteria)], 7 of them 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 higher band number in summer (e.g. Jun, Aug, and Oct of 2004 and 2005), resulting a seasonal variation (One-way ANOVA, $p<0.05$). However, only samples collected in April always showed 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 showed 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 observed for samples collected in February 2006 as well (Fig. 2).

Generally, bacterial community structures, revealed by cluster analysis of 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). Bacterial community structure was more stable in summer than in winter. Among 10 temporal dynamic patterns of bacterial community structure investigated (5 stations × 2 populations), samples collected in October and August clustered together in 6 and 5 patterns, respectively. Only 2 patterns showed that the samples collected at the same winter time (e.g. Dec, Feb or Apr) formed 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 bacterial community structure in 9 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 BOD_5 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 6 correlations. Nitrogen nutrient (NH_4^+, NO_2^-, 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 relatively abundance, compared to 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 belonged to the gamma subgroup, two to the alpha subgroup and one to an uncultured delta subgroup. Other two DGGE bands were affiliated in *Cyanobacteria* (*Synechococcus* sp.) and *Bacteroidetes* (*Cytophaga* sp.). Relative density of six out of eight bands (except M-1 and M-2) showed significant seasonal pattern (Fig. 5; One-way ANOVA, $p<0.05$).

Sequence of the DGGE band M-1 showed 93% and 91% similarities to the 16S rDNA sequence of an uncultured and a cultured *Legionella* sp., respectively. Phylogenetic analysis based on the ARB database also indicated that it was closely related with a group of *Legionella* spp. (Fig. 4). M-1 showed a lower occurrence in particle-attached bacteria of 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 PCR-amplified 16S rRNA gene 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 percentage of 15.3% of total amplicon in most of both particle-attached and free-living bacterial communities in all the five stations (Fig. 5). Sequences of M-3 and M-4, which shared 97.9% in sequence identity but were clearly separated on DGGE gel, were 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 *Cytophaga* spp. and *Bacteroidetes* spp. from hypersaline ecosystem (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 a group of cultured *Synechococcus* spp.. Significantly, it bloomed in summer, especially for the particle-attached bacterial populations, with the highest percentage (30%) recorded at TLC in August 2004 (Fig. 5; One-way ANOVA, $p<0.05$). For free-living bacteria, no signal of M-6 was detected for samples from three stations at Victoria Harbor (Fig. 5). M-7 did not have any cultivated relatives with BLASTN search in the Genbank and showed 99% sequence identity with a clone from HOT station, USA. It was abundant in summer season (One-way ANOVA, $p<0.001$) and showed the highest percentages in 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 (Supplementary material Fig. S1), which was also recorded in previous studies (Yin, 2002; Yin, 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, northeasterly 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 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, meso-trophic 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 the bacterioplankton in Hong Kong waters showed clear seasonal patterns as well.

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 variation of temperature (Supplementary material 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 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, e.g. the Blanes Bay (Temperate Mediterranean Sea; Alonso-Sáez*, et al.*, 2007; Schauer*, et al.*, 2003, Pinhassi*, et al.*, 2006), the Gulf of Trieste (Temperate Adriatic Sea; Celussi & Cataletto, 2007); the Chesapeake Bay (Subtropical-temperate Atlantic; Kan*, et al.*, 2006; Crump*, et al.*, 2007; Kan*, 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), 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 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 versus 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). Due to the fact that TLC is the cleanest station, we supposed that the clear seasonal pattern of bacterial apparent 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 similarity matrix from DGGE gel pattern, considered the band intensity on DGGE gel (e.g. abundant bacterial groups might show 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. pollutions, 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 facts that bacterial diversity displayed on DGGE gel was not representative of all bacterial community due to limitation of DGGE gel resolution.

Effects of anthropogenic pollutions on bacterial communities in Hong Kong waters

Since 1970s, the Hong Kong waters, especially in 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 tons, 280 tons, and 3000 kg per day, 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} 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, showed possible effects of pollution as well. 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 VHW was the only station in which 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 Victoria Harbor area. TLC was the least affected by the pollution discharge and might be a reference site in comparison to 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 presence or absence of weak bands on 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 our best knowledge, the present study, for the first time, documented the long-term effects (disturbing seasonal pattern of bacterial community structure) of pollutions to marine bacterioplankton. Furthermore, our study indicated that BOD_5 (combined with other nutrient parameters) maybe 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

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 *Roseobacter* sp. was rather consistently distributed within two summer-winter cycles with a clear temperature variation, which was differed 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 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 area (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 population (>1.0 μm) and at TLC were in good agreement with their cell size and aggregation in *in situ* environments and the facts that TLC is the most oceanic environments with the least effects of fresh water discharge (Fig. 1). Furthermore, seasonal patterns showed they always appeared in summer in Hong Kong waters (Fig. 5). Previous studies on spatial diversity of total bacteria and temporal dynamics of cyanobacteria using clone library analysis verified the conclusion from DGGE pattern (Zhang, *et al.*, 2007). A recent multiyear investigation

in the Chesapeake Bay revealed 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 intensity in each samples, although their sequences had 98% of 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. averagely accounted for 21% of all bacterial signals on DGGE gel and in some samples (e.g. free-living bacteria at VHW), the percentages were higher than 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 sub-polar 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 north-west 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 adaption of *Glaciecola* spp. for less than 2 % sequence difference of their 16S rRNA genes. Similar to previous study of *Prochlorococcus*, the diversification of different “ecotypes” in the same “species” of *Glaciecola* might help them confounding 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 utilizer 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* spp. 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 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. Although 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. Oct, 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 community at different sites with contrasting environments in a subtropical coastal area. Possible combined effects of hydrological conditions and anthropogenic pollutions on bacterial community 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 area.

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Table 1. BIOENV analysis showed the correlations (Corr.) between bacterial community structure and environmental (Env.) factors. T: temperature, Sal: salinity, Turb: turbidity, SS: suspended solid, Chl *a*: chlorophyll *a*, DO: dissolved oxygen, BOD_5: 5-day biochemical oxygen demand, DN: dissolved nitrogen, TN: total nitrogen, TP: total phosphate.

	Particle-attached bacteria		Free-living bacteria	
	Corr.	Env. factors	Corr.	Env. factors
TLC	0.551	T, Sal, NH_4^+, NO_2^-, Turb	0.705	T, NH_4^+, Turb
	0.493	T, NH_4^+, Turb	0.693	T, NH_4^+, Si, Turb
	0.491	T, NH_4^+, NO_2^-, Turb	0.670	NH_4^+, Turb
VHE	0.701	T, SS	0.755	T, NO_2^-, DN, SS
	0.643	T, Si, SS	0.754	T, NO_2^-, Si, TN, SS
	0.630	T, NO_2^-, DN, SS	0.751	T, Sal, NO_2^-, DN, SS
VH	0.500	T	0.706	T, Sal, NO_2^-, SS
	0.467	T, Sal	0.703	T, NO_2^-, TP, SS, Chl *a*
	0.449	T, Sal, NO_2^-	0.687	T, Sal, pH, NO_2^-, SS
VHW	0.727	T, DO, NH_4^+, TP, Chl *a*	0.802	DO, NH_4^+, TP, Turb, BOD_5
	0.727	T, TP	0.787	DO, NH_4^+, NO_2^-, TP, BOD_5
	0.727	T, DO, TP	0.779	T, NH_4^+, NO_2^-, TP, BOD_5
PC	0.512	T	0.528	T, NO_2^-
	0.506	T, Si	0.502	T, Sal, NO_2^-
	0.467	T, Sal	0.484	T, Sal, NO_2^-, Si

Fig. 1. Map showing five sampling stations in Hong Kong waters. The large sewage treatment works is indicated with ★ (near VHW). TLC: Tung Lung Chau; VHE: Victoria Harbor East; VH: Victoria Harbor; VHW: Victoria Harbor West; PC: Peng Chau.

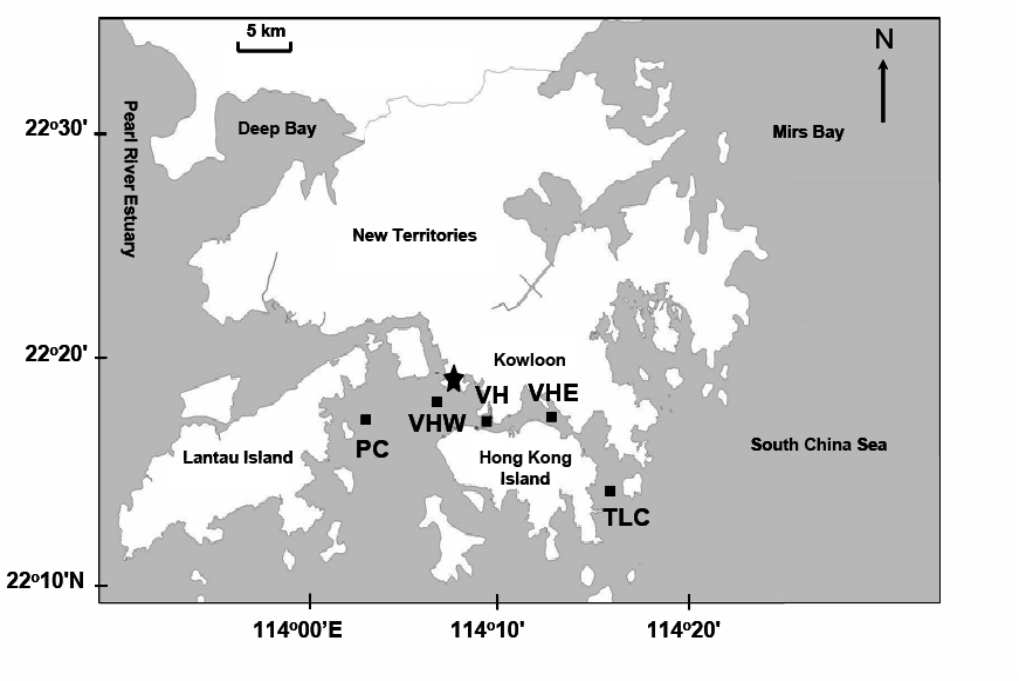
Fig. 2. Temporal variations of particle-attached bacterial apparent diversity (number of DGGE bands) at Tung Lung Chau (TLC) and Victoria Harbor (VH) of Hong Kong waters.

Fig. 3. DGGE patterns and cluster analyses of free-living bacterial community structure at Tung Lung Chau (TLC) and Victoria Harbor West (VHW) stations in Hong Kong waters from 2004 to 2006. Samples collected in summer (Jun, Aug, and Oct) and winter (Dec, Feb, and Apr) were indicated.

Fig. 4. Neighbour-joining phylogenetic tree for bacterial 16S rRNA gene sequences retrieved from DGGE. Bootstrap values (other than 100% were indicated at nodes) were based on an analysis of 1,000 re-sampling using ARB software. The scale bar represents 10% nucleotide sequence difference. *Bacillus naganoensis* (Firmicutes) was used as an out-group.

Fig. 5. Temporal patterns of relative abundance of sequenced DGGE bands at five stations in Hong Kong waters. 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 were indicated. Refer to Fig. 1 for site abbreviations.

1 Fig. 1.


5 km
N
22°30'
22°20'
22°10'N
Pearl River Estuary
Deep Bay
Mirs Bay
New Territories
Kowloon
VH
VHE
VHW
PC
Lantau Island
Hong Kong Island
South China Sea
TLC
114°00'E
114°10'
114°20'

2

3

4

Fig. 2.

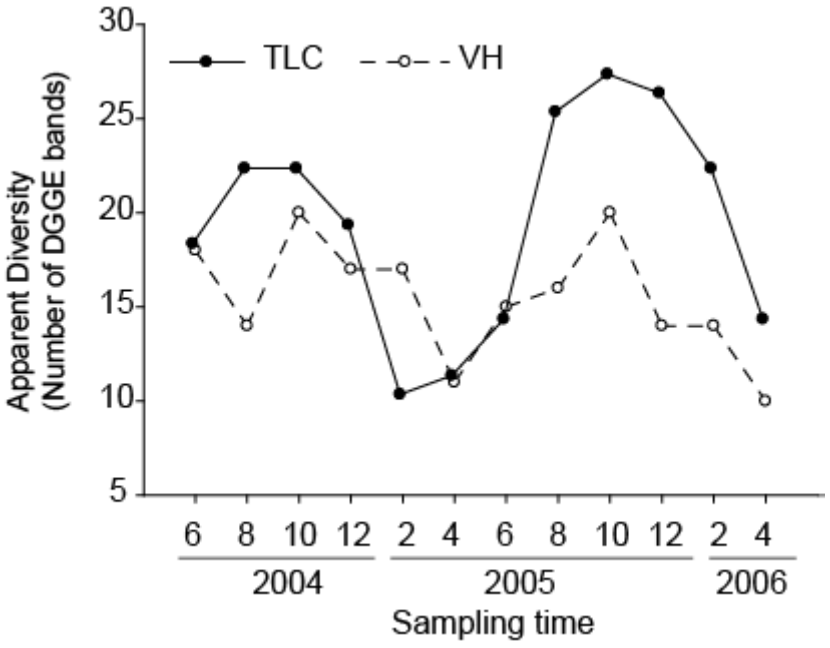
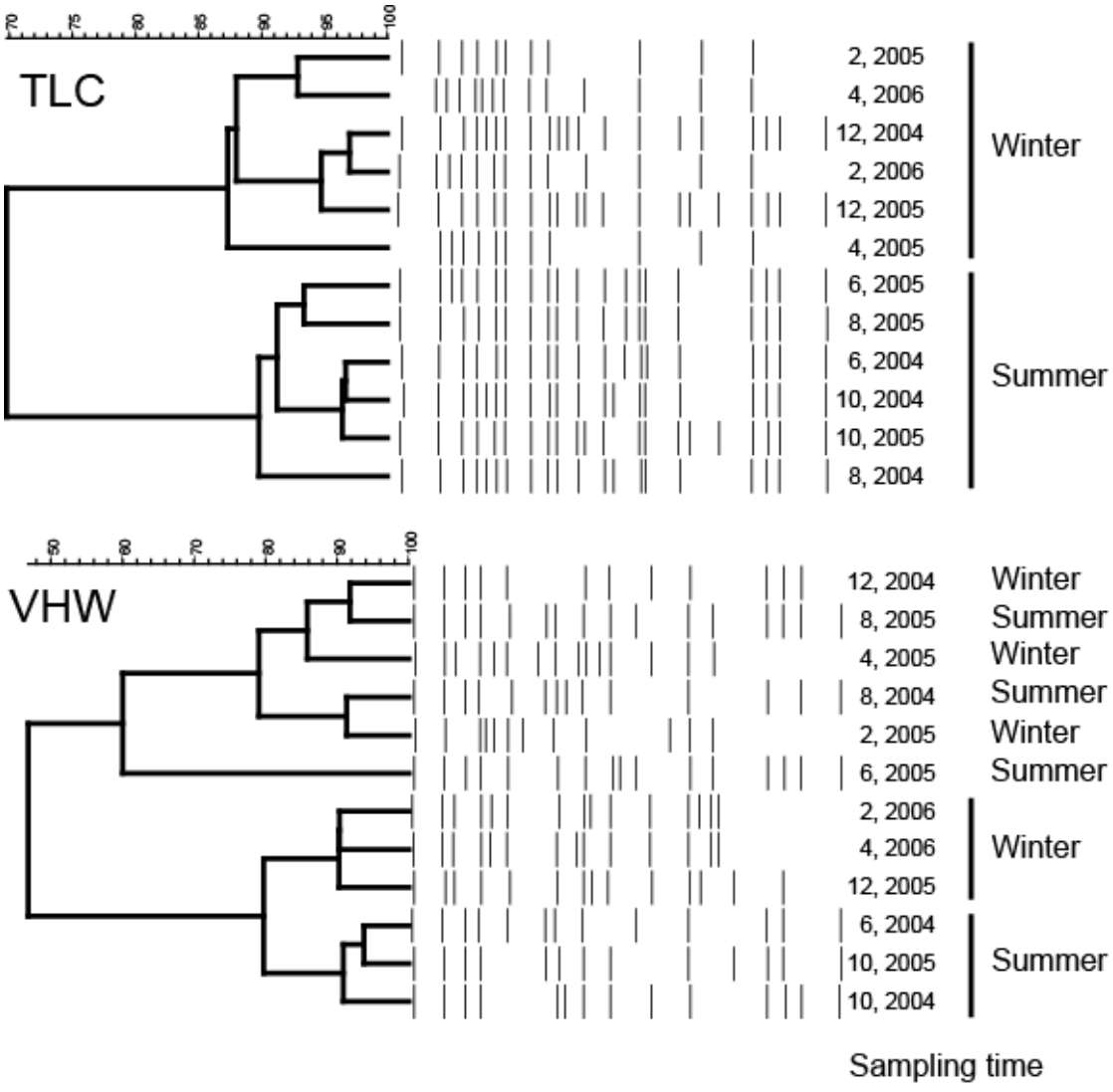
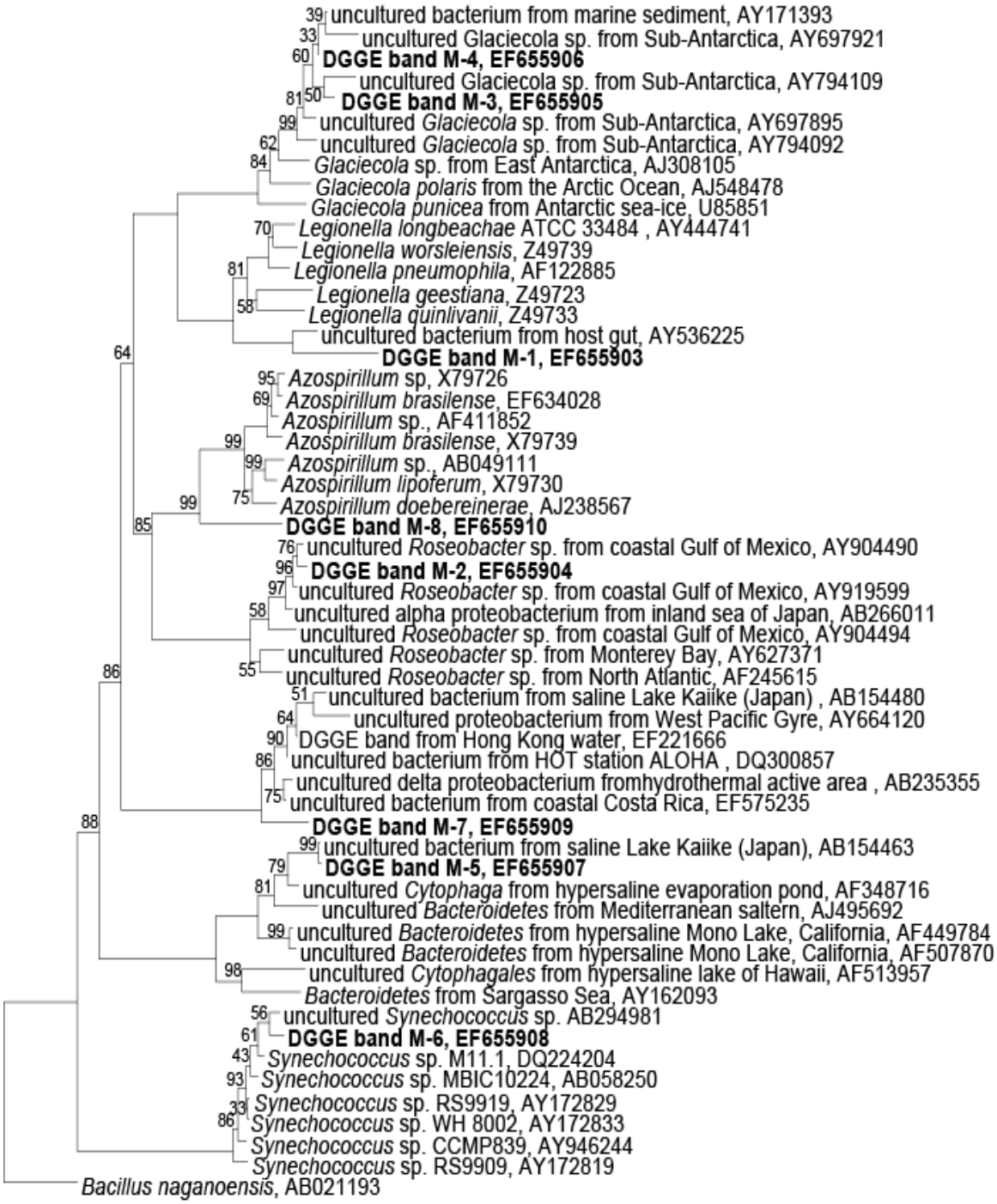

TLC
VH
Apparent Diversity
(Number of DGGE bands)
30
25
20
15
10
5
6 8 10 12 2 4 6 8 10 12 2 4
2004
2005
2006
Sampling time

Fig. 3.


70
75
80
85
90
95
100
TLC
2, 2005
4, 2006
12, 2004
2, 2006
12, 2005
4, 2005
Winter
6, 2005
8, 2005
6, 2004
10, 2004
10, 2005
8, 2004
Summer
50
60
70
80
90
100
VHW
12, 2004
Winter
8, 2005
Summer
4, 2005
Winter
8, 2004
Summer
2, 2005
Winter
6, 2005
Summer
2, 2006
4, 2006
12, 2005
Winter
6, 2004
10, 2005
10, 2004
Summer
Sampling time

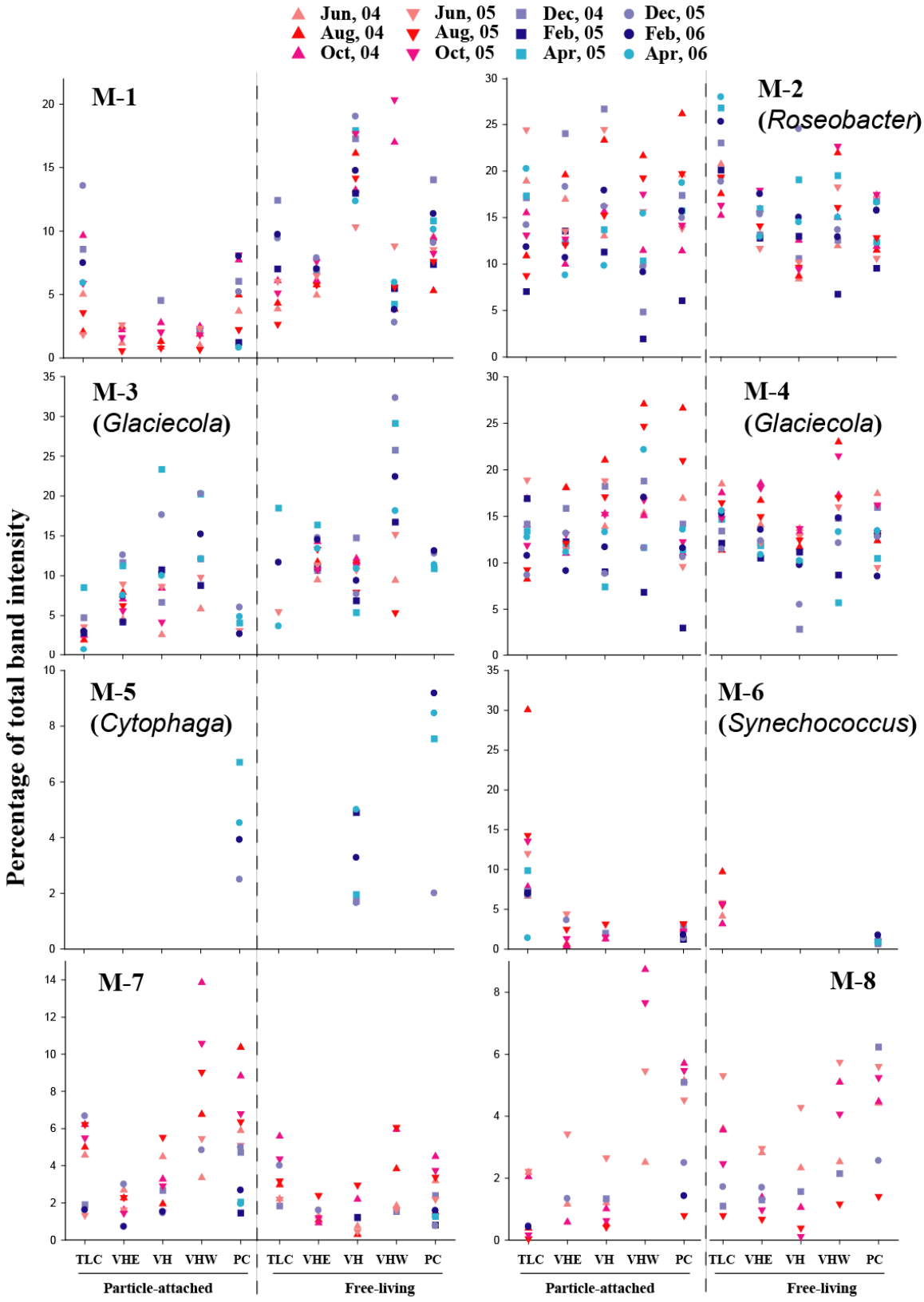
1 Fig. 4.


39 uncultured bacterium from marine sediment, AY171393
33 uncultured Glaciecola sp. from Sub-Antarctica, AY697921
60 DGGE band M-4, EF655906
uncultured Glaciecola sp. from Sub-Antarctica, AY794109
81 50 DGGE band M-3, EF655905
99 uncultured Glaciecola sp. from Sub-Antarctica, AY697895
62 uncultured Glaciecola sp. from Sub-Antarctica, AY794092
84 Glaciecola sp. from East Antarctica, AJ308105
Glaciecola polaris from the Arctic Ocean, AJ548478
Glaciecola punicea from Antarctic sea-ice, U85851
70 Legionella longbeachae ATCC 33484 , AY444741
Legionella worsleiensis, Z49739
81 Legionella pneumophila, AF122885
Legionella geestiana, Z49723
58 Legionella quinlivanii, Z49733
uncultured bacterium from host gut, AY536225
64 DGGE band M-1, EF655903
95 Azospirillum sp, X79726
69 Azospirillum brasilense, EF634028
Azospirillum sp., AF411852
99 Azospirillum brasilense, X79739
99 Azospirillum sp., AB049111
Azospirillum lipoferum, X79730
99 75 Azospirillum doebereinerae, AJ238567
85 DGGE band M-8, EF655910
76 uncultured Roseobacter sp. from coastal Gulf of Mexico, AY904490
96 DGGE band M-2, EF655904
97 uncultured Roseobacter sp. from coastal Gulf of Mexico, AY919599
58 uncultured alpha proteobacterium from inland sea of Japan, AB266011
uncultured Roseobacter sp. from coastal Gulf of Mexico, AY904494
uncultured Roseobacter sp. from Monterey Bay, AY627371
86 55 uncultured Roseobacter sp. from North Atlantic, AF245615
51 uncultured bacterium from saline Lake Kaiike (Japan) , AB154480
64 uncultured proteobacterium from West Pacific Gyre, AY664120
90 DGGE band from Hong Kong water, EF221666
86 uncultured bacterium from HOT station ALOHA , DQ300857
uncultured delta proteobacterium fromhydrothermal active area , AB235355
75 uncultured bacterium from coastal Costa Rica, EF575235
88 DGGE band M-7, EF655909
99 uncultured bacterium from saline Lake Kaiike (Japan), AB154463
79 DGGE band M-5, EF655907
81 uncultured Cytophaga from hypersaline evaporation pond, AF348716
uncultured Bacteroidetes from Mediterranean saltern, AJ495692
99 uncultured Bacteroidetes from hypersaline Mono Lake, California, AF449784
uncultured Bacteroidetes from hypersaline Mono Lake, California, AF507870
98 uncultured Cytophagales from hypersaline lake of Hawaii, AF513957
Bacteroidetes from Sargasso Sea, AY162093
56 uncultured Synechococcus sp. AB294981
61 DGGE band M-6, EF655908
43 Synechococcus sp. M11.1, DQ224204
93 Synechococcus sp. MBIC10224, AB058250
33 Synechococcus sp. RS9919, AY172829
86 Synechococcus sp. WH 8002, AY172833
Synechococcus sp. CCMP839, AY946244
Synechococcus sp. RS9909, AY172819
Bacillus naganoensis, AB021193
0.10

2

3

Fig. 5


Jun, 04
Aug, 04
Oct, 04
Jun, 05
Aug, 05
Oct, 05
Dec, 04
Feb, 05
Apr, 05
Dec, 05
Feb, 06
Apr, 06
M-1
M-2 (Roseobacter)
M-3 (Glaciecola)
M-4 (Glaciecola)
M-5 (Cytophaga)
M-6 (Synechococcus)
M-7
M-8
Percentage of total band intensity
TLC VHE VH VHW PC
Particle-attached
Free-living