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The Response of Bacterial Community Structure to Ecological Environment Changes in Daya Bay between Winter and Spring

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ABSTRACT

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The distribution patterns of the bacterial biomass and its diversity were investigated in Daya Bay in winter and spring 2008. Based on the acridine orange direct count, the mean bacterial biomass (0.31±0.20 μg L^{-1}) in spring was 1.5 times more than that (0.21±0.07 μg L^{-1}) in winter. Spatially it showed the trend that the mean bacterial biomass at station surface and bottom layers in the south of the bay was higher than in the north of the bay. The results based on PCR-DGGE and DNA sequence analysis showed that the *Proteobacteria* group dominated in bacterial communities of Daya Bay and other groups included the *Cyanobacteria*, *Actinobacteria* and *Verrucomicrobia*. The mean bacterial apparent species richness (No. of DGGE bands) was distinctly higher in winter (18) than in spring (12). It was concluded that phytoplankton controlled bacterioplankton biomass by utilizing dissolved inorganic nitrogen (DIN) in winter and spring. The observed seasonal variations of bacterioplankton community composition in Daya Bay was mainly associated with the high-in-winter- and -low- in- spring availability of nutrients like DIN and SiO_3^{2-}-Si.

INTRODUCTION

Bay is a typical ecosystem which has the highest productivity in the world and has been strongly influenced by coastal human activities. Located in the northern South China Sea, Daya Bay is a subtropical drowned valley bay in Guangdong Province in China (Song *et al.*, 2004). The area of this bay is about 600 km^2. The east-west width and north-south length of the bay are about 15 and 30 km, respectively. The water depth in the most area of the bay is lower than 10 m (Xu, 1989). The ecological environment in this marine area has been deteriorated to some extent (Wang *et al.*, 2008). Generally, in inner bay and bay head of Daya Bay the water quality is mainly associated with anthropogenic activities, but in the bay mouth and mid bay it is affected by seawater exchange (Wu *et al.*, 2011).

The microbial food chains were discovered more than 30 years ago (Azam *et al.*, 1983). Since then the functions of marine bacteria were investigated extensively for a long time all over the world. The amount distribution and species composition of marine microorganisms have been investigated using culture techniques (Shen, Cai, and Zhou, 1989). However, the traditional culturing techniques are time-consuming and laborintensive and cannot correctly reflect the phase of in situ microbial community because of nonculturability of many bacterial species (Fry, 2000). It is necessary to use culture-independent methods to investigate environmental microorganism communities. Denaturing gradient gel electrophoresis (DGGE) technique is widely used in investigating microbial communities. It is rapid, sensitive, visual and reliable in supplying information about environmental communities.

In this study, four typical ecological niches including the subtropical coastal environment were explored: bay mouth, inner bay (cage aquaculture area), mid bay and bay head. The authors expected that different hydrodynamic conditions and human activities such as cage aquaculture might induce microbial differences among the four stations which represent a basic ecological situation of Daya Bay. The acridine orange direct count (AODC), PCR-DGGE and sequencing were used to analyze the abundance and diversity of bacterioplankton in winter and spring. The authors revealed their physiochemical characteristics and discussed the relationship between bacterioplankton community and environmental variables in Daya Bay. The results of the present study displayed that the seasonal differences in nutrients availability led to variations in bacterioplankton community structure (including bacterial abundance and diversity) in Daya Bay between winter and spring.

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METHODS

In this study, two seasonal investigations were carried out in winter (January 21-22) and spring (April 28-29) 2008, respectively. Four sampling stations from south to north Daya Bay were employed by Key Lab of Field Marine Biology Station of Chinese Ecosystem Research Network for long-term ecological environment monitoring: bay mouth (WK), inner bay (YZ), mid bay (WZ) and bay head (WD) (Figure 1).

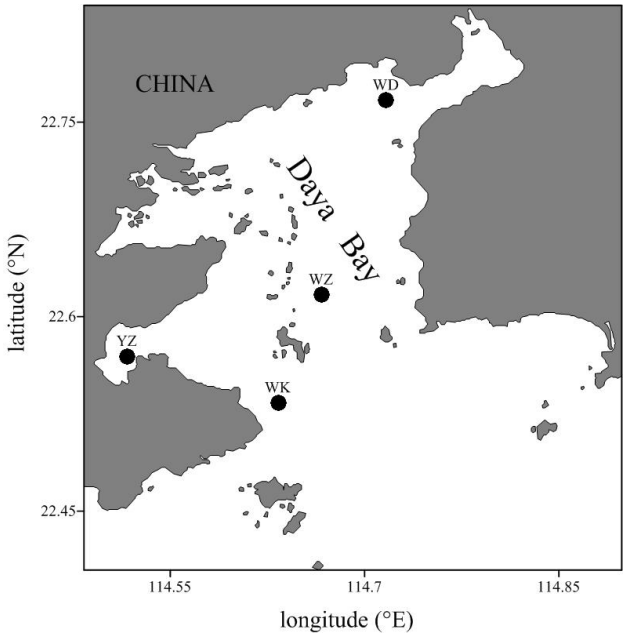

Figure 1. Location of the sampling stations in Daya Bay. WK: bay mouth; YZ: inner bay (cage aquaculture area); WZ: mid bay; WD: bay head.

Sample Collection

Surface and bottom layer seawater samples were collected at each station according to the method described by Zhang, Weinbauer, and Qian (2007). The seawater was filtered first through a 5.0 μm poresize polyethylene terephthalate membrane (47 mm diameter) to remove large suspended substances and subsequently through a 0.22 μm pore size polycarbonate membrane (47 mm diameter, Whatman) to collect bacterioplankton cells. The membranes were stored in liquid nitrogen and delivered to the laboratory at -80 ℃ until DNA extraction.

Environmental Parameter

All the environmental parameter data including temperature, salinity, pH, dissolved oxygen content (DO), ammonia (NH_4^+-N), nitrate (NO_3^--N), nitrite (NO_2^--N), phosphate (PO_4^{3-}-P), silicate (SiO_3^{2-}-Si), total phosphorus (TP), total nitrogen (TN), total alkalinity (ALK), suspended substance (SS), turbidity (TURB), chlorophyll a (Chl-a), chemistry oxygen demand (COD) and 5 day biochemical oxygen demand (BOD_5) were provided by the Key Lab of Daya Bay Field Marine Biology Station of CERN, SCSIO, CAS (http://mbrs.scsio.ac.cn/). Dissolved inorganic nitrogen (DIN) is NH_4^+-N+NO_3^--N+NO_2^--N. Phytoplankton biomass (PB, mg C m^{-3}) was calculated according to a reduction coefficient of 50 mg C mg Chl-a^{-1} (Harris, 1986, Krempin and Sullivan, 1981).

Bacterial Abundance and Biomass

50 ml seawater samples were fixed by formaldehyde (4% final concentration). After that, 1 ml of fixed samples was filtered to capture the bacterioplankton cells using a 0.22 μm poresize 25 mm polycarbonate membrane filter (Whatman). Then bacterioplankton cells captured on filter membranes were dyed with acridine orange (AO) for 10 min in the dark (Boetius and Lochte, 1996). Finally bacterial cells were counted by means of epifluorescence microscope (Leica DMR, German) with excitation wavelength of 450-490 nm.

Ten visual fields of each sample were selected for cell counts based on the standard of 15-30 cells per field. Bacterioplankton abundance (BA, cell m^{-3}) was calculated by the following equation (Zheng *et al.*, 2002):

$$BA = A \times S_1 / (S_2 \times V) \quad (1)$$

where, A is the mean number of cells in 10 fields; S_1 is the membrane effective filtration area; S_2 is the area of the microscope's visual field; V is the volume of filtered water sample. Bacterioplankton biomass (BB) was determined with a carbon reduction coefficient of 20 fg C $cell^{-1}$ (Lee and Fuhrman, 1987).

DNA Extraction

Total bacterioplankton DNA extraction and purification from the filter membranes were performed using the typical phenol-chloroform-isopropanol method described by Rivera *et al.*, (2003).

PCR

PCR amplification of bacterial 16rRNA genes was done with a combination of the primer 968f (including a 40 bp GC clamp sequence) and 1401r by the procedure of Evans *et al.*, (2004) with modifications. 50 μl total reaction volume of PCR amplification system included 1 μl of template DNA and 20 pmol of each primer with the *TaKaRa Ex Taq*™ (TaKaRa Biotechnology (Dalian, China) Co., Ltd.) according to the manufacturer's protocol. PCR amplification was performed according to the denaturation procedure for 4 min at 94°C, 35 cycles for 1 min at 94°C, annealing for 1 min at 55°C and extension for 1 min at 72°C, a final extension for 8 min at 72°C. Electrophoresis experiment with 1.5% (w/v) agarose gel dying with Gold View (0.5% v/v) was used to detect PCR products.

DGGE and DNA Sequencing

DGGE was performed to separate the specific sequences of the above PCR products using a D-code System (Bio-Rad, USA). DGGE bands should refer to apparent bacterial diversity. The denaturing gradient of polyacrylamide gel (6% w/v) ranged from 40% to 65% of denaturing (100% corresponds to 7 M urea

and 40% [v/v] formamide). PCR products were applied directly onto polyacrylamide gels in a 0.5×TAE buffer. Electrophoresis was done under the condition of a constant voltage of 80 V at 60°C for 15 h. Gels were dyed in 0.5×TAE buffer containing ethidium bromide (0.5 mg L^{-1}) for 20 min, prior to photography with UV transillumination.

For sequencing and identification, the main DGGE bands were selected and cut from the gels. If some bands were equally distant from the well in different lanes, only one band representing the same bacterial 16S rDNA was selected. After the DGGE band was removed from the gel, it was re-suspended in 20-30 μl sterilized ddH_2O (double distilled water) and kept at -20℃ overnight for DNA elution. After centrifugation at 12000 rpm for 3-5 min, the supernatant was used as a template and amplified again with the same primer set 968f (without GC clamp) and 1401r. The PCR products were purified, ligated into the pMD 18-T vector and subsequently transformed into *E. coli* DH5α according to the manufacturer's instructions (TaKaRa Biotechnology (Dalian, China) Co., Ltd.). The positive clones were screened and cultured. The bacteria cultures were used for extraction and purification of plasmid DNA by Shanghai Invitrogen Biotechnology Co., Ltd. DNA sequencing was then done by using an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, USA).

Data Analysis

The patterns of the DGGE band were analyzed by scanning DGGE maps using BioCaptMW software (Vilber Lourmat, France) combined with a careful manual check. The peak areas of the fingerprint patterns represent the intensities. Low-intensity bands were discarded if their relative intensity less than 0.5% of the sum of all band intensities. Band position data of all samples were exported into an Excel spreadsheet for further statistical analyses. The images were analyzed for the number of bands per sample (presence versus absence). The patterns of DGGE bands were transformed into a binary data matrix. The DGGE bands patterns were analyzed for similarity by the Nei & Li's coefficient method and the results presented as a tree diagram. The dendrograms were calculated using the unweighted pair-group clustering algorithm with arithmetic averages (UPGMA). The MVSP package version 3.1 was employed for cluster analyses. The differences in seasonal changes of BB and bacterial apparent richness (BAR, calculated as no. of DGGE bands) were examined by the ANOVA test. The relationship of environmental parameters BB and BAR was tested by Pearson correlation analysis. The statistic software SPSS version 15.0 was used for all the statistical analyses.

All the obtained sequences were directly submitted to GenBank. The similarity searches for determination of the closest relatives and phylogenetic affiliation of these sequences were carried out using the blast program (Altschul *et al.*, 1990). The Neighbor-Joining method was used to construct the phylogenetic tree (Saitou and Nei, 1987). The percentage of replicate trees in which the associated data clustered together in 1000 bootstraps is shown next to the branches (Felsenstein, 1985). The Maximum Composite Likelihood method is used to compute the evolutionary distances (Tamura, Nei, and Kumar, 2004) expressed in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were performed using MEGA version 4.1 software (Tamura *et al.*, 2007).

RESULTS

In this study, tempo-spatial distribution of bacterioplankton biomass and bacterioplankton apparent richness was investigated. Bacterioplankton community composition was disclosed, environmental parameters were characterized, and the correlation of bacterial communities and environmental parameters was analyzed.

Tempo-spatial Distribution of Bacterioplankton Biomass

Bacterial biomass (BB) values in Daya Bay in winter (January) and spring (April) in 2008 are shown in Figure 2. The bacterial biomass ranged from 0.12 to 0.20 μg L^{-1} in winter and from 0.15 to 0.66 μg L^{-1} in spring. The highest BB was recorded in surface seawater sample of the bay mouth in spring and the lowest at the bottom of the bay head in winter. The mean BB was approximately 1.5 times higher in spring (0.31±0.20 μg L^{-1}) than in winter (0.21±0.07 μg L^{-1}) with a weak trend toward the value of $P = 0.16$. There was no significant difference in BB between the bay surface and the bottom in both seasons ($P = 0.84$ in winter and $P = 0.07$ in spring). The spatial distribution difference of BB in Daya Bay in both seasons was analyzed by comparing the mean BB of surface and bottom samples at each station as shown in Figure 2. The mean BB on the surface and bottom was ranked as follows: bay mouth > mid bay > inner bay > bay head in winter and bay mouth > inner bay > mid bay > bay head in spring with the trend of increase in the south relative to the north of the bay.

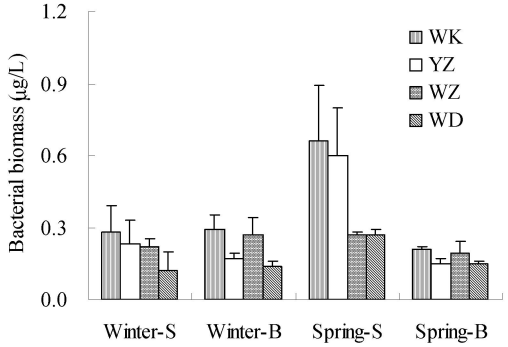

Figure 2. The distribution of bacterioplankton biomass (mean ± SD; n = 6) at the surface (S) and bottom (B) of each station in Daya Bay in winter (January) and spring (April). WK: bay mouth; YZ: inner bay (cage aquaculture area); WZ: mid bay; WD: bay head.

Tempo-spatial Distribution of Bacterioplankton Apparent Richness

In the preliminary experiments the same DGGE profile was observed in all six replicates from each sampling (data not shown). The DGGE band patterns obtained in this study are shown in Figure 3. A total of 42 different bands were observed in DGGE profile through the image and statistical analysis. The community similarity in samples was obtained by the cluster analysis of the DGGE band patterns (Figure 4). High similarity (70%-100%) of bacterial community structure in the surface and bottom samples at each station during the winter and spring was observed (Figure 4) with much less vertical variation (≤ 30%) probably due to vertical mixing in shallow Daya Bay. The similarity of spring and winter patterns in all the sampling sites was below 50%, except 55% similarity in the bottom of the bay head, indicating the higher seasonal difference (> 50%) of the community composition which may follow from the difference in the availability of nutrients between seasons. Consequently, two main clusters were observed in the clustering dendrogram: (1) the first cluster containing all spring samples with 65% similarity; (2) the second cluster including all winter samples with 55 % similarity.

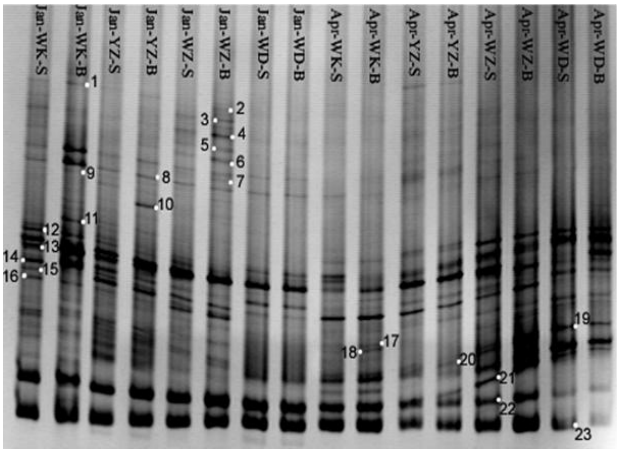

Figure 3. DGGE profiles of 16S rDNA fragments amplified from culture-independent bacteria in Daya Bay in winter and spring. Jan: January; Apr: April; WK: bay mouth; YZ: inner bay (cage aquaculture area); WZ: mid bay; WD: bay head; B: bottom; S: surface.

The number of DGGE bands representing the total bacterial apparent richness is shown in Figure 5. There was no significant difference in BAR between the bay surface and bottom in winter ($P = 0.88$) and spring ($P = 0.50$). The mean BAR was 18 in winter and 12 in spring that is 1.5 times less, with the considerable statistical difference ($P = 0.06$). The bacterial species richness in all the samples except those from the bay head and mid bay surface was apparently higher in winter than in spring. Spatial BAR sequence may be ranked as inner bay > bay mouth > mid bay > bay head in winter. In spring the results were mid bay > bay head > bay mouth or inner bay. The spatial distribution variation of bacterial apparent richness in each season may be attributed to different environmental conditions around the stations.

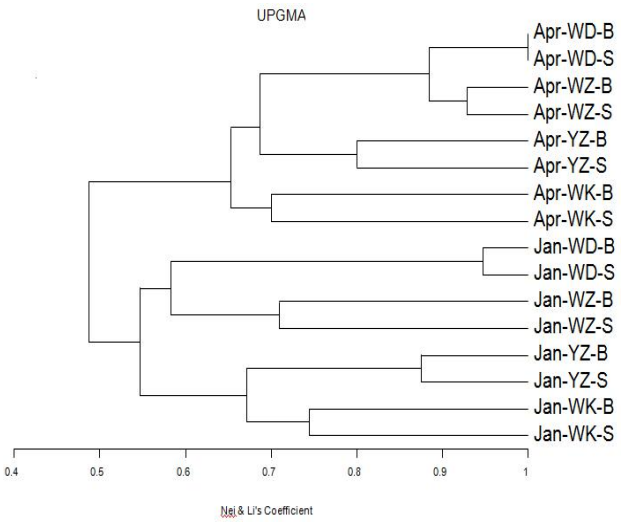

Figure 4. Unweighted pair-group clustering dendrogram of the DGGE profiles using arithmetic average linkage. Apr: April; Jan: January; WK: bay mouth; YZ: inner bay (cage aquaculture area); WZ: mid bay; WD: bay head; B: Bottom; S: Surface.

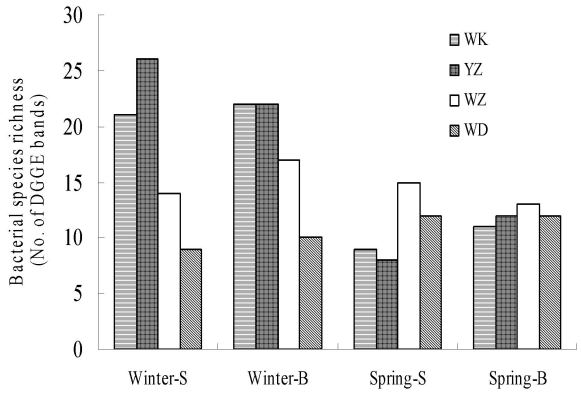

Figure 5. Bacterial species richness in different populations of Daya Bay. Species richness is defined as the number of bands on the DGGE gel. DGGE was performed for PCR products from DNA templates combined from six replicates of the same samples which individually generated the same DGGE patterns (data not shown).

Bacterioplankton Community Composition

The sequences of 23 bands from 42 different DGGE bands were obtained (Figure 3). The phylogenetic tree was constructed using these sequences (labeled DY1 to DY23, corresponding to

bands from 1 to 23) and each of their closest sequences obtained using the BLASTN program (Figure 6). The online classifier tool in RDP was used to compare these sequences (about 430 bp) (GenBank accession numbers GU562831-GU562853). Nine sequences had no more than 97% similarities with their respective closest relatives obtained from GenBank database, revealing that they might be new species (39% of total bacterial sequences). The bacteria identified from Daya Bay were closely associated with four groups including *Proteobacteria* (35%, 8 species), *Cyanobacteria* (30%, 7 species), *Actinobacteria* (26%, 6 species) and *Verrucomicrobia* (9%, 2 species) (Figure 6). However, most bacteria accounting for 78% (18 species) were uncultured.

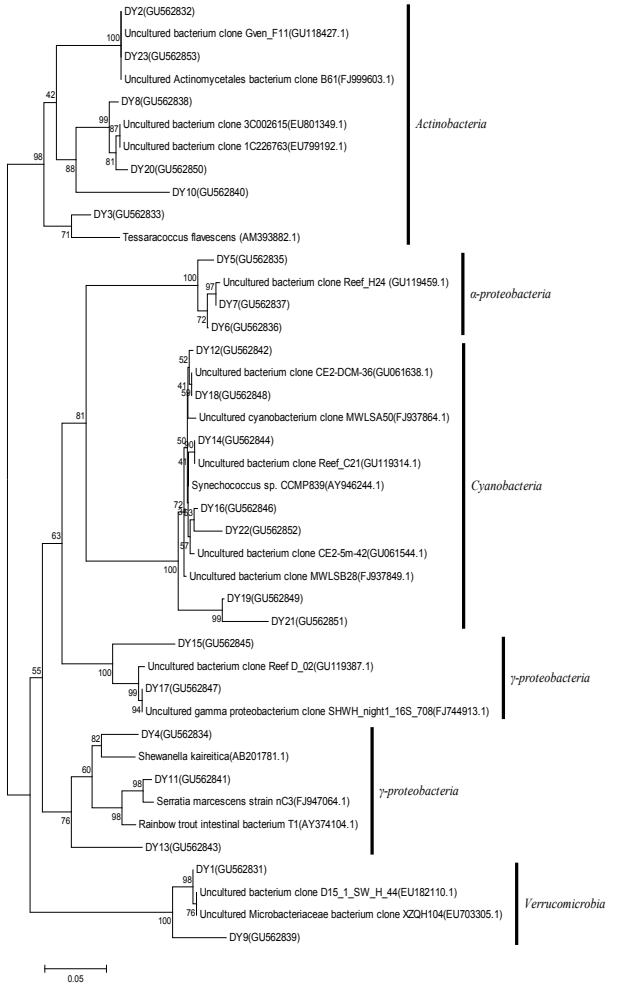

Figure 6. Neighbour-joining phylogenetic tree showing the relationship of bacterial 16S rDNA gene sequences retrieved from DGGE profiles in Daya Bay. The scale bar represents 5% sequence variation.

The *Proteobacteria* group included *Gammaproteobacteria* and *Alphaproteobacteria*. The former comprised three known cultured species: *Shewanella kaireitica* (AB201781.1), *Serratia marcescens* strain nC3 (FJ947064.1) and Rainbow trout intestinal bacterium T1 (AY374104.1) which formed a cluster, and two uncultured bacterium clones (GU119387.1 and FJ744913.1) forming another cluster. *Shewanella kaireitica*, a facultative anaerobe, has both respiratory and fermentative metabolism types, was isolated for the first time in the deep-sea sediment of Suruga Bay, Japan. The bacterium with the ability of nitrate reduction and H_2S-production is chemo-organotrophic and has the activity of catalase and cytochrome oxidase (Miyazaki *et al.*, 2006). *Serratia marcescens* is also a facultative anaerobe. This kind of bacterium occurred in varied environments such as soil, water, air, plants and animals (Grimont and Grimont, 1984). A study by Chen *et al.* (2001) revealed that these species as biological control agents had the potential in a field of plant pest's neutralization. It also occurs as a human pathogen which can result in chronic debilitating disorders and diverse infections comprising respiratory tract infection, urinary tract infection, septicaemia, meningitis, wound infections and infective endocarditis (Cox, 1985; Gouin *et al.*, 1993; Hejazi and Falkiner, 1997; Komer *et al.*, 1994; Mills and Drew, 1976). Rainbow trout intestinal bacterium T1 was isolated from rainbow trout (*Oncorhynchus mykiss*, Walbaum) in a freshwater fish farm in Denmark (Huber *et al.*, 2004). *Alphaproteobacteria* consisted of three uncultured bacterium clones isolated from threatened corals. As widespread marine plankton, *Alphaproteobacteria* constitutes an essential portion of the marine microbial community and dominates in an uptake of labile DOM (Church, 2008).

Phylogenetic analysis showed that seven Daya Bay clones belonged to *Cyanobacteria* group as the most important N_2-fixing microbe in the oceans, containing the sequence of only one cultured organism, *Synechococcus* sp. CCMP839. Physiological experiments indicated that *Synechococcus* sp. CCMP839 had low requirements for N, P and Fe concentrations or illumination levels. This species maybe utilize both inorganic and organic N and P sources (Timmermans *et al.*, 2005). It is commonly considered that the small-size phytoplankton species have an ecological advantage (*i.e.* a low nutrient requirement and a high efficiency of nutrient absorption) due to a large surface to volume ratio (Raven, 1994; Veldhuis *et al.*, 2005). In fact, the genus *Synechococcus* as oxygenic photoautotrophs provides a significant contribution to primary production. It belongs to the most abundant members of the picophytoplankton in the world's oceans and plays a key role in the marine base food web (Scanlan and West, 2002). Moreover, since the genome sequencing of *Synechococcus* strains has been completed and the full set of genetic information is available at present (Rocap *et al.*, 2003), more data on physiology, biology, and ecology of the genera may be obtained in future. We observed high richness and abundance (the intensity of DGGE bands) of *Cyanobacteria*, which may be attributed to the limiting role of DIN in the water column.

A total of 6 clones were affiliated with the *Actinobacteria*, accounting for 26% of the total clones. The phylogenetic group has only one known cultured species, *Tessaracoccus flavescens* (AM393882.1). *Tessaracoccus flavescens*, isolated firstly in

marine sediment, is a facultative anaerobe with the capacity of nitrate reduction (Lee and Lee, 2008).

Phylogenetic analysis revealed diverse sequences retrieved from the water column in Daya Bay and they were grouped into previously recognized eubacterial divisions. Most sequences were closely related to other 16S rDNA clones recovered from marine environments rather than cultured species. That was in agreement with results of other studies on marine bacterial ecology (Bano and Hollibaugh, 2002; Gallagher *et al.*, 2004).

Environmental Characterization

Environment variables in Daya Bay in winter and spring are shown in Tables 1 and 2. An average seawater temperature was 18.16±0.39 ℃ in winter and 22.99±0.41 ℃ in spring, and the difference between the two seasons was 4.73 ℃. The surface seawater temperature was 0.3 and 0.6 ℃ higher than at the bottom in the inner bay in winter and mid bay in spring, respectively, and the corresponding temperature difference at every other station in both seasons was within 0.1 ℃. In winter the seawater temperature in mid bay and inner bay was higher than in the bay head and bay mouth. In spring the seawater temperature was in the order of bay mouth < mid bay < inner bay or bay head. The mean salinity was 0.62‰ higher in winter than in spring, which may be attributed to spring rainfall. In winter the salinity was in the order of inner bay < bay mouth or mid bay < bay head. In contrast, spring salinity was in the order of mid bay < inner bay < bay mouth or bay head.

Table 1. *Data of environment variables in Daya Bay in winter (January).*

Parameters	Unit	Min.	Max.	Mean±SD*
T	℃	17.7	18.8	18.16±0.39
Sal	‰	32.91	34.05	33.62±0.46
pH		8.25	8.3	8.28±0.02
DO	mg/L	6.42	7.95	7.28±0.54
NH_4^+-N	μmol/L	0.07	3.58	1.17±1.33
NO_3^--N	μmol/L	1.37	5.81	3.00±1.42
NO_2^--N	μmol/L	0.2	0.66	0.51±0.19
DIN	μmol/L	1.68	10.04	4.68±2.72
PO_4^{3-}-P	μmol/L	0.05	0.49	0.18±0.15
SiO_3^{2-}-Si	μmol/L	3.47	8.43	6.05±1.58
TP	μmol/L	0.45	1.71	0.84±0.41
TN	μmol/L	9.5	36.77	20.33±8.35
SS	mg/L	1.4	7.62	5.16±2.03
ALK	mmol/L	2.02	2.15	2.09±0.06
TURB	mg/L	1.28	12.45	4.40±3.99
BOD_5	mg/L	0.3	0.46	0.39±0.07
COD	mg/L	0.63	2.1	1.13±0.49
Chl-a	μg/L	1.34	3.31	2.06±0.78
PB	μg/L	67.05	165.35	102.98±38.98

*T: temperature, Sal: salinity, DO: dissolved oxygen content, NH_4^+-N: ammonia, NO_3^--N: nitrate, NO_2^--N: nitrite, DIN: dissolved inorganic nitrogen, PO_4^{3-}-P: phosphate, SiO_3^{2-}-Si: silicate, TP: total phosphorus, TN: total nitrogen, ALK: total alkalinity, SS: suspended substance, TURB: turbidity, Chl-a: chlorophyll a, COD: chemistry oxygen demand, BOD_5: 5 day biochemical oxygen demand, PB: phytoplankton biomass, *calculated from all samples data in each season.*

DO concentration was higher in the winter than in spring, which may be attributed to seasonal temperature variations. In winter DO was higher in the surface water than at the bottom of the bay head, but the situation at the other three stations was quite the opposite. DO was in the order of inner bay > bay mouth > mid bay > bay head. In spring DO was higher in the surface layer than at the bottom of bay mouth or head, while the situation at the other two stations was the opposite. DO was in the order of mid bay > bay mouth > bay head > inner bay.

Table 2. *Data of environment variables in Daya Bay in spring (April).*

Parameters	Unit	Min.	Max.	Mean±SD*
T	℃	22.4	23.3	22.99±0.41
Sal	‰	32.86	33.51	33.00±0.30
pH		7.78	8.25	8.12±0.16
DO	mg/L	5.08	7.86	6.90±1.00
NH_4^+-N	μmol/L	1.57	13.28	5.78±4.61
NO_3^--N	μmol/L	0.86	5.78	3.23±1.81
NO_2^--N	μmol/L	0.07	0.64	0.32±0.24
DIN	μmol/L	3.64	16.99	9.33±5.35
PO_4^{3-}-P	μmol/L	0.06	0.29	0.12±0.08
SiO_3^{2-}-Si	μmol/L	6.36	14.5	9.77±3.30
TP	μmol/L	0.36	1.26	0.64±0.34
TN	μmol/L	4.35	24.7	14.97±8.23
SS	mg/L	3.6	9.6	6.80±1.92
ALK	mmol/L	2.08	2.22	2.14±0.05
TURB	mg/L	1.28	4.13	2.65±0.96
BOD_5	mg/L	0.69	1.57	1.17±0.35
COD	mg/L	0.9	1.25	1.09±0.12
Chl-a	μg/L	0.07	0.88	0.19±0.28
PB	μg/L	3.35	44.2	9.38±14.10

*T: temperature, Sal: salinity, DO: dissolved oxygen content, NH_4^+-N: ammonia, NO_3^--N: nitrate, NO_2^--N: nitrite, DIN: dissolved inorganic nitrogen, PO_4^{3-}-P: phosphate, SiO_3^{2-}-Si: silicate, TP: total phosphorus, TN: total nitrogen, ALK: total alkalinity, SS: suspended substance, TURB: turbidity, Chl-a: chlorophyll a, COD: chemistry oxygen demand, BOD_5: 5 day biochemical oxygen demand, PB: phytoplankton biomass, *calculated from all samples data in each season.*

The mean DIN concentration was about 2 times larger in spring than in winter. The highest DIN was observed in the inner bay, it was 1.5-5 times larger than at other stations. SS in all of the seawater samples investigated was below 10.0 mg l^{-1}.

The mean pH was higher in winter than in spring. This may occur due to the seasonal difference in rainfall. In both seasons the lowest pH occurred in inner bay. Especially in spring pH at the bottom was 0.21 units lower than in the surface layer in the inner bay. This possible acidification phenomenon may be attributed to cage aquaculture. The mean PO_4^{3-}-P was higher in the winter than in spring. Based on the monitoring results, the Daya Bay seawater met the national seawater quality standards for Grade I marine water (≤ 0.015 mg l^{-1}) except minor pollution (0.0153 mg l^{-1}) in the surface layer of the inner bay in the winter. As for another environmental factor COD, according to the national standards mentioned above, it also indicated good water quality (< 2.0 mg l^{-1}), excluding minor pollution (2.1 mg l^{-1}) in the surface water of the bay head in the winter. The BOD_5 index also showed good water quality in the winter (< 0.5 mg l^{-1}). But in the spring it showed minor pollution in all

the seawater samples in Daya Bay (1.0-1.6 mg l^{-1}), except the surface of inner bay and bottom of bay mouth (< 1.0 mg l^{-1}).

Table 3. *Relationship between bacterial biomass (BB) and apparent species richness (BAR, calculated as no. of DGGE bands), and environment variables in Daya Bay in winter (January) and spring (April).*

Environmental	Winter		Spring	
Parameters	BB	BAR	BB	BAR
T	0.21	0.62	-0.32	0.01
Sal	0.15	-0.80*	0.18	0.48
Depth	0.37	-0.2	-0.52	-0.1
DO	0.57	0.93**	-0.26	0.56
pH	0.2	-0.55	0.01	0.26
NH_4^+-N	0.04	0.66	0.07	-0.45
NO_3^--N	0.52	0.91**	-0.26	-0.1
NO_2^--N	0.81*	0.92**	-0.15	-0.12
DIN	0.19	0.77*	-0.04	-0.45
PO_4^{3-}-P	0.2	0.79*	0.43	-0.62
SiO_3^{2-}-Si	0.46	0.85**	-0.1	0.21
TP	-0.03	0.36	0.06	-0.24
TN	-0.05	-0.38	0.27	-0.5
SS	0.69	0.47	-0.3	-0.25
ALK	0.72*	0.58	-0.27	-0.22
Chl-a	-0.72*	-0.80*	0.69	-0.43
TURB	-0.04	0.54	-0.34	0.34
BOD_5	-0.21	-0.01	-0.42	0.64
COD	-0.72*	-0.54	-0.22	0.62

*T: temperature, Sal: salinity, DO: dissolved oxygen content, NH_4^+-N: ammonia, NO_3^--N: nitrate, NO_2^--N: nitrite, DIN: dissolved inorganic nitrogen, PO_4^{3-}-P: phosphate, SiO_3^{2-}-Si: silicate, TP: total phosphorus, TN: total nitrogen, ALK: total alkalinity, SS: suspended substance, TURB: turbidity, Chl-a: chlorophyll a, COD: chemistry oxygen demand, BOD_5: 5 day biochemical oxygen demand, ** and * represent correlation values at P < 0.01 and P < 0.05 level, respectively.*

Chl-a concentration was about 10 times higher in the winter (2.06±0.78 μg l^{-1}) than in spring (0.19±0.28 μg l^{-1}). To be precise, there was no significant difference between bay surface and the bottom. Chl-a concentration was the highest in the bay head (about twice more than at the other stations), moderate in the bay mouth and the lowest in the mid bay and inner bay. By comparison, in spring Chl-a concentration was in the order of surface of bay mouth (0.88 μg l^{-1}) > bottom of the inner bay and surface of mid bay (0.11-0.12 μg l^{-1}) > all the other samples (< 0.10 μg l^{-1}). The difference of Chl-a concentrations between the surface and the bottom was more than 13 times at bay mouth and less than 2 times at other stations.

As mentioned above, PO_4^{3-}-P and BOD_5 indexes revealed minor pollution in the inner bay which should be attributed to cage aquaculture. The relatively lower DO, pH and higher DIN level compared with other stations investigated may further strengthen this viewpoint. The minor pollution resulting from cage aquaculture caused the difference of bacterial community composition between inner bay and the other Daya Bay stations.

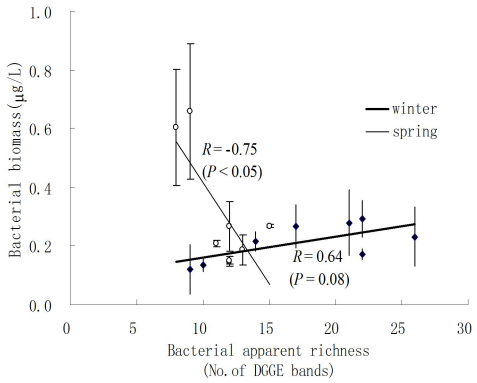

Figure 7. The relationship between bacterial biomass (mean±SD) and bacterial apparent species richness in Daya Bay in winter and spring.

The Correlation of Bacterial Communities and Environmental Parameters

The relation between bacterial community (including the bacterial biomass and the apparent species richness) and different ecological environment variables of Daya Bay was analyzed using the statistic software SPSS version 15.0. The results are shown in Table 3. In winter ALK and NO_2^--N had significant positive correlations with BB ($P < 0.05$), and PB (Chl-a) while COD correlations were negative to BB ($P < 0.05$). This indicated that the four parameters mentioned above were the main controlling factors of BB. DO, NO_3^--N and SS had a strong correlation with BB ($r > 0.5$), indicating these parameters were important factors controlling bacterial biomass. NO_2^--N, NO_3^--N, DIN, SiO_3^{2-}-Si and DO had a highly significant correlation with BAR, PO_4^3-P had a significant correlation with BAR, and Sal and Chl-a had significant negative correlation with BAR, indicating these parameters were major controlling factors of bacterial diversity. T, pH, NH_4^+-N, ALK, TURB and COD had a strong correlation with BAR ($r > 0.05$), indicating their important effects on bacterial diversity. In the spring, the high correlation ($r = 0.69$) was obtained between BB and PB, indicating phytoplankton was an important controlling factor of bacterioplankton biomass. BB had the high negative correlation ($r = -0.52$) with the depth, indicating the increase in BB as the seawater depth decreased, which to some extent may be a result of illumination limit at deeper regions. DO, PO_4^{3-}-P, TN, BOD_5 and COD had a strong correlation with BAR ($r > 0.5$), indicating their important impacts on bacterial diversity. There existed strong correlation ($r = 0.64$, $P = 0.08$) between BB and BAR in winter, and contrary, significant negative correlation ($r = -0.75$, $P < 0.05$) in spring (Figure 7).

DISCUSSION

The nutrient competition exists prevalently between phytoplankton and bacterioplankton (Joint *et al.*, 2002; Rivkin and Anderson, 1997), and ultimately it determines their respective availability and affects the bacterial community structure. Marine bacteria can inhibit phytoplankton growth by

nutrient competition and even lyse their cells by excreting special compounds (Zhou *et al.*, 2001). In contrast, marine bacterial growth is top-down controlled by phytoplankton's predation (Zubkov and Tarran, 2008). Marine bacteria have stronger competition capacity for nutrients at low concentrations. Phytoplankton has the highest uptake potentials and may dominate under higher or fluctuating nutrient conditions (Cho and Azam, 1990; Kononen, 2001). The diversity of bacterioplankton community composition may be influenced by combinations of environmental parameters (Ghiglione *et al.*, 2008; Signori *et al.*, 2014). Here, the correlations between environment variables, bacterioplankton biomass, and apparent richness were discussed. Moreover, possible ecological environment regulation mechanism of bacterioplankton community structure was revealed in Daya Bay in winter and spring, mainly according to the theories of resource competition and nutrient limit.

The Correlation between Bacterioplankton Biomass and Environment Variables

In winter NO_2^--N and NO_3^--N were the key limiting factors of bacterioplankton biomass in consistence with the previous declaration (Howarth, 1988), *i.e.* in marine ecosystems the limiting role was commonly attributed to nitrogen. The significant negative correlation between BB and PB (Chl-a) (Table 3) indicated that under conditions of limited nutrients there existed competition for nutrients between bacterio- and phytoplankton.

According to the present study, in winter phytoplankton growth in Daya Bay was stimulated by the nutrients transported continually up from marine sediment by intensive vertical mixing driven by a strong northeast monsoon. Consequently, phytoplankton biomass was more than ten times higher in winter than in spring (Tables 1 and 2). Since most N and P in the water column was sequestrated by phytoplankton, both TN and TP concentrations were higher in winter than in spring (Tables 1 and 2). Phytoplankton depleted most DIN (with its mean concentration below the half of that in spring) and especially NO_2^--N (the concentration ≤ 0.66 μmol l^{-1} in each seawater sample was determined). Phytoplankton dominated ultimately through the nutrient competition with bacterioplankton (the BB/PB value was only 0.21% in winter), which made inorganic nitrogen (NO_2^--N and NO_3^--N) become the most limiting nutrient of bacterial biomass. Thus it appears that the nutrient competition between phyto- and bacterioplankton mentioned above might explain the significant negative relationship between PB and BB (Table 3).

At the same time, other factors can also play important role in BB. BB showed a negative correlation with DO as bacteria consume oxygen at organic matter decomposition. Similar to PB, COD also had significant negative correlation with BB. Generally, it represents the content of the reduced organic matter which may reflect the magnitude of PB in seawater. ALK, representing the total concentration of all kinds of feeble acid ions in seawater, was also one of the important factors controlling BB. Phytoplankton and photosynthetic bacteria use CO_2 for photosynthesis. There existed a balance among CO_2, CO_3^{2-} and HCO_3^- and so ALK (may be considerably attributed to CO_3^{2-} and HCO_3^-) played an important role in BB. TURK reflected to some extent the degree of water column mixing and thus correlated with BB.

In spring the seawater column had a poor mixing due to northeast monsoon decline. This resulted in nutrients transport reduction from marine sediments. The decreased nutrients further led to reduced PB accompanied by the decrease in TN and TP. An uptake of inorganic nutrients by phytoplankton reduced, that followed by a substantial change in DIN (NH_4^+-N and NO_3^--N) and perhaps DOM (mainly produced by phytoplankton residues) concentrations in seawater. DIN (NH_4^+-N and NO_3^--N) and possibly DOM were used by marine bacteria and provided high BB (mean BB value was 1.5 times more than in winter). As the time passed the temperature and illumination (light) became better, and so PB began to increase. During the certain period of investigation growth of BB followed a growth in PB. Thus, a strong positive correlation (r = 0.69) was observed between PB and BB. This very likely indicated that PB limits BB. It may explain results of winter observations in Daya Bay. That is, the change of PB from winter to spring was responsible for winter to spring variation of BB as well.

The Correlation between Bacterioplankton Community Composition and Ecological Environment

The results of the present study indicated that the diversity of bacterioplankton community composition was regulated and controlled by combinations of ecological environment factors. That supports the conclusions of the previous studies (Ghiglione *et al.*, 2008; Signori *et al.*, 2014). In detail, although NO_2^--N and PO_4^{3-}-P levels enhanced in winter, phytoplankton with higher biomass in winter assimilated most of the nutrients (NO_3^--N, NH_4^+-N and SiO_3^{2-}-Si). This led to the decrease of the part of nutrients fed by marine bacteria and consequently the reduction of BB. The reduced BB and the discreteness of bacterioplankton distributions (Siegel, 1998), in addition to relatively lower nutrients (DIN, NH_4^+-N, NO_3^--N and SiO_3^{2-}-Si) availability mentioned above resulted in the decrease of rates of competitive displacement. The decrease of rates of competitive displacement caused the decrease of the portion of absolute dominant bacteria species and the increase of the portion of relative dominant bacteria species, *i.e.* the increase of the apparent species diversity (No. of DGGE bands). However, at these tropic conditions bacterial biomass had an obviously positive correlation with apparent species richness, with a borderline value at P = 0.08 (Figure 7). In winter higher salinity favored growth and reproduction of halophilic and halotolerant bacteria, leading to the decrease of bacterial species diversity. Nevertheless, the relatively lower temperature was unfavorable for the formation and maintenance of the dominant species, thus promoting the growth of bacterial diversity.

In spring BAR had a strong correlation with DO, PO_4^{3-}-P, TN, BOD_5 and COD ($r > 0.5$), indicating their important impacts on bacterial diversity. There was a strong negative correlation between bacterial biomass and apparent diversity (Figure 7). Regarding the response of the bacterial community to environmental changes in this season, a possible explanation is as follows. High DIN (NH_4^+-N and NO_3^--N) and SiO_3^{2-}-Si concentrations may lead to an increase of BB, in turn, the increase of BB accelerated the rates of competitive

displacement of bacterioplankton populations. The rates of competitive displacement further induced the increase of the proportion of absolute dominant bacterial species and the decrease of the percent of relative dominant bacterial species, *i.e.* the decrease of the BAR. In the present study, it appears that DIN, SiO_3^{2-}-Si and PO_4^{3-}-P strongly affected BAR.

The Response of Bacterioplankton Community to Ecological Environment Variation in Winter and Spring

In summary, possible response processes (mechanisms) of bacterioplankton communities on ecological environment changes from winter to spring in Daya Bay were revealed in the present study. They are the following. Strong in winter and weak in spring mixing degrees of water column gave rise to high-in-winter-and-low-in-spring (HWLS) values of PB, TN and TP. The DIN availability for phytoplankton resulted in the low-in-winter-and-high-in-spring (LWHS) DIN availability for bacterioplankton as well, which further provided LWHS bacterial biomass. The LWHS nutrients (DIN, NO_3^--N, NH_4^+-N, SiO_3^{2-}-Si) availability to bacterioplankton led to LWHS rates of competitive displacement among bacterioplankton populations. That caused LWHS absolute dominant bacterioplankton populations, HWLS relative dominant bacterioplankton populations and HWLS apparent bacterioplankton diversity.

CONCLUSIONS

The response mechanisms of bacterial communities including bacterial biomass and diversity on ecological environment changes from winter to spring in Daya Bay were investigated and discussed for the first time in the present study. It was found that phytoplankton controlled bacterioplankton biomass by utilizing dissolved inorganic nitrogen (DIN) (NH_4^+-N and NO_3^--N) in Daya Bay. The observed seasonal variations of bacterioplankton community composition in Daya Bay were mainly associated with the different availability of nutrients (DIN and SiO_3^{2-}-Si) in winter and spring. The distinctive community composition in inner bay relative to other stations in Daya Bay may be attributed to minor seawater pollution resulting from cage aquaculture. It was found that some bacterial species identified had individual metabolism capacity and may have bioremediation function. Further study should be performed to obtain their cultured strains, reveal their biochemical characteristics and develop helpful ecological restoration strategies for environment protection.

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