Bacterial composition and spatiotemporal variation in sediments of Jiaozhou Bay, China

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Abstract

Purpose Although coastal marine sediments harbor diverse bacteria with important ecological and environmental functions, a comprehensive view of their community characteristics is still lacking in typical environments along the China coast. We studied the diversity and composition of bacterial community in the sediment of Jiaozhou Bay and characterized their spatiotemporal patterns, aiming to analyze the effects of geographic heterogeneity, seasonal difference, anthropogenic activity, and eutrophication gradient on the bacterial ecology. **Materials and methods** Sixteen full-length bacterial 16S rRNA gene clone libraries were constructed from the sediment bacteria at four stations (A5, B2, D1, and D7) of Jiaozhou Bay over four seasons (February, May, August, and November). The bacterial diversity and community structure variation were analyzed.

Results and discussion A total of 1088 16S rRNA gene sequences and 746 distinct operational taxonomic units (OTUs) (at 97 % sequence similarity level) were retrieved from the 16 clone libraries. Libraries of each sample revealed a high diversity, and a total of 17 bacterial phyla were identified.

Bacteria lineages *Proteobacteria* (mostly γ -*Proteobacteria* and δ -*Proteobacteria*), *Actinobacteria*, *Bacteroidetes*, and *Planctomycetes*, which are commonly found in marine sediments, were the dominant divisions in our samples. Meanwhile, some uncommon phyla, such as *Deinococcus-Thermus* and *Lentisphaerae*, were also detected. Distinct spatial patterns and noticeable seasonal variations of bacteria community were observed, indicating that environmental heterogeneity in spatial variation other than in temporal variation may be more important in determining the bacterial communities. Most of the abundant OTUs were related to sequences retrieved from the heavy-metal-polluted environments, indicating that the Jiaozhou Bay might be influenced by pollution. **Conclusions** The bacterial communities in Jiaozhou Bay sediments are highly diverse. The four typical stations that we chose might experience different anthropogenic effects and eutrophication gradients, which shaped their bacterial community composition. The environment of Jiaozhou Bay may be under pressure exerted by multi-dimension anthropogenic activities. This study provided a novel insight into the bacterial community variations in Jiaozhou Bay, enriching knowledge about ecological bioindicators and mechanisms that maintain bacterial diversity and shape bacterial community composition.

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1 Introduction

Surface sediment bacteria play a significant ecological and biogeochemical role in marine ecosystems due to their high abundance relative to the overlying water column and their key role in organic matter decomposition and nutrient cycling (Wang et al. 2012). As one of the most variable marine

habitats, coastal sediments are generally characterized by a high biodiversity and complicated community structure and distribution in response to environmental heterogeneity, seasonal variation, and anthropogenic effects (Danovaro and Pusceddu 2007; Aguiló-Ferretjans et al. 2008). Moreover, it was demonstrated that bacterial communities have great potential to be used as sensitive indicators of variation and contamination in coastal aquatic sediments (Lachmund et al. 2003; Sun et al. 2012). Although large populations of bacteria are well documented in coastal surface sediments, their biogeographical patterns under anthropogenic disturbances and environmental gradients, as well as temporal variations remain largely unexplored (Bissett et al. 2006; Bertics and Ziebis 2009), especially in heterogeneous marine ecosystems such as Jiaozhou Bay (JZB).

Jiaozhou Bay is a large semi-enclosed water body of the temperate Yellow Sea in China. It covers an area of about 390 km² with an average depth of about 7 m, connecting to the outside seawater via a narrow opening about 2.5 km in width (Dai et al. 2007). The environmental quality of Jiaozhou Bay has deteriorated dramatically in the past three decades due to rapid development in agriculture, industry, urbanization, and mariculture in the surrounding areas (Dang et al. 2008b; Gao and Chen 2012). Several small rivers form the major sources of discharged industrial and sewage wastewater bring excessive nutrients into the northeast of the bay (Shi et al. 2011). Meanwhile, sporadic oil spilling from the commercial ports near the bay mouth, intensive maricultural practices at the north area, along with red tides all make the situation even worse (Wang et al. 2008; Dang et al. 2010b; Deng et al. 2010). A wide spectrum of environmental conditions and accordingly a widely varying bacterial community structure have already drawn the JZB many research interests in recent years (Dang et al. 2008a, b, 2009b, 2010b). To date, however, studies that describe the spatiotemporal variations of the bacterial communities in JZB sediments are still limited. In this study, four stations were chosen with different sources of pollution and various levels of eutrophication, representing typical habitats of JZB to study the structure and dynamics of coastal bacterial communities.

This study used 16S rRNA clone library analysis to determine the diversity and spatiotemporal distribution of sediment bacteria in different JZB environmental conditions. Our primary focus was to find the bacterial biogeographical patterns in the four distinctive stations over different seasons, which is the study of “who is where and at what abundance and why,” providing knowledge about ecological mechanisms that maintain diversity and shape sediment bacterial composition in JZB. In our previous research by denaturing gradient gel electrophoresis (DGGE), we investigated the sediment bacterial community composition in ten stations of JZB over four seasons (Liu et al. 2011). Here, we can also compare the bacteria community structures revealed by DGGE and by

clone library analysis to provide useful information for future research.

2 Materials and methods

2.1 Site description and sampling

2.1.1 Sampling sites

Jiaozhou Bay is located in northern China (35° 43′–36° 18′ N, 120° 04′–120° 23′ E) and is the largest semi-enclosed water body along the southwest coast of the Yellow Sea. The bay is dominated by intertidal and shallow subtidal areas. JZB tides induce strong turbulent mixing, resulting in nearly homogeneous vertical profiles of seawater temperature and salinity. More than 10 small seasonal rivers enter JZB with varying water and sediment loads, including the Yanghe, Dagou, and Licun Rivers (Liu et al. 2005). Because of the increases in the population and commerce in the Qingdao city, the majority of these rivers have become canals for industrial and domestic waste discharge. In recent years, certain areas of JZB have been affected by continuously increasing anthropogenic activities such as land reclamation, port construction, landfill, and reservoir construction (Shi et al. 2011).

2.1.2 Sample collection and physicochemical properties

Sediment samples were collected from four stations (A5, B2, D1, and D7) of JZB (Fig. 1). Stations A5 and B2 are located in the inner bay (characterized by yellow-gray sandy mud), station D1 is located in the mouth of the bay (characterized by gray-black ooze), and station D7 is in the outer bay (characterized by yellow-gray muddy sand). The four stations can also be grouped by the pollution status. B2 is a less polluted station, A5 is a heavily polluted station, and D1 and D7 represent the seaport-influenced station and the station near the outside sea, respectively. Samples were collected in May (spring), August (summer), and November (fall) 2008 and in February (winter) 2009, using a stainless-steel 0.05 m² Gray O'Hara box corer. Only undisturbed surface samples with clear overlying water were used to ensure the integrity of the surface sediment structures. Surface sediment samples (5 cm depth) were taken aseptically with sterile 60-ml syringes (luer end removed), homogenized, and stored in airtight sterile plastic bags at –20 °C and stored at –80 °C after transported to the laboratory. We obtained the following sediment physicochemical parameters from the Ecological Environment Monitoring Station in JZB: total nitrogen (TN), total phosphorus (TP), organic nitrogen (ON), organic carbon (OC), organic matter (OM), and moisture content (MC) (Table 1). In some cases, we were unable to obtain data due to engineering failure or substrate characteristics (station D1). Some basic

Fig. 1 Location of the Jiaozhou Bay and the four sampling stations

texture, such as the particle size, of the sediment samples was limited because the monitoring station in JZB only measured this parameter in February and August of each year.

2.2 DNA extraction and PCR amplification

DNA was extracted from sediment samples (0.5 g) in triplicate with the Power Soil DNA Isolation kit (MoBio, USA) according to the manufacturer's instruction, and the extracted DNA concentration was determined with Nanodrop® ND-1000 Spectrophotometer (NanoDrop Technologies). The presence of high molecular weight DNA (ca. 15,000 bp) in the extracts was verified by comparing the banding pattern of the sample with DNA molecular weight standard electrophoresed on 0.8 % agarose gel. The DNA was stored at -80°C until amplification by polymerase chain reaction (PCR).

The bacterial 16S rRNA genes were amplified (Premix Ex Taq™, Takara) using the universal bacterial 16S rDNA gene primer set 27F (5'-AGAGTTTGATCACTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGAC-3' (Baker et al. 2003). Bovine serum albumin was added to reduce inhibition by humic substances. Amplification was performed with an initial denaturation step at 95°C for 5 min, 30 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min, and a

final extension at 72°C for 10 min. All PCR products were checked via 1 % agarose gel electrophoresis.

2.3 Construction of clone libraries

The PCR products from the three experimental replicates were combined (to reduce stochastic PCR bias) and gel purified. The cleaned PCR products were cloned into Takara pMD-18T simple cloning vector (Takara, Japan), and the ligation products were transformed into competent *Escherichia coli* TOP10 cells. Recombinants were isolated on Luria-Bertani (LB) indicator plates supplemented with X-Gal-IPTG and $100\text{ }\mu\text{g ml}^{-1}$ ampicillin at 37°C for 12–14 h (Dang et al. 2010a). Approximately 180 randomly selected white colonies per sample were cultured overnight in 1.5 ml Luria Broth media at 37°C placed on an orbital shaker at 250 rpm. Representative genotypes were determined via restriction fragment length polymorphism (RFLP). All positive colonies were checked with the M13 and RV-M primers. Amplimers of correct size were separately digested using *MspI* (NEB) and *HhaI* (NEB). Restriction fragments were screened on 3 % agarose gel, running at 100 V in $1\times$ Tris-borate-EDTA (TBE) buffer for 50 min.

Table 1 Sediment physicochemical parameters at the sampling sites in the Jiaozhou Bay over different seasons

Station		A5	B2	D7
Longitude (E)		E120 °19'48"	E120 °11'12"	E120 °25'30"
Latitude (N)		N36 °09'18"	N36 °08'00"	N35 °59'00"
February	Moisture content (MC) (%)	43.6	34.3	19.3
	Total phosphorus (TP) (mg/kg)	554.7	361.9	377.6
	Total nitrogen (TN) (mg/kg)	1700	600	100
	Organic nitrogen (ON) (N%)	0.17	0.06	0.01
	Organic carbon (OC) (C%)	1.04	0.32	0.36
	Organic matter (OM) (%)	6.72	2.68	3.35
	Mean grain size (Mz) (μm)	5.58	22.86	50.10
	Moisture content (MC) (%)	44.2	32.1	23.7
	Total phosphorus (TP) (mg/kg)	611.4	383.2	440.3
	Total nitrogen (TN) (mg/kg)	1800	700	700
May	Organic nitrogen (ON) (N%)	0.18	0.07	0.07
	Organic carbon (OC) (C%)	1.35	0.28	0.29
	Organic matter (OM) (%)	6.71	2.96	3.58
	Mean grain size (Mz) (μm)			
	Moisture content (MC) (%)	47.2	30.8	24.7
	Total phosphorus (TP) (mg/kg)	609.5	402.1	545.1
	Total nitrogen (TN) (mg/kg)	1500	700	800
	Organic nitrogen (ON) (N%)	0.15	0.07	0.08
	Organic carbon (OC) (C%)	1.05	0.33	0.29
	Organic matter (OM) (%)	4.93	1.78	1.88
August	Mean grain size (Mz) (μm)	7.14	19.79	49.28
	Moisture content (MC) (%)	45.5	35.7	21.9
	Total phosphorus (TP) (mg/kg)	522.6	323.4	381.6
	Total nitrogen (TN) (mg/kg)	1800	1200	400
	Organic nitrogen (ON) (N%)	0.18	0.12	0.04
	Organic carbon (OC) (C%)	1.03	0.54	0.31
	Organic matter (OM) (%)	5.07	2.23	1.76
	Mean grain size (Mz) (μm)			
	Moisture content (MC) (%)			
	Total phosphorus (TP) (mg/kg)			
November	Total nitrogen (TN) (mg/kg)			
	Organic nitrogen (ON) (N%)			
	Organic carbon (OC) (C%)			
	Organic matter (OM) (%)			
	Mean grain size (Mz) (μm)			
	Moisture content (MC) (%)			
	Total phosphorus (TP) (mg/kg)			
	Total nitrogen (TN) (mg/kg)			
	Organic nitrogen (ON) (N%)			
	Organic carbon (OC) (C%)			

2.4 Clone library analysis

Restriction profiles were digitally photographed and visually recognized using ChemiDoc™ XRS imaging system (Bio-Rad). All profiles were further sorted into patterns differentiated by the number and size of fragments using Quantity-One software (Bio-Rad). Clones with the same RFLP pattern were grouped into the same phylotype. Full-length 16S rRNA region of at least one phylotype-representing clone was then sequenced. The number of redundant copies was also included in all the statistical calculations to avoid the potential biases in the microbial community parameters due to this pre-screening of representatives of each RFLP pattern. Poor-quality sequences and suspected chimeras were screened out using the Chimera check program (Maidak et al. 2001). Sequences were further assigned to operational taxonomic units (OTUs) at a cutoff value of 97 % using DOTUR (Schloss and Handelsman

2005). For each non-chimera sequence, the GenBank BLAST program was used to determine the nearest phylogenetic neighbor sequence (Altschul et al. 1997).

2.5 Statistical analysis

The coverage of each 16S rDNA clone library was calculated as $C = [1 - (n_1/N)] \times 100$, where n_1 is the number of unique OTUs, and N is the total number of clones in a library. This value approximated the probability that all the unique OTUs in a given sample were represented at least once in the library (Dang et al. 2008c). Estimations of the 16S rDNA phylogenetic diversity (Shannon–Wiener H' and Simpson D) and Pielou's evenness (J) indices were calculated with the OTUs of each library. The equations were as follows: $H' = -\sum P_i \ln P_i$, where $P_i = n_i/N$, n_i is the number of clones in the i th OTU, and N is the total number of clones in the sample; $D = 1$

$-\sum P_i^2$; $J=H'/H_{\max}$, where $H_{\max}$ is the theoretical maximum value for H' if all taxa in the sample were equally abundant. Rarefaction analysis and two nonparametric richness estimators, the abundance-based coverage estimator (S_{ACE}) and the bias-corrected Chao₁ (S_{Chao1}), were calculated using the DOTUR software (Schloss and Handelsman 2005). These diversity indices and richness estimators are useful statistical tools to compare the relative complexity of communities and to assess the degree to which sampling effort is saturated.

The bacterial community classification was determined using the Unique Fraction (UniFrac) environmental clustering method (Lozupone et al. 2007), which takes the molecular evolutionary distances of the sequences for community similarity analysis. This multivariate statistical analysis of clone libraries is based on the different bacterial distributions of each sample and can show the differences of bacterial community structure among different sites and seasons. Canonical correspondence analysis (CCA) was performed to determine the environmental parameters best linked to community separation. A matrix of OTU abundance ratios for each sample was used as the basis of the community analysis. A matrix of environmental values (environmental factors $\times$ quadrats) was also established, which were applied into analyzing the relationships between the bacterial community structure and environmental conditions using CCA. These calculations were carried out with the CANOCO 4.5 software (Microcomputer Power, USA) (ter Braak and Smilauer 2002).

3 Results

3.1 Physicochemical characteristics of sediment samples

The physicochemical characteristics of sediment samples from the stations in four seasons were presented in Table 1. The sampling sites in JZB differed in trophic status. The highest average values for all parameters throughout the four seasons were found at station A5, which is located near the Licun River. The B2 station had the second highest average values for all parameters, and the lowest values were recorded in D7 station. Physicochemical characteristics in all stations also showed seasonal variations. For instance, concentrations of TP and OM were high in May and August, while concentrations of TN and OC were relatively higher, except for station D7, in November.

3.2 RFLP and clone library analysis of the bacterial community

For each of the 16 sediment samples collected from the four typical stations in four seasons, full-length 16S rRNA gene clone libraries were built and analyzed by the RFLP method.

A random of 180 clones in each library was selected for the RFLP analysis, and finally, 2560 positive and proper clones were screened from the total 2880 clones. Restriction enzyme digestion using *MspI* and *HhaI* showed 1231 different RFLP patterns occurred in the total 5120 RFLP patterns. Most of the RFLP patterns were comprised by only one or two clones, suggesting a high degree of interspecific genetic diversity (Michaud et al. 2004). In each RFLP pattern, both the phylotype-representing clone and the redundant copies were included in the calculations of the microbial community parameters. After sequencing, 1088 clones were valid for further genetic analysis. Based on a cutoff of 97 % similarity level, in total, 746 unique operational taxonomic units (OTUs) were assigned. When all libraries were assembled separately, the numbers of OTUs ranged from 76 (station D7 in February) to 155 (station D1 in August), with an average of 123 (Table 2). Among all the 746 OTUs, 412 OTUs occurred in only one library, while the others existed in at least two libraries, indicating a heterogeneous distribution of most of the bacteria in the spatiotemporal profile of the Jiaozhou Bay.

The coverage of the recovered 16S rRNA phylotypes in clone libraries ranged widely from 22.4 to 47.6 % and was not correlated with library size (Table 2). The highest and the lowest coverage were in Aug-A5 and Aug-D7, respectively, which was obtained near the mouth of Licun River and the open sea area, respectively. The rarefaction curves in Fig. 2 revealed high levels of bacterial complexity for marine sediment samples, as none has reached the curvilinear or plateau phase. The low coverage on average was consistent with the rarefaction results, indicating that the actual microbial community was far more diverse and all samples had potentially much more undetected OTUs. To compare the diversities and richness of the bacterial communities, the diversity indices and richness estimators were also calculated (Table 2). Since the Shannon index is always underestimated in the case of incomplete sampling (Hill et al. 2003), it is not an appropriate choice for this dataset because of the incomplete sampling as mentioned. However, each library's Shannon index in this study exhibited similar trend with the reciprocal Simpson index, and the most complex diversity was observed in Aug-D7 and the least in Aug-A5. The average reciprocal Simpson indexes results revealed a more even distribution of the phylotypes for the bacteria communities in B2 (74.12) and D7 (62.74) stations than in contaminated stations A5 (58.40) and D1 (56.91), which suggested that the diversity and evenness of bacterial community may have been negatively affected by sediment contaminations. Among the four seasons, the highest average Shannon index for all the sites was in November (4.58), followed by May (4.28), August (4.25), and the lowest February (4.19), showing a variation that possibly be associated with sea water temperature. The Simpson index values in the four seasons are highly consistent with the Shannon index analysis (Table 2).

Table 2 Analyses of the 16 bacterial clone libraries in the Jiaozhou Bay

Sample	Shannon–Wiener (H')	Evenness (J)	Simpson's diversity (1/dominance)	No. of OTUs 97 %	Single OTU (nl)	Coverage (%)	S_{ACE}	S_{Chao1}
Feb-A5	4.255	0.971	58.320	108	61	0.435	248.80	194.37
Feb-B2	4.268	0.966	57.075	110	69	0.373	388.25	343.66
Feb-D1	4.196	0.984	60.714	85	60	0.294	271.90	267.66
Feb-D7	4.068	0.986	54.491	76	49	0.355	174.51	152.46
May-A5	4.558	0.977	80.772	133	88	0.338	452.35	379.42
May-B2	4.347	0.968	63.259	125	66	0.472	256.25	215.17
May-D1	4.120	0.963	47.751	95	60	0.368	367.59	293.25
May-D7	4.112	0.968	47.604	87	61	0.299	490.19	331.42
Aug-A5	3.736	0.828	10.689	149	78	0.477	397.66	391.30
Aug-B2	4.606	0.974	83.765	145	94	0.352	489.91	449.23
Aug-D1	3.843	0.846	14.587	155	84	0.458	675.00	675.00
Aug-D7	4.791	0.991	112.978	143	111	0.224	589.69	562.07
Nov-A5	4.566	0.979	83.834	133	88	0.338	415.22	405.46
Nov-B2	4.669	0.980	92.495	142	101	0.289	593.46	537.83
Nov-D1	4.749	0.987	104.602	145	104	0.283	404.89	404.89
Nov-D7	4.337	0.932	35.875	144	90	0.375	492.84	505.50

OTU operational taxonomic units

3.3 Taxonomic identification and phylogenetic analysis

Diverse 16S rRNA gene sequences were recovered from the clone libraries. Although more than half (55 %) of the OTUs had quite high identities (≥ 97 % identity) with known GenBank sequences, most of the nearest neighboring GenBank sequences were originally recovered from uncultured environmental bacteria. About 16.5 % OTUs had quite low identities (< 94 %) with known GenBank sequences, and the remaining OTUs (28.5 %) had moderate (≥ 94 and < 97 %) identities with known GeneBank sequences. Due to low identity with known bacterial sequences of our 16S rRNA gene sequences, these sequences may represent the as-yet-uncultivated novel bacteria in this typical semi-enclosed bay. The RDP classifier was also used to assign 16S rRNA gene sequences (Wang et al. 2007). The benthic bacterial communities in the JZB sediments displayed a highly diversified composition including members from seventeen bacterial phyla and some unclassified sequences (0.85 %). *Proteobacteria* (61.3 % of the total sequences), *Acidobacteria* (9.06 %), *Bacteroidetes* (7.54 %), *Planctomycetes* (5.72 %), *Actinobacteria* (3.65 %), *Verrucomicrobia* (3.04 %), *Chloroflexi* (2.48 %), and *Nitrospirae* (1.22 %) were the rather dominant groups in all samples. Members of *Firmicutes*, *Fusobacteria*, *Cyanobacteria*, *Gemmatimonadete*, *Spirochaetes*, *Elusimicrobia*, *Deferribacteres*, *Lentisphaerae*, and *Deinococcus-Thermus* were also found as minor phyla (ranging from 0.05 to 1 %) in the JZB sediments. Five subdivisions of the *Proteobacteria* presented in our libraries,

including the γ - (32.8 %), δ - (22.68 %), α - (4.15 %), ε - (0.91 %) and β -*Proteobacteria* (0.76 %) (Fig. 3).

3.4 Spatiotemporal distribution of the sedimentary bacterial assemblages

The abundance and distribution of all bacterial assemblages in the 16 libraries, as well as their spatial and temporal variations, are illustrated in Fig. 3. Microbial community experienced more obvious changes in D1 and D7 sites than in B2 and A5 sites. Site D1 exhibited the most diverse microbial communities. The 16S rRNA gene sequences affiliated within the *Proteobacteria* were the most diverse and abundant in all JZB sediment bacteria clone libraries, showing a consistence with typical marine sediment environment. In total, 173, 135, and 38 OTUs were affiliated within the γ -, δ -, and α -*Proteobacteria* subdivision, respectively, representing the three most abundant subdivisions. Although these dominant bacterial communities were present in all the stations studied and throughout the year, in comparison, γ - and δ -*Proteobacteria* were slightly less abundant at the outer bay stations. For example, γ -*Proteobacteria* was less distributed in D7 station, and δ -*Proteobacteria* was less in D1 station, especially in the Aug-D1 sample. α -*Proteobacteria* were rarely observed in A5 stations. On the contrary, the *Acidobacteria* occurred more frequently in the D1 and D7 samples, especially in Nov-D1 sample. The *Planctomycetes* bacteria were also more prevailing in D1 samples. Finally, among the abundant genera, the *Bacteroidetes* were distributed relatively less in A5 and D1 station.

Fig. 2 Rarefaction curves of bacterial 16S rRNA gene clone libraries in the Jiaozhou Bay sediment (samples from four stations in four seasons). The phylotypes were determined with a 97 % similarity cutoff value over 1088 nucleotides

No significant seasonal variation in bacterial community composition could be observed in the four sampling locations. The samples collected in February tended to be the most different (the least in abundance and diversity) from those collected in other months (Fig. 3). The four predominant phyla, *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, and *Planctomycetes*, were widely distributed in all months, and some seasonal differences were still observed. The γ -*Proteobacteria* occurred much more in August than in the other three seasons. The OTU percentage of the γ -*Proteobacteria* was 55 % in the total OTUs of the Aug-D1 samples and was 52 % in the Aug-A5 samples. The δ -*Proteobacteria* as the second largest community member presented more in May samples. The bacteria related to α -*Proteobacteria* could hardly be found in February samples. The *Acidobacteria* were more abundant in November. The *Planctomycetes* bacteria were obviously more abundant in August, and the *Bacteroidetes* were distributed relatively evenly over the temporal scale.

In addition to those predominant bacteria groups, some relatively minor bacterial groups were also observed to shift in response to spatial and temporal variations, indicating different environmental adaptation strategies of various bacteria. For instance, *Chloroflexi* was found in all the stations, but were less in B2 samples. *Gemmatimonadetes* was found in all libraries except the A5 and February samples. *Fusobacteria* lost its occurrence in May. *Elusimicrobia* occurred only in August and only in the two inner bay stations A5 and B2. Members of *Cyanobacteria* occurred only in B2 and D7 clone libraries in November. *Lentisphaerae* occurred only in May and in station D7. *Deinococcus-Thermus* occurred only in August in station D1.

3.5 Community classification of bacterial assemblages

The community assemblages were further compared using the obtained 16S rRNA gene sequences by the online program UniFrac. As shown in Fig. 4, the 16 samples clustered separately according mainly to the different stations, thereby

Fig. 3 Representation of different bacteria distribution and abundance at phylum levels with regard to sampling months and stations. Different colors correspond to the number of samples where the clones have been identified

illustrating a relatively more obvious spatial difference (Fig. 4). Bacterial assemblages of the D1 station appeared to represent the most distinct cluster, and the D1 samples in different seasons were separated in three main clusters. The D7 samples represented the second distinct cluster, in which all its libraries in four seasons were strictly classified into one distinct group. In the remaining stations, nearly all samples from the A5 and B2 station, except for the Feb-A5 and Feb-B2

samples, grouped together and finally formed one main cluster. Unlike the spatial distribution, seasonal variations were less pronounced in the bacterial communities and only slightly affected clustering. The May and August samples seemed to cluster more closely and that the bacterial community structure in February was pretty much different from the other three months. This is in agreement with general observations of seasonality of annual marine microbial communities.

Fig. 4 Dendrogram of the hierarchical clustering analysis of the Jiaozhou Bay bacterial assemblages, constructed using the UniFrac normalized and weighted jackknife environment clusters statistical method

We used CCA to evaluate the effect of environmental variables on the bacterial compositions (Fig. 5). Results of CCA illustrated that the differences in bacterial community between the four sampling stations are not related to any of the environmental factors. None environmental parameters contributed significantly ($P < 0.05$) to variance in bacterial community. The eigenvalues of the first and second axis were 0.518 and 0.474, respectively, and the first two ordination axes explained only 15.8 % of the total variance in the bacteria composition.

4 Discussion

4.1 Bacterial taxonomic diversity in Jiaozhou Bay sediments

As a mixing environment between terrestrial and marine habitats, the coastal area is thought to have a significantly higher diversity of bacterial community than marine sediment (Allen et al. 2009; Zinger et al. 2011). Our results identified 17 bacterial phyla in the sediments of Jiaozhou Bay. To our knowledge, this is among the most diverse coastal sediment habitats in clone library studies so far (Zhang et al. 2008; Feng et al. 2009). The bacterial diversity in this study is also higher than that of our previous DGGE study, in which the bacterial composition in the sediments collected from ten different Jiaozhou Bay stations in four seasons were evaluated. In comparison, several bacterial groups were only found in the current study, such as the *Planctomycetes*, *Verrucomicrobia*,

Deferribacteres, *Lentisphaerae*, *Deinococcus-Thermus*, and *Elusimicrobia*, while the *Bacillariophyta* and *Tenericutes* previously detected by DGGE (Liu et al. 2011) were not found here. For the limited sensitivity of detection of rare community members by DGGE (Vallaey et al. 1997), the absence in DGGE results may be attributed to the variation of species abundance. Moreover, the bacterial species in our current study were more diverse than those in other previous studies, which worked on the same Jiaozhou Bay sampling stations and also via clone library method (Doctoral thesis, unpublished data). Compared to other relevant reports, here we found for the first time the occurrence of *Spirochaetes*, *Nitrospirae*, *Elusimicrobia*, *Deferribacteres*, and *Lentisphaerae* bacteria groups in Jiaozhou Bay sediments (Jing et al. 2006; Bai et al. 2009). This difference in diversity may not only be due to PCR primer coverage and specificity but also may result from some experimental details that might improve the quality of our experiment (Dang et al. 2008c; Li et al. 2010). Indeed, some of the sediment bacterial groups uniquely found in the current study were also detected in previous surface-colonizing bacterial community analyses in Qingdao coastal seawater (Dang et al. 2008a, 2011). In the present study, the Power Soil DNA Extraction Kit was used, which has been demonstrated to be an efficient DNA extraction method for marine sediments. Three tubes of PCR products were pooled to reduce the biases of each individual reaction. The clones of each library were also selected from a single indicator plate to reduce the opportunity of sampling artificially redundant clones (Dang et al. 2009a). Thus, it further proved that the diversity of sediment bacteria in Jiaozhou Bay may have been underestimated in previous studies. For most of the time, it cannot be guaranteed that different methods will produce consistent results, as every approach has its technical advantages and disadvantages (Zeng et al. 2009; Ying et al. 2013). The size of clone library may be an important limit for its resolution (Sogin et al. 2006). Advances in sequencing technology are expanding our view of the microbial communities in unprecedented ways. New high-throughput pyrosequencing methods are increasing dramatically the number of sequences, allowing deeper coverage and improving our statistical power as well (Wang et al. 2012; Hu et al. 2013). A combination of more advanced methods may provide a comprehensive and complementary view in understanding of the marine sediment bacteria community.

Among the three predominant bacterial groups in our study, most of the Jiaozhou Bay sediment bacterial sequences had their closest matches originally detected in marine surface sediments and estuarine (Table S1, Electronic Supplementary Material), especially from the Yellow Sea, the Suez and Tokyo Bay, and the heavy metal contaminated marine sediments, indicating a significant similarity of the bacterial communities in these sediment environments. Our results here not only reflected a specific relationship between Jiaozhou Bay and

Fig. 5 Multivariate statistical analysis of correlations between environmental variables and distribution of bacteria community by CCA

the Yellow Sea but also supported the hypothesis of environmental control on the composition and distribution of microbial communities, especially the key bacteria groups. In comparable environments, similar bacterial communities might exist (Dang et al. 2009b).

4.2 Factors shaping the bacterial community structure

The UniFrac method combined the advantages of the molecular evolutionary information and the traditional ecological studies (Lozupone et al. 2007). In the present study, both the UniFrac environmental clustering (Fig. 4) and the composition of bacterial community (Fig. 3) revealed considerable heterogeneity of the sediment bacterial community in the four stations. By UniFrac analysis, the bacterial assemblages were divided into four main clusters corresponding to the four stations (Fig. 4). However, in our previous study including ten different Jiaozhou Bay stations in four seasons, the seasonal variation had stronger influence on the bacterial community composition than did the spatial variation in that the bacterial communities clustered according to seasons (Liu et al. 2011). Such season-driven shift in microbial composition was consistent with another bacterial spatiotemporal investigation, in which sediment samples from eight Jiaozhou Bay stations over four seasons were collected (Doctoral thesis, unpublished data). Therefore, it may possibly concluded that, if the whole Jiaozhou Bay areas are under consideration, season might be the driving force in variation of the sediment bacterial community structure under this large scale. While, as far as the four stations in this study are considered, it might be their geographic heterogeneity, containing strong physicochemical and biological gradients, that mainly shapes the benthic microbiomes. On the other hand, the UniFrac clustered mainly by stations, rather than seasons, reflected the distinction of the four sampling sites we chose in this study, and these four stations may symbolize four typical heterotypical environments in the Jiaozhou Bay.

Both our current and previous studies (Liu et al. 2011) showed that the bacterial community in sediments of A5 and B2 firstly grouped into one cluster, reflecting a similar bacterial community structure in these two stations. Similarly, Ren et al. (2006) divided the water bacterial community in Jiaozhou Bay into three distinct classes according to the three geographical types of the sampling locations: the innermost (i.e., station A5 and B2), the outside (i.e., station D7), and the transitional in the middle (i.e., station D1) of the Jiaozhou Bay. On the contrary, Dang et al. (2009b) found that the A5 station could be clearly distinguished from the B2 station based on total sedimentological, geochemical and physicochemical parameters, and the A5 and B2 stations might represent the worst and the best environmental conditions in Jiaozhou Bay, respectively. Their results further suggested that the *nirS*-encoding bacterial assemblages of the A5 and D1 stations

grouped together, showing clearly difference from that of the B2 station. Such differences between our results and theirs might due to the total bacterial community and the functional denitrifying community that the two studies had differently focused on. Hybrid environmental conditions possibly have more complicated influences on the hybrid total bacterial communities than on certain functional bacterial communities. However, Fig. 4 shows that the A5 and D1 samples clustered together in February, which was consistent with the results by Dang et al. (2009b). One explanation may be that both the sea water and the sediment are the cleanest in February in Jiaozhou Bay. Low sea temperature and less active biologic metabolism resulted in a more important influence by pollutions. Thus, the polluted situations caused a similar effect on these two stations, which receive most of the inflow contaminations in the Jiaozhou Bay.

Interestingly, nearly all studies on the microbial communities in Jiaozhou Bay sediment indicated that the bacterial assemblage in the D1 station was the most distinct from other stations (Dang et al. 2009b, 2010a). Our current study here proved one more time the most special features of bacterial structure in the D1 station. An anticlockwise eddy was found in the north of Huangdao at station D1 (Liu et al. 2004), which might make the in situ hydrological conditions more stagnant than those of the other stations in Jiaozhou Bay (Dang et al. 2009b). This may reduce material transport and water exchange between D1 station and the Yellow Sea outside. Station D1 is also the heaviest contaminated station by oil from the nearby crude oil refining plants (Dang et al. 2010a). It was reported that there had been about 208 sporadic oil-spilling events with 5810-t oil leakage from 1974 since the first use of Huangdao oil port started (Jing et al. 2006). Therefore, the unique sediment bacterial assemblage in station D1 (Figs. 3 and 4) might be a result of the most unique in situ hydrological, geochemical, or pollution condition.

There are a variety of environmental conditions that might influence the sedimentological condition and thus the sedimentary bacterial assemblages, including the in situ hydrological regime, such as currents (Hamdan et al. 2013), tides (Gobet et al. 2012), upwelling (Kuyper et al. 2005), lateral transport (Dalsgaard et al. 2005), water mixing and exchange, and the intensity and dynamics of these activities (Dang et al. 2008d, 2013). Certain geochemical background, such as sediment NH_3 , OrgC (Jorgensen et al. 2012) and OrgN (Martiny et al. 2011), mineral (Jorgensen et al. 2012), salinity (Bolhuis and Stal 2011), and pH (Lozupone and Knight 2007); and many sedimentological factors, such as median grain size (Jackson and Weeks 2008) and the electrical conductivity, had also been identified as potentially the key regulators of the bacterial composition, community structure, abundance, and distribution in the marine sediment. Dang et al. (2010a) found that the sedimentological factors dominated the geochemical and physicochemical factors for the distinction of

sediment anammox bacterial community between station B2 and D1 in Jiaozhou Bay, and have significant impacts on the spatial distribution between B2 and the other stations. In this study, none of the present environmental parameters contributed significantly ($P < 0.05$) to variance in bacterial community. The differences in bacterial community are not related to any of those measured environmental factors. The low coverage of clone libraries might be one reason for the complicated CCA results we had got. Rarefaction curves showed that the diversity of our sediment samples was quite high (Fig. 2). Therefore, the diversity suggested by the data was not necessarily representative of the in situ bacteria communities in the sediments (von Wintzingerode et al. 1997). It may be biased to compute statistical correlations between the environmental variables and the bacteria community structure based on libraries with low coverage. Besides, the lack of the environmental parameters of D1 station probably also contributed to the difficulty in pointing out the most influential environmental factor clearly. To gain definite conclusions, more samples, more extensive sequences, and also more complete environmental parameters should be analyzed in future studies.

4.3 Bacterial communities in comparable habits

Microbial environment-specificity indicates that the bacterial community may provide a bioindicator of the shift of the sedimentary physicochemical and geochemical characteristics (Dang et al. 2009a, 2010b). *Proteobacteria*, *Acidobacteria*, and *Bacteroidetes* were present in all stations and all seasons in our study. These phyla have been always identified as dominant groups in sediment environments and were generally important contributors to biogeochemical processes (Durbin and Teske 2011; Wang et al. 2012). *Proteobacteria* were dominant in most surface marine sediments, comprising >50 % of the microbial biomass (Bowman and McCuaig 2003), and it seems that γ -*Proteobacteria* was the most significant clade in marine sediments (Feng et al. 2009; Wang et al. 2012). Global distribution might be an important ecological characteristic of these marine bacteria groups, and many corresponding clusters included might have flexible adaptation to the various marine sediment environments. Most members of δ -*Proteobacteria* are chemoorganotrophs related to the sulfate-reducing bacteria (SRB), which play an important role for sulphur cycling in global marine sediments (Ravenschlag et al. 2000). Besides SRB, potential sulfur-oxidizing bacteria (SOB), which belong to ϵ -, γ -, and α -subdivision of *Proteobacteria* also constitute an important fraction in bacterial communities of marine sediments (Zeng et al. 2009). Similar SOB sequences have also been detected abundantly in the surfaces of submerged carbon steel coupons in Qingdao coastal seawater (Dang et al. 2011). In the eastern part of the Jiaozhou Bay, increasing nutrient input due to intensive agriculture and industry causes serious

eutrophication, which may be related to the high levels of δ - and ϵ -*Proteobacteria* in the A5 station. However, in another contaminated D1 station, a possible explanation is needed for the conflicting result that δ -*Proteobacteria* was found significantly less in Aug-D1 samples. It has been demonstrated that the β -*Proteobacteria* usually constitute a more common fraction in freshwater environment and negatively related to water salinity, as well as they are believed to share the ability to degrade complex organic macromolecules with the *Bacteroidetes* (Eiler and Bertilsson 2004; Feng et al. 2009). In our study, the β -*Proteobacteria* was the least abundant in station D7, which belongs to the outer bay area and meet the least freshwater input. Previous findings have highlighted the importance of *Bacteroidetes*, formerly known as the *Cytophaga*–*Flavobacteria*–*Bacteroidetes* phylum, on submerged surfaces, suspended particles, and during phytoplankton blooms (Eiler and Bertilsson 2004). *Bacteroidetes* has been shown especially abundant in samples derived from *Cyanobacterial* aggregates (Tang et al. 2009). In this study, the higher percentage of *Bacteroidetes* and the only observation of *Cyanobacterial* in B2 and D7 stations might be reasonable.

All the obvious spatial heterogeneity will make the microbe–environment relationship even clearer. If the suite of environmental factors responsible for structuring the Jiaozhou Bay sediment bacterial communities is known, then samples with similar values for these variables would be expected to contain rather similar bacteria communities.

5 Conclusions

Bacterial community composition in sediments of four typical stations in Jiaozhou Bay over four seasons were assessed by clone libraries. Full-length bacterial 16S rRNA gene sequences and the taxonomical analyses showed the existence of extremely diverse bacterial communities in this hypenutrient semi-enclosed bay. UniFrac analysis revealed that the community structures clustered with respect to different stations. Data from this study and our previous work were compared to address this geographic heterogeneity, which might shape the benthic microbiomes. Moreover, Jiaozhou Bay may be affected by multi-dimension anthropogenic activities. Our study provides new information and is a useful indicator for environmental assessment of Jiaozhou Bay and will help to enhance the overall understanding of the microbial ecology.

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