**Ultradeep 16S rDNA sequencing** **analysis of the bacterial communities** **in seagrass sediment and water, Xincun Bay, South China Sea**

**Yufeng Jiang1#, Biao Chen2,3#, Yanying Zhang4,5,** **Juan Ling4,5, Hongyan Sun6, Junde Dong4,5\***

1 Department of Medical Laboratory, Jining No. 1 People's Hospital, Jining, 272111, China;

2 Postdoctoral of Shandong University of Traditional Chinese Medicine，Jinan 210355, China;

3 The Laboratory of Medical Mycology, Jining No. 1 People's Hospital, Jining 272111, China;

4 Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Provincial Key Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China;

5 Tropical Marine Biological Research station in Hainan, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Sanya 572000, China;

6 College of Animal Science, South China Agricultural University, Guangzhou 510642, China.

**#** these authors contributed equally to this study.

\* correspondence: E-Mail: dongjunde@vip.163.com.

Abstract: An intense interest in exploration of bacterial communities’ distribution and function in seagrass habitats has been rising due to the critical role of bacteria in coastal marine chemistry and biogeochemical cycling. In the present study, we molecularly characterized the bacterial assemblages in rhizosphere sediment and nearby water of three seagrass species (*Thalassia hemprichii*, *Enhalus acoroides* and *Cymodocea rotundata*) in Xincun Bay, South China Sea through highthrough sequencing method. The quantity of bacteria and *Synechococcus* were measured by Flow cytometry (FCM). As well, canonical correspondence analysis (CCA) was employed to further investigate the relationship between bacterial distribution pattern and environmental variables. Analysis of sequencing results indicated high abundance of *Gammaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria* in sediment, while a majority of bacteria belonged to *Alphaproteobacteria*, *Gammaproteobacteria* and *Flavobacteria* groups in water. The comparison of bacterial communities composition among the three linked sites implies that bacterial distribution may be structured by multiple factors in terms of oxygen release from the nearby seagrass, the exchange of marine water and the nitrate level. Additionally, the unit quantity of bacteria in water was two orders of magnitude higher of the *Synechococcus* less than the ratio in sediment.

Keywords: bacterial communities; seagrass; *Epsilonproteobacteria* ; South China Sea

1 Introduction

Seagrass habitat, typically in coastal area, is an important ecotone between terrestrial and marine ecosystem. The nutrients concentration of seagrass habitat are often richer than unvegetated regions due to the reduction waves, deposition sediment, and mineralization organic particles([Caffrey and Kemp 1990](#_ENREF_6)，Furman 2019). As well, with the release of oxygen and dissolved organic carbon, higher abundance of bacterial populations are present in seagrass meadows([Caffrey and Kemp 1990](#_ENREF_6), [Miyajima *et al.,* 1999](#_ENREF_35), [Sun *et al.,* 2015](#_ENREF_46), Lima *et al*.,2020).Seagrass habitats are tending to be hot spots in biochemical ecological processes with highly microbial activity and various community assemblages([McRoy and Goering 1974](#_ENREF_34), [Herbert 1999](#_ENREF_24), [Sun *et al.,*2015](#_ENREF_46), Henderson *et al.,*2019). While, bacterial communities in seagrass are quite different temporally and spatially ascribe to the oxygen penetration, the quantity of organic matter and the inorganic nutrient levels([Devereux 2005](#_ENREF_13), [Duarte, Holmer *et al.*, 2005](#_ENREF_14), Trevathan-Tackett *et al.,* 2020). Unravel the factors which influence the bacterial communities has been thriving nowadays. Studies indicate the nutrient availability of phosphorus (P), nitrogen (N), sulfate (S) and organic carbon (OC) were important factors in determining the distribution and community of bacteria in aquatic ecosystem([Jiang *et al.,*2015](#_ENREF_26)a, [Jiang *et al.,*2015](#_ENREF_27)b).

The advent of next generation sequencing method has provided an effective alternative method in identifying the microbial community structure and there is an increasing researches focused on the composition of microorganism in variety ecological scales([Caporaso *et al.,*2010](#_ENREF_9), [Zhang *et al.,*2014](#_ENREF_54)).Further, flow cytometry (FCM) is extremely sensitive in identifying and enumerating fluorescent-labelled cells, which can avoid the needs for cultivation or enrichment and is commonly use to study the ecology of microbial communities in aquatic environments([Shapiro 2005](#_ENREF_43), [Morono *et al.,*2013](#_ENREF_37)). Also, an improved technique for separating microbial cells from marine sediments has been established by Morono([Morono *et al.,*2013](#_ENREF_37)).

In this study, 16S rRNA gene was used for analyzing the bacterial constitution and proportion, as they may varies under the condition of different sites and seagrass species. Also, the unit quantity of the bacteria and *Synechococcus* among the three sites were measure in order to have a scene of their relatively quantity distribution. We also detected several environmental factors for predicting whether they have significant influence on the distribution of bacteria or the bacterial community. The present work aims (1) to depict the bacterial constitution and proportion; (2) to compare the quantity of bacteria in different sites; as well as (3) to explore the relationship between bacterial communities and the explanatory environment variables.

**2 Materials and Methods**

2.1 Sampling and DNA extraction

In the present study, three geographically linked marine sites were selected, each sampling sites have distinctive feature in terms of seagrass diversity, abundance, anthropogenic activities and pollution status as previously described([Jiang *et al.,*2015](#_ENREF_26)a). Ten tripled samples including water, sediments were collected on July 20th, 2013. Filtered water and sediment samples were transported to lab and stored at -20℃. Sediment samples of *E. acoroides* in three sites were labeled with HS1, HS2 and HS3, respectively. Sediments of *T. hemprichii* in three sites were labeled with TS1, TS2 andTS3, respectively. Water samples from three sites were labeled with W1,W2 and W3, respectively. In addition, *C. rotundata* only exist in site 1 and the sediments sample of *C. rotundata* was marked as CS1.

2.2 Environmental parameters

Abiotic environment parameters are the indicators of biochemical ecological processes in seagrass ecosystem. The filtered water samples in bottles were kept homogeneous and analyzed for nitrate (NO3-N/mg. L−1), nitrite (NO2-N/mg. L−1) , silicate ([SiO](http://link.springer.com/search?dc.title=SiO&facet-content-type=ReferenceWorkEntry&sortOrder=relevance)3-Si/ mg. L−1), ammonium (NH4-N/mg.  L−1) and phosphorus (PO4-P/mg. L−1) with Spectrophotometer according to “Specification for Oceanography Survey” (GB/T 12763.4-2007, China). The intertidal sediment were analyzed for the environmental variables including total carbon (TC/%), total nitrogen (TN/%), total phosphas (TP/%), available soil potassium (AK/mg·kg-1), nitrate (NO3-N /mg·kg-1), nitrite (NO2-N/mg·kg-1) and (NH4-N/mg·kg-1) according to Bao (1999)([Bao 2000](#_ENREF_2)).

2.3 Bacteria and *Synechococcus* counting by FCM

Water samples were fixed with formalin in a final concentration of 2%. About the sediments, 0.1 ml sediments samples were dissolved into 1.5 ml sterile seawater, then 30 ul formalin was added. For efficient extraction the samples were sonicated for 3 minutes, interrupt for 30 seconds and then centrifuged at 800 rpm for 1minute([Danovaro and Serresi 2000](#_ENREF_12), [Duhamel and Jacquet 2006](#_ENREF_15)). Bacterial samples were stained with the SYBR Green I (Molecular Probes Inc.,USA). FCM analyses were performed with a FACS flow cytometer (Becton Dickinson, San Jose, USA) equipped with standard filter setup. The FCM instrumentation and methodology followed by Pan (2007)([Pan *et al.,*2007](#_ENREF_39)).

2.4 DNA extraction, Bioinformatic analysis

The DNA from the water samples was extracted by the recommended method of Ling([Ling, Dong *et al.,*2012](#_ENREF_29)), and DNA from sediment samples were extracted with E.Z.N.A Soil DNA (OMEGA, USA) according to the manufacturer instruction. The extracted DNA was diluted in TE buffer. Triplicate samples were mixed and quantified.

Bacterial V4 hypervariable region of the 16S ribosomal RNA genes was amplified, based on the PCR primers F515: GTGCCAGCMGCCGCGGTAA and R806: GGACTACHVGGGTWTCTAAT. Adapter sequences and barcode sequences were added for multiplexed sequencing([Caporaso, Lauber *et al.,*2012](#_ENREF_10)). Parallel tagged sequencing was performed using a Miseq Benchtop for 2 × 150 bp paired-end sequencing (Illumina, San Diego, USA). Data preprocessing was performed mainly with software (<http://ieg.ou.edu/>). The operational taxonomic units (OTUs) were classified using Uclust at 97% similarity level. The OTUs that were only present in a single sample were removed. The taxonomic assignment was conducted by RDP classifier (release 5.0)([Wang *et al.,*2007](#_ENREF_50)). All Illumina sequence data were submitted to the NCBI Sequence Read Archive (SRA) under accession number SRP064563.

Less new sequences were detected, indicating a good coverage of each library to estimate the sequence composition. The diversity index were calculated by PAST3([Hammer *et al.,*2009](#_ENREF_23)). Multivariate data analysis software Canoco 4.5 was generated to link the variables within the samples taxonomic to the explanatory environment variables([Braak and Šmilauer 2012](#_ENREF_5)).

**3 Results**

3.1 Diversity Index

The diversity index of bacteria in sediments was much higher compared with water(Table 1). The values of the Shannon index were ranged from 3.385 to 3.45 in water, while they were ranged from 4.963 to 6.692 in sediment. The Simpson index showed the sediment had a higher diversity than water. The Shannon index was ranged from 5.86 to 6.208 in *E. acoroides*sediment, while it was ranged from 4.963 to 6.528 in *T. hemprichii* sediment.

3.2 Taxonomic assignment at Phylum level

Targeting the hypervariable V4 region, a total of 213232 reads were recovered through quality filtering and clustered into 4888 different OTUs. The numbers of qualified reads were ranged from 17080 to 22379 within sediment samples, while they were ranged from 22472 to 28939 within water samples. These qualified sequences could be assigned into 26 phyla and 54 classes.

Table 1. Numbers of sequences, OTUs and diversity estimates

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The top nine predominant phyla observed in all sediment samples were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria*, *Chloroflexi*, *Euryarchaeota*, *Actinobacteria*, *Deferribacteres* and *Verrucomicrobia*. *Proteobacteria* was the major group, with an average of 61.54%. Additionally, high abundance of *Bacteroidetes* was detected in HS1 compared with CS1 and TS1. Site 3 has the lowest percentage of *Firmicutes*, which was much rarer detected in TS3. *Spirochaetes*, *Chlorobi*, *Cyanobacteria*/*Chloroplast*, WS3, *Planctomycetes*, *Crenarchaeota* and *Fusobacteria* were ubiquitous in sediment with less abundant (<1%).

In water, the top eight abundant phyla were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Cyanobacteria*/*Chloroplast*, Tenericutes, *Planctomycetes,* *Firmicutes* and *Verrucomicrobia*. *Bacteroidetes* was more predominant in W1 (35.16%) and W2 (35.00%) compared to W3 (19.75%).In W2, the spike of *Cyanobacteria*/*Chloroplast* (4.41%) resulted from an increasing of both *Cyanobacteria* and *Chloroplast*. The proportion of *Tenericutes* was nearly 0.60-2.29% in water. The SR1, OD1, WS3, *Chlorobi*, *Nitrospira*, *Chlamydiae*, *Deinococcus*-*Thermus*, *Chloroflexi* and *Fusobacteria* were rarely detected phyla in water.

3.3 Taxonomic assignment at class level

At class level, the main groups of all sediment samples were *Deltaproteobacteria*, *Gammaproteobacteria*, *Epsilonproteobacteria*, *Acidobacteria*, *Alphaproteobacteria.*, *Flavobacteria*, *Clostridia*, *Actinobacteria*, *Sphingobacteria*, *Anaerolineae* and *Deferribacteres*, which were accounted for 92.41%-97.23% of the bacterial sequences(Fig. **1**). The predominate *Deltapoteobacteria* was ranged from 16.75% to 38.19% form all sites, and TS3 maintain the least percentage of it. The average of *Gammaproteobacteria* was 12.36% and much was detected in *T. hemprichii* in contrast to *E. acoroides* and *C. rotundata*. *Epsilonproteobacteria* was diverse with an average of 11.45%, and it reached 47.52% in TS3. However, classified reads affiliated with *Alphaproteobacteria* were abundant group in site 1 compared to site 2 and site 3. *Flavobacteria*, *Clostridia*, *Actinobacteria*, *Sphingobacteria*, *Anaerolineae* and *Deferribacteres* were found at high frequency in *E. acoroides* compared to *T.hemprichii*.

The predominant phyla and its proportions were variety among different water samples. As it was observed *Alphaproteobacteria* were ranged from 28.12% to 34.68% and *Gammaproteobacteria* from 16.84% to 32.64%. *Flavobacteria* were predominant in W1(30.61%) and W2(31.42%) compared to W3(14.03%). Additionally, *Actinobacteria* was a major group in W1(11.71%) and less found in W2(7.81%) as well as in W3 (4.98%). Compared with sediments, less classified reads affiliated with *Deltaproteobacteria* (0.22%-1.07%), *Epsilonproteobacteria* (0.30%-1.15%) and *Acidobacteria* (0.01%-0.05%) were obtained in water. A total of 1.90%-4.25% and 0.60%-2.29% of sequences were related to *Cyanobacteria* and *Mollicutes* were exist in water, respectively.

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Fig. 1 Relative abundance of the dominant bacterial phyla in the ten samples

3.4 Unit quantity of bacteria and *Synechococcus*

FCM was used to analyze the unit quantity of bacteria and *Synechococcus* in sediment and water. The bacteria were in the same magnitude among all sediment samples with the quantity of bacteria was highest in S2 (2.72× 106), compared to S1(1.87 × 106) and S3(1.23 × 106). In water, W1 showed the highest concentration of bacteria which could reach 4.34 × 106 compared with less bacterial were detected in W2(1.76× 106) and W3(1.16× 106).

However, the unit quantity of bacteria was two or three orders of magnitude much than *Synechococcus* in water and sediment separately. Much quantity of *Synechococcus* were detected in W1(5.92 × 103) compared with W3(1.24 × 103). Few amount of *Synechococcus* in S2 (3.04 × 102) and they were rarely found in site 3(0.70 × 102) of sediment samples. In total, the number of bacteria was nearly 102 times of *Synechococcus* in water compared with the value about 103 times in sediment.

*3.5.Environmental parameters and bacteria distribution analysis*

The highest concentration level of TC,TP, TN, nitrate and k+ were found in S1. The dissolved ammonium and DIN were found at high amount in site 2 sediment (S2), and nitrite in the mouth of the Bay (S3) was higher than inside of the Bay (Table 2, 3). There were different characteristic of nutrient level in water samples, W1 was featured with high level of nitrite, nitrate, ammonium, DIN and phosphate.

**Table 2** Physicochemical parameters of sediment in Xincun Bay

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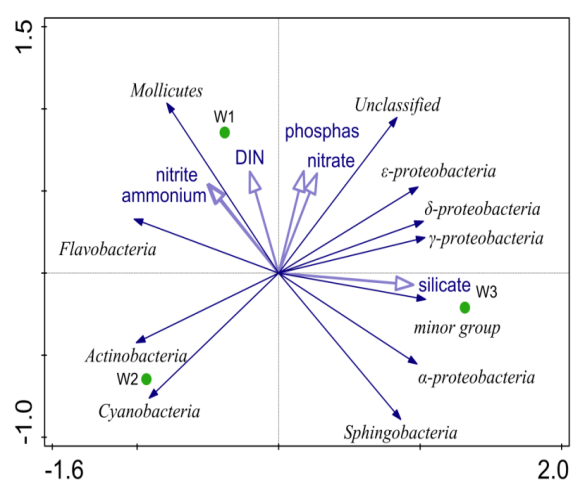
**Table 3** Physicochemical parameters of water in Xincun Bay

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The constrained ordination redundancy analysis (RDA) method was used for describing the relationship between the bacteria and environment factors. RDA figure showed the eigenvalues for the first two multivariate axes which were 0.84 and 0.16 in sediment samples(Fig. 2). While Axes 1 and 2 were found to explain 84.1 and 15.9 of the overall variance (100) in water samples, respectively(Fig. 3). We found in sediments samples, *Gammaproteobacteria* had a strong positive correlation with DIN and ammonium, while, nitrate, TC and TN was positive related with *Alphaproteobacteria. Epsilonproteobacteria* was less correlated with nitrite and ammonium. In water samples, nitrate, phosphas and silicate were environmental factors influencing the distribution and community of the *Epsilonproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria* .

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**Fig. 2** Redundancy Analysis (RDA) diagram illustrating the relationship between the main bacterial community structure (>1%) at class level from sediment samples and environmental variables(the feint arrows are represent the environment factors, the solid arrows are represent the bacterial species and green dots represent the samples)



**Fig. 3** Redundancy Analysis (RDA)）diagram illustrating the relationship between the main bacterial community structure (>1%) at class level from water samples and environmental variables(the feint arrows are represent the environment factors, the solid arrows are represent the bacterial species and green dots represent the samples)

**4 Discussion**

4.1Biogeographical distribution of bacterial communities

A comparison of the bacterial diversity in different seagrass species sediment and ambient water showed the average of Shannon index and the bacterial constitution were variety. Even among the same seagrass species the bacterial communities was quite difference, which was consistent with the study about the bacteria in the Mediterranean *Posidonia oceanic* seagrass habitats, where environmental heterogeneity influenced the structure of prokaryotic assemblages and biodiversity patterns([Lunab*et al.,*2013](#_ENREF_33), Flavia *et al.,*2019).

Interestingly, the abundance of *Epsilonproteobacteria* in water and sediment was positive correlated with the level of nitrate. *Epsilonproteobacteria* has a capacity involved in multiple biogeochemical processes, such as carbon, nitrogen and sulfur cycles([Gupta 2006](#_ENREF_21)). *Campylobacterales* was the second most abundance of *Epsilonproteobacteria* and can be detected in seagrass and mangrove, members of this order are important player in the process of sulfide-oxidization and denitrification in marine environment([Campbell *et al.,*2006](#_ENREF_7), [Zhang *et al.,*2009](#_ENREF_53)), they may active involved in the nitrite cycling of seagrass ecosystem under the condition of high level of nitrite. Thus, the proportion of the *Epsilonproteobacteria* in seagrass sediment may depending on the nitrate level.

The redundancy analysis showed the level of TC, nitrate and TN were positive related with the distribution of *Alphaproteobacteria.* *Alphaproteobacteria* bacterial populations(*Rhizobiales*  and *Rhodospirillales*) in sediment including nitrogen fixation, organic matter decomposition, and plant growth promotion populations, and they are necessary roles in marine biogeochemistry([Im *et al.,*2006](#_ENREF_25)). Correspondingly, the distribution of *Alphaproteobacteria* might be driven by high level of TC, nitrate and TN.

*Flavobacteria* were mainly feathered with aerobic, and were found at high frequency in *E. acoroides* compared to *T. hemprichii* ([Reichenbach 1992](#_ENREF_42)). This shift might be explained in terms of low levels of oxygen and DOC released from *T. hemprichii* both in sediment and water, as the seagrass often possess oxygen diffusing from an internal oxygen source, and the photosynthetic production of oxygen taking play in the leaves during daytime ([Armstrong *et al.,*1996](#_ENREF_1), [Stapel 1997](#_ENREF_45), [Teske *et al.,*2000](#_ENREF_47), [Pedersen *et al.,*2004](#_ENREF_41), [Borum *et al.,*2005](#_ENREF_4), [Teske *et al.,*2011](#_ENREF_48)). *C. rotundata* has been found to release oxygen from the roots, with the concentration up to 75%, and the DOC released from the roots nearly 3 times of the *T. hemprichii*([Stapel 1997](#_ENREF_45)). Thus, *Flavobacteria* around *C. rotundata*, *E. acoroides* and *T. hemprichii* were inclined to be shaped by the unmeasured factors, such as oxygen and organic root exudates.

The minima of bacteria was appeared in the mouth of the Bay, which is similar to the Liu's reports([Liu *et al.,*2003](#_ENREF_32)). Seaward site 3, as reactor that regulates and modifies natural and anthropogenic materials transferred from the continents to the open sea and imposed by high turbidity so the most abundance of bacteria does not occur in this region([Chen *et al.,*1999](#_ENREF_11), [Gao and Song 2005](#_ENREF_19)).The bacteria can be affected by the nutrient level and seagrass hydrodynamics properties([Pan, Zhang *et al.,*2007](#_ENREF_39)) and which may critically related to their inhabiting environment([Wang *et al.,*2012](#_ENREF_51))**.**

4.2 Comparisons of microbial quantity in different sites

Our study showed the total suspended bacteria was two orders of magnitude higher than those of *Synechococcus* in water which is concord with the study in the Changjiang river mouth which featured with the interface between freshwater and seawater ([Pan *et al.,*2007](#_ENREF_39)). As bacteria has been reported to be dependent on dissolved organic substrates produced by phytoplantkton( [Liu *et al.,*2004](#_ENREF_30)), so there is a positive correlation between bacteria and *Synechococcus*. In sediment, we found the dissolved bacteria was three orders of magnitude higher of *Synechococcus* in coastal marine seagrass ecosystem, which was rarely reported before.

Least bacterial communities were recorded in Site 3, which are comparable to previous findings that least quantity of bacterial communities were detected in here([Jiang *et al.,*2015](#_ENREF_27)b). In another study carried out by Campbell that bacteria have low abundance in transition zone([Campbell and Kirchman 2013](#_ENREF_8)). This may induced by the exchanging of water near the site and anthropogenic activities.

As seagrass root exudates, fallings and phytoplankton debris provides organic matter in sediments was readily used by bacteria and a relative fraction was retained in sediments([Thayer, Kenworthy *et al.,*1984](#_ENREF_49)).The C/N ratio was 11.3 on average in unvegetated sediments, whereas the organic matter in Xincun sediment (10.2-21.4) showed a higher proportion([Gacia and Duarte 2001](#_ENREF_18), [Duarte, Holmer *et al.,*2005](#_ENREF_14)) and TC/TN ratio of S2 (21.4) was higher than S1 (18.4) and S3 (10.2). Correspondingly, the relative abundance of the bacterial and *Synechococcus* was higher at the sediment of high TC/TN ratio and medium compared to the low one. This finding may suggest that the bacterial abundance was related to the sediment C/N ratio, however, the factors involved in bacterial distribution patterns within a small scale may not be necessarily important in the large scale.

4.3 Comparison of microbial composition

Consistent with previous reports, *Gammaproteobacteria* was ubiquitous in coastal seagrass water and sediment, with its ability to oxidize thiosulfate and probably also other reduced sulfur compounds([Teske *et al.,*2000](#_ENREF_47)). Sulfide nutrient were deposit inside the habitat by the mineralization and transportation through waves and currents in the coastal areas. Further, *Gammaproteobacteria* and *Deltaproteobacteria* which hosts most sulfate-reducing bacteria in sulfate cycling were predominated in seagrass sediment, and more researches are need to confirm their function.

*Firmicutes* and *Actinobacteria* were better competitors in natural environments([Pandey *et al.,*2013](#_ENREF_40)). *Firmicutes* extensively exist in seagrass sediment as one kind of producers of enzyme inhibitors and antibiotics. *Firmicutes* act like cell factories hidden in deep sea, now they were beginning to be appreciated for the precious natural products([Haefner 2003](#_ENREF_22)).Ultimately, our understanding about the distribution and relative abundance of *Firmicutes* in sediment and nearby seawater will accelerate the exploring of marine resources with the background information.

*Methanobacteria* and *Methanomicrobia* are methanogens([Embley and Finlay 1994](#_ENREF_16), [Yu, García-González *et al.,*2008](#_ENREF_52)), which could be detected in seagrass sediment and involved in the carbon cycle through methanogenesis, as they are solely dependent on methane and methanol for survival and cannot catabolize acetate, formate and dimethyl sulfide([Boone *et al.,*1993](#_ENREF_3), [Singh *et al.,*2005](#_ENREF_44), [Mochimaru *et al.,*2009](#_ENREF_36)). *Halobacteria* tend to live in high salinity environments, which could be found in sediment. *Thermoplasmata* were acidophilic and also exist in sediment samples, which contains the function species involved in iron and sulfur cycling([Golyshina 2005](#_ENREF_20), [Oren 2006](#_ENREF_38), [Falb *et al.,*2008](#_ENREF_17)). High abundance of Archaeal communities exists in seagrass sediments, while it was rarely reported before.

In general, coastal seagrass ecosystem showed a unique pattern of bacterial distribution and structure in sediment and water shaped by environmental heterogeneity and seagrass species in Xincun Bay. The relation among bacterial communities, the seagrass nutrient concentrations and the coastal physiochemical characteristic along the coastal Bay areas in tropical areas can be attractive and requires further investigation.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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