THE INFLUENCE OF EELGRASS (*ZOSTERA MARINA* L.) ON THE ENVIRONMENTAL FACTORS AND THE MICROBIAL COMMUNITIES IN SEDIMENT FROM SHUANGDAO BAY OF CHINA

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Abstract. *Zostera marina* is a perennial seagrass that is widespread on temperate coasts around the world. Some studies have showed that the presence of *Z. marina* in marine environments influences the microbial communities in sediment collected from Shuangdao Bay of China. To investigate the impact of *Z. marina*, the environmental factors and microbial communities in sediments collected in the eelgrass beds and adjacent unvegetated area, were examined. Several environmental factors and microbial communities composition differed significantly between the two areas. The content of total-phosphate and NH_4^+-N in the sediments of eelgrass beds were lower than the sediments of the unvegetated area, while the bacterial abundance and diversity in eelgrass bed were greater than that of unvegetated area. The most abundant bacteria found in sediments were Proteobacteria, Bacteroidetes, Planctomycetes and Acidobacteria, while the bacterial community structures were significantly different between the two areas. The changes found in the environmental factors and microbial communities were mainly attributed to the presence of *Z. marina*. This study may provide further understanding of the effects of *Z. marina*, which may be vital to the regulation of the marine environment.
Keywords: *Zostera marina, environmental factors, microbial communities, marine sediments, marine environment*

Introduction

Seagrasses are a unique group of plants that comprise 60 monocotyledonous angiosperm species (Hemminga and Duarte, 2002) that grow in coastal sediments in shallow coastal zones (Fonseca, 1998; Lebreton et al., 2012). *Zostera marina* is one of the most common seagrasses and distributed mainly in temperate regions of the northern hemispheres (Short et al., 2007; Zhou et al., 2015). Eelgrass meadow can provide a valuable habitat and food source for many invertebrates and fishes (Gillanders, 2006).

In recent years, many studies on *Z. marina* have focused on its biological processes that are related to photosynthesis, nitrogen fixation and restoration (Goodman et al., 1995; McGlathery et al., 1998; Marion, 2010). Eelgrass link sediment and nutrient cycles providing several vital coastal ecosystem functions (Harlin et al., 1981), so their protection and restoration are important. Many bacterial species found in the sediments of eelgrass bed are absent in sediment habitats outside of eelgrass bed (Campbell et al., 1990; Shieh et al., 1997). Several studies showed that bacteria inhabit the roots of the eelgrasses (Kuo et al., 1981; Kurtz et al., 2003; Küsel et al., 1999; Nielsen et al., 1999) and may form synergistic relationships. Eelgrass can excrete amino acids and sugars from their roots, thereby create suitable niches for a wide variety of microorganisms in the rhizosphere (Lee et al., 2000; Holmer et al., 2001; Jensen et al., 2005). Double

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gradient denaturing gradient gel electrophoresis (DG-DGGE) was used to investigate several factors' impact on bacterial community diversity, and the results suggests that vegetation and season exert stronger controls on microbial community structure (James et al., 2006). Jensen et al., found that eelgrass can affect microbial processes in the rhizoplane via the excretion of amino acids and sugars. Significant differences in the bacterial communities associated with the roots and the bulk sediment associated with *Z. marina* have been found (Jensen et al., 2007), and the presence of roots could favour the growth of particular bacteria. Few studies to date have examined the effects of the eelgrass meadow on the environmental factors and bacterial communities.

In this study, we used a variety of methods to measure the environmental factors in sediments collected from eelgrass meadow and unvegetated area. We then characterized the richness, diversity, similarity, and composition of the microbial communities. In addition, root-specific bacteria were also analysed.

Materials and methods

Study area and sampling

Shuangdao Bay is located in Weihai, which lies within the Shandong Peninsula in northern China (N37°28.5′-37°29.2′, E121°57.5′-122°58.5′). The sample S1 (N37°29.087′, E121°58.267′) was collected from the sediments in eelgrass meadow, while sample S2 (N37°29.045′, E121°58.286′) was collected from the sediments in adjacent unvegetated area (*Fig. 1*). S0, S40, and S80 were collected from the sediments of a single plant. The sediment taken from the root of *Z. marina* was designated S0 (N37°29.073′, E121°58.306′), and the sediments taken from 40 and 80 cm away from the root were designated as S40 and S80, respectively. The S40 and S80 samples are a mixture of four samples taken from the circumference of the sampling zone. Samples were collected in April 2015.

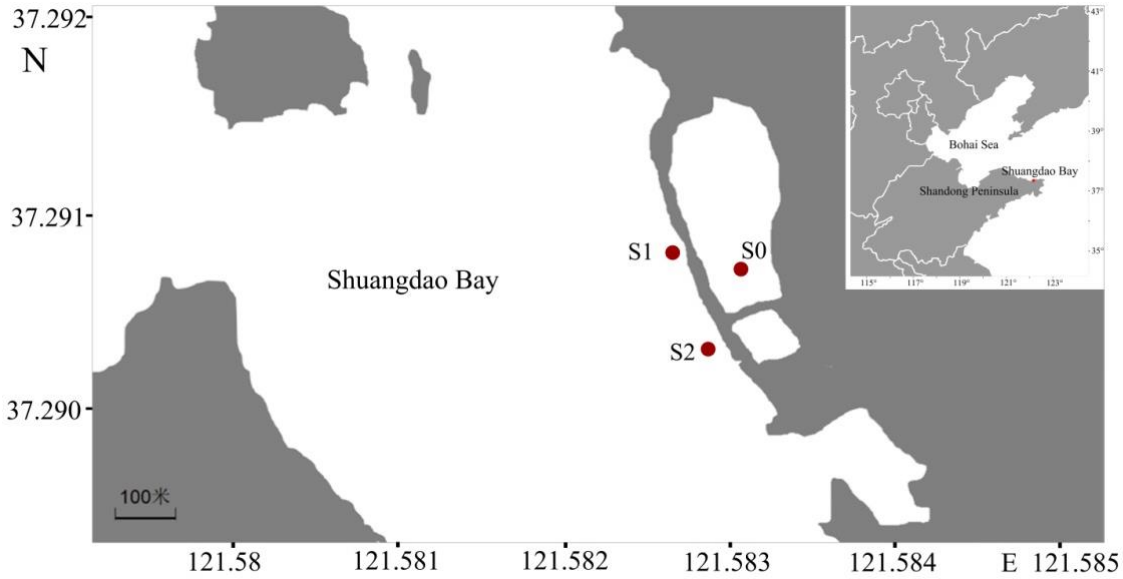

Figure 1. *Sampling area in Shuangdao Bay, Shandong Peninsula, China. Sediments were sampled in eelgrass beds (S1) and in adjacent unvegetated area (S2)*

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In each sampling location, a composite sediment sample was collected at a depth of approximately 0–10 cm using a bottom sampler. Each fresh composite sample was subdivided into two subsamples, one of which was stored at -80 °C prior to bacterial 16S ribosomal ribonucleic acid (rRNA) gene sequencing, while the other was freeze-dried and tested to determine the levels of elemental factors that included salinity and nutrition factors such as sediment organic matter (SOM), total-nitrogen (TN), total-phosphate (TP), NO_3^-, NO_2^-, NH_4^+, PO_4^{3-} and water-soluble sulfide (WS).

Determination of environmental parameters in sediment

The contents of SOM and WS were determined by using the muffle furnace heating methodand $BaSO_4$ turbidimetry (Lv et al., 2018), respectively; three replicates were performed for each measurement. The TN/TP extract solution was obtained using a simultaneous preparation method (Li et al., 2007) and the contents of TN and TP were determined by using zinc cadmium reduction and the phosphomolybdenum blue method, which utilizes the reduction of ascorbic acid for specific ions according to the oceanographic survey GB17378.5-2007, respectively. NO_3^- was determined using zinc cadmium reduction, while NH_4^+ was detected using the indophenol blue method described in GB17378.5-2007. One-way ANOVA (Wolfram Mathematic 11.3) was used and the LSD-t tests were performed to compare TN, TP, WS, SOM, NH_4^+ and NO_3^- between different samples. Differences were deemed significant at $P < 0.05$ and extremely significant at $P < 0.01$. Values are means ± SD ($n = 3$).

DNA extraction, clone library construction, PCR amplification and high-throughput sequencing

The PowerSoil DNA Isolation Kit (TineGene Limited Company, Shanghai) was used to extract the genomic DNA. The DNA library was established using a two-step PCR amplification method, and the sequencing was conducted on an Illumina Miseq 16S rDNA system. The PCR primer sequences used to amplify the V4-V5 region within the bacterial DNA are shown in *Table 1*. The 16S rDNA sequences of S1 and S2 have been deposited in the NCBI Sequence Read Archive under accession number PRJNA506488. The 16S rDNA sequences of S0, S40 and S80 have been deposited in the NCBI Sequence Read Archive under accession number SRP158072.

Table 1. *The PCR primers sequences for amplifying bacterial DNA*

Primer name		Sequence (5'-3')
16SrDNA (1st amplification)	515F	AATGATACGGCGACCACCGAGATCTACACNNNNNNNNTCTTTCCCTAC ACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA
	926R	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCCTT GGCACCCGAGAATTCCACCGTCAATTCMTTTGAGTTT
16SrDNA (2nd amplification)	515F (Inner)	TTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA
	515F (Outer)	AATGATACGGCGACCACCGAGATCTACACNNNNNNNNTCTTTCCCTAC ACGACGCTC
	926R (Inner)	GAGTTCCTTGGCACCCGAGAATTCCACCGTCAATTCMTTTGAGTTT
	926R (Outer)	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCCTT GGCACCCGAGA

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Data analyses

Paired-end reads were assigned to each sample based on their unique barcode and were truncated by cutting off the bar code and primer sequence. Split sequences for each sample were merged using FLASH V1.2.7 (Magoc and Salzberg, 2011), and the splicing sequences were called raw tags. Quality filtering on the rawtags was performed under specific filtering conditions to obtain the high-quality clean tags according to the QIIME quality-controlled process (Caporaso et al., 2011). The tags were compared with the reference database using UCHIME algorithm (Edgar et al., 2011; UCHIME, 2011) to detect chimera sequences, and then the chimera sequences were removed, finally obtaining the effective tags. Using the USEARCH and Perl scripts, the sequences retained were analyzed following the UPARSE pipeline to generate an operational taxonomic unit (OTU) table and pick representative sequences (Edgar, 2013). The sequences with a length shorter than 200 bp and singletons were removed. The retained sequences were were aligned with the 16S rDNA sequences using Silva 119 (http://www.arb-silva.de/) in order to annotate the species based on 97% sequence similarity using the average neighbour method. Taxonomical assignments of OTUs were executed using MOTHUR 1.33.3. OTU abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences. Rarefaction analysis was performed to compare the relative levels of OTU richness across all sediment samples at an OTU cutoff of 0.03 (Xu et al., 2017). Principal components analysis (PCA) of the PCORD5 is used to examine the relationship between the microbial community (phyla) and environmental factors. All data are tested for normal distributions before ANOVA. Figures are prepared by PCORD5.

Results

Determination of environmental factors in sediment

Compared with S2, the contents of TP (LSD-t = 113.12, $p < 0.01$) and NH_4^+ (LSD-t = 7.41, $p < 0.01$) in S1 were significantly reduced, while the contents of other factors have no significant differences (*Fig. 2*). To explore whether these differences were caused by the presence of eelgrass, we examined these environmental factors in the sediment near its roots (S0), as well as 40 cm (S40) and 80 cm (S80) from the roots. The contents of NH_4^+ (LSD-t = 0.007825, $p < 0.05$) and TP (LSD-t = 0.02093, $p < 0.05$) in S0 were lower than that of S40 and S80 (*Fig. 3*). In conclusion, the sediment in eelgrass meadow contains lower levels of TP and NH_4^+ than that of unvegetaed area.

Microorganism richness and diversity

After processing, 33424 and 27009 high-quality sequences were obtained from S1 and S2, respectively. A total of 9640 OTUs were obtained for the samples at 3% dissimilarity (*Table 2*). The abundance indices (Chao and Ace indexes) of S1 were both higher than that of sample S2, while the diversity indices (Shannon and Simpson indexes) were lower than that of sample S2. These results suggested that the bacterial abundance and diversity in the sediment of eelgrass meadow were greater than that of unvegetated area. In the same way, we measured the microorganism richness and diversity in S0, S40 and S80. After processing, 72720, 53733 and 25842 high-quality sequences were obtained from these samples, and a total of 15232 OTUs were obtained

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(*Table 2*). As shown in *Table 2*, the bacteria diversity and richness in the sediment near the roots (S0 and S40) were greater than that of sediment far from the roots (S80).

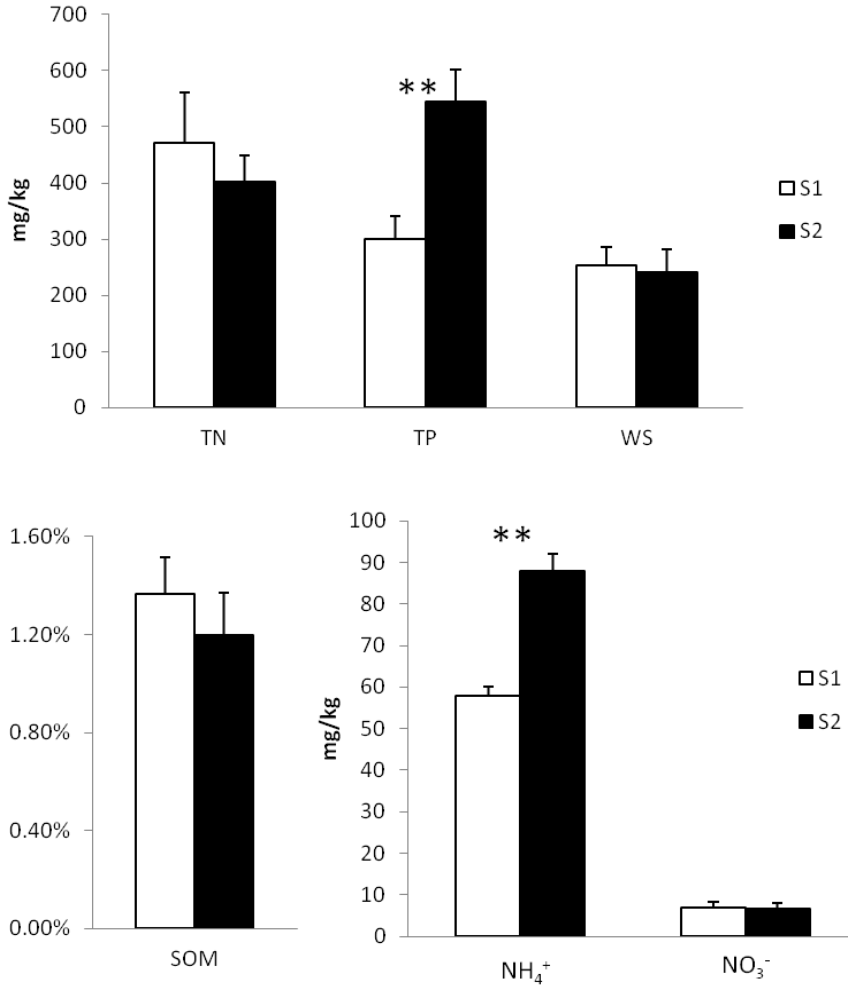

Figure 2. *The change of environmental factors in the sediments. Values are means ± SD (n = 3). *P < 0.01. TN: total-nitrogen; TP: total-phosphate; WS: water-soluble sulfide; SOM: sediment organic matter*

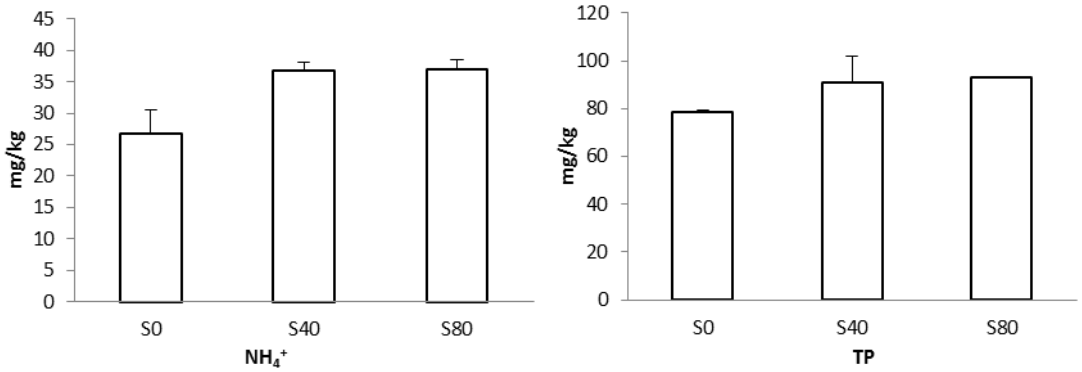

Figure 3. *The contents of NH_4^+ and TP in the S0, S40 and S80. Values are means ± SD (n = 3). * P < 0.05. TP: total-phosphate*

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Table 2. *The community richness and diversity in different samples*

Sample	OTU numbers	Coverage	Chao index	Ace index	Shannon index	Simpson index
S1	5260	84.3	11668	24097	7.05	0.0042
S2	4380	85.8	10315	16090	7.03	0.0036
S0	5735	92.1	11957.1	16241.4	6.9	0.0049
S40	5455	88.3	12717.0	19374.8	7.0	0.0046
S80	4042	90.1	9225.0	14475.2	6.5	0.0067

Comparison of microbial communities among different samples

To assess the variation in the microbial communities, the compositions of taxa at the phyla and class levels are represented in a pie chart (*Figs. 4* and *5*). A significant difference in bacterial community was found between eelgrass beds and adjacent unvegetated area. At the level of phyla (*Fig. 4)*, the most abundant phylum represented within each sample was Proteobacteria, which comprised 48% and 53% of the reads from S1 and S2.

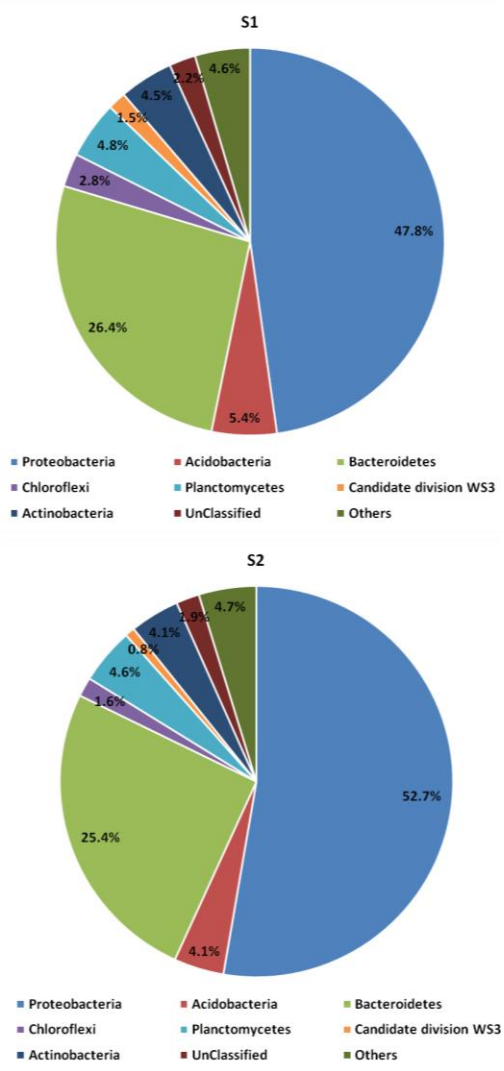

Figure 4. *The diagram for different samples's microbial communities at phylum level*

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The relative abundances of Proteobacteria, Gemmatimonadetes, Cyanobacteria and Chlamydiae were higher in the sediment of eelgrass meadow, while the relative abundances of Bacteroidetes, Firmicutes, Deferribacteres, Planctomycetes and Chlorobi were higher ($P < 0.05$) in the sediment of adjacent unvegetated area. At the level of class (*Fig. 5)*, the relative abundance of Gemmaproteobacteria was higher in the presence of eelgrass, while the relative abundances of Alphaproteobacteria, Flavobacteriia, Sphingobacteriia and Planctomycetacia were higher ($P < 0.05$) in the absence of eelgrass.

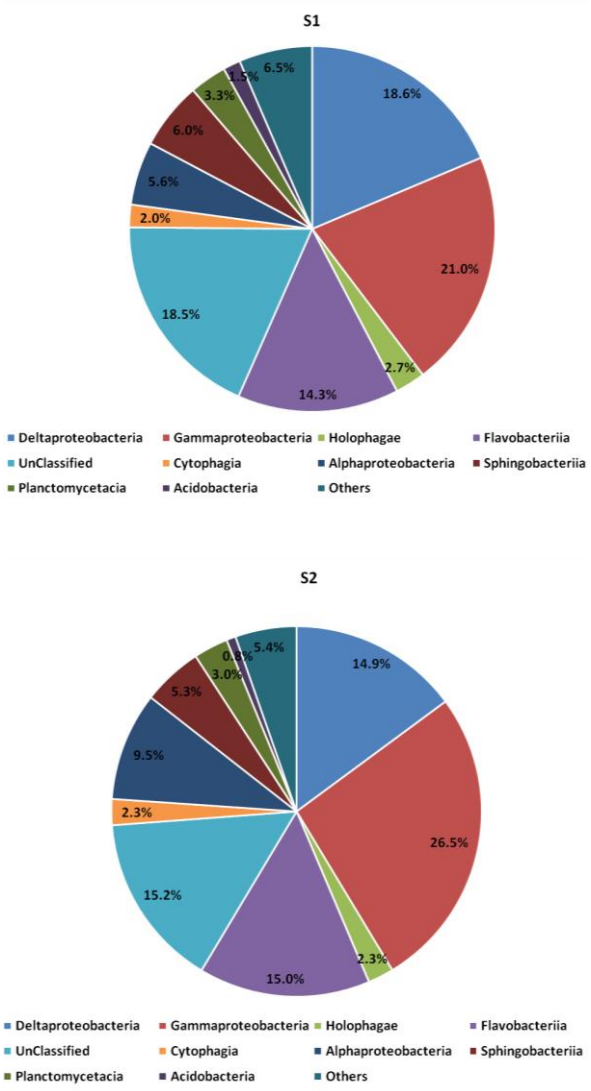

Figure 5. *The diagram for different samples's microbial communities at class level*

Microbial community and environmental conditions

Sediment properties are listed in *Figures 2* and *3*. The sediment in eelgrass meadow had much lower TP and NH_4^+ contents. The PCA analysis showed that PC1 and PC2 explained 56.246% and 18.277% of the bacterial phyla, respectively. The changes of microbial community composition in S1 and S2 were mainly attributed to NH_4^+, NO_3^- and TN (*Fig. 6*).

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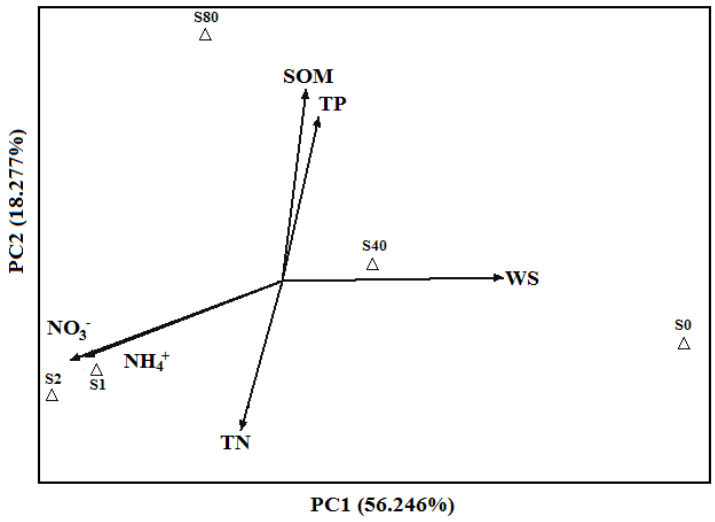

Figure 6. *Principal components analysis (PCA) of the microbial community composition and sediment properties on the basis of the relative abundance of bacterial phyla*

Root-specific bacteria

To obtain the root-specific bacteria of eelgrass, we analysed the OTUs specifically detected in S0. The results showed that there were 2654 unique OTUs detected within the roots of *Z. marina*, and the OTU size was 3464. Among these, 601 OTUs were identifiable at the family level, 302 at the genus level, and 11 at the species level. At the family level, the identified bacteria were Alicyclobacillaceae, Anaerolineaceae, Desulfobacteraceae, Desulfobulbaceae, Flavobacteriaceae, Planctomycetaceae and Spirochaetaceae. At the genus level, the identified bacteria were *Coxiella*, *Desulfococcus*, *Desulfobulbus*, *Acidiferrobacter*, *Geobacter*, *Sulfurovum*, *Rhodopirellula* and *Spirochete*. The eleven identified species were *Bacillus hwajinpoensis*, *Bacillus algicola*, *Brevundimonas bullata*, *Brevundimonas vesicularis*, *Paenibacillus provencensis*, *Roseiovarius aestuarii*, *Labrenzia marina*, *Lewinella nigricans*, *Sphingomonas paucimobilis*, *Ruditapes philippinarum*, and *Stenotrophomonas acidaminiphila*.

Discussion

Influence on environmental factors

The sediment in eelgrass meadow contains low levels of TP and NH_4^+ (*Figs. 2* and *3*). In this study, the content of NH_4^+ was significantly lower in eelgrass meadow which may be due to the vigorous ammonium absorption process in the roots (Hemminga et al., 1994). The contents of the other environmental factors such as organic matter have no significant difference in eelgrass meadow and unvegetated area which is consistent with previous research (Xu et al., 2016). From the above results, we speculate that nitrogen metabolism and phosphorus metabolism in the sediments inside eelgrass meadow are more vigorous than those outside eelgrass meadow, while there is no significant difference in carbon metabolism and sulfur metabolism. These environmental factors are considered to have been influenced by the presence of eelgrass, then influence the growth of particular bacteria.

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Variation in the bacterial community and ecological functioning

The coverage index for all of the samples in this study was estimated to be between 84.3% and 92.1%, which indicated that the results of the high-throughput sequencing most likely encompass most of the microbial organisms in the sediment. The richness and diversity analysis indicated that the bacterial abundance and diversity in eelgrass meadow were greater than in the unvegetated areas (*Table 2*), which may be related to the metabolic cycle or root exudates of *Z. marina*.

Proteobacteria is the most abundant phylum of bacteria in many coastal areas (Hu et al., 2014; Li et al., 2016). In the present study, this phenomenon is laso observed, and the dominance of *Proteobacteria* does not change in different samples (*Fig. 4*). In this study, the microbial community structures were analyzed on the basis of two approaches: eelgrass meadow analysis (*Figs. 4* and *5*) and single eelgrass analysis (*Fig. 7*). The two different approaches did not return similar result. The results suggested that the bacteria communities in sediment were complex. The results indicate that the first approach is more representative of the effect of eelgrass on microbial communities in sediments, as the effects of individual plant is limited.

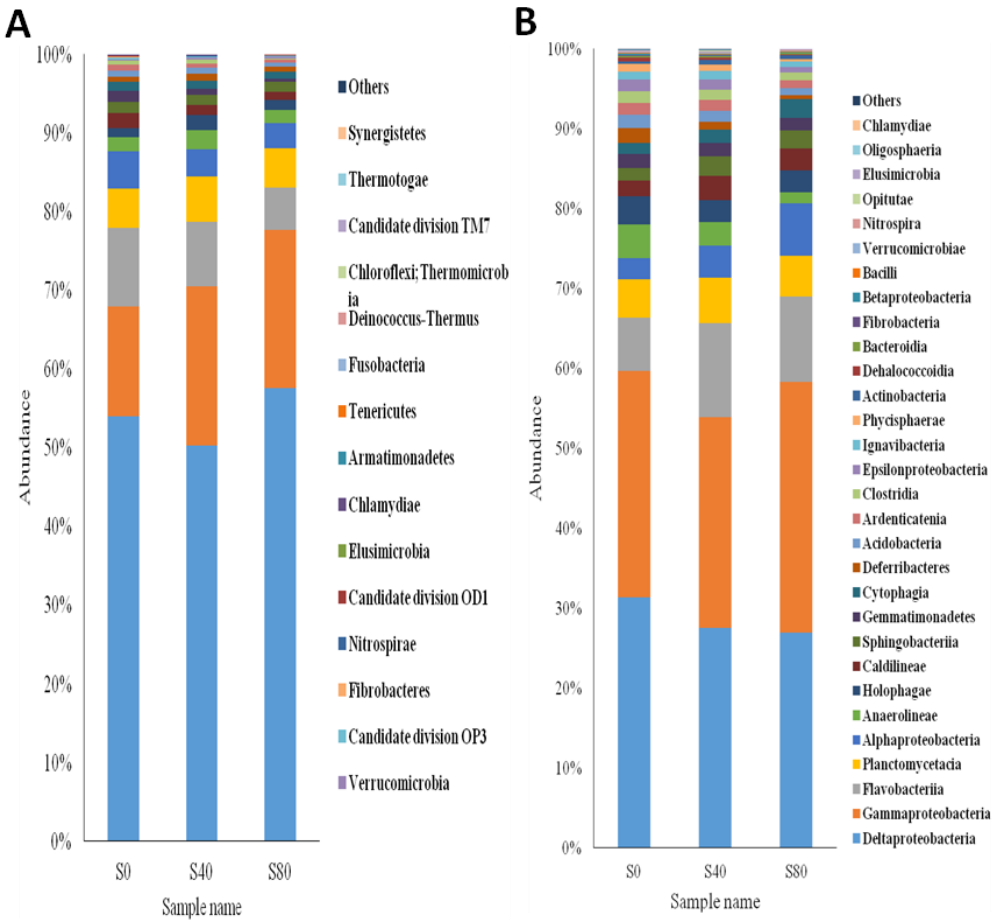

Figure 7. *The diagram for different samples' microbial communities at phylum level (A) and class level (B)*

Nutrient loading was reported to regulate the shifts in microbial structure and diversity in sediment along the river (Wang et al., 2018). Previous studies reported that

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microbial biomass in saline environments was increased by an addition of easily decomposable substrate (Yan and Marschner, 2012), and TN is the main factor increasing the microbial biomass (Li et al., 2016). In this study, the S1 (in eelgrass meadow) and S2 (in unvegetated area) sites had apparently different bacterial phylum structure, which was found to be attributed to NH_4^+, NO_3^- and TN (*Fig. 6*). On the basis of the environmental properties results, we believe that NH_4^+ content in sediment influence the microbial community structure of eelgrass meadow.

Root-specific microorganisms

Root-specific microorganisms were analyzed through single eelgrass analysis. The results showed that there were 2654 unique OTUs detected within the roots of *Z. marina*, while there were 5735 OTUs detected. The result indicated that the microbial community structure and diversity in the area near the roots of eelgrass are significantly different from the surrounding environment. There abundance of *Rhodopirellula* in habitats with eelgrass was lower than that of unvegetated area. As a kind of Planctomycetes, *Rhodopirellula* has an anaerobic ammonium oxidation function (Wecker et al., 2010). It is speculated that the nitrogen cycle in sediments with eelgrass is more vigorous, and the conversion of ammonium is more rapid, which is also consistent with the ammonium content of the sample S1 below the sample S2 (*Fig. 2*). The microorganisms specifically detected in the roots of eelgrass may be closely related to their growth, which reflect the influence of eelgrass meadow on environmental factors and microbial community.

Based on the current and previous studies, we proposed that microbial community composition was closely related to environmental factors which may be influenced by the presence of eelgrass. However, more studies are needed on whether the sediments' microbial community compositions in different areas have the same trend.

Conclusions

Based on the measurements of the environmental factors, the sediments in eelgrass meadow have low levels of NH_4^+ and TP. The abundance and diversity of the microorganisms in the sediment with eelgrass were greater than in the sediment without eelgrass. In addition, significantly differences were observed in the microbial community composition between eelgrass meadow and unvegetated aera. Therefore, we speculate that the changes in the structure of the microbial community in the sediments with eelgrass are closely related to the vigorous N and P metabolism.

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