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Comparative analysis of microbial communities between water and sediment in Laoshan Bay marine ranching with varied aquaculture activities

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ABSTRACT

We profiled and compared the bacterial and protist community compositions and dynamics in the Laoshan Bay marine ranching involving varied aquaculture activities. The dominant species, differential species and community compositions among the five aquaculture areas, two habitats and two periods were significantly different. The relationships between microbial communities and environmental factors were analyzed. We found that microbial communities in the water were more sensitive to the environmental changes than sediment, and the responses of bacterial and protist communities to the disturbances were varied. To meet the challenges of higher aquaculture density, the proportion of the positive correlations among co-occurrence networks in the water increased markedly from July to November; while the positive proportion in the sediment was stable. Potential ecological interactions and keystone taxa between bacteria and protists were studied. These results advanced our understanding of how mariculture stressors affect microbial communities in marine ranching.

1. Introduction

Marine ranching is referred to as stock enhancement through releasing eggs, larvae, or juveniles into the environment, which is an important tool for resource restoration and ecologically sustainable development (Moksness and Stole, 1997; Moberg and Salvanes, 2018; Yu and Zhang, 2020). Because of overfishing, habitat destruction, and marine pollution, fishery resources have been declining in recent decades (Xue and Jiang, 2015). Marine ranching is an effective aquaculture process to cultivate and recover offshore fishery resources in China, which the Chinese government has advocated since the 1970s (Zhou et al., 2019). Over the decades of development, China's marine ranching has been designed and constructed by deploying artificial reefs and raft cultures in specific marine areas (Ministry of Agriculture of China, 2017).

Marine microbes, including bacteria and protists, play crucial roles in global biogeochemical processes such as energy, carbon, and nutrient cycles (Sunagawa et al., 2015). Studies revealed that one-half of the global primary production occurs in the oceans, which urges marine ecologists to understand how marine organisms involve in the spatial-temporal patterns of the carbon and energy fluxes (Falkowski et al., 1998; Field et al., 1998; Azam and Malfatti, 2007). Thus, studying the microbial community structure and dynamics is essential to comprehend these processes (Jonathan and Ward, 2002; Mccalley et al., 2014). Aquaculture is an important high value-added marine industry that provides good-quality seafood worldwide (Campbell and Pauly, 2013). Studies on the impacts of aquaculture on microbial communities have been extensively conducted (Allison et al., 1999; Naoki et al., 2009; Liu et al., 2015). Eddy et al. (2015) elucidated the impacts of organic enrichment on bacterial communities in the sediment of the chinook salmon (*Oncorhynchus tshawytscha*) farms in New Zealand and showed that bacterial compositions were strongly influenced by organic enrichment. The effects of mariculture on the distributions and seasonal variations of seawater picoplankton in Sanggou Bay, China, with two aquaculture species (bivalves and macroalgae) were investigated; the study showed that several physicochemical factors and protist grazing were the most important variables (Zhao et al., 2016). The cumulative impacts of the long-term intensive mariculture on the bacterial community in the sediment of Ailian Bay, China were studied; the results demonstrated that the influence on the total bacterial communities was more significant than that on the active bacterial communities (Liang et al., 2019). Artificial reefs are marine structures deployed along the

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coast mainly to enhance the fish stock production (Lima et al., 2019), while there has been little concern about their impacts on the microbial communities. Wang et al. (2019) pointed out that the bacterial communities in the sediment were significantly different between the reef area and control area in Bohai Bay. The dynamic responses of the protist communities to the artificial reefs in Daya Bay, China was observed, and the results concluded that the protist communities in the artificial reefs had more complex interactions or broader niches than those in the open waters (Zhu et al., 2020). Above all, an understanding of microbial responses to aquaculture activities is essential to reveal the impact of mariculture on marine ecosystems. However, studies related to the microbial communities in the water and sediment with different aquaculture activities in marine ranching are limited.

Laoshan Bay marine ranching is located in the Laoshan Bay, Yellow Sea, China with high-quality aquatic environment and fishery resources (Sheng et al., 2018). For many years, Laoshan Bay marine ranching has developed mixed aquaculture modes, including artificial reefs and raft cultures. Rock reefs were deployed to farm sea cucumber (*Apostichopus japonicus*) in 2005, which is the highest economic-value species in China; concrete reefs were arranged for recreational fishing aiming to fish black rockfish (*Sebastes schlegelii*) and perch (*Lateolabrax maculatus*) in 2018; laver (*Porphyra yezoensis*) is cultivated from September to May annually; polyculture of oyster (*Crassostrea gigas*) and scallop (*Argopecten irradians*) is practiced every year. Because of the high water temperature in the Yellow Sea, the aquaculture density is lowest in the summer, and the highest density occurs in the latter part of the autumn. The Laoshan Bay marine ranching is a typical operation among the marine ranching has been constructed in the Yellow Sea, China, and this current study will meaningfully understand the mechanisms of aquatic microbial ecosystems in a bay with small-scale integrated aquaculture activities in the water and sediment.

High-throughput sequencing, including 16S rRNA and 18S rRNA sequencing, has greatly facilitated the understanding of the mechanism of marine microbial ecology and is widely applied to profile microbial communities (Steele et al., 2011; Langille et al., 2013). In this study, we elucidated the bacterial and protist communities in the water and sediment of marine ranching with varied aquaculture activities using Illumina MiSeq sequencing methods for the first time. The primary objectives were (1) to provide a comprehensive understanding of bacterial and protist community structure in the marine ranching, (2) to compare the bacterial and protist communities between two habitats (water and sediment) and two periods (with the lowest and highest culture densities in July and November, respectively), (3) to reveal which environmental factors mainly shaped the bacterial and protist communities, and (4) to evaluate the interactions among bacterial and protist communities and identify the keystone species in the marine ranching.

2. Material and methods

2.1. Study areas and sample collection

The two habitats (water and sediment) samples were collected from the Laoshan Bay Marine ranching in two periods (July and November) 2020 (Fig. S1). Sixty samples from five sampling areas in the marine ranching were studied: rock reefs area (RR), concrete reefs area (CR), laver culture area (LC), shellfish culture area (SC), and the control area (CA). In each area, approximately 1 L of water and ample sediment samples within a depth range of 0–10 cm were randomly collected by a water sampler and a grab sediment sampler, respectively. Both water and sediment samples had three replicates from each area. The sample weights were sufficient for DNA extraction and analysis of physicochemical parameters. After sampling, the water and sediment samples were kept on ice and transported to the laboratory immediately. Each water sample was divided into two parts: one was filtered through a 0.2 μM pore size 47 mm polycarbonate filter (PF, Millipore) and then stored at −80 °C for DNA extraction; the other was used to assess physical and chemical factors. Each sediment sample was divided into two parts: one part was stored at −20 °C for physical and chemical analyses, and the second part was stored at −80 °C for DNA extraction.

2.2. Measurements of environmental factors

Water environmental factors, such as sampling depth (Dep), temperature (Temp), pH were measured by YSI PRODSS multi-parameter water quality meter (Yellow Springs, OH, USA). Transparency (Trans) was measured with a Secchi Disk. Turbidity (Turb) was measured by Turb 430 IR (Xylem Analytics, Germany). Total organic carbon (TOC) was measured by a TOC-L series total organic carbon analyzer (Shimadzu, Japan). Chemical oxygen demand (COD), dissolved inorganic nitrogen (DIN), active phosphate (PO_4-P), active silicate (SiO_3) and particulate organic matter (POM) were measured according to the GB/T 12763-2007 (State Bureau of Quality and Technical Supervision of China, 2007).

For the sediment samples, sediment bulk density (BD) was determined by dividing oven-dry mass obtained after drying at 105 °C for 72 h by the volume of the sediment. Water content (WC) was obtained by measuring the weight loss after the sediment was dried at 70 °C for 72 h until a constant weight was attained. Organic matter content (OM) was measured by burning sediment to ash at 550 °C for 4 h. The sediment pH and electrical conductivity (EC) were measured in a 1:2.5 (w/v) mixture of sediment and deionized water.

2.3. DNA extraction, PCR amplification, and Illumina MiSeq sequencing

Genomic DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's instructions. The DNA extract was checked on a 1% agarose gel, and the DNA concentration and purity were determined using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The extracted DNA samples were used for PCR amplification using two sets of primers. One was 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), which amplified the 468 bp V3-V4 hypervariable region of the 16S rRNA gene from bacteria; and the other was V4F (5′-CCA GCA SCY GCG GTA ATT CC-3′) and V4RB (5′-ACT TTC GTT CTT GAT YRR-3′) (Balzano et al., 2015), which amplified the 300 bp V4 hypervariable region of the 18S rRNA gene from protists.

The PCR amplification was performed as follows: initial denaturation at 95 °C for 3 min, followed by 27 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s, a single extension at 72 °C for 10 min, and a final extension at 4 °C. The PCR reaction consisted of a 20 μL mixture containing 4 μL of 5 × *TransStart* FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of forward primer (5 μM), 0.8 μL of reverse primer (5 μM), 0.4 μL of *TransStart* FastPfu DNA Polymerase, 10 ng template DNA, and ddH_2O up to 20 μL. The PCRs were performed in triplicate. The PCR product was run on a 2% agarose gel, and then, the amplicon was extracted and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. Subsequently, the DNA was quantified using the Quantus™ Fluorometer (Promega, Madison, WI, USA).

2.4. Sequence analysis

Raw sequence reads were analyzed using the QIIME 1.9 bioinformatics pipeline. Sequences shorter than 200 bp, quality scores lower than 20, and mismatched bases in barcodes or primers were removed. The operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE 7.0, and chimeric sequences were identified and removed using UCHIME. The taxonomy of each sequence was analyzed by RDP Classifier 11.5 against the Silva database (v138). For downstream analyses, a randomly selected subset of the lowest sequencing number per sample was used to compare the relative

differences between samples. The sequencing data reported in this study have been deposited in the NCBI Sequence Read Archive database under the accession number PRJNA714974.

2.5. Statistical analysis

Alpha diversity estimators (OTUs, Shannon, Simpson, Chao 1, and Good's coverage) were calculated and compared between two time periods, two habitats and five areas. Principal coordinate analysis (PCoA) was performed with the relative abundance data based on a Bray-Curtis distance matrix using the "*phyloseq*" package. Temporal and spatial effects on the microbial communities were tested using permutation multivariate analysis of variance (PERMANOVA) and analysis of similarity (ANOSIM). Statistical differences were analyzed using the Kruskal-Wallis test, and the differences were considered significant at $P < 0.05$. The relationships between the environmental parameters and community compositions were explored by the distance-based redundancy analysis (db-RDA) based on a Bray-Curtis distance matrix using the "*vegan*" package. Linear regression analysis was performed to investigate the influence of environmental factors on microbial communities. Statistical analyses were conducted in R 4.0.2.

Co-occurrence networks were built using Cytoscape 3.8.2 for bacterial and protist communities in each period of the water and sediment samples, respectively. Network topology parameters were calculated using the Analyze Network plugin to identify the keystone phyla/OTUs in the networks. Three main network topology parameters were as follows: (1) degree: the number of edges that a node has; (2) betweenness centrality: the number of shortest paths between any two nodes in the

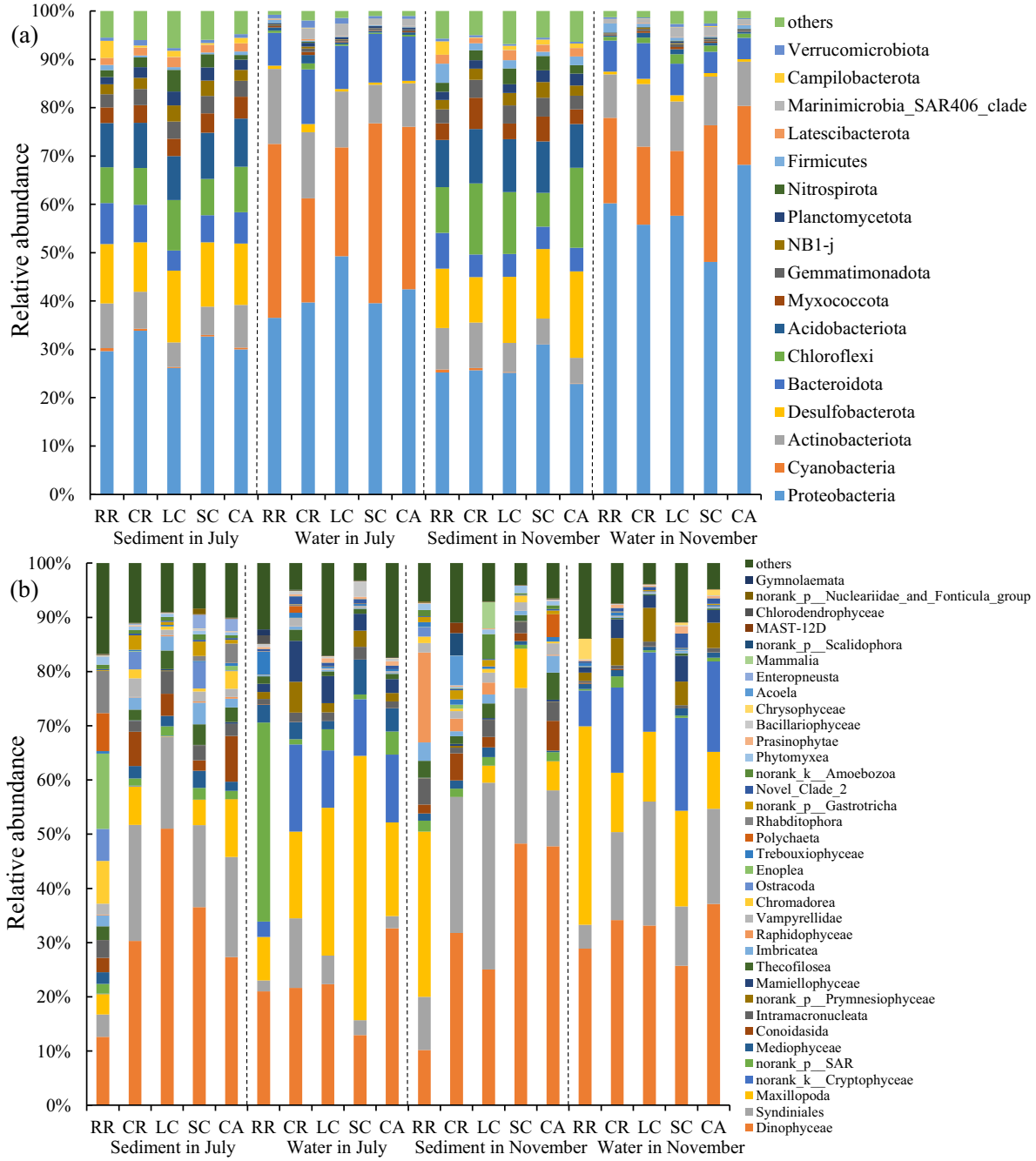

Fig. 1. Relative abundance of (a) bacteria communities at phylum level and (b) protist communities at class level of five areas in the two habitats and two periods. Five sampling areas were rock reefs (RR), concrete reefs (CR), laver culture (LC), shellfish culture (SC), and control area (CA).

graph passing through a node; (3) closeness centrality: the average distance of a node to any other node. The keystone phyla/OTUs were identified by calculating a keystoneness score defined as the average of degree, 1-betweenness centrality, and closeness centrality after Min-Max scaling (Berry and Widder, 2014).

3. Results

3.1. Microbial community compositions

In total, 3,227,550 bacterial 16S rRNA gene sequences for bacteria (5077 OTUs) and 3,457,380 protist 18S rRNA gene sequences for protists (7304 OTUs) were obtained from 60 water and sediment samples. Good's coverage values for bacteria and protists were 0.964 ± 0.011 and 0.992 ± 0.002, which indicated that the current sequencing depth was sufficient to represent the microbial communities in the marine ranching.

The bacterial communities were dominated by the phyla Proteobacteria, Cyanobacteria, and Actinobacteriota in the water samples, comprising 49.7%, 23.9%, and 10.9% of relative abundance, respectively (Fig. 1a). The relative abundance of Proteobacteria in July was lower than in November, whereas the abundance of Cyanobacteria in July was higher compared with that in November. The dominant phyla in the sediment were Proteobacteria (28.2%), Desulfobacterota (13.1%) and Chloroflexi (10.3%), and there were no dominant phyla that showed significant differences between the two periods. The dominant classes of protist communities in the water were Dinophyceae, Maxillopoda and Crptophyceae, which comprised 27.0%, 20.6% and 12.3% of relative abundance (Fig. 1b). The relative abundances of Dinophyceae, Syndiniales and Crptophyceae in November were significantly higher than those in July. Contrarily, the abundance of Maxillopoda in July was higher than that in November. In the sediment, the dominant species were Dinophyceae (32.1%) and Syndiniales (18.4%). The abundance of Syndiniales in November was higher than those in July.

The majority of microorganisms (bacterial phyla and protist classes) with abundance more than 1% were significantly different among the five areas, especially for those with abundance over 5% (Table S2a). For the water samples among the five areas, Syndiniales was the only protist without significant difference. As for the sediment samples, the abundance of Campilobacterota was consistent; class Maxillopoda had similar abundance with the highest abundance (9.3%). Compared with the difference among the five areas, the number of differential microorganisms between four aquaculture areas (RR, CR, LC, SC) and control area (CA) were less (Table S2b, c). For bacteria (Table S2b), the abundance of the differential phyla between the water samples was more abundant than the sediment samples. For example, the abundance of Proteobacteria in the water of SC was significantly lower than that in CA in July, while the abundance of Cyanobacteria in SC was much higher compared with CA. As for the sediment samples, the number of differential phyla in November was largest, and Desulfobacterota in SC was the most abundant differential phyla. For protists (Table S2c), the differential classes always were the dominant classes like Dinophyceae and Syndiniales. For instance, the abundance of Dinophyceae in RR and SC of water samples in July were lower than CA, while its abundance in the sediment was higher than CA. Mamiellophyceae observed in CR of water samples in July was the only differential class compared with CA (2.6%) with relatively lower abundance.

3.2. Alpha diversities and community compositions

Among all water and sediment samples, the bacterial OTU numbers ranged from 829 to 1332 for the water samples in July and from 756 to 1182 in November; and ranged from 1851 to 2248 for the sediment samples in July and from 1867 to 2188 in November (Table 1). The bacterial OTUs in the water samples contained 72.8% of the total OTUs, and 11.0% of which were unique to water samples. The OTUs in the sediment samples accounted for 89.0% of the total OTUs and 27.2% of which were unique. The number of protist OTUs ranged from 577 to 967 for water in July and from 419 to 596 in November; and ranged from 680 to 911 for sediment in July and from 579 to 872 in November (Table 1). The protist OTUs from the water samples contained only 43.1% of the total OTUs and 23.5% of which were unique to water samples. The sediment protist OTUs accounted for 76.5% of the total OTUs, and almost 56.9% were unique.

Table 1
Alpha diversity of microbial communities using Illumina MiSeq sequencing in the water and sediment of marine ranching. Five areas were: rock reefs (RR), concrete reefs (CR), laver culture (LC), shellfish culture (SC) and control area (CA). The significant differences among five areas were analyzed by *P* values with bold font. Asterisk with the bold font represented that the difference between aquaculture area and control area was significant, * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$.

Kingdom	Area		Water				Sediment			
			OTUs	Shannon	Simpson	Chao 1	OTUs	Shannon	Simpson	Chao 1
Bacteria	July	RR	915	4.226	**0.056****	1509	2180	**6.393***	**0.0048****	2936
		CR	**1332***	5.015	0.026	**2247***	2229	6.313	0.0074	3113
		LS	829	4.435	0.035	1418	2248	6.310	0.0074	3055
		SC	849	4.553	0.028	1457	2246	6.309	0.0072	3164
		CA	934	4.627	0.029	1677	1851	5.987	0.0081	2673
		P	**0.001**	**0.001**	**0.003**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
	November	RR	756	4.049	0.085	1350	2188	**6.332***	**0.005****	**3195***
		CR	941	4.339	0.049	1564	1867	5.983	0.009	2727
		LS	**1182***	4.941	0.027	**2066***	**2174***	6.232	0.007	**3157***
		SC	919	4.582	0.034	1572	1900	5.972	0.010	2707
		CA	773	3.986	0.074	1275	1974	6.099	0.007	2788
		P	**0.001**	**0.001**	**0.009**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
Protist	July	RR	**967***	3.769	0.073	**1288***	807	3.733	0.120	951
		CR	794	**4.201***	0.051	1147	867	4.424	0.043	1035
		LS	673	3.503	0.127	1012	680	4.277	0.041	821
		SC	577	3.031	0.252	821	**911***	4.551	0.037	**1055***
		CA	666	3.645	0.087	1031	736	4.329	0.043	862
		P	**0.001**	**0.001**	**0.030**	**0.001**	**0.001**	**0.001**	**0.023**	**0.001**
	November	RR	**419****	**2.468*****	**0.254*****	**608***	872	3.483	0.188	1136
		CR	596	3.926	0.060	**771***	579	3.518	**0.099***	729
		LS	568	3.751	0.084	842	791	3.900	0.091	1007
		SC	529	3.757	0.068	755	676	3.440	0.100	906
		CA	579	3.961	0.055	825	770	4.130	0.048	931
		P	**0.001**	**0.001**	0.051	**0.001**	**0.001**	**0.001**	**0.010**	**0.001**

No significant difference between the two periods was observed for the alpha diversity indices, but the difference between the two habitats was significant ($P < 0.001$). Not only that, the alpha diversity indices of the bacterial and protist communities among the five areas were significantly different, and some differences between the aquaculture areas and control area were observed (Table 1). For Shannon diversity, there was no difference of bacterial water samples between aquaculture areas and CA; while for the sediment sample, it was higher in RR than that in CA in two periods. By contrary, there was no difference in Shannon diversity for the sediment protists between aquaculture areas and CA. For protist water samples, Shannon diversity was higher in CR than CA in July; while it was significantly lower in RR than CA in November. For Simpson diversity and Chao 1 diversity, the differences between aquaculture areas and control area both for the water and sediment samples were shown in Table 1.

The distinctions of the bacterial and protist communities among the two periods, two habitats and five areas were compared based on the principal coordinates analysis (PCoA), analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA). The PCoA for the bacterial and protist communities both showed that two distinct groups existed between the water and sediment samples ($P < 0.001$), and the difference for the water samples between the two periods was larger than that in the sediment samples (Figs. 2a, b; S2).

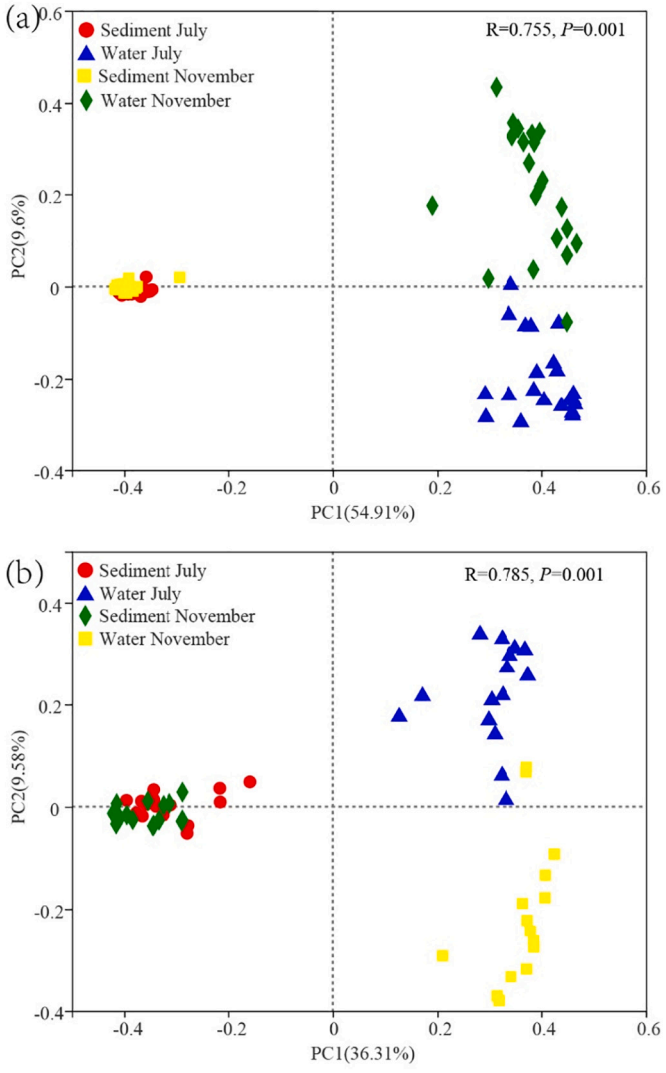

Fig. 2. Principal coordinates analysis (PCoA) plots of (a) bacterial and (b) protist communities of marine ranching in the two habitats and two periods.

The first and second principal coordinates explained 54.91% and 9.60% of the variations in the bacterial community compositions; and 36.31% and 9.58% of the variations for the protist community compositions, respectively. For the five areas, differences were observed by ANOSIM in the water and sediment samples both for the bacterial (water: $P < 0.05$, sediment: $P < 0.001$) and protist communities (water: $P < 0.05$, sediment: $P < 0.01$). The results of the PERMANOVA revealed that period, habitat, area, and their interactions on the bacterial and protist communities were significant (Table 2).

3.3. Relationships between microbial communities and environmental factors

Distance-based redundancy analysis (db-RDA) was employed to illustrate the relationships between microbial communities and environmental factors. In July, the CAP1 axis and CAP2 axis of the db-RDA explained 36.23% of the variations for the water bacterial communities and explained 20.06% of the variations for the sediment bacterial samples (Fig. 3a, b). For the water samples, COD ($P < 0.05$) was positively related with the bacteria in RR, like OTU9784 (Proteobacteria); while POM ($P < 0.05$) and DIN ($P < 0.05$) were negatively correlated with bacteria in RR, like OTU11546 (Cyanobacteria) and OTU12154 (Cyanobacteria). For the sediment samples, pH ($P < 0.001$) had the longest arrow in the db-RDA plot, indicating that pH significantly influenced the bacterial community in SC. In November, 24.93% of the variations for the water bacterial communities were explained, and 21.74% was explained for the sediment samples (Fig. 3c, d). For the water samples, no environmental factor significantly impacted the bacterial communities. For the sediment samples, negative correlations were found between WC ($P < 0.001$), EC ($P < 0.05$) and communities in RR, like OTU4124 (Acidobacteriota).

For protists, the CAP1 axis and CAP2 axis of the db-RDA explained 39.86% and 20.08% of the variations for the water and sediment samples in July (Fig. 3e, f). For the water samples, the protist community in the SC was influenced by POM ($P < 0.01$) and Turb ($P < 0.05$) significantly, especially OTU7611 (Dinophyceae); the community in the RR was shaped considerably by COD ($P < 0.01$) and TOC ($P < 0.05$), especially OTU8008 (Dinophyceae). For the sediment samples, negative correlation between the community in the RR and OM ($P < 0.05$) was observed. In November, 40.23% and 22.17% of the variations for the water and sediment protist communities were explained (Fig. 3g, h). For the water samples, no environmental factor significantly impacted the bacterial communities. For the sediment samples, OM ($P < 0.01$) was highly related with protist community in SC; and EC ($P < 0.05$) was correlated with community in CR, especially OTU4103 (Syndiniales).

Linear regressions on the alpha diversity and beta diversity of the microbial communities versus environmental factors were analyzed. For bacteria (Fig. S3), the Shannon diversity was negatively associated with TOC ($P < 0.05$) for the water samples and pH ($P < 0.01$) for the sediment samples in July. Turb was the only factor that positively correlated with Shannon diversity for the water samples in November. In July, the beta diversity (PC1) was positively correlated with pH ($P < 0.01$), while was

Table 2
PERMANOVA of temporal and spatial effects on microbial communities. *P* values are significant at $\alpha = 0.05$.

Effect	Bacteria		Protist	
	F	*P*	*F*	*P*
Area	3.107	0.003	2.564	0.002
Habitat	132.409	0.001	51.42	0.001
Period	11.551	0.001	8.373	0.001
Area × Habitat	2.986	0.003	2.462	0.001
Area × Period	1.986	0.043	1.984	0.007
Period × Habitat	10.868	0.002	8.061	0.001
Period × Habitat × Area	1.863	0.067	1.904	0.012

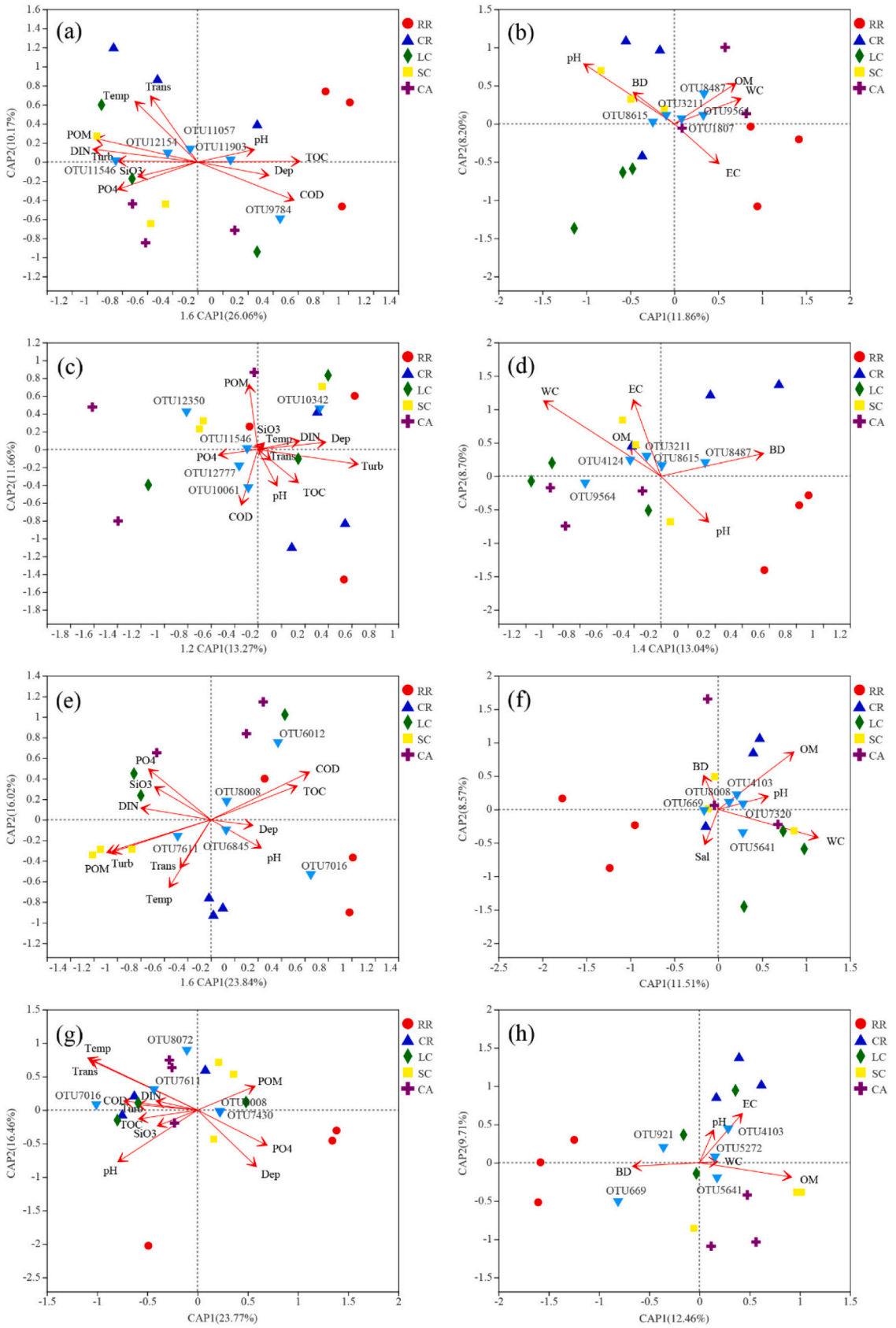

Fig. 3. Distance-based redundancy analysis (db-RDA) of water bacterial community in (a) July and (c) November, sediment bacterial community in (b) July and (d) November, water protist community in (e) July and (g) November, sediment protist community in (f) July and (h) November. Five sampling areas were rock reefs (RR), concrete reefs (CR), laver culture (LC), shellfish culture (SC), and control area (CA). Light blue inverted triangle represented the top five abundant OTUs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

negatively correlated with silicate ($P < 0.01$) and POM ($P < 0.05$) for the water samples; WC ($P < 0.01$) showed a positive correlation with PC1 for the sediment samples. In November, PC1 was only positively correlated with POM ($P < 0.05$) for the water samples; whereas no significant correlation was detected for the sediment samples. For protists (Fig. S4), only in the water samples in July, the Shannon diversity showed a positive correlation with Turb ($P < 0.05$) and negative correlation with PO_4-P ($P < 0.05$). In July, PC1 was positively correlated with pH ($P < 0.01$) and negatively correlated with the SiO_3 ($P < 0.01$) in the water samples; and a positive correlation between PC1 and WC ($P < 0.05$) for the sediment samples was found. In November, OM ($P < 0.01$) was the only environmental factor positively correlated with the water protist communities.

3.4. Co-occurrence network analysis and keystone species

To evaluate the co-occurrence patterns of microorganisms in the water and sediment of Laoshan Bay marine ranching, four co-occurrence networks based on the most abundant 50 bacterial phyla and 50 protist classes were constructed. With the increase of the aquaculture activities from July to November, the proportion of the positive correlations raised from 76.1% to 89.8% in the water and from 55.3% to 73.7% in the sediment. The significant correlations among bacteria and protists identified in the water and sediment samples showed different patterns. For water samples, the proportion of the correlations between bacteria and protists were 38.3% and 18.1% in July and November; the proportion between bacteria were 31.4% and 50.9%; and the proportion between protists were about 30% both in two periods. For sediment samples, the proportion of the correlations between bacteria decreased from 46.0% to 36.5%; the proportion between protists increased from 17.9% to 29.2%; and the proportion between bacteria and protists were nearly 35% in two periods.

Moreover, another four co-occurrence networks based on the top abundant 50 bacterial and protist OTUs were constructed to examine the core interactions ($R > 0.8$ and $P < 0.05$) among microbes. In July, network for the water samples comprised of 81 OTUs mainly from the bacterial (43 OTUs) phyla Proteobacteria, Cyanobacteria and Actinobacteria, and protist (38 OTUs) classes Dinophyceae and Mamiellophyceae (Fig. 4a). In the sediment, only 47 OTUs were observed in the network, mainly from bacterial (33 OTUs) phyla Planctomycetota, Desulfobacterota, Acidobacteriota, and Chloroflexi and the protist (14 OTUs) class Dinophyceae (Fig. 4c). In November, the water network contained 65 OTUs mainly from the bacterial (33 OTUs) phyla Proteobacteria, Cyanobacteria, and Actinobacteriota and protist (32 OTUs) classes Syndiniales and Dinophyceae (Fig. 4b). In the sediment, only 48 OTUs were detected in the network, mainly from the bacterial (34 OTUs) phyla Planctomycetota, Desulfobacterota, and Acidobacteriota and the protist (14 OTUs) class Dinophyceae (Fig. 4d). In the water samples, the proportion of the positive correlations was over 56.1% in July, while in November, the proportion of the positive correlations was up to 97.3%. In the sediment, the proportion of the positive correlations was over 74.2% and 73.5% in July and November, respectively.

For every network, the OTUs with keystoneness >0.6 and relative abundance larger than 1‰ were selected to be identified as keystone taxa. For bacteria, the top ten keystone OTUs with a total relative abundance of 9.3% belonged to two phyla of the water samples in July (Table S3a); and in the sediment samples, the ten keystone OTUs with a total relative abundance of 7.3% were affiliated to eight phyla (Table S3b). In November, the ten keystone OTUs with total relative abundances of 8.8% and 8.2% of three phyla and five phyla were the keystone OTUs in the water and sediment samples, respectively. For protists, the top ten keystone OTUs with a total relative abundance of 13.0% belonged to six classes of the water samples in July (Table S3c); and in the sediment samples, the ten keystone OTUs with a total relative abundance of 18.1% were affiliated to three classes (Table S3d). In November, the ten OTUs with total relative abundances of 9.7% and 18.0% of one and three classes were the keystone OTUs in the water and sediment samples, respectively.

4. Discussion

4.1. Differential taxa analysis

The aquaculture activities impacted the microbial communities in the water and sediment of marine ranching at different degrees. The number of bacterial and protist OTUs in the sediment were significantly higher than those in the water, which was agreed with some similar studies (Feng et al., 2009; Ye et al., 2009; Cole et al., 2013). However,

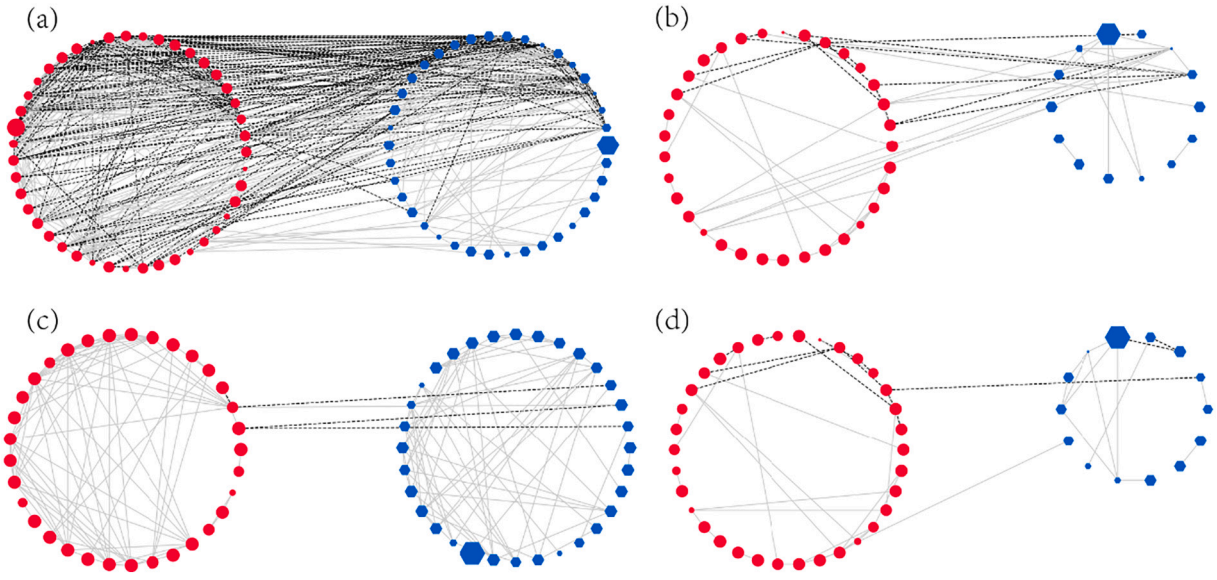

Fig. 4. Co-occurrence networks built from major bacteria and protist OTUs of (a, c) water and (b, d) sediment in July and November. Red circular nodes represented bacterial OTUs, blue hexagonal nodes represented protist OTUs. The size of nodes is proportional to the relative abundance of OTUs. Only edges with $r \geq 0.8$ and $P \leq 0.05$ are shown. Positive and negative lines are represented by grey solid lines and black dotted lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the proportion of shared protist OTUs between water and sediment (19.5%) were significantly lower than those of bacteria (61.8%), showing that the correlations of protists between water and sediment were lower than that of bacteria. For the abundance of the dominant bacterial phyla between the water and sediment samples (Fig. 1a), the inconformity was similar with previous studies (Fang et al., 2015; Sun et al., 2019). As a critical component of marine primary productivity, Cyanobacteria showed a significantly higher abundance in the water samples. In the sediment, higher abundance of Desulfobacteria, Bacteroidia and Chloroflexi were observed. One important reason was that these phyla are closely related to the anaerobic processes in the marine sediment (Roussel et al., 2015; Pelikan et al., 2021). With the increase of the culture density from July to November, the number of differential OTUs between water samples in November were enormous than those in July, which revealed that the effects of higher aquaculture density on the microbial communities were apparent (Halpern et al., 2008; Nogales et al., 2011).

Among the five areas, the number of the differential OTUs for sediment samples in RR and CR was greater than that in other areas; while in LC and SC, the results for the water samples were significant. These findings showed that ARs (rock reefs and concrete reefs) have more remarkable effects on the microbial communities in the sediment (Wang et al., 2019), while the impacts of raft cultures on the communities are more partial to the water environment (Stoeck et al., 2018). Differential taxa between the four aquaculture areas and the control area were identified to investigate the effects of aquaculture activities on the microbial communities (Table S2). For bacteria in the water samples, a lower abundance of Proteobacteria and a higher abundance of Cyanobacteria in SC compared with that in CA were observed. Meantime, their sum abundance in SC was consistent with that in CA. Many studies revealed that the aquaculture activities of bivalves could change the microbial communities in the surrounding seawater (Vezzulli et al., 2018). Therefore, a closing correlation between Proteobacteria and shellfish aquaculture maybe exist. For bacteria in the sediment samples, we found that the relative abundance of Bacteroidota in RR was significantly higher than that in CA. Artificial rock reefs were deployed in RR, where are ideal habitats for macroalgae; and Bacteroidota was reported that significantly enriched in the inshore-reefs, and played crucial roles in degrading macroalgae-derived polysaccharides via unique machinery (Glasl et al., 2020).

4.2. The influence of environmental factors on the microbial communities

The microbial community structure in the marine ranching was shaped by the different environmental factors, such as POM, pH and Turb (Fig. 3). The marine particles of the organic matter are ideal habitat for microbes to absorb nutrients, avoid predators and are also an important factor shifting the bacterial community (Kramer et al., 2013; Yustian et al., 2018). Our results confirmed these hypotheses. For the other important factor, COD had significant effects on the bacterial and protist communities in RR in July (Fig. 3a, e). Area RR is closest to the shore and fishery pier, where was influenced by the terrestrial organic pollutants. COD is an organic pollution index in water, this result showed that microbial communities played important roles in the degradation of organic matter (Shao et al., 2021). For protist communities in the sediment, the impacts of OM were non-negligible (Fig. 3f, h). The Shannon diversity of water protist communities in July also showed a positive correlation with OM (Fig. S4). Many studies reported similar results, showing that OM changed the microbial community compositions and altered bacterial production in the sediment (Hunting et al., 2013).

With the enhancement of aquaculture activities from July to November, the environmental disturbances in the marine ranching have been constantly aggravated. In particular, higher aquaculture density in November significantly influenced the microbial communities in the water. For instance, no significant environment factor was found based on db-RDA (Fig. 3c, g), which revealed that environmental conditions in the marine ranching tended to be more complex in November owing to a higher aquaculture density. Two possible explanations were as follows. On the one hand, the abiotic factors we sampled were not comprehensive enough. For example, organic carbon, total nitrogen and median size for sediment (Liu et al., 2015; H. Hu et al., 2017), total carbon, total nitrogen and total phosphorus for water (Sun et al., 2019) were also regarded as important factors in building microbial communities. On the other hand, biotic factors like inter-taxa relationships based on the trophic traits were crucial to understand the dynamics of the microbial communities (Gilbert et al., 2015). In addition, a lower explanation of environmental factors for bacterial communities was observed, while not found in protist communities (Fig. 3c, g). These outcomes indicated that the capacity of the bacteria to resist the environmental change to affect their community structure was lower than that of the protist community. Protists play multiple roles in the marine microbial loop, such as producers, consumers, degraders, and parasites (Genitsaris et al., 2015). Multiple ecological roles of protists might enhance the ability to recover from disturbance compared with bacteria. Such as protistan grazing, a vital process of microbial loop, higher trophic level and close association with other microorganisms of protist community resulted in a more stable community structure (Livingston et al., 2017). What's more, the concentration of POM in RR and CR was higher than other three areas both in two periods (Fig. S3). However, the relationships between bacterial communities and POM in July and November were absolutely opposite. Intensification of aquaculture activities in SC and LC probably was the main source for the awkward outcomes.

Water temperature is widely accepted as a vital factor in constructing microbial communities (Gilbert et al., 2015). The temperature was found to be highly correlated with the microbial community richness (Thompson et al., 2017), biological processes (Azam and Malfatti, 2007) and biogeochemical cycles driven by the microbiomes (Sunagawa et al., 2015). In this study, the obvious change of water temperature (from 17 °C to 24 °C) in Laoshan Bay marine ranching seems to strongly influence the microbial communities. The microbial difference between the two periods maybe attribute to the variation of temperature. However, we focused more on the impacts of varied aquaculture activities and different aquaculture densities on the microbial communities. What's more, the studying areas were small, and the differences in water temperature among the five areas could be ignored. Therefore, we did not reflect the effects of temperature, and later studies with long time series should consider this question seriously.

4.3. Interaction patterns of microorganisms

The structure of the co-occurrence networks provided insights into the organization and interactions of the microbial communities (Barberan et al., 2012). In the present work, we evaluated the co-occurrence patterns of bacterial and protist communities in the marine ranching at phylum and OTU two levels, and explored the keystone taxa based on the topological parameters of network analysis. There were significant differences in the structures of the co-occurrence networks in the water and sediment, indicating that the marine habitats had significantly affected the co-occurrence networks of the microorganisms (Zhang et al., 2018). For example, the proportions of the bacterial OTUs in the water and sediment networks were over 50% and 70% in the two periods. These results indicated that the interactions of the bacteria in the sediment were much higher than those in the water, which was consistent with Zhang et al. (2020). As for the increasing proportion of significant correlations between bacteria or protists in November compared with that in July, suggesting that the cooperative interactions among the microorganisms predominated in the response to the more complicated aquatic environment. The microbes of the same kingdom usually had significant and predominantly positive correlations with each other (Cheung et al., 2018; Mikhailov et al., 2019), which supported our hypothesis. Reducing nutrients concentrations and

improving water quality by the aquaculture activities of shellfish and laver may also be conducive to reduce competitions among microorganisms (Liang et al., 2019).

The keystone species played essential roles in the co-occurrence network relative to their abundance (Power et al., 1996), such as mediating biogeochemical processes and maintaining ecosystem stability (Michael et al., 2010; Berry and Widder, 2014). For the networks at phylum level, the keystoneness of many phyla was less than 0.6 (Table S4), which indicated that the ecological roles of phyla played in the networks were balanced. For the networks at OTU level, the number of keystone OTUs with keystoneness >0.6 were more abundant (Fig. S3). For bacteria, the most abundant keystone OTUs of the water samples were OTU11696 and OTU10831 in the two periods, belonging to the phyla Proteobacteria and Cyanobacteria, respectively. The OTU11696 was observed as a keystone OTU in both the water and sediment and was most closely related to the genus Rhodospirillum, which has been widely studied for hydrogen production and water purification (Zürrer and Bachofen, 1979; C. Hu et al., 2017). The OTU3211 and OTU8615 were the most dominant OTUs of the sediment in July and November, belonging to the phyla Myxococcota and Desulfobacterota. The Myxococcota has been well studied in the terrestrial habitats with the function of producing fruiting bodies and other secondary metabolites (Shimkets et al., 2006), while their ecosystem function remains unknown in the marine habitats (Brinkhoff et al., 2012). The OTU8615 was most closely related to the genus Desulfobacterota, which plays a key role in anaerobic food webs (Finke et al., 2007). For protists, the OTU6215 was most closely related to the order Noctilucales, which is the most abundant red tide organism in China (Harrison et al., 2011) and was the most abundant OTU in the water.

Co-occurrence network analysis is meaningful to uncover the specific but not apparent correlations among microorganisms (Zhang et al., 2020), which mediate the biodiversity and functions of marine ecosystem (Mikhailov et al., 2019). However, diverse trophic modes (e.g., phototrophic, parasitic and consumers) of protists complicate the analytical process (Singer et al., 2021). Draw on the results of Singer et al. (2021) and Table S3, the keystone OTUs in the water samples in July belonging to phototroph (e.g., Cryptomonadales) and consumers (e.g., Noctilucales); while in November, half of keystone OTUs were classified as parasites (e.g., Syndiniales). Many studies reported seasonal parasite cycling (e.g., Arzul et al., 2009). The parasitoid strategy, means killing the hosts to complete their life cycles, which were widely found in Syndiniales. Mangot et al. (2013) also pointed out that aquatic parasites would attack most of other microorganisms and lysed the hosts rapidly, which might create bloom dynamics of their abundance. Although tremendous advancements have achieved in the studies of the diversity of protists based on high-throughput sequencing, our understanding of their taxonomic affiliations and trophic modes is still partial and limited. Therefore, in-depth researches are encouraged in future studies.

We explored the co-occurrence networks based on the two classification levels (phylum and OTU), while parts of results were inconsistent. For one thing, equal numbers of bacterial phyla and protist classes included in the four networks were observed; while for the other four networks based on OTUs, the ratio of OTUs for bacteria and protists in the sediment samples was 7:3. For another, with higher disturbances of aquaculture activities in November, the networks based on phylum showed that the proportion of significant correlations between bacteria and between protists increased in the water and sediment samples, respectively; while the networks based on OTU revealed that the proportion of significant correlations between bacteria and protists declined significantly to less than 5%. We supposed that the responses of higher-level (phylum) and lower-level (OTU) microorganisms to the aquaculture activities in November may vary due to the different sensitivities and tolerances.

Disaccord of the phyla for bacterial keystone taxa based on the two levels (phylum and OTU) was evident (Tables S3 and S4). For phylum level, the abundance of keystone phyla was lower, including Nitrospinota ranking 19th (Table S4a). For OTU level, the abundance of keystone OTUs was also lower, however, these OTUs belonged to the most abundant phyla like Proteobacteria (Table S3a, b). Two phyla, Actinobacteriota and Nitrospirota, were simultaneously identified as keystone species at two levels for the sediment samples in July. Previous studies about networks were more focused on OTUs (Barberan et al., 2012; Zhang et al., 2020; Zhu et al., 2020), this inconformity was rarely discussed. Two possible explanations could be used as references. First, the ecological functions and the responses of phyla and OTUs to the environmental changes were varied. Second, species identification and function analysis at OTU level were more precise.

4.4. Management and enlightenment

The coastal waters are the highly productive areas that provide various socio-cultural and economic ecosystem services (Barbier et al., 2011). However, with the increase of anthropogenic stressors like marine aquaculture activities, the biotic habitats on the coast have been degraded above our expectations (Beck et al., 2011; Dance and Rooker, 2015). For example, deoxygenation in the aquatic environment resulting from the large amounts of wastes released from fish cages (Yokoyama et al., 2009; Sutherland et al., 2018), usually reshaped the microbial biomass, diversity, and community composition (Li et al., 2017; Xu et al., 2019). The aquaculture activities in the Laoshan Bay marine ranching are small-scale anthropogenic activities, and the main targets are algae and shellfish, whose influence on the marine environment is relatively limited compared with fish farming (Richardson et al., 2008; Wang et al., 2016). Our results showed that the bacterial and protist communities in the marine ranching were sensitive to the aquaculture activities, especially in November with higher mariculture density. We also observed some dissimilarities among five aquaculture areas and two habitats. Despite some disturbances, a healthy balance between the aquaculture activities and ecosystem functions in Laoshan Bay was still maintained. Overall, our study confirmed that microbial communities are effective indicators to check whether the marine ecological environment is healthy or not, which will be of great significance in ecological environment monitoring for management.

5. Conclusions

In summary, this study revealed the first detailed profiles of the bacterial and protist community structure and interactions in the water and sediment from a representative of marine ranching with small-scale aquaculture activities in the Yellow Sea, China. Overall, different microbial communities were observed between the water and sediment habitats. With the intensification of the aquaculture activities in the late autumn, the aquatic environment tended to be more complicated, and the positive correlations among microbial co-occurrence networks were significantly increased, while those were not changed in the sediment. The distance-based redundancy analysis showed that the response of the bacterial communities in the water was more sensitive than that of the protist communities, indicating that apart from abiotic factors, some biotic factors are potential variables in mediating the dynamics of the bacterial communities. Microorganisms are sensitive indicators of the influence of aquaculture activities, and their community dynamics can provide insights to evaluate whether the density of aquaculture activities in marine ranching is reasonable. The effects of different aquaculture modes and farming scales should be assessed in future studies, which will help enhance our understanding of the patterns by which anthropogenic stressors affect microbial communities.

CRediT authorship contribution statement

Guangjie Fang: Conceptualization, Investigation, Methodology, Writing – original draft. **Haolin Yu:** Investigation, Data curation.

Huaxiang Sheng: Investigation, Data curation. **Yanli Tang:** Conceptualization, Writing – review & editing, Supervision. **Zhenlin Liang:** Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2021.112990.

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