



# Cumulative impact of long-term intensive mariculture on total and active bacterial communities in the core sediments of the Ailian Bay, North China

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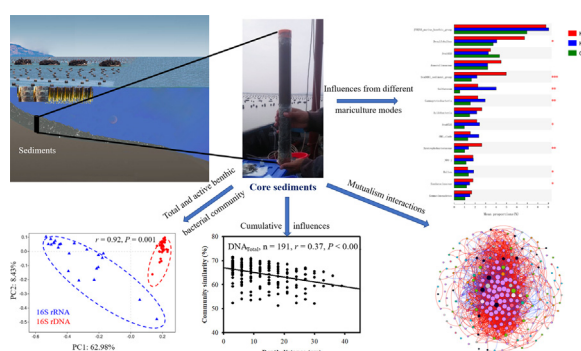
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## HIGHLIGHTS

- Significant differences between total (DNA) and active (RNA) benthic bacterial communities were observed.
- Long-term mariculture showed only slightly cumulative influences on the benthic bacterial communities.
- Bacterial genera in the sulfide cycling and organic consumption were enriched in the total bacteria of the IMTA areas.
- Variations of both total and active bacterial community were influenced by grain sizes, total organic carbon and nutrients.
- Mutualism effect dominated in the total and active bacterial networks in the Ailian Bay.

## GRAPHICAL ABSTRACT



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## ABSTRACT

The exponential growth of off-shore mariculture worldwide over the last 20 years has had significant impact on coastal sediment biogeochemistry. However, there are no long-term records of the cumulative impacts of mariculture on the benthic bacterial community. Here, total (DNA) and active (RNA) bacterial community compositions were characterized using MiSeq sequencing of 16S rRNA gene in four core sediments of the Ailian Bay, one of the typical intensive mariculture areas in China with more than fifty-year history of kelp and scallop cultivation. The  $\gamma$ -Proteobacteria,  $\delta$ -Proteobacteria, Acidobacteria and Actinobacteria were more abundant in the total bacterial communities, while  $\beta$ -Proteobacteria, Anaerolineae, Clostridia, Spirochaetes and Cyanobacteria were enriched in the active bacterial communities. Significant differences were observed between total and active benthic bacterial communities. The influences of different mariculture modes on the total bacterial

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communities were more significant than those on the active bacterial communities. Only limited groups of the total bacterial communities were significantly influenced by the cumulative effects of the long-term mariculture. The bacterial genera with the function in the sulfide cycling and organic consumption were enriched in the total bacterial population of the integrated multi-trophic aquaculture (IMTA) areas. The variations of both total and active bacterial communities were significantly influenced by grain sizes, total organic carbon and nutrients. Both total and active bacterial communities exhibited a slightly stronger response to environmental factors than to spatial (distance) factors. The effects of mutualism might dominate the total and active bacterial networks in the Ailian Bay. The present study demonstrated that the cumulative influences of the long-term and intensive IMTA mariculture on total benthic bacterial communities in the sub-surface sediments of the Ailian Bay were stronger than those on the active benthic bacterial communities, which provided some insights into the potential ecological roles of specific taxa in the sediments of the IMTA ecosystems.

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## 1. Introduction

As land resources decrease and the human population booms, aquaculture has been rapidly grown to meet global demands for food and it is predicted that it will be entirely responsible for the increase of global seafood production by 2030 (FAO, 2018; Kobayashi et al., 2015). However, the flourishing mariculture of economical animals (such as fish, shellfish, etc.) has generated massive quantities of biological deposits enriched with organic matter (OM) (Carlsson et al., 2009), causing severe environmental problems in coastal areas (i.e. eutrophication and harmful algal blooms) and risking environmental sustainability (Cao et al., 2007). To improve the degrading environmental conditions caused by the intensive mariculture of economical animals, the large-scale co-cultivation of seaweed with fish and shellfish (also called integrated multi-trophic aquaculture, IMTA for short) was proposed as an essential bioremediation approach (Fang et al., 2016) and has been widely used in the coastal mariculture areas of North China. In recent years, the environmental and biological changes of coastal areas after long-term intensive mariculture activities have been extensively studied (Duarte et al., 2003; Fang et al., 2016), involving nutrient concentrations and fluxes (Li et al., 2018; Li et al., 2016; Ning et al., 2016), microbial community and phytoplankton in the seawater (Yuan et al., 2014; Zhao et al., 2016), and organic carbon and sulfate concentrations in the sedimentation (Kang et al., 2016; S. Liu et al., 2015; Liu et al., 2016a; Xia et al., 2014; Zhao et al., 2016). In the intensive mariculture zones, like the Ailian Bay in this study, the water exchange in the upper layers could be hindered to a great extent (Zeng et al., 2015). The intensive mariculture activities not only influence the water environment remarkably, but also result in a very high burial rate of organic matter in the sediments (S. Liu et al., 2015; Liu et al., 2016a; Xia et al., 2014). The deposit thickness increases with the mariculture history and can reveal well the cumulative impacts of the long-term mariculture (S. Liu et al., 2015). Meanwhile, accompanied with the burial of rich organic matter in the sediment, the bacterial communities, a main decomposer (Azam and Malfatti, 2007; Jiao et al., 2010), play vital roles in changing the sediment biochemistry during the long-term mariculture activities. However, our knowledge about the long-term cumulative impacts of mariculture on microbial communities in the sediments is very limited.

Sediments are one of the largest reservoirs of OM in the ocean (Coppola et al., 2018; Falkowski et al., 2000; Keil, 2017). The intensive mariculture system is one of the hotspots with the most intensive biological deposit rates (S. Liu et al., 2015; Xia et al., 2014; Zhang et al., 2017b). The organic-rich deposits are rapidly accumulated in the sediments of the mariculture regions, stimulating benthic mineralization and oxygen depletion (Carlsson et al., 2009). Since microorganisms play essential roles in marine ecosystems through the recycling of dissolved nutrients and the mediating of global biogeochemical flux of carbon (C), nitrogen (N), phosphorus (P), sulfur (S) and iron (Azam and Malfatti, 2007; Jiao et al., 2010), it is important to understand how

benthic microbial communities respond to environmental changes such as the perturbations from anthropogenic maricultural activities (e.g., fishing and mariculture) (Halpern et al., 2008; Nogales et al., 2011). Previous studies about the sedimental microbial community mainly focused on the dynamics of microbial communities across time and space in natural marine ecosystems (Fuhrman et al., 2015; J. Liu et al., 2015; Zhou et al., 2016). The response of sedimental microbes to long-term mariculture, especially in the seaweed dominated IMTA ecosystems, is still unresolved. Only one study reported the total microbial communities in the surface water and sediment of one short-term IMTA system for three months in which IMTA system was practiced by passing aquaculture effluent from three individual ponds for fish, clam and seaweed (*Gracilaria* sp.), respectively. They found that the IMTA system maintained microbial community structure and Proteobacteria and Bacteroidetes were the two dominant phyla in the short-term IMTA system (Ying et al., 2018). In a seaweed (*Gracilaria lemaneiformis*) monocultivation area near the Nan'ao Island, South China Sea, the total microbial communities in the water and surface sediment in the cultivation zone and a control zone were analyzed. They found that the *G. lemaneiformis* cultivation led to different microbial communities between the cultivation zone and the control zone in both water and sediment environments, and seaweed-associated microbial groups (such as *Arenibacter*, *Croceitalea*, *Glaciecola*, *Leucothrix* and *Maribacter*) were enriched in the seaweed cultivation zone (Xie et al., 2017). The active benthic microbes in the IMTA ecosystems have not been studied before. Whether total and active bacterial communities exhibit similarities or differences in diversity and assemblage mechanisms is still unrevealed. To reveal the possible shaping mechanisms of the diversity and biogeography of benthic microbial communities in the long-term IMTA mariculture area, the relative importance of two major processes (environmental filtering versus dispersal limitations) was analyzed (Green and Bohannan, 2006; Hanson et al., 2012; Y. Zhang et al., 2014). Therefore, we hypothesized that total and active benthic microbial community diversity, composition and structure changed greatly owing to the long-term IMTA mariculture and were significantly influenced by different mariculture styles.

In this study, we aimed to understand the cumulative influences of long-term mariculture with two different intensive culture modes, i.e. kelp-scallop co-culture (KS) and kelp mono-culture (KM) on the total and active bacterial communities along the vertical gradient of sediment, as well as the microbial responses to different intensive mariculture modes (KS, KM and out-culture (OC)). The Ailian Bay is a sub-bay of the Sanggou Bay in the Yellow Sea. The kelp *Saccharina japonica* and scallop *Chlamys farreri* have been co-cultured there since 1958 with a cultivation area of approximately 100 km<sup>2</sup> (Fang et al., 2016; Wu et al., 2017). A total of four core sediments were collected in the Ailian Bay. Two were from KS area, and the other two were from the KM and OC area respectively. The total and active bacterial community structure in different layers of core sediments were revealed using the MiSeq sequencing of the 16S rRNA and 16S rRNA gene (V3–4 regions). The

relationships between the bacterial community and environmental factors were analyzed for the assessment of the factors that regulate bacterial dynamics and bacterial–environmental interactions in the core sediments of the intensive mariculture environments with IMTA cultivation system.

## 2. Materials and methods

### 2.1. Site and sampling description

The Ailian Bay (37°09′–37°11′ N, 122°32′–122°35′ E) is a coastal embayment of 5.56 km<sup>2</sup> in Weihai City, Shandong Province, North China. The bay has been heavily exploited for the long-line culture of scallops and kelp for >20 and 50 years, respectively. Four core sediments were taken on 24 April 2015: two cores (S1 with the depth of 39 centimeter below subsurface (cmbsf) and S3 with the depth of 33 cmbsf) were from the kelp-scallop co-culture area (KS) near the shore, one core (S5 with the depth of 30 cmbsf) was from the kelp mono-culture area (KM) further out of the KS area in the bay, and one control core (S8 with the depth of 9 cmbsf) was from the out-culture area (OC) further out of the KM area in the bay (Fig. S1). Undisturbed sediment was retrieved with a KC - Kajak Sediment core sampler (Kajak, Denmark). Sediment was sampled with PVC cores and immediately subsampled at 3 cm depth intervals. Sediment subsamples were frozen at –80 °C for further processing (Guardiola et al., 2016; Hurt et al., 2001; Lever et al., 2015).

### 2.2. Sediment properties and environmental factor measurements

Sedimental grain size was measured using a laser diffraction particle size analyzer (CILAS, CILAS1190L) for size classes ranging from 0.04 to 2500 μm. Grain size was separated as clay, fine silt, and coarse sediments, corresponding to the size range of 0–4 μm, 4–63 μm and >63 μm, respectively. NO<sub>3</sub>-N, NO<sub>2</sub>-N, NH<sub>4</sub>-N and PO<sub>4</sub>-P in sediment samples were extracted with 1 M MgCl<sub>2</sub> using a 1:30 sediment: extractant ratio from lyophilized sediments, and then measured with a nutrient AutoAnalyser (QuAAtro, Bran-Luebbe Inc., Germany). Dissolved inorganic nitrogen (DIN) was the sum of NO<sub>3</sub>-N, NO<sub>2</sub>-N and NH<sub>4</sub>-N. Total organic carbon, total nitrogen and total sulfide of sediments were determined with a Vario EL III Elemental Analyzer (Elementar, Germany). The ages of the core sections were estimated based on the date of the Sanggou Bay using the sedimentation fluxes reported by S. Liu et al. (2015).

### 2.3. DNA and RNA extraction, sequencing and sequence processing

DNA and RNA were extracted using a FastDNA® SPIN Kit for Soil and a FastRNA® Pro Soil Direct Kit (MP Biomedicals, USA), respectively with a Fast Prep-24 Homogenization System (MP Biomedicals, USA) following the manufacturer's protocols. DNA was digested with a RNase-Free DNase Set (Qiagen, Germany) during RNA purification. RNA samples were reverse transcribed into cDNA using the SuperScript First-Strand synthesis system with random hexamers for RT-PCR (Invitrogen, USA). Hypervariable regions V3–V4 of bacterial 16S rRNA genes were amplified using barcode and adaptor added primer 338F (5'-ACTCCTACGGGAGGAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCRs were performed in triplicate 20 μl reactions containing 20–50 ng of template DNA or cDNA. Reaction condition was set as an initial denaturation at 95 °C for 3 min followed by 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and then a final extension was carried out at 72 °C for 10 min. The PCR products were purified using an AxyPrep DNA Gel Extraction Kit (Axygen, USA) and quantified with a QuantiT PicoGreen double-stranded DNA kit (Invitrogen, CA). Equimolar amounts of the purified PCR amplicons from different samples were mixed and sequenced on an Illumina MiSeq PE 300 platform (2 × 300

bases, paired-end) at Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China.

Sequencing paired-end reads from the original DNA/cDNA fragments was trimmed and merged using Trimmomatic (v. 0.32) and FLASH (Fast Length Adjustment of Short reads, v. 1.2.11) software, respectively (Bolger et al., 2014; Magoc and Salzberg, 2011). Previously reported criteria (Sogin et al., 2006) were applied for the assessment of the quality of sequencing reads. We eliminated those sequences that contained more than one ambiguous nucleotide (N), those which did not have a complete barcode, with more than one mismatch to the primer at one end, and those which were shorter than 200 bp after the removal of the barcode and primer sequences. The remaining sequences passing the quality control were assigned to samples by examining the barcode. Quality-controlled sequences were analyzed using USEARCH (v. 7.0) following the standard operating procedure (Edgar, 2013). Briefly, sequences were classified with the SILVA database SSU Ref NR, release 128 (<http://www.arb-silva.de>), and clustered in operational taxonomic units (OTU) at 97% sequence divergence using the furthest neighbor algorithm. To reduce any sequencing- and/or PCR-derived biases we included a chimera removal step to remove unique sequences. Finally, all 16S rRNA gene sequences classified as mitochondria, chloroplasts and Archaea were removed from the dataset. Illumina sequencing raw data of DNA and cDNA have been deposited at the National Center for Biotechnology Information (NCBI) Short Read Archive database under accession number PRJNA524261 and PRJNA524262, respectively.

### 2.4. Statistical analysis

Principal co-ordinate analysis (PCoA) (Anderson and Willis, 2003; Borcard and Legendre, 2002) and analysis of similarity (ANOSIM) (Clarke et al., 2014) were used for the determination of the similarity of samples to each other based on weighted UniFrac distance matrices (Lozupone et al., 2011). One-way ANOVA was used for the determination of the differences of relative abundance of dominant bacterial communities at phylum, class, genus and OUT levels between different mariculture modes (Qu et al., 2018). Spearman correlation analysis was used for the determination of the correlations between bacterial communities and environmental factors. Linear regression was used for the analysis of the relationships between depth and Bray-Curtis similarity of bacterial communities. Canonical correspondence analysis (CCA) was used for the further analysis of variations in total and active bacterial communities under the constraint of environmental factors with CANOCO 5.0 software (Microcomputer Power, Ithaca, USA) (Ter Braak, 1989). A total of 13 environmental variables were used for the assessment of the variations of total and active bacterial communities, including longitude, latitude, depth, TOC, TN, TS, NO<sub>3</sub>-N, NO<sub>2</sub>-N, NH<sub>4</sub>-N, PO<sub>4</sub>-P, clay, fine silt and coarse sediment. All variables were logarithmically (base 10) transformed before CCA to reduce the influence of extreme values on ordination scores and guarantee normal data distribution. Stepwise procedure 'forward selection' was used to select the significantly explanatory variables ( $P < 0.05$ ). Only significant explanatory parameters ( $P < 0.05$ ) without multicollinearity (variance inflation factor < 20) were used for the CCA analysis (Ter Braak, 1986).

The relative importance of the spatial and environmental factors as well as their covariations, responsible for explaining differences in community composition were identified using variation partitioning analysis (VPA) with adjusted  $r^2$  coefficients based on CCA with stepwise procedure 'forward selection' (Mo et al., 2018; Økland and Eilertsen, 1994; Peres-Neto et al., 2006). In this analysis, the residual fraction represents the unexplained variance. The set of spatial variables was calculated based on the longitude, latitude and depth coordinates of each sampling station following the approach of the principal coordinates of neighbor matrices (PCNMs) analysis (Dray et al., 2006; Legendre et al., 2008).

## 2.5. Network analysis

Potential interactions between bacterial taxa were determined through the modelling of the microbial community in a network structure to decipher the structure of complex communities across spatial gradients (Deng et al., 2012; Zhou et al., 2010; Zhou et al., 2011). To reduce the complexity of the data sets, OTUs present in more than eight samples with >20 sequences were retained for the construction of networks. A total of 255 OTUs were used in the network analysis. Subsequently, all possible pairwise Spearman's rank correlations ( $r$ ) between those OTUs were calculated within the “psych” R package. Only robust ( $r > 0.6$  or  $r < -0.6$ ) and statistically significant ( $P$ -value  $< 0.05$ ) correlations were incorporated into the network analysis. Network visualization and modular analysis were performed with Gephi version 0.9.2. In a network graph, each node represents an OTU indicating an individual taxon. The edge between every two nodes represents positive or negative interactions between those two taxa.

## 3. Results

### 3.1. Physicochemical characteristics of sediments

A total of 38 samples from 4 core sediments were collected from the Ailian Bay, of which 25 samples were from the KS area while ten were from the KM area and three from the OC area. The length of the core sediments ranged from 9 (S8 in OC) to 39 cm (S1 in KS). Fine silt sediments made up the prominent component of the grain size ( $0.77 \pm 0.06$ ) in the study area. Coarse sediments were detected in the KM and OC areas with small sizes ( $0.01 \pm 0.02$ ). TOC ranged from 3.1 to  $11.1 \text{ mg g}^{-1}$  in the study area (Fig. 1A), which was significantly higher in the KS area than in the KM and OC areas (ANOVA,  $P < 0.001$ ). DIN ranged from 0.38 to  $0.75 \text{ } \mu\text{M g}^{-1}$  and was mainly composed of  $\text{NH}_4\text{-N}$  (averaging 79.7%, Fig. 1E).  $\text{PO}_4\text{-P}$  ranged from 0.17 to  $0.76 \text{ } \mu\text{M g}^{-1}$  and was significantly higher in the KS area than in the KM and OC areas (ANOVA,  $P < 0.001$ , Fig. 1F).

The sedimentation rates at the two stations in the adjacent Sanggou Bay were  $0.726$  and  $0.593 \text{ cm year}^{-1}$ , respectively (S. Liu et al., 2015). As

the Ailian Bay is one of the sub-bays of the Sangou Bay with similar mariculture history (Xia et al., 2014) and our sampling stations are only three to five kilometers from the Sangou Bay, we assumed that the sedimentation rates in the Ailian and Sanggou Bays were similar (on average  $0.6595 \text{ cm year}^{-1}$ ), the estimated ages of the core sediments S1, S3, S5 and S8 in the Ailian Bay were on average approximately 64 (ranging from 54 to 66), 50 (ranging from 45 to 56), 50 (ranging from 45 to 56) and 14 (ranging from 12 to 15) years, respectively. The core sediments covered the long-term mariculture history (approximately 50 years) (Fang et al., 2016), which could reflect the influences of the mariculture on the benthic microbial communities in the Ailian Bay.

### 3.2. Total and active benthic bacterial diversity and community structure

In total, 61 bacterial communities (38 total and 23 active) were analyzed through MiSeq sequencing of 16S rRNA gene amplicons. Over 2.05 million high quality sequences were obtained across all the 61 samples. After the random resampling of 20,000 reads per sample, a total of 8281 OTUs were assigned at 3% dissimilarity threshold. These OTUs were classified into 61 phyla, 157 classes, 313 orders, 610 families and 1326 genera, indicating taxonomically diverse microbial communities in the sediments of the Ailian Bay. 4062 OTUs (49.0%) shared by the total and active bacterial community, and 4219 unique OTUs were detected with 1347 OTUs (16.3%) in the total bacterial community and 2872 OTUs (34.7%) in active bacterial community. Both the Shannon and Simpson diversity indexes showed noticeable differences ( $P < 0.001$ , Wilcoxon's rank test) between the total and active bacterial communities; however, the two diversity indexes showed no remarkable differences ( $P > 0.05$ , Wilcoxon's rank test) across different mariculture modes for the total and active bacterial community, respectively (Fig. S1).

Variations in the total and active bacterial community structure were analyzed based on PCoA. Total and active bacterial communities were separated into two clusters (Fig. 2A), while the bacterial communities could be divided into three clusters according to different mariculture modes at the total and active levels, respectively (Fig. 2B and

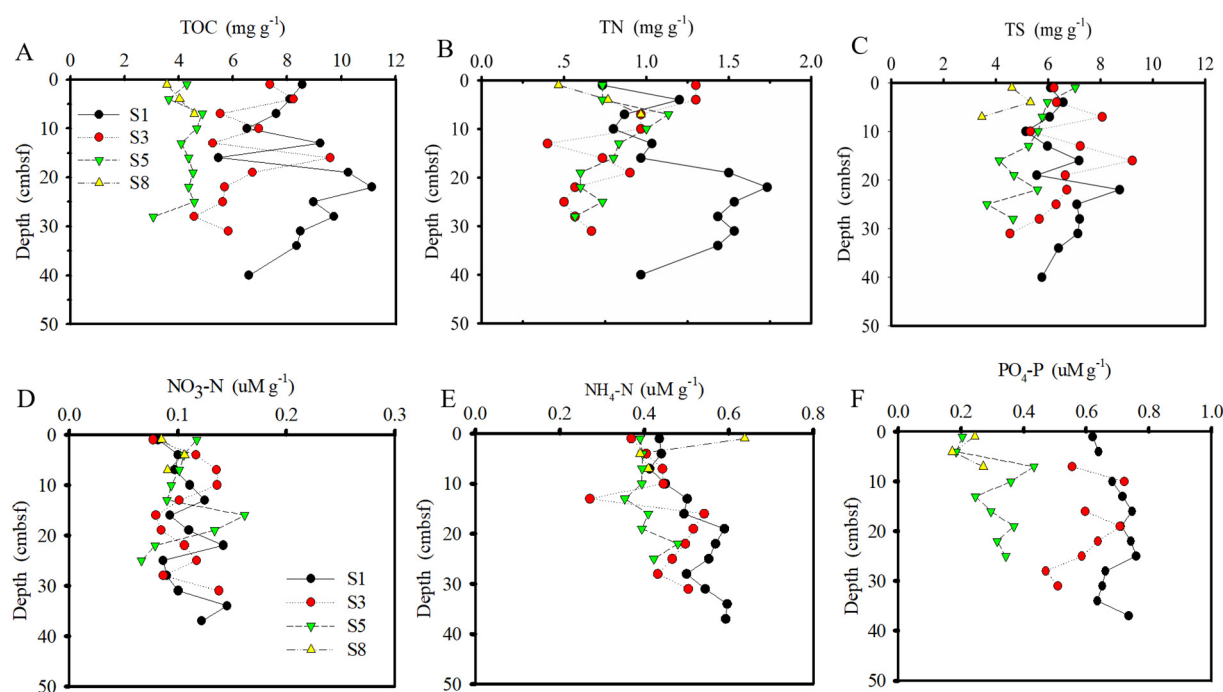
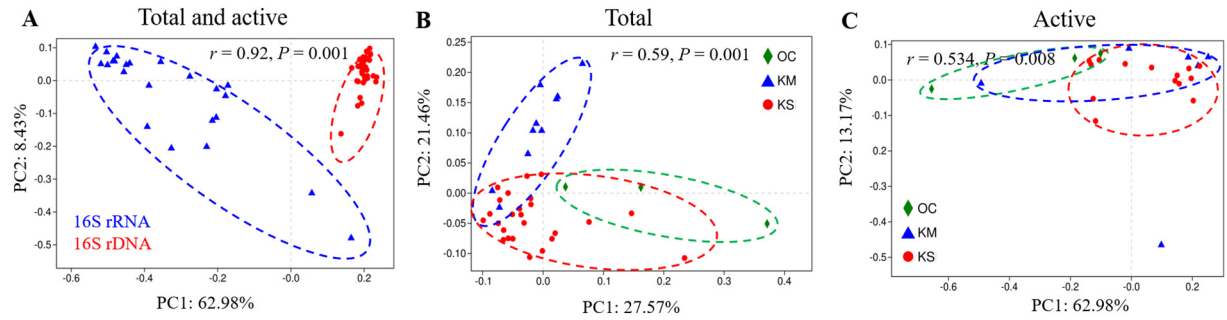


Fig. 1. Vertical distributions of the environmental factors in the sediments of the Ailian Bay, China.





**Fig. 2.** Results of principal co-ordinate analysis (PCoA) of the total and active bacterial communities (A), of the total bacterial community in different mariculture modes (B) and of the active bacterial community in different mariculture modes (C). The  $r$  and  $P$  values of the analysis of similarity (ANOSIM) were shown based on weighted UniFrac distance matrices. Abbreviations: KS: kelp-scallop co-culture, KM: kelp mono-culture culture, OC: out-culture.

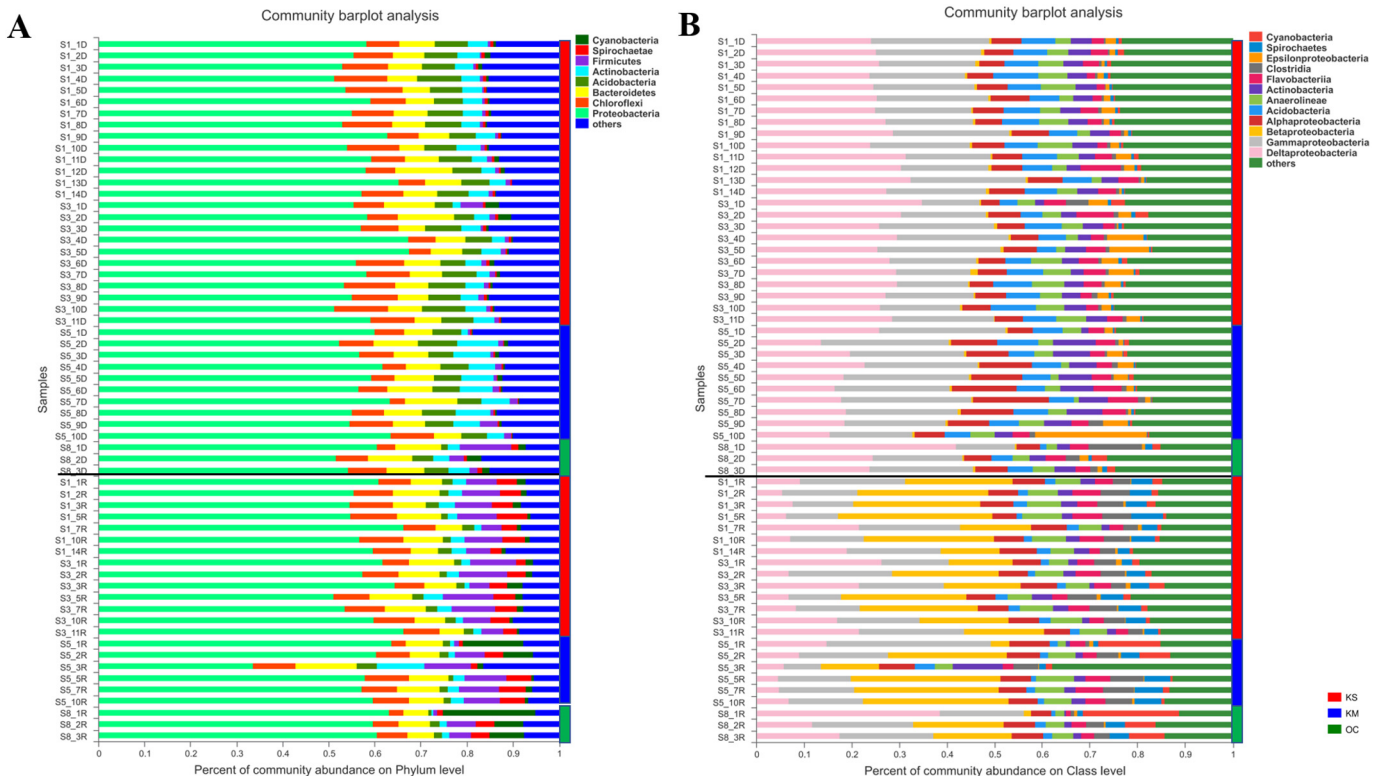
C). Significant differences between the total and active bacterial communities ( $P = 0.001$ ) and among the KS, KM and OC modes of the total and active bacterial communities were obtained using ANOSIM ( $P = 0.001$  and  $P = 0.008$ , respectively, Fig. 2).

The dominant phyla (plus proteobacterial classes, relative abundance > 5%) of the total and active bacterial communities were Proteobacteria ( $\delta$ -Proteobacteria,  $\beta$ -Proteobacteria,  $\gamma$ -Proteobacteria and  $\alpha$ -Proteobacteria), Chloroflexi, Bacteroidetes, Acidobacteria, Actinobacteria and Firmicutes (Fig. 3A). Most of the dominant phyla and classes of the total and active bacterial communities showed significant differences ( $P < 0.01$ , Fig. 4).  $\gamma$ -Proteobacteria,  $\delta$ -Proteobacteria, Acidobacteria, and Actinobacteria were more abundant in the total bacterial community than in the active bacterial community, while  $\beta$ -Proteobacteria, Anaerolineae, Clostridia, Spirochaetes and Cyanobacteria were enriched in the active bacterial community ( $P < 0.01$ , Fig. 4).

### 3.3. Changes of dominant microbial taxa in response to mariculture modes

For the dominant phyla (plus proteobacterial classes, relative abundance > 5%) of the total bacterial community, the relative abundance of  $\delta$ -Proteobacteria was higher in the OC than that in the KS and KM ( $P < 0.01$ ) and  $\alpha$ -Proteobacteria was more abundant in the KM ( $P < 0.05$ ) (Fig. S3A) than that in the others. For the dominant phyla or classes (relative abundance > 5%) of the active bacterial community, there were no significant variations in the active bacterial community (Fig. S3B).

At the genus level, higher abundances of *Desulfobulbus* and *Sva0081* sediment group in the total bacterial community were observed in the KS than in the KM and OC ( $P < 0.05$ ), while those of *Sulfurovum* and unclassified group of *Sva0725* were higher in the KM than those in the KS and OC ( $P < 0.05$ ) (Fig. 5A). In the active bacterial community, unclassified group of Anaerolineaceae, *Pseudoalteromonas*,



**Fig. 3.** Bacterial community composition in the sediment samples at the phylum (A) and class (B) levels. Abbreviations: KS: kelp-scallop co-culture, KM: kelp mono-culture culture, OC: out-culture.

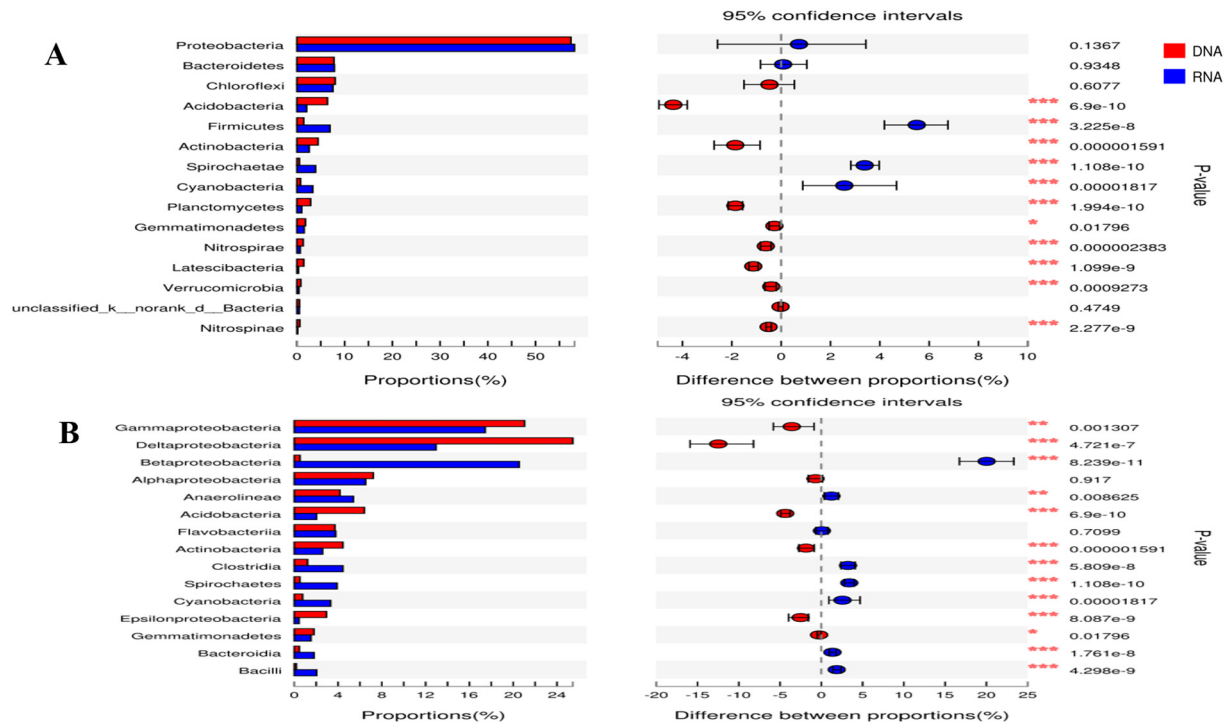


Fig. 4. Comparisons of dominant total and active benthic bacterial community at phylum (A) and class (B) level.

*Desulfobulbus*, *Leptonema* and unclassified group of ns9 marine group were the dominant genus, and higher abundances of *Desulfobulbus* and *Woeseiaceae/JTB255 marine benthic group* were observed in the OC than those in the KS and KM ( $P < 0.05$ ) (Fig. 5B).

To demonstrate the effects of different mariculture modes on total and active bacterial community composition, further analyses were

performed for the identification of the significantly changed shared OTUs and unique OTUs in the KS, KM and OC. For the fifteen abundant shared OTUs of the total bacterial community, long-term mariculture caused an increase in the relative abundances of OTU4105, OTU7279, OTU126, OTU1931, OTU1274, OTU1166, OTU6776, OTU4085, OTU6375 and OTU6631 associated with genus *Sulfurovum*, *Woeseiaceae/JTB255*

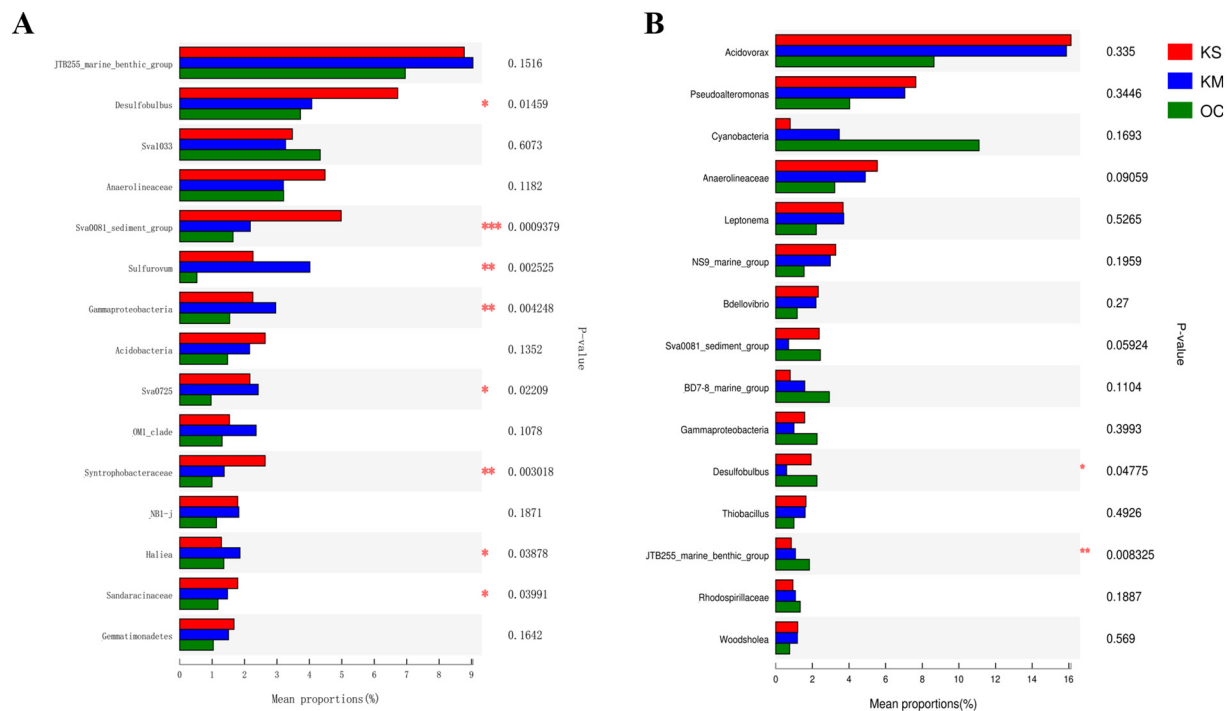


Fig. 5. Changes of dominant genus in different mariculture modes at the total (A) and active (B) bacterial community level. The names of the lowest certain taxonomic level were showed for the unclassified taxonomic groups at the genus level. Abbreviations: KS: kelp-scallop co-culture, KM: kelp mono-culture culture, OC: out-culture.

marine benthic group, *Desulfobulbus*, unclassified group of Sandaracinaceae, unclassified group of Syntrophobacteraceae and *Methyloceanibacter* in the KS and KM core sediments ( $P < 0.05$ , Fig. S4A). For the fifteen abundant shared OTUs of active bacterial community, only OTU6870 associated with genus unclassified group of Anaerolineaceae increased in the KS and KM core sediments ( $P < 0.05$ , Fig. S4B). Among the unique OTUs of the total bacterial community, higher abundances of OTU4687, OTU6948, OTU2781, OTU5208 and OTU572 associated with genus *Brevundimonas*, *Achromobacter*, *Coxiella*, *Lactobacillus* and unclassified group of Aminicenantes were observed in the KS core sediments; the unique OTUs in the KM core sediments were associated with genus *Paenibacillus*, *Sphingomonas*, *Halocella*, and *Formosa* (OTU5527, OTU1014, OTU5781 and OTU7164); while for the OC core sediments, the unique OTUs were associated with genus unclassified group of *Desulfobacteraceae*, unclassified group of NB1-n, unclassified group of WS6, unclassified group of Gracilibacteria and *Sphaerochaeta* (OTU7334, OTU7625, OTU7311, OTU7396 and OTU7953).

### 3.4. Cumulative impacts of long-term mariculture on the benthic bacterial community in the core sediments

The relationships between depth and beta-diversity of benthic bacterial community were analyzed to reveal the cumulative impacts of long-term mariculture on the benthic bacterial community in the core sediments of the Ailian Bay, North China. The regression analysis of total and active benthic bacterial communities showed that only the total bacterial community had significant negative slopes, indicating that the total rather than the active bacterial community was significantly influenced by the cumulative impacts of the long-term mariculture (Fig. 6). For the rare OTUs of active benthic bacterial community, there was no significant relationship between similarity and depth (data not shown).

For the dominant total benthic bacterial community, only limited bacterial populations showed significant variations among the surface, middle and bottom samples of the KS core sediments at the phylum (Spirochaetae,  $P < 0.01$ ), genus (*Sva0081* sediment group,  $P < 0.01$ ;

unclassified group of Gammaproteobacteria,  $P < 0.01$ ; and unclassified group of Sandaracinaceae,  $P < 0.01$ ) and OTU (OTU 1931,  $P < 0.05$ ; OTU 1274,  $P < 0.01$ ; OTU 3334,  $P < 0.001$ ; and OTU 4630,  $P < 0.05$ ) levels (Fig. S5), while no significant variations were observed among the surface, middle and bottom samples of the KM core sediments (Fig. S6). For the dominant active benthic bacterial community at the phylum, genus and OTU levels, only the phylum Cyanobacteria ( $P < 0.05$ ) showed significant variations among the surface, middle and bottom samples of the KS core sediments (Fig. S7), while no significant variation was observed between the surface and middle samples of the KM core sediments (Fig. S8).

### 3.5. Relationships between the total and active bacterial community and environmental factors

For the identification of how environmental factors affecting the sedimental bacterial community composition and structure, Spearman correlation analysis and CCA analysis were conducted. The heatmap plots based on the Spearman correlations showed that the dominant bacterial genus and OTUs exhibited a generally positive correlation with  $\text{PO}_4\text{-P}$ , TOC, clay, TS, depth, latitude and  $\text{NH}_4$  ( $r > 0.5$ ,  $P < 0.01$ ), and a negative correlation with coarse sediment, fine silt and longitude ( $r < -0.5$ ,  $P < 0.01$ ) at the total bacterial community level (Figs. 7A and S9A). For the active bacterial community, only several dominant bacterial genus and OTUs exhibited a positive correlation with  $\text{NH}_4\text{-N}$ , clay and TOC ( $r > 0.5$ ,  $P < 0.05$ ), and a negative correlation with  $\text{PO}_4\text{-P}$  and coarse sediment ( $r < -0.5$ ,  $P < 0.05$ ) (Figs. 7B and S9B).

The CCA results showed that the first CCA axis explained 10.0% and 14.4% of the variations of the total and active bacterial community, respectively. The first two CCA axis explained 17.3% and 21.2% of the variations of the total and active bacterial community, respectively (Fig. 8). The bacterial community was significantly correlated with coarse sediment, longitude, latitude,  $\text{NH}_4\text{-N}$  and  $\text{PO}_4\text{-P}$  at the total bacterial community level and with coarse sediment and  $\text{NH}_4\text{-N}$  at the active bacterial community level (Fig. 8), which is consistent with the Spearman correlation analysis (Figs. 7 and S9). Though TOC is a significant factor correlated with the total and active bacterial

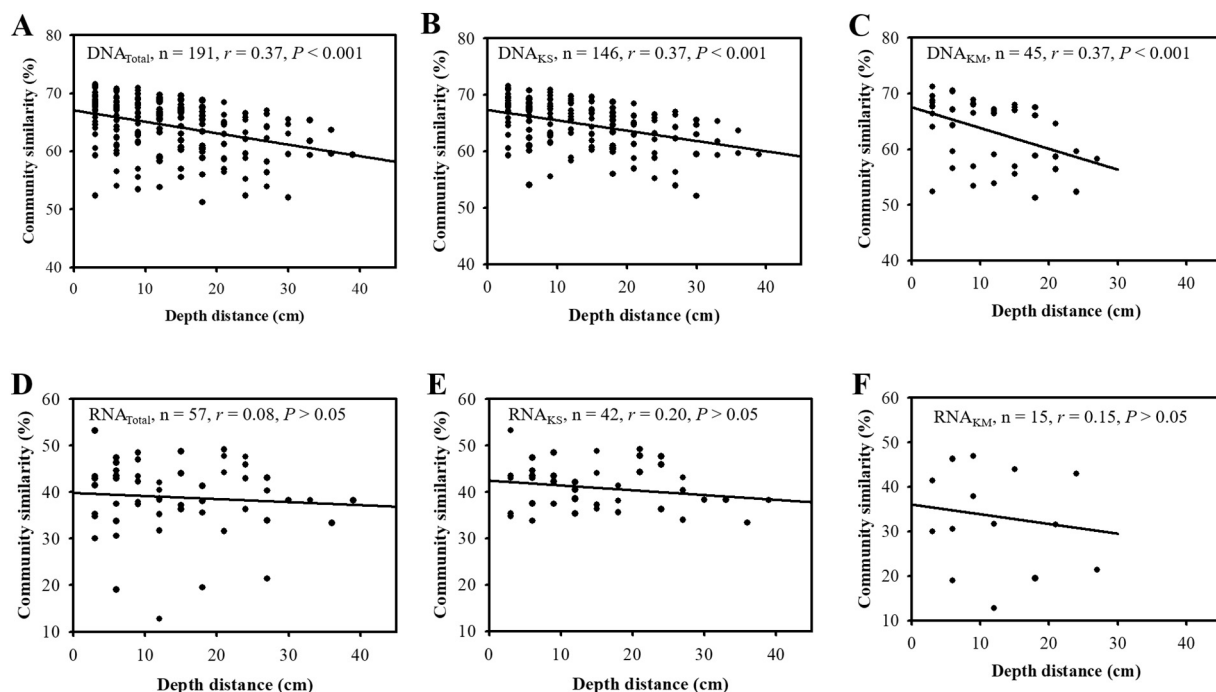
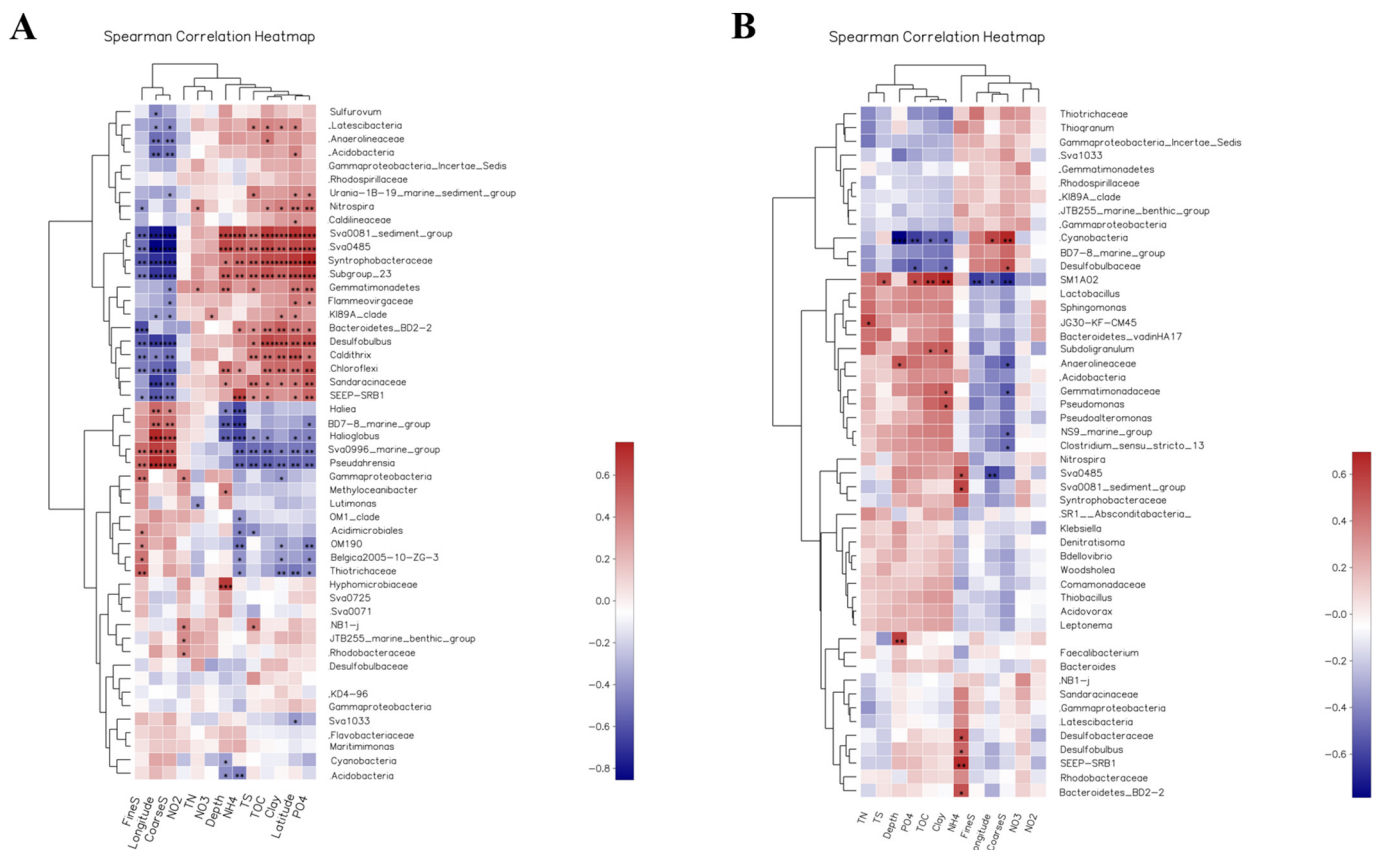


Fig. 6. Regression analysis of total beta-diversity for total (A–C) and active (D–E) bacterial communities. Abbreviations: KS: kelp-scallop co-culture, KM: kelp mono-culture culture, Total: the datasets of KS and KM.

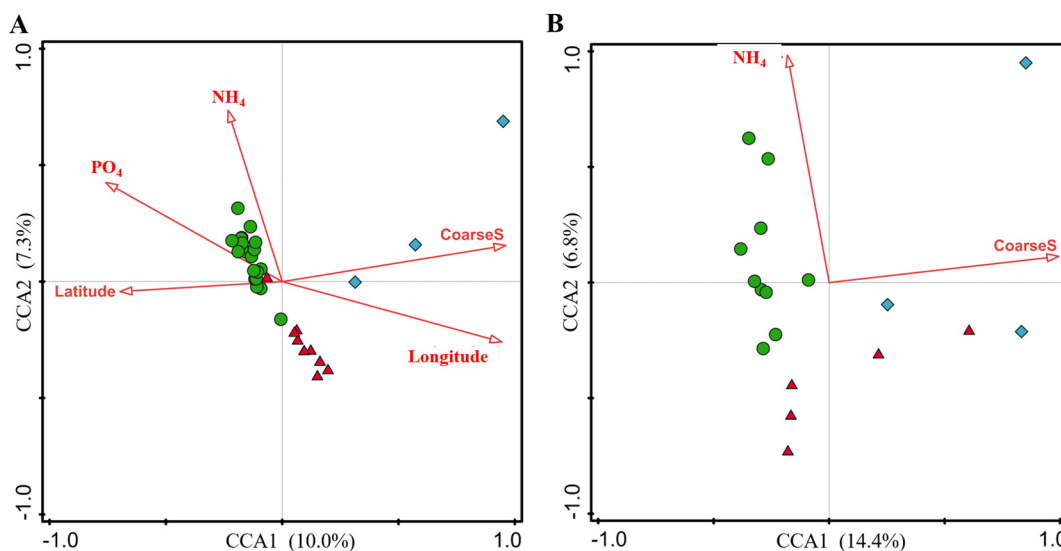


**Fig. 7.** Heatmaps indicate the correlations between dominant genus (The names of the lowest certain taxonomic level were showed for the unclassified taxonomic groups at the genus level) and environmental factors at the total (A) and active (B) bacterial community level. The color of the scale bar indicates the *r* value. \* indicates  $0.01 < P \leq 0.05$ , \*\* indicates  $0.001 < P \leq 0.01$ , \*\*\* indicates  $P \leq 0.001$ .

community ( $P < 0.01$ ), it was not used into the optimal models after the stepwise forward selections.

For a better assessment of the effects of spatial distances (as a proxy for dispersal barriers) and sediment environmental factors on the variations of the bacterial communities, variance partitioning analysis (VPA) was conducted and it showed that both total and active bacterial communities exhibited a slightly stronger response to environmental factors than to spatial (distance) ones (Fig. S10). For the total bacterial

community, the sediment environmental factors explained 7.9% of the variations, the spatial variables alone explained 0.3%, and the environmental and spatial variables co-explained 2.5% (Fig. S10A). For the active bacterial community, the sediment environmental factors explained 8.0% of the variations, the spatial variables alone explained 0.1%, and the environmental and spatial variables co-explained 2.7% (Fig. S10B). However, large fractions of the variations of total and active bacterial communities (89.2% and 89.2%, respectively) were



**Fig. 8.** Canonical correspondence analysis (CCA) of OTUs and environmental factors at the total (A) and active (B) bacterial community level.



**Table 1**

The topological properties of the networks of total and active benthic bacterial communities in the Ailian Bay, North China.

Network metrics	Groups	
	Total	Active
Number of nodes	195	251
Total number of edges	1136	3804
Number of positive correlations	631	2674
Number of negative correlations	310	879
Average path length	3.101	2.57
Network diameter	8	8
Average clustering coefficient	0.624	0.639
Modularity	1.388	0.442
Average degree	11.651	30.311
Average weighted number	6.733	22.489
Connect components	28	22

unexplained (Fig. S10). Overall, sediment environmental factors are more important than geographic distance in shaping both total and active bacterial community structure in the Ailian Bay.

### 3.6. Distinct relation network for the total and active benthic bacterial community

Ecological network analysis was applied to the total and active benthic bacterial communities. All curves of network connectivity were fitted with the power-law model ( $r > 0.6$ ). Substantial differences between the total and active bacterial libraries were observed regarding network size and structure (Fig. S11 and Table 1). The active bacterial community displayed tighter interactions than the total bacterial community as revealed by average path distance (Fig. S11 and Table 1). Notably, there were contrasting interactions among bacterial taxa within the total and active communities. The linkages in the active bacterial networks (number of nodes, edges, positive and negative correlations were 251, 3804, 2674 and 879, respectively) were significantly higher than those in the total bacterial ones (number of nodes, edges, positive and negative correlations were 195, 1136, 631 and 310, respectively) (Fig. S11 and Table 1). Positive connections dominated the interactions between taxa in the total and active bacterial networks (55.55% and 70.29%, respectively) (Fig. S11A and Table 1), suggesting that the effects of mutualism might dominate the total and active bacterial networks in the Ailian Bay. Negative connections were more abundant in the total bacterial networks (27.29%) than those in the active bacterial networks (23.11%) (Fig. S11B and Table 1), suggesting there were more competitive effects in the former and more effects of mutualism in the latter ones.

## 4. Discussion

Microbes which are sensitive to environmental changes play a significant role in mediating biogeochemical cycles in the pelagic and benthic ecosystems (Azam and Malfatti, 2007; Jiao et al., 2010). Though the bacterial community structures are closely related to the changes in the mariculture species and environments (Auffret et al., 2013; Dowe et al., 2015; Xie et al., 2017; Ying et al., 2018; H. Zhang et al., 2016), we have little knowledge about the influences of long-term IMTA mariculture on microbial communities, especially with regards to the sediment microbiota. In this study, we demonstrated that the scallop and seaweed mariculture changed the diversity, composition and structure of the total and active benthic bacterial communities in a long-term IMTA mariculture system in the Ailian Bay, North China. This study provides new insights into benthic microbial communities in IMTA mariculture systems and the impact of shellfish and seaweed mariculture on coastal benthic ecosystems.

### 4.1. Influences of long-term mariculture of kelp and scallop on the environments

Physical and chemical characteristics of sediments can provide essential information about the impact of long-term intensive mariculture on benthic ecosystems and reflect the environmental conditions influenced by the benthic microbial geochemical processes (Li et al., 2013). Large-scale kelp cultivation has been developed as a healthy and ecologically beneficial mariculture mode in the amelioration of the environmental problems caused by the intensive mariculture of commercial animals (e.g., scallop, abalone and fishes, etc.) (Fang et al., 2016). Kelp cultivation could decrease large amounts of nutrients related to eutrophication, release dissolved oxygen and increase total alkalinity to buffer ocean acidification (Li et al., 2017; Li et al., 2018; Li et al., 2016; J. Zhang et al., 2016). Kelp and seaweed cultivation could inhibit the growth of harmful algae through nutrient competition and the excretion of algal-inhibiting allelochemicals (Lu et al., 2011; Tang and Gobler, 2011; Wang et al., 2007). Because of the high bioremediation efficiency and large cultivation area in China, kelp has been introduced into the IMTA systems as the prominent primary producer (Fang et al., 2016; J. Zhang et al., 2016), which could reduce the concentrations of nutrient and pollutants and improve the water quality (Li et al., 2017; Li et al., 2016; Mao et al., 2009).

Scallop and kelp co-cultivation increased the TOC concentration and decreased the grain size in the sediments (Fig. 1), which is similar with the research results in the Sanggou Bay (S. Liu et al., 2015; Ning et al., 2016; Xia et al., 2014). The accumulation of fecal pellets from the scallop could result in high TOC concentration in the KS seawater and sediments (Li et al., 2018; Ning et al., 2016; Xia et al., 2014). The concentrations of TOC and nutrient in the KM area were similar with those of the surface sediments in the Yellow Sea, Bohai Sea and Jiaozhou Bay (Hu et al., 2016; Kang et al., 2017; S. Liu et al., 2015), indicating that the kelp culture did not significantly change the physical and the chemical characteristics of sediments, which is similar with the research results in the Sanggou Bay (S. Liu et al., 2015).

### 4.2. Total and active benthic bacterial communities displayed different diversity and structure

The diversity and structures between the total and active benthic bacterial communities differed substantially (Figs. 2–4, S2). There was a significant division between the total and active benthic bacterial communities and higher species richness in the bacterial DNA libraries than in the RNA libraries was observed (Fig. S2), which is consistent with the results of the study in the salt marsh sediments and seawaters of South China Sea (Kearns et al., 2016; Y. Zhang et al., 2014). The bacterial diversity in sediments was usually higher than that in the seawaters of the mariculture systems (Xie et al., 2017; Ying et al., 2018). The high diversity of the total benthic bacterial community indicated that the sediments might act as a 'seed-bank' of microorganisms (Xie et al., 2017; Y. Zhang et al., 2014), and thus influence the dynamics of pathogenic and probiotic bacterial genera in the mariculture environments (H. Zhang et al., 2016). The high diversity of the total benthic bacterial community may be determined by the accumulated genetic material and slow selection under low evolutionary pressure induced by constant environmental conditions over long timescales in the sediments (Ghiglione et al., 2012).

The dominant total bacterial phyla or classes are in sync with the results in natural surface sediments of the adjacent Yellow Sea (Fig. 3A) (J. Liu et al., 2015), while the knowledge about the active bacteria phyla in the sediments of the adjacent seas is limited. In this study, most of the dominant total and active bacterial communities showed significant differences at both phylum and class level (Fig. 3B), suggesting that the structure of the active community does not reflect the total community and different mechanisms controlling the total and active bacterial community diversity (Kearns et al., 2016; Y. Zhang et al., 2014). The results

of network analysis also showed that the community network size and structure differed greatly between the total and active communities (Fig. S11 and Table 1), showing that the total and active bacterial communities might be shaped by different community assemblage mechanisms. A more complex and tighter network was observed in the active bacterial community than in the total bacterial community (Table 1), which is similar with the results of the study in the seawaters of South China Sea (Y. Zhang et al., 2014). Positive interactions dominated both the total and active communities in the core sediments of the Ailian Bay, which might be owing to the effects of mutualism between species in the stable and nutrient-rich environments.

The relative importance of environmental filtering versus dispersal limitations for shaping the diversity and biogeography of microbial communities was analyzed through VPA (Fig. S10) (J. Liu et al., 2015; Y. Zhang et al., 2014). In the Ailian Bay, the total and active bacterial communities were strongly influenced by the environmental filtering but weakly influenced by the dispersal limitations (Fig. S10), which has also been shown in the past studies in the East China Sea (Xiong et al., 2014). The shorter distance among the three sample stations might account for the weak connection between the bacterial community and dispersal limitations (distance factor). The total bacterial community showed more close relation to the environmental factors, e.g., PO<sub>4</sub>-P, NH<sub>4</sub>-N, depth, TOC and grain size, while the active bacterial community was associated with TOC, NH<sub>4</sub>-N and coarse sediment (Figs. 7, 8 and S9). Grain size was correlated to the total bacterial community in the Ailian Bay, which is similar to that in the surface sediments of north Chinese marginal seas (J. Liu et al., 2015). TOC, PO<sub>4</sub>-P and NH<sub>4</sub>-N, which are the resource supplies to the benthic microbial communities, are correlated to the total bacterial community (Figs. 7A and S9A), which is similar with the results of the surface sediments in East China Sea (Xiong et al., 2014). The active bacterial community is more likely to be related to the heterogeneity of available organic substrates introduced by both the solubilization of particulate organic matter and the release of dissolved organic matter (Azam and Malfatti, 2007; Dang and Lovell, 2016; Jiao et al., 2010) within the sediments. Though it was reported that nitrogen addition could decrease the diversity of the active microbial community (Kearns et al., 2016), the active bacterial community was not well related to the nitrogen concentration in the core sediments of the Ailian Bay (Figs. 7B and S9B). Anaerobic bacteria taxa were abundant and ubiquitous in the core sediments (Figs. 3 and 5). Hence, the oxygen concentration in the core sediments might be an important environmental factor in influencing the assembly of the benthic bacterial community. In the future, research on more environmental factors is supposed to be carried out for a better understanding of the influences of the unobserved sedimental environmental factors on microbial communities, such as oxygen concentration, sulfate concentration and heavy metals, etc.

#### 4.3. Long-term cultivation of kelp and scallop altered the benthic microbial community composition and structure

The long-term intensive culture of kelp and scallop significantly altered the  $\beta$ -diversity of the total and active bacterial community structure in the sediments with different mariculture modes (Fig. 2). Certain microbial groups were stimulated in response to kelp and scallop cultivation (Figs. 5, S2 and S3). For the total bacterial community,  $\delta$ -Proteobacteria was higher in the OC and generally functioned as anaerobic sulfate- and sulfur-reduction, while  $\gamma$ -Proteobacteria and  $\alpha$ -Proteobacteria were abundant in the KM and had the potential of sulfur- and methane-oxidization, nitrate assimilation and nitrogen fixation (Fig. S3A). Similarly,  $\gamma$ -Proteobacteria and  $\delta$ -Proteobacteria were the dominant total bacterial groups in other coastal sediments all over the seas in China (Kim et al., 2008; J. Liu et al., 2015; Wang et al., 2013; Xiong et al., 2014; Zhang et al., 2017a; Zhu et al., 2013). Based on the functional study of the microbial community using metagenomic sequencing, COG and SEED annotations and network analysis, it was

found that the microbial community assemblage was based mainly on functions rather than phylogenetic similarity and could adapt to new environments by changing community structure and composition (Burke et al., 2011; Fuhrman et al., 2015; Langenheder and Székely, 2011; Xie et al., 2017; Yooseph et al., 2010). For the active bacterial community, no significant variations in the bacterial communities at the phylum level (plus proteobacterial classes) were observed along the culture mode, suggesting that the high concentrations of organic matter and nutrient in the Ailian Bay could not constrain the growth of the benthic bacteria and thus significantly influence the variations of the active bacterial community (Fig. S3B).

Similarly, among the three studied zones, there was a significant discrepancy of genera in the total bacterial community rather than in the active bacterial community (Fig. 5). For the total bacterial community, KS had larger percentages of *Desulfobulbus* and *Sva0081 sediment group* (Fig. 5A), which play an essential role in reducing sulfite, sulfate, nitrate, carbon dioxide, hydrogen consumption and carbon remineralization in marine sediment (Dykma et al., 2018; Probandt et al., 2017) and may be correlated with high concentrations of TOC, TS and TN in the KS sediments (Figs. 1, 7A). The abundances of *Sulfurovum* and unclassified group of *Sva0725* were higher in the KM sediments (Fig. 5A). *Sulfurovum* within the  $\epsilon$ -Proteobacteria was firstly isolated from the Okinawa Trough hydrothermal sediments (Inagaki et al., 2004), and are the mesophilic chemolithoautotrophic microorganisms capable of oxidizing sulfur coupled to the reduction of nitrate (Giovannelli et al., 2016; Inagaki et al., 2004; Mori et al., 2018). They are also capable of reducing sulfur, nitrate, thiosulfate and oxidizing hydrogen (Mino et al., 2014). As intensive mariculture activities in the Ailian and Sanggou Bays significantly promoted sulfide accumulation (Kang et al., 2016), the bacterial genera with the function in the sulfide cycling, e.g. *Desulfobulbus*, *Sva0081 sediment group* and *Sulfurovum*, were enriched in the KS and KM zones compared with the OC station (Fig. 5), which is similar with the results of the sediments in the large-scale seaweed cultivation area of the Nanao Island, South China Sea (Xie et al., 2017). *Sva0725*, belonging to the phylum Acidobacteria, was firstly detected in permanently cold shelf sediments off Svalbard (Ravenschlag et al., 1999) but also detected in the coastal and deep sea sediments (Edgcomb et al., 2013; Polymenakou et al., 2015; Zeng et al., 2011). The genomic information of marine Acidobacteria is minimal comparing to that of the soil habitats. The only cultured aerobic marine strains of Acidobacteria are attached to algal and sponge surfaces and can utilize several amino acids (Fukunaga et al., 2008; Green et al., 2015; Mohamed et al., 2008). The sinking kelp debris in the sediments might provide a unique and suitable habitat for the growth of *Sva0725*.

Most enriched OTUs in the total bacterial community at KS and KM (Fig. S4A) were affiliated to microorganisms for sulfide-reduction or oxidization (*Sulfurovum* OTU4105, *Desulfobulbus* OTU126/OTU1931, unclassified group of Syntrophobacteraceae OTU4085/OTU6631) (Kuever, 2014; Liu and Conrad, 2017), sulfite-oxidization, chemolithoautotrophy, denitrification, organic matter consumption (*Woeseiaceae/JTB255 marine benthic group* OTU7279/OTU1166/OTU6776) (Du et al., 2016; Mußmann et al., 2017), low molecular weight organic matter consumption (unclassified group of Sandaracinaceae OTU1274) (Mohr et al., 2012; Probandt et al., 2017), and methanol-oxidization (*Methyloceanibacter* OTU6375) (Takeuchi et al., 2014). Only one enriched OTU in the active bacterial community (unclassified group of Anaerolineaceae OTU6870) was observed (Fig. S4B) and it had the potential metabolic ability of methanogenic alkanes-degradation (Liang et al., 2016). The enriched bacterial groups at the genus and OTU levels were consistent with the promoted sulfide and organic matter accumulation in the intensive mariculture areas in the Ailian and Sanggou Bays (Figs. 5, S3) (Kang et al., 2016). The unique OTUs at KS have the metabolic potentials for denitrification (*Brevundimonas* OTU4687 and *Achromobacter* OTU6948) (Tsubouchi et al., 2014; Vandamme et al., 2016; Z. Zhang et al., 2014), probiotic *Lactobacillus* OTU5208 (H. Zhang et al., 2016), potential anaerobic/facultative metabolism unclassified group of *Aminicenantes* OTU572 (Farag

et al., 2014) and potential animal pathogen (*Coxiella* OTU2781) (Eldin et al., 2017). The unique OTUs at KM have the metabolic potential for algal, cellulose and polysaccharide degradation (*Formosa* OTU7164, *Halocella* OTU5781 and *Paenibacillus* OTU5527) (Choi et al., 2004; Tanaka et al., 2015; Xu et al., 2018), antimicrobial substances production and plant growth enhancement (*Paenibacillus* OTU5527) (Ryu et al., 2003; Weid et al., 2010), and bioremediation of environmental contaminants and degradation of plastics (*Sphingomonas* OTU1014) (Yabuuchi and Kosako, 2015). For unique OTUs at OC, many of them exhibited sulfur/sulfate reduction potentials (unclassified group of Desulfobacteraceae OTU7334) and the anaerobic fermentation of products from the degradation of organic matter to produce lactate (unclassified group of NB1-n OTU7625, unclassified group of WS6 OTU7311, unclassified group of Gracilibacteria OTU7396 and *Sphaerochaeta* OTU7953) (Conthe et al., 2018; Miyazaki et al., 2014; Rinke et al., 2013; Skennerton et al., 2016; Wrighton et al., 2016).

Variations of bacterial communities along depth might indicate the long-term cumulative anthropogenic influences on the bacterial communities (Liu et al., 2016b), so the cumulative impacts of long-term mariculture on the benthic bacterial community in the core sediments were illustrated from the following two aspects: 1) the regressions between depth and beta-diversity of benthic bacterial community; 2) the differences in the dominant total and active benthic bacterial communities among the surface, middle and bottom layers of the KS and KM core sediments, respectively. The relationships between depth and beta-diversity of the benthic bacterial community showed that only the total bacterial community was significantly related to the depth, indicating that the total rather than the active bacterial community was significantly influenced by the cumulative impacts of the long-term mariculture (Fig. 6), which is similar with the results of the spearman correlation heatmap analysis (Fig. 7). Depth is significantly correlated to the total bacterial community (Figs. 6A–C, 7A, 8A, S9A), which is similar with the results in the soil, water column and core sediments (Gobet et al., 2012; Zhang et al., 2017a; Y. Zhang et al., 2014). The comparisons of the dominant total and active benthic bacterial communities among the surface, middle and bottom samples of the KS and KM core sediments also indicated that most of the bacterial populations did not show significant variations at the phylum, genus and OTU levels (Figs. S5–S8). These results indicated that the benthic bacterial communities were only slightly influenced by the cumulative impacts of the long-term mariculture (Figs. 6, S5–S8). We speculated that the constant mariculture species (kelp and scallop) during the past fifty years might induce similar carbon components within the core sediments (S. Liu et al., 2015), which could induce similar benthic bacterial communities along the depth in the core sediments (Figs. 1, 6, S5–S8).

## 5. Conclusions

The present study provided a detailed comparison of both the total and active benthic bacterial communities using high-throughput sequencing in the core sediments of a long-term IMTA ecosystem, the Ailian Bay, North China. The total benthic bacterial community composition was significantly different from the active benthic bacterial community composition among the different mariculture modes. However, for the cumulative impacts of the long-term intensive mariculture, only limited groups of the total benthic bacterial taxa showed significant responses. The multivariate analysis provided evidence that both the total and active bacterial communities exhibited a slightly stronger response to environmental factors than to spatial (distance) ones. Grain size,  $PO_4$ -P,  $NH_4$ -N and total organic carbon are critical environmental factors in influencing the total and the active benthic bacterial community variations in the Ailian Bay. The effects of mutualism might dominate the total and active bacterial networks in the Ailian Bay based on the network analysis of total and active bacterial taxa. However, a significant number of variations of the bacterial community could not be explained by the environmental factors measured in this study, suggesting that

the influences of other sedimental environmental factors such as oxygen concentration, sulfate concentration and heavy metals on microbial communities should be evaluated in the future. Moreover, like the routine methods, RNA was extracted directly from deep-frozen sediments for the analysis of active microbial communities in this study. Since RNA is easy to degrade, it might lead to some biases in the results. The addition of RNA protector such as RNAlater before deep freezing should be a better method for the RNA preservation in sediment samples in future studies. In addition, the accurate measurement of the sedimentation rates of each core sediment and the intensified study of more core sediments could provide insight into the probable linkage between the mariculture intensity of every year during the past half-century with the variations of the benthic bacterial community.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2019.07.200>.

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