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Disentangling the abundance and structure of *Vibrio* communities in a semi-enclosed Bay with mariculture (Dongshan Bay, Southern China)

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ABSTRACT

The genus *Vibrio* contains a diverse group of heterotrophic bacteria, which are members of ubiquitous and abundant microbial communities in coastal ecosystems. *Vibrio* has been frequently found in a wide range of marine environments either by employing *Vibrio*-specific 16S rRNA sequencing or culturing methods. A combination of molecular and cultivation-dependent methods was developed to more precisely discriminate between different members of the genus *Vibrio* in seawater. This newly developed assay was subsequently applied to characterize *Vibrio* community composition in surface water at 18 mariculture sites. It Substantially improved the taxonomic resolution of *Vibrio* species when compared to traditional 16S rRNA analysis. Our qPCR and cultivation analyses revealed that average *Vibrio* abundance (*Vibrio* 16S rRNA gene copy numbers: 3.46×10^6 to 6.70×10^6 copies L^{-1}) and live cell numbers (5.65×10^4–5.75×10^5 cfu mL^{-1}) are significantly related to pH. Total bacteria and *Vibrio*-specific 16S rRNA metabarcode sequenceing resulted in a total of 10 and 32 operational taxonomic units (OTUs), respectively, and 15 *Vibrio* species were identified by targeted cultivation of *Vibrio* strains, with *Vibrio fortis* and *V. brasiliensis* dominating in the mariculture areas. The purpose of this study was to combine several analytical methods to improve current sequence-based *Vibrio* community surveys, and to prove for the effectiveness of this methodological approach comprehensively testing for *Vibrio* dynamics in different coastal environments.

1. Introduction

Over the last decades, it has become apparent that utilization and development of coastal and marine resources have profound impacts on coastal environments. Microorganisms are regarded as one of the major food web components of coastal ecosystems controlling biogeochemical cycles in coastal environments to a large extent [51]. Thus, exploring the spatial pattern of microbial communities related to environmental factors is crucial to reveal the role of microbial communities for the entire coastal food web and biogeochemical cycles [15,18,40].

Some coastal bacteria are capable of responding to changes of environmental conditions quickly, and thereby serve as reliable indicators of water pollution and hydrological changes [21,41]. *Vibrio* species are a dominant group of cultivable bacteria in coastal waters and play an important biochemical role in coastal waters due to their capability, e.g., to fix nitrogen, degrade chitin and metabolize algal polysaccharides [21,26,42,55]. On the other hand, several *Vibrio* species are to a certain extent pathogenic, and thus constitute important microbial risk agents in water threatening environmental and human health. For example, *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus*, *V. harveyi* and *V. fluvialis* are pathogens

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that cause gastroenteritis, sepsis and necrosis in animals and humans [14,19,36,54]. Hence, in-depth knowledge on biogeographic patterns of *Vibrio* species helps to predict the response of coastal water ecosystems to changing environmental factors. Moreover, it may also help to identify potential pathogens and new organisms that pose a serious threat to environmental and human health.

In the laboratory, only a small number of microorganisms including *Vibrio* can be cultured. Thus, monitoring of these species relies to a large extent on ribosomal DNA (rDNA) analysis [16,17,21,34,55], as the specificity of the thiosulfate citrate bile salt sucrose (TCBS) medium to cultivate *Vibrio* species is solely 60% [33]. Amplicon sequencing has uncovered the existence of *Vibrio* in a wide range of marine environments and shows that *Vibrio* community patterns in coastal areas are related to specific environmental factors [3,34,45,48]. Fernandes et al. [9] used amplicon sequencing of the V3-V4 region of the 16S rRNA gene to investigate bacterial community composition of tarball-contaminated seawater of Vagator Beach in Goa, India. They found that *Vibrio* abnormally increased in tarball-contaminated seawater (16.16% OTU), but only three *Vibrio* species were detected, i.e. *V. shilonii* (1.95%), *V. fortis* (1.59%), and *V. harveyi* (0.0033%). During recent years, however, *Vibrio*-specific primers have been widely used to study the assembly of *Vibrio* communities, verifying the predictive power of environmental conditions such as temperature, salinity, and pH on *Vibrio* community structure in several coastal areas, such as the Sydney Harbor estuary [39], the tropical North Atlantic [16,9], Chinese marginal seas [25,26,31,46], and Beibu Gulf [7,24]. Therefore, an in-depth understanding of *Vibrio* community composition, their main driving factors, and responses to nutrient variation is crucial to better reveal their role for functions and ecology of marine coastal ecosystems.

The coastline of Dongshan Bay is one of the most vulnerable areas to climate change, with frequent occurrences of natural disasters. Stronger and more frequent coastal storms or cyclones, higher temperatures, floods and droughts, salinization of upstream rivers, eutrophication due to excess nutrients input from an increase in population size pose a considerable threat to water and food security [54]. Yet, impacts of environmental factors on *Vibrio* population dynamics in mariculture areas have been rarely studied [31,52]. Our research in Dongshan Bay reveals that diversity and abundance of *Vibrio* species usually fluctuate seasonally with an increasing abundance in summer [52]. Similar results have been reported for other *Vibrio* studies using either culture dependent or independent methods [7,21,39,47]. To better understand the role of *Vibrio* species in mariculture-influenced areas and to explore effects of spatial variability of environmental factors on *Vibrio* community abundance and structure, a comprehensive evaluation that characterizes this important bacterial group is required. We combined culturing approaches, amplicon sequencing and quantitative polymerase chain reaction (qPCR) assays to studying the summer *Vibrio* community structure and its relationship with a series of environmental variables in an integrative manner. The objective of this research was to utilize some comprehensive technical measures to improve sequence-based surveys of *Vibrio* communities and to demonstrate the usefulness of this approach by presenting an analysis of *Vibrio* dynamics in relation to environmental conditions, with a particular focus on the *Vibrio* community structure in mariculture-influenced areas. These data deepens our insight into *Vibrio* community dynamics in different coastal waters and thus serve as a solid basis for proper health risk assessment.

2. Materials and methods

2.1. Study area, sampling, and analyses of physicochemical properties

Sampling sites were situated near Tai 'o Central Fishing Port (~117.5°E, ~ 23.6°N), which is an important coastal aquaculture zone in Dongshan Bay in Fujian provinces, Southern China. Two representative sampling areas (DS1and DS2) for aquatic farming animals dominated by Abalone (mainly *haliotis discus hannai ino*) and Epinephelus (mainly *Epinephelus coioides*) were chosen and surface water was collected (~0.5 m depth). A total of 10 water samples were taken on August 18th, 2019 (DS1) with 4 samples from the fish farm area (DS1-FF), 4 samples from the water channel area (DS1-FW), and 2 samples from the control zone (DS1-CN). While 8 water samples were taken on July 31st, 2019 (DS2), with 3 samples from the fish farm area (DS2-FF), 3 samples from the water channel area (DS2-FW), and 2 samples from the control zone (DS2-CN). Specifically, DS1-FF (DS1-FF1, DS1-FF2, DS1-FF3, and DS1-FF4) and DS2-FF (DS2-FF1, DS2-FF2, and DS2-FF3) have significantly different farming practices, including stocking density and production. The sample from the water channel of the farm zone (DS1-FW and DS2-FW) without any net cage served as a control for just marine traffic. Additionally, both of these four CN sites (DS1-CN1, DS1-CN2 and DS2-CN1, DS2-CN2) were situated near the Tai 'o Central Fishing Port without any aquaculture activity present. Details of the location and the distribution of the sampling sites are given in Fig. S1. Briefly, water samples were taken at 0.5 m depth below the water surface using a 5 L Niskin bottle (General Oceanics, Miami, FL, USA), transferred into appropriate sterile plastic bottles and immediately transported to the laboratory in a cooler. Analysis of major environmental parameters was performed (Fig. 1 and Table S1). Water temperature, pH, salinity, dissolved oxygen (DO), and total dissolved solids (TDS) were measured *in situ* at each sampling site with a multiparameter water quality checker (Horiba U-50, Horiba Co., Kyoto, Japan). Chemical parameters comprised concentrations of nitrate (NO_3^-), nitrite (NO_2^-), ammonium nitrogen (NH_4^+), and phosphate (PO_4^{3-}) which were measured using standard protocols [52].

2.2. Quantitative PCR analysis

The universal bacterial primer set A-967F/B-1046R (A-967F:5′-CAA CGC GAA GAA CCT TAC C-3′,and B-1046R:5′-CGA CAG CCA TGC ANC ACC T-3′) and the *Vibrio*-specific primer set 567F/680R (567F: 5′-GGC GTA AAG CGC ATG CAG GT-3′, and 680R:5′-GAA ATT CTA CCC CCC TCT ACA G-3′) were used for qPCR to amplify the 16S rRNA gene as previously described [26] and in order to detect the abundance of total bacteria and *Vibrio* spp. Genomic DNA extracted either from *Vibrio alginolyticus* (Marine Culture Collection of China, strains MCCC1A06468 and MCCC1A07292) or *Escherichia coli* was used for PCR standard curves, with an equivalent of 10^2–10^8 gene copies per reaction. For quantification, environmental and control DNA were used as templates in the qPCR reactions on a LightCycler® 480II Real-Time PCR system (Roche Life Science, Swiss). With the LightCycler480 software, the final abundance was analyzed and displayed as gene copies L^{-1} of seawater. Abundance of bacteria and *Vibrio* was log 10 $^{(x+1)}$ transformed before analyses. Variations in *Vibrio* abundance among different groups were tested for significance using a Mann-Whitney test. In additional, according to a previous study, the average 16S rRNA gene copy number of a *Vibrio* cell was nine [2].

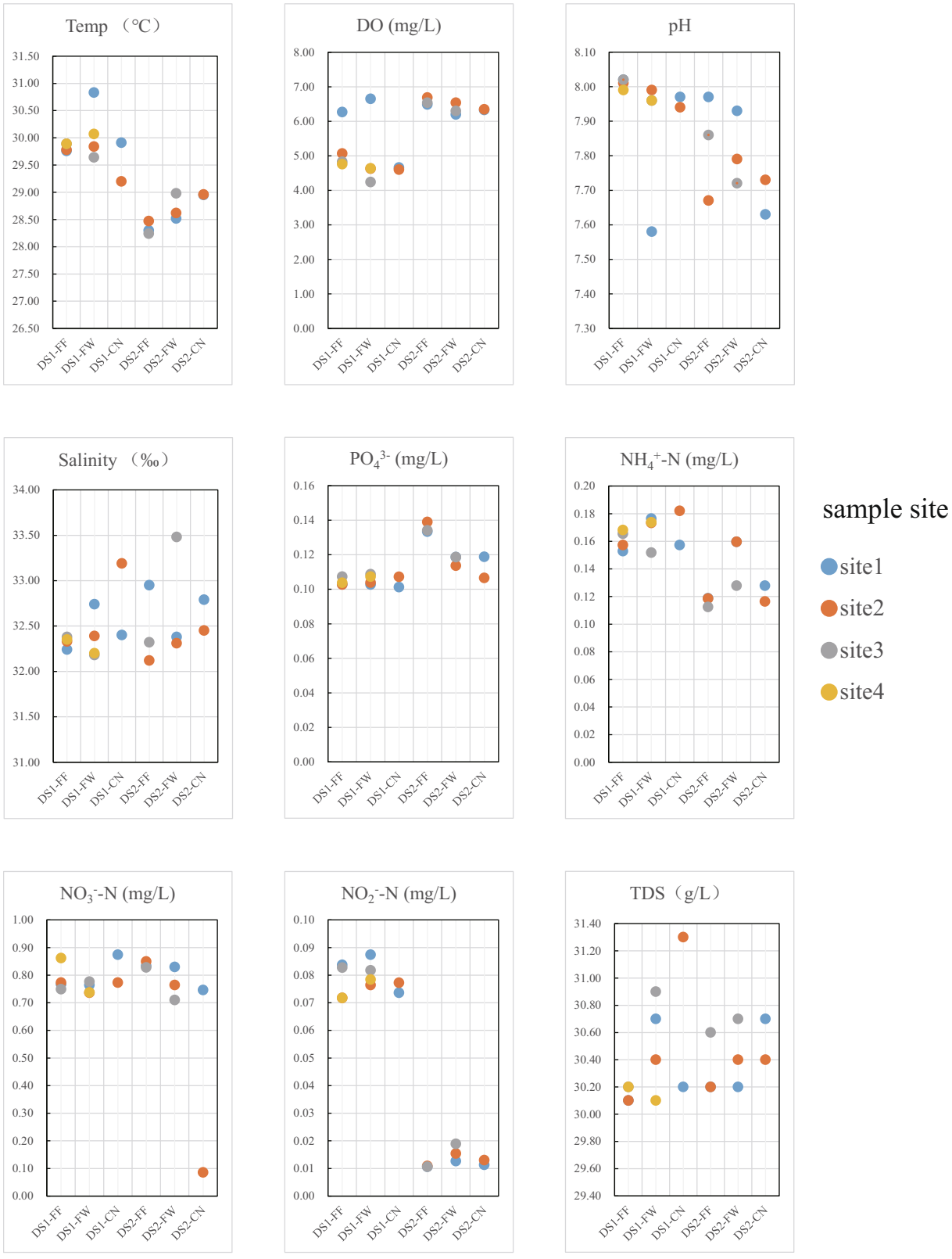

Fig. 1. Physical and chemical index of the sample site.

2.3. Vibrio isolation and 16S rRNA identification

Triplicates (200 μl) of each water sample were plated on Thiosulfate Citrate Bile salt Sucrose (TCBS) agar (Oxoid, Basingstoke, England) and incubated at 25 ± 3 °C for 48 h to retrieve culturable heterotrophic bacteria capable of growing on TCBS agar. Numbers of colony forming units (cfu) were manually enumerated for each plate, while green and yellow colonies with different sizes and shapes were picked with a sterile tooth pick and streaked onto marine 2216E Agar (Hopebiol, Qingdao, China) plates to obtain

pure bacterial cultures for further molecular identification. Genomic DNA extraction was carried out with the EasyPure® Genomic DNA Kit (TransGen Biotech, Beijing, China). Sequencing of the 16S rRNA was performed with the specific primer pair 27F/1492R (27F: 5′- TGG CTC AGA TTG AAC GCT GGC GG-3′, and 1492R: 5′-TAC CTT GTT ACG ACT TCA CC-3′) for molecular identification (Hayashi et al., 2002). The EzTaxon-e server was used for sequence comparison with closely related reference strains. CLUSTAL W was used for multiple sequence alignments. The nucleotide sequence of almost the entire 16S rRNA gene of *Vibrio* sp. isolates was deposited in GenBank under accession numbers: MT269634-MT26941 (DS1 area August 2019) and MT269602-MT269610 (DS2 area July 2019).

2.4. DNA extraction and 16S rRNA sequencing

One L of seawater was filtered through 0.22 μm filter units (GSWG047S6, Millipore, Burlington Unites States) and stored at − 80 °C for later DNA extractions [52]. Total DNA from filters was extracted using the FastDNA Spin Kit for Soil and a FastPrep-24 Instrument (MP Biomedicals, Irvine, United States) according to the manufacturer's protocol. After quality verification via a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, Unites States), the extracted DNA served as a template for PCR amplification. Amplicons were generated using two primer pairs, namely 338F/860R (341F: 5′- ACTCCTACGGGAGGCAGCA-3′ and 806R: 5′-GGACTACNNGGGTATCTAAT-3′) [4] and 169F/680R (169F: 5′-GGATAACC/TATTGGAAACGATG-3′ and 680R: 5′-GAAATT CTACCCCCCTCTACAG-3′) [26,39] targeting the V3–V4 variable regions of bacteria and the V2-V4 hypervariable regions of the *Vibrio* 16S rRNA gene, respectively. PCR amplification was performed in triplicates for each sample (Three samples per sampling point). PCR reactions were conducted as follows: 95 °C of denaturation for 5 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, with a final extension of 72 °C for 5 min. Amplicons were purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) and pooled in equimolar concentrations for sequencing on an Illumina HiSeq 2500 platform (2 × 300 bp) at Majorbio BioPharm Technology (Shanghai, China). The raw reads were submitted to the NCBI Sequence Read Archive (SRA) database (Accession Number: SUB6820618 for bacteria and *Vibrio*).

2.5. 16S rRNA gene diversity analysis

FLASH and Trimmomatic software were used to process and quality-filter the raw sequence files. The USEARCH software (version 7.1) was used to identify the operational taxonomic units (OTUs) with a sequence similarity cutoff at 97%, while the UCHIME software (version 4.2) was used to identify and remove chimeric sequences. On the basis of the OTU dilution curve analysis, Shannon and Chao indices were calculated. The RDP Classifier (http://rdp.cme.msu.edu/) was used to analyze the taxonomy of each representative 16S rRNA gene sequence against the SILVA (SSU132) database with a confidence threshold of 70%. In addition, representative sequences of each OTU were further compared against the EzBioCloud and National Center for Biotechnology Information (NCBI) database to determine their taxonomic status more accurately. On the other hand, to obtain alpha and beta diversity indices, the quality-checked sequence data were then analyzed on the Shanghai Majorbio I-Sanger Cloud Platform (www.i-sanger.com) based on the QIIME 1.9.0 pipeline.

In order to compare diversity and richness of bacterial communities in different samples, alpha diversity indices including PD Whole Tree value, Chao 1 value, Simpson and Shannon indices were calculated. Alpha diversity indices among different groups were compared through a Wilcoxon's test. Differences in relative abundance of taxa among different groups were evaluated by the Wilcoxon Rank Sum test. According to the sampling sites, the Redundancy Analysis (RDA) and Principal Component Analysis (PCA) were employed to evaluate the *Vibrio* OTU distribution and the impact of key environmental parameters on *Vibrio* OTU distribution, respectively. Correlations between community richness, diversity and relative abundance of phylum/gene, and environmental factors were analyzed by a Spearman correlation analysis.

3. Results

3.1. Vibrio abundance and cultivable diversity

Bacteria and *Vibrio* abundances were estimated via qPCR (Fig. 2), revealing that copy numbers of the bacterial 16S rRNA gene ranged from 1.37×10^8 to 5.83×10^8 with an average of 3.22×10^8 copies L^{-1}. *Vibrio* 16S rRNA gene copy numbers ranged from 3.46×10^6 to 6.70×10^6 copies L^{-1}. Predicted cell numbers of *Vibrio* species varied between 3.84×10^5 cells L^{-1} to 7.44×10^5 cells L^{-1}. The relative proportion of *Vibrio* species was on average 1.79% in all samples, and the highest proportion was 2.81% in DS1-FW2 and lowest in DS1-FW2 (0.92%).

Vibrio abundance ranged at all coastal sampling sites between 4.19×10^6 to 6.70×10^6 copies L^{-1} with an average of 5.66×10^6 copies L^{-1} in area DS1 and an average *Vibrio* abundance of 3.46×10^6 to 6.35×10^6 copies L^{-1} in area DS2. In general, qPCR assays revealed no significant differences in total *Vibrio* abundance between the two areas (DS1 versus DS2, $p > 0.05$; Fig. 3c). Specifically, *Vibrio* abundance in the FF zone of DS1 was significant higher than in the FW and CN zones ($p < 0.05$), while in the DS2 area no significant differences occurred between these three different zones ($p > 0.05$). On the other hand, *Vibrio* spp. counts in DS1 ($5.65 \times 10–5.75 \times 10^2$ cfu ml^{-1}) were roughly in the same range as in DS2 ($9.35 \times 10–2.49 \times 10^2$ cfu ml^{-1}) ($p > 0.05$). A decreasing trend in *Vibrio* abundance was observed from FF to CN, in both areas (DS1 and DS2) ($p < 0.05$) (Fig.3). Spearman's rank correlation analysis showed that *Vibrio* abundance (both molecular abundance and cfu count) showed a significant positive correlation with pH (Table 1).

The diversity of *Vibrio* strains isolated on TCBS medium revealed 15 different species across all samples. Spatial and temporal changes in cultivable *Vibrio* numbers were observed (Fig. S2). Area DS1 displayed a higher diversity (12 species) than DS2 (10 species). Based on ANOSIM analysis, no significant differences in cultivable *Vibrio* spp. community composition between DS1 and DS2 were observed. Yet, the dominant species in area DS1 were *Vibrio fortis* (19.15%), followed by *V. harveyi* (17.02%), *V. alginolyticus* (14.89%), and *V. neocaledonicus* (10.64%), whereas the most abundant species in DS2 were *Vibrio campbellii* (26.32%), followed by *V. alginolyticus* (15.79%), *V. harveyi* (13.16%), *V. neocaledonicus* (13.16%), and *V. owensii* (13.16%). Further analysis revealed that the dominant cultivable *Vibrio* species were basically the same as those detected by *Vibrio*-specific 16S rRNA sequencing.

3.2. Richness and relative abundance of Vibrio (total bacterial 16S rRNA sequencing)

Sequencing of the bacterial V3-V4 region of the 16S rRNA gene yielded a total of 717, 718 quality-filtered reads, ranging from 22,992 to 62,332 sequences between samples. Rarefaction plots (data not shown) indicate that our sampling effort was sufficient to capture most of the total bacterial diversity. Overall, 1580 OTUs were obtained at a 97% taxonomic cut-off level. Of these, only 4478 sequences and 10 OTUs were chosen and then classified into the *Vibrio* genus. The ratio of *Vibrio* to bacteria species ranged from

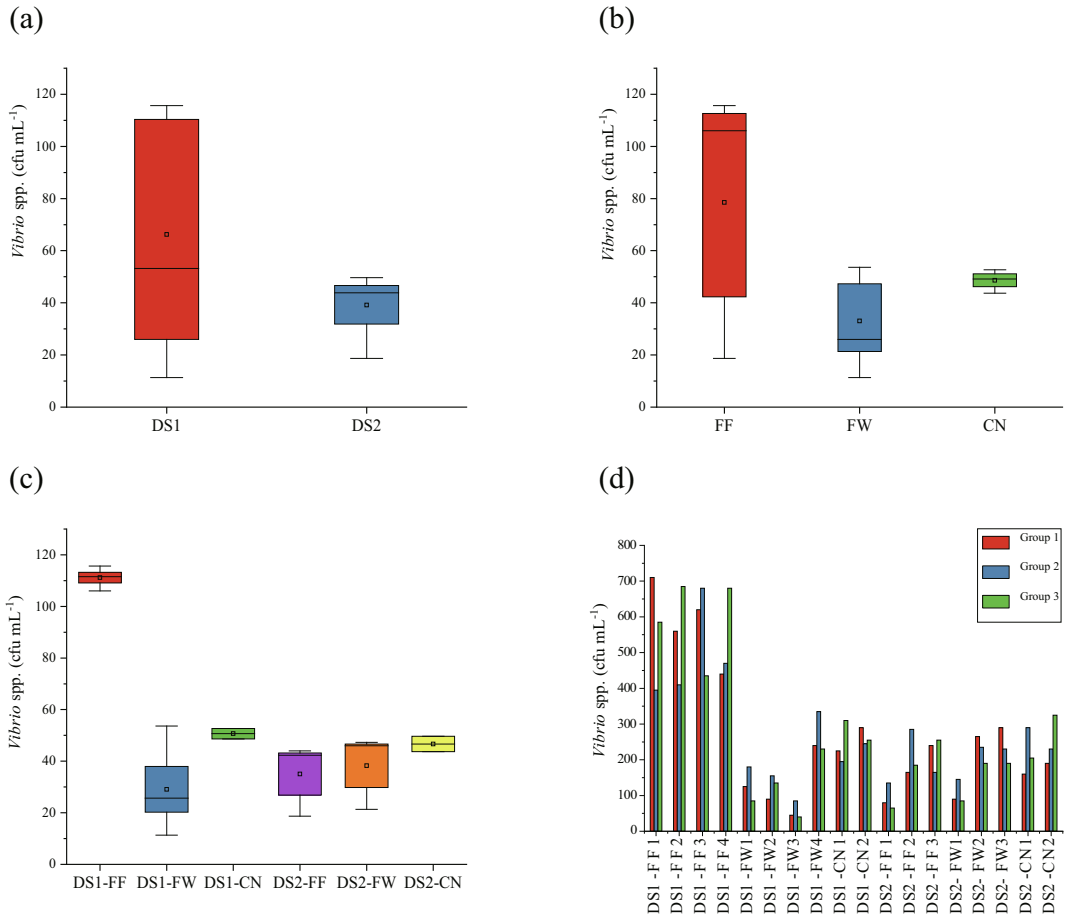

Fig. 2. The cfu counts of cultivated *Vibrio* in different collected areas (a), different aquaculture-influenced zones (b) and in varied sites (c, d).

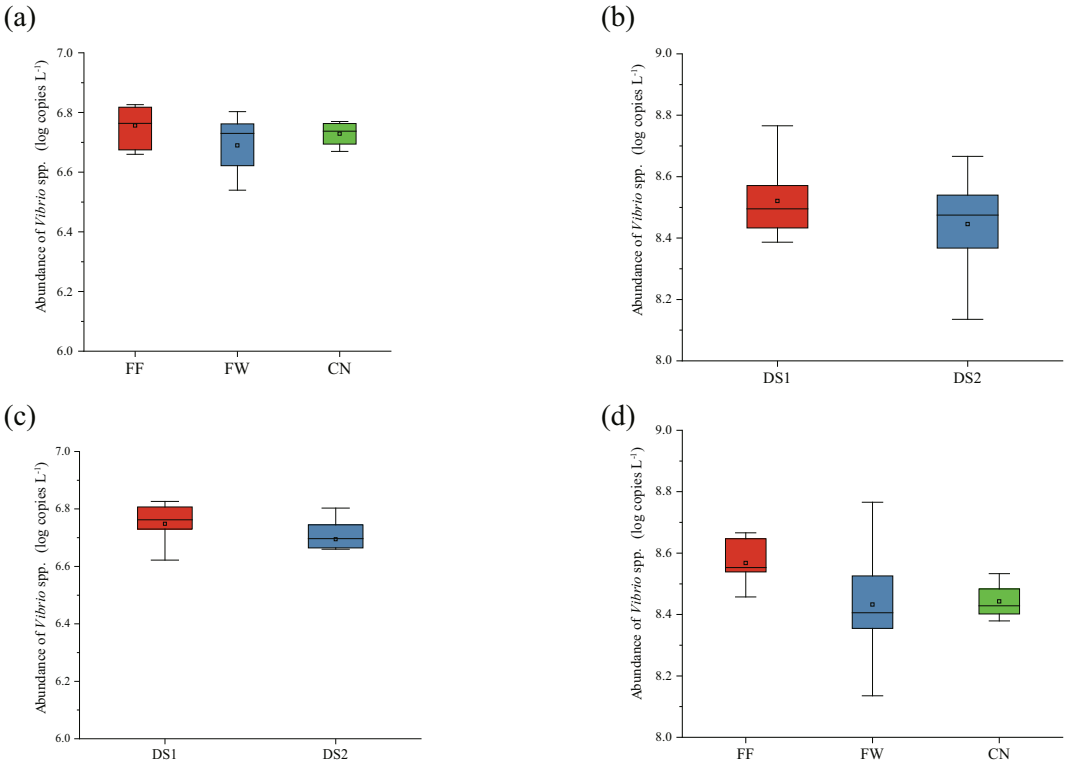

Fig. 3. Total *Vibrio* and bacteria abundance (log copies ml−1) in different areas (a, b) and different aquaculture-influenced zones (c, d).

Table 1
Relationship between *Vibrio* abundance or biomass and the measured environmental variables.

Factor	Spearman's rank correlation coefficient	
	CFU	log10 (Abundance)
Longitude	−0.0468	−0.1031
Latitude	−0.2100	−0.2665
Temperature	0.4316	0.3634
pH	0.4705*	0.5233*
DO	−0.2044	−0.2395
Salinity	−0.0867	−0.1415
TDS	−0.4506	−0.4274
NH_4^+	0.2355	0.3729
PO_4^{3-}	−0.4523	−0.3273
NO_3^-	−0.1682	0.0485
NO_2^-	0.3705	0.2632

* $p < 0.05$

0.05% to 5.20% among different sample sites. Except for a few samples in DS1_CN1 (5.20 %) and DS2_FW3 (1.59%), the relative abundance of *Vibrio* never exceeded 0.8%. After quality filtering and subsampling (22,922 reads per sample), only 2513 sequences and 10 OTUs were related to the *Vibrio* genus. The mean (±SD) relative abundance of *Vibrio* spp. in the coastal water samples from areas DS1 and DS2 was 0.56% (±0.73%) and 0.67% (±0.97%), respectively. Relative *Vibrio* abundances in the FF (0.63 ± 1.07%) and FW (0.79 ± 0.76%) zones were significantly higher than that in the CN zone (0.26 ± 0.07%). However, due to the limited number of sequences and OTUs obtained for each sample, our statistical approaches didn't allow for comparison of *Vibrio* community pattern between the various samples.

Major OTUs classified by the Silva database belong to *Vibrio brasiliensis*, *V. fortis*, *V. harveyi*, *V. nigripulchritudo*, *V. ponticus*, and five unclassified *Vibrio* species. The NCBI and EzBioCloud databases were used to analyze the representative sequences of each OTU to determine their taxonomic status, which clustered with *Vibrio brasiliensis*, *V. caribbeanicus*, *V. cidicii*, *V. fortis*, *V. maritimus, V. nigripulchritudo*, *V. ponticus, V. profundi*, *V. natriegens* and one unclassified *Vibrio* species. The most dominant and common species were *V. fortis* OTU496, *V. harveyi* OTU566, *V. brasiliensis* OTU41, and *V. ponticus* OTU708, which jointly accounted for 97.41% of all retrieved sequences (Fig. 4).

3.3. Diversity and composition of total Vibrio community (Vibrio-specific 16S rRNA)

Relative to the total bacterial 16S rRNA gene sequencing, the *Vibrio*-specific 16S rRNA primer set identified a greater number of species in the *Vibrio* community. In total, 732,734 quality-filtered reads, ranging from 23,196 to 61,238 were generated through Illumina sequencing and clustered into 81 OTUs at a 97% similarity level. Rarefaction curves of all stations reached an asymptote while the Good's coverage values ranged from 99.95% to 99.99% across the entire data set, indicating the detected sequences represented the majority of the *Vibrio* community. Most of the 16S rRNA sequences (78.08%) belonged to the *Vibrionaceae* family, and 76.13% were assigned to the *Vibrio* genus.

For each sample, an average of 20,618 sequences and 32 *Vibrio* OTUs were obtained after quality control and rarefication. We analyzed the representative sequence of each OTU to determine its taxonomic status and relative abundance. Chao I (a measure of richness), phylogenetic distance, evenness and Shannon (including both evenness and diversity) indices of the *Vibrio* communities indicated an approximate 99.9% coverage suggesting that out sequencing results are representative for the "real" *Vibrio* occurrence. According to OTU richness and phylogenetic diversity, the phylogenetic diversity and OTU richness were not significantly different between the two sample areas (DS1 and DS2), but remarkable differences were observed in disparate mariculture-influenced zones ($P < 0.05$) (Fig.5 a and b). More specifically, alpha diversities of *Vibrio* communities in FF were significantly higher than in FW of the DS1 area ($P < 0.05$). However, no significant differences in these two indices among the study zones in area DS2 were observed (Fig. S3a and b).

Taxonomic status was determined via comparing the representative sequences of each *Vibrio* OTU with the NCBI and EzBioCloud databases suggesting 26 known and two unclassified *Vibrio* species (Fig.6). Fourteen *Vibrio* species were identified in all water samples,

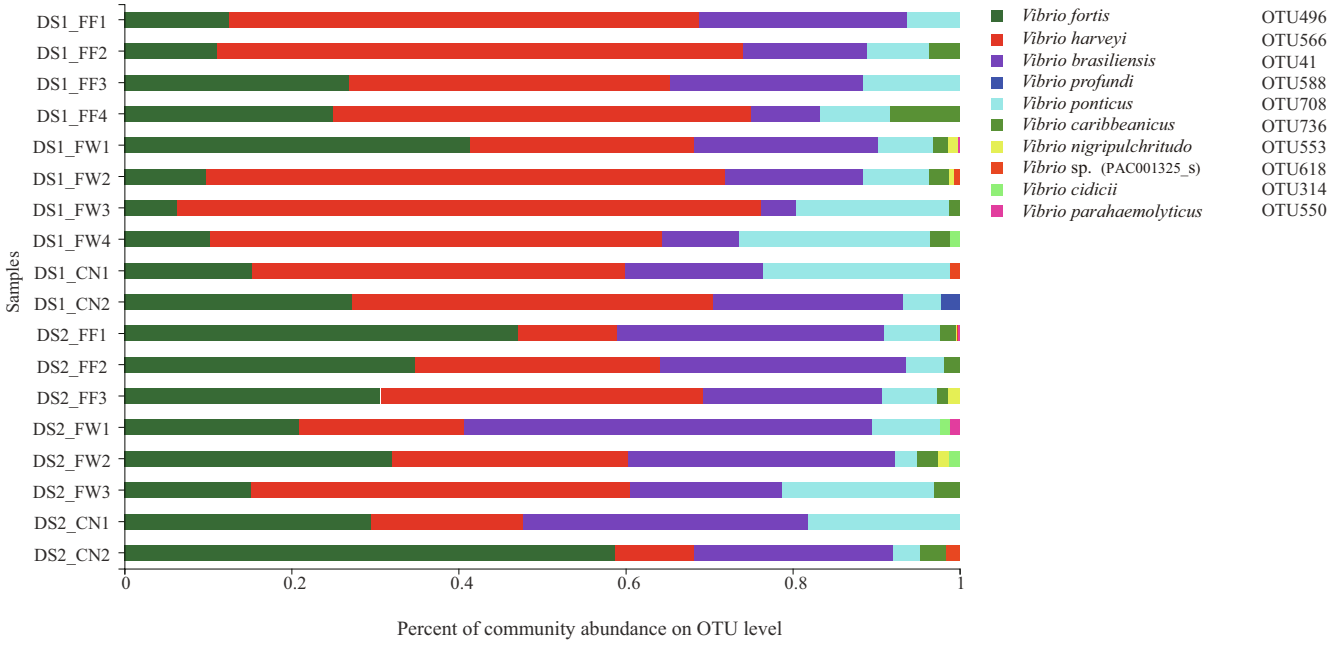

Fig. 4. *Vibrio* community compositions at the species level across all samples (total bacterial 16SrRNA).

whereby *Vibrio ponticus* OTU56, *V. caribbeanicus* OTU2, *V. nereis* OTU20, *V. parahaemolyticus* OTU80, *V. rotiferianus* OTU63, *V. rotiferianus* OTU15, and *V. brasiliensis* OTU9 dominated in all water samples. *Vibrio brasiliensis, V. rotiferianus,* and *V. parahaemolyticus* alone contributed to > 70% of all *Vibrio* OTUs in each sample. OTU63, OTU56, OTU64 and OTU76 were remarkably more prevalent in the area DS1 compared to DS2 ($P < 0.05$), while abundances of OTU9 and OTU15 were remarkably lower in area DS1 compared to DS2 ($P < 0.05$) (Fig.7). In contrast to differences in the *Vibrio* community between areas (DS1 and DS2), relative abundances of *Vibrio* communities in FF, FW and CN only differed significantly between OTU2 and OTU30. Among the different zones of area DS1, differences in specific relative *Vibrio* abundances were statistically significant, but not for zones of area DS2 (Fig. S4).

PCA analysis was used to compare the *Vibrio* community composition of different samples at the OTU level. The samples from different areas (DS1 and DS2) were clearly separated on the first axis, and all three mariculture affected areas of the DS1 sample showed some degree of spatial heterogeneity (ANOSIM, $P < 0.01$) (Fig. 8a). RDA analysis revealed that *Vibrio* communities were positively correlated with NH_4^+, NO_2^- and NO_3^-, and negatively with DO and latitude ($P < 0.05$; Fig.8b). The Spearman correlation heatmap showed that the dominant *Vibrio* OTUs had a strong correlation with numerous environmental factors (Fig. S5).

In order to determine the specific taxa that contributed to the observed temporal and spatial dynamics of the *Vibrio* community and their interrelationships, Spearman correlations between 32 OTUs and the measured environmental factors were calculated (Table 2). The most abundant species, i.e., *Vibrio rotiferianus* OTU63, was positively related to temperature (r = 0.631, p = 0.005), pH (r = 0.494, p = 0.037) and NO_2^- (r = −0.614, p = 0.007), but negatively to Latitude (r = −0.678, p = 0.002) and DO (r = −0.645, p = 0.004). The second most abundant species, i.e. *Vibrio brasiliensis* OTU9, exhibited inverse relationships with these factors. It was negatively correlated with temperature (r = −0.642, p = 0.004), pH (r = −0.642, p = 0.004), and NO_2^- (r = −0.610, p = 0.007), but positively correlated with Latitude (r = 0.684, p = 0.002) and DO (r = 0.677, p = 0.002). And the third most abundant species, i.e. *Vibrio parahaemolyticus* OTU80, didn't show any significant correlation. *V. gallaecicus* OTU211 was the only dominant group of DS1, which was positively correlated with DO and $NH4^+$, and negatively correlated with latitude ($p < 0.05$).

3.4. Taxonomic richness determined by multiple methods

In order to expand the taxonomic richness of the *Vibrio* community, we integrated the taxonomic coverage and complementarity of the two sets of primers for the total number of bacteria and *Vibrio* 16S rRNA gene sequences as well as cultivable species. A total of 717,718 bacterial reads, 732,734 *Vibrio* reads, and 2, 926 isolates were obtained by using the primer sets 338F/860R (total bacteria), 168F/680R (*Vibrio*-specific), and the culture method, respectively. After removing the non-*Vibrio* reads, 2, 513 and 371, 124 *Vibrio*-associated sequences, accounting for 10 and 32 *Vibrio* OTUs were obtained via bacterial and *Vibrio* 16S rRNA gene analyses, respectively. Further comparison of *Vibrio* sequence counts between the

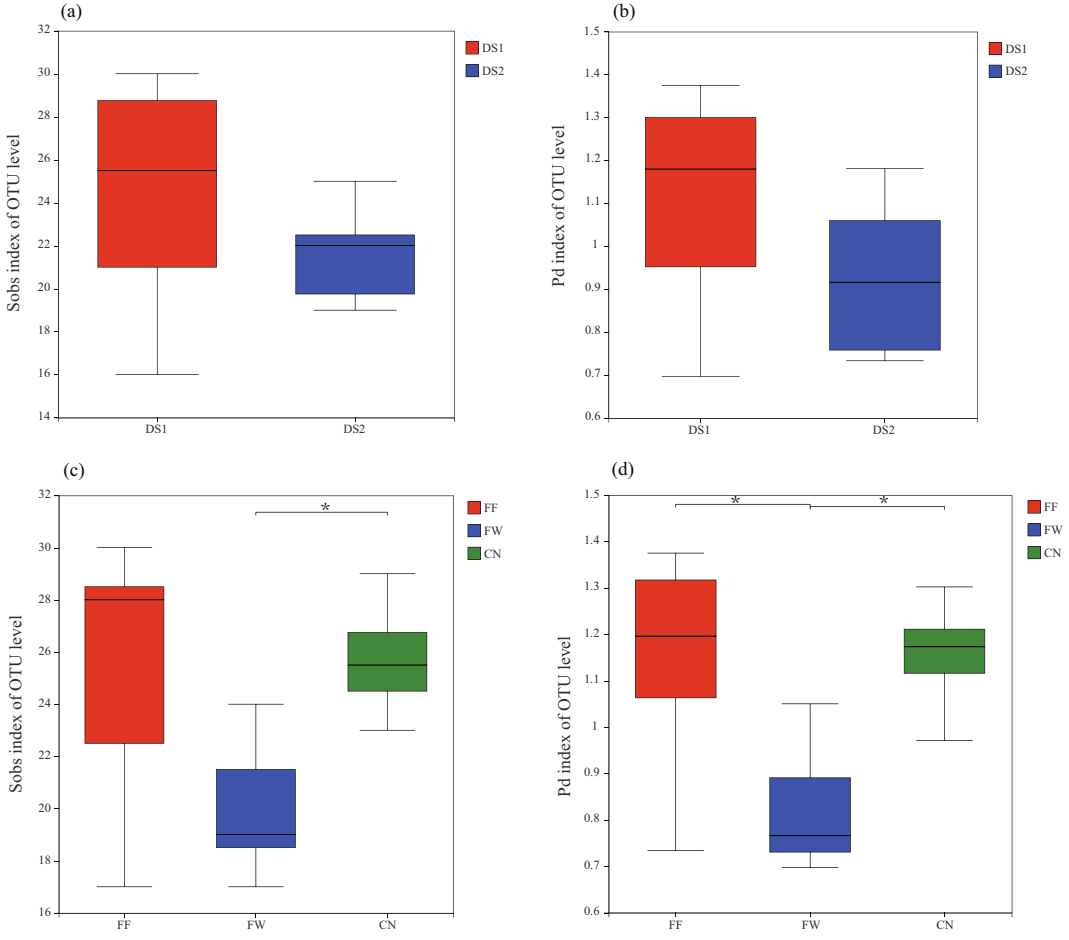

Fig. 5. Richness and diversity of the bacterial communities across different areas (a, c) and zones (b, d) FF, Fish farm; FW, ecological restoration zone; CN, control zone

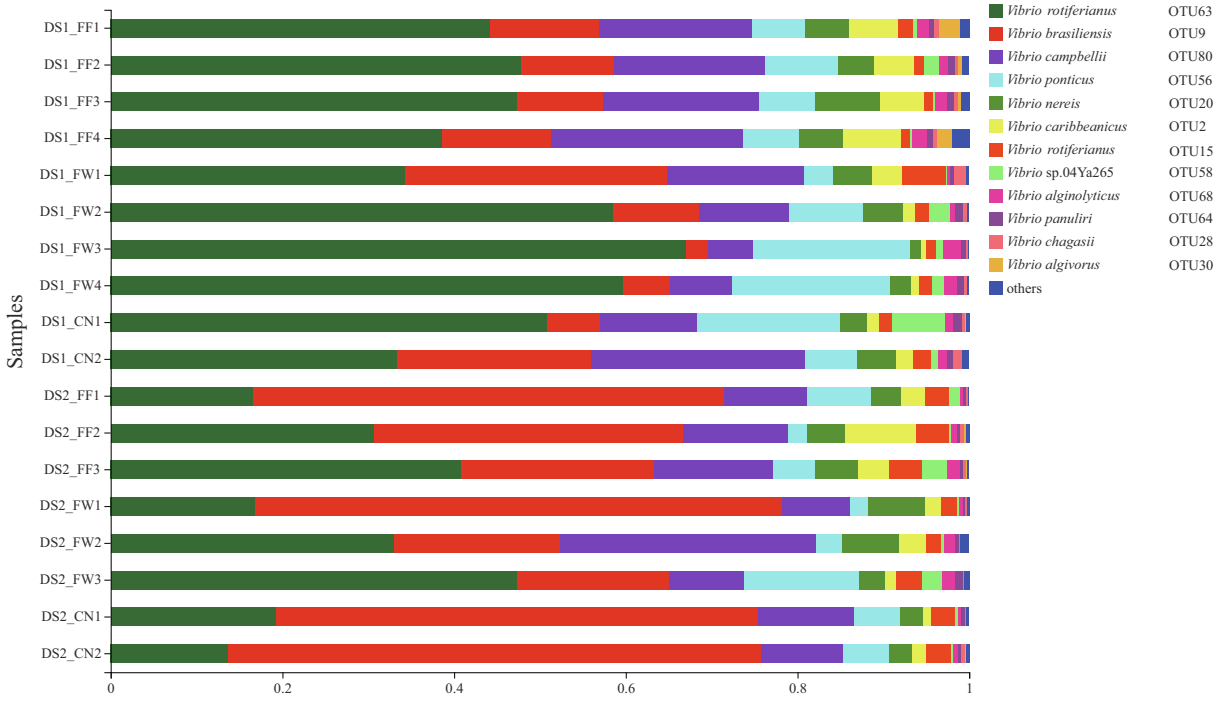

Fig. 6. Top 12 abundant OTUs of total *Vibrio* spp. across all samples by *Vibrio*-specific 16SrRNA sequencing. Other, the species occupied < 0.1% in total samples.

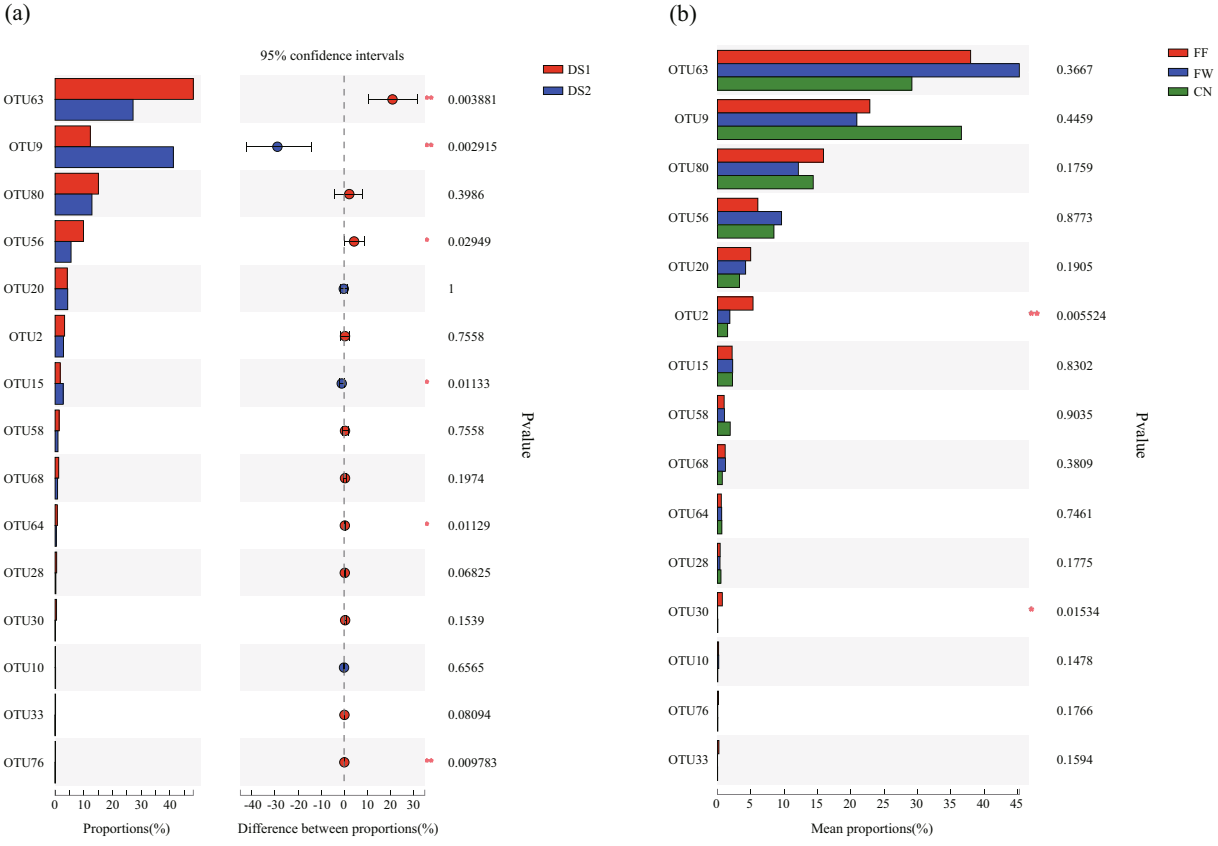

Fig. 7. The difference in taxa relative abundance between different sample (a) areas and zones (b) * indicates $0.01 < P \leq 0.05$, ** indicates $0.001 < P \leq 0.01$

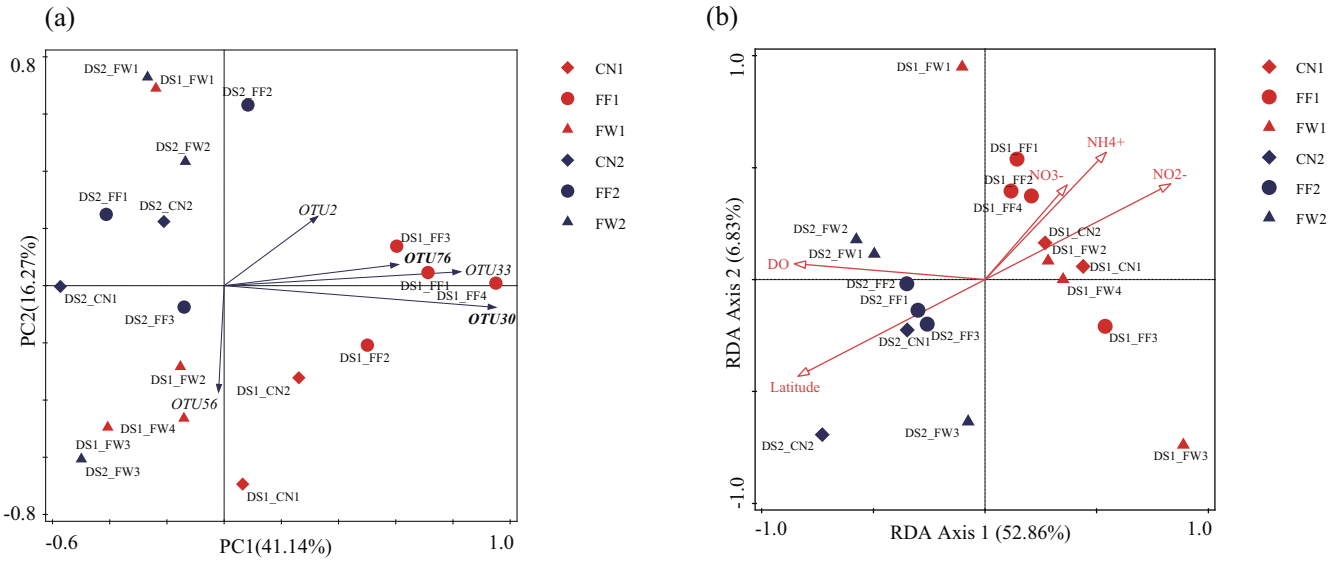

Fig. 8. Community analysis of *Vibrio* at the OTU level. (a) Unweighted PCA plot with PC1 and PC2. (b) RDA analysis illustrating the relationship between *Vibrio* community at the OTU level and top environmental variables

two primers sets and the culture approach indicates that the *Vibrio*-specific 16S rRNA primer set resulted in much higher *Vibrio*-specific read counts than those obtained by the two other methods. At the species level, the *Vibrio*-specific primer set recovered up to 26 *Vibrio* species and two unclassified *Vibrio* species. When using multi-marker gene sequencing, a total of 38 *Vibrio* species and three unclassified *Vibrio* species were recovered, significantly improving the covered diversity of *Vibrio*. Only a single *Vibrio* species, i.e., *V. brasiliensis* was shared by all three data sets indicating that the classification coverage of the three data sets differed. Comparatively, the compositions of the bacterial 16S rRNA, *Vibrio*-specific 16S rRNA, and cultivation characterized communities were on average 24.4, 78.0, and 36.6% similar to the whole *Vibrio* community, respectively

Table 2
Correlations between percentage composition of OTU (Vibrio-specific) and environmental factors.

OTU	Correlation coefficient[1]										
	Longitude	Latitude	Temperature	pH	DO	Salinity	TDS	NH_4^+	PO_4^{3-}	NO^{3-}	NO^{2-}
Vibrio caribbeanicus OTU2											
Vibrio algivorus OTU3											
Vibrio maritimus OTU8				0.469							
Vibrio brasiliensis OTU9		0.684	−0.642	−0.608	0.677					0.61	
Vibrio areninigrae OTU10											
Vibrio maritimus OTU12											
Vibrio stylophorae OTU13											
*Vibrio rotiferianus OTU*15			−0.504	−0.782	0.733						
Vibrio cholera OTU16											
Vibrio porteresiae OTU17		−0.761	0.731	0.629	0.772			0.602	0.585		0.639
Vibrio pectenicida OTU18											
Vibrio nereis OTU20											
Vibrio gazogenes OTU22											
Vibrio harveyi OTU24											
Vibrio chagasii OTU28									−0.509		
Vibrio furnissii OTU29											
Vibrio algivorus OTU30				0.671							
Vibrio sp. OTU33				0.691			−0.677				
Vibrio quintilis OTU38				0.532							
Vibrio hannami OTU39											
Vibrio maritimus OTU45											
Vibrio aphrogenes OTU47		−0.506			0.488			0.537			
Vibrio coralliilyticus OTU49											
Vibrio ichthyoenteri OTU54											
Vibrio ponticus OTU56		−0.583	0.52	0.509							
Vibrio rumoiensis OTU57				0.5			−0.609			0.521	
Vibrio sp. OTU58											
Vibrio rotiferianus OTU63		−0.678	0.631	0.494	0.645						0.614
Vibrio panuliri OTU64		−0.585	0.676	0.492	0.692			0.48	0.528		0.558
Vibrio alginolyticus OTU68						0.528					
Vibrio mexicanus OTU76			0.588	0.498				0.548	0.689	0.556	
Vibrio campbellii OTU80											

Only significant correlations ($P < 0.05$) are shown. - : negative; boldface: $P < 0.01$; lightface: $P < 0.05$.

4. Discussion

Mariculture represents one of the fastest growing industrial sectors in the world, but from the perspective of nutrient load and structural damage, the impact of mariculture on the environment is considerable. Microorganisms form the basis of marine food webs and are crucial for biogeochemical cycles [15,18,51]. Yet, there are many unknowns on how microorganisms respond to environmental interferences from mariculture activities [2,27,38,47]. In coastal environments, for example, it still remains unclear how mariculture changes the composition and abundance of potentially pathogenic bacteria such as *Vibrio* species. The abundances and community composition of *Vibrio* populations have been reported previously along estuaries and coasts [7,20,26,39,45,47,46], however, how environmental heterogeneity affects the *Vibrio* communities in marine mariculture-influenced ecosystems is little studied. Therefore, by using three different analytic methods (culturing, bacterial universal 16S rRNA and *Vibrio*-specific 16S RNA molecular methods), the present study aimed to analyze the influence of mariculture-induced spatial heterogeneity in environmental variables on the spatial and temporal dynamics of the genus *Vibrio*. Our results suggest differences in abundance and composition of *Vibrio* communities in relation to mariculture-impacts on a set of environmental factors.

The average abundance of 16S rRNA gene copy number of *Vibrio* in this area is 3.46×10^6–6.70×10^6 copies L^{-1}, which is basically the same as the *Vibrio* abundance reported earlier in various coastal areas of China (5.1×10^5–7.54×10^8 copies L^{-1}) [24,26,46,47]. On the other hand, the numbers of cultivable *Vibrio* spp. were 5.65×10^4–5.75×10^5 cfu L^{-1} in the two studied areas of Dongshan Bay. A similar or even higher range has been found in other estuaries and coastal waters (10^3–10^6 CFU L^{-1} and 10^4–10^8 16S rRNA gene copies L^{-1}) [39,47,55]. In general, live *Vibrio* species were detectable by cultivation. Overall, this study shows that the qPCR method is more sensitive and hence better suited for targeted *Vibrio* detection than the culture-based approach when analyzing low-biomass seawater samples.

Primers targeting bacterial or *Vibrio*-specific 16S rRNA gene regions are usually used to assess the diversity of *Vibrio* in coastal and marine waters through high-throughput sequencing [31,46,20,43,28,56,8], and also cultivation-dependent methods have been used [47,50]. In our study, we combined all three methods to evaluate consensus and differences between the obtained results. The combination of all methods revealed 38 different *Vibrio* species. Sequence analysis of bacterial 16S rRNA genes showed that *V. fortis* had the largest proportion of the total *Vibrio* community, whereby *V. harveyi* and *V. brasiliensis*, comprised the highest proportions in areas DS1 and DS2, respectively. In contrast, *V. rotiferianus* and *V. brasiliensis* dominate in the *Vibrio*-specific community. In the cultivation-dependent approach, however, *V. fortis* and *V. campbellii* were found to be dominant in both areas (DS1 and DS2). Relative to both of the 16S rRNA based sequencing assays, the *Vibrio*-specific 16S rRNA primer set identified the greatest number of species in the *Vibrio* community, with all of the species present in the water *Vibrio* community correctly identified by this assay. Thus, the choice of a specific analysis method greatly impacts the detected dynamic in *Vibrio* community composition with important implications for subsequent ecological interpretations.

Sequencing of the *Vibrio*-specific 16S rRNA gene has been widely used to describe the diversity and composition of *Vibrio* communities, but it usually provides a poor species-level resolution, especially of phylogenetically highly related *Vibrio* species [5,12,32,35]. Most importantly, this method does not include the highly variable domains required for analyzing the relationships of closely related *Vibrio* species. This notion suggests that also *Vibrio*-specific 16S rRNA gene sequencing must be further improved to access a high *Vibrio* species diversity. In previous studies using *Vibrio*-specific 16S rRNA gene primers, the taxonomic resolution of the method has been gradually improved [53], yet the proportion of sequences that can be clearly assigned to given *Vibrio* species is usually very low [39]. In this study, we removed sequences that were not at least 90% similar to any *Vibrio* 16S rRNA sequence in our database, while the number of sequences not assigned to a *Vibrio* species in our samples was low, it may be possible that some of these sequences could be unidentified *Vibrio* species or *Vibrio* species not in our *Vibrio* database. To determine the identity of these sequences assigned to the "*Vibrio*" genus, the representative sequence can be BLASTed against the EzBioCloud and NCBI database. Based on the above mentioned measures, our systematic method significantly improved the taxonomic diversity of *Vibrio*, rather than targeting a single marker region. In addition, housekeeping genes can be used as surrogate markers for *Vibrio* species, e.g. heat shock protein 60 (*hsp60*) and other protein coding marker genes [20]. This approach allows for better defining the correlation between *Vibrio* species and the presence of specific environmental factors. Although our sequencing method using multiple gene markers seems to be ideal to addressing taxonomic coverage, the use of *Vibrio*-specific primers accurately describes the characteristics of the *Vibrio* community since the higher obtained read numbers enable a better quantification of all *Vibrio* cells.

The objectives of this study were two-fold. First, we aimed to evaluate the use of a combination of culturing and 16S rRNA amplicons for environmental surveys of *Vibrio* community and abundance. In order to demonstrate the utility of this approach, our second objective was to conduct an analysis of *Vibrio* communities and abundance in the context of environmental parameters within natural surface seawater samples. To better understand the influence of environmental heterogeneity in shaping taxonomic richness, the dataset of the *Vibrio*-specific 16S rRNA marker region was further analyzed. The finding revealed that mariculture activities significantly influence *Vibrio* composition in Dongshan Bay. Comparisons of dominant *Vibrio* communities between areas DS1 and DS2 indicate that the abundant *Vibrio* populations were diverse, including several dominant species evenly distributed in the sampling areas, i.e. *Vibrio brasiliensis*, *V. caribbeanicus*, *V. nereis*, *V. parahaemolyticus*, *V. rotiferianus*, and *V. ponticus*. The dominance of a particular *Vibrio* genus seems to be site-specific, because different species dominate in other studies (e.g. *Vibrio alginolyticus*, *V. anguillarum*, *V. brasiliensis*, *V. campbellii*, *V. harveyi*, *V. lentus, V. neptunius,* and *V. parahaemolyticus*) [7,20,26,39,44,46,47]. Generally, ß-diversity and dominance of *Vibrio* species were significantly different in areas DS1 vs. DS2, which may be caused by a greater adaptability and faster growth of these species at local, site-specific environmental selective pressure. The observed difference in Bray-Curtis dissimilarity fluctuations between areas DS1 and DS2 could be related to differences in presence and activity of different mariculture animals as well as an increase in summer sea temperature which may potentially stimulate the rapid growth of members of the genus *Vibrio*. Yet, the relatively small differences between disparate mariculture-influenced zones ($P < 0.05$) in area DS1 suggest that long-term mariculture leads to a strong homogenization in environmental variables and hence a minor effect on differences in *Vibrio* community composition and dynamics among the sampled sites (zones). A previous study supports this notion and indicates that the site-specific, cumulative impact of long-term mariculture on the benthic bacterial community is small [26].

In contrast to previous reports emphasizing the significant role of salinity and temperature for coastal *Vibrio* abundance and beta-diversity, our summer study was restricted in the tested range

width of temperature and salinity and hence the pattern was less clear. Therefore, incorporating a wider range of environmental parameters (such as DO, pH and organic nutrients) for further environmental monitoring will help to better understand the physical and chemical factors that regulate *Vibrio* abundance in the coastal environment, in particular in dependence of mariculture activities. Our analysis shows that pH is significantly positively correlated with the abundance of the genus *Vibrio*. This is in accordance to a previous study suggesting that a higher pH may enrich *Vibrio* species relative to total bacteria [22]. RDA was used to identify key environmental factors that governed the total Vibrio community. The distinction of the *Vibrio* components in DS1 and DS2 area were mainly divided by Latitude, DO and nutrient concentrations (NO_2^-, NO_3^- and NH_4^+, Fig. 8B). DO is positively correlated with the heterogeneity in the *Vibrio* community composition, indicating that increasing dissolved oxygen concentrations in seawater promotes the heterogeneity in the *Vibrio* community [27,46]. Few studies have looked at the correlation between NO_2^-, NO_3^- and NH_4^+ and *Vibrio* spp. A study by Asplund et al. [3] found a slight positive correlation with NO_3^- and *Vibrio* spp. Several previous investigation demonstrated that *Vibrio* spp. are able to reduce nitrate to nitrite or ammonia and are often the dominant nitrate-reducing group [29,37,23,54,10]. This finding suggests apart from temperature and salinity, inorganic and organic nutrients may play an important role in maintaining their activity, and thus more efforts are needed to estimate the correlations between *Vibrio* diversity and these parameters as suggested by Wang et al. [47].

Our study highlights the presence of several potentially pathogenic *Vibrio* isolates, including *V. parahaemolyticus* and *V. harveyi*, which possess a number of virulence factors. *Vibrio* communities in Dongshan Bay differ from those of other coastal environments, i.e. *V. fluvialis, V. gigantis, V. parahaemolyticus, and V. vulnificus* in the Maowei Sea, Beibu Gulf of China [7,6], as well as *V. parahaemolyticus*, *V. vulnificus* and *V. cholera* in the German Bight of the southeastern North Sea [13]. These differences may depend on variable source communities and environmental niches resulting in different *Vibrio* communities being well-adapted to changes in the respective surrounding environments. For example, several pathogenic *Vibrio* species such as *V. alginolyticus*, *V. campbellii*, and *V. fortis* lead to frequent outbreaks of diseases in farmed fishes in many tropical countries and thereby cause severe economic setbacks to the mariculture industry and increased health risks for the environment and humans [1,30]. *Vibrio alginolyticus* is an important opportunistic pathogen in maricultures, which is related to the prevalence of mariculture animals such as shellfish, fish and crustaceans [28,56]. *V. campbellii* is an important pathogen in intensive earing of shrimp, molluscs and finfish [8], and *V. fortis* causes enteritis in cultured seahorses [43]. Consequently, specific environmental factors shape *Vibrio* communities with profound consequences for economy as well as environmental and human health. The longest routine monitoring project of *Vibrio* spp. takes place at the Neuse River Estuary in North Carolina, USA (>10 years) [11,10,17,20,49]. This project reveals a clear pattern in *Vibrio* community composition and specific environmental factors such as nutrients. Concentrations of nutrients in Dongshan Bay show pronounced seasonal changes, greatly affected by seasonal urban and agricultural inputs. Mariculture is one of the main reasons due to low water exchange and large inputs of animal food and excrements in this area. Our previous study [52] confirmed that input of nutrients resulted in the highest diversity and significant temporal changes of the *Vibrio* community in summer. As a consequence, factors affecting the health of this important coastal ecosystem are constantly changing, urging to better understand the dynamics of especially pathogenic *Vibrio* species. The limited time scale of the present work (i.e. summer 2019) provides an indicative snapshot and hence insight for future investigations. Extended monitoring periods of *Vibrio* observation will show whether the annual burden of *Vibrio* populations is a general trend, increasing year by year due to global warming and increasing human activities. Therefore, it is necessary to identify specific "hot spots", such as mariculture areas that are conducive to the growth of *Vibrio* species, and strengthen monitoring of these pathogens in subtropical coastal areas to develop mitigation strategies to reduce their potential future impact on public health.

In summary, most standard methods for detecting *Vibrio* diversity are limited in their taxonomic resolution beyond the genus level. Therefore, we have investigated the dynamics of *Vibrio* spp. in surface waters of Dongshan Bay by combining qPCR, high-throughput sequencing (total bacterial and *Vibrio*-specific), and targeted cultivation approaches with each other. This combination of methods allowed us to detect a significantly higher taxonomic diversity of *Vibrio* species and to better relate changes in *Vibrio* communities to specific environmental drivers. Effects of spatial heterogeneity in coastal environmental variables on *Vibrio* abundance, diversity and community composition were identified and revealed temperature, DO and pH as major environmental drivers during the summer season. Despite an exaggeration in the relative abundance of some species, the *Vibrio*-specific 16S RNA sequencing assay provided greatly superior taxonomic resolution when compared to conventional bacterial 16S rRNA sequencing and culturing methods. *Vibrio*-specific 16S rRNA gene sequencing was successfully applied and, in comparison to rRNA gene amplicon sequencing and cultivation, it provided a better identification of overall *Vibrio* diversity and highlights its applicability for seawater samples. In conclusion, our study promotes our current understanding on marine *Vibrio* taxonomic and functional characteristics in mariculture-impacted coastal waters. This knowledge is urgently needed to alleviate the deteriorating negative impact on the environment and human health caused by the increasing number of pathogenic bacteria such as *Vibrio* due to human activities in marine coastal areas.

CRediT authorship contribution statement

Wei Xu: Conceptualization, Funding acquisition, Validation, Visualization, Writing - original draft, Writing - review & editing. **Wenzhen Lin:** Conceptualization, Visualization, Writing - original draft, Writing - review & editing. **Zhichao Wang:** Investigation, Methodology, Software, Supervision. **Yuanhao Gao:** Data curation, Investigation. **Yu Luo:** Software, Supervision, Validation. **Hans-Peter Grossart:** Writing - original draft, Writing - review & editing. **Ying Guo:** Project administration. **Qiancheng Gao:** Project administration, Resources. **Lixing Huang:** Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Zhuhua Luo:** Funding acquistion, Writing - original draft, Writing - review & editing.

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.csbj.2021.07.040.

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