



Vertical variation in *Vibrio* community composition in Sansha Yongle Blue Hole and its ability to degrade macromolecules

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Abstract

With the advantages of wide distribution, fast growth, and broad metabolic spectrum to organic carbon compounds, *Vibrio* may play an important role in organic carbon cycling. However, the ecological roles of *Vibrio* in many marine environments have not been explored. Here, the world's deepest 'blue hole', the Sansha Yongle Blue Hole (SYBH) in the South China Sea, which is a geographically semi-enclosed environment featuring unique chemical characters, was investigated. The abundance, diversity and carbon source utilization capability of *Vibrio* were studied by quantification and high-throughput sequencing of *Vibrio* specific 16S rRNA genes and cultivation methods. The abundance of *Vibrio* in water column of the SYBH ranged from 3.78×10^4 to 7.35×10^6 16S rRNA gene copies L⁻¹. Free-living *Vibrio* was more abundant than particle-associated *Vibrio* ($\sim 1.20 \times 10^6$ versus $\sim 2.68 \times 10^5$ gene copies L⁻¹), indicating that *Vibrio* prefers a free-living life style. The *Vibrio* assemblages showed clear vertical stratification and could be divided into three groups: aerobic-transition, middle anaerobic and bottom anaerobic zones. Dissolved oxygen (DO), temperature, pH and salinity were the main environmental factors affecting the abundance and community composition. Cultivated *Vibrio* demonstrated a degrading capability to various macromolecular substrates, including starch, Tween 20/40/80, DNA, gelatin, alginate, casein, chitin, lecithin, κ -carrageenan, mannan, xylan and hyaluronic acid. This suggests that *Vibrio* could produce a variety of highly active extracellular enzymes. Our study provides new insights into the distribution pattern and possible role in carbon cycle of *Vibrio* in the unique environment of a 'blue hole'.

Keywords *Vibrio* community · Vertical stratification · Carbon source utilization · Sansha Yongle Blue Hole

Introduction

The genus *Vibrio* is a group of gram-negative bacteria belonging to the *Vibrionaceae* family of the *Gammaproteobacteria*, which are usually mesophilic and heterotrophic motile rods (Thompson et al. 2004a). *Vibrio* species are ubiquitous in estuarine and marine habitats worldwide,

even including deep-sea hydrothermal vents and sediments at a depth of more than 6000 m, and seawater at a depth of 10,500 m in the Mariana Trench (Hasan et al. 2015; Hsieh et al. 2008; Li et al. 1999; Liu et al. 2019). In addition, species within the *Vibrionaceae* family show two alternative niche specializations, i.e., free-living forms or attached to particles (such as biotic and abiotic surfaces) (Reen et al. 2006). Currently, the genus *Vibrio* contains more than 110 species, many of which are known as causative agents of foodborne related illnesses, which have a huge impact on public health and management (Lee and Raghunath 2018; Letchumanan et al. 2015; Pruzzo et al. 2005). The major pathogenic *Vibrio* species include *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Letchumanan et al. 2016).

Despite the importance of microbially driven carbon cycling, the exact mechanisms of carbon cycling in the ocean are poorly understood. Due to wide distribution, very short replication times (as short as ~ 10 min) and an

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ability to utilize a wide range of organic carbon compounds, *Vibrio*, one of the best model marine heterotrophic bacterial groups, is of great value in understanding marine organic carbon cycling (Zhang et al. 2018). *Vibrio* spp. show distinct abilities to break down and utilize common carbon substrates via extracellular enzymes, such as chitinase (Svitil et al. 1997), protease (Venugopal and Saramma 2006), xylanase (Araki et al. 1999b), mannanase (Tamaru et al. 1995), κ -carrageenanase (Araki et al. 1999a), fucoidanase (Furukawa et al. 1992), laminarinase and alginase (Davis 1992). The production and secretion of these enzymes could provide access to abundant nutrients that are unavailable to other organisms (Mansergh and Zehr 2014; Thompson and Polz 2006). In addition, *Vibrio* spp. are copiotrophic bacteria that have the capacity to increase rapidly in abundance, shifting from a relatively rare component to being dominant in response to environmental perturbations and climate changes (Gilbert et al. 2012; Oberbeckmann et al. 2012; Takemura et al. 2014).

Blue holes are underwater caves, found in shallow marine and fresh waters (Whitaker and Smart 1990). Blue holes have been widely studied over decades, and have provided information on global climate change, marine ecology and carbonate geochemistry (Myroie et al. 1995). Blue holes differ from other marine habitats in that they have little to no photosynthetic oxygen production and restricted vertical mixing, which results in anoxic or microoxic conditions. They also have further distinct biogeochemical reactions compared to other marine environments (Gonzalez et al. 2011). In many blue holes, microbial metabolism of organic matter, which might have originated from in situ bacterial primary production coupled with oxidization of inorganic sources (e.g., sulfide, ammonium and methane; Brankovits et al. 2017), leads to anoxic conditions. For example, chemoautotrophic bacteria discovered in a ground-water blue hole ecosystem in southern Romania and an anchialine cave in Yucatan Peninsula might provide sufficient organic matter to the food web (Brankovits et al. 2017; Pohlman et al. 1997; Sarbu et al. 1996). Unique microbial communities have been reported from oxic zones to anoxic zones in the Jewfish Sink of the Gulf of Mexico (Garman and Garey 2005). In this blue hole, sulfate reduction appears to be the major metabolic process in the anoxic zones, the walls of which have characteristic microbial mats, whereas bacterial activity in the oxic zones appears to be phosphate-limited (Garman and Garey 2005).

Sansha Yongle Blue Hole (SYBH, 16°31'30"N, 111°46'05"E), also called the Dragon Hole, is located on Yongle Atoll in the Paracel Islands of the South China Sea and is the deepest (~ 300 m) blue hole in the world (Li et al. 2018). The lack of fresh water input or a subsurface connection to the sea makes SYBH an isolated and still ecosystem (Xie et al. 2019), representing a natural

laboratory for the study of microbial communities. SYBH contains multiple pycnoclines, each associated with complex physicochemical profiles (Bi et al. 2018), which may strongly affect the vertical distributions of indigenous microbial communities. Research on SYBH is still at the initial stage. Although some water column chemistry data have been reported recently (Bi et al. 2018; Xie et al. 2019), little is known about the biogeochemical cycles in this blue hole.

In this study, the abundance and community structure of *Vibrio* population at different depths within SYBH were investigated by *Vibrio*-specific 16S rRNA gene high-throughput sequencing and quantitative PCR (qPCR), as well as traditional cultivation methods. The key environmental factors driving the *Vibrio* community changes were evaluated by statistical analysis. The organic carbon utilization characteristics of *Vibrio* isolates were explored through extracellular enzymatic activity experiments. A significant vertical variation of the *Vibrio* community in SYBH in relation to various environmental factors was discovered, and the *Vibrio* isolates were able to degrade a variety of macromolecules.

Results

Environmental characterization of SYBH in March 2017

In this ~ 300 m deep blue hole, dissolved oxygen (DO) decreased from 6.34 mg/L at 0 m to zero at 100 m, indicating that the environment above 100 m was oxic and below was anoxic; the concentration of DO dropped sharply (from 4.28 to 0.05 mg/L) between 80 and 100 m, indicating a DO-transition/suboxic zone. In addition, most physical and chemical parameters remained relatively uniform below the depth of 160 m (Yao et al. 2018). The water column could thus be divided into three zones; an oxic–suboxic zone (including the oxic zone and the suboxic zone, i.e., 0–90 m), a middle anoxic zone (100–160 m) and a bottom anoxic zone (160–230 m).

Water temperature decreased from 27.05 °C at the surface to 15.27 °C, salinity increased from 33.4 to 34.6 PSU, while pH ranged from 7.26 to 8.14 (Table S1) (Yao et al. 2018). Generally, pH, DO and NO_2^- decreased with depth, whereas salinity, NH_4^+ , PO_4^{3-} and SiO_3^{2-} showed opposite trends (Yao et al. 2018). DO was considered to be the main cause for these changes, while the other parameters, such as NH_4^+ , NO_3^- and PO_4^{3-} , co-varied with DO (Yao et al. 2018). Therefore, there are significant connections between DO and other environmental factors.

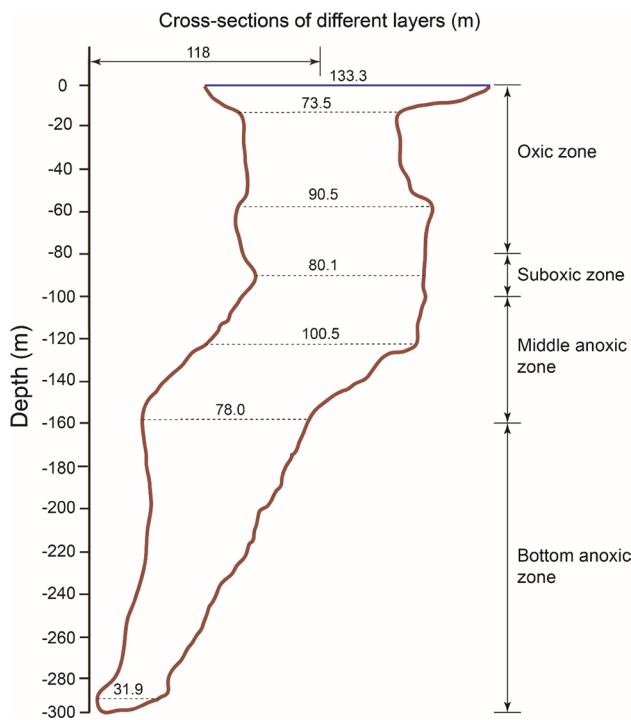


Fig. 1 Vertical section of SYBH at an azimuth of 30° (modified from Xie et al. 2019)

Abundance of *Vibrio* population in March 2017

Because *Vibrio* spp. exhibit two alternative growth strategies, microorganisms were serially collected by 3- μm -pore size and 0.2- μm -pore size polycarbonate membranes, which were designated _F (free-living *Vibrio*) and _P (particle-associated *Vibrio*), respectively in the following analyses (Fig. 1).

The *Vibrio* population size ranged from 3.78×10^4 to 7.35×10^6 16S rRNA gene copies $\cdot \text{L}^{-1}$ in the blue hole seawater (Table 1). The highest abundance was measured at 100_F (7.35×10^6 gene copies $\cdot \text{L}^{-1}$) while the lowest was recorded at 170_P (3.78×10^4 gene copies $\cdot \text{L}^{-1}$). The abundance of free-living *Vibrio* population ranged from 1.21×10^5 to 7.35×10^6 gene copies $\cdot \text{L}^{-1}$, while the particle-associated *Vibrio* population varied from 3.78×10^4 to 9.08×10^5 gene copies $\cdot \text{L}^{-1}$. The abundance of free-living *Vibrio* population ($\sim 1.20 \times 10^6$ gene copies $\cdot \text{L}^{-1}$) was significantly higher than that of particle-associated *Vibrio* population ($\sim 2.68 \times 10^5$ gene copies $\cdot \text{L}^{-1}$) ($P < 0.05$) (Fig. 2). It was found that the abundance of free-living *Vibrio* population in the samples had a positive correlation to salinity, PO_4^{3-} and SiO_3^{2-} , and a negative correlation to DO and pH. No parameters showed significant correlation with the abundance of particle-associated *Vibrio* population (Table 2).

The abundance of both the free-living and particle-associated *Vibrio* populations had no significant differences among

Table 1 Richness and diversity estimates of total free-living and particle-associated *Vibrio* communities at SYBH based on 97% OTU clusters

	Free-living <i>Vibrio</i>						Particle-associated <i>Vibrio</i>					
	gene copies $\cdot \text{L}^{-1}$	Observed OTUs	Chao 1	Shannon	Coverage (%)		gene copies $\cdot \text{L}^{-1}$	Observed OTUs	Chao 1	Shannon	Coverage (%)	
0_F	6.76×10^5	40	66	1.342	99.96	0_P	7.84×10^5	36	38	1.264	99.98	
10_F	2.54×10^5	69	69	2.089	100.00	10_P	7.11×10^4	60	60.75	1.608	99.99	
40_F	3.72×10^5	39	39	1.514	100.00	40_P	4.68×10^4	33	33	1.381	100.00	
70_F	1.21×10^5	29	29.5	1.419	99.99	70_P	1.51×10^5	23	23	1.356	100.00	
80_F	1.69×10^5	34	35	1.492	100.00	80_P	7.72×10^4	20	21	0.978	99.99	
85_F	7.89×10^5	25	25.5	1.428	99.99	85_P	1.69×10^5	25	25	1.618	100.00	
90_F	8.75×10^5	32	32	1.613	100.00	90_P	9.47×10^4	26	26	1.420	100.00	
100_F	7.35×10^6	18	18.3	0.474	99.99	100_P	8.68×10^5	26	26	1.402	100.00	
120_F	9.30×10^5	27	30	1.321	99.99	120_P	9.75×10^4	24	24	1.709	100.00	
140_F	1.45×10^6	37	40	1.444	99.99	140_P	9.08×10^5	32	32	1.741	100.00	
170_F	1.79×10^6	54	60	1.799	99.99	170_P	3.78×10^4	33	33	0.943	100.00	
190_F	4.21×10^5	65	66.5	2.199	99.99	190_P	1.01×10^5	41	41	1.284	100.00	
230_F	3.77×10^5	66	66	2.018	100.00	230_P	8.30×10^4	45	46	1.313	99.99	

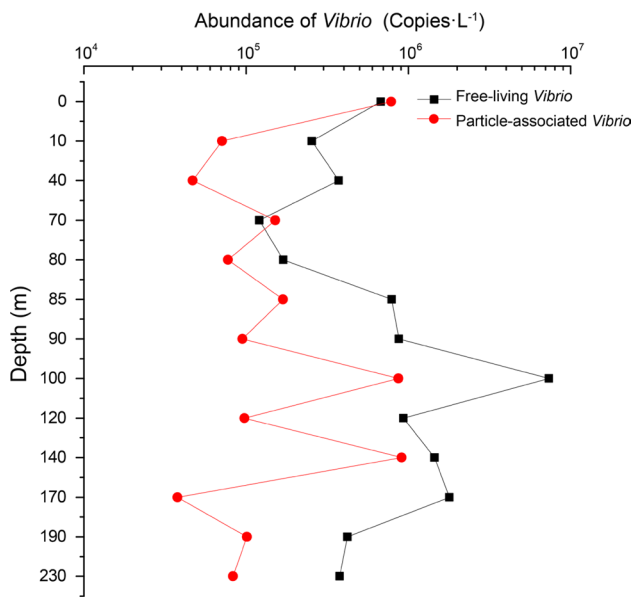


Fig. 2 The abundance of free-living and particle-associated *Vibrio* populations at different water depths of SYBH

the three depth-zones (Kruskal–Wallis test, $P > 0.05$). Comparison of the relative proportions of *Vibrio* (i.e., proportion of *Vibrio* to total bacteria) in different sized fractions showed that *Vibrio* occur naturally and are an abundant group of marine microorganisms (0.15%–15.94% of total bacteria) in SYBH (Fig. S1).

***Vibrio* diversity and richness**

The samples studied here yielded high-quality sequences ranging from 25,645 to 43,959 reads, of which 25,094

sequences were left for each sample after normalizing. The average length of the obtained sequences was 512 base pairs. In total, 136 OTUs were clustered after randomly resampling, ranging from 18 to 69 OTUs per samples (Table 1), at a 97% similarity level. Good's coverages ranged from 99.96% to 100% in all the samples, indicating that most of the species in the study areas could be represented by the libraries generated by high-throughput sequencing.

Both the observed OTUs and Chao 1 index of free-living *Vibrio* were significantly higher than those of particle-associated *Vibrio* ($P < 0.05$, Wilcoxon's rank test), but there were no significant differences in the Shannon index between them. Moreover, the observed OTUs were highest in the oxic–suboxic zone (102 OTUs), while the middle anoxic zone and bottom anoxic zone contained 49 and 97 OTUs, respectively. The Chao 1 index was significantly higher in the bottom anoxic zone than in the middle anoxic zone ($P < 0.05$). In addition, there were no significant differences in diversity measures (including observed OTUs, Chao 1 index and Shannon index) between the oxic–suboxic zone and the middle anoxic zone ($P > 0.05$). The correlation coefficients between diversity indices and environmental factors are shown in Table 2; the observed OTUs and Chao 1 index of free-living and particle-associated *Vibrio* communities had consistently negative relationships with NO_3^- and NO_2^- .

***Vibrio* community structure**

The taxonomy of each OTU was assigned against the Ezbiocloud database, with the 30 most abundant OTUs being shown in Fig. 3. Among the *Vibrio* OTUs (representing $> 0.05\%$ of the total normalized sequences), almost all sequences (98.89%) belonged to the *Vibrionaceae* family

Table 2 Correlation coefficients between abundance/diversity indices of *Vibrio* population and environmental factors at SYBH

Environmental factors	Free-living <i>Vibrio</i>				Particle-associated <i>Vibrio</i>			
	Abundance	Observed OTUs	Chao 1	Shannon	Abundance	Observed OTUs	Chao 1	Shannon
Depth	0.473	0.099	0.072	0.291	0.027	0.124	0.124	-0.022
Temperature	-0.473	-0.099	-0.072	-0.291	-0.027	-0.124	-0.124	0.022
Salinity	0.615*	-0.045	-0.028	0.109	0.168	-0.006	-0.006	0.173
pH	-0.559*	-0.198	-0.193	-0.391	0.066	-0.247	-0.247	-0.008
DO	-0.577*	-0.057	-0.068	-0.226	-0.051	-0.091	-0.091	-0.147
NO_3^-	-0.066	-0.554*	-0.646*	-0.264	-0.121	-0.605*	-0.605*	-0.129
NO_2^-	-0.301	-0.657*	-0.689**	-0.546	0.218	-0.607*	-0.607*	0.199
NH_4^+	0.429	0.448	0.477	0.256	-0.036	0.503	0.503	-0.234
PO_4^{3-}	0.571*	0.055	0.041	0.231	0.027	0.069	0.069	-0.104
SiO_3^{2-}	0.571*	0.055	0.041	0.231	0.027	0.069	0.069	-0.104

Correlation coefficient is a positive value, indicating a positive correlation; correlation coefficient is a negative value, indicating a negative correlation

*Significantly related

**Extremely significant related

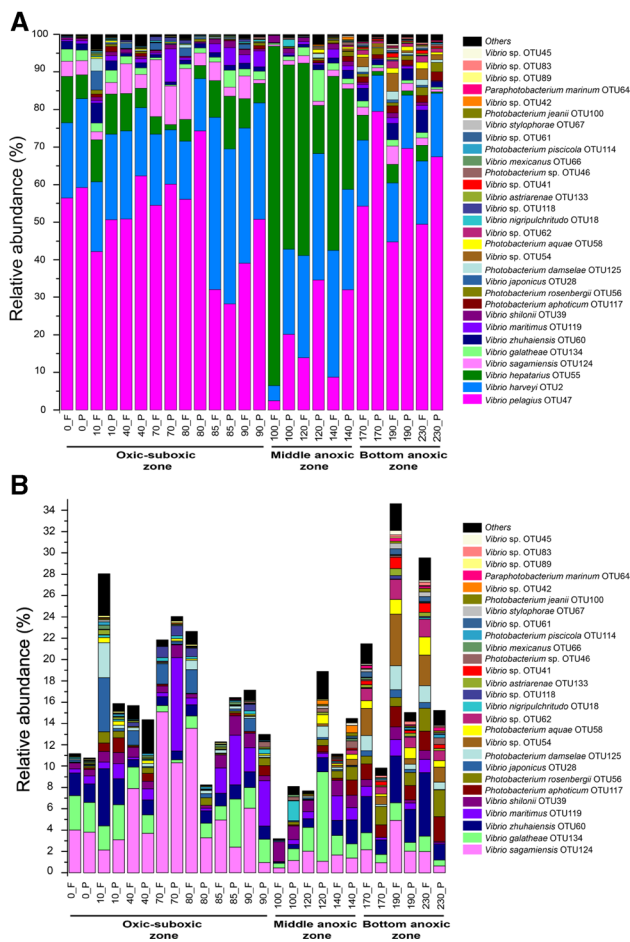


Fig. 3 *Vibrio* community compositions at OTU level across all samples. _F and _P stand for free-living *Vibrio* and particle-associated *Vibrio*, respectively: (a) the most abundant 30 OTUs; (b) the most abundant 30 OTUs except the dominant 3 OTUs

and 96.34% were assigned to the genus *Vibrio*. Seven OTUs (composed of all OTUs with > 1.0% of relative abundance in the total sequences) representing the most abundant species in the population, comprised 93.01% of the sequences among all samples. These seven OTUs included OTU47 (most similar to *V. pelagius*; accounting for 45.93%), OTU2 (most similar to *V. harveyi*; 22.40%), OTU55 (most similar to *V. hepatarius* 15.49%), OTU124 (most similar to *V. sagamiensis*; 3.91%), OTU134 (most similar to *V. galathea*; 1.96%), OTU60 (1.88%; most similar to *V. zhuhaiensis*) and OTU119 (most similar to *V. maritimus*; 1.45%).

The dominant groups differed significantly between each zone (Fig. 3); *V. pelagius* OTU47, *V. harveyi* OTU2, *V. hepatarius* OTU55 and *V. sagamiensis* OTU124 dominated the oxic–suboxic zone, *V. hepatarius* OTU55 and *V. harveyi* OTU2 dominated the middle anoxic zone, whereas *V. pelagius* OTU47 and *V. harveyi* OTU2 were the dominant groups in the bottom anoxic zone. To investigate the differences in free-living and particle-associated *Vibrio*, the absolute

abundance of dominant OTUs was calculated by multiplication of the total abundance of *Vibrio* and their relative proportions in the *Vibrio* communities (Table S3). It was found that the abundance of the dominant groups in the free-living fraction was greater than in the particle-associated fraction. No significant differences were observed among the three depth zones for *V. pelagius* OTU47 and *V. harveyi* OTU2, but the abundance of *V. hepatarius* OTU55 was higher in the middle anoxic zone than in the other two depth zones.

While there were no marked differences in the *Vibrio* community compositions between free-living and particle-associated fractions, significant vertical shifts were observed among the three depth-zones (Fig. 4a). The first two principal components explained 49.89% of the total community variation. Clear variations between the depths below 170 m and other depths were found along the first axis, whereas 0–90 m and 100–140 m could be separated by the second axis.

The relationship between *Vibrio* community compositions and environmental factors at the OTU level was analyzed by db-RDA (Fig. 4b); RDA1 and RDA2 together explained 43.88% of the total variance among the samples. Two size fractions (_F and _P) at each depth were similar (Fig. 4b), so environmental factors shaped the same distribution of free-living and particle-associated *Vibrio* community at the same depth. Monte Carlo permutation tests showed that NH_4^+ ($F=9.3$, $P=0.002$), PO_4^{3-} ($F=8.8$, $P=0.002$), SiO_3^{2-} ($F=7.2$, $P=0.002$), temperature ($F=6.4$, $P=0.002$), pH ($F=6.4$, $P=0.002$), DO ($F=3.9$, $P=0.002$), salinity ($F=4.3$, $P=0.004$), and NO_2^- ($F=2.2$, $P=0.03$) significantly contributed to the *Vibrio* community structure.

Spearman’s correlations between the relative abundance of the 30 most abundant OTUs and environmental factors were calculated. For both the free-living (Table S4) and particle-associated (Table S5) *Vibrio* communities, the three most dominant OTUs (*V. pelagius* OTU47, *V. harveyi* OTU2, and *V. hepatarius* OTU55) had no correlations with the tested environmental factors; many OTUs, which included most OTUs comprising < 4.0% of total sequences, demonstrated correlations with some of the environmental factors. For free-living *Vibrio* communities, the relative abundance of *V. sagamiensis* OTU124 was positively related to DO and NO_3^- , and negatively related to NH_4^+ ; *V. zhuhaiensis* OTU60 and *V. maritimus* OTU119 were negatively correlated with NO_2^- and DO, respectively. For particle-associated *Vibrio* communities, *V. sagamiensis* OTU124 was negatively correlated to depth, PO_4^{3-} , SiO_3^{2-} and salinity ($P < 0.01$), and positive correlated to temperature, DO, pH, and NO_2^- ($P < 0.01$). In addition, the relative abundance of *V. zhuhaiensis* OTU60 and *V. maritimus* OTU119 were negatively correlated with NO_3^- and NO_2^- , and NH_4^+ and DO, respectively. Within the top 30 OTUs, *Vibrio* sp. OTU61 and *Vibrio* sp. OTU45 occurred only in the anoxic water

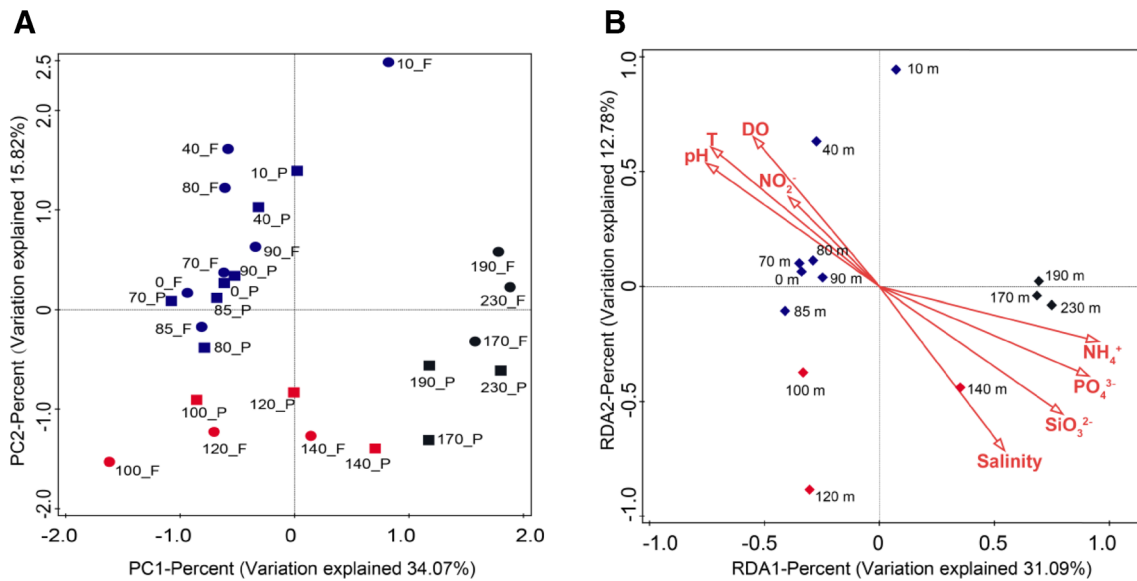


Fig. 4 Community analysis of *Vibrio* and the relationship with environmental factors: (a) UniFrac principal coordinate analysis (PCoA) of different samples based on OTUs assigned at 97% sequence similarity; (b) db-RDA diagram illustrating the relationship between *Vibrio* community and environmental variables based

below 140 m, and *Vibrio* sp. OTU83 was present solely below 170 m.

Cultivable *Vibrio* spp. and their abilities to digest various macromolecules

To understand the ability of *Vibrio* spp. to degrade various macromolecules, an attempt was made to isolate *Vibrio* cultures from seawater at different depths of SYBH. From the two cruises, 19 species of *Vibrionaceae* (in total 178 isolates) were cultured, including *V. antiquaries* (79 isolates), *V. neocaledonicus* (19 isolates), *V. parahaemolyticus* (17 isolates), *V. owensii* (8 isolates), *V. azureus* (7 isolates), *V. harveyi* (6 isolates), *V. tubiashii* (5 isolates), *V. alginolyticus* (5 isolates), *V. nigripulchritudo* (4 isolates), *V. japonicas* (4 isolates), *V. galathea* (2 isolates), *V. maritimus* (2 isolates), *V. fluvialis* (2 isolates), *V. proteolyticus* (1 isolate), *V. hyugaensis* (1 isolate), *V. atypicus* (1 isolate), *Catenococcus thiocycli* (10 isolates), *Photobacterium damsela* (1 isolate) and a potential new *Vibrio* species (4 isolates, isolated from the anaerobic microorganisms incubation media). All *Vibrionaceae* isolates (except the four potential new *Vibrio* isolates) and the depths isolated are shown in Fig. S2; the 16S rRNA genes of 91 isolates were sequenced and their GenBank accession numbers are shown in Table S6.

Media supplemented with a variety of macromolecules were used to detect extracellular enzymes. Of the 178 *Vibrionaceae* isolates cultured, 46 isolates consisting of 15 *Vibrio* species, were selected to test their extracellular

on OTUs assigned at 97% sequence similarity. Blue, oxic–suboxic zone (0–90 m); red, middle anoxic zone (100–140 m); black, bottom anoxic zone (170–230 m). Dot, free-living *Vibrio*; square, particle-associated *Vibrio*; rhombus, free-living *Vibrio* and particle-associated had the same distribution in the db-RDA

enzymes, and 45 isolates were found to display at least one hydrolytic enzymatic activity. The ratio of hydrolysis circle diameter and colony diameter in the media indicated activity strength (Table S7). A total of 15 types of extracellular enzymatic activities detected at 28 °C were summarized in Fig. 5. Amylase, gelatinase, Tween 40 lipase and κ -carrageenanase producing strains (45 out of 46 isolates) were prevalent. Caseinase and lecithinase positive strains comprised a considerable proportion (39 and 38 out of 46 isolates, respectively), while none of the isolates displayed cellulolytic activity. The numbers of isolates that could degrade DNA, Tween 20 and Tween 80 at 28 °C were 35, 33 and 18, respectively. The numbers of isolates that were

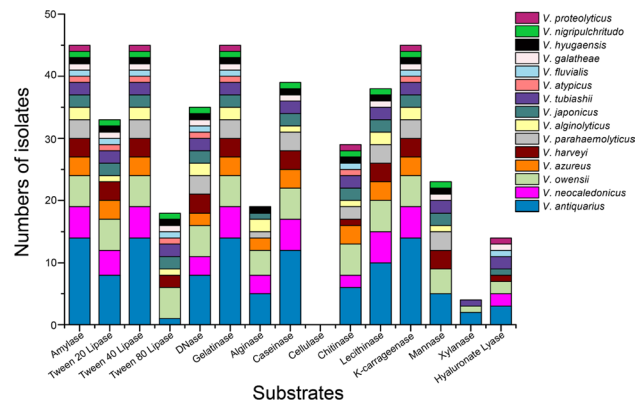


Fig. 5 Hydrolytic enzyme activities at 28 °C among all 46 isolates from the water collected in SYBH

able to degrade chitin and alginate were 29 and 19, respectively. A *V. owensii* isolate cultured from the seawater at the depth of 170 m had no tested extracellular enzymes activities. Generally, *Vibrio* responded rapidly to all these macromolecules. For organic carbon sources readily available (including starch, Tween 20/40/80, gelatin, casein, DNA, lecithin, κ -carrageenan and hyaluronic acid), the transparent zones or hydrolytic circles could be observed after 1 day of culture. However, typically 1–2 weeks were required for other bacteria to utilize chitin (Zhang et al. 2016), while the transparent zones could be observed after 3 days' incubation.

Discussion

Vibrio is a ubiquitous group of metabolically flexible marine heterotrophic bacteria, that play an important role in biogeochemical cycling in the ocean. There have been many studies on the diversity and dynamics of *Vibrio* populations in a variety of marine environments, including the Mid-Atlantic, Mediterranean coast, Australian coast, Japan surrounding seawater, and Chinese marginal seas (Amin et al. 2016; Girard et al. 2017; Liang et al. 2019; Mansergh and Zehr 2014; Siboni et al. 2016; Wang et al. 2019; Westrich et al. 2018). The distribution of *Vibrio* population in marine environment is influenced by many environmental factors including salinity, temperature, and in some cases the abundance of host organisms (Thompson et al. 2004b). However, little is known about *Vibrio* diversity and abundance in the unique environment of blue holes. To the best of our knowledge, this is the first investigation of a *Vibrio* community in a blue hole.

Environmental parameters (such as water chemistry) may strongly affect the spatial and taxonomic distributions of indigenous microbial communities and the trophic levels of food webs in blue holes (Gonzalez et al. 2011; Hoffman et al. 2018; Seymour et al. 2007). However, previous studies have either provided microbial profiles at a broad taxonomic level or have focused mainly on the water samples collected from specific depths within the cave (Gonzalez et al. 2011; Hoffman et al. 2018). A more resolved study targeting a finer taxonomic level can enhance our understanding of accurate microbial distribution patterns in response to environmental changes (Liu et al. 2015). Here, as a complement to previous studies, the vertical distribution patterns of *Vibrio* communities as well as the abilities to digest various macromolecules by the cultivable *Vibrio* was investigated.

Characteristics of *Vibrio* communities within the SYBH

In this study, *Vibrio*-specific 16S rRNA gene primers were used to sequence *Vibrio* communities in seawater samples

from different depths of SYBH, South China Sea. The variation of *Vibrio* community structure was mainly caused by the pronounced vertical layering, and the whole community could be divided into three groups: an oxic–suboxic zone (0–90 m), a middle anoxic zone (100–140 m) and a bottom anoxic zone (170–230 m). A similar structure was found in the Cariaco Basin, which is the second largest permanently anoxic marine water in the world (Garcia-Amado et al. 2011). There also, *Vibrio* could be divided into three groups: an oxic zone (0–200 m), a redox zone (230–280 m) and an anoxic zone (400–1300 m), according to the OTU composition and the water depth. The vertical change in SYBH occurred at two distinct depths; from oxic to anoxic (90–100 m) and within the anoxic water from depths where environmental parameters stopped changing (140–170 m).

The abundance of *Vibrio* was high at 100 m depth (Fig. 2). At this depth (the interface of oxic–anoxic) both aerobic and anaerobic *Vibrio* spp., with two kinds of metabolism modes (i.e., respiration and fermentation modes) existed. However, higher nutrient levels occurred at the interface of oxic–anoxic zone (Yao et al. 2018), which may have caused the increase in *Vibrio* abundance. The high abundance at this depth indicates that there might be organic substances preferred by *Vibrio* here. However, little is known about the composition of the organic matters in SYBH, and it is worth further investigation.

The sequencing analysis of the 16S rRNA gene showed that *V. hepatarius* was the dominant species at depths between 100 and 140 m but was most abundant at 100 m. *V. hepatarius* was originally isolated from the hepatopancreas of shrimp (Thompson et al. 2003) and since then has been found in the haemolymph of the spider crab (Gomez-Gil et al. 2010), the digestive tract of *Holothuria scabra* (Plotieau et al. 2013) and diseased tissue of coral reefs (Séré et al. 2013), suggesting that *V. hepatarius* may prefer utilizing specific substrates derived from animal hosts. In addition, the micro-oxic environment of these animal tissues is in line with the facultative anaerobic metabolic mode of *V. hepatarius* as previously reported (Thompson et al. 2003). This may indicate that animal-derived organic matter accumulates in the transition oxic–anoxic zone; however, this requires further confirmation.

The abundance of free-living *Vibrio* population was significantly higher than that of particle-associated *Vibrio* population, suggesting that *Vibrio* spp. prefer a free-living style. Similar results have also been observed in other marine environments, including the Baltic and Skagerrak Seas and the northern Chinese marginal seas (Eiler et al. 2006; Liang et al. 2019). It was reported that the number of particle-associated bacteria was low in the open ocean, primarily due to the low concentration of particulate material (Unanue et al. 1992). Similarly, free-living *Vibrio* populations are more abundant than particle-associated ones, most

likely because of the low particle concentrations in SYBH. Unfortunately, CFUs values on TCBS were not counted, so qPCR and CFU values could not be compared.

The *Vibrio* community in this special habitat of SYBH in terms of diversity and species richness is significantly different from other marine environments, such as estuaries, offshore and open sea ecosystems. To withstand the variable and complex conditions, it was necessary for *Vibrio* to develop a high species diversity (Liang et al. 2019). At SYBH, *Vibrio*-specific 16S rRNA gene-based sequencing retrieved a total of 136 OTUs, while a similar study at 21 sites of the northern Chinese marginal seas retrieved only 74 OTUs (Liang et al. 2019).

One potentially novel *Vibrio* species (*Vibrio* sp. JC009) from 130 m was cultured by the anaerobic cultivation technique and the full 16S rRNA gene sequence (1515 bp) showed the highest similarity to *Vibrio olivae* IGJ1.11 (95.60%). Interestingly, strain JC009 was strictly anaerobic and could not grow in the presence of oxygen, whereas all known *Vibrio* species are facultative anaerobic (Farmer et al. 2015). Thus, it is possible that the strictly anaerobic property of this new strain might have resulted from long term adaptation to the permanently anoxic environment of the SYBH.

Controls on *Vibrio* abundance and community composition in the SYBH

The results of qPCR assays indicated that *Vibrio* populations were present in all samples, suggesting that *Vibrio* was an abundant and natural component in SYBH. The abundance of *Vibrio* has been demonstrated to be largely related to temperature and salinity (Caburlo et al. 2012; Siboni et al. 2016; Takemura et al. 2014). Salinity was likewise found to be significantly influential on the abundance of free-living *Vibrio* population of this study. Indeed, several *Vibrio* species with a higher salinity preference (high-salinity populations, such as *V. pelagius*) were found in the blue hole (Eiler et al. 2006) and some species (such as *Vibrio* sp. OTU54) were positively correlated with salinity, suggesting that they have evolved to adapt to the high salinity. However, no clear correlations between the abundance of *Vibrio* and temperature in SYBH was observed (Table 2), possibly due to the strong DO gradient. Similarly, it was found that cyanobacterial-derived organic matter played a more important role than temperature in a previous study (Eiler et al. 2007).

DO is an important hydrological parameter that affects the abundance of *Vibrio* population by affecting their metabolic processes. Due to oxygen depletion, *Vibrio* populations would switch from respiration to fermentation mode (Garcia-Amado et al. 2011). The strong stratification in SYBH limits the vertical exchange of oxygen, resulting in two distinct water bodies, an upper oxic region and a bottom anoxic region (Bi et al. 2018). Turner et al. (2009) found that

Vibrio abundance in both free-living and particle-associated fractions were inversely correlated with DO (48.7%–105.8% saturation) along the coast of Georgia, USA. A negative correlation between the abundance of *Vibrio* and DO (~5 to 11 mg L⁻¹) has been found in North Carolina estuaries (Blackwell and Oliver 2008). Similar results were obtained in the present study. It should be noted that all the previous studies that investigated the correlation between *Vibrio* abundance and DO focused on oxic seawater; this study is the first to explore the correlation between *Vibrio* abundance and DO in an oxic–anoxic gradient seawater.

DO (together with its co-varied parameters, such as NH₄⁺, NO₃⁻ and PO₄³⁻), temperature and salinity were found to control the vertical structuring of the *Vibrio* community, although *Vibrio* abundance and temperature were not significantly correlated. Generally, the persistence of *Vibrio* communities depends on many factors, but the most important factors are salinity and temperature (Matteucci et al. 2015; Siboni et al. 2016). Since many *Vibrio* species can tolerate a wide range of temperature and salinity, these two factors may not lead to significant *Vibrio* species changes. In fact, DO has been reported as one of the major environmental parameters shaping *Vibrio* community composition in the Cariaco Basin (Garcia-Amado et al. 2011). In this study, the concentration of DO varied greatly from the surface to the bottom and this may have caused the significant metabolic alternation and thus species variations. Many heterotrophic bacteria are capable of anaerobic growth with nitrogen oxides (such as NO₃⁻) as electron acceptors instead of oxygen, while *Vibrio* spp. demonstrated the most efficient anoxic growth (Lacko et al. 2003). In anoxic water, the availability of an electron acceptor may be a key factor determining the survival and growth of *Vibrio* species. Concomitantly, correlations between *Vibrio* species and nitrogen oxides were found, indicating that nitrogen oxides may act as electron acceptors for *Vibrio* species dwelling in the anoxic water. Although this hypothesis needs further verification, the discovery of a diverse *Vibrio* community in anoxic water is very interesting, since no *Vibrio* species have previously been found to live strictly anaerobically.

Chemical elements, such as N, P, and Si, are essential components for the growth of marine organisms, and PO₄³⁻ and SiO₃²⁻ in particular were found to contribute to the observed variations in the *Vibrio* community (Jahid et al. 2006; Paranjpye et al. 2015; Takemura et al. 2014). This suggests that nitrogen oxides and NH₄⁺ may alternatively serve as important resources for *Vibrio* species, thus affecting their abundance and diversity (Machado and Bordalo 2014; Siboni et al. 2016). The db-RDA analyses undertaken here indeed identified NH₄⁺ as the most important factor with the greatest explanatory power. The importance of NH₄⁺ in explaining *Vibrio* distribution has also been observed in previous studies (Kopprio et al. 2017; Machado and Bordalo

2014). Thus, nutrients (as well as organic matter) are important in shaping the *Vibrio* diversity and distribution (Koprio et al. 2017). Considering that NH_4^+ , PO_4^{3-} , etc. were co-varied with DO, DO may contribute mostly to the vertical structure of the *Vibrio* spp. community.

The abilities of *Vibrio* isolates to digest various macromolecules

In this study, 167 *Vibrio* strains (belong to 17 species) from two cruises at the SYBH were isolated using different culture media. Four *Vibrio* species, including *V. harveyi*, *V. maritimus*, *V. japonicus* and *V. nigripulchritudo*, were identified by both cultivation and high-throughput sequencing methods. One reason that only four species were identified by both methods was that it was impossible to isolate all the *Vibrio* species as all the cultivation methods were selective. Another reason was that the average length of the 16S rRNA sequences obtained from the high-throughput sequencing was only ~ 500 bp, so many OTUs could not be assigned to a specific species. In addition, as primer mismatches may exist in the 16S rRNA gene high-throughput sequencing, not all the *Vibrio* species could be generated from the amplicons.

Vibrio species are suggested to play important roles in marine biogeochemical cycles, especially the organic carbon cycle (Simidu and Tsukamoto 1980; Zhang et al. 2018). *Vibrio* plays a role in nutrient cycling in aquatic environments by uptaking dissolved organic matter, or degrading many types of organic carbon by producing extracellular enzymes (Thompson et al. 2004a). Small molecules produced by macromolecular hydrolysis may be utilized by other marine bacteria, which may provide the basis for the formation of nutrient chains in microbial communities.

The *Vibrio* species isolated from SYBH in this study were able to produce a variety of highly active extracellular enzymes with generally larger hydrolytic circles, capable of breaking down 14 out of 15 macroorganic substrates tested. The reasons for having highly diverse extracellular enzymes in *Vibrio* might be as following. Firstly, there might be a range of substrates in SYBH, leading *Vibrio* isolates to produce a diverse range of extracellular enzymes here. Secondly, the genomes of *Vibrio* spp. are highly plastic, enabling them to have a broad metabolic range and produce a range of extracellular enzymes (Zhang et al. 2018). *Vibrio* isolates were able to make use of high diverse commonly existing organic substrates, indicating that they might play an important role in driving organic carbon cycling in SYBH.

As the detection of carbon substrates in natural environments is still technically difficult, no attempt was made to detect the carbon substrates in SYBH. Fifteen commonly used substrates were selected to test the abilities of *Vibrio* in SYBH to degrade macromolecules. To make this study more environmentally relevant, the organic matter present

in SYBH should also be examined in future studies. In addition, although the results from the degradation of macromolecules are preliminary, this screening may provide a resource to discover novel biocatalysts (Zhang et al. 2016). The enzymes produced from *Vibrio* may have a great potential for biotechnology, due to the fast growth rate with shorter generation-times of the organisms (Zhang et al. 2018). An isolate of *V. fluvialis*, which can produce an alkaline extracellular protease with high efficiency and good stability, was considered to be in industrial detergents (Venugopal and Saramma 2006).

Conclusions

In summary, abundant, unique and highly diverse *Vibrio* communities were present in the SYBH. The vertical variations and diversity of the *Vibrio* community seemed to be influenced simultaneously by a range of environmental factors (such as DO, temperature, salinity and pH) rather than an individual one and different *Vibrio* species responded to the environmental factors differently. The cultivated *Vibrio* isolates were able to degrade many different macro organic substrates efficiently, suggesting their potential important roles in the organic carbon cycle. In the future, a more extensive analysis of the effect of environmental parameters, such as organic carbon availability, on *Vibrio* community compositions may provide a better insight into how these factors affect *Vibrio* population dynamics. Since *Vibrio* populations might have significant influence over nutrient availability through utilizing chitin and other carbon substrates, understanding the role of the *Vibrio* populations in the SYBH may help to better constrain the carbon cycle in this unique environment.

Materials and methods

Water sampling and environmental factors

Water sampling at SYBH (Fig. 1) was conducted onboard the R/V Qiongqionghai 03138 during a spring and a summer cruise. During the two cruises, a Niskin bottle (5 L) was used to collect the water samples from depths between 0 and 190 m; an underwater robot (VideoRay Pro4 ROV) with the same Niskin bottle was used to collect water deeper than 190 m, due to the curved shape of the walls of the blue hole at depth. In the spring cruise (14–20 March, 2017), water samples from 13 depths (0, 10, 40, 70, 80, 85, 90, 100, 120, 140, 170, 190 and 230 m) were collected. One liter water sample from each depth was pre-filtered through

3- μm -pore size polycarbonate membranes (Millipore Corporation, Billerica, MA, USA) to collect particle-associated microorganisms. The free-living microorganisms were then collected through 0.2- μm -pore size polycarbonate membranes (Millipore). The filters were frozen at $-80\text{ }^{\circ}\text{C}$ until DNA extraction. The water samples were immediately inoculated into anoxic liquid media contained in acid-washed, sterile ($121\text{ }^{\circ}\text{C}/20\text{ min}$) 200 mL glass bottles for enrichment of the anaerobic microorganisms; additional water samples were stored in sterile closed bottles (acid-washed, 100 mL) at $4\text{ }^{\circ}\text{C}$, and inoculated for other types of bacterial cultivation within 1 h of sampling. In the summer cruise (14–15 June, 2017), water samples were collected from 27 depths (15, 20, 30, 50, 60, 75, 95, 105, 110, 130, 150, 160, 180, 270 m, plus 13 depths as in March); these samples were only used for bacterial cultivation.

Environmental parameters of water samples were detected by either CTDs or AA3 Auto-Analyzer. Briefly, water chemistry such as salinity, temperature, pH and DO were monitored with Alec ASTD102 CTD (JFE Advantech Co., Japan) at 0–190 m, and YSI EXO CTD (YSI Co., USA) at depths greater than 190 m, respectively. Samples for nutrients, including nitrite (NO_2^-), nitrate (NO_3^-), ammonium (NH_4^+), silicate (SiO_3^{2-}) and phosphate (PO_4^{3-}), were filtered with 0.45- μm cellulose acetate membranes and analyzed with spectrophotometric and colorimetric methods, using an AA3 Auto-Analyzer (Seal Analytical, UK) (Yao et al. 2018).

Bacterial isolation

For isolation of *Vibrionaceae* strains, 200 μL of seawater sample was spread onto marine agar 2216E (Becton–Dickinson), marine R2A agar (Fluka R2A agar was prepared with seawater instead of distilled water) and *Vibrio* selective medium TCBS (thiosulfate-citrate-bile salt-sucrose) agar, and inoculated into media for anaerobic microorganisms (Wang et al. 2015) and sulfur-oxidizing bacteria (Du et al. 2019). The anaerobic bacteria were enriched for 1 month in liquid media under an atmosphere of mixed gas (10% H_2 , 20% CO_2 and 70% N_2) at $16\text{ }^{\circ}\text{C}$, then the bacterial suspension was spread on marine agar 2216E in an anaerobic packet of Aneropack-Anaero (Mitsubishi Gas Chemical Co., Japan) for bacterial purification (Fu et al. 2018). The sulfur-oxidizing bacteria were enriched in liquid medium at $28\text{ }^{\circ}\text{C}$ for 1 month in dark, and then the bacterial suspension was spread on solid medium (Du et al. 2019). The bacterial colonies were purified, preserved and sequenced to determine the phylogenetic characteristics according the method described by Yin et al. (2013).

DNA extraction and quantitative PCR (qPCR)

The filtered membranes were cut into small pieces and DNA on the filters were extracted by chemical (phenol–chloroform method) and mechanical lysis according to the method described by Yin et al. (2013), with additional steps to facilitate cell lysis by a Fast Prep-24 Homogenization System (MP Biomedicals, Irvine, CA, USA) and the addition of lysozyme and protease K. DNA quantity and purity was evaluated using a Nanodrop-1000 Spectrophotometer.

The abundance of the total *Vibrio* population was quantified by a SYBR Green qPCR method utilizing *Vibrio*-specific 16S rRNA gene primers V-567F (5'-GGCGTAAAGCGCATGCAGGT-3') (Thompson et al. 2004b) and V-680R (5'-GAAATTCTACCCCCCTCTACAG-3') (Thompson et al. 2004b). The reactions were performed in triplicate with negative controls at the following condition: an initial denaturation at $95\text{ }^{\circ}\text{C}$ for 5 min, followed by 35 cycles of a 2-step reaction at $95\text{ }^{\circ}\text{C}$ for 30 s and $64\text{ }^{\circ}\text{C}$ for 60 s. The 20 μL qPCR experiments were run involved: 10 μL SYBR Premix Ex Taq II (2 \times), 0.4 μL ROX Reference Dye II (50 \times), 0.8 μL forward and reverse primers (10 μM), 6 μL sterile double-distilled water and 2 μL DNA samples. To quantify the total bacteria, the 16S rRNA gene universal primer sets Eub338F/518R (Yin et al. 2013) were used. Each 20 μL of quantitative PCR reaction solution contained the components described as Zheng et al. (2017), and the PCR protocol was at $95\text{ }^{\circ}\text{C}$ for 3 min, followed by 35 cycles of $95\text{ }^{\circ}\text{C}$ for 30 s, $56\text{ }^{\circ}\text{C}$ for 30 s, and $72\text{ }^{\circ}\text{C}$ for 30 s.

All qPCR assays performed on StepOne™ Real-time PCR System (Applied Biosystems) and StepOne software version 2.2, and standard curves (equivalent to 10^1 – 10^7 gene copies L^{-1}) for each qPCR assay were prepared according to Liang et al. (2019), obtained by amplifying pUCm-T (a plasmid, Sangon Co., China, and the inserted sequence of 16S rRNA gene was shown in Table S2). The efficiencies of the qPCR reactions varied from 95 to 105%, with R^2 values $>99\%$. In addition, a melting curve was added to the end of each qPCR assay, to confirm that each primer pair produced only a single specific product and agarose gels were generated if variance was observed among target sequences.

PCR amplification and high-throughput sequencing

The *Vibrio*-specific 16S rRNA gene primers V-169F (5'-GGATAACC/TATTGGAAACGATG-3') (Liu et al. 2006) and V-680R (Thompson et al. 2004b) were used to detect *Vibrio* community compositions of the water samples at different depths. The PCR reactions and cycling conditions were performed as described by Liang et al. (2019). After confirming positive amplification, the PCR products from the samples were used to prepare DNA libraries with the Illumina TruSeq DNA library preparation protocol.

Sequencing was performed on the Illumina MiSeq platform (Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China).

Sequence processing and operational taxonomic unit (OTU) assignment

The 16S rRNA gene data were analyzed on the online platform of Majorbio I-Sanger Cloud Platform (<http://www.i-sanger.com>). The SILVA database (SSU128) was used for taxonomic identification. The sequences were clustered into OTUs at the level of 97% and the classification confidence threshold was 70%. The representative sequences of the top 30 OTUs were blasted against the EzBioCloud database (<https://www.ezbiocloud.net>), and the top-hit sequence was downloaded for each OTU, and OTUs with less than 97% 16S rRNA gene similarities to known *Vibrio* species may be new species.

Screening for the abilities of *Vibrio* isolates to digest various macromolecules

For cultivated *Vibrio* isolates, the ability to digest macromolecules was detected by using different culture media with the method described by Zhang et al. (2016). The substrates included starch, Tween 20/40/80, DNA agar, gelatin, alginate, casein, chitin and lecithin. Mannan, κ -carrageenan, xylan and hyaluronic acid were used as substrates in marine agar 2216E plates to detect the additional extracellular enzymes. The appearance of transparent zones or hydrolytic circles in the media demonstrated the activities of a series of extracellular enzymes (Zhang et al. 2016).

Statistical analysis

The distribution and abundance matrix of OTUs was randomly resampled to equalize sampling efforts, and the observed OTUs, Chao 1 richness, Shannon diversity index and Good's coverage were calculated in I-sanger website. The normalized Shannon and Chao 1 index were used as proxies for diversity and richness and differences in the alpha diversity indices were tested using student's *t* test. For the beta diversity, a principal coordinate analysis (PCoA) bi-plot was utilized to visualize relationships of *Vibrio* populations in the water samples. Distance-based-redundancy analyses (db-RDA) with 999 Monte Carlo permutation tests were conducted on Canoco (version 5) to evaluate the relationship between samples and environmental factors. Spearman's rank analysis was calculated with IBM SPSS statistics 22.0 software (SPSS Inc., Chicago, IL, USA) to define statistically significant relationships between environmental factors and diversity indices, and between environmental factors and *Vibrio* abundance.

Availability of data

The 16S rRNA gene high-throughput sequencing data were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (accession number: PRJNA531757). The 16S rRNA gene sequencing data of 91 *Vibrionaceae* isolates were deposited at GenBank under the accession numbers MN028428 to MN028517 and MF942360.

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Author contributions XHZ designed the experiments and analyzed the data. LF and ZY designed the cruise and provided the water samplers. BL analyzed the environmental sequencing data, and performed qPCR and the experiments for the abilities of *Vibrio* isolates to digest various macromolecules. JL analyzed the environmental sequencing data. SZ extracted the community DNA and isolated the strains from seawater. PY and LC analyzed the environmental factors. XHZ, BL, JL and XW wrote the manuscript. All authors edited and approved the final manuscript.

Compliance with ethical standards

Conflict of interest All the authors declare that there are no conflicts of interest.

Animal and human rights statement This article does not contain any studies with human participants or animals performed by any of the authors.

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