Spatiotemporal dynamics of the total and active *Vibrio* spp. populations throughout the Changjiang estuary in China

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Summary

***Vibrio* is ubiquitously distributed in marine environments and is the most extensively characterized group within *Gammaproteobacteria*. Studies have investigated *Vibrio* spp. worldwide, but mostly focused on pathogenic vibrios and based on cultivation methods. Here, using a combination of molecular and culturing methods, we investigated the dynamics of the total and active *Vibrio* spp. throughout the Changjiang estuary in China. The total *Vibrio* abundance was higher in summer (∼6.59×10^3 copies ml^{-1}) than in winter (∼1.85×10^3 copies ml^{-1}) and increased from freshwater to saltwater (e.g. 8.04×10^1 to 9.39×10^3 copies ml^{-1} in summer). The ratio of active to total *Vibrio* (Va/Vt) revealed a high activity of vibrios, with remarkable differences between freshwater and saltwater ($p < 0.05$). Based on the community compositions of the culturable, total and active *Vibrio*, *Vibrio atlanticus* and *Vibrio owensii* were the dominant and active species in winter and summer, respectively. The distribution of *Vibrio* was governed by the effects of diverse environmental factors, such as temperature, salinity, pH, dissolved oxygen and SiO_3^{2-}. Our study clearly demonstrates the spatiotemporal dynamics of total and active *Vibrio* spp. and lays a foundation for fully understanding the ecological roles of marine *Vibrio*.**

Introduction

Vibrio species are Gram-negative bacteria that belong to *Gammaproteobacteria* and comprise more than 120 species (Table S1). Several well-known *Vibrio* spp. are pathogenic to human and marine animals, e.g. *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio anguillarum* and *Vibrio harveyi* (Daniels and Shafaie, 2000; Farmer *et al*., 2005; Zhang *et al*., 2020; Phillips and Satchell, 2017; Hickey and Lee, 2018). However, most *Vibrio* species are saprophytes (Table S1) (Takemura *et al*., 2014; Zhang *et al*., 2018). The common characteristics of the genus *Vibrio* are halophilic nature, fast growth and broad metabolic activity with the capability in production of various extracellular enzymes (Asplund *et al*., 2011; Zhang *et al*., 2018). In addition, most *Vibrio* species are motile by means of polar flagella and grow very fast with much shorter generation-times (Farmer *et al*., 2005; Zhang *et al*., 2018).

In general, *Vibrio* species have comparatively low abundance within the natural microbial community. Nevertheless, they are considered to play important roles in the marine carbon cycle, especially in marginal seas and estuarine ecosystems (Takemura *et al*., 2014; Vezzulli *et al*., 2016; Zhang *et al*., 2018). *Vibrio* spp. have flexible physiology and relatively short generation time, allowing them to rapidly increase in number and become dominant during phytoplankton (e.g. diatom and *Phaeocystis*) and micronutrient (e.g. iron) 'bloom' events (Baffone *et al*., 2006; Westrich *et al*., 2016; Zhang *et al*., 2018). For example, explosive *Vibrio* blooms have been observed at a temperate coastal site off Plymouth (UK) (Gilbert *et al*., 2012) and in the Caribbean Sea and subtropical Atlantic Ocean (Westrich *et al*., 2016). Such a rapid growth of *Vibrio* spp. may be achieved by their capacity to consume a wide range of marine organic matter, including chitin, alginate and agar derived from marine algae and animals, which may exert large impacts on the marine carbon cycling (Farmer *et al*., 2005;

Takemura *et al*., 2014; Zhang *et al*., 2018). Consistently, *Vibrio* species are intimately linked to the transformation of organic and inorganic nutrients and are involved in both the uptake and remineralization of carbon, nitrogen and phosphorus compounds (Urdaci *et al*., 1988; Takemura *et al*., 2014; Kopprio *et al*., 2016; Jesser and Noble, 2018).

The occurrence and distribution of *Vibrio* spp. in natural environments has been well investigated either by culturing methods (Hsieh *et al*., 2007; Hsieh *et al*., 2008; Kopprio *et al*., 2016; Zhang *et al*., 2018) or by molecular methods at the DNA level using *Vibrio*-specific 16S rRNA gene primers (Vezzulli *et al*., 2009; Siboni *et al*., 2016). However, the DNA-based studies cannot discern vibrios that are active, dead, or in a viable but non-culturable (VBNC) state (i.e. dormant) (Miskin *et al*., 1999; Eiler *et al*., 2006). As active microbes usually contain a higher number of ribosomes than quiescent cells, analysis of sequences derived from RNA may provide information on the active community (Nomura *et al*., 1984; Griffiths *et al*., 2000; Nogales *et al*., 2001; Sessitsch *et al*., 2002; Liu *et al*., 2019). Previous reports have revealed that the total and active bacterial communities exhibited contrasting ecological distributions driven by different environmental factors (Miskin *et al*., 1999; Nogales *et al*., 2001; Zhang *et al*., 2014). However, the ecology of active *Vibrio* in natural environments is limited. With the aim of better constraining the ecological role of *Vibrio*, it is of great value to explore the distribution pattern of the active *Vibrio* (*V*a) and compare it to that of the total *Vibrio* (*V*t).

Estuaries are dynamic environments where freshwater meets seawater, thereby creating gradients of various environmental factors, including salinity, temperature, dissolved oxygen (DO), chlorophyll *a* (Chl *a*), inorganic/organic nutrients, as well as toxic chemicals. In addition, estuaries normally contain a high amount of organic matter due to river runoff and suffer from physical disruptions caused by human intervention and storm events (Elliott and McLusky, 2002). These environmental characteristics shape the unique estuarine microbial communities that are distinct from the adjacent coastal communities and make estuaries particularly suitable for the study of ecological pattern of microorganisms. With a high level of eutrophication and diverse environmental niches (Nixon, 1995; De Jonge *et al*., 2002; Fricke *et al*., 2016; Kopprio *et al*., 2016), many copiotrophic bacteria, such as *Vibrio* spp., are abundant in estuarine ecosystems (Vezzulli *et al*., 2016; Jesser and Noble, 2018).

Indeed, vibrios were found to be abundant in estuaries (Vezzulli *et al*., 2016). In the Sydney Harbour estuary, several human pathogens (i.e. *V. vulnificus* and *V. parahaemolyticus*) were particularly abundant in summer, and their abundance and community composition displayed clear spatiotemporal heterogeneity in response to temperature and salinity (Siboni

et al., 2016). In two Patagonia estuaries, Argentina, the distribution of pathogenic *Vibrio* species (i.e. *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*) was linked to eutrophication and enriched by high concentrations of ammonium, salinity and organic nutrients (Kopprio *et al*., 2016). In the Neuse River Estuary, USA, the abundance of cultivable *Vibrio* spp. in surface waters increased downstream, and were positively correlated with the phytoplankton population and salinity (Hsieh *et al*., 2007). These previous studies on *Vibrio*, however, mainly focused on pathogenic vibrios (Zhang *et al*., 2018) and most of them were based on culturing (Hsieh *et al*., 2007; Hsieh *et al*., 2008; Kopprio *et al*., 2016) rather than molecular methods (Siboni *et al*., 2016). Further studies are necessary to unravel the dynamics of *Vibrio* spp. in estuarine ecosystems and to explore their activities.

The Changjiang (Yangtze) estuary is located offshore from the mouth of the Changjiang River in China, which is the longest river in Asia (Feng *et al*., 2009; Ye *et al*., 2016). Many industrial and urban centres are located in the watersheds of the Changjiang River, especially along its lower reaches and estuary. Thus, emission of industrial and domestic waste has led to the high nutrient inputs to the estuary (Chai *et al*., 2006), which results in frequent occurrence of noxious algal outbreaks (Han *et al*., 2003; Zhou *et al*., 2003). The complex nature of the Changjiang estuary makes it an ideal area to examine the microbial ecology, and an increasing number of studies have been conducted along the Changjiang estuary (Chai *et al*., 2006; Feng *et al*., 2009; Nie *et al*., 2009; Li *et al*., 2012; Hou *et al*., 2013; Hu *et al*., 2014; Ye *et al*., 2016). Nevertheless, little is known about the ecology of *Vibrio* communities throughout the estuary. We hypothesize that the total and active *Vibrio* exhibit clear seasonal and spatial dynamics in the Changjiang estuary and are affected by diverse environmental factors (e.g. temperature and salinity). In this study, we investigated the dynamics and environmental drivers of the total and active *Vibrio* spp. along the Changjiang estuary through four research cruises between 2016 and 2017. A combination of culturing, quantitative polymerase chain reaction (qPCR), amplicon sequencing and statistical methods were implemented to study the *Vibrio* community structure and relate it to a range of environmental variables.

Results

Environmental conditions

The environmental parameters measured during the study period are summarized in Table 1. All samples were divided into two groups: summer (collected in July 2016 and July 2017) and winter (collected in March 2016 and March 2017). The water temperature, DO, Chl *a* and

Table 1. Environmental parameters throughout the Changjiang estuary.

Physicochemical parameter	Value[a]							
	Summer				Winter			
	Total samples	Freshwater	Transitional sites	Saltwater	Total samples	Freshwater	Transition sites	Saltwater
Temperature (°C)	25.69 ± 3.38	28.38 ± 0.61	27.08 ± 1.94	22.26 ± 2.95	10.66 ± 1.78	10.04 ± 0.59	9.04 ± 0.86	11.62 ± 1.74
Salinity (PSU)	20.69 ± 13.71	0.12 ± 0.01	21.64 ± 7.85	33.19 ± 1.77	24.26 ± 12.83	0.30 ± 0.23	23.68 ± 5.77	32.95 ± 1.41
Depth (m)	15.83 ± 20.05	6.74 ± 6.79	2.60 ± 3.78	35.48 ± 20.43	14.99 ± 19.89	6.64 ± 6.72	4.16 ± 4.95	22.89 ± 23.54
pH	7.97 ± 0.19	7.80 ± 0.04	8.08 ± 0.22	7.96 ± 0.13	8.05 ± 0.08	8.00 ± 0.11	8.05 ± 0.06	8.08 ± 0.07
DO (mg L⁻¹)	4.72 ± 1.56	4.60 ± 1.03	5.69 ± 1.38	3.80 ± 1.47	9.58 ± 1.33	10.84 ± 0.51	9.84 ± 1.65	9.00 ± 0.99
Chl *a* (μg L⁻¹)	1.41 ± 1.56	1.07 ± 0.41	2.10 ± 2.23	0.91 ± 0.76	0.52 ± 0.36	0.85 ± 0.36	0.71 ± 0.42	0.32 ± 0.15
NO_3^- (μmol L⁻¹)	51.01 ± 40.56	107.25 ± 9.29	47.11 ± 30.24	17.04 ± 12.32	43.36 ± 48.47	132.90 ± 12.02	46.60 ± 20.97	10.40 ± 6.87
NO_2^- (μmol L⁻¹)	0.64 ± 0.39	0.28 ± 0.13	0.78 ± 0.38	0.62 ± 0.39	0.71 ± 0.96	2.48 ± 0.61	0.42 ± 0.47	0.21 ± 0.08
NH_4^+ (μmol L⁻¹)	4.31 ± 2.27	0.20 ± 0.07	3.98 ± 2.19	5.34 ± 1.55	4.50 ± 3.29	5.68 ± 6.14	6.29 ± 3.16	3.32 ± 0.97
PO_4^{3-} (μmol L⁻¹)	0.98 ± 0.51	1.45 ± 0.13	0.88 ± 0.63	0.75 ± 0.31	0.98 ± 0.60	1.99 ± 0.24	1.10 ± 0.36	0.57 ± 0.18
SiO_3^{2-} (μmol L⁻¹)	53.12 ± 42.57	111.83 ± 1.52	48.85 ± 34.61	19.07 ± 13.54	37.31 ± 38.32	108.18 ± 5.91	39.99 ± 16.85	11.18 ± 5.97

[a]Statistical differences after chi-square test.

SiO_3^{2-} varied significantly between seasons ($p < 0.05$; Fig. 1), with decreasing mean water temperature (15.03 °C) and Chl *a* (0.89 μg L⁻¹) and increasing DO (4.86 mg L⁻¹) and SiO_3^{2-} (15.81 μmol L⁻¹) from summer to winter. Based on the salinity series, all samples were separated into three groups, i.e. freshwater (salinity < 1), transitional (1 ≤ salinity < 30) and saltwater (salinity ≥ 30) areas (Liu *et al*., 2015). In additional to salinity, other physicochemical attributes also showed variations between areas (Table S3). Briefly, in summer, the freshwater sites had the lowest level of pH but the highest level of temperature, NO_3^- and SiO_3^{2-} ($p < 0.01$, one-way ANOVA; Fig. 1). In winter, the freshwater sites had the highest DO and nutrients (i.e. NO_3^-, SiO_3^{2-} and PO_4^{3-}), whereas the saltwater sites showed the highest temperature but lowest nutrient concentrations ($p < 0.01$; Fig. 1).

Total Vibrio spp. abundance

qPCR assays revealed significant differences in total *Vibrio* abundance between the two seasons (summer versus winter, $p < 0.05$; Fig. 2A). Overall, *Vibrio* were more abundant in summer than in winter, with the mean 16S rRNA gene copy numbers of 6.59×10^3 and 1.85×10^3 copies ml⁻¹, respectively across the entire estuary. More than half of the sites exhibited higher *Vibrio* spp. abundance in summer than winter (Fig. S1A). Interestingly, when samples collected from March 2016 and July 2016 were compared, no obvious difference in *Vibrio* abundance was found (Fig. 2A). Spatially, the freshwater sites were populated by a lower *Vibrio* abundance than the transitional and saltwater sites ($p < 0.05$). This phenomenon was observed in both summer (increased from 8.04×10^1 to 9.39×10^3 copies ml⁻¹) and winter (increased from 3.77×10^1 to 2.96×10^3 copies ml⁻¹) (Fig. 2B). In the transitional zone, the temporal variation trend of *Vibrio* abundance was not uniform; higher winter *Vibrio* abundance was observed at sites CE12 and CE13, but not at others (Fig. S1A). Similar to total *Vibrio*, there was a higher abundance of total bacteria in summer than in winter ($p < 0.05$; Fig. 2C and S1B), whereas no clear difference between freshwater and saltwater was observed (Fig. 2D).

Diversity and composition of total Vibrio community

Next, we examined the dominant *Vibrio* spp. in different seasons and regions, in view of the spatiotemporal differences in absolute abundance. The high-throughput sequencing yielded 350 571 clean reads from 27 DNA libraries (no freshwater samples were successfully sequenced likely due to the low abundance) with 8443 to 18 328 reads per sample. The average sequence length

was 513 bp. The read numbers in each sample were limited to 8443 after rarefaction. All sequences were clustered into 40 OTUs at a 97% sequence similarity cut-off. The rarefaction curves of all stations reached an asymptote (Table S4), and the Good's coverage values ranged from 99.91% to 100% across samples, indicating that the sequences generated from these samples represented most of the *Vibrio* diversity at the studied sites. The phylogenetic distance, Chao I (a measure of richness), evenness and Shannon (including both richness and

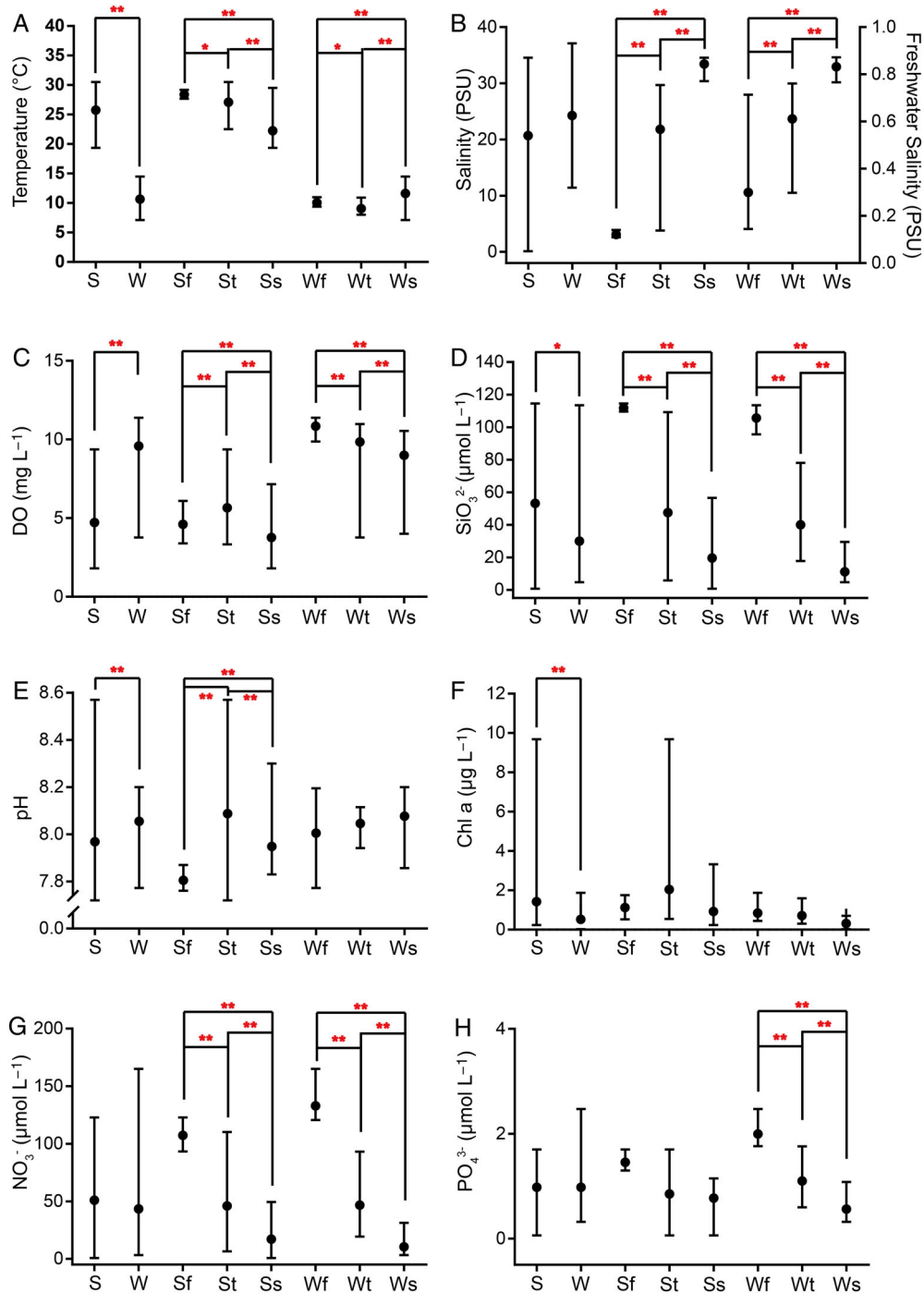**Fig. 1.** Spatiotemporal dynamics of significantly varied environmental parameters among different seasons and sites. S, summer; W, winter. Wf, freshwater in winter; Wt, transitional sites in winter; Ws, saltwater in winter; Sf, freshwater in summer; St, transitional sites in summer; Ss, saltwater in summer. *, $p < 0.05$; **, $p < 0.01$. [Color figure can be viewed at wileyonlinelibrary.com]
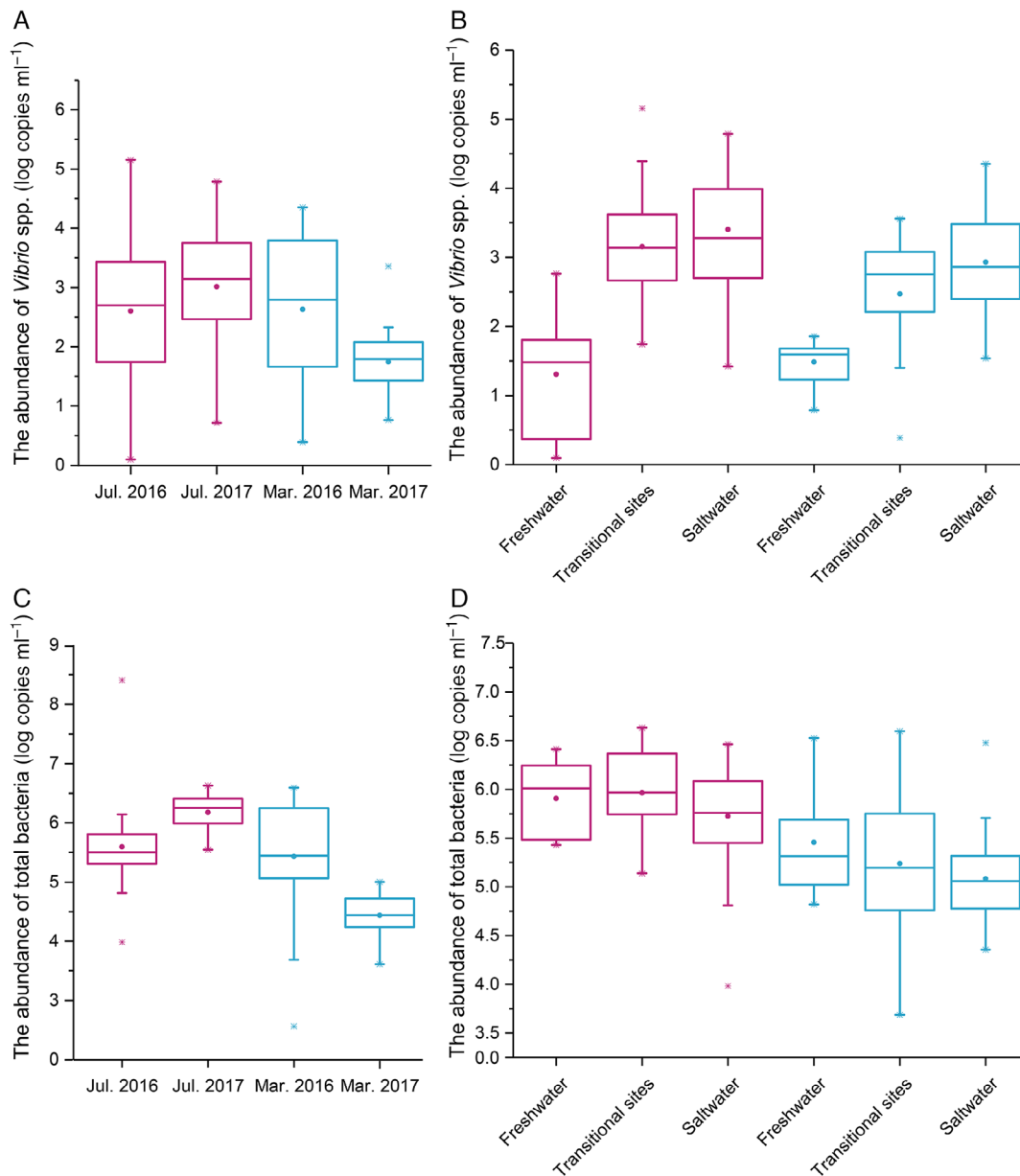
Fig. 2. Total *Vibrio* and bacteria abundance (log copies ml^{-1}) in different periods (A, B) and different areas (C, D). Vermilion boxplots denoted summer samples, and blue boxplots denoted winter samples. Freshwater, salinities < 1 ppt; transitional sites, 1 ppt ≤ salinities < 30 ppt; and saltwater, salinities ≥ 30 ppt. [Color figure can be viewed at wileyonlinelibrary.com]

evenness) indices were calculated, and all of these indices showed higher values in summer than in winter (Wilcoxon's test, $p < 0.01$; Fig. S2). No significant differences in alpha diversity were found between saltwater and transitional sites.

A comparison of *Vibrio* community composition between different samples was performed with principal component analysis (PCA) at the OTU level. Both spatial and seasonal shifts in the *Vibrio* assemblages were observed (Fig. 3A). A clear separation of samples from different seasons (summer and winter) was observed along the first axis (ANOSIM, $p < 0.01$), and spatial heterogeneity displayed within both the summer and winter samples (ANOSIM, $p < 0.01$). Redundancy analysis (RDA) showed that the *Vibrio* communities were influenced by various environmental factors, including temperature, pH, DO, Chl a, NO_2^-, SiO_3^{2-} as well as latitude and longitude ($p < 0.05$; Fig. 3B). Temperature was the main factor separating the summer and winter populations, and salinity may explain the spatial changes from freshwater to saltwater.

To identify specific taxa that contributed to the observed spatiotemporal dynamics of *Vibrio* communities (Fig. 4), representative sequences of each OTU were compared against the EzBioCloud database to determine their taxonomic identities. Almost all sequences (99.66%) belonged to the *Vibrionaceae* family, and 94.44% were assigned to the *Vibrio* genus. The dominant OTUs, i.e. OTU 26 was most similar to *Vibrio atlanticus*, OTU 35 to *Vibrio caribbeanicus* and OTU 8 to *Vibrio campbellii*. These three OTUs made up 84.12% of all sequences. *V. atlanticus* (OTU 26) was the dominant group in winter, whereas *V. caribbeanicus* (OTU 35) and *V. campbellii* (OTU 8) were abundant in summer (Fig. S3). The summer and winter samples clearly differed in *Vibrio* community components at the species level, and 68.18% of the species were shown to be distinctly distributed across these two seasons (Fig. S3). However, no significant differences were found between the saltwater and transitional areas in either summer or winter.

The abundance and community structure of active Vibrio *spp.*

One major aim of this study was to investigate the active *Vibrio* diversity and their spatiotemporal dynamics. RNA based quantification analysis showed that the abundance of active *Vibrio* was consistent in different seasons, unlike the scenario seen for total *Vibrio*. In contrast, a clear spatial variance was observed, with an overall lower abundance in freshwater sites than others. Specifically, there were significant differences between saltwater, freshwater and transitional sites in winter ($p < 0.01$), whereas no similar variations were recorded in summer. An analysis of the ratio of the active: total *Vibrio* abundance (Va/Vt) showed that the copy number of vibrionic 16S rRNA was higher than that of 16S rDNA (Va/Vt; Table S5), indicating a high *Vibrio* activity in this region. Nevertheless, the culturable vibrios only accounted for 0.12%–6.26% of the active or total *Vibrio* (Vc/Va and Vc/Vt), suggesting a large number of species are yet to be cultured (Table S5). Although the mean values of active *Vibrio* abundance were 7.68×10^3 and 9.93×10^4 copies ml^{-1}, no significant differences were recorded between saltwater and transitional sites in summer (Table S5). Predictably, vibrios in transitional sites and saltwater areas exhibited high activities, and the lowest *Vibrio* spp. activity was found in freshwater sites in winter (CE2SW and CE2BW) (Fig. 5). In addition, the total bacterial activities showed a tendency similar to that of *Vibrio*.

High-throughput sequencing of the cDNA libraries generated 31 080–62 249 reads across all samples. Twenty-nine thousand two hundred and three reads were left for each sample after rarefaction, yielding a total of 156 OTUs. The Good's coverage values were >99.9%

across samples, indicating that sequences generated from these samples could represent most of the active *Vibrio* in the studied sites. All the samples were divided into three groups based on the ACE index differences ($p \leq 0.05$, Student's t test), including Wf, Ws and Ss. Similar to the total community, clear variations in the active *Vibrio* community between summer and winter samples were observed (Fig. 3C, $p < 0.05$). In addition, the samples collected from different salinities in winter were separated by the second axis (Fig. 3C). RNA-based RDA was used to identify key environmental factors that governed the active vibrios; similar results as the DNA-based RDA were obtained. The summer samples, despite from different salinity ranges, showed a close clustering relationship and were distinct from those collected in winter due to the high temperature (Fig. 3D). The distinction of the *Vibrio* components in winter were mainly divided by salinity and nutrient concentrations (e.g. NO_2^-, SiO_3^{2-} and PO_4^{3-}; Fig. 3D).

The dominant OTUs in the RNA libraries were OTU 97, 30 and 10. They made up 59.82% of all sequences and were most similar to *V. atlanticus*, *V. caribbeanicus* and *Vibrio owensii*, respectively. The community compositions of active *Vibrio* spp. varied among the Wf, Wt, Ws, St and Ss groups (Fig. 4B). *V. atlanticus* OTU 97 was the dominant group of Ws, whereas *V. caribbeanicus* OTU 30 and *V. owensii* OTU 10 were abundant in summer (Fig. 4B). Regarding the transitional samples, i.e. CE7SW and CE7BW in winter and CE15SS and CE17SS in summer, the dominant groups varied among each site, such as *Vibrio hippocampi* at CE7BW and *V. owensii* at CE15SS (Fig. 4B). Interestingly, no *Vibrio* species were dominant in the Wf group.

Cultivable Vibrio

As evidence, the temporal and spatial variations in the counts of cultivable vibrios are shown in Fig. 6. The number of culturable vibrios accounted for ∼0.12%–6.26% of the total and active *Vibrio* derived from qPCR (Table S5). The counts of culturable *Vibrio* spp. in summer (8.33×10^0–5.43×10^2 cfu ml^{-1}) were approximately twice as high as those in winter (1.67×10^0–2.25×10^2 cfu ml^{-1}; Fig. 6A). Indeed, except four samples from the transitional sites (CE6S, CE6B, CE7S and CE7B), the numbers of colony-forming units (cfu) in most sites (66.67%) were higher in summer than that in winter in the samples collected from March and July 2016 (Fig. 6B). In addition, the cfu counts showed an increasing trend from freshwater to saltwater in both summer and winter (Fig. 6B).

The diversity of isolated *Vibrio* strains that grew on *Vibrio*-selective thiosulfate-citrate-bile salts-sucrose (TCBS) medium were analysed (Fig. 4C). Sixteen species were identified across all samples, and the summer samples
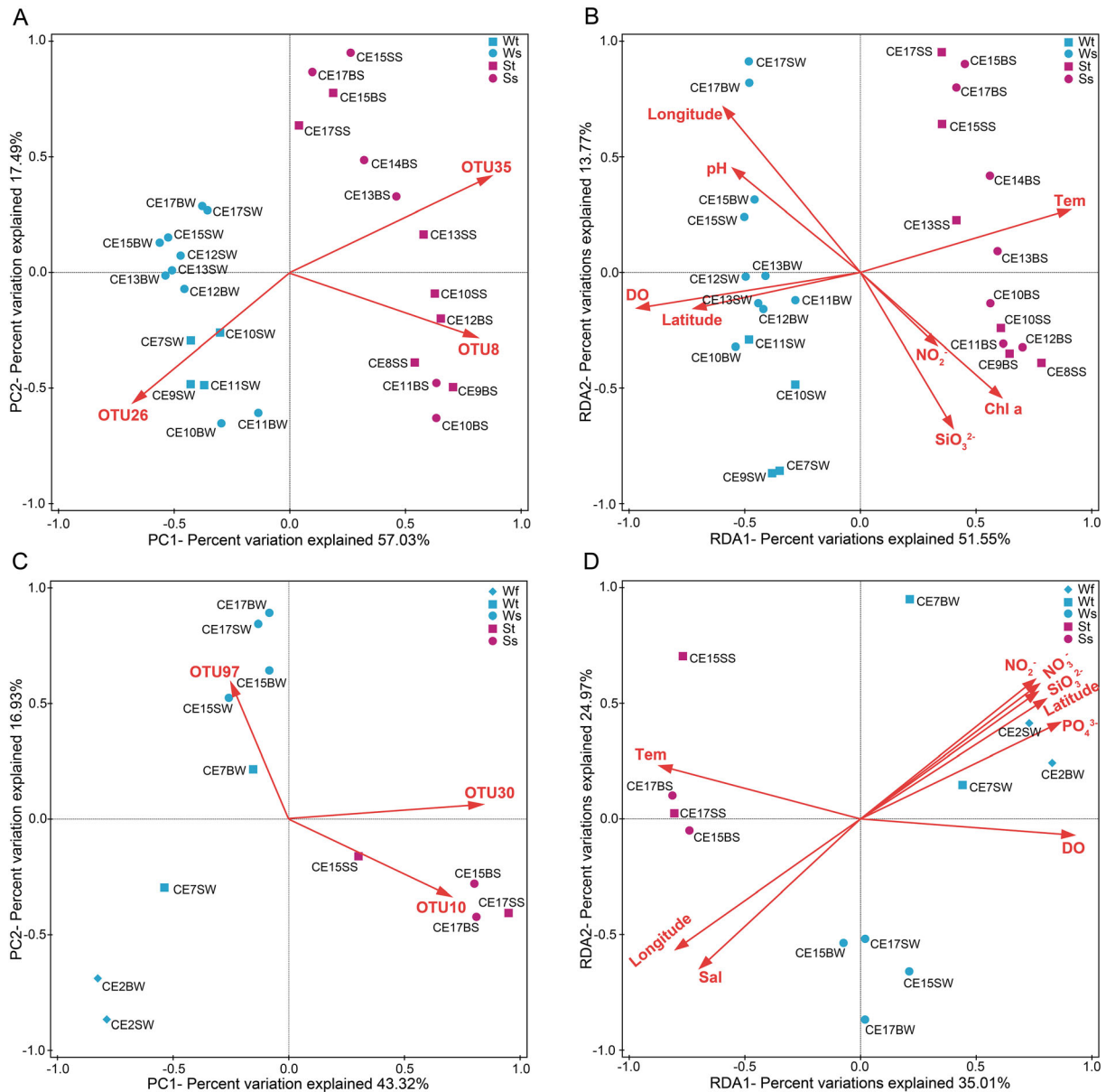
Fig. 3. Community analysis of *Vibrio* at the OTU level. A and C, Unweighted PCA plot with PC1 and PC2. B and D, RDA analysis illustrating the relationship between *Vibrio* community at the OTU level and top environmental variables. Tem is the abbreviation for temperature, and Sal for salinity. [Color figure can be viewed at wileyonlinelibrary.com]

showed a higher diversity (12 species) than the winter samples (5 species). In summer, *V. campbellii* (26.97%) was the dominant group, followed by *V. harveyi* (15.73%), *Vibrio rotiferianus* (13.48%), *Vibrio fortis* (7.87%) and *Vibrio azureus* (7.87%). The most abundant species in winter were *V. atlanticus* (60.00%), *Vibrio gallaecicus* (18.00%), *Vibrio hemicentroti* (12.00%), *Vibrio kanaloae* (8.00%) and *Vibrio hangzhouensis* (2.00%). The dominant species of cultivable vibrios were generally consistent with those occupying the highest proportion in the total and active *Vibrio* (Fig. 4).

The correlation with environmental parameters

Across the entire data set, total *Vibrio* abundance demonstrated a positive correlation with salinity ($p < 0.01$), depth, pH and NH_4^+ ($p < 0.05$) and a negative correlation with NO_3^-, PO_4^{3-}, SiO_3^{2-} ($p < 0.01$), DO and Chl *a* ($p < 0.05$). In summer, positive correlations with salinity, depth and pH ($p < 0.01$), and negative correlations with temperature, Chl *a*, NO_3^-, PO_4^{3-} and SiO_3^{2-} ($p < 0.01$) were recorded. The relationships in winter were similar to those in summer, but additionally, *Vibrio* abundance was positively correlated
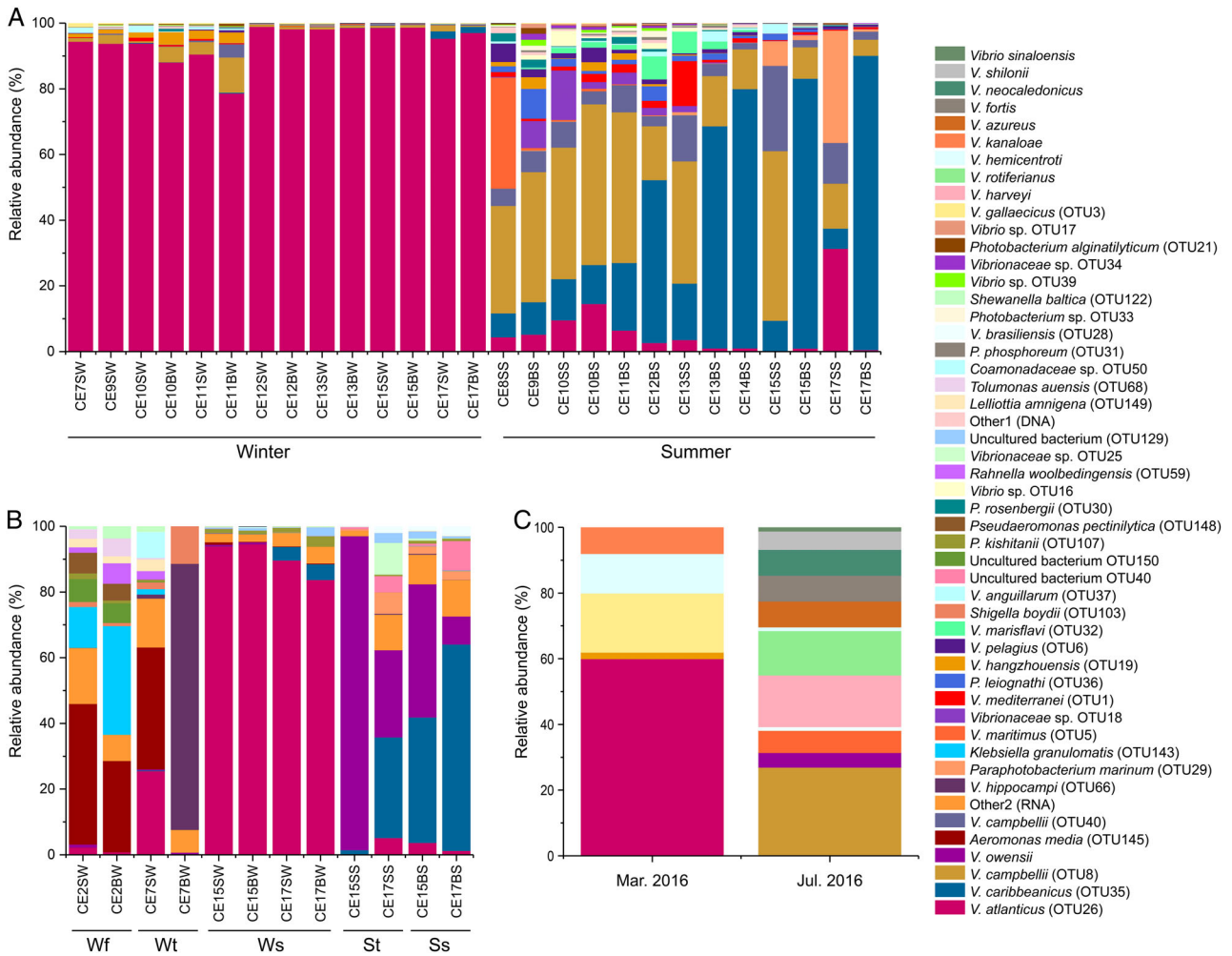
Fig. 4. Community component of culturable *Vibrio* spp., total and active vibrios at the species level which re-annotated by the EzBioCloud database. Sample names are defined by sampling depth and season, e.g. CE7SW is the surface seawater (S) collected in winter (W) at the station CE7.

A. Top 22 abundant OTUs of total *Vibrio* spp. across all samples. Other, the species occupied < 0.1% in total samples.

B. Most abundant 20 OTUs of active *Vibrio*, representing 67.5% of the total normalized sequences. Wf, freshwater in winter; Wt, transitional sites in winter; Ws, saltwater in winter; St, transitional sites in summer; Ss, saltwater in summer. Other, the species occupied < 0.5% in total samples.

C. The relative abundance of cultivated *Vibrio* in different collected periods. [Color figure can be viewed at wileyonlinelibrary.com]

with temperature ($p < 0.05$) and negatively correlated with DO and NO_2^- ($p < 0.01$). However, when the groups were divided based on the salinity gradient, the correlations between *Vibrio* abundance and environmental factors were rare. In addition, total bacterial abundance was positively correlated with temperature, Chl *a*, NO_2^-, NH_4^+, NO_3^-, PO_4^{3-} and SiO_3^{2-} ($p < 0.01$), but negatively correlated with salinity, depth, pH and DO ($p < 0.01$). Total bacterial abundance was correlated with only DO ($r = 0.555$, $p < 0.01$) in summer, whereas it was positively correlated with Chl *a* and nutrients ($p < 0.05$) and negatively correlated with salinity ($r = -0.282$, $p < 0.05$) in winter.

Spearman's correlations between the 22 most abundant *Vibrio* species and the environmental factors were calculated (Table 2). In general, the most abundant species exhibited close correlations with temperature, pH, DO, Chl *a*, NO_2^-, PO_4^{3-} and SiO_3^{2-}. The relative abundance of *V. atlanticus* OTU 26 was positively correlated with pH and DO ($p < 0.01$) and negatively correlated with temperature, Chl *a* and NO_2^-, whereas *V. caribbeanicus* OTU 35 and *V. campbellii* OTU 8 exhibited inverse relationships with these factors. Moreover, *V. caribbeanicus* OTU 35 was positively correlated with temperature, salinity and depth, and *V. campbellii* OTU 8 showed a positive correlation with Chl *a*, NO_3^- and SiO_3^{2-} (Table 2).

The abundance of active *Vibrio* showed a positive correlation with temperature and salinity ($p < 0.05$), whereas it was negatively correlated with DO, Chl *a* and nutrients ($p < 0.05$). Additionally, the analyses between the 20 most abundant species of active *Vibrio* and environmental

parameters are summarized in Table 2. *V. atlanticus* OTU 97 was negatively correlated with Chl *a*, NO_3^-, NO_2^- and SiO_3^{2-}, whereas *V. caribbeanicus* OTU 30 was negatively correlated with DO, NO_3^-, NH_4^+ and SiO_3^{2-} but positively correlated with temperature and salinity. *V. owensii* OTU 10 showed a positive correlation with temperature, whereas it was negatively correlated with DO and NH_4^+ (Table 2).

Discussion

Vibrio spp. are autochthonous and ubiquitous marine heterotrophic bacteria that have been extensively studied,
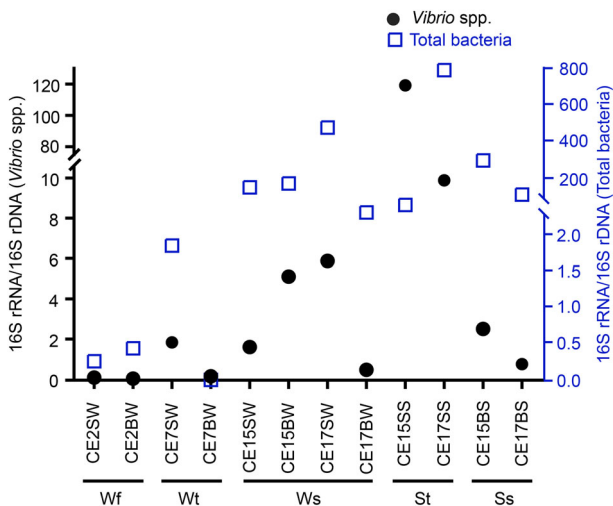

Fig. 5. The ratio of 16S rRNA/16S rDNA indicative of microbial activities among different seasons and sites. Wf, freshwater in winter; Wt, transitional sites in winter; Ws, saltwater in winter; St, transitional sites in summer; Ss, saltwater in summer. [Color figure can be viewed at wileyonlinelibrary.com]

especially in recent years (Vezzulli *et al.*, 2009; Amin *et al.*, 2016; Kopprio *et al.*, 2016; Siboni *et al.*, 2016). Most of these works have focused on the effects of environmental factors and have been conducted by culturing or molecular methods at the DNA level (Amin *et al.*, 2016; Siboni *et al.*, 2016). However, studies on the spatiotemporal dynamics of *Vibrio* communities in estuaries are rare, and little is known about the activity of *Vibrio* in environmental samples. Here, as a complement to previous studies, we assessed the spatiotemporal distribution of *Vibrio* communities along the Changjiang estuary with a combination of culturing and molecular methods at both the DNA and RNA levels. To the best of our knowledge, this is the first investigation regarding the distribution pattern of active *Vibrio*. We observed similar spatial variations in total and active *Vibrio* from freshwater to saltwater sites. Additionally, we showed that *Vibrio* displayed a consistently high activity across the seasons, although the abundance was higher in summer than in winter.

Vibrio *as an indicator of global warming: not only abundance but also community composition*

Climate changes, such as global warming, have direct impacts on the marine ecosystem (Baker-Austin *et al.*, 2016). A recent study in the North Atlantic found that ocean warming resulted in a significant increase in *Vibrio* abundance and associated human diseases during the 1980s onwards (Vezzulli *et al.*, 2016). This finding suggests the potential of *Vibrio* abundance as a climate change microbial barometer as suggested by Baker-Austin and colleagues (2016). In the present study, the *Vibrio* abundance was within the average range in the global ocean (10^4–10^8
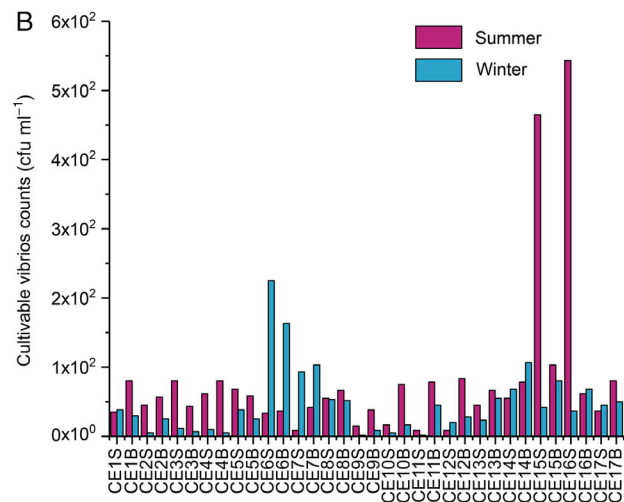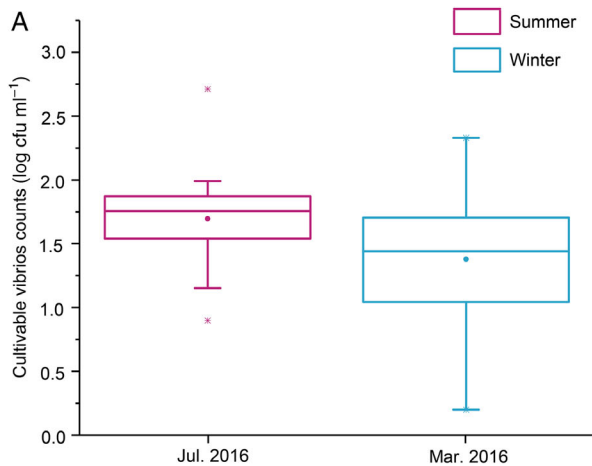

Fig. 6. The cfu counts of cultivated *Vibrio* in different collected periods (A) and in varied sites (B). The figure displays the distribution of species in different periods, including summer (Jul. 2016) and winter (Mar. 2016). [Color figure can be viewed at wileyonlinelibrary.com]

Table 2. Spearman's rank correlation coefficients between percentage composition of taxa and environmental factors using STATISTICA version 22.0. [Color table can be viewed at wileyonlinelibrary.com]

Vibrio taxa	Temperature	Salinity	Depth	pH	DO	Chl α	SPM	NO$_3^-$	NO$_2^-$	NH$_4^+$	PO$_4^{3-}$	SiO$_3^{2-}$
At DNA level												
Vibrio atlanticus OTU26	**-0.630**			0.474	**0.737**	-0.435				-0.416		
V. caribbeanicus OTU35	**0.668**		0.393	**-0.491**	**-0.890**							
V. campbellii OTU8	**0.748**			-0.448	**-0.672**	**0.590**			**0.532**			0.420
V. campbellii OTU40	**0.869**				**-0.660**	**0.541**	-0.405					
Paraphotobacterium marinum OTU29	**0.697**				-0.627		**-0.679**					
V. maritimus OTU5				**-0.732**		**0.602**		**0.627**			**0.693**	**0.720**
Vibrionaceae sp. OTU18	**0.643**			**-0.599**	**-0.715**	0.485			**0.593**	-0.451		0.430
V. mediterranei OTU1a	**0.514**			**-0.623**	**-0.669**	0.477			0.445			0.473
Photobacterium leiognathi OTU36a	**0.738**			**-0.554**	**-0.738**	**0.615**			0.475			0.416
V. hangzhouensis OTU19a		-0.447		**-0.584**		0.478	**0.526**	**0.713**			**0.697**	**0.778**
V. pelagius OTU6	**0.605**			**-0.671**	**-0.678**	**0.571**			0.468			0.404
V. marisflavi OTU32a	**0.600**			**-0.604**	**-0.790**	0.410			**0.519**			
V. anguillarum OTU37a				**-0.524**		0.479		**0.516**			**0.507**	**0.597**
P. rosenbergii OTU30				**-0.700**	**-0.500**						0.390	
Vibrio sp. OTU16	0.465			**-0.522**	**-0.644**				**0.578**			
P. phosphoreum OTU31a						0.390						
Photobacterium sp. OTU33				**-0.636**	-0.479	0.435			0.404			**0.496**
Vibrio sp. OTU39				**-0.700**		**0.626**		**0.671**			**0.640**	**0.770**
Vibrionaceae sp. OTU34	**0.621**			**-0.578**	**-0.762**				0.435	-0.408		
P. alginatilyticum OTU21		**-0.561**		-0.447		**0.615**		**0.697**			**0.530**	**0.664**
Vibrio sp. OTU17				**-0.678**	**-0.620**	**0.507**			0.496		0.390	0.400
V. gallaecicus OTU3	**-0.618**	-0.387			**0.516**		**0.510**	**0.499**			**0.636**	0.432
Other1	**0.440**			**-0.714**	**-0.522**	**0.531**					0.394	0.482
At RNA level												SiO$_3^{2-}$
Vibrio atlanticus OTU97					-0.699			-0.587	-0.618			-0.594
V. owensii OTU10	0.655				-0.613					-0.641		
V. caribbeanicus OTU30	0.676	**0.715**			**-0.729**				-0.581	**-0.774**		-0.606
Aeromonas media OTU145												
V. hippocampi OTU66												
Klebsiella granulomatis OTU143												0.660
Shigella boydii OTU103	**-0.923**	-0.613			**0.923**					**0.972**	**0.768**	
Uncultured bacterium OTU40	**0.799**				**-0.799**					**-0.774**		
Uncultured bacterium OTU150		**-0.757**				0.628		**0.757**	**0.769**			**0.803**
Paraphotobacterium marinum OTU67	**0.815**				**-0.782**					-0.766		
Photobacterium kishitanii OTU107						-0.678						
Pseudaeromonas pectinilytica OTU148												0.678
Rahnella woolbedingensis OTU59	-0.621				0.625					0.610	0.614	
Vibrionaceae sp. OTU25	0.652				-0.670					-0.624		
Uncultured bacterium OTU129	0.580	0.703			-0.587	-0.609		-0.616	-0.610	-0.631		**-0.841**
Lelliottia amnigena OTU149											0.657	
Tolumonas auensis OTU68										0.632	0.632	0.577
Comamonadaceae sp. OTU50												
V. brasiliensis OTU28	**0.739**				**-0.739**					-0.662		
Shewanella baltica OTU122	-0.631				0.631					0.602	0.624	
Other2												

Only significant correlation ($p < 0.05$) were shown in table. Bold, $p < 0.01$; regular, $p < 0.05$. Red, positive correlation; blue, negative correlation.

16S rRNA copies L^{-1}) (Zhang *et al.*, 2018), and the *Vibrio* populations were present year-round throughout the Changjiang estuary, with an expected elevation in population size during summer. *Vibrio* spp. were more abundant in summer (6.59×10^3 copies ml^{-1}) than in winter (1.85×10^3 copies ml^{-1}) across the entire estuary. In light of these observations, temperature is likely the most important driver of the overall change in *Vibrio* abundance in temperate coastal waters. Increased sea surface temperature has been shown to explain 45% of the variance in *Vibrio* data among the environmental variables, supporting the view that ocean warming favours the spread of vibrios (Vezzulli

et al., 2012). Although previous reports have revealed that the spread of *Vibrio* spp. may be exacerbated by global warming, the biocomplexity of interactions between *Vibrio* and the surrounding environment in a climate change context is still poorly understood (Vezzulli *et al*., 2015). Long-term studies, such as the establishment of a system for public health (including the environmental monitoring of *Vibrio* levels) (Martinez-Urtaza *et al*., 2010) should be performed.

Supporting this view, we found that the peaks of *Vibrio* abundance and temperature were not always well fitted, which may be due to the complex nutrient conditions. 16S rRNA gene copies of total *Vibrio* in March 2016 were obviously higher than those in March 2017 (Fig. 2A). High concentrations of inorganic nutrients and Chl *a* were detected in 2016, suggesting a possible linkage between *Vibrio* abundance and phytoplankton. In addition, narrow temperature range may also result in the statistical uncorrelation between temperature and *Vibrio* distribution (Takemura *et al*., 2014). Similarly, we found that the abundance of total *Vibrio* showed no significant difference between summer and winter in 2016 (Fig. 2A). This might be explained by the presence of complex nutrient combinations and the changes in freshwater discharge (Asplund *et al*., 2011; Liu *et al*., 2015). Compared with summer, the seawater is colder in winter, but there is a substantial decrease in freshwater discharge. Thus, the salinity was significantly elevated, as well as slight increases in pH, DO, NO_3^-, NH_4^+ and PO_4^{3-}.

The observed increase in *Vibrio* abundance under a long-term effect of global warming may alter its natural community composition (Vezzulli *et al*., 2012; Tout *et al*., 2015; Vezzulli *et al*., 2015). For example, increasing water temperature has been linked to increased *Vibrio* occurrence in the North and Baltic Seas (Vezzulli *et al*., 2012), and alterations to the structure of the natural *Vibrio* populations has been associated with the coral species *Pocillopora damicornis* within the Heron Island lagoon (Tout *et al*., 2015). In this study, the dominant *Vibrio* species changed dramatically between seasons (Fig. 4). The absolute abundance of summer species (i.e. *V. caribbeanicus* and *V. campbellii*) decreased significantly with the decrease in seawater temperature, whereas *V. atlanticus* increased in winter. It has been reported that *V. campbellii* and *V. caribbeanicus* prefer to grow at higher temperatures, i.e. 35 °C (Borrego *et al*., 1996; Ishimaru *et al*., 1996; Macian *et al*., 2004; Yoshizawa *et al*., 2010; Hoffmann *et al*., 2012), but the growth of *V. atlanticus* occurs at 4–25 °C and not at 3 or 44 °C (Dieguez *et al*., 2011). The fact that few *Vibrio* species can endure the low temperature in winter may have resulted in a higher *Vibrio* diversity in summer than in winter. This is in contrast to the total bacterial community, which was always more diverse in winter than in winter (Grzymski *et al*., 2012; Ladau *et al*., 2013). The disappearance of the

predominant *Vibrio* species in summer and the increase of *V. atlanticus* in winter thus drove the succession of the *Vibrio* spp. community in different seasons.

V. campbellii, an important pathogen in the intensive rearing of molluscs, finfish, and shrimp (Defoirdt *et al*., 2006), *V. anguillarum*, a pathogen to a number of aquatic organisms (Hickey and Lee, 2018), and *V. harveyi*, a serious pathogen of marine fish and invertebrates (Zhang *et al*., 2020) occurred in our community results, and might be the major foodborne pathogens in the Changjiang estuary. This was unlike those in other estuaries, such as *V. vulnificus* and *V. parahaemolyticus* in the Sydney Harbour estuary (Siboni *et al*., 2016), and *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* in two Patagonia estuaries (Kopprio *et al*., 2016). The reason for this difference may be that there are natural variations in the fundamental niche shape and the changes in species adapt to the ambient environment (e.g. temperature) (Materna *et al*., 2012). Furthermore, *Vibrio* species represent a highly diverse genetic reservoir linked to phenotypic polymorphisms, which promotes adaptation to diverse habitats and the occupation of different micro-ecological niches (Pretzer *et al*., 2017). Because of the preference for warm and saline waters, rapid growth, and pathogenic character, vibrios are a useful barometer of the change of marine environmental conditions, particularly in temperate and mid-latitude areas that are undergoing rapid warming (Baker-Austin *et al*., 2016; Vezzulli *et al*., 2016). Further studies should be systematically assessed to decrease the detrimental effects on human and animal health.

Salinity gradient altered the community composition of Vibrio *from freshwater to saltwater*

Several previous studies in temperate estuaries reported that the spatial shifts in the composition of the *Vibrio* community were primarily governed by salinity (Asplund *et al*., 2011; Siboni *et al*., 2016). Indeed, the abundance of *Vibrio* spp. showed an increasing trend from freshwater to saltwater along the Changjiang estuary (Fig. 2B) and had a strong positive correlation with salinity in winter and summer, suggesting a preference of *Vibrio* spp. for a high salinity within the sampled range in this study. Also, salinity gradient altered the communities of *Vibrio* spp. from freshwater to saltwater. Different species may become the dominant groups at various salinity conditions (Simonin *et al*., 2019) and individual *Vibrio* species has its own salinity range for growth. The proportion of *V. campbellii* was high in coastal sites (from CE8SS to CE13SS as well as CE15SS), whereas *V. caribbeanicus* was high in open sea areas (from CE13BS to CE17BS) in summer (Fig. 4A). A similar trend was found in winter, in which *V. campbellii* decreased from freshwater to

saltwater sites (Fig. 4A). The growth of *V. caribbeanicus* occurred at NaCl concentrations of 8.0%, whereas *V. campbellii* cannot grow at NaCl concentrations ≥8.0% (Yoshizawa *et al*., 2010; Hoffmann *et al*., 2012). Thus, *V. campbellii* might survive in low salt environments and its abundance may decrease with salinity, whereas *V. caribbeanicus* may represent indigenous species showing a high tolerance to salinity. No freshwater samples were successfully sequenced, which might be due to the low salinity and low abundance of *Vibrio*.

Though spatial variations in total and active *Vibrio* from freshwater to saltwater sites were mainly driven by salinity, the combination and interaction of other environmental factors (except for temperature and salinity) may also influence *Vibrio* communities (Hsieh *et al*., 2008; Turner *et al*., 2009). However, these factors have shown no well-resolved trends on the effect of *Vibrio* abundances across studies (Takemura *et al*., 2014; Amin *et al*., 2016; Jesser and Noble, 2018). Our results revealed that DO, pH (Fig. 3B) and inorganic nutrients (SiO_3^{2-} and NO_3^-; Fig. 3D) also had significant effects, but these effects varied among different groups (seasons, spatial groups and individual site). Organic matter also has a strong effect on *Vibrio* ecology and cell metabolism (Grimes *et al*., 2009; Kopprio *et al*., 2016; Zhang *et al*., 2018), as *Vibrio* spp. can secrete various extracellular hydrolytic enzymes to degrade polysaccharides (Zhang *et al*., 2018). In the present study, *V. campbellii*, the most abundant species in winter, showed a positive correlation with Chl *a*, which might indicate the relationship between *Vibrio* and phytoplankton abundance (Table 2). In addition, compared with only 2.5% in other marine bacteria (Zhang *et al*., 2018), most *Vibrio* cultures (56.06%, 162 out of 289 isolates; data not shown) can degrade chitin, and some strains (3.46%; data not shown) have also the ability to degrade alginate. The analysis of the correlation between the distribution of the *Vibrio* population and organic carbon will provide more information regarding the ecology of *Vibrio* spp. (Jesser and Noble, 2018; Zhang *et al*., 2018). Our study helps reveal the distribution patterns and potential functions of *Vibrio*, but more efforts should be conducted to elucidate their accurate roles in marine biogeochemical cycling.

The active Vibrio community was analysed for the first time at the RNA level

The diversity and biogeographic patterns differed substantially between the total and active bacterial communities, and different mechanisms controlled them (Zhang *et al*., 2014). Thus, it is important to examine both bacterial 16S rRNA and 16S rDNA to understand community biogeography and diversity and its ecological driving forces. In this study, RNA was extracted from the

environmental samples, and *Vibrio* activities were analysed for the first time. Consistent with the total vibrios, the 16S rRNA/16S rDNA (*Vibrio* spp.) (Fig. 5) and cultivable vibrios (Fig. 6B) increased from freshwater to saltwater, suggesting that *Vibrio* spp. in saltwater possessed high activities. However, although high cfu occurred in summer (Fig. 6A), the relative abundance of active *Vibrio* showed no remarkable differences in seasonal patterns (Fig. 5). The reason for this result may be only 12 samples (8 in winter and 4 in summer) were analysed in this research. A high proportion of *V*a/*V*t was found in most samples (7/12; Table S5) suggesting that the metabolism of vibrios in this region is active. However, the counts of culturable vibrios accounted for only ∼6% of total vibrios, indicating that a large number of *Vibrio* was not culturable on TCBS agar. In addition, the proportion of *V*a/Ba is lower than that of *V*t/Bt (Table S5), which may reflect that the increased range of other active bacteria is higher than that of vibrios.

The comparison of the community structure based on the OTU percentages between the total and active communities indicated a divergent distribution pattern of individual vibrio. In winter, the dominant groups of active *Vibrio* were consistent with total *Vibrio* spp. *V. atlanticus* occupied a high relative percentage and could be isolated from seawater (Fig. 4), indicating that it was the most abundant active species in winter. No species of *Vibrio* dominated the freshwater area (Fig. 4B), which can be explained by the low total and active *Vibrio* abundance. Interestingly, the dominant group in CE7BW was *V. hippocampi*, which was different from the dominant group in every other sample
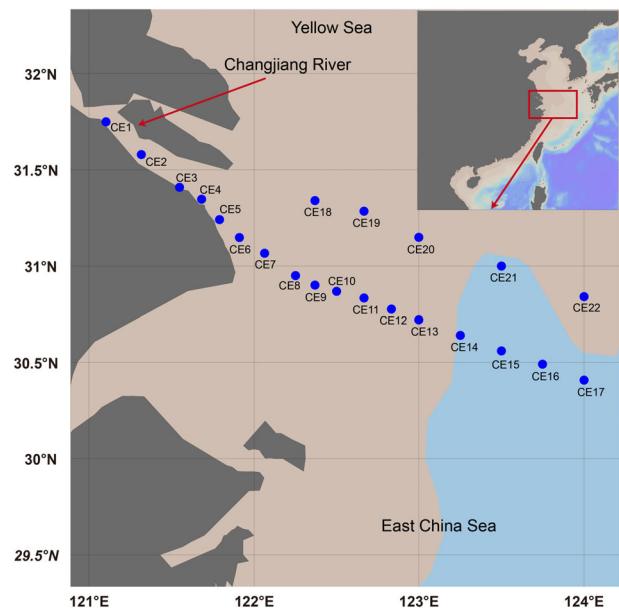

Fig. 7. Map showing locations of Yangtze River estuary and the sampling sites. Details are shown in Table S2. [Color figure can be viewed at wileyonlinelibrary.com]

Table 3. 16S rRNA oligonucleotide primers for qPCR amplification and sequencing.

Primers	Sequences (5′–3′)	Information on target gene	Reference
V567F/V680R	GGCGTAAAGCGCATGCAGGT/ GAAATTCTACCCCCCTCTACAG	General *Vibrio* spp. (113 bp)	Thompson *et al*., 2004; Vezzulli *et al*., 2012
B967F/B1046R	CAACGCGAAGAACCTTACC/ CGACAGCCATGCANCACCT	Total bacteria (79 bp)	Vezzulli *et al*., 2012; Sogin *et al*., 2006
V169F/V680R	GGATAACCTATTGGAAACGATG/ GAAATTCTACCCCCCTCTACAG	General *Vibrio* spp. (511 bp)	Siboni *et al*., 2016

(Fig. 4B). This difference might be due to the special environments (high concentrations of DO, Chl *a*, NO_3^-, NO_2^-, PO_4^{3-}, SiO_3^{2-}) and extensive substrate utilization of *V. hippocampi* (Balcázar *et al*., 2010). However, in summer, there were several differences between the active and total communities, as *V. owensii* instead of *V. campbellii* become the other abundant group in the RNA library and cultured results (Fig. 4B). The reason for this difference might be that the low-abundance OTUs in the DNA libraries may have high activities (Zhang *et al*., 2014), and *V. owensii* was the dominant species in summer and grew quickly at warmer sites (Cano-Gomez *et al*., 2009). Moreover, *V. caribbeanicus* was abundant in the RNA library but was not cultured, possibly because *V. caribbeanicus* originated from an oligotrophic environment, and TCBS is a eutrophic medium. Indeed, the *V. caribbeanicus* strain was first isolated from a marine sponge (*Scleritoderma cyanea*) collected from a depth of 242 m off the west coast of Curaçao (Hoffmann *et al*., 2012). Apart from temperature and salinity, inorganic (Table 2) and organic nutrients may play an important role in maintaining their activity, and thus more efforts are needed to estimate the correlations between *Vibrio* activity and these parameters.

Conclusion

The dynamics of the total and active *Vibrio* spp. throughout the Changjiang estuary were investigated by DNA-based and RNA-based qPCR and high-throughput sequencing as well as culturing approaches. The abundance of total *Vibrio* was higher in summer than in winter and showed an increasing trend from freshwater to saltwater, which indicated that vibrios represent an important and tangible barometer of climate change in marine systems, particularly in temperate and mid-latitude areas. A decrease in the dominant *Vibrio* species in summer and an increase in *V. atlanticus* in winter may promote the succession of *Vibrio* spp. between seasons. Our study highlights the importance of examining both vibrionic 16S rRNA and 16S rDNA to understand the diversity of the community and ecological driving forces. *V. owensii* was the dominant species throughout the Changjiang estuary in summer because of its high proportions in the RNA library and culturable

community components. *V. atlanticus* was the most abundant active species in winter due to its occurrence in DNA, RNA library and culturable results. Further work is necessary to provide a better understanding of the global distribution of *Vibrio* spp. and their roles in biogeochemical cycling. House-keeping genes could be used as alternative markers to differentiate closely related *Vibrio* species, e.g. heat shock protein 60 (*hsp*60) (Jesser and Noble, 2018), and the correlation could be detected between *Vibrio* spp. and organic nutrients.

Experimental procedures

Site description, sampling and isolation of Vibrio *strains*

Waters from the Changjiang estuary were retrieved aboard the R/V *Runjiang I* from a total of 22 stations in four cruises, including March and July 2016, March and July 2017 (Table S2). Surface water (S) and bottom water (B) samples were collected using a Sealogger CTD (SBE25, Electronic, USA) rosette water sampler (Liu *et al*., 2014). Sampling stations among the different periods are shown in Fig. 7, and the detailed analyses for each station are summarized in Table S2.

Water chemistries such as salinity, temperature, pH and DO were monitored with the CTD. Samples for dissolved inorganic nutrients (NO_2^-, NO_3^-, NH_4^+, SiO_3^{2-} and PO_4^{3-}) were filtered with 0.45-μm cellulose acetate membranes and analysed by a nutrient auto-analyser (AA3, Seal Analytical, UK) (Liu *et al*., 2015). Samples for Chl *a* analysis were collected on 0.7-μm GF/F filters (Whatman, UK). Chl *a* was extracted with 90% acetone for 24 h in dark and determined using a Turner-Designs Trilogy Laboratory® Fluorometer (Zhang *et al*., 2014).

One litre of water for DNA or RNA analyses was filtered in sequence through 3 μm (TSTP, 142 mm, Millipore) and 0.22 μm (GTTP, 142 mm, Millipore) polycarbonate membranes. Samples for RNA extraction were collected within 30 min and stored in 5 ml RNase-free tubes with 1 ml RNA*later* RNA stabilization solution (Ambion, USA). All filters were stored in liquid nitrogen onboard and transferred to −80 °C in the laboratory until DNA or RNA extraction. Twenty-seven DNA samples, including samples from transitional sites in winter (Wt)

and summer (St) and saltwater sites in winter (Ws) and summer (Ss); and 12 RNA samples, including Wt, St, Ws, Ss and freshwater in winter (Wf), were analysed for diversity and abundance of the total and active *Vibrio* (Table S2).

Triplicate 200 μl of each water sample (Table S2) was spread onto TCBS agar plate (HopeBiol, Qingdao, China). After incubated at ambient temperature for 48 h onboard, the numbers of individual cfu were manually enumerated; and colonies were randomly picked and purified on the ZoBell's 2216E agar (MA; Becton Dickinson) plate at 28 °C in the laboratory. Taxonomic identity was assigned in Ezbiocloud database (https://www.ezbiocloud.net/) on the basis of 16S rRNA gene (Zheng *et al*., 2016). These strains were preserved at −80 °C with 15% (v/v) glycerol.

Nucleotide acid extraction and qPCR

DNA extraction was performed according to Yin and colleagues (2013) with few modifications. The mixture was vigorously shocked on a FastPrep-24 homogenization system (MP Biomedicals, Irvine, California, USA) twice (60 s for each time at a speed of 6.0 m s^{-1}) to facilitate cell lysis. The extracted DNA was resuspended in 50 μl TE buffer (1 M Tris–HCl, 0.5 M EDTA, pH 8.0). RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany), followed by an RNase-Free DNase set (Qiagen, Hilden, Germany) to digest DNA during RNA purification (Zhang *et al*., 2014). The SuperScript RT-PCR system with random hexamers (Invitrogen, Carlsbad, CA, USA) was used to perform the reverse transcription reaction (Zhang *et al*., 2014). The genomic DNA and synthesized RNA were stored at −80 °C.

The patterns in the abundance of total and active *Vibrio* and the bacterial community were tracked using 16S rRNA gene-targeted qPCR with SYBR-green detection. qPCR and quantitative reverse transcription PCR (RT-qPCR) were performed using the StepOnePlus™ Real-Time PCR system (Applied Biosystems) and STEPONE software version 2.2. V567F and V680R were used as the specific 16S rRNA oligonucleotide primers in the qPCR and RT-qPCR for the *Vibrio* genus (Thompson *et al*., 2004; Vezzulli *et al*., 2012), whereas B967F and B1046R (Sogin *et al*., 2006; Vezzulli *et al*., 2012) specific to the *Bacteria* domain were used to quantify the total bacterial community (Table 3). Each 20-μl reaction mixture contained 5.0 mM of MgCl$_2$ and 0.2 μM of each primer for *Vibrio* (0.4 μM for bacteria). Cycling conditions for *Vibrio* were modified from Siboni and colleagues (2016): an initial denaturation at 95 °C for 3 min; followed by 35 cycles of followed by 35 cycles of a two-step reaction incorporating: denaturation at 95 °C for 30 s, annealing/extension step at 64 °C for 60 s. Cycling parameters for total bacteria (Bt) were the same as reported by

Sogin and colleagues (2006) except that the cycling number was set to 35 in this study. All extracted DNA was diluted fivefold to reduce pipetting errors, and all of the above tests were performed in triplicate. The standards were prepared from 16S rDNA nucleic acid templates of *V. rotiferianus* WXL191 (our laboratory) and referred to the methods from Zheng and colleagues (2017). All amplification efficiencies were between 95% and 105% with R^2 values > 0.99.

Vibrio diversity analysis via high-throughput sequencing

To determine the community composition of the total and active *Vibrio*, we used the *Vibrio*-specific 16S rRNA gene primers V169F and V680R (Table 3) that target the variable regions of V2-V4 (Siboni *et al*., 2016). The PCR reaction system (20 μl) contained 1× Fast Pfu Buffer, 0.25 mM of dNTPs, 0.2 μM of each primer, 1 U of FastPfu polymerase, 10 ng of template DNA or cDNA, and 0.2 μl of BSA. PCR cycling conditions were 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 45 s at 72 °C (Siboni *et al*., 2016). After confirming positive amplification, purified amplicons were pooled in equimolar and paired-end sequences (2 × 300) on the Illumina MiSeq platform (Illumina, San Diego, USA) following the manufacturer's guidelines. Raw data files were deposited in NCBI Sequence Read Archive (SRA) (accession numbers SRP159304 and SRP166911) under bioproject numbers PRJNA488569 and PRJNA497918, respectively.

Vibrio 16S rDNA and cDNA sequences (Table S2) were analysed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (version 1.9.1) (Caporaso *et al*., 2010). Reads were assigned to samples according to their barcodes without mismatch. The raw reads that had a quality score higher than 20 over a 5-bp window size and a minimum length of 100 bp were retained (Kong, 2011). Paired-end DNA sequences were joined with at least a 50-bp overlap and less than 5% mismatches using FLASH (Magoč and Salzberg, 2011). A Perl script, i.e. daisychopper.pl, was used to randomly subsample sequences from each sample according to the lowest read numbers to equalize sampling efforts (Gilbert *et al*., 2009). OTU clustering and taxonomic assignment were also performed in QIIME. Specifically, OTUs were defined at a 97% sequence similarity level, and then chimera sequences were detected and removed with UCHIME (Edgar *et al*., 2011), as recommended by QIIME tutorials. Taxonomy was assigned using the RDP Classifier v2.2 (Wang *et al*., 2007) against the SILVA v128 16S rRNA gene reference database (http://www.arb-silva.de) with a minimum support threshold of 70%, and *Vibrio* sequences were reassigned against the EzBioCloud database to obtain a more accurate classification. To remove the effect of sampling effort upon analysis, sequences were then rarefied to the

lowest read number for all samples with a 'single rarefaction' QIIME script (Caporaso *et al*., 2010; Wang *et al*., 2016).

Statistical analysis

Seasonal differences in environmental parameters were tested with the non-parametric Mann–Whitney test. To compare the total abundances of *Vibrio* spp. and bacteria, the data were log [$x + 1$] transformed, and the Mann–Whitney test and Kruskal–Wallis test were performed. Correlations between qPCR results and environmental parameters were assessed using Spearman's rank correlation analysis package. All analyses were performed using STATISTICA version 22.0 (StatSoft, Tulsa, OK, USA).

The diversity indices for alpha diversity analysis, including Good's coverage, phylogenetic diversity, Chao I, equitability (a Shannon index-based measure of evenness) and Shannon, were calculated using MOTHUR software packages (Schloss *et al*., 2009). Wilcoxon's test was used to compare the alpha diversity indices between different groups of samples. For beta diversity, PCA was performed using CANOCO v 5.0 software (Microcomputer Power) at the species level. The relationships between phylotypes and environmental factors were evaluated by RDA in CANOCO v 5.0 with 9999 Monte Carlo permutation tests using square-root-transformed data. Spearman's rank correlation coefficients were calculated to determine the relationship between environmental factors and diversity indices or the bacterial community at the species level.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information.

Fig. S1. The difference of total *Vibrio* spp. (A) and total bacteria (B) abundance between summer (Jul. 2016 and 2017) and winter (Mar. 2016 and 2017). Vermilion and pink columns denote summer samples; dark and light blue columns denote winter samples.

Fig. S2. Histogram indicated the richness, evenness and diversity estimators of *Vibrio* spp. among the YS, ECS and SCS. Asterisks denote significant differences between each area. *, $0.01 < P \leq 0.05$; **, $0.001 < P \leq 0.01$; ***, $P \leq 0.001$. The richness and evenness of *Vibrio* spp. among different groups were calculated by Chao 1 (A) and Shannoneven indices (B), while Shannon index and phylogenetic distance were used to estimate the diversity of *Vibrio* spp. (C, D). Blue columns represented samples collected in winter, while vermilion columns denoted samples from summer, respectively.

Fig. S3. Heatmap generated in R with function "pheatmap" of the top 22 abundant species at DNA level in all samples based on the EzBioCloud database annotation. The top tree showed the cluster relationship of samples. Other, <0.1% across all the sequences. *P*-values were calculated via Kruskal-Wallis test. Asterisks indicate the significant difference between sampling groups. *P* value of <0.5 (*), <0.01 (**), and <0.001 (***).

Table S1. The information of total species in the genus *Vibrio* till Jun. 2020.

Table S2. Sampling location and information for each samples.

Table S3. Summary for environmental parameters of each sites.

Table S4. Observed richness and diversity estimates of *Vibrio* spp. based on 97% OTU clusters.

Table S5. The abundance of culturable, active and total *Vibrio* and bacteria, and the ratios.