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# Microbial Communities in Water during Red Tides along the Coast of China-A Case Study of *Prorocentrum Donghaiense* Red Tide in the East China Sea

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ABSTRACT

Red tides are a major public hazard in the global oceans. The coast of the East China Sea is the sea area where red tide disasters are the most frequent and serious in China. In order to accurately grasp the occurrence of red tides in the coastal waters of the East China Sea, and to understand the microbial communities in the waters during the occurrence of red tides in the East China Sea, a special survey of red tides in the coastal waters of Zhejiang, China was carried out in June 2018. The results showed that nutrient concentrations of N and P were generally high in this area, DIN concentrations in most areas exceeded the permitted limit of Chinese seawater quality grade I. There were significant differences in dissolved oxygen, pH, COD, chlorophyll and phytoplankton abundance of red tides. During the investigation, red tides were found in the waters near the Yushan Islands. The content of chlorophyll a was 42.12mg/m<sup>3</sup>, the cell abundance of phytoplankton was 8.16×10<sup>8</sup>/L, and the abundance of *Prorocentrum edulis* accounted for 98.5%. The Illumina MiSeq sequencing platform was used for 16s high-throughput sequencing of water microorganisms, and a total of 16 bacteria were identified. Proteobacteria is the first dominant phylum, followed by Cyanobacteria and Bacteroides. Some differences in bacterial community compositions between HAB and the nearby seawater were observed. The predominant bacteria in the red tide occurrence area were Proteobacteria, comprising 46.1% of the relative abundance; while the predominant bacteria in the nearby sea area, comprising 42.0% of the relative abundance.

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## 1. Introduction

Red tides are a major public hazard in the global oceans today. With the economic development of coastal areas, the occurrence of red tides in coastal waters has become more and more frequent, and the scope of occurrence and the degree of harm are also increasing. The ecological environmental damage caused by red tides has become more and more serious. It has become a marine ecological environment of general concern in the world today<sup>[1]</sup>. Influenced by several main rivers, such as the Yangtze River and Qiantang River in the north, the Taiwan warm current from the South and the southerly wind in summer, the dynamic environment of the coastal water in Zhejiang is complex, and the nutrients and biomass in the water are high, which is the most frequent and serious red tide disaster in China. The earliest record of red tide in China is the *Noctiluca* red tide which occurred along the coast of Zhejiang Province, China in 1933. According to the *Zhejiang Marine Environment Bulletin* (2000-2010) published by the Zhejiang Ocean and Fisheries Bureau and related documents, from 2000 to 2010, a total of 316 red tides were recorded in Zhejiang sea area, with a cumulative area of more than 91,000 km<sup>2</sup>. There were 57 toxic and harmful red tides, with a cumulative area of more than 2.2 km<sup>2</sup>. In 2011, the direct economic losses caused by red tide disaster in Zhejiang Province exceeded 243 million yuan. It is especially worth noting that the occurrence of toxic red tides in recent years has shown a rapid growth trend, which has caused long-term potential adverse effects on people's lives and health and the marine ecological environment, and at the same time has brought great threats to the sustainable development strategy of the marine economy<sup>[3-5]</sup>.

The growth and proliferation of red tide algae are not only closely related to environmental factors, but also closely related to surrounding microorganisms. The growth and elimination of red tide algae is accompanied by the joint action of many microorganisms. Marine bacteria and algae are closely combined in space and time. They can synthesize different types of metabolites, and these metabolites can have beneficial or harmful effects on both sides<sup>[6]</sup>. Bacterial metabolites can specifically hinder or even stop the propagation of algae<sup>[7]</sup>, and may also promote the proliferation of algae. There may be some feedback mechanisms which play an important role in controlling the population dynamics of algae and marine bacteria.

Most microorganisms in the marine environment are in an unculturable state, which affects the in-depth study of environmental microorganisms<sup>[8]</sup>. Among the numerous

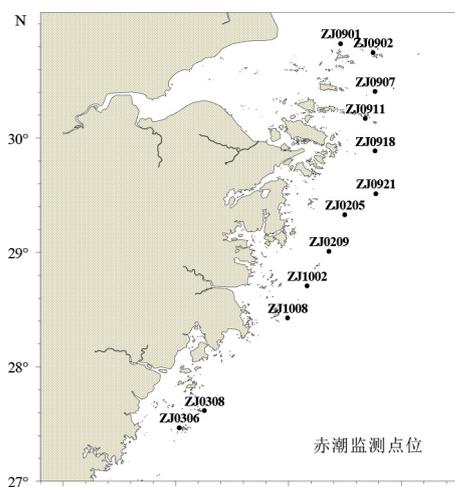
microorganisms, less than 1% of them can be separated by culture method<sup>[9]</sup>. Therefore, this traditional plate culture method can only be used as an auxiliary tool, and it needs to be combined with modern biotechnology methods to reflect the real information of microbial community structure more objectively and comprehensively. With the popularization of molecular biology technology in recent years, studying the diversity of microorganisms at the DNA level has become the most important technical means of current research, and has promoted the rapid development of microbial oceanography<sup>[10]</sup>. The paper uses the MiSeq high-throughput sequencing platform to conduct a comprehensive and in-depth investigation of the microbial diversity of sea water when red tides occur, and uses a variety of statistical software and other common community analysis tools to conduct in-depth research on the potential relationship between microbial communities and environmental factors, with a view to providing technical support for China's offshore ecological research and environmental management.

## 2. Materials and Methods

### 2.1 Sampling Overview

According to the perennial monitoring results of the Zhejiang Sea by the Zhoushan Marine Ecological Environment Monitoring Station in Zhejiang Province: the turn of spring and summer is the period of high incidence of red tide disasters in the Zhejiang sea area. The sea areas with high frequency of occurrence are mainly concentrated in the sea area near Shengsi Sea Area, Dongji Sea Area, Zhujiajian Sea Area, Xiangshan Harbo, Yushan Islands, Taizhou Islands and Nanji Islands.

In June 2018, the professional marine environment survey ship "Zhehai Environmental Supervision" was used to conduct a patrol survey of the marine ecological environment in the above sea areas. According to the occurrence of red tides at the scene over the years, a total of 12 stations have been set up. The specific locations of the survey stations are shown in Figure 1. During the investigation, a red tide was suspected to have occurred in the waters of the Yushan Islands. The water on site was dark brown, distributed in blocks, accompanied by a fishy smell.



**Figure 1.** Sampling sites of high frequency area of red tide in Zhejiang sea area

## 2.2 Sampling and Analysis Methods

Ecological environment survey: the sampling analysis and phytoplankton analysis methods of the investigated sea area ecological environment parameters are shown in Table 1.

## 2.3 Microbial Community Analysis

Sampling water samples in areas where red tides occur and nearby sea areas to conduct microbial community analysis. The total DNA was extracted with TIANGEN TIANamp Soil DNA Kit and operated according to standard procedures. The sequencing samples were constructed by Hangzhou G-BIO Biotechnology Co., Ltd. in accordance with the 16S metagenomic sequencing standard process to construct the 16S rRNA gene V3-V4 variable

**Table 1.** Analyzing methods and equipment of marine ecological environmental investigations

Item	Sampling method	Analysis method	Method	Analytical Instruments
Chlorophyll a	surface water sampling	Spectrophotometry	GB17378.7-2007(8.2)	Cary50 spectrophotometer
Water temperature	GO-FLOW bottle	CTD Method	GB/T12763.2-2007	YSI6600 multi-parameter water quality instrument
Salinity	GO-FLOW bottle	CTD Method	GB/T12763.2-2007	YSI6600 multi-parameter water quality instrument
COD	GO-FLOW bottle	Basicity KMnO <sub>4</sub>	GB17378.4-2007(32)	VITLAB automatic titrator
pH	GO-FLOW bottle	Glass Electrode Method	GB17378.4-2007(26)	PB-21pH meter
Labile Phosphate	GO-FLOW bottle	Flow injection colorimetric method	EPA 365.5-1997	QuAAtro Flow Analyzer
Nitrite Nitrogen	GO-FLOW bottle	Flow injection colorimetric method	EPA 353.4-1997	QuAAtro Flow Analyzer
Nitrate Nitrogen	GO-FLOW bottle	Flow injection colorimetric method	EPA 353.4-1997	QuAAtro Flow Analyzer
Ammonia nitrogen	GO-FLOW bottle	Flow injection colorimetric method	EPA 349.0-1997	QuAAtro Flow Analyzer
Silicate	GO-FLOW bottle	Flow injection colorimetric method	EPA366.0-1997	QuAAtro Flow Analyzer
Phytoplankton	GO-FLOW surface water extraction	Quantity: concentrated counting method	GB17378.7-2007(5)	Leica DM4000B microscope
	Shallow water type III vertical trawl	Type: Microscope		

region (primer sequence: 341F-CCTACGGGNGGCWGCAG; 805R-GACTACHVGGGTATCTAATCC) sequencing library of environmental microorganisms, perform corresponding quality control, and use Illumina MiSeq double-end (250 bp×2) sequencing platform to complete the sequence determination. Specific experimental procedures, quality control and sequence analysis methods can refer to Reference 11<sup>[11]</sup>.

## 2.4 Data Processing

The analysis results of various environmental factors and phytoplankton in the seawater environment are used to draw the planar distribution map of each parameter using ODV4.0 software. Based on the 16S high-throughput sequencing results, the species composition map and Venn diagram were drawn by R language. The canonical Association (CCA) analysis was carried out with the software of Past to explore the potential relationship between phytoplankton and the environment.

## 3. Results

### 3.1 Sea Water Quality

A water environment survey was conducted in the surveyed sea area in June 2018. The monitoring results of the main water quality factors are shown in Table 2. The plane distribution of each factor is shown in Figure 2.

According to the national “Sea Water Quality Stan-

dard” (GB3097-1997), the measured parameters of inorganic nitrogen and active phosphate are the factors that exceed the standard of sea water quality. Among them, the concentration of inorganic nitrogen is generally higher, with an average value of 0.414 mg/L, and 91.7% of the samples exceed the first-class seawater standard. From a planar distribution, the inorganic nitrogen content in the northern waters at the mouth of Hangzhou Bay is significantly higher than other regions. The average value of active phosphate is 0.013 mg/L, and 41.7% of the samples exceed the first-class seawater standard, and the planar distribution is the same as that of inorganic nitrogen.

The single factor T test was used to investigate the results of the water quality factors to investigate the differences between the various indicators of the red tide water body and the nearby sea area. It was found that the dissolved oxygen, pH, COD, chlorophyll and phytoplankton abundance of the red tide water body were significantly different ( $p < 0.01$ ).

### 3.2 Phytoplankton

A total of 56 species of phytoplankton belonging to 38 genera, 20 families and 6 phyla were identified, including 30 species of Bacillariophyta (53.6%), 21 species of dinoflagellate (37.5%), 1 specie of Chrysophyta, Euglena, Cryptophyta, Rhaphidophyta and Dinoflagellate, accounting for 1.8% respectively. The main species are *Hillea* sp., *Prorocentrum donghaiense*, *Skeletonema costatum*

**Table 2.** Statistical results of major monitoring indicators

Water quality factors	Sample quantities	Range	Average value	Variance	P value (T test)
Chlorophyll a(mg/m <sup>3</sup> )	13	0.36~42.12	6.18	127.688	8.00×10 <sup>-8</sup>
Number of phytoplankton species	13		75 (Total)	84.97436	0.950
Phytoplankton Abundance (×10 <sup>4</sup> /L)	13	0.4~816	162	5922555	5.06×10 <sup>-7</sup>
Shannon index	13	0.58~3.12	1.86	0.6508231	0.182
Water temperature (°C )	13	21.2~23.7	22.3	0.925	0.467
Salinity	13	20.7~32.3	29.3	10.04333	0.507
Dissolve oxygen (mg/L)	13	6.54~15.1	8.30	5.18309	1.6×10 <sup>-7</sup>
pH	13	8.03~8.60	8.20	0.0207641	5.90×10 <sup>-7</sup>
Active phosphate (mg/L)	13	<0.001~0.030	0.008	9.48E-05	0.012
COD (mg/L)	13	0.36~8.29	1.31	4.603581	6.25×10 <sup>-8</sup>
Dissovled inorganic nitrogen (mg/L)	13	0.051~0.998	0.414	0.09032924	0.024
Active silicate (mg/L)	13	0.311~1.26	0.648	0.09146092	0.108

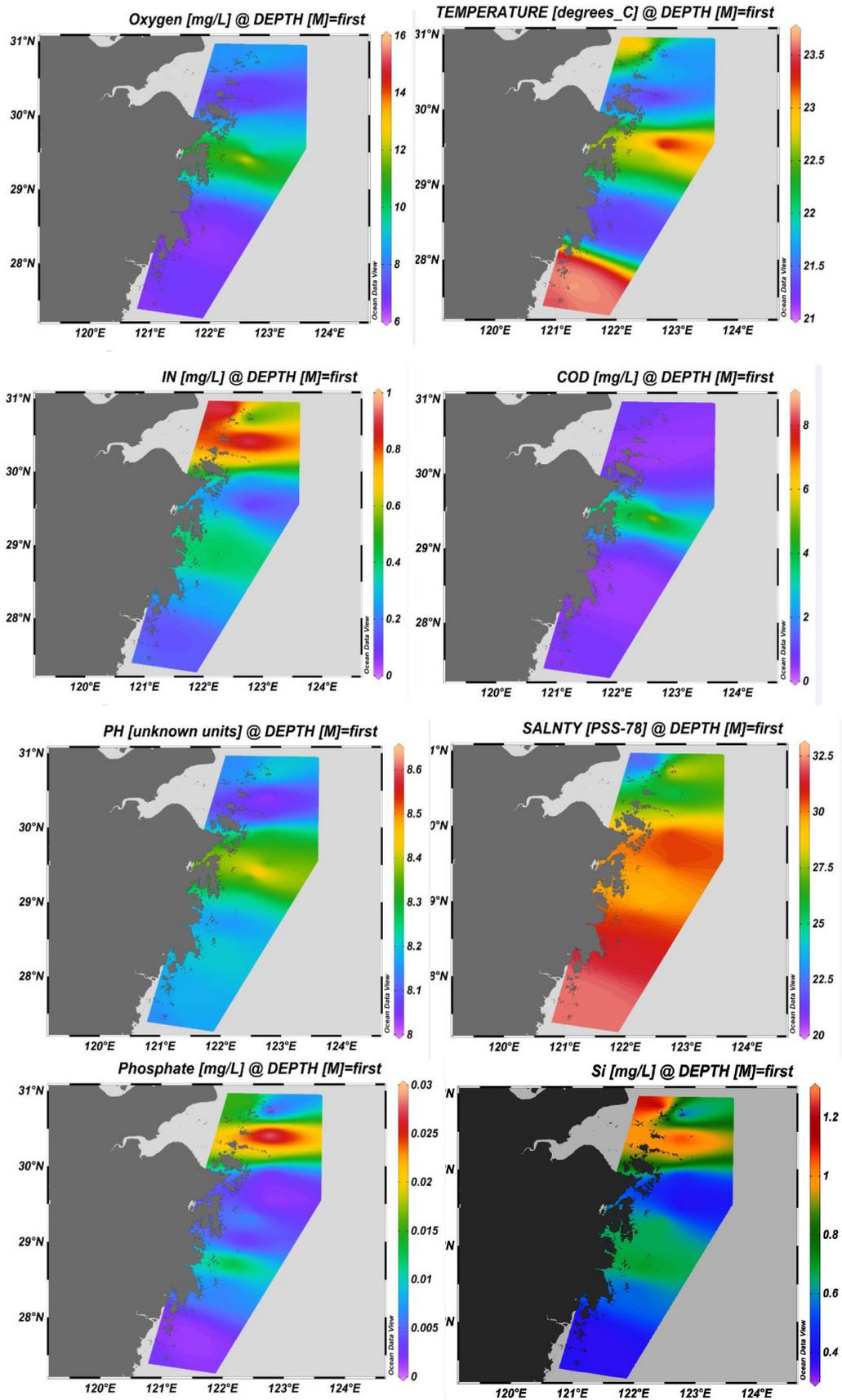


Figure 2. Distribution of main environmental factors

and so on. The average cell abundance is  $1.03 \times 10^5/L$ . The average content of chlorophyll a is  $3.16 \text{ mg/m}^3$ , and the middle part of the survey area is the high value sea area of chlorophyll a. The red tide was found in the sea area near Yushan Islands. In the red tide area (J2018), the content of chlorophyll a is  $42.12 \text{ mg/m}^3$ , and the cell abundance of phytoplankton is  $8.16 \times 10^8/L$ , of which the abundance of *Prorocentrum donghaiensis* accounts for 98.5%. It can be considered that this is a *Prorocentrum donghaiense* event.

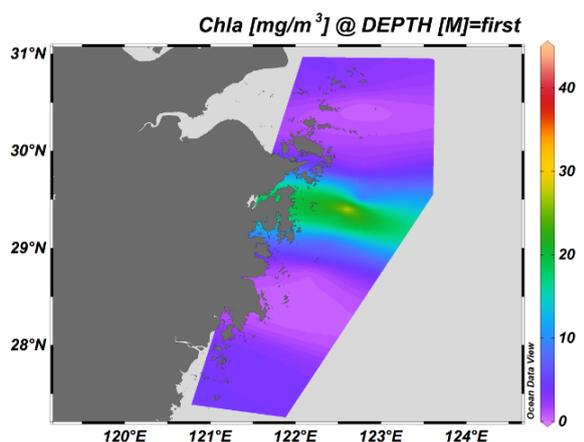


Figure 3. Distribution of chlorophyll

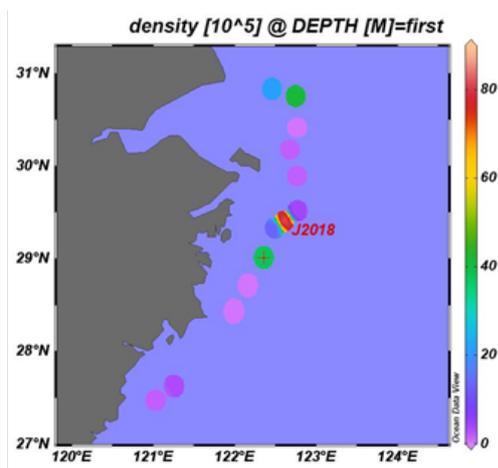


Figure 4. Phytoplankton density of monitoring sites

### 3.3 Microbial community

#### 3.3.1 Sequence Statistics

High throughput sequencing based on Illumina platform was carried out to collect microorganisms in surface water of the sea area where red tide occurs (HAB01) and the sea area where no red tide occurs (ZJ0205). A total of 75451 pairs of microbial original sequences (pair-end reads) were obtained, with an average sequence length of 412 bp. After quality control and sequence optimization, 77430 high-quality sequences were selected. Considering

the large differences of sequencing data at each station, further random sampling was conducted to standardize the data to balance the impact of sampling error on subsequent analysis. After preprocessing, 595 OTUs were obtained according to 97% sequence similarity. The detailed distribution of OTUs is shown in Table 3.

Table 3. Summary of sequence information of marine sediment microbes

Station	Original sequence	Effective sequence	Optimized sequence	Operational taxonomic units(OTUs)
HAB01	38203	37983	37540	420
ZJ0205	37248	37045	36890	505

Using mothur software to randomly sample the optimized sequences, and construct the dilution curve with the number of sequences drawn and their Chao1 index as shown in Figure 5. The results show that the curve of each station tends to be flat, indicating that the number of sequencing in this experiment is reasonable, the OTU sampling has reached or close to saturation, and the experimental results basically reflect the species diversity of the samples.

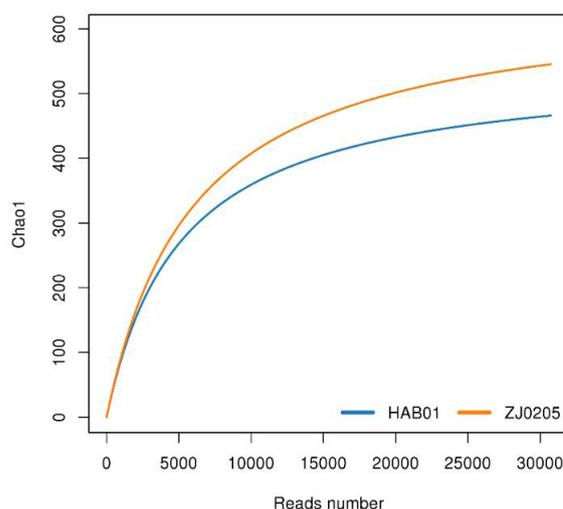


Figure 5. Rarefaction of marine sediment microbes

#### 3.3.2 Microbial Species Composition

A total of 16 phyla, 82 families and 126 genera of bacteria were identified in this survey. Among them, Proteobacteria is the first dominant group, accounting for 38.4% of the total, followed by Cyanobacteria and Bacteroidetes, accounting for 29.9% and 24.3% of the total. In addition, Actinobacteria, Verrucomicrobia and Planctomycetes were also detected. The composition of microflora in the

red tide occurrence area (HAB01) and the nearby sea area (ZJ0205) is shown in Figure 6. At the level of subordination, some genera of Bacillariophyta, Candidatus Pelagibacte and Flavobacteriaceae are the main dominant genera. The composition of the main genera is shown in Figure 7.

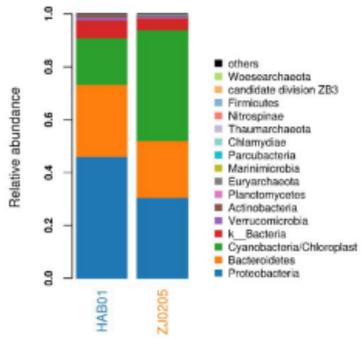


Figure 6. Species composition (phylum level)

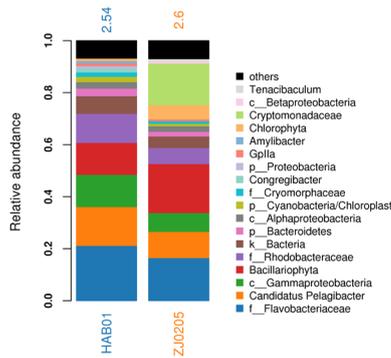


Figure 7. Species composition (genera level)

At the subordinate level, there are 75 genera of microorganisms in the two regions, accounting for 59.5%, and more than half of the total species

The Venn-Diagram diagram at the genera level was used to analyze the differences in the microbial communities between the red tide occurrence point and the nearby sea waters (Figure 8). From a genera level, there are 75 genera of microorganisms in the two regions, accounting for 59.5%, and a total of more than half of the species; 11 genera of microorganisms in the in the sea area where red tide occurs, accounting for 8.7%; and 40 genera of microorganisms in nearby waters, accounting for 31.7%. In

terms of species composition, there are nearly half of the two different species, and there is a certain degree of difference in water microorganisms between the two sides.

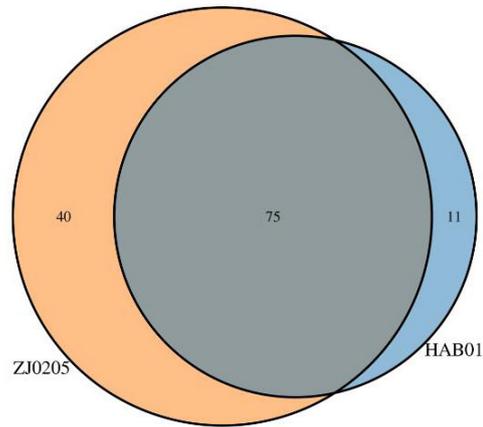


Figure 8. The variance analysis of microbial community (genera level)

Table 4 is the Comparison of main dominant categories of microorganisms in Case2 waters. The first dominant bacteria in red tide water and the second dominant bacteria in nearby waters are Proteobacteria. The second dominant bacteria in red tide water and the third dominant bacteria in nearby waters are Bacteroides. However, the proportion of each dominant bacteria is quite different between the two sides. For example, the first dominant bacteria in the red tide area were Proteobacteria (46.1%), accounting for 30.6% in the nearby sea area, while cyanobacteria (42.0%) were the first dominant bacteria in the nearby sea area, accounting for 17.7% in the red tide area.

Huang Bei and other scholars used culturable technology to isolate and purify bacteria from Proocentrum bloom in the East China Sea and the control waters in Taizhou sea area, and identified the strains based on biochemical reaction [13]. The results showed that the dominant bacteria in red tide water were Vibrio and Pseudomonas, all belonging to the Proteobacteria, which was consistent with the survey results. However, the main dominant bacteria in Zhujiajian sea area without red tide are Flavobacterium and Aeromonas, the results of this investigation are cyanobacteria, which are quite different. This is due to the

Table 4. Dominant species of marine microbes (phylum level)

	First dominant bacteria	Second dominant bacteria	Third dominant bacteria	Fourth dominant bacteria
HAB01	Proteobacteria, 46.1%	Bacteroidetes, 27.2%	Cyanobacteria, 17.7%	Verrucomicrobia, 1.0%
ZJ0205	Cyanobacteria, 42.0%	Proteobacteria, 30.6%	Bacteroidetes, 21.4%	Actinobacteria, 0.4%.

different technical means used in the second survey. The high-throughput sequencing method used in this survey is quite different from the traditional biochemical identification of bacteria. In addition, the sampling sea area of the two methods is also different, so the results are quite different. According to the 16S rDNA sequence of cultivable bacteria in different periods of red tide, Wang Jian and other scholars carried out molecular classification and identification of bacteria, and found that most of the bacteria are bacterial groups of the Proteobacteria and Bacteroides [14].

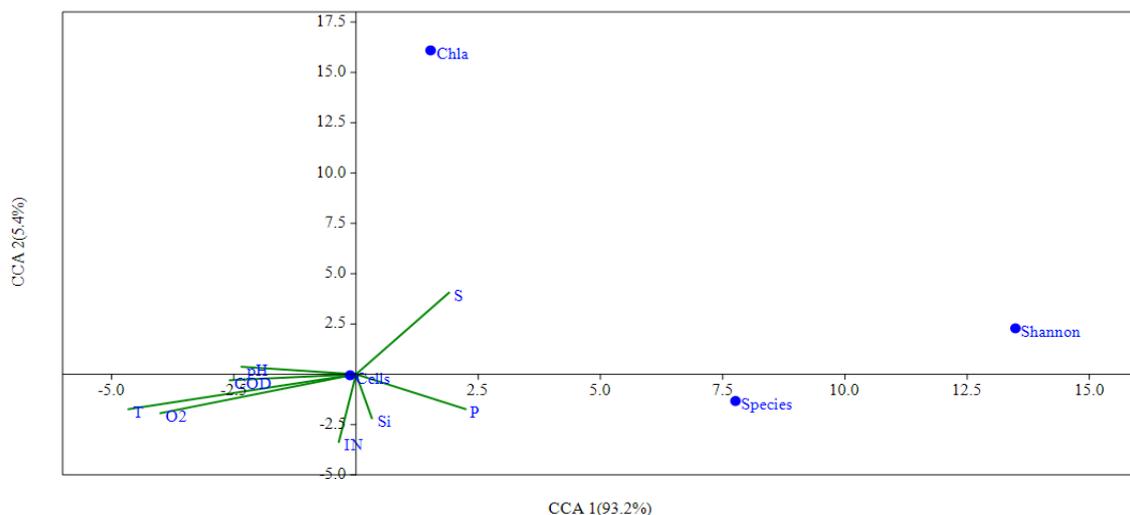
Chang Hong and other scholars used molecular biology methods such as terminal restriction fragment length polymorphism (T-RFLP) analysis, through principal component analysis and cloning library construction, to study the bacterial community structure of *S. conidus* during the red tide in the Shenzhen Dapeng Bay. It was found that the types of bacteria mainly belong to the Proteobacteria, Bacteroides and Firmicutes [15]. Hye Eun Kang and other scholars carried out molecular biology research on the microbial community during the occurrence of *Heterosigmaakashiwo* red tide in Juji Island, South Korea. The 16S rDNA sequencing and species identification results showed that Proteobacteria and Bacteroides are the two most important bacterial groups in red tide waters, followed by Firmicutes and Actinomycetes [16]. It can be seen that using molecular biology techniques to study water microbial communities when red tides occur, although the causes of red tides and the studied sea areas are different, the structure of the water microbial community is relatively similar at the phylum level. Proteobacteria, Bacteroides, Actinobacteria and Firmicutes are the main bacterial groups. The results of this study also confirmed this con-

clusion.

### 3.4 Correlation Analysis of Water Quality and Phytoplankton

In order to directly reflect the correlation between phytoplankton and environmental factors, the Past software and CCA analysis method were used for sequencing. Through the analysis of the correlation between phytoplankton species number, cell abundance, chlorophyll, Shannon index and environmental factors, it was found that the first axis and the second axis explained 93.2% and 5.4% of the total variation respectively in CCA ordination chart (Figure 9), which indicated that the environmental factors in this study explained the variation of phytoplankton community to a great extent. The dots in the figure represent the number of phytoplankton species, cell abundance, chlorophyll, Shannon index, and the rays represent environmental factors.

The dots in the figure represent the number of phytoplankton species, cell abundance, chlorophyll, and Shannon index, and the rays represent environmental factors. The longer the radiation of environmental factors, the greater the impact of the factor on the research object; and the length of the vertical distance from the microorganism to the environmental factors determines the correlation between them, the shorter the distance, the greater the correlation, and the longer the distance, the smaller the correlation. It can be seen from Figure 9 that, in general, the environmental factors in this survey have little effect on the phytoplankton community. For example, each factor has little effect on cell abundance. Relatively speaking, the number of plant species, chlorophyll and Shannon index were significantly affected by active phosphate and



**Figure 9.** CCA ordination of phytoplankton and environmental factors

salinity in seawater.

Salinity is a key factor in the growth of marine phytoplankton. Dr. Zhang and other scholars studied the phytoplankton and its water quality physical and chemical parameters in Hangzhou Bay in the summer from 2004 to 2010. The survey results showed that salinity was the main variable related to phytoplankton community<sup>[17]</sup>. In addition, Dr. Jiang and other scholars studied the biomass and physical and chemical indicators of phytoplankton in Xiangshan Bay, Zhejiang Province, and found that phytoplankton also showed spatial heterogeneity along the salinity gradient, which was driven by marine environmental factors<sup>[18]</sup>. Phosphorus is one of the essential nutrients for the growth of microalgae, and most of the metabolic structure and functions require phosphorus<sup>[19,20]</sup>. Many scholars have studied the estuarine areas of the East China Sea and the South China Sea, and found that the high nitrogen concentration in the coastal areas of China leads to serious phosphorus limitation<sup>[21,22]</sup>. The results of this survey are consistent with the above-mentioned research results. In this survey, the concentration of inorganic nitrogen in seawater is generally high, and the CCA ranking shows that active phosphate has a relatively large influence on the number of plant species, chlorophyll, and Shannon index.

#### 4. Conclusion

(1) In June 2018, a patrol survey of the marine ecological environment was carried out in the sea areas with high incidence of red tides along the coast of Zhejiang, China. It was found that the nitrogen and phosphorus nutrient contents in the surveyed seas were generally high, and the inorganic nitrogen of most samples exceeded the first-class sea water quality standards. There are significant differences in dissolved oxygen, pH, COD, chlorophyll and phytoplankton abundance in the water body when red tide occurs. The active phosphate and salinity in seawater have a greater impact on the number of plant species, chlorophyll and Shannon index.

(2) During the investigation, a red tide was found in the sea area near Yushan Islands. The chlorophyll *a* content was 42.12 mg/m<sup>3</sup> and the cell abundance of phytoplankton is 8.16×10<sup>8</sup>/L, of which the abundance of *Prorocentrum donghaiensis* accounts for 98.5%. It can be considered that this is a *Prorocentrum donghaiense* event.

(3) The Illumina MiSeq sequencing platform was used to perform 16s high-throughput sequencing on the red tide occurrence area and nearby sea waters, and a total of 16 phyla, 82 families and 126 genera of bacteria were identified. At the phylum level, the Proteobacteria is the first dominant phyla, with 38.4% of the total sequence number;

followed by the cyanobacteria and Bacteroides.

(4) Comparing the microbial species composition of the red tide occurrence point and the nearby sea area, there is a certain degree of difference between the two water bodies. The proportion of dominant bacteria between the two groups was quite different. The most dominant bacteria in the red tide area was Proteobacteria, accounting for 46.1%; Cyanobacteria was the first dominant bacteria in the nearby sea area, accounting for 42.0%.

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