

Ecological assessment of phytoplankton community via microscopic method and 18S rRNA gene sequencing in Pearl River Estuary

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Abstract. Monitoring phytoplankton community underpins our understanding of water quality and ecological functions. In this study, we approached phytoplankton abundance, community composition, and diversity by both microscopy and 18S rRNA gene sequencing. Environmental variances influencing the phytoplankton were evaluated as well. There were 6 phyla and 62 species identified by microscopy, and the diversity index Shannon-Wiener and evenness index Pielou index indicated phytoplankton community had high diversity; however, the high density of dominance genus suggested that our research region had potential red tide effects. The canonical correspondence analysis illustrated that suspended solids, phosphate and temperature were three major factors that affected the distribution and components of phytoplankton community. The DNA barcoding sequencing of 18S rRNA gene supported the main results via microscopic methods while providing more identified community components, which implied that 18S rRNA gene sequencing can be used as a supplemental method for fast ecological assessment of phytoplankton community.

1 Introduction

Phytoplankton composes a group of key ecological organisms in aquatic ecosystems. Like the functions of plants in terrestrial ecosystems, phytoplankton usually contains chlorophyll, requires sunlight to grow, and plays the character of primary producer in the aquatic systems [1, 2]. In a balanced ecosystem, phytoplankton absorbs nutrients to grow and forms the base of foodweb by providing food sources to upper layer aquatic creatures such as larval fish, zooplankton or other bigger size animals. When too many nutrients are observed, phytoplankton may grow out of control and form harmful algal blooms, i.e., red tide, which consumes marine oxygen and produces extremely toxic compounds that have harmful effects on aquatic ecosystems and even human [3]. In this perspective, phytoplankton community composes a more direct index to the health of aquatic systems than traditional physical and chemical index. Thus, both US EPA's Clean Water Act and the European Water Framework Directive (EU WFD) utilize phytoplankton as a biological quality element to assess the ecological condition of aquatic systems, in which the components, abundance, and diversity of phytoplankton were evaluated [4, 5].

Traditional method for the identification of phytoplankton community relies on taxonomic expertise

and microscope-based morphological research, which is time-consuming and labor-intensive. Meanwhile, this method is limited to a subset of phytoplankton species (usually around 100) [6, 7]. Despite this, the framework of ecological assessment by phytoplankton identification has been established. For instance, Diatoms and Dinoflagellates are two phytoplankton groups that most identified and used to predict water quality. Diatoms are estimated to be responsible for 20% of the global carbon fixation and main source of oxygen in the marine ecosystem, but the sudden blooms of certain diatom species may cause marine red tide [8, 9]. Dinoflagellates are perhaps best known as causers of harmful algal blooms, and about 75-80% of toxic phytoplankton species belonged to dinoflagellate groups [10]. Meanwhile, the evaluation index, such as diversity index Shannon-Wiener (H'), evenness index Pielou index (J), or integrated index like phytoplankton index of biotic integrity are also used to evaluate the health level of phytoplankton community [11].

Recently, the high-through sequencing represents a new method to overcome the identification of phytoplankton organisms [1], but the overestimated diversity index and conflicts regarding the morphologies and functions of phytoplankton are also announced [12]. Therefore, the studies linking the microscopic results and

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sequencing results to assess the phytoplankton community are necessary.

In this study, we aimed to (1) achieve phytoplankton community by both microscopic methods and high-throughput sequencing in a typical estuary region; (2) analyze the environmental factors and their influence on the phytoplankton community; and (3) assess the ecological status of phytoplankton community.

2 Methods and materials

2.1 Sampling and chemical analyses

A typical estuary was chosen in Pearl River Estuary, which represents the estuary that linking to the most developed region with huge population as well as complex contaminants from upstream urban rivers. 8 sample locations were selected within the region of longitude 116°45'E-117°50'E and latitude 23°20'N-23°30'N (Fig. 1).

Temperature, pH, salinity and dissolved oxygen (DO) of the estuarine water were measured in situ, using a Hach HQ40d multi-probe meter (Hach, Loveland Co., USA). Biochemical oxygen demand (BOD), chemical oxygen demand (COD_{Mn}), suspended solids (SS), were measured in estuarine water samples using procedures described in APHA (1998) [13]. The dissolved nitrogen of ammonium (NH₄⁺) and nitrate (NO₃⁻), sulfite (S²⁻), phosphate (PO₄³⁻) were analyzed using a Dionex™ Ion Chromatograph (IC3000) with an AS-22 and CS-16 column.

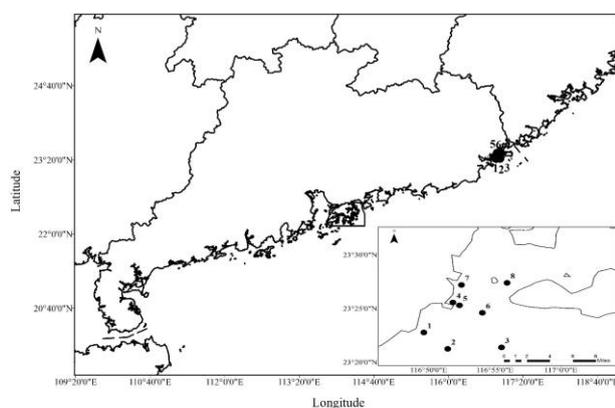


Fig. 1. Location of sampling sites in the Pearl River Estuary.

2.2 Collection and identification of phytoplankton

For chlorophyll a concentration, water samples were filtered through Whatman GF/F filters which were frozen at -20°C immediately. Chlorophyll a was extracted in 90% acetone and quantified by a spectrophotometric procedure.

For microscopic phytoplankton taxonomic analysis, 100 mL water was fixed with buffered formaldehyde to obtain a final concentration of 2.5%. Fixed samples were concentrated and then qualitative and quantitative

analyses by microscope. A minimum of 50 random visual fields and at least 400 cells in total were counted for each sample.

Biological diversity (H') and evenness (J) were calculated according to the following equation [14, 15]. Correlations between phytoplankton community structure and environmental factors were analyzed using the canonical correspondence analysis (CCA) [16].

2.3 DNA extraction and amplicon amplifications

Sea water for DNA sequencing was taken triplicately from two river outflow station, S4 and S7. For each water sample, 1L water was first filtered through 5- μ m microporous filter paper (Millipore, USA) and then extracted DNA by PowerWater DNA Isolation Kit (MOBIO Co., USA) with the recommended manuscript methods. The extracted DNA was amplified with primers 528f/706r targeting the V4 region of 18S rRNA gene, and then sequenced on Illumina Hiseq platform (Illumina Inc.) [17]. All amplicon sequencing was generated in Guangdong Magigene Biotechnology Co., Ltd., Guangzhou, China.

A total of 200,680 raw reads was generated. Paired-end reads were split, assembled and filtered to achieve 155,065 clean tags. Operational taxonomic units (OTUs) were clustered with a 97% similarity cut-off using the Uparse software (Uparse v7.0.100, <http://drive5.com/uparse/>) [18], which resulted in 852 OTUs in S4 and 815 OTUs in S7. A representative sequence for each OTUs was screened for annotation. Numbers of observed-species, Chao1, Shannon, Simpson, ACE, Goods-coverage were estimated using Qiime (Version 1.7.0).

3 Results and discussion

3.1 Environmental variables

The environmental variables of sea water were listed in Table 1. The water temperature was $19.6 \pm 0.2^\circ\text{C}$ and pH was neutral; seawater had high DO with the fluctuation from 7.57 to 8.16. Salinity of this region ranged from 32.24 to 32.45 that depended on the distance from the coastline. The similar trend was found in COD_{Mn} and S²⁻ which decreased from river flow to sea area. NH₄⁺, however, accumulated in the sea side. Basically, the concentrations of nutrients were not high in this sea region, with the average COD_{Mn}, BOD, NO₃⁻, NH₄⁺, PO₄³⁻, S²⁻ of 1.12 mg L⁻¹, 1.40 mg L⁻¹, 0.33 mg L⁻¹, 0.03 mg L⁻¹, 0.01 mg L⁻¹ and 0.45 mg L⁻¹, respectively. Chlorophyll a, an index linking to marine primary production, had the content of 0.97 $\mu\text{g L}^{-1}$ to 3.56 $\mu\text{g L}^{-1}$, which equaled to 87.62-273.33 mgC m⁻² d⁻¹ primary production in our study.

Table 1. Sea water properties. DO: dissolved oxygen; SS: suspended solids; COD: chemical oxygen demand; BOD: biological oxygen demand; Chl a: chlorophyll a.

Sampling Sites	Salinity	pH	DO (mg L ⁻¹)	SS (mg L ⁻¹)	COD _{Mn} (mg L ⁻¹)	BOD (mg L ⁻¹)	NO ₃ ⁻ (mg L ⁻¹)	NH ₄ ⁺ (mg L ⁻¹)	PO ₄ ³⁻ (mg L ⁻¹)	S ²⁻ (mg L ⁻¹)	Chl a (μg L ⁻¹)
S1	32.25	8.24	7.62	18.80	1.29	0.84	0.33	0.02	0.01	0.50	3.56
S2	32.44	8.26	7.72	9.60	1.03	1.61	0.36	0.04	0.02	0.40	3.03
S3	32.41	8.32	7.82	9.40	0.81	1.75	0.32	0.04	0.01	0.00	1.67
S4	32.19	8.15	8.16	10.40	1.34	2.18	0.30	0.01	0.01	0.70	3.56
S5	32.35	8.22	7.57	10.00	1.20	1.13	0.27	0.03	0.01	0.80	1.89
S6	32.42	8.31	7.72	11.20	0.79	1.32	0.28	0.04	0.01	0.20	2.43
S7	32.24	8.22	7.86	11.10	1.29	1.26	0.40	0.04	0.01	0.60	1.20
S8	32.44	8.29	7.77	15.40	1.20	1.11	0.34	0.05	0.02	0.40	0.97

3.2 Species composition by microscopic methods

In this sea region, there were a total of 39 genus and 62 phytoplankton species, which belonged to 6 phyla. Within 39 genus, *Bacillariophyta* (diatoms) had 27 genus (69.3%), which was more than *Dinoflagellata* (dinoflagellates) (8 genus, 20.5%), *Chlorophyta* (green algae) (1 genus), *Charophyta* (1 genus), *Cryptophyta* (1 genus) and *Heterokont* (1 genus). The dominant classes were *Bacillariophyceae* and *Coccolodiscophyceae* that affiliated with *Bacillariophyta* phylum. Besides, the dominant *Dinophyceae* was the only class within *Dinoflagellata* phylum. All of the phyla and classes were widely found in marine or estuary ecosystems [6, 7].

The genus number did not change among sampling sites, with 12-17 genus found at each location. However, the density of phytoplankton fluctuated among sampling sites. 3 sampling sites had a high phytoplankton density that higher than 300×10^3 cells m⁻³ (S4, S5 and S7). The highest phytoplankton density of 628.6×10^3 cells m⁻³ was found in S5, which was 8.4 times in S3 (75.2×10^3 cells m⁻³) and 8.0 times in S8 (78.3×10^3 cells m⁻³). The average phytoplankton density in this region was 266.3×10^3 cells/m³. Basically, the phytoplankton density was higher in the river flow side than the sea side, which was coincidence with the nutrient distribution (Table 1 and Fig. 2).

Dominant genus was identified as species dominance index $Y > 2.0\%$. There were 7 dominant genus in our research region. *Pseudo-nitzschia*, a dinoflagellate, had the highest species dominance of 18.7%. The highest genus density of 233.3×10^3 cells m⁻³ appeared in S5 and an average of 49.7×10^3 cells m⁻³ was found among all sampling locations. *Chaetoceros* (*Bacillariophyta*, *Coccolodiscophyceae*) was the second dominant genus ($Y = 11.0\%$, average density of 29.3×10^3 cells m⁻³). High number of *Chaetoceros* occurred in S5, S6 and S7. Other dominant genus included *Prorocentrum* (*Chlorophyta*), *Rhizosolenia* (*Dinoflagellata*) *Thalassionema* (*Dinoflagellata*), *Thalassiosira* (*Dinoflagellata*), and *Thalassiothrix* (*Heterokont*) (Fig. 2).

The community composition of marine phytoplankton can indicate the health level of sea region. 4 out of 7 dominant genus, *Pseudo-nitzschia*, *Chaetocero*, *Prorocentrum*, and *Thalassiosira*, are best recognized as common red tide genus. For instance, series studies have reported the density over 10^4 cells L⁻¹ of *Pseudo-nitzschia* can target red tide [19]. In our

region, besides S8, other 7 locations had the *Pseudo-nitzschia* density more than 10^4 cells L⁻¹, and S5 had the highest density over 10^6 cells L⁻¹. These results implied that our research region had potential red tide effects by *Pseudo-nitzschia*.

Diversity index Shannon-Wiener (H') and evenness index Pielou index (J) were used to evaluate the community diversity. H' ranged 3.36-4.10 and J ranged 0.82-0.94 suggested that the phytoplankton community had great diversity level and evenness level.

The CCA result of phytoplankton communities at the class level in response to environmental variables was shown in Fig. 3. The first two CCA axes (CCA1 and CCA2) explained 51.8% of the total variance in phytoplankton composition and 81.8% of the cumulative variance of the phytoplankton-environmental relationship. The influence of the environmental factors in decreasing order was $SS > PO_4^{3-} > \text{temperature} > NO_3^- > DO > BOD > NO_2^- > \text{other environmental variables}$. These results were coincidence with other studies that temperature and nutrient concentrations could affect the phytoplankton density and community in marine [7, 11].

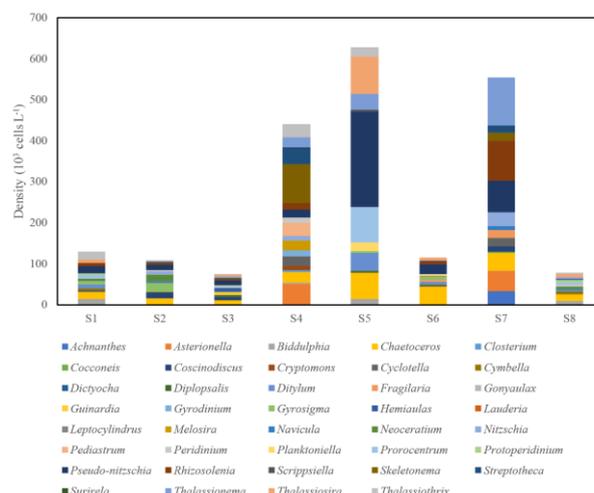


Fig. 2. The recognized phytoplankton and their biomass density at the genus level.

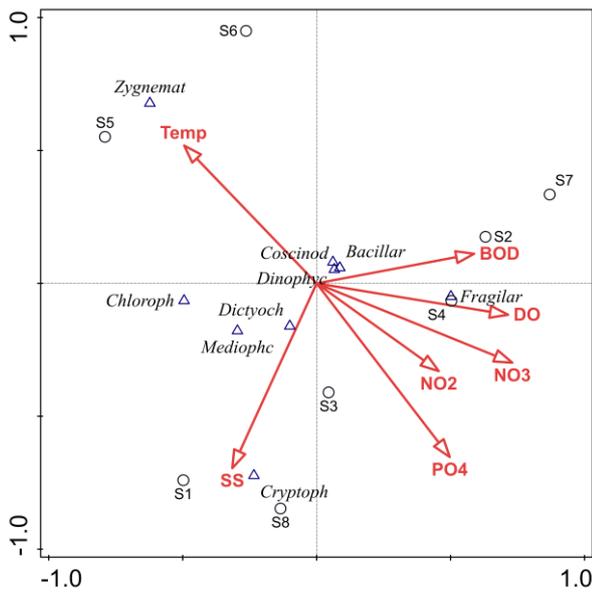


Fig. 3. Canonical correspondence analysis (CCA) demonstrated the relationships of sampling sites, environmental factors and phytoplankton community composition at the class level.

3.3 Community Structure by sequence results

S4 and S7 were selected for 18S rRNA sequencing. A total of 25 phyla and over 800 species was identified via sequencing method. *Chlorophyta* and *Bacillariophyta* had the highest abundance, which accounted for 17.6% and 10.9% in S08 as well as 11.5% and 13.6% in S17, respectively. It is worth mentioning that *Dinoflagellata* and *Cryptophyta* were not classified in the phylum level but identified in the class level. More phyla were identified than microscopic method, such as *Ascomycota*, *Absidiomycota*, *Annelida*, whereas they cannot be grouped into phytoplankton.

There were 75 identified classes in this study, and 16 dominant classes more than 1% were illustrated in Fig. 4. Within them, *Dinophyceae* had the highest number in S4 (26.5%), followed by *Chlorophyceae* (11.1%) and *Coscinodiscophyceae* (9.1%). The order was a little change in S7, as the most dominant classes in S7 were *Coscinodiscophyceae* (10.9%), *Chrysophyceae* (7.6%) and *Dinophyceae* (7.5%). Some classes had not been recognized by microscopic methods appeared in sequencing data, such as *Mamiellophyceae* and *Trebouxiophyceae*. These results were coincident with other research that sequencing data can over report the phytoplankton community [1].

Basically, the results of 18S rRNA sequencing were coincidence with the microscopic methods, but with more demonstration. *Bacillariophyta*, *Dinoflagellata* were still the main phyla by sequencing, while the importance of *Chlorophyta* and *Cryptophyta* were emphasized. The similar results were found in lower levels. For instance, *Mamiellophyceae* and *Trebouxiophyceae* were counted as dominant classes (>1%) by sequencing method, but they were not recognized by microscopy. Over 800 identified species appeared in sequencing data, which was much more than

62 identified species by microscopic methods. In terms of diversity index, the diversity index indicated in our water samples owned high community diversity (Chao1, Shannon, and Simpson were 934, 6.35, and 0.952, respectively). All of these results suggested that sequencing data can use as supplement results to support the ecological assessment of phytoplankton community.

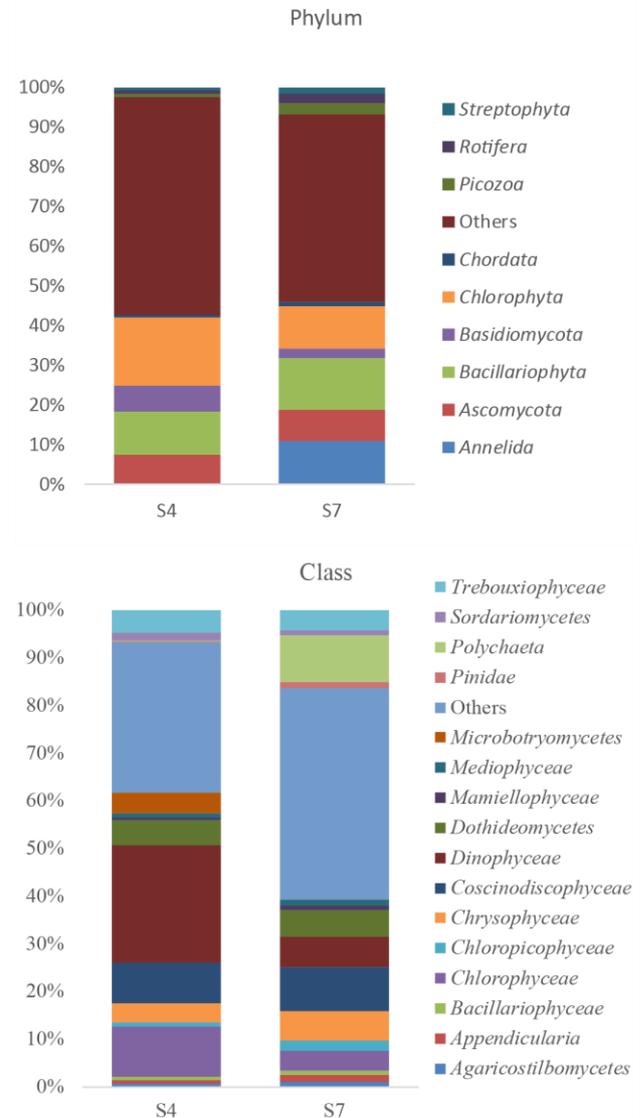


Fig. 4. Dominant (>1%) phylum (top) and class (down) via sequencing method

4 Conclusions

The ecological assessment by phytoplankton community was evaluated in a Pearl River Estuary region. Based on the microscopic methods, Shannon-Wiener and evenness index Pielou index indicated that phytoplankton community had high diversity in our research region, while the dominance genus suggested that our regions had potential red tide effects. The CCA results demonstrated that SS, PO_4^{3-} and temperature were three major factors that affected the phytoplankton community distribution and components. The DNA barcoding sequencing by 18S rRNA gene supported the main results of microscopic methods but with more taxonomic

data, which implied that 18S rRNA sequencing can be used as a supplemental method for ecological assessment.

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