Metabarcoding analysis of harmful algal species in Jiaozhou Bay

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ABSTRACT

Accurate detection of the composition and dynamics of harmful algal bloom (HAB) species is critical for studying the mechanisms of HAB formation and for developing means for predicting the occurrences of HABs. Jiaozhou Bay is an epitome of China’s coastal ecosystem and an ideal site for HAB research with the accumulation of decades of historical investigation records. Nevertheless, most of these earlier studies on phytoplankton communities applied primarily morphology-based approaches with limited resolution in phytoplankton species identification, especially for those with small-sized cells and for cryptic species. Through analyzing samples collected at 12 spatially isolated locations using metabarcoding methods, 89 phytoplankton species, including 34 Bacillariophyta, 25 Dinoflagellata, 7 Cryptophyta, 11 Chlorophyta, 8 Ochrophyta and 2 Haptophyta species were detected. Of those, 70 species had never been reported in Jiaozhou Bay in the previous expedition investigations, demonstrating the strength of the metabarcoding analysis approach. The distribution of many algal species demonstrated unique patterns, which were likely influenced by interactions among phytoplankton species or by predation by groups such as Ciliophora and Cercozoa, in addition to environmental factors such as temperature and nutritional conditions. Among these algal species, 28 were annotated as HAB species, among which 13 were reported for the very first time in Jiaozhou Bay including a mixotrophic dianoflagellate Heterocapsa rotundata and a chain-forming diatom Skeletonema marinoi, both ranked among the top 10 most abundant ASVs. The present study represents a first attempt to study HAB species and other phytoplankton species in Jiaozhou Bay using the metabarcoding approach, which revealed substantially more algal species in Jiaozhou Bay than previously identified and sets a solid foundation for further research on the mechanisms of HAB formation.

1. Introduction

Marine phytoplankton are diverse and influenced by environmental factors and, in return, their dynamic changes directly affect the structure and function of ecosystems (Silkin et al., 2013). Harmful algal blooms (HABs) are natural phenomena resulting from extreme successions of phytoplankton communities (McNamee et al., 2016). The frequency and scale of HABs, as well as the damage caused by HABs, have become increasingly serious in the past decades along the coastal areas of the world (Anderson et al., 2012) including China (Yan et al., 2002). Due to climate changes and intensified human activities, HABs have gained new characteristics including increased outbreak scale, longer duration, severer consequences and more significant global expansion (Yu and Chen, 2019). However, details associated with HAB processes and the mechanisms underlying HAB formation remain poorly understood. Accurate and quantitative knowledge of the HAB species composition and dynamics are critical for studying HAB outbreak mechanisms. The emergence of metabarcoding methods makes it possible to accurately study the composition, diversity and dynamic distribution of phytoplankton including HAB species (Lima-Mendez et al., 2015), which in turn greatly promotes the understanding of the mechanisms of HAB formation.

Jiaozhou Bay is an epitome of China’s offshore ecosystem and is significantly affected by climate changes and human activities (Liu

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et al., 2004). As a result, the nutrient level in Jiaozhou Bay has experienced substantial changes, leading to great changes in the phytoplankton species and structure (Shen, 2001; Shen et al., 2006). It is also an ideal research site for studying HAB species composition and dynamic changes (Guo et al., 2019). The first record of a HAB in Jiaozhou Bay was caused by *Mesodinium rubrum* in 1990 (Wu et al., 2005). Since the mid-1990s, occurrences of HABs in Jiaozhou Bay have greatly intensified in frequency, with wider outbreak areas and more HAB species (Wu et al., 2005). The common algae species, *M. rubrum, Skeletonema costatum, Eucampia zodiacus, Noctiluca scintillans, Coscinodiscus asteromphalus* and *Thalassiosira nordenskioldii*, were identified as causative of HABs in Jiaozhou Bay (Liu et al., 2004; Wu et al., 2005; Sun et al., 2011; Yuan et al., 2017).

Although long-term research activities carried out on phytoplankton in Jiaozhou Bay have accumulated extensive data, much of this data was obtained using morphology-based methods including light microscopy (Wu et al., 2005; Sun et al., 2011; Guo et al., 2019). Despite their ease of use, microscopy requires expert knowledge and experience in algae identification that involves extensive training. Additionally, water impurities may interfere with the sample analysis, especially for fragile algae, which are difficult to collect and to fix (Zarauz and Irigoien, 2008). Moreover, it is challenging to observe and identify species with small cell diameters (under 10 μm, especially those under 5 μm) (Xu et al., 2017) such as *Aureococcus anophagefferens* (Gobler et al., 2011), *Phaeocystis globosa* (Rousseau et al., 2013), *Emiliania huxleyi* (Hansen et al., 1996) and *Teleaulax acuta* (Xing et al., 2008). Furthermore, cryptic species, which are different species with remarkably similar morphologies, can form species complexes that are difficult to distinguish morphologically. This has been clearly demonstrated by the "*Alexandrium tamarense* species complex" which actually represents five unique species, including *A. fundyense, A. mediterraneum, A. tamarense, A. australiense* and *A. pacificum* (John et al., 2014).

Molecular biology techniques have been demonstrated to be effective in analyzing phytoplankton qualitatively and quantitatively and can be successfully applied alone or in combination with microscopy to study algal species. DNA metabarcoding has been successfully applied to study microbial communities and has been demonstrated to be effective in various applications (Streit and Schmitz, 2004). The metabarcoding technique has also been applied in many global marine environment projects including the Tara Oceans Expedition (Sunagawa et al., 2015) and the Ocean Sampling Day (Kopf et al., 2015). A metabarcoding analysis was also effective in revealing that protist species could establish complex interactions including predator-prey, host-symbiont, and host-parasite relationships (Gross, 2003; Katechakis, 2005; Montagnes et al., 2008), which together form complicated interaction networks that play a critical role in controlling HAB outbreaks (Berdjeb et al., 2018). For example, many toxic bloom-forming dinoflagellates can be infected by parasitic *Amoebophrya* species, which are themselves small species that are difficult to identify and track by light microscopy (Montagnes et al., 2008). Although Syndiniophyceae (e.g., the parasitic dinoflagellates *Amoebophrya* species) are not autotrophic phytoplankton species, because of their close relationship with bloom-forming dinoflagellates (Li et al., 2014; Li and Chen, 2017), they were also included in this study.

In this project, metabarcoding methods were applied to analyze the composition, relative abundance and distribution of algal species in Jiaozhou Bay, with a focus on the accurate identification of HAB species. Compared to results obtained in previous expedition studies using morphology-based methods, the metabarcoding analysis identified many species, most of which had small cell sizes, and found that some HAB species were previously incorrectly identified. Moreover, this research examined potential species-species interactions by examining their relative distribution patterns in Jiaozhou Bay. This study represents the first attempt to study the phytoplankton systematically in Jiaozhou Bay using a metabarcoding approach and lays a solid foundation for further research on HABs in Jiaozhou Bay and other ocean regions.

2. Materials and methods

2.1. Sample collection

Jiaozhou Bay is a semi-enclosed bay in Qingdao, Shandong Province, China, connecting with the Yellow Sea through a narrow opening, with developed port, shipping, coastal tourism and fishery facilities (Liu et al., 2004). This project was carried out during January 9–10 in 2019 on the RV *Chuangxian*, which was operated by The Jiaozhou Bay Marine Ecosystem Research Station. Details of the sampling stations and environmental factors are shown in Fig. 1, Table 1 and Table S1. The ranges of salinity, PO4³⁻, NH4⁺, NO3⁻, NO2⁻ and SiO3²⁻ concentrations at the sampling sites are 30.794–31.651, 0.133–1.03 μmol/L, 1.411–8.088 μmol/L, 4.726–18.693 μmol/L, 0.839–1.467 μmol/L and 1.955–7.569 μmol/L, respectively (The data were provided by Jiaozhou Bay National Marine Ecosystem Research Station, Institute of Oceanology, Chinese Academy of Sciences).

At each sampling site, 1 L surface seawater sample was collected and filtered using 200 μm mesh (Hebei Anping Wire Mesh Co., Ltd, China) to remove larger zooplankton and phytoplankton, followed by a second filtration using 10 μm polycarbonate membranes (Millipore, USA), before filtering using 0.2 μm polycarbonate membranes.
and 4.4 μL ddH₂O. The reaction was denatured at 95°C for 3 min. Then, 1 μL KOD FX Neo (Toyobo, Japan), 10 μL KOD FX Neo Buffer, 2 μL dNTP DNA (50 ng), 0.5 μL Vn forward primer, 0.5 μL Vn reverse primer, 0.4 μL V4F/R and overhang adapter was called Vn. The reaction was performed in a final volume of 20 μL containing 2 μL diluted template DNA. The reaction was denatured at 95°C for 3 min. Then, reactions were run for 20 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 40 s and a final extension at 72°C for 7 min. The PCR products were purified by VAHTSTM DNA Clean Beads (Vazyme, China). The second-round of PCR (called Solexa PCR) was carried out in 20 μL reactions containing 5 μL purified PCR products as templates, 2.5 μM adapter primers (including index) and 10 μL 2 × Q5 HF MM (NEB, USA). The Solexa PCR reactions were run as follows: initial denaturation at 98°C for 30 s, followed by 10 cycles of 10 s denaturation at 98°C, annealing at 65°C for 30 s, extension at 72°C for 30 s and a final 5 min extension at 72°C. The PCR products were run on a 1.8% agarose gel to check amplicon lengths, purified by the Cycle-Pure Kit (Omega, USA), and sequenced using the Illumina platform (Illumina, USA; Biomarker Technologies, China).

2.2. DNA extraction, PCR amplification and sequencing

DNA was extracted from the samples using the HP Plant DNA kit (Omega, USA) according to the manufacturer’s instructions with some modifications. Briefly, after the samples were taken out of the liquid nitrogen, 500 μl CSPL buffer (Omega, USA) was immediately added so that membranes were completely immersed. The membranes were cut with scissors 50 times then the samples were crushed by a cell crusher (MP, USA) for 5 s at a speed of 4 m/s after adding 20 mg glass beads. The remaining procedure followed the manufacturer’s instructions. Briefly, the library preparation protocol was based on two amplification steps (Miya et al., 2015). The first-round of PCR amplified the V4 region of the 18S rDNA sequence, using the V4F forward primer, CCAGCA(G/T)GA(A/G)C(C/T)GCGGTAATTCC, and the V4R reverse primer, ACTTTCGTTC(A/G)TCCGCT(G/T)(A/G)C(C/T)(A/G). The second-round of PCR (called Solexa PCR) was carried out in 20 μL reactions containing 5 μL purified PCR products as templates, 2.5 μM adapter primers (including index) and 10 μL 2 × Q5 HF MM (NEB, USA). The Solexa PCR reactions were run as follows: initial denaturation at 98°C for 30 s, followed by 10 cycles of 10 s denaturation at 98°C, annealing at 65°C for 30 s, extension at 72°C for 30 s and a final 5 min extension at 72°C. The PCR products were run on a 1.8% agarose gel to check amplicon lengths, purified by the Cycle-Pure Kit (Omega, USA), and sequenced using the Illumina platform (Illumina, USA; Biomarker Technologies, China).

2.3. Sequence and bioinformatics analysis

Sequencing results were deposited to National Center for Biotechnology Information (NCBI) under the project number PRJNA577777. The sequencing results were analyzed using the software package DADA2 in R (Callahan et al., 2016, 2017). We set the parameters as follows: maxEE = c (2, 2); minLen = 200; truncLen = c (220, 220); minBoot = 80; Min overlap = 12 bases. Taxonomical assignment was done in two steps. In step 1, an initial taxonomical assignment was performed using the SILVA database and BLAST annotation using the NCBI NT database. There were 326 ASVs that were annotated as phytoplankton plus Syndiniophyceae, of which 166 ASVs were annotated to species and 160 ASVs could not to be annotated to species level. After filtering the ASVs corresponding to two or more species, 119 ASVs remained. Finally, 89 potential phytoplankton species were identified.

<table>
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<th>Latitude (°N)</th>
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<th>Temperature (°C)</th>
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The sites with asterisk symbols (*) are located outside the Jiaozhou Bay; The sites with hash symbols (#) are located inside the Bay; The sites without symbol are close to the opening of the Bay.

(Millipore, USA). The membranes were then transferred into liquid nitrogen for brief storage. In this project, only samples on the 0.2 μm polycarbonate membranes were used for metabarcoding analysis. Thus, cells analyzed in this project have diameters roughly between 0.2 μm and 10 μm.

Fig. 2. A flowchart describing the ASV annotation and processing procedure. 1276 ASVs were obtained from analyzing 1,255,230 sequences using DADA2. After applying the filtering threshold for details see Material and methods, 730 ASVs were retained. The next step was taxonomical assignment with DADA2 using the SILVA database and BLAST annotation using the NCBI NT database. There were 326 ASVs that were annotated as phytoplankton plus Syndiniophyceae, of which 166 ASVs were annotated to species and 160 ASVs could not to be annotated to species level. After filtering the ASVs corresponding to two or more species, 119 ASVs remained. Finally, 89 potential phytoplankton species were identified.
belong to Ochrophyta; Coccolithophyceae belongs to Haptophyta; Katablepharidophyceae belongs to Katablepharidophyta.

In this study, we filtered ASVs for those that made up at least 0.01% of the total reads of at least one of the 12 samples. Altogether, 730 ASVs were found to satisfy this criterion in the 12 samples collected in Jiaozhou Bay during the January 2019 expedition. The alpha diversity of algae species (and Syndiniophyceae), including richness (ASV richness), Chao1 (Chao, 1984), ACE (Chao and Yang, 1993), Pielou index (Pielou, 1969), Shannon diversity (Shannon and Weaver, 1949), Simpson diversity (Gini-Simpson; Simpson, 1949), and Good’s coverage (Good, 1953), were analyzed using the R package vegan (Dixon, 2003) after rarefying each sample to the number of reads in the sample with the fewest reads. We tested for differences in the alpha indices between sites inside and outside Jiaozhou Bay using unpaired t-tests with P ≤ 0.05 as the significance threshold. In addition to analyzing richness indices at the ASV level, we also analyzed the richness indices of these samples at the phylum and the class level.

Correlation analysis of the ASVs was carried out using the Pearson correlation coefficient in the R package psych (Revelle, 2018). Phylogenetic trees were generated in MEGA (version 7.0.26; Kumar et al., 2016) using the Maximum Likelihood (ML) method and the Tamura-Nei genetic distance model (Tamura and Nei, 1993) with 1000 bootstrap replicates. The bubble chart was drawn with the R package ggplot2 (Wickham, 2009), the network picture was generated with Gephi (version 0.9.2; Bastian et al., 2009), and the histogram figures was drawn with Origin (version 8.5.1; OriginLab, USA).

3. Results
3.1. Phytoplankton community composition and relative abundance in Jiaozhou Bay

We obtained 1276 unique ASVs from analyzing 1,255,230 sequences using DADA2 (Callahan et al., 2016). Among the 730 ASVs that passed the filtering threshold (for details see Materials and Methods), 326 belonged to eukaryotic algal species (plus Syndiniophyceae; Fig. 2; Figure S1-S3), 111 were Ciliophora, 161 were Cercozoa and 41 were Opisthokonta.

The alpha diversity indices of phytoplankton at the twelve sampling sites in Jiaozhou Bay are shown in Table 2. According to the Good’s coverage index, the sequencing depth is adequate for this study. The Richness, Chao1, ACE, Pielou, Shannon and Simpson indices from different ASVs that were not annotated to a specific algal species level, 34 were annotated as Syndiniophyceae, including 16 candidate parasitic Amoebophrya species.

3.2. Differential distribution patterns of phytoplankton species in Jiaozhou Bay

This analysis revealed that different phytoplankton species have unique geographical distribution patterns in Jiaozhou Bay (Figs. 4, 6 and 7).

Among annotated Bacillariophyceae species, different species had unique patterns of distribution. The distribution of the highly abundant S. marinoi at different sites in Jiaozhou Bay was relatively uniform (Fig. 4). In contrast, many ASVs annotated to Bacillariophyceae showed differential distribution patterns. For example, ASV84 (T. aestivalis/T. pacifica), ASV129 (Nitzschia paleaformis) and ASV448 (Paribellus de-legen) were more abundant inside the Bay, while ASV50 (T. curviser-iata), ASV72 (T. prochkiniae) and ASV104 (T. concaviuscula) were more

### Table 2

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<th>Site</th>
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<th>ACE</th>
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S. Liu, et al. Harmful Algae 92 (2020) 101772


Fig. 3. ASV richness and relative abundance (A and B for the level of phylum and superclass; C and D for the level of class) of eukaryotic phytoplankton (plus Syndiniophyceae) in Jiaozhou Bay.

Fig. 4. The distribution of identified Bacillariophyta in Jiaozhou Bay. The phylogenetic tree was constructed using the Maximum Likelihood (ML) method and the Tamura-Nei genetic distance model with 1000 bootstrap samplings using MEGA.
abundant outside of the Bay. Most of Dinoflagellata ASVs showed higher abundance outside of the Bay (Fig. 6), especially ASV6 (Heterocapsa rotundata), ASV17 (N. marinoi) and ASV18 (K. glaucum).

The distribution patterns of Cryptophyta, Chlorophyta, Ochrophyta and Haptophyta in Jiaozhou Bay are more complex (Fig. 7). While for Bacillariophyceae and Dinoflagellata species, closely-related species showed rather different distribution patterns, with some species more abundant inside the Bay, while others were more abundant outside of the Bay. Some Cryptophyta and Chlorophyta ASVs were exceptionally more abundant in Jiaozhou Bay, including ASV4 (T. amphioxeia/T. acuta), ASV10 (Bathycoccus prasinus), ASV12 (Micromonas pusilla) and ASV19 (T. amphioxeia; Fig. 7).

3.3. A putative community network suggesting potential species-species interactions

To explore potential species-species interaction relationships among phytoplankton species in Jiaozhou Bay, the correlation between the 730 ASVs detected in the Bay was examined using their distribution patterns. As illustrated in Fig. 8, some phytoplankton groups had positive correlations (shown in pink), while others had negative (green) correlations. Overall, there were 31 significantly negative correlations and 50 significantly positive correlations between groups of phytoplankton. For example, the distribution of Dinophyceae and Syndiniophyceae had a significant positive correlation ($r = 0.99$, Fig. 8B).

Notably, the distribution of Ciliophora, which showed very high abundance (reflected by the sizes of the circles), was negatively correlated with many algal groups (Dinophyceae, Syndiniophyceae, Coscinodiscophyceae, Katablepharidophyceae, Pyramimonadophyceae and Coccolithophyceae; Fig. 8A). Fig. 8B shows the spatial distribution of Ciliophora and other phytoplankton groups. The distribution of Cercozoa was positively correlated with Coscinodiscophyceae, Coccolithophyceae, Katablepharidophyceae, Dinophyceae (Fig. 8B) and Syndiniophyceae (Fig. 8B), but negatively correlated with Mamiellaphyceae and Chrysophyceae.

Because the above analysis of correlation at higher taxonomy levels (phytoplankton at the class level, Ciliophora, Cercozoa, Telonemia and Bigya at the phylum level, Opisthokonta at the kingdom level) might not reveal detailed interactions at the species-level, the correlations at the ASV-level were further analyzed (Fig. 9). 79,328 significant correlations between 727 ASVs were identified, among which 86.93 % were positively correlated (Fig. 9A), demonstrating that many species had similar distribution patterns. Taking typical and abundant species as an example (Fig. 9B), the distribution of ASV5 (Bacillariophyta) and ASV22 (Cercozoa) had a significant positive correlation ($r = 0.80$). ASV2 (Ciliophora), ASV4 (Cryptophyta) and ASV12 (Chlorophyta) had significant positive pairwise correlations among them (ASV2-4: $r = 0.87$; ASV2-12: $r = 0.72$; ASV4-12: $r = 0.87$). However, there was a significant negative correlation between ASV2 and ASV6 (Dinoflagellata, $r = 0.75$), and between ASV4 and ASV6 ($r = 0.76$).

3.4. Identification of HAB species in Jiaozhou Bay

This analysis identified 28 potential algal species that have been previously reported to cause HABs (Table S2), including Dinoflagellata (8 species), Bacillariophyta (17 species), Chlorophyta (1 species), Ochrophyta (1 species) and Haptophyta (1 species). Some of these HAB species, such as S. marinoi (ASV5), H. rotundata (ASV6), N. scintillans (ASV17) and K. glaucum (ASV18), had high abundance during the sampling time.

Among these 28 HAB species, 13 were never reported in previous voyage studies to be present in Jiaozhou Bay, including S. marinoi, H. rotundata, K. glaucum, K. veneficum, T. curviserata, Polyrhizos kofoidii, T. mala, P. globosa, K. bidigirata, V. globosus, Gymnodinium aureolum, Ditylum brightwellii and Nephroselms pyriformis.

4. Discussion

In this project, we conducted a metabarcoding analysis of phytoplankton in Jiaozhou Bay, a well-known and well-studied embayment in Qingdao, Shandong Province, located in Northern China, targeting the V4 region of the 18S rDNA. In addition to identifying a large number of previously unreported phytoplankton species in Jiaozhou Bay, this study also revealed interesting distribution patterns of various phytoplankton, suggesting the existence of important and complex species-species interactions in Jiaozhou Bay.
4.1. Neglected phytoplankton species

Through metabarcoding analysis of samples, this study demonstrates the underestimated richness of algal species in Jiaozhou Bay. Of the 326 identified phytoplankton ASVs, 166 ASVs were annotated to 89 potential phytoplankton species (Fig. 2; Table S2). The actual number of phytoplankton species should be substantially higher than 89 for the following reasons. First, all the samples analyzed in this study were collected in a single sampling time point, which was January of 2019 (winter). Many phytoplankton species that are dominant in other months might be too rare in January to be detected. Second, this study only focused on small algal species (<10 μm) with larger species excluded. Larger algal species, including many dinoflagellates, were not sampled. Third, during the processing and filtering of ASVs, only ASVs that made up at least 0.01 % of the reads in at least one sample were considered and thus some rarer species were not included. Fourth, we annotated species based on the PID between the V4 segment of the 18S rDNA sequences of sampled species to their corresponding sequences in the reference database. The low PID (lower than 100 %) probably suggests that many more species could be better identified with improved databases. Fifth, 18S rDNA sequences are so conserved (Baverstock and Johnson, 1990) that closely related species may share identical V4 sequences and thus one ASV may represent more than one species. Taken together, the number of species annotated in this study was most likely an underestimate of the number of species in Jiaozhou Bay.

The list of phytoplankton species identified in this study showed dramatic difference from the species reported previously for Jiaozhou Bay voyage investigations (Sun et al., 2011; Shi et al., 2015; Guo et al., 2019). One remarkable difference between this study and previous studies is that many additional phytoplankton species were identified, including those in Cryptophyta, Chlorophyta, and Ochrophyta. In contrast, previous studies essentially only identified Bacillariophyta (including Bacillariophyceae, Coscinodiscophyceae and Mediophyceae) and Dinoflagellate (including Dinophyceae and Noctilucopeae) species, with Cryptophyceae, Mamiellophyceae and Chrysophyceae species largely undetected. The most likely explanation for this surprising difference is that the vast majority of the species missed by previous studies are relatively small in size (<10 μm) (Clarke and Pennick, 1981; Sekiguchi et al., 2002; Zingone et al., 2002; Lane and Archibald, 2008; Rousseau et al., 2013; Baren et al., 2016), mostly belonging to Cryptophyta, Chlorophyta, Ochrophyta and Haptophyta, which are difficult to detect and identify using light microscopy. For example, *Bathycoccus* and *Micromonas* (Mamiellophyceae) are 1.5–2.5 μm (Eikrem and Throndsen, 1990) and 1.0–3.0 μm (Tomas, 1997), respectively. The cell sizes of *Teleaulax* (Cryptophyceae) (Hill, 1991) and *Pyramimonas* (Pyramimonadales) (Daugbjerg, 2000) are in general rather small. Results from this metabarcoding analysis are generally consistent with results obtained from a recent flow cytometer study in Jiaozhou Bay, which identified chain-forming eukaryotes (including Dinoflagellata and Bacillariophyta, 7.06 ± 3.40 μm) and picoeukaryotes (1.93 ± 0.39 μm) as the most abundant species and Cryptophyta as another significant group (Chen et al., 2017b). Thus, the metabarcoding analysis is more effective at detecting and differentiating phytoplankton species, especially those with small cell sizes (Massana, 2011).

In addition to small cell sizes, many other factors could limit the resolution power of morphology-based methods in identifying species in Jiaozhou Bay. Among these groups of species, Syndiniophyceae, which include parasitic dinoflagellates, have been recently isolated and identified in coastal waters in the Atlantic Ocean, Mediterranean Sea...
and Red Sea (Guillou et al., 2008; Pearman et al., 2017) and in China (Li and Chen, 2017; Chen et al., 2019). The Syndiniophyceae strains are not only generally small, but are endoparasitic and thus cannot be easily identified using light microscopy (Gran-Stadniczenko et al., 2019). Furthermore, cryptic species with similar morphologies may not be distinguishable using light microscopy (De Luca et al., 2019).

In this study, the phytoplankton analysis was limited to small phytoplankton cells (0.2−10 μm). In future research, it is planned to explore the composition of phytoplankton species with cell sizes that span a full-size range and to use full-length 18S rDNA sequences to improve the resolution of phytoplankton species annotation. To help enrich molecular marker databases and validate metabarcoding results, the phytoplankton cells will also be isolated, and molecular markers such as the full-length 18S rDNA sequence will be sequenced and analyzed.

4.2. Potential algal species interaction networks

The unique distribution patterns of various phytoplankton species in Jiaozhou Bay (Fig. 4, 6 and 7) suggests that different species may be influenced or dictated by species-species interaction relationships such as predator-prey, host-symbiont, and host-parasite relationships (Gross, 2003; Katechakis, 2005; Berdjeb et al., 2018; Montagnes et al., 2008). This is consistent with the idea that phytoplankton communities are controlled by biotic interactions in addition to environmental factors such as nutrients and temperature, which control the growth rates of protists (Goldman, 1977; Rose and Caron, 2007) and modulate the seasonal changes of algal community composition (Langer et al., 2017). Our results are consistent with previous research, which shows that despite the obvious effect of environmental conditions, a complex network interaction is also an important factor controlling microbial communities (Fuhrman et al., 2015).

An important group of microzooplankton, Ciliophora, can feed on pico- and nano-phytoplankton, which plays an indispensable role in the material circulation and energy flow of the marine plankton ecosystem (Pierce and Turner, 1992). The same is true for Cercozoa (Ward et al., 2003). In this study, the distribution patterns of Ciliophora and Cercozoa were significantly correlated with that of many phytoplankton species in Jiaozhou Bay (Fig. 4, 6 and 7).
species, suggesting that the growth and composition of phytoplankton species may be tightly controlled by zooplankton through predator-prey relationships.

Interspecific competition between phytoplankton, such as allelopathy, parasitism or different trophic modes (autotrophy, heterotrophy and mixotrophy), is common, but their interactions can also be synergistic or promoting (Gross, 2003). In this study, we identified the presence of 34 potential Syndiniophyceae species (corresponding to 34 ASVs), among which 16 were identified to be *Amoebophrya* sp.. This analysis is consistent with recent studies that isolated *Akashiwo sanguinea* infected with *Amoebophrya* sp. from Jiaozhou Bay (Chen et al., 2017a). The positive correlation between Syndiniophyceae and Dinophyceae indicates that Syndiniophyceae may have a significant influence on the distribution and abundance of Dinophyceae in Jiaozhou Bay (Montagnes et al., 2008). Additionally, many phytoplankton have been shown to produce allelopathic substances that inhibit other species growth by changing physiological functions, inhibiting enzyme activity, and destroying cell structure (Legrand et al., 2003). Thus, there exists complex relationships between different planktonic species. To explore species-species interactions with improved precision, additional samples (such as time-series samples) are needed.

**4.3. The HAB species in Jiaozhou Bay**

In this project, 28 potential HAB species were identified, among which five species (*T. nordenskioeldii*, *E. zodiacus*, *Rhizosolenia delicatula*, *Odonella regia* and *N. scintillans*) have been found to cause HABs previously in Jiaozhou Bay (Wu et al., 2005; Yuan et al., 2017). The co-existence of these HAB species suggests that there is a high risk of HAB outbreaks in Jiaozhou Bay with suitable ecological conditions.

Among the HAB species, 8 Dinoflagellata were detected, including the mixotrophic and cosmopolitan species *H. rotundata* (ASV6) (Millette et al., 2016), which was very abundant in Jiaozhou Bay in January 2019. In recent years, *Amoebophrya* sp. have been found to infect and parasitize harmful dinoflagellates (Montagnes et al., 2008). These parasitic species, which are themselves dinoflagellates, can eventually lyse and break up the host cells to block the formation of HABs (Montagnes et al., 2008). In this study, four hosts of *Amoebophrya* sp. were detected: *H. rotundata* (ASV6) (Chambouvet et al., 2008), *K. veneficum* (ASV47) (Bai et al., 2007), *P. kofoidii* (ASV68) (Park et al., 2004) and *A. sanguinea* (ASV88) (Mazzillo et al., 2011). Thus, even though *H. rotundata* showed high abundance in January in Jiaozhou Bay, its abundance may have been suppressed by the presence of parasites.

*Skeletonema* sp. is the dominant species in the coastal waters of China and is distributed globally with wide adaptability to different temperature and salinity. It is reported that both *S. marinoi* (ASV5) and *S. costatum* have caused severe HABs previously (Gu et al., 2012). Sarno et al. (2005) demonstrated that *S. costatum* comprises a complex of cryptic species which includes *S. marinoi*. Further studies revealed that...
the cryptic species \textit{S. marinoi} and \textit{S. dohrnii} (Godhe et al., 2006), \textit{S. menzelii} and \textit{S. tropicum} (Kooistra et al., 2008) cannot be adequately distinguished using light microscopy alone. Therefore, molecular biology methods such as metabarcoding are needed to help resolve them. Through combined metabarcoding, molecular and morphological approaches, we confirmed that the most abundant diatom species in Jiaozhou Bay was \textit{S. marinoi}, rather than \textit{S. costatum} as reported in previous studies.

\textit{P. globosa} (ASV142) is a cosmopolitan algal species in the coastal waters of China that has been implicated in HABs in the Bohai Sea (Lin et al., 2008), Zhujiang estuary (Wang et al., 2010), Beibu Gulf (Hu et al., 2019) and Maowei Sea (Liu et al., 2015). It has a complex life cycle that includes forms of solitary cells and gelatinous colonies. Its cell sizes are small (3–10 μm) during the solitary stage before bloom formation (Peperzak et al., 2000; Rousseau et al., 2013), making it difficult to be accurately detected using microscopy. Metabarcoding analysis successfully resolved this problem. This finding may guide future research to isolate \textit{P. globosa} strains in Jiaozhou Bay to support further investigation.

In summary, through applying metabarcoding methods, this study substantially enriched our understanding of the phytoplankton composition of Jiaozhou Bay. Metabarcoding detected many phytoplankton species, especially those with small cell sizes, that have been neglected in previous morphology-based studies and corrected the misclassification of some species. This study demonstrates that the metabarcoding approach is a strong method for detecting and monitoring HAB species in Jiaozhou Bay and other coastal regions.

With the improvement in metabarcoding analysis, including the enrichment of databases with molecular markers representing more phytoplankton species, molecular marker sequence variants from the same species, and molecular marker copy number information, together with the application of third-generation DNA sequencing technologies for sequencing full-length molecular markers, we expect to see increased resolution of phytoplankton species annotation in the near future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hal.2020.101772.
References


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