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Phytoplankton community structure and diversity in the indoor industrial aquaculture system for *Litopenaeus vannamei* revealed by high-throughput sequencing and morphological identification

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Abstract

The explosive multiplication of phytoplankton caused by water eutrophication often occur in the intensive shrimp aquaculture. To comprehensively assess the diversity and community structure of phytoplankton in the waters of typical indoor industrial aquaculture system for *Litopenaeus vannamei*, a combination of high-throughput sequencing and morphological identification methods were used in the present study. A total of 41 genera belong to nine phyla were detected by both methods. Chlorophyta, Cyanophyta and Bacillariophyta were found to be three dominant phyla. The high-throughput sequencing revealed that green algae and cyanobacteria were the most dominant phytoplankton; however, diatoms were the first dominant phytoplankton by using the morphological identification. At the genus level, *Picochlorum* and *Synechococcus* were dominant, accounting for 20.94%–97.19% and 0.01%–52.81% of total phytoplankton, revealed by the high-throughput sequencing. Therefore, more attention should be paid to their ecological impacts on the surrounding sea areas or potential toxicity to shrimp. *Cyclotella* was the most dominant genus revealed by the morphological identification. High-throughput sequencing revealed a high diversity and small-sized phytoplankton which were undetected by microscopy. Both methods provide similar information on the environmental drivers of phytoplankton community. NO_3^-, NH_4^+, DIP, DSi, DON and DOP concentrations were the main factors influencing the phytoplankton community structure and diversity.

KEYWORDS
aquaculture, high-throughput sequencing, morphological identification, nutrients, phytoplankton

1 | INTRODUCTION

Over the years, shrimp aquaculture has evolved into an intensive industrial activity and massive corporate investments in the sector have led to considerable increase in production volumes and profits. In 2017, the total production of shrimp was 1,345,154 tons, accounting for 82.46% of crustacean yields (CFSY, 2018). *Litopenaeus vannamei* was the major species of shrimp aquaculture, accounting for 80.35% of shrimp yields in 2017 (CFSY, 2018). However, intensive aquaculture systems with high stocking densities face water quality

problems resulting from residual feeds and faces, which in turn has an effect on the shrimps predisposing them to disease (Liu & Chen, 2004; Tseng & Chen, 2004). In addition, the aquaculture wastewater was directly discharged into the sewers or the surrounding sea areas, which not only contaminates the environment, but also damages the coastal ecological balance (Grosholz et al., 2015).

Phytoplankton play very important roles in an aquaculture ecosystem. It helps to improve the recirculation of nutrients and to maintain water quality (Harrison, Perry, & Li, 2005), and another role of phytoplankton is to serve as a direct or indirect (through enrichment of zooplankton) food source for the cultured organisms (Martins, Odebrecht, Jensen, D'Oca, & Wasielesky, 2016; Pulz & Gross, 2004; Webb & Chu, 1983). Some phytoplankton groups, such as diatoms and green algae, are desired for their high nutritional value and contribution to water quality (Brito et al., 2016; Godoy, Odebrecht, Ballester, Martins, & Wasielesky, 2012; Guedes & Malcata, 2012; Raja, Hemaiswarya, Kumar, Sridhar, & Rengasamy, 2008; Roy & Pal, 2015). Other groups, such as cyanobacteria and dinoflagellates, are undesired because of their low nutritional value and capacity of producing toxins (Campa-Cordova, Nunez-Vazquez, Luna-Gonzalez, Romero-Geraldo, & Ascencio, 2009; Paerl & Tucker, 1995; Perez-Linares, Ochoa, & Gago-Martinez, 2008; Pérez-Morales, Band-Schmidt, & Martínez-Díaz, 2017; Sinden & Sinang, 2016). So, monitoring and assessing the phytoplankton community structure and diversity is key for managing aquaculture systems.

Previous investigations on phytoplankton composition and diversity in the aquatic water mainly used traditional optical microscopy (Chen et al., 2016; Soares et al., 2011). The microscopic method mainly relies on identification of the cell morphology, which makes it difficult to recognize and distinguish similar taxa and small cells (Manoylov, 2014). In addition, morphological features are not always stable as they can change in response to environmental factors (Albrecht, Pröschold, & Schumann, 2017), which increases the difficulty of microscopic identification. Therefore, microscopy is potentially vulnerable to subjective judgments and requires advanced taxonomic skills and expertise. With the development of DNA sequencing technology (Sundermann, Kushnir, & Schulz, 2010), the high-throughput sequencing approach has already been successfully applied for the assessment of phytoplankton community structure and diversity (Mäki, Salmi, Mikkonen, Kremp, & Tiirola, 2017; Oliveira et al., 2018; Penna, Casabianca, Guerra, Vernesi, & Scardi, 2017). High-throughput sequencing surveys have advantages over traditional morphological methods due to their cost and time savings, higher resolution and lower detection threshold (Reuter, Spacek, & Snyder, 2015). This approach has ability to differentiate taxa and detect low abundance and small size cells, and even discover the new species (de Vargas et al., 2015; Marcelino & Verbruggen, 2016; Oliveira et al., 2018). However, the high-throughput sequencing approach has some shortcomings. On the one hand, PCR amplification bias can result in a large amount of missing data or an overestimation of diversity and may lead to a misinterpretation of species composition (Kunin, Engelbrektson, Ochman, & Hugenholtz, 2010; Quince et al., 2009; Shokralla, Spall, Gibson, & Hajinanaei, 2012). On the other hand, gene copy number variation may affect interpretations based on operational taxonomic unit (OTU) relative abundance (Huse, Welch, Morrison, & Sogin, 2010; Quince et al., 2009; Reeder & Knight, 2010). Therefore, combined use of morphology and phylogenetically informative genetic markers will be useful to get a comprehensive understanding of plankton communities (Bazin et al., 2014; Lejzerowicz et al., 2015; Liu, Deng, et al., 2017; Monchy et al., 2012).

A number of marker genes have been employed to describe the structure and diversity of microbial populations. 16S rRNA is commonly used for bacterial identification (Liu et al., 2018; Xu, Lu, et al., 2017). The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA is employed in studies of fungi (Liu, Song, Chen, & Li, 2017; Shi et al., 2017). Amplicons of 18S rDNA were used to investigate eukaryotes diversity (Yi et al., 2017). Several studies have analyzed phytoplankton community and diversity by examining 18S rDNA amplicons (Gerikas, Lopes, Marie, Pereira, & Vaulot, 2018; Tragin, Zingone, & Vaulot, 2018; Xu, Yu, et al., 2017), but the cyanobacteria could not be detected simultaneously. Fortunately, the universal plastid amplicon (UPA, a fragment of the plastid 23S rRNA gene) is suitable for environmental sequencing with the ability to identify both eukaryotic algae and cyanobacteria (Li et al., 2016; Sherwood & Presting, 2007).

In this study, we conducted environmental sampling to characterize the phytoplankton community structure and diversity using traditional morphological identification and high-throughput sequencing, and measured the water quality parameters of the industrial aquaculture waters. Our primary objectives were to (a) compare high-throughput sequencing data on phytoplankton community with a traditional morphological identification performed on the same samples; (b) investigate the dominant species and their potential ecological roles; and (c) explore the effects of water quality parameters on the phytoplankton community.

2 | METHODS

2.1 | Field survey

Field observations and samples collections were conducted at Haiyang Yellow Sea Aquatic Products, located in Shandong Province in eastern China (Figure S1a). Thirty indoor cement tanks (S = 80 m^2, h = 1.0 m) were used for *L. vannamei* culture in three plants (Figure S1b). The aquaculture water was the mixture of seawater and underground brine, whose salinity was about 30 g/L.

Field observations were conducted at six cement tanks (3–1, 3–10, 4–5, 4–6, 5–3, and 5–7) (Figure S1b) on September 11, 2018. In cement tank 3–1, 4–6 and 5–7, the water colour was brown; however, the water colour was green in cement tank 3–10, 4–5 and 5–3. Water temperature, salinity, dissolved oxygen (DO) and pH in each tank were measured using an YSI Model Handheld Instrument (YSI Incorporated). Five litres of water samples were first prefiltered through a sieve with a pore size of 200 μm to remove large suspended particles, microzooplankton, and other large cells. One

litre of prefiltered water added 5 ml of Lugol's solution was used for algae identification and counting. 500 ml of prefiltered water was filtered through 0.22 μm Millipore membrane. The 0.22 μm Millipore membranes containing the algae were flash frozen and stored at −80°C until DNA analysis. 500 ml of prefiltered water was filtered through 0.45 μm membranes. A portion of the filtered water was stored at −20°C without any treatment to measure the ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), phosphate (dissolved inorganic phosphorus, DIP), dissolved total nitrogen (DTN) and dissolved total phosphorus (DTP) contents, and an additional portion was stored at room temperature with a drop of chloroform to analyze the silicate (dissolved silica, DSi) content.

2.2 | Physico-chemical parameter analysis

Concentrations of NH_4^+, NO_3^-, NO_2^-, DIP, and DSi were measured using a QuAAtro nutrient auto analyzer (Seal Analytical; Parsons, Maita, & Lalli, 1984). DTN and DTP were determined with alkaline persulfate digestion (SAC, 2007) and measured using a QuAAtro nutrient auto analyzer (Seal Analytical). The dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) values were calculated as the difference between DTN and DIN and between DTP and DIP, respectively.

2.3 | Morphological identification

The water samples for phytoplankton analysis were preserved with 0.5% Lugol's solution. Each sample was concentrated to 50 ml, and then stored in darkness at 4°C until analysis. Phytoplankton species were identified and cell numbers were counted using a phytoplankton enumeration chamber under an inverted microscope (Olympus CKX41; Olympus Corporation).

2.4 | DNA extraction and sequencing

The total genomic DNA was extracted from all samples using the FastDNA spin kit for soil (MP Biomedicals), following the manufacturer's instructions. DNA quality and concentration were measured by gel electrophoresis and a Nanodrop spectrophotometer (NanoDrop Technologies), respectively.

PCR was performed with the 23S rDNA gene primer pair p23SrV_f1 (5′-GGA CAG AAA GAC CCT ATG AA-3′) and p23SrV_r1 (5′-TCA GCC TGT TAT CCC TAG AG-3′) (Sherwood & Presting, 2007). The 20 μl PCR reaction mixture consisted of 4 μl of 5× FastPfu Buffer, 2 μl of 2.5mM dNTPs, 0.8 μl of Forward primer (5 μM), 0.8 μl of Reverse primer (5 μM), 0.4 μl of FastPfu Polymerase, 0.2 μl of BSA and 10 ng of template DNA. PCR cycling was performed under the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 10 min. The PCR products were extracted from a 2% agarose gel and further purified using the AxyPrepDNA Gel Extraction Kit (Axygen Biosciences) referring to the manufacturer's instruction, and quantified using QuantiFluor™-ST (Promega). Purified amplicons were pooled in equimolar and paired-end reads (PE300) on an Illumina MiSeq platform (Illumina) referring to the standard protocols by Majorbio Bio-Pharm Technology.

2.5 | Bioinformatics

Sequences from the Illumina MiSeq platform were processed using the QIIME (version 1.70) software package. Raw fastq files were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the following standards: (a) the reads were truncated at any site receiving an average quality value <20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50 bp; (b) exact barcode matching, two nucleotide mismatch in primer matching, reads containing ambiguous characters were removed; (c) sequences with an overlap longer than 10 bp were merged according to their overlap sequence.

Operational taxonomic units (OTUs) were clustered with 99% similarity cutoff using UPARSE (version 7.1, http://drive5.com/uparse). The chimeric sequences were identified and removed using UCHIME. The taxonomic assignment was determined for the representative sequence of each OTU using the Basic Local Alignment Search Tool (BLAST) in the NCBI database (http://www.ncbi.nlm.nih.gov). Following the exclusion of bacteria (all non-cyanobacteria) and unclassified sequences, phytoplankton sequences were selected for analysis of community structure and diversity based on the taxonomic information. To perform statistical analyses and compare the data across all samples, the samples were resampled randomly to the same size because an uneven sequencing depth could result in inflated microbial diversity.

2.6 | Phytoplankton community analysis

Phytoplankton community diversity was evaluated using the Shannon-Wiener diversity index (*H*). The calculation formula is $H = -\sum_{i=1}^{S} P_i \ln P_i$, where *S* is the total number of OTUs or species, and P_i is the relative abundance of OTU*i* or species *i*. The β diversity used in the comparison of phytoplankton community structure between samples was estimated by non-metric multidimensional scaling (NMDS) analysis. Correlations between phytoplankton community and water quality parameters were identified by linear model-based redundancy analysis (RDA) using CANOCO for Windows (version 4.5). The relative abundance of the phytoplankton at the genus level were used as the species input, respectively, and the water quality parameters entered into RDA were normalized by logarithmic transformation. An automatic forward selection that was found to be significant in optimal tests involving Monte Carlo permutations was used to build optimal models of the phytoplankton-environment relationship.

2.7 | Data accessibility

The paired-end Illumina sequencing data from this study were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number SRP174205.

3 | RESULTS

3.1 | Water quality parameters

The water quality parameters in the six tanks are summarized in Table 1. The average values of the temperature, salinity and pH were 28.35°C, 30.45 g/L and 7.98, respectively. The DO concentrations varied from 5.81 to 6.95 mg/L. The NH_4^+, NO_3^- and NO_2^- concentrations ranged from 1.50 to 3.29, 0.48 to 0.70, and 0.32 to 1.00 mg/L, respectively. The average NH_4^+, NO_3^- and NO_2^- concentrations in brown water tanks (3–1, 4–6 and 5–7) were lower than those in green water tanks (3–10, 4–5 and 5–3). The DON concentrations ranged from 0.17 to 0.60 mg/L, and the average concentration in brown water tanks were higher than those in green water tanks. The DIP concentration ranged from 0.12 to 0.45 mg/L, and the average concentration in brown water tanks were lower than those in green water tanks. The DOP concentration ranged from 0.001 to 0.04 mg/L, and the average concentration in brown water tanks were higher than those in green water tanks. The concentrations of nitrogen and phosphorus in all tanks were higher than those in raw water. The DSi concentrations in brown water tanks were all below detectable limit. The DSi concentrations in green water tanks were lower than that in raw water (Table 1).

3.2 | High-throughput sequencing data statistics

A total of 257,637 raw reads were obtained using high-throughput Illumina sequencing for all samples, and the number of reads for each sample ranged from 30,689 to 63,791. After quality and chimera checking and removal of the low-quality reads, a total of 193,518 clean reads were obtained. The average reads length was 388 nucleotides. The reads for all samples were classified into 336 OTUs, ranging from 105 to 203 OTUs, at a 99% similarity level (Table 2).

Taxa were assigned to the representative sequence of each OTU using the NCBI database. According to the taxonomic information, the sequences that were annotated as eukaryota and bacteria accounted for 65.84% and 34.01%, respectively. At phylum level, Chlorophyta sequences accounted for the greatest proportion of the total sequences (46.34%), followed by Bacillariophyta (18.18%), Cyanobacteria (17.02%), Verrucomicrobia (8.50%), Planctomycetes (4.74%), Proteobacteria (3.68%) and Cryptophyta (0.97%). The sequences of Haptophyta, Raphidophyta, Bacteroidetes, Phaeophyta, Euglenophyta and Dinophyta accounted for less than 1% of the total sequences (Figure 1).

Following the exclusion of bacteria (all non-cyanobacteria) and unclassified sequences, 160,349 sequences, ranged from 18,001 to 343,325, were assigned to phytoplankton (Table 2). The number of phytoplankton sequences were randomly rarefied to 17,986 per sample, which were used in further analyses of community structure and diversity.

3.3 | Phytoplankton diversity

In the high-throughput sequencing analysis, Shannon indices was ranged from 1.72 to 3.05, with maximum in sample 4–6 and minimum

TABLE 1 Water quality parameters of industrial aquaculture for *Litopenaeus vannamei*

Samples	T (°C)	S (g/L)	DO (mg/L)	pH	NH_4^+ (mg/L)	NO_3^- (mg/L)	NO_2^- (mg/L)	DON (mg/L)	DIP (mg/L)	DOP (mg/L)	DSi (mg/L)
3–1	28.54	30.38	6.92	8.10	1.502	0.571	0.343	0.516	0.117	0.035	B.D.L.
3–10	28.60	30.12	5.81	7.86	2.367	0.548	0.324	0.191	0.300	0.001	0.364
4–5	27.99	30.85	6.57	7.92	3.191	0.701	0.797	0.172	0.378	0.004	0.216
4–6	27.80	30.45	6.95	8.14	1.639	0.579	0.589	0.367	0.184	0.014	B.D.L.
5–3	28.37	30.66	6.48	7.90	3.291	0.668	1.000	0.597	0.446	0.041	0.141
5–7	28.78	30.21	6.20	7.95	2.384	0.481	0.489	0.404	0.324	0.007	B.D.L.
Raw water	N.D.	N.D.	N.D.	N.D.	0.201	0.374	0.003	0.137	0.027	0.004	0.780

Abbreviations: B.D.L., below detectable limit; DIP, dissolved inorganic phosphate; DO, dissolved oxygen; DON, dissolved organic nitrogen; DOP, dissolved organic phosphate; DSi, dissolved silicate; N.D., not detected; NH_4^+, ammonium; NO_2^-, nitrite; NO_3^-, nitrate; S, salinity; T, temperature.

TABLE 2 Numbers of sequences in quality control analysis

Samples	Raw reads	Clean reads	Total OTUs	Phytoplankton reads
3–1	33,919	25,835	198	23,132
3–10	30,689	22,944	203	18,001
4–5	33,370	27,617	105	27,614
4–6	34,798	23,458	183	23,166
5–3	63,791	49,416	187	34,111
5–7	61,070	44,248	178	34,325

Abbreviations: OTUs, operational taxonomic units.

in sample 5–7. In the morphological analysis, Shannon indices was ranged from 0.10 to 2.32, with maximum in sample 3–10 and minimum in sample 5–7. Comparing with morphological identification, high-throughput sequencing revealed higher phytoplankton diversity in all samples except for the sample 4–5 (Figure 2).

Non-metric multidimensional scaling (NMDS) analysis based on the OTUs and species identified by the high-throughput sequencing and morphological identification, respectively, was shown in Figure 3. In the high-throughput sequencing analysis, the samples 3–1 and 4–6 had a similar phytoplankton community structure and samples 3–10 and 4–5 had a similar phytoplankton community structure. However, each of samples 5–3 and 5–7 had a different phytoplankton community structure with other samples (Figure 3a). In the morphological analysis, the samples 3–1, 4–6 and 5–7 had a similar phytoplankton community structure and samples 3–10 and 5–3 had a similar phytoplankton community structure. However, the phytoplankton community structure in sample 4–5 was different to other samples (Figure 3b).

3.4 | Phytoplankton community structure

At the phylum level, 9 phyla were identified by high-throughput sequencing (Figure 4a). Chlorophyta was the most dominant group, which accounted for 22.42%–97.38% of total phytoplankton. Cyanophyta and Bacillariophyta also represented large proportions of phytoplankton, with 0.57%–52.91% and 0.31%–63.74% of relative abundances, respectively. Cryptophyta and Haptophyta accounted for 0.04%–6.16% and 0.01%–1.16% of total phytoplankton cells, respectively. The relative abundance of Raphidophyta, Phaeophyta, Euglenophyta and Dinophyta were below 1%. The abundance pattern derived from morphological identification was distinct from that of high-throughput sequencing analysis. A total

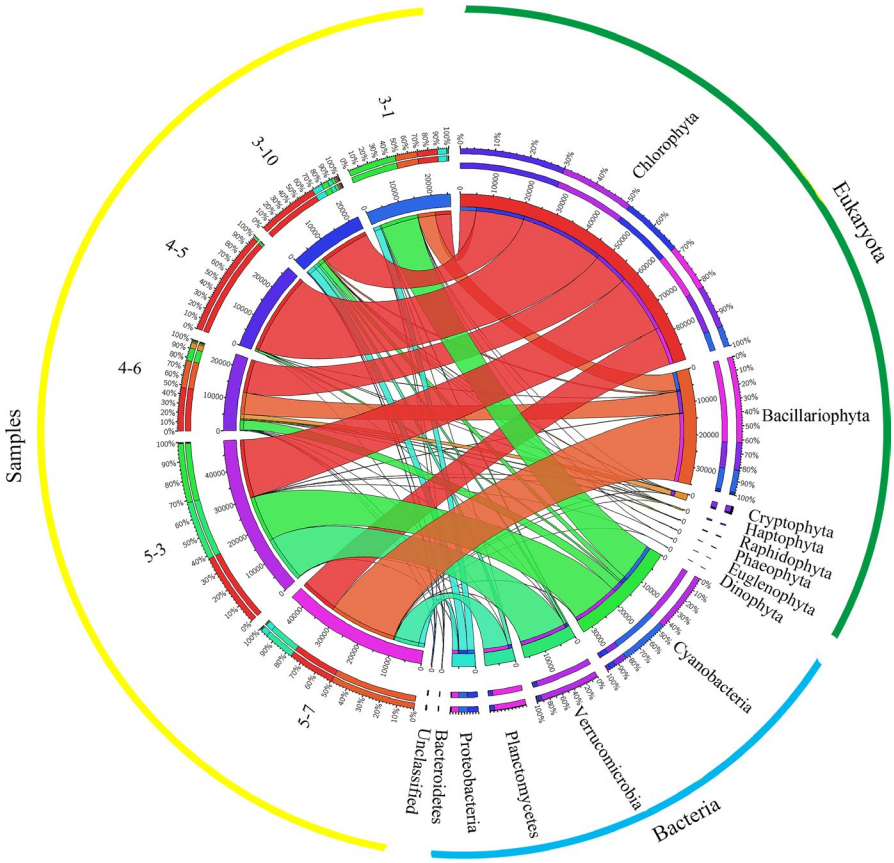

FIGURE 1 Circular representation of sequences assigned to eukaryota, bacteria and unclassified [Colour figure can be viewed at wileyonlinelibrary.com]

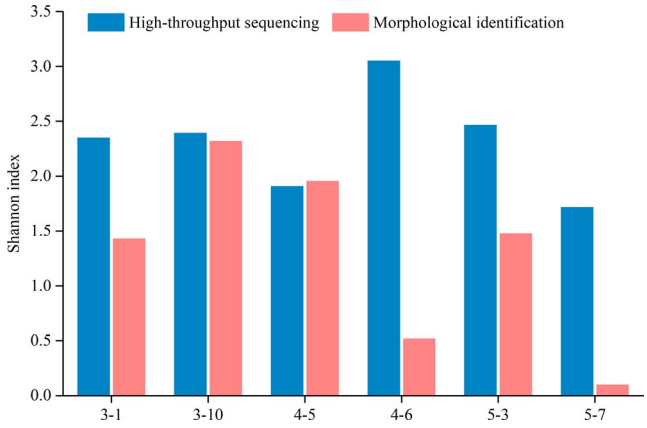

FIGURE 2 Shannon index for each sample analyzed by high-throughput sequencing and morphological identification [Colour figure can be viewed at wileyonlinelibrary.com]

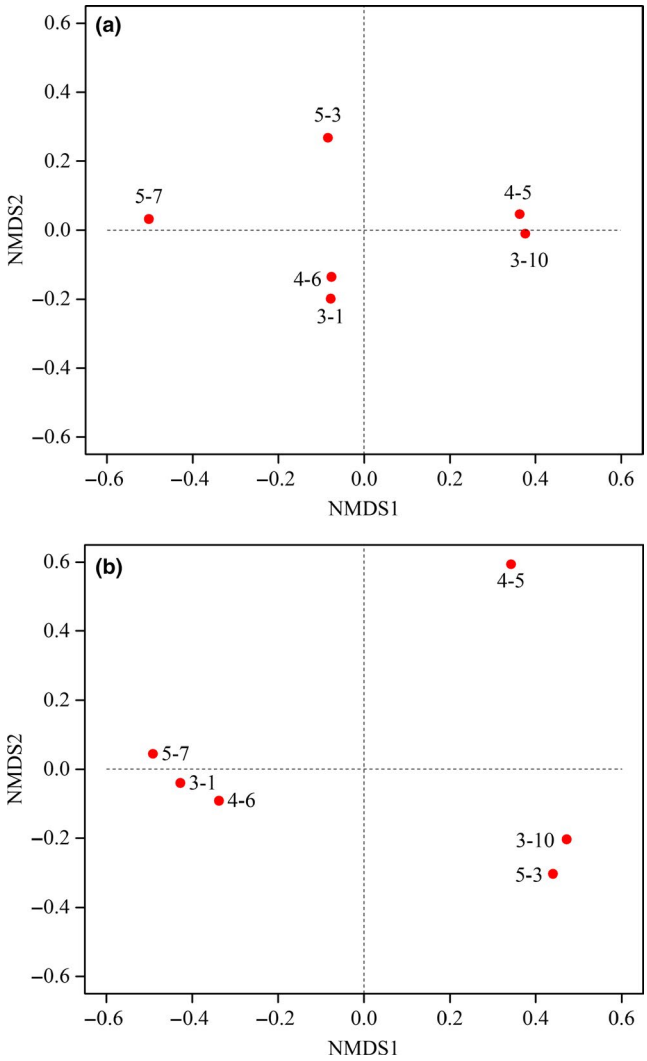

FIGURE 3 Non-metric multidimensional scaling (NMDS) analysis of phytoplankton community identified using high-throughput sequencing (a) and traditional morphological identification (b) methods [Colour figure can be viewed at wileyonlinelibrary.com]

of 35 taxa, belonging to six phyla, were identified by morphological observations in all samples (Figure 4a). Bacillariophyta was the most dominant group (26.35%–99.57%), followed by Cyanophyta (0.03%–45.95%), Cryptophyta (0.37%–27.02%), Dinophyta (0.02%–15.76%), Euglenophyta (0.02%–4.73%) and Chlorophyta (0%–3.04%).

At the genus level, a total of 26 genera were identified by high-throughput sequencing. Among of them, the relative abundance of 11 genera accounted for over 1% at least in one sample (Figure 4b). *Picochlorum* was the most dominant group, which accounted for 20.94%–97.19% of total phytoplankton. *Synechococcus* and *Cyclotella* also represented large proportions of phytoplankton, with 0.01%–52.81% and 0.01%–63.44% of relative abundances. Large proportions of *Pyramimonas* (2.20%), *Rhodomonas* (4.74%), *Skeletonema* (2.35%), *Chroomonas* (1.37%) and *Cerataulina* (2.41%) were present in the sample 4–6, while large proportions of *Chondrocystis* and *Coscinodiscus* were present in the sample 3–10 and 3–1, respectively. In the morphological observations, a total of 29 genera were identified. Among of them, the relative abundance of 14 genera accounted for over 1% at least in one sample (Figure 4b). *Cyclotella* was the most dominant group, which accounted for 17.92%–99.56% of total phytoplankton. *Cerataulina* also represented large proportions of phytoplankton, with 0%–56.26% of relative abundances. Large proportions of *Teleaulax*, *Chroomonas*, *Melosira*, *Gyrodinium*, *Dactyliosolen* and *Protoperidinium* were present in the sample 3–10, with 15.83%, 11.89%, 20.87%, 1.37%, 7.20%, 1.19% and 1.58% of relative abundances, respectively. Large proportions of *Eutreptiella*, *Gymnodinium*, *Oscillatoria*, *Pyramimonas* and *Nitzschia* were present in the sample 4–5, with 4.73%, 10.81%, 45.95%, 2.36% and 5.07% of relative abundances, respectively. Large proportion of *Hemiaulus* were present in the sample 5–3, with 10.56% of relative abundances.

3.5 | Correlations between phytoplankton community and water quality parameters

RDA was performed using CANOCO (ver. 4.5) to examine the relationships between phytoplankton community and water quality parameters in the waters of industrial aquaculture for *L. vannamei*. In the high-throughput sequencing analysis, the first two axes of the RDA explained 60.0% and 39.1% of the cumulative variance in the species-environment relationship (Figure 5a). The first axis was positively correlated with the DSi (0.8953), NO_3^- (0.6217), NH_4^+ (0.6108) and DIP (0.5368) concentrations and negatively correlated with the DON concentration (−0.7058), and the second axis was negatively correlated with the DOP (−0.8547) and DON (−0.5532) concentrations. These results showed that the DSi, NO_3^-, NH_4^+, DIP, DOP and DON concentrations had the greatest influences on the phytoplankton community. The dominant genus *Picochlorum* was positively correlated with the dissolved inorganic nitrogen (DIN, the sum of NH_4^+, NO_3^- and NO_2^-), DIP and DSi concentrations, and negatively

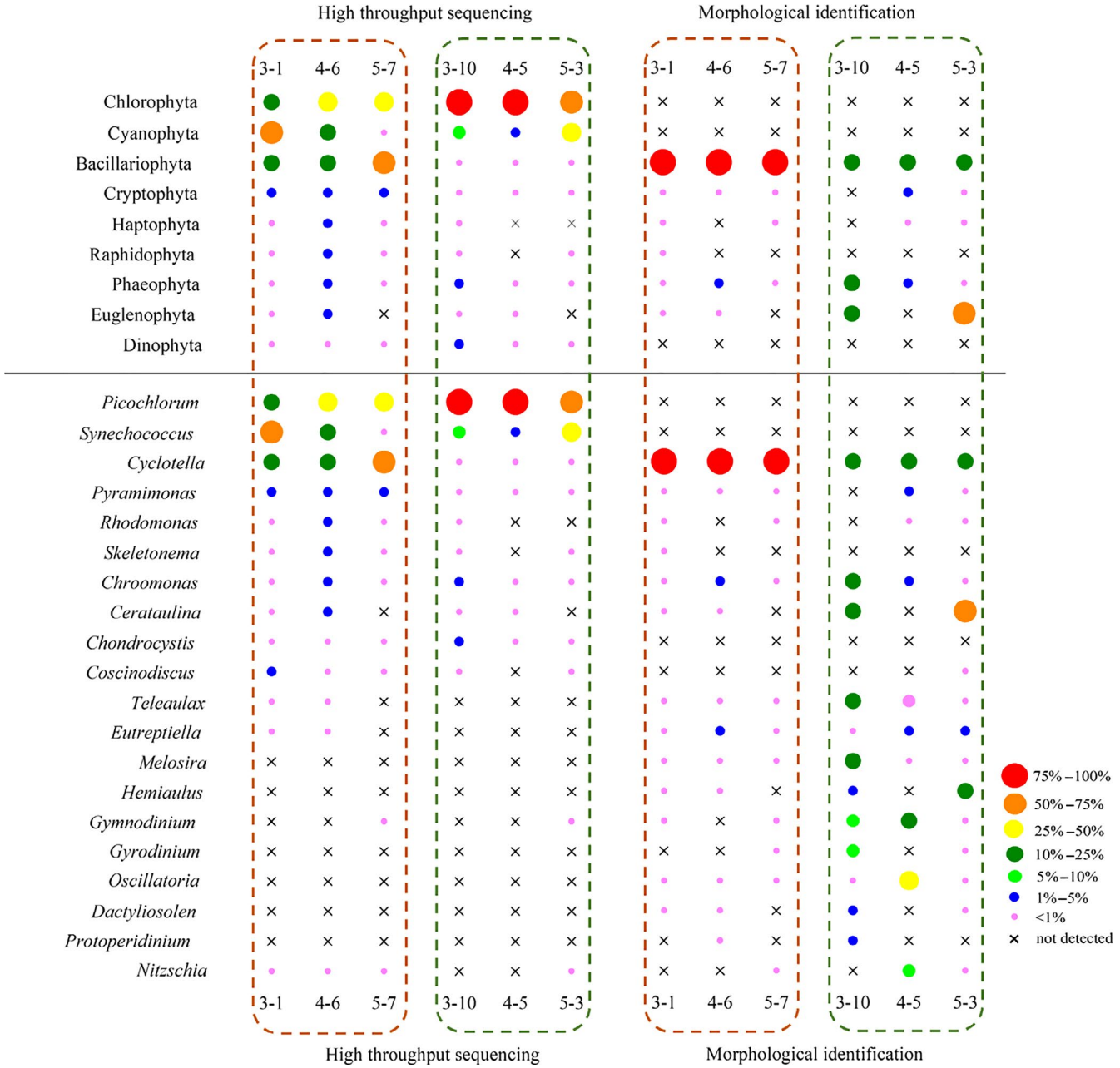

FIGURE 4 Relative abundance of phytoplankton community at the phylum (a) and genus (b) levels identified using high-throughput sequencing and traditional morphological identification methods. The size of the circles shows proportions of sequences or cells from each sample. Brown water samples (3–1, 4–6 and 5–7) were framed in the brown dashed box, and green water samples (3–10, 4–5 and 5–3) were framed in the green dashed box [Colour figure can be viewed at wileyonlinelibrary.com]

correlated with DON and DOP concentrations; however, diatoms (*Cyclotella*, *Skeletonema* and *Coscinodiscus*) showed negative correlations with the DIN, DIP and DSi concentrations. The cyanobacteria, *Synechococcus* was positively correlated with DON and DOP concentrations. In the morphological analysis, the first two axes of the RDA explained 64.8% and 25.4% of the cumulative variance in the species-environment relationship (Figure 5b). The first axis was positively correlated with the DSi (0.8902), NH_4^+ (0.8053), DIP (0.7440) and NO_3^- (0.6578) concentrations, and the second axis was negatively correlated with the DON (−0.7501) and DOP (−0.6407) concentrations. These results showed that the DSi, NH_4^+, DIP, NO_3^-, DON and DOP concentrations had the greatest influences on the phytoplankton community. The dominant genus *Cyclotella* was negatively correlated with the DIN, DIP and DSi concentrations, and positively correlated with the DON and DOP concentrations; however, cryptophytes (*Teleaulax* and *Chroomonas*), euglenophyte (*Eutreptiella*), dinoflagellate (*Gymnodinium*) and cyanobacteria (*Oscillatoria*) showed negative correlations with the DON and DOP concentrations, and positive correlations with the DIN, DIP and DSi concentrations.

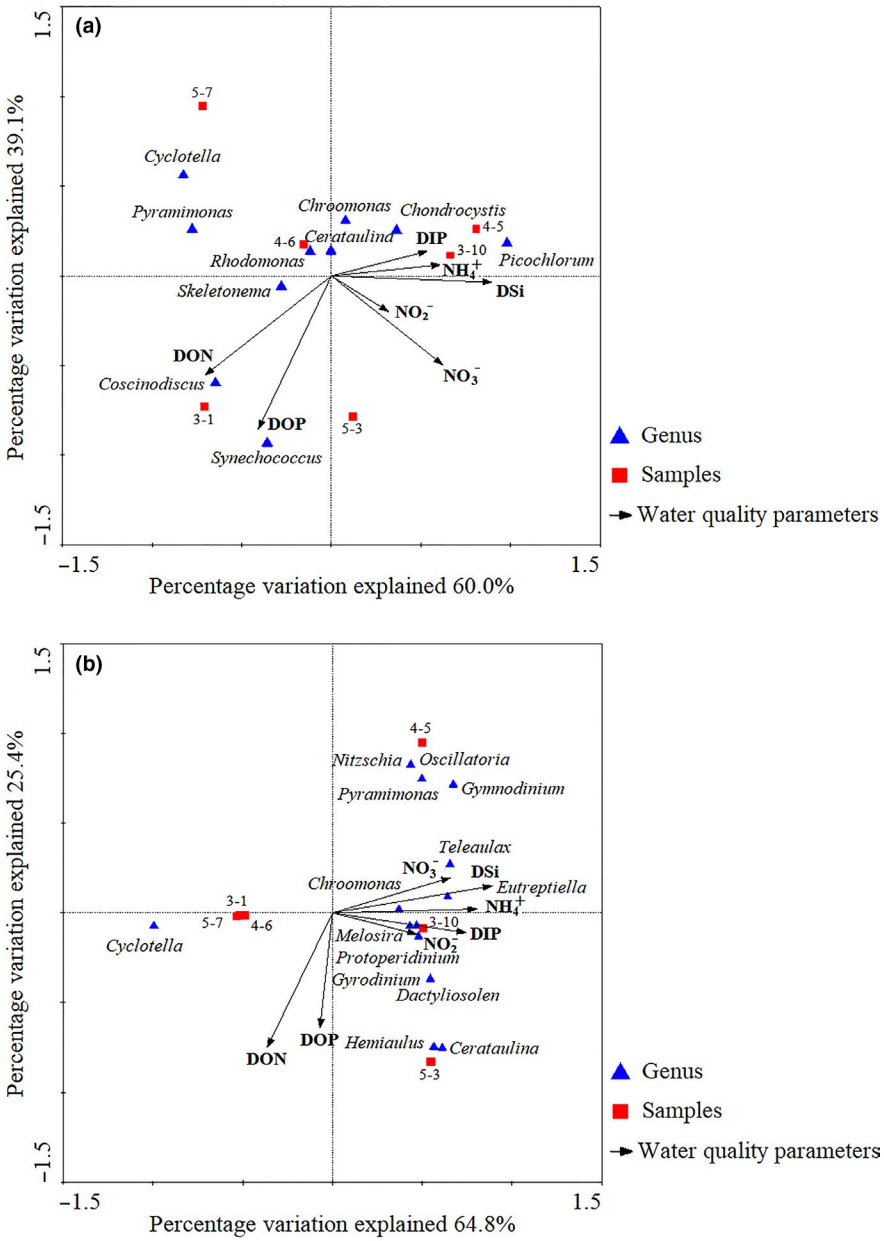

FIGURE 5 Redundancy analysis (RDA) of phytoplankton community at the genus level identified using high-throughput sequencing (a) and traditional morphological identification (b) methods and water quality parameters [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

4.1 | Discrepancy in phytoplankton communities inferred from the two methods

In this study, nine phyla of phytoplankton were identified by high-throughput sequencing, which was much higher than the parallel morphological identification. Chlorophyta and Cyanophyta were the dominant group, with the relative abundances higher in high-throughput sequencing than those in morphological identification. Unlike Chlorophyta and Cyanophyta, the relative abundances of Bacillariophyta and Dinophyta were lower in the DNA-inferred communities. Particularly, the phyla, Haptophyta, Raphidophyta and Phaeophyta, were not found by morphological identification (Figure 4a). A total of 41 genera were detected by both methods, but only 14 genera were consistent. On the whole, high-throughput sequencing revealed higher phytoplankton diversity comparing with morphological identification (Figure 2).

Discrepancy between high-throughput sequencing and morphological identification may result from many aspects. First of all, morphological identification is not suitable for diagnosis or quantification of pico-sized and small nano-sized phytoplankton. In this study, *Picochlorum* and *Synechococcus* were not observed morphologically. However, abundant of sequences affiliated to *Picochlorum* and *Synechococcus* were found in the high-throughput sequencing data (Figure 4b). Secondly, the low-abundance phytoplankton might escape detection with the use of light microscopy, but they could be detected by the high-throughput sequencing because of their higher throughput and lower detection threshold (Reuter et al., 2015). *Heterosigma akashiwo* was detected, with the relative

abundance of 0%–0.98% by the high-throughput sequencing, but it was not observed morphologically. Thirdly, the taxonomic coverage in the BLAST-nr database of 23S rDNA is incomplete. The 23S rDNA sequences were lacking in the database for some diatoms (such as *Hemiaulus* and *Dactyliosolen*) and dinoflagellates (such as *Gyrodinium* and *Protoperidinium*; Figure 4b), which might be the reason of their absence in the high-throughput sequencing data. The diversity of these groups maybe underestimated in the high-throughput sequencing. Therefore, further efforts are required to continue to develop the 23S rDNA reference library to improve its taxonomic coverage. Fourthly, dead cells, dormant cells, or molecular debris of rare taxa could be detected by the high-throughput sequencing, which might overestimate the richness and diversity of phytoplankton. In this study, *Lobophora variegate*, a foliose brown macroalga, was detected with low relative abundance by the high-throughput sequencing. More research is needed to determine whether these sequences came from the spore or molecular debris of this macroalga. Lastly, rDNA sequence of phytoplankton genomes consists of numerous repeats (Schippers & Neretin, 2006). rDNA copies vary in different taxonomic groups. Consequently, the relative abundance of taxa from the high-throughput sequencing may not exactly reflect their actual abundance. It may have to be combined with estimation of species abundance proportions with microscopy.

4.2 | The high relative abundance of phytoplankton and their potential ecological roles

Picochlorum was the most dominant genus revealed by the high-throughput sequencing in our study, but not detected by morphological identification (Figure 4b). *Picochlorum* is a slightly oval, nonflagellated green alga with an approximate mean size of 2 μm (De la Vega, Diaz, Vila, & Leon, 2011; Wang, Lambert, Giang, Goericke, & Palenik, 2014). Studies have shown that this genus is remarkably robust in the face of environmental perturbations, thriving in distilled water as well as in seawater supplemented with 0.5 M of NaCl, light intensities between 100 and 1,200 $\mu E\ m^{-2}\ s^{-1}$, and temperatures that range from 15–40°C (Huesemann et al., 2016; De la Vega et al., 2011). In addition, *Picochlorum* has higher growth rates and biomass production, and is rich in protein, carotenoid and lipid content (von Alvensleben, Stookey, Magnusson, & Heimann, 2013; Dahmen et al., 2014; Pereira et al., 2013; De la Vega et al., 2011; Zhu & Dunford, 2013), and can be harvest through common flocculation methods (Zhu, 2012), Therefore, *Picochlorum* has been investigated and is considered suitable for biofuel, nutritional, nutraceutical, and waste water remediation applications (von Alvensleben et al., 2013; Dahmen et al., 2014; Dinesh Kumar, Santhanam, Park, & Kim, 2016; Dinesh Kumar et al., 2018; Pereira et al., 2013; De la Vega et al., 2011; Zhu & Dunford, 2013). Studies demonstrated that the total *Vibrio* count in aquaculture water could be limited to a low level through the introduction of *Picochlorum* strain S1b (Chen, Chen, Lin, Lin, & Lu, 2012; Kuo, Chang, Chen, & Chen, 2015). Therefore, *Picochlorum* might serve as algal material for a biological method to control *Vibrio* in aquaculture rearing systems.

Synechococcus was the second dominant genus revealed by the high-throughput sequencing in our study (Figure 4b). This genus is spherical to rod shaped, 0.6–2.1 μm in diameter (Herdman, Castenholz, & Waterbury, 2001), which was too small to observe by microscope. *Synechococcus* is ubiquitous and abundant in major oceanic regimes contributing approximately 17% of net primary production, which plays important roles in global productivity and biogeochemical cycles (Flombaum et al., 2013; Partensky, Blanchot, & Vaulot, 1999). It also plays a key role in marine food-web structure via energy transfer within the microbial loop through heterotrophic flagellate, ciliate and copepods grazing (Apple, Strom, Brian, & Bianca, 2011; Christaki et al., 2002; Christaki, Jacquet, Dolan, Vaulot, & Rassoulzadegan, 1999; Motwani, Duberg, Svedén, & Gorokhova, 2018; Yoo et al., 2015). Because of its fast growth and tolerant to a wide range of environmental stress conditions, *Synechococcus* has been widely used in aquaculture and biotechnology. Studies have showed that *Synechococcus* could be used to remove ammonium, nitrate and phosphorus from aquaculture wastewater (Aguilar-May & Sánchez-Saavedra, 2009; Ruiz-Güereca & Sánchez-Saavedra, 2016; Srimongkol, Thongchul, Phunpruch, & Karnchanatat, 2019), and be applied to the treatment of heavy metals from industrial wastewater (Croot, Bengt, Elteren, & Kroon, 2003; Srivastava & Majumder, 2008). In addition, *Synechococcus* was also used as a platform to generate a range of commercially significant products, including Omega-3 fatty acids (Dong et al., 2016; Yoshino et al., 2017), Mannitol (Jacobsen & Frigaard, 2014), Hydrogen (McNeely, Xu, Bennette, Bryant, & Dismukes, 2010), limonene and bisabolene (Davies, Work, Beliaev, & Posewitz, 2014). However, some species of *Synechococcus* (e.g. *Synechococcus lividus*) were found to produce microcystins (Mohamed, 2008). Toxicity assessment revealed the ability of some marine *Synechococcus* crude and partially purified extracts to be toxigenic to marine invertebrates, such as *Artemia salina*, *Paracentrotus lividus* and *Mytilus galloprovincialis*, resulting in an inhibition of embryogenesis or development of smaller larvae (Frazão, Martins, & Vasconcelos, 2010). In addition, some marine *Synechococcus* extracts also can induce apoptosis in eukaryotic cells and cause inhibition of Gram-positive bacteria (Martins et al., 2008).

Cyclotella was the most dominant genus revealed by morphological identification. The highest density of *Cyclotella* was detected in sample 5–7 with 1.16×10^8 cells/L, accounting for 99.56% of total phytoplankton (Figure 4b). *Cyclotella* species are major components of the phytoplankton in various types of freshwater, brackish and marine ecosystems worldwide, which are strong indicators of global change (Malik, Northington, & Saros, 2017; Malik & Saros, 2016; Saros & Anderson, 2015). *Cyclotella* is nontoxic, suitably sized, growing fast and rich in nutrition, which has previously been used as a feed source within aquaculture (Huang, Iang, Lu, & Deng, 2011; Laing & Millican, 1992; De Pauw & Persoone, 1988; Webb & Chu, 1983). Investigations revealed that *Cyclotella* was the dominant species at a

later stage of shrimp aquaculture, and it could improve aquaculture water quality and was helpful for shrimp growth (Laokiatsophon, Limsuwan, Taparhudee, & Chuchird, 2006; Zeng & Huang, 2011). In addition, studies have shown that *Cyclotella* sp. is the potential to produce chitin microfibers (Chiriboga & Rorrer, 2018; Rorrer et al., 2016), which have applications in biomedical, cosmetics, food preservation, dietary supplements, paper making, and in wastewater treatment (Bhatnagar & Sillanpaa, 2009; Elwakeel, 2010; Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010; Jeon, Shahidi, & Kim, 2000; Morganti & Morganti, 2008; No, Meyers, Prinyawiwatkul, & Xu, 2007).

4.3 | Effects of water quality parameters on the phytoplankton community

Phytoplankton can adapt to fluctuations in aquatic environments and result in changes in community structure accordingly, which could be used as a biological indicator of water quality and ecosystem change (McCormick & Cairns, 1994; Villegas & Biner, 1973; Wu et al., 2017). Our results indicated that NO_3^-, NH_4^+, DIP, DSi, DON and DOP concentrations can affect the structure of the phytoplankton communities inferred from both morphological identification and high-throughput sequencing methods.

The intensive shrimp aquaculture can make nutrient levels rise leading to eutrophication (Chen et al., 2018), which cause an explosive multiplication of phytoplankton. In this study, the concentrations of nitrogen and phosphorus in all tanks were higher than those in raw water (Table 1), and the water colours of shrimp tanks showed green or brown. Investigate showed that green water was mainly caused by green algae, and brown water was mainly caused by diatoms (Figure 4). *Picochlorum* dominated the green algae community, which was positively correlated with the DIN and DIP concentrations, and negatively correlated with DON and DOP concentrations. However, *Cyclotella* dominated the diatoms community, which showed negative correlations with the DIN and DIP concentrations, and positive correlations with the DON and DOP concentrations (Figure 5). The results indicated that *Picochlorum* might be able to utilize dissolved organic nitrogen and phosphorus (Wang, Shi, & Palenik, 2016), and *Cyclotella* might mainly utilize dissolved inorganic nitrogen and phosphorus (Pahl, Lewis, King, & Chen, 2012; Saros et al., 2012; Winder & Hunter, 2008). The DSi concentration in all tanks were lower than that in raw water (Table 1). Studies found that groundwater is characterized by high silica content, because of the lixiviation of biogenic silica from Gramineae (Sebastia & Rodilla, 2013; Sebastiá et al., 2012; Sospedra et al., 2018). The aquaculture water was the mixture of seawater and underground brine in this study. Therefore, biogenic silica precipitated from plant into underground brine, which might be the reason why the DSi concentration was higher in raw water. The DSi concentrations in brown water tanks were all below detectable limit, which was related to diatoms. Diatoms are a clade of microalgae that possess intricately patterned porous cell walls of biosilica called frustules, and require dissolved silicon in the form of $Si(OH)_4$ for frustule formation (Martin-Jézéquel, Hildebrand, & Brzezinski, 2000). In addition to the diatoms, silicon accumulation was also observed in cultured *Synechococcus* strains (Baines et al., 2012). This maybe the reason why *Synechococcus* was negatively correlated with DSi concentrations in this study (Figure 5a).

4.4 | Comparative analysis of phytoplankton community between the indoor industrial aquaculture system and the Yellow Sea

Investigations on phytoplankton community in the Yellow Sea in autumns from 2007 to 2017 found that a total of 371 species belong to 108 genera and four phyla were identified, and the total number of phytoplankton species was significantly increased since 2007 (Huang, Wei, Tang, & Jin, 2018). In this study, a total of 41 genera were detected by both high-throughput sequencing and morphological identification. Among these genera, 13 genera, including *Picochlorum*, *Synechococcus*, *Rhodomonas*, *Chondrocystis*, *Isochrysis*, *Lobophora*, *Emiliania*, *Desmochloris*, *Guillardia*, *Teleaulax*, *Cyanobacterium*, *Dactyliosolen*, *Pinnularia*, were not found in the Yellow Sea in autumns from 2007 to 2017. Whether they really don't exist in these waters of the Yellow Sea need further investigations combined with molecular methods. Diatoms and dinoflagellates were the most dominant groups in the Yellow Sea in autumn. The dominated species were *Gymnodinium* sp. *Nitzschia delicatissima*, *Chroomonas* sp. *Thalassionema nitzschioides*, *Scrippsiella trochoidea*, and *Gyrodinium* spp. (Huang et al., 2018). *Gymnodinium*, *Nitzschia*, *Chroomonas* and *Gyrodinium* were also detected in this study, but had a relatively low degree of dominance, while *Picochlorum*, *Synechococcus* and *Cyclotella* were the dominated species in this study (Figure 4). In addition, the average density of phytoplankton was 10^7 cells/L revealed by morphological identification in this study, far higher than those with 10^4 cells/L in the sea (Huang et al., 2018). If the aquaculture wastewater containing lots of nutrients and phytoplankton was directly discharged into the surrounding sea areas, it will contaminate the coastal environment, and even may damage the ecological balance (Grosholz et al., 2015). Therefore, it is worth mentioning that the aquaculture wastewater must be treated before discharging to meet the effluent COD standard.

5 | CONCLUSIONS

The community structure and diversity of phytoplankton in the waters of the industrial aquaculture for *L. vannamei* was analyzed by both high-throughput sequencing and morphological identification. Phytoplankton communities inferred from the two methods were both influenced by NO_3^-, NH_4^+, DIP, DSi, DON and DOP concentrations. Chlorophyta, Cyanophyta and Bacillariophyta were the three dominant phyla as revealed by the two methods. High-throughput sequencing revealed that green algae and cyanobacteria were the most dominant phytoplankton; however, diatoms were the first dominant phytoplankton in the morphological identification. High-throughput sequencing revealed a high diversity and small-sized phytoplankton

which were undetected by microscopy. However, the absolute abundance of species cannot be obtained by high-throughput sequencing, but can be quantified by morphological identification. Therefore, the combination of molecular and morphological techniques, in contrast to one single approach, will be useful to get a comprehensive understanding of phytoplankton communities and provide valuable information for healthy and sustainable aquaculture.

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DATA ACCESSIBILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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