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Phytoplankton diversity in a tropical bay, North Borneo, Malaysia as revealed by light microscopy and Next-Generation Sequencing

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Abstract

Assessments of phytoplankton diversity in Sabah waters, North Borneo, have primarily relied on morphology-based identification, which has inherent biases and can be time-consuming. Next-Generation Sequencing (NGS) technology has been shown to be capable of overcoming several limitations of morphology-based methods. Samples were collected from the Sepanggar Bay over the course of the year 2018 in different monsoon seasons. Morphology-based identification and NGS sequencing of the V8–V9 region of the 18S LSU rDNA were used to investigate the diversity of the phytoplankton community. Microscopy and NGS showed complementary results with more diatom taxa detected by microscopy whereas NGS detected smaller and rarer taxa. The harmful algal genera in the study site comprised of *Skeletonema*, *Margalefidinium*, *Pyrodinium*, *Takayama*, and *Alexandrium* as detected by NGS. This study showed that that an integrative approach of both morphological and molecular techniques could provide more comprehensive information about the phytoplankton community as the approach captured quantitative variability as well as the diversity of phytoplankton species.

Key words: phytoplankton community, Next-Generation Sequencing (NGS), Sabah, South China Sea

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1 Introduction

Phytoplankton are important primary producers in the ocean, yet their diversity in the tropics is not well known. Changes in the diversity of phytoplankton community has greater repercussions for organisms at higher trophic levels (Lewandowska et al., 2012; Vallina et al., 2017). With mounting anthropogenic influences on coastal environments, it is important to understand its effects on the diversity of the phytoplankton community, which can be an important measure of ecosystem health (Ptacnik et al., 2008).

Monitoring of the phytoplankton community has been of particular interest in Asia due to the deleterious effects that harmful algal blooms (HABs) might bring to the fisheries industry and human health. In East Asia, HABs have been recorded from as early as the 1970s with data suggesting that the phytoplankton community assemblage has shifted (e.g., from dominance by diatoms to dinoflagellates in Korea) (Sakamoto et al., 2021). New harmful algal bloom (HAB) species that were previously undetected at particular sites (e.g., *Takayama xiamenensis, Coolia malayensis*) have also been reported, suggesting a recent expansion or introduction of HAB species into the region by increased human activities (Furuya et al., 2018).

In Malaysia, the first paralytic shellfish poisoning (PSP) event caused by *Pyrodinium bahamense* was reported in Sabah in 1976 (Roy, 1977). Although there was no increasing trend in the frequency of toxic blooms over the past three decades since 1976, the presence of paralytic shellfish toxin (PST) producers such as *Alexandrium minutum* and *Alexandrium tamiyavanichii*, among others, are of concern to the country (reviewed in Lim et al., 2012; Yñiguez et al., 2021). In Sabah, regular monitoring along the coast has been conducted since 2000 and several other HAB species (*Gymnodinium catenatum*, *Gonyaulax polygramma*, *Margalefidinium polykrikoides*, and *Noctiluca scintillans*) were reported from Sabah waters (Jipanin et al., 2019).

Phytoplankton diversity has been traditionally studied by examining and identifying species based on morphological characteristics under the microscope (Savin et al., 2004). Such methods are often tedious and time-consuming especially in the case of extensive, long-term monitoring programs with many sampling sites. Identification of small naked dinoflagellate species such as *Karlodinium* (Leong et al., 2015) is also hardly possible without the help of SEM observation. Common preservation methods using Lugol's iodine in preparing cells for microscopic observation have been shown to produce artefacts, including changes in cell size and morphology, in diatoms and dinoflagellates (Montagnes et al., 1994; Menden-Deuer et al., 2001). Hence, alternative methods are needed to provide more precise assessments of phyto-

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plankton diversity.

Molecular methods have shown much promise in the identification, detection, and enumeration of phytoplankton species. Phylogenetic analysis has been useful in confirming the species (Hong et al., 2008; Kadar et al., 2018), detecting cryptic species (López-García et al., 2001), and revising taxonomic classifications (Gómez et al., 2017). The most common application is the use of molecular methods to detect HAB causative species at subbloom densities. For instance, real-time polymerase chain reaction (qPCR) assays which allows for the rapid and accurate detection of HAB species have been developed for toxic dinoflagellates such as *Pfiesteria piscicida* (Bowers et al., 2000), *Alexandrium minutum* (Galluzzi et al., 2004), *Alexandrium tamarense* and *Alexandrium catenella* (Hosoi-Tanabe and Sako, 2005), *Alexandrium tamiyavanichii* (Kon et al., 2015; Hii et al., 2019), *Karlodinium australe* (Kon et al., 2017), the toxic haptophyte *Prymnesium parvum* (Zamor et al., 2012), and the toxic diatom *Pseudo-nitzschia* spp. (reviewed in Bates et al., 2018; Ajani et al., 2021). The changes of the HAB community assemblage in the Johor Strait have also been revealed by using the metabarcoding approach targeting the 18S ribosomal RNA (rRNA) gene marker region (Hii et al., 2021).

Previous studies on species ribotypes conducted in Malaysia have shown that molecular methods are essential in distinguishing different clades. For instance, phylogenetic analysis based on the large subunit ribosomal deoxyribonucleic acid (LSU rDNA) of *M. polykrikoides* have revealed that the ribotype of this species found in Sabah, Malaysia belonged to the Malaysian/American clade rather than the Philippines clade (Iwataki et al., 2008). Another study conducted in Kuantan Port, Pahang, also showed that molecular techniques targeting LSU rDNA sequences is critical in confirming the identity of a toxic dinoflagellate such as *Alexandrium tamiyavanichii* (Liow et al., 2019). Sequences from the D1–D3 regions of the LSU rDNA and the second internal transcribed spacer (ITS2) region have also aided researchers in establishing a new species, *Prorocentrum malayense*, with samples collected from the Perhentian Islands Marine Park, Terengganu (Lim et al., 2019). Toxic diatoms such as *Pseudo-nitzschia* spp. found in Malaysian waters have also been characterised by targeting the LSU rDNA gene or the ITS region (Bates et al., 2018 and references therein; Teng et al., 2021).

Studies on the phytoplankton diversity in Sabah coastal waters are mainly based on morphological observations (Sidik et al., 2008; Mohammad-Noor et al., 2014; Chong et al., 2020). Molecular techniques are usually used to examine and study harmful algal species such as *M. polykrikoides* (Anton et al., 2008) and *A. tamiyavanichii* (Kon et al., 2015). Identification of species based on microscopy alone may lead to misleading inferences of ecological studies when cryptic species are present in the phytoplankton community (Verma et al., 2016). However, there is a considerable lack of studies examining the phytoplankton diversity in these waters using molecular techniques. Hence, there is a need to obtain genomic sequences of the phytoplankton community from the coastal waters of Sabah to uncover cryptic diversity in the phytoplankton community.

For diatoms and dinoflagellates, the 18S LSU ribosomal rRNA gene is often used as a metabarcoding marker (Penna et al., 2017; Gran-Stadniczeñko et al., 2019). The hypervariable regions of V1–V9 have been targeted for amplicon sequencing in eukaryotic phytoplankton with the V4 and V9 regions often used in tandem (Stoeck et al., 2010; Lohan et al., 2016). Commonly used primer sets provide convenient means to achieve the sequencing of eukaryotic phytoplankton. However, a recent study found degeneracies on the 3′ end of V4-specific primers which impacted read length and mean relative abundance (Bradley et al., 2016). The PCR error was also found to be higher for communities with rich GC content compared to the communities with balanced GC content (Bradley et al., 2016). While the V4 region failed to reliably capture a portion of the mock community, the V8–V9 primers more accurately represented the mean relative abundance, alpha and beta diversity of the mock community. Hence, this study employed the developed primer which targeted the V8–V9 region.

The availability of high-throughput sequencing technologies such as Next Generation Sequencing (NGS) technologies have enabled rapid and more economic analyses of genomic samples. The Illumina sequencing platform is an example of NGS sequencing technologies which have been applied in phytoplankton studies (Chen et al., 2019). The succession and community composition of phytoplankton have been characterised with this platform (Elferink et al., 2017; Yoshida et al., 2018; Hii et al., 2021), which was also capable of identifying species that were too small to be identifiable with light microscopy (Chen et al., 2019; Sildever et al., 2019).

Studies have shown that both morphological and molecular methods may be complementary in supplying information for different phylogenetic groups (Edvardsen et al., 2000; Savin et al., 2004; Giribet and Wheeler, 2005). A combination of both morphological and molecular approaches in the study of phytoplankton can better capture the true diversity of the phytoplankton community, especially in the waters of Sabah, where such information is limited. There is also a need to assess the suitability of NGS in determining the diversity of phytoplankton species in these coastal waters. Hence, the current study was carried out to compare the ability of the morphology- and NGS-based techniques in identifying the diversity of phytoplankton species in the coastal waters of Kota Kinabalu.

2 Materials and methods

2.1 *Sample collection*

Surface seawater samples were collected with a clean bucket from the Sepanggar Bay (6.05°N, 116.14°E) on the north-western coast of Sabah (Fig. 1). Five samples were collected in February (northeast monsoon 1; NEM1), April (intermonsoon period 2; IMP2), June (southwest monsoon; SWM), October (IMP3), and December (NEM2) 2018, coinciding with the different monsoon periods, namely, the NEM, SWM, and IMP. Samples for both morphology- and molecular-based analyses were collected concurrently from surface waters at 10 am local time.

2.2 *Morphology-based analysis*

One litre of surface seawater was sieved through a 5 μm mesh size plankton net and concentrated to a final volume of 300 mL, identified based on morphology following phytoplankton identification keys (Cupp, 1943; Hartley, 1996; Tomas, 1997; Lund and Hendey, 1965; Omura et al., 2012), and counted to the genus level under a light microscope (Olympus CX31) at 100× magnification in a Sedgewick Rafter chamber (Pyser-SGI) in triplicates.

2.3 *Samples for NGS-based analysis*

Surface seawater samples of 1.5 L were filtered onto 0.2 μm nylon membranes (Millipore) at low pressure (maximum 100 mm Hg) in the laboratory shortly after collection. The nylon membranes were rinsed with distilled water to remove excess

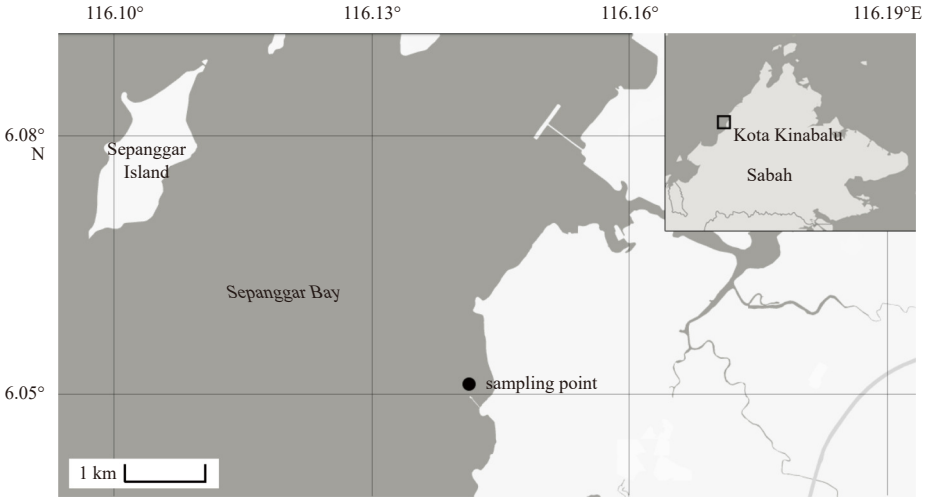

Fig. 1. Map of sampling location.

salts and pump-dried as much as possible. The membranes were then folded in half, placed in aluminium foil pre-cleaned with 70% ethanol, folded and placed in a clean zip-lock bag. The membranes were then stored at –80℃ until DNA extraction was carried out.

2.4 *DNA extraction*

The membranes were cut into small pieces and inserted into 2 mL microcentrifuge tubes. Extraction of genomic DNA (gDNA) from the total biomass collected on the membrane filters was carried out using the DNeasy Plant Mini Kit (QIAGEN) and the instructions from the manufacturer were followed, except centrifugation was carried out at 4℃ as opposed to 15–25℃ as stated in the kit's manual. The eluted gDNA were kept in 2 mL microcentrifuge tubes, sealed with parafilm, and stored at –20℃. The gDNA samples were then sent to Apical Scientific Sdn. Bhd. (Malaysia) for 18S amplicon sequencing.

The quantity of DNA was measured using a BioTek FLx800 fluorescence microplate reader with PicoGreen dye. The quality of the gDNA was evaluated with standard agarose gel electrophoresis with 50 ng of bacterial gDNA template as the positive control. The purity of the DNA samples was evaluated using a NanoDrop™ spectrophotometer at 260 nm, 280 nm, and 230 nm.

2.5 *DNA amplification*

Established primers targeting the hypervariable V8–V9 region of the 18S rRNA gene were used in this study (Bradley et al., 2016). The primers used were the forward primer V8f (5′–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATAACAGGTCTGTGA TGCCCT–3′) and the reverse primer V9r (5′–GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTCYGCAGGTTCACCTAC–3′).

The PCR cocktail comprised of 15–20 ng/μL of DNA, 1 U/μL of *Taq* DNA polymerase, 10 μmol/L of each primer, 12.5 μL of 5× PCR Buffer (Invitrogen, Life Technologies, Waltham, MA, USA), and 10 μL of nuclease-free water. PCR cycling parameters consisted of an initial denaturation step at 94℃ at 120 s, followed by 35 amplification cycles of 98℃ for 20 s, 65℃ for 15 s, and 68℃ for 30 s. The PCR products were purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA) following the manufacturer's protocols. The quality of the libraries was measured using the 4200 TapeStation System (Agilent Technologies, Santa Clara, CA, USA) and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a paired-end MiSeq Reagent Kit v2 (2×250 bp) following the manufacturer's instructions.

2.6 *NGS sequence processing*

Sequence adapters and low-quality reads were first removed from the paired-end reads using BBDuk from the BBTools package (https://sourceforge.net/projects/bbmap/). Then, the forward and reverse reads were merged using USEARCH v11.0.667 (https://www.drive5.com/usearch/). Sequences shorter than 150 bp or longer than 600 bp were removed from downstream processing. Reads were then aligned with the SILVA database (release 132) and chimeric errors were inspected using VSEARCH v2.6.2 (Rognes et al., 2016).

After quality assessment, reads were clustered *de novo* into operational taxonomic units (OTUs) at 97% similarity using UPARSE v11.0.667. Rare OTUs with less than two reads (doubleton), were removed from downstream processing. Taxonomic assignment of the OTUs was achieved using QIIME V1.9.1 against the SILVA database. Sequences obtained in this study are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under the accession numbers SAMN22930037–SAMN22930041 (BioProject PRJNA778196).

3 Results

A total of 440 014 reads were obtained from the five samples sequenced in this study, of which 47 354 reads or 11.4% are assigned to the phytoplankton group (Table 1). Unassigned reads constituted less than 2% of the total reads except for the IMP2 sample where they constituted about 23% of total reads. Sequencing effort was uniform in all samples with an average of (88 003±73) reads per sample. Non-phytoplankton organisms occurred in higher numbers than phytoplankton, which is reflected in the ratio between phytoplankton reads and total reads (Table 1).

In total, 394 phytoplankton OTUs were identified using the NGS platform with an average of (218.2±47.7) OTUs per sample. The taxonomic resolution varied between OTUs, with the raphidophytes and silicoflagellates resolved to class level while the other phytoplankton groups could largely be resolved to genus level (Table 1). Overall, *Chaetoceros* was the most abundant (36.9% of reads), followed by *Skeletonema* (17.8%), and *Minutocellus* (15.7%), all three being diatom genera. The most abundant dinoflagellate was *Blixaea* (2.1%) (Table 1).

Table 1. Taxonomic composition as revealed by amplicon sequencing

Group	Rank	Taxon	Relative abundance/%				
			NEM1	IMP2	SWM2	IMP3	NEM2
Diatom	Subphylum	Coscinodiscophytina	0.0	0.0	0.0	0.0	<0.1
	Class	Bacillariophyceae	0.9	0.0	0.0	4.3	1.0
	Class	Mediophyceae	<0.1	<0.1	<0.1	1.4	0.5
	Order	Fragilariales	0.0	0.0	0.0	<0.1	<0.1
	Genus	*Chaetoceros*	17.3	22.0	2.5	55.7	87.1
	Genus	*Corethron*	<0.1	0.0	0.0	<0.1	<0.1
	Genus	*Coscinodiscus*	0.0	0.0	0.0	<0.1	<0.1
	Genus	*Cyclotella*	0.9	1.0	2.5	1.4	1.0
	Genus	*Leptocylindrus*	<0.1	<0.1	<0.1	1.4	<0.1
	Genus	*Minutocellus*	1.8	3.0	72.5	<0.1	1.0
	Genus	*Navicula*	<0.1	0.0	0.0	<0.1	<0.1
	Genus	*Odontella*	0.0	0.0	0.0	<0.1	<0.1
	Genus	*Rhizosolenia*	<0.1	<0.1	0.0	<0.1	0.0
	Genus	*Skeletonema*	63.6	14.0	2.5	7.1	1.9
	Genus	*Thalassiosira*	<0.1	1.0	0.0	<0.1	<0.1
Dinoflagellate	Infraphylum	Dinoflagellata	2.7	12.0	5.0	4.3	1.0
	Sub-class	Gymnodiniphycidae	<0.1	<0.1	<0.1	<0.1	<0.1
	Order	Dinophysiales	0.0	0.0	<0.1	<0.1	<0.1
	Order	Gonyaulacales	0.9	0.0	0.0	<0.1	0.0
	Genus	Peridiniales	0.0	0.0	0.0	<0.1	<0.1
	Family	Suessiaceae	<0.1	<0.1	0.0	0.0	<0.1
	Family	Thoracosphaeraceae	<0.1	0.0	0.0	<0.1	0.0
	Genus	*Akashiwo*	0.0	<0.1	0.0	0.0	0.0
	Genus	*Alexandrium*	<0.1	<0.1	<0.1	1.4	<0.1
	Genus	*Amphidiniopsis*	0.0	0.0	<0.1	0.0	<0.1
	Genus	*Amphidinium*	0.9	<0.1	<0.1	<0.1	<0.1
	Genus	*Azadinium*	0.0	0.0	<0.1	0.0	0.0
	Genus	*Blixaea*	<0.1	<0.1	<0.1	8.6	1.9
	Genus	*Cochlodinium*	<0.1	<0.1	<0.1	2.9	0.5
	Genus	*Diplosalis*	0.0	0.0	0.0	<0.1	0.0
	Genus	*Erythropsidinium*	0.0	0.0	<0.1	2.9	<0.1
	Genus	*Fragilidium*	<0.1	0.0	0.0	<0.1	<0.1
	Genus	*Gonyaulax*	<0.1	1.0	<0.1	<0.1	<0.1
	Genus	*Gymnodinium*	0.9	<0.1	<0.1	<0.1	0.5
	Genus	*Gyrodinium*	1.8	0.0	<0.1	2.9	<0.1
	Genus	*Haplozoon*	<0.1	1.0	<0.1	<0.1	<0.1
	Genus (cyst)	*Islandinium*	0.0	0.0	0.0	<0.1	0.0
	Genus	*Katodinium*	0.0	<0.1	0.0	<0.1	<0.1
	Genus	*Oodinium*	<0.1	<0.1	5.0	0.0	<0.1
	Genus	*Paragymnodinium*	0.9	6.0	2.5	<0.1	<0.1
	Genus	*Polykrikos*	0.0	<0.1	0.0	<0.1	<0.1
	Genus	*Posoniella*	0.0	<0.1	0.0	0.0	0.0
	Genus	*Prorocentrum*	0.0	0.0	0.0	<0.1	<0.1
	Genus	*Protoperidinium*	<0.1	1.0	<0.1	1.4	0.5
	Genus	*Ptychodiscus*	<0.1	<0.1	<0.1	<0.1	<0.1
	Genus	*Pyrodinium*	<0.1	0.0	<0.1	<0.1	0.5
	Genus	*Sinophysis*	<0.1	0.0	<0.1	<0.1	<0.1
	Genus	*Symbiodinium*	0.9	1.0	<0.1	<0.1	0.5
	Genus	*Takayama*	<0.1	1.0	<0.1	<0.1	<0.1
	Genus	*Tripos*	4.5	1.0	<0.1	1.4	0.5
	Genus	*Woloszynskia*	<0.1	0.0	0.0	<0.1	<0.1
	Clade	Gymnodinium clade	1.8	<0.1	2.5	2.8	1.4
Eustigmatophyte	Order	Eustigmatales	<0.1	<0.1	<0.1	<0.1	0.0
Ochrophyte	Phylum	Ochrophyta	0.9	5.0	2.5	1.4	0.5
Raphidophyte	Order	Chattonellales	<0.1	<0.1	<0.1	<0.1	<0.1
	Class	Raphidophyceae	<0.1	<0.1	<0.1	<0.1	<0.1

to be continued

Continued from Table 1

Group	Rank	Taxon	Relative abundance/%				
			NEM1	IMP2	SWM2	IMP3	NEM2
Silicoflagellate	Class	Dictyophyceae	0.0	<0.1	0.0	<0.1	<0.1
Stramenopile	Genus	*Phaeomonas*	0.0	1.0	0.0	0.0	0.0
	Genus	*Pinguiochrysis*	0.0	<0.1	0.0	0.0	0.0
Chrysophyte	Class	Chrysophyceae	0.0	<0.1	<0.1	<0.1	<0.1
	Genus	*Ankylochrisis*	0.0	0.0	0.0	0.0	<0.1
	Genus	*Chrysowaernella*	0.0	0.0	0.0	<0.1	0.0
	Genus	*Ochromonas*	0.0	0.0	0.0	0.0	<0.1
	Genus	*Paraphysomonas*	0.0	0.0	0.0	<0.1	<0.1
Cryptophyte	Class	Cryptophyceae	<0.1	0.0	<0.1	<0.1	<0.1
	Order	Cryptomonadales	<0.1	2.0	<0.1	<0.1	0.5
	Genus	*Leucocryptos*	<0.1	1.0	<0.1	<0.1	<0.1
	Genus	*Proteomonas*	0.9	1.0	<0.1	<0.1	<0.1
	Genus	*Teleaulax*	0.0	<0.1	<0.1	0.0	<0.1
	Species	*Leucocryptos marina*	<0.1	<0.1	0.0	<0.1	<0.1
	Species	*Hemiselmis virescens*	0.0	0.0	0.0	0.0	<0.1
	Species	*Proteomonas sulcata*	<0.1	1.0	<0.1	<0.1	<0.1
Chlorophyte	Phylum	Chlorophyta	0.9	7.0	5.0	<0.1	1.0
	Class	Mamiellophyceae	<0.1	<0.1	<0.1	0.0	<0.1
	Order	Chlorodendrales	<0.1	2.0	7.5	1.4	<0.1
	Order	Mamiellales	<0.1	8.0	<0.1	<0.1	<0.1
	Order	Ulvales	<0.1	<0.1	<0.1	<0.1	<0.1
	Genus	*Bathycoccus*	0.0	0.0	0.0	<0.1	0.0
	Genus	*Coccomyxa*	0.0	<0.1	0.0	<0.1	<0.1
	Genus	*Crustomastix*	0.0	<0.1	0.0	0.0	0.0
	Genus	*Mamiella*	<0.1	<0.1	<0.1	0.0	<0.1
	Genus	*Nephroselmis*	<0.1	2.0	<0.1	<0.1	<0.1
	Genus	*Prasinoderma*	0.0	<0.1	0.0	0.0	<0.1
	Genus	*Tetraselmis*	0.0	<0.1	<0.1	0.0	<0.1
Total phytoplankton reads			10 046	8 363	3 869	6 331	18 745
Total reads			88 124	88 028	87 927	87 928	88 007
Unassigned reads			1 374	20 327	128	189	1 026

Note: Values with "<" indicate sequences that have at least one read.

From the aligned reads, 11 genera of diatoms, 29 genera of dinoflagellates, four genera of chrysophytes (golden algae), five genera of cryptophytes (cryptomonads), and seven genera of chlorophytes (green algae) were identified. Based on the number of reads, diatoms were the most dominant group (>50%) in all five samples sequenced. Relative abundance of *Chaetoceros* spp. was mostly higher than 20% except for the NEM1 sample, where *Skeletonema* spp. was dominant (63.6%), and in the SWM sample where *Minutocellus* spp. was dominant (72.5%). Relative abundances of dinoflagellates were mostly below 10% for all five samples. Sequences of the genera *Pyrodinium* and *Margalefidinium* (dubbed *Cochlodinium* from the database) were successfully detected from the samples.

The phytoplankton community can be largely divided into three clusters with the IMPs being closely grouped with the NEM samples (Fig. 2). Highest relative abundance of the diatom *Chaetoceros* spp. was found during NEM2, based on number of reads from the NGS data (Fig. 2). However, cell counts of the same period revealed that the dinoflagellate *Gonyaulax* spp. was more abundant in the community compared to *Chaetoceros* spp. (Fig. 3), while the relative abundances of *Gonyaulax* spp. in the NGS data were relatively low (Fig. 2). Results of amplicon sequencing also revealed a high diversity of other rare groups (e.g., chlorophytes and chrysophytes) that were not detected by microscopy.

Overall, 21% of total phytoplankton genera were detected by both morphology-based (light microscopy) and NGS-based methods (Fig. 4). Of the total phytoplankton genera, 18% was detected by morphological identification only whereas 33% was detected by amplicon sequencing. In addition, amplicon sequencing also detected other phytoplankton groups besides diatoms and dinoflagellates (grouped as "others"), which accounted for 28% of taxa.

Between the monsoons, the NGS-based method consistently detected more taxa compared to the morphology-based method with a difference of at least 10 taxa for each monsoon (Fig. 5). More diatom genera were detected by morphological identification compared to amplicon sequencing (18 and 11, respectively), whereas more dinoflagellate genera were detected by amplicon sequencing compared to morphological identification (28 and 10, respectively).

4 Discussion

The diversity of the phytoplankton community as revealed by microscopy- and NGS-based methods were shown to be different but not mutually exclusive (Fig. 2). Diatoms were largely dominant in all five samples sequenced (Fig. 2), a trend which corresponded to the relative abundance revealed by cell counts

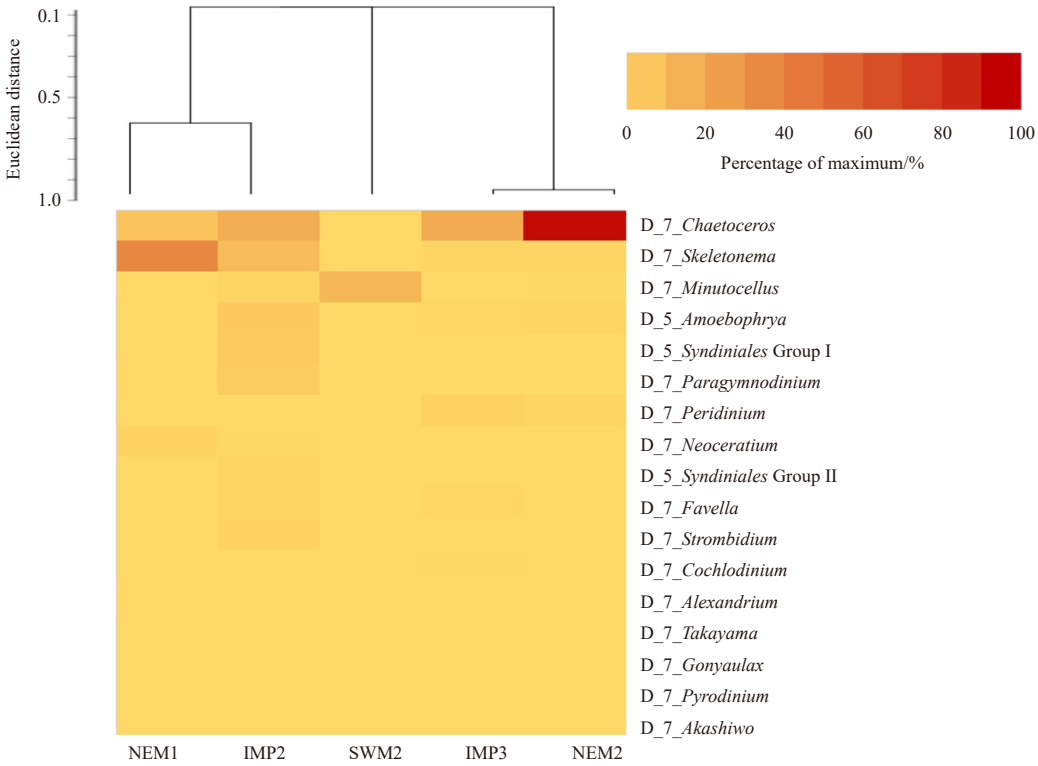

Fig. 2. Heat map of dominant and harmful phytoplankton taxa during the different monsoonal periods. Colours indicate the relative abundance of each taxon at each time-point.

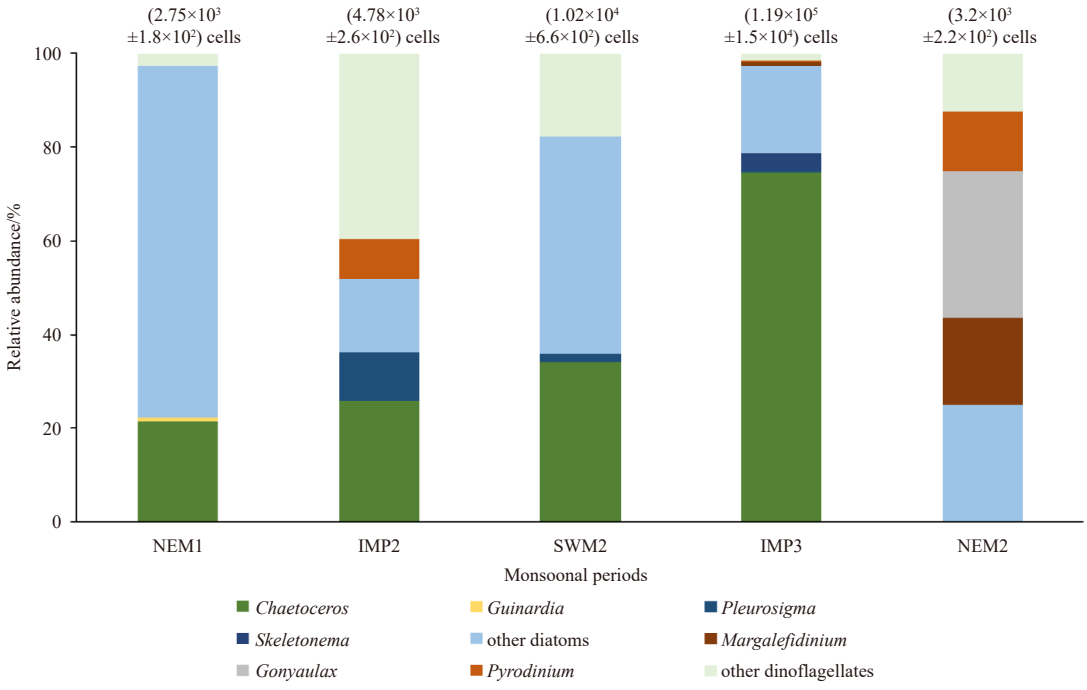

Fig. 3. Bar chart of relative abundances of phytoplankton based on cell counts. Total cell counts are provided in brackets above the respective bars. The group "other diatoms" include *Asteromphalus*, *Bacteriastrum*, *Coscinodiscus*, *Cylindrotheca*, *Dactyliosolen*, *Ditylum*, *Eucampia*, *Lauderia*, *Leptocylindrus*, *Navicula*, *Nitzschia*, *Rhizosolenia*, *Thalassionema*, and *Pseudo-nitzschia*. The group "other dinoflagellates" include *Akashiwo*, *Ceratium*, *Dinophysis*, *Gymnodinium*, *Gyrodinium*, and *Prorocentrum*.

(Fig. 3). Both methods were also capable of detecting harmful algal taxa (Table 1, Fig. 3), which implies the suitability of their use in monitoring programs.

With the primer pairs employed in this study, several phytoplankton taxa were resolved to the species level (such as *Proteomonas sulcata*) and most of the OTUs were resolved to the genus

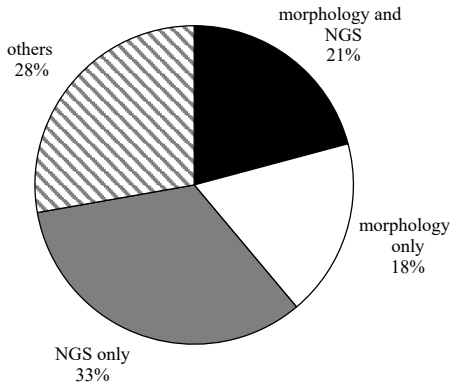

Fig. 4. Overall number of taxa found using morphology- and NGS-based methods. The group "others" refer to the non-diatom and non-dinoflagellates detected by the NGS-based method only.

level. However, there were also some unresolved taxa (annotated as ambiguous taxa) or some OTUs that were annotated as "uncultured marine eukaryote", which reveals that more work can be done in sequencing marine phytoplankton and building a more complete database. The low resolution of the raphidophytes (only to class level) might be addressed by applying primers that target the V4 region (Tyrrell et al., 1996) or other regions of DNA such as the 5.8S gene (Band-Schmidt et al., 2012). For example, the phylogenetic position of the marine harmful raphidophyte, *Chattonella subsalsa*, have been successfully determined by using primers that target the LSU rDNA (D1–D3) and ITS regions (Lum et al., 2019). The LSU rDNA is also useful in identifying the harmful dinoflagellate, *Karlodinium australe* (Lim et al., 2014).

The NGS-based method was also more successful in differentiating different genera of dinoflagellates that have similar morphologies as observed with light microscopy. For instance, *Azadinium* spp. could be mistaken as *Heterocapsa* spp. as they bear resemblance in shape (having an apical spine) and size (Tillmann et al., 2014). Another dinoflagellate, *Takayama* spp. also resembles *Gymnodinium* spp. in morphology (de Salas et al., 2003). As a result, these genera were most likely counted as the latter two genera due to the difficulty of differentiating them under the light microscope. Amplicon sequencing has also detected chlorophytes and chrysophytes that were not detected via light microscopy (Fig. 3). Some taxa, such as *Crustomastix* and *Mamiella* are too small and difficult to sample and distinguish by microscopic identification (Xu et al., 2017). This implies that morphologically-similar species and species with small cell sizes can be better characterised with NGS-based methods, giving a more comprehensive representation of the community assemblage.

About 21% of taxa were detected in both morphology and molecular-based surveys (Fig. 4), indicating that there is a considerable difference between the detection capabilities of the two methods. Small protists may remain undetected or unrecognised with morphological methods due to their cryptic nature (Boenigk et al., 2005; Šlapeta et al., 2006). This was also shown in this study with several taxa or lineages (e.g., within the chrysophytes, cryptophytes, and chlorophytes) being absent from the list of microscopic identification but were revealed by the molecular approach (Table 1). The differences in sampling strategies (filtration by 5 μm mesh and 0.2 μm membrane) and the magnification used to enumerate the cells (100×) may also partially account for the absence of smaller cells detected by light microscopy. The greater number of taxa detected by amplicon sequencing (Fig. 4) further highlights the advantage of molecular methods of identifying the rarer components of the phytoplankton community assemblage.

However, microscopic identification detected more diatom taxa compared to amplicon sequencing in some instances, a trend which was also observed in other studies (Savin et al., 2004; Bazin et al., 2014). The diatoms that were undetected by the molecular method (e.g., *Guinardia* spp. and *Pleurosigma* spp.) comprised of larger cells with more distinguishable features compared to picoplankton (e.g., *Crustomastix* spp.), which might explain why they were better represented by the morphological approach (Savin et al., 2004). However, these large taxa had low densities within the community (i.e., *Pleurosigma* spp. with 6.10×10^2 cells/L out of 1.19×10^5 cells/L) which probably led to the dilution of their sequences in the template DNA pool (Bazin et al., 2014). The non-detection or underrepresentation of some taxa in the amplicon sequencing results could also be explained by the lack of reference sequences in the Silva database for several taxa (e.g., *Gonyaulax* spp.), the underlying biases of the meth-

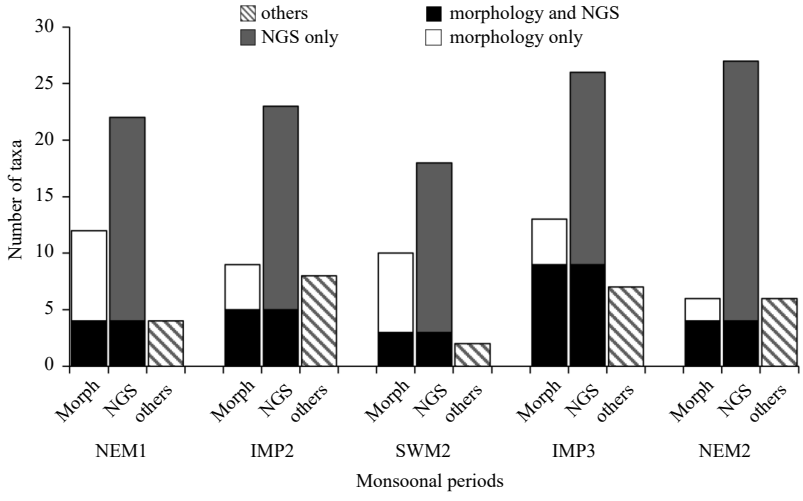

Fig. 5. Number of taxa found using morphology- and NGS-based methods relative to the monsoons. The group "others" refer to the non-diatom and non-dinoflagellates detected by the NGS method only.

od such as incompatibilities of the PCR primers (Stoeck et al., 2006; Liu et al., 2009), and competition for primers (Potvin and Lovejoy, 2009) due to the large variations in 18S rRNA gene copy numbers between different taxa (Zhu et al., 2005).

Although the 18S rRNA gene provides high resolution of the phytoplankton taxa, it does not capture the cyanobacterial community, since the gene is only present in eukaryotes. Some cyanobacteria have symbiotic relationships with other phytoplankton and often serve as nitrogen fixers (Wilkerson and Grunseich, 1990; Carpenter et al., 1999; Bergman et al., 2013). Hence, it is important to also monitor these smaller-sized organisms as their seasonal distribution might have an impact on the seasonality of the micro-phytoplankton. A combination of primers targeting the 16S rRNA and 18S rRNA gene could be used to obtain a clearer picture of the phytoplankton community in future studies. Different primer pairs which target different regions of the genome, such as the 28S rRNA region (Lim et al., 2014, 2019) or the ITS region (Yuan et al., 2015) for metagenomic analysis of the phytoplankton community should also be considered.

The taxa revealed by molecular methods provides new insight to the phytoplankton community composition in the study site. Toxic species found in this study such as *Takayama* spp., have been reported to cause fish kills elsewhere (Steidinger et al., 1998; de Salas et al., 2005). The chlorophyte *Coccomyxa* spp. are commonly found in acidic or neutral habitats with increased heavy metal concentrations (Řezanka et al., 2019) and have been reported as parasites on marine mussels (Rodríguez et al., 2008; Syasina et al., 2012). This further highlights the potential for HAB occurrences in these waters and reveals taxa that can be used as indicator species to monitor pollution in the environment.

The integrated approach of conventional microscopy and NGS amplicon sequencing, both with their own advantages and limitations, were clearly complementary and provided access to greater phytoplankton diversity than when observed by a single method. In general, larger cells of diatoms and dinoflagellates could be detected by microscopy, highlighting their application in the detection of larger phytoplankton taxa that have significant ecological functions while amplicon sequencing may reveal the overall diversity of the phytoplankton community especially when rare taxa are present in low abundances. The detection of previously unreported taxa in Sabah waters using the NGS-based method also implicates the potential for future studies in these waters. Evidently, both approaches, when used in tandem, would provide a better representation of the richness of taxa in the samples analysed.

5 Conclusions

This study showed that the morphological and molecular-based approaches were complementary. Application of any single method will only capture a fraction of the whole phytoplankton community. The combination of methods enables a more thorough understanding of the phytoplankton diversity in coastal waters, especially with regard to the distribution of harmful algal species. The higher diversity of HAB-forming species found here warrants more monitoring of these waters. For the sequencing of low abundance taxa and the prokaryotic component of the phytoplankton, it is suggested that a multi-primer PCR strategy targeting different genes and/or regions be applied to increase the probability of detecting a broad variety of taxa and to avoid undersampling due to overrepresentation of some OTUs.

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