

# Diversity of *Heterocapsa* (Dinophyceae) and the algal bloom event in the mariculture areas of Johor Strait, Malaysia

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**Abstract:** In November 2020, a high biomass multi-species algal bloom caused heavy water discoloration in the fish and mussel farm areas of the Johor Strait, Malaysia. A total of 19 microalgal taxa were identified from the plankton samples collected during the bloom event. Eleven genera were diatoms, and eight genera were dinophytes. The microalgal composition was dominated by the diatom *Guinardia* sp., with an average cell density of  $1.7 \times 10^6$  cells  $L^{-1}$ , making up 65–80% of the phytoplankton composition. Concomitantly, high densities of the dinophytes *Heterocapsa minima* ( $3.8\text{--}5.3 \times 10^5$  cells  $L^{-1}$ ) and *Karlodinium* spp. ( $3.5\text{--}6.6 \times 10^3$  cells  $L^{-1}$ ) were found. This is the first record of the occurrence of *H. minima* in Malaysian waters. Detailed morphological observations of *H. minima* based on scanning electron microscopy are presented in this study. To have a better insight into the *Heterocapsa* species assemblages in the Johor Strait, the diversity of *Heterocapsa* species assemblages along the strait was investigated based on a metabarcoding approach. Environmental DNA collected between 2018 and 2019 was used for high throughput amplicon sequencing targeting the small subunit (SSU) ribosomal RNA gene marker. The metabarcoding analysis detected three rare *Heterocapsa* species in the waters, *H. niei*, *H. rotundata*, and *H. steinii*. The results showed that *Heterocapsa* species assemblages varied temporally across the strait, with higher species diversity and amplicon sequence variant (ASV) read abundances detected in the Eastern Johor Strait. Although no fish/shellfish kills were sighted during the 2020 bloom event, the presence of harmful microalgal species, such as *Heterocapsa minima* and *Karlodinium* spp., urged the need for a comprehensive HAB monitoring program in the Strait to safeguard the aquaculture industry in the areas.

**Key words:** diatom, dinoflagellates, harmful algal bloom, *Heterocapsa*; shellfish

## Introduction

Phytoplankton plays an important role in marine and freshwater ecosystems as they serve as a primary producer and support higher trophic levels of aquatic ecosystems. These microscopic organisms also provide dissolved oxygen essential for marine organisms as part of the by-prod-

uct of the photosynthesis process (Falkowski et al. 2003). However, Harmful algal blooms (HAB), an increase in the microalgal biomass, can cause deleterious effects on the environment and its organisms. In other words, HAB is a natural phenomenon due to the rapid proliferation of one or more species of microalgae in the environment (Lim et al. 2012). The impact of HABs has been accelerated by the rapid development and expansion of aquaculture areas that disrupt the nutrient balance of aquatic ecosystems (Bukola et al. 2015, Er et al. 2018).

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HAB not only affects human health (Hallegraeff et al. 2021), but it also causes a tremendous impact on the aquaculture industry by causing massive fish mortality. Many past studies have demonstrated that some HAB species produced neurotoxins that killed marine organisms (e.g., Lim et al. 2014, Furuya et al. 2018) while others promoted hypoxic/anoxic conditions (e.g., Escobar et al. 2013, Mohd-Din et al. 2020). HAB species that caused mass fish mortality have been well documented in the coastal waters of Malaysia (Yñiguez et al. 2021). These included the raphidophytes *Chattonella* B.Biecheler (Choo 1994, Leaw et al. 2018, Lum et al. 2021), the dinophytes *Ceratium* (*Tripes*) *furca* (Lemmerman) Jörgesen (unpublished data), *Margalefidinium polykrioides* (Margalef) F.Gómez, Richlen & D.M.Anderson (Anton et al. 2008, Harun et al. 2015, Mohd Razali and Shahuntalla 2017), *Noctiluca scintillans* (Macartney) Kofoid & Swezy (Choo 1994, Mohd Razali et al. 2016), and *Karlodinium australe* Salas, Bolch & Hallegraeff (Lim et al. 2014, Teng et al. 2016). Blooms of *Margalefidinium* sp. along the coast of northwestern Peninsular Malaysia in 2020 were among the massive blooms that caused massive fish kills in the aquaculture areas (Mohd Razali et al. 2020).

HABs have also been reported to impact shellfish aquaculture worldwide (Shumway 1990, Matsuyama et al. 1997a). The dinophyte species *Heterocapsa circularisquama* Horiguchi, *Gymnodinium aureolum* (E.M.Hulburt) Gert Hansen, and the raphidophyte *Chattonella marina* (Subrahmanyam) Y.Hara & M.Chihara have been reported to kill shellfish (Matsuyama 1999, Basti et al. 2011, Kim et al. 2011b). However, to our knowledge, there is no information on the occurrence of this type of HAB species, particularly the species of *Heterocapsa* F.Stein, in Malaysian waters. This study documented, for the first time, a bloom event associated with high abundances of *Heterocapsa* species in the proximity of shellfish farms in the Johor Strait, Malaysia. In addition, to fill the gaps, this study aims to provide insights into the spatial and temporal distribution of *Heterocapsa* species in the Johor Strait by applying next-generation sequencing using the metabarcoding approach.

## Materials and Methods

### Study sites and sampling strategies

On 13 November 2020, a field survey was undertaken at three locations along the Johor Strait: A1 (1°27'19.1"N 103°41'31.1"E), A2 (1°28'9.2"N 103°50'58.7"E), and A3 (1°27'41.0"N 103°51'40.1"E) (Fig. 1). Duplicate water samples were collected using a Van Dorn water sampler from each station. One liter of water sample was filtered through a 10 µm-mesh sieve. Plankton samples that were retained on the sieve were washed back into a 100 mL sampling bottle and preserved with 1% acidic Lugol's solution for cell enumeration and identification. Water quality param-

eters of salinity, pH, temperature, total dissolved oxygen (DO; mg L<sup>-1</sup>), and total suspended solids (TSS) were measured *in situ* using a YSI ProPlus multiparameter water quality probe (Yellow Spring, Ohio, USA). Dissolved inorganic macronutrients (nitrate, nitrite, ammonia, and phosphate) were measured using the HACH DR3900 Spectrophotometer (HACH, USA) according to the manufacturer's protocols.

### Phytoplankton cell enumeration and species identification

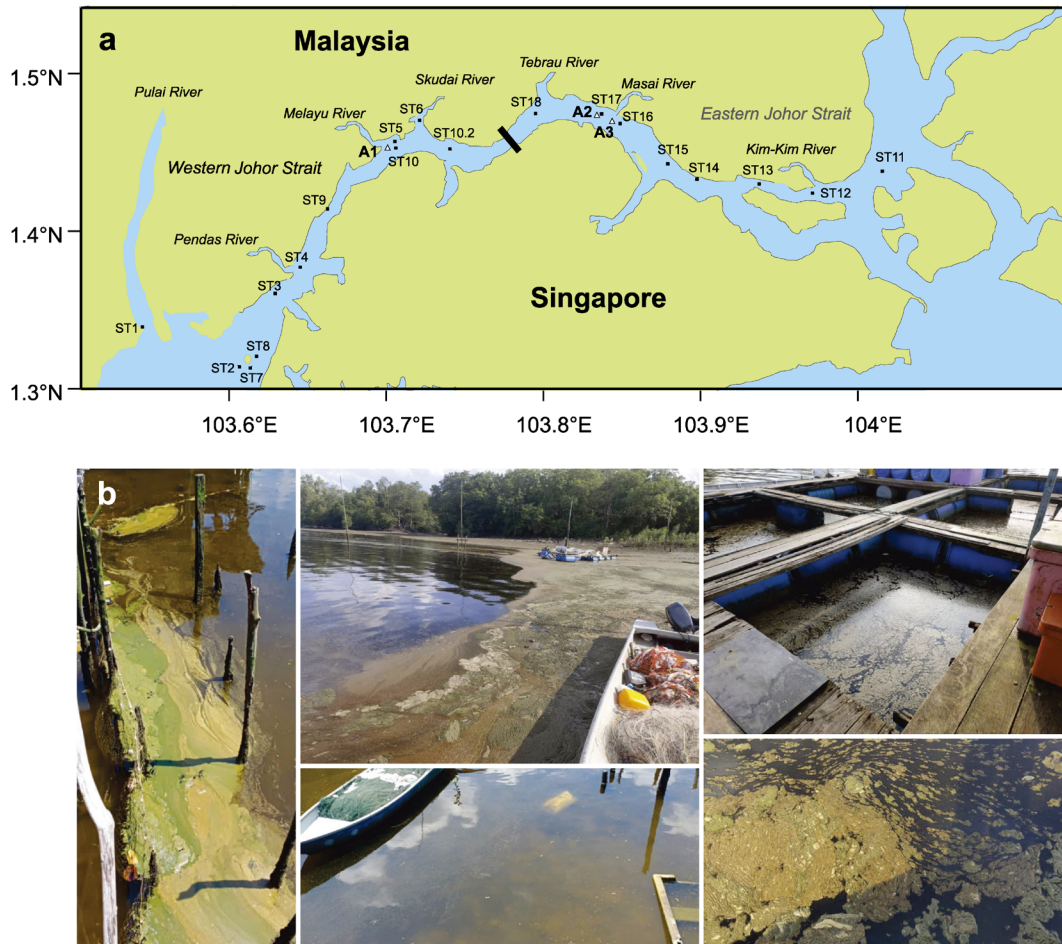
Phytoplankton cell densities were determined by counting 1 mL of samples using a Sedgewick Rafter slide under a Leica CME compound microscope (Buffalo, NY, USA). Phytoplankton cell densities (in cells L<sup>-1</sup>) were determined from triplicate counts.

For species identification, cells were observed under an Olympus IX70 inverted microscope (Tokyo, Japan) at a 100× magnification. Digital micrographs were captured using a CCD camera and Analysis<sup>®</sup> software (Soft Imaging System Inc., USA). Phytoplankton taxa were identified to genus and/or species levels according to Round et al. (1990), Hasle et al. (1996), Tomas (1997), Omura et al. (2012). The taxonomic assignments were further confirmed by referring to AlgaeBase (Guiry and Guiry 2021; <http://www.algaebase.org>) and the World Register of Marine Species (WoRMS Editorial Board 2022; <http://www.marinespecies.org>).

### Morphological observations of *Heterocapsa* species

Preserved cells of *Heterocapsa* species were examined with light microscopy (LM) using an Olympus IX51 inverted light microscope, equipped with an Olympus DP72 digital camera (Olympus, Tokyo, Japan). Digital images of cells were captured to record cell shape, the position of pyrenoids, and position and shape of the nucleus.

For scanning electron microscopy (SEM), cells were pre-treated with 60% ethanol by centrifugation at 800×g for 5 min; the supernatant was discarded. These pre-treatment steps were repeated twice. Distilled water was added to re-suspend the pelleted cells. The cells were then filtered onto a 3-µm polycarbonate membrane filter using a vacuum manifold. The dehydration steps were started with 30% ethanol and gradually increased to 50%, 75%, 90%, 95%, 99%, 99.5% and 100% ethanol. Each of the dehydration steps was carried out for at least 15 min. The membrane was folded and transferred into a 1.5 mL centrifuge tube containing 300 µL of 100% ethanol. The sample was kept at -20°C for 15 min, then freeze-dried at -115°C with a pressure of 0.2 bar for 40 min. The treated samples were mounted onto a stub and coated with a thin layer of gold-palladium. The samples were observed under a JSM-6510 SEM (JEOL, Japan).



**Fig. 1.** (a) Map showing the sampling locations in Johor Strait, Malaysia, during the bloom events on 12 November 2020 (triangle symbols) and monthly sampling locations between May 2018 and September 2019 (square symbols). (b) Water discoloration in the aquaculture areas of Station A1.

### Assessing *Heterocapsa* diversity assemblages by DNA metabarcoding

In order to have better insight into the *Heterocapsa* species assemblages in the Johor Strait, this study has retrieved the environmental DNA (eDNA) samples as described in Hii et al. (2021). The samples were collected between May 2018 and September 2019 at 19 sampling stations across the Johor Strait (Hii et al. 2021; as illustrated in Fig. 1). In brief, the plankton samples were collected using a 15  $\mu\text{m}$ -mesh size plankton net, followed by vacuum-filtration onto a 0.2  $\mu\text{m}$ -mesh pore-size nylon filter membrane (Johnson<sup>®</sup>, Tividale, England) and preserved with 10 mL of modified saline ethanol (Miller and Scholin 2001) in a sterile conical tube and kept at  $-20^{\circ}\text{C}$ .

Genomic DNA was extracted from the saline ethanol-preserved samples using the TOYOBO Magextractor plant genome extraction kit (Tokyo, Japan). The V9 region of the small subunit ribosomal RNA gene (SSU rDNA) was amplified using a pair of eukaryotes-universal primers, 1391F (5'-GTACACACCGCCCGTC-3') and EuKB (5'-TGATCC TTCTGCAGGTT-CACCTAC-3') (Stoeck et al. 2010) as

described in Hii et al. (2021). The amplicons were then sequenced with an Illumina MiSeq platform (Illumina, San Diego, USA) using a paired-end MiSeq Reagent Kit v2 ( $2 \times 250$  bp) following the manufacturer's instructions.

Sequences obtained in this study are available in the Sequence Read Archive, NCBI (<http://www.ebi.ac.uk/ena>) under the accession numbers BioProject PRJNA727463. The sequencing results were quality filtered, merged, dereplicated, and the chimeras removed using the DADA2 pipeline in R (Callahan et al. 2016). The parameters were set as follows: maxEE=c (2,2); minLen=200; truncLen=c (90,90); minBoot=80; and Min overlap=12 bases. The taxonomy of the amplicon sequence variants (ASVs) was then annotated with a naïve Bayesian classifier (Wang et al. 2007) as implemented in the DADA2 pipeline against the Protist Ribosomal Reference (PR2) database (Guillou et al. 2013) and own in-house *Heterocapsa* curated database (available upon request). The spatial and temporal distribution of *Heterocapsa* species (in ASV reads) in the Johor Strait were visualized by a bubble chart plotted using *ggplot2* (Wickham 2016) in R package (R Core-team 2021).

## Results and Discussion

### The 2020 bloom event

On 12 November 2020, the Fisheries Department of Johore State (Malaysia) received a report from the local green mussel farmers regarding water discoloration around the water of Melayu River (station A1; Fig. 1a). Intense green to brown patches of discoloration had been sighted covering an area of about 10 km in length from Melayu River to Pendas. The local folks also noticed waters turned slimy with odor for several days (Fig. 1b). There are 20 mussel farms and six caged fish breeders in the Melayu River area. Fortunately, no mussel mortality or fish kills were reported during the bloom event.

A total of 19 taxa of microalgae were documented at the three sampling stations (A1–A3) during the bloom event, with 11 genera of diatoms and eight genera of dinophytes (Figs 2, 3). The microalgal composition was dominated by the diatom *Guinardia* sp., with cell densities ranging between  $1.2 \times 10^6$  cells  $L^{-1}$  and  $2.4 \times 10^6$  cells  $L^{-1}$  (67–80% of the relative abundance; Fig. 3). Bloom-forming and harmful species of dinophytes, *Karlodinium* sp. and *Heterocapsa minima* A.J.Pomroy, were found at considerably high cell densities, ranging from  $3.5\text{--}6.6 \times 10^3$  cells  $L^{-1}$  and  $3.8\text{--}5.3 \times 10^5$  cells  $L^{-1}$ , respectively (Fig. 3a). Other harmful or potentially harmful dinophyte taxa, *Alexandrium*, *Ceratium* (*Triplos*), *Dinophysis*, *Gyrodinium*, *Margalefidinium*, and *Protoperidinium* were also recorded in the samples but composed only a minority of the overall phytoplankton (<0.2%; Fig. 3c).

The environmental properties of temperature, pH, and salinity recorded at the sampling stations during the bloom event ranged from 30.5–32.4°C, pH 8.21–8.82, and salinity of 14.3–18.1, respectively. The DO levels of 7.18–10.57 mg  $L^{-1}$  were within the recommended range for aquaculture. The concentrations of ammonia, nitrate, and phosphate were in the ranges of 0.13–0.19 mg  $L^{-1}$ , 0.3–1.2 mg  $L^{-1}$ , and 0.77–1.76 mg  $L^{-1}$ , respectively; which exceeded the acceptable values of the Malaysian Marine Water Quality Criteria and Standard (Class 2; Department of Environment Malaysia 2021). A lower nitrite level was recorded, 0.008–0.03 mg  $L^{-1}$ . TSS concentrations ranged from 231 mg  $L^{-1}$  to 468 mg  $L^{-1}$ , also exceeding the acceptable values of Marine Water Quality Criteria and Standard.

It is well recognized that environmental factors such as temperature, salinity, light penetration, and unbalanced nutrient conditions affect both the growth of phytoplankton and alter the phenology of phytoplankton blooms (Anderson et al. 2002, Granéli and Flynn 2006, Trombetta et al. 2019). Excess of macronutrients such as nitrogen and phosphorus are the primary drivers for coastal eutrophication (Glibert and Burford 2017) and this has caused the phytoplankton community to shift from a diverse assemblage to the proliferation of harmful microalgal species (Anderson et al. 2012, Hallegraeff et al. 2021). Previous

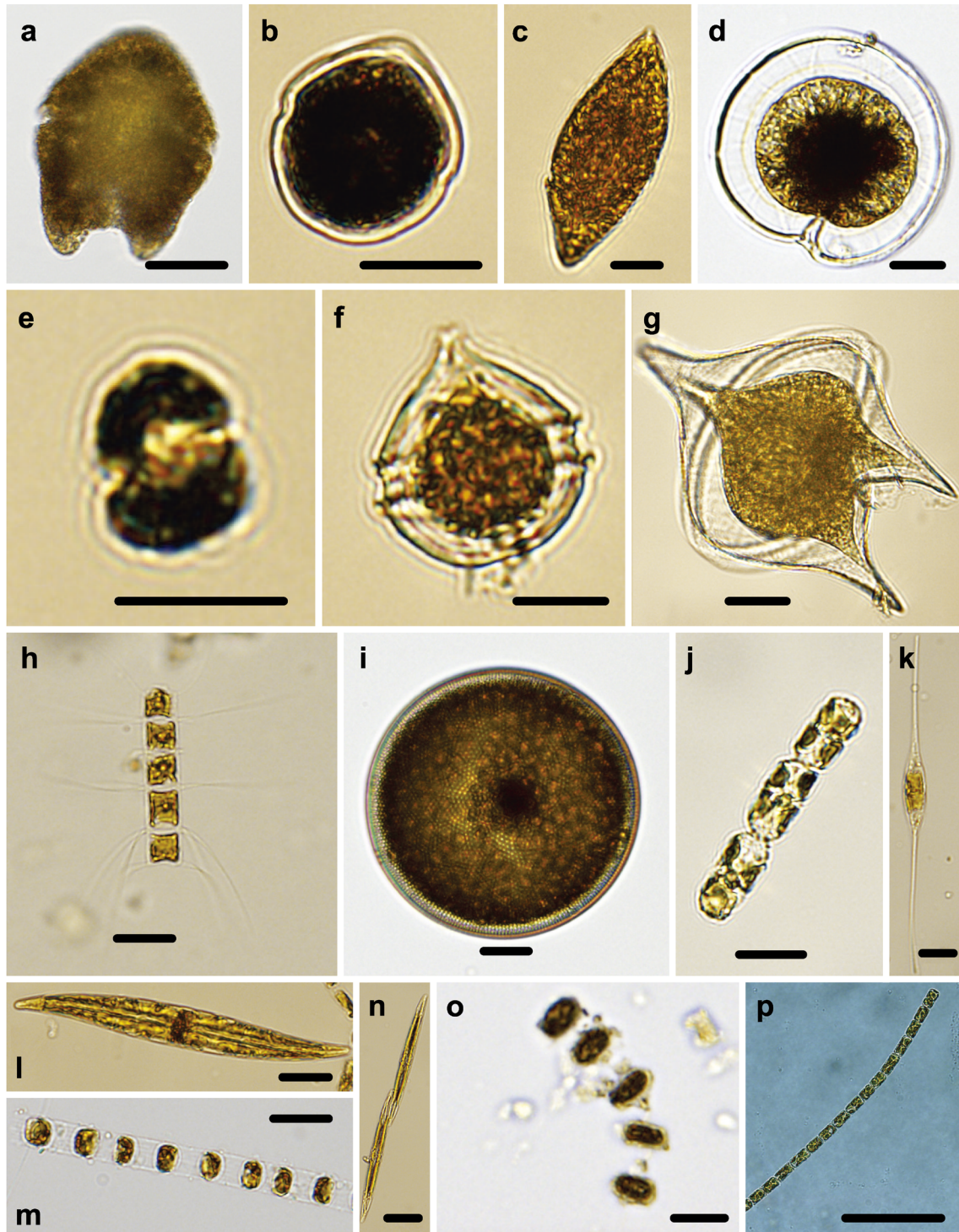
studies have shown that the nutrient dynamics and environmental variabilities significantly influenced the spatio-temporal shifts of phytoplankton community assemblages in the Johor Strait (Chai et al. 2021, Mohd-Din et al. 2020, 2022), and likely promoted HAB events in the aquaculture sites (Lim et al. 2014, Kok and Leong, 2019, Hii et al. 2021). For instance, enrichment of inorganic nitrogen shifted the community from the dominant diatom *Rhizosolenia* to the chain-forming diatoms like *Skeletonema*, *Pseudo-nitzschia*, and *Thalassiosira* in the Johor Strait (Hii et al. 2021, Mohd-Din et al. 2022), particularly in the freshwater-influenced environment (Mohd-Din et al. 2022). Several past studies also claimed that the harmful dinoflagellate blooms in the area were attributable to eutrophication, especially during the high availability of phosphate (Bužančić et al. 2016, Mohd-Din et al. 2020). The nutrient phosphate is required by dinoflagellates (Temperton et al. 2011, Sahraoui et al. 2013) for their growth metabolic structures and functions (Lin et al. 2016).

*Heterocapsa* species are small thecate dinophytes, ranging from 6 to 45  $\mu m$  (Iwataki 2008). To date, there are 25 species of *Heterocapsa* that have been described, 21 species are taxonomically accepted (Guiry and Guiry 2021). Species of *Heterocapsa* are distributed worldwide (Boonyapiwat 1999, Matsuyama 1999, Iwataki 2008, Hernández-Becerril et al. 2010, Baek et al. 2011, Salas et al. 2014, Tan et al. 2016, Lee et al. 2019, Balci et al. 2020). The species *Heterocapsa minima* was found, for the first time, in bloom samples collected from the Johor Strait. This represents the first record of the species occurrence in Malaysian waters. The species has a wide geographical distribution, as it has been reported in the Northeast Atlantic Ocean (Iriarte et al. 2003, Salas et al. 2014), Gulf of Naples, the Tyrrhenian Sea (Percopo et al. 2011), Korean (Lee et al. 2019), and New Zealand waters (Balci et al. 2020).

Cells of *H. minima* collected from Johor Strait resembled *H. minima* described previously (Pomroy 1869, Lee et al. 2019). Morphologically, the cells have a conical shape of epitheca, rounded hypotheca with a larger epitheca (Fig. 4). The cells ranged from 6.5–9.2  $\mu m$  long ( $7.76 \pm 0.98 \mu m$ ;  $n=5$ ) and 5–6  $\mu m$  wide ( $5.21 \pm 0.32 \mu m$ ;  $n=5$ ). The large and ellipsoid nucleus is in the hypotheca, whereas the spherical pyrenoid is positioned in the epitheca. The thecal plate arrangement is as follows: Po, cp, X, 5', 3a, 7'', 6c, 5s, 5'', 2'''. No cell with a red accumulation body was observed in the preserved environmental samples. Cells of *H. minima* collected from Johor Strait also possess the thecal pores found surrounding the cell (Fig. 4) as described in the cells of *H. minima* (HMMJ1604) collected from Korea (Lee et al. 2019).

### Spatio-temporal distribution of *Heterocapsa* species in Johor Strait

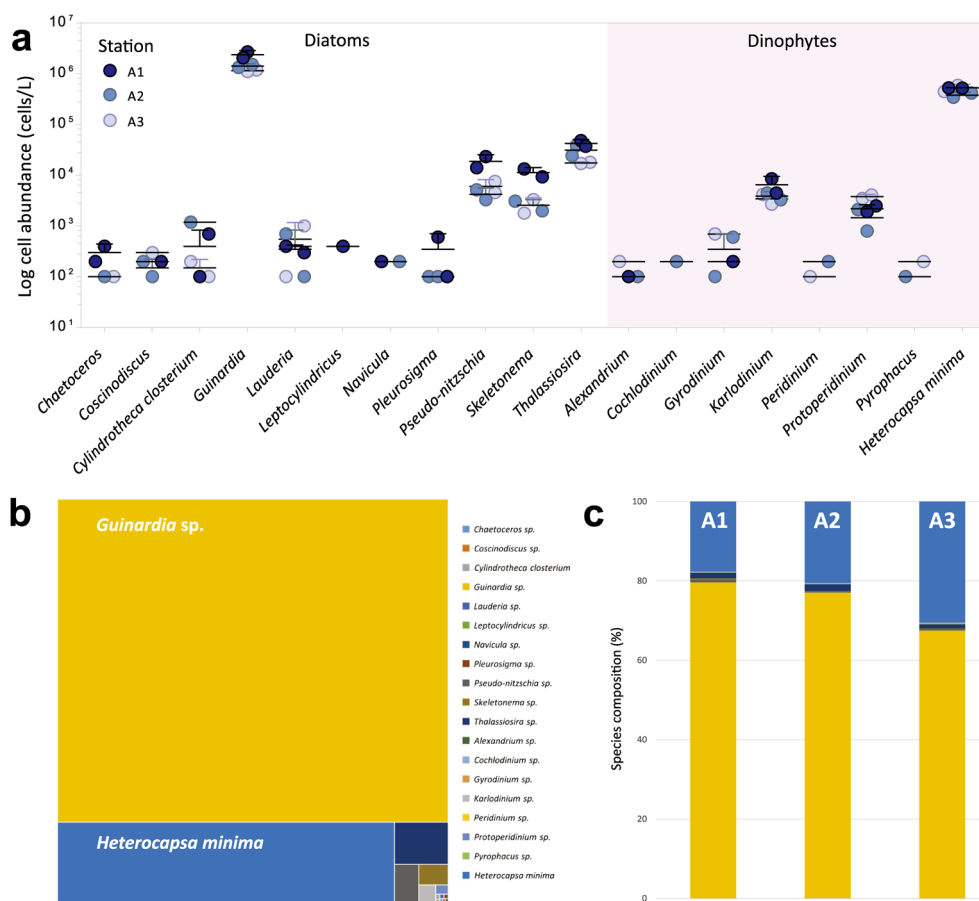
Blooms of *Heterocapsa* have been reported in several Asia Pacific countries, including Japan, China, and Korea



**Fig. 2.** Common diatoms and dinoflagellates observed during the bloom event on November 2020 in the Johor Strait. (a) *Akashiwo sanguinea*. (b) *Alexandrium* sp. (c) *Gyrodinium* sp. (d) *Pyrophacus* sp. (e) *Karlodinium* sp. (f) *Peridinium* sp. (g) *Protoperidinium* sp. (h) *Chaetoceros* sp. (i) *Coscinodiscus* sp. (j) *Lauderia* sp. (k) *Cylindrotheca* sp. (l) *Pleurosigma* sp. (m) *Skeletonema* sp. (n) *Pseudo-nitzschia* sp. (o) *Thalassiosira* sp. (p) *Guinardia* sp. Scales, 20  $\mu\text{m}$  (a–o), 100  $\mu\text{m}$  (p).

(Horiguchi 1995, Matsuyama 1999, Iwataki et al. 2002, Baek et al. 2011, Sakamoto et al. 2021). *Heterocapsa* species that were associated with red tides were *H. circularisquama* and *H. rotundata* (Lohmann) Gert Hansen (Boonyapiwat 1999, Matsuyama 1999, Kamiyama et al.

2001, Iwataki et al. 2002, Litaker et al. 2002, Hernández-Becerril et al. 2010, Baek et al. 2011). These bloom-forming species caused water discoloration, and some of the bloom events have been associated with mollusk kills (Matsuyama 1999, Iwataki et al. 2002, Matsuyama 2003a,



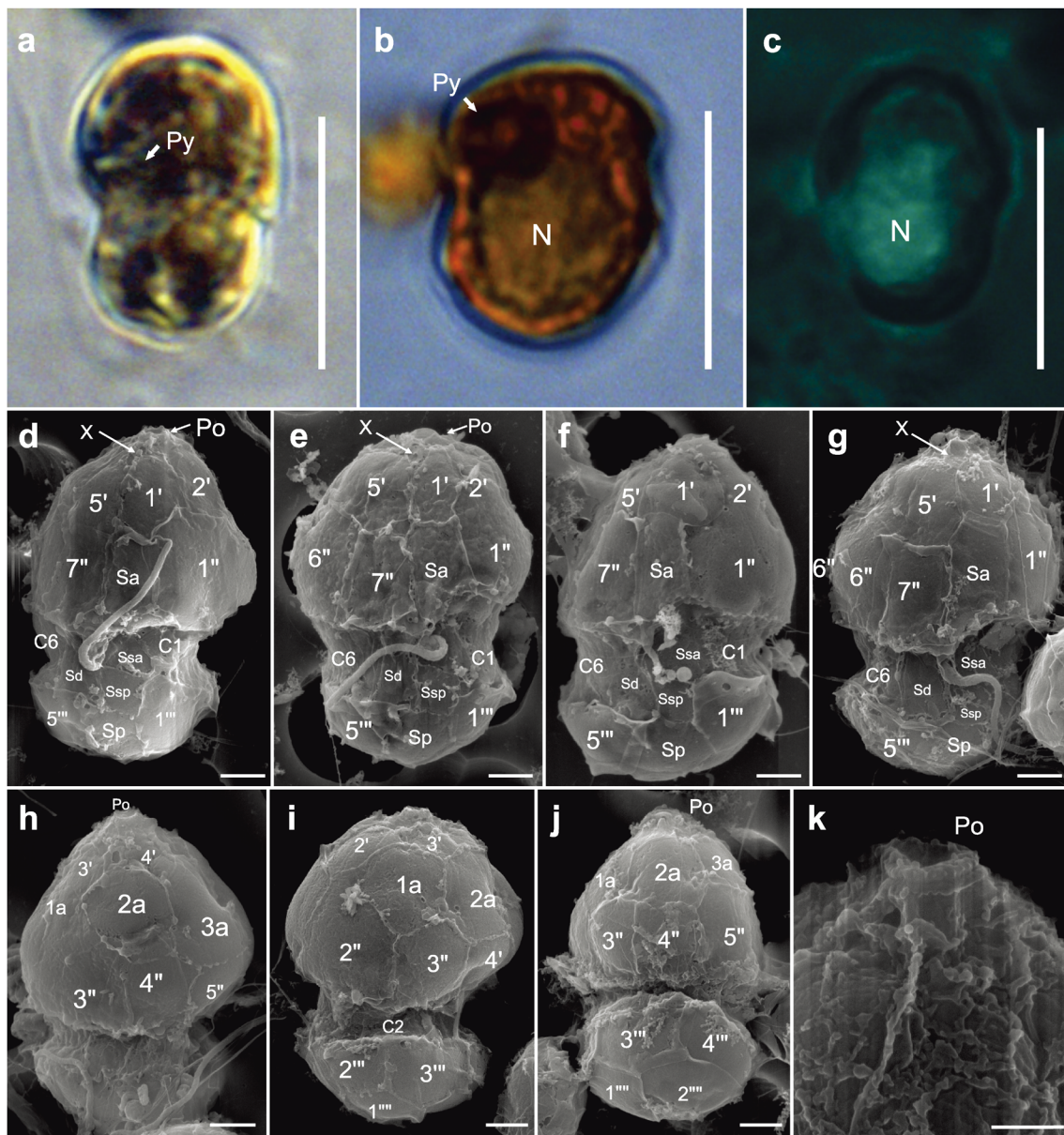
**Fig. 3.** Phytoplankton abundances (a) during the bloom event on November 2020 at the three sampling stations in Johor Strait. Tree map (b) showing the dominant groups of phytoplankton and (c) species compositions in percentages.

Basti et al. 2009, Basti et al. 2011). For instance, a *H. circularisquama* bloom that reached up to  $25 \times 10^4$  cells  $\text{mL}^{-1}$  resulted in the mortality of shellfish in Japan (Matsuyama 1999, Matsuyama 2003a, Basti et al. 2009). In a laboratory experiment, bivalves showed behaviors such as vigorous clapping and shrinkage of the mantle after exposure to *H. circularisquama* (Nagai et al. 1996, Basti et al. 2009, Kim et al. 2011a, Matsuyama 2012). Cells of *H. circularisquama* might cause damage to several organs of shellfish, thus leading to physiological stress and ultimately resulting in death (Kim et al. 2011a, Basti et al. 2011, 2016). Besides, *H. circularisquama* also adversely affects the development of larvae of several bivalves, and the reproduction of bivalves (Matsuyama 2003b, 2012, Basti et al. 2011, 2013). While *Heterocapsa* blooms caused massive shellfish kills, there have been no reports on harmful effects on wild and cultured finfishes (Horiguchi 1995, Matsuyama 1999, Iwataki et al. 2002, Baek et al. 2011, Sakamoto et al. 2021).

Since most *Heterocapsa* species are relatively small and morphologically similar, they are difficult to differentiate from each other by conventional light microscopy (Horiguchi 1995, Iwataki 2008). The presence of *Heterocapsa* species in the tropical Asian region is limited to a few records with new species descriptions (Iwataki et al. 2009, Xiao et

al. 2018, Benico et al. 2021, Choi and Kim 2021) and they have never been previously encountered in Malaysian waters. Given the sparse knowledge of this harmful species, particularly of the tropical populations, it is thus, important to gain more information regarding the species occurrence, distribution, and the environmental driving factors that permit *Heterocapsa* to bloom at unpredictable intervals of time.

High throughput sequencing with a metabarcoding approach applied on the 194 plankton eDNA samples collected between May 2018 and September 2019 from the Johor Strait revealed a total of 211 ASVs that were classifiable as *Heterocapsa* species, including 131 ASVs corresponding to *H. niei* (A.R.Loeblich) L.C.Morrill & A.R.Loeblich, 30 ASVs corresponding to *H. rotundata*, and 50 ASVs as *H. steinii* Tillmann, Gottschling, Hoppenrath, Kusber & Elbrächter. However, the newly identified species *H. minima* was not detected in our metabarcoding data. One possible explanation is that *H. minima* is relatively smaller in size ( $< 10 \mu\text{m}$ ), and the sampling approach adopted in this study by using a  $15 \mu\text{m}$ -mesh plankton net most likely did not retain the cells in the samples, thus hampering the detection of this species in these metabarcoding datasets. Furthermore, currently there is no SSU rDNA sequence yet avail-



**Fig. 4.** *Heterocapsa minima*. LM. (a–b) Lugol's fixed cells showing pyrenoids (Py) and nucleus (N). Scales, 5  $\mu$ m. (c) SYBR-stained cells showing rounded nucleus in the hypotheca. Scale, 5  $\mu$ m. SEM. (d–k). Ventral (d–g) and dorsal views (h–k) showing the thecal plate arrangement. Po, apical pore plate; X, canal plate; 1'–5', apical plates; 1a–3a, anterior intercalary plates; 1''–7'', precingular plates; C1–C6, cingular plates; Sa, anterior sulcal plate; Ssa, left anterior sulcal plate; Ssp, left posterior sulcal plate; Sd, right sulcal plate; Sp, posterior sulcal plate; 1'''–5''', postcingular plates; 1''''–2'''', antapical plates. Scales, 1  $\mu$ m. (k) Close up of the apical pore plate, Po. Scale, 0.5  $\mu$ m.

able for this species in the public databases for sequence verification and metabarcoding data validation. Detection of harmful microalgae based on a metabarcoding approach is dependent on the availability of reference sequences in database libraries. Therefore, it is important to obtain sequence information for HAB species to improve the sequence database library for future molecular detection of HAB species using a metabarcoding approach (Nagai et al. 2017, Hii et al. 2021).

Nonetheless, the metabarcoding approach detected three rare *Heterocapsa* species in these waters, i.e., *H. niei*, *H. rotundata*, and *H. steinii*. The *Heterocapsa* species assem-

blages varied temporally across the strait (Fig. 5). Higher species diversity and ASV read abundances were detected in the Eastern Johor Strait (EJS). *Heterocapsa niei* was the most abundant and was detected in March 2019, followed by *H. rotundata*. A high ASV read abundance of *H. rotundata* was observed in July 2019 (ST14, EJS), and gradually decreased toward the outermost station, ST18 (Fig. 5). Slightly higher ASV reads of *H. rotundata* (1607 reads) were also observed at WJS (ST7) in July 2019. Considerably high ASV read abundances of *H. steinii* were also observed at ST14 and ST15 during May, August, and September 2019, and ST18 in April 2019, where August

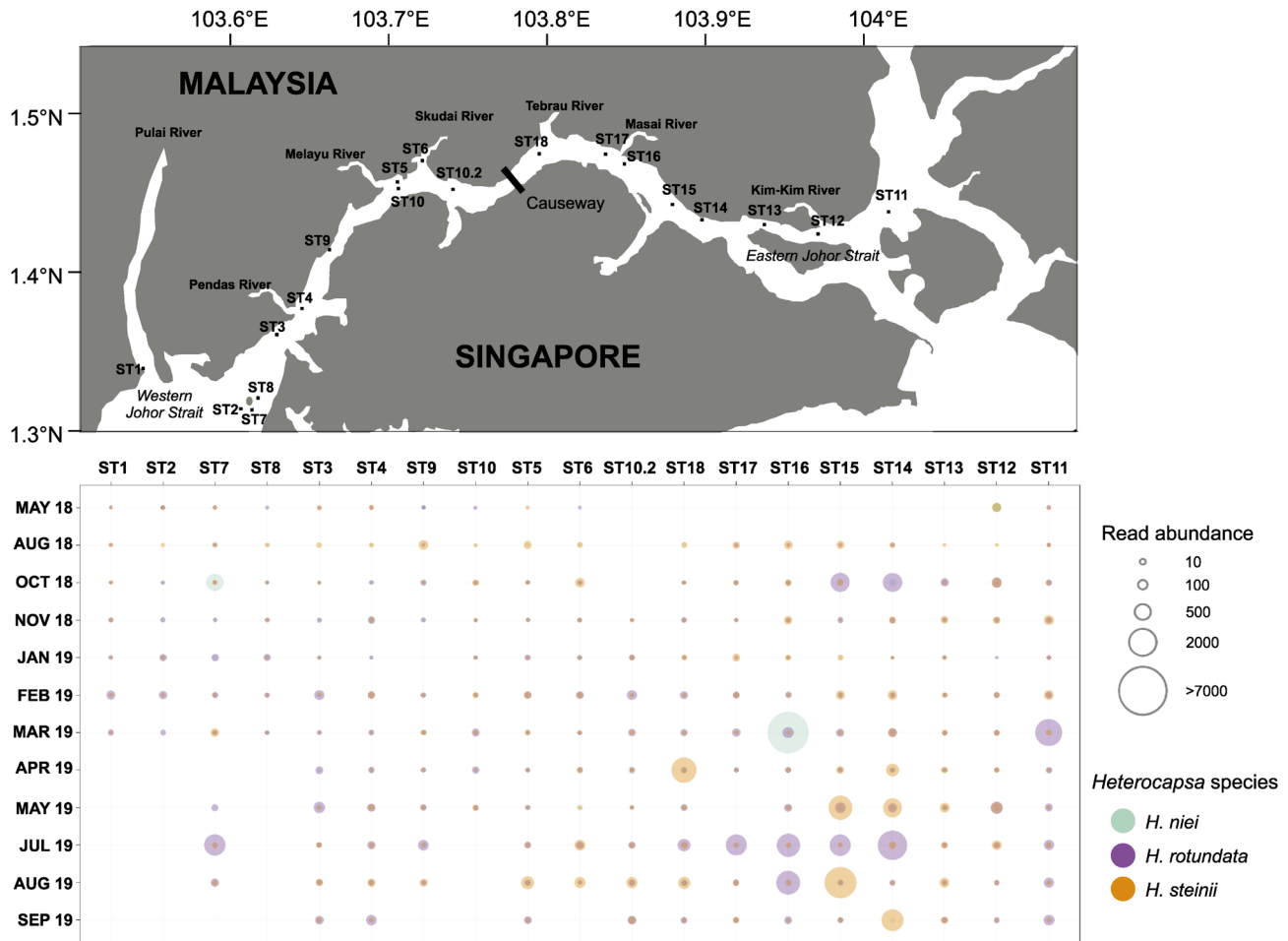


Fig. 5. Spatio-temporal information on *Heterocapsa* species assemblages in the Johor Strait between May 2018 and September 2019.

2019 (ST15) recorded the highest ASV reads (3,940 reads) throughout the sampling period.

### Conclusion

The bloom event in the West Johor Strait in November 2020 was attributed to a multispecies bloom comprised of the diatom *Guinardia* sp., and the dinophytes *Heterocapsa minima* and *Karlodinium* spp. This is the first report of the presence of *H. minima* in Malaysian waters. Although no fish/shellfish mortality was reported during the bloom event, the presence of HAB species, in this case, *Heterocapsa minima* and *Karlodinium* spp., urged the need for a comprehensive monitoring program to safeguard the aquaculture industry in the area.

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