Report of a Fish Kill Due to a Dinoflagellate Bloom in Perak and Penang, Malaysia

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Abstract

A fish kill incident was reported at the marine fish culture areas north of Perak and south of Penang, Malaysia, on 26 May 2020. An investigation was carried out at 10 stations in Kerian, Perak and Seberang Perai Selatan, Penang. Seawater samples were collected to identify microalgae species and determination of dissolved inorganic nutrients. The physical parameters of water such as salinity, pH, temperature and dissolved oxygen were measured *in situ* while the dissolved inorganic nutrients were analysed spectrophotometrically. The dominant microalga was identified as *Margalefidinium fulvescens* (Iwataki, Kawami & Matsuoka) Gómez, Richlen & Anderson, 2017, based on the morphological and molecular characterisation of the large subunit ribosomal gene. Long rounded and ellipsoidal cells, 30-43 µm in length, appeared in chains of single, two, four or eight cells. The sulcus was slightly narrow surrounding the cell about one turn, but the cingulum was rather deep, encircling the cell approximately twice, and the chloroplasts were brownish, granular and scattered peripherally. The highest *M. fulvescens* cell counts were recorded at 6.22×10^5 cells L^{-1} and 4.61×10^5 cells L^{-1} in Kerian, Perak and Seberang Perai Selatan, Penang, respectively. The physical parameters of the seawater from the affected sites were within the Malaysian Marine Water Quality Standard (MMWQS) for aquaculture. However, slightly higher levels of nitrate, phosphate and ammonia were noted at several stations. Although the exact cause of the bloom was undecided, it could be due to nutrient discharge along the coasts, which also concurred with the transition phase of the northeast to the southwest monsoon.

Keywords: LSU rDNA, fish mortality, fish kill, microalgae bloom, *Margalefidinium fulvescens*

Introduction

Perak and Penang are two main marine fish producing states in Peninsular Malaysia. Intensive fish farming areas are situated in Kerian and Larut Matang districts in Perak (Figs. 1, 2). Meanwhile, for Penang, the Seberang Perai Selatan district is a significant culture area (Figs. 1, 2). In 2019, aquaculture production from Perak and Penang contributed about 6,948.16 and 21,329.19 tonnes of marine fish, respectively, with an approximate value of MYR687.58 million (USD154.40 million) (Department of Fisheries, 2019). This constituted about 76.4 % of the total cultured marine fish in Malaysia. The main species cultured are barramundi (*Lates calcarifer* (Bloch, 1790)), brown-marbled grouper (*Epinephelus fuscoguttatus* (Forsskål, 1775)), snubnose pompano (*Trachinotus blochii* (Lacépède, 1801)), giant trevally (*Caranx ignobilis* (Forsskål, 1775)) and mangrove red snappers (*Lutjanus argentimaculatus* (Forsskål, 1775)).

Unfortunately, harmful algal blooms (HABs) often affect these two states. HABs are commonly associated with public health impact, commercial fisheries, and tourism, leading to economic losses (Shumway, 1990; Hallegraeff, 2003; Basti et al., 2019). Several types of microalgae are involved in HAB events. Some HAB species include *Alexandrium* spp., *Dinophysis* spp., *Pyrodinium bahamense var compressum* (Böhm) Steidinger, Tester & Taylor, 1980,

Gymnodinium catenatum Graham, 1943 and *Pseudo-nitzchia* spp. are toxin producers and may intoxicate humans. On the other hand, some species of the genera *Chatonella*, *Heterosigma*, *Karlodinium* and *Margalefidinium* could kill fish through the production of ichthyotoxic compounds such as reactive oxygen species (ROS) and polyunsaturated fatty acids, irritate the gills and trigger the secretion of mucus that damages or clogs the gill and leads to suffocation (Dorantes-Aranda et al., 2015; Hallegraeff et al., 2017). Non-toxic species such as *Chaetoceros* spp., *Rhizosolenia* spp., *Skeletonema* spp. and *Tripos* spp. may also cause fish kill due to oxygen depletion in the water column during the decomposition of the microalgae, thus creating hypoxic or anoxic conditions (Rensel and Whyte, 2003; Lopez et al., 2008).

Fig. 1. Map showing locations (red circle) of harmful algal blooms associated with fish kills reported in Peninsular Malaysia.

Table 1 summarises the HABs associated with fish kill events in Peninsular Malaysia. The earliest HABs case was reported in 1978 in Teluk Kumbar, Penang, with *Noctiluca scintillans* (Macartney) Kofoid & Swezy, 1921, as the culprit (Choo, 1994) (Fig. 1). In 2005, mortality of cultured fish worth around MYR20 million (USD4.49 million) was reported in Penang with an unknown causative organism (Lim et al., 2012). The recurrence of fish kills in Sungai Udang, Penang was reported in January 2016 by an unknown causative organism (Roziawati and Shahunthala, 2018). In addition, the *Tripos furca* (Ehrenberg) Gómez, 2013, bloom that caused red discolouration and farmed fish kill along the coastal waters of Sungai Dinding, Lumut, Perak was described in 2007 (Department of Fisheries, 2007) and again in June 2008 (DOF, unpublished data). Later, a fish kill due to *Margalefidinium polykrikoides* (Margalef) Gómez, Richlen & Anderson, 2017, bloom was detected in Perak coastal waters in March 2013 (Siti NorRohaida et al., 2015; Roziawati and Shahuntala, 2018), followed by *N. scintillans* bloom at Kuala Gula, Perak in 2016 (Roziawati et al., 2016). The most recent case of HAB associated with mass mortality occurred on 26 May 2020, in Kerian district, Perak and Penang, with estimated losses of about MYR11.0 million (USD2.47 million). In addition, the presence of fish-killing microalgae species such as *Akashiwo sanguinea* (K. Hirasaka) Gert Hansen & Moestrup, 2000, *Margalefidinium* spp., *Karlodinium australe* Salas, Bolch & Hallegraeff, 2005 and *N. scintillans* in marine fish cage culture areas of Pulau Aman and Sungai Udang, Penang was observed by Usup et al. (2002), Roziawati et al. (2015) and Roziawati et al. (2021).

This paper presents an overview of the HABs event in Perak and Penang in May 2020, particularly the quantification of cells and identification of the causative species. Microalgae species identification was confirmed with molecular tools owing to the challenges of using a microscope for identification.

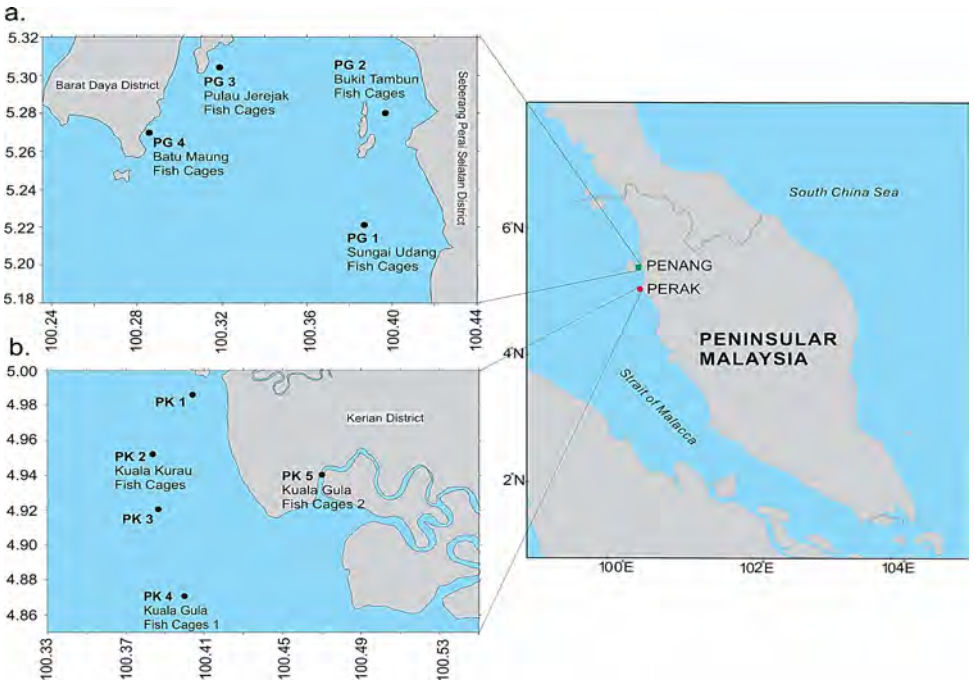

Fig. 2. Map of Peninsular Malaysia showing the seawater sampling locations at Penang (a) and Kerian, Perak (b) during the harmful algae bloom events in May 2020.

Table 1. A summary of harmful algal blooms (HABs) associated fish kill events in Peninsular Malaysia.

Timeline	Harmful algae	Location	Impact	References
1978	*Noctiluca scintillans* (Macartney) Kofoid & Swezy, 1921	Teluk Kumbar, Penang	Mortality of fish	Choo (1994)
2005	unknown causative organism	Penang	Red tide and mortality of cultured fish	Lim et al. (2012)
2007	*Tripos furca* (Ehrenberg) Gómez, 2013	Sg. Dinding, Perak	Water discolouration	Department of Fisheries (2007)
2008	*T. furca*	Sg. Dinding, Perak	Mortality of cultured fish	Department of Fisheries, unpublished data
2013	*Margalefidinium polykrikoides* (Margalef) Gómez, Richlen & Anderson, 2017	Kerian, Perak	Mortality of cultured fish	Siti NorRohaida et al. (2015); Roziawati and Shahuntala (2018)
2014	*Karlodinium australe* Salas, Bolch & Hallegraeff, 2005	West Johor Strait	Mortality of cultured fish	Lim et al. (2014)
2015	*K. australe*	Johor Strait	Mortality of cultured fish	Teng et al. (2016)
2016	*N. scintillans*	Kuala Gula, Kerian, Perak	Mortality of cultured fish	Roziawati et al. (2016)

The actions taken, and management measures for fisheries and aquaculture stakeholders are also highlighted.

Materials and Methods

Harmful algal bloom events

On 26 May 2020, the Kerian, Perak Fisheries District Office, Department of Fisheries (DOF), received a report from local farmers concerning the occurrence of a fish kill at cage culture areas in Kuala Kurau and Kuala Gula, Perak. The farmers noticed sea water discolourations (brownish-red) around the cages a few days before the fish died. They also witnessed fish swimming near the surface, appearing breathless, and swimming erratically. The most affected fish at the farm was the mangrove red snapper. The event became more serious during the subsequent 3 days, continued for another 2 weeks and subsided by 3 June 2020. On 29 May 2020, the fish kill spread north to Tanjung Piandang, Perak and Seberang Perai Selatan District, Penang (adjacent to Kerian) particularly Sungai Udang, Bukit Tambun and Batu Maung. At this point, more species were affected, including barramundi, groupers, snubnose pompano and giant trevally. There was no observation of wild fish or other marine life killed.

Sample collection

A field investigation was carried out at Kerian, Perak (6 stations) and Seberang Perai Selatan (4 stations), as specified in Table 2 and shown in Figure 2. Dead fish were collected and put in an ice-cooled insulated box. Live microalgae samples (100 mL) were collected using a 20 µm mesh-size plankton net for species identification and molecular characterisation. Sub-surface seawater samples (1 L) were collected using Van Dorn water sampler to quantify microalgae species and determine the inorganic nutrient levels. Dead fish were sent to the Fisheries Research Institute's Fish Health Research Division laboratory for post-mortem.

Physico-chemical analysis of the seawater samples

Seawater's physical parameters, including pH, salinity, temperature and dissolved oxygen (DO), were measured *in situ* using YSI Pro DSS Multi-parameter Water Quality Probe (Yellow Spring, USA). Sub-surface seawater samples (1 L) were brought back to the laboratory and immediately analysed spectrophotometrically for dissolved inorganic nutrients (nitrate, nitrite, ammonia and phosphate) using HACH DR3900 spectrophotometer (HACH, USA) according to the manufacturer's instruction. The total suspended solids (TSS) were determined with photometric method (HACH, USA) according to the manufacturer's instruction.

Microalgae identification and quantification

Cells were observed under an inverted microscope (IX70, Olympus, Japan) and digital images of cells were captured using CCD camera and Analysis (R) software (Soft Imaging System Inc., USA). Seawater samples (1 L) for microalgae quantification were concentrated by filtering through a 20 µm-mesh sieve and subsequently preserved in Lugol's iodine solution. A concentrated microalgae sub-sample (1 mL) was placed in the Sedgewick-Rafter counting chamber and the cell density (L^{-1}) of each phytoplankton was enumerated under a microscope (Leica CME, USA) in three replicates. Microalgae identification was performed following Tomas (1997), Backer et al. (2003), Iwataki et al. (2007), Omura et al. (2012) and Iwataki et al. (2015).

Molecular characterisation

A single dinoflagellate cell was picked using a micro-

Table 2. Locations of seawater samples collected during the harmful algal bloom events.

Sampling stations	Location coordinates	Location details	Sampling dates (2020)
PK 1	N4°59'11.6", E100°24'13.9"	River mouth of Kuala Kurau, Perak	27 May, 29 May
PK 2	N4°57'10.4", E100°23'01.4"	Kuala Kurau cage culture area	27 May, 29 May, 11 June
PK 3	N4°55'15.0", E100°23'11.1"	Outside fish cages area, Kuala Kurau	27 May, 29 May, 11 June
PK 4	N4°52'15.0", E100°24'01.1"	Kuala Gula, Perak cage culture 1	27 May, 29 May, 11 June
PK 5	N4°56'23.3", E100°28'13.0"	Kuala Gula, Perak cage culture 2	29 May, 11 June
PK 6	N5°05'28.6", E100°21'11.7"	Tg. Piandang cage culture	11 June
PG 1	N5°13'15.8", E100°23'13.3"	Sungai Udang, Penang cage culture	29 May, 2 June, 3June, 11 June
PG 2	N5°16'48.0", E100°23'48.8"	Bukit Tambun, Penang cage culture	29 May, 2 June, 3 June, 11 June
PG 3	N5°18'14.7",E100°19'08.3"	Pulau Jerejak, Penang cage culture	11 June
PG 4	N5°16'10.9", E100°17'09.2"	Batu Maung, Penang cage culture	2 June, 3 June, 11 June

pipette under an inverted microscope (IX70, Olympus, Japan) and rinsed in droplets of sterile-filtered seawater. The cell was transferred into a 0.2 mL PCR tube containing 1 µL of TE buffer (Tris 1 M, EDTA 0.5 M, pH 8). Genomic DNAs (gDNAs) of the cell were extracted by Proteinase K (20 mg.L^{-1}) at 55 °C for 30 min, followed by 10 min at 95 °C. The gDNAs were then used as a template for gene amplification. The gene amplification was performed using the universal primer pair of D1R (5'-ACC CGC TGA ATT TAA GCA TA-3') and D3Ca (5'- ACG AAC GAT TGC ACG TCA G-3'), targeting the large subunit ribosomal DNA gene (LSU rDNA) (Scholin et al., 1993). Amplification was performed in 25 µL reaction volumes containing 12.5 µL of MyTaq Red mix, 2× (Bioline, USA), 0.1 µM of each primer, 5.0 µL of the template using a thermal cycler (Kyratec, Australia), with the following cycling condition: pre-denaturation at 94 °C for 4 min, 35 cycles of 94 °C for 10 s, 55 °C for 10 s and 68 °C for 15 s, a modification from Crenn et al. (2018).

The size and quality of PCR products were assessed in 1.5 % agarose gel electrophoresis. The PCR products were sent to Apical Scientific Sdn Bhd for purification and Sanger sequencing. The purified products were used as the template DNA for cycle sequencing reactions performed using BigDye® Terminator v3.1 cycle sequencing kit chemistry (Applied Biosystems, USA), and sequencing was conducted on an ABI PRISM 3730 (Applied Biosystems, USA) automatic sequencer. Both DNA strands were sequenced to ensure accuracy. For species identification, steps involving a similarity-based method were employed. Each sequence was compared to available GenBank sequences using the Basic Local Assignment Search Tool (BLAST) search routine implemented in GenBank (default parameters) as measured by percentage maximum sequence identity; the best hit was retained.

Molecular phylogenetic analyses

The obtained sequences of LSU rDNA were aligned with the related sequences from NCBI Genbank using the clustalW algorithm (Thompson et al., 1994), and were subsequently edited manually at the part that was misaligned. The LSU rDNA dataset of this study comprised nine nucleotide sequences of *Margalefidinium fulvescens* (Iwataki, Kawami & Matsuoka) Gómez, Richlen & Anderson, 2017 and 11 other close taxa sequences, which were used for phylogenetic analyses (as the accession numbers of each taxon are shown in the phylogenetic tree; Fig. 4). *Karenia mikimotoi* (Miyake & Kominami ex Oda) Gert Hansen & Moestrup, 2000, *G. catenatum* and *A. sanguinea* were set up as an outgroup in the analyses. Maximum parsimony (MP) and maximum likelihood (ML) were conducted using PAUP ver. 4.0a169 (Swofford, 2000). Hierarchical likelihood ratio test and tree-bisection-reconnection were performed in ML analyses. The best model fit of evolution was generated from jModelTest 2.1.9 (Darriba et al., 2012), with the best substitution and rate heterogeneity model of TIM3 + I + G for ML and the Bayesian inference (BI) analyses. Maximum parsimony was performed by a heuristic search of 1000 random addition and TBR branch swapping. Bootstrapping for ML and MP was analysed with 100 and 1000 replication, respectively. Bayesian inference (BI) was examined using MrBayes 3.2 (Ronquist et al., 2012) with four simultaneous MCMC chains of 1 × 10^6 generations each run and sampled at every 1000 generations. Posterior probabilities (PP) were estimated at 25 % burn-in with a majority rule consensus tree.

Results

Phytoplankton composition

A total of 59 phytoplankton taxa were identified from the sampling areas in Kerian, Perak, belonging to three classes, i.e., Bacillariophyceae (diatoms) (41 taxa), Dinophyceae (dinoflagellates) (16 taxa) and Cyanophyceae (blue-green algae) (2 taxa). Meanwhile, a total of 48 taxa were identified from the sampling areas in Seberang Perai Selatan District, Penang, belonging to three classes, i.e., Bacillariophyceae (32 taxa), Dinophyceae (14 taxa) and Cyanophyceae (2 taxa).

Causative species description

Seawater samples, observed under the microscope, showed a high number of unarmoured dinoflagellate cells resembling *M. fulvescens,* as described by Iwataki et al. (2007, 2015). The species exist in single-cell forms and chains of 2, 4 and 8 cells which were rounded to ellipsoidal, ranging from 30.78–43.86 µm in length and 26.35–36.90 µm in width (Fig. 3). *Margalefidinium fulvescens* had a spherical nucleus at the central epicone and an eyespot in the anterior dorsal part of the cell. The sulcus was relatively narrow, surrounding the cell about one turn. The chloroplasts of *M. fulvescens* were granulated and distributed along the cingulum and periphery of the cell, brownish in colour, and scattered peripherally. The cingulum was rather deep, encircling the cell approximately twice, but the sulcus was rather narrow, surrounding the cell about one turn. An orange pigmented body also appeared in the epicone. The sulcus of *M. fulvescens* is located at the intermediate position of the cingulum on the dorsal side.

Molecular identification of the causative species

A fragment of about 699 bp was obtained from the single cell, and the BlastN search hits showed high similarity with *M. fulvescens* isolate ZG-28 in GenBank (Sequence ID: MN828327.1) with an identity score of 99 %. The sequence was registered with the GenBank database with the accession number MW646468. In phylogenetic analysis, all the MP, ML and BI analyses revealed the same tree topology with two monophyletic clades, *M. polykrikoides* and *M. fulvescens* groups. Both *Margalefidinium* clades were strongly supported by MP, ML and BI (97/97/1) (Fig. 4). *Margalefidinium fulvescens* sequence from this study and other Asian *M. fulvescens* strains from China and Japan were clustered together with the strong support of MP, ML and BI (91/95/97) (Fig. 4). In contrast, the Europan *M. fulvescens* from California, Spain and Canada formed paraphyletic clades with the Asian strain (Fig. 4), where the strain from Canada and Spain were strongly supported by MP, ML and BI (100/100/1). Whereas the strain from California was only relatively supported by BI (0.81). The sequence divergence values between this study's *M. fulvescens* sequences and both sequences from China and California strains were 0.0059 % (3/507 bp) and 0.0216 % (11/507 bp), respectively.

Phytoplankton abundance

Figure 5 illustrates the sampling stations, phytoplankton density (cells L^{-1}) and relative abundance (%) in Perak on 27 May 2020 and 29 May 2020. The highest counts of *Margalefidinium* spp. was observed at station PK 4 on 27 May 2020, ranging from 3.35×10^5 cells L^{-1} to 6.22×10^5 cells L^{-1} (70.6 % of total phytoplankton) followed by station PK 1 (1.51×10^4 cells L^{-1}, 23.6 % of total phytoplankton), station PK 3 (1.87×10^4 cells L^{-1}, 20.5 % of total phytoplankton) and station PK 2 (8.85×10^3 cells L^{-1}, 8.89 % of total phytoplankton). On 29 May 2020, *Margalefidinium* spp. counts were much lowered at station PK 1 (3.30×10^2 cells L^{-1}), slightly reduced at station PK 4 (1.03×10^4 cells L^{-1}), remained around the same density at station PK 3 (1.07×10^4 cells L^{-1}) and increased at station PK 2 (2.63×10^4 cells L^{-1}). *Margalefidinium* spp. was detected at station PK 5 on 29 May 2020 but in low abundance (5.1×10^3 cells L^{-1}). Other dinoflagellates include *Tripos* spp., *Dinophysis* spp., *Gyrodinium* spp., *Prorocentrum* spp., *Protoperidinium* spp., *Phyrophacus* spp. and *Scripsiella* spp. were observed in Kerian on 27 May and 29 May 2020, but only in minority (<8.0 % of the overall phytoplankton composition).

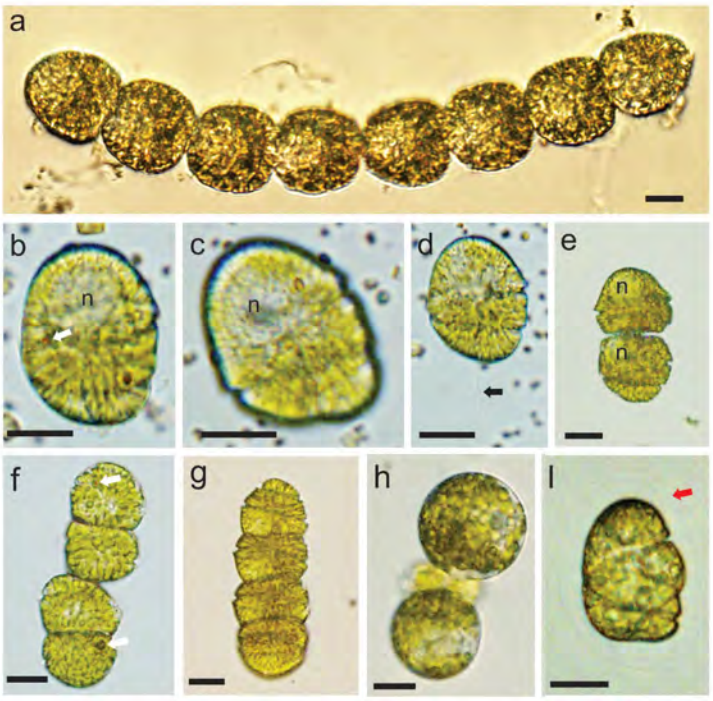

Fig. 3. Light micrographs of *Margalefidinium fulvescens*. (a) chain of eight cells fixed with Lugol's solution, (b–d) three single cells showing reddish orange pigmented body (white arrow), a spherical nucleus (n) located in the central epicone, sulcus position and flagellum (black arrow); (e) chain of 2 cells, (f, g) chain of four cells; white arrow indicate eyes spot, (h, i) Cysts of *M. fulvescens*, red arrow indicate hyaline membrane. Scale = 20 µm.

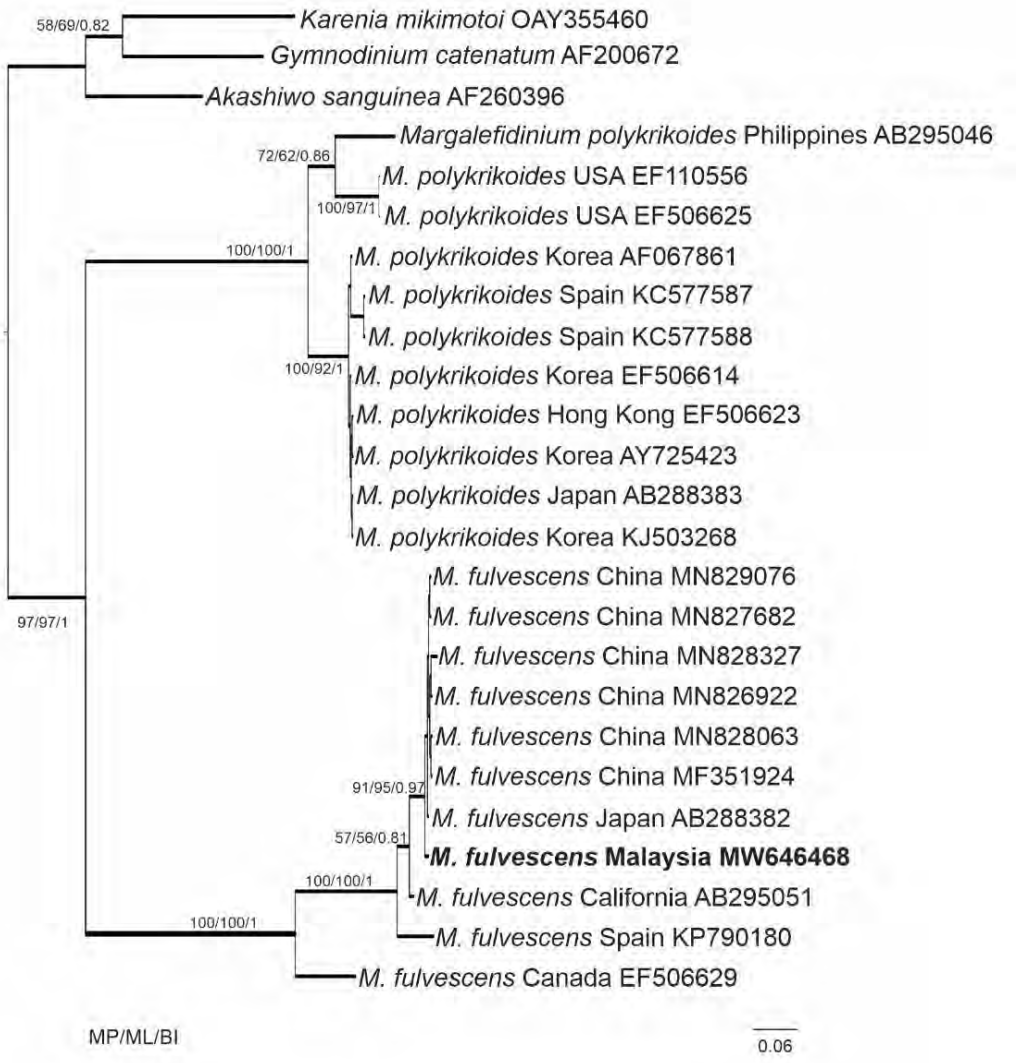

Fig.4. *Margalefidinium* Bayesian tree based on large subunit ribosomal DNA gene (LSU rDNA) sequences. Values at nodes are maximum parsimony (MP)/maximum likelihood (ML)/Bayesian inference (BI), bootstrap value and Bayesian Markov chain Monte Carlo (BMCMC) posterior probabilities. Sequences obtained in this study were in bold. Scale bar indicates a branch length corresponding to 0.06 substitutions per site.

Algal bloom and fish kill were also reported at Tanjung Piandang, Perak, north of Kuala Gula and Kuala Kurau on 29 June 2020, however, seawater samples were not collected for observation. Seawater samples collected on 11 June 2020 at Tanjung Piandang indicated a low number of *Margalefidium* spp. (<100 cells L^{-1}) and phytoplankton counts were dominated by diatoms (98.7 %), particularly *Bacillaria* spp. (57.0 %). On the same date, the phytoplankton composition at Kuala Kurau and Kuala Gula was also dominated by diatoms, but *Thallassiosira* spp. was dominant with 20.9 % and 37.1 %, respectively. *Margalefidinium* spp. was very low, indicating that the bloom was over in Kerian coastal waters.

The bloom also spread adjacent to the district, i.e., Seberang Perai Selatan Penang. As presented in Figure 6, on 29 May 2020, a high number of *Margalefidinium* spp. were observed in seawater samples collected from station PG 1 (Sungai Udang fish cages area) with counts up to 1.44×10^5 cells L^{-1} (46.9 % of total phytoplankton count). Within days, *Margalefidinium* spp. density at station PG 1 was reduced to only 0.4 %, 0.6 % and 0.8 % of total phytoplankton counts on 2, 3 and 11 June, respectively. Meanwhile, further north at station PG 2 (Bukit Tambun fish cages), the *Margalefidinium* spp. cell density gradually increased from about 2.4 % of total phytoplankton (1.02×10^4 cells L^{-1}) on 29 May 2020 to 46.6 % of total phytoplankton (2.79×10^5 cells L^{-1}) on 2 June reduced to only 0.8 % and 0.7 % of total phytoplankton on 3 and 11 June respectively. *Margalefidinium* spp. was detected at station PG 3 (Batu Maung fish cages), which is situated east of Bukit Tambun, on 2 June 2020, with the highest cell count, which comprised 73.2 % of total phytoplankton (4.61×10^5 cells L^{-1}) and it caused the mass fish kill, but subsequently decreased on 3 June 2020 to 3.58×10^4 cells L^{-1} (18.3 %).

Other dinoflagellates such as *Alexandrium* spp., *Ceratium* spp., *Dinophysis* spp., *Gonyaulax* spp.,

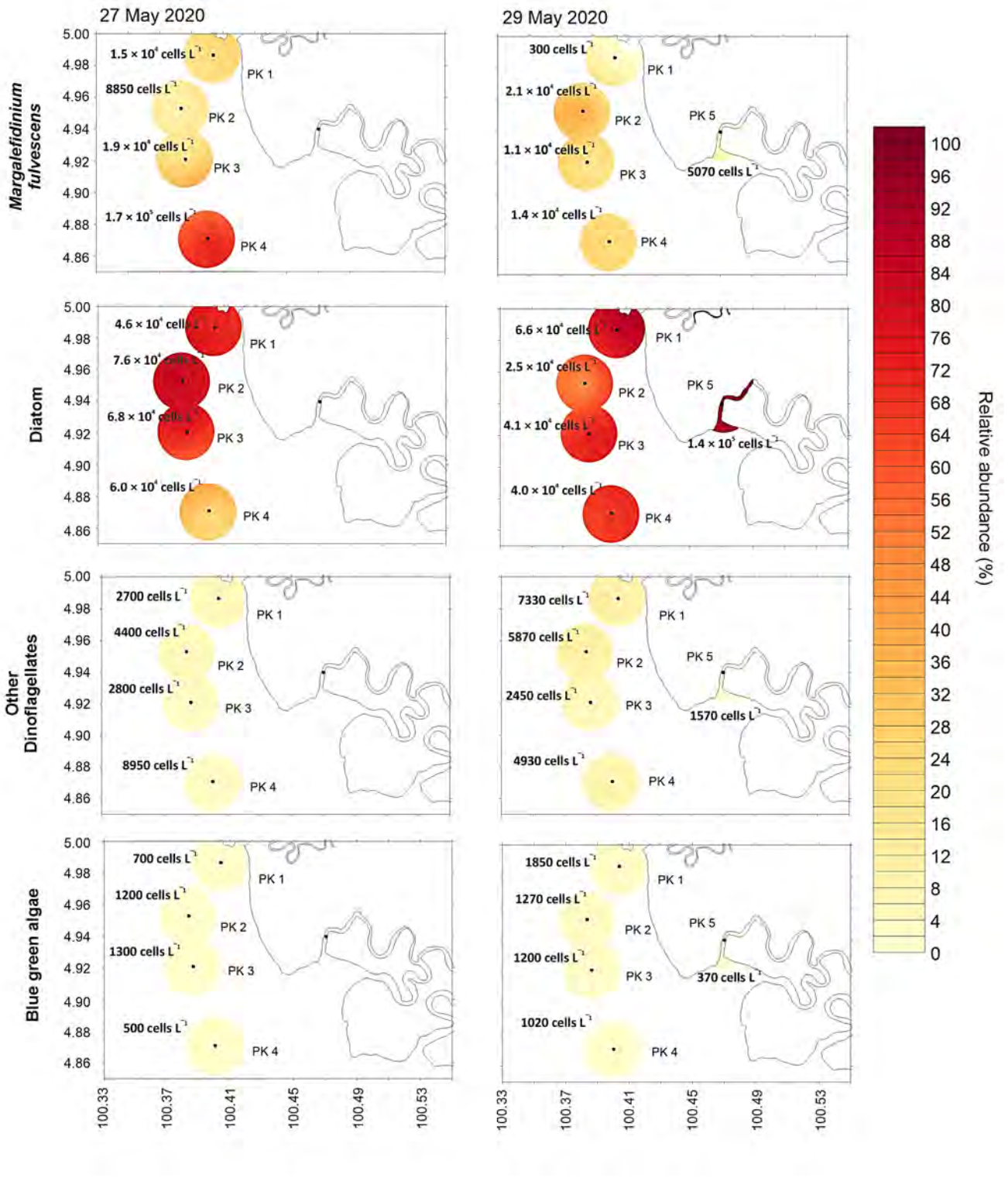

Fig. 5. Contour map showed the cell density (cells L^{-1}) related to the bloom of dinoflagellate, *Margalefidinium fulvescens* and relative abundance (%) of phytoplankton at each station in Kerian, Perak on 27 May 2020 and 29 May 2020.

Gymnodinium spp., *Gyrodinium* spp., *Karlodinium* spp., *N. scintillans*, *Prorocentrum* spp., *Protoperidinium* spp., *Phyrophacus* spp. and *Scripsiella* spp. were recorded in Penang samples but comprised only a minority of the overall phytoplankton composition (<9.0 %). No "red-tide" or mass mortality of fish was reported in Pulau Jerejak fish cages area even though the location is close to the area affected by the blooms. The cells were almost diminished (<300 cells L^{-1}) by 11 June 2020 in all sampling stations suggesting that the bloom was over.

Physico-chemical parameters of seawater

All physical parameters of seawater in Perak and Penang were within the acceptable range for aquaculture activities, as indicated in the Malaysian

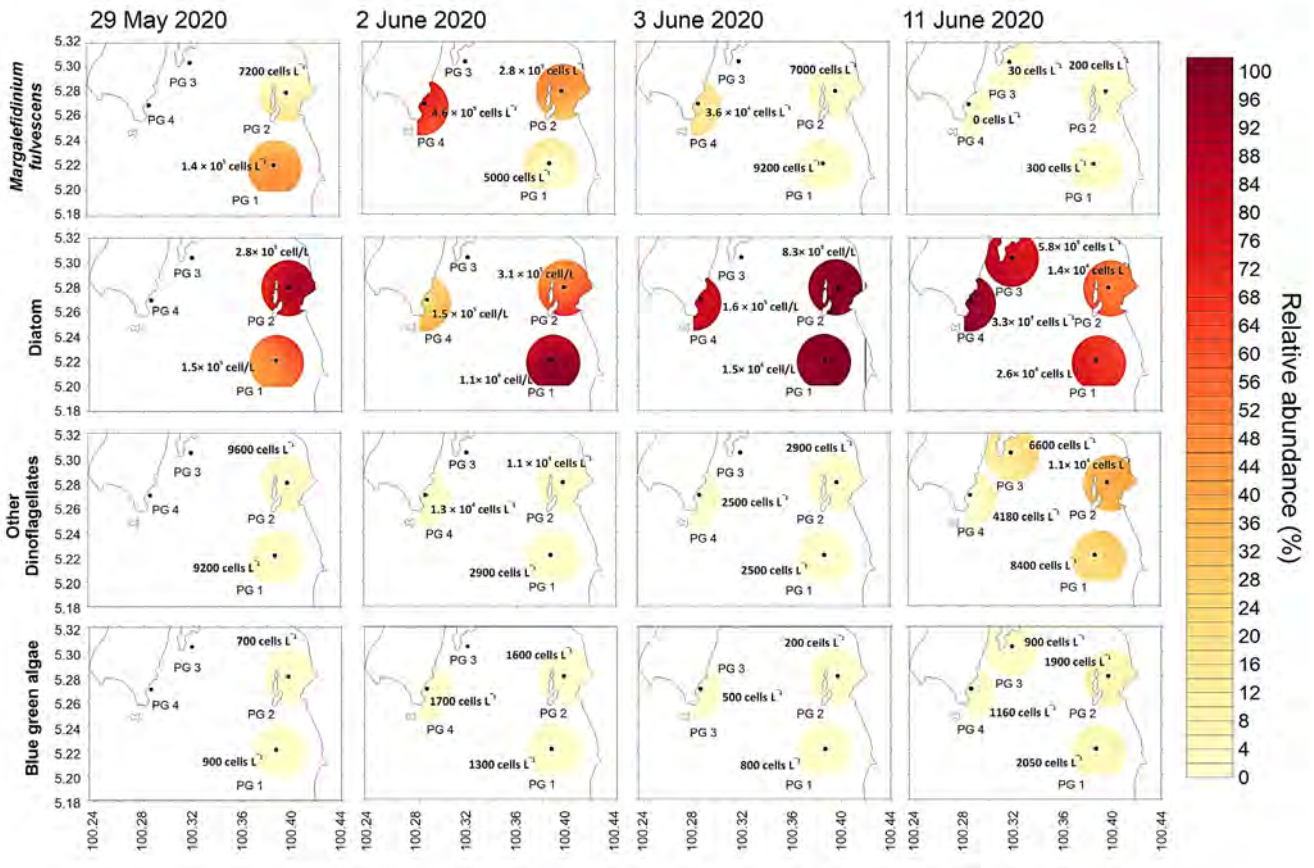

Fig. 6. Contour map showed the cell density (cells L^{-1}) related to the bloom of dinoflagellate, *Margalefidinium fulvescens* and relative abundance (%) of phytoplankton at each station in Penang on 29 May 2020, 2 June 2020, 3 June 2020 and 11 June 2020.

Marine Water Quality Standard (MMWQS). The sea-surface temperature and pH in the vicinity of the sampling stations in Kerian waters ranged from 30.47–31.23 °C and 7.54–8.20, respectively. The salinity was lower at the river mouth of Kuala Kurau (PK 1) (14.69–20.20 ppt), compared to three other sampling locations further out to the sea (25.19–31.05 ppt). The dissolved oxygen (DO) levels ranged from 4.26–6.31 mg.L^{-1}. Meanwhile, in Penang, the physical parameters recorded were temperature (30.7–31.50 °C), pH (7.54–8.20), salinity (22.67–29.80 ppt) and DO (5.20–9.05 mg.L^{-1}).

Table 3 and 4 present the nutrients level examined at the sampling stations in Perak and Penang, respectively. Nitrate and phosphate levels were higher than the MMWQS acceptable level of 0.06 mg.L^{-1} and 0.075 mg.L^{-1}, respectively, in some stations and on certain dates in Perak and Penang sampling locations. Some stations in Penang record slightly higher levels of ammonia than the MMWQS recommended levels of 0.05 mg.L^{-1}. On the other hand, the total suspended solid (TSS) was low at all stations.

Discussion

In the current study's phylogenetic analysis of the LSU rDNA gene, *M. fulvescens* was closely related to *M. fulvescens* strains reported from China and Japan. However, the Canadian *M. fulvescens* strain has the greatest genetically distinct from all the *M. fulvescens* stains, including the Malaysian strain. This finding supports the study of Hu et al. (2018), suggesting it could be due to the inter-populational diversity between Canadian species with other regional species. In addition, the close relation of *M. fulvescens* to a clade based on partial LSU rDNA sequences was also reported by Iwataki et al. (2015), Gomez et al. (2017) and Hu et al. (2018). The taxonomy of the *Cochlodinium* genus was revised in 2017, where *C. polykrikoides* and *C. fulvescens* were put under the *Margalefidinium* genus. *Margalefidinium polykrikoides* and *M. fulvescens* are the two main harmful algae species associated with fish kill under this new genus (Gómez et al., 2017). *Margalefidinium fulvescens* was first described in 2007 from Asian waters (Tachibana Bay, west Japan, and Hurun Bay in south Sumatra of Indonesia) (Iwataki et al., 2007). After that *M. fulvescens* was associated with HAB in coastal waters of Canada (Bates et al., 2020), USA (Anderson et al., 2021), the Gulf of California, Mexico (Gárate-Lizárraga et al., 2008, 2009, 2014), China (Zhangxi et al., 2018), Japan, Indonesia (Iwataki et al., 2007), Korea (Kudela and Gobler, 2012), Pakistan (Munir et al., 2012) and the southern coast of Vietnam (Iwataki et al., 2015). This dinoflagellate was described as a fish-killer and exhibited ichthyotoxic properties similar to *M. polykrikoides* that caused mortalities of wild and cultured fish (Whyte et al., 2001; Iwataki et al., 2008; Howard et al., 2012).

Table 3. The nutrient levels in seawater in Perak on 27 and 29 May 2020 during the harmful algae bloom events.

Date	Location	Nitrate ($mg.L^{-1}$)	Nitrite ($mg.L^{-1}$)	Ammonia ($mg.L^{-1}$)	Phosphate ($mg.L^{-1}$)
27 May 2020	PK 1	0.06 ± 0.00	0.03 ± 0.00	0.0 ± 0.00	0.06 ± 0.00
	PK 2	0.16 ± 0.00	0.08 ± 0.00	0.0 ± 0.00	0.07 ± 0.00
	PK 3	0.06 ± 0.00	0.04 ± 0.00	0.0 ± 0.00	0.03 ± 0.00
	PK 4	0.19 ± 0.00	0.08 ± 0.003	0.0 ± 0.00	0.06 ± 0.00
29 May 2020	PK 1	0.08 ± 0.006	0.03 ± 0.002	0.0 ± 0.00	0.12 ± 0.006
	PK 2	0.18 ± 0.006	0.07 ± 0.008	0.0 ± 0.00	0.08 ± 0.006
	PK 3	0.19 ± 0.012	0.09 ± 0.001	0.0 ± 0.00	0.15 ± 0.006
	PK 4	0.08 ± 0.006	0.04 ± 0.014	0.0 ± 0.00	0.06 ± 0.012
	PK 5	0.18 ± 0.006	0.08 ± 0.002	0.0 ± 0.00	0.11 ± 0.006
Malaysian Marine Water Quality Standard		0.06	N/A	0.05	0.075

*N/A: Not available.

Table 4. The nutrient levels in seawater in Penang on 29 May 2020, 2 and 3 June 2020 during the harmful algae bloom events.

Date	Location	Nitrate ($mg.L^{-1}$)	Nitrite ($mg.L^{-1}$)	Ammonia ($mg.L^{-1}$)	Phosphate ($mg.L^{-1}$)
29 May 2020	PG 1	0.09 ± 0.006	0.04 ± 0.002	0.09 ± 0.00	0.05 ± 0.07
	PG 2	0.02 ± 0.004	0.06 ± 0.01	0.03 ± 0.00	0.07 ± 0.02
2 June 2020	PG 1	0.04 ± 0.00	0.02 ± 0.001	0.00 ± 0.07	0.05 ± 0.07
	PG 2	0.04 ± 0.006	0.02 ± 0.001	0.00 ± 0.00	0.08 ± 0.00
	PG 3	0.19 ± 0.002	0.07 ± 0.00	0.12 ± 0.00	0.09 ± 0.00
3 June 2020	PG 1	0.0 ± 0.00	0.0 ± 0.00	0.00 ± 0.07	0.03 ± 0.07
	PG 2	0.02 ± 0.00	0.02 ± 0.001	0.93 ± 0.00	0.49 ± 0.00
	PG 3	0.18 ± 0.002	0.08 ± 0.00	0.06 ± 0.01	0.09 ± 0.02
Malaysian Marine Water Quality Standard		0.06	N/A	0.05	0.075

*N/A: Not available.

Margalefidinium fulvescens had been reported to cause substantial mortality to farmed salmon on the west coast of Canada (Whyte et al., 2001; Bates et al., 2020) and farmed abalone in Monterey Bay, California (Howard et al., 2012). The mortality of fish due to *Margalefidinium* is caused by gill hyperplasia, haemorrhaging, squamation and apoptosis of fish gills, and digestive tracts cells damage (Gobler et al., 2008). Bloom of *M. fulvescens* with density ranging from 3.8×10^5 cells L^{-1} to 4.9×10^5 cells L^{-1} has been documented in Bahía de Mazatlán, Sinaloa, Mexico but no adverse effects were reported during the bloom (Gárate-Lizárraga et al., 2008). For the record, this is the first documented *M. fulvescens* bloom in Malaysian coastal waters. *M. fulvescens* were found in high density at most of the sampling stations in Perak and Penang from 27 June 2020 until 3 June 2020. The mortality of fish was probably due to gill clogging, as indicated in the dead fish's post-mortem results, which indicate the presence of whitish mucus on the gills.

This is the second report of a fish kill due to *Margalefidinium* spp. in Peninsular Malaysia. The first fish kill event due to *M. polykrikoides* was reported in March 2013 in Perak Coastal Waters (Siti NorRohaida et al., 2015). In the state of Sabah, fish kill events due to *M. polykrikoides* are more common. Bloom with a density of up to 6.0×10^6 cells L^{-1} and fish mortality due to *M. polykrikoides* was first reported in Sepanggar Bay, off Kota Kinabalu, Sabah, in 2005 (Anton et al., 2008). Until 2007, the blooms of *M. polykriokides* continued to dominate the area and nutrients from heavy rainfall supported the prevalence of this species (Adam et al., 2011). Since the mass fish kill due *M. polykrikoides* blooms in Perak waters in 2013 and the importance of this area to Malaysia fish production, the FRI had carried out phytoplankton monitoring in Sungai Udang fish cages for 12 months (2016–2017) and Kuala Gula, for 19 months (2017–2018). Although HAB incidents were not reported during this period, the presence of potential HAB species, *Tripos* spp., *Karlodinium* sp., *N. scintillans*, and *Margalefidinium* spp. had been recorded (Roziawati et al., 2021).

The factor that triggers *M. fulvescens* bloom and its spread is unknown. However, it may be attributed to an increase in nutrients discharged along the coasts from riverine and other anthropogenic activities, including mariculture. The nutrient levels such as ammonia, phosphate and nitrate at some stations in the affected

areas in Perak and Penang during the event exceeded the limits recommended by the MMMQS. One station in Penang recorded a high level of phosphate (0.93 ± 0.00 $mg.L^{-1}$) and ammonia (0.49 ± 0.00 $mg.L^{-1}$). It was reported that dinoflagellate groups were more dominant in the phytoplankton composition when the level of phosphate in seawater was high (Wang et al., 2009; Lau et al. 2017). Phosphate and ammonia were responsible for triggering the bloom of *Scrippsiella* in the East of Johor Strait (Mohd-Din et al., 2020). The bloom occurred after transition of northeast to the southwest monsoon at the end of the third week of May. During this period, the weather conditions are irregular, especially along the west coast of Peninsular Malaysia, with a high frequency of rainfall in the evening (Wong et al., 2016). As widely known, HAB events are often followed by intense rainfall and terrestrial runoff from rivers or river mouths into the water column (Glibert, 2020). Heavy rains were also associated with nitrification, which provided nutrients for plankton's rapid growth and caused HAB to occur (Glibert, 2020). Blooms of *M. polykrikoides* in Latin America have been associated with periods of heavy rain that cause an increase in nutrients in coastal waters due to runoff from rivers. Moreover, coastal upwellings associated with wind patterns, mixing of the water column and rain, all of which can create nutrient enrichment of surface waters, seem to favour the proliferation and recurrence of this dinoflagellate (López-Cortés et al., 2019).

Conclusion

Margalefidinium fulvescens was the main species responsible for the plankton bloom in the Kerian, Perak and Seberang Perai Selatan, Penang, in May 2020. *Margalefidinium fulvescens* was identified based on its morphology and confirmed molecularly. This is the first report of this ichthyotoxin producer species in Malaysian coastal waters associated with the fish kill. The physical water quality of the seawater during the event was within the recommended levels. However, dissolved nutrients such as nitrate, phosphate and ammonia were slightly higher than recommended levels. More information is needed for this species, particularly on the counts that could lead to fish kill and spatial-temporal dynamics as it threatens the fisheries' economy. Because of this, a surveillance program should be implemented to provide early warning and minimise the impact of harmful algae blooms (HABs). In addition, there should be an awareness program for fish farmers/aquaculture operators about HABs to educate them on how to respond effectively to safeguard their fish and operation.

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