

## Growth of Harmful Dinoflagellate *Margalefidinium polykrikoides* in Different Nutrient Concentrations

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*Margalefidinium polykrikoides* blooms resulted in massive fish mortality in many countries around the world. The prevalence of booms is believed to be associated with the eutrophication of the coastal area. Therefore, this study aims to determine the effects of different nitrogen (N) and phosphorus (P) concentrations on the growth of *M. polykrikoides*. Experiments were conducted on *M. polykrikoides* isolated from the affected area by exposing the cell to various N and P ratio: seawater without the addition of N and P (SW), equal N:P ratio (NP), trial ratio of N and P (TR), F/2 medium (+NP), deficient N (-N), and deficient P (-P). The experiments were carried out in triplicates for 20 d in similar environmental conditions with the determination of density, specific growth rate, and cell size during the study period. Furthermore, nutrient concentrations nitrite+nitrate ( $\text{NO}_2^- + \text{NO}_3^-$ ), phosphate ( $\text{PO}_4^{3-}$ ), and ammonia ( $\text{NH}_4^+$ ) were measured initially in the medium and both nutrients and chlorophyll were determined at the end of the experiment. The results revealed that *M. polykrikoides* grew in all the experiments, but the growth patterns differed between the treatments. The highest specific growth rate ( $0.1901 \pm 0.017 \text{ d}^{-1}$ ) with the highest cell density ( $1709 \pm 68.21 \text{ cells mL}^{-1}$ ) with a long chain of cells was observed in TR during the exponential phase. However, cell size was significantly smaller in SW compared to other nutrient conditions during the exponential phase. This study shows that *M. polykrikoides* a flexible species in nutrient uptake, thus allowing the species to survive in different nutrient conditions. The understanding of this bloom mechanism is important in monitoring and management of this harmful species, particularly in Sabah coastal waters.

Keywords: harmful algae, nutrients, *Margalefidinium polykrikoides*, nitrate, phosphate, red tide

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## INTRODUCTION

Toxic blooms of *Margalefidinium polykrikoides* (Margalef) are widely distributed worldwide since it was first reported and described as *Cochlodinium polykrikoides* by Margalef in 1961 (Gómez *et al.* 2017). The blooms of *M. polykrikoides* have been reported in various countries in America, Europe, and Asia (Iwataki *et al.* 2008; Kudela and Gobler 2012; Gómez *et al.* 2017; Sakamoto *et al.* 2021). Bloom expands widely, occurs at a longer period, and reoccurs yearly in some countries. The number of cells could reach  $10^3$ – $10^5$  cells mL<sup>-1</sup> and cause harm – particularly to fishes, shellfish, and marine organisms (Kudela and Gobler 2012; Gobler *et al.* 2012; Yñiguez *et al.* 2021; Sakamoto *et al.* 2021). Aquaculture industries in East Asian countries such as Japan and Korea are the most affected by *M. polykrikoides* blooms, resulting in significant economic losses amounting to millions of United States Dollars (USD) (Sakamoto *et al.* 2021). Hence, field and laboratory studies on *M. polykrikoides* are actively ongoing regarding its growth characteristics, migration, and nutrition to understand the bloom formation. This is important for the development of monitoring and management programs to minimize the impact of blooms on fish species.

*Margalefidinium polykrikoides* is a chain-forming species, which mostly occur in two or four-celled chains and occasionally as single. *M. polykrikoides* is ellipsoidal with lengths ranging from 25–40 µm (Gómez *et al.* 2017). Several studies have shown that the growth of *M. polykrikoides* is influenced by environmental factors such as temperature, salinity, and nutrients (Kudela and Gobler 2012). For instance, *M. polykrikoides* can grow at 21–26 °C and salinity of 25–40 (Kudela and Gobler 2012). However, the species do not grow at a temperature less than 10 °C, therefore, they form vegetative cells during winter. In addition, *M. polykrikoides* increases its number of cells per chain to adapt to stress conditions (Jiang *et al.* 2010; Tang *et al.* 2010).

Nitrogen (N) and phosphorus (P) have been identified as limiting nutrients that affect the bloom of harmful algal species (Smayda 1997; Kudela and Gobler 2012), including *M. polykrikoides* (Lee 2008; Gobler *et al.* 2012). Both field and laboratory studies have revealed that *M. polykrikoides* utilize different types of nutrients for bloom formation (Lee and Lee 2006; Al-Azri *et al.* 2014). Similarly, Al-Azri *et al.* (2014) found that bloom formation occurs following the increase of nutrients attributed to anthropogenic activities near coastal areas. Other studies reported that blooms of *M. polykrikoides* were enhanced by runoff after the rainy season (Adam *et al.* 2011; Al-Azri *et al.* 2014; Chong *et al.* 2020) and coastal upwelling. The different types of nutrients that can be taken up by the species include dissolved organic N and P (Gobler *et al.* 2012) and various forms of N such

as nitrate (NO<sub>3</sub>), ammonium (NH<sub>4</sub>), urea, and glutamic acid (Gobler *et al.* 2012). Furthermore, it is generally accepted that dissolved organic substances from land runoff, causing eutrophication contributes to the initiation of harmful algal blooms (HABs) (Smayda 1997; Gobler *et al.* 2012), including *M. polykrikoides*. Laboratory studies on the growth of *M. polykrikoides* show that the species utilizes N compounds and they can grow at high concentrations of NH<sub>4</sub> ranging from 50–80 µM with 20 µM of NO<sub>3</sub> (Lee 2008). However, high concentrations of NH<sub>4</sub> yield a fewer number of *M. polykrikoides* cells compared to a low NH<sub>4</sub> concentration (*i.e.* 5 µM of NH<sub>4</sub>). In addition, Gobler *et al.* (2012) found that *M. polykrikoides* from the New York estuaries grew faster on glutamic acid media than other cultures grown on NH<sub>4</sub>, NO<sub>3</sub>, and urea.

HAB species have special mechanisms such as mixotrophic (a combination of phototrophic and phagotrophic nutrition) and can migrate vertically to absorb light during the day and migrate to the bottom layers for nutrient uptake during the night (Smayda 1997). Likewise, *M. polykrikoides* is a mixotrophic alga and they prey on smaller phytoplankton such as *Amphidinium carterae* and *Isochrysis galbana* (Jeong *et al.* 2004). Mixotroph ability among dinoflagellates and raphidophyte are believed to be influenced by the nutrient limiting condition (Park *et al.* 2013). By having the mixotroph characteristic, HABs species could acquire other substances by ingesting the prey instead of relying only on sunlight for photosynthesis to grow, thus contributing towards better adaptation in bloom formation mechanism.

In Malaysia, *M. polykrikoides* was first observed in the Sepanggar Bay, Sabah in 2005 (Anton *et al.* 2008) before it was reported in peninsular Malaysia in 2013 (Harun *et al.* 2015). Based on large the subunit ribosomal RNA gene (LSU rDNA), *M. polykrikoides* found in Malaysia belong to the American ribotype (Iwataki *et al.* 2008). The blooms reoccurred yearly and have caused massive mortality of caged fish (Adam *et al.* 2011; Mohammad-Noor *et al.* 2014; Yñiguez *et al.* 2021). Nevertheless, there are limited studies to elucidate the factors contributing to bloom formation in *M. polykrikoides* in these areas. Besides, more studies have been conducted in temperate regions compared to tropical regions. Based on the annual occurrence of *M. polykrikoides* bloom and the potential of the increasing fish mortality to spread to new areas in Malaysia, it is pertinent to identify the factors influencing the event. Hence, the study aimed at comparing the growth of *M. polykrikoides* in terms of their cell densities, specific growth rate (µ), and sizes at different nitrogen (N) and phosphorus (P) ratios. Results from this study will provide useful information on the nutrient requirement by *M. polykrikoides* to form a bloom. Such information can be used for monitoring and managing *M. polykrikoides* bloom, particularly in Sabah's coastal water, to minimize the impact.

## MATERIALS AND METHODS

### Algal Cultures

Unialgal non-axenic culture of *Margalefidinium polykrikoides* strain (HABSMP-01) from the Borneo Marine Research Institute (BMRI) was used in this study. The cultures were isolated from Sepanggar Bay, Kota Kinabalu, Malaysia in July 2019, and they were grown in F/2 medium (Guillard and Ryther 1962). All the culture media were prepared in an autoclave containing filtered seawater using a filter with 0.1- $\mu\text{m}$  pore size plus a salinity of 30 and pH of  $8 \pm 0.1$ . Thereafter, the cultures were incubated at 25 °C and a light-dark cycle of 12:12 under  $150 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$  (provided by cool white LED bulb). The cultures were left to acclimatize for 3 mo in the original F/2 medium (nitrogen at 883  $\mu\text{M}$ ; phosphorus at 32  $\mu\text{M}$ ), before inoculating about 100 cell  $\text{mL}^{-1}$  into 150-mL media with several N:P ratios, as shown in Table 1. The experiments were acclimatized for 1 d, and the experiments were conducted in triplicates in a 250-mL Schott flask for 20 d.

*M. polykrikoides* was exposed to several N and P ratios – including seawater (SW), equal N:P ratio (NP), trial ratio of N and P (TR), F/2 medium (+NP), deficient N (–N), and deficient P (–P). For groups NP and TR, the mediums were prepared as per the F/2 recipe with the same volume of trace metal and vitamin, whereas the concentration of N and P were prepared, as shown in Table 1. The NP concentration for the *M. polykrikoides* TR experiment was adjusted, as described in a previous study conducted in the same area, *i.e.* Sepanggar Bay (Weliyadi 2012). The deficient experiments (–N and –P) were performed according to Wang *et al.* (2020). The concentration of N and P were determined based on the N and P ratio in f/2 medium, whereby the concentrations were diluted 1000 times.

### Culture Samplings and Growth Measurement

An aliquot of 3 mL of the sample was collected every second day and fixed with Lugol's iodine for cell enumeration (cells  $\text{mL}^{-1}$ ). Cell densities were determined

microscopically using a Sedgewick Rafter chamber under Zeiss Axiostar compound microscope at 100x magnification. The enumeration was performed twice using 1 mL of the sample. At the end of the experiment, a 110-mL aliquot of the samples were filtered using GF/F filter paper to determine the chlorophyll *a*, where the filtered aliquot was used for nutrient analysis.

Specific growth rates ( $\mu$ ) were determined according to (Guillard and Ryther 1962):

$$\text{Growth rate } (\mu) = \ln(N_t/N_0) / \Delta T \quad (1)$$

where  $N_t$  is the population size at the end of the time interval,  $N_0$  is the population size at the beginning of the time interval, and  $\Delta T$  is the length of the time interval.

### Morphology and Cell Sizes

A 2.0 dino-eye photo camera mounted on an Olympus light microscope was used to record the morphological characteristics of *M. polykrikoides*. The width and length of the cells ( $n = 15\text{--}30$ ) were measured using the Dinolite USB microscope 2.0 at a magnification of 400x, which were then presented in pictorial forms. Based on the cell length and width, the cell shapes were drawn using illustration shape tools in Microsoft PowerPoint. Additionally, the cell size of *M. polykrikoides* collected from the field was determined.

### Chlorophyll $\alpha$ Assays

Chlorophyll  $\alpha$  was measured using a spectrophotometer (DR3900 Hach Laboratory VIS Spectrophotometer) at 665, 645, and 470 nm wavelengths after the cells were extracted with 95% acetone overnight at 4 °C (Strickland and Parsons 1972).

The following equations were used to calculate the pigment content:

$$\text{Chlorophyll-}\alpha \text{ } (\mu\text{g/L}) = [(11.9 \times A_{665}) - (1.31 \times A_{645}) - (0.14 \times A_{630})] \times 1000 \times V/S \quad (2)$$

**Table 1.** The various treatments showing the N and P ratios and experimental conditions used to grow and culture *M. polykrikoides*.

Experiment type	Label	Ratio (N:P)	Nitrogen N ( $\mu\text{M}$ )	Phosphorus P ( $\mu\text{M}$ )
Seawater without N and P added	SW	0:0	0	0
Equal N:P ratio	NP	1:1	6	5
Trial ratio	TR	6:1	30	5
F/2 medium	+NP	28:1	882	32
Deficient N	-N	1:36	0.882	32
Deficient P	-P	1:0.0003	882	0.032

Keys: [SW] seawater, [NP] equal N:P ratio, [TR] trial ratio, [+NP] medium f/2, [–N] deficient N, and [–P] deficient P

where A is the absorbance at 665, 645, and 630 nm; V is the volume of acetone used (mL), and S is the volume of sample filtered (mL).

### Nutrient Analysis

The nitrite+nitrate-N ( $\text{NO}_2^- + \text{NO}_3^-$ ), phosphate-P ( $\text{PO}_4^{3-}$ ), and ammonia ( $\text{NH}_4^+$ ) were determined during the initial experiment (before the inoculation of *M. polykrikoides*) and at the end of the experiment (*i.e.* at Day 20). The phenol hypochlorite method was employed to measure ammonia ( $\text{NH}_4^+$ ), whereas nitrite+nitrate-N ( $\text{NO}_2^- + \text{NO}_3^-$ ) and phosphate-P ( $\text{PO}_4^{3-}$ ) were quantified according to the standard procedure described by Strickland and Parsons (1972).

The sum of nitrite+nitrate-N ( $\text{NO}_2^- + \text{NO}_3^-$ ) concentration was obtained from the diazotization of sulfanilamide with N-1-naphthalenediamine to form a colored azo dye, and the absorbance was measured under a wavelength of 543 nm. Orthophosphate concentration was measured using the ascorbic acid method. Specifically, the ascorbic acid was quantified under a wavelength of 640 nm, followed by the reaction between ammonium molybdate and orthophosphate in the sample to form a blue compound. The resulting absorbance was measured under 885 nm using a spectrophotometer (DR3900 Hach Laboratory VIS Spectrophotometer).

### Data Analysis

Statistical analyses were done using the Statistical Software for Social Science (IBM SPSS Statistic, Version 20.0). The data were checked for normality using the level of skewness and kurtosis. Descriptive statistics were applied to compute the mean and standard errors for each group. All nutrient experiments were analyzed using analysis of variance (ANOVA) to determine significant differences, followed by Tukey's *post hoc* test. A *p*-value less than 0.05 ( $p < 0.05$ ) was considered for statistical difference between the treatments.

## RESULTS

### Growth of *Margalefidinium polykrikoides* in Different Nutrient Conditions

Generally, the growth *Margalefidinium polykrikoides* under the tested nutrient concentrations passed through all the growth phases, *i.e.* lag, exponential, stationary, and death phases. However, the growth patterns differed between the groups, as shown in Figure 1. The duration of the lag phase of *M. polykrikoides* was similar in the NP, TR, +NP, and -N, which lasted for 4 d (Days 0–4). On the other hand, the lag phase was longer for the SW

and -P groups, which took place for 8 d (Days 0–8). All the treatment groups except SW, +NP, and -P underwent an accelerated exponential growth. Furthermore, NP, TR, and -N reached stationary phases at Day 14 before slowly decreasing until Day 20. In contrast, SW, +NP, and -P reached the stationary phase much later (*i.e.* Day 18) before showing a sharp drop (Figure 1). The highest mean cell density was recorded in TR ( $1709 \pm 68.21$  cells  $\text{mL}^{-1}$ ), whereas the -P recorded the lowest mean cells ( $523 \pm 85.88$  cells  $\text{mL}^{-1}$ ). However, when *M. polykrikoides* was cultured in sufficient nutrient condition (+NP), the specific growth rate was  $0.1142 \pm 0.023$   $\text{div d}^{-1}$  significantly lower (ANOVA,  $p \leq 0.05$ ) compared to NP, TR, and -N ( $0.192 \pm 0.025$ ,  $0.190 \pm 0.017$ , and  $0.166 \pm 0.005$ , respectively) during exponential growth at Day 14. There was no significant difference in the specific growth rate of *M. polykrikoides* in all of the nutrient treatments at Day 20. At the end of the experiment (*i.e.* Day 20), *M. polykrikoides* cultured in sufficient NP (+NP) recorded a cell density and chlorophyll a of  $1164 \pm 189.98$  cells  $\text{mL}^{-1}$  and  $1.31 \pm 0.18$   $\mu\text{gL}^{-1}$ , respectively, which were significantly higher than other treatments (Figure 2). The lowest concentration of chlorophyll a ( $0.22 \pm 0.04$   $\mu\text{gL}^{-1}$ ) was recorded in SW.

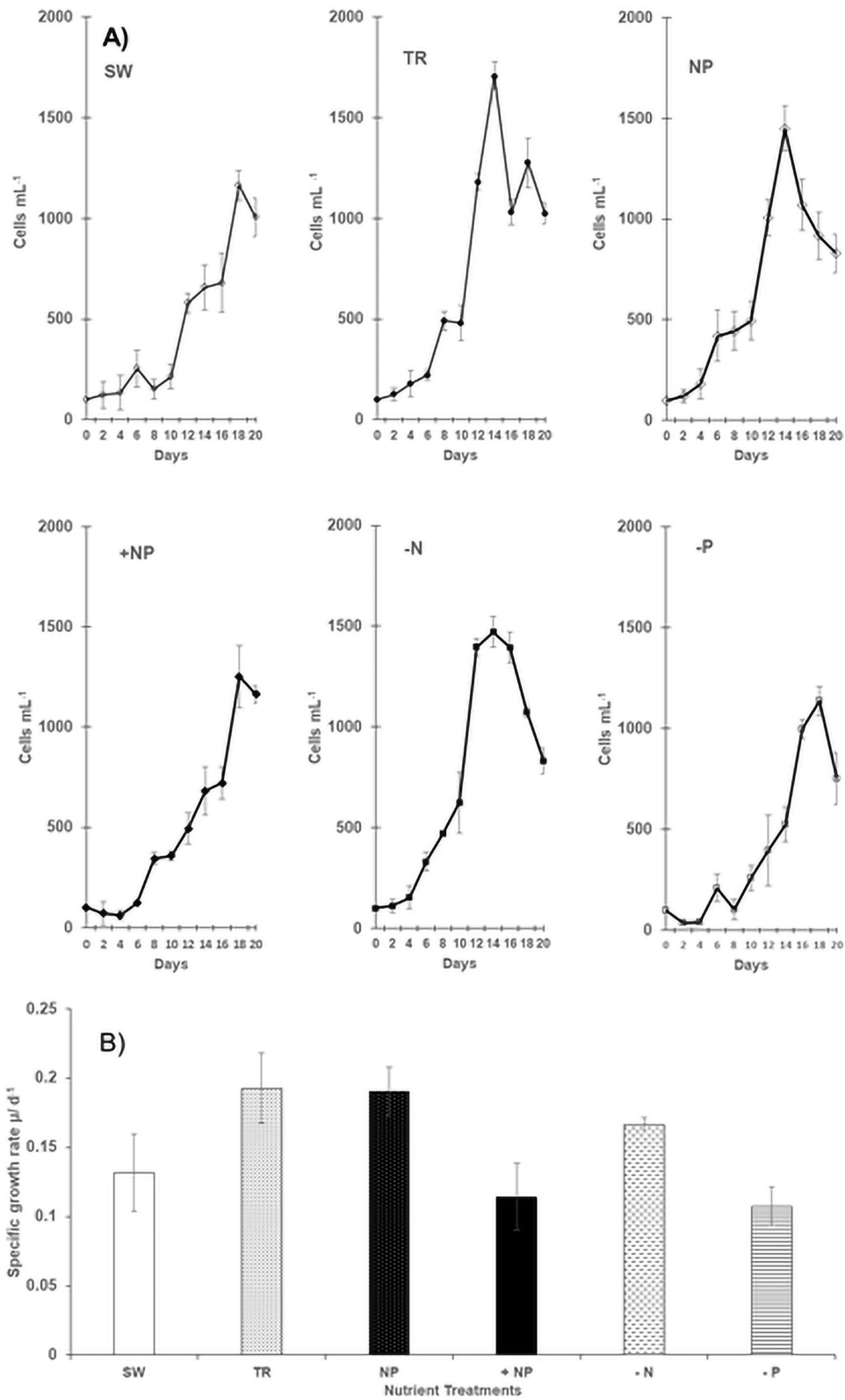
### Nutrient Analysis

Table 2 shows the nutrient concentrations at the onset (Day 0) and end (Day 20) of all the experiments conducted. The concentration of nitrite+nitrate ( $\text{NO}_2^- + \text{NO}_3^-$ ) in all nutrient conditions were slightly similar during initial and at the end of experiments except for +NP, whereby the nitrite+nitrate-N ( $\text{NO}_2^- + \text{NO}_3^-$ ) concentration at Day 20 ( $0.1092$   $\mu\text{M}$ ) was significantly higher ( $p < 0.05$ ) compared to the initial concentration ( $0.0456$   $\mu\text{M}$ ). Consequently, the cell density of *M. polykrikoides* was highest towards the end of the experiment (death phase). However, the concentration of phosphate ( $\text{PO}_4^{3-}$ ) decreased on Day 20 in all treatments, whereas ammonia ( $\text{NH}_4^+$ ) concentration was very low both at the initial and end of the experiment.

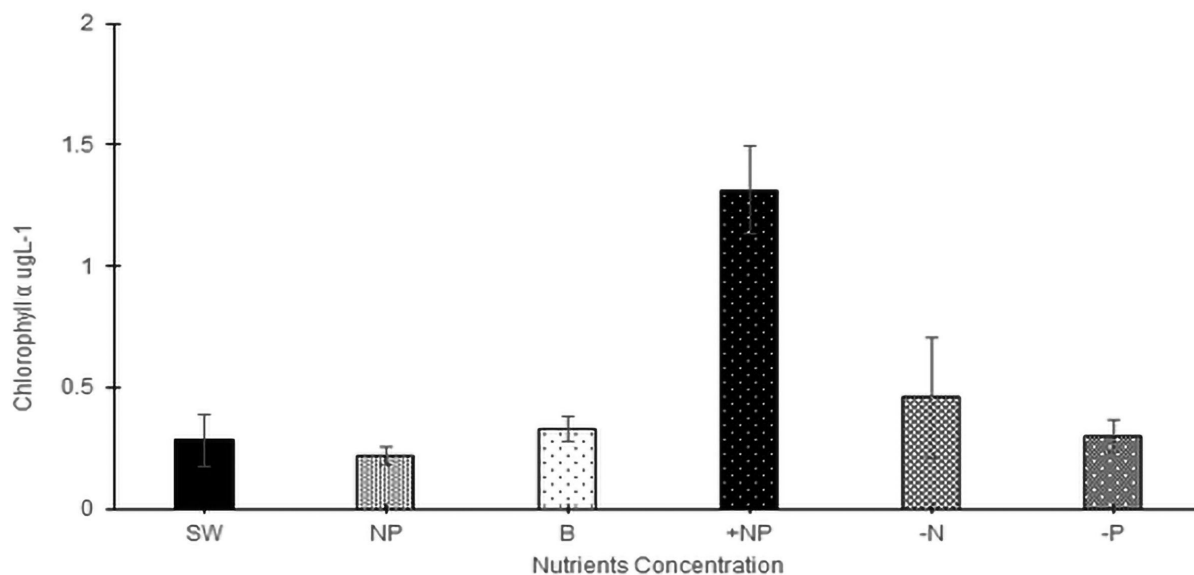
### Morphology and Sizes of *M. polykrikoides* in Different Nutrient Conditions

The length and width of *M. polykrikoides* during each growth phase were measured for all experiments. The cell shapes were drawn based on the cell length and width (Figure 3). *M. polykrikoides* cells demonstrated an oblique shape in all experimental conditions. However, the cell size in SW during the exponential phase was significantly smaller ( $p < 0.005$ ) compare to other nutrient conditions. In addition – during the lag, early exponential, and stationary phases – the cells in all the experimental conditions except B(TR) existed as a single cell, a chain of two cells, and a chain of four cells, respectively. However, only B exhibited long chains of eight cells, particularly during the exponential phase.





**Figure 1.** [A] Cell densities (cells mL<sup>-1</sup>) and [B] specific growth rate of *M. polykrikoides* grown under different N and P; seawater (SW), trial ratio of N and P (TR), equal ratio of NP (NP), sufficient NP (+NP), deficient N (-N) and deficient P (-P). Standard deviations are indicated by error bars.



**Figure 2.** Total chlorophyll a (ch α μg L<sup>-1</sup>) of *Margalefidinium polykrikoides* grown under different N and P; seawater (SW), equal ratio of NP (NP), trial ratio of N and P (TR), sufficient NP (+NP), deficient N (-N), and deficient P (-P). Standard deviations are indicated by error bars.

**Table 2.** Nutrient concentration for experiment condition before *M. polykrikoides* was inoculated (initial) and at the end of the experiments for seawater (SW), equal ratio of NP (NP), trial ratio of N and P (TR), sufficient NP (+NP), deficient N (-N), and deficient P (-P).

Experiment conditions (N uM, P uM concentration)	Initial of experiment (uM)			End of experiment (uM)		
	Nitrite +Nitrate (NO <sub>2</sub> <sup>-</sup> +NO <sub>3</sub> <sup>-</sup> )	Phosphate (PO <sub>4</sub> )	Ammonia (NH <sub>4</sub> )	Nitrite +Nitrate (NO <sub>2</sub> <sup>-</sup> +NO <sub>3</sub> <sup>-</sup> )	Phosphate (PO <sub>4</sub> )	Ammonia (NH <sub>4</sub> )
SW (0,0)	0.0425	0.0652	0.0010	0.0440	0.0850	0.0012
NP (1,1)	0.0468	0.1302	0.0022	0.0424	0.0729	0.0008
TR (30,5)	0.0451	1.0566	0.0006	0.0433	0.1016	0.0011
+NP (882,32)	0.0456	6.8250	0.0016	0.1092	2.7300	0.0008
-N (0.882,32)	0.0421	6.6856	0.0009	0.0418	5.8176	0.0032
-P (882,0.32)	0.060	0.1853	0.0020	0.0913	0.0751	0.0024

## DISCUSSION

In this study, *M. polykrikoides* grew at all experiment conditions with different N:P ratios for 20 d. However, in SW, the numbers of cells were less than 1500 cells mL<sup>-1</sup> throughout the phases and a significant small cell size was observed in the exponential phase. *M. polykrikoides* SW had a high specific growth rate during the exponential phase, but a low chlorophyll a (0.22 ± 0.04 μg L<sup>-1</sup>) was detected at the end of the experiment. Although a low amount of nutrients was recorded during the initial and end of the experiments, a considerable amount of growth was observed for 20 d. This result indicates that the cells used up all vitamin and trace metals required for cell growth under the experimental condition. It also highlights that

























nutrient was not a limiting factor under such a condition. Previously, the growth of *M. polykrikoides* was reported to be influenced by vitamin B<sub>1</sub> (Jiang *et al.* 2010; Tang *et al.* 2010), whereas trace metal and inorganic nutrients had no significant effect on the growth of the species (Jiang *et al.* 2010).

The small cell size of *M. polykrikoides* in SW during the exponential phase might be an adaptation to the environmental condition. Microalgae size is associated with environmental parameters such as the levels of nutrient uptake, light absorption, and specific growth rate. For instance, small-sized algae can uptake nitrogen faster than larger-sized algae at both low and high substrate concentrations (Hein *et al.* 1995). Therefore, *M.*

**Table 3.** Comparison of *Margalefidinium polykrikoides* size and nutrient concentrations at different locations.

Location	Size (µm)	Nutrient concentration (µM)	References
Nakdong River, South Korea	Length: 25–40	NO <sub>2</sub> +NO <sub>3</sub> : 0.01–5.16 PO <sub>4</sub> : 0.05–0.56	Baek <i>et al.</i> (2019)
Lampung Bay, Indonesia	20–30	NO <sub>2</sub> : 0.003–0.02 NO <sub>3</sub> : 0.032–0.413 NH <sub>4</sub> : 0.085–1.241 PO <sub>4</sub> : 0.075–0.080	Puspasari <i>et al.</i> (2018)
Peninsular Malaysia, Malaysia	Width: 20–24 Length: 30–40	NO <sub>2</sub> : 0.6949–0.8165 NH <sub>4</sub> : 2.78–55.50 PO <sub>4</sub> : 0.00–0.0009	Harun <i>et al.</i> (2015)
Gulf of California, Mexico	Width: 22–30 Length: 30–42	NO <sub>2</sub> +NO <sub>3</sub> : 0.165–0.897 PO <sub>4</sub> : 0.16–3.25	Gárate-Lizárraga <i>et al.</i> (2004)

\*[NO<sub>2</sub>] nitrite, [NO<sub>3</sub>-] nitrate, [NH<sub>4</sub>-] ammonia, [PO<sub>4</sub>-] phosphate, and [DON] dissolved organic nitrogen

Experiment type	Lag phase	Exponential phase	Stationary phase	Death phase
Seawater without N and P were added (SW)				
Length (µm)	20.00 ± 1.85	10.10 ± 4.70	15.55 ± 4.20	17.83 ± 4.05
Width (µm)	13.40 ± 1.37	8.90 ± 4.85	13.23 ± 2.88	15.13 ± 2.30
Equal N: P ratio (NP)				
Length (µm)	18.48 ± 3.15	16.00 ± 2.22	19.15 ± 3.40	17.35 ± 3.58
Width (µm)	15.30 ± 2.69	12.85 ± 2.55	14.90 ± 2.98	13.18 ± 3.32
N:P ratio during bloom (B)				
Length (µm)	15.63 ± 0.64	16.80 ± 5.15	16.83 ± 3.42	18.60 ± 3.05
Width (µm)	12.58 ± 1.98	11.31 ± 2.69	12.48 ± 2.83	15.25 ± 3.14
1/2 medium (+NP)				
Length (µm)	17.90 ± 4.67	16.10 ± 2.51	16.58 ± 2.88	15.82 ± 2.29
Width (µm)	11.92 ± 2.71	12.88 ± 1.59	13.78 ± 2.26	12.36 ± 2.26
Deficient N (-N)				
Length (µm)	19.20 ± 4.31	16.53 ± 3.71	17.31 ± 2.68	14.41 ± 3.14
Width (µm)	14.01 ± 2.55	12.99 ± 2.08	13.25 ± 3.33	12.05 ± 1.80
Deficient P (-P)				
Length (µm)	17.21 ± 2.44	16.85 ± 2.62	16.81 ± 2.62	18.07 ± 4.56
Width (µm)	13.27 ± 1.43	14.63 ± 2.51	14.93 ± 3.00	13.50 ± 3.87

Scale: 10 µm

**Figure 3.** Pictogram and length and width (mean ± standard deviation, µm) of *M. polykrikoides* during lag, exponential, stationary, and death phases for all experiments conducted.

*polykrikoides* in SW were smaller in size at the exponential phase compared to other phases, demonstrating the highest specific growth rate because the cells were actively dividing (Figure 3).

In this study, significant differences in sizes of *M. polykrikoides* were observed between lag and exponential phases but not at stationary and death phases (Table 3; Figure 3). According to a laboratory study by Lee and Lee (2006), an increased amount of ferum (fe) in trace metal of f2/media resulted in an increased size of *M. polykrikoides* (31 µm in width, 48 µm in length), but no cell division was observed. However, cells from chains of *M. polykrikoides* were reported to be influenced by vitamin B<sub>1</sub> and B<sub>12</sub> concentrations (Jiang *et al.* 2010; Tang *et al.* 2010). In this experiment, a long chain of cells and the highest cell density were observed for TR (N and P during the bloom) during the exponential phase. Since the concentration of vitamins used in this study is similar to the aforementioned studies; therefore, the ratio of N and P concentration provides better growth conditions for *M. polykrikoides*. From field observation, the cell sizes of *M. polykrikoides* differed in various bloom phases. During the first peak of bloom, the recorded size of *M. polykrikoides* was 18.40 ± 4.26 µm in width and 24.36 ± 5.44 µm in length. During the second phase of bloom, the cell size was smaller with a width of 13.82 ± 1.30 µm and 14.08 ± 4.27 µm in length (Roselli *et al.* 2020). These discrepancies in cell size have also been reported for the cells cultured in the laboratory and those collected from the field. Table 3 shows different cell sizes of *M. polykrikoides* collected from different locations. The size of cultured *M. polykrikoides* in this study was also smaller compared to those collected from field bloom (18.79 ± 3.63 µm length, 16.0 ± 2.25 µm width).

The highest cell densities of *M. polykrikoides* was found for TR with a ratio of N:P; 6:1. This ratio was estimated based on the previous study by Weliyadi (2012), whereby a positive correlation was reported between NO<sub>3</sub> (> 30

$\mu\text{M}$ ) and *M. polykrikoides* occurrences in Sepanggar Bay, Sabah. This finding corroborates the study by Gobler *et al.* (2012), where the bloom *M. polykrikoides* occurred at a high  $\text{NO}_3^-$  ( $>20 \mu\text{M}$ ) in the USA estuaries. Hence, it suggests that the high concentration of  $\text{NO}_3^-$  (*i.e.*  $> 30 \mu\text{M}$ ) in this study favored the growth of *M. polykrikoides*. Towards the end of the experiment (Day 20), the +NP cultured *M. polykrikoides* had a high cell density and chlorophyll  $\alpha$  compared to other experiments. Since the +NP treatment was performed using the original F/2 medium without modification, the results indicate that the medium is suitable to maintain the culture in the laboratory as the cell growth can be prolonged.

Nutrient dynamics play an important role in shaping the phytoplankton community. This effect was demonstrated in a study conducted by Chong *et al.* (2020) on phytoplankton communities in coastal areas at Sepanggar Bay, Kota Kinabalu. Besides, HABs in coastal areas are positively influenced by nutrient supply from sources such as coastal upwelling and runoff from adjacent lands (Adam *et al.* 2011; Chong *et al.* 2020). The occurrences of *M. polykrikoides* blooms at Sepanggar Bay have been associated with the high nutrient concentration after the rainy season (Anton *et al.* 2008; Adam *et al.* 2011; Mohammad-Noor *et al.* 2014; Yñiguez *et al.* 2021). The uptake of nutrients, especially  $\text{NO}_3^-$ , was observed in this study as their concentrations decreased in all the treatments at the end of the experiment (Table 2). Baek *et al.* (2019) found that blooms of *M. polykrikoides* occurred during summer with the lowest of  $\text{NO}_2^- + \text{NO}_3^-$  concentration (0.01–5.16  $\mu\text{M}$ ) compared to that during winter (6.48–41.9  $\mu\text{M}$ ). Where else, the occurrence of *Cochlodinium* spp. (including *M. polykrikoides*) in several countries indicated that the species preferred a high N:P ratio, *i.e.* 14.1 in the Philippines (Vicente *et al.* 2002) and 35:5 in Lampung, Indonesia (Puspasari *et al.* 2018) (Table 3).

Another advantage of *M. polykrikoides* is that the species can grow by utilizing different types of N substances (Lee 2008; Gobler *et al.* 2012). Moreover, the main source of N for *Cochlodinium* sp. is the dissolved inorganic nitrogen form (*e.g.*  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ ) (Kudela and Gobler 2012). In the same vein, previous studies found that *M. polykrikoides* preferred N substances such as ammonia, glutamic acid,  $\text{NO}_3^-$ , and urea (Lee 2006; Gobler *et al.* 2012; Park *et al.* 2013), whereas others favor both  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  (Al-Azri *et al.* 2014). However, the growth of *M. polykrikoides* is inhibited by a concentration of  $\text{NH}_4^+$  ( $> 50 \mu\text{M}$ ) (Lee 2008). However, a high concentration of ammonia (2.78–55.50  $\mu\text{M}$ ) was recorded during the first occurrence of *M. polykrikoides* in peninsular Malaysia (Harun *et al.* 2015). However, a high concentration of phosphate (0.16–3.25  $\mu\text{M}$ ) was found during blooms of *M. polykrikoides* in Mexico (Gárate-Lizárraga *et al.* 2004) (Table 3).

To maintain growth in the natural environment, *M. polykrikoides* may use its capability to obtain inorganic nutrients (N and P) through mixotrophic by the ingestion of prey (Kudela and Gobler 2012). Based on the experiments conducted in this study, *M. polykrikoides* can grow in all nutrient concentrations, but nutrient increment (*i.e.* as seen in TR and +NP) may trigger bloom formation. Nevertheless, the growth of *M. polykrikoides* is also influenced by other factors such as light, temperature, salinity, and trace elements (Lee and Lee 2006; Kudela and Gobler 2012; Chong *et al.* 2020). The combination of the right environmental conditions, including high nutrient concentration, is needed for the species to form a bloom. However, the exact and optimum conditions are difficult to identify. Hence, further studies are recommended to elucidate the factors contributing to bloom formation by considering the ratio of N and P during bloom in combination with other factors such as salinity and light intensity.

## CONCLUSION

This study shows that *M. polykrikoides* can grow in different N to P ratios, including when the nutrients are deficient (–N and –P). Thus, the findings reinstate the flexibility of *M. polykrikoides* in nutrient uptake as reported by previous studies. This feature increases its survival and might be a vital factor that facilitates its dominance over other phytoplankton during blooms. The best growth of *M. polykrikoides* was observed upon using the N and P ratio according to the TR condition, which was characterized by significant cell growth, high cell number, and formation of a long chain during the exponential phase. Nevertheless, N and P alone are probably insufficient to sustain the growth for a long period. In addition, *M. polykrikoides* can adapt to the environment by changing their size. In this study, the cell sizes of *M. polykrikoides* in SW were smaller in the exponential phase compared to other phases. Similar results were observed in the other experiments. Hence, cell size plays a significant role in the bloom formation of *M. polykrikoides*. Other important factors contributing to the bloom formation of *M. polykrikoides* could be determined by designing an experiment to determine the combined role of the ratio based on TR and other environmental parameters such as salinity and light. Besides, future studies may focus on identifying the relationship between nutrient requirement and toxicity; in this case, ichthyotoxin of *M. polykrikoides* can be considered.

The source of nutrients in Sabah coastal areas is mainly from anthropogenic activities – including deforestation, sewage, and aquaculture. In order to minimize the



recurrence of *M. polykrikoides* in the areas, there is a need to control the release of the nutrient required for the species to proliferate. On that note, an awareness program should be conducted on the impact of human activity and its relationship on the occurrence of HAB in Sabah.

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## REFERENCES

- ADAM A, MOHAMMAD-NOOR N, ANTON A, SALEH E, SAAD S, MUHD SHALEH SR. 2011. Temporal and spatial distribution of harmful algal bloom (HAB) species in coastal waters of Kota Kinabalu, Sabah, Malaysia. *Harmful Algae* 10: 495–502.
- AL-AZRIAR, PIONTKOVSKI SA, AL-HASHMI KA, GOES JI, GOMES H, DO R, GLIBERT PM. 2014. Mesoscale and nutrient conditions associated with the massive 2008 *Cochlodinium polykrikoides* bloom in the Sea of Oman/Arabian Gulf. *Estuaries and Coasts* 37: 325–338.
- ANTON A, TEOH PL, MUHAMAD SHALEH SR, MOHAMMAD-NOOR N. 2008. First occurrence of *Cochlodinium* blooms in Sabah, Malaysia. *Harmful Algae* 7: 331–336.
- BAEK SH, KIM D, KIM YO, SON M, KIM Y, LEE M, PARK BS. 2019. Seasonal changes in abiotic environmental conditions in the Busan coastal region (South Korea) due to the Nakdong River in 2013 and effect of these changes on phytoplankton communities. *Continental Shelf Research* 175: 116–126.
- CHONG BWK, SANDRIC CYL, VICTOR SK, YOSHIDA T. 2020. Monsoonal variation of the marine phytoplankton community in Kota Kinabalu, Sabah Reg Stud Mar Sci 37: 101326.
- GÁRATE-LIZÁRRAGA I, LÓPEZ-CORTÉS DJ, BUSTILLOS-GUZMÁN JJ, HERNÁNDEZ-SAN-DOVAL F. 2004. Blooms of *Cochlodinium polykrikoides* (Gymnodiniaceae) in the gulf of California, Mexico. *Revista de Biología Tropical* 52: 51–58.
- GOBLER CJ, BURSON A, KOCH F, TANG Y, MULLHOLLAND MR. 2012. The role of nitrogenous nutrients in the occurrence of harmful algal blooms caused by *Cochlodinium polykrikoides* in New York estuaries (USA). *Harmful Algae* 17: 64–74.
- GÓMEZ F, RICHLLEN ML, ANDERSON DM. 2017. Molecular characterization and morphology of *Cochlodinium strangulatum*, the type species of *Cochlodinium*, and *Margalefidinium* gen. nov. for *C. polykrikoides* and allied species (Gymnodiniales, Dinophyceae). *Harmful Algae* 63: 32–44.
- GUILLARD RR, RYTHER JH. 1962. Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Can J Microbiol* 8: 229-239.
- HEIN M, PEDERSEN MF, SAND-JENSEN K. 1995. Size-dependent nitrogen uptake in micro- and macroalgae. *Mar Ecol Prog Ser* 118: 247–253.
- HARUN SN, MOHAMMAD-NOOR N, AHMAD Z, CHU KB, SAAD S, HIDAYAT NSM, MUKAI Y. 2015. First report of *Cochlodinium polykrikoides* (Dinophyceae), a harmful algal bloom (HAB) species in the coastal waters of Peninsular Malaysia. *Mal Jour of Sci* 34: 87–92.
- IWATAKI M, KAWAMI H, MIZUSHIMA K, MIKULSKI CM, DOUCETTE GJ, RELOX JR JR, ANTON A, FUKUYO Y, MATSUOKA K. 2008. Phylogenetic relationships in the harmful dinoflagellate *Cochlodinium polykrikoides* (Gymnodiniales, Dinophyceae) inferred from LSU rDNA sequences. *Harmful Algae* 7: 271–277.
- JEONG HJ, YOO Y DU, KIM TH, KIM JH, KANG NS, YIH W. 2004. Mixotrophy in the Phototrophic Harmful Alga. *J Eukaryot Microbiol* 51: 563–569.
- JIANG X, LONSDALE DJ, GOBLER CJ. 2010. Grazers and vitamins shape chain formation in a bloom-forming dinoflagellate, *Cochlodinium polykrikoides*. *Oecologia* 164: 455–464.
- KUDELA RM, GOBLER CJ. 2012. Harmful dinoflagellate blooms caused by *Cochlodinium* sp.: global expansion and ecological strategies facilitating bloom formation. *Harmful Algae* 14: 71–86.
- LEE YS. 2008. Utilization of various nitrogen, phosphorus, and selenium compounds by *Cochlodinium polykrikoides*. *J Environ Biol* 29: 799–804.
- LEE YS. 2006. Factors affecting outbreaks of high-density *Cochlodinium polykrikoides* red tides in the coastal seawaters around Yeosu and Tongyeong, Korea. *Mar Pollut Bull* 52: 1249–1259.
- LEE YS, LEE SY. 2006. Factors affecting outbreaks of *Cochlodinium polykrikoides* blooms in coastal areas of Korea. *Mar Pollut Bull* 52: 626–634.

- MOHAMMAD-NOOR N, WELIYADI E, AUNG T, ADAMA A, HANAN DSM. 2014. Effects of meteorological conditions on the occurrence of *Cochlodinium polykrikoides* and *Pyrodinium bahamense* var. *compressum* in coastal waters of Kota Kinabalu, Sabah, Malaysia. *Sains Malaysiana* 43: 21–29.
- PARK J, JEONG HJ, YOO YDU, YOON EY. 2013. Mixotrophic dinoflagellate red tides in Korean waters: Distribution and ecophysiology. *Harmful Algae* 30S: S28–S40.
- PUSPASARI R, SUGIANTI Y, RUSTAM A, ADI RA, SAGALA SS, PRANOWO WS. 2018. The outbreak of *Cochlodinium* sp.: the red tide maker in the coastal of Lampung Bay. *IOP Conf. Series. Earth and Environmental Sci.* 176: 1–11.
- ROSELLI L, VADRUCCI MR, BELMONTE M, CIRCIELLO P, RUBINO F, UNGARO N, CAROPPO C. 2020. Two-stages bloom of *Margalefidinium* cf. *polykrikoides* in a Mediterranean shallow bay (Ionian Sea, Italy). *Marine Pollution Bulletin* 151: 110825.
- SAKAMOTO S, LIM WA, LU D, DAI X, ORLOVA T, IWATAKI M. 2021. Harmful algal blooms and associated fisheries damage in East Asia: Current status and trends in China, Japan, Korea, and Russia. *Harmful Algae* 102: 101787.
- SMAYDA TJ. 1997. Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. *Limnol Oceanogr* 42: 1137–1153.
- STRICKLAND JDH, PARSONS TR. 1972. A practical handbook of seawater analysis.
- TANG YZ, KOCH F, GOBLER CJ. 2010. Most harmful algal bloom species are vitamin B1 and B12 auxotrophs. *Proc Natl Acad Sci* 107: 20756–20761.
- WANG R, XUE Q, WANG J, TAN L. 2020. Competitive interactions between two allelopathic algal species: *Heterosigma akashiwo* and *Phaeodactylum tricoratum*. *Mar Biol Res* 16: 32–43.
- VICENTE HJ, GAID RD, DEJARME HE. 2002. Harmful algal bloom in Iligan Bay, Southern Philippines. *Science Diliman* 14: 59–65.
- WELIYADI E. 2012. The effects of physico-chemical parameter on *Cochlodinium polykrikoides* abundance in Sepangar and Likas Bays, Sabah [MS thesis]. Universiti Malaysia Sabah. 71p (available at the Universiti Malaysia Sabah).
- YÑIGUEZ AT, LIM PT, LEAW CP, JIPANIN SJ, IWATAKI M, BENICO G, AZANZA RV. 2021. Over 30 years of HABs in the Philippines and Malaysia: what have we learned? *Harmful Algae* 102: 101776.