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A Study on Allelopathy Effects of Dinoflagellate *Margalefidinum (Cochlodinium) polykrikoides* on *Pyrodinium bahamense*

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ABSTRACT

The dinoflagellate *Margalefidinium polykrikoides*, a fish killer, is found all over the world and is associated with harmful algal blooms (HABs). In this study, we investigated the allelopathy effects of *M. polykrikoides* on the growth of toxic dinoflagellate *Pyrodinium bahamense*. Allelopathy effects of *M. polykrikoides* were studied using different volumes (10 mL, 20 mL, and 50 mL) of filtrate filtered from *M. polykrikoides's* bloom collected from the field and cultured medium. The results showed that *P. bahamense* was inhibited significantly ($p<0.05$) in 50 mL cell-free culture filtrate of *M. polykrikoides* bloom with an inhibition rate of >80%, but not in *M. polykrikoides* cell-free culture filtrate. In a bi-algal, non-contact culture experiment of *M. polykrikoides* and *P. bahamense* at three different ratios was also conducted, separated by a 5µm membrane. The observation showed that at a ratio of 1:5 (100 cells mL^{-1} of *M. polykrikoides*: 500 cells mL-1 *P. bahamense*), *P. bahamense* cell number increased significantly ($p<0.05$) after Day 4. This study provides useful information on competing with *P. bahamense* and forming bloom to dominate the area occupied by *M. polykrikoides.*

Keywords: Allelopathy, *Margalefidinium polykrikoides*, *Pyrodinium bahamense*, Bloom

ABSTRAK

Dinoflagellate Margalefidinium polykrikoides, pembunuh ikan, ditemui di seluruh dunia dan dikaitkan dengan bunga alga yang berbahaya (HAB). Dalam kajian ini, kami menyiasat kesan alelopati M. polykrikoides terhadap pertumbuhan toksik dinoflagellate Pyrodinium bahamense. Kesan alelopati M. polykrikoides dikaji menggunakan isipadu berbeza (10 mL, 20 mL, dan 50 mL) turasan yang ditapis daripada bunga M. polykrikoides yang dikumpul dari ladang dan medium kultur. Keputusan menunjukkan bahawa P. bahamense telah dihalang dengan ketara (p<0.05) dalam 50 mL turasan kultur bebas sel M. polykrikoides mekar dengan kadar perencatan >80%, tetapi tidak dalam turasan kultur bebas sel M. polykrikoides. Dalam eksperimen bi-algal, kultur bukan sentuhan M. polykrikoides dan P. bahamense pada tiga nisbah berbeza turut dijalankan, dipisahkan oleh membran 5µm. Pemerhatian menunjukkan bahawa pada nisbah 1:5 (100 sel mL-1 M. polykrikoides: 500 sel mL-1 P. bahamense), bilangan sel P. bahamense meningkat dengan ketara (p<0.05) selepas Hari 4. Kajian ini menyediakan maklumat berguna tentang bersaing dengan P. bahamense dan membentuk mekar untuk menguasai kawasan yang diduduki oleh M. polykrikoides.

Kata Kunci: Alelopati, *Margalefidinium polykrikoides*, *Pyrodinium bahamense*, mekar

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1. INTRODUCTION

Marine harmful algal blooms (HABs) phenomena exhibit impacts on aquaculture and marine aquatic ecosystems, and they are toxic to human and other organisms. One of the HAB species is the fish killer, dinoflagellate *Margalefidinium* (=*Cochlodinium*) *polykrikoides* and this species is widely distributed in temperate, subtropical, and tropical coastal waters (Tang & Gobler, 2010; Band-Schmidt et al., 2020; Sakamoto et al., 2021; Tang et al., 2022). It has gained much attention from scientists because it produces a toxin that is harmful to aquatic organisms (e.g. fish, shellfish, and phytoplankton) (Tang & Gobler, 2010; Band-Schmidt et al., 2020; Tang et al., 2022), which has caused economic losses in the aquaculture industry (Sakamoto et al., 2021). Toxic dinoflagellate *Pyrodinium bahamense* produces saxitoxin that causes severe paralytic shellfish poisoning (PSP) (Usup et al., 2012; Ching et al., 2015; Jipanin et al., 2019). The species' distribution is restricted to the Indo-Pacific and Caribbean Seas (Usup et al., 2012). Nevertheless, this species produces saxitoxin which is higher in concentration than other saxitoxin producers such as *Alexandrium tamiyavanichii* and *Alexandrium minutum* (Usup et al., 2006).

Many factors influence the HABs phenomenon such as change in temperature, global warming, and eutrophication, and these factors have contributed to the expansion of HABs' distribution (Usup et al., 2012: Glibert, 2020), including *M. polykrikoides.* Another factor that has been strongly suggested to play an important role in HAB occurrences is allelopathy. Allelopathy is the ability of phytoplankton to release toxic metabolites into the environment during the bloom that will affect other organisms surrounding it by inhibiting their growth or causing mortality (Legrand et al., 2003; Band-Schmidt et al., 2020). Allelopathy involves donor and target cells whereby donor cells will release allelochemicals to inhibit the growth of target cells. Many factors contribute to the success of allelopathy, i.e., the ratio between each species and direct or indirect contact between both species. Many studies on the allelopathy of *M. polykrikoides* on other phytoplankton have been carried out, but not on *P. bahamense.* Therefore, understanding the bloom mechanism of this species is crucial due to the huge impact of *M. polykrikoides'* bloom on the fish industry. Sakamoto et al. (2021) reported that the species has caused millions of United States Dollars (USD) in losses to aquaculture industries in East Asian countries. Previous studies showed that *M. polykrikoides* inhibited the growth of other harmful dinoflagellate species such as *Akashiwo sanguinea* and *Gymnodinium catenatum* (Tang & Gobler, 2010; Band-Schmidt et al., 2020).

A harmful algal bloom caused by the toxic *Pyrodinium bahamense* was first reported in the Sabah coastal waters in 1976 (Roy, 1977). *Pyrodinium bahamense* is a toxin producer that causes paralytic shellfish poisoning in humans through consuming contaminated shellfish (Usup et al., 2012). In 2005, the first bloom of *M. polykrikoides* was reported in Sepanggar Bay, Sabah, and since then, the co-occurrences of both species have been observed in the area (Adam et al., 2011; Mohammad-Noor et al., 2014: Chong et al., 2020; Lorons et al., 2022). During certain period, *M. polykrikoides* dominated and *P.bahamense* occurred at low cell density (Mohammad-Noor et al., 2014; Chong et al., 2020) or they form monobloom at certain time (Lorons et al., 2022). The blooms of these species in the Sepanggar coastal waters are monitored by the Fisheries Department of Sabah (DOFs), and a warning will be issued when both species

reach a certain cell density (Jipanin et al., 2019). To date, several laboratory studies have been conducted to increase the understanding of the ecology and physiology of both species, which include the growth of *P. bahamense* (Usup, 1995; Mustakim et al., 2019), the toxicity of *P. bahamense* (Usup et al., 1994; Usup et al., 2006; Al-Has et al.,2023), and the growth of *M. polyrkrikoides* (Tang & Gobler, 2010; Aquino-Cruz et al., 2020; Al-Has et al., 2022a). Nevertheless, a study on the interaction between *M.polykrikoides* and *P. bahamense* is still limited (Shaleh et al., 2010; Al-Has et al., 2022b).

Previous study by Al-Has et al. (2022b) showed that *M. polykrikoides* inhibited the growth of *P. bahamense* through direct contact. Therefore, this study was conducted to understand the allelopathic interaction of *M. polykrikoides* and *P. bahamense* in non-contact conditions and to compare the allellopathic effect using cell-free filtrate samples collected from the field and laboratory. Hence, additional information on these two harmful species could be utilized to understand the bloom mechanisms of these two important species and could be used to monitor the occurrences of these species, particularly at Sepanggar Bay, Sabah.

2 MATERIAL AND METHODS

2.1 Cultures and culturing conditions

The strain of *Margalafedinium polykrikoides* and *Pyrodinium bahamense* were obtained from the Borneo Marine Research Institute. The cells were cultured in sterile f/2 medium with a salinity of 30 ppt, made with autoclaved filtered seawater. Cultures were maintained at a temperature of 25ºC with a 12- hour light/ 12 hour- dark cycle.

2.2 Allelopathy Experiments

2.2.1 Filtrate experiment.

The experiment was carried out using the bloom of *M. polykrikoides* that occurred in Sepanggar Bay in October 2020 (1,116 cells mL^{-1}) and a culture of *M. polykrikoides*. The culture of *P.bahamense* was maintained until the exponential phase. The *M. polykrikoides* cells were gently removed by filtration using glass fiber membranes (GF/F Whatman) (Band-Schmidt et al., 2020). Three volumes (10, 20 and 50 mL) of *M. polykrikoides* bloom and culture filtrates were inoculated in 250 mL Erlenmeyer flasks containing 150 mL of *P. bahamense* (500 cells mL^{-1}). *P. bahamense* (150 mL) cultures with an initial cell abundance of 500 cell mL^{-1} in f/2 medium were used as controls. All experiments were carried out in triplicate.

2.2.2 Non-contact experiment.

Experiments were conducted by exposing *P. bahamense* to M. *polykrikoides* and they were then separated with a membrane in three different ratios. To create a physical barrier between them, 5 µm nylon membranes were used by fitting them at the bottom of 15 mL falcon tubes. Within the tube, 15 mL of *M. polykrikoides* were inoculated in a 1:5 ratio (100 cells mL^{-1}: 500 cells mL^{-1}); a 5:1 ratio (500 cells mL^{-1}: 100 cells mL^{-1}); and a 5:5 ratio of *M. polykrikoides*: *P. bahamense* (500 cells mL^{-1}: 500 cells mL^{-1}). Cultures of *P. bahamense* at initial cell abundance of 100 and 500 cell mL^{-1} in f/2 medium were used as controls. All experiments were carried out in triplicate.

The effect of *M. polykrikoides* in both experiments on *P. bahamense* was examined by monitoring the *P. bahamense* cells daily. A three mL aliquot sample was collected daily and preserved in Lugol-iodine. The samples were collected for seven days and the cell enumeration was conducted using Sedgwick-Rafter cells

under light microscopy. Allelopathic growth inhibition was measured by the following equation: allelopathic effect (AE) (%) =[(N_0-N)/N_0)]100, where N_0 and N are the number of cells in the control and the number of cells in the treatment, respectively (Fistarol et al., 2004).

2.2.3 Statistical analysis.

One-way analysis of variance (ANOVA) was applied after the normality test. Statistical Package For Social Science (SPSS) version 21 was used to test the significant difference ($p \leq 0.05$) between treatments using ANOVA and a Tukey post hoc test.

3. RESULTS AND DISCUSSION

In the filtrate experiment, the inhibition rate of *P. bahamense* was low (<50%) when introduced to the volumes of 10 mL (Figure 1a,1d) and 20 mL (Figure 1b, 1e) filtered from the bloom and culture of *M. polykrikoides*. However, the 50 mL filtrate bloom of *M. polykrikoides* (Figure 1c,1f) exhibited significant allelopathic effects on *P. bahamense* (one-way ANOVA: $F_{6,35}$ = 41.76, $p<0.05$) with a high percentage of allelopathic effect (> 90%) (Figure 1). Both the culture and bloom of *M. polykrikoides* can exhibit high inhibitory effects on many phytoplankton (Tang & Gobler, 2010; Band-Schmidt et al., 2020). This study shows that the bloom of *M. polykrikoides* has a higher allelopathic effect on *P. bahamense* and clearly can be seen when exposed to a higher volume of filtrate but not to the culture filtrate (Figure 1). However, Band-Schmidt et al. (2020) reported that when using the culture filtrate of *M. polykrikoides*, the cell density of *Gymnodinium catenatum* decreased when a higher volume of culture filtrate of *M. polykrikoides* was used, and this might be due to a higher secondary metabolite that was released when the cells disrupted. According to Aquino-Cruz et al. (2020), *M. polykrikoides* produced a significant concentration of hemolytic activities (50-60%) at high abundances (1.0-2.0 x10^4 cells mL^{-1}). However, the average allelopathic effects of filtrate *M. polykrikoides* in the present study range from 20-50%.

Tang et al. (2022) suggested that the allelochemicals and toxicity of *M. polykrikoides* are caused by the same chemical. In addition, the toxic substance does not require direct physical contact to cause fish mortality (Tang & Gobler, 2009). A study by Tang & Gobler (2010) found that allelopathy effects of *M. polykrikoides* cultures and bloom could cause about 60-100 % cell mortality within 24 hours. However, the filtration could lead to the complete loss of the allelopathic effect of *M. polykrikoides* due to the short-lived allelochemicals dissolving in the culture medium (Tang & Gobler, 2010; Wang et al., 2020). The degradation of phytoplankton allelochemicals can happen, and the interaction is influenced by surrounding factors such as bacteria, light, and pH (Legrand et al., 2003: Granéli et al., 2008). In addition, water turbulence and the ability of the dinoflagellates to swim away can alter allelopathy interaction under field conditions (Jonsson et al., 2009). Furthermore, allelochemicals released by organisms are difficult to prove to have allelopathic activities in nature, as these allelochemicals are influenced by the mode of release, concentration, phytotoxic action, and target response to the allelochemical released (Inderjit & Duke, 2003).

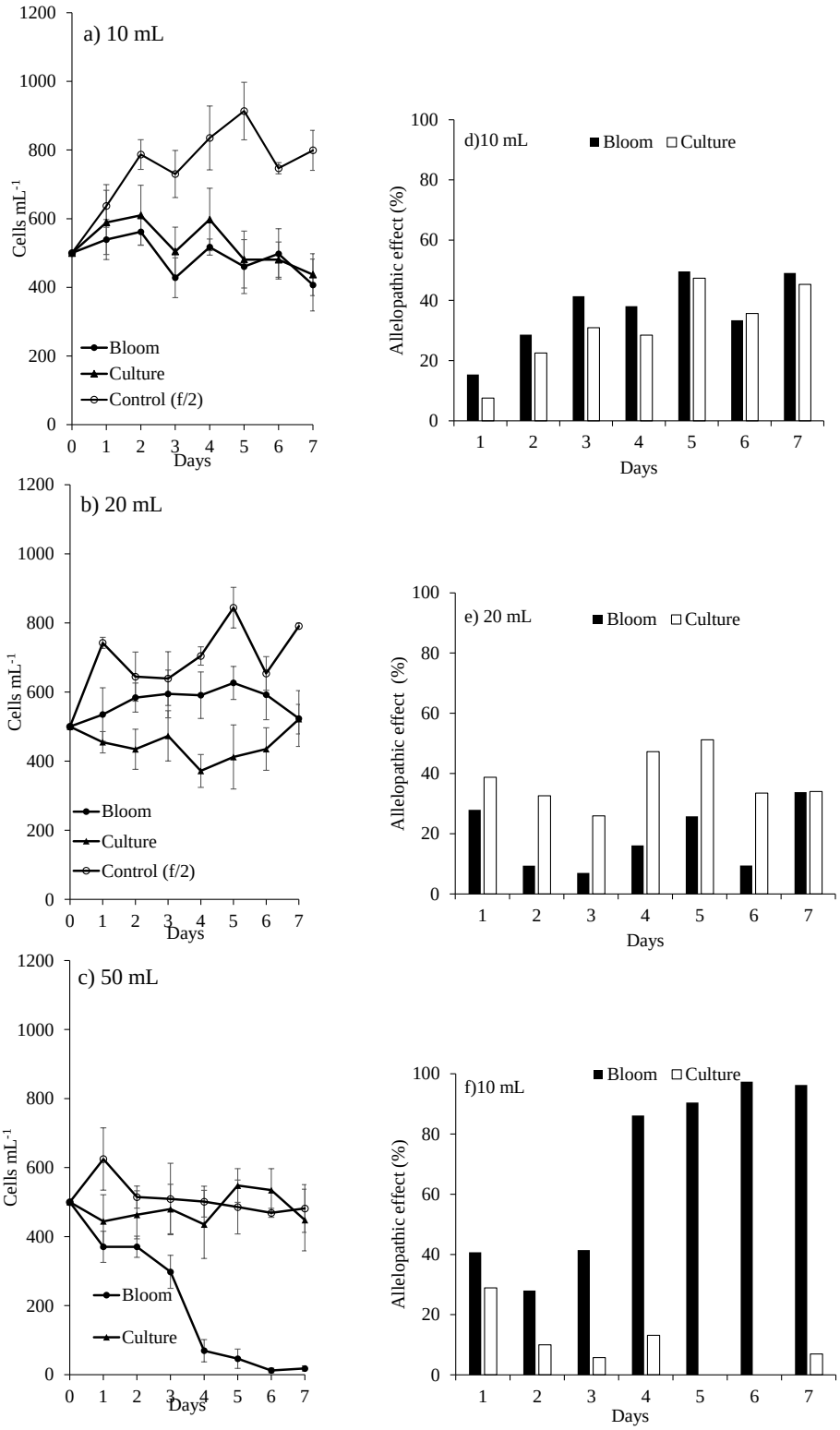

Figure 1. Cell abundance (cells mL-1) and inhibition percentange (%) of *Pyrodinium bahamense* exposed to different volumes of filtrate a,d) 10 mL b,e) 20 mL and c,f) 50 mL.

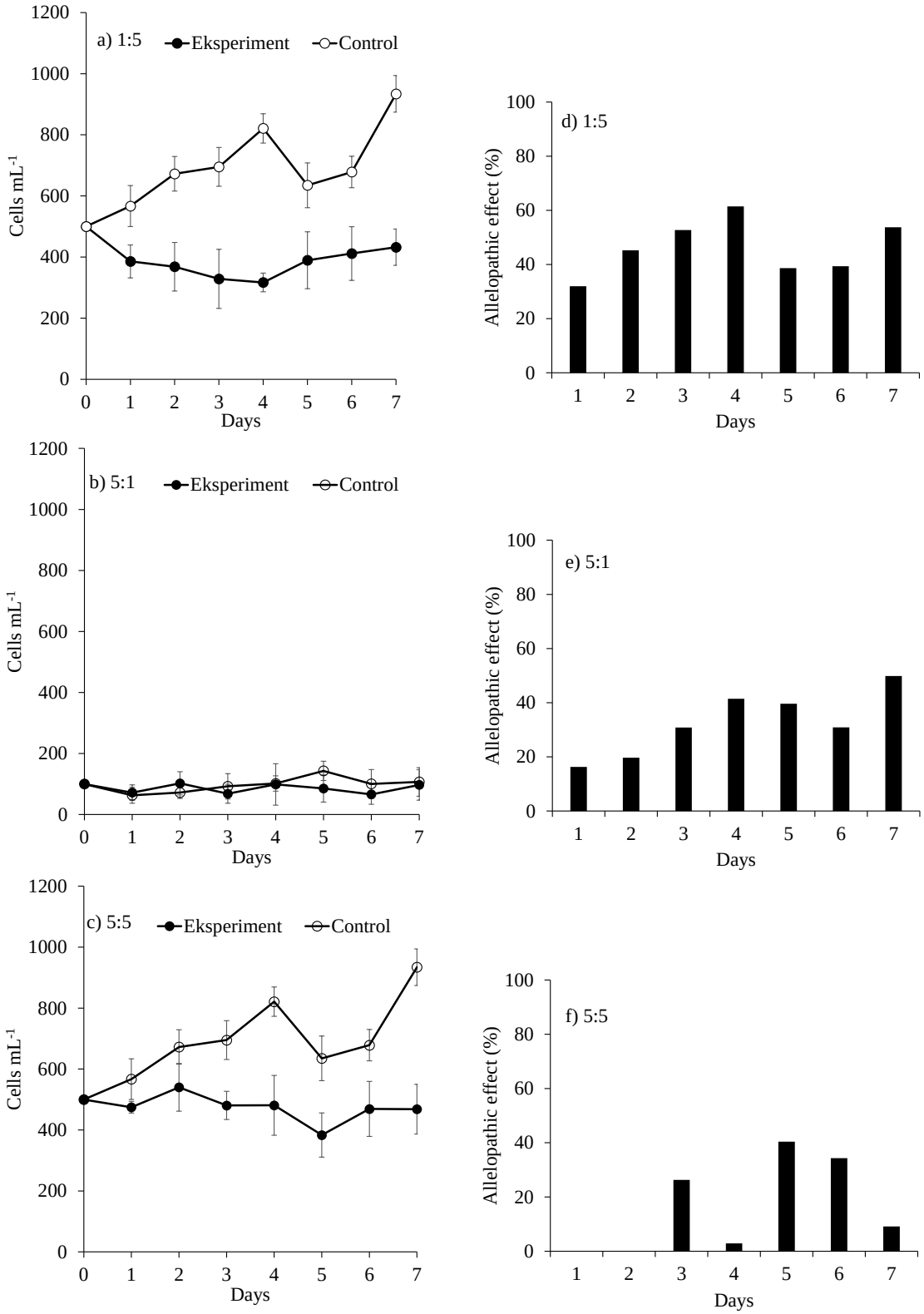

Figure 2. Cell abundance (cells mL-1) and allelopathic effect (%) of *P. bahamense* exposed to different ratios of *M. polykrikoides* in a non-contact experiment. The ratios of abundance (a,d) 1:5, (b,e) 5:1, and (c,f) 5:5 indicate the ratio of *M. polykrikoides* to *P. bahamense*.

The initial number of cells influences the allelopathy impact of *M. polykrikoides,* with more cells producing a stronger allelopathy effect (Tang & Gobler, 2010; Band-Schmidt et al., 2020). However, this situation was not seen in the experiments conducted (Figure 2). In the non-cell contact experiments, indirect exposure of the ratio of 100 cells mL^{-1} of *M. polykrikoides* to 500 cells mL^{-1} of *P. bahamense* (1:5; Figure2 a,d) showed an inhibitory cell number (inhibition rate of 60%) at the beginning (Day 4), but led to a significant increase (one way ANOVA: $F_{6,77}=3.74$, $p<0.05$) of *P. bahamense* afterward until it reached 432 cells mL^{-1} at Day 7. This indicates that *P. bahamense* may release allelochemicals and be able to recover its cell number. The allelopathic effect of *M. polykrikoides* requires direct exposure of the cells, as seen in a research done under varied nutrient conditions at a ratio of 500 cells mL^{-1} of *M. polykrikoides* and 100 cells mL^{-1} of *P. bahamense* via a direct contact experiment (Al-Has et al., 2022b).

Studies have shown that allelopathic activity is essential for a species to survive and increase its cell number. Allelochemicals may target specific species and may not be similar to the toxin produced, and the effects do not correlate with saxitoxin levels (Fistarol et al., 2004). For example, allelopathy studies of saxitoxin producer *Alexandrium catenella* reported the production of Alexandrolide, an allelochemical compound that can inhibit the growth of diatoms, e.g., *Skeletonema costatum*, but not other dinoflagellates such as *Amphidinium carterae* and *Karenia mikimotoi* (Satake et al., 2019). Therefore, it is crucial to identify the allelochemical produced by *M. polykrikoides* and *P. bahamense* to increase our understanding of the interaction between these species and with other phytoplankton species.

Previous studies have shown that allelopathic effects have been observed in natural phytoplankton but are difficult to measure (Hakanen et al., 2014). Allelopathy's ability helps the slow-growing dinoflagellate compete for nutrients in the community. *P. bahamense* is difficult to cultivate in the laboratory and has a slow growth rate (Usup et al., 2012). The new findings on the potential allelopathic ability of *P. bahamense* explain why the species dominate in the presence of *M. polykrikoides*.

Another reason for not detecting allelopathic effects is the size of the membrane. However, Tang & Gobler (2010) reported that *M. polykrikoides* inhibited the growth of *A. sanguinea* when using 5 µm mesh nylon. Others studies reported different results using different membrane sizes. Study by Yamasaki et al. (2007) found that *M. polykrikoides* did not inhibit the growth of *A. sanguinea* using a 3 µm mesh membrane filter. Another study showed that *M. polykrikoides* did not inhibit the growth of *G. catenatum* using a 10 µm nylon membrane (Band-Schmidt et al., 2020). This concludes that the membrane's size is important as it will permit the flow-through of the allelochemical to the target cells. Nevertheless, these results must be interpreted with caution as different experiments were conducted in different environmental conditions, thus influencing the results obtained.

4. CONCLUSION

The allelopathic effect of *M. polykrikoides* on the growth of *P. bahamense* is higher when using filtrate from the bloom samples collected from the field compared to *M. polykrikoides* culture medium. This indicates the complexity of allelopathic

interaction between phytoplankton species and is governed by many biological, chemical, and physical factors. The new findings on the potential ability of *P. bahamense* to produce allelochemicals to maintain its bloom indicate a critical strategy for the species to survive. Considering the species is toxic and has caused thousands of human deaths in Sabah, this finding warrants further attention. Although allelopathy is a complex study, understanding the bloom mechanism, including donor and target ratios, contact versus non-contact, and laboratory versus field study, will shed light on the bloom mechanisms of these two important species, as well as contribute towards harmful algal bloom studies in general.

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