Allelopathic effects of *Margalefidinium polykrikoides* on the growth of *Pyrodinium bahamense* in different nutrient concentrations

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Abstract

Co-occurrence of the dinoflagellates *Margalefidinium polykrikoides*, a known fish killer, and the neurotoxic species *Pyrodinium bahamense* is commonly observed in the coastal waters of Sabah, Malaysia. During most of these events, *M. polykrikoides* dominated the bloom, apparently suppressing the growth of *P. bahamense*. To increase our understanding on the nutrient conditions of this phenomenon, a study was conducted to explore the interaction between these species. The specific aim was to document the allelopathic effects, if any, of *M. polykrikoides* on *P. bahamense* when varying ratios of the two species were co-cultured under different nitrogen (N) and phosphorus (P) concentrations. The bioassay experiments started with three cell abundance proportions, which were 5:5 (500 cells mL^{-1} of each species, *M. polykrikoides*, and *P. bahamense*); 1:5 (100 cells mL^{-1} of *M. polykrikoides* and 500 cells mL^{-1} of *P. bahamense*); and 5:1 (500 cells mL^{-1} of *M. polykrikoides* and 100 cells mL^{-1} of *P. bahamense*). Additionally, culture filtrates (10, 20 and 50 mL) from the late exponential phase of *M. polykrikoides* were added to 150 mL of *P. bahamense* to determine if cell filtrates were allelopathic. Results indicate that *M. polykrikoides* was allelopathic to *P. bahamense* when nutrients were abundant, but not when nutrients were limiting or N was limiting relative P. The production of allelopathic compounds was supported by abnormal morphological changes in *P. bahamense* when co-cultured with *M. polykrikoides*. This capacity to suppress *P. bahamense* growth, combined with the inherently faster growth rate of *M. polykrikoides* relative to *P. bahamense* can account for why *M. polykrikoides* forms nearly monospecific blooms when nutrients are high. The filtration studies indicated the allelopathic capacity of *M. polykrikoides* required direct cell contact or that the allelopathic compounds degraded rapidly and were inactive when added to *P. bahamense* cultures. These results are important in understanding the bloom mechanisms of these two harmful algal blooms (HABs) species.

Keywords: Allelopathy, bi-algal cultures, *Margalefidinium polykrikoides*, nutrients, *Pyrodinium bahamense*

Introduction

Harmful algal blooms (HABs) are natural events where increased phytoplankton biomass can result in adverse impacts on both public and ecosystem health. In recent years, *Margalefidinium* (*Cochlodinium*) *polykrikoides* blooms have gained attention because of their increasing geographical distribution worldwide (Kudela and Gobler, 2012). This species has been reported to produce cysts, is a fast swimmer, mixotrophic, and has Allelopathic properties (Kudela and Gobler, 2012).

Allelopathy is a phenomenon where a species produces chemicals that inhibit the growth of, or kill, other competing species (Hattenrath-Lehmann and Gobler, 2011). Species which have been adversely effected by *M. polykrikoides* include *Gymnodinium catenatum* (Band-Schmidt *et al*., 2020), *Chattonella subsalsa*, *Isochrysis galbana*, *Heterocapsa rotundata*, *Thalassiosira weissflogii* (Tang and Gobler, 2010) and *Akashiwo sanguinea* (Yamasaki *et al*., 2007). These studies indicate the allelopathic effects of *M. polykrikoides* are not species-specific. The impacts of other allelopathic species has been shown to be influenced by nutrient limitation, i.e. *Alexandrium tamarense* (Zhu and Tillmann, 2012) and *Prymnesium parvum* (Uronen *et al*., 2005). In contrast, the effects of nutrient availability on the allelopathic impacts of *M. polykrikoides* are less studied.

Another HAB species of concern, which often co-occurs with *M. polykrikoides* is *Pyrodinium bahamense*. This species is a saxitoxin producer reported from the Indo-Pacific and Caribbean Sea (Usup *et al*., 2012). Although the distribution of *P. bahamense* is more limited compared to *M. polykrikoides*, the species can have devastating impacts where it blooms due to its ability to produce higher intracellular saxitoxin concen-trations relative to other saxitoxin producers which cause paralytic shellfish poisoning (PSP) (Usup *et al*., 2012).

The co-occurrence of *M. polykrikoides* and *P. bahamense* has been monitored by the Sabah Fisheries Department of Malaysia since 1976 (Jipanin *et al*., 2019). Since 2005, blooms of *M. polykrikoides* have also been observed in the coastal waters adjacent to Kota Kinabalu, the capital city of Sabah (Anton *et al*., 2008). These later blooms sometimes co-occur with *P. bahamense* (Adam *et al*., 2011; Chong *et al*., 2020). Shaleh *et al*. (2010) found that in the laboratory, salinity and pH did not significantly affect interactions between these species. However, field reports indicate elevated nutrient concentrations (N and P) can trigger blooms dominated by *M. polykrikoides* blooms and containing only a small subpopulation of *P. bahamense* (Mohammad-Noor *et al*., 2014). The present study used bi-species cultures to simulate bloom conditions where either *M. polykrikoides* or *P. bahamense* dominated, or co-dominated the bloom population. These different simulated populations were further allowed to develop under different nutrient conditions. The goal was to quantify how the relative cell densities of *M. polykrikoides* versus *P. bahamense* in conjunction with different nutrient levels might affect the allelopathic interactions between these two species. Also, the effects of cell free filtrates from *M. polykrikoides* on the growth of *P. bahamense* were investigated to evaluate if

direct cell contact was required to achieve any observed allelopathic effects.

Materials and Method

Strains of *M. polykrikoides* and *P. bahamense* were obtained from the Borneo Marine Research Institute (BMRI), University Malaysia Sabah, Malaysia. Strains of *M. polykrikoides* and *P. bahamense* were isolated during blooms in Sepanggar Bay in 2019 and 2013, respectively, and established into unialgal non-axenic cultures in f/2 media. The experiments were conducted by inoculating *M. polykrikoides* into f/2 media containing three nutrient concentrations: (a) f/2 medium, 882 µM NO_3-N and 32 µM PO_4-P, N:P = 27.6 (b) nutrient enriched media (NE), 30 µM NO_3-N and 5 µM PO_4-P, N:P = 6 and (c) cells were added from the late log stock cultures without nutrient addition, low concentration (LC). *Pyrodinium bahamense* was grown in f/2 medium. Trace metals and vitamins were the same as listed in Guillard and Ryther (1962). All media were prepared using autoclaved filtered seawater. The cultures were maintained at 25-26 ^{o}C with a 12:12 light–dark cycle illuminated by LED light with an intensity of 100 µmol quanta m^{-2} s^{-1}.

Cell contact experiments Bi-algal culture experiments were conducted in 250 mL flasks containing 150 mL of f/2 medium which augmented with nutrients as described above. Both species were harvested during their late exponential phase from the unialgal cultures and mixed at three cell abundance ratios, which were 1:5 (100 cells 100 mL^{-1} of *M. polykrikoides* and 500 cells mL^{-1} of *P. bahamense*); 5:1 (500 cells mL^{-1} of *M. polykrikoides* and 100 cells mL^{-1} of *P. bahamense*); and 5:5 (500 cells mL^{-1} of each species). Treatments were run in triplicate.

Culture filtrate experiments Cell-free filtrates of *M. polykrikoides* cultured in different nutrient concentrations (f/2, EN and LC treatments) were harvested at the late exponential phase and filtered through 47 mm GF/F filter (Whatman). Three volumes (10, 20 and 50 mL) of the culture filtrates of *M. polykrikoides* were added to 250 mL Erlenmeyer flasks containing 150 mL of *P. bahamense* containing an initial density of *P. bahamense* was 500 cells mL^{-1}. A three mL aliquot was collected from each experiment daily during seven days for cell enumeration and morphological observations. One milliliter of each sample was counted twice using a Sedgewick Rafter cell under a light microscope (Zeiss, Axiostar). Growth rate (µ) was determined based on the following formula (Guillard and Ryther, 1962).

Results and Discussion

In the f/2 nutrient treatment, *P. bahamense* was able to initially compete well with *M. polykrikoides* when it started with a 5:1 concentration advantage. However, by day four of the experiment, *P. bahamense* began declining relative to the control as *M. polykrikoides* cell concentration began slowly increasing. In the corresponding treatment where *M. polykrikoides* had a 5:1 starting advantage, *M. polykrikoides* increased slowly relative to the control whereas *P. bahamense* failed to increase in the presence of *M. polykrikoides*. Interpretation of these experimental results is complicated because the control culture of *P. bahamense* did not grow well. The treatment starting with the 5:5 (equal concentration of both

species) showed that *M. polykrikoides* cell concentrations steadily increased while the *P. bahamense* cell population steadily declined, with both controls showing steady growth. Cumulatively, these results are consistent with *M. polykrikoides* inhibiting the growth of *P. bahamense* either through allelopathy or an ability to outcompete *P. bahamense* for nutrients. Filtrates from *M. polykrikoides* cultures did not effect the cell morphology or the growth of *P. bahamense.*

Evidence for the production of allelopathic com-pounds is supported by abnormal changes in the morphology of *P. bahamense* after two more days with direct cell? contact with *M. polykrikoides*. The main changes observed were loss of the thecate plate and cell lysis (Fig. 2). These observations were consistent with allelochemicals increasing the cell membrane penetrability thereby suppressing cell growth (Xue *et al.*, 2018). Future studies where ambient nutrient concentrations are monitored closely are needed to further separate the extent to which the inhibition is related to nutrient competition versus production of allelo-chemical(s).

Interestingly all three experiments showed that *M. polykrikoides* growth in the bi-species cultures was slower than that in the control. This reduced growth rate indicates a cost for competing with *P. bahamense*, but that cost is not likely due to the production of allelochemicals (Zhu and Tillman 2012). Another observation from the control cultures is that *M. polykrikoides* has an inherently faster growth rate than *P. bahamense*. This would give *M. polykrikoides* a growth advantage when nutrient levels are elevated.

The high nutrient treatment corresponds to field conditions where nutrient concentrations are elevated, but there is an excess of P relative to N (N:P = 6). In the treatment where there were five *P. bahamense* relative to one *M. polykrikoides*, the population of *P. bahamense* steadily declined while the *M. polykrikoides* cell counts gradually increased. The 5:1 *M. polykrikoides*: *P. baha-mense* experiment failed after three days and provided no results. In contrast, the 5:5 experiment showed that under the more N-limited conditions, growth of both species in the bi-cultures was very similar to the 1:5 treatment. The only difference from that initial 1:5 treatment is that the control population rapidly increased indicating the media is capable of supporting rapid growth of *M. polykrikoides*. Though these nutrient conditions were not optimal for *P. bahamense* growth, at high enough cell concentration this species inhibited the growth of *M. polykrikoides*. Because *P. bahamense* growth was exceeding slow this inhibition is unlikely due to direct nutrient competition indicating under certain nutrient regimes *P. bahamense* may inhibit *M. polykrikoides*, instead of the reverse situation.

The low nutrient condition, where cells grown in f/2 media, with an N:P ratio closer to the Redfield ratio, and then allowed to become more nutrient limited represents what might happen more toward the end of a bloom. In the 1:5 *M. polykrikoides*: *P. bahamense* treatment the controls grew while the populations in the bi-species cultures steadily declined. In the 5:1 treatment the growth of both species in the controls and the cells in the bi-species cultures were essentially the same (Fig. 1) as was also true for the 5:5 treatment. These results are consistent with cells growth largely

dependent on internally stored nutrient with no indication of any allelopathic interactions.

Cumulatively these results suggest elevated nutrient concentrations favor *M. polykrikoides* over *P. bahamense* because of its inherently higher growth rate. The data are further consistent with high densities of nutrient replete *M. polykrikoides* having the ability to produce allelopathic compounds that impeded the growth of *P. bahamense*. This allelopathic inhibition of *P. bahamense*, however, did not occur at higher nutrient concentrations where the N:P ratio was skewed toward an excess of phosphate or when the cells were nutrient limited (Fig. 1). This may indicate N is a structural component of the allelopathic compound itself. Similarly observed high allelopathic activity of *A. sanguinea* when nutrient concentrations were high. Tang and Gobler (2010) also demonstrated the allelopathic effect of *M. polykrikoides* was dependent on the initial cell abundance and Band-Schmidt *et al.*, (2020) showed the duration of the inhibition was proportional to cell density.

In summary, *M. polykrikoides* likely dominates over *P. bahamense* when nutrients are high due to its inherrently higher growth rate (Kudela and Gobler, 2012). Evidence from this and other studies futher support allelopathic supression of *P. bahamense* when *M. polykrikoides* cell concentration are high. This would account, at least in part, for why there are relatively few *P. bahamense* present when *M. polykrikoides* cell densities are high and nutrients abundant.

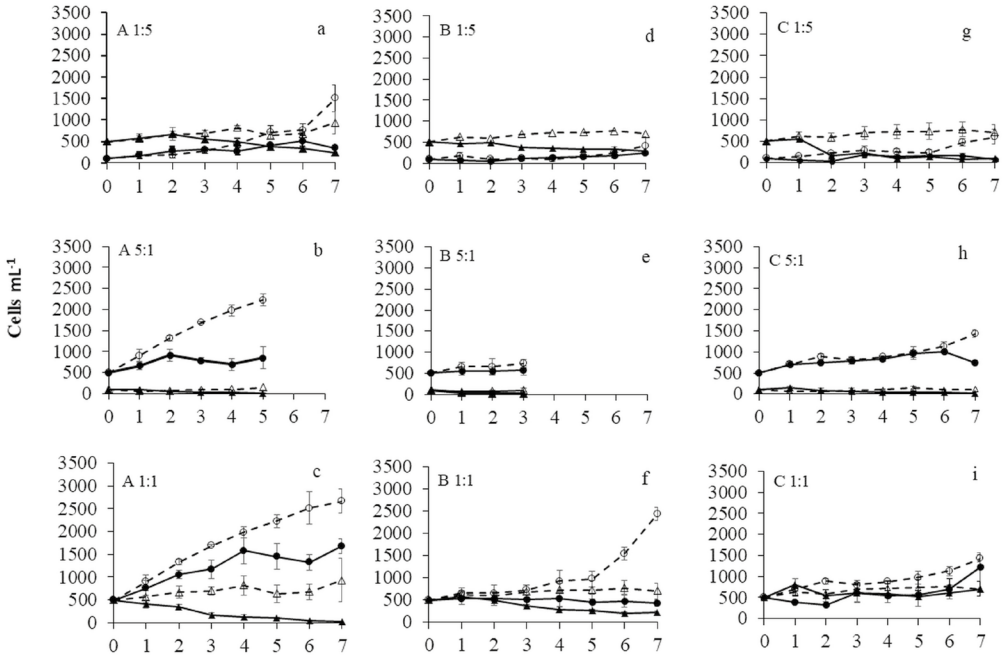

Fig. 1. Cell abundance proportions of *M. polykrikoides* (mean ± standard deviation) and *P. bahamense* in co-cultured cell contact experiments: (i) 1:5, (ii) 5:1, (iii) 5:5 at different treatments (A) f/2 medium (f/2); (B) nutrient enriched (EN); and (C) low concentration (LC). Closed circles: *M. polykrikoides* in bi-algal culture; open circles: *M. polykrikoides* control; closed triangles: *P. bahamense* in bi-algal culture; open triangles: *P. bahamense* control.

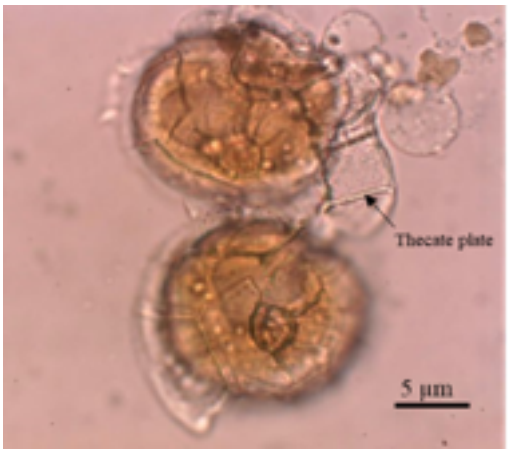

Fig. 2. Detachment of the thecal plate of *P. bahamense* when exposed to *M. polykrikoides*.

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