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Paralytic Shellfish Profiles Produced by the Toxic Dinoflagellate *Pyrodinium bahamense* from Sepanggar Bay, Malaysia

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ABSTRACT

Pyrodinium bahamense var *compressum* is a harmful dinoflagellate that produces saxitoxin, which causes paralytic shellfish poisoning (PSP) that is deadly to humans. A non-axenic culture of *P. bahamense* was established using f/2 media from samples collected from Sepanggar Bay, Kota Kinabalu, Sabah. Toxin analyses of cultures harvested on days 60, 120, 180, and 360 were performed using high-performance liquid chromatography with a fluorescence detector and compared with samples collected at the same location during the bloom in 2021. The highest cell toxin content was found in the bloom sample (86.2 fmole/cell), and no toxin was detected in the culture 60 days old. In addition, cell toxin content for the *P. bahamense* culture was low (9.4-16.5 fmole/cell). Based on the toxin profile, *P. bahamense* comprises 84-98% of gonyautoxin 4. In summary, the current findings add to the existing knowledge of the toxin profiling of *P. bahamense*, a toxic, harmful algal bloom species, thus, leading to better toxin management.

Keywords: Gonyautoxin, HPLC, PSP, *Pyrodinium bahamense*, saxitoxin

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INTRODUCTION

Pyrodinium bahamense var. *compressum* is a thecate dinoflagellate and one of the most harmful algal bloom (HAB) organisms. The *P. bahamense* bloom is mainly distributed in the tropical Indo-Pacific and the pacific Atlantic coasts of Central America, including Florida (Usup et al., 2012). Paralytic shellfish poisoning (PSP) consists of more than 57 saxitoxin

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analogues (Oyaneder-Terrazas et al., 2017). The highest PSP concentration is usually recorded during or after an algal bloom. PSP is an illness caused by consuming shellfish contaminated with toxic dinoflagellates, a vector of PSP in humans due to the presence of saxitoxin (STX) in their tissue (Wiese et al., 2010). The STX PSP outbreak is usually associated with the algal bloom of toxic dinoflagellates, such as *P. bahamense*, *Gymnodinium catenatum*, and *Alexandrium* spp., often responsible for the paralytic shellfish poisoning toxin (PST) that can impact human health. Saxitoxin was isolated and named after the Alaskan butter clam (*Saxidomus gigantes*) in 1957 (Schantz et al., 1957).

Pyrodinium bahamense bloom has been a constant occurrence in Sepanggar Bay, Sabah, Malaysia, for decades. This species is the main cause of harmful algal blooms (HABs) in Sabah coastal waters besides *Margalefidinium polykrikoides*. Therefore, this area is regularly monitored for PSP by the Department of Fisheries, Sabah (DOFS). A warning will be released to the public once the *P. bahamense* population exceeds 7,000 cells/L and the shellfish toxicity level exceeds 80 µg poison 100/g of meat (Jipanin et al., 2019). In 2013, 64 patients were hospitalised, and four deaths were recorded (Jipanin et al., 2019; Suleiman et al., 2017) due to the consumption of contaminated shellfish, such as mussels (*Atrina fragilis*), green mussels (*Perna viridis*), and oyster (*Crassostrea belcheri*) collected from Kota Kinabalu (Suleiman et al., 2017). In addition, PSP cases have become increasingly common along the west coast of Sabah, including Tuaran, Kuala Penyu, and Membakut (Suleiman et al., 2017). Generally, shellfish toxins, especially in green mussels (Montojo et al., 2006; Suleiman et al., 2017), remain in their tissue as decarbomyl and other STX derivatives for up to two years before being released as waste (Mustakim et al., 2016).

Besides *P. bahamense*, other marine dinoflagellates, such as *Alexandrium minutum*, *Alexandrium tamiyavanichii*, and *G. catenatum*, are also associated with PSP in Malaysia (Mohammad-Noor et al., 2018; Usup et al., 2006). Despite that, *P. bahamense* has caused more fatalities than other species (Usup et al., 2006, 2012). The *P. bahamense* is challenging to culture in the laboratory and is not widely distributed, hence the lack of studies on the physiology of this species. In the laboratory, *P. bahamense* can grow in the enriched seawater media (ES-DK) (Usup et al., 1994) and f/2 medium (Gedaria et al., 2007; Mustakim et al., 2019), yielding less than 10,000 cells/mL; much lower than other saxitoxin producers, such as *Alexandrium* spp. (Usup et al., 2012). Since 1976, there has been no record of *P. bahamense* blooming in other Malaysian coastal waters (Yñiguez et al., 2021). Moreover, field data obtained during *P. bahamense* bloom suggests that this species thrives in waters with high salinity and temperature (Adam et al., 2011; Banguera-Hinestroza et al., 2016; Lorons et al., 2022; Mohammad-Noor et al., 2014; Morquecho, 2019; Phlips et al., 2006). Meanwhile, the specific growth rate of *P. bahamense*

increased when cultured at higher salinities under laboratory conditions (Gedaria et al., 2007; Muhammad Shaleh et al., 2010). *Pyrodinium bahamense* also coexists with *Margalefidinium polykrikoides* and *G. catenatum*; hence salinity, temperature, and pH may not be limiting factors of the bloom (Adam et al., 2011).

Generally, PSP is caused by exposure to STX (an alkaloid) and other analogues, such as gonyautoxins (GTXs), neosaxitoxin (NeoSTX), dicarbamoyl-saxitoxin (dcSTXs), decarbamoyl-neosaxitoxin (decneoSTX), and decarbamoyl-gonyautoxins (dcSTXs) through the consumption of contaminated shellfish (Farabegoli et al., 2018). Therefore, it is essential to discover the toxin produced by various algae to protect consumers from lethal food poisoning (Farabegoli et al., 2018; Hummert et al., 1997). Natural and cultured *P. bahamense* samples from the Indo-Pacific coasts contain dc-STX, STX, neo-STX, B1, and B2 (Usup et al., 2012). Meanwhile, the toxin content of *P. bahamense* batch culture from Kota Kinabalu and the Philippines is higher during the exponential phase based on the high-performance liquid chromatography (HPLC) analysis (Gedaria et al., 2007; Usup et al., 1994; Yahumin et al., 2022).

The cellular toxin content of *P. bahamense* might increase with a lower growth rate and remains unaffected by different growth conditions (Usup et al., 2012). Microalgae release toxins in the water body and toxicity levels of shellfish are affected by the abundance and duration of exposure to the toxic microalgae (Tang et al., 2021). In this study, a post-column oxidation HPLC-FLD method was performed according to Oshima (1995) to analyse the toxin level and profile of *P. bahamense* at different culture ages (60, 120, 180, and 360 days) after the death phase to see how long the toxin can be sustained and bloom according to the AOAC Official Method 2011.02 (AOAC International, 2011). The findings will act as additional information regarding the toxin content of *P. bahamense*.

MATERIALS AND METHODS

Cultures and Field Sample Collection

Pyrodinium bahamense culture (CC-UHABS-040(M)) was obtained from the Borneo Marine Research Institute (BMRI), isolated during blooms in Sepanggar Bay in 2012, and established into unialgal non-axenic cultures in f/2 media (Guillard & Ryther, 1962). The media was prepared using autoclaved filtered seawater, with a salinity of 30 and pH of 8 ± 0.1. The culture was maintained at 25–26^{o}C with a 12:12 light-dark cycle illuminated by LED lights with an intensity of 100 µmol quanta/$m^{2/}$s. For the experiment, *P. bahamense* was cultured for 360 days at a similar condition. In addition, *P. bahamense* was collected from the field during the bloom in December 2021 at Sepanggar Bay using a plankton net (20 µm) and transported to the lab for further analysis.

Sample Preparation for Toxin Analysis (Figure 1)

For toxin extraction, about 1 to 2 L of *P. bahamense* at different culture ages (60,

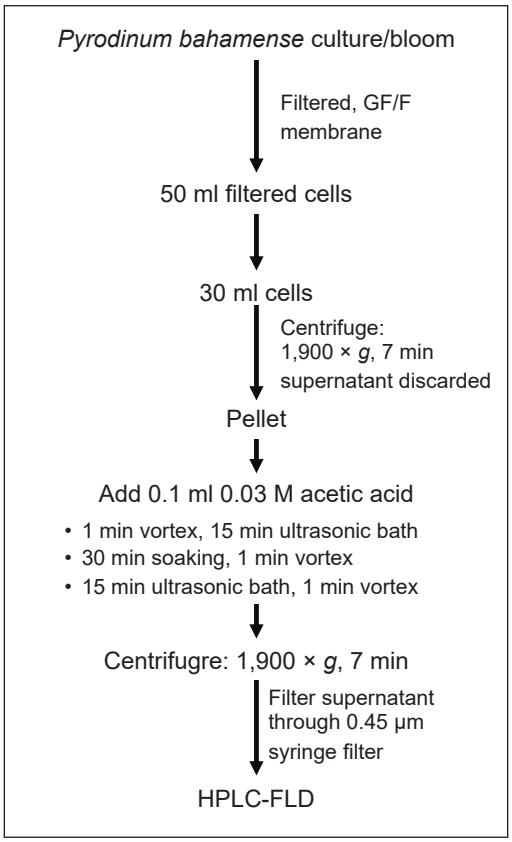

Figure 1. Sample preparation for toxin analysis

120, 180, and 360 days) and bloom samples were harvested and filtered using 45 mm glass-fibre GF/F filter paper (Whatman) to obtain a volume of 50 ml. A volume of 30 ml filtered cells was used for toxin extraction. Pellets were obtained by centrifuging the filtered cells at 1,900 × *g* for 7 min and discarding the supernatant. Afterwards, the pellets were mixed with 1 ml 0.03 M acetic acid (Merck, Germany) using a vortex for 1 min and homogenised in the ultrasonic water bath (Branson 2510, USA) for 15 min. Next, the samples were soaked in the mixture for 30 min and homogenised again for 15 min, followed by centrifugation at 1,900 × *g* for 7 min. Next, the supernatant was filtered using the 0.45 µm syringe filters (Whatman). The protocol was repeated on bloom samples collected from the field. Finally, cell densities were calculated using the Sedgewick Rafter chamber for the cultured and field samples at 400× magnification under the light microscope (Zeiss Axiostar, Germany), and the cell sizes (n = 20) were recorded.

Toxin Analysis by HPLC

For PSP toxin calibration, gonyautoxin-4/1 (GTX4/GTX1), gonyautoxin-3/2 (GTX3/GTX2), gonyautoxin-5 (GTX 5), dcSTX, and STX standards were purchased from the National Research Council (NRC), Halifax, Canada. The PSP toxins were analysed via HPLC (Shimadzu, Japan) with the post-column device and fluorescence detector utilising the isocratic post-column derivation with a slight modification from Oshima (1995). First, the samples were separated using a Luna C18(2) column (150 mm × 4.6 mm inner diameter, 120 Å, 5 µm) (Phenomenex) with a security guard cartridge (C18, 4.0 mm × 3.0 mm inner diameter) (Phenomenex, USA) at a flow rate of 0.8 ml/min. The column temperature was kept at 27ºC, while the post-column temperature was set at 65ºC for all runs. Toxin verification was performed in non-oxidising post-column conditions by substituting distilled water for the oxidising reagent. The reaction coil was kept in an ice bath during the analysis.

The chromatographic conditions are as follows: 1) STX = the mobile phase was 2

mM heptanesulfonate (Fisher Scientific, USA) in 30 mM ammonium phosphate buffer (Fisher Scientific, USA) and 5% (v/v) of acetonitrile (v/v, pH 7.1; 2) (J. T. Baker®, USA), and for the GTXs mobile phase was 2 mM heptanesulphonate (Fisher Scientific, USA) in 10 mM ammonium phosphate buffer (Fisher Scientific, USA) and 1% of acetonitrile (v/v, pH 7.1) (J. T. Baker®, USA). The acidifier was 0.5 M acetic acid (J. T. Baker®, USA), and the post-column oxidising reagent was 7 mM periodic acid (J. T. Baker®, USA) in 10 mM sodium phosphate buffer (Fisher Scientific, USA) at pH 9.0. The sample injection volume was 10 to 20 µl at a flow rate of 0.4 ml/min for each post column. Detection wavelengths were set at 330 nm for excitation and 390 nm for emissions. Toxin identification and quantification were carried out via comparisons with standard toxin materials. The concentrations of each toxin or epimeric pair (GTX1/4, GTX2/3, GTX5, STX, and dcSTX) were calculated with linear calibration curves achieved using PSP-certified references standards. The results were expressed in relative amounts of each toxin on a molar basis (mole %) and cellular toxin content as fmole/cell.

Statistical Analysis

After the data normality was tested, a one-way analysis of variance (ANOVA) was conducted, with a significance level of $p \leq 0.05$, followed by a Tukey post hoc test using Statistical Package for Social Science (SPSS) ver. 21.

RESULTS

The PST profile in the *Pyrodinium bahamense* culture isolated from Sepanggar Bay in 2012 and the seawater sample collected during a bloom of *P. bahamense* in 2021 from the same area was characterised by HPLC-FLD. The data obtained show that the culture and field bloom of *P. bahamense* produces dcSTX, STX, GTX1, GTX2, GTX3, GTX4, and GTX 5 (Figure 2). No toxin was detected on day 60, and GTX4 was the major toxin, constituting about 84-98 mole% among whole PSP toxins in other samples (Figure 3). The SXT toxin was absent in 120 days culture but comprised 4.3 mole% in the field bloom sample. However, no dcSTX toxin was detected in the field bloom sample, but high dcSTX was detected in the 360 days culture (5.4%). The proportion of GTX 1 in 180 and 360 days of culture was 5.4 and 5.9 mole%, respectively. The contribution of other toxins, such as GTX 2, GTX 3, and GTX 5 was less than 5 mole% in all samples.

The study findings also show that the cell size of cultured *P. bahamense* was not significantly different ($p < 0.05$) from the field bloom sample (Table 1). The highest cell toxin content was found in *P. bahamense* of the field bloom sample at 86.2 fmol/cell, and no cell toxin content was found in a culture of 60 days. The high number of *P. bahamense* cells (18,000 cells/mL) from the field bloom contains a high toxicity potential per cell at 63.92 fmol STXequiv./cell. There were no significant differences in the total

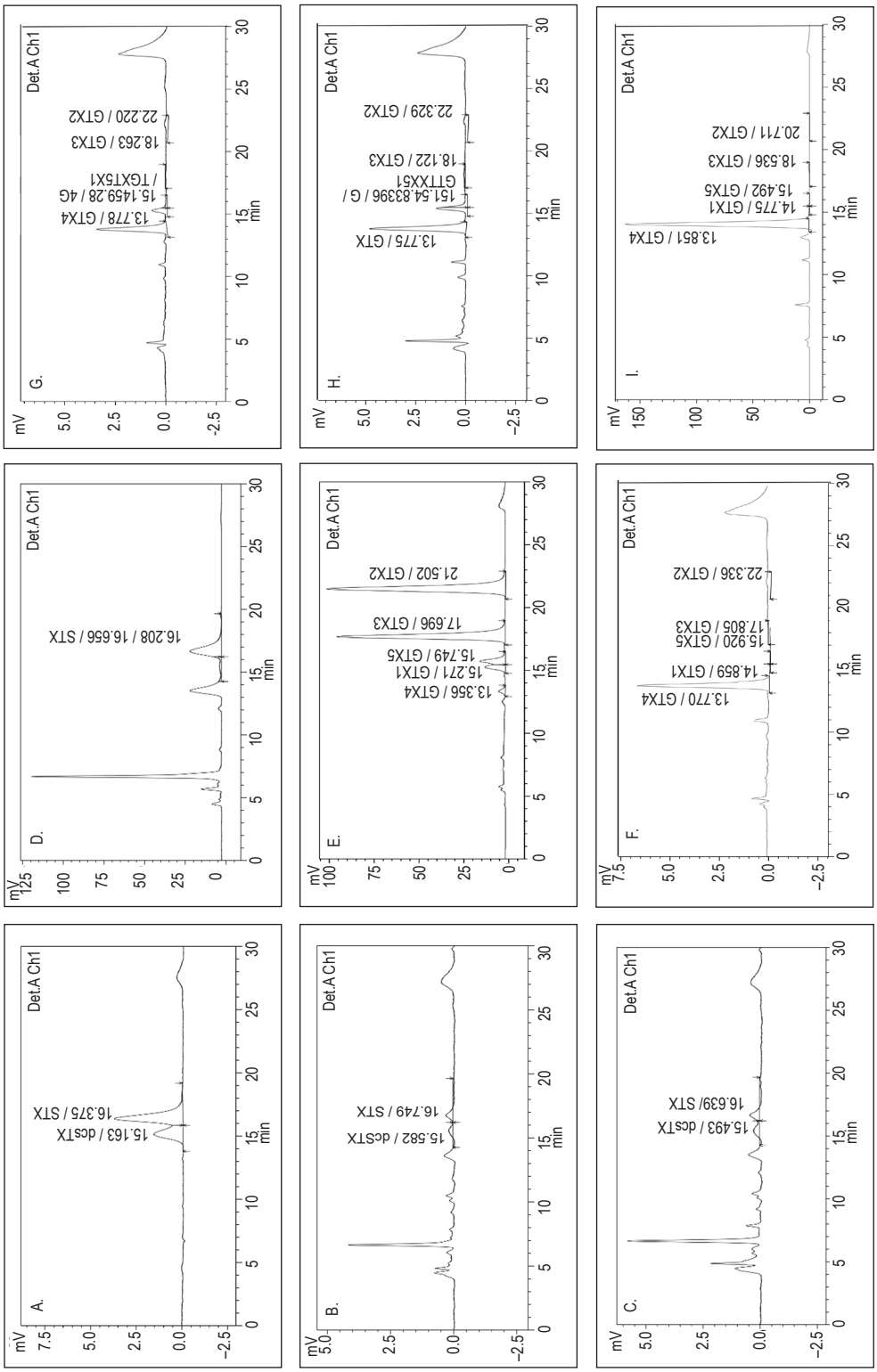

Figure 2. HPLC chromatogram profile of the culture and field bloom of *Pyrodinium bahamense*. A) STX standard; B-C) STX at 180 and 360 days, respectively; D) STX at field bloom sample; E) GTX standard; F-H) GTX at 120, 180, and 360 days, respectively; I) GTX at field bloom sample

toxicity potential per cell of *P. bahamense* samples at different culture ages (120, 180, and 360 days) ranging from 6.7-12.0 fmole STXequiv./cell.

DISCUSSION

This study detected six toxin compounds from *P. bahamense* sampled at Sepanggar Bay: GTX1, GTX2, GTX3, GTX4, GTX5,

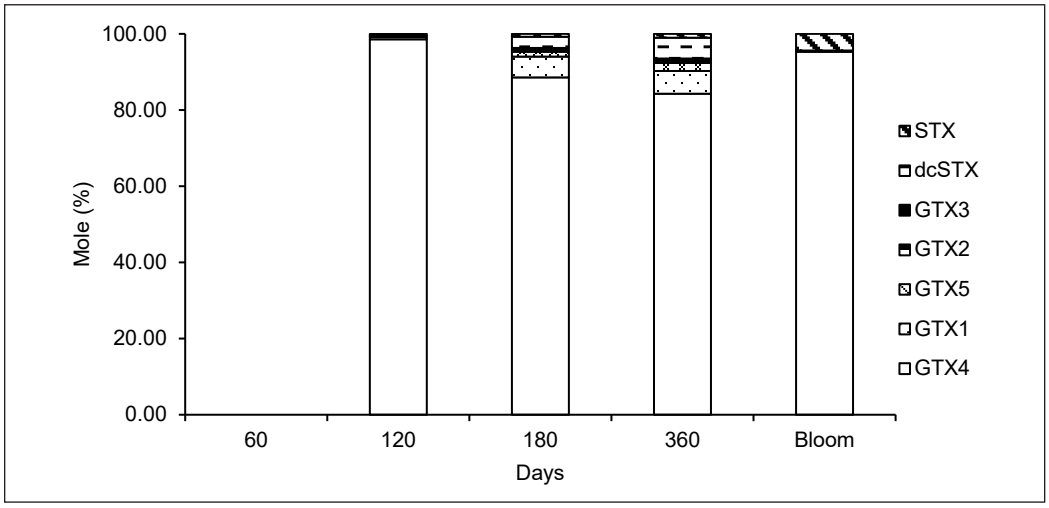

Figure 3. Toxin profile (mole%) of *Pyrodinium bahamense* from the Sepanggar Bay cultivated in f/2 media at different culture ages and field bloom sample

Table 1
Cell size (µm), cell count (cells/mL), toxin profile, and toxin content (f mole/cell) of the Pyrodinium bahamense *in different culture ages and field bloom*

	Culture age (days)				Bloom
	60	120	180	360	
Size (µm)					
Width	14.17 ± 1.42	14.34 ± 1.67	14.25 ± 2.33	14.35 ± 1.99	14.17 ± 2.43
Length	13.65 ± 1.80	14.36 ± 1.57	13.43 ± 1.65	14.29 ± 1.70	14.10 ± 2.03
Cell count (cells/mL)	1,866	2,910	4,050	4,116	18,000
Toxin (fmol/cell)					
GTX1	n.d.	0.10 (0.09)	0.52 (0.51)	0.78 (0.78)	0.16 (0.15)
GTX2	n.d.	0.08 (0.03)	0.10 (0.03)	0.12 (0.04)	0.03 (0.009)
GTX3	n.d.	n.d.	n.d.	0.04 (0.03)	0.05(0.03)
GTX4	n.d.	16.24 (11.85)	8.37 (6.11)	11.15 (8.14)	82.22 (60.02)
GTX 5	n.d.	0.06 (0.003)	0.12 (0.007)	0.28 (0.02)	0.06 (0.004)
dcSTX	n.d.	n.d.	0.28 (0.14)	0.72 (0.37)	n.d.
STX	n.d.	n.d.	0.07 (0.06)	0.13 (0.13)	3.71 (3.71)
Total toxin/cell	0	16.47	9.45	13.23	86.22
STXequiv./cell	0	11.97	6.86	9.51	63.92

Note. n.d. = Not detected. Figure in brackets is the STX equivalent value for the derivative

dcSTX, and STX via HPLC-FLD analysis (Figure 2). Based on a previous study, these compounds also were detected in *P. bahamense* from nature, cultures, and vectors (shellfish, fish) exposed to *P. bahamense* (Table 2). The GTX4 was the major toxin compound found in different culture ages and field bloom samples collected. However, GTX3 was found as the primary toxin using the same culture but at an exponential phase (Yahumin et al., 2022). It indicates that growth phases will determine the type of toxin produced besides other factors, such as temperature, medium, and chain length (Band-schmidt et al., 2006). In the *P. bahamense* batch culture, GTX 5 increased from 25 to 55% as the temperature increased from 22 to 34°C, but NeoSTX decreased from 70 to 40%. In addition, the high light intensity can cause an inversion of the NEO/B1 ratio (Usup et al., 1994), while STX and dcSTX decrease by approximately 20 mole% when *P. bahamense* is cultured in a high salinity environment (Gedaria et al., 2007). However, there was also the biotransformation of the of PSTs where less toxic PSTs into analogues of greater toxicity has been reported, such as C-toxin conversion into GTXs or GTX to STX (Wiese et al., 2010). The most toxic analogues are STX, NeoSTX, and gonyautoxin (GTX 1-4), followed by the decarbamoyl group consisting of the decarbamoyl derivatives of STX, GTX 1-4, and Neo (Oshima, 1995).

During the bloom of PSP producers, shellfish concentrate the toxins in their tissue from the water they filter when feeding (Montojo et al., 2006; Wiese et al., 2010). For instance, *Alexandrium catenella* at a low density (10 cells/ml) can accumulate up to 80 ug toxin 100/g in mussel tissue (Nishitani & Chew, 1984). During the *P. bahamense* bloom in 2013, the shellfish toxin level was 360–2920 µg STXequiv. 100/g meat with a population of 34, 200 cells/L (Suleiman et al., 2017). Furthermore, GTX 4 was found in green mussels two years after the *P. bahamense* bloom in Sabah (Mustakim et al., 2016). It is probably due to toxins that can be maintained in the cells even after cell death and the main toxin compound in GTX4, as observed in this study. Some bivalve species can maintain toxicities in their tissues for a long time after exposure to algal bloom (Mustakim et al., 2016; Oyaneder-Terrazas et al., 2022). For instance, STX, NeoSTX, GTX 5, and GTX 6 were found in bivalves after exposure to *P. bahamense* bloom. The exact analogues were present in *P. bahamense* in nature and cultures, but the toxin levels differ depending on the bivalve species (Montojo et al., 2006). The varying toxin profiles among shellfish may be attributed to the selective retention or elimination of toxins or enzymatic conversions by the molluscs (Oyaneder-Terrazas et al., 2022). Determining toxin profile or PST analogues in shellfish, fish, and organisms accumulating the toxin is crucial due to the association with human health (Vilariño et al., 2018). Meanwhile, the green mussel showed high toxicity during the *P. bahamense* bloom, and the bivalve toxicity receded instantly when the bloom subsided (Montojo et al., 2006). The human intestinal

Table 2
Toxin profile and toxicity of Pyrodinium bahamense *in bloom, culture, shellfish, and fish exposed to* P. bahamense

	Paralytic shellfish toxin (PST)																			
	Carbamate						N-sulfocarbamoyl						Decarbamoyl							
Reference/ Medium	STX	Neo STX	GTX1	GTX2	GTX3	GTX4	GTX5 (B1)	GTX6 (B2)	C1	C2	C3	C4	dsSTX	dcneo STX	dc GTX1	dc GTX2	dc STX3	dc STX4	Toxin level	Region
Bloom																				
Montojo et al. (2006)	X	X					X	X											1624 fmole/cell	Philippines
Landsberg et al. (2006)	X						X						X						3.28 pg STXeq/cell	USA
Culture																				
Usup et al. (1994)	X	X					X	X					X						200 - 400 fmol/cell	Malaysia
Montojo et al. (2006)	X	X					X	X											165-402 fmole/cell	Philippines
Landsberg et al. (2006)	X						X												2.02 - 12.74 pg STXeq/cell	USA
Usup et al. (2006)	X	X					X	X					X						59 fmole STXeq/cell	Malaysia
Gedaria et al. (2007)	X						X						X						50 to 250 fmole/cell	Philippines
Yahumin et al. (2022)	X	X	X		X	X	X						x						None	Malaysia
Shellfish																				
Montojo et al. (2006)	X	X					X	X											500-2916 mg STXeq 100/g	Philippines
Mustakim et al. (2016)						X													30 µgeq 100/g	Malaysia
Fish																				
Landsberg et al. (2006)	X						X						X						6.25 - 9,039 µg STXeq 100/g	USA

epithelium can absorb almost all PST analogues after consuming the contaminated shellfish (Rodrigues et al., 2021). Multiple factors must be considered in analysing the toxin content and toxic profile of shellfish; thus, Hayashi et al. (2006) recommend using a cell bioassay for routine monitoring.

This study shows that *P. bahamense* can survive in low cell numbers for up to 360 days. After the cell entered the death phase, the morphology remained the same, and the cell size did not experience significant changes. This observation indicates the cell's ability to utilise the nutrients from degraded cells and store them for later use (Phlips et al., 2006). Meanwhile, the *P. bahamense* from the Philippines had a low growth rate (0.2 div/d) that declined on day 35 and entered the death phase on day 43 (Gedaria et al., 2007). There were significant differences in toxin levels between field-collected and cultured *P. bahamense*, which aligned with previous reports. Toxin production rate is related to production of arginine (Arg) within the cells due to cell division (Anderson et al., 1990). PSP toxin content of a cell also relates to nitrogen within the cells (Usup et al., 2006). In this study, the toxin content of culture *P. bahamense* was constant. However, Usup et al. (2006) reported that the total toxicity potential per cell was higher in the field bloom sample with a toxin of 63.92 fmole STXequiv./cell compared to 59 fmole STXequiv./cell of *P. bahamense* culture. The high *P. bahamense* cell numbers reflect the high STX levels (Lopez et al., 2021). Moreover, Usop et al. (1994) found that the toxin level of isolated *P. bahamense* from Sabah increased at the beginning of the exponential phase and achieved maximum toxin content during the mid-exponential phase (400 fmole/cell), followed by a rapid decrease and plateau at 200 fmole/cell. Contrary to *G. catenatum*, no significant changes in toxin content with culture age were observed (Band-schmidt et al., 2006). In contrast, Montojo et al. (2006) reported no significant difference in toxin content from five strains of *P. bahamense* harvested at the late exponential phase in the Philippines. *Pyrodinium bahamense* toxin content is not significantly influenced by different growth conditions but could affect the toxin profile in terms of the ratio of different PSTs (Usop et al., 2012). Furthermore, minimal differences were identified in PSTs detected in *P. bahamense* and shellfish (Montojo et al., 2006). Since there are discrepancies in the existing literature, it is essential to monitor *P. bahamense* bloom regularly to understand better the ecology and toxin mechanism of these STX producers. Furthermore, the findings can be utilised in developing a HAB programme to preserve human health and food safety.

CONCLUSION

This preliminary study showed that *P. bahamense* could sustain its growth for up to 360 days and produce toxins in low concentrations. Toxins GTX 4 is the main analogue found in *P. bahamense*, and a constant toxin cell content was found during the death phase. Furthermore, fresh

samples may contain more analogues than cultured cells, as observed in the bloom sample collected from the field. The study results align with previous findings that toxins are retained in the cell for a long time, although at low concentrations. Consequently, the vectors, such as the shellfish, will continuously accumulate toxins through filter-feeding after a bloom (death phase). Therefore, it is crucial to identify the environmental factors that trigger toxin production in harmful algae, such as *P. bahamense*, to ensure human and food safety and security.

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