[BIO34] Occurrence of potentially toxic benthic marine dinoflagellate in Malaysia

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Introduction

Malaysia is one of the maritime countries affected by seafood poisoning events associated with toxins produced by marine dinoflagellates. In Malaysia the most significant form of seafood toxicity due to dinoflagellate toxins is paralytic shellfish poisoning (PSP). The history of PSP in the country dates back to 1976 and to date has resulted in at least 350 intoxications including at least 50 fatalities. So far, three PSP-producing marine dinoflagellate species have been confirmed in Malaysian waters, namely *Pyrodinium bahamense* var. *compressum*, *Alexandrium tamiyavanichii* and *A. minutum* Most of these toxic species are planktonic.

Another group of potentially toxic dinoflagellates, but have not been studied in Malaysia previously, are the benthic or epiphytic species. These are species that live attached to sand particles, coral, seaweeds and mangroves in tropical and subtropical waters. Studies that have been carried out elsewhere showed that many of these benthic dinoflagellates are capable of producing bioactive compounds, including those that can cause seafood toxicity. For example, palytoxin which is considered the most potent toxin of marine origin is produced by the benthic dinoflagellate *Ostreopsis siamensis*. The most well known human intoxication due to benthic dinoflagellates is ciguatera fish poisoning (CFP). The toxins responsible for CFP reach humans through the food chain that begins with herbivorous reef fishes that are in turn eaten by larger carnivorous species such as barracudas, groupers and jacks. Other seafood poisoning caused by benthic dinoflagellates are diarrhetic shellfish poisoning (DSP) and clupeotoxicity.

In coastal waters of Malaysia habitats suitable for benthic dinoflagellates abound. To date, however, there has been no documentation on the occurrence or toxicity of these species in Malaysia. This study was carried out to determine the species present and establish laboratory cultures for diversity, physiological and toxicity studies.

Materials and methods

Samples

Seaweeds, coral fragments, seagrasses and sand were collected by snorkeling or SCUBA. Sampling was carried out in Kota Kinabalu, Pulau Redang, Pulau Langkawi and Port Dickson (Figure 1). Samples were placed into separate plastic bags while still underwater and added with a little seawater. Samples were brought back to the laboratory without preservation. In the laboratory the samples were shaken vigorously to dislodge attached dinoflagellate cells. The suspension was then passed through a 120 µm mesh sieve to remove large debris. The material that passed through was sieved again with a 20 µm mesh sieve. Material retained by the sieve was resuspended in sterile filtered seawater for examination and cell isolation. Cells of interest were isolated singly for culture using a very finely-drawn Pasteur pipet. Cultures were established in ES-DK medium (Kokinos & Anderson, 1995) at 26 ºC under a 14:10 hour light-dark photoperiod.

FIGURE 1. Map of Malaysia showing the collection sites.

Species identification

For identification, cells were examined under a normal light microscope, under epifluorescence microscope with calcofluor staining, and for some under scanning electron microscopy (SEM). For SEM, cells were initially fixed with 4% glutaraldehyde for 24 h and post-fixed with 1% osmium tetraoxide for 1 h at room temperature. Cells were rinsed three times with ddH_2O to remove salt and fixatives, followed by dehydrating in a graded series of ethyl alcohol concentration, filteredmounted to a stub and dried in a critical point dryer. The sample was coated with gold using a BioRad SC500 sputter coater and observed with a Philips XL30 SEM. Cell dimensions were determined from SEM and LM micrographs of 20 to 200 cells.

Results

Benthic dinoflagellates were found at all the locations sampled. However, not all species were present at all sites. The dinoflagellates were particularly common in association with brown and red seaweeds such as *Sargassum*, *Padina* and *Turbinaria*. None were found on green seaweeds examined or on seagrass, and very few were found associated with coral fragments and sand. A list of the benthic dinoflagellates species encountered is shown in Table 1. The morphological characteristics of the important species are also provided.

TABLE 1. Benthic dinoflagellate species found in Malaysia. kk=Kota Kinabalu; pr=Pulau Redang; pd=Port Dickson; pl=Pulau Langkawi.

Species	Location			
	kk	pr	pd	pl
Gambierdiscus toxicus	+	-	-	-
G. belizeanus	+	-	-	-
Ostreopsis siamensis	-	+	-	-
Ostreopsis ovata	+	+	+	+
Coolia monotis	+	-	+	+
*Prorocentrum lima**	+	+	+	+
P. emarginatum	+	-	-	-
Amphidinium steinii	-	+	-	-

* Not available in culture

Gambierdiscus toxicus **Adachi & Fukuyo (Fig. 2)**

Cells are rounded to obliquely ellipsoidal in apical view and lenticular in ventral view. Cells are anterior-posteriorly compressed. The thecal plate formula is: Po, 3’, 7’’, 6c, 8s, 5’’’, 1p, 2’’’’. The thecal surface is smooth covered by small pores. The APC is located centrally in the epitheca. The apical pore is shaped like an apostrophe and is surrounded by 40 tiny pores. The intercalary bands and the cingulum lists are smooth. In antapical view, the 1p is pentagonal adjoining the 1’’’’, 2’’’’, 2’’’, 3’’’ and 4’’’. Cells ranged from 61-74 µm in length to 62-71 µm width. This species was found only in samples from Kota Kinabalu.

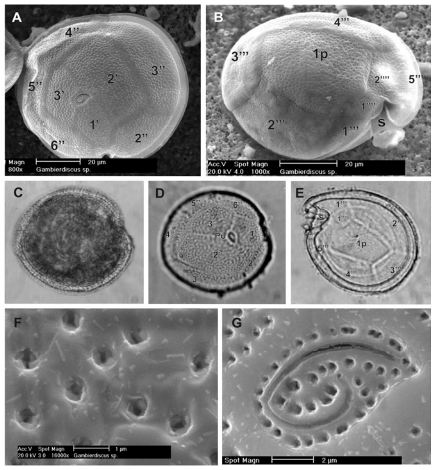

FIGURE 2. *Gambierdiscus toxicus*. A) Apical view of cell; B) antapical view showing a wide pentagonal posterior intercalary plate (1p); C) heavily pigmented living cell; D) empty epitheca; E) empty hypotheca; F) close-up of theca showing the smooth surface with evenly distributed round pores; G) close-up of APC with an apostrophe-shaped Po surrounded by 40 pores.

Gambierdiscus belizeanus **Faust (Fig. 3)**

Cells are orbicular in apical view and anterior-posteriorly compressed. The overall shape is nearly identical to *G. toxicus*. The theca surface has areolae and is covered with numerous small pores. The intercalary bands are striated. The cingulum lists are supported by ridges. The APC is suppressed. An apostrophe-shaped Po is located in the APC with 28 pores surrounding the Po. The number of pores is fewer than in *G. toxicus*. The 1’’ and 7’’ are small and hardly visible in apical view. The plates are immersed into the ascending cingulum. The 1p is pentagonal but relatively small. The sulcus is deep and short, covered by sulcus lists. Cells ranged from 61-74 µm in length to 61-71 µm in width. This species was found only in samples from Kota Kinabalu.

Ostreopsis siamensis **Schmidt (Fig. 4)**

Cells are ovate with blunt tip at the ventral point and anterior-posteriorly compressed. The thecal plate formula is: Po, 3’, 7’’, 5’’’, 1p, 2’’’’. The epitheca and hypotheca are virtually equal in size. The Po is curved and narrow and tightly joined to the long narrow 2’ plate. Trichocyst pores are present, round in shape and uniform size. The anterior margin of 1p is straight, bordering the 1’’’’. Cells heavily pigmented.

The morphology of these isolates of *Ostreopsis* are somewhat different from the

O. siamensis previously described and more similar to *O. lenticularis*. Fukuyo (1981) distinguished *O. lenticularis* from *O. siamensis* by the presence of fine pores in the former. The absence of two different sizes of trichocyst pores in our isolate affirmed the species as *O. siamensis*. Cells ranged from 68-79 in dorso-ventral diameter to 51-61 µm in transdiameter. This species was found only in samples from Pulau Redang.

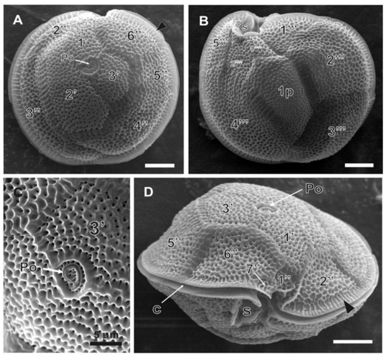

FIGURE 3. *Gambierdiscus belizeanus*. A) apical view of cell; B) antapical view of cell showing a deep sulcus. Note that 1p is relatively small compared to *G. toxicus*; C) closeup of the APC; D) partial apical-ventral view of cell showing tiny 1'' and 7'' immersed into the ascending cingulum. Note the striation of the intercalary bands and the ridged margin of cingulum list (arrowhead).

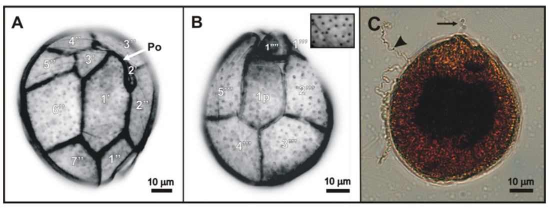

FIGURE 4. *Ostreopsis siamensis*. A) Apical view showing a curved Po adjoining the narrow second apical plate, 2'; B) antapical view of a cell. The anterior margin of 1p is straight adjoining the 1'''' (insert: uniform size of pores); C) heavily pigmented cell showing the transverse flagellum (arrowhead) and short longitudinal flagellum (arrow).

Ostreopsis ovata Fukuyo (Fig. 5)

The thecal plate formula is similar to *O. siamensis*. Cells are relatively small and heavily pigmented. Cells oval to tear-drop in shape with an acute tip at the ventral point. The hypotheca is somewhat broader than the epitheca. Trichocyst pores are round and sparsely distributed on the thecal plates. The Po is situated in the APC and partly covered by lists. The lists were heavily stained by calcofluor and can be easily observed by epi-fluorescence microscopy. The 2' is very small and hardly visible by epi-fluorescence microscopy. However, in SEM, the plate is found adjacent to the Po with almost the same length. The Po is curved for Port Dickson isolates and straight for Kota Kinabalu and Redang isolates. The 1' is also slightly different between these two isolates. The left margin of 1' is straight in Port Dickson isolates whereas it is rather concave in Kota Kinabalu isolates. Both isolates share the same hypotheca profile except the anterior margin of 1p for Kota Kinabalu isolates has a curved margin slanting towards the left margin while the margin of Port Dickson isolates is straight. Cells ranged from 38-44 µm in dorsoventral diameter to 28-35 µm in transdiameter. This species found was in samples from all sampling sites.

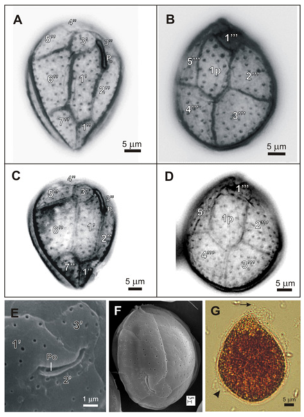

FIGURE 5. *Ostreopsis ovata*. A-B) Kota Kinabalu isolate showing a straight Po, a concave left margin of first apical plate (1') and a curved anterior margin of the posterior intercalary plate (1p); C-D) Port Dickson isolate showing a curved Po, straight left margin of 1' and straight anterior margin of 1p; E) apical pore complex with strong list covered the narrow apical pore (Po); F) theca surface of *O. ovata*; G) cell showing the transverse flagellum (arrowhead) and short longitudinal flagellum (arrow).

Coolia monotis Fukuyo (Fig. 6)

Cells are round in dorso-ventral view and ellipsoid in lateral view. The thecal plate formula is: Po, 3', 7'', 7c, 8s, 5''', 2''''. The nucleus is located in the dorsal part of the hypotheca. Theca surface is smooth with round and large pores of 0.4 µm in average diameter. In apical view, the right margin of the first apical (1') is straight and adjoins the sixth precingular plate (6''). The 1' is narrow and oblong. The 6'' is the widest plate in the epitheca, occupying nearly half of the epitheca.

Apical pore complex (APC) is short (approximately 5 µm long) and relatively straight, contiguous to the first, second and third apical plates (1’, 2’ and 3’). The narrow apical pore is located in the APC. The third apical plate (3’) is pentagonal bordering the 1’, 2’ and APC, 4’’, 5’’ and 6’’. Some cells have a quadrilateral-shaped 3’ without touching the 5’’. The epitheca plate shapes and tabulation are similar to previously described *C. monotis* (Faust, 1992) except for the relatively few pores on the theca, the short APC and cell shape. The plate’s layout however is significantly different from *C. tropicalis* described by Faust (1995).

In antapical view, the postcingular profile is distinctively different from the holotype of *C. monotis* (Faust, 1992) and *C. tropicalis* (Faust 1995). The third postcingular plate (3’’’) is very wide and occupies the central part of the hypotheca. The first and second antapical plates (1’’’’ and 2’’’’) are separated by the sulcus and do not touch each other. The sulcus is short. Sulcal lists extend from the 5’’’, 1’’’’ and 2’’’’, covering the left, right, and posterior part of the sulcus respectively. The intercalary bands are smooth and hardly visible under electron microscopy, but the bands can be easily distinguished by epi-fluorescence microscopy. Cells ranged from 28-33 µm in length to 27-32 µm in width. This species was found in samples from Kota Kinabalu, Port Dickson and Pulau Langkawi.

Prorocentrum emarginatum **Fukuyo (Fig. 7)**

Cells are ovate in valve view and both valves are concave in lateral view. The valve surface is smooth and has pores arranged radially. The center of both valves is flattened and pores are absent. The right valve has a deep, V-shaped indentation. Cells are heavily pigmented. The nucleus is located posteriorly. Cells ranged from 37-41 µm in length to 32-37 µm in width. This species was found only in samples from Kota Kinabalu.

Prorocentrum lima **Ehrenberg (Fig. 7)**

Cells are oval to oblong in valve view. Both valves are concave in lateral view. The thecal surface is smooth and punctuated by pores except in the centre of the valves. The perifalgellar is V-shaped and located on the right valve. The intercalary band is smooth. Pyrenoid and chloroplasts are present. Cells ranged from 59-63 µm in length to 36-42 µm in width. This species was found in samples from all sampling sites.

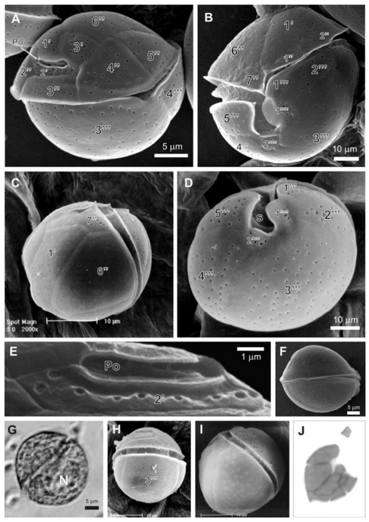

FIGURE 6. *Coolia monotis*. A) dorso-apical view of a cell showing the position of apical pore complex (APC) and the apical pore (Po); B) ventral view of cell showing the short sulcal surrounded by the sulcus lists. Note the straight right margin of first apical plate (1’); C) ventro-apical view showing the shape of 1’ and the wide sixth precingular plate (6’’); D) antapical view of cell, showing the deep sulcus separating the first antapical plate (1’’’’) and 2’’’’. The third postcingular plate (3’’’) is the largest plate, occupying almost two-thirds of the hypotheca; E) closeup of apical pore complex; F) lateral view showing the ellipsoidal shape of cell; G) position of nucleus; H) dorsal view of cell showing the position of 3’’’; I) the big 6’’; J) epi-fluorescence micrograph of epitheca showing the theca arrangement.

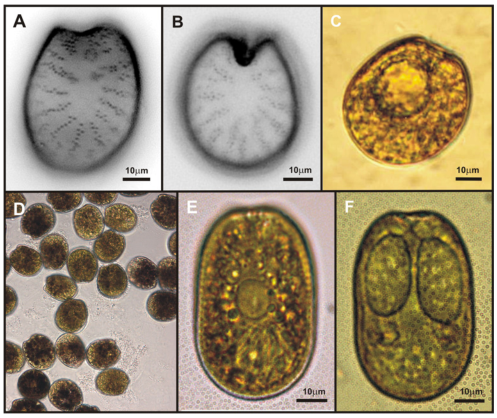

FIGURE 7. A-D) *Prorocentrum emarginatum*. A) left valve; B) right valve showing the deep and V-shaped indentation; the valve pores are arranged radially in both valves; C) cell with centrally located nucleus; D) vegetative cells in culture; E-F) *P. lima*. E) cell with centrally located pyrenoid; F) binucleated vegetative cell prior to cell division.

Amphidinium steinii **Hulburt (Fig. 8)**

Unarmored dinoflagellate found in Pulau Redang. Cells are round with premedian cingulum in a Y-configuration with sulcus. The epitheca is tongue-shaped. In ventral view, the epitheca is pointed to the left. The nucleus is round to oval and located in the posterior of the hypotheca. The plastid ropes radiate from the central pyrenoid. Cells ranged from 24-30 µm in length to 23-28 µm in width.This species so far was found only in sand samples from Pulau Redang.

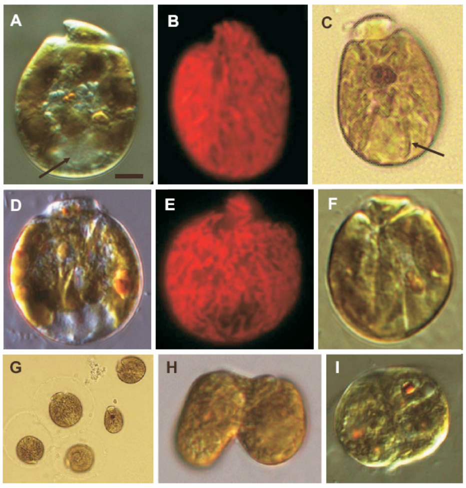

FIGURE 8. *Amphidinium steinii.* A) cell in dorsal view showing the position of nucleus (arrow); B) the same cell under epifluorescence showing plastids radiating; C) cell with thick plastid ropes radiating from the center; D-E) cell observed under DIC and epifluorescence; F) ventral view of cell showing premedian cingulum in a Y configuration with sulcus; G) vegetative cells in culture; H) binary division of vegetative cell; I) zoosporagia/cyst.

Discussions

Only a very small portion of Malaysia's marine waters have been sampled in this study, but even so there is now evidence of the occurrence of potentially toxic benthic dinoflagellates at the locations sampled. The study needs to be extended over a much wider area in order to gain a better understanding of the distribution of these species and their biogeography.

Arguably the most significant species found in this study was *G. toxicus*. Somewhat surprising was the absence of the species in all samples from Peninsula Malaysia that have been examined to date. This is the main species considered responsible for ciguatera fish poisoning worldwide. Studies that have been carried out elsewhere showed that *G. toxicus* produced two types of toxins. The first is the water soluble maitotoxin which acts on calcium channels. The second toxin is the lipid-soluble polyether ciguatoxin. This is a neurotoxin that acts on the sodium channel and can lead to paralysis. CFP is considered the most important form of seafood poisoning in the United States. In Malaysia, no CFP has been reported, although there were a few suspected cases in Sabah. Previous studies have shown that the toxicity potential of *G. toxicus* varied according to geographical origin. The isolate from Malaysia has not yet been tested for toxin production and this should be considered a priority.

Another important species was *G. belizeanus*, discovered from the same location as *G. toxicus*. Morhologically this is similar to *G. toxicus*, and can be misidentified as *G. toxicus* under light microscopy. However, they differed in some features such as smooth thecal surface in *G. toxicus* with scattered pores whereas *G. belizeanus* has a deeply areolated surface. The posterior intercalary plate (1p) is broad and pentagonal in *G. toxicus* whereas it is long and narrow in *G. belizeanus*. To date, the toxic potential of *G. belizeanus* is not known although other species within this genus have been reported as toxic (Chinain *et al*., 1999).

Ostreopsis siamensis was found only in Pulau Redang. This species was previously misidentified as *O. lenticularis* (Leaw *et al*., 2001). This is the species that produces palytoxin and has caused fatal clupeotoxicity in Madagascar (Usami *et al*., 1995). Another closely related species, *O. ovata* was also very common in Malaysian waters. No toxicity data has been reported for either of these two species, but it was evident from this study that cell extracts contained biological activity, particularly hemolytic activity. This was similar to what has been reported previously (Nakajima *et al*., 1981). Studies to characterize the bioactive products from this two species are currently underway. Interestingly, the *O. ovata* populations in Malaysian waters seemed to be divided into two distinct geographical groups, namely the South China Sea group and a Straits of Malacca group (Leaw *et al*. 2001).

Another important species that was found in the samples and has been successfully cultured is *Coolia monotis*. Previous studies have shown that the species produces cooliatoxin. Again, the toxicity of the Malaysian clones have not been established other than demonstration of high hemolytic activity. Somewhat surprising was the genetic homogeneity of isolates from all the locations studied, which was different from the results obtained for *O. ovata* (Leaw *et al*., 2001).

Another important species commonly encountered but has proved very difficult to establish in culture is *Prorocentrum lima.* This species is well known for its ability to produce okadaic acid which is responsible for diarrhetic shellfish poisoning. The common occurrence of this species together with several species of *Dinophysis* suggests that DSP could become a significant problem in Malaysia particularly with expansion of mussel farming.

The benthic dinoflagellate species tend to occur together in a particular habitat and indeed on the same host. Combined actions of the different toxins produced by these species are considered responsible for the highly complex symptoms of ciguatera fish poisoning, ranging from neurological to gastrointestinal maladies.

Considering the common occurrence of these benthic dinoflagellates in the waters of Malaysia the apparent absence of CFP in the country is surprising. Several reasons could be proposed. First, the species that are present in Malaysia are non-toxic. Second, densities of the species never achieve a level that would cause toxicity. Third, not many reef fishes are caught for consumption and those that are caught are often small and may not contain enough toxins to intoxicate humans. Finally, there may be occasional and sporadic occurrences of CFP that go unreported or misdiagnosed. The third possible reason, pertaining to fish size is quite important. In the Caribbean for example, fishes that are often toxic and are banned from consumption are large individuals. More studies are required to determine the toxicity potential of the benthic dinoflagellate species that are found in Malaysia. It is also important to try to ascertain whether there has been past events of CFP and locations from which toxic fish originated. While it would seem that CFP is not a problem in the country, it should be noted that under the HACCP, ciguatera toxins are one of the items that need to be tested for in fishes for export.

Acknowledgements

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