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Harmful Algae 1 (2002) 265–275

HARMFUL ALGAE

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Alexandrium (Dinophyceae) species in Malaysian waters

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Received 8 July 2002; received in revised form 2 August 2002; accepted 22 August 2002

Abstract

A study was carried out to determine the presence of paralytic shellfish poisoning (PSP) toxin-producing dinoflagellates in the coastal waters of Peninsula Malaysia. This followed first ever occurrences of PSP in the Straits of Malacca and the northeast coast of the peninsula. The toxic tropical dinoflagellate *Pyrodinium bahamense* var. *compressum* was never encountered in any of the plankton samples. On the other hand, five species of *Alexandrium* were found. They were *Alexandrium affine*, *Alexandrium leei*, *Alexandrium minutum*, *Alexandrium tamarense* and *Alexandrium tamiyavanichii*. Not all species were present at all sites. *A. tamiyavanichii* was present only in the central to southern parts of the Straits of Malacca. *A. tamarense* was found in the northern part of the straits, while *A. minutum* was only found in samples from the northeast coast of the peninsula. *A. leei* and *A. affine* were found in both the north and south of the straits. Cultured isolates of *A. minutum* and *A. tamiyavanichii* were proven toxic by the receptor binding assay for PSP toxins but *A. tamarense* clones were not toxic. Mean toxin content for the *A. tamiyavanichii* and *A. minutum* clones were 26 and 15 fmol per cell STX equivalent, respectively. This study has provided evidence on the presence of PSP toxin-producing *Alexandrium* species in Malaysian waters which suggests that PSP could increase in importance in the future.

Keywords: *Alexandrium*; Harmful algal blooms; Malaysia; Paralytic shellfish poisoning

1. Introduction

Malaysia is one of many countries affected by harmful algal blooms (HABs) and associated seafood poisoning. Currently, paralytic shellfish poisoning (PSP) is the only HAB-related shellfish poisoning that has been documented in the country. Until 1990, PSP was confined to the west coast of Sabah, where the dinoflagellate *Pyrodinium bahamense* Plate var. *compressum* Böhm form blooms almost annually. This species has long been considered the most important PSP toxin-producing species in southeast Asia (Malaysia and The Philippines) and along the Pacific coastline of central America (Rosales-Loessener et al., 1989; Orellana-Cepeda et al., 1998; Usup and Azanza, 1998). In Malaysia alone, *P. bahamense* has caused many poisoning events including several fatalities.

In early 1991, PSP occurred for the first time outside Sabah. Three people were poisoned after consuming mussels from a mussel farm in Sebatu in the Straits of Malacca. Naturally *P. bahamense* was suspected to be the toxin producer, but to date the species has never been found in plankton samples collected from several locations in the Straits of Malacca. In the

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1568-9883/02/$ – see front matter
PII: S1568-9883(02)00044-6

most recent event in September 2001, six people were poisoned, including one fatally, after consuming the benthic clam *Polymesoda* sp. collected from a coastal lagoon in Tumpat on the northeast coast of Peninsula Malaysia. All victims displayed symptoms typical of PSP. Analysis of clam samples collected from the site during the event using the live mouse bioassay indicated very high levels of PSP toxins (Malaysia Department of Fisheries, personal communication).

The PSP events referred to suggest the expansion of the problem to other coastal areas of Malaysia. PSP, and possibly other HAB-related intoxications, could become more significant in the country with intensification of aquaculture activities. It is thus important to identify potentially toxic phytoplankton species that are present and if possible the extent of their distributions. This report provides for the first time evidence on the occurrence of several species of *Alexandrium* in Malaysian waters.

2. Materials and methods

2.1. Samples

Three locations in the coastal water of Peninsula Malaysia were sampled (Fig. 1). Sebatu, in the Straits of Malacca, and Tumpat on the northeast coast were locations where there have been PSP cases, while Pulau Aman in the northern part of the Straits of

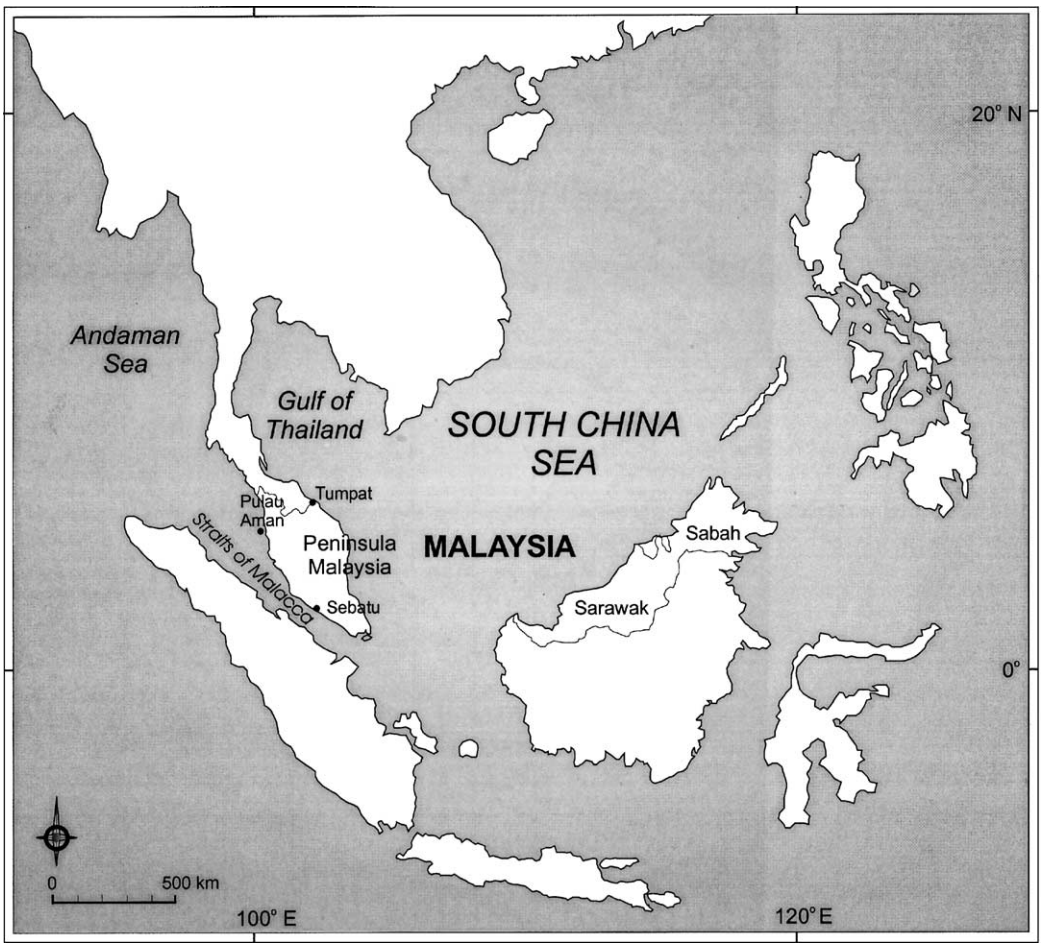

Fig. 1. Map showing locations of Sebatu, Tumpat and Pulau Aman in Peninsula Malaysia from where samples were collected.

Malacca is a very important finfish and shellfish aquaculture area. Non-quantitative samples were collected with 20 μm mesh size plankton nets. Samples were preserved in Lugol's iodine solution, in formaldehyde or in glutaraldehyde according to need. Live samples were also obtained for isolation of cells to establish laboratory cultures. Clonal cultures were established by single cell isolation. All cultures were grown in ES-DK medium (Kokinos and Anderson, 1995) at 26 °C under a 14 h:10 h light:dark photoperiod.

2.2. Identification

Field and cultured specimens were examined under a microscope using normal light and epifluorescence (Olympus BX51). For epifluorescence, fixed samples were stained with calcofluor white (Sigma) and viewed under UV with a UV filter set. Images were captured with a cooled CCD camera (SIS Colorview F12, Germany). Some of the samples were also examined on a scanning electron microscope (Philips XL30). Identification of the genus was based on overall cell shape and Kofoidan theca plate tabulation. Identification of *Alexandrium* species was based primarily on the morphology of the following plates: posterior sulcal (S.p.), second antapical (2′′′′), first apical (1′), anterior sulcal (S.a.), third apical (3′), sixth precingular (6′′) and the apical pore complex (APC). Other features used in identification were the presence and location of the ventral pore as well as the anterior and posterior attachment pores. Identification was based on Balech (1995) and Fukuyo (2001).

2.3. Toxicity

Clones of the *Alexandrium* species in laboratory cultures were tested for PSP toxin content by the receptor binding assay. All clones were tested in triplicate. The assay protocols were as described by Doucette et al. (1997) with slight modifications. The receptor was prepared from brains of 6-week-old male Spraque–Dawley rats. Tritiated STX-diacetate standard with specific activity of 777 GBq mmol^{-1} was obtained from Amersham while unlabeled STX-dihydrochloride standard (25 mg ml^{-1}) was obtained from the NRC Halifax. Cultures for toxin analysis were harvested at mid-exponential growth phase by centrifugation at 2000 × *g* for 5 min. The cell pellet was suspended in 0.05 M acetic acid and sonicated on ice. The supernatant was collected by centrifugation at 4000 × *g* for 10 min. Assays were carried out in Millipore multiscreen plates (MAFBNOB50). The activity of bound ^{3}H-STX was measured on a Perkin-Elmer–Wallac Microbeta 1450 plate reader. A standard curve based on data points obtained from the competitive binding of labeled and unlabeled STX standards was fitted in Sigmaplot v6 (SPSS Inc.) using the sigmoidal four-parameter logistic equation. A separate standard curve was prepared for each assay plate.

3. Results

3.1. Species presence

Analyses of the plankton samples showed that at least five species of *Alexandrium* are present in coastal waters of Peninsula Malaysia. They are *Alexandrium affine*, *Alexandrium leei*, *Alexandrium minutum*, *Alexandrium tamarense* and *Alexandrium tamiyavanichii*. Significant morphological characteristics of Malaysian isolates of these species are given below.

3.1.1. *A. affine* (Inoue and Fukuyo) Balech

3.1.1.1. Material examined. Wild and cultured cells from Sebatu and Pulau Aman in the Straits of Malacca (Fig. 2).

3.1.1.2. Morphology. Cells approximately pentagonal in shape, with a spherical anterior and flat posterior. Chains of several cells are common in culture. The species is easy to identify when diagnostic plates are visible. S.p. is longer than wide, with both anterior ends well projected. Sulcal list not well-developed. 1′ is wide with a sharply tapering anterior end. 1′ touches the APC directly. A ventral pore is present, located towards the anterior of 1′ along the right margin. The main diagnostic feature is the location of the anterior attachment pore in the APC. The pore is located directly above the apical pore along the same axis.

3.1.1.3. Dimensions. Length: 29–33 μm; transdiameter: 31–36 μm.

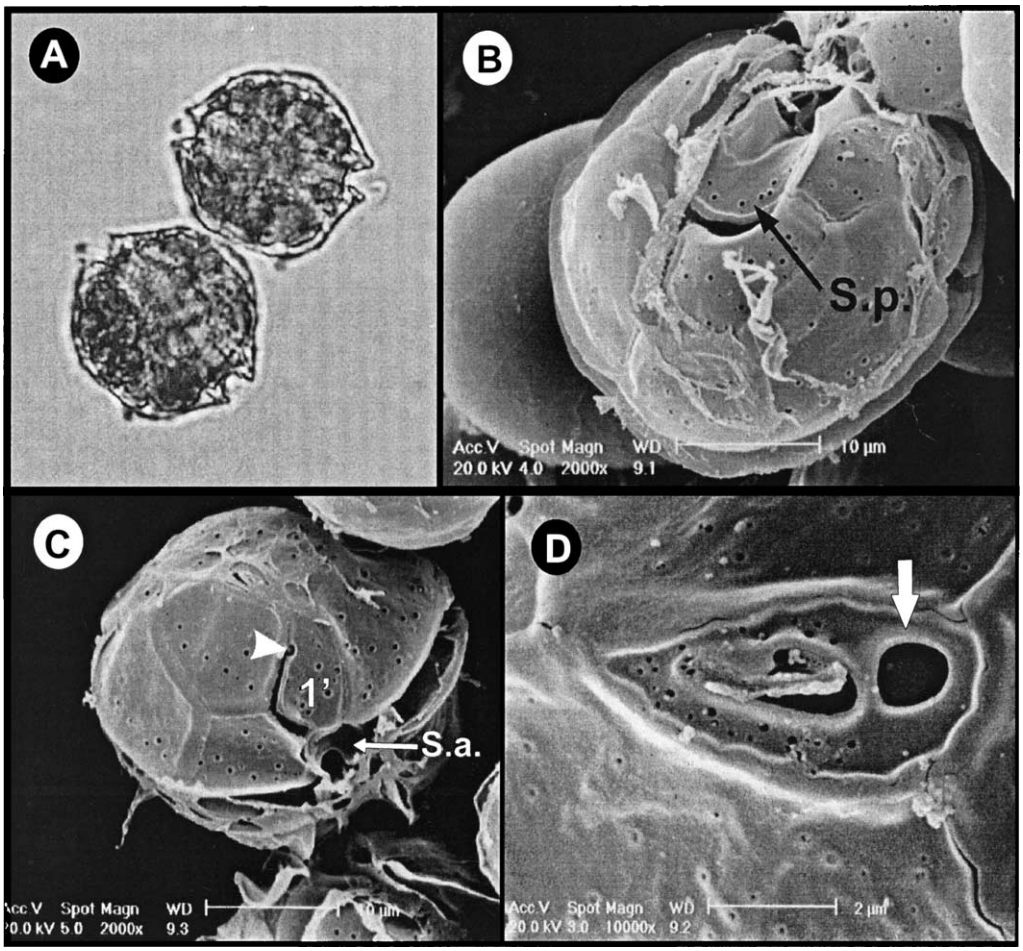

Fig. 2. *A. affine*: (A) a pair of vegetative cells; (B) antapical view of a cell by SEM, showing the elongated posterior sulcal plate (S.p.); (C) ventro-apical view by SEM, showing the first apical plate (1′), the anterior sulcal plate (S.a.) and location of the ventral pore (arrowhead); (D) close up of the apical pore complex showing the location of the anterior attachment pore (arrow) relative to the apical pore.

3.1.1.4. Distribution in Malaysia. The species has been found in samples from Sebatu and Pulau Aman in the Straits of Malacca.

3.1.2. A. leei Balech

3.1.2.1. Material examined. Wild and cultured cells from Sebatu and Pulau Aman, Straits of Malacca (Fig. 3).

3.1.2.2. Morphology. Cells are large, roundish, with a conical epitheca. The epitheca is slightly longer than the hypotheca. The cingulum is shallow, without lists. 1′ is quite wide and tapers to a point where it touches the APC. A small ventral pore is present, located centrally between the left and right margins of 1′. A groove connects the pore to the right margin of 1′. 6″ is wide. The S.p. is wider than long, with a curvy posterior margin. The left posterior lateral plate is wide. Sulcal lists are absent, but sulcal edges are reinforced. The S.a. is just slightly longer than wide, with a straight anterior margin. In most of the cells examined, the sutures are quite wide. Thecal plates are perforated by many round, variable-sized pores.

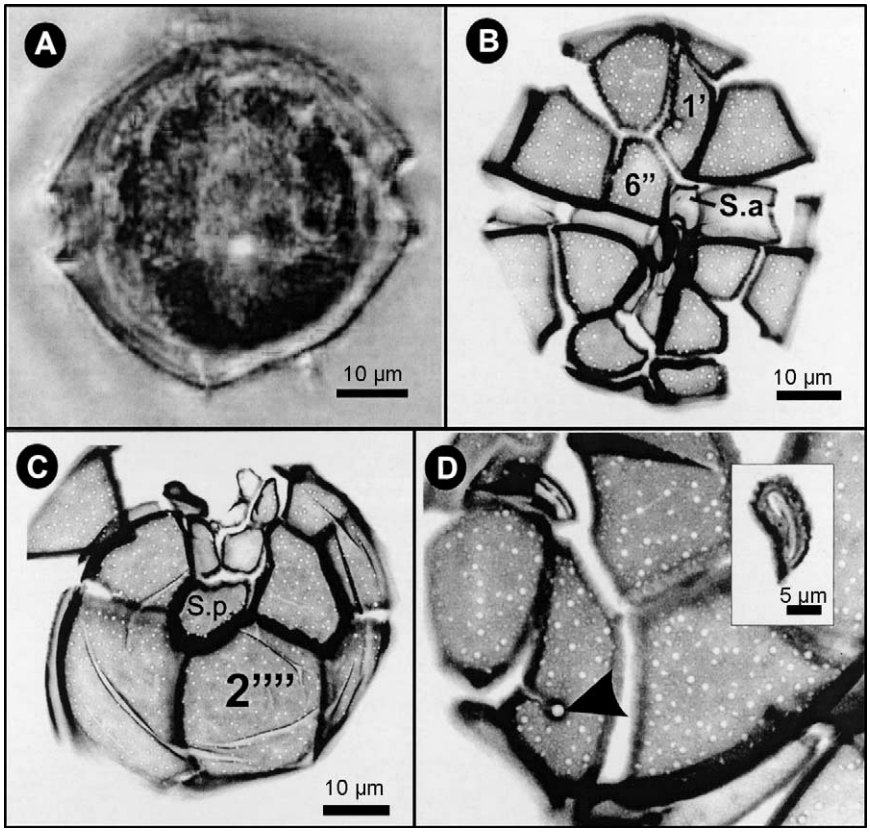

Fig. 3. *A. leei*: (A) a roundish vegetative cell with conical epitheca; (B) ventral view of a cell showing the first apical plate (1′), sixth precingular plate (6″) and the wide anterior sulcal plate (S.a.); (C) antapical view showing the wide posterior sulcal plate (S.p.); (D) the ventral pore (arrowhead) is in the middle of 1′ (inset: the P_0 plate).

3.1.2.3. Dimension. Length: 43–50 μm; transdiameter: 43–52 μm.

3.1.2.4. Distribution in Malaysia. The species has been found in samples from Sebatu and Pulau Aman in the Straits of Malacca.

3.1.3. A. minutum Halim

3.1.3.1. Material examined. Wild and cultured cells originating from the waters around Tumpat, northeast coast of Peninsula Malaysia (Fig. 4).

3.1.3.2. Morphology. Cells are roughly oval in shape with a deep cingulum. S.p. is much wider than long, with curved margins. The anterior margin is slightly wavy in appearance. 1′ is of medium width with slightly curved left and right margins. The plate touches the APC. A ventral pore is located midway down the right margin. 6″ is narrow and long. The APC is large, with straight right and dorsal margins. The left margin tapers to a point on the ventral end. The apical pore is centrally located. 3′ is asymmetrical, with the left anterior margin longer than the right. Other characteristic features are the rhomboidal shape of the left anterior lateral plate (s.a.a.) and the short left posterior lateral plate (s.a.p.) in the sulcus.

3.1.3.3. Dimensions. Length: 26–30 μm; transdiameter: 24–30 μm.

3.1.3.4. Distribution in Malaysia. The species has only been found in samples from the northeast coast of Peninsula Malaysia to date.

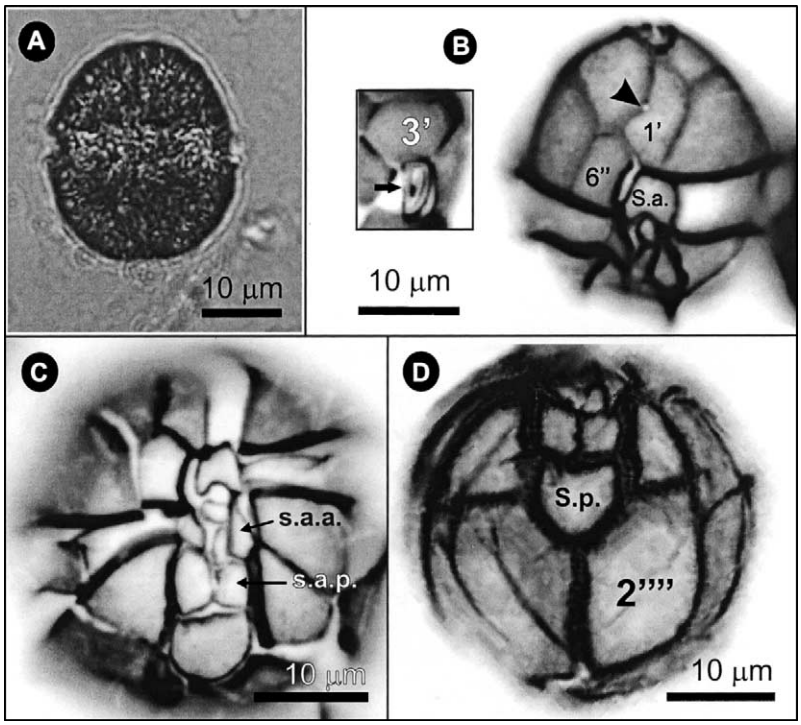

Fig. 4. *A. minutum*: (A) an oblong-shaped vegetative cell; (B) partial ventral view showing the first apical plate (1′), a tall sixth precingular plate (6″), the anterior sulcal plate (S.a.) and location of the ventral pore (inset: details of the APC and third apical plate (3′)); (C) individual sulcal plates showing the rhomboidal left anterior lateral plate (s.a.a.) and short left posterior lateral plate (s.a.p.); (D) antapical view showing a wide posterior sulcal plate (S.p.) and second postcingular plate (2‴′) typical of the species.

3.1.4. A. tamarense (Lebour) Balech

3.1.4.1. Material examined. Clonal cultures established from Pulau Aman plankton sample (Fig. 5).

3.1.4.2. Morphology. Cell shape is approximately pentagonal. The epitheca and hypotheca are of the same height. Cells in culture often exist in pairs. S.p. is longer than wide and both anterior corners are projected. A posterior attachment pore may or may not be present. When present it is located near the right margin, to which it is connected by a groove. Sulcal lists are quite well-developed. 2‴′ is large and pentagonal. In most cells it is dorsoventrally extended. The right and posterior margins tend to be straight. 1′ is wide and touches the APC directly. A ventral pore is located midway on the right margin. In most cells the left and right margins are straight and parallel. In some cells the plate widens to the posterior from the point of the ventral pore. 6″ is about as long as it is wide and is concave on the left margin. 3′ is hexagonal and asymmetrical, with the anterior left margin always longer than the right. The APC is medium-sized. The left and dorsal margins are straight. The right margin tapers quite sharply towards the ventral. The apical pore and platelet are located equidistant from the margins. When an anterior attachment pore is present, it is located to the right of the apical pore and the dorsal end of the APC is wider. The S.a. is about as wide as it is long, with a deep posterior sinus. The anterior margin is straight, with a small groove directed to the right. The right foot of the plate is pointed.

3.1.4.3. Dimensions. Length: 28–34 μm; transdiameter: 30–36 μm.

3.1.4.4. Distribution in Malaysia. To date the species has only been found in samples from waters around Pulau Aman and Penang in the Straits of Malacca.

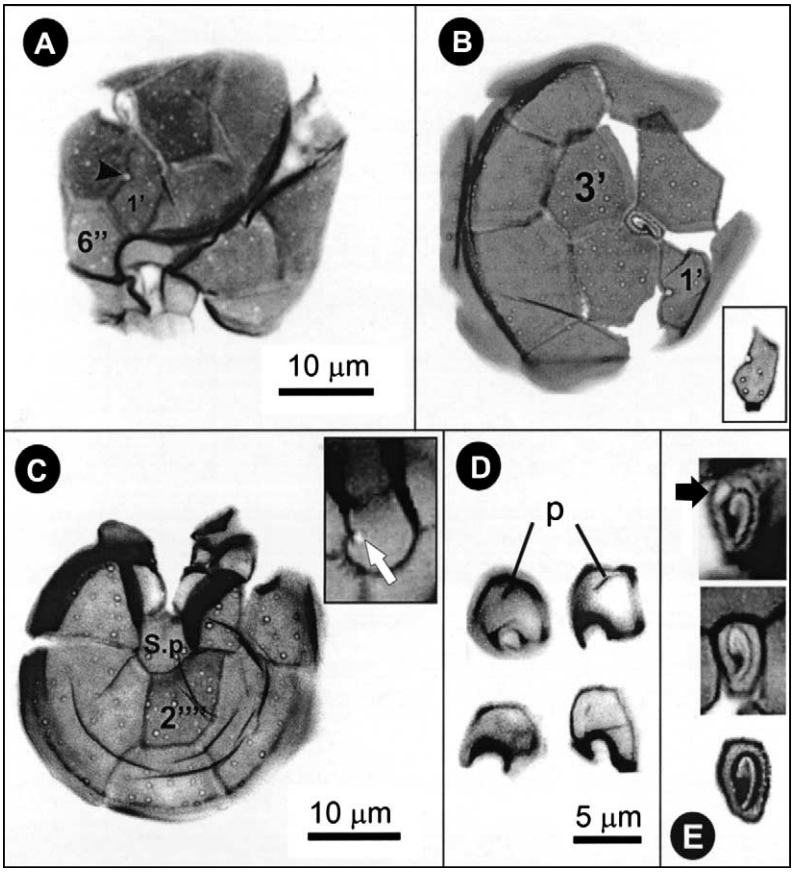

Fig. 5. *A. tamarense*: (A) ventral view of a vegetative cell showing the first apical plate (1′), the sixth precingular plate (6″) and location of the ventral pore (arrowhead); (B) apical view showing an APC without an attachment pore and the third apical plate (3′) (inset: first apical plate (1′)); (C) antapical view showing the posterior sulcal plate (S.p.) and second antapical plate (2″″) (inset: a posterior sulcal plate with an attachment pore (arrow)); (D) detail of the anterior sulcal plate (S.a.) showing the right-directed plica (p); (E) apical view showing an APC with an anterior attachment pore (arrow).

3.1.5. *A. tamiyavanichii* Balech

3.1.5.1. Material examined. Wild and cultured specimens originating from the waters around Sebatu in the Straits of Malacca (Fig. 6).

3.1.5.2. Morphology. Cells are round and quite small. Forms long chains in culture. Cingulum is shallow. Sulcal lists are well developed. 1′ is wide and touches the APC directly. Left and right margins are straight and almost parallel. A ventral pore is present, formed by notches on both the 1′ and 4′. 3′ is asymmetrical, with the anterior left margin about twice as long as the anterior right margin. A large anterior attachment pore is located to the right of the apical pore. The S.p. is much longer than wide. A large posterior attachment pore is located centrally in the S.p. with a groove connecting the pore to the right margin of the plate. Even when a pore is absent, a groove may also be present in the S.p. 6′ is wide and is bell shaped. The diagnostic feature of the species is the S.a. It is longer than wide and has a precingular part that extends into the epitheca. Even in single clonal culture two variations of the precingular part are commonly observed. In the first type (*tamiyavanichii*-type), the precingular part is dome-shaped and the anterior left margin adjoins the posterior right margin of 1′. In the second type (*cohorticula*-type), the anterior tip of the

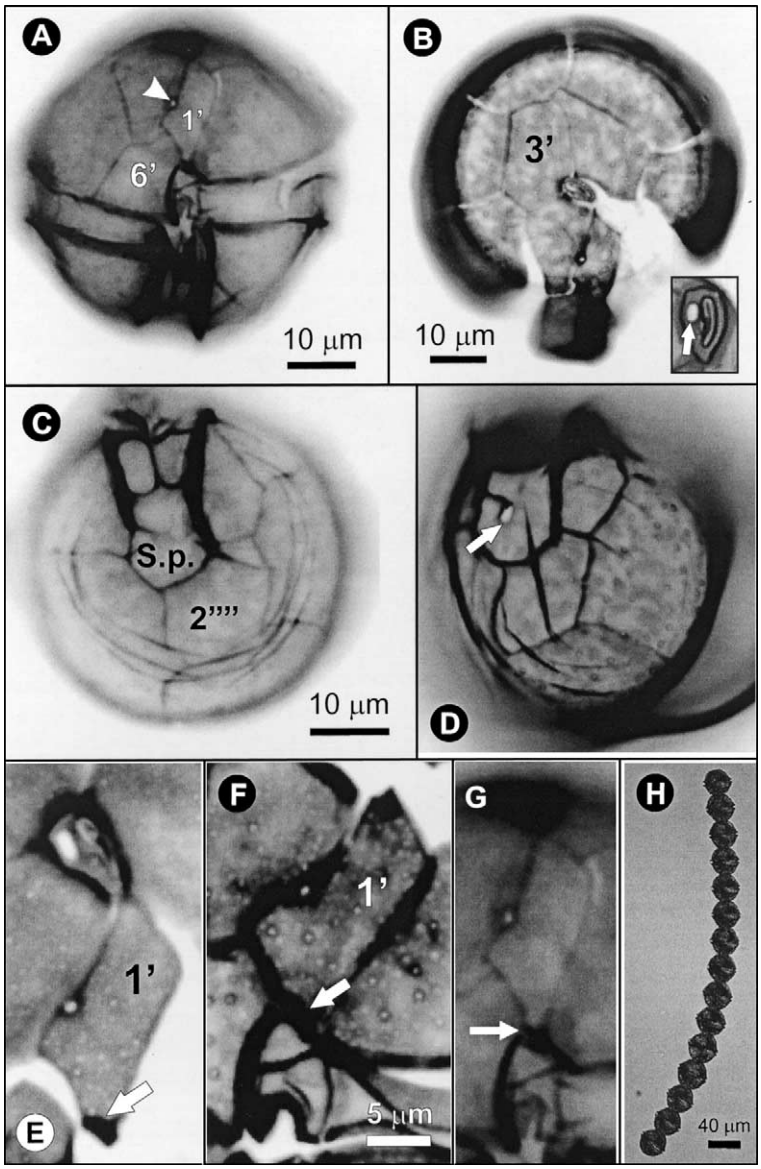

Fig. 6. *A. tamiyavanichii*: (A) ventral view of a cell showing the first apical plate (1′), wide sixth precingular plate (6″), and location of a ventral pore (arrowhead); (B) apical view showing the third apical plate (3′) and the APC without an attachment pore (inset: APC with an attachment pore (arrow)); (C) antapical view showing the long posterior sulcal plate (S.p.); (D) posterior sulcal plate with a large attachment pore; (E and F) the '*tamiyavanichii*-type' anterior sulcal plate where margins of the precingular part and 1′ are oblique to the horizontal plane (arrow); (G) the '*cohorticula*-type' anterior sulcal plate where margins of the precingular part and 1′ are parallel to the horizontal plane (arrow); (H) chain of *A. tamiyavanichii*, cells in culture.

precingular part is slightly truncated and abuts the posterior tip of 1′.

3.1.5.3. Dimensions. Length: 32–40 μm; transdiameter 34–40 μm.

3.1.5.4. Distribution in Malaysia. The species has only been found in samples from Sebatu to date.

3.2. Toxicity

Only the *A. minutum* and *A. tamiyavanichii* clones contained PSP toxins as revealed by the receptor binding assay. All three *A. tamarense* clones were negative. In the *A. tamiyavanichii* clone tested the mean toxin content was 26 fmol per cell STX equivalent. In the two *A. minutum* clones, the mean toxin content was 12 and 19 fmol per cell STX equivalent.

4. Discussion

The presence of HABs and related seafood toxicity imposes a heavy burden on the affected country. An effective seafood toxicity monitoring program needs to be established to safeguard public health and fulfill seafood export requirements. Such a program depends in a large part on knowing HAB species that are present and locations where toxicity problems are most likely to arise.

In Malaysia, up to 1990, the situation was relatively simple, with monitoring activity being necessary only for the west coast of Sabah, where *P. bahamense* var. *compressum* regularly blooms. However, there is now strong evidence that more areas in the country could be affected by PSPs, involving toxin producers other than *P. bahamense*. Indeed, the current situation warrants the setting up of monitoring programs for both the east and west coasts of Peninsula Malaysia.

It took several years after the initial PSP event in Sebatu before the most likely toxin producer was identified to be *A. tamiyavanichii*, although it is not entirely certain that *A. tamiyavanichii* was indeed responsible for the PSP event in 1991. However, this is the only toxic species that we have found in plankton samples collected from the area to date and the density tends to peak in September–November. Initially we identified the isolates from Sebatu as *A. cohorticula*, but more detailed examination of the sulcal plates showed that they are *A. tamiyavanichii* based on descriptions in Balech (1995). However, the situation is not so straightforward. In both field and cultured material, cells possessing typical '*tamiyavanichii*-type' and '*cohorticula*-type' precingular of the anterior sulcal plate were observed. Both cell types can occur in a clonal culture, indicating that this is not a stable character. Our opinion is that the two should be regarded as a single species, in which case the name *A. cohorticula* should be used. Analysis of the rRNA nucleotide sequences showed very close affiliation of the species to the *A. tamarense*/*catenella*, *fundyense* group, and in particular to an *A. tamarense* isolate from Thailand (Usup et al., 2002). This is not the first record of *A. tamiyavanichii* from the region, since there was a report of its occurrence in Thailand waters much earlier (Kodama et al., 1988). There is no published *A. tamiyavanichii* toxicity for comparison. However, the toxin content obtained in this study is similar to those obtained for *A. cohorticula* isolates from Japan (Ogata et al., 1990).

Three species were identified from Pulau Aman material. While *A. affine* and *A. leei* were simple to identify, the third species was quite problematic. Cells in the field samples were very difficult to observe even with calcofluor staining since the thecae were very thin. Good images were only obtained from cultured material. A potential problem for microscopic examination is that folding of a thin membrane covering the theca could give a distorted view of the theca plates. The species found in the Pulau Aman samples are very similar to those described from Thailand and designated as *Alexandrium* sp. cf. *tamarense* by Balech (1995). The main difference between this isolate and typical temperate *A. tamarense* is that in the tropical isolates 1′ is wide and the cells are smaller. Cells with narrow, sharply tapering 1′ were observed in culture but were rare. This could represent aberration from the normal morphology. The isolates described by Fukuyo et al. (1988) originated from the Gulf of Thailand which adjoins the northeast coast of Peninsula Malaysia. This suggests that *A. tamarense* is present on both coasts of Peninsula Malaysia, although it has yet to be found in any of the samples from the east coast. At least two toxicity studies on isolates collected from different periods have shown that *A. tamarense* from the Gulf of Thailand do not

produce PSP toxins (Piumsomboon et al., 2001). Results from this study suggest that *A. tamarense* isolates from the Straits of Malacca are also probably non-toxic. It has been suggested much earlier that typical *A. tamarense* do not produce PSP toxins (Boyer et al., 1985; Balech, 1995). However, one clone from Thailand was reported as toxic by Scholin (1993). Further studies need to be carried out to establish whether temperate and tropical *A. tamarense* isolates should be taxonomically separated, and if non-toxicity is a common feature of the tropical isolates. In this respect, taxonomic re-examination of the toxic *A. tamarense* clone CU-13 from Thailand would be very valuable.

In contrast to *A. tamarense* and *A. tamiyavanichii*, *A. affine*, *A. leei* and *A. minutum* were much easier to identify since the diagnostic features were readily visible. The occurrence of *A. minutum* on the east coast of Peninsula Malaysia is not totally unexpected considering that the species has been reported from the Gulf of Thailand (Fukuyo et al., 1988) and more recently from the waters of Vietnam (Yoshida et al., 2000). It is still not known whether or not *A. minutum* is present in the Straits of Malacca. The toxin content obtained for the *A. minutum* isolates in this study is very similar to those reported for isolates from other parts of the world (Chang et al., 1997; Hwang and Lu, 2000). In general the toxin content of both *A. tamiyavanichii* and *A. minutum* are lower than that of *P. bahamense* var. *compressum* and other *Alexandrium* species.

The presence of toxic *A. minutum* on the east coast and *A. tamiyavanichii* on the west coast of Peninsula Malaysia raises at least two important points. One is regarding the origin of these populations, and two is the apparent absence of PSP cases in the peninsula until relatively recently. With regards to origin, it would be tempting to speculate that these species could have been introduced via ships' ballast water, considering the fact that the South China Sea and the Straits of Malacca are among the busiest sea lanes in the world. Ballast water has been shown to be a significant means of dispersion of HAB species (Hallegraeff and Bolch, 1992). However, this speculation would be very difficult to prove. It is very likely that these species have been present in Malaysian waters for a long time, since they have been reported from adjoining Thailand waters about two decades earlier, albeit only from the Gulf of Thailand and not the Andaman Sea.

The apparent absence of PSPs in Peninsula Malaysia until recently could be attributed to several factors. Firstly, there may well have been PSP incidences but were not reported or were misdiagnosed. Secondly, the toxic dinoflagellate species very rarely achieve densities sufficient to cause toxicity. Indeed there has never been any report of red tides in coastal waters of the peninsula, although it should be noted that *Alexandrium* spp. very seldom, if ever, form visible blooms. The third and probably most likely reason has to do with the pattern of shellfish resource utilization. In Peninsula Malaysia, the only bivalve mollusk that is consumed on a wide scale is the mud-dwelling blood cockle *Anadara granosa*. Mussels are still not so popular, and mussel farming is still on a very small scale compared to other countries. The only significant mussel farming areas are in the Johore Straits, in Sebatu and in Port Dickson, and these cater mainly for export. On the east coast of the peninsula, mussel farming is non-existent. It is noteworthy that the first incidence of PSP in Sebatu in 1991 resulted from consumption of mussels from the newly established mussel farm there. This suggests that PSPs, particularly in the Straits of Malacca, could become more significant if mussel farming is expanded.

In conclusion, this study is significant in at least two main respects. First, it has provided evidence that PSPs in Malaysia could involve at least three toxin producers and may affect more areas. This would require a significant expansion of the existing shellfish toxicity monitoring program in the country. Secondly, through this study laboratory cultures of tropical isolates of *Alexandrium* spp. have been successfully established. These are invaluable resources for comparative studies on the taxonomy, physiology, toxicity, as well as biogeography of this important genus.

Acknowledgements

This study was funded by the Malaysia government through research grants IRPA 08-02-02-0018 and IRPA 09-02-02-0058.

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