

Identification of *Alexandrium halim* (Dinophyceae) using EPI-fluorescence microscopy

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ABSTRACT

Alexandrium species is a group of dinoflagellates comprise of more than 20 different toxic and non toxic species More than half of them are capable in producing a type of neurotoxins called saxitoxins which act as a sodium channel blocker in mammalian nerve system. The occurrences of toxic *Alexandrium* blooms have increased tremendously over the past decade throughout the world. Lack of distinctive characteristic such as horn, spine or heavy ornamentation of the thecal plates caused the difficulty in identification of this species using ordinary light microscope. In this present paper, an epi-fluorescence microscopy with an image analysis system was applied to identify several species of *Alexandrium* found in the Straits of Malacca and South China Sea. Five species of *Alexandrium* viz. *Alexandrium tamarense*, *A. minutum*, *A. tamiyavanichii*, *A. leei* and *A. affine* have been identified based on the ultrastructure and thecal plate tabulation of the cells using calcofluor white staining. The observation of those fine morphological characteristics such as apical pore (P_o), anterior sulcal plate (S.a.), posterior sulcal plate (S.p.) and ventral pore (pv) were enhanced by using this method.

KEYWORDS: *Alexandrium*, Dinoflagellates, EPI-fluorescence microscopy.

INTRODUCTION

The genus *Alexandrium* comprises more than twenty species and more than half of them are capable to produce a group of neurotoxins called saxitoxins, which act as a sodium channel blocker in mammalian nerve system. Increased in cell number of these microalgae in the water column promote toxins accumulation in filter feeder organisms such as oyster and clam, and hence cause paralytic shellfish poisoning (PSP) through consumption of contaminated organisms. Recently harmful algal blooms events in Peninsula Malaysia [1, 2] that were caused by *Alexandrium* species have changed the perception of the HAB and PSP research in the country, which was only confined to the west coast of Sabah since the first events due to *Pyrodinium bahamense* var. *compressum* occurred in the 1976 [3].

Identification of *Alexandrium* is mainly based on the Kofoidan thecal plate tabulation as suggested by Kofoid [4] (Figure 1). However, morphological identification of this genus using normal light microscope had faced numerous problems, due to lack of distinctive characteristic in *Alexandrium* such as horn, spine or heavy ornamentation of the thecal plates. These include inability to recognize the ultrastructures such as the anterior and posterior sulcal plate, apical pore complex under light microscopy.

In this paper, we would like to present our data on the application of an epifluorescence approach coupled with image analysis system to examine the outer morphological characteristics of *Alexandrium* species found in various locations in Malaysia water.

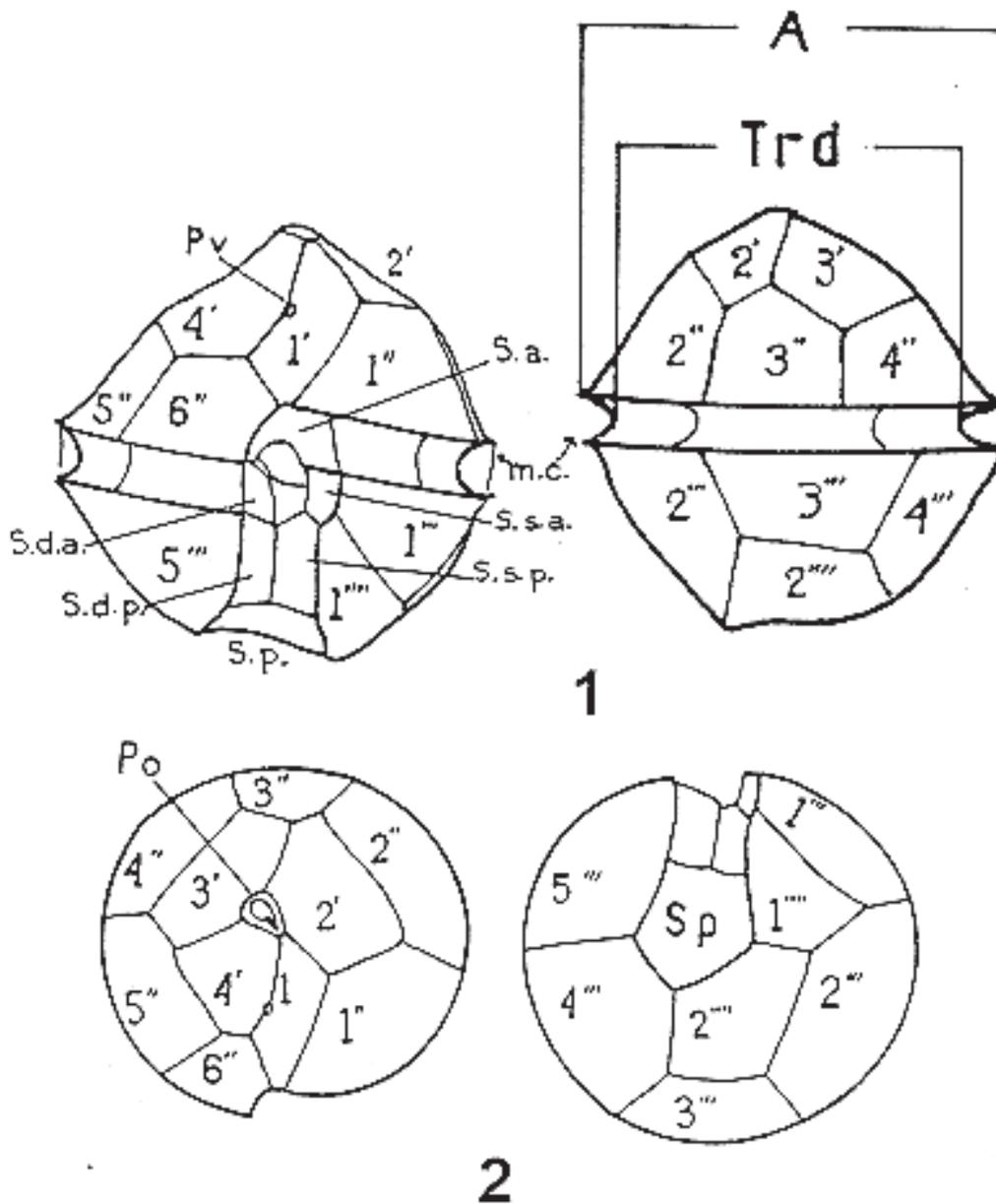


Figure 1. Thecal plates tabulation of *Alexandrium*. pv, ventral pore, m.c., curtain fins, A, amplitude, Trd, transdiameter, adapted from [5].

MATERIAL AND METHODS

1. Cultures conditions and sample preparation

The dinoflagellate *Alexandrium* species were collected from various locations of the Straits of Malacca and South China Sea and clonal cultures of these species were established in the laboratory. The cultures were maintained in ES-DK medium [6] with soil extract at 26 ± 0.5 °C under a 14:10 hour light dark cycle. Port Dickson seawater (0.2 μm filtered, 28 ‰ salinity) was used as the medium base. The samples for morphological observation were harvested from mid-exponential growth phase cultures. Cells were preserved in neutral Lugol's iodine solution or fixed in 1% glutaraldehyde (BDH) and incubated for 1 h at 4°C.

2. Morphological observation

For epifluorescence microscopy, cells were stained with 1 % calcofluor white (Sigma) solution. Ten microliters of stained cells solution was placed on a microscope slide and covered with a coverslip (22 × 22 mm). The coverslip was sealed with nail polish and the stained cells were analysed immediately on an Olympus BX51 epifluorescence microscope (Olympus, Melville, NY) using 200, 400, and 600 × objectives. Samples were illuminated by a 100-W mercury burner USH102D (Ushio). Excitation occurred through bandpass excitation filters for calcofluor (BP330-385), and chlorophyll autofluorescence (BP520-550) and emitted light was collected through double pass dichroic mirrors and barrier filters (calcofluor, DM400, BA420; chlorophyll, DM565, BA580IF).

Digital images were captured using a ColorView F12 cooled CCD camera (1300 × 1030 pixels of 6.7 × 6.7 μm, Soft Imaging System GmbH, Germany) which was operated in 12 bit (3 RGB) mode. The camera functions were controlled by analySIS v. 3.0 (Soft Imaging System GmbH, Germany).

Identification of *Alexandrium* species was based 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 such as ventral pore as well as the anterior and posterior attachment pores were used in species identification [5,7].

3. Image processing

The captured images were saved in tagged image file format (TIFF). The images were then extracted by blue channel using color separation function available in the analySIS package. The gray scale images were then inverted and the quality of images was enhanced by intensity adjustment.

The cell dimensions were estimated from the images using analySIS. Total of twenty randomly selected cells were measured and the value presents were expressed as mean.

RESULTS AND DISCUSSION

Method applied using fluorostain calcofluor White has been successfully identified the genus *Alexandrium* to the species level. At least five species of *Alexandrium* have been identified from the coastal waters of Peninsula Malaysia. They are *A. minutum*, *A. tamiyavanichii*, *A. leei*, *A. tamarensis* and *A. affine*.

A PSP event has been reported in Tumpat, Kelantan in September, last year. The investigation that has been carried out after the event concluded the causative species as *Alexandrium minutum* [1]. The diagnostic characters of this species are the wide posterior sulcal plate and narrow and long sixth precingular plate. The anterior sulcal plate is longer than wide with a straight anterior margin (Figure 2).

The causative species responsible for PSP in Melaka in 1991 was only been able to identify as one of the *Alexandrium* species. The species designation remain undissolved until the recent study on this particular species had been carried out and also the success in bringing the species into culture [8]. The species was identified as *Alexandrium tamiyavanichii*, based on the following morphological characters; the cells are round and quite small, chain forming, well developed sulcus list, 1' is wide and touched the APC directly, present of ventral pore and a large anterior attachment pore (Figure 2). Evident from toxins composition analysis of the species and mussel extracts also proved that *A. tamiyavanichii* as the responsible species (unpublished data).

Alexandrium leei is the largest *Alexandrium* found among the species. The species is easy to distinguish from other *Alexandrium* spp. by its shape and the location of ventral pore. The hypotheca have asymmetrical lobules with larger lobules on the left. The ventral pore is located centrally with a groove connected to the right margin of 1' (Figure 2).

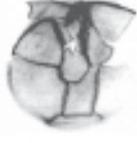
	<i>A. minutum</i>	<i>A. tamiyavanichii</i>	<i>A. jeel</i>	<i>A. tamarensis</i>	<i>A. affine</i>
Posterior sulcal plate (S.p.) and second antapical plate (2'')					
P _o plate and anterior attachment pore (apc)					
First apical plate (1') and sixth precingular plate (6''); Location of ventral pore (pv)					
Anterior sulcal plate (s.a.) *					
Chain forming	No	Yes	No	No	Yes
Cell dimension (μm) **	L: 26-30 TD: 24-30	L: 32-40 TD: 34-40	L: 43-50 TD: 43-52	L: 28-34 TD: 30-36	L: 28-34 TD: 30-36

Figure 2. The diagnostic morphological characteristic of the thecal plates of *Alexandrium* species. Abbreviation: * p.pr., precingular part, p, plica; ** L, length; TD, transdiameter.

Alexandrium tamarensis were identified from Pulau Aman samples. The cells are smaller than typical *A. tamarensis* but have a very similar characteristic with *A. cf. tamarensis* described from Thailand specimens [3]. The 1' is wide with straight margins. The S.a. is wide and the anterior margin is straight, with a groove (plica) directed to the right (Figure 2).

A. affine has been found in the samples from Sebatu and Pulau Aman. This species is easy to identify based on the location of anterior attachment pore in the APC (Figure 2). The shape of the cells is approximately pentagonal. The S.p. is longer than wide. Cells in chain have been observed in culture (Figure 3).

Application of fluorostain, calcofluor white method provides an alternative to electron microscopy, at the same time it reduces significantly the amount of time needed for samples processing. In addition, it also provides an edge over normal light microscopy that is able to provide sufficient in the identification of the species concerned. This method also provided a high resolution and enhancement on fine structures and characters that need to be observed, for instances, plate suture, which separate between different plate.

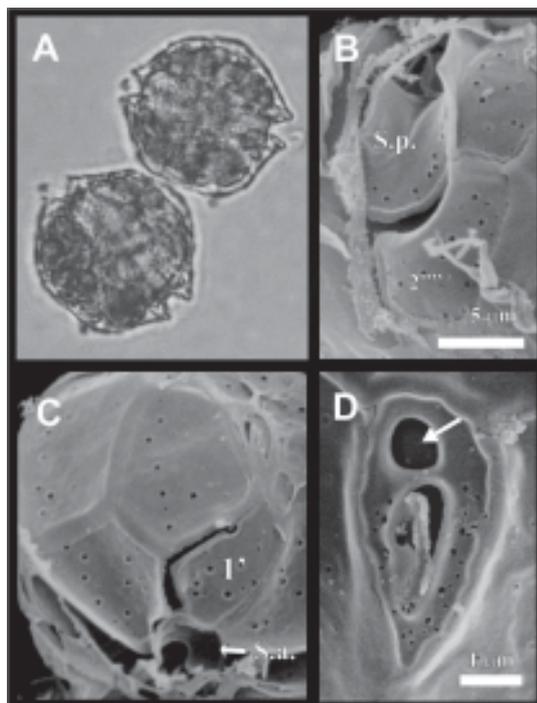


Figure 3. (A) Typical vegetative cells of *A. affine*. (B) Scanning electron micrograph of cell, showing posterior sulcal plate (S.p.) and second antapical plate (2'''), (C) electron micrograph of cell showing the first apical plate (1'), anterior sulcal plate (S.a.) and ventral pore (v.p.), (D) Apical plate with a large anterior attachment pore (arrow).

Identification of five *Alexandrium* species in Malaysian waters should serve as a warning sign on the expansion of HAB in the country. It also provide a strong evident on potentially wide spread of the shellfish poisoning in the near future, which in need of urgent attention from relevent authority. Information on the distribution of these *Alexandrium* species becomes critically important in areas associated with mussel or cockle farming activities. In addition, it is needed for better management and mitigation of HAB problems in the country.

Precise identification of these species is also crucial, especially in differentiating the toxic and non-toxic species of *Alexandrium* spp. at the preliminary stage. This also provide useful information before proceed to further toxin analysis.

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