STUDY ON MORPHOLOGY, GROWTH PHYSIOLOGY OF *Alexandrium* AND TRANSFORMATION OF PSP TOXINS IN BENTHIC CLAM, *Polymesoda similis*

Priscilla Simba anak Daniel Ngilek

Master of Science
2008
STUDY ON MORPHOLOGY, GROWTH PHYSIOLOGY OF *ALEXANDRIUM*
AND TRANSFORMATION OF PSP TOXINS IN BENTHIC CLAM,
*POLYMESODA SIMILIS*

PRISCILLA SIMBA ANAK DANIEL NGILEK

A thesis submitted in fulfillment of the requirements for the degree of

Faculty of Resource Science and Management
UNIVERSITI MALAYSIA SARAWAK
2008
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my Lord and Saviour for His amazing love and grace. My utmost appreciation and gratitude to my supervisors, Dr. Lim Po Teoh for his exceptional supervision and advice, Dr. Othman Baja for his concern and special assistance, and Dr. Gires Usup for his patience and his generosity in providing all the equipments, samples and training. I would also like to thank everyone of the UAM laboratory for their assistance. I also thank my family, especially my mother Mei Yee and my brother and also fellow researches and lab assistants of the Unimas lab and my postgraduate students and research support of UNIMAS and the universities. In summary, it is mainly to thank my beloved family for their support and encouragement, friends like Suharia, Dacitin, Saladin, Arshad and many others who have directly or indirectly been involved with the success of this thesis and last but not the least, my faithful companion, Pedja for being there for me.

28 MAY 2008

PRISCILLA SIMBA AK. DANIEL NGILEK

02-02-0788
ACKNOWLEDGEMENTS

First and foremost I would like to thank my Lord and Saviour for His amazing love and grace. My utmost appreciation and gratitude to my supervisors; Dr. Lim Po Teen for his exceptional supervision and advice, Dr. Othman Bojo for his concern and special assistance, and Dr. Gires Usup for his patience and his generosity in providing all the equipments, samples and training. I would also like to thank everyone of the UKM laboratory for their assistance, especially Cheah Mei Yee and Boon Khoon and also fellow researchers and lab assistants of the Unimas laboratory, staff of the postgraduate studies and research support of UNIMAS and not forgetting Ministry of Science and Technology for providing me with substantial financial assistance throughout the study.

I want to thank my beloved family for their support and encouragement; friends, Marjorie, Farrah, Darfrina, Sally, Alwani, Norli, Agatha, Marilyn and Jefferine who has directly or indirectly been involved with the completion of the thesis and last but not the least, my faithful companion, Pudun for being there for me.
ABSTRACT

A morphology, growth physiology and toxicity study was carried out on five clonal cultures of *Alexandrium* species i.e. *A. minutum, A. tamarense, A. leei, A. affine* and *A. tamiyavanichii*. These species were originally established from various coastal and estuarine waters in Peninsula Malaysia. The five *Alexandrium* species showed high similarity in morphology that it is difficult to correctly identify under normal light microscope. However, these species can be distinguished based on outer thecal tabulation that can be stained using Fluorostain, Calcoflour White MR2 and observed under epi-fluorescence microscope. The different species can be differentiated by cell size, shape and special characteristics possessed by each species which includes shape of 1', 6'' and 2''' plates, position of the ventral pore and also shape of anterior and posterior sulcal plates. Of the five species, *A. minutum* can be easily distinguished based on its small size and round to oval shape. Some of the species has variable characteristics such as the two variant of precingular part in *A. tamiyavanichii*. The five *Alexandrium* species showed different growth responses under different salinity and nitrate condition. *Alexandrium minutum* showed the widest salinity tolerance compared to other species. It was able to survive in salinity ranging from 5 to 35 psu. Salinity tolerance of the four species in decreasing order was *A. minutum, A. leei, A. affine* and *A. tamarense*. The study suggests that *A. minutum* is an euryhaline species while *A. tamarense* is more stenohaline type. Overall, nitrate seems to be a limiting factor for growth of all the cultured *Alexandrium* species at low concentrations. Nitrate is a limiting factor for growth of *A. tamarense* when nitrate concentration is less than 300 µM; and for *A. tamiyavanichii* it becomes a limiting factor at much lower nitrate concentration (< 100 µM). Nitrate requirement of the five species in decreasing order was *A. tamarense, A. affine, A. leei, A. minutum* and *A. tamiyavanichii*. The five species also showed that varied nitrate uptake and growth response might imply the adaptation strategy of the species in changing estuarine environment. The clam, *Polymesoda similis* that was fed with toxic *A. minutum*, had four types of gonyautoxins in its gut tissues when analysed by HPLC. Results showed that toxin accumulated very slowly in clam gut tissue and it took a long period of time to depurate the toxins. Gonyautoxins in the gut tissue were also able to go through transformation from the less toxic form to the more toxic type; which was from GTX 1 and GTX 4 to GTX 2 and GTX 3.
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### LIST OF ABBREVIATIONS

- NVSP: Neurotic Shellfish Poisoning
- EHSP: Diarrhetic Shellfish Poisoning
- Ciguateric Fish Poisoning
- Azaspiracid Poisoning
- PSU: Pūr Salinity Unit
- Div: Division
- ppm: Parts Per Million
- EnfSW: Enriched natural seawater
- RPM: Rotations Per Minute
- HAs: Hydromeloric Acid
- Sax: Saxitoxin
- Gln: Glutamine
- MUN: Mouse Unit
- UVP: Ultra Violet
- GR: Growth Rate
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<td>AOAC</td>
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<td>AIMS02</td>
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<td>HAB</td>
<td>Harmful Algal Bloom</td>
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<td>UNIMAS</td>
<td>Universiti Malaysia Sarawak</td>
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<td>UKM</td>
<td>Universiti Kebangsaan Malaysia</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>APC</td>
<td>Apical Pore Complex</td>
</tr>
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<td>Po</td>
<td>Apical Pore Plate</td>
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<tr>
<td>s.p.</td>
<td>Posterior Sulcal Plate</td>
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<td>s.a.</td>
<td>Anterior Sulcal Plate</td>
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<td>v.p.</td>
<td>Ventral Pore</td>
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<td>UNESCO</td>
<td>United Nation Education, Science and Cultural Organisation</td>
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<td>GEOHAB</td>
<td>Global Ecology and Oceanography of Harmful Algal Blooms</td>
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<td>Azaspirazid Poisoning</td>
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<td>psu</td>
<td>Per Salinity Unit</td>
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<td>ES-DK</td>
<td>Enriched natural seawater</td>
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<td>rpm</td>
<td>Revolutions Per Minute</td>
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CHAPTER I

GENERAL INTRODUCTION

1.1 Harmful Algal Blooms

For the past few decades, harmful algal blooms (HABs) have attracted global attention due to its huge impact on aquatic life and human health (Li et al., 2001). Occurrences of HABs have increased in frequency, intensity and geographical distribution (Hallegraeff, 2003a). Natural and anthropogenic activities have been frequently related as the cause of the occurrence of HABs. Nutrient inputs to coastal areas, transportation and discharge of ballast water are among the factors (Hallegraeff & Bolch, 1992). Occurrence of HABs is a complex phenomenon. It was believed to be caused by various physical and biological factors such as temperature of the water, light, salinity, nutrients and trace minerals, runoff, winds, and other environmental conditions (Yentsch, 1984).

Generally, HAB organisms are classified into two main groups; the toxin producers that can contaminate seafood and high-biomass producers that are not toxic but causes anoxia that leads to mortalities of marine life after reaching dense concentration (GEOHAB, 2001). Concentration of cells can reach up to more than 75 million cells per litre during bloom incidents (Dodge, 1982). High density algal blooms may or may not be visible and coloration depends on the species involved and environmental conditions. These may be non-toxic, but under exceptional conditions, blooms may cause indiscriminate kills of fish and invertebrates (Dodge, 1982). Likewise, visibility does not
reflect the potential for toxicity. Blooms of *Alexandrium* spp. are not characteristically found at sea surfaces, but occur as near-surface aggregations between 3-15 metres under stratified conditions (Cembella & Therriault, 1989; Parkhill & Cembella, 1999). Adverse effects can occur with toxic species, even when cell concentrations are low and the water is clear. Conversely, non-toxic species can dominate the plankton community, depleting oxygen and nutrients and discolouring the water with their pigments (Huntley, 2000).

One of the first recorded fatal cases of human poisoning after eating shellfish contaminated with dinoflagellate toxins was in 1793 when Captain George Vancouver and his crew landed in British Columbia in an area known as Poison Cove. He noted that for local Indian tribes it was taboo to eat shellfish when the seawater became bioluminescent due to dinoflagellate blooms (Dale & Yentsch, 1978). The link between shellfish toxicity and dinoflagellates was first recognized in 1937, following a poisoning outbreak in San Francisco Bay (Sommer et al., 1937), but was not ascribed to a specific organism until 1961 (Prakash et al., 1971; Quayle, 1969).

Among the impact of HABs are severe economic losses to aquaculture, fisheries and tourism operations. Impacts of bloom phenomena include mass mortalities of wild and farmed fish and shellfish, alterations of marine trophic structures, deaths of marine mammals, seabirds, and may cause severe poisoning in humans (UNESCO, 1995). Humans can be exposed to these toxins through the consumption of contaminated shellfish products, which can cause a variety of human gastrointestinal and neurological illnesses. Filter-feeding shellfish, such as clams, mussels, scallops and oysters may ingest toxic algal cells. These toxins can bioaccumulate within flesh tissues to levels that can be lethal to humans.
Approximately 60 of the 2000 extant species of marine dinoflagellates, are known to produce potent water- or lipid-soluble toxins; these may be cytolytic, hepatotoxic or neurotoxic (Graham & Wilcox, 2000). Majority of toxin-producing dinoflagellates are photosynthetic, estuarine or coastal shallow-water forms that are capable of producing benthic resting cysts, and which tend to form monospecific populations (Graham & Wilcox, 2000). Presently, six human syndromes are recognized caused by algal toxins accumulated in fin- or shellfish, namely paralytic shellfish poisoning, diarrhetic shellfish poisoning, amnesic shellfish poisoning, neurotropic shellfish poisoning, ciguateric fish poisoning and azaspirazid poisoning.

1.2 Paralytic Shellfish Poisoning (PSP)

PSP is a marine toxin disease with both gastrointestinal and neurological symptoms reported worldwide. It is caused predominantly by the consumption of toxic shellfish that have ingested large quantities of toxic microalgae by filter feeding. As a result of continuous filtration of these organisms, paralytic shellfish toxins or saxitoxins, are concentrated in the shellfish, particularly in the digestive glands (Lehane, 2000).

Gonyaulacoid dinoflagellates are known as the source of PSP toxins. These dinoflagellates produce at least twenty toxins that are tetrahydropurines, and heat and acid stable. The toxins may be divided into 3 groups (Shimizu, 2000):

1. The highly toxic carbamate toxin
2. The decarbamoyl toxins of intermediate toxicity
3. The weakly toxic N-sulfocarbamoyl toxin.

The PSP toxins block the Na\(^+\) channels of neuronal and muscular cells, thereby preventing depolarisation and propagation of the action potential, which is essential for
the transmission of nerve impulses and muscle contraction. This results in paralysis and
other illness in victims that consumed contaminated shellfish. The peripheral nervous
system is particularly affected (Shimizu, 2000). Primary sources of saxitoxins include
three morphologically distinct genera of marine dinoflagellates: Alexandrium spp.,
Pyrodinium spp. and Gymnodinium spp. (Hall et al., 1990) and four species of freshwater
blue-green algae: Aphanizomenon flos-aquae, Anabaena circinalis, Lyngbya wollei and
Cylindrospermopsis raciborskii (Humpage et al., 1994; Falconer, 1996; Onodera et al.,
1997; Lagos et al., 1997).

At present, the dinoflagellate Pyrodinium bahamense is confined to tropical,
mangrove-fringed coastal waters of the Atlantic and Indo-West Pacific (Hallegraeff,
2003a). The first harmful implications of Pyrodinium blooms became evident in 1972 in
Papua New Guinea. Red-brown water discolorations coincided with the fatal food
poisoning of three children and mouse bioassays on shellfish from the affected village
subsequently established P. bahamense as a source of paralytic shellfish poisons
(MacLean, 1977). Since then, toxic Pyrodinium blooms have been reported in Brunei and
Sabah (1976), the central Philippines (1983), the northern Philippines (1987) and
Indonesia (North Mollucas) (Hallegraeff, 2003a).

The present day distribution of the paralytic shellfish poison-producing
dinoflagellate, Gymnodinium catenatum includes the Gulf of California, Gulf of Mexico,
Venezuela, Argentina, Japan, Korea, China, the Philippines, Palau, Tasmania (Australia),
New Zealand, the Mediterranean and the Atlantic coast of Spain, Portugal and Morocco
(Hallegraeff & Fraga, 1998). The known global distribution of G. catenatum has
increased dramatically in the past few decades and is now reported from every continent
(Bolch, 2000). In 1996, G. catenatum was first identified at Port Lincoln, South Australia,
and may have been introduced from other sites in Australia, such as Tasmania or Victoria (McMinn et al., 2000).

Species from the genus *Alexandrium* represent the largest species group known to cause HABs (30 identified species) though not all are toxin producers (Anderson, 1998). The genus *Alexandrium* currently consists of more than 27 species worldwide, 9 of which are currently known to be toxic (Balech, 1995; Lilly et al., 2005).

Distribution of PSP is increasing in frequency and geographical distribution. Until 1970, toxic dinoflagellate blooms of *Alexandrium tamarense* and *A. catenella* were only known from temperate waters of Europe, North America and Japan (Dale & Yentsch, 1978). By 1990, this phenomenon was well documented throughout the Southern Hemisphere, in South Africa, Australia, New Zealand, India, Thailand, Brunei, Sabah, the Philippines and Papua New Guinea (Hallegraeff, 2003a).

The first PSP case in Malaysia occurred in Sabah, in East Malaysia in 1976 (Roy, 1977). Since then, hundreds of poisoning cases and 22 fatalities have been reported (1976 – 1990). The first PSP incidence in Peninsula Malaysia was reported in 1991 in Sebatu, in the Straits of Malacca (Usup et al., 2002). Three people were taken ill after consuming green mussels from a mussel farm. The following incidence of PSP in Malaysia occurred in September 2001 in Tumpat, Kelantan on the east coast of Peninsula Malaysia. Six people were hospitalized and one fatality was reported following consumption of contaminated clam known locally as 'lokan' (*Polymesoda* sp.). Those affected showed symptoms of PSP (Lim et al., 2004).
1.3 General morphology of dinoflagellates

Dinoflagellates have a common thecal or cell covering structure that, along with their flagellar and nuclear characters, differentiates them from other algal groups. The theca can be smooth and relatively unornamented, as in some *Gymnodinium*, or it can constitute a cell wall of polysaccharide plates with spines and flanges, as in *Pyrodinium*. Its basic structure is a series of membranes, sometimes with a pellicle layer and microtubules. Thecal vesicles usually constitute the second and third membranes (outer to inner), and can be empty, contain additional membranes, or, in the case of most armoured forms, contain polysaccharides such as cellulose, mannose, or galactose (Steidinger & Tangen, 1996). Species in which the wall material consists of cellulose plates that can be observed in the light microscope are colloquially called 'armoured' or 'thecate' as opposed to 'unarmoured' or 'naked' species without cellulose plates. Species belonging in the orders Prorocentrales, Dinophysiales, Gonyaulacales are armoured while species of the Gymnodiniales and the Noctilucales are unarmoured. Tabulation and plate pattern are important for species identification. This system recognizes plate series based on the position on the cell. Tabulation refers to the number of plates in the specific series of plates, while plate pattern refers to the specific morphology of particular plates.
1.4 Morphology of *Alexandrium* species

The genus *Alexandrium* is composed of two related subgenera; *Alexandrium* and *Gessnerium* (Balech, 1995). The species assigned to *Alexandrium* have previously been referred to *Gonyaulax* Diesing 1866, *Goniodoma* Stein 1883, *Gessnerium* Halim 1967, or *Protogonyaulax* Taylor 1979. Thecal plates are thin and difficult to observe under a regular light microscope. Staining solutions, which dye plates by iodine or brighter fluorescence colours help with the observations.

For identification purposes, the artificial system introduced by Kofoid (1909, 1911) and modified by Balech (1980) is generally used. The criteria used for species differentiation are the shape of the apical pore complex (APC), position of the ventral pore, position of the anterior and posterior attachment pores, and the shape of the sulcal anterior plate. The apical pore plate (Po) has a comma-shaped hole covered by a tiny plate and may have an anterior attachment pore and a number of marginal pores; these features together form APC (Balech, 1995).
1.5 Physiology of *Alexandrium* species

1.5.1 Salinity

Dinoflagellates are widely distributed in both fresh and marine water. Some species are euryhaline and can withstand a range of wide salinity changes, whereas others are stenohaline and are sensitive to salinity variations (Iwasaki, 1979). Most red tide flagellates are euryhaline, except subtropical and oceanic species. *Pyrodinium bahamense*, *Gonyaulax monilata*, *G. tamarensis* and *Gymnodinium breve* prefer high salinities and red tide blooms occur in salinities higher than 30 psu (Aldrich & Wilson, 1960; Steidinger & Williams, 1970; Buchanan, 1971; Wardle *et al.*, 1975; Yentsch *et al.*, 1975; Maclean, 1977). Ryther (1955) reported that salinity seemed not to be a limiting factor for dinoflagellate blooms. It is likely that salinity does not restrict the distribution, except in marine waters of extremely low salinity, of euryhaline species. However, salinity seems to be an important factor for stenohaline species.

The fact that most *Alexandrium* spp. blooms are found within estuaries or are largely confined within coastal zones under the influence of river plumes suggests that salinity may be an important biogeographical determinant (Parkhill & Cembella, 1999). The survival tolerance of *Alexandrium* spp. ranges between 5 - 42 psu salinity and wide salinity ranges are commonly found in estuarine environments (Cembella & Therriault, 1989). Optimum salinity for *Alexandrium* spp. growth is between 20-30 psu (Prakash, 1967; White, 1978). Parkhill and Cembella (1999) proposed that ambient salinity may determine the mean growth rate, yet found no significant effects on growth rates between 20-30 psu salinity. Growth was inhibited at low salinities (10 psu) and maximum growth rates of 0.50 divisions per day occurred at 25 psu salinity (Parkhill & Cembella, 1999).
1.5.2 Temperature

According to Anderson (1984), water temperature is a particularly critical environmental factor influencing the development of blooms, and in most cases, must be 5–8 °C. Sanders (1987) reported that optimal maturation of motile cells occurs when the water temperature reaches 16 °C, and that this is normally achieved during spring warming and fall cooling. The temperature window for germination of *A. tamarense* is about 10–15 °C (Itakura & Yamaguchi, 2000). The temperature of deep ocean waters is rather constant at 4 °C, which is too cold to encourage algal blooms (Taylor, 1988). Under laboratory culture, individual dinoflagellate cells tend to have a higher toxin concentration when grown at lower temperatures (Lehane, 2000). The higher concentration may be caused by toxin production continuing during low temperature conditions while low temperatures reduce the rate of cell reproduction (Lehane, 2000).

1.5.3 Nutrient

Harmful Algal Blooms (HABs) species, just like all plants, require certain major and minor nutrients for their nutrition. These can be supplied either naturally from marine and freshwater biogeochemical processes, or through human activities, such as pollution. One of the explanations given for the increased incidence of HAB outbreaks worldwide is that these events are a reflection of increased pollution and nutrient loading in coastal waters (Smayda, 1990). At the simplest level, HAB species may increase in abundance due to nutrient enrichment but remain as the same relative fraction of the total phytoplankton biomass (i.e. all phytoplankton species are stimulated proportionally by the enrichment). More often, this enrichment results in the dominance of particular groups of algae that are best able to capitalize on the enrichment (Anderson *et al.*, 2001). Certain species could grow well even in a nutrient deficient environment while others could not survive when
vital nutrients are scarce. It is therefore important to determine the nutrients requirement of HAB algae such as *Alexandrium* spp.

1.6  **Toxin profiles in *Alexandrium* species**

More than 20 analogues of naturally occurring saxitoxins (STXs) have now been described (Figure 1.1) (Oshima, 1995b). The most commonly encountered are STX, neosaxitoxin (neoSTX), gonyautoxins (GTXs) I, II, III, IV (GTX1-4), B1 (GTX5), B2 (GTX6), and C-toxins, C1-4 (Hall & Reichardt, 1984). PSP can be caused by a combination of any of these toxins, depending on the species of dinoflagellate, geographic area and type of marine animal accumulating the toxin. In addition, toxin profiles in dinoflagellates, shellfish and other animals may change as a result of chemical conversions (Shimizu, 1987).

![Figure 1.1 Structures of the saxitoxins analogues (Oshima, 1995).](image)

1.6.1  **Transformation of STXs in shellfish**

Chemical transformations of STXs can occur in the shellfish during storage, shellfish as food in processing or via food digestion. As well, there is considerable variation in human susceptibility (Bond & Medcof, 1958; Gessner & Middaugh, 1995). Since the hydrolysis of the 21- sulfo group is facile, converting sulfocarbamoyl toxins to their carbamate...
counterparts, the sulfocarbamoyl toxins, when present in seafood, constitute a reservoir of latent toxicity. Although the sulfocarbamoyl toxins themselves probably have low human oral potency, they can hydrolyse at low rates to the more toxic carbamate toxins under conditions of food storage, preparation or digestion. They are readily converted to the corresponding carbamate toxins under acidic conditions, with increases in toxicity up to 40-fold (Kao, 1993). Such conversion has some potential clinical and public health significance, because weakly toxic shellfish containing sulfocarbamoyl toxins could cause disproportionately severe poisoning once ingested (Oshima, 1995b).

1.7 Objectives of the study

A better understanding of important characteristics of *Alexandrium* species will be used to improve strategies for monitoring, prediction, and mitigation of HABs. Knowledge on the identification of species, quantification of the factors that regulate the dynamics of blooms and toxicological characteristics are the fundamental keys for better management of the HAB species in the future.

The objectives of this study were:

1. To identify the *Alexandrium* species based on morphological characteristic.

2. To determine the effect of salinity and nutrient on growth of several *Alexandrium* species.

3. To determine the bioaccumulation and bioconversion of toxin in clam, *Polymesoda similis*, fed with toxic *Alexandrium minutum*.
CHAPTER II

MORPHOLOGY OF ALEXANDRIUM SPECIES (DINOPHYCEAE)

2.1 INTRODUCTION

Harmful algal blooms are often almost monospecific events. Correctly assessing the precise taxonomic identity of the causative organism is crucial in deciding whether knowledge on toxicology, physiology and ecology gained from similar blooms elsewhere can be reliably applied to the species at hand (Hallegraeff, 2003b). Morphology of an organism is the complex expression of its genotype, subject to phenotypical change due to the environment, life-cycle transformations and other influence. (Hallegraeff, 2003b).

Certain taxonomic criteria are used in harmful dinoflagellate identification and classification. Cell size and shape are commonly used features and surface ornamentation (pores, spines, ridges, etc.) is also important if present (Taylor et al., 2003).

Alexandrium cells are typically spherical to hemispherical to oval to slightly biconical, but without horns or spines. (Steidinger & Tangen, 1996). The tabular formula is as follows: Po, 4' (3'+1'), 6", 5"", 2"", 6c, and 9-10s (Balech, 1995).
2.2 MATERIALS AND METHODS

2.2.1 Samples
Unialgal cultures of *A. affine*, *A. minutum*, *A. tamiyavanichii*, *A. tamarense* and *A. leei* used in this study (designated AaMS02, AmKB01, AcMS01, AtPAOI and AIMS02 respectively) were isolated from plankton samples which were obtained using plankton net sized 20 μm as reported earlier (Usup et al., 2002). All cultures have been maintained at the Unimas Aquatic Toxicology Laboratory. *A. minutum* culture was obtained from the bloom which occurred in Tumpat, Kelantan, the east coast of Peninsula Malaysia in September 2001 (Lim et al., 2004). *A. tamiyavanichii* and *A. affine* culture were obtained from a green mussel farm in Sebatu in the Straits of Malacca on the west coast of Peninsula Malaysia where three people were taken ill after consuming green mussels in 1991. *A. tamarense* and *A. leei* were taken from Pulau Aman, Penang in May 2002 (Usup et al., 2002). All cultures were grown in ES-DK medium (Kokinos & Anderson, 1995) at 26°C under a 12h:12h light:dark photoperiod. Samples for identification were harvested during the late exponential phase of culture and then immediately preserved in Lugol’s iodine solution.

2.2.2 Morphological observation

Cultured specimens were examined under a microscope using normal light and epifluorescence (Olympus IX51) microscope. For epifluorescence, fixed samples were stained with Calcofluor white M2R (Sigma) and viewed under UV with a UV filter set. Images were captured with a cooled CCD camera (SIS Colorview, Japan). *Alexandrium* cells were measured using the application available in the Olympus IX51 software.