THE LIFE CYCLE OF TWO PARALYTIC SHELLFISH TOXIN-PRODUCING DINOFLAGELLATES, *Alexandrium minutum* AND *Alexandrium tamiyavanichii* (Dinophyceae) IN MALAYSIA

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ABSTRACT

Hundreds of marine and brackish water dinoflagellates are associated with the natural phenomenon called harmful algal blooms (HABs). HAB is known to cause negative impacts to coastal ecosystems and threaten human lives by contaminating seafood. The dinoflagellates *Alexandrium minutum* and *A. tamiyavanichii* are capable of producing the sodium channel-blocking neurotoxins, saxitoxins (STXs). The purpose of this study is to investigate the dynamics and life cycle transitions of these two species in Malaysian waters in order to understand the triggering environmental factors in formation of blooms. Field sampling was undertaken at two paralytic shellfish poisoning (PSP) hotspots, Tumpat, Kelantan and Kuantan Port, Pahang. Clonal cultures of *A. tamiyavanichii* were established from Kuantan Port, and *A. minutum* from Tumpat. Microscopic enumeration coupled with quantitative qPCR assay was used to detect the low cell abundance of toxic *Alexandrium* species in both the motile vegetative cell and dormant resting-cyst phases in Kuantan Port. The results from a 14-months survey showed that cell abundance up to 17 cells m^{-3} of *A. tamiyavanichii* was present between April 2015 and May 2016. In order to understand the bloom dynamics in relation to the life cycle transitions, *A. minutum* were used in cross-mating and cyst germination experiments. The results revealed that the period of encystment-excystment for *A. minutum* were relatively short (~10 days). This study provides baseline data for future predictive modelling study and early warning of HABs, particularly *A. minutum* and *A. tamiyavanichii*.

ABSTRAK

Beratusan spesies dinoflagelat marin dan air payau adalah berkait rapat dengan fenomena semula jadi yang dipanggil ledakan alga berbahaya (HABs). HABs diketahui menyebabkan impak negatif kepada ekosistem persisiran pantai dan mengancam nyawa manusia melalui makanan laut yang tercemar. Dinoflagelat *Alexandrium minutum* dan *A. tamiyavanichii* mampu menghasilkan neurotoksin penghalang saluran ion sodium, saxitoxins (STXs). Tujuan kajian ini adalah untuk menyiasat dinamik dan peralihan kitaran hidup dua spesies ini di perairan Malaysia untuk memahami faktor-faktor persekitaran yang mecetus pembentukan ledakan. Kerja lapangan telah dijalankandua kawasan panas keracunan kerang-kerangan peralitik (PSP), Tumpat, Kelantan dan Kuantan, Pahang. Kultur klon *A. tamiyavanichii* telah didirikan dari Kuantan dan *A. minutum* dari Tumpat. Penghitungan microskopik berserta cerakin kuantitatif qPCR digunakan untuk mengesankan kelimpahan sel rendah *Alexandrium* spesies dari kedua-dua peringkat hidup sel vegetatif dan sista yang tidak beraktif di Pelabuhan Kuantan Keputusan penyelidikan selama 14-bulan telah menunjuk kelimpahan sebanyak 17 sel m^{-3} *A. tamiyavanichii* dari April 2015 sehingga Mei 2016. Untuk memahami dinamik ledakan yang berhubung dengan peralihan kitaran hidup, *A. minutum* telah digunakan dalam eksperiment mengawan silang dan percambahan sista. Hasil keputusan menunjukkan bahawa tempoh pembentukan-pencambahan sista bagi *A. minutum* adalah agak pendek (~10 hari). Kajian ini telah menyediakandata asas dalam kajian model ramalan dan amaran awal HAB untuk spesies *A. tamiyavanichii* and *A. minutum.*

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LIST OF SYMBOLS AND ABBREVIATIONS

ASP	:	Amnesic shellfish poisoning
AV	:	Average vigor
AZP	:	Azaspiracid shellfish poisoning
CFP	:	Ciguatera fish poisoning
CI	:	Compatibility index
DSP	:	Diarrheic shellfish poisoning
gDNA	:	Genomic deoxyribonucleic acid
HAB	:	Harmful Algal Bloom
IF	:	Immunofluorescence
ITS	:	Internal transcribed spacer
LSU	:	Large subunit
N	:	Nitrogen
NH_3^-	:	Ammonia
NSP	:	Neurotoxic shellfish poisoning
PCR	:	Polymerase Chain Reaction
PO_4	:	Phosphorous
PSP	:	Paralytic shellfish poisoning
PST	:	Paralytic shellfish toxin
PSU	:	Practical salinity unit
qPCR	:	Quantitative real-time polymerase chain reaction
RC	:	Reproductive compatibility
rDNA	:	Ribosomal deoxyribonucleic acid
SiO_2	:	Silicate
STX	:	Saxitoxin
TN	:	Total nitrogen

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CHAPTER 1:

GENERAL INTRODUCTION

1.1. Introduction

Harmful algal blooms (HABs) are extraordinary phenomena of high proliferation of harmful algae that prevail in the coastal zone, where most of the global seafood production, fish resources, and maricultures are situated (Rossi & Fiorillo, 2010; Alvarez et al., 2011; Moore et al., 2015). High density of toxin-producing algal species may produce high concentration of toxins and cause negative impacts to the environment and human health (Rossi & Fiorillo, 2010; Moore et al., 2015). Some marine dinoflagellates in the genus *Alexandrium* tend to produce a group of neurotoxins, collectively named saxitoxins (STXs) (Anderson, 1998), that are responsible for paralytic shellfish poisoning (PSP) in humans (Lim et al., 2012). STXs cause abnormal function of neurons through the voltage-sensitive sodium channels blockage (Catterall et al., 1979) and occasionally cause death (Hall et al., 1990).

Internationally, a wide range of coastal hydrographic regimes is suffering from PSP events. Incidents of PSP related to *Alexandrium* spp. were reported in Thailand (Fukuyo et al., 1988; Kodama et al., 1988), Japan (Hashimoto et al., 2001; Oh et al., 2009), northeastern Brazil (Menezes et al., 2010), and Mediterranean Sea (Vila et al., 2005). In Malaysia, paralytic shellfish poisoning (PSP) event is a severe issue and found frequently associated with *Alexandrium* spp. and *Pyrodinium bahamense* (Usup et al., 2002a; Usup et al., 2002c; Lim et al., 2012). *Alexandrium tamiyavanichii* caused PSP event with three people poisoned after consuming mussels from Sebatu, Strait of Malacca in 1991 (Usup et al., 2002a; Usup et al., 2002c; Lim et al., 2006). In September 2001, six people were hospitalized and one fatally after consuming clams *Polymesoda* spp. contaminated by *A. minutum* for the first time in Tumpat, Kelantan (Usup et al., 2002a; Usup et al., 2002c;

Lim et al., 2004). The bloom of *A. tamiyavanichii* in Kuantan, Pahang was reported for the first time by Mohammad-Noor et al. (2017). Blooms of *P. bahamense* in Sabah recurred almost annually (Usup et al., 2002a; Lim et al., 2004) and resting cysts were found at the surface sediment (Furio et al., 2006). These resting cysts may be viable and play an important role in bloom initiation or decline, and dispersal or depopulation of a particular area (Furio et al., 2012). Information known about the dynamics and life cycle of cysts and factors promoting the bloom formation of harmful species have been well documented in several regions, particularly the temperate regions (Kim et al., 2002; Garces et al., 2004; Richlen et al., 2016), but not been clearly defined in the tropical Asian Pacific region. Therefore, monitoring and understanding toxic species cysts abundance and distribution in relation to its planktonic motile form are essential to provide early warning and prediction (Furio et al., 2012; Usup et al., 2012).

In this study, environmental factor (nutrient sources) triggering the bloom of harmful (paralytic shellfish toxin) PST-producers in Malaysia were investigated. This information will lead to better understanding on the initiation and development of blooms and future socio-economic implication. In brief, the methodology involved collection of plankton and hydrographic data along transect lines. Plankton and sediment samples were collected for quantitative assessments at monthly interval along transects. Vertical profiles of *in situ* salinity and chlorophyll *a* were determined. Water samples were taken for nutrient concentration determined. Vegetative cells and dormant cysts were identified and enumerated microscopically in conjunction with the molecular approach of quantitative real-time PCR (qPCR). Cyst encystment and excystment were studied by cross-mating experiment and observed daily to determine the dormancy period of the cysts.

1.2. Research Objectives

The main aim of this study is to investigate the life cycle of PST-producers in bloom dynamics. The specific objectives are as below:

1. To investigate the dynamics of encystment and excystment of *A. minutum*.
2. To determine the spatial abundance and distribution of *A. tamiyavanichii* in term of planktonic and cyst stages in Kuantan Port, Pahang.
3. To investigate the triggering physico-chemical water parameters of *A. tamiyavanichii*.

1.3. Thesis Structure

This dissertation is compiled into four chapters. **Chapter 1** emphasized on the gap lack of tropical PST-producers in life cycle and bloom dynamics. Research aim and objectives was also stated. A brief background studies about PST-producers in relation with environmental factors and pollutions were made in **Chapter 2**. In addition, qPCR assay as an advanced molecular approach was brief introduced in this chapter. In **Chapter 3**, sexual reproduction of a tropical toxic dinoflagellate, *A. minutum* was investigated. Cross-mating (encystment) and cyst germination (excystment) experiments were developed to investigate the sexual mechanisms of *A. minutum*. The rate of encystment-excysment of tropical cysts was determined in this study. **Chapter 4** investigated the abundance and spatial distribution of a PST-producer, *A. tamiyavanichii* in Kuantan Port, Malaysia. This chapter discussed about the factors influenced the abundance of phytoplanktons, particularly *Alexandrium* spp. Both motile and cyst forms of *A. tamiyavanichii* were detected by using qPCR assay to determine the cell density. The findings of this study were concluded in the last chapter (**Chapter 5**).

CHAPTER 2:

LITERATURE REVIEW

2.1. Harmful Algal Blooms (HABs)

Phytoplankton is a microscopic marine photosynthetic organism, plays important roles in marine food web as a primary food source, and in global carbon cycle as oxygen producer by removing inorganic carbon dioxide. Some of these algae are recorded toxic species and their high proliferation lead to harmful algal bloom (HAB) (Alvarez et al., 2011). HAB is also known as "red tide" which carries the meaning of discolored seawater with red-brown pigments of some algae (Camacho et al., 2007). Usually, the proliferation of algae is greatly influenced by water temperature, dissolved oxygen (DO) concentration, salinity, light intensity and nutrient concentration (Rodriguez et al., 2009). Many negative effects are brought to the coastal areas as well as aquaculture. Therefore, this frequent and harmful phenomenon has led to the concern to monitor the quality and quantity of seafood for human consumption, especially in spring and summer (Rossi & Fiorillo, 2010).

2.2. Shellfish and Fish Poisoning

These algae are categorized into toxic, potentially toxic, and non-toxic, high biomass producers as they cause harm in multiple level (Ignatiades & Gotsis-Skretas, 2010). Among 2,000 living and 2,500 fossil species described, there are more than 70 species involved in HABs and produced biotoxins (Taylor, 2004). The toxins produced are small molecular weight guanidium-containing neurotoxins and polyethers (Taylor, 2004). Consumption of contaminated seafood and direct exposure to HABs might cause shellfish poisoning and fish kill (Taylor, 2004; Camacho et al., 2007). Paralytic shellfish poisoning (PSP) were mainly contributed by genera of *Alexandrium* spp. (Anderson, 1998), *P.*

bahamense (Gacutan et al., 1985), and *Gymnodinium catenatum* (Dolah, 2000), diarrheic shellfish poisoning (DSP) by *Dinophysis* spp. (Lee et al., 1989), amnesic shellfish poisoning (ASP) by *Pseudo-nitzschia* spp. and *Nitzschia* spp. (Bates, 2000; Van Dolah, 2000), neurotoxic shellfish poisoning (NSP) by *Karenia brevis* (Kirkpatrick et al., 2004; Watkins et al., 2008), azaspiracid shellfish poisoning (AZP) by *Protoperidinium crassipes* (Furey et al., 2010) and *Azadinium* spp. (Magdalena et al., 2003), and ciguatera fish poisoning (CFP) by *Gambierdiscus* spp. (Van Dolah, 2000), *Prorocentrum* spp., and *Ostreopsis* sp. (Fukuyo et al., 2011).

2.3. Paralytic Shellfish Toxin (PST)-Producers and Events

The marine dinoflagellates, *Alexandrium* spp. (Anderson, 1998), *Pyrodinium bahamense* (Gacutan et al., 1985), and *G. catenatum* (Hallegraeff, 1993; Van Dolah, 2000) were highly contributed to PSP cases. They produced neurotoxin, STXs and the toxins were accumulated in filter feeders, such as mussels and scallops (Lim et al., 2012). STXs block the voltage-sensitive sodium channels and caused to abnormal function of neurons (Catterall et al., 1979). The symptoms of the PSP were diarrheal, vomiting, nausea, numbness, muscle paralysis, and respiratory difficulty (Yasumoto et al., 1978; Hallegraeff, 1993; Costa et al., 2015). The symptoms of intoxication were shown within 30 minutes or up to hours after consumption (Yasumoto et al., 1978).

PSP cases have brought into public health concerns and economic impacts globally. In Philippines, frequent blooms of *P. bahamense* (135 times) were occurred between 1983 and 2005 (Bajarias et al., 2006). There were 2,162 PSP cases recorded with 123 fatalities at 135 times of blooms (Bajarias et al., 2006). Besides that, blooms of *P. bahamense* were also found in the coastal waters of Florida, USA (Phlips et al., 2006).

In addition, blooms of *G. catenatum* and PSP outbreaks were frequently reported in the Portuguese waters in late 1980s to early 1990s, and recurrent in 2005 (Costa et al.,

2015). Several PSP outbreaks that associated *with G. catenatum* were first found in 1976 in Spain and the production of blue mussels from this region were detected high concentration of STX (Anderson, 1989). Since then, *G. catenatum* was expanded world widely, and suggested this species might have been introduced artificially in Tasmania, Australia by ballast water, where there was no bloom and incident before 1975 (Hallegraeff, 1992; Matsuoka et al., 2006). In 1979, PSP cases in Mexico with 28 persons affected and three fatalities by consumption of contaminated oysters and coquina clams (Cortes-Altamirano & Nunez-Pasten, 1992; Ang, 2012). Besides that, PSP event was also first reported in Japan in 1986 and its resting cysts were also found in the sediment even though in low concentration (Matsuoka et al., 2006).

Furthermore, PSP cases of *A. tamarensis* were reported in the waters of Gulf of Thailand (Fukuyo et al., 1988; Kodama et al., 1988). In early December of 1999, bloom of *A. tamiyavanichii* caused PSP outbreak and high toxin contents was found in the contaminated mussel *Mytilus galloprovincialis*, the Pacific oyster *Crassostrea gigas*, and the ark shell *Scapbarca broughtonii* in Seto Inland Sea, Japan (Hashimoto et al., 2001; Oh et al., 2009). The toxic marine dinoflagellate, *A. tamiyavanichii* were also found in northeastern Brazil (Menezes et al., 2010). Several PSP events that associated with *A. minutum* were also reported in Northern Adriatic Sea, Eastern Aegean, Tyrrhenian Sea, and Catalan-Balearic Basin (Vila et al., 2005). In Arenys de Mar harbour, resting cysts were found and determined as the main recurrence factor of *A. minutum* blooms (Vila et al., 2005).

In September, 2001, *A. minutum* was first known in Malaysian waters after a PSP incident reported in Tumpat, Kelantan (Usup et al., 2002a); six people were hospitalized with one fatality (Lim et al., 2004). Annual blooms of *P. bahamense* were recurred from the west coast of Sabah and high toxin concentration of this species was detected (Usup et al., 2002a). Besides that, in 1991, three persons were poisoned after consumption of

contaminated mussels by *A. tamiyavanichii* from Sebatu, Strait of Malacca (Usup et al., 2002a; Usup et al., 2002c; Lim et al., 2006). While in November, 2013, bloom of *A. tamiyavanichii* was reported for the first time from the east coast of Kuantan, Pahang, with ten person were hospitalised (Mohammad-Noor et al., 2017).

2.4. Life Cycle of *Alexandrium* Species

In the life histories of some toxic species such as *A. minutum* and *A. tamiyavanichii*, were involving asexual and sexual reproductions in the life cycle transformations (Fig. 2.1) (Anderson, 1998). Binary division or asexual division of the cells helps in proliferation of vegetative cells which might cause to HAB (Anderson, 1998). In sexual reproduction, compatible gametes were undergone sexual induction and sometimes they were performed a unique swimming behaviour (Smith & Persson, 2005; Persson et al., 2013). The compatible gametes were conjugated and formed planozygotes and settled down as resting cysts (Figueroa & Bravo, 2005; Figueroa et al., 2007). The resting cysts play an important role in bloom initiation or termination, and dispersal or depopulation of a particular area (Furio et al., 2012). In addition, temporary cysts can be also formed sometimes through sexual or asexual reproductions (Bravo et al., 2010). However, the information about temporary cysts is limited to understand its role in the life cycle and natural population (Bravo et al., 2010). Hence, monitoring and understanding toxic species cysts abundance and distribution in relation to its planktonic motile form are essential as the life cycle transitions highly influence the bloom dynamics (Anderson, 1998).

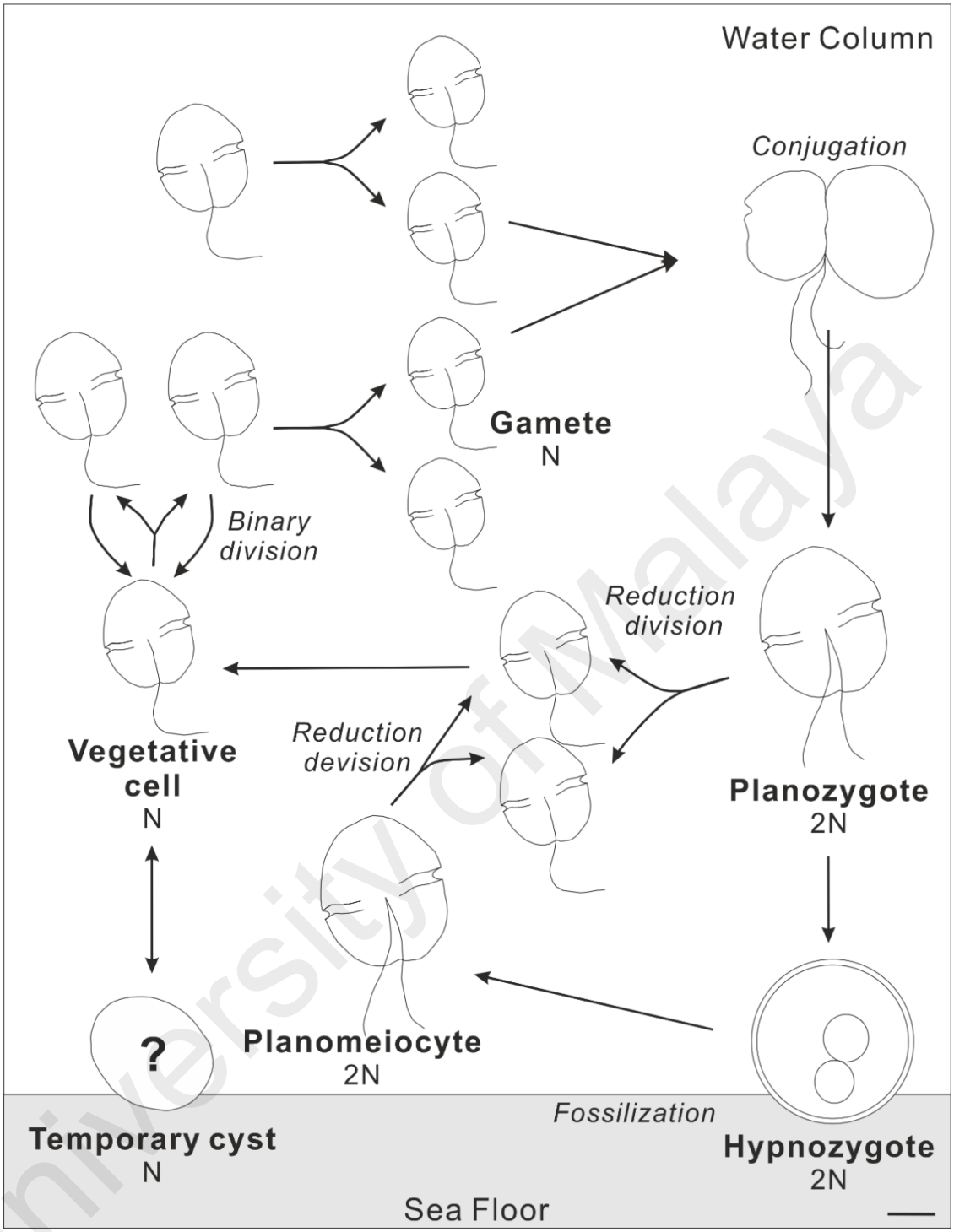

Figure 2.1: Life cycle of *Alexandrium minutum* that involve sexual and asexual reproductions.

The vegetative cells and gametes have similar morphological characteristics. The motile planktonic was identified based on their overall shape and Kofoidian thecal plate tabulation (Fig. 2.2) (Usup et al., 2002a). In contrast, cysts formed in an immotile form and settle down on the sediment or bottom of attachment (Matsuoka & Fukuyo, 2000; Bravo et al., 2010). According to Matsuoka & Fukuyo (2000), cyst was identified based on their cyst body, wall structure and colour, surface ornamentation, and archeophyle (Fig. 2.3). Occasionally, dormancy period of cyst was used as a identify feature of cyst type (Matsuoka & Fukuyo, 2000; Bravo et al., 2010). Resting cysts have dormancy period whereas pellicle cysts have no mandatory dormancy period (Bravo et al., 2010). For temperate *A. minutum* resting cyst, it has dormancy period of approximately 1.5 months (Bravo et al., 2010) whereas *A. tamiyavanichii* has no dormancy period and could germinate within 1 week (Nagai et al., 2011).

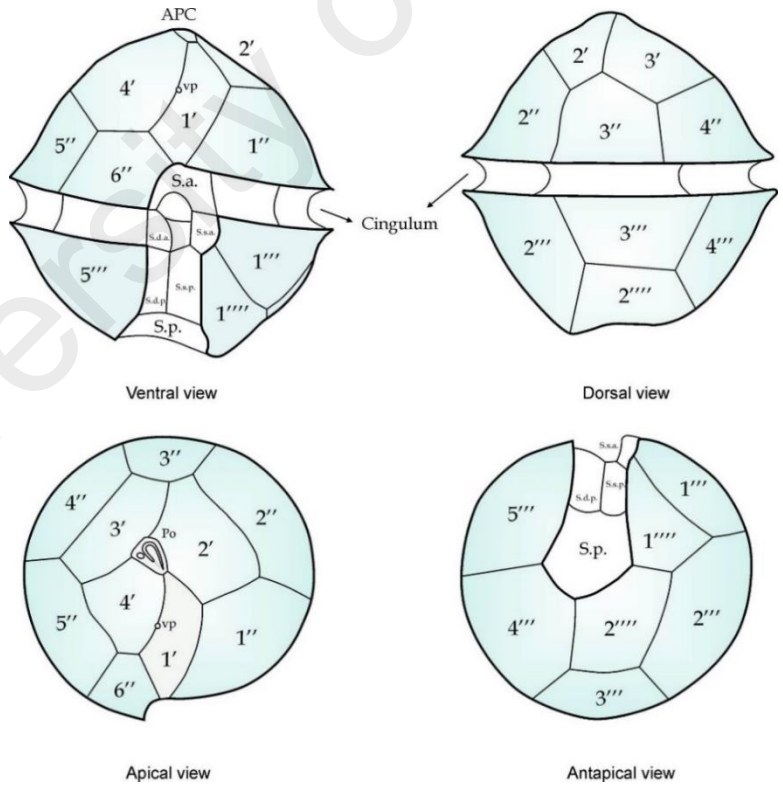

Figure 2.2: Kofoidian thecal plate tabulation of *Alexandrium* species showing the ventral, dorsal, apical and antapical views. Apical plates are represented as ('), precingular plates as (''), postcingular (''') and antapical plates (''''') (Source: Taylor et al., 1995).

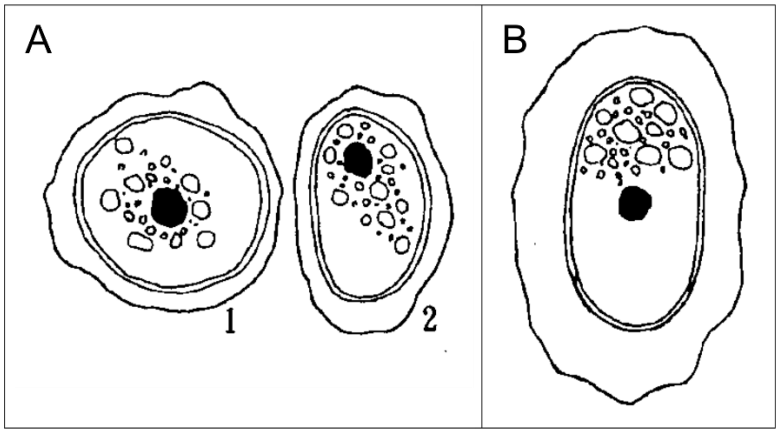

Figure 2.3: Morphology of resting cyst of *Alexandrium minutum and Alexandrium tamiyavanichii.* Ventral view of *Alexandrium minutum*, showing spherical (A1), lateral view, showing bean-like shape (A2), and *Alexandrium tamiyavanichii*, showing ellipsoidal (B) (Source: Matsuoka and Fukuyo, 2000).

The encystment (cyst formation) and excystment (cyst germination) of dinoflagellate are highly influenced by environmental regimes such as nutrient sources, salinity, temperature (Figueroa et al., 2011), water turbulence (Maia-Barbosa & Bozelli, 2006), grazing, competition (Furio et al., 2012), eutrophication and pollution conditions (Maia-Barbosa & Bozelli, 2006; Satta et al., 2014). Therefore, expression of tropical cysts and temperate cysts in encystment-excystment were believed that having differ environmental conditions and acclimation. Besides that, previous studies have shown some of the resting cysts were also regulated by their own endogenous clock (Genovesi et al., 2009; Bravo et al., 2010; Moore et al., 2015). Under optimal environmental conditions, dormant resting cysts are not influenced by these factors to germinate; the resting cysts endogenous clock-controlled germinate even under conditions with limited growth factors, such as limited light intensity and cold temperature (Anderson, 1998; Bravo et al., 2010). Until now, the main factors and mechanisms of encystment-excystment of resting cyst were not well understood (Furio et al., 2012).

2.5. Environmental Factors

The growth physiology and bloom dynamics of dinoflagellate are highly related to both internal (endogenous) and environmental (exogenous) factors (Kremp & Anderson, 2000; Moore et al., 2015). However, the current understanding on environmental factors that regulating the life cycle of the dinoflagellate cells transitions is still remain poor (Figueroa et al., 2011).

The environmental factors that affect the bloom dynamics of *Alexandrium* spp. are salinity (Figueroa et al., 2011; Lim et al., 2011), temperature (Kremp & Anderson, 2000; Figueroa et al., 2011; Moore et al., 2015), concentration of nitrogen and phosphate (Figueroa et al., 2011; Lin et al., 2016), cell density (Figueroa et al., 2011), oxygen conditions (Kremp & Anderson, 2000), and light intensity (Kremp & Anderson, 2000; Moore et al., 2015). These factors induce *Alexandrium* spp. in encystment (Figueroa et al., 2011) and excystment (Moore et al., 2015), might initiate or terminate blooms. Nevertheless, most of the studies are derived from temperate regions, this might likely showed different growth physiology from tropical counterparts (Lim et al., 2011). Temperature and light intensity in tropical rainforest climate are always optimum and unlimited. Therefore, studies on dynamics of tropical *Alexandrium* spp. associated with environmental factors such as salinity, nutrient source and oxygen conditions are interesting to be investigated.

2.6. Anthropogenic Activities and Pollutions

Anthropogenic activities brings negative impacts to human health and environmental indirectly or directly. The incidence of HABs recorded in recent years increased and highly related to the anthropogenic activities (Gowen et al., 2012; Louzao et al., 2015). The examples of human activities are industry, agriculture, shipping, and navigation (Dailianis, 2011). The inputs of anthropogenic nutrients (Davidson et al., 2014) and

environmental pollutants (Dailianis, 2011) have changed the water conditions physically and chemically.

Non-native species was introduced into a new region in the forms of motile cells and resting cyst by ballast tank waters and sediments, respectively (Hallegraeff & Bolch, 1992). Diatom resting spores (e.g. *Chaetoceros* spp.) and dinoflagellate resting cysts (e.g. *Alexandrium* spp.) were carried in the ballast tank sediment (Hallegraeff & Bolch, 1992). The aquatic non-indigenous species were potentially toxic and altered the ecosystem structure and diversity (Burkholder et al., 2007). The viable cysts have long term survival ability may deposited in the sediments for years (Furio et al., 2012; Miyazono et al., 2012). Thus, no matter the invasive species in motile or cyst form, they were high risk to bloom and dominant in the conducive environment (Burkholder et al., 2007).

Bauxite is an alumina ore (Al_2O_3) which is contains mixtures of various minerals such as kaolin and quartz (Donoghue et al., 2014). It is a main source of manufacturing aluminium (Al), sandpaper, polishing powders. The first discovered and mined of bauxite in Malaysia is Johor since 2000 (Noor Hisham Abdullah et al., 2016), and Kuantan Port in April 2014 (Lines, 2015). In early 2014, Indonesia banned exportation of bauxite and India raised ore tariffs, thus increased demanding resource from China and created some economic opportunities. In a short period of time, mining activities such as transporting and stockpiling of bauxite in huge quantities in Kuantan Port had led to environmental issues such as air, river and sea pollutions (Noor Hisham Abdullah et al., 2016). This also brought high risks to the public health and living quality (Donoghue et al., 2014). Due to the extensive and aggressive mining activities in Kuantan that caused community outrage, mining moratorium were imposed from 15 January to 31 December 2016 (Radhi, 2016).

Crude oil and petroleum products content differ chemical compositions (Wang et al., 1999), but mainly contain hydrogen and carbons (hydrocarbons) which are chronically polluting waters (Teal & Howarth, 1984). Oil is an important energy source involved in

industrial development and urbanization (Dailianis, 2011). The spillage of oils and petroleum formed oil slick in the water column and sank on the sediment (Brooks et al., 2015). This oil slick might cause anoxic condition at the bottom of the seafloor and decreased abundance of benthic communities (Teal & Howarth, 1984; Brooks et al., 2015).

2.7. Quantification Real-Time Polymerase Chain Reaction (qPCR) Assay

One of the advanced molecular techniques is qPCR assay. It involves in various important fields to identify, monitor and quantify (Klein, 2002; Antonella & Luca, 2013; Park et al., 2016). The qPCR assay is a high specificity, sensitivity, simplicity and less time-consuming (Maeda et al., 2003; Peirson et al., 2003). The qPCR assay amplifies the targeting genomic DNA by using fluorophore-labeled primers, sequence-specific probe, and general nonspecific DNA binding fluorophores (Bustin, 2005). It combines the applications of nucleic acid amplification and detection in a single step (Bustin, 2005; Bustin et al., 2005). Besides that, it allows to monitor the reaction of amplification products (Klein, 2002), and eliminate the need for gel electrophoresis (Bustin, 2005; Bustin et al., 2005). The qPCR assay able to detect the targeting species despite the concentration of DNA is low (Peirson et al., 2003). During the amplification, fluorescence intensity which is linear correlation to amplification products is also measured for quantification (Klein, 2002; Bustin, 2005). In past, many researches were successfully enumerate vegetative cells (Antonella & Luca, 2013; Kon et al., 2015) and cysts (Kim et al., 2016; Park et al., 2016) of specific species by using qPCR assay. A simple method for removing DNA debris from sediment samples was developed (Kim et al., 2016) to avoid false positive results that caused by the not degraded DNA of dead cells (Antonella & Luca, 2013).

The common fluorophores used are SYBR green-based and Taqman hydrolysis probe-based assay. For SYBR green-based detection, it is a cheaper assay as no probes are required, but it may generate false positive results from primer dimer or non-specific amplified sequence (Maeda et al., 2003). In contrast, Taqman probe-based detection required specific probe in order to generate fluorescent signals and significantly reduced the false positive results (Maeda et al., 2003).

CHAPTER 3:

SEXUAL REPRODUCTION OF A TROPICAL TOXIC DINOFLAGELLATE *ALEXANDRIUM MINUTUM* (DINOPHYCEAE)

3.1. Introduction

A. minutum is one of the toxic species associated with PSP events. It produces voltage-gated sodium channel-blocking neurotoxins, collectively called STX, leading to paresthesia, coordination loss, nausea, vomiting, diarrhea and occasionally death by asphyxiation in the victims due to consumption of contaminated shellfish (Llewellyn, 2006; Kodama, 2010; Burrell et al., 2013).

Blooms of *A. minutum* and PSP events are frequently reported from the Asia Pacific region (Usup & Azanza, 1998; Usup et al., 2002a). Malaysia is no exception, PSP cases were reported since the mid 1970s (Roy, 1977; Lim et al., 2006). In September, 2001, HAB was first encountered in Tumpat, Kelantan which is a semi -enclosed lagoon (Usup et al., 2002a; Lim et al., 2004; Lim et al., 2006). Outbreak of toxic *A. minutum* bloom caused PSP incidents with six people being hospitalized and one casualty after consuming contaminated benthic clam *Polymesoda* spp. (Usup et al., 2002a; Lim et al., 2004). Since then, no recurrence of blooms and *A. minutum* was found to be a common species in the waters. Till end of August, 2015, HAB was occurred and sustained approximately four months and high toxicity was detected in the clams (Law et al., In prep.). Local shellfish collector and traders faced losses of income from this event due to ban of shellfish collection and trading in the area.

The accumulation rate of resting cyst was strongly affected by the environmental regimes (Pospelova et al., 2004; Elshanawanya et al., 2010). In temperate region, the process of encystment-excystment was coincided with seasonal bloom and changes in water temperature (Garces et al., 2004). Encystment occurred with the present of gametes

from compatible mating types (Garces et al., 2004) and was further induced under environmental stressors (e.g. nutrient depletion, low temperature, darkness, dissolved oxygen, salinity) (Blackburn et al., 1989; Garces et al., 2004; Figueroa et al., 2011). Excystment was considered to be regulated by both internal and environmental factors (Figueroa & Bravo, 2005; Genovesi et al., 2009; Moore et al., 2015). Nevertheless, this process could potentially occur any time (even under unfavourable conditions) without specific requirements (e.g. nutrient rich, room temperature, light intensity, dissolved oxygen and salinity) (Blackburn et al., 1989; Figueroa & Bravo, 2005; Moore et al., 2015).

Different from temperate Pacific region, Malaysia has a tropical rainforest climate. Most of the studies were done in temperate or subtropical counterpart, might showed dissimilar physiological adaptation [e.g. *A. minutum*, *A. tamiyavanichii* (Lim et al., 2006) *A. tamarense*, and *A. peruvianum* (Lim & Ogata, 2005), *P. bahamense* var. *compressum* and *Alexandrium* spp. (Furio et al., 2012)]. Biogeographical distribution and cyst assemblages were described in tropical coastal marine waters in order to highlight the importance of cyst mapping in relation to HAB phenomenon (Furio et al., 2012). Better understanding on existing toxic species cysts formation and germination are essential to provide early warning and prediction (Furio et al., 2012; Usup et al., 2012). However, the information about the encystment-excystment and factors promoting the bloom formation of the tropical species, *A. minutum* were limited.

Present study was carried out to investigate the sexual reproduction of *A. minutum* under culture conditions to understand: 1) sexual behaviour and development of cyst formation and germination; 2) factor (nutrient) promoting excystment; and 3) determine the mating types of each culture strains.

3.2. Materials and Methods

3.2.1. Algal Cultures and Natural Cyst Collection

Cells of *A. minutum* were collected from a semi-enclosed lagoon, Sungai Geting, Kelantan, Malaysia (N 6 °13'31.13", E 102 °6'44.79") by 20-µm mesh plankton net hauls. Live samples were brought back to the laboratory for single-cell isolation and culture establishment. Fifteen cultures were established and used in this study (Appendix B). The cultures were maintained in ES-DK medium (Kokinos & Anderson, 1995) at 25 ± 0.5 °C, salinity of 15, pH 7.8, and 12:12 h light:dark photoperiod (Lim et al., 2011).

Sediment samples were collected from the same site, using a flow-through Ekman grab sampler or a sediment corer. Undisturbed surface sediment of 2 cm thickness was taken by pooling, and placed into tightly-sealed dark containers (Matsuoka & Fukuyo, 2000; Miyazono et al., 2012). The sediment samples were placed at room temperature and brought back to the laboratory. Ten grams of sediment were immediately processed by suspending in filtered seawater, and sonicated for 1 min (operated at 10% amplitude) in ice bath using a QSonica Q55 ultrasonic processor (QSonica LLC, CT, USA), followed by fractionation using Nitex mesh sieves to obtain 20–125 µm fractions. The samples were examined under a Leica DM750 compound microscope (Leica, Germany). Viable cysts were isolated by micropipetting for later excystment experiments.

3.2.2. Cross-Mating Experiment and Encystment

Cross-mating experiment was performed in a pairwise combination in a 24-wells sterile tissue culture plate. Clonal cultures were harvested at mid-exponential phase and cross-mating was conducted by mixing two clonal cultures in each well. Monoclonal cultures were self-crossed for homothallism test (Mardones et al., 2014). The plates were incubated at culture conditions as described above.

Samples were examined under an Olympus SZX10 stereo-microscope (Olympus, Tokyo, Japan) daily. Cells at different life stages were further identified by using a Leica DM3000 LED compound research microscope (Leica), and images captured by DFC450 digital camera (Leica). The cell sizes at each life cycle stage were measured, with means and standard deviations presented.

Swimming behavior of cells were recorded on an Olympus SZX10 stereo-microscope with a DP21 digital camera (Olympus). The video recording was taken under 63× magnification. The footages were acquired using VirtualDub (www.virtual dub.org) in a continuous mode, time period of 2 s, resolution of 400 × 300 pixels, and frame rate of 15 frames s^{-1}. Cell movements were tracked by LabTrack (www.bioras.com), with a threshold of average background subtraction, for tracking rapid moving objects.

3.2.3. Reproduction Compatibility and Mating Types

The number of resting cysts formed in each pairwise combination was quantified. A cross-mating matrix was developed for sexual compatibility analysis (Blackburn et al., 2001; Figueroa et al., 2010; Mardones et al., 2014). The mating types of the clonal cultures were categorized by fitting in an incompatibility group system as described in Blackburn et al. (2001). The indices of reproductive success were measured and estimated as described in Blackburn et al. (2001):

Strain reproductive compatibility (RC) = CI × AV

where,

CI, compatibility index, the number of successful crosses resulting in a score of ≥1 divided by the total number of possible crosses, with exception of self-crosses;

AV, average vigour, the average of scores (0–3) for cyst production per cross in successful crosses involving a particular strain.

3.2.4. Cyst Germination Experiment

In laboratory setup, compatible strains of *A. minutum* cultures were selected for the subsequent germination experiment. A total of 100 laboratory-produced cysts were successfully isolated for each treatment (100 cysts for filtered seawater treatment and 100 cysts for ESDK enriched medium. The isolated cysts were observed daily (Bolch et al., 1991; Matsuoka & Fukuyo, 2000). The changes in cell morphology and cellular content of resting cysts to germination of motile planomeiocytes were observed microscopically. Viability of cysts was determined by cyst germination to planomeiocytes, that later yielded the germling cells that were able to produce vegetative progeny (Vahtera et al., 2014).

For wild cyst germination experiment, natural cysts were isolated individually from sediment samples collected from Sungai Geting during two bloom events: November 23, 2015 (n = 30) and March 5, 2016 (n = 100). The isolated cysts were then transferred into 96-well plates containing filtered seawater and enriched medium. The cysts were incubated under the same culture conditions as described above. Cyst germination was observed daily as described earlier. The frequency of successful excystment in both laboratory-produced and natural cysts was determined (Destombe & Cembella, 1990).

3.3. Results

3.3.1. Mating Compatibility and Encystment

Both asexual and sexual reproductions were observed in the mating cultures. Binary fission was observed (Fig. 3.1. E–F) in single-strain (clonal) cultures and mixed-cultures of non-compatible strains, with increase in cell densities through cell division. However, cell density decreased in the culture mixture of compatible strains. The mixed-cultures of compatible strains remained viable for longer duration (6–8 weeks) compared to clonal cultures or mixed cultures of non-compatible strains (3 weeks).

The laboratory-induced sexual life cycle stages of *A. minutum* are depicted in Fig. 3.1. Two singlets with distinct cell sizes were observed in the mixed cultures of compatible strains (Fig. 3.1 A–D).

Mating pairs that attached to each other at the sulcal region were observed after 24 h of mixing. The mating pairs were found fusing with either identical size of cells (isogamous) (Fig. 3.1 G, J, K, L) or with different sizes of singlets (anisogamous) (Fig. 3.1 H−I). Fusion of more than two cells was also observed, but rare. The mating pairs moved in a whirling pattern.

Planozygotes were observed on the second day after mixing, the planozygotes can be distinguished by two longitudinal flagella (Fig. 3.1 M−R). Movement of planozygotes was much slower when compared to the vegetative cells and mating cells. It lost its flagella gradually and the theca shed off, and encysted into a cyst. A process-like ornament planozygote was also found in cultures (Fig. 3.1 O). Occasionally, theca remained without rupture (Fig. 3.1 T−X). It was observed that not all planozygotes encysted. Some were also observed sporadically in both clonal and non-compatible cross-mating cultures. The zygotes appeared transparent, with scattered chlorophyll contents, and lost the longitudinal flagella (Appendix C).

The resting cysts were observed settling down at the bottom of culture plate in day 3−5. The cyst is spherical at the ventral view (Fig. 3.1 U−X), and ellipsoidal or bean-like shape at the lateral view (Fig. 3.1 S−T). The resting cysts formed are with transparent double-walls, and the surface is smooth (Fig. 3.1 S−X). Its content appeared granular with a condensed amber-colored accumulation body. Sometimes, a mucilaginous material was found covering the cysts. The resting cysts in the wild have similar features as those of the laboratory-produced cysts, and they were found mostly aggregated or attached to particles (Fig. 3.2).

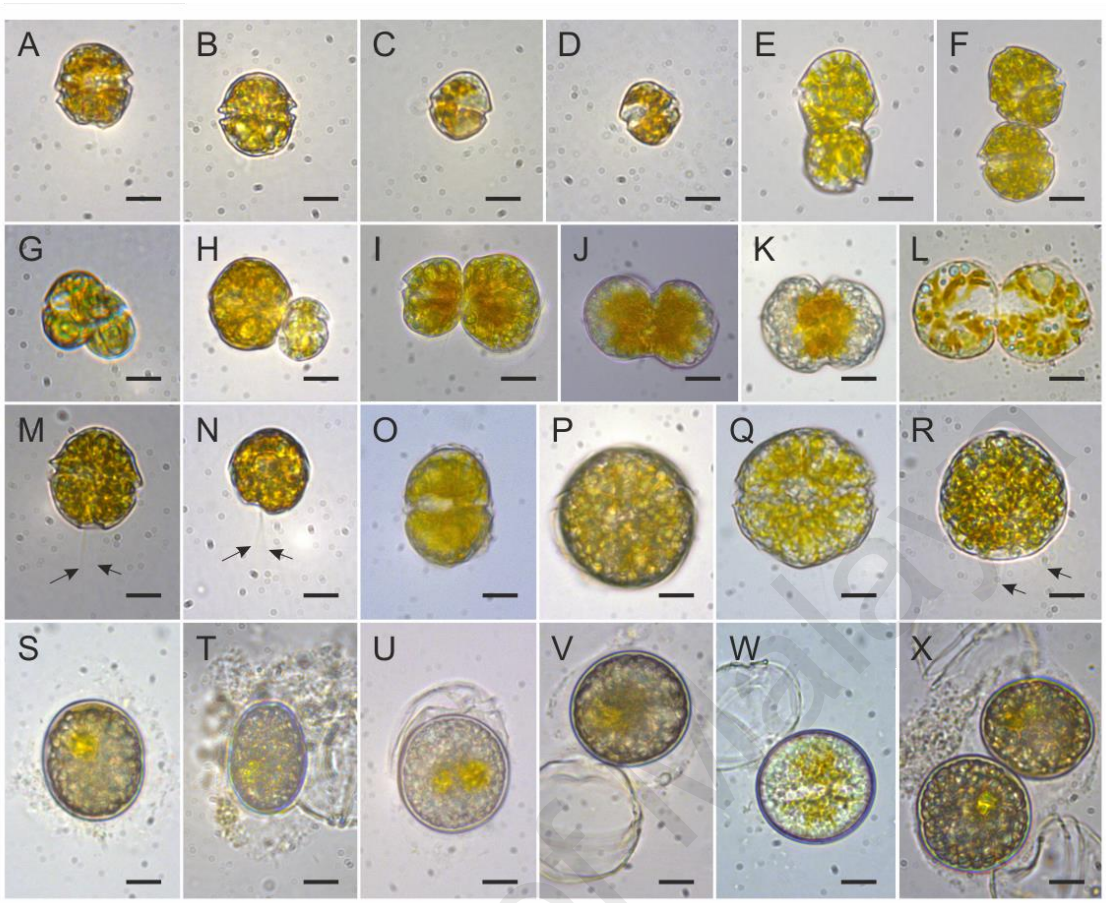

Figure 3.1: Light micrographs of *Alexandrium minutum*. Vegetative cell with a longitudinal flagellum (A, B), gamete (C), gamete with a moving longitudinal flagellum (D), vegetative division (E, F), isogamous (G, J, K, L), anisogamous (H, I), planozygote showing two longitufinal flagella (arrows) (M−N), process-like ornament planozygote (O), planozygote with big cell size (P−R), resting cyst or hypnozygote with red bodies and a mucilaginous material surrounding at lateral view (S, T), resting cyst or hypnozygote with two red bodies and condensed chloroplast (U), resting cyst with red bodies and uncondensed chloroplast (V−X). Scale bars, 10 µm.

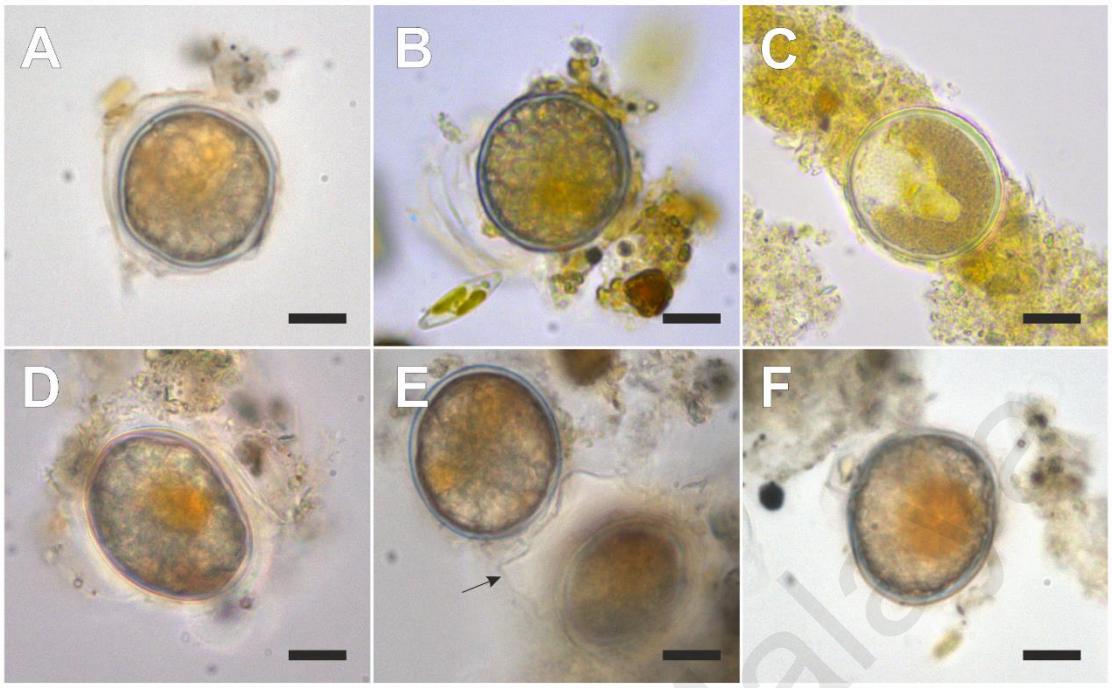

Figure 3.2: Natural cysts of *Alexandrium minutum* found in Sungai Geting, Malaysia. Resting cyst with unshed theca (A). Newly-formed resting cyst with sheded theca (B). Resting cysts with red bodies and condensed chloroplasts (C). Resting cysts with uncondensed chloroplasts (D−F), arrow shows a mucilaginous material surrounding the resting cyst. Scale bars, 10 µm.

Sexual induction was detected immediately on the day of culture mixing. Sexual induction behavior was observed in the compatible singlets; where the singlets swam/danced and accumulated in "spots" with circulation motion (Fig. 3.3 A–B), and sometimes changed in direction suddenly without interference. The movement of the dancing singlets is faster than of the vegetative cells. Giant spot of accumulated dancing cells were found in the water column (Fig. 3.3 A), but scattered small spots of accumulated dancing cells usually observed in the bottom layer of the culture wells (Fig. 3.3 B). Unlike compatible cells, the motility patterns of non-compatible cells were random and disorder (Fig. 3.3 C), which is similar to those in clonal cultures (Fig. 3.3 D); where they moved forward with a self-rotating pattern at different directions and changed their ways with or without interference.

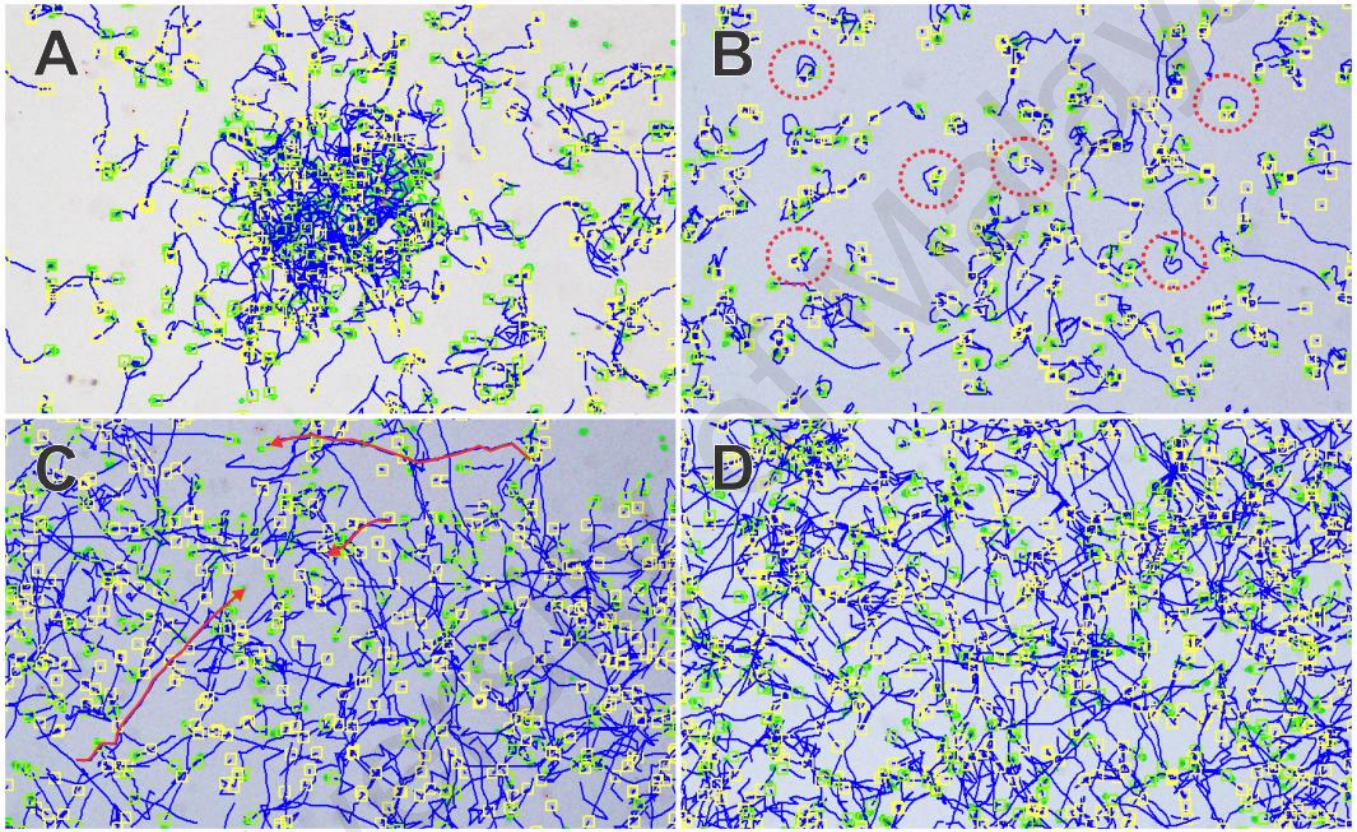

Figure 3.3: Trajectories of *Alexandrium minutum* cells in compatible mating cultures (A−B), non-compatible mating cultures (C), and single clonal culture (D). Yellow and green squares are the beginning and final configuration of cells, blue lines show the paths of each tracking point across frames. The footages are with continuous mode, time period of 2 s, and frame rate of 15 frames s^{-1}.

A. minutum sizes varied at different life-history stages (Fig. 3.4). Vegetative cells and gametes are observed in two size ranges; big cells are in the range of 26.5 ± 1.8 μm long, 24.4 ± 2.2 μm wide (*n* = 21), while smaller cells are 21.4 ± 2.4 μm long and 19.0 ± 2.3 μm wide (*n* = 20). However, sizes of gametes and vegetative cells were not precluded from being smaller and bigger. The obvious morphological distinction of gametes was the slightly protruding narrow epitheca and lesser chlorophyll contents (Fig. 3.1 C–D). Planozygotes was larger: 32.9 ± 3.5 μm in length, 30.8 ± 3.5 μm in width (*n* = 24), likely due to the fusion of two basal bodies. The sizes of planozygotes were slightly smaller than the laboratory-produced resting cyst (33.8 ± 3.7 μm in diameter; *n* = 28), even though it was observed that planozygotes can be sometimes larger than the laboratory-produced resting cysts. Some planozygotes were also found smaller in size, which had similar cell size and morphology to vegetative cells, but can be distinguished by having biflagella (Fig. 3.1 M–N).

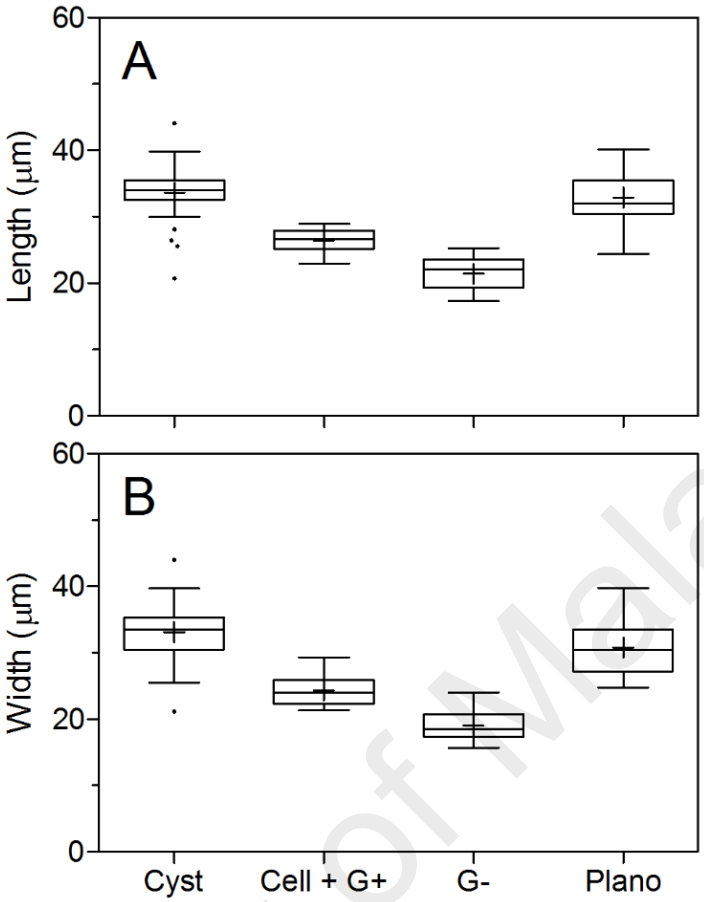

Figure 3.4: Cell dimensions of *Alexandrium minutum* different life-history stages. Cyst, resting cysts or hypnozygotes. Cells/ G+, vegetative cells or big-sized gametes. G–, small-sized gametes. Plano, planozygotes and planomeiocytes.

3.3.2. Mating Compatibility of *Alexandrium minutum* Cultures

A matrix of cross-mating compatibility of cultures established in this study is presented in Table 3.1. The intercross experiments showed 50.5% (53 of 105 combinations, $n = 2$) of positive mating compatibility and resting cysts formation.

The cross-mating results revealed a multiple mating systems in *A. minutum* from Sungai Geting Lagoon. Strains AmTm01 and AmTm05 were likely the same mating type because of similar mating compatibility, while AmTm09 was a mating type that could mate with most strains studied (Table 3.1). By fitting an incompatibility group system, the crossing matrix was categorized into four, five, six or seven mating types (Table 3.1).

Gamete fusion and small amount of transparent planozygotes were observed in individual strains AmTm06, AmTm08, AmTm09, AmTm13, AmTm14, and AmTm15, but no cyst was found in the self-crossing experiments. Cyst formation was only observed in cross-mating cultures, indicating that the species is heterothallic.

Table 3.1: Cross-mating of *Alexandrium minutum* strains in a pairwise combination. Scoring criteria for encystment: 0, unsuccessful crosses; 1, 1–99 cysts ml^{-1}; 2, ≥100 cysts ml^{-1}. Highlighted and lined boxes show the categorization of mating types according to incompatibility group system.

Strains	AmTm 01	AmTm 05	AmTm 10	AmTm 15	AmTm 04	AmTm 13	AmTm 11	AmTm 02	AmTm 03	AmTm 06	AmTm 08	AmTm 12	AmTm 14	AmTm 07	AmTm 09
AmTm01	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1
AmTm05	0	0	0	0	0	0	2	1	1	1	1	2	2	2	1
AmTm10	0	0	0	0	0	0	2	0	2	2	1	2	2	2	1
AmTm15	0	0	0	0	0	0	1	1	1	1	0	1	1	1	1
AmTm04	0	0	0	0	0	0	0	1	1	1	1	0	1	2	1
AmTm13	0	0	0	0	0	0	0	0	0	0	1	1	1	2	1
AmTm11	1	2	2	1	0	0	0	0	0	0	0	0	0	0	2
AmTm02	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0
AmTm03	1	1	2	1	1	0	0	0	0	0	0	0	0	0	0
AmTm06	1	1	2	1	1	0	0	0	0	0	0	0	0	1	1
AmTm08	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1
AmTm12	1	2	2	1	0	1	0	0	0	0	0	0	0	0	1
AmTm14	1	2	2	1	1	1	0	0	0	0	0	0	0	0	1
AmTm07	1	2	2	1	2	2	0	0	0	1	0	0	0	0	1
AmTm09	1	1	1	1	1	1	2	0	0	1	1	1	1	1	0

The strain AmTm05 alone showed 64% (9 of 14 combinations) successful cross-mating, but each positive cross showed different mating compatibility and efficiency in cyst formation (Fig. 3.5). Some crosses (e.g. [AmTm05 × AmTm14]) produced >100 cysts in a week, while some crosses (e.g. [AmTm05 × AmTm07]) produced low number of cysts (<20 cysts formed in a week) (Fig. 3.5, Table 3.2).

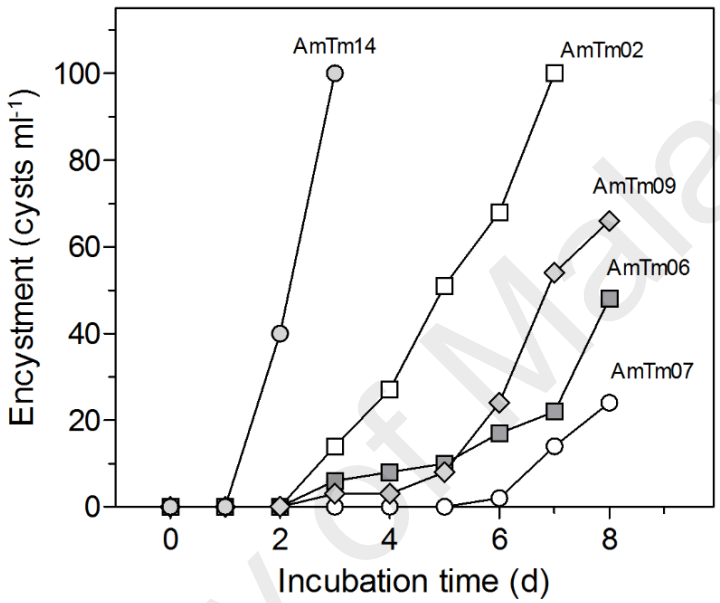

Figure 3.5: The daily encystment (cysts ml^{-1}) of *Alexandrium minutum* AmTm05 with other cross-mating strains (AmTm02, 06, 07, 09, and 14).

All the strains showed successful crosses ($CI_s > 0$) but low cyst production ($AV_s \leq 2$). Reproductive compatibility index (RC) of all strains ranged from 0.29 – 1.00, with the strain AmTm02 the lowest (Table 3.2).

Table 3.2: Reproductive compatibility of each *Alexandrium minutum* strain measured by compatibility index (CI), average vigor (AV) and reproductive compatibility (RC).

Strain	CI	AV	RC
AmTm01	0.64	1.00	0.64
AmTm02	0.29	1.00	0.29
AmTm03	0.36	1.20	0.43
AmTm04	0.50	1.14	0.57
AmTm05	0.64	1.44	0.93
AmTm06	0.43	1.00	0.43
AmTm07	0.50	1.14	0.57
AmTm08	0.43	1.33	0.57
AmTm09	0.86	1.08	0.93
AmTm10	0.57	1.75	1.00
AmTm11	0.36	1.60	0.57
AmTm12	0.50	1.29	0.64
AmTm13	0.36	1.20	0.43
AmTm14	0.57	1.50	0.86
AmTm15	0.57	1.00	0.57

3.3.3. Cyst Dormancy and Germination

The dormancy period of *A. minutum* cysts from Sungai Geting Lagoon was relatively short, estimated to be less than a week. In laboratory-produced cysts, excystment was first observed 3–5 days after encystment, either in enriched seawater medium or filtered seawater (Fig. 3.6 A–B), while natural cysts collected from the wild had shorter dormancy of 2 days.

In the laboratory-produced cysts, the higher number of excystments was observed in enriched medium (cumulative excystment rate, 40 – 60 %) compared to filtered seawater (cumulative excystment rate, 10 – 20 %) (*t*-test, $P<0.0001$). The success rate of excystment differed among different crosses (Fig. 3.6 A–B). For example, cysts obtained from the cross [AmTm10 × AmTm07] exhibited higher cumulative excystment rates

(62.9% in enriched medium, 20% in filtered seawater) compared to the cross [AmTm10 × AmTm11] (34.3% and 11.4% in enriched medium and filtered seawater, respectively).

Natural cysts of *A. minutum* collected from different bloom events exhibited different excystment rates (Fig. 3.6 C–D). Cysts collected from November 2015 bloom exhibited lower success rates (27 – 33%) as compared to the cysts collected from March 2016 bloom (70 – 77%). Incubation under enriched medium did not significantly influence germination for 2015 bloom-collected cysts (*t*-test, $P>0.05$), but showed slight difference for 2016 bloom-collected cysts (*t*-test, $P = 0.006$). Generally, the natural cysts had higher success rates under incubation with filtered seawaters (Fig. 3.6 C–D), indicating that excystment of natural cysts was not affected by nutrient availability.

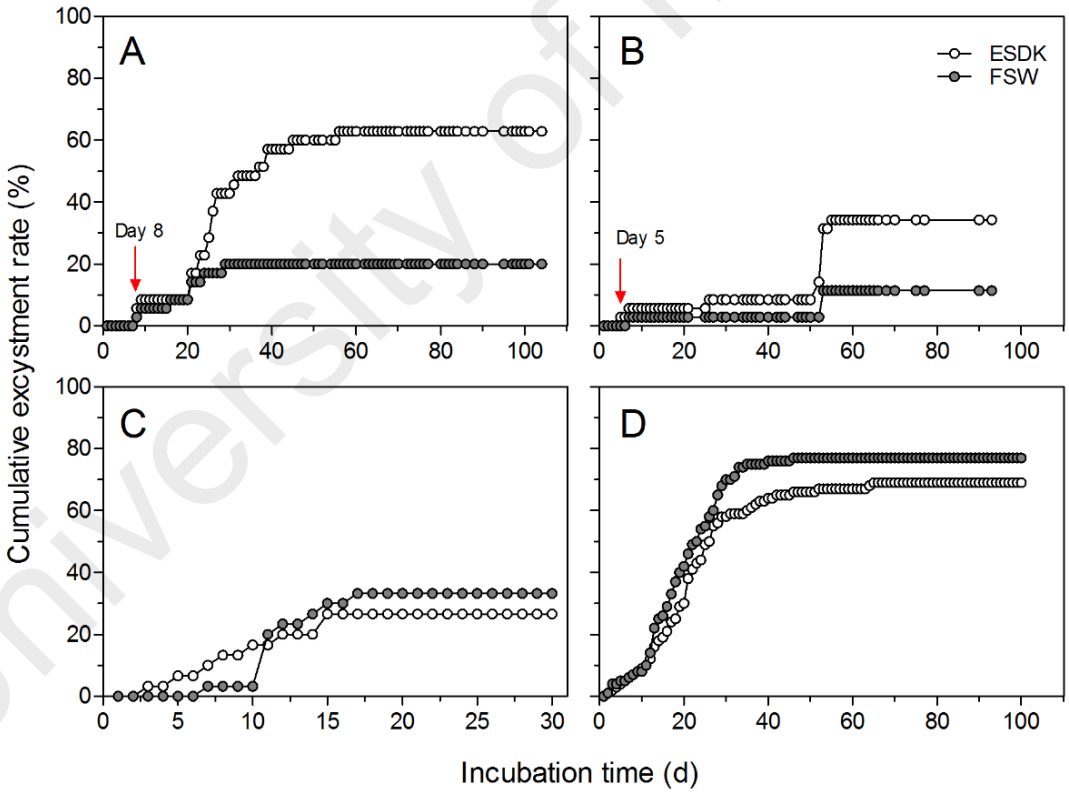

Figure 3.6: Cumulative percentage excystment of *Alexandrium minutum* over time in the ES-DK enriched medium (open circles) and filtered seawater (grey circles). (A–B) Laboratory-produced cysts from cross-mating strains of [AmTm10 × AmTm07] (A), and [AmTm10 × AmTm11] (B). (C–D) Natural cysts collected from November 2015 bloom (C) and March 2016 bloom events (D).

When the cyst germinated, planomeiocyte with two longitudinal flagella was observed; its morphology was similar to planozygote. The state of planomeoicyte remained for a day (sometimes less than a day), and followed by emergence of two or four germling cells. Among the 200 natural cysts isolated, 89 cysts germinated into two germling cells and 57 cysts germinated into four germling cells. In enriched medium, the germling cells were sustained for approximately two months without adding additional nutrients.

3.4. Discussion

Like many dinoflagellates, the life cycle of *A. minutum* comprised two types of reproduction, i.e. asexual and sexual reproductions (Anderson, 1998, Probert et al., 2002). These reproduction systems were highly affecting their growth dynamics in the environment. In asexual reproduction, binary fission was performed to increase the cell population; this rapid increment of cell population may cause abrupt proliferation of cells, but ceased if sexual reproduction was induced and formed resting cysts (Anderson, 1998), this process was interpreted to cause bloom termination (Kremp & Anderson, 2000).

Despite the numerous studies on dinoflagellate sexuality and cyst formation, the processes of gamete formation have been inadequately described, partly owing to the fact that many species are hologamous, of which the vegetative cells and gametes are morphologically indistinguishable (Kremp & Anderson, 2004). However, several studies have demonstrated that these two life-history forms (vegetative cells vs. gametes) of *Alexandrium* cells, like many dinoflagellates species, exhibit distinctive swimming patterns and behaviors (e.g., Probert et al., 2002; Persson et al., 2013). These features thus were used to examine the life history of dinoflagellates in laboratory setting (Smith and Persson, 2005; Persson et al., 2013). The distinctive motion characteristic of *Alexandrium* gametes allows the investigations of the processes involving sexual induction of this bloom-forming species. The movements of gametes observed in this study (Fig. 3.3) were

in agreement with the previously described dinoflagellate mating behaviors; displaying the "swarming" or "dancing" patterns, circular movements and frequent directional changes without interference as elucidated in Smith and Persson (2005) and Persson et al. (2013). The swimming behaviors were postulated to increase the cell-to-cell contacts for mating purpose (Persson & Smith, 2013, Persson et al., 2013). In the wild, several studies have demonstrated that the mating cells were found in the thin layers of pycnocline, with giant spot of accumulated dancing cells observed (Persson et al., 2008; Persson et al., 2013). In our laboratory observations, giant spot of accumulated dancing cells were detected in the water column (Fig. 3.3 A), while small spots of accumulated dancing cells were usually found in the bottom layer of culture plate (Fig. 3.3 B). The swimming pattern and accumulation behavior during sexual induction might explain the formation of patches during blooms in the field (Persson & Smith, 2013). Gamete expression and sexual induction were detected in all the cross-mating experiments (disregarding successful mating), indicating that all the strains were readily searching for the compatible complementary singlets with which to mate. However, this action did not warrant a successful encystment, as only compatible strains will produce cysts.

The fusion of mating pairs was believed only contribute by gametes, likely from the vegetative cells that had undergone some physically and metabolically transformation (Persson & Smith, 2013). Some studies postulated that gametes might produce pheromone-like chemical compounds such as protoplast release-inducing protein (PR-IP) and agglutinin that promote gamete-gamete recognition (Sawayama et al., 1993a; Sawayama et al., 1993b; Kremp & Anderson, 2004; Kobiyama et al., 2007). Kremp and Anderson (2004), on the other hand, reported that cell wall of gametes contained specific chemical structures that helped in gametes fusion and conjugation. Sexual induction of *A. minutum* in this study was observed within 24 h after inoculation of compatible strains in a laboratory setting. The change of behaviors of cells was somehow immediate, and

relatively shorter than those observed in *Lingulodinium polyedrum* (Figueroa & Bravo, 2005), *A. tamutum* and the temperate *A. minutum* (Figueroa et al., 2007), which was 2−4 days after inoculation. Given the shorter time needed for sexual induction and cyst formation in the tropical *A. minutum*, it is crucial to investigate the factors triggering sexual induction in the wild and laboratory setting to better understand the bloom dynamics of this tropical species.

The size ranges of tropical *A. minutum* and temperate *A. minutum* differed, where the tropical *A. minutum* vegetative cells were larger than gametes, while temperate *A. minutum* vegetative cells (20.1 ± 2.4 μm length, 17.8 ± 2.1 μm width, n = 65) were found smaller than gametes (21.4 ± 2.0 μm length, 18.8 ± 1.9 μm width, n = 13) (Figueroa et al., 2007). In addition, the tropical *A. minutum* planozygotes and resting cysts were larger than the temperate planozygotes (22.8 ± 1.4 μm length, 20.2 ± 1.3 μm width, n = 6) and the resting cyst (30.0 ± 2.9 μm diameter, n = 135) (Figueroa et al., 2007). The presence of biflagella in planozygotes was used to distinguish planozygote from the vegetative cells that had similar cell sizes. Bigger planozygotes might be due to fusion of two basal bodies of compatible vegetative cells.

The features such as angle of cells attachment and position of longitudinal flagella were used to distinguish the mating pairs from dividing cells (Persson et al., 2013). The movement of planozygotes was slow, even though with a biflagellate structure but it did not contribute to an expected fast motion. Apart from the flagella arrangement, swimming speed decreased as cell size increased (Lewis et al., 2006). The slow movement of planozygotes decreased the cell-to-cell contact (Persson & Smith, 2013, Persson et al., 2013) and some eventually encysted into cysts (Figueroa & Bravo, 2005, Figueroa et al., 2007); while some cysts formed without rupturing their thecae (Gribble et al., 2009).

The cyst wall of dinoflagellate was used to differentiate pellicle cysts and resting cysts (Bravo et al., 2010). Morphologically, resting cysts were defined as cysts with double-

layered cyst walls whereas pellicle cysts were with single-layered cyst wall (Bravo et al., 2010). The resting cysts produced in the laboratory and of those found in the wild had similar morphological features as depicted in Bravo et al. (2010). Both pellicle cysts and resting cysts were observed morphologically in the present study. In addition, Matsuoka and Fukuyo (2000) showed that most *Alexandrium* resting cysts have similar morphology: spherical cyst body with single or usually double layer-transparent cyst walls, and was covered by a transparent mucilaginous material, this was observed in the present study for both laboratory-produced cysts and wild cysts. The sticky mucus layer of *Alexandrium* cysts was speculated to aid sinking by attaching to heavier particles, to prevent grazing or assisted in greater dispersion (Smith et al., 2009). However, chemical composition and production of the cyst mucilage is not known.

A. minutum culture strains in this study exhibited higher number of successful crosses but relatively low cyst production, the temperate Chilean *A. catenella* also showed higher successful crosses (24 of 45 combinations) and low cysts production, but cyst formation occurred 26−45 days after inoculation (Mardones et al., 2014). In contrast, formation of *A. minutum* cysts in this study was relatively fast, 3−5 days after incubation. The reproductive compatibility of the tropical *A. minutum* was slightly higher when compared to *G. catenatum* (Blackburn et al., 2001; Figueroa et al., 2010). *A. minutum* in this study demonstrated multiple heterothallic mating system, this was commonly observed in the mating-types of *Alexandrium* spp. [*A. tamerense* (= *A. excavatum*) (Destombe and Cembella, 1990); *A. tamutum, A. minutum* (Figueroa et al., 2007), *A. fundyense* (Persson et al., 2013) and *A. catenella* (Mardones et al., 2014)].

The encystment and excystment processes that the tropical *A. minutum* cells passed through, including the mandatory dormancy period, were relatively short, estimated within a period of 5 – 10 days. This is in agreement with the findings of some studies of temperate *Alexandrium* species: *A. catenella* on the Catalan coast (Figueroa & Bravo,

2005); *A. tamarense*/*A. catenella* in Thau Lagoon (Genovesi et al., 2009). A slightly longer mandatory dormancy period of 15 – 18 days has been observed in *A. catenella* on the west coast of South Africa (Joyce & Pitcher, 2006). The relatively shorter mandatory dormancy period of the tropical *Alexandrium* clearly showed that vernalization is not required for excystments. While some temperate *Alexandrium* species possessed vernalization period for excystments (Montresor & Marino, 1996), some do not require vernalization period (Joyce and Pitcher, 2006), but this overwintering strategy has served to synchronize excystments (Genoversi et al., 2009). Excystment of benthic resting cysts was always linked to bloom initiation (Kremp & Anderson, 2000). If vernalization is not the prerequisite for the tropical *Alexandrium* cysts to undergo excystment, it is postulated that excystments of this tropical *Alexandrium* could occur throughout the entire year without circannual rhythm. Conversely, natural cysts of a tropical species *P. bahamense* has a mandatory dormancy period of ~90 days under laboratory setting (G. Usup, per. comm.). It is believed that cyst germination and dormancy period is regulated by a cyclic endogenous clock (Matsuoka & Fukuyo, 2000; Itakura & Yamaguchi, 2001; Matrai et al., 2005; Genovesi et al., 2009; Moore et al., 2015). Dormancy periods of temperate *A. minutum* cysts with <3 months (Garces et al., 2004) and 1.5 months (Bravo et al., 2010) had been reported. This further supported the species-dependent dormancy period in PST-producing dinoflagellates.

While environmental conditions are believed to trigger excystments and cyst germination, many studies have shown strong dependency on species, biogeographical and ecotypic adaptations (Canon, 1993; Itakura & Yamaguchi, 2001; Kim et al., 2002; Anderson et al., 2005a; Anderson et al., 2005b; Fauchot et al., 2005; Joyce & Pitcher, 2006; Fauchot et al., 2008; Genovesi et al., 2009). The experimental results in this study indicated that both wild and laboratory-produced resting cysts were viable and capable of germinating under incubation in either enriched seawater medium or filtered seawater.

This finding revealed that cyst germination is not affected by nutrient availability as has been demonstrated in other studies (Binder and Anderson, 1987; Genovesi et al., 2009).

3.5. Conclusion

In conclusion, the rapid encystment-excystment processes of the tropical *A. minutum* observed in this study, and the high success rates of excystments and shorter cyst dormancy period are believed to play a crucial role in the bloom dynamics of this species in tropical coastal region.

CHAPTER 4:

ABUNDANCE AND SPATIAL DISTRIBUTION OF A PARALYTIC SHELLFISH TOXIN-PRODUCER, *ALEXANDRIUM TAMIYAVANICHII* IN KUANTAN PORT, PAHANG, MALAYSIA (DINOPHYCEAE)

4.1. Introduction

The well-known PST-producer, *A. tamiyavanichii* is one of the species that involved in HABs, which can cause tremendous impact to socio-economy and human health. Several reported PSP cases in Malaysia were found associated with *Alexandrium* spp. (Usup et al., 2002b). In 1991, *A. tamiyavanichii* has caused PSP event with three victims poisoned after consuming mussels from Sebatu, the Straits of Malacca (Usup et al., 2002a; Usup et al., 2002c; Lim et al., 2006). On the other hand, an incident of PSP has been reported for the first time in Kuantan Port, Pahang in November 2013, where ten people were hospitalized after consuming shellfish contaminated by PSTs from *A. tamiyavanichii* (Mohammad-Noor et al., 2017).

Blooms of *Alexandrium* species were believed to strongly influence by nutrient sources and water salinity (Figueroa et al., 2011). Nutrient such as low phosphate/nitrogen ratio have played an important role in inducing or shifting sexuality and cyst formation (Figueroa et al., 2011). While for the salinity, it affected the growth rates, biochemical components, and cellular pigment concentrations; Leong et al. (2006) reported that outbreak of a bloom was observed at low salinity. Under the unfavourable conditions such as nutrient depletion and low salinity, *Alexandrium* species formed dormant resting cysts (Anderson, 1989). It is common that resting cyst was found at the surface sediment in the blooming areas (Furio et al., 2006). Cysts might be viable (Furio et al., 2012) as it plays an important role in dispersal and depopulation (Furio et al., 2012). Thus, spatial abundance and distribution of dormant cysts and its planktonic motile form

are crucial to be investigated by both microscopic and molecular enumeration for the better understanding of bloom development.

Presence of the toxic species, particularly *A. tamiyavanichii* in Kuantan Port has initiated public health concerns. In this study, an investigation of the environmental parameters of nutrients, salinity and temperature that may trigger the bloom of this PST-producer were conducted, and the relationship of these abiotic stressors and the phytoplankton assemblages in the water was examined.

4.2. Materials and Methods

4.2.1. Study Site

The study area was conducted in Kuantan Port, Pahang, which is a multi-cargo port facing the South China Sea. Four sampling sites were sampled in this port with water depth of approximately 13 m (Fig. 4.1), which placed in the inner (KP 3 and 4) and the closed-outer (KP 1 and 2) part of the deep sea Kuantan Port. Monthly samplings were undertaken fourteen times by collecting plankton, sediment, and hydrographic samples from April, 2015 to May, 2016. The environments were polluted by the bauxite activities (Fig. 4.7 A−B) and oil spill can be seen in the surface of the waters (Fig. 4.7 C−D).

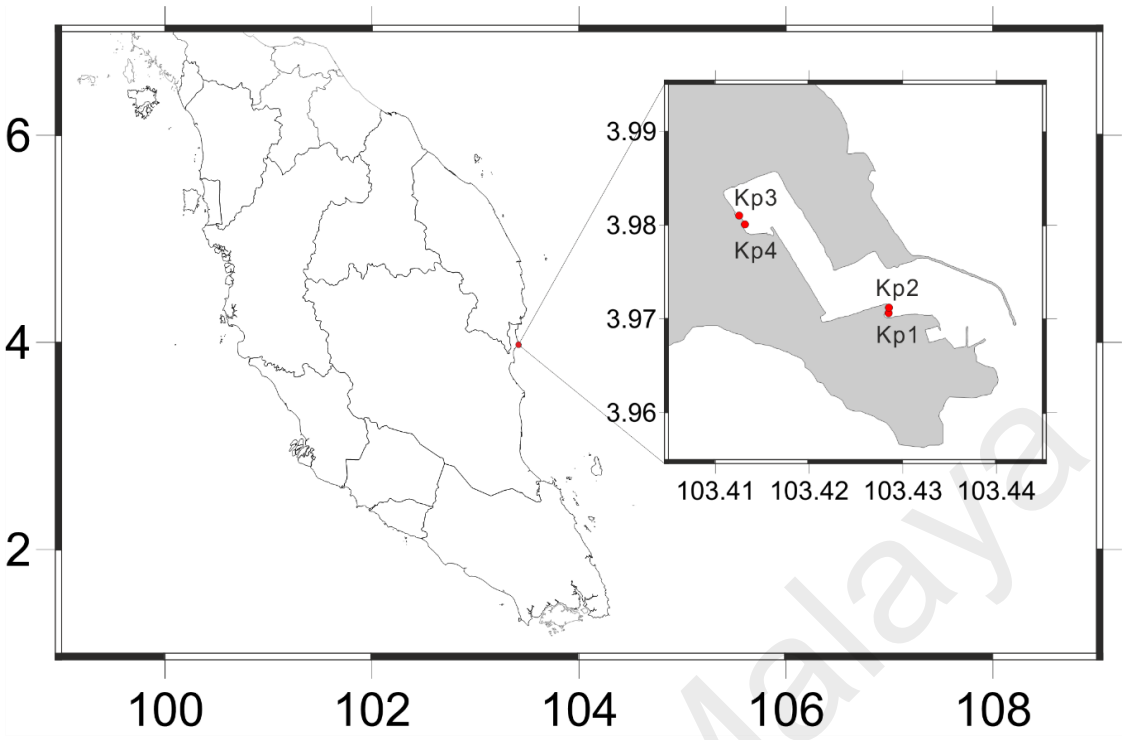

Figure 4.1: Map of Kuantan Port, Pahang, showing the four sampling sites in the close-outer part (KP1 and KP2) and inner port (KP3 and KP4).

4.2.2. Algal Cultures

Single cell isolation was performed by micropipetting technique (Hoshaw & Rosowski, 1973). A fine capillary pipette was prepared by taking aseptically a sterile Pasteur pipette and a Latex tubing was used to attach with the wide end of the capillary pipette for cell isolation. The isolated cell was transferred into a sterile 96-wells tissue-culture plate containing 0.2 μm-filtered seawater. The isolates were transferred into culturing tubes containing ES-DK medium (Kokinos & Anderson, 1995) after it reached >100 cells. Cultures was maintained at 25 ± 0.5℃, pH 7.8, and 12:12 h light:dark photoperiod. The medium was prepared and adjusted to desired salinity of 30 PSU by adding distilled water in natural filtered seawater (Lim et al., 2006).

4.2.3. Species Identification

Water samples collected from the field were preserved in acidic Lugol's solution. Plankton cells were then identified under a Leica compound microscope (DM3000 LED, Leica, Germany) with 20−100× magnifications. Aliquot of 1 mL samples were used for phytoplankton enumeration at 20× magnification using a Sedgewick-Rafter counting chamber.

Alexandrium species were further identified by thecal plate tabulation (Balech, 1995). Cells were stained with Imamura-Fukuyo (IF) staining solution (Yuki & Fukuyo, 1992) after treated with freshly prepared 10% hypochroric acid solution. The morphological characteristics of cell shapes were determined, and cell dimensions measured by using an image processing program, Image J (*ver.* 1.50d).

For molecular analysis, about 100 mL of clonal cultures were harvested by using sieving method during mid-exponential phase. The cells were further concentrated by centrifugation (580 ×*g*, 10 min) to precipitate cell pellets. Genomic DNA (gDNA) was extracted by using Mo-Bio PowerPlant DNA isolation kit (Mo-Bio, USA) by following the manufacturer's instruction.

Gene amplification was carried out by using a peqSTAR thermocycler (peqSTAR 96× Universal Gradient, peqLab, Germany). A 25-µL PCR master mixture included 1× *Taq* buffer, 0.2 mM dNTPs, 2 mM $MgCL_2$, and 1 mM each primer were first prepared before adding 2 µL gDNA and 0.2 µL *Taq* polymerase (Invitrogen, Life Technologies, USA). The primer pair, ITS1F (5'-TCGTAACAAGGTTTCCGTAGGTG-3') and ITS1R (5'-ATATGCTTAAGTTCAGCGGG-3') (Leaw et al., 2001) were used to amplify the internal transcribed spacer (ITS) region of the ribosomal RNA gene (rDNA), wherease the primer pair, D1R (5'-ACCCGCTGAATTTAAGCATA-3') and D3Ca (5'-ACGAACGATTTGCACGTCAG-3') (Scholin et al., 1994) were used to amplify the large subunit (LSU) rDNA. PCR conditions were as follow: initial denaturing at 94 ℃ for

4 min, 35 cycles of primer annealing at 50 ℃ for 45 s, and primer extension at 72 ℃ for 1 min, and followed by substances clearance at 72 ℃ for 7 min before kept at 4 ℃. PCR products were further purified by QIAquick PCR purification kit (Qiagen, Hilden, Germany) and later sequenced by the First Base private sequencing laboratory (Selangor, Malaysia) using an ABI 3770XL automated sequencer.

4.2.4. Phytoplankton Spatial Distribution

Aliquot of 1 mL of Lugol-preserved samples that collected by using Van Dorn sampler were used for phytoplankton enumeration at 20× magnification using a Sedgewick-Rafter counting chamber under compound microscope (DM750, Leica, Germany). The total number of diatoms and dinoflagellates enumerated were then used for the determination of Diatom:Dinoflagellate (D:D) ratio and relative abundance of each dinoflagellate species.

For chlorophyll *a* determination, water samples were processed and extraction in the dark or dim condition. Phytoplankton samples were harvested by filtration with glass-fibre filters and followed by acetone (90%) extraction for 18 h before analyses. The absorbance of spectrophotometer was read at wavelengths of 750, 664, 647 and 630 nm. Total chlorophyll a concentration was calculated by using the following equation (Parsons et al., 1984):

$$[Chl\ a]\ (\mu g\ L^{-1}) = \frac{[\ 11.64\ (Abs\ 663) - 2.16\ (Abs\ 645) + 0.10\ (Abs\ 630)\ ]\ (E)(F)}{V(L)}$$

where, F is the dilution factor;

E is the volume of acetone used (mL);

V is the volume of water filtered (L);

L is the cuvette path length (cm)

4.2.5. Spatial Distribution of *Alexandrium tamiyavanichii* by qPCR Assay

Saline ethanol-preserved planktonic samples were undergone genomic DNA isolation using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The gDNAs extracted were amplified by qPCR (Applied Biosystems® 7500 Fast Real-time PCR System ver. 2.0.6) using *A. tamiyavanichii* species-specific primer pairs and Taqman probe targeting internal transcribed spacer ribosomal II (ITS2) rDNA (Table 4.1) (Kon et al., 2015).

Table 4.1: *Alexandrium tamiyavanichii* species-specific qPCR primer-probe set (Kon et al., 2015).

Primer	Primer sequences (5' – 3')
TamiaiiF	GCATTGATGTGCTTGACTGCATTGC
TamiaiiR	GCAACACACACCAATGTACAACCAC
Tamia-probe	TGAGCTGTAAGGGTCAATGTGTATGCA

The region was amplified in a total reaction volume of 10 µl containing the 1× Taqman® Fast Advanced Master Mix (Applied Biosystems, California, United States), 200 nM probes, 300nM of each primers, and 2 µl genomic DNA template in each reaction. The qPCR amplification was performed with the following thermal-cycling conditions: holding stages at 50 ℃ for 2 min and 95 ℃ for 20 s, followed by 40 cycles of denaturation at 94 ℃ for 3 s, and extension at 60 ℃ for 30 s.

A synthetic gene fragment-based calibration curve was constructed with 10-fold serial dilutions to determine total extractable gene copy number per cell in the samples. The assay was run with no template control (NTC) and 10-fold serial dilutions of *A. tamiyavanichii* synthetic gene fragment as the positive controls.

4.2.6. Spatial Abundance and Distribution of *Alexandrium tamiyavanichii* Cysts

Sediment samples were collected using a flow-through Ekman grab sampler (Matsuoka & Fukuyo, 2000). Replicate samples were taken from top 2 cm undisturbed sediment surface, pooled and placed into tightly-sealed containers to prevent germination (Matsuoka et al., 1988; Matsuoka & Fukuyo, 2000; Miyazono et al., 2012). Samples were kept dark at 4 ℃ until processing (Matsuoka et al., 1988).

Sediment samples (10 g) were suspended in filtered seawater and sonicated for 1 min (operated at 10% amplitude) using a QSonica Q55 sonicator ultrasonic processor (QSonica, LLC, USA). The sonified sediments were fractionated through Nitex screens (Endecotts Ltd, UK) to obtain a 20 – 53 µm size component (Matsuoka et al., 1988). The processed samples were stained with acidic Lugol's solution to enumeration in Sedgewick-Rafter counting chamber at 20 × magnification using a compound microscope (DM750, Leica, Germany) (Vila et al., 2005).

For molecular-based enumeration, 1 mL of processed sediment samples were undergone mechanical breakage prior to genomic DNA isolation by using Mo-Bio PowerSoil Extraction Kit (Mo-Bio Laboratories, USA) (Erdner et al., 2010). Assay of qPCR were also performed by using *A. tamiyavanichii* species-specific primers/probes that listed in Table 4.1 (Kon et al., 2015).

4.2.7. Cross-Mating Experiment

Cross-mating experiment was performed by using clonal cultures of *A. tamiyavanichii* in 24-wells tissue culture plate. Clonal cultures in exponential phase were cross-mating in a pairwise combination and monoclonal cultures were also self-crossed. The cross-mating cultures were incubated at culture conditions as described in section 3.2.2. Samples were examined under an Olympus SZX10 stereo-microscope (Olympus, Tokyo, Japan) daily. Micrographs of resting cysts formed were taken by using a Leica DM3000

LED compound research microscope attached with DFC450 digital camera (Leica). Using same method described above, the cross-mating experiment was also performed by using clonal cultures of *A. leei*.

4.2.8. Physico-Chemical Data

Salinity and temperature were determined *in situ* using a multiparameter water quality sonde (HI9829 multiparameter, Hanna instruments, Italy). Water pH was also determined using pH meter (LAQUAtwin pH33 Compact, Horiba, Japan).

Biochemical samples were collected by using Van Dorn sampler in 2-3 m depth and 20 µm mesh plankton net, respectively. Water samples were kept frozen for nutrient analyses of ammonia, phosphorus, and silica by spectrophotometer following manufacturer's instructions (DR3900, Hach Company, USA) and total nitrogen (TN) by Total Organic Carbon Analyzer (TOC-L Analyzer, Shimadzu, Japan). Filtered water sample of each station (10 mL) was used as negative control in spectrophotometer, and calibration curve prepared from ultrapure water and serial dilutions of 5 mg L^{-1} potassium nitrate (KNO_3) was used as standard in TN analysis. The obtained readings (mg L^{-1}) of dissolved inorganic nutrients, ammonia nitrogen (NH_3-N), phosphate phosphorus (PO_4-P), and silicate (SiO_2), were further converted into nitrogen (N), phosphorus (P) and silica (Si) (µM L^{-1}), respectively by the following equations (Parsons et al., 1984).

$$[Nutrient]\ (\mu M\ L^{-1}) = \frac{x\ (mg\ L^{-1}) \times 1000}{MW_{nutrient}}$$

where, MW is the molecular weight

MW of nitrogen (N) is 14.006720,

MW of phosphorus (P) is 30.973762,

MW of silica (Si) is 28.085530

4.2.9. Statistical Analyses

Canonical correlation analysis (CCA) was carried out to analyse the relationship of planktonic abundance to other parameters. Prior to CCA, phytoplankton cell abundance was performed in logarithm transformation [log (x+1)] to decrease the variability of data by minimize the influence of prevalent groups and increase the weight of rare group.

4.3. Results

4.3.1. Algal Cultures

Several species of *Alexandrium* were encountered from the sampling sites. A total of 32 clonal cultures of *A. tamiyavanichii* and 43 cultures of *A. leei* were established. *A. tamiyavanichii* were reported as PST-producing species whereas *A. leei* as a non-toxic species. Both species formed long chain in the cultures.

4.3.2. Species Identification

A total of six genera of dinoflagellates, eight genera of diatoms, and one genus of raphidophyte were recorded. There were eleven species of harmful dinoflagellates recorded in Kuantan Port (Fig. 4.2). Harmful dinoflagellates consists of *A. tamiyavanichii, Prorocentrum micans, P. sigmoides, Dinophysis acuminate, D. caudata, D. miles, Ceratium furca, C. fusus, Akashiwo sanguinea, Noctiluca scintillans* and *Chatonella* spp. Non-harmful dinoflagellates and diatoms found in Kuantan Port were *A. leei, Protoperidinium* spp., *Ceratium* spp., *Coscinodiscus* spp., *Pleurosigma* spp., *Navicula* spp., *Eucampia* spp., *Ditylum* spp., *Odontella* spp., *Rhizosolenia* spp. and *Guinardia* spp.

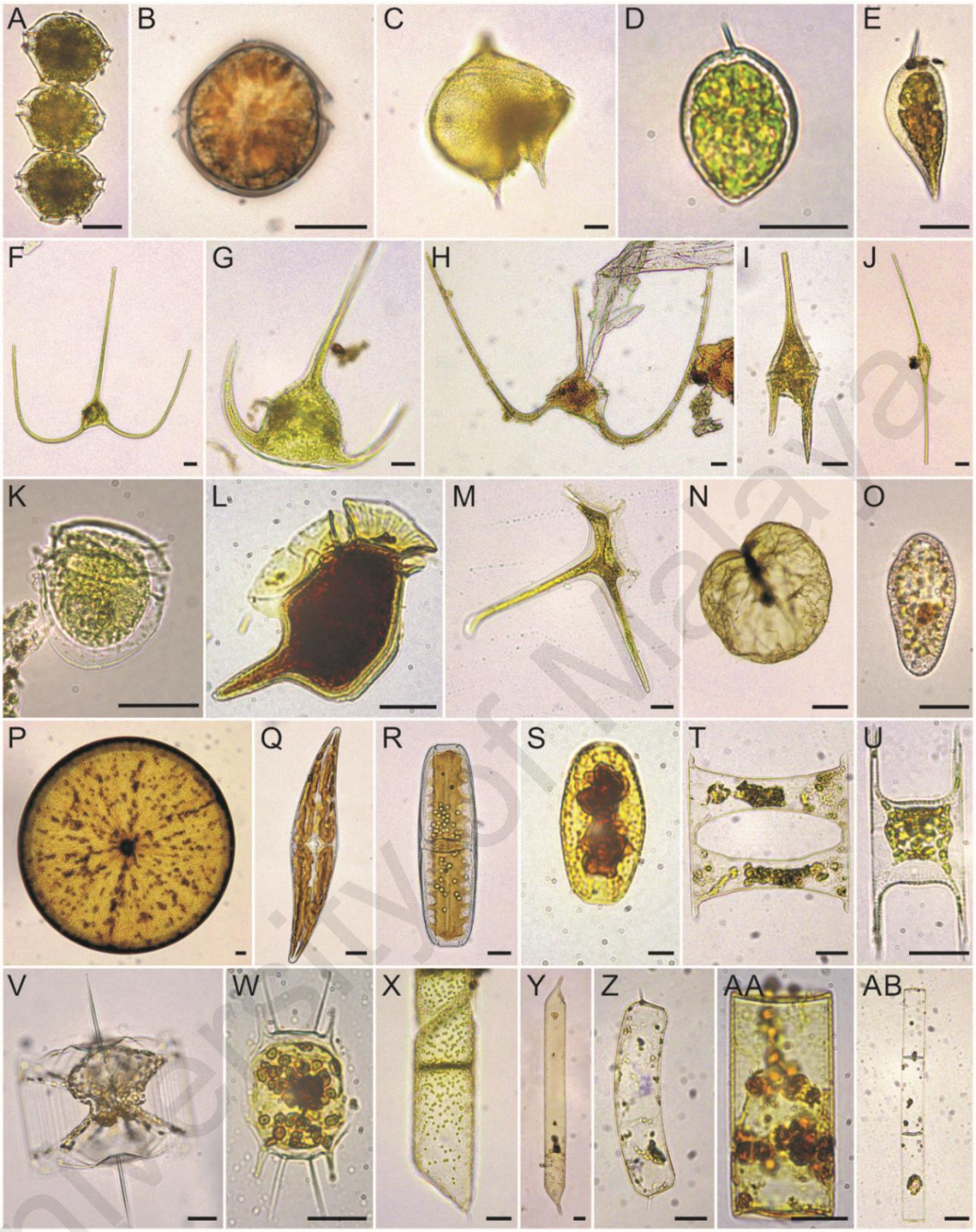

Figure 4.2: Micrographs of the phytoplankton that collected from field. *Alexandrium* spp. (A−B). *Protoperidinium* spp. (C). *Prorocentrum* spp. (D−E). *Ceratium* spp. (F−J). *Dinophysis* spp. (K−M). *Noctiluca* spp. (N). *Chatonella* spp. (O). *Coscinodiscus* spp. (P). *Pleurosigma* spp. (Q). *Navicula* spp. (R−S). *Eucampia* spp. (T−U). *Ditylum* spp. (V). *Odontella* spp. (W). *Rhizosolenia* spp. (X−Y). *Guinardia* spp. (Z−AB). Scale bar, 10µm.

A. tamiyavanichii and *A. leei* were confirmed by their thecal plate tabulation (Fig. 4.3) and further confirmed molecularly (Appendix D). Both plate 1' of *A. tamiyavanichii* and *A. leei* were directly connected to the apical pore complex (APC) and both species showed anterior attachment pore in their APC. Ventral pore (v.p.) of *A. tamiyavanichii* were connected in between plate 1' and 4', whereas v.p. of *A. leei* were located in the plate 1', connected with a groove from right margin of 1'. A triangle precingular part (p.pr.) was found connected with anterior plate (S.a.), which was a unique characteristic only presented in *A. tamiyavanichii*. In *A. tamiyavanichii*, posterior plate (S.p.) is longer than wide, whereas posterior plate (S.p.) of *A. leei* is wider than long. A groove was presented in the right margin of S.p. plate of *A. tamiyavanichii* and posterior attachment was found in central in some cells but these features absence in *A. leei*.

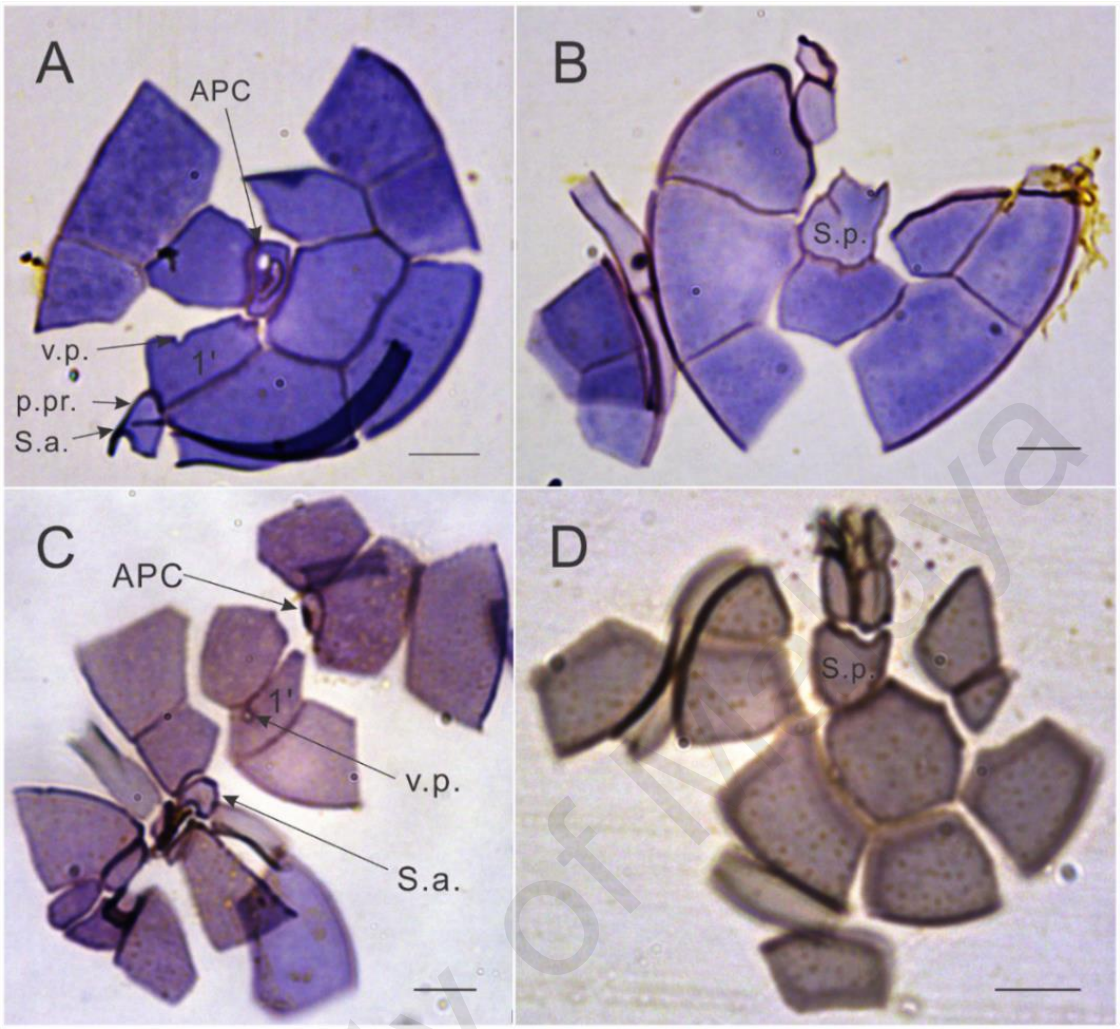

Figure 4.3: Thecal plate tabulation of *Alexandrium* species. (A) Apical view of *Alexandrium tamiyavanichii*, showing apical pore complex (APC), anterior plate (S.a.), precingular part (p.pr.), first apical plate (1'), and location of ventral pore (v.p.). (B) Antapical view of *Alexandrium tamiyavanichii*, showing posterior plate (S.p.). (C) Apical and ventral view of *A. leei*, showing APC, S.a., plate 1', and location of v.p. (D) Antapical view of *A. leei*, showing S.p. Scale bar, 10 µm.

4.3.3. Phytoplankton Spatial Distribution

Total chlorophyll *a* was measured to represent the whole phytoplankton assemblages throughout the study areas (Fig. 4.4 A). Densities of dinoflagellates and diatoms were correlated with the total chlorophyll *a* concentration. Microscopic enumeration which only encountered up to genus level enumerated the cell density of *Alexandrium* spp. (*A. tamiyavanichii* and *A. leei*) (Fig. 4.4 B) whereas qPCR assay which is species-specific and high sensitivity quantified the cell density of *A. tamiyavanichii* (Fig. 4.4 C).

In April 2015, chlorophyll *a* concentration was ~1.07 µg L^{-1} and the abundance of *Alexandrium* spp. were moderately high (241 cells L^{-1}). However, the species-specific qPCR assay revealed that no *A. tamiyavanichii* cell was detected in the sample.

In August 2015, cell density of phytoplankton was moderate (~0.99 µg L^{-1}); densities of *Alexandrium* spp. in the inner port and outer port were ~7932 cells L^{-1} and ~111 cells L^{-1}, respectively. By qPCR quantification, *A. tamiyavanichii* was detected, and the cell density was ~17 cells m^{-3}. Relative abundance of *Alexandrium* spp. was the highest among dinoflagellates in the inner port, with the Diatom:Dinoflagellate (D:D) ratio of ~0.26.

The highest cell density of phytoplankton in January 2016 and March 2016 were ~1.74 µg L^{-1} and ~1.44 µg L^{-1}, respectively. It was dramatically decreased in February 2016 with density of~0.66 µg L^{-1}. Coincidentally, a bauxite mining moratorium was enforced almost a year (11 months) from January 2016 onwards. Cell density of *Alexandrium* spp. enumerated through the microscope in March 2016 was ~91 cells L^{-1} and the qPCR quantification of *A. tamiyavanichii* was ~5 cells m^{-3}. The D:D ratio of the January 2016 and March 2016 were 182.11 and 70.71, respectively, where higher densities of diatoms were observed compared to dinoflagellates.

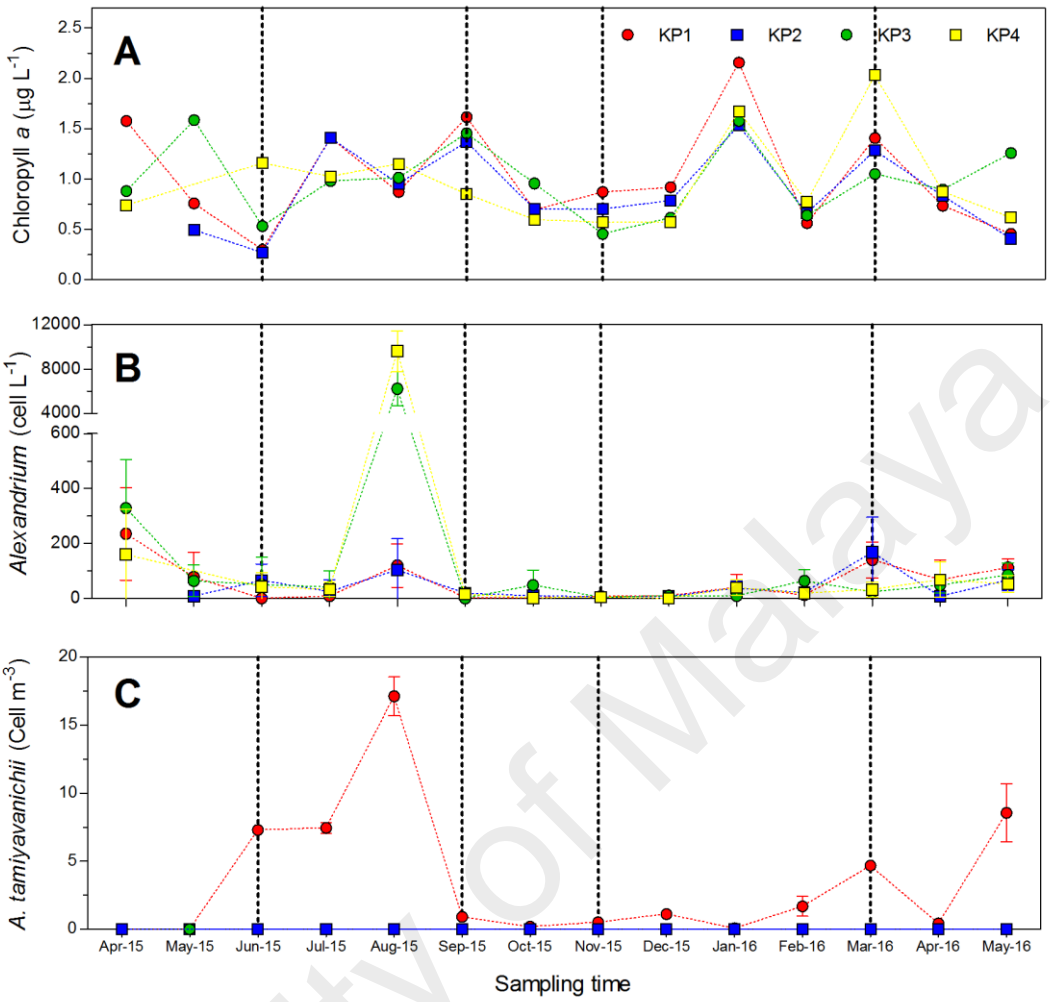

Figure 4.4: Phytoplankton spatial distribution. Total chlorophyll *a* (A). Microscopic enumeration of *Alexandrium* species (B). The qPCR quantification of *Alexandrium tamiyavanichii* (C).

In the microscopic enumeration, *Alexandrium* spp., *Dinophysis* spp., *Ceratium* spp., *Prorocentrum* spp., and *Protoperidinium* spp. were the five dominant dinoflagellates commonly found in Kuantan Port waters. *Protoperidinium* spp. was the most abundant species and frequently detected in all sampling dates. In April 2015, enormous cell densities of dinoflagellates decreased.

In the beginning and end of dry seasons (June and September 2015), all the cell abundance of five dominant dinoflagellates were the lowest. However, during the dry seasons (July and August 2015), cell abundance of these five dominant dinoflagellates

increased and showed higher cell densities. In between the inter-monsoon and wet seasons, low cell densities of five dominant dinoflagellates were slightly fluctuated, and increased again in May 2016.

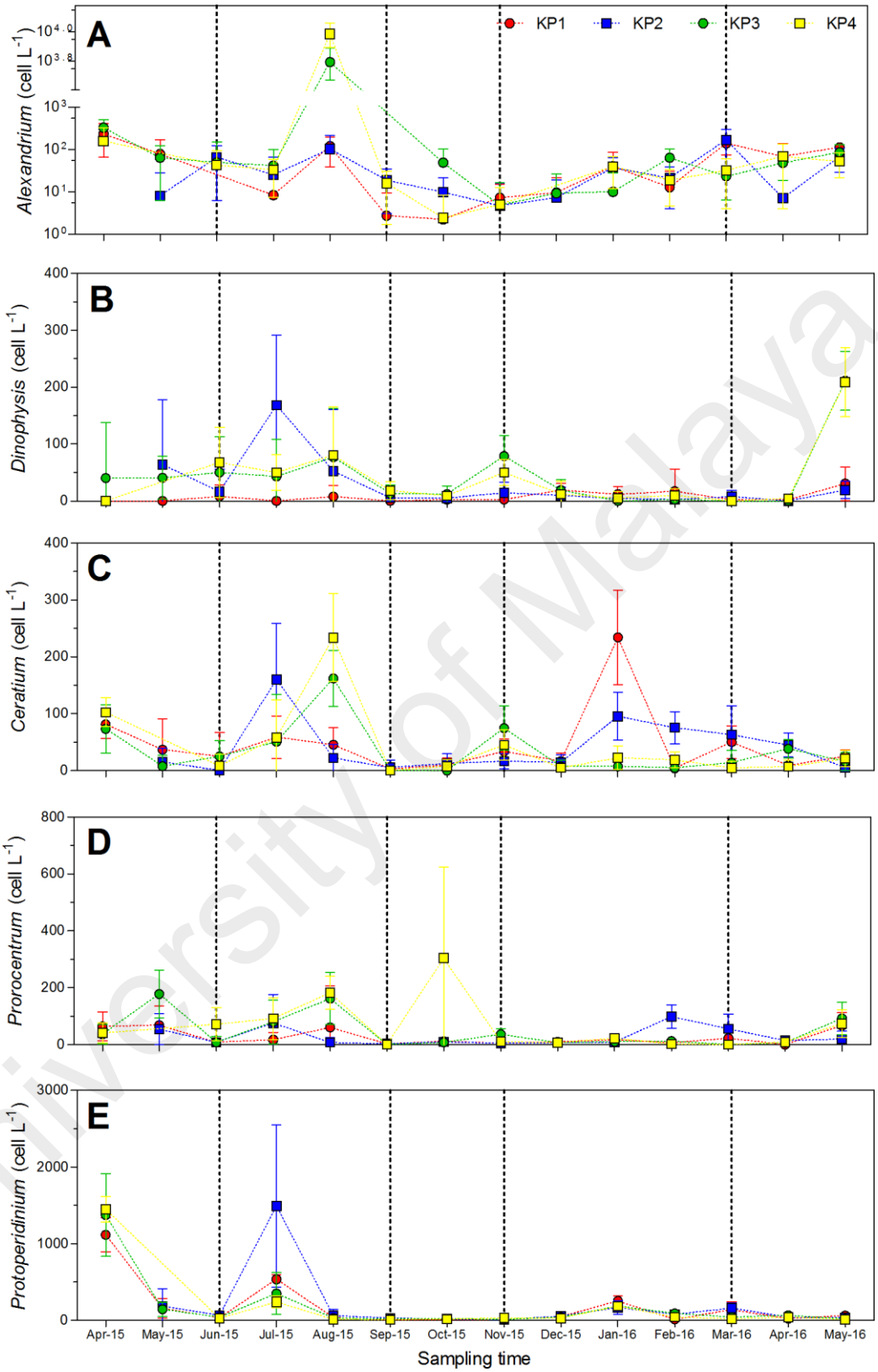

Fig. 4.5. Cell densities of five dominant dinoflagellates enumerated by microscopic count. *Alexandrium* spp. (A). *Dinophysis* spp. (B). C*eratium* spp. (C). *Prorocentrum* spp. (D). *Protoperidinium* spp. (E).

Similar cell abundance trend with dinoflagellates, enormous cell densities of diatom were decreased in April 2015 and showed lowest cell densities in the beginning and end of dry season (June 2015 and September 2015) with 958−3686 cells L^{-1} and 743−2153 cells L^{-1}, respectively. Abundance of diatom showed high densities in July 2015 with 17507−47670 cells L^{-1} and highest densities in January 2016 with 36085−70236 cells L^{-1}. Nevertheless, zooplankton density was observed constantly throughout the sampling dates.

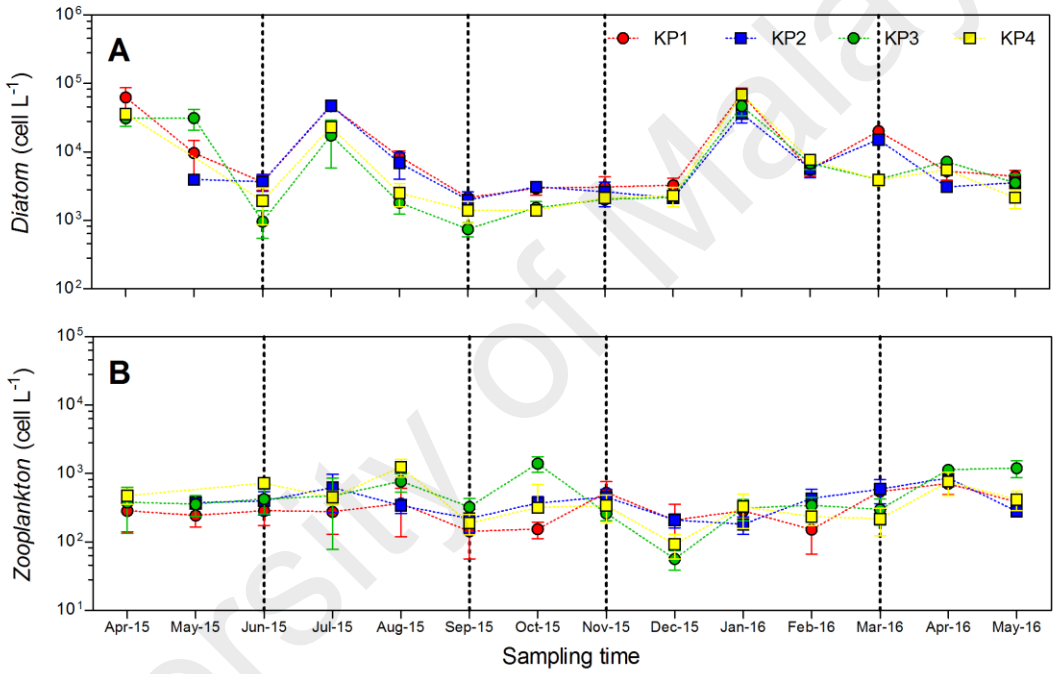

Fig. 4.6. Cell density of other phytoplankton and zooplankton. Diatom (A). Zooplankton (B).

4.3.4. Cyst Spatial Abundance and Distribution

Sediments were collected from each of the stations were in different compositions (Fig. 4.7). The sediments from the inner port were covered by a thick layer of bauxite, whereas sediments from outer port were covered by a thick layer of black sludge.

In qPCR quantification, very low *A. tamiyavanichii* cysts were detected; 1 cyst g^{-1} from both KP2 and KP4 in August 2015, and 3 cysts g^{-1} from KP1 in May 2016. No cyst was found under microscopic enumeration.

Figure 4.7: Sampling environment and sediment collected from Kuantan Port. Inner port (KP 3 & 4) (A−D). Sediment collected from the inner port (E). Outer port (KP 1 & 2) (F). Sediment collected from outer port (G).

Cross-mating experiment of *A. tamiyavanichii* showed low reproduction compatibility and cyst reproduction. The experimental data showed that some crosses (e.g. [AcKP02 × AcKP09] and [AcKP02 × AcKP16]) produced less than three cysts in 2-weeks incubation. Cysts of *A. tamiyavanichii* appear elongated and oval (Fig. 4.8 A−C).

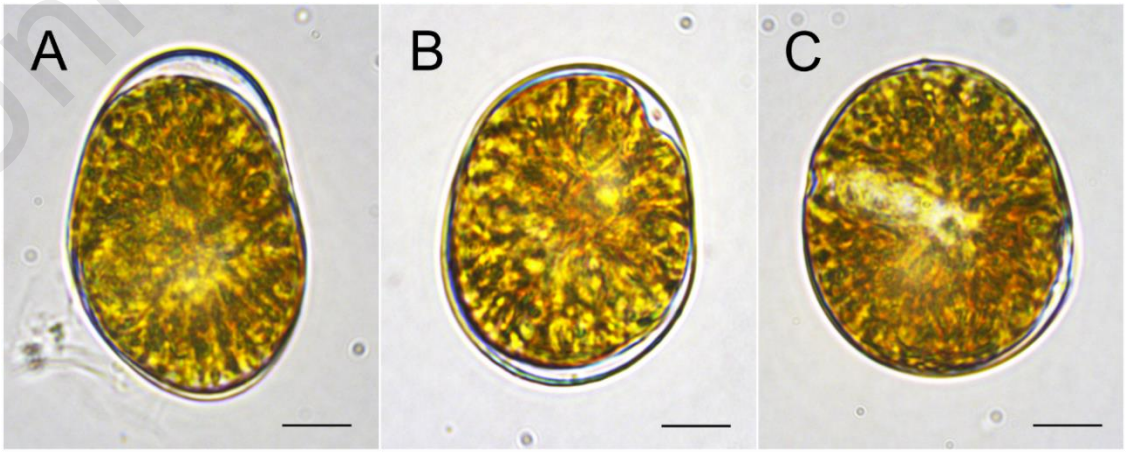

Figure 4.8: Micrographs of laboratory-produced cyst of *Alexandrium tamiyavanichii*.

4.3.5. Physico-Chemical Environmental Variability

Hydrographic data such as salinity, water temperature and pH of water samples were recorded (Fig. 4.9). Salinity from April 2015 to August 2015 fluctuated between 32−35 PSU. Salinity decreased from 32 PSU to 28.5 PSU in the subsequent months (Fig. 4.9 A). The water temperature and pH in Kuantan Port was constant (26.62−31.89 °C; pH 7.82−8.30) except in months of October (pH 8.87) and December 2015 (pH 7.28). Data of temperature and pH were not obtained in the first few monthly samplings (April − September 2015) due to the breakdowns of instruments (salinometer and pH meter).

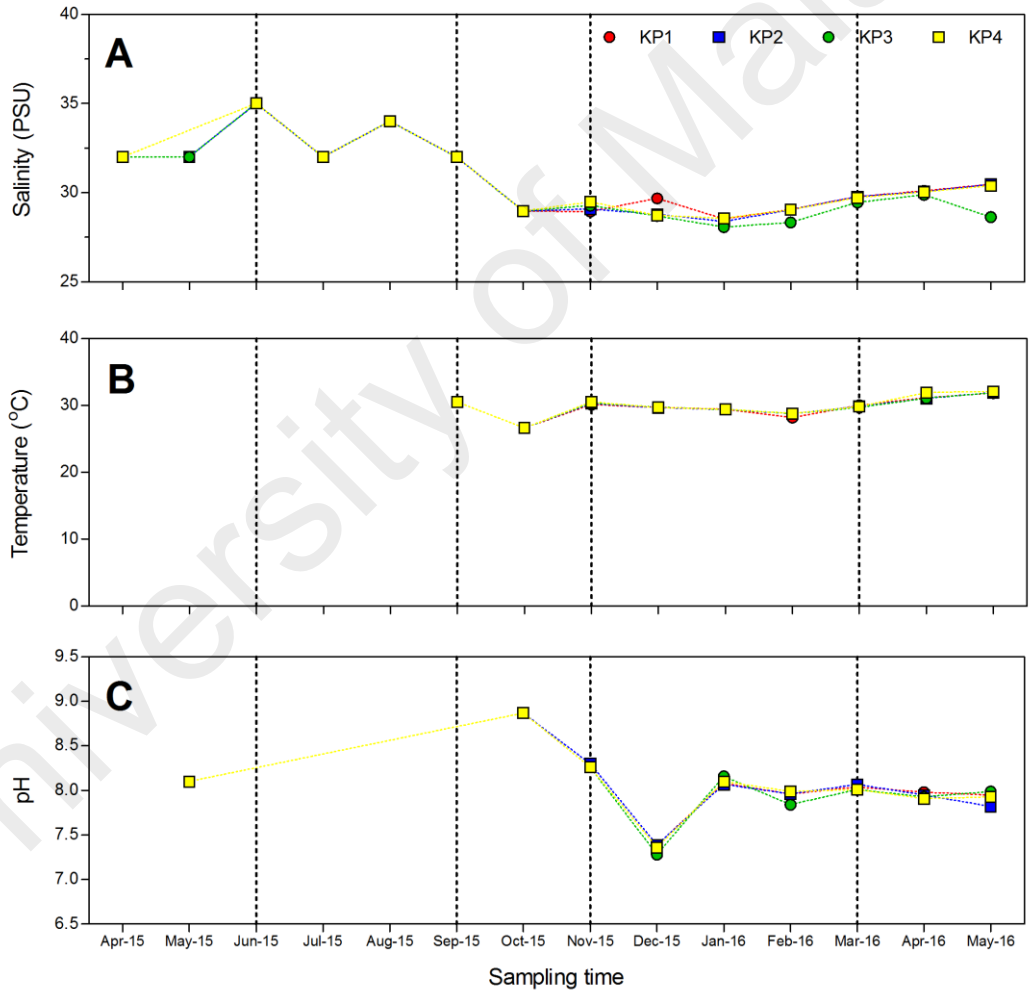

Figure 4.9: Hydrographic data. Salinity (A). Water temperature (B). Water pH (C).

Concentrations of total nitrogen (TN) was relatively constant except a sudden increment (66.33 ~ 141.57 μM) in May 2016. The high concentration of TN was contributed by other N-compounds such as nitrite and nitrate as the concentrations of ammonia was low. From April 2015 until the end of the dry season (September 2015), concentration of ammonia slightly increased. In September 2015, concentration of ammonia in the outer port (6.43 μM) was higher than inner port (14.28~16.06 μM). In between inter-monsoon and wet seasons, concentration of ammonia decreased until January 2016. A dramatically increase in concentration of ammonia (6.78 μM to 14.99 μM) in February 2016. In August 2015, concentration of phosphate was the lowest (2.10 μM) and dramatically increased (6.94 μM) in following month. By entering the inter-monsoon season, concentration of phosphate was relatively constant and showed higher than previous months. Concentration of silica fluctuated less. In January 2016, all the nutrient concentrations were slightly increase.

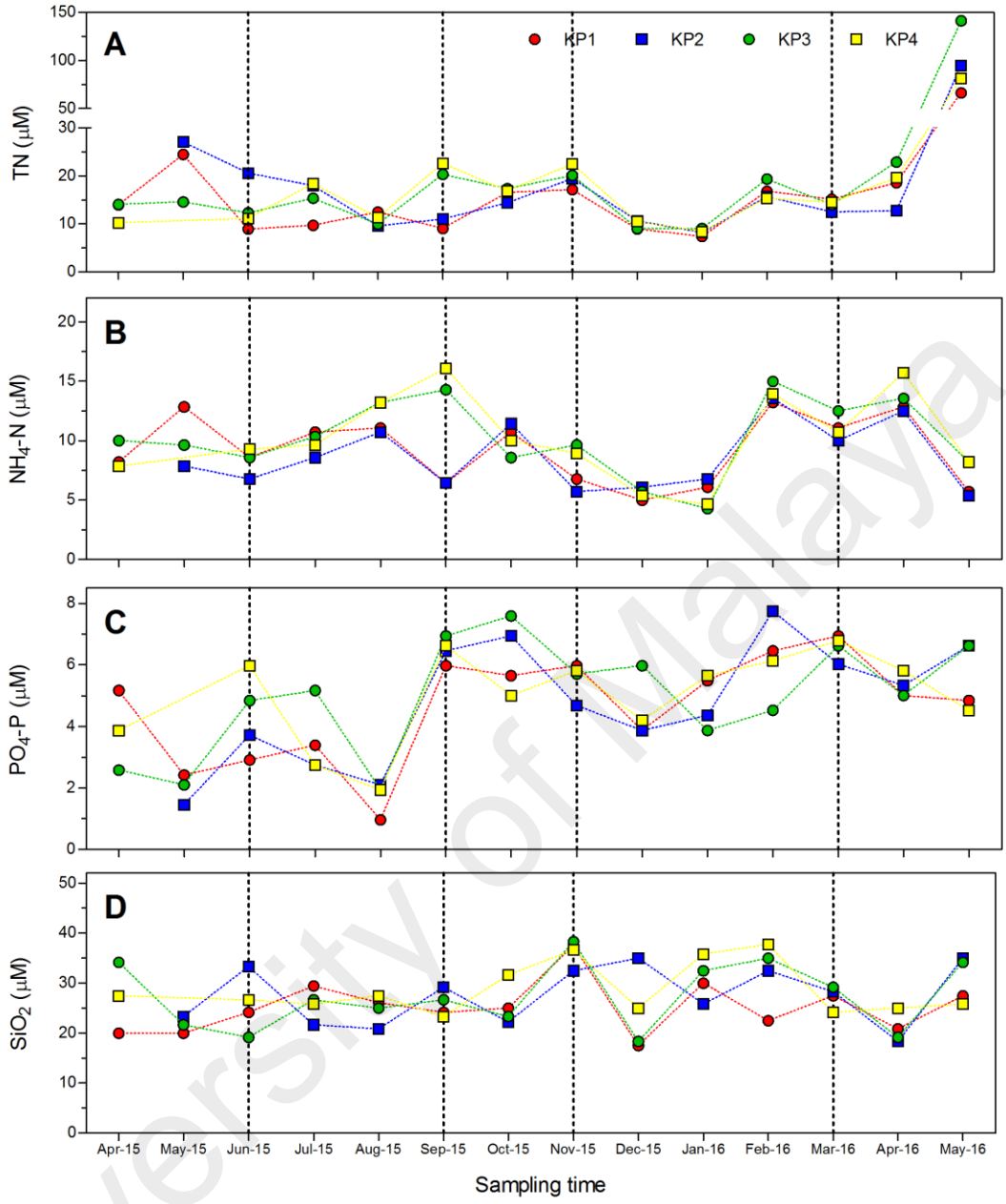

Figure 4.10: Chemical data. Total nitrogen (A). Ammonia (B). Phosphate (C). Silica (D).

CCA plot revealed the relationships of phytoplankton abundances and the environmental parameters (Fig. 4.11). In the triplot, cell density of both *Alexandrium* spp. and *Prorocentrum* sp. were strongly influence by salinity and N:P ratio, whereas *Dinophysis* spp. was affected by TN. Diatom abundance was correlated with high phosphate concentration and P:N ratio. Phosphate in conjugation to ammonia was

contributed to density of zooplankton. Total chlorophyll *a* representing the whole assemblage of phytoplankton showed high contribution by P:N ratio.

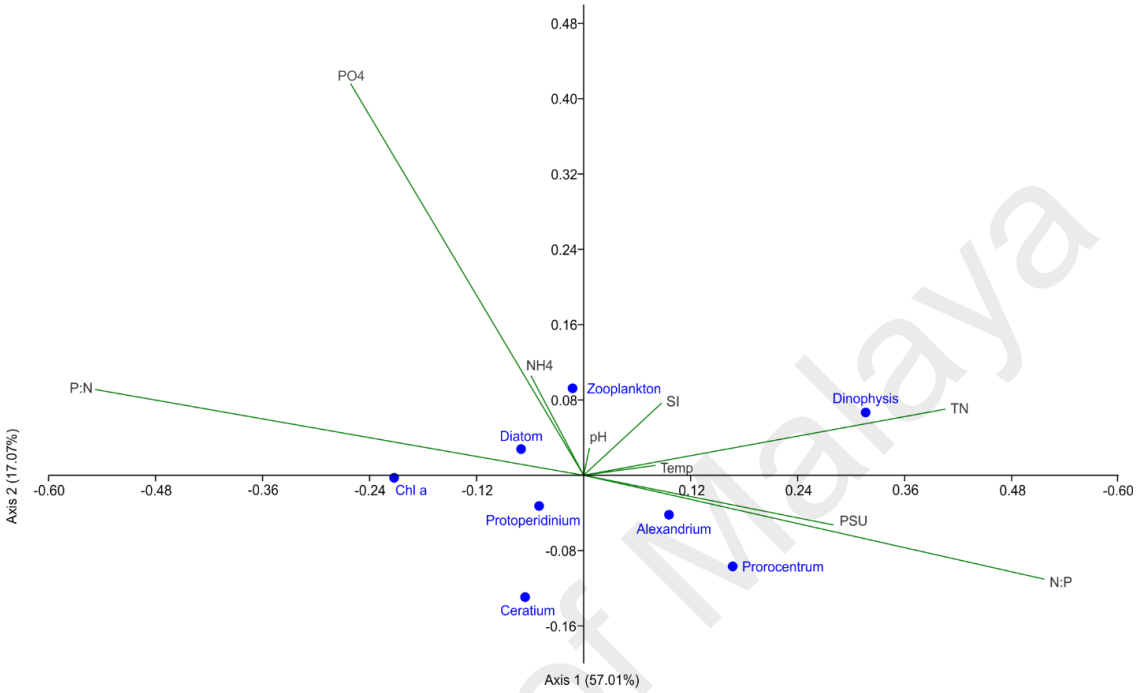

Figure 4.11: Canonical correlation analysis (CCA) showed the relationship of planktonic abundance to environmental factors.

4.4. Discussion

In this study, samplings in Kuantan Port were conducted during the peak season of bauxite mining activities such as shipments and stockpiles. The air, river and seawater were seriously been polluted by bauxite (as showed in Fig 4.7). The seawater was changed to brownish red and the sediment in the Kuantan Port was covered by a thick layer of bauxite. Due to the bauxite pollution, the Kuantan government had restricted the regulation for bauxite-related activities on January 2016 and temporary moratorium of the port was also been implemented (Radhi, 2016). By the time, cell density of phytoplankton was increased (Fig. 4.4 A). This has shown that the bauxite tailing in Kuantan Port was acted as clay dispersion and flocculation. Clay dispersal is an effective HAB-control treatment that frequently used in Japan, South Korea, and China (Seo et al.,

2008; Louzao et al., 2015) to mitigate phytoplankton blooms by aggregation and sedimentation of the phytoplankton (Sengco & Anderson, 2004; Seo et al., 2008; Louzao et al., 2015). In Batata Lake, Brazil, the bauxite taillings that accumulated from year 1979 till 1989 was acted as an Amazonian crystalline system, in which it has affected the cell density of phytoplankton were decreased by 60% (Roland & de Assis Esteves, 1998; Maia-Barbosa & Bozelli, 2006). According to Melack (1985), the electrical interactions between bauxite tailing and cells was explained as the reason of descending cell density of phytoplankton (Melack, 1985; Roland & de Assis Esteves, 1998). The collisions of particles caused by Brownian motion had induced the electrical double layer and reduced the electrostatic repulsion between particles (Avnimelech et al., 1982). Besides that, electrolyte between the particles had reduced the electrostatic repulsion too (Avnimelech et al., 1982). Thus, the electrical interactions help to coagulate tailings with two or more algal cells. In addition, spilled oil was also observed in the Kuantan Port surface water (Fig. 4.7 C−D) and caused to the formation of dirty blizzard or oil-associated marine snow (Brooks et al., 2015; Passow, 2016). The oily particulate matter aggregated with phytoplankton through physical coagulation and flocculent materials rapidly sank on the seafloor (Joye et al., 2014; Passow, 2016). The sedimentation of algal-pollutants flocculation reduced the seawater turbidity, particularly in the euphotic zone (Melack, 1985; Roland & de Assis Esteves, 1998). The reduction of turbidity had increased the light intensity in the water. The increased light intensity as the one of important phytoplankton growth factor had increased the possibility of HAB. However, this phenomenon has been reported that it was able to reduce approximately 60% of phytoplankton (Brooks et al., 2015). The aggregation and flocculation of algal and pollutants increased the sedimentation rates (Melack, 1985). Due to the sedimentation, benthic organisms such as clams, crabs, seagrass, coral and microalgae, were potentially in the risk of temporary anoxic (Brooks et al., 2015). Anoxic of the benthic organisms

might cause imbalance of biodiversity and destroy the marine ecosystems. Besides that, the pollutions also led to the sudden changes of environmental conditions (Melack, 1985; Louzao et al., 2015). The nutrient interchange between sediment and water column was impeded by bauxite tailings (Roland & de Assis Esteves, 1998) and oily particulate matter. Thus, pollutions in Kuantan Port was the factor that cause the cell density of phytoplankton become low and barely find cyst in this study.

In this study, the CCA have shown that the nutrient sources affected the cell density of phytoplankton whereas salinity was highly influenced the cell density of *Alexandrium* spp. In August 2015, both total nitrogen and phosphate concentration were low but high concentration of ammonia and salinity. This environment condition had given an advantage for the dinoflagellate a better growth condition, especially *Alexandrium* spp. Under the low P and N, dinoflagellate was the late succession species in Kuantan Port, it is well defended from grazing and enable to survive for growth (Lin et al., 2016). This showed the nutrient sources were one of the main factors caused bloom of HAB in August 2014 (Mohammad-Noor et al., 2017). Besides that, ecophysiology and toxin production of *Alexandrium* spp. were also affected by salinity. *A. tamiyavanichii* is a salinity-dependent growth, it will be grew better in 20 – 30 PSU with the optimum salinity was 25 PSU (Lim & Ogata, 2005).

Preview phytoplankton diversity study from Mohammad-Noor et al. (2013) have shown the *A. tamiyavanichii* was absent in Kuantan, which it was incongruence with the findings in present study. In this study, *A. tamiyavanichii* can be commonly found in Kuantan Port waters with low abundance (Fig. 4.4). Therefore, *A. tamiyavanichii* can be suspected introduction to Kuantan Port through ballast water of shipments and it also had caused to the first HAB case in Kuantan Port in 2014 (Mohammad-Noor et al., 2013). Besides that, this study also found the high cell density of *Alexandrium* spp. in August 2015, with the cell density up to 9637 cells L^{-1}. This might likely had exceeded the

regulatory threshold of bioaccumulate PSP toxins by shellfish at 80 μg STX eq. 100 g^{-1}, which it will be equivalence with 20−40 cells L^{-1} of *A. tamiyavanichii* (Kon et al., 2015). Besides that, other potential harmful species also has been found in this study. The potential harmful dinoflagellate such as *Prorocentrum micans* (putative palytoxin and ovatoxin-a producer) (Ignatiades & Gotsis-Skretas, 2010), *P. sigmoides* (cause fish killing) (Lu & Hodgkiss, 2004), *Dinophysis acuminata* (okadaic acid and Dynophysis-toxin producer) (Ignatiades & Gotsis-Skretas, 2010; Hattenrath-Lehmann et al., 2013), *D. caudata* (okadaic acid and palytoxin producer) (Ignatiades & Gotsis-Skretas, 2010; Munir et al., 2010), *D. miles* (okadaic acid producer) (Munir et al., 2010), *Ceratium furca* (cause fish killing) (Munir et al., 2010), *Ceratium fusus* (cause fish killing) (Munir et al., 2010), *Akashiwo sanguinea* (cause fish killing) (Badylak et al., 2014), *Noctiluca scintillans* (cause fish killing) (Lu & Hodgkiss, 2004; Xu et al., 2017), and *Chattonella* spp. (fish killer) (Imai & Yamaguchi, 2012) were also regularly found in the waters.

Cyst carried a crucial role in bloom initiation, termination, dispersion, and depopulation in an area (Kremp & Anderson, 2000). Among the phytoplankton that found in this study, *A. tamiyavanichii* (Nagai et al., 2003), *Protoperidinium* spp. (Lewis et al., 1984; Matsuoka & Fukuyo, 2000), *Akashiwo sanguinea* (Tang & Gobler, 2015), and *Chattonella* spp. (Imai & Yamaguchi, 2012) were the species that are capable to produce cyst in their life cycle. In this study, cross-mating experiment was carried out with *A. tamiyavanichii* strains isolated from Kuantan Port. The reproduction compatibility and cyst germination experiments were failed to further investigate in this study due to the low successful encystment of a mating pair (<10 cysts). The calculation of cyst production in reproductive compatibility was performed in logarithmic scale (Blackburn et al., 2001) and formation of 0−10 resting cysts in logarithms were determined as 0 cyst production. While for the environment samples, the qPCR have detected very low number of *A. tamiyavanichii* cysts, 1–3 cycts g^{-1}. Nevertheless, there have some cases such as in Arenys

de Mar harbor, the presence of resting cysts in that harbor has become the recurrence factor of *A. minutum* bloom (Vila et al., 2005). Possibility of recurrence of *A. tamiyavanichii* or other harmful dinoflagellates at Kuantan port in future due to resting cyst germination could not be ruled out. Therefore, a routine monitoring of the harmful planktonic and cysts was still necessary conducted on this area.

4.5. Conclusion

This study showed the effects of pollution and physiochemical on the abundance of planktonic dinoflagellates and cysts in Kuantan Port. The bauxite related activities and oil spill in Kuantan Port have reduced the cell density of the phytoplankton and impeded nutrient interchange between sediment and water column. The limited phosphate and nitrogen concentration had favoured the late succession species from HAB. In this study, *A. tamiyavanichii* as a PST-producer was strongly influenced by physiochemical variability (high N:P ratio and optimum salinity) and pollutions. However, a continuous and long term monitoring in this area would better explain the dynamics of phytoplankton and factors promoting blooms, particularly *A. tamiyavanichii*. In addition, further study about *A. tamiyavanichii* cyst and completion of ecophysiological would provide better understanding of HABs. Hence, HABs are anticipated given the ideal conditions in Kuantan port.

CHAPTER 5:

CONCLUSIONS AND RECOMMENDATION

The resting cyst of harmful dinoflagellates plays an important role in the bloom dynamics. The formation and germination of cysts are crucial processes in the life cycle transitions of the species that might trigger blooms initiation and termination in a region. The tropical cyst of *A. minutum* had a relatively short encystment-excystment period (<10 days) in Malaysian waters, with no significant dormancy period. On the other hand, the experimental data in this study revealed that the encystment rate of *A. tamiyavanichii* is relatively low. Studies on encystment and excystment of *A. tamiyavanichii* resting cysts are very limited, thus it is interesting to further investigate the processes of this species.

In tropical regions, environment factors such as nutrient sources and water temperature are probably not the main factor that triggering bloom initiation but might be the factor that caused the bloom to develop. In this study, anthropogenic activities that caused the changes of environmental factors and pollutions have influenced the bloom dynamics of phytoplankton in the waters and also in the sediments. Particularly for *Alexandrium* spp., environmental conditions with low P:N ratios and optimum salinity helped to increase the inoculum of the cells and potentially caused blooms. On the other hand, bauxite mining and oil spill had severely polluted the water and sediment. They not only reduced the cell density of the phytoplankton in the water column, but also impeded cyst germination and cell growth in the sediment. This study has provided valuable information that will be used as the early warning for HABs, in both the life stages of motile vegetative cells and dormant resting cysts.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Several parts of this study have been accepted or published in relevant journals whereby the remaining parts are now in the process of peer-review.

1. **Liow, G.R.**, Lim, P.T., Tan, S.N., Leaw, C.P. (2016). Encystment and excystment of a toxic tropical dinoflagellate, *Alexandrium minutum* (Dinophyceae). ***Harmful Algae News. 52***:8.
2. **Liow, G.R.**, Lau, W.L.S., Law, I.K., Leaw, C.P. & Lim, P.T. Short cyst dormancy of a tropical toxic dinoflagellate, *Alexandrium minutum*, revealed by its sexual processes and life-history stages. ***Harmful Algae*** **(under reviewed).**
3. **Liow, G.R.**, Lau, W.L.S., Law, I.K., Hii, K.S., Leaw, C.P. & Lim, P.T. Abundance and spatial distribution of a paralytic shellfish toxin-producer, *Alexandrium tamiyavanichii* (Dinophyceae) in Kuantan Port, Pahang, Malaysia. ***Marine Pollution Bulletin*** **(in prep).**
4. Mohammad-Noor, N., Aimimuliani A., Lim, P.T., Leaw, C.P., Lau, W.L.S., **Liow, G.R.**, Mohammad Bunnori, N., Nurul Ashima Hamdan, Md Noor, A., Norazizah K., Devaraj M. **2017.** First report of paralytic shellfish poisoning (PSP) caused by *Alexandrium tamiyavanichii* in Kuantan Port, Pahang, East Coast of Malaysia. ***Phycological Research*** **(in press).**

Some parts of the study were presented in relevant seminars or conferences.

1. **Liow G.R.**, Lau, W.L.S, Law, I.K., Leaw, C.P., Lim, P.T. (2016, Dec). Gamete expression and swimming behaviors at different life cycle stages of the toxic tropical dinoflagellate, *Alexandrium minutum* (Dinophyceae). Paper presented at the 21st Biological Sciences Graduate Congress (BSGC), University of Malaya, Kuala Lumpur, Malaysia.

2. **Liow G.R.**, Lau, W.L.S, Law, I.K., Gu, H.F., Leaw, C.P., Lim, P.T. (2017, Jan). Encystment-excystment of a tropical toxic dinoflagellate, *Alexandrium minutum* (Dinophyceae). Paper presented at the Second Xiamen Symposium on Marine Environmental Sciences, Xiamen University, Xiamen, China.
3. **Liow G.R.**, Law, I.K., Tan, S.N., Lau, W.L.S, Leaw, C.P., Lim, P.T. (2017, Apr). Monitoring of a paralytic shellfish toxin-producer, *Alexandrium tamiyavanichii* (Dinophyceae) in Kuantan Port, Pahang using the qPCR detection. Western-Pacific (WESTPAC) International Scientific Conference, Qingdao, China.