DEVELOPMENT OF MOLECULAR PLATFORM FOR THE DETECTION OF HARMFUL ALGAL BLOOM SPECIES, *Alexandrium minutum*

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ABSTRACT

Toxic marine dinoflagellate *Alexandrium minutum* is widely associated with paralytic shellfish poisoning (PSP) in the Asia Pacific region. This species have been reported for PSP cases at east coast of Peninsular Malaysia. In this study, a powerful and advanced method was demonstrated for the detection of *A. minutum* using SYBR Green I-based quantitative real-time PCR (qPCR) and investigates the life cycle of *A. minutum* in natural bloom using flow cytometry. For qPCR detection, species specific primers were designed from second internal transcribed spacer (ITS2) region of ribosomal RNA gene (rDNA) which had been showed to be the excellent molecular signature for species identification and discrimination. The qPCR assay showed high specificity and sensitivity in detecting *A. minutum* from the environmental samples. This assay allow quantification of target cells by convert total extractable gene copies into cell number based on the extractable gene copy number per cell estimated in the studies. Gene-based calibration curve with AE 97.6% was served as an alternative calibrator in *A. minutum* qPCR quantification. Based on the calibration curve, the assay estimated $7,584 \pm 1,139$ extractable gene copies per cell of *A. minutum*. However, the gene copy number per cell determined is only an estimate to convert the total extractable gene copies in a sample to cell estimates. In addition, SYBR Green I-based qPCR assay was applied to the *A. minutum* bloom samples. However, overestimation and underestimation in some samples were observed due to different DNA content at different stage of life cycle. Specificity and sensitivity of the assay provide an effective method for *A. minutum* monitoring especially in the site with different *Alexanderium* spp. coexist. In early September 2015, a remarkably high density of *A. minutum* occurred in Sungai Geting, Tumpat, a semi-enclosed lagoon at north-eastern Peninsular Malaysia, causing severe discoloration and contaminated the benthic clam, *Polymesoda*

similis. Samplings were undertaken over the duration of four months between the bloom periods from September to December 2015. Plankton samples were collected and used to investigate the mechanisms of blooms initiation, development and termination by flow cytometry based detection. A 48-h sampling was conducted in March 2016 when the bloom recurred to investigate the life cycle stage. Results from flow cytometric analysis showed that planomyocetes (4C) were observed (88%) in the early bloom, suggesting that the bloom was initiated by cyst excystment. Increase in planozygotes (2C) was signified in the middle of the bloom, coincided with an abrupt decrease in salinity and elevated N:P ratio. Gamate expression and mating process were induced among the population under phosphorous limitation. The results also confirmed that the bloom was initiated at the inner part of the lagoon and slowly dispersed to the river mouth. The blooms were sustained for four months by 72– 92% of vegetative cells (1C) although 36% of planozygotes (2C) were observed in the early of the bloom. Results from the 48-h diurnal sampling confirmed that cells undergo consistent cell cycle in natural environment. Increase of precipitation and freshwater intrusion from the Golok River might cause the bloom to terminate. This event provides a fundamental understanding of the life cycle of this tropical toxic dinoflagellate.

Keywords: *Alexandrium minutum*, paralytic shellfish poisoning, real-time quantitative PCR, second internal transcribed spacer (ITS2), flow cytometry, life cycle, cyst, planozygote, planomyocete.

ABSTRAK

Dinoflagellat marin bertoksik *Alexandrium minutum* merupakan spesies yang berkaitan secara meluas dengan keracunan kerang-kerangan (PSP) di kawasan Asia Pasifik. Dalam kajian ini, satu kaedah yang sangat berguna dan termaju dalam pengesanan *A. minutum* yang berasaskan SYBR Green I PCR masa sebenar (qPCR) dan menyiasat kitaran hidup *A. minutum* secara semula jadi dengan menggunakan kaedah sitometrik aliran. Penjujuk spesies-spesifik telah direkabentuk berasaskan transkrip spacer dalaman kedua (ITS2) dari gen ribosom RNA (rDNA) di mana ia telah ditunjukan sebagai penanda molekular yang terbaik bagi pengenalpastian dan diskriminasi spesies. Cerakinan qPCR telah menunjukan kekhususan dan kepekaan yang tinggi dalam pengesanan *A. minutum* dalam sampel-sampel lapangan. Cerakinan qPCR membenarkan kuantifikasi berdasarkan nombor salinan gen ekstrak setiap sel yang dianggar dalam kajian ini dengan menukarkan jumlah salinan gen kepada anggaran sel nombor. Lengkung penentukuran berasaskan gene dengan AE 97.6% telah berfungsi sebagai pengayuh alternatif dalam kuantifikasi qPCR *A. minutum*. Kaedah SYBR Green I qPCR menganggarkan 7,584 ± 1,139 salinan gen ekstrak setiap sel. Yang pentingnya, nombor salinan gen setiap sel yang didapati dari kajian adalah hanya satu anggaran dan digunakan pada cerakinan masing-masing untuk menukarkan jumlah salinan gen ekstrak dari sampel kepada nombor sel anggaran. Di samping itu, aplikasi berasaskan-SYBR Green I qPCR telah digunakan untuk menilai *A. minutum* semasa ledakan yang berlaku. Tetapi, di sesetengah sampel, penilaian berlebihan dan penyusutan nilai telah diperhatikan kerana kandungan DNA yang berbeza dalam kitaran hidup sel tersebut. Kekhususan dan kepekaan cerakinan ini merupakan satu kaedah yang berkesan untuk memantau *A. minutum* di mana beberapa jenis *Alexandrium* terjumpa di tempat yang sama. Pada awal September 2015, kejadian ledakan *A. minutum* telah berlaku di Sungai Geting, Tumpat, lagun yang terletak di pantai timur laut Semenanjung

Malaysia, menyebabkan perubahan warna air yang mendadak dan mencemarkan kerang-kerangan, lokan, *Polymesoda similis*. Aktiviti persampelan telah dijalankan selama 4 bulan dari bulan September ke Disember 2015. Sampel telah dikumpul dan diguna untuk menyiasat mekanisma permulaan, pembangunan dan penamatan ledakan dengan menggunakan kaedah sitometrik aliran. Persampelan 48 jam juga telah dijalankan pada bulan Mac 2016, apabila ledakan berulang untuk menyiasat peringkat kitaran hidup. Analisa sitometrik aliran bagi sampel plankton menunjukkan ledakan ini bermula dengan pencambahan sista kerana kehadiran 88% planomiosetes (4C) telah diperhatikan dalam sampel. Planozygotes (2C) yang semakin meningkat, bertepatan dengan penurunan salinity dan peningkatan nishab N: P. Ekspresi gamet proses pembiakan seksual dalam populasi di bawah keadan ketandusan fosforus telah mendorong kepada pembentukan planozygotes. Keputusan daripada sitometri aliran mengesahkan bahawa ledakan tersebut bermula dari bahagian dalaman lagun dan disebarkan ke arah muara sungai. Kejadian ini telah dikekalkan selama empat bulan dengan peratusan sel vegetatif (1C) dalam julat 72−92% berbanding dengan 36% planozygotes (2C) pada awal ledakan. Keputusan daripada 48 jam persampelan mengesahkan bahawa sel menjalani kitaran sel dengan tetap dalam persekitaran semulajadi. Peningkatkan hujan dan kemasukan kepulan air tawar Sungai Golok ke dalam lagun dalaman menyebabkan penamatan ledakan. Kajian ini telah membawa kepada kefahaman asas mengenai kitaran hidup dinoflagellat toksik tropika yang penting ini.

Kata kunci: *Alexandrium minutum*, keracunan kerang-kerangan melumpuhkan, PCR masa sebenar, transkrip spacer dalaman kedua, sitometrik aliran, kitaran hidup, sista, planozygote, planomyocete.

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LIST OF SYMBOLS AND ABBREVIATIONS

ASP   :   Amnesic shellfish poisoning

CFP   :   Ciguatera fish poisoning

CV   :   Coefficient of variation

DSP   :   Diarrheatic shellfish poisoning

gDNA   :   Genomic deoxyribonucleic acid

FISH   :   Fluorescence *in situ* hybridization

HAB   :   Harmful algal bloom

ITS   :   Internal transcribed spacer

ITS2   :   Second internal transcribed spacer

LSU   :   Large subunit

N   :   Nitrogen

NSP   :   Neurotoxic shellfish poisoning

P   :   Phosphorous

PCR   :   Polymerase chain reaction

PSP   :   Paralytic shellfish poisoning

PST   :   Paralytic shellfish toxin

qPCR   :   Quantitative real-time PCR

rDNA   :   Ribosomal deoxyribonucleic acid

rRNA   :   Ribosomal ribonucleic acid

SSU   :   Small subunit

STX   :   Saxitoxin

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CHAPTER 1: GENERAL INTRODUCTION

Harmful algal bloom (HAB) is a phenomenon that commonly known as red tide, occurs when a group of harmful algae grow in high biomass in the marine or freshwater environments. There are three different types of HABs, which are algae that produce toxins and caused human seafood poisoning that lead to human illness and death; certain phytoplankton produce toxic that only harmful to fish by clogging and damage their gills; and algae which are non-toxic but caused harm by oxygen depletion that lead to massive fish kills (Hallegraeff, 1993; Anderson et al., 2002).

In Malaysia, several marine algal bloom events occurred has resulted in massive fish kills and shellfish poisoning that lead to human illness (Lim et al., 2012). Over the years, HABs have been increasingly reported in Malaysia, this included *Pyrodinium bahamense* bloom in 2001 and 2009 at Kota Kinabalu that caused shellfish contamination (Usup et al., 2002a; Lim et al., 2012), *Cochlodinium polykrikoides* bloom in 2006 at Kota Kinabalu with fish kill event in finfish maricultures (Anton et al., 2008) and blooms of *Karlodinium australe* at the western Johor Strait in early 2014 and 2015 which resulted in massive fish kills in the aquaculture farm (Lim et al., 2014; Teng et al., 2015). Massive fish kills in aquaculture due to HABs has resulted in economic losses to fisherman and shellfish poisoning such as paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP) may lead to human illness and death (Lim et al., 2012). In September 2001, *Alexandrium minutum* bloom at Tumpat cause six persons hospitalized including one fatality due to PSP after consumed benthic clams that collected from this coastal lagoon (Usup et al., 2002b; Lim et al., 2004).

The increasing case of HABs in Malaysia that affect public health and also economic losses to finfish maricultures introduced the important of HABs monitoring in the country. Nowadays, molecular tools have been widely applied in the efforts for rapidly

detection and estimate the cell concentration for monitoring purpose (Sellner et al., 2003; Kamikawa et al., 2006). Quantitative real time PCR (qPCR) is one of the approaches that have been widely used for detection of HABs species (Dyhrman et al., 2006; Kamikawa et al., 2007; Perini et al., 2011; Howard et al., 2012; Penna & Galluzzi, 2013). Recently, this method is also adopted in toxin gene detection for PSP producer species (Murray et al., 2011; Gao et al., 2015). qPCR is known to be rapid, highly specific and sensitive which can detect down to single vegetative cell in field sample (Sellner et al., 2003; Kon et al., 2015). Therefore, qPCR detection can alert the abundance of HAB species in water before seafood contamination and fish kills happen.

Furthermore, flow cytometry is another approach that used to study the life cycle of HAB species. This technique also widely used in cell cycle analysis and measurement of DNA content by stain the cell with fluorescent dye (Nunez, 2001). SYTOX Green is one of the DNA binding dyes that used to stain the nucleus of the cell and the stained cell will merged an amount of dye proportional to the amount of DNA in the nucleus (Nunez, 2001; Haberkorn et al., 2011). Flow cytometry was then measured the cell through the amount of fluorescent signal that emitted by the cell. In addition, in cell cycle analysis, flow cytometry able to estimate various cell cycle phases which are the G1-, S- (DNA synthesis phase), G2- and M-phase (mitosis) (Nunez, 2001). With the advantages of flow cytometry, this technique is widely used in cell cycle analysis of dinoflagellate (Bhaud et al., 2000; Figueroa et al., 2010) such as *Alexandrium fundeyense* (Taroncher-Oldenburg et al., 1997), *Alexandrium minutum* (Dapena et al., 2015; Figueroa et al., 2015), *Prorocentrum lima* (Pan et al., 1999) and also to investigate the distribution of different cell phase during HAB (McGillicuddy et al., 2014).

In this study, we focus on developing molecular detection tool of *A. minutum* for the monitoring at Sungai Geting, a semi-enclosed lagoon where *A. minutum* bloom exist. Besides that, investigations on life cycle of *A. minutum* in natural bloom by flow cytometry analysis enable to identify the distribution of the cells in various phases. By investigate the life cycle of bloom species in natural bloom, it provides better understanding of the relationship of bloom initiation (Anderson & Wall, 1978; Anderson & Morel, 1979), bloom termination (Probert, 1999), species dispersal (Anderson & Stolzenbach, 1985) and survival during forced resting periods (Probert, 1999).

The main aim of this study is to develop a molecular platform for the detection of harmful algal bloom species. The specific objectives of this study are as belows:

    i. To characterize the harmful and red tide-forming algal species, *Alexandrium minutum*, molecularly;

    ii. To develop species-specific primers of harmful blooming species, *Alexandrium minutum*, for molecular detection;

    iii. To investigate the relationship of different life cycle stages of *Alexandrium minutum* on bloom initiation and termination;

Thesis structure

This thesis was prepared in an 'Article Style Format' (as in Guidelines for the Preparation of Research Reports Dissertations and Theses, 2015), and consists of a total of five chapters. Chapter 1 introduces the general background of the study, explaining the occurrence of HABs in Malaysia that were associated with human intoxification and massive fish kills. This chapter also explained the approaches of qPCR and flow cytometry analyses that were developed for *Alexandrium minutum* in this study. Chapter 2 includes literature review that provided the scientific information of this study. In Chapter 3, the development of a SYBR Green I-based qPCR assay to detect the toxic *Alexandrium minutum* is detailed. A set of qPCR species-specific primers targeting the cells of *Alexandrium minutum* was designed, and the assay tested on the bloom samples collected from Sungai Geting, Tumpat. The potential application of this assay in HAB monitoring is discussed. Chapter 4 describes a study on investigating the life-history stages of *Alexandrium minutum* in natural bloom by flow cytometry analysis. Lastly, the general discussion of both methods is summarized in Chapter 5, with recommendation provided for future investigations.

CHAPTER 2: LITERATURE REVIEW

2.1 Harmful algae blooms (HABs)

Phytoplankton refers to microscopic organisms that are found in marine and freshwater ecosystems. They are free-floating plants that transported by current and tides. These microalgae are just like terrestrial plant; they contain chlorophyll pigments that enable them to undergo photosynthesis process (Hester & Harrison, 2011). They form the base of marine and freshwater food chain and serve as main food for shellfishes and also larvae for crustaceans and finfishes (Hester & Harrison, 2011). Besides that, they also play important role in regulating atmospheric carbon dioxide and transport them to the deeper water (Patricia et al., 2005). However, these microalgae may have negative impact to the ecosystem when they grow in mass in water or release certain toxin. These phenomena are known of HABs.

HAB can cause severe fish kill in finfish maricultures and human intoxification due to consumption on contaminated shellfish. Toxins that produced by HAB species associated with shellfish contamination are Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Amnesic Shellfish Poisoning (ASP), and Neurotoxic Shellfish Poisoning (NSP) (Anderson et al., 2012b). Exposure of these toxins not only will cause harmful to human but also cause illness or death to marine mammals, seabirds and other animals.

Apart from shellfish poisoning, some of the algae such as marine raphidophyte will cause mass mortality on wild and farmed fish due to the ichthyotoxin (Marshall et al., 2003; de Boer et al., 2012). These algae species will damage the gill of the fish by certain mechanism or through production of haemolytic substances (Marshall et al., 2003; Branco et al., 2014). This damage can cause great economic losses to aquaculture farm. Example of fish kill event that happened in 2014 and 2015 caused by *Karlodinium*

australe in Johor Straits, Malaysia, caused mass mortality in farmed and wild fishes (Lim et al., 2014).

2.2   Paralytic shellfish poisoning (PSP)

PSP is one of the effects of HAB which contributed by some of the dinoflagellates especially *Alexandrium* spp.. PSP are derivatives of alkaloid saxitoxin (STX) with at least 21 derivatives of this compound exist with different concentration and combinations (Hester & Harrison, 2011). In 1975, structure of STX was first studied and later found that this toxin are heat stable in acidic condition but unstable in alkaline condition (Bower et al., 1981). Paralytic shellfish toxins (PSTs) are highly lethal through consumption of contaminated shellfish (Brosnahan et al., 2014). Bivalve shellfish that feed by filtering food particles in water such as mussels, clams, oysters, scallops and cockles were identified in cases of PSP. When these shellfishes exposed to a toxic dinoflagellate bloom, they become the concentrators of toxin and clean themselves of saxitoxin through depuration. However, rates of intoxification and detoxification for these shellfishes are species specific. Symptoms of PSP that reported included feeling of lightness, dizziness, weakness, drowsiness, incoherence and headache (Acres & Gray, 1978; Backer & McGillicuddy, 2006). In mammals, this toxin will block the voltage-gated sodium channels in nerve cell membranes without affected the potassium channel and cause inhibition of impulses along the nerves that lead to paralysis, respiratory depression, and circulatory failure (Kao, 1993; Backer & McGillicuddy, 2006; Hester & Harrison, 2011). Marine dinoflagellates which involved in PSP are several *Alexandrium* spp. (*A. minutum*, *A. tamiyavanichii*, *A. fundyense*, *A. tamarense*, and *A. catenella*), *Pyrodinium bahamense* and *Gymnodinium catenatum* (Hester & Harrison, 2011).

In Malaysia, the earliest PSP event that had been documented was caused by *Pyrodinium bahamense* at Sabah since 1976 (Roy, 1977; Usup et al., 2012; Lim et al., 2012). In 1991, bloom of toxic *A. tamiyavanichii* in Sebatu, the Straits of Malacca contaminated mussels from a mussel farm and caused three people poisoned (Usup et al., 2002a). In 2001, an incident of human intoxication was reported due to *A. minutum* bloom in a semi-enclosed lagoon which situated at north-eastern of Peninsular Malaysia (Usup et al., 2002b; Lim et al., 2004).

2.3   The genus *Alexandrium*

Alexandrium is one of the genus under marine dinoflagellate which first discovered by Halim in Alexandria harbor, Egypt (Halim, 1960; Balech, 1989). This genus was originally described under a different genus name such as *Goniodoma*, *Gessnerium*, *Protogonyaulax*, *Gonyaulax* and *Pyrodinium* (Balech, 1989; Anderson et al., 2012a). After Halim discovered a small dinoflagellate and named the genus as *Alexandrium*, this name was adopted to the species that formerly referred as *Gonyaulax* due to similar morphology (Probert, 1999). For morphological description, Kofoidean plate pattern with tabular formation of Po, 4′, 6″, 5‴, 2⁗, 6c, and 9-10s was used (Balech, 1995). This genus has the largest number of toxic species which are responsible to paralytic shellfish poisoning (PSP) (Anderson, 1998; Dyhrman & Anderson, 2003). Of the more than 30 species described, at least half were known to be PST producer or have other harmful effect (Anderson et al., 2012a). Species that produced PST include *A. acatenella*, *A. catenella*, *A.cohorticula*, *A. fundyense*, *A. ostenfeldii*, *A. minutum*, *A. tamarense* and *A. tamiyavanichii*. These toxic species produce neurotoxin, saxitoxin and the derivatives (STXs) that associated with PSP (Anderson et al., 1990). Toxin production of *Alexandrium* differs among the species. In Malaysia, toxic *Alexandrium* that have been reported are *A. minutm* and *A. tamiayavanichii* associated with PSP cases (Usup et al., 2002a).

CHAPTER 3: DEVELOPMENT OF SPECIES-SPECIFIC QUANTITATIVE REAL-TIME PCR ASSAY FOR THE DETECTION OF TOXIC *Alexandrium minutum* (DINOPHYCEAE)

3.1 Introduction

HAB is a natural phenomenon due to the increase in density of microalgal species or some highly toxic cells that cause problems at low cell concentration in water environment (Anderson et al., 2012b; Lim et al., 2012). HABs are common in coastal marine ecosystems, but some HAB events have been encountered in open ocean. Blooms of several toxigenic algae can cause illness, death of fishes, seabirds, mammals and other marine life (Anderson et al., 2012b). Consumption of these contaminated shellfish and fishes by human caused poisonings, [i.e. NSP, ciguatera fish poisoning (CFP), DSP, PSP and ASP], suffering severe toxic symptoms and fatalities in extreme cases (Anderson et al., 2012b). Besides that, impacts of HABs not only constrained to public health issues, but also economic losses that attributed to reduced tourism and seafood related industries.

In Southeast Asia countries, PSP is a predominant threat to seafood consumers, and Malaysia has no exception. In Malaysia, marine dinoflagellates that produced PST included *Pyrodinium bahamense*, and several species in the genus of *Alexandrium* (Usup et al., 2002a; Lim et al., 2012). The toxicity mechanism of this group of toxins is to block the sodium-channel of mammalian nerve cells, inhibiting the transmission of action potentials in nerve axons and skeletal muscle fibres.

Alexandrium minutum Halim is one of the PST producers in the genus *Alexandrium*. This species was distributed globally and frequently associated with PSPs events (Mediterranean Sea: Garcés et al., 2004; Vila et al., 2005). In Malaysia, *A. minutum* bloom was reported in 2001 (Lim et al., 2004) and 2015 with PSP event recorded in

2001. Previous studies indicated that the tropical *A. minutum* have higher tolerance toward salinity changes, and the toxicity was higher in low salinity (Lim & Ogata, 2005).

The conventional method that used to identify *Alexandrium* spp. was direct observation under microscope, and identify based on their thecal morphological characteristics (Balech, 1995; Sellner et al., 2003). However, this technique is tedious and time-consuming, and required trained personale in phytoplankton identification. Therefore, some of the alternative molecular approach such as fluorescence *in situ* hybridization (FISH) coupled with FLOWCAM fluid imaging system or flow cytometry, and real-time quantitative PCR (qPCR) detection were developed. Most of the techniques used include FISH and qPCR detections required species-specific primers and probe to specifically identify the species. Ribosomal RNA (rDNA) gene region included small subunit (SSU), internal transcribed spacer (ITS), and large subunit (LSU) were the regions that commonly used to select the species-specific primers and probe sites. These regions were widely chosen due to their universally highly conserved nucleotide regions coupled with the variable regions, as well the availability of huge database for sequence comparative analysis (Amann & Ludwig, 2000; Ki & Han, 2006; Litaker et al., 2007). The second internal transcribed spacer (ITS2) that exhibits conserved secondary structure has made it a valuable marker and phylogenetic inferences (Keller et al., 2009; Ankenbrand et al., 2015). Due to the conserved structure in ITS2, it has been proposed in species barcoding (Litaker et al., 2007). Therefore, ITS2 secondary structure for *A. minutum* was constructed in this study to determine the potential site for species-specific primer design.

At present, qPCR assay is an extremely sensitive molecular tool compares to FISH that used identify and estimated the density of targeted phytoplankton species in natural

samples (Penna & Galluzzi, 2013). This method was widely applied in detecting toxic marine microalgae and also toxin gene (sxtA4) in marine dinoflagellates (Murray et al., 2011; Gao et al., 2015). Galluzzi et al. (2004) developed a genus-specific primer based on the 5.8S region of *Alexandrium* spp. and applied for *A. minutum* quantification in field samples. As the assay developed previously was not discriminative enough to the species level, the assay may have limitations when analysing samples collected from areas where several *Alexandrium* spp. co-existed.

In this study, a SYBR Green I-based qPCR assay was developed to detect and quantify the toxic *A. minutum* cells, with the species-specific primers developed from a specific fragment of the ITS2 rDNA. The specificity of the primer pair was tested *in silico* and further confirmed by applying the assay on clonal cultures of different *Alexandrium* species and other phytoplankton species. The accuracy of the assay was further evaluated by using natural seawater samples that spiked with known number of target cells. The assay was later used to test on bloom samples during an event in 2015 at Sungai Geting, Tumpat.

3.2 Literature Review

In the past decade, *Pyrodinium bahamense* is the main PSP toxin-producing species that found in Malaysia. Until 1991 and 2001, *A. minutum* and *A. tamiyavanichii* were found as PSP toxin-producer and cause human intoxification. Thus, it is important to identify and monitor the potential toxic phytoplankton species that are present.

Nowadays, various molecular method such as whole-cell FISH, sandwich hybridization assay, biosensor detection, and quantitative real time PCR assay were developed for HAB species identification and monitoring purpose (Humbert et al., 2010; Penna & Galluzzi, 2013). Among current technologies, real-time PCR assay was proved to be an effective tool for rapid detection and enumeration on harmful phytoplankton species (Handy et al., 2008; Ebenezer et al., 2012). This assay was mostly applied to *Alexandrium* spp. due to the PSP cases that happen frequently (Penna & Galluzzi, 2013).

In silico species specific primer design is a technique that needed for polymerase chain reaction (PCR). A good pair of primer is especially important in qPCR to amplify targeted DNA and prevent amplification of unintended targets (Ye et al., 2012). Primer should design with amplicon length less than 150 bp for efficient amplification in qPCR (Arya et al., 2005; Arvidsson et al., 2008; Ye et al., 2012). Major steps that involved in primer design are identifying short region which is usually between 18 to 30 nucleotides in length in a sequence alignment unique to target group of interest, 40 to 60% of GC content, with more than one mismatches to non-target organism, both forward and reverse primer should have similar melting temperature or within 2°C, formation of secondary structure and primer complementarity should minimize to prevent primer dimer (Arya et al., 2005; Penna & Galluzzi, 2008; Ye et al., 2012).

Nuclear-encoded ribosomal RNA gene (rDNA) was used as a region for primer design due to the highly conserved and variable sequence regions (Adachi et al., 1996).

In addition, rDNA contains large sequence of data bases which are publicly available and it is also a naturally amplified target molecule (Wallner et al., 1993). Nuclear-encoded rDNA contain 18S rDNA, large subunit (28S), internal transcribe spacer (ITS1 and ITS2) and 5.8S rDNA. These regions were used to determine the divergence at inter- and intragenic levels of phytoplankton population (Penna & Magnani, 1999; Litaker et al., 2007). Among these regions, ITS region was found to have higher variability and use as a marker in phylogenetic analysis and also species specific molecular assay since last decade (Adachi et al., 1996; Leaw et al., 2005; Litaker et al., 2007). Therefore, ITS region was adopted for species specific primer design. For primer design process, there are a number of public software tools available such as Primer-Blast, Primer3 program, and Primer express. These programmes help to design target-specific primers rapidly with all parameters include. Primer that developed was then applied in qPCR assay for detection and quantification.

Quantitative qPCR assay was carried out by amplification of specific genomic DNA sequences with specific primer and the formation of product was monitored by measuring the fluorescence signal (Arya et al., 2005; Penna & Galluzzi, 2008; Penna & Galluzzi, 2013). There are two approaches in qPCR assay which are SYBR green based and Taqman probe-based assay. The fluorescence in SYBR green-based qPCR assay was generated by using an intercalating fluorescent dye which is cheaper and simplest approach. SYBR green-based qPCR assay are highly sensitive, however, the disadvantage is false positive results such as primer dimer or non-specific amplified sequence might obtaine if the primer is not specific (Saunders, 2004; Penna & Galluzzi, 2008; Penna & Galluzzi, 2013).

The Taqman probe-based qPCR assay is based on design of oligonucleotide probe that located between two primers for qPCR amplification. The Taqman probe required a

fluorescent reporter dye and a quencher dye that conjugated at 5′ and 3′ end (Livak et al., 1995; Arya et al., 2005). As the proximity of these two dyes is close, the fluorescent of the reporter dye is quenched by other through fluorescent resonance energy transfer. At the extension step of qPCR, the probe was degraded by DNA polymerase and separates the reporter from quencher with increase in fluorescence emission (Arya et al., 2005; Penna & Galluzzi, 2008; Penna & Galluzzi, 2013). The additional specificity that provided by the probe reduce the false positive but is more costly compare to SYBR-based assay. Multiplex PCR can be carried out using Taqman probe-based assay by label the probe with different fluorophores. qPCR technique can be applied to all phytoplankton species with DNA sequence data available for primer and probe designed. The main advantage for this technique is the sensitivity and specificity to monitor the harmful phytoplankton by using preserved environmental sample. Besides that, this method also reduced working time for species identification and cell enumeration on certain species using microscope.

3.3 Materials and Methods

3.3.1 Algal cultures

Clonal cultures of *Alexandrium* species and microalgae used in this study are listed in Table 3.1. The cultures were maintained in ES-DK medium (Kokinos & Anderson, 1995) (Appendix A). Cultures were maintained at $25.0 \pm 0.5°C$ with a 12:12h light: dark cycle regime, and light intensity of ~100 µmol photons $m^{-2} s^{-1}$.

Table 3.1: Clonal culture of algae used in this study.

Species	Strain	Locality
Alexandrium affine	AaBK06	Bachok, Kelantan
A. leei	AlKK01	Kota Kinabalu, Sabah
A. minutum	AmTm15	Tumpat, Kelantan
	AmTB01	Tok Bali, Kelantan
	AmKB02	Tumpat, Kelantan
A. tamiayavanichii	AcTm01	Tumpat, Kelantan
A. tamutum	AuBK03	Bachok, Kelantan
Chattonella marina v. *antiqua*	ChMi01	Miri, Sarawak

3.3.2   Genomic DNA extraction

Genomic DNAs (gDNAs) of *A. minutum* cultures were extracted as described in Leaw et al. (2010). Approximately 150−200 mL mid-exponential phase cultures were harvested by centrifugation (2,800 ×*g*, 10 min). Cell pellets were rinsed with ddH$_2$O and resuspended in 60 µL of 10× NET lysis buffer [3 mL of 5 M NaCl, 20 mL of 0.5 M EDTA, 10 mL of 1 M Tris-HCl (pH 8.0) and 40 mL ddH$_2$O], 60 µL of 10% sodium dodecyl sulphate (SDS), and 480 µL of sterile ddH$_2$O. The mixtures were vortexed and incubated at 65°C for 30 min. Total of 100 µL of 10% cetyltrimethylammonium bromide (CTAB) and 80 µL of 5 M NaCl were added to the sample. The mixture was well mixed and incubated in water bath for 10 min at 65°C.

Subsequently, 700 µL of Chloroform/Isoamyl alcohol (C:I; 24:1) were added. The viscous and clear supernatant (aqueous DNA layer) were carefully transferred and precipitated for two to three times using 700 µL of Phenol/Chloroform/Isoamyl alcohol (P:C:I; 25:24:1) and followed by cold C:I extraction. Then, genomic DNA was precipitated by addition of one volume of absolute ethanol (EtOH) and 0.1 volume of 3 M sodium acetate (NaOAc, pH 5.0). The mixture was stored in 20°C for >3 h. DNA pellet was rinsed with cold 70% ethanol and dissolved in TE buffer (10mM Tris-HCl, pH 7.4 and 1mM EDTA, pH 8.0) for further analysis (Leaw et al., 2010).

3.3.3   Amplification of ITS2 rDNA region

Polymerase Chain Reaction (PCR) was carried out to amplify the targeted ribosomal DNA (rDNA). The internal transcribed spacer (ITS) region for rDNA was amplified by using the genus specific primer pair, Alex_ITSf1 and Alex_ITSr1 (Table 3.2). The PCR mixtures of 25 µL contained 1× PCR buffer, 2 mM MgCl$_2$, 0.02 µM of each primer, 0.2 mM of each dATP, dTTP, dCTP and dGTP, 2.5 U *Taq* polymerase and <100 ng µL of gDNA. Amplification was performed by using the peqSTAR thermal cycler (PEQlab,

Germany) with optimal PCR conditions (Table 3.3). Amplicons were further purified by MoBio UltraClean® PCR clean up kit (Mo Bio laboratories, USA) according to the manufacturer's instruction. Purified amplicons were sent to private sequencing laboratory for DNA sequencing (First Base, Selangor, Malaysia). Sequencing was performed using an ABI 3700XL automated DNA sequencer (Applied Biosystems, USA), with both strands sequenced.

Table 3.2: Primer pairs used in this study for ITS rDNA amplification.

Primer	Sequence (5'–3')	Direction
Alex_ITSf1	GAG GAA GGA GAA GTC GTA ACA AGG	Forward
Alex_ITSr1	GCA TTC AAT GC CRA GGA RTG	Reverse

Table 3.3: Optimal condition of PCR for Alex_ITSf1/ Alex_ITSr1 primers.

Process	Temperature (°C)	Duration (min)
Initial denaturation	94.0	5.0
Denaturation	94.0	0.3
Annealing	54.0	0.45
Extension	72.0	1.30
Final extension	72.0	7.0

3.3.4 Development of *Alexandrium minutum* species-specific qPCR primers

3.3.4.1 ITS2 secondary structure prediction

The sequence of ITS rDNA of *A. minutum* that obtained in this study was used to construct ITS2 secondary structure. The ITS2 secondary structure of *A. minutum* was modelled using RNAStructure v. 5.7 (Reuter & Mathews., 2010) and illustrated by VARNA (Darty et al., 2009). The sequence structure modelled was used as a template

for homology modelling, using the ITS2 Database V (Koetschan et al., 2010; Merget et al., 2012; Koetschan et al., 2012; Ankenbrand et al., 2015).

3.3.4.2 Species-specific qPCR primer design

The ITS rDNA sequences of *A. minutum* obtained in this study was multiple aligned with other *Alexandrium* species that retrieved from NCBI nucleotide database, GenBank and also in this study. Potential species-specific primer regions with amplicons size of ≤150 bp, melting temperature (Tm) of 58–60 °C, GC content of 45–65 % were chosen. Selected primer set was then analyzed *in silico* using IDT OligoAnalyzer 3.1 (http://sg.idtdna.com/calc/analyzer). BLAST search was performed to determine potential cross-reactivity of the primers with non-target species. The potential primer set was then synthesized by private laboratory (IDT Inc., Singapore) for qPCR detection.

3.3.5 qPCR primer specificity

Specificity of *A. minutm* primer pairs was determined by conventional PCR and quantitative real-time PCR (qPCR) using the gDNAs from cultures of *A. minutum* and non-target species (Table 3.1). The gDNAs were amplified using ITS rDNA primer pairs, Alex_ITSf1 and Alex_ITSr1, to confirm the positive amplification. Amplification was performed by using the peqSTAR thermal cycler (PEQlab, Germany). The optimal PCR conditions for the *A. minutum* species-specific primer pair was 95 °C for 4 min, 35 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 90 s, final extension of 72 °C for 7 min. Amplicons were purified using MoBio UltraClean® PCR clean up kit (Mo Bio laboratories, USA) prior to DNA sequencing. In addition, the specificity of the primer pair was also tested in qPCR assay.

3.3.7 SYBR Green I-based qPCR assay

The qPCR assay was performed on MyGo Pro real-time PCR instrument v.3.0.31 (IT-IS Life Science Ltd, Ireland). No-template control (NTC) was included in each assay. All samples were run in triplicate. The qPCR assay was run using KAPA SYBR® FAST qPCR kit Master Mix Universal (KAPA Biosystems) in a total reaction volume of 20 µl. The optimized qPCR mixtures contained 250 nM of each forward and reversed primer and incorporated 2 µl of gDNA template in each reaction. The qPCR cycling condition was as follows: 3 min hold time at 95 °C, followed by 50 cycles of 10 s at 95 °C, and 30 s at 60 °C.

3.3.8 Construction of gene-based calibration curve

A calibration curve using synthetic GBlocks® gene fragment (also referred as gene-based calibration curve) was constructed by using 10-fold serial dilutions of the synthetic gene fragment of target species. The gBlocks® gene fragment containing the target sequence of *A. minutum* ITS2 rDNA, was synthesized (Integrated DNA Technologies, IA, USA). The synthetic gene fragment flanked the *A. minutum* species-specific primer pair with extra 10 bp long at both ends and re-suspended in 250 µl TE buffer (Tris 1 M, EDTA 0.5 M, pH 8) to obtain a stock concentration of 1 ng µl⁻¹. The copy number of gene fragment in 1 ng µl⁻¹ was calculated as:

$$\frac{PCR\ amplicon\ concentration\ \left(\frac{ng}{\mu L}\right) \times 6.022E23\ \frac{molecule}{mole} \times 1\ target\ sequence\ per\ molecule}{Amplicon\ length\ (bp) \times 650\ \frac{g}{mole}\ for\ 1\ bp\ of\ doube\ stranded\ DNA\ \times 1E9\ \frac{ng}{g}}$$

Stock solution of gene fragment was diluted to 1×10^8 gene copies, followed by ten-fold serial dilutions (1×10^2 to 1×10^8 gene copies), were performed in triplicate qPCR runs.

The calibration curve was constructed using GraphPad Prism v. 5.03 (GraphPad Inc., USA) by plotting triplicate C_q values against the log-transformed copy numbers. A linear regression was performed onto the calibration curve to determine the R^2 value and slope. Amplification efficiency (AE) was calculated as $AE=[10^{(-\frac{1}{slope})} - 1] \times 100\%$.

The gene-based calibration curve was used to determine the total extractable gene copies per cell in the samples.

3.3.9 Determination of extractable gene copies per cell

The extractable mean of ITS2 copies per cell of *A. minutum* was determined using qPCR based on 22 independent samples ranging from 3 to 4806 cells ml^{-1}. Microscopic cell count was carried out to determine the cell densities of the samples. Copy number of ITS2 was defined as the slope of linear regression that detected by qPCR based on cell densities by microscopic count and gene-based calibration curve. The extractable gene copy number per cell was defined based on the slope of the linear regression.

3.3.10 Field application of qPCR assay

Five samples were collected from Sungai Geting, Tumpat, during *A. minutum* bloom between September to November 2015 (Figure 3.1). Water samples were collected using 20μm mesh plankton net that hauled on the surface of the water. Water samples collected was then sieved through 10 μm mesh sieve and preserved in modified saline ethanol (Miller & Scholin, 2000). Samples were kept at −20 °C until further analysis.

Aliquot sample of 300 μl were extracted using PowerPlant® Pro DNA isolation kit (Mo Bio laboratories, USA). Known copies of synthetic DNA was included as a positive control in every qPCR run to assess if C_q was delayed. Total cell abundance for every stations was calculated by included the preserved volume and total sample volume to determine the potential cell densities during *A. minutum* bloom.
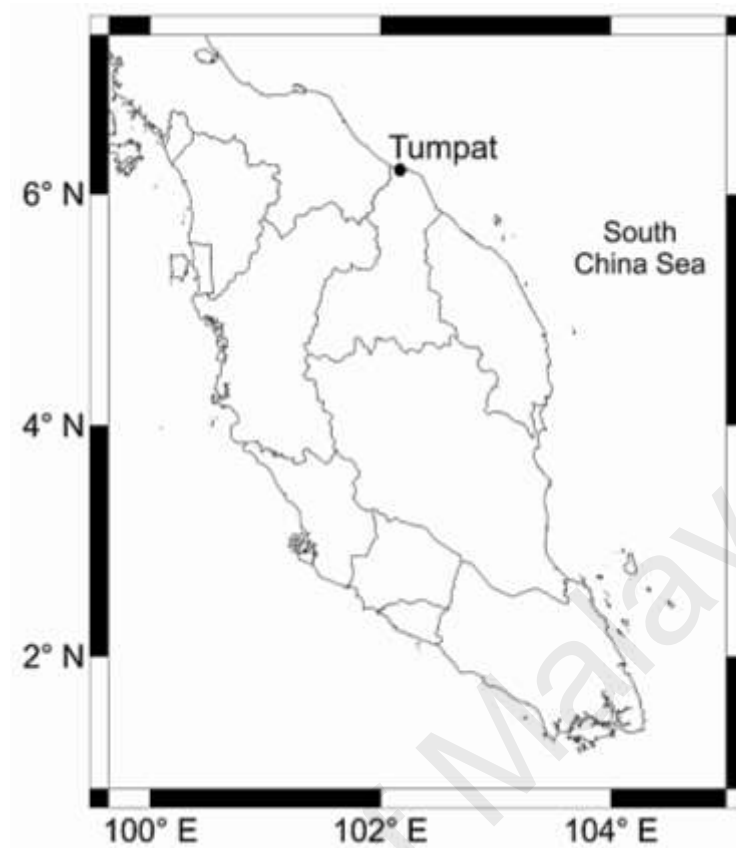
Figure 3.1: Map showing the sampling site of Tumpat, Malaysia

3.4 Results

3.4.1 *Alexandrium minutum* ITS2 RNA transcript and molecular signature

The ITS2 secondary structure for *A. minutum* was evolutionarily maintained and conserved with a total sequence length of 215 bp (Figure 3.2). The structure of *A. minutum* was confirmed by examine the 3′ and 5′ termini of ribosomal 5.8S and 28S rRNA, also known as proximal stem. The ITS2 universal motifs were confirmed with the presence of universal conserved motif U–U mismatch at helix II, AAA motif located in between Helix II and helix III, and UGGU motif along the apex of helix III. Molecular signature for *A. minutum* was selected in ITS2 region as this region showed high sequence variability. Species-specific primers site for qPCR detection were identified in between helix I to helix IV with maximum 14 mismatches to other *Alexandrium* spp..

Figure 3.2: Secondary structure of ITS2 and the molecular signatures. Locations of species-specific primer set (AmSF2, AmSR2) for qPCR show high variability.

3.4.2 *Alexandrium minutum* species-specific qPCR primer

Sequences of *A. minutum* (AmTm15 and AmTB01) that obtained in this study were multiple aligned (Appendix B) with their closely related species using MUSCLE program (https://www.ebi.ac.uk/Tools/msa/muscle/) and edited using Cluster X (Thompson et al., 1997). A total of 60 ITS rDNA sequences from different *Alexandrium* species (*A. minutum* from Mediterranean, *A. affine*, *A. andersoni*, *A. catenella*, *A. tamarense*, *A. diversaporum*, *A. fraterculus*, *A. fundyense*, *A. insuetum*, *A. leei*, *A. lusitanicum*, *A. margalefii*, *A. mediterraneum*, *A. ostenfeldii*, *A. peruvianum*, *A. pacificum*, *A. pseudogoniaulax*, *A. tamiyavanichi*, *A. taylori*, *A. tamutum*) were retrieved from Genbank database (NCBI) and in this study (Appendix C).

Potential species-specific primers site were selected in ITS2 region with total amplicon length 147 base pairs (bp). Length of the selected primers was in the range of 18 − 21 bp. *In silico* analysis for both forward and reversed primer show that GC content of the primers were in the range of 47−50% and Tm in the range of 60−62.3°C. Confirmatory test of primers specificity with other non-target species was performed by blasting in nucleotide databases. The Blastn results showed that the primers selected were specific to *A. minutum* with 100% coverage and identity with *A. minutum* from China (Genbank accession: JF906998 and DQ176668). Sequence alignment of both forward and reversed primers with European *A. minutum* sequences showed 7 to 8 mismatch. The selected primer pair that used in this study was listed in Table 3.4.

Table 3.4: Primer sequences for qPCR assay used in this study.

Primer	Sequences (5´→3´)	Length	Melting temperature (Tm ,°C)	GC content (%)
AmSF2	ACCTTCCCTTGCTGTGTA	18	60.4	50
AmSR2	CTACATGCCCAAACACATTGC	21	62.3	47.6

3.4.3 Assay specificity

Specificity test of *A. minutum* species-specific primer pair using the conventional PCR showed that only gDNA from *A. minutum* produced amplicons with bands clearly seen in the agarose gel (Figure 3.3), while no amplification was observed for gDNAs of other non-target species and the negative control (NTC) (Figure 3.3B). The gDNAs used in this test were initially confirmed by amplifying the ITS rDNA using Alex_ITSf1 and Alex_ITSr1 primer pair, and all gDNAs showed positive amplification (Figure 3.3A). The amplicons with expected size were further confirmed by DNA sequencing.
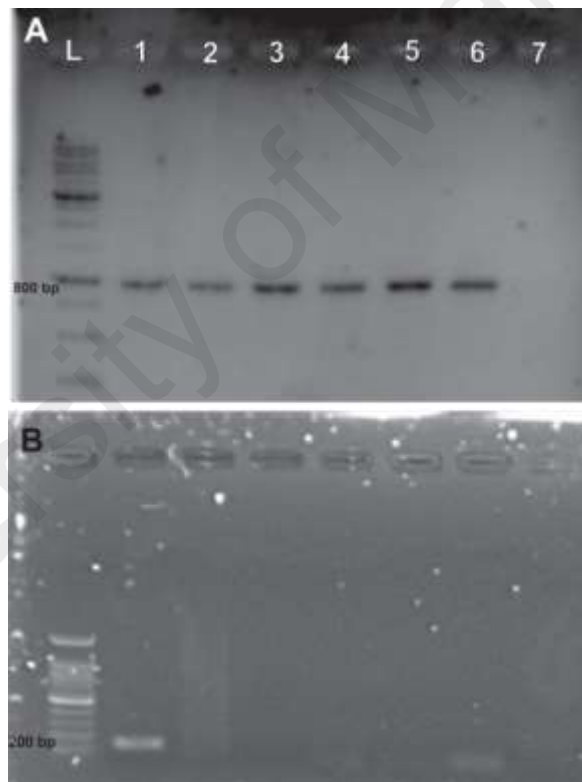
Figure 3.3: Primers cross-reactivity test by the conventional PCR using gDNAs of (1) *Alexandrium minutum*, (2) *A. tamiyavanichii*, (3) *A. affine*, (4) *A. leei*, (5) *A. tamutum*, (6) *Chattonella marina* var. *antiqua*, and (7) negative control (NTC). (A) PCR amplification using the ITS rDNA primer pair confirmed the presence of gDNAs from all species tested, 1kb DNA ladder was used. (B) Cross-reactivity test using *Alexandrium minutum* species-specific primer pair designed in this study showed only gDNAs from target species produced amplification, 100bp DNA ladder was used.

A. *minutum* species-specific primer pair was then tested with the qPCR assay with target and non-target species. Result showed that only gDNAs from A. *minutum* were successfully amplified (Figure 3.4).
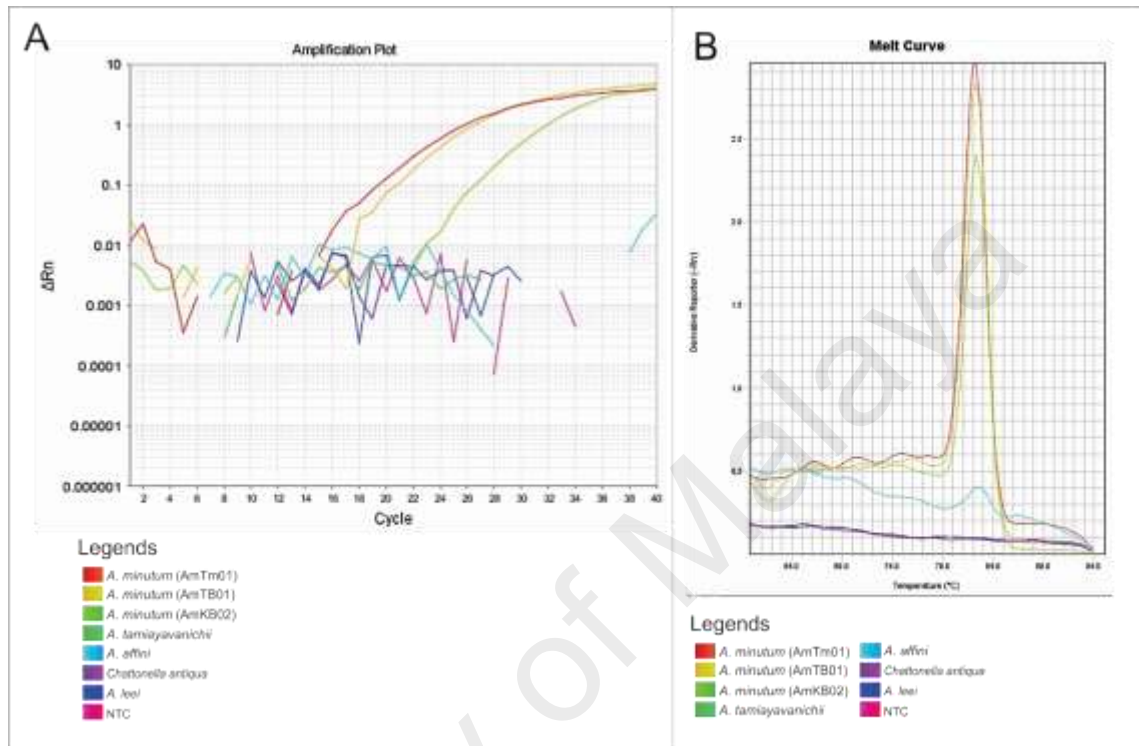
Figure 3.4: Validation of *Alexandrium minutum* species-specific primer pair by qPCR analysis. Only templates containing *Alexandrium minutum* showed positive amplification. (A) Amplification plot of *Alexandrium minutum* specificity test. (B) Melting curve analysis of targeted and non-targeted species.

3.4.4 Gene-based calibration curve

The gene-based calibration curve was constructed with 10-fold serial dilutions with dynamic range from 10^8 to 100 copies. The slope produced from gene-based calibration curve had a strong linear relationship between C_q and the log of copy number ranging over seven-orders of magnitude ($R^2 = 0.993$, AE = 97.6%; Figure 3.5). Although a slight deviation was observed at lower gene copy number (100 copies), the qPCR assay still can detect as low as 100 gene copies.
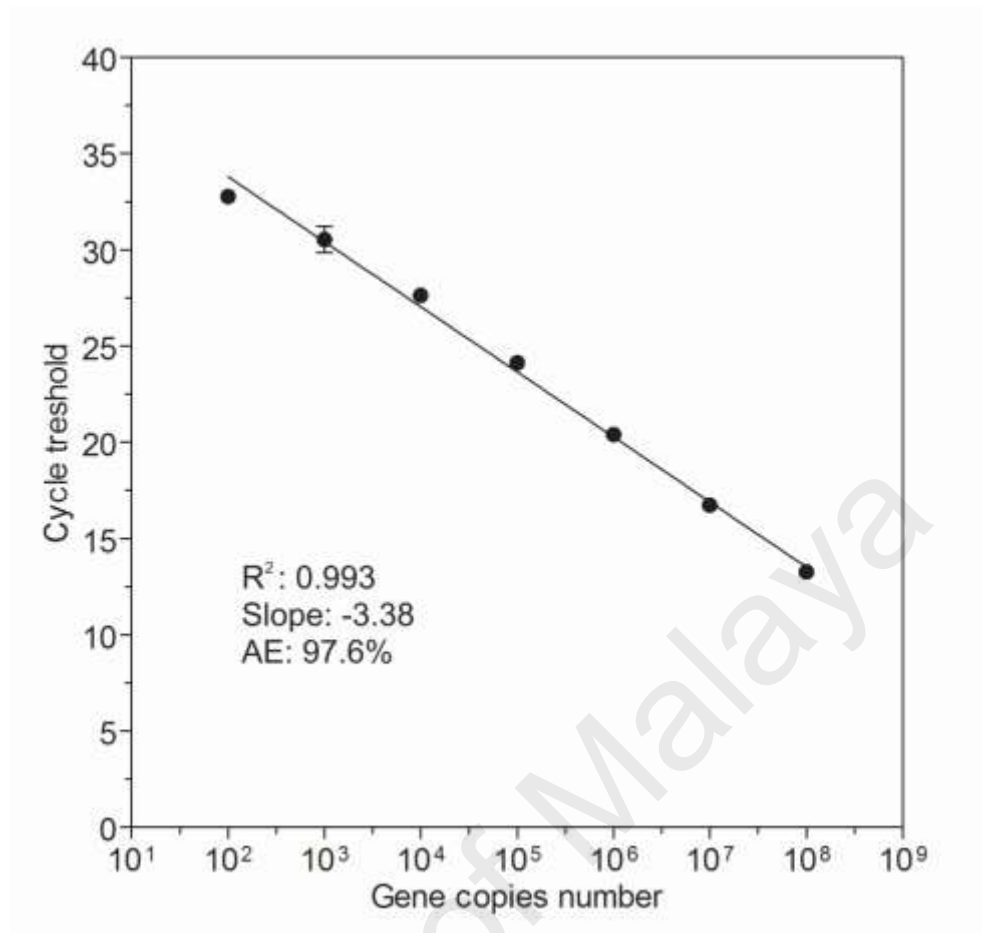
Figure 3.5: Gene-based calibration curves of the cycle threshold generated using 10-fold serial dilutions of *Alexandrium minutum* ITS2 synthetic gBlock® gene fragment.

3.4.5 Determination of extractable gene copies per cell

To obtain the gene copies per cell of *A. minutum* and evaluate the assay sensitivity, a lower range of cell dilutions (1, 3, 5, 7, and 10 cells ml^{-1}) were prepared. However, the C_q for a single cell detection was not consistent and fell beyond 40 reaction cycle. Samples with 3 cells were consistently amplified with a C_q within the linear dynamic range (30.58±0.22). Therefore, the lower limit of detection (LOD) of this assay was 3 cells. The mean of extractable ITS2 copy numbers per *A. minutum* cell was 7,584 ± 1,139 (slope ± SD) ($R^2 = 0.99$, *P*<0.0001).

3.4.6 Assay accuracy

The assay accuracy was tested using samples with known target cell densities through microscopic cell counts. *A. minutum* cultured cells were spiked into environmental samples and extracted using MoBio PowerPlant® Pro DNA isolation kit based on the procedure specified. The actual *A. minutum* cell densities obtained by microscopic counts was correlated with the potential cell densities obtained based on the gene-based calibration curves ($R^2 = 1.00$, $P < 0.0001$) (Figure 3.6). Deviation between actual cell and potential cell density were observed when the cell is equal or more than 5 with correction factor calculates as 0.5 and coefficient variation for actual and potential cell density are 257.6 and 259.6. Therefore, the quantification in this study was performed by considered the correction factor.
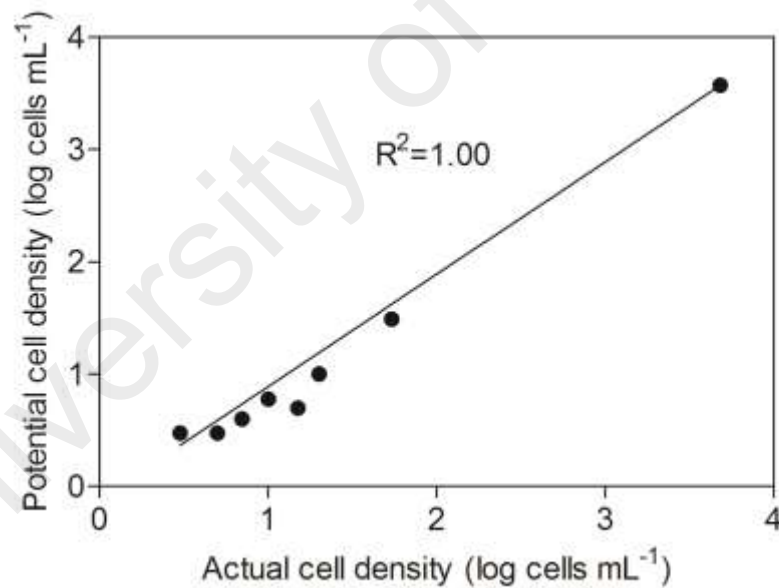
Figure 3.6: Correlation between the actual cell densities based on microscopic cell counts and the potential cell densities determined by the qPCR assay.

3.4.7 Field application of qPCR assay

Alexandrium minutum bloom was observed in Sungai Geting lagoon from September to November 2015. Five samples that collected randomly during the bloom period were used to examine the efficiency of *A. minutum* SYBR Green-I based qPCR assay that developed to the field samples. The density of *A. minutum* in field samples were determined by qPCR assay based on the number of ITS2 gene copies per cell. The results showed that the density of *A. minutum* estimated by qPCR after considered the correction factor was significantly correlated with the microscopic counts (Spearman r = 0.9, P = 0.0417*). Overestimation and underestimation on cell densities based on qPCR were observed in samples 2, 3, 5 (CV, 4.7−8.3%) and samples 1, 3 (CV, 6.4−13.1%), respectively (Figure 3.7).
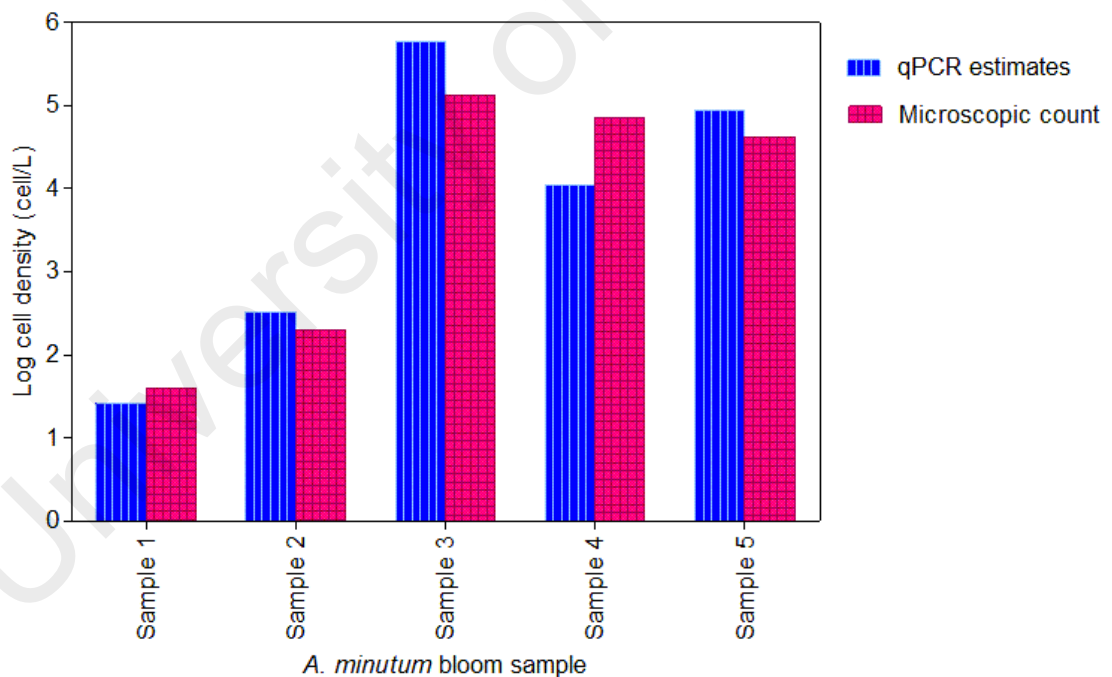
Figure 3.7: Cell densities of *Alexandrium minutum* based on qPCR (blue) and microscopic (red) cell count.

3.5   Discussion

3.5.1   Development of *Alexandrium minutum* qPCR assay

A species-specific primer was successfully developed based on ITS2-rDNA region for the detection of *A. minutum*. ITS2-rDNA gene was selected due to its universally high conserved nucleotide regions coupled with the variable regions and also constantly used as a marker to discriminate microalgae at the interspecies level (Connell, 2002; Ankenbrand et al., 2015). The higher divergence in this region may reduce false positive when applied to environmental samples (Park et al., 2007; Park et al., 2009; Yuan et al., 2012; Kon et al., 2015).

The specificity of the primer was confirmed by demonstrating no amplification on other *Alexandrium* spp. (*A. leei*, *A. tamiyavanichii*, *A. affine*, and *A. tamutum*) that found in Sungai Geting. Screening of the primer sequences in Genbank database also confirmed that the primers did not show any cross reactivity with other species, particularly *A. minutum* from European waters. According to Leaw et al. (2005), Malaysian *A. minutum* were clustered with New Zealand strains that formed a monophyletic group.

In this study, gene-based calibration curve using the synthetic gene fragment was constructed and served as an alternative calibrator in qPCR quantification (Kon et al., 2015). The gene copy number per cell for *A. minutum* was estimated based on single cell isolation by isolating different number of cells and triplicate run was performed. It is important to note that the average gene copy number per cell for *A. minutum* estimated in this study was not an absolute value; as an empirical relationship was applied to convert the total extractable gene copies to cell estimates with the same gDNA extraction method applied to all samples throughout this study. Therefore, we assumed that the extraction

efficiency were normalized throughout the treatment and provided a comparable gene copies per cell and estimated cell density.

3.5.2 Quantification of *Alexandrium minutum* in field bloom sample

A. minutum is one of the PST producing species that found in a semi-enclosed lagoon at east coast of Peninsular Malaysia since 2001. This species was responsible to HAB event that reported in 2001 cause six person hospitalized with one fatality after consumed benthic clam, *Polymesoda* sp., that collected in the lagoon (Usup et al., 2002b; Lim et al., 2004). In 2015, the bloom was observed in the lagoon and contaminated shellfishes. The real-time PCR-based assay described in this study was proved to be specific and sensitive for detection of *A. minutum* in cultured and environmental samples.

The cell density for environmental samples that examined in this study was estimated by qPCR and microscopic cell count. Spearman correlation analysed showed that there is no significantly different between qPCR counts and microscopic counts (P <0.05). Besides that, coefficient of variation (CV) of qPCR and microscopic counts between 5 samples with the value ranging from 5% to 13% also supported that they are not significantly different, a factor that used to explained overestimation and underestimation in qPCR count is changes of cellular DNA content during *A. minutum* bloom (Galluzzi et al., 2004; Galluzzi et al., 2010).

In natural bloom, DNA content of single cell can be higher or lower depend on the stage of cell in life cycle. This finding was confirmed by previous evidence (Anglès et al., 2012; Dapena et al., 2015) and also in my following chapter that cell with high DNA content such as planozygote and planomeiocytes cells were detected using flow cytometry on certain date during the bloom.

In between the bloom, some of the diatoms (*Skeletonema* spp. and *Chaetocerus* spp.) and also green algae (*Tetraselmis* spp.) were found coexist in the water. Coexist of these phytoplankton may affect the DNA extraction efficiency which high concentration of non-target DNA was obtained compared to targeted DNA. The limitation of using column-based DNA extraction kit was the DNA tends to loss in the column and gives insufficient yield of DNA (Rohland & Hofreiter, 2007; Rohland et al., 2010). This is because DNA tends to loss during centrifugation and washing step (Abdel-Latif & Osman, 2017). Therefore, magnetic-bead based extraction method was suggested which the DNA will bind to the bead using a permenant magnet. This method reduced the contamination and also yields high quality and quantity of DNA (Berensmeier, 2006). Besides that, surface water that contained humic acid, phenolic compounds and heavy metals that known to be PCR inhibitors also one of the main factor that affect the accuracy of qPCR (Wilson, 1997; Park et al., 2007; Park et al., 2009; Staggs et al., 2013).

In this study site, different *Alexandrium* spp. (4−5 species) were found co-existed in the water. Therefore, possible misidentification of *Alexandrium* under light microscope during the process of cell enumeration might over-estimate cell densities of *A. minutum* in plankton samples. The qPCR assay that developed in this study was optimized with species-specific primers for *A. minutum* and able to detect cells at extremely low abundance (3 cells per reaction). In conclusion, the specificity and sensitivity of the assay provides a rapid detection method to identify and enumerate this toxic species especially during the bloom initiation stage for HAB monitoring.

CHAPTER 4: FLOW CYTOMETRY ANALYSIS OF A TOXIC *Alexandrium minutum* BLOOM: INSIGHTS INTO THE BLOOM DYNAMICS OF A TROPICAL DINOFLAGELLATE SPECIES

4.1 Introduction

Harmful algal bloom (HAB) is a natural phenomenon which had been increasingly reported in Malaysia over the years. Paralytic Shellfish Poisoning (PSP) is one of the effects of HAB which contributed by some of the dinoflagellates that produce saxitoxins (STXs) (Anderson, 1998). PSP toxin that contaminated shellfish can cause paralysis or death through consumption (Brosnahan et al., 2014). In Malaysia, PSP events have been documented since last few decades. In 2001, PSP event reported due to *A. minutum* bloom at north-eastern of Peninsular Malaysia caused six persons hospitalized including one fatality after consumed contaminated benthic clams (Lim et al., 2004)..

Growth of *A. minutum* is mainly achieved by asexual division. In bloom condition, sexual behaviour is necessary for the species to form cyst when the environmental condition is insufficient (Garcés et al., 2004). Sexual transition of *A. minutum* in bloom condition plays an important role during initiation and termination of the bloom (Anderson et al., 1983; Brosnahan et al., 2014). Therefore, detection of sexual transition of the cells through different DNA content is important to predict the different life cycle stages of the cells. In *A. minutum* life cycle, sexual reproduction was induced by several environmental factors such as nutrients, salinity and temperature (Ellegaard et al., 2013). These factors induce gametes expression, and subsequently forming diploid planozygote through gamete conjugation (Ellegaard et al., 2013). Motile planozyogtes will remain in water column for some times, then either divided into haploid vegetative cells or transformed into resting cysts (Figueroa et al., 2015). After several months or years, these resting cysts will germinate and form planomeiocytes through meiotic

division (Blackburn et al., 1989). Motile planomeiocytes will divide into haploid vegetative cells through mitosis and may develop into another bloom.

The common method that used to observe cells in different life cycle stages is by light and fluorescent microscope. Anderson and Wall (1978) used to use light microscope to observe the different life cycle stages of cells. Nowadays, DNA probes and staining dye have been widely used to stain the nuclear of the cells to observe the changes of DNA content, and thus confirmed their different life stages (Figueroa et al., 2007). At present, flow cytometry is the technique that used to analyze the DNA content and quantify the cells in different life cycle stage either in culture or monospecific bloom samples (Figueroa et al., 2010). Previously, this technique was mostly applied in biomedical field to characterize and identify the cancerous cells through DNA content (Robinson & Grégori, 2007). Now, this technique had become useful in ecology, evolutionary biology and systematics and also to study the life cycle of dinoflagellates (Kron et al., 2007; Figueroa et al., 2010).

In this study, a rapid response sampling effort was carried out in between three to five day interval, from September to November 2015, during a massive bloom of *A. minutum* in Sungai Geting, Tumpat. Flow cytometry was used to identify the life cycle stages of the cells. Samples collected during the *A. minutum* bloom from September to December 2015 were gone through flow cytometry analysis to observe the life cycle of *A. minutum*. Detection of changes in DNA content of the cells was used to predict the stages of the bloom.

4.2   Literature Review

Alexandrium spp. is one of the harmful marine phytoplankton which at least 8 species from this genus are associated with PST and form HAB (Figueroa et al., 2007; Anderson et al., 2012a). Marine dinoflagellate includes *Alexandrium* spp. undergo their life cycle through sexual and asexual reproduction. Study on the life cycle of harmful algae bloom species is essential to understand the development of the blooms as different life stages have their roles at different phases of the blooms (Garces et al., 2001; Persson et al., 2008).

In dinoflagellates, as in eukaryotes, population growth for a species undergoes binary division to produce two daughter cells of equal size. The cell division cycle consists of G1, S, G2 and M (mitosis) phases. In G1 phase, the vegetative cell (1C genome content) exhibit high metabolic activity that allow all cellular components, except DNA, to build up; S phase is the beginning of DNA synthesis and duplication; G2 phase is the gap that the cell will last until mitosis occur and the vegetative cell is now with 2C genome content; mitosis (M phase), where the nuclear division occurs (Figure 4.1) (Taroncher-Oldenburg et al., 1997; Pan et al., 1999; Harashima et al., 2013). S phase and M phase are two important check points to ensure the cell receive full complement of the hereditary material (Nurse, 1994). However, after cytokinesis, cells may enter a quiescent stage (G0) with same DNA content as in G1 phase and stop dividing (Pan et al., 1999; Dapena et al., 2015). According to Pardee (1989), there is possibility to activate G0 cells and re-enter cell cycle at G1.

Unlike other eukaryote cell, cell cycle in dinoflagellate is tightly related to light dark cycle. Different dinoflagellate species have their own division pattern. In some *Alexandrium* spp., S phase start before the beginning of dark period, G2+M phase normally occur at night and cytokinesis normally occur 3 to 6 hours after the cell enter

dark period or occur early in the light period (Taroncher-Oldenburg et al., 1997; Dapena et al., 2015). In natural environment, vegetative cells can form pellicle cyst which is a temporary resting stage without sexual behaviour when the environmental condition is unfavorable and recover back to vegetative cells once the suitable condition restored (Figueroa et al., 2006; Bravo et al., 2010).
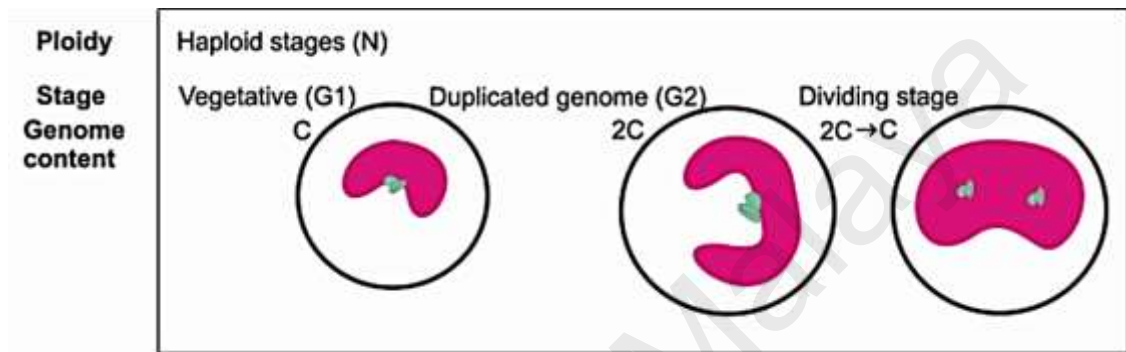
Figure 4.1: Two vegetative stages (G1 and G2) and a mitotic stage (dividing cell). Stages with duplicated genome (2C genome content) are known as G2 (Source: Figueroa et al., 2014).

Sexual reproduction is a common feature in many dinoflagellate include *Alexandrium* spp.. Sexual behavior in bloom species is one of the factors that are important to promote and sustain the blooms (Garces et al., 2001). Sexuality begins when two gamete cells fused and form motile planozygote with two longitudinal flagella. Gamete cell was divided mitotically from vegetative cell under appropriate condition (Probert, 1999). Planozygote may remain motile for some time before they undergo meiosis and transformed into benthic resting cyst that go through mandatory dormancy period. In some cases, planozygote may bypass cyst formation and undergo division (Uchida et al., 1996; Figueroa & Bravo, 2005; Figueroa et al., 2015). After dormancy period, these resting cysts will germinate and form planomeiocyte through meiotic division and become an important factor to promote a bloom (Blackburn et al.,

1989). However, the dormancy period of the cyst is highly variable between species (Probert, 1999).

In natural environmental condition, sexual behavior was induced by environmental factors such as salinity, temperature and nutrients (nitrogen (N) and phosphorus (P)) (Anderson & Lindquist, 1985; Coats & Tyler, 1985; Garcés et al., 2004; Ellegaard et al., 2013). Previous studies in some *Alexandrium* spp. showed that low N and P especially P induced gamete expression and increase in planozygote formation (Anderson et al., 1984; Pfiester & Anderson, 1987; Figueroa et al., 2011). Besides that, salinity and temperature also affected planozygote and cyst formation (Figueroa et al., 2011). Figure 4.2 shows the life cycle of *A. fundyense* which also applied to most of the *Alexandrium* species.
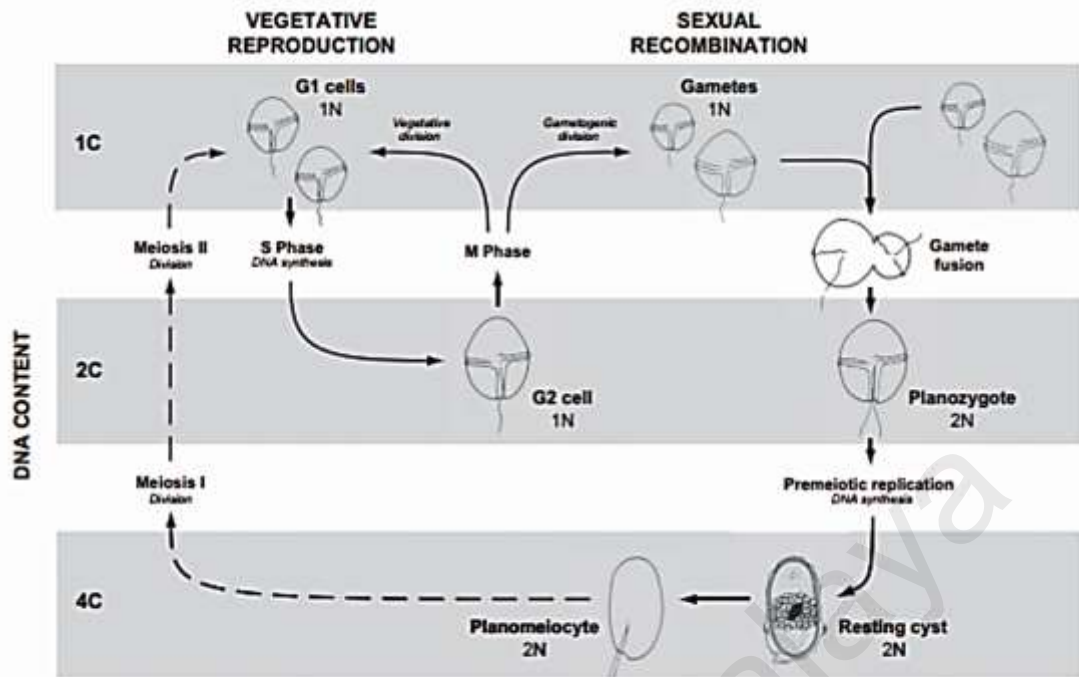
Figure 4.2: Life cycle diagram of *Alexandrium fundyense*. The life cycle is comprised of asexual and sexual reproduction. The mitotic cycle consists of an initial growth period (G1 phase), DNA replication (S phase), a short growth phase (G2), then nuclear division (M phase) and cytokinesis (cell division). The sexual cycle through gametogenesis, a division that yields two gametes. Cells were classified according to their total DNA content which was quantified in increments'C', where 1C is the DNA content of a haploid cell immediately after division. Each stage in the mitotic cycle is haploid (1N) and contains either a single or double complement of chromosomes (1C or 2C DNA content). Sexual stages may contain 1C, 2C, or 4C DNA content. Gametes are haploid but the planozygote and resting cyst stages are diploid (2N). Premeiotic replication would give rise to 4C DNA content cells (Source: Brosnahan et al., 2014).

Flow cytometry is a high-throughput analytical tool which contains fluorescence and light scatter properties in order to detect and quantify single particles. Cells stained with fluorescent probe or nuclei acid stain will pass through a narrow liquid stream and quantify the cells by side and forward scatter and all fluorescent channels. Flow cytometry has two main advantages, the first is large quantity of particles can evaluated in short time and produce strong and representative results of the whole population; second is cell sorting process in flow cytometry able to separate single particles or cells physically from a mixed population at rate up to 70,000 cells per second (Robinson & Grégori, 2007). Now, this application was widespread in aquatic microbiology for DNA quantification by using DNA fluorochromes (Collier, 2000; Kron et al., 2007). Information of DNA content provided by flow cytometry enables the study of life cycle on aquatic microorganism included dinoflagellate.

In marine dinoflagellate, flow cytometry was widely used to study the importance of different sexual pathway. PicoGreen, SYTOX green and SYBR Green I are nuclei acid stain that proved to be useful with flow cytometry for quantitative detection of cellular DNA (Veldhuis et al., 1997; Collier, 2000). Measurement of DNA content in large amount of cells by flow cytometry enable examination of cell at different life stage such as gametes, planozygotes with a standard of known DNA content (Collier, 2000; Figueroa et al., 2010).

Besides that, this technique also used to identify and classified pico- and nanophytoplankton which the cell range from 0.2 to 2 µm and from 2 to 20 µm in diameter by scattering and autoflorescence detection (Collier, 2000). This phytoplankton can be distinguished and identified by their unique pigmentation and red fluorescent from chlorophyll. Previous study also proved that fluorescent measurement

and scatter provide detailed insights of natural picophytoplankton assemblages (Jacquet et al., 1998; Vaulot & Marie, 1999).

To date, flow cytometry had become common in phytoplankton study and still forging for new research program in different aspect.

4.3 Materials and Methods

4.3.1 Field sampling

Alexandrium bloom samples were collected from a semi-enclosed coastal lagoon, Sungai Geting, Tumpat, located at the northeastern Peninsular Malaysia between September 2015 and March 2016. Samples were collected from our sampling stations (S1, S2, S4 and S6) along the lagoon (Figure 4.3). The lagoon is brackish, receives freshwater discharges from Golok River that bordering Malaysia and Thailand. Benthic clams (*Polymesoda similis*) are collected from the intertidal mudflats for local consumption.

Quantitative water samples were collected at each station at 1m depth using a 4-L Van Dorn water sampler. One-liter samples were concentrated by sieving through a 10-μm mesh nylon sieve, and concentrated samples were preserved with 1% acidic Lugol's solution for cell enumeration. Cell counts (in triplicate) were performed using a Sedgwick-Rafter counting chamber under a Leica DM3000 LED microscope (Leica Microsystems GmBH, Wetzlar, Germany).

Plankton samples for flow cytometric analysis were collected using a 20-μm plankton net that hauled vertically in the water column (<3 m). Concentrated samples were fixed in saline ethanol (Miller & Scholin, 1998), transported in cooler box with ice packs, and stored at −20°C in the laboratory until further analysis. Live samples were brought back to the laboratory for culture establishment and molecular characterization.

A 48-h sampling was carried out during a recurrent bloom in March 2016. Samples were collected at station 6 for every two hours between 07:30 on 3rd of March and 05:30 on 5th of March, 2016. Samples were collected using 20 μm-mesh plankton net, and kept in saline ethanol as described above. Temperature and salinity of seawater were measured in situ.
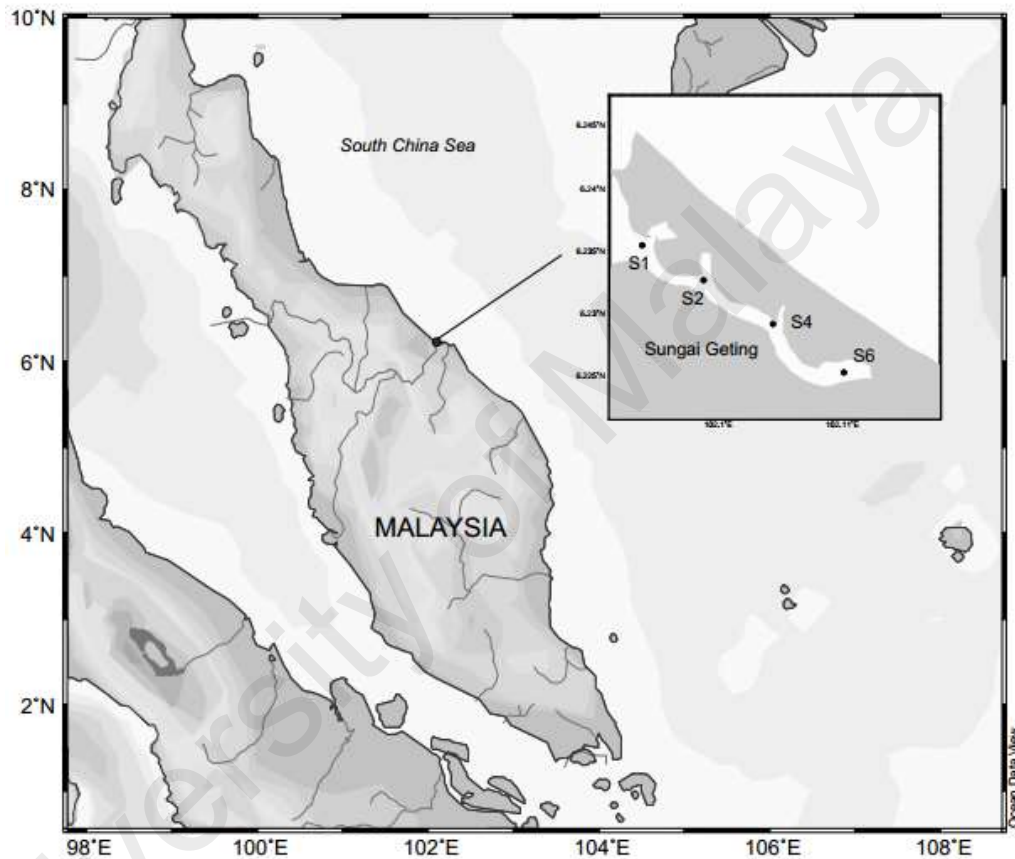
Figure 4.3: Map showing the sampling sites of Tumpat, Malaysia with four stations (S1, S2, S4, S6) labelled.

4.3.2   Single-cell PCR, DNA sequencing and qPCR detection

Single cells collected from the field were isolated by micropipetting technique and used as template for gene amplification (Lim et al., 2014). Gene amplification was performed using the universal primer pairs targeting domain 1–3 of the large subunit ribosomal RNA gene (LSU rDNA, D1–3) (Scholin et al., 1993) and the internal transcribed spacer region (ITS) (Leaw et al., 2001). Amplification was performed using a peQSTAR Thermal Cycler (Peqlab Biotechnologie GmbH, Erlangen, Germany). Purified amplicons were directly sequenced for both strands.

The cell ability to produce PSTs was further confirmed by real-time quantitative PCR (qPCR) assay using the specific primers targeting *sxtA4* gene (Murray et al., 2011; Hii et al., 2016). Genomic DNA (gDNA) from the field samples was extracted using PowerPlant® Pro DNA isolation kit (Mo Bio Laboratories Inc., CA, USA). The assay was performed by using two positive controls: gDNAs from clonal cultures of *A. minutum* and *A. tamiavanichii* from Sungai Geting, while a non-PST producing species, *Coolia malayensis* was used as negative control.

4.3.3   Microscopic observation

Live and preserved field samples at different cell stages (vegetative cells, gametes, fused cells, planozygotes and cysts) were observed under a Leica DM3000 LED research microscope (Leica Microsystems, Wetzlar, Germany), under 400× magnifications, and micrographs were captured with Leica DFC450 camera (Leica Microsystems). Species was identified by examining the thecal plates after staining with Imamura-Fukuyo stain (Balech, 1995; Fukuyo, 2001). Cell nuclei were stained with SYTOX® Green nuclei acid stain (Invitrogen, Life Technologies, CA, USA), observed under an Olympus BX53 fluorescence compound microscope and images captured with Olympus DP73 camera (Olympus, Tokyo, Japan).

4.3.4 Sample preparation and flow cytometric analysis

Saline ethanol-preserved samples (1 mL) were harvested by centrifugation (700 $\times g$, 4 min) and cell pellet rinsed once with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Cells were then resuspended in TE, and stained with SYTOX® Green nuclei acid stain (Invitrogen) for 30 min in the dark at 4°C. Excess stain was removed by rinsing in TE. Cells were resuspended again in TE, and kept in the dark at 4°C until further flow cytometric analysis.

SYTOX Green-stained cells were acquired by an ACEA Novocyte™ flow cytometer (ACEA Biosciences, CA, USA) with an argon laser of 488 nm excitation. Samples were run at a flow rate of 10 µL min^{-1} using ACEA NovoFlow™ solution (ACEA Biosciences) as a sheath fluid, and 200,000 events were acquired for each sample. Fluorescence arbitrary values of channels BL1 (FITC, 530/30 nm), BL3 (PerCP, 675/30 nm) and the forward scatter (FSC) were recorded (in logarithmic mode). Data analysis was performed using NovoExpress™ (ACEA Biosciences); signal detection of up to 10^7 dynamic ranges was used in this study.

For flow cytometric calibration, exponential-phase culture of *A. minutum* strain AmTm02 was synchronized in total darkness for 48 h. Culture was then collected by a10-µm sieve and sample was analysed flow-cytometrically as described above.

4.4 Results

4.4.1 2015-bloom of *Alexandrium minutum* and the environmental conditions

On 30th August 2015, *A. minutum* bloom was discovered at Sungai Geting, Tumpat. Reddish-brown discoloration was clearly visible in whole lagoon (Figure 4.3) and the benthic clams were contaminated with PSP toxins. Maximum cell density during the bloom period up to 10^7 cell L^{-1} was observed, prompting an investigation on the bloom species and its dynamics. The samples were immediately undergone thecal observations (Figure 4.4) and single-cell PCR, and the results confirmed the species as *Alexandrium minutum*. Furthermore, qPCR detection of the toxin gene *sxtA4* confirmed the toxic population (Figure 4.5).
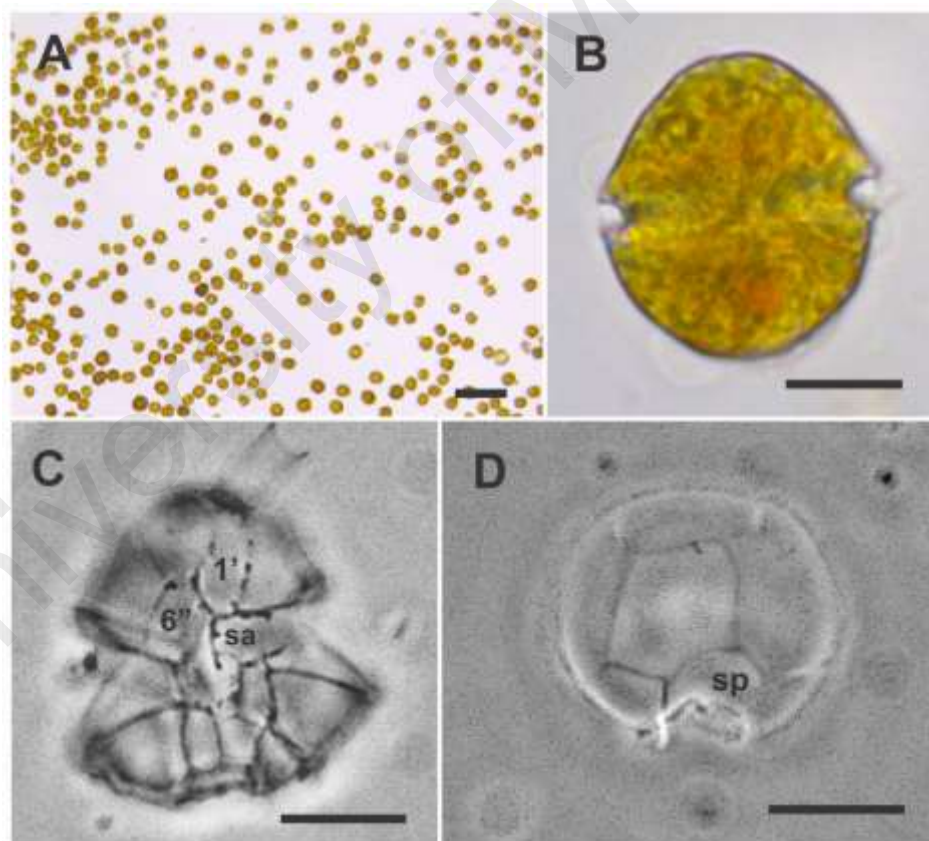
Figure 4.4: Cells of *Alexandrium minutum* from bloom sample (a-b). Ventral view (c) Antapical view (d). Scale bars, 100 μm (a), 10 μm (b-d).
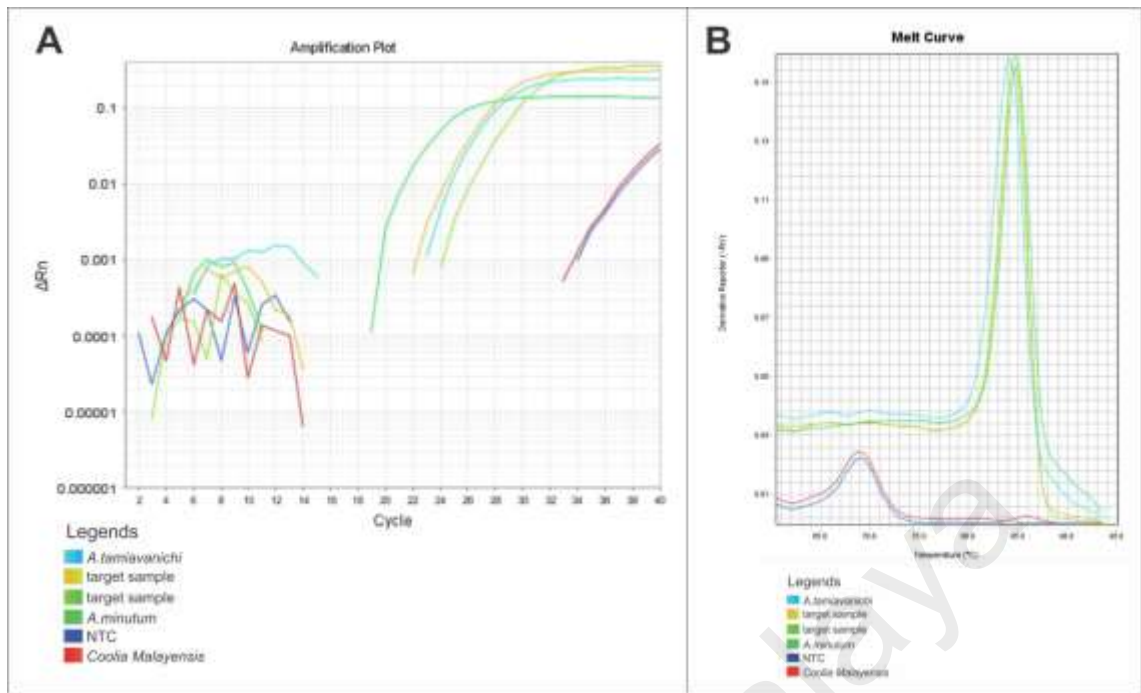
Figure 4.5: Toxin gene qPCR assay on the bloom samples. (a) qPCR amplification plot of *sxtA4*. (b) Melting curve analysis of *sxtA4*.

4.4.2   Observation of *Alexandrium minutum* life stages during the bloom

In the field samples, vegetative cells were observed in single cells (Figure 4.6 A, B), but rarely with vegetative two-cell chains. Cells are oval, with numerous globe-shaped chloroplasts, cingulum is deeply excavated. Cells varied greatly in size (16 – 33 μm length, 15 – 30 μm width; *n* = 17), but similar in cell shape and appearance (Figure 4.6 A–C). Two longitudinal flagella-cells were observed in the field samples (Figure 4.6 D–F), with cell length of 30 – 42 μm, and width of 28 – 36 μm (*n* = 7). Under epi-fluorescent microscope, nucleus of *A. minutum* cyst and normal cell were observed through SYTOX green nuclei acid stain (Figure 4.7). The nucleus of the cyst was in circular shape (Figure 4.7 A, D), while for vegetative or gamete cells, DNA content filled up the cells equally (Figure 4.7 B, E). Some of the *A. minutum* cells with "U" shape condensed DNA content also observed at early stage of the bloom (Figure 4.7 C-D, F-G).

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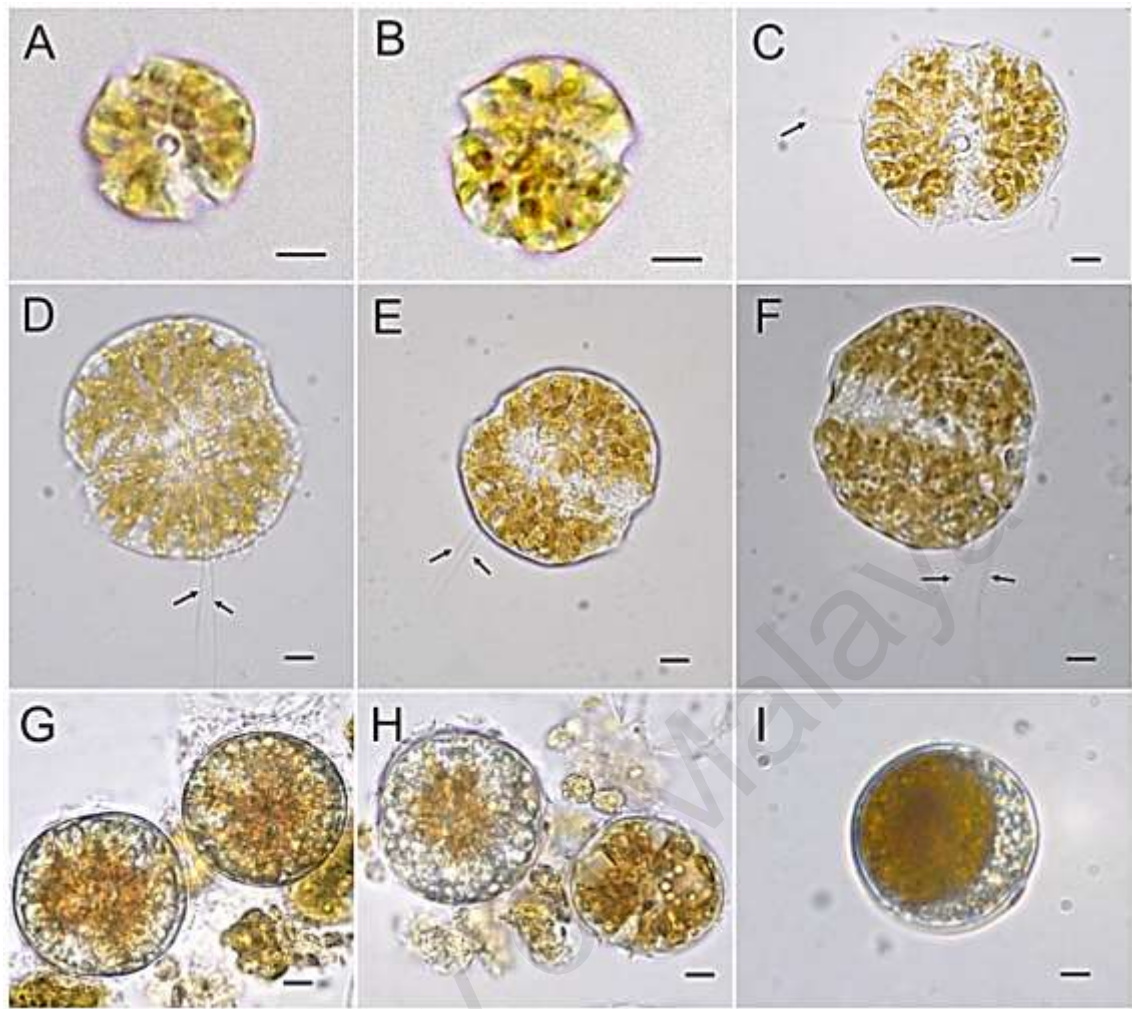
Figure 4.6: Different life cycle stages of *Alexandrium minutum* observed during the bloom in Sungai Geting. (A−C) Vegetative cells, note that cells are in various sizes; (C) cell with a longitudinal flagellum (arrows). (D−F) Cells with two longitudinal flagella (arrows). (G, H) Pellicle cysts with a thin pellicle layer. (I) Double-walled resting cyst. Scale bars, 5 μm.
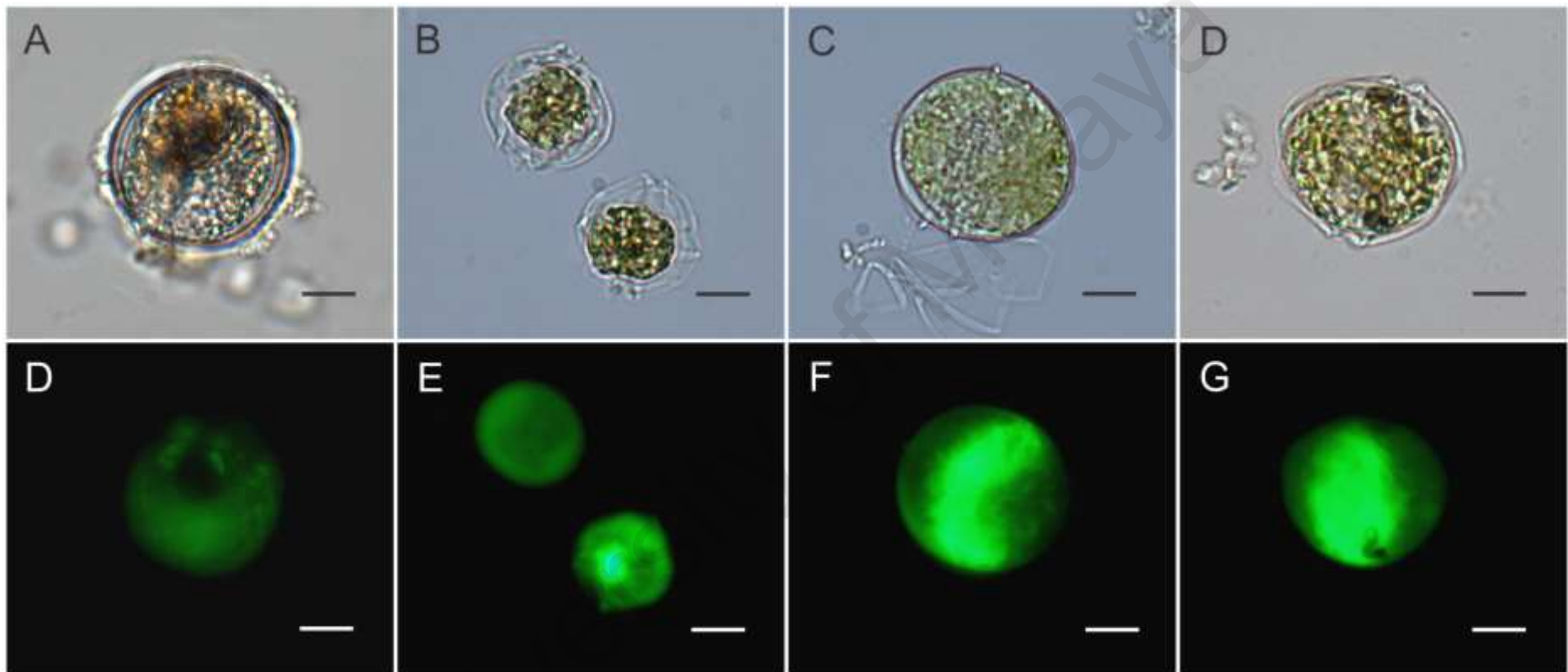
Figure 4.7: Light and epi-fluorescent micrograph of SYTOX green nuclei acid stain of *Alexandrium minutum* from bloom samples. (A, D) Resting cyst with circular DNA content. (B, E) Vegetative/gamete cells that filled with DNA. (C-D, F-G) *Alexandrium minutum* cells with "U" shape or condensed DNA content. Scale bars, 10 μm.

4.4.3 Cell cycle analysis

The frequency distribution plot obtained from flow cytometry analysis showed three peaks: 1C (gamete or vegetative cells), 2C (planozygotes) and 4C (planomeiocytes or cysts) (Figure 4.8). The dominant 2C peak (G2 phase) of cultured cells arrested in the dark was corresponded to the 2C peak in the field samples; no 4C cells were detected in the culture samples (Figure 4.9A).

At early stage of the bloom (30th August) high proportions of 1C (61%) and 4C (34%) cells were detected. On the following days, 4C cells increase continuously until 89% on 3rd of September, with 7% 1C and 4% 2C cells (Figure 4.9, 4.10). In the middle stage of the bloom (6th-27th September), 2C (12%-43%) and 1C (41%-72%) cells start to increase with the decrease of 4C (8%-15%) cells (Figure 4.9, 4.10). On 6th and 13th of September, there was a peak between 2C and 4C cells (Figure 4.9). At the late stage of the bloom (4th October onwards), 2C (1%-7%) and 4C (0.3%-6%) cells decrease to the lowest number with abundance of 1C (86%-98%) that sustained the bloom for one month before it terminated (Figure 4.9, 4.10). However, on 25th October, low number of 2C (6%) and 4C (2%) cells were observed together with abundance of 1C (91%) cells (Figure 4.9, 4.10). Same condition was repeated on second bloom which 4C cells also in abundance at the beginning of the bloom and when 2C and 4C cells decrease, 1C cells increase (Figure 4.10). However, the bloom does not sustained for long period and it terminated due to environmental factors.
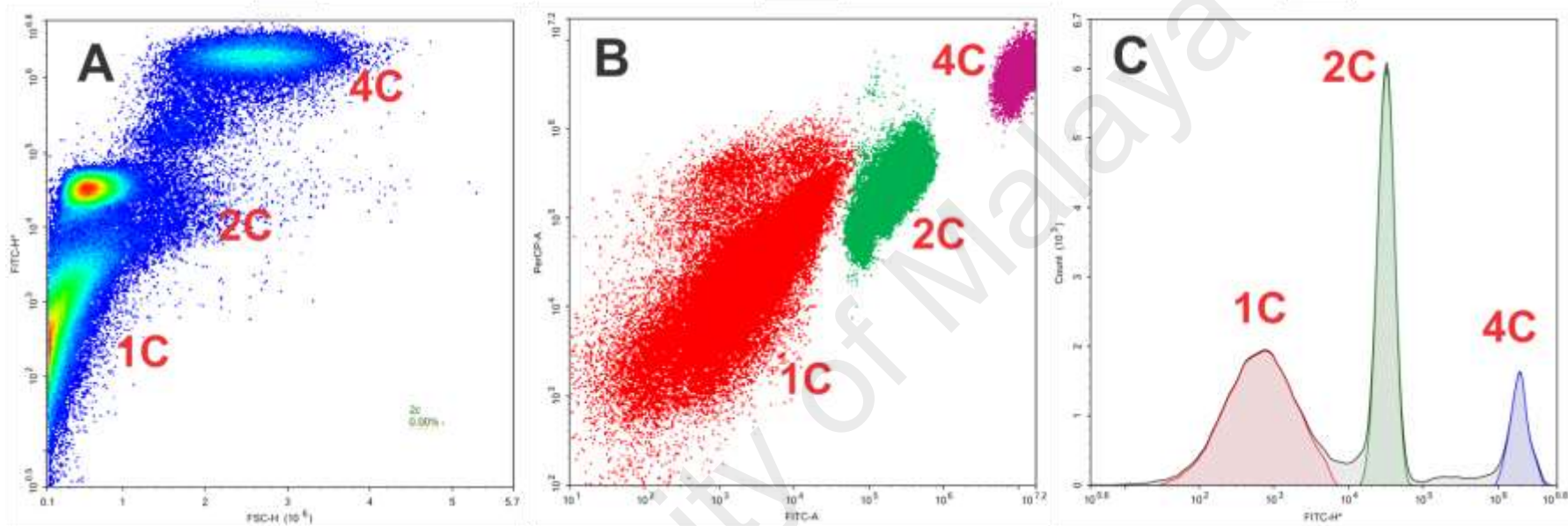
Figure 4.8: (A) Cytogram of forward scatter (FSC) versus SYBR Green® fluorescence (FITC, channel BL1). (B) Cytogram of chrolophyll-related pigment red fluorescence (PerCP, channel BL3) versus SYBR Green® fluorescence. (C) Relative frequency distribution of particle counts from field samples showing distinct peaks of 1C, 2C, and 4C.
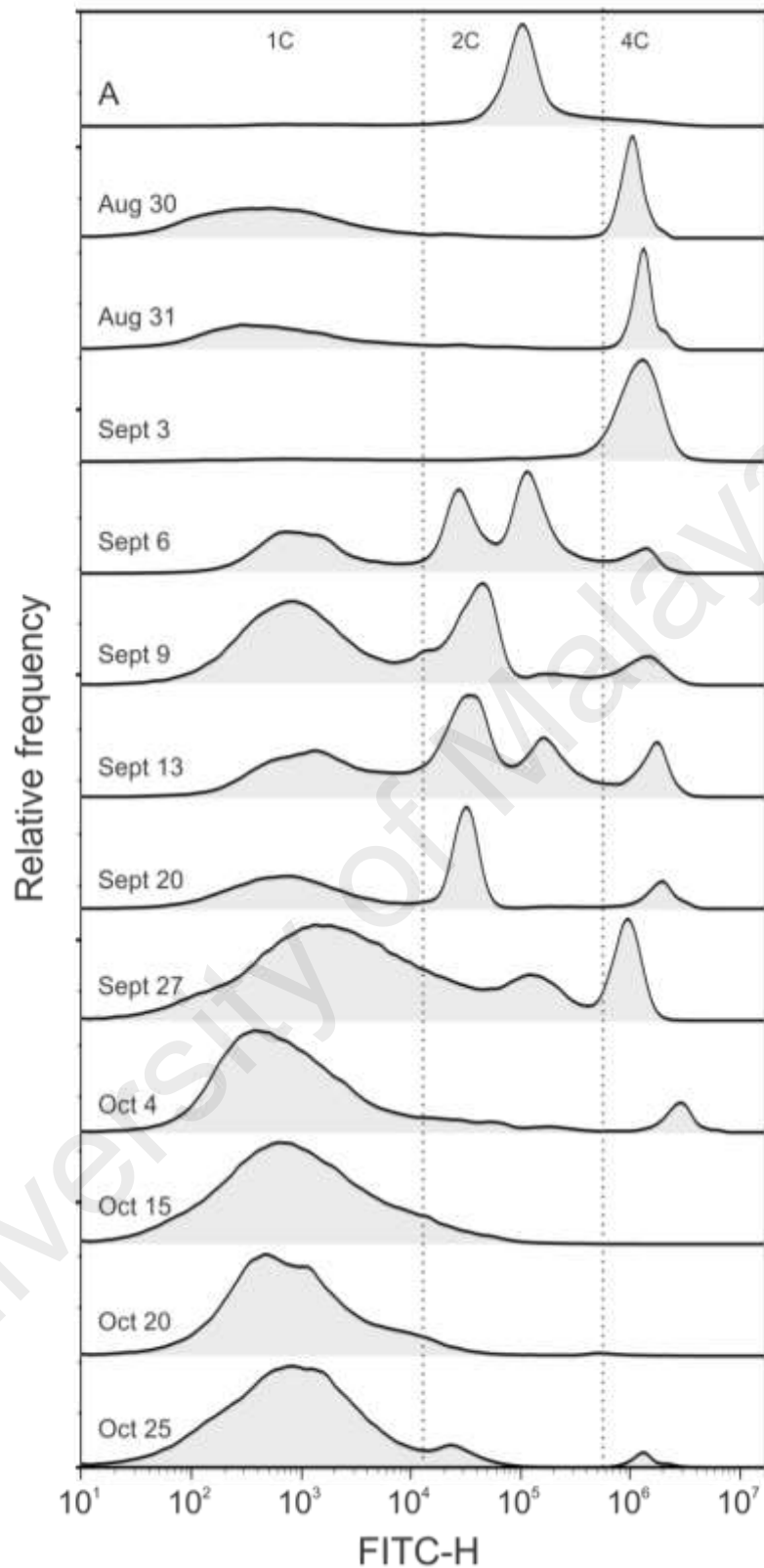
Figure 4.9: Relative frequency of SYBR Green®-DNA associated fluorescence (in FITC-H channel) of a synchronized log-phase culture of *Alexandrium minutum* showing the 2C peak (A) and bloom samples collected from station S6 (see Figure 4.3) during the 2015 blooms.
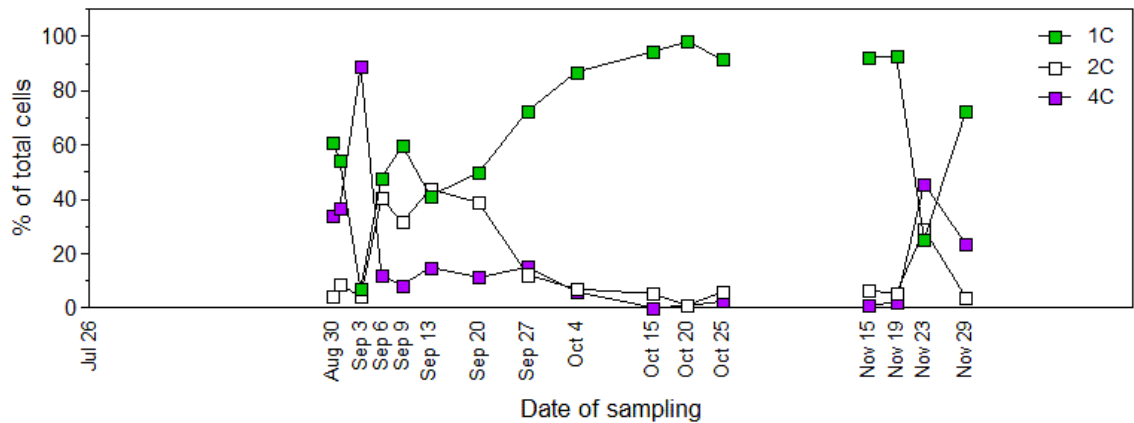
Figure 4.10: Relative compositions (in percentage) of *Alexandrium minutum* from station S6 during the bloom period.

4.4.4   Investigation on the station where bloom initiated

Flow cytometry determination of samples showed that on the onset of bloom (30th August), the highest composition of 4C (34%) cells at station S6, with the cell density of 2.3×10^6 cells L^{-1}; while proportion of 1C cells were dominated at stations S1 (99%), S2 (98%) and S4 (89%), respectively (Figure 4.11).

The results showed that they had same trend that 4C cells only found at station 6 which it slowly dispersed to other station and only 1C cells exist at station 1.
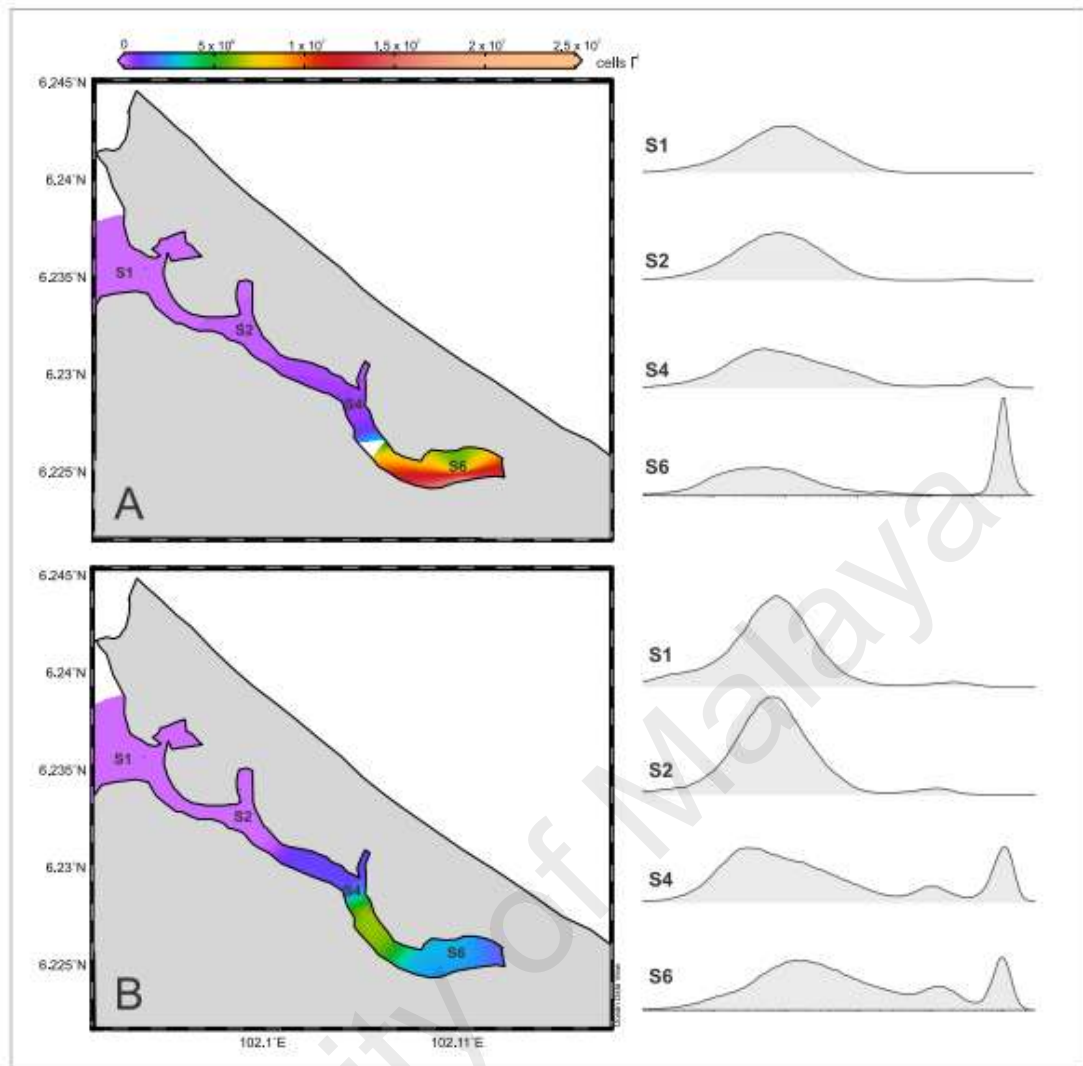
Figure 4.11: Relative frequency of *Alexandrium minutum* cells at DNA content of 1C, 2C and 4C peaks on station S1, S2, S4 and S6 on August 30 (A) and September 27 (B), 2015.

4.4.5 Diurnal sampling

Our results demonstrated typical phases of eukaryotic cell cycle where the *A. minutum* cells proceed through the 48-hr observations (Figure 4.12), with cells at G1 phase (1C DNA content), and the S phase separating the G2+M phase (2C populations at dark periods). Increases in the 2C cells were followed by concomitant decreases in 1C proportions.

From the graph, we found that 1C cells were higher than 2C cells in water column once day break. Slowly, 1C cells started to decrease and 2C cells increase. In afternoon around 3pm, 1C cells started to increase in the water column with decrease of 2C cells. On the second day, 1C cells increase at 5pm, two hours delay compare to first day. 1C cells reach maximum density at 7pm for both days with lowest density of 2C cells at the same time. After 7pm, the 1C cells started to decrease and 2C cells start to increase. At 1am, both 1C and 2C cells have the same quantity in the water column. Then, 2C cells continue to increase with decrease of 1C cells until daybreak which 1C cells start to increase in water column. From the result, S phase was observed started in the afternoon until night. In these samples, amount of 4C cells detected was only 1 to 2% although we observed a number of temporary cysts in the samples.
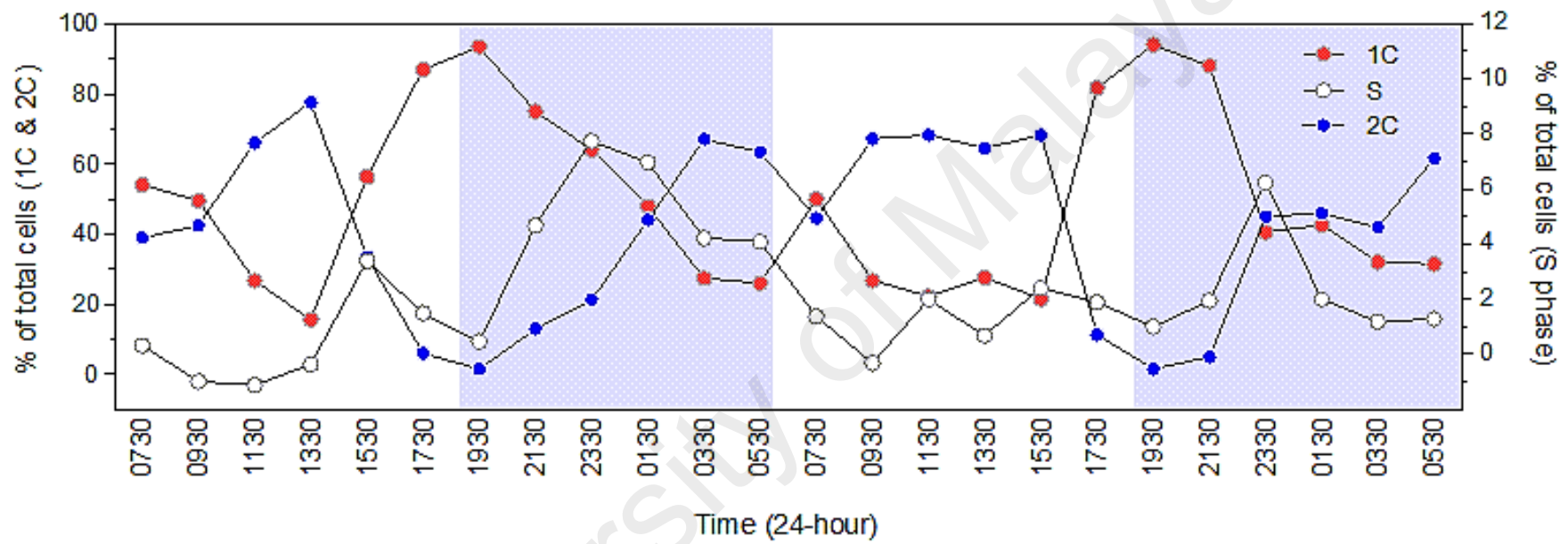
Figure 4.12: 48 hours sampling with 1C, S and 2C cells ploted from first day 0700 to third day 0530.

4.5   Discussion

In this study, flow cytometry successfully distinguished DNA content in different stages of the cells in bloom condition. From the results, DNA content of *A. minutum* was classify as 1C, 2C and 4C, where 1C population belongs to vegetative or gamete cells in G1 phase after dividing from G2 phased vegetative cell, planozygote or planomeiocyte; 2C DNA content found in vegetative cells with G2+M phase, planozygote or cyst that formed by sexual behavior of the cell and planomeiocyte that germinate from cyst with 2C DNA content; 4C cells found in cyst those go through DNA replication by meiosis or planomeiocyte germinate from cyst with 4C DNA content before division occurred (Figueroa et al., 2006; Brosnahan et al., 2014; Figueroa et al., 2015).

Alexandrium minutum cultures were synchronized in the dark for 48 h as cells arrested in G2 phase with 2C DNA content. Corresponded 2C peak as showed in field samples and cultures confirm that the 2C peak show in field samples is cells with 2C DNA content.

Several studies indicated that most of the toxic dinoflagellate blooms are initiated from benthic resting cysts (Persson et al., 2008; Anglès et al., 2012). These cysts will undergo excystment when maturation is complete or when the environment factors reach optimal (Bravo et al., 2010; Haberkorn et al., 2011). From the results, we know that at the beginning of the bloom, 1C and 4C cells are predominance in water column. Cysts for *A. minutum* were with 2C, 4C or more than 4C DNA content (Figueroa et al., 2015). Therefore, germination of cysts into planomeiocyte also contains 2C, 4C or more than 4C DNA content. Although there is also possibility for flow cytometry to detect cyst, but, no cyst was found in the field sample under microscopic observation. Therefore, 4C cells found in the samples were confirmed as planomeiocyte. At the

beginning of the bloom, most of the 4C cells detected could be planomeiocyte as the bloom suspected initiated by massive excystment from the cyst bed. Cyst goes through planomeiocyte and divide into vegetative or gamete cells which was 1C cells that contribute to part of the bloom at the beginning (Figueroa et al., 2007). On 3rd September, 81% of 4C cells found in the sample of station 6. This could be the time that massive excystment happen. In the middle of the bloom, from 6th to 27th September, 2C cells appeared with decrease of 4C cells. This condition could be the conversion of the cells to sexual phase of life cycle due to their sexual behaviour. However, not all of the 2C cells detect by flow cytometry were planozygote due to *A. minutum* is haploid microalgae that also undergo mitosis and the DNA content tends to change from 1C G1 phase to 2C G2 phase (McGillicuddy et al., 2014). Therefore, *A. minutum* may have 2C DNA content in G2 phase by asexual reproduction or as a planozygote during sexual reproduction. In *A. minutum*, cells entered G2 phase at night and lasted until daybreak which phase division occurred at daybreak and cells enter 1C G1 phase (Dapena et al., 2015). In this study, samples were collected in the late-morning and afternoon between 1000 to 1300 local time, when most of the cells were in 1C G1 phase and we can confirm that the cells with 2C DNA content were mainly planozygote. Besides that, result from 48 hours diurnal sampling also confirmed that 2C cells found in morning are planozygote as cells observed in S phase was almost 0% (Figure 4.12). Previous study indicated that formation of planozygote in bloom is the sign of bloom decline (Persson et al., 2008), but, in this study, *A. minutum* bloom sustained for two months after observed high density of planozygote in the early stage of the bloom. In this study, 1C vegetative cells played an important role to sustain the bloom.

On 6th and 13th September, there was a peak in between 2C and 4C. This peak could be the cells which undergo 2C to 4C process. According to Figueroa et al. (2015), they proposed a new model for *A. minutum* cell cycle. In this model, planozygote (2C cells)

undergoes division by mitosis or meiosis. Planozygote that go through mitosis, DNA content was transited from 2C to 4C and divided from 4C to two 2C cells. During meiosis division, DNA content of the cell might shift from 2C to 4C, to two 2C cells and divide to four 1C cells. We suspected that the peak between 2C and 4C represent the intermediate cells which is going to enter 4C stage as division of planozygote is light-dependent and diel process (Figueroa et al., 2015).

In dinoflagellate bloom, most of the bloom was sustained by asexual reproduction while sexual reproduction in the bloom is to produce cyst (Dale, 1983; Persson et al., 2008). In this study, the result showed that planomeiocyte contributed to the initiation of the bloom. Slowly, with the decrease of 4C cells, 1C and 2C cells increase. Mating process of gamete increases the amount of 2C planozygote in the sample (Persson et al., 2008). Nutrient, temperature and salinity were the factors that induced gamete expression. According to Figueroa et al. (2011), high N/P ratio tends to induce gamete expression and increase the amount of planozygote while low salinity and high temperature also increase the sexuality in the population significantly. From the result obtained, on 6th September, planozygote increase and at the same time N/P ratio increase (increase in nitrate, Figure 4.13C) (Figure 4.13D). On the following days (9th to 20th September), no significant increase in N/P ratio was observed but the amount of 2C cells still remain high in water column (Figure 4.13E). Therefore, high temperature and low salinity in the environment could be the main factor that causes high density of 2C cells in this duration, especially on 13th September, water temperature reach 38°C with low salinity (8 psu) (Figure 4.13A, B). Subsequently, bloom was sustained by 1C cells (Figure 4.9, 4.13E). These 1C cells were donated by germination of pellicle and resting cyst and some planozygote that did not go into encystment but divided through meiosis or mitosis (Figueroa et al., 2015). Then the bloom was terminated due to high precipitation (Figure 4.13A) and fresh water plumes from Golok River.

The cytometric data at stations 1, 2, 4, and 6 showed that the bloom started from inner part of the lagoon which is station 6. Station 1 was dominated by 1C cells and some 2C and 4C cells on station 2 and 4 through fluctuation of water from inner lagoon to river mouth. Absence of 4C cells at station 1 and 2 at the beginning of the bloom confirm that the cyst bed was located at station 6 which abundance of excystment happen and expand to whole lagoon.

Furthermore, to look into detail on the pattern of cell cycle in bloom, 48 hours sampling was carried out during the early stage of the bloom. Sampling site for 48 hours was set at the inner part of the lagoon with low water movement and constant salinity at water column throughout the sampling period. Therefore, the population of the cell is believed to be consistent in water column. Sampling started at 7a.m. when the time daybreak. From the results, we observed the trend of *A. minutum* life cycle that involved sexual and asexual behaviour. Cells enter G2 phase with 2C DNA content after 7p.m. when the nightfall and enter G1 phase with 1C DNA content once daybreak. Flow cytometry data clearly showed that after 7p.m., 1C cells start to decrease while 2C cells start to increase with the increase of S phase also observed. These 2C cells might be G2 pahased vegetative cells as S phase was observed. In the morning, after division of the cell in daybreak, 2C cells increase continuously in the morning while 1C cells decrease until afternoon. Planozygote formation through mating of the gametes promoted the increase of 2C cells at day time. This was confirmed due to low amount of S phased cells (~0%) were observed. The consistent pattern of life cycle in this 48 hrs confirmed that the cell in bloom condition was synchronized.

4.6   Conclusion

We summarize the life cycle of *A. minutum* bloom in this study in Figure 4.14. The bloom was initiated by massive excystment (1) and go through asexual division (2 – 4) at the early stage of the bloom. Environmental factor such as temperature, salinity and nutrients induced sexual reproduction (5, 6), thus increase the number of planozygote (7). These planozygote will either become cyst (8) or go through division to produce vegetative cells. Besides that, environmental stress also will cause vegetative cell to settle down become pellicle cyst (10). Heavy precipitation and fresh water plumes from Golok River cause the bloom to terminate.

We concluded that *A. minutum* bloom in this study was (i) initiated by massive excystment at inner lagoon; (ii) bloom sustained for two months after increase of planozygotes; (iii) nutrients, salinity and temperature play an important role in *A. minutum* life cycle and sustained the bloom.
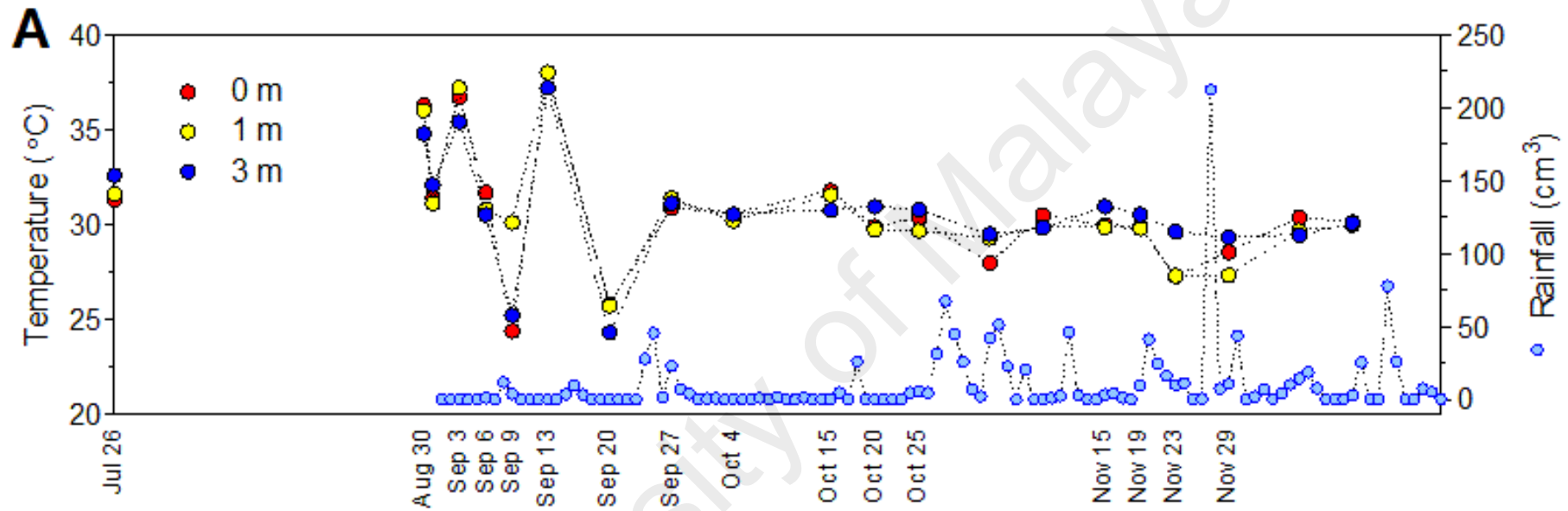
Figure 4.13: Physical-chemical parameter that obtained from station S6 of the lagoon during *Alexandrium minutum* bloom. (A) Temperature from surface (0 m), 1 m and 3 m water associated with rainfall data; (B) Salinity from different layer (surface, 1 m and 3 m); (C) Nutrients (Ammonia, Nitrate, Phosphorous) from 1 m water; (D) N/P during *Alexandrium minutum* bloom; (E) Relative compositions (in percentage) of *Alexandrium minutum* in different life cycle stage associated with cell density at different layer (surface, 1 m and 3 m).
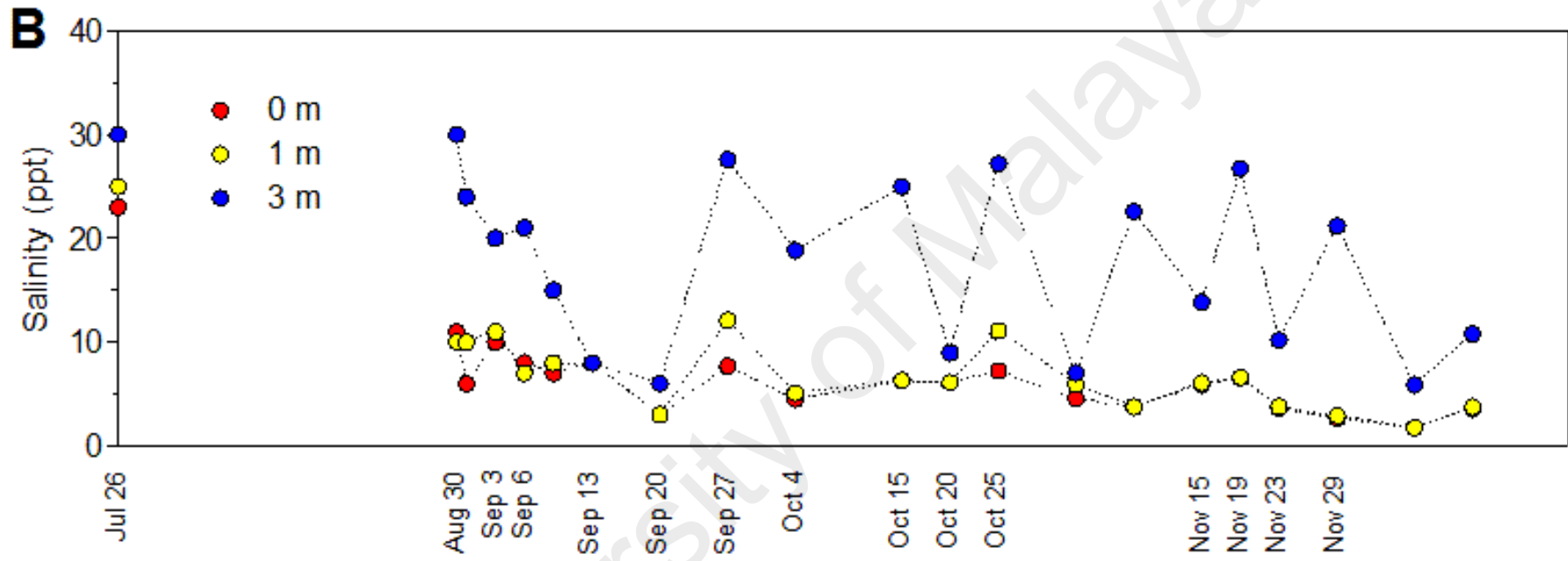
Figure 4.13: Continued.
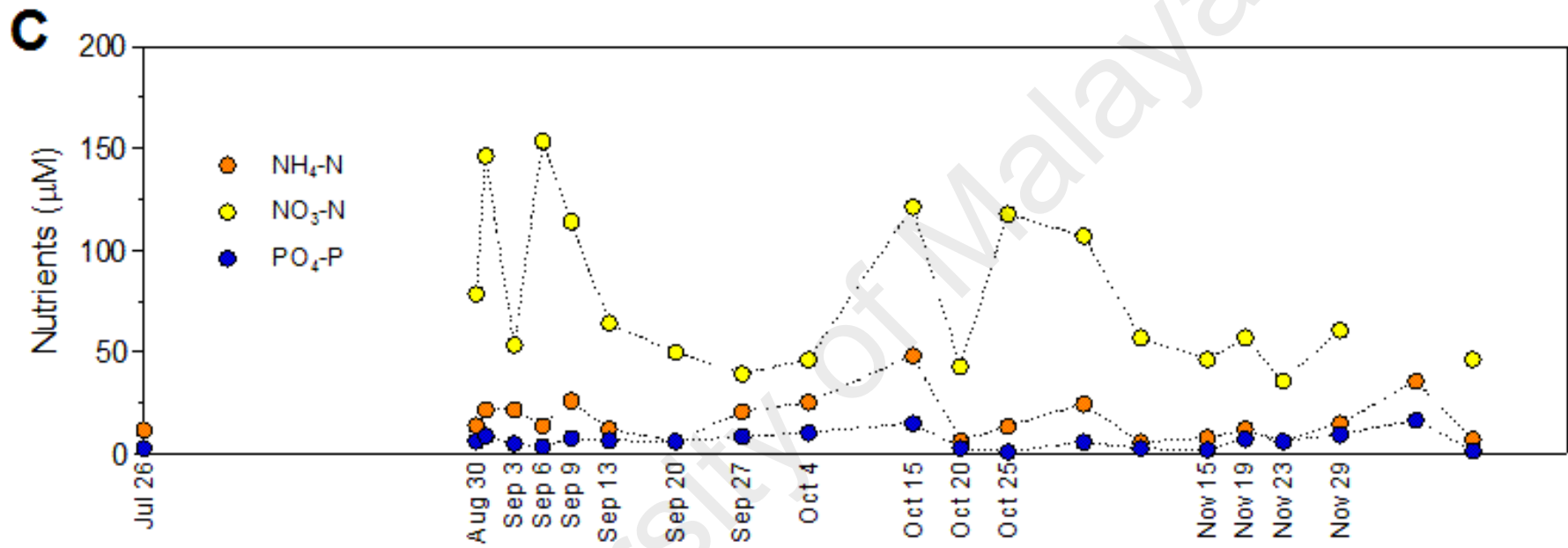
Figure 4.13: Continued.
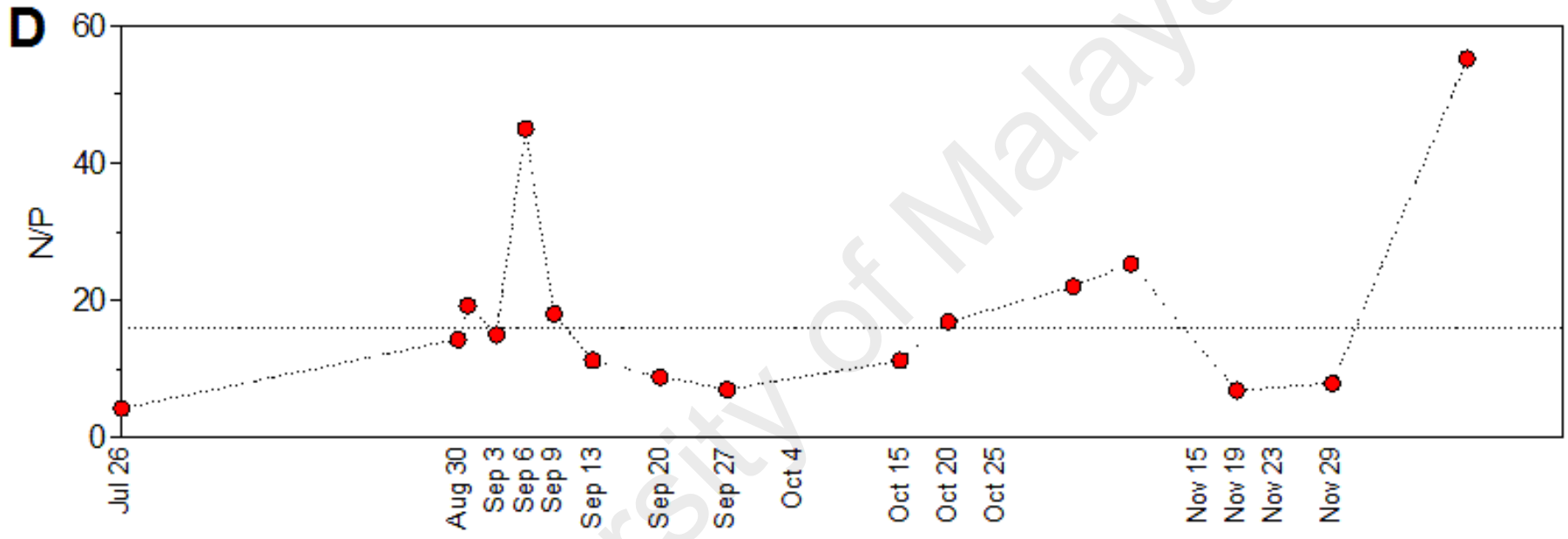
Figure 4.13: Continued.
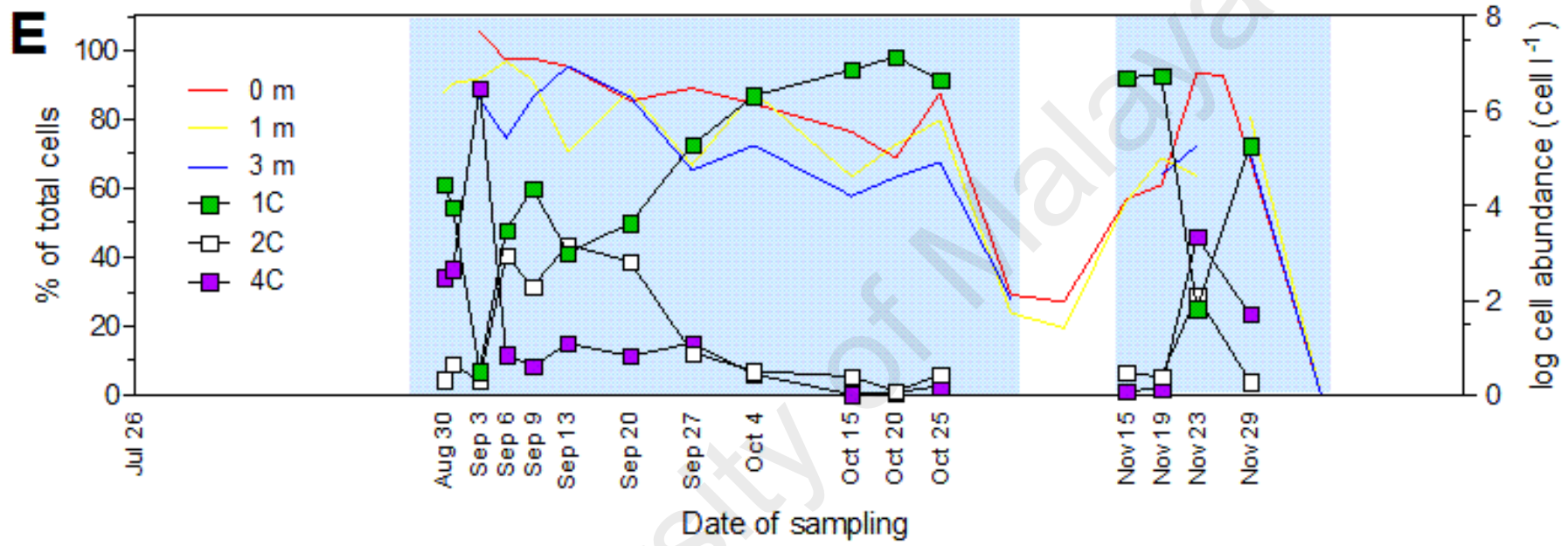
Figure 4.13: Continued.
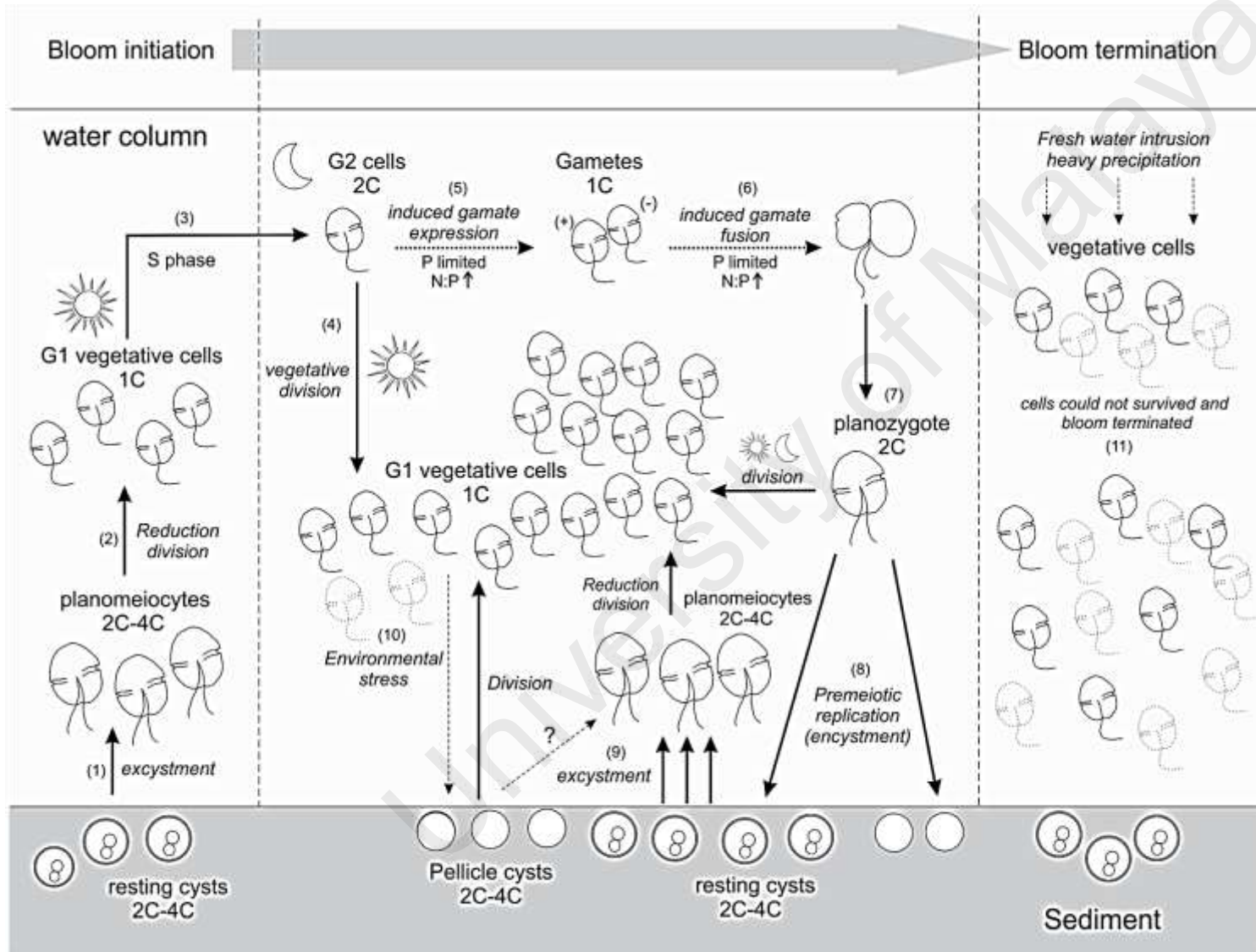
Figure 4.14: Life cycle of *Alexandrium minutum* in natural bloom.

CHAPTER 5: CONCLUSION AND RECOMMENDATION

Toxic marine dinoflagellate such as *A. minutum*, *A. tamiyavanichii*, and *Pyrodinium bahamense* are widely associated with PST and cause PSP cases in Malaysia since last four decades. In early September 2015, a remarkably high density of *A. minutum* occurred in Sungai Geting, Tumpat, a semi-enclosed lagoon situated at the north-eastern Peninsular Malaysia, causing severe discoloration and contaminated the benthic clam, *Polymesoda*. Samplings were undertaken over the duration of four months between the bloom periods from September to December 2015. It is important to note that the bloom was first recorded in 2001 with human fatality due to PSP, and the blooms recurred after a time gap of 14 years. With the negative impact brought by HABs to the human health and aquaculture industries, it is crucial to elucidate the bloom mechanism of this HAB species.

At present, molecular tool has emerged as an effective platform for species detection and quantification. Therefore, SYBR Green I-based quantitative real-time PCR (qPCR) was developed for *A. minutum* in this study. Species-specific primer was designed from second internal transcribed spacer (ITS2) region of ribosomal RNA gene (rDNA). The qPCR assay showed high specificity and sensitivity in detecting *A. minutum* by validated in the cross-reactivity test using cultured and environmental samples. This assay allows detection of as low as three cells. However, SYBR Green I-based qPCR may lead to false positive results as the assay is based on the intercalating dye, SYBR Green, which binds to any double-stranded DNA and emits fluorescent signals. The specific and non-specific PCR products could be both detected as shown in the formation of smaller melt peaks such as primer-dimers in the melting curve analysis.

On the other hand, investigation on the life cycle of *A. minutum* in natural bloom was carried our using flow cytometry analysis. Study on the life cycle of HAB species

enables the investigation on the mechanisms of blooms initiation, development and termination. Results from the flow cytometric analysis showed that the bloom was initiated by cyst excystment. Increase in planozygotes (2C) was signified in the middle of the bloom, might due to an abrupt decrease in salinity with high water temperature and elevated N:P ratio. The flow cytometric results also confirmed that the bloom was initiated at the inner part of the lagoon and slowly dispersed out to the river mouth. The blooms events were sustained for four months by vegetative cells (1C) although increase in planozygotes (2C) was observed in the early of the bloom. Increase of precipitation and freshwater intrusion from the Golok River plumes into the inner lagoon might cause the bloom to terminate.

In conclusion, the qPCR assay and flow cytometry were two different molecular platforms that used to detect the harmful algal bloom species. These molecular tools, especially qPCR demonstrate high specificity and rapidity in detection of harmful species for monitoring purpose while flow cytometry serve as a platform to investigate the development of blooms. Besides that, flow cytometry analysis also help to determine the DNA content of the cells in order to verify the overestimation in *A. minutum* qPCR assay. Future direction on the development of molecular approach will be more optimized molecular assays should be developed in future to target other emergence HAB species. Multiplex assays that target multiple species in one reaction can be developed, particularly to those with the occurrence of different type of harmful species in an area. Besides that, qPCR with high resolution melting curve (HRM) is strongly suggested to overcome the shortage of using SYBR green-based qPCR assay by observe the melting curve to avoid false positive results. Hence, with the development of molecular techniques with its wide application, a comprehensive monitoring of HABs and investigation on bloom development can be achieved.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications:

1. Kon, N. F., **Lau, W.L.S.**, Law, I. K., Lim, P. T. & Leaw, C. P. 2016. On-site rapid detection of toxic *Alexandrium tamiyavanichii*: integrating the species-specific hydrolysis probe in insulated isothermal polymerase chain reaction (iiPCR). **Journal of Applied Phycology. 28**:2815-2820.

2. Kon, N. F., **Lau, W.L.S.**, Hii, K. S., Law, I. K., Teng, S. T., Lim, H. C., Takahashi, K., Gu, H. F., Lim, P. T. & Leaw, C. P. 2017. Quantitative real-time PCR detection of a harmful unarmored dinoflagellate, *Karlodinium australe* (Dinophyceae). **Phycological Research.** Doi:10.1111/pre.12186

3. **Lau, W.L.S.**, Law, I. K., Liow, G. R., Hii, K.S., Usup, G., Leaw, C. P. & Lim, P. T. **Submitted**. Using flow cytometry for determining life-history stages of natural bloom populations and understanding the bloom dynamics of a tropical *Alexandrium minutum*. **Harmful Algae**.

Papers presented at conferences/ seminars/symposiums:

1. **Lau, W.L.S.**, Law, I.K., Liow, G.R., Lim, P.T. & Leaw, C.P. 2017. Flow cytometry analysis of toxic *Alexandrium minutum* bloom at east cost of Peninsular Malaysia. The Second Xiamen Symposium on Marine Environmental Sciences.