Faculty of Resource Science and Technology

Benthic Epiphytic Dinoflagellate Assemblages in the Fringing Reefs of Sampadi Island and Satang Besar Island, Sarawak

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A final report submitted in fulfilment of the Final Year Project 2013 (STF 3013)

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DECLARATION

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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LIST OF ABBREVIATIONS

CFP	Ciguatera Fish Poisoning
HAB	Harmful Algal Bloom
ITS	Internal Transcribed Spacer
LSU	Large Subunit
PCR	Polymerase Chain Reaction
SEM	Scanning Electron Microscope
LM	Light Microscope
PLTX	Palytoxin

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ABSTRACT

This study presents a comprehensive examination of the taxonomy of the genus *Ostreopsis*. The genus *Ostreopsis* includes several species capable of producing various palytoxin-like compounds which have harmful effects on human and marine fauna. Species in this genus are regular members of the epiphytic community in tropical seas but their geographical range have shown an apparent expansion towards temperate regions in recent years. In this study, clonal cultures of *Ostreopsis* from Sampadi and Satang Besar Island, Sarawak were established. The morphology of the strains was described from light, epi-fluorescence and scanning electron micrographs. Based on detailed morphological observation and morphometric measurements, the strains were designated as two *Ostreopsis* species; *O*. cf. *ovata* and *O*. cf. *lenticularis*. The benthic harmful dinoflagellate (BHAB) assemblages of Sampadi and Satang Besar Island were investigated and compared. The results showed that all five major genera of BHAB species, viz. *Amphidinium, Coolia, Gambierdiscus, Ostreopsis* and *Prorocentrum* were found in Sampadi Island, whilst in Satang Besar Island, *Ostreopsis* spp. was dominated the site. This could be due to several environmental variables among which light intensity, temperature, hydrodynamism, and substrate typology seem to display major roles, while links with salinity and nutrient levels are less clear. Less research has been conducted on *Ostreopsis* than other benthic dinoflagellates, but the future path of research on *Ostreopsis* should follow a similar trajectory; which is the revision of the taxonomy, genotyping and physiological studies.

Key words: *Ostreopsis ovata*, *Ostreopsis lenticularis*, benthic dinoflagellates, HAB species, Malaysia

ABSTRAK

Kajian ini membentangkan pemeriksaan komprehensif taksonomi genus *Ostreopsis*. Genus *Ostreopsis* merangkumi beberapa spesies yang mampu menghasilkan pelbagai sebatian seperti palytoxin yang mempunyai kesan buruk kepada manusia dan fauna di dalam laut. Spesies dalam genus ini merupakan epifit di laut tropika tetapi julat geografi mereka telah menunjukkan peningkatan yang ketara ke arah kawasan sederhana sejak kebelakangan ini. Morfologi dua spesies dinoflagellates marin, *Ostreopsis ovata* Fukuyo 1981, *Ostreopsis siamensis* Schmidt 1902, *Ostreopsis lenticularis* Fukuyo 1981, *Ostreopsis heptagona* Norris, Bomber & Balech 1985, O*streopsis mascarenensis* Quod 1994 dan *Ostreopsis labens* Faust et Morton 1995 dari Pulau Sampadi dan Pulau Satang Besar daripada habitat utama iaitu makroalga telah dibincangkan dengan menggunakan mikrograf cahaya, mikrograf epi-pendarfluor dan imbasan elektron mikrograf. Kajian ini juga menunjukkan kesemua genus iaitu Genus *Amphidinium, Coolia, Gambierdiscus, Ostreopsis* dan *Prorocentrum* telah dijumpai di Pulau Sampadi manakala di Pulau Satang Besar, hanya genus *Ostreopsis* dijumpai. Penyebabnya berkemungkinan mengenai pelbagai faktor seperti keamatan cahaya, suhu, hydrodynamism, dan tipologi substrat. Penyebab seperti kemasinan dan tahap nutrient merupakan faktor sampingan. Kajian mengenai genus *Ostreopsis* masih tidak mencukupi setakat ini.

Kata-kata kunci: *Ostreopsis ovata*, *Ostreopsis lenticularis*, dinoflagellate bentik, spesies HAB, Malaysia

1.0 INTRODUCTION

In warm tropical waters toxic benthic dinoflagellates occurs as assemblages of unicellular, photosynthetic, thecated species belonging to four genera: *Gambierdiscus* (Adachi & Fukuyo, 1979), *Ostreopsis* (Fukuyo, 1981), *Coolia* (Besada *et. al.*., 1982), *Prorocentrum* (Fukuyo, 1981). Some of the species are toxic, and are potential source of toxins present in herbivorous fish and molluscs (Fukuyo, 1981). The polyether toxins (maitoxin, oetreopsin, cooliatoxin etc.) produced by these species may cause ciguatera fish poisoning (CFP).

The multiple toxins produced by the benthic dinoflagellate, *Ostreopsis lentricularis, Gambierdiscus toxicus* and their associated bacterial flora enter into the tropical marine toxin food chain when the macroalgae on which they live as epiphytes are eaten by invertebrates and herbivorous fishes associated with shallow reef environments (Legrand *et al.*, 1992, Tosteson, 1995; Tosteson *et al.*, 1995). These toxins are then passed through the "food chain" when these organisms are consumed by larger carnivores that visit those reefs in search of food (Helfrich & Banner, 1963; Tosteson *et al.*, 1992; Winter & Tosteson, 1992; Lewis & Holmes, 1993; Tosteson, 1995; Tosteson *et al.*, 1998, Pottier *et al.*, 2002).

Thus far, approximately 50 dinoflagellate species are known to produce toxins or poisons that cause marine animal or bird deaths, toxicity in bivalves, and seafood poisoning in humans (Steidinger, 1993). Biotoxins produced by dinoflagellates accumulate via the food chain by herbivorous and carnivorous fish (Quod & Turquet, 1995)

Research interest in benthic dinoflagellates is rapidly increasing because the invasion of several tropical or subtropical species into temperate waters, which can be a signal of global warming (Shears & Ross, 2009). Higher abundances of *Ostreopsis* and *Coolia* species are usually recorded during warmer periods, and they can associate with human health problems such as respiratory and skin irritations (Sansoni *et al.*, 2003). A

more recent series of case reports of symptoms such as general malaise and weakness, associated with myalgia, respiratory effects, impairment of the neuromuscular apparatus and abnormalities in cardiac function were known causes of PLTX intoxication. Systemic symptoms are often recorded together with local damages whose intensity varies according to the route and length of exposure. Such symptoms were obtained through oral intake of contaminated seafood and also inhalation and cutaneous/systemic exposures after direct contact with aerosolized seawater during *Ostreopsis* blooms (Tubaro *et al.*, 2010).

In this study, species composition of BHABs species in Sampadi Island will be determined, their morphology will be investigated and molecular information characterized. Live samples will be collected from seaweed, coral and sand sediment of Sampadi Island. Samples will then be isolated and established into clonal cultures, follow by observations under fluorescence and scanning electron microscopes. Genomic DNAs of the clonal cultures will be extracted and gene amplification of the large subunit (LSU) ribosomal DNA and the internal transcribed spacer (ITS) region will be conducted.

Thus, the specific objectives of this study are:

1. To establish clonal cultures of wide diversity of benthic dinoflagellates;
2. To observe the morphology of benthic dinoflagellates under fluorescence microscopy and SEM;
3. To determine the species composition and distribution of benthic dinoflagellates in Sampadi and Satang Besar Island.

2.0 LITERATURE REVIEW

2.1 Benthic dinoflagellate

Benthic marine dinoflagellates are species that distributed in warm, shallow, coastal marine waters and live attached to macrophytes, detritus, sand particles, corals, seaweeds and mangroves. They occur as assemblages of unicellular, photosynthetic, thecated species belonging to four genera: *Gambierdiscus*, (Adachi & Fukuyo, 1979), *Ostreopsis* (Fukuyo, 1981), *Coolia* (Besada *et al*., 1982), *Prorocentrum* (Fukuyo, 1981).

Benthic dinoflagellates contribute significantly to the production of the coral reef community. These species are toxic and are the potential source of toxins present in herbivorous fish and molluscs (Fukuyo, 1981). Many benthic dinoflagellates are capable of producing bioactive compounds, including those that can cause seafood toxicity. The most well-known human intoxication due to benthic dinoflagellates is ciguatera fish poisoning (CFP) where the responsible toxins occur in species of the genus *Gambierdiscus* (Leaw *et al.*, 2010).

2.2 Ciguatera fish poisoning

Ciguatera fish poisoning is a food-borne disease widespread in tropical and sub-tropical marine areas (Lewis, 2006). It is a well-documented disease that causes gastro-intestinal, neurological and cardiovascular symptoms which occurs after the consumption of fish contaminated with ciguatoxins (Lewis, 2001; Alfonso *et al.*, 2005).

It is also the most frequently reported marine toxin-derived disease worldwide resulting in illness affecting more than 50,000 people each year and also affecting fish industry with consequent economic losses (Glaziou & Legrand, 1994; Lewis, 2001).

In 1977, Yasumoto *et al.* reported the discovery of a benthic dinoflagellate responsible for ciguatera, the most common form of phycotoxin-borne seafood illness across the globe. So far approximately 50 dinoflagellate species are known to produce toxins or poisons that cause marine animal or bird deaths, toxicity in bivalves, and seafood poisoning in humans (Steidinger, 1993).

Ciguatera fish poisoning is caused by multiple toxins produced by benthic dinoflagellate, *Ostreopsis lentricularis*, *Gambierdiscus toxicus* and their associated bacterial flora enter into the tropical marine toxin food chain when the macroalgae on which they live as epiphytes are eaten by invertebrates and herbivorous fishes associated with shallow reef environments (Legrand *et al.*, 1992; Tosteson, 1995; Tosteson *et al.*, 1995). These toxins are then passed through the "food chain" and accumulated (Quod & Turquet, 1995) when these organisms are consumed by larger carnivores that visit those reefs in search of food (Helfrich & Banner, 1963; Tosteson *et al.*, 1992; Winter & Tosteson, 1992; Lewis & Holmes, 1993; Tosteson, 1995; Tosteson *et al.*, 1998; Pottier *et al.*, 2002).

2.3 Benthic epiphytic marine dinoflagellates

Dinoflagellates are among the major components of marine ecosystems (Jeong, 1999; Lessard, 1991; Sherr & Sherr, 2007; Terrado *et al*., 2009). They are important as primary producers and play an important role in marine food webs (South & Whittick, 1987). They are able to live in diverse environments (Faust, 1995; Lan *et al*., 2009; Richlen & Lobel, 2011) and play diverse roles in marine food webs, feeding on prey and in turn, serving as a prey for a range of different predators (reviewed by Jeong *et al*., 2010).

Ostreopsis species were initially reported mostly from tropical and subtropical seas, where they are associated with other epiphytic dinoflagellates such as *Gambierdiscus*, *Coolia* and *Prorocentrum* species (Besada *et al*., 1982). These organisms form epiphytic communities associated with coral reefs, or rather with macroalgae attached to coral surfaces. These assemblages may vary in species composition and cell concentration between sites (Tindall & Morton, 1998).

2.3.1 Ostreopsidaceae

Benthic dinoflagellates belonging to the family Ostreopsidaceae are common members of benthic microalgal communities in both tropical and temperate areas (Faust *et al*., 1996; Rhodes, 2011). The dinoflagellate genus *Ostreopsis* (Schmidt, 1901) belongs to the family Ostreopsidaceae (Lindeman, 1928).

Ostreopsidaceae species are widespread in most epiphytic and benthic dinoflagellate communities from ciguatera-endemic regions of the world (35°N to 35°S) (Tindall & Morton, 1998).

There are nine known species of the genus *Ostreopsis.* They are *O. siamensis* (Schmidt, 1902), *O. ovata* (Fukuyo, 1981), *O. lenticularis* (Fukuyo, 1981), *O.heptagona* (Norris *et al.*, 1985), *O. mascarenensis* (Quod, 1994), *O. labens* (Faust & Morton, 1995), *O. belizeanus* (Faust, 1999), *O. caribbeanus* (Faust, 1999) and *O. marinus* (Faust, 1999) (Figure 2.1). The tenth species, isolated from Hawaiian waters in 2001 is currently being described (Morton *et al*., in preparation; Rhodes, 2011)

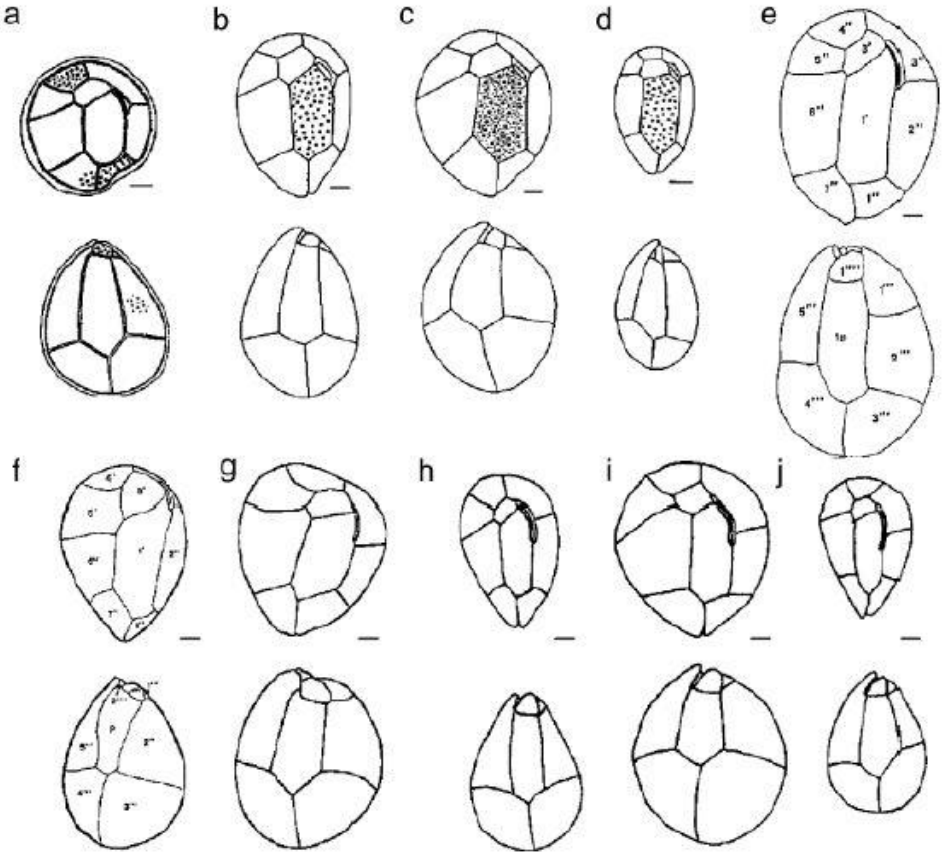

Figure 2.1: Line drawings of the nine described *Ostreopsis* species in epithecal (upper) and hypothecal (lower) view. (a) *Ostreopsis siamensis*, after Schmidt (1901); (b–d) *Ostreopsis siamensis*, *O. lenticularis*, and *O. ovata*, respectively, after Steidinger and Tangen (1996); (e) *Ostreopsis mascarenensis*, after Quod (1994); (f) *Ostreopsis heptagona*, after Norris et al. (1985); (g) *Ostreopsis labens*, redrawn from Faust and Morton (1995); (h–j) *Ostreopsis belizeanus*, *O. marinus*, and *O. caribbeanus*, respectively, after Faust (1999). Scale bars, 10 µm. (adopted from Penna *et al*., 2005)

These species are described as epiphytic on macroalgae and seagrasses, or attached to coral rubble or on sand. They are also known as a 'bloom former' (Faust *et al.*, 1996; Rhodes *et al.*, 2000; Mangialajo *et al.*, 2008, 2011; Totti *et al.*, 2010).

Ostreopsis is known to produce palytoxin (PTX) and analogues (Cohu *et al*., 2011). Six of the nine currently recognised species are toxic, producing palytoxin-related

compounds (Rhodes, 2011). They are *O. siamensis* (Schmidt, 1902), *O. ovata* (Fukuyo, 1981), *O. lenticularis* (Fukuyo, 1981), *O. heptagona* (Norris *et al.*, 1985), *O. mascarenensis* (Quod, 1994), *O. labens* (Faust & Morton, 1995).

The toxins produced by *O.* cf. *ovata* have been initially identified as a putative palytoxin (PLTX) in small amount and ovatoxin-a (OVTX-a) as the major toxin (Ciminiello *et al.*, 2008; Guerrini *et al.*, 2010); recently the presence of putative PLTX and OVTX-a was confirmed and the occurrence in the extract of four new palytoxin-like compounds, OVTX-b, -c, -d, and -e, was highlighted (Ciminiello *et al.*, 2010). *Ostreopsis siamensis* and *O. mascarenensis* were found to produce the ostreocin-D (Usami *et al.*, 1995; Ukena *et al.*, 2001) and mascarenotoxins (Lenoir *et al.*, 2004), respectively.

In the Mediterranean Sea, neurotoxic effects due to toxin accumulation in food web have not yet been reported and *Ostreopsis* species are implicated thus far only in respiratory affections and skin or eyes irritations, in events in Italy and Spain (Simoni *et al.*, 2003; Brescianini *et al.*, 2006; Ciminiello *et al.*, 2006; Barroso Garcia *et al.*, 2008; Tichadou *et al.*, 2010).

These syndromes may be caused by simple contact and/or inhalation of cells (or toxins), and can affect people near the shore exposed to marine aerosols during *Ostreopsis* bloom events (Gallitelli *et al.*, 2005; Kermarec *et al.*, 2008; Tubaro *et al.*, 2011). Since the end of the 1990s massive blooms of *O. ovata* (Fukuyo, 1981) have been reported in a number of coastal areas of the temperate regions with increasing frequency, intensity and distribution.

The effects of environmental factors on *Ostreopsis* blooms are somewhat unclear. It has been suggested that temperature is a key factor for bloom development, attributing the recent *Ostreopsis* expansion in the Mediterranean Sea to global warming (Granéli *et al.*,

2011). However, it has also been recognized that the role of temperature is not the same in all Mediterranean areas (Mangialajo *et al.*, 2011).

Hydrodynamics have been shown to play an important role in regulating benthic/planktonic dinoflagellate abundances, especially in shallow coastal areas, enhancing the resuspension of benthic cells in the water column (Totti *et al*., 2010; Vila *et al*., 2001), although the fate of planktonic *Ostreopsis* cells is still poorly known. The relationship between benthic dinoflagellate blooms and nutrient concentration has been only sporadically investigated, although (Armi *et al*, 2010) found a positive correlation between nutrients and abundances for *Coolia monotis*.

The taxonomy of the species is based on some morphological characters as thecal plates, shape, size, trichocyst pores and thecal pore size (Monti *et al*., 2007). Characterisation of *Ostreopsis* species has traditionally been by morphology, but in recent years phylogenetic studies have been helpful in determining species identifications, although more sequences need to be deposited in web-based sequence databases (for example, GenBank) to facilitate this approach (Penna *et al*., 2005).

The identification of the *Ostreopsis* morphotypes at the species level continues to be problematic, at least in routine microscopy analysis, because of the species' high morphometric variability, especially in terms of antero-posterior and dorso-ventral diameter ratios. Therefore, alternative diagnostic methods based on rapid, accurate and less time-consuming molecular methodologies can be highly advantageous. Recently, molecular analyses based on the PCR assay, which was developed and validated using species specific *Ostreopsis* primers, were performed on environmental samples collected in different areas of the Mediterranean Sea (Accoroni *et al*., 2011).

Leaw *et al.* (2001) stressed that morphology alone was insufficient to differentiate these strains, particularly given the plasticity that can occur in the species, whereas the ITS regions proved useful for highlighting genetic diversity and are therefore potential markers for biogeographic studies.

2.4 Palytoxin (PLTX)

Palytoxin (PLTX) is one of the most potent non-protein marine toxins (Deeds & Schwartz, 2010) known so far. It is a complex macromolecule whose chemical structure was elucidated in the 1980s (Moore & Bartolini, 1981; Uemura, 1981; Moore, 1985). There are at least 8 different PLTX analogues known, which are PLTX, ostreocin D, ovatoxin-A, homopalytoxin, bishomopalyloxin, neopalytoxin, deoxypalytoxin and 42-hydroxypalytoxin (Riobo & Franco, 2011).

PlTX was first isolated from the marine zooxanthid *Palythoa toxica* (Moore & Scheuer, 1971) and since then has been found in numerous soft corals of the genus Palythoa (Deguchi *et al.*, 1974; Castineiras *et al.*, 1975; Beress *et al.*, 1983; Hirata & Uemura, 1985; Uemura *et al.*, 1985). However, PlTX origin in the *Palythoa* zooxanthids is not clear and has been suggested to derive from bacteria or symbiotic microorganisms (Moore *et al.*, 1982; Uemura *et al.*, 1985).

Several marine organisms have been reported to produce PlTXs and the analog toxins encountered in vector species including seafood (Aligizaki *et al*., 2011). *Ostreopsis siamensis* was the first dinoflagellate associated with the production of toxic compounds analog to PlTX (Usami *et al.*, 1995), whereas other species of the genus were later shown to also produce PlTX analogs (Ukena *et al.*, 2002; Taniyama *et al.*, 2003).

Over the years, records of toxic *Ostreopsis* species have increased and new compounds similar to PlTX have been described. Ostreocins were the first characterized analog (Ukena *et al.*, 2001, 2002), followed by mascarenotoxins (other PlTX analogs) detected in a *Ostreopsis mascarenensis* bloom in the southwest Indian Ocean (Lenoir *et al.*, 2004). Ovatoxin-a (OVTX-a) was isolated from field samples during an *Ostreopsis ovata* bloom along the Ligurian coasts (Italy) and from *O. ovata* cultures (Ciminiello *et al.*, 2008). At first, a study conducted on cultured strains isolated from the Adriatic and Tyrrhenian Seas (Guerrini, 2010) showed the presence of ovatoxin-a (OVTX-a) as the main toxin and putative palytoxin (pPLTX) as the minor component. Further investigations highlighted the presence of four additional ovatoxins (OVTX-b, c, d, e) produced by *O.* cf. *ovata* (Ciminiello, 2010).

Several other new PlTX compounds have been very recently characterized (Ciminiello *et al.*, 2010; Rossi *et al.*, 2010).

PLTX has been the cause of human poisoning through the consumption of contaminated seafood such as crabs (Alcaca *et al.*, 1988), parrotfish (Noguchi *et al.*, 1988) and clupeotoxic sardine (Onuma *et al.*, 1999). This indicates that the toxins produced by *Ostreopsis* cells can be transferred to higher trophic levels via food chains (Granéli *et al.*, 2011).

PLTX shows remarkable biological activity even at very low concentration (Moore & Scheuer, 1971). It is known that the presence of palytoxin in seafood can lead to clupeotoxism, i.e., an illness linked to human mortality (Onuma *et al.*, 1999). These neurotoxins cause a type of poisoning in humans called palytoxicosis, characterized by symptoms including: salivation, abdominal cramps, nausea, severe diarrhoea, muscle

spasms and breathing difficulties, followed by death in the most severe cases (Alcala *et al.*, 1988; Yasumoto *et al.*, 1986; Yasumoto, 1998).

However, the most commonly reported complications of PTX poisoning appears to be rhabdomyolysis (Kodama *et al.*, 1989; Taniyama *et al.*, 2002; Okano *et al.*, 1998), a syndrome injuring skeletal muscle, causing muscle breakdown, and leakage of large quantities of intracellular (myocyte) contents into blood plasma (Deeds & Schwartz, 2010).

Other symptoms associated with PTX poisoning in humans are characterized by a bitter/metallic taste, abdominal cramps, nausea, vomiting, diarrhea, paresthesia, bradycardia, renal failure, cyanosis and respiratory distress (Alcala *et al.*, 1988; Kodama *et al.*, 1989; Taniyama *et al.*, 2002). The latter two precede death in fatal cases (Munday, 2008).

These cases describe the intoxication that may occur after handling toxic organisms or inhalation of steam after cleaning the aquarium from these organisms with boiling water (Tubaro *et al.*, 2011).

PTX impairs the function of the Na^+/K^+-ATPase (Habermann, 1989; Kim *et al.*, 1995; Wu, 2009; Rossini & Bigiani, 2011) by binding to the extracellular part of the Na^+/K^+-ATPase, and thereby inhibits the active transport of Na^+ and K^+ across the cell membrane by transforming the pump into a non-specific permanently open ion channel. The membrane depolarization generated and the massive increase of Ca^{2+} in the cytosol (Satoh, 2003) interferes with some vital functions of cells.

3.0 MATERIALS AND METHODS

3.1 Study sites

Macroalgae was collected from the sampling sites, Sampadi Island and Satang Besar Island, Sarawak. Sampadi Island (N01°44´04.5´´, E110°05´06.5´´) and Satang Besar (N01°47´08.8´´, E110°09´29.7´´) are located in Sarawak, east Malaysia, to the southwest of Borneo (Figure 3.1). The islands are located around 3 km from the mainland. These islands have a typical equatorial climate affected by the northeast and southwest monsoon with surface water of 27 to 29°C.

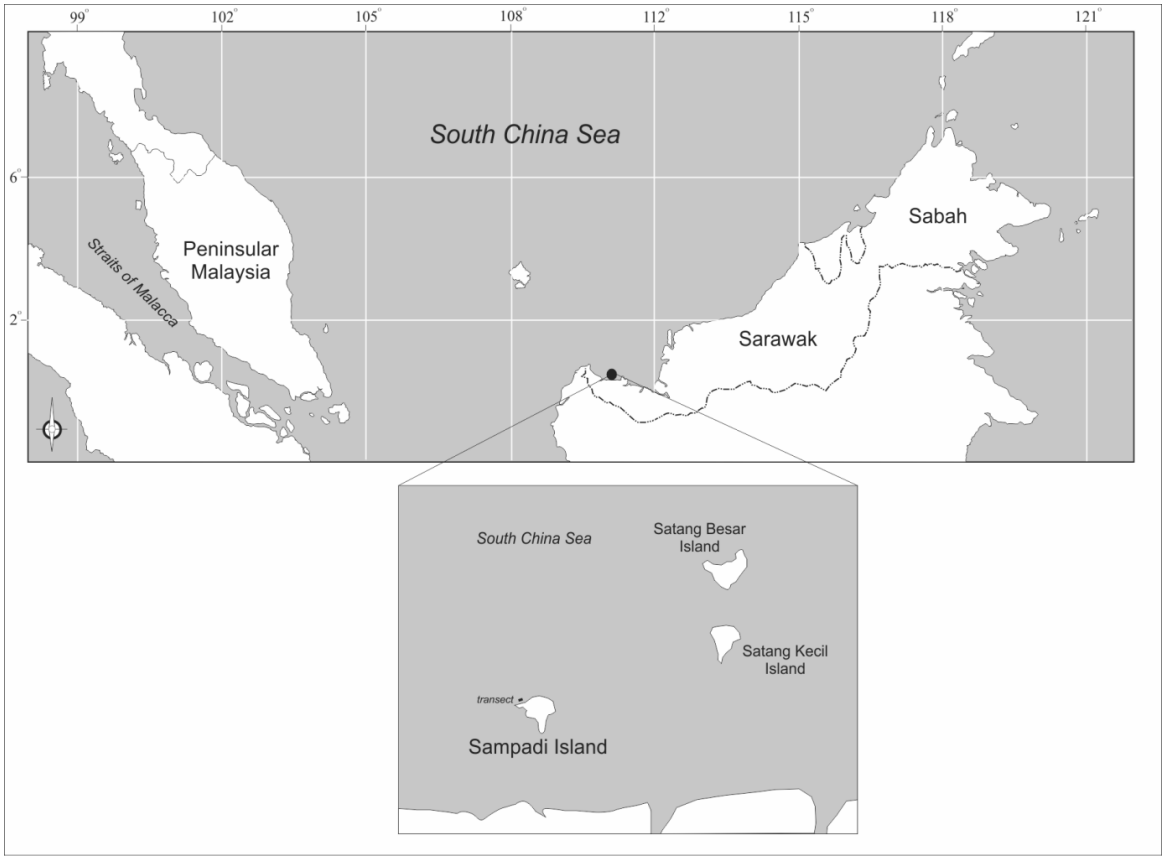

Figure 3.1: Malaysia map showing the location of Sampadi Island and Satang Besar Island, Sarawak.

3.2 Sample collection

Macroalgal samples were hand collected and placed in 1 L jars or zip lock bags by snorkelling or SCUBA diving. In the laboratory, dinoflagellate cells attached to the macroalgae were dislodged by physical means such as vigorously shaken by hand into a plastic jar for 1 min. The materials were sieved through 250 and 100-µm mesh sieves, and cells retained in a 20-µm mesh sieve were back-washed into a 50 mL centrifuge tube.

Samples were preserved in acidic Lugol's solution or buffered paraformaldehyde for cell enumeration, in 4% glutaraldehyde for SEM observation (Leaw *et al.* 2001, 2010).

3.3 Cell isolation and clonal culture establishment

Clonal culture were established from the live samples collected using capillary pipette method (Hoshaw & Rosowski, 1973) into a 96 multiple well plate chamber containing filtered seawater from the sampling site under the observation of inverted microscope. The 96 multiple well plate chambers were kept in an incubator and observations under inverted microscope are made every two days. The cells were transferred into a 24 multiple well plate chambers containing a small amount of ES-DK medium when the number of cells exceed 100 cells per well (Kokinos & Anderson, 1995). Once the cell multiplied into more than 500 cells per well, the cultures were transferred into test tubes and the samples were maintained at 25°C and 12:12 light: dark photoperiod with light intensity of 110–140 µ mole photon m^{-2} s^{-1}. Clonal culture were maintained in test tubes and sub-cultured every week using ES-DK medium.

3.4 Morphological observation

3.4.1 Epifluorescence microscopy

Species identification was based on morphological descriptions given by Fukuyo (1981) and Faust *et al.* (1996). For normal light microscopy, the cultures were fixed in 4% glutaraldehyde. Cell dimensions were determined by measuring the dorsoventral diameter and transdiameter of fixed cells using Image J (National Institute of Health, USA).

Thecal morphology and plate tabulation was examined under an epifluorescence microscope with calcofluor-white staining. Sample preparation begins with staining fixed samples with 1% Calcoflour white solution (Fluka, Japan). Nucleus was stained with SYTOX Green (Invitrogen, USA). Stained samples were viewed under an Olympus IX51 inverted epifluorescence microscope (Olympus, Melville, USA) with UV filter set. Images were captured with a cooled CCD camera (SIS Colorview F12, Germany).

3.4.2 Scanning electron microscopy

Cultures of cells were fixed with 5% glutaraldehyde for 1 h at room temperature. Samples were then rinsed three times with cacodylate buffer (0.1M) and post-fixed in 1% OsO_4 solution. Dehydration was performed with a graded series of ethanol (30, 50, 70, 80, 90, and 100 %). The inter-medium was substituted with amyl acetate and finally preceded to critical point drying.

After drying, the samples were carefully mounted on an aluminum stub using double stick carbon tape. Samples were then introduced into the chamber of the sputter coater and coated with a very thin film of gold-palladium by a sputter coater (JEOL, JFC-1600). Samples examination were performed with a JEOL scanning electron microscope