SPATIO-TEMPORAL HETEROGENEITY OF BENTHIC HARMFUL DINOFLAGELLATE ASSEMBLAGES AT THE FRINGING REEFS OF RAWA ISLAND, MALAYSIA

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ABSTRACT

Ciguatera Fish Poisoning (CFP) is regarded as the most common seafood intoxication in human, involving the neurotoxins produced by some species of epiphytic and benthic dinoflagellates found in coral reefs and inshore habitats of tropical and subtropical regions. The benthic harmful dinoflagellates, *Gambierdiscus* spp., *Fukuyoa* spp., *Ostreopsis* spp., *Prorocentrum* spp., *Coolia* spp. and *Amphidinium* spp. are predominantly epiphytic in nature, forming mucilaginous layers to attach onto macrophytes and epi-benthic layers of substratum. The phase shifts of coral-dominated reefs to algal dominated reefs may favor proliferation of these benthic dinoflagellate assemblages. The effects of bottom substrate complexity and host selectivity factors on host colonization by these benthic dinoflagellates are still unknown. This study aimed to investigate benthic harmful dinoflagellate assemblages in relation to reef microhabitats by adopting a non-destructive sampling technique. Rawa Island, Terengganu was selected based on healthy and unhealthy coral reefs at the study site. Hierarchical cluster analysis and non-metric multidimensional scaling (MDS) was adopted to define biologically distinct regions with respect to the reef microhabitats characters. A total of 115 artificial screen samples collectors were deployed underwater and successfully retrieved by SCUBA after 24 hours deployment period. Average daytime temperature and salinity of shallow sea surface water were recorded between 30 – 33 ℃ and 30 – 32 PSU respectively, with consistent daily light intensity in a range of 1000 – 2500 µmol $m^{-2}s^{-1}$ recorded. The resultant data of this study clearly indicated benthic dinoflagellate assemblages were prominently distributed in disturbed reef microhabitats and dispersed in a patchy distribution pattern among distinct habitat types. *Ostreopsis* was known as predominant species among other five benthic epiphytic genera and perennially present in all bottom microhabitats investigated at each sites. It was more tolerate to slightly shaken microhabitats rather than calm sheltered areas probably due to their abilities to secrete mucilage layers in order to associate with bottom substratum. *Gambierdiscus* population was likely attributed to the presence of filamentous turf algae population appeared on dead coral fragments in shallow sheltered reefs area (0.5 – 3 m). Tuft algal mat on dead coral fragments provides favorable dense and fine branches of adherent microhabitats for *Gambierdiscus* to overcome strong water movement, supporting larger surface area for cell attachment. Majority of *Prorocentrum* spp. and *Amphidinium* spp. were significantly higher at inshore sheltered associated with heavy sand sediments but less at macrophyte. The presence of *Coolia* spp. was scarce in almost all samples collected from sites. This phenomenon can be explained that sudden water disturbance may breakdown the close attachment between benthic dinoflagellates and bottom substratum, prompting to reintroduction of benthic dinoflagellates populations into water column. As a conclusion, data analyzed in this study indicated disturbed coral reefs environment could attribute to proliferation of benthic harmful dinoflagellate communities despite of possible circumstances of environmental variations. Preliminary investigation on host preference of benthic harmful dinoflagellates is important to discover their species diversity and distribution and their possible impacts on marine organisms as well as human intoxication risks.

ABSTRAK

Ciguatera Fish Poisoning (CFP) merupakan salah satu keracunan makanan laut yang melibatkan neurotoksin yang dihasilkan oleh spesies epifitik dan bentik dinoflagelat yang boleh dijumpai di kawasan terumbu karang dan pesisiran laut tropika dan subtropika. Enam genera bentik dinoflagelat yang lazim diketahui, iaitu *Gambierdiscus* spp., *Fukuyoa* spp., *Ostreopsis* spp., *Prorocentrum* spp., *Coolia* spp. dan *Amphidinium* spp., merupakan dinoflagelat epifitik yang terdapat dalam ekosistem marin. Mereka mampu membentuk lapisan bermusilaj secara semulajadi untuk melekat pada permukaan makrofit dan substratum yang terdapat di bahagian epi-bentos. Kemusnahan terumbu karang akan mencetuskan anjakan fasa yang menggalakkan pertumbuhan makroalga secara berleluasa. Kerumitan bentik substratum dan faktor-faktor pemilihan himpunan dinoflagelat bentik masih lagi tidak diketahui selain daripada sifat epifitik yang diperihalkan. Kajian ini bertujuan untuk memahami perumah himpunan dinoflagelat bentik dengan menggunakan teknik substrat gantian. Pulau Rawa telah dipilih sebagai tapak kajian dengan kewujudan terumbu karang sihat dan tidak sihat. Hierarchical cluster analisis dan non-metric multidimensional scaling (MDS) telah diaplikasikan untuk mengaitkan perbezaan biologi habitat berdasarkan ciri-ciri bentik substratum. Sebanyak 115 sampel skrin telah berjaya diaplikasikan di tempat kajian dan dikumpul melalui kaedah SCUBA setelah 24 jam. Purata suhu dan saliniti yang direkodkan dalam ringkungan 30 – 33 ℃ and 30 – 32 PSU di mana maksimum keamatan cahaya mencecahi bacaan yang stabil 1000 – 2500 µmol $m^{-2}s^{-1}$. Hasil kajian menunjukkan kepadatan dinoflagelat bentik lebih tinggi di kawasan terumbu karang yang teruk dimusnahkan serta menyerak secara bertompok di habitat yang berbeza. Ostreopsis merupakan bentik dinoflagelat yang pradominan dan lazim dijumpai di kebanyakan dasar mikrohabitat. Dinoflagelat ini lebih cenderung di mikrohabitat yang lebih rekah disebabkan rembesan musilaj yang membantu mereka berlekat pada dasar

permukaan substratum. *Gambierdiscus* spp. boleh didapati di kawasan serpihan terumbu karang mati (0.5 – 3 m) yang penuh ditumbuhi dengan hamparan alga. Hamparan hamparan alga di permukaan terumbu karang mati menyediakan tempat pelekatan mikrohabitat bagi *Gambierdiscus* supaya mampu menahani pengaliran air yang deras dan memberikan ruang tempat yang luas sebagai tempat pelekatan. *Amphidinium* spp. dan *Prorocentrum* spp. tertumpu ke kawasan berpasir dan/atau terumbu karang mati yang dilitupi oleh kawasan pasir, namun begitu kurang makrofit yang didapati di kawasan tersebut. Kehadiran *Coolia* jarang dijumpai dalam kajian ini. Ini boleh dijelaskan bahawa gangguan air secara tiba-tiba berkemungkinan akan menyebabkan bentik dinoflagellate tertanggal daripada dasar substratum dan berada dalam turus air. Kesimpulannya, kemusnahan terumbu karang dipercayai akan mencetuskan pembesaran makroalga dan benthik dinoflagellate secara berleluasa selain daripada faktor-faktor perubahan persekitaran alam. Kajian ini terhadap keutamaan perumah bentik dinoflagellate adalah sangat penting untuk memahami kepelbagaian spesies dan impak terhadap marin organisma dan risiko keracunan manusia akibat bawaan makanan laut.

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LIST OF SYMBOLS AND ABBREVIATIONS

HAB : Harmful algal bloom

BHABs : Benthic harmful algal blooms

CFP : Ciguatera fish poisoning

DSP : Diarrhetic shellfish poisoning

CTX : Ciguatoxin

MTX : maitotoxin

PTX : Palytoxin

McTX : Mascarenotoxin

OvTx : Ovatoxin

CCA : Crustose calcerous algae

SCUBA : Self-contained underwater breathing apparatus

CCD : Cooled charge-couple device

SEM : Scanning electron microscopy

TIFF : Tagged image file

EDTA : Ethylenediaminetetraacetic acid

Tris-HCl : Tris-hydrochloride

CTAB : cetyltrimethylammonium bromide

C:I : chloroform:isoamyl

P:C:I : phenol:chloroform:isoamyl

PCR : Polymerase chain reaction

dNTP : deoxynucleoside triphosphate reagent

BLAST : Basic local alignment search tool

NCBI : National Center of Biotechnology Institute

PAUP* : Phylogenetic Analysis Using Parsimony*

MP	:	Maximum parsimony
ML	:	Maximum likelihood
TBR	:	tree-bisection-reconnection
GTR	:	General-time-reversible
PAST	:	Paleontological statistics software package
nMDS	:	non-Metric multidimensional scaling
ANOSIM	:	One-way analysis of similarity
SIMPER	:	Similarity percentage
CCA	:	Canonical correspondence analysis
LM	:	Light microscope
L_{AP}	:	Anterioposterior length
W_{AP}	:	Anterioposterior width
L_{DV}	:	Dorsoventral depth
Po	:	Apical pore
APC	:	Apical pore complex

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Appendix A	List of culture strains of benthic dinoflagellates established in this study

CHAPTER 1: INTRODUCTION

1.1 Source and fate of ciguatera fish poisoning (CFP)

The occurrence of ciguatera fish poisoning (CFP) and ciguatoxin-associated field studies have been globally studied for years ever since the first event of CFP outbreak reported on suspected benthic epiphytic dinoflagellate described as *Gambierdiscus toxicus* (Adachi, & Fukuyo, 1979, Yasumoto et al., 1977). In early years, the genus *Gambierdiscus* was recognized as toxic benthic dinoflagellate producer of ciguatoxin affecting human health and even cause fatal through consumption of contaminated seafood (Gómez et al., 2015, Parsons et al., 2012, Yasumoto et al., 1995). The infected victims may experience gastrointestinal symptoms such as vomiting, diarrhea, nausea, abdominal pain as well as neurological symptoms such as tingling of lips and extremities, reversal perception of temperature, localized itch of skin, hypotension, respiratory difficulties and paralysis. Yet, little is known on the origin of ciguatoxin related to benthic harmful dinoflagellates in term of ecology, life cycles as well as their taxonomy. Recent studies believed that a close relationship between naturally assemblages benthic epiphytic dinoflagellates genus *Ostreopsis*, *Prorocentrum* and *Amphidinium* might also one of the key point contributing to CFP illness, releasing a numbers of unrelated biotoxins and accumulate and/or biomagnifying throughout food web transfers (Gómez et al., 2015, Shears, & Ross, 2009, Tindall, & Morton, 1998).

Up to date, six genera of benthic harmful algal blooms (BHABs) dinoflagelllates are reported mainly from tropical and subtropical regions of the Pacific Ocean, Indian Ocean, and Caribbean Seas (Bomber et al., 1989, Tan et al., 2013, Tindall, & Morton, 1998, Vila et al., 2001, Yasumoto et al., 1980a), namely as *Gambierdiscus* Adachi & Fukuyo, *Ostreopsis* Schmidt, *Fukuyoa* Gómez, Qiu, Lopez & Lin, *Prorocentrum* Ehrenberg, *Amphidinium* Claparède & Lachmann, and *Coolia* Meunier. BHABs dinoflagellates are often co-existed among different benthic dinoflagellates and typically associated with bottom natural substratum in ciguatera-endemic areas. They are known to be benthic epiphytic dinoflagellates as well, forming a close association with host macroalgae and attach on bottom substratum such as dead corals, sand and detritus (Rains, & Parsons, 2015, Vila et al., 2001). Common natural substrates dominated by BHAB dinoflagellates including seagrasses, seaweed bed, coral rubble, rocks and sediment (Adachi, & Fukuyo, 1979, Tan et al., 2013, Tester et al., 2014). For instance, *Ostreopsis* spp. was abundantly found on the surface of macrophytes, *Sargassum* spp., *Turbinaria* spp. and *Halimeda* spp. (Sidabutar, 1996, Tan et al., 2013, Vila et al., 2001).

The nature of microhabitat host preference studied on benthic epiphytic dinoflagellates is still unknown and requires more in-depth ecological investigations. Certain field studies pointed out that benthic epiphytic dinoflagellate species might revealed in totally different host preference on macroalgae and hard bottom substratum even within similar reef environments. This preference might be due to possible functions of existence of surface areas for attachment (Bomber et al., 1989, Rains, & Parsons, 2015, Tindall, & Morton, 1998), types of macroalgae (Bomber et al., 1988, Vila et al., 2001, Yasumoto et al., 1979), types of benthic substratum (e.g., dead corals, rocks, sand, water column and macroalgae) (Chinain et al., 1999a, Faust, 1995, Fukuyo, 1981, Nishimura et al., 2014, Vila et al., 2001), and/or presence of chelating factors excused by macroalgae host (Bomber et al., 1989, Parsons et al., 2012).

Relevant ecological and environmental studies have been conducted simultaneously to understand possible stimulating factors on the outbreaks of *Gambierdiscus* and other ciguatoxin associated benthic dinoflagellates (Bomber et al., 1988, Bomber et al., 1989, Fraga et al., 2012, Kim et al., 2011, Shah et al., 2013, Yasumoto et al., 1979, Yasumoto et al., 1980a). As per mentioned in Gómez et al., (2015), coral degradation results in dead coral surface provide a favorable colonization of host macroalgae, in return this phase shift of reef system have promoted increasing numbers of potential ciguatoxin related benthic epiphytic dinoflagellate communities. These benthic epiphytic dinoflagellate species might consume by herbivorous fish and invertebrates that grazing upon macroalgae host. Indirectly, ciguatoxin may bioaccumulate and biomagnify into higher trophic levels through food wed transfer, and eventually cause human intoxication (Rains, & Parsons, 2015). Moreover, environmental physical changes (e.g. coral bleaching and global warming) happened within the reef ecosystem might contribute towards sudden outbreaks of ciguatera as a consequence. A close monitoring on the assemblages of possible toxic or non-toxic BHABs dinoflagellates might be useful in relation of providing as bio-indicator purposes of coral reef ecosystem health (Gómez et al., 2015).

In Malaysia, very limited benthic epiphytic dinoflagellates relevant studies were carried out particularly on ecological variations of benthic epiphytic dinoflagellates in relation to their host preferences. The non-destructive artificial substrate method was recently introduced widely and applied in BHABs ecological studies (Tan et al., 2013, Tester et al., 2014) for effective cell quantification purposes. The quantification of cell abundance of BHAB dinoflagellates was initially expressed as cells g^{-1} after samples are concentrated and enumerated using standard light microscopy method, where wet weight of macrophytes collected is measured (Adachi, & Fukuyo, 1979, Koike et al., 1991, Tan et al., 2013, Tester et al., 2014, Vila et al., 2001). Nevertheless, complex morphologies of natural substrates have restricted researchers to compare BHAB dinoflagellate cell abundances among different substrates due to variant of surface area to mass ratios.

Artificial substrate method is only applicable for benthic epiphytic dinoflagellates that prompt to attach onto surfaces of macroalgae and/or substratum. The surface area of artificial screen is standardized with a known surface area (10.2 × 15.2 cm) and ready to deploy onto potential targeted environmental habitats. The sample collected is much cleaner compared to the natural substrates. Yet, the deployed artificial screen substrates have to be retrieved from each sampling site the next day after adequate incubation period of 24 hours (Tan et al., 2013, Tester et al., 2014).

1.2 Aims and objectives of the study

This study aimed to carry out an ecological investigation of possible toxic benthic epiphytic dinoflagellates in term of spatial and temporal distributions located in Rawa Island, Kuala Terengganu (Malaysia). The relationship of benthic epiphytic dinoflagellates with their bottom microhabitats and environmental physical variations was monitored and analyzed accordingly in an attempt to determine their specific host preferences. The specific objectives of this study are:

i. To determine species distribution of benthic epiphytic dinoflagellate assemblages,

ii. To determine diversity of benthic epiphytic dinoflagellate species in term of morphological and molecular characterization,

iii. To investigate environmental physical variation on species distribution of benthic epiphytic dinoflagellate community, and

iv. To define the relationship between benthic epiphytic dinoflagellate community and their microhabitat host preferences.

1.3 Thesis structure

The outline of research approach as well as the structure of this study was presented with general introduction in Chapter I, Literature review in Chapter II, follows by Material and methods in Chapter III. All results obtained in this study will be presented in Chapter IV. This will be followed by Discussion in Chapter V. The report will end with a conclusion and recommendation of in Chapter VI.

CHAPTER 2: LITERATURE REVIEW

2.1 Marine benthic dinoflagellate

Recent advances in population and species identification for phytoplankton have revealed immense biodiversity at different taxonomic levels. There are vast numbers of novel species been well documented and described as marine benthic dinoflagellates globally, aided by rapid development of molecular methods. Marine benthic dinoflagellates are described as marine tycoplanktonic habitants, either benthic or epiphytic, in the tropical and subtropical coral reef ecosystems. Most of the prominent benthic harmful algal blooms (BHABs) dinoflagellates species were epiphytic in nature by forming coating layers of mucus and loosely attached to the surface of specific macroalgae. To date, six major genera associated with marine benthic dinoflagellate includes *Gambierdiscus* Adachi & Fukuyo 1979; *Fukuyoa* Gomez, Qiu, Lopes & Lin 2015; *Ostreopsis* Johs.Schmidt 1901; *Prorocentrum* Ehrenberg 1834; *Coolia* Meunier 1919; and *Amphidinium* Claperède & Lachmann 1859 have been well documented and are summarized here in the following sections.

2.1.1 *Gambierdiscus* Adachi & Fukuyo (1979) and *Fukuyoa* Gomez, Qiu, Lopes & Lin (2015)

The genus *Gambierdiscus* was formerly identified as thecated species namely *G. toxicus* with its heavily anterior-posteriorly compressed morphologies in Adachi, and Fukuyo, (1979). Following in 1990's years until today, this ciguateric genus was widely documented into thirteen species from ciguatera endemic areas mainly from tropical and subtropical marine areas e.g. Carribean Sea, Polynesia, Pacific Ocean and Indian Ocean. *G. belizeanus* (Faust, 1995) was observed in sand dwelling samples and described as second species, following by *G. australes* Faust et Chinain, *G. pacificus* Chinain et Faust, *G. polynesiensis* Chinain et Faust (Chinain et al., 1999a), *G. caribaeus*

Vandersea, Lintaker, Faust, Kibler, Holland & Tester, *G. carolinianus* Lintaker, Vandersea, Faust, Kibler, Holland & Tester, *G. carpenteri* Kibler, Litaker, Faust, Holland, Vandersea & Tester (Litaker et al., 2009), *G. excentricus* S. Fraga (Fraga et al., 2011), *G. scabrosus* T. Nishimura, Shin. Sato & M. Adachi (Nishimura et al., 2014), and *G. silvae* S. Fraga & F.Rodríguez (Fraga, & Rodríguez, 2014).

In recent years, another two novel species *G. balechii* sp. nov and *G. cheloniae* sp. nov are newly described as potent benthic toxic dinoflagellate from Celebes Sea and Cook Islands located at Pacific Ocean respectively (Fraga et al., 2016, Smith et al., 2016). Most *Gambierdiscus* species are difficult to distinguish due to high morphological similarity. Nonetheless, more accurate and details identification of species in term of specific morphological characterization and phylogenetical identification, together with their possible associated toxicity are therefore crucial.

The *Gambierdiscus* species exhibiting globular morphologies was erected as a newly described genus, *Fukuyoa* Gomez, Qiu, Lopes & Lin in Gómez et al., (2015) from the formerly documented globular *Gambierdiscus* species e.g. *G. yasumotoi* and *G. ruetzleri* (Faust, & Morton, 1995). This genus pointed out to be morphologically and genetically distinct to the typical lenticular *Gambierdiscus* species. Their distinct morphologies were distinguishable from other benthic *Gambierdiscus* species based on their significant morphological characteristics, either anterio-posteriorly compressed or globular body shaped (Adachi, & Fukuyo, 1979, Chinain et al., 1999b, Faust, 1995, Fraga, & Rodríguez, 2014, Fraga et al., 2011, Litaker et al., 2009, Parsons et al., 2012). Hereby, three morphologically distinct globular-shaped *Fukuyoa* species are established, *F. paulensis*, *F. yasumotoi* and *F. ruetzleri*. *Fukuyoa* species, with their remarkable smaller cell size with the previously described *Gambierdiscus* species, a descending cingular displacement, distinguishable shape of the apical pore plate and different arrangement of sulcus plates (Faust, & Morton, 1995, Gómez et al., 2015).

2.1.2 *Ostreopsis* Johs.Schmidt (1901)

In 1900, the genus *Ostreopsis* was first described as *O. siamensis* Schmidt identified from plankton samples collected from The Gulf of Siam by Schmidt, (1900). Based on Schmidt's description, *O. siamensis* was observed as strongly anterio-posteriorly compressed cells with its remarkable tear-like shaped in apical view. The cell surface was smooth and covered with randomly spaced identical pore sizes observed on the surface structure of plates. Following that, Fukuyo, (1981) has introduced two morphologically distinguished *Ostreopsis* species, namely *O. lenticularis* Fukuyo and *O. ovata* Fukuyo. *O. lenticularis* was claimed to be similar with *O. siamensis* in term of cell size and shapes. However, *O. lenticularis* was distinguishable from the latter based on the body undulation and presence of fine pores and larger pores widely scattered all over the thecal plates (Fukuyo, 1981).

To date, six new benthic *Ostreopsis* spp. were recognized at different marine areas in worldwide, including *O. heptagona* Norris, Bomber and Balech (1985), *O. mascarenensis* Quod (1994), *O. labens* Faust and Morton (1995), *O. belizeanus* Faust (1999), *O. caribbeanus* Faust (1999), and *O. marinus* Faust (1999). The taxonomical characters to discriminate all described *Ostreopsis* species were still very confusing and scarce. Penna et al., (2005) encountered that almost all *Ostreopsis* species appeared in similar plate patterns and could match with original description of *O. siamensis,* except *O. heptagona*. The morphological plasticity or ambiguous characteristics displayed in *Ostreopsis* species both collected from fields and in culture conditions showed the necessity to revise the taxonomic characters of *Ostreopsis* spp. Several studies have begun and on-going to gather more accurate morphological data and genetic information in relation to geographic distribution for the specific types of *Ostreopsis* spp. (Leaw et al., 2001, Penna et al., 2005).

2.1.3 *Coolia* Meunier (1919)

The genus *Coolia* was originally described by Meunier (1919) as *C. monotis* from samples collected from Nieuport, Belgium. This species is well illustrated as spherical shaped cell from ventral view with its smooth and scattered round small pores of thecal surface. *Coolia* species showed its remarkable distinct morphological characteristics in which their epitheca is slightly smaller than the hypotheca. *Coolia* was remained monospecific until possible toxic species *C. tropicalis* Faust (Faust, 1995) and *C. areolata* Ten-Hage, Turquet, Quod et Couté (Faust, 1999) were discovered from sand samples, in addition to a new nontoxic epiphytic *C. canariensis* Fraga (Fraga et al., 2008). These species are further differentiated by their notable thecal plate arrangement and ornamentation, following by incorporation of comprehensive molecular phylogenetic approach to distinguish between closely related species (Muller et al., 2007).

C. malayensis Leaw, Lim & Usup (Leaw et al., 2010) was described as the smallest known species of *Coolia* collected from tropical Malaysian waters. It was shown to be morphologically varied with *C. monotis* Meunir in term of comparison of its significant smaller in cell size, largest hypothecal plate of third posticingular plate (3′′′) and the presence of fine perforations within the pores. Apart from detailed morphologically comparisons between this two particular *Coolia* species, Leaw et al., (2010) had proven *C. malayensis* was genetically considered as a distinct species based on their secondary structure of ITS2, which was also further supported in Leaw et al., (2016). In recent years, two novel *Coolia* species, *C. palmyrensis* Karafas, Tomas, York and *C. santacroce* Karafas, Tomas, York were newly described by Karafas et al., (2015) in which contributing to a total of seven species under the genus *Coolia*.

2.1.4 *Prorocentrum* Ehrenberg (1834)

The dinoflagellates genus *Prorocentrum* was one of the most diverse genus in marine tropical areas (Faust, & Gulledge, 2002). Species of *Prorocentrum* can existed as either planktonic, sand dwelling or benthic/epiphytic in nature and some are known to be toxic blooming species, as described in Faust, and Gulledge, (2002). These species are morphologically small to medium in cell size with two dissimilar flagella emerging from the anterior part of the cell and varied in shape from spheroid to pyriform in valve view. The micromorphology of valve surface and pattern of the intercalary band was observed and described in details for specific morphological characters of species identification purposes (Faust, 1990a, 1990b, 1993, 1994, 1997, Hernández-Becerril et al., 2000). The genus *Prorocentrum* was described by Ehrenberg (1834) and has been studied extensively since year 1990s with *P. micans* Ehrenberg (1833) was firstly described species. More than 70 species of *Prorocentrum* have been introduced in the following years with the discovery of some potent toxin species producers.

2.1.5 *Amphidinium* Claperède & Lachmann (1859)

The genus *Amphidinium* Claparède & Lachmann (1859) is one of the widespread dinoflagellate genus and cosmopolitan in marine environments. It belongs to naked dinoflagellates with no thecal plates can be observed. The type of species was originated by formerly described *A. operculatum* obtained from its type locality at west coast of Norway. A huge number of 171 species of *Amphidinium* have been reported from freshwater and marine environments. They can be found either as benthic/epiphytic, planktonic, sand-dweling or symbiotic dinoflagellates. The taxonomy of *Amphidinium* is complicated by morphological interspecific similarities and intraspecific cell variability (Dolapsakis, & Economou-Amilli, 2009, Maranda, & Shimizu, 1996, Murray et al., 2012). Hence, accurate species identification should be characterized by carried out phylogenetic analysis to effectively exclude the possibilities of confusion between close species identification.

2.2 Benthic dinoflagellate-associated human illness

The frequent occurrence and magnitude of harmful algal blooms (HABs) events have increased in worldwide leading to fatal food-borne illness affecting human health and loss of economics and marine resources. The first report by Yasumoto et al., (1977) on the involvement of a benthic dinoflagellate responsible for ciguatera fish poisoning (CFP) have successfully triggered research attention on the importance studies of ecological and taxonomy with regards to potent benthic dinoflagellates and other ciguatera-associated genera. *Gambierdiscus toxicus* Adachi and Fukuyo was described and proven to be responsible on the outbreak of CFP incident happened in Gambier Island. The seafood poisoning illness related to BHABs was subsequently linked to other marine benthic harmful dinoflagellates (e.g. *Ostreopsis*, *Prorocentrum*, and *Coolia*), in which later also found to be toxic and threaten to human health (Aligizaki et al., 2011, Fukuyo, 1981, Holmes et al., 1995, Nakajima et al., 1981, Yasumoto et al., 1980b). Most relevant food poisoning cases related to BHAB are ciguatera fish poisoning (CFP), palytoxin (PTX) fish poisoning, clupeotoxism and diarrhetic shellfish poisoning (DSP).

2.2.1 Ciguatera fish poisoning (CFP)

Ciguatera fish poisoning (CFP) is the most common nonbacterial food-borne illness associated with consumption of ciguatoxin-contaminated fish resources. Around 25,000 – 50,000 people are estimated to suffer from CFP intoxication annually with gastrointestinal, and neurological symptoms especially reversal of temperature sensation. Lipid soluble ciguatoxins (CTX) are originally produced by benthic dinoflagellate genus *Gambierdiscus* in which can be found in close association with a variety of microhabitats including marcoalgae and sediments on coral reef ecosystem in tropical and subtropical waters (Parsons et al., 2012, Yasumoto et al., 1977). *Gambierdiscus toxicus* is known as the most common harmful species linked with ciguatera, in which it produces gambiertoxins that contribute to a range of lipid soluble CTX along with water soluble maitotoxins (MTX) (Yasumoto et al., 1995).

Species of *Gambierdiscus* are responsible to produce the principal toxin lipid-soluble neurotoxins CTX and second major water-soluble MTX which have been implicated as the cause of CFP (Anderson, & Lobel, 1987, Parsons et al., 2012). These toxins are readily transferred into coral reef food web when grazing activities by smaller herbivores reef fishes occurred on algal colonized by potent ciguatoxin benthic dinoflagellates (e.g. *G. toxicus*). Consequently, CTX-contained fish flesh was accumulated and biomagnified to non-toxin larger predators, and ultimately to cause mass mortality of fishes and human intoxication (Anderson, & Lobel, 1987, Scheuer et al., 1967).

CTX are odorless, tasteless, lipid-soluble and heat-stable which bio-transformed from gambiertoxin in larger fishes (Lehane, & Lewis, 2000). Therefore, the significant vectors of CFP cases are coral reef fishes, such as red snapper, moral eel and amberjacks, which are common to be frequent sources of ciguatera. Despite principal neurotoxin CTX and MTX responsible for CFP illness, the resultant intoxication signs appeared in mice suggested that cooliatoxin might be a mono-sulphated analogue of yessotoxin that is also related to ciguatoxins (Holmes et al., 1995). Cooliatoxin was preliminary isolated from culture strains of *C. monotis* obtained from Queensland (Australia) and described as likely mono-sulphated, polyether toxin. This toxin is a potent cardiac stimulant that induces hypothermia and respiratory failure but the toxin alone is unlikely to cause fatality in mice (Holmes et al., 1995).

Ciguatera is endemic in subtropical and tropical regions of the Caribbean Sea western Indian and Pacific Ocean regions (Lewis, 2001). Infected victims may experience gastrointestinal symptoms such as vomiting, diarrhea, nausea, abdominal pain as well as neurological symptoms such as tingling of lips and extremities, reversal perception of temperature, localized itch of skin, hypotension, respiratory difficulties and paralysis. Typically, ciguatera infected victims may experience a burning sensation in contact with cold objects and this symptoms may continue for several months or even years (Shoemaker et al., 2010). Although death case are rare, symptoms may reappear when the toxin are release from the lipid into the blood via alcohol and exercise (Tilman, & Lewis, 1994). The impacts of ciguatera on marine resource development have been examined in the Pacific and Caribbean regions respectively. The most obvious impacts of ciguatera impacts was typically restricted to small-scale fisheries for local consumption and for export, in which marine fish supplies have been a primary source of protein (Anderson, & Lobel, 1987).

2.2.2 Palytoxin fish poisoning (PTX)

Palytoxin (PTX) is a group of complex marine toxins primarily isolated from the marine zoanthid, *Palythoa toxica* (Moore, & Scheuer, 1971). This potent neurotoxin is a very complex molecule consisting both lipophilic and hydrophilic areas and slightly less toxigenicity than maitotoxin in total potency. These toxins are capable to cause severe impacts on membrane sodium-potassium pumps (Na+/K+-ATPase) responsible for maintaining ionic gradients (Artigas, & Gadsby, 2003, Parsons et al., 2012), leading to delayed hemolysis with a loss of potassium, converting Na/K pump into nonspecific ionic channels. Ultimately, this fatal intoxication results in nausea, vomiting, hypersalivation, abdominal cramps, diarrhea, numbness of extremities, severe muscular spasms and respiratory distress (Yasumoto et al., 1986).

Several species of *Ostreopsis* are responsible in producing a number of PTX-analogs that believed to have similar chemical structure as the parent PTX as well as a similar mode and site of actions. For instance, cultures of *O. siamensis* was first successfully isolated a kind of potent PTX-analogs namely as ostreoxin-D (Usami et al., 1995) following by second PTX-analog, mascarenotoxin (McTX), obtained from *O. mascarenensis* (Lenoir et al., 2004) and ovatoxin (OvTx) isolated from *O.* cf. *ovata* (Ciminiello et al., 2010). Cultures of *O. lenticularis* isolated from the Caribbean were found to synthesize totally distinct chemical compounds known as ostreotoxin-1 and ostreotoxin-3 (Mercado et al., 1995) that do not display the same mode and site of action similar to PTX-analogs.

The vectors of exposure to palytoxin and palytoxin analogs were confirmed mainly through the consumption of marine reef fishes and animals, such as crabs, triggerfish, mackerel, sardines and parrotfish (Randall, 2005, Yasumoto et al., 1986). These toxins can be accumulated in shellfish (Aligizaki et al., 2008) and herbivore reef fish, and have been associated with clupeotoxism that lead to human fatality.

Clupeotoxism is referred as one of the symptomology of PTX intoxication of fish which is also similar to that of ciguatera but exhibited higher mortality rate than ciguatera (Onuma et al., 1999). Similarly, infected victims suffered from clupeotoxism are typically because of their accidentally consumption on clupeoid or soft-finned fishes such as sardines and herrings or anchovies contaminated with palytoxins (PlTX). This particular toxin may blocks sodium and potassium ions that are complimentary for cell homeostasis, ending up with human illness symptoms of weakness, fever, nausea and vomiting (Mart ńez et al., 2015).

Aside from that, inhalation of released toxic aerosols from *Ostreopsis* bloom events happened in Italy and Spain have attributed to respiratory problems and skin or eyes irritations in humans (Ciminiello et al., 2006). A drastic decline in population of sea urchins was also reported during blooms of *O. siamensis* on shallow reefs in northern New Zealand (Shears, & Babcock, 2003, Shears, & Ross, 2009), indicating PTX toxin effects on the mass mortality of other marine organisms. However, it is still unclear if the toxin involved can be attributed to PTX-analogs produced by *Ostreopsis* species.

2.2.3 Diarrhetic shellfish poisoning (DSP)

Diarrhetic shellfish poisoning (DSP) toxins are known as heat-stable and lipophilic polyether toxin compound. Hence, cooking or freezing contaminated shellfish may not eliminate the potent DSP toxins. In early stage of investigation, dinoflagellate *Dinophysis fortii* was the origin of DSP-contaminated shellfish producer, namely as dinophysistoxins (DTX). Likewise, the acidic toxin okadaic acid (OA) purified from benthic dinoflagellate species *Prorocentrum lima* culture strains was found to be structurally related with DTX (Yasumoto et al., 1984). Both DTX and OA share the similar skeleton and are responsible for diarrhea and other gastrointestinal disorder (Terao K. et al., 1986).

DSP involves gastrointestinal disturbance to humans after ingestion of toxic shellfish infested with potent dinoflagellates toxins within 30 minutes to few hours (Yasumoto et al., 1989). More than 1,300 people were hospitalized because of consumption of DSP-contaminated food in Japan during the period of 1976 – 1982 (Yasumoto et al., 1984). Most of the infected patients were diagnosed with DSP illness with common gastrointestinal disorder symptoms including diarrhea, nausea, vomiting, abdominal pain and decline of body temperature. The vectors of DSP exposure were majority marine filter feeders including clams, mussels, oysters, geoduck, and scallops that able to accumulate DSP toxin in their guts.

2.3 Formation of benthic algal-dominated reefs and their effects on proliferation of BHABs dinoflagellates

In recent decades, coral reefs ecosystem has been severe disturbed and degraded at global scale, either due to direct impacts from natural phenomena or anthropogenic activities. Reef degradation are usually manifested as a marked failure in live coral population (e.g. mass coral bleaching events) and decline numbers of marine herbivores (e.g. overfishing of herbivorous fishes, crown-of-thorns starfish outbreaks etc.), and localized nutrient enrichment (Birrell et al., 2008, Guillermo Diaz-Pulido Laurence, 2002, Hughes, 1994, Ilsa et al., 2006). In Caribbean Sea, as reported in Hughes, (1994), coral reef of Jamaica islands are critically threatened under increasing and frequent disturbance due to a combination factors including climatic changes, chronic coral mortality, exploitation of natural resources and overfishing etc., contributing to extensive phase shifts in reef community structure replaced by drastic growth of benthic algae.

Coral mortality is usually followed by colonization of benthic algae of various functional groups, in which this proliferation of benthic algae referred to as a phase shift (Guillermo Diaz-Pulido Laurence, 2002, Hughes, 1994, Ilsa et al., 2006). The continued depression of entire reef ecosystem may result in spectacular growth of benthic algal on the newly available substratum provided by dead coral residues. It is important to know that re-establishment of coral reef after disturbance is often a must to go through a "phase shift" in which the abundance of coral declines, and experiences a pre-colonization background of a variety functional group of benthic algal dominance (Birrell et al., 2008, McCook, 1999). Nonetheless, some reef ecological studies showed the widespread effect of common macroalgae species (e.g. *Lobophora* spp. and *Dictyota* spp.) and cyanobacteria (e.g. *Lyngbya* spp.) on adult coral covers may have predominantly negative impacts to the maintenance and recovery of coral reef ecosystem. Prolonged competitive interaction between benthic algal and coral, including space occupation, eutrophication and sediment inputs, can be the suppression factor on the successful rates of coral replenishment within marine ecosystem, particularly during the larval and immediate post-settlement processes (Birrell et al., 2008, Ilsa et al., 2006).

Despite the effect of benthic algal inhibition on reef disturbance and degradation, benthic algal-dominated reef environment is also assumed as one of the triggering factors on proliferation of benthic harmful dinoflagellates within reef ecosystem. The occurrence of benthic harmful algal bloom is predicted to increase remarkably along with the colonization of macrophytes population within degraded coral reef conditions. However, their effects on other marine organisms and ecosystem dynamics are still inadequately studied. For instance, certain observation studies on bloom event of *Ostreopsis siamensis* on shallow waters have been reported on mortality and sudden decline in numbers of sea urchins population located in northern New

Zealand (Shears, & Babcock, 2003, Shears, & Ross, 2009). Preliminary toxicological studies of *O. siamensis* have confirmed the presence of palytoxin analogue that potentially poses a threat to coastal food webs and marine organisms (Rhodes et al., 2002).

In term of the possible interaction between benthic algal population and occurrence of benthic harmful dinoflagellate assemblages, many previous studies have summarized and generalized characteristics as well as common species-specific ecological differences on host preferences. Hereby, the influence of benthic algal-dominated reef environment has been focused as the key aspects relevant to elevating factors for BHABs dinoflagellates distribution, by focusing on (i) physical aspects exhibited on macroalgal populations, (ii) possible impacts of macroalgal to resist water flow and turbulence and finally (iii) effects of sedimentation rates.

2.3.1 Physical aspects of macroalgae

Benthic macroalgae are referred as individual algae that are visible to naked eyes ranging from crustose calcareous and tufting of surface covering algae forms, following by thick cushion-like mats of robust algae, filamentous, as well as the larger, fleshy, up-right algal functional groups (Birrell et al., 2008, McCook, 1999). Most of the bare coral substratum are often preliminary colonized with variable mixture of crustose calcerous algae (CCA) and countless numbers of very short, finely filamentous algal turfs (1 – 5 mm in height), which are generally compatible with coral recruitment (Birrell et al., 2008, Guillermo Diaz-Pulido Laurence, 2002).

The nature of macroalgal colonization on degraded coral reef can be widely diverse in term of their relevant physical aspects, involving height, function of structure and growth patterns. In some cases, for example, benthic macroagal growth rates might turn out to be uncontrollable forming cushion-liked dense mats of larger, more robust macroalagae (50 – 150 mm in height) over coral reef skeletons under extreme condition, such as experiencing drastic physical stress and increasing nutrient and/or sediment inputs. Overgrowth of damaged corals by fine filamentous algae creating a low turf algal mat may generate a function of surface area available for benthic dinoflagellates to attach themselves to macroalgal hosts. Clearly, the presence of complex physical aspects of benthic macroalgal population have provide a stable protection space in relation to close association between epiphytic dinoflagellates with macroalgae surfaces and/or hard substratum (e.g. rocks, sand, and coral rubbles).

Benthic dinoflagellates are typically described as marine tycoplanktonic habitants, either benthic or epiphytic, in the tropical and subtropical coral reef systems. Some prominent epiphytic benthic dinoflagellates tend to loosely attached on the surface of macroalgae by embedding themselves around layers of mucilage coating formation. The most notable epiphytic benthic dinoflagellates species have been addressed and reviewed in literatures, including *G. toxicus*, *O. lenticularis*, *O. cf. ovata*, *O. siamensis*, *C. malayensis*, *P. lima*, *P. concavum*, *A. carterae* etc. (Aligizaki, & Nikolaidis, 2006, Grzebyk et al., 1994, Parsons et al., 2012, Tindall, & Morton, 1998, Vila et al., 2001, Yasumoto et al., 1980a).

The nature of specific host preferences in relation to benthic harmful dinoflagellates assemblages have been reviewed elsewhere, likely reflecting their preferences onto greater surface areas and opted for ideal protections from the distinct physical appearances of macroalgae (Bomber et al., 1989, Lobel et al., 1988, Parsons et al., 2012, Tindall, & Morton, 1998). Since then, benthic macroalgae do play important roles on the abundance and composition of benthic harmful dinoflagellates, by providing the cells an ideal host attachment.

2.3.2 Impacts of water flow and turbulence

Of the few ecological survey focused on algal substrate selection on benthic epiphytic dinoflagellate assemblages, there is evidence that most varieties of epiphytic dinoflagellate species may have different algal host preferences in which a widespread of BHABs ecological studies have been demonstrated on distinct macroalgae class (Rhodophytes, Chlorophytes, and Phaeophytes). Certain studies are still considered on the possible factors that might influence specific host preferences of BHABs dinoflagellates, since their regional distributions could be remarkable varied in abundance even in such a small area, so-called as “micro-regionality”. In Tahiti Island, the algal host preference of *G. toxicus* was commonly found on *Turbinaria ornata* (Yasumoto et al., 1980a), whereas Yasumoto et al., (1979) concluded that similar *Gambierdiscus* species was varied regionally among each station and highly abundance on red calcacerous algae, *Jania* sp. in Gambier Island. Ballantine et al., (1988) noted that dense population of *G. toxicus* was encountered on *Dictyota* sp. samples collected from Caribbean Island which showed different algal morphological appearance.

The general consensus is that epiphytic dinoflagellates communities are much more conspicuous in relatively calm areas that experienced minimal current. Richlen, and Lobel, (2011) suggested that total benthic dinoflagellates abundance was primarily influenced by the degree of water motion and showed the effects at different levels among four genera surveyed (*Gambierdiscus, Ostreopsis, Prorocentrum,* and *Amphidinium*). Some epiphytic dinoflagellates such as *Ostreopsis* and *Gambierdiscus* may have behaviors and mechanisms to protect themselves from extreme water disturbance. This can be related to their ability to produce mucilage matrix enabling the cells well attached on host algal surfaces, which may shield the cells from releasing into water column (Ballantine et al., 1988, Lobel et al., 1988, Vila et al., 2001). To some extents, most epiphytic dinoflagellate species are quite vulnerable to sudden water turbulence happened within marine environment because of reduced physically pressure obtained from benthic macroalgae, especially at shallow water areas exposed to moderate to strong water movements (Aligizaki, & Nikolaidis, 2006, Tindall, & Morton, 1998, Vila et al., 2001).

A combination factor of different macroalgal assemblage patterns especially the displays of physical structure and texture may have major effects on the water flow speeds, gradients and turbulence, followed by different scales of epiphytic dinoflagellate compositions. Birrell et al., (2008) hypothesized that the impacts of macroalge on degree of water motion can be vastly diverse and significantly determine by their height and structure of the macroalgal formation. Fleshy macrophytes (such as D*ictyota* spp. and *Padina* spp.) are able to minimize water flow within metres to decimetres of the substratum which are compatible to enhance stable proliferation of epiphytic dinoflagellates. In contrast, water flow over fine filamentous algal turf with lower canopy heights is influenced by more viscous forces at level within a range of microns to millimetres of substratum layers (Birrell et al., 2008, Carpenter, & Williams, 1993). Those macrophytes with their highly defined three dimensional structures play important roles in providing shelter regions from water disturbance, and may therefore be preferred hosts for that reason (Ballantine et al., 1988, Lobel et al., 1988, Nakahara et al., 1996, Richlen, & Lobel, 2011, Vila et al., 2001).

The variation in flow speeds are also drastically affected by physical morphology and texture complexity of distinct functional group of macroalgae (Birrell et al., 2008, Carpenter, & Williams, 1993). Macroalgae are well-adapted to strong water turbulence in nature by creating rough surface features in order to minimize water pressure created in water. It is notable that epiphytic dinoflagellates do not simply attach to a host macroalgae, but might detach and release into water column when disturbed. A widespread of case studies focused on preliminary effects of water motion on the abundance and distribution of epiphytic dinoflagellates have been published for future understanding. Vila et al., (2001) believed that *Ostreopsis* was well adapted to slightly shaken reef habitat compared to relatively calm sheltered areas that experienced high sedimentation rate. Some species of benthic dinoflagellates were described as epiphytic and loosely attach on the surface of macroalgae by forming mucilaginous coating in order to reduce physical pressures of the water turbulence (Aligizaki, & Nikolaidis, 2006, Holmes, & Teo, 2002, Tindall, & Morton, 1998).

2.3.3 Impacts of sedimentation rate

The direct and indirect impacts on benthic macroalgae formation with different degree of water flow are also attributed to changes of sediment transportation and depositions. The rates of sedimentation may remarkably influenced by wave energy exposure across different geomorphic zones of coral reef. Indeed, the sedimentation rate in minimal water turbulent areas, such as reef flat, inner lagoon and middle reef flat, is inversely proportional with wave energy in which increased sediment load was observed in filamentous turf here. Small scale complexity of macroalgae surface has their advantages to trap sediment particles coupled with consequence nutrient enhancement for macroalgal growth on newly exposed substrate which probably resulted in coral mortality (Gowan et al., 2014, McCook, 1999). Therefore, sediment inputs arisen from lands run-off and eutrophication may affect directly on coral-algal interaction either by damaging or killing coral tissues and trigger the overgrowth of macroalgal via sediment trapping mechanisms.

The influence of sedimentation rates and nutrient levels in manipulating epiphytic dinoflagellates composition and their growth rates is still unclear. Most of the studies found no significant correlation between epiphytic dinoflagellate cell densities and nutrients concentration (Parsons et al., 2012, Tindall, & Morton, 1998, Vila et al., 2001, Yasumoto et al., 1980b), where cell densities do not appear to be well respond to nutrient limitation and enrichment. In fact, sediment accumulation by algal turf is likely existed parallel with increasing gradient of nutrients supply associated with sediments, in return promotes rapid macroalgal growth rates. Under a slow water flow of reef condition, sediment and/or nutrient inputs tend to remain static and accumulate by potential macroalgal population, particularly the macronutrients nitrogen and phosphorus, may turn out stimulating macroalgal overgrowth on exposed corals.

Consequently, a dramatic explosion of macroalgal abundances will suppress defensive mechanism addressed on living corals and hinder survival of existing corals, (Birrell et al., 2008, Gowan et al., 2014, Guillermo Diaz-Pulido Laurence, 2002, Hughes et al., 2007, McCook, 1999). Guillermo Diaz-Pulido Laurence, (2002) described that most of the dead corals were preliminary colonized by diverse community of CCA encrusting and algal turf at the early stage of coral degradation, but rapidly shifted to an assemblages dominated by upright and branched filamentous algae and also fleshy macroalgae. However, the competitive interaction between algal growth and living corals and the impacts of algal overgrowth on the corals are still not well understood.

The consequence of uncontrollable algal abundance on degraded reefs was not the only cause contribute to coral mortality, rather anthropogenic eutrophication phenomenon credited to escalation of sediment inputs associated may also contributes to imbalance coral reef ecosystem, such as decline of marine herbivores organisms (Guillermo Diaz-Pulido Laurence, 2002, Hughes, 1994, McCook, 1999). In contrast, heavy loaded sediment algal turfs can suppress herbivory on coral reef and that may be one of the critical factors of declining numbers in reef-related herbivores (Bellwood, & Fulton, 2008, McCook, 1999).

Asides form negative impacts onto marine herbivores, anthropogenic eutrophication is also commonly linked to harmful algal blooms (HABs) incidents (Anderson et al., 2002), however, it might not appear to be the important contributing factor. In Taylor (1985), a clear seasonal distribution pattern was typically displayed in *G. toxicus* compositions found in Caribbean Island but less significant correlated with heavy loaded sediment areas. Grzebyk et al. (1994) also pointed out extremely low benthic dinoflagellates abundance reflected in areas subjected to elevated sedimentation rates due to the presence of muddy materials on dead corals or trapped on algal turf that reduce cell compositions. Given that, most ecological studies related to distribution of benthic epiphytic dinoflagellates agreed to the fact of increasing sediment deposition may lead to growth limitation of relevant dinoflagellates.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study site

Rawa Island (5 °57'44.45" N 102 °40'53.26" S) located offshore of Terengganu in the east coast of Peninsular Malaysia was selected as a study site (Figure 3.1). The island was known with its biotic abundance and diversity of corals and the tropical marine resources that formed the fringing reef. In the inner reef area, benthic community was generally dominated by dead corals, coral rubbles and massive turf algal mats, while hard corals and various types of macroalgae were common at the outer part of the reef.

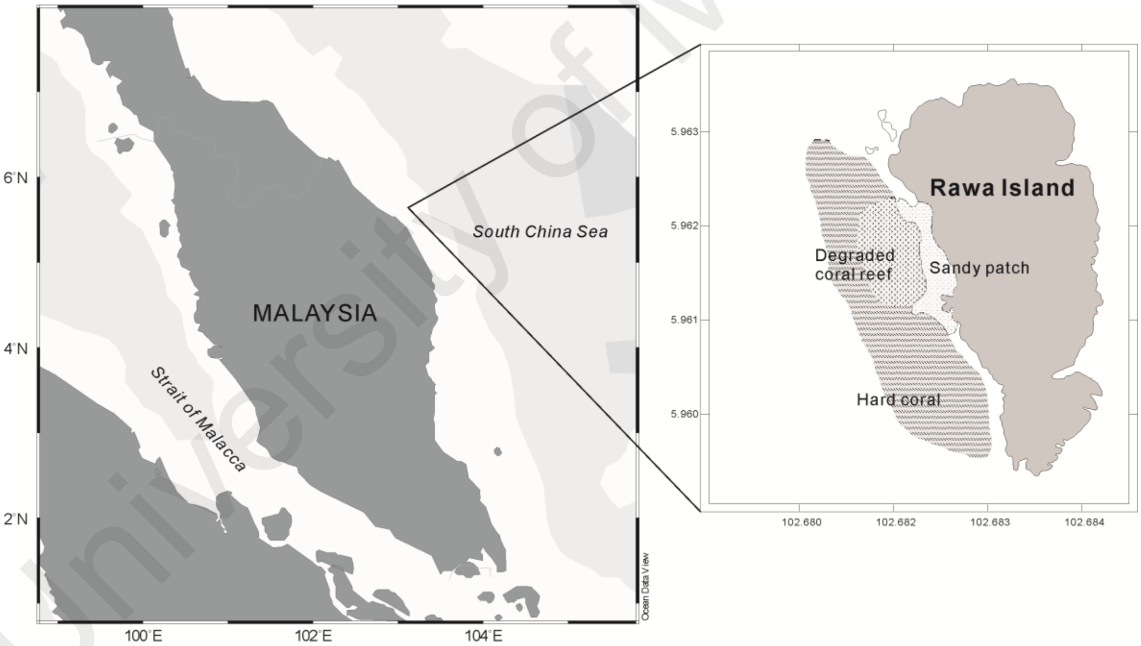


Figure 3.1: Malaysia map indicating the sampling location of Rawa Island, Terengganu.

3.2 Sampling approaches

3.2.1 Physical data collection

Fortnightly sampling was undertaken across the shallow fringing reefs of the island (depth of <3 m) between April and September, 2015 (dry Southwest Monsoon), and one sampling taken in January, 2016 (wet Northeast Monsoon). Seawater salinity (roughly 0.5 m) was recorded using a HI 96822 seawater refractometer (HANNA Instrument Incorporation, USA) whereas water depths were measured with a portable depth sounder (Speedtech Instruments, USA). The HOBO Pendant Temperature/Light data loggers were deployed at sampling sites to record the water temperature (℃) and light intensity (µmol photon m^{-2} s^{-1}).

3.2.2 Microalgae sample collection and processing

An artificial substrate method was applied in this study to quantify the abundance of benthic dinoflagellates without disturbing natural benthic substrates of coral reefs (Tester et al., 2014). A fiberglass window screen was used and cut into a standard measurement of 10.2 cm × 15.2 cm as an artificial screen substrate (Figure 3.2A). Each screen was connected to a weight (< 200 g) with monofilament fishing line as basement and submerged in water column using a small subsurface buoy. The screen was estimated to be 20 cm above the seafloor (Figure 3.2B) to avoid disturbance to the screens.

In the field, artificial screens were placed underwater by SCUBA and/or snorkeling. The screens were sampled from bottom vegetative environments including hard coral patch reefs, seaweed mats (Rhodophyta, Chlorophyta and Phaeophyta) and sandy areas, as well as those area disturbed. After deployment, the incubation time of screen was suggested to be within 24 h (Tester et al., 2014). The screen was gently retrieved into a one-litre wide mouth plastic bottle filled with ambient seawater.

Collected screen samples must be stored under ambient temperature and transported back to the laboratory.

Prior to detach targeted cells from screen samples, approximately 20% of seawater in the plastic bottle was poured through a 200 µm-mesh nylon sieve into a one litre beaker. The screen was shaken vigorously for 30 - 60 seconds to dislodge epiphytic dinoflagellate cells. The suspended seawater sample was then sieved through 200 µm-mesh nylon sieve again to remove coarse sediment, detritus and other unwanted large particles with their total volume were recorded accordingly. A 0.2 µm pore nylon membrane filter was applied as final filtration stage of sample processing. The sampling screen was discarded for each treatment. The sample filtrate was then transferred into a 50 ml conical tube, and filled up with 30 ml of filtered seawater. One drop of 1% acidic Lugol's iodine fixative solution was added into 30 mL sample solution and kept in the dark under 4 ℃ until processed. Make sure the volume of end sample and total filtered volume were recorded in data collection.

Natural substrates, such as dead corals and seaweed, were collected for cell isolation and preliminary cell identification purpose. Likewise, natural samples were collected in the plastic bottle that was filled with ambient seawater, and returned to laboratory processing. Samples were shaken vigorously in ambient seawater and subsequently sieved through a 200 µm-mesh sieve to remove large particles. Epiphytic dinoflagellate cells retained were used for single-cell isolation and culture establishment. Clonal cultures of cell interests were establish from specimens retained in 20 µm-mesh sieve as described in Leaw et al., (2010).

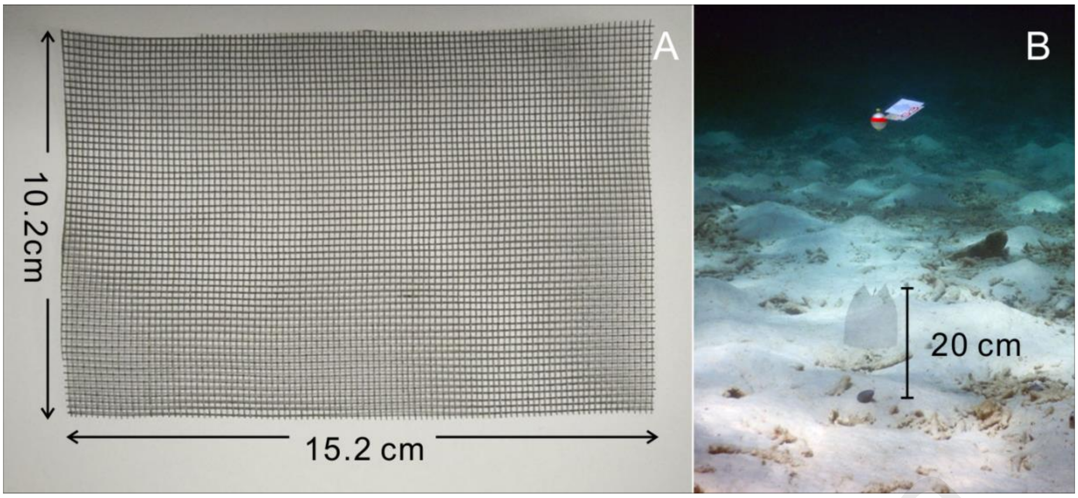


Figure 3.2: Artificial substrate method was applied in this study. (A) Black fiberglass window screen that cut into a standardized measurement of 10.2 cm × 15.2 cm. (B) Fiberglass window screen placed 20 cm above the seabed.

3.2.3 Cell enumeration of benthic dinoflagellates

Quantitative analysis of benthic dinoflagellates was proceeded subsequently to determine cell densities at distinct bottom environment. In this study, cells were enumerated using a clean Sedgwick-Rafter slide under a Leica DM750 light microscope (Leica Microsystems Ltd., Heerbrugg) with a 100× magnification**.** The Lugol's fixed cell sample was inverted for several times to suspend the cells. 1 mL of fixed cell sample was withdrawn evenly using pipetting method and transferred onto the Sedgwick-Rafter slide slowly. The benthic dinoflagellates of interest were counted and recorded in data accordingly. At least triplicates were performed to minimize any bias or statistical errors. For cell enumeration, the benthic dinoflagellate cells were identified up to genus level based on their basic morphological features such as cell outlines and cell sizes under customized light microscopy observations (Figure 3.3).

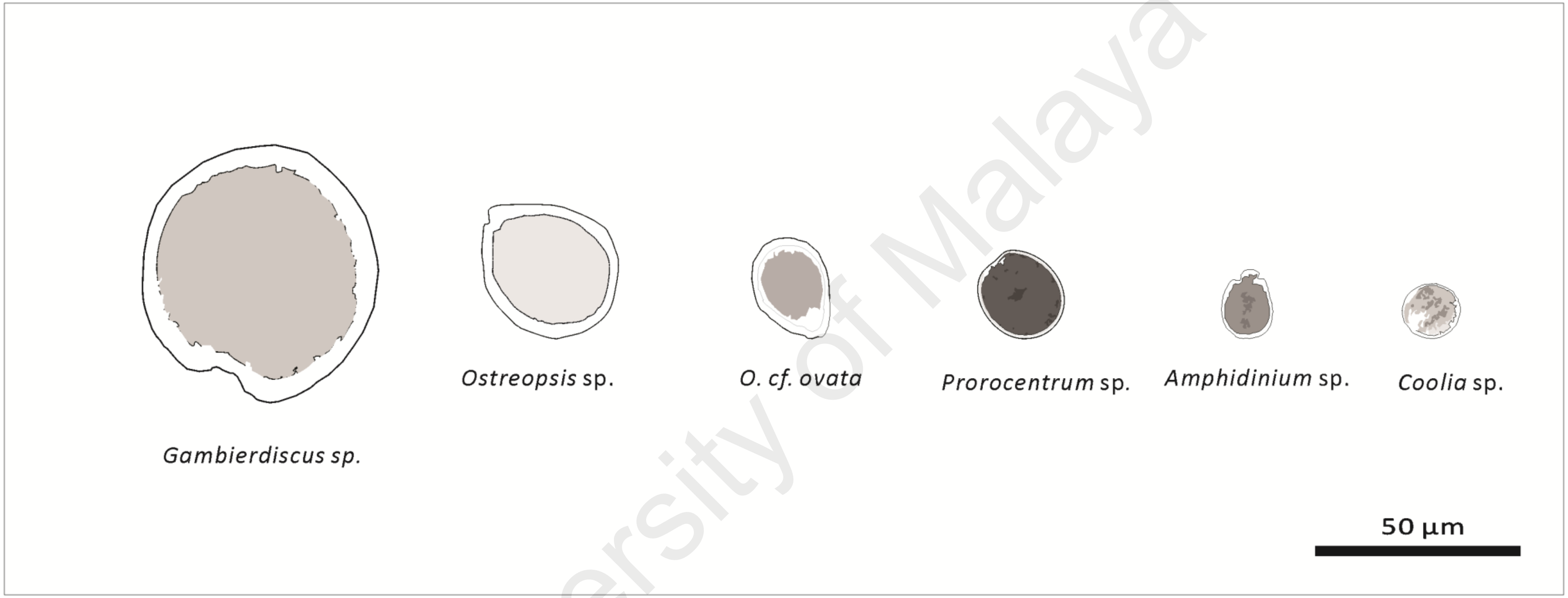
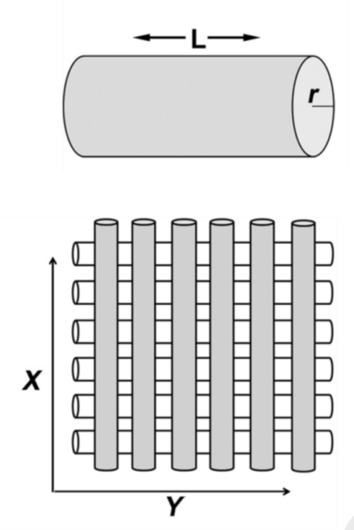


Figure 3.3: General cell outline illustrations of five main benthic harmful dinoflagellates under light microscope (scale bar = 50 µm).

The actual cell densities of benthic dinoflagellates was calculated by estimating the number of cell present on screen sample and divide with surface area of measured screen. Three dimensional cylindrical structures of screen filaments and corresponding spaces should be taken into account to estimate the true surface area, as shown in Figure 3.4.



$A_x = 2\pi r L_x$

$A_y = 2\pi r L_y$

$A_{scr} = A_x N_x + A_y N_y - N_x N_y 16r^2$

Where
A_x = Area of x filaments
A_y = Area of y filaments
N_x = Number of x filaments
N_y = Number of y filaments
r = the radius of filament

Figure 3.4: Determination of actual screen surface area considering series of cylindrical structured screen filaments (adopted from Weisstein, 2013).

3.3 Single-cell isolation and algal culture maintenance

Single-cell isolation was performed with capillary micropipette-washing method (Guillard, 1975) under a Leica DM750 light microscope (Leica Microsystems, Wetzlar, Germany) to effectively obtain cells of interest from the field samples. A clean Pasteur pipette was heated in flame, and quickly removed from flame with a gentle pull action to form a fine tube. A flexible latex tube was provided to connect to a mouthpiece on one end and a micropipette tube at another end. Under observation of light microscope, cell of interest was gently picked up from sample through capillary action and deposited onto a readily prepared sterile droplet placed in plastic dish. Cleaning step by transferring cell into second sterile droplet was repeated at least twice to ensure cell of interest was free of other contaminants, preferably with minimal stress.

After all, the cell of interest was ready to transfer into sterile culture medium provided in a 48-well culture plate. The clonal cultures were subsequently maintained and observed in 48-well culture plate with addition of sterile ES-DK medium occasionally. Successful clonal cultures were later established and maintained in ES-DK medium (Kokinos, & Anderson, 1995) under salinity of 33 PSU and 25 ℃, under a 12:12 light:dark (L:D) photoperiod cycle. The algal cultures were maintained and went through routine sub-culturing process fortnightly where the cells may have reached an exponential cell stage.

3.4 Species characterization

3.4.1 Morphological observation

Cell observation of targeted benthic dinoflagellates under conventional light microscopy was restricted only up to genus level from collected live field samples, by observing their swimming patterns and apparent cell outline. Both wild and cultured cells were examined under a Leica DM3000 LED inverted microscope (Leica Microsystems, Wetzlar, Germany) and digital images captured with a Leica DFC450 cooled charge-couple device (CCD) camera (Leica Microsystems, Wetzlar, Germany).

For preparation of scanning electron microscopy (SEM), an equal volume of 5% glutaraldehyde fixative was added into cultured samples and fixed at room temperature for an hour. The fixative was then discarded carefully with a pipette followed by rising process with equal amount of 0.1 M Na-cacodylate buffer washing solution repeated at least three times. Fixed samples were post-fixed with small amount of 1% osmium tetroxide (OsO_4) fixative solution to cover the cell pellet under room temperature for another one hour. The buffer washing step was repeated for three times to remove excess fixatives. The dehydration steps were carried out with a graded series of ethanol concentration (30%, 50%, 70%, 80%, 90%, and 95%) for 15 minutes in each separated dehydration step with an exemption of 30 minutes of incubation period for 100% of ethanol solution. The dehydrated samples were preceded with freeze-drying process using 6-liter console freeze dryer with defroster system (LABCONCO, USA) upon SEM observations. Samples were mounted to a stub before coated with a thin layer of gold-palladium by using a JEOL JFC-1600 magnetron sputter coating instrument (JEOL, Japan). Coated samples were then observed under a JEOL JSM-6510 analytical scanning electron microscope (JEOL, Japan). Detailed ultrastructural morphological micrographs were then ready to be captured and digital images saved in Tagged image file format (.TIFF).

3.4.2 Molecular characterization

3.4.2.1 Genomic DNA extraction

Culture strains were harvested at mid-exponential stage (approximately 12 – 14 days) by centrifugation at 2,800 rpm for 10 min. Excess seawater was discarded carefully using pipetting method. Cell pellet was suspended in NET lysis buffer containing 1% SDS, 15 mM NaCl, 10 mM EDTA (pH 8.0) and 10 mM Tris-HCl (pH 7.5) follow by incubation step under a tempearature of 65 ℃ for 30 min. The mixture was then resuspended with cetyltrimethylammonium bromide (CTAB) solution following addition of chloroform:isoamyl (C:I) alcohol (24:1) and standard phenol:chloroform:isoamyl (P:C:I) alcohol (25:24:1) respectively. Two volumes of absolute ethanol and 1/10 volume of 3M sodium acetate were added into mixture products to precipitate the resultant genomic DNA (gDNA). Centrifugation method was applied on mixture products to obtain gDNA pellet after >3 h of incubation time under –20 ℃. The gDNA pellet was rinsed with 70% cold ethanol, and dissolved in TE buffer (pH 8.0) (Leaw et al., 2005, Penna et al., 2005).

3.4.2.2 Gene amplification, purification and DNA sequencing

Gene amplification of rDNA was performed by polymerase chain reaction (PCR), as described in Leaw et al. (2001). In this study, the internal transcribed spacer (ITS) regions were amplified with universal primers originally developed by Adachi et al., (1996) and further modified by Leaw et al., (2001) (Table 3.1). Whereas D1/D2 region of 28S rDNA were amplified with primer pairs D1R and D3Ca (Scholin, & Anderson, 1994) (Table 3.1).

Genomic DNA was amplified in a 25 µL reaction mixture containing 1× reaction buffer (Invitrogen, Life Technologies), 2mM $MgCl_2$, 0.2µM deoxynucleoside triphosphate reagent (dNTP), *Taq* DNA polymerase (5 unit/µL), correspondent primers and template DNA product. PCR amplification condition was performed in 35 cycles using peQSTAR Thermal Cycler (Peplab, Germany) as illustrated in Table 3.2. Single pass DNA sequencing was carried for both strands of each sample. PCR products were visualized on 1% agarose-gel electrophoresed stained with SYBR safe DNA stain (Invitrogen, Life Technologies, USA) for 30 mins (70 V). PCR products were further purified using QIAquick purification columns (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The purified products should be kept in – 20 ℃. DNA sequencing was evaluated using an ABI 377 automated sequencer (PE applied Biosystems, Foster City, CA, USA).

Table 3.1: Oligonucleotide primers used for amplification of ITS and 28S rDNA regions.

Primer	Sequence (5'-3')	Direction	Target region	Reference
ITS1F	TCGTAACAAGGTTTCCGTAGGTG	Forward	ITS	Leaw et al., 2001
ITS1R	ATATGCTTAAGTTCAGCGGG	Reverse	ITS	Leaw et al., 2001
D1R	ACCCGCTGAATTTAAGCATA	Forward	LSU	Scholin et al., 1993
D3Ca	CTTGGTCCGTGTTTCAAGA	Reverse	LSU	Scholin et al., 1993

Table 3.2: PCR amplification conditions for ITS and LSU rDNA respectively.

PCR condition		Temperature (℃)		Duration time	
		LSU	ITS	LSU	ITS
Initial denaturation		94	94	4 min	2 min
Denaturation	35 cycles	94	94	35 sec	30 sec
Annealing		55	50	50 sec	30 sec
Elongation		72	72	35 sec	30 sec
Final extension		72	72	7 min	5 min
Hold		4	4	∞	∞

3.4.2.3 Molecular phylogenetic analysis

The resultant nucleotide sequences were initially evaluated by comparing them through BLAST program (Altschul et al., 1990) available in National Center of Biotechnology Institute (NCBI), following by sequences analysed by ABI Sequence Scanner ver 1.0 (Applied Biosystems, USA). Taxon sampling was performed to obtain related sequences and selected sequences were saved as FASTA format. Multiple sequence alignment was constructed and aligned by eyes using Clustal-X (Thompson et al., 1997) and subsequently aligned by eye for further phylogenetic analyses. Ambiguous bases were edited manually by BioEdit version 7.0.9.0 (Hall, 1999).

Phylogenetic analyses were performed using Phylogenetic Analysis Using Parsimony* (PAUP*) ver. 4.0b10 (Swofford, 2002) with maximum parsimony (MP) and maximum likelihood (ML) algorithms used to estimate the phylogeny. MP analysis was performed with heuristic search and tree-bisection-reconnection (TBR) swapping with 1000 bootstrap replications. ML analysis was performed using a general-time-reversible (GTR) evolutionary model, with the estimated parameters. A heuristic search and TBR swapping with 100 bootstrap replications were conducted for the ML analysis.

Bayesian inference (BI) was performed by MrBayes 3.2.4 (Ronquist and Huelsenbeck, 2003) using GTR substitution model. Four simultaneous Markov chain Monte Carlo (MCMC) chains of 1×10^6 generations each were sampled every 100 generations. The posterior probabilities (PP) were estimated from the last 7500 trees, and majority-rule consensus tree was then constructed from the trees.

3.5 Habitat mapping and classification

The spatial distribution of benthic microhabitats was defined by determining visual appearance of surficial biotic and physical substrata. The underwater photoquadrat survey was conducted by SCUBA (Figure 3.4) to provide general data on each microhabitat type present. A 5cm × 5cm quadrat was customized prior to allow more accurate estimation of bottom vegetative substratum coupled with photographic techniques. The images of photoquadrat were reviewed accordingly and bottom substrate coverage in surface area was estimated in percentage coverage (%). A scientific image processing software of ImageJ 1.50b (Wayne Rasband, National Institutes of Health, USA) was adapted to estimate the surface area of bottom substratum coverage in coverage percentage (%) observed from the photoquadrat captured from the fields.

Figure 3.5: An underwater photoquadrat survey conducted to determine bottom biota and physical substratum coverage in percentage cover (%).

3.6 Data analysis

A cluster analysis with group-average sorting was computed to evaluate the degree of heterogeneity of benthic habitat character groups with Bray-Curtis dissimilarity coefficient using program PAST 3.08 (Hammer et al., 2001). This analysis was organized hierarchically and presented as a dendogram grouping of relatively discrete habitat groups based on their corresponding biological and substratum similarities. Habitat characteristics data sets were also analysed using non-metric multidimensional scaling (*n*MDS) ordination in attempt to emphasize the correspondence between distinct major clusters representing diverse benthic classes in a low-dimensional space.

One-way analysis of similarity (ANOSIM) was performed with permutations of 5000 to evaluate for differences in similarities occurred among microhabitat groupings (Clarke, 1993); and also similarity percentage (SIMPER) to determine the average percent contribution of characteristic substrata of each group contributing towards the dissimilarity between major clusters performed in cluster analysis and NMDS (Clarke, 1993).

Prior to canonical correspondence analysis (CCA), a logarithm transformation log(x+1) was performed on benthic dinoflagellate cell abundance data to lessen the influence of prevalent groups and to increase the weight of rare groups. This analysis was conducted with the inclusion of nine environmental variables (i.e. hard corals, degraded corals, sands, rubbles with turf algae, rubbles covered with sands, green fleshy algae, brown fleshy algae, red filamentous algae and others (e.g. clams and rocks) to reveal the relationships among benthic dinoflagellates and the benthic microhabitat characteristics. The analysis was performed with 1000 permutations.

CHAPTER 4: RESULTS

4.1 Environmental data of Rawa Island

In this study, the average daytime temperatures and salinity of shallow sea surface water were recorded between 30 – 33 ℃ and 30 – 32 PSU, respectively (Table 4.1). The degree variation of temperature exhibited completely constant readings and did not show much change throughout seasonal transition periods. Overall salinity readings were slightly decreasing by two PSU from June 2015 to January 2016 due to consequent rainfall input during wet season. Light intensity data collected was consistent as well with a range of 1000 – 2500 µmol m^{-2} s^{-1} recorded (Figure 4.1). This suggested that light penetration within a shallow depth of 2 – 5 m provide substantial sunlight to the bottom vegetative such as healthy reef growth and macroalgal abundance.

Table 4.1: Details of the occasionally physiological data taken occasionally from Rawa Island, showing the sampling date, salinity (PSU), maximum light intensity (µmol photon m^{-2} s^{-1}), as well as average and minimum temperature (℃).

Date	Salinity (PSU)	Maximum light intensity (µmol m^{-2} s^{-1})	Average Temperature (℃)	Minimum Temperature (℃)
June 2015	32	2446.95	32.56	27.67
July 2015	30	1937.17	32.70	22.91
August 2015	31	2039.13	30.84	26.98
September 2015	31	2141.08	32.16	27.96
January 2016	30	1019.56	30.03	29.25

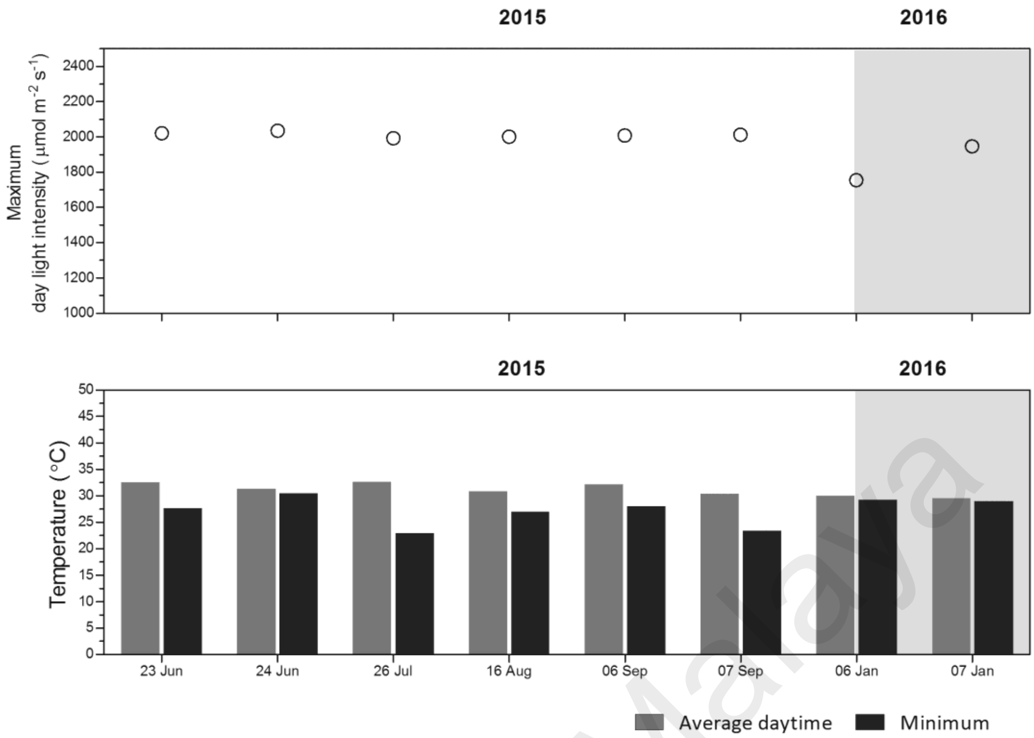


Figure 4.1: Environmental data indicated maximum day light intensity (µmol m^{-2} s^{-1}) and temperature readings collected at similar depth (2 – 5 m) from June 2015 until early January 2016.

4.2 Benthic dinoflagellates of Rawa Island

4.2.1 Diversity of benthic dinoflagellates

Morphological observation of five major genera benthic dinoflagellates was carried out in field specimens and cultured strains (Figure 4.2). Nine most abundant benthic dinoflagelate taxa were observed preliminary under LM, and species identity was confirmed based on detailed thecal morphological observation and molecularly by single-cell PCR of the ribosomal RNA genes. The taxa confirmed are: *Gambierdiscus* spp., *Ostreopsis* cf. *ovata*, *Ostreopsis* cf. *lenticularis*, *Coolia malayensis*, *Coolia tropicalis*, *Prorocentrum emarginatum, P. lima, P. maculosum*, and *Amphidinium* spp.

4.2.2 Morphology of *Coolia* and *Ostreopsis*

Cells of *Coolia* and *Ostreopsis* from the field samples were single-cell isolated by micropipetting technique, and this yielded a total of 32 strains of *Coolia* and 35 strains of *Ostreopsis* isolates. These isolates were then established and successfully maintained into 17 *Coolia* and 13 *Ostreopsis* clonal cultures (Appendix A). The cultures were then proceeded with detailed observation of outer morphology and theca ornamentation by SEM. The morphological descriptions are detailed below.

4.2.2.1 *Coolia tropicalis*

Cells of *C. tropicalis* observed under SEM revealed the general outline of ovoid-subsperical shaped cells with numerous fine scattered pores seen on smooth theca surface (Figure 4.3.1B–H). The cells were slightly displaced when observed from ventral view (Figure 4.3.1B). Cells are 28–33 µm (30.68 ± 3.68 µm, *n* = 2) in anterioposterior length (L_{AP}), 31–34 µm (32.70 ± 1.78 µm, *n* = 2) in width (W_{AP}), and 32 µm in dorsoventral depth (L_{DV}). Plate tabulation formula is Po, 4´, 6´´, 6-7c, 6-7s, 5´´´, 2´´´´. The apical pore (Po) was located at the left dorsal side of epitheca with its measured length of 7.4 ± 0.12 µm (*n* = 2). Cells were enclosed with round to oval pores (0.27 ± 0.04 µm) encountered on each thecal plates. The fourth apical plate (4´) was the largest among the epithecal plates and appeared in slightly broader toward the ventral side of theca plates (Figure 4.3.1F). This 4´ plate was positioned on the central of the epicone from ventral and apical views (Figure 4.3.1B and F). The 3´ apical plate was described as pentagonal in shaped, in contact with plates 2´, 4´, 3´´, 4´´ and 5´´ (Figure 4.3.1F). In hypotheca plate ornamentation, both postcingular plates 3´´´ and 4´´´ were larger in size and almost equal in size (Figure 4.3E).

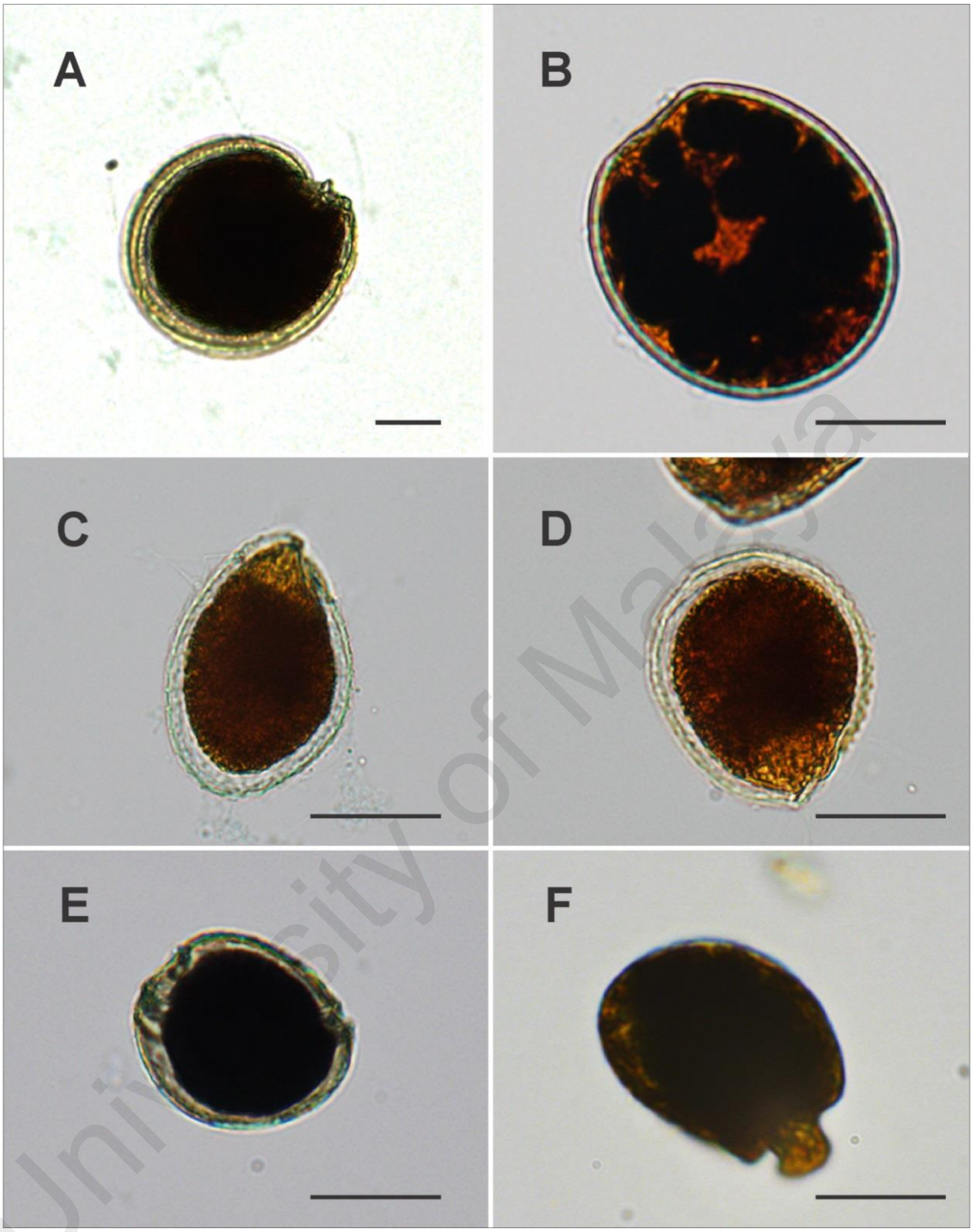


Figure 4.2: Five main genera of benthic harmful dinoflagellates obtained from Rawa Island were observed under LM. (A) *Gambierdiscus* sp., (B) *Prorocentrum* sp., (C) *Ostreopsis* cf. *ovata*, (D) *Ostreopsis* cf. *lenticularis*, (E) *Coolia malayensis*, and (F) *Amphidinium* sp. (Scale bar = 25 µm).

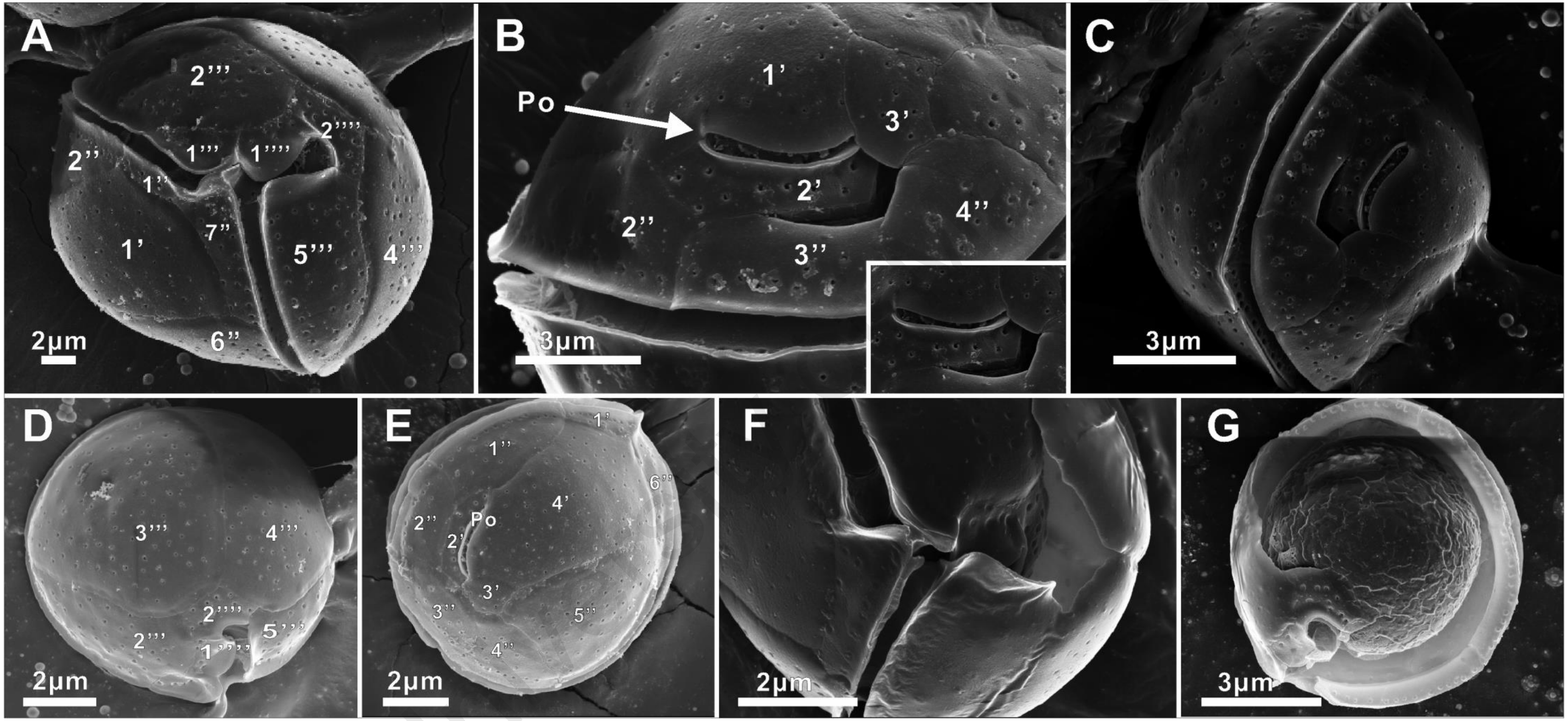


Figure 4.3.1: *Coolia tropicalis* SEM micrographs. (A) Ventral view, (B) Dorsal view showing a close-up of APC (inset), (C) Lateral view, (D) Antapical view, (E) Apical view, (F) A close up view of sulcul plates, (G) Cingular plates disclosed internal cell contents.

4.2.2.2 *Ostreopsis* cf. *ovata*

Under an epifluorescence microscope observation, cells were observed to be markedly anterio-posteriorly compressed and pointed to the ventral side (Figure 4.3.2. A–C). The cells showed in oval to lentil-shaped from apical view. However, the morphological characters including general cell shape and sizes were highly variable due to cell morphologically plasticity limitations. Thecal plates displayed the formula Po, 4´, 0s, 6´´, 6c, ?s, 5´´´, 0p, 2´´´´. Thecal surface was smooth and covered with scattered pores under SEM observation (Figure 4.3.2A). The epitheca and hypotheca showed similar in size (Figure 4.3.2A&C). Plate 4´was hexagonal in view and located at the centre of the epitheca plate observations. It was connected with plates Po, 1´, 3´, 1´´, 5´´and 6´´(Figure 4.3.2A&C). Apical view indicating the epithecal plates of cell with the pentagonal 3´ plate touched relevant plates 4´, 2´´, 3´´, 4´´ and 5´´ (Figure 4.3.2A). The APC plate was slightly curved and enclosed by elongated rectangular 2´ plate (Figure 4.3.2A&C). The antapical plate 1´´´´ was much smaller compared to others from antapical view of hypotheca plate ornamentation (Figure 4.3.2B). The large pentagonal 2´´´´ plate was surrounded by all the other plates from antapical view (Figure 4.3.2B).

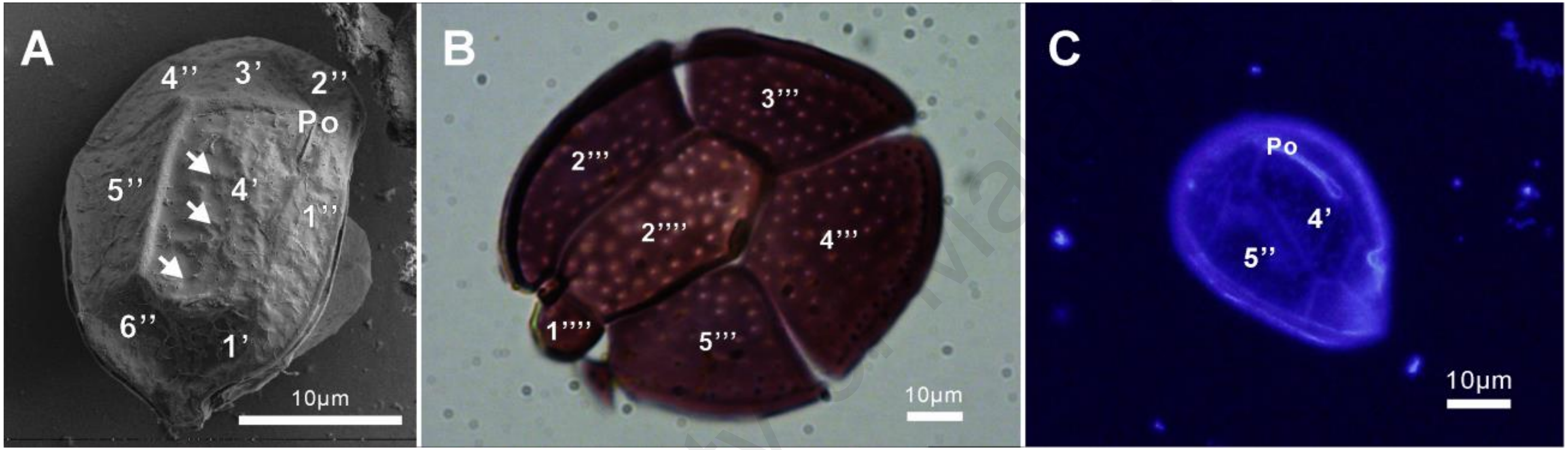


Figure 4.3.2: *Ostreopsis* cf. ovata. (A) SEM. Apical view (arrowheads indicate fine pores on theca plates), (B) Thecal morphology observation under LM. Antapical view showing clear hypotheca plates ornamentation, (C) Epi-fluorescence observation. Apical view cells presenting apical pore (Po) and pentagonal 4 ´plate.

4.2.3 Species composition of benthic dinoflagellates

Benthic epiphytic dinoflagellates were present and reported in reef microhabitats of Rawa Island provided with their distinctive spatial distribution patterns. The relative abundance of benthic dinoflagellates for shallow sites (roughly 2 – 5 m) was exhibited chronologically following comparable values of species relative abundance (Table 4.2): *Ostreopsis* > *Prorocentrum* > *Gambierdiscus* > *Amphidinium* > *Coolia*. Considering the relative abundance of each benthic dinoflagellates species at each microhabitats sites (Clade I – V) (Figure 4.4), *Ostreopsis* was the dominant species with its average relative abundance 54%, indicating its occurrence at almost all sampling sites followed by a drastic decline in average relative value of *Prorocentrum* (27%). Relative high relative abundance of *Ostreopsis* was recorded in overall selected study sites with its highest value of 100% and lowest value of 3%. The other three harmful benthic dinoflagellates: *Gambierdiscus* (8%), *Amphidinium* (9%) and *Coolia* (2%) did not showed high average relative abundance in which mostly less than 10% of average relative abundance was observed in current study. Both *Gambierdiscus* and *Amphidinium* only reached a maximum relative abundance value of 35% and 38% respectively.

The potentially toxic dinoflagellate genera *Ostreopsis* was known as predominant species and perennially present among the five benthic epiphytic genera, with its highest mean cell composition (1633 $\pm$ 3705 cells/100 cm^2) (Figure 4.5; Table 4.2). Apart from that, *Ostreopsis* was presumably to be well adapted to seasonality changes compared to other benthic dinoflagellates when its species composition reported to be relatively high cell densities (12,500 cells/100 cm^2) with its stable cell numbers obtained during a rough sea condition during January 2016 (wet season) (Figure 4.6). A drastic decline of mean cell composition was reported on *Prorocentrum* spp. with only 299.7 $\pm$ 234.8 cells/100 cm^2 (Figure 4.5; Table 4.2) marked as the second highest of species assemblages. Only two samples were reported to have more than

1000 cells/100cm^2 of *Prorocentrum* spp. among all collected samples from sites, achieving total cell densities of 1147 cells/100 cm^2 and 1022 cells/100 cm^2 collected from benthic environment respectively (Figure 4.5). The species composition of *Prorocentrum* showed distinct abundance pattern by its gradual increases of cell numbers along dry seasons (April – September 2015) but sudden decrease in abundance on January 2016 (wet season) (Figure 4.6).

Gambierdiscus was observed with remarkable lower in mean cell composition of 103.8 ± 161.7 cells/100 cm^2 (Figure 4.5; Table 4.2), recorded as a minor component of existing BHAB communities. Two major cell densities were identified occasionally on April and May 2015 with maximum cell densities of 832 cells/100 cm^2 and 1,182 cells/100 cm^2 detected respectively (Figure 4.5). However, overall cell densities of *Gambierdiscus* were reported to be only less than 500 cells/100 cm^2 from the sampling sites. No *Gambierdiscus* was visually detected from at least 27 screen samples data obtained from degraded reef areas covered by fine sand particles. A remarkable exemption of *Gambierdiscus* occurrence in January 2016 was identified in which no cell was encountered during this particular period (Figure 4.6).

Amphidinium spp. and *Coolia* spp. were likely co-occurred in the benthic dinoflagellate assemblages in such a low composition. *Amphidinium* spp. showed poor mean species composition value of 97.38 ± 96.85 cells/100 cm^2 (Figure 4.5; Table 4.2). Maximum total cell count of *Amphidinium* spp. was encountered as 507 cells/100 cm^2 whereas remaining collected samples only appeared less than 100 cells/100 cm^2 of existing total cell counts. Unlike others epiphytic dinoflagellates, *Coolia* spp. was almost absent in most of the samples resulting in extremely low mean species composition of 12.50 ± 15.47 cells/100 cm^2 (Table 4.2) throughout overall sampling months. In fact, total cell concentration usually did not exceed 60 cells/100 cm^2 among overall screen samples collected.

Table 4.2: Benthic epiphytic dinoflagellates assemblage composition obtained from sampling sites ($n = 115$): Average cell composition (cells/100 cm^2 ± SD) of each genus; relative abundance (%).

Genus	Relative abundance (%)	Species composition (cells/100 cm^2) ($n = 115$)		
		Average	Maximum	Minimum
Gambierdiscus	8	103.8 ± 161.7	1182	0
Ostreopsis	54	1633 ± 3705	34,114	10
Prorocetrum	27	299.7 ± 234.8	1147	0
Amphidinium	9	97.38 ± 96.85	507	0
Coolia	2	12.50 ± 15.47	60	0

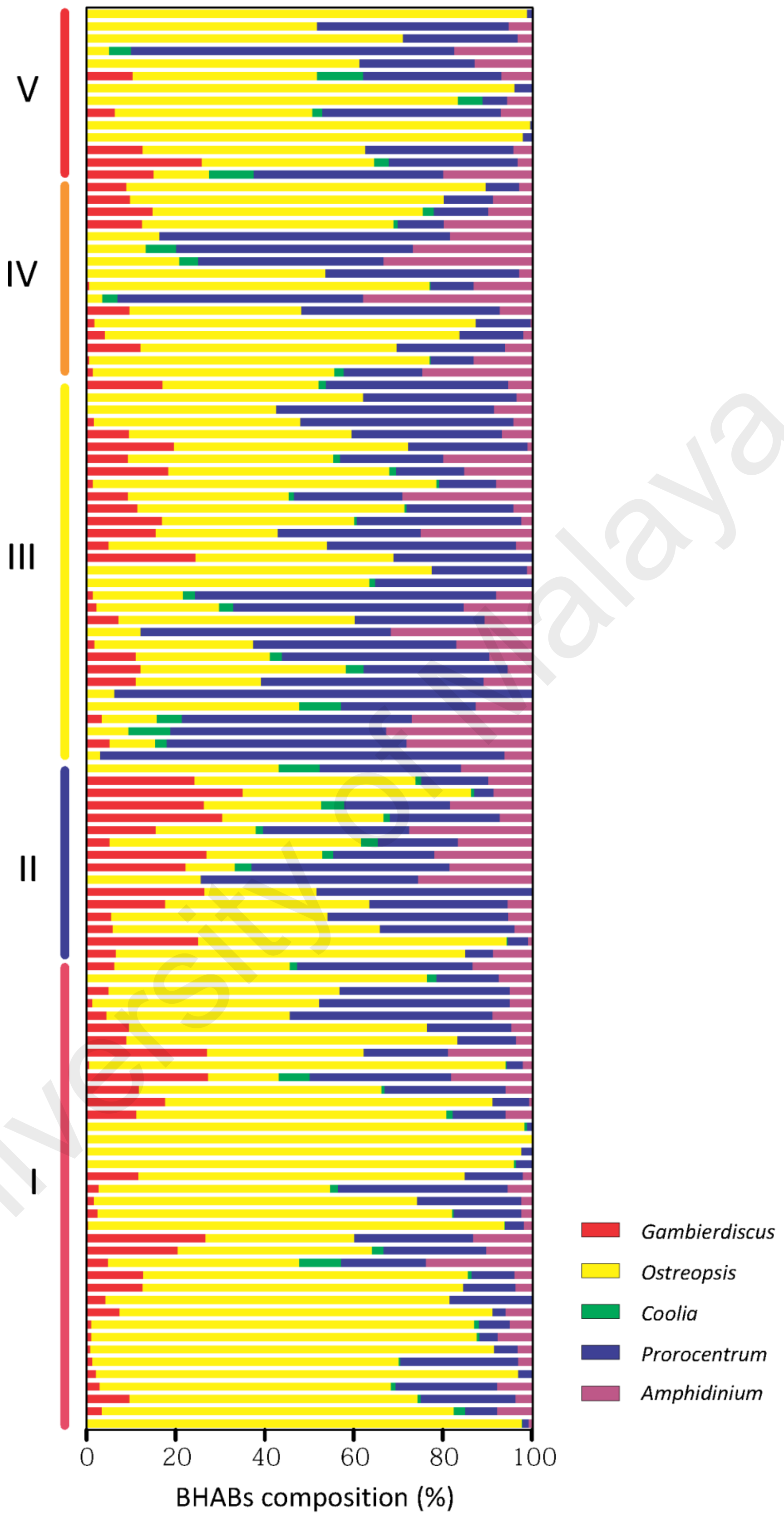


Figure 4.4: Relative abundance of BHAB compositions (in percentage, %) in different reef environment (Clade I – V). *Ostreopsis* abundance represented 54% of relative abundance of BHAB assemblages, following by *Prorocentrum* (27%). Less species assemblages were encountered on *Gambierdiscus* (8%), *Amphidinium* (9%) and *Coolia* (2%).

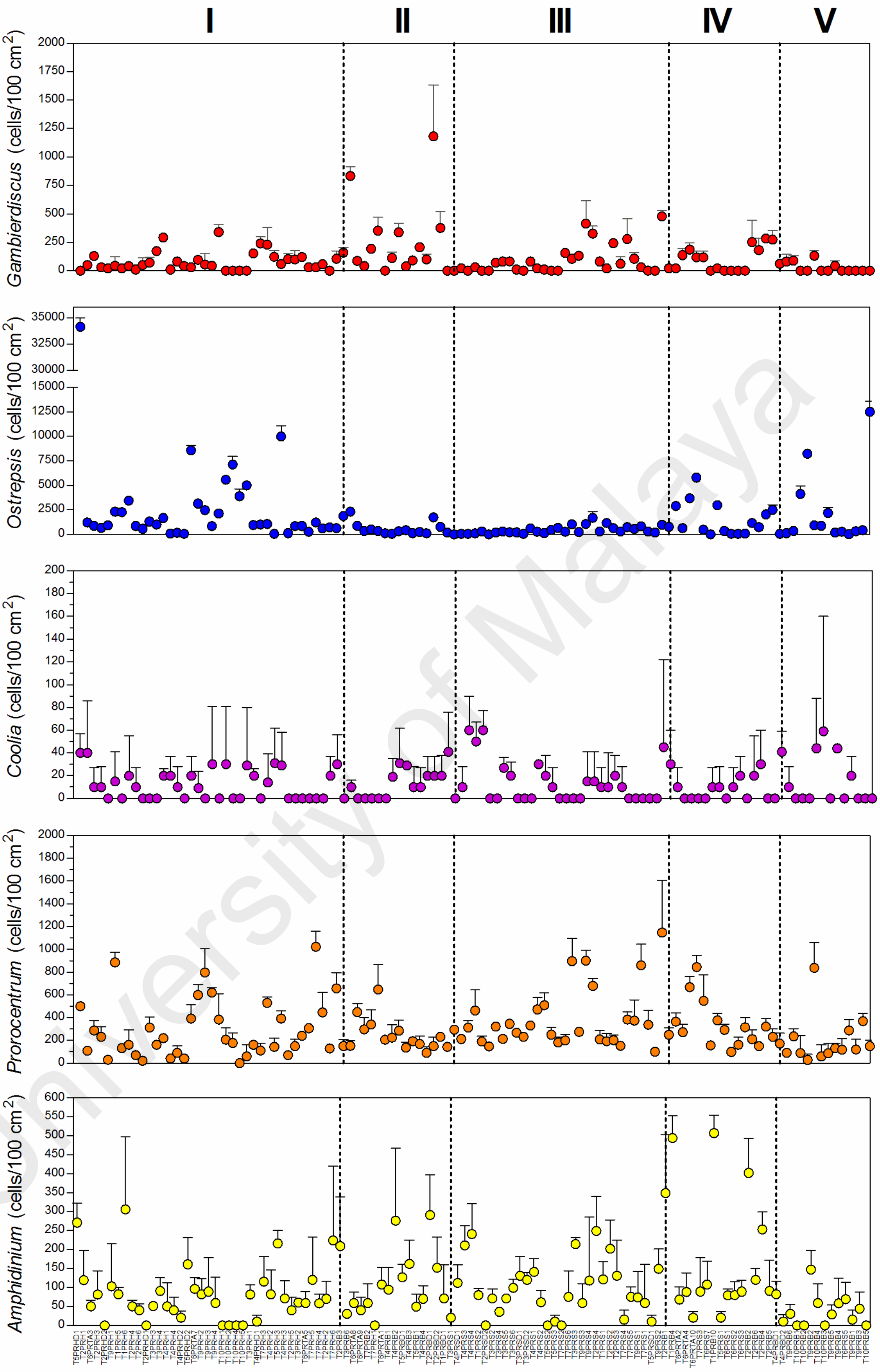


Figure 4.5: Species composition (cells/100 cm^2) of benthic epiphytic dinoflagellates in relation to variation of bottom microhabitats. Dotted line represented microhabitat separation between each clade (Clade I – V).

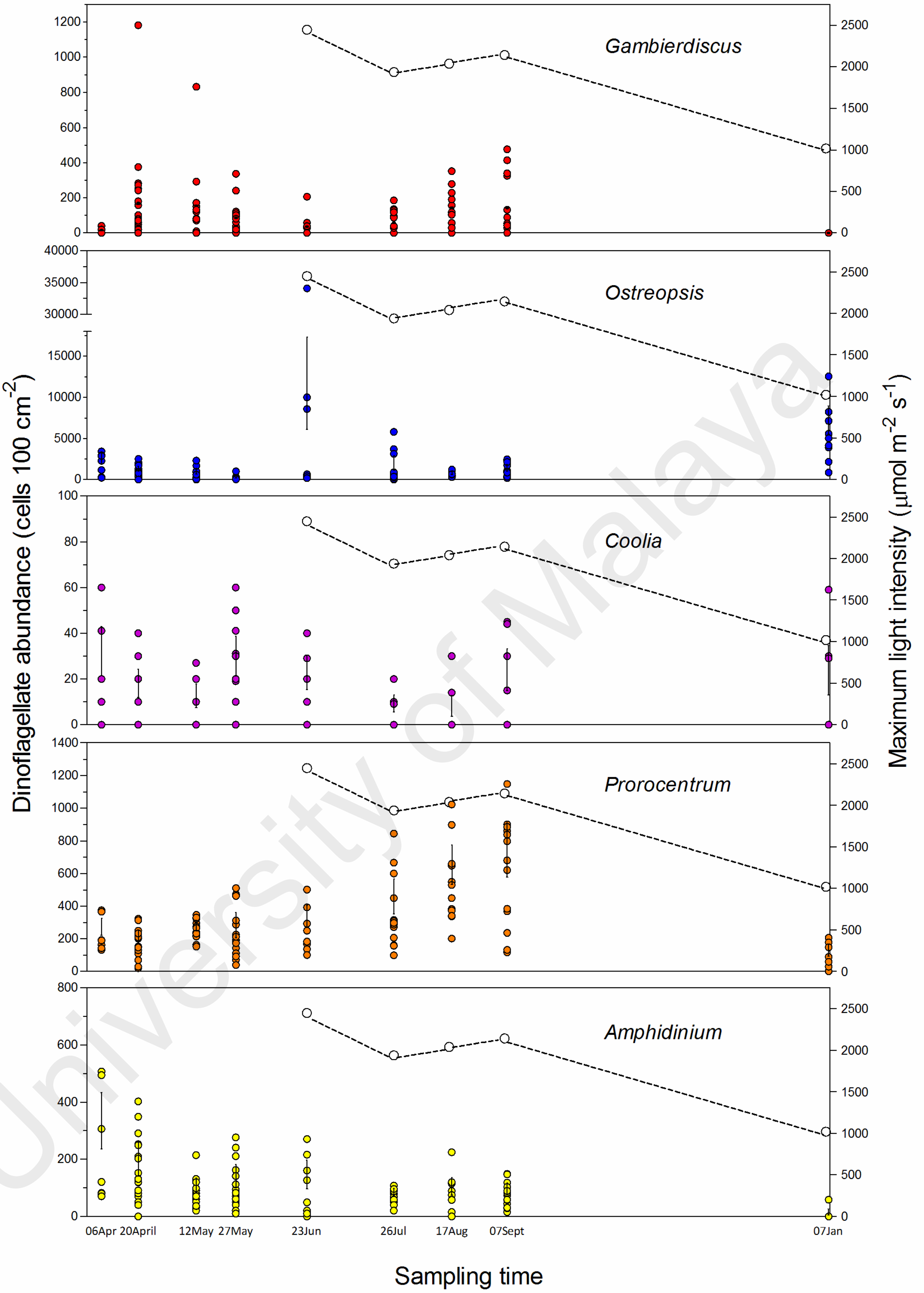


Figure 4.6: The influence of seasonal changes (from dry season to wet season) on monthly variation of benthic epiphytic dinoflagellates species abundance obtained from Rawa Island.

4.3 Natural benthic substrata of Rawa Island

4.3.1 Description of benthic substrata

The outermost fringing reef of Rawa Island was occupied with different growth forms of hard corals (Figure 4.7). Shallow reef communities (0.5 – 5 m) were prominently built up with branching corals and thin, plate-like corals (Figure 4.7 A, C), such as *Acropora* spp. and *Montipora* spp., with less/scattered tabulated-form and massive corals found at this site (Figure 4.7 E–G). Certain coral colonies exhibited as encrusting growth form which grew in lumpy surfaces and became flatter on bottom substrates like huge rocks and dead coral seabed (Figure 4.7 H).

The coral reef ecosystem was destructed in term of community structure because of natural and anthropogenic impacts involving heavy boat traffic and unlimited flows of tourism activities such as snorkeling with fins in coral reef ecosystem. Under such condition, the effect of reef disturbance events had alternately stressed out and slowed down or even limiting recovery rate of natural reef habitats at Rawa Island. Despite of the healthier reefs at the deeper sites, massive distribution of frondose macroalgae and hair-like algae can be visually identified at the internal shoreline due to the decrease of coral covers.

Massive growth of macroalgae especially *Jania* spp. and *Lobophora* spp. (Figure 4.8 A and C respectively) was found on degraded coral covers in the middle reef area. This may contribute to a phase shift of coral-dominated reefs to algal dominated reefs condition. For instances, red calcareous *Jania* spp. was found in sheltered reef habitats and it distributed with massive coverage growth rates on destructed reef areas (Figure 4.8 A). Tones of dead corals had been washed off toward the inner calm area prompting to exaggerated growth of microalgae communities, especially associated with varieties of turf algal. Detailed structural features of turf algal associated on dead corals was then observed under light microscope and recorded (Figure 4.9).

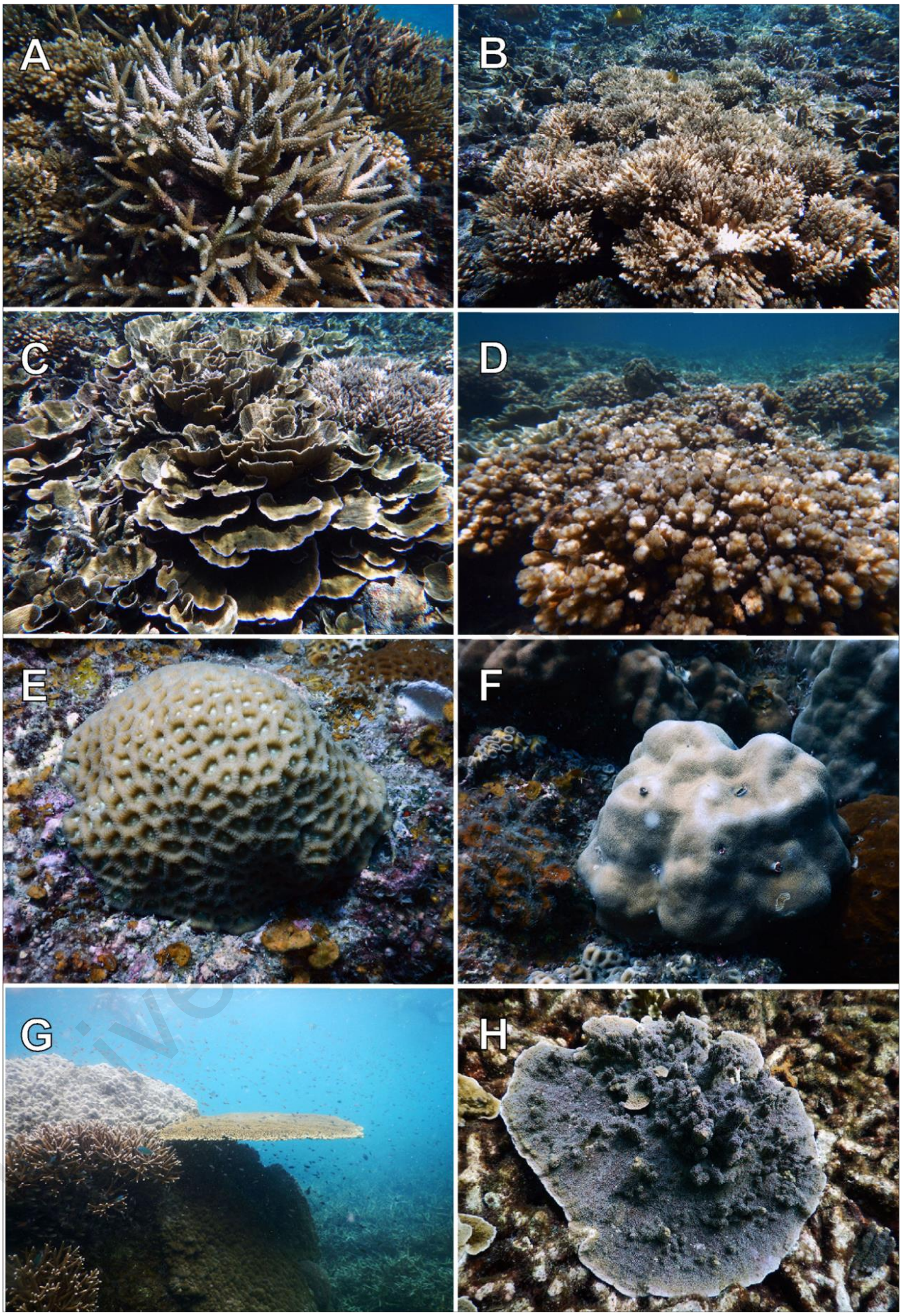


Figure 4.7: Various growth forms of hard corals found in Rawa Island. (A-B) *Acropora* spp. displayed in (A) branching and (B) digitated growth forms. (C) *Montipora* spp. in laminar or thin, plate-liked appearance. (D) *Pocillopora* spp. in digitates-formed. (E-F) *Favites* spp. and *Porites* spp. showed in sub-massive form respectively. (G) *Acropora* spp. grew horizontally in tabulated shape. (H) Encrusting corals appeared on dead coral seabed.

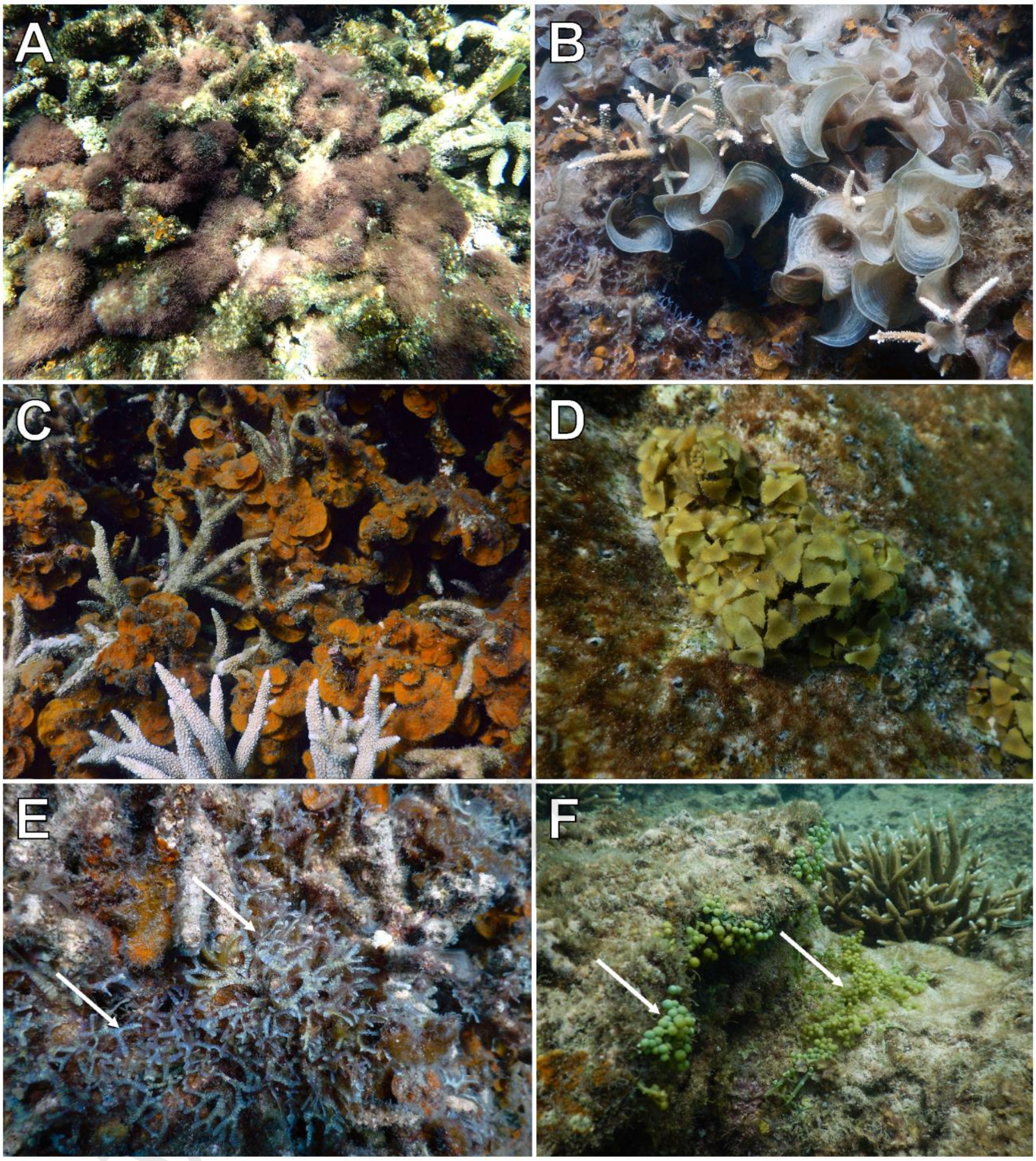


Figure 4.8: Common seaweed observed in Rawa Island. (A) *Jania* spp., (B) *Padina* spp., (C) *Lobophora* spp., (D) *Turbinaria* spp., (E) *Dictyota* spp. (arrows), and (F) *Caulerpa* spp. (arrows).

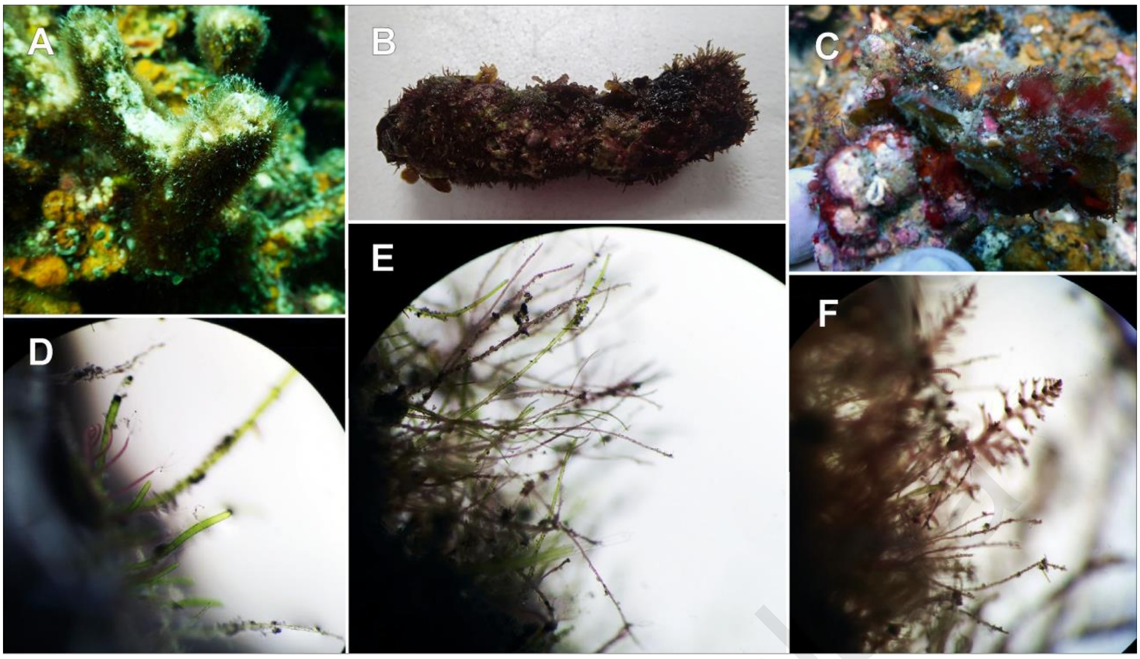


Figure 4.9: A close-up observation of turf agal under light microscope was carried out accordingly. (A – C) Dead corals were collected as natural substrates from study sites and observed under stereo microscope. (D – F) Under microscopic observation, hair-like green and red turf algae were finely attached on dead rubbles. The natural population of benthic dinoflagellates may form a mucilaginous matrix surround turf algae and remained motile within the matrix to seek for shelter microhabitats, as revealed under light microscopy.

4.3.2 Characterization of benthic substratum

A list of bottom microhabitats characteristics representing variety of benthic biological and substratum characters were described in Table 4.3. Cluster analysis of the coverage percentage for 115 photoquadrats obtained from the sites representing existence of five major distinct geomorphic zones at Rawa Island (herein referred as Clade I – V; Figure 4.10).

The comparison of these five distinct geomorphic zones was interpreted based on a combination of one-way ANOSIM analysis, non-metric multidimensional scaling (*n*MDS) ordination and cluster dendogram (Figure 4.10). Individual pairwise r-values between sites within geomorphic zones using one-way ANOSIM indicating majority of sites within each geomorphic zone (Clade I – V) to be statistically dissimilar where overall r-value achieved 99.28% with a p-value of 0.0002 under permutation (*n*) of 5000. This separation was further confirmed with two-dimentional (2D) *n*MDS ordination (stress level = 0.4) based on benthic substratum characterization data with little chance of misinterpretation (Figure 4.10), indicating no overlapping among these five geomorphic zones (Clade I – V).

Based on the result obtained, two major zones can be identified (refer to Table 4.3): a healthy hard coral reef zone (Clade I) and a degraded coral reef zone (Clade II – V) characterized by existence of massive dead corals seabed and overgrowth of macroalgae. Clade I was clearly distinguished from other benthic substratum covers mainly because of their relative high coverage of hard corals communities (> 90% coverage; Type A) with less coverage of macrophytes (0 – 35% of macrophytes). Total representative 38 screen samples were retrieved solely from hard coral reef community composition. Less than 40% of rubbles with turf algae were encountered from six screen samples (Type B) out of this Clade I.

Table 4.3: Benthic biological and physical substratum of Rawa Island were briefly explained and classified into five major distinct geomorphic zones (Clade I – V). Variation of benthic substrata was denoted as type A – I to clearly differentiate their surface features within bottom marine reef environment.

Type	Major substratum	Macrophyte coverage (%)	Habitat description	Screen (*n*)	Clade
A	Hard corals	Less (0 – 35%)	>90% of hard corals coverage	32	I
B	Hard corals	Less (0 – 35%)	60-80% covered by hard corals; consisted of less than 40% of rubbles with turf algae	6	I
C	Rubbles with turf algae	Abundant (>90%)	>90% are rubbles with turf algae	11	II
D	Rubbles with turf algae	Abundant (>90%)	Approximately 70-80% of rubbles with turf algae and roughly 30% coverage of sands and macrophytes	5	II
E	Sand	None	High coverage of sandy areas (>90%)	25	III
F	Sand	None	Moderate coverage (>50%) of sandy area and slightly increase of rubbles with sand (20-50%)	6	III
G	Sand with rubbles	Less (0 – 35%)	Drastic increase coverage of rubbles covered with sand (70-100%) with less sand coverage (<30%)	8	IV
H	Sand with rubbles	Moderate (<50%)	Combination of rubbles with sand coverage (~60%) and less than 40% of turf algae / macrophytes	8	IV
I	Macrophytes (green/brown/red algae)	Abundant (>90%)	>80% dominated with Rhadophyta macrophytes (*Jania* spp.)	14	V

As for degraded coral reef zone (Clade II – V), the bottom biological vegetative and physiological substratum was progressively differentiated from Clade I in term of proliferation of macroalgae growth and disturbance process toward reef communities. Majority of bottom environmental surface areas under Clade II were covered with 70 – 90% and above of coral residues attached with hair-like turf algae together with abundant diversity of visible macrophytes (> 90%). Roughly 30% are synchronized with fine sand particles and scarce vegetative classified as Clade II (Type C and D).

The grouping of Clade III was revealed as sandy areas with relative low number of macroalgae coverage (%). When come to the inner shore sites, 25 screen samples (Type E) were collected from sandy patches (> 90% sand coverage) whereas six screen samples (Type F) was classified as benthic habitats consisted of moderate coverage of sandy patches (> 50%) and slightly increase of rubbles with fine sand particles (20 – 50%). Another eight samples were taken from further shoreline where a drastic increase of rubbles overlaid with fine sand particles (70 – 100%) can be found but decreasing areas of bare sand (<30%) (Type G). Approximately 60% of rubbles with fine sand particles coverage was found coexisted with moderate coverage of macroalgae (<40%) under Type H. Overall, no significant occurrence of macroalgae domination was observed in Clade III and IV.

Corresponding to the postulation of coral covers shift from coral to macroalgal domination, Clade V was further identified by cluster analysis due to excessive growth of macroalgal (>90% of macrophyte coverage), such as Phaeophyta, Rhadophyta and Chlorophyta in general. Over 80% of Rhodophyta macrophyte (*Jania* spp.) was found to be seasonally predominant compared to others (Type I). Massive growth of macrophytes was seen to be well adapted with coral rubbles near to inner shallow reef areas, which achieving a dominant range of more than 90% coverage.

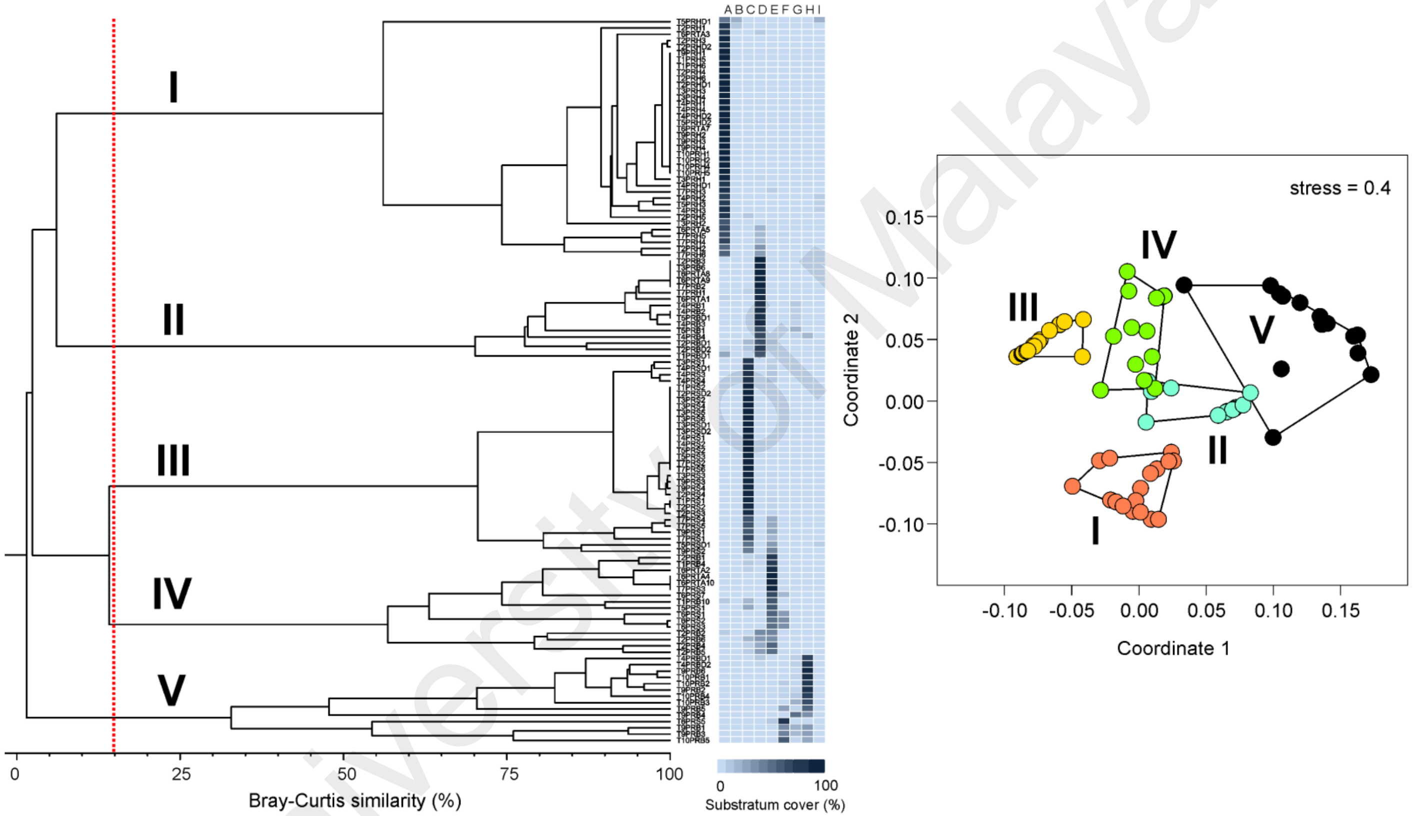


Figure 4.10: Clustering dendrogram showed the dissimilarities relationship of each microhabitat sites based on benthic biological and physical substratum characters covers (Types A – I), separating into five distinguishable geomorphic zones (Clades I – V). The quadrat-substratum heatmap explained the range of substratum cover in percentage (%). Non-metric multidimensional scaling (*n*MDS) ordination (on the right) illustrated five geomorphic zones (Clade I – V) were distinguishable, showing relationship between individual sites.

4.4 Habitat preference of epiphytic benthic dinoflagellates

At most microhabitat sites, overall epiphytic benthic dinoflagellate assemblages did not show any noticeable spatial variation in term of average species composition. Only species abundance of total epiphytic benthic dinoflagellates classified under hard corals microhabitats (Clade I) showed slight positive significant difference with expansive sandy microhabitat (Clade III) due to lacking of bottom vegetative (One-way ANOVA: Kruskal-Wallis test; *n* = 115; *p-value* = 0.05). Highest total abundance of benthic dinoflagellates occurred at live hard coral microhabitats (Clade I) with an average mean value of 663.4 ± 1158 cells/100cm^2, followed by mean value of 493.8 ± 731.2 cells/100 cm^2 happened at enclosed inner reef area with excessive coverage of macroalgae (Clade V), comprising mostly Rhodophyta and Phaeophyta. The lowest total abundance was recorded on sandy areas (Clade III) located at intertidal zones with a mean value of 205.2 ± 140.3 cells/100 cm^2 (Table 4.4) where non-living substratum such as sand particles and fine rocks were localized here.

The species diversity of benthic dinoflagellates demonstrated distinct species preference patterns across five major microhabitat classification (Clade I – V) clearly shown in Figure 4.11. *Gambierdiscus* spp. was highly visible and abundant at sheltered inner areas composited of coral rubbles with highest filamentous turf algal populations (Clade II) highlighted with its average value of 256.8 ± 322.5 cells/100 cm^2. This observation was strongly supported with one-way ANOVA statistical analysis carried out on comparison of *Gambierdiscus* species composition between all major microhabitat speciation (Clade I – V). The resultant statistical differences clearly visualized a strong significant difference of *Gambierdiscus* abundance under Clade II with microhabitat Clade V (One-way ANOVA: Kruskal-Wallis test; *n* = 115; *p-value* = 0.01). Dead corals were washed off and settled down at this site due to less exposure of wave motions and prompted to inhibition of tiny turf algae.

The species composition of *Ostreopsis* spp. appeared negative significant difference between comparisons of five distinct benthic geomorphic classification with exemption between Clade I and Clade III showing positive significant difference (One-way ANOVA: Kruskal-Wallis test; *n* = 115; *p-value* = 0.001). *Ostreopsis* were major species assemblages at hard corals microhabitats (Clade I) (Figure 4.11). Maximum *Ostreopsis* species densitiy was obtained from hard corals microhabitat categorized under Type A (Clade I, as shown in Table 4.3), achieving total cell counts of 34,114 cells/100cm^2. Aside from that, *Ostreopsis* spp. did not show high preference toward dead coral residues overlaid with turf algal population as well as sandy areas (Clade II, III and IV); where most of the total cell counts under these two clades were less than 2500 cells/100cm^2. Highest *Ostreopsis* average value of 2860 ± 5717 cells/100cm^2 was reported at hard coral reef areas (Clade I) following second highest peak observed under Clade V (2186 ± 3733 cells/100cm^2).

Prorocentrum spp. and *Amphidinium* spp. were the key benthic dinoflagellate genus used to define remarkable preference toward expansive sandy areas (Clade III) and sand with rubbles (Clade IV) (Figure 4.11.). Maximum mean value of *Prorocentrum* spp. was recorded majority at sandy areas (Clade III) and/or rubbles covered with fine sand particles(Clade IV) (376.2 ± 258.5 cells/100cm^2), following by 329.0 ± 201.4 cells/100cm^2 of mean species composition respectively. The percent abundance of *Prorocentrum* at macroalgal covers (Clade V) was relatively low where Clade V was reported to positively significant difference with Clade III (One-way ANOVA analysis: Kruskal-Wallis test; *n* = 115; *p-value* = 0.001).

Majority of *Amphidinium* spp. appeared higher abundance at degraded reef areas occupied with dead rubbles overgrown with turf algae and excessive fine sand coverage (112.3 ± 85.83 cells/100 cm^2 and 178.6 ± 166.2 cells/100 cm^2, respectively). Similar with other benthic dinoflagellates except *Ostreopsis* spp., species composition of

Amphidinium only showed slightly positive significant difference with Clade V (One-way ANOVA: Krustal-Wallis test; n = 115; p-value = 0.001) when compared with Clade IV.

Abundance of *Coolia* species was among the lowest of the five major epiphytic benthic dinoflagellates and hardly be found from screen samples collected. The resultant quantitative data showed low cell abundance among overall samples collected from all sites. *Coolia* was statistically emphasized that no significant differences between each microhabitat speciation (Clade I – V), suggesting lack of specific habitat preference on species distribution of *Coolia*. Extreme low species composition ranged from zero to less than 60 cells/100cm^2 in agreement with the hypothesis of *Coolia* spp. may either planktonic cells swim freely within water column or loosely attached with bottom substrate.

Table 4.4: Abundance (cells/100 cm^2) of benthic dinoflagellates species of interest at each classified microhabitat speciation (Clade I – V) from April 2015 to January 2016. Values were reported as average ± standard deviation.

	Dinoflagellates (cells/100 cm^2)					
Clade	*Gambierdiscus*	*Ostreopsis*	*Prorocentrum*	*Amphidinium*	*Coolia*	Total dinoflagellates
I	78.32 ± 83.28	2860 ± 5717	286.9 ± 255.7	80.18 ± 72.76	12.03 ± 13.10	663.4 ± 1158
II	256.8 ± 322.5	647.6 ± 704	241.5 ± 141.5	112.3 ± 85.83	13.13 ± 13.13	254.3 ± 194.9
III	91.35 ± 127.2	450 ± 406.3	376.2 ± 258.5	95.32 ±72.40	13.29 ± 18.28	205.2 ± 140.3
IV	100.6 ± 107.1	1513 ± 1662	329 ± 201.4	178.6 ± 166.20	8.75 ± 10.88	425.9 ± 377.8
V	29.07 ± 44.49	2186 ± 3733	198.4 ± 206	38.79 ± 41.95	15.57 ± 21.73	493.8 ±731.2

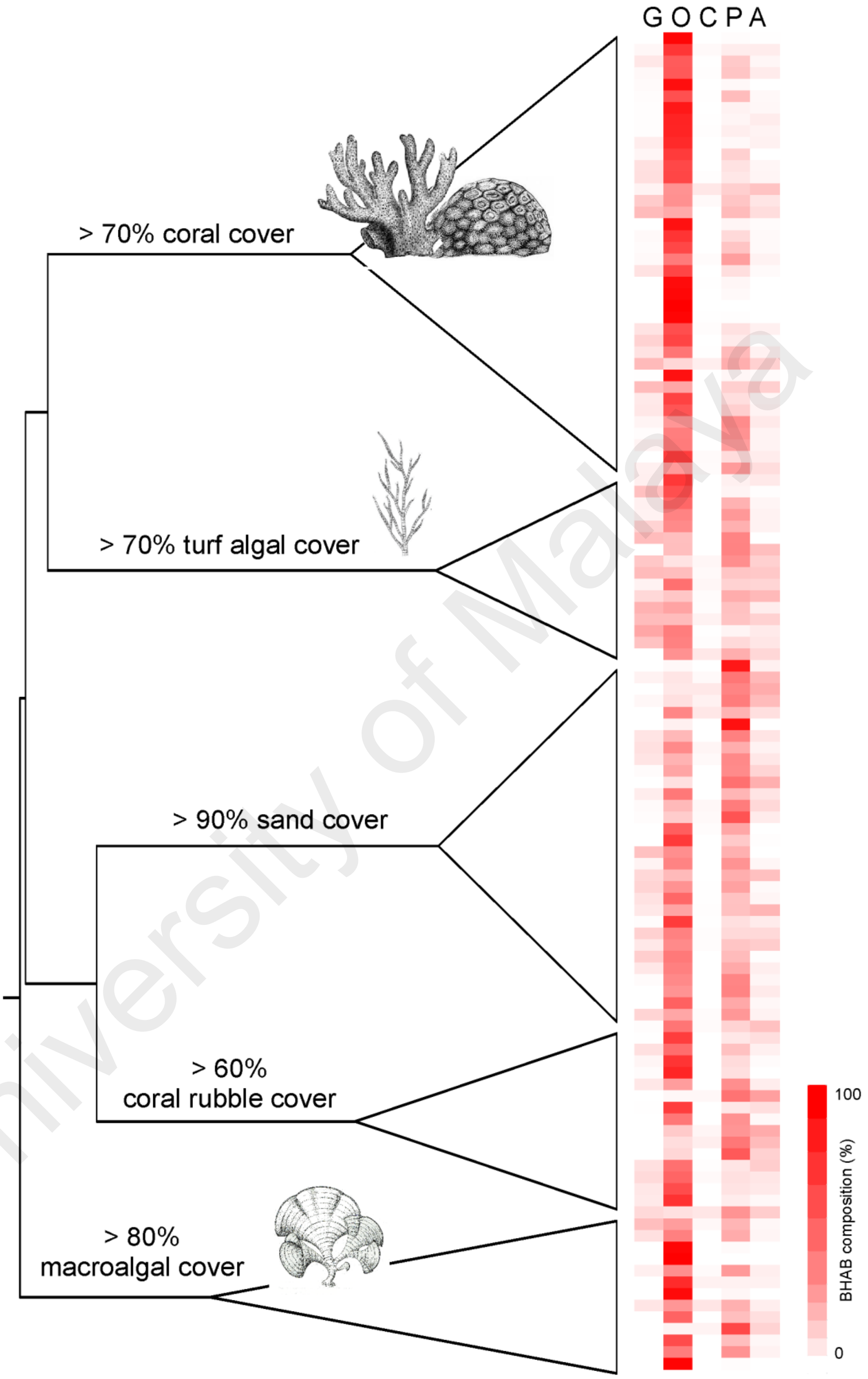


Figure 4.11: Species distribution of benthic dinoflagellates in relation to benthic microhabitat speciation. *Ostreopsis* was the most dominant species at most of the sites; occupying more in hard coral sampling sites (Clade I).

4.5 Canonical corresponding analysis (CCA)

In CCA ordination diagram of environmental variables (Figure 4.12), the upper right quadrat, healthy reef microhabitat (hard corals) was clearly distinguished from the degraded reef microhabitats (turf algae, red filamentous algae, brown fleshy algae, green fleshy algae, sand, sand-covered rubbles, bleached corals and others). The eigenvalues of the first two canonical axes indicated as 0.015 and 0.0059 respectively, explaining in 66% of the total variance. These results suggested that degraded reef microhabitats did play important roles in the distribution of the majority of BHAB dinoflagellates.

On axis 1, the species distribution of *Gambierdiscus* spp. appeared in the positive side of this axis with highly association toward rubbles associated with turf algae and hard corals but less found on various macrophyte species, sands and also sand-covered rubbles. Majority of *Ostreopsis* spp. and *Coolia* spp. indicated strong correlation with hard corals and red filamentous algae, but low species abundance on turf algal population. *Prorocentrum* spp. and *Amphidinium* spp. were located in the lower left quadrat, where these species were dominant in sand and sand-covered rubbles. Species compositions of *Prorocentrum* spp., *Ostreopsis* spp. and *Coolia* spp. only revealed moderate correlation with green fleshy macroalgae.

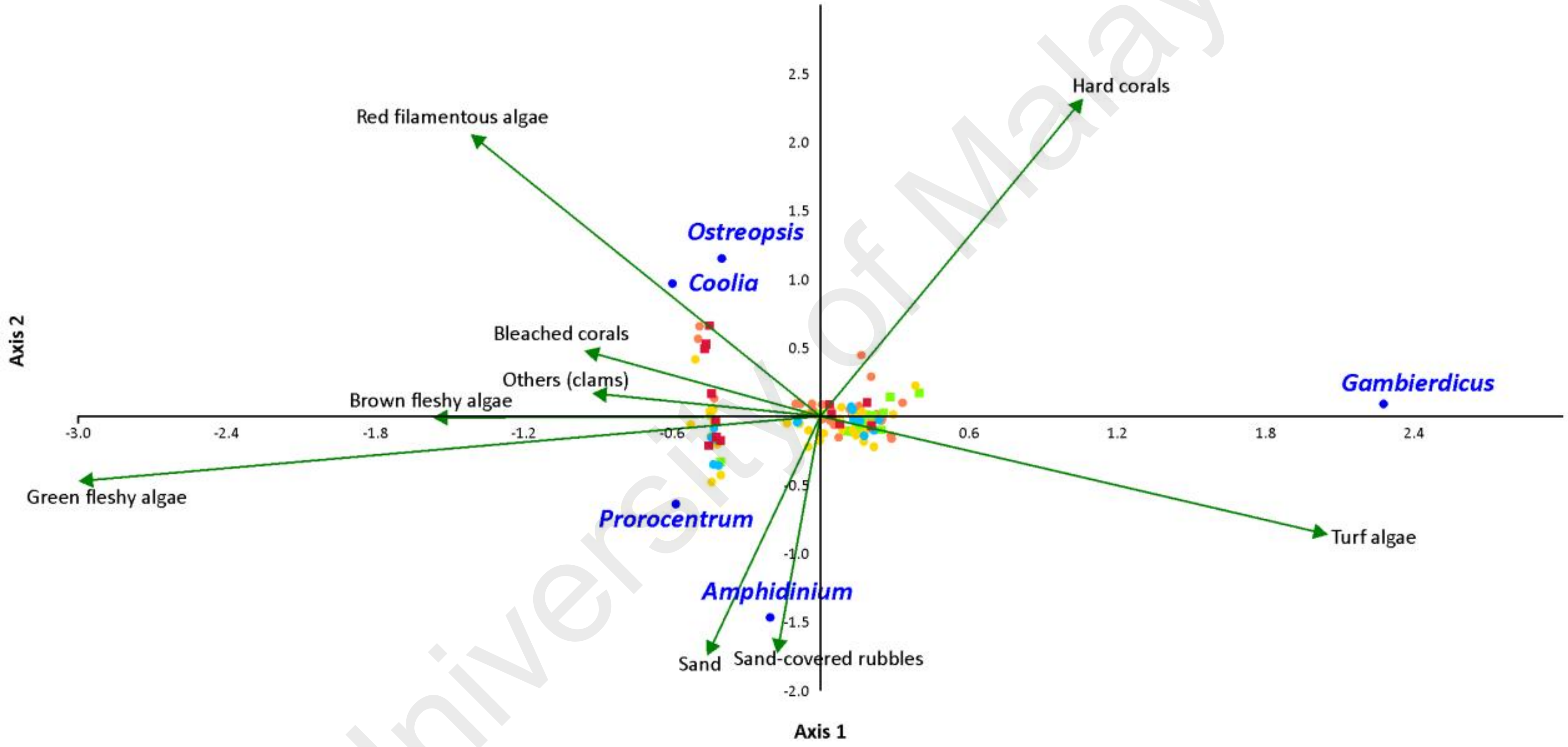


Figure 4.12: Canonical correspondence analysis (CCA) indicated the relationship between benthic dinoflagellates and microhabitat speciation (66% of total variance), revealing preferences of benthic dinoflagellates toward distinct microhabitat (labeled on arrowed lines).

CHAPTER 5: DISCUSSION

5.1 Occurrence of benthic marine dinoflagellates in Malaysian waters

The number of species identification in this study was comparable to the ciguatera-related dinoflagellates species reported in Malaysian waters since early 2000s (Caillaud et al., 2010, Fraga, & Rodr ǵuez, 2014, Leaw et al., 2001, Mohammad-Noor et al., 2004, Mohammad-Noor et al., 2013, Rhodes, 2011, Tan et al., 2013). The present study successfully isolated two species of *Ostreopsis, O.* cf. *ovata* and *O.* cf. *lenticularis* from Rawa Island. Leaw et al., (2001) have genetically described two significant *Ostreopsis* species known as *O. ovata* and *O. lenticularis* that apparently exists in the bottom substratum and fresh macrophytes samples collected from Malacca Straits of Peninsular Malaysia.

Similar to the current finding, the thecated species *Coolia malayensis* described by Leaw et al., (2010) and *Coolia tropicalis* reported by Mohammad-Noor et al., (2013) were both identified from the studied sites. *Gambierdiscus* spp. found in this study was not identified up to species level because of the morphological complexity. In the east coast of Sabah, cells resembled *Gambierdiscus belizeanus* was first recorded in the Asian Pacific region based on morphological feature observed using LM, epi-fluorescence, and scanning electron microscopy, however, lack of molecular data to confirm the cell identities (Leaw et al., 2011). Eleven *Prorocentrum* species were reported by (Mohammad-Noor et al., 2004), with four out of these eleven species were potentially toxic i.e. *P. arenarium, P. lima, P. concavum, and P*. cf. *faustiae*. Three significant *Prorocentrum* species were found in this study including the potent toxic species, *P. lima*, that suspected to produce diarrhetic shellfish poisoning (DSP) toxins (Yasumoto et al., 1984).

5.2 Benthic epiphytic dinoflagellate species abundance

Ostreopsis spp. was predominated in abundance and occupied an average relative abundance of 54% far exceeding *Prorocentrum* spp. and *Gambierdiscus* spp. The other ciguatera-associated benthic dinoflagellates *Prorocentrum* (27%), *Gambierdiscus* (8%), *Amphidinium* (9%) and *Coolia* (2%) only contributed low cell abundance to overall benthic dinoflagellate community. Similarly, Rahman Shah et al., (2013) claimed that *O. ovata* as the dominant species in the benthic dinoflagellates assemblages accounted for its slightly higher maximum relative abundance value (57.4%) achieved in August 2012 in Jeju Island of Korea, comparing to *Ostreopsis* relative abundance (54%) recorded in current study. The prevalence of *Ostreopsis* spp. and *Prorocentrum* spp. abundance occurred in shallow coral reef lagoon from Indian Ocean was reported with *Gambierdiscus toxicus* as the lowest relative abundances obtained in Grzebyk et al., (1994). The occurrence and proliferation of *Ostreopsis* was known as one of the wide spread phenomena overwhelmed by its extreme high abundance among benthic dinoflagellates ever since 1990s (Tognetto et al., 1995).

The peak concentration of *Ostreopsis* in Rawa Island was lower than previous records of *Ostreopsis* in different regions (i.e. 2541×10^3 cells/100cm^2 in Geneo, Italy (Mangialajo et al., 2008); and 1406×10^3 cells/100cm^2 in New Zealand (Shears, & Ross, 2009), with its maximum cell densities of 34×10^3 cells/100cm^2. A close association of benthic epiphytic dinoflagellate communities dominated by *Ostreopsis* was also reported by Vila et al., (2001) in NW Mediterranean, indicating much higher maximum *Ostreopsis* cell concentration of 596×10^3 cells g^{-1} on macroalgal samples. Conversely, low occurrence of *Ostreopsis* was reported in several studies, with only 9.05×10^3 cells g^{-1} of *Ostreopsis* in Mediterranean waters (Ismael, & Halim, 2012) and 9×10^3 cells g^{-1} in Jeju Island, Korean waters (Kim et al., 2011).

Prorocentrum was known to be the second highest in species abundance (299.7 ± 234.8 cells/100cm^2) among benthic dinoflagellate assemblages obtained from coral reef of Rawa Island (Table 4.2). In the previous studies, most of the cigua-associated benthic dinoflagellates *Gambierdiscus, Ostreopsis* and *Prorocentrum* were mostly co-existed in a close association with macroalgae where *Prorocentrum* was reported to the be dominant species in previous studies (Delgado et al., 2006, Okolodkov et al., 2014, Richlen, & Lobel, 2011). Overall, the maximum species abundance of *Prorocentrum* in this study only comprised of 1.2 × 10^3 cells/100cm^2 observed across samples with extremely low cell abundance among other related studies (mostly >10^4 cells g^{-1}) (Delgado et al., 2006, Okolodkov et al., 2014). Okolodkov et al., (2014) pointed out *Prorocentrum* as the dominant species with its highest abundance up to 2.41 × 10^4 cells g^{-1} in shallow subtropical waters and mangrove lagoon located at Gulf of Mexico.

In the earliest ecological studies on ciguatera dinoflagellates, *Gambierdiscus* always appeared in remarkable low cell abundance throughout coral reef ecosystem in shallow coastal areas. The maximum abundance of *Gambierdiscus* sp. (1.2 × 10^3 cells/100cm^2) in present study was relatively lower than those of 500 × 10^3 cells g^{-1} of *G. toxicus* from Pacific Ocean (Yasumoto et al., 1980a); 1.6 × 10^3 cells g^{-1} from Indian Ocean (Grzebyk et al., 1994), and <400 cells g^{-1} on fresh macroalgal *Dictyota* spp. from Caribbean Sea (Ballantine et al., 1988). (Kim et al., 2011) and Grzebyk et al., (1994) also reported lower *Gambierdiscus* composition found on macroalgae and dead coral residues samples with higher densities of *Ostreopsis* and *Prorocentrum,* indicating similar species abundances ratio in present study. One potential factor that might influence the distribution of *Gambierdiscus* population even in such a small area, is so-called as “micro-regionality”. In Tahiti Island, the algal host preference of *G. toxicus* was commonly found on *Turbinaria ornata* (Yasumoto et al., 1980a), whereas Yasumoto et al., (1979) concluded that similar *Gambierdiscus* species was varied among each station and highly abundant on red calcacerous algae, *Jania* sp. in Gambier Island.

The average species composition of *Amphidinium* spp. reached its highest population of 97.38 ± 96.85 cells/100 cm^2 whereas *A. carterae* and *A. operculatum* were reported with their maximum values of 76.39 ± 37.21 cells g^{-1} and 103.04 ± 37.60 cells g^{-1} respectively (Rahman Shah et al., 2013). Present study reported the maximum species composition of *Amphidinium* spp. could be up to 507 cells/100 cm^2 that mostly found in sandy bottom microenvironment. In Gulf of Mexico, the abundance of *A.* cf. *carterae* was much lower with 3.69×10^3 cells g^{-1} reported (Okolodkov et al., 2014).

In contrast with major species composition of benthic dinoflagellates assemblages, *Coolia* was occasionally observed from collected samples, as minor components of the community with its average cell density 12.50 ± 15.47 cells/100cm^2 recorded. This particular genus was assumed to be not preferable toward bottom substratum and shifted more in the water column, where less cell density was encountered on macroalgae so far (< 60 cells/cm^2). *Coolia monotis* was found to be well adapted both in water column and closely associated with macroalgal (Vila et al., 2001). The former probably due to disturbance happened onto macroalgae and resuspended into water column under shaken water environment. With exceptional records taken in Sampadi Island (Malaysia), benthic dinoflagellates assemblages in fringing reef complex seemed to be dominated by *Coolia* and *Prorocentrum* coupled with occasionally occurrence of *Ostreopsis* and *Gambierdiscus* (Tan et al., 2013). To some extents, *Coolia* could be quite vulnerable to sudden water turbulence happened within marine environment especially when exposed to moderate to strong water movements at shallow water areas because of reduced physically pressure supports from bottom substratum (Aligizaki, & Nikolaidis, 2006, Tindall, & Morton, 1998, Vila et al., 2001).

5.3 Hydrodynamic influence on benthic dinoflagellate assemblages

Both benthic dinoflagellate species abundances and community composition had demonstrated significant variation towards water motion and depth. In present study, the outermost back reef sites possessed relatively distinct changes of moderate to strong flow of water motion, expressing similar physical and chemical characteristics of those in oligotrophic. This high energy barrier had supported relatively high abundance dinoflagellates abundance (Table 4.4; Clade I with average cell composition value of 663.4 ± 1158 cells/100cm^2), conversely, total dinoflagellates abundance was the lowest in calm inshore sandy area that subjected to less water motion.

These spatial variability pattern observed in this study was in congruent with the studies of Grzebyk et al., (1994) and Vila et al., (2001). High densities of total benthic dinoflagellates communities were significantly found on middle and outer lagoonal reef habitats given similar hydrodynamics condition of sampling sites, where less terrigenous sediment inputs and were moderately exposed to wave action (Grzebyk et al., 1994). Vila et al., (2001) showed that *Ostreopsis* sp. was well adapted to slightly shaken reef habitat compared to relatively calm sheltered areas that experienced high sedimentation rate. Some species of benthic dinoflagellates were described as epiphytic and loosely attach on the surface of macroalgae by forming mucilaginous coating in order to reduce physical pressures of the water turbulence (Aligizaki, & Nikolaidis, 2006, Grzebyk et al., 1994, Tindall, & Morton, 1998, Vila et al., 2001). Hence, this could be likely the reason of high *Ostreopsis* spp. abundance was observed in high energy barrier of reef habitat located in the outermost back reef sites in current studies.

In contrast, total benthic dinoflagellate composition among reef habitats at Johnson Atoll (Richlen, & Lobel, 2011) and Virgin Islands (Calson and Tindall, 1985) were in contrast with present studies. Richlen, and Lobel, (2011) reported that larger composition of benthic dinoflagellates communities was supported in sampling sites located in calm lagoon areas that received limited water movement but extending in drastic decline numbers of total abundance at shallow back reef sites/reef crest exposed to greater wave motions. These pattern was also observed in Tindall, and Morton, (1998) on the effect of hydrodynamic variations on development of epiphytic benthic dinoflagellates communities.

Similar to our studies, the proliferation of *Prorocentrum* spp. was prominently found at calm and less water movement reef environment where sedimentation rate was greater in Johnson Atoll (Richlen, & Lobel, 2011). Both abundance of *Prorocentrum* spp. and *Amphidinium* spp. in present study were significantly high at inshore sheltered reef habitats with less occurrence of macrophyte. Conversely, *Ostreopsis* spp. comprised the largest proportion of benthic dinoflagellates abundance at persistently moderate to strong water flows reef environments.

5.4 Preference of host substratum

This study revealed that overall ciguatera dinoflagellates assemblages at each sampling sites was not randomly dispersed rather proliferated in a patchy distribution pattern among distinct habitat types. Each identified ciguatera dinoflagellates species composition were presented at majority of sampling sites and contributed minor proportion of total epiphytic ciguatera dinoflagellates assemblages. For instance, *Ostreopsis* composition was mainly encountered at healthy hard corals area (Clade I) in where strong water flow was expected here, whereas other ciguatera dinoflagellates assemblages tend to accumulate at moderate water motions and degraded coral reef zones (Clade II – V). Population of epiphytic ciguatera dinoflagellates was explained as ‘micro-regionality’ in which wide variations of total cell densities were reported accordingly even within such a short distance small area.

This phenomenon was well reflected and supported in the earliest environmental studies of ciguatera dinoflagellates (Ballantine, 1985, Bomber et al., 1989, Richlen, & Lobel, 2011, Taylor, 1985, Vila et al., 2001, Yasumoto et al., 1979, 1980a). Yasumoto et al., (1980a) reported regional distribution of *G. toxicus* was highly varied in term of cell densities at distinct spatial levels taken in Gambier Islands coupled with limited observation periods. Richlen, and Lobel, (2011) clearly support the fact of ‘micro-regionality’ dispersion pattern encountered on ciguatera dinoflagellates communities in single sites. They observed a comparable high number of dinoflagellates cell densities in relatively calm lagoon and channel sites instead of back reef and reef crest habitats located in Johnston Atoll of Pacific Ocean, likely subject to potential differences in preferable surface area of bottom substratum and influence of water motion. These ecological studies were explicable when abundance of epiphytic benthic dinoflagellates at each studied sites varied markedly due to lack of specificity on bottom substratum.

The maximum cell abundance of *Gambierdiscus* spp. was likely attributed to the presence of filamentous turf algae population appeared on coral fragments in shallow sheltered reefs area (0.5 – 3 m). Only moderate cell composition of *Gambierdiscus* spp. was observed at certain areas comprising of hard corals, heavy coated of sand particles on dead corals and benthic macroalgae such as *Jania* sp., *Dictyota* sp. and *Lobophora* sp. With respect to possible factors that trigger accumulation of *Gambierdiscus*, tuft algal mat on corals fragments somehow provides favorable dense and fine branches of adherent microenvironment for dinoflagellate to overcome strong water movement, given larger surface area for cell attachment. Previous researches believed that epiphytic dinoflagellates densities have less significant difference with any particular macroalgae types rather more preferred 3-dimensional, flexible and high surface area algae, prompting to a likely close association between cells and macroalgae (Bomber et al., 1989, Taylor, 1985, Tindall, & Morton, 1998, Vila et al., 2001).

Similar host preference pattern of *Gambierdiscus* was supported in Taylor, (1985) where *G. toxicus* concentration was abundantly found on coral fragments coupled with finely branched turf algae and less land run-off in the Caribbean sea regions. These epiphytic dinoflagellates population might be diminished due to significant exposure to muddy sediments covered on dead corals or even on turf algae surface and extreme strong wave action. These patterns was in agreement with the ecological studies of ciguatera dinoflagellates in south-west Indian Ocean that documented the number of ciguatera dinoflagellates was adversely influenced by areas subjected to sediment inputs and water motions (Grzebyk et al., 1994).

Ostreopsis spp. was exclusively found on nearly all substrates investigated at both healthy and degraded coral reef zone in which maximum species composition was dominated in healthy hard coral reef areas exposed to moderate flows of water currents. It was virtually unknown why *Ostreopsis* spp. appeared to be highly visible on hard corals surface areas, whereas others are more susceptible probably due to prolonged exposure to strong passage currents. Aside from water turbulence effects in coral reef habitats, relevant studies have been proposed aggregation of benthic dinoflagellates on macrophytes surface might be influenced by the excretion of stimulatory or inhibiting compound in algal extracts (Aligizaki, & Nikolaidis, 2006, Grzebyk et al., 1994, Parsons et al., 2012). *Ostreopsis* was preferentially epiphytic on macrophytes, hard bottom substratum (e.g. rocks, sands and dead coral fragments) and in water column (Aligizaki, & Nikolaidis, 2006, Faust, 1999, Faust, & Morton, 1995, Kim et al., 2011, Rhodes, 2011, Vila et al., 2001). Rapid cell proliferation may form a dense mucilaginous matrix and loosely aggregated on macrophytes surfaces , in which cells may tend to detach from substrates in a mucilage form and were found in water column due to disturbance or resuspension from macrophytes surfaces (Aligizaki, & Nikolaidis, 2006, Grzebyk et al., 1994, Vila et al., 2001). This is compatible with the observation documented in Aligizaki, and Nikolaidis, (2006) in which cell densities in the water column and sediments were relatively positive correlation with those on macrophytes.

In present study, majority of benthic dinoflagellates genera *Prorocentrum* and *Amphidinium* were significantly found to be associated with sand sediments. Under conditions of relatively low turbulence at calm areas, sand particles tend to settle down in which heavier sand coverage on dead corals may provide ideal microhabitat for benthic dinoflagellates assemblages. It was proven that *Prorocentrum* and *Amphidinium* were very common identified from sand sediments in previously published literatures (Faust, 2009, Rahman Shah, 2014, Saburova et al., 2009). Based upon the observation reported in Faust, (2009), four out of eight known toxic benthic dinoflagellates species were identified from brownish coloured sand sediment: *P. lima*, *P. mexicanum*, *A. carterae*, and *A. klebsii*. Aside from that, some of the *Prorocentrum* species, e.g. *P. mexicanum* and *P. rhathymum* were indicated as potentially bloom-forming dinoflagellates in The Lair of Belize waters, reported by Faust, (2009). A number of *Amphidinium* species was also identified in sand and muddy sand samples collected from intertidal coastal areas of Kuwait, provided with taxonomic characterization under light microscopy examinations of living dinoflagellates cells (Saburova et al., 2009). Hence, sand sediment may assumed as potential seed bank for slow swimmer benthic dinoflagellates like *Prorocentrum* and *Amphidinium* species to seek for shelter from either predators or sudden water turbulence.

The presence of *Coolia* spp. was scarce in almost all samples collected from sites. This phenomenon can be explained that *Coolia* species may appeared as either epiphytic on macrophytes surface or planktonic species in water column. Benthic dinoflagellates were potentially swift into the water column due to substantial flows of water currents, and establish new benthic dinoflagellates populations, as supported by Vila et al., (2001) and Faust, (2009). Apart from that, limited occurrence of *Coolia* population in present study might be attributed to possible pre-sampling and post-sampling precautions (disturbance onto screen samples during sample collections, sample processing methods etc.).

CHAPTER 6: CONCLUSION

Ciguatera Fish Poisoning (CFP) is regarded as the most common seafood intoxication in human, involving the neurotoxins produced by some species of epiphytic and benthic dinoflagellates found in coral reefs and inshore habitats of tropical and subtropical regions. The benthic harmful dinoflagellates, *Gambierdiscus* spp., *Fukuyoa* spp., *Ostreopsis* spp., *Prorocentrum* spp., *Coolia* spp. and *Amphidinium* spp. are predominantly epiphytic in nature, forming mucilaginous layers to attach to macrophytes and epi-benthic layers of substratum. The phase shifts of coral-dominated reefs to algal dominated reefs may favor proliferation of these dinoflagellates. The effects of bottom substrate complexity and host selectivity factors on host colonization by these benthic dinoflagellates are still unknown. This study has investigated the benthic harmful dinoflagellates assemblages in relation to reef microhabitats by adopting a non-destructive sampling technique.

A total of 115 artificial screen samples collectors were deployed underwater and successfully retrieved by SCUBA after 24 hours deployment period. Average daytime temperature and salinity of shallow sea surface water were recorded between 30 – 33 ℃ and 30 – 32 PSU, with consistent daily light intensity in a range of 1000 – 2500 µmol $m^{-2}s^{-1}$ recorded. Current study clearly indicated the targeted interest genera benthic harmful dinoflagellates were prominently distributed in destroyed reef microhabitats and dispersed in a patchy distribution pattern among distinct habitat types. *Ostreopsis* was known as predominant species among other five benthic epiphytic genera and perennially present in all bottom microhabitats investigated at each sites. It was more susceptible to slightly shaken microhabitats rather than calm sheltered areas probably due to their abilities to secrete mucilage layers in order to associate with bottom substratum. *Gambierdiscus* spp. was likely attributed to the presence of filamentous turf algae population appeared on dead coral fragments in shallow sheltered reefs area (0.5 – 3 m). Tuft algal mat on dead coral fragments provides favorable dense and fine branches of adherent microhabitats for *Gambierdiscus* to overcome strong water movement, supporting larger surface area for cell attachment.

Majority of *Prorocentrum* spp. and *Amphidinium* spp. were significantly higher at inshore sheltered associated with heavy sand sediments but less occurrence of macrophyte. The slow swimmer benthic dinoflagellates like *Prorocentrum* and *Amphidinium* were commonly found in sand sediment as shelter purpose either from predators or sudden water turbulence. The presence of *Coolia* spp. was scarce in almost all samples collected from sites. This phenomenon can be explained that *Coolia* species may appeared as either epiphytic on macrophytes surface or planktonic species in water column. Sudden water disturbance may breakdown mucilage attachment between benthic dinoflagellates and bottom substratum, prompting to the reintroduction of benthic dinoflagellates populations into water column.

Current study has demonstrated that disturbed coral reefs environment were most likely attributed to proliferation of benthic harmful dinoflagellate communities despite of possible circumstances of environmental changes (light intensity, water temperature and seawater salinity). Preliminary investigation on natural habitat preference of benthic harmful dinoflagellates is important to discover more knowledge concerning species diversity and distribution of benthic dinoflagellates and their possible impacts on marine organisms as well as human intoxication risks. Hence, more sample replicates of different locations are needed to reveal the differences in species diversities of benthic dinoflagellate communities.

In this study, data analyzed on benthic dinoflagellates species diversity patterns was mainly concentrated on physical appearance of bottom substratum neglecting the possible manipulative factors including water depth, chemical excretion from macroalgal, and nutrients in relation to benthic dinoflagellates. Furthermore, ecological studies on the toxin transfer pathway from the causative organisms to human consumption may help the public to understand more on seafood intoxication risks of BHABs dinoflagellates. Rapid and reliable toxin detection methods could help the government to pre-alert the public on cigua-contaminated marine food supplies either in local or imported markets whenever there is a possible occurrence of BHABs bloom events.

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