BENTHIC DINOFLAGELLATES ASSEMBLAGES ASSOCIATED WITH CIGUATERA FISH POISONING (CFP) AT FRINGING CORAL REFF ECOSYSTEM OF PERHENTIAN ISLANDS, MALAYSIA

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INSTITUTE FOR ADVANCED STUDIES
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2019

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

INSTITUTE FOR ADVANCED STUDIES
UNIVERSITY OF MALAYA
KUALA LUMPUR

2019

UNIVERSITY OF MALAYA

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Matric No: HGT150006

Name of Degree: Master Degree

Title of Project Paper/Research Report/Dissertation/Thesis:

Benthic Dinoflagellates Assemblages Associated with Ciguatera Fish Poisoning (CFP) at Fringing Coral Reef Ecosystem of Perhentian Islands, Malaysia

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BENTHIC DINOFLAGELLATES ASSEMBLAGES ASSOCIATED WITH CIGUATERA FISH POISONING (CFP) AT FRINGING CORAL REEF ECOSYSTEM OF PERHENTIAN ISLANDS, MALAYSIA

ABSTRACT

Ciguatera fish poisoning (CFP) is a foodborne disease associated with seafood contamination by ciguatoxins (CTXs) produced by *Gambierdiscus* species, which known to assimilate and metabolize through multiple trophic levels from herbivorous fish to larger finfish predators. However, the source and fate of CTXs into the marine food web remained ambiguous. Benthic dinoflagellates are known to be closely associated with the benthic biotic substratum such as seaweed, seagrass, turf algae and corals where these substrata are served as a feeding ground for reef inhabitants (reef fishes, invertebrates). Thus, the distribution and natural assemblages of benthic dinoflagellates on the bottom substratum of coral reef ecosystem becomes one of the key elements to trace the origin of ciguatoxin transfer. This study aims to understand the CTX transfer into the marine food web by investigating the distribution and natural assemblages of benthic harmful dinoflagellates in the different substratum. The diversity of benthic dinoflagellate was investigated. The study was conducted in Perhentian Islands (5°54'13.44"N, 102°44'49.27"E), located off the coast of Terengganu, Malaysia. A total of 243 samples were collected from five sampling sites over the period of April 2016 to May 2017 using an artificial substrate sampling method (fibreglass screens with a dimension of 10.2 × 15.2 cm). The benthic habitats were characterized by using CoralNet. Cells of benthic dinoflagellates, *Gambierdiscus*, *Ostreopsis*, *Coolia*, *Amphidinium* and *Prorocentrum* were enumerated microscopically. The species were further identified by advanced morphological and molecular characterizations. The results revealed the presence of three species of *Gambierdiscus*, four species of *Coolia*, two species of *Amphidinium*, five species of *Prorocentrum,* a

species of *Ostreopsis*, and a species of *Gymnodinium*; this included the first record of *C. palmyrensis*, *C.* cf. *canariensis*, *Gymnodinium dorsalisculcum*, and *A.* cf. *massartii* in our waters. The results showed a depth gradient of benthic dinoflagellate distribution and abundance, where *Gambierdiscus, Ostreopsis* and *Amphidinium* abundances decreased with depth (>10 m). *Coolia* and *Prorocentrum* were commonly found distributed throughout the depths investigated. Results of the Kruskal-Wallis test revealed that benthic dinoflagellates demonstrated different habitat preferences spanning from areas with sandy patches and corals to macrophyte coverages. *Prorocentrum* was the dominant group; it was found across various types of the substratum but particularly preferred the substratum with high sand covers. In contrast, *Ostreopsis* was abundant in shallower water which showed preference towards macrophyte-covered substratum and turf algae assemblage. *Gambierdiscus* demonstrated a preference towards macroalgae such as *Jania* spp. and turf algae assemblages. Ciguatoxicity of two species of *Gambierdiscus, G. caribaeus* and *G. balechii*, were confirmed through a cytotoxicity assay, neuroblastoma-2a assay. In conclusion, benthic dinoflagellates assemblages displayed distinct community structure and compositions across different bottom substrates. Habitat preferences of *Gambierdiscus* on substratum with high turf algal covers may promote ciguatoxin flux from the bottom substrates into the marine food web as turf algae have high colonization rate and high palatability.

Keywords: Artificial substrate; benthic harmful algae; Ciguatera Fish Poisoning; coral reefs; Perhentian Islands.

PERHIMPUNAN DINOFLAGELAT BENTIK DENGAN KERACUNAN IKAN CIGUATERA (CFP) DI EKOSISTEM TERUMBU KARANG DI PULAU PERHENTIAN, MALAYSIA

ABSTRAK

Keracunan ikan Ciguatera (CFP) adalah penyakit bawaan makanan yang dikaitkan dengan pencemaran makanan laut oleh ciguatoksins (CTXs) yang dihasilkan oleh *Gambierdiscus* spp., yang akan berasimilasi dan metabolisma melalui pelbagai peringkat trofik dari ikan herbivora kepada ikan karnivor. Walau bagaimanapun, pemindahan CTX ke dalam jaringan makanan marin masih kurang jelas. Dinoflagelat marin bentik selalu bersekutu dengan substratum biotik bentik seperti rumpai laut, rumput laut, alga turf dan karang-karang di mana akan sebagai bahan makanan untuk ikan karang dan invertebrata. Oleh itu, taburan dan himpunan semulajadi dinoflagelat bentik di ekosistem terumbu karang adalah penting untuk menjejaki asal-usul dan pemindahan ciguatoxin. Kajian ini bertujuan untuk memahami pemindahan CTX ke dalam siratan makanan marin dengan mengkaji taburan dan himpunan semulajadi dinoflagelat bentik dalam substratum yang berbeza. Kepelbagaian dinoflagelat bentik di Pulau Perhentian juga diselidik. Kajian ini dijalankan di Pulau Perhentian yang terletak di luar persisiran perairan Terengganu, Malaysia. Sejumlah 243 sampel dikumpulkan dari lima lokasi persampelan sepanjang tempoh April 2016 hingga Mei 2017. Substrat tiruan skrin gentian kaca (dimensi 10.2 × 15.2 cm) telah digunakan dan habitat bentik dicirikan dengan menggunakan CoralNet. Kelimpahan sel-sel dinoflagelat bentik genus *Gambierdiscus*, *Ostreopsis*, *Coolia*, *Amphidinium* dan *Prorocentrum* telah ditentukan. Dalam kajian kepelbagaian dinoflagelat epifit, kehadiran tiga spesies *Gambierdiscus*, empat species *Coolia*, dua spesies *Amphidinium*, lima spesies *Prorocentrum*, satu spesies *Ostreopsis* dan *Gymnodinium* telah disahkan dengan kaedah genetik dan morfologi termasuk laporan yang pertama di perairan Malaysia untuk spesies *C.*

palmyrensis, *C*. cf. *canariensis*, *C*. cf. *massartii* dan *Gymnodinium dorsalisulcum*. Hasil kajian ini menunjukkan kecerunan kedalaman pengedaran dinoflagelat bentik dan kelimpahan *Gambierdiscus*, *Ostreopsis* dan *Amphidinium* menurun dengan kedalaman (>10 m). *Coolia* dan *Prorocentrum* dijumpai di seluruh kedalaman yang dikaji. Hasilan ujian Kruskal-Wallis menunjukkan permilihan habitat dinoflagelat bentik yang berbeza merangkumi pasir, batu karang dan rumpai laut. *Prorocentrum* adalah kumpulan yang dominan, ia didapati merentas pelbagai jenis substrat, tetapi lebih tertumpu di substrat pasir. Sebaliknya, *Ostreopsis* banyak terdapat di dalam air yang lebih cetek yang memperlihatkan keutamaan ke atas substrat yang dilapisi dengan makroalga dan karang. *Gambierdiscus* diperlihatkan dengan keutamaan terhadap makroalga (*Jania* spp.) dan kompleks alga turf. Dua spesies *Gambierdiscus*, *G. caribaeus* dan *G. balechii*, telah dikesan dengan ciguatoxin melalui bioesei neuro-2a. Kesimpulannya, perhimpunan komuniti dinoflagelat bentik adalah berbeza mengikut substrat dasar, kecenderungan *Gambierdiscus* pada habitat substrat alga turf dengan kadar kolonisasi yang tinggi dan makan pilihan pemakan adalah mekanisma fluks pemindahan ciguatoksin dari substrat dasar ke dalam jaringan makanan.

Kata kunci: bentik alga berbahaya; Keracunan ikan Ciguatera; Pulau Perhentian; substrat artifisial; terumbu karang

ACKNOWLEDGMENTS

Foremost, I would like to express my heartfelt gratitude to my supervisors, Assoc. Prof. Dr. Lim Po Teen, Assoc. Prof. Dr. Leaw Chui Pin, and Assoc. Prof. Dr. Leo Lai Chan for their altruistic guidance, wisdom, inspiration, and liberty given to me throughout the whole project.

Secondly, I would like to applause my seniors, lab mates and interns for their continuous support and motivation in assisting data collection and analysis whereby it would be insurmountable without unselfish and great team effort of Dr. Tan Toh Hii, Yong Hwa Lin, Er Huey Hui, Lim Zhen Fei, Nurin Izzati Mustapa, Dr. Hii Kieng Soon, Tan Suh Nih, Liow Guat Ru, Law Ing Kuo, Winnie Lau Lik Sing and Dr. Teng Sing Tung. I thank Assoc. Prof. Dr. Leo Lai Chan, Dr. Maggie Mak and members from State Key Laboratory in Marine Pollution, City University of Hong Kong and Dr Lu Chung Kuang and members from National Yang-Ming University, and Prof. Dr. Haifeng Gu from Third Institute of Oceanography, China for their hospitality and sharing of knowledge for my work.

Thirdly, I would like to reach out to the former Director of Institute of Ocean and Earth Sciences (IOES), Prof. Dr. Phang Siew Moi, for her endorsement of my work. I would like to express my appreciation towards staff and officers of Bachok Marine Research Staton (BMRS), IOES, Kelantan for their helping hands given during the duration of the project.

Lastly, the journey to my higher education would be unrealistic without unyielding love and blessing from my family and friends, especially my brother, Lee Li Chuen for his camaraderie and advices given.

I am obliged for the MyBrain 15 scholarship awarded by MOHE for my Master's project. This work was funded by MOSTI Sciencefund (04-01-03-SF1010) to

Dr. Leaw; MOSTI ICF (UM0042224) and MOHE HICoE Fund (IOES-2014C) to Dr. Lim.

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CFP	:	Ciguatera Fish Poisoning
CTXs	:	Ciguatoxins
P-CTX	:	Pacific ciguatoxins
VGSC	:	Voltage-gated sodium channel
CBA	:	Cell-based assay
Neuro-2a	:	Mouse neuroblastoma cell line
PCR	:	Polymerase chain reaction
LSU	:	Large subunit
rDNA	:	Ribosomal deoxyribonucleic acid
nMDS	:	Non-metric multi-dimensional scaling
ANOSIM	:	Analysis of Similarity
SIMPER	:	Similarity Percentages

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CHAPTER 1: GENERAL INTRODUCTION

Benthic marine dinoflagellates are common microalgae associated with macrophytes or on epi-benthic layers of coral reef in tropical and subtropical coastal waters. They were defined as epiphytic benthic microalgae due to their close association with the natural substrates such as sand particles, coral rubble, and macroalgae such as seaweed (Parsons et al., 2011). The study of harmful benthic microalgae did not arise until the mid-1970s when Yasumoto et al. (1977) reported the discovery of a species of benthic dinoflagellate responsible for ciguatera fish poisoning (CFP). CFP is the most common form of phycotoxin-borne seafood illness across the globe. CFP is widespread, with an estimated 25,000–50,000 poisonings annually (Parsons et al., 2012). The causative toxins for CFP are known as ciguatoxins (CTXs), produced by the dinoflagellate *Gambierdiscus toxicus*. The toxins are accumulated and magnified through the trophic interaction among micro/macroinvertebrate and reef fishes, causing human intoxication via consumption of the contaminated reef fishes in the ciguatera-epidemic region.

The benthic harmful dinoflagellates *Gambierdiscus* and *Fukuyoa* were closely associated with other epiphytic dinoflagellates such as *Ostreopsis*, *Prorocentrum*, *Coolia*, and *Amphidinium* are also known to produce bioactive substances (Hoppenrath et al., 2014).

Of the known toxigenic benthic microalgae, the genus *Gambierdiscus*, which is the main culprit for CFP, has been on the spotlight since the discovery of Yasumoto et al. (1977). CFP was initially considered to be endemic to tropical and subtropical coral reef regions, however, due to international trading of live seafood, it is now the most common non-bacterial illness associated with seafood consumption. To date, a total of 14 *Gambierdiscus* species (Adachi & Fukuyo, 1979; Chinain et al., 1999a; Litaker et al., 2009; Fraga et al., 2011; Fraga & Rodríguez, 2014; Nishimura et al., 2014; Fraga et al.,

2016; Smith et al., 2016; Kretzschmar et al., 2017; Rhodes et al., 2017a) and 3 species of Genus *Fukuyoa* (Gómez et al., 2015) have been described.

Most species of *Gambierdiscus* were known as ciguatoxin producers (Dickey & Plakas, 2010). Ciguatoxins (CTXs) are a family of heat-stable, lipid-soluble, highly oxygenated, cyclic polyether molecules with more than 30 congeners or isomers have been identified (EFSA Panel on Contaminants in Food Chain, 2010), it also resemblance of brevetoxins in the structural framework (Lewis, 2001). Based on geographic origins and structural variants, ciguatoxins can be classified into Pacific (P-CTXs), Caribbean (C-CTXs) and Indian (I-CTXs) ciguatoxins (Lehane & Lewis, 2000). Out of the three variants, P-CTXs are the most potent toxins (Caillaud et al., 2010a). Studies reported that the safety level of consumption of ciguateric fish is no more than 0.01 ppb P-CTX equivalent toxicity in fish (Dickey & Plakas, 2010). Ciguatoxins are difficult to detect by a conventional method such as UV-absorption due to their chemical structure properties (Dickey & Plakas, 2010).

The screening and detection of ciguatoxins are needed for numerous applications including seafood screenings, environmental monitoring and risk assessment (Caillaud et al., 2010a). For biological methods, neuro-2a mouse neuroblastoma cell assay has been used frequently in laboratories for screening and detection of CTXs (Dickey & Plakas, 2010). Another type of biological assay is the competitive receptor binding assay (RBA). Asides from the biological approaches, analytical approaches also have been incorporated such as physicochemical analysis using HPLC (High-Performance Liquid Chromatography) and LC/MS (Liquid Chromatography-Mass Spectrometry) for structure elucidation of CTX congeners (Caillaud et al., 2010a). Immune-assays for CTXs such as enzyme-linked immunosorbent assay (ELISA) provides rapid and accurate screening in toxicity (Dickey & Plakas, 2010).

In the Malaysian waters, long-term data on Benthic Harmful Algal Bloom (BHAB) occurrence and its environmental conditions were scarcely documented even though the occurrence of BHAB species was reported in some selected reefs and islands (Leaw et al., 2001; Leaw et al., 2010; Leaw et al., 2011). Several ciguatera-related dinoflagellates have been studied in Malaysian waters which focus on the taxonomic aspect, such as *Ostreopsis ovata* (Leaw et al., 2001), *Coolia malayensis* (Leaw et al., 2010; Leaw et al., 2016), *Gambierdiscus* cf. *belizeanus* (Leaw et al., 2011) and *G. balechii* (Dai et al., 2017). In Malaysia, ciguatera was first reported in September 2010, 22 members from 5 families were affected with consumption of red snapper (Nik Khairol Reza et al., 2011).

There was a hypothesis that linked reef disturbance to increase in ciguatera incidence which suggested reef disturbance can be good predictors on potential CFP events (Rongo & van Woesik, 2011, 2013). This was based on the disturbances of reef that commenced ecological succession whereby opportunistic macroalgae and turf algae with higher colonization rates will out-compete settlement of reef-building coralline algae (McCook et al., 2001). The succession of the turf algae which was the preferred substrate to ciguatoxic dinoflagellate would attract grazing activity by herbivorous fish or invertebrates, herein inadvertently allowed convey of algal-origin CTX into the marine food webs (Bagnis et al., 1980; Bagnis & Rougerie, 1992; Kohler & Kohler, 1992; Rongo & van Woesik, 2013). Moreover, there were reports on potential linked between mass mortalities and or damage of benthic organisms with benthic dinoflagellates such as *Ostreopsis* (Shears & Ross, 2010; Totti et al., 2010)

The aims of this study are to investigate the diversity of benthic harmful dinoflagellates in the coral reef ecosystem of Malaysia through a molecular and morphological diagnostic. This study also emphasized on the distribution and assemblages of benthic harmful dinoflagellates on a wide range of reef substratum with

a non-destructive approach such as artificial substrates. Information gathered allowed researchers to gain more insight into the diversity and ecological niche of harmful benthic dinoflagellates in order to understand the bloom dynamics of harmful dinoflagellates in the marine benthic system. The specific objectives of this study are as below:

1. To explore the diversity of epiphytic benthic harmful dinoflagellates in the Perhentian Islands, Terengganu, Malaysia.
2. To investigate the distribution and assemblages of benthic harmful dinoflagellates with the emphasis on species-environment relationships in the fringing coral reef system of Perhentian Islands, Terengganu, Malaysia.
3. To conduct preliminary ciguatoxicity screening on potential CTX-producing *Gambierdiscus* from Perhentian Islands.

CHAPTER 2: LITERATURE REVIEW

2.1 Harmful benthic dinoflagellates

The first studies of benthic dinoflagellates started in the last century with samples first discovered in sandy sediments (Kofoid & Swezy, 1921; Herdman, 1922, 1924a, 1924b; Balech, 1956). Benthic dinoflagellates are since termed as it occurred in different types of benthic habitats ranging from sediments of beaches, intertidal flats, subtidal areas, tidepools, and are epiphytic on seaweeds, seagrass, and corals (Hoppenrath et al., 2014). The study of harmful benthic dinoflagellates intensified in the late 1970s with the discovery of benthic species which responsible for ciguatera fish poisoning (Yasumoto et al., 1977).

Progression of molecular technologies in protist systematics has help scientist to decipher and delineate the perplexity of taxonomy structure in benthic dinoflagellates, especially benthic dinoflagellates which bear various types of phycotoxins. Combination of morphology-based taxonomy and molecular phylogenetic hypotheses and character evolution in dinoflagellate has helped to revise and summarize taxonomical complex in several genera (Hoppenrath, 2017). Among the harmful benthic dinoflagellates, genus *Gambierdiscus* has been the focus for its notorious ciguatoxins which cause ciguatera fish poisoning. The other common known harmful benthic dinoflagellates were *Ostreopsis*, *Coolia*, *Prorocentrum*, and *Amphidinium.* According to AlgaeBase (Guiry & Guiry, 2017), current taxonomically accepted number of species of *Prorocentrum*, *Ostreopsis*, *Amphidinium*, and *Coolia* on the basis of listed literature were as follow, *Prorocentrum* (71), *Ostreopsis* (11)*, Amphidinium* (110) and *Coolia* (7).

Out of all the benthic dinoflagellates described, seven genera of dinoflagellates: *Vulcanodinium,* unarmoured dinoflagellates *Amphidinium*, prorocentroid dinoflagellates *Prorocentrum*, and notably the Gonyaulacalean taxa *Gambierdiscus*, *Ostreopsis*, *Coolia*, and *Alexandrium* were known to produce numerous noxious and bioactive compounds

(reviewed in Hoppenrath et al., 2014). The unarmoured dinoflagellate *Amphidinium* Claparède & Lachmann is able to produce amphidinols, polyketide metabolites with antifungal properties and a wide range of bioactive compounds (Houdai et al., 2001; Echigoya et al., 2005; Meng et al., 2010; Rhodes et al., 2010). Numerous species in genus *Prorocentrum* Ehrenberg were known to produce okadaic acid (OA) and its analogues (Murakami et al., 1982; Dickey et al., 1990; Morton et al., 1998). At least four putative species from genus *Ostreopsis* Schmidt are able to produce potent toxins of palytoxins group and the toxins can aerosolized cause mass casualties (Taniyama et al., 2003; Ciminiello et al., 2012; Crinelli et al., 2012). The genus *Coolia* Meunier produces cooliatoxin, analogues of yessotoxin (Holmes et al., 1995; Holmes, 1998; Penna et al., 2005; Aligizaki & Nikolaidis, 2006). Lastly, *Gambierdiscus* Adachi & Fukuyo produces lipid soluble ciguatoxins and also two other water-soluble toxins, maitotoxins and gamberic acid (Parsons et al., 2012). Ciguatoxins (CTXs) are polyether toxins and potent sodium channel agonist which have a similar chemical structure to brevetoxins (Nicholson & Lewis, 2006). Ciguatoxins are known to be metabolized into different forms where the toxicity can be escalated along the food web. The bioaccumulated toxins shift along through trophic interaction from primary producer to herbivorous grazing fish and end up in carnivorous fish before being consumed by human causing ciguatera fish poisoning (Lewis & Holmes, 1993; Lewis, 2001).

2.1.1 Taxonomy review of CTX producer: Genus *Gambierdiscus* and *Fukuyoa*

Until now, in the genus *Gambierdiscus* and its closely related genus *Fukuyoa*, there are currently 14 and three recognized species respectively, with the recognition of five ribotypes. Chronologically, the type species described in the genus was *Gambierdiscus toxicus* Adachi and Fukuyo (Adachi & Fukuyo, 1979) from the Gambier Islands reported by Yasumoto et al. (1977). *Gambierdiscus toxicus* was described as

large and anterior-posteriorly compressed (lenticular = lens-shaped) where the cell size was highly variable (average of 100 µm depth) (Litaker et al., 2009). The second species was described by Faust (1995) as *Gambierdiscus belizeanus* Faust where the species was isolated from coastal waters of Belize. It is distinguished from *G. toxicus* being smaller in size and heavily areolated thecal surface. In 1998, Holmes (1998) isolated and described the third species *Gambierdiscus yasumotoi* from samples collected from the fringing reef of Pulau Hantu, Singapore. *G. yasumotoi* was far more different than the previous two species by having more globular shape and smaller in size (Parsons et al., 2012).

Another three new species were described by Chinain et al. (1999a) isolated from French Polynesia as *G. polynesiensis* Chinain and Faust, *G. pacificus* Chinain and Faust, and *G. australes* Faust and Chinain. *Gambierdiscus polynesiensis* has smooth cell surface, a large triangular apical pore plate (P_o), a narrow fish-hook opening, and large, broad posterior intercalary plate (1p), which the plate 1p make up 60% of the width of hypotheca. While for *G. australes,* they are identified by broad ellipsoid apical pore plate (P_o), the 1p plate is long and narrow, make up 30% of the width of hypotheca. The third one, *G. pacificus* have four-sided apical pore plate, the 1p plate is narrow and occupied about 20% width of hypotheca (Chinain et al., 1999a).

Four species of *Gambierdiscus* were described by Litaker et al. (2009). *Gambierdiscus caribaeus* Vandersea, Litaker, Faust, Kibler, Holland, and Tester, *G. carolinianus* Litaker, Vandersea, Faust, Kibler, Holland, and Tester, *G. carpenteri* Kibler, Litaker, Faust, Holland, Vandersea, and Tester, *G. ruetzleri* Faust, Litaker, Vandersea, Kibler, Holland, and Tester. A dichotomous tree was constructed for species identification using cell size and shape, the architecture of thecal plates and cell surface morphology (Litaker et al., 2009). *Gambierdiscus caribaeus*, *G. carpenteri,* and *G. carolinianus* are anterior-posteriorly compressed and have broad 1p while *G. ruetzleri* is

the globular type. Both *G. caribaeus* and *G. carpenter* have broad 1p, have rectangular shaped 2′. They can be differentiated by the 4′′ plate as *G. caribaeus* have symmetric 4′′ while *G. carpenteri* has asymmetric 4′′. For *G. carolinianus,* it almost has similar characteristics with *G. polynesiensis* like hatchet shaped 2′ and oblique dorsal end 1p. It can be distinguished from *G. polynesiensis* by the absence of distinct fold along the juncture with 1′, 1′′ and 2′′ plate and shorter rectangular 1′′ (Litaker et al., 2009).

Gambierdiscus excentricus was described by Fraga et al. (2011) isolated from seaweed samples in the Canary Islands, Atlantic Ocean. It was described as lenticular species with smooth thecal plates and evenly distributed round to oval pores. The Po plate is ventrally displaced in relation to other described species. Another main feature of *G. excentricus* standout from the rest of the species is the high ratio (around 2.3) between the 2′/3′ and 2′/4′ suture length where other species ranges between only 1.0 and 1.6 (Fraga et al., 2011).

In 2014, another two new species were described by Fraga and Rodríguez (2014) and Nishimura et al. (2014) as *Gambierdiscus silvae*. Fraga & Rodriguez and *Gambierdiscus scabrosus* Nishimura, Sato & Adachi. *Gambierdiscus silvae* was first reported as *G.* sp. ribotype 1 by Litaker et al. (2010). Generally, *G. silvae* was very similar to *G. polynesiensis* in shape and tabulation but differs from it in lack of distinct fold formed by 1′, 1′′ and 2′′ in *G. polynesiensis* as reported in Litaker et al. (2009).

Gambierdiscus scabrosus Nishimura, Sato & Adachi was reported as *Gambierdiscus* sp. type 1 in Nishimura et al. (2013). *Gambierdiscus scabrosus* was morphologically reminiscent of *G. belizeanus* with narrow 2‴ plate, and areolated surface but the distinguishable features of *G. scabrosus* was the presence of the asymmetric shaped 3″ plate and the rectangular shaped 2′ plate (Nishimura et al., 2013).

In 2016, two species of *Gambierdiscus, Gambierdiscus balechii* Fraga, Rodriguez & Bravo (Fraga et al., 2016) from Manado, Indonesia and *Gambierdiscus*

cheloniae Smith, Rhodes & Murray (Smith et al., 2016) from Rarotonga, Cook Islands were described. *Gambierdiscus balechii* has a very ornamented theca, a hatchet-shaped second apical plate, a narrow second antapical plate, and an asymmetrical third precigular plate. Cells size range was wide from 36 to 88 µm (Fraga et al., 2016). On the other hand, *G. cheloniae* are morphologically similar to *G. pacificus, G. toxicus* and *G. belizeanus* but smaller in term of depth and length than *G. toxicus* has characteristics of hatchet shaped 2′ plate, dorsal end of 1p is pointed and relatively narrow 1p plate. The apical pore plate size was between *G. belizeanus* and *G. pacificus* (shorter and narrower) and *G. toxicus* (larger) (Smith et al., 2016).

Gambierdiscus lapillus Kretzschmar, Hoppenrath & Murray was described in Kretzschmar et al. (2017). The strain was isolated from Heron Island, Australia. *G. lapillus* cells are closer morphologically to *G. belizeanus* and *G. scabrosus* with a narrow 1p plate and heavily areolated cell surface (strong reticulate-foveate thecal ornamentation), but the distinguishing difference is the diminutive size. *G. lapillus* also differs from *G. scabrosus* due to its symmetric 4″ plate and 2′ plate differs from *G. belizeanus* (Kretzschmar et al., 2017). The most recently described species of genus *Gambierdiscus* was *G. honu* Rhodes, Smith & Murray isolated from Meyer Island, Kermadec Islands (Rhodes et al., 2017a). The characteristic morphological features of this species were smooth thecal surface, equal sized 1′′′′ and 2′′′′ plates together with its relatively small short dorsoventral length and width and the shape of the individual (Rhodes et al., 2017a).

In 2015, a new genus *Fukuyoa* was introduced by Gómez et al. (2015) to differentiate globular type and anterior-posteriorly compressed type of *Gamberdiscus* spp. A previous molecular phylogeny study of Litaker et al. (2009) showed that *F. yasumotoi* and *F. ruetzleri* formed a separate clade basal to the typical lenticular species of *Gambierdiscus.* This lead to speculation of globular type species as evolutionary

intermediates in the transitional phase between more ancestral globular morphology and lenticular shapes of *Gamberidiscus s.s.* (Litaker et al., 2009). Hence, *Gambierdiscus* sp. was characterized with lenticular shapes, highly compressed anterioposteriorly, with short-shank fishhook apical pore plate, large 2' plate, low and ascending cingular displacement and pouch-like sulcal morphology. Meanwhile, the new genus *Fukuyoa* should be applied to the globular species, slightly laterally compressed, with long-shank fishhook apical pore plate, large 1' plate, greater and descending cingular displacement, and not pouch-like vertically-oriented sulcal morphology (Gómez et al., 2015).

The introduction of genus *Fukuyoa* successfully transferred the previously globular type species described as *G. yasumotoi* Holmes and *G. ruetzleri* into the genus as *F. yasumotoi* and *F. ruetzleri* with *Fukuyoa paulensis* as the type species. The type species *Fukuyoa paulensis* Gomez, Qiu, Lopes and Lin was isolated from coasts of Ubatuba, Brazil with globular in shape and can be distinguished from *F. yasumotoi* and *F. ruetzleri* by its broader first apical plate that occupies a larger portion of the epitheca (Gómez et al., 2015).

In Dai et al. (2017) studies, the authors manifested the phylogenetic relevance of *Gambierdiscus* morphological trait characters by mapping trait on the phylogenetic tree with informative insights on the evolutionary shift of important morphological traits within the lineage (Hoppenrath, 2017). Based on *Gambierdiscus* SSU (small subunit ribosomal) phylogeny, the species of *Gambierdiscus* was claded into three major clades; X, Y, and Z based on morphologically distinct characteristics of Plates 2‴ and 2′. The monophyly of clade X which comprised of *G. polynesiensis* and *G. carolinianus* was corroborated by the broad oblique end of Plate 2‴ and hatcher-shaped 2′. Species in clade Y comprises of *G. caribaeus, G. carpenteri* and *G.* sp. type 2 have broad 2‴ ′ but pointed end and rectangular 2′. Species in clade Z hold remaining species such as *G. australes, G. belizeanus, G.* sp. ribotype 2*, G. scabrosus*, *G. balechii, G.* sp. type 5, *G.*

lapillus, G. pacificus and *G. toxicus* where the clade is likely supported by synapomorphic oblong 2‴ but broad and oblique end Plate 2‴ of *G. toxicus* showed homoplasy. Clade Z was mainly characterized by hatchet-shaped 2′ but showed variability from hatchet-shaped to rectangular (Dai et al., 2017).

2.2 Ciguatera fish poisoning

2.2.1 Ciguatera in the world versus Asia

Ciguatera fish poisoning is a foodborne illness related to phycotoxin contamination of fishes which transform from endemic to global menace through increased in reef fish trading. The disease has been noticed in the Caribbean and South Pacific as described in the literature since the 18th century, with mentions of illness consistent with ciguatera dating back to the 16th century (Halstead, 1967). Although ciguatera distributed circumtropically, it is largely confined to islands in the Pacific Ocean, the western Indian Ocean and the Caribbean Sea (Lehane & Lewis, 2000; Lewis, 2001). Islands in central Pacific, especially French Polynesia, have arguably more cases of ciguatera poisoning than other regions around the world (Lewis, 1986). To date, the annual prevalence of CFP worldwide has been estimated to be around 50,000–100,000 cases, but these statistics could be underestimated due to misdiagnosis and under-reporting (Lehane & Lewis, 2000). The epidemiology of CFP has been investigated and reviewed in several articles from different regions such as Caribbean (Tester et al., 2010; Radke et al., 2015), Pacific (Lewis, 1986; Chateau-Degat et al., 2007), and Asia (Chan, 2015a). A summary of ciguatera incidence report from 1946 to 2015 around the world was presented in Table 2.1.

Table 2.1: Reports of ciguatera incidence around the world from 1946 to 2015 (extracted from Chan, 2016).

Country	Years	Incidence per 100000 people
Hong Kong	1989-2008	1.6
Japan (Okinawa)	1997-2006	0.77
Cook Islands (Rarotonga)	1993-2006	1760
French Polynesia (Raivavae Island)	2007-2008	1400
South Pacific Islands	1998-2008	194.6 (104.3 in 1973-1983)
Montserrat	1996-2006	586
U. S Virgins Islands	2007-2011	1200
Caribbean Islands (18 countries)	2000-2010	45.2(34.2 in 1980-1990)
United States (Florida)	2000-2011	5.6

Focusing in Asia region, Chan (2015a) compiled a comprehensive ciguatera epidemiology of East Asia (China including Hong Kong and Macau (Chan, 2014, 2015b), Japan (Hashimoto et al., 1969; Yasumoto et al., 1984; Taniyama, 2008; Oshiro et al., 2010; Oshiro et al., 2011; Toda et al., 2012; Yogi et al., 2013), South Korea (Cha et al., 2007; Oh et al., 2012), North Korea and Taiwan (Hsieh et al., 2009; Liang et al., 2009; Tsai et al., 2009; Chen et al., 2010; Lin et al., 2012) and Southeast Asia (Brunei, Cambodia, East Timor (Infectious Disease Surveillance and Epidemic Preparedness Unit, 2000), Indonesia, Malaysia (Nik Khairol Reza et al., 2011), Myanmar, Philippines (de Haro et al., 2003; Azanza, 2006; Mendoza et al., 2013), Singapore (The Communicable Disease Surveillance in Singapore, 2000), Thailand (Sozzi et al., 1988; Saraya et al., 2014) and Vietnam (Dao & Pham., 2017; Gascón et al., 2003). Brunei, Cambodia, Indonesia, Myanmar, and North Korea were without any reports of ciguatera incident, whereas at least one report of ciguatera was found in remaining countries (Chan, 2015a). Among the East Asian countries, Japan and China including Hong Kong

and Macau were at the top of the list where China alone has 24 reports of ciguatera and three major outbreaks affecting more than 100 victims from 1994 to 2008, while Hong Kong has 3–117 outbreaks affecting 19–425 persons each year in 1989-2008 (Chan, 2015b).

In Japan, ciguatera was first thought to be restricted to the subtropical region of the country until the 1980s when it developed into a nationwide concern (Yasumoto et al., 1984; Toda et al., 2012). There were 99 outbreaks affecting ~477 individuals reported from Ryukyu and Amami Islands from 1930 to 1968, two-thirds of which occurred after 1950 (Hashimoto et al., 1969). Two nationwide outbreaks occurred from 1949 to 1980 and 1989 to 2010, with a total of 101 outbreaks affecting over 1000 personnel (Yasumoto et al., 1984; Toda et al., 2012). In Malaysia, ciguatera was first reported in September2010, affecting 22 members from 5 families (Nik Khairol Reza et al., 2011).

2.3 Nature of ciguatoxins (origin, structure, pharmacology)

Ciguatoxins (CTX) was first named to major toxin present in the flesh of ciguateric moray eels by Scheuer et al. (1967). The complete assignment of the stereo-chemistry structure of ciguatoxins was obtained after comparing structural elucidation of Pacific ciguatoxins and its precursor (CTX-4B) from moral eel viscera and *Gambierdiscus* culture material (Murata et al., 1989; Murata et al., 1990). Hence, it provided solid evidence on the source of ciguatera fish poisoning. Ciguatoxins were lipophilic polyether compounds with skeletal structures comprised of 13 – 14 transfused ether rings. Approximately 29 congeners have been identified from toxins precursor produced by *Gambierdiscus* (Lewis et al., 1991; Lewis & Holmes, 1993). An annotation system for ciguatoxins was proposed based on region and structural variation which can differentiate into P-CTX (Pacific), C-CTX (Caribbean) and I-CTX (Indian) (Lewis &

Holmes, 1993). Besides ciguatoxins, *Gambierdiscus* was known to produce water-soluble toxins, maitotoxins (MTXs). The toxins were considered as one of the most potent marine toxins which were of the non-proteinaceous natural compound and have a cytotoxic effect (Parsons et al., 2012). Currently, there are four types of maitotoxins been elucidated (Pisapia et al., 2017b).

There was a comprehensive study on pharmacology and mode of action of ciguatoxins on the cellular level. In general, ciguatoxins were branded as the most potent sodium channel toxins known (Lewis, 2001), which renowned for indicative of central and peripheral nervous system injury (Dickey & Plakas, 2010). Ciguatoxins and brevetoxins were similar groups of toxins known as VGSC activating toxins, which prompt membrane depolarization (Lewis, 2001). Ciguatoxins and brevetoxins selectively target and compete binding for "site 5" on voltage-gated sodium channel, spontaneously activate voltage-sensitive sodium channels (VSSC) and caused a hyperpolarising shift in the voltage-dependence of channel activation, hence force opened the sodium channels at resting membrane potentials (Nicholson & Lewis, 2006). These set off cascading cellular effects of elevation of intracellular calcium levels, induction of tetrodotoxin-sensitive leakage current in dorsal root ganglion neurons and reference therein (as reviewed in Dickey and Plakas (2010) and Nicholson and Lewis (2006)). Ciguatoxins have unique effect distinguished from brevetoxins which elicit distinctive spontaneous single-channel events in sensory neurons (Hogg et al., 1998).

2.3.1 Symptoms, diagnosis and treatment of ciguatera

CFP was generally registered with gastrointestinal, neurological and cardiovascular symptoms; however, clinical features can vary among patients from different geographical regions (Lehane & Lewis, 2000). These are mainly due to structure-activity and pharmacokinetic variations between different CTX congeners

(Lewis et al., 1991; Lewis & Sellin, 1992). The variant of clinical symptoms of reported ciguatera fish poisoning from different regions was presented by Friedman et al. (2017; Table 2). Ciguatera fish poisoning starts off with gastrointestinal symptoms (nausea, vomiting, abdominal pain, and diarrhea) which usually onset within 6–12 hrs of fish consumption and resolved spontaneously within 1–4 days (Dickey & Plakas, 2010). Neurological symptoms can be presented concurrently or after the gastrointestinal symptoms. Wide-ranges of mild to severe neurological symptoms have been reported. Mild symptoms included paresthesias (numbness or tingling) in hands and feet or oral region, metallic taste, the sensation of loose teeth, generalized pruritus (itching), myalgia (muscle pain), arthralgia (joint pain), headache and dizziness (Friedman et al., 2017). More severe symptoms involved cardiovascular problems such as bradycardia and hypertension, may progress into respiratory distress and coma, but death is uncommon in CFP (Dickey & Plakas, 2010). The pathognomonic of ciguatera fish poisoning was cold allodynia (hot-cold reversal), a delusion of temperature sensitivity in which touching cold surface gave burning sensation or a dysesthesia (abnormal sensation) (Pearn, 2001). Chronic illness in the form of neuropsychological symptoms from ciguatera fish poisoning also been reported where the patient suffered from fatigue, anxiety, depression, hysteria and memory disturbances (Friedman et al., 2017). Recurrence of symptom due to physical or dietary behaviour such as physical over-exertion, alcohol consumption, and excessive caffeine also had been reported (Dickey & Plakas, 2010).

Currently, the antidote for ciguatera fish poisoning has yet to be devised where most of the treatment only involves symptomatic and supportive care (Friedman et al., 2017). Intravenous mannitol is considered the only treatment recommended for reduction of acute neurological symptoms and prevention of chronic neurologic symptoms (Dickey & Plakas, 2010).

2.4 Ciguatoxins detection

One of the main challenges regarding CFP management was the detection of CTXs from the fish specimen with precision and sensitivity. Detection of CTXs was exorbitant and time-consuming procedures from extraction, purification, and determination of CTXs. Moreover, the reference materials such as CTXs standard are limited due to difficulty in recovery and purification of high purity CTXs standard where commercial CTXs standard did not exist. Knowing ciguatera fish poisoning was the result of simultaneous exposure to distinct CTXs congeners with different intrinsic potencies at very low concentration, setting a Maximum Permitted Level (MPL) by regulatory authorities was vital (Caillaud et al., 2010a). Example of MPL was at 0.01 ng g^{-1} P-CTX-1 equivalent toxicity for fishery product caught in Pacific using mouse bioassay (Lehane & Lewis, 2000). Hence, before the outbreak of CFP in the region, screening of CTXs in phytoplankton samples containing *Gambierdiscus* spp. was crucial for ciguatera risk assessment (Chinain et al., 1999b; Rhodes et al., 2010; Roeder et al., 2010). The Neuro-2a bioassay for the screening of ciguatera in fish has been broadly endorsed and proven to be reproducible and comprehensive in the case study (Dechraoui et al., 2005; Caillaud et al., 2012; Mak et al., 2013). The application further extends into a screening of CTXs originate from microalgae (Manger et al., 1993; Cañete & Diogène, 2008; Caillaud et al., 2009; Caillaud et al., 2010).

The methodologies for CTXs determination were collated in Caillaud et al. (2010a) from the protocol of sample preparation (fish samples and microalgal samples) to methods of determination. Sample preparation mainly consists of extraction and purification steps, these two steps differ significantly depends on the nature of the sample as well as the grade of purity of extracts required for different analysis. Current widely used methods for CTX determination include mouse bioassay, bioassay on

animal tissues, *in vitro* neuroblastoma CBA (Neuro-2a CBA), pharmacological RBA, immunological assays and analytical methods (high performance liquid chromatography coupled with spectroscopic (UV, FLD) or spectrometric (MS/MS) methods (reference therein Caillaud et al. (2010a)). More recent advances on detection of CTXs include ciguatoxins rapid extraction method (CREM) developed by Lewis et al. (2016) coupled with new functional bioassay that detects intracellular calcium changes in response to sample addition in SH-SY5Y cells (Lewis et al., 2016; Coccini et al., 2017). SH-SY5Y cells are human brain-derived cell line applied to explore the mechanisms of neurotransmission and nociception, it was considered as a new CBA model of a neuroblastoma cell line of human origin which gave a more realistic physio-pathological response of CTXs in human compared to murine Neuro-2a cells (Coccini et al., 2017). Another novel model for CTXs determination was presented by Martin-Yken et al. (2018) using engineered yeast strains which CTXs exposure activate calcineurin signalling pathway. Besides laboratory detection, a field detection devices of ciguatoxins by *Gambierdiscus* using solid phase adsorption toxin tracking (SPATT) was demonstrated by Roué et al. (2018) highlighted the suitability of the SPATT technology for routine in-situ monitoring for ciguatera risk assessment.

The example of official protocols for CTX detection in fish was by the Food and Drug Administration (FDA) of the United States. The FDA's CTX testing protocol utilized two-tiered protocol involving: (1) *in vitro* neuroblastoma (N2a) cell assay as semi-quantitative toxicity screening and (2) LC-MS/MS for molecular confirmation of CTX (Friedman et al., 2017). The two-tiered protocols were integrated with species identification of fish remnants through DNA barcoding, which allows the official to regulate fish consumption (Schoelinck et al., 2014). To date, no rapid and cost-effective CTX-testing product was commercially available which provide reliability or accuracy in detection.

2.4.1 Distribution and toxicity of *Gambierdiscus* and *Fukuyoa*

The reviews of the toxicity and distribution of all described species of *Gambierdiscus* and *Fukuyoa* were presented in Table 2.2. The compilation mainly focused on reported isolates from different localities and regions together with toxicity data using numerous methods comprises of neuroblastoma assay (Neuro-2a), receptor binding assay, mouse bioassay, erythrocyte lysis assay, fluorescent calcium flux assay, LC-MS/MS, LC-LRMS/MS.

Table 2.2: Compilation of distribution together with toxicity data available of ciguatoxins producer, *Gambierdiscus* and *Fukuyoa* species. "+" indicates positive, "-" indicates a negative result, "+/-" indicates mixed results from different strains in the same locality, "ND" no data available

Species	Geographical distribution	Region	Toxicity data		Reference for toxicity
			Ciguatoxic (Ciguatoxins)	Cytotoxic (Maitotoxins)	
G. toxicus	Tahiti, French Polynesia	SW Pacific	+/-	+	Chinain et al. (1999a); Chinain et al. (2010a); Pisapia et al. (2017b),
	La Reunion Island	Indian Ocean	-	+	Chinain et al. (2010a)
G. pacificus	Society Islands, French Polynesia	SW Pacific	+	+	Roeder et al. (2010); Holland et al. (2013); Pisapia et al. (2017a)
	Rarotonga, Cook Islands	SW Pacific	-	+/-	Rhodes et al. (2014); Munday et al. (2017)
	Hao/Moruroa/Tubuai, French Polynesia	SW Pacific	+/-	ND	Chinain et al. (2010a)
	Marakei, Republic of Kiribati	Central Pacific	+	ND	Xu et al. (2014)
	Malaysia	West Pacific	+	+	Caillaud et al. (2011); Pisapia et al. (2017b)
G. lapillus	Heron Islands, Australia	SW Pacific	-	+	Kretzschmar et al. (2017)

Table 2.2 continued

Species	Geographical distribution	Region	Toxicity data		Reference for toxicity
			Ciguatoxic (Ciguatoxins)	Cytotoxic (Maitotoxins)	
G. balechii	Manado, Indonesia	West Pacific	+	+	Fraga et al. (2016); Pisapia et al. (2017a); Pisapia et al. (2017b)
	Perhentian Islands, Malaysia	West Pacific	-	ND	Dai et al. (2017)
	Marakei, Kiribati	SW Pacific	+	ND	Dai et al. (2017)
G. cheloniae	Rarotonga Island, Cook Islands	SW Pacific	-	+/-	Smith et al. (2016); Munday et al. (2017)
G. scabrosus	Kochi, Japan	West Pacific	+	+/-	Pisapia et al. (2017a)
G. belizeanus	St. Barthelemy, Collectivity of France	Caribbean	+	+	Chinain et al. (2010a); Roeder et al. (2010); Holland et al. (2013); Lewis et al. (2016); Pisapia et al. (2017b); Litaker et al. (2017)
	St. Maarten, Collectivity of France	Caribbean	+	ND	Litaker et al. (2017)
	Florida Keys, USA	North Atlantic	+	+	Holland et al. (2013); Litaker et al. (2017); Pisapia et al. (2017b)
	St. Thomas, US Virgin Islands	Caribbean	+	+	Holland et al. (2013); Litaker et al. (2017); Pisapia et al. (2017b)

Table 2.2 continued.

Species	Geographical distribution	Region	Toxicity data		Reference for toxicity
			Ciguatoxic (Ciguatoxins)	Cytotoxic (Maitotoxins)	
	Southwater Cay, Belize	Caribbean	+	ND	Litaker et al. (2017)
	Turks and Caicos	North Atlantic	+	ND	Litaker et al. (2017)
G. honu	Kermadec Island, New Zealand	SW Pacific	-	+/-	Munday et al. (2017); Rhodes et al. (2017a)
	Rarotonga, Cook Islands	SW Pacific	-	+	Munday et al. (2017); Rhodes et al. (2017a)
G. caribaeus	Hawaii, USA	Central Pacific	+/-	+	Holland et al. (2013); Pisapia et al. (2017a); Lewis et al. (2016); Pisapia et al. (2017b)
	Florida, USA	North Atlantic	ND	+	Holland et al. (2013); Pisapia et al. (2017b)
	Florida Keys, USA	North Atlantic	+	+	Holland et al. (2013); Litaker et al. (2017); Pisapia et al. (2017b)
	US Virgin Islands, USA	Caribbean	ND	+	Holland et al. (2013)
	Flower Garden Banks National Marine Sanctuary, Gulf of Mexico	North Atlantic	ND	+	Holland et al. (2013)

Table 2.2 continued.

Species	Geographical distribution	Region	Toxicity data		Reference for toxicity
			Ciguatoxic (Ciguatoxins)	Cytotoxic (Maitotoxins)	
	Carrie Bow Cay/Norval Cay/Southwater Cay/Twins Cay, Belize	Caribbean	+/-	+	Holland et al. (2013); Lewis et al. (2016); Litaker et al. (2017); Pisapia et al. (2017b)
	Grand Cayman Islands	Caribbean	+	+	Lartigue et al. (2009); Roeder et al. (2010); Litaker et al. (2017); Pisapia et al. (2017b)
	Ocho Rios, Jamaica	Caribbean	ND	+	Holland et al. (2013)
	Cancun, Mexico	Caribbean	+	+	Holland et al. (2013); Litaker et al. (2017); Pisapia et al. (2017b)
	Tahiti, French Polynesia	SW Pacific	+	ND	Roeder et al. (2010)
	Dry Tortugas, Gulf of Mexico	North Atlantic	ND	+	Holland et al. (2013)
	Gulf of Thailand, Thailand	West Pacific	+	+	Tawong et al. (2016)
G. carpenteri	Hawaii, USA	Central Pacific	+	+	Holland et al. (2013); Lewis et al. (2016); Pisapia et al. (2017a); Pisapia et al. (2017b)
	New South Wales, Australia	SW Pacific	-	-	Munday et al. (2017)

Table 2.2 continued.

Species	Geographical distribution	Region	Toxicity data		Reference for toxicity
			Ciguatoxic (Ciguatoxins)	Cytotoxic (Maitotoxins)	
	Aruba	Caribbean	+	ND	Litaker et al. (2017)
	Carrie Bow Cay/Southwater Cay, Belize	Caribbean	+/-	+	Roeder et al. (2010); Holland et al. (2013); Lewis et al. (2016); Litaker et al. (2017); Pisapia et al. (2017b)
	Ocho Rios, Jamaica	Caribbean	+	+	Holland et al. (2013); Litaker et al. (2017); Pisapia et al. (2017b)
	Cancun, Mexico	Caribbean	+	ND	Litaker et al. (2017)
	Flower Garden Banks National Marine Sanctuary, USA	North Atlantic	+	+	Holland et al. (2013); Litaker et al. (2017); Pisapia et al. (2017b)
	Guam, USA	North Pacific Ocean	+	+	Roeder et al. (2010); Holland et al. (2013)
	Dry Tortugas, Gulf of Mexico	North Atlantic	ND	+	Holland et al. (2013)
G. australes	Hawaii, USA	Central Pacific	+/-	+	Roeder et al. (2010); Holland et al. (2013); Lewis et al. (2016); Pisapia et al. (2017a); Pisapia et al. (2017b)

Table 2.2 continued.

Species	Geographical distribution	Region	Toxicity data		Reference for toxicity
			Ciguatoxic (Ciguatoxins)	Cytotoxic (Maitotoxins)	
	Canary Islands, Spain	NE Atlantic	+	+	Pisapia et al. (2017a); Pisapia et al. (2017b)
	Rarotonga, Cook Islands	SW Pacific	-	+	Rhodes et al. (2014); Munday et al. (2017)
	Kermadec Islands, New Zealand	SW Pacific	-	+/-	Munday et al. (2017); Rhodes et al. (2017b); Rhodes et al. (2017c)
	Mangareva/Moruroa/Raivavae/Tubuai, French Polynesia	SW Pacific	+/-	+	Chinain et al. (1999a); Chinain et al. (2010a)
	Kochi, Japan	North Pacific	+	+	Nishimura et al. (2013); Pisapia et al. (2017b)
G. excentricus	Canary Islands, Spain	NE Atlantic	+	+	Fraga et al. (2011); Pisapia et al. (2017a)
	Florida, USA	North Atlantic	+	+	Litaker et al. (2017); Pisapia et al. (2017b)
	Southern Gulf of Mexico	North Atlantic	ND	+	Pisapia et al. (2017b)
	Rio de Janeiro, Brazil	South Atlantic	ND	+	Pisapia et al. (2017b)

Table 2.2 continued.

Species	Geographical distribution	Region	Toxicity data		Reference for toxicity
			Ciguatoxic (Ciguatoxins)	Cytotoxic (Maitotoxins)	
G. silvae	Canary Islands, Spain	NE Atlantic Ocean	+	+	Pisapia et al. (2017a)
	Curacao	Caribbean	+	ND	Litaker et al. (2017)
	Brazil	South Atlantic	ND	+	Pisapia et al. (2017b)
G. polynesiensis	Rarotonga, Cook Islands	SW Pacific	+	+/-	Rhodes et al. (2014); Munday et al. (2017)
	Mangareva/Raivavae/ Tuamotu Archipelago/ Tubuai Island, French Polynesia	SW Pacific	+	+	Chinain et al. (1999a); Chinain et al. (2010a)
G. carolinianus	Aruba	Caribbean	+	ND	Litaker et al. (2017)
	Ocho Rios, Jamaica	Caribbean	+	+	Litaker et al. (2017)
	St Maarten	Caribbean	+	ND	Litaker et al. (2017)
	US Virgin Islands, USA	Caribbean	ND	+	Holland et al. (2013)

Table 2.2 continued.

Species	Geographical distribution	Region	Toxicity data		Reference for toxicity
			Ciguatoxic (Ciguatoxins)	Cytotoxic (Maitotoxins)	
	Flower Garden Banks National Marine Sanctuary, Gulf of Mexico	North Atlantic	ND	+	Holland et al. (2013)
	North Carolina, USA	North Atlantic	-	+	Holland et al. (2013); Lewis et al. (2016); Litaker et al. (2017)
	Florida, USA	North Atlantic	ND	+	Holland et al. (2013)
	Hawaii, USA	Central Pacific	-	+	Lewis et al. (2016)
	Puerto Rico, USA	Caribbean	ND	+	Holland et al. (2013); Pisapia et al. (2017b)
	Crete, Greece	Mediterranean	+	+	Holland et al. (2013), Pisapia et al. (2017a); Pisapia et al. (2017b)
	Carrie Bow Cay/Elbow Cay, Belize	Caribbean	+	+	Holland et al. (2013); Lewis et al. (2016)
	Dry Tortugas, Gulf of Mexico	North Atlantic	ND	+	(Holland et al. (2013); Lewis et al. (2016)
	Mexico	North Atlantic	ND	+	Holland et al. (2013)

Table 2.2 continued.

Species	Geographical distribution	Region	Toxicity data		Reference for toxicity
			Ciguatoxic (Ciguatoxins)	Cytotoxic (Maitotoxins)	
G. sp. ribotype 2	Martinique	Caribbean	+	+	Lartigue et al. (2009); Litaker et al. (2017); Pisapia et al. (2017b)
	Puerto Rico, USA	Caribbean	+	+	Holland et al. (2013); Lewis et al. (2016); Litaker et al. (2017); Pisapia et al. (2017b)
	US Virgin Islands	Caribbean	+	+	Holland et al. (2013); Litaker et al. (2017)
	St Maarten, Collectivity of France	Caribbean	+	+	Lewis et al. (2016); Litaker et al. (2017); Pisapia et al. (2017b)
	Southwater Cay, Belize	Caribbean	+	+	Litaker et al. (2017); Pisapia et al. (2017b)
G. sp. type 2	Kochi, Japan	North Pacific	-	-	Nishimura et al. (2013)
G. sp. type 3	Wakayama, Japan	North Pacific	-	+/-	Nishimura et al. (2013)
G. sp. type 4	Marakei, Kiribati	SW Pacific	+	ND	Xu et al. (2014)
G. sp. type 5	Marakei, Kiribati	SW Pacific	+/-	ND	Xu et al. (2014); Dai et al. (2017)

Table 2.2 continued.

Species	Geographical distribution	Region	Toxicity data		Reference for toxicity
			Ciguatoxic (Ciguatoxins)	Cytotoxic (Maitotoxins)	
F. yasumotoi	Pulau Hantu, Singapore	West Pacific	ND	+	Holmes (1998)
F. paulensis	Northland, New Zealand	South Pacific	-	-/+	Munday et al. (2017)
	Formentera Islands	Mediterranean	+	+	Laza-Martínez et al. (2016)
	Ubatuba, Brazil	South Atlantic	-	ND	Gómez et al. (2015)
F. ruetzleri	Carrie Bow Cay/Southwater Cay, Belize	Caribbean	+	+	Roeder et al. (2010); Lewis et al. (2016); Litaker et al. (2017); Pisapia et al. (2017b)
	North Carolina, USA	North Atlantic	+	+	Holland et al. (2013); Litaker et al. (2017)
	Flower Garden Banks National Marine Sanctuary, USA	North Atlantic	+	+	Litaker et al. (2017); Pisapia et al. (2017b)

2.5 Ciguatera food webs

The food chain transfer of ciguatoxins was first hypothesised by Randall (1958) which the author noticed few premises in ciguatera: (1) toxicity of fish are associated with their dietary, (2) large predaceous species are most poisonous, (3) herbivorous and detritus-feeding fishes may be poisonous, (4) the origin of ciguatoxins may come from algal origin (primary succession in ecological succession). Helfrich and Banner (1963) further strengthened the hypothesis and demonstrated that the key of transfer lies on the toxins acquired by dietary exposure and persisted in the vector as moving up the trophic level through predation without lethal effect on the vectors. The ingress of ciguatoxins into marine food webs through herbivores fish which grazed on macrophytes and turf algae resides with ciguatoxins producer, *Gambierdiscus* was later discovered by Yasumoto et al. (1977). Lewis and Holmes (1993) laid out factors influencing the concentration of ciguatoxins, including the rate of dietary intake, efficiency of assimilation, degree, and nature of toxin biotransformation, the rate of depuration and growth rate of fish. The ciguatera food web was further refined in Cruz-Rivera and Villareal (2006) with the amalgamation of other elements such as differential epiphytism of benthic dinoflagellate and macroalgal palatability in order to comprehend the flux of ciguatoxins through marine food webs.

The ciguatoxins vector can be differentiated into three groups: mesograzer (invertebrates), herbivorous/corallivorous and carnivorous/piscivorous. Among these three groups, the detection and transfer of toxins of the herbivorous/corallivorous and carnivorous/piscivorous vector were well-documented (Lewis, 2001; Chan et al., 2011; Mak et al., 2013). Example of common ciguatoxic fish species was presented in Friedman et al. (2017) and a catalogue of ciguateric fish (Laurent et al., 2005). The abundance and potent assemblages of ciguatoxin congeners in finfish occupying higher trophic level are due to assimilation and biotransformation of ciguatoxins through

multiple trophic levels. According to Lewis and Holmes (1993), the precursor of P-CTX-1 (dominant ciguatoxins in carnivorous fish) originated from *Gambieridiscus* which produced a less-oxidised form of ciguatoxins (CTX-4B), as it went through trophic interaction of marine food webs, the potency amplified up to 10-fold more toxic than its precursor. Mak et al. (2013) investigated the distribution, transfer and trophic magnification of Pacific ciguatoxins using food webs component of coral reef systems in the Republic of Kiribati showed a weak but significant positive correlation between Pacific ciguatoxins concentration in fish and their estimated trophic level ($\delta^{15}N$ measure). Another study on ciguatoxins trophic transfer implication using mullet (a second trophic level in Pacific coral reef ecosystems) as model organism by dietary exposure to *Gambierdiscus* pellet showed that no accumulation of toxin in mullet and further proposed that the time-dependent transformation of oxopene ciguatoxins may be necessary for the concentration of ciguatoxins through higher trophic levels (Ledreux et al., 2014).

Other than teleost fish, elasmobranchs such as shark, reef apex-predators also alleged to be ciguatera vector due to high-order in trophic level. Meyer et al. (2016) found that no detectable CTXs presence in liver and muscle of shark species from Australia waters. However, Diogène et al. (2017) discovered the presence of ciguatoxins in bull sharks that caused poisoning and death of 11 people in Madagascar in 2013. The findings included identification of two new I-CTX analogues, the first observation of gambierdic acid D in shark flesh other than in *Gambierdiscus* cell, and report of CTX concentration well exceed the guidance level concentration of P-CTX set by FDA (Diogène et al., 2017). This further strengthened Randall's hypothesis that apex-predator such as shark will be most poisonous and enlisted sharks as high-risk ciguatera vector.

The role of mesograzers such as marine invertebrates as sink and vectors of ciguatoxins was confirmed in giant clams (*Tridacna maxima*) (Roué et al., 2016), trochus (*Tectus niloticus)* (Darius et al., 2017), starfish (*Ophidiaster ophidianus* and *Marthasterias glacialis)* (Silva et al., 2015). This also confirmed that the ciguatera-like intoxication attributed to consumption of marine invertebrates that were documented in several PICTs (Pacific Island Countries and Territories) (Chinain et al., 2010b; Rongo & van Woesik, 2011; Pawlowiez et al., 2013). Giant clams and trochus are considered local delicacy in the South Pacific and are extensively consumed (Pawlowiez et al., 2013). While giant clam has a passive accumulation of ciguatoxins due to partial filter-feeding behaviour (Roué et al., 2016), trochus and starfish are active grazers which feed on macroalgae or turf algae containing *Gambierdiscus* population (Silva et al., 2015; Darius et al., 2017). Despite cells of *Gambierdiscus* can induce severe effects on behaviour and survival of brine shrimp and marine copepod, making them more susceptible to predation. The role of zooplankton in ciguatoxins transfer is less clear as there is no bioaccumulation data to ascertain the transfer of the toxins (Lee et al., 2014, Neves et al., 2017).

2.6 Environmental factors affecting the population of harmful benthic dinoflagellates in coral reefs

The five genera comprises of *Gambierdiscus, Ostreopsis*, *Coolia*, *Prorocentrum,* and *Amphidinium* have been heavily featured in benthic dinoflagellate assemblages in natural substrate are due to the fact that these organisms commonly form epiphytic communities associated with coral reefs and most of the genera have been reported in the ciguatera endemic areas (Ballantine et al., 1985; Carlson & Tindall, 1985; Bomber & Aikman, 1989; Bourdeau et al., 1995; Faust, 1995). Similar to planktonic dinoflagellates, the benthic dinoflagellates dynamics also influenced by environmental

changes and seasonality such as light (depth), water temperature, salinity, nutrients and hydrodynamic, but with the additional factors of the benthic substratum due to their epiphytic nature.

Depth distribution

One of the key interest in understanding the distribution of harmful benthic dinoflagellates in coral reef environments is the depth distribution. Depth distribution of benthic dinoflagellates has been recorded in numerous study across the different region such Pacific Ocean (Richlen & Lobel, 2011), Caribbean Sea (Boisnoir et al., 2018) and Gulf of Mexico (Okolodkov et al., 2014) with contradictory results as summarized in Table 2.3.

Table 2.3: A summary of the depth-related studies of harmful benthic dinoflagellates in field observations.

Genus	Location	Depth range	Depth distribution	Reference
Gambierdiscus	Caribbean Sea	0.5 – 3.0 m	Decrease with depth	Taylor (1985)
	Johnston Atoll, Pacific Ocean	2 – 13 m	Increase with depth	Richlen & Lobel (2011)
	Northern Gulf of Mexico	20 – 46 m	Detected, but no abundances stated	Tester et al. (2013)
	Gulf of Mexico	0.4 – 4	Negative correlate with depth	Okolodkov et al. (2014)
	Caribbean Sea	~ 10 m and ~ 20 m	No correlation	Loeffler et al. (2015)
	Caribbean Sea	Sub-surface to 20 m	Higher in shallow	Boisnoir et al. (2018)
Ostreopsis	Northern Adriatic Sea	0.5 – 10 m	Decrease with depth	Totti et al. (2010)
	Johnston Atoll, Pacific Ocean	2 – 13 m	Decrease with depth	Richlen & Lobel (2011)
	Mediterranean Sea	0.5 – 20 m	Decreases with depth	Cohu & Lemee (2012)
	Mediterranean Sea	0.5 – 3 m	Decrease with depth	Cohu et al. (2013)

Table 2.3 continued

Genus	Location	Depth range	Depth distribution	Reference
Ostreopsis	Gulf of Tunis	0.5 – 3 m	Decrease with depth	Hachani et al. (2018)
	Caribbean Sea	0 – 20 m	Decrease with depth	Boisnoir et al. (2018)
Prorocentrum	Johnston Atoll, Pacific Ocean	2 – 13 m	Increase with depth	Richlen & Lobel (2011)
	Mediterranean Sea	0.5 – 20 m	Increase with depth	Cohu & Lemee (2012)
	Gulf of Mexico	0.4 – 4	Increase with depth	Okolodkov et al. (2014)
	Gulf of Tunis	0.5 – 3 m	Decrease with depth	Hachani et al. (2018)
	Caribbean Sea	Sub-surface to 20 m	Decrease with depth	Boisnoir et al. (2018)
Coolia	Mediterranean Sea	0.5 – 20 m	Increase with depth	Cohu & Lemee (2012)
	Gulf of Tunis	0.5 – 3 m	Decrease with depth	Hachani et al. (2018)
Amphidinium	Johnston Atoll, Pacific Ocean	2 – 13 m	No correlation	Richlen & Lobel (2011)
	Gulf of Mexico	0.4 – 4	Negative correlate with depth	Okolodkov et al. (2014)
	Caribbean Sea	Sub-surface to 20 m	Positive correlate with depth	Boisnoir et al. (2018)

Generally, the field observation of depth distribution of benthic dinoflagellates was not homogenous except for *Ostreopsis* which recorded a general decreasing trend in abundances with depth. Most of the author relates this observation with the potential effect of irradiance as *Ostreopsis* blooms mainly develop in shallow waters (Accoroni & Totti., 2016), but with the exception of the shallow site with high hydrodynamics (Totti et al., 2010). The mixed observation in the depth distribution of benthic dinoflagellates is mainly due to numerous environmental parameters changes accordingly with depth such as salinity, hydrodynamic action, temperature, and irradiances. Besides, availability of substratum in different area or depth and the inherent weakness in the

quantification of abundances from different natural substrates may result in contradict observation in the depth distribution of benthic dinoflagellates.

Water temperature/Seasonality

The role of temperature and seasonal pattern in population dynamics of benthic dinoflagellates has been well studied by several researchers throughout different region, particularly genus *Gambierdiscus* (Bomber et al., 1988a; Bagnis et al., 1990; Morton et al., 1992; Hokama et al., 1996; Turquet et al., 1998; Chinain et al., 1999b; Chateau-Degat et al. 2005) and *Ostreopsis* (as reviewed in Accoroni & Totti, 2016 and references therein). Although conflicting results were observed, most authors relate it to the species-specific/strain-specific interaction with the temperature or other driving factor being prominent. This is further supported by more recent laboratory experiment of different species/strains which resulted different optimum growth temperature such as *Gambierdiscus* (Kibler et al., 2012, 2015, 2017; Xu et al., 2016; Sparrow et al., 2017), *Ostreopsis* (Guerrini et al., 2010; Pezzolesi et al., 2012; Scalco et al., 2012; Yamaguchi et al., 2012; Tawong et al., 2015), *Coolia* (Larsson et al., 2019), *Prorocentrum* (Nascimento et al., 2005; Vale et al., 2009; López-Rosales et al., 2014). The general trend of the role of temperature in population dynamic of *Gambierdiscus* and *Ostreopsis* were well defined. For *Gambierdiscus*, the population likely to peak around dry season or summer when water temperature was higher and between 24–30°C in tropical areas (Chinain et al., 1999b; Jean Turquet et al., 2000) and *Gambierdiscus* cell abundances was positively correlate with temperature in Caribbean (Tester et al., 2010) whereas exception was recorded in temperate area which abundances peak when temperature below 20°C (Kohli et al., 2014). For *Ostreopsis*, the proliferation needs relatively high temperature which coincides with the warming of sea surface temperature, especially in a temperate area such as the Mediterranean Sea. *Ostreopsis* blooms were recognized as

the summer event although the peaks can occur from spring to autumn with water temperature ranged from 9 to 30°C and with certain inter-annual variability (Accoroni & Totti, 2016). Summary of maximum proliferation period, temperature and abundances of benthic dinoflagellates recorded in field observation around the world were compiled in Table 2.4.

Table 2.4: Summary of maximum proliferation period, temperature and abundances of benthic dinoflagellates recorded in field observation; data not explicated in the main text of the references, wherever possible, have been extrapolated from tables or figures"-" mean no data stated in reference. "ww"; wet weight; "fw": fresh weight.

Genus	Region	Maximum proliferation period	Temperature (°C)	Abundances (maximum)	References
Gambierdiscus	Florida Keys	-	29	-	Bomber (1985)
	Caribbean	-	-	-	Carlson & Tindall (1985)
	Queensland, Australia	September	Below 22	-	Gillespie et al. (1985)
	Puerto Rico	October to November	29 to 30	Above 2000 cells g^{-1}	Ballantine et al.(1988)
	Florida Keys	September	30	4774 cells g^{-1}	Bomber et al. (1988a)
	French Polynesia	October, April (begin and end of hot season)	28.2 to 30.9	4992 cells g^{-1}	Chinain et al. (1999b)
	Mayotte, Indian Ocean	October	32	6×10^5 cells g^{-1}	Jean Turquet et al. (2000)

Table 2.4 continued

Genus	Region	Maximum proliferation period	Temperature (°C)	Abundances (maximum)	References
	Cuba	March to July	26.4 to 30.8	10^4 - 10^5 cell g^{-1}	Delgado et al. (2005)
	French Polynesia	-	-	876.1 per g^{-1}	Chateau-Degat et al. (2005)
	Hawaii	-	25.4 to 26.6	$> 1 \times 10^4$ cells m^{-2}	Parson et al. (2010)
	Jeju Island, Korea	October	21.0 to 23.6	4,871 cells g^{-1} wet ww	Kim et al. (2011)
	Jeju Island, Korea	September and June	20 to 25	Average 7.351 cells g^{-1}	Shah et al. (2013); Shah et al. (2014)
	Australia	May	16.5 to 17	8,255 cells g^{-1} wet ww	Kohli et al. (2014)
	Gulf of Mexico	May	25.5 to 29.2	9988 cells g^{-1}	Okolodkov et al. (2014)
	Canary Islands, North Atlantic Ocean	May to July	More than 20	4938 cells g^{-1} ww	Rodríguez et al. (2017)

Table 2.4 continued

Genus	Region	Maximum proliferation period	Temperature (°C)	Abundances (maximum)	References
	Red Sea	February and May	29.5	< 40 cells g^{-1} ww	Catania et al. (2017)
	Perhentian Islands, Malaysia	April	-	1200 cells 100 cm^{-2}	Yong et al. (2018)
	Japan	November (Autuum)	22 to 27	232.2 cells g^{-1} ww	Nishimura et al. (2018)
	Guadeloupe, Caribbean Sea	September (Wet season)	above 30	113 cells·g^{-1} fw	Boisnoir et al. (2018)
	Lesser Antilles, Caribbean Sea	September	29	301 cells g^{-1} ww	Boisnoir et al. (2019)
	Wakasa Bay, Japan	October	~ 20	262 cells g^{-1} ww	Nakada et al. (2018)
Ostreopsis	Florida Keys	-	Less than 26	3.0×10^2 cells g^{-1} fw	Bomber (1985)
	French Polynesia	-	-	4.0×10^3 cells g^{-1} fw	Bagnis et al. (1985)
	Caribbean	-	-	2.1×10^4 cells g^{-1} fw	Carlson & Tindall (1985)

Table 2.4 continued.

Genus	Region	Maximum proliferation period	Temperature (°C)	Abundances (maximum)	References
	Puerto Rico	July to September	29.5 to 30	~ 1.5×10^3 cell g^{-1}	Ballantine et al. (1988)
	Tyrrhenian Sea	August – early October	24.5-28	800 cells L^{-1}	Tognetto et al. (1995)
	New Zealand	March	22.1	1095.0 cells g^{-1} fw	Chang et al. (2000)
	NW Mediterranean	July	24	5.9×10^5 cells g^{-1} fw	Vila et al. (2001)
	Cuba	-	-	Below 10^3 cell.g^{-1}	Delgado et al. (2005)
	Gulf of Tunis	August to September	20 to 27	3.6×10^5 cells 100 g^{-1} ww	Turki (2005)
	North Aegean coast	September to October	13.9 to 29.7	4.05×10^5 cells g^{-1} fw	Aligizaki & Nikolaidis (2006)
	Gulf of Mexico	-	-	1202 cells g^{-1}	Okolodkov et al. (2007)
	Ligurian Sea	July to August	26 to 30	~ 2.5×10^6 g^{-1} fw	Mangialajo et al. (2008)

Table 2.4 continued

Genus	Region	Maximum proliferation period	Temperature (°C)	Abundances (maximum)	References
	New Zealand	February	17 to 18	1.4×10^6 g^{-1} ww	Shears & Ross (2009)
	Sea of Japan	Late September	9	1×10^4 cells g^{-1} dry weight	Selina & Orlova (2010)
	Nothern Adriatic Sea	October	16.8 to 21.8	1.7×10^6 cells g^{-1} fw	Totti et al. (2010)
	Northern Adriatic Sea	September	22.7 to 22.9	1.3×10^6 cells g^{-1} fw	Accoroni et al. (2011)
	North-western Mediterranean and Northern Adriatic Sea	October	20.4	7.2×10^6 cells g^{-1} fw	Mangialajo et al. (2011)
	Gulf of Lions and Ligurian Coasts	July	23 to 27.5	8.54×10^6 cells g^{-1} fw	Cohu et al. (2013)
	Sea of Japan	September	17.2 to 21	2.3×10^5 cells g^{-1} dry weight	Selina et al. (2014)
	Catalan Sea	July to August	28.7	~ 1.4×10^6 cells g^{-1} fw	Carnicer et al. (2015)

Table 2.4 continued

Genus	Region	Maximum proliferation period	Temperature (°C)	Abundances (maximum)	References
	Perhentian Islands, Malaysia	June	32	3.4×10^4 cells 100 cm^{-2}	Yong et al. (2018)
	Guadeloupe, Caribbean Sea	September (Wet season)	26.8 to 31.4	1669 cells g^{-1}fw	Boisnoir et al. (2018)
	Lesser Antilles, Carribean Sea	August (Dry season)	28 to 29	1×10^5 cells g^{-1}	Boisnoir et al. (2019)
Prorocentrum	Florida Keys	-	More than 26	-	Bomber (1985)
	NW Mediterranean	July	22	Around 10^3 to 10^4 cells/g fw	Vila et al. (2001)
	Gulf of Tunis	September to November and May to June	20 to 27	1.6×10^5 cells. 100 g^{-1} ww	Turki (2005)
	Cuba	-	-	10^4 - 10^5 cell.g^{-1}	Delgado et al. (2005)
	Gulf of Mexico	May	-	2.9×10^4 cells g^{-1}	Okolodkov et al. (2007)
	Gulf of Mexico	May	28 to 30	2.4×10^4 cells g^{-1}	Okolodkov et al. (2014)

Table 2.4 continued

Genus	Region	Maximum proliferation period	Temperature (°C)	Abundances (maximum)	References
	Perhentian Islands, Malaysia	September	32	1022 cells 100 cm^{-2}	Yong et al. (2018)
	Guadeloupe, Caribbean Sea	February (Dry season)	27.4	939 cells·g^{-1} fw	Boisnoir et al. (2018)
	Lesser Antilles, Carribean Sea	March	31 to 33	6.6×10^4 cells g^{-1}	Boisnoir et al. (2019)
Coolia	NW Mediterranean	April	17	Between 10^3 to 10^4 cells/g fw	Vila et al. (2001)
	Cuba	-	-	Below 10^3 cell.g^{-1}	Delgado et al. (2005)
	Gulf of Tunis	September to May	20 to 27	4×10^5 cells. 100 g^{-1} ww	Turki (2005)
	North Aegean coast	August	13.9 to 29.7	1.6×10^4 cells g^{-1} fw	Aligizaki & Nikolaidis (2006)
	Gulf of Mexico	July	-	2724 cells g^{-1}	Okolodkov et al. (2007)
	Perhentian Islands, Malaysia	April, May, January	-	62 cells 100 cm^{-2}	Yong et al. (2018)
	Guadeloupe, Caribbean Sea	September (Wet season)	29.4 to 31	60 cells·g^{-1} fw	Boisnoir et al. (2018)

Table 2.4 continued

Genus	Region	Maximum proliferation period	Temperature (°C)	Abundances (maximum)	References
	Lesser Antilles, Carribean Sea	-	-	1464 cells g^{-1}	Boisnoir et al. (2019)
Amphidinium	Florida Keys	-	27	-	Mitchell (1985)
	Gulf of Mexico	-	-	4.1×10^4 cells g^{-1}	Okolodkov et al. (2007)
	Gulf of Mexico	August	Above 20	$3.69x10^3$ cells g^{-1}	Okolodkov et al. (2014)
	Perhentian Islands, Malaysia	April	-	507 cells 100 cm^{-2}	Yong et al. (2018)
	Guadeloupe, Caribbean Sea	September (Wet season)	Above 29	31 cells·g^{-1} fw	Boisnoir et al. (2018)
	Lesser Antilles, Carribean Sea	-	-	163 cells g^{-1}	Boisnoir et al. (2019)

Substratum preference

Since the origins and food chain hypothesis for ciguatera proposed by Randall (1958), numerous ecological studies of *Gambierdiscus* have pointed out the epiphytic nature of the organism as it was strongly associated with various bottom substrate such as rock and sediment, macrophytes, seagrass denuded coral rubble, algal turf (Yasumoto et al., 1979; Yasumoto et al., 1980; Ballantine et al., 1988; Bomber et al., 1988b; Bomber & Aikman, 1989; Bomber et al., 1989; Faust, 1995). Likewise, Nakahara et al. (1996) captured the epiphytic behaviour of *Gambiediscus* cell where the cells actively swim or attach under the macroalgae for shelter during daylight hours or sensed disturbance suggest a form of evasion instinct. Other studies observed the formation of mucilage matrix over either thallus of host macroalgae or sand surface where dinoflagellates cell aggregates within the mucilage matrix (Ballantine et al., 1988; Faust, 1995; Parsons et al., 2011).

To understand the primary mechanism of toxin transfer from benthic dinoflagellates into consumers via herbivory, many studies have explored the relationship between algal host and benthic dinoflagellates. Cruz-Rivera and Villareal (2006) compiled a comprehensive review of *Gambierdiscus* abundances found from 56 algal genera, two cyanobacteria, one diatom, and one seagrass. Subsequently, a survey on substrate preference of benthic dinoflagellates was conducted based on three hundred and sixty nice macroalgal and non-algal samples collected in Hawaii with evidence of host morphologies preferences (Parson & Preskitt, 2007). Numerous studies have been looking into the host preference in laboratory condition such as Parson et al. (2011) focusing on *Gambierdiscus toxicus* with twenty fours specimen of macroalgae and Rains and Parsons (2015) experiment with the pairing of eight macroalgae with five different species of *Gambierdiscus.* The result of Rains and Parsons (2015) showed a host pairing between *G. belizeanus-Polysiphonia* and *G. belizeanus-Dictyota* with

highest growth and attachment combinations, providing hypothetical vectors in the transfer of ciguatoxins. In contrast, host preference experiment conducted between *Ostreopsis* and macroalgae found negative effects towards the benthic dinoflagellates with highest inhibitory effect observed in *Dicyota* and lowest in *Rhodymenia* host macroalgae (Accoroni et al., 2015b). Study of Ben Gharbia et al. (2017) provide new insight on species-specific allelopathic interactions between macrophytes and three harmful benthic dinoflagellates (*Ostrepsis* cf. *ovata*, *Prorocentrum lima* and *Coolia monotis*). Besides macrophytes, benthic dinoflagellates such as *Ostreopsis* are known to colonise a wide range of reef benthic substratum such as rocks, coral bubble, soft sediments and invertebrates (as reviewed in Accoroni et al., 2016 and reference therein). However, the assemblages and abundances of benthic dinoflagellates on substratum other than macroalgae are scarcely studied even though blooms of benthic dinoflagellates been recorded in non-algal substratum such as *Gambierdiscus* blooms on dead coral rubbles covered with algal turfs (Jean Turquet et al., 2000) and mat-formation of *Ostreopsis* on rocks (Totti et al., 2010).

2.7 Approach in monitoring benthic dinoflagellates

One of the aspects in CFP risk management is the estimation of abundance and population of benthic dinoflagellates especially *Gambierdiscus* in the high-risk area. Theoretically, the "bloom" of epibenthic *Gambierdiscus* may increase the flux of ciguatoxins into food webs, resulting in CFP events. With this assumption, the abundance of *Gambierdiscus* cells becomes the first indicative marker in mitigating CFP. The threshold of cell densities have been proposed regarding the potential of ciguatera outbreak using the macroalgal hosts expressed in cells g^{-1} wet weight of algae, where ~1000–10000 cells g^{-1} represented the level of concern (Litaker et al., 2010). Lobel et al. (1988) suggested that normalizing cell abundance to algal surface area (cells

cm^{-2}) would be more informative; however, most of the colonised substrates (macroalgae) possess complex morphologies that make the measurement of algal surface areas impractical. Tester et al. (2014) discussed that macrophyte sampling method evinces complication towards standardization due to inconsistency in the spatial and temporal distribution of macroalgae host and patchy distribution of benthic dinoflagellate populations.

Many studies have focused on the distribution and abundances of *Gambierdiscus* on macroalgae or other substrates by quantifying dinoflagellate cells over a surface area or wet weight of macroalgae (Ballantine et al., 1988; Lobel et al., 1988; Chinain et al., 1999b; Vila et al., 2001). However, quantifying population density over macroalgae surface area or weight has its shortcoming. Cruz-Rivera and Villareal (2006) reasoned that the assumption of algae sampled has equal palatability in the environment was fairly unrealistic due to complex survival strategies of macroalgae in the form of structural or chemical defence. Moreover, seasonality and sporadic distribution of macroalgae likely to create erraticism during measurement of cell abundance with a low number of sample replicate (Lobel et al., 1988; Tester et al., 2014). Hence, artificial substrate sampling method was introduced to overcome the flaw in macroalgae sampling method (Tester et al., 2014; Tan et al., 2015; Jauzein et al., 2016). The advantages of utilizing artificial substrates were demonstrated in the study of Tester et al. (2014) as it offered normalization of cell abundances to known surface area which allowed comparison among studies. In term of sampling effort, the artificial substrate was more promptly deployed and ease in sample processing compare to the natural substrate.

A number of alternative approaches have been introduced for macrophytes substrate methods such as fabric strips (Caire et al., 1985), test tube brushes (Bomber & Aikman, 1989), fabric tubes (Ishikawa et al., 2011), fiberglass screen (Tester et al., 2014;

Tan et al., 2015; Jauzein et al., 2016) and BEDI (Benthic Dinoflagellates Integrator) (Mangialajo et al., 2017). Among the replacement of macrophytes sampling method, fiberglass screen with fixed area as artificial substrates for estimating abundances of benthic dinoflagellates was well studied by Tester et al. (2014), it allows normalisation of cell abundances to a surface area expressed in cell/100 cm^2, comparison among locality and eliminates complication related to mass variation of macrophytes and substrate preferences. The fibreglass screen artificial substrate method was adopted and improvised by Jauzein et al. (2016) with the easy clip-in system and optimum suggestion of porosity for fibreglass screen. A different approach was taken by Mangialajo et al. (2017) wherein the device allowed quantification of abundances as cells per surface unit area of the seabed (cells mm^{-2}) or expressed as Potentially Resuspended cells per unit of volume (PR cells ml^{-1}). This approach allowed for the possibility of integrating both cells that are trapped in biofilm and suspended in surrounding water with mechanical resuspension (Mangialajo et al., 2017). Efficiency assessment of artificial substrate for *Gambierdiscus* cell monitoring was conducted by Parsons et al. (2017) and pointed out the flaw of artificial deployment on large scale due to lack of consistency correlation among cell densities on macrophytes versus artificial substrates. The authors suggested the utilisation of different methodologies should be considered and cautioned during interpreting data garnered from the deployments. All the methods mentioned above were part of the progression towards non-destructive and standardised cell monitoring effort in ciguatera risk management as well as bloom event of other harmful benthic dinoflagellates (Berdalet et al., 2017).

CHAPTER 3: METHODOLOGY

3.1 Study site

The coral-fringed Perhentian Islands Marine Park is located ~19 km off the northeastern coast of Peninsular Malaysia. The marine park consists of two main islands (Perhentian Besar and Perhentian Kecil Islands) and three small uninhabited islands (Susu Dara, Serenggih and Rawa Islands) (Fig. 3.1). Field sampling was conducted at five sampling sites: Rawa Island (5°57'41.28"N, 102°40'57.25"E), Serenggih Island (5°56'30.99"N, 102°40'3.46"E), Tokong Laut (5°57'39.49"N, 102°39'18.26"E), D. Lagoon (5°55'42.34"N, 102°43'26.78"E) and Batu Nisan (5°55'16.19"N, 102°43'40.50"E) (Fig. 3.1). Rawa Island experiences higher wave action at the windward site while the leeward site is sheltered. Serenggih Island is relatively sheltered with lower wave action. Both the reef crests are shallow (~3–5 m) and the slopes reach down to 20 m. Tokong Laut is a pinnacle island which rises straight from the ocean floor with the depth of ~25 m. D. Lagoon and Batu Nisan are located on Perhentian Kecil Island; D. Lagoon is sheltered and shallower (average depth of 5 m); Batu Nisan is relatively exposed with an average depth of 10 m.

The main temporal changes in the Perhentian Islands were driven by reversing monsoonal winds accompanied by corresponding changes in precipitation. The northeast monsoon (wet season) usually starts in early November and ends in mid-March. The southwest monsoon (dry season), begins in late May or early June and ends in September. The wet season is characterised by frequent and heavy rainfall whereas little rainfall and arid weather are typical in the dry season (Suhaila et al., 2010). The islands of Perhentian Marine Park experience heavy storms with rough sea condition during the wet monsoon that closes tourism activities.

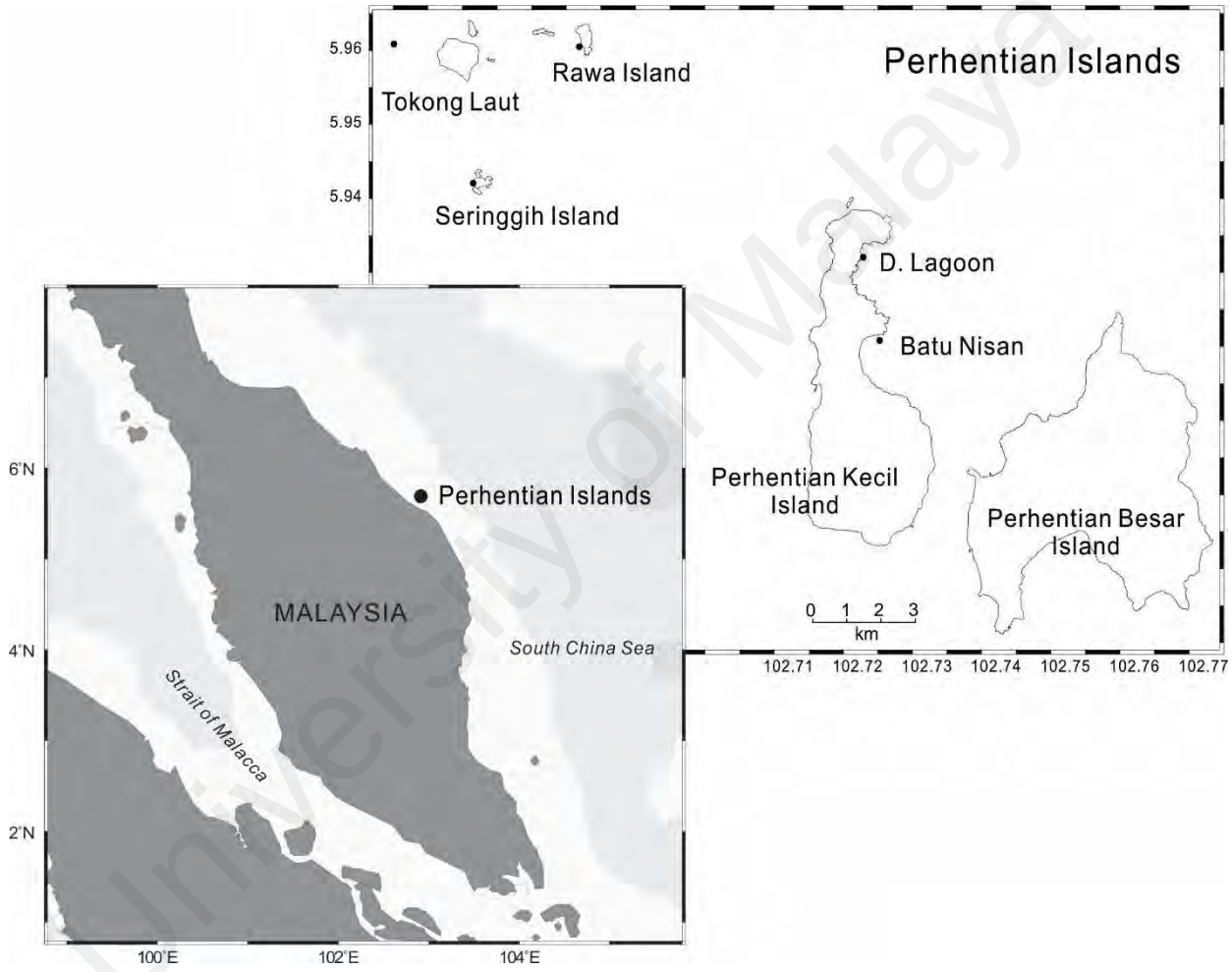

Figure 3.1: The study was conducted in the Perhentian Islands located approximately 19 km off the coast of Peninsular Malaysia. The sites selected were Rawa Island, Tokong Laut, Seringgih Island, D. Lagoon and Batu Nisan.

3.2 Sample collection, isolation and maintenance

For clonal cultures establishment, various types of macrophytes and turf algae complex which can be found on coral rubbles were collected from five sites (Rawa Island, Seringgih Island, Tokong Laut, Batu Nisan and D. Lagoon) in Perhentian Islands. Samples were agitated and sieved using 250 μm mesh sieve, the filtrate was subsequently concentrated using 20 μm mesh sieve. Single cell isolation approach was used to establish clonal cultures of epiphytic benthic dinoflagellates which obtained from various types of macrophytes and turf algae complex through micropipette technique. A single cell of benthic dinoflagellates was isolated and transferred into a 24 or 48-well tissue culture plate containing ambient syringe-filtered seawater. Clonal cultures were maintained in tissue culture wells with ES-DK medium (Kokinos & Anderson, 1995). Prior to the full establishment of cultures in larger volumes (25 mL tubes), single cell PCR was performed to obtain DNA sequences (detailed in section 4.3.2). All clonal cultures were cultivated at 25 ± 0.5 °C, 12:12 h light: dark photocycle, with a light intensity of 70 μmol photon $m^{-2}s^{-1}$. Algal cultures established in this study were deposited in the Harmful Algae Culture Collection of Bachok Marine Research Station, Institute of Ocean and Earth Sciences, UM.

3.3 Sampling design: Artificial substrate method

An artificial substrate sampling method of Tester et al. (2014) was employed in this study. Sampling was undertaken fortnightly or monthly if weather is unfavourable by scuba diving between April 2016 and May 2017. The artificial substrate consists of a piece of fibreglass window screen of 10.2 cm × 15.2 cm (porosity of 1.0 mm) in size, attached to a fishing line with a weight and a sub-surface buoy, and lifted 20-cm above the bottom floor (Tester et al., 2014). The screens were deployed by scuba, retrieved 24 h later by placing each one in a wide-mouth 1L bottle underwater. The feasibility of

artificial substrate sampling method has been highlighted in Tester et al. (2014), Jauzein et al. (2016, 2018) and Yong et al. (2018) to enable normalisation of benthic dinoflagellate abundances on a known surface area for comparative studies. This method also highlighted the non-destructive approach toward the fragile coral reef ecosystem (Tan et al. 2013; Tester et al., 2014; Berdelet et al., 2017; Yong et al., 2018).

To characterise the benthic dinoflagellates assemblages in relation to microhabitat variability in the bottom substratum, where the screens were deployed, were characterised simultaneously using a photo-quadrat method. This method utilised a waterproof digital camera mounted perpendicularly to a 0.25 m^2 quadrat (50 cm × 50 cm) to photograph the substratum at 1 m distance. The digital underwater images were then analysed for percentage coverage of various bottom substrates using CoralNet, an online repository and sources for benthic image analysis (Beijbom et al., 2015; http://coralnet.ucsd.edu). The images were uploaded and annotated with a total of 100 uniform annotation points. The annotation was based on general benthic reef community characterisation and the bottom substrates were grouped into the defined [labelset] as shown in Table 3.1.

3.3.1 Temperature and irradiance with data logger

The depths of each sampling location where the screens were deployed were recorded using a dive computer. Seawater temperatures and irradiances at the depths of 3–5 m and 10 m were recorded using HOBO Pendant temperature/light data loggers (Onset Computer Corporation, MA, USA). The data loggers were deployed on the seafloor by scuba diving and replaced during every sampling trip (fortnight) for data collection and to prevent excessive fouling. Data were downloaded using an optic USB base station and analyzed by HOBOware 2.1 (Onset Computer Corporation). Daily maximum temperature and light intensity were filtered. Light intensity data in

illuminance (lux) was converted to photosynthetic photon flux density (PPFD) by a conversion factor of 0.0185.

Table 3.1: Labelset used for annotation of benthic substratum images with respective short code and description.

Short code	Description
HC	Hard coral colonies
Invt	Other invertebrates including giant clams, corallimorph and sea anemone
Sft	Soft coral including sea feather
Spg	Sponges
Mm	Microbial mats including diatom and cyanobacteria
Sd	Fine sand and silt
Rub	Coarse rubble and rock
Fles	Upright fleshy macroalgae
Turf	Turf algal assemblages

3.3.2 Sample processing and cell enumeration

In the laboratory, the screens were shaken vigorously for 5–10 s to dislodge the attached cells. Samples were passed through a 200 µm sieve to remove detritus or particles. The filtrates were then filtered onto a 0.2-µm nylon membrane filter. The membrane filter was transferred into a 50-ml tube, filled with 30 ml of filtered seawater, and preserved with 1% acidic Lugol's iodine solution for cell enumeration.

Abundances of five benthic harmful dinoflagellates *Gambierdiscus*, *Ostreopsis*, *Coolia*, *Prorocentrum*, and *Amphidinium* were enumerated (3–5 replicate counts) at the genus-level using a Sedgewick Rafter counting chamber under a Leica DME750

compound microscope (Leica, Germany) at 100–200× magnifications. Cell abundance was expressed as [cells/100 cm^2] based on the calculation described in Tester et al. (2014).

3.4 Molecular characterization: Genomic extraction and single-cell PCR

The protocols for molecular characterization from extraction until DNA sequencing have adhered to methodology outline in Leaw et al. (2010) where nuclear-encoded ribosomal DNA genes (rDNAs) were amplified. Cell materials were harvested at exponential growth phase into a 50 mL centrifuge tube and centrifuged at 2,800 ×*g* for 10 min. The supernatant was discarded with the cell pellet retained. The cell pellet first went through lysis process with 60 µL of 10× NET lysis buffer and 10% SDS (sodium dodecyl sulfate) then extracted using cetyltrimethylammonium bromide (CTAB)(10%) and 5 µL proteinase K (20 mg mL^{-1}) which incubate at 65 °C for 1hr. Chloroform: Isoamyl alcohol (C:I; 24:1) and phenol: chloroform: isoamyl alcohol (P:C:I; 25:24:1) were used for the organic extraction phase. 700 µL of C:I was added into the CTAB mixture, vortexed and centrifuged at 10,000 rpm, 4 °C for 10 min. The upper aqueous phase was transferred into a new labelled microcentrifuge tube with 700 µL of P:C:I, vortex for 2 min and centrifuged at 10,000 rpm, 4 °C for 10 min. Extraction with an equal volume of P:C:I was repeated for 2 to 3 times to ensure eradicate of organic material. The upper clear aqueous phase was transferred into another new microcentrifuge tube with 700 µL C:I and centrifuged at 10,000 rpm, 4 °C for 10 min. During alcohol precipitation phase, the aqueous phase was transferred into a new microcentrifuge tube, subsequently mixed inversely with 500 µL of ice-cold absolute EtOH and 25 µL of 3 M sodium acetate, pH 5.0 and let stand at -20 °C for 3 hr. Thereafter, the sample was centrifuged at 13,000 rpm, 4 °C for 10 min and excess ethanol were removed by micropipette. Another 700 µL of cold 70% ethanol was added,

mixed inversely and centrifuged at 13,000 rpm, 4 °C for 10 min. Excess ethanol was removed as much as possible and DNA pellet was allowed for air-dried at room temperature before re-dissolved with 30 µL of TE buffer and deposited at -20 °C. DNA yield and quality were assessed with gel electrophoresis.

Single-cell PCR technique as detailed in Kim and Kim (2007) was adopted. A single cell was isolated with a micropipetting technique and undergo serial dilution washing with either distilled water or TE buffer. A single cell was then transferred into a PCR tube with 1.0 µL of TE buffer and proteinase K, further incubated for 42 min before PCR amplification.

3.4.1 PCR amplification, purification and sequencing

Amplification of the D8-D10 region and D1-D3 region of the large subunit (LSU) ribosomal DNA gene (rDNA) were performed using primer pair FD8 (5'-GGA TTG GCT CTG AGG GTT GGG-3') and RB (5'-GAT AGG AAG AGC CGA CAT CGA-3') (D8-D10 region) (Chinain et al., 1999a) for *Gambierdiscus*. Meanwhile, the D1-D3 region was amplified by using primer pair D1R (5'-ACC CGC TGA ATT TAA GCA TA-3') and D3a (5'-ACG AAC GAT TTG CAC GTC AG-3') for other benthic dinoflagellates (Scholin et al., 1994; Hansen et al., 2000).

For *Gambierdiscus*, PCR was carried out in a total volume of 25 µL comprised of 1× KOD FX Neo buffer, 0.4 mM deoxynucleoside triphosphate reagent (dNTPs), 0.5 U KOD FX Neo (TOYOBO, Japan), 2.5 mM of each primer and 2 µL of genomic DNA template. For other benthic dinoflagellates, PCR mixture was in a total volume of 25 µL contained 1× PCR reaction Taq buffer (Invitrogen, Life Technologies), 0.2 mM $MgCl_2$, 0.2 µM dNTPs, *Taq* DNA polymerase (0.5 unit/µL), correspondent primers and DNA product. All PCR amplification was carried out in peQSTAR Thermal Cycler (Peplab, Germany) with PCR cycle as follow: denaturation at 94 °C for 4 min, 30 cycles of 30s

denaturing at 94 °C, followed by annealing at 55 °C for 55 min, 2 min elongation at 72 °C and 10 min of final elongation at 72 ℃ .

Amplicons were electrophoresed in 1% agarose gel and run simultaneously with a 1kb DNA ladder (GeneDireX, Taiwan). The gel was stained with SYBR Safe DNA stain (Invitrogen, USA) followed by visualisation under blue light transilluminator. The amplicons were then purified by using UltraClean® PCR Clean-Up Kit (MoBio, QIAGEN, USA) or Wizard® SV Gel & PCR clean-up system (Promega, USA). DNA sequencing was carried out on an ABI 3700XL automated DNA sequencer (Applied Biosystems, USA) by a private sequencing laboratory (1st Base, Selangor, Malaysia).

3.4.2 LSU rDNA phylogenetic analyses

Nucleotide sequences obtained in this study were first inspected using Sequence Scanner v1.0 (Applied Biosystem), cleaned using BioEdit ver. 7.0.9.0 (Hall, 1999) and Clustal-X 2.0 (Thompson et al., 1997). Taxon sampling was conducted by arraying related sequences from NCBI Genbank nucleotide database with strain designation, locality, and accession number. Sequences were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) (Edgar, 2004) in EMBL-EBI and region of sequences were selected using Clustal-X 2.0 as alignment file for phylogenetic tree reconstruction, Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI).

Phylogenetic reconstruction for *Gambierdiscus, Coolia*, *Prorocentrum*, and *Amphidinium* were performed. Details of datasets and parameters used in each analysis are shown in Table 3.2. MP and ML were performed using Phylogenetic Analysis Using PAUP (PAUP* 4b10) (Swofford, 2002). In ML, the parameters were gauged based on the best model calculated by Akaike information criterion using jModelTest 2.1.10 (Darriba et al., 2012) where the best fitted evolutionary model was selected. In Bayesian

inference (BI) analyses (sequence-only information), parameters for the LSU rDNA dataset were fixed based on the same model implemented in ML analysis. Markov Chain Monte Carlo (MCMC) was used to estimate the posterior probability (PP) distribution executed using MrBayes v3.2.6 (Huelsenbeck & Ronquist, 2001). The effective sample size (ESS) value was examined in Bayesian Evolutionary Analysis Sampling Trees BEAST v1.8.4 (Drummond et al., 2012) and Tracer v1.6 (Rambaut et al., 2014). The LSU rDNA dataset (sequence-only information) was performed at selected generations per run, the sampling frequency of 100 and PP was estimated with 25% burn-in. The phylogenetic tree generated was viewed using FigTree v1.4.3, and the best-represented tree was selected and edited using CorelDraw. Pairwise genetic distances were estimated using MEGA7 (Kumar et al., 2016), the intraspecific and interspecific divergence of selected species were compiled.

Table 3.2: Detail of datasets and parameters used in LSU rDNA phylogenetic reconstruction of *Gambierdiscus*, *Coolia*, *Prorocentrum* and *Amphidinium* for maximum parsimony (MP), maximum-likelihood (ML) and Bayesian Inference (BI).

	Gambierdiscus	*Coolia*	*Prorocentrum*	*Amphidinium*
The region of Ribosomal DNA large subunit	D8-D10	D1-D3	D1-D3	D1-D3
Operational taxonomic units (OTUs)	95	33	56	38
Character	753	474	592	891
Outgroups	*F. paulensis; F ruetzleri; F. yasumotoi*	*Ostreopsis ovata*	*Karenia brevis; Brachidinium capitatum; Takayama helix*	*Heterocapsa arctica; Karlodinium armiger;*
JModelTest (best fitted evolutionary model)	GTR+I+G	TIM1+I+G	GTR+G	TIM2+G
Maximum Parsimony (MP)				
Branch swapping	Tree-bisection reconnection (TBR)	Tree-bisection reconnection (TBR)	Tree-bisection reconnection (TBR)	Tree-bisection reconnection (TBR)
Heuristic search	1000	1000	1000	1000
Boostrap	1000	1000	1000	1000

Table 3.2 continued

	Gambierdiscus	*Coolia*	*Prorocentrum*	*Amphidinium*
		Maximum likelihood (ML)		
Branch swapping	Nearest neighbor interchange (NNI)	Tree-bisection reconnection (TBR)	Tree-bisection reconnection (TBR)	Tree-bisection reconnection (TBR)
Heuristic search	100	1000	100	100
Boostrap	100	100	100	100
		Bayesian Inference (BI)		
Generation per run	3.0×10^6	1.2×10^6	1.5×10^6	2.0×10^6
Sample frequency	100	100	100	100
PP Burn in value	25%	25%	25%	25%

3.5 Morphological observations

For armoured benthic dinoflagellate, live culture samples were stained with 1% calcofluor white solution and the thecal tabulation was observed using epi-fluorescence under Olympus BX53 digital microscope with Cellsens digital image software (Olympus American, Inc., Center Valley, PA, USA).

For scanning electron microscopy (SEM), clonal cultures at exponential phase were concentrated by centrifugation at 1250 ×*g* for 10 min at room temperature using a Sorvall Biofuge Primo R (Thermo Scientific, Massachusetts, USA). The supernatant was removed and the cell pellet is suspended in 60% ethanol for 1h at 8 °C. Cells were centrifuged again and ethanol was removed. Pellet was fixed for 3h at with 5% glutaraldehyde in prepared filter seawater. The cell pellet was washed twice and fixed with 2% OsO_4 overnight. The supernatant was removed and the cell pellet was placed on a coverslip coated poly-L-lysine (molecular weight 70,000–150,000). Cells were washed with Milli-Q water for 10 min and undergo series of dehydration through graded series ethanol (10, 30, 50, 70, 90 and 3× in 100%) for 10 min each step. Samples are later critical point dried on K850 Critical Point Dryer (Quorum/Emitech, West Sussex, UK), sputter-coated with gold, and examined with a Zeiss Sigma FE (Carl Zeiss Inc., Oberkochen, Germany) or a Zeiss Ultra 55 FE (Zeiss, Jena, Germany) scanning electron microscope as described in (Luo et al., 2017).

3.6 Toxicity screening with Neuro-2a cells assay

Selected strains of *Gambierdiscus* sp. were undergone mass cultivation in 4 L volume of ES-DK medium. The culture was harvested in exponential growth phase through 10 µm mesh sieve and transferred into a 15 mL centrifuge tube. One millilitre of cell-matrix was subsampled and fixed with Lugol's solution for enumeration.

Cultures were then centrifuged at 2800 ×*g* for 10 min and the supernatant was removed before stored in -80 °C prior freeze-drying process.

The cell pellet was extracted with methanol and sonicated for 10 min then centrifuged at 10,000 rpm for 10 min. The upper layer supernatant was then transferred into rotary evaporator flask for drying. The extracts were then partitioned with 1:1 ratio of 60% methanol (MeOH) and dichloromethane (DCM). The lower DCM phase layer was then collected and evaporated with a rotary evaporator. SPE fractioning of the extract were performed using 1 g Florisil cartridge with silica gel (40-60µm) (Merck) and C18 gel (Nacalai Tesque). Prior fractioning, the column was pre-conditioning with solvent (dichloromethane). The mobile phase was dichloromethane (DCM) and methanol (MeOH) with four different ratios: DCM, DCM:MeOH (95:5), DCM:MeOH (90:10), MeOH. Each fraction was collected consecutively using SPE vacuum manifold by elution of solvent with the respective ratio. During SPE fractioning with C18 gel, only the DCM:MeOH (95:5) fraction from previous fractioning was chosen for the C18 fractioning. The mobile phase was methanol (MeOH), ultrapure water (H_2O) and dichloromethane (DCM) with five different ratios: MeOH:H_2O (1:1), MeOH:H_2O (7:3), MeOH:H_2O (9:1), MeOH and DCM. The MeOH:H_2O (9:1) fraction were collected and dried with rotary evaporator before Neuro-2a cell assay.

Neuro-2a cells (ATCC) were maintained according to Caillaud et al. (2012) in 10% fetal bovine serum (FBS) RPMI medium (Sigma) at 37 °C in a 5% CO_2 chamber (Binder, Tuttlingen, Germany). Culture medium RPMI-1640 was supplemented with 1% sodium pyruvate solution (100 mM), 1% L-glutamine solution (200 mM) and 0.5% antibiotic solution (10 mg/mL streptomycin and 1000 U/mL penicillin) (Cañete & Diogène, 2008). The Neuro-2a cell was subcultured for at least 3 to 4 generations before subjected to experiment. Trypsin solution (0.5 g/L) was used to dislodge cells from the petri dish. The cell matrix was subsampled for cell counting under hemocytometer to

check for cell conditions as well to obtain an optimum number of cells for the experiment. Prior experiments, cells were conditioned 5% FBS RPMI-1640 medium and seeded in a 96-well microplate in at an approximate density of 35,000 cells per well. Cells were incubated 24 h before exposure in the same conditions of temperature and atmosphere as described for cell maintenance (Caillaud et al., 2010b; Caillaud et al., 2012)

According to the study of Cañete and Diogène (2008), voltage-gated sodium channel (VGSC) activating toxins (in this case is ciguatoxin), ouabain and veratridine (O/V) concentration that produces around 20% mortality were used to detect the effect by causing the increase in cell mortality. In the O/V 20% mortality treatment, a positive O/V control (O/Vt) was used to determine the 100% of viability, equivalent to the 0% toxic effect. 0% viability corresponds to a 100% (toxic effect) response. The viability of cells was expressed in relation to the viability of the corresponding cell control (with or without O/V treatment) (Caillaud et al., 2012)

Ten microliters of 2 mM ouabain were added followed by an equal volume of 0.2 mM veratridine. The dichloromethane extracts retained from the SPE fractioning was diluted in different dosage and 10 µL of each designated dosage was added into the culture well containing Neuro-2a cell and incubated for 24 hours. Cell viability was estimated through MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium] assay using microplate reader under 570 ~ 630 nm absorbance.

3.7 Statistical analysis and data visualisation with R package

Data analysis and visualisation were completed using R ver. 3.5.0 (R Development Core Team, 2018). Analysis of microhabitat characterisation was performed using a community ecology package 'vegan' (Oksanen et al., 2017). Non-metric multidimensional scaling (*n*MDS) was employed to visualise the correspondence

between distinct major clusters of benthic substrates. One-way analysis of similarity (ANOSIM) was conducted to test significant difference between benthic microhabitat clusters (Clarke, 1993). SIMPER analysis helped assess the average percent contribution of microhabitat characteristics towards dissimilarity between clusters formed in *n*MDS and identified probable major contributors to differences between clusters detected in ANOSIM (Clarke, 1993).

Computation of the Bray-Curtis dissimilarity matrix on the benthic substratum percent coverage was performed prior to the cluster analysis. A heatmap of the benthic substratum coverage was generated using 'Heatplus' (Ploner, 2015) and the heat map of benthic dinoflagellate relative abundances between defined clusters were generated by 'gplots' (Warnes et al., 2016).

The distribution of benthic harmful dinoflagellates at each sampling site, in different benthic microhabitats and depths, were conceptualised through bubble plots using 'ggplot2' (Wickham, 2009). A non-parametric one-way ANOVA on rank (Kruskal-Wallis) with a Dunn's multiple comparison tests was used to test for significant differences in the data set of benthic harmful dinoflagellate assemblages against locality, temporal variability, and microhabitat clusters. Analyses were performed in GraphPad Prism 5.02 (GraphPad Inc., USA).

Canonical correspondence analysis (CCA) was used as an exploratory tool to infer the underlying relationship between the benthic dinoflagellate assemblages and environmental variables such as benthic microhabitat characteristics, depths, irradiances and temperature. Cell abundances and environmental data were log-transformed [log(x+1)] prior to CCA (to ensure the data meet the statistical assumptions of normality and linearity). The analysis was performed using the "vegan" package in R (Oksanen et al., 2017). The significance of variation in species composition (benthic dinoflagellates assemblages) explained by explanatory variables (environmental variables) was tested

using ANOVA like Monte Carlo permutation test (randomisation test) implemented in "vegan" package of R.

CHAPTER 4: RESULTS

4.1 Diversity of benthic harmful dinoflagellates in the Perhentian Islands

A total of 80 strains of epiphytic benthic dinoflagellates were isolated and established from macrophytes collected from Perhentian Islands: 54 strains of *Gambierdiscus* and 26 strains of other benthic dinoflagellates. A total of 16 species from six genera (*Coolia*, *Prorocentrum*, *Gambierdiscus*, *Gymnodinium*, *Ostreopsis*, and *Amphidinium*) were successfully identified with sequence data obtained (Table 4.1).

Four species of *Coolia*: *Coolia tropicalis* Faust (Faust, 1995), *Coolia canariensis* Fraga (Fraga et al., 2008), *Coolia malayiensis* Leaw, Lim & Usup (Leaw et al., 2010) and *Coolia palmyrensis* Karafas, Tomas & York (Karafas et al., 2015) were identified. Three species of *Gambierdiscus*: *Gambierdiscus caribaeus* Vandersea, Litaker, Faust, Kibler, Holland & Tester (Litaker et al., 2009), *Gambierdiscus pacificus* Chinain & Faust (Chinain et al., 1999a) and *Gambierdiscus balechii* Fraga, Rodriguez & Bravo (Fraga et al., 2016). A total of five species of *Prorocentrum* were identified: *Prorocentrum lima* Ehrenberg, *Prorocentrum concavum* Fukuyo (Fukuyo, 1981), *Prorocentrum mexicanum* Osorio-Tafall, *Prorocentrum fukuyoi* Murray & Nagahama (Murray et al., 2007b), *Prorocentrum emarginatum* Fukuyo (Fukuyo, 1981). Two species of *Amphidinium* were identified as *Amphidinium massartii* Biecheler and *Amphidinium operculatum* Claparède & Lachmann. One species of *Gymnodinium, Gymnodinium dorsalisulcum* Murray, de Salas & Hallegraeff (Murray et al., 2007a) and one species of *Ostreopsis, Ostreopsis* cf. *ovata* were identified molecularly. Selected sequences were included in each dataset for phylogenetic reconstruction to further verify species identity with morphological observations.

Table 4.1: Taxonomic list of epiphytic benthic dinoflagellates recorded in this study, with nucleotide sequences obtained in this study.

Genus	Species	Strain designation	LSU rDNA region
Gambierdiscus	*G. caribaeus*	BNSGam05	D8-D10
		BNSGam06	D8-D10
		DLGam03	D8-D10
		T3Gam07	D8-D10
		PRGam14	D8-D10
	G. pacificus	TLGam01	D8-D10
	G. balechii	PRGam20	D8-D10
Coolia	*C. malayensis*	SS09H3	D1-D3
	C. canariensis	BNS001	D1-D3
		SS06S8	D1-D3
	C. palmyrensis	SS09H2	D1-D3
		SS1203	D1-D3
		SS0707	D1-D3
	C. tropicalis	SS0706	D1-D3
Prorocentrum	*P. mexicanum*	BNS003	D1-D3
	P. emarginatum	SS06S7	D1-D3
		SS15S3	D1-D3
	P. lima	SS0905	D1-D3
	P. concavum	SS1201	D1-D3
	P. fukuyoi	BNS002	D1-D3
Amphidinium	*A.* cf. *massartii*	SS11H1	D1-D3
	A. operculatum	SS06S5	D1-D3
		SS09H1	D1-D3
		SS15S4	D1-D3
Gymnodinium	*G. dorsalisulcum*	SS10H1	D1-D3
		SS13H1	D1-D3
Ostreopsis	*Ostreopsis* cf. *ovata*	SS06H3	D1-D3

4.1.1 Morphological observation of epiphytic benthic dinoflagellates

Genus *Amphidinium* is dorsoventrally flattened with minute, crescent-shaped or triangular, left deflected epicone. Two species of *Amphidinium* Claparède & Lachmann, *Amphidinium operculatum* Claparede & Lachmann and *Amphidinium* cf. *massartii* Biecheler were identified in this study.

In Figure 4.1, *A. operculatum* can be easily differentiated from *A.* cf. *massartii* from the cell size. Cells of *A. operculatum* are 38.7 ± 2.8 µm long and 27.9 ± 1.4 µm wide while cells of *A.* cf. *massartii* are 12.8 ± 1.6 µm long and 12.1 ± 1.0 µm wide. Cells of *A. operculatum* are ovoid, wide and anteriorly flattened epicone and rounded hypocone with slight asymmetry (Figure 4.1A), chloroplasts are in long thin strands. Cells of *A.* cf. *massartii* are ovoid to round shape, minute epicone is crescent-shaped, flattened slightly and deflected to the left (Figure 4.1B). The chloroplast arrangement is different from *A. operculatum* where plastid radiating from the centre of the cell with several narrow lobes.

Gymnodinium dorsalisulcum (Hulburt, McLaughlin and Zahl) Murray, de Salas & Hallegraeff is oval to elongate oval and dorso-ventrally flattened (Figure 4.1C). Cell length is 29.6 ± 1.8 µm and width of 22.1 ± 2.2 µm. The epicone is longer than hypocone. The cingulum is wide, relatively deep and descending with a displacement of one cingulum width. Narrow sulcus extending onto the epicone and continuing as apical groove and reaching antapex, where it slightly widen (Figure 4.1C). Mucus production in culture was a typical observation. Pyrenoids with starch sheaths are observed.

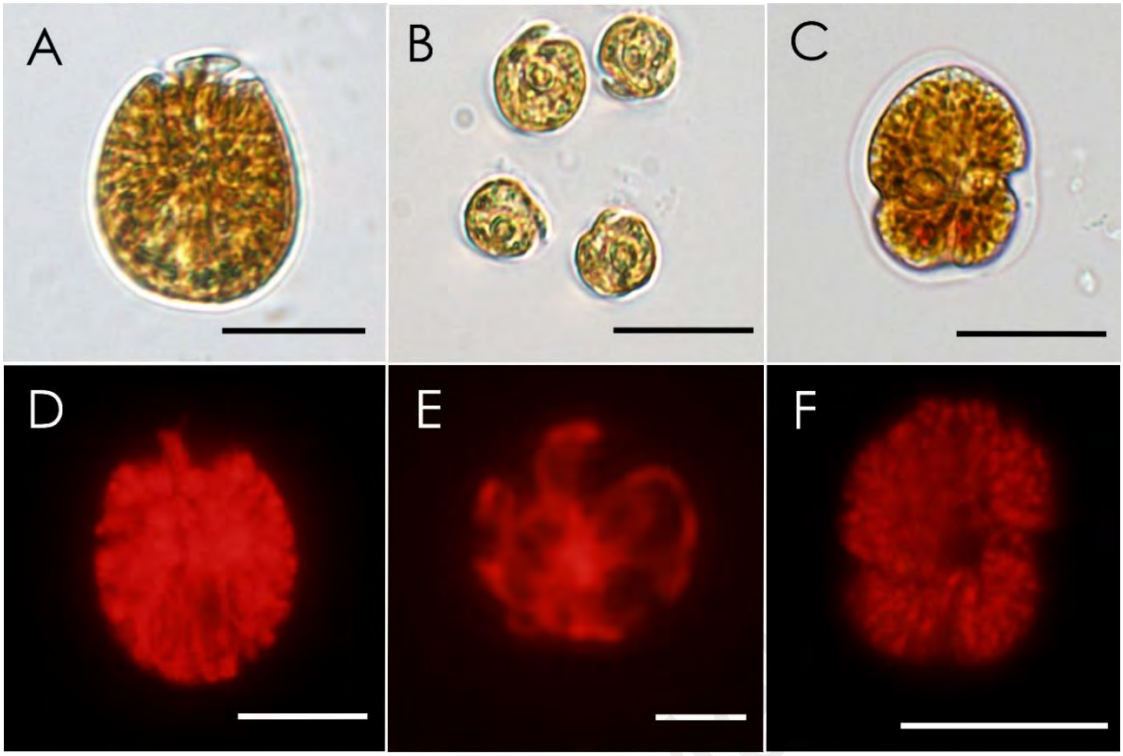

Figure 4.1: Epiphytic athecate benthic dinoflagellates found in Perhentian Islands. *Amphidinium operculatum* (A and D), *A.* cf. *massartii* (B and E), *Gymnodinium dorsalisulcum* (C and F). A-C: Light micrograph, D-F: Epifluorescent plastids. Scale bars: 20 µm (A-D, F), 10 µm (E).

Prorocentroids dinoflagellates, *Prorocentrum* Ehrenberg are defined as lacks of cingulum and a sulcus (prorocentroid tabulation) and display an apical, rather than a ventral, insertion of flagella resulting in changes swimming direction (desmokont flagellation) (Hoppenrath et al., 2013). Theca of *Prorocentrum* consists of two major plates (valves) that meet in a sagittal suture and an apical cluster of 5 to 14 platelets around two pores in the periflagellar area (Faust et al., 1999).

The morphological description of *Prorocentrum* species documented in this study was based on Hoppenrath et al. (2013) on the taxonomy and phylogeny of benthic *Prorocentrum*. The main morphological features of five *Prorocentrum* species: *P. concavum* Fukuyo (1981), *P. mexicanum* Osorio-Tafall (1942), *P. lima* (Ehrenberg) Stein (1878), *P. emarginatum* Fukuyo (1981), *P. fukuyoi* Murray & Nagahama (2007) are presented in Table 4.2.

Cells of *P. concavum* (Figure 4.2A, F, K) are 36 ± 1.7 µm in length, 33 ± 1.7 µm in width, cell shape is broad oval to ovoid. Pyrenoids with the starch ring can be observed in LM (Figure 4.2A). The thecal surface is with reticulate-foveate ornamentation where pore pattern was scattered and dense towards margin. Plate centre is devoid of pores and may also devoid of depression. The periflagellar area is in V-shaped (Figure 4.2F).

Cells of *P. mexicanum* (Figure 4.2F, G, L) are 31 ± 0.7 µm in length, 22 ± 1.1 µm in width, cell shape is in oval to oblong asymmetrically. Theca surface is smooth and unique pore patterns with one apical row, posterior radial rows in shallow thecal furrows, large pores can be observed in depression. Plate centre is devoid of pores. Periflagellar area was wide V-shaped, the apical wing-shaped spine can be observed from the periflagellar area.

Cells of *P. emarginatum* (Figure 4.2D, I, N) are 36 ± 1.7 µm in length, 33 ± 2.0 µm in width, round to oval, theca surface was smooth with radial rows or double rows of pores, plate centre devoid of pores. The periflagellar area is deep and narrow V-shaped, a wing-shaped spine in the periflagellar area. A thick flange extends around the tip of the area.

Prorocentrum fukuyoi (Figure 4.2E, J) are 31 ± 0.8 µm in length, 27 ± 1.2 µm in width, oval or oblong asymmetric, periflagellar area was deep and narrow V-shaped. The thecal surface is with radial rows of pores. A thick flange extends around the tip of the area. The spine can be observed in the periflagellar area.

Cells of *P. lima* (Figure 4.2C, H, M) have great plasticity in cell shape from almost round to oblong-ovoid, cell size is 36 ± 0.7 µm in length, 28 ± 0.5 µm in width. Pyrenoid with starch sheath, visible as a ring in the centre of the cell is prominent in LM. Smooth thecal surface with scattered large pore and a marginal row of large pores. Plate centre is devoid of pores. The periflagellar area is wide V-shaped.

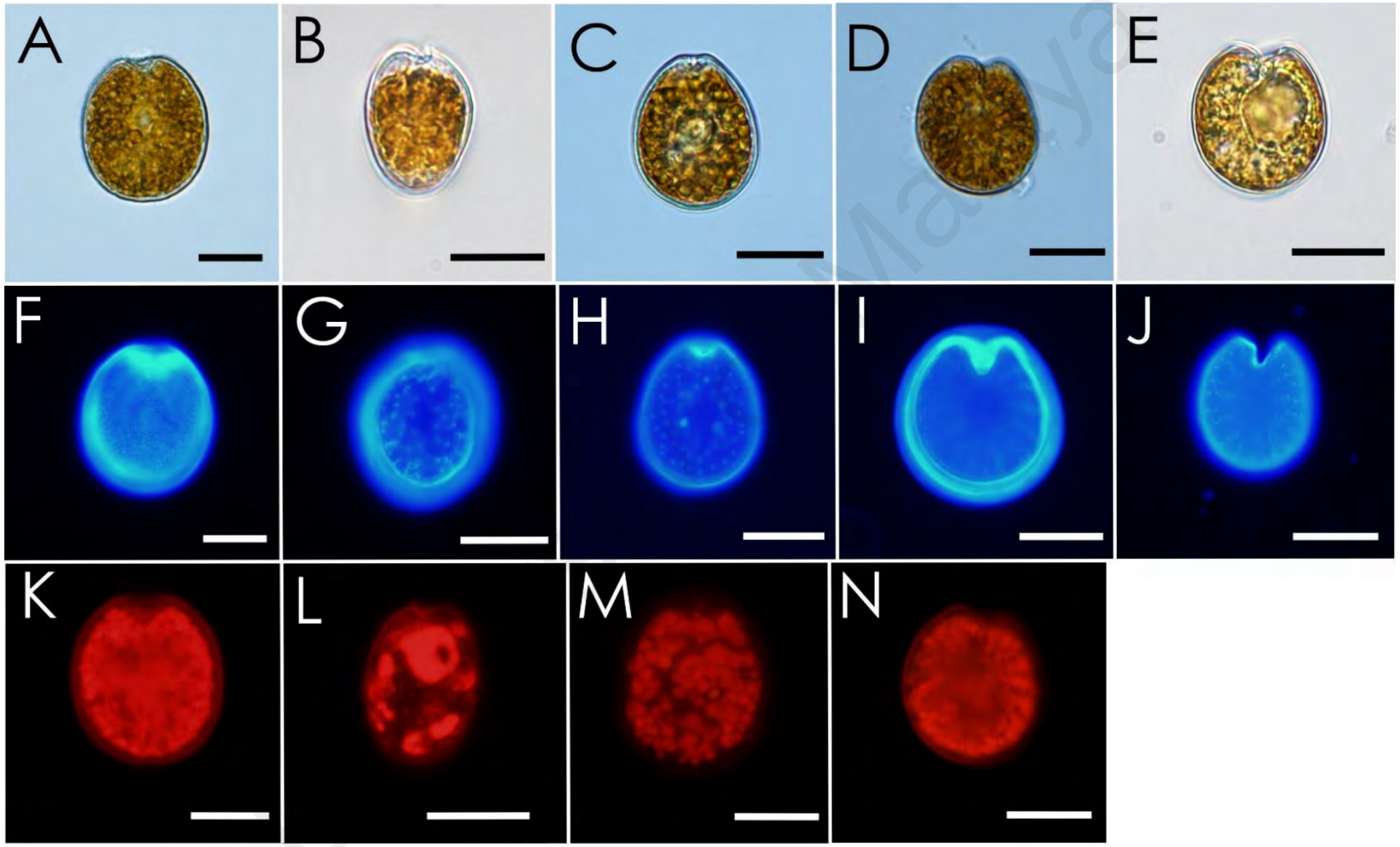

Figure 4.2: *Prorocentrum* species found in Perhentian Islands. *P. concavum* (A, F, K), *P. mexicanum* (B, G, L), *P. lima* (C, H, M), *P. emarginatum* (D, I, N), *P. fukuyoi* (E, J). A-E: LM observation, F-J: Epifluorescent observation of valves, K-N: Epifluorescent plastids. Scale bar: 20 µm.

Table 4.2: Morphological features including morphometric measurement and reported toxicity among five *Prorocentrum* species found in the Perhentian Islands.

	P. mexicanum	*P. concavum*	*P. lima*	*P. fukuyoi*	*P. emarginatum*
Cell shape	Oval to oblong, asymmetric	Broad oval to ovoid	Ovoid to round, oval to oblong-oval	Oval to oblong, asymmetric	Round to oval, asymmetric
Cell size					
Length (μm)	31.1 (0.7)	45.1 (1.0)	36.3 (0.7)	31.9 (0.8)	36.4 (1.7)
Width (μm)	22.2 (1.1)	39.0 (0.8)	29.0 (0.5)	27.4 (1.2)	33.2 (2.0)
Theca ornamentation	Smooth (radial furrow)	Foveate (depression)	Smooth	Smooth	Smooth
Periflagellar Shape	Protusion, Wide V-shaped	V-shaped	Wide V-shaped	Deep, Narrow V-shaped	Deep, Narrow V-shaped
Pore pattern	Radial rows posterior	Scattered	Scattered	Scattered	Radial rows posterior
Toxicity	Okadaic acid (OA)	Okadaic acid (OA)	Okadaic acid (OA)	Not Toxic	Not Toxic

For some genera of gonyaulacoid dinoflagellates (i.e. *Alexandrium*, *Gambierdiscus, Coolia,* and *Ostreopsis*), the thecal plate tabulation system followed the modified Kofoidian nomenclature system which was implemented in Besada et al. (1982), Fraga et al. (2011), Leaw et al. (2016).

For *Ostreopsis* cf. *ovata* Fukuyo, the cells are ovate and ventrally slender in tear-drop shape with many yellow-brown peridinin-chloroplasts (Figure 4.3). *Ostreopsis* cf. *ovata* had plate formula of Po, 4′, 0s, 6′′, 6c, 5′′′, 0p, 2′′′′. Dorsoventral diameter (DV) is 38.8 ± 5.6 µm, trans-diameter (W) is 26.9 ± 4.0 µm, DV/W ratio of 1.44 ± 0.09 µm (n = 5). Thecal plates are with numerous minute pores.

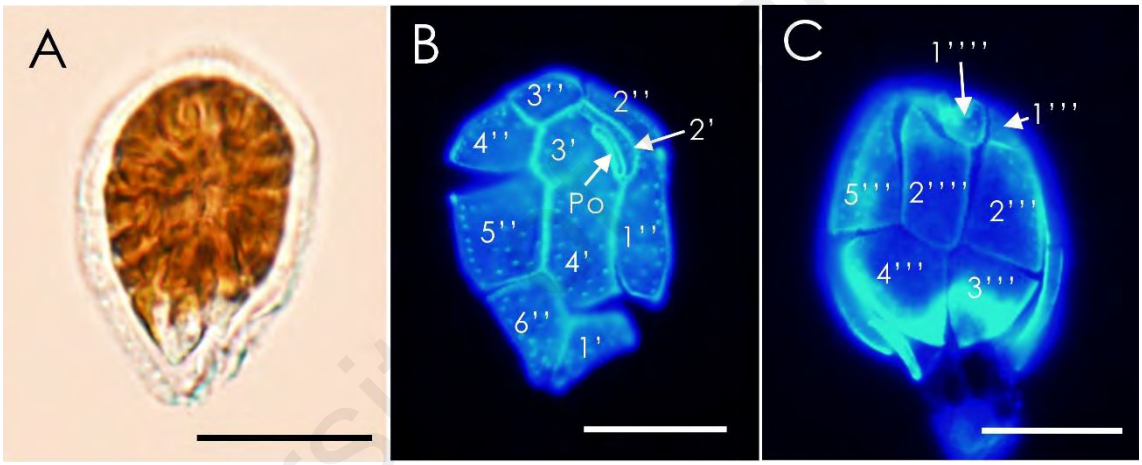

Figure 4.3: Morphology observation of *Ostreopsis* cf. *ovata*. A: Light micrograph showing typical teardrops shape for Genus *Ostreopsis*. B-C: Epifluorescence showed epitheca (B) and hypotheca (C) tabulation. Scale bars: 20 µm.

For *Coolia* Meunier**,** the plate formula is Po, 4′, 6′′, 6-7c, 6-7s, 5′′′, 2′′′′. Cells are almost oval in apical or antapical view, slightly anterior-posteriorly compressed. The most notable feature of *Coolia* is the slanted apex-antapex axis in regard to cingulum plane (apex eccentric, located on the left dorsal side of the epitheca, antapex towards the ventral side). Epitheca is smaller than hypotheca, cingulum is narrow and deep. The sulcus is narrow and excavated. The apical pore plate bears peculiar slit-like pore similar to *Ostreopsis*. The thecal surface is covered with well-defined plates delineated by a network of intercalary bands. A total of four species of *Coolia* were

identified, they are *C. canariensis* Fraga (2008), *C. tropicalis* Faust (1995), *C. malayensis* Leaw, Lim and Usup (2010), and *C. palmyrensis* Karafas, Tomas, York (2015).

The morphological features comparison among four species of *Coolia* is presented in Table 4.3. Cells of *C. canariensis* are an almost spherical shape. Apical plate (4′) was the largest epitheca, and hexagonal shape, the sixth precingular plate (6′′) is long and rectangular-shaped, with L:W ratio of 2.3 ± 0.2 (Figure 4.4E, F), ornamentation by small pits is observed in hypotheca in fifth postcingular plate (5′′′) and second antapical plate (2′′′′) (Figure 4.5B). Cells of *C. tropicalis* are spherical and the largest epitheca is 4′ plate where it widened towards ventral side, 6′′ plate is long, narrow and five-sided, L:W ratio was 3.6 ± 0.8 (Figure 4.4G, H, 4.5D - F). Cells of *C. malayensis* is round and smooth theca surface, 4′ plate is oblong, hexagonal and positioned left of centre, 6′′ plate is smaller with LW ratio of 1.1 ± 0.06 and 5′′ plate is the largest in epitheca (Figure 4.4K, L). Cells of *C. palmyrensis* are nearly spherical and similar size range to *C. malayensis*. The 4′′ plate is hexagonal, elongated and positioned left of centre, the 5′′ plate is the largest in the epitheca, 6′′ plate is small with LW ratio of 1.2 ± 0.1. The pores density on theca surface is low (Figure 4.4I, J, 4.5G-I).

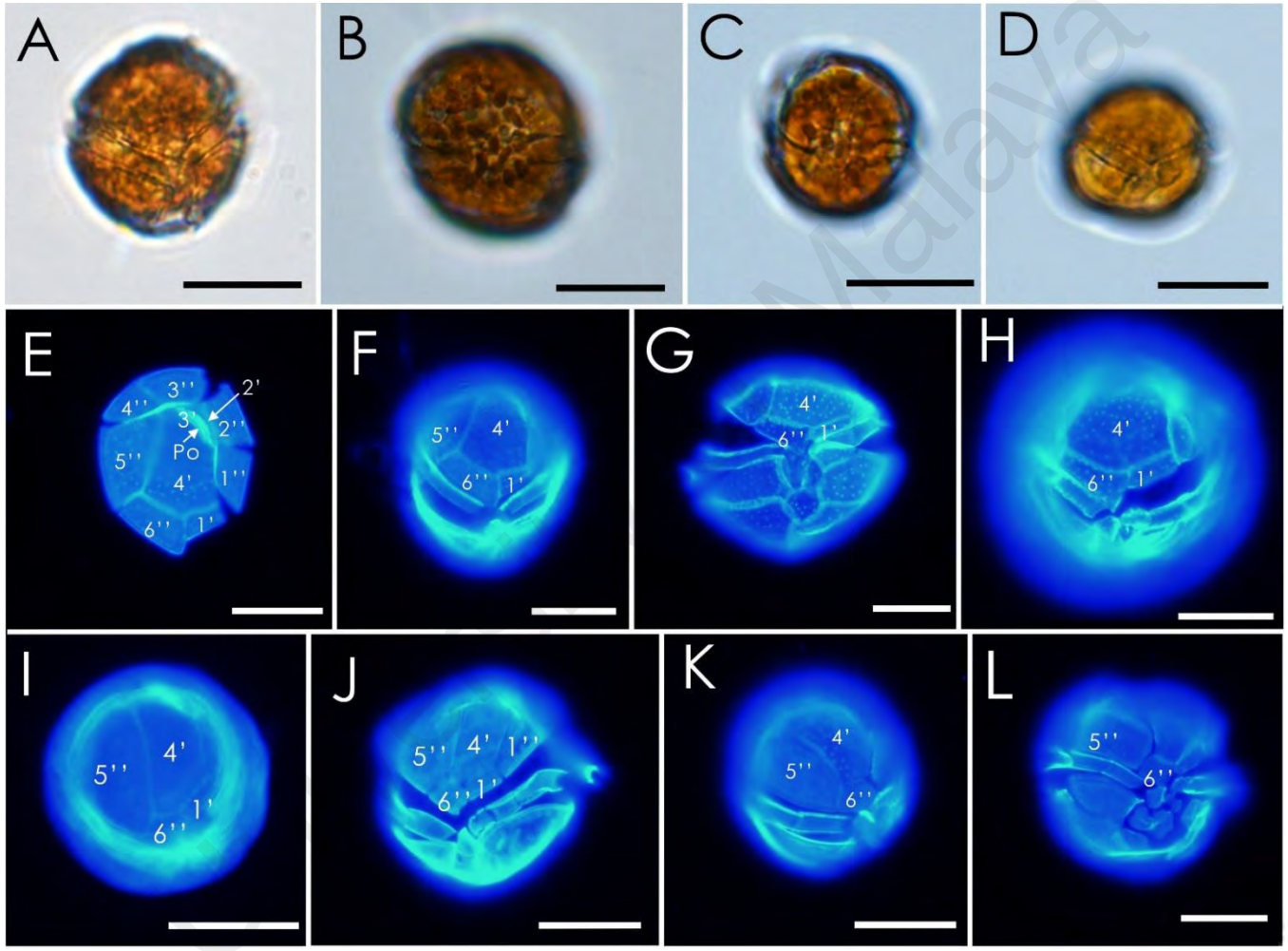

Figure 4.4: Morphology of four *Coolia* species recorded in the Perhentian Islands. *C. canariensis* (A, E, F), *C. tropicalis* (B, G, H), *C. malayensis* (C, K, L), *C. palmyrensis* (D, I, J). A-D: LM observation, E-L: Epifluorescent observation of thecal tabulation. Scale bars: 20 µm.

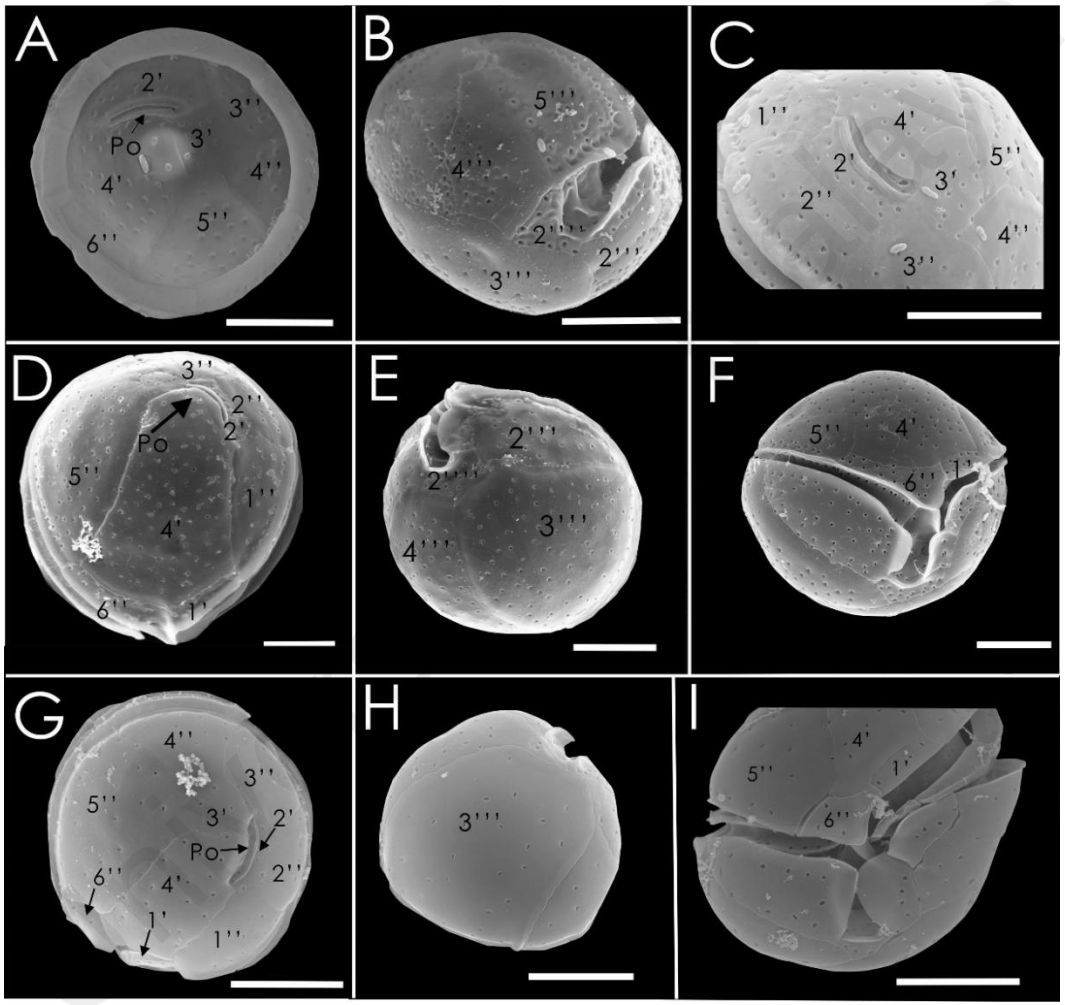

Figure 4.5: SEM micrographs of three *Coolia* species. *C. canariensis* (A-C), *C. tropicalis* (D-F), *C. palmyrensis* (G-I). A, D, G: Apical view of epitheca plate, B, E, H: Antapical view of hypotheca plate. C, F, I: Ventral view. Scale bars: 10 µm.

Table 4.3: Comparison of morphological features and measurements of four *Coolia* species documented in this study.

	C. malayensis	*C. palmyrensis*	*C. tropicalis*	*C. canariensis*
Ornamentation	Smooth	Smooth	Smooth	Hypothecal plate pitted
AP length	24.9 (0.6)	23.2 (0.9)	38.1 (2.1)	32.5 (1.8)
DV width	22.5 (1.9)	23.0 (2.0)	36.3 (2.6)	30.0 (1.4)
Largest epithecal plate	5′′	5′′	4′	4′
4’ location/shape	Left of centre; narrow; oblong; hexagonal	Left of centre; elongated and narrow; hexagonal	Central; pentagonal wedge-shaped or not	Central; hexagonal
6’’ W:L ratio	1.1 (0.06)	1.2 (0.1)	3.6 (0.8)	2.3 (0.2)

***Gambierdiscus* Adachi & Fukuyo (1979)**

Gambierdiscus general plate tabulation is Po, 4′, 6′′, 6c, 8s, 5′′′, 2‴ . *Gambierdiscus* cell is typically large and heavily armoured, in lenticular shape and highly anterior-posteriorly compressed. Apical pore was short-shank fishhook shape. Largest plate in epitheca was 2′. Useful morphological features to delineate between species was the size and shape of antapical plate 2‴ , smooth or areolated theca surface, the shape of apical plate 2′ by quantifying the ratio of the 2′/1′′ suture length and 2′/3′′ suture length. Three *Gambierdiscus* species were identified in this study, they were *G. balechii* Fraga, Rodriguez & Bravo, *G. pacificus* Chinain & Faust, and *G. caribaeus* Vandersea, Litaker, Faust, Kibler, Holland & Tester.

Cells of *G. balechii* are anterioposteriorly compressed with a length/width ratio of 0.76 ± 0.07, the average depth of 59 ± 4.5 μm and width 56 ± 3.4 μm . Thecal plates are heavily ornamented. Apical pore plate Po is oval with fishhook-shaped slit. Second apical plate 2′ is the largest of the epitheca, in hatchet-shaped and has a ratio of sutures 2′/1′′ to 2′/3′′ average 0.57 ± 0.06. Antapical plate 2‴ is small and narrow with LW

ratio of 1.69 (Figure 4.6D, G). *Gambierdiscus pacificus* cells are anterioposteriorly compressed with length/width (LW) ratio of 0.69 ± 0.04, the average depth of 63 ± 3.4 µm and width 59 ± 4.2 µm . Smooth thecal plate with fine pores, apical pore plate oval with the fish-hook shaped pore. Apical plate 2′ is the largest in epitheca with hatchet-shaped with a ratio of sutures 2′/1′′ to 2′/3′′ average 0.43 ± 0.06. Antapical plate 2′′′ is long and narrow with LW ratio of 2.21 (Figure 4.6E, H). Cells of *G. caribaeus* cells are anterioposteriorly compressed and relatively larger with an average depth of 82 ± 1.6 µm and width 80 ± 6.5 µm , LW ratio of 0.58 ± 0.05. The thecal surface is smooth with fine pores. Apical pore plate oval with fishhook-shaped slit. Apical plate 2′ is in rectangular shape with a ratio of sutures 2′/1′′ to 2′/3′′ average 0.88 ± 0.13. Antapical plate 2′′′′ is comparatively broad with LW ratio of 1.27 (Figure 4.6F, I).

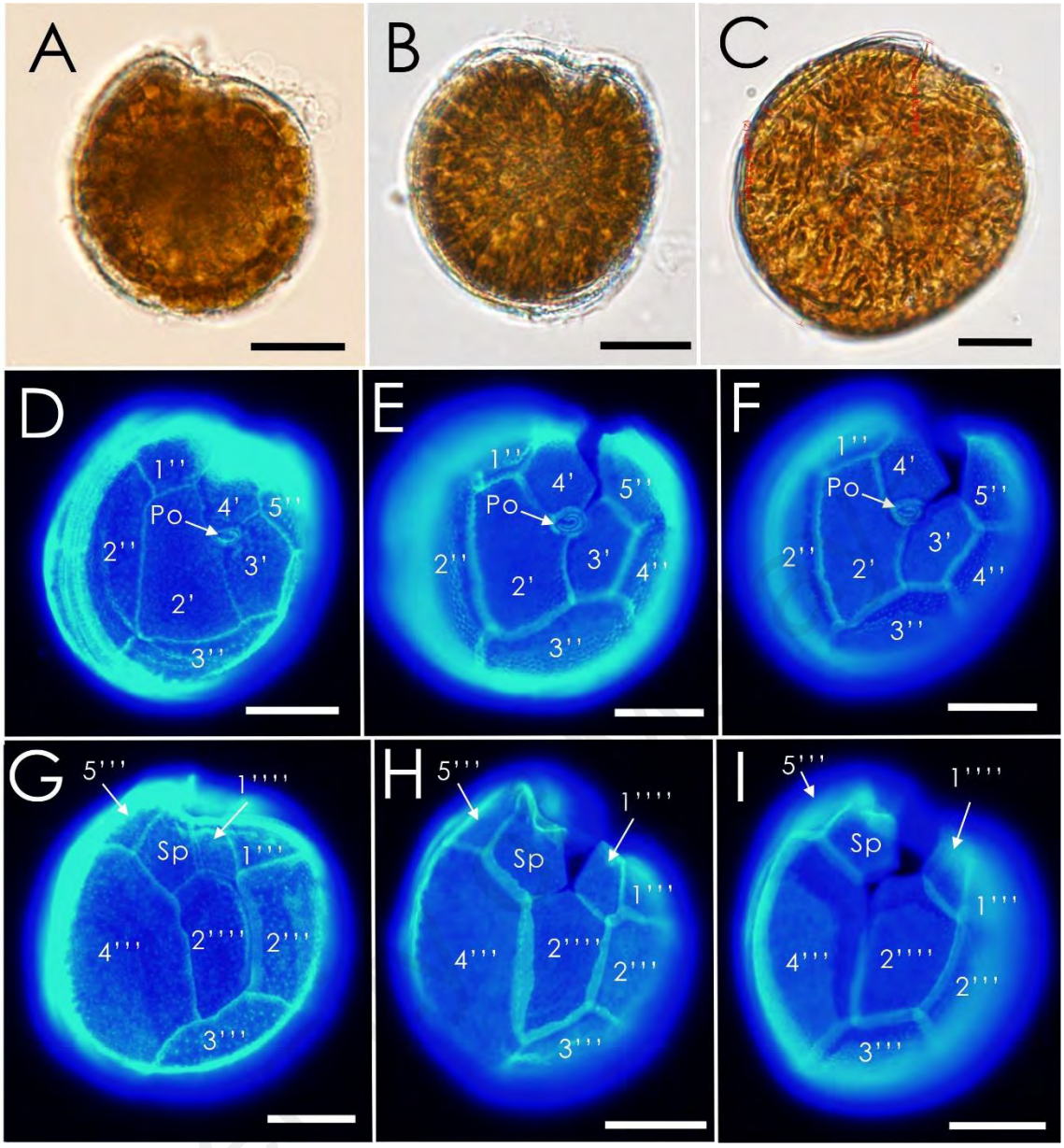

Figure 4.6: Morphological observation of three Gambierdiscus species recorded in Perhentian Islands. *G. balechii* (A, D, G), *G. pacificus* (B, E, H), *G. caribaeus* (C, F, I). A-C: LM observation, D-F: Epifluorescent apical view, G-I: Epifluorescent antapical view. Scale bars: 20 μm

4.1.2 Phylogenetic inferences of *Amphidinium*, *Coolia, Prorocentrum,* and *Gambierdiscus* from Perhentian Islands

Genus *Amphidinium* Claparede & Lachmann

Four strains of two species of *Amphidinium* were successfully identified morphologically and further supported by molecular phylogenetic inferences. Strains SS11H1 claded into *A.* cf. *massartii* clade with strong nodal supports (MP/ML/BI, 100/100/1.0). Three strains of *Amphidinium* (SS09H1, SS15S4, SS06S5) were clade

into *A. operculatum* clade with strong support in MP and ML but not in BI (100/100/-) as shown in Figure 4.7. Uncorrected pairwise genetic divergences of selected *Amphidinium* species are shown in Table 4.4. Intraspecific divergence of *A. opeculatum* and *A.* cf. *massartii* was 0.2 – 3.6 % and 0.2 – 0.6 %, respectively. The intraspecific divergences of four selected *Amphidinium* species ranged from 0 – 42.9 % with the largest range between *A. operculatum* and other species. Interspecific divergences between sister taxa *A. massartii* and *A.* cf. *massartii* range from 4.4 – 5.3 %.

Verification of *Gymnodinium dorsaliculcum* (SS10H01, SS13H01) and *Ostreopsis* cf. *ovata* (SS06H3) was based on the results of BLAST search through the Genbank nucleotide database. Both strains of *Gymnodinium dorsalisulcum* have query coverage of 99 – 100 % and identity of 99 % towards sequence with the accession number of DQ336190. *Ostreopsis* cf. *ovata* has query coverage of 100 % and identity score of 97 % towards sequence with the accession number of KX781270.

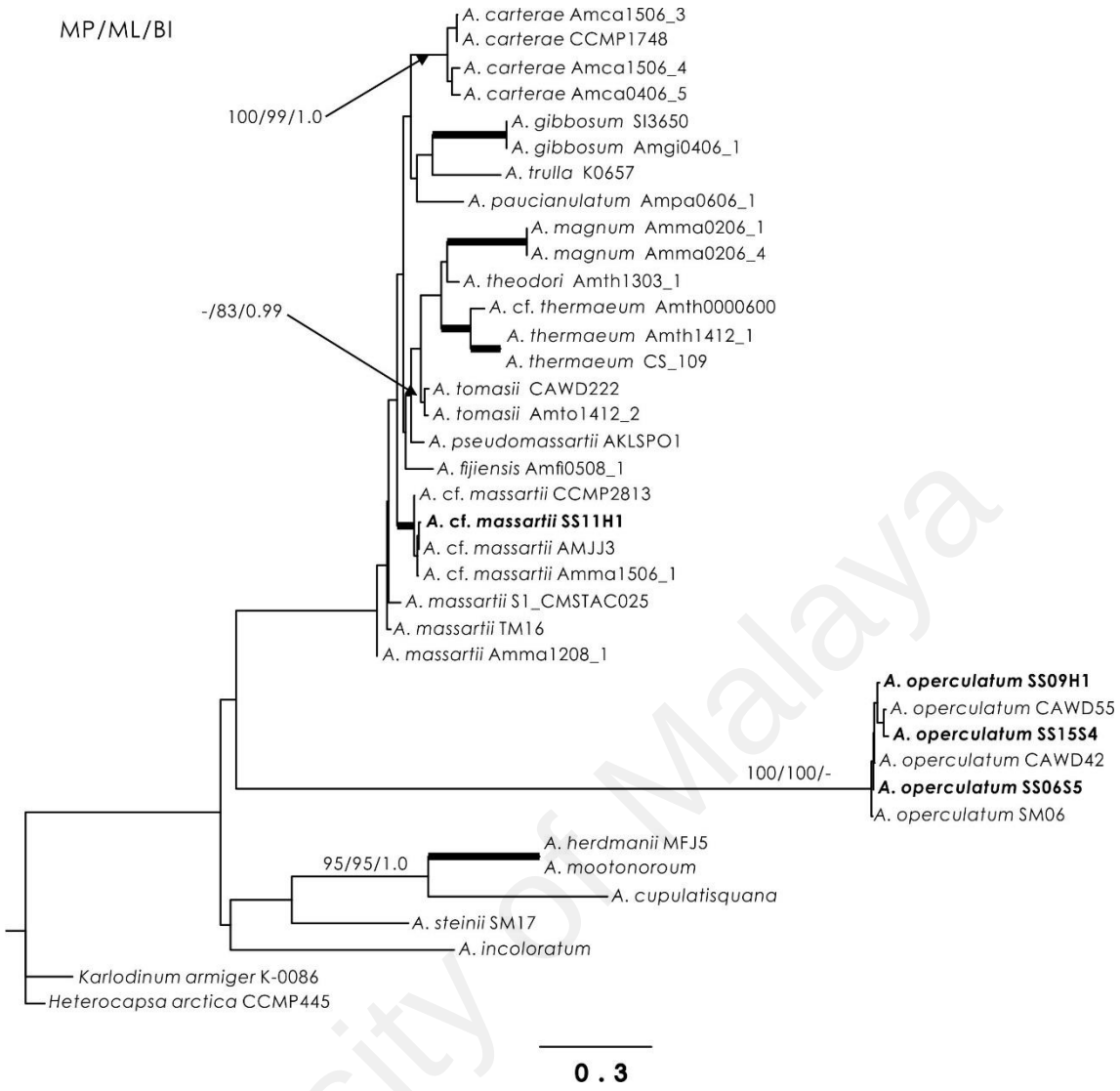

Figure 4.7: ML tree based on the D1-D3 LSU rDNA dataset of *Amphidinium* species in this study. The thick line indicates MP/ML bootstrap of 100% and PP at 1.00. Taxa in bold indicate sequences obtained in this study.

Table 4.4: Uncorrected pairwise divergence range of LSU rDNA data of four selected *Amphidinium* species included in this study. Bold on the diagonal is the intraspecific divergence.

	A. operculatum	*A. massartii*	*A. cf. massartii*	*A, carterae*
A. operculatum	**0.002-0.036**			
A. massartii	0.410-0.429	**0.025-0.034**		
A. cf. massartii	0.412-0.425	0.044-0.053	**0.002-0.006**	
A, carterae	0.416-0.427	0.082-0.101	0.099-0.108	**0.000-0.036**

Genus *Prorocentrum* Ehrenberg

Five species of benthic *Prorocentrum* from six strains were documented in this study, *P. concavum*, *P. lima*, *P. mexicanum*, *P emarginatum, P. fukuyoi*. Identification of four species (*P. concavum*, *P. lima*, *P. mexicanum*, *P emarginatum*) further supported by using LSU phylogeny analysis as shown in Figure 4.8. Strongly supported clade was attained in MP/ML/BI for *P. concavum* (100/100/1.0), *P. lima* (95/97/1.0), *P. mexicanum* (92/88/1.0) and *P. emarginatum* (100/100/1.0).

Uncorrected pairwise genetic divergences of selected *Amphidinium* species are shown in Table 4.5. The intraspecific divergences of *P. concavum, P. lima, P. mexicanum, P emarginatum* were 0.2 – 1.2 %, 0 – 2.2 %, 0.2 – 1.2 %, 0 – 5.5 % respectively. For interspecific divergences, closely related groups like *P. concavum* and *P. foraminosum* have a larger divergence of 11.9 – 12.5 % whereas *P. emarginatum* was sister taxa to *P. fukuyoi* with even larger divergence ranged from 26.9 – 30.6 %. *P. mexicanum* and *P. koreanum* was sister taxa where interspecific divergence ranged from 2.8 – 3.2 %. In the *P. lima* species complex (*P.lima, P.* cf. *lima*, *P. caipirignum, P. hoffmannium* "species complex"), *P. lima* was with interspecific divergence of 4.0 – 5.7 %, 3.8 – 5.3 %, 4.2 – 5.5 % to *P. hoffmannium* "species complex", *P. caipirignum* and *P.* cf. *lima* accordingly.

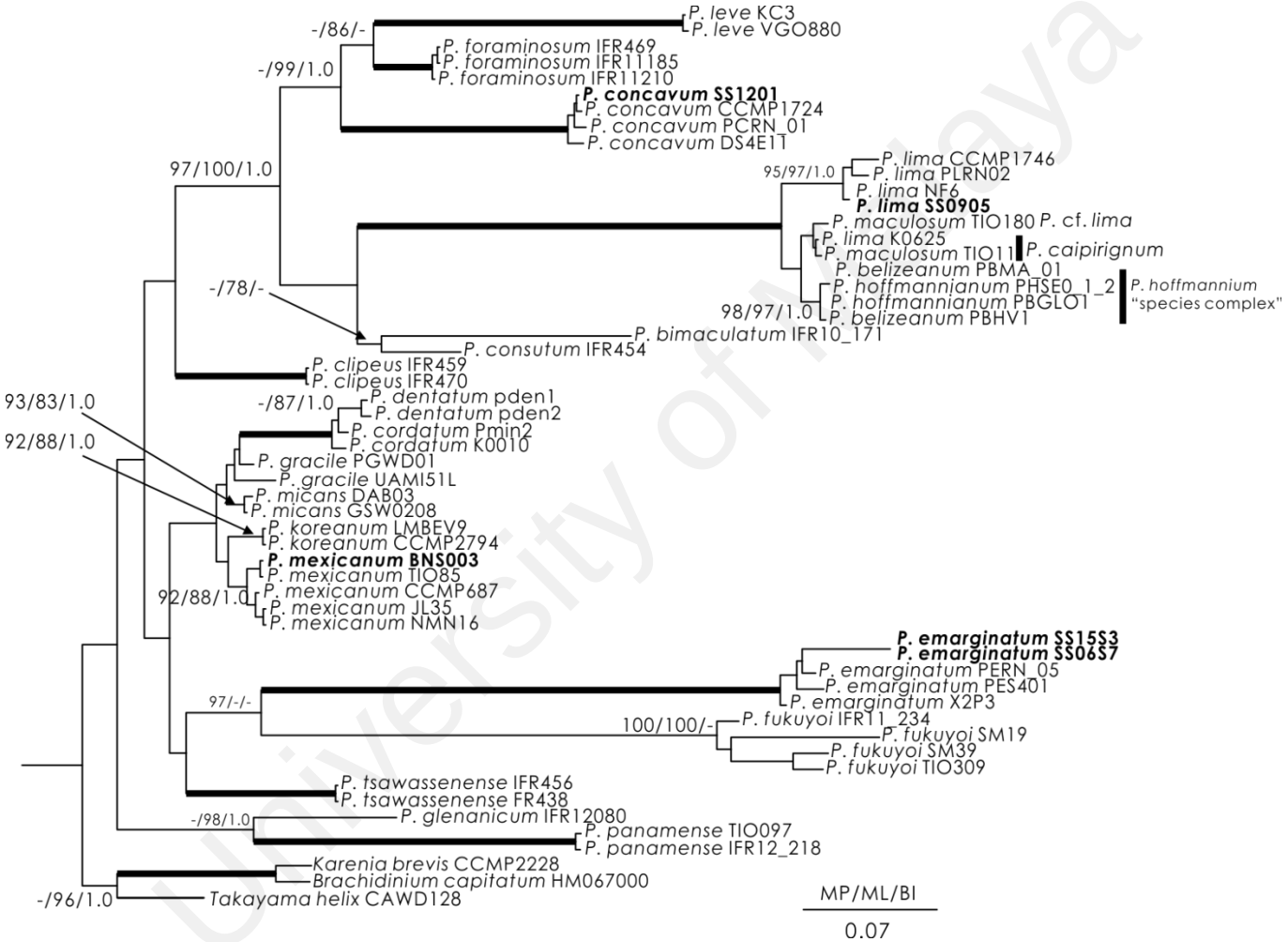

Figure 4.8: ML tree based on the D1-D3 LSU rDNA dataset of *Prorocentrum* species. The thick line indicates MP/ML bootstrap of 100% and PP at 1.00. Taxa in bold indicate sequences obtained in this study.

Table 4.5: Uncorrected pairwise divergence range of LSU rDNA data of selected *Prorocentrum* species included in this study. Bold on the diagonal are the intraspecific divergences.

	concavum	*foraminosum*	*lima*	*hoffmannium species complex*	*caipirignum*	*cf. lima morphotype 5*	*mexicanum*	*koreanum*	*emarginatum*	*fukuyoi*
concavum	**0.002-0.012**									
foraminosum	0.119-0.125	**0.000-0.002**								
lima	0.241-0.253	0.225-0.233	**0.000-0.022**							
hoffmannium species complex	0.241-0.255	0.229-0.235	0.040-0.057	**0.000-0.008**						
caipirignum	0.231-0.243	0.219-0.223	0.038-0.053	0.016-0.022	**0.002**					
cf. lima morphotype 5	0.229-0.237	0.215-0.217	0.042-0.055	0.020-0.024	0.004-0.006	**0**				
mexicanum	0.172-0.180	0.140-0.142	0.227-0.229	0.227-0.233	0.217-0.223	0.217-0.221	**0.000-0.012**			
koreanum	0.170-0.178	0.144-0.146	0.225-0.227	0.229-0.231	0.223-0.225	0.223	0.028-0.032	**0**		
emarginatum	0.273-0.294	0.243-0.263	0.316-0.328	0.322-0.334	0.316-0.328	0.324-0.330	0.206-0.227	0.200-0.219	**0.000-0.055**	
fukuyoi	0.251-0.285	0.233-0.261	0.300-0.326	0.304-0.322	0.304-0.320	0.304-0.322	0.206-0.245	0.208-0.247	0.269-0.306	**0.028-0.117**

Genus *Coolia* Meunier

Four species of genus *Coolia* out of seven species described were documented in this study from seven strains of cultures, namely, *C. malayensis, C. canariensis, C. tropicalis* and *C. palmyrensis*. LSU phylogenetic analysis showed strong support in MP/MP/BI for *C. tropicalis* clade (100/100/1.0), *C. canariensis* (100/100/1.0), *C.* cf. *canariensis* (100/97/1.0), *C. malayensis* (100/82/1.0), but low support for *C. palmyrensis* (45/-/0.64) (Figure 4.9). Isolates of *C. canariensis* obtained in this study were clade into another well-supported subclade of *C. canariensis*, hence the isolate been assigned as *C.* cf. *canariensis*. Uncorrected pairwise divergences of six *Coolia* species are presented in Table 4.6. For intraspecific divergences, only *C. palmyrensis* has relatively higher intraspecific divergence (0 – 11.3 %) compared to other *C. tropicalis* (1.1 – 4.8 %), *C. malayensis* (0 – 0.3%), *C. monotis* (0.3 – 0.8 %). Sister clades were formed between *C. canariensis* and *C.* cf. *canariensis* due to relatively significant divergences between these two with 16.1 – 16.6 %.

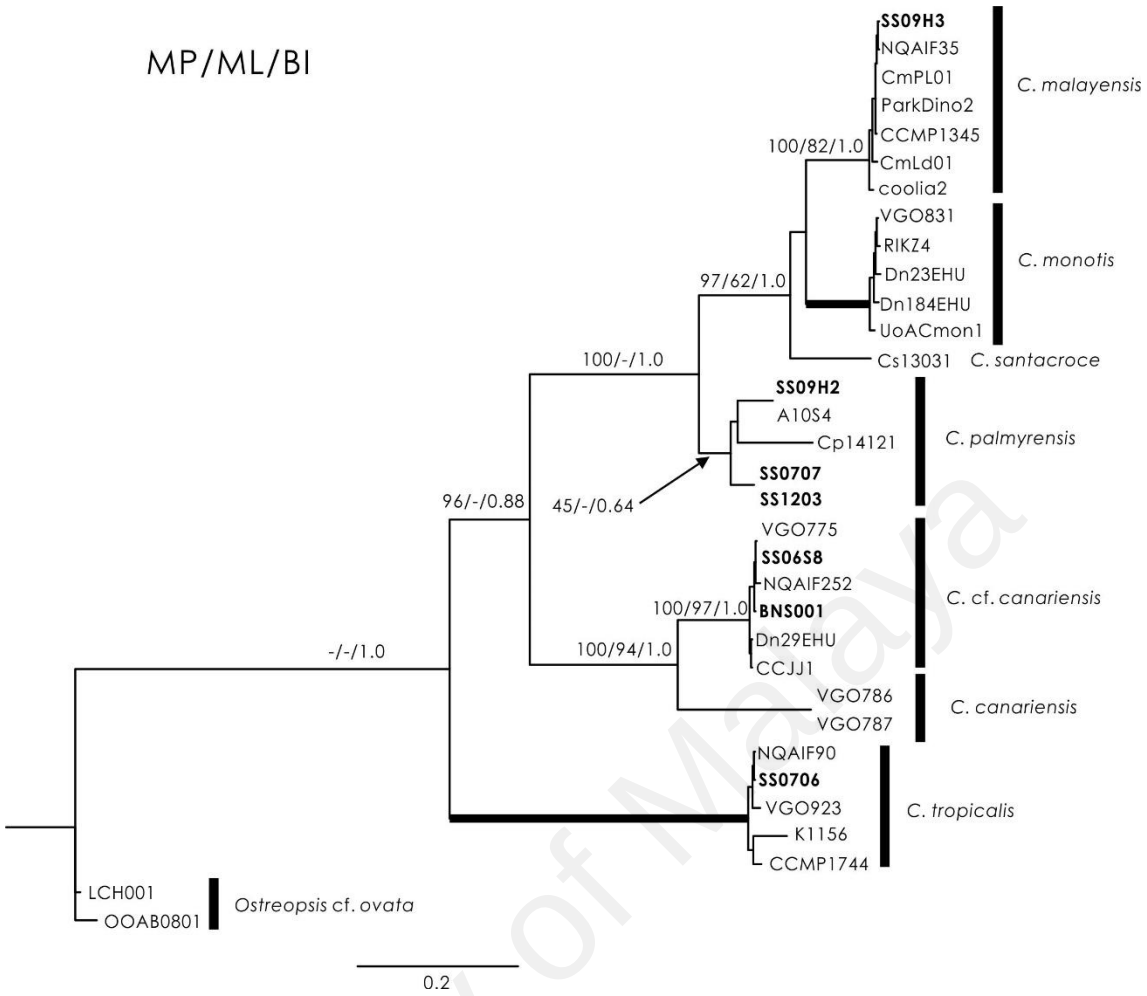

Figure 4.9: ML tree based on the D1-D3 LSU rDNA dataset of *Coolia* species. The thick line indicates MP/ML bootstrap of 100% and PP at 1.00. Taxa in bold indicate sequences obtained in this study.

Table 4.6: Uncorrected pairwise divergence range of LSU rDNA data of seven *Coolia* species included in this study. Bold on the diagonal is the intraspecific divergence.

	Tropicalis	*canariensis*	*cf. canariensis*	*palmyrensis*	*santacroce*	*monotis*	*malayensis*
tropicalis	**0.011-0.048**						
canariensis	0.363 – 0.372	**0.000**					
cf canariensis	0.335- 0. 346	0.161 – 0.166	**0.003 – 0.008**				
palmyrensis	0.335-0.394	0.299 – 0.330	0.296 – 0.313	**0.000-0.113**			
santacroce	0.397-0.406	0.372	0.369 – 0.375	0.169-0.183	**0**		
monotis	0.383-0.394	0.355 – 0.361	0.344 – 0.355	0.149-0.183	0.113-0.121	**0.003-0.008**	
malayensis	0.372-0.383	0.335 – 0.338	0.330 – 0.338	0.163-0.194	0.132-0.135	0.101-0.110	**0.000-0.003**

Genus *Gambierdiscus* Adachi & Fukuyo

Three species of *Gambierdiscus* were successfully identified molecularly from seven clonal culture strains. Sequences of six strains were included in phylogenetic reconstruction. The *Gambierdiscus* LSU phylogeny generated in this study similar tree topology as Dai et al. (2017) with strong supports in MP, ML bootstraps and Bayesian Inference posterior probability as shown in Figure 4.10.

Five strains of *G. caribaeus* and one strain of *G. balechii* isolated from Perhentian Islands were grouped together with respective clades, with strong supports in MP, ML and BI (*G. caribaeus*, 91/83/0.96; *G. balechii*, 91/87/1.0). For *G. pacificus*, the sequence was excluded from our phylogenetic analysis but was verified by BLAST search in Genbank. Blast search results returned the sequence in 92 % query coverage, with 98 % identity to *G. pacificus* KR229998.

The intraspecific divergence of *G. balechii* in this study was 0.2 – 5.4 % whereas *G. caribaeus* has 0 – 0.6 % (Table 4.7). The interspecific divergence of *G. balechii* with sister taxa of *G. lapillus, G. pacificus, G. toxicus,* and *G. cheloniae* ranged of 6.1 %, 1.6 – 7.2 %, 1.6 – 7.0 % and 1.9 – 5.9 %, respectively. *G. caribaeus* was sister taxa of *G. carpenteri* and *G.* sp type 2 where the interspecies divergence ranged in 0.5 – 1.0 % and 1.6 – 1.8 % for the latter.

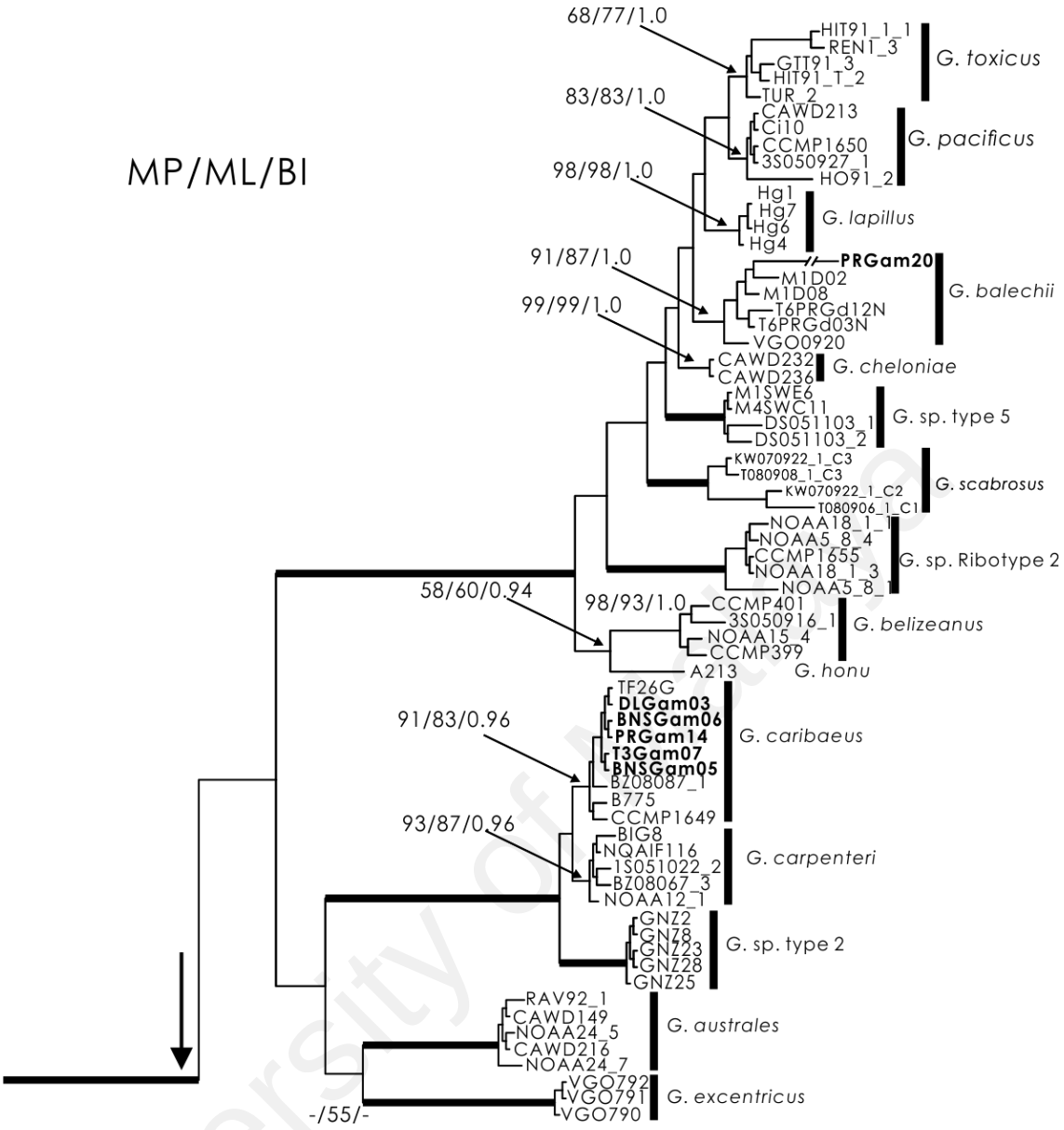

Figure 4.10: ML tree based on the D8–D10 LSU rDNA dataset of *Gambierdiscus* species. The thick line indicated MP/ML bootstrap of 100% and PP at 1.00. Taxa in bold indicate sequences obtained in this study.

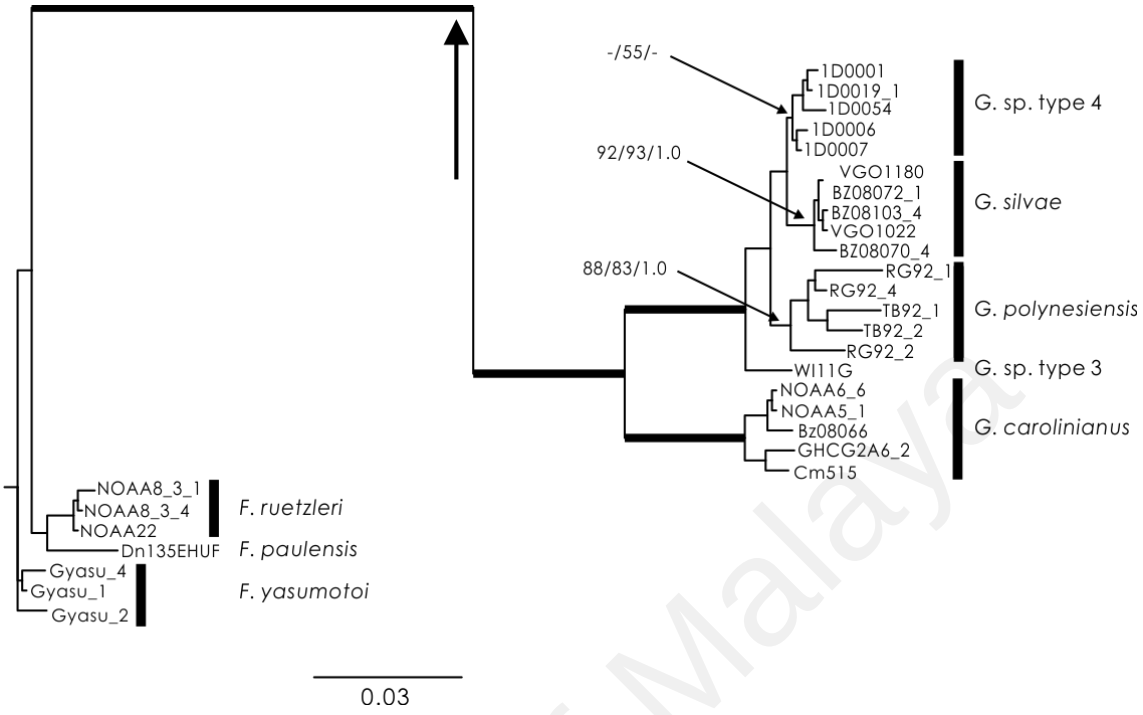

Figure 4.10: Continued.

Table 4.7: Uncorrected pairwise divergence range of D8–D10 region LSU rDNA data of eight *Gambierdiscus* species included in this study. Bold on the diagonal is the intraspecific divergence.

	G. balechii	*G. lapillus*	*G. pacificus*	*G. toxicus*	*G. cheloniae*	*G. caribaeus*	*G. carpenteri*	*G.* ribotype 2
G. balechii	**0.002 – 0.018 (0.002-0.054)***							
G. lapillus	0.061	**0.000-0.003**						
G. pacificus	0.016-0.072	0.016-0.037	**0.000-0.019**					
G. toxicus	0.016-0.070	0.032-0.034	0.019-0.038	**0.003-0.018**				
G. cheloniae	0.019-0.059	0.021	0.016-0.035	0.016-0.029	**0**			
G. caribaeus	0.107-0.138	0.104-0.109	0.104-0.118	0.115-0.118	0.109-0.110	**0.000-0.006**		
G. carpenteri	0.102-0.136	0.099-0.106	0.099-0.115	0.115-0.118	0.107-0.110	0.005-0.010	**0.002-0.006**	
G. ribotype 2	0.107-0.141	0.104-0.107	0.102-0.115	0.120	0.112	0.016-0.018	0.011-0.014	**0**

4.2 Ciguatoxin screening of *Gambieridiscus* species in Perhentian Islands

A total of seven strains of *Gambierdiscus* consists of *G. caribaeus, G. balechii,* and two unidentified *Gambierdiscus* sp. were screened for ciguatoxin with neuroblastoma 2A assay using brevetoxins as standard. Out of seven strains, only two strains of *Gambierdiscus,* BNSGam06 and PRGd07N showed CTX-like activity in the dichloromethane fractioned extracts (Table 4.8). In neuroblastoma assay for VSGC activating toxins such as brevetoxins (PbTXs) (as standard) and ciguatoxins (CTXs) (samples), O/V concentration that produces around 20% cell mortality were used to detect the VSGC activating type effect that would increase cell mortality (Cañete & Diogène, 2008). In this study, dichloromethane fraction extracts of BNSGam06 and PRGd07N showed a decrease of cell viability in Neuro-2a cells with positive O/V treatment signaled present of ciguatoxins as shown in Figure 4.11. The cell mortality in negative O/V treatment may suggest presence of cytotoxicity effect as *Gambierdiscus* sp. was also known for producing putative maitoxins.

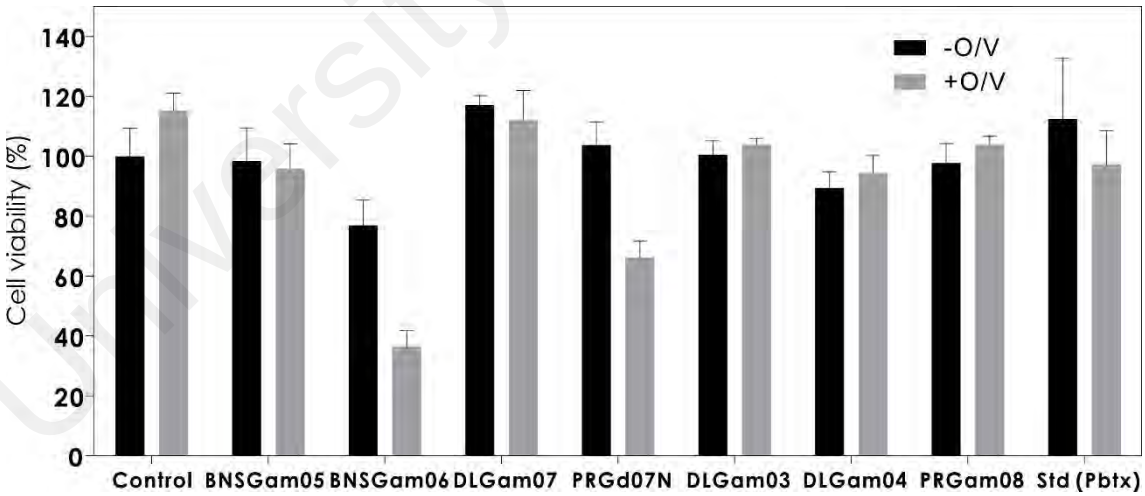

Figure 4.11: Neuro-2a bioassay screening for ciguatoxins-like activity of *Gambierdiscus* with O/V treatment using brevetoxins as standard. –O/V indicates absence of O/V, +O/V indicates the presence of O/V.

Table 4.8: The list of *Gambierdiscus* culture strains including strains code, identification, and the result for ciguatoxins screening.

Identification	Strains code	Ciguatoxin screening
G. caribaeus	BNSGam05	Negative
G. caribaeus	BNSGam06	**Positive**
Gambierdiscus spp.	DLGam07	Negative
G. balechii	PRGd07N	**Positive**
G. caribaeus	DLGam03	Negative
Gambierdiscus spp.	DLGam04	Negative
Gambierdiscus spp.	PRGam08	Negative

4.3 Distribution and assemblages of benthic harmful dinoflagellates in the Perhentian Islands

4.3.1 Temporal variation in water temperature and light intensity

Water temperatures of Perhentian Islands exhibited larger fluctuation in shallow depths (3 m) where they varied between 28.06 and 33.74 °C (average of 30.66 °C) while temperatures at the water depth of 10 m were more stable ranging between 30.15–32.18 °C (average of 31.01 °C). Temporal variation in water temperatures is depicted in Fig. 4.12A. During the dry season (southwest monsoon), between March and late October, water temperatures varied from 30.35 to 33.63 °C, with an average of 31.52 °C. As the northeast monsoon (wet season) commenced from early November to March, water temperatures dropped and remained between 28.06 and 30.76 °C, with an average of 29.32 °C (Fig. 4.12A).

Maximum irradiance intensities observed in the shallow (3–5 m) and deeper (10 m) depths of Perhentian Islands were 2345 and 382 μmol photons m^{-2} s^{-1}, respectively Monthly average irradiance intensity over the studied period was higher in the dry season, while a long period of low irradiance intensities was observed during the wet season due to cloud cover (Fig. 4.12B).

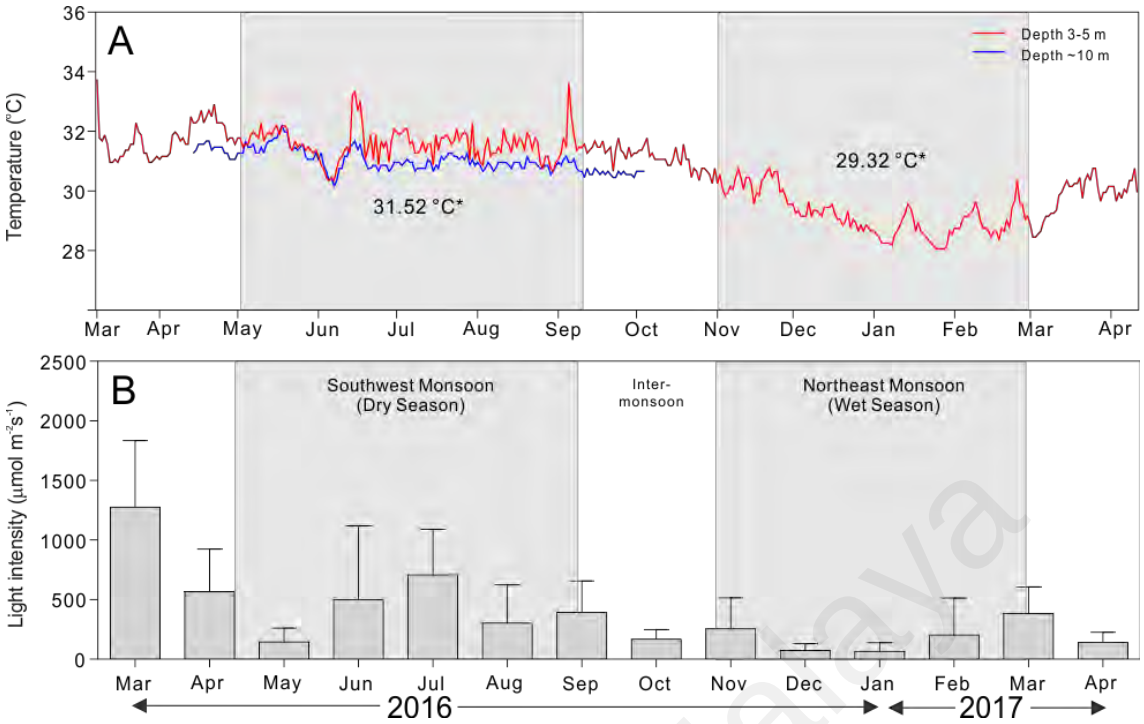

Figure 4.12: Physical water parameters. (A) Water temperature recorded at shallow (3–5 m) and deeper depths (10 m) from March 2016 to April 2017. * indicates average water temperatures in the dry and wet seasons. (B) Monthly average irradiances (PPFD, photosynthetic photo flux density) recorded at 3–5 m depth from March 2016 to April 2017.

4.3.2 Benthic dinoflagellates distribution in different sites of Perhentian Islands

Five locations in Perhentian Islands were chosen as study site: Rawa Islands (PR), Seringgih Islands (PS), Batu Nisan (BNS), D.Lagoon (DL) and Tokong Laut (TL). Figure 4.13 depicted the overall relative abundances of the five benthic dinoflagellates, *Gambierdiscus*, *Ostreopsis*, *Coolia*, *Prorocentrum* and *Amphidinium* in Perhentian Islands. *Prorocentrum* was the most dominant group (49.4 %) comprising almost half of the composition; followed by *Ostreopsis* (35.1 %). The remaining three minor groups were *Coolia* (8.8 %), *Amphidinium* (5.00 %), and *Gambierdiscus* (1.7 %). In term of locality, *Prorocentrum* was the dominant genus in two sampling sites, Batu Nisan (57.4 %) and Tokong Laut (67.2%), while *Ostreopsis* dominated in three other sampling sites: Rawa Island (51.4 %), Seringgih Island (60.4 %) and Batu Nisan (57.4 %).

Kruskal-Wallis test on the cell abundances of benthic dinoflagellates and sampling sites was shown in Fig 4.14. No significant difference was detected for cell

abundances of *Gambierdiscus* and *Coolia* among five localities. Cell abundances of *Ostreopsis* were significantly different between TL and four other sites, PR-TL ($p < 0.0001$), PS-TL ($p \leq 0.01$), DL-TL ($p < 0.0001$), BNS-TL ($p \leq 0.01$). Significant difference was observed in cell abundances of *Prorocentrum* in site comparison of PR-BNS ($p \leq 0.05$), PR-TL ($p < 0.001$), DL-TL ($p < 0.0001$) and BNS-TL ($p < 0.0001$). For cell abundances of *Amphidinium*, a significant difference was observed in site comparison between PR-DL ($p \leq 0.05$), PR-BNS ($p \leq 0.01$), PR-TL ($p < 0.001$) and PS-TL ($p \leq 0.05$).

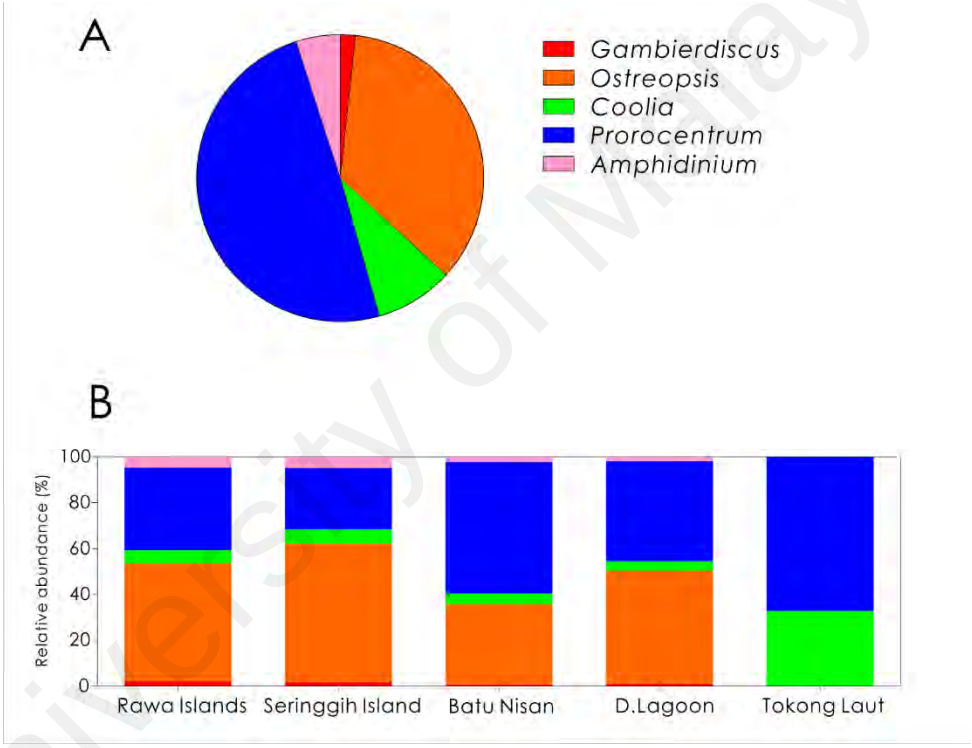

Figure 4.13: (A) Overall relative abundance of the five genera of benthic dinoflagellates from the Perhentian Islands ($n = 234$). (B) Relative abundances of the five benthic dinoflagellates from the respective sampling sites; Rawa Island ($n = 105$), Seringgih Island ($n = 35$), Batu Nisan ($n = 44$), D.Lagoon ($n = 40$), Tokong Laut ($n = 10$).

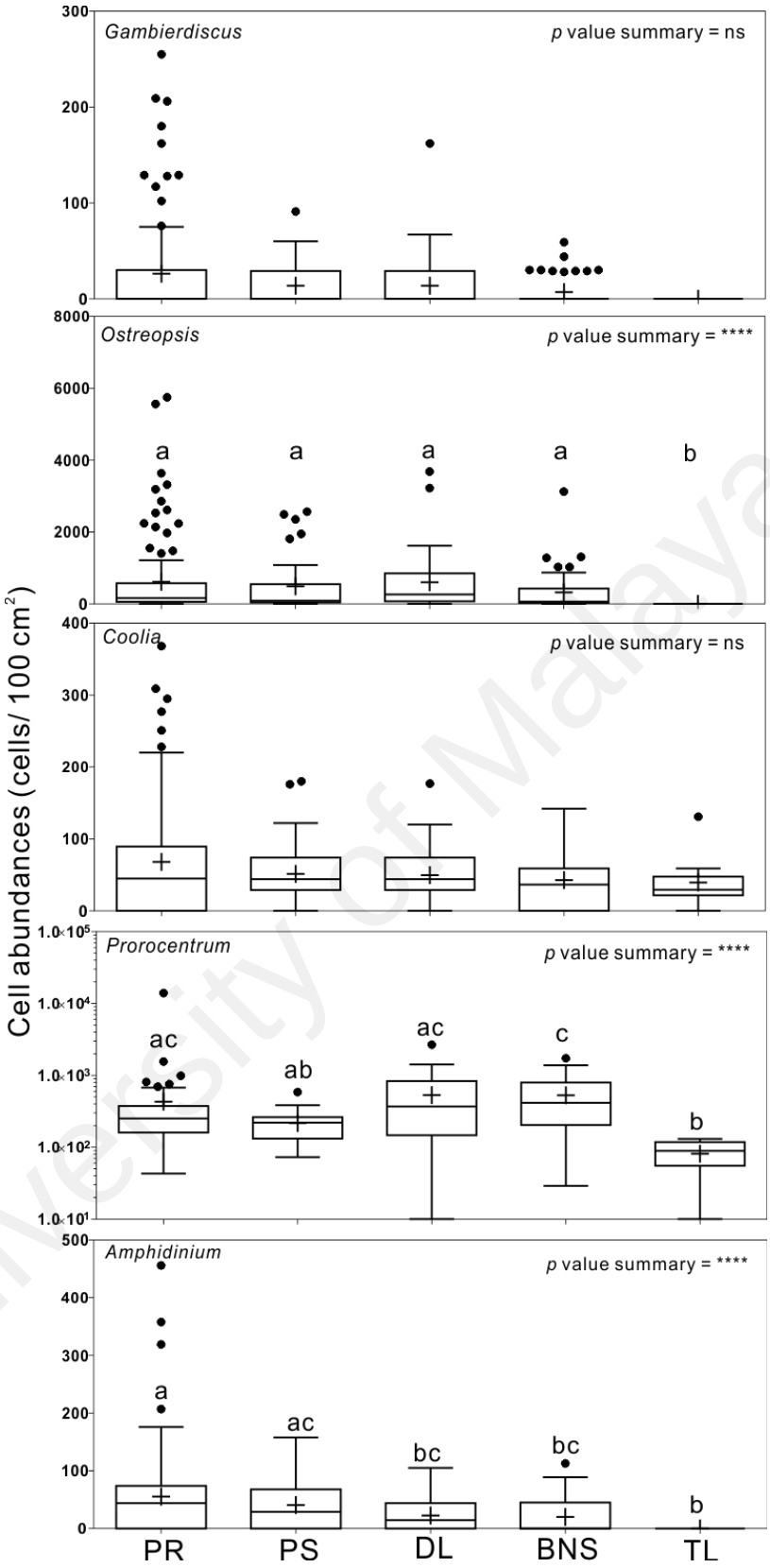

Figure 4.14: Tukey plot of benthic dinoflagellates cell abundances in different localities of the Perhentian Islands with *p*-value summary of Kruskal-Wallis test and alphabet indicate the outcome of Dunn's multiple comparisons test. "+" showed mean; horizontal line in box showed median; Box ends at the quartiles Q_1 and Q_3. Whiskers showed the upper and lower extreme; Dots represents outliers. Ns: $p > 0.05$; "*": $p \leq 0.05$; "**": $p \leq 0.01$; "***": $p \leq 0.001$; "****": $p \leq 0.0001$.

4.3.3 Benthic substratum structure of Perhentian Islands

The Bray-Curtis dissimilarity cluster analysis systematised the characteristics of benthic microhabitats into eight clusters (75% similarity; Fig. 4.15A); the description of each cluster is presented in Table 4.9. Benthic substratum coverage (% cover) where the screens were deployed was visualised in a heatmap as shown in Fig. 4.15A. The pattern of benthic substratum clustering was further reflected in the *n*MDS ordination plot (Fig. 4.15B), obtaining a stress factor of 0.0594 in the reduced dimensions. A one-way Analysis of Similarity test (ANOSIM) showed that the benthic substratum structure between the defined clusters was significantly different (Global $R = 0.9857$, $p < 0.0001$). The results of the SIMPER test further revealed the overall average dissimilarity between the defined clusters and the major contributors of benthic substratum characteristics (See Appendix I).

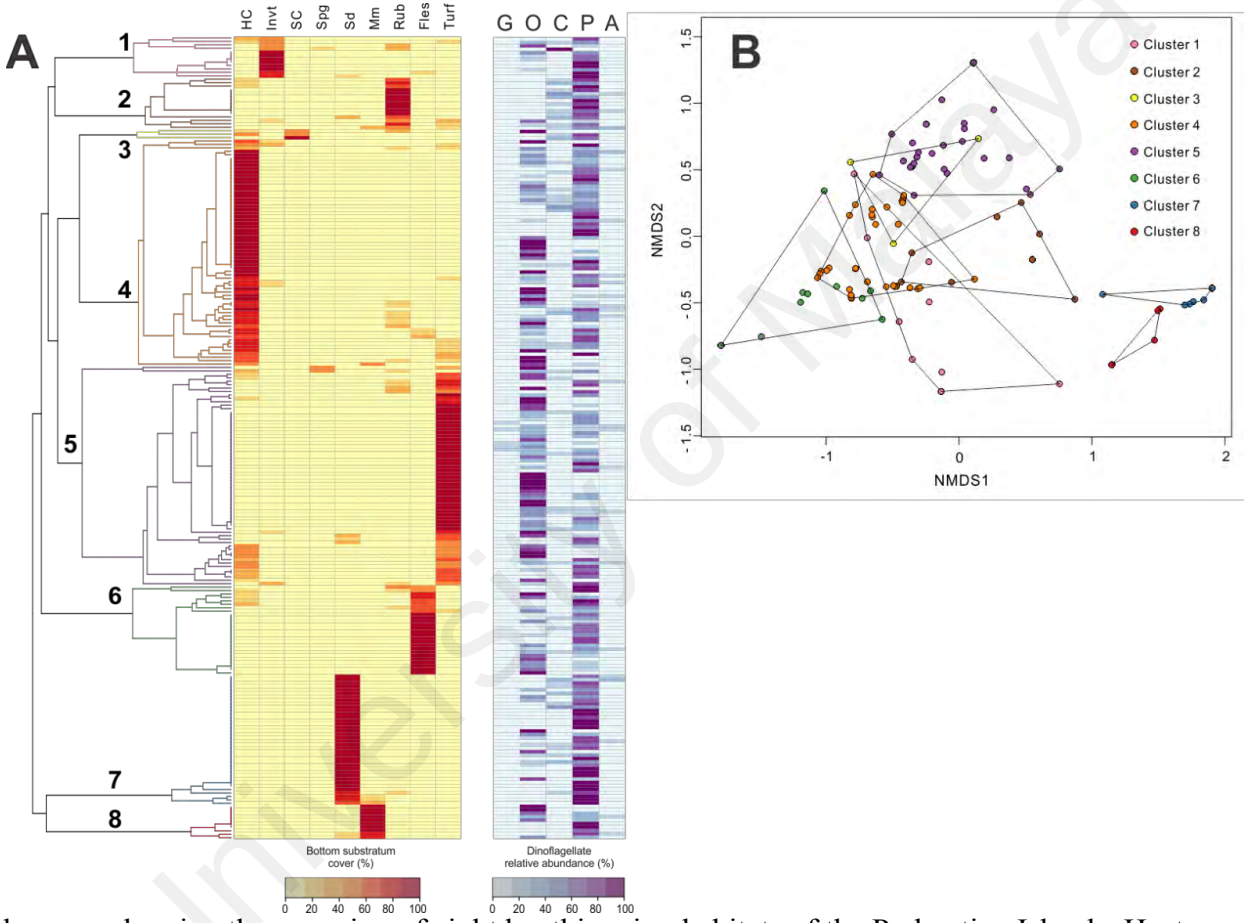

Figure 4.15: (A) Dendrogram showing the grouping of eight benthic microhabitats of the Perhentian Islands. Heatmap (left panel) of benthic substratum coverage (%) (see Table 4.9 for substratum descriptions). Heatmap on the right panel presents the relative abundances of benthic dinoflagellates corresponding to the defined clusters. G, Gambierdiscus; O, Ostreopsis; C, Coolia; P, Prorocentrum; A, Amphidinium. (B) Non-metric multidimensional scaling (nMDS) ordination plot based on the Bray-Curtis similarity of the log-transformed actual abundances. Dimension = 3, stress = 0.0594. Symbols differentiate the eight microhabitat clusters

Table 4.9: Description of defined clusters from cluster analysis microhabitat characteristics of Perhentian Islands.

Cluster	Microhabitat characteristics
Cluster 1	Mainly comprised of other invertebrates such as giant clams, sea anemone and corallimorph (Appendix A)
Cluster 2	Coarse rubbles and rock (Appendix A)
Cluster 3	Soft coral (Appendix A)
Cluster 4	Variety of hard coral regardless of growth formation (Appendix A)
Cluster 5	Mixed assemblages of microfilamentous turf algae such as *Polysiphonia, Ceramium, Neosiphonia* and *Lyngbya* (Appendix C)
Cluster 6	Variety of upright and fleshy macroalgae including *Padina* sp., *Dictyota* sp., *Jania* sp. and *Lobophora* sp (Appendix B)
Cluster 7	Sand and silt substrate (Appendix A)
Cluster 8	Mats-forming or slime formation which colourised on sand substratum or coral rubbles that commonly associated with cyanobacteria or diatoms (Appendix B)

4.3.4 Benthic dinoflagellates assemblages on benthic microhabitat in coral reefs

In this study, comparison of the benthic dinoflagellates assemblages present at different microhabitats of Perhentian Islands was made. The relative abundances differed over the benthic microhabitats examined (Fig. 4.15A). The assemblages were dominated by *Prorocentrum* (48–71%), except in cluster 5 (turf algae; 34%) where *Ostreopsis* was the most abundant group (51%). *Gambierdiscus*, *Coolia,* and *Amphidinium* remained minor elements in the assemblages throughout the microhabitat clusters examined (0–3.7%; 3.5–20.3%; 0–7.3%, respectively). Overall, the benthic microhabitat clusters 4 (hard corals) and 5 (turf algae) supported the highest abundances of benthic harmful dinoflagellates as compared to other benthic microhabitat types (Fig. 4.16). Clusters 1 (invertebrates), 2 (rubble and gravel), 3 (soft corals), and 8 (microalgal mats) were less preferred by these five groups of benthic harmful dinoflagellates.

Among the benthic harmful dinoflagellates, the distribution of *Prorocentrum* over the benthic microhabitats investigated was relatively homogenous, as no

significant difference was observed in cell abundances among the benthic microhabitat clusters (p = 0.1585). It is noted that the abundance of *Prorocentrum* reached a maximum of 1.4×10^4 cells/100 cm^2 in Cluster 1 (invertebrates) (Figs. 4.16). In contrast, *Ostreopsis* was strongly associated with Cluster 4 (hard corals) and Cluster 5 (turf algae) with the maximum cell abundances of 5750 and 5562 cells/100 cm^2, respectively, and with a high level of occurrences among samples (89.4 and 98.4%) (Fig. 4.16). This observation was supported by a non-parametric one-way ANOVA showing a significant difference in the *Ostreopsis* assemblages among different microhabitat clusters (p < 0.0001) (Fig. 4.17) *Ostreopsis* was also found frequently in habitat Cluster 6 (fleshy algae; 88.5% of occurrence, n = 26), but the maximum abundance was lower (2531 cells/100 cm^2) than found in Clusters 4 and 5. *Gambiediscus* showed a preference for habitat Clusters 5 (turf algae) and 6 (fleshy algae) (>40% of occurrence; Fig. 4.16), with maximum abundances (255 cells/100 cm^2) found in Cluster 5 (turf algae). Habitat specificity of *Gambierdiscus* is especially clear, as cells were not found in Clusters 1 (invertebrates) and 3 (soft corals), and at much lower occurrences in other clusters (6.7–28.8%). *Coolia* did not show significant differences (p = 0.1756) in their abundances among microhabitat clusters (>70% of occurrences in all substratum types); the highest cell abundance (368 cells/100 cm^2) was observed in Cluster 2 (rubble and gravel). Although abundances of *Amphidinium* were low (0–7.3%), the frequency of occurrences in some microhabitats was relatively high (27–81%), except Cluster 3 (soft corals) where no cells were detected. The highest occurrence of *Amphidinium* (81%) was attributed to Cluster 6 (fleshy macroalgae).

Benthic harmful dinoflagellates abundances and compositions varied greatly with depths (Fig. 4.16). With regard to the depth distribution, benthic harmful dinoflagellates were relatively abundant at intermediate depths, where the average maximum abundances were observed at depths of <10 m (Fig. 4.16). *Prorocentrum* and

Coolia have broader depth distribution ranges as compared to *Gambierdiscus, Ostreopsis,* and *Amphidinium. Prorocentrum* and *Coolia* were ubiquitous, occurred at all depths along the entire depth gradient investigated (<25 m), while *Ostreopsis*, *Gambierdiscus* and *Amphidinium* are more aggregated at the depths of <10 m and scarcely found in the water depth of >10 m. The deepest habitable depth for *Ostreopsis*, *Gambierdiscus* and *Amphidinium* was 16 m, while *Prorocentrum* and *Coolia* were able to inhabit as deep as 25 m (Fig. 4.16).

Ostreopsis aggregated between the depths ranged in 1 to 10 m at Cluster 4 (hard corals) but absent in the same microhabitat at >15 m; likewise, the populations were rarely found at deeper depths (>15 m) in most of the microhabitat clusters. *Gambierdiscus* displayed similar depth distribution pattern as in *Ostreopsis* where they are rarely observed at depth >15 m but was found colonising different microhabitats at different depths: Cluster 4 (hard corals) at depths <5 m and Cluster 5 (turf algae) between the depth ranges of 5 and 10 m. *Prorocentrum* has dominated all microhabitat clusters throughout the depth range investigated.

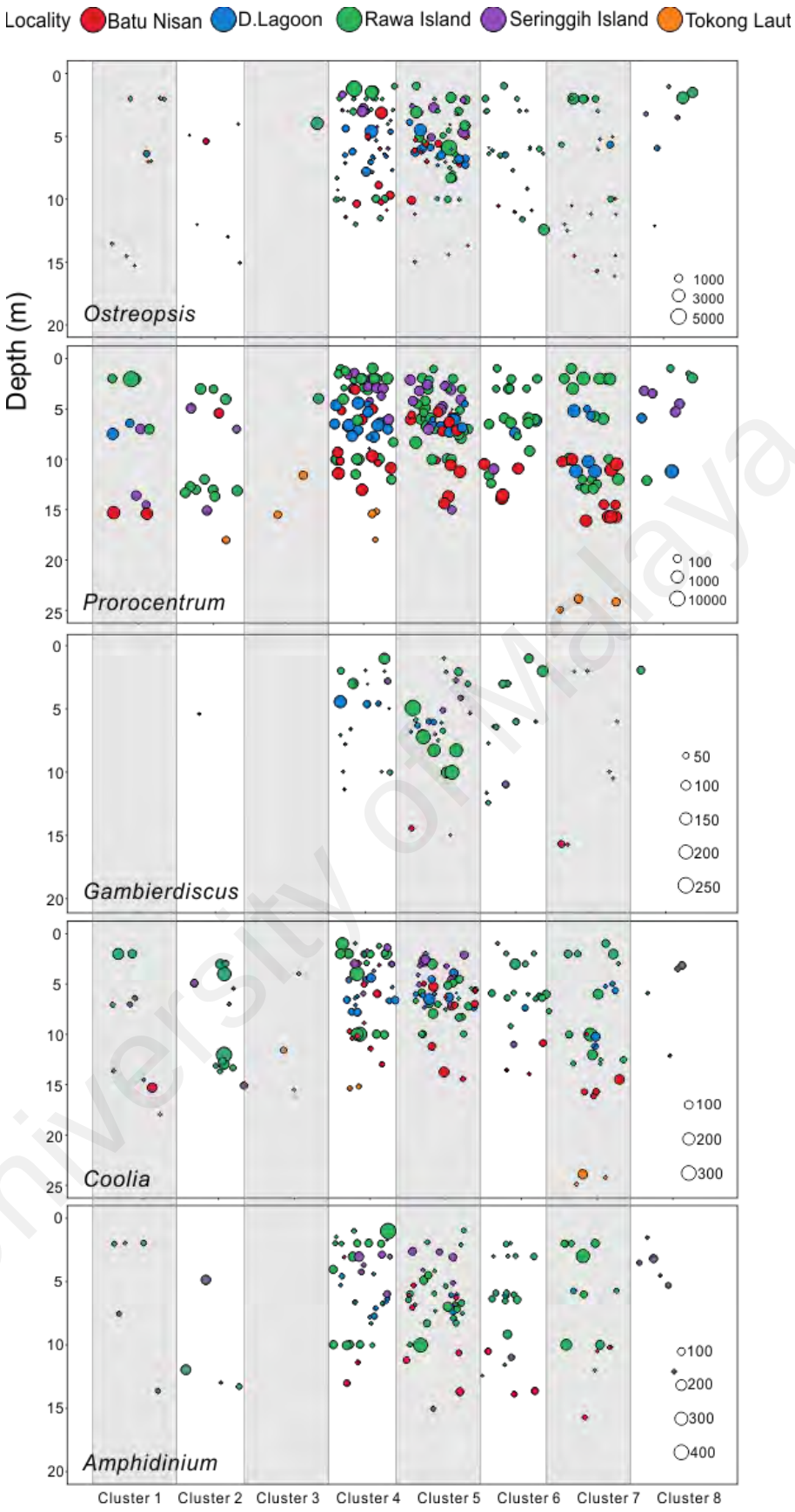

Figure 4.16. Bubble chart visualised the concomitant of benthic harmful dinoflagellates with the reef benthic microhabitat clusters (refer to Table 4.9) and depth profile across the five sampling sites in Perhentian Islands, with the respective size of circles representing cell abundance ranges [cells 100 cm^{-2}].

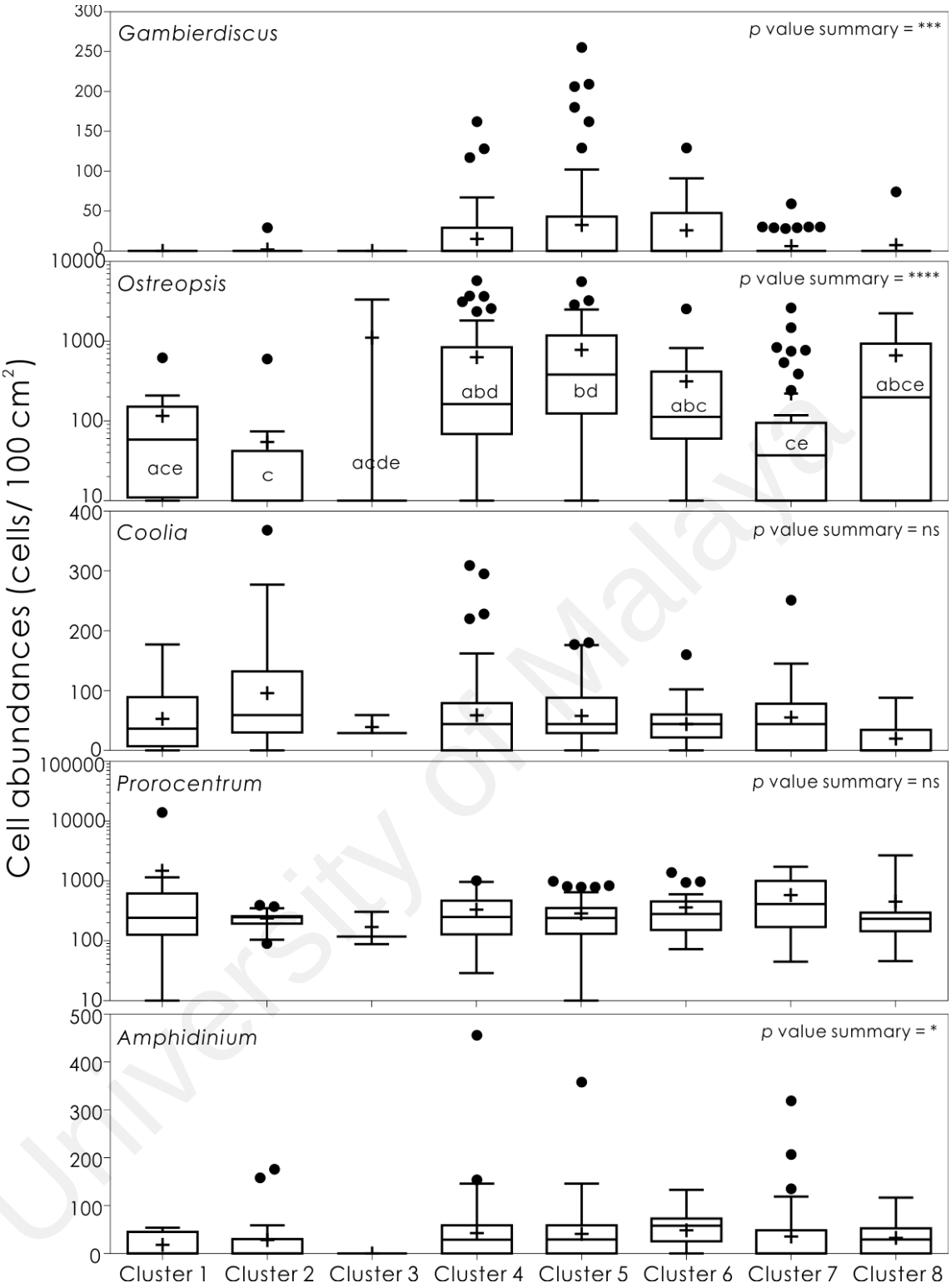

Figure 4.17: Tukey plot of benthic dinoflagellates cell abundances reflect on benthic microhabitat clusters with *p*-value summary of Kruskal-Wallis test and alphabet indicate the outcome of Dunn's multiple comparisons test. "+" showed mean; horizontal line in box showed median; Box ends at the quartiles Q_1 and Q_3. Whiskers showed the upper and lower extreme; Dots represents outliers. Ns: $p > 0.05$; "*": $p \leq 0.05$; "**": $p \leq 0.01$; "***": $p \leq 0.001$; "****": $p \leq 0.0001$.

4.3.5 Temporal discrepancies in assemblages of benthic dinoflagellates

A total of 234 artificial substrates were deployed using SCUBA diving over the 14-month survey from April 2016 to May 2017 in Perhentian Islands. Data on cell abundances of benthic dinoflagellates were charted from April 2016 to May 2017 (Figure 4.18). Data gaps existed from November to early February due to unfavourable weather condition for data collection during the northeast monsoon (wet season).

The highest abundance of *Gambierdiscus* was observed in June 2016, with 255 cells/100 cm^2. Both *Coolia* and *Amphidinium* attained highest abundances in April with 367 and 456 cells/100 cm^2. *Prorocentrum* cell abundances achieved the highest abundances in April (13,949 cells/100 cm^2). *Ostreopsis* attained the highest abundances in February, with the abundances of 5,750 cells/100 cm^2. No clear temporal pattern was observed for abundances of benthic dinoflagellates in Perhentian Islands. However, higher abundances were observed in the month with higher average water temperature and irradiances compare to month with lower temperature and low irradiances such as *Gambierdiscus* in June with a monthly water temperature of 31.5 °C and irradiances of 500 µmol photons m^{-2} s^{-1}. The abundances of *Coolia*, *Prorocentrum* and *Amphidinium* peak in April when the average water temperature was 32 °C and irradiances 672 µmol photons m^{-2} s^{-1} (Figure 4.12). Only *Ostreopsis* showed a contrary trend where two similar peak abundances were detected when average water temperature and irradiances were relatively lower in October (31°C; 168 µmol photons m^{-2} s^{-1}) and February (29 °C; 203 µmol photons m^{-2} s^{-1}). The Kruskal Wallis test showed that no significant difference was detected among the monthly abundances for *Gambierdiscus*, but highly significant for *Ostreopsis* ($p \leq 0.0001$), *Coolia* ($p \leq 0.0001$), *Prorocentrum* ($p \leq 0.0001$) and *Amphidinium* ($p \leq 0.001$). The multiple comparison tests for benthic dinoflagellates abundances between months was presented in Appendix J. For *Ostreopsis*, the monthly abundances between May-16, Sep-16, Oct-16, Feb-17, Mar-17 was highly significant (*p*

≤ 0.0001 or $p \leq 0.001$). *Coolia* abundances were highly significant between Apr-16, Jun-16, Oct-16 and Apr-17. A highly significant difference was detected in abundances of *Prorocentrum* between monthly comparison except for Jun-16, Aug-16 and May-17.

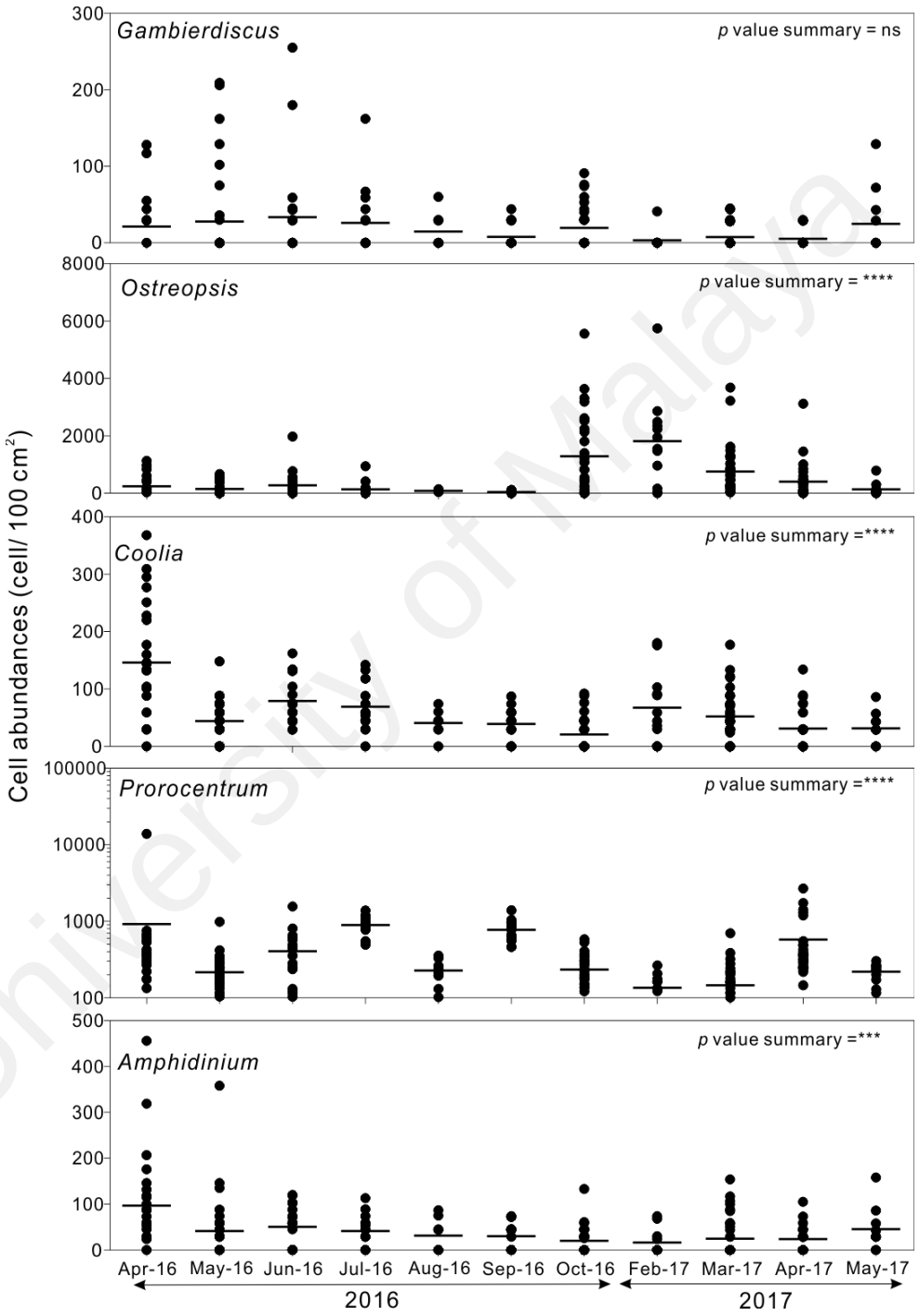

Figure 4.18: Temporal variations of benthic dinoflagellate abundances from April 2016 until May 2017 in Perhentian Islands with *p*-value summary of the Kruskal-Wallis test. The horizontal line indicates the mean value. Ns: $p > 0.05$; "*": $p \leq 0.05$; "**": $p \leq 0.01$; "***": $p \leq 0.001$; "****": $p \leq 0.0001$.

4.3.6 Relationship of benthic dinoflagellate assemblages with benthic substratum characteristics, depth, irradiances, temperature and temporal variability

A significant difference was recorded in term of the relationship between benthic harmful dinoflagellate (BHD) abundances and microhabitat characteristics and depth (CCA, F= 3.647, $p < 0.001$) indicating a strong correlation between BHD abundances and the microhabitat characteristics and depth. The horizontal axis (CCA1) explained 68.1% (eigenvalue, 0.036, $p = 0.001$) of this constrained variation, and the vertical axis (CCA2) explains 18.9 % (eigenvalue, 0.01, $p = 0.093$) (Fig. 4.19), taken together, both axes of the data set explained 87.1 % of total inertia.

The CCA clearly separated *Prorocentrum–Coolia* (CCA1<0) and *Gambierdiscus–Ostreopsis–Amphidinium* (CCA1>0) (Fig. 4.19) which implied the pairing of *Prorocentrum–Coolia* and *Gambierdiscus–Ostreopsis–Amphidinium* have a more similar ecological niche with each other respectively. Significant correlation was detected between BHD abundances and microhabitat characteristics and depth such as soft coral (CCA, F = 2.196, $p = 0.043$), sponge (CCA, F = 2.350, $p = 0.042$), highly significant was detected for sand (CCA, F = 7.098, $p = 0.001$), rubble (CCA, F = 7.581, $p = 0.001$), turf algae (CCA, F= 4.874, $p = 0.001$) and depth (CCA, F= 5.813, $p = 0.001$). *Prorocentrum* and *Coolia* seem to be positively correlated with rubble and sandy substrate microhabitat, as well as substrate dominated by invertebrates, sponges, soft corals and microbial mats. The pairing of *Prorocentrum* and *Coolia* also positively correlated with depth. This partially supported the observation of *Prorocentrum* and *Coolia* abundances in deeper water. *Prorocentrum* showed a stronger association with sand and silt while *Coolia* has a stronger affinity towards rubble/rock substratum.

The trio of *Gambierdiscus–Ostreopsis–Amphidinium* were more positively correlated with hard coral, turf algae assemblages and flesh macroalgae, and negatively correlated with depth. These supported the observation of higher abundances of

Gambierdiscus–Ostreopsis–Amphidinium in shallower water and likely to be associated with microhabitat characteristics aforementioned. Both abundances of *Gambierdiscus* and *Amphidinium* showed affinity toward high turf algae and fleshy macroalgae Likewise, *Ostreopsis* abundances were associated with hard corals and turf algae assemblages.

The effect of temporal changes (wet and dry season) on BHD abundances was further explained in Fig. 4.20 with relation to temperature and irradiance variability. The effect of irradiances and temperature on BHD abundances were highly significant (CCA, F = 7.493, p = 0.001) where horizontal axis (CCA1) significantly explains 84.8 % of variances (eigenvalue = 0.020, p = 0.001) but not vertical axis (CCA2, eigenvalue = 0.004, p = 0.057). The effect of temperature fluctuation significantly affect the changes in BHD abundances (CCA, F = 12.668, p = 0.001) than effect of irradiances (CCA, F = 2.317, p = 0.059). *Gambierdiscus*, *Amphidinium* and *Coolia* were positively correlated with temperature and irradiances while *Ostreopsis* were negatively correlated with temperature but show a positive association with irradiance. *Prorocentrum* has a positive correlation with temperature but a negative correlation with irradiance.

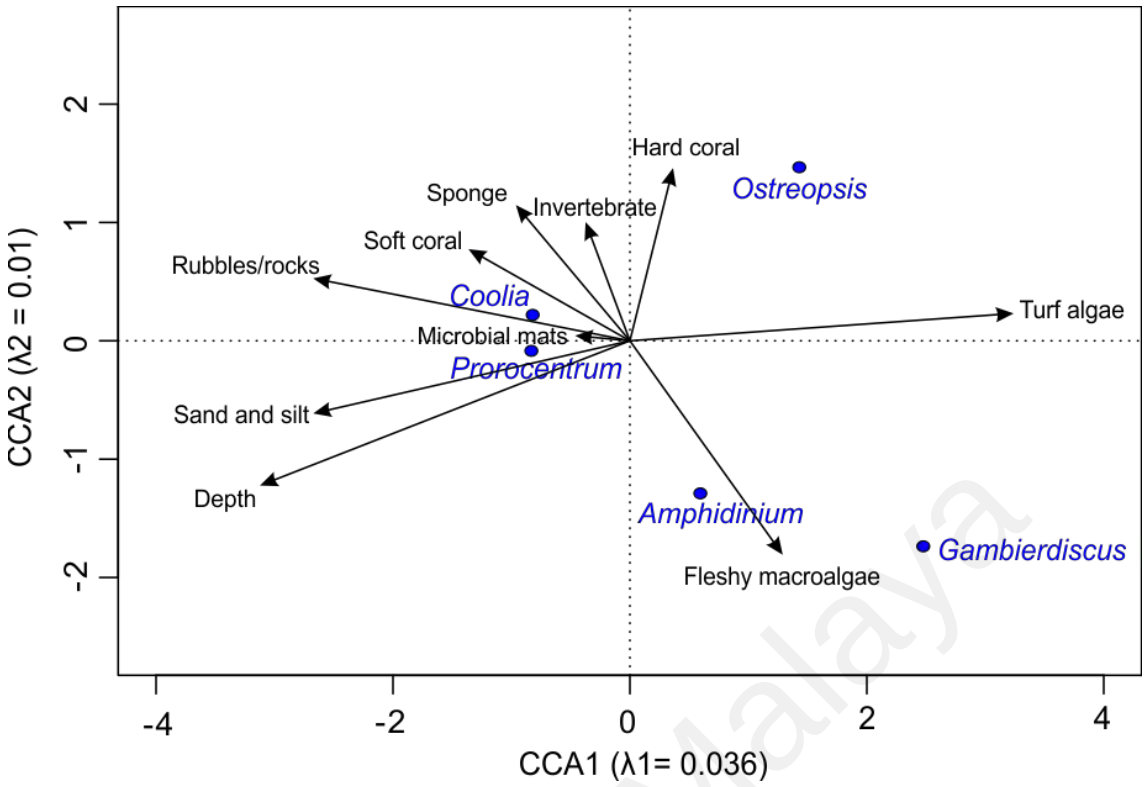

Figure 4.19: CCA TriPlot depicting association between benthic dinoflagellates assemblages, reef benthic microhabitat and depth in all sampling sites in Perhentian Islands. The eigenvalue of the first two axed is indicated by *λ1 and λ2.*

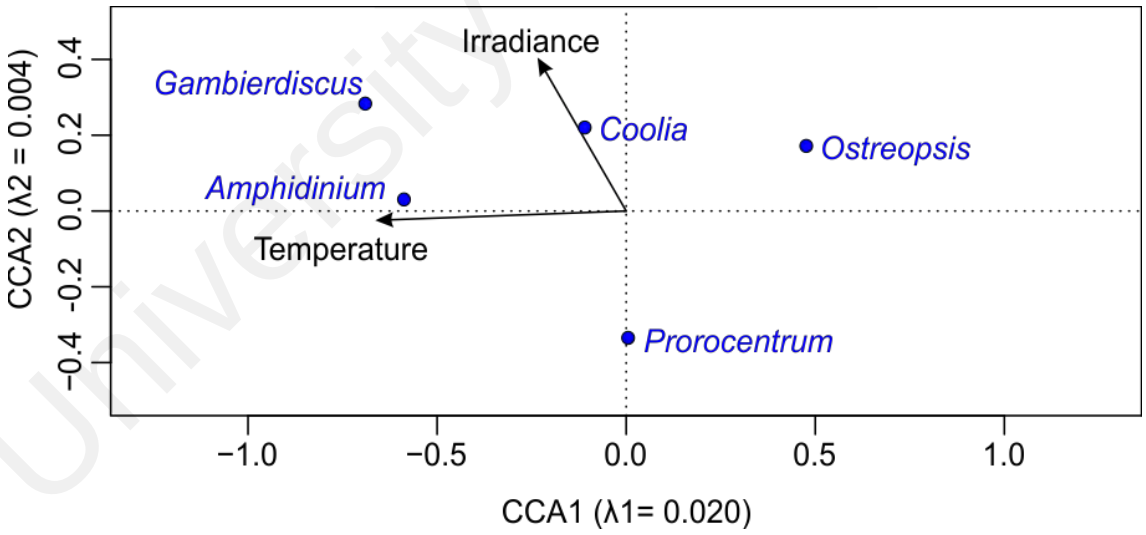

Figure 4.20: CCA TriPlot depicting association between benthic dinoflagellates assemblages and environmental factors (irradiance and temperature) (April 2016 to April 2017) in the Perhentian Islands. The eigenvalue of the first two axed is indicated by *λ1 and λ2.*

CHAPTER 5: DISCUSSION

5.1 Diversity of athecate benthic dinoflagellate in the Perhentian Islands

A wide variety of morphological features were used to identify *Amphidinium* species in addition to size and shape, such as presence, location and number of nuclei, pustules, pyrenoids, chloroplasts, eyespots, scales and life cycle stages (Karafas et al., 2017). In this study, three species of benthic athecate dinoflagellates have been documented for the first time in Malaysia with both morphological and molecular records. *Amphidinium operculatum* was mostly reported in the temperate region, Norway, Japan, New Zealand, France, Canada, Korea (Murray et al., 2004; Shah et al., 2014). Although *A. operculatum* has been reported in Sabah, Malaysia (Mohammad-Noor et al., 2004), no molecular data was available for phylogenetic reconstruction. Nevertheless, the morphological observation was similar to that observed in Mohammad-Noor et al. (2004). Morphologically, *A. massartii* and *A. carterae* are very difficult to distinguish with certainty based on LM as both overlaps completely in size range but differ by the shape of plastid (Murray et al., 2004). Hence, in this study, the phylogenetic approach was conducted to differentiate the species. The strains obtained that have a similar morphological observation to *A. carterae* somehow claded differently in our phylogenetic tree (Figure 4.9), and the interspecific divergence between *A. carterae/A. massartii* and *A. carterae/A.* cf. *massartii* were 8.2 – 10.1 % and 9.9 – 10.8 % respectively. Moreover, strains from this study claded together into *A.* cf. *massartii* clade as presented by Karafas et al. (2017). *A. massartii* and *A.* cf. *massartii* were hard to tell apart morphologically, but genetic diversity between two clades of *A. massartii* (in this study, the interspecific divergence ranged in 4.4 – 5.3 %) suggested that *A. massartii* and *A.* cf. *massartii* may represent two different species but no unique morphological trait was to be observed to warrant the separation of the species (Karafas et al., 2017).

Another athecate benthic dinoflagellate, *Gymnodinium dorsalisulcum* is reported for the first time in Malaysia, conciling the molecular data. The species was first described by Murray et al. (2007a) from tropical northern Australia. The morphological observation in this study agreed with the previous studies. *Gymnodinium dorsalisulcum* differs with other *Gymnodinium* species with similar morphological observation in term of behavioural aspects such as benthic habitat, mucus production (observed in culture in this study) and non-chain forming (Murray et al., 2007a).

5.2 Diversity of thecate benthic dinoflagellates in Perhentian Islands: Genus *Prorocentrum*

Prorocentrum was the dominant genus in terms of distribution and assemblages of benthic dinoflagellates in Perhentian Islands. Five species of *Prorocentrum* have been recorded in this study. In Mohammad-Noor et al. (2004), eleven species of *Prorocentrum* were identified morphologically without molecular data, namely *P. arenarium*, *P. concavum*, *P. emarginatum*, *P.* cf. *faustiae*, *P. foraminosum*, *P. formosum*, *P. lima*, *P. norrisianum*, *P. rhathymum*, *P.* scuptile, and *P. sipadanensis*. Among the eleven species, some of them were extremely similar morphologically and had been described without molecular evidence. For example, *P. arenarium* was determined to be within the range of morphological variation of *P. lima* and currently has been synonymised with *P. lima* (Nagahama et al., 2011). The strain that has been described by Mohammad-Noor et al. (2004) as *P. arenarium* was now described as *P. caipirignum* (Nascimento et al., 2017). *Prorocentrum concavum* was similar to *P. faustiae* morphologically and needed molecular reinvestigation to clarify whether they are conspecific. As for *P. rhathymum,* the name has been synonymised by Gómez et al. (2017) as the junior synonym of *P. mexicanum*, although *P. rhathymum* is still widely used in literature.

In this study, all four species of *Prorocentrum* recorded in this study has been determined morphologically and molecularly to confirm the position of strains in the phylogenetic topology, except for *P. fukuyoi* due to lost of cultured strain. *P. concavum* has the largest cell size and in a broad oval shape, reticulate-foveate ornamentation can be observed in theca plates which make it stand out from other *Prorocentrum* species. Cells of *P. mexicanum* can be easily distinguished among the five species with wide V-shaped and present of the protrusion in the periflagellar area. Cells of *P. emarginatum* and *P. fukuyoi* were both with deep and narrow V-shaped. The periflagellar area has thick flange extended and wing-shaped spine bordering the platelet in the periflagellar area which visible in LM. Both also observed with the thecal surface of radial rows of pores. However, the reliability of the features to distinguish these species are not clear yet where the species complex contains a considerable level of genetic diversity among strains and species (Hoppenrath et al., 2013). But the identification of *P. emarginatum* was supported in LSU phylogenetic tree and considerable wide interspecific divergences between *P. emarginatum* and *P. fukuyoi*. Cells of *P. lima* have great plasticity in cell shape from almost round to oblong oval to ovoid cell. Pyrenoid with starch sheath, visible as a ring in the centre of the cell was prominent in LM. However, due to the wide range of morphological variability has been reported for *P. lima*, it was proposed to be *P. lima* complex which contained cryptic diversity (Hoppenrath et al., 2013). Nevertheless, the strains from Perhentian Islands was successfully claded together with other *P. lima* and clade out with other sister taxa such as *P. hoffmannium* species complex, the newly described *P. caipirignum* (Nascimento et al., 2017) and one *P.* cf. *lima* morphotype 5 in LSU phylogeny with considerable interspecific divergence between *P. lima* to *P. hoffmannium* species complex, *P. caipirignum* and *P.* cf. *lima* morphotype 5 with 4.0 – 5.7 %, 3.8 – 5.3 % and 4.2 – 5.5 % respectively.

5.2.1 Diversity of *Ostreopsis* and *Coolia* in Perhentian Islands with the first report of *C. palmyrensis* and *C. cf. canariensis*

Only one species of *Ostreopsis* was documented in this study, the cell size of *O.* cf. *ovata* recorded in this study overlapped and was similar in morphological observation to strains found in Thailand waters (Tawong et al., 2014) and in Malaysia (Leaw et al., 2001; Mohammad-Noor et al., 2004) but smaller compared to *O. ovata* originally described by Fukuyo (1981) which have DV of 50-56 µm and 25-35 µm of trans-diameter. Mohammad-Noor et al. (2004) samples have cell size ranges from 25 – 35 µm dorsoventrally and 15 – 30 µm in transdiameter. Leaw et al. (2001) documented a wider cell size range from 33 to 55 µm dorsoventrally, 22 to 39 µm in transdiameter. Tawong et al. (2014) presented two subclades of *O.* cf. *ovata* and assigned them into Thailand subclade and South China Sea subclade with phylogenetic support. However, no distinctive characters were presented to support the introduction of novel species as the thecal pattern was similar and cell size range overlapped.

The comparison of major morphological features of seven *Coolia* species including *C.* cf. *canariensis* was compiled in Karafas et al. (2015). In term of cell size, the four species of *Coolia* documented in this study fitted with the range of AP length and DV width as presented in Karafas et al. (2015). Generally, *C. tropicalis* was largest among the four species, followed by *C. canariensis*, *C. malayensis* and *C. palmyrensis*. In term of apical plate differences, *C. tropicalis* and *C. canariensis* are closely related with the largest plate of epitheca in 4′ and occupied a central position. In contrast, *C. malayensis* and *C. palmyrensis* share similar apical plate features by having 4′ that was oblong and narrow, positioned to left of centre, while the largest plate of epitheca was 5′′. *C. tropicalis* can differentiate from *C. canariensis* by the 6′′ LW ratio where *C. tropicalis* have larger ratio compare to *C. canariensis*. Another key feature of *C. canariensis* is the ornamentation in the hypothecal plate which is pitted resembles

ornamentation of *C. areolata* but smooth apical plate while *C. tropicalis* has a smooth hypothecal surface (Fraga et al., 2008). For *C. malayensis* and *C. palmyrensis,* density and size of pores on apical plates were used to tell apart the two species where *C. palmyrensis* has relatively low pore density in theca surface compare to other *Coolia* species (Karafas et al., 2015).

Coolia palmyrensis was described with type locality in Palmyra Atoll which was isolated from sandy sediments and macroalgae (Karafas et al., 2015). A similar observation was first reported by Momigliano et al. (2013) with isolates from Great Barrier Reef which resembles the species described; the strains were later assigned as *C. palmyrensis* (Leaw et al., 2016). Morphologically, *C. palmyrensis* was closest to *C. santacroce, C. monotis, C. malayensis* which all share similar features such as apical plate 4' was oblong and narrow, positioned to the left of center (Karafas et al., 2015) and sharing of common traits were corresponding in the phylogenetic tree as these four species formed a monophyletic group (Leaw et al., 2016). Nevertheless, *C. palmyrensis* cladded out from its closest sister taxa (*C. santacroce, C. monotis* and *C. malayensis*) even though with low supports in MP/ML/BI but high interspecific divergence. The key features that can be used to differentiate morphologically between the four species were cell size, apical pore size, size and density of pores/poroids (Karafas et al., 2015) where *C. palmyrensis* was the smallest among *Coolia* species and have the lowest density of pores.

For *C. canariensis,* the existence of two distinct lineages of *C. canariensis* has been reported ever since the species was first described by Fraga et al. (2008) with isolates from Tenerife, Canary Islands. With the increased number of isolates discovered elsewhere, the divergences of this two lineage become clearer and hypothesised that it represents cryptic species (Fraga et al., 2008; Jeong et al., 2012b; Momigliano et al., 2013; David et al., 2014). Jeong et al. (2012b) and David et al.

(2014) noticed though divergence between *C. canariensis* clades was less than that of *C. monotis* and *C. malayensis* but acknowledge that the separation was adequate to delineate the clades. In this study, the range of interspecific divergence that delineates closely related group yet been taxonomically recognised as two separate species, *C. monotis* and *C. malayensis* was 10.1 – 11.0 % and the interspecific divergence between the two lineages in *C. canariensis* has a range of 16.1 – 16.6 % in LSU rDNA. This indicated no overlapping of divergence range and further strengthened the hypothesis of two unique taxa exist in *C. canariensis* clades. However, no distinctive morphological features were available to differentiate the two subclades, and acquisition of more resolute molecular marker dataset such as ITS2 was vital to solve the puzzle (Leaw et al., 2016).

5.2.2 Diversity of *Gambierdiscus* in Malaysian waters

In Asia, a total of five species and two ribotypes of *Gambierdiscus* species, and one species of *Fukuyoa* were reported in the region, namely, *G. balechii* (Dai et al., 2017), *G. caribaeus* (Jeong et al., 2012a; Tawong et al., 2016; Zhang et al., 2016), *G. pacificus, G. australes.* (Zhang et al., 2016), *G. scabrosus, G.* sp type 2 and *G.* sp. type 3 (Nishimura et al., 2013; Nishimura et al., 2014), *F. yasumotoi* (Holmes, 1998).

In this study, three *Gambierdiscus* species were documented in the Perhentian Islands, the comparison of cell sizes and thecal morphometric measurement among three species with the previous study is presented in Table 5.1. Among the three species, *C. caribaeus* has the biggest cell size, postcingular plate 2′′′′ was broad with LW ratio of 1.27, apical plate 2′ has a rectangular shape with suture length ratio between 2′/1′′ and 2′/3′′ of 0.88, highest suture ratio among the three species documented. *Gambierdiscus balechii* and *G. pacificus* were considered closely related taxa, with similar hatchet shaped apical plate 2′, narrow postcingular plate 2′′′′, but *G. balechii*

has heavily ornamented theca surface while *G. pacificus* has smooth theca surface. Cells of *G. pacificus* have higher LW ratio in antapical plate 2′′′′ (2.21) compare to *G. balechii* (1.69). The apical plate 2' of *G. pacificus* has more clear hatchet-shaped with lower suture length ratio between 2′/1′′ and 2′/3′′ of 0.43 compare to the ratio of 0.57 in antapical plate 2′ of *G. balechii*.

The discovery of *Gambierdiscus* species in the Southeast Asia region was first reported by Holmes (1998) from coral reef surrounding Pulau Hantu, Singapore. The reported species was *G. yasumotoi* with a globular shape which been rectified by Gómez et al. (2015) into a new genus as *Fukuyoa yasumotoi*. Mohamma-Noor et al. (2004) reported *G. pacificus* and Leaw et al. (2011) reported *G. belizeanus* from Sabah, Malaysian Borneo based only on morphological observation. The *G. pacificus* described in Mohammad-Noor et al. (2004) has average cell size of 61 ± 1.5 µm in length and in width 55 ± 2.5 µm, the thecal surface is perforated, the second apical plate, 2′ is narrow and rectangular and the second antapical plate, 2′′′′ is narrow. However, no suture length ratio of 2′/1′′ and 2′/3′′ was recorded for comparison. With re-examination of the micrograph (Figs 26a-g) presented in the paper of Mohammad-Noor et al. (2004), the theca surface looks ornamented and plate 2′ was in hatchet-shaped rather than rectangular. These features put the species reported closer to *G. balechii* as *G. pacificus* has smooth theca surface as presented by Chinain et al. (1999a). In the case of *G. belizeanus*, the strains reported has high resemblance in thecal morphology and morphometric measurement to *G. balechii* as pointed out by Dai et al. (2017). However, high thecal shape variations may cause confusion in identification when only using morphological diagnostic approach. For example, Fraga et al. (2016) highlighted the variation of plate 2′ in strains of *G. balechii* from clear-hatchet shaped to almost rectangular. Another variation was noticed in plate 2′′′′ by Dai et al. (2017). Hence,

with a lack of genetic data available, no further rectification can be done with phylogenetic analysis.

Phylogenetic inferences of *Gambierdiscus* species was well documented and was refined with each increase in number of described species and discovery of ribotype in the recent years (Xu et al., 2014; Fraga et al., 2016; Smith et al., 2016; Kretzschmar et al., 2017; Rhodes et al., 2017a). Currently, most comprehensive phylogenetic analysis of *Gambierdiscus* species based on D8-D10 region of the LSU rDNA dataset was documented in Dai et al. (2017) with a total of 230 available sequences except those with ambiguous nucleotide. The verification of *G. caribaeus* and *G. balechii* was further supported by phylogenetic analysis and uncorrected pairwise divergence in this study.

However, due to ambiguous nucleotides in the sequences of some strains obtained in this study, an anomaly of Long Branch effects (Bergsten, 2005) was observed in *G. balechii* clade and resulted in high variation in intraspecific divergence; however, the strains were still clustered together with other *G. balechii* forming the monophyletic clade. The inclusion of *G. balechii* strains from Perhentian Islands in the uncorrected pairwise divergence yielded intraspecific divergence of 0.2 – 5.4 %, while without the Perhentian Islands strains yielded 0.2 – 1.8 %. Further inspection of sequence alignment among *G. balechii* sequences included in this study showed that SNP (single nucleotide polymorphism) and INDEL (insertion or deletion of bases) were presenced as this likely explained the anomalies in divergence and the Long Branch effect in our phylogenetic tree. The ambiguous nucleotides may indicate the presence of pseudogenes which were defined as defective copies of ribosomal genes that are retained in the genome (Litaker et al., 2009). The existence of pseudogenes was well known for dinoflagellates and complication imposed by pseudogenes can be problematic when interpreting of variation in the rDNA sequence (Santos et al., 2003; Litaker et al., 2007).

In this study, the intraspecific and interspecific divergences of *G.balechii* and its sister taxa (*G. lapillus, G. pacificus, G. toxicus, G. cheloniae*) was shown to be overlapping (intraspecific ranged in 0 to 1.9 %, interspecific ranged in 1.6 to 7.2 %). As for *G. caribaeus* and its sister taxa (*G. carpenteri* and *G.* sp type 2) has intraspecific divergence range of 0 to 0.6 %, interspecific divergence range of 0.5 to 1.8 % which shown to be overlapping as well. In Dai et al. (2017) study, the overall intraspecific divergence range from 0 to 3.6 % in comparison to divergences of *G.balechii* and its related sister taxa ranged in 1.7 to 4.9 % and *G. caribaeus* with its sister taxa ranged in 0.8 to 2.5 % for LSU dataset which also shows overlapping in range of value. In proposition of Litaker et al. (2007), uncorrected genetic distance (*p*) values ≥ 4 % can be used as a threshold for species-level boundary based on ITS/5.8s variant analysis for most free-living dinoflagellate species. Yet, estimation of genetic variation for different regions in rDNAs that can be used for species boundary still remain indecisive (Nishimura et al., 2013; Fraga et al., 2016; Smith et al., 2016; Dai et al., 2017).

Hence, Dai et al. (2017) demonstrated the combination of comprehensive phylogenetic analyses with nucleotide sequence and sequence-structure information. The pairing of morphological systematic and maximum likelihood tree inferred using SSU rDNA sequence-structure information of *Gambierdiscus* species provide an informative, accurate and robust explanatory framework for evolutionary insight (Dai et al., 2017). Similar approaches have been presented in other dinoflagellates such as *Coolia* (Leaw et al., 2016).

Table 5.1: Comparison of average cell size, a thecal morphometric measurement of three species of *Gambierdiscus* recorded with previous studies. Bold indicate data recorded in this study. n = 10. Bracket indicate standard deviation, "-" data unavailable.

Species	Cell Size (µm)			Plate 2″ (µm)			Apical pore plate (Po) (µm)			Sutural ratio	Reference
	Depth	Width	L:W	Length	Width	L:W	Length	Width	L:W	2′/1″ :2′/3″	
G. balechii	59 (6.3)	60 (6.7)	0.69	30 (3.2)	18 (2.4)	1.72	5.6 (1.1)	4.1 (0.8)	1.4	0.64 (0.11)	Dai et al. (2017)
	57 (0.3)	60 (0.3)	0.65	28	14	2	5.3	3.5	1.51	0.64 (0.14)	Fraga et al. (2016)
	59.7 (4.5)	**56.7 (3.4)**	**0.76 (0.07)**	**28.1 (1.4)**	**16.6 (0.4)**	**1.69 (0.12)**	**6.2 (0.2)**	**4.34 (0.6)**	**1.47 (0.16)**	**0.57 (0.06)**	
G. pacificus	70 (4.7)	63 (3.6)	0.71	36 (3.5)	14 (3.5)	2.57	5.2 (0.3)	4.1 (0.4)	1.27	0.36 (0.10)	Litaker et al. (2009), Chinain et al. (1999a)
	65.8 (5.1)	60.7 (4.5)	0.73	31.1 (2.1)	13.6 (0.9)	2.3 (0.2)	5.9 (0.4)	4.3 (0.3)	1.4 (0.1)	-	(Zhang et al., 2016)
	63.5 (3.4)	**59.1 (4.2)**	**0.69 (0.04)**	**31.3 (2.3)**	**14.2 (1.1)**	**2.21 (0.25)**	**6.4 (0.8)**	**5.1 (0.8)**	**1.26 (0.10)**	**0.43 (0.06)**	
G. caribaeus	77 (6.1)	79 (8.4)	0.73	43 (6.4)	34 (6.8)	1.26	8.3 (1.3)	5.3 (0.8)	1.59 (0.26)	0.91 (0.09)	Litaker et al. (2009)
	87.6 (6.8)	84.7 (6.5)	0.64	51.0 (3.3)	35.0 (2.1)	1.5 (0.1)	8.4 (0.6)	5.5 (0.5)	1.5 (0.2)	-	(Zhang et al., 2016)
	82.6 (1.6)	**80.6 (6.5)**	**0.58 (0.05)**	**39.4 (5.1)**	**31.2 (6.1)**	**1.27 (0.12)**	**9.9 (0.8)**	**7.2 (0.2)**	**1.41 (0.21)**	**0.88 (0.13)**	

5.3 Ciguatoxicity of *Gambierdiscus* in different regions

The preliminary ciguatoxicity screening of *Gambierdiscus* from Perhentian Islands showed strain-specific toxicity scenario, strains of *G. balechii* and *G. caribaeus* were tested positive in ciguatoxin with neuro-2A bioassay but not all. Strains of *G. balechii* from Manado, Indonesia (Fraga et al., 2016) has been tested positive in ciguatoxicity while Dai et al. (2017) recorded mixed result where strains from Marakei, Kiribati showed positive but not from Perhentian Islands, Malaysia strains. The strain-specificity in toxin production was noted in Xu et al. (2014) where strains of *G.* sp type 5 from the same location with a mixed result in the ciguatoxicity test. The similar observation was also reported for *G. caribaeus* from different locality as reviewed in Litaker et al. (2017) with the closest locality was *G. caribaeus* found in Thailand which tested positive in ciguatoxicity using mouse bioassay (Tawong et al., 2016). Larsson et al. (2018) provided another curious insight where no current characterised microalgal CTXs were detected with LC-MS/MS analyses but CTX-like activity was detected using functional assay, a similar observation was reported by Kohli et al. (2014) and Kretzschmar et al. (2017) as well. This fuelled speculation of unrecognised novel ciguatoxin which structurally similar yet with different masses existed as currently characterised ciguatoxins of microalgal origin was still progressing (Larsson et al., 2018).

Based on thorough compilation by Litaker et al. (2017) and with few supplementary in this study (In Table 4.1) validated the production of ciguatoxin was not species-specific exclusively as almost all species described were capable of producing ciguatoxin or related congener that was undisclosed, but it was cleared that some species were more predominant in ciguatoxin production from different region (Chinain et al., 2010a; Litaker et al., 2017; Pisapia et al., 2017a). Chinain et al. (2010a) demonstrated the existence of "super-producing strains" in *G. polynesiensis* clones

which comparatively has high ciguatoxin activity than other species. The authors further deduced that *G. polynesiensis* regarded as the predominant ciguatoxin producer in the South Pacific region (Chinain et al., 2010a; Rhodes et al., 2014). While (Litaker et al. (2017)) and Pisapia et al. (2017a) inferred that *G. excentricus* likely to be primary ciguatoxin producing species in the Caribbean and Eastern Atlantic Oceans as *G. polynesiensis* has not been identified in the region. Both species were remarked as high toxicity species among 13 described species. The hypothesis was reinforced with a concomitantly high incidence rate of ciguatera fish poisoning in both South Pacific and Caribbean region (Chateau-Degat et al., 2007; Tester et al., 2010; Friedman et al., 2017)

The five species and two ribotypes of *Gambierdiscus* as well as *F. yasumotoi* that was recorded in the region as mentioned above held the potential to be the contributor to ciguatera fish poisoning in the Southeast Asia region. All the aforementioned species from other region has been tested with a detectable amount of ciguatoxin in numerous studies (Roeder et al., 2010; Nishimura et al., 2013; Rhodes et al., 2014; Litaker et al., 2017; Pisapia et al., 2017a). Interestingly, a strain of unidentified *Gambierdiscus* sp. from Vietnam exhibited relative high toxicity as it ranked first among the Pacific species tested in Pisapia et al. (2017a). This may indicate that Vietnam isolates of *Gambierdiscus* reckoned as highly toxic species in the Southeast Asia region. However, most of the strains from Southeast Asia yet to be tested with a more standardised method such as Neuro-2a bioassay and analytical method such as LC-MS/MS for quantitative and qualitative assessment of toxicity with strains from other regions.

5.4 Distribution and assemblages of benthic dinoflagellates in relation to temporal changes, depth profile and benthic substratum.

Rawa Islands hosted high abundances of all five groups of benthic dinoflagellates. This may due to the availability of benthic substrates such as profusion of macrophytes in Rawa Islands (field observation). In Tokong Laut, only *Prorocentrum* and *Coolia* were detected in low abundances. Tokong Laut was a pinnacle which rises from seafloor approximately 25 m with rock platform forming around 15 m where coral colonies existed with a strong current. The distinct geomorphology and water current of Tokong Laut lead to scarcity of macrophytes; made it a least favourable habitat for colonisation of benthic dinoflagellates as compared to other sites.

5.4.1 Temporal variation affecting benthic dinoflagellate assemblages

It is well known that phytoplankton dynamics was largely affected by seasonal changes in term of temperature, light intensity and hydrodynamics. While the effects were much more profound in planktonic dinoflagellates, benthic dinoflagellates assemblages were also affected by the seasonal changes. Seasonality abundances of benthic dinoflagellates were recorded in numerous studies in the temperate region and sub-tropical region (Parsons et al., 2012). In this study, benthic dinoflagellate assemblages were affected by temporal changes in term of temperature as supported by CCA analysis in this study. *Gambierdiscus, Ostreopsis* and *Prorocentrum* showed prominent temporal changes compare to other benthic dinoflagellates.

Generally, cell abundances of *Gambierdiscus* observed in this study was relatively low compared to other studies (maximum cell abundances of 255 cells/100 cm^2). Yet, *Gambierdiscus* have relatively higher abundances in May and June compared

as average temperature before from May to September was 31.5 °C and plunged to 29.3 °C from November to March. The abundances of *Gambierdiscus* peaked when average water temperature was 31.5 ℃ and CCA analysis showed a positive correlation with water temperature. This observation was similar to observation in the tropical region. For example, Bomber et al. (1988a) presented *Gambierdiscus* abundances peaked in September (summer) when temperature approximately 30 ℃ in the Florida Keys. In Puerto Rico, the study of Ballantine et al. (1988) showed that *Gambierdiscus* population was correlated with temperature where it abundances tend to peak in late summer and fall. Elsewhere, in Tahitian waters, Chinain et al. (1999b) showed that *Gambierdiscus* achieved the highest abundances at the beginning and end of the hot season with temperature maxima (28.2 to 30.9 °C). Seasonally comprehensive data on *Gambierdiscus* cell abundances and physico-chemical parameter from Hawaii in Parsons et al. (2010) also displayed its abundances peak in summer time with a temperature range of 28 to 29 ℃ . The highest abundances of *Gambierdiscus* occurred in May and November with temperature range from 24.5 to 30.2 ℃ in the northern coast of the Yucatan Peninsula (Okolodkov et al., 2014). However, studies of Ballantine et al. (1985) and Hokama et al. (1996) found no seasonal pattern in *Gambierdiscus* cell densities in the Caribbean or Hawaii. A rather anomaly event of high abundances of *Gambierdiscus* in the temperate region of Australia was reported in Kohli et al. (2014) with water temperatures of 16.5 – 17.0 ℃ . Nevertheless, more recent studies agreed that *Gambierdiscus* abundances were positively correlated with water temperature with regard to rising sea surface temperature while following a Gaussian curve from laboratory experiments (Parsons et al., 2012). In earlier studies, Morton et al. (1992) found that *Gambierdiscus* have an optimum growth temperature of 29 °C. Bomber et al. (1988a) reported limited growth of *Gambierdiscus* with temperature exceeded 29 °C and lower than 26 °C. Tester et al. (2010) reported thermal optimum tested for five

species of *Gambierdiscus* was ≥ 29 °C, with one exception of *Gambierdiscus* species which have an optimum temperature of 25 °C. Another study with eight species of *Gambierdiscus* shows that optimum temperature for maximum growth was rather species-specific, varied between 26.5 and 31.1 °C with different upper and lower thermal limits (Kibler et al., 2012). Yoshimatsu et al. (2014) tested four species of *Gambierdiscus* isolates from coastal water of Japan and found that different optimal temperature existed among the four species. In the much closer region to Malaysia, the study of Tawong et al. (2016) found that Thai's isolates of *G. caribaeus* from the Gulf of Thailand which is north of Perhentian Islands in this study can maintain growth in the temperature range of 20 to 35 °C, with optimum at 25 °C. This partly explained the low abundances in Perhentian Island compare to other regions as average water temperature in Perhentian Islands is 30.7 ℃ and minimum temperature of 28 ℃ , higher temperature all year round might inhibit the proliferation of *Gambierdiscus* in Perhentian Islands. The variability of optimum temperature for maximum proliferation of *Gambieridiscus* can be explained by a comprehensive study on the influence of environmental variables by Xu et al. (2016) showed that growth of *Gambierdiscus* eight species/phylotype to temperature variables displayed a pattern in near-Gaussian, non-linear manner, with optimal and suboptimal growth occurred in the range of 21.0 to 32.5 °C. The growth pattern varied among and within species, with species showing wider ranges of tolerance than others.

Differ from *Gambierdiscus*, *Ostreopsis* population in Perhentian peak in the month of October and February where the average water temperature was relatively lower. While similar observation was reported in studies from other regions, contradiction still exists among studies. Most of the studies on *Ostreopsis* population were conducted in the temperate region such as the Mediterranean Sea where many authors suggested relatively high temperatures were needed for the proliferation of

Ostreopsis and global warming may have influenced in the expansion (Accoroni & Totti, 2016). A review on *Ostreopsis* in the temperate region by Accoroni and Totti (2016) summarized that *Ostreopsis* flourished in summer time although the trend of blooming may differ from spring to autumn. The highest peak of *Ostreopsis* population recorded differed in a different area at a different time in the Mediterranean. Northern Adriatic Sea (September-October) with temperature range of 16.8 to 27.9 °C (Monti et al., 2007; Totti et al., 2010; Accoroni et al., 2011; Mangialajo et al., 2011; Accoroni et al., 2012; Accoroni et al., 2015a), Ligurian Sea (mid-summer, July-August) with temperature range of 22.6-30 °C (Mangialajo et al., 2008; Cohu et al., 2011; Mangialajo et al., 2011), Costa Brava, North Western Mediterranean (spring and summer) with temperature range from 18 to 28.3 °C (Vila et al., 2001). While in the Aegean Sea, two different times of peak abundances were reported in Aligizaki and Nikolaidis (2006), from mid-summer to late fall, while Spatharis et al. (2009) observed a peak in May, temperature ranging from 13.9 to 29.7 °C. In the Sea of Japan, *Ostreopsis* thrived in the time range of August-October with temperature range from 9 to 25 °C (Selina & Orlova, 2010; Selina et al., 2014). In the temperate region of New Zealand, *Ostreopsis* bloom events happened from February to April with temperature ranged from 17.8 to 22.1 °C (Chang et al., 2000; Shears & Ross, 2009). In general, *Ostreopsis* in the temperate region showed a positive correlation with rising water temperature. However, in tropical water, different maximum proliferation temperature and period were reported (Selina at el., 2014). For example, Ballantine et al. (1988) reported blooms from July to September in Puerto Rico with temperature range from 29.5 to 30 ℃ . In Caribbean Sea, maximum proliferation period of two different period seasons (dry season and wet season) were reported (Boisnoir et al., 2018; 2019). In exact same sampling site, Yong et al (2018) reported a bloom of *Ostreopsis* in June with water temperature of 32 ℃ .

Experiments on growth response of *Ostreopsis* to temperature using strains isolated from the region have given possible explanation to the various temporal trend in different area, where the strains from different area showed different growth optimum temperature which parallels to *in situ* temperature measured during the blooming periods (Guerrini et al., 2010; Pezzolesi et al., 2012; Scalco et al., 2012). While *Ostreopsis* population dynamics in the temperate region may behave differently compared to *Ostreopsis* from the tropical region in term of temperature response, experiment documented in Tawong et al. (2015) using strains from the South China Sea and Gulf of Thailand regions may clue in the dissimilarity observed between studies in Perhentian Islands. In the experiment, two different subclades of *Ostreopsis*; Thailand subclade and South China Sea subclade, show different optimal temperature conditions. Thailand subclade show semi-optimal temperature ranges from 22.7 to 27.4 °C, the optimum temperature at 25 °C, whereas South China Sea subclade ranges from 27.9 to 30.7 °C with an optimum temperature of 30 °C (Tawong et al., 2015). In this study, two peak abundances of *Ostreopsis* were observed in the month of October (31 ℃) and February (29 ℃) and Yong et al. (2018) study which the bloom occurred in June (32℃). These probably indicates that the bloom-forming *Ostreopsis* exist in Perhentian might belong to South China Sea subclade as the optimal growth temperature was similar and their optimal growth temperature in the natural environment might be higher than what been measured in laboratory condition.

While no clear seasonal variation of *Coolia, Prorocentrum* and *Amphidinium* abundances were recorded in this study, studies on seasonal abundances of these three species were relatively meagre as compared to *Gambierdiscus* and *Ostreopsis*. In case of *Coolia*, more abundances detectable in spring and winter, peak abundances were detected in August in North Aegean Sea with temperature range from 13.9 to 29.7 °C (Greece) (Aligizaki & Nikolaidis, 2006). On the contrary, the study of Armi et al. (2010)

discovered the proliferation of *Coolia* during spring in North Lake of Tunis, a natural lagoon in Tunisia where temperature higher than 22 °C. Another study by Rhodes and Thomas (1997) found *Coolia* bloom associated with calm and warm weather with a temperature of 20 – 22.8 °C. A laboratory experiment showed that *Coolia* cells can have optimum growth with broad temperature from 10 to 35 °C (Rhodes et al., 2000). Perhaps, the lack of seasonality variation in a population of tropical *Prorocentrum, Amphidinium* and *Coolia* in Perhentian Islands can be enlightened by Tindall and Morton (1998) theory's on the classification of tropical ecosystem-based primarily on flow dynamics and nutrient enrichment rather than temperature (Glibert et al., 2012).

The four types of tropical ecosystems where ciguatera dinoflagellates and associated species occurred were Type 1 (high flow, oligotrophic), Type 2 (moderate flow, ~mesotrophic), Type 3 (limited flow, eutrophic), Type 4 (very slow flow, high organic load) (Tindall & Morton, 1998). While in a temperate region, high seasonal variation was reported focusing only particular species complex of *Prorocentrum* where abundances can peak in a different season in different site with temperature ranging from 10 to 29.5 °C (Glibert et al., 2012).

To conclude, although benthic dinoflagellates such as *Prorocentrum, Gambierdiscus* and *Ostreopsis* showed temporal discrepancies on population response to temperature, yet no abundance data on the peak of northeast monsoon such as November, December and January (wet season) to further reaffirm the monsoonal pattern of the benthic dinoflagellates abundances. Hence, more long-term studies and icorporating other environmental factors such as salinity, hydrodynamic and nutrients are crucial to validate and distinguish the monsoonal pattern or interannual variability.

5.4.2 Depth profile of benthic dinoflagellates assemblages implies light-dependent responses

Two major observations from this study were (1) *Gambierdiscus*, *Ostreopsis* and *Amphidinium* have narrower range in term of depth distribution, and preferred to colonise Cluster 4 (hard coral), Cluster 5 (turf algae assemblages) and Cluster 6 (fleshy macroalgae) benthic substratum; (2) *Prorocentrum* and *Coolia* have wider ranges in term of depth distribution and able to colonise a variety of benthic substrate. This may give hints on *Coolia* and *Prorocentrum* adaptability to a different depth related factors such as wave action, light intensity and temperature. In term of light intensity and depth influences toward a natural population of benthic dinoflagellates, Richlen and Lobel (2011) reported a positive correlation of total dinoflagellate abundances with depth, with *Prorocentrum* and *Gambierdiscus* recorded the highest abundances at 13 m, while *Ostreopsis* was negatively correlated given the absence in samples collected from 13 m in Johnson Atoll, Pacific Ocean. Another study by Loeffler et al. (2015) in the US Virgin Islands found that the effect of depth on *Gambierdiscus* abundances was not significant. While no water motion was measured in the present study sampling site to evaluate the effect of turbulence towards population, Richlen and Lobel (2011) suggested 'opportunistic' behaviour of *Gambierdiscus* may help them to settle in deep and calm water (channel/lagoon) rather than rough and shallow water (back reef/reef crest). The discrepancy of observation rooted in water motion of sampling sites as pointed out by Loeffler et al. (2015) where water motion effect may mask the effect of light variability between depths. Another possible explanation to the inconsistencies among present and past ecological studies may due to substrate availability or substratum preference which undermined the ecological interpretation.

Gambierdiscus, *Ostreopsis* and *Amphidinium* were observed from shallower water of 1 m to deeper water of 16 m, with more abundances detected around 5 m.

Laboratory experiments on the response of *Gambierdiscus* and *Ostreopsis* towards light intensity have been well documented (Parsons et al., 2011). For *Gambierdiscus,* many studies concluded that it preferred low light intensities with optimum growth achieved only at ~2.5 to 10 % of surface irradiance (Yasumoto et al., 1980; Morton et al., 1992; Kibler et al., 2012). Kibler et al. (2012) found that maximum growth rates for different *Gambierdiscus* species were between 49 and 231 µmol photons m^{-2} s^{-1}. Interestingly, that study also observed that *Gambierdiscus* maintained positive growth at irradiances between 6 and 17 µmol photons m^{-2} s^{-1} which only represent less than 1 % of ambient surface irradiances, further estimated that *Gambierdiscus* could survive down to a maximum projected depth of 150 m. Hitherto, the recorded deepest depth in field observation of *Gambierdiscus* was 45.7 m, found in the Gulf of Mexico (Tester et al., 2013). Studies of Xu et al. (2016) determined the optimum light intensity for the growth of different *Gambierdiscus* species extended to ~ 4.4 - 16 % (110 – 400 µmol photons m^{-2} s^{-1}) of full sunlight irradiance (2500 µmol photons m^{-2} s^{-1}). All these studies explained the existence of *Gambierdiscus* at 16 m in this study. Likewise, most of the field observation or isolated clonal cultures of *Gambierdiscus* were obtained from shallow depth of coral reef (1 - 5 m), mangrove, tidal pond or in some case drifting algae where can be exposed to inhibiting irradiance limit (Ballantine et al., 1988; Bomber et al., 1988b; Faust, 1995; Tindall & Morton, 1998; Faust, 2009; Fraga et al., 2011) same as observed in this study where *Gambierdiscus* cells still existed around 1 m. Many authors were congruent with the idea that formation of mucus to attach and ability to seek shelter using finely-branched and thalli structure of host algae as a form of adaptive strategy implemented by *Gambierdiscus* to survive in high irradiances shallow water (Yasumoto et al., 1980; Ballantine et al., 1988; Bomber et al., 1988b; Nakahara et al., 1996; Villareal & Morton, 2002).

5.4.3 Assemblages of benthic dinoflagellates in relation to benthic substratum

Benthic dinoflagellates are known to epiphytically associate with a wide range of natural substrates, however, most of the studies have fixated on distribution and tenacity of benthic dinoflagellates on macroalgae while little studies had thoroughly explored vast numbers of reef benthic substratum besides macroalgae. This study pondered over the substrate preference of benthic dinoflagellates by using the artificial substrate to sample across various reef benthic substrates. This study successfully captured the estimated cell abundances of five genera of benthic dinoflagellates compare among different benthic substratum.

The natural population of *Gambierdiscus* in Perhentian Island showed a predilection towards turf algae assemblages, hard coral colonies and fleshy macroalgae. The epiphytic behaviour of *Gambierdiscus* was first documented in Yasumoto et al. (1979) and the preferences for certain algal hosts have been reviewed in Cruz-Rivera and Villareal (2006) by compiling cell abundances data available over 56 algal genera, two cyanobacteria, one diatom and one seagrass. Although the essentiality of the preference remains ambiguous, investigator speculated that the behaviour may tie to the function of surface area (Lobel et al., 1988; Bomber et al., 1989), class of algae (Taylor, 1979; Yasumoto et al., 1979; Yasumoto et al., 1980; Bomber et al., 1989), algal structure (Parsons & Preskitt, 2007) and stimulatory compounds from algal extracts/exudates (Carlson et al., 1984; Carlson & Tindall, 1985; Bomber et al., 1989; Grzebyk et al., 1994). In this study, no significant difference was observed in abundance of *Gambierdisucs* on turf algae assemblages (255 cell/100 cm^2) and fleshy macroalgae substrate cluster (129 cell/100 cm^2) comprised of *Jania*, *Dictyota*, *Padina* and *Lobophora*. Regarding higher *Gambierdiscus* abundances observed in turf algae assemblages, Grzebyk et al. (1994) study's found high variability of dinoflagellates numbers and species ratio particularly *Gambierdiscus* on dead coral rubble which

colonised by algal turf. Study of Jean Turquet et al. (2000) found that monospecific bloom of *Gambierdiscus toxicus* was detected after a coral bleaching event that causes mass mortality of coral lead to colonisation of multispecies algal turf on coral fragments which provides surface area for proliferation. Parsons and Preskitt (2007) showed that *Gambierdiscus* have a preference towards microfilamentous algae (turf algae) by comparing abundances among four groups of host macroalgae based on morphologies (microfilament, macrofilament, microblade and macroblade). Besides, in experimenting epiphytism of *Gambieridiscus* species on the macroalgal host, Rains and Parsons (2015) reported epiphytic behaviour differed among *Gambierdiscus* species and in particular saw higher attachment rate and stimulate growth. The host pairings with *Polysiphonia* and *Dictyota* were further proposed by Rains and Parsons (2015) as potential hypothetical vectors for trophic transfer of ciguatoxins based on their palatability and demonstrated as good host for *Gambierdiscus*. This supported the observation in this study as *Polysiphonia* was one of the components in turf algal assemblages observed in the Perhentian Islands.

Genus *Ostreopsis* were able to colonise all cluster of benthic substratum reported in this study with maximum cell abundances ranges from 598 to 5750 cells/ 100 cm^2. This observation generally agreed with other studies as *Ostreopsis* natural population has been reported on a wide range of benthic substratum from macroalgae, seagrass, rocks, coral rubbles, soft sediments, even on invertebrates as reviewed in Accoroni and Totti (2016). Cells of *Ostreopsis* showed strong inclination in term benthic substratum, the natural population observed in hard coral colonies (Cluster 4) and turf algae assemblages (Cluster 5) was greatly significant compare to rubbles (Cluster 2) and sand and silt (Cluster 7) substratum. Interestingly, no study reported the natural population of *Ostreopsis* on hard coral colonies, as this study detected a population of *Ostreopsis* in the proximity of a variety of hard coral colonies. This

confirmed the speculation that *Ostreopsis* able to colonise a variety of benthic substratum living as epiphytic, epilithic and epizoic (Totti et al., 2010; Accoroni & Totti, 2016). Study on substratum preference of *Ostreopsis* does not always consistent. In term of macrophyte morphology, Vila et al. (2001) reported *Ostreopsis* preferred three-dimensional flexible thalli of macroalgae while Parsons and Preskitt (2007) found two different species of *Ostreopsis* displayed dissimilar partiality on macroalgae with different morphology. Aligizaki and Nikolaidis (2006) discovered that *Ostreopsis* abundances can peak at the same time on the different substratum, on algae, sediment substrates and in the water column but the degree of preference on different macrophyte groups was in ordered from rhodophytes, phaeophytes, ulvophytes, and seagrasses. Monti et al. (2007) reported similar aspect of preference, that brown and red algal host was more preferential owning to the class of algae. However, significantly higher abundances of *Ostreopsis* cf. *ovata* were reported on pebbles and sand substrates than macroalgae (Totti et al., 2010; Accoroni et al., 2011). A study to address this matter confirmed that allelopathic interactions exist between *O.* cf. *ovata* and different class of macroalgae (green, brown and red alga) which result in inhibiting growth and induce cyst formation (Accoroni et al., 2015b). Likewise, *Ostreopsis* are not obligate epiphyte, though some study showed preferentiality in macroalgae, they can be found as free-living in plankton too (Chang et al., 2000; Totti et al., 2010; Accoroni et al., 2011). The current study showed that *Ostreopsis* can have peak abundances in non-algal samples and it is concurrent with observation in other studies (as reviewed in Accoroni and Totti, 2016).

Amphidinium generally behaved like *Gambierdiscus* and *Ostreopsis* in term of distribution on benthic substratum with higher abundances were detected in turf algae assemblages and hard coral colonies. While abundances of *Amphidinium* are a relatively small component in this study, only 5 % in relative abundances, other studies reported a

disparate observation. Shah et al. (2014) reported that *Amphidinium* was the dominant genera in Jeju Island, Korea, where it was observed on thalli of a different class of macroalgae investigated including Chlorophytes, Rhodophytes and Phaeophytes. Species of *Amphidinium* has been regarded as a sand-dwelling as most of them typically found in shallow marine sediment, yet some may undergo vertical migration to proliferate in the water column when conditioned was favourable (Murray & Patterson, 2002; Murray et al., 2015). *Amphidinium* bloom has been reported in Murray et al. (2015) which caused an extensive yellow-brown water discolouration in shallow sandy lagoon system in Sydney, Australia. Similar bloom has also been reported in Pakistan (intertidal pool), Portugal (estuary) and Mexico (intertidal pool) (Sampayo, 1985; Baig et al., 2006; Gárate-Lizárraga, 2012). All these bloom events have rather a resemblance as it occurred in a location with low water flow, high organic loads and sandy patch as *Amphidinium* was regard as eurytopic (Lee et al., 2003). This was another example of non-obligate epiphytic nature of benthic dinoflagellate which does not necessarily attached to macrophytes.

Generally, *Coolia* and *Prorocentrum* were able to occupy a wider ecological niche by colonising a wide range of benthic substratum. Yet, this study revealed that the population of *Coolia* and *Prorocentrum* were more likely associated in proximity to rubbles, sand and sediment benthic substratum. The report on existence of *Prorocentrum* and *Coolia* on rubble and sand substratum was not rare as numerous of studies reported isolation of benthic *Prorocentrum* from sand and sediment substratum, hence, most benthic *Prorocentrum* also known as "sand-dwelling" or epipelic dinoflagellate (Faust, 1994, 1995, 1997; Faust, 2009; Hoppenrath et al., 2014). While two species of *Coolia* described were isolated from sediment samples (Faust, 1995; Ten-Hage et al., 2000). "Sand-dwelling" behaviour may be interpreted as an interstitial space between sand grains providing sanctuary for benthic dinoflagellates from

meiofauna predator (Faust, 2009). Another theory was that "sand-dwelling" dinoflagellates such as *Coolia* and *Prorocentrum* will loosely attach to detrital particle associated with sand grains, which the aggregation of detritus facilitate vertical migration of dinoflagellates (Faust, 2009). Other studies have reported *Coolia* was more abundant on microfilamentous algae, though this may simply reflect more surface area of the host (Parsons & Preskitt, 2007). Aligizaki and Nikolaidis (2006) found that abundances of *Coolia* were remarkably lower in less leafy Chlorophyceae and seagrass, while maximum abundances were recorded in branched macroalgae.

The interpretation of habitat preferences by benthic dinoflagellates was intricating as many factors contributing to epiphytic behaviours such as chemical cue, light, wave disturbance as well as co-habitant such as bacteria and fungi (Parson et al., 2012). The representation of benthic dinoflagellate assemblages with the application of artificial substrate generally mirrored the estimated abundances with the assumption of non-obligate epiphytism in benthic dinoflagellate on selected habitat (Tester et al., 2014).

5.5 Role of benthic substratum in ciguatoxin food webs

This study successfully captured the temporal abundances of benthic dinoflagellates as well as their natural assemblages across different benthic substratum and depth profile in the fringing coral reef ecosystem. The understanding of the role of benthic substratum in the ciguatera food web is the first step in contemplating the flux of ciguatoxin. This study clearly demonstrated each genus have a certain degree of preference towards various benthic substratum other than macrophytes. Surprisingly, the population of benthic dinoflagellates on fleshy macrophytes was much lesser compared to turf algae assemblages.

There are several possibilities to explain such observation in the current study. First, in term of surface area coverage, turf algae assemblages which defined as dense, multi-species assemblages and microfilamentous, which are typically less than 1 cm in height (Connell et al., 2014), have higher colonisation rates and growth which likely to outcompete settlement of reef-building crustose coralline algae (McCook et al., 2001; Diaz-Pulido & McCook, 2002; Littler et al., 2006), thus provide more surface area for attachment as compared to fleshy macroalgae. Second, most of the fleshy macroalgae are known to produce allelochemical that comes with algaecide effect (Gross, 1999; Jeong et al., 2000) and it has been proven in laboratory experiment that existential of allelopathy interaction between macroalgae and harmful dinoflagellates (Accoroni et al., 2015b; Tang et al., 2015; Ben Gharbia et al., 2017). However, the study of Ben Gharbia et al. (2017) noticed that benthic dinoflagellates such as *Prorocentrum, Ostreopsis* and *Coolia* seem to be more resistant to allelochemical releases by macrophytes which may see as part of the co-evolutionary process of epiphytism (Hilt, 2006). Besides epiphytism of benthic dinoflagellates, Cruz-Rivera and Villareal (2006) pointed out marine algal-herbivore interactions that involved palatability and complex defences and adaptive strategies of macroalgae/turf algae which have important consequences in understanding the ciguatera flux pathway. A conceptual model of potential ciguatera food webs was illustrated in Cruz-Rivera and Villareal (2006) based on the palatability of different algal sources to further hypothesised more realistic ciguatera pathway. To conclude, while highly palatable turfs with rapid colonisation may result in a large flux of ciguatoxins into marine food webs, the fleshy macroalgae which have much complex defence strategy may provide passive accumulation and may act as "sink" of ciguatoxins or a safe harbour for other harmful benthic dinoflagellates.

5.6 Limitation of artificial substrates in monitoring benthic dinoflagellates and possible improvements

The most common way of monitoring abundances of benthic dinoflagellates is through collection of natural substrates where abundances can be estimated directly according to weight or size of the material collected such as quantification of cells in the biofilm, expressed as cell g^{-1} of collected macroalga or per surface area of different substrates (pebbles, corals, shells) and quantification of cell concentrations in the surrounding water, usually expressed as cell L^{-1} (Mangialajo et al., 2017). Alternative methods of cell abundances quantification which independent of their natural substrates have been tested such as suction apparatus which gives representative abundances of cell in biofilm (Parsons et al., 2010; Abbate et al., 2012) or deployment of artificial substrates which integrate over time the cells in surrounding water (Tester et al., 2014; Jauzein et al., 2016).

Although the advantages of artificial substrates have been demonstrated in numerous studies (Tan et al., 2013; Tester et al., 2014; Jauzein et al., 2016; Jauzein et al., 2018), the artificial substrates method for monitoring and assessment of harmful benthic dinoflagellates still required optimisation and the efficiency yet to be proven over a large scale. Possible limitation and inherent weakness of the deployment of artificial substrates been discussed in detail by Parson et al. (2017). The authors concluded the limitation as follow:

(1) Lack of consistency of correlation in cell density with those on macrophyte hosts;

(2) Question on a high degree of variability displayed in the triplicate samples collected

(3) Risk of artificial substrate failure

(4) The inherent weakness in large scale study (numerous of evaluation is needed for a specific site with one or several macrophytes before widely deployed for monitoring purposes)

(5) Even if the significant result obtained, it may be necessary to retest the approach in different years or different times of the same year

Other authors also agreed upon the major disadvantages of the artificial substrate such as time-consuming and labour intensive due to two trips needed for deployment and retrieval (Tester at al., 2014; Jauzein et al., 2018).

Possible improvements regarding artificial substrates method have been highlighted in Jauzein et al. (2016) and Jauzein et al. (2018) study where optimisation on sampling, cell collection and counting were conducted. In Jauzein et al. (2016), the improvement of the artificial substrate method including collection protocol (positioning of fibreglass screen), optimal porosity and mesh size for efficient cell abundances estimation. In Jauzein et al. (2018), the optimisation of sampling effort, cell collection and counting based on the macroalgal collection and artificial substrates were both introduced. The authors also pointed out the improved collection efficiency of artificial substrates regarding the incubation time and fibreglass screen positioning after modified set-up based on the protocol described in Jauzein et al. (2016). Other improvements include changes in collection mechanism such as BEDI (Benthic dinoflagellate Integrator) which modified from artificial substrates. The major improvement of BEDI was mechanical resuspension of cells enables the quantification of cell abundances in both biofilm and surrounding water. This method is independent of substratum and potentially allows the comparison of benthic dinoflagellate blooms over broad temporal and spatial scales (Mangialajo et al., 2017).

CHAPTER 6: CONCLUSION

In this study, the diversity of epiphytic benthic dinoflagellates from Perhentian Islands were documented with both morphology and molecular evidence as the first step to understand the risk of ciguatera fish poisoning and potential outbreak causes by other harmful benthic dinoflagellates in Malaysia as well as Southeast Asia. Total of 16 species from six genera of harmful benthic dinoflagellates (*Amphidinium*, *Coolia*, *Gambierdiscus*, *Gymnodinium*, *Prorocentrum* and *Ostreopsis*) were documented in this study. Two species of *Coolia*, *C. canariensis* and *C. palmyrensis* and *Gymnodinium dorsalisulcum* were the first to report in Malaysian water. High diversity was observed in *Prorocentrum* and *Coolia* where five species of *Prorocentrum* and with four out of seven described species of *Coolia* was reported. Three species of ciguatoxin producer, *Gambierdiscus* has been recorded, the morphological observation and phylogeny were consistent with other studies. This study also confirmed the existence of ciguatera producer in Malaysian waters as two of species recorded, *G. balechii* and *G. caribaeus* were tested positive in ciguatoxicity screening.

The distribution and natural assemblages of epiphytic benthic dinoflagellates in Perhentian Islands were estimated with artificial substrates approach. The sparsity and heterogeneity of benthic dinoflagellate assemblages over a wide range of benthic substratum in reef environment were also highlighted. An indication of microhabitat preferences by benthic dinoflagellates species as an adaptive response to natural disturbance such as wave and high light exposure was important for understanding the flux of ciguatoxins. For example, *Gambierdiscus* and *Ostreopsis* have high tendency resides in turf algal assemblages compare to other benthic substratum types. Turf algae which have a high surface area and high colonisation rate would become a perfect shelter for the concentration of cell abundances. Besides, this study also demonstrated the strength of utilising artificial substrates: (i) estimation of abundances are not

dependent on variation in composition and distribution of macrophytes in time and space, (ii) data can be easily standardised per unit of surface area over different kind of benthic substratum for comparisons, (iii) versatility of artificial substrate deployment enable investigation of benthic dinoflagellate abundances in other potential benthic host other than macrophytes such as turf algae and coral rubbles.

In conclusion, while the reported cases of CFP in Malaysia was scarce, the risk remained as toxic species was found in the region. Monitoring the abundances and ascertaining the composition of *Gambierdiscus* species was vital to estimate the ciguatoxins flux coupled with ecological models based on physiological and ecological preferences of predominant toxin-producing species. Artificial substrate method has proven to be noteworthy in the assessment of population dynamics of harmful benthic dinoflagellates due to the versatility and standardisation of the approaches. Besides, with modification and alternative method such as BEDI (Benthic dinoflagellate Integrator), artificial methods can be considered in the framework of routine monitoring abundances of harmful benthic dinoflagellates as well as for alert system where good estimations of population abundances can be calculated in less than a day. Whilst, similar sampling technique permitted molecular-based monitoring such as species-specific qPCR may provide an edge for accurate assessment of the diversity of ciguatoxin-producing species. Standardised protocol in toxicity testing such as Neuro-2a bioassay and erythrocyte lysis assay which proved to be accurate and rapid in screening ciguatoxins needed to be adopted for routine monitoring and produce comparable toxicity data to other studies in other regions. Besides, with the knowledge on distribution and assemblages of benthic dinoflagellates on reef substratum, screening of potential ciguatoxin vectors in marine food web from different trophic level such macrograzer, herbivorous fishes and carnivorous fishes can provide depiction ciguatoxins flux in marine food webs.

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LIST OF PUBLICATION AND PAPERS PRESENTED

Papers presented at conferences/seminars/symposiums:

1. **Li Keat Lee[1]**, Zhen Fei Lim[1], Hwa Lin Yong[1], Nurin I. Mustapa[1], Gires Usup[2], Leo Lai Chan[3], Po Teen Lim[1], Chui Pin Leaw[1] Benthic dinoflagellate assemblages associated with Ciguatera Fish Poisoning (CFP) at fringing coral reef ecosystem of Perhentian Islands. 3rd Xiamen Symposium on Marine Environmental Sciences, Xiamen University, China, 9-11 January 2017. **[Poster]**
2. **Li Keat Lee[1]**, Zhen Fei Lim[1], Hwa Lin Yong[1], Nurin I. Mustapa[1], Gires Usup[2], Leo Lai Chan[3], Po Teen Lim[1], Chui Pin Leaw[1] Primary ciguatoxin transfer and benthic harmful dinoflagellate assemblages at the fringing coral reef ecosystem of Perhentian Islands. 10th WESTPAC International Scientific Conference Qingdao, China, 17-20 April 2017 pg. 132. **[Oral]**
3. **Lee, L. K**, Lim, Z. F., Yong, H. L., Mustapa, N. I., Chan, L, L., Gu, H., Lim, P. T., & Leaw, C. P. 2019. Benthic harmful dinoflagellate assemblages of Perhentian Islands (South China Sea): the relationships between benthic substratum characteristics, depth and monsoonal shift. 4th Xiamen Symposium on Marine Environmental Sciences, 2019, Xiamen, China, Xiamen University. 6-9 Jan 2019. **[Poster]**