#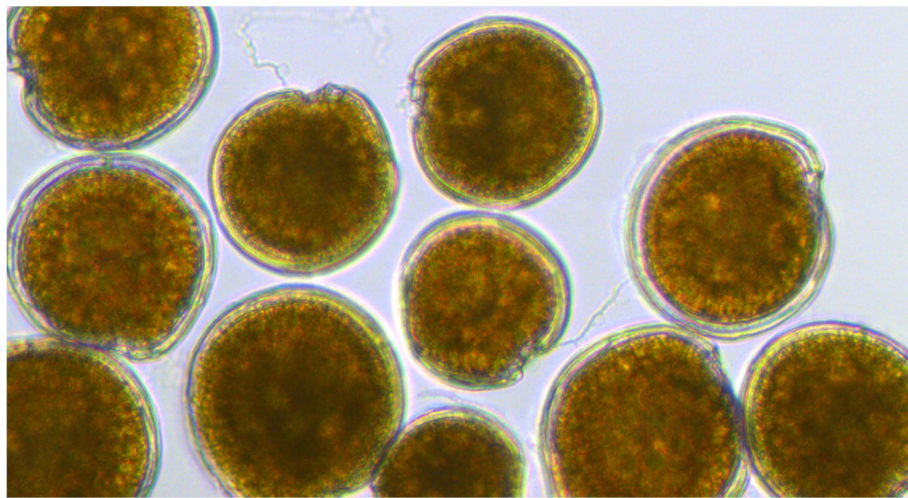 Toxicology and Ecology of *Gambierdiscus* from Australia: A Dinoflagellate Genus Associated with Ciguatera Fish Poisoning

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B.Sc. (Hons)

Submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy in Science.

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Certificate of Authorship/Originality

I, Michaela E. Larsson declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution

This research is supported by the Australian Government Research Training Program.

Signature:

Date: 14th February 2019

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Publications and Research Outputs

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Larsson, M. E., Smith, K. F., Doblin, M. A. First description of the environmental niche of the dinoflagellate species *Coolia palmyrensis*, *C. malayensis* and *C. tropicalis* (Dinophyceae) from eastern Australia. (Journal of Phycology; in press).

Larsson, M. E., Laczka, O. F, Harwood, D. T., Lewis, R. J., Himaya, S. W. A., Murray, S. A., Doblin, M. A. (2018) Toxicology of *Gambierdiscus* (Dinophyceae) from tropical and temperate Australian waters. Marine Drugs. 16(1):7, doi:10.3390/md16010007.

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Munday, R., Murray, S., Rhodes, L. L., Larsson, M. E., Harwood, D. T. (2017). Ciguatoxins and maitotoxins in extracts of sixteen *Gambierdiscus* isolates and one *Fukuyoa* isolate from the South Pacific and their toxicity to Mice by intraperitoneal and oral administration. Marine Drugs 15(7):208, doi: 10.339/md15070208.

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Other outputs arising directly from this doctoral thesis

Australia's Science Channel (2018). Marine microalgae 'rafting' into new territory – research inspired by famous Kon-Tiki expedition.
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Table of Contents

List of Figures

this study are shown in bold. Values at nodes represent Bayesian posterior probability and Maximum Likelihood bootstrap support.

Figure 2.3. Ciguatoxin-like (red) and maitotoxin-like (blue) activities of the High-Performance Liquid Chromatography (HPLC) fractionated (black trace) dichloromethane phase of extracts from *Gambierdiscus* strains established in this study.

Figure 2.4. Ciguatoxin-like (red) and maitotoxin-like (blue) activities of the High-Performance Liquid Chromatography (HPLC) fractionated (black trace) methanol phase of extracts from *Gambierdiscus* strains established in this study.

Figure 3.1. Linear relationship between the *in vivo* chlorophyll *a* fluorescence (RFU, Relative Fluorescence Units) and cell abundance (cells mL^{-1}) for *Gambierdiscus* species included in this study (a) tropical *G. carpenteri*, (b) temperate *G. carpenteri*, (c) *G. lapillus*, (d) *G.* cf. *pacificus* and (e) *G.* cf. *silvae.*

Figure 3.2. Temperature reaction norms for strains of *Gambierdiscus* established from tropical and temperate Australia in this study. Dots indicate the measured growth rates; the blue line indicates the line of best fit using the function described in Thomas et al. (2012) and the red lines represent the 95% confidence band.

Figure 3.3: Salinity reaction norms for strains of *Gambierdiscus* established from tropical and temperate Australia in this study. Dots indicate the measured growth rates; the blue line indicates the line of best fit using Thomas et al. (2012) function and the red lines represent 95% confidence band.

Figure 3.4. Irradiance reaction norms for strains of *Gambierdiscus* spp. established from tropical and temperate Australia in this study. Dots indicate the measured growth rates;

the blue line indicates the line of best fit using Thomas et al. (2012) function and the red lines represent 95% confidence band.

Figure 3.5. Principle Coordinate Analysis (PCO) of the collective estimated growth parameters (e.g. μ_{max}, opt, niche min, niche max, niche width) from temperature, salinity and irradiance experiments. Each symbol represents an individual strain of *Gambierdiscus* and the shape and colour of the symbols represent different *Gambierdiscus* species. Groupings represent 90% similarity (solid line) and 95% similarity (broken line).

Figure 3.6. Dots represent the temperature at which maximum growth was achieved (T_{opt}) (blue), and the minimum (T_{min}) (green) and maximum (T_{max}) (red) temperature for growth estimated from temperature reaction norm experiments. These data have been fitted with a polynomial curve to demonstrate the relationship of the thermal performance traits with latitude. Data sourced from Kibler et al. 2012; Yoshimatsu et al. 2014, 2016, Tawong et al. 2016; Xu et al. 2016 and this study.

Figure 3.7. Monthly average temperature for the Heron Island, Queensland Australia (tropical) and Merimbula, New South Wales (temperate). Data sourced from the IMOS-Weather Station (**http://www.aims.gov.au**) and the New South Wales Food Authority.

Figure 3.8. Temperature, salinity and irradiance niche of *Gambierdiscus* strains in this study compared to other studies. Black bar indicates μ_{max} , grey bar indicates region of maximum growth (>80% of μ_{max}) and white bar indicates region of growth. Y-axis shows *Gambierdiscus* species name and abbreviation of isolation locations. Note: irradiance values up to 400 µmol photons m^{-2} s^{-1} only included in figure.

Figure 4.1. (a) Map showing the location of the Port Hacking 100 m Station (PH100) and the sampling location of rafts collected in the East Australian Current (EAC) and an eddy and (b) map of the Sea Surface Temperature (SST) during collection of raft samples on the oceanic voyage between Brisbane and Sydney in June 2015. Data for (b) sourced from IMOS http://oceancurrent.imos.org.au/sst.

Figure 4.2. Non-metric Multi-Dimensional Scaling (nMDS) plot showing differences in the microalgal community composition associated with different rafts based on raft type.

Figure 4.3. Relationship between (a) the richness of microalgal taxa and (b) microalgal abundance associated with rafts according to the raft size and complexity (raft index score).

Figure 4.4. Richness (a) and abundance (b) of the microalgal communities associated with rafts drifting in the East Australian Current (EAC) and associated water masses.

Figure 4.5. Images of clonal epibenthic dinoflagellates cultures established from a macrophyte raft samples of *Sargassum* sp. collected drifting in the East Australian Current (EAC), a) *Coolia palmyrensis* b) *Prorocentrum lima*, c) *Amphidinium* cf. *operculatum* and d) *Amphidinium* cf. *carterae*. Scale bar = 10 µm.

Figure 4.6. *Sargassum* sp. raft from which the *Coolia* sp., *Amphidinium* spp., and *Prorocentrum* sp. strains were isolated for cell viability assessment. Image (a) shows raft prior to processing and image (b) shows a close-up image of the raft.

Figure 4.7. Maximum likelihood tree of the *Coolia* culture (UTSR7) isolated from *Sargassum* sp. raft sample collected drifting along the East Australian Current (EAC), showing alignment of the partial LSU rDNA sequences (D1-D3 region). Values at the

nodes represent Maximum Likelihood bootstrap and Bayesian posterior probability support.

Supplementary Figure 4.1. Percent occurrence of epibenthic dinoflagellates from the genera *Gambierdiscus, Ostreopsis, Coolia, Amphidinium* and *Prorocentrum* as determined from five datasets spanning 50 years from a coastal monitoring station (PH100) influenced by the East Australian Current (EAC) in south east Australia (1965 to 2015). Red colour shows occurrence of *Prorocentrum lima* as differentiated from unconfirmed species of *Prorocentrum* (*Prorocentrum* spp.) in blue.

Supplementary Figure 4.2. Images of raft samples included in this study as examples.

Figure 5.1. Map showing the isolation location for epibenthic dinoflagellate strains used in this study, Heron Island, Queensland and Merimbula New South Wales (triangles) and the sampling for sites for the natural community experiments, Lake Macquarie, Brisbane Water and Narrabeen Lagoon, New South Wales, Australia (solid circles).

Figure 5.2. Mean (±SD) abundance of *Gambierdiscus* cells within the natural epiphytic community experiment at Lake Macquarie (a, d, g, j), Brisbane Water (b, e, h, k) and Narrabeen Lagoon (c, f, i, l) for the tropical strain of *G. carpenteri* (d to f), *G. lapillus* (g to i) and the temperate strain of *G. carpenteri* (j to l). Each line in a-c represents the abundance of *Gambierdiscus* cells in the controls with no epibenthic community for each species at each site and in d to l represents the abundance of *Gambierdiscus* cells for each concentration originally added.

Figure 5.3. Mean (±SD) growth rate of individual *Gambierdiscus* species controls at each site.

Figure 5.4. Mean (±SD) abundance of *Gambierdiscus* cells within the artificial community colonisation experiment through time for the tropical strain of *G. carpenteri* (a, d, g), *G. lapillus* (b, e, h) and the temperate strain of *G. carpenteri* (c, f, i). The lines in a-c represent the abundance of healthy and stressed cells for each species of *Gambierdiscus*.

Figure 5.5. Mean (±SD) growth rate for control treatment for each individual genus.

Figure 5.6. Summary of abiotic and biotic conditions within natural epibenthic communities used in experiments. The gradient of optimum to stressful refers to the physicochemical environment only, as the nature of biotic interactions (that may involve competition, resource partitioning, predation etc) are currently unknown.

Supplementary Figure 5.1. Mean (±SD) F_v/F_m (indicator of health) measurements for each *Gambierdiscus* species included in the artificial community colonisation experiment.

Supplementary Figure 5.2. Mean (±SD) concentrations of dissolved nitrate (mol L^{-1}) in the growth medium through time for the Lake Macquarie (a, d, g, j), Brisbane Water (b, e, h, k) and Narrabeen Lagoon (c, f, I, l) treatments and for the tropical strain of *G. carpenteri* (d to f), *G. lapillus* (g to i) and the temperate strain of *G. carpenteri* (j to l) for the natural community colonisation experiment. Each line in a to c represents the concentration of nitrate dissolved in the growth medium in the natural epiphytic community control (no added *Gambierdiscus* cells) and in d to l, represents the concentration of nitrate dissolved in the growth medium for each treatment when *Gambierdiscus* cells were added.

Supplementary Figure 5.3. Mean (±SD) concentrations of dissolved nitrate (moles L^{-1}) in the growth medium through time for the tropical strain of *G. carpenteri* (a, d, g), *G. lapillus* (b, e, h) and the temperate strain of *G. carpenteri* (c, f, l) during the artificial community colonisation experiment. Each line in plots a to c represents the concentration of nitrate dissolved in the growth medium in the *Gambierdiscus* cell only controls and in d to l represents the concentration of nitrate dissolved in the growth medium for each treatment when *Gambierdiscus* cells were added.

Figure 6.1. Conceptual diagram summarising the design and major findings of this thesis.

List of Tables

(μ_{max}), the salinity at which maximum growth was reached (S μ_{max}), the optimum salinity range for growth (S_{opt}) and the minimum and maximum salinity where growth terminated (S_{min} and S_{max}).

Table 3.4. Summary of calculated growth parameters calculated from the irradiance reaction norm function for each strain of *Gambierdiscus* measured in this study, including maximum growth rate (μ_{max}), the irradiance (μmol photons m^{-2} s^{-1}) at which maximum growth was reached (I μ_{max}), the optimum irradiance range for growth (I_{opt}) and the minimum and maximum irradiance where growth terminated (I_{min} and I_{max}).

Table 4.1. Type of rafts collected in the East Australian Current (EAC) and associated oceanographic features (e.g. eddies), with the environmental conditions and location of sampling sites. Note that some samples contained more than one type of raft and were identified to genus level when possible.

Table 4.2. Microalgal taxa associated with rafts collected in the East Australian Current (EAC) (Table 4.1) and associated water masses. The taxa classified as epibenthic are indicated with + symbol, planktonic with a – symbol, and NA denotes when information was not available or applicable.

Table 5.1. Location and water quality information for sites used in natural epiphytic community colonisation experiment.

Supplementary Table 5.1. Microalgal taxa identified from resident epibenthic communities of the three sites sampled for the natural community colonisation experiments (Lake Macquarie, Brisbane Water and Narrabeen Lagoon). + indicates the taxa was present and – indicates the taxa were absent.

Abstract

ANOVA	Analysis of Variance
ASP	Amnesic Shellfish Poisoning
aq	Aqueous
AZP	Azaspiracid Shellfish Poisoning
BHAB	Benthic Harmful Algal Bloom
bp	Base Pair
BSA	Bovine Serum Albumin
CFP	Ciguatera Fish Poisoning
CTX	Ciguatoxin
CO_2	Carbon Dioxide
DNA	Deoxyribonucleic Acid
DSP	Diarrhetic Shellfish Poisoning
EAC	East Australian Current
FBS	Foetal Bovine Serum
FLIPR	Fluorescent Imaging Plate Reader
F_v/F_m	Maximum quantum yield of photosystem II
GTR	General Time Reversible
GTR+G	General Time Reversible with Gamma-shaped among site variation
HA	Haemolytic Assay

HAB	Harmful Algal Bloom
HPLC	High Performance Liquid Chromatography
LC-MS/MS	Liquid Chromatography Tandem-Mass Spectrometry
LD	Lethal Dose
LSU	Large ribosomal subunit gene
MBA	Mouse Bioassay
nMDS	Non-metric Multidimensional Scaling
MTX	Maitotoxin
NSP	Neurotoxic Shellfish Poisoning
N2a	Mouse Neuroblastoma Assay
PCO	Principle Coordinate Analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational Analysis of Variance
PSP	Paralytic Shellfish Poisoning
RBA	Receptor Binding Assay
RPMI	Roswell Park Memorial Institute medium
SH-SY5Y	Human Neuronal Cell Line
sp.	Species (singular)
spp.	Species (plural)
TFA	Trifluoroacteic Acid
UPLC	Ultra Performance Liquid Chromatography

Abstract

Some species from the epibenthic marine microalgal genus *Gambierdiscus* produce potent neurotoxins, such as ciguatoxins (CTXs) and maitotoxins (MTXs), which can accumulate in the marine food web and cause the human illness Ciguatera Fish Poisoning (CFP). The genus typically has a tropical distribution and is known to occur in the Great Barrier Reef region of north east Australia, although recently, populations have been documented in more temperate locations. In this thesis, a toxicological and ecological approach was used to investigate CFP causing organisms in Australia, with an emphasis on assessing the potential for temperate range extension of the genus in this region. Monoclonal isolates of *Gambierdiscus* were established from a tropical and a temperate location in eastern Australia and formed the foundation of experimental work. Four species of *Gambierdiscus* (*G.* cf. *pacificus, G.* cf. *silvae, G. carpenteri* and *G. lapillus*) were identified from the tropical location and only *G. carpenteri* was identified at the temperate location. Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS) was used to assess whether isolates produced known microalgal CTXs (P-CTX-3B, 3C, 4A and 4B) and MTX-1, but these characterised toxins were not detected in any of the *Gambierdiscus* strains. Putative MTX-3, however, was detected in all strains, except the temperate *G. carpenteri* isolates. Using the novel Ca^{2+} influx bioassay, compounds displaying CTX-like activity were identified in extracts of *G.* cf. *pacificus, G.* cf. *silvae* and *G. lapillus*, and compounds displaying MTX-like activity were detected in all species tested. Fitness curves across environmental gradients of temperature, salinity and irradiance showed *Gambierdiscus* species can grow across a broad range of environmental conditions. The environmental niche of the tropical strains was not

significantly different from that of the temperate strains, suggesting that tropical toxin producing *Gambierdiscus* species also have the capacity to occupy temperate locations. Rafting on detached macrophyte fragments that are transported poleward in the East Australian Current was identified as the likely natural long-distance dispersal mechanism for *Gambierdiscus* species in eastern Australia. The ability of *Gambierdiscus* to colonise new temperate locations was examined by studying the growth of different strains co-cultured within both natural and artificial epibenthic microalgal communities. These experiments confirmed that it may only require a single pulse of very few cells for successful colonisation of *Gambierdiscus*. This thesis advances knowledge about the diversity and toxicology of *Gambierdiscus* in eastern Australia, identifies the potential cause of CFP from this region and provides experimental evidence of the mechanisms that could facilitate temperate range extension of the genus. Results from this thesis therefore provide fundamental information for developing a management strategy to mitigate the risk of human exposure to CFP in eastern Australia.

Chapter 1 General Introduction

1   General Introduction

1.1.   Microalgae

Microalgae are photosynthetic, microscopic, unicellular organisms that occupy a variety of niches in freshwater, estuarine and marine habitats. They occur in both pelagic and benthic environments, can live in symbiotic relationships with other organisms (e.g. coral, Muscatine & Porter (1977); sponges, Sara & Liaci (1964)) or epiphytically on both organic (e.g. macrophytes, Shah, An & Lee (2013); seagrass, Mabrouk et al. (2014)) and inorganic substrates (e.g. coral rubble, Ballantine et al. (1985); sand, Faust (1995)) . Microalgae require light, nutrients and inorganic carbon (CO_2) in combination with specific physicochemical variables such as temperature, pH and salinity in order to photosynthesise and grow. Microalgal species that occupy benthic and epiphytic niches (epibenthic) also require space on a substrate in order to sustain growth.

1.1.1   Harmful Algal Bloom (HAB) species

The term Harmful Algal Bloom (HAB) species describes any microalgal species that has a negative impact on human activities and/or aquatic communities. The term therefore incorporates microalgal species that cause mechanical clogging of gills, through to species that produce potent toxins which can cause illness and death when accumulated in seafood consumed by humans or marine life (see review by Zingone & Enevoldsen (2000)). The class Dinophyceae comprises the majority of toxin producing marine microalgal species (Smayda & Reynolds 2003; Zingone & Enevoldsen 2000). Species within this class have been identified as the source of a variety of human food related illnesses including Paralytic Shellfish Poisoning (PSP) (Wiese et al. 2010), Diarrhetic Shellfish Poisoning (DSP) (Murata et al. 1982), Neurotoxic Shellfish Poisoning (NSP)

(Baden & Adams 2000), Amnesic Shellfish Poisoning (ASP) (Jeffery et al. 2004), and Azaspiracid Shellfish Poisoning (AZP) (Satake et al. 1998).

1.1.2   Benthic Harmful Algal Bloom (BHAB) species

HAB species that occupy benthic and epiphytic niches are known as Benthic Harmful Algal Bloom (BHAB) species. Common BHAB genera include *Gambierdiscus* Adachi & Fukuyo (Adachi and Fukuyo 1979), *Fukuyoa* Gómez, Qui, Lopes & Lin (Gomez et al. 2015), *Ostreopsis* Schmidt (Schmidt 1901), *Coolia* Meunier (Meunier 1919), *Amphidinium* Claparède & Lachmann (Claparede & Lachmann 1859) and *Prorocentrum* Ehrenberg (Ehrenberg 1834). BHAB species have been implicated in a number of human related illnesses including respiratory (Durando et al. 2007) and dermatologic (Tubaro et al. 2011) conditions, through to clupeotoxism (Aligizaki et al. 2011), and Ciguatera Fish Poisoning (CFP) (Yasumoto, Inoue & Bagnis 1979; Yasumoto et al. 1979). Toxins produced by BHAB species are introduced to the food web through filter feeding activity of shellfish or grazing by herbivorous fish and invertebrates on macrophyte substrates harbouring the toxin producing organisms (Cruz-Rivera & Villareal 2006; Lewis & Holmes 1993; Yasumoto 2005).

1.2   Ciguatera Fish Poisoning (CFP)

Ciguatera Fish Poisoning (CFP) arises when humans consume marine fish whose flesh and viscera have been contaminated with ciguatoxins (Lehane & Lewis 2000) and/or maitotoxins (Kohli, Papiol, et al. 2014). Globally, it is estimated that between 50,000 and 200,000 people each year are affected by CFP (Dickey & Plakas 2010; Lehane & Lewis 2000), making it the most prevalent nonbacterial human illness associated with the consumption of seafood globally (Friedman et al. 2017). However, it is thought that many

individuals with CFP often do not seek medical attention. When they do, symptoms including nausea, vomiting, diarrhoea, abdominal pain, sensory changes, numbness, weakness and fatigue, blurred vision and temperature dysesthesia (Dickey & Plakas 2010; Friedman et al. 2017; Gillespie, Lewis, et al. 1985) are often not recognised as CFP (Friedman et al. 2017;), leading to significant under-reporting (Skinner et al. 2011).

Cases of CFP are prevalent in nations of the South Pacific (Bagnis, Kuberski & Laugier 1979; Lewis 1986), affecting up to 2,000 per 100,000 people each year (Skinner et al. 2011). There have also been cases documented from fish caught in the Indian Ocean (Quod & Turquet 1996), Atlantic Ocean (Boada et al. 2010; Lawrence et al. 1980; Pérez-Arellano et al. 2005; Pottier, Vernoux & Lewis 2001), the Caribbean (Czernichow et al. 1984; Radke 2013; Radke, Reich & Morris Jr 2015; Tester et al. 2010) and Australia (Gillespie, Lewis, et al. 1985) and the incidence and worldwide distribution of CFP appears to be increasing (Levine 1995; Oshiro et al. 2010; Pérez-Arellano et al. 2005; Skinner et al. 2011). Additionally, international importation of seafood has caused cases of CFP to occur in non-endemic areas such as the United States (Geller, Olson & Senécal 1991; Zlotnick et al. 1995), Canada (Pilon et al. 2000) and Hong Kong (Sadovy 1998; Sadovy 1999; Wong et al. 2005), meaning this foodborne syndrome has become a global problem.

1.3   The causative organisms of Ciguatera Fish Poisoning (CFP)

Ciguatoxins and maitotoxins are produced by the BHAB genera *Gambierdiscus* and *Fukuyoa.* These genera have therefore been identified as the causative organisms of CFP (Yasumoto et al. 1977).

1.3.1 Taxonomy of *Gambierdiscus* and *Fukuyoa*

Adachi & Fukuyo (1979) originally described *Gambierdiscus toxicus*, the first species in the *Gambierdiscus* genus, from samples collected in the Gambier Islands, French Polynesia. For many years, the genus was thought to be monotypic, however increased interest and the application of sequencing approaches to taxonomic identification has caused significant reclassification of the genus. Gómez et al. (2015) used a DNA sequencing approach to re-classify some morphologically distinct taxa with globular shaped cells into a new genus, *Fukuyoa* F.Gómez, D.X.Qiu, R.M.Lopes & Senjie Lin, which now includes the species *F. yasumotoi*, *F. ruetzleri* and *F. paulensis* (see Table 1.1). Thus, the genus *Gambierdiscus* is now comprised of 15 formally classified species; *G. australes, G. balechii, G. belizeanus, G. caribeaus, G. carolinianus*, *G. carpenteri*, *G. cheloniae, G. excentricus, G. honu, G. lapillus, G. pacificus, G. polynesiensis, G. silvae, G. scabrosus, G. toxicus,* and 5 sub-groups that do not fit any published descriptions and are yet to be formally classified (*G.* ribotype 2, *G.* sp. 2, 3, 4, 5) (Table 1.1).

1.3.2 Toxins produced by *Gambierdiscus* and *Fukuyoa*

Not all species of *Gambierdiscus* or *Fukuyoa* produce ciguatoxins (Table 1.2), the causative agent of CFP (Yasumoto et al. 1977). These highly potent, lipophilic, polyether toxins affect mammalian cells by activating voltage gated sodium channels (Benoit, Legrand & Dubois 1986; Legrand et al. 1989; Lombet, Bidard & Lazdunski 1987). Ciguatoxins produced by *Gambierdiscus* species occur in a number of structural forms (e.g. P-CTX-3B, 3C, 4A, 4B), each displaying different toxicities (Chinain et al. 2010) (summarised in Table 1.2)

Table 1.1. Species within *Gambierdiscus* and *Fukuyoa* genera.

Gambierdiscus and *Fukuyoa* **Species**

G. australes Chinain & M.A.Faust, G. balechii S.Fraga, F.Rodríguez & I.Bravo

G. balechii S.Fraga, F.Rodríguez & I.Bravo

G. belizeanus M.A.Faust

G. caribeaus Vandersea, Litaker, M.A.Faust, Kibler, W.C.Holland & P.A.Tester

G. carolinianus Litaker, Vandersea, M.A.Faust, Kibler, W.C.Holland & P.A.Tester

G. carpenteri Kibler, Litaker, M.A.Faust, W.C.Holland, Vandersea & P.A.Tester

G. cheloniae K.F.Smith, L.Rhodes & S.A.Murray

G. excentricus S.Fraga

G. honu Rhodes, Smith & Murray

G. lapillus Kretzschmar, Hoppenrath & Murray

G. pacificus Chinain & M.A.Faust

G. polynesiensis Chinain & M.A.Faust

G. silvae S.Fraga & F. Rodríguez

G. scabrosus T.Nishimura, Shinya Sato & M.Adachi

G. toxicus Adachi & Fukuyo

G. **ribotype 2**

Gambierdiscus **sp. 2**

Gambierdiscus **sp. 3**

Gambierdiscus **sp. 4**

Gambierdiscus **sp. 5**

F. yasumotoi (M.J.Holmes) F.Gómez, D.X.Qiu, R.M.Lopes & Senjie Lin

F. ruetzleri (M.A.Faust, Litaker, Vandersea, Kibler, W.C.Holland & P.A.Tester) F.Gómez, D.X.Qiu, R.M.Lopes & Senjie Lin

F. paulensis F.Gómez, D.X.Qiu, R.M.Lopes & Senjie Lin

Maitotoxins are also produced by species of *Gambierdiscus* and *Fukuyoa* (Yasumoto et al., 1976) (summarised in Table 1.2) and also occur in a variety of structural forms (e.g. MTX-1, MTX-2, MTX-3, MTX-4). These large, extremely potent hydrophilic polyether toxins enhance intracellular calcium levels (Gusovsky & Daly 1990; Murata et al. 1992; Trevino et al. 2008) and are amongst the most lethal non-proteinaceous natural compounds known (Murata et al. 1993). It was originally thought that these hydrophilic compounds did not contribute to human poisonings due to their water solubility (Lewis & Holmes 1993), however recent work has confirmed that maitotoxins can be retained in the viscera, liver and flesh of fish (Kohli, Papiol, et al. 2014) and may therefore contribute to the syndrome of CFP in humans.

Other toxic compounds identified from *Gambierdiscus* and *Fukuyoa* include Gambierol (Satake, Murata & Yasumoto 1993), Gambieroxide (Watanabe et al. 2013), Gambieric acid (Nagai et al. 1992) and Gambierone (Rodríguez et al. 2015). Gambierol is a lipophilic compound that is a potent blocker of voltage-gated potassium channels (Cuypers et al. 2008). Gambieroxide, is another lipophilic compound for which the mode of activity remains unidentified (Watanabe et al. 2013). Gambieric acid, is hydrophilic and known to have antifungal properties (Nagai et al. 1992) but the mode of activity is also undefined. Finally, Gambierone is another hydrophilic compound known to activate voltage-gated sodium channels much like maitotoxins but is far less potent (Rodríguez et al. 2015). Many of these toxic compounds were described from strains of *Gambierdiscus* when the species was still thought to be monotypic, therefore their presence and the role of each in CFP is yet to be verified.

Table 1.2. Toxin composition of *Gambierdiscus* and *Fukuyoa* species. Positive symbol (+) and green colour indicates positive results, negative symbol (-) and red colour indicates toxins were not detected, both positive and negative symbols with orange colour indicates both positive and negative results have been reported and empty cells indicate no data is available.

Species	CTX					MTX					Crude Extract		References
	LC-MS/MS CTX	MBA	N2a	RBA	FLIPR	LC-MS/MS MTX-1	LC-MS/MS MTX-3	MBA	FLIPR	HA	MBA	N2a	
G. australes	-	- +	+	- +	+	- +	+	+	+	+	+	+	1, 2, 3, 4, 5, 6, 7, 10, 14, 15, 17, 18, 19, 20, 21
G. balechii		+	+					+		+			9, 7
G. belizeanus	-			+	+			+	+		+		4, 5, 10, 13, 14
G. caribaeus	-		+		+			+	+			+	10, 12, 14, 17
G. carolinianus			+		+			+	+				10, 14, 17
G. carpenteri	-		+		+			+	+				10, 14, 17
G. cheloniae	-					-	+				+		25
G. excentricus			+						+			+	8, 17
G. honu	-					-	+				+		21
G. lapillus	-					-	+				+		11
G. pacificus	-	+	- +	-		-	+	+		+	+		5, 6, 7, 10, 16, 17, 19, 20, 25
G. polynesiensis	+	+	+	+		-	+	+			+	+	5, 6, 16, 19, 20, 25
G. ribotype 2					+			+	+			+	10, 12, 14
G. scabrosus (G. sp. type 1)		+	+					+		+			15, 17
G. silvae			+							+			17

													References
(G. ribotype 1*)*													
G. sp. type 2		-						-					15
G. sp. type 3		-						-/+					15
G. sp. type 4			+										25
G. sp. type 5			+										25
G. toxicus		-/+	-	-/+				+			+	-	4, 5, 6, 7, 16
F. paulensis	-	+	-			-	+	+					26, 28
F. ruetzleri	-				+			+	+	+			10, 14, 27, 29
F. yasumotoi													
Fukuyoa cf. *yasumotoi*													

Toxicology method codes: Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS); Mouse Bioassay (MBA); Mouse Neuroblastoma Assay (N2a); Receptor Binding Assay (RBA); Ca^{2+} influx SH-SY5Y cell Fluorescent Imaging Plate Reader bioassay (FLIPR); Haemolytic Assay (HA); CTX and MTX refer to the CTX and MTX fraction following liquid partitioning and Crude Extracts refers to studies that did not separate CTXs and MTXs using liquid partitioning.

References: 1. Babinchak, Jollow et al. 1986; 2. Bomber et al. 1989; 3. Caillaud et al. 2011; 4. Chinain et al. 1997; 5, Chinain et al. 1999; 6. Chinain et al. 2010; 7, Darius et al. 2007; 8, Fraga et al. 2011; 9. Fraga et al. 2016; 10. Holland et al. 2013; 11. Kretzschmar et al. 2016; 12. Lartigue et al. 2009; 13. Leaw et al. 2011; 14. Lewis et al. 2016; 15. Nishimura et al. 2013; 16. Pawlowiez et al. 2013; 17. Pisapia et al. 2017; 18. Rhodes et al. 2010; 19. Rhodes, Gimènez Papiol, et al. 2014; 20. Rhodes, Harwood, et al. 2014; 21. Rhodes et al. 2017; 22. Rhodes et al. 2017; 23. Roue et al. (2016); 24. Smith et al. (2016); 25. Xu et al. (2014); 26. Gomez et al. (2015); 27. Holmes et al. (1998); 28. Laza-martinez et al. (2015); 29. Lyu et al. (2017).

1.3.3   Detection of toxins produced by *Gambierdiscus* and *Fukuyoa*

Detection of toxins produced by *Gambierdiscus* and *Fukuyoa* is complicated by the many structural differences in the natural products produced, typically low observable quantities, and the lack of adequate reference standards (Lehane & Lewis 2000). Consequently, there is, no unified approach for evaluating the toxicology of *Gambierdiscus* and *Fukuyoa* strains.

Selected known toxins (P-CTX-3B, 3C, 4A, 4B, MTX-1, putative MTX-3) can be detected and quantified using Liquid Chromatography Mass Spectrometry (LC-MS) (Chinain et al. 2010) but the most common assessment of toxicity is via the mouse bioassay (e.g. Chinain et al. 2010; Chinain et al. 1999; Kohli, Murray, et al. 2014; Nishimura et al. 2013). This involves administering the extracted toxin both intraperitoneally and orally to Swiss albino mice, observing the symptomatology and calculating the median lethal dose (LD_{50}) (Hoffman, Granade & McMillan 1983). The mouse bioassay remains the only validated method for ciguatoxin detection and is still widely used, however it is non-specific and does not allow identification of the compounds responsible for the observed toxicity.

More recently, cell-based assays have been developed which assess the toxicology of an extract and have the advantage of allowing some degree of differentiation of the class of toxin. These cell-based assays include the Receptor Binding Assay (Chinain et al. 2010), Neuroblastoma (Neuro-2a) Cell Based Assay (Fraga et al. 2011; Pisapia et al. 2017; Xu et al. 2014), Human Erythrocyte Lysis Assay (Holland et al., 2013) and Ca^{2+} influx SH-SY5Y cell Fluorescent Imaging Plate Reader (FLIPR) bioassay (Lewis et al. 2016).

1.3.4   Geographic distribution of *Gambierdiscus* and *Fukuyoa*

Species of *Gambierdiscus* are primarily distributed in tropical and sub-tropical regions with some evidence of geographic endemism (Figure 1.1). For example, *G. excentricus* has only been reported from the Atlantic Ocean (Fraga et al. 2011; Nascimento et al. 2015), *G.* ribotype 2 and *G. silvae* from the Caribbean and Atlantic Ocean (Fraga & Rodríguez 2014; Litaker et al. 2010). *Gambierdiscus toxicus* has been reported from the South Pacific Ocean and Indian Ocean (Chinain, Faust & Pauillac 1999), while *G. pacificus* has only been reported from the Pacific Ocean (Dai et al. 2017; Litaker et al. 2010; Rhodes, Harwood, et al. 2014; Xu et al. 2014; Zhang et al. 2016). *Gambierdiscus balechii* and *G. scabrosus*, appear to be restricted to the North Pacific Ocean (Dai et al. 2017; Fraga et al. 2016; Nishimura et al. 2014; Xu et al. 2014) and *G. cheloniae*, *G. honu*, *G. lapillus*, *G. polynesiensis* are restricted to the South Pacific Ocean (Chinain et al. 2010; Kretzschmar et al. 2017; Rhodes et al. 2017; Smith et al. 2016), although many of these latter species have only recently been described and thus there is incomplete information about their distribution. In comparison, *G. australes*, *G. belizeanus*, *G. caribeaus*, *G. carolinianus* and *G. carpenteri* seem to have a global distribution (Jeong et al. 2012; Leaw et al. 2011; Litaker et al. 2009; Tawong et al. 2015; Xu et al. 2014). There are also reports of unidentified species of *Gambierdiscus* and *Fukuyoa* in many locations worldwide (e.g. Litaker et al. (2010); Nishimura et al. (2013)), highlighting that we still have an incomplete understanding of their biogeography.
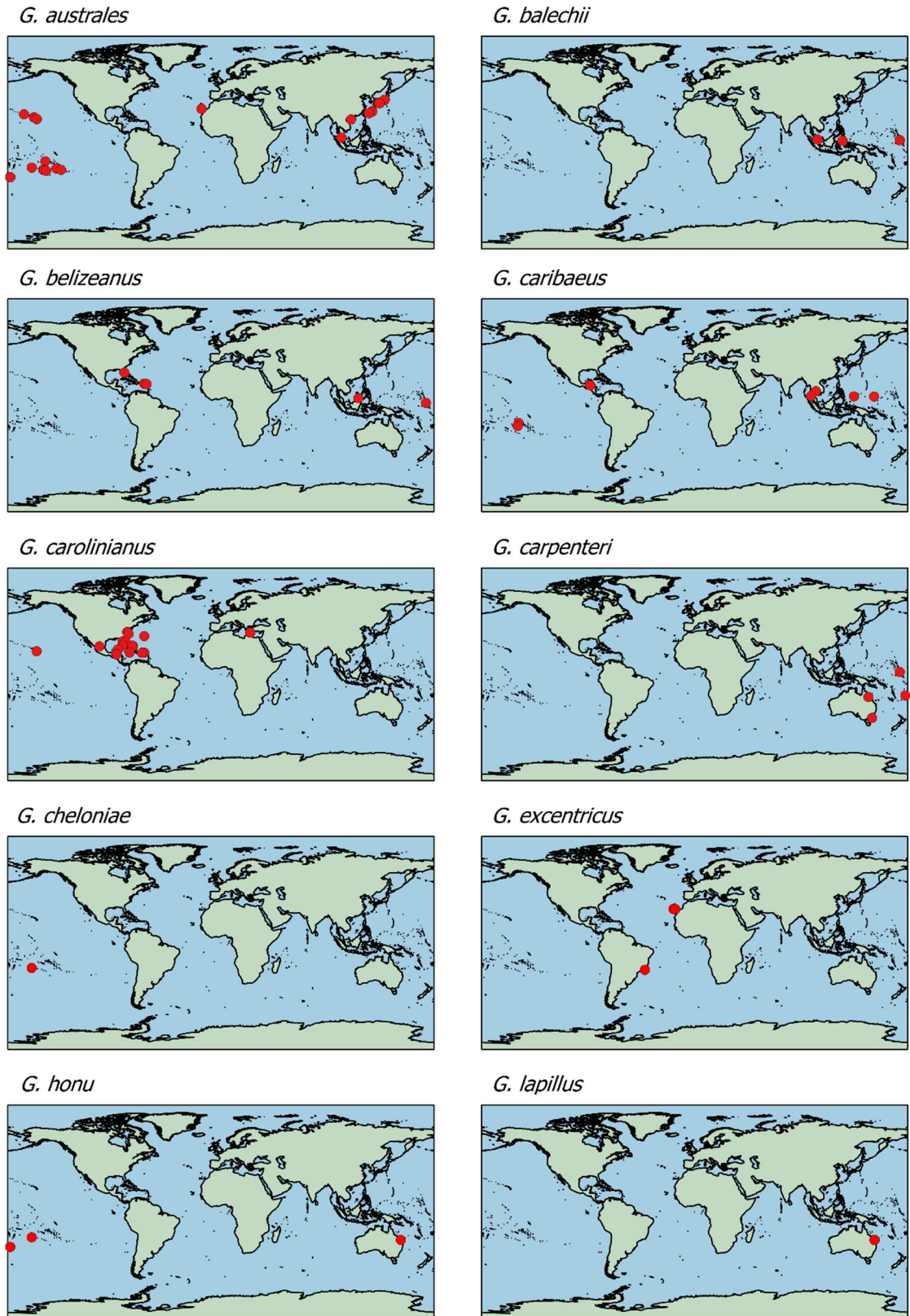
Figure 1.1. Global distribution of the 15 *Gambierdiscus* species and 5 sub-groups as listed in Table 1.1. *Gambierdiscus* strains that were not identified to species level were not included in this figure.
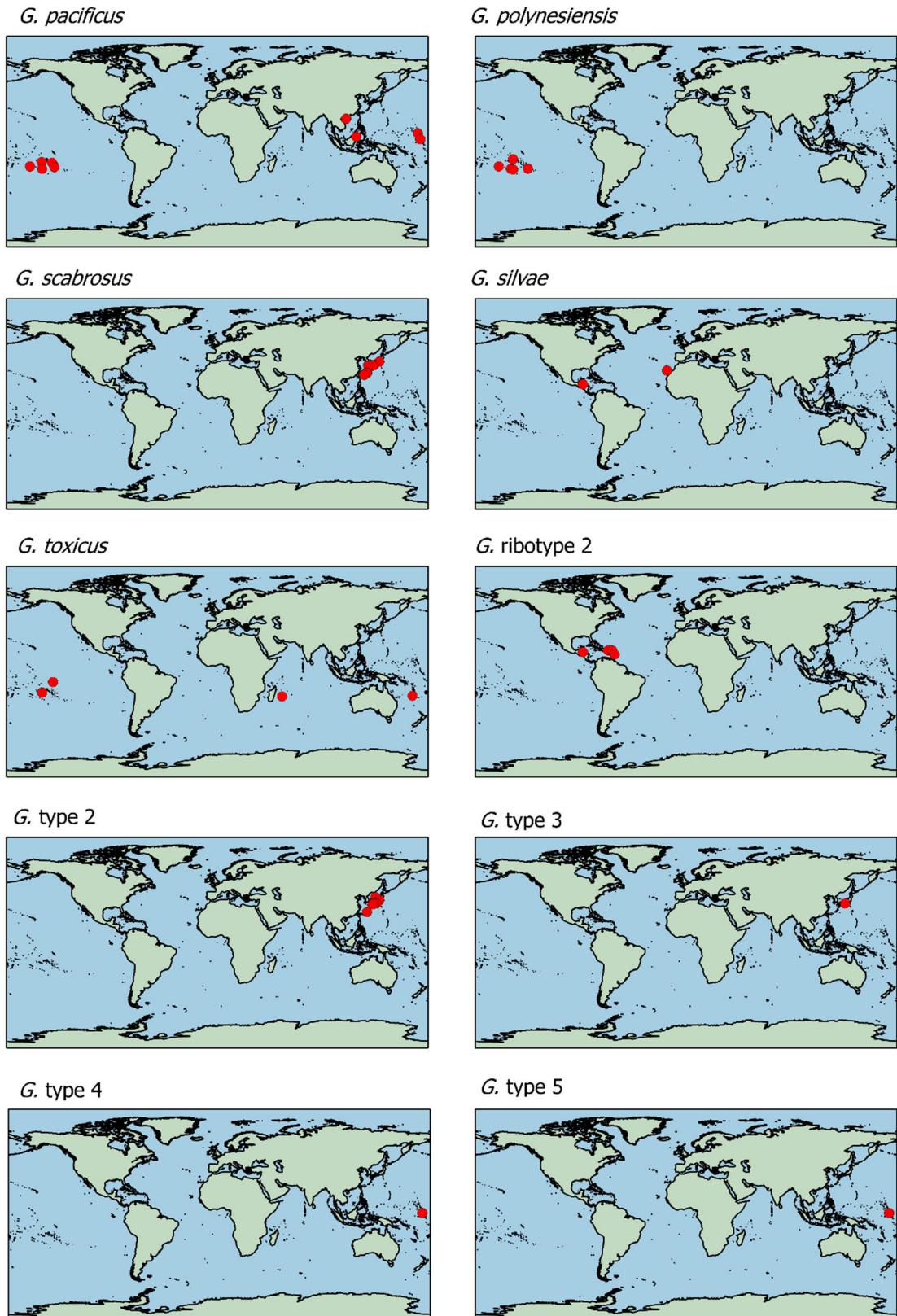
Figure 1.1. continued. Global distribution of the 15 *Gambierdiscus* species and 5 sub-groups as listed in Table 1.1. *Gambierdiscus* strains that were not identified to species level were not included in this figure.

Literature sources for *Gambierdiscus* species distribution maps: Argyle et al. 2016; Caillaud et al. 2011; Chinain et al. 1997; Chinain, Faust & Pauillac 1999; Chinain et al. 2010; Dai et al. 2017; Darius et al. 2007; Fraga & Rodrígues 2014; Fraga et al. 2011; Fraga et al. 2016; Holland et al. 2013; Jeong et al. 2012; Kibler et al. 2012; Kohli, Murray, et al. 2014; Kretzschmar et al. 2017; Lartigue et al. 2009; Leaw et al. 2011; Lewis et al. 2016; Litaker et al. 2009, Litaker et al. 2010; Mohammad-Noor et al. 2005; Murray et al 2014; Nascimento et al. 2015; Nishimura et al. 2013; Pawlowiez et al. 2013; Pisapia, Holland, et al. 2017; Rhodes, Gimènez Papiol, et al. 2014; Rhodes, Harwood, et al. 2014; Rhodes, Smith et al. 2014; Rhodes, Smith, Verma, Curley, et al. 2017; Richlen et al. 2008; Roeder et al. 2010; Smith et al. 2016; Tawong et al. 2015; Xu et al. 2014; Yoshimatsu et al. 2014; Zhang et al. 2016.

There are generally very few reports of *Fukuyoa* species, but some patterns of distribution are emerging (Figure 1.2). *Fukuyoa ruetzleri* has only been documented from the Caribbean and North Atlantic Ocean (Holland et al. 2013; Lewis et al. 2016; Lyu et al. 2017). *Fukuyoa yasumotoi* and *F.* cf. *yasumotoi* have so far only been identified from the northern hemisphere in the Florida Keys, USA (Lyu et al. 2017), Singapore (Holmes 1998; Litaker et al. 2009; Litaker et al. 2010) and Japan (Nishimura et al. 2013), while *F. paulensis* appears to have a much wider distribution across both the northern and southern hemispheres including the Mediterranean Sea (Laza-Martínez et al. 2016), Brazil (Gómez et al. 2015), New Zealand (Rhodes, Giménez Papiol, et al. 2014) and northern Australia (Murray et al. 2014). There have also been reports of *Fukuyoa* (not classified to species level) from Kuwait, Jordan (Saburova, Polikarpov & Al-Yamani 2013) and Mexico (Hernández-Becerril & Almazán 2004).
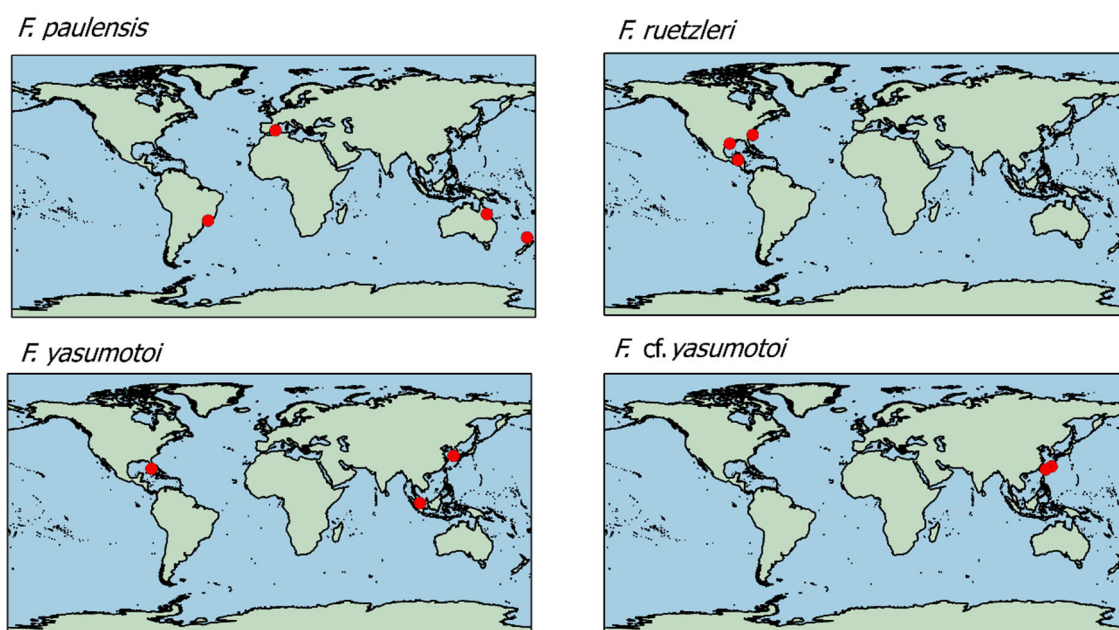
Figure 1.2. Global distribution of *Fukuyoa* species as listed in Table 1.1.

Literature sources: Gómez et al. 2015; Holland et al. 2013; Holmes 1998; Laza-Martínez et al. 2016; Lee et al. 2014; Litaker et al. 2009; Litaker et al. 2010; Murray et al. 2014; Nishimura et al. 2013; Rhodes et al. 2014.

Like cases of CFP, the causative organisms were thought to have a tropical to sub-tropical distribution between the latitudes of 32 °N and 35 °S (Lehane & Lewis 2000; Lewis 2001; Tindall & Morton 1998). However, in recent years, temperate populations of *Gambierdiscus* and *Fukuyoa* have been documented in the Eastern Mediterranean Sea (Aligizaki, Nikolaidis & Fraga 2008), North Pacific Ocean (Nishimura et al. 2013) and the South Pacific Ocean (Kohli, Murray, et al. 2014). This suggests the distribution of *Gambierdiscus* and *Fukuyoa*, and risk of exposure to CFP, may be expanding. It has been suggested that the presence of these temperate populations represents a range extension of the genus in response to or facilitated by global climate change (Aligizaki, Nikolaidis & Fraga 2008; Heimann, Capper & Sparrow 2011).

1.4 Co-occuring toxin producing epibenthic dinoflagellates

Gambierdiscus and *Fukuyoa* are not sole members of natural epibenthic assemblages. Instead, they coexist with many other microalgal taxa including other toxin producing dinoflagellates from the following genera *Ostreopsis*, *Coolia*, *Prorocentrum* and *Amphidinium* (Tan et al. 2014; Shah et al. 2014; Xu et al. 2014). Despite the considerable potential for interspecific competition, anecdotal observations by many researchers confirm that these taxa seem to form a consistent consortium in benthic habitats globally. The mechanisms that facilitate coexistence are currently unknown but may influence range extensions.

1.4.1 *Ostreopsis*

Species from the genus *Ostreopsis* frequently coexist with species of *Gambierdiscus* and *Fukuyoa*, although often occur in higher densities (Tindall and Morton, 1998). The genus currently consists of eleven species, *O. belizeanus* M. A. Faust (Faust 1999), *O. caribbeana* M. A. Faust (Faust 1999), *O. fattorussoi* Accoroni, Romagnoli & Totti (Accoroni et al. 2016), *O. heptagona* D. R. Norris, J. W. Bomber and Balech (Anderson, White & Baden 1985), *O. labens* M. A. Faust & S. L. Morton (Faust & Morton 1995), *O. lenticularis* Y. Fukuyo (Fukuyo 1981), *O. marina* M. A. Faust (Faust 1999), *O. mascarenensis* Quod (Quod 1994), *O. ovata* Y. Fukuyo (Fukuyo 1981), *O. rhodesiae* Verma, Hoppenrath & S. A. Murray (Verma, Hoppenrath, Dorantes-Aranda, et al. 2016) and *O. siamensis* Johs. Schmidt (Schmidt 1901). However, ambiguities in defining morphological characteristics mean this group is currently under phylogenetic review (Penna et al. 2010; Penna et al. 2005; Pin et al. 2001).

Five of the currently recognized eleven species of *Ostreopsis* are thought to produce analogues of palytoxins, while the remaining six are yet to be analysed (reviewed in

Parsons et al., 2012). Palytoxins are large, water soluble polyalcohols, which affect the function of cellular sodium pumps (reviewed in Vale 2008) and are another of the most potent natural toxins known, second only to maitotoxins (Riobó et al. 2008). Human poisonings after consumption of fish affected with palytoxins, thought to have originated from species of *Ostreopsis,* have been documented, including fatalities (Onuma et al. 1999; Taniyama et al. 2003). There was also a case in the Mediterranean in 2005, where hundreds of people required medical attention for respiratory problems after toxins became aerosolized during a large *Ostreopsis* bloom (Brescianini et al. 2006; Ciminiello et al. 2006).

Species of *Ostreopsis* are distributed widely across the globe (see review by Rhodes 2011) with the latitudinal distribution ranging from 45 °N (Monti et al. 2007) to 45 °S (Rhodes 2011). In Australia, species of *Ostreopsis* have been found from tropical Queensland (Hallegraeff 2002; Holmes et al. 1988; Murray 2010) to the temperate coastal waters of New South Wales (Kohli, Murray et al. 2014; Verma, Hoppenrath, Harwood, et al. 2016) and Tasmania (Pearce et al., 2001) yet much work needs to be done to characterise the toxicity and distribution of this genus in Australia.

1.4.2 *Coolia*

As with other epibenthic dinoflagellates, there has been considerable taxonomic confusion regarding the genus *Coolia*. It was originally described by Meunier (1919) as a separate genus and then grouped with the genus *Ostreopsis* by Lindemann (1928) due to morphological similarities. Later it was transferred to the genus *Glenodinium* by Biecheler (1952) before being reinstated to the genus *Coolia* after more detailed morphological examination (Balech 1956) and phylogenetic analysis (Dolapsakis et al. 2006; Penna et al. 2005). For more than 90 years, the genus *Coolia* was thought to be

monotypic, consisting only of *Coolia monotis*. Since 1995 however, an additional six species have been described. The genus now consists of *C. areolata* L. Ten-Hage, J. Turquet, J. P. Quod & Couté (Ten-Hage et al. 2000), *C. canariensis* S. Fraga (*Fraga et al. 2008*), *C. malayensis* Leaw, P. T. Lim & Usup (Leaw et al. 2010), *C. monotis* Meunier (Meunier 1919), *C. palmyrensis* Karafas, Tomas & York (Karafas, York & Tomas 2015), *C. santacroce* Karafras, Tomas & York (Karafras, York & Tomas 2015) and *C. tropicalis* M. A. Faust (Faust 1995).

A strain of *Coolia monotis*, isolated from Queensland, Australia, was found to produce Cooliatoxin (Holmes et al. 1995) a lipophilic, polyether toxin similar to yessotoxins (which are structurally alike to ciguatoxins) (Tubaro et al. 2010). However, the strain in Holmes' study is now suspected to have been *C. tropicalis* (Mohammad-Noor et al. 2013). Similarly, a strain of *C. monotis* isolated from Japan also produced cooliatoxin (Nakajima, Oshima & Yasumoto 1981) but a New Zealand strain did not, although the latter strain is now thought to have been *C. malayensis* (Rhodes, Smith et al. 2014). Despite toxins isolated from species of *Coolia* proving lethal to mice (Carlson et al. 1984), and invertebrates (Rhodes & Thomas 1997), these lipophilic polyether toxins have not yet been implicated in human illnesses. Further work to confirm identification and assess toxicity of *Coolia* species and strains as they are discovered is required.

As currently understood, the distribution of species from the genus *Coolia,* appears to be worldwide, including both tropical and temperate regions, although *C. tropicalis*, *C. areolata* and *C. palmyrensis* seem to be restricted to tropical regions (Faust 1995; Fraga et al. 2008; Mohammad-Noor et al. 2013; Rhodes et al. 2000). In Australia, there has been very limited work evaluating the distribution, species identity and toxicity of *Coolia.* Early work began with Holmes et al. (1995) characterising a strain collected from Platypus Bay, Queensland. More recently, the genus was assessed in more detail in the

Great Barrier Reef region (Momigliano et al. 2013) and some species were detected during sampling in Exmouth, Western Australia and temperate regions of New South Wales (Kohli, Neilan et al. 2014).

1.4.3 *Prorocentrum*

Prorocentrum is another genus of epibenthic dinoflagellate which co-occurs with *Gambierdiscus, Ostreopsis* and *Coolia.* This genus is very large, consisting of 56 species (Gómez 2005) with representatives occupying planktonic, benthic and epiphytic habitats (see review by Hoppenrath et al. 2013). Species from this genus continue to be discovered and their toxicity assessed (e.g. David, Laza-Martínez et al. 2014; Henrichs et al. 2013; Lim et al. 2013) with several species shown to produce Okadaic Acid and similar analogues (e.g. Heredia-Tapia et al. 2002; Morton, Bomber & Tindall 1994; Ten-Hage et al. 2000), although it is not known if these toxins contribute to human poisonings. Epibenthic species of *Prorocentrum* are distributed widely throughout Australia (e.g. Heil et al. 2004; Morton 1998; Murray, Nagahama & Fukuyo 2007) and the world (see review by Hoppenrath et al. 2013).

1.4.4 *Amphidinium*

Amphidinium is a very diverse genus which includes species that are planktonic, benthic, epiphytic and symbiotic (Jørgensen et al. 2004; Taylor 1971; Trench 1993). This genus is currently undergoing major reclassification with many species formerly classified under the genus *Amphidinium*, now being transferred to genera of their own (e.g. Sparmann, Leander & Hoppenrath 2008; Hoppenrath et al. 2012; Horiguchi et al. 2012). Epibenthic species of *Amphidinium* are widely distributed throughout Australia (e.g. Murray et al. 2004; Murray & Patterson 2002) and the world (e.g. Berland, Grzebyk & Thomassin 1992; Lee et al. 2003; Dolapsakis & Economou-Amilli 2015) with some

species known to produce bioactive compounds such as amphidinols (Murata et al. 1999; Paul et al. 1995; Satake et al. 1991). However, there is no evidence linking the production of toxins from species of *Amphidinium* to human poisonings, although considerable work further characterising species and the toxins is still required.

1.5   An Australian Perspective
1.5.1   Ciguatera Fish Poisoning (CFP) in Australia

CFP has been an issue in Australia for many years. There were two human fatalities and more than 1400 documented cases between 1965 and 2010 (Gillespie, Lewis, et al. 1985; Hamilton et al. 2010). Outbreaks have primarily occurred in Queensland (Togue et al. 1967; Clark & Whitwell 1968; Lewis & Endean 1984; Gillespie, Lewis, et al. 1985; Lewis et al. 1988; Lewis & Sellin 1992; Endean et al. 1993) and the Northern Territory (Lucas, Lewis & Taylor 1997), with one case from Victoria traced to consumption of a Maori Wrasse (*Cheilinus undulates*) imported from the Great Barrier Reef (Lucas, Lewis & Taylor 1997). More recently, there have been multiple cases of CFP in northern New South Wales, linked with the consumption of locally caught Spanish mackerel (*Scomberomorus commerson*) contaminated with P-CTX-1B (Farrell et al. 2016) (Figure 1.3).
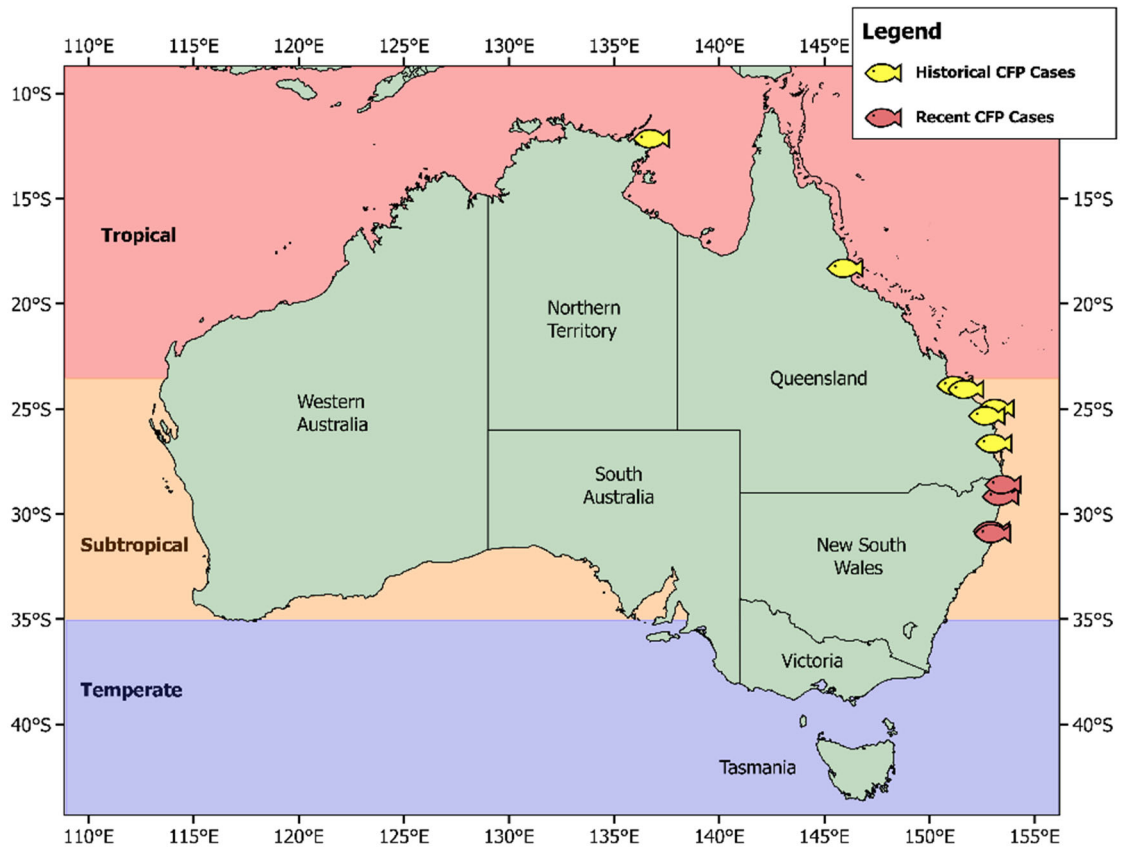
Figure 1.3. Map of Australia showing the locations of historical (1965 – 1985) and recent (2002 – 2017) reported cases of Ciguatera Fish Poisoning (CFP).

Literature sources: Endean et al. 1993; Farrell et al. 2016; Kohli et al. 2017; Lewis and Endean 1984; Lewis & Sellin 1992; Lucas, Lewis & Taylor 1997; Tonge et al. 1967

1.5.2 *Gambierdiscus* and *Fukuyoa* in Australia

Several species of *Gambierdiscus* and *Fukuyoa* have been documented from the Great Barrier Reef region of tropical eastern Australia (Figure 1.1). Sparrow et al. (2017) identified *G. carpenteri* from the central Great Barrier Reef region. Similarly, Murray et al. (2014) identified *G. carpenteri* and a species similar to *G. belizeanus* from the same region. Murray et al. (2014) also identified a species similar to *G. yasumotoi* (*G.* cf. *yasumotoi*) from this region but this taxon has been reclassified by Gómez et al. (2015)

as *Fukuyoa paulensis*. More recently, Kretzschmar et al. (2017) described *G. lapillus* collected from Heron Island within the southern Great Barrier Reef region. In addition, a previously unidentified strain of *Gambierdiscus* from the same location (Richlen et al. 2008) was reclassified as a new species, *G. honu*, found to be widely distributed across the South Pacific Ocean (Rhodes et al. 2017). There were also numerous reports of *Gambierdiscus* with unconfirmed species designations from other sites in the Great Barrier Reef region from early studies before the genus was reclassified (Gillespie, Holmes, et al. 1985; Hallegraeff 1993; Holmes & Lewis 1994; Holmes, Lewis & Gillespie 1990; Holmes et al. 1991; Skinner, Lewis & Morton 2013; Sparrow & Heimann 2016).

A temperate population of *Gambierdiscus* has also been identified from eastern Australia. Kohli, Murray, et al. (2014) provided the first description of this population at Merimbula Lake and other nearby inlets in southern New South Wales and identified the species as *G. carpenteri* (Figure 1.1). It is not known if this temperate population of *G. carpenteri* is a uniquely adapted strain of the species or is evidence of an extension of the range of the tropical population into higher latitudes.

There is only one report of *Gambierdiscus* from the west coast of Australia. Kohli (2013), identified a *Gambierdiscus* species similar to *G. carpenteri* in samples analysed with *Cob* gene pyrosequencing from Exmouth. Despite the limited investigations of *Gambierdiscus* and *Fukuyoa* diversity in Australia, *G. carpenteri* appears to be most frequently recorded, suggesting it has a broad distribution and is well adapted to coastal benthic habitats.
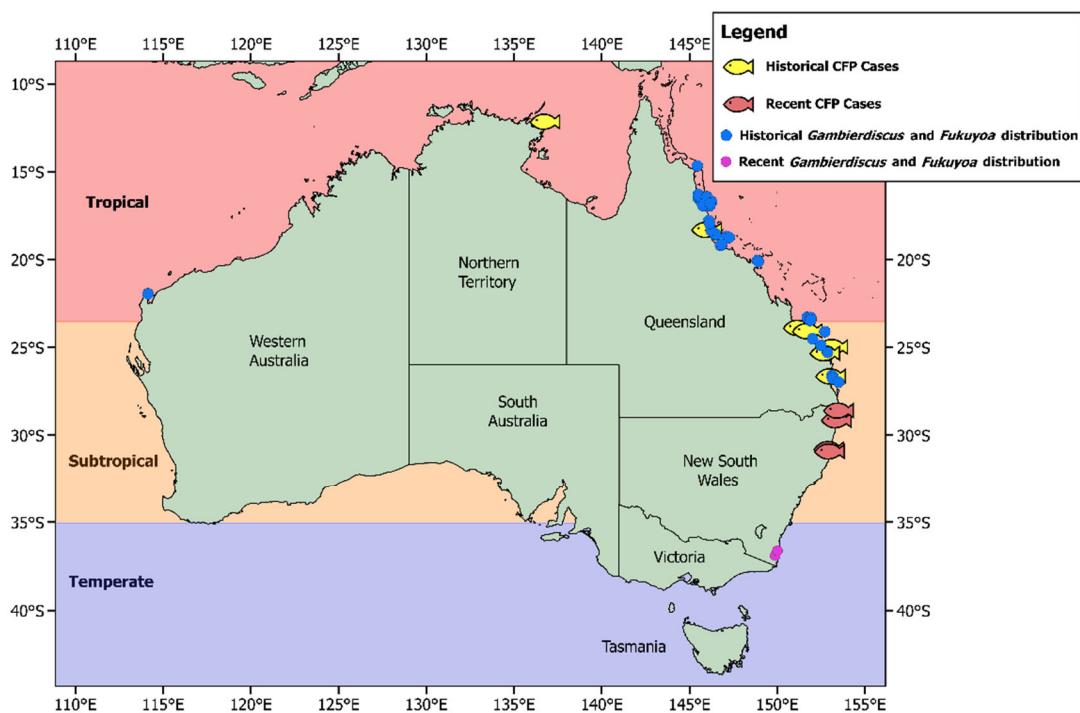
Figure 1.4. Map of Australia showing the locations of historical and recent reported cases of Ciguatera Fish Poisoning (CFP) in humans overlayed with the historical (< 2014) and recent (> 2014) distribution of *Gambierdiscus* and *Fukuyoa*.

Literature sources: Gillespie et al. 1985; Holmes, Lewis and Gillespie 1990; Holmes et al. 1991; Holmes 1992; Holmes and Lewis 1994; Kohli, Murray, et al. 2014; Kretzschmar et al. 2017; Murray et al. 2014, Richlen et al. 2008; Skinner et al. 2013; Sparrow et al. 2016.

1.5.3   Toxicology of *Gambierdiscus* and *Fukuyoa* from Australia

The first toxicological analyses of Australian strains of *Gambierdiscus* started in the early 1990's, with strains collected from Queensland (Holmes 1992; Holmes & Lewis 1994; Holmes, Lewis & Gillespie 1990; Holmes et al. 1991). Examination of strain NQ1 isolated from Hastings Reef, yielded the description of MTX-2 but no ciguatoxins were detected (Holmes, Lewis & Gillespie 1990). Further examination of two other strains,

NQ2/7 and WC1/1, isolated from Arlington Reef and Platypus Bay respectively, identified the presence of toxicity detected using the mouse bioassay, which was attributed to compounds called gambiertoxins, which were described as being precursors to CTXs (Holmes et al. 1991). The term gambiertoxins was used to discern between the compounds isolated from fish (ciguatoxins) and those of microalgal origin (gambiertoxins), but this terminology was eventually abandoned and now all compounds are known as ciguatoxins. Holmes et al. (1991) were the first to recognise toxicity likely associated with CTXs in strains of *Gambierdiscus* isolated from Australia. Unfortunately, the taxonomic identity of the strains used in these studies (described as *Gambierdiscus toxicus*) is not confirmed, as identification occurred prior to reclassification of the genus.

More recently, Kretzschmar et al. (2017), used LC-MS/MS methods to test strains of *G. lapillus* for production of characterised CTXs (P-CTX-3B, 3C, 4A, 4B) and MTXs (MTX-1, putative MTX-3) for which standards are available. No CTXs were detected, although unassigned peaks were reported in the CTX transition zone. The putative MTX-3 was the only MTX detected using this method. Results from the mouse bioassay showed toxicity via intraperitoneal injection but were not as clear for oral administration. However, crude extracts without separation of CTXs and MTXs were used for this study. Without separating the CTXs from MTXs, the toxic effects cannot be assigned to a particular toxin class. This is important because current data suggests it is primarily CTXs responsible for CFP but the toxic effects identified in Kretzschmar et al. (2017), may be a result of high MTX toxicity and therefore the actual contribution of *G. lapillus* to cases of CFP cannot be determined. This study by Kretzschmar and colleagues represents the only toxicological assessment of *Gambierdiscus* or *Fukuyoa* species from Australia reflecting the lack of laboratory cultures of Australian origin.

Kohli, Murray, et al. (2014) performed toxicological analyses on the temperate population of *G. carpenteri* from southern New South Wales, using cells collected directly from the field. Using the same LC-MS/MS and mouse bioassay approach as Kretzschmar et al. (2017), no characterised CTXs or MTXs were detected, though toxicity was detected via intraperitoneal injection and not via oral administration. Just as in Kretzschmar et al. (2017), it is not possible to determine whether CTXs or MTXs were responsible for the toxicity as crude extracts were also used in this study.

To date, these are the only investigations into the toxicology of Australian *Gambierdiscus* and there are no studies investigating the toxicology of Australian *Fukuyoa*. This thesis sought to close that knowledge gap by examining the toxicology of *Gambierdiscus* and *Fukuyoa* from Australia and identify the species likely responsible for cases of CFP in this region.

1.6   Thesis outline

The goal of this thesis was to provide new understanding of the CFP causing taxa present in Australia. Within this context, this thesis aimed to:

1. Investigate the diversity and toxicology of *Gambierdiscus* from eastern Australia.

2. Understand the ecology of the unique temperate population of *G. carpenteri.*

3. Assess if the temperate *G. carpenteri* population represents an extension of the distribution of the tropical *G. carpenteri* population.

4. Evaluate the mechanisms involved in range extension of *Gambierdiscus* in eastern Australia.

5. Consider if other *Gambierdiscus* species also have the capacity for temperate extension of their distribution.

These specific aims are addressed in the following chapters.

Chapter 2 describes the identity and toxicology of *Gambierdiscus* monoclonal isolates established from a tropical and a temperate location in eastern Australia.

Chapter 3 investigates how environmental variables influence the growth of the established tropical and temperate *Gambierdiscus* isolates and characterises the ecological niche for each species.

Chapter 4 examines long distance dispersal mechanisms for BHAB species.

Chapter 5 evaluates the capacity for *Gambierdiscus* cells to colonise novel temperate locations.

Finally, to conclude, Chapter 6 summarises the key findings and highlights new insights that this thesis has contributed to our understanding of CFP causing organisms in eastern Australia, and their capacity for temperate range extension in this region.

The implications of these findings are evaluated in relation to changing climatic conditions from both an Australian and global perspective, and future research directions are identified.

Chapter 2 Diversity and Toxicology of *Gambierdiscus* spp. (Dinophyceae) from tropical and temperate Australian waters

2 Diversity and toxicology of *Gambierdiscus* spp. (Dinophyceae) from tropical and temperate Australian waters

Further publications arising from or related to this chapter:

Larsson, M. E., Harwood, D. T, Lewis, R. J., Himaya, S. W. A., Doblin, M. A. (2018). Toxicological characterisation of *Fukuyoa paulensis* (Dinophyceae) from temperate Australia. Phycological Research. 67(1):65-71, doi: 10.1111/pre.12349

Kretzschmar, A. L. and Larsson, M. E., Hoppenrath, M., Doblin, M. A., Murray, S. A. Characterisation of two toxic *Gambierdiscus* spp. (Gonyaulacalas, Dinophyceae) from the Great Barrier Reef (Australia): *G. lewisii* sp. nov. and *G. holmesii*, sp. nov. (Protist; in review).

Munday, R., Murray, S., Rhodes, L. L., Larsson, M. E., Harwood, D. T. (2017). Ciguatoxins and maitotoxins in extracts of sixteen *Gambierdiscus* isolates and one *Fukuyoa* isolate from the South Pacific and their toxicity to mice by intraperitoneal and oral administration. Marine Drugs 15(7):208, doi: 10.339/md15070208.

2.1   Abstract

Ciguatera Fish Poisoning (CFP) is a human illness caused by the consumption of marine fish contaminated with ciguatoxins (CTX), and possibly maitotoxins (MTX), produced by species from the epibenthic dinoflagellate genus *Gambierdiscus*. This study describes the identity and toxicology of some species of *Gambierdiscus* established from tropical and temperate eastern Australia. Based on newly cultured strains, four *Gambierdiscus* species were present at the tropical location, including *G. carpenteri*, *G. lapillus*, *G.* cf. *pacificus* and *G.* cf. *silvae*. Only *G. carpenteri* was identified from the temperate location. Characterised microalgal CTXs (P-CTX-3B, 3C, 4A and 4B) and MTX-1; were not detected in any species when tested using LC-MS/MS analyses. However, putative maitotoxin-3 (MTX-3) was present in all species except for the temperate strains of *G. carpenteri*. The presence of compounds displaying CTX-like activity in extracts of *G.* cf. *pacificus* and *G.* cf. *silvae* and trace level activity in strains of *G. lapillus* were detected using the Ca^{2+} influx SH-SY5Y cell Fluorescent Imaging Plate Reader (FLIPR) bioassay. While no detectable CTX-like activity was observed in tropical or temperate strains of *G. carpenteri*, all species showed strong MTX-like activity. This study, which represents the most comprehensive analysis of the toxicology of *Gambierdiscus* strains established from Australia to date, suggests that CFP in this region may be caused by currently undescribed CTXs and MTXs.

2.2   Introduction

Ciguatera fish poisoning (CFP) is a human illness of global importance. It occurs when marine fish contaminated with ciguatoxins (CTXs) and maitotoxins (MTXs) are consumed (Friedman et al. 2017). These potent neurotoxins are produced by species of

the epibenthic dinoflagellate genus *Gambierdiscus* (Yasumoto et al. 1977). There has been considerable taxonomic confusion within this genus. The application of molecular sequencing techniques, toxicity studies, more detailed scanning electron microscopy and a careful re-analysis of morphological characteristics, has led to reclassification of the genus into at least 15 species and five sub-groups (Litaker et al. 2009; Nishimura et al. 2014; Richlen et al. 2008; Xu et al. 2014). However, targeted sampling and continued research will likely reveal more diversity in the future.

As the increase of taxonomic resolution in the *Gambierdsicus* genus continues, it is also important to characterise the toxicology of each species to better understand which species are contributing to cases of human illness. CTXs are the primary toxins linked with cases of CFP (Yasumoto et al. 1977). These are highly potent, lipophilic, polyether toxins which affect mammalian cells by activating voltage gated sodium channels (Benoit, Legrand & Dubois 1986; Legrand et al. 1989; Lombet, Bidard & Lazdunski 1987) and can occur in a variety of different structural forms (P-CTX-3B, P-CTX-3C, P-CTX-4A, P-CTX-4B), each displaying differing toxicities (Chinain et al. 2010). MTXs are large (3, 424 dalton), potent polyether toxins which enhance intracellular calcium levels (Gusovsky & Daly 1990; Murata et al. 1992; Trevino et al. 2008), can occur in a variety of structural forms (Holmes & Lewis 1994; Holmes, Lewis & Gillespie 1990) and are produced by species of *Gambierdiscus* (Yasumoto et al. 1977).

Detection of toxins produced by *Gambierdiscus* species is complicated by the many structural forms produced, low observable quantities and a lack of adequate reference standards (Lehane & Lewis 2000). There is currently, no validated unified approach for evaluating the toxicity of *Gambierdiscus* species. Traditionally, *Gambierdiscus* toxicity was assessed via the mouse bioassay (Chinain et al. 2010; Chinain, Faust & Pauillac 1999), which involves administering the extracted toxin intraperitoneally or orally to

Swiss albino mice, observing the symptomatology and calculating the median lethal dose (LD50) (Hoffman, Granade & McMillan 1983). This method however is non-specific and does not allow identification of the compound responsible for the observed toxicity. Therefore, more recently, mammalian cell-based assays have been developed which assess the toxicity of a microalgal extract by utilizing the mode of activity of the toxins and provide some degree of differentiation amongst toxin classes. These include neuroblastoma (Neuro-2a) cytotoxicity (Pisapia, Holland, et al. 2017) and the Ca^{2+} influx SH-SY5Y cell Fluorescent Imaging Plate Reader (FLIPR) bioassay (Lewis et al. 2016). However, the most specific evaluation of the toxicology of *Gambierdiscus* species currently available is a Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS) approach which can detect selected toxins for which standards are available (P-CTX-3B, P-CTX-3C, P-CTX-4A, P-CTX-4B, MTX-1, putative MTX-3) (e.g. Rhodes et al. (2014); Rhodes, Smith, Verma, Curley, et al. (2017); Smith et al. (2016)), but is limited to the detection of only these specific compounds.

CFP has been an issue in Australia for decades (Clark & Whitwell 1968; Gillespie, Lewis, et al. 1985; Hamilton et al. 2010; Lucas, Lewis & Taylor 1997) and continues to be a contemporary issue (Farrell et al. 2016). However, to date, the species of *Gambierdiscus* producing the toxins responsible are yet to be identified. The presence of *Gambierdiscus* has been documented throughout the Great Barrier Reef region of tropical eastern Australia (Gillespie, Holmes, et al. 1985; Hallegraeff 1993; Holmes & Lewis 1994; Skinner, Lewis & Morton 2013). Many of these early reports however, were documented prior to the reclassification of the genus and therefore an accurate identification of the species is not available. Work to characterise the taxonomy of *Gambierdiscus* within Australia is progressing with the occurrence of *G. carpenteri* and a species very similar to *G. belizeanus* described at several locations in the Great Barrier Reef (Murray et al.

2014) as well as identification of a temperate variant of *G. carpenteri* at Merimbula, New South Wales (Kohli, Murray, et al. 2014). Kretzschmar et al. (2017) described *G. lapillus*, from samples collected at Heron Island and a previously unidentified strain of *Gambierdiscus* (Richlen et al. 2008) was reclassified as a new species distributed widely in the Pacific Ocean, *G. honu* (Rhodes, Smith, Verma, Curley, et al. 2017). Although our understanding of the identity of *Gambierdiscus* species in Australia is advancing, very little is known of their toxicology.

In this study, single cells of *Gambierdiscus* from tropical and temperate Australia were isolated and monoclonal cultures established. To verify their identity to species level, DNA was extracted from cultures and the D1-D3 and D8-D10 regions of the large subunit ribosomal rDNA gene were sequenced. Toxicity was assessed using LC-MS/MS and the Ca^{2+} influx SH-SY5Y cell FLIPR bioassay, advancing our understanding of the organisms which contribute to CFP in Australia.

2.3 Methods

2.3.1 Sampling and isolation

Epiphytes, including associated epibenthic microalgae, were removed from the surface of seagrass (*Zostera* sp.) collected from the temperate sampling site at Merimbula Inlet, New South Wales, Australia (36.8979°S, 149.9044°E) and macroalgae (*Padina* sp., *Laurencia* sp. and *Chnoospora* sp.) collected from the tropical sampling site at Heron Island lagoon, Queensland Australia (23.4423°S, 151.9148°E) on 7th April 2014 and 27th July 2014, respectively (Figure 2.1).
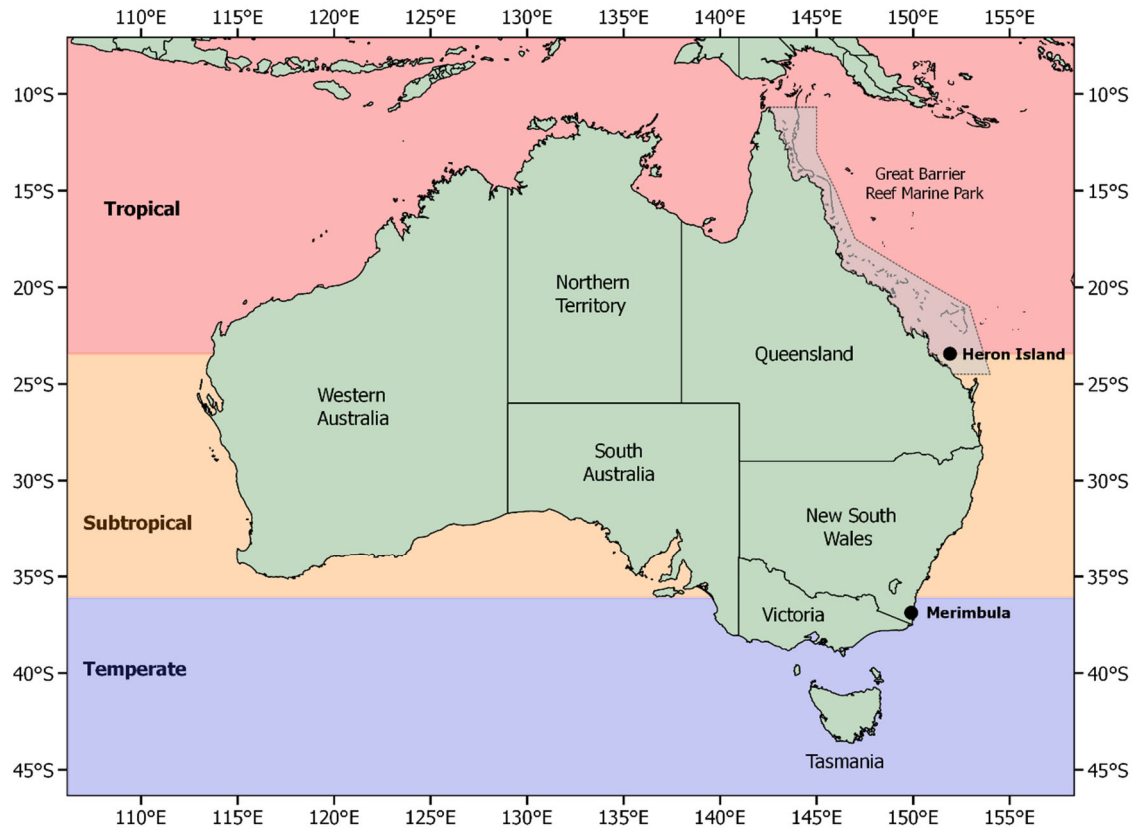
Figure 2.1. Map showing the location of the sampling sites, Heron Island, Queensland and Merimbula, New South Wales, Australia.

Single cells of *Gambierdiscus* spp. were isolated using the micropipette technique (Andersen & Kawachi 2005) and placed in individual wells of a 48-well clear microplate with 0.2 μm filtered sterilised and autoclaved seawater collected from each site and incubated in a plant growth chamber (Labec, Australia) at 20 °C under ~100 μmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle. Modified K medium (Litaker et al. 2009) was gradually introduced as cells began to grow. Once isolates reached a concentration of approximately 20 cells per well, they were transferred to 25 cm^2 (50 mL) sterile vented polystyrene tissue culture flasks (Falcon), oriented horizontally. Established cultures were then maintained in these vessels in modified K medium made from sterile oceanic

seawater (salinity was approximately 32, under ~100 µmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle.

2.3.2   Strain identification

Cells from approximately 100 mL of each *Gambierdiscus* culture were harvested by centrifugation at 600 x g for 10 min. DNA was extracted using a MoBio Soil DNA Extraction Kit following the manufacturer's instructions and sent to a commercial service (Australian Genomic Research Facility (AGRF), Queensland, Australia) where the D1-D3 region of the Large Subunit (LSU) rDNA was amplified using the primers D1R-F (Scholin et al. 1994) and D3-R (Nunn et al. 1996), and the D8-D10 region was amplified using the primers D8F and D10R (Litaker et al. 2009). These genetic markers were selected because they are commonly used for the *Gambierdiscus* genus and many sequences are publicly available for comparison on GenBank (www.ncbi.nlm.nih. gov). PCR amplifications were carried out in 50 µL reaction volumes containing AmpliTaq Gold 360 master mix, both forward and reverse primers (2.5 µm) and template at a concentration of 1 ng $µL^{-1}$. Thermocycling conditions for the D1-D3 region were 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 60 °C for 2 min, with a final step at 72 °C for 10 min.   Thermocycling conditions for the D8-D10 region were 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min, with a final step if 72 °C for 5 min. Amplification products (~ 950 bp) were purified and sequenced in both directions using the Sanger sequencing platform. Phylogenetic analyses were conducted in Genious v9.1.5 (Kearse et al. 2012). Publicly available sequences of *Gambierdiscus* spp., and other dinoflagellates used as out-groups (*Akashiwo sanguinea, Prorocentrum micans* and *Alexandrium affine, A. catenella* and *A. tamarense*) were downloaded from GenBank (www.ncbi.nlm.nih.gov) and aligned with the sequences obtained from this study using the MUSCLE algorithm (maximum number of iterations 8) (Edgar 2004). Sequences

from the D1-D3 and D8-D10 regions were truncated to 979 bp and 764 bp, respectively. Maximum Likelihood (ML) phylogenetic trees were generated for both regions with PHYML with 1,000 bootstraps (Guindon & Gascuel 2003) using a GTR substitution model and an estimated gamma distribution. Bayesian analysis was performed for both regions using MrBayes 3.2.6 (Huelsenbeck & Ronquist 2001) by means of the GTR+G (general-time reversible with gamma-shaped among-site variation) model. Bayesian analyses were carried out in four simultaneous runs with four chains each for 3.1×10^6 generations, sampling every 1,000 trees and 1,000 trees were discarded as burn in.

2.3.3 Toxicology

2.3.3.1 Detecting P-CTX-3B, 3C, 4A and 4B using Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS)

To test for the presence of these CTX compounds commonly linked with CFP, strains of *Gambierdiscus* established in this study were cultured in larger volume triplicate 75 cm^2 (250 mL) sterile vented polystyrene tissue culture flasks (Falcon, Corning, USA), at 24°C in a temperature controlled room under the maintenance conditions described above. To test the effect of temperature on toxin production by the tropical and temperate strains of *G. carpenteri* , an experiment was performed where cultures of the tropical and temperate strains (UTSHI2C4 and UTSMER9A3, respectively) were grown at 18 and 27 °C in temperature controlled plant growth cabinets (Climatron®, Plant Growth Cabinet, Australia).

Cell growth was monitored using *in vivo* chlorophyll *a* fluorescence measurements. A 1 mL aliquot was taken every 3 to 4 days from each flask and *in vivo* chlorophyll *a* fluorescence was measured for each sample using a fluorometer with the blue filter "*in vivo* chlorophyll *a*" module (Turner Designs, Trilogy®, USA) and then samples were

preserved with 1% Lugols iodine solution. Cultures were harvested in early stationary phase by centrifugation (3000 x g for 10 mins) and the three replicates were pooled to yield sufficient biomass before being freeze dried until further LC-MS/MS analyses.

Counts of Lugols-preserved cells were used to estimate the number of cells in each cell pellet. The final 1 mL aliquot of culture collected prior to harvesting was counted using a Sedgewick Rafter Counting Chamber under an inverted light microscope (x 100 magnification) (Nikon Eclipse TS100, Japan).

Analysis of selected CTXs was carried out using a quantitative LC-MS/MS method developed at the Cawthron Institute (full method details will be disclosed in an upcoming manuscript by T. Harwood). Microalgal pellets containing between 2.0×10^5 and 2.0×10^6 cells were extracted in 1 mL of 100 % MeOH in glass tubes, then mixed and sonicated for 5 minutes. Samples were transferred to glass auto sampler vials using a glass pipette and 2 μL was injected for analysis. LC-MS/MS analysis was performed on an Ultra Performance Liquid Chromatograph (UPLC) coupled to a mass spectrometer with electrospray ionization. Chromatographic separation used a BEH Phenyl phenyl column eluted with ammoniated mobile phases; A) Milli-Q and B) acetonitrile. Starting conditions were 25 % B followed by a stepped gradient to 95 % B after 8 min, with re-equilibration to 25 % B between 8-9 min. A flow rate 0.55 mL min^{-1} was used and the total run time was 9 minutes. Microalgal CTX reference material (P-CTX-3B; P-CTX-3C; P-CTX-4A; P-CTX-4B) was supplied by Institut Louis Malardé, French Polynesia.

MTX-1 (limit of detection of 1 ng mL^{-1}) and putative MTX-3 were monitored as intact structures using methods developed at Cawthron Institute (Selwood et al., 2014; Argyle et al., 2016). Briefly, for MTX-1 a pseudo multiple reaction monitoring (MRM) transition (*m/z* 1689.6>1689.6) was acquired for the intact di-anion with the electrospray ionization

source being operated in negative-ion mode. It was also possible to monitor the presence of the sulphated polyether analyte known as MTX-3 using a specific MRM transition (m/z 1037.6>96.8).

2.3.3.2 Ciguatoxin (CTX) and maitotoxin-like (MTX) activities

Selected strains of *Gambierdiscus* (tropical *G. carpenteri* UTSHI2C4, UTSHI6C3; temperate *G. carpenteri* UTSMER8B4, UTSMER9A3; *G. lapillus* UTSHI6B5, UTSHI2B6; *G.* cf. *pacificus* UTSHI6A6; *G.* cf. *silvae* UTSHI 6B1) were analysed for CTX and MTX-like activities. This subset of strains was chosen for analysis due to limited resources and selected based on the primary criterion of capturing species diversity across both locations.

Strains were again grown under standard conditions at 24 °C in a temperature controlled room in two x 2 L glass bottles. *In vivo* chlorophyll *a* fluorescence was used as a proxy for cell abundance to track growth and duplicate flasks were pooled and harvested in the early stationary phase by first concentrating the cells on a 20 µm sieve into a small volume, followed by centrifugation (3000 x g for 10 min). Total cell abundances ranged between 1.0×10^6 and 3.5×10^6.

CTX and MTX-like activities were determined following the functional bioassay described by Lewis et al. (2016). Briefly, cell pellets were extracted twice in a mix of analytical grade methanol:ultrapure water:hexane (2:1:1) at a concentration of 10 mL per 1.0×10^6 cells, and subsequently sonicated twice for 1 min using a sonication probe (Q125, QSonica, Newtown, Connecticut) at an amplitude of 50%. The extract was then centrifuged to remove cell debris (600 x g for 10 mins) and the supernatant layers sampled. The hexane layer was discarded and the remaining extract dried under N_2 gas, then reconstituted in 10 mL dichloromethane (DCM), and extracted twice with 5 mL 60 %

methanol (MeOH). The DCM lipophilic phase containing CTXs (top layer) was then separated from the MeOH hydrophilic phase containing MTXs (bottom layer) and both phases were dried separately under N_2 gas in 20 mL amber glass vials.

The dried extracts were reconstituted in 30% acetonitrile (ACN)/0.1% formic acid (FA) and approximately 1.0×10^6 microalgal cell equivalents were used for fractionation. Extracts were fractionated on an UltiMate 3000 Rapid Separation Liquid Chromatography System (Dionex, Illinois, USA) with a FC 204 Fraction Collector (Gilson, Middleton, USA). A Grace Vydac C18 (218TP) Reverse-Phase HPLC Column (250 x 4.6mm, 5 μm) (Grace Hichrom, Berkshire, UK) was used to separate the extracts with 0.043 % trifluoroacetic acid/90 % acetonitrile (aq) as elution buffer B and 0.05 % trifluoroacetic acid (aq) as buffer A. Initial elution was at 5 % B for 5 mins and then increased linearly to 90% B over 60 mins at a flow rate of 0.7 mL min^{-1}. Seventy-seven fractions were collected, freeze dried and reconstituted in 30 μL physiological salt solution (PSS; composition NaCl 140mM, glucose 11.5 mM, KCl 5.9 mM, $MgCl_2$ 1.4 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 5mM, CaCl2 1.8 mM, HEPES 10 mM) containing 0.1% bovine serum albumin (BSA) just prior to FLIPR assay analysis.

Functional activity of extracts was then determined following Lewis et al. (2016). Briefly, SH-SY5Y human neuronal cells (ECACC, Salisbury, Wiltshire, UK) were maintained in RPMI medium containing 15% Foetal Bovine Serum (FBS) and 2 mM L-glutamine, 100 units mL^{-1} penicillin and 0.1 mg L^{-1} streptomycin at 37 °C, under 5 % CO_2 atmosphere. Cells were routinely split once a week at a 1:5 dilution using 0.25% trypsin with ethylenediaminetetraacetic acid (EDTA) (Gibco, Thermofisher Scientific, Australia).

For the Ca^{2+} influx FLIPR bioassay, SH-SY5Y cells were planted into 384-well black walled imaging plates (Corning, Australia) at a density of 50, 000 cells per well and

cultured for 48 h under the conditions described above. Fluorescent responses (excitation, 470-495 nm; emission, 515-575 nm) were assessed using the $FLIPR^{TETRA}$ fluorescent plate reader (Molecular Devices, Sunnydale, California, USA) after a 30 min incubation with a fluorescent Ca^{2+} dye (Calcium 4 No Wash Dye, Molecular Devices, San Jose, California, United States) diluted in Physiological Saline Solution (PSS) containing 0.1 % BSA. Ten microliters of reconstituted HPLC fractions were added to each well and the fluorescence response recorded for 5 mins prior to stimulation with 5 µM veratridine. Signals were interpreted as follows: if MTX-like activity was detected in the sample, calcium influx would be evident in this early stage of the assay. If a second peak was detected in response to veratridine addition, this was interpreted as an effect of CTX-like activity, which acts to enhance an otherwise sub-effective dose of veratridine. Positive control standards of P-CTX-2 and P-CTX-3 were used to verify CTX-like activity. The assay detected P-CTX-2 at 9.51±0.13, and P-CTX-3 at 9.26±0.14, (pEC_{50}, n=2) in the presence of 5 µm veratridine, consistent with previous results using this assay by Lewis et al. (2016).

FLIPR assay data were analysed using ScreenWorks software version 3.2.0.1.4 (Molecular Devices, Sunnydale, California, USA). For each fraction, the MTX-like activity was normalised to the baseline and the maximum peak height in the following 300 reads represented the MTX-like response. CTX-like activity was normalized to the veratridine response and the maximum peak height after the veratridine addition represented the CTX-like response.

2.4   Results

2.4.1   Strain identification

Maximum Likelihood and Bayesian analyses of the LSU rDNA D1-D3 and D8-D10 regions provided evidence to support the clades described in Nishimura et al. (2013) for *Gambierdiscus* sp. All five strains of *Gambierdiscus* established from temperate eastern Australia (UTSMER8B4, UTSMER7A1, UTSMER1A3, UTSMER9A3, UTSMER8A4) and four strains from tropical eastern Australia (UTSHI2C4, UTSHI6C3, UTSHI6A1, UTSHI6D2), group with high support with other strains of *G. carpenteri* in both phylogenies (Figure 2.2 a and b). Therefore, most of the isolates collected from eastern Australia were identified as *G. carpenteri* (Table 2.1).

Three strains (UTSHI6B5, UTSHI2B6, UTSHI2B5) grouped with high support in the LSU rDNA D8-D10 region phylogeny with *G. lapillus* (Figure 2.2 b), a new species described from the same tropical location in eastern Australia (Heron Island) (Kretzschmar et al. 2017). Sequences were not available however, to compare the LSU rDNA D1-D3 region phylogeny. Strain UTSHI6A6 grouped within clade V, the most diverse *Gambierdiscus* clade (Nishimura et al. 2013), in both LSU gene phylogenies. This strain is closely related to, but distinct from, *G. pacificus* strains from around the world (Figure 2.2 a and b). Similarly, strain UTSHI6B1 grouped within clade III (Nishimura et al. 2013) and was closely related to *G. silvae* in both phylogenies but also formed a distinct cluster (Figure 2.2 a and b). More analyses are required to fully describe these strains but are referred to here as *G.* cf. *pacificus* and *G.* cf. *silvae* respectively.

Table 2.1. Geographic origin and toxicity of *Gambierdiscus* strains established in this study. All strains were grown at 24 °C for toxin analysis. Strains listed with temperatures (e.g. 18 °C and 27 °C), indicate additional growth temperatures that were tested. For the LC-MS/MS analysis, ND denotes toxins not detected. For the Ca^{2+} influx SH-SY5Y cell FLIPR bioassay, results shown as the number of CTX- and MTX-like peaks of activity and NA signifies strains which were not analysed.

Species Name	Site of Isolation	Strain Number	LC-MS/MS		Ca^{2+} influx SH-SY5Y cell FLIPR Bioassay	
			CTX	MTX	CTX-like activity	MTX-like activity
Tropical *G. carpenteri*			ND	MTX-3	0	3
Tropical *G. carpenteri* (18°C)	Heron Island Lagoon, Australia	UTSHI2C4	ND	MTX-3	NA	NA
Tropical *G. carpenteri* (27°C)			ND	MTX-3	NA	NA
Tropical *G. carpenteri*	Heron Island Lagoon, Australia	UTSHI6C3	ND	MTX-3	0	4
Tropical *G. carpenteri*	Heron Island Lagoon, Australia	UTSHI6A1	ND	MTX-3	NA	NA
Tropical *G. carpenteri*	Heron Island Lagoon, Australia	UTSHI6D2	ND	MTX-3	NA	NA
Temperate *G. carpenteri*	Merimbula Lake, Australia	UTSMER8B4	ND	ND	0	4
Temperate *G. carpenteri*	Merimbula Lake, Australia	UTSMER7A1	ND	ND	NA	NA
Temperate *G. carpenteri*	Merimbula Lake, Australia	UTSMER1A3	ND	ND	NA	NA
Temperate *G. carpenteri*			ND	ND	0	4
Temperate *G. carpenteri* (18°C)	Merimbula Lake, Australia	UTSMER9A3	ND	ND	NA	NA
Temperate *G. carpenteri* (27°C)			ND	ND	NA	NA
Temperate *G. carpenteri*	Merimbula Lake, Australia	UTSMER8A4	ND	ND	NA	NA
G. lapillus	Heron Island Lagoon, Australia	UTSHI6B5	ND	MTX-3	1	3
G. lapillus	Heron Island Lagoon, Australia	UTSHI2B6	ND	MTX-3	1	2
G. lapillus	Heron Island Lagoon, Australia	UTSHI2B5	ND	MTX-3	NA	NA
G.* cf. *pacificus	Heron Island Lagoon, Australia	UTSHI6A6	ND	MTX-3	1	2
G.* cf. *silvae	Heron Island Lagoon, Australia	UTSHI6B1	ND	MTX-3	1	3

2.4.2   Detection of characterised ciguatoxins and maitotoxins

Analysis of the *Gambierdiscus* culture extracts using LC-MS/MS did not reveal the presence of the microalgal-derived ciguatoxins P-CTX-3B, P-CTX-3C, P-CTX-4A and P-CTX-4B for which we had calibration standards, or MTX-1, in any of the strains established in this study. Putative MTX-3 was detected in all tropical strains of *Gambierdiscus* including *G. carpenteri* but was absent from all temperate strains of the same species (Table 2.1). Interestingly, these toxin profiles did not change when the cultures were grown at 18 or 27 °C (Table 2.1). Tropical strains of *G. carpenteri* continued to produce MTX-3 at temperatures as low as 18 °C and at the standard tropical water temperature of 27 °C, and the temperate cultures produced MTX-3 at either temperature.
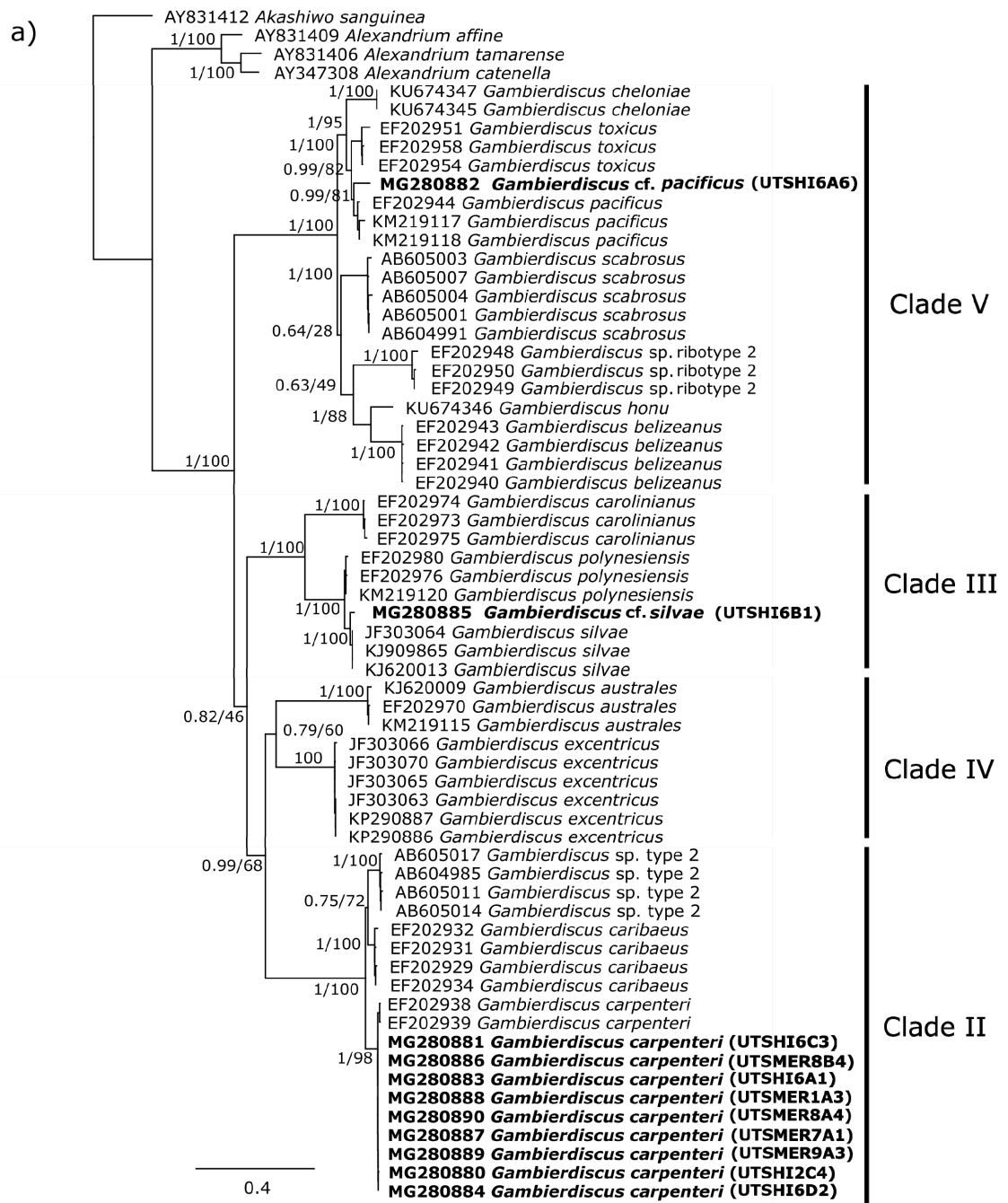
Figure 2.2. Maximum Likelihood phylogenetic tree showing alignment of the D1-D3 region (a) and the D8-D10 region (b) of the LSU rDNA sequences. Strains from this study are shown in bold. Values at nodes represent Bayesian posterior probability and Maximum Likelihood bootstrap support.

Figure 2.2. *continued.* Maximum Likelihood phylogenetic tree showing alignment of the (a) D1-D3 region and the (b) D8-D10 region of the LSU rDNA sequences. Strains from this study are shown in bold. Values at nodes represent Bayesian posterior probability and Maximum Likelihood bootstrap support.

2.4.3 Presence of ciguatoxin (CTX) and maitotoxin-like (MTX) activities in established strains

The Ca^{2+} influx SH-SY5Y cell FLIPR bioassay (Lewis et al. 2016) showed distinct CTX-like activities (i.e. post veratridine addition) in several of the HPLC fractions from the dichloromethane phase of three of the four *Gambierdiscus* species. Two species, *G.* cf. *pacificus* and *G.* cf. *silvae*, showed clear CTX-like activities (Figure. 2.3 g-h, Table 2.1), and low levels of CTX-like activity was detected in both strains of *G. lapillus* (Figure 2.3 e and f). No distinct CTX-like activity was detected for tropical or temperate strains of *G. carpenteri* (Figure 2.3 a-d). MTX-like activity was detected in fractions eluted at 42-45 min in extracts of all species except UTSMER9A3, one of the temperate strains of *G. carpenteri* (Figure 2.3 d). CTX-like activity was also detected in the fractions eluted prior to, during, and following this MTX-like activity peak (Figure 2.3).

In comparison to the variable CTX-like activities amongst strains, strong MTX-like activities were detected in all *Gambierdiscus* strains tested. There was a distinct peak in the earliest fractions of the HPLC-fractionated methanol phase, and large activity in the fractions eluted between 30-60 min for all extracts (Figure 2.4). All the strains *of G. carpenteri* from both tropical and temperate Australia showed additional MTX-like activity peaks (Table 2.1) in fractions eluted between 10 and 30 min (Figure 2.4). CTX-like activity was also detected in most extracts, prior to, and following strong MTX-like activity (Figure 2.4).
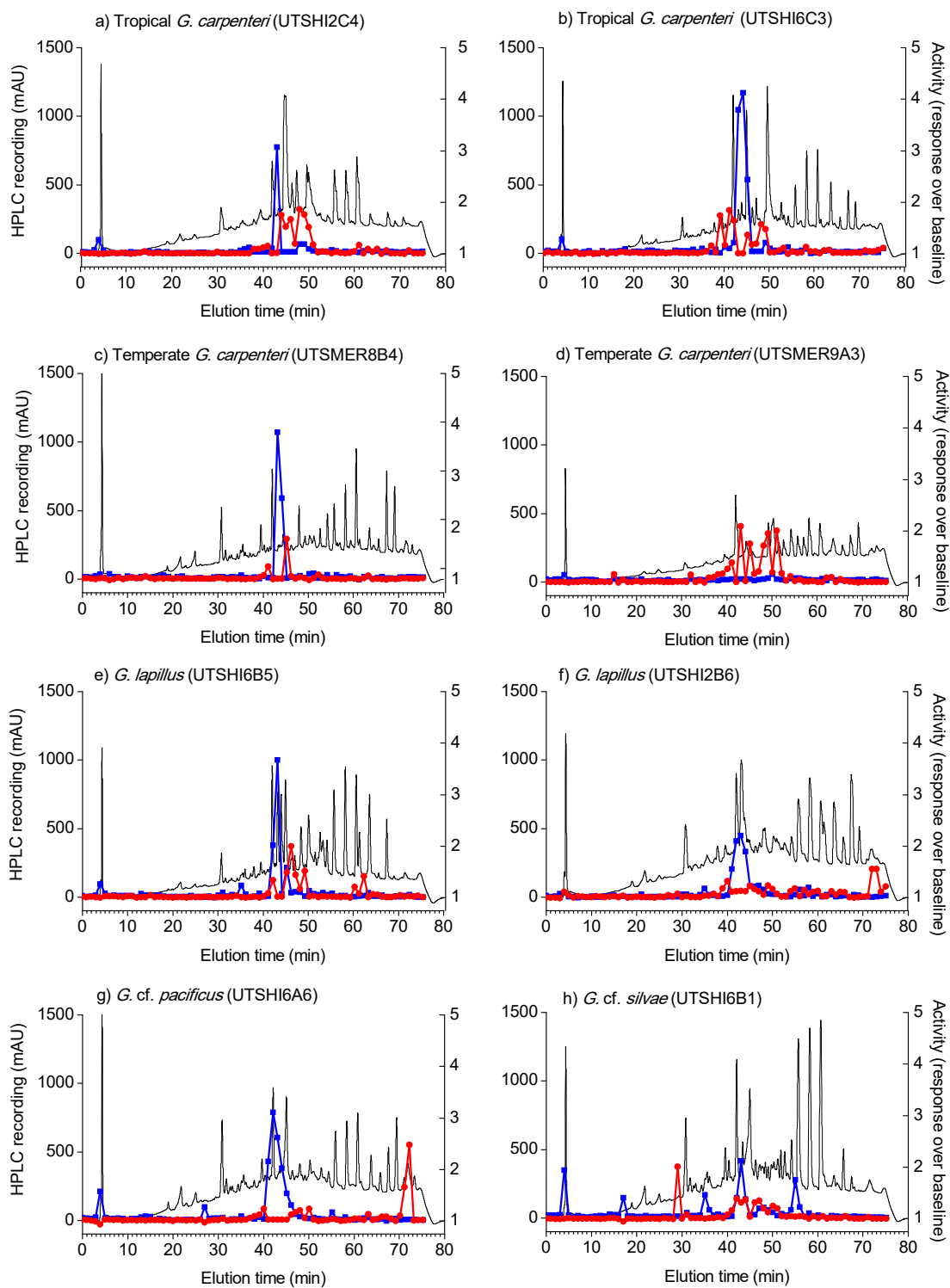
Figure 2.3. Ciguatoxin-like (red) and maitotoxin-like (blue) activities of the High-Performance Liquid Chromatography (HPLC) fractionated (black trace) dichloromethane phase of extracts from *Gambierdiscus* strains established in this study.
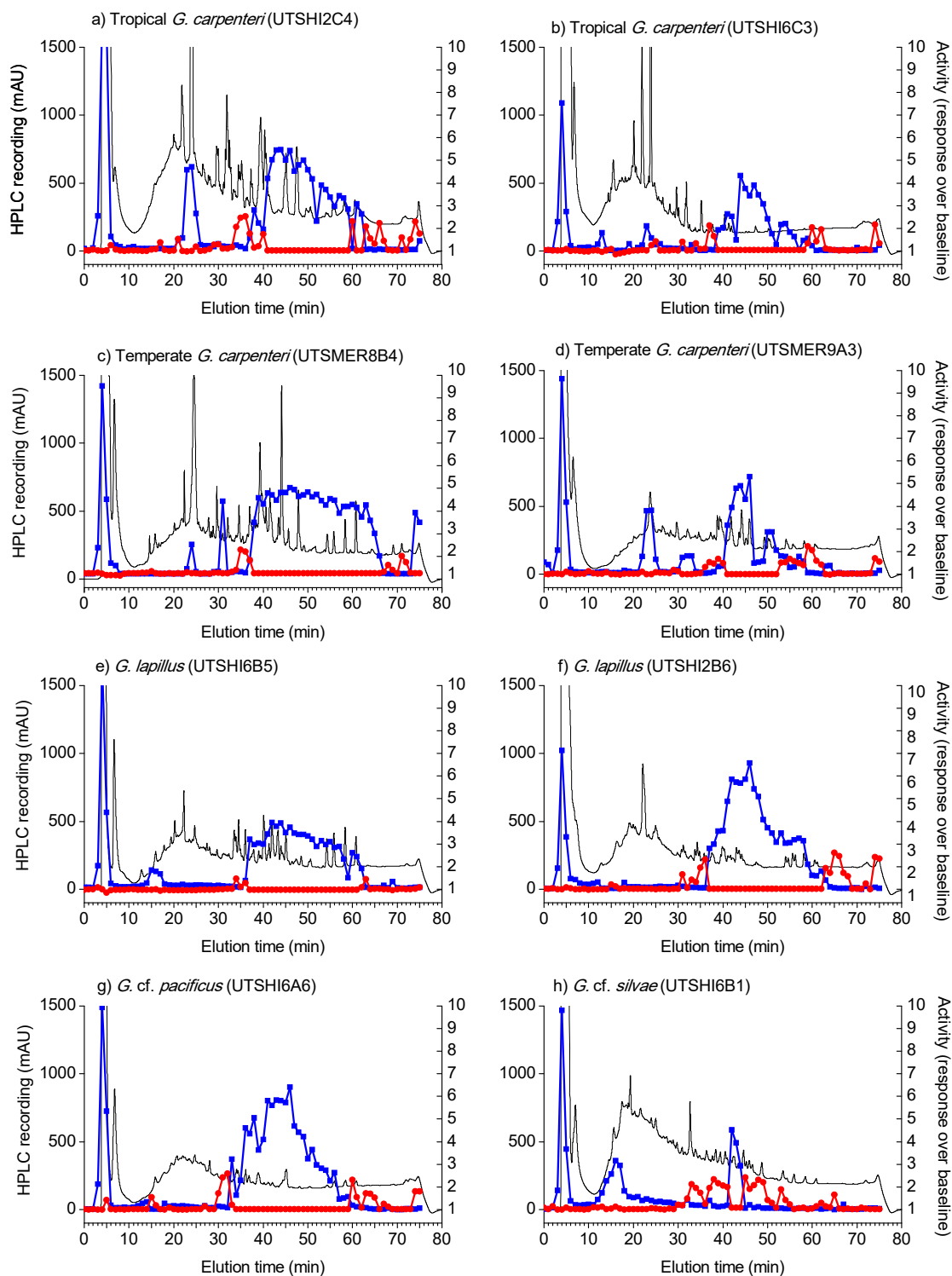
Figure 2.4. Ciguatoxin-like (red) and maitotoxin-like (blue) activities of the High-Performance Liquid Chromatography (HPLC) fractionated (black trace) methanol phase of extracts from *Gambierdiscus* strains established in this study.

2.5   Discussion

CFP has been an issue in Australia for some time but very little is known about the toxicology of the causative organism/s in this region. Prior to this study, strains from various Australian locations had been identified, but a thorough analysis of their toxicology had not been undertaken. Identifying the *Gambierdiscus* species and compounds responsible for CFP in Australia is a critical first step toward improving risk management strategies and helping safeguard recreational fishers and the seafood export industry. In this study, 14 strains of *Gambierdiscus* were established from two locations in eastern Australia. Four *Gambierdiscus* species were identified from a site located in tropical eastern Australia, two of which could represent new species, and one species at a temperate location. Using a functional bioassay approach, *G. lapillus* and two other *Gambierdiscus* taxa (*G.* cf. *pacificus, G.* cf. *silvae*) were found to produce compounds with CTX-like activities. Chemical techniques were also used to verify that these compounds likely differ from the currently characterised CTXs produced by *Gambierdiscus* species (P-CTX-3C, 3B, 4A, 4B).

This study uncovered considerable diversity of *Gambierdiscus* at the tropical location; recording four co-occurring species from Heron Island, Queensland. *G. carpenteri* was the only species found to occur at both the tropical and temperate locations (Merimbula, New South Wales). This species is one of the most widely distributed in the genus, occurring in the North Atlantic Ocean (Holland et al. 2013; Lyu et al. 2017), North Pacific Ocean (Holland et al. 2013; Kibler et al. 2012; Lewis et al. 2016; Litaker et al. 2009; Litaker et al. 2010; Pisapia, Holland, et al. 2017), South Pacific Ocean (Lyu et al. 2017) and the Caribbean Sea (Holland et al. 2013; Lewis et al. 2016). In Australia, *G. carpenteri* has previously been documented in the central Great Barrier Reef in Townsville (Murray et al. 2014; Sparrow et al. 2017) and at Merimbula, New South Wales (Kohli, Murray, et

al. 2014). Our results, therefore, verify these earlier reports of *G. carpenteri* from tropical and temperate Australia.

Gambierdiscus lapillus was also identified from the tropical location of Heron Island. This species was described by Kretzschmar et al. (2017) from the same collection site in the southern Great Barrier Reef and is currently the only other record of this species. However, increased sampling and identification of *Gambierdiscus* species will likely reveal a much larger distribution. Two additional strains of *Gambierdiscus* established in this study (*G.* cf. *pacificus, G.* cf. *silvae*), were not genetically identical to other currently described species within the genus. These may represent additional undescribed species from Heron Island, although further morphological and molecular identifications are required to verify this. A recently described species of *Gambierdiscus* (*G. honu*) (Rhodes, Smith, Verma, Curley, et al. 2017) has a wide distribution across the South Pacific Ocean, including Heron Island (Richlen et al. 2008), but this species was not isolated in our study. This result highlights that a single sample collection and subsequent isolation process does not necessarily reveal all the diversity of a genus at a specific location.

Using LC-MS/MS analyses, characterised CTXs of microalgal origin (P-CTX-3C, 3B, 4A and 4B) were not detected in any of the culture extracts from this study. However, compounds with CTX-like activity were identified in three of the four species tested. Previous studies of the toxicology of *Gambierdiscus* from Australia are consistent with these results. Using the same LC-MS/MS method, Kretzschmar et al. (2017) did not detect characterised microalgal CTXs in strains of *G. lapillus* established from Heron Island but noted the presence of unassigned peaks in the CTX transition zone. Similarly, Kohli, Murray, et al. (2014) did not detect characterised microalgal CTXs in a collection of *G. carpenteri* cells taken directly from the field at Merimbula. The LC-MS/MS method used in all studies were targeted at identification of P-CTX-3C, 3B, 4A and 4B, as these

are the currently characterised forms of microalgal origin for which standards are available. Therefore, this approach will not detect structurally related compounds with different masses. This indicates that the CTX-like activity detected using the functional assay in our study; is likely casused by compounds which differ from the currently characterised CTXs of microalgal origin.

Gambierdiscus cf. *pacificus* produced the most distinct CTX-activity peak eluting at approximately 72 min (Figure 2.3). In a study using the same cell based functional assay, Lewis et al. (2016) identified CTX-like activity eluting at the same time in strains of *G. ruetzleri* (now *Fukuyoa ruetzleri*), *G. carolinianus* and *G.* ribotype 2. However, LC-MS/MS analyses had not been performed on the strains from Lewis et al. (2016) so it is not known whether this activity is linked with characterised forms of CTXs. The results from this study suggest that this compound may be a novel type of lipophilic toxin with CTX-like activity, and its characterisation should be the target of future research. Both strains of *G. lapillus* also showed CTX-like activity although the elution time of the compounds differed between strains. These differences may result from the fact that the strains produce different congeners of a toxin; however, understanding the reasons for such differences requires further investigation.

Gambierdiscus cf. *silvae* showed distinct CTX-like activity, from a low polarity compound eluting at approximately 29 min (Figure 2.3). This compound was identified in the dichloromethane (DCM) phase of the cell extract, where lipophilic compounds like CTXs accumulate; however, known ciguatera toxins typically have higher polarities so further research is needed to accurately classify this toxin. *G. carpenteri* strains from tropical and temperate Australia did not show any distinct CTX-like activities, consistent with Lewis et al. (2016) who tested two strains of *G. carpenteri* (originally isolated from Belize in the Caribbean Sea and Hawaii in the eastern North Pacific Ocean), using the

same functional cell based Ca^{2+} influx SH-SY5Y cell FLIPR bioassay. These results suggest that *G. carpenteri* does not produce detectable quantities of ciguatera causing sodium channel activation compounds and therefore, may not contribute to the occurrence of CFP.

Many *Gambierdiscus* species display toxicity in the lipophilic phase, following partitioning in assays such as the mouse bioassay and the mammalian cell based neuroblastoma (N2a) assay, suggesting that CTXs or compounds with the same mode of action are present (Pisapia, Holland, et al. 2017; Xu et al. 2014). Efforts should focus on testing whether the toxicity of these strains can be attributed to the CTXs characterised from French Polynesia, or whether toxins responsible for CFP differ between locations, as our findings seem to suggest. Certainly, the results from this study indicate the CTX-like toxins from Australia may differ from those found in French Polynesia. Geographic differences in the structure of CTXs isolated from marine fish have been identified. For example, CTXs in fish from the Pacific Ocean are structurally distinct from those recovered in fish from the Caribbean and Indian Ocean (Lewis & Holmes 1993). CTXs isolated from marine fish do differ in structure from the CTXs produced by *Gambierdiscus* species, a result of bioaccumulation and biomagnification, however the geographic differences in the CTXs isolated from marine fish originating from different locations could suggest that the precursor toxins produced by *Gambierdiscus* sp. are just as diverse.

Large MTX-like activities were identified in the DCM phase of all *Gambierdiscus* cell extracts, except for one strain of *G. carpenteri* established from temperate Australia. This was unexpected, as MTXs should only be present in the methanol phase following liquid partitioning of the microalgal cell extracts. However, the elution time of the MTX-like activity peak in the DCM phase corresponds to a compound with strong MTX-like

activity in the methanol phase and therefore, likely represents a carryover of MTXs. Although liquid partitioning of lipophilic (CTX) and hydrophilic (MTX) compounds in cell extracts using DCM and aqueous methanol is effective, some MTX carryover can occur and was also observed at the same elution time in the original Ca^{2+} influx SH-SY5Y cell FLIPR bioassay method description by Lewis et al. (2016). CTX-like activity was also detected on the shoulders of this MTX-like activity. This is likely a result of sensitisation of the sodium channels on the mammalian cell membrane in response to the addition of veratridine, resulting in a CTX-like activity peak and therefore, was not interpreted as an indicator of the presence of potential CTXs in this study. The MTX peak was not detected in *G. carpenteri* strain UTSMER9A3; however the CTX-like activity was. This was likely due to a lower level of MTX carryover from the methanol phase, only high enough to show a response after the addition of veratridine.

Yasumoto et al. (1977) were the first to establish the link between CFP and the dinoflagellate *Gambierdiscus.* The structure of the microalgal derived CTXs responsible were later elucidated from a strain (RG1-1) of *Gambierdiscus* (reported as *G. toxicus* but taxonomic identity yet to be confirmed) isolated from the Gambier Islands in French Polynesia (Murata et al. 1990; Murata et al. 1989; Satake et al. 1997; Satake, Murata & Yasumoto 1993; Yasumoto et al. 2000). Chinain et al. (2010) then went on to complete the first comprehensive characterisation of CTX compounds from two highly toxic strains of *G. polynesiensis* (TB-92 and RG-92) from French Polynesia and the toxins characterised in these studies remain the primary toxins linked with CFP today.

Gambierdiscus polynesiensis has been consistently found to exhibit considerable CTX-like activity (receptor binding assay, Chinain et al. (2010); mouse bioassay, Rhodes et al. (2014); Neuro-2a assay, Pawlowiez et al. (2013) and is the only species shown to produce the characterised CTX compounds using LC-MS/MS analyses (Chinain et al. 2010;

Rhodes et al. 2014). *Gambierdiscus polynesiensis* is therefore thought to be an important contributor to CFP in the Pacific. Interestingly, a strain of *G. polynesiensis* was recently established and showed no production of CTX compounds when tested with LC-MS/MS but this is highly unusual for this species (Rhodes, Smith, Murray, et al. 2017). Presently, only six strains of *G. polynesiensis* have been reported in the literature, four from French Polynesia (Chinain et al. 2010), one from the Cook Islands (Rhodes et al. 2014), and one from the Kermadec Islands (Rhodes, Smith, Murray, et al. 2017). These locations are all located within the central South Pacific Ocean but CFP occurs across a much larger distribution (Friedman et al. 2017) and CTXs are confirmed to be present in fish from many locations (Bienfang et al. 2008; Boada et al. 2010; Lewis & Holmes 1993). Therefore either the distribution of *G. polynesiensis* is much larger than presently acknowledged, or there are other currently unidentified precursor toxins that contribute to CFP in other locations.

Four congeners of MTX have been described, MTX-1, MTX-2, MTX-3 and MTX-4 (Holmes & Lewis 1994; Holmes, Lewis & Gillespie 1990; Pisapia, Sibat, et al. 2017); and chemical detection methods exist for the two disulphated forms, MTX-1 and MTX-3. Using LC-MS/MS analyses, we did not detect MTX-1 in any of the strains established from eastern Australia in this study. MTX-1 was originally described from a strain of *Gambierdiscus* (FP) isolated from French Polynesia (Holmes, Lewis & Gillespie 1990). The species used for this original description is not known, but MTX-1 has since only been identified in strains of *G. australes* from the Cook Islands (Rhodes et al. 2014), the Kermadec Islands (Rhodes, Smith, Verma, Murray, et al. 2017) and Japan (Pisapia, Sibat, et al. 2017).

MTX-3 is a putative MTX first described by Holmes & Lewis (1994). It is structurally smaller than MTX-1 and MTX-2, but its complete structure, potency, and mode of action

remains unknown. Since the original description, MTX-3 has been found in all *Gambierdiscus* strains tested (T. Harwood pers. comm.), except for the temperate *G. carpenteri* strains established in this study. The factors driving the differences in MTX-3 production between the tropical and temperate populations of *G. carpenteri* established in this study are unknown; however, the temperature experiment showed that temperature is not one of them. MTX-4 is a recently described congener of MTX, found only to be present in strains of *G. excentricus* isolated from a variety of locations and is highly potent (Pisapia et al. 2017).

The Ca^{2+} influx SH-SY5Y cell FLIPR bioassay (Lewis et al. 2016) used in this study, showed that all strains of *Gambierdiscus* established from eastern Australia produce between two and four compounds displaying MTX-like activities. As LC-MS/MS analyses did not detect the presence of MTX-1 in these strains, the toxicity must therefore be attributed to other types of MTX. Pisapia, Sibat, et al. (2017) tested a wide range of *Gambierdiscus* strains for the presence of the newly described toxin MTX-4 and found that only *G. excentricus* produced this compound. It is therefore unlikely the MTX-like activity in the strains tested in this study is related to the presence of MTX-4. Alternatively, MTX-2 was originally described from a strain of *Gambierdiscus* (NQ1) isolated from the central Great Barrier Reef, Australia (Holmes, Lewis & Gillespie 1990). As there is no chemical detection method for this type of MTX, it is not known if any of the compounds showing MTX-like activity in this study are from MTX-2, however it is likely.

MTXs are hydrophilic compounds, and the likelihood of their contribution to human illness events was originally discarded due to their water solubility (Holmes & Lewis 1994). However recent work has confirmed that MTXs can be retained in the viscera, liver, and flesh of fish (Kohli, Papiol, et al. 2014). These recent findings, coupled with

the consistent detection of MTX-like activity in all *Gambierdiscus* strains tested in this study and others (e.g. haemolytic activity, Holland et al. (2013); Pisapia, Holland, et al. (2017), mouse bioassay, Chinain, Faust & Pauillac (1999); Nishimura et al. (2013) and Ca^{2+} influx SH-SY5Y cell FLIPR bioassay, Lewis et al. (2016)), as well as the extremely high potency of these toxins, represent compelling arguments toward the need for further investigations into the role of MTXs in CFP.

2.6   Conclusion

In this study, 14 strains of *Gambierdiscus* were established from eastern Australia, representing four species. Three species were found to produce compounds displaying CTX-like activities, which are distinct from those able to be detected using current chemical analytical techniques. These results suggest that toxins produced by these Australian strains of *Gambierdiscus* likely differ from those currently linked with CFP identified in extracts of *G. polynesiensis* from French Polynesia, though characterisation of these compounds responsible for the CTX-like activity is necessary to evaluate this. Although this study was limited to Australian strains of *Gambierdiscus*, the findings are likely universal and therefore a concentrated effort should be made toward testing and characterising the toxicology of strains from other locations. Future work should also include establishing a baseline of *Gambierdiscus* species distribution in Australia, understanding the relative abundance of each species, and finding out how these organisms are influenced by environmental conditions, particularly in view of a changing climate.

Chapter 3 Environmental niche of *Gambierdiscus* spp. (Dinophyceae) from tropical and temperate Australian waters

3 Environmental niche of *Gambierdiscus* spp. (Dinophyceae) from tropical and temperate Australian waters

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3.1 Abstract

Some species of the dinoflagellate genus *Gambierdiscus* produce potent neurotoxins which can cause Ciguatera Fish Poisoning (CFP) in humans. The genus is known to occur in benthic habitats throughout tropical and subtropical latitudes, although populations are being increasingly reported from temperate locations. In Australia, several *Gambierdiscus* strains established in culture from the tropics were shown to produce compounds with ciguatoxin-like activity (Chapter 2). In this study, we define the environmental niche of these and other *Gambierdiscus* strains from tropical and temperate Australia firstly to understand how environmental variables influence growth dynamics and secondly, evaluate if temperate range extension of the genus is restricted by the ecophysiological requirements of the species. Growth was measured across a gradient of temperature (15-30 °C), salinity (20-38) and irradiance (10-200 µmol photons m^{-2} s^{-1}) for twelve strains of *Gambierdiscus* representing four genetically distinct species, including the newly described *G. lapillus*, two potentially new species, *G.* cf. *pacificus* and *G.* cf. *silvae*, and tropical and temperate strains of *G. carpenteri*. Irrespective of their isolation locality, all strains showed maximum growth at ~25 °C and showed growth within 80% of maximum at temperatures between 20 and 28 °C. Maximum growth occurred at a salinity of ~32, although the optimal range was between 25 and 40. Cells divided at irradiance levels > 10 µmol photons m^{-2} s^{-1} but maximum growth occurred at levels greater than 100 µmol photons m^{-2} s^{-1}. The environmental niche of tropical strains of *Gambierdiscus* did not significantly differ from temperate strains, suggesting that tropical toxin producing *Gambierdiscus* species also have the capacity to occupy temperate locations. These results provide valuable insights into the potential for range extension of *Gambierdiscus* to temperate areas and can assist with forecasting the risk of exposure to CFP in temperate Australia.

3.2   Introduction

Ciguatera Fish Poisoning (CFP) is a human illness caused when marine fish contaminated with ciguatoxins and potentially maitotoxins are consumed (Kohli, Papiol, et al. 2014; Lehane & Lewis 2000). Cases of CFP are prevalent in island nations of the Pacific Ocean (Anderson et al. 1983; Chateau‐Degat et al. 2007; Lewis 1986) but have also been documented in the Indian Ocean (Quod & Turquet 1996), as well as the Caribbean sea and other parts of the tropical and subtropical Atlantic Ocean (Boada et al. 2010; Lawrence et al. 1980; Pérez-Arellano et al. 2005; Pottier, Vernoux & Lewis 2001; Czernichow et al. 1984; Radke 2013; Radke, Reich & Morris Jr 2015; Tester et al. 2010). The incidence and worldwide distribution of CFP is also thought to be expanding (Oshiro et al. 2010; Pérez-Arellano et al. 2005; Skinner et al. 2011).

Ciguatoxins (CTX) and maitotoxins (MTX) are produced by species of the epibenthic dinoflagellate genus, *Gambierdiscus* (Yasumoto et al. 1977). The genus *Gambierdiscus* was originally described by Adachi & Fukuyo (1979) with samples collected from the Gambier Islands in French Polynesia. The genus consisted of a single species (*G. toxicus*) for many years until detailed research and the application of molecular identification techniques discovered finer taxonomic detail (Litaker et al. 2009). This led to the description of many new species (Fraga et al. 2016; Kretzschmar et al. 2017; Rhodes et al. 2017; Smith et al. 2016), as well as the reclassification of some species into a new genus, *Fukuyoa* (Gómez et al. 2015).

The biogeographic distribution of *Gambierdiscus* is estimated to be between the latitudes of 32 °N and 35 °S (Lehane & Lewis 2000; Lewis 2001; Tindall & Morton 1998) although more recently, populations have been documented from temperate locations in the Mediterranean Sea, (Aligizaki, Nikolaidis & Fraga 2008), the North Pacific Ocean

(Nishimura et al. 2013) and the South Pacific Ocean (Kohli, Murray, et al. 2014; Chapter 2). Speculation of the cause of this expanding distribution includes increased awareness and sampling effort and/or range extension due to climatic and anthropogenic pressures (Bienfang et al. 2008; Hallegraeff 2010; Villareal et al. 2007).

Gambierdiscus, like many other marine photosynthetic organisms, requires suitable physicochemical conditions in terms of temperature, pH and salinity, in combination with carbon, nutrients and light to support growth. Determining how environmental variables influence growth will therefore allow greater understanding of the potential distribution of *Gambierdiscus* species. Early work describing the ecology of the genus identified its greatest abundance when water temperatures were between 21 and 31 °C (Bagnis, Legrand & Inoue 1990; Ballantine, Bardales & Alvey 1993; Chateau-Degat et al. 2005; Chinain et al. 1999; Hales, Weinstein & Woodward 1999; Hokama et al. 1996; Morton, Norris & Bomber 1992; Rongo, Bush & Van Woesik 2009), salinity was stable and high (Ballantine, Tosteson & Bardales 1988; Carlson & Tindall 1985; Delgado et al. 2006; Grzebyk et al. 1994; Morton, Norris & Bomber 1992), and irradiance levels were < 10 % of incident light (Ballantine, Bardales & Alvey 1993; Bomber, Guillard & Nelson 1988; Villareal & Morton 2002). Unfortunately, those studies preceded the reclassification of the genus so the identity of the *Gambierdiscus* species used remains unconfirmed. Furthermore, the environmental niche of many of the newly described species of *Gambierdiscus* has yet to be evaluated.

The early ecological studies however, established the foundations of our understanding of the optimum growth conditions for the *Gambierdiscus* genus and its likely distribution. The preference for temperatures ranging from 21 and 31 °C (Bagnis, Legrand & Inoue 1990; Ballantine, Bardales & Alvey 1993; Chateau-Degat et al. 2005; Chinain et al. 1999; Hales, Weinstein & Woodward 1999; Hokama et al. 1996; Morton, Norris & Bomber

1992; Rongo, Bush & Van Woesik 2009), coupled with the higher incidence rate of CFP in tropical locations, suggest the distribution of *Gambierdiscus* would be restricted to lower latitudes. However, recent discoveries of temperate populations of *Gambierdiscus* suggest these limits may need to be revised, or niche preferences for individual species and strains determined.

Several species of *Gambierdiscus* have been reported from tropical eastern Australia, including *G. carpenteri* (Murray et al. 2014; Sparrow et al. 2017; Chapter 2), *G. lapillus* (Kretzschmar et al. 2017; Chapter 2), *G. honu* (Rhodes et al. 2017), and three currently unclassified strains which may represent new species*, G.* cf. *belizeanus* (Murray et al. 2014), *G.* cf. *pacificus and G.* cf. *silvae* (Chapter 2). Three of these species are known to produce compounds with CTX-like activity and therefore may be contributing to cases of CFP in this region (Chapter 2). A temperate population of *G. carpenteri* has also been identified (Kohli, Murray et al. 2014; Chapter 2) at a latitude of 37 °S, the highest latitude reported for a species of *Gambierdiscus*. Having established clonal cultures of both tropical and temperate populations (Chapter 2) the aim of this study was to investigate and compare the physiological requirements of *Gambierdiscus* species and determine if temperate strains are uniquely adapted to conditions characteristic of higher latitude locations.

3.3   Methods

3.3.1   Cell culture

Twelve strains of *Gambierdiscus* representing four species *(G. carpenteri, G. lapillus, G.* cf. *pacificus* and *G.* cf. *silvae)* were used for experiments in this study (Table 3.1). Strains were established by isolating single cells of *Gambierdiscus* from a tropical location in the

Great Barrier Reef region of eastern Australia (Heron Island) and the temperate location of Merimbula, New South Wales, as described in Chapter 2. Stock cultures of each strain were grown in modified K medium (Litaker et al. 2009) made from sterile aged natural seawater at a salinity of 32 and maintained at 24°C under ~100 μmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle in 25 cm^2 (70 mL) sterile vented polystyrene tissue culture flasks (Falcon, Corning, New York, USA), oriented horizontally.

3.3.2 Growth estimates

In vivo chlorophyll *a* fluorescence was used as a proxy for cell abundance to measure growth of *Gambierdiscus* strains during experiments. Strains were grown in 75 cm^2 (250 mL) sterile vented polystyrene tissue culture flasks (Falcon, Corning, New York, USA) under stock culture maintenance conditions. A 1 mL aliquot was removed from each flask every 6 days until the cultures reached the stationary phase of growth. The *in vivo* chlorophyll *a* fluorescence was measured for each sample using a fluorometer (Turner Designs Trilogy Laboratory Fluorometer®, USA) and was then preserved with 1% Lugol's iodine solution. To confirm *in vivo* chlorophyll *a* fluorescence is correlated with *Gambierdiscus* cell abundance, the cell abundance for each strain was enumerated using a Sedgewick Rafter counting chamber under an inverted light microscope (Nikon Eclipse TS100, Japan). Results from strains of the same species were combined (except for *G. carpenteri* for which the tropical and temperate strains were kept separate) and the correlation between *in vivo* chlorophyll *a* fluorescence and cell abundance was calculated for each species using linear regression analyses (OriginPro 8, OriginLab Corporation, Massachusetts, USA). *In vivo* chlorophyll *a* and cell abundance was shown to be linearly related for all species (Figure 3.1; $p < 0.05$), therefore *in vivo* chlorophyll *a* fluorescence was used as a rapid method to estimate cell growth in all experiments in this study.

Table 3.1: Information for strains of *Gambierdiscus* used in temperature, salinity and irradiance experiments for this and other studies. Additional strains are included for comparative purposes and form the basis of data in Figure 3.8.

Species	Strain	Location	Latitude	Longitude	Reference
G. carpenteri	UTSHI2C4	Heron Island, Australia	23°26'32 S	151°54'53 E	This study
	UTSHI6C3	Heron IslandAustralia	23°26'32 S	151°54'53 E	This study
	UTSHI6A1	Heron Island, Australia	23°26'32 S	151°54'53 E	This study
	UTSHI6D2	Heron Island, Australia	23°26'32 S	151°54'53 E	This study
	UTSMER8B4	Merimbula Lake, Australia	36° 53'48 S	149°54'68 E	This study
	UTSMER1A3	Merimbula Lake, Australia	36° 53'48 S	149°54'68 E	This study
	UTSMER9A3	Merimbula Lake, Australia	36° 53'48 S	149°54'68 E	This study
	UTSMER8A4	Merimbula Lake, Australia	36° 53'48 S	149°54'68 E	This study
	NOAA 1, CCMP 1654	Guam, Northern Mariana Islands	13°26'39 N	144°47'37 W	Kibler et al. (2012)
	KML1	Long Key, Florida Keys	24°49' 36 N	80°48'51 W	Xu et al. (2016)
G. lapillus	UTSHI6B5	Heron Island, Australia	23°26'32 S	151°54'53 E	This study
	UTSHI2B6	Heron Island, Australia	23°26'32 S	151°54'53 E	This study
G. cf. *pacificus*	UTSHI6A6	Heron Island, Australia	23°26'32 S	151°54'53 E	This study
G. pacificus	NOAA 9, MR1, CCMP 1650	Moorea, Society Islands, Pacific Ocean	17°32'19 S	149°49'46 W	Kibler et al (2012)
	3S0509-27	Marakei, Kiribati	02°00'00 N	173°16'0 E	Xu et al. (2016)
	3S0510-19	Marakei, Kiribati	02°00'00 N	173°16'0 E	Xu et al. (2016)
G. cf. *silvae*	UTSHI6B1	Heron Island, Australia	23°26'32 S	151°54'53 E	This study
G. silvae	FC May10_9	St. Thomas, US Virgin Islands	18°20' 70 N	64°57'12 W	Xu et al. (2016)
G. australes	S080911_1	Coasts of Susaki, Japan	33°23'38 N	133°20'29 E	Yoshimatsu et al. (2014) Yoshimatsu et al. (2016)

	NOAA 25	Honolulu Hawaii, USA	21°18'24 N	157°51'29 W	Kibler et al (2012)
G. belizeanus	NOAA 2, SB03, CCMP 399	St. Barthelemy Island, Caribbean	17°54'10 N	62°49'14 W	Kibler et al (2012)
	BP Mar10_6	St. Thomas, US Virgin Islands	18°20' 70 N	64°57'12 W	Xu et al. (2016)
	BP Mar10_7	St. Thomas, US Virgin Islands	18°20' 70 N	64°57'12 W	Xu et al. (2016)
	BP Mar10_22	St. Thomas, US Virgin Islands	18°20' 70 N	64°57'12 W	Xu et al. (2016)
	FC Dec10_13	St. Thomas, US Virgin Islands	18°20' 70 N	64°57'12 W	Xu et al. (2016)
G. caribaeus	NOAA 19	Carrie Bow Cay, Belize, Caribbean	16°48'00 N	88°04'60 W	Kibler et al (2012)
	BP Aug08	St. Thomas, US Virgin Islands	18°20' 70 N	64°57'12 W	Xu et al. (2016)
	FC Nov09_4	St. Thomas, US Virgin Islands	18°20' 70 N	64°57'12 W	Xu et al. (2016)
	SH Nov09_3	St. Thomas, US Virgin Islands	18°20' 70 N	64°57'12 W	Xu et al. (2016)
	TF9G	Koh Wai, Trat, Gulf of Thailand, Thailand	11°54′07′ N	102°24′23 E	Tawong et al. (2016)
G. carolinianus	NOAA 6, CCMP 3144	Cape Fear, North Carolina, Atlantic Ocean	33°50'25 N	77°57'36 W	Kibler et al (2012)
	SH Mar10_12	St. Thomas, US Virgin Islands	18°20' 70 N	64°57'12 W	Xu et al. (2016)
	BB Apr10_3	St. Thomas, US Virgin Islands	18°20' 70 N	64°57'12 W	Xu et al. (2016)
	BP May10_1	St. Thomas, US Virgin Islands	18°20' 70 N	64°57'12 W	Xu et al. (2016)
G. ribotype 2	MQ2, CCMP 1655	Martinique, Caribbean	14°38'29 N	61°01'27 W	Kibler et al (2012)
G. scabrosus (*G.* sp. type 1*)*	KW070922_1	Otsuki, Japan	32°46'22 N	132°37'31 E	Yoshimatsu et al. (2014) Yoshimatsu et al. (2016)
G. sp. type 2	M080828_2	Muroto, Japan	33°14'47 N	134°10'42 E	Yoshimatsu et al. (2014) Yoshimatsu et al. (2016)
G. sp. type 3	WI11G	Kushimoto, Japan	33°27'10 N	135°47'34 E	Yoshimatsu et al. (2014) Yoshimatsu et al. (2016)
G. sp. type 4	1D0509-16	Marakei, Kiribati	02°00'00 N	173°16'0 E	Xu et al. (2016)
	1D0510-22	Marakei, Kiribati	02°00'00 N	173°16'0 E	Xu et al. (2016)
G. sp. type 5	DS0511-03	Marakei, Kiribati	02°00'00 N	173°16'0 E	Xu et al. (2016)

3.3.3   Niche estimate experiments

For each experiment, established strains of *Gambierdiscus* were inoculated into triplicate 25 cm^2 sterile vented polystyrene tissue culture flasks (Falcon, Corning, New York, USA), with 50 mL fresh standard modified K medium prepared from natural aged seawater, at a standardised cell density of approximately 10 cells mL^{-1}. Flasks were acclimated to the experimental conditions for seven days before the 60-day growth assays. A 1 mL aliquot was removed from each flask every 3 to 4 days, and the i*n vivo* chlorophyll *a* fluorescence measured, until the cultures reached the stationary phase of growth. The maximum growth rate was calculated using the slope of natural logarithm transformed cell abundance data in the linear portion of the growth curve.

For temperature experiments, flasks were placed horizontally under ~100 μmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle and incubated under different temperatures (15, 18, 21, 24, 27, 30 °C) in controlled-temperature incubators (Climatron® Plant Growth Cabinet, Australia). For salinity experiments, the salinity of natural aged seawater (~35) was adjusted to the experimental treatments of 20, 26, 32 and 38 by diluting with sterile MilliQ water or by adding sterile artificial seawater of relatively high salinity prepared as per Berges, Berges, Franklin & Harrison (2001). Nutrients were added as per standard modified K medium and flasks were incubated at a constant temperature of 24 °C under ~100 μmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle. For irradiance experiments, flasks were incubated at a constant temperature of 24 °C on a 12:12 light:dark cycle under different irradiance levels (10, 50, 100 and 200 μmol photons m^{-2} s^{-1}), verified using a light meter (WALZ Universal Light Meter-500, Germany) and a 4π light sensor (WALZ, Model US-SQS/L, Germany) in air.
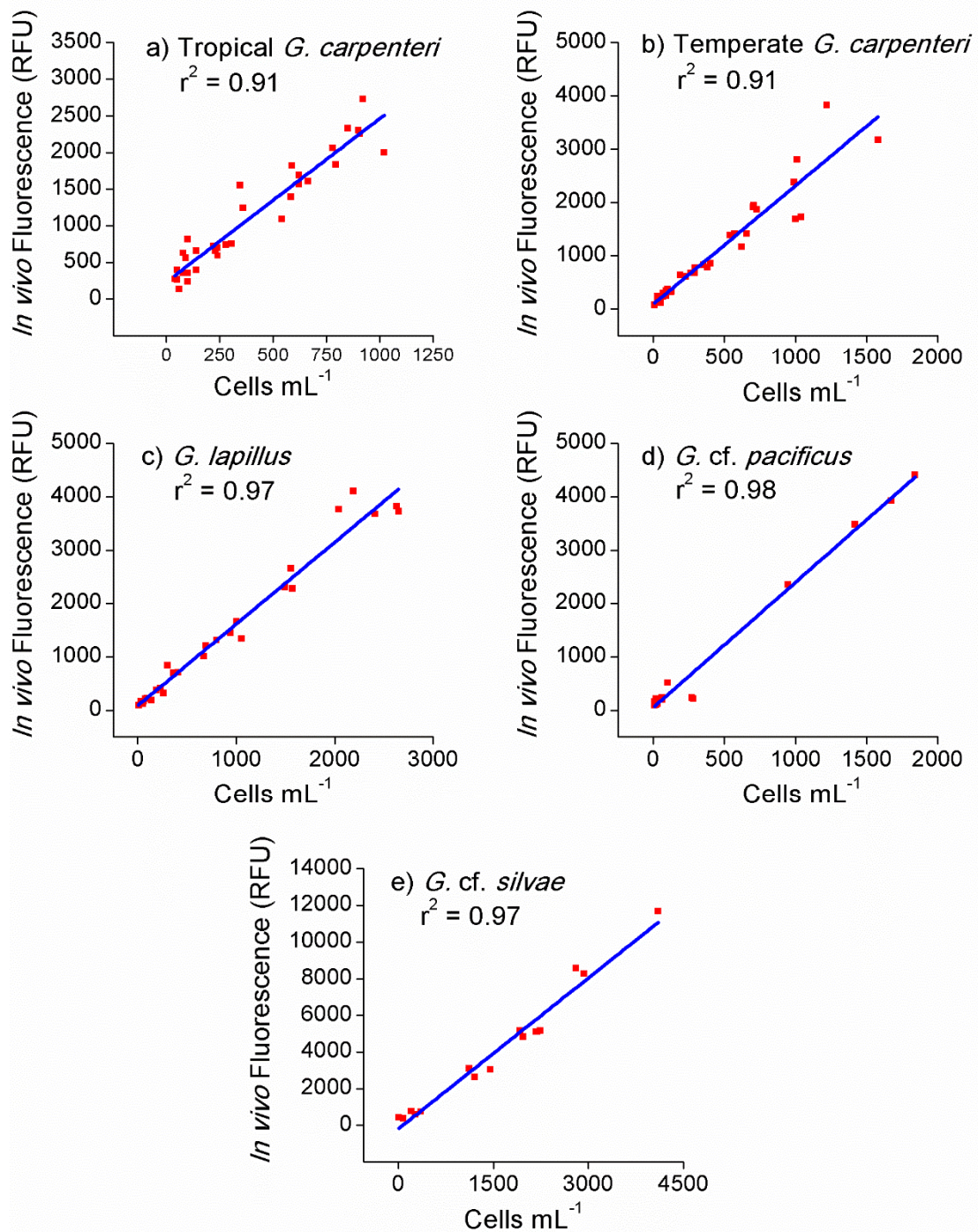
Figure 3.1. Linear relationship between the *in vivo* chlorophyll *a* fluorescence (RFU, Relative Fluorescence Units) and cell abundance (cells mL⁻¹) for *Gambierdiscus* species included in this study (a) tropical *G. carpenteri*, (b) temperate *G. carpenteri*, (c) *G. lapillus*, (d) *G.* cf. *pacificus* and (e) *G.* cf. *silvae.*

3.3.4 Estimating optimum growth conditions and niche width

The calculated maximum growth rate for each replicate of each strain was plotted as a function of the environmental variable (e.g. temperature, salinity, irradiance) to form a reaction norm. The shape of the reaction norm was then fitted to estimate growth parameters such as the conditions under which maximum growth occurred and the upper and lower limits for growth (e.g., for temperature, T_{opt}, T_{max}, T_{min}, respectively). Parameter estimation was achieved in R version 3.2.3 (R Core Team, 2012).

Temperature and salinity reaction norms were both fitted with a function first applied to phytoplankton by Eppley (1972) and modified by Thomas et al. (2012). This function describes the temperature or salinity dependent specific growth rate using the following equation:

$$f\,(\mathrm{E}) = ae^{bE}\left[1 - \left(\frac{E-z}{w/2}\right)\right]^2$$

where, growth rate f is a function of either temperature or salinity (E). The shape of the reaction norm is controlled by species traits, z and w. The niche width is given by w and species trait z determines the location of the maximum of the quadratic portion of the function.

Irradiance reaction norms were fitted with a Monod function with a photoinhibition term as was used in Megard, Tonkyn & Senft (1984) and Litchman (2000).

$$\mu = \mu_{max}\frac{I}{I + k + \dfrac{I^2}{k_{inh}}} - r$$

where, μ is the specific growth rate (day^{-1}), μ_{max} is the maximum specific growth rate (day^{-1}), I is the irradiance (μmol photons m^{-2} s^{-1}), k is a half-saturation constant (μmol photons m^{-2} s^{-1}), r is the metabolic loss rate and k_{inh} is the photoinhibition constant (μmol photons m^{-2} s^{-1}).

For temperature, salinity and light, the optimum range for growth was estimated to be the threshold at which 80% of the maximum growth was achieved as described in Kibler et al. (2012). The upper and lower limits for growth were estimated at the point at which growth was below zero. Other studies have used a polynomial curve fit to estimate these parameters (Kibler et al. 2012; Xu et al. 2016), so for comparison, data from this study were also fitted with a polynomial curve. There were no significant differences in the growth parameter estimates between methods (data not shown).

Growth rates across environmental gradients of temperature, salinity and irradiance were statistically analysed individually using parametric methods. They were also analysed collectively using a multivariate approach (PRIMER-E v6; Clarke & Gorley 2006) to compare fitness of individual *Gambierdiscus* strains. Analysis of similarity (ANOSIM) was used as the data were not normally distributed and showed heteroscedasticity.

To test whether tropical and temperate strains of *G. carpenteri* had different environmental niches, an Analysis of Variance (ANOVA) was used to statistically analyse differences between the estimated niche parameters (SPSS v24 IBM Corporation, Armonk, New York, USA). Analyses were performed individually for parameters including T μ_{max}, T_{opt}, T_{min}, T_{max}, T niche width (calculated from the difference between T_{min} and T_{max}), S μ_{max}, S_{opt}, S_{min}, S_{max}, S niche width and I μ_{max}, I_{opt}, S_{min}, I_{max}, I width. This type of statistical comparison was only possible for *G. carpenteri* as there were multiple strains of the species.

The environmental niche of *Gambierdiscus* species was also statistically analysed using Permutational Analysis of Variance (PERMANOVA) of a Bray-Curtis resemblance matrix in PRIMER-E v6 (Clarke & Gorley 2006) for temperature, salinity and irradiance individually and collectively through comparison of the estimated growth parameters for each strain (e.g. μ_{max}, opt, niche min, niche max, niche width). Individual strains could not be analysed using this approach as only a single estimated growth parameter is produced from the three replicates. Therefore, this could only be analysed at a species level where there were multiple strains of the same species. The collective estimated environmental growth parameters were visualised using Principle Coordinate Analysis (PCO) in PRIMER-E v6 (Clarke & Gorley 2006) and the similarity displayed using a Bray-Curtis resemblance matrix.

3.4 Results

3.4.1 Thermal niche estimates

Maximum growth rates (μ_{max}) of *Gambierdiscus* across the tested temperatures ranged between 0.10 and 0.20 day^{-1} (Table 3.2 and Figure 3.2). Tropical *G. carpenteri* strains had higher growth rates (0.16 ± 0.04 day^{-1}; n = 4) compared to temperate strains (0.14 ± 0.04 day^{-1}; n = 4) although considerable intraspecific variation was observed (Table 3.2 and Figure 3.2;). *Gambierdiscus lapillus* strains (n = 2) had the highest growth rate of all taxa (0.20 and 0.19 day^{-1}), followed by *G.* cf. *silvae* (0.18 day^{-1}) and *G.* cf. *pacificus*, which had the lowest rate of growth (0.13 day^{-1}) although only a single strain of the latter species was tested (Table 3.2 and Figure 3.2; $p < 0.05$).

The temperature at which maximum growth occurred (T μ_{max}) across all strains ranged between 23.8 and 27.2 °C (Table 3.2 and Figure 3.2). The tropical strains of *G. carpenteri*

generally had a T μ_{max} 1 °C lower than the temperate strains (T μ_{max} = 25.4 ± 1.2 °C and 26.4 ± 0.8 °C, respectively) and exhibited a wider thermal optimum range (> 80 % of μ_{max}) of 4.5 to 6.6 °C, compared with 4.1 to 4.9 °C for the temperate strains (Table 3.2 and Figure 3.2), however these differences were not statistically significant (p > 0.05). *Gambierdiscus lapillus* showed the highest growth (~0.20 day^{-1}) at ~24 °C, the lowest T μ_{max} for all species tested, however the optimum range extended from 20.0 to 27.0 °C. *Gambierdiscus* cf. *pacificus* and *G.* cf. *silvae* reached maximum growth at 25 °C and had an optimum range from ~22.0 to 27.7 °C (Table 3.2 and Figure 3.2).

The minimum temperature where growth terminated (T$_{min}$) was estimated to be ~15.0 °C for all species of *Gambierdiscus* tested, except for a single temperate strain of *G. carpenteri* (UTSMER8B4) which had a T$_{min}$ of 0.7 °C (potential outlier due to the lack of convergence during curve fitting; Figure 3.2). Similarly, the maximum temperature where growth terminated (T$_{max}$) was estimated to be ~30.0 °C for all *Gambierdiscus* species tested, including the temperate *G. carpenteri* strains (Table 3.2 and Figure 3.2).

Table 3.2: Summary of calculated growth parameters from temperature reaction norms for each strain of *Gambierdiscus* measured in this study, including maximum growth rate (μ_{max}), the temperature (°C) at which maximum growth was reached (T μ_{max}), the optimum temperature range for growth (T_{opt}) and the minimum and maximum temperatures where growth terminated (T_{min} and T_{max}).

Species	μ_{max} (d^{-1})	T μ_{max} (°C)	T_{opt} (°C)	T_{min} (°C)	T_{max} (°C)
Tropical *G. carpenteri* (UTSHI2C4)	0.20	26.5	23.8 - 28.3	14.9	30.0
Tropical *G. carpenteri* (UTSHI6C3)	0.17	24.2	20.7 - 27.2	15.1	30.3
Tropical *G. carpenteri* (UTSHI6A1)	0.12	25.2	22.1 - 27.7	15.3	30.0
Tropical *G. carpenteri* (UTSHI6D2)	0.15	25.5	22.3 - 27.9	14.8	30.2
Temperate *G. carpenteri* (UTSMER8B4)	0.10	27.2	24.7 - 28.8	0.7	30.1
Temperate *G. carpenteri* (UTSMER1A3)	0.12	26.0	23.3 - 28.0	15.4	29.9
Temperate *G. carpenteri* (UTSMER9A3)	0.16	26.1	23.2 - 28.1	13.4	30.0
Temperate *G. carpenteri* (UTSMER8A4)	0.18	26.1	23.2 - 28.1	15.1	30.0
G. lapillus (UTSHI6B5)	0.20	23.8	20.3 - 26.8	14.8	30.0
G. lapillus (UTSHI2B6)	0.19	24.1	20.6 - 27.0	15.0	30.1
G. cf. *pacificus* (UTSHI6A6)	0.13	25.4	22.4 - 27.7	16.0	30.0
G. cf. *silvae* (UTSHI6B1)	0.18	25.3	21.9 - 27.7	14.1	30.0
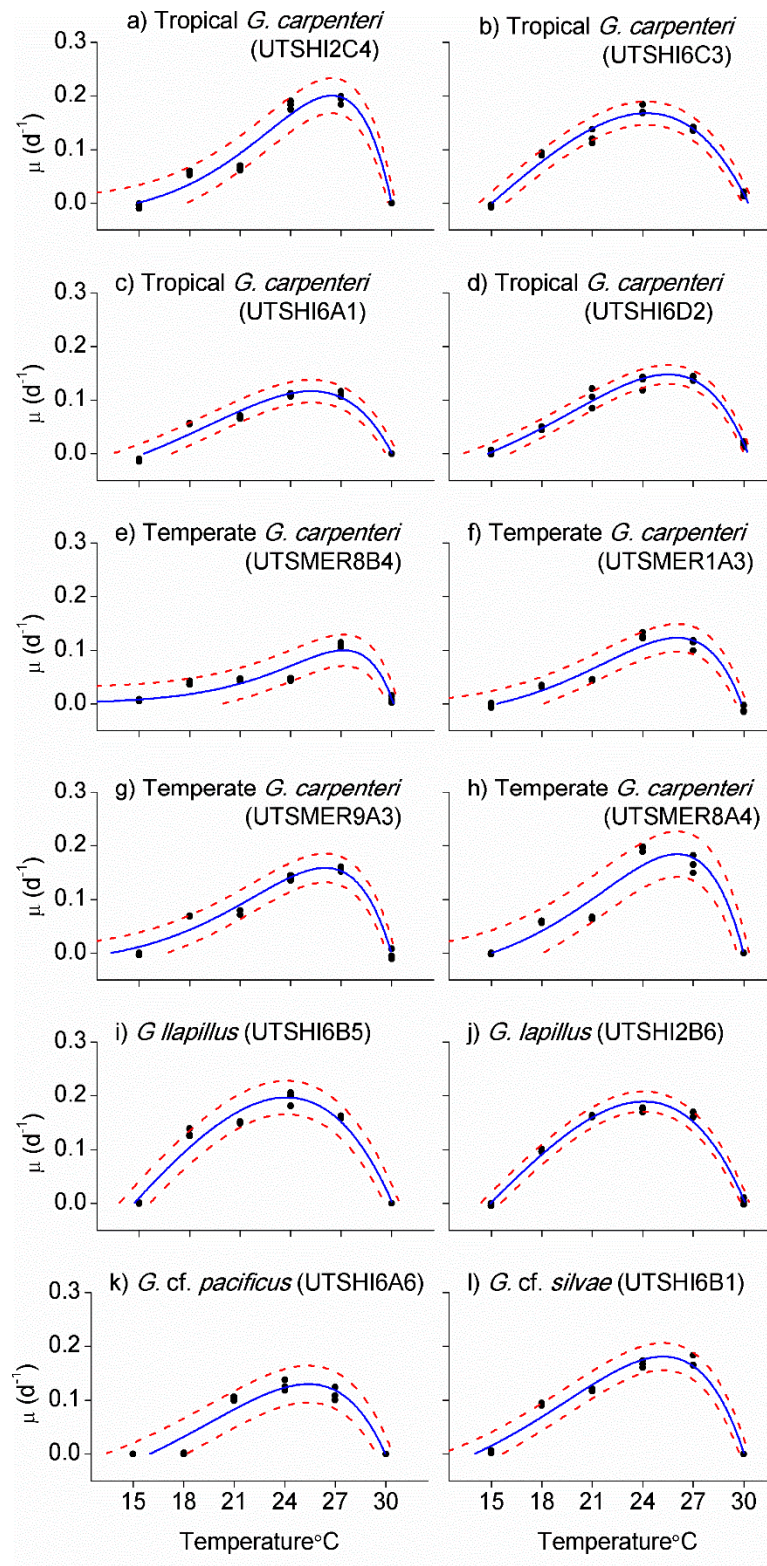
Figure 3.2. Temperature reaction norms for strains of *Gambierdiscus* established from tropical and temperate Australia in this study. Dots indicate the measured growth rates; the blue line indicates the line of best fit using the function described in Thomas et al. (2012) and the red lines represent the 95% confidence band.

3.4.2   Salinity niche estimates

In general, maximum growth rates (μ_{max}) under different salinity conditions were lower than under different temperatures, ranging from 0.04 to 0.18 day^{-1} (Table 3.3 and Figure 3.3) and were significantly different between strains ($p < 0.05$). Maximum growth was reported at salinities (S_{opt}) ranging from 26 to 35 (Table 3.3 and Figure 3.3) and the shape of the salinity reaction norms differed between species and strains of *Gambierdiscus* (Figure 3.3). Both the tropical and temperate strains of *G. carpenteri* had an S_{opt} of 33.0, compared with 31.0 for *G.* cf. *pacificus* and a more brackish 30.2 for *G.* cf. *silvae.* The two strains of *G. lapillus* tested had highest growth at contrasting salinities, with μ_{max} of strain UTSHI6B5 at 31.5, similar to the other *Gambierdiscus* species, whereas for *G. lapillus* strain UTSHI2B6, growth reached a maximum at 26.0 (Table 3.3 and Figure 3.3).

The S_{min} differed enormously between strains (Table 3.3 and Figure 3.3), ranging from < 10 to 20. It was similar among tropical *G. carpenteri* strains (~20), but ranged from < 10 to 20.4 for the temperate *G. carpenteri* strains (Table 3.3 and Figure 3.3). This variation led to no significant effect of strain location for S_{min}. Growth terminated for the *G.* cf. *silvae* strain at 19.6 and < 10 for the *G.* cf. *pacificus* strain. The S_{min} for strains of *G. lapillus* were also quite different: < 10 for strain UTSHI6B5 and 16.1 for strain UTSHI2B6 (Table 3.3 and Figure 3.3). All strains could tolerate hypersalinity to some degree, with growth terminating at salinities (S_{max}) between 38 and 45 (Table 3.3 and Figure 3.3). There was no significant difference in S_{min} or S_{max} between the tropical and temperate strains of *G. carpenteri* ($p > 0.05$)

Table 3.3. Summary of calculated growth parameters from the salinity reaction norms for each strain of *Gambierdiscus* measured in this study, including maximum growth rate (μ_{max}), the salinity at which maximum growth was reached (S μ_{max}), the optimum salinity range for growth (S_{opt}) and the minimum and maximum salinity where growth terminated (S_{min} and S_{max}).

| Species	μ_{max} (d^{-1})	S μ_{max}	S_{opt}	S_{min}	S_{max}
Tropical *G. carpenteri* (UTSHI2C4)	0.09	32.1	25.7 - 40.9	19.8	> 60
Tropical *G. carpenteri* (UTSHI6C3)	0.13	34.2	30.5 - 36.7	19.8	39.1
Tropical *G. carpenteri* (UTSHI6A1)	0.07	32.2	27.6 - 36.1	20.3	40.1
Tropical *G. carpenteri* (UTSHI6D2)	0.08	33.7	29.2 - 37.0	19.8	40.3
Temperate *G. carpenteri* (UTSMER8B4)	0.04	29.4	24.0 - 35.7	18.4	44.0
Temperate *G. carpenteri* (UTSMER1A3)	0.06	31.5	24.6 - 36.7	< 10	40.9
Temperate *G. carpenteri* (UTSMER9A3)	0.09	35.0	25.5 - 41.7	< 10	47.7
Temperate *G. carpenteri* (UTSMER8A4)	0.06	34.6	31.0 - 37.0	20.4	39.2
***G. lapillus* (UTSHI6B5)**	0.18	31.5	24.9 - 35.8	< 10	39.6
***G. lapillus* (UTSHI2B6)**	0.12	26.0	21.0 - 32.8	16.1	46.9
***G.* cf. *pacificus* (UTSHI6A6)**	0.14	31.0	24.8 - 35.0	< 10	38.6
***G.* cf. *silvae* (UTSHI6B1)**	0.14	30.2	25.2 - 35.6	19.6	43.0

Figure 3.3: Salinity reaction norms for strains of *Gambierdiscus* established from tropical and temperate Australia in this study. Dots indicate the measured growth rates; the blue line indicates the line of best fit using Thomas et al. (2012) function and the red lines represent 95% confidence band.

3.4.3 Irradiance niche estimates

Maximum growth rates (μ_{max}) measured during irradiance experiments were significantly different ($p < 0.05$) among *Gambierdiscus* strains, ranging from 0.05 to 0.14 day^{-1} (Table 3.4 and Figure 3.4). Growth was measured across four irradiance levels (10, 50, 100, 200 µmol photons m^{-2} s^{-1}) and the irradiance level at which maximum growth occurred (I_{opt}) was similar for all species and strains at ~100 µmol photons m^{-2} s^{-1} (Table 3.4). The shape of the irradiance reaction norms were very similar (Figure 3.4) which is reflected in similar estimates of the I_{min} (~10 µmol photons m^{-2} s^{-1}) and I_{max} (>200 µmol photons m^{-2} s^{-1}) amongst all strains tested (Table 3.4 and Figure 3.4). There was no significant difference between the tropical and temperate *G. carpenteri* strains for each of the estimated irradiance growth parameters.

3.4.4 *Gambierdiscus* environmental niche

The ecophysiological requirements of *Gambierdiscus* species were not significantly different ($p > 0.05$) from one another when estimates of growth parameters (e.g. μ_{max}, opt, niche min, niche max, and niche width) were compared for temperature, salinity and irradiance individually or when they were analysed collectively. When the overall estimated environmental niche (temperature, salinity and irradiance estimated growth parameters combined) of *Gambierdiscus* strains measured in this study were compared using Principle Coordinate Analysis (PCO) analysis, there was over 90% similarity amongst isolates (Figure 3.5).

Table 3.4. Summary of calculated growth parameters calculated from the irradiance reaction norm function for each strain of *Gambierdiscus* measured in this study, including maximum growth rate (μ_{max}), the irradiance (μmol photons m^{-2} s^{-1}) at which maximum growth was reached (I μ_{max}), the optimum irradiance range for growth (I_{opt}) and the minimum and maximum irradiance where growth terminated (I_{min} and I_{max}).

Species	μ_{max} (d^{-1})	I μ_{max} (μmol photons m^{-2} s^{-1})	I_{opt} (μmol photons m^{-2} s^{-1})	I_{min} (μmol photons m^{-2} s^{-1})	I_{max} (μmol photons m^{-2} s^{-1})
Tropical *G. carpenteri* (UTSHI2C4)	0.09	132.6	66.0 - > 200	12.0	> 200
Tropical *G. carpenteri* (UTSHI6C3)	0.07	173.3	84.1 - > 200	11.1	> 200
Tropical *G. carpenteri* (UTSHI6A1)	0.08	95.7	49.0 - 186.1	10.0	> 200
Tropical *G. carpenteri* (UTSHI6D2)	0.09	108.5	54.9 - > 200	9.7	> 200
Temperate *G. carpenteri* (UTSMER8B4)	0.05	99.9	51.1 - 195.0	10.6	> 200
Temperate *G. carpenteri* (UTSMER1A3)	0.05	114.5	55.7 - > 200	8.4	> 200
Temperate *G. carpenteri* (UTSMER9A3)	0.12	120.9	68.1 - > 200	10.8	> 200
Temperate *G. carpenteri* (UTSMER8A4)	0.05	92.6	48.2 - > 200	10.6	> 200
G. lapillus (UTSHI6B5)	0.05	106.8	54.6 - > 200	10.3	> 200
G. lapillus (UTSHI2B6)	0.08	117.7	58.9 - > 200	11.0	> 200
G. cf. *pacificus* (UTSHI6A6)	0.09	125.8	60.7 - > 200	8.6	> 200
G. cf. *silvae* (UTSHI6B1)	0.14	105.3	53.3 - > 200	10.3	> 200

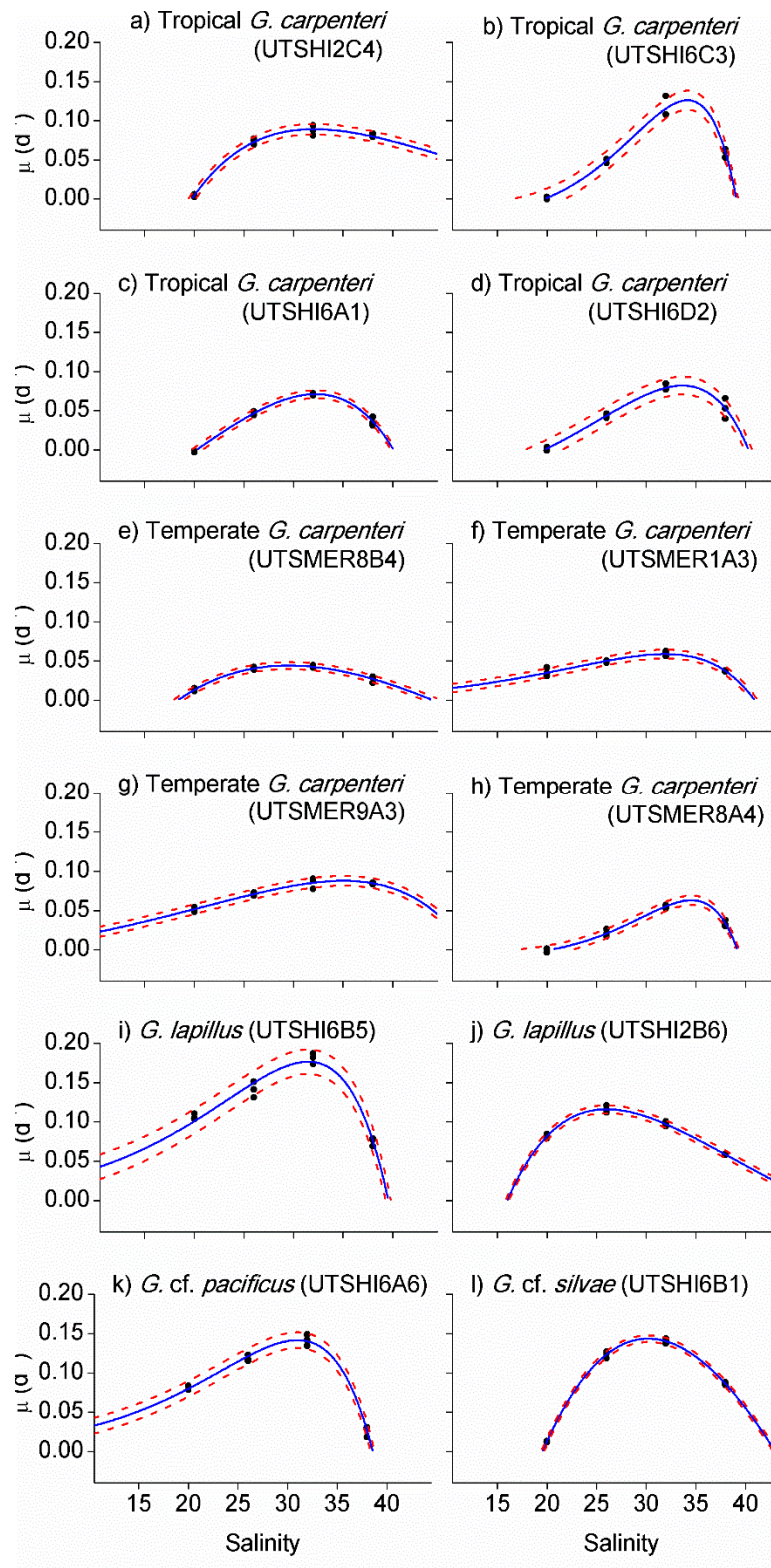**Figure 3.4.** Irradiance reaction norms for strains of *Gambierdiscus* spp. established from tropical and temperate Australia in this study. Dots indicate the measured growth rates; the blue line indicates the line of best fit using Thomas et al. (2012) function and the red lines represent 95% confidence band.

Figure 3.5. Principle Coordinate Analysis (PCO) of the collective estimated growth parameters (e.g. μ_{max}, opt, niche min, niche max, niche width) from temperature, salinity and irradiance experiments. Each symbol represents an individual strain of *Gambierdiscus* and the shape and colour of the symbols represent different *Gambierdiscus* species. Groupings represent 90% similarity (solid line) and 95% similarity (broken line).

3.5   Discussion

In Australia, *Gambierdiscus* species are known to be distributed throughout the tropical waters of the Great Barrier Reef (Gillespie et al. 1985; Holmes, Lewis & Gillespie 1990; Holmes et al. 1991; Kretzschmar et al. 2017; Murray et al. 2014; Rhodes et al. 2017; Skinner, Lewis & Morton 2013; Sparrow & Heimann 2016; Sparrow et al. 2017; Chapter 2) and at the temperate location of Merimbula, New South Wales (Kohli, Murray, et al. 2014; Chapter 2). Strains established from both the tropical and temperate part of the distribution provided a unique opportunity to compare their environmental niche and evaluate whether tropical taxa could occupy temperate habitats, thereby assessing the potential for range extension of the ciguatera causing genus in this region. Here we show that 12 Australian *Gambierdiscus* isolates have potentially similar environmental niches based on their physiological response to three environmental parameters: temperature, salinity and light.

There was no significant difference in the environmental niche of the tropical and temperate strains of *G. carpenteri*, and there was over 90% similarity in the environmental niche of all strains tested. These results suggest that strains of *G. carpenteri* established from the temperate location are not specifically adapted to these environmental conditions and therefore, other strains or species of the genus may also have the capacity to occupy temperate locations without the need for vast adaptation. This is particularly important for future management of CFP, as it indicates that species which produce compounds with CTX-like activity and are potentially contributing to cases of CFP, such as *G. lapillus, G.* cf. *pacificus* and *G.* cf. *silvae* (Chapter 2)*,* could have a much wider distribution than is presently documented.

3.5.1 Comparison of the fitness of *Gambierdiscus* strains

The maximum growth rates (μ_{max}) measured for *Gambierdiscus* strains in this study were consistent with other studies (Kibler et al. 2012; Tawong et al. 2016; Xu et al. 2016; Yoshimatsu et al. 2016; Yoshimatsu et al. 2014). Of the tropical species in this study, *G. lapillus* had the highest fitness, followed by *G.* cf. *silvae*, *G. carpenteri* and *G.* cf. *pacificus*.

Overall, *Gambierdiscus* growth rates are generally low compared with other microalgal genera. For example, Morton, Norris & Bomber (1992) compared the growth of multiple genera of epibenthic dinoflagellates under a gradient of environmental conditions and found *Amphidinium* had the highest (~0.45 division rate), followed by *Ostreopsis* (~0.35 division rate), *Prorocentrum* (~0.3 division rate), *Coolia* (~0.3 division rate) and finally *Gambierdiscus* (~0.2 division rate). This indicates that high cell abundances of *Gambierdiscus* species would likely take several months to develop (Litaker et al. 2010), and therefore bloom formation requires prolonged favourable environmental conditions.

3.5.2 Temperature

Tropical and temperate Australian strains of *Gambierdiscus* had similar optimum temperatures for growth (T μ_{max}). Thomas et al. (2012) found that T μ_{max} is related to the latitude of the isolation location, which directly reflects the mean annual water temperature (lower water temperatures at higher latitudes results in a lower T μ_{max}). Given the vast differences in water temperature between Heron Island and Merimbula (Figure 3.7) and 13 degrees of latitudinal difference; we anticipated T μ_{max} for the temperate *G. carpenteri* strains would be lower. To explore this and other hypotheses more broadly across the *Gambierdiscus* genus, growth data from similar experiments conducted globally were compiled (Table 3.1; Figure 3.6 and 3.8)

The relationship between T μ_{max} and latitude for *Gambierdiscus* (Figure 3.6) shows a weak positive correlation with latitude, compared with the strong correlation found in Thomas et al. (2012). There are two likely reasons for the differences. Firstly, the range of latitudes used by Thomas et al. (2012) was much broader than the latitudinal range in this study (~70 versus 13 degrees). Secondly, the taxa used in Thomas et al.'s comparison were isolated from ocean environments. In contrast, *Gambierdiscus* are epibenthic species and often occur in shallow water habitats which may have local thermal conditions that are not representative of annual mean offshore ocean temperatures (e.g. the benthos of shallow environments may reach warmer temperatures). A relationship between mean annual water temperature and T μ_{max} may still exist for *Gambierdiscus*, but it may not be well represented by latitude, as opposed to local water temperatures.

Local conditions at the sites where strains were isolated from in this study were therefore considered. The mean annual water temperatures at Heron Island, and Merimbula, the tropical and temperate locations from this study, are 24.4 °C and 17.6 °C respectively (Figure 3.7). Indeed, the water temperatures at Merimbula rarely reach 24 °C or above so it is somewhat surprising that the temperature for maximum growth of *Gambierdiscus* strains isolated from this location is ~25 °C, with the population persisting during winter when temperatures are as low as 12 °C and even reaching "bloom" proportions annually (Kohli, Murray, et al. 2014).

Figure 3.6. Dots represent the temperature at which maximum growth was achieved (T μ_{max}) (blue), and the minimum (T_{min}) (green) and maximum (T_{max}) (red) temperature for growth estimated from temperature reaction norm experiments. These data have been fitted with a polynomial curve to demonstrate the relationship of the thermal performance traits with latitude. Data sourced from Kibler et al. 2012; Yoshimatsu et al. 2014, 2016, Tawong et al. 2016; Xu et al. 2016 and this study.
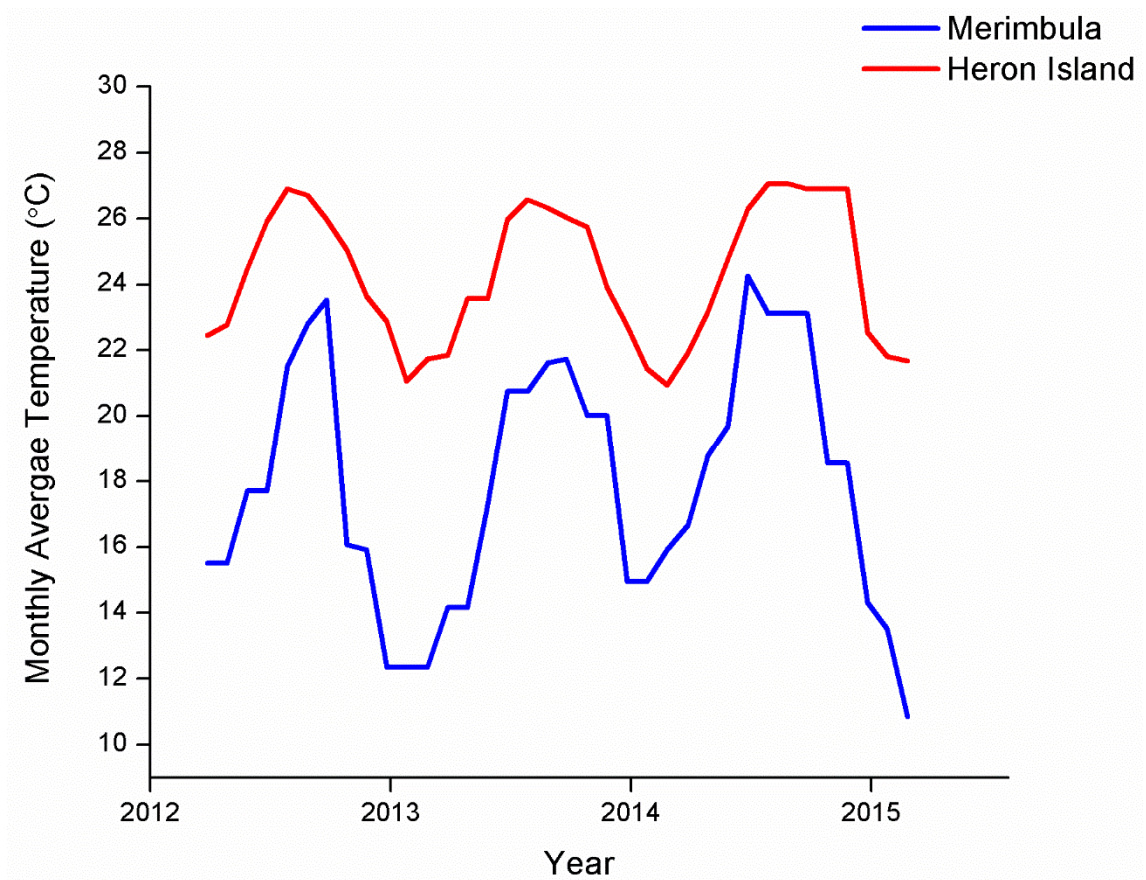
Figure 3.7. Monthly average temperature for the Heron Island, Queensland Australia (tropical) and Merimbula, New South Wales (temperate). Data sourced from the IMOS-Weather Station (**http://www.aims.gov.au**) and the New South Wales Food Authority.

One possible explanation for the poor relationship between habitat temperature and T μ_{max}, could be that the *G. carpenteri* temperate population represents a relatively recent range extension of a *G. carpenteri* population with tropical origins and has not yet thermally adapted to the local conditions. *Gambierdiscus* has a known tropical distribution but has been reported from temperate locations in recent years (Aligizaki, Nikolaidis & Fraga 2008; Kohli, Murray, et al. 2014; Nishimura et al. 2013). The marine environment along the east coast of Australia is highly connected by a polewards flowing western boundary current, the East Australian Current (EAC), which delivers warm water southward from the tropics year-round (Ridgway 2007). It has been suggested that the EAC could disperse epibenthic harmful algal bloom species to temperate locations (Heimann, Capper & Sparrow 2011; Murray et al. 2014; Sparrow et al. 2017). However, the genetic relatedness of the Australian tropical and temperate populations of *G. carpenteri* are yet to be evaluated.

Other parameters derived from thermal reaction norms are the T_{min} and T_{max} temperature for growth and may prove more valuable when evaluating the potential for *Gambierdiscus* species to extend their range into temperate latitudes. Indeed, the relationship between latitude and T_{min} temperature for growth of *Gambierdiscus* species is stronger than with the T μ_{max} or T_{max} temperatures for growth (Figure 3.6). The average T_{min} of *Gambierdiscus* species included in our study was 17.1 °C and 18.0 °C for other studies (Figure 3.8), which is much lower than temperatures experienced in tropical marine habitats. Interestingly, monthly average temperatures at the temperate isolation location from this study (Merimbula), can drop as low as 12 °C (Figure 3.6), suggesting *Gambierdiscus* species can survive temperatures well below their estimated minimum temperature for growth.

Overall, maximum growth of Australian strains of *Gambierdiscus* occurred at temperatures between 23.8 and 26.5 °C (Table 3.2). In comparison, other studies have found maximum growth at temperatures between 23.8 and 31.1 °C (Figure 3.8; Kibler et al. 2012; Tawong et al. 2016; Xu et al. 2016; Yoshimatsu et al. 2016; Yoshimatsu et al. 2014). Despite this variation in the temperature at which maximum growth occurs, the thermal niche of *Gambierdiscus* species across the genus is fairly consistent. Generally, growth can occur at temperatures between 15 and 30 °C (Figure 3.8), reflecting the typical tropical distribution of the genus.

Figure 3.8. Temperature, salinity and irradiance niche of *Gambierdiscus* strains in this study compared to other studies. Black bar indicates μ_{max} , grey bar indicates region of maximum growth (>80% of μ_{max}) and white bar indicates region of growth. Y-axis shows *Gambierdiscus* species name and abbreviation of isolation locations. Note: irradiance values up to 400 μmol photons m^{-2} s^{-1} only included in figure.

Isolation location abbreviations are as follows: HI, Heron Island, Australia; MER, Merimbula, Australia; MI, Mariana Islands; USA, United States of America; SI, Society Islands; KIR, Kiribati; VI, Virgin Islands; JP, Japan; HAW, Hawaii; BEL, Belize; TL, Thailand; NC, North Carolina; MAR, Martinique
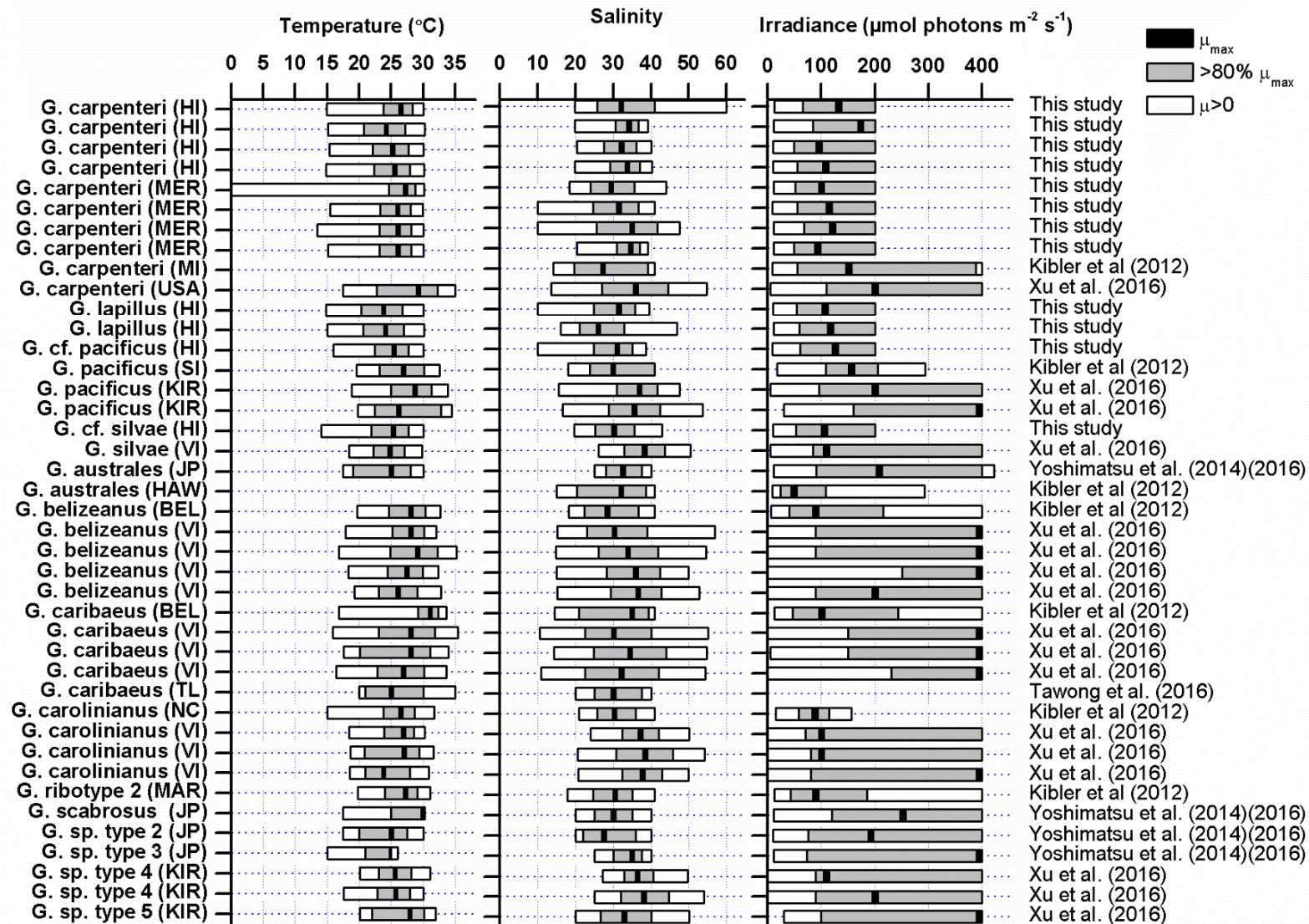
3.5.3   Salinity

Defining the salinity niche of a marine microalgal species can provide insight into habitat preferences and when considering HAB species, can identify potential high-risk environments (e.g. reef flats, island lagoons, estuaries that have a gradient in salinity). *Gambierdiscus* species are traditionally thought to inhabit tropical reef environments (e.g. Litaker et al. (2010)), although have been increasingly reported from seagrass dominated lake or estuarine systems (Chapter 2; Lugomela 2006; Shah et al. 2010). In our study, S μ_{max} was between 31.0 and 33.0, except for one strain of *G. lapillus* (UTSHI2B6) that had a brackish water preference (26.0). The cause of this disparity between strains of *G. lapillus* is not known but highlights intraspecific differences that can occur between strains isolated from the same location at the same time. Interestingly, the S μ_{max} of *Gambierdiscus* strains occurs at salinities lower than oceanic 35 (Figure 3.8) but taxa have an optimum range (S_{opt}) which extends well beyond this value (i.e. 26 to 40) (Figure 3.8).

The S_{min} and S_{max} is also important in predicting habitats where *Gambierdiscus* can occur. The S_{min} of *Gambierdiscus* species from our study was between 10 and 20 (Figure 3.3). Although these values show divergence, they demonstrate that *Gambierdiscus* species isolated from Australia can grow at salinity levels well below seawater and can therefore also occupy habitats with freshwater inputs such as estuaries. The S_{max}, although variable, was greater than 38 for all strains (Figure 3.3), indicating these species can also occur in habitats typical of the distribution of the genus (e.g. marine reef habitats). There were also no differences in the salinity niche found between strains of *Gambierdiscus* established from the tropical reef habitat and the temperate seagrass dominated coastal embayment in this study (Figure 3.3). The salinity niche estimated from other studies is

also very broad ranging from 10 to 57 (Figure 3.8), suggesting these findings are not unique to Australian strains of *Gambierdiscus*.

For the interpretation of these laboratory data, it is important to consider that reaction norms and model fits produce optimal results when there are large differences in growth between treatments. The capacity to accurately extrapolate the minimum and maximum niche for growth is therefore hindered when growth responses are low or the number of treatments are limited. Evidence of this can be seen in some of the salinity niche width estimates from this study where a greater resolution of tested salinities would have more accurately defined the niche limits. Nonetheless, reaction norms and model fits have accurately captured optimum growth conditions for the taxa in this study and are a valid method for investigating how environmental variables influence growth of microalgal species.

3.5.4   Irradiance

Microalgae are photosynthetic organisms that derive energy from light. Light or irradiance levels can vary enormously with water depth and can ultimately regulate the vertical distribution of epibenthic marine microalgae. In our study, growth was measured across a gradient of irradiance ranging from 10 to 200 photons m^{-2} s^{-1} and maximum growth (I μ_{max}) was achieved at ~100 µmol photons m^{-2} s^{-1} (Figure 3.4). There is enormous diversity in the growth response of *Gambierdiscus* strains to irradiance reported in the literature (Figure 3.8). Some studies find maximum growth occurs at irradiance levels as low as 49 µmol photons m^{-2} s^{-1} (*G. australes*, Kibler et al. (2012)) and as high as 427 µmol photons m^{-2} s^{-1} (*G.* sp. type 3, Yoshimatsu et al. (2016)). However, direct comparisons are made difficult by the different designs of irradiance experiments (e.g. different light sources, treatment levels and methods of irradiance

measurement, growth medium, temperature and light interactions). A general comparison of all *Gambierdiscus* strains and species reveals that the optimum range for growth is between 50 and 400 μmol photons m^{-2} s^{-1} and growth can occur at irradiance levels as low as 10 μmol photons m^{-2} s^{-1} (Figure 3.8).

Gambierdiscus species are commonly found in shallow water habitats where irradiance levels are high, therefore the relative preference for low irradiance levels is interesting. It is not known why *Gambierdiscus* species favour the benthic environment but some hypothesise it is advantageous for optimising exposure to irradiance. Villareal & Morton (2002), speculate that substrate association may allow epibenthic dinoflagellates to occupy microhabitats within the thalli of macrophytes allowing for adjustment to the level of irradiance as required. However, others have suggested that the large amount of mucus produced by epibenthic dinoflagellates is a mechanism to shield cells under high irradiance conditions (Ballantine, Tosteson & Bardales 1988; Heil, Maranda & Shimizu 1993).

Another important implication of the ability of *Gambierdiscus* species to grow under low irradiance conditions is that deep water habitats are also able to be occupied. Based on irradiance reaction norm experiments, Yoshimatsu et al. (2016) concluded *Gambierdiscus* can grow at depths of up to 87 m. This expansive vertical distribution of *Gambierdiscus* species has vast implications for the area that can be occupied in a given location and the maximum population size of a given region and could influence the type of marine fish species able to accumulate the CFP causing toxins produced by the microalgal cells.

3.5.5 Future work

The environmental niche of 41 strains of *Gambierdiscus* representing nine described species and seven ribotypes have now been described (this study, Kibler et al. 2012; Tawong et al. 2016; Xu et al. 2016; Yoshimatsu et al. 2016; Yoshimatsu et al. 2014). Although this is an enormous effort, many species remain to be examined and the huge diversity in growth responses of *Gambierdiscus* strains from the same species, mean species-specific niches remain difficult to accurately define. Nonetheless, genus specific environmental niche estimates are broadly consistent and support the currently recognised tropical to subtropical distribution. They do however also show, that occupation of temperate habitats is possible. To further refine our current understanding of the distribution of the organisms involved in CFP, it will be important to focus on defining the environmental niches of highly toxic *Gambierdiscus* species in the future. These results can be then be used to model the risk of CFP under current and future climate conditions (Kibler et al. 2017).

There are some important considerations which must be made when transferring the results of environmental niche estimates to evaluations of species ditributions. The first is that laboratory experiments were completed under nutrients replete conditions which can affect how species respond to environmental extremes and may not reflect responses in nature where resources are limiting. The second, is that the laboratory exeriments were also conducted under constant temperature, salinity and irradiance levels. Again, this may not reflect natural conditions which are variable and therefore may overestimate growth. It is therefore anticipated that the described fundamental niche may differ from the realised niche and should be considered when assessing the risk of CFP in a location. It is ultimately toxin production which will affect the risk of CFP in a particular area so

future work should also focus on the relationship between environmental parameters, including biological interactions, and toxin production.

Chapter 4 Hitchhiking in the East Australian Current – rafting as a dispersal mechanism for harmful epibenthic dinoflagellates

4   Hitchhiking in the East Australian Current – rafting as a dispersal mechanism for harmful epibenthic dinoflagellates

Author contributions:

M.E.L conceived the idea, completed microalgal cell identification and enumeration, isolated, established and maintained all microalgal cultures, carried out DNA extractions, data analysis, designed the study and wrote the manuscript. O.F.L, I.M.S and M.A.D participated in the design of the study, and collected and processed raft samples on board the RV Investigator. P.A.A assisted with microalgal identifications and performed the analyses with the long-term coastal phyoplankton monitoring data series (PH100). All authors revised the manuscript.

4.1   Abstract

Due to their small size, drifting marine microorganisms have large dispersal capacity in the global ocean. However, it is not known how epibenthic microalgae disperse across long distances, because they are generally associated with a substrate. This study examined a long-term data series (~50 years) of microalgal composition from a coastal station in south east Australia, for the presence of epibenthic dinoflagellates in the plankton. In addition, drifting macrophytes and plastic debris were collected from the East Australian Current, the associated microalgal taxa identified, their viability assessed, and phylogenetic analyses to identify cryptic harmful epibenthic dinoflagellate species were used. There were no occurrences of epibenthic dinoflagellates from the genera *Gambierdiscus*, *Fukuyoa, Ostreopsis,* and *Coolia* at the long-term coastal station. It was therefore concluded that entrainment of cells in ocean currents is an unlikely mechanism for transportation of these taxa. The epibenthic microalgal community associated with macrophyte rafts and plastic debris was primarily comprised of diatom taxa. However, intact cells of potentially harmful epibenthic dinoflagellates from the genera *Coolia, Amphidinium,* and *Prorocentrum* were also observed, and their viability confirmed by division of isolated cells and establishment into clonal cultures. Phylogenetic analyses confirmed the presence of *Coolia palmyrensis* on a drifting *Sargassum* sp. raft, which constitutes the first report of this potentially harmful epibenthic species in temperate Australian waters. This study shows that harmful epibenthic dinoflagellates can attach to, and remain viable when associated with macrophyte fragments that drift in the open ocean, therefore revealing rafting as a potential vector for dispersal of these organisms.

4.2   Introduction

Dinoflagellates are a frequent part of microalgal communities in the photic and benthic zones of the world's coastal waters and oceans. A range of species, known as 'harmful' dinoflagellates, can produce potent toxins that accumulate in the food web (e.g., filter-feeding shellfish) and when consumed by humans, can cause food related illnesses (Van Dolah 2000). Epibenthic dinoflagellates occupy the benthic environment, living in close association with the benthos or substrates such as a macrophytes. These organisms have been implicated in several human related illnesses ranging from respiratory (Durando et al. 2007) and dermatologic (Tubaro et al. 2011) conditions and Ciguatera Fish Poisoning (CFP) (Yasumoto et al. 1979).

Over the past decade, there has been considerable research focus on understanding the taxonomy of the main epibenthic dinoflagellate genera *Gambierdiscus* Adachi & Fukuyo (Adachi & Fukuyo 1979), *Fukuyoa* Gómez, Qui, Lopes & Lin (Gómez et al. 2015), *Ostreopsis* Schmidt (Schmidt 1901), *Coolia* Meunier (Meunier 1919), *Prorocentrum* Ehrenberg (Ehrenberg 1834) and *Amphidinium* Claparède & Lachmann (Claparède & Lachmann 1859). Each can easily be identified to genus level based exclusively on cell morphology using a light microscope, however large morphological similarities between species within some genera (e.g. *Gambierdiscus, Fukuyoa, Ostreopsis* and *Coolia*) mean phylogenetic analyses are often necessary to resolve identification to species level (Accoroni et al. 2016; Karafas, York & Tomas 2015; Litaker et al. 2009).

More recently, epibenthic dinoflagellate research has shifted to ecological studies aimed at understanding current and future species distributions (Kibler et al. 2012; Kibler et al. 2015; Xu et al. 2016). As a result, there is growing evidence that harmful epibenthic dinoflagellates are extending their range from tropical to more temperate locations in both

the northern and southern hemispheres (Aligizaki, Nikolaidis & Fraga 2008; Berdalet, Vila & Abós-Herrandiz 2015; Kohli et al. 2014). However, the natural and human-assisted vectors facilitating this range extension remain relatively unexplored.

Due to their small size, marine micro-organisms have the potential to be dispersed long distances, suspended in ocean currents (McManus & Woodson 2012). It has been suggested that harmful epibenthic dinoflagellates may also be dispersed in this way (Heimann, Capper & Sparrow 2011; Murray et al. 2014; Sparrow et al. 2017); however, their association with a substrate makes planktonic drift of free-living cells an unlikely mechanism of transport. A more likely method of dispersal is 'rafting'. This involves organisms being transported while attached to floating materials and is already a recognised long distance dispersal mechanism for many sentinel marine species (Thiel & Gutow 2005a, 2005b). These floating materials, or rafts, can have a biotic origin (e.g. macrophytes) or an abiotic origin (e.g. litter and plastics) (Thiel & Gutow 2005a, b), and both have the potential to disperse harmful epibenthic dinoflagellates (Besada et al. 1982, Bomber et al. 1988, Masó et al. 2003, Masó et al. 2016). After observing the poor swimming ability and nonplanktonic habit of epibenthic dinoflagellates, Besada et al. (1982) were the first to propose rafting as the most likely dispersal mechanism for these organisms. Investigations then followed and epibenthic dinoflagellates were found to be associated with drifting macrophyte fragments collected in the Florida Straits (Bomber et al. 1988), and marine plastic debris collected along the Catalan coast in the north-western Mediterranean (Masó et al. 2003, Masó et al. 2016). However, whether viable epibenthic dinoflagellates could remain associated with, and be transported by, rafts in open ocean environments is not known.

The East Australian Current (EAC) is the western boundary current of the South Pacific Ocean, originating in the tropical Coral Sea and flowing southward along the edge of the

eastern Australian continental shelf (Ridgway 2007). Marine organisms such as reef fish are frequently transported from tropical to temperate latitudes in eastern Australia *via* the EAC (Booth et al. 2007). Like other western boundary currents, the EAC is increasing its poleward extension (Suthers et al. 2011; Wu et al. 2012; Yang et al. 2016), transporting more tropical water into temperate latitudes. Range extensions facilitated by this strengthening of the current have already been reported for a number of marine organisms including coastal fish (Last et al. 2011), invertebrates (Banks et al. 2007; Ling et al. 2009), zooplankton (Johnson et al. 2011) and kelp (Coleman et al. 2011) and could also aid range extensions of epibenthic dinoflagellates. This current was therefore used as a case study to investigate the potential for long distance dispersal of epibenthic dinoflagellate species, both suspended in the plankton and *via* rafting.

The influence of the EAC on the temperate southeast Australian pelagic ecosystem has been monitored at a historic time series station, Port Hacking (PH100), for over 70 years (Thompson et al. 2009). PH100 is located ~5 km from the coast at a depth of 100 m (Figure 4.1), and is routinely sampled for hydrographic and biological parameters, including phytoplankton species composition (http://imos.org.au). Given that the fraction of EAC water at PH100 increased during the decade 1997 to 2007 (Thompson et al. 2009), this station provides a relevant point of reference to assess the abundance of epibenthic dinoflagellates in the water column versus their abundance on rafts.

In this study, our objective was to sample rafts of biotic and abiotic origin during an oceanographic voyage, and quantify their capacity to transport viable cells of epibenthic dinoflagellates. We then assessed this against the backdrop of epibenthic dinoflagellate diversity and abundance detected in the plankton at the PH100 station over the previous 50 years, to evaluate rafting as a natural dispersal vector for epibenthic dinoflagellates.

4.3 Methods

4.3.1 Presence of epibenthic dinoflagellates in the Port Hacking (PH100) time-series (1965-2013)

Port Hacking 100 m Station (PH100) is a long-term observing station located on the continental shelf of south east Australia (34.120 °S, 151.224 °E) (Figure 4.1). This station is regularly influenced by the East Australian Current (EAC) and can therefore be used to investigate microalgal species composition and transport in this dynamic western boundary current system (Thompson et al. 2009, Ajani et al. 2014a, b). The time-series used in this study was a combination of datasets from the Port Hacking National Reference Station (1965-2009) and the Integrated Marine Observing System (IMOS) National Reference Station (NRS) Network (same location, 2009-2013), and was combined and curated as described in Ajani et al. (2016). The five datasets included weekly sampling between April 1965 - April 1966 (Grant and Kerr 1970); weekly sampling between April 1978 - April 1979 (Hallegraeff 1981); weekly sampling between April 1997 - April 1998 (Ajani et al. 2001); monthly sampling between September 1998 - December 2009 (Ajani et al. 2014a, b); and monthly sampling between February 2009 - December 2013 (imos.aodn.org.au). For a detailed description of the sample collection methodology, see Ajani et al. (2016). Briefly, samples were collected either as discrete bottle samples from 0 - 50 m (Grant & Kerr 1970, Hallegraeff 1981, Hallegraeff & Reid 1986) or by 50 m or 100 m vertical haul with a 37 μm or 20 μm mesh net (Ajani et al. 2001, Ajani et al. 2014a, b). Samples were preserved, and microscopic examination was performed to identify and enumerate microalgal taxa.

This extensive 50-year database consisting of 267 individual samples, was searched for occurrences of epibenthic dinoflagellate cells from the genera *Amphidinium*, *Ostreopsis*,

Coolia, *Prorocentrum, Gambierdiscus* and *Fukuyoa.* Their presence in the plankton at PH100 would therefore suggest that cells had either been advected to the site from local shallow water habitats after being suspended from the benthos or transported from more distant sites via the plankton. As the genus *Prorocentrum* comprises some species which are pelagic and others which are epibenthic, only species which were described as benthic/epibenthic in Hoppenrath et al. (2014) or Hallegraeff et al. (2010) (e.g. *Prorocentrum lima, P. rhathymum and P. clipeus*), or were not defined as being either benthic/epibenthic or pelagic (*Prorocentrum* spp.) were included in the analyses. The percent occurrence rate of epibenthic dinoflagellates was calculated by dividing the number of samples where the genus occurred with the total number of samples in the 50-year record and multiplying by 100.
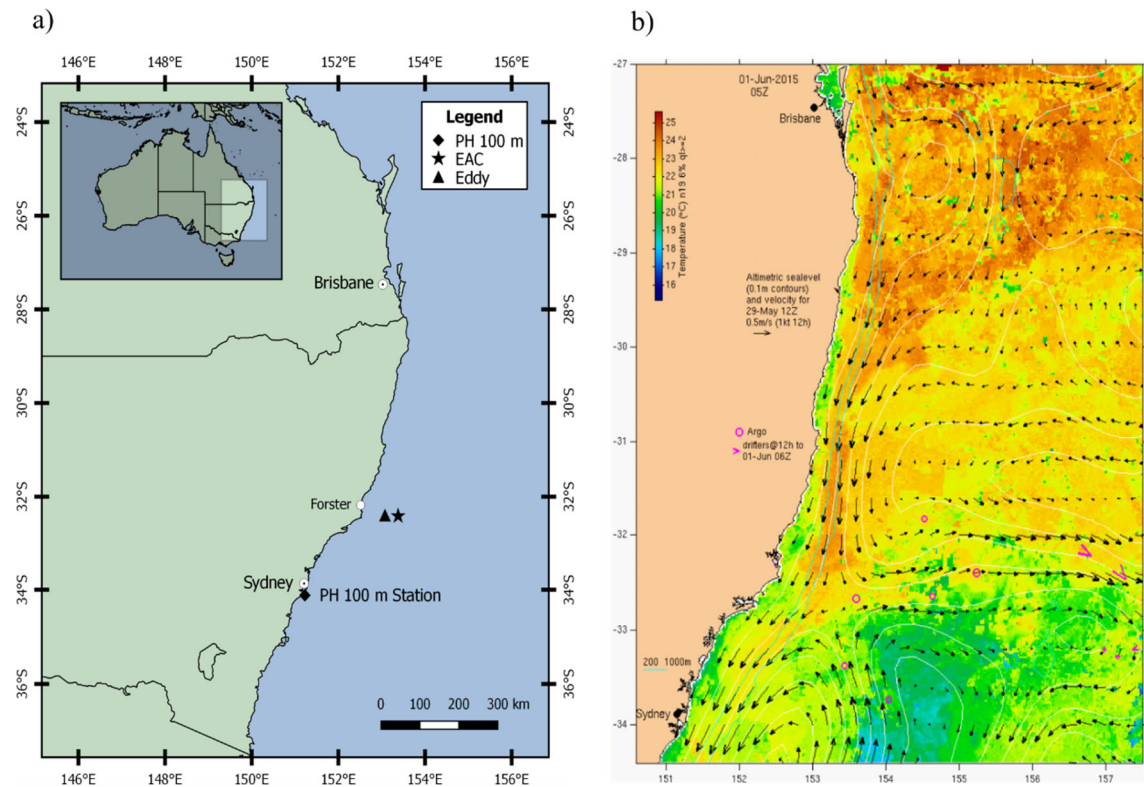
Figure 4.1. (a) Map showing the location of the Port Hacking 100 m Station (PH100) and the sampling location of rafts collected in the East Australian Current (EAC) and an eddy and (b) map of the Sea Surface Temperature (SST) during collection of raft samples on the oceanic voyage between Brisbane and Sydney in June 2015. Data for (b) sourced from IMOS http://oceancurrent.imos.org.au/sst.

4.3.2 Presence of epibenthic dinoflagellates on ocean rafts

4.3.2.1 Raft collection and sample processing

Drifting macrophyte and plastic debris rafts were collected in horizontally towed 1 m^2 neuston nets and occasionally from a 1 m^2 towed multiple opening and closing net in the upper 100 m, at sites within the EAC and associated oceanographic features (e.g. mesoscale eddies) during a voyage (IN2015_V03) from Brisbane to Sydney on the RV *Investigator* in June 2015 (Figure. 4.1). Floating debris in a single horizontal net tow,

sometimes comprising multiple macrophyte species, was combined into a single sample and recorded as a single raft. Rafts were then processed by being placed into a plastic sealable bag with a volume (150 mL to 500 mL) of 0.2 µm filtered seawater. Epiphytes were vigorously shaken from the surface of the rafts for approximately one minute and 15 mL of the suspended organisms were preserved with Glutaraldehyde solution to a final concentration of 1% (v/v). Macrophyte raft samples were oven dried for preservation. The rest of the suspension was incubated at 20 °C, under ~50 µmol photons m^{-2} s^{-1} light before being examined under the microscope for cell isolations.

To evaluate the relationship between the type and size of rafts and associated microalgae abundance and diversity, raft samples were graded with a size and complexity score to take into account differences in surface area and texture. Each sample was given a score ranging from 1 to 4, describing the raft size (1 = very small (< 5 cm^2), through to 4 = large (> 20 cm^2)), and a score describing raft complexity (1 = single species with flat blades, through to 4 = complex leaf structure such as *Sargassum* sp.). Scores for individual rafts were then combined to get a total raft index score (see Supplementary Figure 4.1. for examples).

4.3.2.2   Identification and enumeration of the microalgal community associated with rafts

Glutaraldehyde preserved samples were concentrated from 15 mL to 2 mL by sedimentation for 48 hours and microalgae taxa identified to the lowest possible taxonomic level using a Sedgewick Rafter counting chamber under an inverted light microscope (maximum magnification x 1000) fitted with phase contrast (Nikon Eclipse TS100, Japan). Cells were counted to a threshold of 100 and the entire chamber scanned for the presence of rare taxa. The number of cells per raft was calculated from the initial

volume of filtered seawater added. Taxa were determined to be benthic/epibenthic based on classification by Hoppenrath et al. (2014) and Hallegraeff et al. (2010). Differences among the epibenthic microalgal community associated with raft samples were visualised using Multi-Dimensional Scaling (MDS) in Primer v6.1.16 (Clarke & Warwick 2001) and significance tested for using ANOSIM (p < 0.01).

To examine whether epibenthic dinoflagellate cells associated with rafts were viable, single cells were isolated from a *Sargassum* spp. raft samples using the micropipette technique (Andersen & Kawachi 2005) under an inverted light microscope (Nikon Eclipse TS100, Japan). Established monoclonal cultures were maintained in modified K medium (Litaker et al. 2009) made from sterile aged natural seawater at a salinity of 32 at 24 °C, under ~100 μmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle.

4.3.2.3 Phylogenetic analysis of cryptic harmful epibenthic dinoflagellate species associated with rafts

Due to the strong morphological similarity amongst some epibenthic dinoflagellate taxa, a detailed phylogenetic analysis was conducted to identify the established strain of *Coolia*. Other monoclonal isolates could be identified by light microscopy using an inverted light microscope (Nikon Eclipse TS100, Japan). Cells from 100 mL of the *Coolia* culture were pelleted by centrifugation at 3000 g for 10 min and DNA was extracted using a PowerSoilDNA Extraction Kit (Mo Bio, USA), following the manufacturer's instructions. Extracted DNA was sent to a commercial service (Australian Genomic Research Facility (AGRF), Queensland, Australia) where the D1-D3 region of the LSU rDNA was amplified using primers D1R-F (Scholin et al. 1994) and D3-R (Nunn et al. 1996) using the conditions described in Rhodes et al. (2014). Amplification products (~ 950 bp) were purified and sequenced in both directions.

Phylogenetic analyses were conducted in Geneious v9.1.5 (Kearse et al. 2012). Publicly available sequences of *Coolia* spp. and *Ostreopsis* spp. were downloaded from GenBank (www.ncbi.nlm.nih.gov) and aligned with the sequences obtained from this study using the MUSCLE algorithm (maximum number of iterations 8) (Edgar 2004), with *Ostreopsis* spp. sequences used as out-groups. Sequences were truncated to 756 bp and a maximum likelihood phylogenetic tree was generated using PHYML with 1,000 bootstraps (Guindon & Gascuel 2003) using a GTR substitution model and an estimated gamma distribution. Bayesian analyses was performed using MrBayes 3.2.6 (Huelsenbeck & Ronquist 2001) by means of the GTR+G (general-time reversible with gamma-shaped among-site variation) model. Alignment analyses were carried out in four simultaneous runs with four chains each for 3.1×10^6 generations, sampling every 1,000 trees and 1,000 trees were discarded as burn in.

4.4 Results

4.4.1 Presence of epibenthic dinoflagellates in the Port Hacking (PH100) time-series (1965-2013)

There were no occurrences of epibenthic dinoflagellates from the genera *Gambierdiscus*, *Fukuyoa, Ostreopsis,* or *Coolia* in the PH100 microalgal database. However, genera such as *Amphidinium* and *Prorocentrum* which contain both pelagic and epibenthic taxa were detected. *Amphidinium spp*. occurred in 17 of 267 (6%) samples and *Prorocentrum* spp. occurred in 78 of 267 (30%) samples (Supplementary Figure 4.1). Epibenthic *Prorocentrum* taxa (e.g*. Prorocentrum lima*), occurred in 3 of 267 samples (1%) (Supplementary Figure 4.1).

4.4.2 Presence of epibenthic dinoflagellates on ocean rafts

Fifteen raft samples were collected from the oceanographic region influenced by the East Australian Current (EAC) and associated ocean water masses off the continental shelf of New South Wales, Australia (i.e. >200 m isobath). Thirteen of the fifteen rafts were collected in an eddy and the remaining two were collected in the EAC (Table 4.1). Twelve samples were macrophytes including seagrasses *(Posidonia* spp. and *Zostera* spp.*)* and macroalgae (*Hormosira* spp. and *Sargassum* spp.). There was one sample of pumice, a terrestrial plant fragment (small tree branch), and a sheet of plastic.

The composition and abundance of microalgal communities associated with rafts of seagrass, macroalgae, and the plastic sheet were very similar, but distinct from the communities present on the pumice and small tree branch (ANOSIM, $p < 0.01$; Figure 4.2). The top six taxa driving these differences included the diatoms *Grammatophora marina, Licmophora* cf. *abbreviata, Navicula* spp.*, Plagiotropis* sp.*, Leptocylindrus* sp.*,* and the dinoflagellate *Amphidinium* spp..

Raft size and complexity was linearly positively correlated with both the abundance (r^2=0.27) and diversity (r^2=0.38) of associated microalgal taxa, with larger and more complex rafts (of any type) harbouring more abundant and diverse microalgal taxa (Figure 4.3 a and b).

Table 4.1. Type of rafts collected in the East Australian Current (EAC) and associated oceanographic features (e.g. eddies), with the environmental conditions and location of sampling sites. Note that some samples contained more than one type of raft and were identified to genus level when possible.

Rafts	Collection Location	Temperature (°C)	Salinity	Latitude (°S)	Longitude (°E)
Seagrass 1	Eddy	21.1	35.5	-32.39563	153.07483
Seagrass 2	Eddy	21.1	35.5	-32.39563	153.07483
Seagrass 3	Eddy	20.7	35.5	-32.39920	153.12806
Seagrass 4	Eddy	20.7	35.5	-32.39920	153.12806
Seagrass 5	Eddy	20.7	35.5	-32.39920	153.12806
Seagrass 6	Eddy	20.8	35.5	-32.35212	153.05226
Seagrass 7	Eddy	20.4	35.6	-32.44710	153.19540
Seagrass 8	Eddy	20.2	35.8	-32.46802	153.23935
Macroalgae/ seagrass 1	Eddy	20.4	35.6	-32.44710	153.19540
Macroalgae/ seagrass 2	Eddy	20.2	35.7	-32.46802	153.23935
Macroalgae /seagrass 3	Eddy	20.4	35.6	-32.44752	153.22234
Sargassum **sp.**	EAC	21.3	35.7	-32.47680	153.42200
Small tree branch	EAC	21.6	35.7	-32.41252	153.37844
Pumice	Eddy	20.7	35.6	-32.43980	153.18450
Plastic	Eddy	20.4	35.6	-32.44710	153.19540
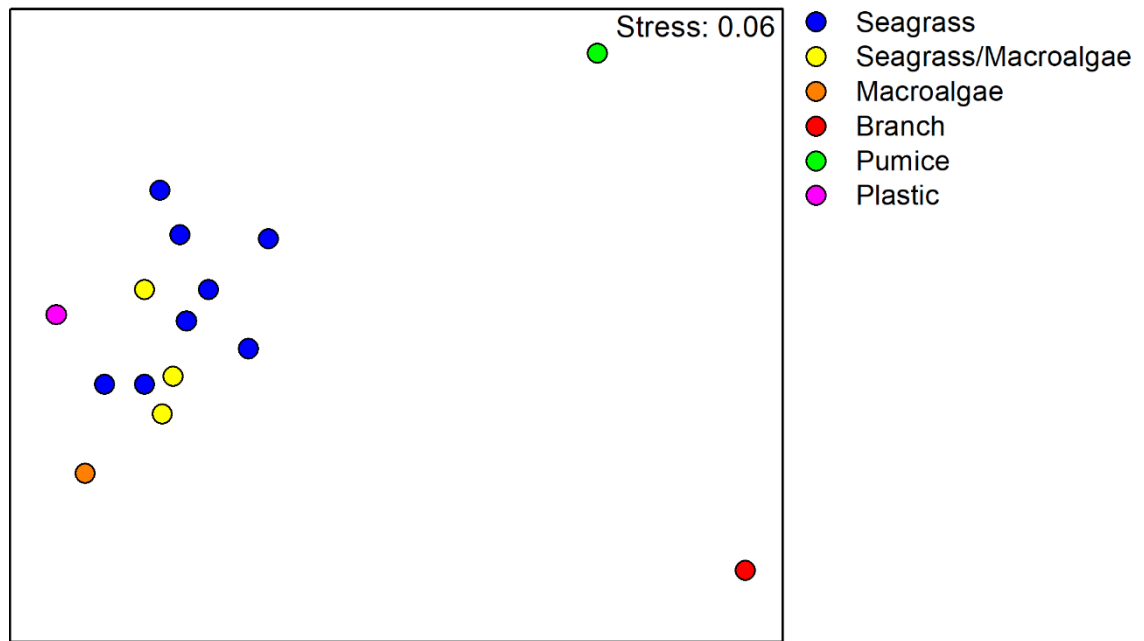
Figure 4.2. Non-metric Multi-Dimensional Scaling (nMDS) plot showing differences in the microalgal community composition associated with different rafts based on raft type.

Overall, the microalgal community associated with rafts was dominated by diatoms (Figure 4.3 a), with many taxa classified as epibenthic (e.g. *Bacillaria paxillifera, Cocconeis* sp*., Cylindrotheca closterium*, *Grammatophora marina*, *Licmophora* cf. *abbreviata, Navicula* spp. and *Striatella* spp.) (Table 4.2 and Figure 4.4 a). Some pelagic taxa were also present, possibly due to entrainment in the complex three dimensioanl structures of some rafts. Potentially harmful epibenthic dinoflagellate taxa were the next most abundant group. *Prorocentrum* cf. *clipeus, Prorocentrum lima, Prorocentrum rhathymum,* and *Coolia* spp. were found to be associated with 5 of 15 rafts (33 %) and *Amphidinium* spp. with all rafts. As clonal isolates of algae were only established from one raft, the overall proportion of rafts from which we have confirmed a toxic epibenthic dinoflagellate is 6%. 'Other' taxa (cyanobacteria) were also present in some samples (Table 4.2 and Figure 4.4), contributing least to microalgae abundance (Figure 4.4 b).

The viability of microalgal cells was confirmed by the establishment of six clonal isolates from raft samples. Cultures of *Amphidinium* cf. *carterae, Amphidinium* cf. *operculatum, Prorocentrum lima,* and *Coolia* sp. (Figure 4.5) were established into culture after being isolated from a macroalgal raft of *Sargassum* sp. (Figure 4.6).

The identity of the *Coolia* isolate was confirmed using the LSU D1-D3 region, a widely used marker region of the rDNA capable of distinguishing *Coolia* species (Jeong et al. 2012; Karafas, York & Tomas 2015; Leaw et al. 2010). Phylogenetic analysis of the LSU D1-D3 rDNA revealed that the raft strain grouped with other strains of *Coolia palmyrensis* from tropical locations around the world (Figure 4.7), confirming its identity and likely low latitude origin.
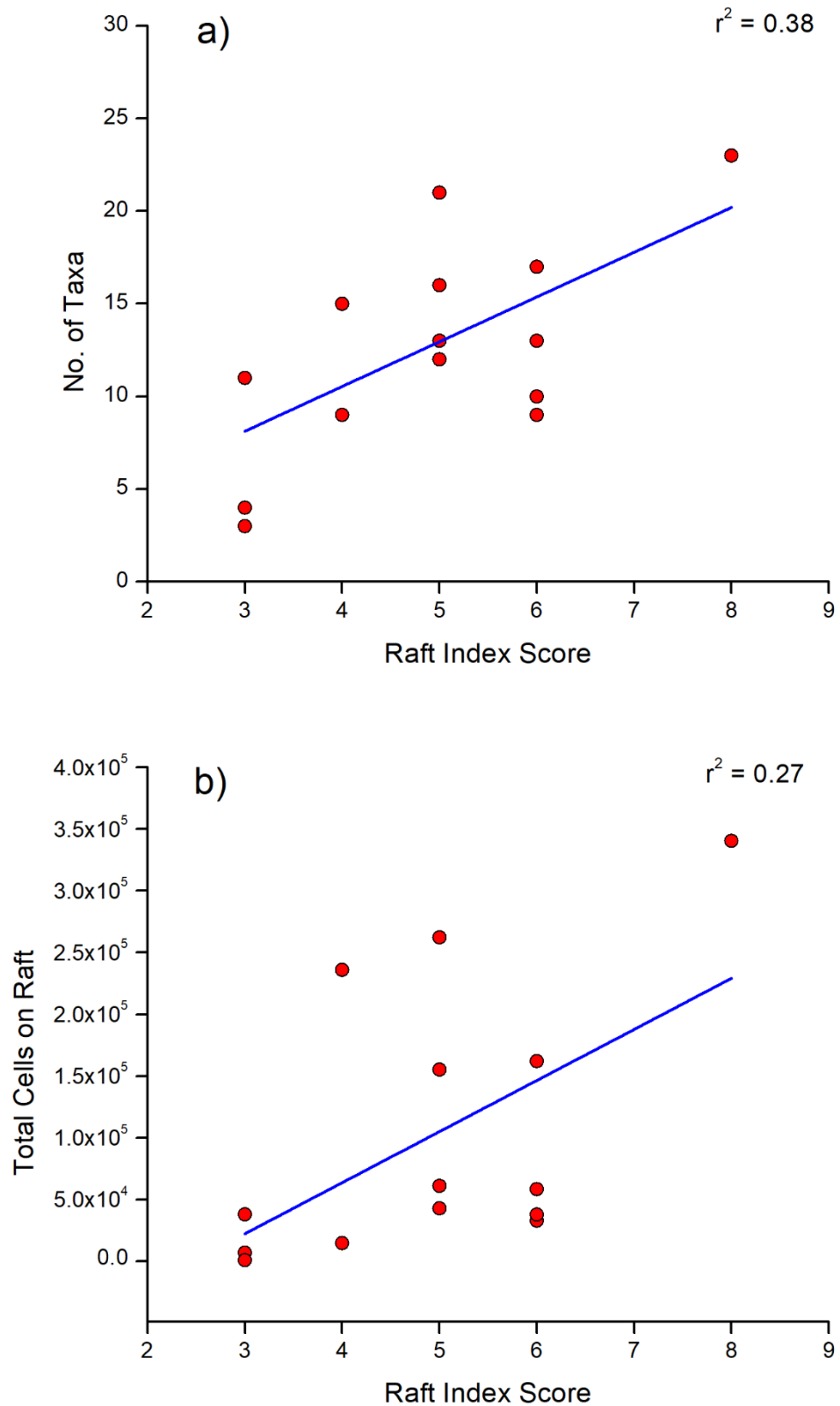
Figure 4.3. Relationship between (a) the richness of microalgal taxa and (b) microalgal abundance associated with rafts according to the raft size and complexity (raft index score).
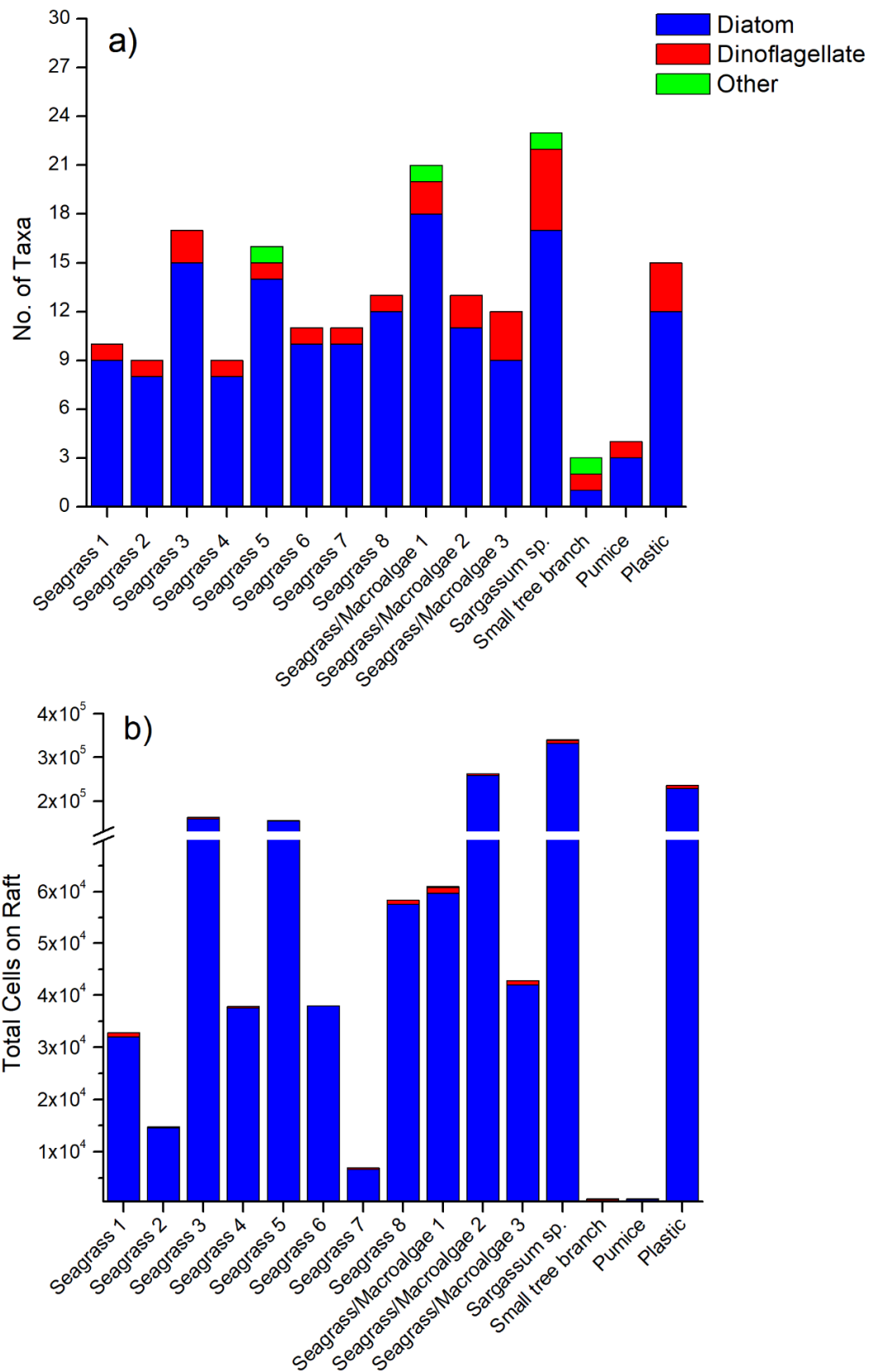
Figure 4.4. Richness (a) and abundance (b) of the microalgal communities associated with rafts drifting in the East Australian Current (EAC) and associated water masses.

Table 4.2. Microalgal taxa associated with rafts collected in the East Australian Current (EAC) (Table 4.1) and associated water masses. The taxa classified as epibenthic are indicated with + symbol, planktonic with a – symbol, and NA denotes when information was not available or applicable.

Class	Genus/Species	Epibenthic
Bacillariophyceae	*Achnanthes longipes* C.Agardh, nom. illeg.	NA
	Amphora spp. Ehrenberg ex Kützing, 1844	+
	Bacillaria paxillifera (O.F.Müller) T.Marsson	+
	Climacosphenia moniligera Ehrenberg	NA
	Cocconeis sp. Ehrenberg, 1836	+
	Cylindrotheca closterium (Ehrenberg) Reimann & J.C.Lewin	+
	Grammatophora marina (Lyngbye) Kützing	+
	Haslea sp. Simonsen, 1974	+
	Lauderia annulata Cleve	-
	Leptocylindrus spp. Cleve, 1889	-
	Licmophora cf. *abbreviata* C.Agardh	+
	Licmophora spp. C.Agardh, 1827, nom. et typ. cons.	+
	Melosira sp. C.Agardh, 1824, nom. cons.	+
	Melosira varians C.Agardh	+
	Navicula spp. Bory, 1822	+
	Nitzschia longissima (Brébisson) Ralfs	+
	Plagiotropis sp. Pfitzer, 1871	NA
	Pleurosigma W.Smith, 1852, nom. et typ. cons.	+
	Striatella sp. C.Agardh, 1832	+
	Striatella cf. *unipunctata* (Lyngbye) C.Agardh	+
	Synedra sp. Ehrenberg, 1830	NA
	Unidentified pennate	NA
Dinophyceae	*Amphidinium* cf. *operculatum*	+
	Amphidinium spp. É.Claperède & J.Lachmann, 1859	+
	Heterocapsa sp. F.Stein, 1883	-
	Prorocentrum cf. *clipeus* Hoppenrath	+
	Prorocentrum lima (Ehrenberg) F.Stein	+
	Prorocentrum rhathymum A.R.Loeblich III, Sherley & Schmidt	+
	Coolia palmyrensis Karafas, Tomas & York	+
Cyanophyceae	Unidentified Oscillatoriales	NA

Figure 4.5. Images of clonal epibenthic dinoflagellates cultures established from a macrophyte raft samples of *Sargassum* sp. collected drifting in the East Australian Current (EAC), a) *Coolia palmyrensis* b) *Prorocentrum lima*, c) *Amphidinium* cf. *operculatum* and d) *Amphidinium* cf. *carterae*. Scale bar = 10 µm.

Figure 4.6. *Sargassum* sp. raft from which the *Coolia* sp., *Amphidinium* spp., and *Prorocentrum* sp. strains were isolated for cell viability assessment. Image (a) shows raft prior to processing and image (b) shows a close-up image of the raft.

Figure 4.7. Maximum likelihood tree of the *Coolia* culture (UTSR7) isolated from *Sargassum* sp. raft sample collected drifting along the East Australian Current (EAC), showing alignment of the partial LSU rDNA sequences (D1-D3 region). Values at the nodes represent Maximum Likelihood bootstrap and Bayesian posterior probability support.

4.5   Discussion

Planktonic microorganisms are not typically considered to be dispersion limited in the ocean, due to their small size, vast population sizes, and pelagic lifestyle, all of which facilitate their transport in ocean currents. Epibenthic dinoflagellates, however, are closely associated with the benthos or substrates such as macrophytes, and therefore, planktonic dispersal of free-living (unattached) cells is less likely. This study used two lines of evidence to show that rafting is a natural mechanism for long distance transport of epibenthic dinoflagellates: (1) the relatively high prevalance of epibenthic taxa associated with drifting macrophytes collected on an oceanographic voyage; and (2) their virtual absence in the water column at a coastal site over 50 years.

Earlier investigations demonstrated the association of epibenthic dinoflagellates with biotic or abiotic rafts in shallow and sheltered waters (Bomber et al. 1988, Masó et al. 2003). However it was not known if these organisms could remain associated with and be transported by rafts in open ocean environments.This study found intact cells of six taxa of epibenthic dinoflagellates from three genera (*Coolia, Prorocentrum* and *Amphidinium*) on macrophyte rafts drifting in a major western boundary current, and confirmed their viability by establishing laboratory cultures of species from all three genera. These findings therefore demonstrate that viable cells of epibenthic dinoflagellates can remain associated with macrophyte rafts as they are transported offshore, confirming that rafting represents a natural means by which these organisms could extend their distribution, particularly from tropical to more temperate locations when entrained in ocean boundary currents.

New cultures of epibenthic dinoflagellate species from the genera *Amphidinium*, *Prorocentrum* and *Coolia* were established from a *Sargassum* sp. raft sample collected in

this study. *Prorocentrum* is a very diverse genus that includes both planktonic and epibenthic species (Hoppenrath et al. 2013) and is widely distributed throughout Australia (Heil et al. 2004; Morton, Norris & Bomber 1992; Murray, Nagahama & Fukuyo 2007) and the world (Hoppenrath et al. 2013). Similarly, *Amphidinium* is broadly distributed in Australia (Murray et al. 2004; Murray & Patterson 2002) and globally (Berland, Grzebyk & Thomassin 1992; Lee et al. 2003). This genus is considered primarily epibenthic but is frequently reported in the plankton, likely due to misidentification or taxonomic uncertainty (Jørgensen, Murray & Daugbjerg 2004; Murray et al. 2004). In comparison, species from the genus *Coolia* have only been found associated with epibenthic environments and although the genus has a seemingly global distribution, some species have a more restricted range (Leaw et al. 2016). There is only one study describing the taxonomic identity of *Coolia* from Australia (Momigliano et al. 2013), thus the culture of *Coolia* isolated from the *Sargassum* sp. raft sample in this study was examined in more detail.

This investigation provided the first reports of *Coolia palmyrensis*, in temperate Australia. *Coolia palmyrensis* is a newly described, mildly toxic species (Karafas, York & Tomas 2015) and has so far only been reported to have a tropical distribution, recorded at Palmyra Atoll in the North Pacific Ocean and the Dominican Republic in the Caribbean Sea (source locations for description) (Karafas, York & Tomas 2015). However, sequences available in Genbank (www.ncbi.nlm.nih.gov) suggest that the distribution might extend to Hong Kong, Fiji, and Spain. *C. palmyrensis* has also been found in a sample from the Central Great Barrier Reef region in Australia (originally described as *Coolia* sp. in Momigliano et al. (2013) but later classified as *C. palmyrensis* (Gómez et al. 2016; Karafas, York & Tomas 2015; Leaw et al. 2016). While there has not been a comprehensive survey of the distribution of *C. palmyrensis* in Australia, this species has

never been described from temperate locations (Leaw et al. 2016), suggesting the origin of the *Sargassum* sp. raft sample collected from the EAC from which the *C. palmyrensis* strain was isolated was probably tropical.

Other raft samples in this study were collected in an oceanic eddy located at approximately 32 °S. At latitude 30 – 32 °S, the EAC generally separates from the coast to form the eastward flowing Tasman Front, with the remaining southwards flow forming a series of mesoscale eddies (Mata et al. 2006). These eddies can entrain coastal water and organisms and transport rafts of marine macrophytes and debris. The implication is that the raft samples collected in the eddy may have been transported directly offshore from temperate latitudes rather than upstream tropical locations. Prevailing wind direction and strength can also influence the dispersal of floating material (Ruiz-Montoya, Lowe & Kendrick 2015) and should be considered when assessing the potential source of raft material.

The size and complexity of raft samples collected in this study were positively correlated with both the abundance and diversity of microalgal taxa, as commonly found in a wide range of studies considering many different taxa (Thiel & Gutow 2005a, 2005b). Furthermore, rafts of macroalgae and seagrass from the genera *Sargassum* spp*.,* *Posidonia* spp*.,* and *Zostera* spp. were the most common and the general abundance and widespread distribution of these macrophytes in Australia (Christianson et al. 1981; Kirkman 1997) suggests that these taxa may have the highest capacity to act as rafts in this region. Furthermore, if storm intensity and frequency increases (Barnard et al. 2015), the rate of detachment of coastal macrophytes may rise (Witman 1987, Seymour et al. 1989), thereby increasing the supply of rafts and potential for transportation of associated organisms (Macreadie et al. 2011).

A study by Masó et al. (2003) was the first to assess whether rafts of abiotic origin, specifically marine plastic debris, could harbour harmful microalgae taxa. They found the epibenthic dinoflagellates *Ostreopsis* spp. and *Coolia* spp. associated with plastic debris collected along the Catalan coast in the western Mediterranean. The microalgal community associated with the plastic raft sample had a very similar composition to that of seagrass and macroalgae, indicating the likely coastal origin of the plastic. This reveals the capacity of anthropogenic marine debris to act in a manner similar to that of macrophyte rafts, a concern given that abiotic materials such as plastic have low decomposition rates, can stay afloat for extended periods and are becoming increasingly more abundant in our oceans (Derraik 2002; Eriksen et al. 2014; Haward 2018; Lebreton et al. 2018). The ability if millimetre sized marine plastics to act as habitat for microorganisms has also recently been investigated (Reisser et al. 2016). Although small, these materials were found to have microalgal cells associated with their surface and may therefore also act as rafts and could also aid dispersal of harmful epibenthic dinoflagellates. Pumice was another type of raft collected during this study. Many small clasts of pumice can be generated in a single volcanic event and can travel vast distances in ocean currents, making this material an effective rafting agent (Bryan et al. 2012) that should also be considered in the future.

This study contrasted the abundance of potentially harmful epibenthic dinoflagellates on rafts with those in the plankton at a time-series location. Epibenthic dinoflagellates were virtually absent from a long-term coastal phytoplankton monitoring site located ~5 km from the coast. Although it seems unlikely that an epibenthic species would be advected in the plankton, it has been the most commonly suggested mechanism for dispersal of these organisms to date (Faust & Tester 2004), particularly along the east coast of Australia (Heimann, Capper & Sparrow 2011; Murray et al. 2014; Sparrow et al. 2017).

This study showed that the epibenthic dinoflagellate *Prorocentrum lima* was documented in only 3 of 267 samples over a fifty-year period (1978, 1979 and 2008). This occurrence rate is very low but shows that some unassociated epibenthic dinoflagellate cells can be found in surface waters. Therefore, we cannot rule out that planktonic transport *via* drifting of free-living cells may occur for these taxa, but it seems a far less likely compared to rafting.

4.6   Conclusion

In this study, it was shown for the first time that epibenthic dinoflagellates remain viable when transported into the open ocean while associated with rafts of both biotic and abiotic origin. Rafting is therefore an effective dispersal mechanism for harmful epibenthic dinoflagellates and could facilitate geographic range extensions. However, whether rafted epibenthic dinoflagellate cells can be delivered to shallow waters and successfully colonise new coastal habitats remains an open question and should be a fruitful area of future research.

4.7   Supplementary Materials
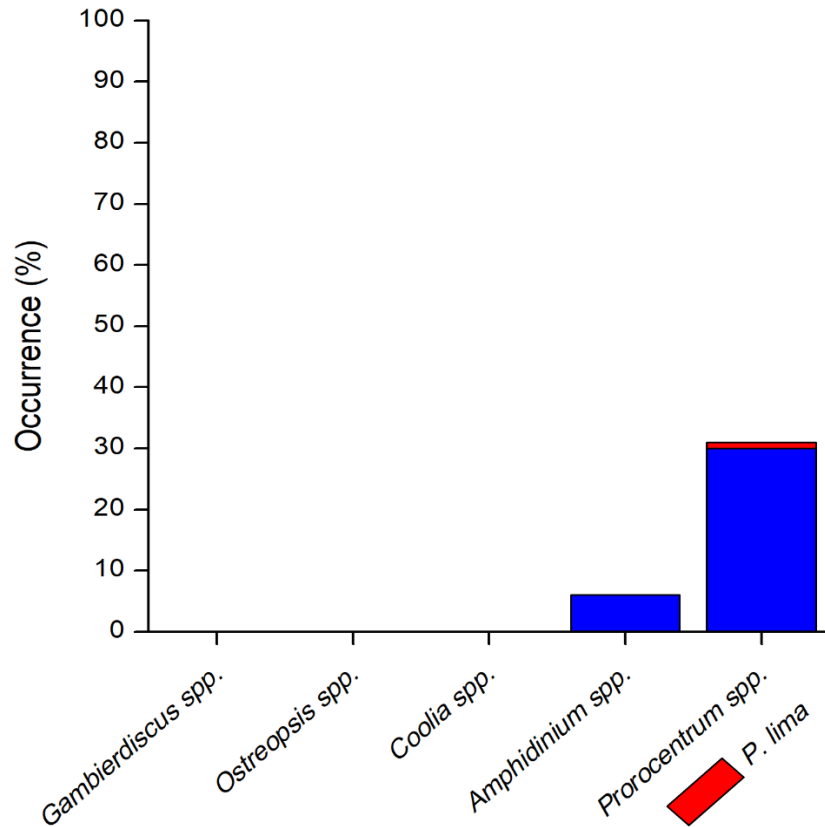
Supplementary Figure 4.1. Percent occurrence of epibenthic dinoflagellates from the genera *Gambierdiscus, Ostreopsis, Coolia, Amphidinium* and *Prorocentrum* as determined from five datasets spanning 50 years from a coastal monitoring station (PH100) influenced by the East Australian Current (EAC) in south east Australia (1965 to 2015). Red colour shows occurrence of *Prorocentrum lima* as differentiated from unconfirmed species of *Prorocentrum* (*Prorocentrum* spp.) in blue.
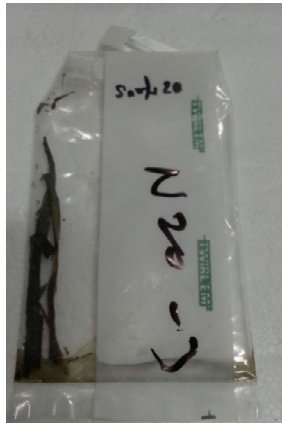
Size score = 2
Complexity score = 1
Total raft index score = 3
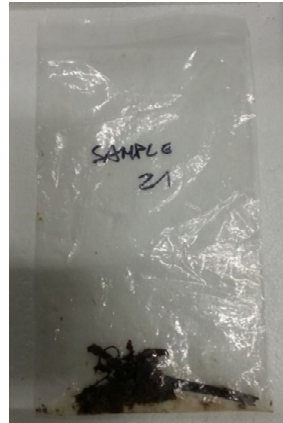
Size score = 2
Complexity score = 3
Total raft index score = 5

Size score = 3
Complexity score = 2
Total raft index score = 5

Size score = 3
Complexity score = 2
Total raft index score = 5

Size score = 3
Complexity score = 2
Total raft index score = 6

Size score = 4
Complexity score = 4
Total raft index score = 8
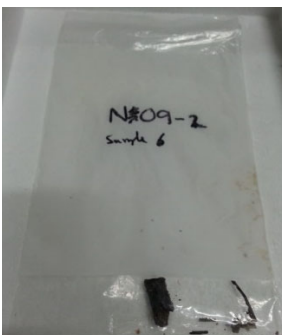
Tree branch

Plastic sheet

Pumice

Supplementary Figure 4.2. Images of raft samples included in this study as examples.

Chapter 5 Capacity for *Gambierdiscus* (Dinophyceae) to successfully colonise novel temperate locations

5 Capacity for *Gambierdiscus* (Dinophyceae) to successfully colonise novel temperate locations

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Author contributions:

M.E.L carried out the field collections and experimental work, data analysis, designed the study and wrote the manuscript; M.A.D participated in the design of the study and helped draft the manuscript. Both authors revised the manuscript.

5.1 Abstract

Gambierdiscus is a genus of epibenthic dinoflagellate, of which some species are associated with the human illness Ciguatera Fish Poisoning (CFP). Species from this genus are typically found in tropical environments though in recent years, populations have been documented in more temperate locations, stimulating concern the genus may be extending its range. Here we evaluate for the first time, the capacity of *Gambierdiscus* to successfully colonise novel temperate locations. Two strains of *Gambierdiscus* established from tropical eastern Australia representing two species (*G. carpenteri* and *G. lapillus*), were compared to a strain of *G. carpenteri* established from a population in temperate eastern Australia. Dose response experiments whereby natural and artificial epiphytic microalgae assemblages were supplemented with *Gambierdiscus* cells showed that very few propagules are required for successful colonisation (survival and subsequent cell division) under optimal environmental conditions for *Gambierdiscus* growth. There was also no difference in the colonisation abilities of the tropical strains of *Gambierdiscus* when compared with the temperate strain, indicating that emigrant populations of the genus have similar capacity to colonise temperate locations as a local population. This study provides important insight into the risk of temperate range extension of organisms that have the potential to cause human harm.

5.2 Introduction

Common epibenthic dinoflagellate genera include *Gambierdiscus* Adachi & Fukuyo, *Fukuyoa* Gómez, Qui, Lopes & Lin, *Ostreopsis* Schmidt, *Amphidinium* Claparède & Lachmann*, Prorocentrum* Ehrenberg and *Coolia* Meunier. The dinoflagellate genera, *Gambierdiscus* and *Fukuyoa*, are of particular interest because some species produce

ciguatoxins (CTXs) and related compounds (Adachi & Fukuyo 1979), which have been implicated in the human illness Ciguatera Fish Poisoning (CFP) (Yasumoto et al. 1979).

Gambierdiscus has a tropical to sub-tropical distribution (Litaker et al. 2010), although more recently, temperate populations of *Gambierdiscus* have been documented in the North Atlantic Ocean (Aligizaki, Nikolaidis & Fraga 2008), North Pacific Ocean (Nishimura et al. 2013) and South Pacific Ocean (Kohli, Murray et al. 2014). It has been suggested that these temperate populations represent a range extension of the genus in response to, or facilitated by, global climate change (Aligizaki, Nikolaidis & Fraga 2008; Heimann, Capper & Sparrow 2011). Certainly, such range shifts have been shown for many other marine organisms including sea urchins (Agatsuma & Hoshikawa 2007; Ling et al. 2009), anemones (Sagarin et al. 1999), intertidal invertebrates (Pitt, Poloczanska & Hobday 2010), fish (Booth et al. 2007; Figueira & Booth 2010; Last et al. 2011) and zooplankton (Johnson et al. 2011). While these range extensions are being documented increasingly, our understanding of the mechanisms involved is limited.

Smayda (2002) conceptualised the process of dinoflagellate expansion into new areas and described three stages that must be successfully completed for an emigrant microalgal species to successfully extend its range. Stage one involves compatibility of the new habitat, meaning the environmental conditions must meet the requirements and lie within the physiological tolerance range of the emigrant species. Stage two refers to persistence of the emigrant species in the new location and is achieved when there is an increase in the abundance of descendants of the original founder population. Successful population growth can be influenced by the number of propagules originally introduced (a concept referred to in the invasion literature as 'propagule pressure' (Carlton 2003)). Stage three refers to entry into the already established microalgal community at the new location and is often achieved when a colonising species is more competitive than at least some of the

resident species, or an unoccupied niche is available. This conceptual framework from arrival, to growth and co-existence in a new location is used in this study to assess the mechanisms involved in temperate range extension of *Gambierdiscus*. Specifically, the ability of *Gambierdiscus* to persist in natural epibenthic microalgal communities from temperate eastern Australia is examined, and the competitive ability of *Gambierdiscus* species in relation to other epibenthic dinoflagellates is evaluated. To our knowledge, this is the first study to empirically measure the colonisation capacity of a marine microalga and assess this aspect of species range extensions.

5.3   Methods

5.3.1   Natural epiphytic community colonisation experiment

Three temperate coastal sites in eastern Australia were chosen for this experiment: Lake Macquarie, (33.0539 °S, 151.6433 °E), Brisbane Water, (33.4641 °S, 151.2510 °E) and Narrabeen Lagoon (33.7231 °S, 151.2952 °E) (Figure 5.1). The sites were selected as they did not have a documented resident population of *Gambierdiscus* but were locations which could feasibly be colonised by *Gambierdiscus* in the future. Sites were sampled on the 13th February 2016, during the austral summer.

Physicochemical parameters such as water temperature (°C), pH, conductivity (mS/cm) and dissolved oxygen (DO) (mg/mL) were measured at each site using a multi-parameter probe (WTW Multi 3430; Xylem Analytics, Germany) and turbidity (ntu) was measured using a turbidity meter (Analite NEP160; McVan Instruments Pty Ltd, Victoria, Australia).
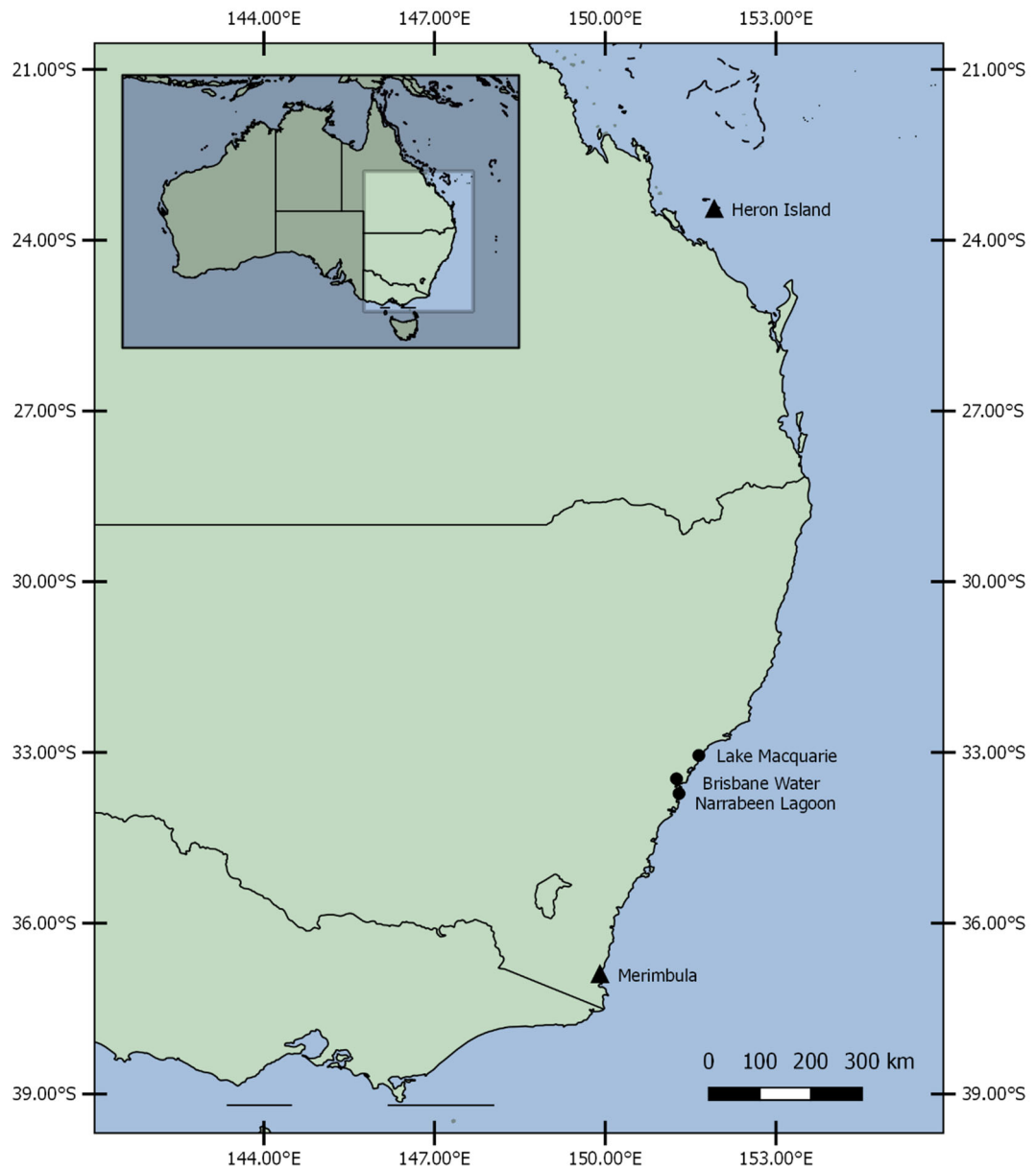
Figure 5.1. Map showing the isolation location of the epibenthic dinoflagellate strains used in this study, Heron Island, Queensland and Merimbula New South Wales (triangles) and the sampling for sites for the natural community experiments, Lake Macquarie, Brisbane Water and Narrabeen Lagoon, New South Wales, Australia (solid circles).

A 30 x 30 cm patch of seagrass (*Zostera* sp.) (~ 500 g seagrass wet weight) was removed from each site and placed in a large ziplock bag. Ambient water was added to the bag and the epiphytic community was detached from the surface of the seagrass by vigorously shaking and kneading. The suspended material was then poured through a 165 µm mesh into a 20 L container to exclude large zooplankton and debris. Ambient water continued to be added until 20 L of water was collected, and all epiphytes had been removed from the surface of the seagrass. This epiphytic material was the natural community used for these experiments.

A 50 mL subsample was removed and preserved with Lugols iodine solution (~1% final concentration) for identification and enumeration of the original epiphytic community. Samples were concentrated by sedimentation for 48 h and microalgal taxa identified to the lowest possible taxonomic level using a Sedgewick Rafter counting chamber under an inverted light microscope (maximum magnification x 1000) fitted with phase contrast (Eclipse TS100, Nikon, Japan). When identification to species level was not possible, cells were assigned to genus level only. Abundance was estimated by counting to 100 cells as per Hötzel & Croome (1999) and each sample was scanned for 1 h to enumerate rare taxa. A 200 mL aliquot of the natural epiphytic community was added to each 75 cm^2 Tissue Culture Flasks (Falcon, Corning, New York, United States) and randomly allocated to treatments that were supplemented with *Gambierdiscus* cells in a gradient of cell concentration (0, 0.1, 1, 2, 5, 10 cells mL^{-1}; n = 3). Macronutrients and trace metals were added to each flask at a concentration of 20 % modified K medium (Litaker et al. 2009) with added silicate at a concentration of f/10 medium (Guillard 1975) at the beginning and every six days of the experiment. Three cultures of *Gambierdiscus* were used for this experiment, a tropical strain of *G. carpenteri* (UTSHI2C4), a temperate strain of *G. carpenteri* (UTSMER9A3) and a strain of *G. lapillus* of tropical origin

(UTSHI2B6). These were selected on the basis that the temperate *G. carpenteri* may represent a population which has already successfully colonised a temperate location and may therefore represent a population with a high colonisation ability. The inclusion of the same species from a tropical location provided a species-specific comparison. A third culture, *Gambierdiscus lapillus* was included as this strain has been found to produce compounds with CTX-like activity (Chapter 2) and has a comparatively fast growth rate relative to the other strains that could influence its colonisation ability (Chapter 3). Flasks were incubated at temperatures corresponding to the collection site (Table 5.1) in a Climatron Plant Growth Cabinet (Thermoline, New South Wales, Australia) under ~100 μmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle.

Table 5.1. Location and water quality information for sites used in natural epiphytic community colonisation experiment.

Site Name	Lake Macquarie	Brisbane Water	Narrabeen Lagoon
Latitude	33.0539 °S	33.4641 °S	33.7231 °S
Longitude	151.6433 °E	151.2510 °E	151.2952 °E
Water Temperature (°C)	26.5	27.6	31.1
pH	8.2	8.5	8.1
Salinity	30.8	29.2	20.1
Dissolved Oxygen (mg/mL)	7.4	10.0	7.8
Turbidity (ntu)	6.7	27.2	300

To test the fitness of *Gambierdiscus* in the absence of the epibenthic community under the same environmental conditions, control flasks containing only *Gambierdiscus* cells in water collected from each site were also included in the experiment. To prepare these

control (absence of biotic interactions) treatments, particulate material was removed from the epiphyte mixture by filtering through glass fibre filter (0.7 μm pore size; Whatman GF/F; GE Healthcare; Kent, United Kingdom) and 200 mL was added to triplicate 75 cm^2 Tissue Culture Flasks (Falcon, Corning, New York, United States). *Gambierdiscus* cells were added to yield a final concentration of 10 cells mL^{-1}. The rate of growth of control cultures was calculated using the slope of natural logarithm transformed cell abundance in the linear portion of the growth curve and a one-way Analysis of Variance (ANOVA) was used to test the difference in *Gambierdiscus* growth between control and community treatments (SPSS v24 IBM Corporation, Armonk, New York, USA).

Gambierdiscus cell abundance in all treatments was estimated every 2-3 days for 18 days. This was done by removing a 1 mL aliquot from each replicate and preserving the sample in Lugols iodine solution (~1% final concentration). Each sample was then loaded into a 24 well glass bottom plate (In Vitro Technologies, United States) and left to settle for 1 h. The base of each well was then imaged using bright field mode and a 20 x objective using a high throughput bioimager (IN Cell Analyzer 2200; GE Healthcare, Kent, United Kingdom). The number of *Gambierdiscus* cells in each image was counted manually and the number of cells per mL calculated.

5.3.2   Artificial community colonisation experiment

To test the competitive ability of *Gambierdiscus* in terms of other co-occurring epibenthic dinoflagellate genera under optimum growth conditions, an artificial community colonisation experiment was performed. Cultures of *Ostreopsis* sp*., Coolia* sp*., Prorocentrum* sp. and *Amphidinium* sp. were established from epibenthic microalgal samples collected from the surface of seagrass collected at Merimbula Inlet, Australia (36.8979 °S, 149.9044 °E) (Figure 5.1). Epibenthic dinoflagellates were initially

identified to genus level using morphological characteristics under an inverted light microscope (Eclipse TS100; Nikon, Japan). Single cells were isolated using the micropipette technique (Andersen & Kawachi 2005) and monoclonal cultures were established in modified K medium (Litaker et al. 2009) made from sterile aged natural seawater at a salinity of 32. The flasks were incubated at 24°C, under ~100 µmol photons $m^{-2} s^{-1}$ on a 12:12 light:dark cycle. Isolates were maintained under the same conditions in 25 cm^2 (50 mL) sterile vented polystyrene tissue culture flasks (Falcon, Corning, New York, United States), oriented horizontally.

The mixed artificial epibenthic dinoflagellate community was prepared by combining established laboratory cultures of *Ostreopsis* sp*., Coolia* sp*., Amphidinium* sp. and *Prorocentrum* sp*.* (Table 5.1), each at a concentration of 10 cells mL^{-1}. The community mixture was made in sterile aged natural seawater supplemented with nutrients every six days at 20% the concentration of modified K medium (Litaker et al. 2009) and flasks were incubated in 25 cm^2 Tissue Culture Flasks (Falcon, Corning, New York, United States). Cells of *Gambierdiscus* were added in a concentration gradient (0, 1, 2, 5, 10, 20 cells mL^{-1}; n = 3) as described above for the natural epiphyte community colonisation experiment.

To examine the effect of condition on the capacity of the founding population to persist in a new habitat, there was an additional treatment involving addition of stressed cells to a parallel set of flasks. As the health of introduced *Gambierdiscus* cells could be compromised during transportation in a dispersal scenario (e.g., via ocean currents or ships' ballast water), this was intended to be more representative of the colonisation process.

The pre-treatment involved growing the cultures for 30 days with no added nutrients. Chlorophyll *a* fluorescence was measured using a Water Pulse-Amplitude-Modulated fluorometer (WATER-PAM, Walz, Germany) and the maximum quantum yield of PSII (F_v/F_m) was used to provide an index of the overall health of the cultures (Schreiber, Bilger & Neubauer 1995). A 2 mL sample of each *Gambierdiscus* culture was measured prior to addition to confirm that healthy and stressed inocula had a significant divergence in F_v/F_m ($p < 0.05$) (Supplementary Figure 5.1).

Controls consisted of each species grown individually (tropical and temperate *G. carpenteri* and *G. lapillus* (both healthy and stressed)*, Ostreopsis* sp.*, Coolia* sp.*, Amphidinium* sp. and *Prorocentrum* sp.) at a concentration of 10 cells mL^{-1}. Flasks were incubated at optimum growth conditions (24 °C, 32 salinity, 100 µmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle) as previously determined (Chapter 3). *Gambierdiscus* abundance was estimated every three days for 30 days using the same method described in the previous experiment, and growth was calculated using the slope of natural logarithm transformed cell abundance data from the linear portion of the growth curve. Differences in growth rates between taxa were tested using One-way Analysis of Variance (ANOVA) in SPSS v24 (IBM Corporation, Armonk, New York, USA).

5.3.3   Dissolved nutrient analysis

Dissolved nutrient concentrations were monitored throughout each experiment to confirm limitation did not occur. Samples were collected prior to nutrient addition at T0 and each subsequent addition (T6, T12, T18 etc) by removing 1 mL from each replicate and placing in a 1.5 mL tube (Eppendorf, Germany). Tubes were centrifuged at 3000 g for 5 minutes to pellet the particulate material and 250 µL of the supernatant was transferred to a 96

multi-well plate (Falcon, Corning, New York, United States) which was stored at - 20°C until analysis.

Nitrate analysis was completed using the colorimetric assay as per Schnetger & Lehners (2014), using a 96 multi-well plate reader (Infinite M100 PRO, TECAN, Hombrechtikon, Switzerland). This was the most practical nutrient to measure, as organic phosphate was used as the phosphate source and cannot be detected using the standard soluble reactive phosphorus method. Samples were diluted using Ultrapure MilliQ water when concentrations were above the detectable range.

5.4   Results

5.4.1   Natural community colonisation experiment

The physicochemical conditions at each of the three sites for the natural community colonisation experiment differed. Lake Macquarie had a water temperature of 26.5 °C, compared with 27.6 °C at Brisbane Water and 31.1 °C at Narrabeen Lagoon (Table 5.1). Salinity also differed among sites, with Lake Macquarie and Brisbane Water having salinity levels closest to seawater at (~ 30) while salinity at Narrabeen Lagoon was quite low (20; Table 5.1). Dissolved oxygen levels were consistent across sites (7.4, 7.8 and 10.0 mg/mL), whereas turbidity varied enormously (Table 5.1). Turbidity levels were 6.7, 27.2 and 300 ntu at Lake Macquarie, Brisbane Water and Narrabeen Lagoon, respectively (Table 5.1).

The epibenthic microalgal communities at each of the three sites varied considerably. Lake Macquarie had the lowest total microalgal cell abundance at ~280 cells mL^{-1}, followed by Brisbane Water (~430 cells mL^{-1}), and Narrabeen Lagoon (~1300 cells mL^{-}

^{1}). The microalgal community was dominated by diatoms at all sites, followed by dinoflagellates, then all other cell types (e.g. cyanobacteria). Species richness was lowest at Narrabeen Lagoon, where seven of nine taxa were diatoms, in addition to one dinoflagellate and one cyanobacterium. The Lake Macquarie epibenthic community comprised twelve taxa in total: seven diatoms, four dinoflagellates and one cyanobacterium. Brisbane Water had the highest species richness with twenty taxa, including 14 diatoms and six dinoflagellates (Supplementary Table 5.1).

Cells within the control treatments (*Gambierdiscus* only) were able to grow and persist at all sites; albeit at different rates (Figure 5.2 a, b, c and Figure 5.3). All three species of *Gambierdiscus* were able to persist for the duration of the experiment when added to the natural Lake Macquarie and Brisbane Water communities, irrespective of the initial concentration of cells added. In comparison, no *Gambierdiscus* species at any cell concentration survived after 18 days when added to the Narrabeen Lagoon community (Figure 5.2). The final yield of *Gambierdiscus* cells after 18 days of incubation was highest when added to the natural Lake Macquarie epibenthic microalgal community.

Growth of all three *Gambierdiscus* taxa was higher in the filtered controls compared to within the epiphytic community. Both the tropical and temperate strain of *G. carpenteri* grew slower in the filtered water from Narrabeen Lagoon versus Lake Macquarie and Brisbane Water (Figure 5.3). *Gambierdiscus lapillus* also grew well at Lake Macquarie (0.15 day^{-1}) and Brisbane Water (0.14 day^{-1}), however growth was negative at Narrabeen Lagoon (Figure 5.3).

Nitrate did not limit growth in any treatment at any stage of the experiment (Supplementary Figure 5.2). Indeed, some accumulation of nitrate was evident, which was consistent across species, sites and treatments.

Figure 5.2. Mean (±SD) abundance of *Gambierdiscus* cells within the natural epiphytic community experiment at Lake Macquarie (a, d, g, j), Brisbane Water (b, e, h, k) and Narrabeen Lagoon (c, f, i, l) for the tropical strain of *G. carpenteri* (d to f), *G. lapillus* (g to i) and the temperate strain of *G. carpenteri* (j to l). Each line in a-c represents the abundance of *Gambierdiscus* cells in the controls with no epibenthic community for each species at each site and in d to l represents the abundance of *Gambierdiscus* cells for each concentration originally added.
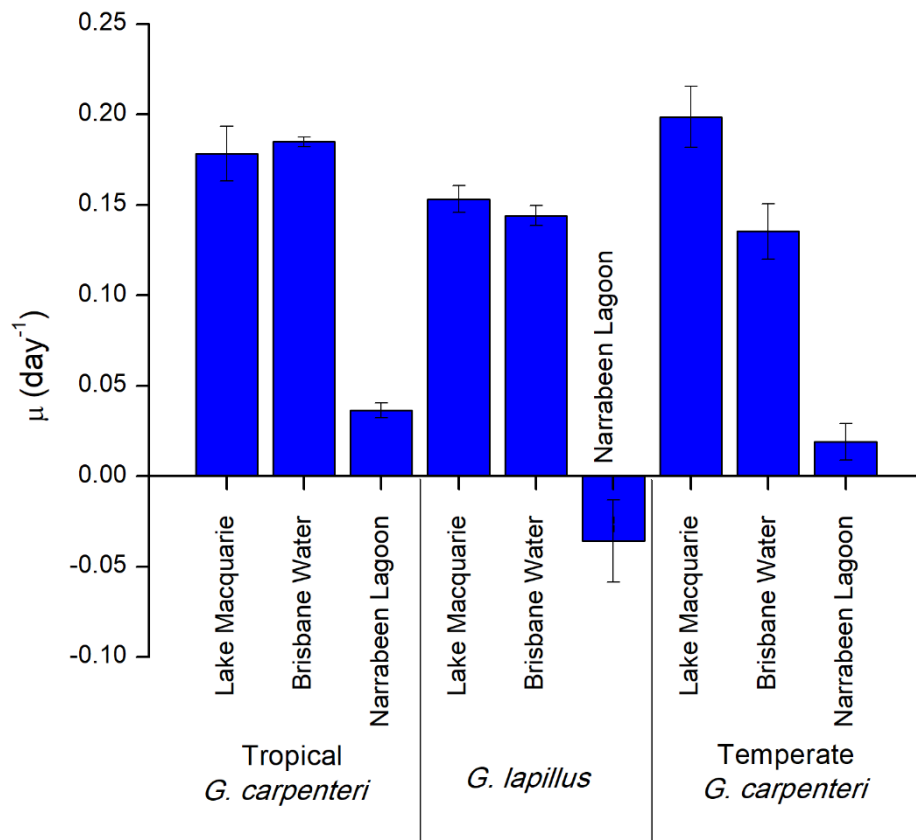
Figure 5.3. Mean (±SD) growth rate of individual *Gambierdiscus* species controls at each site.

5.4.2   Artificial community colonisation experiment

The abundance of healthy *Gambierdiscus* cells increased in all treatments, regardless of the initial concentration added. In contrast, stressed cells only persisted, and in some cases grew, but at very slow rates (Figure 5.4).

The growth rate of each species differed significantly ($P < 0.05$). *Amphidinium* sp. had the highest growth rate at 0.77 day^{-1}, followed by *Coolia* sp. (0.47 day^{-1}), *Ostreopsis* sp. (0.26 day^{-1}) and *Prorocentrum* sp. (0.15 day^{-1}) (Figure 5.5). Tropical *G. carpenteri* cells with high F_v/F_m (i.e. healthy inoculum) grew the fastest at 0.25 day^{-1}, while healthy cells of *G. lapillus* and the temperate strain of *G. carpenteri* both grew at ~0.16 day^{-1} (Figure 5.5). The stressed cells of each *Gambierdiscus* species grew at approximately half the rate of the healthy cells of the same species (Figure 5.5). Interestingly, *Ostreopsis* sp. and *Prorocentrum* sp. grew at a similar rate to that of the *Gambierdiscus* species (Figure 5.5). As with the natural community experiment, dissolved nutrient concentrations were not limiting (Supplementary Figure 5.3).
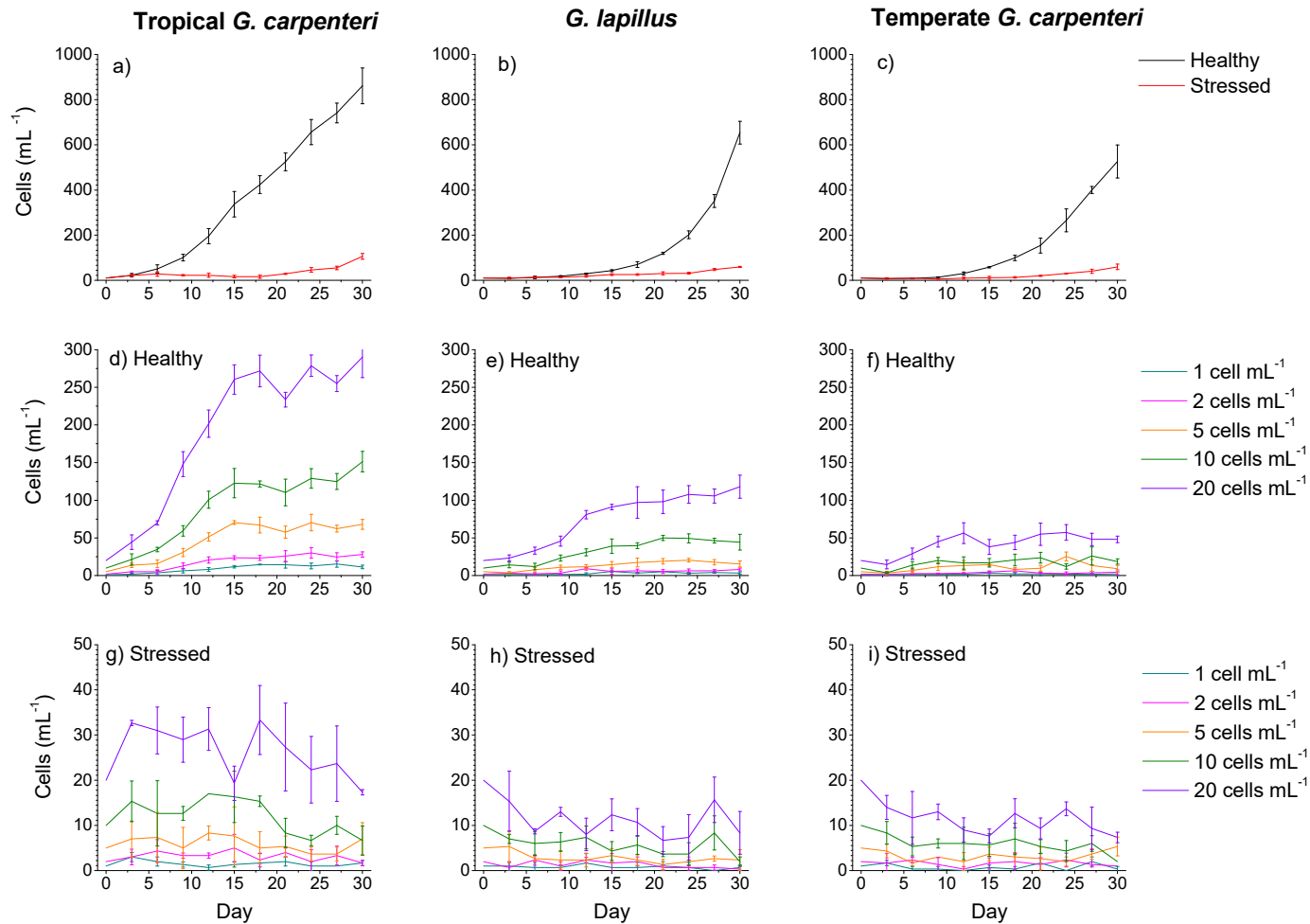
Figure 5.4. Mean (±SD) abundance of *Gambierdiscus* cells within the artificial community colonisation experiment through time for the tropical strain of *G. carpenteri* (a, d, g), *G. lapillus* (b, e, h) and the temperate strain of *G. carpenteri* (c, f, i). The lines in a-c represent the abundance of healthy and stressed cells for each species of *Gambierdiscus*.
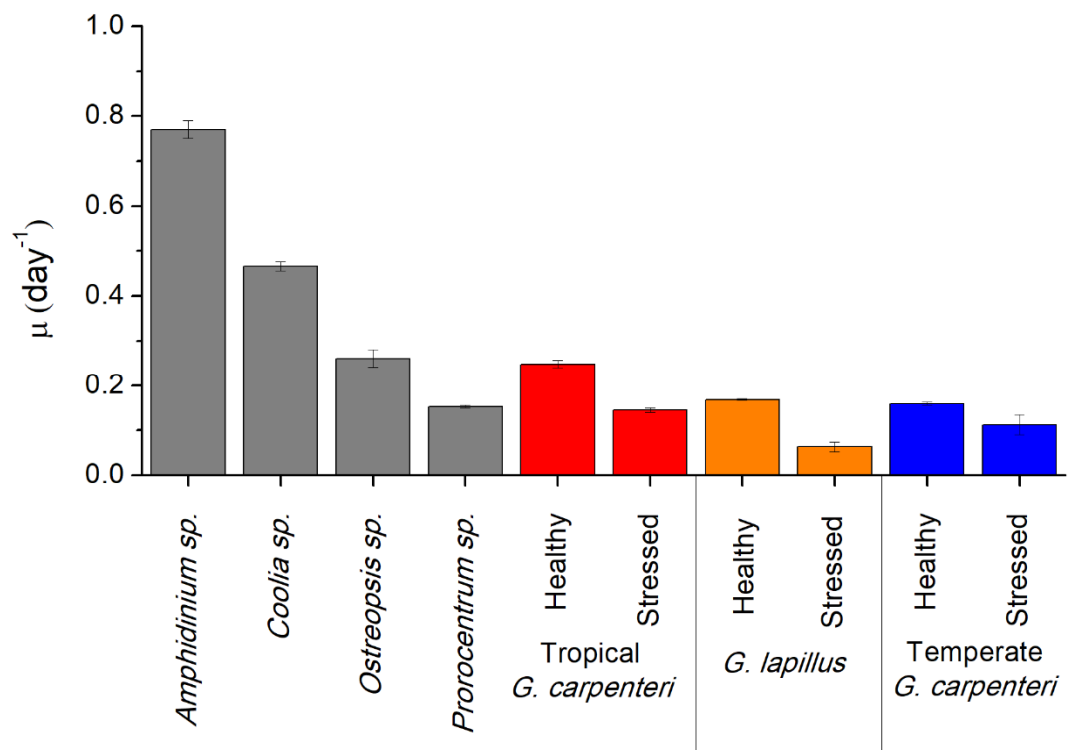
Figure 5.5. Mean (±SD) growth rate for control treatment for each individual genus.

5.5   Discussion

Two approaches were used to evaluate the capacity for successful colonisation of *Gambierdiscus* into epibenthic habitats in this study. The first involved adding *Gambierdiscus* cells in a gradient of abundance to natural epibenthic microalgal communities collected from temperate locations. Provided abiotic conditions were suitable, all *Gambierdiscus* strains were able to persist and grow at all cell abundances tested. The second approach involved assessing the competitive ability of *Gambierdiscus* strains in relation to other epibenthic dinoflagellates under optimal environmental conditions and showed that despite having the slowest growth rates, *Gambierdiscus* cells were able to compete effectively with other epibenthic dinoflagellate genera. The results from this study demonstrate that the presence of *Gambierdiscus* in communities is more strongly influenced by abiotic conditions than competition for resources amongst the resident epibenthic microalgal community. This is an important finding when evaluating the possible range extension of the genus into more temperate locations.

The locations chosen for the natural epiphytic community colonisation experiment represent a gradient of environmental conditions and resident epibenthic microalgal community taxon diversity. Lake Macquarie had a temperature (27 °C) and salinity (30) within the optimum range for growth of the *Gambierdiscus* strains (Chapter 3), but a high diversity and abundance of resident epiphytic microalgal taxa to compete with. Brisbane Water had less favourable environmental conditions (temperature of 28 °C and salinity of 29), although conditions remained within the tolerable range for *Gambierdiscus* species (Chapter 3) and had a slightly lower diversity and abundance of the resident epiphytic microalgal community compared to Lake Macquarie. Narrabeen Lagoon had temperature (30 °C) and salinity (20) conditions that were on the edge of the tolerable

range for the *Gambierdiscus* strains (Chapter 3), but had relatively low cell abundance and diversity of resident epiphytic microalgae. These differences are summarised below in a conceptual diagram (Figure 5.6).
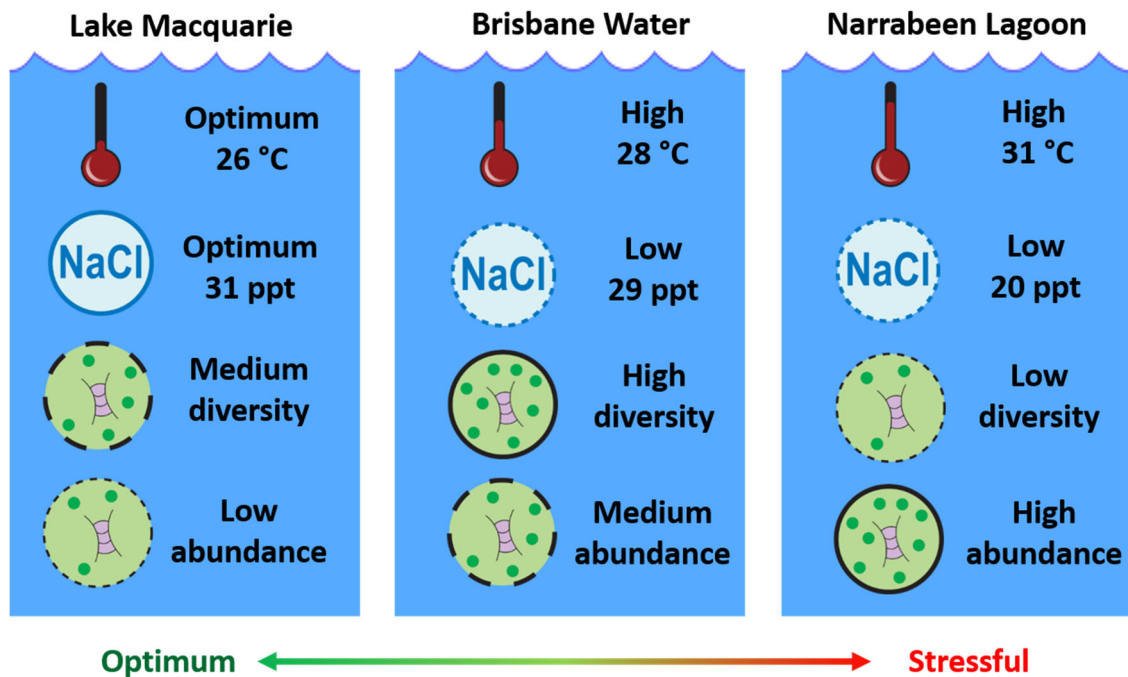
Figure 5.6. Summary of abiotic and biotic conditions within natural epibenthic communities used in experiments.

The basic principles of colonisation outlined in Smayda (2002) highlight the importance of compatible habitat for a colonising emigrant dinoflagellate species and suggest locations with lower resident species diversity would be easier to colonise than locations with higher diversity because there would be fewer taxa to compete with. This was not the case in this study. The presence of *Gambierdiscus* in communities was more related to favourable environmental conditions than the abundance and diversity of other microalgal epiphytes. *Gambierdiscus* cells added to the Narrabeen Lagoon community

(which had moderate diversity) were not able to persist because of sub-optimal environmental conditions, suggesting that the environment is the primary filter for colonisation. This finding highlights the importance of determining compatible habitat for *Gambierdiscus* when assessing the potential for range extension of the genus.

Epibenthic dinoflagellates exist in a surprisingly consistent consortium globally. There are often representatives from the genera *Amphidinium, Coolia, Ostreopsis* and *Prorocentrum* in benthic samples (Besada, Loeblich & Loeblich III 1982; Faust 1995; Vila, Garcés & Masó 2001). These genera are very widespread and are the most likely competitors of *Gambierdiscus* species. The size of the cells in each genus ranges enormously with *Amphidinium carterae* for example having relatively small cell diameter (~15 μm), while *Prorocentrum lima* is much larger (50 μm). The size ranking of the consortium is therefore *Amphidinium < Coolia < Ostreopsis < Prorocentrum*, with *Gambierdiscus* cells the largest (between 50 and 100 μm) (Hoppenrath et al. 2014).

Cell size is strongly correlated with growth rate (Banse 1976), which can ultimately affect competitiveness. One would therefore predict *Amphidinium carterae* to have a much faster growth rate than *Gambierdiscus* and could potentially outcompete *Gambierdiscus* in co-culture. Consequently, to further examine the capacity for temperate range extension of *Gambierdiscus*, we tested the ability of three *Gambierdiscus* species to grow in the presence of other members of the epibenthic dinoflagellate consortium in an artificial community colonisation experiment. The results of our study show that growth rates were generally in accordance with the cell size-growth rate relationship. All *Gambierdiscus* species were able to survive for the length of the experiment at all initial cell concentrations suggesting *Gambierdiscus* can compete with other epibenthic dinoflagellate genera despite having the slowest growth rates. We note however, that

these taxa may not be competing if there is resource partitioning, as has been elegantly demonstrated for co-ocurring diatoms (Alexander et al. 2016).

Eastern Australia was a relevant case study to evaluate the range extension of tropical *Gambierdiscus* as there is already a resident population of *G. carpenteri* in a temperate location at Merimbula in New South Wales (Kohli, Murray et al. 2014; Chapter 2). It has been suggested that this population represents a temperate range extension of the genus from the central population in northeastern Australia (Heimann, Capper & Sparrow 2011; Murray et al. 2014; Sparrow et al. 2017). Whether the finding of G. *carpenteri* in that location indicates a recent range extension remains to be elucidated, however the very presence of this population outside the known distribution of the genus suggests that colonisation occurred at some stage in the past. This scenario therefore provides a reference for evaluating the colonisation success of tropical *Gambierdiscus* strains in temperate locations. The current study included strains of *Gambierdiscus* established from both tropical and temperate eastern Australia and therefore allowed comparison of colonisation success of the contrasting populations. Interestingly, there were no differences between the tropical and temperate strains of *G. carpenteri* and *G. lapillus* in their ability to persist in natural epiphytic communities from temperate eastern Australia and compete with co-occurring epibenthic microalgae. Both *G. carpenteri* and *G. lapillus* have a widespread distribution in the Great Barrier Reef Region, however the temperate population of *Gambierdiscus* at Merimbula consists only of *G. carpenteri*. While our experiments do not cover the full scope of processes (e.g., predation, disease etc) that would occur when *Gambierdiscus* is introduced to novel environments, they showed no difference in the persistence of taxa within epiphytic communities (Figure 5.2 d, g, j), suggesting *G. lapillus* has as much potential to colonise temperate Australia as *G. carpenteri*.

5.6   Conclusions

In this study, in the context of temperate range extension, the ability of three strains of *Gambierdiscus* to colonise novel locations was evaluated. The ability of the strains to persist and grow in natural epibenthic microalgal communities and the competitive ability of *Gambierdiscus* in relation to other epibenthic dinoflagellate taxa was assessed. Even though species of *Gambierdiscus* have some of the slowest growth rates amongst the epibenthic microalgal taxa examined in this study, cells were able to survive at concentrations tested in both experiments. The results of the experiments demonstrated that, at least under laboratory conditions, the local epibenthic community and an artificial dinoflagellate community did not outcompete *Gambierdiscus* populations. Unfortunately, there are environmental and moral contraints that limit our ability to validate the outcomes of our laboratory experiments in natural settings, but they suggest that it may only take a single pulse of very few *Gambierdiscus* cells for this genus to colonise new habitats and potentially extend its range into more temperate locations. Some *Gambierdiscus* species have been recently found to produce compounds with CTX-like activities (*G. lapillus*, *G.* cf. *pacificus* and *G.* cf. *silvae*) (Chapter 2). Understanding the capacity for temperate range extension of these species will be crucial for evaluating the risk of CFP in these regions and should be the focus of future research.

5.7 Supplementary Materials

Supplementary Table 5.1. Microalgal taxa identified from resident epibenthic communities of the three sites sampled for the natural community colonisation experiments (Lake Macquarie, Brisbane Water and Narrabeen Lagoon). + indicates the taxa was present and – indicates the taxa were absent.

Class	Genus/Species	Lake Macquarie	Brisbane Water	Narrabeen Lagoon
Bacillariophyceae	*Amphora* spp. Ehrenberg ex Kützing, 1844	+	+	+
	Cocconeis sp. Ehrenberg, 1836	+	+	+
	Grammatophora marina (Lyngbye) Kützing	+	+	+
	Leptocylindrus spp. Cleve, 1889	+	+	+
	Licmophora spp. C.Agardh, 1827, nom. et typ. cons.	+	+	-
	Melosira sp. C.Agardh, 1824, nom. cons.	-	+	-
	Navicula spp. Bory, 1822	+	+	+
	Nitzschia longissimi (Brébisson) Ralfs	+	+	+
	Pleurosigma sp. W.Smith, 1852, nom. et typ. cons.	+	+	+
	Striatella sp. C.Agardh, 1832	-	+	-

Dinophyceae	*Amphidinium* spp. É.Claperède & J.Lachmann, 1859	+	+	+
	Coolia sp. A.Meunier, 1919			
	Fukuyoa sp. F.Gómez, D.X.Qiu, R.M.Lopes & Senjie Lin, 2015	-	+	+
	Prorocentrum lima (Ehrenberg) F.Stein	-	+	+
	Prorocentrum rhathymum A.R.Loeblich III, Sherley & Schmidt	-	+	+
	Unidentified Dinoflagellate	-	+	-
Cyanophyceae	Unidentified Oscillatoriales	+	-	+

Supplementary Figure 5.1. Mean (±SD) F_v/F_m (indicator of health) measurements for each *Gambierdiscus* species included in the artificial community colonisation experiment.

Supplementary Figure 5.2. Mean (±SD) concentrations of dissolved nitrate (mol L^{-1}) in the growth medium through time for the Lake Macquarie (a, d, g, j), Brisbane Water (b, e, h, k) and Narrabeen Lagoon (c, f, I, l) treatments and for the tropical strain of *G. carpenteri* (d to f), *G. lapillus* (g to i) and the temperate strain of *G. carpenteri* (j to l) for the natural community colonisation experiment. Each line in a to c represents the concentration of nitrate dissolved in the growth medium in the natural epiphytic community control (no added *Gambierdiscus* cells) and in d to l, represents the concentration of nitrate dissolved in the growth medium for each treatment when *Gambierdiscus* cells were added.

Supplementary Figure 5.3. Mean (±SD) concentrations of dissolved nitrate (moles L^{-1}) in the growth medium through time for the tropical strain of *G. carpenteri* (a, d, g), *G. lapillus* (b, e, h) and the temperate strain of *G. carpenteri* (c, f, l) during the artificial community colonisation experiment. Each line in plots a to c represents the concentration of nitrate dissolved in the growth medium in the *Gambierdiscus* cell only controls and in d to l represents the concentration of nitrate dissolved in the growth medium for each treatment when *Gambierdiscus* cells were added.

Chapter 6 General Discussion

6   General Discussion

6.1   Overview

This thesis has significantly advanced our understanding of the toxicology and ecology of *Gambierdiscus* strains isolated from tropical and temperate Australia (summarised in Figure 6.1). It has shown that there is a large diversity of *Gambierdiscus* species in eastern Australia and identified some that are likely contributors to cases of CFP in the region (Chapter 2). This work also evaluated the potential for temperate range extension of epibenthic dinoflagellates, including CFP causing genera, showing that the environmental niche of strains is not likely restricting the distribution of *Gambierdiscus* in eastern Australia (Chapter 3). Furthermore, this study identified rafting as the most likely natural long-distance dispersal mechanism for epibenthic dinoflagellates (Chapter 4) and that relatively slow growth of colonising *Gambierdiscus* cells did not preclude it from persisting amongst natural temperate epiphytic communities (Chapter 5). Overall, results from this thesis provide fundamental information for developing a management strategy to mitigate the risk of human exposure to CFP, in eastern Australia. Below, the key findings and implications of this work are discussed.
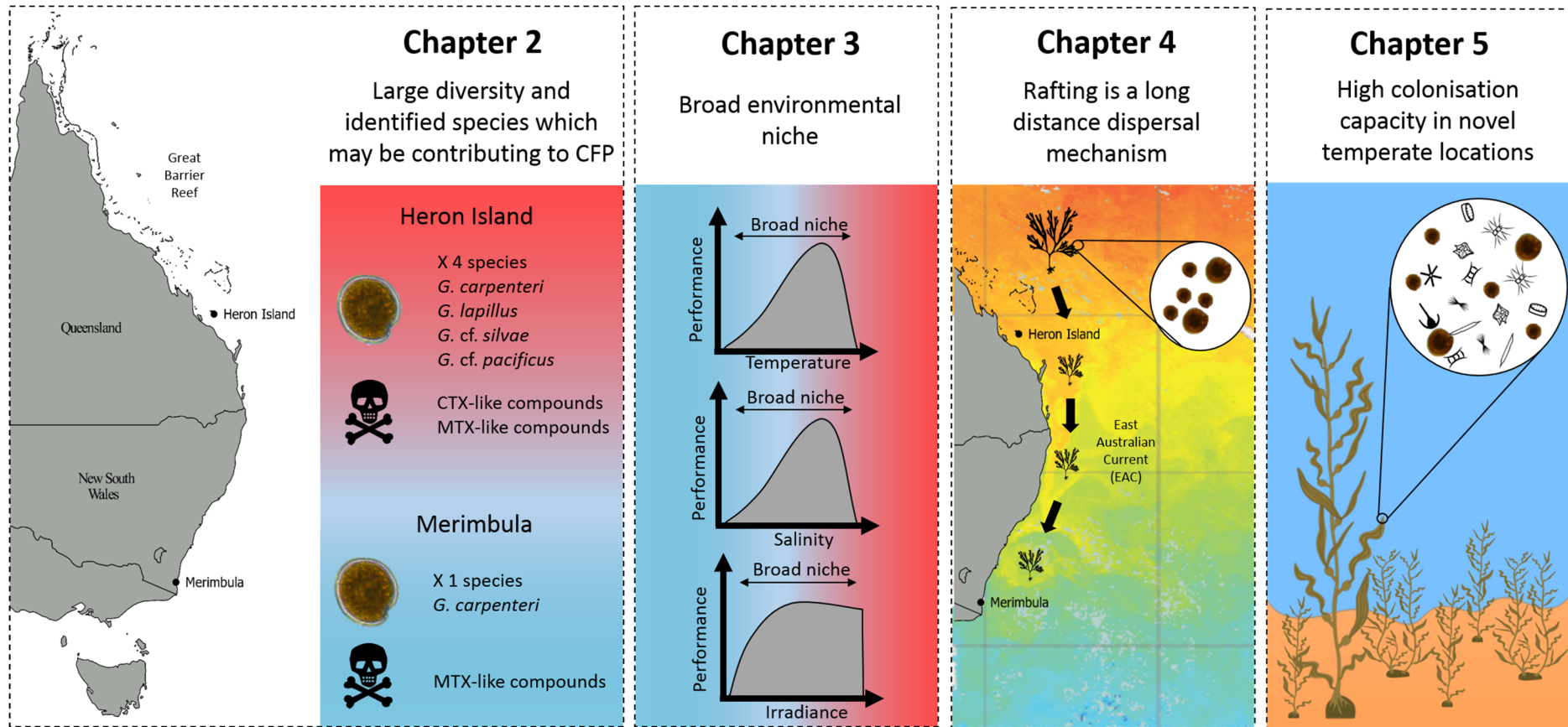
Figure 6.1. Conceptual diagram summarising the design and major findings of this thesis.

6.2 Diversity of *Gambierdiscus* and *Fukuyoa* in eastern Australia

Cases of CFP have been reported in Australia as far back as the 18th century (Lewis 2006). Since then, there have been large outbreaks of the syndrome and two human fatalities (Gillespie et al. 1985; Hamilton et al. 2010). CFP in Australia is primarily caused by fish caught in the tropical waters of eastern Australia (Clark & Whitwell 1968; Gillespie et al. 1985; Tonge et al. 1967), although cases are beginning to be reported from fish caught in more sub-tropical locations (Farrell et al. 2016).

Despite the widespread occurrence of CFP in Australia, very little is known of the causative organisms in this region. Holmes, Lewis and Gillespie were the first to isolate strains of *Gambierdiscus* from Australia (Holmes & Lewis 1994; Holmes, Lewis & Gillespie 1990; Holmes et al. 1991). These studies remained the only published investigations into CFP causing organisms from this region for the next 20 years, and thus comprise the foundations of our current understanding. Unfortunately, the identity of the species from these older studies remain unconfirmed as they preceded the taxonomic reclassification of the genus (Litaker et al. 2009).

Murray et al. (2014) provided the first glimpse of the diversity of *Gambierdiscus* and *Fukuyoa* species from tropical eastern Australia. Sampling at multiple sites in the central Great Barrier Reef region, three species were identified, *F. paulensis* (originally reported as *G.* cf. *yasumotoi* but later reclassified by Gómez et al. (2015) as *F. paulensis*), *G. carpenteri* and a species similar to *G. belizeanus* (*G.* cf. *belizeanus*). Following this, a unique temperate population of *G. carpenteri* was described from Merimbula and Wapengo, New South Wales (Kohli, Murray et al. 2014), further contributing to our understanding of the distribution of potential CFP taxa along the east coast of Australia. Then very recently, two new species of *Gambierdiscus* were formally described from Heron Island in the southern Great Barrier Reef region, *G. lapillus*

(Kretzschmar et al. 2017) and *G. honu* (Rhodes et al. 2017) and another study confirmed the presence of *G. carpenteri* in the Great Barrier Reef region (Sparrow et al. 2017).

The findings of this thesis further contribute to this growing understanding of the diversity of CFP causing genera in Australia (Chapter 2). In a single sampling event at Heron Island, four species of *Gambierdiscus* were established into culture, two of which may represent new taxa (*G.* cf. *silvae* and *G.* cf. *pacificus*). These results indicate that there are likely many species of *Gambierdiscus* in Australia and the true extent of the diversity within this region is yet to be elucidated.

Currently, accurate identification of *Gambierdiscus* species is challenging, relying on establishing laboratory cultures, a process that is inherently difficult for this genus and seems to target the dominant and resilient species – i.e., those that survive the cell isolation and culture process. This has been a major limitation to CFP research, and resolving diversity in the field without the need for cultivating significant biomass for scanning electron micrograph and phylogenetic analyses will be the key to advancing understanding of their ecology. Rapid screening assays which can identify cells directly from environmental samples utilising techniques such as quantitative Polymerase Chain Reaction (qPCR), Fluorescent In-situ Hydridisation (FISH) or metabarcoding may be the way forward, alongside continued growth of sequence databases. These techniques rely on a baseline understanding of the diversity of species and often need to be developed for each region individually. Nonetheless, rapid detection techniques such as these will enable investigations into the relative abundance of different species in natural communities relative to abiotic and biotic conditions; a vital next step to improve our ability to predict the spatio-temporal distribution of potential CFP causing organisms.

6.3   Determining the species contributing to CFP in eastern Australia

Understanding the diversity of *Gambierdiscus* and *Fukuyoa* provides valuable insight into the organisms potentially contributing to CFP in a region, but investigations into the toxicology of the species are necessary to accurately attribute the source of CFP toxins. There is currently no unified approach for the detection of toxins produced by *Gambierdiscus* and *Fukuyoa* species, largely because of the many structural forms and congeners that exist. This is the second major limitation to progress in the field of CFP research. The early study by Holmes et al. (1991), identified compounds with CTX-like activity (originally referred to as Gambiertoxins (GTXs)) in the lipophilic phase of a strain of *Gambierdiscus* (reported as *G. toxicus*, though species identification remains unconfirmed) from tropical eastern Australia. Thus, this study provided the first evidence of CFP causing organisms in Australia. Holmes & Lewis (1994) and Holmes, Lewis & Gillespie (1990), also determined significant toxicity to mice in the hydrophilic phase of the partitioned crude microalgal extract of the same *Gambierdiscus* strain, and went on to describe three chemical structures, MTX-1, MTX-2 and MTX-3. This work provided the foundation for our understanding of the toxicology of *Gambierdiscus* from the Australian region but only a limited number of strains with unconfirmed identities were investigated.

At approximately the same time, the structure of microalgal derived CTXs was elucidated from a strain of *Gambierdiscus* (RG1-1) (reported as *G. toxicus* but species identity unconfirmed) isolated from the Gambier Islands in French Polynesia (Murata et al. 1990; Murata et al. 1989; Satake, MacKenzie & Yasumoto 1997; Satake, Murata & Yasumoto 1993). Chinain et al. (2010) completed the first comprehensive characterisation of CTX compounds from two highly toxic strains of *G. polynesiensis* (TB-92 and RG-92) from French Polynesia. The toxins characterised in these studies (P-CTX-3B, 3C, 4A and 4B) remain the primary toxins linked with CFP today (Yogi et al. 2014). These compounds can now be detected using Liquid

Chromatography-Mass Spectrometry (LC-MS), one of the approaches used to investigate the toxicology of Australian *Gambierdiscus* isolates in this thesis (Chapter 2). None of the characterised toxins were detected in any of the newly established strains of *Gambierdiscus* isolated from tropical or temperate Australia, supporting the findings of Kretzschmar et al. (2017) and Kohli, Murray et al. (2014), who tested a tropical *G. lapillus* strain and a temperate *G. carpenteri* population (respectively) for toxicity. However, this LC-MS/MS approach is limited to detecting CTXs with known chemical structures, thus a mammalian cell-based assay approach which utilises the known mode of activity of the toxins, was used to further characterise the toxicology of established Australian isolates of *Gambierdiscus* in this thesis (Chapter 2).

Compounds displaying CTX-like activity were identified in strains of *G.* cf. *pacificus, G.* cf. *silvae* and *G. lapillus,* as indicated by the accumulation of Ca^{2+} in SH-SY5Y cells detected using a Fluorescent Imaging Plate Reader (FLIPR) (Lewis et al. 2016). Applied to Australian isolates of *Gambierdiscus* for the first time (Chapter 2), the FLIPR assay results have significantly contributed to our understanding of the taxa which may be causing cases of CFP in the Australian region. The cell-based FLIPR approach to toxicological investigations of Australian *Gambierdiscus* isolates has also shown that the active compounds likely differ from the currently characterized CTXs of microalgal origin for which standards are available (P-CTX-3C, 3B, 4A, 4B).

These results highlight two things, firstly, that multiple methods are required to fully characterise the toxicology of species from the genera *Gambierdiscus* and *Fukuyoa* and that a single technique provides an incomplete description of the contribution of a certain species to CFP. Further research needs to not only assess the toxicity of established strains of *Gambierdiscus* and *Fukuyoa* from a region but to also characterise the structure of the compounds involved. Linking the taxonomic identity of *Gambierdiscus* and *Fukuyoa* species

with their toxicology and understanding their relative abundance in natural communities will also need to be coupled with understanding how toxin production is influenced by environmental factors. Chapter 3 showed how environmental variables influence growth of *Gambierdiscus* strains but toxin production was not quantified. It is likely that certain environmental conditions stimulate or regulate toxin production but there are currently no studies addressing this for CFP causing organisms. Clearly, understanding toxin production in the field will be essential for managing CFP in the future.

6.4   Potential for range extension of *Gambierdiscus* in eastern Australia

Gambierdiscus and *Fukuyoa* are known to be distributed throughout tropical and sub-tropical latitudes (Litaker et al. 2010). However, populations are increasingly being reported from more temperate locations (Aligizaki, Nikolaidis & Fraga 2008; Kohli, Murray et al. 2014; Nishimura et al. 2013), suggesting the genus is extending its range. In Chapter 3 of this thesis, the environmental niche of tropical and temperate *Gambierdiscus* strains was compared to characterise the physiological limits for growth and better understand potential constraints on species distributions. *Gambierdiscus* was able to grow across a very broad range of environmental conditions, with strains of tropical and temperate origin having a similar environmental niche. These results demonstrate that the tropical strains of *Gambierdiscus*, including those which produce CFP causing toxins, have the capacity to occupy temperate locations, but are potentially limited in their current distribution by other, yet to be tested factors.

An inherent aspect of range extension of an organism is dispersal. The organism must be dispersed from a population within its range, before being transported to a novel location. Due to their small size, marine microorganisms are known to have large dispersal capacity in the

global oceans (McManus & Woodson 2012). Current driven long-distance dispersal has been suggested as a vector for temperate range extension of *Gambierdiscus,* particularly along the east coast of Australia (Heimann, Capper & Sparrow 2011; Murray et al. 2014; Sparrow et al. 2017). Chapter 4 of this thesis has shown that pelagic dispersal of *Gambierdiscus* is most likely *via* rafting in association with macrophytes and other substrates, not as free-floating cells but may include other mechanisms not assessed in this thesis, such as ballast water dispersal.

Following dispersal, an organism must also successfully colonise a novel location to extend its range. Despite *Gambierdiscus* having one of the slowest documented growth rates amongst epibenthic microalgal taxa, colonisation experiments investigating growth of *Gambierdiscus* strains within natural and artificial communities showed that immigrants can persist (Chapter 5). Although these experiments did not address all aspects which may be influencing prospective colonisation success of *Gambierdiscus*, they demonstrated that the genus has a high capacity for potential colonisation but only under optimal environmental conditions.

The results of this thesis show that tropical *Gambierdiscus* strains could (1) occupy temperate locations (Chapter 3), (2) be dispersed long distances *via* rafting (as was shown for *Coolia*; Chapter 4) and (3) persist when introduced to epibenthic temperate seagrass communities (Chapter 5). Together, these data indicate that temperate range extension of *Gambierdiscus* in eastern Australia is feasible. This does not, however, provide empirical evidence that the population of *G. carpenteri* at Merimbula in southern NSW is a result of a tropical range extension. Verifying the relatedness of these populations would require application of more advanced molecular techniques than were applied in this study. For example, microsatellite markers have recently been used to compare the population dynamics of *Gambierdiscus* in the US Virgin Islands (Sassenhagen & Erdner 2017). Application of this technique amongst the Australian strains of *G. carpenteri* may similarly demonstrate genetic relatedness and should be considered in the future. A fundamental issue when evaluating whether a species has

extended its range, is that the organism may have been present at the "new" location all along but remained undetected. This is a possibility for the newly identified temperate populations of *Gambierdiscus* in Australia and elsewhere. However, results from this thesis establish that temperate range extension of *Gambierdiscus* is possible and if it hasn't yet occurred, it may in the future. Furthermore, the very existence of temperate populations of *Gambierdiscus* in Australia, and the growth of tropical strains under a broad range of conditions, provide enough evidence to suggest that our current understanding of the distributional limits of the genus needs to be revised.

Given that tropical populations of *G. carpenteri* could be transported to temperate locations, colonising and occupying them, this raises the question of why *Gambierdiscus* is not already found throughout temperate latitudes in eastern Australia. The most likely explanation may be that propagules of the species have not yet been dispersed from tropical to temperate locations, or that when they have been delivered, they were unable to persist. Without a comprehensive survey to document the distribution and abundance of benthic dinoflagellate species in Australia, this is a challenging question to answer. An obvious recommendation emerging from this thesis is that a baseline diversity survey of benthic dinoflagellates in Australia is conducted.

While this thesis has provided considerable insight into the causative organism/s of CFP in Australia, it has not addressed the ultimate vector of toxin transfer to humans, marine fish. Very little is known about how toxins are transferred from the microalga to marine fish (e.g., how many cells need to be consumed for CTXs to accumulate above threshold levels, over what time etc), but this process is ultimately what controls the risk of CFP in a region. Understanding these trophic transfer processes will be key to advancing our current understanding of the risk of CFP in humans, and eventually developing a risk framework for how to manage this public health issue in tropical to temperate locations globally.

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