Development of a quantitative PCR assay for the detection and enumeration of toxic *Gambierdiscus lapillus* (Gonyaulacales, Dinophyceae).

Key words:Ciguatera fish poisoning, *Gambierdiscus lapillus*, Quantitative PCR assay

A. L. Kretzschmar
Climate Change Cluster (C3), University of Technology Sydney, Ultimo, 2007 NSW, Australia
ithree institute (i3), University of Technology Sydney, Ultimo, 2007 NSW, Australia, anna.kretzschmar@uts.edu.au

A. Verma
Climate Change Cluster (C3), University of Technology Sydney, Ultimo, 2007 NSW, Australia

G. S. Kohli
Climate Change Cluster (C3), University of Technology Sydney, Ultimo, 2007 NSW, Australia
Department of Ecological Chemistry, Alfred Wegener Institute (AWI), Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570, Bremerhaven, Germany

S. A. Murray
Climate Change Cluster (C3), University of Technology Sydney, Ultimo, 2007 NSW, Australia

Abstract

Ciguatera fish poisoning is an illness contracted through the ingestion of seafood containing ciguatoxins. It is prevalent in tropical regions worldwide, including in Australia. Ciguatoxins are produced by some species of *Gambierdiscus*. Therefore rapid *Gambierdiscus* species identification through quantitative PCR (qPCR), along with its toxicity, are required to screen for potential ciguatera risk development. In Australia, the identity, distribution and abundance of species of *Gambierdiscus* that produce ciguatoxins is largely unknown. In this study we developed a rapid qPCR assay to quantify the presence and abundance of *Gambierdiscus lapillus*, a ciguatoxic species. We assessed the specificity and efficiency of the qPCR assay targeting *G. lapillus*. The assay was tested on 33 sites around Heron Island in the southern Great Barrier Reef, to which ciguatera is endemic, in triplicate to determine the presence and patchiness of these species across samples from several macroalgal hosts.

Introduction

Benthic dinoflagellates of the genus *Gambierdiscus* Adachi & Fukuyo produce ciguatoxins (CTX), which can accumulate in humans via consumption of contaminated seafood and cause ciguatera fish poisoning (CFP) (Fig. 1). Symptoms of CFP are largely gastrointestinal and neurotoxic however in severe cases humans can develop cardiovascular symptoms [55]. Species of *Gambierdiscus* can be epiphytic, growing on macroalgae and other substrates such as dead coral, as well as traverse the water column. Species of *Gambierdiscus* can vary in the production of ciguatoxins and/or maitotoxins [7, 27]. If a particular *Gambierdiscus* sp. is a CTX producer, and inhabit a palatable macroalgal substrate, the toxins bioaccumulate in herbivorous fish and filter feeders with the potential to travel up the food chain to cause CFP in humans [9, 24].

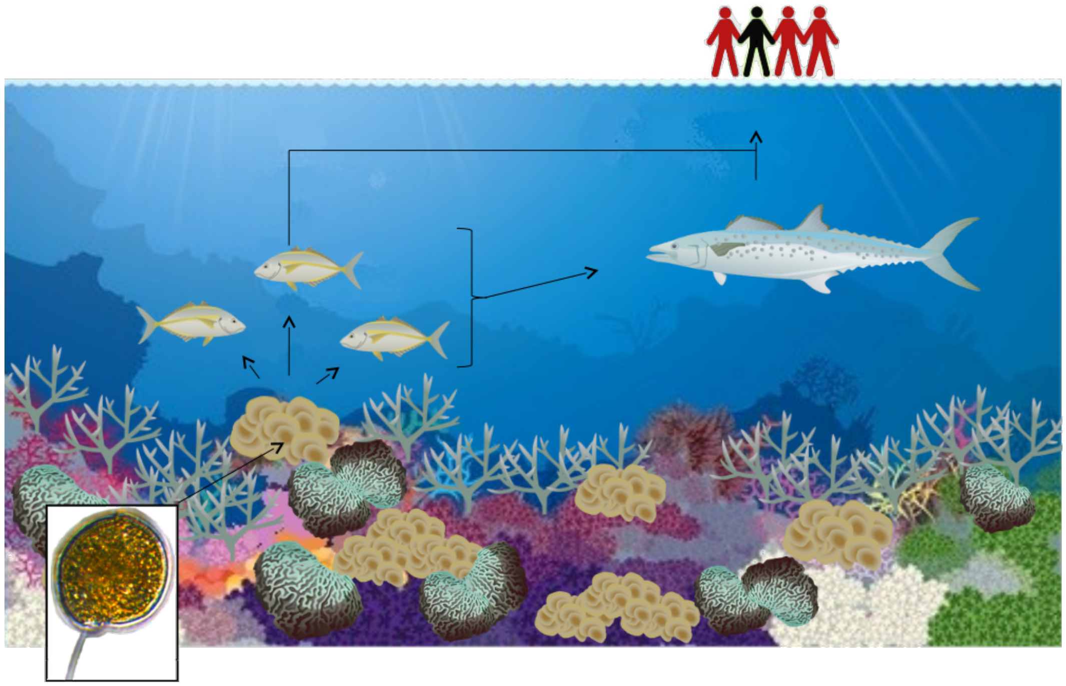

Figure 1: The mechanism of bioaccumulation of CTXs, with *G. polynesiensis* at the base of the food web inhabiting the macroalgae *Padina* spp. [66]. A herbivore, here a white trevally (*Pseudocaranx dentex*) [30] consumes CTX from *G. polynesiensis* along with the macroalgae, which is then either passes directly to humans through consumption, or through an intermediary piscivorous vector such as Australian spotted mackerel (*Scomberomorus munroi*) [54]. Image of *G. polynesiensis* (strain CG15) taken by A. L. Kretzschmar, 2016, Nikon Eclipse TS100 equipped with an Infinite Luminera 1 camera.

Gambierdiscus was first identified in 1977, with the type species *G. toxicus* Adachi & Fukuyo [1]. The genus remained monotypic for 18 years until the discovery of a second species *G. belizeanus* Faust [17]. To date, the genus comprises 14 described species and 6 ribo/species types [1, 8, 11, 17, 18, 19, 20, 31, 34, 35, 43, 48, 58, 67] . A major revision of the *Gambierdiscus* species taxonomy was undertaken by Litaker et al. (2009). Reports of *Gambierdiscus* spp. identified based on morphology alone, prior to this revision, need to be considered with caution as several new *Gambierdiscus* spp. were defined. Further, intra-species variation and inter-species similarities can cause misidentification [5, 27, 31]. Hence molecular genetic tools are important for determining the distribution and abundance of *Gambierdiscus* species and assess the risk of CFP in that region [27, 31].

Gambierdiscus spp. produce a suite of different polyketide compounds - CTX, maitotoxin (MTX), gambierone, gambieric acid and gambierol have been characterised to date [37, 38, 41, 51, 53]. While any of these can contribute to toxicity, only CTX has been clearly linked to CFP in humans [9, 24]. The toxin profile of many *Gambierdiscus* species is not well understood, and many different assays have been used to determine CTX toxicity [25]. Bioassays, such as mouse bioassays and neuroblastoma cell-line bioassays are good indicators of the toxicity of an organism, however species/strain specific toxin profiles needs to be elucidated with LC-MS/MS in order to characterise individual toxin congeners [13]. The toxin profile of *Gambierdiscus polynesiensis* Chinain & Faust is the only of *Gambierdiscus* spp. whose production of CTX congeners (P-CTX-3B, P-CTX-3C, P-CTX-4A, P-CTX-4B and M-seco-CTX-3C) has been verified by LC-MS/MS in isolates from French Polynesia and the Cook Islands, and is thought to be the principal cause of CFP in the Pacific region [6, 47]. However recently, a *G. polynesiensis* strain isolated from the Kermandec Islands, Pacific Ocean, did not exhibit CTX toxicity detectable by LC-MS/MS [49]. An uncharacterised peak in the CTX phase of several strains of *Gambierdiscus lapillus* extracts was reported via LC-MS/MS, which did not match any available CTX standards (CTX-3B, CTX-3C, CTX-4A, CTX-4B) [31]. Further, Larsson et al. (2018) found that *G. lapillus* extracts showed ciguatoxin-like activity when investigated with a bioassay. Therefore, this species likely produces previously uncharacterised CTX congener(s), and its production of CTX compounds requires further investigation. Determining the toxin profile of *Gambierdiscus* species requires toxin standards for comparative peak analysis. However, these are currently not commercially available. Therefore, progress in determining the toxins produced by species of *Gambierdiscus* has been comparatively slow, though bioassays provide a strong indicator for toxin production.

CFP was given the "neglected tropical disease" status by a panel of experts co-ordinated by the Intergovernmental Oceanographic Commissions (IOC) Intergovernmental Panel on Harmful Algal Blooms (IPHAB), as part of the United Nations Educational, Scientific and Cultural Organization), and a global ciguatera strategy was developed [25]. One element of the IOC/IPHAB Global Ciguatera Strategy is to investigate various species of the genus *Gambierdiscus*, determine which species produce CTXs through LC-MS/MS and other means, and develop efficient and reliable molecular monitoring tools for the species of interest [25]. Quantitative PCR (qPCR) is a useful molecular genetic screening tool, as it can give species-specific and quantitative results from DNA samples extracted from environmental samples [25].

qPCR is a variant of PCR in which a fluorescent agent is included in the PCR mix. Assays using qPCR have been extensively developed for the quantification of species of phytoplankton, particularly those involved in harmful algal blooms, utilizing methods such as SYBRgreen, Taqman assays, and others e.g. [2, 23, 40, 42, 56, 63].
Currently there is one qPCR assay to identify the presence of the genera *Gambierdiscus/Fukuyoa* [57]. Assays for species specific identification are available for 9 of the 14 described *Gambierdiscus* spp. and 3 out of 6 undescribed *Gambierdiscus* sp. types/ribotypes (Table 1). It is noteworthy that the qPCR assays described by Darius et al. (2017) rely on species identification based on the melt curve of the qPCR product, which requires any subsequent users of these assays to have a reference culture for positive identification rather than rely on a positive result being linked to the species investigated. Assays are available for 2 of the 3 species of *Fukuyoa* (Table 1), which seceded from *Gambierdiscus* as their own genus in 2015 [21]. *Fukoyoa* spp. are of interest for monitoring purposes as MTX producers, as the involvement of that toxin in CFP has not been resolved [29].

Table 1: Published qPCR assays for *Gambierdiscus* and *Fukoyoa* spp.

Species	**Method**	**Reference**
***Gambierdiscus* spp.**		
G. australes	TaqMan Probes & SYBR Green	[12, 42]
G. belizeanus	SYBR Green	[63]
G. caribaeus	SYBR Green	[63]
G. carolinianus	SYBR Green	[63]
G. carpenteri	SYBR Green	[63]
G. pacificus	SYBR Green	[12]
G. polynesiensis	SYBR Green	[12]
G. scabrosus	TaqMan Probes	[42]
G. toxicus	SYBR Green	[12]
Gambierdiscus sp. ribotype 2	SYBR Green	[63]
Gambierdiscus sp. type 2	TaqMan Probes	[42]
Gambierdiscus sp. type 3	TaqMan Probes	[42]
***Fukuyoa* spp.**		
Fukoyoa ruetzleri	SYBR Green	[63]
Fukoyoa cf. *yasumotoi*	TaqMan Probes	[42]

In Australia, outbreaks of CFP occur annually in Queensland [45]. However, due to the complicated presentation of symptoms, the predicted report rate is less than 20% [33]. Annually, there have been 7-69 reported cases between 2011 and 2015 (considering the report rate, > 35-345 cases, see Table 2), with 2 fatalities reported in the state [62]. Cases of ciguatera from Spanish Mackerel fish caught in NSW have been reported since 2014 [16], with five separate outbreaks affecting a total of 24 people since then [15]. Farrell et al. (2017) put forward a recommendation on how to manage the emerging ciguatera risk in NSW.

Despite the prevalence of CFP in Australia, the characterization of *Gambierdiscus* population is on going work. A causative species for CFP has not been identified and verified by LC-MS/MS to date, though recent bioassays by Larsson et al. has identified several species, two of which yet uncharacterised, that showed ciguatoxin-like activity which could all contribute to the ciguateric web [32]. Over 50% of Australia's expansive coastline (total 66,000 km) is tropical or subtropical, and may be considered potential habitat for *Gambierdiscus* spp. [31]. The 7 species of *Gambierdiscus* that have been identified from Queensland and New South Wales are as follows: *G. belizeanus* [39], *G. carpenteri* [27, 59], *G. honu* (based on D8-D10 LSU sequence matching to a study by Richlen et al. [50]) [48], *G. lapillus* [31, 32], *G. toxicus* [22] and two uncharacterized potentially new species [32], as well as *F. yasumotoi* [39]). Using pyrosequencing, *Gambierdiscus* was identified to the genus level in Western Australia [28], indicating that this is a coastline that should be examined further for CFP risk. qPCR primers that can be used for identification in Australia for potential monitoring purposes, have been developed for *G. belizeanus*, *G. carpenteri* and *F. yasumotoi* [42, 63]. Therefore, in order to assess the distribution and abundance of species that may produce CTXs in Australia, it is necessary to be able to assay the presence of other species in this region that are known to produce CTXs.

Table 2: Cases of Ciguatera Fish Poisoning reported to health authorities in Queensland, Australia, between 2011 and 2015, by Queensland Health [45].

Year	2011	2012	2013	2014	2015
Recorded CFP cases	18	7	25	69	11
Extrapolated CFP indcidences	∼90	∼35	∼125	∼345	∼55

The aim of this study was to develop and test a qPCR assay to detect *G. lapillus* which exclusively amplifies the target species without requiring the operator to have a positive control for comparison. *G. lapillus* is a recently identified species from the GBR with an unresolved toxin profile that indicates the possibility of CTX production.

Materials and methods

Clonal strains and culturing conditions

Three strains of *G. lapillus* and one strain of *G.* cf. *silvae* were isolated from Heron Island, Australia, as previously described [31]. Two strains of *G. polynesiensis* were isolated from Rarotonga, Cook Islands (table 3). The cultures were maintained F•-10 medium at 27 °C, 60mol•-m^2 •-s light in 12hr:12hr light to dark cycles.

Table 3: List of *Gambierdiscus* clonal strains used for the qPCR assay.

Species	**Collection site**	**Collection date**	**Latitude**	**Longitude**	**Strain name**
G. lapillus	Heron Island, Australia	July 2014	23° 4420' S	151° 9140' E	HG4
					HG6
					HG7
G. polynesiensis	Rarotonga, Cook Islands	November 2014	21° 2486' S	159° 7286' W	CG14
					CG15
G. cf. *silvae*	Heron Island, Australia	July 2014	23° 4420' S	151° 9140' E	HG5

DNA extraction and species specific primer design

Genomic DNA was extracted using a modified CTAB method [64]. The purity and concentration of the extract were measured using the Nanodrop (Nanodrop2000, Thermo Scientific), and the integrity of the DNA was visualised on 1% agarose gel. A unique primer set was designed for the small-subunit (SSU) rDNA region of *G. lapillus* based on sequences available in the GenBank reference database. The target sequences were aligned against sequences of all other *Gambierdiscus* spp. that were available on GenBank reference database, with the MUSCLE algorithm (maximum of 8 iterations) [14] used through the Geneious software, version 8.1.7 [26]. Unique sites were determined manually (Table 4). Primers were synthesised by Integrated DNA Technologies (IA, USA). The primer set was tested systematically for secondary product formation for all 3 strains of *G. lapillus*(Table 3) via standard PCR in 25μl mixture in PCR tubes. The mixture contained 0.6 μM forward and reverse primer, 0.4 μM BSA, 2 - 20 ng DNA, 12.5 μl 2xEconoTaq (Lucigen) and 7.5 μl PCR grade water. The PCR cycling comprised of an initial 10 min step at 94 °C, followed by 30 cycles of denaturing at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 1 min, finalised with 3 minutes

Table 4: G. lapillus specific qPCR primer set for SSU rDNA.

Primer name	**Amplicon size**	**Synthesis direction of primer**	**Sequence (5'-3')**
qGlapSSU2F	138bp	Forward	TTTTTGTCCCAGGAGGGTGA
qGlapSSU2R		Reverse	TGAGGCCAAAACTCGAAAATC

of extension at 72 °C. Products were visualised on a 1% agarose gel.

Evaluation of primer specificity

To verify primer set specificity as listed in Table 4, DNA was extracted via CTAB from *G. australes* (CCMP1650 and CG61), *G. belizeanus* (CCMP401), *G. carpenteri* (UTSMER9A3), *G. pacificus* (CAWD149) and *G.* cf. *silvae* (HG5). *G. cheloniae* (CAWD232) DNA was extracted using a PowerSoil DNA isolation kit (Mo Bio Inc., CA, USA). *G. scabrosus* (KW070922_1) DNA was extracted using DNeasy Plant Mini Kit (Quiagen, Tokyo, Japan) according to the manufacturer's protocol. For all extracted samples, the presence and integrity of genomic DNA was assessed on 1% agarose gel. The primer set designed for *G. lapillus* was tested for cross-reactivity against all other *Gambierdiscus* spp. available via PCR (BioRadT100 Thermal Cycler (CA, USA)), appropriate positive and negative controls were applied. PCR amplicons were visually assessed on 1% agarose gel.

Evaluation of primer sensitivity

The qPCR reaction mixture contained 10 μl SYBR Select Master Mix (Thermo Fisher Scientific), 7 μl MilliQ water, 0.5 μM forward and reverse primers and 2 - 20 ng DNA template, for a final volume of 20 μl. Cycling conditions consisted of 10 min at 95, then 40 cycles of 95 °C for 15 seconds and 60 °C for 30 seconds, followed by a temperature gradient for melt curve construction.

Calibration curve construction

Standard curves were constructed to determine the efficiency of the assay, using a synthetic gene fragment approach, and also to use to quantify species presence, using calibration curves based on DNA extracted from known cell numbers. For curves based on synthetic gene fragments, a 10-fold serial dilution of a synthesised fragment containing the SSU target sequence, forward and reverse primer sites and 50bp flanking both primer sites matching sequencing results was generated. Cell-based standard curves were constructed using 10-fold dilutions of gDNA extract of known cell concentrations. The calibration curves for both methods were calculated (R^2, PCR efficiency and regression line slope) and graphed in R version 3.2.3 [46], using R studio version 1.0.136 [52] and the ggplot2 package [65].

Gene based calibration curve

For the target amplicons of *G. lapillus*, a DNA fragment spanning the target sequence, the reverse and forward primer sites and an extra 50bp on either end was synthesised called gBlocks ® by Integrated DNA Technologies (IDT, IA, USA). Lyophilized gBlocks ® was re-suspended in 1x TE (Tris 1M, EDTA 0.5 pH8) to a concentration of 1 ng.μl. The copy number of gene fragment was then calculated as 28,850,000,000 for *G. lapillus*. The stock solution was 10-fold serially diluted and dilutions between 10^3 and 10^8 were amplified by qPCR (on StepOnePlus System by Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA) in triplicate.

Cell based calibration curve

Two strains of *G. lapillus* (HG4 and HG7) were used to construct cell based standard curves. Cells were counted using a Sedgwick Rafter counting chamber as viewed under a Nikon Eclipse TS100 (Australia) microscope. DNA was extracted with the FastDNA spin kit for soil by MP Biomedicals (CA, USA), as per the manufacturer's instructions. The gDNA extracts were 10-fold serially diluted. Dilutions ranging from 3880 to 0.0388 cells and 5328 to 0.05328 for HG4 and HG7 respectively. Samples were amplified via qPCR (on StepOnePlus System by Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA) in triplicate.

Determination of extractable gene copies per cell for *G. lapullis*

To determine the extractable mean SSU rDNA copies per cell, the known cell counts for the cell based calibration curve were used as input for calculation. Copy number was defined as a linear regression of the gene based calibration curve using the input cell counts to determine extractable SSU rDNA copy number per cell.

Screening environmental samples for *G. lapillus*

Around Heron Island and Heron Reef (Fig. 2) 33 sites (within 1km of the shore) were sampled in October 2015, in spatial replicates (A, B, C) within a 2m radius. Two species of macroalgae that commonly grow on this reef, *Padina* sp. and *Saragassum* sp., were sampled for the presence of epiphytic *Gambierdiscus* spp. For each sample, about 200 g of macroalgae was collected from approximately 1 m deep water at low tide and briefly placed in plastic bags containing 200 to 300 ml of ambient seawater. They were shaken vigorously for 5 min to detach the epiphytic dinoflagellates from the macroalgae. This seawater was passed through $>$ 120 m mesh filter to remove any remaining larger fauna and debris. The collected seawater was centrifuged at 1000 rpm. The supernatant was discarded and the pellet was dissolved in 10 ml RNAlater (Ambion, Austin, TX, USA) for preservation and stored at 4°C. Samples were screened in triplicate for both *G. lapillus* on a StepOnePlus System by Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA).

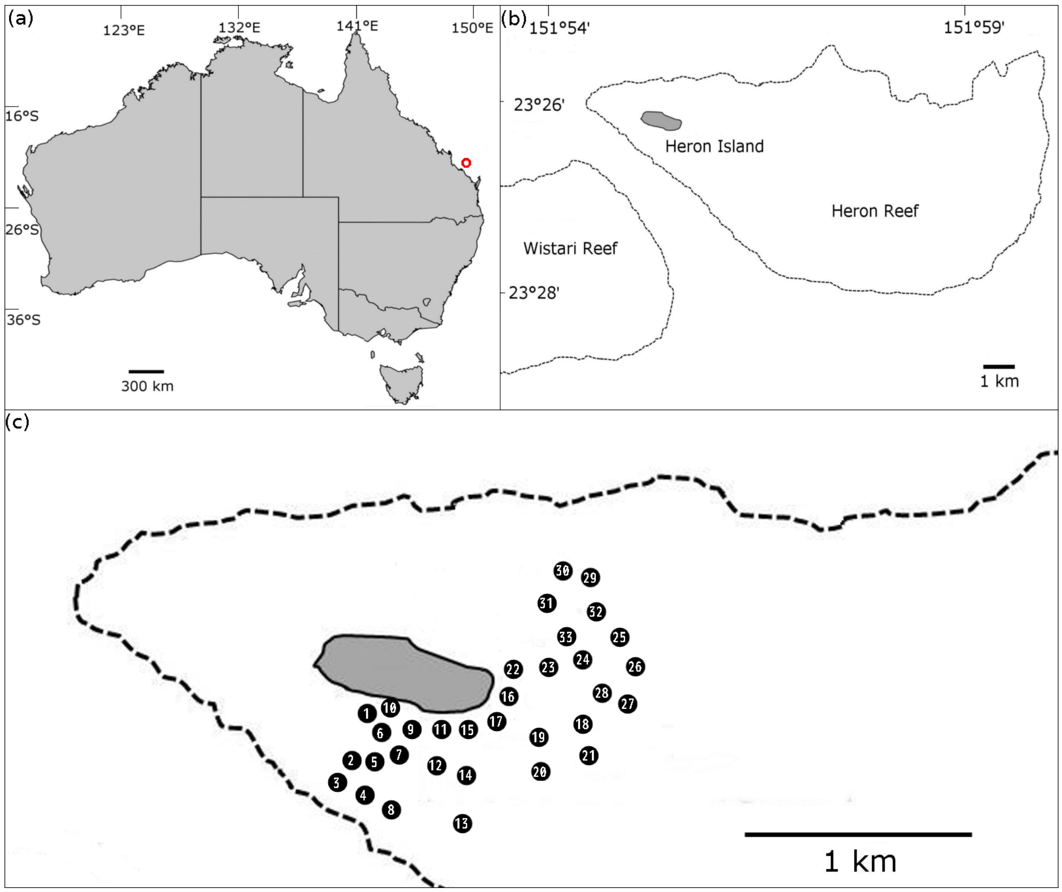

Figure 2: (A) Map of Australia, with the position of Heron Island (red circle); (B) Heron Island including surrounding reefs; (C) Approximate location of sampling sites around Heron Island.

Results

Evaluation of primer specificity

The qGlapSSU2F - qGlapSSU2R primer pair (Table 4) amplified in PCRs of all five strains of *G. lapillus*, while no amplification was observed for genetically closely related species *G. belizeanus*, *G. cheloniae*, *G. pacificus* and *G. scabrosus*. Other species of *Gambierdiscus* from different clades, *G. australes*, *G. carpenteri*, *G. polynesiensis* and *G.* cf. *silvae* (Table 5) were not amplified using this primer set [31, 58].

Table 5: Cross-reactivity of the qPCR primer set.

Template	**Strain name**	**gDNA gel band**	**GlapSSU2F-GlapSSU2R**
G. australes	CCMP1650	+	-
	CG61	+	-
G. belizeanus	CCMP401	+	-
G. carpenteri	UTSMER9A3	+	-
G.cheloniae	CAWD232	+	-
G. lapillus	HG1	+	+
	HG4	+	+
	HG6	+	+
	HG7	+	+
	HG26	+	+
G. pacificus	CAWD149	+	-
G. polynesiensis	CG14	+	-
	CG15	+	-
G. scabrosus	KW070922_1	+	-
G. cf. *silvae*	HG5	+	-

Evaluation of primer sensitivity

The cell-based standard curves for *G. lapillus* (HG4 and HG7, Fig. 3a) showed high linearity with R^2 approaching 1.00. The slope for the Ct vs. $\log_{10}$ cell number for HG4 was -3.4, efficiency 96.8 %; and -3.51, efficiency of 92.7 % for HG7. The linear detection for both *G. lapillus* isolates covered six orders of magnitude. The lowest number of cells detected were 0.04 and 0.05 cells for HG4 and HG7 respectively (Fig. 3a).

The gene based standard curve for *G. lapillus* covered linear detection over 7 orders of magnitude, with a slope of -3.42, R^2 equals 0.99 and a PCR efficiency of 96 %. The detection limit tested was less than 10^5 gene copy numbers. The Ct for the lowest gene copy number tested was less than 25, so it is likely that the sensitivity is lower than 10^5 gene copy numbers.

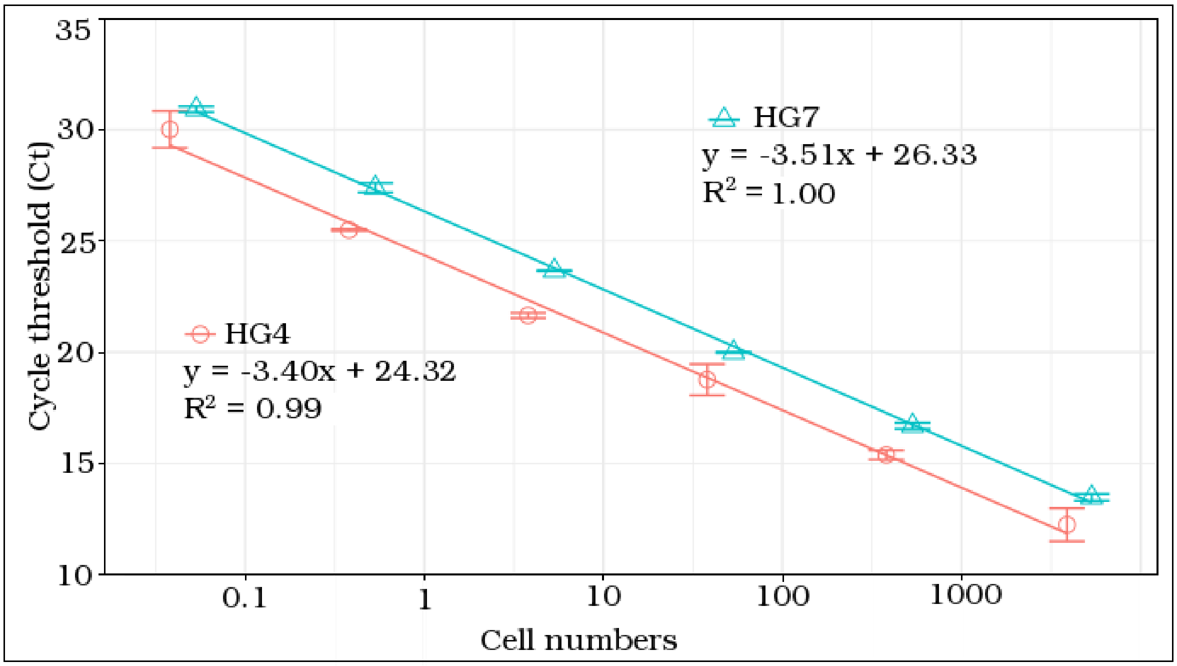

Figure 3: qPCR cell based standard curves of *G. lapillus* strains HG4 (circle) and HG7 (triangle). Error bars represent the deviation of technical replicates during reactions.

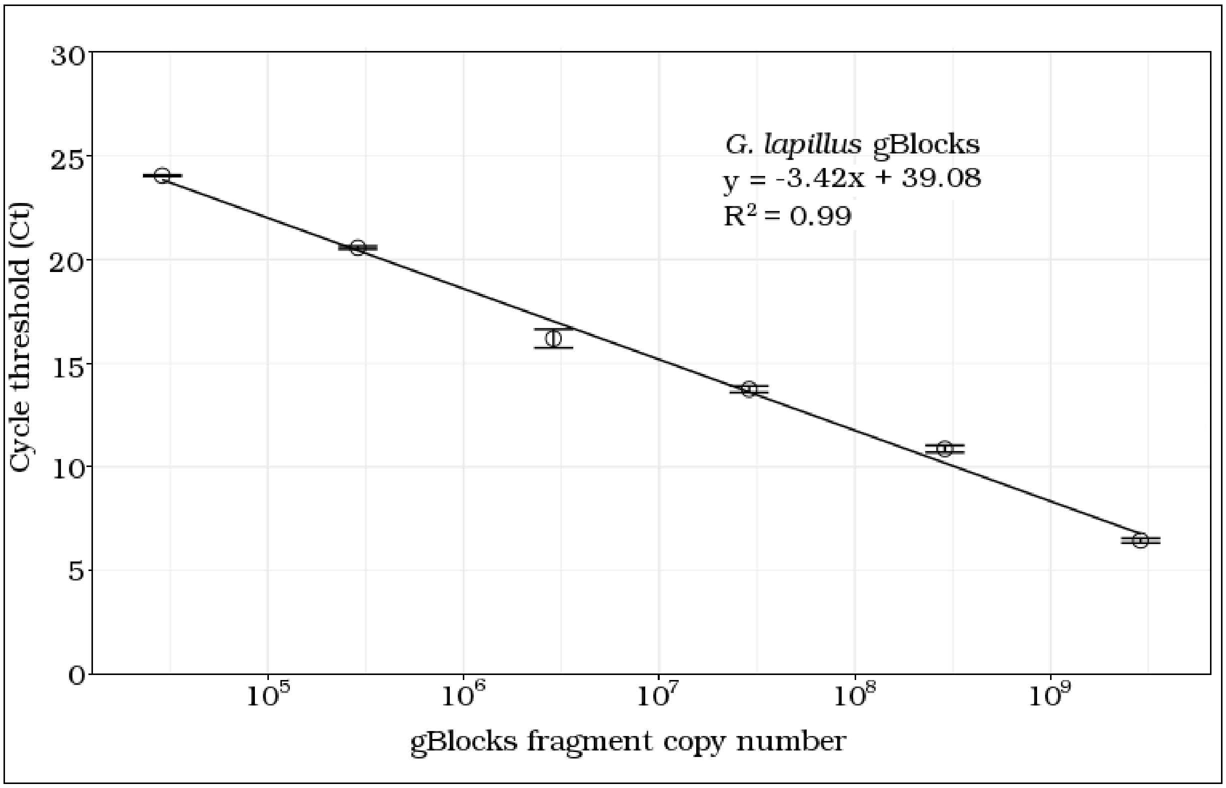

Figure 4: qPCR gene based standard curves of *G. lapillus*. Error bars represent the deviation of technical replicates during reactions.

Quantification of extractable SSU rDNA copy number per cell of *G. lapillus*

The detectable SSU copies for *G.lapillus* were 22,430 and 5,855 copies per cell for HG4 and HG7 respectively.

Screening environmental samples for *G. lapillus* abundance

To evaluate the adequacy of the *G. lapillus* qPCR assay for environmental screening, the assay was applied to environmental community DNA extracts collected around Heron Island. Low cell numbers were detectable for *G. lapillus*. Ct values for *G. lapillus* detection in environmental samples were calibrated to the HG7 standard curve and calculated as cells per gram wet weight macroalgae (Table 6). *G. lapillus* was detected across 32 of the 33 sampling sites. Sites at which *G. lapillus* was present, it showed a patchy distribution, being present at two of the three spatial replicates in the majority of samples (23 of 33 sample sites), followed by all three spatial replicates testing positive (8 out of 33 sites) and at one site only one of the spatial replicates was positive (Fig. 5).

G. lapillus was detected at 71 out of the 99 spatial replicates, specifically at 23/30, 18/28 and 9/10 samples from *Chnoospora* sp., *Padina* sp. and *Saragassum* sp. as substrate respectively, as well as 26/31 sites sampled from mixed macroalgae (Table 6). Patchiness was also found in the abundance as well as the distribution of *G. lapillus*, from 0.24 cells.g^{-1} wet weight macroalgae to 49.51 cells.g^{-1} wet weight macroalgae, with a mean of 5.84 cells.g^{-1} wet weight macroalgae. For example (6A - *Chnoospora* sp.) and (6B - *Padina* sp.) hosted comparable cell numbers (1.12 cells and 1.65 cells per g.ww algae respectively) while no *G. lapillus* cells were detected on (6C - *Padina* sp.). Only at one of 33 sampling sites, no *G. lapillus* presence was detected across all three spatial replicates (25A, B, C). At all other sites, the presence of *G. lapillus* varied between spatial replicates of but did not significantly differ between macroalgal host or location (Fig. 6).

Table 6: Screening of macroalgal samples for *G. lapillus* and cell density estimates via qPCR. Cell numbers were modeled on the type strain HG7. N/D denotes not detected; N/A denotes not attempted due to loss of sample.

Sample ID	**Spatial replicate**	**Macroalgal substrate**	***G. lapillus* cell number**
1	A	*Padina* sp.	N/D
1	B	*Sargassum* sp.	10.55
1	C	*Padina* sp.	2.75
2	A	*Padina* sp.	N/D
2	B	*Padina* sp. & *Chnoospora* sp.	4.33
2	C	*Padina* sp.	4.27
3	A	*Padina* sp.	0.62
3	B	*Padina* sp.	N/D
3	C	*Padina* sp.	1.99
4	A	*Padina* sp. & *Chnoospora* sp.	6.13
4	B	*Chnoospora sp.*	0.62
4	C	*Padina* sp. & *Chnoospora* sp.	N/D
5	A	*Padina* sp.	N/D
5	B	*Padina* sp.	2.41
5	C	*Padina* sp.	3.81
6	A	*Chnoospora* sp.	1.12
6	B	*Padina* sp.	1.65
6	C	*Padina* sp.	N/D
7	A	*Padina* sp.	9.35
7	B	*Padina* sp.	N/D
7	C	*Padina* sp.	N/D
8	A	*Chnoospora* sp.	N/D

8	B	*Padina* sp.	1.67
8	C	*Padina* sp. & *Chnoospora* sp.	3.61
9	A	*Padina* sp. & *Chnoospora* sp.	N/D
9	B	*Padina* sp.	1.69
9	C	*Padina* sp.	1.92
10	A	*Padina* sp. & *Chnoospora* sp.	12.6
10	B	*Padina* sp. & *Chnoospora* sp.	4.01
10	C	*Padina* sp.	N/D
11	A	*Padina* sp. & *Sargassum* sp.	N/D
11	B	*Padina* sp. & *Sargassum* sp.	0.26
11	C	*Padina* sp. & *Sargassum* sp.	1.29
12	A	*Chnoospora* sp.	N/D
12	B	*Chnoospora* sp.	17.09
12	C	*Chnoospora* sp.	4.27
13	A	*Chnoospora* sp.	N/D
13	B	*Padina* sp. & *Chnoospora* sp.	49.51
13	C	*Padina* sp. & *Chnoospora* sp.	18.58
14	A	*Padina* sp. & *Chnoospora* sp.	0.91
14	B	*Padina* sp. & *Chnoospora* sp.	N/D
14	C	*Chnoospora* sp.	5.95

15	A	*Padina* sp. & *Chnoospora* sp.	2.01
15	B	*Chnoospora* sp.	4.89
15	C	*Chnoospora* sp.	N/D
16	A	*Chnoospora* sp.	6.70
16	B	*Chnoospora* sp.	8.83
16	C	*Chnoospora* sp.	3.08
17	A	*Chnoospora* sp.	2.58
17	B	*Chnoospora* sp.	9.39
17	C	*Padina* sp. & *Chnoospora* sp.	N/D
18	A	*Chnoospora* sp.	0.02
18	B	*Chnoospora* sp.	N/D
18	C	*Chnoospora* sp.	9.24
19	A	*Chnoospora* sp.	5.27
19	B	*Padina* sp. & *Chnoospora* sp.	48.46
19	C	*Padina* sp. & *Chnoospora* sp.	2.71
20	A	*Padina* sp. & *Chnoospora* sp.	2.81
20	B	*Padina* sp. & *Chnoospora* sp.	10.26
20	C	*Chnoospora* sp.	N/D
21	A	*Padina* sp. & *Chnoospora* sp.	5.50
21	B	*Chnoospora* sp.	1.23
21	C	*Padina* sp.	10.32
22	A	*Chnoospora* sp.	N/D
22	B	*Chnoospora* sp.	37.68
22	C	*Chnoospora* sp.	5.57

23	A	*Chnoospora* sp.	7.86
23	B	*Chnoospora* sp.	N/D
23	C	*Chnoospora* sp.	3.14
24	A	*Padina* sp. & *Chnoospora* sp.	0.72
24	B	*Padina* sp. & *Chnoospora* sp.	0.58
24	C	*Padina* sp.	3.68
25	A	*Padina* sp.	N/D
25	B	*Padina* sp.	N/D
25	C	*Padina* sp.	N/D
26	A	*Sargassum* sp.	N/D
26	B	*Sargassum* sp.	0.19
26	C	*Sargassum* sp.	0.18
27	A	*Sargassum* sp.	N/D
27	B	*Sargassum* sp.	2.11
27	C	*Sargassum* sp.	2.05
28	A	*Padina* sp.	7.17
28	B	*Padina* sp.	2.67
28	C	*Padina* sp.	8.64
29	A	*Chnoospora* sp.	1.24
29	B	*Chnoospora* sp.	5.90
29	C	*Chnoospora* sp.	N/D
30	A	*Padina* sp. & *Sargassum* sp.	4.09
30	B	*Padina* sp. & *Sargassum* sp.	2.58
30	C	*Padina* sp. & *Sargassum* sp.	N/D
31	A	*Sargassum* sp.	1.91
31	B	*Sargassum* sp.	2.90

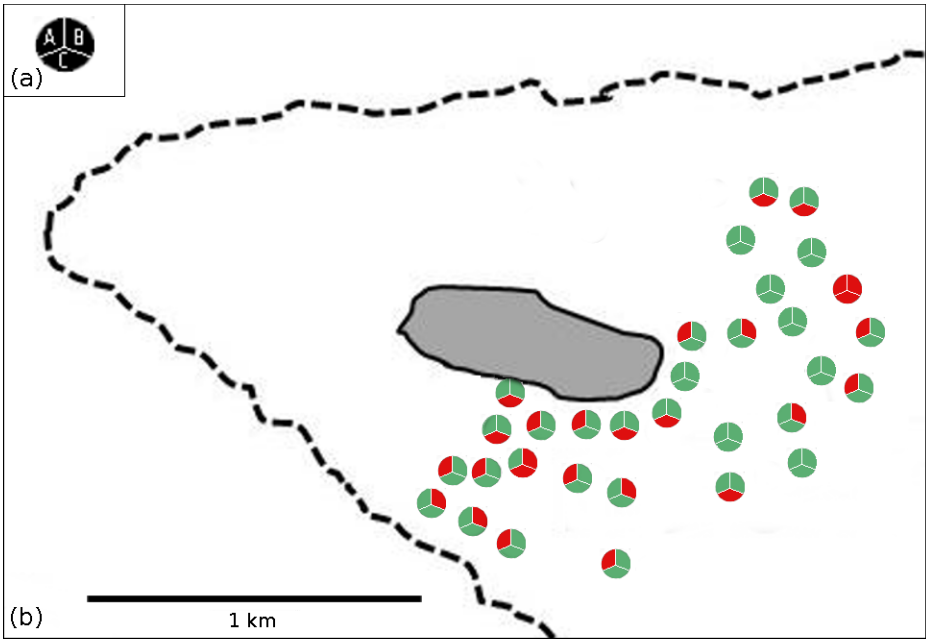

Figure 5: *G. lapillus* presence at the macroalgal sampling sites around Heron Island. The spatial replicates for each site are set up as shown in (A); the sites in (B) linked to numbering in Fig. 2 where positive (green) and negative (red) as per Table 6.

31	C	*Sargassum* sp.	3.97
32	A	*Padina* sp. & *Sargassum* sp.	2.24
32	B	*Chnoospora* sp. & *Sargassum* sp.	1.36
32	C	*Padina* sp. & *Sargassum* sp.	2.00
33	A	*Padina* sp.	4.73
33	B	*Padina* sp. & *Chnoospora* sp.	0.07
33	C	*Padina* sp. & *Chnoospora* sp.	2.44

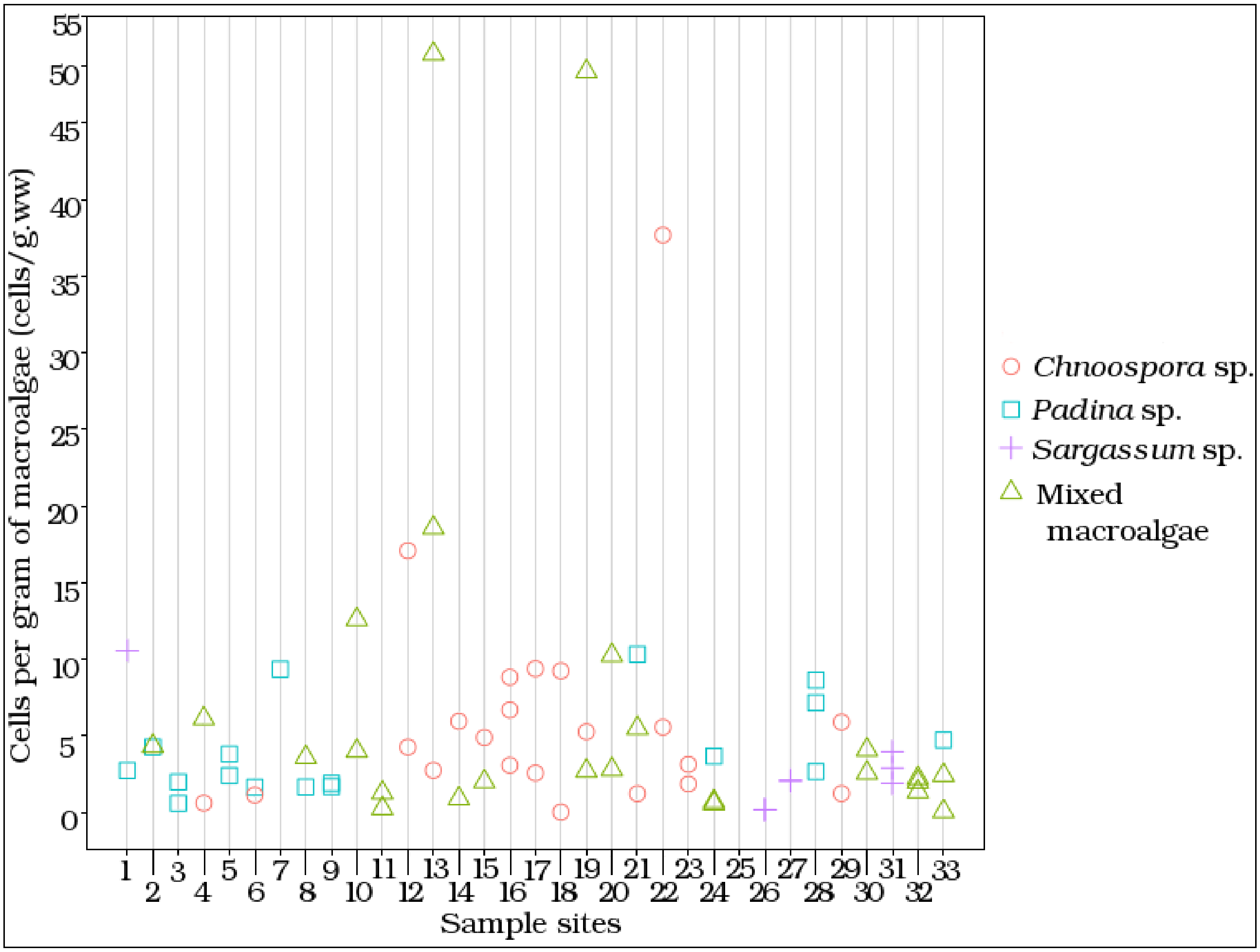

Figure 6: Detection of *G. lapillus* per spatial replicate at each macroalgal sampling site, cell numbers as normalised to HG7 standard curve (Fig. 3A). Spatial replicates cper macroalgal substrate where *Sargassum* sp. are squares, *Padina* sp. are circles and mixed macroalgal substrates are triangles (Table 6).

Discussion

The aim of the study was to design and validate a species-specific qPCR assay to quantify *G. lapillus* which may produce CTX toxins in the Australian GBR region. Species-specific PCR primers with high specificity and sensitivity were developed and the SSU copy number for two strains were determined. We also established that this primer set were effective in measuring the abundance and distribution of *G. lapillus* at the Heron Island reef. The cross-reactivity of primers designed in this study showed high specificity for both *G. lapillus* while not amplifying when tested against other *Gambierdiscus* spp. The species tested for cross-reactivity were chosen because they represented species that are most genetically similar to each target species for the SSU region (as per Fig. 2 in [31]). Standard curves were constructed for two strains of *G. lapillus* for which the primers showed high linearity and amplification efficiency (Fig. 3). Hence this primer set is an accurate and reproducible molecular tool to enumerate the target species exclusively from environmental community DNA extracts. Importantly, this assay does not require the operator to rely on melt curves to identify species, or to have access to *G. lapillus* DNA extracts as a positive control. Due to the potential CTX production of *G. lapillus* [31, 32] the presence and distribution of this species is of interest in Australia where the causative organism(s) for CFP is yet to be established, though several potential candidates have been identified by Larsson et al. (2018).

As ciguatera risk is linked to the abundance of *Gambierdiscus* species producing CTXs, it was important to establish a quantitative assay for detection. We validated a synthetic gene fragment standard curve of the target region (gBlocks ®) and compared this to cell standard curves to establish an 'absolute' qPCR assay [23, 42]. Further, we determined the extractable copy SSU rDNA number for two strains of *G. lapillus* (HG4 and HG7). The copy number for *G. lapillus* (5,855.3 to 22,430.3 rDNA copies per cell) were comparable to the copy numbers determined by Vandersea et al. (2012), which ranged from 690 rDNA copies for *G. belizeanus* to 21,498 copies for *G. caribaeus*. In comparison the cell copy numbers determined by Nishimura et al. (2016) ranged from 532,000 copies for *G. scabrosus* and 2,261,000 for *G.* sp. type 3. While the difference in rDNA copy numbers may be due to inter-species differences, or even intra-species as per the *G. lapillus* results, Nishimura et al. argue that the difference could be underestimation of rDNA copy numbers due to 'ghost' cells counted for total cell number which do not contribute to amplification [23, 42]. The difference in extractable SSU rDNA copies between the two strains of *G. lapillus* is intriguing. As the variation between the two strains tested is within the observed variation reported by Nishimura et al. (2016) from single cell qPCR experiments for rDNA copy number elucidation, the difference reported here is likely representative of biological intra-strain variation rather than methodological artifacts. A 5-fold difference in toxicity between the same HG4 and HG7 strains for *G. lapillus* was also reported by Kretzschmar et al. (2017), and there was a noticeable difference in growth rate between the two strains observed (but not quantified) in this study. The amounting evidence of intra-strain variability in toxicity, detectable rDNA copy numbers and potentially growth rate could have severe implications for qPCR based cell enumeration of environmental samples when attempting to extrapolate ciguatera risk and requires further investigation.

The qPCR assay was successfully tested on environmental DNA extracts from around Heron Island, and gave some insight into *G. lapillus* distribution and abundance. The qPCR assay detected *G. lapillus* at all of the sites tested (Fig. 5). Within the spatial replicates, the distribution of *G. lapillus* was patchy, as 25 of the 32 sites included at least one replicate with no *G. lapillus* present (Fig. 5). Patchiness in the distribution of *Gambierdiscus* species has previously been found in a study of 7 *Bryothamnion* macroalgae spaced 5 to 10 cm apart, in which 5 to 70 cells g-1 algae were found [60]. There was no significant difference in the presence/absence of *G. lapillus* cells observed as per the macroalgal host, *Padina* sp. or *Sargassum* sp.

Motile behaviour has been observed previously in the field at various time points [3, 68]. Parsons et al. (2011) reported *Gambierdiscus* sp. behaviour as facultative epiphytes during lab scale experiments, as cells showed attachment as well as motile stages over time in the presence of different macroalgae [44]. Taylor & Gustavson (1983) reported that *Gambierdiscus* cells were captured in plankton tows by de Silva in 1956 but reported as *Goniodoma* [60]. Motility could be a factor for the patchy distribution observed in the spatial replicates. Across spatial replicates where *G. lapillus* was detected, cell densities were consistent (Fig. 6). The average cell density of *G. lapillus* 5.84 cells^{-1}g wet weight macroalgae, which is comparable to the cell densities recorded by Nishimura et al. (2016) in their environmental screening (Table 4 in [42]).

As many authors have pointed out (e.g. [4, 10, 25, 35, 36, 44, 61]), there are several difficulties in determining precise quantification of *Gambierdiscus* species on macroalgae in order to assess potential ciguatera risk. Due to the difference in habitable surface area between samples taken from structurally diverse macroalgae, including those sampled in this study (*Chnoospora*, *Padina* sp. and *Sargassum* sp.), the potential habitable space is difficult to compare. Further, in order to assess ciguatera risk in a given area, the properties of the macroalgae with *Gambierdiscus* epiphytes need to be considered. If the macroalgae is structurally or chemically defended against herbivory, any CTX produced by the epiphytes is unlikely to enter the food chain and cause CFP [10]. Due to the difficulty in quantifying *Gambierdiscus* on a particular substrate, Tester et al. (2014) proposed have the use of an artificial substrate (commonly available black fibreglass screen of a known surface area) and a standardised sampling method [61].

Conclusion

The qPCR assay developed in this study is an expedient and accurate molecular tools to detect and enumerate the present of *G. lapillus* in environmental samples. The assay was shown to be highly sensitive and accurately detected cell 0.05 to over 4000 cells for *G. lapillus*. *G. lapillus* may produce CTXs, but regardless is a part of the ciguateric web in Australia and was detected at most sites sampled. The assay was applied to spatial replicates from 33 sites around Heron Island on the GBR, which found that proximity is a poor predictor of *G. lapillus* presence. The development and validation of a quantitative monitoring tool presented here for *G. lapillus* is in line with Element 1 of the Global Ciguatera Strategy [25].

Acknowledgements

Gratitude to Dr. Adachi and Dr. Nishimura for supplying *G. scabrosus*, Michaela Larsson for supplying *G. carpenteri* as well as Dr. Kirsty Smith and Dr. Lesley Rhodes for supplying *G. cheloniae*, all three species were used for DNA for cross reactivity assessment.

Conflict of interest

The authors report no conflict of interest in conducting this study.

Author contribution

The environmental samples from Heron Island were collected by G. Kohli. DNA from environmental samples was extracted by A. L. Kretzschmar, A. Verma and G. Kohli. The *G. lapillus* assay was designed by A. L. Kretzschmar. The assay was tested for specificity, sensitivity, standard curves generated and environmental screening conducted by A. L. Kretzschmar. The manuscript was drafted by A. L. Kretzschmar and revised by all authors.

References

[1] Adachi, R., and Fukuyo, Y. The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et sp. nov. collected in a ciguatera-endemic area. *Bulletin of the Japanese Society of Scientific Fisheries (Japan) 45* (1979), 67–71.

[2] Antonella, P., and Luca, G. The quantitative real-time PCR applications in the monitoring of marine harmful algal bloom (HAB) species. *Environmental Science and Pollution Research 20*, 10 (2013), 6851–6862.

[3] Bomber, J. W. *Ecology, genetic variability and physiology of the ciguatera-causing dinoflagellate* Gambierdiscus toxicus *Adachi & Fukuyo*. 1987.

[4] Bomber, J. W., Rubio, M. G., and Norris, D. R. Epiphytism of dinoflagellates associated with the disease ciguatera: substrate specificity and nutrition. *Phycologia 28*, 3 (1989), 360–368.

[5] Bravo, I., Figueroa, R. I., and Fraga, S. Cellular and nuclear morphological variability within a single species of the toxigenic dinoflagellate genus *Gambierdiscus*: Relationship to life-cycle processes. *Harmful Algae 40* (2014), 1–8.

[6] Chinain, M., Darius, H. T., Ung, A., Cruchet, P., Wang, Z., Ponton, D., Laurent, D., and Pauillac, S. Growth and toxin production in the ciguatera-causing dinoflagellate *Gambierdiscus polynesiensis* (Dinophyceae) in culture. *Toxicon 56*, 5 (2010), 739–750.

[7] Chinain, M., Darius, H. T., Ung, A., Fouc, M. T., Revel, T., Cruchet, P., Pauillac, S., and Laurent, D. Ciguatera risk management in French Polynesia: the case study of Raivavae Island (Australes Archipelago). *Toxicon 56*, 5 (2010), 674–690.

[8] Chinain, M., Faust, M. A., and Pauillac, S. Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp. nov., *G. australes*, sp. nov., and *G. polynesiensis*, sp. nov. *Journal of Phycology 35*, 6 (1999), 1282–1296.

[9] Chinain, M., Germain, M., Sako, Y., Pauillac, S., and Legrand, A.-M. Intraspecific variation in the dinoflagellate *Gambierdiscus toxicus* (Dinophyceae). i. isozyme analysis. *Journal of Phycology 33*, 1 (1997), 36–43.

[10] Cruz-Rivera, E., and Villareal, T. A. Macroalgal palatability and the flux of ciguatera toxins through marine food webs. *Harmful Algae 5*, 5 (2006), 497–525.

[11] Dai, X., Mak, Y. L., Lu, C.-K., Mei, H.-H., Wu, J. J., Lee, W. H., Chan, L. L., Lim, P. T., Mustapa, N. I., Lim, H. C., et al. Taxonomic assignment of the benthic toxigenic dinoflagellate *Gambierdiscus* sp. type 6 as *Gambierdiscus balechii* (Dinophyceae), including its distribution and ciguatoxicity. *Harmful Algae 67* (2017), 107–118.

[12] Darius, H. T., Roué, M., Sibat, M., Viallon, J., Vandersea, M. W., Tester, P. A., Litaker, R. W., Amzil, Z., Hess, P., Chinain, M., et al. *Tectus niloticus* (Tegulidae, Gastropod) as a novel vector of ciguatera poisoning: detection of Pacific ciguatoxins in toxic samples from Nuku Hiva Island (French Polynesia). *Toxins 10*, 1 (2017), 2.

[13] Diogènèd, J. The chemistry of ciguatoxins: From the first records to current challenges of monitoring programs. *Toxins and Biologically Active Compounds from Microalgae 1* (2014), 176.

[14] Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research 32*, 5 (2004), 1792–1797.

[15] Farrell, H., Murray, S. A., Zammit, A., and Edwards, A. W. Management of ciguatoxin risk in Eastern Australia. *Toxins 9*, 11 (2017), 367.

[16] Farrell, H., Zammit, A., Harwood, D. T., McNabb, P., Shadbolt, C., Manning, J., Turahui, J. A., van den Berg, D. J., and Szabo, L. Clinical diagnosis and chemical confirmation of ciguatera fish poisoning in New South Wales, Australia. *Communicable Diseases Intelligence 40*, 1 (2016).

[17] Faust, M. A. Observation of sand-dwelling toxic dinoflagellates (Dinophyceae) from widely differing sites, including two new species. *Journal of Phycology 31*, 6 (1995), 996–1003.

[18] Fraga, S., and Rodríguez, F. Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp. nov., a new potentially toxic epiphytic benthic dinoflagellate. *Protist 165*, 6 (2014), 839–853.

[19] Fraga, S., Rodríguez, F., Caillaud, A., Diogène, J., Raho, N., and Zapata, M. *Gambierdiscus excentricus* sp. nov.(Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). *Harmful Algae 11* (2011), 10–22.

[20] Fraga, S., Rodríguez, F., Riobó, P., and Bravo, I. *Gambierdiscus balechii* sp. nov (Dinophyceae), a new benthic toxic dinoflagellate from the Celebes Sea (SW Pacific Ocean). *Harmful Algae* (2016).

[21] Gómez, F., Qiu, D., Lopes, R. M., and Lin, S. *Fukuyoa paulensis* gen. et sp. nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). *Journal of Molecular Evolution 10*, 4 (2015), e0119676.

[22] Hallegraeff, G. M., Bolch, C., Hill, D., Jameson, I., LeRoi, J., McMinn, A., Murray, S., De Salas, M., Saunders, K., de Salas, M., et al. *Algae of Australia: phytoplankton of temperate coastal waters.* 2010.

[23] Hariganeya, N., Tanimoto, Y., Yamaguchi, H., Nishimura, T., Tawong, W., Sakanari, H., Yoshimatsu, T., Sato, S., Preston, C. M., and Adachi, M. Quantitative pcr method for enumeration of cells of cryptic species of the toxic marine dinoflagellate Ostreopsis spp. in coastal waters of Japan. *PloS one 8*, 3 (2013), e57627.

[24] Holmes, M. J. *Gambierdiscus yasumotoi* sp. nov.(Dinophyceae), a toxic benthic dinoflagellate from southeastern Asia. *Journal of Phycology 34*, 4 (1998), 661–668.

[25] Intergovernmental Oceanographic Commission of UNESCO. *Global Ciguatera Strategy*, 2015 (accessed January 7, 2016). `http://hab.ioc-unesco.org/index.php?option=com_oe&task=viewDocumentRecord&docID=15111&allversions=0`.

[26] Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., et al.

Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics 28*, 12 (2012), 1647–1649.

[27] Kohli, G. S., Murray, S. A., Neilan, B. A., Rhodes, L. L., Harwood, D. T., Smith, K. F., Meyer, L., Capper, A., Brett, S., and Hallegraeff, G. M. High abundance of the potentially maitotoxic dinoflagellate *Gambierdiscus carpenteri* in temperate waters of New South Wales, Australia. *Harmful Algae 39* (2014), 134–145.

[28] Kohli, G. S., Neilan, B. A., Brown, M. V., Hoppenrath, M., and Murray, S. A. Cob gene pyrosequencing enables characterization of benthic dinoflagellate diversity and biogeography. *Environmental microbiology 16*, 2 (2014), 467–485.

[29] Kohli, G. S., Papiol, G. G., Rhodes, L. L., Harwood, D. T., Selwood, A., Jerrett, A., Murray, S. A., and Neilan, B. A. A feeding study to probe the uptake of maitotoxin by snapper (*Pagrus auratus*). *Harmful Algae 37* (2014), 125–132.

[30] Kraeer, K., and van Essen-Fishman, L. *Pseudocaranx dentex* (white trevally). `ian.umces.edu/imagelibrary/`, 2010. Integration and Application Network, University of Maryland Center for Environmental Science.

[31] Kretzschmar, A. L., Verma, A., Harwood, T., Hoppenrath, M., and Murray, S. Characterization of *Gambierdiscus lapillus* sp. nov.(Gonyaulacales, Dinophyceae): A new toxic dinoflagellate from the Great Barrier Reef (Australia). *Journal of phycology 53*, 2 (2017), 283–297.

[32] Larsson, M. E., Laczka, O. F., Harwood, D. T., Lewis, R. J., Himaya, S., Murray, S. A., and Doblin, M. A. Toxicology of *Gambierdiscus* spp.(dinophyceae) from Tropical and Temperate Australian Waters. *Marine drugs 16*, 1 (2018), 7.

[33] Lewis, R. J. Ciguatera: Australian perspectives on a global problem. *Toxicon 48*, 7 (2006), 799–809.

[34] Litaker, R. W., Vandersea, M. W., Faust, M. A., Kibler, S. R., Chinain, M., Holmes, M. J., Holland, W. C., and Tester, P. A. Taxonomy of *Gambierdiscus* including four new species, *Gambierdiscus caribaeus*, *Gambierdiscus carolinianus*, *Gambierdiscus carpenteri* and *Gambierdiscus ruetzleri* (Gonyaulacales, Dinophyceae). *Phycologia 48*, 5 (2009).

[35] Litaker, R. W., Vandersea, M. W., Faust, M. A., Kibler, S. R., Nau, A. W., Holland, W. C., Chinain, M., Holmes, M. J., and Tester, P. A. Global distribution of ciguatera causing dinoflagellates in the genus *Gambierdiscus*. *Toxicon 56*, 5 (2010), 711–730.

[36] Lobel, P. S., Anderson, D. M., and Durand-Clement, M. Assessment of ciguatera dinoflagellate populations: sample variability and algal substrate selection. *The Biological Bulletin 175*, 1 (1988), 94–101.

[37] Murata, M., Legrand, A. M., Ishibashi, Y., and Yasumoto, T. Structures of ciguatoxin and its congener. *Journal of the American chemical Society 111*, 24 (1989), 8929–8931.

[38] Murata, M., Naoki, H., Iwashita, T., Matsunaga, S., Sasaki, M., Yokoyama, A., and Yasumoto, T. Structure of maitotoxin. *Journal of the American Chemical Society 115*, 5 (1993), 2060–2062.

[39] Murray, S., Momigliano, P., Heimann, K., and Blair, D. Molecular phylogenetics and morphology of *Gambierdiscus yasumotoi* from tropical eastern Australia. *Harmful Algae 39* (2014), 242–252.

[40] Murray, S. A., Wiese, M., Stüken, A., Brett, S., Kellmann, R., Hallegraeff, G., and Neilan, B. A. sxta-based quantitative molecular assay to identify saxitoxin-producing harmful algal blooms in marine waters. *Applied and environmental microbiology 77*, 19 (2011), 7050–7057.

[41] Nagai, H., Murata, M., Torigoe, K., Satake, M., and Yasumoto, T. Gambieric acids, new potent antifungal substances with unprecedented polyether structures from a marine dinoflagellate *Gambierdiscus toxicus*. *The Journal of Organic Chemistry 57*, 20 (1992), 5448–5453.

[42] Nishimura, T., Hariganeya, N., Tawong, W., Sakanari, H., Yamaguchi, H., and Adachi, M. Quantitative pcr assay for detection and enumeration of ciguatera-causing dinoflagellate *Gambierdiscus* spp.(gonyaulacales) in coastal areas of japan. *Harmful Algae 52* (2016), 11–22.

[43] Nishimura, T., Sato, S., Tawong, W., Sakanari, H., Yamaguchi, H., and Adachi, M. Morphology of *Gambierdiscus scabrosus* sp. nov.(Gonyaulacales): a new epiphytic toxic dinoflagellate from coastal areas of Japan. *Journal of Phycology 50*, 3 (2014), 506–514.

[44] Parsons, M. L., Settlemier, C. J., and Ballauer, J. M. An examination of the epiphytic nature of *Gambierdiscus toxicus*, a dinoflagellate involved in ciguatera fish poisoning. *Harmful algae 10*, 6 (2011), 598–605.

[45] Queensland Government, Queensland Health. *Notifiable conditions annual reporting*, 2016 (accessed December 30, 2016). `https://www.health.qld.gov.au/clinical-practice/guidelines-procedures/diseases-infection/surveillance/reports/notifiable/annual`.

[46] R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, 2013.

[47] Rhodes, L., Harwood, T., Smith, K., Argyle, P., and Munday, R. Production of ciguatoxin and maitotoxin by strains of *Gambierdiscus australes*, *G. pacificus* and *G. polynesiensis* (Dinophyceae) isolated from Rarotonga, Cook Islands. *Harmful Algae 39* (2014), 185–190.

[48] Rhodes, L., Smith, K. F., Verma, A., Curley, B. G., Harwood, D. T., Murray, S., Kohli, G. S., Solomona, D., Rongo, T., Munday, R., and Murray, S. A. A new species of *Gambierdiscus* (Dinophyceae) from the south-west Pacific: *Gambierdiscus honu* sp. nov. *Harmful Algae 65* (2017), 61–70.

[49] Rhodes, L. L., Smith, K. F., Murray, S., Harwood, D. T., Trnski, T., and Munday, R. The epiphytic genus *Gambierdiscus* (Dinophyceae) in the Kermadec Islands and Zealandia regions of the southwestern Pacific and the associated risk of ciguatera fish poisoning. *Marine drugs 15*, 7 (2017), 219.

[50] Richlen, M. L., Morton, S. L., Barber, P. H., and Lobel, P. S. Phylogeography, morphological variation and taxonomy of the toxic dinoflagellate *Gambierdiscus toxicus* (Dinophyceae). *Harmful Algae 7*, 5 (2008), 614–629.

[51] Rodriguez, I., Genta-Jouve, G., Alfonso, C., Calabro, K., Alonso, E., Sanchez, J. A., Alfonso, A., Thomas, O. P., and Botana, L. M. Gambierone, a ladder-shaped polyether from the dinoflagellate *Gambierdiscus belizeanus*. *Organic letters 17*, 10 (2015), 2392–2395.

[52] RStudio Team. *RStudio: Integrated Development Environment for R*. RStudio, Inc., Boston, MA, 2015.

[53] Satake, M., Murata, M., and Yasumoto, T. Gambierol: a new toxic polyether compound isolated from the marine dinoflagellate *Gambierdiscus toxicus*. *Journal of the American Chemical Society 115*, 1 (1993), 361–362.

[54] Saxby, T. *Scomberomorus munroi* (australian spotted mackerel). `ian.umces.edu/imagelibrary/`, 2004. Integration and Application Network, University of Maryland Center for Environmental Science.

[55] Sims, J. K. A theoretical discourse on the pharmacology of toxic marine ingestions. *Annals of emergency medicine 16*, 9 (1987), 1006–1015.

[56] Smith, C. J., and Osborn, A. M. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS microbiology ecology 67*, 1 (2009), 6–20.

[57] Smith, K. F., Biessy, L., Argyle, P. A., Trnski, T., Halafihi, T., and Rhodes, L. L. Molecular identification of *Gambierdiscus* and *Fukuyoa* (Dinophyceae) from environmental samples. *Marine Drugs 15*, 8 (2017), 243.

[58] Smith, K. F., Rhodes, L., Verma, A., Curley, B. G., Harwood, D. T., Kohli, G. S., Solomona, D., Rongo, T., Munday, R., and Murray, S. A. A new *Gambierdiscus* species (Dinophyceae) from Rarotonga, Cook Islands: *Gambierdiscus cheloniae* sp. nov. *Harmful Algae 60* (2016), 45–56.

[59] Sparrow, L., Momigliano, P., Russ, G. R., and Heimann, K. Effects of temperature, salinity and composition of the dinoflagellate assemblage on the growth of *Gambierdiscus carpenteri* isolated from the Great Barrier Reef. *Harmful Algae 65* (2017), 52–60.

[60] Taylor, F., and Gustavson, M. An underwater survey of the organism chiefly responsible for ciguatera fish poisoning in the eastern Caribbean region: the benthic dinoflagellate *Gambierdiscus toxicus*. In *Proceedings of the 7th. International Diving Science Symposium* (1986), CMAS, University of Padua, pp. 95–111.

[61] Tester, P. A., Kibler, S. R., Holland, W. C., Usup, G., Vandersea, M. W., Leaw, C. P., Teen, L. P., Larsen, J., Mohammad-Noor, N., Faust, M. A., et al. Sampling harmful benthic dinoflagellates: Comparison of artificial and natural substrate methods. *Harmful Algae 39* (2014), 8–25.

[62] Tonge, J., Battey, Y., Forbes, J., Grant, E., et al. Ciguatera poisoning: a report of two out-breaks and a probable fatal case in Queensland. *Medical Journal of Australia 2*, 24 (1967), 1088–90.

[63] Vandersea, M. W., Kibler, S. R., Holland, W. C., Tester, P. A., Schultz, T. F., Faust, M. A., Holmes, M. J., Chinain, M., and Wayne Litaker, R. Development of semi-quantitative pcr assays for the detection and enumeration of *Gambierdiscus* species (Gonyaulacales, Dinophyceae) 1. *Journal of Phycology 48*, 4 (2012), 902–915.

[64] Verma, A., Hoppenrath, M., Dorantes-Aranda, J. J., Harwood, D. T., and Murray, S. A. Molecular and phylogenetic characterization of *Ostreopsis* (dinophyceae) and the description of a new species, *Ostreopsis rhodesae* sp. nov., from a subtropical Australian lagoon. *Harmful algae 60* (2016), 116–130.

[65] Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York, 2009.

[66] Woerner, J. *Padina* (brown algae)). `ian.umces.edu/imagelibrary/`, 2011. Integration and Application Network, University of Maryland Center for Environmental Science.

[67] Xu, Y., Richlen, M. L., Morton, S. L., Mak, Y. L., Chan, L. L., Tekiau, A., and Anderson, D. M. Distribution, abundance and diversity of *Gambierdiscus* spp. from a ciguatera-endemic area in Marakei, Republic of Kiribati. *Harmful Algae 34* (2014), 56–68.

[68] Yasumoto, T., Nakajima, I., Bagnis, R., and Adachi, R. Finding of a dinoflagellate as a likely culprit of ciguatera. *Bulletin of the Japanese Society of Scientific Fisheries (Japan)* (1977).