Harmful Algae 97 (2020) 101853

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

The role of 44-methylgambierone in ciguatera fish poisoning: Acute toxicity, production by marine microalgae and its potential as a biomarker for *Gambierdiscus* spp.

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ARTICLE INFO

Keywords:
Ciguatoxin
Benthic dinoflagellate
Liquid chromatography-tandem mass spectrometry
Maitotoxin-3
Quantitative nuclear magnetic resonance spectroscopy

ABSTRACT

Ciguatera fish poisoning (CFP) is prevalent around the tropical and sub-tropical latitudes of the world and impacts many Pacific island communities intrinsically linked to the reef system for sustenance and trade. While the genus *Gambierdiscus* has been linked with CFP, it is commonly found on tropical reef systems in microalgal assemblages with other genera of toxin-producing, epiphytic and/or benthic dinoflagellates – *Amphidinium, Coolia, Fukuyoa, Ostreopsis* and *Prorocentrum*. Identifying a biomarker compound that can be used for the early detection of *Gambierdiscus* blooms, specifically in a mixed microalgal community, is paramount in enabling the development of management and mitigation strategies. Following on from the recent structural elucidation of 44-methylgambierone, its potential to contribute to CFP intoxication events and applicability as a biomarker compound for *Gambierdiscus* spp. was investigated. The acute toxicity of this secondary metabolite was determined by intraperitoneal injection using mice, which showed it to be of low toxicity, with an LD_{50} between 20 and 38 mg kg^{-1}. The production of 44-methylgambierone by 252 marine microalgal isolates consisting of 90 species from 32 genera across seven classes, was assessed by liquid chromatography-tandem mass spectrometry. It was discovered that the production of this secondary metabolite was ubiquitous to the eight *Gambierdiscus* species tested, however not all isolates of *G. carpenteri*, and some species/isolates of *Coolia* and *Fukuyoa*.

1. Introduction

Ciguatera fish poisoning (CFP) is the most common non-microbial, food-borne illness in the world. It can be extremely debilitating, with symptoms potentially lasting years. Intoxications manifest as a wide array of symptoms including gastrointestinal discomfort (e.g. nausea and diarrhoea), neurological impairment (e.g. parathesia and dysaesthesia) and/or cardiovascular complications (e.g. hypotension and bradycardia) (Friedman et al., 2017; Diogene, 2018). The syndrome is prevalent in the circumtropical regions of the world, including areas of the Pacific Ocean, Indian Ocean, Caribbean Sea and the Gulf of Mexico (Friedman et al., 2017). While the existence of CFP has been known for centuries (Friedman et al., 2008), the true level of incidence is not known. It is estimated that 25,000–50,000 people are affected annually, with epidemiological studies indicating that < 20% of actual cases are reported (ILM, 2014).

Ciguatera fish poisoning is caused by the consumption of reef fish contaminated with ciguatoxins (CTXs) and possibly other compounds, including maitotoxins (MTXs). These compounds are produced by the epiphytic, benthic dinoflagellate genus *Gambierdiscus* and are some of

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https://doi.org/10.1016/j.hal.2020.101853
Received 21 April 2020; Received in revised form 1 June 2020; Accepted 7 June 2020
Available online 19 June 2020

the most potent non-peptide toxins known (Friedman et al., 2008). In addition to CTXs and MTXs (Rhodes et al., 2014), other bioactive, ladder-shaped polyether secondary metabolites such as gambieric acids (Morohashi et al., 2000), gambierol (Morohashi et al., 1999), gambieroxide (Watanabe et al., 2013) and gambierones (Murray et al., 2019) are produced by *Gambierdiscus*. However, the role that these compounds play in intoxication events is currently unknown.

Ciguatoxins have been demonstrated to bioaccumulate and biotransform into more toxic analogues as they move up the marine food chain, from herbivorous fish grazing on coral (e.g. parrot fish, *Scaridae* spp.) or macroalgae (e.g. mullet, *Mugil cephalus*) (Yasumoto et al., 1971, 1977; Ledreux et al., 2014; Clausing et al., 2018), to the higher trophic level omnivorous (e.g. wrasse, *Cheilinus* spp.) and carnivorous reef fish species that predate upon them (e.g. Spanish mackerel, *Scomberomorus* spp.) (Murray et al., 2016; Kohli et al., 2017). While CTXs have been shown to bioaccumulate in fish of all trophic levels, with published estimates of CFP vectors ranging from 60 (Gaboriau et al., 2014) to 90 (Kohli et al., 2015) to over 400 species (FAO, 2014), it is the carnivorous fish species that are most commonly implicated in CFP cases as they are often targeted by commercial and recreational fishers. As a result, carnivorous fish species are responsible for 68% of intoxication events in French Polynesia and 85% in New Caledonia (Caillaud et al., 2010). The role of the additional secondary metabolites produced by *Gambierdiscus* in intoxication events is currently unknown.

Ciguatera fish poisoning is particularly prolific throughout the tropical and sub-tropical waters of the South Pacific and affects many indigenous island communities intrinsically linked to the reef system for sustenance and trade. In addition, climate change is causing an increase in global ocean temperatures, resulting in an expansion of the sub-tropical latitudes (Rhodes et al., 2020). Consequently, the habitable regions for *Gambierdiscus* are expanding. These regions now include the New South Wales coastline (Australia), the Rangitāhua/Kermadec Islands (a New Zealand territory) and mainland Aotearoa/New Zealand (Rhodes and Smith, 2019). To date, only one *Gambierdiscus* species has been reported from New Zealand's mainland and was reported from a single cell attached to floating macroalgae, *Sargassum* (Chang, 1996). No culturing was undertaken with the single cell and therefore no chemical analysis nor phylogenic investigation was performed. It is however noted that Pacific-CTX (P-CTX) producing strains of *Gambierdiscus polynesiensis* have been isolated from the Kermadec Islands (Rhodes et al., 2020).

Gambierdiscus is found attached to macroalgae (e.g. in the Pacific region it favours filamentous red and calcareous green species), coralline turfs, dead corals and volcanic sands around the world (Rhodes et al., 2017a). It is regarded as an opportunistic dinoflagellate that proliferates following damage to the reef system from tropical hurricanes, crown of thorn starfish outbreaks or coral bleaching events (Rongo and van Woesik, 2013; Xu et al., 2016). Adding to the complexity of CFP is that *Gambierdiscus* is commonly found co-habitating in assemblages with other toxin-producing benthic dinoflagellates from the genera *Amphidinium, Coolia, Fukuyoa, Ostreopsis* and *Prorocentrum* (Hachani et al., 2018; Yong et al., 2018; Rhodes and Smith, 2019). These genera of microalgae produce multiple toxic secondary metabolites including palytoxins (Ishii et al., 1997), okadaic acid (Malagoli et al., 2008), dinophysistoxins (Carmody et al., 1996) and amphidinols (Echigoya et al., 2005). While significant research has been conducted on these metabolites to determine their toxicity, they are currently not considered to play a role in CFP events.

The World Health Organisation considers the CFP syndrome a neglected disease worldwide and in 2015, to help promote research activities on CFP, the United Nations Educational, Scientific and Cultural Organisation (UNESCO) formulated a global research strategy (IOC/IPHAB Global Ciguatera Strategy 2015–2019) through its Intergovernmental Oceanographic Panel on Harmful Algal Blooms (IPHAB). This document highlighted several priority research areas, including the urgent need for improved monitoring capabilities for CTXs and MTXs, along with the development of tools for early warning/monitoring of toxic *Gambierdiscus* blooms (IOC/UNESCO, 2015). One such tool could be the identification of a biomarker compound(s) in the environment, as this would enable the development of better management and mitigation strategies.

Biomarker compounds have been used for epidemiological investigations for decades and in more recent years have been adopted as an early detection method for the planktonic paralytic shellfish poisoning dinoflagellate genus *Alexandrium* (Chan et al., 2006). However, identifying a biomarker for CFP is complicated as the causative CTXs are produced at very low levels, are analytically difficult to detect and their production has only been confirmed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in one species, *G. polynesiensis* (Chinain et al., 2010; Rhodes et al., 2014). As a result, there is currently no biomarker compound that can be used to confirm CFP intoxication in humans nor has one been identified for the early detection of toxic *Gambierdiscus* blooms in the environment (Friedman et al., 2008). In an effort to develop biomarkers for CFP, progress has been made in animal studies using blood samples, however, these are not appropriate in human clinical scenarios (Friedman et al., 2017).

One secondary metabolite that is more easily detectable by LC-MS/MS than CTXs is 44-methylgambierone (Fig. 1). This compound has previously been reported as putative maitotoxin-3 (MTX-3) and was recently structurally characterised from *G. australes* (Murray et al., 2019) and *G. belizeanus* (Boente-Juncal et al., 2019). To better understand whether 44-methylgambierone plays a role in CFP intoxication events, its acute toxicity to mice was determined by intraperitoneal injection. To evaluate the potential of 44-methylgambierone to be used as a biomarker for the presence of *Gambierdiscus* spp. in areas of high CFP risk, this study screened its production in 252 microalgal isolates consisting of 90 species from 32 genera across seven classes using LC-MS/MS.

2. Materials and methods

2.1. Chemicals and reagents

High purity methanol (MeOH) and acetonitrile (MeCN) were obtained from Thermo-Fisher (Fisher-Optima). Purified water (18.2 MΩ) was produced with a Milli-Q system (Millipore, Canada). Ammonium hydroxide (≥ 25%) was from Honeywell Research Chemicals.

2.2. Acute toxicity of 44-methylgambierone by intraperitoneal injection

2.2.1. Quantitative nuclear magnetic resonance spectrometry

44-methylgambierone reference material was produced in-house from a cultured *G. australes* isolate (CAWD149) using the purification procedure described in Murray et al. (2019). The reference material was dissolved in 0.5 mL of deuterated methanol (CD_3OD) and transferred to a 5 mm NMR tube. The millimole level of the reference sample, and subsequently the mg mL^{-1} concentration, of 44-methylgambierone in

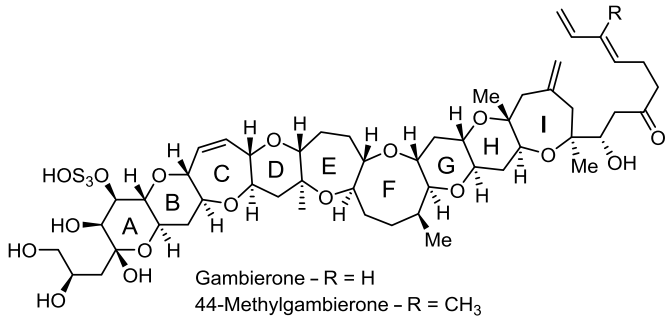

Fig. 1. Structures of gambierone and 44-methylgambierone, previously reported as maitotoxin-3 (MTX-3; Murray et al., 2019).

the reference solution was determined by quantitative nuclear magnetic resonance spectroscopy (QNMR) using a Bruker AVIII-HD 8000 MHz NMR spectrometer and electronic reference to assess *in-vivo* concentrations (ERETIC2) QNMR software (Wider and Dreier, 2006). External standard quantification was performed using a dioxane solution (1.58 mg mL^{-1} in CD_3OD). Eight sets of triplicate ERETIC2 QNMR analyses were performed over several days using 90- or 30-degree pulses with 60- or 30 s pulse delays respectively (total $n = 24$). Quantification was performed using the integrated peak areas of the four methyl proton signals of 44-methylgambierone which occurred at 1.23 (s), 1.21 (s), 1.15 (s) and 1.02 (d) ppm (equivalent to 12 protons) and the dioxane proton signal at 3.68 ppm (equivalent to eight protons). The concentration of the 44-methylgambierone reference solution was determined to be 2.33 ± 0.02 mg mL^{-1}, with a precision of 0.9% relative standard deviation.

2.2.2. Animals

Female Swiss albino mice (18–22 g) were bred at AgResearch, Ruakura, New Zealand. The mice were housed individually during the experiments and were allowed unrestricted access to food (Rat and Mouse Cubes, Speciality Feeds Ltd., Glen Forrest, Western Australia) and water. All experiments were approved by the Ruakura Animal Ethics Committee established under the Animal Protection (code of ethical conduct) Regulations Act, 1987 (New Zealand), Project Number 14,320, approval date 2 November 2017.

2.2.3. Toxicity assessment

Acute toxicity was determined using the principles of Organisation for Economic Co-operation and Development (OECD) guideline 425 (OECD, 2006). This guideline employs an up-and-down procedure whereby one animal is dosed and if it survives the dose for the next animal is increased, whereas if it dies, the dose for the next animal is decreased. To determine the LD_{50}, dosing is continued until four live-death reversals have been achieved.

Toxicity was determined by intraperitoneal injection. Each mouse was weighed prior to dosing and the appropriate quantities of test compound calculated to yield the required dose on a mg kg^{-1} basis. The dose was prepared by taking the appropriate volume of stock solution (pure 44-methylgambierone in 90% aq. MeOH), drying it down under nitrogen and immediately re-dissolving in 1% Tween 60 in normal saline (1 mL) with the aid of sonication. This solution was injected into mice. All dosing was conducted between 8 and 9.30 am to avoid any diurnal variations in response. Mice were monitored intensively during the day of dosing and any that survived were monitored for a 14-day period which included a daily measurement of food consumption and bodyweight. After 14 days, the animals were euthanized by carbon dioxide inhalation and necropsied. The weights of the liver, kidneys, spleen, heart, lungs, stomach (full and empty) and the whole gut were measured and expressed as a percentage of bodyweight.

2.3. Screening of microalgal cultures for 44-methylgambierone production

2.3.1. Microalgal culturing and sample extraction

Microalgal isolates (252 in total) consisted of 90 species from 32 genera across seven classes. Depending on the specific nutritional requirements of each genus, cultures were grown in either 25% f/2, 33% f/2, f/2 (Guillard and Ryther, 1962), GP, 50% GP, K, modified K, L1 (Bigelow, 1985) or metals mix SWII medium (Matsuda et al., 1996; Nishimura et al., 2019) diluted in sterile seawater (autoclaved and filtered; 0.22-μm). Depending on the origin of the isolate, the cultures were grown at either 17 °C (± 2 °C; for temperate isolates) or 25 °C (± 2 °C; for sub-tropical and tropical isolates) with 40–70 μmol m^{-2} s^{-1} photon irradiance and a 12:12 h light:dark cycle. Isolates were sourced from previous research expeditions; the Cawthron Institute Culture Collection of Microalgae (CICCM); or donated as frozen cell pellets by researchers from French Polynesia, Hong Kong, Spain and Australia. Cultures were harvested in the late exponential or stationary phase and contained at least 1×10^6 cells. The cells were harvested by centrifugation (3200 × g, 4 °C, 10 min), the growth medium decanted, and the resulting cell pellets frozen at −20 °C.

Each cell pellet was extracted twice with 90% aq. MeOH at a ratio of 1 mL per 2×10^5 cells using ultrasonication for 10 min in a 59 kHz water bath (model 160HT, Soniclean Pty, Australia). Cellular debris was pelleted by centrifugation (3200 × g, 4 °C, 5 min) and the supernatant was transferred to another vial before the cell pellets were re-extracted in the same manner. The resulting extract supernatants were pooled to give a final extract equivalent to approximately 1×10^5 cells mL^{-1}. The combined extracts were stored at –20 °C for 24–48 h to precipitate insoluble matrix co-extractives, which were removed using centrifugation (3200 × g, 4 °C, 5 min) prior to analysis. An aliquot of the clarified extract was transferred into a 2 mL glass autosampler vial for analysis by LC-MS/MS using a modified version of the method described in Murray et al. (2018).

2.3.2. Liquid chromatography-tandem mass spectrometry conditions

Analysis was performed on a Waters Xevo TQ-S triple quadrupole mass spectrometer coupled to a Waters Acquity UPLC i-Class with flow-through needle sample manager. Chromatographic separation used a Waters Acquity UPLC BEH phenyl column (1.7-μm, 100 × 2.1 mm column) held at 50 °C. The column was eluted at 0.55 mL min^{-1} with Milli-Q water (A) and acetonitrile (B) mobile phases, each containing 0.2% (v/v) of a 25% ammonium hydroxide solution. Fresh mobile phases were prepared daily to ensure optimal sensitivity and stable retention times. The initial solvent composition was 5% B with a linear gradient to 50% B from 0 − 2.5 min, ramped up to 95% B by 3 min and held at 95% B until 3.2 min, followed by a linear gradient back to 5% B at 3.5 min. The column was then re-equilibrated with 5% B until 4 min. The autosampler chamber was maintained at 10 °C and the injection volume was 1 μL. The mass spectrometer used an electrospray ionisation source operated in negative-ion mode. Other settings were: capillary voltage 3.0 kV, cone voltage 40 V, source temperature 150 °C, nitrogen gas desolvation flowrate 1000 L h^{-1} at 600 °C, cone gas 150 L h^{-1} and the collision cell was operated with 0.15 mL min^{-1} argon. 44-Methylgambierone was monitored using the following transitions: *m/z* 1037.6 > 96.8 (Channel 1) and 899.6 > 96.8 (Channel 2), with collision energies of 60 and 48 eV respectively, and using a dwell time of 30 ms.

Data acquisition and processing were performed with TargetLynx software (Waters, Milford, US). 44-Methylgambierone was identified in sample extracts based on the retention time (2.61 min) and a fragment ion ratio of 3:1 (Channel 1 / Channel 2; as determined using reference material). The isolates were analysed qualitatively yielding a result of presence/absence for 44-methylgambierone, with an 'absent' result equating to less than 2 ng mL^{-1} in an extract generated from a cell pellet of 1×10^5 cells mL^{-1}, or 0.02 pg $cell^{-1}$.

3. Results

3.1. Acute toxicity of 44-methylgambierone by intraperitoneal injection

Single mice were dosed with 44-methylgambierone at dose rates of 0.89, 4.45, 20 and 38 mg kg^{-1}. The mice dosed at 0.89, 4.45 and 20 mg kg^{-1} showed no adverse effects on the day of dosing and their movement, behaviour, appearance and respiration appeared normal throughout the 14 days of the study. At necropsy, none of these mice showed any abnormalities and the organ weights, as expressed as% of bodyweight, were all normal. On the day of dosing, the mouse dosed at 38 mg kg^{-1} also looked normal. However, over the first 24 h period, it was found to have eaten very little and although it was moving normally, its behaviour was slightly abnormal (hunched posture) by day two. At 48 h post-dosing, the mouse was alert and moving normally but

it still had a very low food intake. At 56 h post-dosing, the mouse was hunched, and its breathing was laboured. To avoid long-term suffering, this animal was euthanised and necropsied in accord with the requirements of the OECD Humane Endpoints Guidance Document (OECD, 2000). Under OECD guideline 425, this euthanised animal can be considered in the same way as an animal which died during the test. The food intake and bodyweight data suggest that this mouse, dosed with 38 mg kg^{-1} of 44-methylgambierone, suffered from anorexia and the reduced hepatic and splenic weights observed were consistent with that diagnosis. Necropsy of this mouse showed that the stomach, caecum and intestines contained a dark green, runny material. Due to the limited availability of 44-methylgambierone, a full LD_{50} determination could not be completed. However, since the mouse dosed at 20 mg kg^{-1} was healthy and death was induced at a dose rate of 38 mg kg^{-1}, the LD_{50} of 44-methylgambierone sits between 20 and 38 mg kg^{-1}.

3.2. 44-methylgambierone production by common co-habitating benthic microalgae

Isolates from the six main dinoflagellate genera that co-habitate in the Pacific region (*Amphidinium, Coolia, Fukuyoa, Gambierdiscus, Ostreopsis* and *Prorocentrum*; 190 isolates in total from 34 species) were collected during previous research expeditions to the Kermadec Islands (Raoul Island, North Meyer Island and Macauley Island), the Cook Islands and the Federated States of Micronesia, or cell pellets were received as gifts from researchers in French Polynesia, Hong Kong, Spain and Australia.

44-Methylgambierone production was ubiquitous to all *Gambierdiscus* species analysed from Australia, the Cook Islands, French Polynesia, the Kermadec Islands and Micronesia. These species included *G. australes, G. caribaeus, G. carpenteri, G. cheloniae, G. honu, G. lapillus, G. pacificus* and *G. polynesiensis* (total 78 isolates; Table 1). However, it is noted that for *G. carpenteri*, isolates from the Cook Islands and French Polynesia produced this secondary metabolite whereas *G. carpenteri* isolates from Australia did not (total 5 Australian isolates; Table 1).

In addition, of the three *Fukuyoa* species tested, a single isolate of *F. paulensis* from New Zealand produced 44-methylgambierone, as did two of the three *F. ruetzleri* isolates from Hong Kong. The two isolates of *F.* sp. HK Type 1 from Hong Kong were negative. Only two of the five species of *Coolia* tested produced 44-methylgamberieone (*C. malayensis* and *C. tropicalis*), while the isolates of *C. canariensis, C. monotis* and *C. palmyrensis* were all negative. For *C. malayensis*, all isolates from Australia produced the secondary metabolite, as did two of the five isolates from New Zealand, but the isolates from the Cook Islands and Hong Kong did not. For *C. tropicalis*, all isolates from the Cook Islands and Hong Kong produced 44-methylgambierone, while only two of the three from Australia did. The isolates of *Amphidinium, Ostreopsis* and *Prorocentrum* tested did not produce 44-methylgambierone (Table 1).

3.3. . 44-methylgambierone production by other marine microalgae genera

The Cawthron Institute curates a unique collection of microalgal isolates that includes many benthic and planktonic dinoflagellates, along with other classes of marine microalgae. To complete this survey of 44-methylgambierone production by marine microalgae, isolates (62 in total) from an additional 56 species spanning 26 genera across seven classes were analysed. None produced 44-methylgambierone (Table 2).

4. Discussion

The acute toxicity assessment of 44-methylgambierone showed that the LD_{50} of this secondary metabolite is between 20 and 38 mg kg^{-1} by intraperitoneal injection. This toxicity is relatively low in comparison to that of the CTXs implicated in CFP such as P-CTX-1B that has an LD_{50} of just 0.00036 mg kg^{-1} by intraperitoneal injection (Yogi et al., 2014). Maitotoxins have previously been considered unlikely to contribute to CFP due to their hydrophilicity and low bioaccumulation in the viscera of reef fish (Kohli et al., 2014). Additionally, although toxic by intraperitoneal injection, MTXs have low oral toxicity. 44-methylgambierone was previously classified as MTX-3. It is also hydrophilic in nature, so is similarly unlikely to bioaccumulate in reef fish flesh. This study has determined that the intraperitoneal toxicity of 44-methylgambierone is very low, demonstrating that it is highly unlikely to play a role in CFP. Anorexia observed in the mouse dosed with 44-methylgambierone at 38 mg kg^{-1}, and liquid stomach contents observed at necropsy, were consistent with observations by Munday et al. (2017) who dosed mice with *Gambierdiscus* species which expressed CTXs and MTXs. Some isolates in that study only expressed 44-methylgambierone, indicating the symptomology observed was a result of this secondary metabolite.

During the present study, the use of 44-methylgambierone as a biomarker was investigated. Preliminary research showed that its production appeared to be widespread in *Gambierdiscus* species and a LC-MS/MS signal was easily observed in culture extracts. However, little research had been conducted on the expression of this secondary metabolite by other marine microalgae. An ideal biomarker is one that is ubiquitous to a single genus, unique and is easily detected. To test the potential use of 44-methylgambierone as a biomarker, LC-MS/MS was used to assess its production amongst a wide range of marine microalgae. The 44-methylgambierone reference material used for the acute toxicity assessment was generated after the microalgal isolates were analysed. 44-Methylgambierone levels were not able to be quantified using the data that was available as the reference standard was not analysed alongside the isolates in order to compensate for day-to-day variations in the LC-MS/MS signal. Therefore, results are expressed as a qualitative measurement only. This study confirmed 44-methylgambierone production in all previously analysed isolates (Munday et al., 2017; Larsson et al., 2018; Leung et al., 2018). At the time of these earlier publications, results were reported as putative MTX-3 with the LC-MS/MS analysis being performed at Cawthron Institute (the isolates are identified in Table 1). It is now possible to report this compound as 44-methylgambierone.

The analysis of 44-methylgambierone from *Gambierdiscus* cultures demonstrated ubiquitous production across all species tested; *G. australes, G. caribaeus, G. carpenteri, G. cheloniae, G. honu, G. lapillus, G. pacificus* and *G. polynesiensis*. However, an interesting observation was that not all isolates of *G. carpenteri* produced it. All *G. carpenteri* isolates from Australia (total 5 isolates) were negative, while other *G. carpenteri* isolates from Pacific locations (the Cook Islands and French Polynesia) were all positive for the production of 44-methylgambierone. It is noted that *G. carpenteri* is not a CTX producer, so depending on the intended application (i.e. identifying CFP hot spots), the negative isolates might not pose an issue. In the literature, additional *Gambierdiscus* spp., not tested during this study, have been shown to produce 44-methylgambierone (reported as putative MTX-3). These include *G. balechii, G. belizeanus, G. carolinianus, G. excentricus, G. scabrosus, G. silvae, G. toxicus* (Pisapia et al., 2017); *G. holmesii* and *G. lewisii* (Kretzschmar et al., 2019). Collectively, these results confirm that 44-methylgambierone production is ubiquitous to the 17 species tested to date, with only *G. jejuensis* remaining untested.

To date, only three *Fukuyoa* species have been described and two were analysed during this study (*F. paulensis* and *F. ruetzleri*). Five of the eight *Coolia* species described were also analysed during this study (*C. canariensis, C. malayensis, C. monotis, C. palmyrensis* and *C. tropicalis*). The results showed that some isolates of *F. paulensis* (New Zealand), *F. ruetzleri* (Hong Kong), *C. malayensis* (New Zealand and Australia) and *C. tropicalis* (Cook Islands, Australia and Hong Kong) produced 44-methylgambierone. The production of this secondary metabolite by the genus *Coolia* supports the findings recently published by Yan et al. (2020) and limits its use as a *Gambierdiscus*-specific biomarker. To complete the study on 44-methylgambierone production by

Table 1
Summary of the 190 benthic dinoflagellate isolates from 34 species spanning 6 genera tested for 44-methylgambierone production.

Genus	Species	Location	Number of isolates	44-methylgambierone production
Gambierdiscus	*australes*	Cook Islands	8[a]	+
		Kermadec Islands	43	+
	caribaeus	Micronesia	1	+
	carpenteri	Cook Islands	4	+
		French Polynesia	3	+
		Australia	5[b]	–
	cheloniae	Cook Islands	2[a]	+
	honu	Cook Islands	2[a]	+
		Kermadec Islands	2	+
	lapillus	Cook Islands	2	+
	pacificus	Cook Islands	3[a]	+
		Australia	1	+
	polynesiensis	Cook Islands	1[a]	+
		Kermadec Islands	1	+
Fukuyoa	sp. HK Type 1	Hong Kong	2[c]	–
	paulensis	New Zealand	1[a]	+
	ruetzleri	Hong Kong	3[c]	+/– (2/3)
Coolia	*canariensis*	Hong Kong	2	–
	malayensis	New Zealand	5	+/– (2/5)
		Australia	3	+
		Cook Islands	3	–
		Hong Kong	1	–
	monotis	Spain	1	–
	palmyrensis	Australia	2	–
		Hong Kong	2	–
	tropicalis	Cook Islands	5	+
		Australia	3	+/– (2/3)
		Hong Kong	2	+
Amphidinium	cf. *boggayum*	New Zealand	1	–
	carterae	Hong Kong	1	–
		New Zealand	1	–
	massartii	Cook Islands	1	–
	thermaeum	Cook Islands	1	–
	trulla	New Zealand	1	–
	sp.	Australia	3	–
		Cook Islands	1	–
Ostreopsis	*rhodesiae*	Australia	7	–
	siamensis	New Zealand	10	–
		Australia	3	–
Prorocentrum	*concavum*	Hong Kong	2	–
	dentatum	Hong Kong	2	–
	gracile	Hong Kong	2	–
	HK sp. 2	Hong Kong	2	–
	koreanum	Hong Kong	2	–
	lima	New Zealand	26	–
		Kermadec Islands	1	–
		Australia	2	–
	cf. *lima*	Hong Kong	2	–
	mexicanum	Hong Kong	2	–
	rhathymum	Australia	2	–
	triestinum	Hong Kong	2	–

[a] Results from these isolates have been previously published as MTX-3 (Munday et al., 2017).
[b] Results from these isolates have been previously published as MTX-3 (Larsson et al., 2018).
[c] Results from these isolates have been previously published as MTX-3 (Leung et al., 2018).

Fukuyoa and *Coolia*, further analyses need to be made on *F. yasumotoi, C. areolata, C. guanchica* and *C. santacroce*. No isolates analysed from the genera *Amphidinium, Ostreopsis* and *Prorocentrum* tested positive.

Fukuyoa and *Gambierdiscus* have a close phylogenetic relationship and in fact *Fukuyoa* was originally classified as *Gambierdiscus* before being reclassified as a separate genus by Gómez et al. (2015). It is therefore not surprising that both genera produce 44-methylgambierone. *Coolia* and *Ostreopsis* are within the same family as *Fukuyoa* and *Gambierdiscus* (Ostreopsidaceae) but not as closely related (Rhodes et al., 2017b). It is interesting that whereas some species of *Coolia* produced 44-methylgambierone none of the *Ostreopsis* species tested did. *Amphidinium* and *Prorocentrum* show even higher genetic divergence away from *Gambierdiscus* (compared to that of *Coolia*) and it was anticipated (correctly) that these commonly found co-habitating genera would not produce 44-methylgambierone. While these benthic genera are producers of other structurally unrelated compounds, their contribution to CFP intoxications is still unclear. Further research into identifying the genes involved in the production of 44-methylgambierone may shed some light on why the production of this secondary metabolite is observed in many isolates of three genera of co-habitating benthic dinoflagellates (*Gambierdiscus, Fukuyoa* and *Coolia*) and not others.

To gain some understanding on the specificity of 44-methylgambierone as a biomarker compound, additional benthic and planktonic dinoflagellates and other classes of marine microalgae from the CICCM were also analysed. A total of 56 species spanning 26 genera across seven classes were assessed and all were negative for 44-methylgambierone production.

While these results show that 44-methylgambierone cannot be used as an exclusive indicator of toxic *Gambierdiscus* blooms, it may still be

Table 2
Summary of the 62 isolates from 56 species spanning 26 genera across 7 classes tested for 44-methylgambierone production from the Cawthron Institute Culture Collection of Microalgae (CICCM).

Class	Genus	Species	Location	Number of isolates	CICCM code	44-methylgambierone production
Bacillariophyceae (Diatoms)	*Paralia*	*marina*	New Zealand	1	CAWB38	–
	Pseudo-nitzschia	*pungens*	New Zealand	1	CAWB125	–
Coccolithophyceae	*Chrysochromulina*	*apheles*	New Zealand	1	CAWP05	–
		camella	New Zealand	1	CAWP16	–
		ericina	New Zealand	1	CAWP31	–
		simplex	New Zealand	1	CAWP20	–
	Phaeocystis	*globosa*	New Zealand	1	CAWP26	–
		cf. *pouchetii*	New Zealand	1	CAWP24	–
	Pleurochrysis	*dentata*	New Zealand	1	CAWP39	–
Cryptophyceae	*Cryptomonas*	sp.	New Zealand	1	CAWCr01	–
Dictyochophyceae	*Pseudochattonella*	*verruculosa*	New Zealand	1	CAWDC03	–
Dinophyceae (Dinoflagellates)	*Akashiwo*	*sanguinea*	New Zealand	1	CAWD01	–
	Alexandrium	*fraterculus*	New Zealand	1	CAWD52	–
		margalefii	New Zealand	1	CAWD10	–
		minutum	New Zealand	1	CAWD11	–
		ostenfeldii	New Zealand	1	CAWD135	–
		pacificum	New Zealand	1	CAWD260	–
		pseudogonyaulax	New Zealand	1	CAWD54	–
	Amphidinium	*carterae*	New Zealand	1	CAWD22	–
		cf. *boggayum*	New Zealand	1	CAWD164	–
		massartii	Cook Islands	1	CAWD231	–
		thermaeum	Cook Islands	1	CAWD265	–
		trulla	New Zealand	1	CAWD68	–
		sp.	Cook Islands	1	CAWD162	–
	Gonyaulax	cf. *elegans*	New Zealand	1	CAWD143	–
		hyalina	New Zealand	1	CAWD100	–
		sp.	New Zealand	1	CAWD141	–
	Gymnodinium	*aureolum*	New Zealand	1	CAWD59	–
		catenatum	New Zealand	1	CAWD102	–
		dorsalisulcum	New Zealand	1	CAWD225	–
		impudicum	New Zealand	1	CAWD139	–
		cf. *microreticulatum*	New Zealand	1	CAWD191	–
		simplex	New Zealand	1	CAWD86	–
		sp.	New Zealand	1	CAWD172	–
	Heterocapsa	*niei*	New Zealand	1	CAWD88	–
		triquetra	New Zealand	1	CAWD36	–
	Karenia	*bidigitata*	New Zealand	1	CAWD80	–
		brevis	USA	1	CAWD08 (Wilson strain)	–
		brevisulcata	New Zealand	1	CAWD82	–
		mikimotoi	New Zealand	1	CAWD192	–
		papilionacea	New Zealand	1	CAWD91	–
		selliformis	New Zealand	1	CAWD79	–
		umbella	New Zealand	1	CAWD131	–
	Karlodinium	*veneficum*	New Zealand	1	CAWD93	–
	Lepidodinium	*chlorophorum*	New Zealand	1	CAWD62	–
	Lingulodinium	*polyedrum*	New Zealand	1	CAWD240	–
	Protoceratium	*reticulatum*	New Zealand	1	CAWD127	–
	Scrippsiella	sp.	New Zealand	1	CAWD67	–
	Takayama	*helix*	New Zealand	1	CAWD128	–
		tasmanica	New Zealand	1	CAWD115	–
	Togula	*jolla*	New Zealand	1	CAWD41	–
	Vulcanodinium	*rugosum*	New Zealand	7	CAWD163	–
					CAWD166	
					CAWD167	
					CAWD168	
					CAWD170	
					CAWD171	
					CAWD178	
Pavlovophyceae	*Pavlomulina*[a]	*kotuku*[a]	New Zealand	1	CAWP21	–
Raphidophyceae	*Chattonella*	*marina* var. *antiqua*	New Zealand	1	CAWR18	–
	Fibrocapsa	*japonica*	New Zealand	1	CAWR02	–
	Heterosigma	*akashiwo*	New Zealand	1	CAWR08	–

[a] The genus and species name referenced are tentative only due to the proposal for these names not being published yet.

useful as a biomarker compound for *in-situ* screening of at-risk areas. While a positive result would most likely indicate a high microalgal abundance of *Gambierdiscus* species, it is noted that a negative result would also be useful in showing an area might be of low risk from toxic CFP blooms. One technique that has shown promise is solid-phase absorption toxin tracking (SPATT) (MacKenzie et al., 2004) for *in-situ* sampling. Proof of concept for the detection of 44-methylgambierone was demonstrated by Roué et al., in 2018, where the secondary metabolite was detected in cultures and from in-field deployment of SPATT bags at a known CFP hotspot (Roué et al., 2018). The use of this environmental sampling technology therefore shows some promise. Further in-field investigations are now required to ascertain if there is a

correlation between the detection of 44-methylgambierone and the microalgal assemblages on the reef system.

5. Conclusion

The acute toxicity of 44-methylgambierone by intraperitoneal injection to mice was low (LD_{50} between 20 and 38 mg kg^{-1}), suggesting that 44-methylgambierone is unlikely to contribute to CFP. Its production was assessed using LC-MS/MS in 252 microalgal isolates consisting of 90 species from 32 genera across seven classes. It was shown to be ubiquitous to all *Gambierdiscus* species tested, and some species/isolates of *Fukuyoa* and *Coolia*. These results indicate that 44-methylgambierone cannot be used as a selective biomarker for toxic *Gambierdiscus* blooms. However, because of its considerably easier detection compared to CTXs and MTXs it may still have use as a screening tool in high risk areas to allow the early identification of benthic microalgal assemblages, that could include toxic *Gambierdiscus* species.

Author contributions

J. Sam Murray designed, carried out and co-ordinated the experiment, performed data analysis and wrote the manuscript; Tomohiro Nishimura and Lesley Rhodes cultured and harvested the microalgal isolates in New Zealand; Sarah Finch performed the toxicity assessment; Frode Rise and Alistair Wilkins performed the qNMR; Michaela Larsson and Martina Doblin provided the isolates from Australia; Priscilla Leung and Meng Yan provided the isolates from Hong Kong; Jonathan Puddick, D. Tim Harwood and Michèle Prinsep assisted with experimental design and revised the manuscript. All authors read, revised, and approved the final manuscript.

Declaration of Competing Interest

The authors have declared no conflict of interest.

Acknowledgements

The authors acknowledge Tom Trnski from the Auckland War Memorial Museum for collecting water samples containing the *Gambierdiscus* cells and Dr Mireille Chinain for the *G. carpenteri* isolates from French Polynesia. We are grateful to Janet Adamson, Sarah Challenger and Krystyna Ponikla for her assistance with culturing. This research was conducted with funding from the Seafood Safety research programme (contract CAWX1801), a doctoral scholarship from the New Zealand Food Safety Science and Research Centre awarded to JSM, a Japan Society for the Promotion of Science (JSPS) overseas research fellowship awarded to TN, and partly supported by the Research Council of Norway through the Norwegian NMR Platform, NNP (226244/F50) and the Collaborative Research Fund of Research Grants Council, Hong Kong (C1012–15 G) [CG].

References

Bigelow, 1985. National Center for Marine Algae and Microbiota.
Boente-Juncal, A., Álvarez, M., Antelo, Á., Rodríguez, I., Calabro, K., Vale, C., Thomas, O.P., Botana, L.M., 2019. Structure elucidation and biological evaluation of maitotoxin-3, a homologue of Gambierone, from *Gambierdiscus belizeanus*. Toxins Basel 11 (2), 79.
Caillaud, A., de la Iglesia, P., Darius, H.T., Pauillac, S., Aligizaki, K., Fraga, S., Chinain, M., Diogène, J., 2010. Update on methodologies available for ciguatoxin determination: perspectives to confront the onset of ciguatera fish poisoning in Europe. Mar. Drugs 8, 1838–1907.
Carmody, E.P., James, K.J., Kelly, S.S., 1996. Dinophysistoxin-2: the predominant diarrhoetic shellfish toxin in Ireland. Toxicon 34 (3), 351–359.
Chan, L.L., Sit, W.-.H., Lam, P.K.-S., Hsieh, D.P.H., Hodgkiss, I.J., Wan, J.M.-F., Ho, A.Y.-T., Choi, N.M.-C., Wang, D.-.Z., Dudgeon, D., 2006. Identification and characterization of a "biomarker of toxicity" from the proteome of the paralytic shellfish toxin-producing dinoflagellate. Alex. Tamarense Dinophyceae 6 (2), 654–666.
Chang, F., 1996. Shellfish Toxin Update. Seafood New Zealand, pp. 26.
Chinain, M., Darius, H.T., Ung, A., Cruchet, P., Wang, Z., Ponton, D., Laurent, D., Pauillac, S., 2010. Growth and toxin production in the ciguatera-causing dinoflagellate *Gambierdiscus polynesiensis* (Dinophyceae) in culture. Toxicon 56 (5), 739–750.
Clausing, R.J., Losen, B., Oberhaensli, F.R., Darius, H.T., Sibat, M., Hess, P., Swarzenski, P.W., Chinain, M., Dechraoui Bottein, M.-.Y., 2018. Experimental evidence of dietary ciguatoxin accumulation in an herbivorous coral reef fish. Aquat. Toxicol. 200, 257–265.
Diogene, J.C., Monica, C., 2018. Comprehensive analytical chemistry. Recent Advances in the Analysis of Marine Toxins. Wilson and Wilsons.
Echigoya, R., Rhodes, L., Oshima, Y., Satake, M., 2005. The structures of five new antifungal and hemolytic amphidinol analogs from *Amphidinium carterae* collected in New Zealand. Harmful Algae 4 (2), 383–389.
FAO, 2014. Application of Risk Assessment in the Fish Industry. Food and Agriculture Organisation of the United Nations Available at: http://www.fao.org/tempref/docrep/fao/007/y4722e/y4722e00.pdf - FAO fisheries technical paper 442.
Friedman, M.A., Fernandez, M., Backer, L.C., Dickey, R.W., Bernstein, J., Schrank, K., Kibler, S., Stephan, W., Gribble, M.O., Bienfang, P., Bowen, R.E., Degrasse, S., Flores Quintana, H.A., Loeffler, C.R., Weisman, R., Blythe, D., Berdalet, E., Ayyar, R., Clarkson-Townsend, D., Swajian, K., Benner, R., Brewer, T., Fleming, L.E., 2017. An updated review of ciguatera fish poisoning: clinical, epidemiological, environmental, and public health management. Mar. Drugs 15 (3), 1–41.
Friedman, M.A., Fleming, L.E., Fernandez, M., Bienfang, P., Schrank, K., Dickey, R., Bottein, M.-.Y., Backer, L., Ayyar, R., Weisman, R., Watkins, S., Granade, R., Reich, A., 2008. Ciguatera fish poisoning: treatment, prevention and management. Mar. Drugs 6 (3), 456–479.
Gaboriau, M., Ponton, D., Darius, H.T., Chinain, M., 2014. Ciguatera fish toxicity in French Polynesia: size does not always matter. Toxicon 84, 41–50.
Gómez, F., Qiu, D., Lopes, R.M., Lin, S., 2015. *Fukuyoa paulensis* gen. et sp. nov., a New Genus for the Globular Species of the Dinoflagellate *Gambierdiscus* (Dinophyceae). PLoS ONE 10 (4), e0119676.
Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt and *Detonula confervacea*. Can. J. Microbiol. 8 (2), 229–239.
Hachani, M.A., Dhib, A., Fathalli, A., Ziadi, B., Turki, S., Aleya, L., 2018. Harmful epiphytic dinoflagellate assemblages on macrophytes in the Gulf of Tunis. Harmful Algae 77, 29–42.
ILM, 2014. Institute Loius Malarde - Ciguatera online. Avaliable at: http://www.ciguatera-online.com/index.php/en/.
IOC/UNESCO, 2015. Intergovernmental Oceanographic Commission of UNESCO - Harmful algal bloom programme - Global ciguatera strategy.
Ishii, K., Ito, K.M., Uemura, D., Ito, K., 1997. Possible mechanism of palytoxin-induced Ca ++ mobilization in porcine coronary artery. J. Pharmacol. Exp. Ther. 281 (3), 1077–1084.
Kohli, G.S., Farrell, H., Murray, S.A., 2015. *Gambierdiscus*, the Cause of Ciguatera Fish poisoning: An increased Human Health Threat Influenced By Climate Change. Climate Change and Marine and Freshwater Toxins, De Gruyter, pp. 273–312.
Kohli, G.S., Haslauer, K., Sarowar, C., Kretzschmar, A.L., Boulter, M., Harwood, D., Laczka, O., Murray, S.A., 2017. Qualitative and quantitative assessment of the presence of ciguatoxin, P-CTX-1B, in Spanish mackerel (*Scomberomorus commerson*) from waters in New South Wales (Australia). Toxicol. Rep. 4, 328–334.
Kohli, G.S., Papiol, G.G., Rhodes, L.L., Harwood, D.T., Selwood, A., Jerrett, A., Murray, S.A., Neilan, B.A., 2014. A feeding study to probe the uptake of maitotoxin by snapper (*Pagrus auratus*). Harmful Algae 37, 125–132.
Kretzschmar, A.L., Larsson, M.E., Hoppenrath, M., Doblin, M.A., Murray, S.A., 2019. Characterisation of Two Toxic *Gambierdiscus* spp. (Gonyaulacales, Dinophyceae) from the Great Barrier Reef (Australia): *G. lewisii* sp. nov. and *G. holmesii* sp. nov. Protist 170 (6), 125699.
Larsson, M.E., Laczka, O.F., Harwood, D.T., Lewis, R.J., Himaya, S., Murray, S.A., Doblin, M.A., 2018. Toxicology of *Gambierdiscus* spp.(Dinophyceae) from tropical and temperate Australian waters. Mar. Drugs 16 (1), 7.
Ledreux, A., Brand, H., Chinain, M., Bottein, M.-Y.D., Ramsdell, J.S., 2014. Dynamics of ciguatoxins from *Gambierdiscus polynesiensis* in the benthic herbivore *Mugil cephalus*: trophic transfer implications. Harmful Algae 39 (0), 165–174.
Leung, P.T., Yan, M., Lam, V.T., Yiu, S.K., Chen, C.-.Y., Murray, J.S., Harwood, D.T., Rhodes, L.L., Lam, P.K., Wai, T.-C., 2018. Phylogeny, Morphology and Toxicity of Benthic Dinoflagellates of the Genus *Fukuyoa* (Goniodomataceae, Dinophyceae) From a Subtropical Reef Ecosystem in the South China Sea. 74, 78–97.
MacKenzie, A.L., Beuzenberg, V., Holland, P.T., McNabb, P., Selwood, A., 2004. Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. Toxicon 44 (8), 901–918.
Malagoli, D., Casarini, L., Ottaviani, E., 2008. Effects of the marine toxins okadaic acid and palytoxin on mussel phagocytosis. Fish Shellfish Immunol. 24 (2), 180–186.
Matsuda, A., Nishijima, T., Fukami, K., 1996. Effects of Nitrogen Deficiency on the PSP Production By *Alexandrium Catenella* Under Axenic Cultures. Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO, France, pp. 305–308.
Morohashi, A., Satake, M., Nagai, H., Oshima, Y., Yasumoto, T., 2000. The absolute configuration of gambieric acids A-D, potent antifungal polyethers, isolated from the marine dinoflagellate *Gambierdiscus toxicus*. Tetrahedron 56 (46), 8995–9001.
Morohashi, A., Satake, M., Yasumoto, T., 1999. The absolute configuration of gambierol, a toxic marine polyether from the dinoflagellate, *Gambierdiscus toxicus*. Tetrahedron Lett. 40 (1), 97–100.
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. Mar. Drugs 15 (7), 208.
Murray, J.S., Boundy, M.J., Selwood, A.I., Harwood, D.T., 2018. Development of an

LC–MS/MS method to simultaneously monitor maitotoxins and selected ciguatoxins in algal cultures and P-CTX-1B in fish. Harmful Algae 80, 80–87.
Murray, J.S., Selwood, A.I., Harwood, D.T., van Ginkel, R., Puddick, J., Rhodes, L.L., Rise, F., Wilkins, A.L., 2019. 44-Methylgambierone, a new gambierone analogue isolated from *Gambierdiscus australes*. Tetrahedron Lett. 60 (8), 621–625.
Murray, S., Kohli, G.S., Harwood, D.T., Boulter, M., 2016. Safeguarding Seafood Consumers in New South Wales from Ciguatera Fish Poisoning. Fisheries Research and Development Corporation.
Nishimura, T., Uchida, H., Noguchi, R., Oikawa, H., Suzuki, T., Funaki, H., Ihara, C., Hagino, K., Arimitsu, S., Tanii, Y., Abe, S., Hashimoto, K., Mimura, K., Tanaka, K., Yanagida, I., Adachi, M., 2019. Abundance of the benthic dinoflagellate *Prorocentrum* and the diversity, distribution, and diarrhetic shellfish toxin production of *Prorocentrum lima* complex and *P. caipirignum* in Japan. Harmful Algae In press.
OECD, 2000. OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs As Humane Endpoints For Experimental Animals Used in Safety Evaluation. Organisation for Economic Co-operation and Development (OECD), Paris, France.
OECD, 2006. OECD Guideline for Testing of Chemicals 425. Acute Oral Toxicity -Up-and-Down-Procedure (UDP). Organization for Economic Co-operation and Development (OECD), Paris, France.
Pisapia, F., Holland, W.C., Hardison, D.R., Litaker, R.W., Fraga, S., Nishimura, T., Adachi, M., Nguyen-Ngoc, L., Séchet, V., Amzil, Z., 2017. Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays. Harmful Algae 63, 173–183.
Rhodes, L., Harwood, T., Smith, K., Argyle, P., Munday, R., 2014. Production of ciguatoxin and maitotoxin by strains of *Gambierdiscus australes, G. pacificus* and *G. polynesiensis* (Dinophyceae) isolated from Rarotonga, Cook Islands. Harmful Algae 39, 185–190.
Rhodes, L., Smith, K., Murray, S., Harwood, D., Trnski, T., Munday, R., 2017a. The epiphytic genus *Gambierdiscus* (Dinophyceae) in the Kermadec Islands and Zealandia regions of the southwestern Pacific and the associated risk of ciguatera fish poisoning. Mar. Drugs 15 (7), 219.
Rhodes, L., Smith, K.F., 2019. A checklist of the benthic and epiphytic marine dinoflagellates of New Zealand, including Rangitāhua/Kermadec Islands. N. Z. J. Mar. Freshw. Res. 53 (2), 258–277.
Rhodes, L.L., Smith, K.F., Murray, J.S., Nishimura, T., Finch, S.C., 2020. Ciguatera Fish Poisoning: The Risk From an Aotearoa/New Zealand Perspective. 12(1), 50.
Rhodes, L.L., Smith, K.F., Verma, A., Murray, S., Harwood, D.T., Trnski, T., 2017b. The dinoflagellate genera *Gambierdiscus* and *Ostreopsis* from subtropical Raoul Island and North Meyer Island, Kermadec Islands. N. Z. J. Mar. Freshw. Res. 1–15.
Rongo, T., van Woesik, R., 2013. The effects of natural disturbances, reef state, and herbivorous fish densities on ciguatera poisoning in Rarotonga, southern Cook Islands. Toxicon 64, 87–95.
Roué, M., Darius, H.T., Viallon, J., Ung, A., Gatti, C., Harwood, D.T., Chinain, M., 2018. Application of solid phase adsorption toxin tracking (SPATT) devices for the field detection of *Gambierdiscus* toxins. Harmful Algae 71, 40–49.
Watanabe, R., Uchida, H., Suzuki, T., Matsushima, R., Nagae, M., Toyohara, Y., Satake, M., Oshima, Y., Inoue, A., Yasumoto, T., 2013. Gambieroxide, a novel epoxy polyether compound from the dinoflagellate *Gambierdiscus toxicus* GTP2 strain. Tetrahedron Lett. 69 (48), 10299–10303.
Wider, G., Dreier, L., 2006. Measuring protein concentrations by NMR spectroscopy. J. Am. Chem. Soc. 128 (8), 2571–2576.
Xu, Y., Richlen, M.L., Liefer, J.D., Robertson, A., Kulis, D., Smith, T.B., Parsons, M.L., Anderson, D.M., 2016. Influence of environmental variables on *Gambierdiscus* spp. (Dinophyceae) growth and distribution. PLoS ONE 11 (4), e0153197.
Yan, M., Leung, P.T.Y., Gu, J., Lam, V.T.T., Murray, J.S., Harwood, D.T., Wai, T.-.C., Lam, P.K.S., 2020. Hemolysis associated toxicities of benthic dinoflagellates from Hong Kong waters. Mar. Pollut. Bull. 155, 111114.
Yasumoto, T., Bagnis, R., Thevenin, S., Garcon, M., 1977. A survey of comparative toxicity in the food chain of ciguatera. Nippon Suisan Gakkaishi 43 (8), 1015–1019.
Yasumoto, T., Hashimoto, Y., Bagnis, R., Randall, J.E., Banner, A.H., 1971. Toxicity of the surgeonfishes. Bull. Jpn. Soc. Sci. Fish. 37 (8), 724–734.
Yogi, K., Sakugawa, S., Oshiro, N., Ikehara, T., Sugiyama, K., Yasumoto, T., 2014. Determination of toxins involved in ciguatera fish poisoning in the Pacific by LC/MS. J. AOAC Int. 97 (2), 398–402.
Yong, H.L., Mustapa, N.I., Lee, L.K., Lim, Z.F., Tan, T.H., Usup, G., Gu, H., Litaker, R.W., Tester, P.A., Lim, P.T., Leaw, C.P., 2018. Habitat complexity affects benthic harmful dinoflagellate assemblages in the fringing reef of Rawa Island, Malaysia. Harmful Algae 78, 56–68.