Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays

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Abstract

Species in the epi-benthic dinoflagellate genus *Gambierdiscus* produce ciguatoxins (CTXs) and maitotoxins (MTXs), which are among the most potent marine toxins known. Consumption of fish contaminated with sufficient quantities of CTXs causes Ciguatera Fish Poisoning (CFP), the largest cause of non-bacterial food poisoning worldwide. Maitotoxins, which can be found in the digestive system of fish, could also contribute to CFP if such tissues are consumed. Recently, an increasing number of *Gambierdiscus* species have been identified; yet, little is known about the variation in toxicity among *Gambierdiscus* strains or species.

This study is the first assessment of relative CTX- and MTX-toxicity of *Gambierdiscus* species from areas as widespread as the North-Eastern Atlantic Ocean, Pacific Ocean and the Mediterranean Sea. A total of 13 strains were screened: (i) seven Pacific strains of *G. australes*, *G. balechii*, *G. caribaeus*, *G. carpenteri*, *G. pacificus*, *G. scabrosus* and one strain of an undetermined species (*Gambierdiscus* sp. Viet Nam), (ii) five strains from the North-Eastern Atlantic Ocean (two *G. australes*, a single *G. excentricus* and two *G. silvae* strains), and (iii) one *G. carolinianus* strain from the Mediterranean Sea. Cell pellets of *Gambierdiscus* were extracted with methanol and the crude extracts partitioned into a CTX-containing dichloromethane fraction and a MTX-containing aqueous methanol fraction. CTX-toxicity was estimated using the neuro-2a cytoxicity assay, and MTX-toxicity via a human erythrocyte lysis assay.

Different species were grouped into different ratios of CTX- and MTX-toxicity, however, the ratio was not related to the geographical origin of species (Atlantic, Mediterranean, Pacific). All strains showed MTX-toxicity, ranging from 1.5 to 86 pg MTX equivalents (eq) cell^{-1}. All but one of the strains showed relative low CTX-toxicity ranging from 0.6 to 50 fg CTX3C eq cell^{-1}. The exception was the highly toxic *G. excentricus* strain from the Canary Islands, which produced 1,426 fg CTX3C eq cell^{-1}. As was true for CTX, the highest MTX-toxicity was also found in *G. excentricus*. Thus, the present study confirmed that at least one species from the Atlantic Ocean demonstrates similar toxicity as the most toxic strains from the Pacific, even if the metabolites in fish have so far been shown to be more toxic in the Pacific Ocean.

52 **Keywords**: Ciguatera Fish Poisoning, *Gambierdiscus*, ciguatoxins, maitotoxins, neuro-2a
53 assay, erythrocyte lysis assay.

1. Introduction

Dinoflagellates in the genera *Gambierdiscus* and *Fukuyoa* produce ciguatoxins (CTXs) and maitotoxins (MTXs), cyclic polyether neurotoxins that rank in the top five most potent natural toxins isolated to date (Fusetani and Kem, 2009).

Ciguatoxins, like brevetoxins, bind voltage-gated sodium channels (VGSCs) at site 5 on the alpha-subunit causing an influx of Na^+ into affected cells that disrupts cellular function, especially in nerve cells (Benoit et al., 1986; Legrand et al., 1982; Lombet et al., 1987). Ciguatoxins are lipophilic and they could readily accumulate in the marine food chain reaching their highest concentration in fish, as hypothesized by Randall (1958), albeit with considerable lag-time between the bloom of *Gambierdiscus* sp. and CTX-related CFP outbreaks (Chateau-Degat et al., 2005). The genera *Gambierdiscus* and *Fukuyoa* are epi-benthic and are found on many substrates including macro-algae, algal turfs, sea grasses and coral rubble (Parsons and Preskitt, 2007; Rains and Parsons, 2015) but they can also be found in near bottom plankton as shown using moored screens (Tester et al., 2014). Algal turfs appear to be very suitable substrates as support for *Gambierdiscus*, even when compared to macrophytes (Leaw et al., 2016). It is commonly assumed that the primary flux occurs from herbivorous grazers of such macro-algae to carnivorous fish (Ledreux et al., 2014), though other vectors such as crustaceans, echinoderms, and bivalves have been implicated (Kelly et al., 1992; Laurent et al., 2008; Roué et al., 2016; Silva et al., 2015). During this accumulation process CTXs are biotransformed, frequently resulting in metabolites of greater toxicities than the algal parent compounds (Lehane and Lewis, 2000). Certain *Gambierdiscus* species also produce other bioactive polyether compounds, such as gambierol (Cuypers et al., 2008; Satake et al., 1993a), gambieric acids (Nagai et al., 1993; Nagai et al., 1992) and gambierone (Rodríguez et al., 2015). The biological activity of gambierone is known to mimic that of CTX3C, although much lower in intensity, whereas the overall toxicity of gambierol and gambieric acids has yet to be characterized. The role, if any, of these three classes of compounds in causing CFP is unknown.

Maitotoxins are amphiphilic molecules that bind non-selective ion channels, causing an influx of Ca^{2+} that significantly raises intracellular Ca^{2+} levels. This is important since Ca^{2+} is one of the major signaling ions in the cell. The increased influx of the ion abnormally activates numerous biochemical pathways, including apoptosis, which disrupt the function of neuronal, muscular and red blood cells (Gusovsky and Daly, 1990; Ogura et al., 1984;

86 Ohizumi and Kobayashi, 1990). Even though MTXs are more toxic than CTXs when injected
87 intraperitoneally into mice, MTXs are less likely to be involved in causing Ciguatera Fish
88 Poisoning (CFP) because of their low capacity to accumulate in fish flesh and their low oral
89 potency as assessed in mice (Yasumoto et al., 1976). Still, a recent study by Kohli et al.
90 (2014) suggests that MTX could accumulate in carnivorous fish (fed in controlled conditions
91 with *Gambierdiscus*-inoculated herbivorous fish), particularly in their digestive tract and
92 liver, and thus MTXs may potentially contribute to CFP. Also, the large diversity of
93 symptoms of CFP observed in different oceans has been suggested to be related to different
94 CTX profiles (Lewis, 2001) but may also potentially be related to differences in consumer
95 habits, e.g. the consumption of the intestinal parts of fish (Gatti et al., 2008; Hamilton et al.,
96 2010). Consequently, the role of MTXs in contributing to CFP still remains to be clarified, in
97 particular whether such contribution may derive from contamination of fish fillets during
98 dissection of ciguateric fish or only from the consumption of visceral tissues of ciguateric
99 fish.

100 In addition to uncertainties regarding different toxin profiles and the routes of accumulation
101 little is known about the degree to which toxicity varies among species. One reason this has
102 proven challenging is that the taxonomy has only recently been sufficiently resolved to
103 examine species-specific toxicity (Fraga and Rodríguez, 2014; Fraga et al., 2011; Fraga et al.,
104 2016; Kretzschmar et al., 2016; Litaker et al., 2009; Nishimura et al., 2014; Smith et al.,
105 2016). This taxonomic work includes the separation of the globular *Gambierdiscus* species
106 into the genus *Fukuyoa* (Gómez et al., 2015).

107 The goal of this study was to characterize the relative toxicity of *Gambierdiscus* strains from
108 the Pacific Ocean, the North-Eastern Atlantic Ocean and the Mediterranean Sea. A total of 13
109 strains were examined, representing ten known species and one strain for which species
110 annotation is not yet complete (Table 1, section 2.2). Except two strains (CCMP1653 and the
111 strain from Viet Nam), none of the strains studied had previously been shown to produce any
112 known CTXs or MTXs. Hence, this study examined the strains with a targeted cellular
113 bioassay approach to detect activity of hitherto undescribed analogs of CTXs and MTXs.

2. Materials and Methods

2.1. Reference toxins and chemicals

CTX3C was kindly provided by Mireille Chinain (Institut Louis Malardé, Tahiti) and used as the reference standard for the neuroblastoma neuro-2a (N2a) cytotoxicity assay. MTX was purchased from Wako Chemicals USA, Inc. (Richmond, Virginia, USA) and used as the reference standard for the erythrocyte lysis assay (ELA). CTX3C was dissolved and stored in pure MeOH prior to utilization in the N2a assay. MTX was stored in MeOH:H_2O (1:1, v/v), dried and re-dissolved in ELA buffer prior to utilization in the ELA. HPLC grade methanol and dichloromethane were purchased from Sigma Aldrich (St. Louis, Missouri, USA).

Eagle's Minimum Essential Medium (EMEM, ATCC® 30-2003) for culture of N2a cells was purchased from the American Type Culture Collection. The following additives to the N2a medium were purchased from Sigma Aldrich (St. Louis, Missouri, USA): sodium pyruvate, streptomycin, penicillin and fetal bovine serum. N2a assay reagents were also purchased from Sigma Aldrich: trypsin-(ethylenediaminetetraacetic acid) (trypsin-EDTA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Saponin for the ELA was purchased from Sigma Aldrich (St. Louis, Missouri, USA).

2.2. Laboratory cultures of *Gambierdiscus* spp.

The strains of *Gambierdiscus* which were examined in this study and their location of origin are listed in Table 1. Strains of *G. scabrosus* (Nishimura et al., 2014), *G. excentricus* (Fraga et al., 2011), *G. silvae* (Fraga and Rodríguez, 2014) and *G. balechii* (Fraga et al., 2016) all belong to recently described species. Molecular analysis of *Gambierdiscus* sp. Viet Nam, previously reported as *G. toxicus* Vietnam by Roeder et al. (2010), still needs to be completed, and hence it was not assigned to this species.

Culture experiments were conducted using a semi-continuous batch method at both the Phycotoxins laboratory at the French Research Institute for Exploitation of the Sea (IFREMER), Nantes, France and at the National Oceanic and Atmospheric Administration, Center for Coastal Fisheries and Habitat Research (CCFHR), Beaufort, NC, USA. Cell densities were maintained at levels to ensure the absence of nutrient or CO_2 limitation. At pH > 8.4 cells become progressively more CO_2 limited. Cells of *Gambierdiscus* were harvested

144 in log phase growth. Slight differences in the experimental protocols necessitated by
145 differences in the equipment available at each location are noted below (sections 2.2.1 and
146 2.2.2). As a control, *G. pacificus* CCMP1650 was grown in both laboratories to determine if
147 growth rate and toxin values obtained in each laboratory were comparable.

148 ***2.2.1. NOAA CCFHR laboratory (Beaufort, NC, USA)***

149 Four strains of *Gambierdiscus* (*G. caribaeus* Bill Hi Gam8, *G. carolinianus* Greece Gam2, *G.*
150 *carpenteri* Pat Hi Jar7 Gam11 and *G. pacificus* CCMP1650) (Table 1) were grown in 75 cm^2
151 tissue culture flasks with vented caps (Falcon®, BD Biosciences, Bedford, MA, USA).
152 Media consisted of 0.2 µm filtered Gulf Stream seawater (at a salinity of 33), vitamins and
153 nutrients were added according to a modified K-medium protocol (Keller and Guillard, 1985;
154 Keller et al., 1987). Phosphate was added in the form of Na_2 β-Glycerophosphoric Acid, 5-
155 Hydrate at twice the concentration used for K-medium preparation. An EDTA-trace metal
156 buffer system was used with the omission of copper as described by Hardison et al. (2012).
157 The media was sterilized via microwave treatment as described in Keller et al. (1988). The
158 culture pH was monitored (Thermo Orion 3 star pH meter, Ross ultra-combination pH
159 electrode) to ensure pH range throughout experiments were between 8.1 and 8.4. This
160 ensured the cells were not CO_2-limited. Cultures were maintained in a Percival Scientific
161 incubator (Boone, IA, USA) at 27°C, under full spectrum lights (Blue Max F20-T12, Full
162 Spectrum Solutions, Mississippi, USA) with an incident photon flux density at
163 100 µmol photons m^{-2} s^{-1} and a daily light-dark cycle of 12h:12h light:dark (LD). Full
164 spectrum light source was placed in illumination cassettes above the culture flasks. Flasks
165 were randomly placed and the position was changed once a day in order to ensure a
166 homogeneous exposure to light. When the culture reached ~1000-2000 cells mL^{-1}, cells were
167 first retained on a 20 µm sieve, washed with sterile seawater and collected by centrifugation
168 (10 min, 1800 *g*, 20°C) in 50 mL Falcon tubes. Cell pellets were stored at -20°C until further
169 extraction for toxicity screening.

170 ***2.2.2. IFREMER laboratory (Nantes, France)***

171 Ten strains of *Gambierdiscus* were cultured in 75 cm^2 culture flasks (Corning® CellBIND®,
172 Grosseron SAS, Coueron, France): *G. australes* CCMP1653, VGO1178 and VGO1181,
173 *G. excentricus* VGO791, *G. pacificus* CCMP1650, *G. scabrosus* KW070922_1, *G. silvae*
174 VGO1167 and VGO1180, *G. balechii* VGO917 and *Gambierdiscus* sp. Viet Nam (Table 1).

Media consisted of filtered (0.2 µm) natural Mediterranean seawater (at a salinity of 33) enriched with L1 nutrients with the exception of silica (Guillard and Hargraves, 1993). Cultures were maintained in a growth chamber incubator (Binder KBW240, Binder GmbH, Tuttlingen, Germany) at 25°C, under the same light conditions described above. After three weeks of semi-continuous culture, cells were harvested by centrifugation (20 min, 3000 *g*, 4°C) in 50 mL Falcon tubes and cell pellets were stored at -20°C until further extraction for toxicity screening.

2.3. Maximum growth rate determination

In order to determine the maximum growth rates during the exponential growth phase, *Gambierdiscus* cells were grown in semi-continuous batch cultures as previously described by Hardison et al. (2012). Briefly, all the cultures were acclimated to the culture conditions specific to each of the two laboratories (sections 2.2.1 and 2.2.2) for several months prior to experimentation. Cultures of *Gambierdiscus* cells were inoculated in 200 mL of culture medium at an initial concentration of ~100-200 cells mL^{-1} and incubated at randomly determined sites in the incubator which were rotated daily. Cells were kept in the exponential growth phase as follows: cultures were transferred to new medium (1 to 10 dilution) when cell concentration reached ~1000-2000 cells mL^{-1} and, thus, they never experienced nutrient or CO_2 limitation. An aliquot of culture was taken every 3-4 days during a period of 53-78 days (n = 13-15 samplings, at least three generations) and analyzed for cell concentration (cells mL^{-1}) and mean cellular biovolume (Estimated Spherical Volumes, ESV, μm^3 $cell^{-1}$) using a Multisizer™ 3 Coulter Counter® (Beckman Coulter, Georgia, USA) particle counter equipped with a 280 µm aperture tube and a 1 mL sample volume. The total volume of cells per liter of culture media (biovolume) was then calculated. Maximum growth rate (μ_{max}, d^{-1}) was the slope calculated by the linear regression of the natural logarithm of the biovolume versus time, after correcting for serial culture dilutions (Sunda and Hardison, 2007). SigmaPlot software (version 12.5) was used to calculate regression slopes and associated relative standard error and R^2 values. Maximum growth rate (μ_{max}, divisions day^{-1}) was then calculated as follows: μ_{max} (divisions day^{-1}) = μ_{max} (d^{-1}) $\ln(2)^{-1}$.

2.4. Toxin extraction and liquid-liquid partitioning

Cultures of each strain have been grown in three separate flasks. After the cells had been harvested in log phase growth, they were suspended in MeOH (30 mL per 1 million cells)

and disrupted using sonication (CCFHR laboratory) or bead beating (IFREMER laboratory). Sonication was conducted twice for 1 min at 50% of total power (500 W) using a 3 mm diameter probe sonicator (Q-Sonica, Q700, Newtown, Connecticut USA). Grinding with the bead-mill was conducted twice for 30 min at a vibration frequency of 30 Hz using a mixer mill (Retsch MM400, Germany) with glass beads (0.25 g, diameter 250-500 μm) (Serive et al., 2012). Completeness of cell disruption was verified using light microscopy. Crude extracts (CEs) were blown dry under N_2 gas at 40°C. The residue was suspended in MeOH:H_2O (3:2, v/v) (25 mL per 1 million cells) and partitioned twice with dichloromethane (DCM) (50 mL per 1 million cells) as previously described by Satake et al. (1993b). The lipophilic CTXs were partitioned into the DCM soluble fraction (DSF) while the amphiphilic MTXs were partitioned into the aqueous methanol (aq. MeOH) soluble fraction (MSF). Once the DSF and MSF fractions were isolated, they were blown dry under N_2 gas at 40°C and stored at -20°C. Just prior to the bioassays, the dried DCM and aq. MeOH residues were re-dissolved in MeOH or MeOH:H_2O (1:1, v/v), respectively. An aliquot of the hydrophilic fraction was then evaporated (N_2 gas at 40°C) and stored at -20°C until use in the human erythrocyte lysis assay (ELA). Just prior to running the ELA the dried residue from the MSF fraction was dissolved in ELA buffer.

2.5. Neuroblastoma neuro-2a assay

The neuroblastoma neuro-2a (N2a) cell line is frequently used to estimate levels of CTXs in fish, shellfish or phytoplankton extracts (Pawlowiez et al., 2013). The N2a cytotoxicity assay developed by Manger et al. (1993) and modified by Dickey et al. (1999) was performed at CCFHR laboratory (Beaufort, NC, USA), with some modification (Hardison et al., 2016). Ciguatoxins do not induce N2a cell death, however, when N2a cells are pre-incubated with ouabain (O) and veratridine (V) they become highly sensitive to sodium channel activator toxins. Assays were set up so that the N2a cells are exposed to partially purified cell extracts with and without O and V. If cell death occurs in the samples without O and V it indicates the presence of a non-specific toxic compound other than a sodium channel activator. The details of the assay were as follows.

The N2a cell line was obtained from the American Type Culture Collection (ATCC, CCL 131). Neuro-2a cells were grown and maintained as described by Hardison et al. (2016). The assay was carried out in 96-well flat-bottom CELLCOAT® tissue culture plates with Poly-D-

Lysine coating (Greiner Bio-One, Kremsmünster, Austria). Plates were seeded with 30,000 N2a cells per well and were incubated for 24 h until they were >90% confluent at the bottom of each well. The CTX3C standard, controls and *Gambierdiscus* samples were added next and incubated for 24 h. The standard curve was added in presence of O/V (250 µM and 25 µM, respectively) at 50% cell viability to increase sensitivity and specificity to CTXs. The CTX3C standard curve for this assay ranged from 0.001 to 2,000 pg mL^{-1}. A sigmoidal dose-response curve was plotted and an EC_{50} of 1.66 ± 0.16 (SD, n=12) pg CTX3C mL^{-1} was calculated using GraphPad Prism 6.0 (Fig. S1) (Hardison et al., 2016). Controls included buffer wells to provide maximum survival estimates and wells with the addition of 1% MeOH (final concentration in well) to identify any cell mortality caused by the presence of MeOH used to dissolve the dried extracts. Half of the sample aliquots (1 µL additions) from each assay were processed in the presence of O/V (O/V^+) so they were directly comparable to the CTX3C standard curve. The other half was incubated without O/V (O/V^-) to identify non-specific mortality caused by other compounds in the sample. Total well volume was 100 µL. No more than 500 *Gambierdiscus* cell equivalents were added to each well to avoid matrix effects or non-specific N2a cell death. Each of the three replicate samples was run in duplicate in the N2a assay. Cell viability was assessed after 24 h incubation using the quantitative colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983) using a FLUOstar® Omega microplate reader (BMG Labtech, Germany) at 544 nm. As the cells treated with O/V showed 50% of cell viability relative to control cells in solvent vehicle (1% MeOH in N2a medium), the viability of cells treated with CTX3C standard or algal extracts was estimated relative to O/V^+ wells. Quantitation of CTX3C eq in the samples using the N2a assay was operated within a range from 20% to 80% N2a cell viability with respect to the O/V^+ wells. The limit of quantification (80% N2a cell viability) was 0.197 ± 0.005 (SD, n=12) ng CTX3C mL^{-1}. When N2a cell viability was lower than 20%, a ten-fold dilution of the sample (in MeOH) was made. Extracts or strains showing activity with this assay will be referred to as "containing CTXs" or showing "CTX-toxicity", even though this is a simplification as other compounds may also show sodium specific activity using this model.

2.6. Erythrocyte lysis assay

The erythrocyte lysis assay (ELA), developed by Eschbach et al. (2001) was performed at the CCFHR laboratory (Beaufort, NC, USA), with some modifications (Holland et al., 2013) to

estimate the hemolytic activity of *Gambierdiscus* strains. This assay is based on lysis of erythrocytes due to hemolytic compounds and subsequent photometrical determination of the released hemoglobin. In the context of marine dinoflagellates, the ELA has broadly been used on different red blood cell lines to detect hemolytic compounds from different microalgae such as *Alexandrium* (Tatters et al., 2012), *Karenia* (Tatters et al., 2010), *Ostreopsis* cf. *ovata* (Nascimento et al., 2012a) and *Gambierdiscus* (Holland et al., 2013).

Human red blood cells (hRBCs) were obtained from the Red Cross (Durham, North Carolina, USA). The hRBCs treated with saponin provided the maximal hemolysis (100% lysis) used to normalize the hemolytic activity of MTX standard or the diluted algal extracts. The hRBCs incubated solely in ELA buffer served as negative control (0% lysis). Details of how the assay was performed can be found in Holland et al. (2013). The 8-point MTX standard curve for this assay ranged from 0.0002 to 2,000 ng mL^{-1} using purified MTX from Wako Chemicals USA, Inc. (Richmond Virginia, USA). Four replicate wells were used for each of the eight concentrations. A sigmoidal dose-response curve was plotted and an EC_{50} of 14.2 ± 3.3 (SD, n=4) ng MTX mL^{-1} was calculated using GraphPad Prism 6.0 (Fig. S2). The minimum hemolytic activity observed was 1.51 ± 0.39 (SD, n=4) % hemolysis when hRCBs were exposed to 1.0 ng MTX mL^{-1}. For each *Gambierdiscus* MSF sample, six dilutions were tested and three replicate wells for each dilution were run. Sigmoidal dose-response curves were plotted and EC_{50} values (cell eq mL^{-1}) were calculated for each strain using GraphPad Prism 6.0. Quantitation of MTX eq in the samples using the ELA was operated converting EC_{50} values (cell eq mL^{-1}) for each strain into toxin equivalent per cell (pg MTX eq $cell^{-1}$) taking into account the EC_{50} value obtained from the MTX standard curve. Erythrocyte lysis was assessed after 24h incubation at 4°C using a FLUOstar® Omega microplate reader (BMG Labtech, Germany) at 415 nm. Extracts or strains showing activity with this assay will be referred to as "containing MTXs" or showing "MTX-toxicity", even though this is a simplification as other compounds may also show hemolytic activity using this model.

2.7. Statistical analysis

Statistical analysis was performed using RStudio (Version 0.99.903) (http://www.rstudio.com) utilizing the R statistical language version 3.3.1 (https://www.R-project.org).

Multiple and linear regression models were obtained using basic functions in R on a data matrix including all qualitative information and quantitative values measured for each replicate on all strains. This corresponded to 42 observations of 7 variables [origin, laboratory, species, μ_{max} (divisions day^{-1}), biovolume (ESV, µm^3 cell^{-1}), fg CTX3C eq cell^{-1} (DSF) and pg MTX eq cell^{-1} (MSF)]. For comparison purpose, the same analyses were performed on the same data matrix with the outlier strain (*G. excentricus* VGO791) excluded, which allowed for showing consistent correlations.

Unsupervised clustering of the strains based on the mean centered and normalized values of biovolume (ESV, µm^3 cell^{-1}), fg CTX3C eq cell^{-1} (DSF) and pg MTX eq cell^{-1} (MSF) was performed by 1) calculating the distance matrix between each observation (strain) using the Euclidean distance 2) executing a hierarchical cluster analysis using the Ward's minimum variance method. The result was displayed as a cluster dendrogram, one replicate of *Gambierdiscus* sp. Viet Nam being excluded for this analysis as it presented very dissimilar results to other two replicates.

3. Results

3.1. Maximum growth rates and cellular biovolumes

Maximum growth rates (μ_{max}) of *Gambierdiscus* in culture ranged from 0.099 to 0.244 divisions day^{-1}, depending on the strain (Table 2). The slowest growing strains were *G. excentricus* VGO791 and *G. balechii* VGO917 (IFREMER laboratory conditions), while the fastest growth was observed for *G. pacificus* CCMP1650 (CCFHR laboratory conditions).

Interestingly, *G. excentricus* VGO791, which was the slowest growing strain, had the largest cellular biovolume (Table 2). *G. pacificus* (CCMP1650), the species with the smallest biovolume (3.8-fold < *G. excentricus*) was the fastest growing species (Table 2). Notwithstanding, overall the correlation was poor between growth rate and cellular volume, e.g. *G. balechii* had a similar growth rate as *G. excentricus* but a substantially smaller cellular biovolume. The overall low growth rates (< 0.5 divisions day^{-1}) observed in this study are consistent with those previously reported in the literature (Kibler et al., 2012; Litaker et al., *submitted to PLoS One on Feb 2017*; Xu et al., 2016; Yoshimatsu et al., 2014). If a comparison could be made, it can be concluded that, in the present study, *Gambierdiscus*

strains behaved as slow-growers when cultured under CCHFR and IFREMER laboratory conditions, with μ_{max} < 0.25 divisions day^{-1}, which appeared similarly low or somewhat lower than those reported in other studies.

3.2. Screening of DSF toxicity using the neuro-2a assay

Figure S1 shows the sigmoidal dose-response curve of CTX3C standard on the neuro-2a assay (Hardison et al., 2016).

For each strain, both dichloromethane soluble fraction (DSF, a fraction expected to contain CTXs) and the corresponding crude extract (CE) were tested on the neuro-2a assay. All DSFs tested were found to enhance the ouabain/veratridine (O/V) mediated cell mortalities consistent with CTX activation of voltage-gated sodium channels (VGSCs). There was no enhanced mortality without addition of O/V, indicating absence of non-specific toxicity after the initial purification step. In contrast, CEs showed substantial non-specific mortality (cell death in absence of O/V), indicating the presence of bioactive compounds other than VGSC activators such as MTXs or other toxic algal compounds. Thus, the quantitative estimation of CTXs was only possible in DSFs. Results were expressed in CTX3C equivalents (eq) per cell (Fig. 1).

Only the one *G. excentricus* (VGO791) strain examined from the Canary Islands exhibited a high level of CTX-type toxicity, i.e. 1,426 fg CTX3C eq cell^{-1}. The CTX content for all the other strains examined fell into the range of 0.6-40.8 fg CTX3C cell^{-1} (Fig. 1).

Among the Pacific strains examined in this study, the Vietnamese strain, representing an as of yet undescribed species, showed the highest N2a cytotoxicity followed by the Japanese *G. scabrosus* strain. The two *G. silvae* strains showed low CTX-type toxicity equivalent to that of *G. pacificus*, i.e. around 10 fg CTX3C eq cell^{-1}. The Mediterranean strain of *G. carolinianus* showed background levels of CTX-type activity (< 4 fg CTX3C eq cell^{-1}) similar to the Pacific *G. caribaeus*, *G. carpenteri* and *G. australes* strains. Interestingly, *G. australes* strains originating either from the Pacific or the North-Eastern Atlantic Oceans, showed similar CTX-type activity (Fig. 1).

The strain of *G. pacificus* (CCMP1650) from French Polynesia showed similar levels of CTX-toxicity in cultures from both IFREMER and CCFHR, despite the differences in culture

conditions and extraction procedure (Table 2). Thus, the differences in culture and extraction techniques between the two laboratories appear to not have affected the results.

3.3. Screening of MSF toxicity using the erythrocyte lysis assay

Figure S2 shows the sigmoidal dose-response curve of MTX standard on the human erythrocyte lysis assay (ELA).

Figure 2 shows the MTX-type activity of the 13 strains of *Gambierdiscus* evaluated using the ELA. The strain of *G. excentricus* exhibited the highest hemolytic activity, followed by the Vietnamese strain. The Mediterranean strain of *G. carolinianus* was intermediate and about 5-fold more toxic than the two Atlantic strains of *G. silvae.* The Japanese strain of *G. scabrosus* showed the lowest hemolytic activity among all the strains tested in this study. Also, *G. australes*, *G. caribaeus*, and *G. carpenteri* species showed low MTX-type activity. Interestingly, among the latter ones, *G. australes* strains, originating either from Pacific or the North-Eastern Atlantic Oceans, showed the same MTX-type activity. Overall, though variable, all strains tested showed measurable MTX-toxicity.

As in the evaluation of CTX3C equivalent toxicity, *G. pacificus* (CCMP1650) showed similar levels of MTX-toxicity in cultures from both IFREMER and CCFHR, despite the differences in culture conditions and extraction procedure (Table 2).

3.4 Relationship between CTX and MTX toxicity

The CTX toxin content per unit biovolume varied over four orders of magnitude compared to a 10-fold variation in MTX toxicity per unit biovolume (Fig. 3). A similar pattern was observed when the data were normalized on a per cell basis. The relationship between CTX and MTX for the various species fell into three groups (Fig. 4) with strains of from a given species classifying into the same group.

4. Discussion

CTX-toxicity. The present study showed that *Gambierdiscus* species examined from the Pacific Ocean, North-Eastern Atlantic Ocean and Mediterranean Sea exhibited marked

differences in toxicity ranging from 0.6 fg to > 1400 fg CTX3C equivalents (eq) cell^{-1} (Fig. 1). The greatest toxicity was exhibited by *G. excentricus* (VGO791) from the Canary Islands. This result is consistent with a previous study reporting *G. excentricus* strains exhibiting between 0.37 - 1.10 pg CTX1B eq cell^{-1} (Fraga et al., 2011), which would be equivalent to 1.17 - 3.49 pg CTX3C eq cell^{-1} according to Bottein Dechraoui et al. (2007). The Canary Islands, where these strains were obtained, are a temperate region (North-Eastern Atlantic Ocean) from which Ciguatera Fish Poisoning (CFP) has recently been reported (Boada et al., 2010; Pérez-Arellano et al., 2005). More recently, *G. excentricus* has been found in Brazil, its contribution to CFP in the region has yet to be evaluated (Nascimento et al., 2012b; Nascimento et al., 2015). The observation of CFP in the Canary Islands is important because CFP is typically considered a tropical disease (Lewis, 2001). Thus, *G. polynesiensis* and *G. excentricus* could be considered as primary toxin producing species in the South Pacific and the Eastern Atlantic Oceans, respectively (Chinain et al., 2010; Rhodes et al., 2014; Rhodes et al., 2016).

In contrast, *G. australes*, *G. balechii*, *G. carolinianus*, *G. carpenteri*, *G. pacificus* and *G. silvae* all had toxicities below 50 fg CTX3C eq cell^{-1} (Fig. 1). In a separate study on Caribbean strains, *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *Gambierdiscus* ribotype II, *G. silvae* and *F. ruetzleri* have similarly low toxicities (< 20 fg CTX3C eq cell^{-1}) (Litaker et al., *submitted to PLoS One on Feb 2017*). Further, *G. carolinianus* and *G. carpenteri* strains from the Caribbean, Mediterranean and Pacific, as well as *G. silvae* and *G. australes* strains originating either from the Caribbean, Pacific or the North-Eastern Atlantic Oceans showed similar CTX-type activity, suggesting that each species produce comparable levels of toxin worldwide. The data presented here also indicate that most *Gambierdiscus* species produce relatively low levels of CTXs. This low level of toxin production raises an important question concerning the degree to which these low toxicity species contribute to the overall toxin flux into the food chain relative to the high toxicity species *G. excentricus* and *G. polynesiensis* (Litaker et al., *submitted to PLoS One on Feb 2017*). As these low toxicity species can only be of public health importance if they are able to bloom, it will be important to map abundances of the different *Gambierdiscus* species in the field.

The CTX-toxicity results from this study were consistent with other studies on individual species. The strain of *Gambierdiscus* sp. Viet Nam (previously reported as *G. toxicus* based

418 on morphology), for example, showed the highest toxicity among all the Pacific strains
419 examined (Fig. 1) and has been shown to produce several CTX analogs (Roeder et al., 2010).
420 Nishimura et al. (2013) similarly detected the CTX-like toxicity of the DSF fraction from the
421 Japanese strain of *G. scabrosus* (species previously reported as *Gambierdiscus* sp. type 1),
422 KW070922_1, using the mouse bioassay (MBA), i.e. 20 x 10^{-4} MU/1,000 cells. These
423 toxicities are relatively high compared to most other species, but still low compared to *G.*
424 *excentricus* or *G. polynesiensis,* consistent with the findings in this study.

425 The three *G. australes* strains in this study were in the same low range of toxicity,
426 independently of their origin (Canary Islands and Hawaii). Of these strains, comparable
427 toxicity data are only available for the Pacific CCMP1653 strain, previously reported as T39.
428 For this particular strain, Babinchak et al. (1986) reported high toxicity of the crude extract
429 using the MBA. Later on, a more specific assay described by Van Dolah et al. (1994) (radio-
430 labelled brevetoxin ([^{3}H]BTX-3) displacement assay) was conducted on CCMP1653,
431 showing from no detectable to low Na^+ ion channel activity indicating low toxicity (Sperr and
432 Doucette, 1996). More recently, LC-MS studies conducted by Roeder et al. (2010) on
433 CCMP1653 showed the presence of one CTX analog. Chinain et al. (2010) reported
434 comparatively low toxicity for six *G. australes* strains originating from French Polynesia,
435 ranging from < 0.016 (LOD) to 0.030 pg CTX3C eq cell^{-1}. Rhodes et al. (2010) found
436 intermediate N2a cytotoxicity at sub-pg range (0.13 pg CTX3C eq cell^{-1}) for the *G. australes*
437 CAWD149 strain (Cook Islands). Nishimura et al. (2013) reported DSF-toxicity (MBA) for a
438 Japanese *G. australes* strain (S080911_1) of 670 x 10^{-4} MU/1,000 cells, comparable to the
439 highly toxic *G. polynesiensis* species, i.e. 800-1500 x 10^{-4} MU/1,000 cells (Chinain et al.,
440 1999). Such difference in toxicity between strains suggests that a larger number of strains are
441 needed to assess intraspecific variations in CTX toxicity.
442
443 Chinain et al. (2010) observed that the slow growing species examined in their study,
444 *G. polynesiensis* exhibited the highest level of toxicity and hypothesized that slower growing
445 species were more toxic. In this study, *G. excentricus* was the slowest growing species and it
446 also showed by far the highest CTX and MTX toxicity (1.4 pg CTX3C eq cell^{-1} and 86 pg
447 MTX eq cell^{-1}) (Figs. 1 and 2, Table 2). A study of *Gambierdiscus* species found in the
448 Caribbean showed an inverse exponential relationship between CTX toxicity on a per-cell
449 and per-biovolume basis consistent with the Chinain et al. (2010) hypothesis (Litaker et al.,
450 *submitted to PLoS One on Feb 2017*). The species *G. balechii* however appears an exception

451 to this rule as it had a comparable growth rate to *G. excentricus* but was substantially less
452 toxic (Figs. 1-3).

453 **MTX-toxicity.** The MTX-toxicity of *Gambierdiscus* strains varied more than 50-fold (1.5 -
454 86 pg MTX eq cell^{-1}). The most maitotoxic species was *G. excentricus* (VGO791; ~80 pg
455 MTX eq cell^{-1}) followed by the Vietnamese strain (*Gambierdiscus* sp. Viet Nam; ~70 pg
456 MTX eq cell^{-1}). The variability was highest for the Vietnamese strain (64% RSD) with one
457 replicate giving a much higher result that the other two, followed by variability for
458 *G. excentricus* (48% RSD). Additional trials with more replication are needed before
459 concluding the toxicity of these species is comparable. The measured MTX-toxicity for *G.*
460 *excentricus* was lower than the ~600 pg MTX eq cell^{-1} estimated for the strains VGO790,
461 VGO791 and VGO792 (Fraga et al. 2011) using a modified neuro-2a assay (Caillaud et al.,
462 2010). The MTX content however was estimated using a crude extract whereas MTX-toxicity
463 in the present study was estimated in the aqueous methanol extracts, i.e. after liquid-liquid
464 partitioning. Possible explanations of this discrepancy include differences in the assay applied
465 (N2a vs. ELA) and sources of standard (MTX from different sources). Similarly, the G10DC
466 strain of *G. pacificus*, isolated from Malaysia, was estimated to have a toxicity of 50.2 pg
467 MTX eq cell^{-1} (Caillaud et al., 2011), 2.5 times more toxic than results from the present study
468 for CCMP1650 strain. The three *G. australes* strains assayed in this study showed low MTX-
469 toxicity (< 5 pg MTX eq cell^{-1}) consistent with the low toxicity of *G. australes* RAV-92
470 strain of from Raivavae Island (Australes Archipelago) measured by MBA (Chinain et al.,
471 1999). *G. scabrosus* strain (KW070922_1) showed a similarly low hemolytic activity (1.5 pg
472 MTX eq cell^{-1}) consistent with the observed MSF fraction toxicity of 67 x 10^{-4} MU/1,000
473 cells (Nishimura et al. 2013).

474 **Ratio CTX/MTX-toxicity**. It should be noted that all the strains examined in this study
475 produced measureable quantities of both CTXs and MTXs (Figs. 1 and 2). The variation
476 among species, however, was not consistent for the two groups of toxins. CTX-toxicity per
477 unit biovolume varied over three orders of magnitude among species whereas the MTX-
478 toxicity varied over one order of magnitude (Fig. 3).

479 Based on a hierarchical cluster analysis (section 2.7), strains could be classified into three
480 different groups: group I (*G. excentricus* VGO791), group II (*G. australes* CCMP1653,
481 VGO1178, VGO1181, *G. caribaeus* Bill Hi Gam8, *G. carolinianus* Greece Gam2 and *G.*
482 *carpenteri* Pat Hi Jar7 Gam11), and group III (*G. balechii* VGO917, *G. pacificus*

CCMP1650*, G. silvae* VGO1167, VGO1180, *G. scabrosus* KW070922_1 and *Gambierdiscus* sp. Viet Nam) (Fig. 4). As visually suggested in Figure 3, there is a correlation between CTX and MTX contents (on a per cell basis, in DSF and MSF fractions, respectively): the Pearson correlation coefficient for linear regression was Rr^2=0.45 ($P < 0.001$) when including *G. excentricus*, and Rr^2=0.23 ($P < 0.01$) when excluding *G. excentricus*. Though the data were limited, different strains of the same species fell into the same grouping indicating that the relationship between CTX and MTX toxicity appears constant for a given species. More data are needed to fully test this hypothesis. It should also be noted that toxicity in this study was assessed using functional assays. Those assays cannot distinguish between production of large amounts of low toxicity CTX or MTX congeners relative to smaller production of high toxicity congeners. *Gambierdiscus* cell extracts that were fractionated and assayed for toxicity using a calcium flux assay indicated that despite strains of a species possessing similar CTX and MTX toxicity, the actual congeners being produced in a given species probably vary (Lewis et al., 2016). How these relative profiles might affect grazing pressure or deter bacterial or fungal infections remains unknown.

Also unidentified is whether the different patterns of CTX and MTX can offer insights into biosynthetic pathways for these studies. Ongoing studies are focusing on growing large-scale cultures of the most toxic strains of *Gambierdiscus* for the purification of the toxic compounds through fractionation and screening using the cellular bioassays.

In summary, it should be noted that only one of the thirteen strains examined has been shown to contain pg amounts of CTX-type toxicity per cell, and this strain of *G. excentricus* needs to be examined in detail for its toxin contents to identify the algal precursor(s) of toxins involved in CFP in the Atlantic Ocean. As this strain of *G. excentricus* exhibits CTX-type toxicity in the same order of magnitude as *G. polynesiensis* from the South Pacific, *Gambierdiscus* species from both oceans should be considered to be a similar potential threat to fish consumers. All strains displayed MTX-toxicity in the pg range (1.5 - 86 pg $cell^{-1}$). Also, the variability of CTX and MTX-type toxicities between species and strains appeared to be similar to those previously reported in literature and was equivalent between Atlantic and Pacific strains of *Gambierdiscus*. Several of the findings, including correlation between growth rate and toxicity or variability within and between species, are only indicative of possible trends and more strains should be examined to corroborate the findings.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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Figure captions

Figure 1. Neuro-2a (N2a) cytotoxicity of dichloromethane soluble fractions (DSFs, n=3) of 13 *Gambierdiscus* strains. Results are expressed in fg CTX3C eq $cell^{-1}$.

Figure 2. Hemolytic activity of aq. MeOH soluble fractions (MSFs, n=3) of 13 *Gambierdiscus* strains evaluated by means of a human erythrocyte lysis assay (ELA). Results are expressed in pg MTX eq $cell^{-1}$.

Figure 3. Plot of log CTX toxicity (fg CTX3C eq μm^{-3}) versus MTX toxicity (pg MTX eq μm^{-3}).

Figure 4. Dendrogram of a hierarchical cluster analysis of 13 *Gambierdiscus* strains based on the following three variables: CTX-toxicity in the DSF (fg CTX3C eq $cell^{-1}$), MTX-toxicity in the MSF (pg MTX eq $cell^{-1}$) and cell biovolume (μm^3 $cell^{-1}$).

Figure S1. Sigmoidal dose-response curve of CTX3C and CTX1B standards on the neuro-2a (N2a) assay plotted using GraphPad Prism 6.0 (Hardison et al., 2016). Error bars represent the standard deviation (SD, n=12 for CTX3C, n=14 for CTX1B). The CTX standard used in this study was CTX3C only.

Figure S2. Sigmoidal dose-response curve of MTX standard on the human erythrocyte lysis assay (ELA) plotted using GraphPad Prism 6.0. Error bars represent the standard deviation (SD) of four replicates.

Table captions

Table 1. Denomination and origin of *Gambierdiscus* strains examined in this study.

Table 2. Maximum specific growth rates (μ_{max}, divisions day^{-1}) and per-cell CTX- and MTX-toxicity of the *Gambierdiscus* strains cultivated in this study.