Rapid detection of ciguatoxins in *Gambierdiscus* and *Fukuyoa* with immunosensing tools

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Abstract

Consumption of seafood contaminated with ciguatoxins (CTXs) leads to a foodborne disease known as ciguatera. Primary producers of CTXs are epibenthic dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa.* In this study, thirteen *Gambierdiscus* and *Fukuyoa* strains were cultured, harvested at exponential phase, and CTXs were extracted with an implemented rapid protocol. Microalgal extracts were obtained from pellets with a low cell abundance (20,000 cell/mL) and were then analyzed with magnetic bead (MB)-based immunosensing tools (colorimetric immunoassay and electrochemical immunosensor). It is the first time that these approaches are used to screen *Gambierdiscus* and *Fukuyoa* strains, providing not only a global indication of the presence of CTXs, but also the ability to discriminate between two series of congeners (CTX1B and CTX3C). Analysis of the microalgal extracts revealed the presence of CTXs in 11 out of 13 strains and provided new information about *Gambierdiscus* and *Fukuyoa* toxin profiles. The use of immunosensing tools in the analysis of microalgal extracts facilitates the elucidation of further knowledge regarding these dinoflagellate genera and can contribute to improved ciguatera risk assessment and management.

Keywords

Ciguatoxins (CTXs), ciguatera, *Gambierdiscus*, *Fukuyoa*, immunoassay, immunosensor.

Introduction

Epibenthic dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa* are known producers of ciguatoxins (CTXs), potent marine toxins responsible for a foodborne disease termed ciguatera.[1-4] CTXs can accumulate in marine food webs, from herbivorous and detritivorous fishes that graze substrates colonized by *Gambierdiscus* and *Fukuyoa* (*e.g.* macroalgae, corals and rocks) to carnivorous fishes.[5] In the process, other organisms like crustaceans, echinoderms and bivalves may also be implicated.[6-8]

Presently, eighteen species of *Gambierdiscus* are recognized worldwide: *G. toxicus, G. belizeanus, G. australes, G. pacificus, G. polynesiensis, G. caribaeus, G. carolinianus, G. carpenteri, G. excentricus, G. scabrosus, G. silvae, G. balechii, G. cheloniae, G. lapillus, G. honu, G. jejuensis, G. lewesii and G. holmesii*.[9-16] Regarding the genus *Fukuyoa*, only three species (*F. ruetzleri, F. yasumotoi* and *F. paulensis*) have been described.[10,17,18] These species have been found mainly in tropical and subtropical areas, but also in temperate areas. Despite the wide distribution, there are zones where the diversity in terms of reported species is higher, such as the Canary Islands, coasts of the Caribbean and adjacent seas, and French Polynesia[19]. *Gambierdiscus* and *Fukuyoa* are all potential producers of bioactive compounds. In fact, in addition to CTXs,[20,21] maitotoxins (MTXs),[22-24] gambieric acids,[25] gambierol,[26] gambieroxide[27] and gambierone [28,29] have also been detected in laboratory cultures of some species. Even if toxicity of these compounds on cell lines has been reported, it is not fully understood yet if they play a role in ciguatera intoxication.[30] During their accumulation through food webs, CTXs are often biotransformed and this may result in metabolites of higher toxicity than the algal parent compounds.[31,32] The CTXs profiles found in fish are determined by the *Gambierdiscus* and *Fukuyoa* species grazed by fishes, the congeners that these microalgae produce and the biotransformation processes occurring through the food web. Therefore, the oxidation of specific CTX algal precursors can lead to species-specific and region-specific toxin profiles in fishes.[33,34] Thus, studies that aim to investigate toxic profiles of *Gambierdiscus* and *Fukuyoa* are extremely important not only to obtain fundamental knowledge about these genera, but also to understand and monitor the presence of CTXs in fishes and, more broadly, to better describe ciguatera intoxication and predict future outbreaks.

Several methods have been used to analyze *Gambierdiscus* or *Fukuyoa* species. The mouse bioassay (MBA) has been very useful during the first steps to identify CTXs and MTXs in microalgae but it has been demonstrated to lack sensitivity and specificity. As a consequence, other methods have been developed, including cell-based assays (CBAs), receptor binding assays (RBAs) and instrumental analysis techniques (*e.g.* liquid chromatography coupled to mass spectrometry, LC-MS/MS).[35] CTXs have not been detected in all existing species of the genera *Gambierdiscus* and *Fukuyoa*, and even when the presence is confirmed, the contents are very low (few fg/cell).[30] Nevertheless, the species *G. polynesiensis* and *G. excentricus* have shown, consistently over the years, a CTX-like toxicity significantly higher than other species, producing up to several pg/cell of CTX compounds.[3,21,36-39] Therefore, these two species are viewed as the most important CTXs producers in the Pacific and Atlantic Oceans, respectively.

Gambierdiscus and *Fukuyoa* cells are armored dinoflagellates with cellulose thecae difficult to disrupt. Therefore, a key point for the correct determination of the toxin content is the extraction

procedure, which usually involves several purification steps to obtain a clean extract.[39] The first step of this procedure is the intrinsic pellet extraction, which is performed in absolute methanol,[3,13,15,38,41-43] aqueous methanol,[44] or a combination of both,[21,45-49] whilst Lewis et al.[49] extracted pellets with a methanol:water:hexane solution. To facilitate cell disruption, sonication is usually involved in the extraction process, through sonicator probes,[3,21,38,43,46,47,49,50] or ultrasonic baths,[13,44,48] or, alternatively, the use of a bead beater.[38] According to the grade of purity needed, crude extracts have to undergo a first purification step that usually involves liquid/liquid solvent partitioning to separate CTXs from MTXs.[3,21,24,38,46,50] If the extracts are highly concentrated in biomass, further purification steps are needed prior to the analysis with LC-MS/MS. These steps include the use of chromatography, either Solid Phase Extraction (SPE)[21,51,52] or High Performance Liquid Chromatography (HPLC).[21] Evidently, this procedure is time consuming, involves the use of several reagents and instrumentation, and requires skilled personnel. Therefore, the development of more rapid, simpler and equally efficient techniques is desirable.

Recently, our group has developed an immunosensor for the detection of CTXs in fish samples.[53] This technique involves the use of monoclonal antibodies (mAbs) showing high specificity and sensitivity for their CTX targets,[54-62] and their exploitation in a sandwich colorimetric immunoassay and electrochemical immunosensor on magnetic beads (MBs). Specifically, the 3G8 mAb has affinity for the left wing of CTX1B and 54-deoxyCTX1B,[60] the 10C9 mAb for the left wing of CTX3C and 51-hydroxyCTX3C,[55] and the 8H4 mAb for the right wing of the four congeners[58] (Fig. 1).

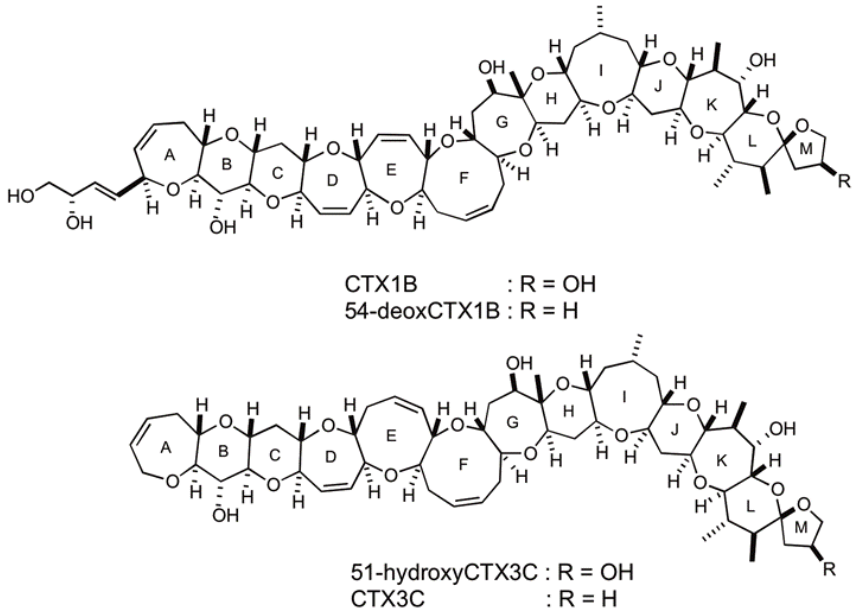

Figure 1. Schematic representation of the four CTXs congeners recognized by the antibodies used in this work.

In this work, the developed MB-based immunoassay and immunosensor have been exploited to investigate the CTXs production of nine *Gambierdiscus* strains belonging to three species (*G. australes, G. excentricus* and *G. caribaeus)* and four *Fukuyoa paulensis* strains (Fig. 2). A rapid CTXs extraction protocol using a bead beater has been evaluated with the intent to accelerate the analytical process. Results have been compared to the CBA. The immunosensing tools provided a

qualitative estimation and discrimination of two series of congeners (CTX1B and CTX3C) of these microalgal strains.

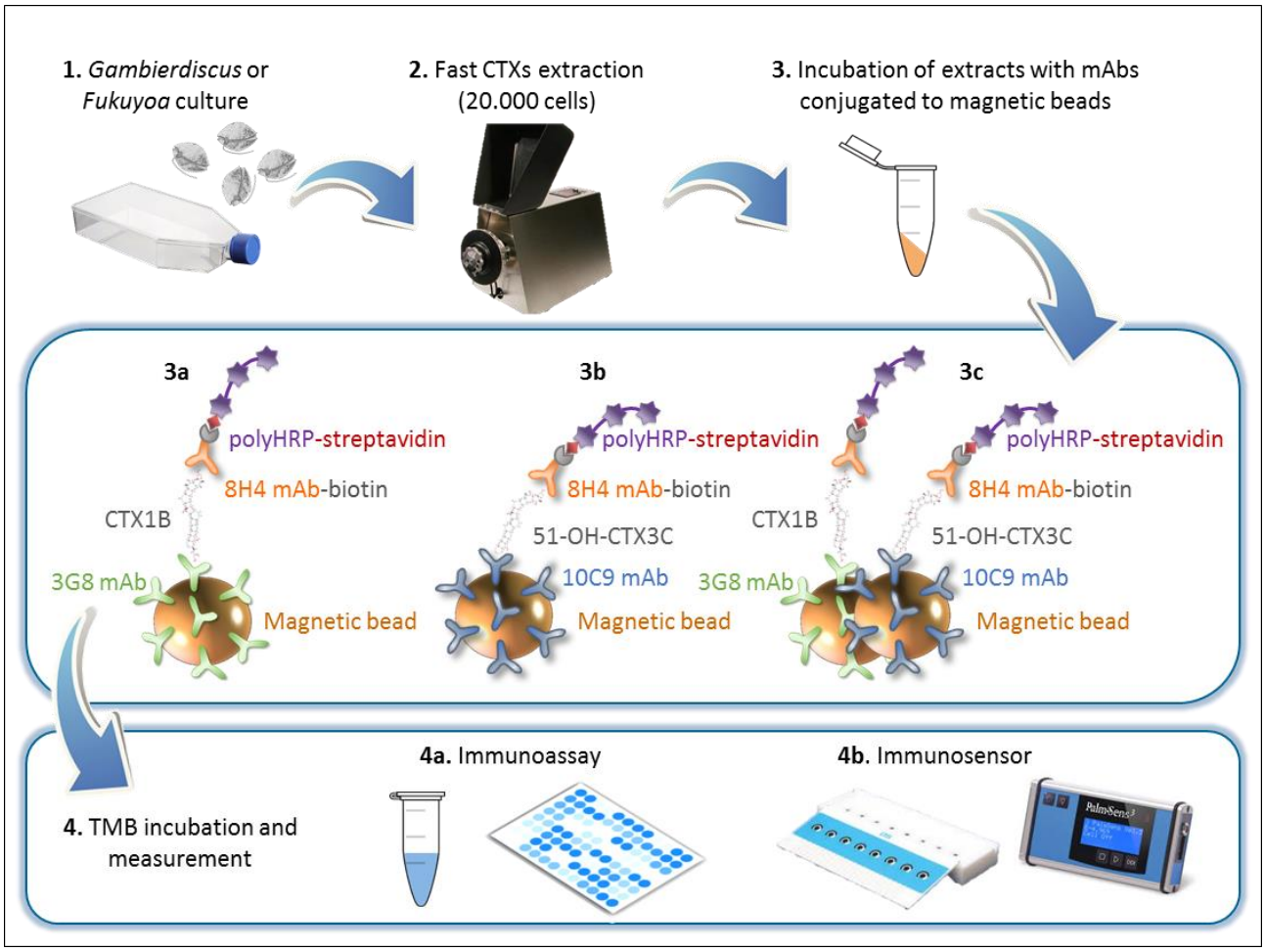

Figure 2. Schematic representation of the *Gambierdiscus* and *Fukuyoa* cultures, rapid CTXs extraction and subsequent analysis of the extracts with the MB-based colorimetric immunoassay and electrochemical immunosensor.

Experimental Section

Reagents and solutions

Dynabeads M-270 Carboxylic Acid (2×10^9 beads/mL) were supplied by Invitrogen (Life Technologies, S.A., Alcobendas, Spain). Potassium phosphate monobasic, potassium phosphate dibasic, potassium chloride, 4-morpholineethanesulfonic acid (MES) hydrate, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), Tween®-20, bovine serum album (BSA), and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate were purchased from Sigma-Aldrich (Tres Cantos, Spain). PolyHRP-streptavidin was obtained from Thermo Fisher (Barcelona, Spain). Milli-Q water (Millipore, Bedford, USA) was used to prepare solutions. For the extractions, methanol was obtained from Honeywell (Barcelona, Spain). CTX1B standard solution was obtained from Prof. Richard J. Lewis (The Queensland University, Australia) and calibrated (correction factor

of 90%) in relation to the NMR-quantified CTX1B standard solution from Prof. Takeshi Yasumoto (Japan Food Research Laboratories, Japan). 51-OH-CTX3C standard solution was kindly provided by Prof. Takeshi Yasumoto (Japan Food Research Laboratories, Japan) and was used as a model for the series of CTX3C congeners. 3G8, 10C9 and 8H4 mAbs had been prepared by immunizing mice with keyhole limpet hemocyanine (KLH) conjugates of rationally designed synthetic haptens[54-62] Biotin labelling of the 8H4 mAb was performed with the EZ-Link™ NHS-PEG4 Biotinylation Kit from Thermo Fisher (Barcelona, Spain) following the manufacturer's instructions. Unreacted NHS-PEG4-Biotin was removed using Zeba Spin Desalting Columns (7 kDa MWCO, 2mL) included in the kit.

Equipment

A Bead Beater (BioSpec, Bartlesville, USA) was used for the extraction of CTXs. An Allegra X-15R (Beckman Coulter, Brea, USA) centrifuge was used to obtain the microalgal pellets and in the CTXs extraction after using the sonicator. An Eppendorf 5415D (Hamburg, Germany) centrifuge was used in the CTXs extraction after using the bead beater. Magnetic separation was performed using a MagneSphere Technology Magnetic Separation Stand (for 12 0.5-mL or 1.5-mL tubes) and a PolyATtract System 1000 Magnetic Separation Stand (for one 15-mL tube) from Promega Corporation (Madison, USA). Colorimetric measurements were performed with a Microplate Reader KC4 from BIO-TEK Instruments, Inc. (Vermont, USA). Gen5 software was used to collect and evaluate data. Arrays of eight screen printed carbon electrodes (DRP-8x110), a boxed connector (DRP-CAST8X) and a magnetic support (DRP-MAGNET8X) were purchased from Dropsens S.L. (Oviedo, Spain). The arrays consist of 8 carbon working electrodes of 2.5 mm in diameter, each with its own carbon counter electrode and silver reference electrode. Amperometric measurements were performed with a PalmSens potentiostat connected to an 8-channel multiplexer (MUX8) (Houte, The Netherlands). Data were collected and evaluated with the PalmSens PC software.

Microalgal cultures

Several *Gambierdiscus* (*N* = 9) and *Fukuyoa* (*N* = 4) strains were used: 1) from IRTA collection (*G. australes* IRTA-SMM-13_07; *F. paulensis* IRTA-SMM-17_206, IRTA-SMM-17_211 and IRTA-SMM-17_220); 2) from Culture Collection of Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo, Spain (*G. excentricus* VGO791; *F. paulensis* VGO1185); and 3) from a sampling performed in the Canary Islands, Spain, in September 2016 and 2017, and recently incorporated to IRTA collection (*G. australes* IRTA-SMM-16_286; *G. excentricus* IRTA-SMM-17_01, IRTA-SMM-17_126, IRTA-SMM-17_407, IRTA-SMM-17_428 and IRTA-SMM-17_432; *G. caribaeus* IRTA-SMM-17_03). In total, 2 *G. australes* strains, 6 *G. excentricus* strains, 1 *G. caribaeus* strain, and 4 *F. paulensis* strains were evaluated. For the sampling, macroalgae were collected, mixed with 1 L of seawater, vigorously shaken and filtered through a 200 µm mesh. Microalgal cells were isolated with a glass pipette following the capillary method (Hoshaw and Rosowski 1973) and cultivated, first in 24-well microplates and then in tissue culture polystyrene flasks.

All the clonal cultures were grown in ES medium[63] containing filtered and autoclaved seawater from L'Ametlla de Mar, Spain, and adjusted to a practical salinity of 36. Cultures were maintained at 24 ± 0.5 °C under a photon flux rate of 100 µmol m^{-2} s^{-1} with a 12:12 h light:dark regime. Culture aliquots were fixed with 3% Lugol's iodine and counted following the Sedgwick-Rafter method[64] using a

Kolkwitz chamber (Hydro-Bios, Altenholz, Germany) under an inverted light microscope (Leica DMIL, Spain). All the cultures were collected at the exponential phase (*ca.* 21 days). Pellets containing 10^4 cells were prepared by centrifugation (3200 g, 20 min) and stored at −20 °C until CTXs extraction. Additionally, pellets of strains from the sampling were prepared and stored at −20 °C for subsequent DNA extraction.

Extraction of genomic DNA was performed using a bead beating system and the phenol/chloroform/isoamylalcohol method.[65] Extracted DNA samples (50 µL) were quantified and checked for their purity using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The D8-D10 domain of the 28S rDNA gene was amplified using PCR and the pair of primers FD8/RB (5-GGATTGGCTCTGAGGGTTGGG-3/5-GATAGGAAGAGCCGACATCGA-3).[9] Each 25 µL reaction mixture contained 600 µM dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 1 U of Taq polymerase, 5% DMSO, and 2 µL of template DNA (10–50 ng). Amplifications were carried out in a Nexus Gradient Thermal Cycler (Eppendorf, Spain) and included 45 cycles of amplification following a three-step protocol (95 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s). Each PCR reaction was checked by agarose gel electrophoresis. PCR products of ~950 bp were purified with QIAquick PCR Purification Kit and bidirectionally sequenced (Sistemas Genómicos, LLC, Valencia, Spain). Forward and reverse sequence reads were edited using BioEdit v7.0.5.2[66] to create consensus sequences for each strain. Sequences were aligned using MAFFT v.7.[67] The phylogenetic relationships were inferred by Maximum Likelihood (ML) using RaxML v.8[68] and Bayesian Inference (BI) using Mr. Bayes v.3.2.2.[69] Sequences were deposited in GenBank (Table S1).

Ciguatoxins (CTXs) extraction

Toxin extraction was performed comparing the use of a sonicator[47] and a bead beater. The sonicator protocol was as follows: 1) two pellets of 10^4 cells were pooled together in a 15-mL tube using 5 mL of MeOH; 2) sonication was conducted for 15 min at 38% of amplitude 3 sec on/2 sec off using a 3 mm diameter sonicator probe (Watt ultrasonic processor VCX 750 (Newton, USA); 3) the extract was centrifuged (3200 g, 10 minutes), transferred to a new tube and dry-blown under N_2 gas at 40 °C; 4) 5 mL of MeOH were added to the first tube and steps 2 and 3 were repeated twice (transferring the supernatants to the tube with the already evaporated extract). The bead beater protocol was as follows: (1) two pellets of 10^4 cells were pooled together into a 2-mL screw-cap cryotube containing ~50 µg of 0.5 mm diameter zirconium glass beads using 1 mL of MeOH; (2) bead beating was conducted for 3 or 6 runs of 40 s each; (3) the extract was centrifuged (3700 g, 1 min), transferred to a glass vial and dry-blown under N_2 gas at 40 °C. Dried extracts were stored at -20 °C until analysis.

Cell-based assay (CBA)

The CBA was performed as previously described.[70] Briefly, neuro-2a (N2a) cells (ATCC, CCL131) were seeded in a 96-well microplate in 200 µL of RPMI medium containing 5% *v/v* fetal bovine serum (RPMI-FBS) at 42,500 cells per well, and incubated under a 5% CO_2 humid atmosphere for 24 h at 37 °C. Prior to exposure to CTX1B standard solution or microalgal extract, some N2a cells were pre-treated with ouabain and veratridine at 1 and 0.1 mM, respectively. CTX1B standard solution or

microalgal extract were dried, reconstituted in 200 µL of RPMI-FBS medium, 1/2 serially diluted (from 575.0 to 4.5 pg/mL for CTX1B standard solution and from 90.000 to 11.250 cells/mL for microalgal extract), and 10 µL were added to the wells with and without ouabain/veratridine pre-treatment (no pre-treatment used as a control to evaluate matrix effects). After 24 h, cell viability was measured using the MTT assay.[71] Measurements were performed in triplicate.

Colorimetric immunoassay and electrochemical immunosensor

Analyses were performed following our previous protocol[53] with some modifications. Briefly, 8 µL of MB suspension were transferred to a tube and activated by incubation with 40 µL of 50 mg/mL EDC and 40 µL of 50 mg/mL NHS (in 25 mM MES, pH 5.0) for 30 min. Subsequently, 80 µL of antibodies (3G8 or 10C9 mAb at 1/50 dilution in MES) were incubated for 1 h. The mAb-MB conjugates were washed, re-suspended in 80 µL of PBS-Tween (0.1 M PBS, 0.05% *v/v* Tween®-20, pH 7.2) and transferred into new tubes either separately (75 µL of conjugate) or mixed together (150 µL containing 75 µL of each conjugate). After supernatant removal, 75 µL of microalgal extract (evaporated extract resuspended in 250 µL of PBS-Tween), CTX standard (CTX1B or 51-OH-CTX3C) or both (for the spiking experiment) were added to the tube and incubated for 30 min. From this step on, the protocol of our previous work was followed without any change. At first, a blocking step was performed with PBS-Tween-BSA. Then, the conjugates were incubated first with 75 µL of biotin-8H4 mAb and afterwards with 75 µL of polyHRP-streptavidin. All the incubations lasted for 30 min, were performed at room temperature with slow tilt rotation, and three washings with PBS-Tween were performed between each step. Finally, immunocomplexes were washed and re-suspended in 75 µL of PBS-Tween. For the analysis two different procedure were followed, for the colorimetric immunoassay: 10 µL of immunocomplexes were transferred to a new tube, the supernatant was removed and 125 µL of TMB were incubated for 10 min. Then, 100 µL of solution were taken for absorbance reading at 620 nm. Measurements were performed in triplicate. Instead, for the electrochemical immunosensor: 10 µL of immunocomplexes were placed on each working electrode of the 8-electrode array, the supernatant was removed and 10 µL of TMB were incubated for 2 min; the TMB reduction current was measured using amperometry (−0.2 V (*vs.* Ag) for 5 s). Measurements were performed in quadruplicate.

Statistical analysis

Multivariate analysis of variance (two-way MANOVA) was first used to analyze differences in CTXs quantifications between the immunoassay and the immunosensor and among strains of different species. MANOVA is used when several dependent variables are measured on each sampling unit instead of only one variable (for more details, see Suarez-Serrano et al.[72] and Rovira et al.[73]). Significances were further explored with two-way analysis of variance (ANOVA). In addition to *P* values, the partial eta squared (η_p^2) was used as a measure of effect size (*i.e.* importance of factors). Similar to regression coefficient (r^2), η_p^2 is the proportion of variation explained for a certain effect, and has the advantage over eta squared of not depending on the number of sources of variation used in the ANOVA, thus it could be compared among different designs.[74] In contrast to *P* value, η_p^2 has the advantage that allows the proper comparison of treatments (*e.g.* a lower *P* value does not

necessarily mean that a factor has stronger effect[75]). Adjusted (or marginal) means of a dependent variable are the means for each level of the factor, and were used to describe the differences among strains and quantification tools. All statistical analyses were performed with SPSS 25.0.

Results

Ciguatoxins (CTXs) extraction

To demonstrate the efficiency of the bead beater protocol to extract CTXs, pellets from a culture of the strain *G. excentricus* IRTA-SMM-17_428 were extracted with two different bead beater settings and also with sonication as a reference method. CTXs extraction was evaluated using CBA (IC_{50} = 0.90 pg/mL, IC_{80} (limit of detection, LOD) = 0.40 pg/mL). Each extraction was performed in duplicate, and each extract was also analyzed in duplicate with the CBA. Observation of the cells under the light microscope indicated that all the protocols caused cell lysis (results not shown). As can be seen in Fig. 3, the different techniques resulted in similar CTXs extraction yields, and no differences were observed between performing 3 or 6 bead beater runs. The bead beater protocol can be considered the most suitable for CTXs extraction because it is more rapid and simpler. Indeed, the time required for the toxin extraction from microalgal pellets is as low as 2 min in comparison to the 60 min used by Pisapia and coworkers[38] or the 45 min required with sonication. Additionally, evaporation of the 15 mL of MeOH required for the sonication protocol takes longer than the evaporation of the 1 mL used with the bead beater. Furthermore, using a bead beater it is possible to extract up to eight samples at the same time, whereas the sonicator can extract only one sample at a time. Therefore, for subsequent experiments, samples were extracted using 3 bead beater runs.

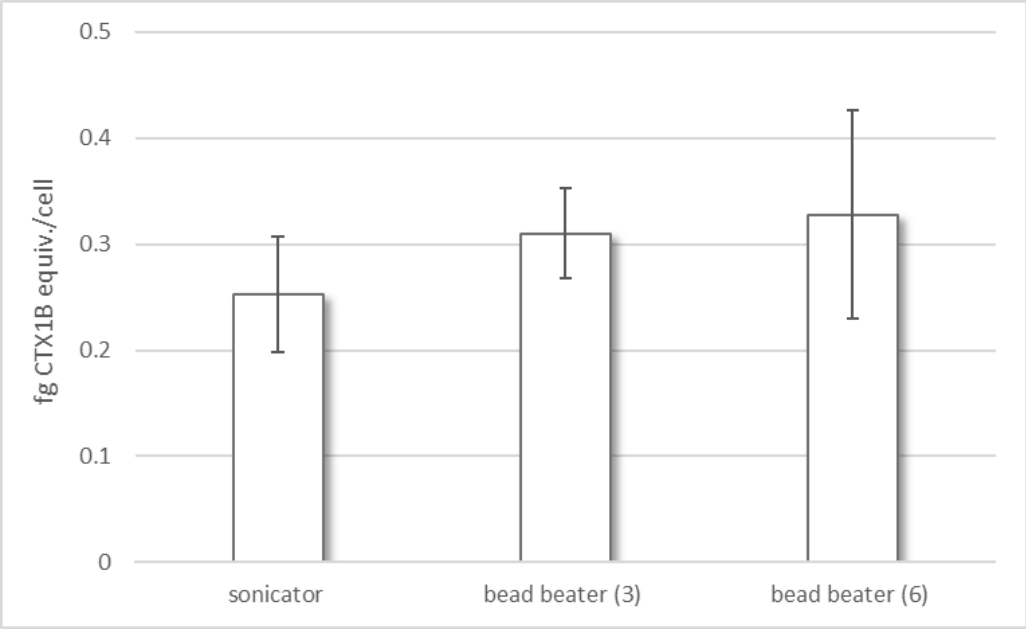

Figure 3. CTXs extracted (fg CTX1B equiv./cell) from *G. excentricus* IRTA-SMM-17_428 using sonicator, bead beater 3 times and bead beater 6 times, and evaluated with CBA.

Colorimetric immunoassay and electrochemical immunosensor

Microalgal extracts from *Gambierdiscus* and *Fukuyoa* cultures were analyzed using the colorimetric immunoassay, the electrochemical immunosensor and the CBA (Fig. 4 and Table S2). Regarding the analysis with the immunoassay and the immunosensor, both approaches should be able to detect at least four congeners among CTXs: CTX1B, 54-deoxyCTX1B, CTX3C and 51-hydroxyCTX3C. This is due to the ability of 3G8 mAb to bind to the left wing of CTX1B and 54-deoxyCTX1B,[60] of 10C9 mAb to bind to the left wing of CTX3C and 51-hydroxyCTX3C,[55] and of 8H4 mAb to bind to the right wing of the four congeners.[58] Whereas in our previous work both capture antibodies (3G8 and 10C9) were used together to analyze fish extracts,[53] thus providing a global response, in this study they have also been used separately to obtain an estimation of the amount of CTX1B or CTX3C series of congeners of several *Gambierdiscus* and *Fukuyoa* strains. Therefore, quantifications are expressed in fg/cell of CTX1B equiv. when the 3G8 mAb was used alone, in fg/cell of 51-OH-CTX3C equiv. when the 10C9 mAb was used alone, and in fg/cell of CTX1B equiv. and 51-OH-CTX3C equiv. when the two antibodies were used together. Regarding the CBA, where the CTXs recognition principle is based on a toxicological effect instead of a structural affinity, quantifications are expressed in fg/cell of CTX1B equiv.

Analyses with the immunoassay and the immunosensor revealed the presence of CTXs in 11 out of 13 extracts (all except for IRTA-SMM-13_07 and IRTA-SMM-17_211). In general terms, as expected, the CTXs contents determined when using two capture antibodies were higher than when using only one. This is certainly explained by the presence of the two different series of congeners, even if one of them was not detected separately because of the LOD of the method. It is also important to note that although in some cases the immunoassay showed higher CTXs contents, the immunosensor was able to detect the presence of CTXs in samples where the immunoassay was not capable. This can be attributed to the lower LODs of the immunosensor (1.96 and 3.59 pg/mL compared to 3.29 and 6.17 pg/mL, for CTX1B and 51-OH-CTX3C respectively.[53]

In order to evaluate the matrix effect, an experiment was performed, where CTX1B and 51-OH-CTX3C standard solutions (at 100 pg/mL) were spiked into the extracts that were negative by the immunoassay, the immunosensor and CBA (IRTA-SMM-13_07 and IRTA-SMM-17_211). Results showed practically no matrix effects in both the immunoassay (87% and 86% CTX1B recovery and 100% and 87% 51-OH-CTX3C recovery for IRTA-SMM-13_07 and IRTA-SMM-17_211, respectively) and the immunosensor (97% and 89% CTX1B recovery and 87% and 102% 51-OH-CTX3C recovery for IRTA-SMM-13_07 and IRTA-SMM-17_211, respectively).

The results obtained show the predominance of CTX1B congeners in 4 out of 6 *G. excentricus* strains (IRTA-SMM-17_126, IRTA-SMM-17_407, IRTA-SMM-17_428 and IRTA-SMM-17_432), ranging from 0.06 to 0.77 fg/cell of CTX1B equiv., and 1 out of 4 *F. paulensis* strains (VGO1185) (0.27-0.33 fg/cell of CTX1B equiv.). In these strains, CTX3C congeners were not detected, or only at very small amounts (0.01-0.04 fg/cell of 51-OH-CTX3C equiv.). Interestingly, 2 out of 4 *F. paulensis* strains (IRTA-SMM-17_206 and IRTA-SMM-17_220) revealed the presence of CTXs only when both capture antibodies were used together in the immunoassay (although due to the lower LODs obtained with the immunosensor, it was able to detect very low amounts in one of them when using the antibodies separately) and 1 out of 4 (IRTA-SMM-17_211) did not show any presence of CTXs at all. On the contrary, CTX3C congeners were the unique or most abundant in 1 out of 2 *G. australes* strains

(IRTA-SMM-16_286, 0.16-0.37 fg/cell of 51-OH-CTX3C equiv. in front of 0.04 fg/cell of CTX1B). In the other *G. australes* strain (IRTA-SMM-13_07), no CTXs were detected. CTX3C congeners were also predominant in 2 out of 6 *G. excentricus* strains (IRTA-SMM-17_01 and VGO791), ranging from 0.16 to 0.54 fg/cell of 51-OH-CTX3C equiv. Regarding the *G. caribaeus* strain (IRTA-SMM-17_03), equal amounts of both CTX congeners were detected (although slightly different depending on the immunosensing tool that was used).

Microalgal extracts were also screened with CBA, in order to compare the presence of CTXs detected with the immunosensing tools with the toxicity. As mentioned above, it must be considered that, even if all the tests have the objective to assess the presence of CTX congeners, their detection principle is different, and so results can differ between them. CTX-like activity was only detected in 4 out of 13 strains (IRTA-SMM-17_407, IRTA-SMM-17_428, IRTA-SMM-17_432 and VGO791) with the CBA, all belonging to the species *G. excentricus*.

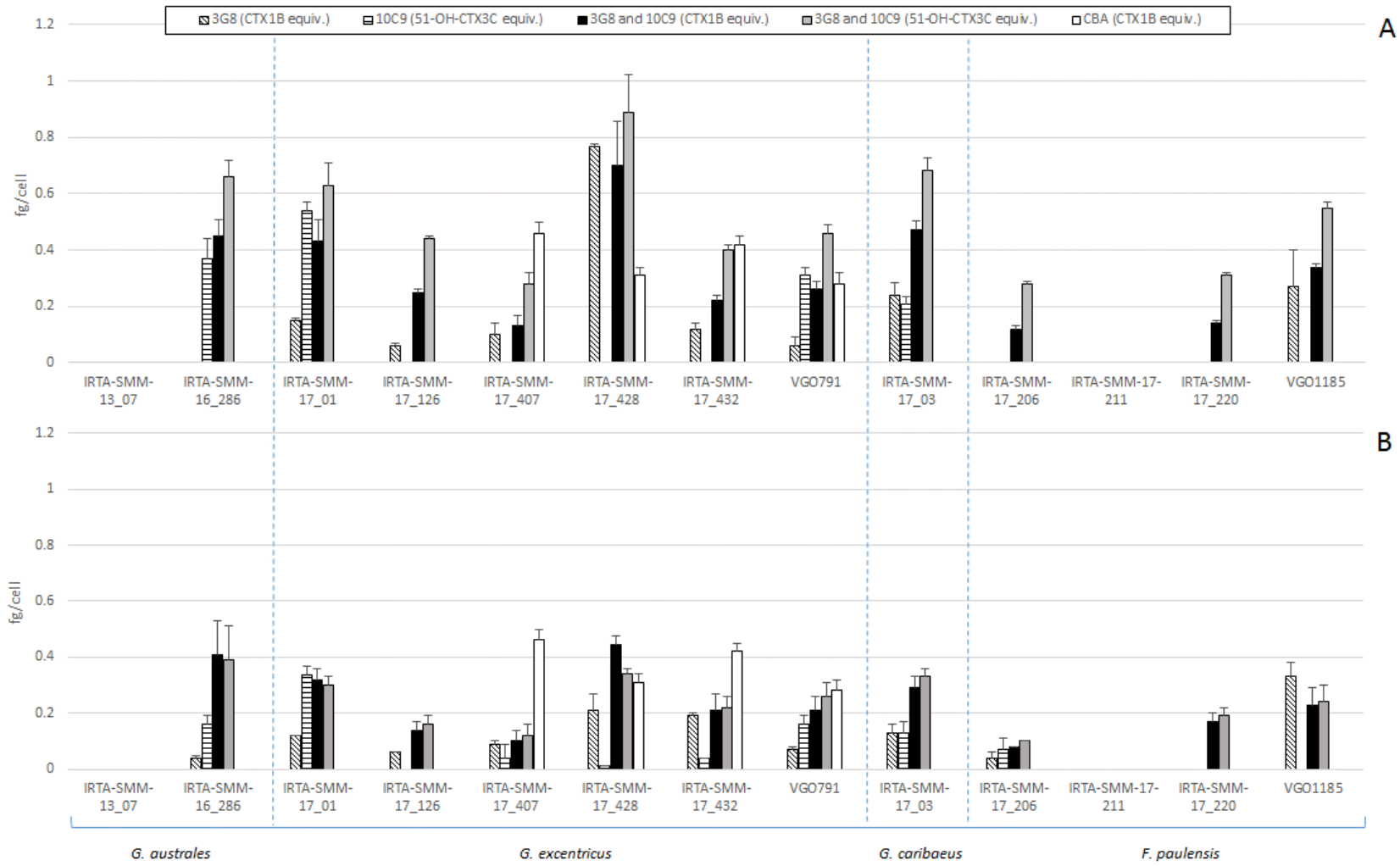

Figure 4. CTXs (fg/cell) extracted from different *Gambierdiscus* and *Fukuyoa* strains using the bead beater protocol, and evaluated with the colorimetric immunoassay (A) and the electrochemical immunosensor (B). CBA results are in both A and B for comparison purposes. Dashed lines separate genera and species.

Statistical analysis

CTXs quantifications significantly differed among strains (MANOVA, Wilks' $\Lambda < 0.001$, $F_{48, 113.75} = 39.20$, $P < 0.0001$), between immunosensing tools (Wilks' $\Lambda = 0.018$, $F_{4, 29} = 387.3$, $P < 0.0001$), and their interaction (Wilks' $\Lambda = 0.001$, $F_{48, 113.75} = 10.78$, $P < 0.0001$). Univariate tests (ANOVA) confirmed this pattern (Table 1). Overall, CTXs quantification differences were mainly explained by strain, followed by tool × strain interaction, tool having a minor weight (see η_p^2 values). The significant interaction between tools and strains (and differences between tools) resulted from a different sensitivity of the immunoassay and the immunosensor in relation to the CTXs contents in the analyzed strains. For strains with higher CTXs contents (*e.g.* IRTA-SMM-17_428), the immunoassay provided higher CTXs quantifications than the immunosensor, as can be observed from the estimated marginal means (Fig. 5). Nevertheless, the immunosensor could detect very low concentrations of CTXs in some strains, for instance CTX1B equiv. in IRTA-SMM-16_286 and IRTA-SMM-17_206, for which the immunoassay did not shown any toxin presence. The same can be observed for the quantification of 51-OH-CTX3C equiv. in IRTA-SMM-17_407, IRTA-SMM-17_428, IRTA-SMM-17_432 and IRTA-SMM-17_206. Therefore, the immunosensor emerged as a better immunosensing tool to analyze samples with low amounts of CTXs, attributable to the lower LOD, as previously mentioned.

Furthermore, the results showed that the variation in the CTXs production among *Gambierdiscus* species was not only interspecific but also intraspecific (Fig. 5). These results are in accordance to the work of Longo et al.,[48] who assessed the intraspecific variation in toxin production of *G. polynesiensis*. Results for the genus *Fukuyoa* showed an intraspecific variability in the CTXs production, identifying more than one strain as a CTXs producer.

Table 1. ANOVAs of the quantifications obtained for the two series of CTX congeners (CTX1B and CTX3C) per immunosensing tool, strain and their interaction.

Variable	Tool			Strain			Tool × Strain		
	$F_{1,32}$	P	η_p^2	$F_{12,32}$	P	η_p^2	$F_{12,32}$	P	η_p^2
3G8 (CTX1B equiv.)	7.05	0.012	0.181	58.55	<0.0001	0.956	14.41	<0.0001	0.844
10C9 (51-OH-CTX3C equiv.)	33.77	<0.0001	0.513	165.52	<0.0001	0.984	17.55	<0.0001	0.868
3G8+10C9 (CTX1B equiv.)	17.20	<0.0001	0.350	41.36	<0.0001	0.939	2.13	0.043	0.445
3G8+10C9 (51-OH-CTX3C equiv.)	246.94	<0.0001	0.885	57.57	<0.0001	0.956	9.02	<0.0001	0.775

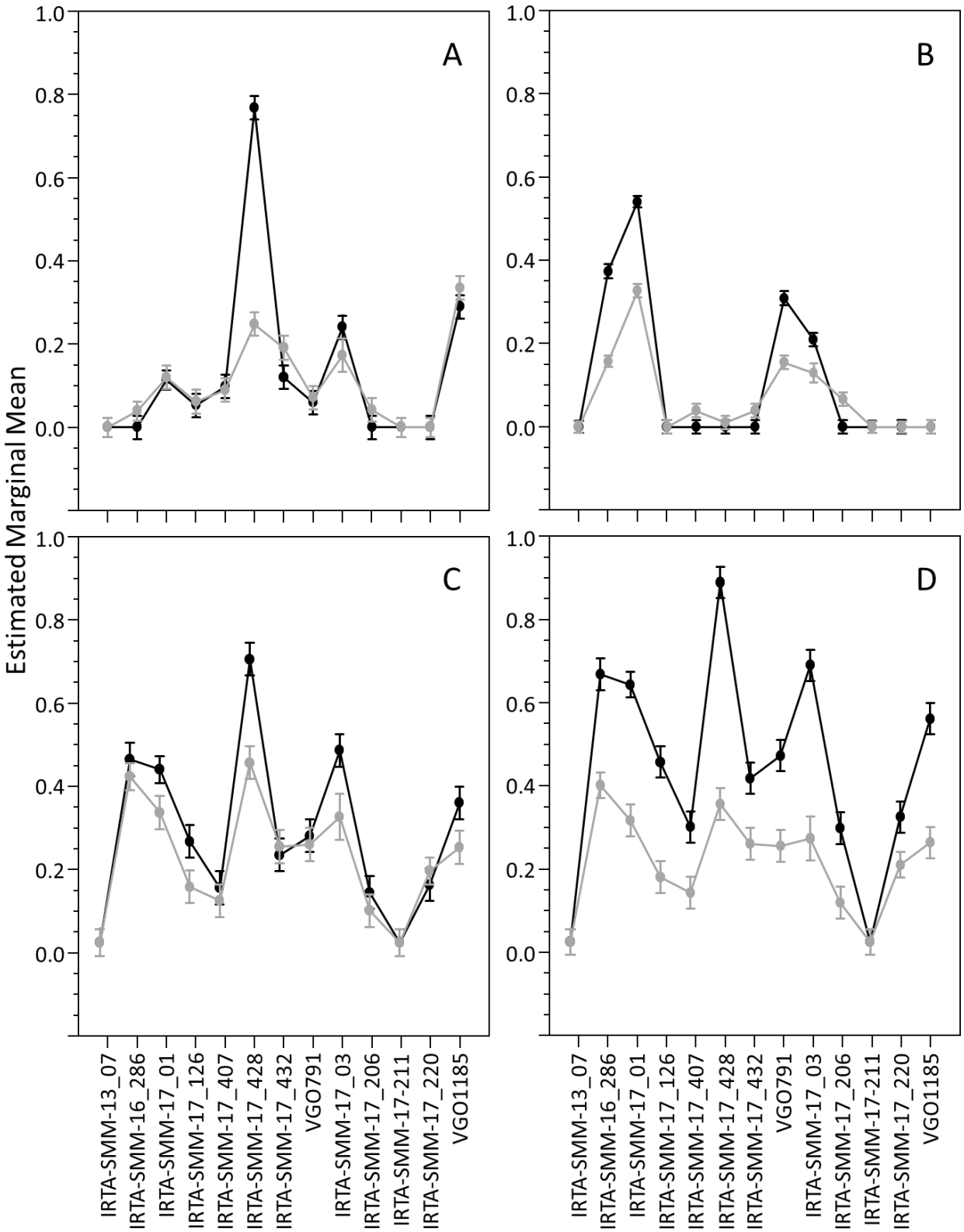

Figure 5. ANOVA adjusted means of CTXs quantifications for *Gambierdiscus* and *Fukuyoa* strains when using the 3G8 mAb and providing the results in CTX1B equiv. (A), when using the 10C9 mAb and providing the results in 51-OH-CTX3C equiv. (B), when using both mAbs and providing the results CTX1B equiv. (C), and when using both mAbs and providing the results 51-OH-CTX3C equiv. (D). Black dots refer to the results obtained with the immunoassay. Grey dots refer to the results obtained with the immunosensor.

Discussion

There is a general lack of studies about toxic profiles of *Gambierdiscus* and *Fukuyoa*, probably due to the complexity of the compounds produced by these genera. Furthermore, cultivating microalgae at a scale large enough to obtain a high cell abundance requires time and space. The subsequent pellet extraction is not straightforward either. In addition, there is a lack of CTXs standards, and the available ones are extremely costly. Regardless, in the past decade, some studies have focused on the identification of CTXs in different strains of *Gambierdiscus* and *Fukuyoa*.

Several *G. australes* strains have been identified as CTXs producers using RBA (17 to 30 fg/cell of CTX3C equiv.) and LC-MS/MS.[44] Lewis and coworkers[50] used a CBA with human neuroblastoma cells to assess the CTX-like activity of a *G. australes* strain without finding any toxicity. Subsequently, Pisapia and coworkers[38] identified other *G. australes* strains as CTX3C equiv. producers (from 0.6 to 2.7 fg/cell) using CBA. CBA was again used to screen other *G. australes* strains, in which the presence of CTX1B equiv. ranging from 200 up to 679 fg/cell[47] and from 31.1 to 107.16 fg/cell[49] was demonstrated. In the present study, CTXs contents are lower and one strain did not show toxicity at all. Discrepancies can arise due to different reasons including, for instance, the age of the strain. In fact, laboratory cultures seem to decrease their ability to produce toxins with the age of the strain, as recorded for one of the *G. australes* strains (IRTA-SMM-13_07), which is the same as that used in Reverté et al.[47]

Owing to its high toxicity, of *ca*. 1000 fg/cell of CTX1B equiv., as observed using a CBA, *G. excentricus* has also attracted attention of researchers worldwide.[36] CBA was also used to determine the CTX-like activity in the study of Pisapia and coworkers,[38] providing similar results (*ca.* 1400 fg/cell of CTX3C equiv.). Other works have reported quantifications of 469 fg/cell of CTX3C equiv.[3] and from 128.2 up to 510.6 fg/cell of CTX1B equiv.[49] in *G. excentricus* strains, also with CBA. It must be underlined that in all these studies, *G. excentricus* strains were identified as the most toxic among other *Gambierdiscus* species. In the current work, the *G. excentricus* strain VGO 791 showed lower CTXs contents than in previous works,[36, 38] again probably due to the age of the strain and the growth conditions. Nevertheless, even if not all the *G. excentricus* strains registered the highest CTXs contents, all of them showed the presence of CTXs (unlike *G. australes* and *F. paulensis*, for which some of them did not), and the strain that showed the highest CTXs contents belongs to the species *G. excentricus*. Additionally, CBA also only showed CTX-like activity in some of the *G. excentricus* strains. These results again place this species among the most toxic known to date, and one of the most important to monitor.

Gambierdiscus caribaeus strains have also been screened for toxicity with CBA, obtaining no CTX-like activity,[50] 1.6 fg/cell of CTX3C equiv.,[38] 0.66 fg/cell of CTX13C equiv.[3] and 2.59 fg/cell of CTX1B equiv.[49] CTXs quantifications obtained in the current work are close to the 0.66 fg/cell value. Here, this strain is much younger compared to that of *G. australes*, and probably

its ability for toxin production has not yet changed in response to the artificial growth conditions.

When considering the genus *Fukuyoa*, the lack of studies is even more evident. The genus *Fukuyoa* was split from *Gambierdiscus* in 2015, when molecular and morphologic criteria from two *Gambierdiscus* species *(G. yasumotoi* and *G. ruetzleri*) were used to define this new genus.[18] Therefore, the *G.* cf *yasumotoi* identified as non-toxic in Rhodes et al.[37] is a *Fukuyoa* species. Subsequent studies on *F. paulensis* did not present any CTX-like activity either, [4, 18, 42] with the exception of the work of Laza-Martínez and coworkers[76] where one *F. paulensis* strain (Dn135EHU) was identified as a 54-deoxy-CTX1B producer by LC-HRMS. *Fukuyoa paulensis* cultures analyzed in our work showed the presence of CTX congeners in the majority of the strains (even in the VGO1185 strain, reported as negative in Gomez et al.[18]), confirming the potential hazard of the genus *Fukuyoa*.

The CTXs production of Gambierdiscus and Fukuyoa is quite complex to evaluate. As it emerges from the literature, CBA has been the most commonly used tool to analyze microalgal extracts. Our results with CBA showed that CTX quantification has been possible only in 4 strains, whereas the immunosensing tools have detected CTXs in 11 out of 13. This can be attributed to the different recognition principles and interfering compounds. Whereas in the immunosensing tools, the detection is based on a structural affinity between CTXs and antibodies, in the CBA, CTXs bind to the voltage-gated sodium channels (VGSCs) of cells and block them, in an open state, causing a toxic effect. Even if in this work the CBA did not show any matrix effects and its LOD was as low as 0.4 pg/mL of CTX1B equiv. (lower than the LODs of the immunosensing tools), the detection of CTXs could be hidden by the presence of other *Gambierdiscus* or *Fukuyoa* toxic compounds. In fact, MTX is known to interfere in the CBA if no additional treatments are performed, but it is not recognized by the antibodies. Specificity of antibodies has always been a crucial issue for their applicability. Whereas the first immunoassays for CTXs showed cross-reactivity towards other marine toxins such as okadaic acid,[77, 78] this problem was overcome by the production of more specific antibodies.[79] The use of these new antibodies in the analysis of fish extracts significantly improved the correlations with other techniques.[80, 81] The antibodies used in the current work are highly specific and do not cross-react with the marine toxins brevetoxin A, brevetoxin B, okadaic acid and MTX.[61] Additionally, two different capture antibodies are used, thus the system is able to detect a higher number of CTXs congeners than in other works where only one antibody is used. It is also important to note that this is the first time that immunochemical approaches have been applied to the analysis of microalgae.

Interestingly, for the 4 strains where CTX-like activity was detected, quantifications obtained using the CBA and immunosensing tools were in the same order of magnitude. Instead, in our previous work,[53] quantifications obtained for fish extracts using CBA were around one order of magnitude higher. These results suggest that whereas the CTXs congeners that are found in microalgal extracts may mostly belong to the two series of CTX congeners detectable by

immunosensing tools, the CTXs congeners in fish may have undergone biotransformation processes.

It is necessary to be aware that we cannot rule out the possibility that the microalgal extracts contain other CTX congeners different from the four targets of this study (*i.e.* with different wings) that may go unnoticed by the immunosensing tools. Instead, LC-MS/MS technique is able to discriminate among all the CTX congeners, provided a standard is available. However, instrumental analysis techniques are strongly affected by the matrix effect caused by other compounds produced by *Gambierdiscus* and *Fukuyoa* (especially MTX), and so, as mentioned above, the extract has to undergo several purifications steps prior to the analysis. These steps usually cause toxin losses during the process. Consequently, pellets with high cell abundances are required to perform this analysis, and obtaining such cultures is costly in terms of time and space. Another important issue here is that whereas the immunosensing tools, like the CBA, provide a global response, LC-MS/MS detects individual CTX congeners. Therefore, the tools of this work may be more useful for the analysis of multi-toxin samples where each CTX congener is at a low concentration.

One particularity of our study is the low concentration of cells that have been used for toxin extraction and analysis (20,000 cell/mL), compared to the concentration in the order of 1,000,000 cell/mL used in the majority of other studies. In fact, during a bloom in the Canary Islands, the concentration of *Gambierdiscus* spp. was estimated to reach 10^4 cells g^{-1} wet weight.[82] Therefore, the fact that the immunosensing tools are able to detect CTXs at such low cell abundances makes them suitable for the analysis of field samples. Additionally, the protocol used herein for the rapid CTXs extraction requires very simple instrumentation, which can be portable and thus appropriate for *in situ* analysis. The results demonstrate that the bead beater protocol is suitable for CTXs extraction, considerably reducing time and costs, since it is possible to extract up to eight samples in 2 min.

More and more *Gambierdiscus* species are being found in non-endemic regions, such as *G. australes* that has been recently identified in the Balearic waters.[83] Whether this is due to an actual increase in their worldwide expansion, or because there are better tools to detect them, global warming will certainly act in favor of their proliferation. Therefore, there is a clear necessity for tools that can detect toxins from extracts with low abundance of microalgal cells, and they must be reliable, rapid, inexpensive and easy to use. The analysis obtained with the immunosensing tools will not only provide information regarding the ecology of *Gambierdiscus* and *Fukuyoa* genera, but will also be important for the socioeconomy and human health, with the ability to predict an intoxication outbreak, facilitating the avoidance of long-term neurological diseases and human fatalities related to ciguatera.

Conclusions

This study examined nine *Gambierdiscus* strains belonging to three species (*G. australes, G. excentricus* and *G. caribaeus)* and four *Fukuyoa paulensis* strains. Microalgal extracts were

obtained using a rapid and efficient CTXs extraction protocol and analyzed with an immunoassay and an immunosensor, which used MBs for the immobilization of antibodies. The unique features of this study are the ability to discriminate between two series of CTX congeners, giving more information on the toxic profile of *Gambierdiscus* and *Fukuyoa* species, the absence of interferences from non-structurally related compounds, and the high sensitivity of the immunosensing tools used for CTXs detection, which has avoided the requirement of large-scale cultures.

The approach presented in this work can be included in the group of methods ready to be used for ciguatera management (such as CBA, LC-MS/MS and RBA), providing a better understanding of CTXs production in the genera *Gambierdiscus* and *Fukuyoa*. The use of the immunosensing tools can open the way for regional and international comparative studies on the CTXs production of those genera and, consequently, on ciguatera as an expanding phenomenon.

Conflict of interest

There are no conflicts of interest to declare.

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Table S1. *Gambierdiscus* and *Fukuyoa* strains used in this study.

Strain	Species	Sampling location and year	Sampling point	Source	GenBank accession number	Sequenced region
IRTA-SMM-13_07	*G. australes*	SGI, Portugal, 2013	30°8'18.00" N, 15°52'4.20" W	Reverté et al.[46]	KY564320	D1-D3
IRTA-SMM-16_286	*G. australes*	Lanzarote, Spain, 2016	28°54'56.48" N, 13°42'38.20" W	This study	MT119197	D8-D10
IRTA-SMM-17_01	*G. excentricus*	Gran Canaria, Spain, 2017	28°9'14.52" N, 15°41'58.78" W	This study	MT119198	D8-D10
IRTA-SMM-17_126	*G. excentricus*	Gran Canaria, Spain, 2017	28°6'24.12" N, 15°42'40.14" W	This study	MT119199	D8-D10
IRTA-SMM-17_407	*G. excentricus*	La Gomera, Spain, 2017	28°4'57.99" N, 17°19'56.00" W	This study	MT119200	D8-D10
IRTA-SMM-17_428	*G. excentricus*	La Gomera, Spain, 2017	28°4'57.99" N, 17°19'56.00" W	This study	MT119201	D8-D10
IRTA-SMM-17_432	*G. excentricus*	La Gomera, Spain, 2017	28°4'57.99" N, 17°19'56.00" W	This study	MT119202	D8-D10
VGO791	*G. excentricus*	Tenerife, Spain, 2004	28°50'2.40" N, 16°49'8.34" W	Fraga et al.[35]	JF303066; JF303075	D1-D3; D8-D10
IRTA-SMM-17_03	*G. caribaeus*	El Hierro, 2017	27°49'26.48" N, 17°53'42.70" W	This study	MT119203	D8-D10
IRTA-SMM-17_206	*F. paulensis*	Mallorca, 2017	39°25'6.43" N, 3°16'15.55" E	Submitted work	MT119204	D8-D10
IRTA-SMM-17_211	*F. paulensis*	Menorca, 2017	39°58'54.18" N, 3°50'3.47" E	Submitted work	MT119205	D8-D10
IRTA-SMM-17_220	*F. paulensis*	Menorca, 2017	39°55'3.13" N, 4°1'51.18" E	Submitted work	MT119206	D8-D10
VGO1185	*F. paulensis*	Ubatuba, Brazil	23°30'3.09" S, 45°7'7.32" W	Gómez et al.[18]	KM886379	18S; D1-D4; ITS

SGI: Selvagem Grande Island

Table S2. CTXs (fg/cell) extracted from different *Gambierdiscus* and *Fukuyoa* strains using the bead beater protocol, and evaluated with the colorimetric immunoassay, the electrochemical immunosensor and the CBA.

Species	Strain	3G8 (CTX1B equiv.)		10C9 (51-OH-CTX3C equiv.)		3G8 and 10C9 (CTX1B equiv.)		3G8 and 10C9 (51-OH-CTX3C equiv.)		CBA (CTX1B equiv.)
		Immunoassay	Immunosensor	Immunoassay	Immunosensor	Immunoassay	Immunosensor	Immunoassay	Immunosensor	
G. australes	IRTA-SMM-13_07	nd	nd	nd	nd	nd	nd	nd	nd	nd
	IRTA-SMM-16_286	nd	0.04 ± 0.01	0.37 ± 0.07	0.16 ± 0.03	0.45 ± 0.06	0.41 ± 0.12	0.66 ± 0.06	0.39 ± 0.12	nd
G. excentricus	IRTA-SMM-17_01	0.15 ± 0.01	0.12 ± 0.00	0.54 ± 0.03	0.34 ± 0.03	0.43 ± 0.08	0.32 ± 0.04	0.63 ± 0.08	0.30 ± 0.03	nd
	IRTA-SMM-17_126	0.06 ± 0.01	0.06 ± 0.00	nd	nd	0.25 ± 0.01	0.14 ± 0.03	0.44 ± 0.01	0.16 ± 0.03	nd
	IRTA-SMM-17_407	0.10 ± 0.04	0.09 ± 0.01	nd	0.04 ± 0.05	0.13 ± 0.04	0.10 ± 0.04	0.28 ± 0.04	0.12 ± 0.04	0.46 ± 0.04
	IRTA-SMM-17_428	0.77 ± 0.01	0.21 ± 0.06	nd	0.01 ± 0.00	0.70 ± 0.16	0.44 ± 0.03	0.89 ± 0.14	0.34 ± 0.02	0.31 ± 0.03
	IRTA-SMM-17_432	0.12 ± 0.02	0.19 ± 0.01	nd	0.04 ± 0.00	0.22 ± 0.02	0.21 ± 0.06	0.40 ± 0.02	0.22 ± 0.04	0.42 ± 0.03
	VGO791	0.06 ± 0.03	0.07 ± 0.01	0.31 ± 0.03	0.16 ± 0.03	0.26 ± 0.03	0.21 ± 0.05	0.46 ± 0.03	0.26 ± 0.05	0.28 ± 0.04
G. caribaeus	IRTA-SMM-17_03	0.24 ± 0.04	0.13 ± 0.03	0.21 ± 0.02	0.13 ± 0.04	0.47 ± 0.03	0.29 ± 0.04	0.68 ± 0.05	0.33 ± 0.03	nd
F. paulensis	IRTA-SMM-17_206	nd	0.04 ± 0.02	nd	0.07 ± 0.04	0.12 ± 0.01	0.08 ± 0.00	0.28 ± 0.01	0.10 ± 0.00	nd
	IRTA-SMM-17_211	nd	nd	nd	nd	nd	nd	nd	nd	nd
	IRTA-SMM-17_220	nd	nd	nd	nd	0.14 ± 0.01	0.17 ± 0.03	0.31 ± 0.01	0.19 ± 0.03	nd
	VGO1185	0.27 ± 0.13	0.33 ± 0.05	nd	nd	0.34 ± 0.01	0.23 ± 0.06	0.55 ± 0.02	0.24 ± 0.06	nd

nd: not detected