Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by Neuro-2a cell-based assays

Laia Reverté[1], Anna Toldrà[1], Karl B. Andree[1], Santiago Fraga[2], Giada de Falco[1], Mònica Campàs[1] and Jorge Diogène[1*]

[1]IRTA, Ctra. Poble Nou, km 5.5, 43540 Sant Carles de la Ràpita, Spain

[2]Instituto Español de Oceanografía, Subida a Radio Faro 50, 36390 Vigo, Spain

[*]Jorge.diogene@irta.cat, Telf.: 00 34 902 789 449 (ext. 1807), Fax: 00 34 977 744 138

Abstract

Within the genus *Gambierdiscus*, several species are well-known producers of ciguatoxins (CTXs) and maitotoxins (MTXs). These compounds are potent marine toxins that accumulate through the food chain, leading to a foodborne disease known as ciguatera when contaminated fish is consumed. Given the threat that the presence of these toxins in seafood may pose to human health and fisheries, there is an evident necessity to assess the potential toxicity of *Gambierdiscus* sp. in a particular area. Thus, the purpose of this work was to evaluate the production of CTX and MTX of 10 strains of *G. australes* isolated from the Selvagem Grande Island (Madeira, Portugal) and El Hierro Island (Canary Islands, Spain). The strains were first characterised by light microscopy and species were confirmed by molecular biology, being identified as *G. australes*. Following the species identification, CTX and MTX production of *G. australes* extracts was evaluated at the exponential growth phase using Neuro-2a cell-based assays. Additionally, the production of MTX was also investigated in two of the G. *australes* strains collected at the stationary growth phase. Interestingly, 9 out of 10 strains were found to produce CTX-like compounds, ranging from 200 to 697 fg equiv. CTX1B · cell^{-1}. None of the *G. australes* strains showed MTX-like activity at the exponential phase, but MTX production was observed in two strains at the stationary growth phase (227 and 275 pg equiv. MTX · cell^{-1}). Therefore, the presence of *G. australes* strains potentially producing CTX and MTX in these Macaronesian Islands was confirmed herein.

Keywords**:** ciguatera; ciguatoxins; maitotoxins; G*ambierdiscus;* Neuro-2a assay.

Abbreviations**:** C-CTX, Caribbean Sea-ciguatoxin; CBA, cell-based assay; CTX, ciguatoxin; DMSO, dimethyl sulfoxide; DR, dose-ratio; EDTA, ethylenediaminetetraacetate; equiv., equivalents; FBS, fetal bovine serum; *G.*, *Gambierdiscus*; HI, Hierro Island; IC, inhibitory concentration; I-CTX, Indian Ocean-ciguatoxin; LC-MS/MS, liquid chromatography tandem mass-spectrometry; LOD, limit of detection; LOQ, limit of quantification; MTT, thiazolyl blue tetrazolium bromide; MTX, maitotoxin; Neuroblastoma murine cells, Neuro-2a; o/v, ouabain/veratridine; P-CTX or CTX1B, Pacific Ocean-ciguatoxin; psu, practical salinity unit; SGI, Selvagem Grande Island.

1. Introduction

Gambierdiscus spp. (Dinophyceae) are marine unicellular algae that reside in epiphytic, benthic and planktonic habitats of tropical and subtropical areas. They are commonly found in shallow and warm waters associated with macroalgae or coral reefs, with low light intensities, elevated salinities and preferably high nutrient contents (Dickey and Plakas 2010, Parsons et al. 2012, Kibler et al. 2015).

The taxonomy of *Gambierdiscus* was initiated by the description of *G. toxicus* in 1979 in the Gambier Islands, French Polynesia (Pacific Ocean) by Adachi and Fukuyo (Adachi and Fukuyo 1979). It was not until 16 years later that another species of *Gambierdiscus* was described from Belize, Central America (Caribbean Sea), named *G. belizeanus* (Faust 1995). Since then, several species have been added to the genus: *G. yasumotoi* from Singapore, Asia (Holmes 1998), *G. australes*, *G. pacificus* and *G. polynesiensis* from the Pacific Ocean (Chinain et al. 1999). In 2009, the genus was revised and 4 new species were discovered: *G. carolinianus* from North Carolina (USA), *G. caribaeus*, *G. ruetzleri* and *G. carpenteri* from Belize (Caribbean Sea) (Litaker et al. 2009). Two years later, Fraga et al. identified *G. excentricus* in the Canary Islands, Spain (Atlantic Ocean) (Fraga et al. 2011) and, in 2014, discovered in the same place the species *G. silvae* (Fraga and Rodriguez 2014). Also in 2014, *G. scabrosus* was described in Japan. In 2016, 3 more species have been described: *G. balechii* from the Celebes Sea (Pacific Ocean) (Fraga et al. 2016), *G. cheloniae*, isolated from the Cook Islands, Pacific Ocean (Smith et al. 2016) and *G. lapillus* from Australia (Kretzschmar et al. 2016). Very recently, a new species designated *G. honu* has been described from the south-west Pacific (Rhodes et al. 2017a). Interestingly, the species *G. yasumotoi* and *G. ruetzleri*, originally described as having a shape distinct from other *Gambierdiscus* (globular rather than lenticular-shaped and smaller in size) (Holmes 1998, Litaker et al. 2009), have been recently assigned to a new genus of the *Gambierdiscus* lineage, designated as *Fukuyoa* gen. (Gómez et al. 2015). The new genus currently contains *F. paulensis*, *F. yasumotoi* and *F. ruetzleri*.

With reference to toxin production, a significant variation has been described within the genus depending on the origin, growth conditions, growth phase and species. Despite few works having addressed this issue in detail, toxicity has been attributed to be species-specific (Chinain et al. 1999, Litaker et al. 2009, Caillaud et al. 2010b, Fraga et al. 2011, Parsons et al. 2012, Holland et al. 2013, Lewis et al. 2016). Benthic dinoflagellates of the genus *Gambierdiscus* are well-known producers of ciguatoxins (CTXs) and maitotoxins (MTXs) (Murata et al. 1990, Holmes et al. 1991, Murata and Yasumoto 2000). CTXs are lipid-soluble polyether compounds with polycyclic backbones, for which numerous congeners have been described differing in toxicity and structure. CTXs can be found in the Pacific Ocean (P-CTX), in the Caribbean Sea (C-CTX) and in the Indian Ocean (I-CTX), although the structure of the latter has not been determined. (Caillaud et al. 2010a). MTXs are potent water-soluble polyether toxins that increase intracellular calcium concentration (Murata and Yasumoto 2000). Certain *Gambierdiscus* species also produce other related compounds,

such as gambierol (Satake et al. 1993, Cuypers et al. 2008), gambierone (Rodriguez et al. 2015) and gambieric acids, the latter having high potent antifungal properties (Nagai et al. 1992). Among the different species described within the genus *Gambierdiscus*, *G. polynesiensis* has been identified as the most toxic, evidenced by the high CTX amounts determined by RBA, while *G. toxicus, G. pacificus and G. australes* showed very low RBA activity (Chinain et al. 2010). *G. excentricus* has also found to produce high CTXs quantity by the Neuro-2a cell-based assay (CBA), compared to *G.australes, G.carolinianus, G. carpenteri, G. balechi, G. caribaeus, G. silvae* and *G. pacificus* (Pisapia et al. 2017).

In the mid-1970s, these toxic microalgae were associated for the first time with ciguatera (Yasumoto et al. 1977), the most common non-bacterial seafood poisoning spread worldwide (Bagnis et al. 1980, Dickey and Plakas 2010, Friedman et al. 2017). Being at the bottom of the food chain, when *Gambierdiscus* species produce CTXs, these are bioaccumulated and undergo biotransformation through the food webs, starting with planktivorous organisms, reaching predator fish, and ultimately, humans (Lewis and Holmes 1993, Parsons et al. 2012). Ciguatera is a seafood-borne illness primarily associated with the consumption of fish containing CTXs that globally affects between 25,000 and 500,000 people per year (Dickey and Plakas 2010). Although little is known about the global distribution, the toxicity and the role that each *Gambierdiscus* species plays in causing ciguatera events, toxin production seems to be enhanced mostly in association to elevated water temperatures and variable environmental conditions (Litaker et al. 2010). Out of all the different toxins produced by *Gambierdiscus* spp., CTXs have been shown to be the main agent involved in ciguatera because of their mechanism of action, lipophilic nature and the likelihood to accumulate through the food chain. In fact, the 3 different MTXs produced by *Gambierdiscus* have not been shown to accumulate to significant levels in fish flesh (Lewis and Holmes 1993), although MTX implication in causing ciguatera may not be totally discarded. Therefore, investigations are warranted to determine whether additional species or strains of Gambierdiscus occur in temperate to subtropical water regions. Despite ciguatera outbreaks being originally reported from tropical and subtropical waters of the Indian, Caribbean and Pacific Oceans, these events have occasionally been reported outside ciguatera endemic areas, such as the Canary Islands and Madeira (Atlantic Ocean) (Dickey et al. 2010). The species of reef fish most commonly linked to ciguatera food poisoning (CFP) are barracuda, red snapper, grouper, amberjack, surgeonfish, sea bass and moray eel (Pearn et al. 2001). Specifically, in the Canary Islands, all documented ciguatera outbreaks since the first report in 2004 (Pérez-Arellano et al. 2005) have been associated with the consumption of large amberjack (*Seriola* spp.), locally known as "Madregal negro". Interestingly, in the same year, *Gambierdiscus* spp. was found adjacent to the islands of Tenerife and la Gomera (Aligizaki et al. 2008). From then, two more ciguatera outbreaks were reported in the Canary Islands involving several individuals, caused by the ingestion of amberjack caught off the northern coast of the Canary Islands, the latter of them near the Selvagem Islands (Portugal) (Boada et al 2010). In Madeira, two species of *Seriola*, captured in waters belonging to the Selvagem Islands, were found to contain

CTXs and were linked to several intoxications reported in this area from 2007 to 2008 (Otero et al. 2010). Along with the presence of *Gambierdiscus* sp. and CTXs in amberjack fish, *Gambierdiscus australes* was first described in the Pacific Ocean in 1999 (Chinain et al. 1999) and its presence in Hawaii and Japan was further confirmed (Litaker et al. 2010, Parsons et al. 2012).

The present work aims at defining the potential production of CTXs by 10 strains of *G. australes* obtained from a sampling carried out at the end of October 2013 in the Canary Islands (Spain) and in the beginning of November 2013 in the Selvagem Grande Island (Portugal), both belonging to the Macaronesian Islands (Atlantic Ocean), in order to better characterise the risk of ciguatera in this area. In addition, the presence of MTXs was also evaluated. To achieve this goal, single cell isolates were grown under specific conditions, cultures were harvested at the exponential phase and finally, toxins were extracted for quantification purposes. Following the identification of the species by light microscopy and further confirmation by molecular biology, cytotoxicity was evaluated for all 10 *G. australes* strains. Specifically, the production of CTX and MTX was assessed by two different approaches previously described using a neuroblastoma (Neuro-2a) CBA to determine CTX and MTX-like toxicity.

2. Materials and methods

2.1. Reagents and equipment

Neuroblastoma murine cells (Neuro-2a) were obtained from de CIC cellular bank of University of Granada. 2 µg of CTX1B standard was provided by R. Lewis, obtained as described in the literature (Lewis et al. 1991), reconstituted in absolute methanol and stored at a concentration of 2 $\mu g \cdot mL^{-1}$ at -20 °C. MTX was purchased from Wako Pure Chemical Industries GmbH (Germany), reconstituted in methanol:H_20 (50:50; v:v) at 20 µg/mL and stored at -20°C. Absolute methanol, biotin, boric acid, cobalt (II) chloride hexahydrate, dimethyl sulfoxide (DMSO), disodium ethylenediaminetetraacetate (EDTA) dehydrate, foetal bovine serum (FBS), fluorescent Brightener 28, iron(III) chloride hexahydrate, L-glutamine solution, manganese (II) chloride tetrahydrate, ouabain, PBS, penicillin/streptomycin, RPMI-1640 medium, SKF-96365, sodium nitrate, sodium phosphate dibasic, sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT), thiamine hydrochloride, trypsin-EDTA, veratridine, vitamin B12 and zinc chloride were all supplied by Sigma Aldrich (Tres Cantos, Spain).

Taq Polymerase was purchased from Invitrogen (Barcelona, Spain). QIAquick PCR Purification Kit was obtained from Qiagen (Hilden, Germany). 175-cm^2 cell culture flasks (vented cap) NUNCLON and surface 96 MicroWell NUNC plates were purchased from VWR International S.L. (Barcelona, Spain).

Neuro-2a cells were maintained at 37 °C and 5% CO_2 in an incubator (Binder, Tuttlingen, Germany). Absorbance was measured with a Microplate reader KC4 from BIO-TEK Instruments, Inc. with the GEN 2.09 software (Vermont, USA).

2.2. Sampling strategy and Gambierdiscus sp. culture

The sampling took place in the Macaronesian Islands, specifically, in El Hierro (Canary Islands, Spain) and in the Selvagem Grande Island (Madeira, Portugal) at the end of October and early in November 2013, respectively. The procedure was carried out as described in the work reported by Carnicer and co-workers (Carnicer et al. 2015). Briefly, macroalgae were collected between 1.5 and 0.5 m depth and placed in plastic bottles containing 200 mL of 0.2-µm filtered seawater. With the aim of releasing the epiphyte community from macroalgae, bottles were vigorously shaken for 1 min and then filtered through a 200-µm mesh to remove detritus and larger grazers (Reguera et al. 2011). The remaining 100 mL of each sample were fixed in 3% of lugol solution at 4 °C in order to evaluate cell abundance. Seawater was taken from the same location where macroalgae were collected and later used for initiation of cultures of *Gambierdiscus* sp. Additionally, water temperature and salinity were measured *in situ* using a portable multi-sensor probe CTD (YSI556 MPS). All samples were collected from shallow waters (<1.5 m), at salinity between 36.2 and 37.3 psu and from warm waters (T ranging from 24 to 27 °C).

In the laboratory, single *Gambierdiscus* sp. cells were isolated under an inverted microscope (Leica, DMIL) using a glass pipette following the capillary method (Hoshaw and Rosowski 1973) and transferred to 12-well microtiter plates (NUNC) containing filtered and autoclaved seawater from the original location: 36 psu modified ES medium (Provasoli 1968) at 24 °C (50:50; v:v). Once culture densities reached approximately 20-35 cells·mL^{-1}, isolates were inoculated in 25-cm^2 glass Erlenmeyer flasks (vented cap) filled with 50 mL of ES medium and maintained at 24 °C, with a 12:12 light:dark regime and a photon flux rate of 80 µmol photons/m^2 s. According to what was claimed by Bomber and co-workers (Bomber et al. 1990), *Gambierdiscus* cultures were acclimated for approximately 1 year prior to experimentation. Then, in order to determine growth rates, cultures were maintained for 40 days and cell densities were monitored during all this period. For testing cytotoxicity, cultures were harvested in the exponential growth phase or the stationary phase as follows: after approximately 15-20 days, cells were reinoculated in 1.8-L glass fernbach culture flask filled with 450 mL of acclimated medium. Finally, cells were reinoculated in 3 L of acclimated medium and transferred to sterile glass bottles under mild aeration (air pump 275R). Cell counts for each culture were obtained every 2-3 days by removing a 10-mL aliquot. Cell abundances (cells·mL^{-1}) were determined per triplicate for each strain removing an aliquot of 10 mL and by settling 3-mL samples for 3 h in Utermöhl Hydrobios chambers for plankton (Tecnicas Ecologicas Indalo S. L., Madrid, Spain). Growth rates of the cultures (µ) were calculated from the

exponential phase portion of the growth curve according to the equation described elsewhere (Guillard et al. 1973): $\mu = [(\ln (N_2 \cdot N_1^{-1})/(T_2-T_1)) \cdot \ln 2^{-1}]$; where N_1 and N_2 indicate the cell density at time corresponding to the starting (T_1) and end (T_2) of the exponential growth phase. Division times (Td) were calculated as the inverse of the growth rate: $T_d=1 \cdot \mu^{-1}$. A total of 3 L per strain of *Gambierdiscus* sp. cultures were harvested in the exponential growth phase or in the stationary phase and collected in sterile 50-mL tubes by centrifugation at 4,500 rpm for 20 min (Alegra X-15R, Beckman Coulter). Supernatants were discarded, pellets were reconstituted in absolute methanol and then pooled. These cell culture homogenates were then stored at -20 °C in sterile glass bottles until toxin extraction.

2.3. Molecular analyses

2.3.1. DNA extraction

Prior to DNA extraction, 50 mL of *Gambierdiscus* cultures were collected at the exponential phase by centrifugation at 2,500 rpm for 20 min (Andree et al. 2011). Cell pellets were resuspended in 200 µL of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6) and transferred to 2-mL screw-up cryotubes containing ~50 µg of 0.5-mm diameter zirconium glass beads (Biospec). Then, 25 µL of 10% DTAB and 200 µL of chloroform were added, and the mixture disrupted using a BeadBeater-8 (BioSpec) pulsed for 45 s at full speed. After centrifugation (5,000 rpm for 5 min), the aqueous phase was transferred to a fresh tube and an equal volume of phenol-chloroform added. DNA was extracted from the aqueous phase using standard phenol-chloroform procedures (Sambrook et al. 1989). Precipitation of the DNA from the final aqueous solution obtained was achieved by the addition of 2 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate (pH=8). The DNA pellet was then rinsed with 70% ethanol and dissolved in 50 µL of molecular biology-grade water. Genomic DNA was quantified and checked for its purity using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and stored at -20 °C for later analysis.

2.3.2. PCR amplification and DNA sequencing

The domain D1-D3 of the LSU rRNA gene was amplified from all strains using the pair of primers D1R/LSUB (5-ACCCGCTGAATTTAAGCATA-3/5-ACGAACGATTTGCACGTCAG-3) (Scholin et al. 1994, Litaker et al. 2003). 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 10-50 ng of template DNA. Amplifications were carried out in an Eppendorf Mastercycler nexus gradient as follows: an initial denaturation step of 5 min at 95 °C, 40 cycles of 30 s at 95 °C, 45 s at 52 °C and 30 s at 72 °C, and a final extension step of 10 min at 72 °C. Each PCR reaction was checked by agarose gel electrophoresis visualized with ethidium bromide stain. PCR products of ~840-910 bp were purified with QIAquick PCR Purification Kit and bidirectionally sequenced using the same primers as those applied in the amplification by an external company

(Sistemas Genómicos, LLC, Valencia, Spain). Consensus sequences obtained from both reads for each strain were manually edited using BioEdit v7.0.5.2 (Hall 1999) to remove primer sequences and terminal artefacts. The D1-D3 sequences obtained in this study were deposited in GenBank under accession numbers: KY564320-KY564329.

2.3.3. Phylogenetic analyses

Gambierdiscus consensus sequences were aligned using the ClustalW algorithm in BioEdit v7.0.5.2 with publicly available *Gambierdiscus* sequences from GenBank (NCBI). Evolutionary analyses were conducted in MEGA v5. All ambiguous positions were removed for each sequence pair. The evolutionary history was inferred from a final data set of 49 sequences, each including 629 positions, using the Maximum Likelihood method (Tamura et al. 2011). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 4.7257)). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was calculated (Felsenstein 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969) and are in the units of the number of base substitutions per site.

2.4. Light microscopy characterisation

Light microscopy observations were carried out under a Leica DMLA light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with epifluorescence, a UV lamp and UV excitation filters. Cultured cells were observed fixed with formalin. For plate pattern identification, cells were stained with Fluorescent Brightener 28 following a modified technique (Fritz and Triemer 1985). Cells were dissected, squashing them by gently pressing the cover slip over them, occasionally with the aid of sodium hypochlorite. Microphotographs were taken with an Axiocam HRC (Carl Zeiss, Jena, Germany) digital camera. When the depth of field was insufficient to obtain clear focus for the whole object, several pictures were taken at a series of different foci and were automatically merged using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA).

2.5. Toxin extraction

Toxin was extracted following the protocol described by Caillaud et al. 2011. In brief, cell pellets containing a total of $7.43x10^6$, $11.66x10^6$, $10.30x10^6$,$7.46x10^6$, $3.93x10^6$, $10.28x10^6$, $13.41x10^6$, $4.09x10^6$, $5.69x10^6$ and $7.89x10^6$ cells of strains IRTA-SMM-13-07, IRTA-SMM-13-08, IRTA-SMM-13-09, IRTA-SMM-13-10, IRTA-SMM-13-11, IRTA-SMM-13-12, IRTA-SMM-13-15, IRTA-SMM-13-16, IRTA-SMM-13-17 and IRTA-SMM-13-18, respectively, were sonicated in absolute methanol with an extraction volume (Ve) proportional to the total cell number (1 mL for 10^3 cells) for 30 min at 38% amplitude (Sonics Vibracell, Newton, USA) keeping temperature low (ice below the bottle). After

the first sonication, extracts were transferred to 50-mL falcon tubes and centrifuged for 5 min at 4 °C and 600 g. Sonication of the cell pellet was repeated twice in methanol:H_2O (50:50; v:v) using the same volume as for the first sonication. Extracts were centrifuged at 600 g and 4 °C for 5 min, supernatants were pooled and evaporated to dryness at 40 °C (Büchi Syncore, Flawil, Switzerland). Once evaporated, pellets were redissolved in absolute methanol, centrifuged at same conditions and filtered through 0.45-µm nylon filters. Methanolic extracts were stored at -20 °C until analysis.

2.6. Toxin analyses

Prior to exposing Neuro-2a cells to microalgal extracts, an aliquot of each *G. australes* extract was evaporated under a nitrogen stream and resuspended in RPMI medium containing 5% FBS.

2.6.1. CTX-like toxicity

CTX-like toxicity was determined in *Gambierdiscus* sp. crude extracts using the CBA reported by Caillaud and co-workers (Caillaud et al. 2009). The addition of the compounds ouabain and veratridine confers specificity to this assay. Whereas ouabain binds to the Na^+/K^+-ATPase pump, blocking it and impeding the sodium efflux of sodium, veratridine increases Na^+ permeability through the selective binding to the voltage-gated sodium channels (VGSCs), blocking them in an open position. Cells exposure to these compounds results in an increase in the intracellular Na^+ concentration, which becomes even higher in the presence of CTX, resulting in higher cell mortality. Neuro-2a cells were exposed for 24 h to 8 concentrations of CTX1B standard (1/2 serial dilutions)/*G. australes* extracts (3/4 serial dilutions) with and without ouabain/veratridine (0.1 mM/0.01 mM) per triplicate. To test cytotoxicity of strain IRTA-SMM-13-16 1/2 serial dilutions were used because Neuro-2a cells were exposed to a higher dose of extract than the others. Absorbance values were recorded following the MTT method described elsewhere (Manger et al. 1993) and expressed as the percentage (%) of viable cells with respect to the controls with and without ouabain/veratridine treatment. Toxin contents were quantified as the ratio between the concentration of CTX1B causing 50% of cell viability inhibition (IC_{50} CTX1B) and the equivalent concentration of *G. australes* cells resulting in 50% inhibition of cell viability (IC_{50} *G. australes* extract) and expressed in pg equiv. of CTX1B · $cell^{-1}$ of *G. australes.* A calibration curve for CTX1B standard was performed ranging from 0.39 to 50 pg·mL^{-1} per well each day of the experiment, and a dose-response curve was fitted to a sigmoidal logistic 4-parameter equation using SigmaPlot software 12.0 (Systat Software Inc., California, US). From this equation, the limit of detection (LOD) was calculated, corresponding to the concentration of CTX1B necessary to inhibit the cell viability by 20% (IC_{20}). Similarly, the IC_{50} and the working range (IC_{20}-IC_{80}) were determined from the equation.

2.6.2. MTX-like toxicity

MTX-like toxicity was determined using the CBA described elsewhere (Caillaud et al. 2010b). Briefly, before exposing Neuro-2a cells to MTX standard or *G. australes* extracts, cells were treated with and without SKF (30 µM per well, made up in miliQ water), a molecule that acts as an inhibitor of MTX cytotoxicity through the blockage of the voltage-gated Ca^{2+} channels (VGCCs), counteracting the increase of intracellular calcium concentration triggered by the exposure of cells to MTX. For each experimental day, a calibration curve was set for the MTX standard, ranging from 0.79 to 100.30 $pg \cdot mL^{-1}$ per well. As for CTX1B dose-response curve, responses obtained with and without SKF treatment were fitted to sigmoidal logistic 4-parameter equations and IC_{50} values and LODs were calculated. In order to determine whether *G. australes* produce MTX-like compounds or not, dose-ratio (DR) was first calculated as the ratio between the IC_{50} provided by *G. australes* extract treated with SKF and the IC_{50} obtained for the extract without SKF treatment. Afterwards, as proposed by Pöch and colleagues (Poch et al. 1995), slope DR was corrected (DR_{corr}) as $\log(DR_{obs})slope_{obs}$, where DR_{obs} is the DR measured from the curve and $slope_{obs}$ is the absolute value of the Hill slope coefficient of the curve with SKF treatment provided by the SigmaPlot software from the adjustment of the curve. Therefore, while DR values above 1 imply the presence of MTX-like compounds, DR values below 1 indicate the absence of these compounds. If DR_{corr} is >1, the MTX equiv. contents determined in *G. australes* extracts are expressed in nmoles per 10^6 cell and calculated according to the following formula:

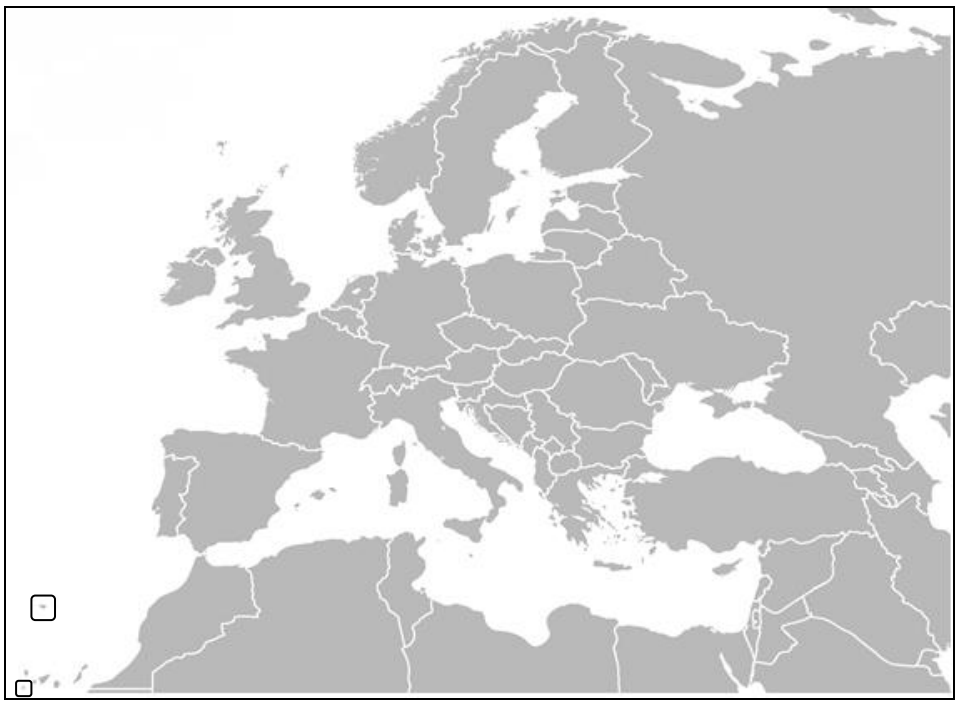
[MTX-like] = ((IC_{50} SKF^+ of MTX (nM)·IC_{50} SKF^+ of *Gambierdiscus* extract $(cells \cdot mL^{-1})^{-1}$) x 10^3

To better compare the amounts of MTX produced by *G. australes* with the estimated CTX equiv. concentrations, MTX equiv. contents were expressed in pg equiv. MTX $cell^{-1}$.

3. Results and discussion

In 2014, *G. australes* was observed for the first time in Tenerife and Gran Canaria (Canary Islands, Spain), Atlantic Ocean (Fraga and Rodriguez 2014). A recent study shows that *G. australes*, along with *G. caribaeus* are the species of *Gambierdiscus* present in El Hierro Island (Rodríguez et al. 2017). The present work reports the presence of G. *australes* in El Hierro (Canary Islands, Spain) and in the Selvagem Grande Island (Madeira, Portugal), both in the Atlantic Ocean. Exact locations from where isolates were obtained are shown in **fig. 1**.

a)



287

288

289

290

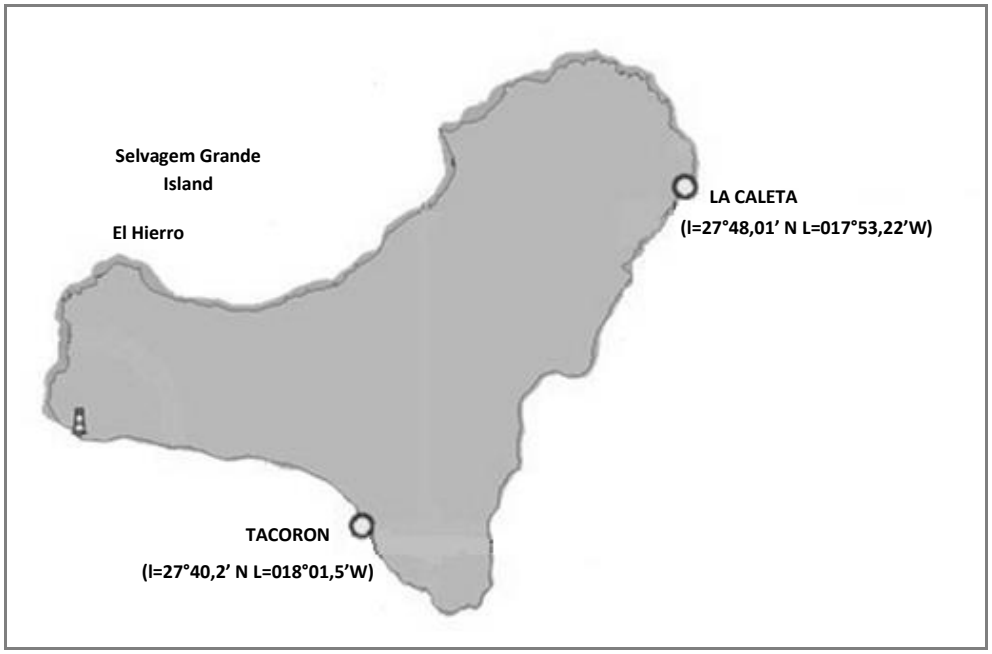
291

292

293

294

295 **b)**



296

297 **c)**

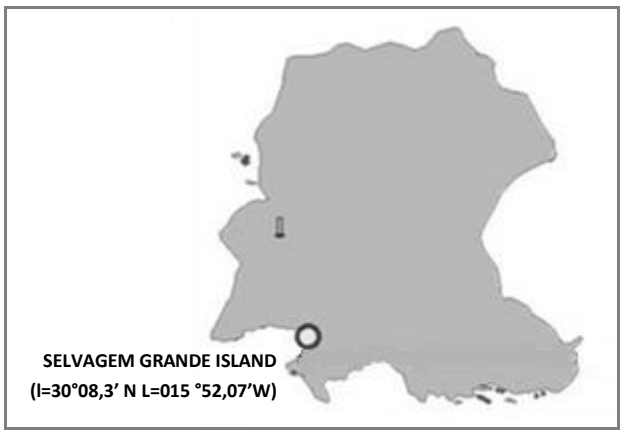


Fig. 1. a) Map of Europe with El Hierro and Selvagem Grande Islands situation. **b)** Map of El Hierro (Canary Islands, Spain). **c)** Map of the Selvagem Grande Island (Madeira, Portugal). Black circles indicate specific locations from where *G. australes* isolates were obtained and coordinates are shown in brackets.

These findings are consistent with the previous reports on CFP events and presence of *Gambierdiscus* sp. in the Atlantic Ocean, as well as with the recent description of the Canary Islands as hotspots of *Gambierdiscus* (Rodríguez et al. 2017). Overall, evidence that Macaronesian Islands may represent new expanding endemic areas for which potential risk of CFP needs to be further evaluated.

3.1. Species identification

Partial LSU rRNA sequences (D1-D3 and D8-D10 region) and partial SSU rDNA sequences are commonly used to delineate *Gambierdiscus* species (e.g. Chinain et al. 1999, Litaker et al. 2009, Nishimura et al. 2013, Fraga et al. 2016, Smith et al. 2016, Rodríguez et al. 2017) and more sequences of this region are publicly available in GenBank. In this study, the D1-D3 domain of the LSU rRNA gene was chosen as a molecular marker. The results of the analysis of the D1-D3 region demonstrate that the sequences of the 10 strains of *Gambierdiscus* were nearly identical. BLAST (Basic Local Alignment Search Tool) analyses showed that the D1-D3 sequences had a high identity with existing *Gambierdiscus* species previously registered in GenBank, with *G. australes* isolates showing the highest similarity. The optimal D1-D3 tree with the highest log likelihood (-4826.1240) is shown in **fig. 2**. The phylogenetic results showed that the 10 isolated *Gambierdiscus* strains are clustered into a well-supported group, which corresponds to *G. australes*.

Notably, the high values of bootstrap (99) obtained in the phylogenetic tree for this group of sequences unequivocally confirmed that they belong to the species *G. australes.*

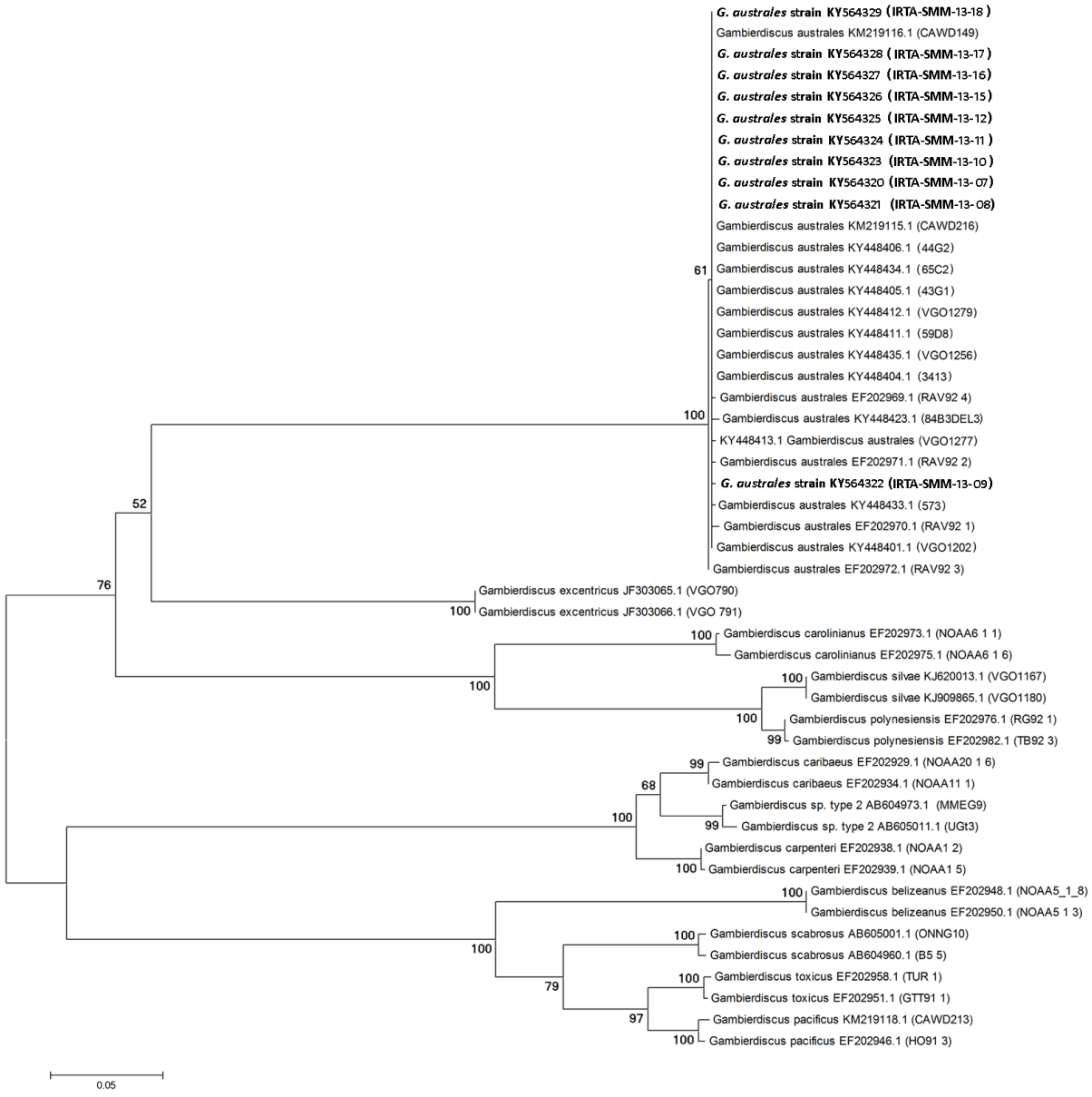


Fig. 2. Phylogenetic analysis of LSU rDNA gene (D1-D3 region) showing the relationship between *Gambierdiscus* strains from this study (in bold) and other *Gambierdiscus* strains. Values at nodes are bootstrap values obtained by the Maximum Likelihood method. Bootstrap values less than 30% are not shown. Scale bar represents substitutions per site.

The 10 strains of this work identified as *G. australes* by molecular biology were morphologically characterised by calcofluor staining method (**fig. 3a,b**). A very high morphological variability was found within each studied strain, mainly in the shape of plate 2' which is used to differentiate species of genus *Gambierdiscus* into two groups: one with a hatchet-shaped plate and another with a rectangular one (Litaker et al. 2009). *G. australes* (Chinain et al. 1999,

Litaker et al. 2009) was described as belonging to the group with a rectangular plate 2', but in the examined strains this was found to be a variable character as many cells showed a hatchet-shaped plate as shown in fig. 3a.

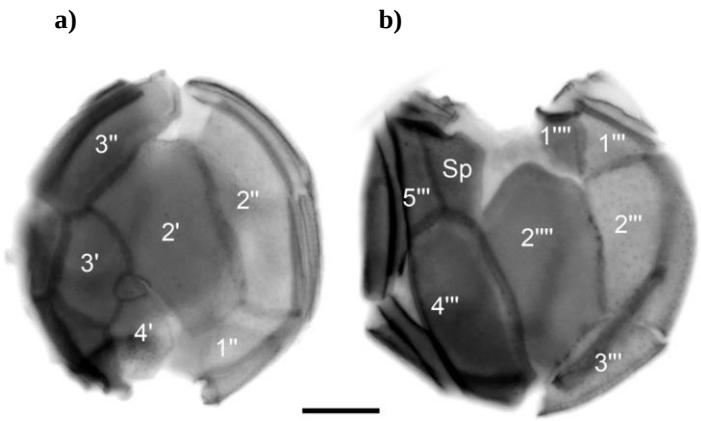
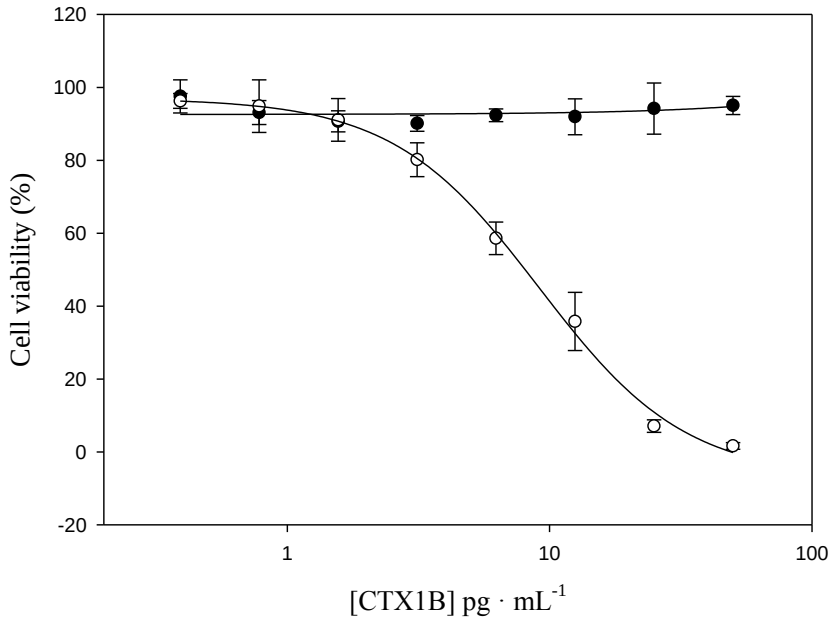


Fig. 3. Fluorescence microscopy images of calcofluor-stained thecae of *Gambierdiscus australes* (strain IRTA-SMM-13-11-12). **a)** Epitheca, **b)** Hypotheca. Scale bar 20 µm.

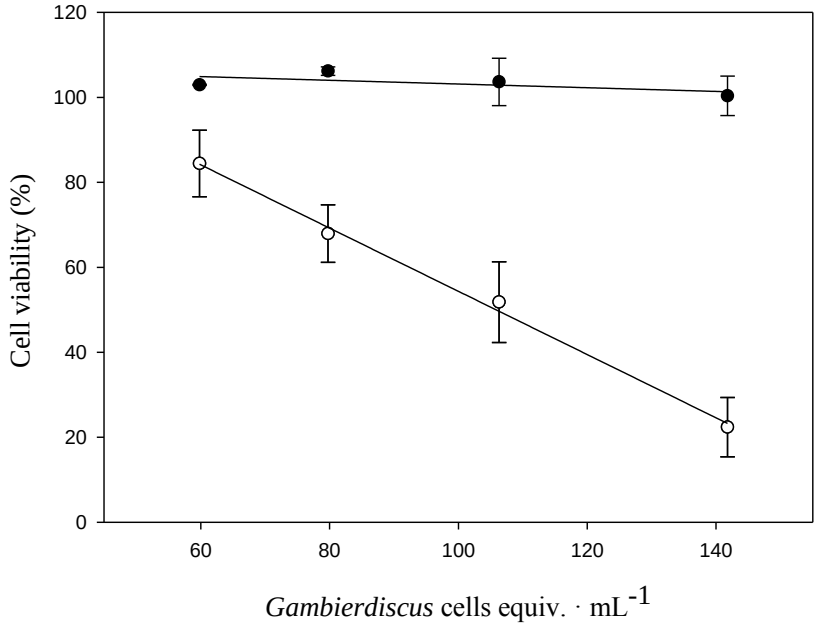
3.2. Determination of CTX-like activity by the Neuro-2a assay

The calibration curve obtained from the exposure of CTX1B to Neuro-2a cells resulted in an IC_{50} of 8.1 ± 0.54 pg·mL^{-1}, an LOD, set at IC_{20}, of 3.2 ± 0.31 pg·mL^{-1} and a working range (IC_{20}-IC_{80}) between 3.2 and 17.9 ± 0.67 pg·mL^{-1} (**fig. 4a**). Since a good sensitivity was attained by the Neuro-2a assay, reliable quantifications could be obtained by the exposure of Neuro-2a cells to *G. australes* extracts with and without the ouabain/veratridine (o/v) treatment. For all the toxic *G. australes* strains, the Neuro-2a cells were exposed up to approximately 260 cells equiv.·mL^{-1} of *G. australes* extract without any observation of matrix effect, and quantification of CTX was assessed according to the IC_{50}. The limit of quantification (LOQ) was calculated as the ratio of the LOD obtained with CTX1B to the maximum concentration of *G. australes* extracts used in the assay, which was of 12.31 fg equiv. CTX1B · cell^{-1}. Among the 10 strains of *G. australes* analysed, 9 strains showed CTX-like toxicity between 200 and 697 fg equiv. CTX1B · cell^{-1} and the IRTA-SMM-13-16 strain was not toxic (**Table 1** and **fig. 4b and 4c**).

a)


120
100
80
60
40
20
0
-20
Cell viability (%)
1
10
100
[CTX1B] pg · mL-1

b)


120
100
80
60
40
20
0
Cell viability (%)
60
80
100
120
140
Gambierdiscus cells equiv. · mL-1

c)

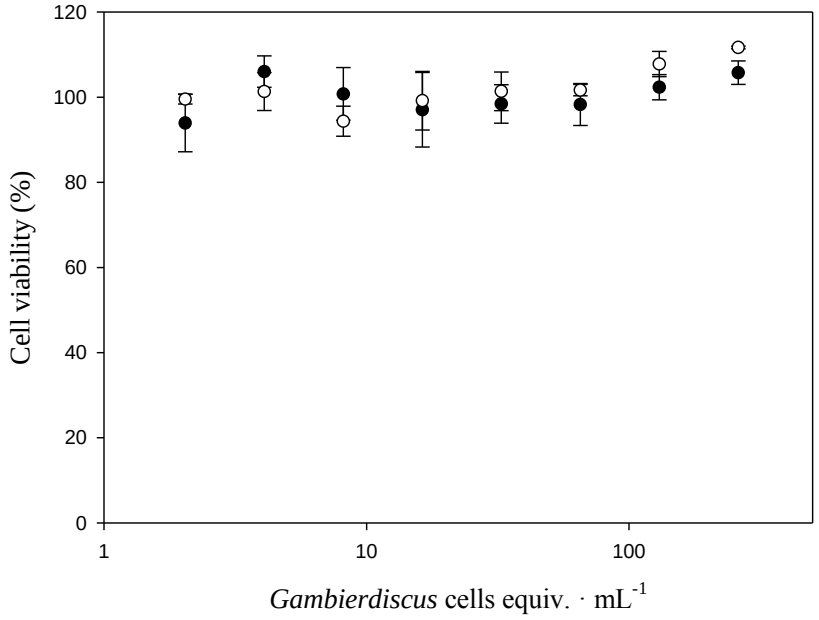


Fig 4. Dose-response curves of Neuro-2a cells exposed to **a)** CTX1B standard, **b)** *G. australes* IRTA-SMM-13-10 extract and **c)** *G. australes* IRTA-SMM-13-16 extract with (**O**) and without (●) o/v treatment.

Although some disparity has been observed for *G. australes* in the literature with reference to toxicity depending on the origin, culture conditions, harvesting growth phase and analytical method, results are partially consistent with data published regarding the toxicity of *G. australes* in exponential phase. In a first attempt to describe the toxin production by three different species of *Gambierdiscus* harvested in the late exponential phase, low/moderate toxicity was reported for *G. australes* strains from the Raivavae Island (Australes Archipelago) measured by the mouse bioassay (MBA), which was described as the least toxic species in both CTX and MTX production, compared to other species such as *G. polynesiensis* ($4 \cdot 10^{-4}$ *vs.* $800 \cdot 10^{-4}$ MU·1,000 cells^{-1}) (Chinain et al. 1999). Later, the same group tested the toxicity of several *G. australes* strains by receptor binding assay (RBA) with respect to P-CTX-3C under the same harvesting conditions and, again, a low CTX-like activity was found, specifically at the fg level (between 17 and 30 fg equiv. P-CTX-3C · cell^{-1}) (Chinain et al. 2010). Similarly, Roeder and colleagues (Roeder et al. 2010) showed the presence of the CTX analogue 2,3-dihydroxy P-CTX-3C by LC-MS/MS in *G. australes* from Hawaii (Pacific Ocean) harvested at the exponential growth phase. Despite the identification of this analogue, quantification was not possible due to the lack

of the corresponding standard. Another work, probably more comparable to our work since it was performed with the Neuro-2a assay, also found low cytotoxicity (Rhodes et al. 2010) at the sub-pg range (40 fg CTX1B equiv. · $cell^{-1}$) for *G. australes* isolated from the Cook Islands. Screening of toxicity in *G. australes* strains from the Atlantic and the Pacific Ocean also showed low toxicity by the Neuro-2a assay (0.6 and 2.7 fg P-CTX-3C equiv. · $cell^{-1}$, respectively) (Pisapia et al. 2017). The CTX amounts determined herein remarkably differed from the results reported by Lewis and co-workers (Lewis et al. 2016). In the latter work, no CTX-like activity was obtained in the screening of the dichloromethane fractions of a *G. australes* strain from Hawaii using an assay with SH-SY5Y cells. HPLC analysis further confirmed that no CTX congeners were present. Unfortunately, further comparisons could not be made since data provided for this strain was qualitative.

For comparative purposes, levels of toxicity of the strains analysed in this work are shown together with the contents reported in the literature for *G. australes* species on **Table 2**. Ciguatoxin equiv. contents determined herein in *G. australes* strains surpass by 10-fold (Chinain et al. 2010, Rhodes et al. 2010) and by 100-fold (Pisapia et al. 2017) the quantities reported in other studies. From our results, it can be concluded that *G. australes* coming from three different locations in Macaronesia (Atlantic Ocean) produce similar amounts of CTXs, with the exception of one strain that did not produce CTX-like compounds. The different CTX levels observed indicate that toxin quantifications could vary depending on the analysis method, the CTX standard and the culture conditions employed. Indeed, it has been demonstrated that media composition can also affect the *Gambierdiscus* physiology and might eventually influence the toxicity (Lechat et al. 1985). Thus, the ES media used herein with a high metallic salt content, the quality of the water or the environmental conditions of the laboratory with influence on the growth rates, among other factors, may account for the high CTX production observed in the strains analysed in this work. Furthermore, difference of toxicity among strains may also be explained by other external factors such as genetic differences or influence of environmental parameters.

3.3. Determination of MTX-like activity by Neuro-2a assay

The MTX calibration curve provided an IC_{50} value of 32.77 ± 1.72 pg MTX·mL^{-1} in cells treated with SKF and an IC_{50} value of 10.89 ± 1.04 pg MTX·mL^{-1} in cells without SKF treatment. Moreover, IC_{20} values (LODs) of 23.22 ± 1.41 pg MTX·mL^{-1} with SKF treatment and 8.72 ± 0.72 pg MTX·mL^{-1} without treatment were obtained. These values were in accordance with the sensitivity reported in the literature (Caillaud et al. 2010b), demonstrating the antagonistic effect rendered by the MTX inhibitor. As can be observed in **fig. 5a,** the pre-treatment of cells with SKF induced a rescue effect on cells: a higher percentage of viable cells at higher MTX concentrations compared to the percentage of viable cells without SKF treatment.

420

421 Exponential phase

422 Neuro-2a cells were exposed to all microalgal extracts with and without SKF, at a maximum concentration of extract of
423 approximately 260 cells equiv.·mL^{-1} per well. The LOQ, calculated as the ratio between the IC_{20} obtained with MTX
424 standard with SKF treatment and the maximum concentration of extract of *G. australes* cells·mL^{-1}, was of 89.31 pg
425 equiv. MTX · $cell^{-1}$. All 10 strains were non-toxic to Neuro-2a cells, as indicated by the 100% of viable cells obtained
426 with and without SKF treatment (results not shown). Since none of the *G. australes* extracts concentrations showed a
427 toxic effect to Neuro-2a cells under these conditions (~100% cell viability), neither DR nor DR_{corr} values could be
428 calculated in exponential phase. These results indicated the lack of production of MTX-like compounds by *G. australes*
429 extracts at the exponential phase. This observation is supported by the statement that *Gambierdiscus* sp. produces *large*
430 amounts of MTX-like compounds in the stationary growth phase, but it is likely to produce CTX-like compounds in the
431 exponential phase (Holland et al. 2013). Due to the different culture conditions and origin despite belonging to the same
432 species, there is some controversy about this issue in the literature (Parsons et al. 2012) (Table 2). While Chinain and
433 co-workers (Chinain et al. 1999) reported higher MTX-like toxicity than CTX-like toxicity of *G. australes* from Hawaii
434 and Japan (Pacific Ocean) in the late exponential growth phase by MBA, the production of 2-3-dihydroxy P-CTX-3C
435 was described for *G. australes* from Hawaii by liquid chromatography tandem mass spectrometry (LC-MS/MS)
436 collected at the same growth phase (Roeder et al. 2010). Similarly, 25 strains of *G. australes* isolated from Macauley
437 Island (Pacific Ocean) showed MTX production ranging from 3 to 36 pg MTX-1 · $cell^{-1}$ but no CTX was detected in
438 any of the strains by LC-MS/MS analysis (Rhodes et al. 2017b). Additionally, different results were obtained in other
439 works (Rhodes et al. 2010, Rhodes et al. 2014), which reported the production of MTX but not CTX of *G. australes*
440 isolated from the Cook Islands by MBA. Nevertheless, the comparison with the later works is difficult since no details
441 are given regarding if these strains were collected in the exponential or in the stationary growth phase. In the work
442 published by Holland and co-workers (Holland et al. 2013), a further comparison was made between the production of
443 MTX and CTX in different growth phases, species, origins and culture conditions. Although little was done for *G.*
444 *australes* isolated from Hawaii, the authors proved that haemolytic activity triggered by MTX significantly increased
445 from 7 to 40% from the exponential to the late exponential-early stationary growth phase. Contrarily, in another work,
446 the presence of MTX (4.7 and 5 pg MTX equiv. · $cell^{-1}$) in *G. australes* strains from the Atlantic Ocean and Pacific,
447 respectively, collected at the exponential phase was determined using the erythrocyte lysis assay (Pisapia et al. 2017).

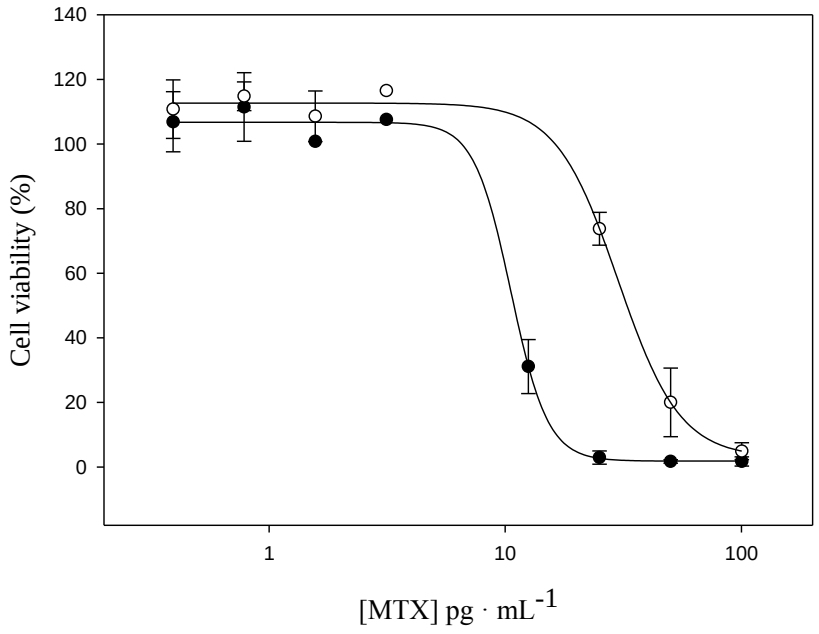
448

449 Stationary phase

450 Although the study of MTXs at different growth phases was beyond the scope of this work, having observed that *G.*
451 *australes* strains were not MTX producers in the exponential phase but CTX producers, two *G. australes* strains (IRTA-

SMM-13-09 and IRTA-SMM-13-10) were additionally tested for MTX production in the stationary phase. As shown for one of the strains (**fig. 5b** and table 2), MTX-like compounds were detected in the *G. australes* strains collected in the stationary growth phase. Estimated MTX equiv. concentrations were 275 and 227 pg equiv. MTX · cell^{-1} for IRTA-SMM-13-09 and IRTA-SMM-13-10 strains, respectively. These results were in agreement with the results reported in the literature (Holland et al. 2013), pointing out the likelihood of MTX production in the stationary phase by *G. australes* isolated from the Macaronesian Islands.

a)



b)

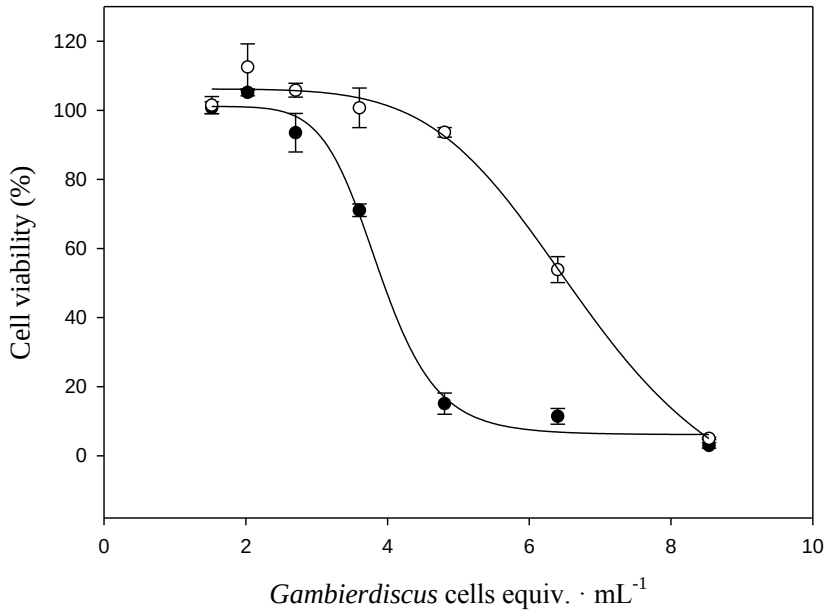


Fig 5. Dose-response curves obtained for Neuro-2a cells exposed to **a)** MTX standard and **b)** *G. australes* IRTA-SMM-13-10 extract at stationary phase with (o) and without (●) SKF treatment.

From a food safety perspective, the levels of toxicity shown by the strains analysed in this work are high enough to be of concern, but still below the levels of CTXs usually found in the "super-producers" *G. polynesiensis* and *G. excentricus* (Chinain et al. 1999, Chinain et al. 2010, Fraga et al. 2011, Pisapia et al. 2017). Thus, in the evaluation of the potential risk of ciguatera in the Canary Islands and Madeira Archipelago (Atlantic Ocean), the presence of *G. australes* species may also be used as an indicator. However, further work is necessary to better characterize the toxicity of the benthic *G. australes* among the different growth phases in ciguateric areas and to overcome the difficulties in comparing cell toxicity results from different studies and *Gambierdiscus* species. This issue could be improved by the thorough standardization of the culture conditions, toxin extraction and analysis method within the research community.

3.4. Growth rates

Growth rates determined in the *G. australes* cultures studied in this work ranged from 0.198 to 0.393 divisions·day^{-1}, and these values are comparable with those reported in the literature for the genus *Gambierdiscus* (Caillaud et al. 2011). Although still below the value of 0.5 divisions·day^{-1}, the growth rates determined herein (Table 1) were somewhat higher than the values of 0.15 divisions·day^{-1} reported for *G. australes* (Chinain et al. 2010, Pisapia et al. 2017). Therefore, the culture conditions used in this study seemed to favor a faster growth of the dinoflagellates, resulting in higher growth rates than those previously reported for the same species. This high growth capacity appeared to have an influence on the toxin production. Thus, the high growth rate could be linked to the high levels of CTX production shown by the present *G. australes* strains compared to other studies. If we divided the strains into two groups according to the CTX toxicity, except for strain IRTA-SMM-13-16, the highest growth rates coincided with the highest toxicity. Notably, whereas in the group of the slow growth rates (≤0.245) the average of fg CTX equiv. · cell^{-1} is of 347 (<350 fg CTX1B equiv. · cell^{-1}), in the high growth rate group (0.245 > 0.400) the mean CTX production value is of 592 fg CTX1B equiv. · cell^{-1}, nearly doubled toxicity.

4. Conclusions

The present study reports the unequivocal identification of ten strains of *G. australes* in El Hierro (Canary Islands, Spain) and in the Selvagem Grande Island (Madeira, Portugal). The species identification has been confirmed by DNA sequence analysis of amplified rDNA fragments. *G. australes* cultures were established and acclimated for one year, and toxin production was assessed at the exponential growth phase. Furthermore, MTX-like activity was tested in two strains collected at the stationary phase. Production of CTX-like compounds was indicated by Neuro-2a cell-based

494 assays in nine out of ten strains in the exponential phase, containing from 200 to 697 fg equiv. CTX1B · $cell^{-1}$. None of
495 the screened strains were found to produce MTX-like compounds at the exponential phase, but two of the *G. australes*
496 extracts analysed at the stationary phase produced MTX (227 and 275 pg equiv. MTX · $cell^{-1}$). The findings obtained
497 herein evidence that in these Macaronesian Islands most of the *G. australes* strains were CTX producers at the
498 exponential phase and suggest the MTX production at the stationary phase.
499 Despite the great strides that have recently been made in this field, further work should be done to provide insight into
500 the relation between toxin production and environmental parameters, to better establish toxin patterns according to the
501 growth phase within specific species and therefore, to predict ciguatera incidence in endemic and non-endemic
502 geographical areas.
503

References

Adachi, R., Fukuyo, Y., 1979. Thecal structure of a marine toxic dinoflagellate *gambierdiscus-toxicus* gen et sp-nov collected in a ciguatera-endemic area. Bull Jpn Soc Sci Fish 45:67-71.

Aligizaki, K., Nikolaidis, G., Fraga, S., 2008. Is *Gambierdiscus* expanding to new areas? Harmful Algae News 36:6-7.

Andree, K.B., Fernandez-Tejedor, M., Elandaloussi, L.M., Quijano-Scheggia, S., Sampedro, N., Garces, E., Camp, J., Diogene, J., 2011. Quantitative PCR coupled with melt curve analysis for detection of selected *Pseudo-nitzschia* spp. (Bacillariophyceae) from the northwestern Mediterranean Sea. J Appl Environ Microbiol 77:1651-1659.

Bagnis, R., Chanteau, S., Chungue, E., Hurtel, J.M., Yasumoto, T., Inoue, A., 1980. Origins of ciguatera fish poisoning-a new dinoflagellate, G*ambierdiscus-toxicus* Adachi and Fukuyo, definitively involved as a causal agent. Toxicon 18:199-208.

Boada, L. D., Zumbado, M., Luzardo, O. P., Almeida-González, M., Plakas, S. M., Granade, H. R., Abraham, A., Jester, E. L. E., Dickey, R. W., 2010. Ciguatera fish poisoning on the West Africa coast: an emerging risk in the Canary Islands (Spain). Toxicon 56:1516-1519.

Bomber, J.W., Tindall, D.R., Venable, C.W., Miller, D.M., 1990. Pigment composition and low-light response of 14 clones of *Gambierdiscus-toxicus*. In Granéli, E., Sundström, B., Edler, L. & Anderson, D. M. [Eds.] Toxic Marine Phytoplankton. Elsevier, New York , pp. 263–8.

Caillaud, A., Canete, E., de la Iglesia, P., Gimenez, G., Diogene, J., 2009. Cell-based assay coupled with chromatographic fractioning: A strategy for marine toxins detection in natural samples. Toxicol in Vitro 23:1591-1596.

Caillaud, A., de la Iglesia, P., Darius, H.T., Pauillac, S., Aligizaki, K., Fraga, S., Chinain, M., Diogene, J., 2010a. Update on methodologies available for ciguatoxin determination: perspectives to confront the onset of ciguatera fish poisoning in Europe. Mar Drugs 8:1838-1907.

Caillaud, A., Yasumoto, T., Diogene, J., 2010b. Detection and quantification of maitotoxin-like compounds using a neuroblastoma (Neuro-2a) cell based assay. Application to the screening of maitotoxin-like compounds in *Gambierdiscus* spp. Toxicon 56:36-44.

Caillaud, A., de la Iglesia, P., Barber, E., Eixarch, H., Mohammad-Noor, N., Yasumoto, T., Diogène, J., 2011. Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices: Application to *Gambierdiscus pacificus* in culture. Harmful algae 10:433-446.

Carnicer, O., Tunin-Ley, A., Andree, K.B., Turquet, J., Diogene, J., Fernandez-Tejedor, M., 2015. Contribution to the genus *Ostreopsis* in Reunion Island (Indian Ocean): molecular, morphologic and toxicity characterization. Cryptogam Algol 36:101-119.

534 Chinain, M., Darius, H.T., Ung, A., Cruchet, P., Wang, Z., Ponton, D., Laurent, D., Pauillac, S., 2010. Growth and toxin
535 production in the ciguatera-causing dinoflagellate *Gambierdiscus polynesiensis* (Dinophyceae) in culture. Toxicon
536 56:739-750.
537 Chinain, M., Faust, M.A., Pauillac, S., 1999. Morphology and molecular analyses of three toxic species of
538 *Gambierdiscus* (Dinophyceae): *G-pacificus*, sp nov., *G-australes*, sp nov., and *G-polynesiensis*, sp nov. J Phycol
539 35:1282-1296.
540 Cuypers, E., Abdel-Mottaleb, Y., Kopljar, I., Rainier, J.D., Raes, A.L., Snyders, D.J., Tytgat, J., 2008. Gambierol, a
541 toxin produced by the dinoflagellate *Gambierdiscus toxicus*, is a potent blocker of voltage-gated potassium channels.
542 Toxicon 51:974-983.
543 Dickey, R.W., Plakas, S.M., 2010. Ciguatera: A public health perspective. Toxicon 56, 123-136.
544 Faust, M.A., 1995. Observation of sand-dwelling toxic dinoflagellates (Dinophyceae) from widely differing sites,
545 including two new species. J Phycol 31:996-1003.
546 Felsenstein, J., 1985. Confidence-limits on phylogenies - An approach using the bootstrap. Evolution 39:783-791.
547 Fraga, S., Rodriguez, F., 2014. Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of
548 *Gambierdiscus silvae* sp nov., a new potentially toxic epiphytic benthic dinoflagellate. Protist 165:839-853.
549 Fraga, S., Rodriguez, F., Caillaud, A., Diogene, J., Raho, N., Zapata, M., 2011. *Gambierdiscus excentricus* sp nov
550 (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). Harmful Algae 11:10-22.
551 Fraga, S., Rodriguez, F., Riobo, P., Bravo, I., 2016. *Gambierdiscus balechii* sp nov (Dinophyceae), a new benthic toxic
552 dinoflagellate from the Celebes Sea (SW Pacific Ocean). Harmful Algae 58:93-105.
553 Friedman, M., et al. 2017. An updated review of ciguatera fish poisoning: clinical, epidemiological, environmental, and
554 public health management. Mar Drugs 15, 72; doi:10.3390/md15030072.
555 Fritz, L., Triemer, R.E., 1985. A rapid simple technique utilizing calcofluor white M2R for the visualization of
556 dinoflagellate thecal plates. J Phycol 21:662-664.
557 Gomez, F., Qiu, D., Lopes, R.M., Lin, S., 2015. *Fukuyoa paulensis* gen. et sp nov., a new genus for the globular species
558 of the dinoflagellate *Gambierdiscus* (Dinophyceae). Plos One 10.
559 Guillard, R.L., 1973. Division rates. In Stein, J. [Ed.], Culture methods and growth measurements. Cambridge
560 University Press, Cambridge, p. 289.
561 Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows
562 95/98/NT. Nucleic Acids Symposium Series 41:95-98.
563 Holland, W.C., Litaker, R.W., Tomas, C.R., Kibler, S.R., Place, A.R., Davenport, E.D., Tester, P.A., 2013. Differences
564 in the toxicity of six *Gambierdiscus* (Dinophyceae) species measured using an in vitro human erythrocyte lysis assay.
565 Toxicon 65:15-33.

566 Holmes, M.J., 1998. *Gambierdiscus yasumotoi* sp. nov. (Dinophyceae), a toxic benthic dinoflagellate from southeastern
567 Asia. J Phycol 34:661-668.
568 Holmes, M.J., Lewis, R.J., Poli, M.A., Gillespie, N.C., 1991. Strain dependent production of ciguatoxin precursors
569 (gambiertoxins) by *Gambierdiscus-toxicus* (Dinophyceae) in culture. Toxicon 29:761-775.
570 Hoshaw, R.W., Rosowski J. R., 1973. Methods for microscopic algae. In Stein, J.R. [Ed.] Handbook of phycological
571 methods, culture methods and growth measurements. Cambridge University Press, Cambridge, pp. 53-67.
572 Jukes, T.H., Cantor, C. R., 1969. Evolution of protein molecules, mammalian protein metabolism (ed. Munro, H. N.).
573 Academic Press, New York, pp. 21-123.
574 Kibler, S.R., Tester, P.A., Kunkel, K.E., Moore, S.K., Litaker, R.W., 2015. Effects of ocean warming on growth and
575 distribution of dinoflagellates associated with ciguatera fish poisoning in the Caribbean. Ecol Modell 316:194-210.
576 Kretzschmar, A.L., Verma, A., Harwood, D.T., Hoppenrath, M., Murray, S., 2016. Characterization of *Gambierdiscus*
577 *lapillus* sp. nov. (Gonyaulacales, Dinophyceae): a new toxic dinoflagellate from the Great Barrier Reef (Australia). J
578 Phycol. doi:10.1111/jpy.12496
579 Lechat, I., Partenski, F., Chungue, E., 1985. *Gambierdiscus toxicus*: culture and toxin production. In Gabrie, C., Salvat,
580 B. [Eds.] Proceedings of the 5th International coral reef congress, Tahiti, vol. 4. Antenne Museum-Ephe, Moorea, 443-
581 448.
582 Lewis, R.J., Holmes, M.J., 1993. Origin and transfer of toxins involved in ciguatera. Comp Biochem Physiol C Toxicol
583 Pharmacol 106:615-628.
584 Lewis, R.J., Inserra, M., Vetter, I., Holland, W.C., Hardison, D.R., Tester, P.A., Litaker, R.W., 2016. Rapid extraction
585 and identification of maitotoxin and ciguatoxin-like toxins from Caribbean and Pacific *Gambierdiscus* using a new
586 functional bioassay. Plos One 11.
587 Lewis, R.J., Sellin, M., Poli, M.A., Norton, R.S., Macleod, J.K., Sheil, M.M., 1991. Purification and characterization of
588 ciguatoxins from moray eel (*Lycodontis-javanicus*, Muraenidae). Toxicon 29:1115-1127.
589 Litaker, R.W., Vandersea, M.W., Faust, M.A., Kibler, S.R., Chinain, M., Holmes, M.J., Holland, W.C., Tester, P.A.,
590 2009. Taxonomy of *Gambierdiscus* including four new species, *Gambierdiscus caribaeus*, *Gambierdiscus carolinianus*,
591 *Gambierdiscus carpenteri* and *Gambierdiscus ruetzleri* (Gonyaulacales, Dinophyceae). Phycol 48:344-390.
592 Litaker, R.W., Vandersea, M.W., Faust, M.A., Kibler, S.R., Nau, A.W., Holland, W.C., Chinain, M., Holmes, M.J.,
593 Tester, P.A., 2010. Global distribution of ciguatera causing dinoflagellates in the genus *Gambierdiscus*. Toxicon
594 56:711-730.
595 Litaker, R.W., Vandersea, M.W., Kibler, S.R., Reece, K.S., Stokes, N.A., Steidinger, K.A., Millie, D.F., Bendis, B.J.,
596 Pigg, R.J., Tester, P.A., 2003. Identification of *Pfiesteria piscicida* (Dinophyceae) and *Pfiesteria*-like organisms using
597 internal transcribed spacer-specific PCR assays. J Phycol 39:754-761.

598 Manger, R.L., Leja, L.S., Lee, S.Y., Hungerford, J.M., Wekell, M.M., 1993. Tetrazolium-based cell bioassay for
599 neurotoxins active on voltage-sensitive sodium-channels - semiautomated assay for saxitoxins, brevetoxins, and
600 ciguatoxins. Anal Biochem 214:190-194.
601 Murata, M., Legrand, A.M., Ishibashi, Y., Fukui, M., Yasumoto, T., 1990. Structures and configurations of ciguatoxin
602 from the moray eel *gymnothorax-javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus-toxicus*. J
603 Am Chem Soc 112:4380-4386.
604 Murata, M., Yasumoto, T., 2000. The structure elucidation and biological activities of high molecular weight algal
605 toxins: maitotoxin, prymnesins and zooxanthellatoxins. Nat Prod Rep 17:293-314.
606 Nagai, H., Murata, M., Torigoe, K., Satake, M., Yasumoto, T., 1992. Gambieric acids, new potent antifungal substances
607 with unprecedented polyether structures from a marine dinoflagellate *Gambierdiscus-toxicus*. J Org Chem 57:5448-
608 5453.
609 NCBI, National Center for Biotechnology Information Search database, U.S. National Library of Medicine. Available
610 online at: https://www.ncbi.nlm.nih.gov/. Accessed by 19th December 2017.
611 Nishimura, T., Sato, S., Tawong, W., Sakanari, H., Uehara, K., Shah, M.M.R., Suda, S., Yasumoto, T., Taira, Y.,
612 Yamaguchi, H., Adachi, M., 2013. Genetic diversity and distribution of the ciguatera-causing dinoflagellate
613 *Gambierdiscus* spp. (Dinophyceae) in coastal areas of Japan. Plos One 8.
614 Parsons, M.L., Aligizaki, K., Bottein, M.-Y.D., Fraga, S., Morton, S.L., Penna, A., Rhodes, L., 2012. *Gambierdiscus*
615 and *Ostreopsis*: Reassessment of the state of knowledge of their taxonomy, geography, ecophysiology, and toxicology.
616 Harmful Algae 14:107-129.
617 Pérez-Arellano, J.-L., Luzardo, O. P., Brito, A. P., Cabrera, M. H., Zumbado, M., Carranza, C., Boada, L. D. 2005.
618 Ciguatera Fish Poisoning, Canary Islands. Emerg Infect Dis 11(12):1981–1982.
619 Pisapia, F., Holland, W. C., Hardison, D. R., Litaker, R. W., Fraga, S., Nishimura, T., Adachi, M., Nguyen-Ngoc, L.,
620 Sechet, V., Amzil, Z., Herrenknecht, C., Hess, P., 2017. Toxicity screening of 13 *Gambierdiscus* strains using Neuro-2a
621 and erythrocyte lysis bioassays. Harmful Algae 63:173–183.
622 Otero, P., Pérez, S., Alfonso, A., Vale, C., Rodríguez, P., Gouveia, N. N., Gouveia, N., Delgado, J., Vale, P., Hirama,
623 M., Ishihara, Y., Molgó, J., Botana, L. M., 2010. First toxin profile of ciguateric fish in Madeira Arquipelago (Europe).
624 Anal Chem 82(14):6032-6039.
625 Poch, G., Reiffenstein, R.J., Baer, H.P., 1995. Quantitative estimation of potentiation and antagonism by dose ratios
626 corrected for slopes of dose-response curves deviating from one. J Pharmacol Toxicol Methods 33:197-204.
627 Provasoli, L., 1968. Media and prospects for the cultivation of marine algae. In Watanabe, A., Hattori, A. [Ed.],
628 Cultures and Collection of Algae, Proceedings of the US-Japanese Conference, Hakone, pp. 63-75.

Reguera, B., Alonso, R., Moreira, A., Méndez, S., 2011. Guía para el diseño y puesta en marcha de un plan de seguimiento de microalgas productoras de toxinas (in Spanish only). COI de UNESCO y OIEA, Paris y Viena. Available online at: http://unesdoc.unesco.org/images/0021/002145/214510s.pdf. Accessed by 19th December 2017.

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.L., Smith, K.F., Munday, R., Selwood, A.I., McNabb, P.S., Holland, P.T., Bottein, M.-Y., 2010. Toxic dinoflagellates (Dinophyceae) from Rarotonga, Cook Islands. Toxicon 56:751-758.

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

Rhodes, L. L., Smith, K. F., Murray, S., Harwood, D. T., Trnski, T., Munday, R., 2017b. 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 15:219; doi:10.3390/md15070219.

Rodríguez, F., Fraga, S., Ramilo, I., Rial, P., Figueroa, R. I., Riobó, P., Bravo, I., 2017. "Canary Islands (NE Atlantic) as a biodiversity 'hotspot' of *Gambierdiscus*: Implications for future trends of ciguatera in the area". Harmful Algae 67:131-143.

Rodriguez, I., Genta-Jouve, G., Alfonso, C., Calabro, K., Alonso, E., Sanchez, J.A., Alfonso, A., Thomas, O.P., Botana, L.M., 2015. Gambierone, a ladder-shaped polyether from the dinoflagellate *Gambierdiscus belizeanus*. Org Lett 17:2392-2395.

Roeder, K., Erler, K., Kibler, S., Tester, P., Ho, V.T., Lam, N.N., Gerdts, G., Luckas, B., 2010. Characteristic profiles of ciguatera toxins in different strains of *Gambierdiscus* spp. Toxicon 56: 731-738.

Sambrook, J., Fritsch, E. F., Maniatis, T., 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Satake, A., Murata, M., Yasumoto, T., 1993. Gambierol: A new toxic polyether compound isolated from the marine dinoflagellate *Gambierdiscus toxicus*. J Am Chem Soc 115:361-362.

Scholin, C.A., Herzog, M., Sogin, M., Anderson, D.M., 1994. Identification of group and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae) II. Sequence-analysis of a fragment of the LSU Ribosomal rRNA gene. J Phycol 30:999-1011.

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

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., and Kumar S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 28:2731-2739.

Yasumoto, T., Nakajima, I., Bagnis, R., Adachi, R., 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. Bull Jpn Soc Sci Fish 43:1021-1026.

Acknowledgements

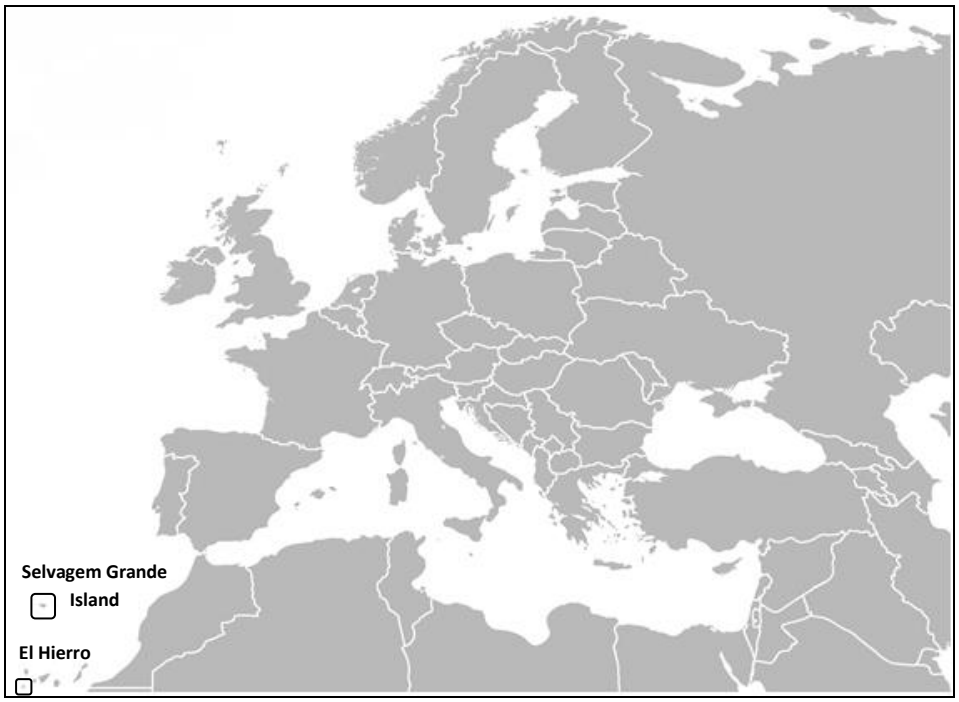
Authors would like to thank Dr. Olga Carnicer for the helpful advices in sampling and culturing of microalgae. Authors also acknowledge Dr. Ana Raquel Díaz from Universidad de la Laguna (Canary Islands, Spain), Dr. Pedro Diniz from ITB - Investigação e Transferência de Biotecnologia (Madeira, Portugal) and personnel from Fisheries and the Parque Natural da Madeira (Portugal) for their collaboration in sampling, processing and shipment of microalgal samples. The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under the ECsafeSEAFOOD project (grant agreement n° 311820), from the CERCA Programme/Generalitat de Catalunya, from the Ministerio de Economía, Industria y Competitividad through the SEASENSING (BIO2014-56024-C2-2-R) and CIGUASENSING (BIO2017-87946-C2-2-R) projects. LR acknowledges the scholarship from the ECSafeSEAFOOD project and AT thanks IRTA-URV-Banco Santander for the PhD grant (2015PMF-PIPF-67). We would like to acknowledge the constructive comments of two anonymous reviewers.

Author's contributions

JD and LR designed the study and carried out the sampling and isolation of microalgae. LR and GdF maintained, scaled up the *Gambierdiscus* cultures and performed the cytotoxicity assays on Neuro-2a cells for CTX and MTX quantification. AT and KA carried out the species identification by molecular biology. SF characterized the species by light microscopy. LR wrote the manuscript. JD and MC participated in the writing and discussion of the manuscript.

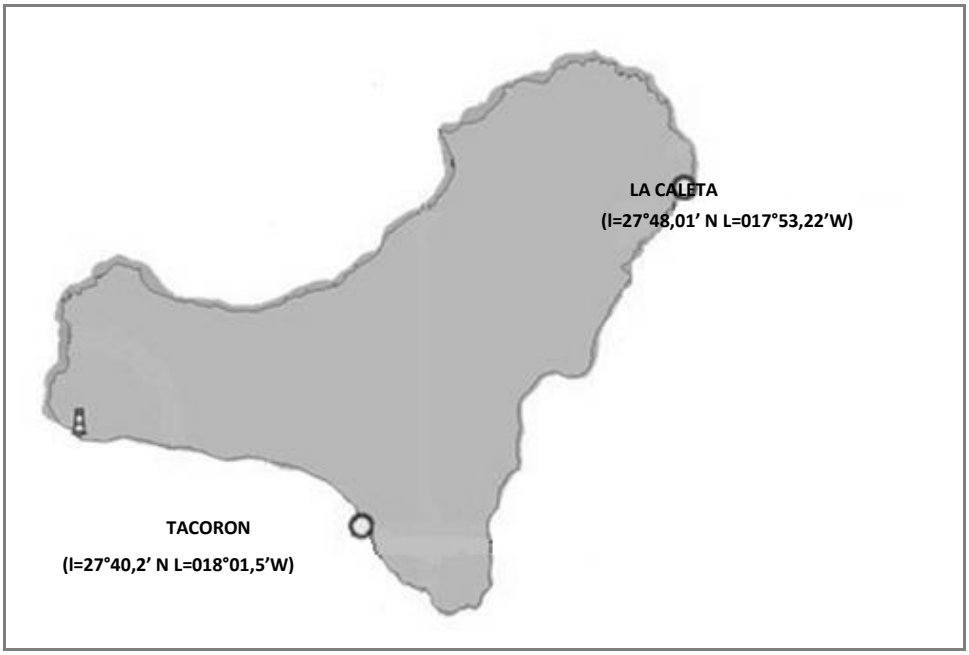
All authors have revised and approved the final version of the article.

687 **a)**



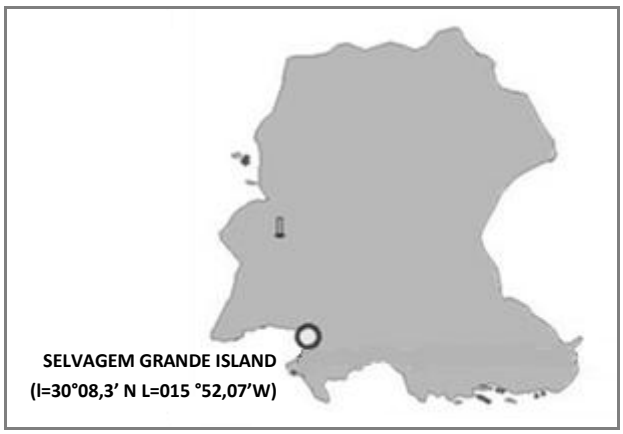
688

689 **b)**



690

691 **c)**



692

693

694 **Fig. 1. a)** Map of Europe with El Hierro and Selvagem Grande Islands situation. **b)** Map of El Hierro (Canary Islands,

695 Spain). **c)** Map of the Selvagem Grande Island (Madeira, Portugal). Black circles indicate specific locations from where

696 *G. australes* isolates were obtained and coordinates are shown in brackets.

697

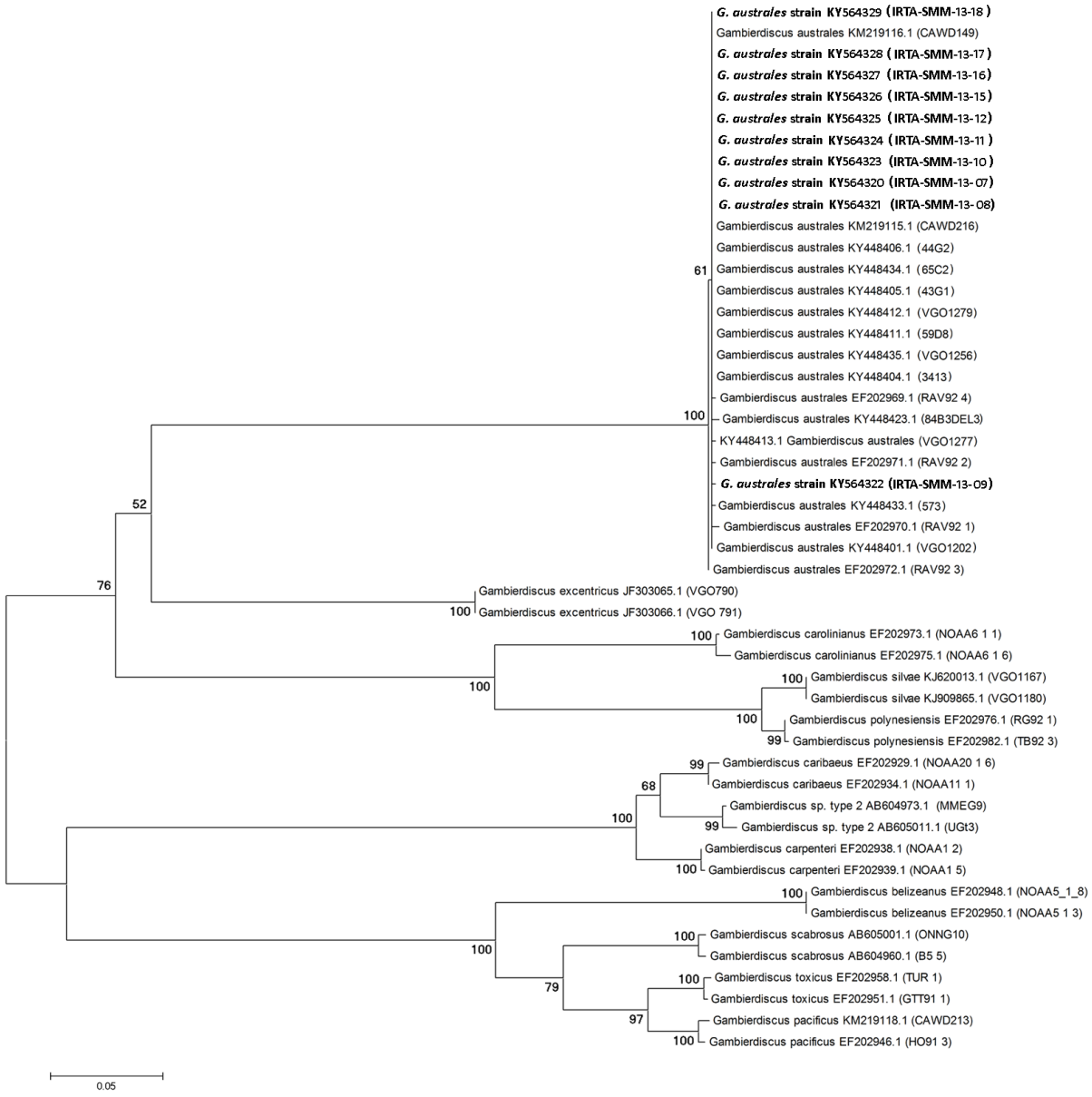


Fig. 2. Phylogenetic analysis of LSU rDNA gene (D1-D3 region) showing the relationship between *Gambierdiscus* strains from this study (in bold) and other *Gambierdiscus* strains. Values at nodes are bootstrap values obtained by the Maximum Likelihood method. Bootstrap values less than 30% are not shown. Scale bar represents substitutions per site.

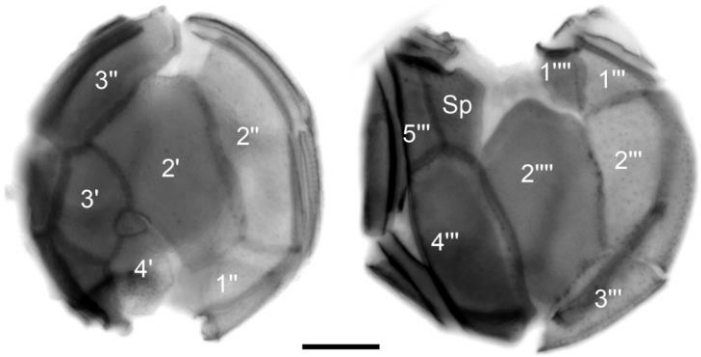
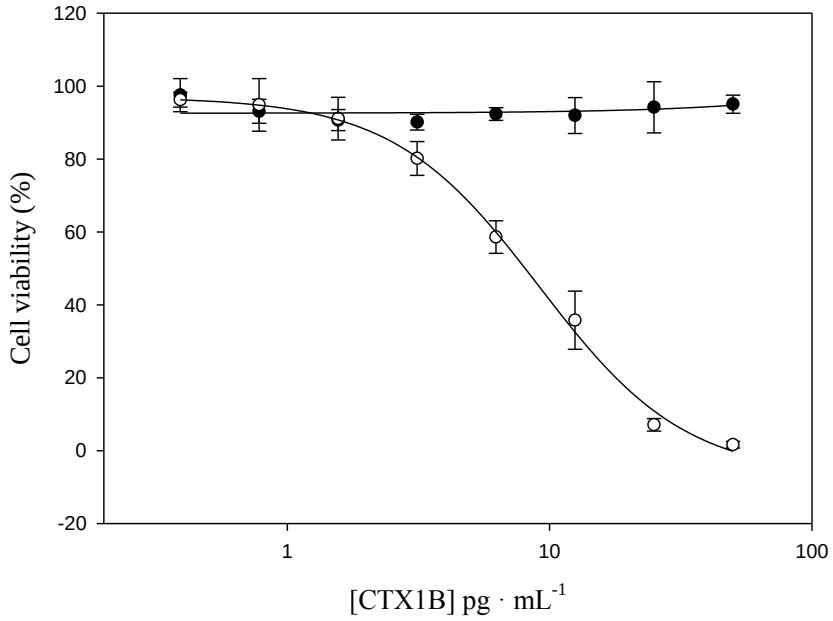


Fig. 3. Fluorescence microscopy images of calcofluor-stained thecae of *Gambierdiscus australes* (strain IRTA-SMM-13-11-12). **a)** Epitheca, **b)** Hypotheca. Scale bar 20 µm.

713

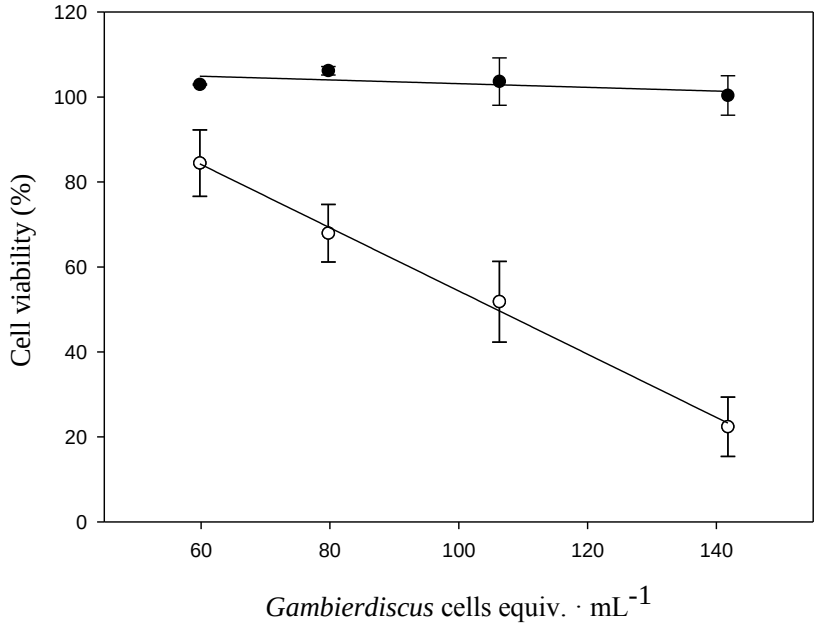
714 **a)**



715

716

717 **b)**



718

719

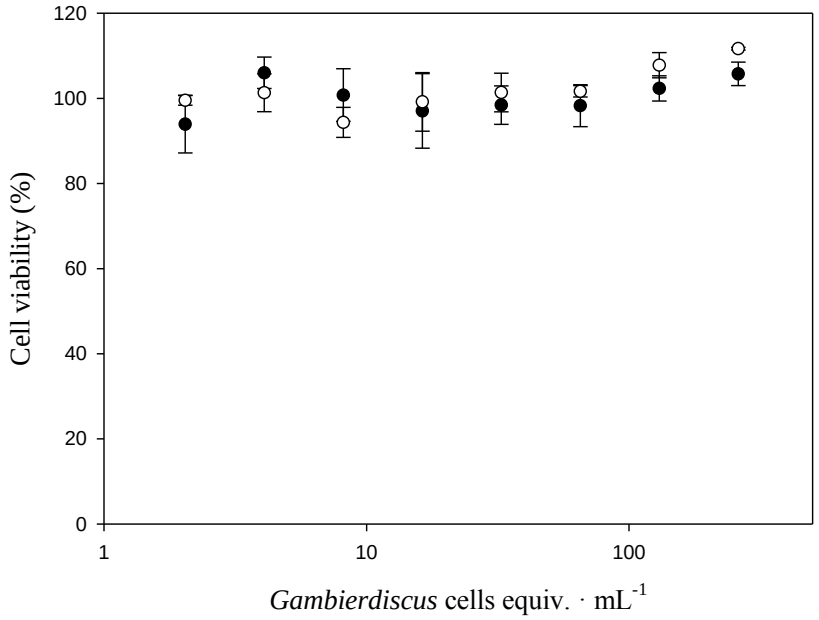
720

721

722

723

724 **c)**



725

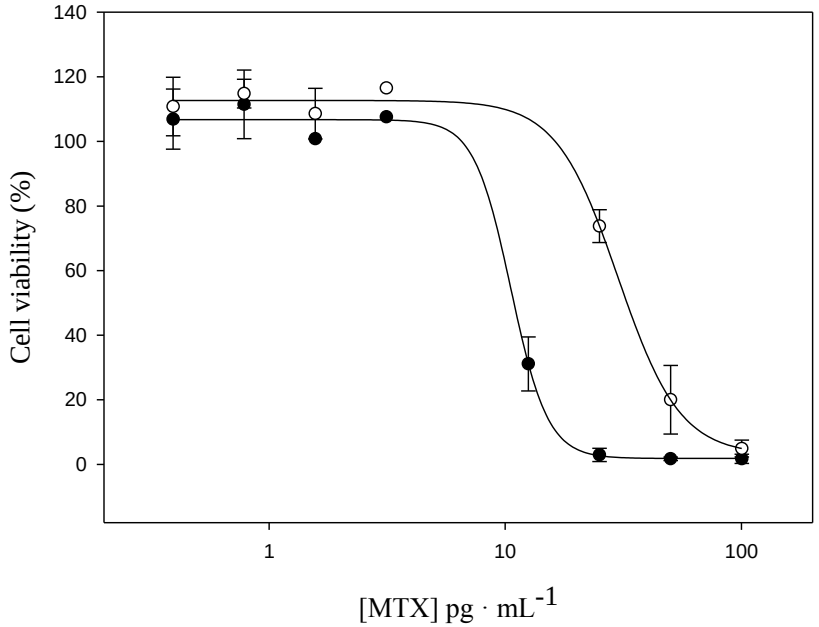
726 **Fig 4.** Dose-response curves of Neuro-2a cells exposed to **a)** CTX1B standard, **b)** *G. australes* IRTA-SMM-13-10

727 extract and **c)** *G. australes* IRTA-SMM-13-16 extract with (**O**) and without (●) o/v treatment.

728

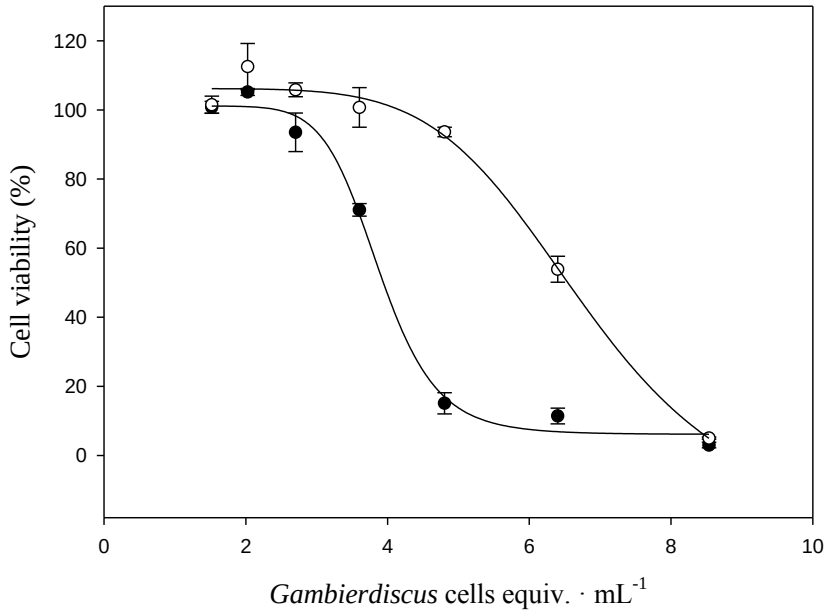
729

730 **a)**



731

732 **b)**



733

734 **Fig 5**. Dose-response curves obtained for Neuro-2a cells exposed to **a)** MTX standard and **b)** *G. australes* IRTA-SMM-

735 13-10 extract at stationary phase with (o) and without (●) SKF treatment.

Table 1. Identification code, origin, sampling point, culture cell density at sampling (exponential growth phase), growth rate, division time and CTX equivalent concentration in 10 strains of *Gambierdiscus australes.*

ID	Origin	Sampling point	Culture cell density (cells·mL^{-1})	μ = growth rate (divisions·day^{-1})	Td = division time (days)	CTX concentration (fg equiv. CTX1B·cell^{-1})
IRTA-SMM-13-07	SGI, Portugal	30°08.3'N, 015°52.07'W	2,475	0.245	4.08	404 ± 20
IRTA-SMM-13-11	SGI, Portugal	30°08.3'N, 015°52.07'W	1,310	0.199	5.02	515 ± 52
IRTA-SMM-13-15	SGI, Portugal	30°08.3'N, 015°52.07'W	4,470	0.298	3.36	388 ± 30
IRTA-SMM-13-16	SGI, Portugal	30°08.3'N, 015°52.07'W	1,362	0.245	4.09	n.d.
IRTA-SMM-13-17	SGI, Portugal	30°08.3'N, 015°52.07'W	1,896	0.198	5.04	200 ± 17
IRTA-SMM-13-18	SGI, Portugal	30°08.3'N, 015°52.07'W	2,630	0.222	4.50	338 ± 38
IRTA-SMM-13-08	HI, Spain	27°40.2'N, 018°01.5'W	3,887	0.306	3.27	697 ± 121
IRTA-SMM-13-09	HI, Spain	27°40.2'N, 018°01.5'W	3,434	0.376	2.66	625 ± 67
IRTA-SMM-13-10	HI, Spain	27°40.2'N, 018°01.5'W	2,488	0.393	2.54	656 ± 62
IRTA-SMM-13-12	HI, Spain	27°48.01'N, 017°53.22'W	3,425	0.242	4.13	280 ± 20

n.d.: not detected (LOQ of 12.31 fg equiv. CTX1B·cell^{-1})

Table 2. Data on the origin, analysis method, CTX and MTX-like toxicity reported for *Gambierdiscus australes* from 1999 to 2017.

Strain ID	Origin	Analysis method	CTX concentration	Growth phase	Analysis method	MTX concentration	Growth phase	Reference

IRTA-SMM-13-07	Selvagem Island (Portugal)	Neuro-2a CBA	404 fg equiv. CTX1B·cell^{-1}	Exponential	Neuro-2a CBA	<89.31 pg equiv. MTX·cell^{-1}	Exponential	Reverté et al. 2018 (this work)
IRTA-SMM-13-11	Selvagem Island (Portugal)	Neuro-2a CBA	515 fg equiv. CTX1B·cell^{-1}	Exponential	Neuro-2a CBA	<89.31 pg equiv. MTX·cell^{-1}	Exponential	Reverté et al. 2018 (this work)
IRTA-SMM-13-15	Selvagem Island (Portugal)	Neuro-2a CBA	388 fg equiv. CTX1B·cell^{-1}	Exponential	Neuro-2a CBA	<89.31 pg equiv. MTX·cell^{-1}	Exponential	Reverté et al. 2018 (this work)
IRTA-SMM-13-16	Selvagem Island (Portugal)	Neuro-2a CBA	<2.8 fg equiv. CTX1B·cell^{-1}	Exponential	Neuro-2a CBA	<89.31 pg equiv. MTX·cell^{-1}	Exponential	Reverté et al. 2018 (this work)
IRTA-SMM-13-17	Selvagem Island (Portugal)	Neuro-2a CBA	200 fg equiv. CTX1B·cell-1	Exponential	Neuro-2a CBA	<89.31 pg equiv. MTX·cell-1	Exponential	Reverté et al. 2018 (this work)
IRTA-SMM-13-18	Selvagem Island (Portugal)	Neuro-2a CBA	338 fg equiv. CTX1B·cell^{-1}	Exponential	Neuro-2a CBA	<89.31 pg equiv. MTX·cell^{-1}	Exponential	Reverté et al. 2018 (this work)
IRTA-SMM-13-08	Hierro Island (Spain)	Neuro-2a CBA	697 fg equiv. CTX1B·cell^{-1}	Exponential	Neuro-2a CBA	<89.31 pg equiv. MTX·cell^{-1}	Exponential	Reverté et al. 2018 (this work)
IRTA-SMM-13-09	Hierro Island (Spain)	Neuro-2a CBA	625 fg equiv. CTX1B·cell^{-1}	Exponential	Neuro-2a CBA	<89.31 pg equiv. MTX·cell^{-1}	Exponential	Reverté et al. 2018 (this work)
			n.a.			275 pg equiv. MTX·cell^{-1}	Stationary	
IRTA-SMM-13-10	Hierro Island (Spain)	Neuro-2a CBA	656 fg equiv. CTX1B·cell^{-1}	Exponential	Neuro-2a CBA	<89.31 pg equiv. MTX·cell^{-1}	Exponential	Reverté et al. 2018 (this work)
			n.a.			227 pg equiv. MTX·cell^{-1}	Stationary	
IRTA-SMM-13-12	Hierro Island (Spain)	Neuro-2a CBA	280 fg equiv. CTX1B·cell^{-1}	Exponential	Neuro-2a CBA	<89.31 pg equiv. MTX·cell^{-1}	Exponential	Reverté et al. 2018 (this work)
RAV-92	Raivavae Island (French Polynesia)	Mouse bioassay	4·10^{-4} MU·1,000 cells^{-1}	Late exponential	Mouse bioassay	0.2 MU·1,000 cells^{-1}	Late exponential	Chinain et al. 1999
RAV-92	Raivavae Island (French Polynesia)	Receptor binding assay	20 fg equiv. P-CTX-3C·cell^{-1}	Early stationary		n.a.		Chinain et al. 2010
MUR-6	Morurora (French Polynesia)	Receptor binding assay	17 fg equiv. P-CTX-3C·cell^{-1}	Early stationary		n.a.		Chinain et al. 2010
MUR-14	Morurora (French Polynesia)	Receptor binding assay	30 fg equiv. P-CTX-3C·cell^{-1}	Early stationary		n.a.		Chinain et al. 2010
TB-1	Tubuai (French Polynesia)	Receptor binding	22 fg equiv.	Early		n.a.		Chinain et al.

		assay	P-CTX-3C·cell^{-1}	stationary				2010
RAI-5	Raivavae Island (French Polynesia)	Receptor binding assay	<16 fg equiv. P-CTX-3C·cell^{-1}	Early stationary		n.a.		Chinain et al. 2010
MG-4	Mangareva (French Polynesia)	Receptor binding assay	<16 fg equiv. P-CTX-3C·cell^{-1}	Early stationary		n.a.		Chinain et al. 2010
CCMP 1653	Hawaii (USA)	LC-MS/MS	CTX analogue 2,3-dihydroxy P-CTX-3C	Exponential		n.a.		Roeder et al., 2010
CAWD149	Cook Islands	Neuro-2a CBA	40 fg CTX1B equiv.·cell^{-1}	Stationary	Mouse bioassay	12 10^{-5} MU·cell^{-1}	Stationary	Rhodes et al., 2010
W B Gam 3	Hawaii (USA)	HPLC	-	Exponential	HPLC	+ (3 congeners)	Exponential	Lewis et al., 2016
VGO1181[b]	Tenerife (Spain)	Neuro-2a CBA	0.6 fg P-CTX-3C equiv.·cell^{-1}	Exponential	Erythrocyte lysis assay	4.7 pg MTX equiv.·cell^{-1}	Exponential	Pisapia et al., 2017
VGO1178[b]	Tenerife (Spain)	Neuro-2a CBA	1.4 fg P-CTX-3C equiv.·cell^{-1}	Exponential	Erythrocyte lysis assay	4.3 pg MTX equiv.·cell^{-1}	Exponential	Pisapia et al., 2017
CCMP1653 (NOAA 24)	Hawaii (USA)	Neuro-2a CBA	2.7 fg P-CTX-3C equiv.·cell^{-1}	Exponential	Erythrocyte lysis assay	5 pg MTX equiv.·cell^{-1}	Exponential	Pisapia et al., 2017
MacX-X	Macaulay Island (New Zealand)	LC-MS/MS	< 1 ng·mL^{-1}	not mentioned	LC-MS/MS	3-36 pg MTX-1·cell^{-1}	not mentioned	Rhodes et al., 2017b

n.a.: not analysed