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Gambierdiscus and *Fukuyoa* as potential indicators of ciguatera risk in the Balearic Islands

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Highlights

- *G. australes* and *F. paulensis* are well distributed and established in the Balearic Islands, a region free of Ciguatera Poisoning.
- Overall, low CTX-like toxicity was detected in *G. australes* and *F. paulensis* strains.
- Presence of MTX-like activity was detected in *G. australes* strains.

Keywords

Ciguatera, ciguatoxins, maitotoxins, Gambierdiscus, Fukuyoa, neuro-2a cell-based assay.

Abstract

Gambierdiscus and *Fukuyoa* are genera of toxic dinoflagellates which were mainly considered as endemic to marine intertropical areas, and that are well known as producers of ciguatoxins (CTXs) and maitotoxins (MTXs). Ciguatera poisoning (CP) is a human poisoning occurring after the consumption of fish or more rarely, shellfish containing CTXs. The presence of these microalgae in a coastal area is an indication of potential risk of CP. This study assesses the risk of CP in the Balearic Islands (Western Mediterranean Sea) according to the distribution of both microalgae genera, and the presence of CTX-like and MTX-like toxicity in microalgal cultures as determined by neuro-2a cell based-assay (neuro-2a CBA). Genetic identification of forty-three cultured microalgal strains isolated from 2016 to 2019 revealed that all of them belong to the species *G. australes* and *F. paulensis*. Both species were widely distributed in Formentera, Majorca and Minorca. Additionally, all strains of *G. australes* and two of *F. paulensis* exhibited signals of CTX-like toxicity ranging respectively between 1-380 and 8-16 fg CTX1B equivalents (equiv.) · cell^{-1}. Four extracts of *F. paulensi*s exhibited a novel toxicity response in neuro-2a cells consisting of the recovery of the cell viability in the presence of ouabain and veratridine. In addition, *G. australes* showed MTX-like toxicity while *F. paulensis* strains did not. Overall, the low CTX-like toxicities detected indicate that the potential risk of CP in the Balearic Islands is low, although, the presence of CTX-like and MTX-like toxicity in those strains reveal the necessity to monitor these genera in the Mediterranean Sea.

1.Introduction

Gambierdiscus (Adachi and Fukuyo, 1979) and *Fukuyoa* (Gómez et al., 2015) (Dinophyceae) are marine benthic dinoflagellates that live attached to different substrates such as macroalgae, corals, rocks and sands in well-illuminated habitats but also at very low light levels (>45 m depth) (Tester et al., 2013). Historically, the genera *Gambierdiscus* and *Fukuyoa* were known to be distributed primarily in tropical and subtropical areas of the Caribbean Sea, the Pacific and Indian Ocean. However, in recent decades, both genera have been reported in warm-temperate areas. The genus *Gambierdiscus* was recently recorded in the North East Atlantic Ocean (Fernández-Zabala et al., 2019; Fraga et al., 2011; Rodríguez et al., 2017), North West Atlantic Ocean (Litaker et al., 2009), South West Atlantic (Nascimento et al., 2015), the Mediterranean Sea (Aligizaki and Nikolaidis, 2008; Tudó et al., 2018), the Red Sea (Catania et al., 2017), Sea of Japan (Jang et al., 2018) and the South Pacific Ocean (Kohli et al., 2014a; Larsson et al., 2018). In contrast species of the genus *Fukuyoa* (formerly within the genus *Gambierdiscus*), have been reported in the Atlantic Ocean (Gómez et al., 2015), the Mediterranean Sea (Laza-Martínez et al., 2016; Aligizaki et al., 2018), the South Pacific Ocean (Rhodes et al., 2017), the China Sea and the Asia Pacific region (Larsson et al., 2019, 2018; Leung et al., 2018).

Gambierdiscus and *Fukuyoa* produce multiple secondary metabolites, among which are included ciguatoxins (CTXs) and maitotoxins (MTXs) (Chinain et al., 2010; Holmes et al., 1990; Lewis and Holmes, 1993; Munday et al., 2017; Satake et al., 1996). CTXs are lipophilic polyethers, that bind to voltage-gated sodium channels (VGSCs), thereby inhibiting the inactivation process of VGSCs resulting in intracellular sodium increase (Hidalgo et al., 2002; Molgó et al., 1993; Nicholson and Lewis, 2006; Strachan et al., 1999). Moreover, CTXs are potassium channel inhibitors (Inserra et al., 2017). MTXs are amphiphilic polyethers that bind to Ca^{2+} independent voltage gated channels and non-selective ion channels causing an increase of intracellular Ca^{2+} (Reyes et al., 2014).

CTXs in fish or shellfish are responsible for the human intoxication known as Ciguatera Poisoning (CP) (Bagnis, 1993; Bagnis et al., 1980). CTXs enter marine food webs through invertebrates and herbivorous fish, where they may be biotransformed along the food webs and bioaccumulated at different trophic levels, eventually reaching humans (Bagnis et al., 1980; Yasumoto et al., 1977). Regarding MTXs, their implication in CP is unlikely. Although its intraperitoneal administration in mice is more toxic than CTXs, their oral potency is almost non-detectable (Munday et al., 2017). In addition, their bioaccumulation along the food webs is low (Litaker et al., 2010; Munday, 2014; Yasumoto et al., 1971) and they have not been found in the tissue of fish involved in CP cases. However, snapper (*Chrysophrys auratus*) (previously *Pagrus auratus*), that had been experimentally fed with *G. australes* contained MTXs in their viscera, liver and muscle (Kohli et al., 2014b).

Although, epidemiological records of CP are not available at a global level, it is estimated that CP affects between 25,000 – 500,000 people per year (Fleming et al., 1998; Friedman et al., 2017; Skinner et al., 2011). CP effects include gastrointestinal, neurological, and cardiovascular symptoms, and the latter two can last for months or years (Friedman et al., 2017). Fatal cases of CP are rare (Chan, 2016; Diogène et al., 2017). CP occurs mainly in tropical and subtropical areas (35 °N - 35 °S), but in more recent decades, CP cases have been reported in temperate areas, previously free of CP (Bravo et al., 2015; Chinain et al., 2019; Gouveia et al., 2010).

In the Mediterranean Sea, the presence of CTXs in fish, or confirmed CP cases have not been demonstrated. Follow-up investigations of previous descriptions of CP cases in the eastern Mediterranean did not find CTXs in fish tissue (Bentur and Spanier, 2007; Herzberg, 1973; Raikhlin-Eisenkraft and Bentur, 2002; Raiklin-Eisenkraft et al., 1988; Spanier et al., 1989). The detection of possible CTX-compounds in *Siganus* sp. by Bentur and Spanier (2007) was performed using a *Cigua-Check* strip test, which was later considered unreliable (Bienfang et al., 2011). In addition, the clinical symptoms described, including hallucinations, are rare in CP cases (Chinain et al., 2019) and they are indicative of ichthyoallyeinotoxism, which is often mistaken for cases of CP (De Haro and Pommier, 2006).

At present, five confirmed species of the genus *Gambierdiscus* and *Fukuyoa* live in the Mediterranean Sea (Aligizaki et al., 2018; Laza-Martínez et al., 2016; Litaker et al., 2009; Tudó et al., 2018). The presence of certain CTX-producing species in the area can be indicative of a higher risk of CP in comparison to areas where they are absent (Chinain et al., 2019, 2010; Friedman et al., 2017). Nonetheless, evaluating CTX-production by these species is important to estimate the risk, since CTX production varies accordingto species, and high and low CTX-producers species have been characterized (Litaker et al., 2017; Pisapia et al., 2017). For the estimation of CTX production in *Gambierdiscus,* growth phases and strain variability among isolates of the same species have to be taken into account (Reverté et al., 2018; Rossignoli et al., 2020).

The goal of this study was to assess the potential risk of CP based on the presence in the Balearic Islands of the genera *Gambierdiscus* and *Fukuyoa* (Western Mediterranean Sea), and their potential production of compounds with CTX-like and MTX-like activity. This is the first study that provides information about the risk of CP in the Balearic Islands, according to the presence of the genera *Gambierdiscus* and *Fukuyoa* in several sampling locations, and their evaluation of toxin production of several strains.

2. Materials and Methods

2.1 Reagents and equipment

CTX1B was provided by Dr. Lewis, University of Queensland (Lewis et al., 1991). Neuroblastoma murine cells (neuro-2a) were purchased from ATCC LGC standards (USA). Poly-L-lysine, foetal bovine serum (FBS), L-glutamine solution, ouabain, veratridine, phosphate buffered saline (PBS), penicillin, streptomycin, RPMI-1640 medium, sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT) and SKF96365 were purchased from Merck KGaA (Germany). Dimethyl sulfoxide (DMSO) and absolute methanol were purchased from Honeywell (Spain) and Chemlab (Spain) respectively. Taq Polymerase was purchased from Invitrogen (Spain). QIAquick PCR Purification Kit was obtained from Qiagen (Germany).

2.2 Sampling, cell isolation and initial culturing

The Balearic Archipelago (North West Mediterranean Sea) is located at 170 km distance from the Iberian Peninsula (Fig. 1). It is characterized by a narrow continental shelf surrounding a rocky coast, with occasional sea grass meadows over a biogenic muddy bottom. Samples from the Balearic Islands were collected at different islands, specifically, in Formentera in late September 2016, in Majorca and Minorca in early September 2017 and in early October 2018. In Minorca an additional sampling was performed in late September 2019. At each sampling point, two different types of samples were collected: 1) epilithic, which were obtained by scraping of the substrate (rocks) with a plastic bottle (Nalgene, HDPE, 1L), and 2) epiphytic, which were obtained from macroalgae that were collected using plastic bottles under water. Macroalgae were identified morphologically at the genus level. Each sample was kept in the container and was intensively shaken by hand to release the dinoflagellates from the substrates. Samples were sieved through a 200 µm nylon mesh. The filtered water was stored in two plastic bottles (Nalgene, HDPE, 125mL), one with 125 mL was kept untreated to isolate live cells and another was preserved in 3% Lugol's iodine solution for further observation in the laboratory. Coordinates of each sampling station were recorded by GPS. Salinity, oxygen (% and mg · L^{-1}), temperature and pH were recorded in *s*itu using a multiparametric probe (YSI 556 MPS).

Samples were observed under an inverted light microscope Leica DMIL (Leica Microsystems GmbH, Germany) and individual microalgal cells were isolated by capillary method (Hoshaw and Rosowski, 1973) to establish clonal cultures. Each cell was inoculated in a well of an untreated Nunc 24 well plate (Thermo Fisher Scientific) with 1 mL of modified ES medium (Provasoli, 1968). Medium was prepared from sterile aged seawater from L'Ametlla de Mar (Spain), Mediterranean Sea (40.8465° N; 0.77243° E) and salinity was adjusted to 36. After 2-3 weeks, when cell abundance of cultures reached 20-30 cells · mL^{-1}, cells were transferred to 28 mL round bottom glass tubes (Thermo Fisher Scientific) containing 10 mL of medium. Cultures were maintained in a culture chamber at a temperature of 24 ± 0.5 °C, which is the average of the range of the optimal temperatures of growth for *G. australes* (Yoshimatsu et al., 2014) and in coherence with our previous studies, Reverté et al. (2018), Caillaud et al. (2010). Illumination in a 12:12 light:dark cycle was provided by fluorescent tubes with white light and with photon irradiance of 100 µmol photons · m^{-2} · s^{-1} measured by an irradiometer (QSL-2100 Radiometer, Biospherical Instruments, San Diego, USA). Preserved field samples were settled in 10 mL sedimentation chambers and observed under an inverted light microscope for microalgal identification.

2.3 Molecular identification

Molecular identification at species level was performed by sequencing the D8-D10 region of the 28S ribosomal large subunit gene (LSU rDNA). Molecular identification was conducted for 34 *Gambierdiscus* strains and 9 *Fukuyoa* strains. To that purpose, strains were inoculated in 50mL of medium at 50 cells · mL^{-1} in 25 cm^2 sterile Nunclon^{TM} culture flasks (Thermo Fisher Scientific), and when cultures achieved the exponential phase, they were harvested by centrifugation at 4300g for 20 min (Allegra X-15R, Beckman Coulter). Genomic DNA was extracted by phenol/chloroform/isoamylalcohol (PCI) extraction following Toldrà et al., (2018). After DNA extraction, genomic DNA was quantified and checked for its purity using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and stored at -20 °C. Afterwards, the region D8-D10 was amplified by PCR using the primers FD8 and RB (Chinain et al., 1999). 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 0.4–2 ng · ul^{-1} of DNA template. Amplifications were carried out in a Mastercycler nexus gradient thermal cycler (Eppendorf, Spain) as follows: an initial denaturation step of 5 min at 95 °C, 40 cycles of 30 s at 95 °C, 45 s at 60 °C, and 30 s at 72 °C and a final extension step of 10 min at 72 °C. Each PCR reaction was verified by agarose gel electrophoresis and visualized with ethidium bromide stain. The resulting PCR products of ~ 840–910 bp were purified with the QIAquick PCR Purification Kit. Purified products were bi-directionally sequenced 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) and deposited in GenBank. Sequences were aligned using MAFFT v.7 (Rozewicki et al., 2019) with G-INS-1 progressive method. The final alignment consisted of 617 positions. The evolutionary model of data was estimated using jModelTest 2.1.10 (Darriba et al., 2012) and the phylogenetic relationships were inferred by Maximum likelihood (ML) using RaxML v.8 (Stamatakis, 2014) and Bayesian inference (BI) using Mr. Bayes v.3.2.2 (Huelsenbeck and Ronquist, 2001). In the BI approach two analyses were run in parallel, 10^6 generations, and four chains in each run. The parameters used for analysis were nst=mixed and rates=gamma. By default, 25% of the trees were discarded. Stability of the chains were checked using Tracer v.1.7.1 (Rambaut et al., 2018).

2.4 Morphological characterization

For morphological characterization strains were acclimated at least one year to avoid stress-induced variance during the adaptation period to laboratory conditions (Bomber et al., 1989).

2.4.1 Light microscopy (LM)

Seven monoclonal cultures of *G. australes* and two of *F. paulensis* were inoculated at 20-30 cells · mL^{-1} in 28 mL round bottom glass tubes. When cultures arrived at final exponential phase (after ± 20 days) cells were stained with Calcofluor White M2R (Sigma Aldrich, Spain) according to Fritz and Triemer (1985). Calcofluor-stained cells were observed using an epifluorescence microscope (LEICA

DMLB and NIKON eclipse 80i) equipped with an Olympus camera (Olympus DP70), and they were measured using the software Olympus DP controller (Olympus Corporation). Morphological characteristics of microalgal cells were based on the tabulation system described in Fraga et al. (2011). Cell length was determined as the apical to antapical distance dimensions, depth as the dorso-ventral distance and width as the transdiameter distance that is the longest distance between opposed sides of the cingulum (Balech, 1989). Cell dimensions were expressed as mean ± standard deviation (SD).

2.4.2 Scanning electron microscopy (SEM)

SEM was used to study two monoclonal cultures of *G. australes* (IRTA-SMM-17-253 and IRTA-SMM-17-164) and one of *F. paulensis* (IRTA-SMM-17-211). For that, ten mL samples of cultures at the initial exponential growth phase were fixed with glutaraldehyde at a final concentration of 4% during 2 h at room temperature. After that, 3 mL of culture were collected with a syringe by applying a low pressure on 5 µm Nuclepore Track-Etch Membrane (Thermo Fisher Scientific) coated by poly-L-lysine and held in a plastic filter mould 13 mm (PALL, life Science). Filters were rinsed twice. Once with seawater (autoclaved and filtered by active carbon 0.2 µm) and a second time with filtered seawater/MilliQ water (50:50, v:v). Afterwards, filters were rinsed in a graded EtOH series of 30, 50, 70, 80, 90 and twice with 96% (v:v). Later, filters were kept in a recipient with absolute EtOH and they were sent to the Scanning Electron Microscopy Service in the Institute of Marine Science (ICM-CSIC). In the facilities, filters were submitted to critical-point drying with liquid carbon dioxide in a BAL-TEC CPD030 unit (Leica Microsystems, Austria). Dried filters were mounted on stubs with colloidal silver, then sputter-coated with gold in a Q150R S (Quorum Technologies Ltd). Cells were observed with a Hitachi S3500N scanning electron microscope (Hitachi High Technologies Co., Ltd, Japan) at an accelerating voltage of 5 kV. Length and width of the Po plate and the second antapical plate, 2'''' plate (Fraga et al. 2011), were measured and the number of pores of the Po plate were counted. Measurements were made using ImageJ software (Schneider et al., 2012).

2.5 Growth dynamics analysis

Before the growth dynamics analysis strains were first acclimated to laboratory conditions for approximately 1 year. To evaluate growth dynamics, three strains of *G. australes* (IRTA-SMM-17-162, IRTA-SMM-17-189, IRTA-SMM-17-271) and one strain of *F. paulensis* (IRTA-SMM-17-209) were randomly selected from the algal collection. For each strain, 500 mL of medium were inoculated into 1.5 L Fernbach flasks at an initial concentration of 50 cells · mL^{-1} in triplicate. Every 2-3 days, at the same time of the day, each culture was vigorously manually homogenized and 3 mL samples from each replicate were collected and preserved with 3% Lugol's iodine solution. Three countings of each sample were conducted under observation in an inverted light microscope using a 0.5 mL Kolkwitz counting chamber (Plankton Chamber acc. to Kolkwitz-Hydro-bios). For each day and replicate, average of the cell abundance (cells · mL^{-1}) and SD were estimated. The growth rate (r) of each replicate was estimated by the equation of a linear regression by the least square fit after logarithmic transformation of the cell abundance vs time considering at least 3 points of the exponential phase. The growth rate was expressed in units of divisions (div.) · day^{-1}. Moreover, the doublings per day (K) were calculated as $K= r/\ln(2)$ (Eq. 1) and expressed as doublings day^{-1} (Guillard, 1973). Besides, the time of division or doubling time (Td) was calculated as $Td =\ln(2)/r$ (Eq. 2) and expressed as day^{-1} (Guillard, 1973). Also, growth phases were defined as Wood et al. (2005) where the exponential phase (log phase) was defined as the period when the slope of the regression line between elapsed time and log cell concentration was maximum. Late-exponential (late log) – early stationary phase was defined when the slope of the regression line between elapsed time and log cell concentration is reduced in comparison to the slope from the log phase. The negative growth was defined by constituent decrease of cells, which was assessed by observation of microalgal cells by light microscope and confirmed by observation of empty thecae. However, cultures did not arrive at significantly negative growth.

2.6 Toxin Analysis

2.6.1 Culture, harvesting and algal extraction

The CTX-like activity was evaluated for 21 strains of *G. australes* (11 strains from Majorca and 10 from Minorca), and 6 strains of *F. paulensis* (2 strains from Majorca and 4 from Minorca) harvested at late log - early stationary phases of culture. For this purpose, strains were inoculated in 500 mL of medium in 1.5 L Fernbach flasks at an initial concentration of 50 cells · mL^{-1}. When culture arrived at late-exponential phase (after 20 ± 3 days), cultures were vigorously shaken, and 15 mL aliquots were fixed using Lugol's iodine solution (3%) to estimate the cell concentration (cell · mL^{-1}) in the culture. Subsequently, the remaining volume was collected in sterile 50 mL Falcon tubes and centrifuged at 4300 g for 20 min. Supernatants were discarded, and pellets were pooled in one 50 mL Falcon tube. Centrifugation was repeated, the supernatant was discarded, and the pellet was kept at -20 °C with absolute methanol (10 mL for 10^6 cells) until toxin extraction.

To prepare microalgal extracts, cell pellets of approximately 5 x 10^5 to 10^6 cells with methanol were sonicated using an ultrasonic cell disrupter (Watt ultrasonic processor VCX750, USA). The tip amplitude was set at 37% 3 sec on/2 sec off for 15 minutes. The sample was then centrifuged at 600 g for 5 min at 4 °C. Supernatant was transferred to a glass vial. Procedure was repeated twice, one with methanol and another with aqueous methanol (50:50; v:v) (10 mL for 10^6cells). The methanol extracts were then evaporated to dryness with a rotary evaporator (Büchi Syncore, Switzerland) or dried under N_2 gas (Turbovap, Caliper, Hopkinton, USA) at 40 °C. The aqueous methanol was evaporated at 70 °C. When dryness was achieved, absolute methanol was added to the glass vials, then extracts were pooled, filtered with PTFE filters (0.2 µm) and stored at -20 °C.

2.6.2 CTX-like toxicity evaluation

The presence of CTX-like activity was evaluated on microalgal pellets of 21 cultures of *G. australes*, and 6 cultures of *F. paulensis* harvested at late log - early stationary phase. The evaluation was conducted using the neuro-2a CBA. This assay is used to detect bioactive compounds which target the voltage gated sodium channels (VGSCs) (Cañete and Diogène, 2008; Manger et al., 2003, 1995, 1993). Ouabain blocks the sodium efflux through the inhibition of the Na+/K+ -ATPase pump

(Catterall and Nirenberg, 1973) whereas, the veratridine blocks the sodium voltage-gate channel in an open position (Catterall, 1986). The cell viability of the neuro-2a cells is affected when the extract contains CTXs or CTX-like compounds (molecules that activates to VGSC) after the ouabain and veratridine treatment (Cañete and Diogène, 2008; Manger et al., 2003, 1995, 1993). Exposure of neuro-2a cells to CTX1B standard (reference) or microalgal extracts was performed following the protocol described in Reverté et al. (2018). Briefly, neuro-2a cells were seeded at a density of 1.4 x 10^5 cells · mL^{-1} in 96-well plates. After 24 h, ouabain and veratridine (O/V) were added to a final concentration at 140 µM and 14 µM respectively, then, 10 µL of each sample (serial dilutions of extract or standard) was added to each well in triplicate. Concentrations of CTX1B ranged between 0.2 to 25 pg · mL^{-1} and concentrations of microalgal extracts ranged between 0.3 to 1000 cells equiv. mL^{-1} for *G. australes* and 10 to 4000 cells equiv. · mL^{-1} for *F. paulensis*. After 24 h, cell viability was measured using a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium (Manger et al. 1993). Absorbance was measured at 570 nm using an automated plate spectrophotometer (Synergy HT, Biotek, USA).

Hence, for every assay a calibration curve of cell viability with the standard was obtained. Curves were adjusted to a sigmoidal logistic 4-parameter regression using SigmaPlot software 12.0 (Systat Software Inc., USA). Limit of detection (LOD) was calculated as the necessary concentration of standard to inhibit the cell viability by 20 % (IC_{20}) (Cañete and Diogène, 2008). Concentrations of CTX-like compounds in microalgal extracts were estimated inferring the concentration from the standard curve based on the viability of neuro-2a cells. The amounts of CTX-like compounds were expressed as femtograms (fg) of CTX1B equiv. per cell. The limit of quantification (LOQ) was calculated as the ratio of the LOD obtained with standard to the maximum concentration of microalgal extract used in the assay with no matrix effect being observed. A matrix effect was considered when toxicity was recorded in the neuro-2a cells after exposure to microalgal extracts without ouabain and veratridine treatment (O/V-).

2.6.3 MTX-like toxicity evaluation

The MTX-like toxicity was evaluated qualitatively for 15 *G. australes* strains following the protocol described by Caillaud et al. (2010). This assay is based on the inhibition of the toxic effect by the addition of SKF96365, which is the 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole hydrochloride to neuro-2a cells. SKF96365 blocks the voltage-gated Ca^{2+} channels (VGCCs) (Singh et al., 2010) counteracting the increase of intracellular calcium levels caused by compounds that target VGCCs.

3. Results

3.1 Presence of *Gambierdiscus* and *Fukuyoa* genera in the western Mediterranean Sea.

Presence of *Gambierdiscus* and *Fukuyoa* genera was assessed using samples from live and Lugol's iodine preserved samples collected during 2016 to 2019. A total of 110 isolates from the genera *Gambierdiscus* and *Fukuyoa* were obtained from the epiphytic samples and 26 isolates from the epilithic samples. Epiphytic samples were obtained from macrophytes of the genera *Lobophora*, *Cystoceira*, *Jania*, *Padina*, and *Dictyota*. Furthermore, other dinoflagellates co-occurred with the *Gambierdiscus* and *Fukuyoa* genera, such as the genera *Prorocentrum*, *Coolia*, *Amphidinium* and *Ostreopsis.* Figure 1 shows stations where the presence of the genera *Gambierdiscus* and *Fukuyoa* were recorded in the Balearic Islands during the entire sampling period. Results of the presence of the genera *Gambierdiscus* and *Fukuyoa* by sampling point and the environmental data (temperature, pH, oxygen and salinity) are provided in supplementary Table 1**.** In Formentera, *Gambierdiscus* cells were present in 5 out of 9 sampling stations, in low amounts in both samples (epilithic and epiphytic). The presence was confirmed only in Lugol's iodine preserved samples; therefore, no live cells could be isolated. In Majorca, *Gambierdiscus* cells were found both in epiphytic and epilithic samples, although in Minorca cells were primarily found in the epiphytic samples. In Majorca and Minorca, in 2017 *Gambierdiscus* cells were present in all sampling stations with the exception of one site. Similar results for both islands were obtained in 2018. In 2019 only Minorca was sampled, and in 2 out of 4 sampling stations *Gambierdiscus* cells were present.

The genus *Fukuyoa* was present in epiphytic and epilithic samples, in 6 out of 9 stations of Formentera. *Fukuyoa* cells were found at very low amounts and only in preserved samples, therefore as with the genus *Gambierdiscus,* no cells could be isolated in culture from Formentera. In 2017 in Majorca and Minorca, *Fukuyoa* cells were present in 5 out of 10 stations and 6 out of 9 stations respectively; while in 2018 cells were found in fewer stations: 4 out of 10 stations and 2 out of 9 stations, respectively. In 2019 in Minorca, cells were not observed in any of 4 sampling stations. *Fukuyoa* isolates from Majorca and Minorca were obtained only from epiphytic samples. During the entire study period, *Fukuyoa* cells were concomitant with *Gambierdiscus* in 4 stations in Formentera, and in all stations in Majorca and Minorca.

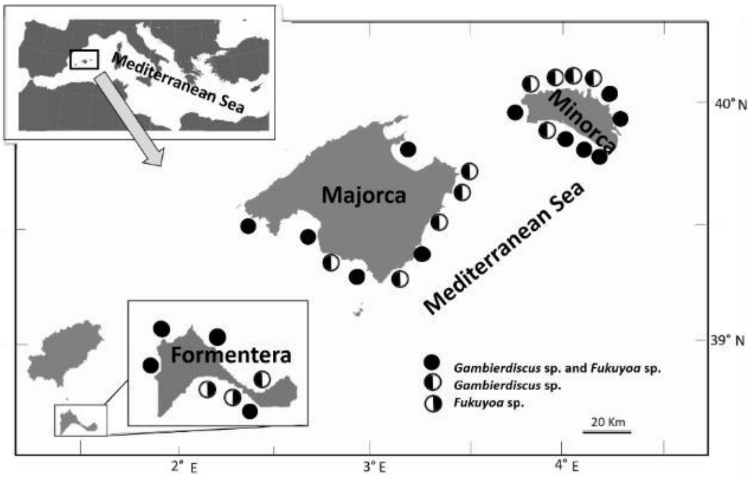

Fig. 1. Presence of the *Gambierdiscus* and *Fukuyoa* genera in the sampling stations in the Balearic Islands (Mediterranean Sea) during 2016-2019.

3.2 Molecular characterization

Species level identification was performed for thirty-four *Gambierdiscus* and nine *Fukuyoa* isolates using the D8-D10 region (LSU) rDNA (Chinain et al. 1999, Litaker et al. 2009). Sequences were matched in GenBank using the BLAST sequence similarity searches (National Center for Biotechnology Information) and they scored the highest identity and similarity with *Gambierdiscus australes* and *Fukuyoa paulensis*. Moreover, further phylogenetic analyses confirmed the identifications (Fig.2).

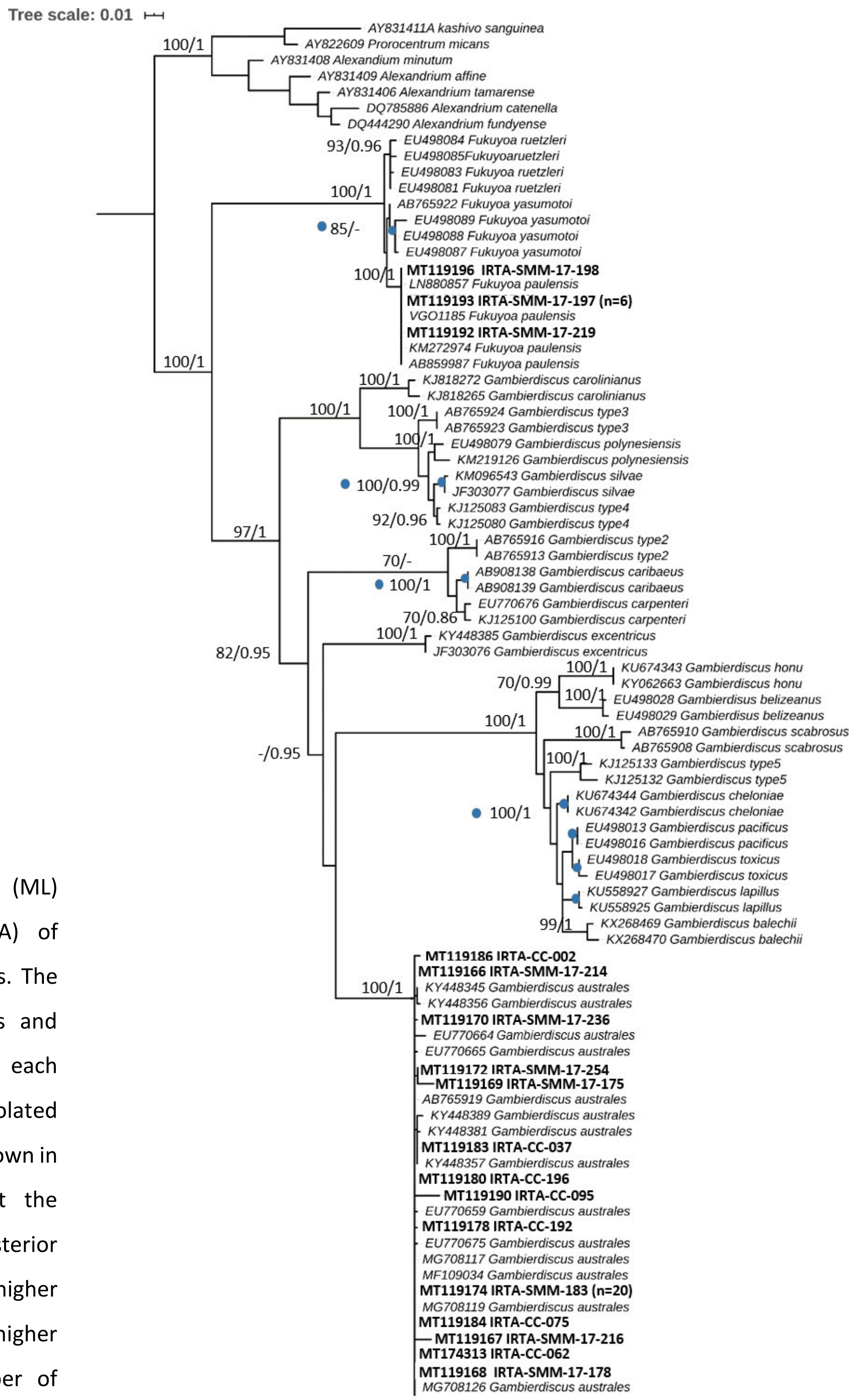

Fig. 2. Maximum likelihood (ML) phylogeny of D8-D10 LSU (rDNA) of *Gambierdiscus* and *Fukuyoa* species. The GenBank code accession numbers and species names are shown for each downloaded sequence. Strains isolated from samples from this study are shown in bold. Values at nodes represent the bootstrap values /Bayesian posterior probability. Only bootstraps values higher than 70 and posterior probabilities higher than 0.95 are shown. The number of clones (n) with the same haplotype is shown in parentheses.

3.3 Morphological characterization

In the present study, cells of *G. australes* were anterior-posteriorly compressed showing a lenticular shape. Table 1 shows the measurements for *G. australes* and *F. paulensis* cells from the Balearic Islands in comparison to the measurements for these species retrieved from the literature. The thecal plate formula was: Po, 4’, 0a, 6’’, 6c, ?s, 5’’’, 0p, 2’’’’. Fig. 3 shows a representative SEM photos for *G. australes*.

F. paulensis cells were globular presenting a lateral compression. Measurements are in Table 1. The thecal plate formula was Po, 4’, 6’’, 6c, ? s, 5’’’, 2’’’’. Fig. 4 shows a representative SEM photos for *F. paulensis*.

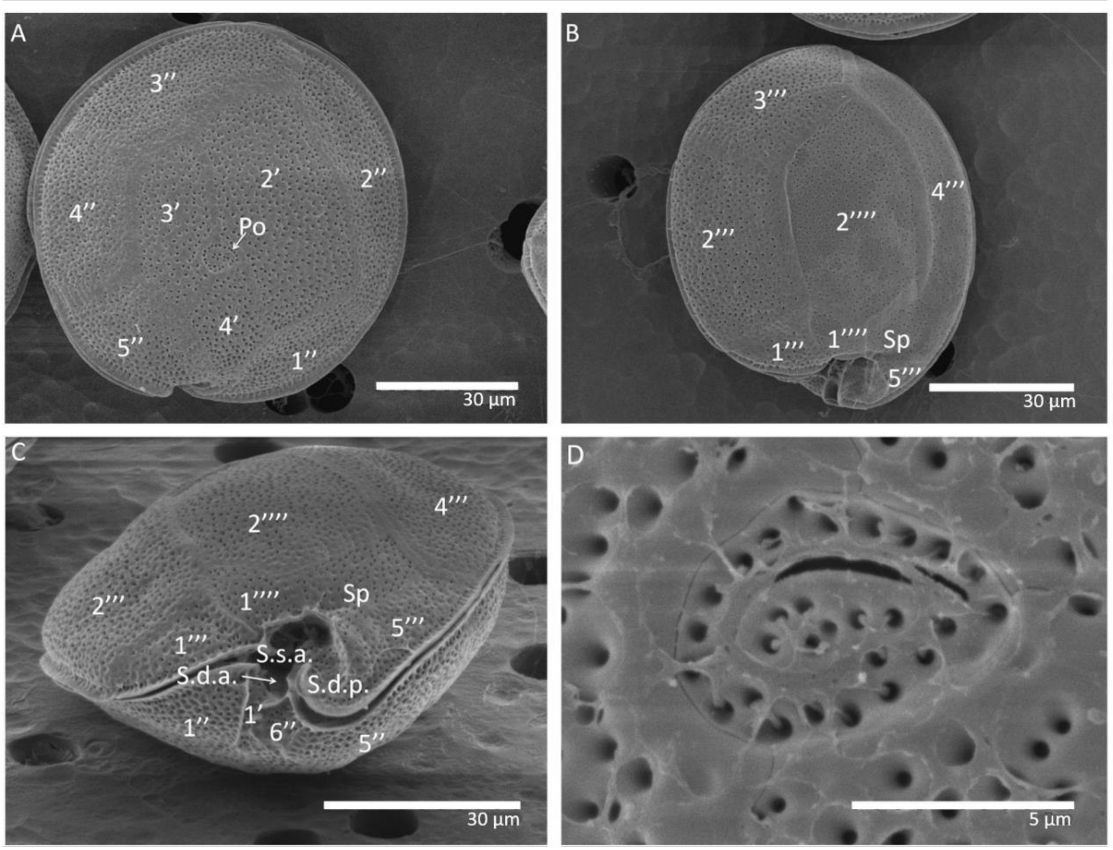

Fig. 3. Images from SEM of *G. australes* (IRTA-SMM-17-253): apical (A), antapical (B), ventral (C) views, detail of Po plate and pores (D).

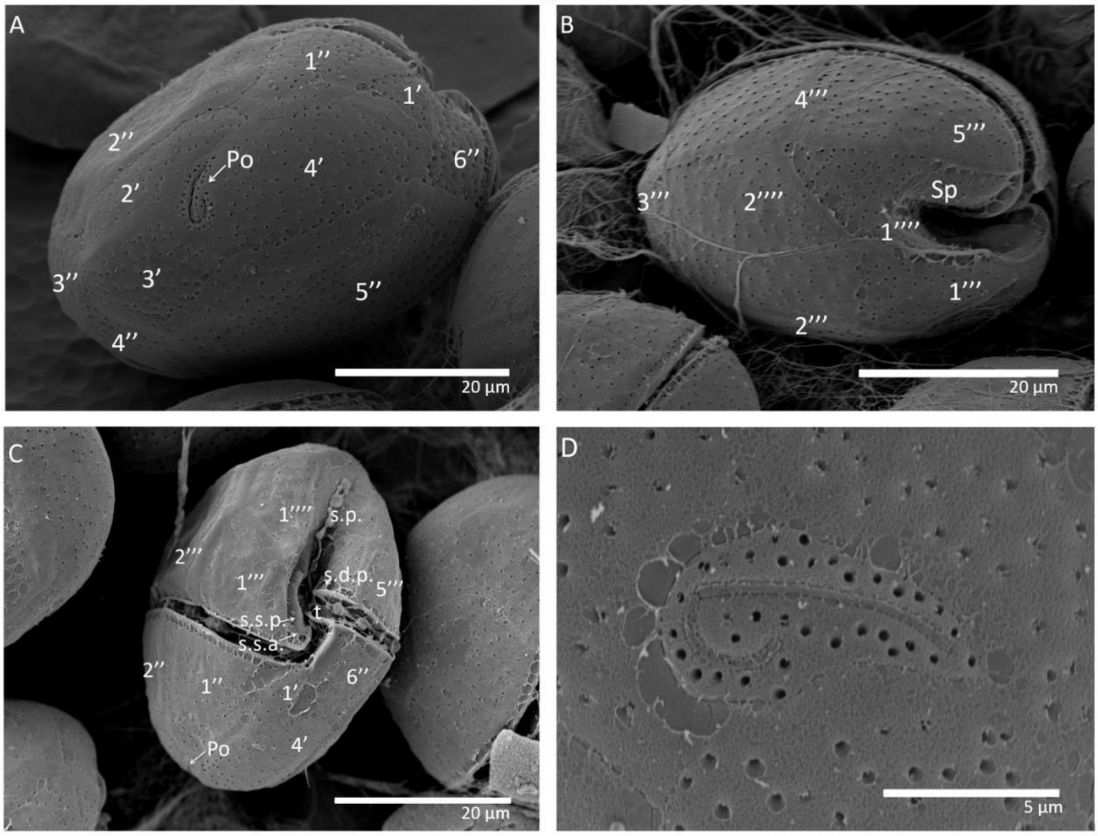

Fig. 4. SEM images of *F. paulensis* (IRTA-SMM-17-211): apical (A), antapical (B) and ventral (C) views and detail of Po plate and pores (D). (s. p.: sulcal posterior, s.s.p.: sulcal left posterior plate, s.d.p.: sulcal right posterior, s.s.a: sulcal left anterior plate).

3.4 Growth dynamics

All the studied strains (three *G. australes* and one of *F. paulensis* strains) displayed a typical growth curve in batch culture conditions. No significant differences were observed among the replicates of the strains. Strains of *G. australes*: IRTA-SMM-17-162, IRTA-SMM-17-189 and IRTA-SMM-17-271 arrived at the stationary phase at the 25th, 22nd and 21st days. For *F. paulensis,* strain IRTA-SMM-17-209 reached the stationary phase at day 21st of culture. The growth curves are provided in supplementary material figure S1 and results of growth rates are shown in Table 2.

3.5 Evaluation of CTX-like and MTX-like toxicity

Exposure of neuro-2a cells to a CTX1B standard was nontoxic. As expected, addition of ouabain/veratridine (O/V+) showed a typical curve of CTX-like toxicity in neuro-2a cells with an average LOD of 0.45 ± 0.24 pg CTX1B equiv. · mL^{-1} and IC_{50} of 1.21 ± 0.48 pg CTX1B equiv. · mL^{-1}.

The maximum concentration of microalgal extract that did not cause any toxicity in the absence of ouabain and veratridine ranged from 10 to 220 and from 40 to 450 cells equiv. · mL^{-1}, for *G. australes* and *F. paulensis,* respectively.

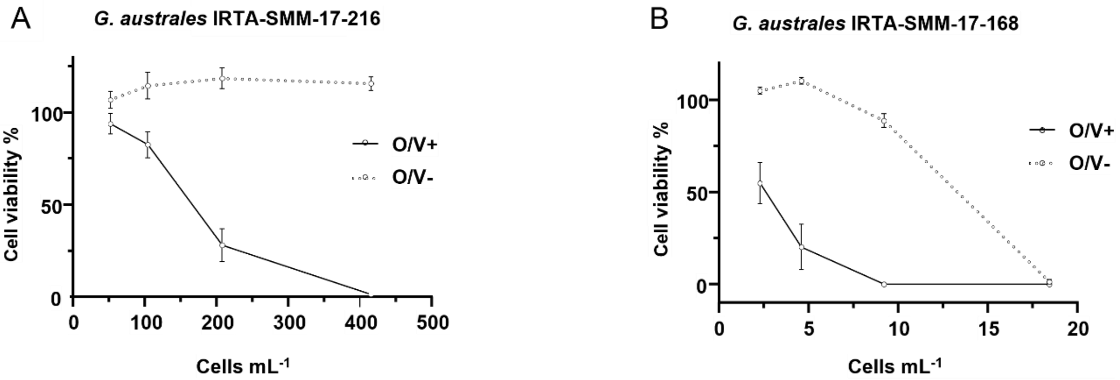

Fig. 5. Dose response curves obtained using neuro-2a CBA for *G. australes* extracts: IRTA-SMM-17-216 (**A**), IRTA-SMM-17-168 (**B**). O/V+: neuro-2a cells exposed to microalgal extract with the ouabain and veratridine treatment. O/V-: neuro-2a cells exposed to microalgal extract without the ouabain and veratridine treatment. Each point is the mean of triplicates and bars represent the SD.

All *G. australes* extracts (n=21) presented CTX-like toxicity and toxicities ranged from 1.38 to 381 fg CTX1B equiv. · $cell^{-1}$ (Table 3). Figure 5 shows representative dose-response curves of the types of neuro-2a cell viability response for *G. australes*. Figure 5A corresponds to *G. australes* extract with low toxicity (IRTA-SMM-17-216). In the O/V+ conditions, the curve showed the typical dose-response curve of CTX-like toxicity with an estimated IC_{50} of 150 cell equiv. · mL^{-1}.

Figure 5B represents *G. australes* with high toxicity (IRTA-SMM-17-168) with an estimated IC_{50} of 2 cell equiv. · mL^{-1}.

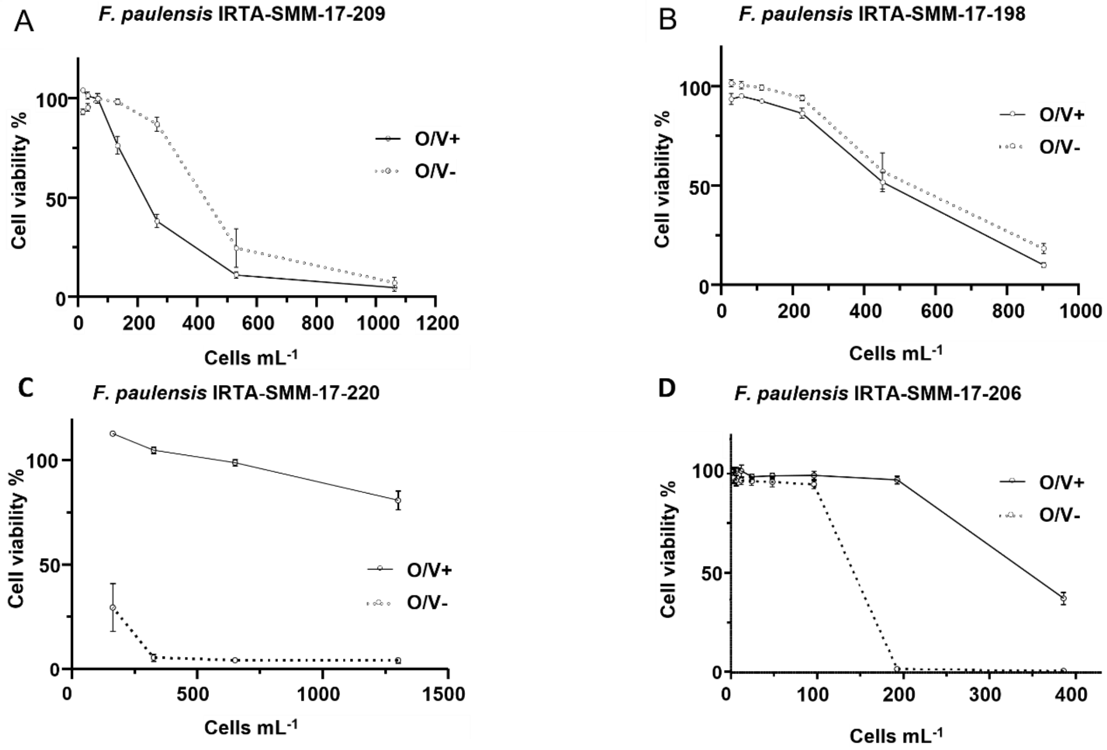

Fig. 6. Dose response curves obtained using neuro-2a CBA for *F. paulensis* extracts: IRTA- SMM-17-209 (**A**), IRTA-SMM-17-198 (**B**), IRTA-SMM-17-220 (**C**), IRTA-SMM-17-206 (**D**). O/V+: neuro-2a cells exposed to microalgal extract with the ouabain and veratridine treatment. O/V-: neuro-2a cells exposed to microalgal extract without the ouabain and veratridine treatment. Each point is the mean of triplicates and bars represent the SD.

Two strains of *F. paulensis* IRTA-SMM-17-209 and IRTA-SMM-17-211 showed CTX-like compounds and the remaining four extracts did not show CTX-like toxicity (Table 3). Figure 6A corresponds to *F. paulensis* extract (IRTA-SMM-17-209). Cell exposure to this extract at <260 cells equiv. · mL^{-1} under O/V- conditions, resulted in no significant toxicity, while at >120 cells equiv. · mL^{-1} in the O/V+ conditions significant toxicity was recorded, indicating a CTX-like effect. Figure 6B corresponds to *F. paulensis* extract (IRTA-SMM-17-198). Under both conditions with and without (O/V), the cell inhibition was significant, therefore no conclusion could be drawn in reference to CTX-like toxicity. Two *F. paulensis* extracts (IRTA-SMM-17-206 and IRTA-SMM-17-220) caused cell mortality of neuro-2a in the absence of O/V (Fig. 6C, 6D). Nonetheless, under O/V+ conditions, the toxicity of these extracts was decreased, and this is a novel toxicity pattern described for this genus.

In order to confirm the presence of MTX-like toxicity, neuro-2a cells were exposed to microalgal extracts in the presence of SKF96365. Twelve out of fifteen *Gambierdiscus* strains showed recovery of the cell viability when SKF96365 was added (Table 3). Figure 7 shows representative dose response curve of a *G. australes* extract with and without SKF96365. On the contrary, two *Fukuyoa* strains did not show recovery of the cell viability when SKF96365 was added.

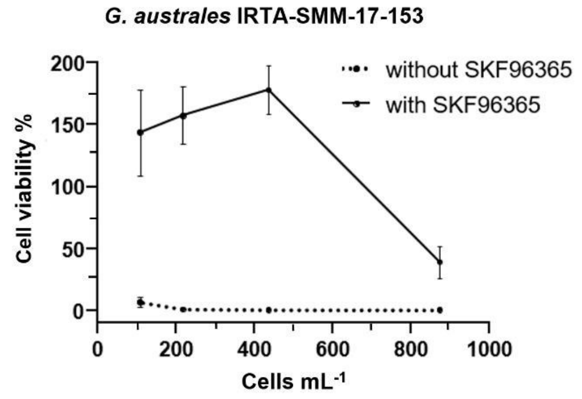

Fig 7. Dose response curve obtained using neuro-2a CBA with the *G. australes* extract (IRTA-SMM-17-153). Without SKF96365: neuro-2a cells exposed to extract in the absence of SKF96365; with SKF96365: neuro-2a cells exposed to toxin extracts in the presence of SKF96365. Each point is the mean of triplicates and the bars represent the SD.

4. Discussion

The presence of the genus *Gambierdiscus* in the Eastern Mediterranean Sea was reported in 2003 (Aligizaki & Nikolaidis, 2008). Reported species include *G. carolinianus* (Holland et al., 2013), *Gambierdiscus* sp., *G.* cf. *belizeanus* and *G. silvae* (Aligizaki et al., 2018). *G. australes* was detected later in the Balearic Islands, as presented in a brief communication (Tudó et al., 2018). The first detection of the genus *Fukuyoa* was in 2016 in the Western Mediterranean Sea (Laza-Martínez et al., 2016) and in 2018 in the Eastern Mediterranean Sea (Aligizaki et al., 2018).

To the best of our knowledge, the Balearic Islands is the location with the highest latitude worldwide, where the *Gambierdiscus* genus has been detected, specifically at 40.06 ° N. In the present study, the presence of the genus *Gambierdiscus* over large areas along the coasts of the Balearic Islands and the recurrence at some stations over three years suggests that this genus is well-established in the archipelago. However, the genus *Fukuyoa* was identified in 2017 and 2018, but not in 2019. Though the absence in 2019 should take into account that only four stations were sampled that year.

Water temperature influences on the growth and cell abundance of microalgae and can predict latitudinal distribution. *Gambierdiscus* species show different thermal limits, and distinct optimal temperatures (Kibler et al., 2012). Other variables such as salinity and irradiance can play an important role in the species distribution, though, their limits may be common for several species (Kibler et al., 2012). During the sampling dates, for Formentera, Minorca and Majorca water temperatures (22.8 and 27.2 °C) were close to optimal temperatures for *Gambierdiscus* species. Generally, for *Gambierdiscus* spp. the optimal temperature range is between 23 and 29 °C and the survival below 15 °C in laboratory conditions is rare (Kibler et al., 2012; Xu et al., 2016). *G. australes* is one of the most cryo-tolerant species in the genus; its optimal temperature for growth is relatively low, at 25 °C (Tester et al., 2020). Besides, *G. australes* cells from the Canary Islands showed the ability to stay alive with no growth for six months at 15 °C, and they resumed growth when the temperature arose to 17 °C (personal communication by Dr. Isabel Bravo https://ciguateravgo.es/). This thermo-physiologic characteristic of *G. australes* could confer upon this species the ability to persist in the Balearic Islands in wintertime when the water temperature drops at 13 °C. Regarding *Fukuyoa* spp., any literature of the optimal temperature for growth is scarce. Nonetheless, *F. paulensis* (classified previously as *G. yasumotoi*) was recorded in New Zealand (Rhodes et al., 2014a), where the water temperature oscillates between 14 and 23 °C. Additionally, one strain (Dn135EHU) from the Balearic Islands showed the formation of cysts (Laza-Martínez et al., 2016), and this could favour the species survival for long periods at low temperatures.

In previous studies of microalgae samples collected in Majorca in 1997-1998 (Vila et al., 2001a), 2001 (Penna et al., 2005) and 2011 (Laza-Martínez et al., 2016) no cells of the genera *Gambierdiscus* or *Fukuyoa* had been detected. In addition, during 2005-2006 an exhaustive sampling was conducted to characterize the phytoplankton communities from 1 to 15 m depth in the entire Balearic Archipelago (Puigserver et al., 2008), and cells of the genera *Gambierdiscus* or *Fukuyoa* were not detected. Although *Gambierdiscus* and *Fukuyoa* are mainly benthic, and Puigserver et al. (2008) was focused on phytoplankton, free-swimming cells could have been observed as was described in Parsons et al. (2011). The recent findings of cells of these genera in the Balearic Islands could be explained by an intense and specific sampling design for benthic species. Although cell abundance was not evaluated in the present work, the recent detection of these genera might be a result of an increase in abundance of endemic populations. The populations could be influenced by climate change (Aligizaki et al., 2008; Kibler et al., 2015; Llewellyn, 2010, Larsson et al., 2019), which in the Mediterranean Sea, is expected to cause an increase in abundance of thermo-tolerant species and a decrease or disappearance of cold-tolerant stenothermal species (Lejeusne et al., 2009). In addition to regional temperature increase as potential cause to changes in microalgal populations, other factors such as storms or anthropogenic activities in coastal regions could be involved. It has been suggested that expansion of benthic dinoflagellates could be attributed to an increase of turf algal mats covering substrates due to environmental changes (storms, currents, acidification) (kohler and Kohler 1992; Rongo and van Woesik, 2013; Turquet et al., 2001), but also to the degradation of the marine environment directly associated to human activities such as bottom dredging for the creation of port structures and other forms of coastal embayments, drag-netting, pollution and over-exploitation of natural resources (Parsons and Preskitt, 2007; Skinner et al., 2013; Vila et al., 2001c; Villareal et al., 2007). The environment of the Balearic Islands, most specifically the coastal areas, has suffered extreme pressures from tourism since the 1960s (Garín-Muñoz and Montero-Martín, 2007). The impact of tourism has caused a clear degradation of the coast by increasing port structures, disturbing the coastal sediments and increasing the eutrophication (Puigserver et al., 2002). In addition, meadows of *Posidonia oceanica* in the Balearic Islands are in decline in favour of colonisation of turf algal mats (Ballesteros et al., 2007; Duarte et al., 2009). It has been suggested that reduction of these disturbances should not be expected in the coming years (Duarte et al., 2009; Garín-Muñoz and Montero-Martín, 2007), so these factors could favour further increase in *Gambierdiscus* populations.

Another explanation for the presence of *Gambierdiscus* cells, may be new colonisations from other regions. For some benthic species of toxic dinoflagellates, the new colonisations may be associated with translocations of organisms by ballast waters (Hallegraeff, 2015). In fact, it has been suggested that *Alexandrium pacificum* (previously identified as *Alexandrium catenella*), a species described as non-native in the Mediterranean Sea, has been introduced by ballast waters of cargo vessels (Vila et al. 2001b, 2001c). It is well reported that the Eastern Mediterranean Basin is suffering a large-scale invasion of tropical and subtropical species. At the moment, more than 700 species of organisms have been identified as having come from the Red Sea through the Suez channel (Zenetos et al., 2012). However, from the genera *Gambierdiscus* and *Fukuyoa*, the only species reported in the Red Sea is *G. belizeanus* (Catania et al., 2017) and *F. yasumotoi* (Saburova et al., 2013). Therefore, the phenomenon of species translocation from the Red Sea to the Mediterranean Sea may not explain the current situation for these species. Considering the possibility that *Gambierdiscus* cells reached the Balearic Islands from the Atlantic Ocean, the genetic information provided by the D8-D10 region (LSU rDNA) shows that *G. australes* populations from the Balearic Islands and the Canary Archipelago are identical. Although this information may establish a link between these populations, more molecular markers should be analysed to determine the relationship between populations in these two areas. Population genetics and phylogeographic studies of these species have to be considered in future studies because they can help to identify the source of populations and reveal expansion patterns, and mechanisms of transfer (Sakai et al., 2001).

Gambierdiscus australes cells from the Balearic Islands show morphological similarities to other *G. australes* described in previous studies (Table 1). Cell size (D and W) from the present study are partially consistent with the range of the first description of *G. australes* for the strain RAV-92 in the Pacific Ocean (Chinain et al., 1999). Later, strains RAV-92 and NOAA2 were measured by Litaker et al., (2009) and their minimum extreme values of D and W were almost the same as in the current study. Cell morphology can change by natural factors, but also, over time of cultures in laboratory conditions (Rhodes et al., 2014b). The maximum values of D and W for *G. australes* were described for strains isolated from the Canary Islands (Atlantic Ocean) in Bravo et al. (2019). Values of Bravo et al. (2019) and the present work show larger sizes than those of Rhodes et al. (2014b), Chinain et al. (1999) and Litaker et al. (2009).

Regarding *Fukuyoa* isolates, the average L and D of cell size in the current study are inside the ranges of previous studies performed in the Mediterranean Sea, the Atlantic and the Pacific Ocean (Gómez et al., 2015, Laza-Martínez et al., 2016, Rhodes et al., 2014a) (Table 1). However, the lowest values for L and W in the present work are smaller than in the previous studies.

The maximum cell yield for *G. australes* cultures in the present study of growth dynamics and toxicity was 2288 to 2274 cells · mL^{-1}, respectively. These values are lower than for *G. australes* strains from the North Atlantic Ocean (Reverté et al., 2018), where the maximum cell yield was 4470 cells · mL^{-1}. Such differences may be attributed to the differences in the culturing conditions. In both works, cells were cultivated at the same temperature and medium, but in Reverté et al. (2018) photon irradiance was lower, a pump supplied the aeration and the vessel was a 3L round-bottom flask.

In the current work, growth rates for *G. australes* were lower than the rates of *G. australes* strains from the Atlantic Ocean reported by Reverté et al. (2018), which ranged from 0.20 to 0.39 div. · day^{-1}, and they are similar to the rates for *G. australes* strains, from the Pacific Ocean described before: 0.12 - 0.19 div. · day^{-1} in Chinain et al. (2010) and 0.149 ± 0.006 div. · day^{-1} in

Pisapia et al. (2017). The growth rate in the *Gambierdiscus* genus has been reported to be in the range of 0.01 to 0.55 div. · day^{-1} (Xu et al., 2016; Whiters, 1981). Some studies for the genus *Gambierdiscus* link high division rates to high toxin production per cell (Chinain et al., 2010, Litaker et al., 2017; Pisapia et al., 2017, Reverté et al., 2018), but in the present study, this relation was not studied.

Regarding *F. paulensis* growth, there was high variability of maximum cell yield among the strains from the current study. The maximum cell yield in the growth dynamics study was 1004 cells · mL^{-1} (Table 2), and for the CTX-like toxicity study, values ranged between 333 and 6636 cells · mL^{-1} (Table 3). These yields were much lower than those achieved at the stationary phase by Laza-Martínez et al. (2016) of 14.800 cells · mL^{-1}. In Laza-Martínez et al. (2016), strains were cultured in culture plastic flasks, with f/4 medium with selenium (Guillard and Ryther, 1962) and salinity was adjusted at 35. Besides, cells were maintained at 25 °C and irradiance of 50-100 µmol · m^{-2} · s^{-1}. Regarding growth rates, the present study provides the first data for *F. paulensis* with 0.24 ± 0.06 div. · cell^{-1}. Within the *Fukuyoa* genus, *F. ruetzleri* (previously *G. ruetzleri*) showed growth rates of 0.17, 0.18 and 0.35 div · cell^{-1} in Litaker et al. (2017), Pisapia et al. (2017), and Kibler et al. (2012), respectively.

In the current paper, *G. australes* strains presented CTX-like activity with quantifications ranging between 1.4 and 380 fg CTX1B equiv. · cell^{-1}. These quantifications are low compared to values for *G. australes* from the Atlantic Ocean reported by Reverté et al. (2018), where values ranged from 200 to 697 fg CTX1B equiv. · cell^{-1}. In both works, strains were acclimated for one year, but as it has been mentioned before, they were cultured in different culturing conditions. Therefore, dissimilar toxin production could be caused by distinct culturing conditions. In contrast, the CTX-like activity was similar to other *G. australes* strains from the Atlantic Ocean (31-107 fg CTX1B equiv. · cell^{-1}) and from the Pacific Ocean (40 fg CTX1B equiv. · cell^{-1}) reported by Rossignoli et al. (2020) and Rhodes et al. (2017), respectively.

Among *Gambierdiscus* species, *G. australes* has intermediate CTX-like toxicity. For instance, by standard mouse bioassay (MBA), *G. australes* extracts presented lower toxicity than *G. pacificus* and *G. polynesiensis* (Chinain et al., 1999), with the latter being the most toxic species in the genus. Furthermore, in Chinain et al. (2010), the CTX-like response for strains from the Pacific Ocean was similar to *G. toxicus*, and 100-fold lower than in *G. polynesiensis.* Moreover, in Pisapia et al. (2017), the CTX-like toxicity of ten strains was evaluated by neuro-2a CBA, and three *G. australes* strains (two from the Atlantic and one from the Pacific Ocean) were placed in the seventh-place, near the bottom of the scale.

Despite several unsuccessful attempts to confirm toxicity in *Gambierdiscus* spp. (Larsson et al., 2018), CTXs have not been confirmed for most *Gambierdiscus* spp., except for *G. australes* (Roeder et al., 2010), *G. pacificus* (Caillaud et al., 2011), *G. polynesiensis* (Chinain et al., 2010) and *G. excentricus* (Paz et al., 2011). A putative CTX (2,3-dihydroxy P-CTX-3C) was detected by liquid chromatography-mass spectrometry (LC-MS/MS) in only one *G. australes* strain (CCMP 1653) from Hawaii (Pacific Ocean), at exponential phase (Roeder et al., 2010). In fact, *Gambierdiscus* species are common producers of MTXs and *G. australes* is one of the top producers (Munday et al., 2017). MTX1 and MTX3 were detected by LC-MS/MS in *G. australes* by Munday et al. (2017). Moreover, in Rhodes et al. (2017), LC-MS/MS confirmed the presence of MTX1 in all tested *G. australes* strains. This is in accordance with the results for the present study in which almost all strains of *G. australes* (12 out of 15) presented MTX-like activity. Nonetheless, a recent study including *G. australes* cultures from the present work, (IRTA-SMM-17-162, IRTA-SMM-17-164, IRTA-SMM-17-189, IRTA-SMM-17-244, IRTA-SMM-17-253, IRTA-SMM-17-271) were analysed by liquid chromatography coupled to low and high resolution mass spectrometry (LC-MS/MS) and (LC-HRMS), and MTX1, desulfo-MTX1 and didehydro-34 desulfo-MTX1 were not detected. By contrast, 44-methylgambierone (MTX3) was present in all of these strains (Estevez et al., 2020).

The MTX family, previously included only molecules with a molecular weight of more than 3000 Da and having no activity on VGSCs (Yokoyama et al., 1988). Recently, 44-methylgambierone has been found in *G. belizeanus* and *G. australes* (Boente-Juncal et al., 2019; Murray et al., 2019). This molecule was previously defined as MTX3 (Holmes and Lewis, 1994). Nonetheless, it is a molecule of 1060 Da which presents structural differences as compared to the previous MTXs (Holmes and Lewis, 1994) and shows CTX-like activity more than MTX-activity (Boente-Juncal et al., 2019). In human cortical neurons, 44-methylgambierone showed no signals of cell mortality at 0.01 to 20 nM for five days, whereas at 0.1 nM of MTX1 significant cell death was observed in 2h (Boente-Juncal et al., 2019). Furthermore, in human neuroblastoma cells, after 24h of exposure of cells to MTX3 at 10 to 50 nM cells did not show signs of toxicity, while with MTX1 at 0.1 nM, complete cell death was observed (Boente-Juncal et al., 2019). Hence, including 44-methylgambierone in the MTX group may lead to confusion on the role of the rest of MTXs in CP. Given that only one strain of *G. australes* strain produced a CTX analogue (Rhoeder et al., 2010), and the effects of 44-methylgambierone in neuro-2a cells could be similar to effects of CTXs, the CTX-like toxicity of the *G. australes* extracts of the present study could be potentially attributed to the effect of 44-methylgambierone. Even so, 44-methylgambierone exhibited very low toxicity by MBA, hence it is unlikely it contributes to CP (Murray et al., 2020).

Concerning *F. paulensis,* the CTX-like toxicities of the current study ranged from 8 to 16 fg CTX1B equiv. · cell^{-1}. These toxicities were low in comparison to the *G. australes* strains. Previously, one *F. paulensis* strain (Dn35EHU) from the Balearic Islands presented low CTX-like toxicity by MBA (Laza-Martínez et al., 2016). In the same study, for the same strain, traces of 54-deoxyCTX1B and gambieric acid A (GA A) were detected by LC-HRMS. Recently, Estevez et. al (2020) detected 44-methygambierone for the *F. paulensis* strain (IRTA-SMM-17-209), which is the same strain used in the current study. This is in accordance with the results of Rhodes et al. (2014a) and Larsson et al. (2019), which detected 44-methylgambierone in *F. paulensis* from the Pacific

Ocean. Therefore, like *G. australes*, the CTX-like toxicity of *F. paulensis* in neuro-2a CBA could be explained by the presence of CTX analogues and the 44-methylgambierone.

F. paulensis presents low toxicity in comparison to other *Fukuyoa* species. Litaker et al. (2017) detected CTX-like toxicity in three *F. ruetzleri* strains by neuro-2a CBA with an average of 24.50 and 6.50 fg CTX3 equiv. · cell^{-1}. Like *G. australes,* it is suggested that some strains of *F. paulensis* are non-CTX-producers because no signal of CTX-like toxicity and no CTX-analogues were found at early stationary phase for a cultured strain (VGO1185) from Brazil (Atlantic Ocean) by neuro-2a CBA (Gómez et al., 2015). Moreover, *F. paulensis* (previously *G. yasumotoi*) CAWD210 from New Zealand did not exhibit CTX-like toxicity by sea urchin embryo assay (SUEA) (Rhodes et al., 2014a). That is in concordance with the results of the present study and the increase in viability observed when neuro-2a cells were exposed to extracts from *F. paulensis*. Laza-Martínez et al. (2016) detected MTX-like activity by MBA. However, in the present work, the MTX-like activity for *F. paulensis* was not detectable (n=2). Furthermore, there is no confirmation of MTX1, desulfo-MTX1 and didehydro-34 desulfo-MTX1 in *F. paulensis* strain (IRTA-SMM-17-209) by analytical methods (Estevez et al. 2020).

Toxicities of several strains of *Gambierdiscus* from the Pacific have been largely studied, but information about the strains from the Mediterranean Sea is scarce despite the increasing identification of species in recent decades. To the best of our knowledge, the presence of CTX-like toxicity in *Gambierdiscus* strains has only been evaluated from the eastern Mediterranean region using three strains of *G. carolinianus, G. silvae* and *Gambierdiscus* sp.; all of them analysed by neuro-2a CBA. The *G. carolinianus* strain showed CTX-like activity in low quantities (< 4 fg CTX3C equiv. · cell^{-1}) (Pisapia et al., 2017), the *G. silvae* showed high CTX-like toxicity and the putative new species *Gambierdiscus* sp. exhibited low CTX-like activity (Aligizaki et al., 2018). The demonstration of CTX-like toxicity in strains in the Balearic Islands, and the fact that no evidence of ciguateric fish or CP has occurred in this area, could suggest that these populations are relatively new residents or that the densities of the populations are probably low.

5. Conclusions

Fukuyoa and *Gambierdiscus* cells found in samples from the Balearic Islands from 2016 to 2019 have been identified as *F. paulensis* and *G. australes.* These two species seem to be well-established in the area. Considering the other studies, CTX-toxicity exhibited by most of the *G. australes* and *F. paulensis* strains was low. However, one strain of *G. australes* (IRTA-SMM-17-168) was classified as a very high producer in comparison to previous studies. In addition, it is not possible to discard that some cells from the Balearic Islands could be a high CTX-producers and could be associated with distinct seasonality. Even though CP cases have not yet been confirmed in the Mediterranean, the CTX-like toxicity present in the strains of *G. australes* and *F. paulensis* from the Balearic Islands may indicate that potential future cases of CP should not be dismissed. There is a clear need for continued studies and monitoring of benthic dinoflagellates in the region.

Conflict of interest

The authors declare that there is no conflict of interest.

Authors contribution

Conceptualization A.T. (Àngels Tudó), M.C. (Mònica Campàs), M.F. (Margarita fernández-Tejedor) and J.D. (Jorge Diogène); methodology A.T., A.T.F. (Anna Toldrà), M.R. (María Rey) and I.T. (Irene Todolí); data curation A.T., K.A. (Karl B. Andree), M.F., J.D. formal analysis A.T., K.A., M.F., M.C., F.S. (Francesc X. Sureda) and J.D.; writing original draft preparation A.T., M.C., A.T.F and J.D. .; writing, review and editing A.T., A.T.F., M.R., M.C., K.A., M.F., F.S. and J.D. All authors have read and agreed to the published version of the manuscript.

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Table. 1. Morphometric comparison of *G. australes* and *F. paulensis* strains of this study with published measurements for those species. Average and standard deviation of depth (D), length (L), width (W), ratio of depth and width (D:W), ratio of length and width (L:W); length of apical pore plate (Po) and surrounding pore numbers (No) and diameter (Ø), size of 2’’’’ plate. Data are expressed as the arithmetic mean, standard deviation (± SD) and number of measured cells (n).

		G. australes **Chinain et al. 1999**	***G. australes*** **Litaker et al., 2009**	***G. australes*** **Rhodes et al., 2014b**	***G. australes*** **Rhodes et al., 2014b**	***G. australes*** **Bravo et al. 2019**	***G. australes*** **This study**	***F. paulensis*** **Rhodes et al., 2014a *(G. yasumotoi)***	***F. paulensis*** **Gómez et al. 2015**	***F. paulensis*** **Laza-Martínez et al. 2016**	***F. paulensis*** **This study**
Isolates		RAV-92	RAV-92/NOAA24	CADW149	CAWD216			CAWD210	VGO1185	Dn35EHU	
Cell size	**L µm**	-	38.7 ± 3.8 (33.4 - 47.3)	32.0 (26.0 - 39.0)	39.0 (32.5 - 45.5)		-	59.8 ± 7.5 (54.3- 67.3) (n=20)	56.0 ± 3.0 (51- 62)	48.9 ± 10.9 (35- 76) (n=100)	46.6 ± 8.7 (32.0- 64.1) (n=21)
	D µm	86.0 ± 5.1 (76.0 - 93.0)	72.5 ± 3.8 (63.8 - 77.4)	44.5 (32.5 - 52.0)	58.5 (45.5 - 65.0)	81 ± 6.3 (68-95)	75.7 ± 6.0 (60.9- 92.3) (n= 112)	54.8 ± 5.7 (49.1- 60.5) (n=20)	50.0 ± 3.0 (45- 56)	40.8 ± 8.2 (31- 67) (n=123)	40.5 ± 4.8 (36.4 - 51.1) (n=14)
	W µm	77.0 ± 3.7 (65.0 - 84.0)	63.4 ± 5 (55.2–73.8)	38.5 (32.5–52.0)	48.0 (40.0–52.0)	78 ± 7.5 (60-95)	78.7 ± 6.6 (54.7 - 90.8) (n= 112)	42.5 ± 4.1 (38.4- 46.6) (n=20)	45.0 ± 2.0 (41- 48)	30.5 ± 6.6 (24- 38) (n=60)	41.1 ± 11.9 (11.9 - 41.1) (n=21)
	L:W		0.61	0.83	0.81			1.41	n.d	1.28 (n=10)	1.44 ± 0.21 (1.1 - 1.7) (n=21)
	D:W	1.12	1.14	1.16	1.22		1.02 ± 0.09 (0.8 – 1.2) (n= 112)	1.29	1.2	1.29 (n=48)	
Po plate	**L µm**	7.1 ± 0.8 (6.3–8.6)					7.2 ± 0.7 (6.2 – 8.4) (n=14)	9.9 (Laza-Martínez et al., 2016)	10-12	7.6	8.2 ± 1.4 (5.9 - 11.4) (n=13)
	W µm	6.1 ± 0.4 (5.7–6.8)					5.7 ± 0.7 (4.8 - 7.8) (n=14)	4.6 (Laza-Martínez et al., 2016)	6-7	4.1	3.0 ± 0.7 (2.2–4.4) (n=13)
	L:W	1.18					1.23 ± 0.1 (1.0- 1.5) (n=14)		n.d		3.1 ± 0.9 (1.8–4.4) (n=13)
	Number pores	31 ± 4.1					29 ± 1.6 (27– 33) (n=14)		23-39	29-39	35.3 ± 1.6 (32– 37) (n=13)
	Diameter pores µm	0.45 ± 0.03					0.39 ± 0.09 (0.2– 0.6) (n=197)			0.35 (n=150)	0.31 ± 0.08 (0.16 - 0.51) (n=52)
2’’’’ antapical	**L 2’’’’ µm**	54 ± 3.1					41.5 ± 4.8 (33.5 - 48.8) (n=14)		33-39		45.3 ± 2.9 (41.7– 48.8)
	W 2’’’’ µm	27 ± 2.7					21.6 ± 3.3 (17.7 - 29.6) (n=14)		19-23		23.7 ± 3.4 (19.4 - 29.6)
	L:W 2’’’’	2.10					1.95 ± 0.28 (1.6 - 2.5) (n=14)				1.95 ± 0.32 (1.7 - 2.5) (n=7)

Table. 2. Growth parameters of *G. australes* (n=3) and *F. paulensis* (n=1) from the Balearic Islands. Averages of the three replicates of: Max. conc.= maximum cell yield (cells · mL^{-1}); r = growth rate (div. · day^{-1}) ± standard deviation (SD), the period when r was calculated (days) is showed in brackets; K= doublings per day (doublings · day^{-1}) ± SD; Td = doubling time ($days^{-1}$) ± SD.

	IRTA-SMM-17-162 ***G. australes***	**IRTA-SMM-17-189** ***G. australes***	**IRTA-SMM-17-271** ***G. australes***	**IRTA-SMM-17-209** ***F. paulensis***
Max. conc.	2288	1451	1244	1004
r	0.12 ± 0.04 (13-20)	0.15 ± 0.04 (13-20)	0.16 ± 0.04 (12-19)	0.24 ± 0.06 (7-14)
K (Eq. 1)	0.17 ± 0.05	0.21 ± 0.06	0.24 ± 0.06	0.34 ± 0.09
Td (Eq. 2)	6.25 ± 1.80	5.85 ± 1.07	4.36 ± 1.01	1.30 ± 1.64

Table 3. Evaluation of the presence of CTX-like and MTX-like toxicity by neuro-2a CBA. Species, code of strain, origin, cell concentration of cultures at harvesting time (cell · mL^{-1}), values of CTX-like toxicity expressed in femtograms (fg) of CTX1B equiv. · cell^{-1} ± SD. n.s.: nonspecific toxicity; +: recovery of the cell viability in the presence of SKF96365; -: non-recovery of the cell viability in the presence of SKF96365; NT: not tested.

Species	**Code**	**Island**	**Cell abundance (cells · mL^{-1})**	**CTX-like toxicity (fg CTX1B equiv. · cell^{-1})**	**MTX-like Toxicity**
G. australes	IRTA-SMM-17-153	Majorca	1750	1.38 ± 0.66	+
G. australes	IRTA-SMM-17-238	Majorca	1632	3.52 ± 0.18	NT
G. australes	IRTA-SMM-17-180	Minorca	613	5.25 ± 0.59	NT
G. australes	IRTA-SMM-17-218	Majorca	1686	9.47 ± 3.18	+
G. australes	IRTA-SMM-17-216[a]	Majorca	1476	13.14 ± 4.50	+
G. australes	IRTA-SMM-17-254	Majorca	1273	13.16 ± 1.34	+
G. australes	IRTA-SMM-17-253	Majorca	1935	13.45 ± 0.97	+
G. australes	IRTA-SMM-17-181	Minorca	1464	13.50 ± 0.80	+
G. australes	IRTA-SMM-17-178	Minorca	2040	14.52 ± 4.31	-
G. australes	IRTA-SMM-17-223	Majorca	1183	14.93 ± 4.69	+
G. australes	IRTA-SMM-17-155	Minorca	332	17.33 ± 1.60	-
G. australes	IRTA-SMM-17-173	Majorca	2087	21.89 ± 9.20	+
G. australes	IRTA-SMM-17-244	Majorca	924	34.33 ± 4.18	+
G. australes	IRTA-SMM-17-256	Majorca	1004	39.17 ± 16.44	NT
G. australes	IRTA-SMM-17-175	Minorca	1498	62.00 ± 0.66	-
G. australes	IRTA-SMM-17-164	Minorca	1022	72.60 ± 43.20	+
G. australes	IRTA-SMM-17-214	Majorca	1694	76.67 ± 29,86	+
G. australes	IRTA-SMM-17-189	Minorca	869	83.39 ± 12.14	NT
G. australes	IRTA-SMM-17-162	Minorca	1390	105.67 ± 18.27	NT
G. australes	IRTA-SMM-17-271	Minorca	843	172.63 ± 5.57	+
G. australes	IRTA-SMM-17-168[a]	Majorca	2274	381.83 ± 91.84	NT
F. paulensis	IRTA-SMM-17-209[a]	Minorca	782	16.30 ± 1.67	NT
F. paulensis	IRTA-SMM-17-211	Minorca	3250	7.96 ± 0.14	NT
F. paulensis	IRTA-SMM-17-198[a]	Majorca	4825	n.s	NT
F. paulensis	IRTA-SMM-17-206	Majorca	2053	n.s	-
F. paulensis	IRTA-SMM-17-220	Minorca	2128	n.s	-
F. paulensis	IRTA-SMM-17-221	Minorca	6636	n.s	NT

[a] response curves of CTX-like evaluation of these strains are shown in Fig. 5 and 6.

Figure 1

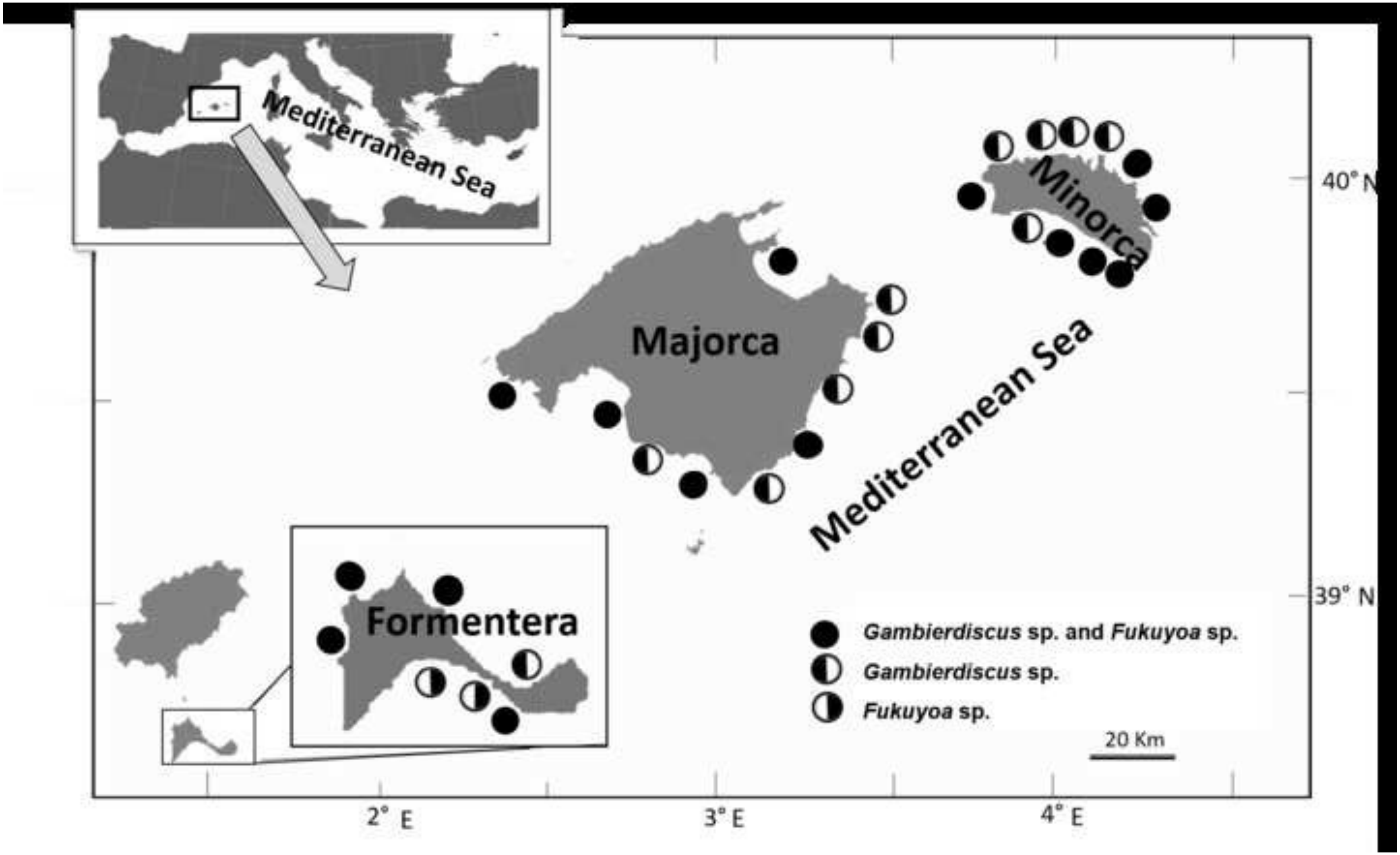
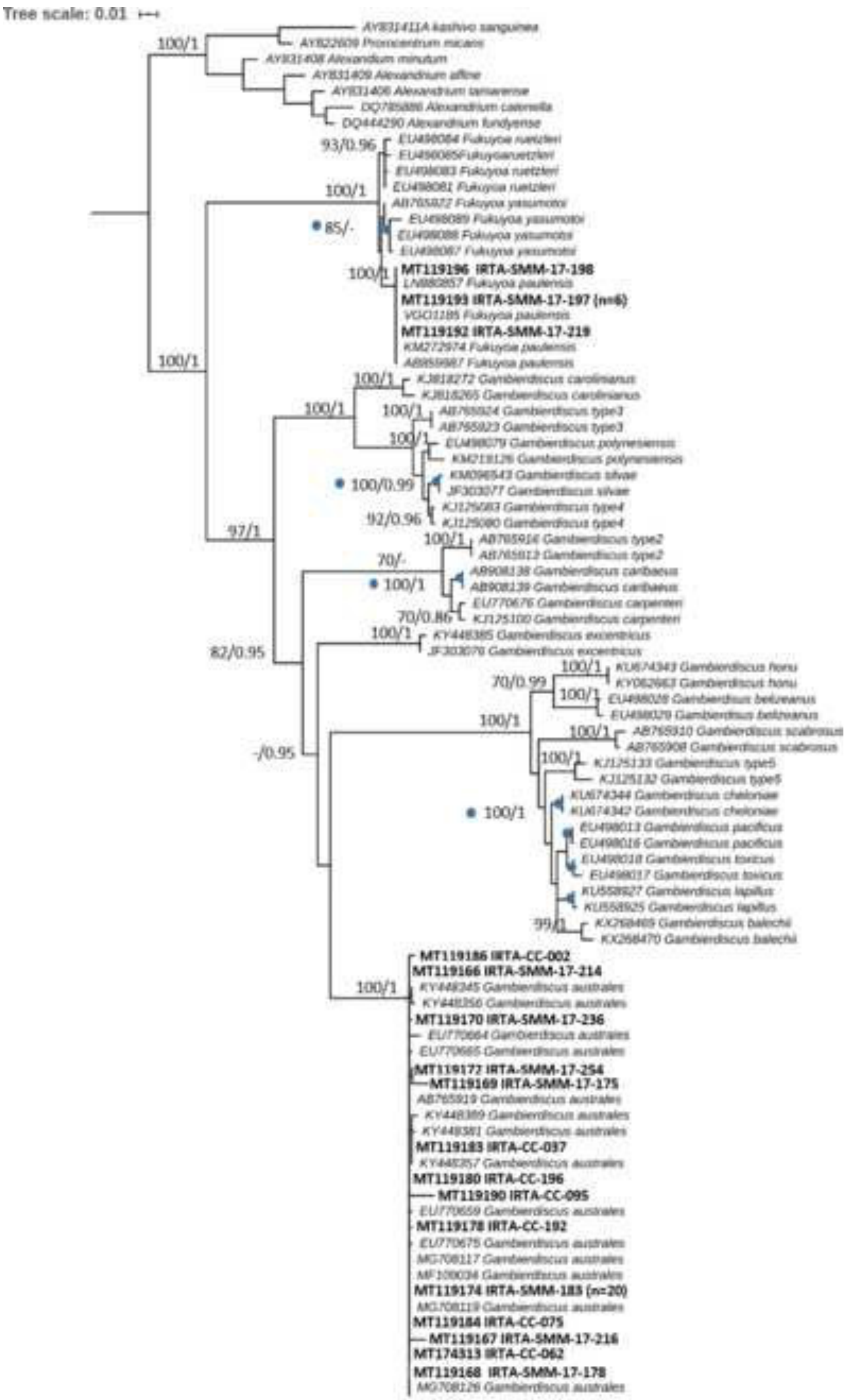

Mediterranean Sea
Minorca
Majorca
Mediterranean Sea
Formentera
● *Gambierdiscus* sp. and *Fukuyoa* sp.
◐ *Gambierdiscus* sp.
◑ *Fukuyoa* sp.
20 Km
40° N
39° N
2° E
3° E
4° E

Figure 2


Tree scale: 0.01
100/1
AY831411A kashivo sanguinea
AY822609 Prorocentrum micans
AY831408 Alexandrium minutum
AY831409 Alexandrium affine
AY831406 Alexandrium tamarense
DQ785886 Alexandrium catenella
DQ444290 Alexandrium fundyense
93/0.96
EU498084 Fukuyoa ruetzleri
EU498085Fukuyoaruetzleri
EU498083 Fukuyoa ruetzleri
100/1
EU498081 Fukuyoa ruetzleri
AB765922 Fukuyoa yasumotoi
85/-
EU498089 Fukuyoa yasumotoi
EU498088 Fukuyoa yasumotoi
EU498087 Fukuyoa yasumotoi
100/1
MT119196 IRTA-SMM-17-198
LN880857 Fukuyoa paulensis
MT119193 IRTA-SMM-17-197 (n=6)
VGO1185 Fukuyoa paulensis
MT119192 IRTA-SMM-17-219
KM272974 Fukuyoa paulensis
AB859987 Fukuyoa paulensis
100/1
100/1
KJ818272 Gambierdiscus carolinianus
KJ818265 Gambierdiscus carolinianus
100/1
100/1
AB765924 Gambierdiscus type3
AB765923 Gambierdiscus type3
100/1
EU498079 Gambierdiscus polynesiensis
KM218126 Gambierdiscus polynesiensis
100/0.99
KM096543 Gambierdiscus silvae
JF303077 Gambierdiscus silvae
KJ125083 Gambierdiscus type4
92/0.96
KJ125080 Gambierdiscus type4
97/1
100/1
AB765916 Gambierdiscus type2
AB765913 Gambierdiscus type2
70/-
AB908138 Gambierdiscus caribaeus
100/1
AB908139 Gambierdiscus caribaeus
EU770676 Gambierdiscus carpenteri
70/0.86
KJ125100 Gambierdiscus carpenteri
100/1
KY448385 Gambierdiscus excentricus
82/0.95
JF303076 Gambierdiscus excentricus
100/1
KU674343 Gambierdiscus honu
70/0.99
KY062663 Gambierdiscus honu
100/1
EU498028 Gambierdiscus belizeanus
100/1
EU498029 Gambierdiscus belizeanus
100/1
AB765910 Gambierdiscus scabrosus
-/0.95
AB765908 Gambierdiscus scabrosus
100/1
KJ125133 Gambierdiscus type5
KJ125132 Gambierdiscus type5
KU674344 Gambierdiscus cheloniae
100/1
KU674342 Gambierdiscus cheloniae
EU498013 Gambierdiscus pacificus
EU498016 Gambierdiscus pacificus
EU498018 Gambierdiscus toxicus
EU498017 Gambierdiscus toxicus
KU558927 Gambierdiscus lapillus
KU558925 Gambierdiscus lapillus
99/1
KX268469 Gambierdiscus balechii
KX268470 Gambierdiscus balechii
MT119186 IRTA-CC-002
MT119166 IRTA-SMM-17-214
100/1
KY448345 Gambierdiscus australes
KY448356 Gambierdiscus australes
MT119170 IRTA-SMM-17-236
EU770664 Gambierdiscus australes
EU770665 Gambierdiscus australes
MT119172 IRTA-SMM-17-254
MT119169 IRTA-SMM-17-175
AB765919 Gambierdiscus australes
KY448389 Gambierdiscus australes
KY448381 Gambierdiscus australes
MT119183 IRTA-CC-037
KY448357 Gambierdiscus australes
MT119180 IRTA-CC-196
MT119190 IRTA-CC-095
EU770659 Gambierdiscus australes
MT119178 IRTA-CC-192
EU770675 Gambierdiscus australes
MG708117 Gambierdiscus australes
MF100034 Gambierdiscus australes
MT119174 IRTA-SMM-183 (n=20)
MG708119 Gambierdiscus australes
MT119184 IRTA-CC-075
MT119167 IRTA-SMM-17-216
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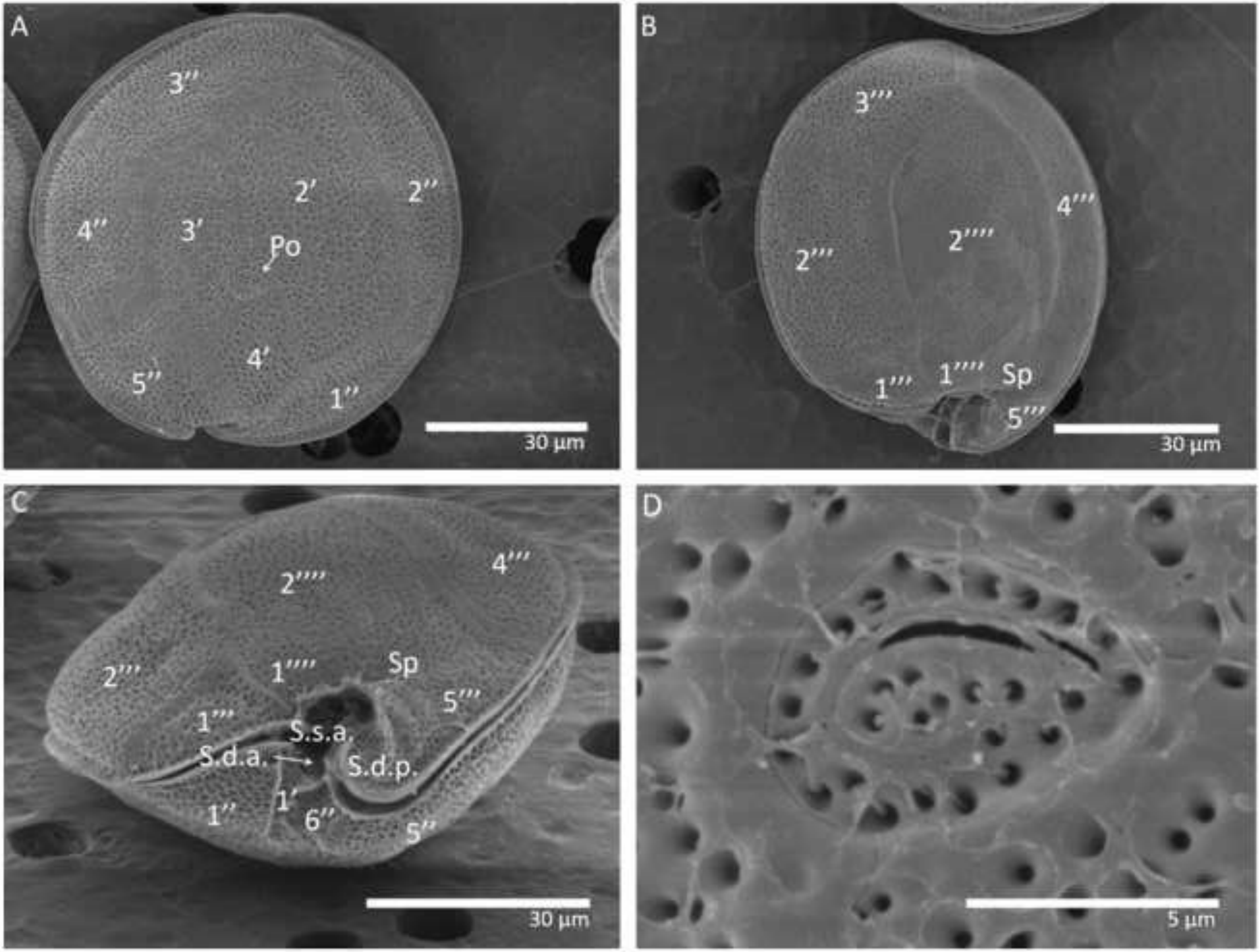
MT119168 IRTA-SMM-17-178
MG708126 Gambierdiscus australes


A
3''
2'
2''
4''
3'
Po
4'
5''
1''
30 µm
B
3'''
4'''
2''''
2'''
1'''
1''''
Sp
5'''
30 µm
C
2''''
4'''
2'''
1''''
Sp
5'''
1'''
S.s.a.
S.d.a.
S.d.p.
1''
1'
6''
5''
30 µm
D
5 µm

[redacted]

Figure 4

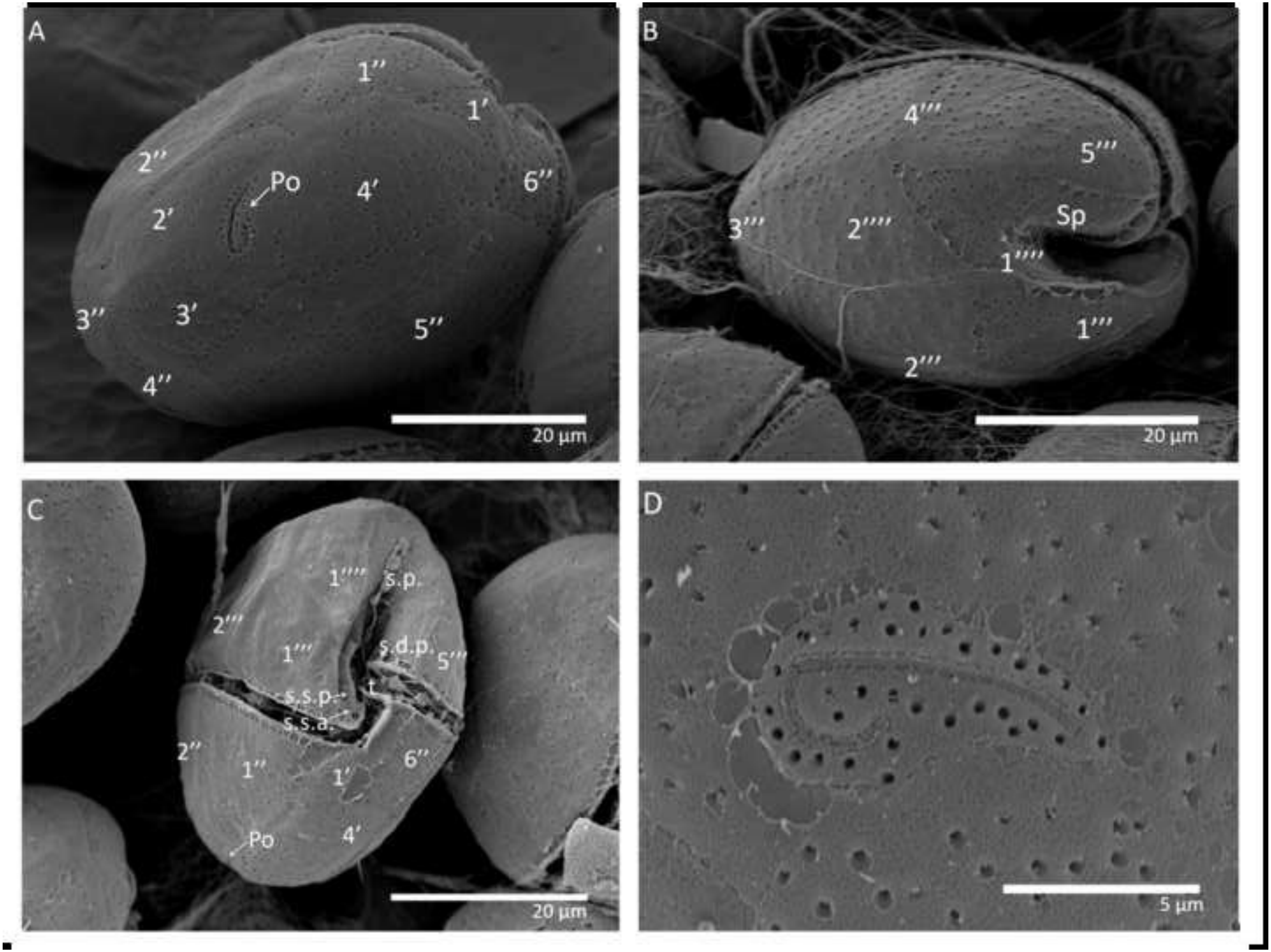

A
1''
1'
2''
Po
4'
6''
2'
3''
3'
5''
4''
20 µm
B
4'''
5'''
Sp
3'''
2''''
1''''
1'''
2'''
20 µm
C
1''''
s.p.
2'''
1'''
s.d.p.
5'''
s.s.p.
t
s.s.a.
2''
1''
1'
6''
Po
4'
20 µm
D
5 µm

Figure 5

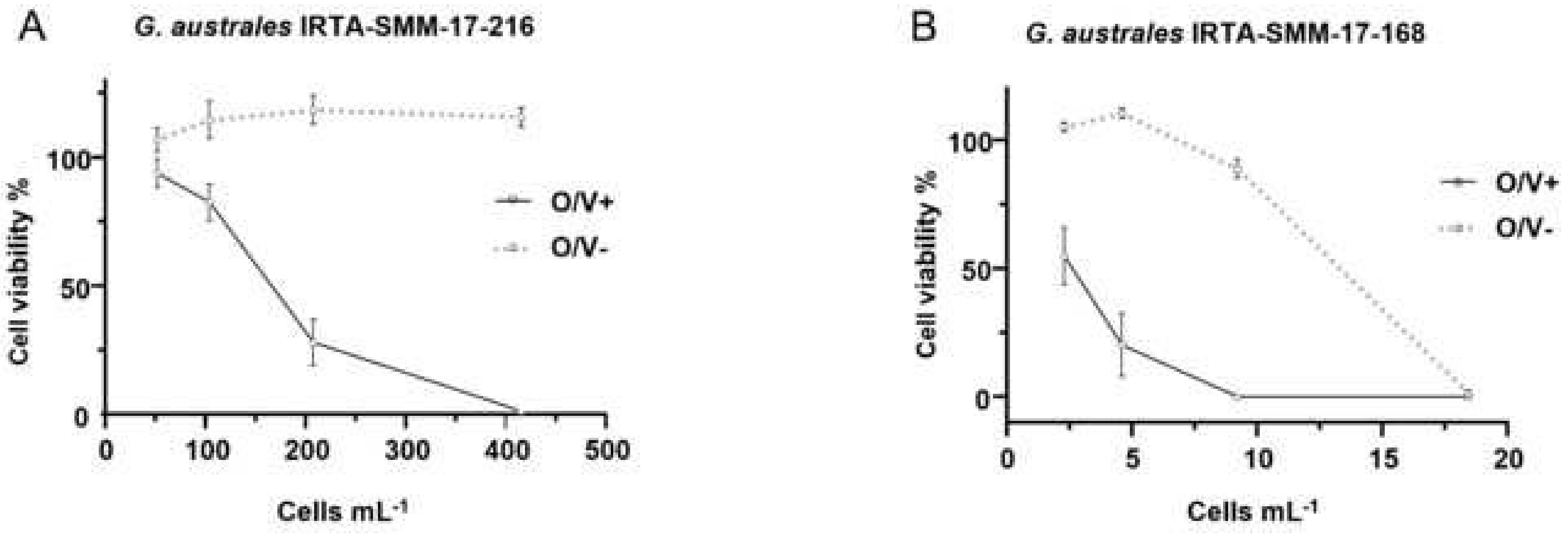
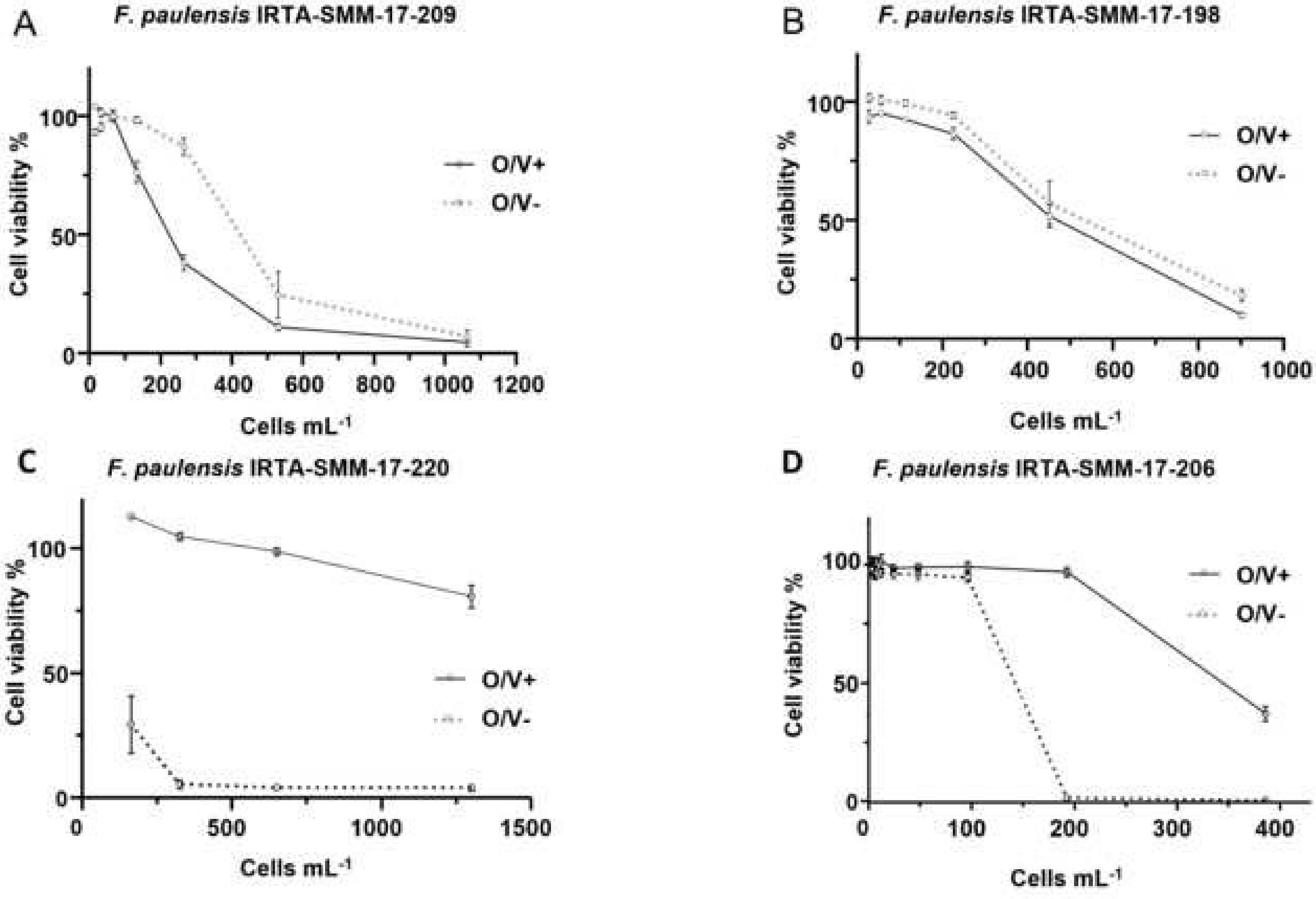

A
G. australes IRTA-SMM-17-216
Cell viability %
100
50
0
0
100
200
300
400
500
Cells mL-1
O/V+
O/V-
B
G. australes IRTA-SMM-17-168
Cell viability %
100
50
0
0
5
10
15
20
Cells mL-1
O/V+
O/V-

Figure 6


A
F. paulensis IRTA-SMM-17-209
Cell viability %
0
50
100
0
200
400
600
800
1000
1200
Cells mL-1
O/V+
O/V-
B
F. paulensis IRTA-SMM-17-198
Cell viability %
0
50
100
0
200
400
600
800
1000
Cells mL-1
O/V+
O/V-
C
F. paulensis IRTA-SMM-17-220
Cell viability %
0
50
100
0
500
1000
1500
Cells mL-1
O/V+
O/V-
D
F. paulensis IRTA-SMM-17-206
Cell viability %
0
50
100
0
100
200
300
400
Cells mL-1
O/V+
O/V-

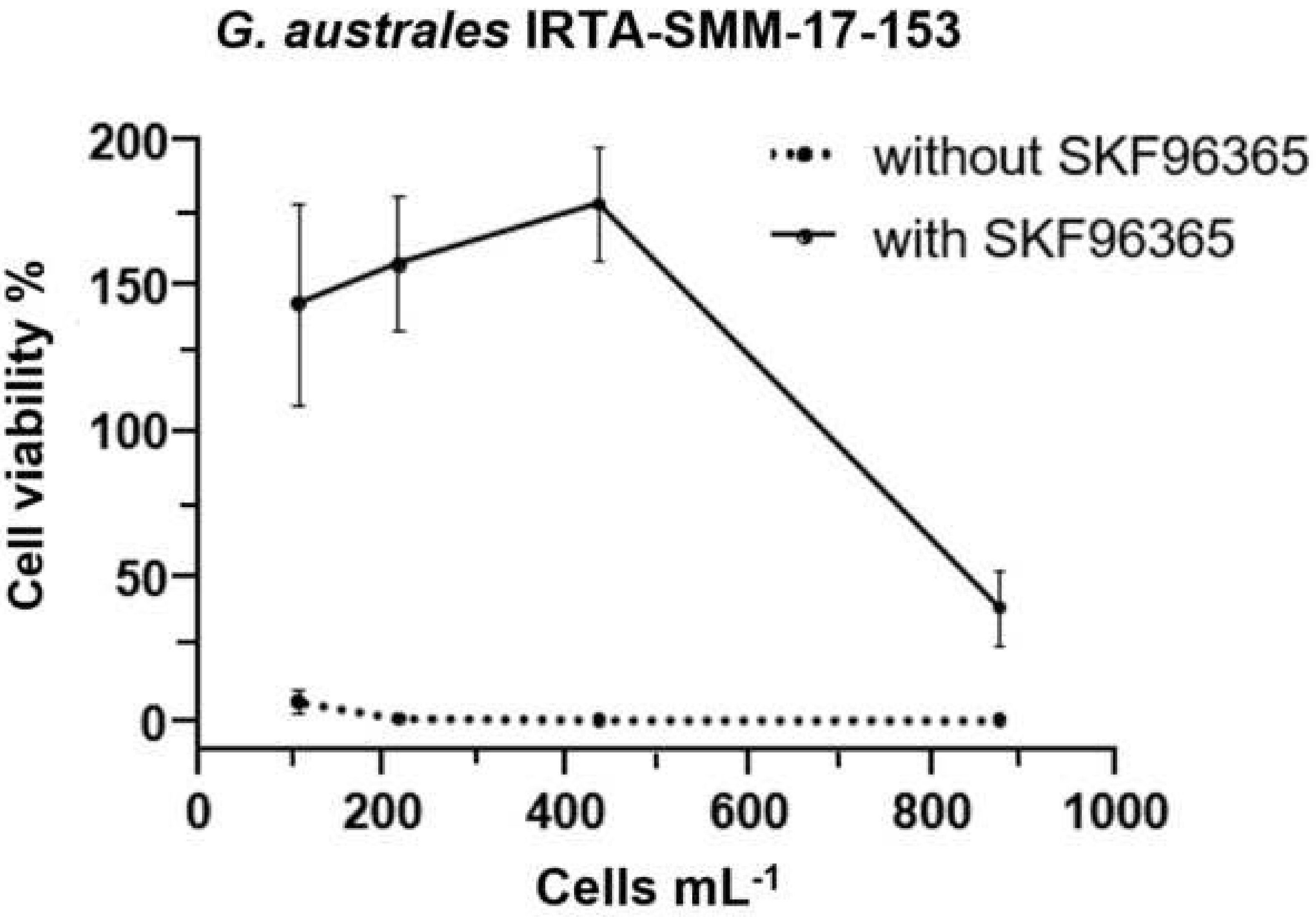

G. australes IRTA-SMM-17-153
without SKF96365
with SKF96365
Cell viability %
200
150
100
50
0
0
200
400
600
800
1000
Cells mL-1

Table 1

		G. australes **Chinain et al. 1999**	***G. australes*** **Litaker et al., 2009**	***G. australes*** **Rhodes et al., 2014b**	***G. australes*** **Rhodes et al., 2014b**	***G. australes*** **Bravo et al. 2019**	***G. australes*** **This study**	***F. paulensis*** **(Rhodes et al., 2014)** ***(G. yasumotoi)***	***F. paulensis*** **Gómez et al. 2015**	***F. paulensis*** **Laza-Martínez et al. 2016**	***F. paulensis*** **This study**
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Cell size	**L µm**	-	38.7 ± 3.8 (33.4 - 47.3)	32.0 (26.0 - 39.0)	39.0 (32.5 - 45.5)		-	59.8 ± 7.5 (54.3- 67.3) (n=20)	56.0 ± 3.0 (51- 62)	48.9 ± 10.9 (35- 76) (n=100)	46.6 ± 8.7 (32.0-64.1) (n=21)
	D µm	86.0 ± 5.1 (76.0 - 93.0)	72.5 ± 3.8 (63.8 - 77.4)	44.5 (32.5 - 52.0)	58.5 (45.5 - 65.0)	81 ± 6.3 (68-95)	75.7 ± 6.0 (60.9- 92.3) (n= 112)	54.8 ± 5.7 (49.1- 60.5) (n=20)	50.0 ± 3.0 (45- 56)	40.8 ± 8.2 (31- 67) (n=123)	40.5 ± 4.8 (36.4 - 51.1) (n=14)
	W µm	77.0 ± 3.7 (65.0 - 84.0)	63.4 ± 5 (55.2–73.8)	38.5 (32.5–52.0)	48.0 (40.0–52.0)	78 ± 7.5 (60-95)	78.7 ± 6.6 (54.7 - 90.8) (n= 112)	42.5 ± 4.1 (38.4- 46.6) (n=20)	45.0 ± 2.0 (41- 48)	30.5 ± 6.6 (24- 38) (n=60)	41.1 ± 11.9 (11.9 - 41.1) (n=21)
	L:W		0.61	0.83	0.81			1.41	n.d	1.28 (n=10)	1.44 ± 0.21 (1.1 - 1.7) (n=21)
	D:W	1.12	1.14	1.16	1.22		1.02 ± 0.09 (0.8 – 1.2) (n= 112)	1.29	1.2	1.29 (n=48)	
Po plate	**L µm**	7.1 ± 0.8 (6.3–8.6)					7.2 ± 0.7 (6.2 – 8.4) (n=14)	9.9 (Laza-Martínez et al., 2016)	10-12	7.6	8.2 ± 1.4 (5.9 - 11.4) (n=13)
	W µm	6.1 ± 0.4 (5.7–6.8)					5.7 ± 0.7 (4.8 - 7.8) (n=14)	4.6 (Laza-Martínez et al., 2016)	6-7	4.1	3.0 ± 0.7 (2.2–4.4) (n=13)
	L:W	1.18					1.23 ± 0.1 (1.0- 1.5) (n=14)		n.d		3.1 ± 0.9 (1.8–4.4) (n=13)
	Number pores	31 ± 4.1					29 ± 1.6 (27– 33) (n=14)		23-39	29-39	35.3 ± 1.6 (32– 37) (n=13)
	Diameter pores µm	0.45 ± 0.03					0.39 ± 0.09 (0.2– 0.6) (n=197)			0.35 (n=150)	0.31 ± 0.08 (0.16 - 0.51) (n=52)
2’’’’ antapical	**L 2’’’’ µm**	54 ± 3.1					41.5 ± 4.8 (33.5 - 48.8) (n=14)		33-39		45.3 ± 2.9 (41.7– 48.8)
	W 2’’’’ µm	27 ± 2.7					21.6 ± 3.3 (17.7 - 29.6) (n=14)		19-23		23.7 ± 3.4 (19.4 - 29.6)
	L:W 2’’’’	2.10					1.95 ± 0.28 (1.6 - 2.5) (n=14)				1.95 ± 0.32 (1.7 - 2.5) (n=7)

Table 2

	IRTA-SMM-17-162 ***G. australes***	**IRTA-SMM-17-189** ***G. australes***	**IRTA-SMM-17-271** ***G. australes***	**IRTA-SMM-17-209** ***F. paulensis***
Max. conc.	2288	1451	1244	1004
r	0.12 ± 0.04 (13-20)	0.15 ± 0.04 (13-20)	0.16 ± 0.04 (12-19)	0.24 ± 0.06 (7-14)
K (Eq. 1)	0.17 ± 0.05	0.21 ± 0.06	0.24 ± 0.06	0.34 ± 0.09
Td (Eq. 2)	6.25 ± 1.80	5.85 ± 1.07	4.36 ± 1.01	1.30 ± 1.64

Table 3

Species	Code	Island	cell abundance (cells ·mL^{-1})	CTX-like toxicity (fg CTX1B equiv. cell^{-1})	MTX-like Toxicity
G. australes	IRTA-SMM-17-153	Majorca	1750	1.38 ± 0.66	+
G. australes	IRTA-SMM-17-238	Majorca	1632	3.52 ± 0.18	NT
G. australes	IRTA-SMM-17-180	Minorca	613	5.25 ± 0.59	NT
G. australes	IRTA-SMM-17-218	Majorca	1686	9.47 ± 3.18	+
G. australes	IRTA-SMM-17-216[a]	Majorca	1476	13.14 ± 4.50	+
G. australes	IRTA-SMM-17-254	Majorca	1273	13.16 ± 1.34	+
G. australes	IRTA-SMM-17-253	Majorca	1935	13.45 ± 0.97	+
G. australes	IRTA-SMM-17-181	Minorca	1464	13.50 ± 0.80	+
G. australes	IRTA-SMM-17-178	Minorca	2040	14.52 ± 4.31	-
G. australes	IRTA-SMM-17-223	Majorca	1183	14.93 ± 4.69	+
G. australes	IRTA-SMM-17-155	Minorca	332	17.33 ± 1.60	-
G. australes	IRTA-SMM-17-173	Majorca	2087	21.89 ± 9.20	+
G. australes	IRTA-SMM-17-244	Majorca	924	34.33 ± 4.18	+
G. australes	IRTA-SMM-17-256	Majorca	1004	39.17 ± 16.44	NT
G. australes	IRTA-SMM-17-175	Minorca	1498	62.00 ± 0.66	-
G. australes	IRTA-SMM-17-164	Minorca	1022	72.60 ± 43.20	+
G. australes	IRTA-SMM-17-214	Majorca	1694	76.67 ± 29,86	+
G. australes	IRTA-SMM-17-189	Minorca	869	83.39 ± 12.14	NT
G. australes	IRTA-SMM-17-162	Minorca	1390	105.67 ± 18.27	NT
G. australes	IRTA-SMM-17-271	Minorca	843	172.63 ± 5.57	+
G. australes	IRTA-SMM-17-168[a]	Majorca	2274	381.83 ± 91.84	NT
F. paulensis	IRTA-SMM-17-209[a]	Minorca	782	16.30 ± 1.67	NT
F. paulensis	IRTA-SMM-17-211	Minorca	3250	7.96 ± 0.14	NT
F. paulensis	IRTA-SMM-17-198[a]	Majorca	4825	n.s	NT
F. paulensis	IRTA-SMM-17-206	Majorca	2053	n.s	-
F. paulensis	IRTA-SMM-17-220	Minorca	2128	n.s	-
F. paulensis	IRTA-SMM-17-221	Minorca	6636	n.s	NT

[a] response curves of CTX-like evaluation of these strains are shown in Fig. 6.

Supplementary Material figure S1