

Identification of *Gambierdiscus* species from La Réunion and evaluation of toxicity and toxin profile

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ABSTRACT

Five different species of *Gambierdiscus* have been identified in La Réunion (*G. belizeanus*, *G. balechii*, *G. pacificus*, *G. silvae* and *G. ribotype* 2) by morphological observations in Scanning Electron Microscopy (SEM) and molecular identification and phylogenetic analysis. Growth rates of cultures have also been evaluated showing values from 0.09 to 0.36 d⁻¹.

The toxicity and toxin profile of thirteen strains have been analysed by a multidisciplinary approach with Neuro-2a cell-based assay (CBA), magnetic bead-based immunoassay, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and LC coupled to high-resolution mass spectrometry (LC-HRMS). *G. balechii* showed the highest toxicity by CBA (~627 fg equiv. CTX1B-cell⁻¹) followed by *G. ribotype* 2 (76 to 13 fg equiv. CTX1B-cell⁻¹), *G. balechii* (63 to 7 fg equiv. CTX1B-cell⁻¹), *G. belizeanus* (30 to 20 fg equiv. CTX1B-cell⁻¹) and *G. pacificus* with values close to LOQ but not conclusive. The toxin profile for the 13 strains was evaluated by LC-MS/MS using seven different methods and being gambierone and 44-methylgambierone the two only known compounds, found in high concentrations in all samples. Gambierone was detected from 2.05 pg·cell⁻¹ in *G. balechii* (P-0414B) to 12.91 pg·cell⁻¹ in *G. balechii* (P-0414A) and 44-methylgambierone was detected from 1.93 pg·cell⁻¹ in *G. belizeanus* (P-0414B) to 14.95 pg·cell⁻¹ in *G. pacificus* (P-0304). These samples were analysed also by LC-HRMS, confirming gambierone and 44-methylgambierone the main compounds detected. Additionally, a potential polyether sulphur-containing compound corresponding to the novel molecular formula C₆₂H₉₄O₂₃S ([M+NH₄]⁺, m/z 1256.6234) were tentatively identified.

This study combining morphological and molecular data is the first to mention such diversity in the area. It is also the first time that toxicity and toxin profile of *Gambierdiscus* from La Réunion have been evaluated.

1. Introduction

Ciguatera poisoning (CP) is one of the most common foodborne

illnesses associated with finfish consumption and characterised by gastrointestinal, cardiological, mild to severe and long-lasting neurological symptoms (Chinain et al., 1999; FAO and WHO, 2020) and

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paradoxical dysesthesia (temperature reversal) (Bagnis et al., 1979). This disease is traditionally linked from tropical and subtropical regions, but during recent decades, CP cases have increased (Llewellyn, 2010; Tester et al., 2010) and they have appeared in temperate zones through the importation of tropical ciguateric fish (Farrel et al., 2019) or by consumption of local ciguateric fish (Bravo et al., 2015; Chinain et al., 2019). Although reliable sources of quantification are lacking regarding CP cases, CP represents an ongoing global public health hazard causing approximately 10,000–50,000 cases annually (Dickey and Plakas, 2010; Friedman et al., 2017; World Health Organization, 2020) and are greatly under-reported due to a lack of diagnostics tools (Chinain et al., 2021).

Species of benthic dinoflagellates have been widely studied because of their role in producing neurotoxins and for being recognized as toxin producers linked to CP (Murray et al., 2020). Toxins or toxin precursors produced by benthic dinoflagellates enter in the food webs through invertebrates, herbivorous and omnivorous fishes (Munday et al., 2017; Soliño and Costa, 2020; Holmes et al., 2021). Although fishes are the primary source of CP, molluscs, crustaceans, and echinoderms are also involved in CP outbreaks but to a minor extent (Munday et al., 2017; Holmes et al., 2021). Climate change could change the geographical and temporal distribution of these dinoflagellates. In addition, the migration patterns of ciguateric fish are contributing to the geographical expansion of CP and increasing population densities of CTX-producing microalgae species in temperate areas (Nishimura et al., 2013; Tester et al., 2013).

Gambierdiscus and *Fukuyoa* are epibenthic dinoflagellates, well known in tropical reef areas where they are found living in association with macroalgae and corals, or attached to the benthos (Parsons et al., 2012; Hoppenrath et al., 2014; Darius et al., 2022). Their presence in temperate environments is increasing (Llewellyn, 2010; Rhodes et al., 2014a; Tester et al., 2018). Furthermore, with the help of morphological characteristics and phylogenetic analysis, 19 different *Gambierdiscus* species have been identified worldwide (Fraga et al., 2016; Smith et al., 2016; Kretzschmar et al., 2017; Kretzschmar et al., 2019; Rhodes et al., 2017; FAO and WHO, 2020; Guiry and Guiry, 2020): *G. australes*, *G. balechii*, *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. cheloniae*, *G. excentricus*, *G. holmesii*, *G. honu*, *G. jejuensis*, *G. lapillus*, *G. lewisii*, *G. pacificus*, *G. polynesiensis*, *G. scabrosus*, *G. silvae*, *G. toxicus* and *G. vietnamensis* (Smith et al., 2016; Rhodes et al., 2016; Rhodes et al., 2017; Kretzschmar et al., 2017; Fraga et al., 2016; Nguyen-Ngoc et al., 2023; Murray et al., 2024). *Fukuyoa* includes four species: *F. koreansis*, *F. paulensis*, *F. ruetzleri* and *F. yasumotoi* (Gómez et al., 2015; Leung et al., 2018; Li et al., 2021). The two toxin groups produced by these dinoflagellates are ciguatoxins (CTXs) (Roue et al., 2018; Sibat et al., 2018; Longo et al., 2019, 2020; T. Yon et al., 2021) (Figure S1a-d) and maitotoxins (MTXs) (Yasumoto and Murata, 1993; Pisapia et al., 2017; Mazzola et al., 2019, 2020; Estevez et al., 2021) (Figures S1-e). Other compounds produced by *Gambierdiscus* and *Fukuyoa* are gambierones (Figure S1-f) (Estevez et al., 2020; Tibirićá et al., 2020; T. Yon et al., 2021; Murray et al., 2021; Mudge et al., 2022; Malto et al., 2022), gambierol (Satake et al., 1993; Cagide et al., 2011) (Figure S1-g), gambieroxide (Watanabe et al., 2013) (Figures S1-h) and gambieric acids (Nagai et al., 1992, 1992b) (Figure S1-i).

CTXs were first reported in the Pacific Ocean (Hawai, French Polynesia) and in the Caribbean (Dickey et al., 1995; Crouch et al., 1995; Pottier et al., 2023), and since these have been reported globally, in new sites such as the Canary Islands (Boada et al., 2010; Sánchez-Henao et al., 2019; Ramos-Sosa et al., 2022). Japan, and Western Gulf of Mexico (Rhodes et al., 2010; Saburova et al., 2013; Uddin et al., 2014; Manche et al., 2014; WHO, 2020), Manora Channel, Pakistan (Munir et al., 2011), Red Sea (Saburova et al., 2013; Uddin et al., 2014; Manche et al., 2014; Catania et al., 2017), Gulf of Aqaba (Saburova et al., 2013), and Indian Ocean (such as the Arabian Sea (Saburova et al., 2013; Uddin et al., 2014; Manche et al., 2014). CTXs are lipophilic (fat-soluble) ladder-shaped polyether marine toxins that are odourless, tasteless, and heat-stable (Lewis, 2001; Friedman et al., 2008). Their structure is

characterized by a long, rigid ladder-like backbone made up of 13 to 14 trans-fused ether rings (typically tetrahydropyran or tetrahydrofuran units) arranged in a continuous, cyclic polyether framework. When these enter the food webs, they can be biotransformed and bio-accumulated in fish. To date, more than 50 CTX analogues have been identified from fishes and benthic dinoflagellates (FAO and WHO, 2020). The group of CTXs traditionally associated to the Pacific Ocean, namely P-CTXs (Satake et al., 1998; Yasumoto et al., 2000), are actually sub-divided into two separate groups (CTX3C-group and CTX4A-group), based on the algal precursors leading to the congeners of the respective groups and their chemical structures (Murata et al., 1990; Satake et al., 1993; Satake et al., 1998; Yasumoto et al., 2000) (Figure S1a-c). The Caribbean CTXs represent the third group (C-CTX1-group) (Vernoux and Lewis, 1997) and the Indian CTXs represent the fourth group (I-CTX1-group) (B. Hamilton et al., 2002; Hamilton et al., 2002b; Soliño and Costa, 2018; Chinain et al., 2020) (Figure S1-d). CTXs interact activating the voltage-gated sodium channels (VGSCs) of cells, resulting in an increase in intracellular sodium, leading to persistent sodium influx and disruption of normal action potential generation and propagation (Molgó et al., 1993; Nicholson and Lewis, 2006). The complex toxin profile of *G. polynesiensis* has so far mostly been characterised with liquid chromatography (LC) coupled to low resolution mass spectrometry, and 12 CTX analogues have been described from their extracts, being to date, the only species capable to produce Pacific ciguatoxin-3B (P-CTX3B), P-CTX3C, isoP-CTX3B/C, P-CTX4A, P-CTX4B and isoP-CTX4 (A/B) (Taiana Darius H. et al., 2022; Longo et al., 2019; Chinain et al., 2010; Yasumoto et al., 2000; Roue et al., 2016; Sibat et al., 2018; T. Yon et al., 2021; Murray et al., 2024). In addition to CTXs, *G. polynesiensis* was also reported as producer of gambierone and 44-methylgambierone (Longo et al., 2019; Roue et al., 2018, 2020; Argyle et al., 2016).

Another class of toxins, from these benthic dinoflagellates is the water-soluble MTXs, which may accumulate in the fish digestive tract and liver. However, the presence of at least one sulphate moiety in the MTX backbone leads to significant hydrophilicity of these toxins and low bioaccumulation in fish flesh (Shmukler and Nikishin, 2017). The low oral bioavailability and bioaccumulation of MTXs suggest that these compounds are not contributors to CP symptomatology (Holmes et al., 2021). There are seven described MTX analogues, and their biological activity is varied (Estevez et al., 2020; Estevez et al., 2021). Maitotoxin-1 (MTX1) is characterized by an extensive polyether backbone consisting of 32 fused ether rings, making it a highly oxygenated and rigid molecule. The structure includes multiple hydroxyl groups and sulphate esters, contributing to its extreme polarity and water solubility (Figures S1-i). MTX1 (the most potent non-peptide toxin (Chinain et al., 2020)), maitotoxin-2 (MTX2), maitotoxin-4 (MTX4) and maitotoxin-5 (MTX5) cause massive Ca^{2+} influx leading to cell death (Estevez et al., 2020). Maitotoxin-3 (MTX3) acts on the same biological target as CTXs, although with lower potency (Boente-Juncal et al., 2019), lately identified as 44-methylgambierone (Murray et al., 2019, 2020; Boente-Juncal et al., 2019). The acute toxicity of maitotoxin-6 (MTX6) and maitotoxin-7 (MTX7) was determined in mice using injection and oral administration. The symptoms observed were similar in both analogues, although the onset of symptoms was much faster for MTX7 (Murray et al., 2022). These later MTXs by injection caused intestinal distension and bloody fluid with a pale green fluid and no oral toxicity was observed at the highest dose are administered (Murray et al., 2022). *G. australes* has been described as producer of MTX1, MTX2, 44-methylgambierone and MTX5, however, not all of them have been found in the same extract from the same culture (Lewis et al., 1994; Pisapia et al., 2017; Estevez et al., 2021), MTX4 is produced by *G. excentricus* from the Canary Islands (Estevez et al., 2021). *G. cheloniae* produced MTX6 (Murray et al., 2022) and *G. honu* produced MTX7 (Murray et al., 2022).

The hydrophilic gambierones (Rodríguez et al., 2015; Murray et al., 2019; T. Yon et al., 2021; Liu et al., 2023) consist of nine continuous trans-fused ether rings and are either mono- or di-sulphated. Some analogues have a terminal diol, 1,3-diene, saturated double bonds and/or

are deoxygenated (Liu et al., 2023). The production of 44-methylgambierone is ubiquitous to all *Gambierdiscus* species tested to date (Murray et al., 2020), while only some of these species produce gambierone (Rodríguez et al., 2015). Boente-Juncal et al. (2019) described similar activities of both gambierone and 44-methylgambierone inducing a small increase in the cytosolic calcium concentration but only 44-methylgambierone caused cell cytotoxicity at micromolar concentrations. Other metabolites produced by *Gambierdiscus* include gambieroxide (Watanabe et al., 2013), a metabolite with twelve continuous trans-fused ether rings, a sulphate ester, an epoxide and an olefinic sidechain (Watanabe et al., 2013) (no toxicity information available); which was detected in *G. lewisii* and *G. pacificus* (Murray et al., 2024). Morohashi et al. (1999) is an analogue with eight continuous trans-fused ether rings and a heptatriene sidechain that displays potent neurotoxicity against mice when administered by injection, with neurological symptoms like those exhibited in CP events. Four analogues of gambieric acids have been characterised: gambieric acid A and B and the 3-methylhemiglutamate forms, and gambieric acid C and D, each containing a carboxylic acid functionality (Morohashi et al., 2000; Diogene and Campas, 2018). These compounds displayed strong antifungal activity (Soliño et al., 2018). Gambieric acid A was detected in ten *Gambierdiscus* species, with *G. australes* (CAWD381) being the only isolate to produce gambieric acids A–D (Murray et al., 2024). Although these other polyether toxins are not as toxic as CTXs, they are structurally related to CTXs and some research suggests gambierones that it is essential in determining if they contribute to CP (Murray et al., 2021).

Traditionally, the analysis of CTXs has employed a mouse bioassay that was first detailed in the late 1960s (Scheuer et al., 1967). Other in vivo assays have been developed using a variety of animal species that include mongooses (Banner et al., 1960), rats, frogs and chickens (Hashimoto and Yasumoto, 1965), cats (Hashimoto et al., 1969) and guinea pigs (Kimura et al., 1982), showing different levels of success. Cell-based assays (CBAs) (Dechraoui et al., 2005; Abraham et al., 2012; Caillaud et al., 2010; Lewis et al., 2016; Pisapaia et al., 2017a; Reverte et al., 2018; Costa et al., 2021; Tudó et al., 2020;) and receptor binding assays (RBAs) (Darius et al., 2007; Hardison et al., 2016) were used as effective methods for monitoring the toxicity of fish and *Gambierdiscus*. The simplicity and low-cost of immunoassay, in addition to the high throughput sample processing, make it an effective method for detecting CTX compounds based on their structure (Tsumuraya et al., 2018; Gaiani et al., 2020; Leonardo et al., 2020; Pasinszki et al., 2020; Perkins et al., 2024). The unequivocal identification of toxins responsible of CP has been conducted with as liquid chromatography-mass spectrometry (LC-MS/MS) (Lewis et al., 2009; Wu et al., 2011; Yogi et al., 2011; Pasinszki et al., 2020; Estevez et al., 2019; Oshiro et al., 2021; Spielmeier et al., 2021, 2022; Estevez et al., 2023; Murray et al., 2024; Perkins et al., 2024). These methods require efficient and efficacious sample extraction techniques, to optimise extraction and concentration of toxins, and remove matrix-derived co-extractives that negatively impact sample analysis (Murray et al., 2018).

Gambierdiscus are endemic to the Atlantic (including the Caribbean and Gulf of Mexico) or to the tropical Pacific (Litaker R.W. et al. 2010). In the Canary Islands (Northeast Atlantic), at least five to six species have been identified (Rodríguez et al., 2017). Furthermore, *Gambierdiscus* species have been documented in temperate waters beyond the tropics, including parts of Australia, Japan and notably in the Mediterranean Sea, suggesting that environmental warming may allow *Gambierdiscus* expansion (Larsson et al. 2018; Wang et al., 2022; Tudó et al. 2022). Although until recently the records of *Gambierdiscus* in the Indian Ocean were scarce and restricted to the western tropical region, whereas now its presence has been recorded in the northern part of the ocean (Saburova et al., 2013; Habibi et al., 2021). Furthermore, toxicities and toxin profiles of *Gambierdiscus* have been poorly documented in the Indian Ocean including the Bay of Bengal, Andaman Sea, the Gulf or La Réunion (Habibi et al., 2021). In this work, a multidisciplinary study on the toxicities by Neuro-2a CBA, the CTX-like compounds by magnetic

bead-based immunoassay and the toxin profile by LC-MS/MS and LC-high-resolution mass spectrometry (HRMS) were implemented for 13 cultures of different *Gambierdiscus* species from La Réunion.

2. Material and methods

2.1. Standards and reagents

The standard solutions used for LC-MS/MS and LC–HRMS analysis used were: CTX1B, CTX3B, CTX3C, 2,3-dihydroxyCTX3C, M-secoCTX3C, and CTX4A were kindly supplied by Dr. Mireille Chinain from Institute Louis Malardé (ILM, Tahiti). Maitotoxin (MTX1) was purchased from WAKO (Richmond, USA). 44-methylgambierone and gambierone were purchased from CIFGA (Lugo, Spain). CTX1B, 52-*epi*-54-deoxyCTX1B (P-CTX2) and 54-deoxyCTX1B (P-CTX3) were purchased from Professor R. J. Lewis (The Queensland University, Brisbane, Australia). 51-hydroxyCTX3C was a gift of Dr. Takeshi Yasumoto from JFRL (Japan Food Research Laboratories, Tokyo, Japan). Different internal reference materials were used: I-CTX1/2 and I-CTX3/4 from a shark described in Diogene et al. (2017), C–CTX1 from a *Seriola* sp. within Eurocigua I project, and C–CTX1/2 and C–CTX3/4 analogues as a gift from Prof. Alison Robertson (University of South Alabama, USA). From the CIGUARISK project were used: MTX4 from a *G. excentricus* culture, MTX5, gambieric acid C and gambieric acid D from a *G. australes* culture. All methanolic extracts and standard solutions were stored in glass vials at -20°C . Mixed standards solutions were prepared in methanol LC-MS grade. The CTX1B standard solution from Prof. R. J. Lewis was used for Neuro-2a CBA and immunoassay quantifications. Gambierone, 44-methylgambierone and CTX1B standard solution from ILM were used for LC-MS/MS quantification purposes. The remain standards and internal reference materials were used to LC-MS/MS identification and retention time confirmation.

Neuroblastoma murine (Neuro-2a) cells (cell line CCL-131) were purchased from ATCC LGC standards (Manassas, VA, USA). Poly-L-lysine, foetal bovine serum (FBS), L-glutamine solution, ouabain (O), veratridine (V), phosphate buffered saline (PBS), penicillin, streptomycin, RPMI-1640 medium, sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT) and hexane, diethyl ether, acetone, methanol, water, and ethyl acetate used for sample preparation were HPLC grade (Merck KgaA, Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from Honeywell (Badalona, Spain).

Dynabeads M-270 Carboxylic Acid, containing 2×10^9 beads·mL⁻¹, were obtained from Invitrogen (Life Technologies, S.A., Alcobendas, Spain). Potassium phosphate monobasic, potassium phosphate dibasic, Tween-20, bovine serum albumin (BSA), 4-morpholineethanesulfonic acid (MES) hydrate, N-(3-(dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). PolyHRP-streptavidin was obtained from Thermo Fisher (Barcelona, Spain). The 3G8, 10C9 and 8H4 monoclonal antibodies (mAb) were produced by immunising mice with synthetic haptens mimicking the wings of CTXs from the CTX1B and CTX3C series (Oguri et al., 2003; Tsumuraya et al., 2006, 2012).

Acetonitrile and water were LC-MS grade (Merck KgaA, Darmstadt, Germany), ammonium formate and formic acid used in mobile phases were LC-MS grade (Sigma Aldrich) (Saint Quentin Fallavier, France).

2.2. Sampling, cell isolation and initial culturing of *Gambierdiscus*

Sampling was carried out in different coastal sites on La Réunion (Fig. 1). The sampled substrate corresponded to macroalgae, rocks or dead corals colonized with biofilm or turf, and was collected at a maximal depth of 1.5 m by snorkelling or walking in small puddles. The substrate was inserted underwater into 500-mL plastic bottles, filled with the surrounding water. Once collected, the sample was immediately processed back on the beach in order to detach the benthic cells

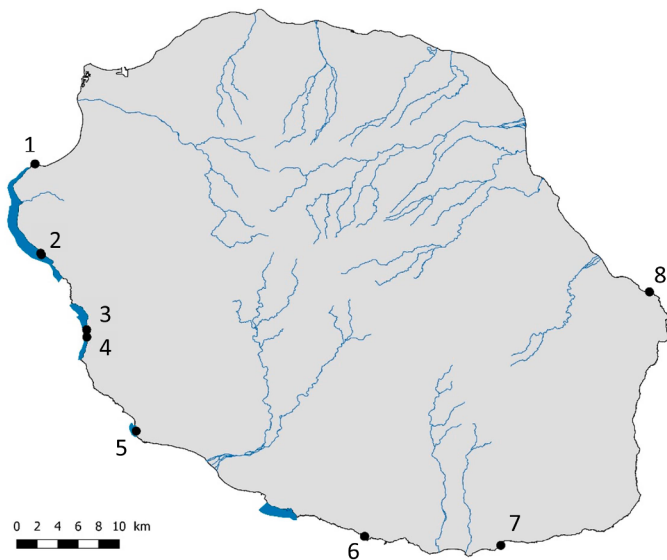


Fig. 1. Location of sampling sites in La Réunion (black dots). The blue areas correspond to back reef depression: 1) Cap Lahoussaye (rocky pools), 2) Ermitage (back reef), 3) Saint Leu North (back reef), 4) Saint Leu South (back reef), 5) Etang-Salé (back reef), 6) Grande Anse (rocky pool), 7) Vincenzo Cap Poisson d'Ail (rocky pools), 8) Point Corail (rocky pools).

from the substrate. The substrate was removed from the sampling bottle after shaking it vigorously for 2 min to detach the cells. The content of the bottle was then filtered through a first sieve (200- μm mesh size) to remove organic matter and the biggest zooplanktonic organisms, and through a 20- μm mesh size sieve in order to collect the targeted *Gambierdiscus* cells. The second sieve was washed using a squeeze bottle with filtered seawater of the sampling site to collect the cells in a bottle filled with 150 mL of filtered seawater. At the laboratory, the samples were examined under the microscope and live *Gambierdiscus* cells were isolated using glass Pasteur pipettes with tips drawn to produce micropipettes. After 3 successive washings in autoclaved seawater droplets, cells were individually transferred into 6-well culture plates with K medium for initiating monoclonal cultures. K medium was prepared with offshore natural seawater (pH = 8.3, on average), previously aged for at least 1 month and filtered through 0.2- μm GF/F Whatman filters (HF Scientific, France) before being autoclaved at 121 °C during 25 min. When isolation was successful (no contaminants, regular cell division, increasing density), the strains were transferred to autoclaved test tubes with K medium, and the cultures were maintained at CITEB microalgae collection Phytobank, at 26 ± 1 °C under 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ irradiance (12:12 light cycle).

2.3. Growth rate estimation

In order to determine the maximum growth rate during the exponential growth phase, the strains were grown in 100-mL Erlenmeyer flasks in sterile K medium (Keller et al., 1987), inoculated to obtain a final concentration of 50 cells $\cdot\text{mL}^{-1}$. Enumeration of the cultures was performed in triplicate by counting *Gambierdiscus* cells on the microscope using 1-mL calibrated Sedgewick-Rafter, every 3 or 4 days. Maximum growth rate (μ_{max} , divisions day^{-1}) was calculated as follows:

$$\mu_{\text{max}} (\text{divisions day}^{-1}) = \mu_{\text{max}} (\text{d}^{-1}) \cdot \ln(2)^{-1} \quad (1)$$

2.4. Identification of strains by molecular sequencing and scanning electron microscope

DNA extraction was performed using PCRbio Rapid extract PCR kit (Eurobio), which combines direct extraction and amplification in the

same tube. The pair of primers selected were D8 (5'-GGATTGGCTCT-GAGGGTTGGG-3') and RB (5'-GATAGGAAGAGCCGACATCGA-3') (~900pb) (Chinain et al., 1999). Obtained amplicons were screened on agarose gel at 1 % and then sequenced by the Sanger method. The sequences were aligned using the BioEdit software. However, due to polymorphism or the occurrence of pseudogenes in some strains, the sequences were not readily exploitable, and a different approach was necessary.

To fix this problem, a “single molecule” sequencing method, such as that developed by Oxford Nanopore Technologies (ONT), was selected and the problematic strains were sequenced using a MinION (ONT) to produce a reference sequence. A new PCR was performed using Long-Amp Hotstart polymerase 2 $\times$ (New England Biolabs, Ipswich, MA, USA) with primers ITSFW (5'-GTAGGTGAACCTGCGGAAGG-3') and RB (5'-GATAGGAAGAGCCGACATCGA-3'). Cycling conditions were as follows: an initial denaturation at 94 °C (30 sec), followed by 30 cycles of the following 3 steps: 94 °C (30 sec), 53 °C (40 sec), and 65 °C (6 min) and, finally, an elongation step of 65 °C (10 min). The PCR products were analysed with the Bioanalyser (Agilent, Santa Clara, CA, USA) in order to display the size of the obtained amplicons. The native barcoding protocol (SQK-NBD112.24) was then used to multiplex the samples and prepare the library subsequently sequenced with kit chemistry 9 and R9 flowcell. Sequences produced were processed by a bioinformatics pipeline Esperanto (<https://github.com/MercierEva/Esperanto>), which allowed to extract the D8-D10 area, used for the identification.

A molecular phylogenetic analysis was inferred from sequences of the D8-D10 domains of the LSU of 75 dinoflagellate species based including 69 sequences of *Gambierdiscus* species, 3 of *Fukuyoa* species and 1 of *Akashiwo*, *Alexandrium*, and *Prorocentrum* as outgroup. Sequences were aligned using MAFFT v. 7.4 algorithm. The final alignment included 965 positions. In order to select the most appropriate model of sequence evolution, the dataset has been processed with jModeltest2 v. 2.1.7 (Darriba et al., 2012) and the General Time Reversible model with invariant sites and gamma distribution (GTR+I + G) was selected. Phylogenetic reconstruction was assessed by two methods. Maximum Likelihood analysis (ML) was performed using PHY-ML v. 3 software (Guindon et al., 2010), and a bootstrap analysis (1000 pseudo replicates) was used to estimate the relative robustness of branches of the ML tree. Bayesian Inference (BI) analysis was realized using MrBayes 3.1.2 software (Ronquist and Huelsenbeck, 2003). Parameters of the models for ML and BI analyses are given in Table S2.

In order to confirm molecular identifications by morphological examination, Scanning Electron Microscopy (SEM) was performed on the different strains. Lugol fixed samples of the cultures were used. A small volume of dense culture was filtered on a polycarbonate membrane with 10- μm pores (Millipore Isopore TCTP) and processed according to Chomérat & Couté (2008). Cells were dehydrated in a graded series of increasing ethanol concentration (15 %, 30 %, 50 %, 70 %, 90 %, 95 %, 99 %, and several baths of absolute ethanol). Then, cells were critical point dried with liquid CO_2 using Quorum EMS850 CPD and coated with gold with a Cressington 108 Auto sputter coater. Observations were performed with a Zeiss Sigma 300 field emission SEM at an acceleration of 1.5 kV.

2.5. Biomass production and harvesting

To produce the biomass required for the further analysis of toxins, the strains were grown in 1-L Erlenmeyer flask in sterile K medium, inoculated to obtain a final concentration of 50 cells $\cdot\text{mL}^{-1}$. The cell density was monitored on the microscope as described above in order to identify the growth phases. Harvesting was performed after 22 to 26 days of culture, at exponential or stationary phase depending on the strain. Before harvesting, an aliquot of 15 mL of culture was sampled and fixed with 3 % Lugol's iodine solution for cell counting under the light microscope to calculate the cellular concentration at harvesting. The remaining volume (985 mL) was centrifuged in several runs in 50-mL

Falcon tubes at 3500 x g during 10 min (Heraeus Megafuge™ 1.0R, Thermo Scientific, Massachusetts, USA). After removing the supernatant, the harvested biomass was weighed in calibrated tubes and stocked at -20°C until extraction.

2.6. *Gambierdiscus* toxin extraction

To obtain the extracts, the frozen biomass of each strain was resuspended in MeOH (10 mL per 1 million cells), then disrupted using sonication during 30 min at 38 % of total power, followed by a centrifugation at 600 x g for 5 min at 4°C . The supernatant was retrieved in a calibrated balloon and the sequence was repeated once. The pellet was then resuspended in MeOH: H₂O (1:1, v/v), sonicated and centrifuged as previously described. The supernatants were then pooled, evaporated at 40°C , weighed and resuspended in MeOH. After a last centrifugation at 600 x g for 10 min, the supernatant was retrieved using a syringe and filtered on a 0.45- μm nylon filter, then it was stocked at -20°C in 25-mL glass bottles. Unfortunately, the strain *G. silvae* (P-0422) shown a very low growth rate and the culture did not reach the number of cells necessary for subsequent extraction and analysis.

2.7. Toxin analysis

2.7.1. Analysis of CTX-like toxicity by neuro-2a CBA

Neuro-2a CBA cells were maintained in RPMI-1640 media supplemented with 10 % (v/v) FBS, 1 % (v/v) penicillin-streptomycin, and 1 % (v/v) sodium pyruvate. All incubation steps were conducted at 37°C in a 5 % CO₂ humid atmosphere. The CBA was performed as previously described (Caillaud et al., 2012; Soliño et al., 2015). Initially, cells were trypsinised and seeded in a 96-well microplate at 34,000 cells/well with 200 μL of culture media containing 5 % (v/v) FBS. Cells were then incubated for 24 h. Prior to extract exposure, half of the wells were treated with 20 μL of an O and V mixture in PBS at 0.12 and 0.012 mM, respectively. Then, *Gambierdiscus* extracts were dried, reconstituted in culture media, $\frac{1}{2}$ serially diluted, and 10 μL of each dilution was added into the wells with and without O/V pretreatment. After a 24 h incubation, cell viability was measured using the MTT assay ((Manger et al., 1993). Full dose-response curves were constructed using different concentrations of CTX1B ranging from 12.5 to 0.10 $\text{pg}\cdot\text{mL}^{-1}$. *Gambierdiscus* extracts were analysed at different cell equiv. concentrations ranging from 0.23 to 257 cells equiv. $\cdot\text{mL}^{-1}$. Measurements were performed in triplicate. Absorbance was measured at 570 nm using an automated plate spectrophotometer (Synergy HT, Biotek, USA). For every assay, a calibration curve of cell viability with CTX1B standard was constructed. 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 % (IC20).

2.7.2. Analysis of CTX-like compounds by magnetic bead-based immunoassay

Gambierdiscus extracts were analysed using a single-step magnetic bead (MB)-based sandwich immunoassay (Reverté et al., 2025), which is a simplified version of a previously developed assay for CTXs (Leonardo et al., 2020; Gaiani et al., 2020). Briefly, the 3G8 and 10C9 mAbs were immobilised on MBs using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-Hydroxysuccinimide (EDC/NHS) chemistry, resulting in two separate conjugates: 3G8-MB and 10C9-MB. For the assay, 35 μL of each conjugate were combined in a tube, and the supernatant was discarded. Then, 30 μL of either CTX1B standard solution or *Gambierdiscus* extract, previously evaporated and resuspended in PBST-BSA (0.1 M PBS, 0.05 % Tween-20 (v/v), 2 % BSA (w/v), pH 7.2), was added to the tube along with 5 μL of PBST-BSA containing biotinylated 8H4 mAb and polyHRP-streptavidin, both at 2 $\mu\text{g}\cdot\text{mL}^{-1}$. After a 30 min of incubation, the MBs were washed with PBST, and 10 μL of the immunoconjugates were transferred to a new tube for colorimetric measurement at 620 nm

following the addition of TMB. All incubations were performed at room temperature and with slow tilt rotation. Measurements were performed in triplicate. Dose-response curves were blank-subtracted and fitted to a sigmoidal logistic four-parameter equation using SigmaPlot software 12.0. The LOQ was calculated using 10 times the standard deviation of the blank value (no CTX) and was determined to be 0.01 fg CTX1B cell⁻¹.

2.7.3. Analysis of CTX, MTX and other related compounds by LC-MS/MS

The LC-MS/MS analysis was conducted using an Acquity UPLC I-Class coupled to a Xevo TQ-XS triple quadrupole mass spectrometer (Waters, Milford, MA, US.). The chromatographic conditions were performed as described in Barreiro-Crespo et al. (2025). Briefly, the chromatographic separation is performed on a reversed-phase BEH C18 (50 mm $\times$ 2.1 mm, 1.7 μm) (Waters) at a flow rate of 400 $\mu\text{L}\cdot\text{min}^{-1}$. Mobile phase A was water and mobile phase B was acetonitrile/water (95:5), both containing 2 mM ammonium formate and 0.1 % formic acid.

Seven LC-MS/MS analysis methods with different transitions were used for CTX identification and confirmatory purposes (Table S4 to S10) in order to cover the main congeners of CTX4A-group and CTX3C-group, C-CTX-group I-CTX-group, gambierone-group and MTX-group. The MS/MS transitions for the MTXs were determined from the injection experiment of MTX1 standard at different cone voltage and collision energy and cone voltage.

The analyses were carried out in positive and negative electrospray ionisation (ESI+ and ESI-) mode. Ion source parameters were performed as described in Barreiro-Crespo et al. (2025) and they were common to both ionization mode. In addition, the voltage parameters in ESI+ and ESI- were: capillary voltage 3.0 kV and 2.0 kV and cone voltage 63 V and 23 V, respectively. The software TargetLynx (Waters) was used for data acquisition and processing.

2.7.4. Analysis of CTX, MTX and other related compounds by LC-HRMS

For the analysis by LC-HRMS, an Orbitrap-Exactive HCD mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with heated electrospray source (H-ESI II), a Surveyor MS Plus pump and an Accela Open AS auto-sampler at 15°C (Thermo Fisher Scientific) were used. The chromatographic separation, positive electrospray ionization, the ion source parameters, and the resolution was performed as described in Barreiro-Crespo et al. (2025). Briefly, the chromatographic separation is performed on a reversed-phase Hypersil Gold C18 (100 mm $\times$ 2.1 mm, 1.9 μm) (Thermo Fisher, Scientific, Bremen, Germany) at a flow rate of 250 $\mu\text{L}\cdot\text{min}^{-1}$. Mobile phase A was water, and B was acetonitrile/water (95:5), both containing 2 mM ammonium formate and 0.1 % formic acid.

The analyses were carried out in positive and negative electrospray ionisation (ESI+ and ESI-) mode, and the instrument was calibrated daily in both ionization modes. In full scan acquisition mode, the mass range were 400–1500 and 600–3800 m/z in ESI+ and ESI-, respectively. In addition, the voltage parameters in ESI+ and ESI- were, respectively: spray voltage of 4.0 and -3.5 kV, capillary voltage of 47.5 and -95.0 V, tube lens voltage of 186 and -190 V and skimmer voltage of 18 and -46 V. The software Xcalibur 2.2 SP1 (Thermo Fisher Scientific) was used for data processing.

The sum of exact mass of $[M + H - H_2O]^+$, $[M + H]^+$, $[M + NH_4]^+$ and $[M + Na]^+$ peaks were extracted from the chromatogram as diagnostic and confirming ions using ± 10 ppm of mass accuracy extraction window. For ESI-, $[M - H]^-$ was monitored. In addition to retention time, mass accuracy and HRMS parameters for accurate mass measurements (AMM) to be confident in the identification and the proposed elemental formula, the described restrictive criteria were used: for CTXs molecular formula and adducts [C: 55 to 70, H: 64 to 110, O: 11 to 25, N: 0 to 1, S: 0 to 1, and cations (Na): 0 to 1]; and MTXs molecular formula [C: 157 to 165, H: 241 to 258, O: 65 to 68, N: 0 to 1, S: 0 to 2, and cations (Na): 0 to 2]; the isotopic pattern was matched to theoretical in silico approach and the ring double bond equivalents (RDBEs), the charge and nitrogen rule were taken into account.

Two selected extracts (one *G. balechii* (P-0370) and one *G. belizeanus* (P-0398)) were also analysed using a UHPLC Vanquish Flex coupled to a Orbitrap ID-XTM TribridTM. HRMS² were acquired in the range of m/z 600–1800 in positive and negative ionization mode. The following source settings were used: spray voltage = 3,2 V; capillary temperature

= 250 °C; sheath = 49 and auxiliary gas flow = 10 (arbitrary units). Resolving power was set at 60,000 FWHM (full width at half maximum) for full MS scans and 15,000 FWHM for full MS² scans. MS² experiments used isolation window width of 1 m/z , High Collision Energy (HCD) of 22 %, maximum injection time of 60 ms and resolving power of 35,000

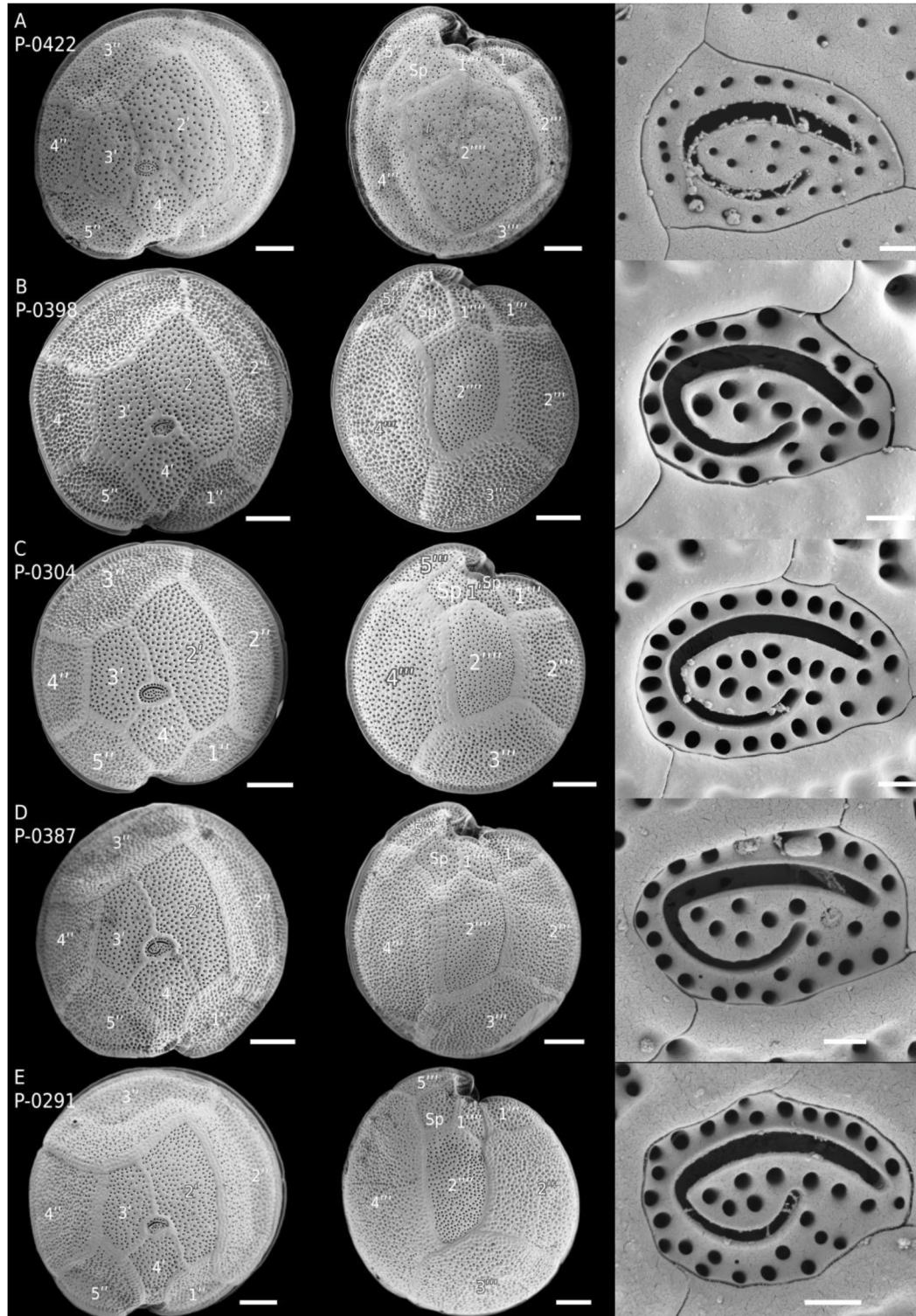


Fig. 2. SEM micrographs of five different strains isolated in the study and identified as separate species. A) *G. silvae* (P-0422), B) *G. belizeanus* (P-0398), C) *G. pacificus* (P-0304), D) *G. ribotype 2* (P-0387), and E) *G. balechii* (P-0291).

FWHM.

3. Results and discussion

3.1. Strains identification

3.1.1. Morphological observations in SEM

Observations of strains with SEM revealed that cells were typical of the genus *Gambierdiscus*, i.e. characterised by an anteroposterior compression, and in apical or antapical views the cells had a roughly

rounded shape indented ventrally, and an apical pore with a fishhook opening (Fig. 2). The plate pattern was similar for all strains: Po, 4', Oa, 6", 6C, 6?S, 5", Op, 2"". From a size point of view, all strains were in a medium-size range with mean DV length (i.e. depth) ranging from 58.3 to 68.1 μm and width from 56.9 to 64.6 μm (Table S1).

Among the 10 strains studied, P-0422 was easily separated from others due to its very peculiar morphology with a smoother thecal surface and a wide plate 2"" contrasting with all other strains in which it was narrow and more pointed posteriorly (Fig. 2a). Only a few *Gambierdiscus* species possess a very broad plate 2"", and the shape observed

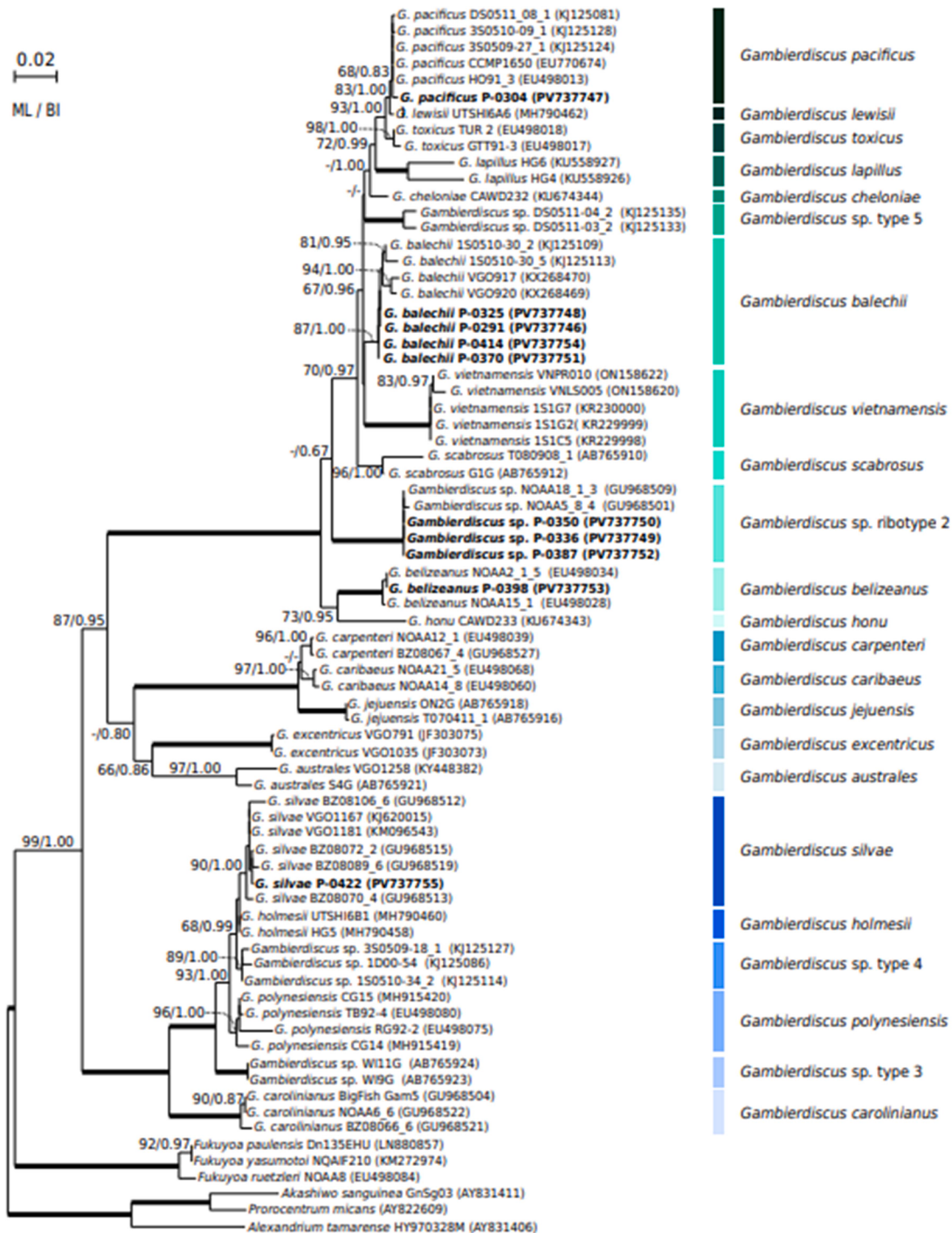


Fig. 3. Phylogenetic tree of *Gambierdiscus* sequences (LSU D8-D10 domains) inferred from maximum likelihood and Bayesian inference. The data set comprised 75 taxa (69 *Gambierdiscus*, 3 *Fukuyoa* and 3 outgroup sequences) and 925 positions including gaps. Thick lines indicate full support. Bootstrap values (>65) and posterior probabilities are indicated on branches. Sequences acquired in the present study are shown with bold face.

in P-0422 fits perfectly with the description of *G. silvae*. Other strains did not show major morphological differences in their plate pattern, and they appear rather similar, all belonging to the group of species with a narrow plate 2^{'''}. Cells of these strains were not completely smooth and different levels of ornamentation (foveated to reticulate-foveated ornamentation) were observed on the different strains. Strain P-0398 (Fig. 2b) appeared to have a stronger ornamentation than all other strains. It was particularly conspicuous on pre- and post-cingular plates which appeared to be deeply reticulate-foveate. To a lesser extent, a similar feature and ornamentation was found in strains P-0304, P-0387 and P-0291 which all appear difficult to distinguish by morphological features (Fig. 2c, 2d and 2e). In all strains, the 2' plate was hatchet-shaped (i.e. with the 2'/1" side smaller than the 2'/3" side, but this feature was variable. Hence, from a morphological point of view, the identification of these strains was not possible as they all appear rather similar, and molecular data were of the utmost importance to resolve their identity.

3.1.2. Molecular identification and phylogenetic analysis

Considering that the production of sequences is particularly complex in *Gambierdiscus* due to the presence of multiple copies of ribosomal genes including pseudogenes, using ONT method coupled to pipeline Esperanto has shown to be a probative approach for poor-quality sequences. Here the assignment rate was very good with similarities to reference sequences above 99 %.

The ML phylogenetic tree inferred from D8-D10 domains of LSU shows that *Gambierdiscus* sequences clustered in 23 well resolved clades corresponding to 19 taxonomically described species and 4 undescribed ribotypes, namely type 3–5 and *G. ribotype 2* (Fig. 3). The 10 sequences obtained clustered in 5 distinct clades of the tree. One sequence (strain P-0304) grouped with other existing sequences of *G. pacificus*. Four sequences (strains P-0291, P-0325, P-370, P-0414) branched basal to the *G. balechii* clade, forming a well-supported clade of 8 sequences (BS 87 PP 1.00). Three sequences (strains P-0336, P-0350, P-0387) clustered with full support with sequences of *G. ribotype 2*. One sequence (strain P-0398) branched within *G. belizeanus* clade, while sequence (P-0422) branched with *G. silvae* with strong support (BS 90 /PP 1.00).

3.1.3. Species diversity around La Réunion

Thanks to molecular data and phylogenetic analysis, five different species could be identified in the strains isolated from La Réunion, namely *G. balechii*, *G. belizeanus*, *G. pacificus*, *G. silvae* and the undescribed *G. ribotype 2*. Examination by SEM was congruent with molecular identification, but it confirmed that variability in morphological features makes the identification of closely resembling species difficult. The morphology of *G. silvae* was peculiar enough allowing its identification from other strains, but strains of *G. balechii*, *G. belizeanus*, *G. pacificus* and *G. ribotype 2* appeared almost similar, with a similar size range. Ornamentation was long considered as feature allowing to identify *G. belizeanus* and the closely related species *G. scabrosus* from other species (Faust, 1995; Litaker et al., 2009; Nishimura et al., 2013), but this feature seems to occur more frequently in several species as we found ornamentation also in *G. ribotype 2*, *G. balechii* and to a lesser extent in *G. pacificus*. Although the latter was long interpreted as species with a smooth thecal surface (Chinain et al., 1999; Litaker et al., 2009), our observations of strain P-0304 showed a clearly foveate ornamented thecal surface. A similar finding was recently reported from Vietnamese strains by Nguyen-Ngoc et al. (2023) who illustrated a foveate ornamentation, especially on precingular and postcingular plates. Although this was not clearly reported by Chinain et al. (1999) in the original description, this is visible on some micrographs and further investigations on this species from the type locality appear necessary to clarify this feature. In addition, *G. balechii* is also a species with an ornamented surface (Fraga et al., 2016) difficult to distinguish from other species in the same size-range. Hence, with morphological data only, the diversity of *Gambierdiscus* species would be difficult to

estimate, as suggested by our data, and phylogenetic data are thus necessary.

While the presence of *Gambierdiscus* in Indian Ocean is well established for more than 20 years (Quod et al., 2000; Ten-Hage et al., 2000a, 2000b; Hansen et al., 2001; Chinain et al., 2020), taxonomic data on species composition are still scarce in the area. Our study reveals the presence of several taxa previously unknown in the Indian Ocean. As reviewed by Habibi et al. (2021), many studies reported *G. toxicus* from Mayotte, Réunion and Mauritius islands, but this identification cannot be considered as specific since no molecular data were available, and at that time, *G. toxicus* was the unique species described. Hence, all these reports should be considered at the genus level only (Chinain et al., 2020). At the species level, *G. belizeanus* appears to be one of the most cited species (i.e. Munir et al., 2011; Catania et al., 2017) and its presence has been recently confirmed by molecular data in Seychelles islands (Lavenu et al., 2018; our study).

Among other species already reported in the Indian Ocean, *G. australes* has been cited from Glorioso Islands, SW Indian Ocean (Chomérat et al., 2008) and from Pakistan waters where it was found together with *G. polynesiensis* (Munir et al., 2011). However, both reports are based on morphological observations only, and they are previous to the descriptions of several species using molecular data. In our opinion, they must be treated cautiously as they may concern resembling species described afterwards. Although SEM constitutes a fantastic tool to examine and study morphological details of dinoflagellates, recent advances in the taxonomy of several benthic genera (i.e. *Ostreopsis*, *Gambierdiscus*) revealed that morphological variations and plasticity in shape, size, ornamentation make the identification at the species level almost impossible, and that several recently described species could only be distinguished genetically. This is perfectly illustrated by *G. vietnamensis*, which cannot be distinguished morphologically from *G. pacificus* but that is genetically a well-divergent species (Nguyen-Ngoc et al., 2023).

From a biogeographic point of view, it is interesting to notice that among the species found in our study, *G. belizeanus* and *G. silvae* correspond to species described from Caribbean Sea and Canary Islands in the Atlantic Ocean, respectively (Faust, 1995; Fraga and Rodriguez, 2014). The *G. ribotype 2* was also identified by Litaker et al. (2010) from Martinique Island (Atlantic Ocean) and was suggested to be a separate species, yet undescribed and restricted to various islands of the Caribbean (Tester et al., 2020). By contrast, *G. balechii* was originally described from Celebes Sea, Indonesia and presently in the Pacific Ocean (Dai et al., 2017; Wu et al., 2020), and *G. pacificus* from the mid-Pacific (Chinain et al. 1999; Litaker et al., 2010). Hence, the assemblage observed around La Réunion comprises taxa also present in both the Atlantic and Pacific Oceans, allowing to extend the geographic distribution of these species.

3.2. Growth dynamics

The growth rate of our strains varied from 0.09 to 0.48 day⁻¹, those values being consistent with the results of previous studies in similar culturing conditions. Results are summarized in Table S1. The lowest growth rate of 0.09 day⁻¹ was recorded for strain P-0398 of *G. belizeanus*. In the literature the growth rate of *G. belizeanus* cultured in the same conditions covers the same range with values from 0.13 - 0.18 day⁻¹ (Litaker et al., 2017) to 0.28 day⁻¹ (Xu et al., 2016). The strain P-0304 of *G. pacificus* had a growth rate of 0.26 day⁻¹ which is in the upper range of values previously reported, 0.18 - 0.21 day⁻¹ (Chinain et al., 2010), 0.22 - 0.24 day⁻¹ (Pisapia et al., 2017) and 0.32 day⁻¹ (Xu et al., 2016). Strains of *G. balechii* exhibited growth rates between 0.21 and 0.35 day⁻¹, which are higher than the value of 0.10 day⁻¹ reported by Pisapia et al. (2017) for strain VG0917. The highest value in our study was observed for strain P-0387 of *G. ribotype 2*, but the growth rate of this species had a wide range 0.14 - 0.48 day⁻¹. These values were higher than reports from the literature ranging between 0.12 and 0.15 day⁻¹

(Litaker et al., 2017). Overall, the growth rates recorded during the present study are of the same order or higher than those described for the highly toxic *G. polynesiensis* (Chinain et al., 2010; Longo et al., 2020) and *G. excentricus* strains (Litaker et al., 2017).

3.3. CTX-like toxicity evaluation by Neuro-2a CBA and CTX-like compounds by immunoassay

Gambierdiscus extracts for toxin analyses included four different species. In total, thirteen strains samples were collected and analysed (Table 1).

Toxicity of *Gambierdiscus* extracts was analysed by Neuro-2a CBA. *G. balechii* (P-0291) showed higher toxicities than the other *Gambierdiscus* species, followed by *G. ribotype 2* (P-0336B), *G. balechii* (P-0414A), *G. balechii* (P-0325), and *G. ribotype 2* (P-0336A). In the other hand, one *G. balechii* (P-0370) showed toxicities lower than LOQ and *G. pacificus* showed a small CTX-like toxicity signal but not conclusive.

Dai et al. (2017) compared the toxicity of several species of *Gambierdiscus* from Kiribati, *G. balechii* being the highest toxic strain (19.9 fg P-CTX1B equiv. cell⁻¹) but presenting lower values than the current study for *G. balechii* (P-0291, P-0414A and P-0325). The values of *G. belizeanus* (P-0398) obtained are consistent with those obtained in studies such as Litaker et al. (2017) and like those obtained by Tudó et al. (2020) using the same method. Also, the CTX-like toxicity values for *G. belizeanus* are higher than the average of the toxicity for the *G. belizeanus* strains from the Red Sea of 0.038 fg CTX1B equiv. cell⁻¹ (Catania et al., 2017). Fraga et al. (2011) observed that *G. excentricus* from the Canary Islands produces compounds with high CTX-like toxicity and Tudó et al. (2020) also observed that *G. excentricus* from the Canary Islands was the highest toxic species with a range of 9.5 – 2566.7 fg CTX1B equiv. cell⁻¹, followed by *G. australes* (1.7 – 452.6 fg CTX1B equiv. cell⁻¹) and *G. belizeanus* (5.6 fg CTX1B equiv. cell⁻¹).

Four strains (P-0414, P-0398, P-0350 and P-0336) were harvested at two different growth phase and a different final extract concentration (first ~100,000 cell mL⁻¹ and the second ~500,000 cell mL⁻¹). In some cases, *Gambierdiscus* harvested in a later growth phase usually showed higher CTX-like toxicity, for example, *G. ribotype 2* in the end of stationary phase (P-0336B) showed higher toxicity than the same strain harvested in an exponential phase (P-0336A). On the other hand, *G. balechii* (P-0414) showed higher toxicity at the beginning of the stationary phase (P-0414A) than at the end of the stationary phase (P-0414B). In addition, no differences were observed for cultures of *G. belizeanus* (P-0398) and *G. ribotype 2* (P-0350). This suggests that higher cell extracts do not always increase the chances to detect a higher toxin concentration in the extract, thus higher possibilities to characterize the toxin profile, however higher matrix effects could be also present.

Some extracts including three *Gambierdiscus* species and different growth phases (P-0414A, P-0414B, P-0398B, P-0325, P-0350B, and P-0336B) were further analysed using the magnetic bead-based immunoassay specifically developed to detect Pacific CTXs from the CTX1B and CTX3C series. None of the samples produced any detectable signal within the tested concentration range (20 to 20,000 cells mL⁻¹), indicating that these specific CTX congeners were absent in the samples (Table 1). Based on these results, we hypothesized that other CTX compounds, structurally different from CTX1B and CTX3C and therefore not recognized by the antibodies, may be responsible for the toxicity observed with CBA.

Previous studies have shown that several *Gambierdiscus* strains from Balearic and Canary Islands tested positive for CTXs of the CTX1B and/or CTX3C-series when assessed with the same immunoassay (Tudó et al., 2020; Gaiani et al., 2020). In these studies, some strains were found to produce up to 0.8 fg CTX1B equiv. cell⁻¹. However, no CTX-like compounds have been detected in the extracts analysed in the present study with the immunoassay. This discrepancy suggests that the production of CTXs from *Gambierdiscus* may not be uniform across all strains and/or

Table 1
CTX-like toxicity evaluation using the Neuro-2a CBA and CTX-like compounds evaluation using a magnetic bead-based immunoassay.

Code	Species	Growth phase at harvesting	Extract concentration (cell mL ⁻¹)	Neuro-2a CBA (fg CTX1B equiv. cell ⁻¹)	Neuro-2a CBA LOQ (fg CTX1B equiv. cell ⁻¹)	Immunoassay (fg CTX1B equiv. cell ⁻¹)
P-0398A	<i>G. belizeanus</i>	exponential	184,322	30	10	-
P-0398B	<i>G. belizeanus</i>	end of stationary phase	521,312	20	7	< LOQ
P-0370	<i>G. balechii</i>	stationary	95,201	< LOQ	3	-
P-0414A	<i>G. balechii</i>	stationary	126,446	63	16	< LOQ
P-0414B	<i>G. balechii</i>	end of stationary phase	651,440	7	2	< LOQ
P-0325	<i>G. balechii</i>	end of exponential phase	121,812	39	8	< LOQ
P-0291	<i>G. balechii</i>	end of stationary phase	684,172	627	170	-
P-0336A	<i>G. ribotype 2</i>	exponential	102,965	32	4	-
P-0336B	<i>G. ribotype 2</i>	end of stationary phase	466,227	76	19	< LOQ
P-0350A	<i>G. ribotype 2</i>	end of exponential phase	71,401	30	3	-
P-0350B	<i>G. ribotype 2</i>	stationary	454,252	26	8	< LOQ
P-0387	<i>G. ribotype 2</i>	end of exponential phase	154,126	13	1	-
P-0304	<i>G. pacificus</i>	end of exponential phase	119,841	no conclusive	-	-

Note: Strains of *Gambierdiscus* in two different biomass production phases with the same cultivation conditions. “-” means not analysed; LOQ immunoassay: 0.01 fg CTX1B equiv. cell⁻¹.

geographical areas (i.e. genetic variability among strains of a particular species or changes in the environmental/culture conditions). These factors can lead to significant variability in CTXs biosynthesis. A more thorough understanding of the complex regulatory mechanisms controlling CTXs production in *Gambierdiscus* is essential for improving the accuracy of CP risk assessments.

3.4. Toxin profile analysis by LC-MS/MS

All thirteen *Gambierdiscus* extracts were analysed by LC-MS/MS using seven different LC-MS/MS methods recorded in Tables S4 to S10. Different extract dilutions (1:100), (1:500) and (1:1000) were analysed and no significant differences were found among concentrations. The main two compounds identified were gambierone and 44-methylgambierone, which were quantified according to their corresponding standard calibration curves (Figure S2). All concentrations are summarized in Fig. 4. Concentrations of gambierone ranged from 2.05 pg·cell⁻¹ to 12.91 pg·cell⁻¹, except for two *Gambierdiscus* (one *G. ribotype 2* and one *G. pacificus*), where gambierone was below the LOQ. Similar range of concentrations were quantified for 44-methylgambierone, showing values from 1.93 pg·cell⁻¹ to 14.95 pg·cell⁻¹.

According to the LC-MS/MS results, *G. belizeanus* (P-0398) and *G. balechii* (P-0414, P-0325 and P-291) produced higher amounts of gambierone. On the other side, *G. balechii* (P-0370), *G. ribotype 2* (P-0350, P-0387 and P-0336) and *G. pacificus* (P-0304) produced higher amounts of 44-methylgambierone.

Four cultures were harvested at different growth phases showing different gambierone and 44-methylgambierone quantities. *G. balechii* (P-0414) and *G. ribotype 2* (P-0336) presented higher amounts of both compounds at the stationary phase (A) in comparison to the end of the stationary (B). In contrast, *G. ribotype 2* (P-0350) showed higher amounts of both compounds at the stationary phase (B) in comparison to the end of the exponential phase (A). In the case of *G. belizeanus* (P-0398), this *Gambierdiscus* presented similar amounts of both compounds at the exponential phase (A) and the end of the stationary phase (B).

Four different cultures of *G. balechii* were cultivated. Most of them were harvested at the stationary phase except for P-0325 which was harvested at the end of exponential phase. Even though all of them produced more gambierone than 44-methylgambierone, P-0414A produced the highest amount of gambierone (12.91 pg·cell⁻¹), while P-0370 produced the highest amount of 44-methylgambierone (11.6 pg·cell⁻¹). Cultures P-0414A and P-0370 produced the highest amount of both compounds followed by P-0325 and P-0291 and P-414B.

Three *G. ribotype 2* cultures were also cultivated and harvested at the end of the stationary phase (P-0350A, P-0387 and P-0336B). All of them showed higher amounts of 44-methylgambierone than gambierone, even though culture P-0387 only produced 44-methylgambierone. The culture P-336A produced the highest amount of 44-methylgambierone followed by P-0387, P-0350B and similar quantities were obtained for P-0350A and P-0336B.

Murray et al. (2024) obtained similar toxin profile results but higher concentration ranges after analysing 13 species of *Gambierdiscus*. They observed that the main producers of gambierone were a *G. belizeanus* (540 pg·cell⁻¹) from St. Barthelémy Island followed by a *G. chelonae* (358 pg·cell⁻¹) from Rarotonga, and a *G. scabrosus* (166 pg·cell⁻¹) from Japan. In contrast, they also observed that *G. carpenteri* from Australia showed the highest production of 44-methylgambierone (441 pg·cell⁻¹), followed by *G. lapillus* (270 pg·cell⁻¹), *G. australes* (259 pg·cell⁻¹) and *G. pacificus* (100 pg·cell⁻¹) from Rarotonga. Studies such as Rhodes et al. (2014) and Pisapia et al. (2017) agree with the study by Murray et al. (2024) and our results, being *G. pacificus* a producer of 44-methylgambierone.

Larson et al. (2018) analysed selected strains of *Gambierdiscus* from Australia (2 tropical *G. carpenteri*, 2 temperate *G. carpenteri*, 2 *G. lapillus* and 2 *Gambierdiscus* sp. (first identification given to *G. lewisii* and *G. holmesii* by Larson et al., 2018) using LC-MS/MS to observe CTX-like and MTX-like activities. Similar CTX-like activities were not detected in any of the samples while 44-methylgambierone was detected only in tropical strains of *Gambierdiscus*.

Subsequently, Munday et al. (2017) also studied the toxicity of sixteen *Gambierdiscus* isolates and one *Fukuyoa* from the South Pacific and observed that *G. pacificus*, *G. honu*, *G. australes*, *G. chelonae*, *F. paulensis* and *G. polynesiensis* produced 44-methylgambierone. This is consistent with our study, the *G. pacificus* (P-0304) also produced 44-methylgambierone and gambierone was not detected.

Only gambierone and 44-methylgambierone could be confirmed by LC-MS/MS by their retention time and ion ratio. In addition, two peaks were also detected showing the possible presence of a potential isobaric compound I/C—CTX and a potential polyether sulphur-containing compound (see Figure S3). This compound was detected in four cultures including *G. balechii* (P-0414, P-0325 and P-0291) and *G. belizeanus* (P-0398). Thus, the identification of these peaks were further evaluated by LC—HRMS.

3.5. Toxin profile analysis by LC-HRMS

For this assessment the 13 *Gambierdiscus* cultures were analysed without dilution. Results are summarized in Table S3. In all of them, the presence of 44-methylgambierone and gambierone was confirmed by meeting the established criteria. Three signals, a diagnostic and two confirming ions, were used for toxin identification. Experimental mass accuracy was < 3 ppm for gambierone and < 3.9 ppm for 44-methylgambierone. The relative ion intensities between the main signals and their *M* + 1 ions were calculated (RA, %) obtaining values < 24 % for gambierone and < 18 % for 44-methylgambierone, matched considering a tolerance of ± 40 % according to the EU Commission SANTE/2021/808 guidance document (EU, 2021). The combination of high resolution, mass accuracy, and restrictive criteria was crucial for the identification of both targeted and unknown compounds. LC—HRMS exact mass spectra of both compounds are shown in standards (Fig. 5a and 5b).

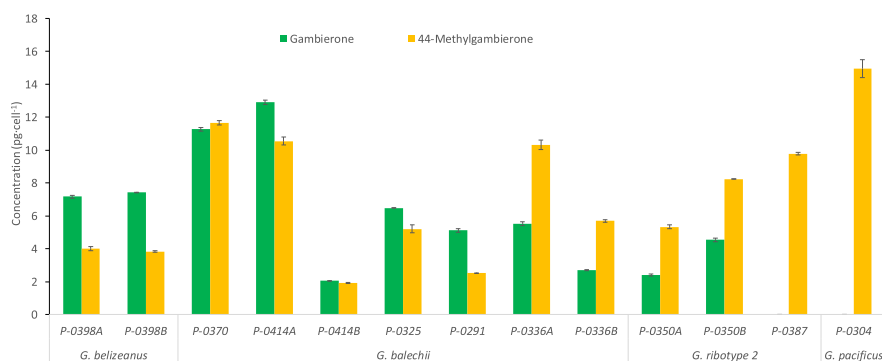


Fig. 4. Concentrations of gambierone and 44-methylgambierone (pg·cell⁻¹) in 13 *Gambierdiscus* extracts from La Réunion.

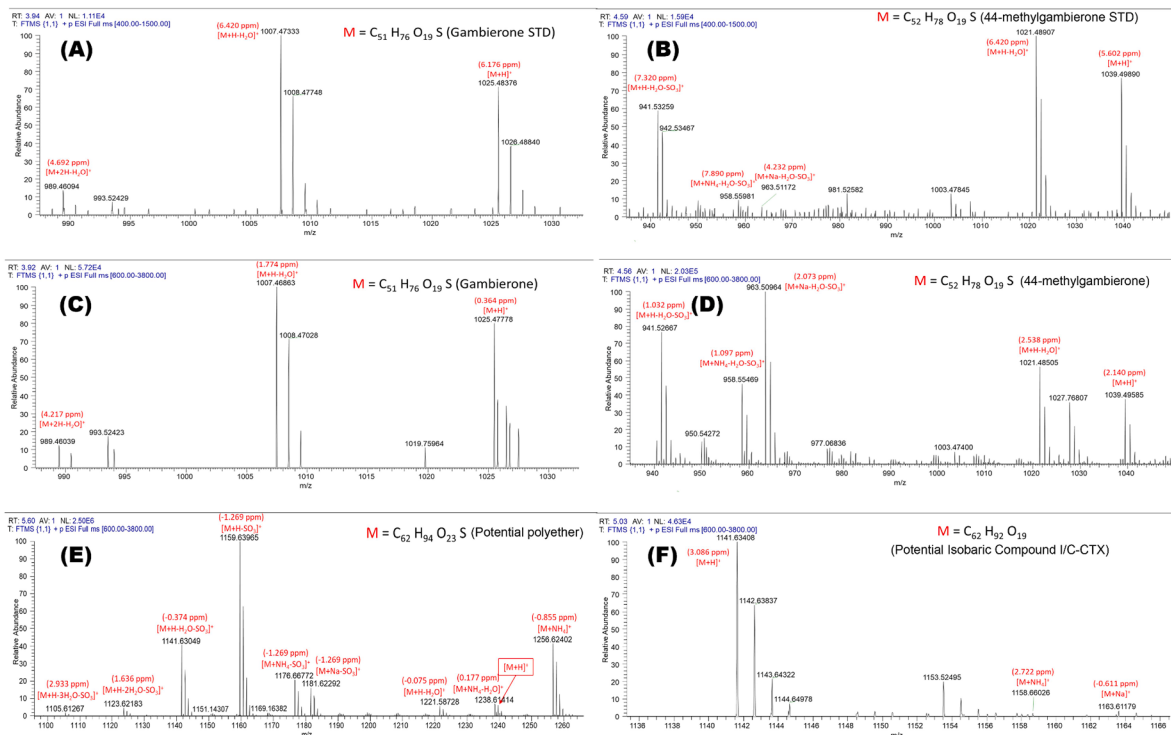


Fig. 5. (a) HRMS exact mass spectra of gambierone (standard); (b) HRMS exact mass spectra of 44-methylgambierone (standard); (c) HRMS exact mass spectra of gambierone in *G. ribotype* 2 (P-0336); (d) HRMS exact mass spectra of 44-methylgambierone in *G. ribotype* 2 (P-0387); (e) HRMS mass spectra of potential polyether C₆₂H₉₄O₂₃S in *G. belizeanus* (P-0398) at 5.59 min and (f) HRMS mass spectra of potential isobaric compound I/C—CTX in *G. balechii* (P-0414) at 5.03 min. The mass accuracy of the main adducts is identified for each compound respectively.

and in *Gambierdiscus* extracts (P-0336 and P-0387) in Fig. 5c and 5d

A potential isobaric compound of I/C—CTX was detected in six culture samples (P-0414A, P-0414B, P-0398A, P-0398B, P-0325 and P-0291) at a retention time of 5.03 min by LC—HRMS (Fig. 5f), which differs from the retention time of C—CTX1/2 an I-CTX1/2 reference materials (4.65 and 6.5 min). Its ionization pattern included two ions: m/z 1141.6305 and m/z 1158.6571 that matched $[\text{C}_{62}\text{H}_{92}\text{O}_{19} + \text{H}]^+$ and $[\text{C}_{62}\text{H}_{92}\text{O}_{19} + \text{NH}_4]^+$ (3.4 and < 4 ppm error, respectively). RDBEs of the main signal was in all cases 16.5 and the monoisotopic pattern ($M + 1$ ion) ratio of the main signal was below 10 % in accordance with the SANTE guidance document (EU, 2021) (See Table S3). Even though the intensity of the precursor ion m/z 1141.6305 did not allow for a high-quality HRMS² analysis (Figure S4), its fragmentation pattern did not match with those of C—CTX1/2 published before (Mudge et al., 2023).

We also detected a potential polyether, sulphur-containing compound in six culture samples (P-0414A, P-0414B, P-0398A, P-0398B, P-0325 and P-0291) at a retention time of 5.59 min by LC–HRMS (Fig. 5e). Its ionization pattern included the ions m/z 1141.6311 and m/z 1159.6411 that may correspond to the $[M + H - H_2O - SO_3]^+$ (0.49 ppm error) and $[M + H - SO_3]^+$ (1.27 ppm error) of the unknown molecular formula $C_{62}H_{94}O_{23}S$ and that can be mistaken by the $[M + H]^+$ and $[M + NH_4]^+$ of C/I-CTX-1/2. RDBEs of the main signal was in all cases 15.5 and the monoisotopic pattern ($M + 1$ ion) ratio of the main signal was below 9 % in accordance with the SANTE guidance document (EU, 2021) (See Table S3). We could also detect the fragment ions m/z 1256.6234 $[M + NH_4]^+$ and m/z 1237.5870 $[M - H]^-$ (Figure S5) at the same retention time. Their fragmentation pattern in positive ionization mode (Figure S5) showed 7 consecutive water losses from the $[M + H - SO_3]^+$, common in other polyethers such as CTXs and in other polyols from dinoflagellate origin (i.e. ovatoxins, amphidinols, etc. (García-Altares et al., 2015)). Its fragmentation pattern in negative mode confirmed the presence of a sulphate group in the molecule (m/z

96.9593, ppm error -8.0) (Figure S6).

The production of 44-methylgambierone and gambierone by *G. belizeanus* was already described and confirmed by [Boente-Juncal et al. \(2019\)](#), which agrees with our results. 44-methylgambierone was identified by also by [Pisapia et al. \(2017\)](#) in *G. balechii* (VGO917 and VGO920), *G. pacificus* (CCMP1650 and G10DC) and *G. ribotype 2* (CCMP1655, Mixed PR, St Maartens Gam10, SW Algae Gam1). In the same study, no gambierone was identified.

C—CTX1 was confirmed by Tartaglione et al. (2023) in a *G. balechii* (VGO920) by LC—HRMS. Our initial results by LC—HRMS agreed with the presence of a potential isobaric compound I/C—CTX according to retention time and the monoisotopic pattern. However, the fragmentation pattern of this potential isobaric compound obtained by Orbitrap ID-XTM TribridTM analysis is not conclusive to identify a ciguatoxin compound analogue (Figure S4).

Gambierone was identified in the G. sp2 from Greece by [Estevez et al. \(2019\)](#). This study also confirmed the presence of gambierone in several *Gambierdiscus* such as *G. balechii*, *G. belizeanus* and *G. ribotype 2*. Gambierone, 44-methylgambierone and C—CTX5 were confirmed in a *G. silvae* (1602 SH-6) from the Gulf of Mexico by [Mudge et al. \(2023\)](#). Gambierone was also detected in two *G. caribaeus* (BPAug08 and USVI-08) from this study and 44-methylgambierone was detected only in *G. caribaeus* (BPAug08). No other reported gambierone-related polyether compounds were detected in the extracts.

4. Conclusions

Compared with previously reported species in the Indian Ocean and around La Réunion, our study combining morphological and molecular data is the first to mention the diversity of *Gambierdiscus* in the area. Five different *Gambierdiscus* species have been identified: *G. belizeanus*, *G. balechii*, *G. pacificus*, *G. silvae* and *G. ribotype 2*, thanks to the morphological identification and the molecular phylogenetic analysis.

The *Gambierdiscus* extracts were analysed by Neuro-2a CBA, immunoassay, LC-MS/MS and LC–HRMS. Results for Neuro-2a CBA showed that *G. balechii* showed higher toxicity than the other *Gambierdiscus* species, followed by *G. ribotype 2*, *G. balechii*, *G. belizeanus*. Some extracts were also analysed by magnetic bead-based immunoassay where no signals were observed suggesting that no Pacific CTXs of the CTX1B and CTX3C series were present in these samples.

LC-MS/MS and LC–HRMS methodology were optimized. The *Gambierdiscus* extracts were analysed by LC-MS/MS using seven different methods. The two main compounds in all samples were gambierone and 44-methylgambierone at very high concentrations. These same samples were also analysed by LC–HRMS, confirming that gambierone and 44-methylgambierone were the only known compounds. A sulphur-containing potential polyether ($C_{62}H_{94}O_{23}S$ m/z 1256.6234 $[M + H]^+$) was also detected by LC–HRMS in four *Gambierdiscus* cultures.

The present study will benefit CP risk assessment in this region since they are providing data on the presence of five different *Gambierdiscus* species in La Réunion and the production of gambierone and 44-methylgambierone for all strains evaluated in this study. Additional systematic studies on a large number of selected *Gambierdiscus* strains and their abundances can significantly improve the risk assessment of CP in the region. Further work should be undertaken to elucidate the chemical structure of novel potential polyether compounds produced by *Gambierdiscus*.

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Research or studies on human subjects or animals

During this study no experiments were conducted on human or animal subjects.

CRedit authorship contribution statement

Lourdes Barreiro-Crespo: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Andres Sanchez-Henao:** Writing – review & editing, Methodology, Formal analysis. **Sandra Gimeno-Monforte:** Writing – review & editing, Methodology, Formal analysis. **Jaume Reverté:** Writing – review & editing, Methodology, Formal analysis. **Mònica Campàs:** Writing – review & editing, Investigation, Funding acquisition. **María García-Altares:** Writing – review & editing, Formal analysis. **Alina Tunin-Ley:** Writing – review & editing, Writing – original draft, Formal analysis. **Fanny Maillot:** Writing – review & editing, Formal analysis. **Cintia Flores:** Writing – review & editing, Formal analysis. **Nicolas Chomérat:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Gwenaél Bilien:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Takeshi Tsumuraya:** Writing – review & editing. **Núria Fontanals:** Writing – review & editing. **Francesc Borrull:** Writing – review & editing. **Jean Turquet:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Jorge Diogène:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Maria Rambla-Alegre:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be made available on request.

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