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Dereplication of *Gambierdiscus balechii* extract by LC-HRMS and *in vitro* assay: First description of a putative ciguatoxin and confirmation of 44-methylgambierone

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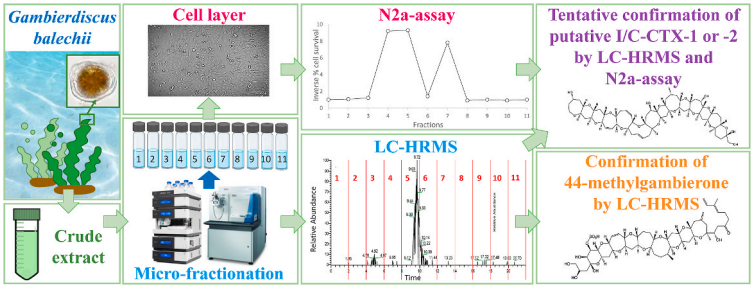
HIGHLIGHTS

- Putative I/C-CTX-1 identified for the first time in a dinoflagellate extract.
- Confirmation of 44-methylgambierone by LC-HRMS^2 in *G. balechii*.
- Interdisciplinary two-tiered approach for the dereplication of toxins.
- Bio-guided fractionation of *G. balechii* extract by LC-HRMS and N2a-assay.

GRAPHICAL ABSTRACT



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ABSTRACT

Marine toxins have a significant impact on seafood resources and human health. Up to date, mainly based on bioassays results, two genera of toxic microalgae, *Gambierdiscus* and *Fukuyoa* have been hypothesized to produce a suite of biologically active compounds, including maitotoxins (MTXs) and ciguatoxins (CTXs) with the latter causing ciguatera poisoning (CP) in humans. The global ubiquity of these microalgae and their ability to produce (un-)known bioactive compounds, necessitates strategies for screening, identifying, and reducing the number of target algal species and compounds selected for structural elucidation. To accomplish this task, a dereplication process is necessary to screen and profile algal extracts, identify target compounds, and support the discovery of novel bioactive chemotypes. Herein, a dereplication strategy was applied to a crude extract of a *G. balechii* culture to investigate for bioactive compounds with relevance to CP using liquid chromatography-high resolution mass spectrometry, *in vitro* cell-based bioassay, and a combination thereof via a bioassay-guided micro-

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fractionation. Three biologically active fractions exhibiting CTX-like and MTX-like toxicity were identified. A naturally incurred fish extract (*Sphyraena barracuda*) was used for confirmation where standards were unavailable. Using this approach, a putative I/C-CTX congener in *G. balechii* was identified for the first time, 44-methylgambierone was confirmed at 8.6 pg cell^{-1}, and MTX-like compounds were purported. This investigative approach can be applied towards other harmful algal species of interest. The identification of a microalgal species herein, *G. balechii* (VGO920) which was found capable of producing a putative I/C-CTX in culture is an impactful advancement for global CP research. The large-scale culturing of *G. balechii* could be used as a source of I/C-CTX reference material not yet commercially available, thus, fulfilling an analytical gap that currently hampers the routine determination of CTXs in various environmental and human health-relevant matrices.

1. Introduction

Ciguatera poisoning (CP) is the most commonly reported non-bacterial seafood-related illness worldwide. CP represents an ongoing global public health hazard and is endemic to various tropical and subtropical coastal regions of the Atlantic, Pacific, and Indian Oceans (FAO and WHO, 2020). Due to the limited surveillance of CP-causative organisms and associated toxins, there is a high degree of uncertainty regarding their impact, however, a total global estimated CP incidence rate of 50,000–500,000 poisonings per year has been proposed by Friedman et al. (2017); FAO and WHO (2020) and the references cited therein. CP can occur following the consumption of seafood containing ciguatoxins (CTXs, Fig. 1) with an elevated CTX content (>0.01–0.1 μg per kg fish tissue) (Friedman et al., 2008, 2017). Predatory species (e.g., barracuda, amberjack, red snapper, and grouper) can bioaccumulate and bio-transform CTXs from the prey they consume (Ikehara et al., 2017). CTXs and their precursors are produced by toxic dinoflagellates in the genus *Gambierdiscus* (18 described species) and *Fukuyoa* (3 described species) (FAO and WHO, 2020; Guiry and Guiry, 2020). Species in these genera are capable of producing bioactive ladder-shaped polyether compounds that, besides CTXs, include gambierol (Satake et al., 1993a), gambieroxide (Watanabe et al., 2013), gambieric acids (Morohashi et al., 2000), gambierone (Rodríguez et al., 2015), gambierone analogs (Holmes and Lewis, 1994b; Boente-Juncal et al., 2019; Murray et al., 2019, 2020; Yon et al., 2021; Mudge et al., 2022; Liu et al., 2023), maitotoxins (MTXs) (Yokoyama et al., 1988; Holmes et al., 1990; Murata et al., 1993, 1994; Holmes and Lewis, 1994a; Pisapia et al., 2017b; Estevez et al., 2021; Murray et al., 2022), as summarized in FAO and WHO (FAO and WHO, 2020). Specifically, within these genera, only *F. paulensis* (tentative identification of 54-deoxyCTX-1B) (Laza-Martinez et al., 2016), *G. australes* (Honsell et al., 2022), *G. toxicus* (CTX-3C, -4A, -4B) (Satake et al., 1993b, 1996), and *G. polynesiensis* (i.e., CTX-3B, -3C, -4A, -4B, 2-hydroxyCTX-3C, M-*seco*-CTX-3C) (Chinain et al., 2010; Munday et al., 2017; Sibat et al., 2018; Longo et al., 2019) have been suggested to produce CTXs using a range of techniques including bioassays.

From a toxicological perspective, CTXs (Fig. 1) are potent toxins, capable of modifying voltage-dependent sodium channels which can result in gastrointestinal, cardiac, and neurological symptomatology in humans (FAO and WHO, 2020). The global distribution, and chemical structural complexity of CTXs, co-occurrence of other compounds (e.g., MTXs, gambierones, and gambieric acids), limited availability of

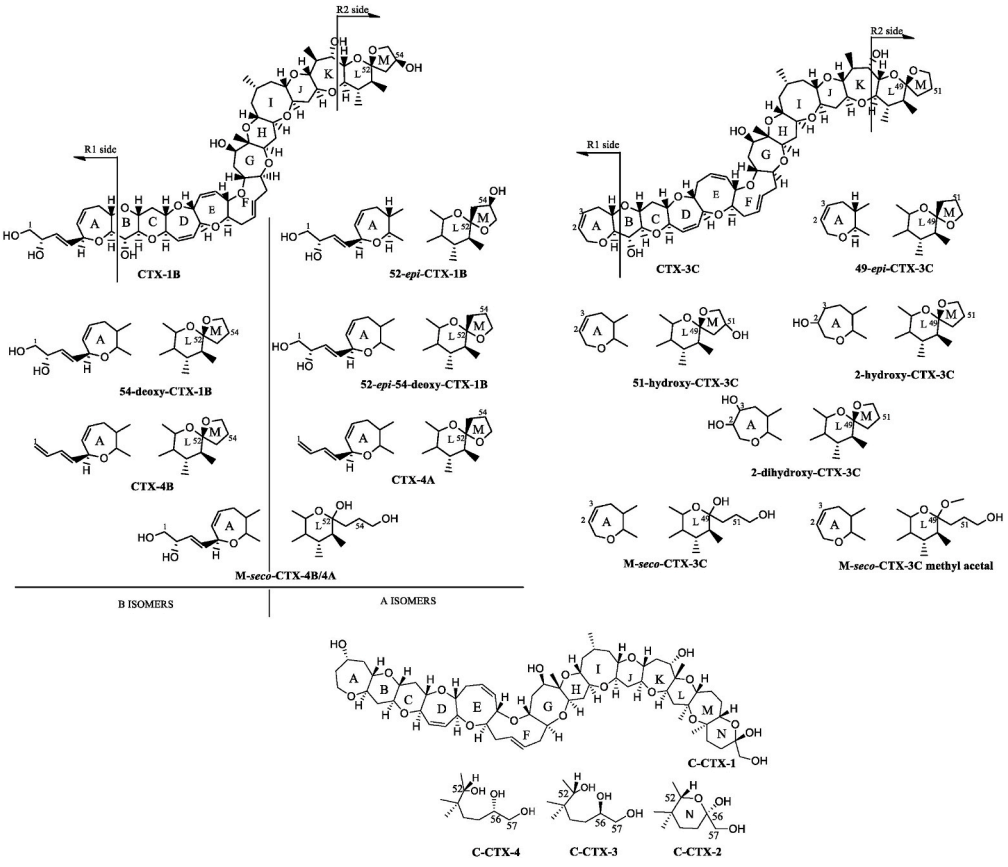

Fig. 1. Chemical structure of Ciguatoxins (CTXs) (Soliño and Costa, 2018; Kryuchkov et al., 2020).

commercially available CTX standards or reference material, the culture scalability and low productivity (in the range of fg cell^{-1}) of CTX-producing dinoflagellates, are recognized challenges inhibiting the identification of chemical profiles, structural assignments, and biological activity assessments of these toxins (Dickey and Plakas, 2010; Friedman et al., 2017; Chinain et al., 2020; Loeffler et al., 2021b). Regardless of these challenges and uncertainties, the United States Food and Drug Administration (FDA) established guidance levels (which include measurement and consumption uncertainties) of 0.10 and 0.01 μg equivalents for C-CTX-1 and CTX-1B per kg fish tissue, respectively (Dickey and Plakas, 2010; U.S. Food Drug Administration, 2020). The detection of CTXs in complex matrices (e.g., fish and shellfish tissues) at human health-relevant concentrations requires advanced analytical methodologies. Currently, routine tests to detect CTXs rapidly and reliably from the environment or in seafood have been established (e.g., those based on immunoassays, immunosensors, receptor binding, mouse, and *in vitro* bioassays), however, none have been validated and the development of analytical methods for the chemical profiling and quantitation of CTXs is difficult (FAO and WHO, 2020). A systematic investigation on CTXs and related compounds using analytical and biological methods has not been conducted yet for all known toxin-producing organisms and suspect seafood, with many endemic CP regions and many *Gambierdiscus* and *Fukuyoa* spp. being currently understudied. Thus, additional research for the discovery and structural characterization of CTXs and their congeners is essential.

In this frame, among the known species of *Gambierdiscus*, *G. balechii* was recently described and reported as toxic by using the mouse bioassay (Fraga et al., 2016). *G. balechii* is widely distributed across the tropical southwest Pacific Ocean where CP-derived food insecurity represents an ongoing endemic issue, especially for Small Island Developing States. Various strains of *G. balechii* have been cultured and shown to sustain the production of 44-methylgambierone (Pisapia et al., 2017b; Malto et al., 2022), a bioactive compound whose direct implication in CP is still debated (Murray et al., 2020), and other undescribed bioactive compounds, as tested by mouse bioassay (Fraga et al., 2016), receptor binding assay (Malto et al., 2022), and *in vitro* neuroblastoma cell-based (N2a) bioassay (Caillaud et al., 2009; Dai et al., 2017; Pisapia et al., 2017b; Wu et al., 2022). However, the description of compounds produced with relevance to CP and a definitive compound responsible for the observed adverse effect or CTX-specific toxicity of *G. balechii* has not yet been elucidated.

In this study, we conducted a chemical investigation into the toxin profile of *G. balechii* to further characterize the compounds produced by the strain VGO920 (origin: Manado, Indonesia). An interdisciplinary two-tiered approach was used, based on liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) and on the N2a-bioassay, a pharmacologically based cytotoxicity method specific for detecting compounds active on the sodium channel (Na_V). A bioassay-guided micro-fractionation approach revealed for the first time the presence of a putative Indian/Caribbean ciguatoxin congener (I/C-CTX-1 or -2) in the crude extract of the *G. balechii* strain; additionally, the presence of 44-methylgambierone was confirmed. CTX profiles in fish within different oceanic regions (i.e., Pacific/Indian/Caribbean) are believed to remain stable over time (Oshiro et al., 2022). As a reference standard for I/C-CTX-1 or -2 is not commercially available, a fish (*Sphyraena barracuda*) commonly associated with CP outbreaks was collected from a well-characterized region for C-CTXs (Virgin Islands, Caribbean Sea) (Dickey, 2008; Abraham et al., 2012; Loeffler et al., 2018). It was used to confirm the identity of the detected compound and ensure correct application of the employed methods.

2. Materials and methods

2.1. Reagents and chemicals

Methanol (MeOH) (HPLC grade, ≥99.9%), water (W) (HPLC grade), acetone (HPLC grade), *n*-hexane (HPLC grade), dichloromethane (DCM) (HPLC grade), chloroform (TCM), ammonium formate (AF) (reagent grade, 97%) and formic acid (FA) (reagent grade, ≥99.5%) were all from Merck KGaA (Darmstadt, Germany). CTX-3C (100 ng LOT# APK4222), CTX-1B (100 ng LOT# ESJ5851), and MTX-1 (10 μg LOT# ESJ5850) standards were purchased from Wako Chemicals GmbH (Neuss, Germany), 44-methylgambierone (10 μg LOT# 20–001) was purchased from Laboratorios CIFGA S.A. (Lugo, Spain). Assorted pacific CTXs – CTX-1B (87 ng mL^{-1}), 51-hydroxyCTX-3C (90 ng mL^{-1}), 52-*epi*-54-deoxyCTX-1B (117 ng mL^{-1}) and CTX-4A (110 ng mL^{-1}) - were kindly provided by Prof. T. Yasumoto from The Japan Food Research Laboratories and used for LC-HRMS and LC-HRMS^2 optimization. Brevetoxin-3 (1.2 μg mL^{-1} LOT# 040728) was from the National Research Council of Canada (NRCC, Halifax, Canada). All standards were dissolved in MeOH. Standard solutions were stored in glass vials at −20 °C.

2.2. G. balechii cultivation and harvest

The *G. balechii* strain was obtained from the Culture Collection of the Spanish Institute of Oceanography (CCVIEO, Vigo, Spain). Strain identification is VGO920 and was originally collected from Manado, Indonesia, East Pacific in 2007. The strain was originally described, isolated, and maintained in culture conditions as defined in Fraga et al. (2016). All cultures strains were grown in medium K2 + L1 (1:1) at 23 ± 1 °C. Light was provided by cool-white fluorescent bulbs (photon flux, 100 μE m^{-2} s^{-1}) in a standard 14:10 h light-dark cycle. A list of the LSU D8-D10 and SSU rDNA sequences can be found using the National Center for Biotechnology Information GenBank Accession number KX268469 and KX384639, respectively (Dai et al., 2017) (https://www.ncbi.nlm.nih.gov/nuccore/KX268469.1/and https://www.ncbi.nlm.nih.gov/nuccore/KX384639.1/). Cells were grown to obtain a total cell count of 1.0 × 10^6 and a cell pellet was produced by centrifugation at 4000 rpm (3800×*g*) at room temperature for 10 min. The cell pellet was stored at −20 °C until chemical analyses.

2.3. Extraction and liquid/liquid partitioning

The cell pellet containing 6.4 × 10^5 cells was extracted by adding 5 mL of MeOH, sonicating in pulse mode at 20% amplitude (Bandelin GmbH 2200.2, Berlin, Germany) for 15 min in an ice bath, and then centrifuging at 6000 rpm (4100×*g*) for 15 min. The final MeOH extract contained the equivalent of 1.28 × 10^5 cells per mL. A 3 mL aliquot of the crude extract was added to 2 mL of water in a 15 mL polypropylene tube to obtain a MeOH: W 3:2 (v/v) solution. A liquid/liquid partition of the obtained aqueous methanol extract was carried out by adding 5 mL of DCM as previously described (Satake et al., 1993b; Chinain et al., 2010). The two layers were separated by centrifugation at 6000 rpm (4100×*g*) for 10 min. The DCM phase was collected by glass pasteur pipette and transferred into a new 15 mL-size PP tube and the aqueous MeOH phase was further extracted by adding 5 mL of DCM. The two DCM extracts were combined and then the DCM and aqueous MeOH extracts were evaporated to dryness under a nitrogen stream, each redissolved in 2 mL of MeOH, and analyzed by the Neuro-2a bioassay and LC-HRMS. The final cell equivalent concentration for DCM and aqueous MeOH was 1.92 × 10^5 cells per mL.

2.4. Caribbean fish extraction and purification

A dehydrated fish (*S. barracuda*) from St. Thomas, United States Virgin Islands (Caribbean Sea) was kindly provided by Anne Tagini (Bioimpact, Inc.) for CTX analysis. Before extraction, the sample muscle tissue (without skin or bone) was further lyophilized using a freeze-dryer (Lyovac GT2, Amsco/Finn-Aqua, Hürth, Germany) for 36 h. Freeze-dried material was ground to a fine powder and 1 g dry tissue equivalent (DTE) was weighed for CTX extraction. The sample was rehydrated with 5 g water for 120 min and then extracted for CTXs, following the

CTX extraction protocol by Dickey (2008) with slight modifications. Muscle tissue in acetone (13 mL) was homogenized by sonicating in a digital ultrasonic bath (ArgoLab DU32) at ultrasonic power control = 5 for 5 min and then centrifuged at 10,000 rpm (16,000×*g*) for 5 min. The supernatant was decanted into a new vessel and the tissue pellet was re-extracted with further 10 mL acetone. Both acetone extracts were combined and dried under a nitrogen stream at 40 °C. The resulting residue was reconstituted in 5 mL of 80% methanol in water and defatted twice with 10 mL *n*-hexane. The aqueous methanol phase was reduced to dryness under a nitrogen stream at 40 °C. The dry residue was reconstituted in 5 mL HPLC-grade water, and CTXs were extracted twice with 5 mL DCM, the organic phases were combined and dried. The dry residue was reconstituted in 0.8 mL TCM and loaded on a pre-conditioned (5% water in MeOH, 100% MeOH, and TCM) Bond Elute SI cartridge (Bond Elut SI, 500 mg 3 mL, Agilent, Waldbronn, Germany). The glass vessel was rinsed with 400 μL TCM and the solvent rinse was loaded on the cartridge and allowed to pass through to waste. This step was repeated twice more, comprising a total of three washes of the glass vessel. The cartridge was then washed with one column volume of TCM (wash was discarded) and then eluted with two column volumes of 10 vol% MeOH in TCM. The eluate was dried and reconstituted in 1 mL MeOH (i.e., 1 g DTE mL^{-1}) and stored in a glass vial at −20 °C until usage.

2.5. Liquid chromatography high-resolution mass spectrometry (LC-HRMS) for G. balechii dereplication

LC-HRMS analyses were carried out on an Ultimate 3000 quaternary LC system coupled to a hybrid linear ion trap LTQ Orbitrap XL™ FTMS equipped with an ESI ION MAX™ source (Thermo-Fisher, USA). A Kinetex C18 column (2.6 μm, 100Å, 2.1 × 75 mm; Phenomenex, USA) kept at 25 °C was used. Mobile phases were water (eluent A) and 95% MeOH (eluent B), both containing 0.1% FA and 5 mM AF. The column flow rate was 0.2 mL min^{-1} with elution performed using the following gradient: t = 0 min 78% B, t = 11 min 100% B, t = 16 min 100% B, t = 22 min 78%B. The column was re-equilibrated for 5 min resulting in a total run time of 27 min. The injection volume was 5 μL.

Positive ions full HRMS experiments were acquired in the range *m/z* 700–1500 at a resolving power (RP) of 30,000 (FWHM at *m/z* 400). Source settings were the following: spray voltage 4.8 kV, capillary temperature 350 °C, capillary voltage 49 V, tube lens voltage 155 V, sheath gas, and auxiliary gas flow 37 and 6 (arbitrary units), respectively. HR collision-induced dissociation (CID) MS^2 experiments were acquired at a RP 60,000 using collision energy of 15% for CTX-3C and 44-methylgambierone, 30% for all the pacific CTX analogs, and 25% for the putative I/C-CTX-1 or -2. Isolation width was set at 4.0 Da, activation Q at 0.250, and activation time was 30 msec. Precursor ions were $[M+H]^+$ of CTX-3C (*m/z* 1023.6), 44-methylgambierone (*m/z* 1039.5), and putative I/C-CTX-1 or -2 (*m/z* 1141.6), $[M+NH_4]^+$ for CTX-1B (*m/z* 1128.6), and 52-*epi*-54-deoxyCTX-1B (*m/z* 1112.6), $[M+H-2H_2O]^+$ for 51-hydroxyCTX-3C (*m/z* 1003.5), and $[M+H-H_2O]^+$ for CTX-4A (*m/z* 1043.6). Xcalibur software v2.0.7 was used to calculate elemental formulae of the mono-isotopic peak (mass tolerance 5 ppm). The isotopic pattern and Ring Double Bond (RDB) equivalents were considered in the formula assignment. A 5-point calibration curve (125, 250, 500, 1000, 4000 ng mL^{-1}) of the 44-methylgambierone standard was used in quantitative analysis with manually integrated chromatographic peak areas used to express peak intensity. Measured limits of detection (LOD) for CTX-3C and CTX-1B were 13 ng mL^{-1} and 25 ng mL^{-1}, respectively.

2.6. Neuroblastoma-2a cell-based assay

2.6.1. Cell handling procedure, materials, and reagents

Mouse (*Mus musculus*) neuroblast type cells, cell line Neuro-2a (ATCC®CCL-131™) were purchased from the American Type Culture Collection (LGC Standards GmbH Wesel, Germany) from the lot numbered 63649750, and modified according to Loeffler et al. (2021a). Cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640, Gibco, Ref. 21875–034) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Millipore, S 0115), 1 mM sodium pyruvate (Gibco, Ref. 11360–039), and penicillin (50 U mL^{-1})/streptomycin (50 μg mL^{-1}) solution (Gibco, Ref. 15140–122). A 0.025% trypsin-EDTA solution (Gibco, Ref. 15400–054) was used to remove the cells from the culture flask (e.g. for passaging or plating). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen M6494) stock solution was prepared by dissolving 500 mg MTT in 100 ml PBS. CTX-3C standard was dissolved in MeOH at 1 ng mL^{-1}.

2.6.2. Toxicity assessment by in vitro Neuro-2a MTT-cytotoxicity assay

Cells were cultured in standard conditions and harvested for the assay when cultures were approximately 85–90% confluent (observed under an inverted microscope at 10× magnification), counted on a Bürker Counting Chamber (Sigma-Aldrich code 718905). Cells were seeded at 4.0 × 10^4 cells/well (200 μL volume) into sterile 96-well microplates and allowed to adhere overnight in the incubator. After the 24 h incubation period, the microplate cultures were examined microscopically to ensure confluent monolayers of cells were present before use.

CTXs are active sodium channel modifiers and N2a cells, when in the presence of ouabain (O) and veratridine (V), can be used to investigate compounds with sodium channel activity (i.e., CTXs). O and V were used at 0.22/0.022 mM O/V to sensitize the N2a cells (+OV) for the detection of sodium channel-specific effects from the sample extracts. Conversely, non-sensitized control cells (-OV) were used to identify the presence of any non-specific cytotoxicity due to other constituents in the sample (e.g., MTXs). Full dose-response curves (8-point dilutions) of sample extracts were prepared with +OV and -OV cells to determine the concentration at which cell viability was reduced by 50% (EC_{50}) compared with a CTX-3C standard (34.8–0.10 pg mL^{-1}) and MTX-1 standard (9.78–0.019 ng mL^{-1}). Samples, standards, and relevant controls were assayed in triplicate. Extracts from the two standards and two sample types (i.e., CTX-3C standard, MTX-1, fish, and *G. balechii*) were analyzed for toxic effects. The range of fish DTE used was 0.30–38.0 mg DTE mL^{-1}. The toxicity of the fish extract was expressed in ng CTX-3C per g DTE, a dry weight measurement. Results for *G. balechii* experiments on the crude extract, fractions, and two solvent partition extracts (DCM and aqueous MeOH) were expressed in cell equivalents (eq.) mL^{-1}.

2.6.3. Bioassay-guided micro-fractionation experiments

The LC-HRMS platform (section 2.5) was used for micro-fractionation experiments of CTX-3C standard, *G. balechii*, and fish DTE extract. Micro-fractionation of *G. balechii* crude extract was conducted by injecting a 10 μL aliquot (four injections, 40 μL in total or 5120 cell eq.) of the extract on the above-mentioned Kinetex column using flow and gradient conditions as reported above (section 2.5). The split ratio was set to 9:1 with 180 μL min^{-1} being collected in glass vials and the remaining 20 μL min^{-1} of the total flow transferred into the ESI source. Fractions were collected at 2 min intervals over a total run time of 22 min, resulting in #11 fractions for each sample type. The collected fractions (e.g., representing 36 μL of the original crude *G. balechii* extract or 4608 cell eq.) were evaporated to dryness under a nitrogen stream and each fraction's dry residue was reconstituted in 340 μL MeOH. The reconstituted samples were divided into two equal aliquots of 170 μL to be used for LC-HRMS and the N2a-assay. The same experimental conditions were used in micro-fractionating of: i) CTX-3C standard with 100 ng mL^{-1} (one single 10 μL injection or 1000 pg) and ii) a fish extract with 1 g DTE mL^{-1} (see section 2.4) (one single 10 μL-injection, i.e., 10 mg DTE). The collected fractions were dried and reconstituted in 200 μL of MeOH. All the reconstituted samples for CTX-3C and the fish were utilized for the N2a-assay (no LC-HRMS analysis of the collected fractions).

The micro-fractionation investigation by N2a-assay was performed as previously described in Loeffler et al. (2022). For each sample type, the following volumes of each collected fraction were reduced to dryness under nitrogen stream, the dry residue was reconstituted in 50 μL 5% FBS RPMI, and tested by the N2a-assay: *G. balechii* 40 μL (i.e., 12% of collected fraction, final concentration in the 96-plate well of 471 cell eq. mL^{-1}), CTX-3C 30 μL (i.e., 15% of the collected fractions), and fish 100 μL (i.e., 50% of the collected fractions, with a final well concentration in the 96-plate of 4.4 mg DTE mL^{-1}).

3. Results and discussion

3.1. Composite toxicity of G. balechii, S. barracuda versus CTX-3C, and MTX-1 standards by Neuro-2a cell-based assay

The composite cytotoxicity and bioactivity of the *G. balechii* crude extract, the DCM and aqueous MeOH (MeOH: W) extracts, the *S. barracuda* fish DTE extract, standards for MTX-1 and CTX-3C were investigated by the recently optimized Neuro-2a cell-based assay (Loeffler et al., 2021a) (Table 1, Fig. S1). The CTX-3C standard produced a response only in +OV conditions with an effective concentration causing a reduction in cell viability at 50% (EC_{50}) of 1.1 pg mL^{-1} (Fig. S1). The LOD for CTX-3C, where 20% cell mortality could be observed (EC_{20}), was 0.42 pg mL^{-1}. The fish (*S. barracuda*) extract elicited a specific response solely in the +OV conditions indicating the presence of a sodium channel activating compound (i.e., CTX) with an EC_{50} of 1.9 mg DTE mL^{-1} or 0.58 pg CTX-3C eq. per mg DTE (Table 1, Fig. S1f).

Full dose-response curve EC_{50} for MTX-1 in the presence of +OV conditions was 0.5 ng mL^{-1} and without –OV was 1.8 ng mL^{-1} (Table 1, Fig. S1a). The N2a-assay was developed to screen for Na_v-specific effects on cell membranes. When a sample (e.g., MTX-1) induces cell death in –OV conditions, then the sample is considered non-specific concerning Na_v activating compounds. All *G. balechii* extracts (i.e., crude, DCM, aqueous MeOH) elicited a cytotoxic response in the cell-based assay, however, they were all non-specific (Table 1, Figs. S1b, c, e). The + OV EC_{50} of the crude extract, DCM, and aqueous MeOH of *G. balechii* were 2.6, 79, and 6.3 cells mL^{-1}, respectively. The non-specific (-OV) condition EC_{50} of the crude extract, DCM, and aqueous MeOH of *G. balechii* were 22, 370, and 60 cells mL^{-1}, respectively (Table 1, Figs. S1b, c, e). All algal extracts (crude, DCM, aqueous MeOH) elicited toxic effects in both the +OV and -OV conditions similar to the MTX-1 standard (Table 1, Fig. S1). Because the obtained results for *G. balechii* extracts were not indicative of CTX-specific toxicity, the need for further sample processing techniques and analytical investigation emerged.

3.2. LC-HRMS optimization and dereplication of known CTXs and gambierones

Only two reference standards (CTX-3C and CTX-1B) out of the >30 known CTX analogs are available and no commercial Indian or Caribbean CTX standard exists (FAO and WHO, 2020). The commercially available standard is cost prohibitive and contains only 0.1 μg of CTX-3C that is insufficient to carry out a complete optimization of all MS parameters by flow injection analysis (FIA) or by mixed infusion/FIA modes. Therefore, the optimization work was conducted directly in LC-HRMS mode. Because of the high structural similarity between brevetoxins (BTXs) and CTXs, BTX-3 standard was used for optimizing source settings, resulting in an increase in detection sensitivity with a 4-fold increase in CTX-3C standard response. This outcome supports the use of BTXs as a relevant and cost-effective alternative to the CTX-3C standard in the optimization of ESI source parameters for CTX detection.

To conduct an in-depth set up of the method, LC-HRMS and MS^2 experiments were also conducted using assorted Pacific CTXs, therein some key issues were identified that were linked to the ionization behavior of the different analogs. The HR full scan MS spectra of CTX-1B standard contained ion peaks due to $[M+Na]^+$, $[M+K]^+$, and $[M+NH_4]^+$ but the protonated ion was absent (Fig. S2). Other CTXs (51-hydroxyCTX-3C, 52-*epi*-54-deoxyCTX-1B, CTX-3C, and CTX-4A) exhibited a pattern of ions that included $[M+H]^+$ and its *in-source* fragments due to the loss of one to three water molecules as well as sodium, potassium and ammonium adducts (Fig. S2). A different relative ion ratio among the above-mentioned ion species in the spectrum was observed for each analog (Figs. S2–S6). Furthermore, the uninformative fragment ions due to sequential loss of water molecules (one to seven) from the precursor ion of each CTX standard were the main distinctive feature observed in CID $HRMS^2$ spectra of most of the analyzed CTX standards. Additionally, the chromatographic peaks of different CTXs spread over a relatively large Rt range (7–15 min, Fig. S7). This is beneficial in terms of chromatographic resolution and facilitates unambiguous ion assignment as it prevents the overlapping of individual CTX MS spectra. Nonetheless, it cannot be excluded that within the same broad chromatographic range many different compounds (other than CTXs) elute, which complicates identification of the unknowns based on the only LC-full scan HRMS data. Overall, the unambiguous identification of CTXs in real samples remains challenging, and therefore in most situations, requires an interdisciplinary approach.

Dereplication is a key screening process, based on hyphenated techniques (such as LC-HRMS, LC-UV, among others) that allow fast and effective chemical profiling of natural samples as reviewed by Gaudêncio and Pereira (2015). When combined with *in vitro* assays, this process can speed up the discovery of unknown biologically active compounds and mitigate time-consuming re-isolation of known compounds or those of poor scientific value. Accordingly, the *G. balechii* crude extract was analyzed by LC-HRMS to identify any CTX-like compounds known to be produced by the genus *Gambierdiscus* (or even previously identified in some CTX-contaminated fish all over the world) that could contribute to the toxicity observed in the Neuro-2a assay.

Chemical dereplication of the crude *G. balechii* extract was thus performed by extracting the exact masses of known CTXs and gambierones (Table S1) for their $[M+H]^+$ ions and relevant adducts from the Total Ion Chromatogram (TIC) based on a mass tolerance of 5.0 ppm. Interestingly, the obtained Extracted Ion Chromatogram (XIC) at *m/z* 1141.6290, which corresponded to the $[M+H]^+$ ion of I/C-CTX-1 or -2 ($C_{62}H_{93}O_{19}^+$, RDB = 16.5, Δ = −1.364 ppm), contained a chromatographic peak at Rt = 8.63 min (Fig. 2). The HRMS spectrum associated to

Table 1
Toxin content of *G. balechii* crude and semi-purified (MeOH: W and DCM) extracts, a reference *S. barracuda* fish extract, and standards (CTX-3C and MTX-1) as measured by the cell-based N2a-assay. Concentration-response curves were generated using sodium channel specific (+OV) or non-specific (-OV) cell death effects. See section 2 for details.[a]

Sample	Na_v-specific (+OV) EC_{50}	Na_vnon-specific (-OV) EC_{50}
CTX-3C standard	1.1 pg mL^{-1}	<LOD
S. barracuda (fish)	1.9 mg DTE mL^{-1} (0.58 ppb CTX-3C eq.)	<LOD
MTX-1 standard	0.5 ng mL^{-1}	1.8 ng mL^{-1}
G. balechii (crude extract)	2.6 cell eq. mL^{-1}	22 cell eq. mL^{-1}
G. balechii (MeOH: W)	6.3 cell eq. mL^{-1}	20 cell eq. mL^{-1}
G. balechii (DCM)	79 cell eq. mL^{-1}	370 cell eq. mL^{-1}

[a] Corresponding concentration-response curve graphs for each sample are provided in Fig. S1.

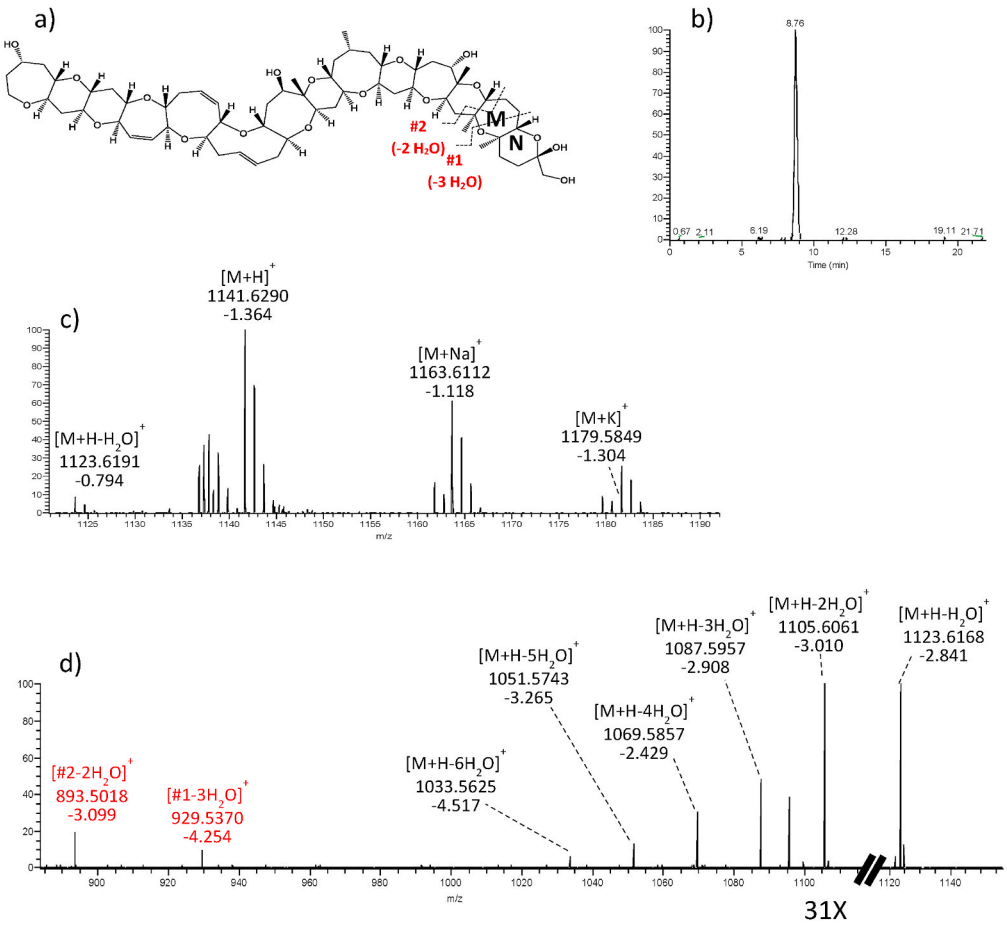

Fig. 2. a) Chemical structure of C-CTX-1. b) to d) LC-HRMS and MS^2 experiments conducted on the *G. balechii* extract: b) Extracted Ion Chromatogram (XIC) at *m/z* 1141.6279, c) the associated HRMS spectrum of the putative I/C-CTX-1 or -2, and d) CID $HRMS^2$ spectrum (precursor ion at *m/z* 1141.6, CE = 25%). Ion assignments and relevant Δ ppm are reported.

this peak contained other adducts/*in-source* fragment ions, namely the $[M+Na]^+$ ion at *m/z* 1163.6112, the $[M+K]^+$ ion at *m/z* 1179.5849, and the $[M+H–H_2O]^+$ ion at *m/z* 1123.6191 (Fig. 2), all being in good agreement with the full scan HRMS spectrum of C-CTX-1, as previously described by Estevez et al. (2020).

Currently, a microalgal source for the Indian or Caribbean CTX-group has not been confirmed. Nevertheless, based on toxicity data (which is presumed to be due to C-CTX-1 or -2 precursors), *G. silvae* and *G. excentricus* have been suggested as the most-likely C-CTX source organisms (Litaker et al., 2017; Pisapia et al., 2017a; Liefer et al., 2021). Confirmation of C-CTX-1 and -2 is routinely conducted in fish from the Caribbean (Yogi et al., 2011; Loeffler et al., 2018; Kryuchkov et al., 2020) and eastern Atlantic regions of the Canary Islands (Spain) and Madeira (Portugal) (Otero et al., 2010; Abraham et al., 2012; Costa et al., 2018), by LC-HRMS or LC-MS, but I/C-CTXs have never been reported from the Pacific Ocean. However, the use of regional (i.e., Caribbean, Pacific, and Indian) nomenclature for CTXs is opportune only to preserve consistency in the literature as algae, fish, and oceanic currents are not always regionally exclusive. The establishment of invasive species and the movement of organisms is demonstrably widespread (Smayda, 2007; Anderson, 2009; Yong et al., 2018), further exacerbated by anthropic pressure and global climate changes affecting the multiple equilibria in the thermohaline ocean circulation (Kasyan et al., 2022; Richter et al., 2022). Therefore, with the distribution and abundance of *Gambierdiscus* and *Fukuyoa* species likely changing (whether newly established or previously unrecognized), so too will the distribution of regionally recognized CTXs (Kryuchkov et al., 2020; Spielmeyer et al., 2022).

In addition to the potential CTX, another peak emerged at Rt = 3.68 min in the XIC at *m/z* 1039.4961 ($C_{52}H_{79}O_{19}S^+$, RDB = 13.5, Δ = 2.908 ppm) suggesting the presence of 44-methylgambierone in the extract (Fig. 3).

An in-depth study including LC-$HRMS^2$ investigation was conducted and reported in the following section to confirm this result.

3.2.1. *Confirmation of 44-methylgambierone in G.balechii extract*

The 44-methylgambierone standard and *G. balechii* extract were analyzed under the same experimental conditions (see section 2.5). The Rt, the full scan HRMS, and CID $HRMS^2$ profiles of the 44-methylgambierone standard and of the compound eluting at 3.68 min in the *G. balechii* extract were indistinguishable (Fig. 3, Table S2). Particularly, in addition to the pseudo-molecular ion the HR full MS spectrum contained a sodium adduct at *m/z* 1061.4766 and a variety of *in-source* fragment ions due to the loss of water molecules and the loss of SO_3 functionality (Fig. 3, Table S2). The CID $HRMS^2$ spectrum generated by fragmenting the $[M+H]^+$ precursor ion contained fragments at *m/z* 959.5358, 941.5283, and 923.5177 that could be assigned to the $[M+H–SO_3]^+$ ion and the subsequent losses of two water molecules, respectively (Fig. 3, Table S2), which confirmed the highly favored *in-source* fragment ions observed in the HR full scan MS spectrum. Furthermore, diagnostic fragments generated through the cleavage of the carbon chain backbone (cleavage #1, at *m/z* 831.3451 and 751.3878) leading to the loss of the side chain of the molecule and the subsequent loss of an SO_3 group were also present (Fig. 3, Table S2).

Comparing the 44-methylgambierone peak area in the *G. balechii* extract with the standard (isolated from *G. belizeanus*, ≥97% purity, non-

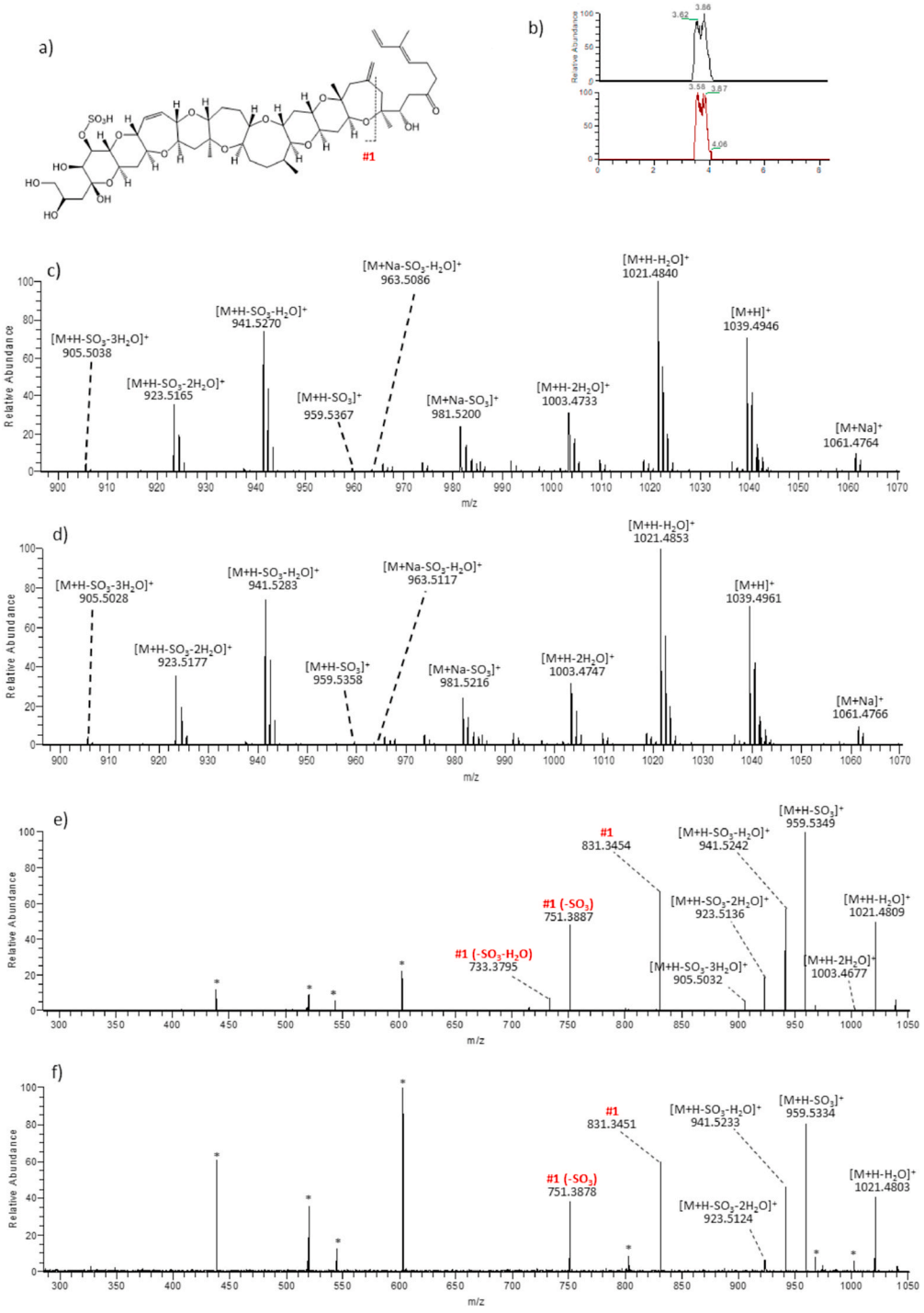

Fig. 3. LC-HRMS and MS^2 experiments conducted on the *G. balechii* extract and 44-methylgambierone standard: a) 44-methylgambierone chemical structure; b) Extracted Ion Chromatogram (XIC) at *m/z* 1039.4961, for 44-methylgambierone standard (up), and *G. balechii* extract (down); c) and d) the associated HRMS spectrum of 44-methylgambierone standard and in *G. balechii* extract, respectively; and e) and f) the CID $HRMS^2$ spectrum (precursor ion at *m/z* 1039.5, CE = 30%) of 44-methylgambierone standard and in *G. balechii*, respectively. Ion assignments and relevant Δ ppm are reported. * = Instrumental noise or impurity.

certified reference material) allowed a quantitative estimation of 8.6 pg $cell^{-1}$. The presence of 44-methylgambierone is in agreement with the literature, indicating that 44-methylgambierone production is so far conserved in this *G. balechii* strain (VGO920) (Pisapia et al., 2017b) and ubiquitous among several *G. balechii* culture isolates (Pisapia et al., 2017b; Malto et al., 2022; Wu et al., 2022). This finding also provides further evidence for the role of 44-methylgambierone as a promising chemotaxonomic biomarker for *Gambierdiscus* spp. to be possibly exploited as an early detection/warning sign of the presence of *Gambierdiscus* spp. in the environment. This has the potential to develop targeted management and mitigation strategies as suggested by Murray et al. (2020). The near-universal production of 44-methylgambierone among the tested *Gambierdiscus* species points to the biological importance of this compound (Pisapia et al., 2017a; Kretzschmar et al., 2019; Murray et al., 2020; Malto et al., 2022), even if its role in relation to CTX production remains undefined. The range of intracellular concentrations of 44-methylgambierone by *Gambierdiscus* spp. are 5–270 pg $cell^{-1}$ (Murray et al., 2021) and although this compound induces acute toxicity in mice by intraperitoneal injection with an LC_{50} of 20–38 mg kg^{-1} (body weight), it is considered to be insufficiently toxic to play an active role in outbreaks of CP (Murray et al., 2020).

3.2.2. *Tentative confirmation of putative I/C-CTX-1 or -2*

I/C-CTX-1 or -2 reference material or standard are not commercially

Table 2
Mono-isotopic ion peaks (*m/z*), elemental formulae, ring double bonds (RDB) equivalents, and errors (Δ, ppm) of the fragment ions contained in CID HRMS^2 spectrum of the putative I/C CTX-1 or -2 (precursor $[M+H]^+$ ion at *m/z* 1141.6) and their assignments to relevant cleavages.

Precursor Ion	*m/z* (theoretical)	*m/z* (experimental)	Elemental composition	RDB	Δ ppm
$[M+H]^+$		1141.6290	$C_{62}H_{93}O_{19}$	16.5	−1.364
Fragment ion/Cleavage#[a]					
$[M+H-H_2O]^+$	1123.6200	1123.6168	$C_{62}H_{91}O_{18}$	17.5	−2.841
$[M+H-2H_2O]^+$	1105.6094	1105.6061	$C_{62}H_{89}O_{17}$	18.5	−3.010
$[M+H-3H_2O]^{1+}$	1087.5989	1087.5957	$C_{62}H_{87}O_{16}$	19.5	−2.908
$[M+H-4H_2O]^+$	1069.5883	1069.5857	$C_{62}H_{85}O_{15}$	20.5	−2.429
$[M+H-5H_2O]^+$	1051.5777	1051.5743	$C_{62}H_{83}O_{14}$	21.5	−3.265
$[M+H-6H_2O]^+$	1033.5672	1033.5625	$C_{62}H_{81}O_{13}$	22.5	−4.517
$[M+H-H_2O-CO]^+$	1095.6251	1095.6222	$C_{61}H_{91}O_{17}$	16.5	−2.626
$[M+H-2H_2O-CO]^+$	1077.6145	1077.6049	$C_{61}H_{89}O_{16}$	17.5	−8.921
$[\#1-3H_2O]^+$	929.5410	929.5370	$C_{55}H_{77}O_{12}$	17.5	−4.254
$[\#2-2H_2O]^+$	893.5046	893.5018	$C_{51}H_{73}O_{13}$	15.5	−3.099

[a] Cleavages refer to Fig. 2.

available, therefore an interdisciplinary approach was required to confirm the identity of the compound eluting at Rt 8.63 min in the *G. balechii* extract. As a first step, the assignment of elemental compositions to all the ions associated with the putative I/C-CTX-1 or -2 was performed, then CID HRMS^2 experiments were conducted by fragmenting the $[M+H]^+$ precursor ion at *m/z* 1141.6 (Table 2).

All the fragment ion assignments were consistent with the chemical structure of the hypothesized putative I/C-CTX-1 (or −2). Particularly, *m/z* 929.5370 and 893.5018 (cleavage #1 and #2, Table 2) occurring on the carbon chain backbone, which were diagnostic and consistent with the characteristic fragmentation pathway of the ladder-frame backbone polyether molecules that require dissociation of one C–C bond and one or two covalent C–O bonds (Yasumoto et al., 2000). Therefore, the obtained data suggest the identity of a CTX-like compound.

Other authors (Kryuchkov et al., 2020) have reported a fragmentation pattern for C-CTX-1 and its epimer C-CTX-2 (indistinguishable using MS) featuring fragment ions which were not observed. All the optimization efforts completed to generate additional ions in HRMS^2 spectra, including exploring the effect of different fragmentation modes (Higher Energy Collisional Dissociation (HCD) fragmentation mode *versus* CID), and an in-depth ramping of collision energy, were unsuccessful. This discrepancy could be attributed to a difference in LC-HRMS instrumentation used (a Q-Exactive mass spectrometer equipped with a HESI-II heated electrospray interface in Kryuchkov et al. (2020), *versus* an LTQ Orbitrap XL™ equipped with an ESI ION MAX™ source in this study) or to the possible structural differences among C-CTX-1 or -2 and the putative I/C-CTX-1 or -2 contained in *G. balechii*.

Furthermore, the putative I/C-CTX-1 or -2 in the extract eluted on the reversed-phase column (see section 2.5) in the proximity of CTX-1B standard analyzed under the same experimental conditions (Rt 8.63 min compared to 7.79 min for CTX-1B). This result is in good agreement with the close similarity in polarity between the C-CTX-1 and CTX-1B (Estevez et al., 2019). However, before considering the data conclusive, some additional identification points were desirable. To this aim, a liquid/-liquid partition of the crude *G. balechii* extract between aqueous MeOH and DCM was conducted as previously described in Chinain et al. (2010). The expected result was recovering the putative I/C-CTX-1 or -2 in the DCM layer, separated by other bioactive components (including 44-methylgambierone) of the extract as previously reported in the literature for the identification of CTX-like compounds (Satake et al., 1993b; Fraga et al., 2016; Dai et al., 2017; Litaker et al., 2017; Pisapia et al., 2017a; Liefer et al., 2021; Mudge et al., 2022; Wu et al., 2022). However, LC-HRMS analyses and N2a-assay of both layers converged in revealing putative I/C-CTX-1 or -2 and 44-methylgambierone equally distributed between DCM and aqueous MeOH layers after partitioning. These results indicated that there is an issue of compound carryover linked to the used partitioning procedure and although both phases have been handled with extreme care as suggested by Fraga et al. (2016) (see section 2.4) the carryover issue remained unresolved. This demonstrates the need for a different strategy in sample preparation. Particularly one that can successfully separate MTXs and CTXs to reduce cross-reactivity in bioassay formats. In this perspective solid phase extraction (SPE) could be a valid alternative for investigating the opportune loading and eluting conditions in order to achieve a complete separation of MTXs from CTXs. Where toxicity is attributed to one class of compounds only, the co-existence of both classes of compounds in a single sample can confound the toxicity conclusions drawn, particularly when both compounds can cause a distinct but compounded reaction in a bioassay type experiments (e.g., the specific mode of action or composite toxicity).

3.2.3. Bioassay-guided LC-HRMS micro-fractionation of CTX-3C, G. balechii crude extract, and S. barracuda

The bioassay-guided micro-fractionation procedure was initiated using the CTX-3C standard. CTX-like toxicity (i.e., +OV specific) was observed only in fraction #8 (time window from 14 to 16 min) corresponding to the retention time observed for the standard by LC-HRMS (14.7 min) (Fig. 4). The same approach was used to support the chemical identification of any components in the eleven fractions generated from the *G. balechii* crude extract. Screening by the Neuro-2a cell-based assay identified some fractions (#4, #5, and #7) which contained bioactive compounds (Fig. 4, Table 3). Fraction #4 elicited a specific response only in +OV conditions with an EC_{50} at 109 cell eq. mL^{-1} (Fig. S8a). Among all fractions, #5 elicited the highest toxicity, however, this was a non-specific response, with an EC_{50} in +OV conditions at

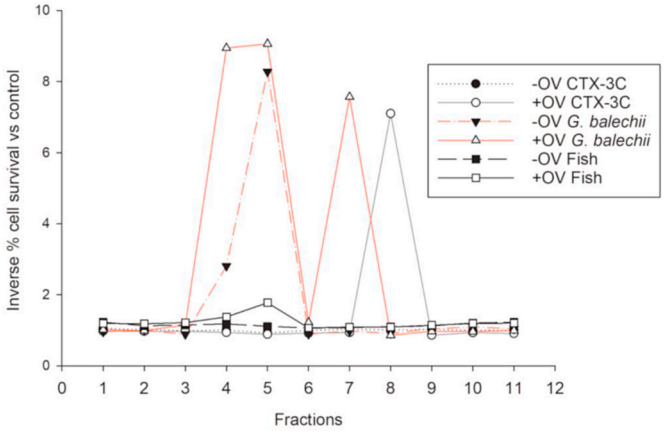

Fig. 4. HPLC fractionation (2 min per fraction) followed by the investigation by *in vitro* cell assay of CTX-3C dosed at 130 pg mL^{-1} (●/○ circle symbol, gray color line), *G. balechii* dosed at 471 cell eq. mL^{-1} (▲/△ triangle symbol, red color line), and *S. barracuda* extract dosed at 4.4 mg DTE mL^{-1} (■/□ square symbol, black color line). All samples were tested in conditions with (+OV) and without ouabain and veratridine (-OV), see sections 2.6.3 and 3.2.3 for additional details.

Table 3
Bioassay-guided fractionation using the crude extract of *G. balechii*, *S. barracuda*, and CTX-3C. Fractions showing Na_v-specific (+OV) and non-specific (-OV) toxicity in the N2a-MTT assay. Results for *S. barracuda* and CTX-3C only showed where a reaction was present (i.e., <LOD not displayed) (see also Fig. 4 and Figs. S1, S10).

Fraction number	Na_v specific (+OV) EC_{50}	Na_v non-specific (-OV) EC_{50}
1- *G. balechii*	<LOD	<LOD
2- *G. balechii*	<LOD	<LOD
3- *G. balechii*	<LOD	<LOD
4- [a]*G. balechii*	109 cell eq. mL^{-1}	<LOD
4-[a]*S. barracuda*	6.4 mg DTE mL^{-1}	<LOD
5- [a]*G. balechii*	53 cell eq. mL^{-1}	131 cell eq. mL^{-1}
5-[a]*S. barracuda*	3.1 mg DTE mL^{-1}	<LOD
6- *G. balechii*	<LOD	<LOD
7- [a]*G. balechii*	117 cell eq. mL^{-1}	<LOD
8- *G. balechii*	<LOD	<LOD
8- [a]CTX-3C	1.1 pg CTX-3C	<LOD
9- *G. balechii*	<LOD	<LOD
10- *G. balechii*	<LOD	<LOD
11- *G. balechii*	<LOD	<LOD

[a] Value generated from collected fractions EC_{50} dose-response composite toxicity results (see section 3.1 and Figs. S1, S8, S10); the assay was performed with fractions collected according to section 2.6.3.

53 cell eq. mL^{-1} and in -OV conditions of 131 cell eq. mL^{-1} (Fig. 5b). Fraction #6 is shown in more detail in Fig. S8b as a representation of a fraction without any bioactivity. Fraction #7 had the lowest response among the three bioactive fractions, however, the response was +OV specific, with an ~ EC_{50} at 117 cell eq. mL^{-1} (Fig. 4, Fig. S8c). Due to the limited amount of material available a full dose-response curve was not possible, and it is unclear if higher concentrations of fraction #7 (Fig. S8c) would induce a non-specific response and thus a higher similar bioactivity to fraction #4 or #5.

LC-HRMS of the collected fractions revealed the presence of the putative I/C-CTX-1 or -2 only in fraction #5, supporting the toxicity data (Figs. 4 and 5). However, the response in the N2a-assay was non-specific (Figs. 4 and 5b), revealing the presence of at least one other compound responsible of the non-specific bioactivity, underlining the importance of the -OV control for identifying unspecific bioactive compounds in a sample. MTXs, while not yet confirmed in *G. balechii* (FAO and WHO, 2020), are produced by *Gambierdiscus* spp. (Pisapia et al., 2017a) and MTX-1 showed non-specific toxicity in the N2a-assay (Table 1). Pisapia et al. (2017a) reported MTX-like toxicity for *G. balechii* at ~20 pg MTX eq. $cell^{-1}$. This study applied a maximum cell eq. of 246 cells mL^{-1} or in terms of MTX eq. per cell, this would equate to a maximum dose of 4.9 ng MTX eq. mL^{-1} (estimate based on the results from Pisapia et al. (2017a)). In this regard, the presence of an MTX-like compound in fraction #5, at the cell eq. applied, could produce a similar result to that observed using the MTX-1 standard, where the non-specific EC_{50} cell death was 1.8 ng MTX-1 eq. mL^{-1} (Fig. S1a and Table 1). MTX-1 standard was analyzed by LC-HRMS under the same chromatographic conditions used in the analysis of *G. balechii* extract and the compound was found to also elute in the proximity of putative I/C-CTX-1 or -2 (Fig. S9). This supports the hypothesis that a possible MTX-like compound and I/C-CTX-1 or-2 can co-elute (in fraction #5) under these chromatographic conditions.

The overall results suggest that fraction #5 may contain both a sodium channel activating compound as well as a non-specific MTX-like cytotoxic compound, thus explaining the non-specific N2a-assay response observed. The previous studies on *G. balechii* are supported by the results of this research regarding the potential for multiple fractions

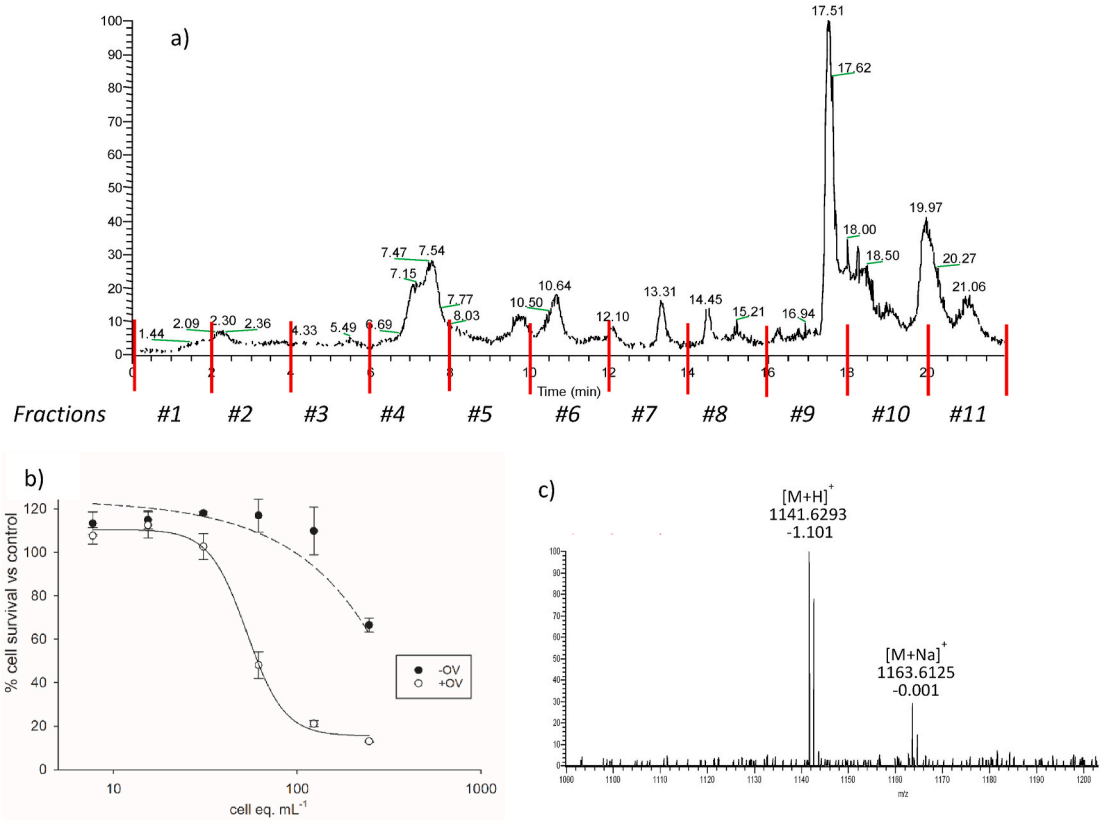

Fig. 5. a) Total Ion Chromatogram (TIC) in *m/z* range 700–1500 of the LC-HRMS micro-fractionation experiment for *G. balechii* crude extract. Red lines indicate the timeframe in which each fraction has been collected. In vitro cell assay in +OV (open circle) and -OV (filled circle) treatment conditions of b) concentration-response curve for fraction #5 representing 246–8 cell eq. mL^{-1} of the original crude extract; error bars indicate standard deviation of 3 replicates. c) HRMS spectrum of fraction #5, ion assignments, and relevant Δ ppm are reported.

with cytotoxic activity in a non-specific response (Caillaud et al., 2009). The original description of toxicity by Fraga et al. (2016) reported that an aqueous MeOH extract and DCM extracts were both toxic by mouse bioassay. We also found both extracts after partition to be toxic by the N2a-MTT assay.

To exhaust all options, given the absence of a commercially available I/C-CTX-1 standard, a naturally incurred extract of a fish from the Caribbean Sea (a geographic region well characterized for C-CTX-1/2 contamination (Abraham et al., 2012)) was analyzed by the bioassay-guided micro-fractionation method. The fish was tested using the N2a-assay and found to contain CTX-like activity (Table 1 and Fig. S1f). Therefore, this fish extract was subjected to chromatographic fractionation and analyzed with the aforementioned micro-fractionation tandem bioassay procedure. Sodium channel-specific toxicity was observed in fraction #4 and more pronounced in fraction #5 (Fig. 4 and Fig. S10). Therefore, the Na_v specific activity observed from the fish crude extract in the N2a-MTT assay (Fig. S1f) was likely the result of the composite toxicity effect due to these two fractions. Unfortunately, LC-HRMS was not able to detect any CTX analog in any collected fraction. Indeed, the combined composite toxicity of fish crude extract measured by the N2a-MTT assay was <1 ppb, and thus the CTXs level was likely < LOD for LC-HRMS.

4. Conclusions

CP research faces many major analytical challenges which inhibit the unambiguous determination of CTXs in environmental and seafood matrices. A critical issue is the lack of commercially available certified CTX standards, as emphasized by the participants at the Expert Meeting on CP, particularly for C-CTX-1 purified toxin standards (FAO and WHO, 2020), that justifies the recent major efforts put forth for the preparation of C-CTX-1 reference materials from naturally incurred fish material (Castro et al., 2022). General information regarding I-CTXs is also a high priority (due to a high fatality rate associated with a CP outbreak (Diogène et al., 2017)) as this group of CTXs lacks data on their structure, toxicity, prevalence, and proposed microalgal source (FAO and WHO, 2020). Therefore, the identification of a microalgal species herein, which can be grown in culture and capable of producing a putative I/C-CTX profile, is an important advancement for CP research, filling several high-priority research gaps. C-CTX production by other *Gambierdiscus* spp. has been implied but not confirmed and the research approach applied herein could be used to re-investigate other species suspected of producing CTXs. Without standards, toxicity and bioactivity-based assays can provide valuable information regarding biological effects from complex mixtures of compounds such as those produced by *Gambierdiscus* spp. and *Fukuyoa* spp. However, toxic effects due to a complex mixture may not be assigned to a specific chemical compound. Therefore, an effect-directed analysis and dereplication approach based on LC-HRMS, *in vitro* cell-based assay, and the bioassay-guided micro-fractionation method can help elucidate or isolate compounds of biological relevance. This approach was successful in confirming the presence of 44-methylgambierone, identifying a putative I/C-CTX, and providing evidence for the potential production of other bioactive compounds (i.e., CTXs in environmental (fish/algae) samples and in fraction #4, #7 and MTX-like compounds in fraction #5). CTX and MTX-like compounds from the crude extract were found to elute nearby under the experimental conditions used and these two types of compounds were present in both extracts after the liquid/liquid clean-up. Thus, the results of this commonly employed separation approach could be affected by the different composition of each single crude extract, which is strictly related to the source biological matrix. This stimulates an in-depth investigation on potential components of the crude extract able to affect the liquid/liquid partitioning outcomes. In future efforts, a larger batch culture coupled with an improved compound separation method and an enrichment such as solid phase extraction (SPE) could be applied for both selective enrichment and the removal of interfering matrix compounds. This would be a first step towards developing the process to generate a source of critically needed I/C-CTX reference material useful for global CP analyses and research.

Credit author statement

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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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.137940.

References

Abraham, A., Jester, E.L.E., Granade, H.R., Plakas, S.M., Dickey, R.W., 2012. Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. Food Chem. 131, 192–198.

Anderson, D.M., 2009. Approaches to monitoring, control and management of harmful algal blooms (HABs). Ocean Coast Manag. 52, 342–347.

Boente-Juncal, A., Álvarez, M., Antelo, Á., Rodríguez, I., Calabro, K., Vale, C., Thomas, O. P., Botana, L.M., 2019. Structure elucidation and biological evaluation of maitotoxin-3, a homologue of gambierone, from Gambierdiscus belizeanus. Toxins 11, 79.

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

Castro, D., Estévez, P., Leao-Martins, J.M., Dickey, R.W., García-Álvarez, N., Real, F., Costa, P.R., Gago-Martínez, A., 2022. Preparation of ciguatoxin reference materials from canary Islands (Spain) and Madeira archipelago (Portugal) fish. J. Mar. Sci. Eng. 10, 835.

Chinain, M., Darius, H.T., Ung, A., Cruchet, P., Wang, Z., Ponton, D., Laurent, D., Pauillac, S., 2010. Growth and toxin production in the ciguatera-causing dinoflagellate Gambierdiscus polynesiensis (Dinophyceae) in culture. Toxicon 56, 739–750.

Chinain, M., Gatti, C.M.i., Darius, H.T., Quod, J.P., Tester, P.A., 2020. Ciguatera poisonings: a global review of occurrences and trends. Harmful Algae, 101873.

Costa, P.R., Estevez, P., Castro, D., Soliño, L., Gouveia, N., Santos, C., Rodrigues, S.M., Leao, J.M., Gago-Martínez, A., 2018. New insights into the occurrence and toxin profile of ciguatoxins in selvagens Islands (Madeira, Portugal). Toxins 10, 524.

Dai, X., Mak, Y.L., Lu, C.-K., Mei, H.-H., Wu, J.J., Lee, W.H., Chan, L.L., Lim, P.T., Mustapa, N.I., Lim, H.C., Wolf, M., Li, D., Luo, Z., Gu, H., Leaw, C.P., Lu, D., 2017. Taxonomic assignment of the benthic toxigenic dinoflagellate Gambierdiscus sp. type 6 as Gambierdiscus balechii (Dinophyceae), including its distribution and ciguatoxicity. Harmful Algae 67, 107–118.
Dickey, R.W., 2008. Ciguatera Toxins: Chemistry, Toxicology, and Detection, second ed. (New York).
Dickey, R.W., Plakas, S.M., 2010. Ciguatera: a public health perspective. Toxicon 56, 123–136.
Diogène, J., Reverté, L., Rambla-Alegre, M., del Río, V., de la Iglesia, P., Campàs, M., Palacios, O., Flores, C., Caixach, J., Ralijaona, C., Razanajatovo, I., Pirog, A., Magalon, H., Arnich, N., Turquet, J., 2017. Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean. Sci. Rep. 7, 8240.
Estevez, P., Castro, D., Leão-Martins, J.M., Sibat, M., Tudó, A., Dickey, R., Diogene, J., Hess, P., Gago-Martinez, A., 2021. Toxicity screening of a Gambierdiscus australes strain from the western mediterranean Sea and identification of a novel maitotoxin analogue. Mar. Drugs 19, 460.
Estevez, P., Castro, D., Pequeño-Valtierra, A., Giraldez, J., Gago-Martinez, A., 2019. Emerging marine biotoxins in seafood from European coasts: incidence and analytical challenges. Foods 8, 149.
Estevez, P., Sibat, M., Leão-Martins, J.M., Reis Costa, P., Gago-Martínez, A., Hess, P., 2020. Liquid chromatography coupled to high-resolution mass spectrometry for the confirmation of Caribbean ciguatoxin-1 as the main toxin responsible for ciguatera poisoning caused by fish from European Atlantic coasts. Toxins 12, 267.
FAO and WHO, 2020. Report of the Expert Meeting on Ciguatera Poisoning. Rome, 19–23. November 2018 Food Safety and Quality No. 9. Rome.
Fraga, S., Rodríguez, F., Riobó, P., Bravo, I., 2016. Gambierdiscus balechii sp. nov (Dinophyceae), a new benthic toxic dinoflagellate from the Celebes Sea (SW Pacific Ocean). Harmful Algae 58, 93–105.
Friedman, M.A., Fernandez, M., Backer, L.C., Dickey, R.W., Bernstein, J., Schrank, K., Kibler, S., Stephan, W., Gribble, M.O., Bienfang, P., Bowen, R.E., Degrasse, S., Flores Quintana, H.A., Loeffler, C.R., Weisman, R., Blythe, D., Berdalet, E., Ayyar, R., Clarkson-Townsend, D., Swajian, K., Benner, R., Brewer, T., Fleming, L.E., 2017. An updated review of ciguatera fish poisoning: clinical, epidemiological, environmental, and public health management. Mar. Drugs 15, 72.
Friedman, M.A., Fleming, L.E., Fernandez, M., Bienfang, P., Schrank, K., Dickey, R., Bottein, M.Y., Backer, L., Ayyar, R., Weisman, R., Watkins, S., Granade, R., Reich, A., 2008. Ciguatera fish poisoning: treatment, prevention and management. Mar. Drugs 6, 456–479.
Gaudêncio, S.P., Pereira, F., 2015. Dereplication: racing to speed up the natural products discovery process. Nat. Prod. Rep. 32, 779–810.
Guiry, M.D., Guiry, G., 2020. AlgaeBase. AlgaeBase. World-wide electronic publication, Galway. National University of Ireland.
Holmes, M., Lewis, R., 1994a. Purification and characterisation of large and small maitotoxins from cultured Gambierdiscus toxicus. Nat. Toxins 2, 64–72.
Holmes, M.J., Lewis, R.J., 1994b. Purification and characterisation of large and small maitotoxins from cultured Gambierdiscus toxicus. Nat. Toxins 2, 64–72.
Holmes, M.J., Lewis, R.J., Gillespie, N.C., 1990. Toxicity of Australian and French Polynesian strains of Gambierdiscus Toxicus (Dinophyceae) grown in culture: characterization of a new type of maitotoxin. Toxicon 28, 1159–1172.
Honsell, G., Gaiani, G., Hirama, M., Pelin, M., Tubaro, A., Tsumuraya, T., Campàs, M., 2022. Cell immunolocalization of ciguatoxin-like compounds in the benthic dinoflagellate Gambierdiscus australes M. Chinain & M.A. Faust by confocal microscopy. Harmful Algae 120, 102353.
Ikehara, T., Kuniyoshi, K., Oshiro, N., Yasumoto, T., 2017. Biooxidation of ciguatoxins leads to species-specific toxin profiles. Toxins 9, 205.
Kasyan, V.V., Bitiutskii, D.G., Mishin, A.V., Zuev, O.A., Murzina, S.A., Sapozhnikov, P.V., Kalinina, O.Y., Syomin, V.L., Kolbasova, G.D., Voronin, V.P., Chudinovskikh, E.S., Orlov, A.M., 2022. Composition and distribution of plankton communities in the atlantic sector of the southern ocean. Diversity 14, 923.
Kretzschmar, A.L., Verma, A., Kohli, G., Murray, S., 2019. Development of a quantitative PCR assay for the detection and enumeration of a potentially ciguatoxin-producing dinoflagellate, Gambierdiscus lapillus (Gonyaulacales, Dinophyceae). PLoS One 14, e0224664.
Kryuchkov, F., Robertson, A., Miles, C.O., Mudge, E.M., Uhlig, S., 2020. LC–HRMS and chemical derivatization strategies for the structure elucidation of caribbean ciguatoxins: identification of C-CTX-3 and -4. Mar. Drugs 18, 182.
Laza-Martinez, A., David, H., Riobo, P., Miguel, I., Orive, E., 2016. Characterization of a strain of Fukuyoa paulensis (dinophyceae) from the western mediterranean Sea. J. Eukaryot. Microbiol. 63, 481–497.
Liefer, J.D., Richlen, M.L., Smith, T.B., DeBose, J.L., Xu, Y., Anderson, D.M., Robertson, A., 2021. Asynchrony of Gambierdiscus spp. abundance and toxicity in the U.S. Virgin Islands: implications for monitoring and management of ciguatera. Toxins 13, 413.
Litaker, R.W., Holland, W.C., Hardison, D.R., Pisapia, F., Hess, P., Kibler, S.R., Tester, P. A., 2017. Ciguatoxicity of Gambierdiscus and Fukuyoa species from the caribbean and gulf of Mexico. PLoS One 12, e0185776.
Liu, X., Ma, Y., Wu, J., Yin, Q., Wang, P., Zhu, J., Chan, L.L., Wu, B., 2023. Characterization of new gambierones produced by Gambierdiscus balechii 1123M1M10. Mar. Drugs 21, 3.
Loeffler, C.R., Bodi, D., Tartaglione, L., Dell'Aversano, C., Preiss-Weigert, A., 2021a. Improving in vitro ciguatoxin and brevetoxin detection: selecting neuroblastoma (Neuro-2a) cells with lower sensitivity to ouabain and veratridine (OV-LS). Harmful Algae 103, 101994.
Loeffler, C.R., Robertson, A., Flores Quintana, H.A., Silander, M.C., Smith, T.B., Olsen, D., 2018. Ciguatoxin prevalence in 4 commercial fish species along an oceanic exposure gradient in the US Virgin Islands. Environ. Toxicol. Chem. 37, 1852–1863.
Loeffler, C.R., Spielmeyer, A., Friedemann, M., Kapp, K., Schwank, U., Kappenstein, O., Bodi, D., 2022. Food safety Risk in Germany from mislabeled imported fish: ciguatera outbreak trace-back, toxin elucidation, and public health implications. Front. Mar. Sci. 9.
Loeffler, C.R., Tartaglione, L., Friedemann, M., Spielmeyer, A., Kappenstein, O., Bodi, D., 2021b. Ciguatera mini review: 21st century environmental challenges and the interdisciplinary research efforts rising to meet them. Int. J. Environ. Res. Publ. Health 18, 3027.
Longo, S., Sibat, M., Viallon, J., Darius, H.T., Hess, P., Chinain, M., 2019. Intraspecific variability in the toxin production and toxin profiles of in vitro cultures of Gambierdiscus polynesiensis (dinophyceae) from French polynesia. Toxins 11, 735.
Malto, Z.B.L., Benico, G.A., Batucan, J.D., Dela Cruz, J., Romero, M.L.J., Azanza, R.V., Salvador-Reyes, L.A., 2022. Global mass spectrometric analysis reveals chemical diversity of secondary metabolites and 44-methylgambierone production in philippine Gambierdiscus strains. Front. Mar. Sci. 8.
Morohashi, A., Satake, M., Nagai, H., Oshima, Y., Yasumoto, T., 2000. The absolute configuration of gambieric acids A–D, potent antifungal polyethers, isolated from the marine dinoflagellate Gambierdiscus toxicus. Tetrahedron 56, 8995–9001.
Mudge, E.M., Robertson, A., Leynse, A.K., McCarron, P., Miles, C.O., 2022. Selective extraction of gambierone and related metabolites in Gambierdiscus silvae using m-aminophenylboronic acid–agarose gel and liquid chromatography–high-resolution mass spectrometric detection. J. Chromatogr. B 1188, 123014.
Munday, R., Murray, S., Rhodes, L.L., Larsson, M.E., Harwood, D.T., 2017. Ciguatoxins and maitotoxins in extracts of sixteen Gambierdiscus isolates and one Fukuyoa isolate from the south pacific and their toxicity to mice by intraperitoneal and oral administration. Mar. Drugs 15, 208.
Murata, M., Naoki, H., Iwashita, T., Matsunaga, S., Sasaki, M., Yokoyama, A., Yasumoto, T., 1993. Structure of maitotoxin. J. Am. Chem. Soc. 115, 2060–2062.
Murata, M., Naoki, H., Matsunaga, S., Satake, M., Yasumoto, T., 1994. Structure and partial stereochemical assignments for maitotoxin, the most toxic and largest natural non-biopolymer. J. Am. Chem. Soc. 116, 7098–7107.
Murray, J.S., Finch, S.C., Mudge, E.M., Wilkins, A.L., Puddick, J., Harwood, D.T., Rhodes, L.L., van Ginkel, R., Rise, F., Prinsep, M.R., 2022. Structural characterization of maitotoxins produced by toxic Gambierdiscus species. Mar. Drugs 20, 453.
Murray, J.S., Finch, S.C., Puddick, J., Rhodes, L.L., Harwood, D.T., van Ginkel, R., Prinsep, M.R., 2021. Acute toxicity of gambierone and quantitative analysis of gambierones produced by cohabitating benthic dinoflagellates. Toxins 13, 333.
Murray, J.S., Nishimura, T., Finch, S.C., Rhodes, L.L., Puddick, J., Harwood, D.T., Larsson, M.E., Doblin, M.A., Leung, P., Yan, M., Rise, F., Wilkins, A.L., Prinsep, M.R., 2020. The role of 44-methylgambierone in ciguatera fish poisoning: acute toxicity, production by marine microalgae and its potential as a biomarker for Gambierdiscus spp. Harmful Algae 97, 101853.
Murray, J.S., Selwood, A.I., Harwood, D.T., van Ginkel, R., Puddick, J., Rhodes, L.L., Rise, F., Wilkins, A.L., 2019. 44-Methylgambierone, a new gambierone analogue isolated from Gambierdiscus australes. Tetrahedron Lett. 60, 621–625.
Oshiro, N., Nagasawa, H., Watanabe, M., Nishimura, M., Kuniyoshi, K., Kobayashi, N., Sugita-Konishi, Y., Asakura, H., Tachihara, K., Yasumoto, T., 2022. An extensive survey of ciguatoxins on grouper Variola louti from the ryukyu Islands, Japan, using liquid chromatography–tandem mass spectrometry (LC-MS/MS). J. Mar. Sci. Eng. 10, 423.
Otero, P., Pérez, S., Alfonso, A., Vale, C., Rodríguez, P., Gouveia, N.N., Gouveia, N., Delgado, J.o., Vale, P., Hirama, M., Ishihara, Y., Molgó, J., Botana, L.M., 2010. First toxin profile of ciguateric fish in Madeira arquipelago (europe). Anal. Chem. 82, 6032–6039.
Pisapia, F., Holland, W.C., Hardison, D.R., Litaker, R.W., Fraga, S., Nishimura, T., Adachi, M., Nguyen-Ngoc, L., Séchet, V., Amzil, Z., Herrenknecht, C., Hess, P., 2017a. Toxicity screening of 13 Gambierdiscus strains using neuro-2a and erythrocyte lysis bioassays. Harmful Algae 63, 173–183.
Pisapia, F., Sibat, M., Herrenknecht, C., Lhaute, K., Gaiani, G., Ferron, P.-J., Fessard, V., Fraga, S., Nascimento, S.M., Litaker, R.W., Holland, W.C., Roullier, C., Hess, P., 2017b. Maitotoxin-4, a novel MTX analog produced by *Gambierdiscus excentricus*. Mar. Drugs 15, 220.
Richter, D.J., Watteaux, R., Vannier, T., Leconte, J., Frémont, P., Reygondeau, G., Maillet, N., Henry, N., Benoit, G., Da Silva, O., Delmont, T.O., Fernàndez-Guerra, A., Suweis, S., Narci, R., Berney, C., Eveillard, D., Gavory, F., Guidi, L., Labadie, K., Mahieu, E., Poulain, J., Romac, S., Roux, S., Dimier, C., Kandels, S., Picheral, M., Searson, S., Tara Oceans, C., Pesant, S., Aury, J.-M., Brum, J.R., Lemaitre, C., Pelletier, E., Bork, P., Sunagawa, S., Lombard, F., Karp-Boss, L., Bowler, C., Sullivan, M.B., Karsenti, E., Mariadassou, M., Probert, I., Peterlongo, P., Wincker, P., de Vargas, C., Ribera d'Alcalà, M., Iudicone, D., Jaillon, O., 2022. Genomic evidence for global ocean plankton biogeography shaped by large-scale current systems. Elife 11, e78129.
Rodríguez, I., Genta-Jouve, G., Alfonso, C., Calabro, K., Alonso, E., Sánchez, J.A., Alfonso, A., Thomas, O.P., Botana, L.M., 2015. Gambierone, a ladder-shaped polyether from the dinoflagellate Gambierdiscus belizeanus. Org. Lett. 17, 2392–2395.
Satake, M., Ishibashi, Y., Legrand, A.-M., Yasumoto, T., 1996. Isolation and structure of ciguatoxin-4A, a new ciguatoxin precursor, from cultures of dinoflagellate Gambierdiscus toxicus and parrotfish Scarus gibbus. Biosci., Biotechnol., Biochem. 60, 2103–2105.
Satake, M., Murata, M., Yasumoto, T., 1993a. Gambierol: a new toxic polyether compound isolated from the marine dinoflagellate Gambierdiscus toxicus. J. Am. Chem. Soc. 115, 361–362.

Satake, M., Murata, M., Yasumoto, T., 1993b. The structure of CTX3C, a ciguatoxin congener isolated from cultured Gambierdiscus toxicus. Tetrahedron Lett. 34, 1975–1978.
Sibat, M., Herrenknecht, C., Darius, H.T., Roue, M., Chinain, M., Hess, P., 2018. Detection of pacific ciguatoxins using liquid chromatography coupled to either low or high resolution mass spectrometry (LC-MS/MS). J. Chromatogr. A. 1571, 16-28.
Smayda, T.J., 2007. Reflections on the ballast water dispersal—harmful algal bloom paradigm. Harmful Algae 6, 601–622.
Soliño, L., Costa, P.R., 2018. Differential toxin profiles of ciguatoxins in marine organisms: chemistry, fate and global distribution. Toxicon 150, 124–143.
Spielmeyer, A., Loeffler, C.R., Kappenstein, O., 2022. Identical Ciguatoxin-3C group profiles in Lutjanus bohar from the Pacific and Indian Oceans - indicating the need to re-evaluate geographical CTX classifications. Front. Mar. Sci. 9.
U.S. Food Drug Administration, 2020. Fish and Fishery Products Hazards and Controls Guidance, p. 498. Fourth Edition. https://www.fda.gov/media/80637/download.
Watanabe, R., Uchida, H., Suzuki, T., Matsushima, R., Nagae, M., Toyohara, Y., Satake, M., Oshima, Y., Inoue, A., Yasumoto, T., 2013. Gambieroxide, a novel epoxy polyether compound from the dinoflagellate Gambierdiscus toxicus GTP2 strain. Tetrahedron 69, 10299–10303.
Wu, Z., Lee, W.H., Liu, Z., Lin, S., Lam, P.K.S., 2022. Microbiome associated with Gambierdiscus balechii cultures under different toxicity conditions. Front. Mar. Sci. 9.
Yasumoto, T., Igarashi, T., Legrand, A.-M., Cruchet, P., Chinain, M., Fujita, T., Naoki, H., 2000. Structural elucidation of ciguatoxin congeners by fast-atom bombardment tandem mass spectroscopy. J. Am. Chem. Soc. 122, 4988–4989.
Yogi, K., Oshiro, N., Inafuku, Y., Hirama, M., Yasumoto, T., 2011. Detailed LC-MS/MS analysis of ciguatoxins revealing distinct regional and species characteristics in fish and causative alga from the pacific. Anal. Chem. 83, 8886–8891.
Yokoyama, A., Murata, M., Oshima, Y., Iwashita, T., Yasumoto, T., 1988. Some chemical properties of maitotoxin, a putative calcium channel agonist isolated from a marine dinoflagellate. J. Biochem. 104, 184–187.
Yon, T., Sibat, M., Robert, E., Lhaute, K., Holland, W.C., Litaker, R.W., Bertrand, S., Hess, P., Réveillon, D., 2021. Sulfo-gambierones, two new analogs of gambierone produced by Gambierdiscus excentricus. Mar. Drugs 19.
Yong, H.L., Mustapa, N.I., Lee, L.K., Lim, Z.F., Tan, T.H., Usup, G., Gu, H., Litaker, R.W., Tester, P.A., Lim, P.T., Leaw, C.P., 2018. Habitat complexity affects benthic harmful dinoflagellate assemblages in the fringing reef of Rawa Island, Malaysia. Harmful Algae 78, 56–68.