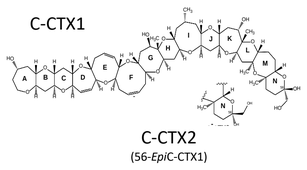
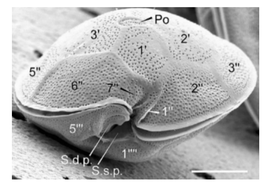


Cell growth and toxin production of *Gambierdiscus* spp. strains from the Macaronesian Region



F. Pisapia[1], C. Marrero Alemán[1*], E. Soler Onis[2], J. Fernández-Zabala[2], F. Acosta[3], J. Bravo[3], E. Portillo Hahnefeld[1] and P. Assunção[1]

[1]Department of Biotechnology, Technological Institute of the Canary Islands (ITC), Playa de Pozo Izquierdo, s/n, 35119 - Pozo Izquierdo, Gran Canaria, Spain
[2]Banco Español de Algas (BEA), FPCT, University of Las Palmas, Muelle de Taliarte, s/n, 35215 - Telde, Gran Canaria, Spain
[3]University Institute of Aquaculture and Sustainable Marine Ecosystems (IU-ECOAQUA), University of Las Palmas, 35200 - Telde, Gran Canaria, Spain
E-mail: carlos1993.18@gmail.com Tel: +34646525648

Introduction

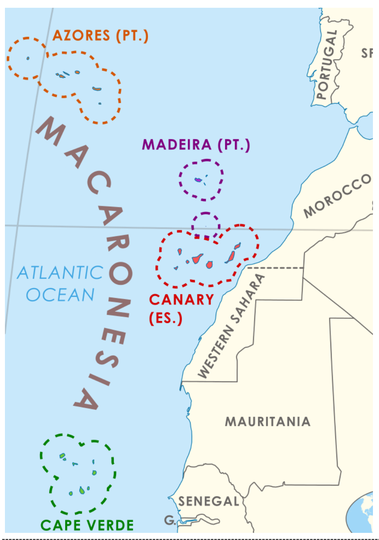


Fig. 1. Map of the Macaronesian Region.

Ciguatera in the Macaronesian Region

- Ciguatera poisoning is the most common non-bacterial food-borne intoxication, mostly distributed in the tropical and subtropical areas.
- Cases of ciguatera have being reported in the Macaronesian Region since 2004 (**Fig. 1**).
- *Gambierdiscus* and *Fukuyoa* spp. are considered the causative agents of ciguatera, as they consitute the primary producers of ciguatoxins (CTXs).
- CTXs are lipophilic toxins which are bioaccumulated and biotransformed in fish through the marine trophic chain (**Fig. 2**).

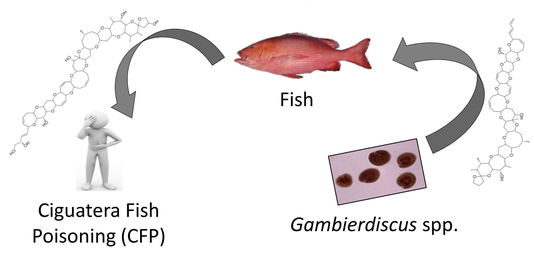


Fig. 2. Schematic route of intoxication of Ciguatera Fish Poisoning.

Gambierdiscus and *Fukuyoa* spp.

- Epi-benthic dinoflagellate genera with huge biological diversity.
- Difficult and slow-growing micro-organisms in laboratory (μ_{max} < 0.3 divisions day^{-1}).
- Considerable differences in toxin production among species (or even strains!) → ≠ amounts and congeners.
- Five species and one phylotype of *Gambierdiscus* have been identified in the Canary Islands since 2004 (**Fig. 3**).
- Three of them have been firstly described from Canarian waters.

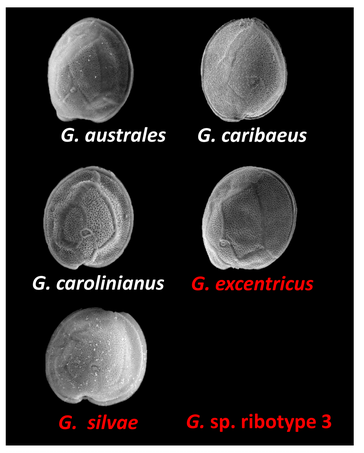


Fig. 3. Species and phylotypes of *Gambierdiscus* reported in the Canary Islands. In red the ones that were firstly described from this region.

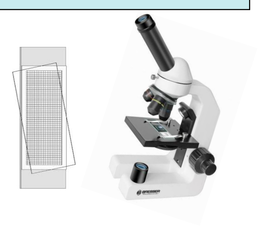
Aims of the study

1. Optimization of *Gambierdiscus* growth under laboratory-controlled conditions:
   – Study of different surfaces for *Gambierdiscus* culturing.
2. Finding the toxin-producing potential of our strains for biotechnological applications:
   – Determination of CTX activity using funcional bioassays.

Material & Methods

Cell growth

Semi-continuous batch cultures



- Five strains of *Gambierdiscus* isolated from the Canary Islands (**Table I**) were cultured under the same laboratory conditions (**Table II**).
- Cell growth was estimated counting the cells once per week, using a 1 mL Sedgewick-Rafter counting chamber under a light microscope.

Table I: List of the strains used in this study, with species identification and geographical origin.

Strain	Species	Origin
OCH85	*G. australes*	**Charcón (Lanzarote)**
OCH141	*G. australes*	**Las Galletas (Tenerife)**
OCH100	*G. carolinianus*	**Charcón (Lanzarote)**
OCH45	*G. excentricus*	**Valle Gran Rey (La Gomera)**
OCH92	*G. excentricus*	**Montaña Amarilla (La Graciosa)**

Table II: Laboratory conditions used for *Gambierdiscus* cultures in this study.

Culture conditions	
Temperature	25 °C
Salinity	32
Culture medium	F/5 (w/ Se, w/o Si)
Photoperiod	12 h/12 h (D/N)
Light intensity	60 µmol m^{-2} s^{-1}

Culture surfaces

- *G. australes* OCH85 and OCH141 were cultivated using 75 cm^2 horizontal flasks presenting four different bottom surfaces made of polystyrene (**Table III**, **Fig. 4**).
- Specific growth rate (µ, d^{-1}) was the slope calculated by the linear regression of the natural logarithm of the cell concentration versus time, after correcting for serial culture dilutions.

Table III: List of the types of polystyrene surface used in this study, with abbreviation code, binding interaction, charge and impact on cell attachment.

Surface type	Code	Binding interaction	Charge	Cell attachment
(a) Non Treated	NT	Hydrophobic	/	/
(b) Tissue-Culture Treated	T	Hydrophilic	-	Favored
(c) CellBIND	CB	Hydrophilic	- -	Improved
(d) Ultra-Low Attachment	ULA	Hydrophilic	/	Prevented (hydrogel layer)

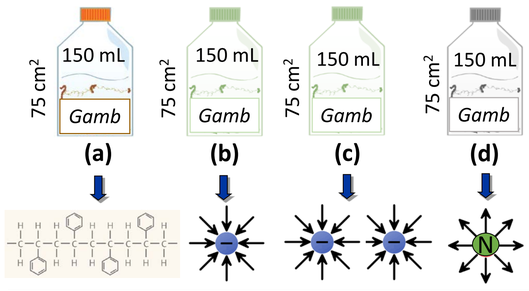


Fig. 4. Types of polystyrene surface used in this study: **(a)** Non Treated (NT), **(b)** Tissue-Culture Treated (T), **(c)** CellBIND (CB), and **(d)** Ultra-Low Attachment (ULA).

CTX extraction and pre-purification

- *Gambierdiscus* cells were harvested in the log-phase growth and ciguatoxins (CTXs) were extracted and pre-purified according to the protocol represented in **Fig. 5**.

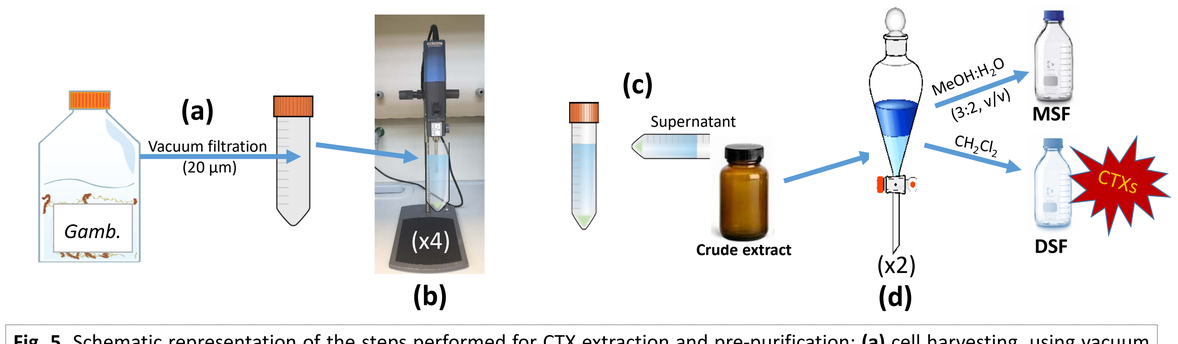
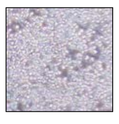


Fig. 5. Schematic representation of the steps performed for CTX extraction and pre-purification: **(a)** cell harvesting, using vacuum filtration on 20 µm nylon disc filters; **(b)** cell disruption in MeOH (x2) and MeOH:H_2O (1:1, v/v) (x2), using a sample homogeneizer (Ultra-Turrax IKA basic T18); **(c)** centrifugation (4000 x *g*, 10', 4 °C) and recovery of the supernatant (i.e. crude extract); **(d)** pre-purification step: liquid-liquid partitioning (x2) between MeOH:H_2O (3:2, v/v) and CH_2Cl_2 (DCM). The lipophilic CTXs were partitioned into the DCM soluble fraction (DSF).

CTX assessment



Neuro-2a bioassay (Caillaud et al., 2010)
Mouse neuroblastoma cells (*Mus musculus*) in 96-well microplates (2.500.000 cells mL^{-1}).

1. **Pre-treatment**: Ouabain (O: 10 mM) and Veratridine (V: 1 mM) → sensitization of the n2a cells to CTXs.
2. **Treatment**: Pacific CTX1B std (R. J. Lewis) or *Gambierdiscus* DSFs.
3. **Incubation**: 24 h (37 °C, 5% CO_2).
4. **Colorimetric MTT assay**: absorbance at 570 nm (formazan) → survival estimation of n2a cells.
5. **Data analysis**: HillSlope model (GraphPad Prism, v 6.01) (**Fig. 6**).

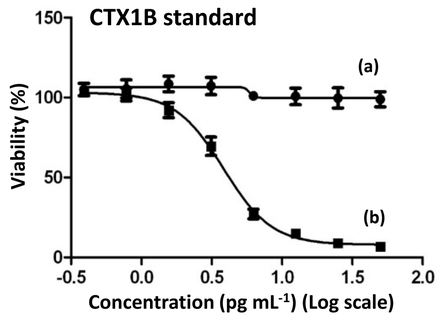


Fig. 6. Viability (%) of neuro-2a cells in response to CTX1B std, in absence **(a)** and in presence **(b)** of the O/V sensitization.

Preliminary Results & Discussion

Cell growth

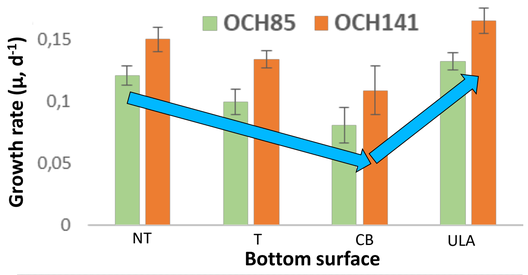


Fig. 7. Specific growth rates (µ, d^{-1}) of *G. australes* OCH85 (in green) and OCH141 (in orange) in Corning® culture flasks with four different bottom surfaces, i.e. Non-Treated (NT), Tissue-Culture Treated (T), CellBIND (CB) and Ultra-Low Attachment (ULA).

- All the different surfaces were suitable for cell growth.
- *G. australes* strains behaved as slow growers (µ < 0.17 d^{-1}).
- *G. australes* OCH141 exhibited slightly higher µ than *G. australes* OCH85 in all the conditions tested (**Fig. 7**).
- The treatments for cell adhesion seem to disfavor growth → lower µ in T and CB flasks (**Fig. 7**).
- The fastest growth was observed in ULA flasks (**Fig. 7**).

→ Statistical analyses are needed to determine whether the different substrates have an authentic impact on cell growth.

→ Estimation of µ of *G. carolinianus* and *G. excentricus* strains is currently ongoing.

CTX assessment

Table IV: Qualitative assessment of the CTX-activity exhibited by our *Gambierdiscus* strains on the n2a assay .

Strain	CTX-activity
G. australes OCH85	+/-
G. australes OCH141	-
G. carolinianus OCH100	-
G. excentricus OCH45	++
G. excentricus OCH92	+

- *G. carolinianus* OCH100 and *G. australes* OCH141 showed no detectable CTX-activity at any of the concentrations tested (**Table IV**).
- Some low CTX activity was observed in *G. australes* OCH85, at the highest concentration only.
- Both *G. excentricus* strains were clearly positive for the presence of CTXs (**Table IV**).

→ *G. excentricus* species is likely to produce more CTXs than the others, in accordance with previous studies (Pisapia et al., 2017; Litaker et al., 2017).

Conclusions

- More investigation is needed in recent hotspots of ciguatera, e.g. the Macaronesian Region.
- The surface of the culture vessel may affect the growth of *Gambierdiscus* in laboratory (what about CTX production?).
- *Gambierdiscus excentricus* seems the most promising species for the discovery of Atlantic CTXs.

Perspectives

- Determination of µ and optimization of cell growth.
- Development of culture scale-up strategies → ↑ biomass.
- Quantification of CTX production using biological assays.
- Discovery of new CTX congeners from the Atlantic: bioguided fractionation + chemical characterization (LC-HRMS/MS, NMR).

References

- Caillaud et al. (2010). Detection and quantification of maitotoxin-like compounds using a neuroblastoma (Neuro-2a) cell based assay. Application to the screening of maitotoxin-like compounds in *Gambierdiscus* spp. *Toxicon*, *56* (1), 36-44.
- Litaker et al. (2017). Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. *PLoS One 12*.
- Pisapia et al. (2017). Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays. *Harmful Algae* 63.

ACKNOWLEDGMENTS. The study presented here is part of the MIMAR project: "Monitoring, control and mitigation of the proliferation of marine organisms associated with human disturbances and climate change in the Macaronesian Region (MAC/4.6D/066)" (2017-2020).