EXTERNAL SCIENTIFIC REPORT

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Evaluation of ciguatoxins in seafood and the environment in Europe

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in collaboration with

Regional Fisheries Management-Madeira Government, DSI-DRP[7], and Instituto das Florestas e Conservação da Natureza, IP-RAM, Secretaria Regional do Ambiente, Recursos Naturais e Alterações Climáticas, Regional Government of Madeira[8]

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Abstract

The present document corresponds to Deliverable No. 10: "Final report (1st of April 2016-31st October 2020)." of the specific agreement "EVALUATION OF CTXs IN SEAFOOD AND THE ENVIRONMENT for the RISK ASSESSMENT OF CIGUATERA FISH POISONING (CFP), with the consequent OBTENTION OF REFERENCE MATERIAL" within the Framework Partnership Agreement GP/EFSA/AFSCO/2015/03 "Risk characterization of ciguatera food poisoning in Europe"

Key words: ciguatera, ciguatoxins, gambierdiscus, fish.

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Summary

Execution of specific grant 3 within the Eurocigua project has found no major difficulty and globally, the project has been run according to the expected timeline. All the agreed deliverables (n=10) have been submitted to EFSA. The major results obtained are described below.

Harmonization of extraction procedures for toxins in microalgae and fish, and of the cell-based assay was achieved between IRTA and ULPGC. A better characterisation of risk posed by microalgae and fish in Macaronesia (Canary Island and Madeira) and the Mediterranean Sea (Crete, Cyprus and Balearic Islands) have been achieved.

Among the studied areas, the Canary Islands constitute by far the area representing the higher risk. Presence of several *Gambierdiscus* species cover the whole archipelago, and the toxicity of the species, particularly *G. excentricus*, indicate their potential as source of CTX-like compounds. As for fish, according to the data on CTX toxicity, there is quite a high incidence of toxic fish (14% of a total of n=746 fish samples) (data from the project).

Regarding Madeira and Selvagens islands, the genus *Gambierdiscus* has been detected in both areas. Toxicity of fish has been identified in 42 out of 128 fish (33%). Primary reference material containing CTXs has been achieved and transferred to U. Vigo (SG4). Efforts in Macaronesia should be centred for a better prediction of ciguatera poisoning cases, and link these to the ecology of microalgae and fish.

From the eastern Mediterranean Sea, a great diversity of *Gambierdiscus* and *Fukuyoa* taxa was detected (at least 6 different taxa) but relatively low cell toxicity was found in the examined isolates. The first fish CTX-like positive by Neuro-2A from the Mediterranean has been detected in Cyprus with a low concentration.

From the Balearic Island, *Gambierdiscus* was identified for the first time in 2017. Up to date, only *Gambierdiscus* australes and *Fukuyoa Paulensis* have been identified. From this area, all fish showed no CTX-like toxicity. Efforts in the Mediterranean Sea should be centred on the ecology of ciguatera. The study of these populations, using quantitative techniques over different spatial and time ranges, is necessary to better acquaint the risk these populations represent.

A complete ENDNOTE database on bibliography of Ciguatera, including more than 2500 references has been submitted, and it includes labels and keywords. From this database, an analysis of references focusing on modelling, and a list of available biological, oceanographic and meteorological data among others has been provided.

It can be concluded that SG3 has succeeded in achieving all the deliverables requested by EFSA, it has provided a very significative advancement on the comprehension of ciguatera in Macaronesia and the Mediterranean, it has provided SG4 with primary reference material needed, and has contributed to conduct an extensive literature and data search for the future modelling and prediction of ciguatera in these regions.

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1. Introduction

In the frame of the EUROCIGUA project, and according to the task defined within the SA3, all the institutions participating in SG3 covered successfully all of the predicted work.

The present document details the main achievements attained during the four years of the project. These achievements include the identification of areas where the genus *Gambierdiscus* is prevalent, the establishment of *Gambierdiscus* strains for algae culture collection, and an array of selected target fish species for subsequent CTX analysis.

1.1. Background and Terms of Reference as provided by the requestor

This contract/grant was awarded by EFSA to: Institute for Research and Technology in Food and Agriculture (IRTA)

Contractor/Beneficiary: Institute for Research and Technology in Food and Agriculture (IRTA), Universidad de Las Palmas de Gran Canarias (ULPGC), Canary Health Service (Servicio Canario de la Salud, SCS), Instituto Português do Mar e da Atmosfera I.P (IPMA), State General Laboratory (SGL) / Ministry of Health, Aristotle University of Thessaloniki (AUTH).

Contract/Grant title: Risk characterization of ciguatera food poisoning in Europe

SPECIFIC AGREEMENT 3: EVALUATION OF CTXs IN SEAFOOD AND THE ENVIRONMENT for the RISK ASSESSMENT OF CIGUATERA FISH POISONING (CFP), with the consequent OBTENTION OF REFERENCE MATERIAL

Contract/Grant number: Framework Partnership Agreement GP/EFSA/AFSCO/2015/03

1.2. Interpretation of the Terms of Reference (if appropriate)

The present document corresponds to Deliverable No. 10:

Final report (1st of April 2016- 31th of October 2020).

1.3. Additional information (if appropriate)

The presentation deliverable regarding the advancement of work within SA3 is displayed according to the order of tasks described in the SA3 Agreement and that have been set according to the following chronogram.

TASK		PARTNER	YEAR 1				YEAR 2				YEAR 3				YEAR 4			
1	**Standardization of the cell-based assay (CBA)**	IRTA, ULPGC																
2	**Sampling, culture, identification and toxicity evaluation of Gambierdiscus spp.**	IRTA, IPMA, AUT																
2.1	Sampling of Gambierdiscus spp.	IRTA, IPMA, AUT																
2.2	Collection of environmental data at sampling sites and from local studies when available	IRTA, IPMA, AUT																
2.3	Establishment of Gambierdiscus spp. low scale- cultures	IRTA, IPMA, AUT																
2.4	Identification of Gambierdiscus spp. by morphological and genetical analysis	IRTA, IPMA, AUT																
2.5	Evaluation of the presence /absence of CTX-like toxicity in Gambierdiscus spp.	IRTA																
2.6	Large scale cultures of selected Gambierdiscus spp. strains producing toxins in order to obtain phase 1 reference material	IRTA																
2.7	Extraction and purification of *Gambierdiscus* spp. biomass	IRTA																
3	**Sampling of fish and CTX evaluation**	IRTA, ULPGC, SCS, IPMA, AUT, SGL, RMFLAW, DRPM																
3.1	Sampling of fish	IRTA, ULPGC, SCS, IPMA, AUT, SGL, RMFLAW, DRPM																
3.2	Extraction and purification of fish	IRTA, ULPGC, IPMA																
3.3	Evaluate the presence of CTXs in different fish tissues	IRTA, ULPGC																
4.	**Define areas at risk for ciguatera**	IRTA, ULPGC, SCS, IPMA, AUT, SGL																
5	**Literature search**	IRTA, ULPGC, SCS, IPMA, AUT, SGL																
5.1	Identify sources of data	IRTA, ULPGC, SCS, IPMA, AUT, SGL																
5.2	Establish a database of scientific literature	IRTA, ULPGC, SCS, IPMA, AUT, SGL																
5.3	Define different modelling strategies	IRTA																
6	**Management**	IRTA																

Advancement of the project is described in order to present the contributions of each partner within SG3. Nevertheless, some results have been obtained through collaboration of different partners.

Partners:
Institut de Recerca i Tecnologia Agroalimentàries (IRTA)
Universidad de Las Palmas de Gran Canarias (ULPGC), Spain;
Canary Health Service (Servicio Canario de la Salud, SCS), Spain;
Instituto Portugues do Mar e da Atmosfera (IPMA), Portugal;
State General Laboratory (SGL), Cyprus;
Aristotle University of Thessaloniki (AUT), Greece;

Subcontractor:
NEXA, La Réunion, France.

Collaborators:
Regional Ministry of Agriculture, Livestock, Fisheries and Water of the Canary Islands Government, Spain (RMALFW)
Direção Regional das Pescas of Madeira (DRPM)

2. Advancement of work

2.1. Standardization of the cell-based assay (IRTA, ULPGC)

2.1.1. Optimization and standardization of the CBA

A standardized method for the evaluation of CTXs with a cell based assay (CBA) has been developed and has been presented in Deliverable 3.1: Description of the extraction procedure for CTXs considering the different matrixes (microalgae, fish flesh and fish liver) and of the standardized screening cell based assay for CTXs to be used by IRTA and partners.

Briefly, the assay consists on the exposure of Neuro-2A cells to a CTX standard and to the extracts of microalgae or fish to be tested. The extracts obtained are dissolved in cell culture medium. Neuro-2A cells are exposed to the medium with extracts and after 24 h evaluation of cell viability is performed. The amount of CTXs in the extract is proportional to a decrease in cell viability. In the presence of ouabaine (O) and veratridine (V), CTXs will reduce the viability of cells proportionally to its concentration. The calibration curve for CTX, obtained after exposing the cells to increasing concentrations of CTX standard, will allow to estimate the concentration of CTXs in the sample according to the effect produced by microalgal or fish extracts on the cells.

This assay is presently implemented at the laboratories of ULPGC and IRTA (within SA3) but also by the Universidad de Vigo (SA4).

At the beginning of the project, IUSA (ULPGC) laboratory was working with two protocols of extraction of CTX from samples. These protocols (P1 and P2) have been included in the deliverable 3.1 with a more detailed description. Only protocol P2 DEE-HX (option 2) is being implemented in IUSA (ULPGC) laboratory.

For the purpose of achieving standardization of CBA, big efforts have been done by IRTA and ULPGC in order to harmonise the results obtained for samples analysed by both laboratories. A positive P-CTX-1 standard of reference (Richard Lewis, University of Queensland, Australia) is always included when implementing the method.

This method was applied for the screening of all the samples received at IRTA and UPLGC's laboratory. In addition, it offers an approximation of the quantity of toxin contained in the sample, based on the curve of cytotoxicity. That means that, the method offers a semi-quantification of the toxin contained in the sample. We are considering a level of toxicity: <20 mg eq/ml (high), 20-40 mg eq/ml (medium) and > 40 mg eq/ml (low). A more accurate analysis in terms of quantification of CTX contained in every sample analysed is subsequently conducted at the University of Vigo (SA4).

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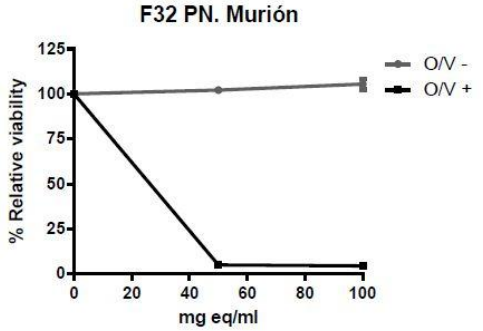

Fig 1. ULPGC: Cytoxicity of positive sample (F32) with the Neuro-2A assay.

The CBA results obtained with the samples from the Canary Islands carried out by ULPGC and IRTA are detailed in the excel_2020_EUROCIGUA_SG3_FISH_TOXICITY, made for the purpose of standardizing the CBA methodology. Results obtained by University of Vigo for some of these samples are included in that file, in order to coordinate the results obtained between specific grant 3 and specific grant 4.

A second Neuro2A cell-based intercomparative assay between IRTA and ULPGC was performed in 2019. Five fish samples were selected to compare the CBA assay results obtained in both laboratories. Samples selected were: EFSA-ULPGC-F0120, EFSA-ULPGC-F0178, EFSA-ULPGC-F0203, EFSA-ULPGC-F0215 and EFSA-ULPGC-F0319. Summarized results obtained can be seen in Table 1.

Table 1. Summary results from N2A cell-based assay 2nd intercomparative exercise.

Id. Code	**EuroCigua No.**	**Species**	**IUSA (µg/kg) CTX-1B**	**IRTA (µg/kg) CTX-1B**
IUSA 1	EFSA-ULPGC-F0120	Dusky grouper	0.077	0.088
IUSA 2	EFSA-ULPGC-F0178	Amberjack	0.761	0.450
IUSA 3	EFSA-ULPGC-F0203	Amberjack	0.207	0.164
IUSA 4	EFSA-ULPGC-F0215	Dusky grouper	0.327	0.312
IUSA 5	EFSA-ULPGC-F0319	Amberjack	0.098	0.059

Results shows that the Neuro-2A cell-based assay is well implemented in both laboratories and results obtained from the five samples are on the same range in both laboratories.

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2.2. Sampling, culture, identification and toxicity evaluation of *Gambierdiscus* spp. (IRTA, IPMA, AUT)

2.2.1. Sampling of *Gambierdiscus* spp. (IRTA, IPMA, AUT)

Methodology of samplings

A total of two different samples in each sampling station (macroalgae and scrap of rocks or sand) were taken. The sample consisted of scrapping on the benthic zone, either on the rocks, sand or macroalgae. Once samples were collected the algae were shaken in seawater. The water of the scraping or that of the algae, was filtered (200 µm mesh) to isolate the communities of epiphytic microalgae from larger particles or organisms.

The filtered water obtained, was stored in two bottles; one with a volume of 60 mL which was preserved in lugol solution and one sample with a volume of 125 mL was kept alive. Finally, each bottle was identified with the corresponding data and station number.

For the abiotic data, we used a multiparameter probe (YSI 556 MPS) to measure salinity, oxygen (% and mg / L), temperature, pH and depth. The coordinates of each sampling station were recorded by GPS.

Fig 2. Filtering water from the scraping of rocks.

Fig 3. Filtering water from the algal sample.

Fig 4. Preservation of the sample in lugol solution.

Fig 5 and 6. Materials used in the sampling.

Fig 7. Sampling point (El Puertillo) Gran Canaria.

CANARY ISLANDS (IRTA)

In October 2016, samples were collected at several locations in the Canary Islands: Gran Canaria, Fuerteventura and Lanzarote. Details are presented in **Annex A**.

During 2017 (April and October), sampling of microalgae was performed in Gran Canaria, La Palma, Tenerife, El Hierro and La Gomera. An intensive campaign was performed and samples and environmental data were recorded (**See Annex B and Annex C**). As a result of the samplings in 2016 and 2017, and the establishment of the number of *Gambierdiscus* spp. cultures set in the work programme, the sampling in the Canary Islands was successfuly concluded.

MADEIRA AND SELVAGENS ISLANDS (IPMA)

Seawater samples were obtained from bottom substrate and macroalgae during October and November, 2016 in different sites of Madeira, Porto Santo and Desertas Island. Information regarding sampling date and sites are presented in **Annex D**.

Initially, most sampling sites are located leeward in the southern area of the islands. Accessible, moderately sheltered areas characterised by smooth and hard substrate with red macroalgae coverage were selected as sampling stations. Two samples were collected in each site and shipped to IPMA: one seawater sample (min. 1.5L), collected from the bottom with a niskin bottle and another of macroalgae, mostly red macroalgae, harvested from the bottom.

Phytoplankton analysis was carried out at IPMA (Lisbon) the day following the arrival. Cells of *Gambierdiscus* were isolated from seawater samples, concentrated by reverse filtration and shaken in plastic bags to release epiphytic cells from the surface of macroalgae. The genus was identified morphologically under a Leica DM80 light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with phase contrast and differential interference contrast. All the water concentrated was screened to count, measure and isolate cells for subsequent culturing. No samples were preserved for other counting approaches yet, like the Utermohl method, because these were the first samples from the project and very low abundances, that would not allow appropriate estimation of cell numbers, were expected. Furthermore *Gambierdiscus*

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cells have very low motility under the microscope which prevents counting duplication and allows immediate isolation. The actual sampling plan already contemplates colleting preserved water samples and larger volumes of unpreserved seawater and macroalgae.

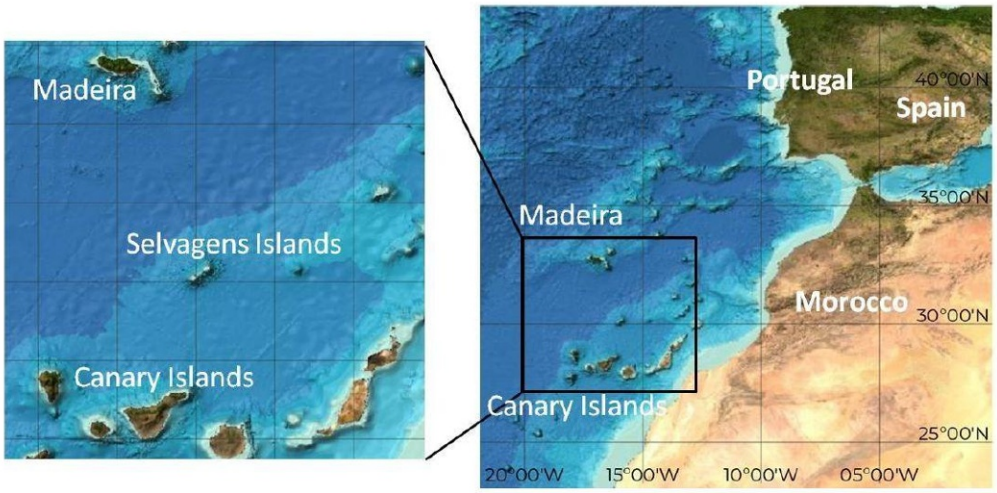

Fig 8. Location of Selvagens Islands.

In 2017, collection of seawater samples for assessing the presence, variability and isolation of *Gambierdiscus* was carried out from early August to late November 2017. Information regarding sampling date and sites are presented in **Annex E**.

The sampling strategy consisted of two steps, being the objective of the first step to identify the potential hot-sposts of *Gambierdiscus* occurrence around Madeira Island. The second step consisted of intensifying the sampling effort on the selected sites, namely Cais do Lazareto, near Funchal Bay (south coast), and Porto Moniz (north coast), both in Madeira Island, and in Cagarras Bay in Selvagem Grande (Fig. 8). Samples of Cais do Lazareto and Selvagens Islands were then tentatively collected on a fortnightly basis, and from Porto Moniz on a more occasional frequency.

Seawater sampling was carried out following the protocol described below:

- A seawater sample was obtained by means of a hand-plankton net (20 um) for isolation of *Gambierdiscus*.
- Seawater samples preserved with a Lugol solution were collected with a hose from the bottom to quantify the *Gambierdiscus* cells abundance
- Samples of macroalgae from the genus *Dyctiota*, *Padina*, *Ulva*, *Jania* and *Corallina*, which had previously shown to hold high abundances of *Gambierdiscus* in areas of the Atlantic and Pacific (Delgado et al., 2006; Parsons et al., 2012; Parsons and Preskitt, 2007; Rains and Parsons, 2015; Yasumoto et al., 1979) were collected and kept in labeled bags.
- Samples were shaken for at least 10 seconds to release phytoplankton from algae and then sieved through a 200 µm filter. Part of the filtrate was preserved with a preservative (lugol) and other was kept alive. Samples from plankton net were concentrated and processed in the same way. These samples were shipped to IPMA laboratories at Lisbon in order to estimate microalgae densities and establish new strains.

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To avoid spoiling of living samples, staff in the Selvagens and Madeira sent samples to Lisbon laboratories as soon as possible after sample collection. In case of Selvagens, samples were sent firstly to Madeira through the Navy ship that periodically dock in the island. Due to inappropriate weather conditions some of the scheduled sampling dates were missed as well as some samples got delayed in transit from Selvagens to Lisbon again due to worse atmospheric conditions, being discarded in the end.

In 2018, collection of seawater samples for assessing the presence, variability and isolation of *Gambierdiscus* was carried out from early May to late November 2018 in several locations around Madeira Island (**Annex F, Table 1**). Two samples from Selvagens Islands (Selvagem Grande) and one from Desertas Islands were also obtained. Seawater samples were collected with a hose from the bottom and preserved with a Lugol solution to afterwards quantify the *Gambierdiscus* cells abundance. Information regarding sampling date and sites are presented in **Annex F.**

A field campaign to Selvagens Islands was carried out in early September 2018. Sampling points were established according to their exposure and bottom features. Information regarding sampling date and sites are presented in **Annex F.** From this field trip 20 samples from macroalgae and surface water were collected for *Gambierdiscus* cell count. Plankton net samples were also obtained in each sampling point for a qualitative estimation of *Gambierdiscus* occurrence (**Annex F, Table 2 and 3**). At 5 sampling points, passive collectors made of a mosquito net were deployed at different depths for 18-96 hours. The deployment was comprised by a rectangle of 300 cm^2 with internal filament squares of 1.6 x 1.8 mm, attached to a rope with a weight and a buoy in each extreme. Two screens were hanged at distances of 0.5 and 3 m from the bottom (Fig. 9).

From this field campaign, 9 preserved samples obtained from macroalgae and 5 from passive collectors have been counted, up to date, to determine *Gambierdiscus* abundances per gram of macroalgae (wet weight) and cm^2 of screen (Tester et al., 2014; Jauzein et al., 2016). Macroalgae samples were collected on the bottom (algae attached to the seafloor) and in the water column (turf algae). Macroalgae species were determinate, when possible. Three additional samples from the water column (surface water) have been checked for *Gambierdiscus* densities (**Annex F, Table 4**).

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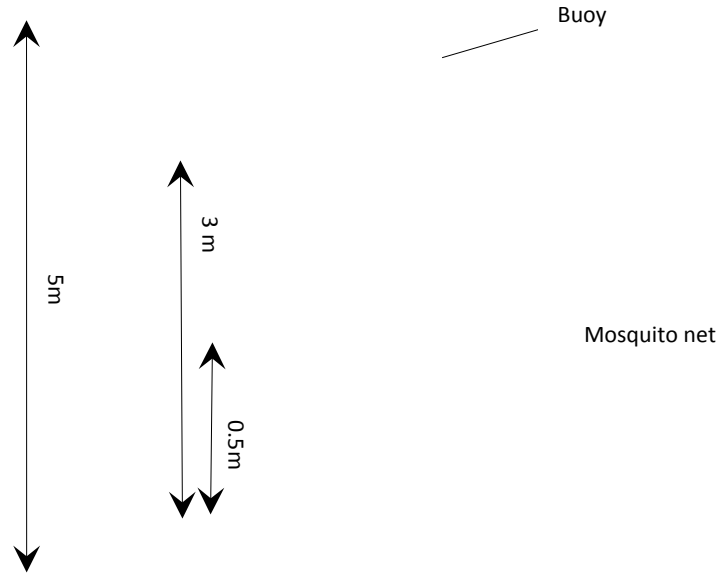

Figure 9. Representation of a passive collector for benthic microalgae sampling.

A third sampling campaing in Madeira Island was carried out in October 2019. Four sampling sites were selected in the north coast (Ponta Delgada, Seixal, Faial and Porto Moniz) and six sampling sites were selected in the south coast of the island (Machico, Caniçal, Funchal –Lido and Santiago –, Ribeira Brava, Calheta and Paul do Mar) (**Annex G**). As described above, seawater samples were collected with a hose from the bottom and preserved with a Lugol solution to quantify the Gambierdiscus cells abundance, and macroalgae samples were collected from the bottom to isolate *Gambierdiscus. Gambierdiscus* cells were also isolated after seawater sampling by means of a hand-plankton net (20 µm).

CRETE (AUT)

Preliminary samplings were carried out already in July and August 2016 in Crete. Information regarding sampling date and sites are presented in **Annex H.**

A second sampling was conducted at the two (2) already defined sampling stations: Kolympari S1, Kissamos S2 in July 2017. Information regarding sampling date and sites are presented in **Annex H.**

An additional sampling was conducted in Samos and Rhodes islands in August and September 2018 in the eastern/south eastern Aegean Sea. Information regarding sampling sites are presented in **Annex I.**

CYPRUS (SGL)

Two sampling efforts were performed, one in October and one in March (10/2016 & 03/2017). Information regarding sampling sites are presented in **Annex J.** The samples were processed at the Ecotoxicology Lab of SGL according to a defined protocol and through training provided

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by Mrs Aligizaki from AUT. In the first attempt it was possible to isolate *Gambierdiscus* spp. and AUT currently is in the process of establishing the cultures.

The second sampling (march, 2017) was carried out in order to try to increase the number of *Gambierdiscus* strains isolated from Cyprus during the 1st year (October, 2016), since the Cypriot strains presented several difficulties in both isolation and growth and was not successful, probably due to the unfavourable (cold) weather during sampling. A third sampling is planned to be carried out in the summer months. The sampling points were located in the coastal area of Cyprus and consisted of Limassol (Ag. Rafail, Ag. Tychon), Zygi (Larnaca District), Ayia Napa & Protaras (Famagusta District).

The first sampling in October 2016 was performed by a common team among AUT-Greece and SGL-Cyprus, with the aim (apart from the collection of samples) to train the local staff to be able to conduct samplings. Five (5) locations along the southern part of the island were sampled during the first day. All samples were examined the same afternoon at the Ecotoxicology lab of SGL and 3 out of 5 stations were selected. The next day the samplings were carried out in these 3 stations. Although it was planned to sample also towards the Ammochostos district (south eastern part), the samplings were restricted in the 3 stations mainly due to weather conditions (wind). In the areas of Ammochostos district, *Gambierdiscus* populations have been recorded in the past (K. Aligizaki, personal information) and for this reason the latter area was sampled in March. No *Gambierdiscus* populations were detected, possibly due to seasonal patterns.

A third sampling in Cyprus was carried out at the end of **September 2017**. The samples were processed at the Ecotoxicology Lab of SGL by K. Aligizaki. Information regarding sampling sites are presented in **Annex K.** Based on the results of the 2016 samplings the station S2-Ag. Tychon-Limassol was not further sampled since *Gambierdiscus* populations were not then detected. On the contrary, the stations S1-Ag. Rafail-Limassol and S2-Ag. Tychon-Limassol were again sampled since both *Gambierdiscus* and *Fukuyoa* populations were detected in 2016.

Furthermore, Ammochostos district (towards the south eastern part of Cyprus island) was selected in order to detect dense populations since *Gambierdiscus* populations have been recorded in the past (K. Aligizaki, personal information).

In this context, three (3) more stations were defined and sampled (S4-Ag. Napa, S5-Konnos bay, S6-Ammos Kampouri bay. In station S2-Ag. Tychon, no *Gambierdiscus* spp. nor *Fukuyoa* spp. populations were detected, while in S1-Ag. Rafail only *Fukuyoa* spp. populations were found. This is the first record of *Fukuyoa* spp. in the eastern Mediterranean Sea. In S3-Zygi only *Gambierdiscus* spp. populations were detected.

<u>BALEARIC ISLANDS (Formentera, Majorca and Menorca) (IRTA)</u>

In 2016, samples were collected at several locations in Formentera in the Balearic Islands Information regarding sampling sites are presented in **Annex L**. Diverse macroalgae and scraps of rocks or sand were collected in polystyrene bottles and shaken. After, the gross particles were removed by filtering (200 µm). Sample was stored in two bottles; one was preserved in lugol solution and one was kept alive for cell isolation.

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In 2017, the Balearic Islands (Majorca and Minorca) were sampled, and as a result of these samplings the presence *of G.australes* in the Balearic Island was reported. Information regarding sampling sites are presented in **Annex M.**

In 2018, the Balearic Islands (Majorca and Minorca) were sampled in order to confirm the reoccurrence of *Gambierdiscus* spp. and *Fukuyoa* spp.), as observed the previous year. Information regarding sampling sites are presented in **Annex N**. As a result*, Gambierdiscus* spp. and *Fukuyoa* spp*.* were recorded in Majorca and Menorca. For the isolates from the sampling in 2018 molecular identification of *Gambierdiscus* spp. showed that 20 isolates this sampling are *G. australes*. Therefore, from our results only one *Gambierdiscus* species have been reported in the Balearic Islands.

2.2.2. Collection of environmental data at sampling sites and from local studies when available (IRTA, IPMA, AUT)

CANARY ISLANDS (IRTA)

In the first sampling in the Canary Islands (Gran Canaria, Fuerteventura and Lanzarote) in 2016, at each sampling station different abiotic parameters were recorded with a multiparameter probe. Sampling data is summarized in **Annex A**.

Collection of environmental data during the second sampling (April 2017) in the Canary Islands (Gran Canaria) and third sampling (October 2017) in the Canary Islands (El Hierro, La Palma, Tenerife and La Gomera) were performed during sampling for microalgae. Different abiotic parameters are summarized in **Annex B and C**, respectively.

MADEIRA AND SELVAGENS ISLANDS (IPMA)

In the first sampling (October and november, 2016), environmental data at each sampling site were recorded by means of portable sensors (temperature, salinity and dissolved oxygen). Different abiotic parameters are summarized in **Table 1 in Annex D**.

Additional literature survey about sampling sites features was carried out. These studies indicate the sea surface temperature in sampling sites, usually ranges from 17ºC in February to 24ºC in September (Kaufmann and Böhm-Beck, 2013). In the three islands wind is predominantly from the north (April and September), northeast (January, February, March, June, July, August, October, November and December) and northwest in April and May. Wind speed varies from a minimum of 0 m s^{-1} in December to a maximum of 8 m s^{-1} in July. Stronger winds are during spring and early summer months (Caldeira et al., 2002). Sea breezes are frequent (84% of the days in 2006) from March to October in site 1 (Lopes et al., 2011).

In Madeira and Porto Santo Islands summertime average wave height were recorded to be 1-2 m with a frequency of 70% and wintertime average values are between 2-4 m with 65% of frequency.

The south of Madeira is general sheltered from the direct action of the waves and the dominant wave direction is from west and propagates in the southern part of the island (Rusu and Soares, 2012).

In the second sampling (early august - late november, 2017), environmental data at each sampling site were recorded whenever was possible and was performed by means of portable

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sensors (temperature, salinity and dissolved oxygen). Temperature varied between 21.9 and 24.2 ºC, salinity varied from 35.0 to 37.5 psu, and oxygen ranged from 6.6 to 7.0 mg L^{-1}. Different abiotic parameters are summarized in Table 2 in **Annex D**.

In the third sampling (early may to late november, 2018), seawater temperature and salinity were recorded in sampling sites of Selvagens Islands, and are summarized in **Annex F**.

Environmental data from field campaign carried out in the north and south coast of Madeira Island, as well as in Porto Santo and Desertas Islands is reported in Table 1 of **Annex G**.

CRETE (AUT)

The environmental data from the 1st sampling (november, 2016), as well as the full details for each sampling station in Crete and Cyprus, are presented in table 1, **Annex H**.

The environmental data from the 2nd sampling (July, 2017), as well as the full details for each sampling station in Crete are presented in the table 2, **Annex H**.

CYPRUS (AUT and SGL)

The environmental data from the 1st sampling (October 2016), as well as the full details for each sampling station in Cyprus, are presented in the tables 1, **Annex J**.

Based on the results of the first year's sampling, the 2nd sampling in 2017 was focused on two areas in Larnaca and Ammochostos district. The sampling was performed on September 2017 by a common team among AUT-Greece and SGL-Cyprus. Full details for each sampling station in Cyprus, are presented in the table 1, **Annex K**.

BALEARIC ISLANDS (Formentera, Majorca and Menorca) (IRTA)

Collection of environmental data during sampling in the Balearic Islands in 2016 was performed during sampling for microalgae. Environmantal data of Formentera in 2016 is summarized in **Annex L**.

Collection of environmental data during sampling in the Balearic Islands in 2017 was performed during sampling for microalgae. Information is summarized in Table 1 (Majorca) and Table 2 (Menorca) in the **Annex M**.

Collection of environmental data during sampling in the Balearic Islands in 2018 was performed during sampling for microalgae. Information is summarized in Table 1 (Majorca) and 2 (Menorca) in the **Annex N**.

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2.3 Establishment of *Gambierdiscus* spp. and *Fukuyoa* spp. low scale-cultures and maintenance cultures. (IRTA, IPMA, AUT)

As reported before, interest exists to isolate species of the genera *Gambierdiscus* and *Fukuyoa*. The latter, is a new genus that includes species previously belonging to the genus *Gambierdiscus*, within the group of Gonyaulacales. Some clarification is needed to understand the presentation of our results.

Gonyaulacales

The globular morphology of *Fukuyoa* species closely resembles that of the *Coolia* species when they are observed at low magnification. A morphological character useful to differentiate *Fukuyoa* from other Gonyaulacales is the shape in apical view: in *Fukuyoa* it is elliptical while in *Coolia*, *Alexandrium*, *Goniodoma* and *Gambierdiscus* it is round. The cell compression in *Fukuyoa* is slightly lateral. Other characteristics need to be observed at a higher magnification and the thecal plates have to be observed using calcofluor or sodium hypochlorite. Some of the strains have been observed using calcofluor but observation for others are pending. The strains pending for observation at higher magnification are temporarily classified as Gonyaulacales in this report.

The major tasks involved in the establishment of microalgal cultures were the following:

- Isolation and culturing of microalgae from the samplings.

- Maintenance and optimization of the culturing of microalgae established previously.

In IRTA, strains were isolated with a glass micropipette by the capillary method (Hoshaw and Rosowski 1973) under an inverted microscope (Leica DM-IL) Fig. 10. Isolated cells were incubated in 24 or 48 well microplates in ES Medium (Provasoli 1968) with salinity adjusted to 36 and incubated at 24 °C and irradiance of 100 µmol m^{-2} s^{-1} under 12:12 h L:D photoperiod Fig. 11. Cells were transferred to non-treated polystyrene flask of 25 mL when the culture reached a density of 20 cels/mL. Succesfully, after few weeks viable cultures were obtained and they were transfer to IRTA collection for duplicate in a glass test tubes of 10mL Fig. 12. Before harvesting alive sample was taken and checked under the microscope to see possible contaminants and irregular shapes or growth. As well as, a sample was fixed by lugol solution for counting the density of cells. Low-scale cultures were harvested in falcon tubes by centrifugation (4500 rpm x 20 min at 20 °C).

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Fig 10. Isolation of microalgae under sterile conditions.

Fig 11. Incubator in IRTA laboratories.

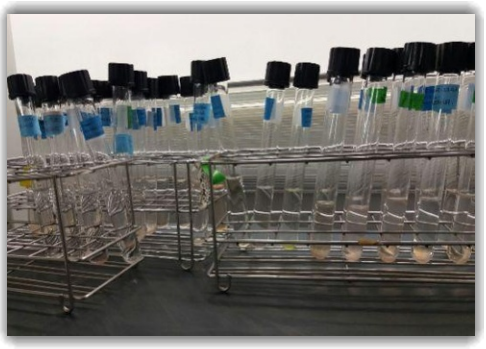

Fig 12. Algal collection in test tubes 10mL.

CANARY ISLANDS (IRTA)

In 2016, samplings in the Canary Islands were in Gran Canaria, Fuerteventura and Lanzarote (Oct. 2016) and in 2017 samplings were in Gran Canaria, La Palma, Tenerife, El Hierro and La Gomera (Abril 2017- Oct. 2017).

Gambierdiscus spp. were found in all the Canary Islands. *G. australes* was detected in all of the islands and *Gambierdiscus excentricus* was detected in Gran Canaria, La Palma, Tenerife and La Gomera. The highest number of recorded strains were in Gran Canaria although all of them were from two sampling points. In Fuerteventura only one *G. australes* strain was detected and isolated, being the island with the lesser number of strains. Currently, 46% of *Gambierdiscus* spp. isolates are alive and a total of 90 strains were established by IRTA laboratories and summarized in Table 2. Identification by morphology and molecular biology was performed (See section 2.4).

Table.2 Isolated and established strains of *Gambierdiscus* spp. by IRTA from samplings in the Canary Islands in October (2016), April (2017) and October (2017).

	Sampling Oct.2016				**Sampling April 2017**	**Sampling Oct.2017**				**Total (all islands)**
	HI	**GC**	**FV**	**LZ**	**GC**	**LP**	**TF**	**HI**	**LG**	
***Gambierdiscus* sp.**	0	0	0	2	18	1	8	7	1	37
G. australes	0	1	1	9	4	4	6	7	2	34
G. excentricus	0	0	0	0	3	3	4	0	7	17
G. caribaeus	1	0	0	0	0	0	0	0	0	1
G. belizeanus	0	0	0	0	0	0	0	1	0	1
Total established	**1**	**1**	**1**	**11**	**25**	**8**	**18**	**15**	**10**	**90**
Total Isolated	**1**	**1**	**1**	**12**	**26**	**40**	**47**	**45**	**23**	**196**

The observation of the samples under light microscopy showed that cells of *Gambierdiscus* spp. were found at 21 stations of the 53 stations sampled in the seven islands in 2016 and 2017. *Gambierdiscus* cells co-occurred with cells of the genera *Coolia, Ostreopsis, Prorocentrum, Amphidinium, Karenia* and *Trichodesmium,* among others. Details of the islands and the number of identifications for each station are shown in Figure 13. Overall*, G. australes* was the most abundant and it was present in all the islands. *G. excentricus* was the second most abundant. It was present in four islands including Gran Canaria, Tenerife, La Gomera, and La Palma, excluding the eastern islands (Lanzarote and Fuerteventura) and the western island (El Hierro). *G. caribaeus* and *G. belizeanus* were identified in El Hierro.

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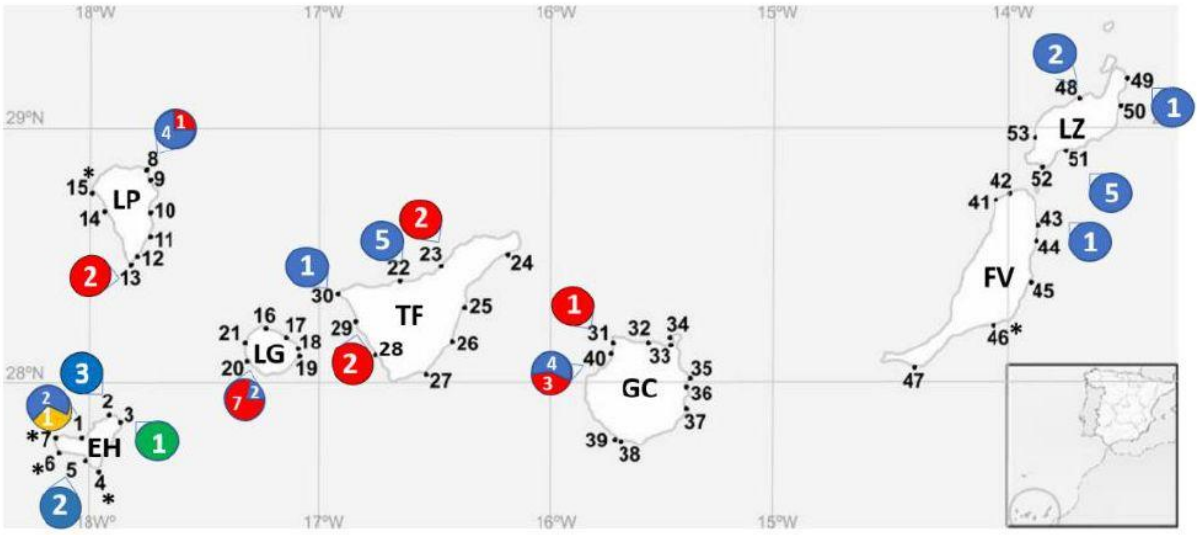

Fig.13 Distribution of each species in the stations of the Canary Islands during 2016-2017. Station numbers are presented in bold. The presence of *Gambierdiscus* species determined by molecular analysis is presented with a circle and includes the number of strains identified for each species. The asterisk representes the precence of *Gambierdiscus* sp. Colors of circles are for *G. australes* (blue), *G. excentricus* (red), *G. caribaeus* (green), and *G. belizeanus* (yellow). EH (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote) and TF (Tenerife).

MADEIRA AND SELVAGENS ISLANDS (IPMA)

In 2017, *Gambierdiscus* cells were isolated and maintained in a 4-well microplate with sterile-filtered seawater enriched with f/2-medium at 24 ºC ± 1 under 50 µmol photon m^{-2} s^{-1} (12:12 L:D). After a few weeks, the cells were transferred to a 25 mL flask and scaled-up once the culture reached a density of 30 cells/mL approximately. Strain data is displayed in Table 3.

Table 3. *Gambierdiscus* strains at IPMA's algal library.

Strain code	Isolation date	Site	Cell density (cell/mL)	Medium	Observations
GAMBI1_MAD_17	04/11/2017	Cais das cagarras Selvagens	36	F/2	All the strains were isolated from water samples
GAMBI2_MAD_17	04/11/2017	Cais das cagarras Selvagens	8	F/2	
GAMBI3_MAD_17	04/11/2017	Cais das cagarras Selvagens	5	F/2	

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GAMBI4_MAD_17	04/11/2017	Cais das cagarras Selvagens	4	F/2	
GAMBI5_MAD_17	23/11/2017	Cais do Lazareto, Madeira	6	F/2	
GAMBI6_MAD_17	23/11/2017	Cais do Lazareto, Madeira	4	F/2	

In 2018, from the samples described in **Table 1 in Annex F**, 68 strains were isolated and were maintained live for cultivation at IPMA facilities in Lisbon (Table 4A). Strains were kept in F/2-Si medium, temperature 23 º C, salinity 37, light:dark cycle of 12:12 h at 50 µmol m^{-2} s^{-1}.

Table 4A. List of *Gambierdiscus* spp strains isolated from Selvagens Islands

Code	Sampling Location	Isolation date
Gambi1	Selvagem Grande (Baía das cagarras (ponto 3, direito)	05/09/2018
Gambi2	Selvagem Grande (Baía das cagarras (ponto 3, direito)	05/09/2018
Gambi3	Selvagem Grande (Baía das cagarras (ponto 3, direito)	05/09/2018
Gambi4	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi5	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi6	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi7	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi8	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi9	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi10	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi11	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi12	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi13	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi14	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi15	Selvagem Pequena (Baía dos espanhóis rede (fundeadouro)	06/09/2018
Gambi16	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi17	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi18	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi19	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi20	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi21	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi22	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi23	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi24	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi25	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi26	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi27	Selvagem grande (Baía das cagarras-rede)	03/07/2018
Gambi28	Selvagem grande (Baía das cagarras-arrasto)	03/07/2018

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Gambi29	Selvagem grande (Baía das cagarras-arrasto)	03/07/2018
Gambi30	Selvagem grande (Baía das cagarras-arrasto)	03/07/2018
Gambi31	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi32	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi33	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi34	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi35	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi36	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi37	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi38	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi39	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi40	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi41	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi42	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi43	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi44	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi45	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi46	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi47	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi48	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi49	Selvagem grande (Baía das cagarras-arrasto)	15/06/2018
Gambi50	Selvagem pequena (Baía dos espanhóis rede (Fora)	07/09/2018
Gambi51	Selvagem pequena (Baía dos espanhóis rede (Fora)	07/09/2018
Gambi52	Selvagem pequena (Baía dos espanhóis rede (Fora)	07/09/2018
Gambi53	Selvagem pequena (Baía dos espanhóis rede (Fora)	07/09/2018
Gambi54	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi55	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi56	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi57	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi58	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi59	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi60	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi61	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi62	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi63	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi64	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi65	Selvagem grande (Baía das galinhas-rede)	07/09/2018
Gambi66	Selvagem grande (Baía das cagarras-7m)	07/09/2018
Gambi67	Selvagem grande (Baía das cagarras-7m)	07/09/2018
Gambi68	Selvagem grande (Baía das cagarras-7m)	07/09/2018

From the 2019 campaing in Madeira Island 12 strains were isolated, mainly from sampling sites in the north coast as indicated in Table 4B.

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Table 4B: List of *Gambierdiscus* spp strains isolated from Madeira Island in 2019.

Code	Sampling Location	Isolation date
GAMBI1_PM_19	Porto Moniz	14/10/19
GAMBI2_PM_19	Porto Moniz	14/10/19
GAMBI3_PM_19	Porto Moniz	14/10/19
GAMBI4_PM_19	Porto Moniz	14/10/19
GAMBI5_PM_19	Porto Moniz	17/10/19
GAMBI6_PM_19	Porto Moniz	17/10/19
GAMBI7_PM_19	Porto Moniz	17/10/19
GAMBI8_PM_19	Porto Moniz	17/10/19
GAMBI1_S_19	Seixal	15/10/19
GAMBI2_S_19	Seixal	15/10/19
GAMBI1_PD_19	Ponta Delgada	16/10/19
GAMBI1_D_19	Desertas	18/10/19

A summary table of the established strains from Madeira and Selvagem Islands is shown in Table 5.

Table 5: Strains isolated and established in Madeira, Desertas and Selvagem Islands between 2017 and 2019.

Sampling Campaigns						Total
2017		2018		2019		
Madeira	Selvagem Grande	Selvagem Grande	Selvagem Pequena	Madeira	Desertas	
2	4	52	16	11	1	86

CRETE AND CYPRUS (AUT)

From the samples collected from Crete (including those collected before the November 2016 campaign), 930 isolations were conducted and 66 *Gambierdiscus* cultures are currently growing (some of them are struggling) in culture conditions (Fig 14).

From the samples collected from Cyprus, 400 isolates were conducted and more than 110 *Gambierdiscus* cultures are currently growing in culture conditions. Twenty *Fukuyoa* cells were isolated, but unfortunately none of them managed to grow. All information is summarized in Table 6.

Fig 14. Collection of acclimated low-scale cultures in AUT, Thessaloniki.

Table 6. Isolated and established strains of *Gambierdiscus* spp. by AUT from sampling in the eastern/south eastern Aegean Sea in Agust/September (2018).

	November 2016		
	Crete	**Cyprus**	**Total (all islands)**
***Gambierdiscus* sp. Total established**	66	110	176
***Fukuyoa* sp. Total established**	0	0	0
***Gambierdiscus* sp. Total Isolated**	930	400	1330
***Fukuyoa* sp. Total Isolated**	0	20	20

EASTERN/SOUTH AEGEAN SEA (SAMOS AND RHODES) (AUT)

From the eastern/south eastern Aegean Sea samples, out of the 37 *Gambierdiscus/Fukuyoa* isolates from Samos 2 *Gambierdiscus* strains are growing in culture conditions, while out of 62 isolates from Rhodes 19 *Gambierdiscus* strains and 1 *Fukuyoa* strain are growing. All information is summarized in Table 7. All strains are studied by morphological and molecular means along with the strains from Crete and Cyprus in order to be characterized in species level.

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Table 7. Isolated and established strains of *Gambierdiscus* spp. by AUT from sampling in the eastern/south eastern Aegean Sea in Agust/September (2018).

	Sampling August/September 2018		
	Samos	**Rhodes**	**Total (all islands)**
***Gambierdiscus* sp. Total established**	2	19	21
***Gambierdiscus* sp. Total Isolated**	37	62	99
***Fukuyoa* sp. Total Isolated**	0	1	1

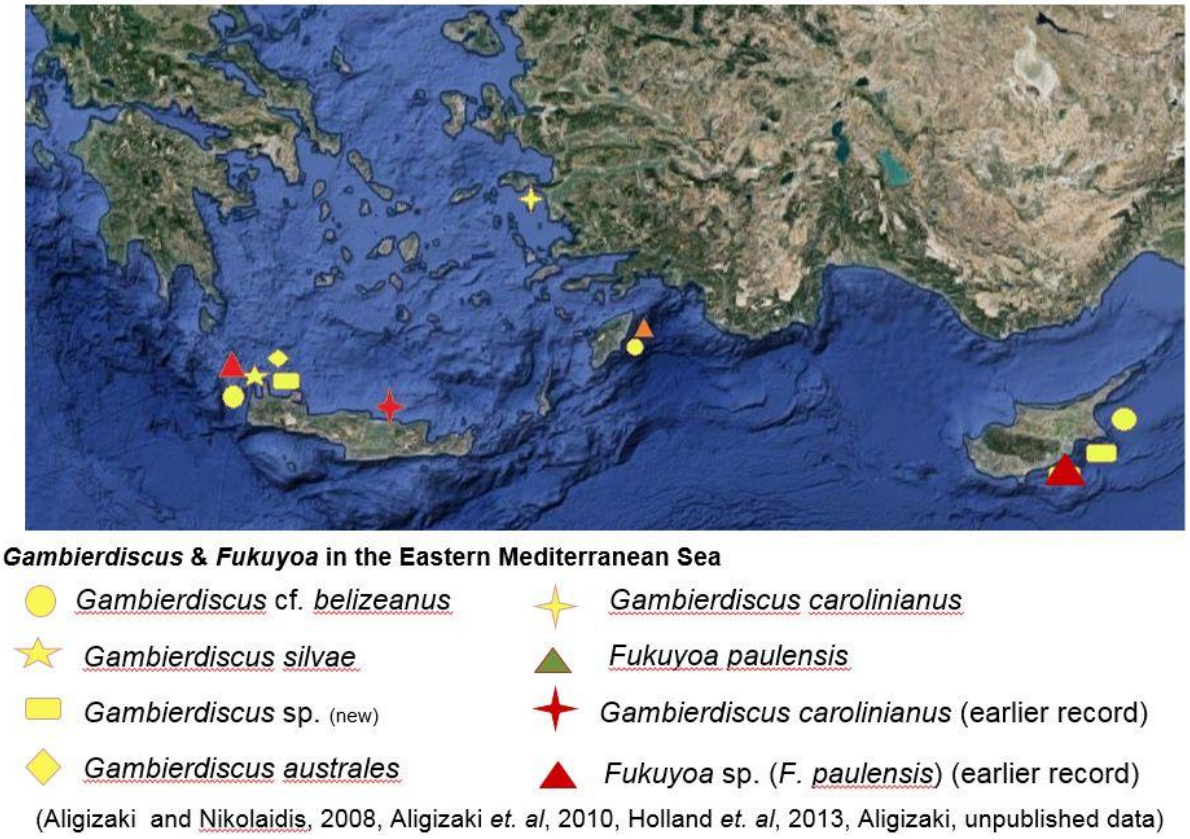

Fig 15. Distribution of each species in the stations of the eastern Mediterranean Sea including previous works and Eurocigua sampings.

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BALEARIC ISLANDS (Formentera, Majorca and Menorca) (IRTA)

Table 8. Isolated and established strains of *Gambierdiscus* spp. and *Fukuyoa* spp. by IRTA from sampling in the Balearic Islands in October (2016-2018).

	Sampling Oct. 2016	Sampling Oct. 2017		Sampling Oct. 218		Total (all islands)
	Formentera	***Mallorca***	***Menorca***	**Majorca**	**Minorca**	
***Gambierdiscus* sp.**	0	4	6	14	6	30
G. australes	0	13	12	13	7	45
***Fukuyoa* sp.**	0	1	12	2	1	16
F. paulensis	0	3	5	0	0	8
Total established	**0**	**21**	**35**	**29**	**14**	**99**
TOTAL isolated	**1**	**39**	**62**	**71**	**25**	**198**

In 2016 in the Balearic Islands samplings were performed in Formentera. In 2017, sampling was performed in Majorca and Minorca. After in 2018 also sampling was performed in Majorca and Menorca to confirm if *Gambierdiscus* spp. and *Fukuyoa* spp. were present in the Balearic Islands. Isolation and culturing processes were the same as the processes described previously for the strains from the Canary Islands.

As a result of our work, *Gambierdiscus* was recorded for the first time in the Western Mediterranean. A new report of *Gambierdiscus australes* was recorded in the Balearic Islands (Majorca and Minorca). 99 strains of *Gambierdiscus* spp. and *Fukuyoa* spp. strains were established from Majorca and Menorca (Table 8). In Formentera, *Gambierdiscus* spp. and *Fukuyoa* spp. were not recorded. Currently, 50% of all isolates are alive.

2.4 Identification of Gambierdiscus spp. and Fukuyoa spp. by morphological and genetical analysis. (IRTA, IPMA, AUT)

There are 15 species described for the genus *Gambierdiscus* and 3 for the genus *Fukuyoa*, 7 of these species have been described in the last 3 years. The genus *Fukuyoa* includes 2 species that taxonomists included before within the genus *Gambierdiscus* (*Fukuyoa ruetzleri* and *Fukuyoa yasumotoi).* The list of species described worldwide within this genus until now in the literature (for the whole planet) is: *Gambierdiscus australes, Gambierdiscus balechii, Gambierdiscus belizeanus, Gambierdiscus caribaeus, Gambierdiscus carolinianus, Gambierdiscus carpenteri, Gambierdiscus cheloniae, Gambierdiscus excentricus, Gambierdiscus honu, Gambierdiscus lapillus, Gambierdiscus pacificus, Gambierdiscus polynesiensis, Gambierdiscus scabrosus, Gambierdiscus silvae, Gambierdiscus toxicus, Fukuyoa ruetzleri, Fukuyoa yasumotoi, Fukuyoa paulensis.*

.

In previous published studies up to five *Gambierdiscus* species were detected based on morphological and molecular data: *G. australes, G. excentricus, G. silvae, G. carolinianus* and *G. caribaeus*. The morphology of species present in the samples from the Balearic Islands corresponds to the genus *Fukuyoa*. The morphology of the species present in the samples from the Canary Islands corresponds to both genera.

The identification to species level requires the use of calcofluor combined with fluorescence microscopy and electronic microscopy. Some of the cultures were processed for electronic microscopy (dehydration and critical point) and were observed also at the technical services of ICM-CSIC in Barcelona.

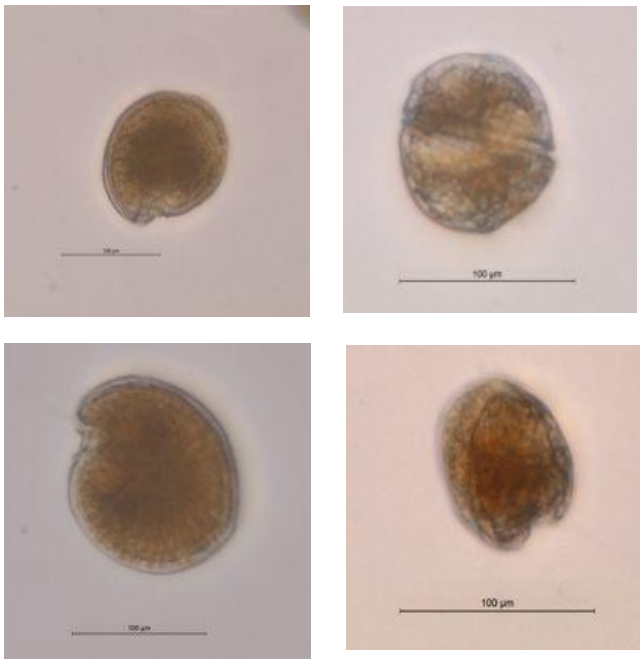

Fig 16. Light microscopy photos of strains corresponding to the genus *Gambierdiscus* (the two images on the left) and to the genus *Fukuyoa* (the two images on the right).

Morphological

CANARY ISLANDS (IRTA)

Identification of *Gambierdiscus* and *Fukuyoa* strains for the isolates for the different islands has been concluded. The approach includes morphological and molecular identification. Morphological identification has been conducted using fluorescence (Calcofluor stain) and electronic microscopy (SEM: Scanning Electron Microscope). For morphological identification we determined the thecal pattern, pore distribution, characteristic of specific plates, and sizes.

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Characterization of strains using SEM: Strains IRTA-SMM-16-286 (Figure 17) and IRTA-SMM-17-03 of *Gambierdiscus australes* from Lanzarote; and IRTA-SMM-17-421 of *G. belizeanus* (Figure 18).

Characterization of strains using fluorescence microscopy was conducted on the following strains:

Strains IRTA-SMM-16-286, IRTA-SMM-16-290, IRTA-SMM-17-04, IRTA-SMM-17-06 (Figure 19 A-B) of *Gambierdiscus australes* from Lanzarote and IRTA-SMM-17-02 (Figure 19 C-D) of the same species from Fuerteventura.

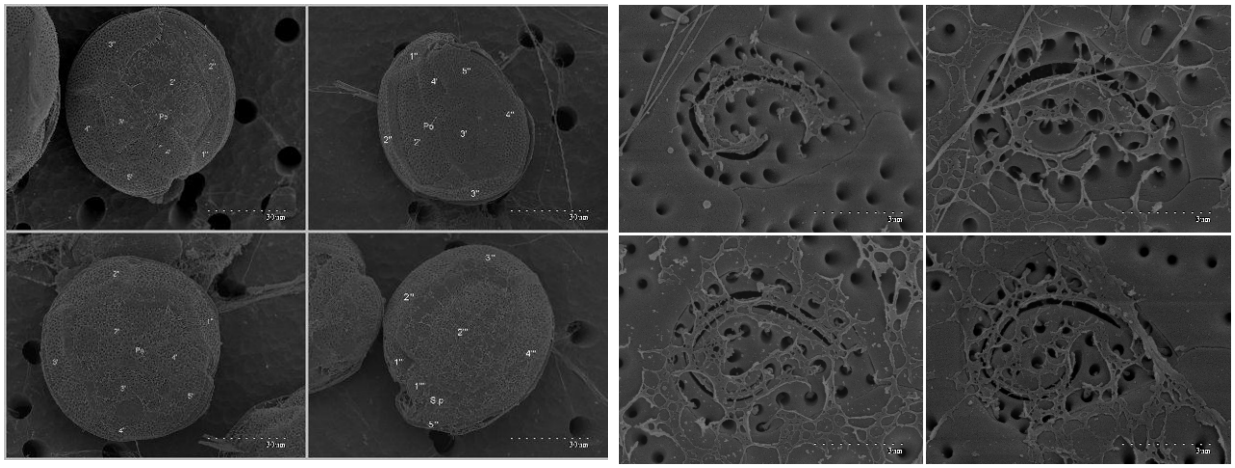

Fig 17. Scanning electron micrographs of thecae (A-D) and Po plates (E-H) of *Gambierdiscus australes* from Lanzarote (Canary Islands).

Cells were anterior–posteriorly compressed. The plate formula of *G. belizeanus* was Po, 4', 0a, 6", 6c, ?s, 5"', 0p, 2"" based on Fraga et al. 2011. The cells were heavily aerolated (Figure 18 A–C). Limits of the thecae are well defined by intercalary bands. These two latter characteristics are typical of *G. belizeanus*. The 20 plate is rectangular (Figure 18A), and the 2' "' plate is pentagonal (Figure 18B). Figure 18D shows the apical pore plate (Po).

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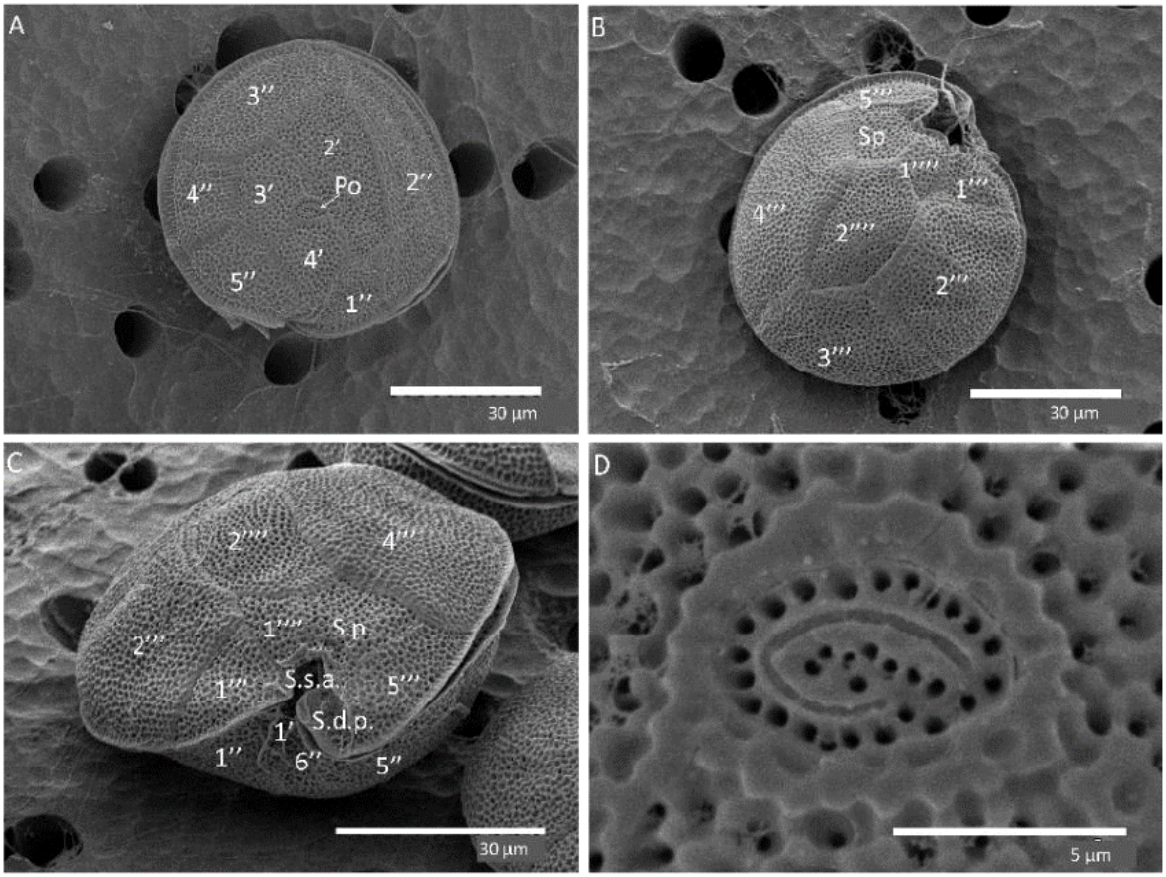
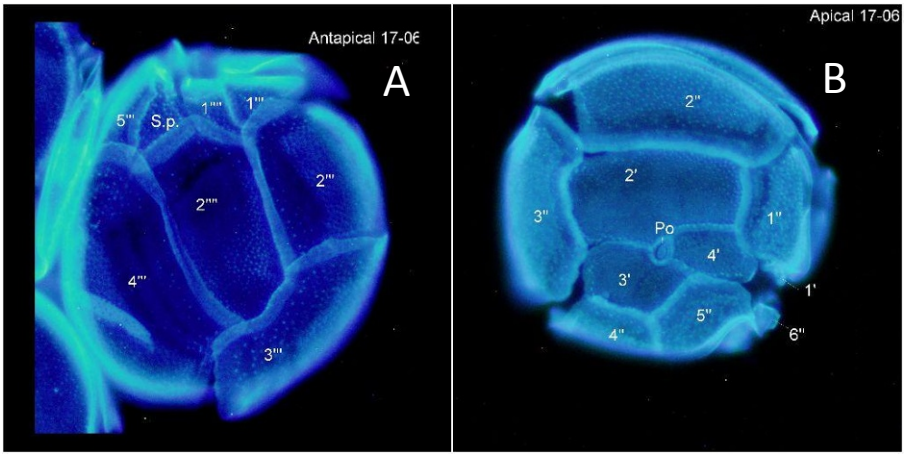


Fig 18. SEM images of *G. belizeanus* (IRTA-SMM-17-421): apical (A), antapical (B), ventral (C) views, detail of Po plate and pores (D).



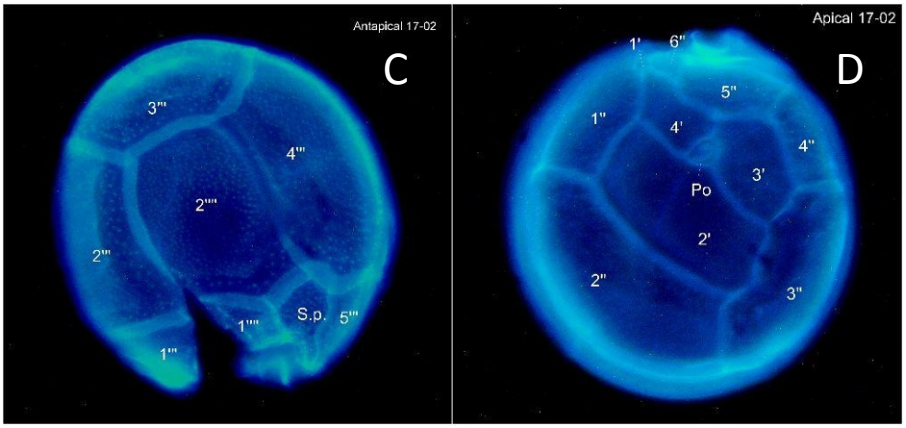


Fig 19. *Gambierdiscus australes* from Lanzarote (A-B) and (C-D) Fuerteventura (Canary Islands) by calcofluor stain.

MADEIRA AND SELVAGENS ISLANDS (IPMA)

Description of species-specific morphological charecters was performed using fluorescence and electronic microscopy (SEM) being determined the thecal pattern, pore distribution, characteristic of specific plates, and sizes. Results from microscopy analysis suggest that *Gambierdiscus* cells observed in Selvagem Islands correspond to one single species, the *G. australes*. Figure 20 shows a SEM image with the apical and antapical view of the epitheca and hypotheca respectively showing the tabulation of *Gambierdiscus* from Selvagem Pequena and S. Grande.

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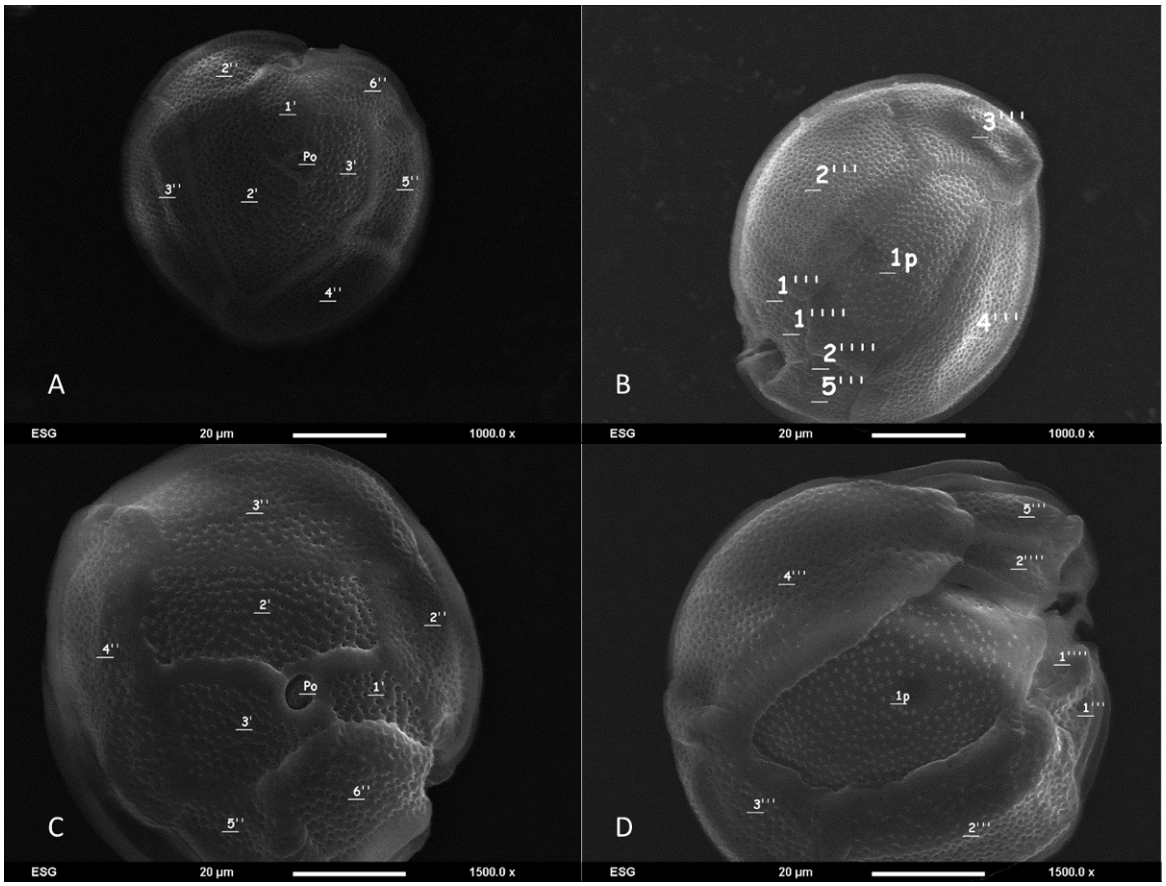

Fig 20. Apical and Antapical view of the epitheca and hypotheca of *Gambierdiscus australes* by scanning electronic microscopy. A,B: *Gambierdiscus* from Selvagem Pequena at 1000x magnification; C,D: *Gambierdiscus* from Selvagem Grande at 1500x magnification.

According to the morphological observations of strains isolated from Madeira Island, Porto Santo and Desertas, *Gambierdiscus excentricus* seems to be the dominant or the single species present in these islands (Fig. 21). The apical pore plate Po is oval with a fishhook-shaped slit and is ventrally displaced. The first apical plate, 1' is very small, and the second apical plate 2' has the suture 2'/3' about twice as long as the suture 2'/4' (Fraga et al., 2011)

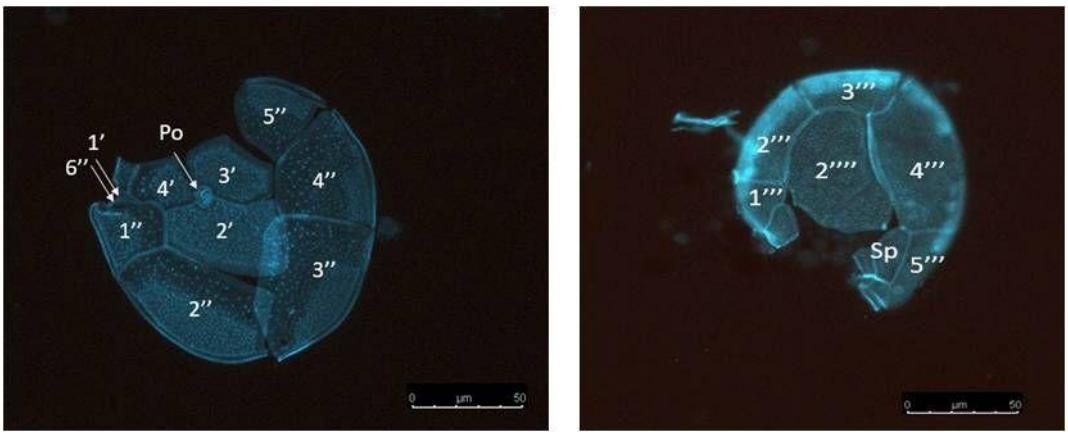

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Fig 21. Apical and Antapical view of the epitheca and hypotheca of *Gambierdiscus excentricus* by scanning electronic microscopy in strain isolated from Madeira Island.

CRETE AND CYPRUS (IP)

The identification of *Gambierdiscus* spp., isolated from Crete and Cyprus by morphological and genetical analyses were performed, while the three implicated institutes collaborated in order to use common approaches. In Cyprus, *Fukuyoa* sp. cells were also detected; however, the isolations did not reach the level of a viable culture.

At the end of the project, a great diversity of *Gambierdiscus* and *Fukuyoa* was detected in the eastern Mediterranean sea, where at least six different taxa were detected: *G. silvae, G. australes, G. carolinianus, G. cf. belizeanus, G. sp* (*sp. nov*) and *Fukuyoa paulensis*.

BALEARIC ISLANDS (IRTA)

There was particular interest in the identification of *Gambierdiscus* from the Balearic Islands (First report in the Western Mediterranean).

Characterization of strains using SEM: strains IRTA-SMM-17-164 of *Gambierdiscus australes* (Figure 22) and IRTA-SMM-17-211 of *Fukuyoa paulensis* from Menorca (Figure 23).

Characterization of strains using fluorescence microscopy: strains of *Gambierdiscus australes* IRTA-SMM-17-173 from Mallorca and IRTA-SMM-17-181 from Menorca were characterized using Calcofluor stain.

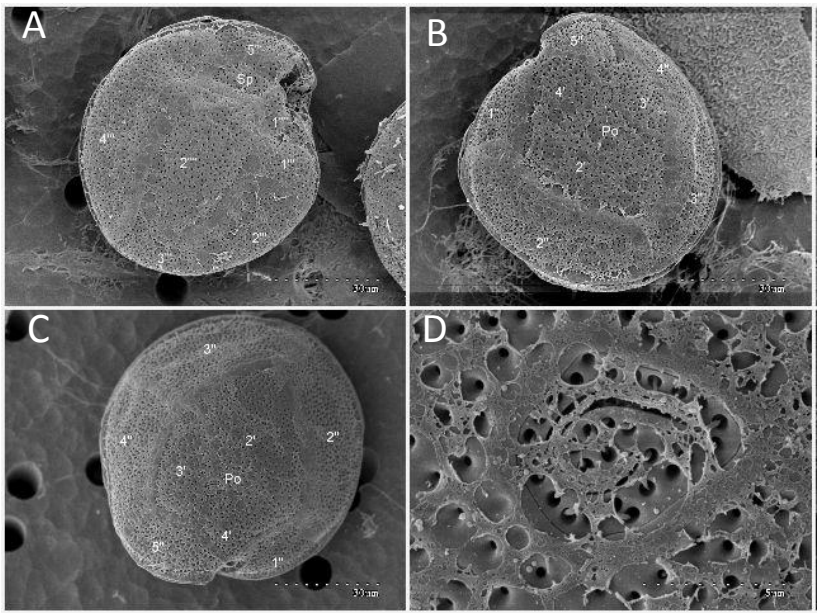

Fig 22. *Gambierdiscus australes* strain IRTA-SMM-17-164 from Menorca, SEM images of (A-C) thecae, (D) Po plate.

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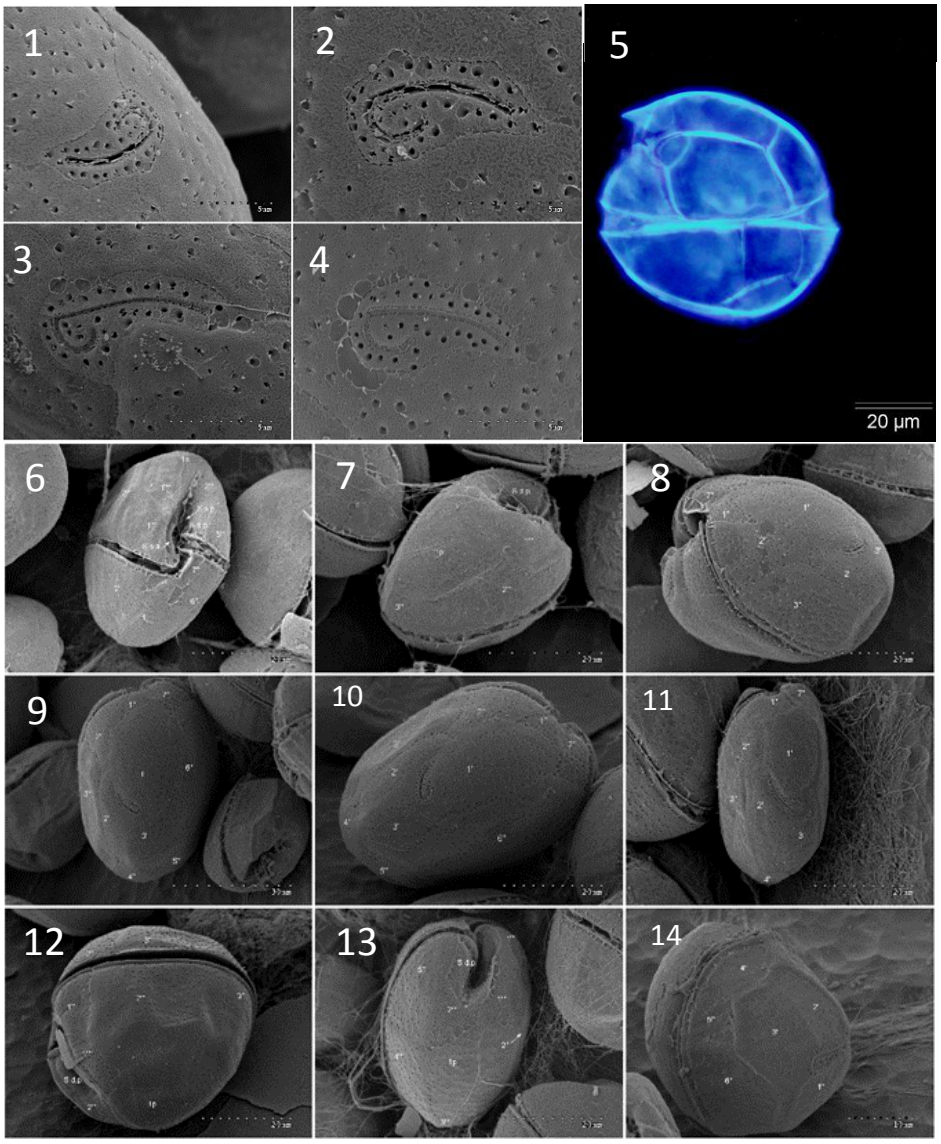

Fig 23. *Fukuyoa paulensis* from Menorca, strain IRTA-SMM-17-211. Po plates (1-4) and (6-14) SEM images of thecae and (5) image of thecae by calcofluor stained.

Molecular genetics

Gambierdiscus spp. from the Balearic Islands was confirmed. All isolated strains evaluated using molecular genetics (n=20) were the same: *Gambierdiscus australes.*

Phylogenetic analysis for species identification is an area of science that continues to evolve as techniques and methodologies improve. The types of genetic marker deemed correct for any given taxa can be a moving target as systematists gain new insights into mating compatibilities and physiologic differences; differences that may not be made manifest by morphological analyses. In the case of *Gambierdiscus* spp. there are two genetic markers that are now widely used for species identification: the LSU rDNA D1-D3 loop and LSU rDNA D8-D10 loop. In this project both of these genetic markers and each requires separate PCR reactions we used. Also, different primer pairs can be useful for amplifying the same region

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from different species when the expected amplification is not obtained with a particular primer set.
To speed the analysis of many samples the extraction and purification of DNA were performed using carefully prepared single cells and utilizing a specific extraction kit (Arcturus Pico Pure Kit, Life Technologies).

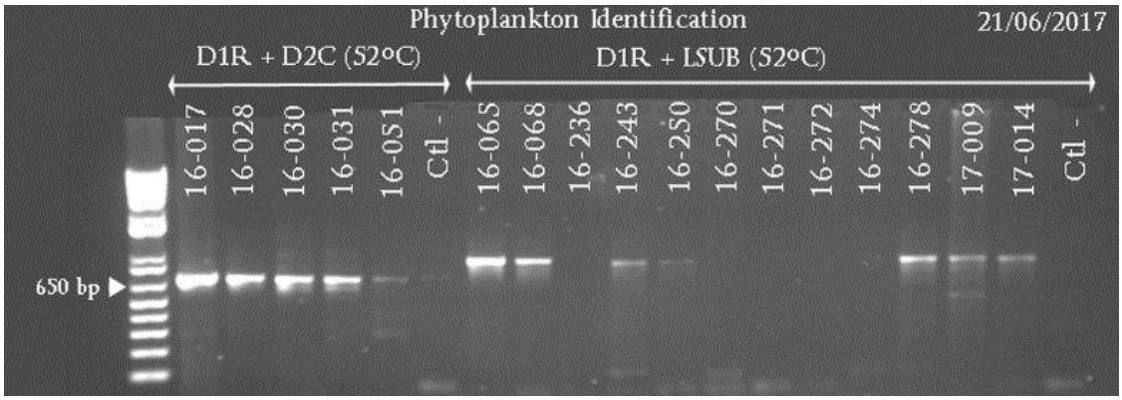

Fig 24. An example of amplification of the LSU rDNA D1-D3 loop using different primer sets. Note: not all amplify equally well since the actual sequences of each strain may not be identical to the primers in use. Expected size of product is ~700 - 800 base pairs depending on primers used.

All samples that amplify are purified by column chromatography (Qiagen) for preparation for sequencing. The sequencing reactions are performed in the laboratories of a contracted external vendor. The raw data is proofed in-house at IRTA. All amplified products that provide complete unequivocal sequence information are subjected to BLAST analysis (NCBI website) to confirm the proximate identity, and full phylogenetic analysis is performed afterward to confirm suspected identity (MEGA ver 6.0).

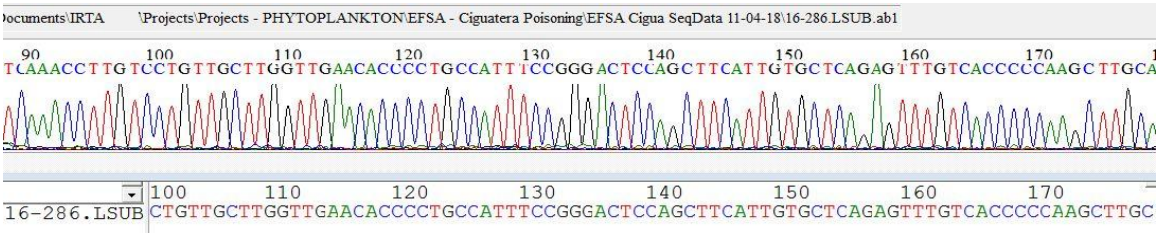

Fig 25. An example of sequence data obtained from DNA extracted from microalgae culture samples. The electropherogram and corresponding sequence are shown for a short representative segment of the amplified LSU rDNA D8-D10 loop.

The samples analysed thus far include strains of *Gambierdiscus sp.*, *Fukuyoa sp.*, and *Coolia sp.* Multiple strains collected from the same site are often the same species, although multiple species of *Gambierdiscus* and *Coolia* are among the samples collected in the Canary Islands and Balearic Islands.

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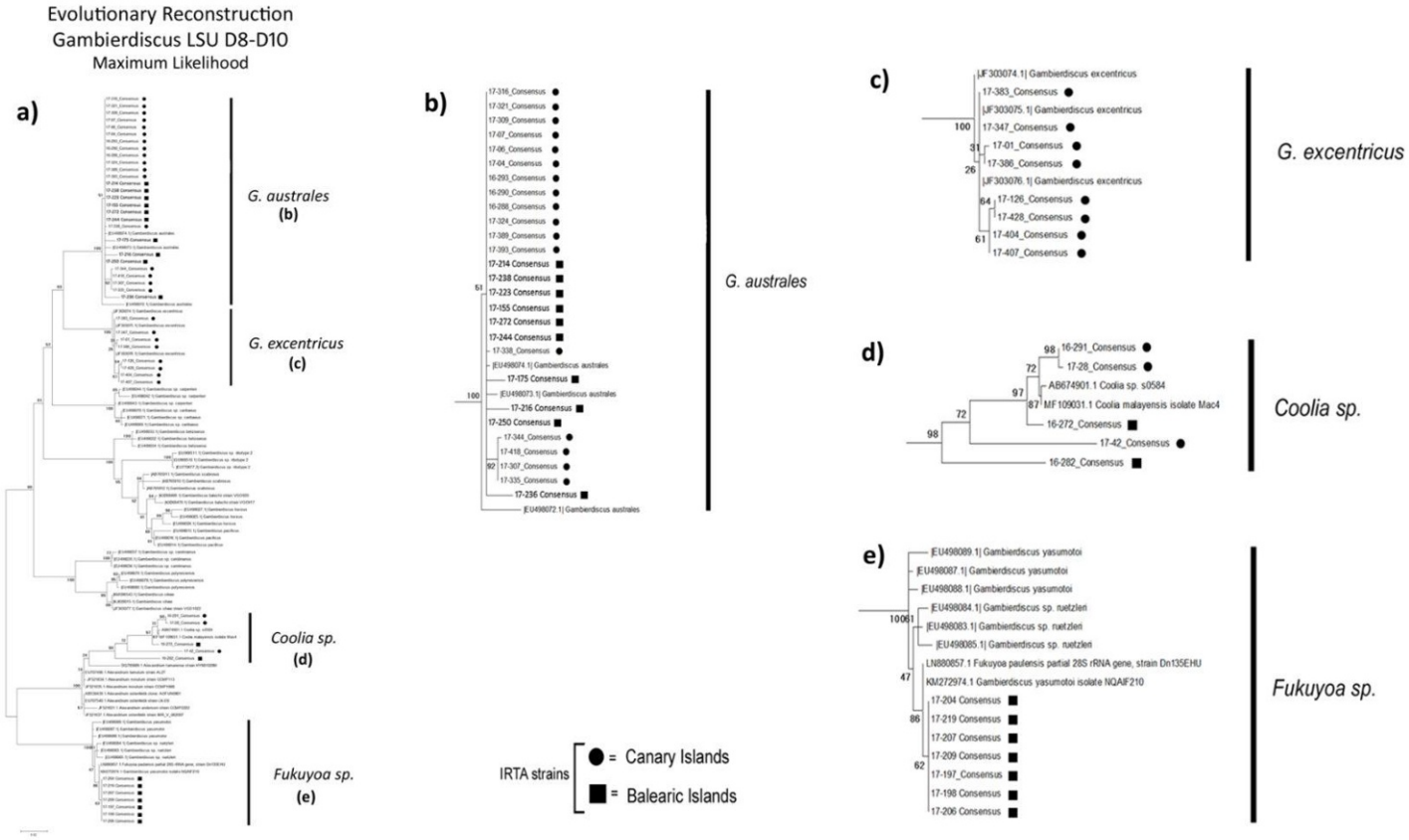

Fig 26. Results obtained from phylogenetic analysis of LSU rDNA (D8-D10 loop) sequence data for identification of *Gambierdiscus*-like cells from culture isolates as of April 2018. IRTA strain isolates are marked with either a circle (Canary Island isolate) or a square (Balearic Island isolate). Maximum Likelihood analysis performed using MEGA ver 6.0. Bootstrap confidence values (1000 replicates) are shown at nodes of the tree branches. a) Complete dendrogram; b) Enlargement of *Gambierdiscus australes* clade; c) Enlargement of *Gambierdiscus excentricus* clade; d) Enlargement of *Coolia* sp. Clade; e) Enlargement of *Fukuyoa* sp. Clade.

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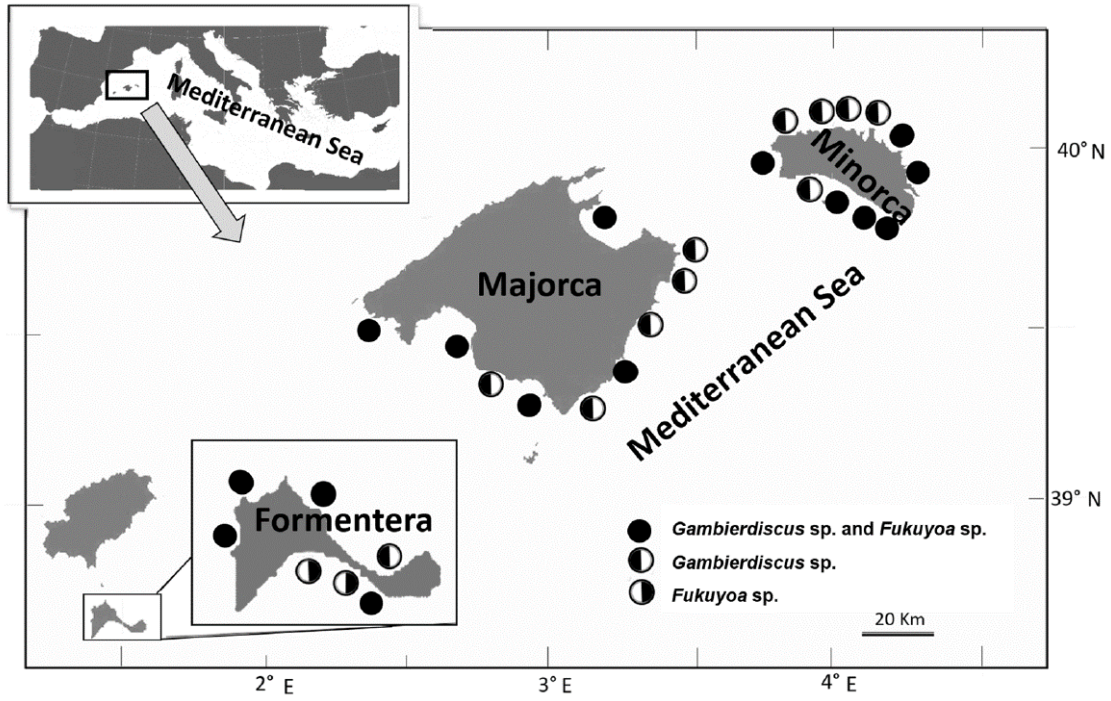

Fig 27. Presence of the *Gambierdiscus* and *Fukuyoa* genera in the sampling stations in the Balearic Islands (Mediterranean Sea) during 2016-2019.

Table 9. Summary of results from dendogram shown above from genetic analysis of strains from IRTA in 2017. The species of *Coolia* sp. shown include *Coolia malayensis*. Long branch lengths of three of the Coolia isolates suggest further analysis is required to confirm identity.

LSU rDNA D8-D10 Sequence Analysis		
Species	**Location**	**n**
Gambierdiscus australes	Balearic Is.	10
G. australes	Canary Is.	17
G. excentricus	Canary Is.	8
Coolia sp.	Balearic Is.	2
Coolia sp.	Canary Is.	3
Fukuyoa paulensis	Balearic Is.	7

MADEIRA AND SELVAGENS ISLANDS (IPMA)

Species identification by molecular techniques was carried out in strains isolated from Selcagens Islands. The obtained sequence of the partial 28S ribosomal RNA gene was Blast against other sequences in the public data bases and a 99% identity with *Gambierdiscus australes* (AB765921.1) was obtained with a coverage of 98 % and 3 gaps over 913 nucleotides.

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Phylogenetic relations based on the LSU D8-D10 region of strains isolated from Selvagens islands within the *Gambierdiscus* genus is presented in Figure 27. The strains clustered with *G. australes* clade with *G. excentricus* as its sister clade. Most of the strains were identical to *G. australes* from the Australes archipelago type material (*G. australes* RAV2, EU498073). However, the strains Gambi 47, 48 and 50) diverge from the other *G. australes* with a bootstrap support for ML analysis of 71.

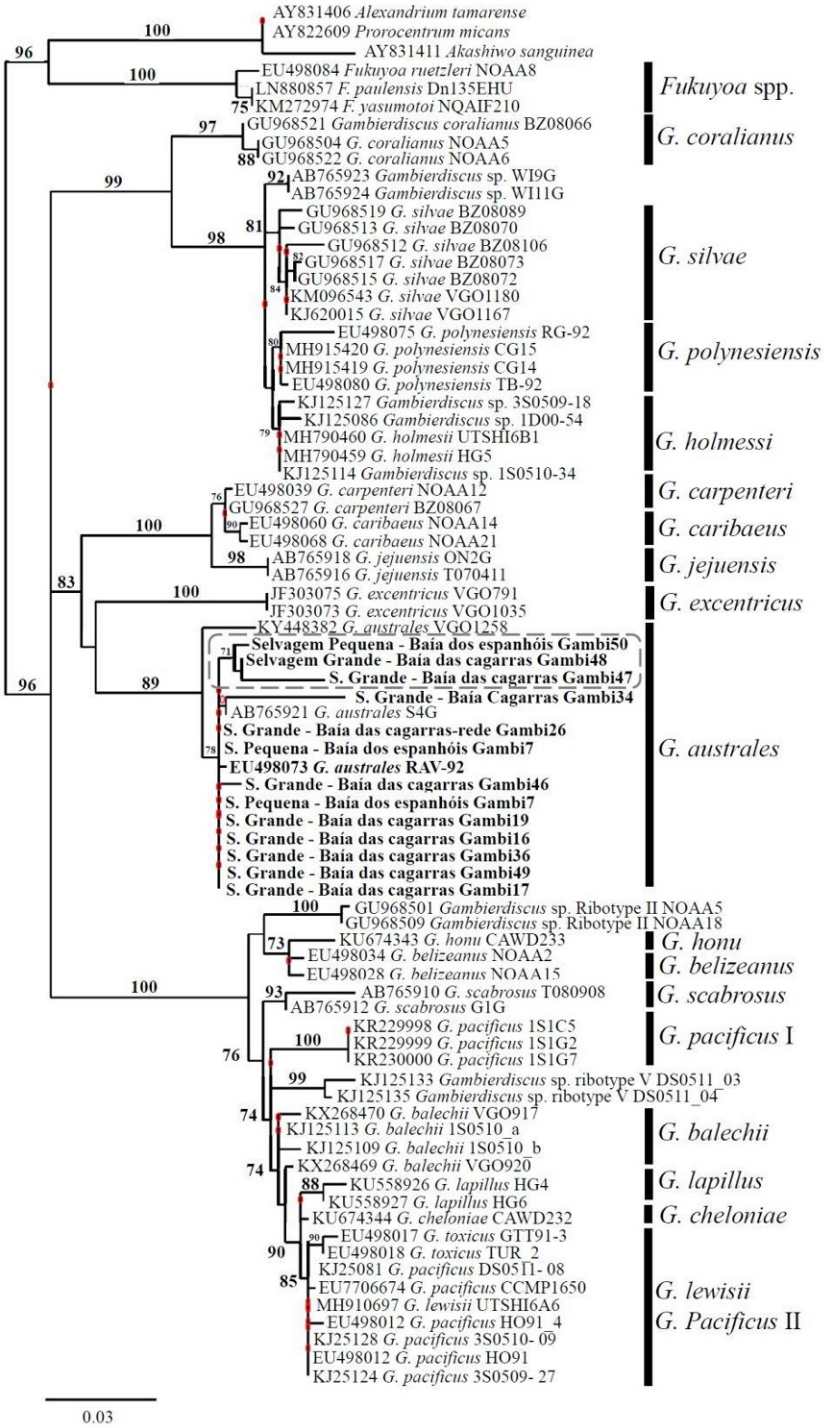

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Fig 28. Phylogeny of *Gambierdicus* genus based on maximum likelihood analysis for the LSUrRNA D8–D10 region, bootstrap values < 70 are not shown. New *Gambierdiscus* sequences obtained in this study are in bold.

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2.5 Evaluation of the presence /absence of CTX-like toxicity in *Gambierdiscus* spp. (IRTA)

2.5.1. Microalgae cultures for toxin evaluation

Microalgal cultures were established from the Canary Islands, Madeira including Selvagens Islands, Crete, Cyprus and the Balearic Islands. These have been described in deliverable D3.4 "Establishment of a culture collection of *Gambierdiscus* spp. and *Fukuyoa* spp. from Macaronesia and the Mediterranean".

The evaluation of toxicity was performed on the microalgae from this collection of the Eurocigua project (EXCEL on Toxicity of microalgae is presented in ANNEX O_2020_EUROCIGUA_SG3_DINOFLAGELLATE_TOXICITY). In addition, among the strains isolated, some where scaled to large volumes obtaining larger amounts of cells (See Table 10). Eight of these cultures were transferred to the U.Vigo (SG4) for the LC-MS/MS analyses.

Table 10. Large scale cultures from the IRTA collection.

Species	IRTA Code	Location	Island	Total cells of extract
G. australes	IRTA-SMM-17-162	St. Adeodat	Menorca	27.810.871
G. australes	IRTA-SMM-17-253	Anguila	Mallorca	13.734.756
G. australes	IRTA-SMM-17-189	Torret	Menorca	17.134.092
G. australes	IRTA-SMM-17-271	Macarella	Menorca	14.007.250
G. australes	IRTA-SMM-17-164	St. Adeodat	Menorca	4.257.199
G. australes	IRTA-SMM-17-178	Torret	Menorca	23.644.800
F. paulensis	IRTA-SMM-17-206	Portocolom	Mallorca	no data*
Gambierdiscus sp.	010G-CR-CCAUTH	Kolimpari	Crete	24.063.250,50
Gambierdiscus cf. *belizeanus*	012G-CR-CCAUTH	Kolimpari	Crete	2.300.000
F. paulensis	IRTA-SMM-17-209	Sacaleta	Menorca	6.964.044
G. australes	IRTA-SMM-17-244	Camp de Mar	Mallorca	41.219.200
G. excentricus	IRTA-SMM-17-407	Playa de Vueltas	La Gomera	6.084.000
G. australes	IRTA-SMM-16-286	Calero	Lanzarote	6.800.000

*used for qualitative evaluation of toxins

2.5.2. Microalgae toxicity

A summary EXCEL on Toxicity of microalgae is presented in ANNEX 2020_EUROCIGUA_SG3_DINOFLAGELLATE_TOXICITY.

2.5.2.1 Canary Islands

2.5.2.1.1 Evaluation of CTX-like toxicity with the Neuro-2a CBA

From the Canary Islands, 45 harvested strains were evaluated by the Neuro-2A assay. Toxicities are summarized in Table 11. Results show CTX-like positive: 43 cases (range: 1.7 - 2566,7 fg of CTX-1B eq. /cell), no quantifiable: 1 cases, CTX-like negative (negative): 1 case.

Table 11. Toxicity of extracts of *Gambierdiscus* spp. from the Canary Islands evaluated by the Neuro-2A assay. (CTX: Ciguatoxin, MTX: Maitotoxin, NQ*: not quantifiable due to matrix effect, NT: not tested).

Extract Code	Strain Code	Species	Island	Archipelago	fg CTX-1B eq. /cell	MTX
E-17-205	IRTA-SMM-16-286	*G. australes*	LANZAROTE	CANARY	33.6 ± 6.5	NT
E-17-220	IRTA-SMM-16-288	*G. australes*	LANZAROTE	CANARY	106,13 ± 75,26	NT
E-17-232	IRTA-SMM-16-290	*G. australes*	LANZAROTE	CANARY	46,10 ± 22,18	NT
E-17-191	IRTA-SMM-16-292	*G. australes*	LANZAROTE	CANARY	39,73 ± 10,54	NT
E-17-237	IRTA-SMM-16-293	*G. australes*	LANZAROTE	CANARY	32,67 ± 10,04	NT
E-17-93	IRTA-SMM-17-01	*G. excentricus*	GRAN CANARIA	CANARY	1149,3 ± 212,3	NT
E-17-244	IRTA-SMM-17-02	*G. australes*	FUERTEVENTURA	CANARY	452,6 ± 23,2	NT
E-17-214	IRTA-SMM-17-03	*G. caribaeus*	EL HIERRO	CANARY	Negative (<0,42 fg/cell eq.)	NT
E-17-245	IRTA-SMM-17-04	*G. australes*	LANZAROTE	CANARY	205,46 ± 34,60	+
E-17-219	IRTA-SMM-17-06	*G. australes*	LANZAROTE	CANARY	127,70 ± 85,03	NT
E-17-209	IRTA-SMM-17-07	*G. australes*	LANZAROTE	CANARY	15,78 ± 1,73	NT
E-17-243	IRTA-SMM-17-102	*Gambierdiscus* sp.	GRAN CANARIA	CANARY	36,85 ± 6,60	NT
E-17-197	IRTA-SMM-17-103	*G. australes*	GRAN CANARIA	CANARY	118,2 ± 30,3	NT
E-17-195	IRTA-SMM-17-106	*G. australes*	GRAN CANARIA	CANARY	1,7 ± 0,1	+
E-17-196	IRTA-SMM-17-107	*G. australes*	GRAN CANARIA	CANARY	12,2 ± 2,1	+
E-17-192	IRTA-SMM-17-112	*G. australes*	GRAN CANARIA	CANARY	1,9 ± 0,6	NT
E-17-255	IRTA-SMM-17-125	*Gambierdiscus sp.*	GRAN CANARIA	CANARY	NQ*	NT
E-17-254	IRTA-SMM-17-126	*G. excentricus*	GRAN CANARIA	CANARY	226,7 ± 22,1	NT

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Extract Code	Strain Code	Species	Island	Archipelago	fg CTX-1B eq. /cell	MTX
E-17-242	IRTA-SMM-17-128	*G. excentricus*	GRAN CANARIA	CANARY	9,5 ± 2,6	+
E-17-218	IRTA-SMM-17-129	*Gambierdiscus* sp.	GRAN CANARIA	CANARY	130,67 ± 13,75	NT
E-18-31	IRTA-SMM-17-287	*G. australes*	LA PALMA	CANARY	11,3 ± 2,3	NT
E-19-26	IRTA-SMM-17-288	*G. australes*	LA PALMA	CANARY	5,7 ± 3,8	NT
E-18-36	IRTA-SMM-17-291	*G. australes*	TENERIFE	CANARY	82,8 ± 22,2	NT
E-18-131	IRTA-SMM-17-307	*G. australes*	TENERIFE	CANARY	37,3 ± 12,6	NT
E-18-129	IRTA-SMM-17-316	*G. australes*	TENERIFE	CANARY	51,5 ± 6,9	NR
E-18-30	IRTA-SMM-17-321	*G. australes*	EL HIERRO	CANARY	31,9 ± 15	NT
E-19-24	IRTA-SMM-17-324	*G. australes*	EL HIERRO	CANARY	160,4 ± 17,2	NT
E-18-33	IRTA-SMM-17-327	*G. australes*	EL HIERRO	CANARY	7,2 ± 0,3	NT
E-18-48	IRTA-SMM-17-330	*G. excentricus*	LA PALMA	CANARY	2566,7 ± 333,3	NT
E-18-62	IRTA-SMM-17-335	*G. australes*	LA PALMA	CANARY	29,1 ± 8,6	NT
E-18-138	IRTA-SMM-17-344	*G. australes*	LA PALMA	CANARY	41,2 ± 0,1	NT
E-18-50	IRTA-SMM-17-358	*G. australes*	TENERIFE	CANARY	138,9 ± 17,7	NT
E-18-77	IRTA-SMM-17-386	*G. excentricus*	TENERIFE	CANARY	12,8 ± 2,8	NT
E-18-128	IRTA-SMM-17-389	*G. australes*	EL HIERRO	CANARY	226,27 ± 24,53	NT
E-18-52	IRTA-SMM-17-393	*G. australes*	LA GOMERA	CANARY	44,6 ± 11,5	NT
E-18-69	IRTA-SMM-17-404	*G. excentricus*	TENERIFE	CANARY	1257,6 ± 319,3	NT
E-18-76	IRTA-SMM-17-405	*G. excentricus*	TENERIFE	CANARY	1153,4 ± 238,8	NT
E-18-74	IRTA-SMM-17-407	*G. excentricus*	LA GOMERA	CANARY	NQ*	NT
E-18-143	IRTA-SMM-17-413	*G. excentricus*	LA GOMERA	CANARY	18,1 ± 5,7	NT
E-18-32	IRTA-SMM-17-417	*Gambierdiscus* sp.	EL HIERRO	CANARY	26,9 ± 7,89	NT
E-19-23	IRTA-SMM-17-418	*G. australes*	EL HIERRO	CANARY	68,3 ± 9,53	NT
E-19-01	IRTA-SMM-17-421	*G. belizeanus*	EL HIERRO	CANARY	5,6 ± 0,1	NT
E-18-51	IRTA-SMM-17-425	*G. australes*	EL HIERRO	CANARY	27,8 ± 3,3	NT
E-18-58	IRTA-SMM-17-427	*Gambierdiscus* sp.	LA GOMERA	CANARY	NQ*	NT
E-18-49	IRTA-SMM-17-428	*G. excentricus*	LA GOMERA	CANARY	NQ*	NT
E-18-90	IRTA-SMM-17-429	*G. excentricus*	LA GOMERA	CANARY	1525,9 ± 634,1	NT
E-19-27	IRTA-SMM-17-432	*G. excentricus*	LA GOMERA	CANARY	962,1 ± 154,7	NT
E-19-25	IRTA-SMM-17-436	*G. australes*	LA GOMERA	CANARY	98,59 ± 25,4	NT

2.5.2.1.2 Geographical distribution and toxicity

Gambierdiscus excentricus exhibited the higest CTX-like toxicity (9.5-2566.7 fg CTX-1B equiv./cell) followed by *G. australes* (1.7 – 452.6 fg CTX-1B equiv./cell). By contrast, the

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toxicity of *G. belizeanus* was low (5.6 fg CTX-1B equiv/cell), and *G. caribeaus* did not exhibit CTX-like toxicity.

The differences of CTX-like toxicity among strains of *G. excentricus* (n=10) and *G. australes* (n=29) from different islands are shown in a boxplot in Figure 29. The highest toxicities are observed in *G. excentricus* strains from the islands placed in the western part of the archipelago, excluding El Hierro, where *G. excentricus* strains were not identified (Fig. 30). The strains producing higher than 500 fg eq. CTX-1B eq. /cell, were from the more western islands of the Archipelago: El Hierro, La Palma, La Gomera and Tenerife.

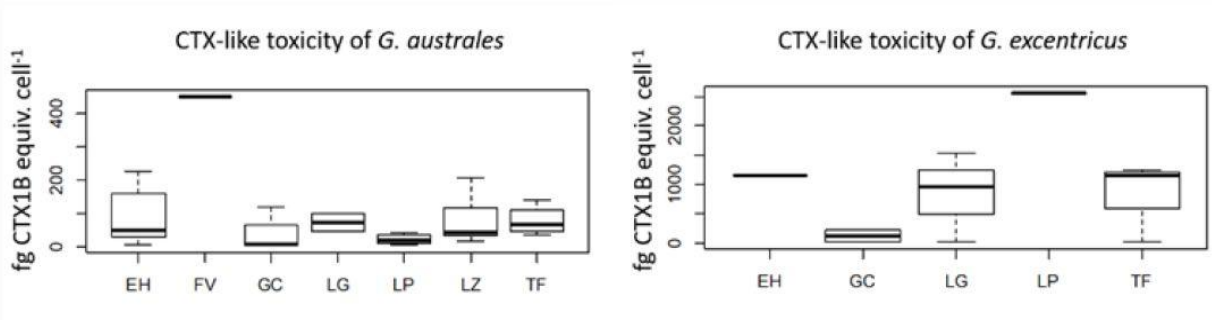

Fig. 29. Distribution of CTX-like toxicity of *G. australes* and *G. excentricus* according to island and origin. EH (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote) and TF (Tenerife).

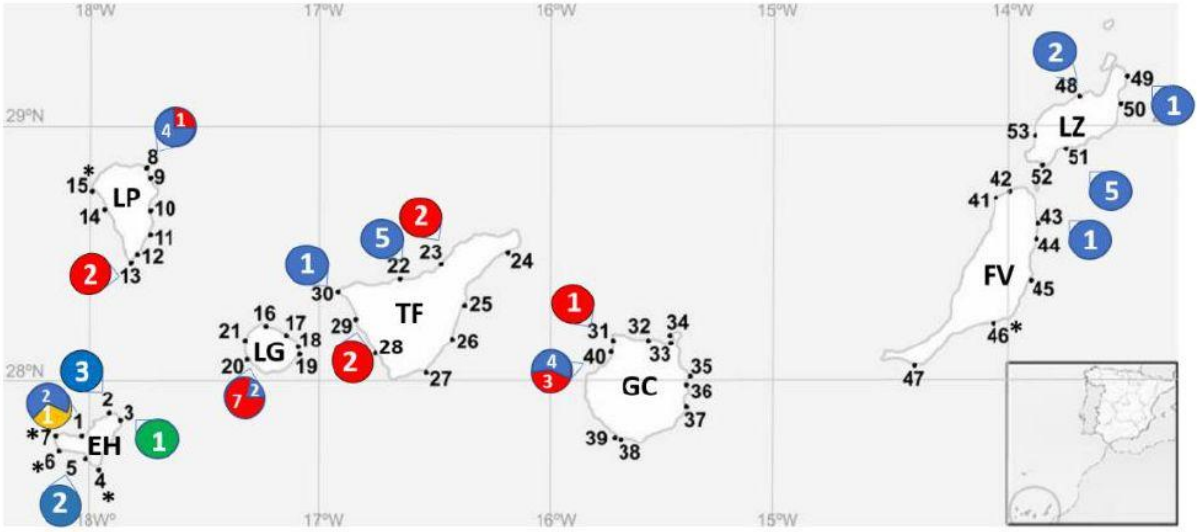

Fig. 30. Distribution of each species in the stations sampled in the Canary Islands during 2016-2017. Station numbers are presented in bold. The presence of *Gambierdiscus* species determined by molecular analysis is presented with a circle and includes the number of strains identified for each species. The asterisk representes the precence of *Gambierdiscus* sp. Colors of circles are for *G. australes* (blue), *G. excentricus* (red), *G. caribaeus* (green), and *G.*

belizeanus (yellow). EH (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote) and TF (Tenerife).

2.5.2.1.3 Evaluation of the presence of two series of CTX congener equivalents (CTX1B and CTX3C)

One *G. australes* strain from Lanzarote (IRTA-SMM-16_286), a *G. excentricus* strain from Gran Canaria (IRTA-SMM-17_01), three *G. excentricus* strains from La Gomera (IRTA-SMM-17_407, IRTA-SMM-17_428, IRTA-SMM-17_432), a *G. caribeanus* strain from El Hierro (IRTA-SMM-17_03) and *the G. belizeanus* strain from El Hierro (IRTA-SMM-17_421) were analysed with the magnetic-bead based immunoassay and immunosensor. Two differerent capture antibodies (3G8 and 10C9) were used to obtain an estimation of the amount of CTX1B or CTX3C series of congeners of the microalgae extracts. Analysis with the immunoassay and the immunosensor revealed the presence of CTXs in all the extracts, including those which could not be quantified by CBA as a consequence of matrix effects (IRTA-SMM-17_407, IRTA-SMM-17_428) and the *G. caribeaus* that did not show toxicity by CBA above the limit of quantification (Fig. 31). In fact, the limit of quantification of CBA was usually lower than the one for the immunosensing tools, probably as a consequence of the presence of other toxic compounds like MTX, that could interfere in the CBA analysis. For the four strains in which CTX-like activity was detected by CBA, quantifications using the immunosensing tools and CBA were in the same order of magnitude.

In general terms, as expected, the CTXs contents determined when using two capture antibodies were higher than when using only one. This is certainly explained by the presence of the two different series of congeners, even if one of them was not detected separately because of the limit of detection of the method. It is also important to note that although in some cases the immunoassay showed higher CTXs contents, the immunosensor was able to detect the presence of CTXs in samples where the immunoassay was not capable. This is attributed to the lower limits of detection of the immunosensor compared to the colorimetric immunoassay.

The results obtained show the predominance of CTX1B congeners in the three *G. excentricus* strains from La Gomera (IRTA-SMM-17_407, IRTA-SMM-17_428, IRTA-SMM-17_432), ranging from 0.06 to 0.77 fg/cell. On the contrary, CTX3C congeners were the most abundant in *G. australes* (IRTA-SMM-16_286) (0.37 fg/cell of 51-OH-CTX3C equiv. in front of 0.04 fg/cell of CTX1B), *G. excentricus* from Gran Canaria (IRTA-SMM-17_01) (from 0.16 to 0.54 fg/cell of 51-OH-CTX3C equiv.), and in *G. belizeanus* (IRTA-SMM-17_421) (0.28 ± 0.02 fg/cell 51-OH-CTX3C equiv.). Regarding the G. caribeanus strain (IRTA-SMM-17_03), equal amounts of both CTX congeners were detected (although slightly different depending on the immunosensing tool that was used).

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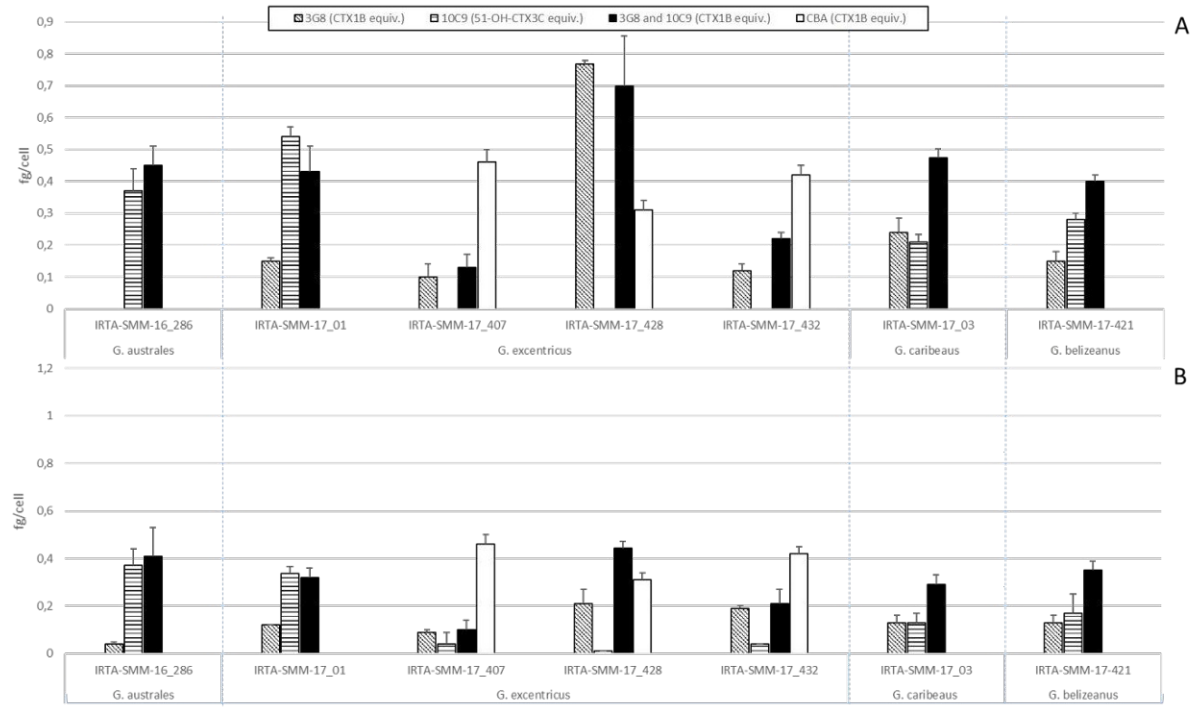

Fig. 31. CTXs (fg/cell) from *Gambierdiscus* strains evaluated with the colorimetric immunoassay (A) and the electrochemical immunosensor (B). CBA results are in both A and B for comparison species. Dashed lines separate species.

2.5.2.2 Madeira and Selvagens Islands

Four *Gambierdiscus* strains from Selvagens Islands, were evaluated by the Neuro-2A assay. Toxicities are summarized in Table 12. Results show all strains were CTX-like positive. Toxicity of the 4 strains evaluated (From Madeira) ranged from 2.46-83 fg eq. CTX1B /cell.

Table 12. Toxicity of extracts of *G. australes* from the Madeira and Selvagens Islands evaluated by the Neuro-2A assay.

Extract Code	Strain Code	Species	Island	Archipelago	fg eq. CTX-1B/cell	MTX
19-EX-119	IPMA-Gambi 7	*G.australes*	Selvagen Pequena, Baia dos Espanhois	Madeira	83	NT
2020-EX-108	IPMA- Gambi 9	*G.australes*	Selvagen Pequena, Baia dos Espanhois	Madeira	9,45	NT
2020-EX-109	IPMA-Gambi 17	*G.australes*	Selvagens Grande, Baia das Cagarras	Madeira	9,21	NT
2020-EX-110	IPMA-Gambi 57	*G.australes*	Selvagens Grande, Baia das Galinhas	Madeira	2,46	NT
Extract Code	**SPATTbag code**	**Resin eq. (g)**	**Exposition time in seawater (h)**		**fg eq. CTX-1B/cell**	**LOQ (µg eq/kg)**

.

2020-EX-111	SPEFC	6,9	24		<LOQ	0,00080
2020-EX-112	SGC18	3	18		<LOQ	0,00080
2020-EX-113	SC3D7M	6	72		<LOQ	0,00323
2020-EX-114	SPEP1	5,18	24		<LOQ	0,00161
2020-EX-115	SGG	19,94	72		<LOQ	0,00020
2020-EX-116	SC3d3m	5	72		<LOQ	0,00161

Six extracts of spattbags immersed in sea water for different times in a *Gambierdiscus* bloom were analysed by neuro-2A assay, all of them showed no CTX-toxicity (see table 12).

2.5.2.3 Mediterranean

2.5.2.3.1 Crete

2.5.2.3.1.1 Evaluation of CTX-like toxicity with the Neuro-2a CBA

From Crete, 19 out of the 20 harvested strains were evaluated with the Neuro-2A assay. Toxicities are summarized in Table 13. Results show CTX-like positive strains: 2 (4.34 and 17.60 fg eq. CTX1B /cell), no quantifiable (NQ): 11 cases, CTX-like negative: 6 cases.

Table 13. Toxicity of extracts of extracts of *Gambierdiscus* spp. from Crete evaluated by the Neuro-2A assay. (for CTXs: NQ*: not quantifiable due to matrix effect. NQ**: not quantifiable due to different toxicity behaviour to be further studied)

Extract Code	Strain Code	Identification	Island	fg eq. CTX-1B/cell	MTX
E-17-263	001G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	NQ*	NT
E-17-207	002G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	NQ**	NT
E-17-225	003G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	NQ*	NQ
E-17-231	004G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	NQ*	NQ
E-17-213	005G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	NQ*	Negative
E-17-210	006G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	NQ**	+
E-17-212	007G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	Negative (LOD: <0.68)	NT
E-17-224	008G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	NQ*	NT

.

E-17-211	009G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	Negative (LOD: not valid)	NT
E-17-152	0010G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	NQ**	NT
E-17-229	011G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	NQ*	NT
E-17-35	012G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	Negative (LOD: <12.86)	NT
E-17-230	013G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	Negative (LOD: <2.98)	NT
E-17-187	016G-CR-CCAUTH	*Gambierdiscus* sp.	CRETE	Negative (LOD: <1.307)	NT
E-17-261	017G-CR-CCAUTH	*Gambierdiscus* sp.	CRETE	17,60	NT
E-17-223	018G-CR-CCAUTH	*G.* australes	CRETE	NQ*	NT
E-17-188	019G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	NQ*	NT
E-17-226	020G-CR-CCAUTH	*Gambierdiscus belizeanus* group	CRETE	4,34	NT

2.5.2.3.1.2 Geographical distribution and toxicity

Strains of *Gambierdiscus* in Crete were isolated from Kolimpari and Kissamos. Toxicity of all strains ranged from 4.34-17.6 fg eq. CTX1B /cell, as we will see quite much lower than values obtained in the Balearic Islands, most probably related to species, as *G. belizeanus* were the dominant ones in Crete versus *G. australes* the only species in the Balearic Islands.

2.5.2.3.2 Cyprus

2.5.2.3.2.1 Evaluation of CTX-like toxicity with the Neuro-2a CBA

From Cyprus, 9 out of the 13 harvested strains were evaluated by the Neuro-2A assay. Toxicities are summarized in Table 14. Results show CTX-like positive (+): 3, no quantifiable (NQ): 2 cases. CTX-like negative: 4 cases. (NT: not tested).

Table 14. Toxicity of extracts of *Gambierdiscus* spp. from Cyprus evaluated by the Neuro-2A. (for CTXs: NQ*: not quantifiable due to matrix effect. NQ**: not quantifiable due to different toxicity behaviour to be further studied)

Extract Code	Strain Code	Identification	Island	fg eq. CTX-1B/cell	MTX
E-17-190	001G-CY-CCAUTH	*Gambierdiscus* sp (new species)	CYPRUS	Negative (LOD:< 0.215)	NT
E-17-260	002G-CY-	*Gambierdiscus* sp (new	CYPRUS	Negative (LOD:	NT

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	CCAUTH	species)		<0.16)	
E-18-137	003G-CY-CCAUTH	*Gambierdiscus* sp (new species)	CYPRUS	0.36 ± 0.04	NT
E-18-145	005G-CY-CCAUTH	*Gambierdiscus belizeanus* group.	CYPRUS	12.2 ± 1.56	NT
E-18-112	006G-CY-CCAUTH	*Gambierdiscus belizeanus* group.	CYPRUS	NQ**	NT
E-18-140	008G-CY-CCAUTH	*Gambierdiscus* sp (new species)	CYPRUS	0,27 ± 0,07	NT
E-18-141	010G-CY-CCAUTH	*Gambierdiscus* sp (new species)	CYPRUS	Negative (LOD: 0,33)	NT
E-18-146	014G-CY-CCAUTH	*Gambierdiscus belizeanus* group.	CYPRUS	NQ*	NT
E-18-134	015G-CY-CCAUTH	*Gambierdiscus belizeanus* group.	CYPRUS	NQ**	NT

2.5.2.3.2.2 Geographical distribution and toxicity

Strains of *Gambierdiscus* in Cyprus were located in Zygi-Larnaca and Konnos-Ammochostos. Toxicity of all strains ranged from 0.27-12.2 fg eq. CTX1B /cell, as we will see quite much lower than values obtained in the Balearic Islands, most probably related to species, as *G. belizeanus* were the dominant ones in Cyprus versus *G. australes* the only species in the Balearic Islands.

In Figure 32, we present data form the Eurocigua project and other previously reported in order to get an update of the present distribution of *Gambierdiscus* and *Fukuyoa* in the Eastern Mediterranean Sea.

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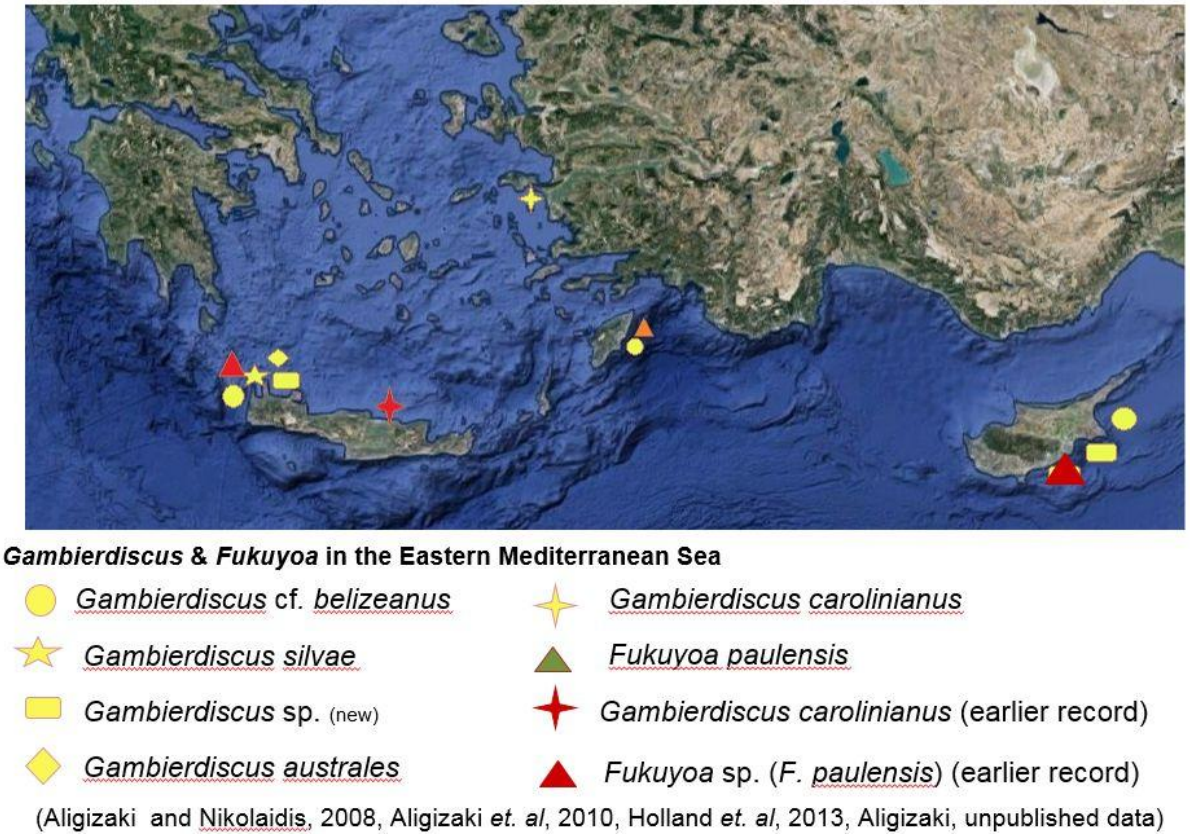

Fig 32. Distribution of each species in the stations of the eastern Mediterranean Sea including previous works and Eurocigua samplings.

2.5.2.3.3 Balearic Islands

2.5.2.3.3.1 Evaluation of CTX-like toxicity with the Neuro-2a CBA

Results of the toxicity evaluated in *Gambierdiscus* sp. from the Balearic Islands indicate that all strains were CTX-like positive: 24 cases (range: 1.38 – 381.83 fg CTX-1B eq./cell) (Table 15). Most strains presented also MTX toxicity.

Table 15. Toxicity of extracts of *Gambierdiscus* spp. from the Balearic Islands evaluated with the the Neuro-2A assay. (NT: not tested)

Extract Code	Strain Code	Species	Island	Archipelago	fg eq. CTX-1B/cell	MTX
E-18-03	IRTA-SMM-17-153	*G. australes*	MINORCA	BALEARIC	1.38 ± 0.66	+
E-18-18	IRTA-SMM-17-155	*G. australes*	MINORCA	BALEARIC	17.33 ± 1.6	+

.

Extract Code	Strain Code	Species	Island	Archipelago	fg eq. CTX-1B/cell	MTX
E-18-21	IRTA-SMM-17-161	*Gambierdiscus* sp.	MINORCA	BALEARIC	14.46 ± 4.43	+
E-18-14	IRTA-SMM-17-162	*G. australes*	MINORCA	BALEARIC	105.67 ± 18.27	+
E-18- 22	IRTA-SMM-17-163	*G. australes*	MINORCA	BALEARIC	14.88 ± 4.69	NT
E-18-15	IRTA-SMM-17-164	*G. australes*	MINORCA	BALEARIC	72.60 ±43.20	+
E-18-135	IRTA-SMM-17-168	*G. australes*	MALLORCA	BALERIC	381.83 ± 91.84	NT
E-18-07	IRTA-SMM-17-175	*G. australes*	MINORCA	BALEARIC	62.00 ± 0.66	NT
E-18-17	IRTA-SMM-17-178	*G. australes*	MINORCA	BALEARIC	14.52 ± 4.31	+
E-18-53	IRTA-SMM-17-180	*G. australes*	MINORCA	BALEARIC	5.25 ± 0.59	+
E-18-12	IRTA-SMM-17-181	*G. australes*	MINORCA	BALEARIC	13.50 ± 0.8	+
E-18-80	IRTA-SMM-17-189	*G. australes*	MINORCA	BALEARIC	83,39 ± 12,14	+
E-18-35	IRTA-SMM-17-173	*G. australes*	MAJORCA	BALEARIC	21.89 ± 9.2	+
E-18-11	IRTA-SMM-17-214	*G. australes*	MAJORCA	BALEARIC	76.67 ± 29.86	+
E-18-55	IRTA-SMM-17-215	*Gambierdiscus* sp.	MAJORCA	BALEARIC	21.42 ± 2.18	+
E-18-04	IRTA-SMM-17-216	*G. australes*	MAJORCA	BALEARIC	13.04 ± 4.5	+
E-18-08	IRTA-SMM-17-218	*G. australes*	MAJORCA	BALEARIC	9.47 ± 3.18	+
E-18-13	IRTA-SMM-17-223	*G. australes*	MAJORCA	BALEARIC	14.93 ±4.69	+
E-18-130	IRTA-SMM-17-238	*G. australes*	MAJORCA	BALEARIC	3.52 ± 0.18	NT
E-18-09	IRTA-SMM-17-244	*G. australes*	MAJORCA	BALEARIC	34.33 ± 4.18	+
E-18-148	IRTA-SMM-17-253	*G. australes*	MAJORCA	BALEARIC	13.45 ± 0.97	NT
E-18-06	IRTA-SMM-17-254	*G. australes*	MAJORCA	BALEARIC	13.16 ± 1.34	+
E-18-133	IRTA-SMM-17-256	*G. australes*	MAJORCA	BALEARIC	39.17 ± 16.43	NT
E-18-24	IRTA-SMM-17-271	*G. australes*	MINORCA	BALEARIC	172.63 ± 5.57	+

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In addition, results of *Fukuyoa* sp. from the Balearic Islands show CTX-like positive: 2 cases (7.96 and 16.3 fg/cell eq.), and CTX-like positive no quantifiable (NQ): 4 cases due to different toxicity behaviour to be further studied (Table 16).

Table 16. Toxicity of extracts of *Fukuyoa* spp. from the Balearic Islands evaluated with the Neuro-2A assay. (for CTXs: NQ**: not quantifiable due to different toxicity behaviour to be further studied)

Extract Code	Strain Code	Species	Island	Archipelago	fg CTX-1B eq./cell
E-18-198	IRTA-SMM-17-198	*F. paulensis*	Majorca	BALEARIC	NQ**
E-18-64	IRTA-SMM-17-206	*F. paulensis*	Majorca	BALEARIC	NQ**
E-18-86	IRTA-SMM-17-209	*F. paulensis*	Minorca	BALEARIC	16.3 ± 1.67
E-18-59	IRTA-SMM-17-211	*F. paulensis*	Minorca	BALEARIC	7.96 ± 0.14
E-18-61	IRTA-SMM-17-220	*F. paulensis*	Minorca	BALEARIC	NQ**
E-18-109	IRTA-SMM-17-221	*F. paulensis*	Minorca	BALEARIC	NQ**

When comparing *Gambierdiscus australes* and *Fukuyoa paulensis* toxicity, we can see that *G. australes* toxicity ranged from 1.38 - 381.83 fg eq. CTX1B /cell and *F. paulensis* toxicity was lower, ranging form 7.96 - 16.3 fg eq. CTX1B/cell.

2.5.2.3.3.2 Geographical distribution and toxicity

In relation to the geographical location of these microalgae in the Balearic Islands in samplings conducted between 2016 and 2019, we can observe that *Gambierdiscus australes* and *Fukuyoa paulensis* have a wide presence in the sampled islands (Majorca, Minorca and Formentera) (Fig. 33)

Among the strains producing higher than 100 fg eq. CTX1B /cell, we identify 3 strains of *G. australes* located in Minorca (St.Adeodat and Macarella) and Majorca (Cala Pi).

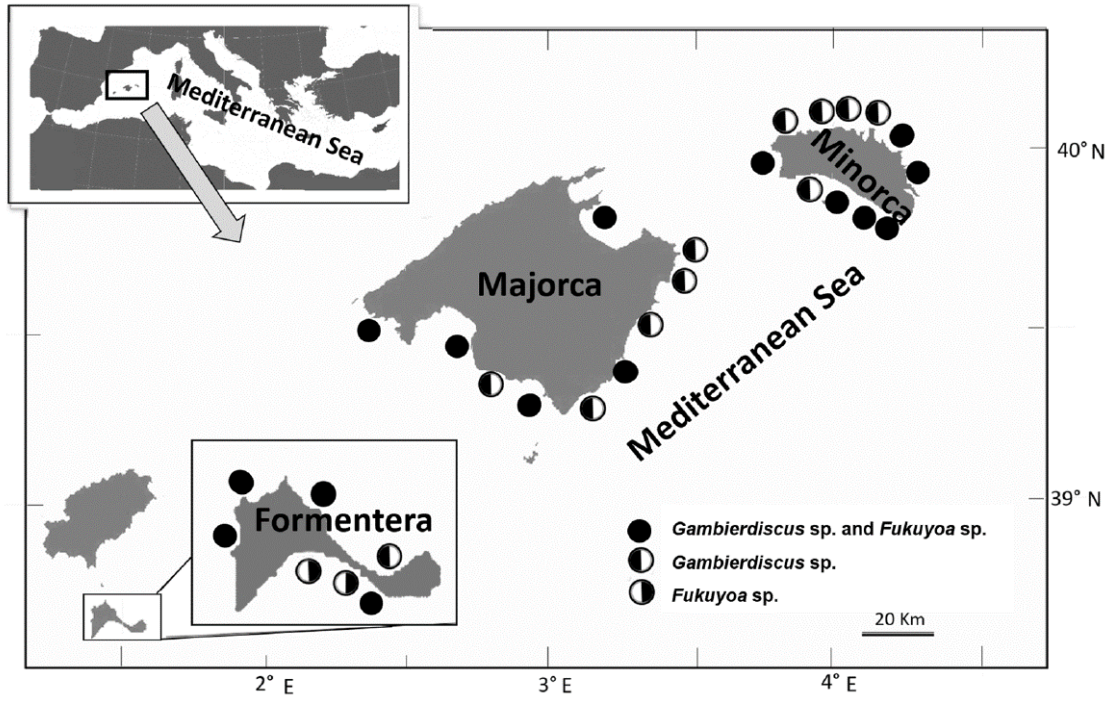

Fig. 33. Presence of the *Gambierdiscus* and *Fukuyoa* genera in the sampling stations in the Balearic Islands (Mediterranean Sea) during 2016-2019.

A specific analysis for toxins was conducted under SG4, and confirmed presence of MTXs. Details are reported in the specific report of SG4.

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3. Sampling of fish and CTX evaluation (IRTA, ULPGC, SCS, IPMA, AUT, SGL, RMLFAW, DRPM)

3.1 Sampling of fish species in areas where locally captured contaminated fish or *Gambierdiscus* spp. has been reported

(Canary Islands, Madeira, Crete, Cyprus and Balearic Islands).

(IRTA, ULPGC, SCS, IPMA, AUT, SGL, RMLFAW, DRPM)

The evaluation of the toxicity of fish is another indicator of the risk of ciguatera in a given area. It is important to state that, since microalgae of the genera *Gambierdisucs* and *Fukuyoa* are benthic algae, their presence, abundance and toxicity may provide an indication of the local risk.

Since most fish have a relative mobility, especially migratory fish, the association of risk considering the origin of the fish and location is less direct, except for some sedentary species. In addition, a certain difficulty rises from the fact that the location of capture is seldom precise.

Below the toxicities of fish evaluated with the Neuro-2a cell based assay within SG3 are presented; they can complement the information retrieved from the toxicities evaluated in microalgae.

3.1.1 Canary Islands

We report here results from two laboratories: ULPGC and IRTA.

As explained in the methodology section, the evaluation of the toxicity of fish with the CBA was harmonized between IRTA and the ULPGC. It is important to state that the ULPGC has analysed many more fish samples from the Canary Islands, as part of their task, providing a scale of toxicity ranging from L1-L5. In addition, IRTA has performed analysis in a selection of these samples in order to initially harmonize activities between the two laboratories, and secondly, to provide additional estimation of the CTX-like toxicity in some samples. IRTA has provided a more detailed quantification of the toxic effect, expressed as CTX-1B equivalents (µg/kg, ppb).

The data of the ULPGC laboratories concern a wider spectrum of fish, and conclusions regarding the geographical distribution of toxic fish may be more accurate than conclusions of IRTA, since the number of fish analysed by IRTA was lower. Noneteheless, the quantification of the toxicity of fish is more accurate in samples analyzed at IRTA, providing a concentration of CTX-1B equivalents.

Hence, three groups of data are presented below:

- ULPGC-EUROCIGUA fish: Data on EUROCIGUA samples analysed by the ULPGC. These cover the whole number of fish (n=746) from the Canary Islands obtained within the project. ULPGC provides toxicity evaluation within a scale from L1 to L5.

- IRTA- selected EUROCIGUA fish: Data on EUROCIGUA samples analysed by IRTA. These cover a selection (n=130) of the whole number of fish from the Canary Islands obtained within the project. IRTA provides an estimation of toxicity expressed in CTX-1B equivalents (µg/kg, ppb).

- 2018 and 2109 OFFICIAL CONTROL fish: Data of the Official Control Program of the Canary Islands government on the toxicity of fish covering the years 2018 (n=1128) and 2019 (n=839). The fish were sampled according to the specific weights above which analysis is obliged in the Canary Islands. These data complement the information obtained within the EUROCIGUA project in order to provide EFSA with a richer synthesis of the situation in the Canary Islands. Analysis of samples from the official control were conducted by the ULPGC using a different extraction method since this was a management decision of the Government of the Canary Islands, and mainly consists of inverting the order of the partition steps, eliminating first very lipophilic compounds with hexane, and later, recovering CTX compounds in the diethylether phase.

3.1.1.1 ULPGC laboratory – EUROCIGUA fish

All fish samples from the sampling in Canary Islands were analysed by CBA at ULPGC. From 746 fish extracts analysed, 105 were positive and 9 were dubious (Table 24, Fig 35).

3.1.1.1.1 Overall data on toxicity of fish

The ULPGC analyzed 746 fish obtained within the EUROCIGUA project. These were distributed along a wide range of Islands, species and weights. Data on the fish caught are reported below and on specific annexes. Out of these 746 fish, 105 were positive for CTX-like toxicity (Table 17 and Fig. 34).

Table 17. Toxicity of the fish and extracts processed from the Canary Islands evaluated with the Neuro-2A CBA.

Results of experimentation	Number
Positives	**105**
Negatives	**632**
Dubious	**9**
Total of extracts	**746**
Total of fish	**746**

Fish were obtained from different sources (Fig. 34). Most of them were directly purchased form the market and local contacts, being 9% of these toxic. Sport fishing was also an important source of fish, with a high percentage of positive fish (27%). The Health Department of the Canary Islands, a member of the project consortium, contributed, through their

PECIGUA program, with additional fish, being 17 % of these toxic. A specific toxic episode of a bloom of *Gambierdiscus* cells was reported in the island of El Hierro, in the harbor of La Restinga, where specific fish during these event were sampled, resulting for 14% as toxic. Other fish analyzed were from ciguatera episodes (CFP), aquaculture and a particular circumstance of fish present in the stomach of a larger fish.

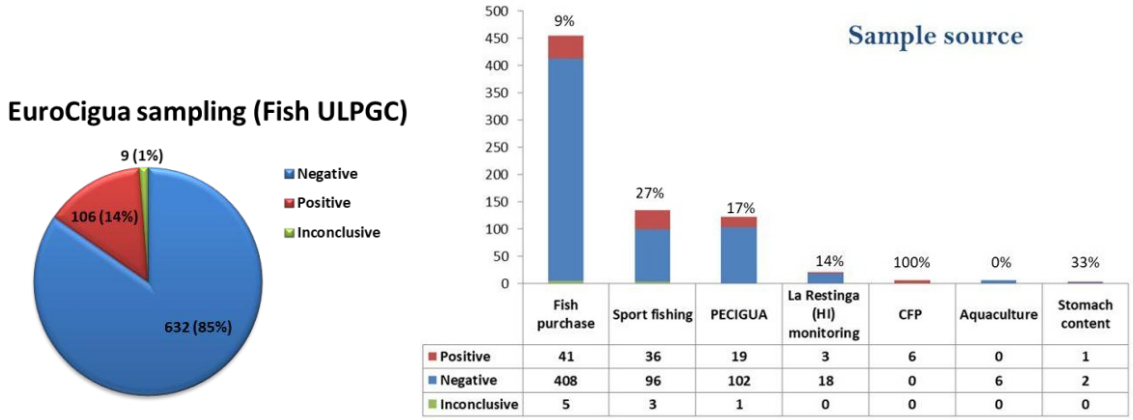

Fig. 34. Toxicity results of the CBA in all fish from the Canary Islands sampled within the Eurocigua project (left) and results according to different sources of sampling (right). La Restinga (El Hierro) monitoring corresponds to a punctual algal bloom occurred in that island. CFP: Ciguatera Fish Poisoning (human poisoning episodes).

When analysing the data according to the island of origin, different percentages of toxic fish were found among islands (Table 18 and Fig. 35, and Fig. 36). When analysing these data, it is important to be cautious. El Hierro island seems to be a hot spot area for ciguatoxin, with greater percentage of toxic samples (34% of positives). As pointed before, a bloom of *Gambierdiscus caribaeus* was detected, resulting in an increased sampling of fish in El Hierro during that episode that may explain this high percentage. Apparently, the incidence of the bloom resulted into presence of toxins in fish in a matter of weeks. It is difficult to provide with clear kinetics, since transfer of toxins will depend on the toxicity and abundance of the microalgae, and fish behaviour, and in this particular case there were not clear data when the bloom had started. It is also important to state that this high percentage in el Hierro is in agreement with the data of the official control (Tables 26 and 28).

Table 18. Toxicity of the fish and extracts processed from the Canary Islands evaluated by the Neuro-2A CBA according to Island.

Island	Total	Inconclusive	Negative	Positive	Positive %
Lanzarote	141	-	124	17	12%
Fuerteventura	59	-	55	4	7%
Gran Canaria	142	1	134	7	5%
Tenerife	150	3	127	20	13%
La Gomera	53	1	51	1	2%
La Palma	73	2	59	12	16%
El Hierro	128	2	82	44	34%
Total	**746**	**9**	**632**	**105**	**14%**

Data on positive fish, are reported in the following figure.

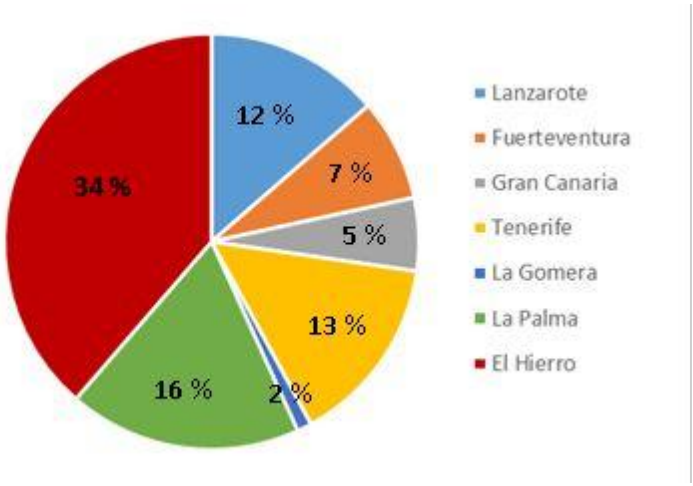

Fig. 35. Percentage of positive fish according to Island from the Canary Islands evaluated by the Neuro-2A CBA.

Fig. 36. Toxicity of fish according to the spatial distribution of samples. The islands were labelled as "1" when the percentage of positives was less than 10%, as "2" when the percentage of positives ranged from 10 to 20% and "3" when it was more than 20%.

Regarding the season of fishing, no significant difference was observed. However, the percentage of positive fish in the warm season with 15% of positives (May-December) was slightly higher than the cold season with 11% of the fish being positive (January-April).

Figure 37 represents positive results according to sample sources and capture season considering 5 toxicity levels (L1 to L5), from very low to very high represented with different grey shades. It can be seen that all samples linked to CFP showed very high level of toxicity, and quite similar percentages of high toxic results were observed in both season categories.

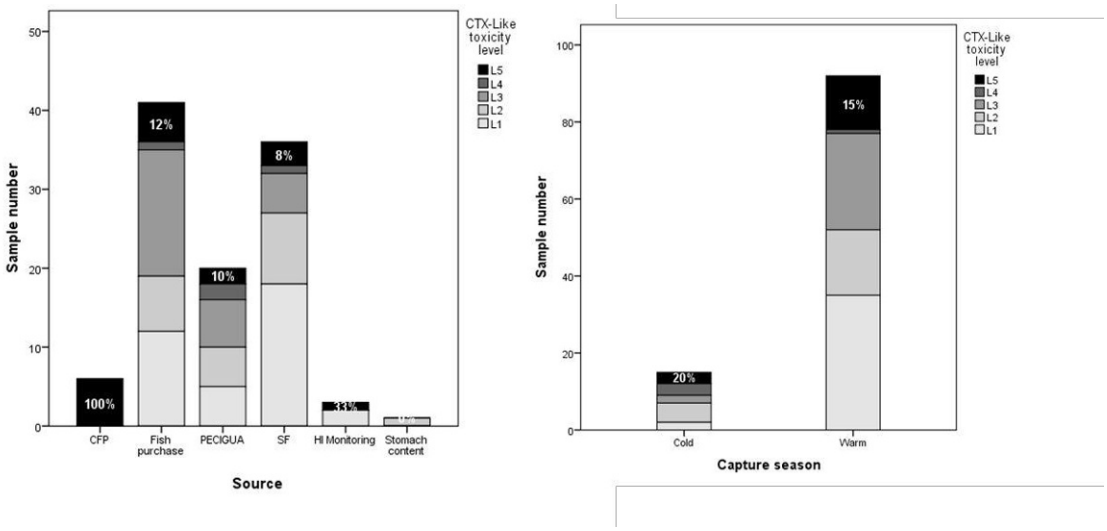

Fig. 37. Bar graphs describe toxicity levels based on sample sources (left) and capture season (right). CTX-Like toxicity levels: L1, very low; L2, low; L3, medium; L4, high; L5, very high.

When comparing the toxicity levels of positive fish from different islands of capture (Fig. 38), El Hierro (HI) was not only the one with higher number of positive fish but also the one where the fish exhibited higher toxicity level (more than 50% of positive samples showed a medium to very high level of toxicity).

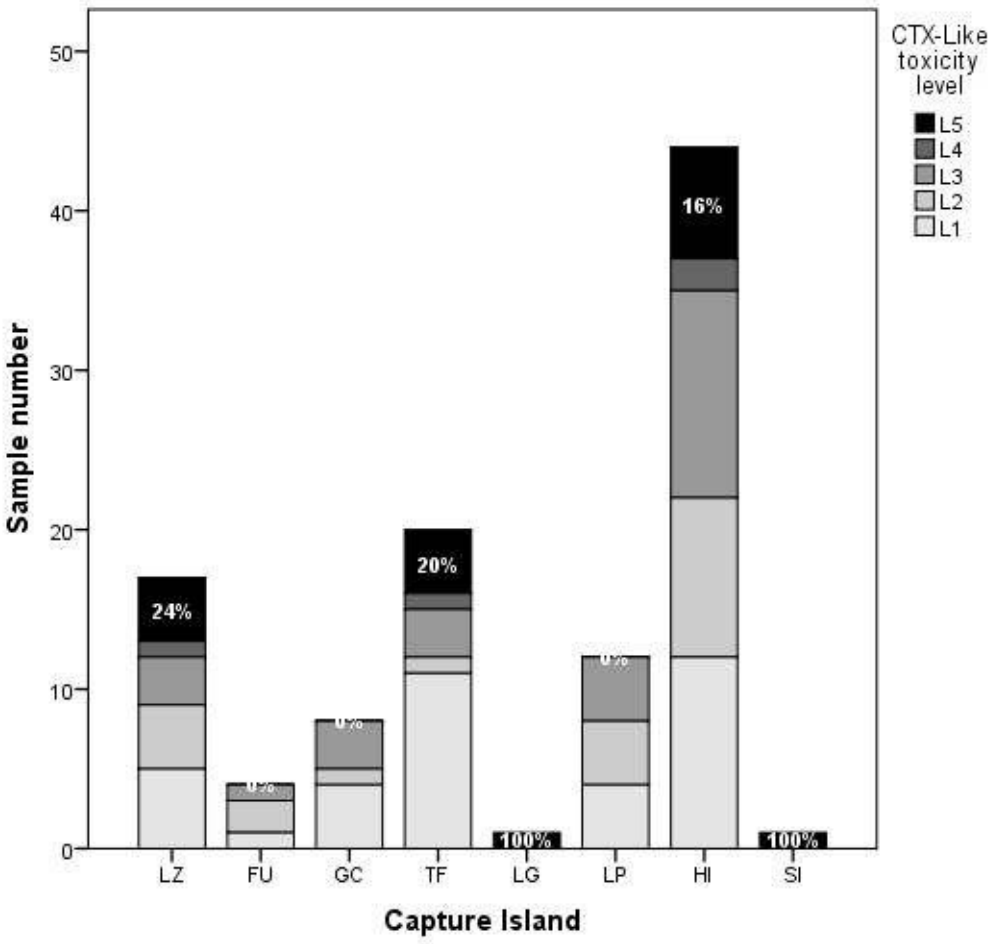

Fig. 38. Toxicity levels of positive samples based on the spatial distribution of sampling. CTX-Like toxicity levels: L1, very low; L2, low; L3, medium; L4, high; L5, very high.

Toxicity also differed between fish species. Table 19 includes all the fish species showing CTX-like toxicity. Regarding the 17 species of fish reported as potentially ciguatoxic, the level of toxicity was significantly different (p=0.012) among species (Fig. 39). More than 20% of the positive samples of amberjack, dusky grouper, black and brown moray, island grouper and red porgy displayed level 5 of toxicity.

Table 19. Toxicity of the fish processed from the Canary Islands evaluated by the Neuro-2A CBA according to fish species.

Fish species	Total	Inconclusive	Negative	Positive	Positive (%)	Weight (kg) Positive fish
Acanthocybium solandri	41	2	31	8	20	14-30
Bodanius scrofa	9	-	8	1	11	1.56
Canthigaster capistrata	1	-	-	1	100	0.03

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Diplodus cervinus cervinus	11	-	9	2	18	0.69-1.15
Diplodus vulgaris	28	-	17	11	39	0.26-0.71
Enchelycore anatina	5	-	-	5	100	0.36-1.44
Epinephelus marginatus	52	1	36	15	29	6-30
Gymnothorax unicolor	16	-	13	3	19	0.51-2.72
Muraena augusti	27	1	14	12	44	0.40-2.81
Muraena helena	41	-	37	4	10	1.05-5
Mycteroperca fusca	11	-	5	6	55	2.5-8
Pagrus pagrus	28	-	26	2	7	1.65-4
Parapristipoma octolineatum	20	-	19	1	5	0.29
Pomatomus saltatrix	17	-	13	4	24	6.10-8.80
Pseudocaranx dentex	31	-	25	6	19	0.23-7
Seriola sp.	86	1	65	20	23	10.2-70
Sparisomam cretense	65	2	59	4	6	0.37-0.48
Other non-toxic fish*	257	2	255	-	-	-
Total	**746**	**9**	**632**	**105**	**7**	

*: data on these fish are included in Annex, 2020_EUROCIGUA_SG3_FISH_TOXICITY

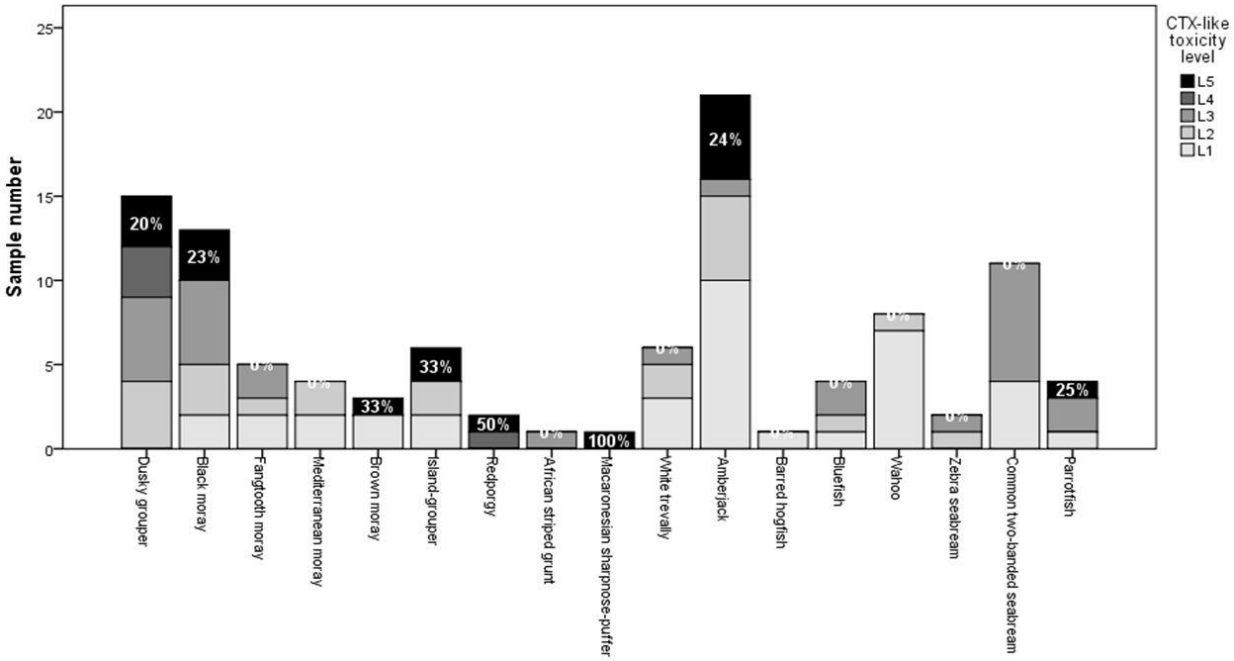

Fig. 39. Toxicity levels of positive samples according to the fish species. CTX-Like toxicity levels: L1, very low; L2, low; L3, medium; L4, high; L5, very high. The percentage of L5 fish is reported.

3.1.1.1.2 Information regarding some remarkable fish species and the specific case of El Hierro island

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Among the samples of the Eurocigua project, Amberjack is one of the most important fish species in the Canary Islands regarding CTX toxicity.

Statistically significant differences have been found with regard to the number of positives and the capture season (warm or cold), as it can be seen in Fig. 37. Therefore, a risk gradient was obtained for amberjack, considering weight of fish and season of capture. Range of positives in amberjack is found between 10,2 -70 kg. For the fish weighing 10,2 kg, only the liver was analyzed. Its feeding habits includes crustaceans, cephalopods and fish.

Another remarkable species is the common two-banded seabream (*D. vulgaris*). Its distribution is surrounding the limit of the cost of the Mediterranean sea and Atlantic Ocean in our region. This species is a carnivorous fish feeding on small crustaceans and worms, although there is no evidence that it has produced ciguatera episodes in the Canary Islands, but this species is known to be carrier of ciguatoxin, 39% of the analyzed fish were positive. Its main feeding habits include crustacea, polychaeta, echinoderms and fish.This species presented the highest contamination rate in El Hierro island (75% of positive) and secondly 22% in Gran Canaria. As it was found with other species of fish, statistically significant relationship between weight and length was observed (Fig. 40 A). Regarding toxicity, negative fish were significantly different from positive fish according to weight data distribution and the median of weight (Fig. 40 B).

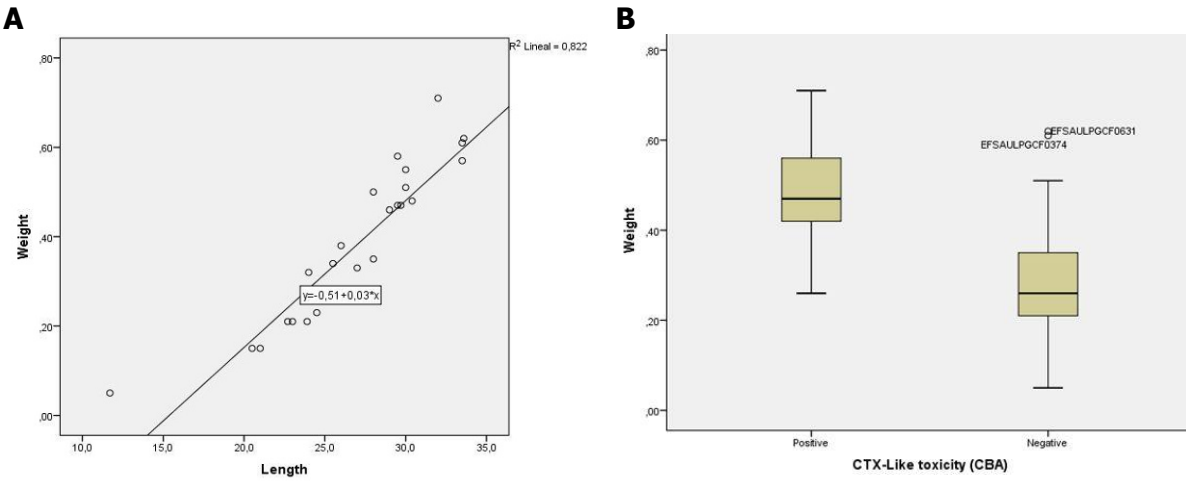

Fig. 40. A) Correlation between weight and length in common two-banded seabream (*D. vulgaris)*, B) Weight for CTX positive and negative two-banded seabream (*D. vulgaris)*

Besides amberjack, dusky grouper (*E. marginatus*) is one of the more important species investigated in this project. Based on the official data provided by the Canary Government, this is one of the species involved in CFP cases in the Canary Archipelago. 32 people resulted poisoned. It can be found all around the seven islands, until 200 m depth. This species is used to be hunted at sunrise and sunset, and its main feeding habits include cephalopods, crustaceans and fish.

Moray eels is a fish species that must be emphasized according to results obtained in this project. Ten moray eel species cohabit in the Canary Islands environment. In addition, there are also important fish species for human consumption in this Region. Among the five more

prevalent moray eel species in the Canary Islands, regarding CTX like toxicity we have found the next ones: fangtooth moray, black moray, brown moray, polygon moray and mediterranean moray. Most of them are nocturnal predators and their feeding habits include crustaceans, shellfish, cephalopods and fish. The relative proportion of positive fish were the following: fangtooth moray (100%), followed by black moray (44%), and occurring less frequently, brown moray (19%) and mediterranean moray (10%).

A very interesting fact is to consider the interaction between dusky groupers and moray eels in the natural environment. Different grouper and moray eel species have been considered responsible of many CFP outbreaks in the Indic, Pacific and Atlantic Regions, and among other carnivorous fish species, moray eels and groupers have been found showing the highest CTX concentration in some places of the world.

In order to document this interaction in the Eurocigua Project, one dusky grouper was submitted to the necropsy service in the Veterinary Faculty of the ULPGC, with the following data: 17,4 kg weight, 93 cm length, it was caught by professional fishermen in august 2017 in "La Santa" (north of Lanzarote).

Necropsy was conducted following standards protocols, and as results, a partially digested body of a black moray (*M. augusti*) was found in the stomach, with 1.03 kg of weight and 82 cm of total length. Other non-identified moray eel rests were also observed in the stomach.

CTX was detected and quantified in flesh from the grouper necropsied and from the moray eel found in its stomach. Quantification was made with the CBA in the IUSA laboratory of the ULPGC. Identification and CTX quantification was also obtained in the University of Vigo with an LC-MS/MS method. As showed in table 20, quantification of C-CTX-1 was very similar for both specimens, showing a high evidence of interaction between both species in the marine environment of the Canary Islands.

At the same time, 46 necropsies of fish from the official control protocol were carried out to prepare 661 kg of fish and has been already sent to Vigo for SG4. Deliverable 3.7 Phase 1 reference material include further information of these necropsies.

Table 20.CTX quantification in flesh from a grouper necropsied and the moray eel found in its stomach.

Species	CTX-like toxicity[c]	CTXs with LC-MS/MS[b]
Dusky grouper (*E. marginatus*)	0.032 ± 0.009 ppb	0.03 ppb C-CTX1
Black Morray (*M. augusti*)	0.037 ± 0.015 ppb	0.05 ppb C-CTX1

[a] Quantification of CTX (CTX-1B eq./kg, ppb) obtained with the CBA in the IUSA laboratory. Average of duplicate results ± standard deviation.

[b] Identification and CTX quantification (ppb) obtained at the University of Vigo using LC-MS/MS method.

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Finally, we present some highlights data regarding the El Hierro Island. A high contamination rate was obtained for some fish species investigated in Eurocigua. For instance, 75% for seabream (*D. vulgaris*), and 33% for moray eel analysed were positive. It must be considered, at the same time, that similar results have been previously reported, showing high percentages (90.9%) of CTX like toxicity in dusky groupers analyzed in the Official Control program established in the Canary Islands. We have to take into account, that an algal bloom of *G. caribaeus* occurred in this island in october 2016. All these facts considered together, suggest El Hierro island as a ciguatoxin hot spot in the Canary Islands Archipelago. This effect should be studied in greater depth.

3.1.1.2 IRTA laboratory - selected EUROCIGUA fish

At IRTA, 157 extracts from 130 fish of the Eurocigua project obtained from different places of the Canary Islands (from the seven islands) were analysed in order to evaluate the presence of CTX and provide a more detailed quantification of toxicity (Table 21). It is important to consider that these fish were selected in order to compare results between the two institutions (ULPGC and IRTA) and to obtain detailed estimations of the toxicity of fish that were previously identified as toxic. Hence, these data should not be used to evaluate incidence of toxicity according to islands or species.

Table 21. Toxicity of the fish and extracts processed from the Canary Islands evaluated by the Neuro-2A CBA and number of fish pending processing (from IRTA).

Results of experimentation	Number of extracts	Number of fish
Positives	**92**	**84**
Negatives	**65**	**46**
Dubious	**0**	**0**
No experimentation	**157**	
Total of extracts	**157**	
Total of fish		**130**

At IRTA, 84 fish out of 130 were reported as CTX-like positives in the Canary Islands. Toxicities ranged from 0.0018-0.5760 µg/kg CTX-1B equivalents (Table 22).

Table 22. Toxicity of the fish from the Canary Islands evaluated by the Neuro-2A CBA by Island (IRTA). LOQ = Limit of quantification.

Island	Total	Negative (<LOQ)	Positive	Positive %	CTX-like toxicity (CTX1B ppb) in positive fish	Average CTX-like toxicity + SD of positive fish
Lanzarote	41	14	27	66	0.0034-0.1440	0.0531 ± 0.0340
Fuerteventura	27	7	20	74	0.0061-0.4136	0.0692 ± 0.0964
Gran Canaria	7	2	5	71	0.0018-0.0718	0.0306 ± 0.0314
Tenerife	20	7	13	65	0.0092-0.5760	0.1068 ± 0.1235
La Gomera	2	2	0	0	-	-
La Palma	7	3	4	57	0.0018-0.0243	0.0100 ± 0.0100
El Hierro	25	12	13	52	0.003-0.3328	0.0487 ± 0.0898
Selvagen Islands*	1	0	1	100	0.1319	-
Total	**130**	**47**	**83**	**53**		

(*:this fish caught outside the Canary Islands was in the samples sent by ULPGC to IRTA)

Table 23. Toxicity and weight of fish extracts according to the fish species. LOQ = Limit of quantification.

Fish species	Negative (< LOQ)	Positive	CTX-like toxicity (CTX-1B eq ppb) in positive fish	Weight (kg) Positive fish	Weight (kg) Negative fish (<LOQ)
Acanthocybium solandri	2	1	0.0034	40	14-42
Canthigaster capistrata	0	1	0.0174	0.03	-
Coryphaena sp.	1	0	-	-	0.95
Diplodus cervinus cervinus	1	0	-	-	0.69
Diplodus vulgaris	1	0	-	-	0.32
Enchelycore anatina	0	1	0.0018	0.82	-
Epinephelus marginatus	6	24	0.0018-0.3328	6-29	19-30.20
Gymnothorax unicolor	1	0	-	-	2.72
Lutjanus cyanopterus	0	1	0.4136	16	-
Muraena augusti	1	3	0.034-0.088	0.41-1.03	0.4
Mycteroperca fusca	1	3	0.045-0.500	2.5-8	4
Pagrus pagrus	0	1	0.1319	4	-
Pseudocaranx dentex	0	1	0.0153	0.23	-
Seriola spp.*	28	47	0.0037-0.5760	16.5-73	14.5-45
Sparisoma cretense	5	0	-	-	0.37-0.48

Fish species	Negative (< LOQ)	Positive	CTX-like toxicity (CTX-1B eq ppb) in positive fish	Weight (kg) Positive fish	Weight (kg) Negative fish (<LOQ)
Total	47	83			

* Includes *Seriola sp.*, *Seriola dumerili* and *Seriola rivoliana*.

Out of these data, we have analysed the weight and CTX-like toxicity for the two groups, amberjacks (*Seriola* species) and dusky grouper(*Epinephelus marginatus*), since these have high number of positive (Fig. 41, Fig. 42).

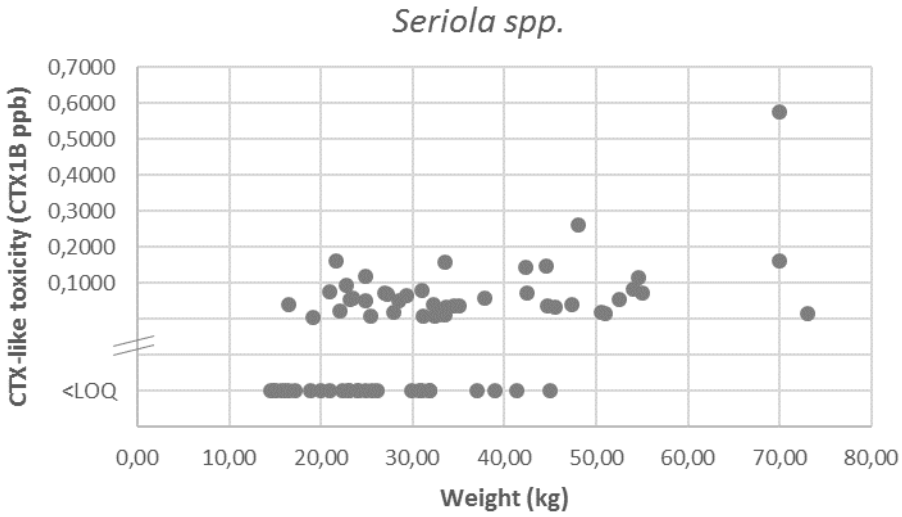

Fig. 41. Weight versus CTX-like toxicity in amberjacks (*Seriola* fish). (Negative fish are reported as <LOQ).

For amberjacks (*Seriola* fish), we can observe that positive fish cover almost the whole range of weights, ant that the heaviest fish have a large range of toxicities.

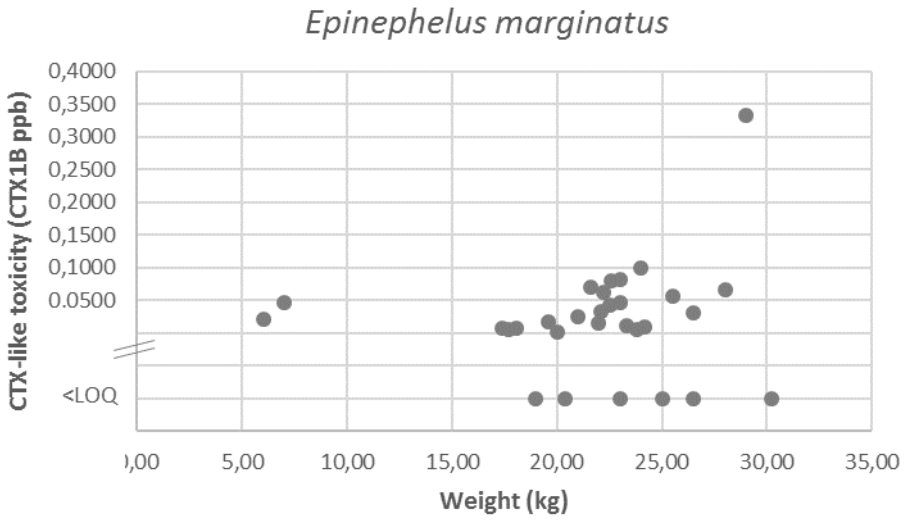

Fig. 42. Weight versus CTX-like toxicity in dusky grouper (*Epinephelus marginatus*). (Negative fish are reported as <LOQ).

For dusky grouper (*E. marginatus)* we can observe toxic fish over a wide range of weights.

Of all the 130 fish analysed at IRTA, 84 presented CTX-like toxicity. Among all toxic fish, 88,8% presented toxicities equal or higher than 0.01 ppb in CTX-1B equivalents, the value presently proposed as to be at risk for consumers by the FDA and EFSA (Table 24).

Table 24. Number of fish according to range of toxicity.

Range of toxicity of positive fish (CTX-1B eq ppb)	Number of fish	%
≥ 0.2	5	6
≥ 0.1 and < 0.2	9	10.7
≥ 0.05 and < 0.1	22	26.2
≥ 0.03 and < 0.05	14	16.7
≥ 0.01 and < 0.03	17	20.2
< 0.01	17	20.2

3.1.1.3 2018 and 2019 OFFICIAL CONTROL fish

In addition to the specific fish analysed within the Eurocigua project, we include herein data of the official control programme of the Canary Islands for the years 2018 and 2019 in order to complement the information on toxic fish.

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It is important to state that, these fish are selected according to a specific weight of fish, and therefore, the results presented below correspond to the following criteria regarding the fish that are analysed upon arrival at land and that are considered of a higher risk:

- Amberjack (*Seriola* spp) > 14 kg
- Wahoo (*Acanthocybium solandri*) > 35 kg
- Bluefish (Pomatomus saltatrix) > 9 kg
- Island grouper (*Mycteroperca fusca*) > 12 kg
- Dusky grouper (*Epinephelus* spp) > 15 kg
- Blue marlin (*Makaira nigricans*) > 320 kg
- Swordfish (*Xiphias gladius*) > 320 kg

3.1.1.3.1 Results of the OFFICIAL CONTROL for 2018

The data are provided from the Official Control program during 2018 and 2019, financed with a specific budget by the Directorate-General for Fisheries of the Canary Government. In the next Tables (25 and 26) the results of 2018 presented according to species and islands are displayed (sample with the presence of CTX-like toxicity is referred as positive). Some fish are reported as "no data". At that time, for some samples received in the lab, the identification of the fish had not been provided:

Table 25. Results of the official control programme of the Canary Islands according to the fish species (from 2018).

SPECIES	**Number Samples**	**Number Positives**	**Number Negatives**	**Positive %**
Amberjack	691	99	592	14,3
Grouper	199	46	153	23,1
Wahoo	31	8	23	25,8
Bluefish	9	5	4	55,6
No data	198	18	180	9,1
TOTAL:	**1128**	**176**	**952**	**15,6**

The % of positivity of some cases is not representative, due to the small size of the sample, as for example for Bluefish.

Table 26. Results of the official control programme of the Canary Islands according to the island (from 2018).

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ISLAND	No. Samples	No. Positives	No. Negatives	Positive %
LZ	191	33	158	17,3
FU	163	23	140	14,1
GC	240	57	183	23,8
TF	181	26	155	14,4
LG	79	5	74	6,3
LP	193	12	181	6,2
HI	81	20	61	24,7
TOTAL:	**1128**	**176**	**952**	**15,6**

As it can be seen, in 2018, 1128 fish samples were received and analysed through the Official Control of CFP with the CBA. The study species included Amberjack (*Seriola* spp.) (n=691), Grouper (*Epinephelus marginatus*) (n=199) and Wahoo (*Acantocybium solandri*) (n=31). Bluefish species displayed the highest percentage of positive samples (55.6%), however it must be highlighted the low number of samples analysed (9), thus this data should be treated with caution. The wahoo and grouper species showed higher percentage of CTX positivity (25.8 % and 23.1% respectively) than the amberjack (14.3%).

According to the island of capture, El Hierro and Gran Canaria account for a higher percentage of CTX positivity (24.7% and 23.8%, respectively) than Lanzarote (17.3 %), followed very closely by Tenerife (14.4 %) and Fuerteventura (14.1%). However, La Gomera and La Palma showed much lower number of CTX positive fish (6.3 % and 6.2 %, respectively) within the Canary Archipelago.

It can be noted that the percentage of samples with CTX-like toxicity from Gran Canaria are mostly due to *Seriola* spp. in contrast to positive samples from El Hierro which mainly belong to grouper species.

3.1.1.3.2 Results of the OFFICIAL CONTROL for 2019

In the next Tables (27 and 28) the results of 2019 presented according to species and islands are displayed (sample with the presence of CTX-like toxicity is referred as positive):

Table 27. Results of the official control programme of the Canary Islands according to the fish species (from 2019).

SPECIES	No. Samples	No. Positives	No. Negatives	Positive %
Amberjack	622	41	581	6.6
Grouper	159	44	115	27.7

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Wahoo	40	7	33	17.5
Bluefish	6	1	5	16.7
No data	12	8	4	66.7
TOTAL:	**839**	**101**	**738**	**12.0**

The % de positivity of some cases is not representative, due to the small size of the sample, as for example for Bluefish. From the 12 samples included with no information, 10 belong to Gran Canaria.

Table 28. Results of the official control programme of the Canary Islands according to the island (from 2019).

ISLAND	No. Samples	No. Positives	No. Negatives	Positive %
LZ	163	14	149	8.6
FU	75	2	73	2.7
GC	51	23	28	45.1
TF	260	15	245	5.8
LG	164	2	162	1.2
LP	33	2	31	6.1
HI	93	43	50	46.2
TOTAL:	**839**	**101**	**738**	**12.0**

In El Hierro the percentage of positive amberjack obtained was 21,9%, and for wahoo it was 22,2%. For dusky grouper, 34 samples were received, and 30 yielded a positive result, which represents a high percentage of dusky grouper obtained in El Hierro (88,2%). The high percentage of positive samples obtained in Gran Canaria was mainly obtained with fish from Castillo del Romeral Fisherman´s guild (GCCR).

As it can be seen, in 2019, 839 fish samples were received and analysed through the official control of CFP with the CBA. The study species included Amberjack *(Seriola spp.*) (n=622), Grouper (*Epinephelus marginatus*) (n=159) and Wahoo (*Acantocybium solandri*) (n=40), bluefish (n=6) and others (n=12). The grouper species and the "no data" species showed higher percentage of CTX positivity (27.7 % and 66.7 % respectively) than the amberjack (6.6 %).

According to the island of capture, El Hierroand Gran Canaria account for a higher percentage of CTX positivity (46.2% and 45.1%, respectively) than Lanzarote (8.6 %), followed very closely by La Palma (6.1%) and Tenerife (5.8 %). However, Fuerteventura and La Gomera showed much lower number of CTX positive fish (2.7 % and 1.2 %, respectively) within the Canary Archipelago.

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3.1.2 Madeira and Selvagens Islands (IPMA)

The toxicity of fish from Madeira were evaluated at IRTA, and the toxicity is reported as CTX-1B equivalents (µg/kg, ppb) (Table 29).

Table 29. Toxicity of the fish from Madeira evaluated by the Neuro-2A CBA.

Results of experimentation	Number of extracts	Number of fish
Positives	**42**	**42**
Negatives	**87**	**86**
Dubious	**0**	**0**
Number experimentation	**129**	
Total of extracts	**129**	
Total of fish		**128**

A summary of the positive in comparison to all fish from fish from Madeira and Selvagens Islands is shown in table 30. The Selvagens appear to be the area with highest percentage of positive fish. In order to understand this high incidence, additional data should be needed, for example, a more complete information on the species of *Gambierdiscus*, their abundance, dynamics and toxicity and also deeper knowledge on the communities of fish.

Table 30. Toxicity of the fish from Selvagens, Madeira and Desertas evaluated by the Neuro-2A CBA by Island (IRTA). LOQ = Limit of quantification.

Location	Total	Negative (<LOQ)	Positive	Positive %	CTX-like toxicity (CTX1B ppb) in positive fish	Mean + SD CTX-like toxicity (CTX1B ppb)
Selvagens	81	42	39	48	0.0039-0.7520	0.0781 ± 0.1386
Madeira	21	18	3	14	0.0270-0.0690	0.0419 ± 0.0235
Desertas	26	26	0	0	-	-
Total	**128**	**86**	**42**	**46**		

From all the positive samples of Madeira and Selvagens Islands, 24 fish were selected to prepare 66,5 kg of fish. These were sent to the University of Vigo for SG4 as reference material for the project. See further details in Deliverable 3.7 Phase 1 - Reference materials.

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A total of 19 species of fish were analysed and 11 of them showed CTX-like toxicity in muscle tissue (Table 31). The fish species showing CTX-like toxicity were the following ones: Island-grouper, dusky grouper, barred hogfish, pink dentex, amberjack, grey triggerfish, parrotfish and blacktail comber.

Table 31. Summary of positive fish from Madeira in comparison to all fish of each species. LOQ = Limit of quantication.

Fish species	Negative (< LOQ)	Positive	% of positive for the most sampled fish	CTX-like toxicity (CTX1B ppb) in positive fish	Weight (kg) Positive fish	Weight (kg) Negative fish (<LOQ)
Aluterus scriptus	1	0		-	-	2.42
Balistes capriscus	5	2		0.0160-0.0200	1.97-2.64	0.59-2.21
Bodianus scrofa	0	20	100 %	0.0039-0.7520	0.77-3.01	-
Dentex gibbosus	2	1		0.0270	8.10	7.54-7.84
Diplodus cervinus	0	1		0.3712	2.84	-
Epinephelus marginatus	0	1		0.0832	19.50	-
Katsuwonus pelamis	9	0		-	-	1.31-3.60
Kyphosus sectatrix	9	0		-	-	0.49-2.39
Makaira nigricans	1	0		-	-	298
Mycteroperca fusca	0	1		0.0677	4.53	-
Pomatus saltatrix	2	0		-	-	7.72-9.41
Seriola dumerili	8	2		0.0298-0.0690	20.12-27.60	0.56-31.48
Seriola fasciata	2	0		-	-	1.60-2.80
Seriola rivoliana	14	1	All *Seriola*	0.1045	12.31	1.22-22.64
Seriola sp.	1	0	10,7%	-	-	1.16
Serranus atricauda	5	5		0.0063-0.0217	0.19-0.34	0.28-0.81
Sparisoma cretense	26	6	18,75%	0.077-0.0421	0.42-0.85	0.23-0.84
Sphyraena viridensis	2	2		0.0768-0.2230	4.14-5.96	1.56-2.25
Total	87	42				

The relation between weight and toxicity was considered and the results presented in figure 43 below for the most representative groups of fish (according to number of sampled individuals presenting toxicity): *Balistes capriscus* (Fig. 43), *Bodanius scrofa* (Fig. 44), *Seriola spp.* (Fig. 45) *Serranus atricauda* (Fig. 46) and *Sparisoma cretense* species (Fig. 47)

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Although more data should be necessary, for three species, *Balistes capriscus, Bodanius scrofa* and *Sparisoma cretense* the most toxic fish were among the heaviest. For the other species, no clear pattern was observed, although the data show that for *Seriola* sp, no toxicity was reported in fish below 6 kg.

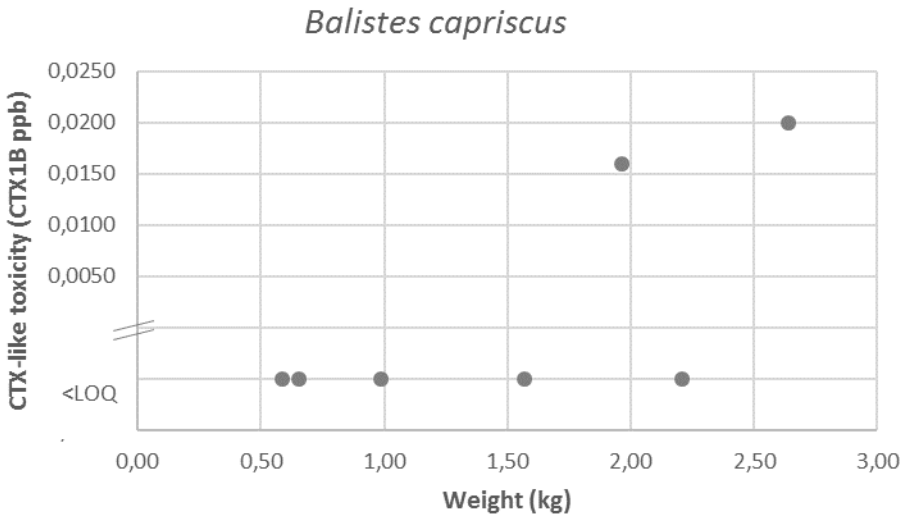

Fig. 43. Weight versus CTX-like toxicity in *Balistes capriscus* (Negative fish are reported as <LOQ).

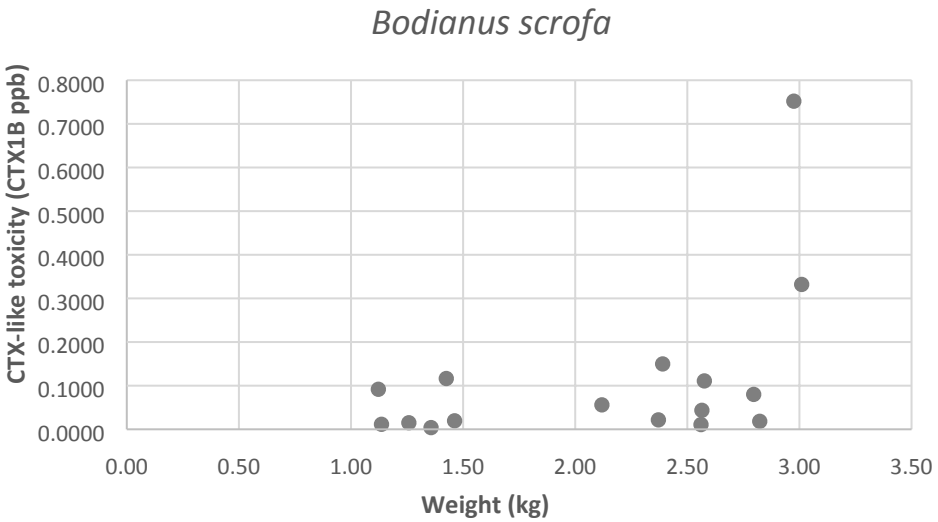

Fig. 44. Weight versus CTX-like toxicity in *Bodanius scrofa*.

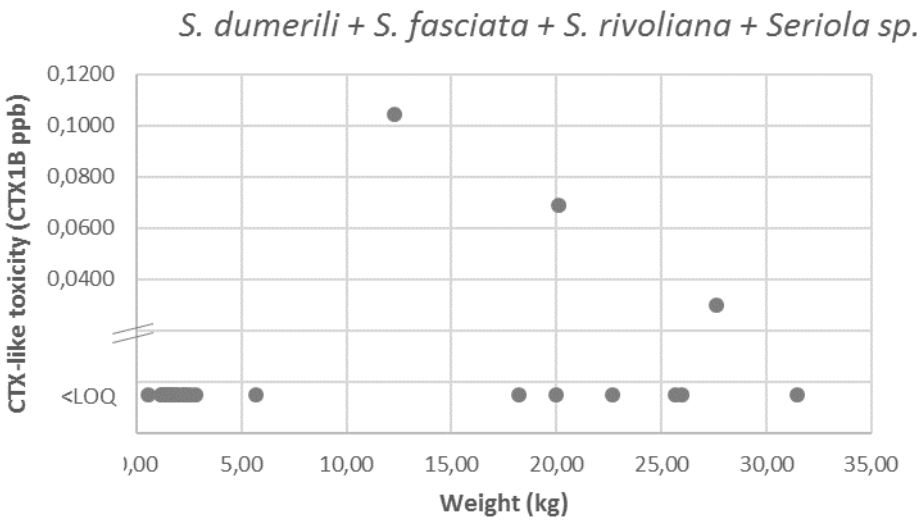

Fig. 45. Weight versus CTX-like toxicity in *S. durmerili, S. fasciata, S. rivoliana andn* Seriola sp. (Negative fish are reported as <LOQ).

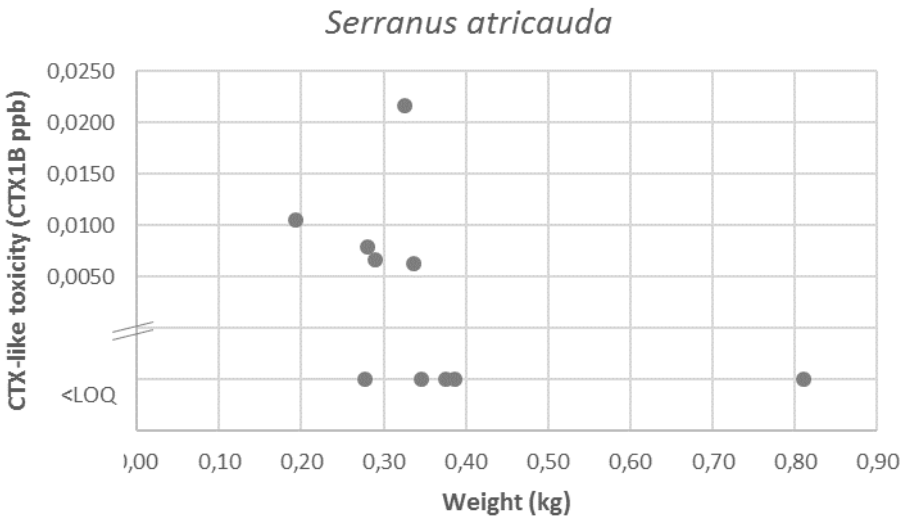

Fig. 46. Weight versus CTX-like toxicity in *Serranus atricauda* (Negative fish are reported as <LOQ).

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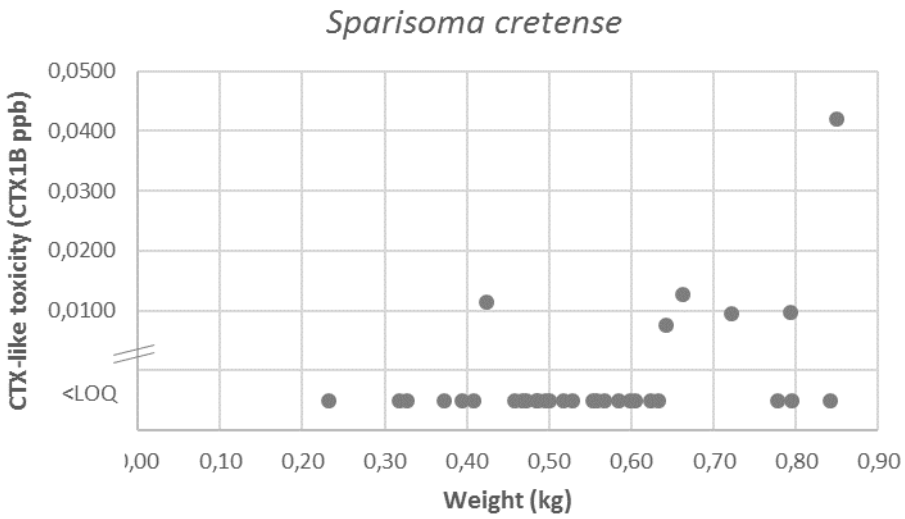

Fig. 47. Weight versus CTX-like toxicity in *Sparisoma cretense* (Negative fish are reported as <LOQ).

3.1.3 Mediterranean

3.1.3.1. Crete

Seventy fish have been received from Crete and extracts of muscle and liver have been obtained. All the extracts have been analysed; none of them has been reported as CTX-like positive (see Table 32).

Table 32. Toxicity of the fish processed from Crete evaluated by the Neuro-2A CBA.

Results of experimentation	Number
Positives	**0**
Negatives	**140**
Dubious	**0**
No experimentation	**140**
Total of extracts	**140**
Total of fish	**70**

The description of the analysed fish is presented in annex: P_2020_EUROCIGUA_SG3_FISH_TOXICITY.

3.1.3.2 Cyprus

As a follow-up action based on discussion in the IV Annual EuroCigua meeting (29-30 May 2019, Madeira), 5 *Muraena Helena* fish samples were sent to IRTA for CTX evaluation in January 2020.

Additionally, following an event of food poisoning in Cyprus due to fish consumption where individuals had symptoms similar to those of ciguatera, 7 additional fish samples were sent to IRTA for ciguatoxin analysis in June 2020.

IRTA has received 82 samples of fishes from Cyprus and 148 extracts of muscle and liver has been obtained and analysed. All of them have a negative result for the CBA assay except 1 muscle extract. (See Table 33).

Table 33. Toxicity of the fish processed from Cyprus evaluated by the Neuro-2A CBA and number of fish pending processing.

Results of experimentation	Number
Positives	**1**
Negatives	**147**
Dubious	**0**
No experimentation	**148**
Total of extracts	**148**
Total of fish	**82**

The identification of one CTX-like positive extracts (muscle) is an important result. This sample was analysed three times by Neuro-2a and the mean result is in Annex P_2020_EUROCIGUA_SG3_FISH_TOXICITY for the sample SGL-EFSA-F-00372.

The flesh crude extract that resulted positive by CBA was analysed with the magnetic bead-based immunoassay using 3G8 and 10C9 capture antibodies together, thus providing a global response of CTX1B, 54-deoxyCTX1B, CTX3C and 51-hydroxyCTX3C congeners. The immunoassay revealed the presence of these structurally-related congeners at a concentration of 4.98 pg CTX1B equiv./g flesh when analysed at 2500 mg flesh eq./mL. This fish sample was analysed by the Universidade de Vigo (See report in SG4), and no toxins were identified with LC-MS/MS.

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3.1.3.3. Balearic Islands

IRTA received 36 samples of fishes from the Balearic Islands that allowed to obtain 70 extracts of muscle and/or liver: 36 muscles extracts and 34 liver extracts
The species of fish evaluated were:

- Yellowmouth barracuda (*Sphyraena viridensis*, n=3)
- Mediterranean barracuda (*S. sphyraena*, n=2)
- White seabream (*Diplodus sargus*, n=4)
- Redporgy (*Pagrus pagrus*, n=3)
- European conger (*Conger conger*, n=5)
- Dusky grouper (*Ephinephelus marginatus*, n=1)
- Common dolphinfish (*Coriphaena hippurus*, n=3)
- Amberjack (*Seriola dumerilli*, n=6)
- Mediterranean moray (*Muraena helena*, n=9)

IRTA analysed all the extracts with the CBA assay and negative results were obtained for all extracts. (See Table 34).

Table 34. Toxicity of the fish processed from the Balearic Islands evaluated by the Neuro-2A CBA.

Results of experimentation	Number
Positives	**0**
Negatives	**70**
Dubious	**0**
No experimentation	**70**
Total of extracts	**70**
Total of fish	**36**

3.2 Smartphone-based electrochemical immunosensor for ciguatoxins detection. (IRTA)

A sandwich electrochemical immunosensor for the detection of CTXs is being developed. Two different capture antibodies able to recognise the left wing of CTX1B and 54-deoxyCTX1B,48 (3G8) and the left wing of CTX3C and 51-hydroxyCTX3C,46 (10C9) are immobilised on screen-printed electrodes modified with carboxyl functionalised multi-walled carbon nanotubes. Following the incubation with the analyte, biotinylated 8H4 antibody, which binds to the right wing of CTX1B, CTX3C, 54-deoxyCTX1B, and 51-hydroxyCTX3C,46, is used as a detector antibody. PolyHRP-streptavidin is used as enzymatic label for signal amplification and detection

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of the biotinylated antibody. Amperometric measurements are performed with Sensit Smart, a small and ready-to-go potentiostat which can be inserted in a smartphone and provide *in situ* measurements.

3.2.1 Optimisation of the experimental variables

Immobilisation of the capture antibodies on multi-walled carbon nanotubes modified screen-printed electrodes

Capture antibodies were immobilised on screen-printed electrodes modified with carboxyl functionalised multi-walled carbon nanotubes through carbodiimide coupling. The amount of 3G8/10C9 was optimised using several 3G8/10C9 dilutions, CTX1B at 500 and 0 pg/mL and biotinylated 8H4 and polyHRP-streptavidin at 1:000 dilution. From 1:50 to 1:500 dilutions, current values did not show significant differences. Current values decreased from 1:1000 3G8/10C9 dilution. Thus, 1:500 dilution was selected for subsequent experiments.

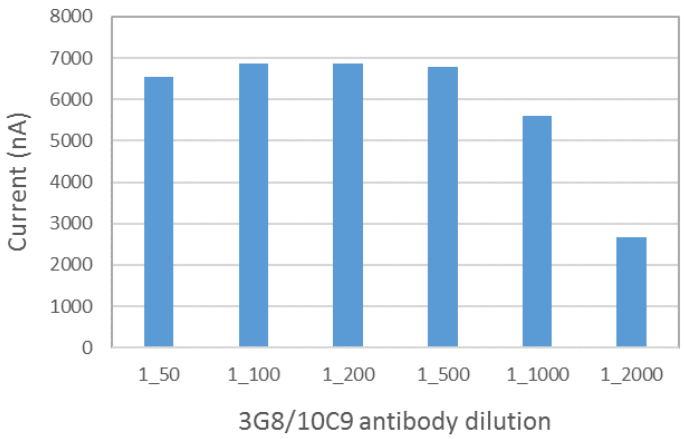

Figure 48. Optimisation of 3G8/10C9 antibody dilution required for the functionalisation of multi-walled carbon nanotube-modified screen-printed electrodes.

Optimisation of the biotinylated detector antibody and polyHRP-streptavidin concentrations

Biotinylated 8H4 concentration was optimised using 1:500 3G8/10C9 dilution, 500 and 0 pg/mL CTX1B and 1:1000 polyHRP-streptavidin dilution. Current values in the presence of 500 pg/mL of CTX1B were similar at all dilutions tested (from 1:100 to 1:2000). However, non-specific current values (in the absence of CTX1B) were higher at the lowest dilutions (1:100 and 1:200) and did not show significant differences from 1:500 to 1:2000. Therefore, 1:1000 biotinylated 8H4 dilution was appropriate for further experiments.

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Finally, polyHRP-streptavidin was optimised to achieve the best signal-to-noise ratio. Previous experiments were performed with a polyHRP-streptavidin conjugate containing a polymer with approximately 20 molecules of HRP per streptavidin. Another polyHRP-streptavidin conjugate containing a polymer with 80 molecules of HRP per streptavidin was tested. No significant differences in signal-to-noise ratios were observed between the two conjugates at the same concentrations. High non-specific currents were achieved with the highest concentrations tested in both cases (from 5 to 1 µg/mL, corresponding to 1:100 and 1:500 dilutions of polyHRP-streptavidin containing 20 HRP molecules per streptavidin and 1:10 and 1:50 dilutions of polyHRP-streptavidin containing 80 HRP molecules per streptavidin). Background currents were stabilised at 1:1000 and 1:100 dilutions of polyHRP-streptavidin containing 20 and 80 HRP molecules per streptavidin, respectively. As a compromise between high current values and low non-specific background, 1:1000 dilution of polyHRP-streptavidin containing 20 HRP molecules was maintained for the development of the immunosensor.

3.2.2 Analytical performance of the immunosensor

CTX1B calibration curve

Under the optimised conditions, a calibration curve for CTX1B from 0 to 1000 pg/mL was constructed. A dose-dependent response was observed with no saturation of the amperometric response at the highest CTX1B concentration tested. A limit of detection (LOD) of 10.44 pg/mL was calculated by applying the 3_{sb} criterion, where $_{sb}$ was the standard deviation expressed in concentration units of the measured blank values (no CTX1B). Relative standard deviations were lower than 15% (n=3).

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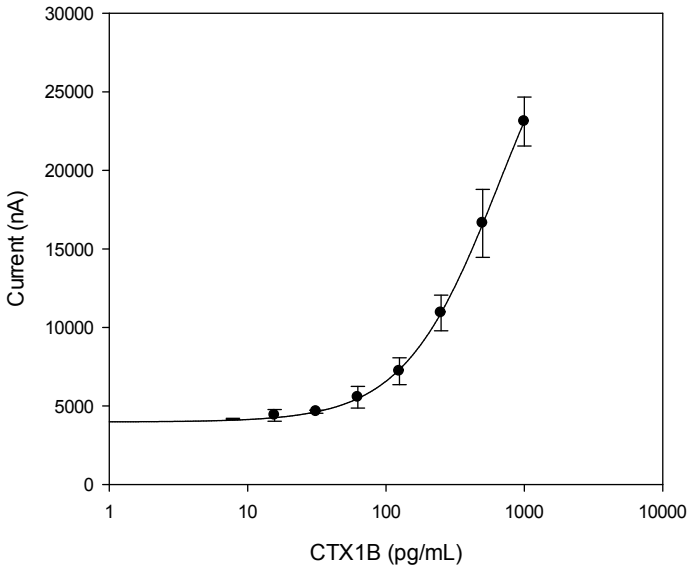

Figure 49. CTX1B calibration curve performed in buffer.

Matrix effect evaluation

To evaluate matrix effects of shark matrix on the response of the immunosensor, CTX1B concentrations (from 0 to 250 pg/mL) were spiked into a noncontaminated shark extract at 2500 mg/mL that had previously determined as negative for CTXs by cell-based assay (CBA). A CTX1B calibration curve was constructed, showing non-significant differences between the one achieved in buffer at the concentrations tested, and attaining an LOD of 10.75 pg/mL.

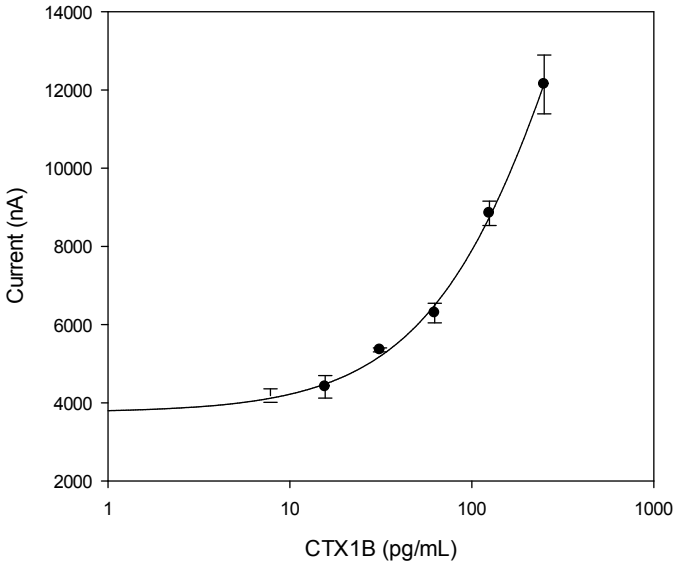

Figure 50. CTX1B calibration curve performed in 2500 mg/mL shark matrix.

3.2.3 Future work: Analysis of naturally contaminated shark samples

As no matrix effects have been observed, naturally contaminated shark samples obtained from *La Reunión* (France) will be analysed at 2500 mg/mL and results will be compared with those obtained by CBA.

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4. Definition of areas at risk for ciguatera: (IRTA, ULPGC, SCS, IPMA, AUT, SGL)

It is very important to state that, as the title of this report specifies, the main focus of the SG3 has been on CTXs in dinoflagellates and in different fish species. Hence it is important to insist that here, any approach to define areas at risk for ciguatera are based on these two criteria. These are crucial data to better characterize the hazard of ciguatera, and therefore to contribute to risk assessment.

Nevertheless, the collection and generation of additional data needed for a more complete risk assessment is beyond the scope of this project. These additional data include, among others: the abundance of the toxic microalgae, their detailed geographical distribution, including also depth and type of substrate, their distribution over time, the species of fish present, the fishing efforts in the regions, with a description of the spectra of available species in the market, their abundance and the fish consumed by the population.

The Eurocigua project has provided a large amount of data that were not previously available in the studied areas, and that allow to better tackle the risk of ciguatera. These large amount of data are presented in annexes in an EXCEL format, that will allow to conduct searches according to any specific criteria such as archipelago, island, location, date, microalgal species, fish species, weight of fish, toxicity of microalgae and fish, and that may contribute to better understand the specificity of ciguatera. By isolating and culturing 472 strains of dinoflagellates, evaluating the toxicity of 102 strains of dinoflagellates and the toxicity of 963 fish, SG3 has succeeded in gathering toxicity data that are crucial to understand the risk ciguatera poisoning may represent in the studied areas.

Below we present a synthesis of the data for the different regions, which are complemented by the conclusions detailed in section 6.

CANARY ISLANDS:

Among the studied areas, the Canary Islands constitute by far the area representing the highest risk taking into consideration the number of ciguatera cases reported. Presence of several *Gambierdiscus* species cover the whole archipelago, and the toxicity of the species, particularly *G. excentricus*, indicate their potential as source of CTX-like compounds. As for fish, according to the data on CTX toxicity, there is quite a high incidence of toxic fish, either from data of the project (14% of a total of n=746 samples) or from the official control in 2018 (15,6% of a total of n=1128 samples) and 2019 (12% of a total of n=839 samples).

The ciguatera poisoning cases occurred in the Canary Islands (as from information provided by the Canary Islands Health Service), have been caused by consumption of the following fish: 55% of the cases by consumption of amberjacks, 20% by dusky groupers, 15% by island groupers and 5% by bluefish.

MADEIRA AND SELVAGENS ISLANDS:

Thus far, fish involved in ciguatera intoxications in Portugal came from Selvagens Islands. For the first time, a *Gambierdiscus* spp. dinoflagellate was detected in Madeira in October 2008 and August 2012 (Kaufman, 2013).

The genus *Gambierdiscus* has been detected in both Madeira and Selvagens islands, in several sampling points during the sampling period. However, preliminary data seems to indicate the highest densities in sites Porto Moniz and Machico in Madeira and Baia das Cagarras in Selvagens. The highest densities recorded were in Selvagens Grande which is in accordance

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with the fact that most toxic fishes have been found nearby Selvagens Archipelagos. Therefore, works addressed to fish caught in this area will be intensified.

Toxicity in fish from Madeira has been identified by IRTA through CBA in 39 fish out of 81 from Selvagens Islands (48%) and 3 fish out of 21 from Madeira (14%) and 0 out of 26 from Desertas. Out of 18 species evaluated, 11 presented CTX- toxicity. Among the most sampled groups, the relative amount of toxic fish was as follows: 100% of *Bodianus* (n=20), 19 % of *Sparisoma cretense* (n=32) and 11 % of *Seriola* sp. (n=28).

CYPRUS AND CRETE:

In the Eastern Mediterranean Sea, the genus *Gambierdiscus* and *Fukuyoa* were identified. Species of this genus include: *G. belizeanus*, *G. australes*, *G. silvae G. carolineanus, Fukuyoa paulensis* and a potential new species of *Gambierdiscus.* sp. The evaluation of their toxicity, mainly from *G. belizeanus*, is quite low in relation to the other region in the Mediterranean, the Balearic Islands. The only toxic fish identified in the Mediterranean corresponds to an amberjack caught in waters of Cyprus. This area of the eastern Mediterranean may have to be considered for further studies on CTXs in fish.

BALEARIC ISLANDS:

Before the start of the Eurocigua project, *Fukuyoa paulensis* was reported previously in Formentera. In the frame of this project, *F. paulensis* was also identified in Minorca, Majorca and Formentera. A very relevant result of the project was that for the first time, the genus *Gambierdiscus* was identified in the Balearic Islands, over successive samplings between 2016 and 2019, indicating their persistence in this region. *Gambierdiscus australes* was the only described species of the genus, and CTX-like toxicity was described in several strains, reaching values that are higher than in strains of the Eastern Mediterranean. Among the sampled fish, no CTX like toxicity was described. Toxic strains of dinoflagellates have a large distribution in Minorca, Majorca and Formentera supporting the region as a potential area of ciguatera poisoning in the future.

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5. Literature search for predictive modelling

5.1 Identify sources of data: (IRTA, ULPGC, SCS, IPMA, AUT, SGL)

There are several sources of meteorological and oceanographic data for the areas investigated in the project. For example, the site puertos http://www.puertos.es/es-es/oceanografia/Paginas/portus.aspx provides forecast as well as historical data related to currents, seawater temperature, waves, wind and sealevel in the Canary Islands. Data from the marine observatory Plocan can be downloaded through https://www.plocan.eu/en/open-ocean-observatory/, the catalogue includes not only data from moorings but also from ship based surveys.

The hydrographic institute of Portugal has a network of buoys that includes two in Madeira archipelago, one in Selvagens five in Açores. The data can be checked on real time at the site https://www.hidrografico.pt/boias, data can be also obtained through the site http://geoportal.hidrografico.pt/geoportal/catalog/main/home.page. A contact person for modelling purposes is Pedro Reis Costa from IPMA.

The Balearic Islands Coastal Observing and Forecasting System (https://www.socib.eu/) provides access to data from different observing facilities including buoys, gliders and ship surveys. Data from buoys can be access on real time, and through the repositories historical data can be accessed.

Meteorological parameters for the Eastern Mediterranean can be obtained http://www.poseidon.hcmr.gr/index.php. From this area, a contact person for modelling, is Dimitrios Moutopoulos from the University of Patras (https://orcid.org/0000-0001-5873-9893) who already has experience in modelling HABs.

Biodiversity data as well as physical data including bathymetry can be obtained through EMODnet https://emodnet.eu/en. Seabed habitats for the whole Europe can be obtained through the European Atlas of The Seas.

These sources of data cover the most important parameters needed for modelling *Gambierdiscus* population and ciguatera. For forecasting purposes it is also necessary to obtain data on the climatic projections for the different geographical areas. Fish landings can be obtained through FAO at national level. For the purpose of ciguatera modelling it will be useful to obtain local data which can be provided by the regional fisheries directorates.

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Environmental and oceanographic data from other institutions

IPMA:
Databases used for scientific research information about *Gambierdiscus* spp. and *Fukujoa* spp. occurrence and toxin profile comprise: Scopus, Science Direct, Web of Kwoldege, among others. Also, a bibliography search of papers describing meteorological and climate phenomenon in the sampling areas has been performed. In addition, the EdModnet (www.emodnet.eu) has been consulted to further define seabed habitats described in literature.

IRTA:
Data providers for the Balearic Islands have been identified. SOCIB (http://www.socib.es/) has several coastal stations with instruments recording seawater temperature, salinity and other parameters. In Menorca the Station Ciutadella records seawater temperature. In Mallorca the station Bahia de Palma records seawater temperature, salinity and current speed, the stations Pollensa and Colonia Sant Pere record seawater temperature.

5.2 Establish a database of scientific literature on modelling for ciguatera and for *Gambierdiscus* spp. population dynamics. (IRTA, ULPGC, SCS, IPMA, AUT, SGL)

In the context of SG3, a literature search has been conducted, which also included our own references. The bibliography also included references retrieved by Dr, Phillip Hess from IFREMER. The references have been inserted in an ENDNOTE database.

The ENDNOTE database contains around 2500 scientific articles and patents. The ENDNOTE database, that includes the abstract for almost all references, allows to conduct specific searches to filter the references according to the user requests.

The references in the ENDNOTE database have been classified according to the following groups:

bioaccumulation in food webs, ciguatera in Europe, environmental variables, epidemiology, extraction methods, geographical distribution, modelling, other toxins, reviews on ciguatera, socioeconomic, taxonomy, toxicity of dinoflagellates, toxin detection, biological effects of toxins, chemistry of toxins, pharmacology of toxins.

We have also included the following labels for the different articles:

Bioaccumulation, Europe, geographical distribution, epidemiology, environmental variables, toxin chemistry, toxin analysis, benthic habitat, toxicology and biological effect, physiology, ecology, other toxins.

Finally, we have added to each reference the following keywords to identify the contents of the articles:

temperature, salinity, light, seasonality, abundance, fish toxicity, Europe, Pacific, Caribbean, Indian, Mediterranean, extraction, macroalgae, coral, anthropogenic

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disturbances, growth rate, nutrients, bioassay, immunoassay, extraction, purification, bacteria, evolution, army, outbreak, traveller.

5.3 Define different modelling strategies to be implemented in the future for the study of ciguatera and for *Gambierdiscus* spp. population dynamics. (IRTA)

Modelling strategies to be implemented in the future in Europe, can consist on similar approaches that have been applied in in other geographical areas. These include strategies that have focused on the time lag between climatic indices such as seawater surface temperature anomalies (SSTA) or severe storm events and the increase of ciguatera cases (Chateau-Degat et al., 2005; Gingold et al., 2014; Lewellyn et al., 2010; Zheng et al., 2020). These modelling strategies have been applied in areas were ciguatera was already studied and cases recorded for long time. Similar modelling strategies could be applied to the Canary Islands where a monitoring program was established some years ago and ciguatera cases are recorded. Tosteson 2004 observed that the number of days in which SST were >29.5 °C correlated with the reported cases and the percentage of captured toxic barracuda in Southwest coast of Puerto Rico. He also observed changes in the seasonality of the events, seasonal fluctuations observed in the period 1985-1988 with higher percentages of toxic barracuda caught in spring and fall were no longer observed in 1990-2000. To associate the increase of seawater temperature to an increase of ciguatera cases has been seen too simplistic. To predict CP incidence in Cook Islands and French Polynesia using SSTA, Zheng et al. 2020 found that autoregressive integrated moving average (ARIMA) models were appropriate. They found positive statistical significance between SSTA and CP incidence rate in Cook Islands 12 months after. A similar result was obtained between SSTA CP incidence in French Polynesia, in this case with a lag of 32 months. Their conclusion was that these warm events produced physical disturbances of the coral reef. This observation was already described by Randall (1958) who pointed out the relation observed by several authors about the fact that ciguatera cases increase after severe storms such as in Bahamas (1908) and Fiji (1929) where local people believed that fishes were poisoned by seaweed that grew after the storm. Randall already suggested in 1958, that the organisms producing ciguatera were one of the first growing in new or denuded surfaces in the ecological succession.

Other modelling strategies have focused on *Gambierdiscus* population dynamics, in some cases based on the experiments conducted by Xu et al. (2016) to find the optimal and suboptimal growth conditions for several *Gambierdiscus* species in relation to salinity, temperature and light. Parsons et al. (2011) included the host macroalgae as a variable in the study of *Gambierdiscus* growth.

The establishment of a new population of *Gambierdiscus* requires the presence of suitable hosts. Parsons et al (2011) tried to find which algae species were suitable for *Gambierdiscus*. They tested the epiphytic relationship between *Gambierdiscus* and algal hosts in laboratory condition. *Jania* and *Galaxaura marginata* stimulated *Gambierdiscus* growth, *Portieria hornemannii*, *Dictyota*, *Microdictyon* inhibited *Gambierdiscus* growth. Studying the habitat suitability has been attempted by Tester et al., (2013) who mapped habitat suitability for *Gambierdiscus* off the coast of Texas (Northern Gulf of Mexico) based on temperature, salinity and light. Maximum depth where growth was possible was set at a depth where light intensity

was 10 umol photons m^{-2} s^{-1}, as the minimum light intensity required for growth of *Gambierdiscus*. Climate models predict and increase in the number of days for optimal *Gambierdiscus* growth and a reduction in the number of days of low temperatures associated to *Gambierdiscus* mortality (<15°C) in the area. They also concluded that shallow water sampling may under-represent *Gambierdiscus* populations, since suitable habitats include depths greater than 50 m. Shifts in benthic communities where coral-sponge assemblages have been replaced by macroalgae dominated community, increase the substrate available for *Gambierdiscus*.

The main factors to consider for modelling purposes are:

- Differences in *Gambierdiscus* population species composition
- Availability of suitable benthic habitats for *Gambierdiscus,* disturbances of coral reefs, coral mortality, coral bleaching.
- Colonization of host macroalgae
- Differences in the thresholds of temperature for maximum growth rate or stop growing or toxin production of the different species of *Gambierdiscus*
- Differences in water motion
- Irradiance
- Salinity

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Table 35. List of suggested modelling strategies to be implemented in the future. (SWT: Sea Water Temperature)

Model description	Type of data needed		Type of modelling strategy	Software required		Examples of other applications	
	Parameters	Format		Name	Provider	Case	References
Time-lag of the impact of a change on SWT	SWT *Gambierdiscus* abundance Cases of ciguatera	Weekly	Autoregressive integrated moving average (ARIMA)	SAS	SAS Institute	Tahiti (French Polynesia)	Chateau-Degat et al., 2005
Time-lag of the impacts of severe storms	SST, SSTA anomaly index Storms Fishing yields	Monthly	Poisson regression	SAS	SAS Institute	United States	Gingold et al., 2014
Time-lags between climate indices and ciguatera cases rate	Ciguatera cases Climate indices SST	Per year	Cross-correlations, Partial autocorrelation,	R	R Development Core Team	South Pacific	Lewellyn et al., 2010
Time-lag SST and ciguatera cases (predictive model)	Ciguatera cases SST, SSTA	Monthly	Cross correlation ARIMA	R 3.5.3	R Development Core Team	South Pacific	Zheng et al., 2020
Gambierdiscus growth models projection	Time series of SWT Projected SWT temperatures (from Global Climate Models) Temperature vs. *Gambierdiscus* spp. growth relationship Bathymetry Light penetration depth	Daily	Curve fitting	MATLAB	The Mathworks, Inc.	Caribbean Sea and Gulf of Mexico	Kibler et al., 2015 Kibler et al., 2017
Gambierdiscus population dynamics	Temperature Salinity Nutrients *Gambierdiscus* abundance *Gambierdiscus* growth rates vs temperature, salinity, light, nutrients Wave height Local precipitation Solar radiation	Monthly	Growth limitation model	SPSS	IBM	Hawaii	Parsons et al., 2010

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Potential modelling perspectives as a response to existing gaps for ciguatera in Macaronesia and the Mediterranean

Regarding modelling as a strategy for understanding processes and for prediction, it is crucial to have enough data of high quality in order to conduct robust data analysis. Based on this postulate, and on the results of the SA3 of the EUROCIGUA project, we present herein some ideas on how modelling strategies may provide with further knowledge on ciguatera in the studied areas.

- The seasonality of *Gambierdiscus* and *Fukuyoa* presence needs to be further studied in order to better understand a pattern of abundance related to climate conditions or time of the year. With the purpose of establishing a predictive model, it will be important to select specific hot spots and do specific sampling in order to estimate microalgae abundances, over time, taking into consideration for example the different substrates (e.g macrophytes), depth and recording environmental and oceanographic parameters including turbulence, for example. The stability of the water column, or excessive mixing, may condition the increase or decrease of *Gambierdiscus* and *Fukuyoa* populations. So could the type of substrate or depth. Such a model should contribute to predict the time of the year presenting a higher risk, and consider in the long term, the potential changes expected in risk intensity.
- For example, a critical seawater temperature may be set, above which the expansion of *Gambierdiscus* and *Fukuyoa* populations is highly probable. In a climate change scenario, where the number of days/weeks for which seawater temperature may be above this reference value increases, not reaching extreme values that may reduce *Gambierdiscus* and *Fukuyoa* growth, then models may be able to predict the impact climate change will have on *Gambierdiscus* and *Fukuyoa* populations in a more detailed approach according to the expected number of days/weeks above that critical temperature.
- The use of devices for the capture of microalgae (plankton nets) or toxins (resins) in the water column may also contribute to better understand patterns of presence of toxins or dispersion of microalgae that may be incorporated in models for a better characterization of risks.
- Selecting one particular species present in different locations in Macaronesia and the Mediterranean, such as *Gambierdiscus australes* may provide with clues on potential patterns of growth, abundance and toxicity over distant geographical areas that may help to better tackle factors that can determine geographical differences, and hence, contribute to conclude on the relevance of these factors to set up predictive models.
- Within the Canary Islands archipelago, the islands located over different longitudes may provide with some interesting information on presence of *Gambierdiscus* and *Fukuyoa* and toxicity in fish in areas closer to Africa and those closer to open Atlantic waters. This particular scenario is worth studying in order to evaluate whether the longitudinal pattern may, to some extent, condition the risk of ciguatera.
- Additional data regarding the physiology of the microalgae, specifically addressing growth rates according to different factors such as temperature, light and nutrient availability, may complement the models as to the potential of synthesis of toxins according to environmental conditions.
- The coverage of the benthic surface by the different substrates where *Gambierdiscus* and *Fukuyoa* grow, may provide further detail on the estimation of the "*Gambierdiscus* and *Fukuyoa* load" at a specific site. Toxicity of the different *Gambierdiscus* and *Fukuyoa* species should be incorporated in models in order to relate abundances of microalgae, species and potential toxicological risk in a given area.
- The potential toxicological risk, estimated by models according to the abundances and toxicity of microalgae can be evaluated against the estimated amount of toxins evaluated in the community of fish. For this purpose, sedentary fish, that may better represent the local transfer and bioaccumulation of toxins are of particular interest. Laboratory work describing rate of accumulation of toxins in fish fed with microalgae can complement the studies.

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- The study of migratory fish, and their toxin content is also of importance, since these specific fish may condition the risk for ciguatera on a given area according to the load of toxins that they may carry from distant areas. In such case, if these fish represent an important part of the fish entering the market, the risk for ciguatera may be less dependent on *Gambierdiscus* and *Fukuyoa* local populations and be more related to migratory patterns.
- A regional approach on the presence and amount of toxins in fish according to species, location, weight and time of the year has been provided in EUROCIGUA in Macaronesia, where enough data were obtained. Nonetheless, these data could further be analysed in detail and complemented by further analysis, in order to identify potential patterns that may have not been evident in a first step.
- Data on ciguatera poisoning cases should be considered in detail, and studied according to several factors such as time of the year when the episode occurred, importance of the described symptomatology and origin of fish. The number of cases is low, but detailed analysis may provide some specific data regarding the episodes that may contribute to test some specific hypothesis.

These are some examples that may be considered in order to define future research to conceive models predicting ciguatera.

6. Communications and outreach:

1- Poster communication at the 18th International Conference on Diseases of Fish and Shellfish.
Location: Belfast, UK.
Date: 4-8 September 2017
Title: EUROCIGUA: Risk characterization of ciguatera food poisoning in Europe
Authors: García-Álvarez, N.; Sánchez-Henao, A.; Silva-Sergent, F.; Gutiérrez-Falcón, A.; Acosta-Hernández, B.; Padilla Castillo, D.; Déniz, S.; Real, F;

2- Poster communication at the 6th International Symposium Marine and Freshwater Toxins Analysis:

Location: Vigo, Spain.

Date: 22-25 October 2017

Title: Risk characterization of ciguatera food poisoning in Europe: First steps from the screening to the confirmation

Authors: García-Álvarez, N.; Sánchez-Henao, A.; Real, F.; Estévez, P.; Castro, D.; Leao, J.M.; Gago-Martínez, A.; P. Aguayo, P.; Diogene, J.

3- Poster communication at the III Congreso Veterinario de Seguridad Alimentaria de Canarias - 2017

Location: Las Palmas de Gran Canaria, Spain.

Date: 17-18 November 2017

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Title: EuroCigua: Caracterización del riesgo de intoxicación por Ciguatera en Europa

Authors: García-Álvarez, N., Sánchez-Henao, A., Silva, F., Gutiérrez-Falcón, A., Acosta, B., Padilla, D., Déniz, S., Real, F.

4- Lecture at the "International day of fishing conference"

Location: Lanzarote, Spain.

Date: 21 November 2017

Title: Ciguatera in the Canaries: consequence for human health.

Author: Fernando Real

5- Oral communication at the *XIII Meeting of Iberic Toxic Alga and Marine Biotoxins*

Location: Vigo, Spain.

Date: 20-22 June 2018

Title: Official control programme for CFP in the Canary Islands: results of CTX analysis in 2017

Authors: N. García-Álvarez, A. Sánchez-Henao, F. Silva, F. Martín, J. Diògene, B. Acosta, M.J. Caballero, D. Padilla, A. Fernández and F. Real.

6- Newsletter communication

Title: First report of *Gambierdiscus* in the Western Mediterranean Sea (Balearic Islands).

Date: January 2018

Newsletter: Harmful Algae News, 59, 22-23

Authors: Tudó A., Toldrà A., Andree K. B., Rey M., Fernández-Tejedor M., Campàs M., Diogène J.

7- Poster communication at the IV Congreso Veterinario de Seguridad Alimentaria de Canarias - 2019

Location: Las Palmas de Gran Canaria, Spain.

Date: 15-16 November 2019

Title: EuroCigua: Presencia de CTX en pescado de las islas canarias: resultados del proyecto EuroCigua

Authors: Silva, F., García-Álvarez, N., Sánchez-Henao, A., Ramos-Sosa, M., Caballero, M., Padilla, D., Fernández, A., Real, F.

8- Predictive score and probability of CTX-like toxicity in fish samples from the official control of ciguatera in the Canary Islands. Sánchez-Henao J. A, García-Álvarez N, Fernández A, Saavedra P, Silva Sergent F, Padilla D, et al. Sci Total Environ; 673: 576-584. doi: 10.1016/j.scitotenv.2019.03.445. (2019).

9- First description of spontaneous granulomatous aerocystitis by Phoma herbarum in a wild greater amberjack (*Seriola dumerili Risso*, 1810). De Sales-Ribeiro C, Sánchez-Henao A,

.

García-Álvarez N, Real F, Rivero M. A, Fernández A, et al. J Fish Dis; 42 (9): 1321-1325. doi: 10.1111/jfd.13045 (2019).

10- Presence of CTXs in moray eels and dusky groupers in the marine environment of the Canary Islands. Sánchez-Henao A., García-Álvarez N., Silva Sergent F., Estévez P., Gago-Martínez A., Martín F., et al. Aquat Toxicol; 221: 105427, (2020).

11- *Gambierdiscus* and *Fukuyoa* as potential indicators of ciguatera risk in Balearic Islands. A. Tudó, A. Toldrà, M. Rey, I. Todolí, K.B. Andree, M. Fernández-Tejedor, M. Campàs, F. X. Sureda, J. Diogène. Harmful Algae 99 (2020)101913.

12- Further advance of Gambierdiscus species in Canary Islands, with the first report of Gambierdiscus belizeanus. A. Tudó, G. Gaiani, M. Rey, T. Tsumuraya, K.B. Andree, M. Fernández-Tejedor, M. Campàs, J. Diogène. Toxins 12 (2020) 692.

13- Rapid detection of ciguatoxins in *Gambierdiscus* and *Fukuyoa* with immunosensing tools. G. Gaiani, S. Leonardo, À. Tudó, A. Toldrà, M. Rey, K.B. Andree, T. Tsumuraya, M. Hirama, J. Diogène, C.K. O'Sullivan, C. Alcaraz, M. Campàs. Ecotoxicology and environmental safety 204 (2020) 111004.

14-Addressing the analytical challenges for the detection of ciguatoxins using an electrochemical biosensor. S. Leonardo, G. Gaiani, T. Tsumuraya, M. Hirama, J. Turquet, N. Sagristà, M. Rambla-Alegre, C. Flores, J. Caixach, J. Diogène, C. K. O'Sullivan, C. Alcaraz, M. Campàs. Anal Chem 92 (2020) 4858-4865.

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7. Conclusions

The goals of the SG3 were correctly achieved. The project has presented the ten deliverables requested by EFSA.

Regarding methodology, the project was sucessful in harmonizing among partners several methodologies including the extraction procedures for toxins in microalgae and fish, and the implementation of the Neuro-2a cell based assay.

Taxonomy studies, which are required for the correct identification of microalgae, included morphological and genetic analysis.

We present herein the main conclusions of the project, divided according to the geographical area.

Main conclusions from the Canary Islands:

- ✓ From the Canary Islands, 196 isolates of *Gambierdiscus* spp were obtained in 2016 and 2017 from the Canary Islands, 90 of which have been successfully established as permanent strains. Out of 45 strains analysed, 43 strains were CTX-like positive and the range of CTX-like content was between 1.7 to 2566.7 fg/cell eq. Among the CTX-like positive samples there were *G. australes*, *G. excentricus* and *G. belizeanus* strains (identified by molecular biology). Toxicities were observed in the strains from all the 7 islands. The most toxic species and strain of *Gambierdiscus* was *G. excentricus* detected in Gran Canaria (E-18-48). One *G. caribaeus* strain was CTX-like negative.
- ✓ Regarding fish samples of the EUROCIGUA project from the Canary Islands, 746 samples of fish analyzed by the Neuro-2A CBA by the ULPGC, from different places of the Canary Islands (from the seven islands). Out of the 747 samples analyzed, 105 resulted positive for CTX-like toxicity, 632 were negative and 9 samples were considered inconclusive.
- ✓ Regarding the incidence of ciguatera in the Canary Archipelago from the results of the EUROCIGUA project analysed by ULPGC, all islands presented positive fish, and the higher incidence was reported in el Hierro followed by La Palma, Tenerife and Lanzarote while La Gomera and Gran Canaria presented the lowest incidence.
- ✓ From the selected fish from the Canary Islands sent to IRTA, 130 fish (157 extracts) were analysed by Neuro-2A at IRTA, 84 were CTX-like positive and 46 were CTX-like negative. Toxicities range from 0.0018 to 0.5760 µg eq./kg of CTX-1B.
- ✓ This work confirms the Canary Islands as an area of expansion of CFP endemicity and highlights the need to monitor CTX accumulation in fish and the presence of *Gambierdiscus* in the Canary Islands marine waters.
- ✓ From data of ULPGC, 14% of the studied fish resulted positive to CTX. A total of sixty different species of fish were evaluated and 17 of them showed CTX-like toxicity.
- ✓ Among the positive fish species, amberjack, dusky grouper, some moray eels and common two-banded seabream are particularly important.
- ✓ For amberjack, several factors were found significantly associated with the probability of contamination by CTX-like toxicity, such as, weight of fish and season of capture.

- ✓ For dusky grouper the island of fishing was significantly associated with CTX-like toxicity.
- ✓ The presence of C-CTX-1 in black moray (*Muraena augusti*), fangtooth moray (*Enchelycore anatina*), mediterranean moray (*Muraena helena*) and brown moray (*Gymnothorax unicolor*) has been found, for the first time, in the Canary Islands Archipelago.
- ✓ Further research is needed to assess the risk that moray eels would represent to human health according to the consumption levels for these fish.
- ✓ A high percentage of fish (34%) from several species captured in El Hierro have showed presence of CTX-like toxicity. This fact, considered together with previous results obtained, suggest this island as a ciguatoxin hot spot in the Canary Archipelago

Main conclusions from <u>Madeira and Selvagens Islands</u>:

In this area, 74 isolates of *Gambierdiscus* spp. were obtained in 2017, 2018 and 2019. Four cultures were established of *G. australes* for the toxicity evaluation. They presented CTX-like positive (ranging from 2.5 – 83 fg eq. of fg/cell eq.). We can conclude:

- ✓ High *Gambierdiscus* cell densities and fish toxicity were observed in samples from Selvagens Islands;
- ✓ *Gambierdiscus australes* was the only and single species identified in Selvagens. Analysis of phylogenetic relations indicates some diverging strains from *G. australes* clade (further studies needed). Ultimately, a new species/ribotype is co-existing in this location;
- ✓ In this study, *Gambierdiscus excentricus* was the only species observed in Madeira. There are evidences for predominance in the north coast (further studies are needed);

Regarding fish sampling, 128 fish have been sampled during the Eurocigua project. Fourty-four CTX-like positive fish were detected out of 129 extracts analyzed by CBA. Toxicities of these fish range from 0.0039 to 0.1253 µg/kg of CTX-1B. Eleven species (*Epinephelus marginatus, Bodianus scrofa, Balistes capriscus, Mictoperca fusca, Serranus atricauda, Dentex gibbosus, Seriola dumerili, Diplodus cervinus, Sparisoma cretense, Sphyreanea viridensis, Seriola rivoliana)* were detected as CTX-like positive species. So, we can conclude:

- ✓ Selvagens Islands constitue the main hotspot for Ciguatera in Portugal
- ✓ CTX-like toxicity was observed in several fish species throughout the marine food web;

Main conclusions from <u>Crete, Samos and Rhodes:</u>

From the samples collected from Crete, 66 *Gambierdiscus* spp strains were established. Nineteen strains were analysed by Neuro-2A: 2 are CTX-like positive ranging from 4.34 to 17.60 fg eq./cell, 6 are negative and 11 are not quantifiable.

An additional sampling was performed in Samos and Rhodes in August-September 2018, 21 *Gambierdiscus* sp. and 1 *Fukuyoa* sp. cultures were stablished. We can conclude:

- ✓ A great diversity of *Gambierdiscus* and *Fukuyoa* taxa was detected in the eastern Mediterranean Sea, where at least 6 different taxa were detected:

1. *Gambierdiscus silvae*
2. *Gambierdiscus australes*
3. *Gambierdiscus carolinianus*
4. *Gambierdiscus* cf. *belizeanus*
5. *Gambierdiscus* sp. (sp.nov) and
6. *Fukuyoa paulensis*

- ✓ The high number of species in the area highlight the potential risk for Ciguatera, despite the relatively low cell toxicity found in isolates examined during this EUROCIGUA project.
- ✓ No CTX-like positive fish was detected from Crete in a total of 140 analysis for a total of 70 fish (140 extracts).

Main conclusions from <u>Cyprus:</u>

- ✓ From Cyprus, 15 *Gambierdiscus* cultures were established. Out of these, 9 were analysed by Neuro-2A: 2 were CTX-like positive, 4 were negative and 3 were no quantifiable. In these non-quantifiable strains possible MTX effect or other toxicological patterns to be studied were identified.
- ✓ From Cyprus, 82 fish have been sampled. Only one CTX-like positive (*S. dumerilii*) was detected out 148 extracts analyzed by Neuro-2A, with an estimation of 0,0113 µg eq CTX-1B/kg. Analysis on LC-MS/MS for the presence of CTXs in this fish was conducted by the Universidad de Vigo within SG4 with no detection of CTXs.However, an inmunoassay indicated the presence of CTX-like compounds in this sample.

Main conclusions from <u>the Balearic Islands:</u>

From the Balearic Islands, *Gambierdiscus* spp and *Fukuyoa* spp were reported and 99 strains were established. Among these, 30 strains were studied for toxicity. We can conclude:

- ✓ *Gambierdiscus* was identified for the first time in the Balearic Island in 2017 confirming the presence of *Gambierdiscus* in the western Mediterranean.
- ✓ *Gambierdiscus* and/or *Fukuyoa* have been reported in samples obtained from 2016 to 2019 for *Gambierdiscus* and before for *Fukuyoa* indicating that these genera are well established in the Balearic Islands.
- ✓ *Gambierdiscus australes* is, up to day, the only species of *Gambierdiscus* reported in the Balearic Islands
- ✓ Cytotoxicity assays showed CTX-like toxicity in *Gambierdiscus* spp strains from the Balearic Islands. Twenty-four *Gambierdiscus* strains have been analyzed by Neuro-2A, all strains were CTX-like positive and the range of CTX-like content was between 1.38 to 381.83 fg eq./cell.Six *Fukuyoa* strains were analysed by Neuro-2A, 2 strains were

CTX-like positive and the range of CTX-like content was between 7.96-16.3 fg eq/cell, 3 were not quantifiable and one was CTX-like negative.

- ✓ Regarding the evaluation for the toxicity in fish, a total of 70 analysis for a total of 36 fish were perforemed showing no toxicity in fish from the Balearic Islands.

Main conclusions from Primary reference material containing CTXs:

- ✓ Thirteen large scale cultures from selected *Gambierdiscus* spp and *Fukuyoa* spp strains were performed for the most interesting toxicogenic strains. Ten of them were sent to the University of Vigo for further study of toxins within the SA4. Phase 1 reference material of fish was obtained from the Canary Island (46 fish, 661 kg) and Madeira and Selvagens Islands (24 fish, 66.5kg). All this material was sent to the University of Vigo to perform further studies and CTX characterization by LC-MS/MS (SG4).

Main conclusions regarding the literature database:

- ✓ An ENDNOTE database contains around 2500 scientific articles and patents. The ENDNOTE database, that includes the abstract for almost all references, allows to conduct specific searches to filter the references according to the user requests.

Main conclusions regarding modelling:

- ✓ A literature review has allowed to define to analyse the litereature specifically focused on modelling strategies.
- ✓ Modelling *Gambierdiscus* populations or ciguatera epidemiology cannot be only based on the increase of average temperature. SST needs to be above a threshold long enough to generate enough ciguatoxin to be observed in human populations.
- ✓ It also happens that if SST exceeds an upper limit long enough, ciguatera occurrence decreases (Hales et al., 1999; Lewellyn et al., 2010). Disturbances of the host population has important effects. There is a delay of several months between the change in the factor that produces an increase in *Gambierdiscus* population and the observed effects in the epidemiological cases.

Overall conclusions:

- ✓ Taxonomy *for Gambierdiscus* spp. in strategic hotspots in Macaronesia and Mediterranean waters, including the morphological and genetic approach was conducted.
- ✓ Evaluation for the toxicity in fish shows toxicity in fish from Madeira and the Canary Islands, CTX-like toxicity (Cell-based assay and immunoassay) in 1 fish from Cyprus and no toxicity in other fish from the Mediterranean Sea.
- ✓ Fish species which represent a risk have been identified including the evaluation of weight.

- ✓ Primary reference material containing CTXs has been achieved and transferred to U. Vigo (SG4).
- ✓ Literature and data search for the future development of models to understand the ecology of ciguatera has been completed.
- ✓ Specific modelling literature review has been conducted for the future development of models.
- ✓ It is necessary to continue advancing in the improvement of detection and confirmation techniquesn for CTXs, and it is also desirable to have rapid screening techniques.

8. Recommendations

A protocol for the extraction of CTXs and a standardized cell-based assay to use in the project implementing the Neuro-2a cell based assay was delivered. Nonetheless, a validation and harmonization of extraction procedures and the cell based assay screening method in the EU is needed.

The recent implementation of immunoassays and immunosensors for CTXs, and the potential increase of available antibodies for a wider spectrum of CTXs in the next years, should be considered in the future as an additional screening tool for CTXs.

These screening methodologies should be complemented with the confirmation of CTXs with instrumental analysis procedures, as described in SG4, in order to better describe the toxicological potency of microalgae and fish for risk assessment studies.

The project succeeded in the identification of several species of *Gambierdiscus* in Macaronesia and in the Mediterranean, and of *Fukuyoa paulensis* in the Mediterranean, being these persistent over the years of the study. Hence, the follow-up of these populations, considering quantitative techniques over different spatial and time ranges should be necessary to better acquaint the risk these populations represent. A more detailed study on the distribution of *Gambierdiscus* and *Fukuyoa* populations according to depth is important since our work was focused in the lower depths to a maximum of 6 m, and populations in deeper waters should be assessed.

The evaluation of toxins in fish, has covered different species, whether migratory or sedentary, considering their weight ranges and has been performed in different tissues, flesh and liver. It will be important when considering risk assessment, to carefully design the selection of sites and fish in order to respond to the specificities of the site.

The selection of hotspots for specific studies and the access to data is crucial for long term studies considering environmental, oceanographic, biological and ecological variables and the impact these may have on the evolution and potential expansion of ciguatera due to climate change. Statistical analysis and modelling should be potentiated in order to develop modelling studies for a better prediction of ciguatera.

Efforts in Macaronesia should be centred for a better prediction of ciguatera poisoning cases, and link these to the ecology of ciguatera involving microalgae and fish. In the Mediterranean, where no cases of ciguatera have been reported, efforts should be oriented to the ecology of ciguatera, and a wider screening of CTXs in fish, whether sedentary, migratory and including evaluation of CTXs in liver and flesh, since liver may me an earlier indicator of CTXs in the food webs.

For modelling purposes about ciguatera in Europe some information is still to be produced through research projects specifically designed for the modelling objective. It is necessary for example to increase the knowledge about the abundance of the different species in the coastal areas, toxin production for each species, the growth rates of local strains at different conditions and the flux of CTX in the food web.

9. Impact Assessment

The project was sucessful in harmonizing among partners several methodologies including the extraction procedures for toxins in microalgae and fish, and the implementation of the Neuro-2a cell based assay. This may have an impact for the development of more efficient monitoring programs for ciguatera. In addition, a magnetic-bead based immunoassay and immunosensor, implementing two different capture antibodies (3G8 and 10C9) were used to obtain an estimation of the amount of CTX1B or CTX3C series of congeners of microalgae extracts and in one fish from Cyprus. This should be considered as an additional screening tool for CTX evaluation.

Eight international scientific publications have been published from 2018 to 2020 including the results of this SG3 grant. Others are in preparation and should be published in the months to come. We expect to reach the scientific community and stakeholders involved in ciguatera management, improve the knowledge and state of the art on ciguatera and open new approaches to handle ciguatera in Europe and worldwide.

A better definition of risk for microalgae and fish in Macaronesia (Canary Island and Madeira) and the Mediterranean Sea (Crete, Cyprus and Balearic Islands) have been achieved. Among the studied areas, the Canary Islands constitute by far the area representing the highest risk. Presence of several *Gambierdiscus* species cover the whole archipelago, and the toxicity of the species, particularly *G. excentricus*, indicate their potential as source of CTX-like compounds. As for fish, according to the data on CTX toxicity, there is quite a high incidence of toxic fish 14% (of a total of n=746) (data from the project). Regarding Madeira and Selvagens islands, the genus *Gambierdiscus* has been detected in both areas. Toxicity of fish has been identified in 42 fish out of 128 fish (33%). Primary reference material containing CTXs has been achieved and transferred to U. Vigo (SG4). Efforts in Macaronesia should be centred for a better prediction of ciguatera poisoning cases, and link these to the ecology of ciguatera involving microalgae and fish.

From the eastern Mediterranean Sea, a great diversity of *Gambierdiscus* and *Fukuyoa* taxa was detected where at least 6 different taxa were detected but relatively low cell toxicity was detected in isolates examined during this Eurocigua project. The first fish CTX-like positive by Neuro-2A from the Mediterranean has been detected in Cyprus with a low concentration. From the Balearic Island, *Gambierdiscus* was identified for the first time in 2017. Up to date, only *Gambierdiscus* australes and *Fukuyoa Paulensis* have been identified. From this area, all fish showed no CTX-like toxicity. Efforts in the Mediterranean Sea should be centred on the ecology of ciguatera. The analysis of these populations, considering quantitative techniques over different spatial and time ranges should be necessary to better acquaint the risk these populations represent.

Literature and data search for the future development of models to understand the ecology of ciguatera has been achieved. Statistical analysis and modelling should be potentiated in order to develop modelling studies for a better prediction of ciguatera.

10. Summary of Deliverables (SG3)

All the deliverables agreed upon signature of the specific agreement 3 have been submitted to EFSA.

This is the list of deliverables.

Deliverable No.	Description
D3.1	Description of the extraction procedure for CTXs considering the different matrixes (microalgae, fish flesh and fish liver) and of the standardized screening cell based assay for Ciguatoxins to be used by IRTA and partners (IRTA).
D3.2	Description of the protocol to be used for the literature search and development of a data collection model (IRTA, ULPGC, SCS, IPMA, SGL, AUT)
D3.3	Definition of the spatial and temporal sampling strategy to be used for collection of *Gambierdiscus* spp. and the different fish species in Macaronesia and Mediterranean (IRTA, ULPGC, SCS, IPMA, SGL, AUT)
D3.4	Report on the establishment of a culture collection of *Gambierdiscus* spp from Macaronesia and the Mediterranean (IRTA, ULPGC, IPMA, SGL, AUT)
D3.5	Report on the collection of fish from Macaronesia and the Mediterranean: (ULPGC, SCS, IPMA, AUT, SGL, IRTA)
D3.6	Report on the environmental data collection from Macaronesia and the Mediterranean. (ULPGC, IRTA, IPMA, AUT, SGC)
D3.7	Phase 1 reference material consisting of fish and biomass from large scale cultures of selected *Gambierdiscus* spp. strains containing CTXs (Linked to Grant 4) 7.1 Fish (IRTA, ULPGC, SCS, IPMA, SGL, AUT) 7.2 *Gambierdiscus* spp. (IRTA, IPMA, AUT)
D3.8	**Three Annual Scientific Progress Reports:** **Report no.1:** Scientific Progress Report summarising activities during the first 12 months following signature of the specific agreement. **Report no.2:** Scientific Progress Report summarising activities during the second year following signature of the specific agreement. This report is linked to the interim payment. **Report no.3:** Scientific Progress Report summarising activities during the third year following signature of the specific agreement.

D3.9	Draft report on the evaluation of CTXs in marine dinoflagellates and in different fish species from Macaronesian and Mediterranean waters, indicating the geographical origin of samples containing CTXs and, for fishes, the weight threshold relevant for human risk assessment. The deliverable shall include a report on the literature and data search for the future development of models to understand the ecological mechanisms leading to toxins accumulation in fish, distinguish the relative importance of local and imported contribution and predict blooms of toxin-producing microalgae and outbreaks of CFP.
D3.10	**Final Report.** The final report should include an impact assessment to be provided to EFSA (EFSA will provide a template) and contain a section to be published on EFSA's website as an External Report.

11. References

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

Caldeira, R.M.A., Groom, S., Miller, P., Pilgrim, D., Nezlin, N.P., 2002. Sea-surface signatures of the island mass effect phenomena around Madeira Island, Northeast Atlantic. Remote Sens. Environ. 80, 336–360.

Delgado, G., Lechuga-Devéze, C. H., Popowski, G., Troccoli, L., and Salinas, C. A. Epiphytic dinoflagellates associated with ciguatera in the northwestern coast of Cuba. Revista de biología tropical 2006, 54(2), 299-310.

Fraga, S., Rodríguez, F., Caillaud, A., Diogène, J., Raho, N., Zapata, M., 2011. Gambierdiscus excentricus sp nov (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). Harmful Algae 11, 10–22

Jauzein, C., Fricke, A., Mangialajo, L. and Lemée, R. 2016. Sampling of Ostreopsis cf. ovata using artificial substrates: Optimization of methods for the monitoring of benthic harmful algal blooms. Marine Pollution Bulletin 107(1), 300-304.

Kaufmann, M., Böhm-Beck, M., 2013. Gambierdiscus and related benthic dinoflagellates from Madeira archipelago (NE Atlantic). Harmful Algae News 47, 18–19.

Lopes, A., Lopes, S., Matzarakis, A., Alcoforado, M.J., 2011. The influence of the summer sea breeze on thermal comfort in Funchal (Madeira). A contribution to tourism and urban planning. Meteorol. Zeitschrift 20, 553–564.

Parsons, M. L., Aligizaki, K., Bottein, M. Y. D., Fraga, S., Morton, S. L., Penna, A., and Rhodes, L. Gambierdiscus and Ostreopsis: Reassessment of the state of knowledge of their taxonomy, geography, ecophysiology, and toxicology. Harmful Algae 2012, 14, 107-129.

Parsons, M. L., and Preskitt, L. B. A survey of epiphytic dinoflagellates from the coastal waters of the island of Hawaii. Harmful algae 2007, 6(5), 658-669.

Provasoli, L., 1968. Media and prospects for the cultivation of marine algae, in: Cultures and Collections of Algae. Proceedings of the US-Japan Conference, Hakone, September 1966. Japanese Society for Plant Physiology, pp. 63–75.

Rains, L. K., and Parsons, M. L. *Gambierdiscus* species exhibit different epiphytic behaviors toward a variety of macroalgal hosts. Harmful Algae 2015, 49, 29-39.

Rusu, E., Soares, C.G., 2012. Wave energy pattern around the Madeira Islands. Energy 45, 771–785.

Tester, P. A., Kibler, S. R., Holland, W. C., Usup, G., Vandersea, M. W., Leaw, C. P., Teen, L.P. Larsen, J., Mohammad-Noor, N. Faust, M. A. and Litaker, R. W. 2014. Sampling harmful benthic dinoflagellates: Comparison of artificial and natural substrate methods. Harmful Algae 39, 8-25.

Yasumoto, T., Nakajima, I., Oshima, Y., and Bagnis, R. A new toxic dinoflagellate (Algae, Gambier Islands) found in association with ciguatera (Ciguatoxin, maitotoxin, French Polynesia). Developments in marine biology 1979.

Glossary [and/or] Abbreviations

Glossary: an alphabetical list of words relating to a specific subject, text, or dialect, with explanations; a brief dictionary.

Abbreviation: a shortened form of a word or phrase (such as Mr., Prof.). It also includes acronyms (a group of initial letters used as an abbreviation for a name or expression, each letter being pronounced separately – such as DVD, FDA – or as a single word – such as EFSA, NATO).

XXX	Dsadsadsadsa
YYY	Sdsdsadsad
ZZZ	Fdsfsafasdf

List of Annexes

Annex A – Report of a mission to Gran Canaria, Fuerteventura and Lanzarote, Canary Islands (October 13 to 17, 2016) for the sampling of microalgae.

Annex B – Report of a mission to Gran Canaria, Canary Islands (April 4 and 6, 2017) for the sampling of microalgae.

Annex C – Sampling of microalgae in the Canary Islands (October 20-28, 2017).

Annex D – Report of a mission to Madeira, Desertas and Porto Santo, (October and November 2016) for the sampling of microalgae *Gambierdiscus* spp.

Annex E – Report of a mission around Madeira and Selvagens Islands (early August - late November 2017) for the sampling of microalgae *Gambierdiscus* spp.

Annex F – Report of a mission to Madeira and Selvagens Islands (Selvagem Grande and Desertas)(early May to late November, 2018) for the sampling of microalgae.

Annex G – Report of a mission to Madeira, Desertas and Porto Santo (October 2019) for the sampling of microalgae *Gambierdiscus* spp.

Annex H – Report of a mission to Crete (November, 2016 and July 2017) for the sampling of microalgae.

Annex I – Report of a mission to Samos and Rhodes Islands (August and , 2017) for the sampling of microalgae.

Annex J – Report of a mission to Cyprus (October, 2016) for the sampling of microalgae.

Annex K – Report of a mission to Cyprus (September, 2017) for the sampling of microalgae.

Annex L – Report of a mission to Formentera, Balearic Islands (September 21 and 22, 2016) for the sampling of microalgae.

Annex M – Sampling of microalgae in the Balearic Islands (September 01-05, 2017).

Annex N – Sampling of microalgae in the Balearic Islands (September 01-05, 2018).

Annex O – Excel 2020_EUROCIGUA_SG3_Dinoflagellate toxicity

Annex P – Excel 2020_EUROCIGUA_SG3_Fish toxicity

Annex Q – Species analysed and overall results from the Canary Islands. The species providing any positive/s result/s are represented in red.

Annex R – Results of bioassay for the detection of CTX-like toxicity from all fish analyzed in Eurocigua (n=746) from the Canary Islands, distributed through the islands, according to the warm or cold season (fish from the official control have not been included).

Annex S – Species analysed in Madeira and Selvagens Islands. The species providing any positive/s result/s are represented in red.

Annex T – Species analysed in Crete. The species providing any positive/s result/s are represented in red.

Annex U – Species analysed in Cyprus. The species providing any positive/s result/s are represented in red.

Annex V – Species analysed in the Balearic Islands. The species providing any positive/s result/s are represented in red.