EXTERNAL SCIENTIFIC REPORT

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Risk characterisation of ciguatera poisoning in Europe

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Abstract

The EuroCigua project main objective is to characterize the risk of Ciguatera Poisoning (CP) in Europe including several specific objectives: to determine the incidence of ciguatera in Europe and the epidemiological characteristics of cases; to assess the presence of ciguatoxin in food and the environment in Europe and to develop and validate methods for the detection, quantification and confirmation of the presence of ciguatoxin contaminated specimens.

This report compiles the activities carried out during the EuroCigua project from the signing in April 2016 until December 2020. The present document corresponds to Deliverable No. 6: "Final Scientific Report" on Risk characterization of ciguatera food poisoning in Europe of the Specific Agreement no. 1 "MANAGEMENT AND SCIENTIFIC COORDINATION" within the Framework Partnership Agreement GP/EFSA/AFSCO/2015/03 "Risk characterization of ciguatera food poisoning in Europe".

Key words: (EuroCigua, ciguatera poisoning, microalgae, ciguatoxins)

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to the design of the protocol for the preparation of reference materials. Acknowledgement and mention to Naomasa Oshiro for his valuable advice during the project. We are also grateful to Dr Phillip Hess from IFREMER who kindly shared his bibliography.

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Summary

The project main objective is to characterize the risk of Ciguatera Poisoning (CP) in Europe including several specific objectives: to determine the incidence of ciguatera in Europe and the epidemiological characteristics of cases; to assess the presence of ciguatoxin in food and the environment in Europe and to develop and validate methods for the detection, quantification and confirmation of the presence of ciguatoxin contaminated specimens.

In order to estimate the incidence and to describe the epidemiological characteristics of ciguatera in the Europe, a protocol for a harmonized ciguatera surveillance in the European Union (EU)/Economic European Area (EEA) was elaborated. This protocol includes a ciguatera case definition and two questionnaires for collecting information on ciguatera cases or outbreak. Around half of the countries answered the data call for reporting cases. Four countries reported 34 outbreaks from 2012 to 2019. Spain and Portugal reported outbreaks due to consumption of fish captured in the Canary Islands and Madeira (autochthonous outbreaks), mainly due to consumption of *Seriola* spp. and *Epinephelus* spp. In more than half of these outbreaks, the fish was captured by sport fishing. Germany and France reported outbreaks due to consumption of fish imported from outside the EU (imported outbreaks); mainly *Lutjanus* spp. Spain, Germany and France reported outbreaks in travellers to tropical endemic territories (travel related outbreaks). The ciguatera cases and outbreaks presented neurological symptoms, most of them had also gastrointestinal symptoms and few outbreak cases mentioned cardiovascular symptoms. Six countries (Austria, France, Germany, Portugal, Spain and Switzerland) reported 34 single cases. As a main result, the incidence rate in the EU/EEA was very low (0.0054 cases per 100,000 inhabitants per year). However, Canary Islands constitute by far the area representing the highest risk with an incidence rate of 0.47 cases/100,000 inhabitants.

Related to the study of the presence of ciguatoxins (CTXs) in the environment, the main objective was to evaluate microalgae associated to the production of CTXs and the evaluation of CTXs in seafood for the risk assessment of CP. The effort consisted on the evaluation of toxicity in 104 strains of microalgae and 1174 fish corresponding to 77 species. Sampling areas included the Canary Islands, Madeira and Selvagens Islands, Balearic Islands, Cyprus and Crete. Particularly, in the Canary Islands the presence of several dinoflagellates of the genus *Gambierdiscus* as the causative organism covered 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).

Regarding Madeira and Selvagens Islands, the genus *Gambierdiscus* was detected in both areas. Toxicity of fish has been identified in 42 fish out of 128 fish (33%). The toxic fish identified with the cell-based assay (CBA), defined as primary reference material was transferred to University of Vigo (UVIGO) to continue the characterization of the toxins present.

From the eastern Mediterranean Sea, a great diversity of six taxa of *Gambierdiscus* and *Fukuyoa* with relatively low cell toxicity was detected. The first fish CTX-like positive by Neuroblastoma Cell-Based Assay (Neuro-2a CBA) from the Mediterranean has been detected in Cyprus with a low toxicity. 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, no fish showed CTX-like toxicity.

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. Efforts in the Mediterranean should be addressed to better understand the dynamics of toxic microalgal populations and tackle the potential presence of toxins in fish.

In order to characterize the risk associated to CP in the EU a sensitive methodology of LC-MS/MS was developed. On the other hand, the preparation of reference materials including the main CTXs responsible for the contamination was considered the secondary objective in order to facilitate the implementation of the Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) methods in the EU laboratories to characterize this emerging risk.

Caribbean ciguatoxin 1 (C-CTX1) has been identified by LC-MS/MS and further confirmed by LC-HRMS as the main responsible for the CTX toxicity in the samples from the areas selected for this study. The low concentration levels of CTXs found on the samples evaluated has been a key limitation on the completion of the objectives, being necessary to establish contingency plans, not only to overcome the problems of sensitivity that might compromise the confirmation of the toxic profiles, but also the accomplishment of the task of preparing reference materials.

The contingency plans involved the development of two complimentary LC-MS/MS approaches, as well as a methodological approach involving LC-MS/MS, Neuroblastoma cell assay and chromatographic fractionations (HPLC and GPC) to be able to characterize the toxins involved in the contaminated extracts. This approach has been also used on the preparation of reference materials to confirm the presence of the C-CTX1 in both pure solutions of C-CTX1 and Fish Tissue Reference Materials (FTRM) containing C-CTX1.

The LC-MS/MS analysis of dinoflagellates samples (*Gambierdiscus* and *Fukuyoa* spp.) allowed to confirm the lack of correlation with the CTXs contamination of the fish samples from the areas were these dinoflagellates were collected. In fact, the *Gambierdiscus* toxicity was attributed to several Maitotoxins (MTXs) analogues as well as gambieric acids C and D, Gambierone, 44-methyl gambierone, gambieric acids (or their analogues) and gambieroxide which were identified by LC-HRMS with varying degrees of confirmation in strains of *Gambierdiscus* and *Fukuyoa* from the Mediterranean Sea and North East Atlantic Ocean.

The EuroCigua project, as seen above, has provided extensive scientific contributions in the fields of public health and epidemiology, environmental assessment of toxins and the associated producing organisms or chemical characterization of CTXs. Hence EuroCigua has contributed significantly to a better characterization of ciguatera in Macaronesia and the Mediterranean Sea. Nonetheless some important issues such as the underreporting of CFP cases, the space and temporal distribution of toxin producing microalgae in some areas, the quantification of their communities, the characterization of the full CTX profiles involved in the CP contamination would require further work for which the availability of reference materials with adequate toxin concentrations is strictly necessary. Capacity building programmes, harmonization of methods, predictive work on ciguatera including modelling, especially focused on the identification of major drivers that may influence ciguatera and better understanding of the toxins involved will improve the assessment and prediction of future scenarios of ciguatera in Europe.

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1. Introduction

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

The present document corresponds to deliverable No. 6, *Final Scientific Report* of Specific Agreement 1 (SA1), Management and scientific coordination and summarises the activities carried out during the project (from 1st April 2016 until 31st December 2020).

This contract/grant was awarded by European Food Safety Authority (EFSA) to Spanish Food Safety and Nutrition Agency (AESAN):

- **Contract/Grant number:** Framework Partnership Agreement (FPA) GP/EFSA/AFSCO/2015/03:
  - **Specific agreement 1 (SA1) description:** Management and scientific coordination.
  - **Specific agreement 2 (SA2) description:** To determine the incidence and epidemiological characteristics of ciguatera cases in Europe.
  - **Specific agreement 3 (SA3) description:** Evaluation of CTX's in seafood and the environment for the risk assessment of ciguatera poisoning (CP), with the consequent preparation of reference material.
  - **Specific agreement 4 (SA4) description:** Characterization of ciguatoxins present in EU contaminated profiles by LC MS/MS and HRMS: Development of standards and secondary reference materials.
- **Contract title**: Framework Partnership Agreement GP/EFSA/AFSCO/2015/03 "Risk characterization of ciguatera food poisoning in Europe" (hereafter named EuroCigua).
- **Contractor/Beneficiary**: Spanish Food Safety and Nutrition Agency (AESAN) and Portuguese Economic and Food Safety Authority (ASAE). National epidemiology centre. Institute of Health Carlos III (CNE-ISCIII), Saúde Doutor Ricardo Jorge, I.P. National Institute, University of Thessaly (UT, Greece) and German Federal Institute for Risk Assessment (BfR). The Institute for Research and Technology in Food and Agriculture (IRTA), Canary Health Service. General Directorate of Public Health (SCS), University Institute of Animal Health and Food Safety – University of Las Palmas Gran Canaria (IUSA-ULPGC), Português Institute do Mar e da Atmosfera (IPMA), State General Laboratory. Ministry of Health from Cyprus (SGL) and Aristotle University of Thessaloniki from Greece. University of Vigo (UVIGO) and French Research Institute for Exploitation of the Sea (IFREMER).
- **Collaborators under SAs:** French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Direção de Serviços de Investigação e Desenvolvimento da Pesca, Direção Regional de Pescas, Secretaria Regional de Agricultura e Pesca, Instituto das Florestas e Conservação da Natureza, IP-RAM, Secretaria Regional do Ambiente, Recursos Naturais e Alterações Climáticas, Regional Government of Madeira and National Institute of Health Science (NIHS, Tokyo).
- **Advisory Board (AB):** Dr Takeshi Yasumoto as expert from Japan Food Research Laboratories (JFRL), Dr Robert Dickey from Marine Science Institute. University of Texas (UTA) and Dr Ronald Manger from Fred Hutchinson Cancer Research Center (FHCRC). European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), Joint Research Centre (JRC) and European Commission.

The EuroCigua Project is a Framework Partnership Agreement in which fourteen European scientific institutions from six Member States (Spain, Portugal, France, Germany, Greece and Cyprus) participate and is coordinated by AESAN. EuroCigua project also has an Advisory Board of excellence, with experts from United States and Japan, and collaborates with European Institutions such as EFSA, European Centre for Disease Prevention and Control (ECDC), Joint Research Centre (JRC) and the European Commission are part of this Advisory Board.

1.2. Interpretation of the Terms of Reference

The project aims to characterize an emerging risk that could be affecting several Member States of the EU. The main goal of Specific Agreement no. 1 is to provide the mechanisms that ensure that the objectives of the Framework Partnership Agreement GP/EFSA/AFSCO/2015/03 "Risk characterization of ciguatera food poisoning in Europe" are accomplished in a global manner and in accordance with the main tasks reflected in the Annex 1 of the Framework Partnership Agreement (FPA). AESAN will specifically facilitate the cooperation and scientific advancement of the Specific Agreements (SAs) led by the Spanish National Centre of Epidemiology – Health Carlos III Institute, CNE-ISCIII (SA2), the Research and Technology Food and Agriculture, IRTA (SA3) and the University of Vigo, UVIGO (SA4). This will foster scientific cooperation among partners within the Governing Board (GB) and facilitate the interchange of information with the members of the Advisory Board (AB). It will also ensure scientific coherence and data integration among the SAs and integrate the different results of the Grants in a final report in order to accomplish the main goals of the FPA.

1.3. Additional information

AESAN is responsible of coordination of the EuroCigua project under SA1. The activities carried out by AESAN are mainly focused on the coordination and management of the project, by providing the means that will ensure the progress and, consequently, the accomplishment of EuroCigua ultimate objective that is the risk characterization of CP in Europe. To facilitate the follow-up of the work entrusted to each SA, a chronogram of activities and deliverables planned for the full duration of the project was designed by AESAN, who updates and adjusts it when required. Subsequently, AESAN integrates the different results of the SAs to provide EFSA with the main goals of the FPA in terms of the risk characterization of CP in the EU.

The activities aimed to ensure scientific coherence and data integration among the SAs and integrate the different results among all SAs in this final report in order to accomplish the main goals of the FPA. In order to facilitate the cooperation and scientific advancement of the project, AESAN has been supporting the work of all SAs by providing and fostering opportunities of collaboration, of communication and of exchange of data with other worldwide entities and experts that would contribute to the project's progress. In this regard, AESAN organized meetings regularly to procure a space for discussion of the tasks at hand and to share the new results and conclusions reached. Through the course of the project, AESAN has organized 28 coordination meetings (with the SAs coordinators), 11 meetings with EFSA and 5 general meetings. The later events are organized on a larger scale, with the attendance of members and collaborators of the EuroCigua project consortium belonging to fifteen scientific institutions from six Member States (Spain, Portugal, France, Germany, Greece and Cyprus)

and other experts on the subject of CP from non-EU countries, such as the USA, Canada, Australia and Japan, to name a few.

The dissemination of information about CP was essential in order to increase the data collection of ciguatera cases and outbreaks. AESAN together with ASAE designed a communication strategy. To do so, AESAN in collaboration with ASAE have elaborated printed and digital materials, such as a leaflet about CP (available in seven languages), a factsheet with a more detailed description of the food poisoning and of the project, and a poster that includes the background, description, objectives, methods and results of the project and all institutions involved in it. These publications are distributed and/or shown in events in which the EuroCigua team presents the project, in fishing associations and health centers, within national and international institutions and authorities, among others. These materials are also available in the project's website, which is managed and updated by AESAN on a regular basis. This webpage contains the most important information about CP and the project and is used to publish the latest relevant news and events, which are also announced through AESAN's social media accounts (Twitter, Facebook, Instagram and YouTube).

2. Methodology

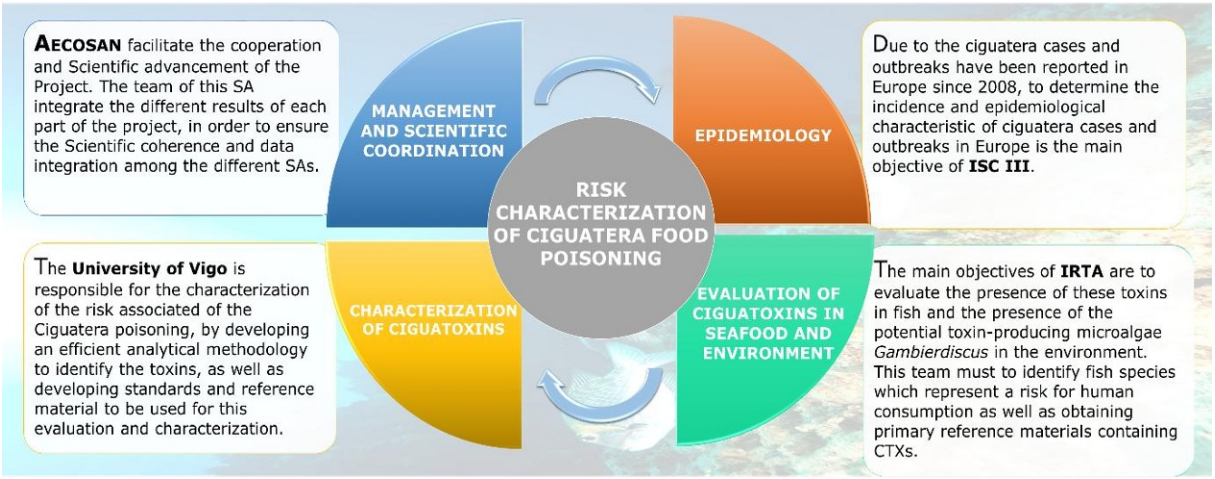
In order to approach the risk characterization of CP in Europe, a consortium of 14 partners was set-up and four SAs within the EuroCigua project coordinated by the Spanish Food Safety and Nutrition Agency, AESAN.

The first Specific Agreement (SA1) "MANAGEMENT AND SCIENTIFIC COORDINATION" was under the responsibility of AESAN and consisted on the management of the project and ensure data integration among the different SAs. Continuous communication and discussions among the different members of the four SAs ensured a coordinated action for the advancement of the project and the success completion of objectives.

The second Specific Agreement (SA2) "EPIDEMIOLOGY" was coordinated by National Epidemiology Centre. Institute of Health Carlos III. The main objective was to determine the incidence and epidemiological characteristics of ciguatera cases and outbreaks in Europe. Members of the other agreements reviewed the documents developed under the SA2, according to the different areas of expertise. They, also, contributed to deliver a ciguatera case definition for the EU/EEA, achieved by consensus.

Agreement number three (SA3) "EVALUATION OF CIGUATOXINS IN SEAFOOD AND THE ENVIRONMENT" was coordinated by the Institute of Agrifood Research and Technology (IRTA). This agreement was oriented to different objectives in order to assess the risk of ciguatera. First, the SA aimed at the sampling and identification of potential toxin-producing microalgae in the environment, characterize these populations, and establish microalgal cultures in order to evaluate the toxicity of the different species. Different institutions participated in the isolation of microalgae and culturing (AUTH, IPMA, IRTA). Toxicity evaluation of microalgae was carried by IRTA. Another objective consisted on the sampling of fish from different sources (IUSA-ULPGC, IPMA, SGL, AUTH and IRTA) and the evaluation of their toxicity (IUSA-ULPGC and IRTA). This agreement was the source of microalgal extracts and fish that were transferred to SA four for toxin characterization.

Specific Agreement number four (SA4) "CHARACTERIZATION OF CIGUATOXINS" was coordinated by the Universidad de Vigo. The IFREMER from France was also involved as partner of this SA. One of the objectives of this SA was focused on the development of an analytical methods based on a liquid chromatography separation, coupled to mass spectrometry (LC-MS/MS and HRMS) for the confirmation of the presence of CTXs and the further characterization of the CTXs analogues involved in the contamination. Fish samples and microalgae received from SA3 were analysed with the methods developed in this SA and the confirmation of the toxicity, as well as the characterization of the main CTX analogues involved in this toxicity was carried out. The preparation of reference materials has been also an important objective of this SA4, to accomplish this objective an optimized analytical procedure for the isolation and purification of the reference materials was developed. The reference materials obtained in this SA will be used for method implementation for the characterization of Ciguatoxins.



Scheme 1. EuroCigua project structure and SAs objectives.

Partners and collaborators:

Agreement number one "MANAGEMENT AND SCIENTIFIC COORDINATION" was under the responsibility of AESAN and included Food Safety and Economic Authority (ASAE) from Portugal as partner and The French Agency for Food, Environmental and Occupational Health & Safety (ANSES) as collaborator.

Agreement number two "EPIDEMIOLOGY" was coordinated by National Epidemiology Centre. Institute of Health Carlos III and included Saúde Doutor Ricardo Jorge, I.P. National Institute from Portugal, University of Thessaly (Greece), Canary Health Service from Spain and German Federal Institute for Risk Assessment as partners.

Agreement number three "EVALUATION OF CIGUATOXINS IN SEAFOOD AND THE ENVIRONMENT" was coordinated by the Institute of Agrifood Research and Technology (IRTA) in Spain, and included as partners the Instituto Universitario de Salud Animal of the University of Las Palmas de Gran Canarias (IUSA-ULPGC), the Canary Health Service (Health Department) of the Government of the Canary Islands, the Instituto Português do Mar e da Atmosfera (IPMA), the State General Laboratory of Cyprus (SGL) and the Aristotle University of Thessaloniki (AUTH) in Greece. Collaboration was also obtained by the Regional Fisheries Management-Madeira Government, DSI-DRP2, and Instituto das Florestas e Conservação da Natureza, IP-RAM, Secretaria Regional do Ambiente, Recursos Naturais e Alterações Climáticas, Regional Government of Madeira.

Agreement number four "CHARACTERIZATION OF CIGUATOXINS" was coordinated by the Universidad de Vigo and included IFREMER from France as a partner.

Advisory Board

EuroCigua project also has an Advisory Board of excellence. Dr Takeshi Yasumoto from Japan Food Research Laboratories who pioneered research on ciguatoxins, Dr Robert Dickey Director and Chairman of the Department of Marine Science at Marine Science Institute from the University of Texas and Dr Ronald Manger, researcher and regional scientific advisor to the US Food and Drugs Administration (FDA). In addition, European institutions such as EFSA, European Centre for Disease Prevention and

Control (ECDC), Joint Research Centre (JRC) and the European Commission are part of this Advisory Board.

2.1. Epidemiological surveillance of ciguatera in the EU/EEA

In order to estimate the incidence and describe the characteristics of ciguatera cases, a surveillance protocol was prepared (Annex I). The surveillance protocol is a guide to carry out retrospective and prospective surveillance of ciguatera in the EU/EEA (excluding tropical overseas territories), from 2012 to 2019, in a harmonized manner and on a voluntary basis for the duration of the FPA. The partners of the SA2, the leaders of the other specific agreements, the advisory board and the EFSA, reviewed the protocol until a final consensus document was achieved. The protocol includes the ciguatera case and outbreak definition, and it defines an outbreak as autochthonous when the fish consumed was harvested within the EU/EEA, and imported when the fish consumed was harvested outside the EU/EEA and imported into the EU/EEA. Moreover, the surveillance protocol includes two questionnaires (Annexes II and III) for collecting information on ciguatera cases and outbreaks. It also includes a non-exhaustive list of fish (Annex IV) previously associated with ciguatera or in which ciguatoxins have been detected (it is an open list and it was updated regularly).

The following list of data sources that may provide information on ciguatera cases and outbreaks was prepared. Depending on the source, the information supply included demographic information, clinical information, food consumption, risk factors, laboratory details, etc.:

<u>At country level</u>

A. Public Health Surveillance System
B. General Practitioners records (outpatients)
C. Hospital Emergency records
D. Hospital Discharge records (inpatients)
E. Poison Centres records
F. Travel or Tropical Medicine Units/Facilities records
G. Food Safety Competent Authorities / Food Surveillance System
H. Vital statistics
I. Laboratories

<u>At European Union level</u>

J. European Union Food-borne Outbreaks Reporting System (EU-FORS). European Food Safety Authority.
K. Epidemic Intelligence Information System (EPIS FWD). European Centre for Disease Prevention and Control.
L. Early Warning and Response System (EWRS). European Commission.
M. Rapid Alert System for Food and Feed (RASFF). European Commission.
N. Rapid Alert System for Chemicals (RAS-CHEM). European Commission.
O. Centralized Information System for Infectious Diseases. World Health Organization. Europe.
P. Alert Systems (IHR, INFOSAN, etc.). World Health Organization. Europe.

<u>List of possible additional ciguatera data sources during an outbreak investigation</u>

1. Fisheries authorities

2. Consumer complaints
3. Restaurants
4. Retail markets
5. Fishery industry
6. Fish importers
7. Wholesalers and intermediate traders
8. Media

To collect homogeneous data, the surveillance protocol was disseminated to different EU/EEA networks, through the EFSA or the European Centre for Disease Prevention and Control (ECDC).

In order to collect the ciguatera information, the structure of a web-based database according to the codes and variables of the two specific questionnaires for ciguatera cases and outbreaks was prepared, considering EFSA and ECDC electronic specifications. The database is allocated in the CNE-ISCIII, within the platform used for epidemiological surveillance in Spain (SiViEs). The platform complies with the European Regulation 2016/679 and the Spanish law 3/2018 on data protection. The metadata for cases include 72 variables, 2.287 code values and 10 internal validation rules and the metadata for outbreaks include 67 variables, 2.299 code values and 13 internal validation rules.

Linear regression was used to estimate the data trend. Statistical estimations were calculated with STATA 16. P values of < 0.05 were considered statistically significant.

2.2. Evaluation of CTXs in seafood and the environment for the risk assessment of Ciguatera Poisoning (CP)

The characterization of the risk of CP was approached by the evaluation of the presence of CTX producing microalgae of the genera *Gambierdiscus* and *Fukuyoa*, and the screening of CTX activity in the microalgae and fish obtained in the different areas studied. The environmental data obtained at the time of microalgal sampling was also reported.

2.2.1. Sampling, culturing and identification of microalgae.

Sampling of microalgae

Since sampling of microalgae was carried out in the Canary Islands, Madeira, Crete, Cyprus and the Balearic Islands, a standardized sampling protocol was developed to harmonize this task among the different partners.

Briefly, 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, algae or sand) and 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. Figure 2 shows the materials used and one of the sampling points.

Environmental data at the different sampling sites where microalgae were obtained, were recorded with a multiparameter probe by means of portable sensors (temperature, salinity and dissolved oxygen). The coordinates of each sampling station were recorded by GPS.

Figure 2. Materials and sampling point.

Culturing of microalgae.

Strains were isolated with a glass micropipette by the capillary method under an inverted microscope (Leica DM-IL, Olympus IX71, and others). Isolated cells were incubated in 24 or 48 well microplates in Provasoli's Enriched Seawater (PES) medium for cultivation of algae or L1/5 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. Cells were transferred to non-treated polystyrene flask of 25 mL when the culture reached a density of 20 cels/mL. Cultures were scaled-up to larger volumes, in order to obtain microalgal biomass for studies of toxicity. Before harvesting, live 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. Cultures were harvested through filtration and centrifugation (4500 rpm x 20 min at 20 °C).

Identification of microalgae

Morphological identification

The identification of microalgae was performed in a first stage with morphological evaluation, aware that the complementary molecular approach was necessary. For morphological evaluation, the use of

calcofluor combined with fluorescence microscopy and Scanning Electronic Microscopy (SEM) was implemented (Fritz and Triemer 1985; Reverté et al., 2018). Figures 3-5 show some examples of morphological evaluation of cells.

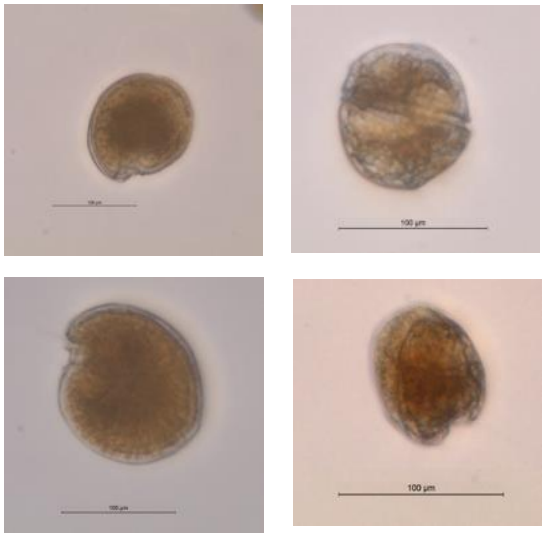


Figure 3. Light microscopy photos of strains corresponding to the genus *Gambierdiscus* (the two images on the left) and to the genus *Fukuyoa* (the two images on the right).

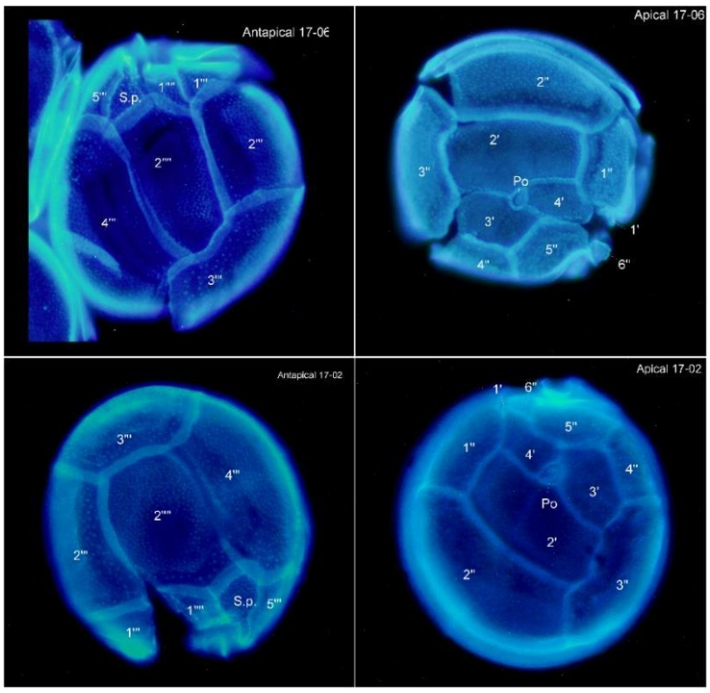


Figure 4. *Gambierdiscus australes* from Lanzarote (A-B) and (C-D) Fuerteventura (Canary Islands) by calcofluor stain.

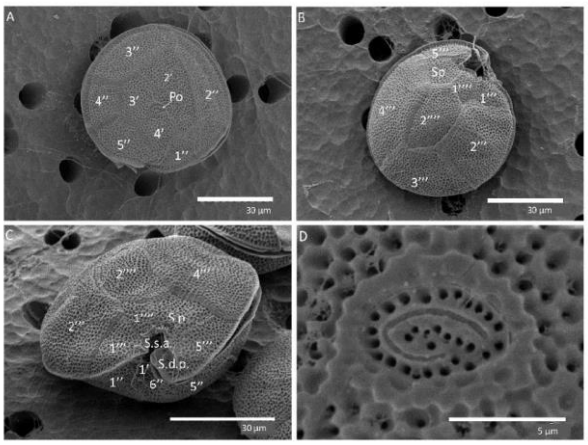


Figure 5. SEM images of *G. belizeanus* (IRTA-SMM-17-421): apical (A), antapical (B), ventral (C) views, detail of Po plate and pores (D).

Molecular genetics

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 Large Sub Unit (LSU) rDNA D1-D3 loop and LSU rDNA D8-D10 loop. In this project both of these genetic markers were used and each one required separate Polymerase Chain Reactions (PCR). Also, different primer pairs can be useful for amplifying the same region from different species when the expected amplification is not obtained with a primer set. Figure 6 shows an example of amplification of the LSU rDNA D1-D3 loop using different primer sets.

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).

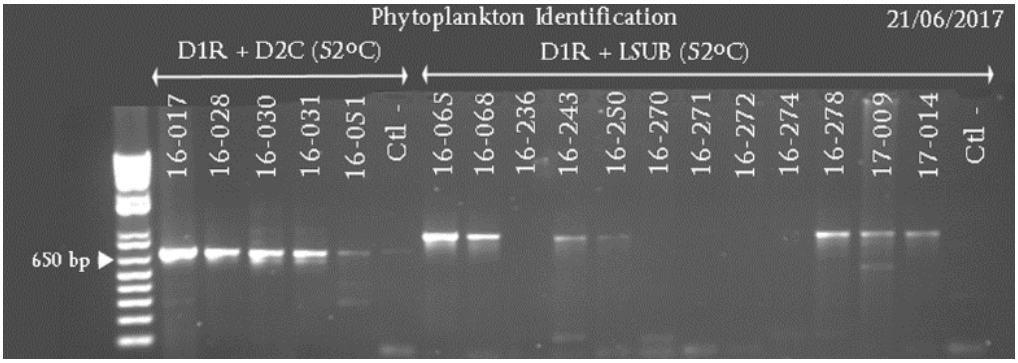


Figure 6. An example of amplification of the LSU rDNA D1-D3 loop using different primer sets.
Note: not all amplify equally well since the actual sequences of each strain may not be identical to the primers in use. Expected size of product is ~700 - 800 base pairs depending on primers used.

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 Basic Local Alignment Search Tool (BLAST) analysis National (Centre for Biotechnology Information, NCBI website) to confirm the proximate identity, and full phylogenetic analysis is performed afterward to confirm suspected identity using the Molecular Evolutionary Genetics Analysis software (MEGA 6.0).

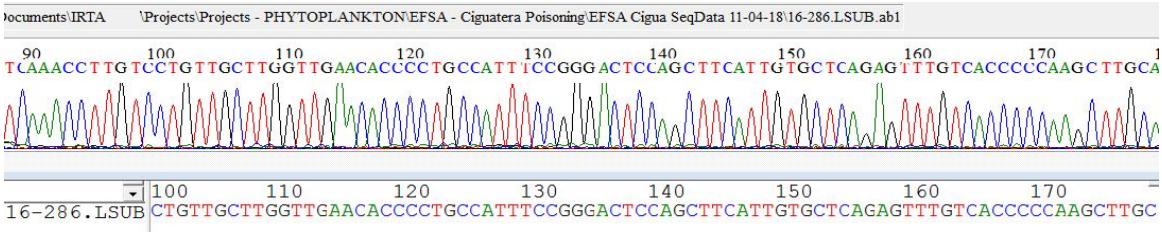


Figure 7. An example of sequence data obtained from DNA extracted from microalgae culture samples. The electropherogram and corresponding sequence are shown for a short representative segment of the amplified LSU rDNA D8-D10 loop.

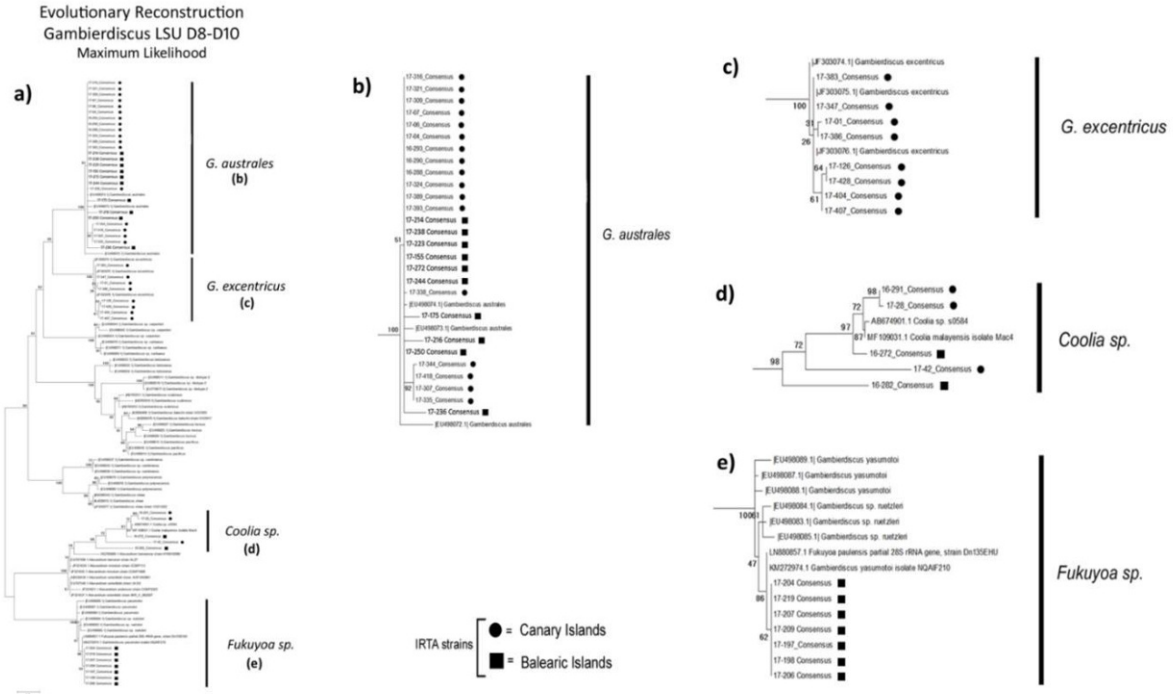


Figure 8. Results obtained from phylogenetic analysis of LSU rDNA (D8-D10 loop) sequence data for identification of *Gambierdiscus*-like cells from culture isolates as of April 2018. IRTA strain isolates are marked with either a circle (Canary Island isolate) or a square (Balearic Island isolate). Maximum Likelihood analysis performed using MEGA 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.

2.2.2. Sampling of fish

The evaluation of the toxicity of fish, is another indicator to establish the risk of ciguatera in each area. It is important to state that, since microalgae of the genera *Gambierdiscus* 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.

A standardized sampling protocol for fish was also developed in order to harmonize work among partners.

In this project, different sampling sources have been used including official control samples, sport fishing samples, monitoring samples, CP outbreak samples, fish purchased, and intoxication episode sources.

Fish were obtained from the Canary Islands, Madeira, Balearic Islands, Cyprus and Crete.

The Canary Islands was the region for which more fish were obtained. Data were obtained from the following three groups of fish from the EuroCigua project and from the Official Control Program to obtain a more complete evaluation of CTX toxicity in fish:

1. IUSA-ULPGC-EuroCigua fish: Data on EuroCigua samples analysed by the IUSA-ULPGC. These cover the whole number of fish (n=746) from the Canary Islands obtained within our project. IUSA-ULPGC provides toxicity evaluation within a scale from L1 to L5.
2. IRTA- selected EuroCigua fish: Data on EuroCigua samples analysed by IRTA. These cover a selection (n=130) 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).
3. 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 were conducted by the IUSA-ULPGC using a different extraction method.

From Madeira and Selvagens Islands, IPMA sampled a total of 128 fish, 81 from Selvagens Islands, 21 from Madeira and 26 from Desertas Islands. From these, 24 fish were selected to prepare reference material in SA4.

From Crete, a total of 70 fish were sampled from the area (Kissamos bay, Gramboussa bay and Kolymbary bay).

From Cyprus, SGL sampled a total of 82 fish from the area (S3-Zygi/Larnaca district, Latsi/Paphos district, Cavo Kiti/Larnaca district, Paphos/Paphos district, Cavo Pyla/Larnaca district, Larnaca/Larnaca district, Zygi area-Limassol).

From the Balearic Islands, IRTA with the help of the Balearic Island government sampled 42 fish from Majorca, Menorca and Ibiza.

Several species of fish were sampled from August to November when *Gambierdiscus* spp. presents higher abundances in the environment. The list of fish species evaluated is presented in Table 1. In the Canary Island, differences in toxicity in fish between winter and summer season were also evaluated.

Table 1. List of fish species evaluated for toxicity

Acanthocybium solandri	*Epinephelus costae*	*Pomatomus saltatrix*
Aluterus scriptus	*Epinephelus marginatus*	*Pontinus kuhlii*
Balistes capriscus	*Gymnothorax polygonius*	*Pseudocaranx dentex*
Beryx splendens	*Gymnothorax unicolor*	*Sarda sarda*
Bodianus scrofa	*Helicolenus dactylopterus dactylopterus*	*Sarpa salpa*
Boops boops	*Katsuwonus pelamis*	*Sciaena umbra*
Canthidermis sufflamen	*Kyphosus sectatrix*	*Scomber colias*
Canthigaster capistrata	*Lithognathus mormyrus*	*Seriola carpenteri*
Chelon labrosus	*Liza aurata*	*Seriola dumerili*
Chromis limbata	*Lutjanus cyanopterus*	*Seriola fasciata*
Conger congre	*Makaira nigricans*	*Seriola rivoliana*
Coryphaena equiselis	*Mullus surmuletus*	*Seriola* sp.
Coryphaena hippurus	*Muraena augusti*	*Serranus atricauda*
Coryphaena sp.	*Muraena helena*	*Serranus cabrilla*
Dentex dentex	*Muraena* sp.	*Shyraena shyraena*
Dentex gibbosus	*Mustelus mustelus*	*Siganus luridus*
Dentex macrophthalmus	*Mycteroperca fusca*	*Sparisoma cretense*
Dicentrarchus labrax	*Oblada melanura*	*Sparus aurata*
Diplodus cervinus	*Pagellus acarne*	*Sphoeroides marmoratus*
Diplodus puntazzo	*Pagellus erythrinus*	*Sphyraena viridensis*
Diplodus sargus	*Pagrus auriga*	*Spondyliosoma cantharus*
Diplodus sargus cadenati	*Pagrus pagrus*	*Stephanolepis hispidus*
Diplodus vulgaris	*Parapristipoma octolineatum*	*Synodus synodus*
Enchelycore anatina	*Phycis phycis*	*Thunnus* sp.
Epinephelus marginatus	*Polymixia nobilis*	*Umbrina canariensis*
Epinephelus aeneus	*Polyprion americanus*	

2.2.3. Cytotoxicity evaluation with the Neuro-2a Cell Based Assay

The evaluation of CTX-like toxicity in microalgae and fish consisted first on an extraction process of the samples followed by an evaluation of CTX-like toxicity implementing the Neuro-2a cell-based assay (Caillaud et al., 2012).

Sample extraction

The extraction of samples was different for microalgae and fish.

Briefly, microalgae were extracted under sonication with pure methanol, followed by methanol (MeOH): water (50/50; v/v) followed by filtration of the extract through PTFE (0,2 µm), and stored at -20ºC (Caillaud et al., 2011).

Fish were cooked for 15 min at 70-95ºC, and then extracted with acetone, followed by two liquid/liquid partition processes (Diogène et al., 2017). First, a diethyl ether water partition, in a proportion 8:2 of diethyl ether relative to the amount of residual water. A second partition consisted on a partition with n-hexane and aqueous methanol:H_2O (4:1, v/v), in a proportion of 4mL of n-hexane and 2 mL of aqueous methanol.

Neuro-2a cell-based assay

A standardized method for the evaluation of CTXs with a Cell-Based Assay (CBA) was established for evaluation of CTX-like toxicity in microalgae and fish.

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 the concentration of CTXs. The calibration curve for CTX (Figure 9), 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.

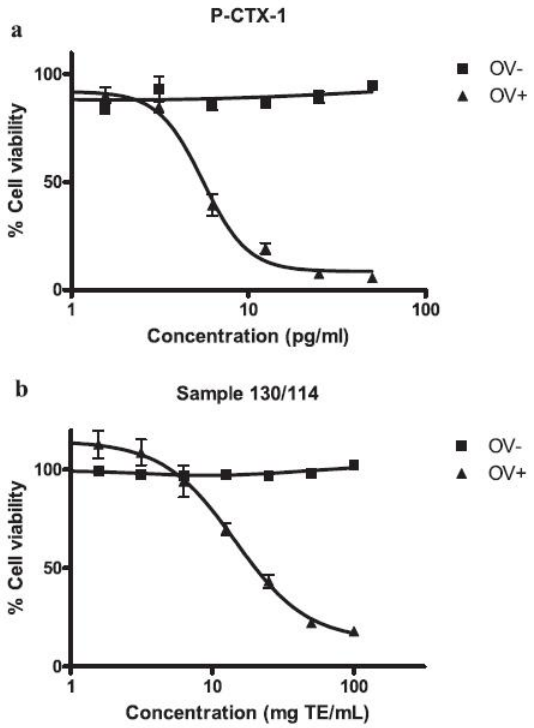


Figure 9. Representative concentration-response curves for a) CTX-1B standard and b) fish extract. Error bars represent the standard deviation from three replicates.

On behalf of standardization of Neuro-2a CBA, big efforts have been done between IRTA and IUSA-ULPGC to harmonize the results obtained for samples analysed by both laboratories. A positive CTX-1B

standard of reference (Richard Lewis, University of Queensland, Australia) was always included when implementing the method.

The Neuro-2a 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 as presented in Figure 10.

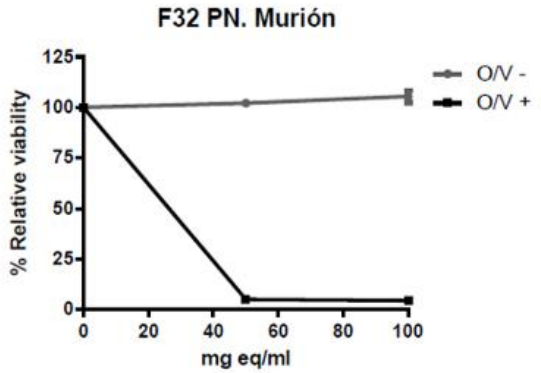


Figure 10. IUSA-ULPGC: Cytotoxicity of positive sample (F32) with the Neuro-2a assay.

Interlaboratory optimization (IUSA-ULPGC - IRTA)

IUSA-ULPGC and IRTA conducted several interlaboratory assays to harmonize methodologies. We present here the example of the second Neuro-2a cell-based assay inter-comparative between IRTA and IUSA-ULPGC performed in 2019. Five fish samples were selected to compare the Neuro-2a CBA 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 2.

Table 2. Summary results from one of the Neuro-2a cell-based assay inter-comparative exercises.

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 showed that the Neuro-2a cell-based assay was well implemented in both laboratories since results obtained from the five samples are on the same range in both laboratories.

2.3. Characterization of CTX in fish and microalgae

2.3.1. Development, optimization and validation of LC-MS (MS/MS and HRMS)

The methods used for the analysis and confirmation of CTXs in the fish samples evaluated in this project are LC-MS/MS and LC-HRMS. These methods are based on previously described methods (Abraham et al., 2012; Yogi et al., 2011) and were optimized in order to be applied to the samples which were studied (Estevez et al., 2019a, 2020a; Sibat et al., 2018). The development, optimization and validation of LC-MS/MS and LC-HRMS was the initial task in this SA. These methods were used, not only for the confirmation of the CTXs toxicity in the selected samples, but also for the selection of contaminated samples to be used as candidates for the preparation of fish tissue reference material (FTRM) and its further characterization.

Extraction and purification

Sample pre-treatment including extraction and purification steps was carried out following the conditions initially proposed in (Yogi et al., 2011) with optimization described in (Estevez et al., 2019a), Briefly: Fish tissue samples (15 g) were extracted twice by homogenizing in 45 mL of acetone during 2 min at 9000 rpm (Ultra Turrax® T25 basic). The combined extracts were concentrated to an aqueous residue and extracted twice with 15 mL of diethyl ether (Et_2O) and evaporated to dryness. The solid residue was dissolved in 4.5 mL 90 % methanol (MeOH). The aqueous MeOH solution was defatted with hexane (9 mL) and evaporated under light nitrogen gas stream (Figure 11).

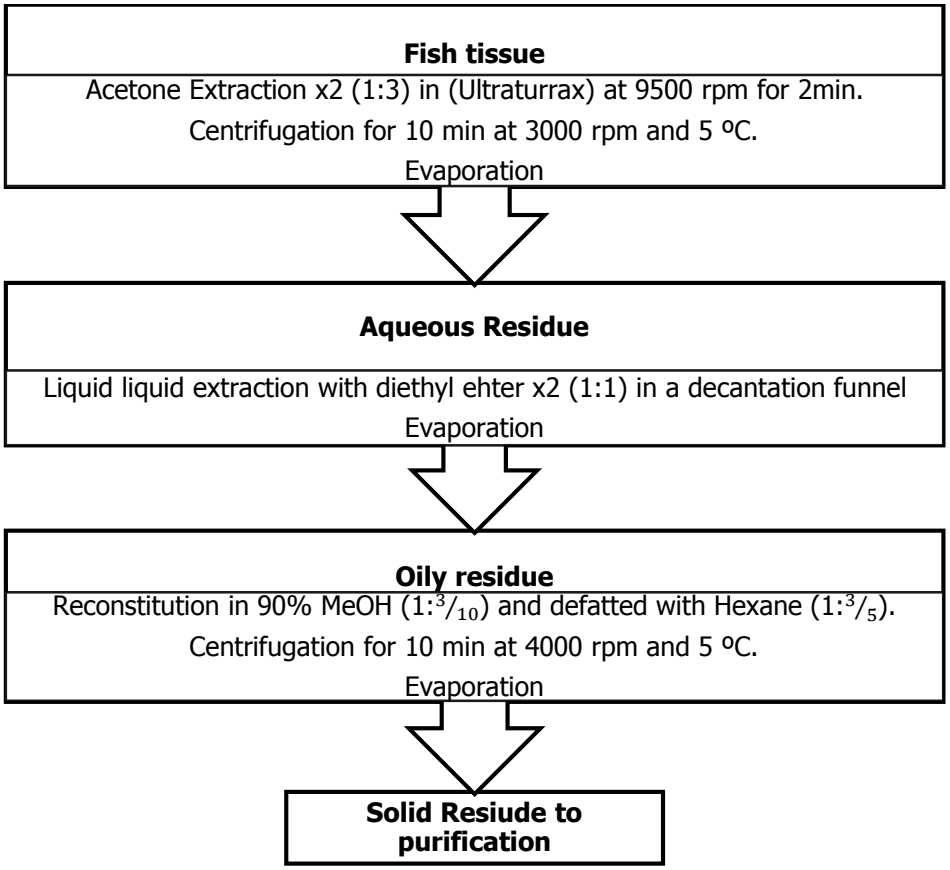


Figure 11: Extraction conditions, in brackets mass/volume relation

The solid residue was dissolved in 2 mL of ethyl acetate (AcOEt) and interfering matrix constituents were removed by using normal and then reversed phase (SPE) cleanup. The normal phase SPE (Florisil), removed polar interferences from the extract. Reverse phase SPE (C18) removed nonpolar and semi polar interfering matrix constituents. The elution process used for the Florisil clean-up was carried out as follows (Table 3): the sample extract in 2 mL AcOEt was passed through a Florisil cartridge (J. Baker, 500 mg) previously conditioned with 3 mL of AcOEt, and eluted in three consecutive steps, with 3 mL of AcOEt, 5 mL of AcOEt-MeOH (9:1) and AcOEt-MeOH (3:1). The toxin, eluting in AcOEt-MeOH (9:1), was dried under nitrogen stream at 50 °C (Murata et al., 1990). The residue from Florisil clean-up was dissolved in 2 mL of 60% MeOH-H_2O and applied to a C18 cartridge (SUPELCLEAN, Supelco, 500 mg) previously conditioned with 3 mL of 60% MeOH-H_2O (Table 3). The cartridge was washed with 3 mL of 60% MeOH-H_2O and the retained toxins were eluted with 5 mL of 90% MeOH-H_2O. The eluate was dried, dissolved in 0.5 mL of MeOH and filtered through a 0.22 µm Polyvinylidene Fluoride (PVDF) filter (Syringe Driver filter Unit, Millex®-CV 0.22 µm, 13 mm) prior to LC-MS/MS analysis.

Table 3. Optimised conditions for the clean-up by solid phase extraction

SPE conditions	
Florisil SPE Bakerbond™ SPE, J.T.Baker ® , 500 mg, 3 mL	**C18 SPE** SupelClean™, Supelco 500 mg, 3 mL
Condition: 3 mL AcOEt	**Condition:** 3 mL MeOH/H_2O (60%)
Load: 2 mL sample extract in AcOEt	**Load:** 2 mL sample extract in MeOH/H_2O (60%)
Wash: 3 mL AcOEt	**Wash:** 3 mL MeOH:H_2O (60%)
Elution: 5 mL AcOEt:MeOH (9:1)	**Elution:** 5 mL MeOH:H_2O (90%)
Second elution: 5 mL AcOEt:MeOH (3:1)	———

2.3.2. Application to the analysis of fish samples and microalgae by LC-MS/MS and LC-HRMS

Confirmation in fish samples

LC-MS/MS

The LC-MS/MS analyses were carried out using the optimized conditions developed in the laboratory, using the LC-MS/MS System described as follows: Agilent 1290 Infinity LC system coupled to an Agilent 6495 Triple Quadrupole LC-MS (Agilent Technologies, CA) equipped with an Agilent Jet Stream electrospray ionization source (iFunnel). The new configuration iFunnel on the electrospray ionization allowed an increased sensitivity. The LC-MS/MS analyses were carried out following two different methods as described by (Estevez et al., 2019a); the first one, focused on the sensitive identification and quantification of the CTXs selecting the $[M+Na]^+$; whilst the second method was focused on the confirmation of specific CTXs by selecting water losses $[M+H-nH_2O]^+$ and specific fragments.

Method for the identification and quantification of CTXs

Liquid Chromatography (LC) conditions

LC separation was achieved using a Poroshell 120 EC-C18 column (3.0 × 50 mm, 2.7 µm, Agilent USA) at 40 °C using a linear gradient of mobile phases: 5 mM ammonium formate and 0.1% formic acid in water (A) and MeOH (B). The concentration of solution B was increased from 78% to 88% in 10 min and held for 5 min. The flow rate was 0.4 mL/min, and the injection volume was 1 µL. The conditions used for LC-separation are summarized in Table 4.

Table 4. Gradient of mobile phase for LC-MS/MS analysis. A: 5 mM ammonium formate and 0.1 % formic acid in water; B: MeOH

Time (min)	% A	% B	Flow (mL min^{-1})
0.00	22.00	78.00	0.40
10.00	12.00	88.00	0.40
15.00	12.00	88.00	0.40
15.01	0.00	100.00	0.40
18.00	0.00	100.00	0.40

Mass spectrometry conditions

The mass spectrometer was operated in positive mode in order to monitor sodium adduct ions ([M+Na]$^+$). The optimized conditions were as follows: collision energy 40 eV and the [M+Na]$^+$ ions were used as precursor ions and product ions. Source and interface conditions were optimized for the analysis of CTXs in positive ionization mode and were adjusted to achieve the optimal sensitivity for the selected ions.

The instrumental parameters were set as follows: Drying gas, 15 L min^{-1} of N^2 at 290 °C; sheath gas flow, 12 L min^{-1} of N^2 at 400 °C; nebulizer gas, N2 at 50 psi; capillary voltage, 5000 V; nozzle voltage: 300 V; fragmentor energy 380 V. This method monitors ions from all CTXs with known structures and all standards available that were kindly provided by Prof. Yasumoto (Mix Pacific Ciguatoxins standard contains: CTX1B, 2,3-dihydroxiCTX3C, 51-hydroxiCTX3C, 52-epi-54-deoxyCTX1B/54-deoxyCTX1B, 49-epi-CTX3C, CTX3C, CTX4A and CTX4B) and Dr. Ronald Manger and Dr. Robert Dickey Caribbean Ciguatoxin-1, (C-CTX1). The optimal MS conditions are summarized in detail in Table 5.

Table 5. Mass spectrometry conditions for CTXs

Toxin	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collision energy (eV)	Cell Acc (eV)
CTX1B	1133.6	1133.6	380.0	40.0	6
C-CTX1	1163.7	1163.7	380.0	40.0	6
2,3-dihydroxi CTX3C	1079.6	1079.6	380.0	40.0	6
51-hydroxi CTX3C	1061.6	1061.6	380.0	40.0	6
52-*epi*-54-deoxy CTX1B/54-deoxyCTX1B	1117.6	1117.6	380.0	40.0	6
49-*epi*-CTX3C/CTX3C	1045.6	1045.6	380.0	40.0	6
CTX4A/CTX4B	1083.6	1083.6	380.0	40.0	6

An example of the LC-MS/MS analysis carried out for the standard mix for Pacific CTXs and C-CTX1 under the optimal conditions is shown in Figure 12.

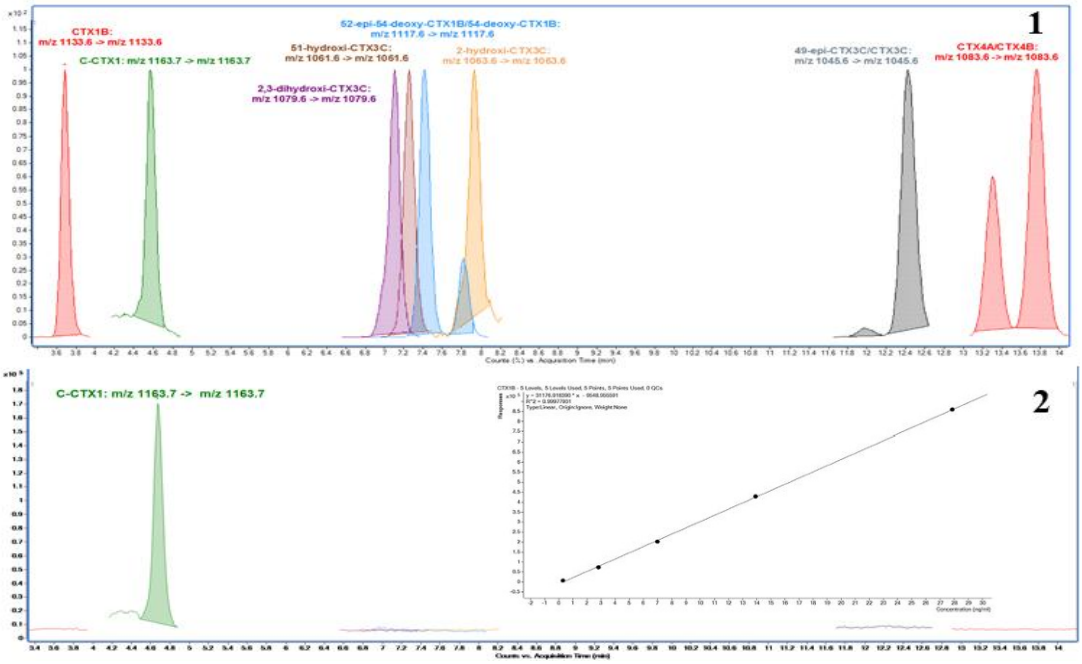


Figure 12. LC-MS/MS chromatogram selecting sodium adduct $[M+Na]^+$ as precursor and product ion of: 1) Mixture of P-CTXs and C-CTX1; 2) C-CTX1 detected in a naturally contaminated sample.

Method for the confirmation of specific CTXs

Liquid Chromatography (LC) conditions

LC separation was achieved using a Poroshell 120 ECC18 (2.1 x 100 mm, 2.7 µm, Agilent USA) at a column temperature of 40 °C. LC mobile phase was 5 mM ammonium formate and 0.1 % formic acid in H_2O (A) and acetonitrile (ACN) (B). The gradient used was 35 % B for 1 min, linear gradient to 80 % B at 15 min, 95 % B at 16 min, hold for 5 min and return gradient to 35 % B at 24 min (Table 6). The flow rate was 0.4 mL/min, and the injection volume was 5 µL.

Table 6. Gradient of mobile phase for LC-MS/MS analysis. A: 5 mM ammonium formate and 0.1 % formic acid in water; B: ACN

Time (min)	% A	% B	Flow (mL min^{-1})
0.00	65.00	35.00	0.40
1.00	65.00	35.00	0.40
15.00	20.00	80.00	0.40
16.00	5.00	95.00	0.40
21.00	5.00	95.00	0.40
24.00	65.00	35.00	0.40

Mass spectrometry (MS7MS) conditions

The mass spectrometer was operated in positive mode in order to monitor water loss ions ([M+H-nH_2O]$^+$) and C-CTX1 fragments by Multiple Reaction Monitoring (MRM) mode. The first water loss, *m/z* 1123.6 ([M+H-H_2O]$^+$), was selected as precursor ion and the following ions were monitored: three water loss ions, *m/z* 1105.6 ([M+H-2H_2O]$^+$), *m/z* 1087.6 ([M+H-3H_2O]$^+$) and *m/z* 1069.6 ([M+H-4H_2O]$^+$); and two C-CTX1 fragments, *m/z* 191.1 and *m/z* 108.9. The collision energy for each precursor/product transition pair were 21 eV for 1123.6/1105.6 and 1123.6/1087.6, 29 eV for 1123.6/1069.6, 40 eV for 1123.6/108.9 and 45 eV for 1123.6/191.1 (Table 7). The instrumental parameters were set as follows: Drying gas, 16 L min^{-1} of N_2 at 250 °C; sheath gas flow, 12 L min^{-1} of N_2 at 400 °C; nebulizer gas, N_2 at 15 psi; capillary voltage, 4500 V; nozzle voltage: 400 V; fragmentor potential 380 V.

Table 7. MMS conditions for the confirmation of specific C-CTXs

Compound	Precursor Ion (Q1)	Product Ion (Q3)	CE (eV)
C-CTX1	[M+H-H_2O]$^+$ *m/z* 1123.6	[M+H-2H_2O]$^+$ *m/z* 1105.6	21
	[M+H-H_2O]$^+$ *m/z* 1123.6	[M+H-3H_2O]$^+$ *m/z* 1087.6	21
	[M+H-H_2O]$^+$ *m/z* 1123.6	[M+H-4H_2O]$^+$ *m/z* 1069.6	29
	[M+H-H_2O]$^+$ *m/z* 1123.6	*m/z* 191.1	45
	[M+H-H_2O]$^+$ *m/z* 1123.6	*m/z* 108.9	40

An example of the LC-MS/MS confirmatory analysis carried out for C-CTX1 standard and C-CTX1 in a naturally contaminated sample is shown in Figure 13.

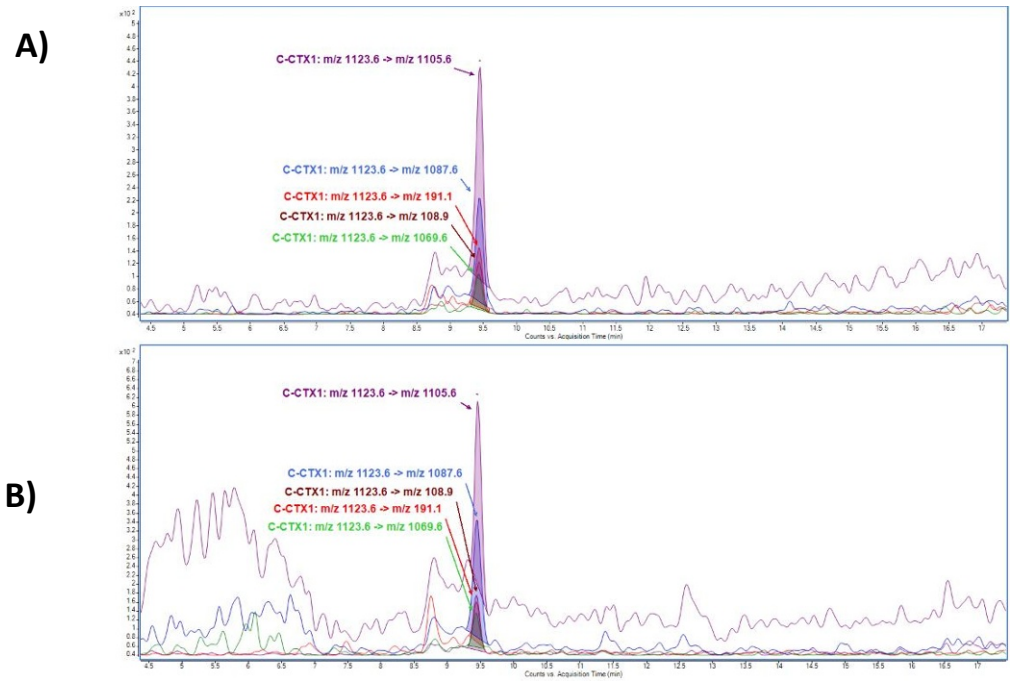


Figure 13. Example of LC-MS/MS confirmatory analysis of: A) C-CTX1 standard; B) C-CTX1 detected in *Bodianus scrofa* from Selvagen Islands

LC-HRMS

CP is primarily caused by consumption of tropical and sub-tropical fish contaminated by Ciguatoxins (CTXs). These lipid-soluble, polyether neurotoxins are produced by dinoflagellates in the genera *Gambierdiscus* and *Fukuyoa*. While there is no regulatory level in Europe for CTXs, the European Food Safety Authority (EFSA) adopted the United States guidance level of 0.01 µg P-CTX1B eq.kg−1 of fish. This limit is extremely low and requires significant improvement in the detection of CTXs. In this study, we compared analytical protocols based on liquid chromatography coupled to tandem low- or high-resolution mass spectrometry (LC-LRMS or HRMS) to find the best conditions for sensitivity and/or selectivity. Different approaches such as LC conditions, ion choice and acquisition modes, were evaluated to detect the Pacific-ciguatoxins (P-CTXs) on a triple quadrupole (API4000 Qtrap, Sciex) or a quadrupole time of flight (QTOF 6550, Agilent Technologies) spectrometer. Moreover, matrix effects were calculated using matrix-matched calibration solutions of P-CTX1B and P-CTX3C prepared in purified fish extract. Subsequently, the method performance was assessed on naturally contaminated samples of seafood and phytoplankton. With LRMS, the ammoniated adduct ion used as a precursor ion showed an advantage for selectivity through confirmatory transitions, without affecting signal-to-noise ratios, and hence limits of detection (LODs). As also reported by some studies in the literature, methanol-based mobile phase gave better selectivity and sensitivity for the detection of P-CTXs. While the LOD for P-CTX1B and P-CTX3C met the EFSA recommendation level when using LRMS, the findings suggested careful evaluation of instrumental parameters for determination of CTXs. LODs were significantly higher for HRMS, which currently results in the need for a significantly higher sample intake. Nevertheless, HRMS allowed for the identification of artefacts and may allow for improved confirmation of the identity of P-CTXs analogues. Consequently, LRMS and HRMS are considered complementary to ensure adequate quantitation and identification of P-CTXs.

The results of this part of the study are fully described by Sibat et al., 2018.

Confirmation of C-CTX1 in a reference material and fish from the North East Atlantic

Our studies showed that Caribbean ciguatoxin-1 (C-CTX1) is the main toxin causing CP through fish caught in the Northeast Atlantic, e.g., Canary Islands (Spain) and Madeira (Portugal). The use of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) combined with neuroblastoma cell assay (Neuro-2a) allowed the initial confirmation of the presence of C-CTX1 in contaminated fish samples from the abovementioned areas, nevertheless the lack of commercially available reference materials for these particular ciguatoxin (CTX) analogues has been a major limitation to progress research. The EuroCigua project allowed the preparation of C-CTX1 laboratory reference material (LRM) from fish species (*Seriola fasciata*) from the Madeira archipelago (Portugal). This reference material was used to implement a liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) for the detection of C-CTX1, acquisition of full-scan as well as collision-induced mass spectra of this particular analogue. Fragmentation pathways were proposed based on fragments obtained. The optimized LC-HRMS method was then applied to confirm C-CTX1 in fish (*Bodianus scrofa*) caught in the Selvagens Islands (Portugal).

The results of this part of the study are fully described by Estevez et al., 2020a.

Confirmation in microalgae

The *Gambierdiscus* and *Fukuyoa* extracts provided by SA3 (IRTA) consisted on MeOH extracts from the *Gambierdiscus* cells. These extracts were filtered through a 0.22 µm PVDF and analysed by LC-MS/MS using the method developed in this SA4. The CTXs (Tudó et al., 2020) monitored on this analysis, were the ones for which standards were available (P-CTXs and C-CTX1). The results of the LC-MS/MS analysis confirmed that none of the CTXs for which standards were available were initially present on the *Gambierdiscus* and *Fukuyoa* extracts analysed in this SA4.

Characterization of *Gambierdiscus* spp. (HPLC/Neuro-2a/LC-MS/MS)

In order to confirm the toxicity of these samples, the approach developed in this SA4 consisting on combining HPLC fractionation with Neuro-2a as published in (Estevez et al., 2019b) was used for characterization. This approach allowed to focus on toxic fractions to facilitate the further confirmation by LC-MS/MS. On the other hand, the evaluation of the Neuro-2a results also allowed to suspect that the toxicity could be associated to toxic compounds other than the CTX-like toxic ones.

Sample fractionation by HPLC-UV

The *Gambierdiscus* extract showing the higher CTX-like activity, (*G. australes* ref: IRTA-SMN-17-271) from Balearic Islands (Spain) was fractionated by following the conditions described in Estevez et al., 2019b. The extracts were submitted to HPLC-C18 fractionation using the conditions described in Estevez et al., 2019b. An Agilent 1100 G1312A LC system including an Agilent 1260 II automatic fraction collector coupled to an Agilent 1260 II UV detector (Agilent Technologies, Waldbronn, Germany) was used for the HPLC fractionation. The separation was performed on a Kinetex® LC-C18 column (4.6 x 250 mm, 5 µm, 100 Å, Phenomenex) and the mobile phases consisted on water with 5 mM of ammonium formate and 0.1 % of formic acid (A) and methanol (B). The gradient conditions started from 60 % of B to 100% B in 85 min at a flow rate of 1 mL/min being the injection volume of 100 µL. A total of 49 fractions were collected, these fractions were evaporated to dryness under N2 at 50 ºC and reconstituted to a final volume of 1 mL of MeOH.

LC-MS/MS analysis

The fractions which toxicity was confirmed by Neuro-2a were analysed by LC-MS/MS, monitoring water losses $[M+H-nH_2O]^+$ and specific fragments for CTXs, the specific ions characteristic for suspected MTXs as hydrogen-sulphate *m/z* 96.5 $[HOSO_3]^-$ were also monitored and by LC-MS/MS. Further confirmation by LC-HRMS was achieved at IFREMER

Confirmation by LC-HRMS

Over the last decade, knowledge has significantly increased on the taxonomic identity and distribution of dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa*. Additionally, a number of hitherto unknown bioactive metabolites have been described, while the role of these compounds in CP remains to be clarified. Ciguatoxins and Maitotoxins are very toxic compounds produced by these dinoflagellates and

have been described since the 1980s. Ciguatoxins are generally described as the main contributors to this food intoxication. Recent reports of CP in temperate waters of the Canary Islands (Spain) and the Madeira archipelago (Portugal) triggered the need for isolation and cultivation of dinoflagellates from these areas, and their taxonomic and toxicological characterization. Maitotoxins, and specifically maitotoxin-4, has been described as one of the most toxic compounds produced by these dinoflagellates (e.g. *G. excentricus*) in the Canary Islands. Thus, characterization of toxin profiles of *Gambierdiscus* species from adjacent regions appears critical. The combination of liquid chromatography coupled to either low- or high-resolution mass spectrometry allowed for characterization of several strains of *Gambierdiscus* and *Fukuyoa* from the Mediterranean Sea and the Canary Islands. Maitotoxin-3, two analogues tentatively identified as gambieric acid C and D, a putative gambierone analogue and a putative gambieroxide were detected in all *G. australes* strains from Menorca and Mallorca (Balearic Islands, Spain) while only maitotoxin-3 was present in an *F. paulensis* strain of the same region. An unidentified *Gambierdiscus* species (*Gambierdiscus* sp.2) from Crete (Greece) showed a different toxin profile, detecting both maitotoxin-3 and gambierone, while the availability of a *G. excentricus* strain from the Canary Islands (Spain) confirmed the presence of maitotoxin-4 in this species. Overall, this study shows that toxin profiles not only appear to be species-specific but probably also specific to larger geographic regions.

The results of this part of the study are fully described by Estevez et al., 2020b.

2.4. Preparation of reference materials

Reference materials were prepared by selecting fish samples in which maximum concentration of C-CTXs had been determined by the LC-MS/MS method developed in the SA4 (Estevez et al., 2019a). A total of 600 Kg of contaminated fish, also including livers, was provided by SA3 (IUSA). Two different types of reference materials, were prepared consisting on pure solutions of C-CTX1 isolated from several fractions and homogenates of fish tissue (FTRM) prepared using initially the autoclave to facilitate the homogenization process also contributing to ensure the stability, indispensable for their use as reference materials. To ensure the efficiency of the extraction step, a continuous Soxhlet extraction with acetone was used allowing to assume an efficient recovery during the extraction process. For the preparation of the isolated fractions of C-CTX1, portions of 1 Kg of contaminated fish were taken in order to ensure the yield of the isolation and also to maximize the production of this toxin taken into account its low concentration on the fish materials available. After the toxin isolation using GPC and HPLC fractionation, combined with an optimised Neuroblastoma cell assay (Neuro-2a) (Castro et al., 2020), as a very useful tool for the confirmation of the toxicity of the fractions, additional purification steps, including, different solid phase extraction (SPE) mechanisms (Florisil, C8) were included in the protocol. A common initial pre-treatment using autoclave (121 ºC, 45 min) was used in the preparation of both purified C-CTX1 solutions and FTRMs with the aim of removing proteins and to facilitate the homogenization also contributing to the preservation of the stability, avoiding possible degradation. The materials were kept at -20 ºC for further distribution.

Preparation of C-CTX1 solutions

Toxin solutions containing C-CTX1 have been prepared by the isolation of C-CTX1 after final HPLC fractionation. The toxicity of the HPLC fractions was confirmed by Neuro-2a before their characterization by LC-MS/MS.

A continuous Soxhlet extraction with acetone was used in order to maximize the extraction efficiency (recovery estimated 99 %). The low concentration of C-CTX1 in the fish samples selected was compensated by increasing the amount of sample taken for further preparation. With this aim 1 Kg of fish homogenate was taken for Soxhlet extraction and the acetone extract was evaporated to an aqueous residue, under reduced pressure being further partitioned twice with diethyl ether. The organic layer was evaporated, and the residue was dissolved in 90% MeOH and defatted with hexane. The methanol extract was evaporated to a solid residue and further purified. The protocol used is described in (Figure 14).

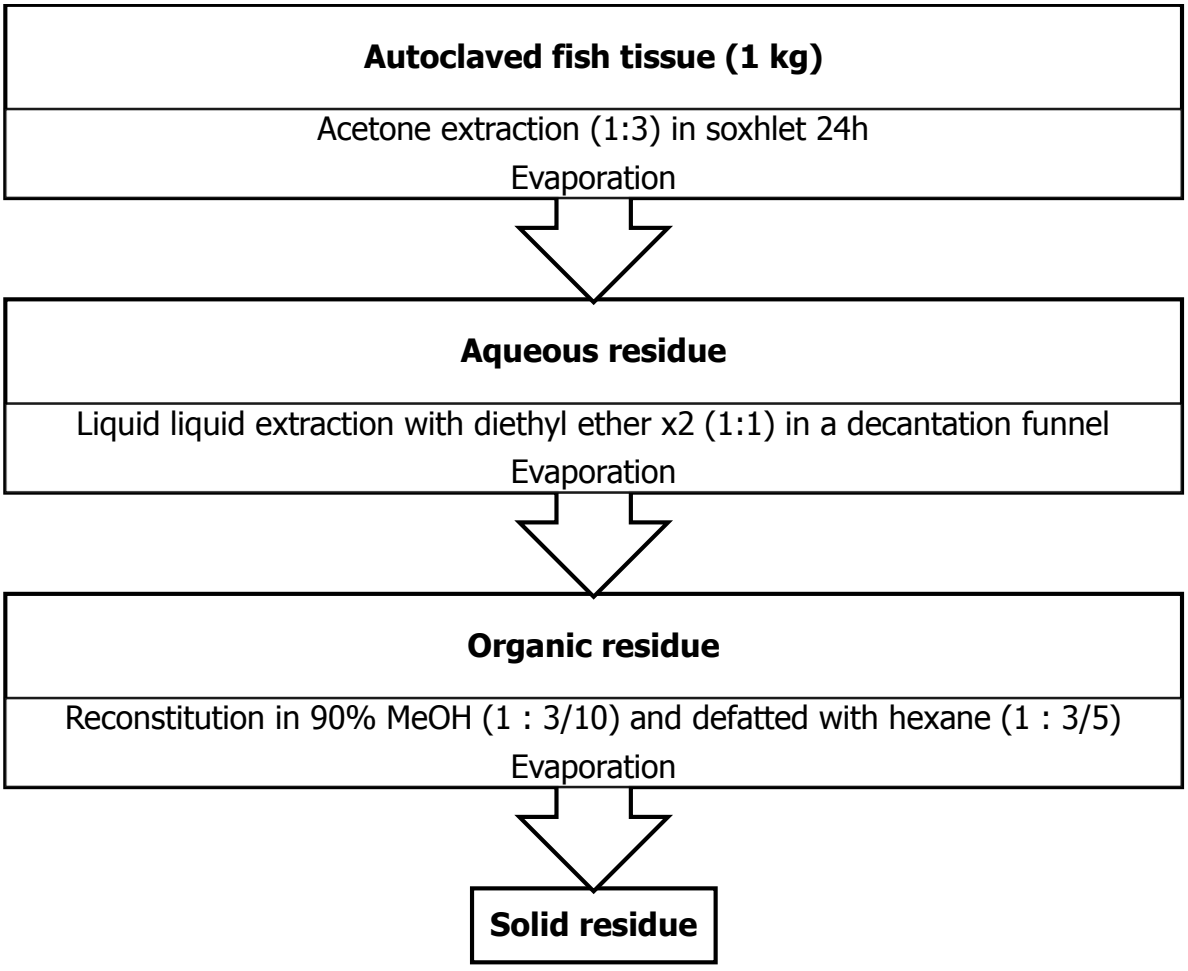


Figure 14. Extraction protocol used for the preparation of the C-CTX1 solutions (in brackets mass/volume ratio)

The purification of the extract was carried out using different Solid Phase Extraction (SPE) mechanisms (Florisil, C8), a further step of purification and fractionation by Gel Permeation Chromatography (GPC) was included followed by an HPLC fractionation to obtain purified fractions of isolated C-CTX1. The protocol used for this purification is described in (Figure 15).

The solid residue obtained after extraction, was dissolved in ethyl acetate and loaded into the Florisil SPE column in which the CTXs were eluted with ethyl acetate/methanol (9:1). The ethyl acetate eluate was evaporated to a solid residue and dissolved in MeOH. The MeOH extract was loaded into a glass

column packed with Sephadex LH-20 (GPC) using MeOH as mobile phase. Fractions from GPC were collected, and the CTX-like toxicity of these fractions was evaluated by Neuro-2a. This toxicity was further evaluated by LC-MS/MS and the presence of C-CTX1 was confirmed. The toxic fractions containing C-CTX1 were combined and evaporated to dryness, being reconstituted in 50% MeOH to be further purified through C8 SPE and eluted with 80 % MeOH. The C-CTX1 eluates were evaporated to dryness and reconstituted in 100 µL of MeOH. An additional fractionation was carried out by HPLC/UV using a C18 column following the optimised protocol developed in this SA4 and published in Estevez et al., 2019b. Isolated fractions of purified C-CTX1 were collected and evaporated to dryness and reconstituted in 1 mL of MeOH and then filtered through 0.22 µm before injection into the LC-MS/MS for final characterization. The full protocol used for purification is described in (Figure 15).

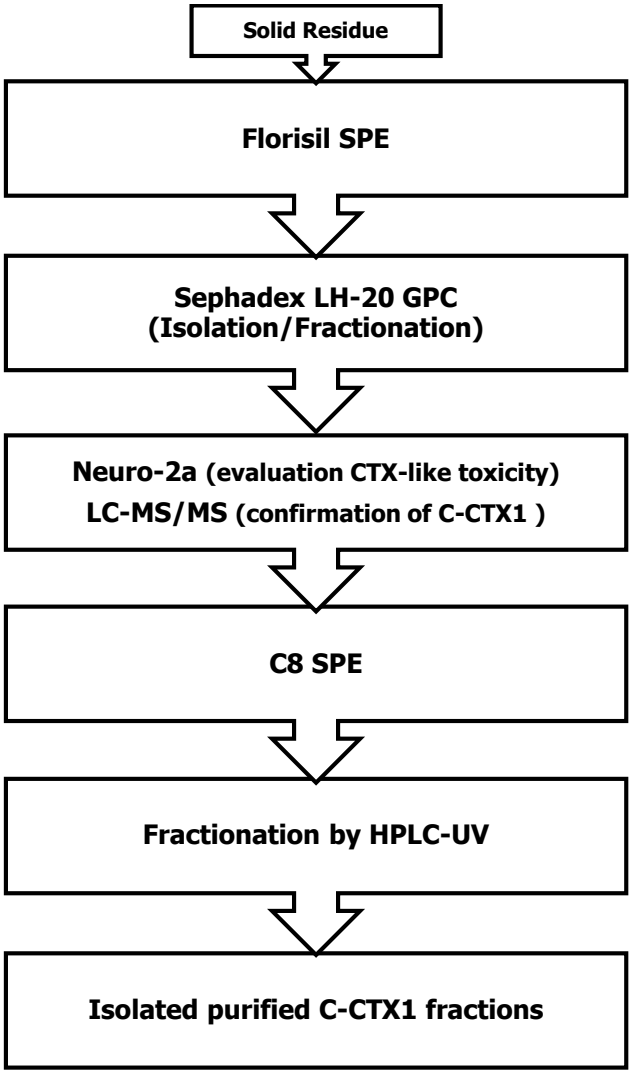


Figure 15. C-CTX1 isolation and purification

Characterization of the FTRM

Fish samples containing C-CTX1 were autoclaved and homogenized being extracted, purified and analysed by LC-MS/MS following the Standard Operating Procedure (SOP) published in Estevez et al., 2019a. The extracts were also evaluated by LC-MS/MS to verify their homogeneity and stability. The quantitative evaluation of C-CTX1 was carried through the calibration curves obtained using the C-CTX1 available kindly provided by Dr Robert Dickey (previously, U.S. Food and Drug Administration) via Dr Ronald Manger (Fred Hutchinson Cancer Research Center, Seattle, USA). This calibration has been compared with the one obtained by using the calibration of the Pacific CTX1B. The homogenates of autoclaved FTRM were kept at – 20 ºC for further distribution.

3. Data/Assessment/Results

3.1. Epidemiology

3.1.1. Collection of outbreak information

Information from the Member States

Data of thirty-four ciguatera outbreaks, occurring from 2012 to 2019 were collected. These outbreaks fulfilled the ciguatera outbreak definition within the EuroCigua Project. The outbreaks were reported by Spain (17), Germany (9), France (6) and Portugal (2). These outbreaks included 209 cases: 83 from Spain, 84 from Germany, 23 from France and 19 from Portugal. Tables 8-11 show the data of the outbreaks from Spain, Germany, France and Portugal.

Table 8. Ciguatera outbreaks reported by Spain from 2012 to 2019, according to the definition for ciguatera outbreak provided in the surveillance protocol document from the EuroCigua project

Date	Country of fishing	N cases	N exposed	Attack rate (%)	Hospitalized cases	Type of fish	Size (Kg)	Fish origin	Place of consumption	CTX detection	Type of outbreak
Jan 2012	Spain	10	12	83	0	*Seriola* sp.	<15	Sport fishing	Restaurant	NA	Autochthonous fish
Apr 2012	Spain	9	9	100	0	*Seriola* sp.	26	Sport fishing	Restaurant	NA	
May 2012	Spain	4	4	100	0	*Seriola* sp.		Local market	Other	NA	
Dec 2012	Spain	12	12	100	0	*Epinephelus* sp.	18	Sport fishing	Household	Yes	
Dec 2013	Spain	15	17	88	0	*Epinephelus* sp.	>29	Local market	Household	Yes	
Feb 2015	Spain	3	3	100	0	*Mycteroperca fusca*	3	Local market	Restaurant	NA	
Mar 2015	Spain	2	2	100	0	*Pomatomus saltatrix*	10	Sport fishing	Household	Yes	
Apr 2015	Spain	3	4	75	0	*Mycteroperca fusca*	3,5		Restaurant	NA	
Nov 2016	Spain	2	5	40	-	*Epinephelus* sp.	7	Local market	Restaurant	Yes	
Dec 2016	Spain	3	3	100	0	*Seriola* sp.	12-20	Street vendor	Household	Yes	
Dec 2016	Portugal*	2	2	100	0	*Pagrus Pagrus*	4	Sport fishing	Ship at sea	Yes	
Apr 2017	Spain	2	2	100	-	*Epinephelus* sp. *Mycteroperca fusca*	29 8	Sport fishing	Household	Yes	
Sep 2018	Spain	4	6	67	1	*Canthidermis sufflamen*	3,2	Sport fishing	Household	NA	
Dec 2019	Spain	6	8	75	-	*Seriola rivoliana*	70	Sport fishing	Household	Yes	
Apr 2015	Dominican Republic	≥2	-	-	-	Unknow	-	-	-	-	Travel related
Aug 2018	Madagascar	2	-	-	-	Unknow	-	-	-	NA	
Sep 2018	Cuba	2	-	-	0	Pargo	-	-	-	NA	

** It was captured in Selvagens Islands, close to the Canary Islands*
**NA: NA: not analysed.*

Table 9. Ciguatera outbreaks reported by Germany from 2012 to 2019, according to the definition for ciguatera outbreak provided in the surveillance protocol document from the EuroCigua project

Date	Country of fishing	Number of cases	Hospitalized cases	Type of fish	Place of consumption	CTX detection	Type of outbreak
Nov 2012/ Nov 2013*	India	24	3	*Lutjanus bohar* *L. argentimaculatus*	Household	Yes	Imported fish
Nov 2014	Indonesia	6	1	*Lutjanus spp.* *(L. bohar* *L. argentimaculatus* *L. erythropterus)* *Pinjalo pinjalo*	Household	Yes	
Nov 2015	India	16	4	*Lutjanus bohar*	Household	Yes	
Jul 2016	India	4	2	*Lutjanus* sp.	Household	Yes	
Mar 2017	Vietnam	15	2	*Lutjanus bohar*	Restaurant	-	
2018	-	2	-	-	-	-	
2012	Caribean	13	0	*Caranx sexfasciatus* *Cephalopholis* sp.	Ship at sea	Yes	Travel related
2016	Cuba	2	-	Red Snapper	Restaurant	-	
2019	Mauritius	2	2	Sparidae	Market	-	

** One case in 2013 related to the outbreak in 2012*

Table 10. Ciguatera outbreaks reported by France from 2012 to 2019, according to the definition for ciguatera outbreak provided in the surveillance protocol document from the EuroCigua project

Date	Country origin of fish	Number of cases	Hospitalized cases	Type of fish	Place of consumption	CTX detection	Type of outbreak
Aug 2015	Guadeloupe	2	-	*Lutjanus* sp.	Household	-	Travel related
Jun 2019	Guadeloupe	3	-	-	Restaurant	-	
Jun 2016	Indian Ocean	7	-	*Lutjanus* sp.	Household	Yes	Imported fish
Jan 2017		2	-	Seabream	Restaurant	-	
Feb 2019	Vietnam	5	-	*Acanthocybium solandri*	Restaurant	Yes	
Oct 2019	India	4	-	Lutjanus sp.	Household	Yes	

Table 11. Ciguatera outbreaks reported by Portugal from 2012 to 2019, according to the definition for ciguatera outbreak provided in the surveillance protocol document from the EuroCigua project

Date	Country of fishing	Number of cases	Hospitalized cases	Type of fish	Size (Kg)	Place of consumption	CTX detection	Type of outbreak
Jun 2012	Portugal	12	12	*Seriola* sp. *Bodianus scrofa*	20	Ship at sea	NA	Autochthonous fish
Sep 2015	Portugal	7	4	*Epinephelus marginatus*	20	Restaurant	NA	

**NA: not analysed.*

Regarding the type of outbreak, they are classified as autochthonous (the fish was captured in EU/EEA waters), imported (the fish was imported into the EU/EEA from outside) or travel related (travellers returning from tropical ciguatera endemic areas). Spain reported 14 autochthonous outbreaks in which the fish had been caught in the Canary Islands (13) and one in the Selvagens Islands (Madeira), and three travel related outbreaks. Germany reported six imported and three travel related outbreaks. France reported four imported and two travel related outbreaks. Portugal reported two autochthonous

outbreaks in which the fish was caught in Madeira. The 16 autochthonous outbreaks included 96 cases. In the ten imported outbreaks, the fish consumed was imported from Asia and the Caribbean (the place was not mentioned in one outbreak) and they accounted for 85 cases. In the eight travel related outbreaks, including 28 cases, travellers went to Cuba, Dominican Republic, Guadeloupe, Madagascar and Martinique. Figure 16 shows a descendent trend in the number of autochthonous outbreaks reported from 2012 to 2019 that was not statistically significant (p=0.13). Figure 17 shows an ascendant trend in the number of imported outbreaks reported from 2012 and 2019 that was not statistically significant (p=0.075).

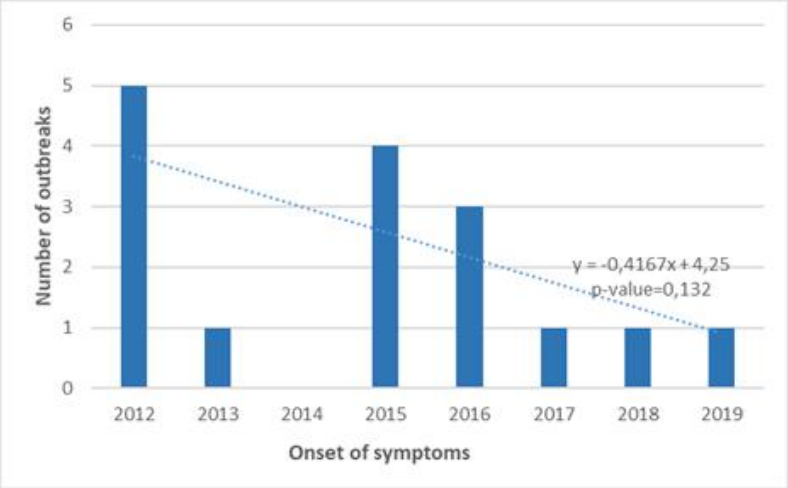


Figure 16. Number of autochthonous outbreaks per year

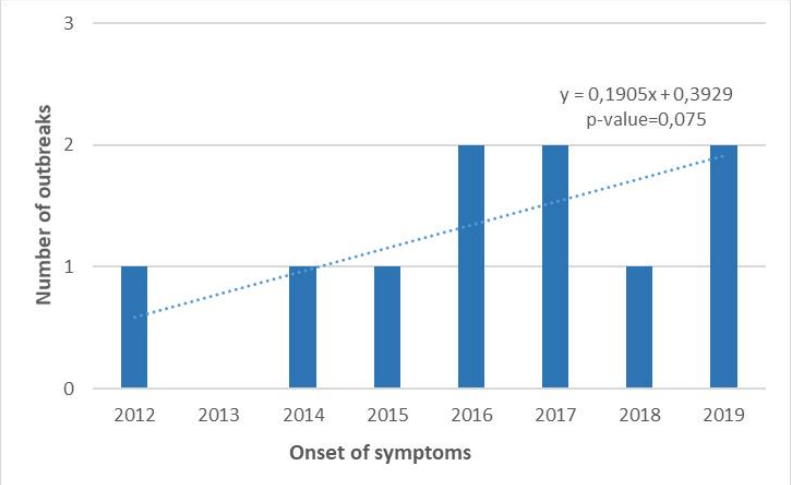


Figure 17. Number of imported outbreaks per year

The median size for all the outbreaks was four cases, with a range from two to 24 cases. It was higher in Germany (median 6.0, range 2-24) and Portugal (median 9.5, range 7-12) than in France (median 3.5, range 2-7) and Spain (median 3.0, range 2-15). Comparing the size of the outbreaks according to

the type it was higher in imported outbreaks (median 5.5, range 2-24) than in autochthonous outbreaks (median 4, range 2-15) or travel related outbreaks (median 2, range 2-13). Differences in the outbreak size could be due to specificities of each outbreak and to differences in ciguatera surveillance, being bigger outbreaks detected more easily; it does not appear to have relation with the consumption of the fish on a restaurant or on the household, or with the type of fish, or the size of the fish consumed. The number of people that consumed the fish (exposed) was only available for the autochthonous outbreaks from Spain, no relation between the number of people that consumed the fish and the size of the fish could be observed. No differences in attack rate (cases/exposed*100) were observed according to the type of fish or the year of outbreak occurrence. Figures 18 and 19 show a descendent trend in the size (median number of cases) of the outbreaks per year from 2012 to 2019 that was not statistically significant.

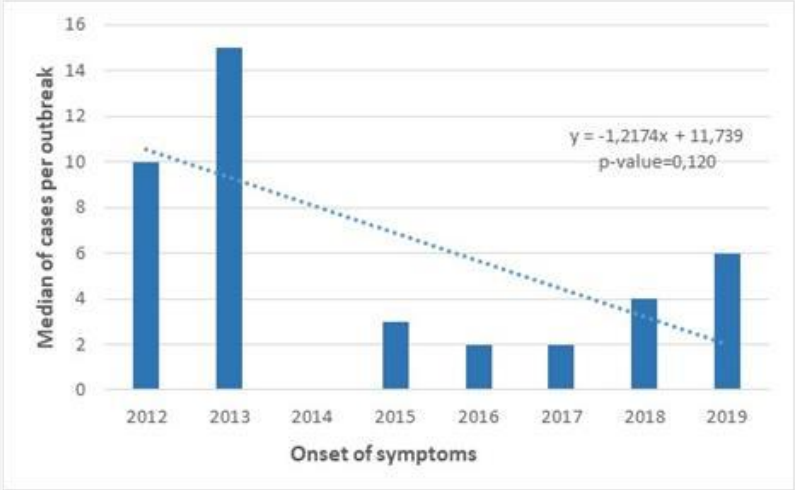


Figure 18. Size (median number of cases) of autochthonous outbreaks per year

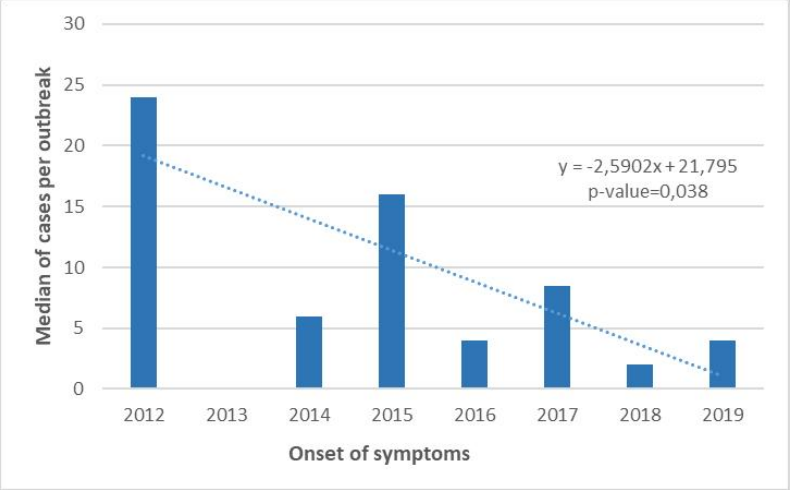


Figure 19. Size (median number of cases) of imported outbreaks

As for seasonality, half of the autochthonous outbreaks occurred in December and April: 5/16 (31%) in December and 3/16 (19%) in April. For imported outbreaks, three occurred in November and the other five occurred in five different months.

CTXs were detected in 16 outbreaks, in 10 outbreaks the analysis was not performed, and the information was not available for the other outbreaks; for some outbreaks, there were not fish leftovers for testing. They were detected in eight autochthonous outbreaks from Spain and in seven imported outbreaks (four from Germany and three from France); therefore, according to the EuroCigua definition, cases from these 15 outbreaks were confirmed cases. Three autochthonous outbreaks from Spain due to *Mycteroperca fusca* and *Canthidermis sufflamen*, one imported outbreak from Germany due to *Lutjanus bohar*, and the two autochthonous outbreaks reported by Portugal due to consumption of *Bodianus scrofa* and *Epinephelus marginatus* were outbreaks with probable cases (based on the consumption of a fish previously associated with ciguatera). Cases of the other 13 outbreaks cases were classified as possible cases as the type of fish consumed was unknown, the common name mentioned corresponds to more than one species or only the genus was mentioned.

With respect to hospitalization, Spain reported only one case hospitalized out of 69, with available information. In Germany 12 out of 80 cases, with available information, were hospitalized (hospitalization rate 15%). Portugal reported 16 out of 19 cases hospitalized (84%) including one outbreak where all 12 cases were hospitalized being all cases hospitalized in one of the outbreaks that included 12 cases. Information on hospitalization from France was not available.

In relation to the type of fish involved in the autochthonous outbreaks reported from Spain, the most frequent genus reported were *Seriola* and *Epinephelus* with 32% and 37%, followed by *Mycteroperca fusca* that accounted for 10%. All the outbreaks reported by Germany, with available information, were due to consumption of *Lutjanus* spp. In France, the most frequent reported genus was *Lutjanus* (three out of six outbreaks). Portugal reported three different fish genera for the two outbreaks: *Seriola* and *Lachnolaimus* for one outbreak and *Epinephelus* for the other. In summary the most frequent fish genus involved in the autochthonous outbreaks were *Seriola* and *Epinephelus* while *Lutjanus* was the most frequent fish genus reported in imported outbreaks. Travel reported outbreaks do not provide enough information on fish type.

The size of the fish consumed in the outbreaks was known for autochthonous outbreaks, the median size of the fish was 15 kg, with a range from three to 70 kg. No trend in the size of the fish per year was observed.

In eight out of 14 autochthonous outbreaks (57%) reported by Spain and in one out of two outbreaks reported by Portugal the fish consumed was captured by sport fishing.

Information on symptoms was not available for one outbreak in France and one in Germany. Neurological symptoms appear in all the outbreaks with symptoms information available; cold allodynia (temperature dysesthesia) appears in all the outbreaks, except in one from Spain and two from France. Gastrointestinal symptoms appear in all the outbreaks except in three outbreaks from Spain. Cardiovascular symptoms were only mentioned in three outbreaks from Spain, two outbreaks from Germany, the two outbreaks from Portugal and two outbreaks from France. As most of the information

was collected retrospectively, the lack of symptoms may be due to some extent to the fact that information for some symptoms (for instance cardiovascular symptoms) was not specifically recorded, rather than to the absence of symptoms.

Information related to the incubation period was available for some outbreaks from Spain and Germany. The incubation period ranges from half an hour to 52 hours. There is only one case with an incubation period above 48 hours (case definition established the incubation period up to 48 hours); this case had paraesthesia and cold allodynia. It was due to the consumption of *Seriola* sp. in the same restaurant as eight other cases. The fish was not tested for CTXs.

Information on age and gender was available for outbreaks reported by Spain, Germany and Portugal. The male/female ratio was 1.83 for the ciguatera cases from the Spanish outbreaks and 0.8 for the ciguatera cases from the German outbreaks. This difference could be due to some extent to the fact that in the Canary Islands some of the outbreaks were related to fish captured by sport fishing and consumed by the fisher persons; men may more frequently practice sport fishing. In the same sense, cases from the two outbreaks reported by Portugal were all male and in one of these outbreaks, the fish was captured by sport fishing. Most of the cases both in Spain and Germany were adults and all the cases from the Portugal outbreaks were 18 years old or older; this may reflect the different food consumer habits of the different age groups.

Moreover, three out of the four cases belonging to the two travel related outbreaks from Spain were male; the age of these four cases was between 36 and 46 years old.

European Union Food-borne Outbreaks reporting System (EU-FORS)

From 2012 to 2018, three countries (France, Germany and Spain) reported 75 ciguatera outbreaks due to consumption of fish to the EU-FORS. Most of the outbreaks were reported by France (63 outbreaks), followed by Spain (nine outbreaks) and Germany (three outbreaks). All the outbreaks reported by Germany and Spain to the EFSA database were reported as well to the EuroCigua project. There were 59 ciguatera outbreaks reported by France to the EU-FORS that were not reported to the EuroCigua project because the outbreaks occurred in EU tropical endemic areas that are outside the scope of the project. Moreover, there are tree outbreaks from Germany (2014, 2016 and 2018), the two outbreaks from Portugal (2012 and 2015) and one from Spain (2012) that were not reported to the EU-FORS, maybe because the national reporting authority did not have the information at the time of the reporting to EFSA.

Rapid Alert System for Food and Feed Network (RASFF)

MS issued four alert notifications through the RASFF network. They correspond to the two outbreaks from 2012 and 2017 reported by Germany to EuroCigua and the two outbreaks from 2016 and 2019 reported to the project from France.

- The fish involved in the 2017 alert was distributed to Austria, Check Republic, Denmark, Germany, the Netherlands and Poland.
- The fish involved in the 2016 alert was distributed to Belgium, France and Germany.
- The fish involved in the 2012 alert was distributed to Check Republic, Germany, Spain and United Kingdom.

Moreover, France has reported five "information for attention" notifications to this network due to detection of ciguatoxins (distribution to other member States was not mentioned):

- One from 2015 in *Caranx* spp. and others, from Sri Lanka.
- Three in 2016: in *Sphyraena jello* from India, *Sphyraena barracuda* from Senegal and *Caranx* spp. from India.
- One in 2017 in *Lutjanus* spp. from India.

Although the fish involved in the alerts was distributed to different countries, only France and Germany reported related cases. This could reflect under diagnosis and under reporting if the fish distributed to the other countries was contaminated, although it may also be that the distribution of CTXs in the fish is heterogeneous.

In addition, the Netherlands issued an alert in 2020 (outside the timeframe of the project). The involved fish was frozen *Lutjanus bohar* from India. Apart from the Netherlands, it was distributed to other countries: Austria, Belgium Finland, France, Germany, Italy, Luxembourg, Sweden, Switzerland, and United Kingdom.

Germany communicated the outbreak from 2012 also to the RAS-CHEM network. No other country communicated any outbreak or case to this network.

No ciguatera events have been reported to the Early Warning and Response System (EWRS) or to the Epidemic Intelligence Information System (EPIS) platform.

3.1.2. Collection of single cases information

Spain, Austria, Portugal, France, Germany, and Switzerland reported 34 ciguatera single cases. No information was available to classify those cases as sporadic or outbreak related or to classify them according to the consumption of autochthonous or imported fish. Cases reported by the travel medicine units were travel related cases and this information was not available for the other cases. These single cases had been recorded as ciguatera. Cases but there was not enough information to know if they fulfilled the EuroCigua case definition, cases with information on symptoms were excluded if neurological symptoms were not mentioned.

The EuroCigua partner from Germany reported one death due to ciguatera. This information was extracted from the governmental vital statistics. Moreover, seven cases (without information whether they were single or outbreak related cases) were retrieved from the German hospitalisation statistics from 2012 to 2018.

The Spanish Poison Centre (Instituto de Toxicología y Ciencias Forenses) reported six ciguatera cases from 2012 to 2019. All the cases had neurological symptoms (one of them mentioned cold allodynia); in addition, three of the cases had also gastrointestinal symptoms. One case was hospitalized.

Two travel medicine units from Spanish hospitals (La Paz-Carlos III and Vall d'Hebron) reported nine ciguatera cases from 2012 to 2019. These travel related cases consumed the fish in Mozambique, Peru, Cape Verde, Cuba, Dominican Republic and Mexico. The type of fish was mentioned only in four cases (corvina, tuna and mere). Cases had gastrointestinal and neurological symptoms, including paraesthesia, moreover four of them had cold allodynia; two cases had hypotension and in one case, painful urination was mentioned.

Spanish discharge hospital records from 2012 to 2018 and Spanish vital statistics from 2012 to 2018 did not yield any ciguatera case.

Switzerland, through the Poison Centre, reported one case in 2014. Epidemiologist, food safety authorities and Reference Laboratory for Marine Biotoxins in Switzerland reported no ciguatera cases.

The Poison Control Centre from Austria reported five possible ciguatera cases in 2015 and 2017. Three of these cases had gastrointestinal symptoms and all had neurological symptoms, including paraesthesia; two of them were hospitalized. The fourth case had arthralgia, headache and breathing difficulties and the fifth case had myalgia and arthralgia. One case mentioned that the fish was consumed in a restaurant in United Kingdom.

Portugal reported two ciguatera cases; the information was obtained from the Portuguese Poison Centre. Both cases were from Madeira. One of the cases was in 2015; it might be link to the 2015 outbreak. The other case occurred in 2016, it was related to the consumption of hogfish.

France reported one ciguatera case in 2019; the information was reported by the Ministry of Agriculture and Food, and the French Agency for Food, Environment, Occupational Health and Safety.

3.1.3. Incidence rates

To calculate the incidence rates, all cases whether outbreak related, or single cases were counted. Eurostat provided the EU/EEA population (excluding inhabitants from EU tropical endemic areas) used for calculations. The incidence rate in the EU/EEA countries was 0.008 (Confidence Interval CI: 0.0000-0.0236) cases per 100,000 inhabitants per year. The incidence rates in the EU/EEA countries, excluding travel related cases, was 0.0054 (CI: 0.0000-0.0236) cases per 100,000 inhabitants per year. According to the literature, it is estimated that only between 10 and 20% of cases are reported.

Moreover, the incidence rate in the Canary Islands was calculated, as ciguatera cases are under surveillance there. The incidence rate in the Canary Islands, excluding travel related cases, was 0.47 (CI: 0.44-0.50).

3.1.4. Additional information

France

The Marseille Poison Centre (MPC) reported aggregated information that could not be added to the individual information from ciguatera outbreaks or single cases. The MPC reported 78 ciguatera cases (73 travel related and five dues to imported fish), some of them related to 14 outbreaks (13 travel related and one due to imported fish) from 2012 to 2019. Cases residing in tropical endemic areas of the EU were excluded. The fish families associated were: Lutjanidae (12), Serranidae (7), Sphyraenidae (3), Scaridae (2), Lethrinidae (1), Scombridae (1), Carangidae (1).

Moreover, France reported 88 outbreaks outside the scope of the project as they occurred in French tropical endemic territories and they were not travel related outbreaks. Those outbreaks occurred in Martinique (46 outbreaks), Guadeloupe (41 outbreaks) and one outbreak in La Reunion Island. They were small outbreaks, the median number of cases per outbreak was 2 (range 2-18). The main fish types involved in the outbreaks were reported as "Carangue" and Lutjanus, these fish types accounted for 42% and 28% of the outbreaks respectively. The place of fishing was only mentioned in 35 outbreaks, being Martinique in 12 of them, Guadeloupe in 12, India in 6, Guyana in 3, Vietnam in 1 and Guadeloupe/Guyana in 1 outbreak. All the outbreaks, except five were family outbreaks. In addition, France reported 6 single cases in these areas: 3 in Martinique and 3 in Guadeloupe.

The Netherlands

According to the scope of the EuroCigua project, only cases or outbreaks that occurred from 2012 to 2019 were collected. However, it is relevant to mentioned that Public health authorities from the Netherlands were contacted for information on the ciguatera alert issued in RASFF in 2020. They reported five cases associated to the consumption of *Lutjanus bohar* imported from India. The fish was consumed at home.

3.2. Environmental assessment and definition of areas at risk

Environmental data were obtained during the sampling of microalgae. An example is presented in table 12.

Table 12. Environmental data obtained during samplings of microalgae. Environmental data for the Balearic Islands in 2017 (Menorca).

SAMPLING STATION	11	12	13	14	15	16
PLACE	*Macarella*	*Sant Adeodat*	*Cala en Porter*	*Cala Torret, Binibeca*	*Es Grau*	*Na Macaret*
DATE	03/09/2017	03/09/2017	04/09/2017	04/09/2017	04/09/2017	04/09/2017
HOUR	17:50	19:30	10:10	11:25	12:20	13:30
TEMPERATURE (Cº)	26,44	26,29	25,45	25,74	25,53	25,86
SALINITY	36,22	37,84	36,58	37,99	37,89	37,89
DISSOLVED OXYGEN %	93,7	91,9	87,7	101,7	88,6	101,8
DISSOLVED OXIGEN mg/L	6,18	5,99	5,84	6,68	5,84	6,68
PH	7,69	8,07	7,93	8,09	8,08	8,13
GPS Lat	39º 56' 25.20"	39º 55' 3.13"	39º 52' 14.19"	39º 48' 57.0"	39º 56' 58.32"	40º 0' 58.54"
GPS Long	3º 56' 4.72"	4º 1' 51.18"	4º 7' 57.83"	4º 14' 22.61"	4º 16' 3.74"	4º 12' 2.58"
DEPTH (m)	0,5	0,5	0,5	0,5	0,5	0,5

SAMPLING STATION	17	18	19
PLACE	*Binimel·là*	*Cala Morell*	*Sa Caleta*
DATE	04/09/2017	04/09/2017	04/09/2017
HOUR	14:30	17:35	19:25
TEMPERATURE (Cº)	26,29	26,46	26,41
SALINITY	37,27	37,1	37,19
DISSOLVED OXYGEN %	106,3	124,7	94,2
DISSOLVED OXIGEN mg/L	2,95	8,14	9,16
PH	8,19	8,23	7,74
GPS Lat	40º 3' 9.68"	40º 3' 13.04"	39º 58' 54.18"
GPS Long	4º 3' 16.46"	3º 52' 59.34"	3º 50' 3.47"
DEPTH (m)	0,5	0,5	0,5

Microalgal cultures allowed to evaluate the presence of toxic strains of dinoflagellates. Evaluation of the toxicity of strains was performed from small (50mL) cultures of strains, but also large-scale cultures reaching up to 4L cultures. Table 10 reports the list of microalgal species harvested in large-scale vessels in order to evaluate toxicity.

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 (SA4).

Table 13. 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
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

Toxic strains of microalgae were identified in all regions. Table 14 summarizes the results obtained regarding the establishment of cultures and toxicity of the different strains. Further details on species identification and toxicity of microalgae are presented in the sections below according to the different regions.

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. In the Canary Islands, 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.

In Madeira and Selvagens Islands, 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.).

Table 14. Microalgal culture strains obtained in the project and number of toxic strains.

	Sampling areas	**Tentative Plan**	**Isolated**	**Established**	**CTX-like toxicity evaluation**
Canary Islands	7	40	332	93	• CTX-like positive: 39 (range: 11,3 - 73557 fg/cell eq.) • CTX-like positive (NQ): 4 • CTX-like negative (negative): 4
Madeira and Selvagens Islands	3	30	86	86	• CTX-like positives: 1 (83 fg/cell eq.)
Cyprus/ Greece (Crete+Samos +Rhodes)	2 / 4	15 / 15	1029/ 420	85/ 110	• CTX-like positive (+): 2 (range: 4.34 -17,6fg/cell eq.) • no quantifiable (NQ): 16 • CTX-like negative: 8
Balearic Islands	2	15	197	113	• CTX-like positive: 26 (range: 1.38 – 381,83 fg/cell eq.), • CTX-like positive (NQ): 4 • CTX-like negative (negative): 0
TOTAL	16	115	2064	487	

In the Mediterranean, 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 established.

From Cyprus, 15 *Gambierdiscus* cultures were established. Out of these, nine were analysed by Neuro-2a: two were CTX-like positive, four were negative and three were no quantifiable. In these non-quantifiable strains possible MTX effect or other toxicological patterns to be studied were identified.

From the Balearic Islands, *Gambierdiscus* spp. and *Fukuyoa* spp. were reported and 99 strains were established. Among these, 30 strains were studied for toxicity. Twenty-four *Gambierdiscus* strains have been analysed 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.

The toxicity of fish in the different areas of Macaronesia (Canary Islands, Madeira and Selvagens) and in the Mediterranean (Crete, Cyprus and the Balearic Islands) was also addressed taking into consideration geographical origin and the weight of fish addressing the relevance for human risk assessment. The relation between toxicity and weight was considered in fish from Macaronesia, since only one toxic fish was reported in the Mediterranean. We also present below the results obtained for the different regions.

Table 15 summarizes the results obtained regarding the toxicity of fish. Further details on toxicity of fish are presented in the sections below according to the different regions.

Table 15. Sampling effort for fish and toxicity.

	Sampling areas (*) (n)	**Tentative Plan Fish (n)**	**Collected fish (n)**	**Weight (kg)**	***CTX-like toxicity***
Canary Islands	7	525	746 fish		• CTX-like positive: 105, • CTX-like negative (negative): 632 • CTX-like dubious: 9
Madeira and Selvagens Islands	3	100	128 fish (131 extracts)	0.2 – 300 kg	• CTX-like positive: 44 • CTX-like negative (negative): 87 • CTX-like dubious: 0
Cyprus	2	70	75 (145 extracts)	0.3 – 30 kg	• CTX-like positive: 1 • CTX-like negative (negative): 144 • CTX-like dubious: 0
Crete	2	70	70 (140 extracts)	0.6 - 12,2 kg	• CTX-like positive: 0 • CTX-like negative (negative): 140 • CTX-like dubious: 0
Balearic Islands	2	40	36 (70 extracts)	0.7 – 3 kg	• CTX-like positive: 0 • CTX-like negative (negative): 70 • CTX-like dubious: 0
TOTAL	16	805	1174		

Regarding fish from the Canary Islands, 746 samples of fish analysed by the Neuro-2a CBA by the IUSA-ULPGC, from different places of the Canary Islands (from the seven islands). Out of the 746 samples analysed, 105 resulted positive for CTX-like toxicity, 632 were negative and nine samples were considered inconclusive.

From the selected fish from the Canary Islands sent to IRTA, 149 fish (157 extracts) were analysed by Neuro-2a at IRTA, 92 were CTX-like positive and 65 were CTX-like negative.

From data of IUSA-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. 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.

Regarding fish in Madeira and Selvagens Islands, 128 fish have been sampled during the EuroCigua project. Fourty-four CTX-like positive fish were detected out of 129 extracts analysed by Neuro-2a CBA. 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.

As for the Mediterranean fish, no CTX-like positive fish was detected from Crete in a total of 140 analysis for a total of 70 fish (140 extracts). From Cyprus, 82 fish have been sampled. Only one CTX-like positive (*S. dumerili*) was detected out 148 extracts analysed 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 SA4 with no detection of CTXs. However, an immunoassay (Leonardo et al., 2020) indicated the presence of CTX-like compounds in this sample. Regarding the evaluation for the toxicity in fish, a total of 70 analyses for a total of 36 fish were performed showing no toxicity in fish from the Balearic Islands.

3.2.1. Macaronesia

***Gambierdiscus* populations: environmental indicators, species, Neuro-2a toxicity and geographical and seasonal analysis. Culture collection.**

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.

- ✓ In the Canary Islands, 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 simples 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.

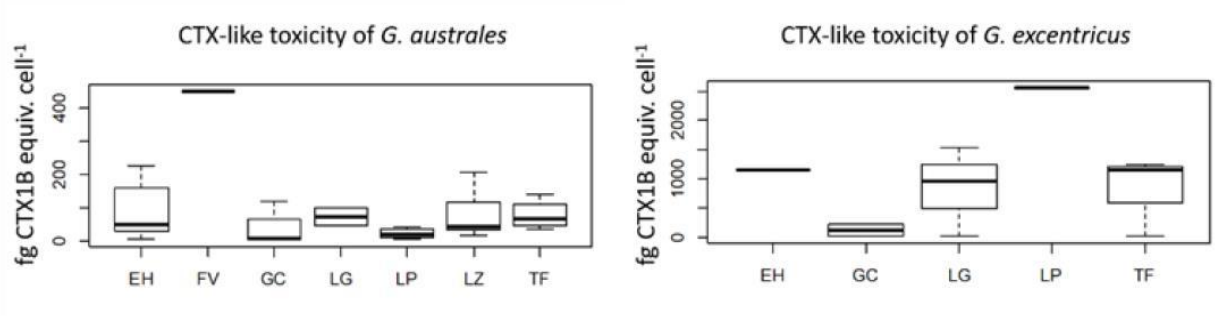


Figure 19. Distribution of CTX-like toxicity of *G. australes* and *G. excentricus* according to island and origin. EH (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote) and TF (Tenerife).

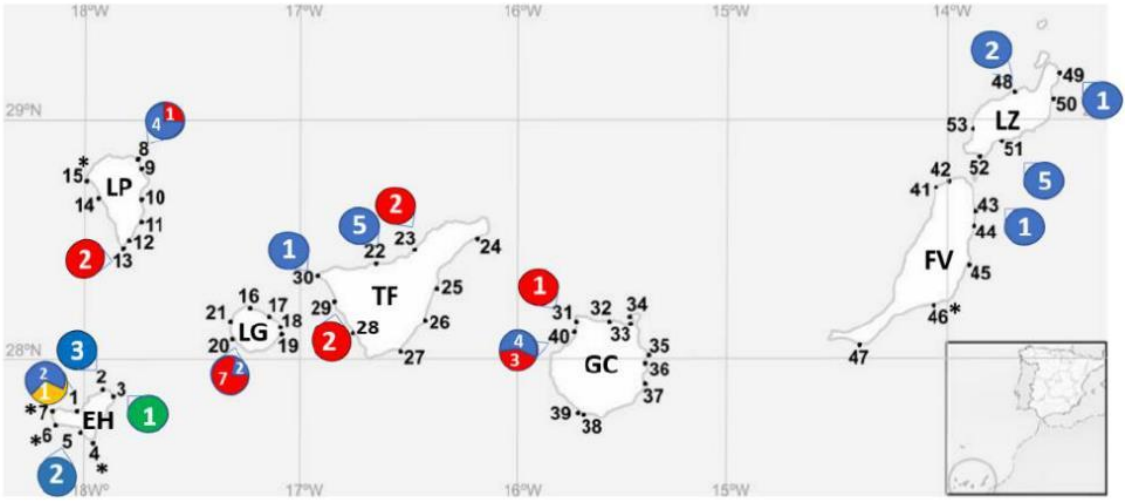


Figure 20. Distribution of each species in the stations sampled in the Canary Islands during 2016-2017. Station numbers are presented in bold. The presence of *Gambierdiscus* species determined by molecular analysis is presented with a circle and includes the number of strains identified for each species. The asterisk represents the presence 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).

From Madeira and Selvagens Islands, 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.).

- ✓ 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).

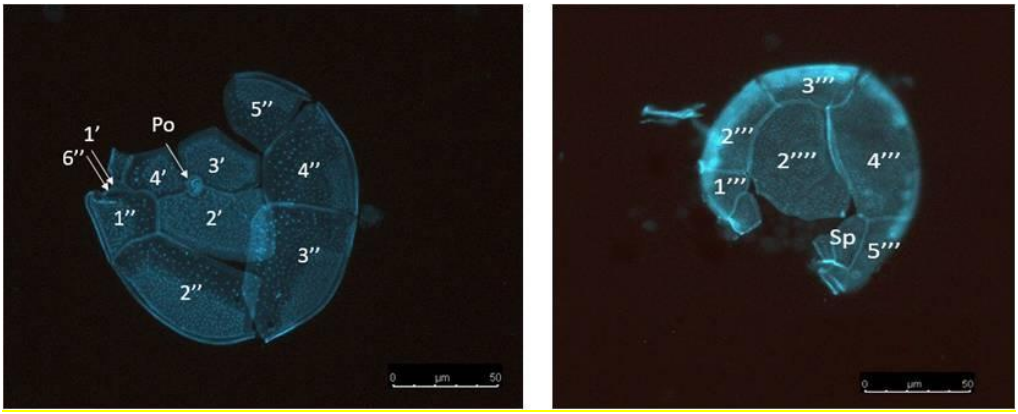


Figure 21. Apical and Antapical view of the epitheca and hypotheca of *Gambierdiscus excentricus* by scanning electronic microscopy in strain isolated from Madeira Island.

Fish: species, Neuro-2a toxicity, geographical and seasonal analysis

Canary Islands:

Regarding fish samples of the EuroCigua project from the Canary Islands, from data of IUSA-ULPGC 746 samples of fish analysed by the Neuro-2a CBA by the IUSA-ULPGC, from different places of the Canary Islands (from the seven islands). Out of the 746 samples analysed, 105 resulted positive for CTX-like toxicity (14%), 632 were negative and 9 samples were considered inconclusive. A total of sixty different species of fish were evaluated and 17 of them showed CTX-like toxicity. Among these positive fish species, amberjack, dusky grouper, some moray eels and common two-banded seabream are particularly important.

From the selected fish from the Canary Islands sent to IRTA, 149 fish (157 extracts) were analysed by Neuro-2a at IRTA, 92 were CTX-like positive (59%) and 65 were CTX-like negative. Toxicities range from 0.0018 to 0.5760 µg eq./kg of CTX-1B.

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. However, 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.

This work confirms the Canary Islands as an area of expansion of CP endemicity and highlights the need to monitor CTX accumulation in fish and the presence of *Gambierdiscus* in the Canary Islands marine waters. Moreover, a high percentage of fish (34%) from several species captured in El Hierro have showed presence of CTX-like toxicity (Figure 22). This fact, considered together with previous results obtained, suggest this island as a ciguatoxin hot spot in the Canary Archipelago.

Table 16. Toxicity of the fish and extracts processed from the Canary Islands evaluated by the Neuro-2a CBA according to Island.

Island	Total fish	Inconclusive	Negative	Positive	Positive fish per island (%)	Total of positive fish (%)
Lanzarote	141	-	124	17	12%	16%
Fuerteventura	59	-	55	4	7%	4%
Gran Canaria	142	1	134	7	5%	7%
Tenerife	150	3	127	20	13%	19%
La Gomera	53	1	51	1	2%	1%
La Palma	73	2	59	12	16%	11%
El Hierro	128	2	82	44	34%	42%
All	**746**	**9**	**632**	**105**	**14%**	**100%**

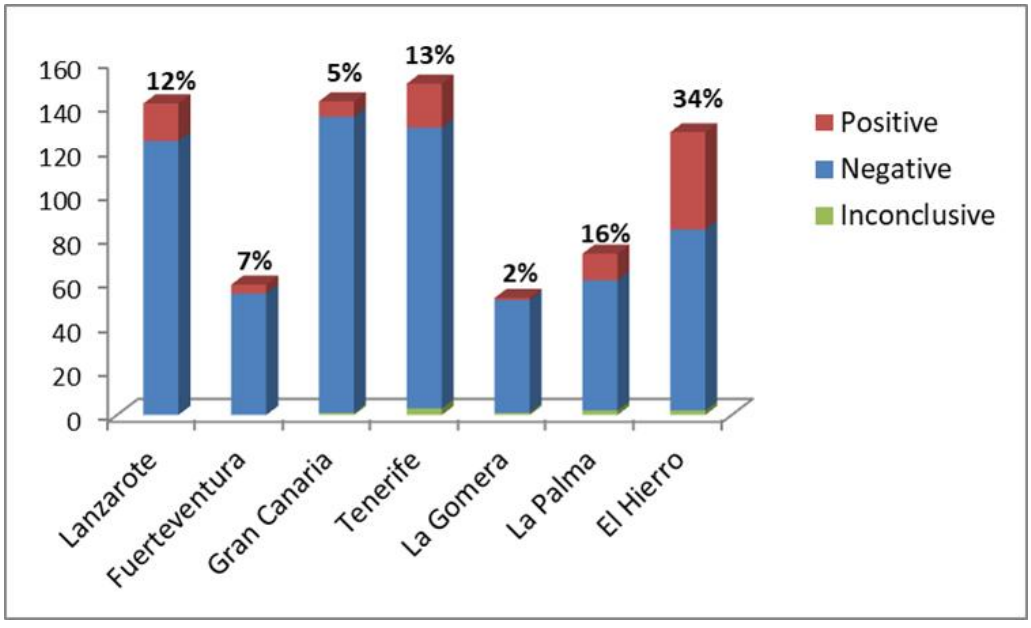


Figure 22. Percentage of positive fish according to Island from the Canary Islands evaluated by the Neuro-2a CBA.

Figure 23. 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%.

Madeira and Selvagens Islands:

Regarding fish sampling from this area, 128 fish have been sampled during the EuroCigua project. Fourty-four CTX-like positive fish were detected out of 129 extracts analysed by CBA. Toxicities of these fish range from 0.0039 to 0.1253 µg/kg of CTX-1B.

CTX-like toxicity was observed in several fish species throughout the marine food web. 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.

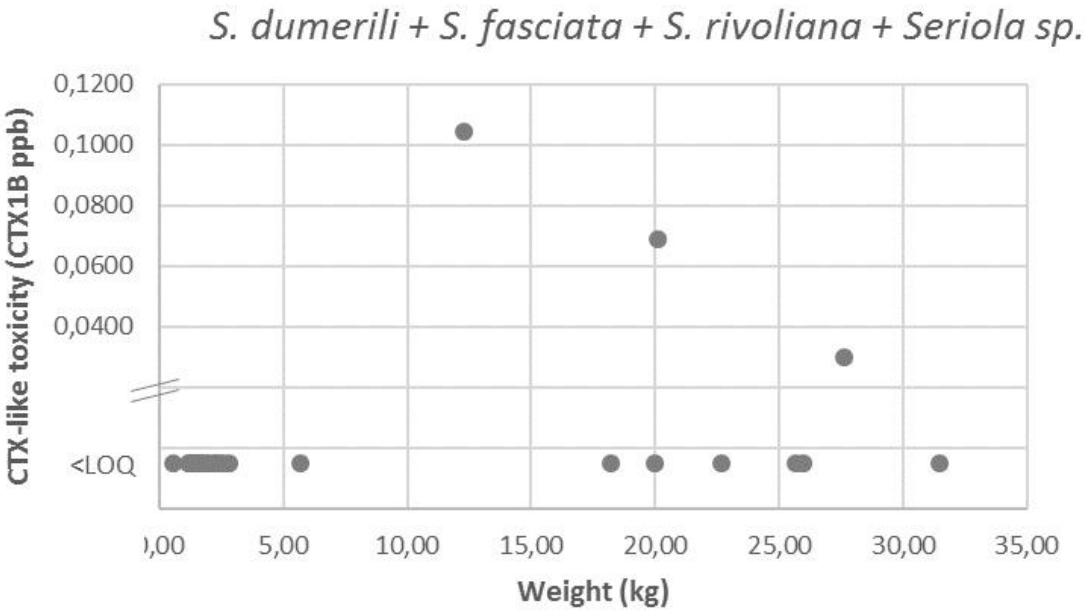


Figure 24. An example of weight versus CTX-like toxicity in *S. durmerili, S. fasciata, S. rivoliana and Seriola* sp. (Negative fish are reported as <LOQ) from Madeira and Selvagens Islands.

Taking into consideration the different toxicities evaluated in fish from Madeira island and from the Selvagens Islands the range of toxicity of fish from Madeira was 0.027-0.069 µg CTX1B eq/kg while in Selvagens it was from 0.0039-0.7520 µg CTX1B eq/kg. Hence Selvagens Islands constitutes the main hotspot for Ciguatera in Portugal.

3.2.2. Mediterranean

***Gambierdiscus* populations: environmental indicators, species and Neuro-2a toxicity geographical and seasonal analysis. Culture collection.**

From the samples collected from Crete, Samos and Rhodes, 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, six are negative and 11 are not quantifiable. An additional sampling was performed in Samos and Rhodes in August-September 2018, 21 *Gambierdiscus* sp. and one *Fukuyoa* sp. cultures were established.

A great diversity of *Gambierdiscus* and *Fukuyoa* taxa was detected in the eastern Mediterranean Sea, where at least six different taxa were detected: *Gambierdiscus silvae*, *Gambierdiscus australes, Gambierdiscus carolinianus, Gambierdiscus* cf. *belizeanus, Gambierdiscus* sp. (sp.nov) and *Fukuyoa paulensis.* The high number of species in this area highlights the potential risk for Ciguatera, despite the relatively low cell toxicity found in isolates examined during this EuroCigua project.

From Cyprus, 15 *Gambierdiscus* cultures were established. Out of these, nine were analysed by Neuro-2a: two were CTX-like positive, four were negative and three were no quantifiable. In these non-quantifiable strains possible MTX effect or other toxicological patterns to be studied were identified.

And, from the Balearic Islands, *Gambierdiscus* spp. and *Fukuyoa* spp. were reported and 99 strains were established. Among these, 30 strains were studied for toxicity.

Gambierdiscus was identified for the first time in the Balearic Island in 2017 confirming the presence of *Gambierdiscus* in the western Mediterranean (Tudó et al., 2018). *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 (Tudó et al., 2020). *Gambierdiscus australes* is, up today, the only species of *Gambierdiscus* reported in the Balearic Islands.

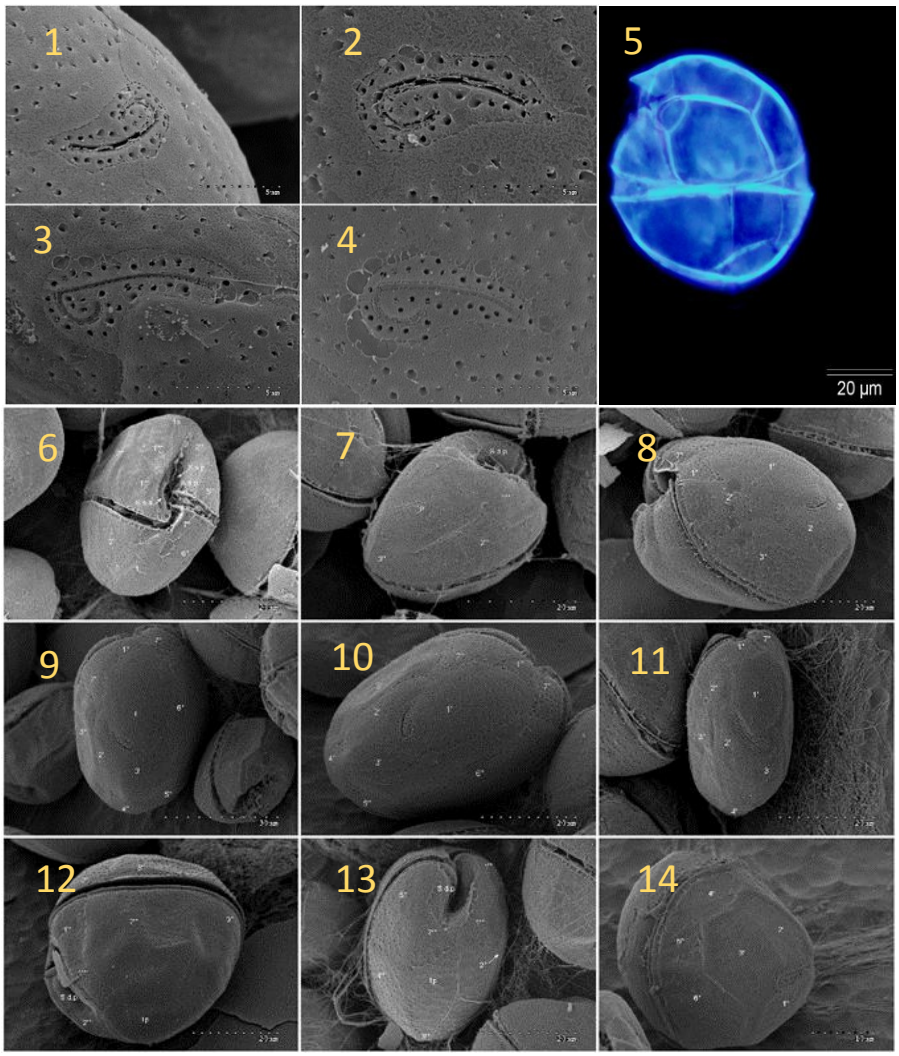


Figure 25. *Fukuyoa paulensis* from Menorca, strain IRTA-SMM-17-211. Po plates (1-4) and (6-14) SEM images of thecae and (5) image of thecae by calcofluor stained.

Cytotoxicity assays showed CTX-like toxicity in *Gambierdiscus* spp. strains from the Balearic Islands. Twenty-four *Gambierdiscus* strains have been analysed 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, two strains were CTX-like positive and the range of CTX-like content was between 7.96-16.3 fg eq./cell, three were not quantifiable and one was CTX-like negative.

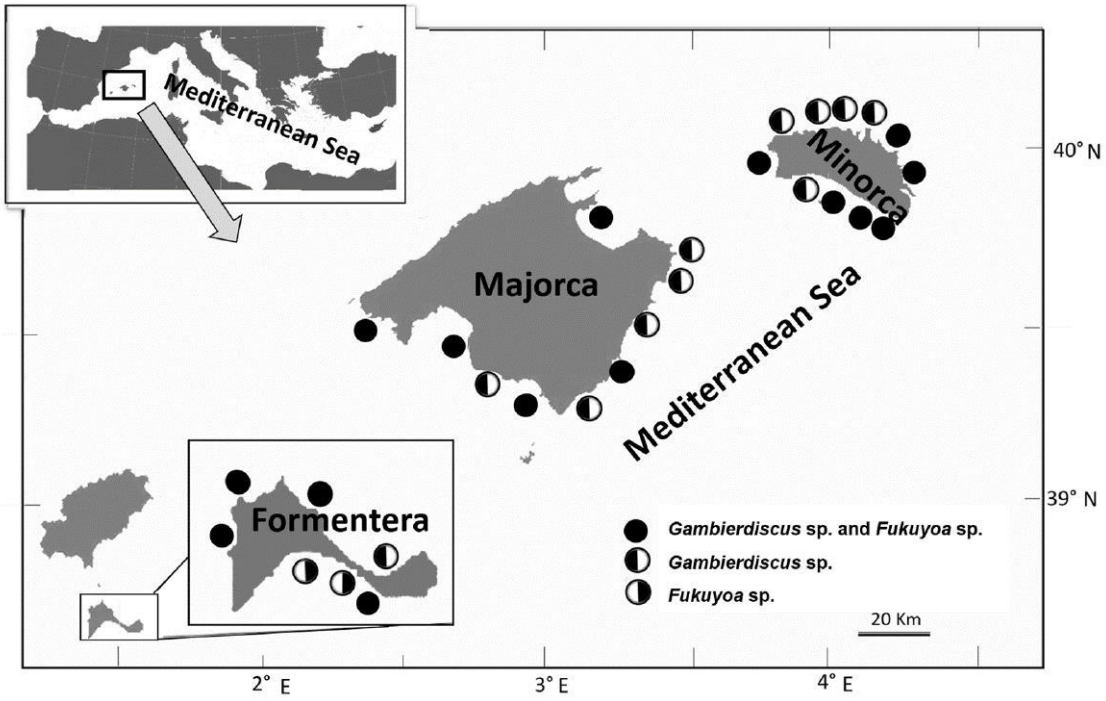


Figure 26. Presence of the *Gambierdiscus* and *Fukuyoa* genera in the sampling stations in the Balearic Islands (Mediterranean Sea) during 2016-2019.

Fish: species, Neuro-2a toxicity, geographical and seasonal analysis

From the samples collected from Crete, no CTX-like positive fish was detected from Crete in a total of 140 analysis for a total of 70 fish (140 extracts).

From Cyprus, 82 fish have been sampled. Only one CTX-like positive (*S. dumerilii*) was detected out of 148 extracts analysed by Neuro-2a, with an estimation of 0.0113 µg eq. CTX-1B/kg. An immunoassay (Leonardo et al., 2020) confirmed the presence of CTX-like compounds in this sample.

From the Balearic Islands, a total of 70 analyses for a total of 36 fish were performed showing no toxicity in fish from the Balearic Islands.

3.3. Confirmation and characterization of CTX by LC-MS (MS/MS and HRMS)

The developed LC-MS/MS methods were applied to the analysis of fish samples from the Canary Islands (Spain) (n = 212) and Madeira archipelago (Portugal) (n = 66). The samples from the Canary Islands and Madeira archipelago were positive in the Neuro-2a for CTX-like compounds. All the samples were extracted, purified and analysed by following the conditions described in section 2.3.1.

Among the monitored CTXs analogues, including P-CTXs and C-CTX1, only C-CTX1 was detected as main compound responsible of the CTX-like toxicity (Figure 27).

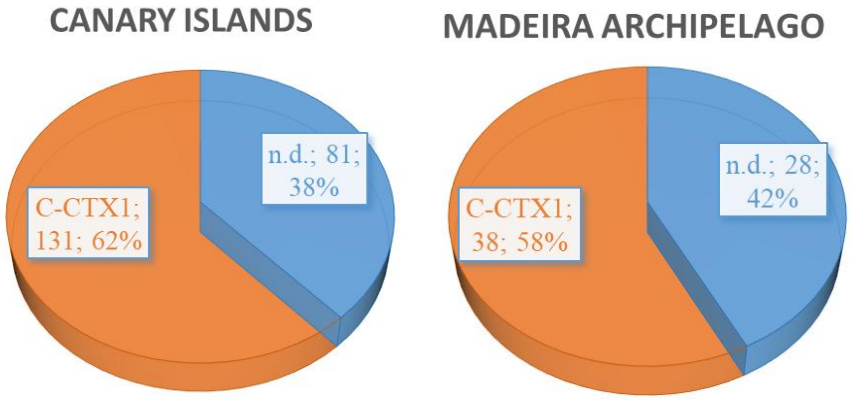


Figure 27. Samples from Canary Islands (Spain) and Madeira archipelago (Portugal) in which C-CTX1 was detected as responsible of the CTX-like toxicity.
*n.d. Non detected.

Application to the analysis of fish samples by LC-MS/MS and LC-HRMS from Canary Islands and Madeira Archipelago

Canary Islands samples

In 131 out of 212, C-CTX1 was detected as main compound responsible of the CTX-like toxicity. In the remaining 81 out of 212 samples neither C-CTX1 nor any other P-CTX with standard available were detected. This discrepancy can be explained due to the low toxicity detected in the Neuro-2a in most of the samples where no CTXs were detected. The higher sensitivity of Neuro-2a limits the detection of CTXs by LC-MS/MS at extremely low concentrations, albeit LC-MS/MS allows the detection of CTXs at lower enough concentrations to protect public health (< 0.1 ng C-CTX1/g). In a discrepant sample but with a high toxicity in the Neuro-2a it was detected a compound at a retention time of 5.6 min with *m/z* 1139 (Putative C-CTX-1157 described by Castro et al., 2020). The analysis in MRM mode reveal the detection of up to three water losses as well as C-CTX1 specific fragment *m/z* 191.1 (Figure 28). Therefore, it should be taken into consideration that despite the presence of C-CTX1 as main compound responsible of the CTX-like toxicity, other C-CTXs may be present in the samples from this geographical region.

Additionally, a possible matrix effect of signal suppression in the LC-MS/MS analysis should be considered. However, the purification using two consecutive SPEs reduce the probability of having the above-mentioned matrix effect.

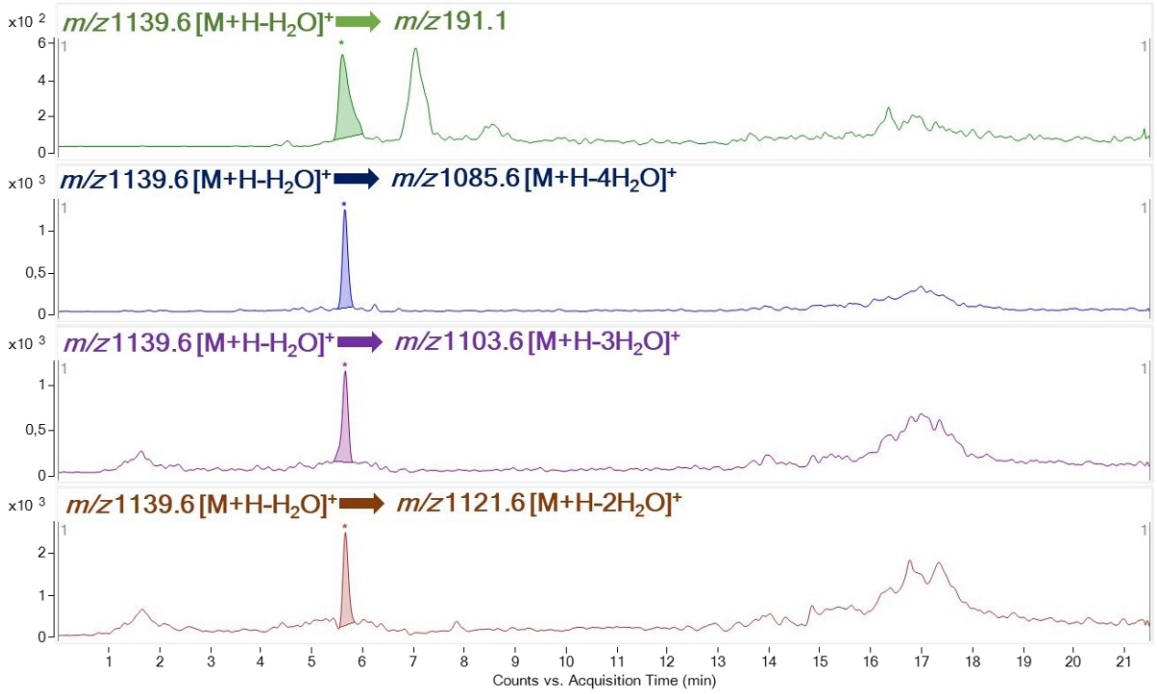
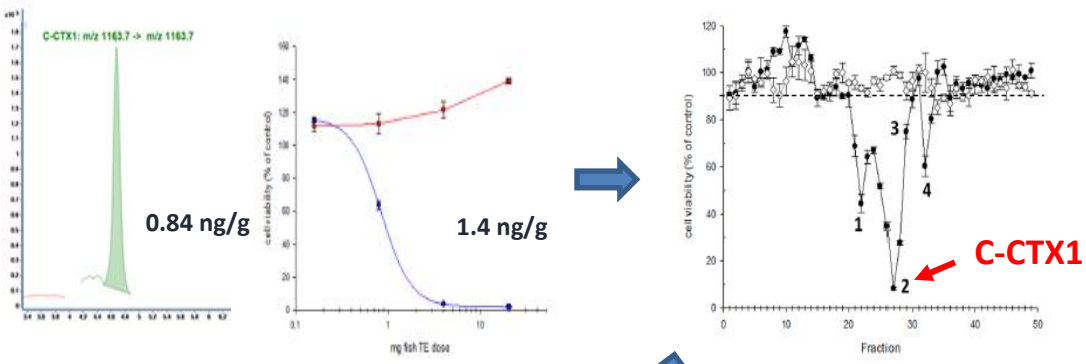


Figure 28. Putative C-CTX-1157 analogue at 5.6 min detected in a black moray (*Muraena augusti*) from El Hierro (Canary Islands, Spain).

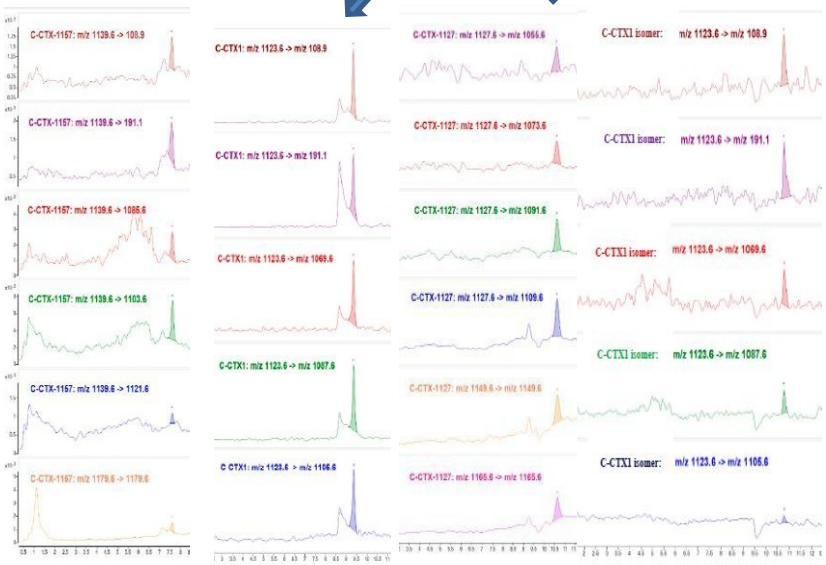
Madeira Samples

In 38 out of 66, C-CTX1 was detected as main compound responsible of the CTX-like toxicity. In the remaining 28 out of 66 samples neither C-CTX1 nor any other P-CTX with standard available were detected. The lack of CTXs in these samples can be justified by considering the same arguments indicated above in section 3.2.1. It is important to highlight that the samples from this region showed a higher concentration of C-CTX1, specifically the samples from Selvagens Islands (Portugal).

The approach carried out to characterize the CTX profile was to couple the sample fractionation through HPLC developed, with the toxic evaluation of each fraction with Neuro-2a to be further analysed by LC-MS/MS to identify the responsible of toxicity (Estevez et al., 2020a). The LC-MS/MS confirmatory method developed was used to characterize these compounds due to the ability of monitoring different ion transition and specific fragments that will allow the confirmation in contrast with the selection of the single sodium adduct. After following these approach, it was concluded that C-CTX1 is the main responsible of the toxicity in the samples fractionated and that several analogues of C-CTXs (C-CTX-1157, C-CTX-1127 and C-CTX1 isomers) seem to be present in this samples, confirming that the profile of the contamination is similar to the detected in the Caribbean Sea Castro et al., 2020 (Scheme 2).



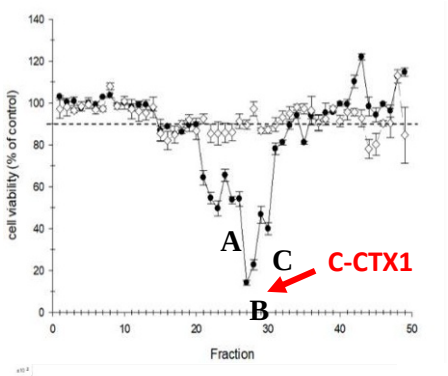
Peak	1	2	3	4
Fractions	19–22	23–28	29	32–33
Retention Time (min)	7.6	9.4	10.6	10.8
$[M+K]^+$	1195.6	1179.6	1165.6	n.d.
$[M+Na]^+$	1179.6	1163.6	1149.6	1163.6
$[M+NH_4]^+$	1174.6	1158.6	1144.6	n.d.
$[M+H]^+$	1157.6	1141.6	1127.6	n.d.
$[M+H-nH_2O]^+$ n range	1–3	1–3	1–4	1–3
C-CTX identification *	C-CTX-1157	C-CTX1	C-CTX-1127	Unknown

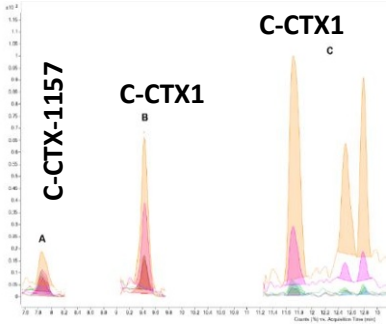


Scheme 2. Example of the approach carried out to characterize the CTX profile of a *Seriola Fasiata* implicated in a CP outbreak in Tenerife 2008.

This approach was extended to other samples from different regions such as Selvagens Islands or Canary Islands, being always C-CTX1 the main toxic analogue (Scheme 3).

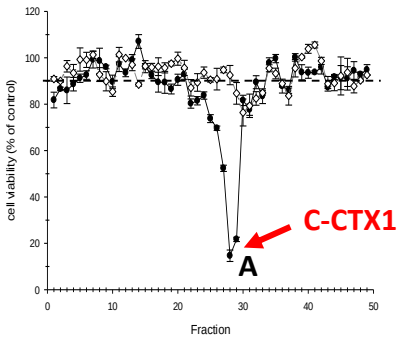
Canary Island sample with 0.49 ng/g (Lutjanus Cyanopterus)

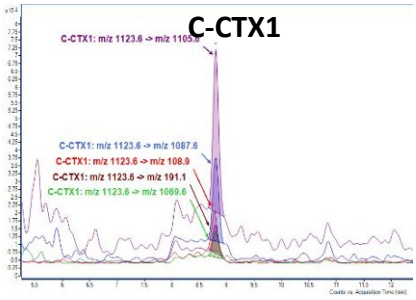




Peak	A	B	C
Fraction	23	25-28	30
Time (min)	7.85	9.4	**11.7**, 12.5, 12.8
$[M+K]^+$	1195.6	1179.6	n.d
$[M+Na]^+$	1179.6	1163.6	1163.7
$[M+NH_4]^+$	1174.6	1158.6	1158.6
$[M+H]^+$	1157.6	1141.6	1141.6
$[M+H-nH_2O]^+$ n range	1-3	1-3	1-3
C-CTX identification*	C-CTX-1157 isomer	C-CTX1	C-CTX1 isomer

Selvagen Islands sample with 0.35 ng/g (Bodianus scrofa)





Peak	A
Fraction	25-28
Time (min)	9.4
$[M+K]^+$	1179.6
$[M+Na]^+$	1163.6
$[M+NH_4]^+$	1158.6
$[M+H]^+$	1141.6
$[M+H-nH_2O]^+$ n range	1-3
C-CTX identification*	C-CTX1

Scheme 3. Other examples of the approach carried out to characterize the CTX profile of the contaminated samples.

Therefore, it is confirmed the CTXs profile of the samples from this geographical region, Canary Islands (Spain) and Madeira archipelago (Portugal), being C-CTX1 the main compound responsible of the contamination.

Application to the analysis of microalgae by LC-MS/MS and LC-HRMS

The LC-MS/MS analysis of the dinoflagellate samples supplied by the SA3 did not reveal a CTX profile since neither P-CTXs nor C-CTX1 were detected (Table 17). As a consequence of that the approach developed by the research group in this SA4 using HPLC/Neuro-2a/LC-MS/MS was used to characterize the toxic profile of these *Gambierdiscus* sp in order to characterize the presence of other toxic analogues also reported in the literature for these particular strains.

Table 17. Detailed information about the dinoflagellate extracts analysed by LC-MS/MS and LC-HRMS

Species	Strain code	Location	Number of cells extracted	Volume of culture (L)	CTX-like (fg CTX1B eq./cell)
G. australes	IRTA-SMM-17-189	Torret, Menorca, Balearic Islands, Spain	17 134 000	20	83 ± 12 [a]
G. australes	IRTA-SMM-17-162	St. Adeodat, Menorca, Balearic Islands, Spain	27 811 000	20	101 ± 7.5
G. australes	IRTA-SMM-17-164	St. Adeodat, Menorca, Balearic Islands, Spain	4 257 000	20	> 62.5
G. australes	IRTA-SMM-17-271	Macarella, Menorca, Balearic Islands, Spain	14 007 000	20	271 ± 29
F. paulensis	IRTA-SMM-17-209	Sacaleta, Menorca, Balearic Islands, Spain	6 964 000	20	16 ± 1.7 [a]
G. australes	IRTA-SMM-17-253	Anguila, Menorca, Balearic Islands, Spain	13 735 000	20	164 ± 16
G. australes	IRTA-SMM-17-244	Camp de Mar, Mallorca, Balearic Islands, Spain	4 121 000	5	155 ± 25
***Gambierdiscus* sp.2**	0010G-CR-CCAUTH	Kolimpari, Crete, Greece	2 300 000	5	NQ
G. excentricus	IRTA-SMM-17-407	Playa de vueltas, La Gomera, Canary Islands, Spain	6 084 000	5	>794 (NQ)

NQ: not quantifiable.
[a] CTX-like toxicity evaluated in Tudó et al., 2020.

Characterization of *Gambierdiscus* extract by HPLC/Neuro-2a/LC-MS/MS

One of the *Gambierdiscus* extracts with the higher CTX-like toxicity in the Neuro-2a (*G. australes* (IRTA-SMN-17-271) (Table 17) was characterized by using the combination of sample fractionation by HPLC with toxicity evaluation by Neuro-2a and final confirmation by LC-MS/MS of the toxic fractions (*Manuscript in preparation*).

The dinoflagellate sample, *G. australes* (IRTA-SMN-17-271) from Balearic Islands (Spain), showed three toxic peaks after the sample fractionation and toxicity evaluation (Figure 28).

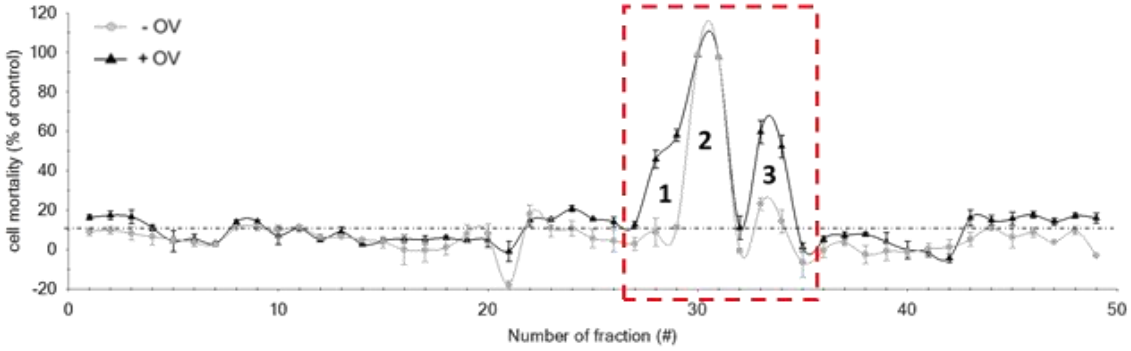


Figure 28. Number of fractions collected after HPLC-C18 fractionation *vs* cell mortality relative to control cells. Cytotoxicity effect of fractions in presence (▲) and absence (○) of OV. The red box highlights the main toxic region including the fractions ranging from 28 to 34, where 3 peaks with a cytotoxic activity can be distinguished.

LC-MS/MS method was used to confirm the presence of compounds responsible for the toxicity found by Neuro-2a. As a result of this initial confirmation MTX3 was detected in the fraction 28 (positive for toxicity by Neuro-2a) whilst gambieric Acid C was detected in fraction 33 and the coelution of gambieric Acid C and D was detected in fraction 34 (Figure 29).

On the other hand, the confirmation of the toxicity corresponding to the chromatographic peak 2 was achieved in both fractions treated and untreated with OV. This toxicity can be atributed to MTXs e.g MTX1 or MTX4). The confirmation carried out by LC-MS/MS allowed to identify the presence of a novel MTX compound in the fractions 30 and 31 (corresponding to the chromatographic peak 2). This novel MTX compound shows similar ion pattern and fragmentation as MTX1 or MTX4.

The toxic profile of the *Gambierdiscus* sp. analysed in this SA includes MTXs and gambieric acids C and D. The presence of these compounds was also confirmed by LC-HRMS. No C-CTX1 or P-CTXs or known potential precursors of these compounds were detected in any of the toxic fractions analysed which make us to think that these compounds might be present at very low levels or that the correlation with the CTXs profile is still unknown for these particular *Gambierdiscus* and *Fukuyoa* strains.

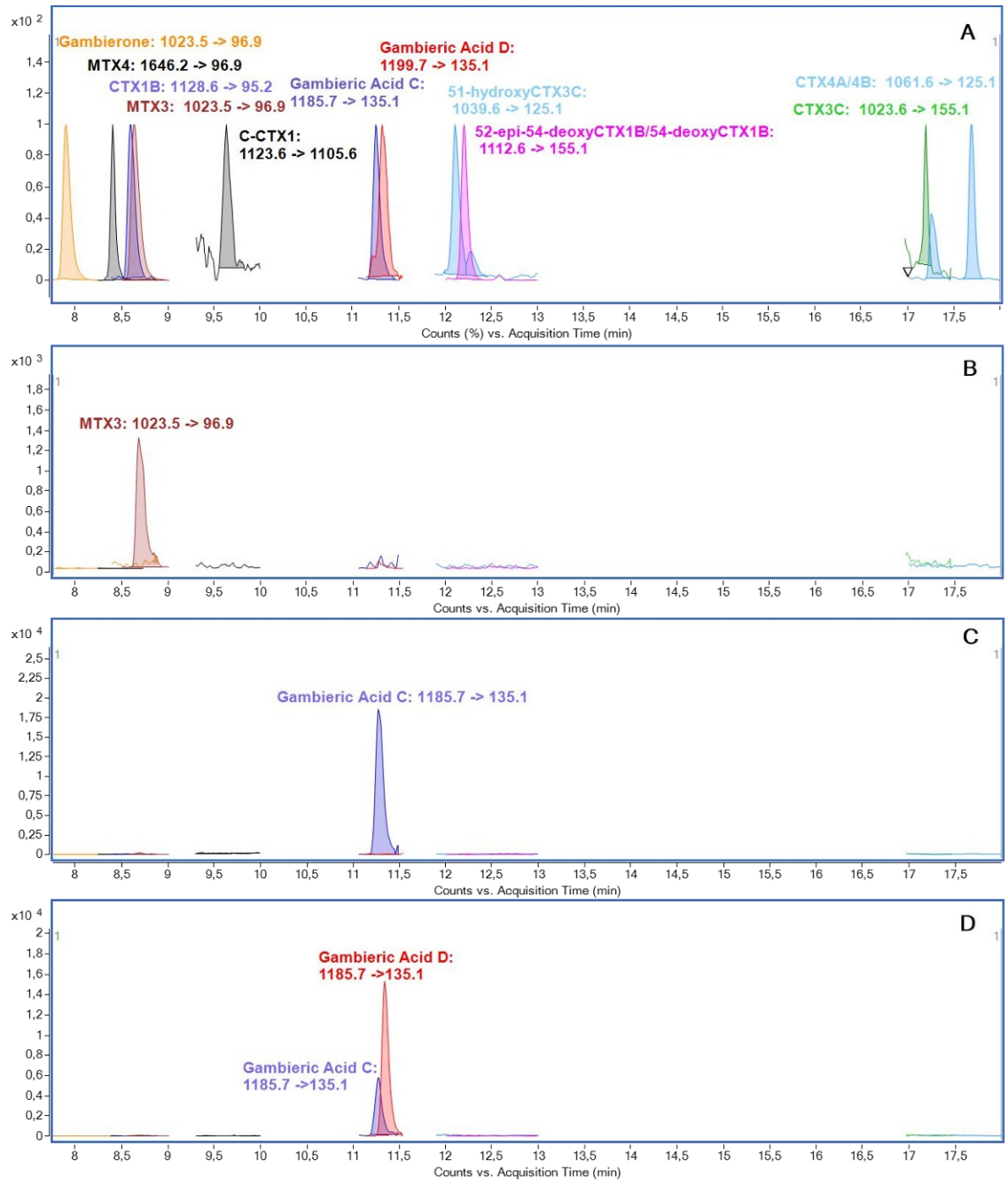


Figure 29. LC-MS/MS analysis in MRM mode showing the most remarkable transition for each compund in order to simplify and having an overview of the compounds present in the *G. australes*. A) Reference Material containing a mixture of P-CTXs, C-CTX1, MTXs, Gambierone and Gambieric Acids. B) MTX3 detected in the CTX-like toxic Fraction 28. C) Gambieric Acid C detected in the CTX-like toxic Fraction 33. D) Coelution of gambieric Acid C and D in the CTX-like toxic Fraction 34.

3.4. Preparation of Reference Materials

Reference materials were prepared by selecting fish samples in which maximum concentration of C-CTXs had been determined by the LC-MS/MS. The generally low concentration of CTXs in the fish

collected, which has been emphasized as an important limitation of this project, prompted the need of also using the fish liver, despite its complexity in order to increase the CTX concentration. Two different reference materials have been prepared consisting on pure solutions of C-CTX1, isolated and purified from fish tissue and liver and homogenates of autoclaved fish tissue (FTRM) containing C-CTX1. This last was considered a useful reference material since includes the matrix the effect of which is relevant on the methodological implementation in particular by LC-MS/MS, for which these materials are going to be used.

Preparation of C-CTX1 solutions

As described above, C-CTX1 was isolated from fish tissue and liver in order to maximize the concentration of C-CTX1, due to the generally low concentration of the toxin in the fish samples collected during this project. The efficiency of the isolation and purification of C-CTX1 was highly dependent on the matrix complexity, this justifies additional steps included on the sample preparation protocol. The full purification of this toxin has been compromised by the limited concentration which also limits a complete purification procedure with multiple steps, compromising the recovery of the toxin, despite of this the optimization carried out on the protocols developed allowed to conclude that the C-CTX1 solutions obtained will allow their use as very valuable laboratory reference materials with an adequate estimation of the concentration, far from being considered certified, but good enough to be used for quantitation purposes. Different concentrations of C-CTX1 have been prepared depending on their source, being more concentrated those ones extracted from livers than the ones extracted from fish tissue. The C-CTX1 solutions extracted from liver are scarce and therefore their adequate use needs to be ensured. An important consideration to consider is the possible presence of a methylated congener of C-CTX1 in some of the C-CTX1 fractions. This C-CTX1-Me is a 56-methoxy congener resulting from the transformation of C-CTX1 during the isolation and purification process as published in Estevez et al., 2020c. This transformation takes place during the HPLC fractionation in which C-CTX1 is selectively collected in a single fraction, albeit the presence of slight acid conditions in methanol from the mobile phase trigger the transformation of C-CTX1 into this compound. This transformation has been also observed in the standard of C-CTX1 kindly provided by Dr Dickey (Figure 30) and used in these studies. Therefore, the concentrations of this methylated congener are also included in the C-CTX1 toxin solutions prepared.

The preparation of these fractions is summarized as follows: 19 kg of fish tissue were obtained from the autoclave and homogenization of 50 kg fish. 1 kg fractions were extracted and purified following the conditions described in section 2.3.1. A total of 48 fractions of toxin solutions of C-CTX1 were finally obtained (Table 18). C-CTX1 concentrations range from 1.350 ng/g to 0.015 ng/g. 20 out of 48 fractions are above C-CTX1 guidance level of 0.100 ng/g proposed by the FDA, USA. 31 out of 48 fractions contained traces of C-CTX1-Me with concentrations ranging from 0.153 ng/g to 0.015 ng/g. The C-CTX1 solutions once characterized were evaporated to dryness and kept at -20 ºC.

A pool of 6 kg of fish liver of positive fish containing C-CTX1 was selected to also produce toxin solutions of C-CTX1. 1 kg fractions were extracted and purified following the conditions described in section 2.3.1. obtaining 41 fractions of C-CTX1 toxin solutions (Table 19). C-CTX1 concentrations range from 1.896 ng/g to 0.015 ng/g. 20 out of 41 fractions are above C-CTX1 guidance level of 0.100 ng/g proposed by the FDA, USA. 29 out of 41 fractions contained traces of C-CTX1-Me with concentrations ranging from 1.341 ng/g to 0.015 ng/g. After the characterization of these toxin solutions of C-CTX1 they were evaporated to dryness and kept at -20 ºC.

Table 18. List of toxin solutions of C-CTX1 obtained from the extraction of autoclaved fish tissue. The dotted line showed the threshold of C-CTX1 guidance level proposed by the FDA, USA (0.1 ng/g). n.d. not detected.

RM ID	C-CTX1 (ng)	C-CTX1 (ng/g)*	C-CTX1-Me (ng)	C-CTX1-Me (ng/g)*
F64-6-Fr2	40.490	1.350	1.353	0.045
F0071-BS-4-Fr2	36.527	1.218	1.047	0.035
F178-1-Fr3	27.748	0.925	3.409	0.114
F178-2-Fr3	27.366	0.912	1.881	0.063
F178-3-Fr3	23.894	0.796	1.098	0.037
F0071-BS-2-Fr2	23.308	0.777	1.320	0.044
F0071-BS-5-Fr2	18.365	0.612	0.623	0.021
F64-4-Fr2	12.982	0.433	0.920	0.031
F0071-BS-3-Fr2	9.644	0.321	1.227	0.041
F349-F350-1-Fr3	8.236	0.275	1.044	0.035
F0071-BS-6-Fr2	7.371	0.246	0.449	0.015
F64-5-Fr2	6.906	0.230	n.d.	n.d.
F350-1-Fr2	6.026	0.201	0.477	0.016
F178-2-Fr2	5.420	0.181	n.d.	n.d.
F0071-BS-1-Fr3	5.087	0.170	2.534	0.084
F64-1-Fr2	4.621	0.154	n.d.	n.d.
F178-3-Fr4	4.583	0.153	0.689	0.023
F64-3-Fr2	3.730	0.124	0.470	0.016
F0071-BS-1-Fr2	3.683	0.123	n.d.	n.d.
F64-2-Fr3	3.037	0.101	1.098	0.037
F0071-BS-6-Fr3	2.924	0.097	1.649	0.055
F0071-BS-4-Fr3	2.750	0.092	3.695	0.123
F350-1-Fr3	2.717	0.091	3.216	0.107
F0071-BS-5-Fr3	2.433	0.081	1.601	0.053
F64-5-Fr3	2.345	0.078	0.688	0.023
F0071-BS-2-Fr3	2.291	0.076	4.605	0.153
F64-3-Fr3	2.218	0.074	3.235	0.108
F64-1-Fr3	2.183	0.073	1.098	0.037
F710-1-Fr2	2.092	0.070	n.d.	n.d.
F349-F350-1-Fr2	2.076	0.069	n.d.	n.d.
F0071-BS-3-Fr3	1.926	0.064	2.225	0.074
F64-4-Fr3	1.671	0.056	1.359	0.045
F64-2-Fr2	1.626	0.054	n.d.	n.d.
F350-1-Fr4	1.098	0.037	n.d.	n.d.
F349-1-Fr3	0.993	0.033	0.436	0.015
F349-1-Fr2	0.715	0.024	n.d.	n.d.
F0071-BS-3-Fr4	0.667	0.022	n.d.	n.d.
F0071-BS-4-Fr4	0.653	0.022	0.905	0.030
F0071-BS-2-Fr4	0.648	0.022	n.d.	n.d.
F178-3-Fr2	0.616	0.021	n.d.	n.d.
F0071-BS-3-Fr1	0.592	0.020	n.d.	n.d.
F710-1-Fr4	0.561	0.019	n.d.	n.d.
F64-6-Fr1	0.536	0.018	n.d.	n.d.
F178-2-Fr4	0.519	0.017	0.622	0.021
F178-1-Fr4	0.481	0.016	1.160	0.039
F64-4-Fr1	0.475	0.016	n.d.	n.d.
F64-6-Fr4	0.454	0.015	n.d.	n.d.
F349-F350-1-Fr4	0.437	0.015	n.d.	n.d.

*If reconstituted in 1 mL // n.d. Non detected

Table 19. List of the toxin solutions of C-CTX1 obtained from the extraction of autoclaved fish liver. The dotted line showed the threshold of C-CTX1 guidance level proposed by the FDA, USA (0.1 ng/g). n.d. not detected.

RM ID	C-CTX1 (ng)	C-CTX1 (ng/g)*	C-CTX1-Me (ng)	C-CTX1-Me (ng/g)*
LOTE-2-LIV-R1-Fr2	56.888	1.896	9.110	0.304
LOTE-2-LIV-R2-Fr2	42.485	1.416	3.806	0.127
LOTE-2-LIV-R3-Fr2	38.856	1.295	3.895	0.130
LOTE-3-LIV-R2-Fr2	25.737	0.858	2.272	0.076
LOTE-4-LIV-R1-Fr2	14.787	0.493	n.d.	n.d.
LOTE-3-LIV-R1-Fr2	10.750	0.358	0.757	0.025
LOTE-4-LIV-R2-Fr2	10.696	0.357	1.889	0.063
RESIDUE FRACT-Fr2	10.518	0.351	0.814	0.027
LOTE-3-LIV-R3-Fr4	7.844	0.261	n.d.	n.d.
LOTE-3-LIV-R3-Fr2	7.462	0.249	0.749	0.025
LOTE-4-LIV-R5-Fr2	6.596	0.220	0.657	0.022
LOTE-3-LIV-R1-Fr4	5.455	0.182	n.d.	n.d.
LOTE-4-LIV-R3-Fr2	4.984	0.166	0.767	0.026
LOTE-3-LIV-R2-Fr4	4.864	0.162	n.d.	n.d.
LOTE-4-LIV-R4-Fr2	4.831	0.161	0.788	0.026
LOTE-2-LIV-R3-Fr4	4.816	0.161	n.d.	n.d.
LOTE-2-LIV-R2-Fr4	4.806	0.160	n.d.	n.d.
LOTE-2-LIV-R1-Fr4	4.310	0.144	n.d.	n.d.
LOTE-4-LIV-R3-Fr4	3.394	0.113	n.d.	n.d.
LOTE-4-LIV-R4-Fr4	3.298	0.110	n.d.	n.d.
LOTE-4-LIV-R5-Fr1	2.207	0.074	n.d.	n.d.
RESIDUE FRACT-Fr3	2.093	0.070	1.827	0.061
LOTE-4-LIV-R1-Fr1	2.084	0.069	2.573	0.086
LOTE-4-LIV-R2-Fr1	1.889	0.063	n.d.	n.d.
LOTE-2-LIV-R3-Fr3	1.640	0.055	18.253	0.608
LOTE-2-LIV-R2-Fr3	1.390	0.046	40.236	1.341
LOTE-2-LIV-R1-Fr3	1.349	0.045	26.234	0.874
LOTE-4-LIV-R3-Fr1	1.243	0.041	n.d.	n.d.
LOTE-3-LIV-R1-Fr3	1.240	0.041	4.455	0.148
LOTE-3-LIV-R2-Fr3	1.061	0.035	3.089	0.103
LOTE-3-LIV-R3-Fr3	1.040	0.035	1.729	0.058
LOTE-3-LIV-R2-Fr1	0.875	0.029	0.457	0.015
LOTE-2-LIV-R1-Fr5	0.633	0.021	3.110	0.104
LOTE-2-LIV-R3-Fr5	0.538	0.018	4.239	0.141
LOTE-3-LIV-R3-Fr1	0.445	0.015	0.749	0.025
LOTE-3-LIV-R1-Fr5	n.d.	n.d.	3.254	0.108
LOTE-4-LIV-R1-Fr3	n.d.	n.d.	3.552	0.118
LOTE-4-LIV-R2-Fr3	n.d.	n.d.	2.661	0.089
LOTE-4-LIV-R3-Fr3	n.d.	n.d.	1.283	0.043
LOTE-4-LIV-R4-Fr3	n.d.	n.d.	1.310	0.044
LOTE-4-LIV-R5-Fr3	n.d.	n.d.	1.528	0.051

*If reconstituted in 1 mL. // n.d. Non detected

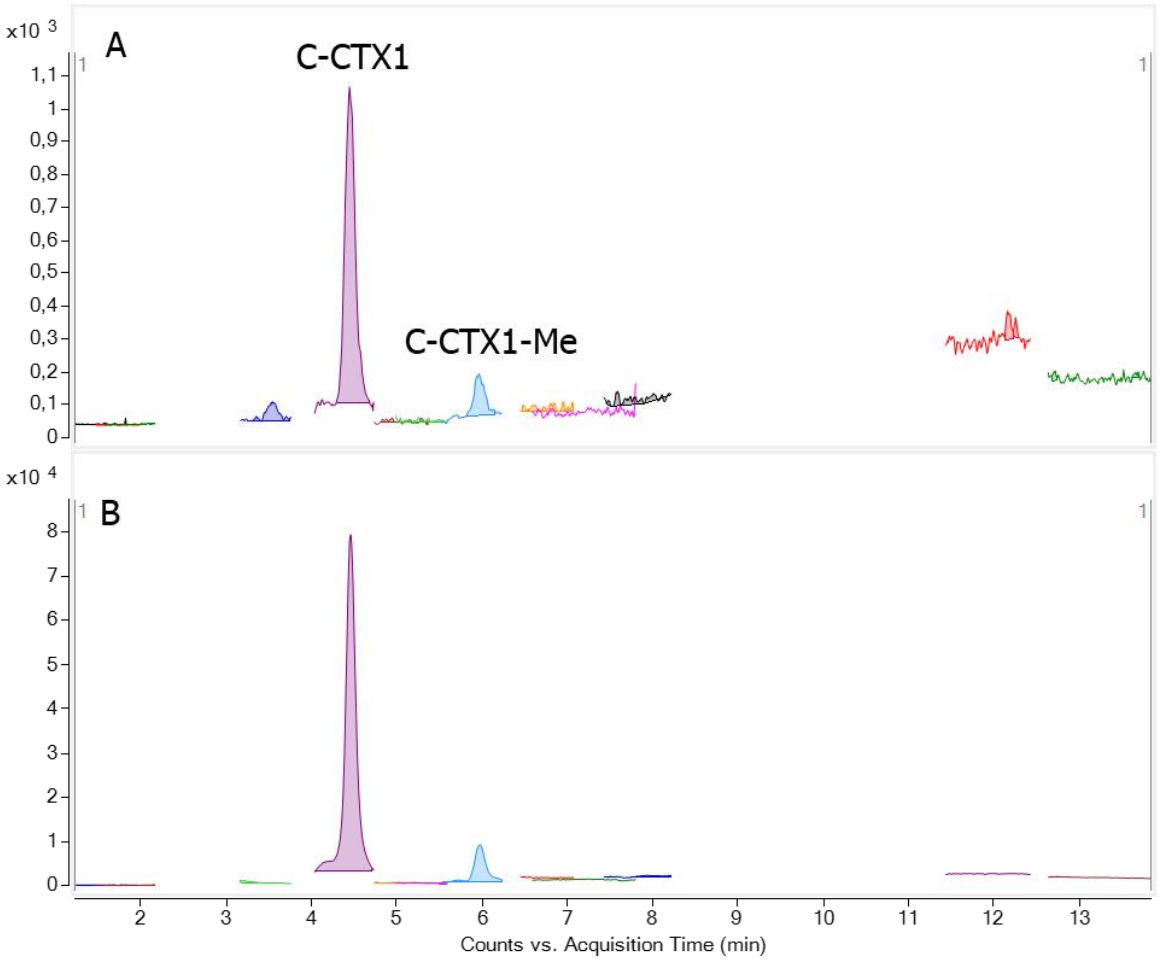


Figure 30. Example of a chromatogram after the LC-MS/MS analysis selecting $[M+Na]^+$ as precursor and product ion of: A) C-CTX1 pure standard (1 ng/mL); B) C-CTX1 toxin solution (LOTE-2-LIV-R1-Fr2).

Characterization of FTRM: Homogeneity and stability studies

The preparation of the FTRM was described above. The selected fish materials consisted on two species prone to contain CTXs in the Canary Islands (Spain), Amberjack (*Seriola sp.*) and Dusky grouper (*Epinephelus marginatus*). Homogeneity and stability studies were performed with several limitations mostly related with the lock down of the laboratories, due to the COVID-19. Despite of this, emphasis was made in ensuring the homogeneity of the materials packed in particular the FTRM and the homogeneity of these materials was evaluated by extracting and purifying three aliquots of each fish sample, selected randomly and analysed in triplicate in repeatability conditions in the same day. The stability of the materials was assessed by analysing triplicates of each fish sample selected randomly three times for one month. A mean concentration valued was assigned to C-CTX1 after this evaluation (Table 20; Figure 31).

Table 20. List of the autoclaved fish tissue reference materials.

Sample ID	Island	Species	Autoclaved FTRM available (kg)	C-CTX1 (ng/g)
FTRM-EFSA-ULPGC-F0178	Tenerife	Amberjack *(Seriola* sp.*)*	6	0.284 ± 0.019
FTRM-EFSA-ULPGC-F0710	Lanzarote	Dusky grouper *(Epinephelus marginatus)*	1.5	0.102 ± 0.017
FTRM-EFSA-ULPGC-F0350	Lanzarote	Dusky grouper *(Epinephelus marginatus)*	15	0.081 ± 0.007
FTRM-EFSA-ULPGC-F0349	Lanzarote	Amberjack *(Seriola* sp.*)*	3	0.039 ± 0.006

Samples were selected to cover a wide range of C-CTX1 concentrations ranging from 0,039 to 0,284 ng/g. The detection of C-CTX1 in the FTRM with lower concentration (e.g. FTRM-EFSA-ULPGC-F0349 or FTRM-EFSA-ULPGC-F0350) will be helpful to assess the sensitivity on the method implementation in the laboratories. On the other hand, the autoclaved FTRM with higher content of C-CTX1, above C-CTX1 guidance level of 0.100 ng/g, (e.g. FTRM-EFSA-ULPGC-F0710 and FTRM-EFSA-ULPGC-F0178) will guarantee that the laboratories can detect samples containing C-CTX1 which might cause CP. The autoclaved FTRM were packed are kept at -20 ºC.

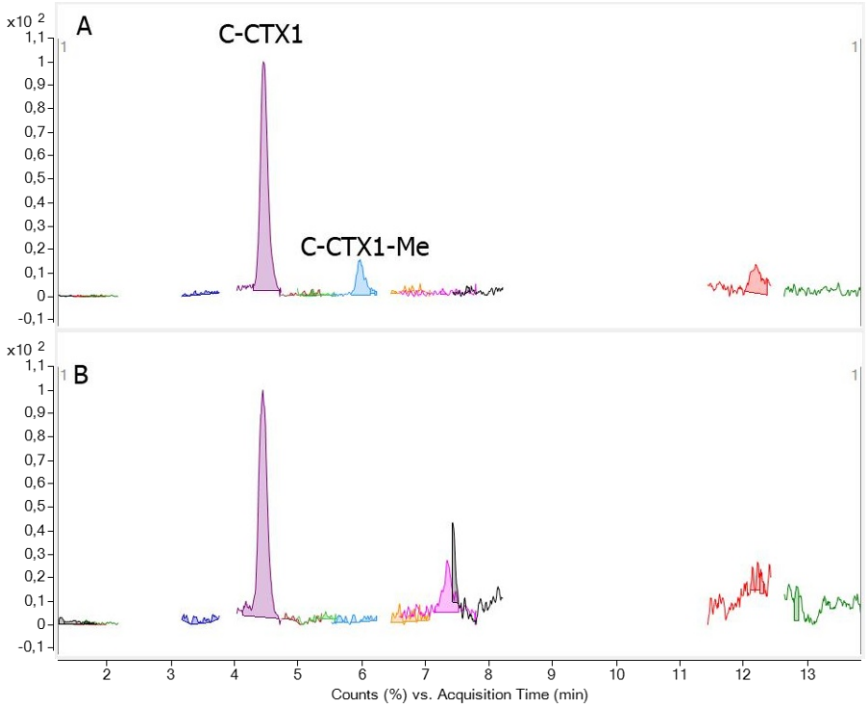


Figure 31. Chromatogram after the LC-MS/MS analysis selecting $[M+Na]^+$ as precursor and product ion of: A) C-CTX1 pure standard (1 ng/mL); B) C-CTX1 FTRM amberjack *(Seriola sp.)* (EFSA-ULPGC-F0349).

3.5. Desk work

Identified sources of biological, oceanographic and meteorological data

There are several sources of biological, oceanographic and meteorological data for the Canary Islands. For example, the site "Portus", 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 sea-level. 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 the European Marine Observation and Data Network (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 to 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.

Environmental and oceanographic data from other institutions

IPMA:

Databases used for scientific research information about *Gambierdiscus* spp. and *Fukuyoa* spp. occurrence and toxin profile comprise Scopus, Science Direct, Web of Knowledge, among others. Also, a bibliography search of papers describing meteorological and climate phenomenon in the sampling areas has been performed. In addition, the EModnet (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.

Database of scientific literature

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

In SA3, a literature search was carried out and references were included in the ENDNOTE database.

The collected references have been classified in the ENDNOTE file 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.

The following labels have also been included 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, some keywords were added to each reference to identify the contents of the articles:

> Temperature, salinity, light, seasonality, abundance, fish toxicity, Europe, Pacific, Caribbean, Indian, Mediterranean, extraction, macroalgae, coral, anthropogenic disturbances, growth rate, nutrients, bioassay, immunoassay, extraction, purification, bacteria, evolution, army, outbreak, traveller.

Modelling strategies for the study of ciguatera

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 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. The 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 appears too simplistic. To predict CP incidence in Cook Islands and French Polynesia using SSTA, Zheng et al., 2020 found that the 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 and 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. This was the case in Bahamas (1908) and Fiji (1929) where local people believed that fish were poisoned by seaweed that grew after the storm. Randall (1958) already suggested, that the organisms producing ciguatera was 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 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 an 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
- ✓ Turbulence

4. Discussion

Ciguatera poisoning (CP) is the most common type of marine biotoxins food poisoning worldwide with an estimation of 50.000 people affected every year, it is caused by the consumption of marine species that have accumulated trace amounts of ciguatoxins. While in some regions, mainly in tropical and subtropical areas, has been known for centuries, since 2008 autochthonous outbreaks have been reported in Europe, specifically in Spain and Portugal, in the Macaronesia area. Furthermore, some studies have identified the presence of *Gambierdiscus* spp. in waters surrounding the Canary Islands and Madeira. In addition to this the increase in import of fish from endemic areas and subsequent rise in cases on Europe make the impact on this research of greater impact than expected.

All these findings suggested that ciguatera was becoming an increasing risk for European countries. Therefore, the main objective of the project EuroCigua was the risk characterization of CP in Europe.

This report summarizes results and findings of the project EuroCigua, consisted on a consortium of 14 organizations from 6 countries, coordinated by the Spanish Food Safety and Nutrition Agency from Spain, witch targeted 3 main objectives: to estimate the incidence of ciguatera in Europe and the epidemiological characteristics of cases, to assess the presence of ciguatoxins in food and the environment in Europe, and to develop and validate methods for the detection.

Autochthonous ciguatera outbreaks due to consumption of fish captured in Macaronesia (Madeira and the Canary Islands) were reported to the project by Portugal and Spain, which was consistent with the already known presence of CTXs in the area. Spain reported outbreaks occurring during the whole period of the study, however Portugal reported outbreaks only from 2012 and 2015 that may reflect under ascertainment (under diagnosis and under reporting) or that implemented measures in Portugal avoid that contaminated fish reach the consumers.

Imported ciguatera outbreaks were reported from France and Germany, although according to the RASFF contaminated fish was distributed to more EU countries; this also point to possible under ascertainment in some countries. Under ascertainment could also be suggested for the travel related ciguatera outbreaks that were only reported by France, Germany and Spain.

No statistically significant trend along the study period, in the number of outbreaks or the size of the outbreaks was observed during the study period. It is not possible to determine if this lack of trend is real or it is due to the under ascertainment or to the effectiveness of the implemented measures.

The most frequent fish genus involved in the autochthonous outbreaks were *Seriola* and *Epinephelus* while *Lutjanus* was the most frequent fish genus reported in imported outbreaks. In almost 60% of the autochthonous outbreaks, the fish was captured by sport fishing. Appropriate risk management measures should consider these fish species and sport fishing as a potential driver.

CTXs were detected in 16 outbreaks. In 10 outbreaks, the analysis was not performed; in some of them, it was mentioned that no fish leftovers were available. This is in line with the foodborne outbreaks reported at EU level. According to the last EU zoonoses report with data from 2018, in 24% of the outbreaks reported to EU-FORS the agent was reported as "unknown" or "unspecified". Moreover, only in 14% of the foodborne outbreaks reported to EU-FORS the evidence of implication of a suspected food as cause of the outbreak was strong. In strong-evidence food borne outbreaks, the evidence supporting the link between a food source and the outbreak has a low level of uncertainty. In contrast, in weak evidence outbreaks this link is much more uncertain.

The hospitalization rate for the ciguatera outbreak related cases was very different among the reporting countries. These differences could be due to different CTXs toxicity profile; differences in surveillance, being severe cases more easily identified and reported; as well as differences in hospitalization policies among the countries and differences in the definition of hospitalization (people attending the hospital emergency room recorded as hospitalized even if they do not overnight).

The incidence rate in the EU/EEA countries, excluding tropical overseas territories, was very low (0.008 cases per 100,000 inhabitants per year). According to the literature, it is estimated that only between 10 and 20% of cases are reported. Under ascertainment is an additional aspect. It may be due to lack of clinicians' awareness, no laboratory test for clinical samples and lack of widely available laboratory test for fish. However, to counterbalance the fact that ciguatera is not a mandatory reporting disease, some countries (mainly the partners of the project) have done an active search at many different data sources.

The incidence rate in the Canary Islands (0.47 cases/100,000 inhabitants) was higher than the one reported by Radke in Florida from 2000 to 2011 (0.2 cases/100,000 inhabitants), where ciguatera cases are also under surveillance. Incidence rate reported in the Canary Islands was similar to the incidence rate reported in the Dominican Republic (0.5 cases/100,000 inhabitants) and lower than the incidence rate reported in Martinique (2 cases/100,000 inhabitants) and the one reported in the French Polynesia (36 cases/100,000 inhabitants).

One of the main limitations in collecting epidemiological information is that the information was collected retrospectively and that ciguatera single cases are not under surveillance in the EU/EEA, except in the Canary Islands. Therefore, relevant information for the characterization of ciguatera in the EU was not available or could not be confirmed. Moreover, in some instances not enough information from single cases was available to assess if they really fulfilled the EuroCigua case definition.

In addition to these, the diagnosis of ciguatera remains difficult as it is a rare disease, there is lack of awareness, and there is not a test available for detection of CTXs in clinical samples and the detection in the fish consumed is not done on a routine basis in the EU/EEA. Ciguatera under ascertainment is known but not to what extent in Europe, therefore the estimation of the incidence rate in the EU/EEA does not reflect its true incidence.

The toxicity evaluation of CTXs in seafood and the environment for the risk assessment and the characterization of CP has been focused on areas from the North Western Atlantic Ocean (Macaronesia) and the Mediterranean Sea. The characterization of this risk has been carried out not only through the toxicity evaluation and further confirmation of the ciguatoxins in fish samples, but also on the microalgae considered as the source of ciguatoxins.

To accomplish this objective an extensive sampling has been carried out in the main areas presumably affected by CP, as the Canary Islands (Spain) and Madeira archipelago (Portugal). Important efforts have been invested on the evaluation of the toxicity of 104 cultures of microalgae and 1174 fish of different species susceptible of containing CP precursors.

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.

The initial evaluation of the toxicity carried out by an implemented neuroblastoma cell assay (Neuro-2a) allowed not only the determination of the positive CTX-like toxicity in the fish samples affected by this contamination but even an initial semi-quantitation of this toxicity.

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.

The implementation of the Neuro-2a cell-based assay facilitated the selection of the primary materials to be considered for further use on the preparation of reference materials.

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/management, to carefully design the selection of sites and fish to respond to the specificities of the site.

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*, indicates 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). 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 (SA4). Efforts in Macaronesia should be centered for a better prediction of CP 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 found with at least 6 different taxa but relatively low cell toxicity detected in 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 focused on the ecology of ciguatera. The follow-up analysis of these populations, considering quantitative techniques over different spatial and time ranges should be necessary to better acquaint the risk these populations represent.

The confirmation of ciguatoxins in samples found toxic by Neuro-2a, was carried out using Mass Spectrometry (MS) as an analytical tool with the ability to provide information regarding the amount and type of contaminant present in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions present in the sample extract.

The method development and optimizations for the identification and confirmation of the toxicity by the characterization of the CTX analogues present in the samples have been a critical step to achieve the main goal of this project. This goal has been successfully accomplished by the implementation of the LC-MS methodologies used in this project (LC-MS/MS and LC-HRMS) to allow the characterization of the CP risk by confirming the presence of Caribbean CTXs, in particular C-CTX1 as the main responsible of the CTX toxicity in the European areas selected as target in this study. The implementation of MS approaches linked to novel strategies such as the combination of Neuro-2a with chromatographic

fractionations through HPLC and GPC allowed not only the characterization of the C-CTX1, but also to resolve most of the discrepancies observed between Neuro-2a and LC-MS/MS. These discrepancies were in some cases justified by the presence of additional C-CTX analogues but also by matrix artifacts affecting both Neuro-2a and LC-MS.

An important limitation was derived from the fact that the concentration of the toxins in the fish samples evaluated in this study was generally low compromising not only an exhaustive evaluation of the efficiency of the analytical protocols developed, but also the accomplishment of the task focused on the preparation of reference materials. In fact, the multiple steps included in the protocols for the isolation and purification of these materials would require higher toxin concentration that could ensure an adequate amount of toxins to be able to be properly quantified. This limitation has been partially overcoming by the preparation of reference materials which were not fully purified but purified enough; they were also evaluated for homogeneity and stability in order to be used as laboratory reference materials in two formats, as purified toxin solutions of C-CTX1 and fish tissue containing C-CTX1, both very useful for method implementation.

An important gap encountered in this project was the unavailability of fish samples directly associated to human cases causing lack of knowledge regarding the correlation between toxic symptoms and toxin responsible for them.

The analysis of the microalgae responsible for the CP in particular the species of *Gambierdiscus* and *Fukuyoa* has been also carried out following the same approach above described for the analysis of the fish samples although in this case the toxicity found by Neuro-2a did not correlate with the CTX toxicity found in the fish samples. This discrepancy could be explained by the fact that the toxicity of both *Gambierdiscus* and *Fukuyoa* was mainly attributed to MTX analogues and not CTX compounds.

Future perspectives

For a better characterization of ciguatera in the EU, it is necessary to collect detailed homogeneous and harmonized information. Investigation (including CTXs detection) and reporting of ciguatera outbreaks should be strengthened.

The future perspectives regarding methodologies would involve not only the development of fast reliable techniques to optimize the screening protocol allowing a faster response in routine control, but also the implementation of the confirmation approaches once the CTX profile has been already identified. The focus on the identification of additional Caribbean analogues also looking for additional Mass Spectrometric strategies would allow to define the CTX profile more accurately and would also help on the toxicological definition which is still not completely understood. All what is above described could be also linked with the need of developing and implementing analytical methods to be applied to the identification of CTXs and/or possible metabolites in human samples. These implementations would be also critical to advance on the CP diagnosis and in the correlation with the epidemiology regarding the identification of human cases associated to CP.

What is above described could not be carried out without adequate standards, taking into account that this project allowed to successfully design an approach for the isolation and purification of CTXs, even at low levels of concentration. This approach could be used for the preparation of reference materials, by applying it to the isolation and purification of primary materials obtained through sampling plans strictly oriented to identified hot spots. This oriented sampling would allow the access materials with higher CTXs concentrations consequently allowing to increase the yield of reference materials. Also

allowing a more accurate quantitation of the CTXs involved in the contamination as well as the application of the isolation and purification protocols can support obtaining adequate amounts of reference material.

Knowledge sharing on the methodologies implemented in this project would support the full characterization of the emerging risk of CP in Europe and also contribute to an expanded and increased knowledge on the worldwide characterization of the CP risk.

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 to develop modelling studies for a better prediction of ciguatera under climate change scenarios.

For the future, efforts in Macaronesia should be focused for a better prediction of CP 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 to a wider screening of CTXs in fish, both sedentary and migratory. This screening should include the evaluation of CTXs in liver, since liver may be an earlier indicator of CTXs in the food webs.

In order to develop predictive tools regarding the risk of ciguatera in Europe some efforts should be carried out through modelling approaches on different topics. The EuroCigua project has provided extensive information that may be used to fulfil modelling objectives. The seasonality of *Gambierdiscus* and Fukuyoa presence needs to be further studied 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 perform 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 SST and turbulence, for example. In a climate change scenario, where the number of days/weeks for which seawater temperature is above a reference value increases (not reaching extreme values that may reduce *Gambierdiscus* and *Fukuyoa* growth), models may be able to predict the impact climate change will have on *Gambierdiscus* and *Fukuyoa* populations according to the expected number of days/weeks above a certain critical temperature. Selecting one 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 different geographical areas. This may help to better understand factors that can determine geographical differences. 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 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 interest. Laboratory work describing rate of accumulation of toxins in fish fed with microalgae can complement the studies. 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 be further analysed in detail, in order to identify potential patterns that may have not been evident in a first step.

5. Conclusions and major achievements

A surveillance protocol including a consensus case definition for ciguatera was created. It is available to carry out ciguatera surveillance in the EU/EEA, in a harmonized way. Around half of the countries answered to the data call for sending information on ciguatera cases or outbreaks.

Public health/food safety authorities provided information on outbreaks. Poison centres and travel and tropical medicine units provided information on single cases. As the data was retrieved retrospectively, there is lack of information for many of the variables requested for the epidemiological characterization of ciguatera. This lack of information was greater on single cases than on outbreaks.

France, Germany, Portugal and Spain have reported thirty-four ciguatera outbreaks, from 2012 to 2019. The outbreaks included 209 cases. Outbreaks due to consumption of autochthonous fish were reported by Spain and Portugal. Germany and France reported outbreaks due to consumption of imported fish. Outbreaks due to travel to tropical endemic areas were reported by Spain, Germany and France. Not all outbreaks reported to the EuroCigua project were reported to the EU-FORS.

The most frequent fish genus involved in the autochthonous outbreaks were Seriola and *Epinephelus* while *Lutjanus* was the most frequent fish genus reported in imported outbreaks. In almost 60% of the autochthonous outbreaks, the fish was captured by sport fishing. CTXs were detected in fish from half of the outbreaks, being the fish in 40% of the outbreaks not analysed.

Neurological symptoms were present in every outbreak; gastrointestinal symptoms appeared in most of them while cardiovascular symptoms were reported in few of them. Thirty-four single cases were reported by five countries: Austria, France, Germany, Portugal, Spain and Switzerland. Nine other EU countries reported zero cases.

The incidence rate in the EU/EEA, excluding tropical overseas territories, was very low (0.0054 cases per 100,000 inhabitants per year), however it was higher in the Canary Islands (0.47 cases/100,000 inhabitants), being more than double the incidence rate reported in Florida from 2000 to 2011 (0.2 cases/100000 inhabitants). The available epidemiological data guide the implementation of prevention and control measures in the Canary Islands. For a better characterization of ciguatera in the EU, it is necessary to collect detailed homogeneous information. Investigation (including CTXs detection) and reporting of ciguatera outbreaks should be strengthened.

Regarding the characterization of risk in the environment according to the presence of toxic microalgae and toxic fish, the project was successful 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.

The identification for *Gambierdiscus* spp. and *Fukuyoa* spp. in strategic hotspots in Macaronesia and Mediterranean waters, based on morphological and genetic approaches, was conducted.

In the Canary Islands, 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. We can conclude that 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 evidence for predominance in the north coast (further studies are needed).

In the eastern Mediterranean Sea, (Crete and Cyprus) a great diversity of *Gambierdiscus* and *Fukuyoa* taxa was detected, with at least 6 different taxa: *Gambierdiscus silvae*, *G. australes*, *G. carolinianus*, *G.* cf. *belizeanus*, *G.* sp. (a new species under current analysis) and *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. In the Balearic Island the EuroCigua project allowed to identify *Gambierdiscus* for the first time. The first identification was obtained in 2017 confirming the presence of *Gambierdiscus* in the western Mediterranean. Later analysis of samples obtained in 2016 also confirmed presence of *Gambierdiscus*. Hence, *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 today, the only species of *Gambierdiscus* reported in the Balearic Islands. Cytotoxicity assays showed CTX-like toxicity in *Gambierdiscus* spp. strains from the Balearic Islands.

Regarding the incidence of ciguatera in the Canary Archipelago from the results of the EuroCigua A project analysed by IUSA-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. 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 CP endemicity and highlights the need to monitor CTX accumulation in fish and the presence of *Gambierdiscus* in marine waters.

In Portugal, Selvagens Islands constitute the main hotspot for ciguatera. CTX-like toxicity was observed in several fish species. Fish toxicity ranges from 0.0039 to 0.1253 µg/kg of CTX-1B.

As for the Mediterranean fish, no CTX-like positive fish was detected from the Balearic Islands and Crete. Only in Cyprus, one CTX-like positive fish (*S. dumerili*) was detected with the Neuro-2a assay. An immunoassay also indicated the presence of CTX-like compounds in this sample.

An optimised LC-MS/MS method was developed in which the optimization of the conditions for the sample pre-treatment, in particular extraction and purification steps, has been critical. The optimized LC-MS/MS method allows the sensitive determination of the CTXs present in fish flesh according to the recommended safety limits described in the literature. The monitoring of sodium adducts, water molecule losses and characteristics fragments could be used as a contingency for monitoring CTXs in absence of adequate standards, but further confirmation by HRMS might be still required.

The optimized LC-MS/MS and LC-HRMS methods developed in this project allowed the characterization of Caribbean Ciguatoxins. C-CTX1 is the main responsible for the CP contamination on the target areas of Canary Islands and Madeira archipelago.

The concentrations of C-CTX1 in the samples analysed in this project was generally low. This resulted in an important limitation for the preparation of reference materials. A contingency plan had to be established by selecting fish with the highest CTX concentration (also including liver), optimizing a novel isolation and purification protocol, including a chromatographic fractionation by HPLC and GPC combined with Neuro-2a for the characterization of the toxicity of the fractions and further confirmation by LC-MS/MS.

The novel protocol designed for the isolation and purification of reference materials, allowed the preparation of pure solutions of C-CTX1. These solutions have been evaporated to dryness and kept at - 20C for further distribution. Additional reference materials consisting on autoclaved homogenates of fish tissue reference materials (FTRM) have been also prepared, including the fish matrix. These materials are very valuable to be used for LC-MS/MS method implementation, in which the evaluation of the matrix effect is critical. These FTRM were also kept at -20 ºC for further distribution.

The LC-MS/MS characterization of the toxic profiles of the *Gambierdiscus* and *Fukuyoa* extracts from the Canary Islands and the Mediterranean Sea, allowed to conclude that their toxicity was mainly associated with MTXs, Gambierone and Gambieric acids, but no precursors of CTXs, in particular C-CTX1, were found in any of the extracts analysed. The LC-MS (MS/MS and HRMS) approaches developed in this project allowed the full characterization of the toxic profiles of *Gambierdiscus* and *Fukuyoa* strains from the Mediterranean Sea (Balearic Islands and Crete) and the results obtained allowed to conclude that while no Ciguatoxins were present in *Gambierdiscus australes*, *G. excentricus* and *Fukuyoa paulensis*, Gambierone, 44-Methyl-Gambierone (= maitotoxin-3), Gambieric acids C and –D and a putative Gambierone analog were tentatively identified for the first time. An important finding was that none of the 6 *G. australes* strains from the Balearic Islands produced maitotoxin (MTX1), previously reported to be produced by *G. australes* strains from Japan.

And 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 and to filter the references according to the user requests.

Regarding existing modelling approaches for CP evaluation, a specific literature review has been conducted. Modelling *Gambierdiscus* populations or ciguatera epidemiology cannot be only based on the increase of average sea surface temperature (SST). 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.

6. Annexes

Annex I.

Surveillance_Protocol.pdf

Annex II.

CFP_case_reporting.pdf

Annex III.

CFP_outbreak_reporting.pdf

Annex IV.

List of fish.pdf

7. References

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

Bagnis R, Kuberski T, Laugier S, 1979. Clinical observations on 3,009 cases of ciguatera (fish poisoning) in the South Pacific. Am J Trop Med Hyg, 28, 1067.

Boada LD, Zumbado M, Luzardo OP, Almeida-González M, Plakas SM, Granade HR, Abrahamb A, Jester ELE, Dickey RW, 2010. Ciguatera fish poisoning on the West Africa Coast: An emerging risk in the Canary Islands (Spain). Toxicon, 56, 1516–1519.

Boucaud-Maitre D, Vernoux JP, Pelczar S, Daudens-Vaysse E, Aubert L, Boa S, Ferracci S, Garnier R, 2018. Incidence and clinical characteristics of ciguatera fish poisoning in Guadeloupe (French West Indies) between 2013 and 2016: a retrospective cases-series. Scientific Reports, 8:3095 | DOI:10.1038/s41598-018-21373-2

Bravo J, Cabrera-Suarez F, Ramirez AS, Acosta F, 2015. Ciguatera, an Emerging Human Poisoning in Europe. J Aquac Mar Biol 3(1): 00053

Caillaud, A., de la Iglesia, P., Barber, E., Eixarch, H., Mohammad-Noor, N., Yasumoto, T., Diogène, J. (2011). Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices: application to Gambierdiscus pacificus in culture. Harmful Algae 10:433–446

Caillaud, A., Eixarch, H., de la Iglesia, P., Rodriguez, M., Dominguez, L., Andree, K.B., et al., (2012). Towards the standardisation of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: application to fish caught in the Canary Islands. Food Addit. Contam.: Part A 29 (6), 1000–1010. https://doi.org/10.1080/19440049.2012.660707.Castro, D.; Manger, R.; Vilariño, O.; Gago-Martínez, A. 2020. Evaluation of Matrix Issues in the Applicability of the Neuro-2a Cell Based Assay on the Detection of CTX in Fish Samples. Toxins (Basel), 12.

Chateau-Degat M-L. Temporal trends and epidemiological aspects of ciguatera in French Polynesia: a 10-year analysis: Trends and epidemiology of ciguatera. Trop Med Int Health. 2007;12:485.

De Fouw JC, Van Egmond HP, Speijers GJA. Ciguatera fish poisoning: a review. RIVM report 388802 021. 2001.

Decision No 1082/2013/EU of the European Parliament and of the Council. Official Journal of the European Union. 2013, L293/1-15.

de Haro L. Ciguatera fish poisoning in France: experience of the French Poison Control Centre Network from 2012 to 2019. Clin Toxicol (Phila). 2020. 7:1.

Develoux M, Le Loup G, Pialoux G. A case of ciguatera fish poisoning in a French traveler. Euro Surveill. 2008; 13(45):pii=19027. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19027

Dickey RW, Plakas SM, 2010. Ciguatera: a public health perspective. Toxicon, 56, 123.

Diogène, J.; Reverté, L.; Rambla-Alegre, .M.; del Río, V.; de la Iglesia, P.; Campàs, M.; Palacios, O.; Flores, C.; Caixach, J.; Ralijaona, C.; Razanajatovo, I.; Pirog, A.; Magalon, H.; Arnich, N.; Turquet, J. 2017. Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean. Scientific Reports 7, 8240.

Directive 2003/99/EC of the European Parliament and of the Council. Official Journal of the European Union. 2003, L325/31-40.

EFSA. European Food Safety Authority (2010). Scientific Opinion on marine biotoxins in shellfish - Emerging toxins: Ciguatoxin group. EFSA Journal, 8(6), 38.

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2018. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSA Journal 2018;16(12):5500, 262 pp. https://doi.org/10.2903/j.efsa.2018.5500

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2018. The European Union One Health 2018 Zoonoses Report. EFSA Journal 2019;17(12):5926

Epelboin L, Pérignon A, Hossen V, et al, 2014. Two clusters of ciguatera fish poisoning in Paris, France, related to tropical fish imported from the French Caribbean by travelers. J Travel Med, 21, 397.

Estevez, P.; Castro, D.; Leao, J.M.; Yasumoto, T.; Dickey, R.; Gago-Martinez, A. 2019a. Implementation of liquid chromatography tandem mass spectrometry for the analysis of ciguatera fish poisoning in contaminated fish samples from Atlantic coasts. *Food Chem. 280*, 8–14.

Estevez, P.; Castro, D.; Pequeño-Valtierra, A.; Leao, J.M.; Vilariño, O.; Diogène, J.; Gago-Martínez, A. 2019b. An Attempt to Characterize the Ciguatoxin Profile in Seriola fasciata Causing Ciguatera Fish Poisoning in Macaronesia. *Toxins (Basel). 11*(4), 221.

Estevez, P., Castro, D., Pequeño-Valtierra, A., Giraldez, J., Gago-Martinez, A. 2019c. Emerging marine biotoxins in seafood from European coasts: incidence and analytical challenges. Foods, 8(5), 149.

Estevez, P.; Sibat, M.; Leão-Martins, J.M.; Reis Costa, P.; Gago-Martínez, A.; Hess, P. 2020a. Liquid Chromatography Coupled to High-Resolution Mass Spectrometry for the Confirmation of Caribbean Ciguatoxin-1 as the Main Toxin Responsible for Ciguatera Poisoning Caused by Fish from European Atlantic Coasts. *Toxins (Basel). 12*, 267.

Estevez, P.; Sibat, M.; Leão-Martins, J.M.; Tudó, A.; Rambla-Alegre, M.; Aligizaki, K.; Diogène, J.; Gago-Martinez, A.; Hess, P. 2020b. Use of Mass Spectrometry to Determine the Diversity of Toxins Produced by *Gambierdiscus* and *Fukuyoa* Species from Balearic Islands and Crete (Mediterranean Sea) and the Canary Islands (Northeast Atlantic). *Toxins (Basel). 12*.

Estevez, P.; Leao, J.M.; Yasumoto, T.; Dickey, R.W.; Gago-Martinez, A. 2020c. Caribbean Ciguatoxin-1 stability under strongly acidic conditions: Characterisation of a new C-CTX1 methoxy congener. *Food Addit. Contam. Part A 37*, 519–529.

Joint FAO-WHO Report of the Expert Meeting on Ciguatera Poisoning, Rome 19–23 November 2018

Friedeman M, 2018. Ciguatera fish poisoning outbreaks from 2012 to 2017 in Germany caused by snappers from India, Indonesia and Vietnam. Journal of Consumer Protection and Food Safety. https://doi.org/10.1007/s00003-018-1191-8

Friedman MA, Fleming LE, Fernandez M, et al, 2008. Ciguatera fish poisoning: treatment, prevention and management. Mar Drugs, 6, 456.

Friedman MA, Fernandez M, Backer LC, Dickey RW, et al, 2017. An updated review of ciguatera fish poisoning: clinical, epidemiological, environmental and public health management. Mar Drugs, 15(3), 72.

Fritz L., Triemer R.E. 1985. A rapid simple technique utilizing calcofluor white M2R for the visualization of dinoflagellate thecal plates. Jouranl of Phycology 21, 662–664.

Gascón J, Macià M, Oliveira I, CorachánM. Ciguatera poisoning in Spanish travellers. Med Clin 2003;120:777-9.

Glaziou P, Legrand AM, 1994. The epidemiology of ciguatera fish poisoning. Toxicon, 32, 863.

Herrero-Martínez JM, Pérez-Ayala A, Pérez-Molina JA y López-Vélez R. A case report of ciguatera fish poisoning in a traveller to Dominican Republic. Enferm Infecc Microbiol Clin. 2011;29(1):70–76.

Leonardo, S.; Gaiani, G.; Tsumuraya, T.; Hirama, M.; Turquet, J.; Sagristà, N.; Rambla-Alegre, M.; Flores, C.; Caixach, J.; Diogène, J.; O'Sullivan, C.K.; Alcaraz, C.; Campàs, M. 2020. Addressing the Analytical Challenges fo the Detection of Ciguatoxins Using an Electrochemical Biosensor. Analytical Chemistry 92, 4858-4865.Ley orgánica 3/2018. Boletin Oficial del Estado. N. 294. Pag. 119788-857.

Li X. Detecting Foodborne Disease Outbreaks in Florida through Consumer Complaints. J Food Prot. 2020. 83:1877.

Mattei C, Vetter I, Eisenblätter A, et al, 2014. Ciguatera fish poisoning: a first epidemic in Germany highlights an increasing risk for European countries. Toxicon, 91, 76.

Murata, M.; Legrand, A.M.; Ishibashi, Y.; Fukui, M.; Yasumoto, T. 1990. Structures and configurations of ciguatoxin from the moray eel Gymnothorax javanicus and its likely precursor from the dinoflagellate *Gambierdiscus* toxicus. *J. Am. Chem. Soc. 112*, 4380–4386.

Nuñez D, Matute P, Garcia A, Garcia P, Abadía N. Outbreak of ciguatera food poisoning by consumption of amberjack (Seriola *spp.*) in the Canary Islands, May 2012. Euro Surveill. 2012;17(23):pii=20188. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20188

Otero, P., Perez, S., Alfonso, A., Vale, C., Rodriguez, P., Gouveia, N. N. et al. (2010). First toxin profile of ciguateric fish in Madeira Arquipelago (Europe). Anal Chem, 82(14), 6032–6039. doi: https://doi.org/10.1021/ac100516q

Perez-Arellano, J. L., Luzardo, O. P., Perez Brito, A., Hernandez Cabrera, M., Zumbado, M., Carranza, C. et al. (2005). Ciguatera fish poisoning, Canary Islands. Emerg Infect Dis, 11(12), 1981–1982. doi: https://doi.org/10.3201/eid1112.050393

Puente Puente S, Cabrera Majada A, Lago Núñez M, Azuara Solís M y González-Lahoz JM. Ciguatera: ocho casos importados. Rev Clin Esp 2005;205(2):47-50.

Radke EG, Reich A, Morris JG, 2015. Epidemiology of Ciguatera in Florida. Am. J. Trop. Med. Hyg., 93(2), 425–432.

-Randall, J.E, 1958. A review of ciguatera, tropical fish poisoning with a tentative explanation of its cause. Bull. Mar. Sci. Gulf Carib. 8: 236–267.

Regulation (EC) No 178/2002 of the European Parliament and of the Council. Official Journal of the European Union. 2001, L31/1-24.

Regulation (EC) No 851/2004 of the European Parliament and of the Council. Official Journal of the European Union. 2004, L142/1-11.

Regulation (EC) No 679/2016 of the European Parliament and of the Council. Official Journal of the European Union. 2016, L119/1-88.

Reverté, L.; Toldrà, A.; Andree, K.B.; Fraga, S.; de Falco, G.; Campàs, M.; Diogène, J. 2018. Assessmenet of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesisan Islands by neuro-2a cell-based assays. Journal of Applied Phycology 30, 2447-2461.

Sanchez-Henao, J.A., García-Álvarez, N., Fernández, A., Saavedra, P., Silva Sergent, F., Padilla, D., Acosta-Hernández, B., Martel Suárez, M., Diogène, J., Real, F. Predictive score and probability of CTX-like toxicity in fish samples from the official control of ciguatera in the Canary Islands. Science of the Total Environment, 2019, 673, 576-584.

Sanchez-Henao, A., García-Álvarez, N., Silva Sergent, F., Estévez, P., Gago-Martínez, A., Martín, F., Ramos-Sosa, M., Fernández, A., Diogène, J., Real, F. Presence of CTXs in moray eels and dusky groupers in the marine environment of the Canary Islands. Aquatic Toxicology 221 (2020) 105427.

Schnorf H, Taurarii M, Cundy T, 2002. Ciguatera fish poisoning: a double-blind randomized trial of mannitol therapy. Neurology, 58, 873.

Schlaich C, Hagelstein JF, Burchard GD, Schmiedel S, 2012. Outbreak of ciguatera fish poisoning on a cargo ship in the port of Hamburg. J Travel Med, 19, 238.

Sibat, M.; Herrenknecht, C.; Darius, H.T.; Roué, M.; Chinain, M.; Hess, P. 2018. Detection of pacific ciguatoxins using liquid chromatography coupled to either low or high resolution mass spectrometry (LC-MS/MS). *J. Chromatogr. A* ,*1571*, 16–28.

Skinner MP. Ciguatera fish poisoning in the Pacific Islands (1998 to 2008). PLoS Negl Trop Dis. 2011. 5

Tester PA, Feldman RL, Nau AW, Kibler SR, Litaker WR. Ciguatera fish poisoning and sea surface temperatures in the Caribbean Sea and the West Indies. Toxicon. 2010 ;56 (5):698-710.

Tosteson TR. Caribbean ciguatera: a changing paradigm. Rev Biol Trop. 2004 Sep;52 Suppl 1:109-13. PMID: 17465123.

Tudó, A.; Toldrà, A.; Andree, K.B.; Rey, M.; Fernández-Tejedor, M.; Campàs, M.; Diogène, J. 2018. First resport of Gambierdiscus in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News 59, 22-23.

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

Yogi, K.; Oshiro, N.; Inafuku, Y.; Hirama, M.; Yasumoto, T. 2011. Detailed LC-MS/MS Analysis of Ciguatoxins Revealing Distinct Regional and Species Characteristics in Fish and Causative Alga from

Abbreviations/Acronyms

AB	Advisory Board
AESAN	Spanish Food Safety and Nutrition Agency
ANSES	French Agency for Food, Environmental and Occupational Health & Safety
ARAE	Regional Authority for the Economics Activities of Madeira
ASAE	Portuguese Economic and Food Safety Authority
BfR	German Federal Institute for Risk Assessment
BLAST	Basic Local Alignment Search Tool
CP	Ciguatera Poisoning
CNE	Spanish National Centre of Epidemiology
CI	Confidence Interval
CINBIO	Biomedical Research Center
CTXs	Ciguatoxins
DARP	Department of Agriculture, Livestock, Fisheries and Food of Catalonia
DMS	Document Management System
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
EFSA	European Food Safety Authority
EREN	Emerging Risks Exchange Network
EPIS	Epidemic Intelligence Information System
EU	European Union
EU-FORS	European Union Food-borne Outbreaks Information System
EURLMB	European Reference Laboratory for Marine Biotoxins
EWRS	Early Warning and Response System
FAO	Food and Agriculture Organization
FEDER	European Regional Development Fund
FP	Focal Points
FPA	Framework Partner Agreement
FSAI	Food Safety Authority of Ireland
FWD	Food and Waterborne Diseases Network
GB	Governing Board
HABs	Harmful Algal Blooms
HRMS	High Resolution Mass Spectrometry
IAEA	International Atomic Energy Agency
ICP	Internal Communication Plan
IFREMER	French Research Institute for Exploitation of the Sea
IHR	International Health Regulations
INFOSAN	International Food Safety Authorities Network
IPHAB	Intergovernmental Panel on Harmful Algal Blooms
IPMA	Portuguese Institute for Sea and Atmosphere
IRTA	Research and Technology Food and Agriculture
IUSA	University Institute for Animal Health and Food Safety

ISCIII	Carlos III Health Institute
JECFA	Joint Expert Committee for Food Additives
JRC	Joint Research Centre
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LIMIA	Marine and Aquaculture Research Laboratory
LSU	Large Sub Unit
MPC	Marseille Poison Centre
MS	Member State
MTXs	Maitotoxins
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PECIGUA	Specific Program of Ciguatoxins
PES	Provasoli's Enriched Seawater
PVDF	Polyvinylidene Fluoride
RASFF	Rapid Alert System for Food and Feed
RAS-CHEM	Rapid Alert System for chemicals
SA	Specific Agreement
SITOX	Italian Society of Toxicology
SOP	Standard Operating Procedure
ULPGC	University of Las Palmas Gran Canaria
UVIGO	University of Vigo
WHO	World Health Organization

List of publications within the EuroCigua project

1. Castro, D.; Manger, R.; Vilariño, O.; Gago-Martínez, A. Evaluation of Matrix Issues in the Applicability of the Neuro-2a Cell Based Assay on the Detection of CTX in Fish Samples. *Toxins (Basel).* **2020**, *12*.

2. Estevez, P.; Castro, D.; Leao, J.M.; Yasumoto, T.; Dickey, R.; Gago-Martinez, A. Implementation of liquid chromatography tandem mass spectrometry for the analysis of ciguatera fish poisoning in contaminated fish samples from Atlantic coasts. *Food Chem.* **2019**, *280*, 8–14.

3. Estevez, P.; Castro, D.; Pequeño-Valtierra, A.; Giraldez, J.; Gago-Martinez, A. Emerging Marine Biotoxins in Seafood from European Coasts: Incidence and Analytical Challenges. *Foods* **2019**, *8*.

4. Estevez, P.; Castro, D.; Pequeño-Valtierra, A.; Leao, J.M.; Vilariño, O.; Diogène, J.; Gago-Martínez, A. An Attempt to Characterize the Ciguatoxin Profile in Seriola fasciata Causing Ciguatera Fish Poisoning in Macaronesia. *Toxins (Basel).* **2019**, *11*.

5. Estevez, P.; Leao, J.M.; Yasumoto, T.; Dickey, R.W.; Gago-Martinez, A. Caribbean Ciguatoxin-1 stability under strongly acidic conditions: Characterisation of a new C-CTX1 methoxy congener. *Food Addit. Contam. Part A* **2020**, *37*, 519–529.

6. Estevez, P.; Sibat, M.; Leão-Martins, J.M.; Reis Costa, P.; Gago-Martínez, A.; Hess, P. Liquid Chromatography Coupled to High-Resolution Mass Spectrometry for the Confirmation of Caribbean Ciguatoxin-1 as the Main Toxin Responsible for Ciguatera Poisoning Caused by Fish from European Atlantic Coasts. *Toxins (Basel).* **2020**, *12*.

7. Estevez, P.; Sibat, M.; Leão-Martins, J.M.; Tudó, A.; Rambla-Alegre, M.; Aligizaki, K.; Diogène, J.; Gago-Martinez, A.; Hess, P. Use of Mass Spectrometry to Determine the Diversity of Toxins Produced by *Gambierdiscus* and *Fukuyoa* Species from Balearic Islands and Crete (Mediterranean Sea) and the Canary Islands (Northeast Atlantic). *Toxins (Basel).* **2020**, *12*.

8. J. Andres Sanchez-Henao, Natalia García-Álvarez, Antonio Fernández, Pedro Saavedra, Freddy Silva Sergent, Daniel Padilla, Begoña Acosta-Hernández, Manuela Martel Suárez, Jorge Diogène, Fernando Real. Predictive score and probability of CTX-like toxicity in fish samples from the official control of ciguatera in the Canary Islands. *Science of the Total Environment*. **2019***, 673; 576–584.*

9. J. Andres Sanchez-Henao , Natalia García-Álvarez, Freddy Silva Sergenta , Pablo Estévez , Ana Gago-Martínez, Francisco Martín, María Ramos-Sosa, Antonio Fernández, Jorge Diogène , Fernando Real. Presence of CTXs in moray eels and dusky groupers in the marine environment of the Canary Islands. *Aquatic Toxicology.* **2020**, 221; 105427.

10. Tudó, A.; Toldrà, A.; Andree, K.B.; Rey, M.; Fernández-Tejedor, M.; Campàs, M.; Diogène, J. First report of *Gambierdiscus* in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News. **2018**, 59, 22-23.

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

12. Tudó, A.; Gaiani, G.; Rey, M.; Tsumuraya T.; Andree, K.B.; Fernández-Tejedor, M.; Campàs, M.; Diogène, J. Further Advance of *Gambierdiscus* Speces in the Canary Islands, with the First Report of *Gambierdiscus belizeanus*. Toxins. **2020,** 12, 692, doi:10.3390/toxins12110692Toxins 2020, 12, 692; doi:10.3390/toxins12110692.